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Tree Species Composition and Diversity in a Secondary Forest along the Sierra Madre Mountain Range in Central Luzon, Philippines: Implications for the Conservation of Endemic, Native, and Threatened Plants

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Abstract: The Sierra Madre Mountain Range (SMMR) is the backbone of the Luzon Islands that contains a high concentration of highly important ecological resources distributed among the 68 protected areas therewith. The present study aimed to assess the composition and diversity of tree species in a secondary forest within the SMMR. A 2.25 km transect with 10 900-m^2 plots were established to record tree species with a diameter at breast height of at least 10 cm. The findings revealed 148 individuals of trees from 38 morphospecies, 28 genera, and 20 families. Importance values unveiled the Aurora endemic *Macaranga stonei* Whitmore as the most important species in terms of the relative values of its abundance, frequency, and dominance. The area was also found to be home to 33 natives, 12 endemics, five IUCN threatened species, and nine Philippine threatened trees. Furthermore, the study site was also found to have considerably high diversity, with a Shannon–Weiner Index value of 3.269 and a relatively even distribution of individuals among species, as supported by the Simpson's Evenness index value of 0.9453. Significant correlational relationships were also found among species richness, Shannon–Weiner index, and Simpson's Evenness index, with correlation coefficients ranging from 0.881 to 0.934, with all significant at $p < 0.001$. Lastly, the study was able to produce a distribution map, which is necessary for implementing targeted conservation strategies. These findings provided valuable implications for future research and implementation of targeted and participatory biodiversity conservation and protection strategies.

Keywords: biodiversity; biodiversity hotspot; correlation analysis; distribution maps; Shannon–Weiner index; Simpson's Evenness index

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1. Introduction

The Philippines, an archipelagic country known for its rich biodiversity, stands proudly as one of the 18 megadiverse nations on Earth [1]. This designation signifies that it harbors over two-thirds of the world's biodiversity resources, which play a crucial role in supporting human well-being and survival while maintaining ecosystem stability [2,3]. Biodiversity generously provides us with essential resources like food, water, raw materials, and clean air. Moreover, it diligently regulates climate and protects us from natural disasters [4]. Ecologically speaking, biodiversity enables vital processes such as pollination, nutrient cycling, water filtration, and soil stabilization and erosion control—all working together to create balanced ecosystems and desirable environmental conditions [5–7].

Unfortunately, biodiversity has long been facing numerous threats that jeopardize its existence and the critical services it provides. Climate change, along with many undesirable human activities such as deforestation, habitat destruction, land use change, and overexploitation, primarily drive biodiversity loss globally [8]. Due to these, scientists were able to identify biodiversity hotspots that contain very high rates of endemism and

drastic loss of vegetation and habitat that threatens various key biodiversity species [9]. At present, there are already 36 biodiversity hotspots, including the Philippines [10]. This signifies the need for immediate planning and implementation of strategies to prevent total biodiversity loss.

In the Philippines, various conservation and rehabilitation efforts are continuously implemented. The establishment and monitoring of protected zones under the National Integrated Protected Areas Systems (NIPAS) Act is considered one of the most important tools in conserving the country's key biodiversity resources, as recommended by the Convention on Biological Diversity [11]. Other conservation and rehabilitation programs, such as the National Greening program under Executive Order No. 26 [12], community-based forest management under Executive Order No. 263 [13], and sustainable ecotourism [14], among others, are recognized as greatly contributing to biodiversity conservation while educating people about its values and services.

However, there were critical issues in some rehabilitation and conservation programs. One of these is the unsuitable choice of plant species to rehabilitate a degraded or disturbed area. Several efforts in the past used exotic and invasive species such as *Gmelina arborea* Roxb. And *Swietenia macrophylla* King [15] in many greening activities. Some used native species, but there was a lack of pre-assessment of the site–species relationships thus introducing the natives to inappropriate habitats and hindering their successful growth and survival [16]. This is where the importance of plant inventory and assessments comes in. The data and findings yielded by these studies provide essential information on the population structure, composition, and ecology of an area and its resources that are beneficial in recovery planning, such as biodiversity conservation and habitat rehabilitation [17].

This current study aims to contribute to the conservation of Philippine biodiversity by assessing tree diversity in the municipality of San Luis in the province of Aurora. The province is a part of the Sierra Madre Mountain Range, the longest mountain range in the country, which is considered a highly important area in terms of valuable ecological resources distributed among its 68 protected areas [18,19]. Furthermore, there are very few studies about the plant composition and diversity in the province, which only cover the tree species in the municipalities of Baler [20] and Dipaculao [21], as well as the diversity of ferns in the municipalities of Maria Aurora [22] and Baler [23]. Hence, this study will pioneer the assessment of plants in the municipality of San Luis, which is beneficial in identifying the area's key biodiversity resources, such as the endemic, native, and threatened species, which is a crucial step in biodiversity conservation. Specifically, the study aimed to determine the tree species composition, including ecological classifications (i.e., indigeneity, endemism, and conservation status), calculate the importance values and diversity indices and explore the underlying relationships among diversity parameters and ecological variables (i.e., elevation).

2. Materials and Methods

2.1. Study Site

The study was conducted in April 2023 in Barangay L. Pimentel in the municipality of San Luis, province of Aurora, situated approximately 15°41′2.94″ N and 121°30′1.23″ E (Figure 1). The barangay is composed of residential, agricultural, and mountainous forest lands. Specifically, the survey was carried out in mountainous forest lands, which is a portion of the Sierra Madre Mountain Range, the backbone of Luzon Island, that serves as a protector and barrier from typhoons coming from the Pacific Ocean [24]. The survey area had a moderately steep topography, with elevations ranging from 273 to 581 masl. Climate-wise, the municipality has average monthly temperatures ranging from 26 °C to 30 °C (high temperature) and 22 °C to 25 °C (low temperature), and average monthly rainfall ranging from 118.3 mm (March: average of 7 rainfall days) to 416.9 mm (October: average of 17 rainfall days) in 2023. In the past 10 years, the average annual temperatures have usually ranged from 26 °C to 28 °C, while rainfall has been 100.23 mm to 624.86 mm. During the study period, the area had an average temperature of 28 °C during daytime and

24 °C during nighttime and there were 8 rainy days, with precipitation of around 300 mm during the month of April [25].

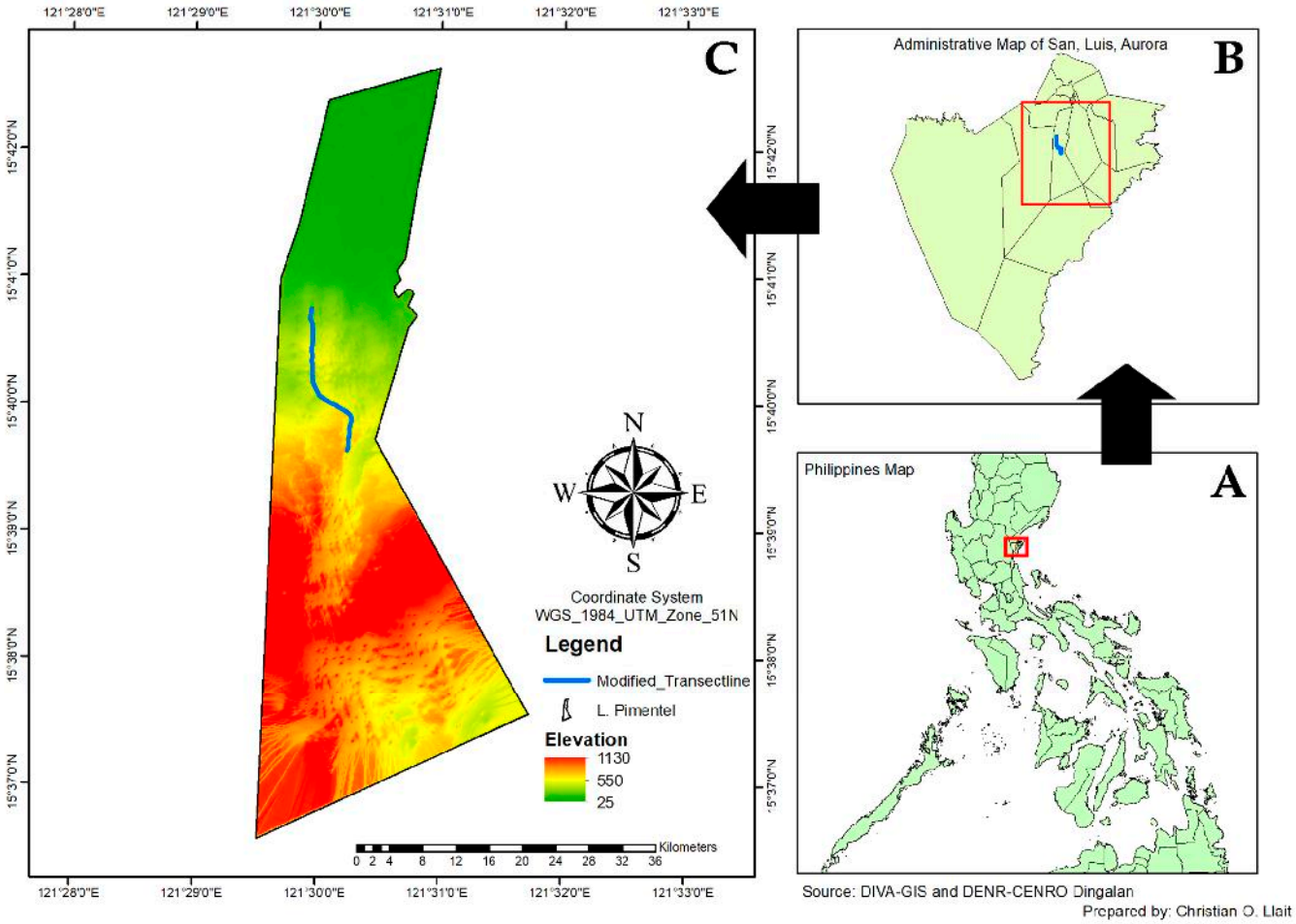

Figure 1. Location map of the study site in San Luis, Aurora: (**A**) study site pointed in the Philippine map, (**B**) location of the site pointed in the map of San Luis, Aurora, (**C**) elevation map of Barangay L. Pimentel showing the location of modified transect.

2.2. Survey and Mapping of Tree Species

The inventory of tree species was carried out along a 2.25 km transect line with 10 30 by 30 m quadrats established at every 250 m point (Figure 2). The transect line was established following the trail while the quadrats were positioned alternately at the left and right of the transect line, with an approximate distance of 5 m away from the trail. The total coverage of all the quadrats was 9000 m^2. The use of transect in conducting this plant inventory was used to ensure that the quadrats were evenly distributed throughout the forest stand [26].

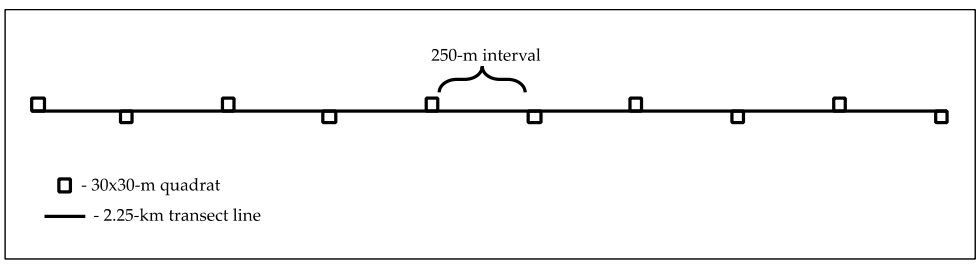

Figure 2. Arrangement of quadrats along the transect line.

After the establishment of the transect and quadrats, the plant survey was carried out. Trees with a diameter at breast height (DBH) of at least 10 cm were included in the study, following the DBH cut-off of many tree species inventories undertaken in the Philippines [27,28]. Plant identities were determined in the field using morphological characteristics. For individuals that were not identified in the field, photos were taken for further verification. References and databases such as Co's Digital Flora of the Philippines [29] and Revised Lexicon of Philippine Trees [30] were used to verify plant identities. Finally, the accepted scientific names of plants were determined using the Plants of the World Online database of the Kew Royal Botanic Gardens [31]. Significant ecological statuses (i.e., indigeneity, endemism, and conservation status) of all species were also assessed. Indigeneity and endemism were obtained from the Co's Digital Flora of the Philippines [29]. Meanwhile, conservation statuses were determined using the IUCN Red List of Threatened Species [32] for the global scale and DAO 2017-11 or the Updated National Checklist of Threatened Plants and their Categories [33] for the national scale.

Mapping was also carried out to visually present the location of each individual tree, which will also serve as the basis for the future implementation of targeted biodiversity conservation and management measures. Initially, the Locus map (a mobile outdoor navigation application) was used to record the location of the transect line and quadrats. Then, the geographic coordinates of each tree were recorded and encoded in Microsoft Excel. Geographic coordinates in decimal degree format were then converted into the Universal Transverse Mercator (UTM) format using the ArcGeek Coordinate Conversion Tool [34] before feeding it to ArcGIS software (v. 10.4). After that, the locations of all trees were plotted on the map. Lastly, final editing was undertaken to produce the final copy of the map in .jpeg format.

2.3. Data Analysis and Interpretation

2.3.1. Species Richness, Abundance, and Importance Values

Species richness, abundance, and importance values were either counted or calculated to discover the species composition in the area. Species abundance refers to the number of individuals of a species in an area [35], while species richness is the number of species or taxa present [36]. Hence, the number of species and its individuals were counted to determine the species richness and abundance. Lastly, importance values (IVs) serve as an index to measure how dominant a certain species is in a forest area through the relative values of its abundance, frequency, and dominance [37]. Thus, IVs were computed using the following equations [38]:

$$Density = \frac{number\ of\ individuals\ of\ a\ \ species}{total\ area\ sampled} \quad (1)$$

$$Relative\ Density = \frac{density\ of\ a\ species}{total\ density\ of\ all\ species} \quad (2)$$

$$Frequency = \frac{number\ of\ plots\ in\ which\ a\ species\ occur}{total\ number\ of\ plots\ sampled} \quad (3)$$

$$Relative\ Frequency = \frac{frequency\ of\ a\ species}{total\ frequency\ of\ all\ species} \quad (4)$$

$$Basal\ Area = 0.7854\ \left(DBH\ of\ a\ species^2\right) \quad (5)$$

$$Dominance = \frac{basal\ area\ of\ a\ species}{total\ area\ sampled} \quad (6)$$

$$Relative\ Dominance = \frac{dominance\ of\ a\ species}{total\ dominance\ of\ all\ species} \quad (7)$$

$$Importance\ Value = Relative\ Density + Relative\ Frequency + Relative\ Dominance \quad (8)$$

2.3.2. Diversity Indices

Biological diversity can be quantified using mathematical functions known as the diversity indices [39]. In this study, the widely accepted Shannon–Weiner (H') and Simpson's Evenness (E) were employed as the species diversity indices and computed through Paleontological Statistics (PAST v 3.18) software. The choice of these indices aligns with the standards set by previous biodiversity studies undertaken in the country and uses the Fernando Biodiversity Scale, which has been widely adopted in diverse ecological investigations in the Philippines to effectively facilitate the interpretation of computed values [40,41] (Table 1).

Table 1. Fernando Biodiversity Scale.

Interpretation	Shannon–Weiner	Simpson's Evenness
Very high	3.5 and above	0.75–100
High	3.0–3.49	0.5–0.74
Moderate	2.5–2.99	0.25–0.49
Low	2.0–2.49	0.15–0.24
Very Low	1.9 and below	0.05–0.14

2.3.3. Correlation Analysis

Exploring intricate relationships among key variables is essential in deeply understanding the dynamics of forest ecosystems. Therefore, Pearson correlation analysis was used to explore the underlying relationship (i.e., monotonic association) among important variables, namely, elevation, species richness, abundance, Shannon–Weiner, and Simpson's Evenness. This was computed at a significance level of $p < 0.05$ through JASP v. 0.16.1, an open-source statistical software package. The results were interpreted using the computed correlation coefficient values (r-values) and their associated p-values, as well as the conventional approach in interpreting r-values, contextualized as a direct or inverse relationship [42] (Table 2).

Table 2. Conventional approach in interpreting correlation coefficient [42].

Absolute Value of r	Interpretation
0–0.09	Negligible correlation
0.10–0.39	Weak correlation
0.40–0.69	Moderate correlation
0.70–0.89	Strong correlation
0.90–1.0	Very strong correlation

3. Results and Discussion

3.1. Tree Species Composition

The study recorded a total of 148 individuals of 38 morphospecies of trees from 20 families and 28 genera. In terms of the families, Dipterocarpaceae and Moraceae were the most speciose with seven and five species, respectively. The most abundant families were Euphorbiaceae, Dipterocarpaceae, and Moraceae, with 29, 28, and 21 individuals, respectively. These families are abundant in the Philippines, especially in tropical lowland evergreen forests that are dominated by dipterocarps [43]. Sadly, dipterocarps are among the most threatened plant species in the Philippines and in Southeast Asia due to deforestation, and their timbers have been massively exported in the past [44,45]. Species-wise, *Macaranga stonei* Whitmore was the most abundant, followed by *Parashorea malaanonan* (Blanco) Merr., with 24 and 9 individuals, respectively. Given that the study plots covered 9000 m^2, which is 9/10 of a hectare, it is estimated that these species, *M. stonei* and *P. malaanonan*, had 26 and 10 individuals in a hectare of the study area, respectively.

The importance values computation also revealed significant findings in terms of the species composition. Eleven (11) species had individual IVs of more than 10 (Figure 3). In

total, these 11 species contributed 55.50% of the total IV of all species in the area. Among them, *M. stonei* had the highest IV of 30.35, which is equivalent to 10.11% of the total IV of all the species recorded, followed by *Parashorea malaanonan* (Blanco) Merr, with 21.63 (7.21%). *M. stonei*'s high IV was related to its high abundance of 24, its occurrence in six plots out of all ten plots, and a total basal area of 136.73 m^2. *M. stonei* is Aurora province-endemic and a critically endangered plant species belonging to the family Euphorbiaceae [29,32]. This keystone species lacks focus in terms of research, thus dictating the need to study this species more and include it as one of the top priorities for conservation due to it being a species restricted to the province of Aurora.

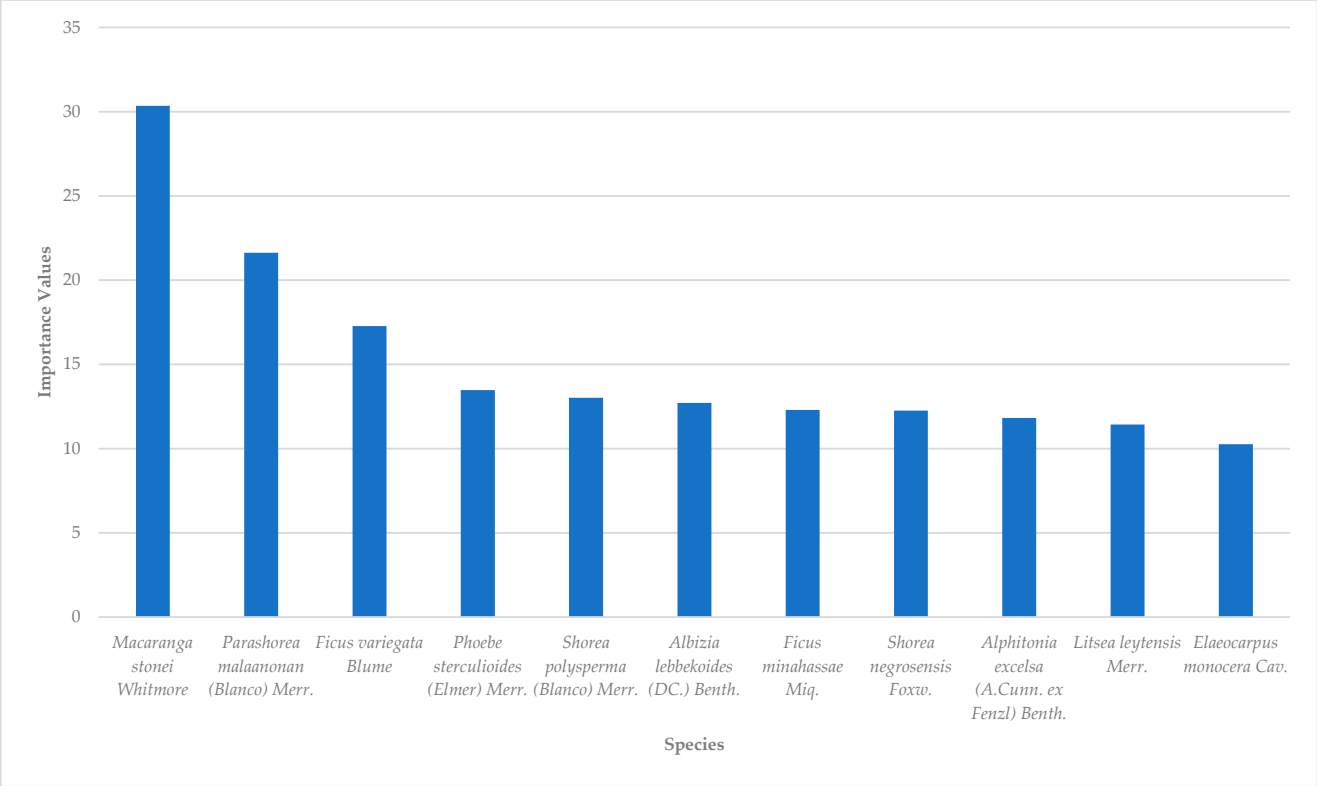

Figure 3. Top eleven species with highest importance values (IVs).

The surveyed forest was also found to be home to ecologically important species, namely, native, endemic, and threatened species (Table 3). Out of the 38 species found, 33 (86.84%) were natives, while five were exotics (with one invasive *Gmelina arborea* Roxb. ex Sm.). The native species were composed of 12 endemics, five IUCN threatened species, and 9 Philippine nationally threatened species. Specifically, there were one critically endangered, two endangered, and two vulnerable species found in the IUCN. Furthermore, there were two endangered, six vulnerable, and one other threatened species found in DAO 2017-11 or the Philippine Red List. The most notable among the Philippine endemic species were the IUCN critically endangered *M. stonei* and the IUCN vulnerable and DAO 2017-11 endangered *Hopea acuminata* Merr, and the IUCN endangered and DAO 2017-11 vulnerable Philippine national tree *Pterocarpus indicus* Willd. The presence of critically important plants in the area dictates the need for immediate action to conserve, protect, and even spread their population. It is emphasized that these species, particularly the endemics, have higher probabilities of extinction because of their narrow and restricted habitat than widespread species [46]. The native and endemic plant species also provide suitable habitats and enough food sources for native and endemic fauna species [47]. In fact, we were able to witness a couple of the Philippine endemic Luzon Rufous Hornbill (*Buceros hydrocorax* Linnaeus) during the survey. However, the presence of invasive species like the *G. arborea*

adds pressure to the survival and propagation of the native and endemic flora and fauna species due to the aggressive nature of most invasive plants [48]. Actual representative photos of some critically important plant species in the area and an image of *B. hydrocorax* individuals are shown in Figure 4.

Figure 4. Representative photos of critically important species in the area. (**A**) *Macaranga stonei* Whitmore (endemic, IUCN critically endangered), (**B**) *Dillenia philippinensis* Rolfe (endemic, IUCN near threatened), (**C**) *Shorea polysperma* (Blanco) Merr. (endemic, DAO 2017-11 vulnerable), (**D**) *Shorea contorta* Vidal (endemic, DAO 2017-11 vulnerable), (**E**) a couple of *Buceros hydrocorax* Linnaeus (endemic, IUCN vulnerable, Philippine Red List endangered).

Table 3. Taxonomic list of native species recorded with their corresponding endemism and conservation statuses.

Family	Species	Endemism [1]	Conservation Status [2]	
			IUCN Red List	DAO 2017-11
Anacardiaceae	*Koordersiodendron pinnatum* (Blanco) Merr.	NE	ND	OTS
Brownlowiaceae	*Diplodiscus paniculatus* Turcz.	PE	LC	ND
Cannabaceae	*Celtis philippensis* Blanco	NE	LC	ND
Dilleniaceae	*Dillenia philippinensis* Rolfe	PE	NT	ND
Dilleniaceae	*Tetracera scandens* (Linn.) Merr.	NE	ND	ND
Dipterocarpaceae	*Dipterocarpus grandiflorus* (Blanco)	NE	EN	VU
Dipterocarpaceae	*Hopea acuminata* Merr.	PE	VU	EN
Dipterocarpaceae	*Parashorea malaanonan* (Blanco) Merr.	NE	LC	ND
Dipterocarpaceae	*Shorea contorta* Vidal	PE	LC	VU
Dipterocarpaceae	*Shorea negrosensis* Foxw.	PE	LC	VU
Dipterocarpaceae	*Shorea polysperma* (Blanco) Merr.	PE	LC	VU
Dipterocarpaceae	*Shorea squamata* (Turcz.) Benth. & Hook.	PE	LC	ND
Elaeocarpaceae	*Elaeocarpus cumingii* Turcz.	NE	LC	ND
Elaeocarpaceae	*Elaeocarpus monocera* Cav.	PE	ND	ND
Euphorbiaceae	*Macaranga grandifolia* (Blanco) Merr.	NE	VU	ND
Euphorbiaceae	*Macaranga stonei* Whitmore	PE	CR	ND
Euphorbiaceae	*Macaranga tanarius* (L.) Muell. Arg.	NE	LC	ND
Euphorbiaceae	*Mallotus paniculatus* (Lam.) Müll. Arg.	NE	LC	ND

Table 3. *Cont.*

Family	Species	Endemism [1]	Conservation Status [2]	
			IUCN Red List	DAO 2017-11
Fabaceae	*Albizia lebbekoides* (DC.) Benth.	NE	LC	ND
Fabaceae	*Pterocarpus indicus* Willd.	NE	EN	VU
Hypericaceae	*Cratoxylum sumatranum* Blume	NE	LC	ND
Lauraceae	*Litsea leytensis* Merr.	PE	NT	EN
Lauraceae	*Phoebe sterculioides* (Elmer) Merr.	PE	LC	ND
Meliaceae	*Aglaia luzoniensis* (Vidal) Merr. & Rolfe	NE	NT	ND
Moraceae	*Artocarpus blancoi* (Elmer) Merr.	PE	LC	ND
Moraceae	*Ficus minahassae* Miq.	NE	LC	ND
Moraceae	*Ficus nota* (Blanco) Merr.	NE	LC	ND
Moraceae	*Ficus variegata* Blume	NE	LC	ND
Myrtaceae	*Syzygium nitidum* Benth.	NE	ND	VU
Myrtaceae	*Syzygium tripinnatum* (Blanco) Merr.	NE	ND	ND
Rhamnaceae	*Alphitonia excelsa* (A.Cunn. ex Fenzl) Benth.	NE	LC	ND
Sterculiaceae	*Sterculia ceramica* R.Br.	NE	ND	ND
Urticaceae	*Leucosyke capitellata* (Poir.) Wedd.	NE	LC	ND

[1] Endemism classifications: PE—Philippine endemic; NE—Not endemic. [2] Conservation status classifications: CR—Critically endangered; EN—Endangered; Vu—Vulnerable; OTS—Other Threatened Species; NT—Near threatened; LC—Least concern; ND—No data.

3.2. Tree Species Diversity

The diversity indices of the secondary forest in San Luis are presented in Figure 5. The Shannon–Weiner index values per quadrat ranged from 1.626 to 2.3384 and were interpreted as very low to low based on the Fernando Biodiversity Scale. In terms of Simpson's Evenness, the values ranged from 0.8182 to 0.9619, which were interpreted as very high. Quadrat 2 had the highest diversity ($H' = 2.384$ and $E = 0.9619$). Overall, the study area had a high Shannon–Weiner index ($H' = 3.269$) and a very high Simpson's Evenness index ($E = 0.9453$), which means that the trees in the area were relatively diverse and had a considerably even distribution of individuals among species. In most ecological studies in the Philippines, H' values generally range from 1.5 to 3.5, wherein higher values dictate higher species diversity [49]. The overall H' value of the present study falls within this range and was interpreted as high, which can possibly be attributed to the variety of native and endemic species that still thrive therewith. This is comparable with some studies undertaken in the Philippines, such as in a lowland forest in Agusan del Sur ($H' = 3.32$, $E = 0.52$) [50], in a secondary forest in Benguet ($H' = 2.40$) [49], and in a secondary forest in Pampanga ($H' = 2.2807$, $E = 0.8549$) [51], which were all categorized as having low to moderate diversity based on the Shannon–Weiner index. Similarly, these study sites were either under the management of upland communities or near their residential or agricultural sites. In contrast, the values are lower than the studies in a private mountainous forest in Baler, Aurora ($H' = 4.096$; $E = 0.9735$) [18], in the Quezon Protected Landscape ($H' = 3.90$, $E = 0.81$) [52], and in the Mt. Makiling Forest Reserve ($H' = 3.50$, $E = 0.91$) [53]. The common characteristics that possibly caused these high values were their classifications as private property, with strict monitoring and considerably high protection for the site in the first study and being classified as protected areas under the law of the second and third study sites, relating to the monitoring and protection activities of the government.

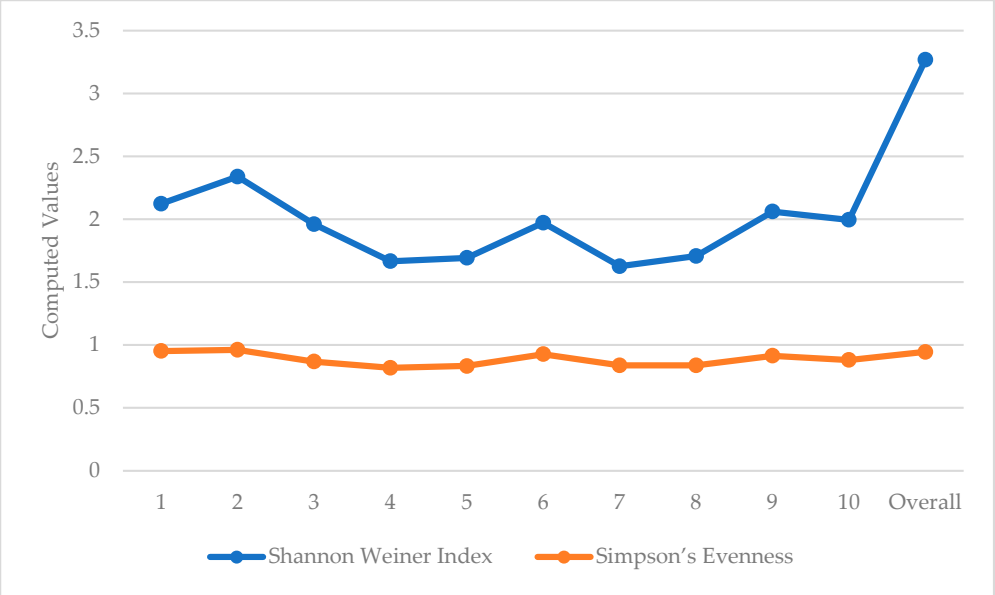

Figure 5. Computed diversity indices per quadrat and for the entire survey area.

3.3. Correlation among Parameters

This study also tested the correlations among elevation, species richness, abundance, Shannon–Weiner, and Simpson's Evenness values. As a result, significant correlational relationships were only observed for the following: (a) Species Richness and Shannon–Weiner (r = 0.881, $p < 0.001$); (b) Species Richness and Simpson's Evenness (r = 0.885, $p < 0.001$); and (c) Simpson's Evenness and Shannon–Weiner (r = 0.934, $p < 0.001$) (Figure 6). Based on the r-values, there was a strong positive correlation between species richness and Shannon–Weiner index as well as between species richness and Simpson's Evenness, as supported by a very high significance value of p that is less than 0.001. This relationship suggests that as species richness increases, the values of the Shannon–Weiner and Simpson's Evenness indices also tend to increase. Thus, this observation indicates that having a greater variety of species can lead to a higher diversity, as measured by using the mentioned indices. Furthermore, there was a very strong positive correlation found between the Simpson's Evenness index and Shannon–Weiner index based on the obtained r-value, which is backed up by a very high statistical significance with $p < 0.001$. This indicates that as the value of Shannon–Weiner index increases, the value of Simpson's Evenness also tends to increase. The findings are corroborated by the study of DeJong, which also found a very strong correlation among species richness, Shannon–Weiner index, and Simpson's Evenness index, with correlation coefficients of more than 0.96 [54]. However, no significant correlational relationships were found between the following: (a) elevation and other variables, and (b) abundance and other variables. A similar finding was found in a study at a mountain range in Southern Mindano, suggesting that elevation did not greatly affect biodiversity parameters such as the diversity indices [55]. In essence, these results are beneficial in understanding the dynamics of an ecosystem, which can be the foundation for implementing management and rehabilitation strategies in different areas within the study site with the goal of improving biodiversity.

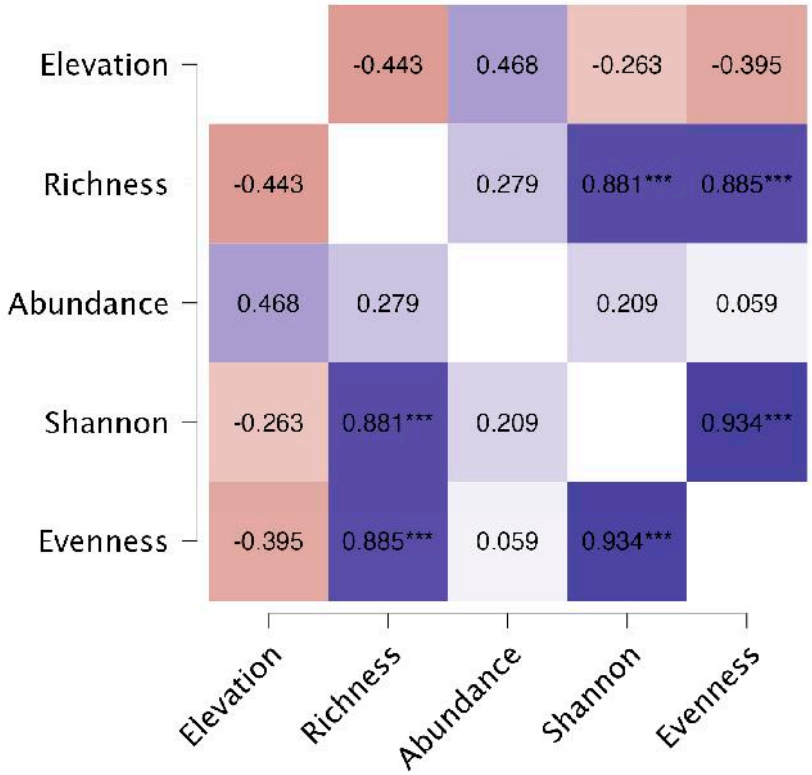

Figure 6. Heatmap of Pearson's *r* correlation among elevation and biodiversity parameters (*** $p < 0.001$).

3.4. Spatial Distribution of Trees

Figure 7 shows the spatial distribution of trees across the sampling plots in the secondary forest of San Luis in Aurora, Philippines. This map shows the position of the transect line and the approximate location of each individual tree, represented by colored dots (legend placed on the right-hand side), based on the recorded coordinates. As observed in the map, the plots were zoomed in to show the locations of the trees more clearly. We can also see in the background of the zoomed image of the plots the actual image of the forest cover in the area, as reflected in the base map used. Mapping the spatial distribution of trees is a crucial element in devising strategies for the sustainable management and conservation of natural resources [56]. For instance, locating the trees can help us identify areas with possible sources of mother trees of the targeted species that we aim to propagate [57]. For example, if we are looking for a source of planting materials for a high-priority species such as *M. stonei*, which is a very important species in the area due to the fact that it is an endemic and critically endangered species, we can refer to the map and see that it can be seen in plots 3, 5, 7, 8, 9, and 10. Furthermore, distribution maps can visually present areas needing attention and immediate measures, such as in the case of our study, the presence of invasive *G. arborea* that poses a threat to the native biodiversity. Knowing the location of its recorded individuals (present in plots 2 and 3) will allow the forest managers to perform targeted measures in managing specific portions of the area where invasion issues arise [48]. Lastly, we can identify micro-biodiversity hotspots among the sampling plots in the study area by determining the number of critically important species [58].

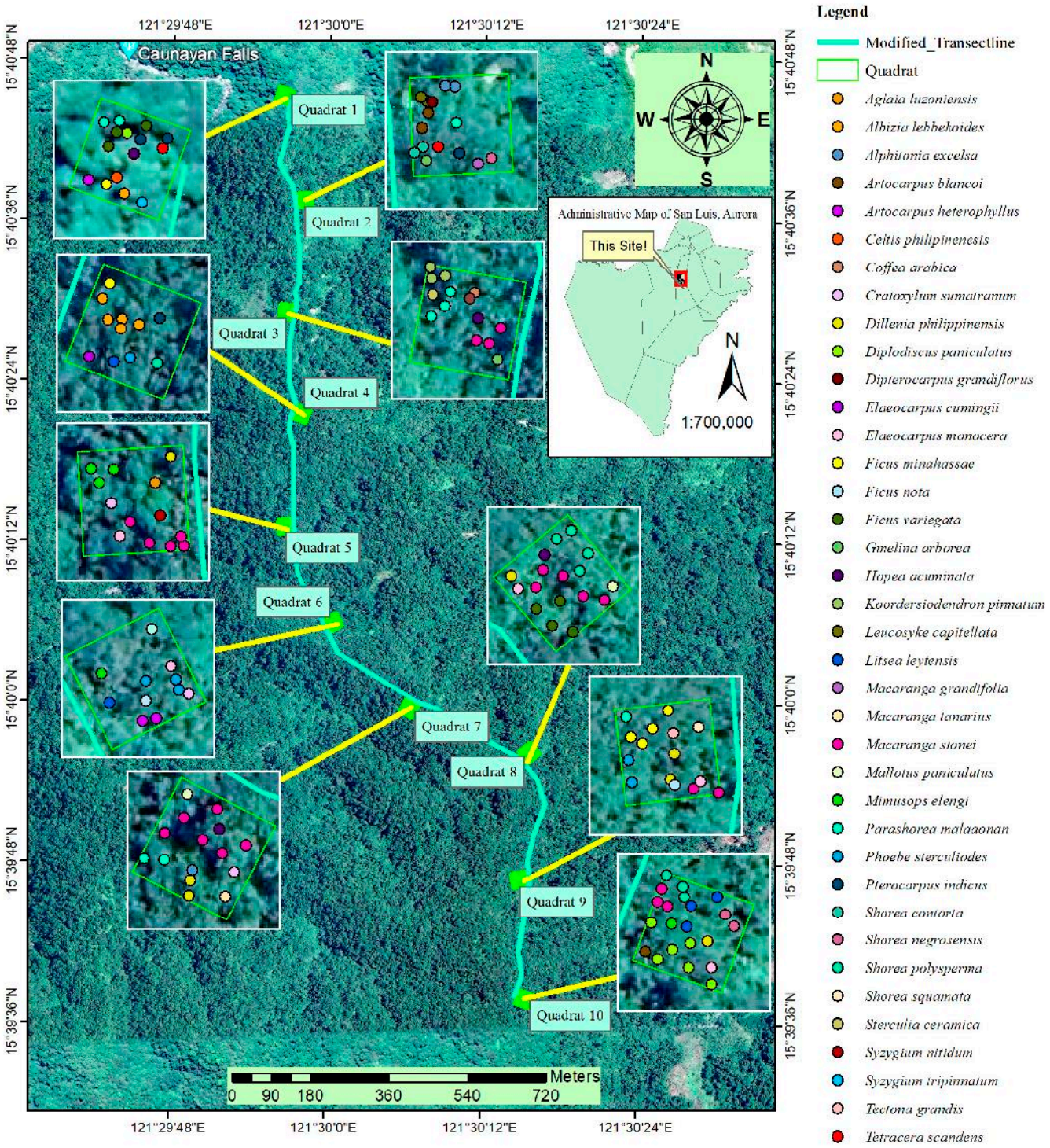

Figure 7. Distribution map of all trees across sampling plots in the secondary forests of San Luis, Aurora.

3.5. Research Limitations

This study provides valuable insights into the composition and diversity of tree species in the San Luis area of the SMMR. However, certain limitations were acknowledged. The research scope was limited to a short duration, and only a specific portion of the secondary forests of San Luis, Aurora, were covered by the transect, where variations in topography, exposure, and elevations, as well as forest dynamics, were not fully explored. Thus, these issues limit the generalizability of the findings in terms of larger ecosystems and dictate

the need for long-term monitoring and the eventual establishment of a protected area. Furthermore, tree species were the only life forms included in the study, opening the door for a more comprehensive assessment of other biodiversity components, such as understorey and ground vegetation, wildlife, and soil characteristics, which were beyond the scope of this study. These limitations are crucial in interpreting the results to guide future research directions and in planning a holistic and more effective biodiversity management and conservation.

4. Conclusions and Implications

This study yielded valuable findings and insights regarding the species composition and diversity of a secondary forest in San Luis, Aurora. Overall, the area had a relatively high diversity and significant conservation, as signified by the recorded 148 individuals of 38 morphospecies belonging to 20 families and 28 genera, with 33 natives, 12 endemics, five IUCN threatened, and nine Philippine threatened species. Furthermore, diversity was found to be high in terms of the Shannon–Weiner index ($H' = 3.269$) and very high in terms of the Simpson's Evenness index ($E = 0.9453$). Significant correlational relationships were also found among species richness, Shannon–Weiner index, and Simpson's Evenness index. Lastly, individual trees were mapped to serve as a guide for targeted conservation measures. These findings are critical in the following applications for the conservation of native, endemic, and threatened species:

1. The presence of many native, endemic, and threatened species underscores the immediate need to prioritize the conservation of these species through the aid of the map produced in locating the micro-biodiversity hotspots in the area. Furthermore, many endemic species lack scientific studies, highlighting the need to conduct focused studies to explore the ecology and distribution of these critically important species. Furthermore, this can serve as a basis for the Department of Environment and Natural Resources to include the forest as one of the high conservation priorities or to expand protected areas to cover the area surveyed.
2. The relatively high diversity values and even distribution of plants calculated for the area somehow indicate a relatively healthy ecosystem. Thus, this underscores the need for intensified law enforcement to protect the remaining forests that serve as habitats for native and endemic wildlife, such as *Buceros hydrocorax* Linnaeus.
3. The presence of introduced and invasive species such as *Gmelina arborea* Roxb. poses a very significant threat to local native biodiversity. Targeted and participatory invasive species management is needed to control and eventually eradicate the impact of invasive plants in the ecosystem.
4. All the implications and conservation strategies discussed above will need the participation of locals and other stakeholders due to the fact that the area is adjacent to residential communities. Thus, information and educational campaigns, as well as a participatory approach in implementing conservation strategies, are ideal tools to ensure more effective biodiversity conservation and protection.

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Research article

Differential expression of *Xoo-induced kinase 1 (XIK1)*, a *Xanthomonas oryzae* pv. *oryzae* responsive gene, in bacterial blight-susceptible and *Xa21*-mediated resistant indica rice cultivars

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Abstract

Bacterial blight (BB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a costly disease in rice that threatens global rice production. The *Xa21* gene is a broad spectrum BB-resistance gene that is extensively used for improving BB resistance in rice. The *Xoo* responsive gene, *Xoo-induced kinase 1* (*XIK1*), recently characterized in japonica rice, was also found to be involved in Xa21-mediated resistance. The current study investigated the differential expression of *XIK1* in the BB-susceptible indica rice cultivar RD47 and its improved BB-resistant progenies BC_3F_3 (*Xa21*/*Xa21*) in various growth stages and during *Xoo* inoculation. The results showed that the expression of *XIK1* was development-dependent and induced earlier in BB-resistant progenies BC_3F_3 than in the susceptible cultivar RD47 after *Xoo* inoculation. However, the expression levels of *XIK1* substantially dropped after a peak of expression. Interestingly, the *XIK1* expression tended to increase again in the succeeding hours post-inoculation in BC_3F_3 but not in RD47. Similar development and induction patterns were also observed when the expression of *XIK1* and *Xa21* was analyzed in the same runs in the BB-resistant BC_3F_3 under different growth stages and during *Xoo* inoculation. The findings suggested that XIK1 may also be involved in the Xa21-mediated resistance pathway of indica rice.

Introduction

Most of the world's population depends on rice (*Oryza sativa*) as the primary food source and this requires large-scale-production volumes to meet the growing demand (IRRI, 2006). However, limiting factors such as insect pests and diseases tend to reduce the yield by 30–80% (Reissig et al., 1985; IRRI, 2018a). Among the major diseases of rice, bacterial blight (BB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the costliest as it reduces production by up to 70% (IRRI, 2018b). Its symptoms include a vascular wilt at the seedling stage, a leaf blight, and unfilled panicles in mature plants which result from the invasion of the vascular system by *Xoo* bacteria (Mew, 1987). At the molecular level, *Xoo* secretes transcription activator-like (TAL) effectors which invade and highjack the host cells by activating the transcription of genes that enhance plant susceptibility and support bacterial virulence (Boch and Bonas, 2010; Bogdanove et al., 2010; Römer et al., 2010).

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In plants, the ability to recognize both general elicitors and specific pathogens through gene-mediated resistance is essential to their defense mechanisms (Andersen et al., 2018). Hence, studying the variations in the expression of various genes provides a perspective of the mechanisms of plant responses to BB. To date, more than 30 BB resistance genes have been identified in *Oryza sativa* and its closely related species; among these, *Xa21* has probably been the most commonly used for rice variety improvement as it provides for high level and broad spectrum BB resistance (Song et al., 1995; Win et al., 2012). *Xa21* codes for a plasma membrane which recognizes the tyrosine-sulfated protein RaxX and triggers the Xa21-mediated immunity (Pruitt et al., 2015). It is known that this response involves Xa21-binding proteins (Wang et al., 2006; Chen et al., 2010; Park et al., 2017) as well as the direct interaction of a cleaved XA21 subunit with the WRKY62 transcription factor (Peng et al., 2008; Park et al., 2012; He et al., 2017). Nonetheless, the precise mechanisms of *Xa21* resistance have not yet been completely elucidated.

The current study centered on the recently discovered *Xoo-induced kinase 1* (*XIK1*, Loc_Os02g4790) which encodes a LRR-RLK protein and is hypothesized to act as a co-receptor of *Xa21* since it positively regulates Xa21-mediated BB resistance (Hu et al., 2015). Since *XIK1* was previously identified and conserved in the japonica rice cultivar Kitaake, the current study characterized the expression levels of this gene in the BB-susceptible cultivar RD47 and its improved BB resistant progenies BC_3F_3 (*Xa21*/*Xa21*) in various growth and developmental stages and during *Xoo* inoculation.

Materials and Methods

Plant samples and growing conditions

Rice (*Oryza sativa* L. ssp. indica) cultivars RD47 and IRBB21 were provided by the Bureau of Rice Research and Development, Phitsanulok, Thailand. The *Xa21* gene from IRBB21 was introgressed in RD47 through backcross breeding and marker-assisted selection (data not shown) until homozygous-*Xa21* BC_3F_3 lines were obtained. Leaf samples were collected from the 2-leaf, 4-leaf, 6-leaf and reproductive stages, respectively.

Xoo isolate and inoculation test

BB-infected leaves were collected from paddy fields in Phitsanulok province and *Xoo* was isolated on nutrient agar (peptone-bovine-agar). The isolated bacteria was identified as *Xoo* through polymerase chain reaction (PCR) assays using *Xoo* specific primers TXT (Sakthivel et al., 2001) and Xoo80 (Lu et al., 2014). Before infection, the *Xoo* isolate labeled as "xoo16PK002" was re-streaked and incubated at 28°C for 48 hr. A *Xoo* inoculum (the optical density at 600 nm (OD600) of 0.2) was prepared and used to inoculate 60-day-old plants using the clipping method of inoculation (Kauffman, 1973). Mock (water) inoculation was used as a control. Samples corresponding to 5 cm of the leaves directly below the inoculation sites were collected at 0 hr, 1 hr, 2 hr, 6 hr and 24 hr post inoculation (hpi); leaf samples were frozen in liquid nitrogen immediately.

RNA extraction and cDNA synthesis

Total RNA was extracted from each 100 mg leaf sample using an RNAprep Pure Kit (Tiangen Biotech Ltd.; China) following the manufacturer's instructions. Each RNA sample was treated with Rnase-Free Dnase I (RBC Bioscience; Taiwan) to remove possible gDNA contaminants. Total RNA samples were quantified using a Synergy H1 microplate reader (Biotek; USA) and their integrity was assessed using agarose gel electrophoresis. The qScript™ XLT cDNA synthesis kit (QuantaBio; USA) was used to reverse transcribe 1 µg of total RNA templates in order to synthesize first strand cDNAs according to the manufacturer's protocol.

Polymerase chain reaction and cloning of XIK1 partial cDNA sequence

PCR was performed using a BioRad T100™ Thermal Cycler for 35 cycles (95°C for 10 s, 60°C for 10 s, 72°C for 20 s). The primer XIK1Ri (Hu et al., 2015) was used to amplify the *XIK1* gene. The PCR products were cloned using RBC TA cloning vector (RBC Bioscience; Taiwan) following the instructions in the manual. Plasmids carrying the *XIK1* gene were extracted and subjected to sequence analysis.

Quantitative real-time polymerase chain reaction

For the quantitative real-time polymerase chain reaction (qPCR) analyses, fast SYBR Green Master Mix (Quantabio; USA) was used to prepare 20 µl qRT-PCR reactions containing 1 µl of the cDNA templates and 0.5 µM of each primer. The specific primers used to amplify the genes of interest (GOIs) evaluated in this study are shown in Table 1. Non-RT PCR was performed to confirm no gDNA contamination. The specificity of the PCR and qPCR products was carefully assessed using gel electrophoresis and melting curve analysis, respectively. Technical triplicates and no template controls (NTCs) were run on an Eco 48 Real Time PCR System (PCR Max; United Kingdom) for 35 cycles (95°C for 10 s, 60°C for 10 s, 72°C for 20 s) followed by melting curve analysis.

Results

Expression of XIK1 and Xa21 in different growth stages

The amplified *XIK1* product using the primers based on japonica rice Kitaake showed 100% nucleotide identicality to the annotated *XIK1* sequence (Fig. 1). The relative expression of *XIK1* in different growth stages (2-leaf, 2S; 4-leaf, 4S; 6-leaf, 6S; reproductive stage, RS) showed that *XIK1* gene expression levels progressively increased from the 2S to 6S stages and eventually decreased in the RS stage in both the BB-susceptible cultivar (RD47) and its improved BB-resistant progenies (BC_3F_3) as shown in Fig. 2A and 2B, respectively. The expression of *Xa21* in the improved BB-resistant (BC_3F_3) progenies showed a similar trend to the *XIK1* gene (Fig. 2C). As expected, no amplification of *Xa21* was detected in the BB-susceptible cultivar RD47 (data not shown).

Table 1 List of genes evaluated in this study

Gene name	Primer sequence (5'-3')	Amplicon length	Reference
Xoo-induced kinase 1 (XIK1)	GACCAGGCGAAATCAACTTT ATGTAAGGCAGTGAGTTTAGTCAA	187 bp	Hu et al., 2015
Xa21	CAGAGTATGGCGTTGGGCT CGGGTCTGAATGTACTGTCA	114 bp	Promma et al., 2016
Triosephosphate isomerase (TI)	CGACATCATCAACTCCGCCAC CCTCTTCAGACATCTTCCCACG	83 bp	Wang et al., 2016
Endothelial differentiation factor (Edf)	TCCGAACCAGCAGATCATCG GCATGGTATCAAAAGACCCAGC	158 bp	Wang et al., 2016
Ubiquitin-5	CCAGTACCTCAGCCATGGA GGACACAATGATTAGGGATC	69 bp	Hu et al., 2015

Gene names and all their details are presented using the style in the reference cited.

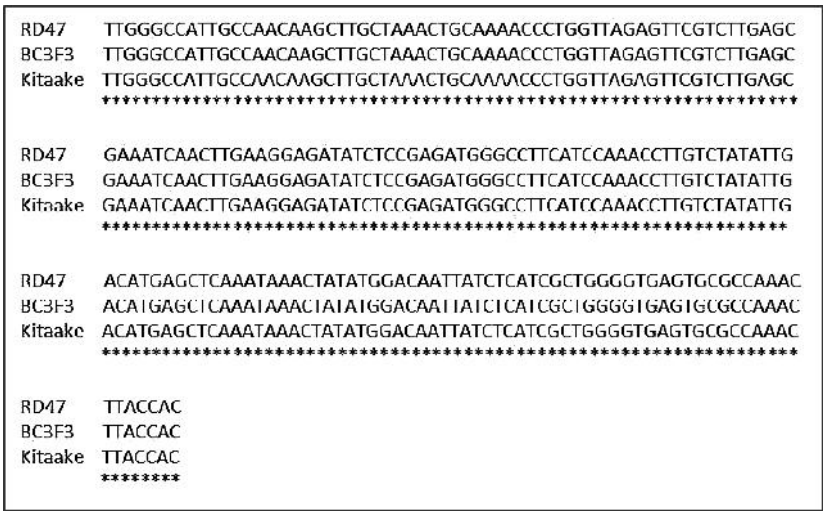

Fig. 1 Multiple sequence alignment of *XIK1* sequences in RD47 and improved BB-resistant BC_3F_3 progenies compared with the japonica cultivar Kitaake (CLUSTAL O 1.2.4).

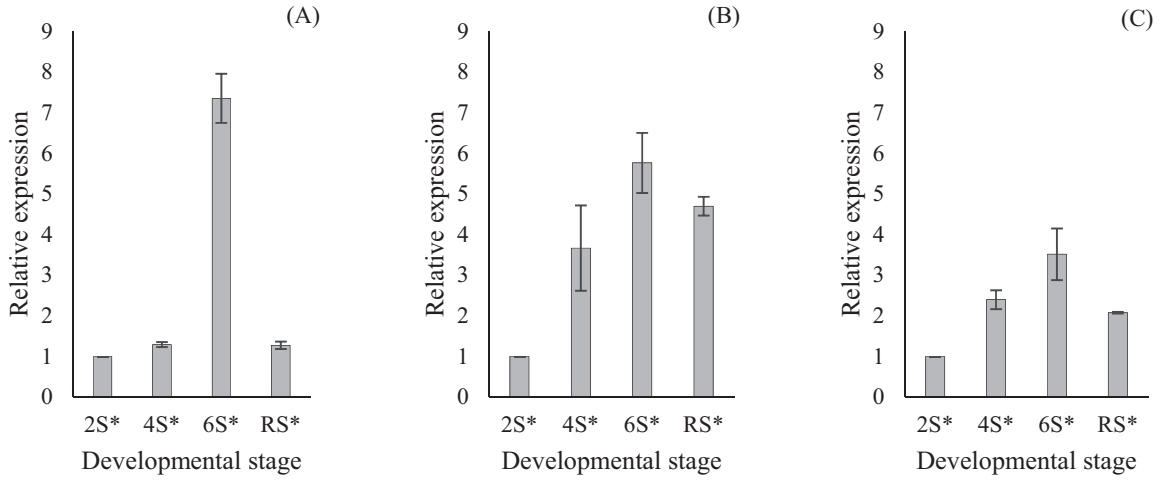

Fig. 2 Relative expression of *XIK1* in: (A) RD47; (B) BC_3F_3 progenies; (C) relative expression of *Xa21* in BC_3F_3 progenies in various growth stages, where 2S, 4S, 6S and RS represent the 2-leaf, 4-leaf, 6-leaf, and reproductive stages, respectively and asterisks indicate significant differences among datasets based on analysis of variance at $p < 0.05$ and error bars indicate SD of the analyzed data that have been normalized relative to *Ubiquitin 5*.

Response of XIK1 and Xa21 to Xoo infection

The 60-day-old RD47 and BC_3F_3 plants corresponding to the 6S stage were subjected to *Xoo* infection and samples were collected at different times post inoculation for qPCR analyses. The results showed that the expression of *XIK1* was induced by *Xoo* inoculation for both the RD47 and BC_3F_3 lines but in different times post inoculation. The expression of *XIK1* in the RD47 cultivar was suppressed in the first hour after *Xoo* inoculation and was later induced after 2 hpi. Notably, the expression level substantially dropped after the time of induction and there was no clear indication of up-regulation in the succeeding time post inoculation (Fig. 3A). On the other hand, the expression of *XIK1* in the BC_3F_3 lines was quickly induced after 1 hpi but the expression level suddenly decreased after 2 hpi. Interestingly, the expression of the gene was up-regulated again after 6 hpi until 24 hpi, thus indicating that the expression of *XIK1* tended to increase again (Fig. 3B). The expression of *Xa21* in BC_3F_3 was also significantly induced after 1 hpi and eventually decreased in the succeeding time post inoculation (Fig. 3C). No signal of *Xa21* up-regulation was observed, even after 24 hpi.

Discussion

The genetics of resistance to bacterial blight has been studied in depth and was first carried out using the resistance (R) gene *Xa21*. As there is diversity in the *Xoo* strains of different rice-producing countries, the mechanism of *Xa21*-mediated resistance has not yet been completely elucidated. Since *Xa21* functions as a pattern recognition receptor and a tyrosine-sulfated protein *RaxX* is required for the activation of Xa21-mediated immunity, the activity of *Xa21* in the plant's plasma membrane may accelerate subsequent responses to the conserved bacterial molecule of *Xoo* (Gómez-Gómez and Boller, 1999). Previous reports have reported that several Xa21-binding proteins are directly involved and play important roles in the early events of the *Xa21* signaling pathways (Wang et al., 2006; Lee et al., 2009; Chen et al., 2010; Park et al., 2010; Park et al., 2012). However, no changes of expression after *Xoo* inoculation were found in the genes encoding these proteins. Hu et al. (2015) found a BB-resistant gene (*XIK1*) that shared a structural motif with *Xa21* and positively regulated Xa21-mediated immunity. However, that study did not indicate whether the expression of the *XIK1*gene was stable in the time post inoculation after *Xoo* infection and whether the gene expression in the various leaf stages was the same for both BB-resistant and BB-susceptible cultivars. The current results showed that the expression levels of *XIK1* were induced earlier post inoculation by *Xoo* in both the BB-susceptible cultivar (RD47) and the BB-resistant BC_3F_3 progenies. However, the induction on the expression was earlier in BC_3F_3 than in RD47. Though the expression of *XIK1* significantly decreased after the induction, its expression tended to accumulate in the succeeding time post inoculation in the resistant BC_3F_3 but not in the susceptible RD47. These results suggested that *XIK1* is activated earlier and is progressively expressed in the succeeding hours after *Xoo* infection in BB-resistant BC_3F_3 but not in the BB-susceptible cultivar RD47. In addition, *XIK1* was either induced by *Xoo* or wounding (data not shown). Considering the reports that *Xa21* expression is development-dependent and its expression is either induced by *Xoo* or wounding (Mazzola et al., 1994; Century et al., 1999), the same findings for *XIK1* expression for both BB-susceptible and BB-resistant cultivars were also presented in this study.

The gradual increase in the expression of *XIK1* during the plant's growth and development shares similarity with the expression of *Xa21* and the responses of both genes to *Xoo* inoculation in the BB-resistant BC_3F_3 progenies carrying the *Xa21* gene are in commonality after 1 hpi and in the succeeding time post inoculation, Thus, *XIK*1 might act as a co-receptor of *Xa21* in regulating the early events of XA21-mediated signaling and thereby conforming

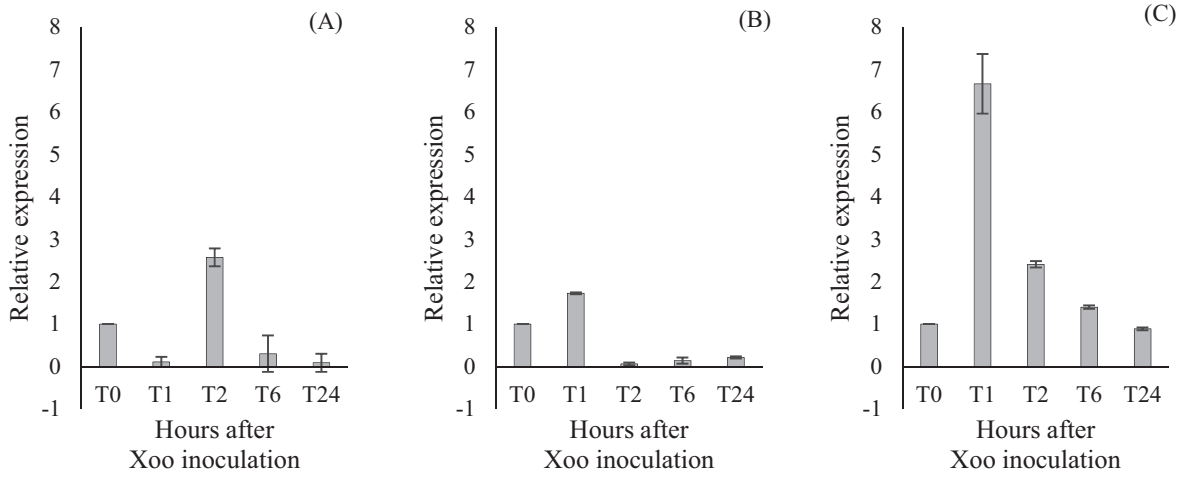

Fig. 3 Relative expression of *XIK1* in: (A) RD47; (B) BC_3F_3 lines; (C) relative expression of *Xa21* in BC_3F_3 lines after *Xoo* inoculation, where 0 hr, 1 hr, 2 hr, 6 hr and 24 hr post inoculation are presented as T0, T1, T2, T6, and T24, respectively and error bars indicate SD of the data analyzed that have been normalized with the reference genes *Edf* and *TI*.

to the findings of Hu et al. (2015). Data on the disease development by *Xoo* in both tested cultivars also confirmed that BC_3F_3 progenies expressing the genes *Xa21* and *XIK1* established resistance against the *Xoo* pathogen (Fig. 4). In this case, the current study supports the mechanism of defense for *Xa21*-mediated rice plants in both indica and japonica subspecies as characterized by *XIK1* and *Xa21* genes expression as being alike.

Fig. 4 Disease development of *Xoo* on indica rice cultivars RD47 and BC_3F_3 progenies 21 d post inoculation, where RD47 shows complete susceptibility while BC_3F_3 shows moderate resistance

The results revealed that the expression of *XIK1* was development-dependent and was induced by *Xoo* in the tested indica rice cultivar RD47 and its improved BB-resistant progenies BC_3F_3 (*Xa21*/*Xa21*). Furthermore, it was shown that the *XIK1* gene was induced earlier in the BB-resistant BC_3F_3 progenies than in the susceptible cultivar RD47 after *Xoo* inoculation, thus indicating that *XIK1* was activated earlier in resistant plants than in susceptible ones. Moreover, similar expression patterns of *Xa21* and *XIK1* were identified in the various growth stages and after *Xoo* inoculation.

Conflict of Interest

The authors declare that there were no conflicts of interest and all ideas reflected herein have the agreement of all authors.

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The potency of *Polylathia longifolia* from Indonesia and the Philippines as therapeutic agents on inflammatory bowel disease (IBD) in Rats *(Rattus norvegicus)* induced by Indomethacin

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Abstract. Herbal medicines have the potential to be used as therapeutic agents. *Polyalthia longifolia* is widely grown in both Indonesia and the Philippines, but it has not commonly used for its therapeutic purposes. Some studies reported that *P. longifolia* leaves extract has anti-inflammatory activity. In this study, the potential of *Polyalthia longifolia* leaves extracts for inflammatory bowel disease (IBD) in Indometachin-induced rats was tested. Four groups of rats were used for this research, were control (T1), rats with IBD with 10 mg/kg BW of sulfasalazine therapy (T2),) rats with IBD treated with 300 mg/kg BW of Indonesia *P. longifolia* leaves extract therapy (T3) and rats IBD treated with 300mg/kg BW of The Philippines *P. longifolia* leaves extract therapy (T4). Histopathology of gastric, duodenum, jejunum, and colon were analyzed, with protein profile and pro-inflammatory cytokines expressions. The results showed that *P. longifolia* leaves extract therapy origin from Indonesia and the Philippines were potent as anti-inflammatory agents comparable to commercially available drugs against IBD. This works proposed the use of *P. longifolia* leaves as IBD therapy.

1. Introduction

Herbal medicines are a prominent part of healthcare whole world [1]. The use of herbal medicine has enhanced rapidly over the past few decades. Therapy with herbal medicine is the primary healthcare for 80% population in the world [2], especially in developing countries, as well as the increasing use of herbal medicine in developed countries [3]. Therapy with herbal medicine has fewer side effects when compared with synthetic drugs. Natural herbs contain phytochemicals that can use to treat various diseases such as inflammatory bowel disease (IBD). One of the plants that apply for this treatment is *Polyalthia longifolia*. This plant belongs to *the Annonacea* family which commonly found in tropical countries [4] such as Indonesia and the Philippines. This plant is widely used as an ornamental plant, reducing sound pollution and reducing fever and tonic. This plant was used to treat fever, diabetes,

hypertension. Some studies indicated that this plant has various classes of compounds terpenoids, alkaloids, and flavonoids. The content of alkaloids and flavonoids has anti-inflammatory activities, including inhibiting infiltration, reducing inflammatory mediators, and reducing oxidative stress [5,6]. Based on the therapeutic effect of the active compounds *P. longifolia* on the inflammatory response, it has potential in inflammatory regulation of intestinal inflammation. Inflammatory bowel disease is a gastrointestinal inflammation that has developed into a global condition with increasing incidents in developed countries and industries in Asia. Long-term treatment with synthetic drugs such as sulfasalazine will cause resistance, aggravate bleeding, male infertility, pulmonary toxicity, and nephrotoxicity [7]. Therefore, it was necessary to expand the knowledge of the use of herbal medicines such as *P. longifolia* leaves as IBD medicine. This study evaluates *P. longifolia* leaf extract used as an IBD drug induced by indomethacin and compared with sulfasalazine as gold standard.

2. Material and Methods

2.1. Plant Material

Leaves of *Polyalthia longifolia* were obtained from Indonesia and the Philippines. Indonesia leaves of *P. longifoia* were obtained from Malang City, while samples from the Philippines from Camiling, Tarlac and were brought to Biosains Institute, Brawijaya University. The taxonomy for plants was identified and authenticated in plant taxonomy laboratory, Brawijaya University.

2.2. Animals

Male Wistar rats aged two months and weighed around 150-200g each. Rats were maintained under standard laboratory conditions of temperature, humidity and 12 h light and dark. Animals have free access to feed and water *(ad libitum)*. Before treatments, rats were acclimatized for a week. The experimental protocol was approved by the Ethic Commission of Brawijaya University (No. 1035-KEP-UB).

2.3. Experimental Design

The animals were in four groups: (T1) negative control, (T2) IBD with Sulfasalazine, (T3) IBD that is treated using *P. longifoia* Indonesian leaves extract, and (T4) treated *P. longifoia* Philippines leaves extract. Inflammatory bowel disease in rats were induced with indomethacin, with a dose of 15mg/kg orally (p.o). Treatment 2 was given 10 mg/kg BW of sulfasalazine as gold standard of IBD. T3 and T4 were given 300mg/kg BW of ethanolic extract of *P. longifoia* leaves from Indonesia and The Philippines, respectively. The therapies were given for 7 days orally. After therapy, rats were sacrificed and gastrointestinal contents from the gastric, duodenum, jejunum, and colon were collected, and organs were preserving in 10% neutral-buffer formalin and others in phosphate buffer saline for further use.

2.4. Histopathological Observations

The gastric, duodenum, jejunum and colon were collected and fixed in 10% neutral-buffered formalin, embedded in paraffin wax and were cut into sections of 3-5µmm thickness. The sections were stained using haematoxylin and eosin for histopathological observation. The effect on the different treatments on inflammation were observed especially the infiltration of the inflammatory cells, desquamation, erosion, and damage of tissue structures microscopically.

2.5. Immunohistochemistry

The paraffin section of the organs also was deparaffinized with xylol and multi-concentrations of alcohol for 15 min. Furthermore, slides were left overnight at 4°C and were washed with distilled water and PBS solution. Slides were incubated with 3% hydrogen peroxide for 40 min then washed for 5 min with PBS solution. Blocking stages were conducted with 1%BSA in PBS for overnight then washed again with PBS. Primary antibody for COX-2 and SMAD3 (Santa Cruz Biotechnology with ratio 1:50000) were added, left overnight and washed with PBS. After this, the secondary antibodies (anti-rabbit biotin conjugated) were added for 1h and washed again with PBS. Drops of *Strepta Avidin-Horseradish Peroxide* (SA-HRP) were made and left for 40 min and washed again with PBS. Drops of DAB was done and left for 10 min and washed with distilled water. Mayer hematoxylin was dropped on the slides, washed with distilled water, dried and then mounted. Slides were observed microscopically.

2.6. Protein Profile Using SDS-PAGE

0.1g of duodenum and colon were crushed and PMSF + PBS-T were added to the solution. The solution was homogenated and sonicated for 10 min and was centrifuged at 6000 rpm at 4 ° C for 15 min. The supernatant was collected, and cold ethanol was added to the solution (1:1), stored at 4 ° C for 12 h. Samples were centrifuged at a 6000 rpm at 4 ° C for 15 min. The pellets were dried until ethanol was lost. Subsequently, Tris-HCl pH 6.8 (1: 1) was added, and stored at -20 ° C.

3. Result and Discussion

3.1. Histopathology

Histopathological analysis of the small intestine was conducted by the Haematoxylin-Eosin (HE) method on all treated groups. Based on previous research, induction of indomethacin caused necrosis in the small intestine, so that the goblet cell and mucus layer were lost, and villi damage (desquamations and rupture) were observed, which was also confirmed in this study. Observation showed the desquamation of epithelia, erosion, congestion, and infiltration of inflammatory cells and villi damages were seen.

3.1.1. Gastric histopathology. The therapy of sulfasalazine as a gold standard has maintained IBD remission [10]. It was observed that the leaf whether from Indonesia and the Philippines have potency as anti-inflammatory [11]. It also reduced the inflammatory symptoms by inhibiting the product in PGE2. As earlier studied [12,13], leaves extract from P. longifolia also had antioxidant activity. One of the compounds was liriodenine [14]. Liriodenine has anti-inflammatory and antioxidant activities that can reduce inflammation and repair tissue damage, as seen in the histopathological picture.

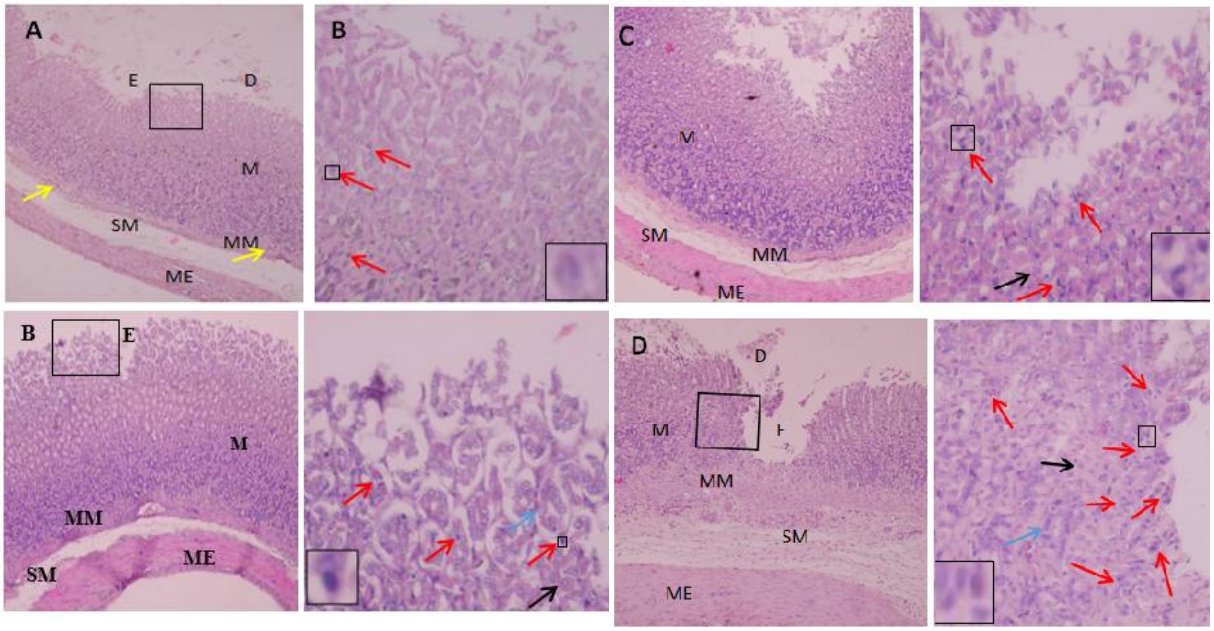

Figure 1. Histopathology of gastric mucosa. (A). Gold Standard group; (B). *P. longfolia* extract from Indonesia therapy; (C). *P. longifolia* extract from The Philippines, and (D) IBD condition.

Legend:

M : Mucosa
MM : Muscularis mucosa
SM : Submucosa
ME : Muscularis external
D : Desquamation of the epithelia
E : Erosion

: Congestion
: Infiltration
: Parietal cells
: Chief cells

3.1.2. Duodenum histopathology. Indomethacin induction in rat caused intestine tissue necrosis so the duodenal villi were eroded, and goblet cells disappeared caused by the villi damages. During inflammatory conditions, indomethacin caused villi desquamation resulted in the loss of goblet cells [15]. Based on Figure 2, the histopathology of the duodenum, group treated with sulfasalazine showed an improvement in the intestinal epithelial structure after treatment, as well as no erosion in the epithelial layer, without the infiltration of inflammatory cells and measured villous forms. The use of leaves extracts from Indonesia group (T3) showed that villous improvement, evidenced by the visible arrangement of columnar epithelial cells arranged by the regular villi and the appearance of goblet cells as mucus secretor for the protection of the villi layer in the duodenum. Histopathological features of the duodenum of rats treated with the Philippine extract showed the repairment of duodenal villi, which was followed by the appearance of goblet cells and the epithelial constituents of the villi.

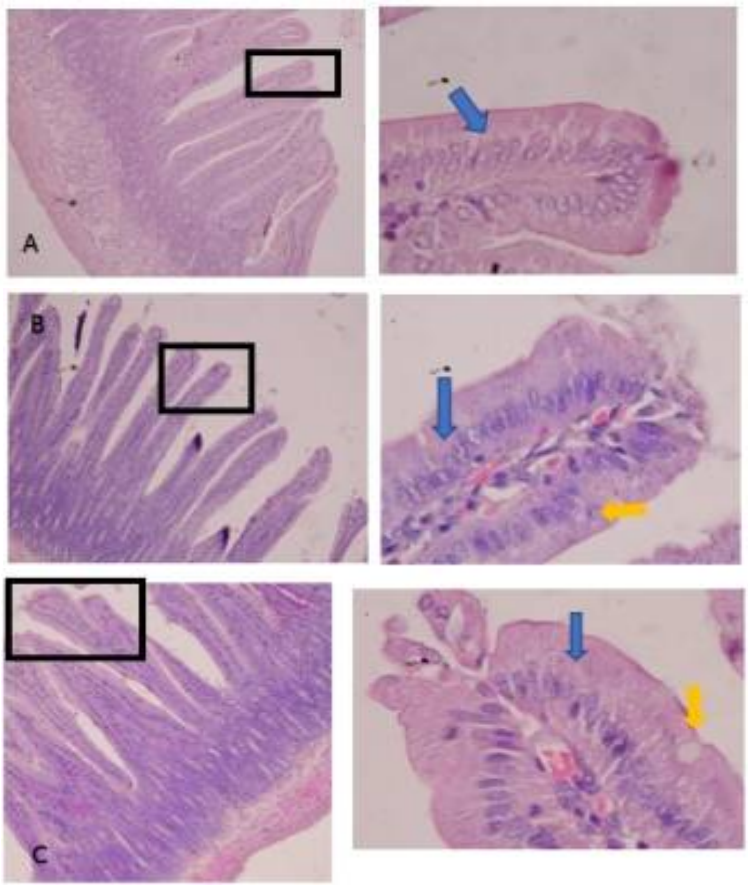

Figure 2. Histopathology of duodenum. (A). Gold Standard group; (B). P. longifolia extract from Indonesia therapy; (C). P. longifolia extract from Philippines.

Legend:

: Columnar epithelial tissue : Goblet cell

3.1.3. Jejunum histopathology. Thick walls and villi characterized the jejunum histology because they contained many blood vessels. Like duodenum, jejunum suffered damage when exposed to toxic substances such as indomethacin. Figure 3 showed the histopathological features of jejunum in the IBD group treated with sulfasalazine (Figure 3A), showed the improvements in villous shape, neat columnar epithelial cell structure and the appearance of goblet cells. However, inflammation cells were still found

coming out of the lymphatic channels and spaces in the Crypt of Lieberkühn, which indicated improvement was not yet completed. The histopathology of jejunum with IBD treated with the leaves extract from Indonesia and the Philippines showed improvement as evidenced by the reduction in inflammatory cell infiltration, neat layer of the columnar epithelial cell structure, goblet cells and no desquamation compared to the control group (Figure 3D).

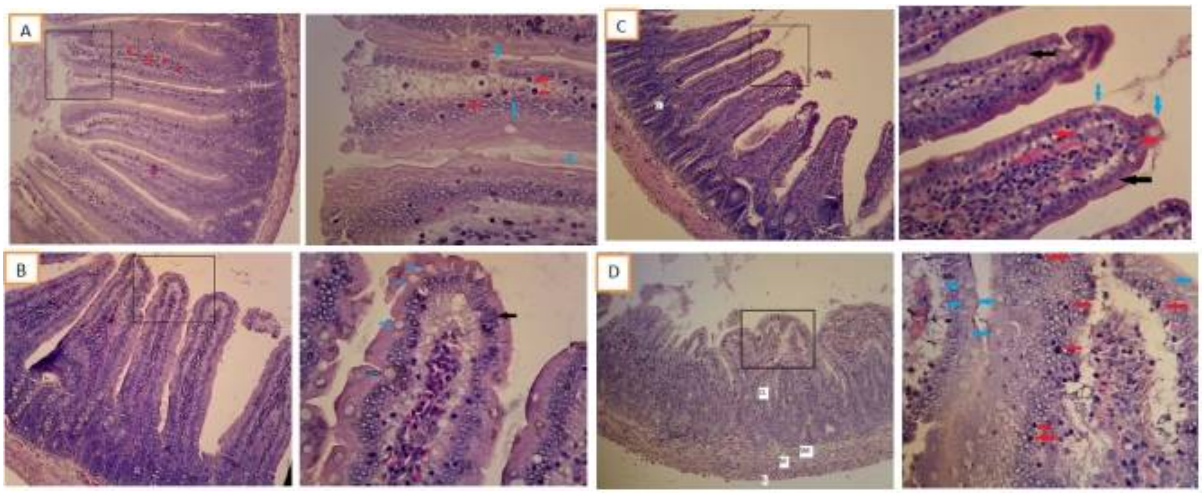

Figure 3. Histopathology of jejunum; (A). Gold Standard group; (B). *P. longifolia* extract from Indonesia therapy; (C). *P. longifolia* extract from Philippines, and (D) IBD condition.

Legend:

S : Serosa
M : Mucosa
SM : Submucosa
CL : Crypt of Lieberkuhn

: Cell infiltration
: Columnar epithelium tissues
: Goblet cells

3.1.4. Colon histopathology. Histopathology analysis showed that the sulfasalazine therapy group has undergone tissue repair in the colonic mucosa marked by the appearance of goblet cells in the mucosal lining of the colonic epithelium. Goblet cells in the colon function as a barrier in the colonic mucosa by removing mucin compounds. As for the colonic histopathology of the leaves extract therapies group from both countries, indicated tissue repairs. However, inflammation cell infiltration was still found, and the surface structure of the epithelium were not yet intact.

The goblet cells in the process of repairing the digestive tract were observed due to sulfasalazine, which has the ability to suppress the production of free radicals thus accelerating the time of inflammation and increasing the work of TGF-β as an anti-inflammatory cytokine for regenerating intestinal stem cells and differentiating into several types of cells namely enterocytes, goblet cells, and cells Paneth [16]. In addition, the content of secondary metabolites in the ethanol extract of *P. longifolia* leaves has quercetin and rutin, which can inhibit the formation of free radicals, reduce pro-inflammatory mediators, improving the protective function of the epithelium in the intestine organ. Flavonoids were known to increase colonic permeability [17].

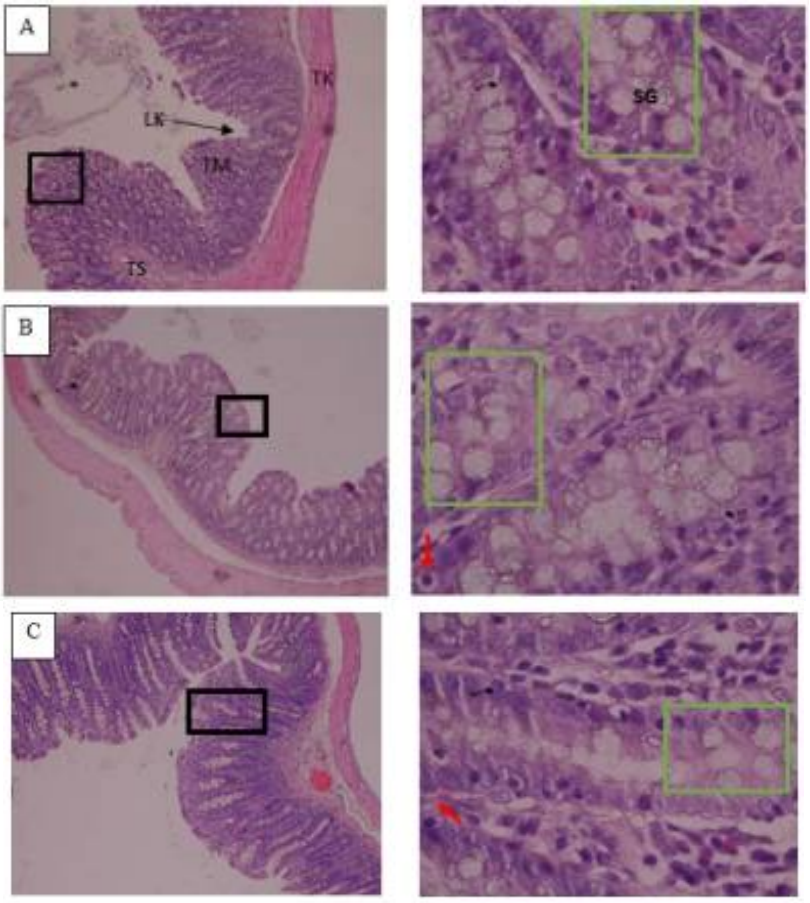

Figure 4. Histopathology of colon. A. Gold Standard group; B. EEPL from Indonesia group; C. EEPL from The Philippines group.

Legend:

= repairing = inflammation cells infiltration

3.2. Immunohistochemistry of COX-2 and Smad3

Inflammatory bowel disease (IBD) is a chronic inflammatory condition that occurs in the gastrointestinal tract. Characterization of the inflammatory process is with the presence of COX-2 expression which increases rapidly and decreases anti-inflammatory cytokines such as Smad3. COX-2 and Smad3 expression were observed by immunohistochemistry methods and analyzed with one-way ANOVA statistics. The results showed that the treatment affected COX-2 and Smad3 expression ($p < 0.05$).

Table 1. Expression of COX-2 on gastric and Smad3 on duodenum in rats' model IBD.

Groups	COX-2	Smad3
T1 (control)	0.6533 ± 0.05465^{a}	14.8000 ± 0.33466^{a}
T2 (Sulfasalazine	1.2433 ± 0.11130^{b}	12.6000 ± 0.56569^{b}
Indonesia	1.3267 ± 0.05164^{b}	12.2000 ± 0.43818^{b}
Philippines	1.5467 ± 0.08262^{c}	8.0333±0.52789c

Note: The notations a, b and c show a significant difference between treatment groups (significance <0.05). While the same notation shows no significant difference between treatment groups.

Table 1 shows the number of COX-2 and Smad3 expressions when Sulfasalazine, leaves extracts from Indonesia and The Philippines as compared to the adverse treatment. Results showed improvement

of IBD by decreasing COX-2 expression and decreasing Smad3 compared with the untreated group. The leaves extract from the Philippines showed significant differences ($P < 0.05$) in treating IBD. The leaves extract from Indonesia has a comparable result as with the gold standard treatment prescribed with sulfasalazine and showed significant differences between the treated and untreated groups. The leaves extract from the Philippines was found better because of increased COX-2 expression and decreased Smad3; this result concurred with the studies of Peng *et al.* [5,17].

Therapy of *P. longifolia* leaves extract from the Philippines and Indonesia has the potential of accelerating healing and cell regeneration in IBD rats. Flavonoid and alkaloid compounds in the leaves of *P. longifolia* can act as antioxidants and anti-inflammatory agents. They play a role in exhibiting COX-2 so that there were limited number of inflammatory cells that migrate to the wound tissue. Furthermore, the inflammatory reaction was shortened and the proliferative ability of Smad3 was not inhibited [16]. Flavonoid and alkaloid compounds stabilize the reactive oxygen species (ROS) by reacting with the compounds from free radicals so that these compounds become inactive [18]. This was evidenced by the histopathological picture of the duodenum which looked normal and has improved.

3.3. Protein profile of duodenum and colon

Table 2. The result of protein profile of duodenum analysis based on SDS-PAGE.

Group	Molecule weight (kDa)							
	289.7	**201.4**	**140.7**	**87.3**	**59.5**	**45.6**	**35.8**	**21.2**
Gold standard sulfasalazine (P1)	√	√	√	-	√	√	-	√
Polyathia longifolia from Indonesia (P2)	√	√	√	-	-	√	√	√
Polyathia longifolia from the Philippines (P3)	√	-	-	-	√	√	√	√

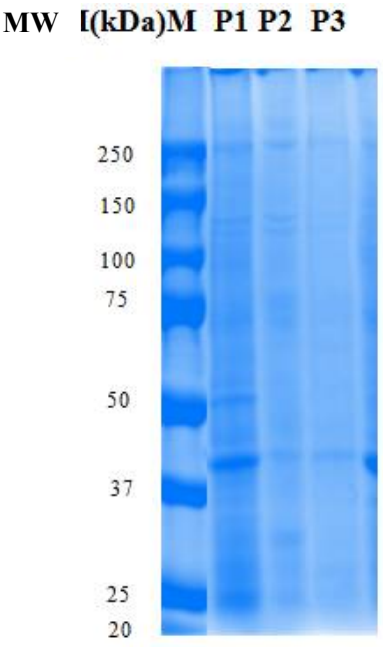

Figure 5. Protein bands of duodenum (SDS PAGE 12%).
Legend: M = marker; P1 = Sulfasalazine group; P2 = EEPL from Indonesia group; P3 = EEPL from Philippines

The 40 kDa protein appeared in sulfasalazine therapy and leaves extract from the Philippines. This protein was thought as Vasoactive Intestinal Polypeptide (VIP) which was secreted by cells in the intestinal mucosa. VIP was produced by inflammatory tissue; VIP increased the permeability of

duodenal tissue stimulated the secretion of fluid and electrolytes from duodenal tissue which triggered watery diarrhea and dehydration [19].

Table 3. The result of protein profile of colon analysis based on SDS-PAGE.

Group	Molecular weight of Protein (kDa)						
	288.2	198.3	167.4	114.6	43.5	37.1	22.0
Negative control (K-)	√	√	√	√	√	√	√
Gold standard sulfasalazine (K1)	√	√	√	-	√	√	√
Polyathia longifolia from Indonesia (K2)	√	√	√	-	√	√	√
Polyathia longifolia from the Philippines (K3)	√	√	√	-	√	√	√

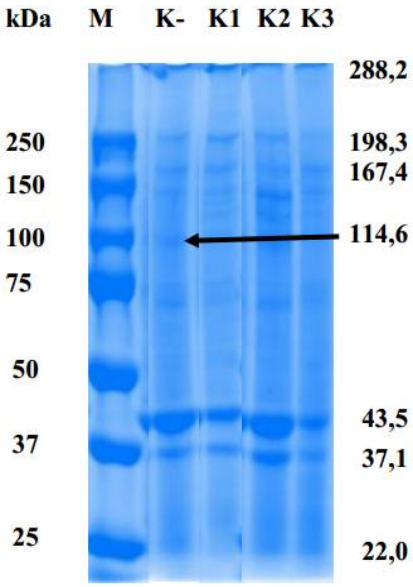

Figure 6. Protein bands of colon (SDS PAGE 12%).
Legend: M = marker; K- = negative control; K1 = Sulfasalazine group; K2 = EEPL from Indonesia group; K3 = EEPL from Philippines

Protein with molecular weights 114.6 kDa were not synthesized in all treatment groups, but it was synthesized in healthy conditioned rats. A protein with molecular weight of 114 kDa is a protein that binds to glycocalyx in blood vessels, namely the β-galactosidase [20]. This β-galactosidase was enzyme that very important to convert carbohydrates into disorders in IBD conditions. This enzyme was located at the peak of the villi to hydrolyze lactose to glucose and galactose [21]

4. Conclusion

Polyalthia longifolia has been proven to improve gastrointestinal tissue in rats with infectious bowel disease-induced indomethacin. It was proven by the histopathological profiles, showed decreasing pro-inflammatory signs, enhanced of anti-inflammatory expression, and improved protein profiles.

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Identification and molecular docking analysis alkaloids *Polyalthia longifolia* leaves from Indonesia and the Philippines as anti-inflammatory

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Abstract. *Polyalthia longifolia* (family: Annonaceae) is widely planted to effectively reduces noise pollution. This plant spread in many countries including Indonesia and the Philippines. Alkaloids are the main active compounds other than terpenoids in *P.longifolia* and it has the potential to be anti-inflammatory. Each leaves considered have different active compounds because of the different geographic factor from each country. To confirm this, we investigated the differences alkaloid compounds from two different sources of plants and predicted their anti-inflammatory potential. Shade-dried leaves from Indonesia and the Philippines were extracted by ethanol 70%. Two extracts were analyzed with LC-MS to ensure alkaloid compounds. Ensured alkaloid compounds further take on molecular docking. The compounds were drawn with ChemDraw then convert to .pdb with Open Babel. The protein COX-2 obtained from .pdb then prepared with PyMol. The docking process held by PyRx and the interaction was visualized by LigPlot+. LC-MS analysis identified 5 alkaloids contained from the ethanol extract of *P.longifolia* leaves from Indonesia and the Philippines. O-methylbulbocapnine-N-oxide was found only in ethanol extract leaves from Indonesia, while N- methylnandingerine-β-N-oxide was only found in ethanol extract leaves from the Philippines. All compounds have the potential as an anti-inflammation. Liriodenine as the most potent compound with binding energy -10.9kcal/mol. O-methylbulbocapnine-N-oxide has lower binding energy than N- methylnandingerine-β-N-oxide. In conclusion, there are differences between the alkaloid compounds and anti-inflammatory potential of the ethanol extract of *P.longifolia* leaves from Indonesia and the Philippines. Moreover, Indonesia's ethanol extract leaves showed more potential than Philippines's.

1. Introduction

Polyalthia longifolia is a plant commonly used to reduce noise pollution [1]. *P.longifloia* can be found in tropical and sub-tropical countries [2] such as Indonesia and the Philippines. This plant has been used as a traditional medicine to treat fever, skin disease, hypertension, diabetes. P.longifolia has pharmacological and biological activities such as anti-oxidant, anti-bacterial, anti-fungal, anti-cancer and anti-inflammatory [3].

Previous studies have reported that this plant contains flavonoids, alkaloids, sesquiterpenes, diterpenes, saponins, quercetin, bulbocapnine [2]. Geographical differences will cause differences in

the content of chemical compounds in plants [4]. *P.longifolia* has the main secondary metabolites of alkaloids and diterpenoids. Various derivatives of the identified alkaloid compounds can be used as an anti-inflammatory [5]. For example, alkaloids are anti-inflammatory for IBD [6].

The anti-inflammatory mechanism can inhibit the enzyme cyclooxygenase (COX) [7]. The cyclooxygenase enzyme has two isoforms, namely COX-1 and COX-2. COX-2 is inducible and responsible for inflammatory [8]. Selective inhibition of COX-2 will reduce inflammation with a low risk of gastrointestinal [9]

In silico methods such as molecular docking are used to predict the ability of active compounds (ligands) to cause a biological effect computationally [7]. This study aims to determine the differences in the active compounds of *P.longifolia* plants from the regions of Indonesia and the Philippines. In addition, molecular docking is used to determine the ability of active compounds as anti-inflammatory compounds.

2. Materials and Methods

2.1. Plant Material

P.longifolia leaves ware collected from two different countries. *P.longifolia* leaves from Indonesia ware collected from Malang, East Java. While *P.longifolia* leaves from the Philippines were collected from Camiling, Tarlac. The Plants identified in plant taxonomy laboratories, Brawijaya University.

2.2. Hardware and Software

The computer used had the specifications of the Inter® Core ™ i3-4005U CPU @ 1.70GHz 1.70 GHz, Random Access Memory (RAM) 6.00 gigabytes. The software used is ChemDraw Ultra 12, PyMol, PyRx 0.8 and LigPlot + v.2.

2.3. Extraction

P.longifolia leaves from Indonesia and the Philippines were shade-dried and powdered. The powder was macerated with 70% ethanol for 72h. The filtrate was evaporated to get the extract. The extracts obtained were stored at 4˚C until further analysis.

2.4. Alkaloid Analysis with LC-MS

LC-MS (TSQ Quantum Access MAX Triple, Thermo-Scientific) with C18 column (1.7 µm 100Å 50x2.1 mm, Kinetex) was adjusted according to the desired conditions (7.50 min separation process and the flow rate of 300 µl/min). The mobile phase was in the form of two solvents (eluent A = water and eluent B = acetonitrile). The gradient elution was carried out as follows, 0-0.60 minutes 90%: 10% (A: B), 5.00-5.50 minutes 25%: 75% (A: B) and 6.00-7.50 minutes 90%: 10% (A: B).

2.5. Preparation of Ligand Structures

The ligands used for molecular docking were alkaloid compounds present in the ethanol extract of leaves of *P. longifolia* from LC-MS. The structure of the ligands was drawn using ChemDraw Ultra 12 and Open Babel is used to convert to .pdb. Alkaloid compounds included in the study were Polylogine, noroliveroline, liriodenine, oliveroline-β-N-oxide, N-methylnandingerine-β-N-Oxide and O-methylbulbocapnine-N-oxide (Results from LC-MS).

2.6. Protein Preparation

Preparation of COX-2 protein begins by selecting an active form protein that binds native ligand (GDP code: 3LN1). Water molecules, ligands and B, C and D chains were removed from proteins using PyMol.

2.7. Molecular docking

Molecular docking proteins with ligands was carried out using AutoDock Vina in PyRx. Docking was carried out on a grid with a center of 31,2552; -23,4834; -16,1872 and Dimensions 17,4839; 17,3785;

21,3620 (Angstrom). Docking results in the form of bond strength interactions between ligands and receptors. Analysis of interactions with amino acids residues was used by the LigPlot + v.2 program.

3. Result and Discussion

3.1. Alkaloids by LC-MS

Alkaloid compounds were identified using LC-MS with 7 targets molecular weight of compounds which included polylongine, polyfothine, liriodenine, noroliveroline, oliveroline β-N-oxide N-methylnandingerine-β-N-oxide and O-methylbulbocapnine-N-oxide. Identification of compounds from the target molecular weight using m/z [M+H]+. Five alkaloid compounds identified on *P.longifolia* leaves from Indonesia and the Philippines. The alkaloid compounds detected are shown in table 1. The structure of the alkaloid compounds is shown in figure 1. There are differences in the compounds found, namely N-methylnandingerine-β-N-oxide in Philippine extracts and O-methylbulbocapnine-N-oxide in Indonesian extracts. Differences in compounds were due to different geographies between Indonesia and the Philippines. This condition would affect environmental factors such as temperature, altitude and sun duration [4].

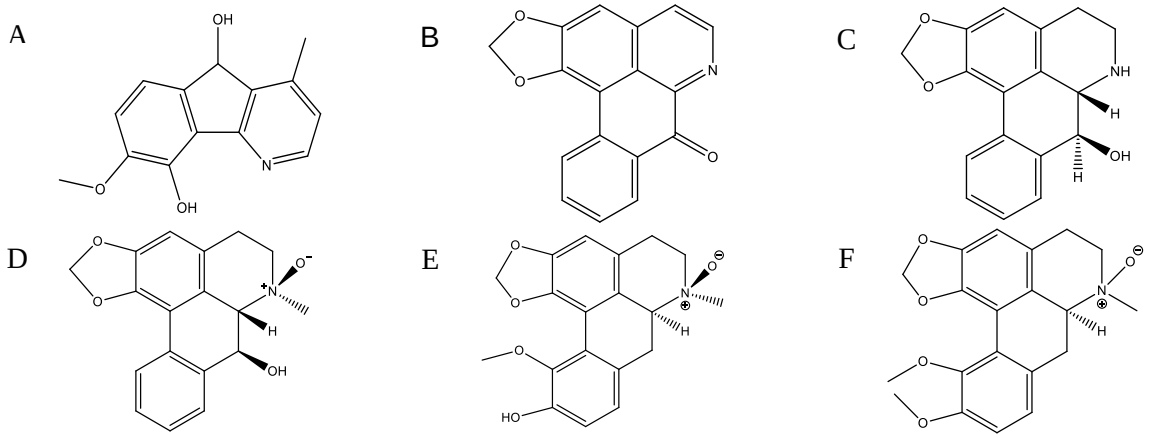

Figure 1. Structure of alkaloid compounds. A) Polylongine, B) Liriodenine, C) Noroliveroline, D) Oliveroline β-N-oxide, E) N-methylnandingerine-β-N-oxide, F) O-methylbulbocapnine-N-oxide.

Table 1. Alkaloid compounds identified in the ethanol extract of *P.longifolia* leaves from Indonesia and the Philippines.

Compounds	MW (g/mol)	From	RT(min)
Polylongine	243.261	Indonesia	0.68
		Filipina	0.71
Liriodenine	275.263	Indonesia	3.09
		Filipina	3.09
Noroliveroline	281.31	Indonesia	2.75
		Filipina	2.75
Oliveroline-β-N-Oxide	311.337	Indonesia	2.99
		Filipina	2.99
N-methylnandingerine β-N-oxide	341.363	Indonesia	-
		Filipina	4.87
O-methylbulbocapnine-N-oxide	355.39	Indonesia	4.92
		Filipina	-

The polarity of alkaloid compounds was indicated by the retention time (RT) resulted. Table 1 showed the polarity of the alkaloid compounds found in *P.longifolia* leaves. Polylongine had the

highest polarity then noroliveroline, oliveroline-β-N-oxide, liriodenine, N-methylnandingerine-β-N-oxide and O-methylbulbocapnine-N-oxide. The greater retention time indicates that the compound was retained by non-polar column. While compounds with the smallest retention time such as polylongine were not held up in column because they were polar in contrast to the column. Liriodenine in previous studies had retention time (RT) 3.51 [10]. Retention time differences were possible due to differences in flow speed and type of column used. Liriodenine was a compound that had been shown antiradical activity [11] and antioxidants [12].

3.2. Docking Results

Alkaloid compounds from *P.longifolia* leaves are thought to have anti-inflammatory effects. The cyclooxygenase-2 enzyme is an inducible enzyme caused by inflammation[8]. Inhibition of COX-2 is clinically effective as an anti-inflammatory agent that does not adversely affect the gastrointestinal tract. Molecular docking was done to determine the potential of alkaloid compounds as an anti-inflammatory by looking at the interaction of compounds with the active site of COX-2. Molecular docking was done with the PyRx program to assess the potential of compounds to be anti-inflammatory.

The 3D structures of alkaloid compounds detected from the ethanol extract of *P.longifolia* leaves were created using ChemDraw (Figure 2).

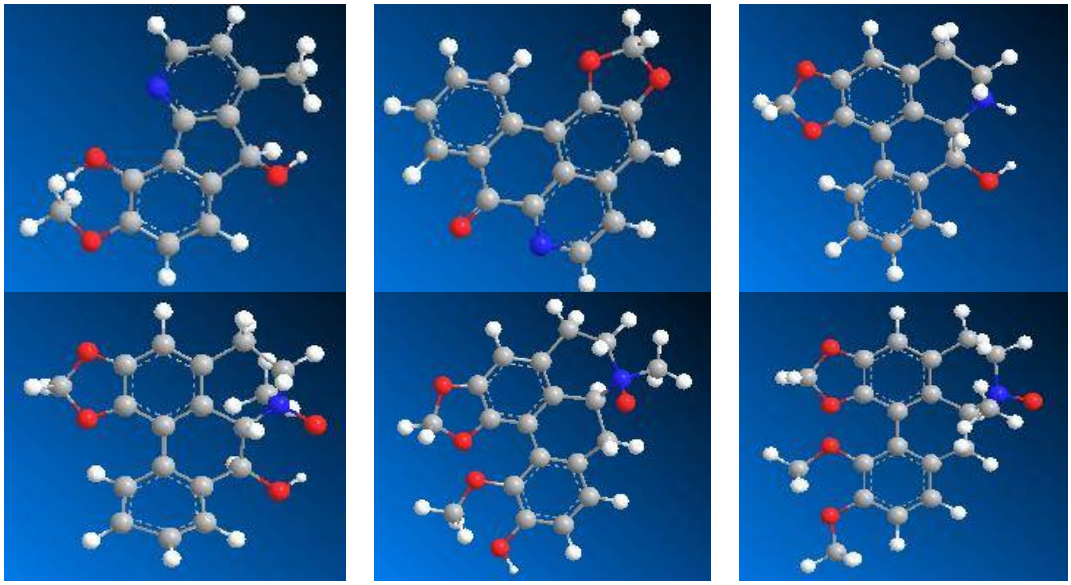

Figure 2. The 3D structure of Alkaloids. A) Polylongine, B) Liriodenine, C) Noroliveroline, D) Oliveroline β-N-oxide, E) N-methylnandingerine-β-N-oxide, F) O-methylbulbocapnine-N-oxide.

The 3D structure of COX-2 proteins that binds to the original inhibitor is obtained at the RCSB GDP online (Figure 3A). Water molecules, other ligands and chain B, C and D removed by PyMol software (Figure 3B).

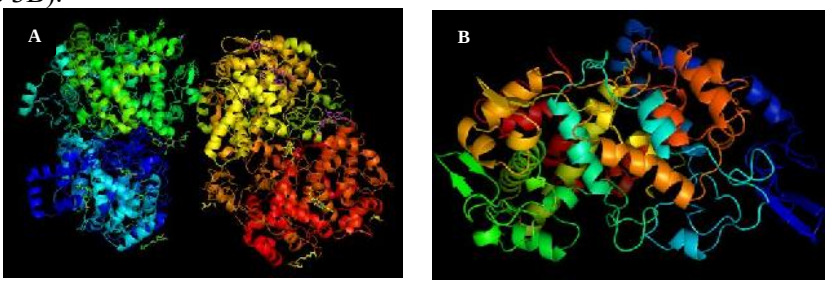

Figure 3. A) The 3D of COX-2 protein, B) The 3D chain A structure of COX-2 protein.

Molecular docking results and interactions with residual amino acids were shown in Table 2. Table 2 showed the potential of each compound as anti-inflammatory seen from the binding energy and amino acid interactions. The total binding energy of the compounds that were docking had a value of RMSD (Root Mean Square Deviation) <2. RMSD was the difference between predictive and experimental observations, the smaller the RMSD value obtained, the predicted position of the compound was closer to native [7]. Therefore, it can be concluded that this molecular docking was valid for predicting COX-2 inhibition.

Table 2. The results of molecular docking between alkaloid compounds in *P.longifolia* leaves with COX-2 enzyme.

Compounds/Ligands	Binding energy (kcal/mol)	Hydrogen bonds distance (Å)	Amino acid residues from hydrogen binding	Amino acid residues from hydrophobic bonds
Polylongine	-8.7	3.29 2.83	Tyr341	Leu517, **Ala513***, Ser516, Val335, **Phe504***, **Leu338***, **Ser339***, **Val509***
Liriodenin	-10.9			**Val335***, **Ala513***, Ser516, **Leu338***, **Val509***, **Phe504***, **Ser339***, **Tyr341***
Noroliveroline	-10.7	3.27 3.12	**Ala513*** Ser516	Val335, Leu338, **Phe504***, **Ser339***, **Val509***, **Tyr341***, Leu517, Arg106
Oliveroline-β-N-oxide	-9.3			**Phe504***, **Val509***, **Met508***, **Leu338***, Tyr371, Trp373, Ser516, Leu517, **Val335***, **Ala513***, **Tyr341***, **Ser339***
N-Methylnandingerine-β-N-Oxide	-7.4	3.05	Ser516	Leu517, **Val335***, **Ala513***, **Gly512***, **Leu338***, **Ser339***, **Phe504***, **Val509***, **Tyr341***, Leu345
O-Methylbulbocapnine-N-oxide	-8.2			Tyr371, **Trp373***, **Phe504***, **Leu338***, **Tyr341***, Ser516, **Ala513***, **Val509***, Arg106, **Ser339***, **Val335***, Leu517, Leu345, Val102

*= same amino acid interactions with reference inhibitor

Binding energy was a conformational stability parameter between receptors (COX-2) and ligands (compounds). All alkaloid compounds that had been docking had low binding energy which ranges from -7.4 to -10.9 kcal/mol. Low binding energy causes the protein-ligand complex (compound) to become stable so that the ability to inhibit COX-2 was getting better [13]. This showed that these compounds had the potential as an anti-inflammatory. Based on the binding energy, it can be classified as anti-inflammatory compounds as follows: liriodenin> noroliveroline> oliveroline-β-N-oxide> polylongine> O-methibulbocapnine-N-Oxide> N-methylnandingerine-β-N-oxide.

The LigPlot+ program was used to visualize protein-ligand interactions. The results obtained illustrate the interaction of amino acid residues with ligands, which were mediated by hydrogen bonds with hydrophobic interactions. The interaction of ligands with amino acid residues of the O-methylbulbocapnine-β-N-oxide compound and N-methylnandingerine-N-oxide was shown in Figure 4.

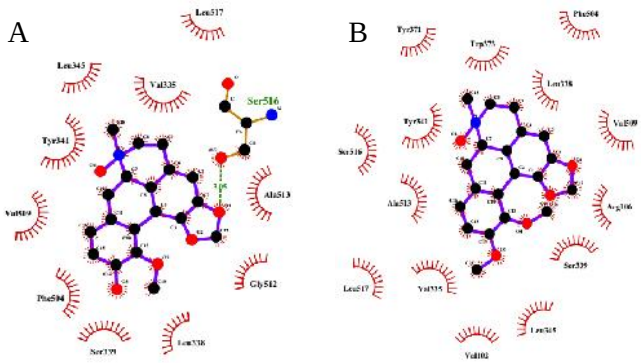

Figure 4. Visualize the results of LigPlot+. A) N-methylnandingerine-β-N-oxide, B) O-methylbulbocapnine-N-oxide.

In addition to binding energy, a comparison of amino acid residue interactions from reference inhibitors was carried out [14], [15]. This was done to see that the alkaloid compounds were on the same side (active side) as celecoxib. Redocking from celecoxib as a reference inhibitor obtained of hydrophobic interaction on the amino acid residues of Met508, Ala513, Gly512, Val509, Trp373, Leu370, Try341, Val335, His75, Phe504, Ile503 and Ala502 and hydrogen bonds acceptor with Ser339, Gln178, Leu338 and Arg499. Based on amino acids binding to the compounds in Table 2, it was found that all alkaloid compounds interacted on the active site of the receptor (COX-2). Hence, it could be concluded that the alkaloid compounds of ethanol extract of *P.longifolia* leaves had the potential as an anti-inflammatory by selectively inhibiting COX-2. Based on the potential of anti-inflammatory compounds O-methylbulbocapnine-N-Oxide was better than N-methylnandingerine-β-N-oxide. Thus, extract ethanol of *P.longifolia* leaves from Indonesia was better than the Philippines.

4. Conclusion

In conclusion, *P.longifolia* leaves from Indonesia and the Philippines contained different alkaloid compounds. N-methylnandingerine-β-N-oxide was only found in extracts from the Philippines, whereas O-methylbulbocapnine-N-oxide was only found in extracts originating from Indonesia. All alkaloid compounds found had anti-inflammatory abilities by inhibiting COX-2 in silico. Molecular docking results showed that compound from Indonesia had better anti-inflammatory potential than the Philippines.

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BIOCONVERSION OF LIGNOCELLULOSIC AGRICULTURAL BY-PRODUCTS BY MICROORGANISMS INTO HIGH MYCOPROTEIN FEEDS

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ABSTRACT

This research study was conducted to produce high mycoprotein feeds (HMPF) from lignocellulosic agricultural by-products through solid-substrate-fermentation. Six species of fungi were used as fermenting organisms and four agricultural wastes as substrates. The nutrient composition of the fermented wastes was determined through proximate analysis. Results of the study showed that the three macrofungi *Pleurotus* spp., *Ganoderma* spp., and *Psilocybe* spp. and the three microfungi *Aspergillus* spp1, *Trichoderma* spp., and *Aspergillus* spp2 were capable of producing HMPF through solid-substrate-fermentation of lignocellulosic agricultural by-products. Further, banana leaves, rice straw, corn cob and sugarcane bagasse are candidate substrates for the production of HMPF. Proximate analysis revealed that the crude protein (CP) of the fermented banana leaves obtained a four- to five-fold increase (24.41%-28.16%) in CP while an eight to nine-fold increase (19.66%-22.63%) in corn cobs after fermentation. The fermented sugarcane bagasse attained 11- to 13-fold (21.37%-25.83%) rise in the CP content while the fermented rice straw obtained two to five-fold increase (18.88%-29.51%) in the CP content. The ash contents (ACs) of the fermented products likewise increased while the crude fiber (CFr) and crude fat (CF) of the lignocellulosic agricultural by-products decreased after fermentation. The present results demonstrate the feasibility of utilizing lignocellulosic agricultural-wastes as substrates of fungal organisms to produce high protein feeds for animals.

Key words: protein biomass, high mycoprotein feeds (HMPF), lignocellulosic, solid-substrate-fermentation, macro and microfungi

INTRODUCTION

Agricultural wastes comprise a major proportion of agricultural production. These crop residues are made up mostly of lignocellulosic materials. These lignocellulosic crop residues are rich in dietary fibers (Valéro *et al*., 2012) which are great potential energy resources for livestock and poultry. However, they are characterized with very poor inherent

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feeding value due to low digestible dry matter and protein content (Nasehi *et al*., 2017). Likewise, these fiber-rich wastes have an enormous potential to be exploited for the production and recovery of several products and ingredients to improve animal nutrition and the worldwide supply of protein and calories for animal production in lesser environmental footprints. By using appropriate technologies, the economic value of lignocellulosic crop residues could be increased through nutrient enrichment and production of products that are safe not only for animal use but also for human feeding.

Animal production in the Philippines is a lucrative viable enterprise and progressively developing. However, the high cost of animal feeds due to the importation of expensive feed ingredients is a major problem that limits the sustainability of animal production. Aside from this, the extensive use of synthetic growth promoters and additives is now a worldwide concern because of its impact on consumers' health and disputes on multidrug resistance. While feed comprises the bulk of the total cost of production, efforts in the production of safe and cost-effective alternative feed ingredients could lead into a cost-efficient and successful animal production by not compromising the quality of feeds, nutrition of animals and health of consumers.

Microbial degradation of lignocellulosic materials brings a variety of changes in their bio-physicochemical properties. Microbial treatment can enhance the digestibility of various agricultural residues. Filamentous fungi are potential candidates that can improve the nutritional quality of lignocellulosic residues by degrading lignin with the use of the complex extracellular cellulolytic enzymes and converting these complex polysaccharides into simple sugars (Singh and Kumar, 2015). The use of fungal fermentation to cycle and recycle these residues will not only result in the reduction of pollution but also help to produce low cost and high-quality protein biomass. Further, production of protein biomass from fibrous wastes is an eco-friendly approach to recovering renewable energy resources from agri-crop production into valuable low-cost products. This research was conducted to produce high mycoprotein feeds from lignocellulosic agricultural by-products through solid-substrate-fermentation. Specifically, it aimed to identify species of filamentous macro and microfungi that could produce mycoprotein feeds and determine the proximate composition of the mycoprotein feeds produced from different agricultural by-products.

MATERIALS AND METHODS

Four independent studies were conducted utilizing four agricultural by-products as substrates and six fungal organisms. The agricultural by-products were sugarcane bagasse, rice straw, corn cobs and banana leaves. Each of the study was set-up with five replications to produce high mycoprotein feeds.

A 39 g potato dextrose agar (PDA) basal medium (TM Media) was weighed using a toploading analytical balance (National LCS-3000, 1200gx0.1g, Nagata Scale Co., Ltd, Taiwan). It was placed in a beaker and dissolved in 1 Li of distilled water. The beaker was placed on top of a hot plate (All American™ Corning™ PC-420 Fisher Scientific, USA) stirred continuously until the PDA was completely dissolved. A 20 ml mixture was dispensed in sterilized flat bottles. The bottles were covered immediately with cotton wool and aluminum foil to reduce contamination. Then, the bottles were sterilized for 15 min using an autoclave (All American™ Stove Top, Wisconsin Aluminum Foundry, USA) maintaining a pressure of 15 psi. After sterilization, the bottles were slanted and allowed the

basal medium to cool down and solidify.

The three macrofungi (*Pleurotus* spp., *Ganoderma* spp., *Psilocybe* spp.) were kindly given by Mr. Benjie L. Garcia of the Central Luzon State University and the three microfungi (*Aspergillus* spp1, *Trichoderma* spp., *Aspergillus* spp2) were obtained from the Department of Agriculture-Philippine Center for Postharvest Development and Mechanization (DA-PhilMech).

Sub-cultures of the six fungal organisms were prepared from pure cultures. Before inoculation, the glass chamber was surface sterilized to minimize contamination. Each pure culture of the fungal organisms was sub-cultured one at a time. The inocula of the pure culture of macro and microfungi were aseptically transferred to flat bottles containing the basal medium using an inoculating loop. After inoculation, the flat bottles containing the inoculants were properly labeled and arranged on the shelves and incubated at room temperature for 5 to 7 days until full mycelial ramification of the fungal mycelium is evident on the surface of the basal medium.

A standard nutrient solution (SNS) based on that used by Pham *et al*. (1992) with some modifications by Demo-os *et al*. (2013) were prepared. Urea, ammonium sulfate, ammonium phosphate, vinegar and sugar were dissolved and mixed in water.

The sugarcane bagasse was obtained from a sugarcane producer and processor in Sta. Ignacia, Tarlac while the banana leaves were obtained in San Jose, Tarlac. The corn cob, on the other hand, was obtained from Pindangan 2nd, Camiling, Tarlac and the rice straw was obtained in Sta. Ignacia, Tarlac. The fibrous agricultural by-products were shredded using a shredding machine, sundried and used as substrates. In the preparation of the substrates, the shredded agricultural by-products were weighed, and the SNS was mixed with the shredded agricultural by-products. For the corn cob, a ratio of 60 SNS:40 corn cob substrate was used while a ratio of 50 SNS:50 substrates was used for sugarcane bagasse, rice hay and banana leaves. The SNS was gradually added to the fibrous sources individually and mixed thoroughly until a uniform mixture was attained. The substrates were packed in an equal quantity of 1 kg in polypropylene bags fitted with polyvinyl chloride (PVC) necks. The mouth of the PVC was covered with cotton wool and used paper and tightly sealed with a rubber band to avoid contamination. The bagged substrates were sterilized in an autoclave for 30 minutes maintaining a pressure of 15 psi. The sterile substrates were placed in a tray to cool down and were arranged on the shelves before inoculation.

The substrates were inoculated using the previously prepared sub-cultures of the six fungal organisms. Each of the cultures was aseptically inoculated in the substrates using an inoculating loop. After inoculation, the substrates containing the inoculum were properly labeled, placed in fermenting shelves and incubated for 21 days at room temperature. Every week, the inoculated substrates were kneaded to mix the fungal colonies in the substrate for efficient colonization and fermentation.

After 21 days of incubation, the resulting products of fermentation were kneaded and were removed individually from the propylene bags, placed in trays and sun-dried to about 90% dry matter.

After sun-drying, a 250 g fermented product was compositely sampled from the five replicates of each substrate, placed in ziplock plastics and labeled for proximate analysis. The finished products were brought to the Department of Agriculture-Regional Feed Chemical Analysis Laboratory in San Fernando City, Pampanga for the analysis of the nutrient composition in terms of moisture content (MC), crude protein (CP), crude fat (CF),

ash content (AC), crude fiber (CFr) and dry matter (DM). Data were gathered from the average of two runs of proximate analyses of the same analytical condition.

RESULTS AND DISCUSSION

The results of the proximate analysis of banana leaves before and after fermentation by macrofungi is shown in Figure 1. It shows that unfermented banana leaves contain 4.07% CP, 1.87% CF, 41.43% CFr, 10.00% AC and 91.00% DM. The fermentation of *Pleurotus* spp. brought a four-fold increase in the crude protein. After fermentation, the CF decreased by 0.70% which is an indication of the use of fats by *Pleurotus* spp. as a source of energy. Moreover, a 0.55% rise in AC indicating an increase in the inorganic components of the fermented banana leaves. A lower %MC brought the high %DM. Similar results were observed in *Ganoderma* spp. and *Psilocybe* spp. when used as fermenting organisms. A remarkable four-fold increase in the protein content was obtained after 21 days of fermentation. The CF decreased by 0.57% and 0.59% for both fungal organisms, respectively. The ACs of the fermented products improved by 0.33% and 0.73%, respectively. DM content was 84.70% and 86.10%, respectively. Proximate analysis of the banana leaves fermented by microfungal species is presented in Figure 2. Fermentation of banana leaves by *Trichoderma* spp. and *Aspergillus* spp2 brought a four-fold increase in the CP of fermented banana leaves while a five-fold CP was attained when *Aspergillus* spp1 was used in fermenting the banana leaves. A slight decline in the %CF

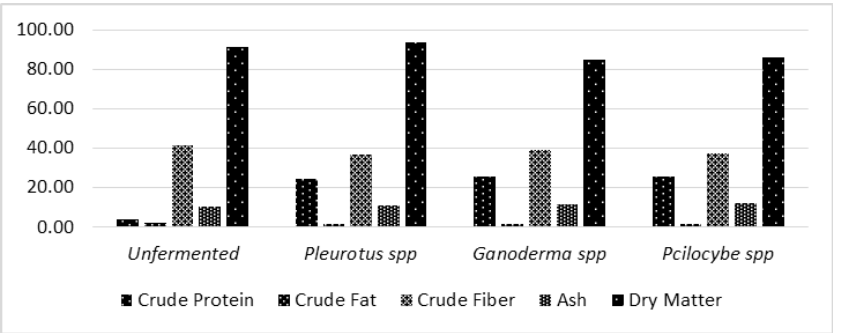

Figure 1. Proximate analysis of banana leaves before and after fermentation by macrofungi for 21 days at room temperature.

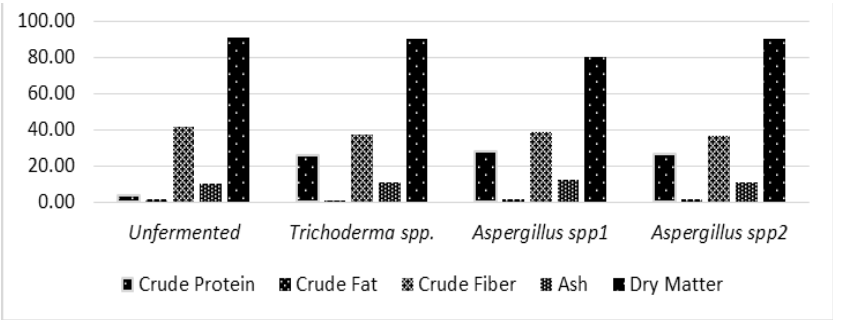

Figure 2. Proximate analysis of banana leaves before and after fermentation of microfungi for 21 days at room temperature.

of banana leaves was observed after fermentation by the three microfungal organisms. The CFr of fermented banana leaves decreased by 3.83% for *Trichoderma* spp., 2.76% for *Aspergillus* spp1 and 5.09% for *Aspergillus* spp2 when compared to unfermented banana leaves having a CFr of 41.43%. The AC increased by 1.23% in *Trichoderma* spp.-fermented banana leaves while 2.02% and 1.15% in the two *Aspergillus*-fermented banana leaves. There was a slight decline in the DM of the banana leaves fermented by *Trichoderma* spp. and *Aspergillus* spp2 while an 11.10% DM was attained in banana leaves fermented by *Aspergillus* spp1.

The results of the proximate analysis of corn cobs before and after fermentation of macrofungi is shown in Figure 3. It shows that unfermented corn cobs contain 2.09% CP, 0.8% CF, 41.90% CFr, 1.87% AC and 90.70% DM. The high organic matter, particularly the CFr, is an indication of high degradability and can be used as substrates for microbial fermentation converting complex carbohydrates into protein by several species of microorganisms. A nine-fold increase in the CP content was attained from the fermented corn cobs after 21 days of fermentation by *Pleurotus* spp. at 21.11%, *Ganoderma* spp. at 21.69% and *Psilocybe* spp. at 19.66%. The CF decreased by 0.63%, 0.49% and 0.35%, respectively, which indicates that the macrofungi utilized CF during the process of fermentation.

Moreover, a 0.5% rise in AC indicates an increase in the inorganic components of the fermented banana leaves. A lower %MC brought the high DM. Similar results were observed in *Ganoderma* spp. and *Psilocybe* spp. when used as fermenting organisms. A remarkable four-fold increase in the protein content was obtained after 21 days of fermentation. The CF of the corn cob decreased by 3.13%, 2.16% and 4.43% after fungal fermentation by *Pleurotus* spp., *Ganoderma* spp. and *Psilocybe* spp., respectively. There was also an improvement in AC of fermented corn cobs at 1.71%, 1.77% and 1.60% respectively, as compared to the unfermented corn cobs. The DM of *Pleurotus* spp. was 81%, 77% for *Ganoderma* spp. and 95% for *Psilocybe* spp.

On the other hand, Figure 4 shows that fermented corn cob by microfungal organisms had resulted in eight- to nine-fold rise in the %CP content of the fermented corn cobs. The CF content decreased by 0.43% when *Trichoderma* spp. was used in fermenting the corn cob while 0.64% and 0.76% decrease in CF when corn cob was fermented by *Aspergillus* spp1 and *Aspergillus* spp2, respectively. The CFr of fermented corn cob decreased by 2.51% for *Trichoderma* spp., 1.90% for *Aspergillus* spp1 and 3.79% for *Aspergillus* spp2 when compared to unfermented corn cob having a CFr of 41.90%. There was also a rise is the AC of the fermented corn cobs by the three fungal organisms compared with the unfermented substrates. The DM of the fermented corn cobs were 86.60% for *Trichoderma* spp., 83% for *Aspergillus* spp1 and 86.60% for *Aspergillus* spp2.

The results of the proximate analysis of unfermented and fermented sugarcane bagasse are shown in Figures 5 and 6. It shows that the unfermented sugarcane bagasse has very low CP, CF and AC but a very high DM constituting about 91.10% DM. After fermentation, there was a 12- to 13-fold increase in the CP content of the fermented sugarcane bagasse. The AC was likewise escalated from 2.41% unfermented sugarcane bagasse to 5.87%, 6.40% and 7.67% after fermentation by *Psilocybe* spp., *Ganoderma* spp., and *Pleurotus* spp., respectively. The CF of the fermented sugarcane bagasse, on the other hand, decreased when compared to the unfermented one whereas similar results were attained in the CFr of the unfermented sugarcane bagasse after fermentation. The fermentation of sugarcane bagasse by the microfungal organisms resulted to an 11- to 13-fold significant

increase in the CP and a 1.5 to 1.8-fold rise in the AC with a percent increase of 3.28% for *Trichoderma* spp., 4.16% for *Aspergillus* spp1 and 3.49% for *Aspergillus* spp2. The CF and the CFr of the fermented sugarcane bagasse also decreased.

Figures 7 and 8 show a snapshot of the proximate analysis of unfermented and fermented rice hay by several species of fungal organisms. The figures reveal that the unfermented rice straw has 5.60% CP, 0.99% CF, 37.14% CFr, 15.16% AC and 91% DM. After 21 days of fermentation, there was a two- to five-fold increase in the CP content of fermented products. Among the macrofungal isolates, *Psilocybe* spp. had produced the highest CP content of the fermented rice straw at 29.51%. This was followed by *Ganoderma* spp. with 22.09% CP and *Pleurotus* spp. with 19.83% CP. The CFr of rice straw fermented by *Pleurotus* spp. was 37.16%, *Ganoderma* spp. was 34.41% and *Psilocybe* spp. was 32.40%. The ACs of the fermented rice straw was 16.28%, 17.84% and 16.09%, respectively, slightly higher when compared to 15.16% of the unfermented rice hay. The DM of the fermented rice straw was 95.60% for *Pleurotus* spp., 86.90% for *Ganoderma* spp. and 93.20% for *Psilocybe* spp. Figure 8 further discloses that the fermentation of the three microfungal isolates resulted in a 2.37 to a 2.73-fold increase in the CP content of the fermented product. The CP of rice straw fermented by *Trichoderma* spp. was 20.19%. *Aspergillus* spp1 fermentation resulted in 18.88% CP and 19.65% CP by *Aspergillus* spp2. There was also an increase in the ACs of the fermented products that ranged from 0.93% to 2.68% by the three isolates. The CFr of rice straw fermented by *Trichoderma* spp. was 34.66% which was lower by 2.46% compared

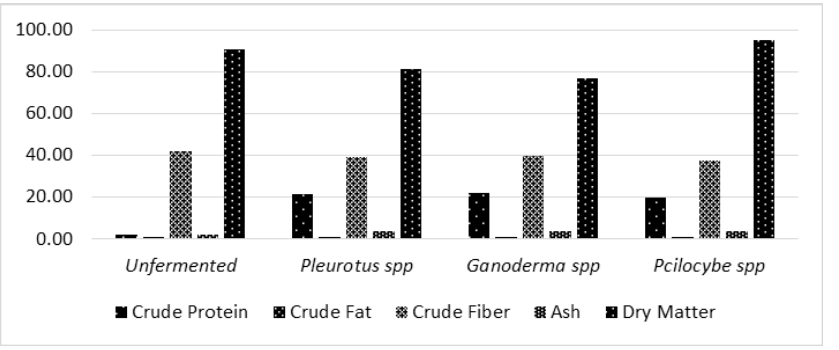

Figure 3. Proximate analysis of corn cob before and after fermentation of macrofungi for 21 days at room temperature.

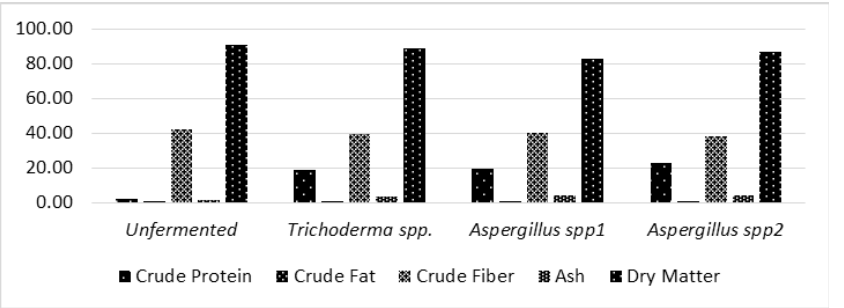

Figure 4. Proximate analysis of corn cob before and after fermentation of microfungi for 21 days at room temperature.

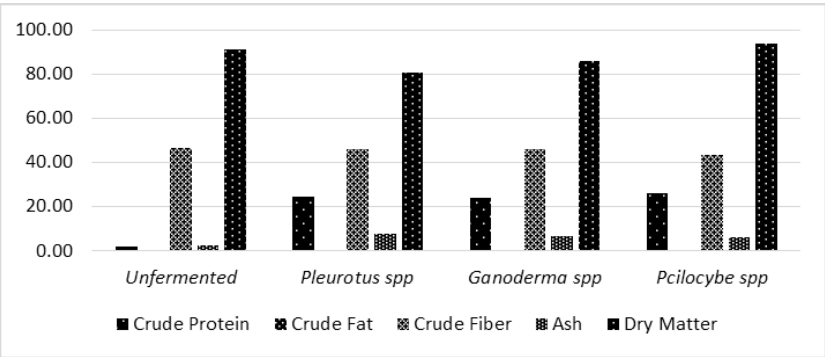

Figure 5. Proximate analysis of sugarcane bagasse before and after fermentation of macrofungi for 21 days at room temperature.

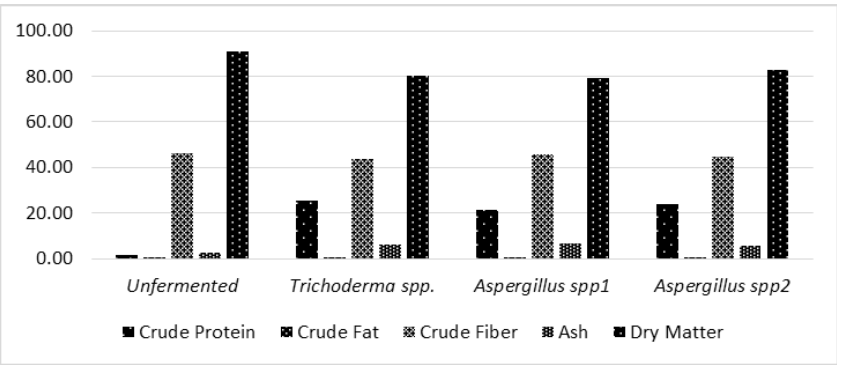

Figure 6. Proximate analysis of sugarcane bagasse before and after fermentation of microfungi for 21 days at room temperature.

to the unfermented one. The CFr obtained from *Aspergillus* spp1 fermented rice straw was 36.29% while rice straw fermented by *Aspergillus* spp2 attained 36.99% CFr.

Production of inexpensive high protein biomass from agricultural crop residues plays a substantial role in improving not only the economic utilization of agricultural by-products but also a tangible solution in replacing imported feed ingredients for animal production. Agricultural by-products like rice straw, sugarcane bagasse, banana leaves and corn cobs contain biologically important nutrients that require microbial bioconversion through biotechnological approaches for the efficient utilization of animals. Most of the agricultural crop residues used in the present study are generally made up of complex carbohydrates like cellulose, hemicellulose and lignin that are difficult to degrade. The physicochemical composition of crops residues is dependent on the types. In the case of rice straw, it is composed mainly of cellulose, hemicellulose, lignin, ash and other extractives. It contains on average between 30% to 45% cellulose, 20% to 25% hemicellulose, 15% to 20% lignin, as well as some minor organic compounds. Rice straw is poor in nitrogen, but relatively high in inorganic compounds, often referred to as ash (Boschma and Kwant, 2013). On the other hand, sugarcane bagasse, a by-product after extracting the juice for sugar production has similar chemical constituents to rice straw. However, it varied in percent composition. The cellulose, hemicellulose and lignin constitute 90% of the dry weight of the fiber (Rezende *et al*., 2011). The ash content is low which implies that the non-fiber extractive compounds

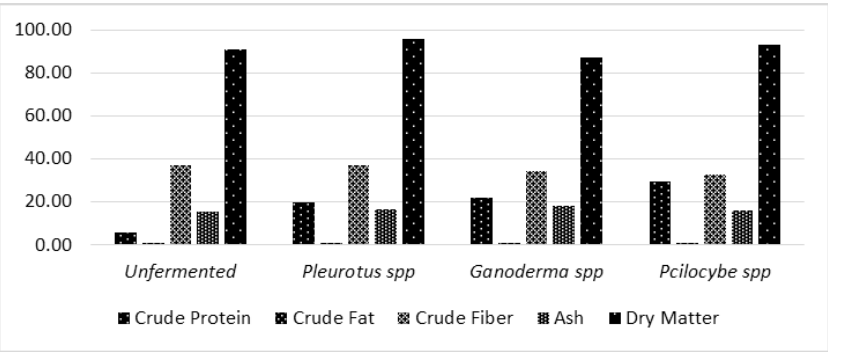

Figure 7. Proximate analysis of rice straw before and after fermentation of macrofungi for 21 days at room temperature.

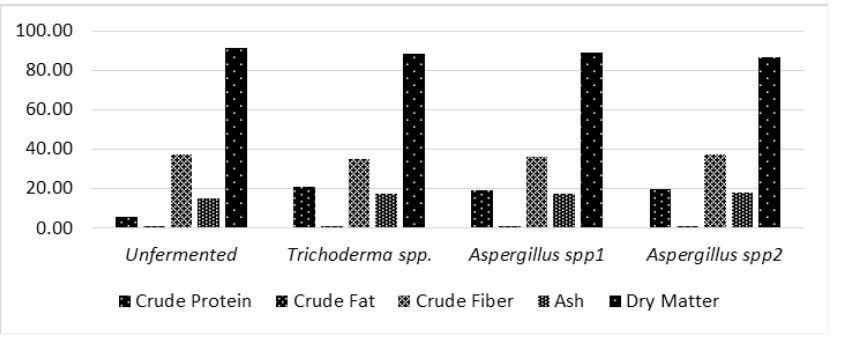

Figure 8. Proximate analysis of rice straw before and after fermentation of microfungi for 21 days at room temperature.

represent most of the dry weight of the sugarcane bagasse.

Furthermore, the polymeric fiber composition of corn cobs is made up mainly of monomeric molecules. Cellulose is made up of C6 sugars while hemicellulose is mainly made-up of the C5 sugars xylose and arabinose. Lignin consists of phenolic macromolecules (Pointner *et al*., 2014). Reports on the proximate composition of banana leaves revealed that it also contains a high amount of lignocellulosic materials mainly cellulose, lignin and hemicellulose. Banana leaves contain approximately 12% ash with very high organic matter of around 83% and around 5% carbon and very low nitrogen at 0.2%. However, the protein contents of these crop residues were found low. The high organic matter of the agricultural crop residues implies their high degradation potentials through enzymatic hydrolysis by several species of microorganisms. However, Mosier *et al*. (2004) and Menon and Rao (2012) revealed that the cellulose, hemicellulose and lignin that are embedded in a complex matrix are very resistant to enzymatic degradation. Screening and selection of several species of fungal organisms that are highly efficient in the biodegradation and conversion of the complex carbohydrates into protein are essential. Filamentous microfungi produce fibrous materials that can be easily converted to textured food products with protein content as high as 30% to 50%. The biomass produced by filamentous fungi can be used as food for animals without any further processing because it provides carbohydrates, lipids, minerals, vitamins and proteins. Also, nucleic acid contents of fungal protein are lower than that of yeast and bacteria (Chahal, 1982). White rot macrofungi, on the other hand, are efficient

lignocellulolytic decomposers capable of metabolizing plant cell constituents particularly cellulose, hemicellulose and lignin by their enzymes (Eriksson *et al*., 1990). Many species are capable of degrading lignin and can improve the nutrient composition of fodder for ruminant nutrition (Howard *et al*., 2003).

The intricate enzyme systems bring the ability of fungal organisms to degrade complex carbohydrates. According to Dashtban *et al*. (2009), the bioconversion of lignocellulosic residues could be done by microorganisms like fungi and bacteria that are capable of degrading lignocellulolytic materials through enzymatic hydrolysis. The conversion of cellulosic biomass to fermentable sugars requires the synergistic action of three cellulolytic enzymes namely 1,4 endoglucanase, -1,4 exoglucanase and -1,4 glucosidase. The most extensively studied cellulases are those produced by efficient lignocellulose-degrading fungi, particularly *Trichoderma* (Narsimha *et al*., 2006) and *Aspergillus* spp. (Baig, 2005). Such innate characteristic is an opportunity in the utilization of these organisms to produce protein biomass. Sibtain *et al*. (2017) revealed that using *Trichoderma harzianum* to produce fungal biomass protein from rice polishing resulted to a maximum of 49.50% crude protein, 32.00% true protein, 19.45% crude fiber, 9.62% ash, 11.50% cellulose content and 0.325% RNA content. The profile of amino acids of the final fungal protein biomass (FPB) exhibited that all essential amino acids were present in great quantities. The *T. harzianum* produces FPB with high nutritional value suitable as supplement for poultry animals.

It was reported by Patyshakuliyeva *et al*. (2016) that fungi such as *Trichoderma reesei* and *Aspergillus niger* produce enormous amounts of extracellular cellulolytic enzymes which is found to have synergistic interaction in degrading cellulose like the endoglucanases, cellobiohydrolases (exoglucanases) and β-glucosidases. Meanwhile, similar enzymes called cellulosome associated with the cell wall are also secreted by bacteria and strains of fungal anaerobes capable of degrading cellulose. In basidiomycetes, lignocellulose degradation could be attributed to having unique oxidative systems and cellulolytic and hemicellulolytic activities (Dashtban *et al*., 2009).

Aspergillus niger and *Aspergillus oryzae* are also the most commonly used industrial *Aspergillus* species for the production of pharmaceuticals, food ingredients and enzymes (Berka *et al*., 1992; Pandey *et al*., 1999). *A. niger* and *A. oryzae* produce a broad range of enzymes related to the degradation of plant polysaccharides, such as cellulose, xylan, xyloglucan, galactomannan and pectin (de Vries and Visser, 2001). These enzymes are essential in converting natural carbon sources into smaller molecules easily permeable to cells. *A. oryzae* also produces some enzymes like proteases which rapidly reduce the viscosity of gelatin solutions and cause rapid digestion of the lower molecular weight components of gelatin have been detected in the culture filtrate. Esterase, phosphatase, amylase, sucrase and catalase were also liberated from the mycelium in sufficient quantity for convenient estimation. Enzymes catalyzing the liberation of inorganic phosphate from phytic acid and lecithin, various dipeptidases, lactase, maltase, salinase, nuclease and urease were also detected. White rot fungi is also an alternative and safe source of biologically active extracellular cellulolytic enzymes. It was reported that *Ganoderma* spp. possesses three major families of fungal lignin-modifying enzymes (LMEs) (Thurston, 1994). These include laccases, manganese-dependent peroxidases (MnPs) and lignin peroxidases (LiPs) (Youn *et al*., 1995). It is believed that these enzymes can degrade lignin that can be used in degrading lignocellulosic material as a renewable resource for the production of paper products, feeds, chemicals and fuels. These LMEs can oxidize phenolic compounds thereby

creating phenoxy radicals, while non-phenolic compounds are oxidized via cation radicals. LiP and MnP oxidize non-phenolic aromatic compounds with high oxidation-reduction potentials, the major components of the lignin polymer. Laccase oxidizes non-phenolic aromatic compounds with relatively low oxidation-reduction potentials (Kirk *et al*., 1987).

Reports also revealed that several species of *Pleurotus* spp. are among the most efficient in utilizing lignocellulosics (Zhang *et al*., 2002; Salmones *et al*., 2005; Albores *et al*., 2006). Similar studies on three *Pleurotus* spp., namely, *P. florida*, *P. ostreatus*, and *P. sajor-caju* for cellulolytic enzymes production showed that *P. florida*, produced the highest levels of enzyme activity, indicating high production of extracellular cellulases under optimum cultural and nutritional parameters using submerged fermentation conditions (Goyal and Soni, 2011).

Based from the results of the study, the three macrofungi *Pleurotus* spp., *Ganoderma* spp., and *Psilocybe* spp. and the three microfungi *Aspergillus* spp1, *Trichoderma* spp., and *Aspergillus* spp2 are capable of producing mycoprotein feeds from agricultural wastes of banana leaves, rice straw, corn cobs and sugarcane bagasse with an increase in CP content through solid-state fermentation. Further study could be conducted by varying moisture content of the substrates, inoculum size, and duration of fermentation, C/N sources concentration, qualitative protein and lipid content analysis, enzymatic analysis and feeding trials in livestock and poultry animals.

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The Effects of Plant Leaves Variants from The Philippines on Infective *Oesophagustonum dentatum* Larvae

Pengaruh Sembilan Tanaman Asal Filipina Terhadap Infeksi Larva Oesophagustonum dentatum

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ABSTRACT

Nine aqueous extracts of plant leaves from the Philippines were evaluated against *Oesaphagustomum dentatum* sensitive (ODSEns) larvae for their anthelmintic effects. The plant species evaluated were *Ananas comosus, Chrysophyllum cainito,Gliricidia sepium, Annona squamosa, Moringa oleifera, Tinospora rumphii* Boerl,*Azadirachta indica, Trichantera gigantea* and *Leuacaena leucocephala*. The leaves of the plants gathered from Camiling, Tarlac, Philippines, were dried under room temperature for 30 days and then ground. Aqueous extracts of these plant leaves were prepared using migration buffer. *In vitro* migration and inhibition assays were used to evaluate the anthelmintic activity against exsheathed third stage larvae of *Oesophagustomum dentatum.* The experiments were put in 24-well plates at room temperature with four replications per treatment using different concentrations of 1, 2.5, 5 and 10 mg/ml. About 120 larvae per well were deposited on a larval migration apparatus consisting of 20 µm nylon mesh filters and incubated 20 hours under different treatments and another 2 hours to allow the migration of active motile larvae. The evaluation criteria were based on the average numbers of inhibited larvae under the different treatments. The percentage inhibition were statistically analyzed using Two-Way ANOVA (P=≤.0001) of the Graph Pad Prizm software (V5 San Diego, Ca). The most effective leaves with their highest inhibition level were from *Ananas comosus* (pineapple, 86.50%), *Gliricidia sepium* (madre de cacao, 84.50%), *Chrysophyllum cainito* (star apple, 83.75%), *Moringa oleifera* (horseradish,58.75%), *Annona squamosa* (sweet sop, 56.00%), *Tinospora rumphii* Boerl (\Makabuhay, 55.75%), and *Azadirachta indica* (Neem, 51.50%), whereby extremely significant (P<0.001) effect against *O. dentatum* larvae. Two leaves extracts, from *Trichantera gigantea* and *Leuacaena leucocephala* have no significant effect on *O. dentatum* larvae. The larval migration assay allows initial studies on the anthelmintic effects of the herbs.

Keywords: plant leaves, anthelmintic property, inhibition assay, Philippines

ABSTRAK

Sembilan ekstrak air daun tanaman dari Filipina dievaluasi terhadap sensitifitas larva *Oesaphagustomum dentatum*yang mempengaruhi anthelmintik. Spesies tanaman yang dievaluasi adalah *Ananas comosus, Chrysophyllum cainito, Gliricidia sepium, Annona squamosa, Moringa oleifera, Tinospora rumphii Boerl, Azadirachta indica, Trichantera gigantea* dan *Leuacaena leucocephala*. Daun tanaman dikumpulkan dari Camiling, Tarlac, Filipina, dikeringkan selama 30 hari dalam suhu kamar dan kemudian ditumbuk. Ekstrak air dari daun tanaman tersebut disiapkan menggunakan buffer migrasi. Migrasi in vitro dan uji penghambatan dilakukan untuk mengetahui aktivitas antelmintik terhadap larva tahap ketiga *Oesophagustomum dentatum.* Eksperimen dilakukan dalam 24-sumuran *plate* pada suhu kamar dengan empat replikasi per perlakuan menggunakan konsentrasi yang berbeda dari 1, 2,5-, 5 dan 10 mg / ml. Sekitar 120 larva per sumur diendapkan pada peralatan migrasi larva yang terdiri dari 20 µm filter mesh nilon dan diinkubasi 20 jam dengan perlakuan berbeda dan 2 jam lagi untuk memungkinkan

migrasi larva motil aktif. Kriteria evaluasi didasarkan pada jumlah rata-rata larva yang dihambat di bawah perlakuan yang berbeda. Penghambatan persentase dianalisis secara statistik menggunakan Two-Way ANOVA (P = ≤,0001) menggunakan *software* Graph Pad Prizm (V5 San Diego, Ca). Daun yang paling efektif dengan tingkat penghambatan tertinggi berasal dari *Ananas comosus* (nanas, 86,50%), *Gliricidia sepium* (daun Gamal, 84,50%), *Chrysophyllum cainito* (daun sawo duren, 83,75%), *Moringa oleifera* (daun kelor, 58,75%), *Annona squamosa* (daun srikaya, 56,00%), *Tinospora rumphii Boerl*(Bratawali, 55,75%), dan *Azadirachta indica* (daun mimba, 51,50%), semuanya menunjukkan efek yang sangat signifikan (P<0,001) terhadap larva *O. dentatum*. Dua ekstrak daun yaitu *Trichantera gigantea*(daun cemara raja) dan *Leuacaena leucocephala*(daun lamtoro) tidak berpengaruh signifikan terhadap larva *O. dentatum*. Uji migrasi larva memungkinkan studi awal tentang efek anthelmintik dari herbal**.**

Kata kunci**:** daun tanaman, antihelmintik, uji penghambatan, Filipina

INTRODUCTION

The high cost of conventional anthelmintic drugs and the development of anthelmintic resistance lead researchers to evaluate the different plants as an alternative source of anthelmintics, especially in tropical countries where many plants abound throughout the year. The use of plants and plant extracts as a possible source of natural anthelmintics has received more interest in the present years.

The use of medicinal plants for the prevention and treatment of gastro-intestinal parasitism has its origin in ethnoveterinary medicine (Athanasiadou *et al*, 2007). For ages, plants have been used to control parasitism, and in many countries of the world, they are still used for this purpose. There have been reports around the world about the use of plants for their anthelmintic properties to treat human and animal parasitic infection (Bahaud, *et al*, 2006; Lopez-Aroche *et al*, 2008; Mali and Mehta, 2008; Bachaya *et al*, 2009; Marie-Magdeleine, *et al*, 2010; Narojini *et al*, 2011; Rabiu and Subhasish, 2011; Reuben *et al*, 2011; Deb Roy *et al*, 2012; Getachew *et al*, 2012 and Ferreira *et al*, 2013). The aim of this study was to test anthelmintic effects of nine plants aqueous extracts from the Philippines under controlled experimental conditions.

MATERIAL AND METHO DS

Plant leaves

Plants leaves were collected fresh, directly from naturally grown plants in Camiling, Tarlac, Philippines during the wet season (July and August). Leaves were dried for 30 days under room temperature and then sent to Iowa State University with an approved permit. The plant leaves were taxonomically identified as *Ananas comosus, Chrysophyllum cainito, Gliricidia sepium, Annona squamosa, Moringa oleifera, Tinospora rumphii* Boerl, *Azadirachta indica, Trichantera gigantea* and *Leuacaena leucocephala.* The scientific names and common names are summarized in **Table 1**.

Plant extract preparations

The leaves were ground using an Oster® processor at high speed for two minutes and stored in 50 ml falcon tubes until later use. One gram of leaves of each species were suspended in 20 ml of migration buffer and were shaken for 24 hours using an Orbit1000® (Labnet Intl). The aqueous solution was then extracted using 20 μm nylon mesh (Elko®) filters thus making the stock solution in equivalent to 0.05 g/ml concentration. Each treatment was prepared adding 0.2 ml, 0.5 ml, 1ml and 2 ml and up to 10 ml of the migration buffer. The

concentrations were prepared at 1 mg, 2.5 mg, 5 mg and 10 mg/ml, respectively.

Oesophagustomum dentatum L_3

The *Oesophagustomum dentatum* larvae were obtained by fecal culture. The feces were mixed daily with wooden spatulas and incubated for 6 days to promote egg hatching and the development of L_3 infective larvae. The L larvae were recovered using the Baerman funnel technique, and filtered and washed by centrifuging in 40% sucrose.Afterwards, they were re-suspended in sterile distilled water and stored in culture flasks and maintained in a low temperature Incubator™ (Fisher Scientific) at 10.5^{o}C.

For exsheathment, the larvae were placed in sterile water in a5 ml Falcon® tube with one ml of 10% of sodium hypochlorite (Vertex CSS-5 Bleach®, St. Louis, MO) for 5-8 minutes at room temperature or as soon as 90-95% of the larvae were exsheathed when seen under a microscope (Nikon TMS™, MVI, Avon, Ma.). The mixture was spun (Eppendorf Centrifuge 5415, Brinkmann™) at 31,000 rpm for 2 minutes, rinsed and re-suspended in sterile water.

Anthelmintic Activity: Larval migration assay

For the larval migration assay, the procedure of Nagamori, *et al* (2013) was used. The concentrations of the larvae were at 120 larvae/well. After the larvae were exsheathed, they were re-suspended in appropriate positive and negative control drugs and with the different leaves extracts in aqueous solution at concentrations of 1mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml, and then incubated at room temperature (25-30^{O}C) for 20 hour treatment exposure time periods.

After twenty hours, the larvae were added to the migration apparatus consisting of 20 µm nylon mesh filters (Elko®) in a 24-well plate (Corning®, Corning, NY) and incubated for another 2 hours to allow migration/inhibition of the L3 active, motile larvae. After 2 hour migration periods, the larvae were washed with migration buffer into 3.5 cm petridishes . Both the number of migrated larvae from the 24-well plate and inhibited larvae from the dishes were recorded under a microscope (Nikon TMS™, MVI, Avon, MA), as well as the total number of larvae. The evaluation criteria were based on the average number of inhibited larvae under the different treatments. The formula was:

$$\% \text{ inhibited} = \frac{\text{No. of inhibited larvae}}{\text{Total No. of Larvae}} \times 100$$

Statistical analysis

Data were statistically analyzed using Two-Way ANOVA (P=≤.0001) of the Graph Pad Prizm software (V5 San Diego, Ca). The differences between the negative control and each of the treatments were analyzed using the Bonferroni post-test.

RESULTS AND DISCUSSION

Table 1 shows the mean percentage inhibition to various concentrations of nine different species aqueous leaves extracts after 20 hours of exposure to *O. dentatum* exsheathed larvae. At the highest leaf extracts concentration of 10mg/ml, *Ananas comosus* (pineapple)leaves produced the highest inhibition of 86.50% (P<0.0001), compared to *Gliricidia sepium* (gliciridia) at 84.50% and *Chrysophyllum cainito* (star apple)*at* 83.75% inhibition (P<0.0001). At the same level of concentration, three other plant extracts, *Annona squamosa*had 56.00% (P<0.001) inhibited larvae in the migration assay,

Moringa oleifera with 58.75% (P<0.001) and *Tinospora rumphii* Boerl with 55.75% (P<0.001).

Ananas comosus and *Chrysophyllum cainito* showed very significant effects even when given at a lower concentration of 1 mg/ml, with 49.25% (P<0.001) and 47.75 % (P<0.001) inhibition, respectively (Figure 1). With the study done by Fernandez *et al* (2013), *Chrysophyllum cainito* extract was mixed with other two leaves extracts, makabuhay (*Tinospora rumphii*), and makahiya (*Mimosa pudica*). Ethanol was the best solvent to extract the bioactive components of the three plants. Eighty percent (80%) of *Haemonchus contortus* larvae died 30 minutes after exposure with the cocktail.Likewise, in a number of tests, tannins were reported in the phytochemical analysis of *C. cainito* as being responsible for its observed anthelmintic effects (Fernandez *et al*, 2013). The beneficial effects of condensed tannins on livestock are the reduction of the detrimental effects of parasitism in grazing livestock (Hoste, *et al*, 2006). The consumption of high concentration of condensed tannins (>7% of DM) has been associated with detrimental effects of ruminants such as reduction of food intake, growth inhibition and interference with the morphology and the proteolytic activity of microbes in the rumen. Low and moderate concentrations of condensed tannins (<6% of DM) have resulted in positive effects in the herbivores like increased growth and increased milk production (Min, *et al*, 2003; Waghorn and McNabb, 2003).

Pineapple leaves have previously been used in the Philippines as anthelmintic preparations for livestock (Jovellanos, 1997; Baldo, 2001). According to Githiori (2004) and Debnath *et al* (2013) the pineapple plant contains cysteine proteases (bromelain), have been reported to have anthelmintic properties.

Table 1. List of medicinal plants used *in vitro* for anthelmintic evaluation against *O. dentatum:* their common name and scientific names

Scientific Name	Common Name
Ananas comosus	Pineapple (Engl); Pina (Spanish); Abacaxi (Portuguese); Annachi pazham (Tamil); Nanas (Malay); Aananas (Many languages)
Chrysophyllum cainito	Caimito (Tag., Span.), Cainito (Engl.) Star apple, Star plum (Engl.) Niu nai guo (Chin.)
Gliricidia sepium	Madre de Cacao (Guatemala) Kakawate (Tag), Cacao de nance, Cacahnanance, Mata Raton, Madriado (Honduras)
Annona squamosa	Custard apple, sugar apple, sweet sop (English); Gishta (Arabic); Kannelappel (dutch), Atis (Tag); Attier, Cachiman canelle, pomme de cannelle (French); Rahmapfel, Süßsack (German); Ata, Sharifa, Sitaphal (Hindi); Buah nona, Nona sri kaya, Sri kaya (Malaysian); Fan-li-chi (Mandarin); Fructa do conde (Portuguese); Anón, Anona, Anona blanca, Candongo, Chirimoya, fructo do conde, pinha, saramuya (Spanish); Lanang, Makkhiap, Noina (Thai); Mang câú ta, na (Vietnamese)
Moringa oleifera	Horseradish tree, Radish tree, Drumstick tree, Mother's Best Friend, West Indian ben (Engl); Bèn ailé, Benzolive, Ben oléifère, Arbre radis du cheval (French); Behenbaum (german); Sàndalo ceruleo (Italian); Acácia branca, Marungo (Portuguese); Árbol del ben, Ben, Morango, Moringa (Spanish); Kachang Kelur (Malasia, Indon); Mulanggay (Tag); Kaanaeng-doeng (Thai)
Tinospora rumphii Boerl	Heavenly elixir (Engl) Makabuhay (Tag., Ilk.); Giloya (Ind.); Fa leng teng (Chin.)
Azadirachta indica	Neem, Nimtree, Indian Lilac, Margosa Tree, Neem Chal (Engl)
Trichantera gigantea	Madre de Agua (Tag), Cenicero, Tuno, Naranjillo, and Palo de agua (Span)
Leuacaena leucocephala	Ipil-ipil (Tag) White Leadtree, White Popinac (Engl) ; Subabool (Indian)

Gliricidia sepium (madre de cacao) was also active with 41.25% inhibition when given at 1mg/ml (P<0.01). Its effect became very significant when exposed at a higher concentration of 5mg/ml, which showed 72.75% inhibition (P<0.001). This validates the efficacy of the leaves extract as it has been used in the Philippines to treat external parasitism. Fresh, clean leaves are pounded, with its juice extracted and applied to affected animal body parts, once to twice a day for one week. Fresh leaves are also used as a fresh feeds for deworming purposes, especially in goats by many farmers in the countryside.

Gliciridia was found to contain 40.7g of condensed tannins/kg dry matter.Tannins have been found to have potential antidiarrheic, antidysenteric, antimutagenic, antinephritic, antioxidant, antiradicular, antiviral, bactericide, cancer-preventive, hepatoprotective, pesticide, psychotropic, and viricide activities (Duke *et al,* 1994). There is little evidence of toxic effects with ruminants fed either fresh or wilted leaves (Stewart, *et al,* 1996). In a reportpublished by the Cornell University (2009), goats safely consumed larger quantities of plants with tannins than cattle and sheep because goats have a salivary protein that binds to the tannins that the other livestock lack. It is reported in Asia that *Gliciridia* has apparent palatability problems (Trung, 1989), but farmers are continuously feeding it, especially in goats, because of its availability. One advantage of *Gliricidia* is having more biomass than *L. leucocephala* (Stewart *et al.* 1992), with annual leaf dry matter production generally ranging from about 2 t/ha/year (Wong and Sharudin, 1986) to 20 t/ha/year (Sriskandarajah, 1987).

Moringa oleifera leaves extract showed 58.75% inhibition, a very significant effect (P<0.001) when given the highest dose of 10mg/ml, and a significant effect of 41.00% inhibition at 5mg/ml concentration (P<0.05). Moringa had been used for a wide range of various ailments (Sreelatha and Padma, 2009). The different parts of this plant such as the leaves, seed, roots, bark, flowers and fruits and immature pods have been found to act as agents for the following effects: cardiac and circulatory stimulus, antiulcer, antispasmodic, diuretic, antihypertensive, possess antitumor, antipyretic, antiepileptic, antiinflammatory, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities, and treatments of other various ailments in South Asia (Anwar *et al,* 2007). Although, there have been no report on its deworming activity, results of this study found that *Moringa* has potential in this regard.

Annona squamosa leaves extract showed 56.00% inhibition at the highest concentration of 10mg/ml and 48.00% at 5mg/ml (P<0.001) against *O. dentatum* larvae. This conforms with other research where *Annona squamosa*was found to reduce faecal egg counts (FEC) and total worm counts in experimental lambs (Githiori *et al,* 2004).The anthelmintic properties of *Annona squamosa* indicated the presence of phenolic compounds in its aqueous leaf extract (Kamaraj and Rahuman, 2011), which was observed also by Fereira *et al* (2013) when they tested the leaves extract against eggs, infective larvae and adult forms of *Haemonchus contortus* in sheep. Plant phenolic compounds are diverse forms of proanthocyanidins and condensed tannins. They were found to prevent bloat in cattle, reduce gastrointestinal nematode numbers and flystrike (Waghorn and McNabb, 2003).

Tinosphora rumphii or makabuhay leaves (*Tag*.) showed 55.75% inhibition at 10mg/ml concentration (P<0.001), an extremely significant effect, but no

significant effectsat lower concentrations against *O. dentatum* larvae. Earlier studies on crude extracts of *Tinosphora rumphii* were highly effective against *Haemonchus* larvae *in vitro* and in reducing worm egg counts and worm numbers in sheep and goats (Sani *et al*, 2004).

Likewise, *Azadiractha indica* or neem tree leaves produced 51.50% inhibition at 10mg/ml (P<0.01), a very significant effect, but similar to *T. rumphii*, no significant effect was seen when applied at lower concentrations. In tropical Asia, fresh neem tree leaves were fed to sheep, which lowered the infection against *H. contortus* fecalysis count (Sani, 2004). Its active compounds were found to be a high content of condensed tannins, which might be responsible for its anthelmintic activity (Chandrawathani *et al*, 2002 and 2006; Githori *et al*, 2004; Costa *et al*, 2006). **Table 2** presents a summary of several plants used in this study as to their naturally occurring agents that possess anthelmintic properties.

Table 2. Aqueous leaves extracts evaluated against *E. dentatum*, and theirmean percentages of inhibition at different concentrations after 20 hours incubation time under room temperature.

Treatments	% Mean Inhibition* at different concentrations			
	1 mg/ml	2.5 mg/ml	5 mg/ml	10 mg/ml
Migration Buffer (-) Control	18.50	23.75	24.50	29.75
Levamisole (+) Control	94.97	96.00	95.50	98.75
Ananas comosus	49.25***	64.00***	84.50***	86.50***
Gliricidia sepium	41.25**	45.25**	72.75***	84.50***
Chrysophyllum cainito	47.75***	52.50***	59.75***	83.75***
Moringa oleifera	28.75 ns	35.25 ns	41.00 *	58.75***
Annona squamosa	19.75 ns	21.00 ns	48.00***	56.00***
Tinospora rumphii Boerl	11.50 ns	25.75 ns	34.00 ns	55.75***
Azadirachta indica	17.00 ns	22.00 ns	35.00 ns	51.50**
Trichantera gigantea	28.50 ns	31.75 ns	37.25 ns	42.25 ns
Leuacaena leucocephala	5.00 ns	12.50 ns	18.00 ns	21.75 ns

* Bonferroni Post tests results as compared to the negative control (P value= <0.01).
** Significant result at P<0.001.
*** Highly significant results at P<0.0001.

In the data analyses, the interaction between the leaves extracts and the concentrations used account for 5.11% of the total variance (P<0.0001) and was extremely significant. The plant leaves extracts account for 71.41% of the total variance (P<0.0001) and the concentrations account for 15.58% (P<0.0001), both effects were also extremely significant. In summary, the most effective plant species leaves in producing the highest *O. dentatum* larvae inhibition levels were from *Ananas comosus* (pineapple, 86.50%), *Gliricidia sepium* (madre de cacao, 84.50%), *Chrysophyllum cainito* (Star apple, 83.75%), *Moringa oleifera* (horseradish,58.75%), *Annona squamosa* (sweet sop, 56.00%), *Tinospora rumphii* Boerl (Makabuhay, 55.75%),and *Azadirachta indica* (Neem, 51.50%), which all proved extremely significant (P<0.001). Two plant species leaves extracts, those from *Trichantera gigantea* and *Leuacaena leucocephala*,had no significant anthelmintic effects on *O. dentatum* larvae.

The active compounds are plant secondary metabolites (PSM) (Githiori et al, 2006), the plant products that have been associated with defensive mechanisms of plants against herbivore grazing (Mueller-Harvey and McAllan, 1992). Saponins, alkaloids, non-protein amino acids, tannins and other polyphenols, lignins, glycosides, are all

PSM and some of them have been considered responsible for the anti-parasitic effect of plants (Githiori, 2006).

Fresh roughages supplied to a 30 kgs goat,with 3% of its body weight dry matter (DM) requirement, needs 0.9 kg DM a day. If the roughage to legumes ratio was 75%:25%, the DM needed for roughages was 0.67 kg and 0.22 kg for legumes. Converted the DM into fresh roughage (20% DM), a goat needs about 3.37 kg of roughage and 0.90 kg fresh legumes (25% DM). The legume part was the replaced components by the different leaves in controlling the parasites. Given 10 mg of leaves (DM)/ml as the effective concentration with the highest inhibition effect to larvae, in a goat's rumen with 3 L capacity, at least 151 grams of fresh leaves must be given daily for three consecutive days to effectively control the parasites.

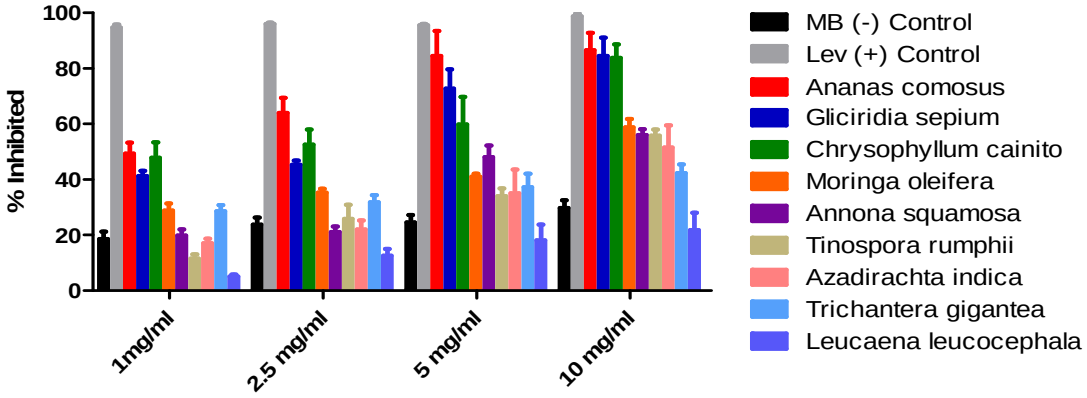

Figure 1. Inhibition percentage of nine aqueous leaves extract on *Oesophagostomum dentatum* larvae using larval migration assay.

CONCLUSIONS

This effort to scientifically screen and evaluate the effect of medicinal plants from the Philippines documented statistically significant inhibition of *O. dentatum* larvae by exposure to the leaf extracts of seven of the nine plant species examined. The results of this study validate the traditional use of these plants as having natural anthelmintic properties and they may be used with its pharmacological potentials for veterinary health management systems, especially in developing countries where these plants abound and the farmers typically lack the financial resources to routinely purchase commercial chemical treatments. The methods of this experiment also provide an additional resource to conduct future *in vivo* investigations on other parasites and with other plant species extracts. Furthermore, combination of DE and plant leaf extracts may worth investigation in the future combining the mechanical damage of the parasites with the natural effect of some plant leaves.

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Validation and molecular analysis of *β-1,3-GLU2 SNP marker* associated with resistance to *anthracnose* in Philippine carabao mango (*Mangifera indica* L. cv. 'Carabao')

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ABSTRACT

In the Philippines, mango anchors a million-dollar industry which is largely dependent on the export of a sole variety, the 'Carabao' mango. Varietal improvement of the 'Carabao' mango involves the introgression of anthracnose resistance to improve its yield and export quality. Molecular genomic tools such as single nucleotide polymorphism (SNP) markers provide a platform to accelerate breeding of resistant varieties through marker-assisted selection (MAS). Here, we developed and analyzed the molecular basis of an SNP marker within the pathogenesis-related β-1,3-glucanase 2 (β-1,3-GLU2) gene putatively linked with resistance to anthracnose. This SNP is an A/G transition causing a missense mutation (I196V) in glycosyl hydrolase family 17 (GH17), a highly conserved domain involved in physiologically important processes in plants, notably in response to biotic and abiotic stresses. Structural analysis suggested that the I196V mutation resulted in conformational changes in the enzyme's (β/α)8 TIM-barrel motif and catalytic groove by causing a steric clash with V261 residue, thereby possibly affecting the overall protein stability or catalytic activity and subsequently inhibit fungal defense response mechanisms. Our findings also demonstrated the association of this SNP marker with anthracnose resistance wherein mango accessions with the mutant allele 'G' showed significantly higher disease severity post-inoculation while those with wildtype allele 'A' showed phenotypic resistance against anthracnose. The positive correlation between the type of SNP allele present and reaction of mango to *C. gloeosporioides sensu lato* coupled with the ability of the marker to discriminate SNP alleles using a simple and cost-effective allele-specific PCR assay make it suitable for MAS. The results of this study support the utilization of the developed β-1,3-GLU2 SNP marker for routine screening of anthracnose resistant phenotypes as early as the seedling stage. This will help improve mango breeding efficiency and significantly reduce the expenses in field inputs, maintenance, and evaluation of material over years.

Keywords: AS-PCR, Carabao mango, *Colletotrichum gloeosporioides sensu lato*, Genotype-by-sequencing, Glucan endo-1,3-beta-glucosidase, Single nucleotide polymorphism

1. INTRODUCTION

Mango (*Mangifera indica* L.) is a commercially important fruit crop in the tropical and subtropical regions, particularly in Asia. It anchors a million-dollar industry ranking sixth in terms global fruit production after bananas, watermelons, apples, oranges, and grapes (FAO, 2019a). In 2018, the global production of mango fruit reached up to over 52 million metric tons, with more than one thousand varieties grown in Asia, Central and South America, and Africa (FAO, 2019b). Although there is increasing demand in developed countries, only 3-4% of the global production is traded internationally and the rest is traded and consumed domestically (Mitra, 2016). Despite its excellent qualities, the export potential of mangoes are not fully attained due to its short shelf-life, thin peel and low quality and production yield attributed to susceptibility to insect pests and pathogenic diseases. The high average temperature and relative humidity in tropical regions favor the rapid development of diseases at both the pre- and postharvest stages, which directly affect fruit quality and yield (Dodd et al., 1991). One of the most serious and destructive diseases of mango is anthracnose, which is primarily caused by the fungus *Colletotrichum gloeosporioides* (Dodd et al., 1991). This fungus causes leaf blight, blossom blight, mummified fruits, and dieback by infecting leaves, flowers and juvenile fruits (Arauz, 2000). During postharvest, germination of dormant fungal spores is induced by ripening and disease development becomes apparent when black, sunken, rapidly proliferating lesions develop on marketable fruits rendering them worthless and non-marketable (Akem, 2006).

Different disease management strategies have been developed to control the disease but these often involve excessive use of fungicides, which are expensive and damaging to the environment (Dodd et al., 1991). Another management method, the hot water treatment (HWT), needs to be revalidated for its fungistatic rather than fungicidal ability (Alvindia & Acda, 2015). In the absence of cheap, safe and efficient protection measures, breeding for resistance is valuable in solving the growing problem in the mango industry. However, traditional mango breeding programs are slow and challenging due to several

factors such as long juvenile stage, long generation time, high heterozygosity, and low crossing rates (0.1%). The detection of the disease poses additional problems in traditional mango breeding programs as infected fruits show no signs of the disease until the onset of ripening.

Advancements in molecular biology provide genomic tools such as DNA markers which can aid in the selection of target traits and to accelerate the breeding process of new varieties of mango. DNA markers are sequences with a known location in the genome and they can assist in breeding selection when found associated with a desired trait. Among the DNA markers, single nucleotide polymorphisms (SNPs) are advantageous in marker-assisted selection (MAS) due to its high density and abundance across the genome (Syvänen, 2005). SNPs are single base changes in the DNA, which allow a higher probability of finding an SNP-based marker within the gene of interest. SNP marker detection can be automated, enabling high-throughput analysis appropriate for breeding programs involving large populations (J. Kumar et al., 2011; Syvänen, 2005; Y. Xu & Crouch, 2008). Although several studies have already used SNPs in linkage mapping and estimation of genetic diversity in mango (Iquebal et al., 2017; D.N. Kuhn et al., 2016; David N. Kuhn et al., 2019; Sherman et al., 2015; Singh et al., 2016; Warschefsky & von Wettberg, 2019), SNP markers associated with important horticultural traits, including disease resistance, are yet to be developed and used for marker-assisted selection in mango breeding.

In the present study, a previously identified SNP marker located within a putative defense-related gene associated with resistance against *Colletotricum gloeosporioides* infection was developed and validated for use in marker assisted selection (MAS) of anthracnose resistant mango phenotypes. We hypothesize that this SNP plays a role in *C. gloeosporioides* pathogenicity on mango fruit that may confer resistance to anthracnose disease.

2. MATERIALS AND METHODS

2.1 Plant materials

A total of 143 mango varieties and strains from commercial growing areas and research institutions in the Philippines were used in the present study. Genetic resources in this collection include 130 'Carabao' mango strains, 9 commercial varieties, and 4 unknown cultivars. Scions of mango collections showing promising traits were collected and asexually propagated by cleft-grafting and maintained at the fruit crops nursery of the Institute of Plant Breeding, University of the Philippines Los Baños (UPLB), Laguna, Philippines.

2.2 Phenotypic screening for anthracnose resistance

Preliminary screening of mango accessions for anthracnose resistance was performed using the pathogen *Colletotrichum gloeosporioides sensu lato* isolated from mango samples collected from various provinces in the Philippines through tissue planting technique. The most aggressive isolate (Mg12) was used in the study (Figure). Molecular identity of this fungal isolate was established through PCR assay using Col1/Col2 (Martinez-Culebras et al., 2003) and CgInt/ITS4 (Mills et al., 1992) primer pairs. Fungal spores were harvested by adding 5 mL sterile distilled water on 7-day old culture and scraped using flamed L-spreader. Spore suspension was filtered on a four-layered sterile gauze cloth to filter only the fungal spores. Spore count was adjusted to 10^6 spores/mL using hemacytometer. Fruits were artificially evaluated *in vitro* via spore suspension droplet inoculation technique. Samples were disinfected using 20% sodium hypochlorite for 10 minutes, rinsed with sterile distilled water twice and blot dried using sterile tissue paper. Disinfected fruits were placed in a moisture chamber with moistened sterile cotton to maintain humid condition (Alcasid et al., 2018; Torres-Calzada et al., 2013). Each fruit was pricked with sterile 1 mL syringe to create wound and inoculated with 20 µL of spore suspension (10^6 spores/mL). Reaction to mango anthracnose was observed after 12 days of incubation following the disease rating scale (Table 1. Disease rating scale used for the phenotypic screening in this study.) and disease severity was calculated using the formula previously described by Paull (2002).

Table 1. Disease rating scale used for the phenotypic screening in this study.

Rating Scale	Description	Reaction
0	No infection	Immune
1	1-5% of the total fruit surface infected	Highly Resistant
3	6-15% of the total fruit surface infected	Resistant
5	16-30% of the total fruit surface infected	Intermediate
7	31-50% of the total fruit surface infected	Susceptible
9	More than 50% infection	Highly Susceptible

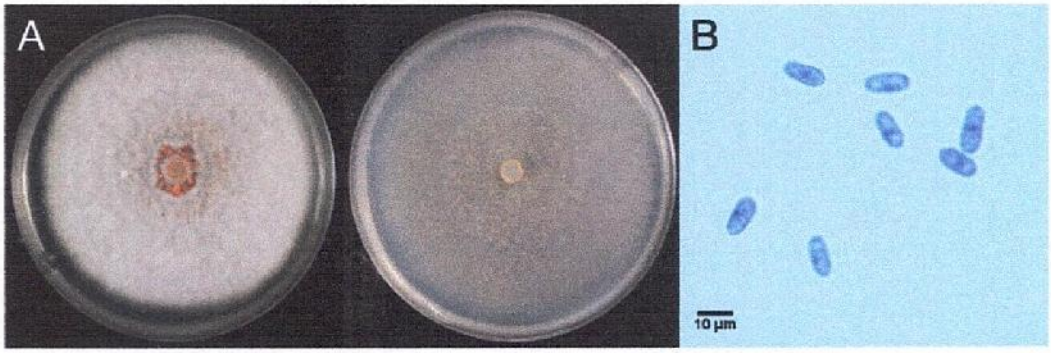

Figure 1. (A) Colony morphology of *C. gloeosporioides sensu lato* isolate MG12 and its (B) spores under 40X magnification.

Confirmatory evaluation of resistance was conducted for accessions that initially showed resistant and intermediate reactions in the preliminary trials. Approximately 120-day old mango fruits were disinfected and reactions were evaluated following the methods described above.

2.3 Genomic DNA isolation

Genomic DNA was isolated from the youngest, fully mature and green leaves following the modified CTAB protocol of Lachica et al. (2019). DNA stock solutions were quantified using Epoch Microplate Spectrophotometer (BioTek® Instruments, Inc., USA) and diluted to 50 ng/µL with sterile nanopure water. All extracted DNA and working stocks were stored at -20°C until use.

2.4 *β-1,3-GLU2* marker design and validation

A significant mango SNP (AlleleID 21881933) was recently identified by the Institute of Plant Breeding–Physiology Laboratory of UPLB (unpublished data) using Genotyping-by-Sequencing (GBS) generated by DArTseq™ platform (Diversity Arrays Technology Pty Ltd., Canberra, Australia). Gene annotation reveals that SNP 21881933 (A>G) is within a putative Glucan endo-1,3-beta-glucosidase 2 or *β-1,3-glucanase 2* (*β-1,3-GLU2*) gene. This SNP was developed into an allele-specific PCR (AS-PCR) marker for experimental validation. Gene-specific primers were designed based on the *Citrus sinensis* glucan endo-1,3-beta-glucosidase 2 (top hit) conserved regions flanking the SNP site using NCBI Primer-BLAST (Altschul et al., 1990). The gene specific forward primer (Glu2-F) was 5'—ACTGCAGCTAATTGGG—3' while the reverse primer (Glu2-R) was 5'—GGTTTGTAGTATCATTTGCT—3'. In addition, an allele-specific forward primer (AS-F), 5'—GTTCGTATCTCATGCTCAAAG—3', with a 3'-terminal nucleotide corresponding to the target mutant SNP allele "G" was designed using the Web-based Allele Specific Primer (WASP) tool (Wangkumhang et al., 2007). All primers were synthesized by Integrated DNA Technologies (IDT), Singapore.

PCR reactions were performed in a final mix of 10 µL using the iNtRON PCR Kit (iNtRON Biotechnology, Inc., South Korea) containing 1X PCR Buffer A, 0.2 mM dNTPs, 0.5 U i-Taq™ DNA Polymerase, 0.2 mM of each primer, 10 ng of template DNA, and sterile nanopure water. The following optimized thermal cycling conditions were performed on T100 Thermal Cyclers (BioRad® Laboratories, USA): 2 min at 94 °C, followed by 30 cycles of 20 s at 94 °C, 10 s at 50 °C (annealing), 30 s at 72 °C, and 1 cycle of 5 min at 72 °C. PCR products were visualized in 2% agarose gel electrophoresis using the GelDoc™ XR+ Gel Documentation System (BioRad® Laboratories, USA). The presence of the SNP allele 'G' was based on the detection of a 507-bp fragment in the AS-PCR assay. Amplifications that did not contain this fragment indicates the presence of the SNP allele 'A'. An 805-bp fragment, serving as internal control, indicates the successful amplification of the *β-1,3-GLU2* gene fragment. Analysis of variance was performed for the available phenotypic data, and the association between the SNP alleles and reaction to anthracnose (% disease severity) was analyzed by performing a Pearson correlation and linear regression analysis using IBM SPSS Statistics v23.0 software (SPSS, Chicago, USA).

2.5 Sequence analysis and molecular modelling of mango *β-1,3-GLU2 and SNP 21881933*

Amplified *β-1,3-GLU2* gene fragments from identified resistant and susceptible accessions were sequenced using Sanger technology (1st BASE, Selangor, Malaysia) to further validate the presence of the SNP and the identity of the target gene. Multiple sequence alignment (MSA) and identification of open reading frames (ORF) were performed using MEGA7 (S. Kumar et al., 2016) and UniPro UGENE v33.0 (Okonechnikov et al., 2012), respectively. Protein sequence analysis utilized the standard protein BLAST (BLASTp) tool and the Conserved Domain Database (CDD) of NCBI. Analysis and identification of conserved and functional residues were performed using ConSurf (Glaser et al., 2003; Landau et al., 2005). ORFs were translated using ExPasy (Gasteiger et al., 2003) and protein structure modeling was performed using Phyre2

(Protein Homology/analogY Recognition Engine version 2.0) (Kelley et al., 2015). The coordinates corresponding to the crystal structure of the glycoallergen endo-beta-1,3-glucanase (Hev b 2) from *Hevea brasiliensis* (top hit) was used to generate the model of *β-1,3-GLU2* in this study. UCSF Chimera software version 1.15 (http://www.cgl.ucsf.edu/chimera) was utilized for 3D model visualization, and analysis of molecular structures and mutation analysis, using default settings and criteria.

3. RESULTS

3.1 Phenotypic evaluation of anthracnose resistance

Isolate Mg12 used in this study amplified a single band product using the Col1/Col2 primer pair establishing that the isolate belongs to genus *Colletotrichum*. Using CgInt/ITS4 primer pair, a 450 bp product was amplified corresponding to the expected amplicon size for *Colletotrichum gloeosporioides sensu lato*. No amplification was observed in the control group.

Of the total 143 mango accessions evaluated, thirty (30) initially exhibited intermediate resistance (IR) and resistant (R) reactions, while the rest showed susceptible (S) and highly susceptible (HS) reactions in the preliminary screenings. Confirmatory evaluations showed that nine (9) accessions exhibited potential resistance to mango anthracnose after three trials. None of the 30 accessions exhibited immune reaction or 0% infection. The remaining 21 accessions showed intermediate and susceptible reactions to mango anthracnose (Table 2).

Table 2. Confirmatory evaluation of 30 mango accessions which initially showed intermediate resistance (IR) and resistant (R) reactions to *Colletotrichum gloeosporioides sensu lato*.

Sample	Accession	Variety	Source	Mean Disease Severity (%)
1	ADMINX	Unknown	Laguna	14
2	12-182	Haden	Laguna	11
3	12-070	Unknown	Laguna	14.2
4	16-016	Haden	Guimaras	11
5	Haden Hawaii	Haden	Laguna	11
6	Tommy Atkins	Tommy Atkins	Laguna	12
7	12-265	Haden	Davao	11
8	Gouveia	Gouveia	Guimaras	15
9	12-112	Carabao	La Union	12.5
10	12-052	Carabao	Laguna	36.67
11	12-080	Carabao	Quezon	30
12	12-088	Carabao	Zambales	29
13	12-171	Carabao	Laguna	25
14	GES 73	GES 73	Guimaras	25
15	12-209	Carabao	Quezon	25.33
16	12-013	Carabao	Laguna	44.67
17	12-014	Carabao	Laguna	43
18	12-027	Carabao	Laguna	31
19	12-075	Carabao	Quezon	36
20	12-081	Carabao	Quezon	33.67
21	12-090	Carabao	Zambales	37
22	12-104	Carabao	La Union	36.67
23	12-106	Carabao	La Union	33
24	12-111	Carabao	La Union	36
25	12-166	Carabao	Laguna	47
26	12-170	Carabao	Laguna	37
27	12-179	Carabao	Laguna	42
28	12-092	Carabao	Zambales	56
29	12-094	Carabao	Zambales	56
30	12-178	Carabao	Laguna	58

3.2 Experimental validation by AS-PCR

The AS-PCR assay optimized in this study yields a polymorphic banding pattern that can be used to differentiate genotypes between mango accessions. Results show that a 507-bp allele G-specific PCR product was amplified in 22 of the 30 accessions tested using the allele-specific primer pair (AS-F and Glu2-R). The remaining 8 accessions, namely 'ADMINX', '12-182', '12-070', '16-016', 'Haden Hawaii', 'Tommy Atkins', '12-265', and 'Gouveia', did not amplify this PCR product (Figure). The specific nucleotide at the 3' end of the AS-F primer will only allow amplification in the presence of the allele G and consequently prevents the amplification of a mismatched allele A. This means that the 22 accessions that amplified the 507-bp PCR product contain the G allele, while the 8 other accessions contain the A allele. To further ensure the accuracy of the AS-PCR, gene-specific primers Glu2-F and Glu2-R were introduced in the reaction to amplify an 805-bp *β-1,3-GLU2* gene fragment. This PCR product was successfully amplified in all tested accessions indicating a successful PCR reaction and confirming the presence of the *β-1,3-GLU2* gene in all samples.

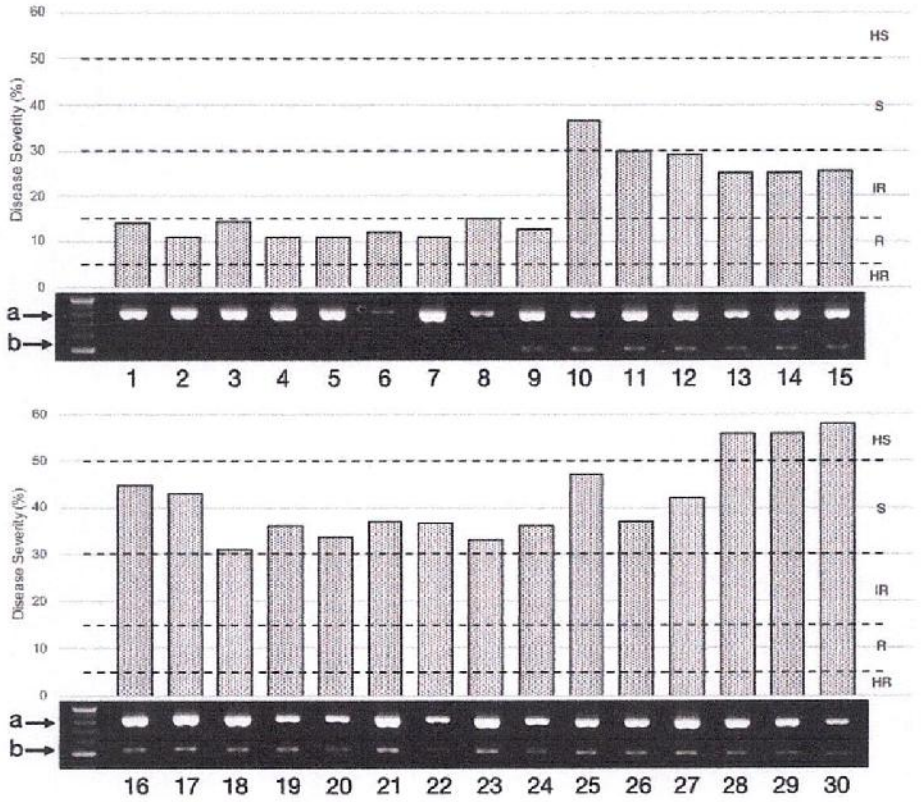

Figure 3. Association of anthracnose resistance phenotypes (% disease severity) with the banding patterns from the SNP 21881933 derived AS-PCR assay of different mango accessions. The upper band (a) is the amplification of the *β-1,3-GLU2* fragment and serves as a positive control. The lower band (b) is an allele-specific amplification. Lanes 1-9 are identified resistant accessions while lanes 10-30 are identified susceptible to intermediately resistant accessions based on Table 2. (HS) highly susceptible; (S) susceptible; (IR) intermediate; (R) resistant; (HR) highly resistant

3.3 Association of SNP 21881933 alleles with phenotypic anthracnose resistance

All but one of the 9 resistant accessions tested showed a consistent genotype–phenotype relationship wherein resistance to anthracnose was associated with the *β-1,3-GLU2* allele A. On the other hand, intermediate resistance and susceptibility to anthracnose were associated with the *β-1,3-GLU2* allele G, as shown by its presence in all accessions with higher % disease severity (>30%). Only the resistant accession '12-112' yielded an inconsistent association between genotype and phenotype, which may be due to a different mode of resistance. Moreover, analysis of phenotype variance (one-way ANOVA) showed that the level of resistance is significantly different between the alleles A and G genotypes ($P < 0.01$). In terms of genotype-phenotype association analysis, Pearson correlation and regression analysis showed that SNP 21881933 alleles is significantly correlated to anthracnose resistance phenotypes ($r = 0.74$; $P < 0.01$).

3.4 Molecular analysis of SNP 21881933 and mango *β-1,3-GLU2* gene

PCR products amplified by the gene-specific primers in this study were sequenced to confirm its identity and the presence of SNP 21881933 in mango accessions. All amplicon sequences significantly matched to a *β-1,3-glucanase 2* (*β-1,3-GLU2*) protein in the NCBI RefSeq database. Sequence comparisons with *β-1,3-GLU2* of closely related fruit tree crops confirms

an A > G nucleotide transition at the SNP 21881933 site (Figure 1-A). This leads to a missense mutation in the translated *β-1,3-GLU2* protein with an isoleucine to valine substitution within the conserved Glycosyl hydrolase family 17 (GH17) domain (Figure 1-B). ConSurf analysis predicts that this specific isoleucine residue (I196) is a structural residue that is highly conserved among closely related fruit tree crops (Figure 1-C, D).

The predicted protein structure of mango *β-1,3-GLU2*, which was modelled based on the crystal structure of *H. brasiliensis* endo-beta-1,3-glucanase (Hev b 2), exhibits the canonical $(\beta/\alpha)_8$ TIM-barrel motif and the catalytic groove across the protein surface found in other glucan endohydrolases (Receveur-Bréchot et al., 2006) (Figure 1-E). Superimposition of the structure models with A and G alleles also revealed changes in the predicted folding pattern of residues 82-90 (α-helix), 119-127 (extended loop), and 293-298 (extended loop) of the mutant *β-1,3-GLU2* (Figure 1-F). Based on the predicted model, the I196V mutation is situated near the catalytic groove where the binding site of substrates containing β-1,3-linked glucose residues is situated (Figure 1-G). Since I196 is predicted to be a structural residue, replacement with a valine residue at this position might result to structural changes in the catalytic groove that may affect the substrate binding specificity of the protein. To investigate the possible effects of this mutation, I196V was introduced using UCSF Chimera v.1.15 and predicted a significant clash with V261 residue having an overlap of 0.780 Å and distance of 2.980 Å (Figure 1-I).

4. DISCUSSION

Fruit tree breeding methods conventionally rely on phenotypic evaluations of parental lines to be used for varietal improvement. Such selection practices are subjective and are highly variable depending on the environment of the plant. Marker-assisted selection (MAS) provides a more rapid, accurate and discriminative way in identifying individuals as parentals for breeding and development of plant varieties with desired agronomic characteristics. In this study, an SNP marker within the β-1,3-glucanase 2 (*β-1,3-GLU2*) gene in mango was found to be associated with resistance to anthracnose and developed for MAS using a simple AS-PCR assay.

β-1,3-glucanases (E.C. 3.2.1.39) are enzymes widely found in bacteria, fungi, viruses, and plants that catalyze the hydrolysis of 1,3-β-D-glucosidic linkages between β-1,3-glucans – a major component of fungal and plant cell walls (Torres et al., 2015; X. Xu et al., 2016). In plants, β-1,3-Glucanase genes (GLUs) forms complex and diverse gene families playing important roles in physiological and developmental processes, including pathogen defense mechanisms (Doxey et al., 2007). GLUs and other pathogenesis-related proteins like chitinase and phenylalanine ammonia-lyase (PAL) are considered key enzymes in the control of plant disease in resistant systems (Zeng et al., 2006). Specifically, plant GLUs defend against pathogen infections by hydrolyzing β-1,3-glucans in fungal cell walls or by promoting the release of cell wall-associated immune elicitors that further stimulate defense reactions (X. Xu et al., 2016).

Previous studies have shown evidences of β-1,3-glucanase's role in *Colletotrichum* infection. In maize, nine GLUs were significantly upregulated in leaves during *Colletotricum graminicola* infection as a result of pathogen-associated molecular patterns (PAMP)-triggered defense response (Oliveira-Garcia & Deising, 2013). Similarly, strawberry plants infected with *C. fragariae* or *C. acutatum* induced the expression of two GLUs to over a thousand fold (Shi et al., 2006). In mango, Zhang et al. (2013) previously reported that β-aminobutyric acid (BABA) treatment in mango fruits significantly enhanced the activities of β-1,3-glucanases and effectively suppressed anthracnose caused by *C. gloeosporioides* during storage at 25°C. Increased β-1,3-glucanase activities was also attributed to reduction of postharvest anthracnose and enhancement of disease resistance in mango fruit after exogenous nitric oxide (NO) treatment (Hu et al., 2014). These previous findings indicate that β-1,3-glucanases significantly respond to *Colletotrichum* infection and possibly elicit defense response mechanisms involving the enhancement of defense-related enzyme activities to confer resistance to the pathogen.

Our results describe a possible mechanism of resistance against *C. gloeosporioides* in mango involving β-1,3-glucanases. A positive correlation between the presence of SNP 21881933 mutation and increased susceptibility to the disease implies that this specific mutation might have significantly reduced the activity of β-1,3-glucanase 2 enzymes, which have a direct role in defense response. As a PR protein, β-1,3-glucanases have been shown to directly defend against fungi infection by hydrolyzing fungal cell walls leading to fungal cell lysis (de la Cruz et al., 1995; Sandhu et al., 2017). While the mutant allele G found in susceptible accessions could have deleterious effect on the enzyme function rendering it vulnerable against infection and disease development, the β-1,3-GLU2 allele A, on the other hand, could be inferred as a biologically-active protein retaining its hydrolyzing function. This conclusion is supported by the fact that there is a significantly higher disease severity in all accessions with the G allele post-inoculation, while accessions with A allele had significantly reduced infection.

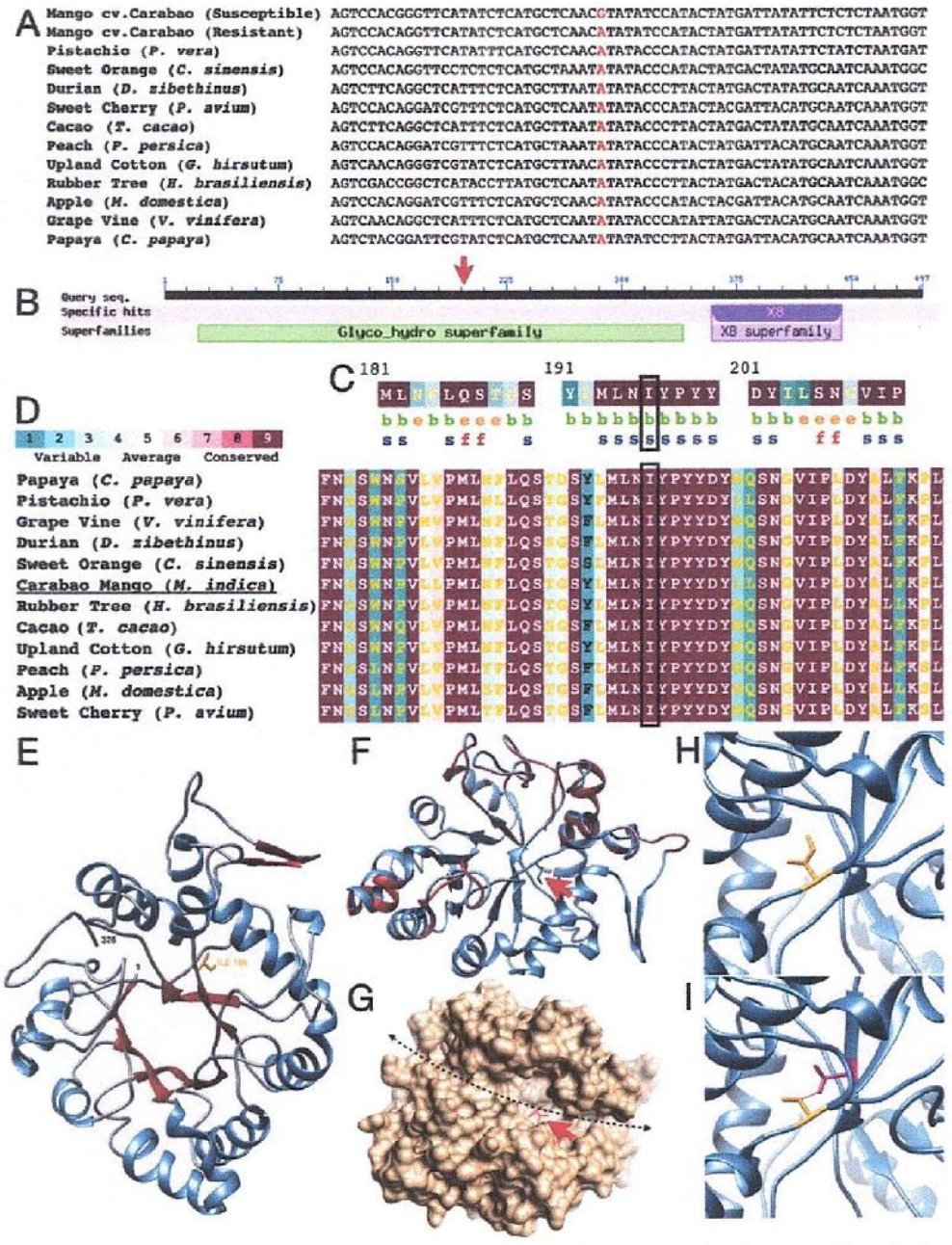

Figure 1. SNP 21881933 is a A > G transition in mango *β-1,3-GLU2* leading to an Isoleucine to Valine substitution. (A) MSA showing the SNP 21881933 site at position 6:17813013 (indicated in red) in mango *β-1,3-GLU2* and its homologs. The adenine [A] base is conserved between mango, apple, cacao and other fruit crops. (B) The isoleucine to valine substitution (I196V) caused by SNP 21881933 is predicted to be within the conserved protein domain family Glycosyl hydrolases family 17 (GH17; pfam00332) through the NCBI conserved domain search service (CD-search). A red arrow indicates site of I196V mutation relative to the GH17 domain. (C) ConSurf analysis of the mango *β-1,3-GLU2* protein sequence shows that 196I is highly conserved and predicted to be a buried residue (b), and thus likely to be a structural residue (s). (D) 196I in mango is highly conserved in β-1,3-glucanases of other fruit tree crops based on ConSurf Color-Coded MSA. Yellow letters in (B,C) indicates insufficient data where calculation of conservation was performed on less than 10% of the sequences. (E) Complete 3D structural model of the wildtype mango *β-1,3-GLU2* protein based on Phyre2 modelling from the *H. brasiliensis* β-1,3-glucanase crystal structure. The β-sheet strands (red) surrounded by the α-helices (blue) form the (β/α)8 TIM-barrel motif referred to in the text. Numbers 1 and 326 indicate the N- and C-terminal ends of the polypeptide chain, respectively. 'ILE 196' indicates the position of the isoleucin residue at the mutation site. (F) Superimposition of the structural models from mango *β-1,3-GLU2* with allele A (blue) and allele G (red) reveals some structural differences, with 97.52% identity. A red arrow points to the I196V substitution caused by SNP 21881933. (G) Molecular surface model of the mango WT *β-1,3-GLU2* protein showing the proximity of 196I residue (red arrow) to the catalytic groove indicated by the curved dashed line. (H,I) The effect of the mutation on the *β-1,3-GLU2* atomic interactions (clashes/contacts) is shown in H and I. (H) In the wildtype A allele, Ile196 residue (yellow) does not interact with any other residues within 5.0 Å. (I) In the SNP 21881933 mutation, Val196 (yellow) forms a steric clash (pink line) with Val261 (magenta) indicating a direct unfavorable interaction.

Missense mutations caused by SNPs occurring at gene coding regions generally affect protein stability, protein-protein interactions, and critical components of biological reaction (Zhe Zhang et al., 2012). SNP 21881933 described in this study has been found to be located within the highly conserved Glycosyl hydrolases family 17 (GH17) domain. This domain family is involved in physiologically important processes in plants, such as response to biotic and abiotic stresses, defense against herbivores, activation of phytohormones, lignification, and cell wall remodeling (Opassiri et al., 2006). Interestingly, another conserved domain called C-terminal X8 family carbohydrate-binding domain is also present in the expressed *β-1,3-GLU2* gene close to the SNP site. This domain is primarily involved in carbohydrate binding and cleaving (1,3)-beta-D-glucosidic linkages along with GH17 family (Marchler-Bauer et al., 2014). These suggest that the SNP is situated near or within the active site region of the enzyme and mutations within this domain could affect any of these functions. Any conformational change altering the active sites of proteins, as well as mutations quite close to it, can affect biochemical reactions because catalytic reactions are very sensitive to the precise geometry of these active sites for both of the products and reactants (Zhe Zhang et al., 2012).

To analyze the possible structural effects of the SNP mutation, we generated the predicted protein structure of the isolated mango *β-1,3-GLU2* and introduced the mutation in silico. Based on the three-dimensional structure model, the resulting I196V mutation is situated within the typical eightfold β/α TIM-barrel motif and the catalytic groove across the protein surface strictly conserved in other glucan endohydrolases of the GH17 family. This motif consists of an internal crown of eight β-strands connected to an outer crown of eight α-helices. According to Receveur-Bréchot et al. (2006), the active site of these enzymes consists of two glutamate residues in strands *β4 and β7* acting as proton donor nucleophile residue, respectively. These correspond to residues E50 and E240 in mango *β-1,3-GLU2*. Moreover, the three-dimensional molecular surface model showed that the I196V substitution constitute a part of the catalytic groove where β-1,3 linked glucan trisaccharide substrates are accommodated (Receveur-Bréchot et al., 2006). These observations suggest that I196, being a conserved structural residue, is necessary in maintaining the precise conformation of the enzyme's active site.

We hypothesized that the replacement of I196 mango *β-1,3-GLU2* with a valine residue caused contacts/clashes with the surrounding residues. Based on mutation analysis, a steric clash between V196 and V261 side chains was predicted with an overlap of less than 0.8 Å. This indicates a direct unfavorable interaction wherein atoms are too close together allowing van der Waals forces between the two residues. Such molecular interactions could result to structural perturbations. Since the I196V mutation is proximal to the active site and catalytic groove of mango *β-1,3-GLU2*, a clash between V196 and V261 could affect conformational stability and topology of the region possibly inhibiting the interaction of the substrate with the active site.

Together, these results suggest that the mutation caused by SNP 21881933 and its predicted structural changes most likely affected the catalytic activity of the wildtype *β-1,3-GLU2* in defense response against *C. gloeosporioides* infection. Although our findings provided an insight in the structural impact of the SNP mutation, functional studies are needed in order to confirm and elucidate further these mechanisms of resistance. Moreover, despite a significant positive correlation between *β-1,3-GLU2* alleles and degree of *C. gloeosporioides* infection, the biological function of β-1,3-glucanases alone cannot be conclusively associated to anthracnose resistance. Plant defense response against fungi is complex and requires involvement of regulatory factors that determines the susceptibility or resistance of plant to a particular pathogen.

The developed *β-1,3-GLU2* SNP marker reported in this study will enable marker-assisted selection of anthracnose-resistant mango as early as the seedling stage. We recommend this functional marker for routine genotyping of parental and hybrid mango to facilitate and improve the efficiency of mango breeding programs.

5. CONCLUSION

In crop production where specific quality traits dictate either significant economic losses or gains, more efficient breeding strategies for food crops are needed. Advancements in molecular biology provide genomic tools such as molecular markers which can aid in the selection of target traits to shorten selection times and to accelerate the breeding process of new varieties of mango. As demonstrated by this study, SNP markers can be used to identify mango with phenotypic anthracnose resistance as early as the seedling stage to significantly reduce the expenses in field inputs, maintenance, and evaluation of material over years. AS-PCR is a simple and effective method to employ such markers, but these can be also used in high-throughput genotyping technologies for large-scale screening and analysis. Our results show that *β-1,3-GLU2* is a reliable marker for screening and developing anthracnose resistant breeding lines that can complement the conventional mango breeding approach. However, due to the limited sample size and phenotypic data available in this study, it is recommended

to further employ the developed marker in a segregating population with a large sample size to strengthen the genotype-phenotype association analysis.

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Molecular characterization and association of lactoferrin gene to subclinical mastitis in goats (*Capra hircus*)

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ABSTRACT. The study characterized the lactoferrin (Lf) mRNA gene in different goat breeds in the Philippines and determined its association with subclinical mastitis (SCM). The study involved collection of milk at second week of lactation (n=75) and blood samples (n=5) to obtain extracted RNA and using cDNA to amplify Lf gene through polymerase chain reaction. The nucleotide and amino acid sequences were determined and used as reference in the evaluation of phylogenetic relationship. Amplified products were utilized for RFLP analysis before determining the association of the gene with SCM. Results of the study demonstrated that Lf gene in goats registered a molecular weight of 2135. Nucleotide and amino acid sequence of Lf gene revealed high similarity (99%) in Saanen, Anglo-Nubian and Philippine native goats with that of *Capra hircus* (U53857) Lf gene submitted to GenBank. Phylogenetic studies showed that Lf gene of Anglo-Nubian, Saanen and Native goats clade together with Lf gene of *C. hircus* (U53857). Three genotypes in goats were documented using the restriction enzymes *AluI* and *HaeIII*. Based on the Statistical analysis, association (comp 5.65, p = 0.0308) has been established between the Lf genes of goats with genotype BB to SCM using *HaeIII* restriction enzyme.

Keywords: Anglo-Nubian; Lf gene; Philippine native goat; Saanen.

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Introduction

Milk coming from dairy animals meets the basic requirements of the body. The significant increase of milk, milk fat, and protein in dairy animals is a result of advanced researches in the past two decades (Tsuda et al., 2000).

Mastitis is an economically significant infectious disease of dairy animals. Losses are due to decreased quantity and quality of milk, heightened by medicine and labor cost (Hogeveen &Van der Voort, 2017; Waminal, Tubalinal, & Mingala, 2019). Limiting the occurrence of subclinical mastitis (SCM), therefore, can be a vital strategy in reducing economic losses. To understand the mechanism on the susceptibility of animals to mastitis, genetic traits for milk production should be considered. The use of genetic markers in the selection of animals for breeding has also be considered (Rupp & Boichard, 2003; Gholizadeh, Mianji, & Zadeh, 2018).

Lactoferrin (Lf) gene, also known as 'red protein from milk', is an iron-binding protein that is found in most fluids in the body (Marchweka, Roterman, Strus, Spiewak, & Majka, 2012) and is the most important iron-binding protein in milk (Collins, Flores, Wang, & Anderson, 2018). Neutrophils and inflamed tissues release Lf and considered to have direct antimicrobial properties (limits bacterial proliferation and adhesion to microbes) and have a role in innate immunity (Van Der Strate, Belijaars, & Molema, 2001; Valenti & Antonini, 2005; Legrand et al., 2008; Walker, 2010; Siqueiros-Cendon et al., 2014). Furthermore, Lf possesses abundant health benefits such as anti-fungal, anti-parasitic, anti-viral, and anti-tumor properties (Niaz et al., 2019).

The potential of Lf gene (Lonnerdal & Iyer, 1995) as genetic marker for mastitis resistance in dairy animals needs to be elucidated in order to validate its role in mastitis (Pawlik, Sender, & Korwin-Kossakowska, 2009). The selection of superior individuals that show resistance or susceptibility to mastitis based on data generated through molecular studies of the Lf gene will be necessary for the selection of animals for breeding (Sharma, Chakraborty, & Gupta, 2015). This strategy may boost the economic condition of the farmer and nurture the dairy industry as a whole while promoting a mastitis-free dairy herd.

Currently, the information about Lf gene in goats (*Capra hircus*) is limited. Genetic characterization of Lf gene would augment their possible role in occurrence, onset and disease resistance to SCM (Pawlik et al., 2009). The potential of Lf gene as a marker for mastitis resistance in dairy animals is important and will augment existing marker-assisted breeding and selection resulting in improved dairy performance of dairy animals (Shimazaki & Kawai, 2017; Waminal et al., 2019). In cattle production, molecular characterization and genetic diversity of Lf gene have been associated with different traits and disease resistance and have been included in their breeding and selection techniques (Sharma et al., 2015).

The main goal of this study is to characterize and identify Lf gene as a genetic marker for SCM resistance in goats through molecular analysis and association of nucleotide polymorphisms with the presence of SCM.

Material and methods

The collection of samples for analysis was conducted from goats in selected provinces in Central Luzon, the Philippines.

Sample collection

A total of 75 goats (30 Anglo-Nubian, 30 Saanen and 15 mixed breeds) on their second week of lactation onwards and 5 Philippine native goats (not lactating) were selected from previously identified goat farms in Luzon (Farm 1 – 15.6310157, 120.5991522; Farm 2 – 15.4557851, 121.3377025; Farm 3 – 15.7409607, 120.9273197). Housing, feeding, and overall management of the animals per farm were not altered or changed for the conduct of this study. In addition, the farms included in the study housed their animals in concrete housing in group pens with elevations. It was during the summer season (March-May 2018) in the Philippines during the collection of the samples. The test animals were manually milked from the udder and pooled to collect 30 mL of milk sample for ribonucleic acid (RNA) extraction. The milk was collected in a 50 mL conical tube and was placed in a cooler with ice transported to the laboratory for processing. In case the milk was not processed immediately, it was placed in a refrigerator and processed the following day. For the five (5) native goats, RNA was extracted from blood as these animals were not lactating. Five (5) mL of blood was collected from the jugular veins of the animals using EDTA tube. Samples were processed that day after collection.

RNA Extraction

RNA extraction from milk and blood samples from the goats was done following the Promega protocol with some modifications. For milk, 30 mL of raw milk was centrifuged at 4,000 rpm for 20 min. After centrifugation, the fat layer and the supernatant were discarded, and afterwards the pellet was suspended in one (1) mL PBS (phosphate-buffer-saline, a pH of 7.4). This suspension was centrifuged again at 4,000 rpm for 10 min. After this step, the pellet was collected into 2 µL microtube after removing the supernatant fluid. One (1) mL of PBS was mixed again with the pellet and was centrifuged for 14,000 rpm for 1 min. This process was repeated twice and the supernatant was removed to collect the pellet.

For blood, 500 µL of the buffy coat was aspired after centrifugation at 4,000 rpm for 5 min and transferred into the 2 µL microtube, and then added with 2-3 volumes of 0.14 NH_4Cl. The mixture was mixed by sonication and centrifuged at 14,000 rpm for 1 min. Supernatant was removed and the process was repeated twice to collect the pellet.

The pellet from the milk and the blood was added with 1 mL cell lysis solution and was subjected to sonication to mix the pellet and cell lysis. After which, it was centrifuged to 14,000 rpm in 1 min. The supernatant was discarded and 500 µL of nuclei lysis and 300 µL protein precipitation solution was added next and centrifuged for 14,000 rpm for 1 min. After discarding the supernatant fluid, isolated cells were dissolved with 1 mL Trizol reagent and incubated for 10 min at room temperature. Then 200 µL of chloroform was added. The mixture was centrifuged at 14,000 rpm for 15 min. at 4°C. The solution was decanted and pellets were washed by 1 mL 75% ethanol and centrifuged for 5 min at 8,000 rpm at 4°C. Pellets were dried and reconstituted with 30 µL diethyl pyrocarbonate (DEPC) treated water.

Reverse-transcription - polymerase chain reaction (RT- PCR)

RT-PCR kit (TaKara™) was used to synthesize the cDNAs from the total RNA samples extracted. One µL of dT random primers were mixed with 1 µL dNTP, 5 µL of RNAse free water and 3 µL of RNA template. The solution was incubated for 5 min at 65°C before running for PCR. The prepared RNA primer mix was mixed

with 4 µL 5x buffer, 0.5 µL RNAse inhibitor, 1 µL reverse transcriptase, and 4.3 µL RNAse free water. This was subjected to PCR run for segment 1, 10 min. at 30°C; segment 2, 45 min. at 50°C; and segment 3, 5 min. at 95°C. The β-actin was used to determine if the DNA has been produced through PCR.

PCR assay

Primers (Table 1) were designed using Primer3 server and Primer-BLAST using the Caprine Lf gene (Accession number: U53857) stored from National Center for Biotechnology Information (NCBI).

Table 1. Primers used for the amplification of cDNA.

Gene	Accession Number	Primer Sequence (5'-3')	Fragment Length (bp)
	Primer 1	Forward- AGACATGAAGCTCTTCGT Reverse- GAGTACGGACAACACCGGGC	834
	Primer 2	Forward- ATCCTTCGCCCGTTCTTGAG Reverse- CGCCGAATCTACTTTTGAGG	561
	Primer 3	Forward- GCCCAGGTCCCTTCTCATG Reverse- GGGCACAGCTCTGACTAAAG	736
	Primer 4	Forward- AGAAAGCAAATGAGGGGCTC Reverse- TTACCTCGTCAGGAAGGCCG	749

All PCR assays were performed in 20 µL reaction volume containing 2 µL of genomic DNA template, 10 pmol of each primer and PCR master mix. The amplification of the Lf gene was carried out in a thermocycler (SimpliAmp, Thermofisher) under the optimized conditions. Briefly, initial denaturation at 94˚C for 5 min., followed by 40 cycles consisting of denaturation at 94˚C for 1 min., annealing at 58˚C for 1 min. and extension at 72˚C for 1 min., with final extension at 72˚C for 5 min.

After amplification, 1 µL of the PCR product was electrophoresed in 1.5 % agarose gel containing 1X TAE buffer at 70 volts for 30 min. and visualized under ultraviolet light using UV transillumination advance imaging system. To ensure that amplification products are of the expected size, a 1 kb plus DNA ladder was run simultaneously as a marker.

Gene sequencing and phylogenetic tree construction

Lf products were submitted for sequencing. Sequences were assembled using Mega 7 software. The forward and reverse sequences for each primer were assembled to form contigs of the respective regions. The gene sequences were compared with the Lf mRNA sequences to annotate different exonic regions putatively to identify SNPs in respective regions. The contiguous Lf gene nucleotide sequence was subjected to Basic Local Alignment Search Tool (BLAST) at NCBI database to determine the sequence similarity with the corresponding regions of other species. DNA nucleotide sequence was also conceptually translated using MEGA 7 software and compared with that of NCBI Genbank for Caprine and Bubaline Lf gene to detect amino acid changes. Phylogenetic trees were derived using MEGA 7 software using Maximum Likelihod method with 1000 bootsrap replications (Tamura et al., 2011).

Restriction fragment length polymorphism (RFLP) analysis

The Lf PCR products using primer 2 (23 µL) were subjected to endonuclease digestion in goat to amplify exon 2, 3 and 4 of Lf gene with an amplicon size of 561 bp.

Restriction enzymes (Table 2) that could cut the fragments were analyzed using Sequence Manipulation Suite: Restriction map (Stothard, 2000). RFLP was conducted by preparing the reaction mixture composed of 7 µL of PCR product, 5.15 µL of sd2H_20, 0.35 RFLP buffer and 0.075 µL for each enzyme. Samples were incubated at 37°C for 4h. Restriction fragments were resolved on 2% agarose gel in horizontal electrophoresis. The restriction-digested gene fragments were visualized and documented using a UV transilluminator (FlourChemE by ProteinSimple ™). The differences in fragment yielded by various restriction enzymes that would indicate polymorphism in a particular gene were analyzed and compared.

Table 2. Restriction enzymes used for the RFLP analysis.

Restriction Enzymes	Lf Gene Loci
AluI (AG/CT)	21, 53, 78, 295, 418, 465, 472
HaeIII (GG/CC)	353, 380, 492

Association of Lf gene with SCM

Samples of amplified Lf gene from the milk of goats where polymorphisms shown by different genotypes of the target gene previously exhibited after RFLP were taken as reference in tracing back the goats suspected for SCM and for California mastitis test (CMT) evaluation utilizing their milk as samples. A total of 69 goat samples were used in the study that associated expression of Lf gene and in the occurrence of SCM. Physical assessment for SCM covered the evaluation of the mammary gland and milk that involved visual observations and palpation.

CMT was used as an indirect method of measuring SCC as the indicator of intramammary infection as it has the advantage of being a quick, cheap, and simple 'animal side' test (Persson & Olofsson, 2011). CMT scores for all animal subjects were classified as non-mastitic if CMT score result is 1 or lower and mastitic if CMT score result is 2 or higher.

Statistical analysis

Univariate analysis on the possible association between the genotypic frequency and the occurrence of SCM was examined using Chi-square ($\chi 2$) by a certified statistician (Petrie & Watson, 2006).

Results and discussion

Of the 80 samples collected, only 4 (5%) samples produced complete Lf mRNA sequences in goats. It has been shown that the mRNA sequence of Lf gene was detected in the milk samples of Anglo-Nubian and Saanen and in the blood samples of the Native goats. Target primers 1, 2, 3, and 4 were amplified and generated an amplicon size of 834 bp, 561 bp, 736 bp, and 749 bp, respectively.

Native goat Lf mRNA sequence resulted in an average of 2133 bp while there were 2134 bp in Anglo-Nubian and 2135 bp in Saanen. Goat Lf nucleotide sequences were aligned with other ruminants and other species. Statistical nucleotide pair frequency of the 14 aligned nucleotide sequences showed an average of 1334 identical pairs, 751 transitional pairs, and 60 transversional pairs. Among the different goat breeds' nucleotide pair frequencies, the average identical pairs were 2118 with 2 transitional pairs and 6 transversional pairs. There was a high similarity of nucleotide sequences between the breeds of goats being studied (Table 3). It shows that there is a high similarity in the nucleotide and amino acid sequence of different goats. This may be due to the highly conserved region that can be found in the Lf gene. This is essential to maintain their distinctiveness from other species.

Table 3. Nucleotide and amino acid sequence percentage similarity of Native, Anglo-Nubian and Saanen breeds in reference to other ruminant species.

Species (Accession No.)	Nucleotide Sequence			Amino Acid Sequence		
	Native	Anglo Nubian	Saanen	Native	Anglo Nubian	Saanen
C. hircus (U53857)	99.24%	99.44%	99.58%	99%	99%	99%
O. aries (NM_001024862.1)	97.74 %	97.97 %	98.03 %	97 %	98 %	98 %
B. grunniens (DQ387455.1)	93.94 %	94.03 %	94.26%	93%	94 %	94 %
B. bubalis (JF825526.1)	94 .09%	94.28%	94.38%	93%	94 %	94 %
B. indicus (GU059864.1)	93.91%	94.14%	94.14%	93%	93%	94 %
B. Taurus (FJ589071.1)	94.04%	94.05%	94.29%	93%	93%	94 %
E. caballus (NM_001163974.1)	77.12%	77.09%	77.19%	77 %	77 %	77 %
S. scrofa (M81327.1)	72.80%	72.97%	73.02%	72%	72%	72%
H. sapiens (U076343)	73.28%	73.11%	73 %	72%	72%	72%

Maximum Likelihood algorithm with 1000 NJ bootstrap resampling revealed the clustering of all breeds of goats' Lf nucleotide sequence with that of *C. hircus* (U53857.1). Anglo-Nubian and Saanen claded together while Native goats claded with other breeds (Anglo-Nubian and Saanen). All breeds of goats' Lf sequences claded together with *C. hircus* (U53857) sequence. *O. aries* (NM_001024862.1) Lf sequence, on the other hand, separated from the clade of goats. While *B. taurus* (FJ589071.1), *B. indicus, B. grunniens,* and *B. bubalis* (JF825526.1) Lf sequences have much greater separation from the clade of small ruminants. *S. scrofa* (M81327.1), *E. caballus* (NM_001163974), and *H. sapiens* (U07643.1) Lf nucleotide sequence have been used to show an outlier from other ruminants (Figure 1).

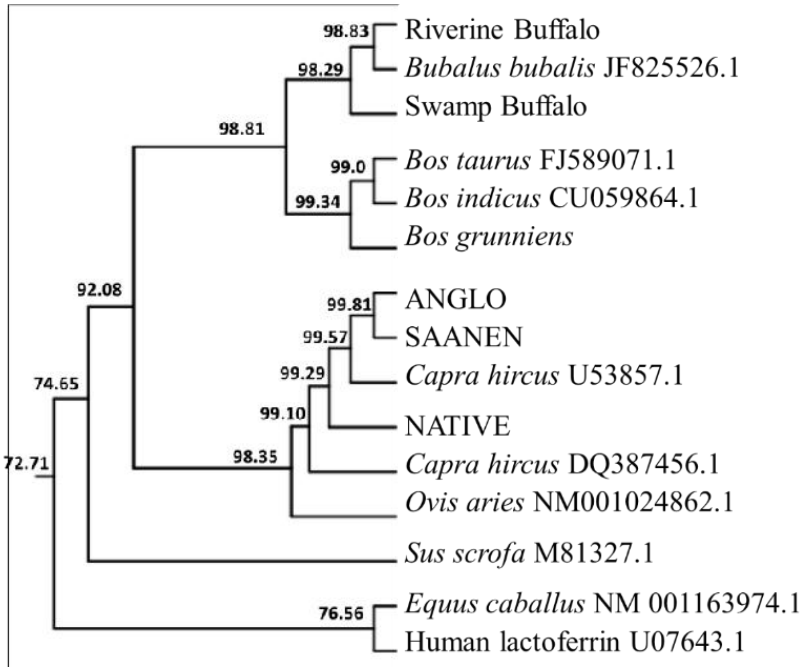

Figure 1. Phylogenetic tree showing the relationship of Lf gene between breed of goats, water buffaloes, other ruminants' and human.

The coding sequence of Lf gene in goats was analyzed for differences in nucleotides and functional amino acid substitution. Furthermore, restriction enzyme capable of digesting the sequence to identify the nucleotide polymorphism and distinguish the genotype has been identified, and the results are herein presented. Sequence analysis revealed seven (7) nucleotide polymorphism in native in the coding sequence of Lf in native goat at locus 551, 552, 553, 701, 910, 939 and 1069 with 5 amino acid substitutions, three (3) in Saanen at locus 1069, 1526 and 1527 with 2 amino acid substitutions and four (4) in Anglo-Nubian breed at locus 910, 1069, 1526 and 1527 with 3 amino acid substitutions.

The nucleotide polymorphism at locus 1069 of Native, Saanen, and Anglo Nubian goat Lf gene sequences resulted in a functional amino acid substitution from leucine to valine. Kaminski et al. (2008) reported that the higher milk protein yield was related to polymorphism at position +216 when it occurs along with another polymorphism. The substitution of valine which is a growth hormone receptor gene can increase the milk yield as well as elevate the milk protein and fat yield.

This is in consonance with the previous studies (Lee et al., 1997; Martin-Burriel, Osta, Baredse, & Zaragosa, 1997; Li & Chen, 1999; Kaminski, Oleński, Brym, Malewski, & Sazanov, 2006; Daly, Ross, Giblin, & Buckley, 2006) that polymorphisms in Lf gene reportedly occur in the coding and regulatory regions and polymorphisms are encoded in exons and introns. To develop rapid recognition of polymorphic site of Lf gene, restriction enzymes *AluI* and *HaeIII* were used to digest 561 bp that was amplified by primer 2. This 561 bp segment covered exons 2 and 3 of the coding region based on the SMS Restriction Digest application of 4 samples with complete Lf gene nucleotide sequence.

The availability of these restriction patterns on extracted Lf gene was examined by using PCR product that was subjected to *AluI* (Figure 2) and *HaeIII* (Figure 3) digestion and electrophoresed. Results showed restriction patterns that were almost the same based on the conceptualized expected pattern (Chang-hong, Gaoming, Yanliang, & Zhaoxia, 2008).

The presence of three restriction patterns using *AluI* seen in gel conferred that there were more polymorphic nucleotides for Lf gene and had to be discovered. High polymorphism in Lf was quite expected because of its function to bind peptides derived from exogenous antigens and in mediating immune response. *AluI* produced cut at AGT/ACT site. Results showed no cut in genotype AA producing 561 bp fragment size of the gene, while genotype AB had one cut producing two bands with 300 bp and 217 bp amplicon size products. Genotype BB produced 3 bands with 561 bp, 300 bp, and 217 bp fragment sizes.

Another restriction enzyme used to test the restriction pattern of Lf gene in goats was *HaeIII* which produced cuts at AGT/ACT site. Results showed no cut in genotype AA producing 561 bp fragment size of the gene, while genotype AB had 5 cuts producing four bands with 561 bp, 312 bp, 122 bp, and 70 bp amplicon size products. Genotype BB produced 3 bands with 312 bp, 122 bp, and 70 bp fragment sizes (Figure 3)

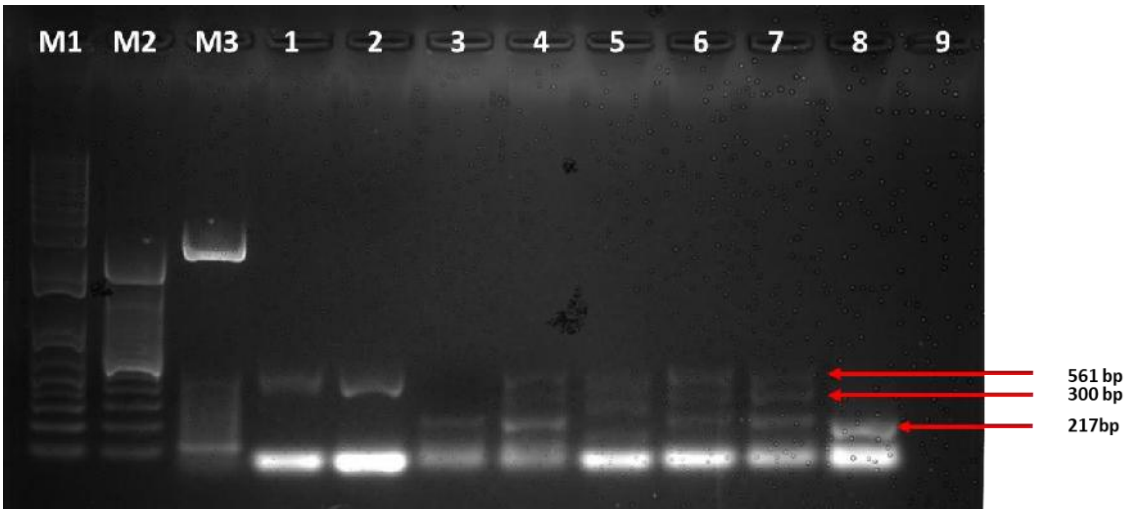

Figure 2. Restriction patterns of caprine Lf gene PCR products using *AluI* in 2% agarose gel. M1. 1000 bp + ladder, M2 100 bp ladder M3 25 bp ladder, Lane 1 and 7-genotype AA, Lanes 2 to 5 genotype AB, Lanes 6 and 8 –genotype BB.

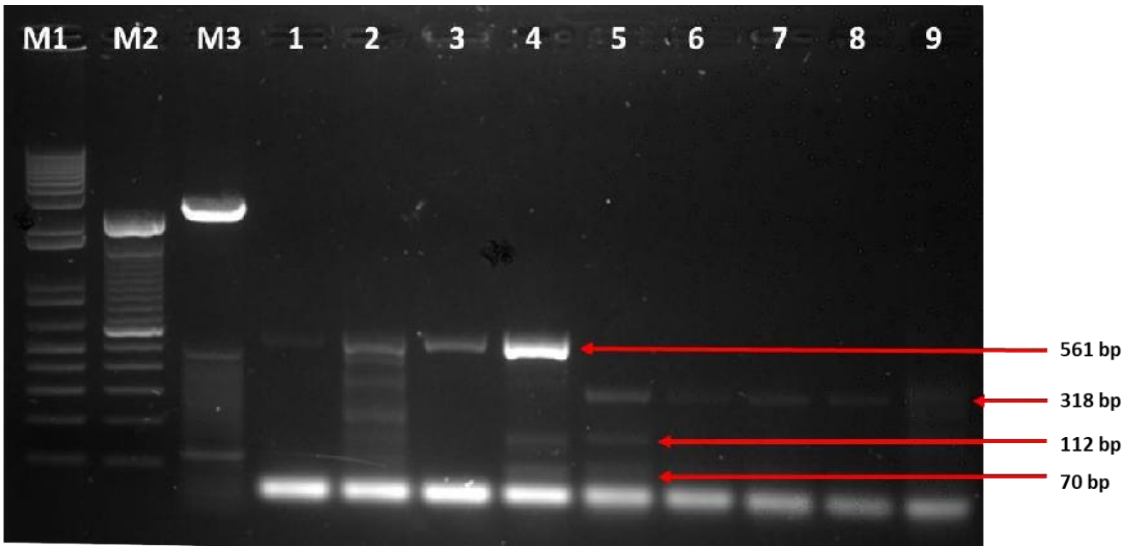

Figure 3. Restriction patterns of caprine Lf gene PCR products using *HaeIII* in 2% agarose gel. M1. 1000 bp + ladder, M2 100 bp ladder. M3 25 bp ladder, Lanes 1 and 3-genotype AA, Lanes 2 and 4-genotype AB, Lanes 5 to 9–genotype BB.

Restriction enzyme *Alul* that can cut AG/CT nucleotide site was selected to examine restriction fragment pattern in the nucleotide sequence of Lf gene based on the SMS Restriction Digest application of the Lf complete nucleotide sequence.

The different genotypes found in the Lf gene are not significantly associated with the occurrence of mastitis using other restriction enzymes. No prior studies have been conducted in support of the three different genotypes found in goats using *HaeIII* and *AluI* restriction enzyme, hence this study will be the baseline for other researchers in identifying mastitic resistance genotypes.

The 561 bp was used for the association study. It showed the frequency of Lf genotypes in non-mastitic and SCM goats. Although chi-square test revealed no significant effect to make an association between genotypes and mastitic incidence (Table 4 and 5), it was clearly presented that genotype BB had a high frequency in SCM compared to non-mastitic animals. Animals having a Genotype BB may or may not increase the tendency on the occurrence of SCM as compared to genotype AA and AB.

Table 4. Chi-Square Analysis on the Association of Genotypes and Sub-clinical mastitis in Goats using Restriction Enzyme *AluI*.

Genotype	Expected Frequencies			Chi-square			Chi-square computed	Chi-square tabulated
	AA	AB	BB					
Non-mastitic	11.88	17.23	11.88	0.10	0.18	0.69	2.43	5.99
Sub-clinically Mastitic	8.11	11.76	8.11	0.15	0.26	1.02	2.43	5.99

Table 5. Chi-Square Analysis on the Association of Genotypes and Sub-clinical mastitis in Goats using Restriction Enzyme *HaeIII*.

Genotype	Expected Frequencies			Chi-square			Chi-square computed	Chi-square tabulated
	AA	AB	BB					
Non-mastitic	10.84	16.57	16.57	0.43	0.35	1.26	5.658	5.99
Sub-clinically Mastitic	6.15	9.42	9.42	0.75	0.62	2.22	5.65	5.99

Percentage frequencies of Lf *AluI* and *HaeIII*-based genotype in non-mastitic and SCM goats are presented in Table 6 and 7.

Table 6. Percentage frequency of Lf *Alu1*-based genotype in non- mastitic and sub-clinically mastitic goats.

Genotype (base pair size)	Category of Animal				Total	
	Non- mastitic		Sub-clinically mastitic			
	%	(n)	%	(n)	%	(n)
AA (561bp)	65.00	13	35.00	7	28.99	20
AB (300 bp, 217 bp)	65.58	19	34.48	10	42.02	29
BB (561 bp, 300 bp, 217 bp)	45.00	9	55.00	11	28.99	20
Total	59.42	(41)	40.58	(28)	100	69

Table 7. Percentage frequency of Lf *HaeIII*-based genotype in non-mastitic and sub- clinically mastitic goats.

Genotype (base pair size)	Category Of Animal				Total	
	Non- mastitic		Sub-clinically mastitic			
	%	(n)	%	(n)	%	(n)
AA (561bp)	76.47	13	23.53	4	24.64	17
AB (561bp, 318bp, 112bp, 70bp)	73.08	19	26.92	7	37.68	26
BB (318bp, 112 bp, 70 bp)	46.15	12	53.85	14	37.68	26
Total	63.77	44	36.23	25	100	69

Association analysis based on the frequency of genotypes revealed that there is an association between genotypes and mastitic incidence. The frequency of the occurrence of digestion patterns were produced by *HaeIII* enzyme with SCM and non- mastitis regardless of breed. The Lf gene in goats has a bactericidal and bacteriostatic activity that could lessen the bacterial population in the milk through phagocytic killing (Tsuda et al., 2000). However, this may not be enough to sequester the microbial flora in the mammary gland of the animals. Underlying factors such as climate, housing system, type of bedding, and rainfall and wetness in the vicinity of dairy premises interact to influence the degree of exposure of teat and tend to increase mastitis pathogens that cause SCM (McEwen & Cooper, 1947).

Conclusion

The whole mRNA coding sequence of goats had an average molecular weight of 2134 bp. Both nucleotide and translated amino acid sequences of Lf were highly similar (98-99%), and phylogenetic analysis found an evolutionary relationship with goats from NCBI GenBank.

The Lf gene of goat established three different genotypes (*AA, AB, and BB*) using restriction enzymes *Alu1* and *HaeIII;* however, Chi-square analysis revealed no association between Lf gene and the occurrence of SCM using *HaeIII* restriction enzymes.

These initial findings are applicable in the field of immunity and disease resistance. The incidence of association between restriction sites and clinical parameters converts PCR-RFLP into a powerful tool in relating specific amino acid substitution at a critical position to possible disease resistance. Moreover, the selection of disease resistance genes will provide potential avenues for improving the health status of the animal and increasing productivity.

The polymorphic nature of the genes merits further investigation for other nucleotide loci and their significance to disease resistance or susceptibility. Identification of the blood composition of the upgraded goats would help establish the bloodline of animals. Expression analysis can also assist in the understanding of how the genes progress in disease protection.

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In Silico Approach and Immunohistochemical Evaluation of *Azadirachta indica* Bioactive Compound as Hsp90 Inhibitor and AIF Inductor in Hepatocellular Carcinoma

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Abstract: Hepatocellular carcinoma is primary malignant liver cancer, leading to the third of cancer death cases. Cancer has insensitivity to growth-inhibitory mechanisms and evasion of programmed cell death. Large numbers of Hsp70 and low expression of AIF play crucial roles in establishing cancer cell hallmarks. Here, we evaluate the effect apoptosis of bioactive compound of *Azadirachta indica* by *in silico* and *in vivo* approach. The protein was docked to the compounds using AutoDock Vina, visualized using Discovery Studio 4.1. *In vivo* experiment of the protein expression in liver organ from rat model hepatocellular carcinoma treated ethanolic extract of *Azadircahta indica* leaves was done by immunohistochemistry staining. Furthermore, the present molecular docking studies of bioactive compounds of *Azadirachta indica* may inhibit cancer development by binding to the active site of Hsp90 comparable to native ligand. An *in vivo* study on rat model of hepatocellular carcinoma by immunohistochemical stainning label with Hsp70 and AIF antibody showed brownish color aggregates on the su. The result of the treatment group was decreasing of Hsp70 and increasing AIF expression. The data obtained in this study demonstrate that ethanolic extract of *Azadircahta indica* leaves exerts a potent anticancer effect against hepatocellular carcinoma by altering key signaling pathways.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy consisting of hepatocyte like cells with varying degrees of differentiation [1]. Chronic liver disease and cirrhosis remain the most important risk factors for the development of liver cancer, where viral hepatitis and excessive alcohol intake are major risk factors worldwide [2]. As for other non-viral causes, namely excessive exposure to chemical compounds, diethylnitrosamine (DEN) initiator is a genotoxic substance that can damage genes and can cause hepatocyte damage in experimental animals and DEN is metabolized by CYP450 in the liver under normal conditions the liver produces free radicals, one of which is Reactive Oxygen Species (ROS) which in excessive amounts can cause oxidative stress and tissue damage [3]. The promoter of carbon tetrachloride (CCl_4) is a hepatotoxic substance that is commonly used as an inducer of liver damage so it is often used in testing the hepatoprotector activity of certain substances, the metabolic activity of CCl4 especially by cytochrome P450 enzymes in the liver, where CCl_4 is converted into more reactive and toxic substances and causes liver damage [4].

Chemotherapy is often an alternative to cancer treatment and often has side effects that can cause different physical conditions such as hair loss and skin rashes, as for other ways to reduce these conditions by using treatments from natural ingredients [5,6]. One of the plants that has the potential for the treatment of hepatocellular carcinoma is

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Azadirachta indica leaf because this plant contains compounds including nimbolide [7], quercetin [8], rutin [9], azadirachtin [10], gallocatechin [11] and nimbine [12]. Some of them are revealed to have anticancer activity [13]. Earlier study crude extract leaves of *Azadirachta indica* has been shown promising a role in the management of tumors through enhanced apoptotic activity including caspase-8, caspase-3 in cervical cancer cell lines [14]. There is continuous search for the efficiency and safety of alternative anticancer drugs from herbal. Therefore, we evaluate which has better potential apoptotic activity between *Azadiracta indica* var. Indonesia and Philippines leaf extract in hepatocellular carcinoma in *Rattus norvegicus.*

This study aimed to evaluate the anticancer effect of *Azadirachta indica* ethanolic extract in diethylnitrosamine and carbon tetrachloride inducing hepatocellular carcinoma in wistar rats through the expression of HSP70 and AIF proteins using immunohistochemical activity and bioactive compound of *Azadirachta indica* binding to induce inhibition of anti-apoptotic receptor Hsp90.

EXPERIMENTAL DETAILS

Subjects

This study used Wistar white rats and was conducted at the Bioscience Experimental Animal Laboratory, Brawijaya University, Malang. The test used was 24 male white rats (*Rattus norvegicus*) Wistar strain with an age range of 2-3 months and an animal body weight of 150-200 grams, obtained from the Biosciences laboratory, Universitas Brawijaya. Before being used for the experiment, 18 rats had hepatocellular carcinoma, and 6 rats were clinically healthy and did not show any symptoms of illness. Nomor etik

Collection of Plant Material and Extraction

Azadirachta indica Treatment

The group of mice were divided into 3; The control group (P0) with distilled water, the induction group Sorafenib (Sigma-Aldrich, St. Louis, Missouri, USA) at a dose of 5 mg/kgBW and given 0.25% CMC-Na suspension (P1) and the treatment group with leaf extract *Azadirachta indica* var. Indonesia and the Philippines at a dose of 500 mg/kgBW orally for 30 days (P2 and P3). Sorafenib induction with a single dose of 5 mg/kgBW intraperitoneally in group P1. On the 31st day, all groups of rats were sacrificed, and liver samples were taken and fixed with 10% neutral formalin buffer for making immunohistochemical preparations.

Immunohistochemistry

The working principle of measuring the expression of AIF and Hsp90 is by indirect immunohistochemistry. Serum was incubated with AIF-specific antibody reagent and Hsp70 with Avidin-Horseradish Peroxidase (HRP) and incubated with the substrate, respectively. Liver AIF and Hsp90 expression were identified by staining the diaminobenzidine substrate with an immunohistochemical kit (BD, Pharmingen). Expressions were seen using a microscope with 1000x magnification in 20 fields of view and analyzed using the immunoration method.

In Silico Analysis

In silico analysis was used to predict the potential activity of bioactive compounds present in the leaves of the *Azadirachta indica* plant and used as a marker compound against the Hsp90 receptor (Heat shock protein 90). The analysis programs used are Autodock Vina 1.5.6. and BIOVIA. The principle of this analysis is by binding a 3-dimensional molecular structure as a ligand to the OH group of the targeted receptor and the energy or interaction of the receptor with the ligand. The smaller the energy required, the stronger and required stable.

Statistical Analysis

Parametric data analysis between the treatment and control groups, if the data were normally distributed and the variance was homogeneous, was performed using One Way ANOVA and followed by Post Hoc Tukey to determine the significant difference between groups. All result was presented in mean ± SD.

RESULTS AND DISCUSSION

Immunohistochemistry

Figure 1 shows the examination of Hsp70 expression by immunohistochemical staining on liver cancer tissue are shown in brown in the cell nucleus. The results of immunochemical staining of liver tissue labeled with monoclonal antibodies against AIF and Hsp70 showed an increase in AIF and Hsp70 expression, which was indicated by brownish color aggregates in hepatocytes in groups P1, P2, and P3. The intensity and distribution of the expression are shown in Table 1.

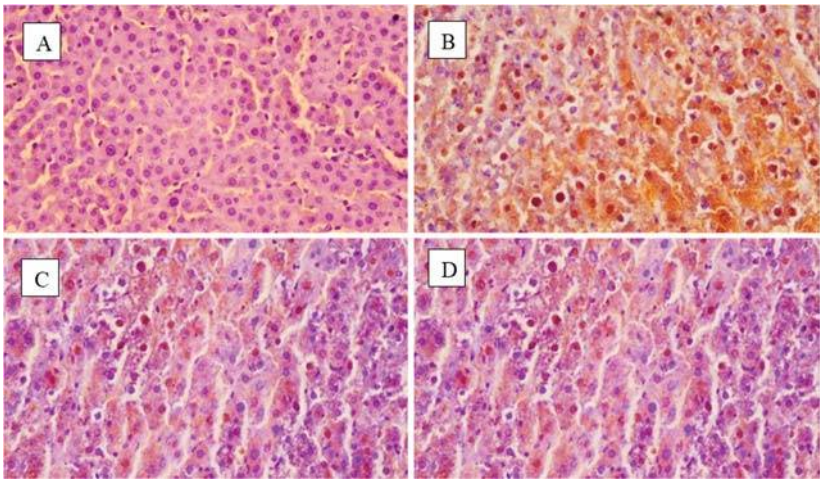


FIGURE 1. The results of the examination of Hsp70 expression by immunohistochemical staining on liver cancer tissue are shown in brown in the cell nucleus. (a) mice that did not get *Azadirachta indica* leaf extract treatment (control); (b) rats receiving 5 mg/kgBW of sorafenib treatment and (c) rats receiving 500 mg/kgBW of EEAII treatment with 400× magnification (d) rats receiving 500 mg/kgBW of EEAIF treatment with 400x magnification

TABLE 1. Comparison of Hsp70 Expression of Control and Treatment Group

Group	Mean ± SD
P0 - Negative control	0.46 ± 0.11^{a}
P1 - Sorafenib treatment	3.25 ± 0.53^{b}
P2 - EAII treatment	1.15 ± 0.18^{a}
P3 - EEAIF treatment	1.16 ± 0.14^{a}

TABLE 2. Comparison of AIF Expression of Control and Treatment Group

Group	Mean ± SD
P0 - Negative control	0.84 ± 0.14^{a}
P1 - Sorafenib treatment	3.45 ± 0.26^{b}
P2 - EAII treatment	2.86 ± 0.15^{c}
P3 - EEAIF treatment	2.91 ± 0.26^{c}

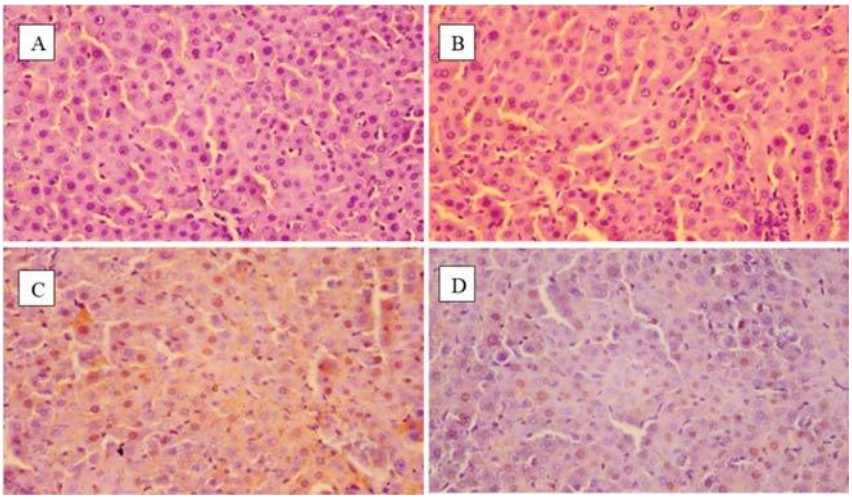


FIGURE 2. The results of the examination of AIF expression by immunohistochemical staining on liver cancer tissue are shown in brown in the cell nucleus. (a) mice that did not get *Azadirachta indica* leaf extract treatment (control); (b) rats receiving 5 mg/kgBW of sorafenib treatment and (c) rats receiving 500 mg/kgBW of EEAII treatment with 400x magnification (d) rats receiving 500 mg/kgBW of EEAIF treatment with 400x magnification

Figure 2 shows the examination of AIF expression by immunohistochemical staining on liver cancer tissue are shown in brown in the cell nucleus. The molecular role of apoptosis protein-inducing factor (AIF) is very important to induce cell apoptosis in the intrinsic pathway. AIF exits the leaky mitochondria, preceded by loss of mitochondrial permeability transition (MPT) [15,16]. The released AIF activates the apoptosome with the help of ATP resulting in apoptosis [17,18]. Subsequently, heat-shock protein 70 (Hsp70) has been reported to prevent apoptosome formation [19,20]. Studies showed that Hsp70 interacts mainly with AIF that Hsp70 can inhibit apoptosis by interfering with AIF targets [21]. The intensity and distribution of the expression are shown in Table 2.

In silico Analysis

Bioactive compounds from the ethanolic extract of *Azadirachta indica* var Indonesia and the Philippines have been shown to potentially cause the release of AIF protein. This can be seen from the increased expression in liver tissue after treatment with *Azadirachta indica*. The results showed that the leaf extract of *Azadirachta indica* var Indonesia and the Philippines 500 mg/kgBW given to white rats (*Rattus norvegicus*) caused an increase in the expression of pro-apoptotic protein (AIF) and a decrease in the expression of anti-apoptotic (Hsp70).

TABLE 3. Results of Native Ligand docking on Target Protein

Protein	Ligand	Binding affinity (kcal/mol)	RMSD	Type of interaction	Amino Acid Residue
3OWD	Gedunin	-6.3	0.0	Conventional hydrogen bond	Mey1, Lys8
				Pi-donor hydrogen bond	Tyr61
	Quarcetin-3-glucoside	-6.1	0.0	Conventional hydrogen bond	Mey1, Ser50, Asn51, Gly132, Gly135
				Pi-anion	Asp54
	Gallocatechin	-6.0	0.0	Conventional hydrogen bond	Asp54, Asn106
				Carbon hydrogen bond	Mey1
	ATP (*native ligand*)	-6.3	0.0	Conventional hydrogen bond	Mey1, Ile26, Glu47, Ser50, Asn51, Ser53
				Pi-anion	Asp54

This is confirmed by the data from an *in silico* study of the inhibition between the bioactive compound ligand of the ethanolic extract of *Azadirachta indica* leaves and Hsp90 protein. Molecular docking method validity was done by docking return (redocking) native ligands to proteins target using the Autodock Vina1.5.6 program. The redocking process is carried out by the semirigid method, which is to arrange so that macromolecules are rigid so there is a change in the shape of the binding site during the redocking process while the ligands will be docked is flexible [22]. The location of the grid box is in the coordinates (X: 29.1679; Y: -44.9965; Z: 65.6034) and dimensions (X: 36.9331; Y 46.9331; Z: 32.8212). Validation parameters in molecular docking in the form of Root Mean Square Deviation (RMSD). RMSD shows the comparison of native conformations docked ligand with native conformation ligands from crystallographic measurements [23]. RMSD value limit that can be accepted is 3Å [24]. The obtained RMSD values for protein 3OWD to ligand was 0 value. Based on these results, the method used can be said to be valid so that the docking process of ligand (*Azadirachta indica* bioactive compound) can be done. RSMD value, binding affinity, type of interaction, and amino acid residues that form bonds hydrogen with native ligands is shown in Table 3.

Based on the results of docking calculations with the Autodock Vina 1.5.6. computer program between the ligand and the Hsp90 receptor, it appears that the binding affinity value of the 3OWD receptor with the sorafenib ligand is -7.6. In contrast, the binding affinity value between the 3OWD receptor and the ligand of the bioactive compound *Azadirachta indica* (gedunin, quercetin-3 -glucoside, gallocatechin) is 6.3 kcal/mol; 6.1 kcal/mol; 6.0 kcal/mol. The lower binding affinity value means that the energy required to form a bond between the receptor and the ligand is lower, which means that the bond formed is more stable, which can predict the compound's activity. The 3OWD protein binds to the native ligand with residues Mey1, Ile26, Glu47, Ser50, Asn51, Asp54, Asp106, Ile110, Ala111, Lys112, Gly132, Gln133, Val136, Phe138, and Tyr139. Visualization of interactions that occur between native ligands with target proteins are shown in Figure 3.

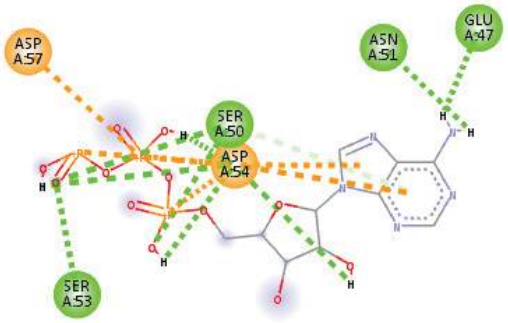
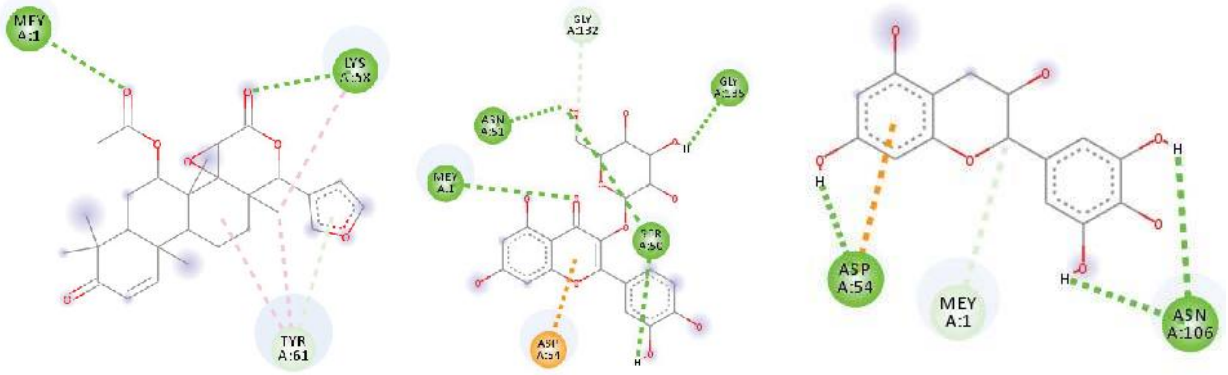


FIGURE 3. Visualization of the Interaction between Native Ligands and Target Proteins



Gedunin Quercetin-3-glucoside Gallocatechin

FIGURE 4. Visualization of the Interaction between Ligands and Target Proteins

The results of the analysis of hydrogen bonds and amino acids residue of 3OWD involved in the ligand-receptor interaction process showed that the gedunin ligand binds 2 amino acids Mey1 and Lys8, the compound quercetin-3-glucoside binds 5 amino acids Mey1, Ser50, Asn51 Gly135, and gallocatechin compound binds 2 amino acids Asp54

and Asn106. Based on this, the conformation of the test compound has low energy and interacts with amino acid residues at the same binding site as the native ligand. Visualization of interactions between ligands with target proteins is shown in Figure 4.

SUMMARY

In summary, according to Tukey's statistical test, there was no significant difference between the leaves of the Indonesian and the Philippines variants in the increase in AIF expression and Hsp70 expression. The results showed a stronger interaction between the ligand and the binding site of the Hsp90 protein compared to the reference ligand. The interactions are mostly through hydrogen bonds. Binding energy of gallocatechin, quercetin-2-glucoside and gedunin. This confirms that the compounds contained in the phytochemical *Azadirachta indica* var Indonesia and the Philippines have the ability to inhibit Hsp90 protein by docking, inhibit Hsp70 and induce AIF expression protein immunohistochemically so that it has the potential for pro-apoptotic compounds, which can therefore be developed as an apoptotic drug.

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AGRICULTURAL CHEMISTRY AND FERTILITY OF SOILS

Delineation of Micronutrient Deficient Zones in Agricultural Soils of Santa Ignacia, Tarlac

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Abstract—Delineation of nutrient-limited zones in agricultural soils is delicate in achieving balanced fertilization and appropriate land management. Due to the lack of detailed information regarding micronutrient availability in Santa Ignacia, Tarlac, Central Luzon Philippines, a study was conducted to investigate the spatial distribution of Zn, Cu, Fe and Mn in the municipality. Soil test results were subjected to descriptive statistics and geo-analytical technique through best fit semivariogram based on highest coefficient of determination. Then, ordinary kriging was employed using the optimum model to generate spatial variability maps and eventually delineate nutrient limited areas. Micronutrient concentration in the area followed the order Fe > Mn > Cu > Zn with moderate to strong variability. Spatial dependence factors (SDf) were found to be moderate for Zn and Cu, while strong SDf were recorded for Fe and Mn. Zn deficient zones accounting to 2517 ha were delineated in the extreme northern and southern region of the municipality. Cu deficient zone covering 1893 ha was delineated at the east-central region of the study area. Meanwhile, no limited zones were delineated for Fe and Mn indicating the relative abundance of these nutrients in Santa Ignacia, Tarlac. Therefore, interventions to increase Zn and Cu availability in deficient zones are recommended to further improve crop yields in the municipality.

Keywords: kriging, micronutrient, semivariogram, spatial variability, Inceptisols

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INTRODUCTION

Essential micronutrients primarily serve as catalyst in enzyme systems and are required in numerous metabolic functions [37]. Although they are needed in minute amounts, the deficiency or abundance of these elements directly affects crop nutrition and environmental pollution [4, 10]. Intensified crop production in past years have resulted in the decline of fertility and overall quality of soils in the Philippines. The Department of Agriculture and prior research have reported cases of micronutrient deficiency in agricultural soils, though its distribution has not been thoroughly analyzed and studied [24, 31]. The lack of micronutrient management system employed in farmlands coupled with indiscreet use of chemical inputs have caused the imbalance of soil nutrients [48]. These issues amplify the call to conduct investigation on the fertility status of soil resources in the country.

The interplay of weathering process, natural events and anthropogenic factors may generate levels of spatial variation among trace elements in different agroecosystems [17, 41]. Zonal delineation on the availability of soil nutrients is a key element of precise agriculture. The identification of nutrient limited and sufficient zones is required to formulate an appropriate and site specific nutrient management scheme [36]. Although fertility maps are available in the region, it is limited to some macronutrients namely, nitrogen, phosphorus and potassium. Characterization and distribution of trace elements in local soils are often neglected. Thus, there is an urgent need to precisely describe the spatial distribution of micronutrients in the study area.

In this regard, geostatistical techniques have become a standard in the analysis of spatial differentiation concerning soil nutrients. Integrating spatial information in geostatistical models can refine estimation and improve map accuracy [26]. Among the various geostatistical methods employed by the researchers for generating soil fertility maps, ordinary kriging remains to be the most commonly utilized interpolation technique [6, 36, 44]. It has the potential to provide the best unbiased linear predictions and generate information regarding estimation errors while reducing the local error variance [27]. It is proven to be fast, cost-efficient and reliable method of producing thematic maps that will serve as basis for appropriate land management. Therefore this study aims to (i) assess the level of micronutrient elements (Zn, Cu, Mn, Fe) in the study area and (ii) delineate micronutrient deficient zones using optimal semivariogram (ordinary kriging) models.

Table 1. Micronutrients rating based on soil test values, mg kg^{-1}

Soil test	Deficient	Sufficient		
		moderate	high	extremely high
Zinc	<0.5	0.5–1.0	1.0–3.0	>3.0
Copper	<0.2	0.2–1.0	1.0–1.8	>1.8
Iron	<4.5	–	–	–
Manganese	<5.0	5.0–15.0	15.0–30.0	>30.0

MATERIALS AND METHODS

Site description. The study was conducted in the Municipality of Santa Ignacia, Tarlac. It is within the Central Luzon region of the Philippines. The capital of the area is geographically located at 15.5841° N and 120.4688° E. It is bounded on the north by the town of Camiling, on the south by San Jose, on the west by Mayantoc and on the east by Gerona, Tarlac. The municipality has a relatively flat terrain in the central and eastern portion, while moderately hilly on the western side. The total land area is 14 189 ha, and most of it is involved in agriculture (Fig. 1). It produces a wide range of crops, including rice, fruits and vegetables. The dominant soil type in the area is fine loamy textured inceptisol characterized by isohyperthermic temperature regime with incipient development towards maturity but not fully developed diagnostic horizons. This soil has minimum complexity towards horizonation which is commonly found in areas with pronounced wet and dry seasons [5, 33]. Soil pH in the area ranges from extremely acidic to slightly alkaline while organic matter (OM) varies from low to medium levels. Finally, the levels of phosphorus (P) and potassium (K) range from low to high concentrations. Based on Corona Climate Classification, Santa Ignacia belongs to Climate Class I which has pronounced wet and dry periods. Annual average precipitation sits at 715 mm where maximum rainfall occurs in the months of July and August. Temperature in the area ranges from 22–31°C, maximum temperature occurs during April and May while minimum temperature is felt in the months of January and February.

Soil sampling and analysis. A total of 336 soil samples at a depth of 0–30 cm were randomly collected in the study area. The geographic location of each sampling point was recorded using a handheld GPS. Distribution of sampling points is shown in Fig. 1. Each soil sample was air dried while impurities were removed by hand before passing through a 2 mm mesh and eventually stored in a clean plastic container. Micronutrients including Fe, Cu, Zn and Mn were analyzed through diethylenetriaminepentaacetic acid extraction–atomic absorption spectrometry (DTPA-AAS) using of 1 : 2 soil:extractant ratio as described in FAO–Standard Operating Procedure for Soil Available Micronutrients and Heavy Metals [8, 18]. In addition, soil pH, organic matter and phosphorus were also analyzed using 1 : 1 soil and water suspension [12], Walkley and Black [45] and Olsen extractant method [30], respectively. Soil test values were interpreted and clustered based on micronutrient fertility standards of Philippine Council for Agriculture and Resources Research and other relevant literature [36, 48].

Data processing and analysis. Descriptive statistical analysis was executed using SPSS 20.0 software. Semivariance analyses and model fitting were conducted using GS + 9.0 software. Models with maximum coefficient of determination (R^2) were identified as optimal model parameters [16]. The best fit model was then used as basis for ordinary kriging interpolation using ArcGIS 10.4 (ESRI, Redlands, CA, USA). Semivariance is expressed as follows:

$$r(h) = \frac{1}{2N(h)} \sum_{i=1}^{n(h)} [z(Xi) - z(Xi + h]^2,$$

where $r(h)$ is the semivariogram, h is the separation distance, $N(h)$ is the pairwise number of data points separated by distance h, $Z(x)$ is the value of a regionalized variable at spatial position x, and $Z(x + h)$ is the value of a regionalized variable at position $x + h$. Nugget, sill and range are key parameters of semivariogram models to describe spatial structure. Furthermore, the nugget/sill ratio and spatial dependence factor (SDf) represents the spatial autocorrelation between variables. SDf < 25% indicates high spatial correlation, in which natural factors are the principal drivers; SDf = 25–75% represents moderate spatial correlation which might be affected by both natural and anthropogenic factors; and SDf > 75% indicates low spatial correlation, where the variation is attributed to random factors [36, 48].

RESULTS AND DISCUSSION

Statistical characteristics of soil micronutrients. Descriptive statistics of the soil test values are presented in Table 2. The micronutrient concentration in the area followed the order Fe > Mn > Cu > Zn. The Zn level in the study area ranges from 0.06–21.16 mg kg^{-1} with a mean value standing at 1.21 mg kg^{-1}. It means that Zn concentrations are qualitatively classified as deficient to extremely sufficient. The Cu concentration ranges from 0.02 to 41.09 mg kg^{-1} with a mean value at

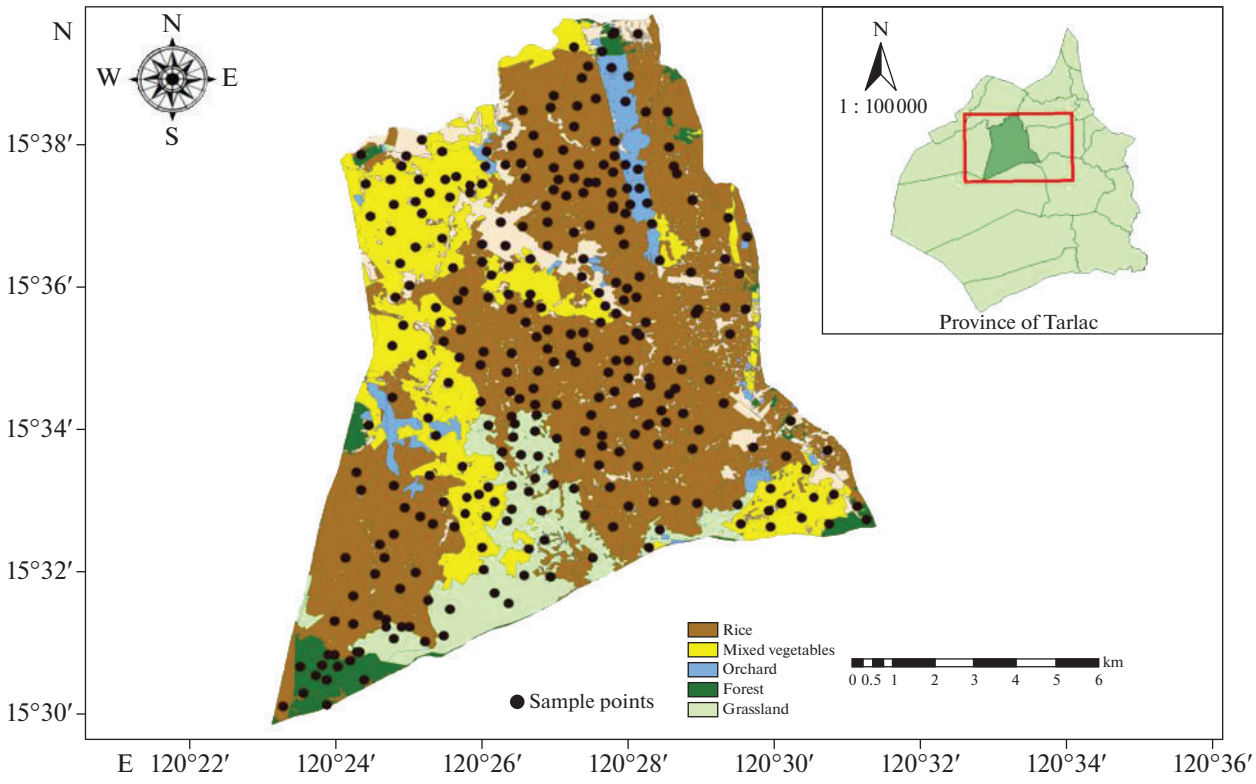

Fig. 1. Land use map and distribution of sampling points in the study area.

5.58 mg kg^{-1}. Likewise, clusters of soil test results are classified as Cu deficient to extremely sufficient. The levels of Fe and Mn ranges 13.97–237.28 and 15.95–246.97 mg kg^{-1} respectively. These values are considered extremely sufficient, which indicates the relative abundance of Fe and Mn in the study area.

One of the very important statistical indices to reflect spatial variation is the CV. A CV of >1 is considered to be strong, while a CV of 0.1–1 and <0.1 is considered moderate and weak variation, respectively [25, 26, 31]. The CV of the subject elements is considered moderate to strong which ranges from 0.6 to 1.97. The order of variation among the nutrients is recorded as Zn > Cu > Mn > Fe. The considerable variation of micronutrients is a common finding especially in agriculture based ecosystems. Aside from parent materials, the nutrient variability in agricultural soils may be attributed to different land utilization types which requires varying fertilizer application, pest control, and other crop management systems [16, 46]. Taking into account the lack of micronutrient fertilization guide and indiscreet use of chemical inputs, it is not surprising to observe moderate to strong variability of soil nutrients in the study area.

Aside from soil micronutrients, different edaphic properties in the study area were also analyzed to determine their possible relationship. The correlation map of Zn, Cu, Fe, Mn, P, K, pH, and OM is summarized in Table 3. Cu concentration is correlated with pH and OM level. Cu concentrations vary with soil pH due to the dissolution of Cu insoluble composts or adsorption on the surfaces of Fe and Al oxides or precipitated in hydroxyl forms depending on the level of acidity [28]. Furthermore, OM has a dynamic relationship with the mobility of Cu in soils. The complex-

Table 2. Descriptive statistics of essential micronutrient elements, mg kg^{-1}

Elements	Max	Min	Mean	Std. Dev.	Skewness	Kurtosis	CV
Zn	21.16	0.06	1.21	2.39	7.18	58.71	1.97
Cu	41.09	0.02	5.85	7.24	2.38	7.15	1.23
Fe	237.28	13.97	89.11	53.13	0.87	0.13	0.60
Mn	246.97	15.95	60.39	43.58	1.61	3.32	0.72

Std. Dev, Standard Deviation; CV, Coefficient of Variation.

Table 3. Correlation matrix of soil trace elements

Variables	pH	OM	P	K	Z	Cu	Mn	Fe
pH	**1**	**0.463**	–0.148	0.185	0.083	**–0.421**	–0.195	**–0.380**
OM	**0.463**	**1**	–0.114	0.119	0.095	**0.337**	–0.100	–0.091
P	–0.148	–0.114	**1**	–0.015	0.133	–0.094	–0.196	0.142
K	0.185	0.119	–0.015	**1**	–0.045	0.081	–0.056	0.036
Z	0.083	0.095	0.133	–0.045	**1**	**–0.216**	**–0.217**	–0.088
Cu	**–0.421**	**0.337**	–0.094	0.081	**–0.216**	**1**	0.185	–0.019
Mn	–0.195	–0.100	–0.196	–0.056	**–0.217**	0.185	**1**	0.062
Fe	**–0.380**	–0.091	0.142	0.036	–0.088	–0.019	0.062	**1**

Values in bold are different from 0 with a significance level $\alpha = 0.05$.

Table 4. Geostatistics of the fitted semivariogram models of micronutrients

Elements	Model	Nugget	Sill	NSR	R^2	SpD
Zinc	Spherical	1.25	2.26	0.55	0.73	55.30
Copper	Spherical	1.19	1.92	0.62	0.69	61.90
Iron	Exponential	115	483	0.23	0.81	23.80
Manganese	Exponential	80	423	0.18	0.89	18.91

NSR—nugget to sill ratio; R^2—R-squared; SpD—spatial dependence factor.

ation of OM with Cu is an important mechanism in its retention and bio-availability [25]. Meanwhile, Fe is negatively correlated with pH. Fe is commonly affected by pH due to the latter's direct effect on the precipitation of soluble Fe into insoluble forms [13]. Interestingly, Zn has negative correlation to Cu and Mn, which suggests negative interactions among these nutrients. Alloway [2] reported that the relationships between Cu and Zn are often antagonistic because of their competitive adsorption on soil colloids and absorption sites of plant roots. Cu fertilization has been shown to alter Zn dynamics in soil or vice versa [20, 21]. Interestingly, Rahman et al. [35] reported that Cu and Zn were mostly speciated as carbonate phases under P-deficient condition. The complexation of these elements with carbonate and phyllosilicate minerals is likely the controlling factor of their bio-availability. In addition, Vasu et al. [43] identified antagonistic nutrient interaction as one of the primary drivers of the concentration and spatial characteristics of micronutrients under semi-arid tropical environment. Thus, co-fertilization trial of Cu and Zn may be explored to further characterize the antagonistic behavior of these nutrients in local soils of Santa Ignacia.

Spatial structure and variability of micronutrients. Spherical model was the optimum semivariogram function for Zn and Cu, while exponential model was optimum for Fe and Mn. The highest coefficient of determination (R^2) was observed for Mn at 0.89, and Cu has the least R^2 at 0.69. A strong spatial dependence factor was observed for Fe (23.80) and Mn (18.91). It indicates that spatial variation of Fe and Mn in the study area is primarily affected by soil forming factors, including parent material, terrain and other structural parameters. Same results were reported by Kavitha et al. [16], Shukla et al. [38] and Zou et al. [48] regarding strong spatial dependence of these nutrients. However, contrary results were reported by Odoi et al. [30] indicating weak SpD of Fe and Mn within the industrial zone of Ghana. They identified the discharge of industrial waste as main factor on the spatial structure of such nutrients. Thus, the absence of heavy industrialization and other similar activities in Santa Ignacia might have caused the dominance of structural factors on the spatial characteristics of Fe and Mn.

On the other hand, moderate spatial dependence factor (SpD) was observed for Zn (55.30) and Cu (61.90). Moderate SpD suggest that spatial variation of these nutrients is primarily affected by the simultaneous action of structural and random factors, including land use, crop management and anthropogenic pollution [48]. Moderate spatial dependence was also observed by Shukla et al. [38] and Liu et al. [19] for Cu and Zn respectively. They hypothesized that the spatial characteristics of these nutrients may be attributed to the imbalance utilization of heavy metal containing inputs in crop production. Furthermore, weak SpD for Zn and Cu was even observed in various crop producing areas which suggest the significant effect of farm management on the variability of these nutrients [34, 38]. Such findings on spatial autocorrelation indicate the need to conduct a deeper analysis on the specific causes of spatial variability of soil micronutrients in the study area.

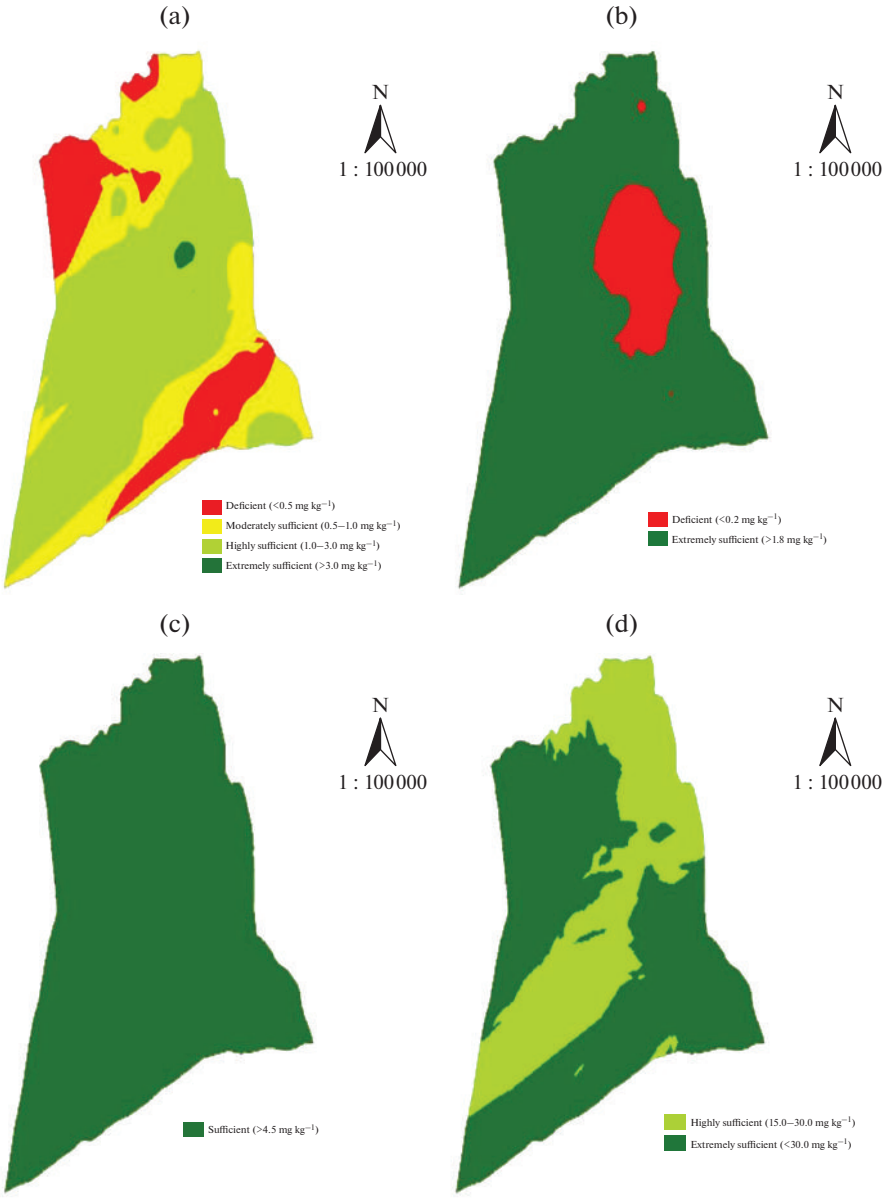

Fig. 2. Spatial variability map of micronutrients (a) zinc, (b) copper, (c) iron, (d) manganese.

The spatial variability of micronutrient concentration in the study area is displayed in Fig. 2. Zn deficient zones accounting to 2517 ha were delineated in the extreme northern and southern region of the municipality, while the remaining portion varies from highly to extremely sufficient. The application of zinc sulphate is recommended to correct the deficiency in the concerned areas. Under flooded conditions, zinc

Table 5. Distribution of micronutrient availability classes

Fertility class	Zn		Cu		Fe		Mn	
	area, ha	%	area, ha	%	area, ha	%	area, ha	%
Deficient	2517	18	1893	13	–	–	–	–
Moderate	3469	24	–	–	–	–	–	
High	8061	57	–	–	14189	100	5788	41
Extremely High	142	1	12296	87	–	–	8401	59

solubility often decreases due to mechanisms associated with soil redox status [1, 3]. Since paddy rice is the dominant crop in the area, water management system to improve aeration and drainage may be employed to increase zinc solubility [13]. Other options, such as utilization of ammonium sulfate and applying organic fertilizer to further increase the availability of zinc may also be considered [11]. Cu deficient zone covering 1893 ha of the study area was delineated at the east-central region. The remaining portion of the area is classified as highly sufficient for Cu. The addition of CuO or $CuSO_4$ in deficient areas is recommended to balance Cu levels. The amendment of Cu-enriched foliar fertilizer and elemental sulfur is likewise recommended to increase Cu absorption by plants [11, 14]. Finally, no deficient zones were delineated for Fe and Mn. The levels of these nutrients are found to be highly sufficient in the study area.

The results of the study concur with prior research regarding micronutrient status of farmlands in the Philippines. Yoshida et al. [47] estimated around 500 000 ha of Zn deficient rice areas alone. Katyal and Vlek [15] also reported Zn deficiency in some hydrosols and gleysols in the country. Mahagud et al. [22] likewise assessed widespread Zn deficiency especially in lowland areas. Furthermore, the Philippines was also included in a group of countries with serious Zn deficiency problem in their agricultural soils [2]. Meanwhile, Descalsota et al. [7] reported Cu deficiency in particular areas of Central Luzon including some parts of Muñoz, Llanera, and Zaragosa in Nueva Ecija; La Paz in Tarlac and Santa Cruz in Zambales. Finally, Mahagud et al. [23] reported high levels of Fe and Mn in agricultural areas of Central Luzon. Toxic accumulations of Fe and Mn (300 and 1000 mg kg^{-1} respectively) are observed in rice plants evidencing the abundance of these elements in the area.

CONCLUSIONS

The study provides the first micronutrient availability map of Santa Ignacia, Tarlac. Moderate to strong variability of micronutrients was observed in the area. Zn (18%) and Cu (13%) deficient zones have been delineated in multiple regions of the municipality. The employment of field measures to increase Zn and Cu availability in deficient areas is recommended to further increase crop yields in the municipality. Meanwhile, Fe and Mn are found to be entirely sufficient suggesting their relative abundance in the study area. Geo-statistical method is indeed a time and cost-effective tool in mapping various soil properties. Finally, the results of this study can be used to formulate site specific nutrient management and other relevant land policies in Santa Ignacia, Tarlac.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest in the conduct of this research.

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Hatchability of duck eggs as affected by types of incubators under varying relative humidity

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Abstract The mean percentage hatchability was not significantly affected by types of incubators, two levels (70% and 80%) of relative humidity, and the interaction effect of types of incubators and relative humidity. However, the percentage of egg hatching was significantly affected by types of incubators such that the means of the percentage hatch in Cabinet-Type Electric Incubator (M=43.95%) and in Bamboo or "Garong"-Type Incubator (M=41.88%) were significantly higher from "Lawanit" Board-Type Incubator (M=27.68%). There was no significant difference in the percentage hatch as affected by two different levels of relative humidity and the interactions of types of incubators and varying percent relative humidity. In this study, the use of Bamboo or "Garong"- Type Incubator indicated the lowest cost (₱0.83) to hatch a duckling, the cheapest (₱17.70) to produce a duckling, and highest ROI of 2.00%.Thus, the Bamboo or "Garong"-Type Incubator was the most economical to use among the three types of incubators.Among the three types of incubators under the two levels of relative humidity, it was observed that the cost to hatch and the cost to produce a duckling is lower under 80% relative humidity with an average cost of ₱1.89 and ₱20.00 respectively. Higher ROI (1.56%) was also observed when the eggs were incubated under 80% RH than 70% RH with an average ROI of 1.54%. Furtheremore, the Cabinet-Type Electric Incubator and Bamboo or "Garong"-Type Incubator were identified to be the most efficient types of incubators. All the hatching parameters were not significantly affected by two levels (70% and 80%) of relative humidity.

Keywords: Bamboo or "Garong" incubator, Duck egg incubation, Parched rice incubation, Rice husk incubation technique

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Introduction

Ducks are commonly raised by rural farmers in the Philippines mostly in Central Luzon and some part of Western Visayas. Around 429,700 families derive their livelihood from it (Santiago, 2018). These farmers, who have less than 100 heads of ducks, contribute to 70% of the ducks in the country (Philippine Statistics Authority [PSA], 2015) as reported by Arrosa (2018). Ducks are preferred by small-holders in the communities compared to other fowls because ducks can adapt and survive under a wide range of climatic conditions, can feed on a variety of feedstuffs, and are resistant to diseases. Raising Mallard Duck (*Anas platyrhynchos*), being known for egg production, provides a good source of income to farmers through its products such as *balut* (embryonated egg) and salted egg. These ducks can also be sold as cull after two years of egg production when their laying performance begins to decline. However, there are some issues and concerns about the duck industry. Results from a farm survey on duck egg production in the Philippines showed that duck farmers generally lack the technical know-how and extension services as well as insufficient supply and high cost of producing good quality ducklings (Chang *et al*., 2008). Most of these problems, particularly on technical and production and supply aspects, are brought about by the issues concerning the type of incubator used and the physical factors to which the eggs were subjected before and during the incubation period.

The duck industry has a promising future given the high demand for salted eggs and *balut* which accounts for 90% of the total egg production in the country (Beltran, 2015). The vast knowledge of local farmers in raising ducks, the availability of complete feeds at different stages of growth (from brooding to laying), and the government programs on strengthening mallard duck production (*Itik Pinas*) all over the country make the duck raising more encouraging. Furthermore, it is one of the special programs of the Bureau of Animal Industry under the Department of Agriculture that aims to contribute to attaining one of the goals of President Duterte on food sufficiency. However, there are other problems with the industry such as the fluctuating prices of eggs, limited space for free-range operations, and inadequate research studies being conducted on duck raising ("Native", 2016).

At present, commercial incubators of varying capacities are being used by *balut* and duck producers in the country. Most of these incubators are operating with electricity for heating and other mechanical functions. However, small-holders experience issues in the cost of procurement, maintenance, and operations. According to Boleli *et al.* (2016), the main focus of research at present is the manipulation of thermal incubation conditions and the integrated

effect of factors that influence incubation. Commercial hatcheries use modern state-of-the-art incubators but one of the questions that need to be answered is how effective and cost-efficient the incubators are in terms of promoting greater hatchability and better chick quality.

Currently, artificial incubators made from locally available materials, using parched rice and rice husk, have been used by hatcheries in Central Luzon and National Capital Region. However, the technology was not widely adopted and little is known about the efficiency of using this type of incubator. Furthermore, literature and studies suggest a wide range of humidity levels inside the incubator and different egg turning frequencies to produce a good hatch. Therefore, it is important to find a method and technique of duck egg incubation that is efficient, less expensive, uses locally available resources, and can easily be adopted by both backyard and commercial raisers.

Thus, this study was conducted to determine the hatchability of duck eggs using different types of incubators under two levels of relative humidity. Also, this study aimed to determine which among the three types of incubators is the most efficient and most economical to be used and be recommended for small-hold, backyard duck raisers, or commercial hatcheries. The results of this study could contribute to the determination of optimum relative humidity which would be needed to attain better hatchability of duck eggs using different types of incubators. Moreover, the results may lead to finding better techniques for hatching duck eggs through the use of locally available resources that would reduce the production cost of quality hatchlings. Through the adoption of technology to be generated, more duck raisers especially the small-hold raisers would be benefited and be able to produce their own ducklings instead of buying them from commercial hatcheries or other suppliers. Furtheremore, the results of this study may be used as a basis for further researches about duck egg incubation.

Materials and methods

Time and place of research

The experiment was conducted at the duck egg hatchery and “balutan” of Mr. Renato C. Ramos in Brgy. Carmen, Zaragosa, Nueva Ecija from June 2020 to July 2020.

Research design

The study was laid out into treatment combinations using a Completely Randomized Design following the 3 x 2 factorial arrangements using three

types of incubators under varying relative humidity. Factor A served as the three types of incubators (Cabinet-Type Electric Incubator, Bamboo or "Garong"-Type Incubator, and "Lawanit" Board-Type Incubator) while the Factor B served as the two levels of relative humidity (70% RH and 80%RH).

Experimental treatments and layout

Five thousand and four hundred duck eggs were used in the study. These were randomly divided into six treatment combinations based on the experimental factor. Each composed of 900 eggs. Each treatment combination was further subdivided into three replications with 300 eggs per replicate.

Setting-up of cabinet-type incubator

Six cabinet-type forced-air electric incubators were used. These were prepared by cleaning especially in the interior area. Thermometers and hygrometers were checked and ensured that these were functioning. The experimental eggs were incubated at a temperature ranging from 37.22-37.78^{o}C.

Setting-up of bamboo or "Garong" and "Lawanit" board incubator

Six "Garong" and six "Lawanit" Board Incubators were also used to complete the types of incubators needed in the study. Each incubator had a diameter of 45-50 cm and a height of 85-90 cm. Nylon net with a size of 75 cm x 70 cm was used to contain 100 eggs and also to contain 1.5 kg of unpolished rice/parched rice as a source of heat.

The rice was heated twice a day to about 42^{0}C to 43^{0}C using a vat or cauldron or "kawa" following the procedures in making *balut* (ATBP.PH, 2016). A pan of water was placed at the bottom of the incubator. Bamboo slats were placed on top of the pan before making a pile of heated rice and duck eggs. Five bags of heated unpolished rice (1.5 kg per bag) and three bags of preheated eggs (100 eggs per bag) were piled in an alternating position having the rice at the bottom and on top when the pile was completed.

The incubators were arranged at a distance of at least four inches from each other. Rice hull was used to fill up spaces between incubators. It served as an insulator and to conserve heat energy.

Egg collection

Large-sized eggs, 65-70 grams, were collected from mated flocks in a selected house on the farm. Eggs laid not later than three days were selected as experimental materials.

Egg setting

The 300 eggs were set in each replication of the three types of incubators. Before setting, eggs were pre-heated under the sun for two to three hours with a temperature ranging from 23.9-26.1^{o}C following the procedures stated in Hatchery Tips (2017).

Incubation duration

Experimental eggs were incubated (with a source of heat) for 15 days. On the 16th day after setting, eggs were transferred onto a table in a closed room (no window) until they were hatched.

Egg turning

Eggs in "Garong" and "Lawanit" board were manually turned two times a day following the procedures in making *balut* by ATBP.PH (2016). On the other hand, eggs in electric incubators were turned by switching on the "turn" button. The eggs were turned twice a day until the 15th day of incubation. From 16 days to hatching, eggs were turned four times within 24 hours.

Candling

The first candling was done on the 10th day after egg setting to determine the number of fertile eggs and infertile eggs. The second candling was done on the 15th day of incubation to select fertile eggs but would fail to hatch due to the following reasons: (1) dead embryo, (2) presence of a red ring or blood around the embryo, (3) enlarged blood vessels, and (4) presence of oozing substance (Smith, 2018).

Relative humidity and temperature control

Relative humidity in the "Garong"-Type and "Lawanit" Board-Type Incubators was controlled by placing a moisture pan inside. Rice was heated two times a day until the 15th day of hatching. After 15 days, rice or palay bags were not heated anymore since the embryos could generate enough heat to keep them warm. However, the humidity in Cabinet-Type Electric Incubator was controlled by placing also moisture pan inside. The temperature was set into 37.5 ^{0}C until 15 days of hatching.

Data analysis

The data from the experiment were subjected to analysis of variance in 3x2 factorial in Completely Randomized Design (Gomez and Gomez, 1984). When significant differences were obtained, means were compared using the Least Significant Difference (LSD) at 5% probability. To facilitate calculations and analysis of experimental data, the computer program Statistical Tool for Agricultural Research was used.

Results

Percentage hatchability

The mean of the percentage hatchability of duck eggs as affected by types of incubators under varying relative humidity is shown in Table 1. The results of the study revealed that percent hatchability of duck eggs was not affected by the types of incubators as indicated by their means (M=48.42%, M=50.40%, and M=37.17%) having no significant difference, $F(2,12) = 2.78$, $p = 0.1020$ when analyzed for variance. The percentage hatchability was not affected also by the two levels (70% and 80%) of relative humidity wherein their means (M=44.98% and M=45.68%) were comparable. As for the effect of the interactions of types of incubators and varying percent relative humidity, it was found out that these interactions did not affect the percent hatchability of duck eggs, $F(2,12) = 1.81$, $p = 0.2057$.

Table 1. Mean of the percentage hatchability of duck eggs as affected by types of incubators under varying relative humidity

Factor A – Types of Incubators	**Factor B – Relative Humidity**		**Factor A Mean**
	70% RH	**80% RH**	
Cabinet-Type Electric Incubator	44.83	52.00	**48.42**
Bamboo or “Garong”-Type Incubator	56.70	44.10	**50.40**
“Lawanit” Board-Type Incubator	33.40	40.93	**37.17**
Factor B Mean	**44.98**	**45.68**	

Percentage hatch

The mean of the percentage hatch of duck eggs as affected by types of incubators under varying relative humidity is presented in Table 2. In this study, the percentage hatch of duck eggs was significantly affected by the types

of incubators, F(2,12) = 5.73, p = 0.0179.The mean percentage hatch in Cabinet-Type Electric Incubators (M=43.95%) and Bamboo or "Garong"-Type Incubators (M=41.88%) was significantly higher than the mean percentage hatch in "Lawanit"Board-Type Incubator (M=27.68%).

However, the percentage hatch was not affected by the two levels (70% and 80%) of relative humidity with which their means (M=38.03% and M=37.65%) were not significantly different, F(1,12) = 0.01, p = 0.9290. As for the effect of the interaction of types of incubators and varying percent relative humidity, it was revealed that these interactions did not affect the percent hatch of duck eggs.

Table 2. Mean of the percentage hatch of duck eggs as affected by types of incubators under varying relative humidity

Factor A – Types of Incubators	Factor B – Relative Humidity		Factor A Mean
	70% RH	80% RH	
Cabinet-Type Electric Incubator	39.00	48.90	**43.95 a**
Bamboo or "Garong"-Type Incubator	48.00	35.77	**41.88 a**
"Lawanit" Board-Type Incubator	27.10	28.27	**27.68 b**
Factor B Mean	**38.03**	**37.65**	

Note: Means followed by the same letter are not significantly different at 5% level of significance by LSD

The most economical type of incubator

The mean of the average cost to hatch a duckling among three types of incubators for 70% and 80% relative humidity is shown in Table 3. In this study, the lowest average cost (₱0.83) to hatch a duckling was determined when using the Bamboo or "Garong"-Type Incubator. It was lower than the cost of using the "Lawanit" Board-Type Incubator and Cabinet-Type Electric Incubator with the average costs of ₱1.12 and ₱4.11, respectively.

Among the three types of incubators under the two levels of relative humidity, it was observed that the lowest average cost (₱0.75) to hatch a duckling was with the use of Bamboo or "Garong"-Type Incubator under 70% while the highest average cost (₱4.47) was by using Cabinet-Type Electric Incubator also under 70% relative humidity. Furthermore, the cost to hatch a ducking was lower (₱1.89) under 80% relative humidity than 70% relative humidity with an average of ₱2.59.

Table 3. Mean average cost (₱) to hatch a duckling using three types of incubators under 70% and 80% relative humidity

Incubator type	Cost to hatch a duckling (₱)		Mean
	70% RH	80% RH	
Cabinet-Type Electric Incubator	4.47	3.75	**4.11**
Bamboo or "Garong"-Type Incubator	0.75	0.91	**0.83**
"Lawanit" Board-Type Incubator	1.22	1.01	**1.12**
Mean	**2.15**	**1.89**	

The mean of the average cost to produce a duckling among three types of incubators for 70% and 80% relative humidity is shown in Table 4. The study revealed that the Bamboo or "Garong"-Type Incubator was the cheapest to use among the three types of incubators to produce a duckling with an average cost of ₱17.70. However, a higher average cost to produce a duckling was realized with the use ofCabinet-Type Electric Incubator and "Lawanit" Board-Type Incubator with the average costs of ₱21.04 and ₱23.72, respectively.

Among the three types of incubators under the two levels of relative humidity, it was observed that the lowest average cost (₱16.02) to produce a duckling was by using Bamboo or "Garong"-Type Incubator under 70% while the highest average cost (₱26.02) was by using the "Lawanit" Board-Type Incubator also under 70% relative humidity. Moreover, it was determined that it was cheaper ((₱20.00) to produce a duckling under 80% relative humidity than under 70% relative humidity with an average cost of ₱21.64.

Table 4. Mean of the average cost (₱) to produce a duckling among three types of incubators for 70% and 80% relative humidity

Incubator type	Cost to hatch a duckling (₱)		Mean
	70% RH	80% RH	
Cabinet-Type Electric Incubator	22.87	19.21	**21.04**
Bamboo or "Garong"-Type Incubator	16.02	19.37	**17.70**
"Lawanit" Board-Type Incubator	26.02	21.42	**23.72**
Mean	**21.64**	**20.00**	

The mean of the average percentage ROI among the three types of incubators for 70% and 80% relative humidity is illustrated in Table 5. Among the three types of incubators, the Bamboo or "Garong"-Type Incubator obtained the highest average percentage return on investment (2.00%) while the "Lawanit" Board-Type Incubator obtained the lowest average ROI (1.21%).

Among the three types of incubators under the two levels of relative humidity, the highest average percentage ROI (2.37%) was obtained when Bamboo or "Garong"-Type Incubator was used under 70%. However, the

lowest average percentage ROI (0.99%) was attained when "Lawanit" Board-Type Incubator was used under 70% relative humidity. On the other hand, higher ROI (1.56%) was realized under 80% relative humidity than under 70% relative humidity with an average ROI of 1.54%.

Table 5. Mean of the average percentage (%) return on investment among three types of incubators for 70% and 80% Relative Humidity

Incubator type	% ROI		Mean
	70% RH	80% RH	
Cabinet-Type Electric Incubator	1.25	1.62	**1.44**
Bamboo or "Garong"-Type Incubator	2.37	1.62	**2.00**
"Lawanit" Board-Type Incubator	0.99	1.43	**1.21**
Mean	**1.54**	**1.56**	

The most efficient type of incubator

Based on the results of the study, only the percentage hatch was affected by a certain factor - the types of the incubators. The analysis of variance yielded a main effect for the type of incubator, $F(2,12) = 5.73$, $p < .05$, such that the average percentage hatch was significantly higher inCabinet-Type Electric Incubator (M=43.95%) and Bamboo or "Garong"-Type Incubator (M=41.88%) than in"Lawanit"Board-Type Incubator (M=27.68%) (see Table 2). The main effects of humidity and interaction were non-significant, $F(1,12) = 0.01$, $p > .05$ and $F(2,12) = 2.27$, $p > .05$, respectively. Therefore, the most efficient types of incubators are the Cabinet-Type Electric Incubator and the Bamboo or "Garong"-Type Incubator.

Discussion

Commercial incubators of varying capacities are being used by *balut* and duck producers. However, the cost of procurement and operation, and the effectiveness and cost-efficiency in terms of promoting greater hatchability of these incubators are the major concerns needed to be answered. Artificial incubators made from locally available materials are being used by hatcheries in Central Luzon and National Capital Region. However, the technology was not widely adopted and little is known about the efficiency of using this type of incubator. The study was conducted to determine the effect of types of incubators under varying relative humidity on the different hatching parameters.

This study demonstrated that the hatchability of fertile duck eggs was not affected by types of incubators ($p = 0.1020$), by the two levels (70% and 80%)

of relative humidity, and the interactions of types of incubators and varying percent relative humidity (p = 0.2057) when analyzed for variance.This result is parallel with the study of Indarsih *et al.* (2019) which revealed that the sawdust incubator gave similar fertility, hatchability, and embryonic mortality values as the electric incubator. Also, this result is associated with the findings of the study Bruzual *et al.* (2000), whichpointed out that fertile hatchability was optimum when incubated at 53% relative humidity. The findings of Bruzual *et al.* (2000), is supported by Hitchener (2017) and Daniels (2020) who recommended that the ideal relative humidity is at 55%.

The percentage hatch of duck eggs was significantly affected by the types of incubators (p = 0.0179). In this experiment, the mean percentage hatch in Cabinet-Type Electric incubators (M=43.95%) and Bamboo or "Garong"-Type Incubators (M=41.88%) was significantly higher than the mean percentage hatch in "Lawanit" Board-Type Incubator (M=27.68%). Boleli *et al*. (2016) explained that this is because the latter provides better incubation physical conditions such as ventilation, egg turning, and egg position, which may affect hatchability. However, the percentage hatch was not affected by the two levels (70% and 80%) of relative humidity and the interaction of types of incubators and varying percent relative humidity. These results could be correlated to the statement of Paniago (2005) as specified by Boleli *et al.* (2016) that despite the technological advances of modern incubation machines, still, the quality of labor both inside and outside the hatcheries determines the success of incubation.

The most economical type of incubator was determined based on the following aspects: (1) cost to hatch a duckling; (2) cost to produce a duckling; and (3) percentage ROI.In this study, the use of Bamboo or "Garong"-Type Incubator indicated the lowest cost (₱0.83) to hatch a duckling, the cheapest (₱17.70) to produce a duckling, and has the highest ROI of 2.00%.

Among the three types of incubators under the two levels of relative humidity, it was observed that the cost to hatch and the cost to produce a duckling was lower under 80% relative humiditywith an average cost of ₱1.89 and ₱20.00 respectively against ₱2.15 and ₱21.64 under 70% relative humidity. Higher ROI (1.56%) was also observed when the eggs were incubated under 80% RH than 70% RH with an average ROI of 1.54%. Moreover, the determination of most economical type of incubator is greatly affected by the hatchability of fertile duck eggs from the specific incubator. This means that the higher the hatchability of an egg from a certain incubator, the lower the cost that may incur to hatch or to produce a duckling. These results are supported by the results of the experiments conducted by El-Hanoun and Mossad (2008) pointing out that the hatchability of fertile Pekin Duck eggs could be improved

by raising the relative humidity (RH) to 80% during the period of 14-28 days of incubation. Their experiments are related to the study of Onbasilar *et al*. (2014) which revealed that hatchability of set and fertile eggs of Pekin Ducks were higher when incubated at 37.5^0C and sprayed with warm water ($25\text{-}28^0$C) from day 4 to day 25 of incubation.

The most efficient type of incubator was determined only when the types of incubators, relative humidity, and the interaction effect of incubator and humidity have a significant effect on the hatching parameters. These conditions were discussed by Boleli *et al.* (2016) in their article regarding optimizing production efficiency that includes manipulation of thermal incubation conditions and the integrated effect of factors that influence incubation. In this present study, only the percentage hatch was affected by a certain factor, the types of the incubators, which revealed that a significantly higher percentage hatch was obtained in Cabinet-Type Electric Incubator and Bamboo or "Garong"-Type Incubator than in "Lawanit" Board-Type Incubator. In addition, all the hatching parameters were not significantly affected by two levels of relative humidity. Therefore, the effects of 70% and 80% relative humiditiesare comparative.

In summary, the study showed that the Cabinet-Type Electric Incubator and Bamboo or "Garong"-Type Incubator yield a significantly higher number of hatch eggs than "Lawanit" Board-Type Incubator. Also, the Cabinet-Type Electric Incubator and Bamboo or "Garong"-Type Incubators were the most efficient types of incubators. Bamboo or "Garong"-Type Incubator was the most economical (lowest cost to hatch and to produce duckling and highest % ROI) type of incubator to use.

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Regulation of the immune system by administering lactic acid bacteria to suppress the effects of aflatoxin B1 in mice (*Mus musculus*)

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ABSTRACT

Aflatoxin B1 (AFB1), which is a toxic compound produced by the filamentous fungus *Aspergillus* sp., is highly carcinogenic, damages vital organs, and may cause death. Prevention of aflatoxin poisoning through proper food storage and physical treatment is an added cost, thus there is a need to identify alternative methods including treatment with probiotic bacteria. We evaluated the effect of *Lactobacillus bulgaricus* on activating immune cells in mice exposed to Aflatoxin B1. The study used a post-test control design consisting of five treatment groups including a negative control, positive control, and T1, T2, and T3 groups treated with lactic acid bacteria at doses of 105 colony forming unit (CFU)/ml, 107 CFU/ml, and 109 CFU/ml, respectively, administered on days 7–28 and AFB1 at a dose of 0.2 mg/kg bw orally on days 15–28. The relative number of lymphocytes consisting of CD11c+transforming growth factor-beta (TGF-β)+, CD4+CD8+, and B220+IgG+, was measured using flow cytometry. The data were analyzed using a one-way analysis of variance test. The results indicated that *L. bulgaricus* bacteria increased the relative number of CD11c+TGF-β+, B220+IgG+, and CD4+CD8+ cells in mice exposed to the mycotoxin. *Lactobacillus bulgaricus* may function as an immunostimulator against mycotoxins by inducing the humoral and cellular immune response.

INTRODUCTION

Mycotoxins, such as aflatoxin B1 (AFB1), are secondary metabolites produced from filamentous fungi that are toxic, carcinogenic, and immunosuppressive to animals and humans. The estimated world production of feed contaminated with fungus is approximately 5%–10% (Oswald *et al*., 2005; Qian *et al*., 2012; Tomkova *et al*., 2001). Aflatoxin reportedly caused up to 100,000 turkey deaths following the consumption of contaminated peanut mushrooms. Broiler feed contaminated with a mycotoxin mixture (3.5 mg/kg diet as 79% of AFB1, 16% AFG1, 4% AFG2, and 1% AFB2) can lead to weight loss and inflammation of the liver and kidneys. Aflatoxin B1 is a potent agent that causes immunosuppression in pigs at a dose of 140–280 µg/kg of feed by inhibiting DNA synthesis and immune cells, such as lymphocytes, but does not affect the humoral immune response (Perczak *et al*., 2018; Pierron *et al*., 2016).

Aflatoxin B1 suppresses the cellular immune system, in particular T lymphocytes, because of decreased complement production by the liver, phagocytosis by macrophages, and neutrophil activity (Perdigon *et al*., 2001). T lymphocytes affected by the toxin, as well as other lymphoid cells, such as cytotoxic T cells and natural killer cells, can promote tumor cell function directly or indirectly. The cellular components of the immune system produce cytokines for protection against tumor progression; however, cytokines may also play a role in the inflammation mechanism that causes damage to various organs (Ibrahim, 2013).

Methods such as heating, chemical treatment, or radiation can destroy and eliminate mycotoxin (Zain, 2011);

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however, the cost is prohibitive and it may impair the nutritional value of the feed. The decline in fungal growth may increase during feed production or storage (Munoz *et al*., 2010). Lactic acid bacteria (LAB) may exhibit antifungal activity, especially *Lactobacillus* sp (Sadiq *et al*., 2019). *Lactobacillus pentosus* and *Lactobacillus brevis* bacterial strains at a concentration of 3.5 × 108 colony forming unit (CFU)/ml can bind and release aflatoxin B1 by 17.4% and 34.7%, respectively, in liquid media as measured by ELISA (Hamidi *et al*., 2013).

Microorganisms, such as *Saccharomyces cerevisiae* and LAB, may be used as biopreservatives in feeds, so it is possible to extend the shelf-life and increase food safety with microflora supplementation. Antimicrobial products of microorganisms also have potential as probiotics and may improve health (Tran *et al*., 2020); however, there is limited data demonstrating the immunostimulating effects of LAB. Therefore, we evaluated the effect of LAB on the cellular and humoral immunity profile of mice.

MATERIAL AND METHODS

Preparation of animal

Male mice (*Mus musculus*), strain Balb/c aged 8–12 weeks (n = 25), with a body weight of 25–30 g, were obtained from Brawijaya University's bioscience laboratory and divided into five groups (five mice/group). The treatment groups consisted of a positive control (mice induced with AFB1 0.2 mg/kg bw on days 15–28), negative control (healthy mice), and treatment groups, T1, T2, and T3 in which mice were administered 1 × 105 CFU/ml, 1 × 107 CFU/ml, and 1 × 109 CFU/ml of LAB on days 7–28, respectively.

Bacterial suspension preparation

Lactobacillus bulgaricus (LAB) was obtained from the Microbiology Laboratory of the Faculty of Medicine, Universitas Brawijaya, and confirmed by biochemical tests and Gram staining. The bacteria were grown on de Man, Rogosa, and Sharpe (MRS) agar media at 37°C for 24 hours. A bacterial suspension was prepared using MRS broth media and the bacterial concentrations were measured using a spectrophotometer. The bacteria were diluted with phosphate buffer saline (PBS) for the experiments.

Aflatoxin B1 preparation

Aflatoxin B1 (Sigma Company catalog: A6636®) is potent with respect to acute toxicity, mutagenicity, and carcinogenicity, and one vial of AFB1 contained 5 mg of powder. AFB1 (0.2 mg/kg BW) was diluted in 1 ml PBS, pH 7.2 (Qian *et al*., 2012).

Flow cytometry

The mice were sacrificed on day 29 by cervical dislocation. Spleens were harvested, placed into a petri dish containing sterile PBS, and crushed with the base of a syringe. 10 ml homogenates of the sample were prepared in a volume of 10 ml, centrifuged, and the pellets were resuspended in 1 ml PBS by pipetting (Ardiana and Rifa'i, 2015).

Then, 100 µl of the suspension was placed into a microtube; 500 µl of PBS was added; and the mixture was centrifuged at 2,500 rpm for 5 minutes at 4°C. Then, 50 µl of PE/Cy5 conjugated rat anti-mouse Cd11c, PECy5 conjugated rat anti-mouse transforming growth factor-beta (TGF-β), fluorescein isothiocyanate (FITC) conjugated rat anti-mouse CD4, PE conjugated rat anti-mouse CD8, FITC conjugated rat anti-mouse B220, and PECy5 conjugated rat anti-mouse IgG (Biolegend®, San Diego) were added to the cells and incubated for 20 minutes in 4°C (8). Afterward, 50 µl of cytofix (BD Biosciences Pharmingen) was added and incubated for 20 minutes in 4°C; wash perm solution (BioLegend®, USA) was added; and the mixture was centrifuged at 2,500 rpm at 10°C for 10 minutes. The cells were analyzed by flow cytometry (BD FACSCalibur, USA) using BD Cellquest ProTM software.

Statistical analysis

The data are presented as the relative number of immune cells (CD11c+TGFβ+, CD4+CD8+, and B220+IgG+). Data were analyzed statistically using one-way analysis of variance (ANOVA) with an error level of $\alpha = 0.05$, followed by Tukey's test.

RESULTS

$CD11c^+TGF\text{-}\beta^+$ cells

The results indicated that supplementation of the mice with LAB induced by AFB1 increased the relative number of CD11c+ cells that produce TGFβ+ (Fig. 1). In the positive control group, the relative number of CD11c+ expressing molecule TGF-β+ (1.75%) was different, but no significance was observed when compared with the negative control (0.91%). All treatment groups (T1, T2, and T3) administered LAB at a 105–109 CFU/ml concentration showed an increase in the relative number of CD11c+TGF-β+ cells by 2.05% 3.14%, and 3.06%, respectively, when compared to the negative and positive controls, as shown in Figure 1.

$CD4^+CD8^+$ cells

The results showed that the supplementation with LAB in mice induced with AFB1 increased the relative number of CD4+CD8+ cells in all treatment groups, but the amount did not significantly differ and was similar to that of the negative control, as shown in Figure 2. The negative control was higher when compared with the positive control.

$B220^+IgG^+$ cells

The results showed significant differences in the relative number of B220+-expressing IgG+ cells in all the treatment groups following supplementation with LAB after induction with AFB1. The negative control group (24.56%) was significantly different from the positive control group (5.76%), which showed a relatively higher number of B220+IgG+ cells compared with the positive control. There was an increase in the relative number in all treatment groups (7.44%, 10.26%, and 7.67% for T1, T2, and T3, respectively), as shown in Figure 3.

DISCUSSION

Effect of LAB on the relative number of $CD11c^+TGF\text{-}\beta^+$ cells

The results indicated that the relative number of CD11c+ cells that expressed TGF-β was different, but there was no significant between the treatment groups; however, a 107 CFU/ml concentration of LAB increased the average of CD11c+TGF-β+

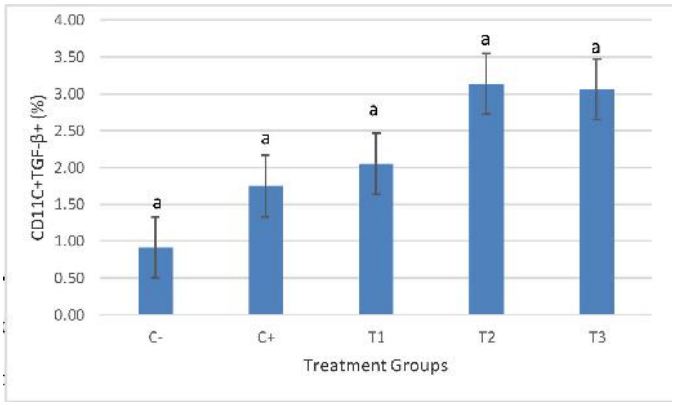

Figure 1. Flow cytometry analysis shows that giving LAB showed an increase in the relative number of CD11c+TGF-β+ cells ($p < 0.05$), but was not significantly different between treatments. There was an increase in the number when compared with the positive control. The highest average increase was seen in the T2 treatment. The treatment groups are: C- (healthy mice), C+ (mice induced with AFB1), and treatment groups, T1, T2, and T3 in which mice were administered with AFB1 and 1 × 105 CFU/ml, 1 × 107 CFU/ml, and 1 × 109 CFU/ml of LAB on days 7–28, respectively.

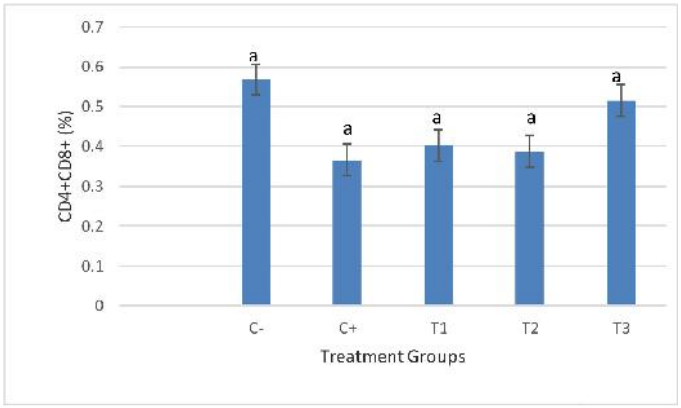

Figure 2. Treatment with LAB showed an increase in the relative number of CD4+CD8+ cells for all treatments, which did not differ significantly ($p < 0.05$). The treatment groups are: C- (healthy mice), C+ (mice induced with AFB1), and treatment groups, T1, T2, and T3 in which mice were administered with AFB1 and 1 × 105 CFU/ml, 1 × 107 CFU/ml, and 1 × 109 CFU/ml of LAB on days 7–28, respectively.

cells in all treatments. Mycotoxin exposure decreased the relative amount of CD11c+TGF-β+ cells. Mycotoxin AF1 altered or decreased anti-inflammatory cytokine synthesis by inhibiting macrophage or T cell activation. In fact, mycotoxin inhibits the synthesis and proliferation of T cells, which prevents macrophage cells from producing anti-inflammatory cytokines. Mycotoxin is cytotoxic to lymphocytes by interfering with lymphocyte receptors or lymphocyte function (Tran *et al*., 2020). A study by Murugesan *et al*. (2015) revealed that mycotoxin was not immunogenic. It could not induce an immune response to pathogens, but interfered with mitogen-activated protein kinase signaling to modulate cell growth, apoptosis, or the immune response. This could expose an individual to a high risk of infection.

Dendritic cells (DC) and CD11c markers are antigen-presenting cells (APCs) that are regulated specifically and nonspecifically by immune cells found in the lamina propria of the small intestine and gut-associated lymphoid tissues, such as

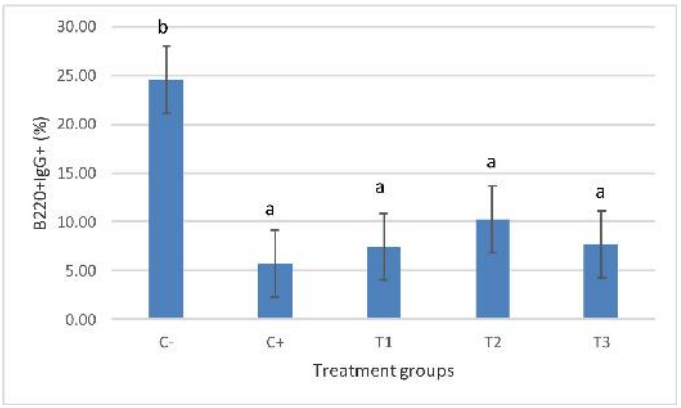

Figure 3. Treatment with LAB showed an increase in the relative number of CD4+CD8+ cells for all treatments, which did not differ significantly ($p < 0.05$). The treatment groups are: C- (healthy mice), C+ (mice induced with AFB1), and treatment groups, T1, T2, and T3 in which mice were administered with AFB1 and 1 × 105 CFU/ml, 1 × 107 CFU/ml, and 1 × 109 CFU/ml of LAB on days 7–28, respectively.

the Peyer's patches. Most dendritic cells (DCs) are present in an immature condition and are less immunogenic because of the low expression of MHC costimulators. Contact with pathogen-associated molecular patterns (PAMPs) or other signals induces the pattern recognition receptor (PRR) signal and activates the NF-κB pathway, resulting in maturation and activation of DC cells (Wells, 2011). Mature DCs may then express high MHC levels, costimulatory molecules, and cytokines which attenuate APC activation and differentiation of T cells to cause inflammation (Mohamadzadeh *et al*., 2005). DCs exposed to AFB1 cannot respond correctly to any invading microbes and fails to initiate antigen presentation to activated T cells that are susceptible to pathogenic agents (Mohammadi *et al*., 2014).

The anti-inflammatory cytokine, TGF-β, inhibits the proliferation of fibroblast epithelial cells, dendritic cells, and macrophages to produce inflammatory cytokines and controls cell growth through adhesion and extracellular matrix formation (Hussain *et al*., 2018). Exposure to mycotoxin could stimulate CD11c+ cells to activate inflammatory pathways that TGF-β may have suppressed. Oral exposure to LAB concentrations of 105 CFU/ml could activate inflammatory cytokines, such as TGF-β, in DCs, which may inhibit CD11c+ cell activity. This was evident in the T1 and T2 group, prior to an observed decrease in the T3 group. According to Vindirelo and Alberto (2015), the higher the concentration of cell bacteria, the greater the binding capacity of AFB1 in liquid media *in vitro*. The concentration of bacteria that can bind AFB1 was 1010 CFU/ml for *L. rhamnosus* GG, *L. casei Shirota*, *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *Escherichia coli*. Probiotics have an immunomodulatory effect on the release of cytokines, interleukins, tumor necrosis factor, transforming growth factor, and chemokines from immune cells that play a role in the innate and adaptive immune systems. LAB may interact with enterocytes and DCs, Th1/Th2 cytokines, or T reg cells in the intestine to stimulate the adaptive immune response into a proinflammatory or anti-inflammatory action (Azad *et al*., 2018; Mohamadzadeh *et al*., 2005).

Mycotoxin AFB1 exerts toxicity because it is readily absorbed by the intestine and rapidly binds to serum protein. AFB1 is

genotoxic and immunogenic in animals (Zimmermann *et al*., 2014). Mycotoxin can activate the microbial intestine, and mycotoxin adsorption–desorption is highly dependent on the intestinal environment and digestive enzymes. The LAB, *L. rhamnosus* RC007, stimulates pH, salts, enzymes, and peristalsis at each stage of AFB1 absorption in the digestive tract. Saliva secretion results in low adsorption and high AFB1 reabsorption. Gastric fluids and intestinal fluids do not decrease the AFB1 adsorption of LAB, rather they stimulate higher AFB1 adsorption (Sadiq *et al*., 2019).

Metabolic LAB products inhibit aflatoxin biosynthesis. Heterofermented LAB, such as *L. bulgaricus*, produce a high level of acetic acid and propionic acid at acidic pH (Vinderola and Ritieni, 2015). The mechanism of action of LAB is to inactivate the fungal membrane and inhibit the absorption of amino acids and inactivated products from fungi, such as acetic acid (Perczak *et al*., 2018). Bacteria and yeasts may neutralize mycotoxins in the body by reshuffling, transforming, and breaking them down into nontoxic metabolic products or inactive forms (Murugesan *et al*., 2015). LAB binds mycotoxins to prevent further absorption by the intestine, which are then secreted with feces (Adilah *et al*., 2018). LAB walls contain peptidoglycans that could interact with mutagenic compounds, including mycotoxins, through binding to reduce stability and bioavailability, and stimulate the secretion of anti-inflammatory cytokines by macrophages (Niderkorn *et al*., 2009; Tabari *et al*., 2018). LAB's capacity to bind mycotoxins would be optimal when the bacteria cells die due to a change in the cell surface. Live LABs require a long time to release mycotoxin from the body (Perczak *et al*., 2018). Cell wall protein denaturation may function by creating a broader area to absorb mycotoxins (Tabari *et al*., 2018). The proteins in the ribosomes, nucleus, chromosomes, cytosol, and cellular cytoskeleton components support the forming of the cell wall of bacteria in the exponential growth phase.

On the contrary, binding between the cell wall of LAB with mycotoxin takes place at the beginning of the end of the bacterial growth cycle (Moller *et al*., 2021). AFB1 could bind to the cell wall β-d-glucan through hydrogen or van der Waals bonds. Absorption of AFB1 toxin depends on the availability of the number of binding sites on the surface of microbes, and the equilibrium constant [K (eq)], which could change as a result of genetic, physical, or chemical alterations (Sadiq *et al*., 2019).

LAB may act as an anti-inflammatory agent, resulting in the reduction of oxidative stress from AFB1 exposure (Abbes *et al*., 2016). Probiotics could also stimulate T cell subsets, humoral immune cells, epithelial-associated dendritic cells, and macrophages to increase anti-inflammatory cytokine products Braat *et al*., 2004. The entry of LABs into the body may increase the capacity and phagocytic receptors of leukocyte cells, especially complement receptor 3 (CR3), for bluffing respiratory bursts (Bravo *et al*., 2019).

Effect of LAB on the relative number of $CD4^+/CD8^+$ cells

Our results showed that the administration of LAB (*L. bulgaricus*) had an effect on the relative number of CD4+/CD8+ immunocompetent cells in mice induced with aflatoxin B1 in the treatment groups ($p > 0.05$) based on a Kruskal–Wallis test. The negative control group had a higher relative number of CD4+/CD8+ T cells than the positive group. This result is consistent with that of Qian *et al*. (2012), in which the administration of AFB1 to mice orally for 24 hours reduced CD3 T cells in the intestinal mucosa. AFB1 caused a decrease in the cellular immune response to the specific dose and duration treatments (Zimmermann *et al*., 2014). A low dose of mycotoxin could induce an inflammatory response if activated by enzymes, such as inflammatory inhibitors (Hussain *et al*., 2018). Mycotoxin AFB1 may undergo systemic hydrolysis and further activate metabolism. Phase metabolism includes conjugation with glucuronic acid and sulfate by the whole-cell biotransformation system during immune cell communication (Tran *et al*., 2020).

High LAB concentrations could increase naive T lymphocyte activation and proliferation as well as memory T cells (Wells *et al*., 2011). Upon entering the body, an antigen is presented by DCs in the digestive tract mucosa (CD11c). Histocompatibility complex class II molecules combined with the expression of costimulatory molecules and cytokines (Gaudinoand Kumar, 2019) and activation of T lymphocytes. As a cellular defense, T cells may differentiate into a subset of T1 that activates macrophages. Macrophages and APCs induce T cells to secrete inflammatory cytokines that cause tissue damage (Mohamadzadeh *et al*., 2005). The T1, T2, and T3 groups had a higher relative amount of CD4+/CD8+ T cells compared with the positive control group is shown in Figure 2.

LAB may act as an antifungal agent because it contains metabolites, such as organic acids, carboxylic acids, phenolic acids, cyclic dipeptides, hydrogen peroxide, and compounds that inhibit sporulation, which may decrease mycotoxin production (Sadiq *et al*., 2019). LAB at concentrations of 105 CFU/ml could activate T lymphocyte cells in all groups compared with the positive control group. These results were consistent with that reported by Tsai *et al*. (2012) in which LAB activates the cellular adaptive immune response.

THE EFFECT OF LAB ON THE RELATIVE NUMBER OF $B220^+IGG^+$ CELLS

Our results showed that $B220^+$ cells expressing IgG in the negative control group was higher compared with the positive group, whereas it was not significantly different among all of the treatment groups. The average of B220 cells expressing IgG increased after LAB administration. Mycotoxin can reduce immunoglobulin production in chicken feed supplemented with AFB1 (Nazarizadeh and Pourreza, 2019). LAB stimulates the humoral immune response by increased circulating antibodies and levels of plaque-forming cells in the host when exposed to mycotoxin (Abbes *et al*., 2015). LAB can absorb and eliminate mycotoxin to prevent intestinal absorption and reduce liver damage, which is a target of mycotoxin. Upon entering the host orally, mycotoxin stimulates the secretion of immunoglobulin A (IgA) in the digestive mucosa and antibody IgG in the circulation. IgG represents a secondary response to protect the body against foreign antigens (Chen and Tsai, 2011).

In the normal intestinal epithelium, microflora bacteria act as a barrier antigen. However, when epithelial cells are degraded by infectious and noninfectious substances, alterations in intestinal permeability and inflammation of the intestinal mucosa occur. The inflammatory response that occurs in the intestine activates IgG antibodies and causes translocation of the microflora bacteria (Paludan *et al*., 2020).

LAB plays a role in the body to activate the immune response by inducing the formation of Secretory IgA (SIgA) and producing vitamins (Wold, 2001). Antibody SIgA is dominant in the

mucous membrane, which is the first defense immune system against a dangerous environment. SIgA antibodies play a role in neutralizing toxins, viruses, salivary exotoxins, and eliminating pathogenic microbes (Hayati *et al*., 2018). *Lactobacillus* bacteria and other probiotics are commensal microorganisms that interact with the mucosa or the immune cells. LAB stimulates specific functions of the mucosal immune system and produces secretory IgA. The presence of receptors, such as Toll-like receptors, nucleotide oligomerization domain-like receptors, and C-type lectin receptors may stimulate *Lactobacillus*. *Lactobacillus* associates with microbe-associated molecular patterns to activate APC and modulate their function through the expression of surface receptors, secretion of cytokines and chemokines, and other nonspecific immune effector cells (Mohamadzadeh *et al*., 2005). Our results indicate that there was an increase in the relative number of IgG+ cells resulting from LAB induction in mice exposed to mycotoxin. This is consistent with the results obtained by Tran *et al*. (2020) in which IgG levels in the serum of Balb/c mice increased when *Lactobacillus* was administered for 7 days (infected with *Salmonella typhimurium* bacteria). DCs play a role in the adaptive immune response. LAB stimulates DC cells to activate specific immune responses in the intestinal mucosa to maintain homeostasis, protect against pathogenic microbes, and maintain intestinal permeability (Mohamadzadeh *et al*., 2005). LAB increases the cellular and nonspecific humoral immune response in mice exposed to mycotoxin AFB1.

CONCLUSION

L. bulgaricus bacteria exhibited a potent effect as an immunostimulator resulting from exposure to mycotoxin AFB1.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the ethics committee (certified no. 012-KEP-UB-2020) of Institut BioSains.

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COMPETING INTERESTS

The authors declare that there are no financial and nonfinancial conflicts of interest.

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DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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AUTHORS' CONTRIBUTIONS

DQS obtained the funding; designed the study, analysis and interpretation of data; and was a major contributor in writing the manuscript. DQS, SM, and IAA analyzed the flow cytometer data, treated the animals, and collected the data. All authors read and approved the final manuscript.

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Epidermal Stem Cell in Wound Healing of *Gliricidia sepium* Leaves from Indonesia and the Philippines in Rats (*Rattus norvegicus*)

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Abstract

AIM: This study intended to investigate the regenerate wound, due to the ointment therapy containing *Gliricidia sepium* leaves that has potential-induced epidermal stem cells producing. It determined its effect on the expression of transforming growth factor-β1 (TGF-β1), Smad-3, β-catenin, LGR-6.

MATERIALS AND METHODS: About 16 Wistar male rats aged approximately 2 months (150–200g) were used and were divided into four treatment groups (T1, positive control; T2, negative control; T3, wounds treated with *G. sepium* from Indonesia; and T4, wounds treated with *G. sepium* from the Philippines). The treatment of ointment was applied to the wound for 3 days. The expression of TGF-β1, Smad-3, β-catenin, and LGR-6 was observed by immunohistochemistry staining.

RESULTS: *G. sepium* leaves significantly ($p < 0.05$) upregulated the expression of TGF-β1, Smad-3, β-catenin, and LGR-6 in the group treated with Indonesian *G. sepium* leaves were higher than that in the group treated with *G. sepium* leaves from the Philippines.

CONCLUSIONS: Both leaves Varian contain flavonoids, saponins, and tannins, which act as producing epidermal stem cell agents to enhance the wound healing process. It can be concluded that both *Gl. sepium* Varian Indonesia and the Philippines have a potential effect on wound healing.

Introduction

Wounds are the destruction of body tissue [1]. Wounds occur in the cutaneous that cause damage to the skin epithelium or the disruption of the normal anatomical structure of the tissue due to trauma [2]. After the injury, cutaneous integrity must be promptly restored to maintain its functions. In this process, cutaneous wound healing is an important step for survival, completing in wound closure [3].

Cutaneous wound healing is a complex process of devitalizing missing cellular structures [4] [5]. The process of tissue repair occurs due to the repair and regenerative abilities of cutaneous tissue. It is related to epidermal stem cells [6]. Epidermal stem cells are multipotent cell types, where the amounts of LGR-6, β-catenin, transforming growth factor-β1 (TGF-β1), and Smad3 protein. These proteins are produced in response to optimally wound healing of tissue damage [7], [8], [9], [10], [11], [12], [13].

A balance of cellular processes is necessary to maintain tissue homeostasis. TGF-β is a cytokine that plays an important role in regulating several cellular processes, including self-renewal and cell differentiation [14]. Smad2 and Smad3 are transcription factors in the TGF- branch through binding between the ligands and the TGF-β1 receptor [15]. TGF-β ligands activate the Smad2/3 intracellular pathway and promote wound contraction resulting in a reduction wound's size area [16], [17].

β-Catenin/Wnt could enhance the healing process. A7B5-Catenin regulates fibroblast behavior during the proliferative phase of dermal wound repair [18]. Lgr6 belongs to the type B family of LGR proteins, which have been intensively studied as markers and regulators of adult stem cells [19].

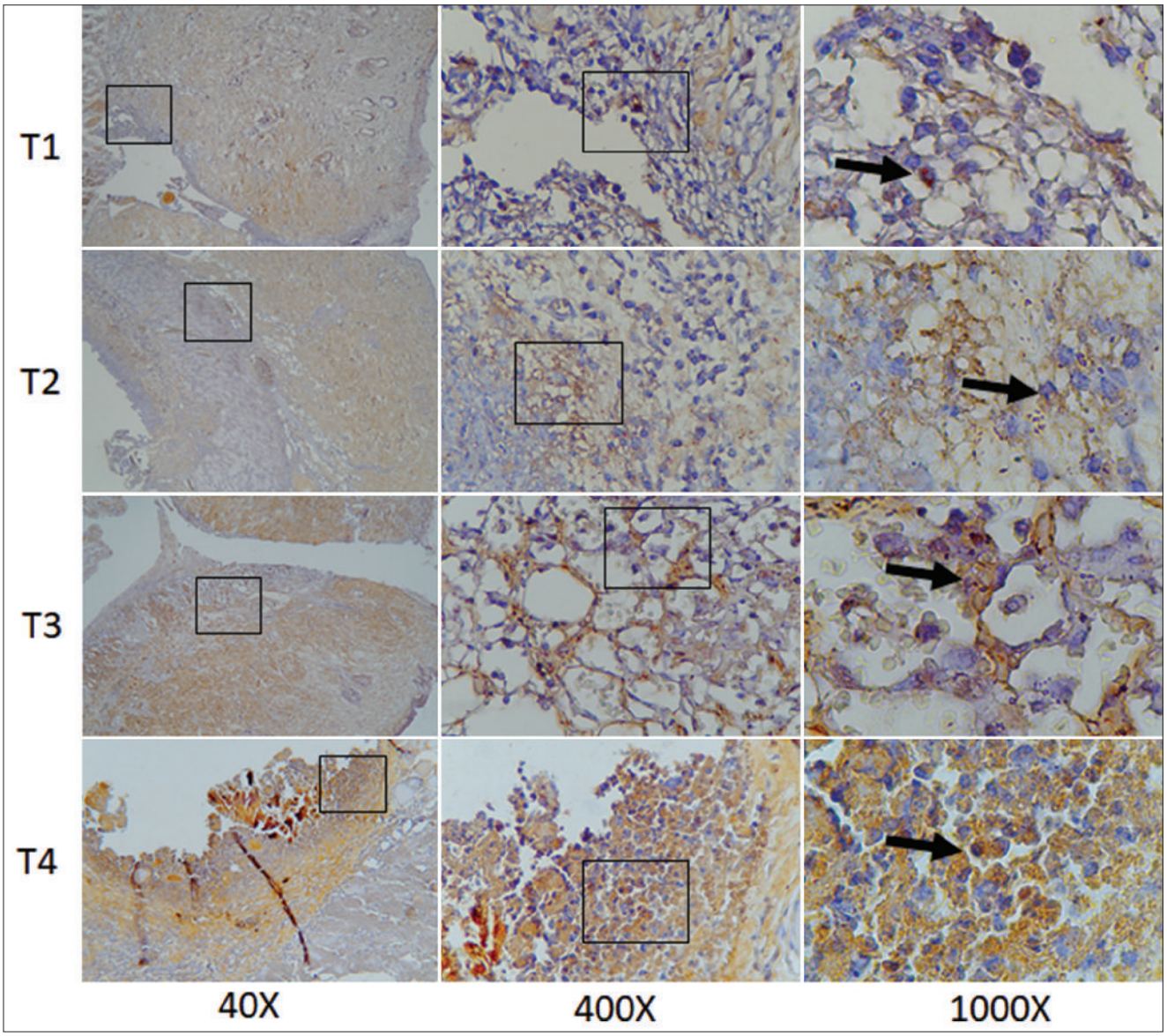

Figure 1: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against transforming growth factor-β1. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated with Gliricidia sepium from Indonesia; (T4) treated with Gliricidia sepium from the Philippines

Enhancing β-Catenin results to strengthen the β-Catenin/Wnt signaling pathway [20].

Nowadays, wound therapies are limited, therefore finding to develop better therapeutic strategies is occurring. According to the World Health Organization, 80% of Asian and African populations use traditional medicine or herbal medicine in their healthcare needs, due to easy and low side effects [21]. Leaves are parts that are often used as herbal medicines, one of which is *Gliricidia sepium (G. sepium)* leaves. *G. sepium* is a legume plant belonging to the family *Fabaceae* and is found widely in subtropical and tropical areas, such as in Indonesia and the Philippines [22]. Molina-Botero *et al.* studied its active substances, including flavonoids, saponins, tannins, alkaloids, polyphenols, hydroxyl acid, and coumarin [23]. Aulanni'am *et al.* use *G. sepium* leaves can heal excision wounds with their anti-inflammatory effect because it contains bioactive compounds to enhance the wound healing process [24]. According to research by Carandang *et al.* wound treated with 7.5% gel *G. sepium* on excision wound is safe, effective, and stable [25].

Hence, this study was performed to further determine the efficacy of *G. sepium* leaves as a wound-healing agent based on the evidence of increased potential of the epidermal stem cells as well as increased expression of TGF-β1, Smad3, β-catenin, and LGR-6 protein.

Materials and Methods

Animals and ethical approval

Inbred male *Rattus norvegicus* used in this study were obtained from Institut Biosains Laboratory.

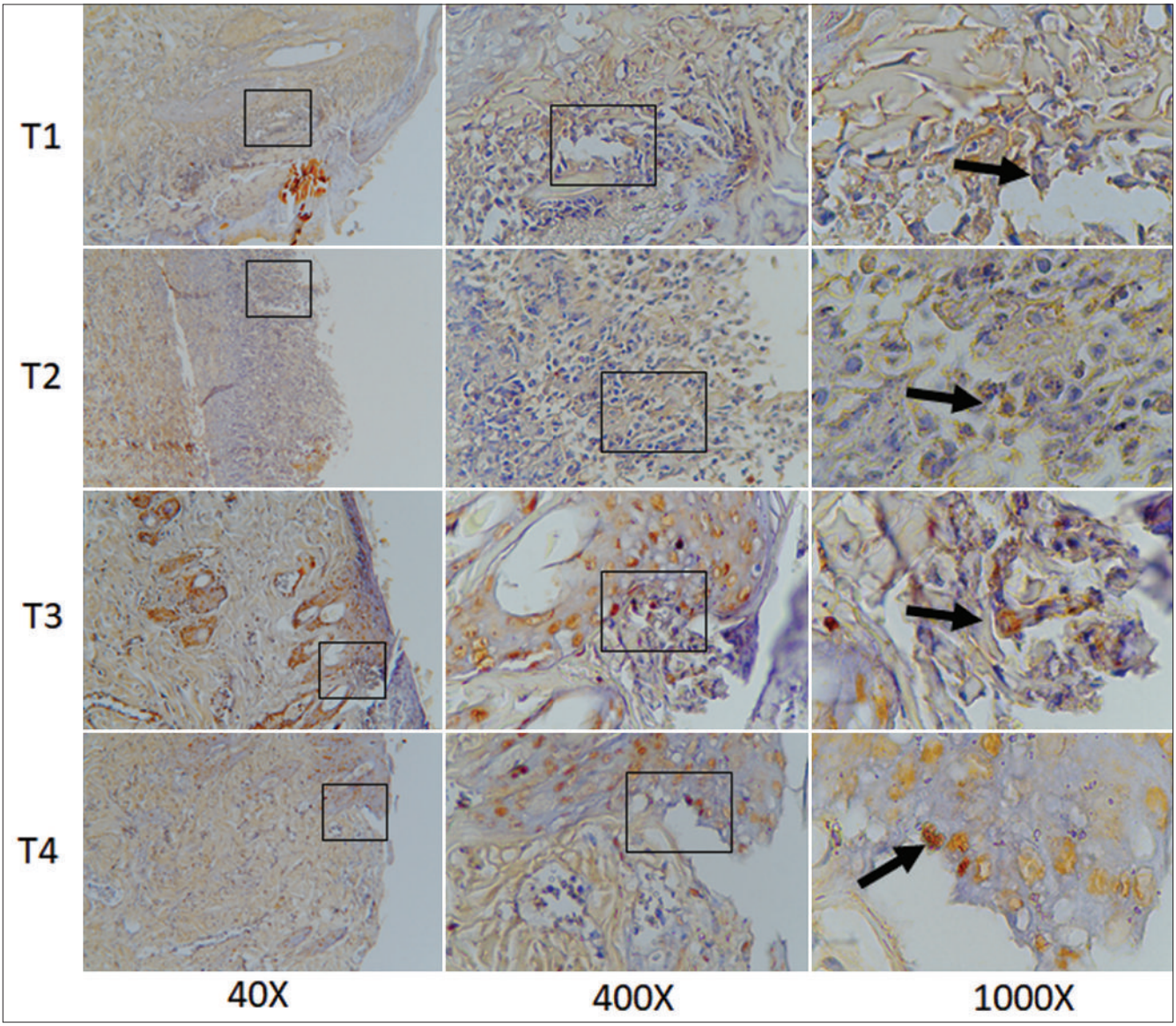

Figure 2: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against Smad3. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated with Gliricidia sepium from Indonesia; (T4) treated with Gliricidia sepium from the Philippines

Rats are approximately 2 months old and weigh 150–200 g. The experimental procedures applied in this study were approved by the Brawijaya University Research Ethics Committee (No. 1004-KEP-UB).

Study period

The research was conducted at the Animal Disease and Diagnostic Laboratory, Faculty of Veterinary Medicine, Brawijaya University, Malang, Indonesia, from May to October 2020.

Experimental design

This experiment used a completely randomized experimental design. Rats were divided into four treatment groups comprising four rats per group as follows: T1, positive control, treated with a commercial wound healing agent; T2, negative control; T3, wounds treated with *G. sepium* from Indonesia; and T4, wounds treated with *G. sepium* from the Philippines. The rats were anesthetized with an intramuscular injection of ketamine (10 mg/kg body weight).

Gliricidia sepium preparation and wound treatment

G. sepium leaves from Indonesia and the Philippines were identified in the Plant Taxonomy Laboratory of the Biology Department, Brawijaya University. All leaves were dry-aired and grounded into a powder. After that, powder the ointment by adding petroleum jelly. The ointment was put into the wounds for 3 days.

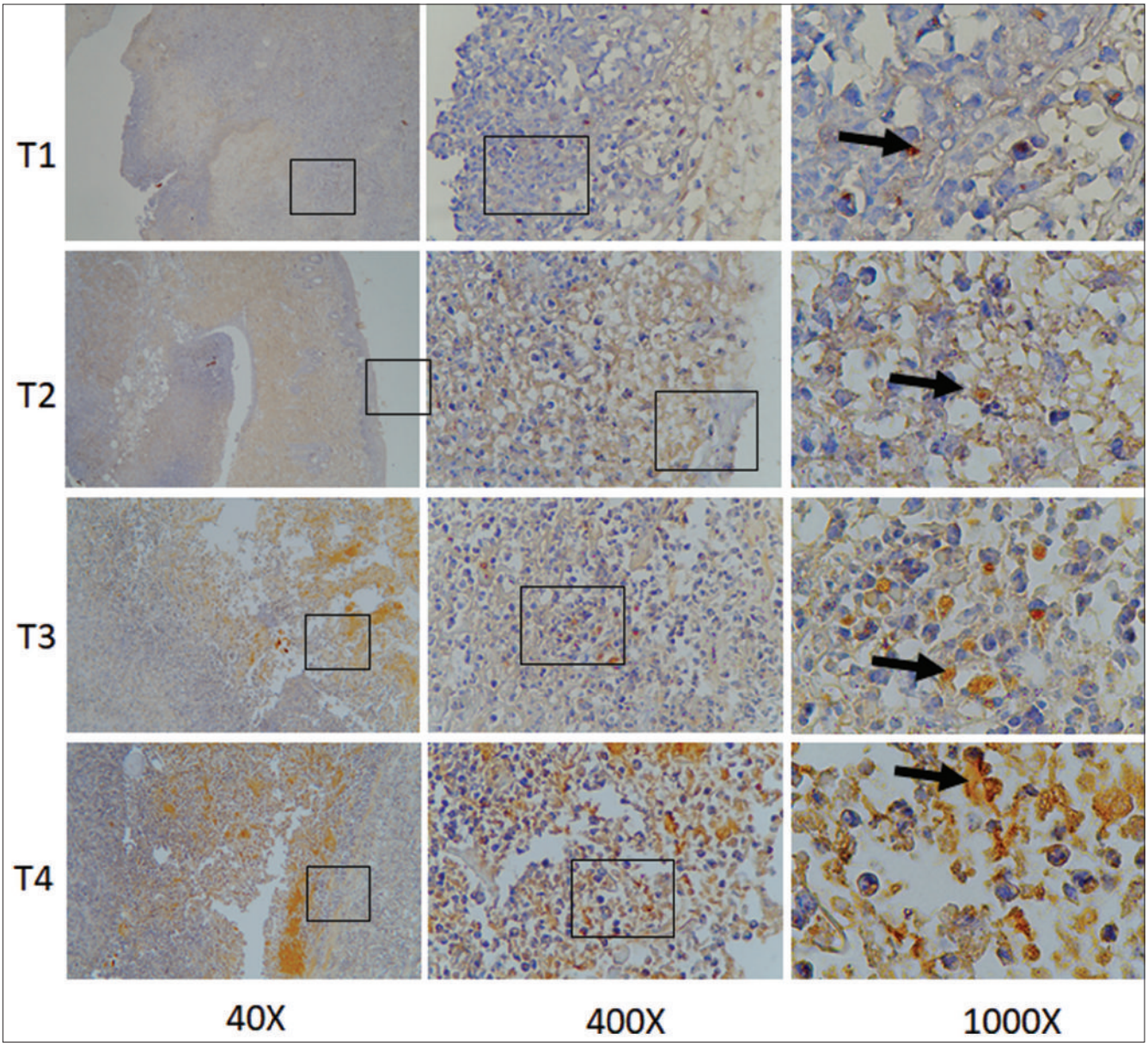

Figure 3: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against β-catenin. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated with Gliricidia sepium from Indonesia; (T4) treated with Gliricidia sepium from the Philippines

Measurement of LGR-6, beta-catenin, transforming growth factor-β1, smad-3 expression by immunohistochemistry

Skin samples were processed in the standard protocol of fixation, embedding, deparaffinization, labeling primary antibody (TGF-β1, Smad-3. β-catenin, Lgr-6) and secondary antibody, counterstaining. An immunohistochemistry technique was performed to analyze TGF-β1, Smad3. β-catenin, LGR-6 expression based on the previous methods [26].

Statistical analysis

Statistical analyses were using SPSS software version 14.0 (IBM, USA). The data were analyzed with a one-way analysis of variance and a Tukey test with $\alpha = 0.05$ to determine differences between the treatment groups.

Results and Discussion

Effect of an ointment containing *G. sepium* leaves on TGF-β1, Smad3, β-catenin, LGR-6 expression in immunohistochemistry evaluations, the positive cells show brown color. Immunostaining intensity for TGF-β1, Smad3, β-catenin, and LGR-6 was moderate to strong for both extracts in the treatment group. As shown in Figures 1-4, TGF-β1, Smad3, β-catenin, and LGR-6 immunoreactivity was higher in both extracts

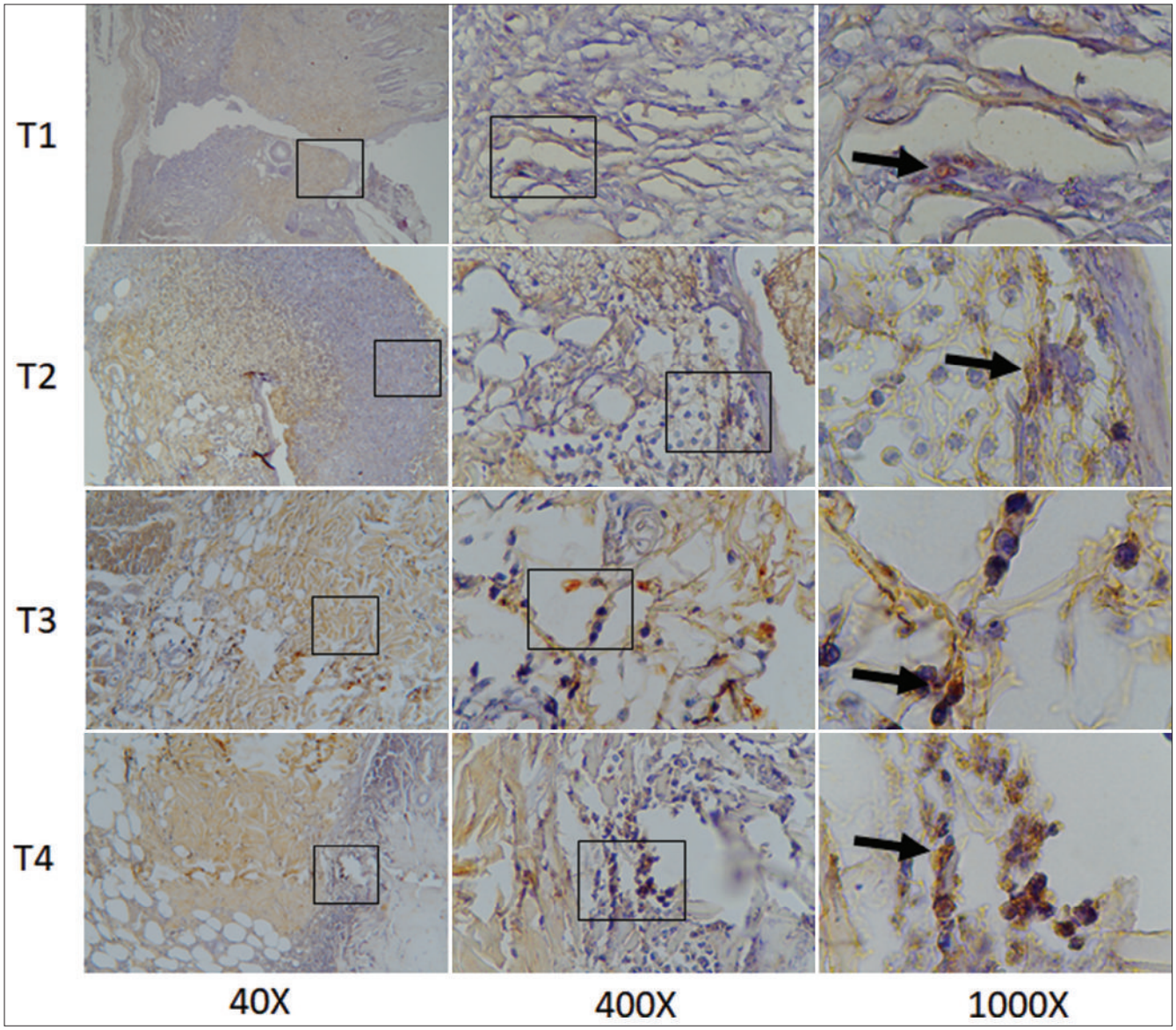

Figure 4: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against LGR-6. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated with Gliricidia sepium from Indonesia; (T4) treated with Gliricidia sepium from the Philippines

treated than in the control group. The treatment group had shown a significant increase in H-SCORE than the control group ($p < 0.05$, Table 1).

Table 1: The expression of transforming growth factor-β1, Smad-3, Beta-catenin and LGR-6

Group	TGF-β1	Smad-3	β-catenin	LGR-6
T1	8.33 ± 2.16^{b}	7.33 ± 2.58^{a}	8.17 ± 1.83^{b}	5.17 ± 2.56^{a}
T2	2.83 ± 1.72^{a}	$3.50 \pm 1.87a^{b}$	3.17 ± 1.94^{a}	4.86 ± 2.48^{a}
T3	9.17 ± 1.60^{b}	13.33 ± 2.42^{c}	12.33 ± 2.58^{c}	12.00 ± 2.10^{b}
T4	8.17 ± 2.32^{b}	$10.00 \pm 2.83^{b,c}$	$10.83 \pm 2.48^{b,c}$	10.33 ± 2.16^{b}

T1: Positive control, T2: Negative control, T3: Wounds treated with *G. sepium* from Indonesia, T4: Wounds treated with *G. sepium* from the Philippines. TGF-β1: Transforming growth factor-β1, *G. sepium: Gliricidia sepium*

The result of Smad3, β-catenin, LGR-6, and TGF-β1 expression in this study are shown in Table 1. The Smad3, β-catenin, LGR-6, and TGF-β1 expression level in the negative control group (T2) were obtained below the expression level in the positive group (T1) and the treated group (T3 and T4). Normally, epidermal stem cells in normal conditions act to maintain the skin homeostasis that displaces the lost keratinocyte through normal differentiation and tissue turnover [27]. After treatment, the treated group with *G.sepium* var. Indonesia and Philippine extract ointment increase TGF-β1 protein expression [28]. The release of TGF-β1 happens at an early stage of the healing process to the recruitment of inflammatory cells into the injury area. TGF-β1 encourages the expression of vascular endothelial growth factor that improves the angiogenic process in the injured area and stimulates the fibroblast to contract for closing the wound [29], [30].

The Smad3 expression of the treatment group (T3 and T4) has significantly increased in this study. Smad family proteins are phosphorylated by TGF-β receptors and will activate Smad 3 pathways [31], [32],

[33]. TGF-β/Smad3 plays a role in the development of vascular reconstruction. It is important in the wound healing process [9].

Epidermal stem cells acquire the re-epithelialization process [34]. The treated group (T3 and T4) showed an increase of β-catenin expression that indicates active Wnt signaling through β-catenin. Wnt signaling through β-catenin plays a crucial role in skin regenerating [35]. Wnt/β-catenin signaling is the first molecular signal that is required to instruct epithelial cells [27].

Protein expression of LGR-6 also enhances after both treatments. LGR-6 is responsible as marker adult stem cells for fueling the renewal of the sebaceous gland and skin [36]. LGR-6 is also a Wnt downstream target gene. LGR-6 cells give rise during homeostatic growth [37], [38], [39]. In this study, the LGR-6 protein significantly increases both the treated group; it indicates that there is enhancement of epidermal stem cells to regenerate wounds.

The wound treated with *G. sepium* leaves Varian Indonesia showed increasing the protein expressions of epidermal stem cells, while wounds treated with *G. sepium* Varian the Philippines (T4). Both therapies showed a significant difference ($p < 0.05$) compared with the positive control (T1). *G. sepium* leaves Varian Indonesia and the Philippines contain active ingredients, such as flavonoids, saponins, tannins, and alkaloids that could enhance the epidermal stem cell function and stimulate healing the wound. Cutaneous wound healing is a vital physiological process that involves the cooperation of a variety of cell strains and their products [40], [41], [42], [43], [44], [45], [46].

We report here that *G. sepium* leaves extract ointment enhances the acquisition of epidermal stem cells in wound healing *in vivo* in a rat model. We demonstrated that *G. sepium* treatment significantly improved the expression of LGR-6, β-catenin, TGF-β1, and Smad3 protein in rat skin cells. These findings imply that *G. sepium* leaves extract to improve reprogramming efficiency and tissue regeneration.

Conclusions

These studies suggest that natural plant products from *G. sepium* leaf exhibit positive histopathological effects on *in vivo* wound healing in a rat model. Based on these findings, we suggest that *G. sepium* extracts potentially represent useful supplements for the regeneration of wound healing direct treatment, but this needs to be studied on tissue before animal models.

Authors' Contribution

AA designed the experiment. RR and WR helped statistically. The study was supervised by DKW, FSP, and MAGB.

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Climate-Resilient agri-fisheries (CRA) assessment, targeting & prioritization for the adaptation and mitigation initiative for tarlac province

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Climate-Resilient Agri-fisheries (CRA) Assessment, Targeting & Prioritization for the Adaptation and Mitigation Initiative for Tarlac Province

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Abstract. This study is part of the Adaptation and Mitigation Initiative in Agri-fisheries (AMIA) Project of the Department of Agriculture (DA) and International Center for Tropical Agriculture (CIAT) to operationalize the goal of making agriculture and its stakeholders adapt and mitigate the effects of climate change. The outputs of this study were vital in the implementation of the next phase of AMIA: climate-risk vulnerability (CRVA) map and investment brief. The CRVA map was developed to identify the vulnerable areas in Tarlac. The CRVA map considered three factors: climate-risks sensitivity, exposure, and adaptive capacity given a 15-15-70 weighted percentages, respectively. The municipalities of Ramos, La Paz, Bamban and Victoria were identified as highly vulnerable to the effects of climate change. Three investment briefs were prepared for policy makers for possible funding and implementation. They were developed by identifying climate-resilient agricultural practices in the province which are the use of climate smart varieties, crop rotation-minimum tillage combination, and alternate wetting and drying method. These practices were evaluated using the CBA Tool and were found to be sustainable. Social net present value was also determined taking into account the externalities, which was given a value through interview of experts.

INTRODUCTION

The Philippines is highly vulnerable to the adverse impacts of climate change as the country's backbone is agriculture [1]. The effect of intense and longer droughts, increase in temperature, erratic rainfall distribution and natural hazards that affects earth's ecosystem as well as health, livelihood, social systems and economy. Agriculture is one of the most vulnerable sectors on the impact of climate change and farmers who are directly dependent on their lands for survival are the most affected. Climate change mitigation and adaptation to climate change is crucial particularly in agriculture sector, thus, different adaptive strategies are being done to cope up with its impacts. One of the activities in the strategic actions of food security for 2011-2028 is to enhance site-specific knowledge on the vulnerability of agriculture and fisheries [2].

The Adaptation and Mitigation Initiative in Agriculture (AMIA) seeks to enable the Department of Agriculture (DA) to plan and implement strategies to support local communities in managing climate risks – from extreme weather events to long-term climatic shifts. Spearheaded by the DA System-wide Climate Change Office (DA SWCCO), AMIA Phase 1 in 2015-16 to implement activities to strengthen DA's capacity to mainstream climate change adaptation and mitigation strategies locally and in national scale. With AMIA Phase 2, making climate-resilient agri-fisheries (CRA) an operational approach through field-level action that directly involves, and influences on the livelihoods of farming communities is one of the next big task for the program. AMIA2 aims to invest in the launching of CRA communities in Tarlac province as the initial target site for action learning, supported by an integrated package of climate services within a broader food value chain setting. The program is launching an integrated and multi-stakeholder effort to operationalize CRA at the community level in 10 target regions. Successful implementation of AMIA2 at the regional level requires the strong collaboration and support of key research and development institutions within the region. This proposed project enables AMIA2 to establish and mobilize regional teams, each led by a local State University/College (SUC), and in partnership with the corresponding Department of Agriculture - Regional Field Office (DA-RFOs).

Climate-resilient agriculture/agri-fisheries (CRA) aims to achieve food security and broader development goals under a changing climate and increasing food demand [3]. With the three pillars: productivity, adaptation, and mitigation, CRA initiates sustainably increase productivity, enhance resilience, and require planning to address tradeoffs and synergies [4]. To enable to assess, plan and pilot climate-risk prone agri-fisheries communities in pursuing sustainable livelihoods while effectively managing the impacts of climate variability. Thus, one of the Region to initiates the CRA strategic framework was Region 3 or Central Luzon specifically in

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Tarlac province to identify key climate risk and vulnerable areas and to assess the current status of CRA as well as the cost and benefits of these practices and technologies. Therefore, the study aims to establish and mobilized team in Region III for AMIA 2 in order to operationalized AMIA strategies in managing climate risk in Tarlac province. Specifically is to enhanced capacities of AMIA partner organizations in the Region, developed geospatially referenced data on climate-risks in Tarlac, generate profile on community's CRA strategies, and perform costs-benefits & trade-offs for these CRA practices. The result of the study will serve as guide in piloting community action research in establishing community-level research and development interventions.

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MATERIALS AND METHODS

Study Area

The study was established in the province of Tarlac. The study covered an area of 273,660 hectare and is located between latitude 15º 10'15" N to 15º 52'52" N, longitude 120º 8'4" E to 120º 46'27" E. The study area has flat to undulating topography, with the eastern part of the province being plain and the western part to be hilly to mountainous. Tarlac has two distinct seasons, the wet and the dry seasons. It has unimodal rainfall pattern, having high monsoon peaks in the wet season (WS) and negligible rainfall in the dry season (DS). Recorded annual rainfall varies from 2,030 mm to 4,060 mm in the northwestern portion [5].

Tarlac is basically an agriculture-based economy, located in the heart of Central Luzon with a total land area of 305,345 ha, constitutes 16.75% of the regional land area and 1.0 % of the total national land area with 112,997.57 hectares concentrated on agricultural production. Rice and corn are the top 2 commodities planted in the province planted in 2 to 3 cropping a year. There are 102,178.06 ha planted to rice, which are irrigated, rainfed and in upland areas. On the other hand, there are 16,458.98 ha planted to corn. Of these, only a small portion, are planted with white corn while the rest are planted with the yellow corn. With this vast track of land concentrated in agriculture, Tarlac likewise grows lowland vegetables and root crops. Of the lowland vegetables grown in the region, tomato occupies the largest area with 215.81 has while sweet potato is the largely grown rootcrops with a production area of 3,641.58 ha. Both crops are grown after rice usually during the onset of the dry season when rice has been harvested. Orchard occupies 10,498.65 hectares planted with our local fruit trees. The most common is mango that is planted in an area totaling to 25,660.03 hectares [6].

Framework of AMIA 2 Project

The project seeks to contribute to the overall AMIA2 program framework, by contributing specific outputs to targeted national-level research projects. It has four key components: (1) Capacity strengthening for CRA research & development, (2) Geospatial assessment of climate risks, (3) Stakeholders' participation in climate adaptation planning, and (4) Documenting & analyzing CRA practices. These project components were designed to be directly aligned with the research agenda of three AMIA2 projects: 1) climate-risk vulnerability assessment (CRVA), 2) decision-support platform for CRA, and 3) institutional and policy innovations. Figure 1 shows the framework for this study.

Component 1 - Capacity strengthening for CRA research & development

The regional project team participated in a series of trainings, workshops and learning events organized by AMIA2 projects. These were focused on three key methodologies: 1) CRVA, 2) CRA prioritization, and 3) CRA M&E. The project provided training support to key research and development stakeholders in the region, by organizing an intra-regional training that covers key learning contents from the national-level trainings. The CRA monitoring and evaluation was later included in the phase two of the AMIA2 which is currently handled by the Department of Agriculture Regional Field Office 3 in Victoria, Tarlac. The town was one of the identified vulnerable areas in the province.

Component 2 - Geospatial assessment of climate risks

The project team collected and organized geo-referenced data on vulnerability to climate risks of the region's agri-fisheries sector. These datasets, from both primary and secondary sources, were used on the methodological guidelines provided by the AMIA2 CRVA project – covering climate-risk exposure, sensitivity and adaptive capacity. Preliminary analysis – using GIS software and climate modelling tools – was undertaken at the regional level. The project team also participated in a national-team level joint analysis of cross-regional data.

Component 3 - Stakeholders' participation in climate adaptation planning

The regional project team organized a series of stakeholders' meetings and focus group discussions to collect supplementary data and validate preliminary results of CRVA, as well as in identifying CRA prioritization and planning. These activities were guided by process facilitation using the MaxEnt and CBA Tool developed by the AMIA2 projects on CRVA and CRA decision-support platform.

Component 4 - Documenting & analyzing CRA practices

A semi-structured survey with local stakeholders was conducted to identify and document CRA practices, as well as collect existing CRA-relevant statistical and other secondary data. Focus group discussion with farmers, farmer-leaders, technician, municipal and provincial agriculturists, and representative of provincial government of Tarlac were done on four separate occasions to gather data, validate and present the results, and revalidation of results. These data was systematized and analysed using cost-benefit and trade-off analyses tools as input to AMIA2 CRA prioritization and investment planning. A CBA Tool was made available online by the CIAT to facilitate the computation of cost, benefits, and others. These contributed to developing knowledge products, such as searchable online portal, under the AMIA2 project on CRVA decision-support platform.

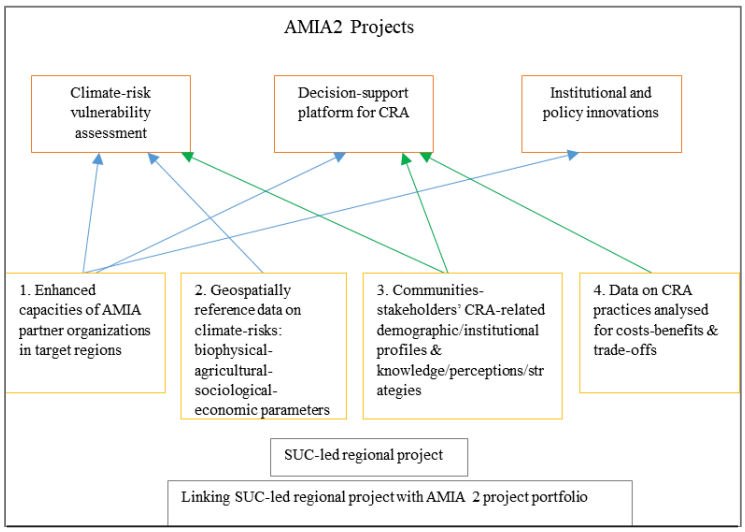

FIGURE 1. Framework of the Study

Data Acquisition

Collection of secondary data for exposure-sensitivity and secondary-primary data for adaptive capacity was done from different agencies such as the Provincial Agriculture Office, Municipal Agriculture Offices, and data from the AMIA2 outputs. Data collection started with gathering of primary and secondary data, that includes those from focus group discussion (FGD), key informant interviews (KII) and municipality surveys.

CRVA Framework

The vulnerability mapping was done which follows the Climate-Risk Vulnerability Assessment (CRVA) framework. It starts with the identification of the vulnerability determinants (hazard, sensitivity, and adaptive capacity) and their respective indicators. This framework was the standardized procedure followed by different SUCs involved in the project. Data collection started in gathering the primary and secondary data, that includes those from focus group discussion (FGD), key informant interviews (KII) and municipality surveys. The identified priority crops of the province are rice, corn, tomato, and mango. Sweet potato was an additional crop prioritized by the team because it is one of the crops planted on the second cropping season after rice and corn.

CRA Practices

Key informant survey on climate-resilient agri-fisheries (CRA) practices in the province of Tarlac. These were gathered from a series of Focus Group Discussions with municipal agriculturists, representatives of the Office of the Provincial Agriculturists and Local Farmer Technicians. From the list of CRA practices, key

informants identified three priority practices. The interview guide provided by CIAT was modified to fit the scope of the study before it was given to the group of key informants. Data gathered were used in the evaluation of CRA practices using the CBA Tool. CRA practices with the incorporation of externalities was given priority in the study.

RESULTS AND DISCUSSIONS

Enhanced Capacities of AMIA Partner Organizations in the Region

Series of trainings, workshops and learning events were organized for AMIA2 project teams in an intra-regional training that covers key learning contents from the national-level trainings as shown in Table 1.

Climate Risk Vulnerability Assessment (CRVA)

The vulnerability mapping was based on the Climate-Risk Vulnerability Assessment (CRVA) framework. It started with the identification of the vulnerability determinants (sensitivity, hazards, and adaptive capacity) and their respective indicators.

Sensitivity Analysis

In developing the impact of climate change to crop suitability, a crop distribution model was used and the factors associated are the 20 bioclimatic variables and the existing crop location. Sensitivity index was used in the sensitivity analysis to determine the sensitivity of crops to changes in temperature and precipitation.

The aggregated sensitivity of crops for rice, maize/corn, tomato, mango, and sweetpotato are given equal weights of 20% as shown in Figure 2 (a). It appears that the sensitivity index of the province generally ranges from -5% to -50% which means that it is sensitive to highly sensitive as influenced by the bioclimatic variables. On the other hand, improved varieties of crops, water conservation and soil conservation technologies that are being practiced by farmers mitigate the effect on crop yield.

Hazards Vulnerability

The natural hazards that were added to come up with the hazard vulnerability index are tropical cyclone/typhoon, flood, drought and erosion which are given the corresponding weights of 35%, 35%, 27% and 3%, respectively. The areas of San Manuel, Anao, Moncada, and La Paz are highly vulnerable to hazards as shown in Figure 2 (b). These areas experiences flooding on the onset of monsoon rain especially during typhoon because of geographically low areas of the province and become the catchment basin of the nearby provinces.

Adaptive Capacity Analysis

The adaptive capacity indicators considered in the assessment of the province's readiness to withstand the effects of climate change. These seven capitals are economic, natural, human, physical, anticipatory, social and institutional. Figure 2 (c) on the adaptive capacity illustrates the economic, natural, human, physical, anticipatory, social and institutional capital of every municipality. These seven (7) capitals of the adaptive capacity were given the same weight to come up with the aggregated adaptive capacity map.

The adaptive capacity of the province shows the readiness to adapt to climate risk. Concepcion and Gerona were found to have very high adaptive capacity while the municipalities of Ramos, San Jose, La Paz, and Bamban have very low adaptive capacity.

Climate-Risk Vulnerability

The climate-risk vulnerability map was developed by adding the sensitivity index, hazard index and the adaptive capacity index with their corresponding weights. A national experts' meeting composed of agriculturists, policy makers, and scientist, agreed the 15-15-70 percentage of weight for the sensitivity, hazard and adaptive capacity, respectively. The formula used in the development of climate-risk vulnerability map is stated in Equation 1:

$$\begin{aligned}\text{Climate - risk vulnerability} = {} & \text{Sensitivity index} * 0.15 + \\ & \text{Hazard index} * 0.15 + \\ & \text{Adaptive capacity index} * 0.70\end{aligned} \quad (1)$$

Figure 3 show the vulnerability to climate-risk is very high in the municipalities of Ramos, Bamban and La Paz; high in San Jose, Victoria and Pura; lowest in Tarlac City. The factor that has the major contribution in the vulnerability assessment is the adaptive capacity given a weight of 70% compared to sensitivity and hazards with 15% weights each. The perspective of giving a high percentage to the adaptive capacity is the thought of the ability of every municipality being able to cope with extreme events like temperature, rainfall, typhoon, flood, drought, erosion and other natural hazards because these municipalities are equipped with facilities and structures, and services for the adaptation.

The maps developed in the CRVA assessment was presented to a focus group discussion (FGD) with the stakeholders from the Provincial Agriculture Office (PAO), Municipal Agriculture Office (MAO), Farmers and other agencies. From the FGD and field visit conducted, the participants agreed that the maps developed are similar to the actual situation in their municipality. Vulnerable areas due to risks of climate was mentioned in the study of Dikitanan, et al. (2017).

TABLE 1. Capability Building Training, Seminars and Workshop Attended by the Regional Team

Title	Date	Venue
Training on Climate Risk Vulnerability Assessment	June 6-8, 2016	Torre Venezia Hotel, Quezon City
Cost- Benefit Analysis (CBA) on Climate Resilient Agriculture Practices	August 6-8, 2016	Torre Venezia Hotel, Quezon City
Methodology for Evaluating Social and Environmental Benefits, in Agricultural Systems	December 2, 2017	Tarlac Agricultural University, Camiling, Tarlac
Climate Risk Vulnerability Assessment (CRVA) Mapping & and Adaptive Capacity Mapping	January 10-12, 2017	SEARCA, UPLB, Los Banos, Laguna
AMIA2-CIAT Project: Results Sharing and Validation Workshop on CRVA & CRA Decision Support	February 6-7,2017	Parklane International Hotel, Cebu City
Workshop on Finalizing Results on CRA and Prioritization and Extended CBA	March 1-3, 2017	B Hotel, Quezon City
Completion Review of BAR Funded Climate Change Projects	May31-June 2, 2017	Partido State University, Goa, Camarines Sur
AMIA2-CIAT Project: Workshop on Outcome Monitoring and Evaluation of Community-Based Action Research	June 21-22, 2017	Sequoia Hotel, Quezon City

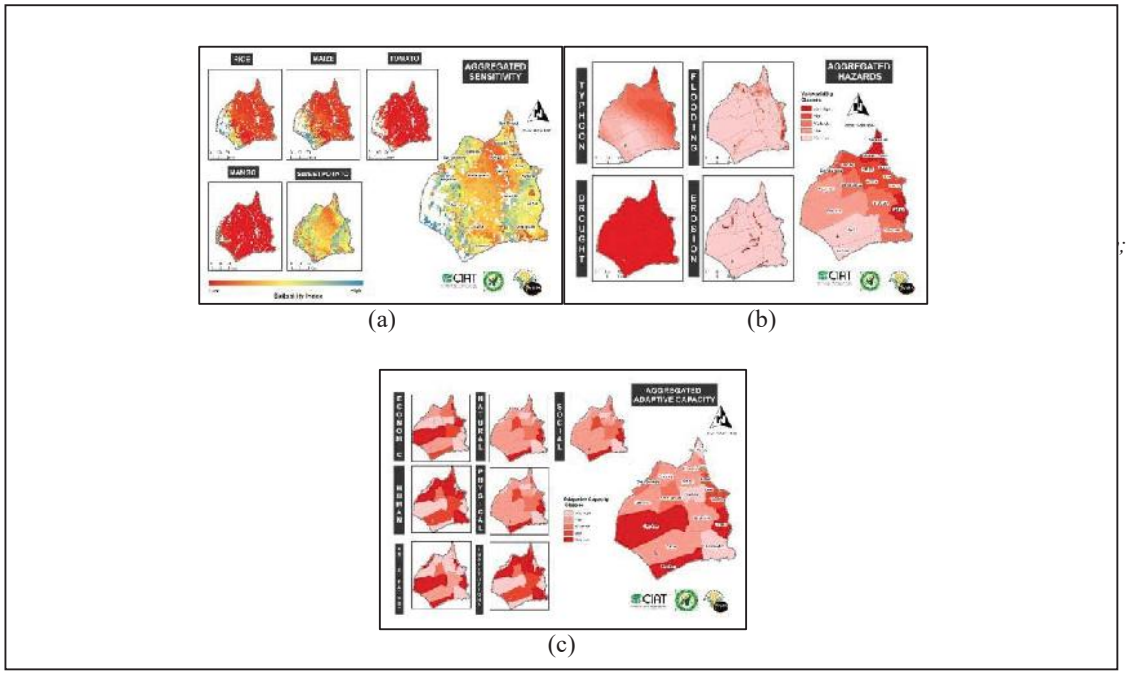

FIGURE 2. Maps on sensitivity, hazards and adaptive capacity to produce CRVA map: (a) Aggregated Sensitivity; (b) Aggregated Hazards; and (c) Aggregated Adaptive Capacity

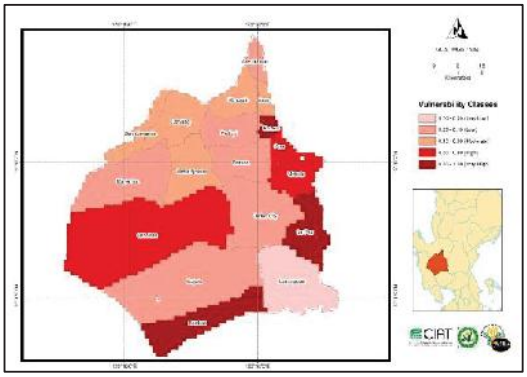

FIGURE 3. Climate-Risk Vulnerability Map of Tarlac

Cost-Benefit Analysis (CBA) of CRA Practices

The identified climate-resilient practices in the province of Tarlac which are the following: Organic agriculture, climate smart varieties/lines, water conservation technology, adaptive crop calendar/crop switching, soil conservation technology, intercropping/crop rotation, community-based management, crop insurance/weather adverse condition insurance, and improved housing for animals. The identification of CRA practices were gathered through interview. The list was trimmed and validated through a series of FGDs with municipal agriculturists, representatives of the Office of the Provincial Agriculturists, local farmer technicians and farmers. CRA practices have also been priorities in the study of Dikitanan, et al. (2017).

From the list of CRA practices, key informants identified three priority practices: climate smart varieties, crop rotation, and water conservation technology particularly the alternate wet and dry method (AWD). The interview guide provided by CIAT was modified to fit the scope of the study before it was given to the group of key informants. Data gathered were used in the evaluation of CRA practices using the CBA Tool. Validation of data was done by seventy one (71) municipal agriculturists, farmer leaders and farmer technicians participated in the Focus Group Discussions. The cost and benefits derived from the CRA practices were confirmed. These data were used to analyse the profitability and sustainability of the CRA practice. The three prioritized practices are the climate smart varieties, crop rotation and water conservation technology because of the immediate effects and perceived potential benefits to the farmers, food security and, mitigation and adaptation to climate change. Investment prioritization brief were also prepared for policy makers to support the climate-resilient agriculture practices in the provinces as shown in Figure 4. Researchers and extensions' personnel must use the investment brief for dissemination and reference for further validation or research in a specific area.

Climate Smart Varieties

Yield of crops is affected by extreme changes in climatic conditions such as flooding, and drought and attack of insect pests and diseases. Farmers and agricultural technologists favor climate smart varieties because of its high yield and its capacity to withstand varied climatic conditions. The Green Super Rice lines (GSR 8, 15, 21, and 22) earned special mention among farmers who have experienced growing it due to its resiliency especially during typhoons and floods, drought, and attack of insect pests and diseases. Farmers who planted GSR lines attested the resilience of these lines to typhoon and submergence. Yield was not likewise affected.

Crop Rotation

Crop rotation was chosen mainly because farmers could switch from rice to other crops (corn, sweet potato, etc.) depending on availability of water, soil conditions, and other climactic factors. Crop rotation also means reduction in pest occurrence due to non-availability of the host.

Water Conservation Technology

To address problems on scarcity of water supply or limited access to water sources in the upland, rainfed, and other areas, water conservation technologies are resorted to. The Alternate Wet and Dry (AWD) Method was specially mentioned because it utilizes materials readily available and can be constructed easily by the farmer. Some parts of the province depend on shallow tube wells and Tarlac Ground (TG) water as their main

source of irrigation. In these areas, one of the main expenses incurred by farmers is gasoline that is used to run pumps. This practice was chosen because it allows farmers to irrigate at the proper time. The intermittent drying of fields enables the farmers to save on time and money.

FIGURE 4. Investment Brief for Climate Smart Agriculture Practices

CONCLUSION

The two major output of this study are: climate-risks vulnerability map and investments briefs. The map was used to identify pilot area of climate smart village. The Department of Agriculture Regional Field Office 3 chose the municipality of Victoria as the pilot climate-smart village in the province of Tarlac. DA RFO3 based the selection on the result of the climate-risk vulnerability mapping and willingness of the local government unit to support the project. Victoria is considered as one of the high vulnerable municipalities that is sensitive to changes in climate, vulnerable to hazards and has less adaptive capacity to cope up with the phenomenon. Victoria is a 2nd class municipality of the province of Tarlac with 26 barangays and is located at the eastern part of the province. Majority of the total land area is devoted to agricultural activities and livestock production.

The climate resilient agriculture practices identified in the prioritization is being adopted in areas in the province for further testing and evaluation in the suitability of a CRA practices in an area. The investment briefs were presented to Department of Agriculture and Office of the President as a support for decision- and policy-making.

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Research Article

An *In Silico* Study of Examining Bioactive Compounds from *Azadirachta indica* Juss. (Neem) as Potential Death Receptor 5 Inductor in Hepatoma Cells

Ricadonna Raissa[1], Anna Safitri[2,3], Masruri Masruri[2], Ma Asuncion Guiang Beltran[5], Aulanni'am Aulanni'am[2,4,*]

ABSTRACT

Hepatocellular carcinoma is a disease that occurs due to the uncontrolled growth of abnormal hepatocytes. While cancer cells will not die by itself, due to resistance to death receptors 5 (DR5)-mediated apoptosis. This study is aimed to investigate *Azadirachta indica* Juss. leaves compound, such as gedunin and nimbolide, in binding DR5 and stimulated the TNF-related apoptosis inducing ligand (TRAIL), native ligand binding to DR5, which has a role of pro-apoptotic by docking simulation. The ligand and protein preparations were done using Discovery Studio 2016 and Hex 8.0.0 for docking. Visualization was done using Discovery Studio 2016. The docking studies revealed that nimbolide has a lower binding energy with the DR5-TRAIL complex than gedunin. According to the findings, nimbolide is a more effective DR5-TRAIL binding inducer than gedunin and has a higher binding affinity for DR5-TRAIL. This interaction has the potential to significantly reduce DR5-TRAIL binding resistance. Nimbolide and gedunin can be considered as drugs that can sensitize TRAIL binding to DR5 and increase the activation of one of hepar cancers signaling apoptosis pathways.

Key words: *Azadirachta indica* Juss., Cancer, Death receptor 5, Apoptosis, *In silico*.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the major contributor to cancer deaths. In 2025, HCC cases are estimated to be about >1 million cases.[1] Predilection of HCC is formed by various factor such as viral infection with hepatitis B or C viruses (HCV), cirrhosis induced by alcohol consumption and other factor.[2] Chronic HCV or cirrhosis lead abnormal proliferation of primary hepatic.[3,4] The liver cancer progression menchanism is cell proliferation. The liver cancer cells keep the abnormal proliferation by defense mechanism to apoptosis.[5]

Death receptor 5 (DR5) is pro-apoptotic protein member of the tumor necrosis factor (TNF) receptor superfamily, localized in cell surface.[6] DR5 bind to its ligand, TNF-related apoptosis inducing ligand (TRAIL) to activate the extrinsic apoptotic pathway.[7] This apoptosis mechanism of DR5-TRAIL binding potential has been the focus of attention, due to selectivity to kill the cancer cell not the normal one.[8] An *in vitro* study show that there is down-regulation expression of DR5 in cancer cells (Elrod et al. 2010). The cell cancer has a defense mechanism against the apoptosis by resisting DR5 to bind the TRAIL.[9,10] Therefore, compounds that can stimulate sensitivity DR5 binding to TRAIL is need to be explored.

Plant derived compounds are promising anti cancer therapies by apoptosis.[11] *Azadirachta indica* Juss. (Neem) plants have been used in ancient herbal medicine for the treatment of various diseases, particularly cancer.[12,13] Compounds such as gedunin and nimbolide have anticancer properties.[14–17] Gedunin and nimbolide, neem limonoid, are one of the main chemical compounds found in the neem tree [14,18–20] Recent studies have shown that gedunin can inhibit the progression of cancer cell proliferation.[21–23] Meanwhile, an *in vitro* study showed that nimbolide induction of growth arrest and apoptosis.[19,24]

In the current study, we used in silico molecular docking method to analyze the interactions between the DR5-TRAIL as an important role in hepatoma cell and neem compounds to induce apoptosis.

MATERIALS AND METHODS

Ligands preparation

The chemical compound of mangosteen which consisted of gedunin (CID: 12004512) and nimbolide (CID: 12313376) were obtained from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in SDF format. Ligands energy were minimized and converted from SDF to PDB format by BIOVIA Discovery Studio Dassault systemes® 2019 (Figure 1).

Protein preparation

The protein structure was obtained from Protein Data Bank (PDB) (https://www.rcsb.org/) as a DR5-TRAIL complex (PDB ID: 1D4V) (Figure…). The protein was then prepared for removing the ligands and water molecules by using BIOVIA Discovery Studio Dassault systemes® 2019.

Chemical interaction and 3D molecular visualization

Docking was conducted by HEX 8.0.0 software to predict the binding energy and possible ligand interactions and its receptor.[25] In this project, we

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Table 1: Molecular interaction of DR5 amino acid residues among ligands.

Interaction	Name	Distance (Å)	Category	Type	Energy (kcal/mol)
DR5 (A) – TRAIL (B)	B:ARG121:NH2 - A:ASP90:OD1	4,15562	Electrostatic	Attractive Charge	-846,53
	B:ARG158:NH2 - A:GLU89:OE2	3,73783	Electrostatic	Attractive Charge	
	B:ARG227:NH1 - A:ASP120:OD2	4,15265	Electrostatic	Attractive Charge	
	B:ARG227:NH1 - A:GLU123:OE2	4,45712	Electrostatic	Attractive Charge	
	A:ARG115:HH11 - B:GLU271:O	2,05744	Hydrogen Bond	Conventional Hydrogen Bond	
	A:ARG115:HH21 - B:GLU271:O	1,66691	Hydrogen Bond	Conventional Hydrogen Bond	
	A:SER121:HG - B:ASN228:O	2,68742	Hydrogen Bond	Conventional Hydrogen Bond	
	B:GLN208:HN - A:GLU151:OE1	2,46377	Hydrogen Bond	Conventional Hydrogen Bond	
	B:LYS224:HZ1 - A:ASP148:O	2,65532	Hydrogen Bond	Conventional Hydrogen Bond	
	B:ARG227:HH21 - A:ARG118:O	2,2749	Hydrogen Bond	Conventional Hydrogen Bond	
	A:GLU151:OE1 - B:TYR209	4,85661	Electrostatic	Pi-Anion	
	A:LEU110:CD1 - B:PHE278	3,53824	Hydrophobic	Pi-Sigma	
	A:LEU111:CD2 - B:PHE163	2,98737	Hydrophobic	Pi-Sigma	
	A:LEU114:CD1 - B:TYR185	3,86078	Hydrophobic	Pi-Sigma	
	A:LEU114:CD2 - B:TYR183	3,15595	Hydrophobic	Pi-Sigma	
	A:ARG115:O - B:TYR243	2,17318	Other	Pi-Lone Pair	
	B:HIS125 - A:PHE112	4,73791	Hydrophobic	Pi-Pi Stacked	
	B:ALA226 - A:MET152	4,24296	Hydrophobic	Alkyl	
	B:TYR243 - A:ARG115	4,45895	Hydrophobic	Pi-Alkyl	
DR5 –Gedunin	A:ARG145:HH12 - :UNK0:O1	2,62927	Hydrogen Bond	Conventional Hydrogen Bond	-237.12
	A:TRP173:HE1 - :UNK0:O4	1,96636	Hydrogen Bond	Conventional Hydrogen Bond	
	A:ARG145:CD - :UNK0:O1	3,67468	Hydrogen Bond	Carbon Hydrogen Bond	
	:UNK0:H46 - A:CYS137:O	2,15221	Hydrogen Bond	Carbon Hydrogen Bond	
	:UNK0:H66 - A:THR135:O	2,55728	Hydrogen Bond	Carbon Hydrogen Bond	
	A:VAL136:CG1 - :UNK0	3,16122	Hydrophobic	Pi-Sigma	
	A:ARG145 - :UNK0	4,4262	Hydrophobic	Alkyl	
	:UNK0:C24 - A:PRO150	4,45623	Hydrophobic	Alkyl	
DR5 (A)-Gedunin-TRAIL (B)	B:ARG121:NH2 - A:ASP90:OD1	4,55422	Electrostatic	Attractive Charge	-868,84
	B:ARG158:NH2 - A:GLU89:OE2	4,44844	Electrostatic	Attractive Charge	
	B:ARG227:NH1 - A:ASP120:OD2	4,26184	Electrostatic	Attractive Charge	
	B:ARG227:NH1 - A:GLU123:OE2	4,50501	Electrostatic	Attractive Charge	
	B:ARG227:NH2 - A:GLU123:OE1	5,58713	Electrostatic	Attractive Charge	
	A:ARG115:HH11 - B:GLU271:O	1,97128	Hydrogen Bond	Conventional Hydrogen Bond	
	A:ARG115:HH21 - B:GLU271:O	1,39071	Hydrogen Bond	Conventional Hydrogen Bond	
	A:SER121:HG - B:ASN228:O	2,44347	Hydrogen Bond	Conventional Hydrogen Bond	
	B:LYS224:HZ1 - A:ASP148:O	2,91092	Hydrogen Bond	Conventional Hydrogen Bond	
	B:ARG227:HH21 - A:ARG118:O	2,32684	Electrostatic	Pi-Cation	
	A:GLU151:OE1 - B:TYR209	3,9936	Electrostatic	Pi-Anion	
	A:LEU110:CD2 - B:PHE278	3,23751	Hydrophobic	Pi-Sigma	
	A:LEU111:CD2 - B:PHE163	3,48296	Hydrophobic	Pi-Sigma	
	A:LEU114:CD2 - B:TYR183	3,58944	Hydrophobic	Pi-Sigma	
	A:ARG115:O - B:TYR243	2,40532	Other	Pi-Lone Pair	
	B:HIS125 - A:PHE112	5,01114	Hydrophobic	Pi-Pi Stacked	
	B:ALA226 - A:MET152	4,11703	Hydrophobic	Alkyl	
	B:TYR185 - A:LEU114	4,66379	Hydrophobic	Pi-Alkyl	
	B:TYR243 - A:ARG115	4,65331	Hydrophobic	Pi-Alkyl	
DR5 - Nimbolide	A:ARG145:HH12 - :UNK0:O6	2,71593	Hydrogen Bond	Conventional Hydrogen Bond	-247,7
	:UNK0:H61 - A:ARG145:O	1,71481	Hydrogen Bond	Carbon Hydrogen Bond	
	:UNK0:H61 - A:PRO172:O	2,01755	Hydrogen Bond	Carbon Hydrogen Bond	
	:UNK0:H64 - A:GLU146:O	2,70192	Hydrogen Bond	Carbon Hydrogen Bond	
	A:CYS139:SG - :UNK0	4,65808	Other	Pi-Sulfur	
	:UNK0 - A:TRP173	5,9592	Hydrophobic	Pi-Pi T-shaped	
	:UNK0:C34 - A:ARG145	3,88385	Hydrophobic	Alkyl	
	:UNK0:C34 - A:PRO150	4,84724	Hydrophobic	Alkyl	
	A:TRP173 - :UNK0	5,45953	Hydrophobic	Pi-Alkyl	
	:UNK0 - A:ARG145	3,45812	Hydrophobic	Pi-Alkyl	

DR5 (A) – Nimbolide – TRAIL (B)	B:ARG121:NH2 - A:ASP90:OD1	4,47138	Electrostatic	Attractive Charge	
	B:ARG158:NH2 - A:GLU89:OE2	4,10473	Electrostatic	Attractive Charge	
	B:ARG227:NH1 - A:ASP120:OD2	4,19337	Electrostatic	Attractive Charge	
	B:ARG227:NH1 - A:GLU123:OE2	4,52691	Electrostatic	Attractive Charge	
	B:ARG227:NH2 - A:GLU123:OE1	5,59371	Electrostatic	Attractive Charge	
	A:ARG115:HH11 - B:GLU271:O	2,00735	Hydrogen Bond	Conventional Hydrogen Bond	
	A:ARG115:HH21 - B:GLU271:O	1,52625	Hydrogen Bond	Conventional Hydrogen Bond	
	A:SER121:HG - B:ASN228:O	2,59169	Hydrogen Bond	Conventional Hydrogen Bond	
	B:GLN208:HN - A:GLU151:OE1	2,4509	Hydrogen Bond	Conventional Hydrogen Bond	
	B:ARG227:HH21 - A:ARG118:O	2,26908	Hydrogen Bond	Conventional Hydrogen Bond	-874,96
	A:GLU151:OE1 - B:TYR209	4,82039	Electrostatic	Pi-Cation	
	A:LEU110:CD1 - B:PHE278	3,91833	Hydrophobic	Pi-Sigma	
	A:LEU110:CD2 - B:PHE278	3,10911	Hydrophobic	Pi-Sigma	
	A:LEU111:CD2 - B:PHE163	3,28832	Hydrophobic	Pi-Sigma	
	A:LEU114:CD1 - B:TYR185	3,9988	Hydrophobic	Pi-Sigma	
	A:LEU114:CD2 - B:TYR183	3,45255	Hydrophobic	Pi-Sigma	
	A:ARG115:O - B:TYR243	2,33367	Other	Pi-Lone Pair	
	B:HIS125 - A:PHE112	4,91888	Hydrophobic	Pi-Pi Stacked	
	B:ALA226 - A:MET152	4,11085	Hydrophobic	Alkyl	

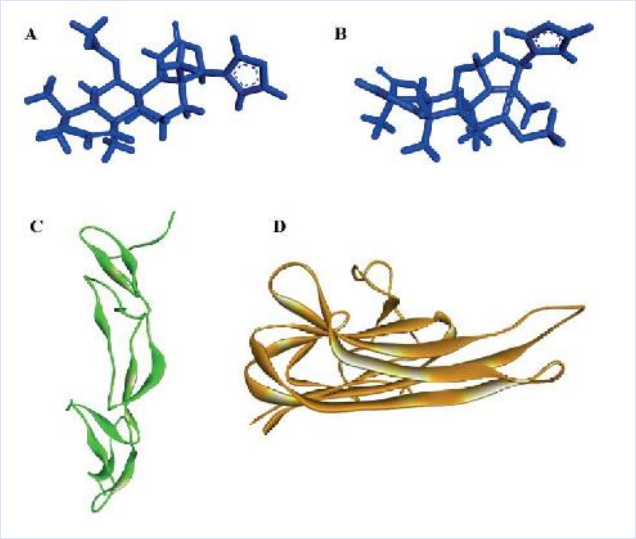

Figure 1: The chemical compound from neem: A) Gedunin (CID: 12004512); B) Nimbolide (CID: 12313376); C) death receptor 5 (DR5) (PDB ID: 1D4V); D) tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (PDB ID: 1D4V).

established several interactions such as DR5-TRAIL, DR5-gedunin. DR5-nimbolide, DR5-gedunin-TRAIL and DR5-nimbolide-TRAIL. The correlation type used in this docking was Shape+Electro+DARS. Docking results were then visualized using BIOVIA Discovery Studio Dassault systemes® 2019 to analyze the interactions.

RESULTS AND DISCUSSION

TRAIL interacted on DR5 amino acid residues includes Glu 89, Asp90, Leu110, Leu111, Leu114, Phe112, Arg115, Arg118, Asp120, Ser121, Glu123, Glu127, Asp148, Glu151, Phe152 and Asn228 with total binding energy -846,53 kcal/mol (Table 1).

Interaction between DR5 and gedunin established 4 hydrogen bonds and 3 hydrophobic bonds with total binding energy -237.12 kcal/mol (Table 1). Those interactions were bound with 5 amino acid residues such as Thr135, Cys137, Arg145, Pro150, and Trp173 outside of the DR5-TRAIL binding site (Figure 2). The DR5-gedunin complex interacted with TRAIL had total binding energy of -868.84 kcal/mol (Table 1). This complex may enhance DR5 activity by forming 4 hydrogen bonds in Arg115, Ser11 and Lys224 and one electrostatic interaction in Glu151 which are the active site of DR5 (Figure 2).

Nimbolide interacted with 4 amino acid residues DR5 with total binding energy -247.7 kcal/mol (Table 1). Nimbolide forms hydrogen bonds with Arg145, Glu146 and Pro172 which indicate that there is a strong bond established between the ligand and active site of DR5 located in Domains II (Figure 2). TRAIL, which interacted with the DR5-nimbolide complex had – 874.96 kcal/mol of total binding energy.

A hydrogen bond was formed in Arg115, Arg118, Ser121, and Glu151 at the active site of DR5 (Figure 3). There are two electrostatic interactions in Glu151, twice the gedunin binding. Electrostatic interactions with each other to form a stable binding.[26] Just like the DR5-gedunin-TRAIL complex, this complex may enhance DR5 activity. The thread of nimbolide before gedunin treatment showed the best result for compound combination to enhance DR5-TRAIL binding.

DR5 is a member of the tumor necrosis factor (TNF) receptor superfamily. DR5 has cytoplasmic death domains to induce cell apoptosis. The extracellular domain of the receptors is characterized by the concatenated cysteine-rich domains (CRDs) that are responsible for ligand binding. DR5 forms a multimer that activates the extrinsic apoptotic pathway.[27,28] Inspection of the DR5 sequences shows in Domain 1 (D1) and in Domain 2 (D2). D2 is implicated as a major focus for TRAIL-binding specificity, with conservation. Domain 2 starts from amino acid 137 until 179.[29]

TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) is the native ligand of receptor DR5, a cytokine that preferentially induces apoptosis in tumor cells compared with normal cells through two receptors (DR4 and DR5).[9,30] TRAIL has selective induction of apoptosis in malignant cells *via* its receptor.[2] TRAIL attracts great research interest for its selective induction of apoptosis in malignant cells *via* its receptors, DR5.[31] The resistance TRAIL to bind the DR5 implicate in a variety of human cancers such as hepatocarcinoma cells.[32]

Lots of studies have evaluated the anti-cancer activity of nimbolide and gedunin. Recent studies have shown that gedunin can inhibit the proliferation of cancer cells including those of the prostate, ovary, and colon.[21,22] Previous studies showed that gedunin and nimbolide increase the occurrence of apoptosis in cell cancer.[23,33] Nimbolide inhibits a number of survival proteins, and upregulates the death receptors (DR) that interact with TRAIL, namely DR4 and DR5. The combined effects of nimbolide's actions increase the apoptotic consequences of TRAIL therapy.[14,18]

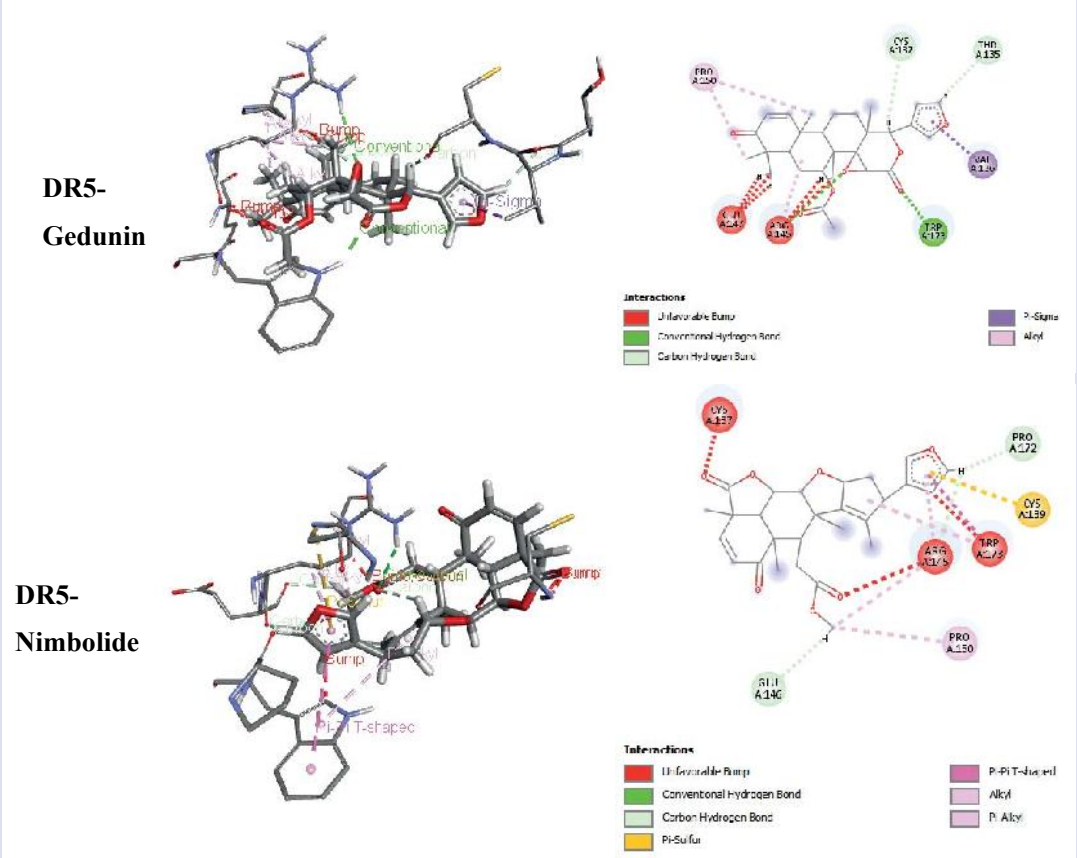

Figure 2: DR5 binding with gedunin and nimbolide in 2D diagram ligand interaction.

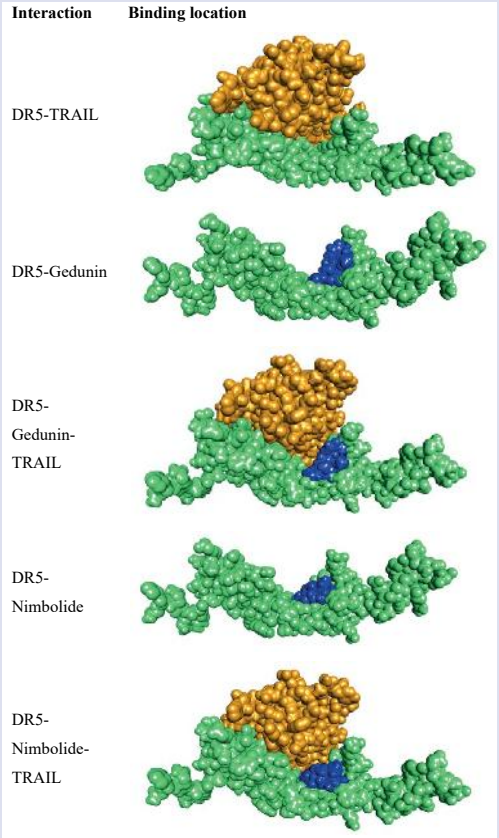

Figure 3: Binding location and interaction DR5 and ligands (DR5: green; TRAIL (native ligand): yellow; ligand: blue).

This study showed that gedunin, nimbolide and a combination of TRAIL can bind in the active site of DR5 which means they can enhance DR5 to bind with its ligands. Nimbolide and gedunin might increase the binding affinity between DR5 and TRAIL. Both nimbolide and gedunin decrease binding energy when DR5 is docked with TRAIL. Although gedunin was not bound in the active site of DR5-TRAIL, the binding site is still in the DR5 Domain II. Nearly all of these interactions bind the DR5 Domain II in amino acids 137-179 which shows that TRAIL binds to the DR5 Domain II participating in ligand binding. Domain 2 is implicated as a major focus for DR5-TRAIL binding specificity.[2] These results indicate gedunin and nimbolide had the stable potential binding to DR5.

The docking results showed that nimbolide has lower binding energy than gedunin with DR-TRAIL complex. The data indicated that nimbolide has a higher potential DR5-TRAIL binding inducer than gedunin and a stronger binding affinity with DR5 (Table 1). The lower binding energy indicates more stable binding between the molecules than the molecule with higher binding energy.[34] This interaction may potentially reduce the resistance of DR5-TRAIL binding.

When TRAIL binds to its receptor, DR5, it causes the receptor to trimerize and the intracellular death domain (DD) of the receptor to cluster, resulting in the development of the death-inducing signaling complex (DISC). The recruitment of FAS-associated death domain protein (FADD), and subsequent binding and activation of caspase-8 and -10. Activated caspase-8 and -10 then cleaved caspase-3, causing the death substrates to be cleaved.[35] However, their potential is stills need to be examined through further analysis to uncover the further potential.

CONCLUSION

In summary, bioactive compounds from neem, such as gedunin and nimbolide have potential as inhibitors of the interaction between DR5 and TRAIL as native ligands. These two compounds were proven to bind with DR5 in their ligand binding site. Nimbolide had shown the best results among other complexes that were tested with threads of nimbolide before TRAIL. Gedunin and nimbolide can be considered as drugs that can sensitize TRAIL binding to DR5 and increase the activation of one of hepar cancers signaling apoptosis pathways.

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DISCLOSURE STATEMENT

The authors declare no conflicts of interests.

ABBREVIATIONS

Caspases: Cysteine-aspartic proteases; CID: PubChem Compound Identifier; CRDs: Concatenated cysteine-rich domains; DD: Death domain; DISC: Death-inducing signaling complex; DR: Death receptor; DR5: Death receptor 5; HCC: Hepatocellular carcinoma; HCV: Hepatitis B or C viruses; PDB: Protein Data Bank; TNF: Tumor necrosis factor; TRAIL: TNF-related apoptosis inducing ligand.

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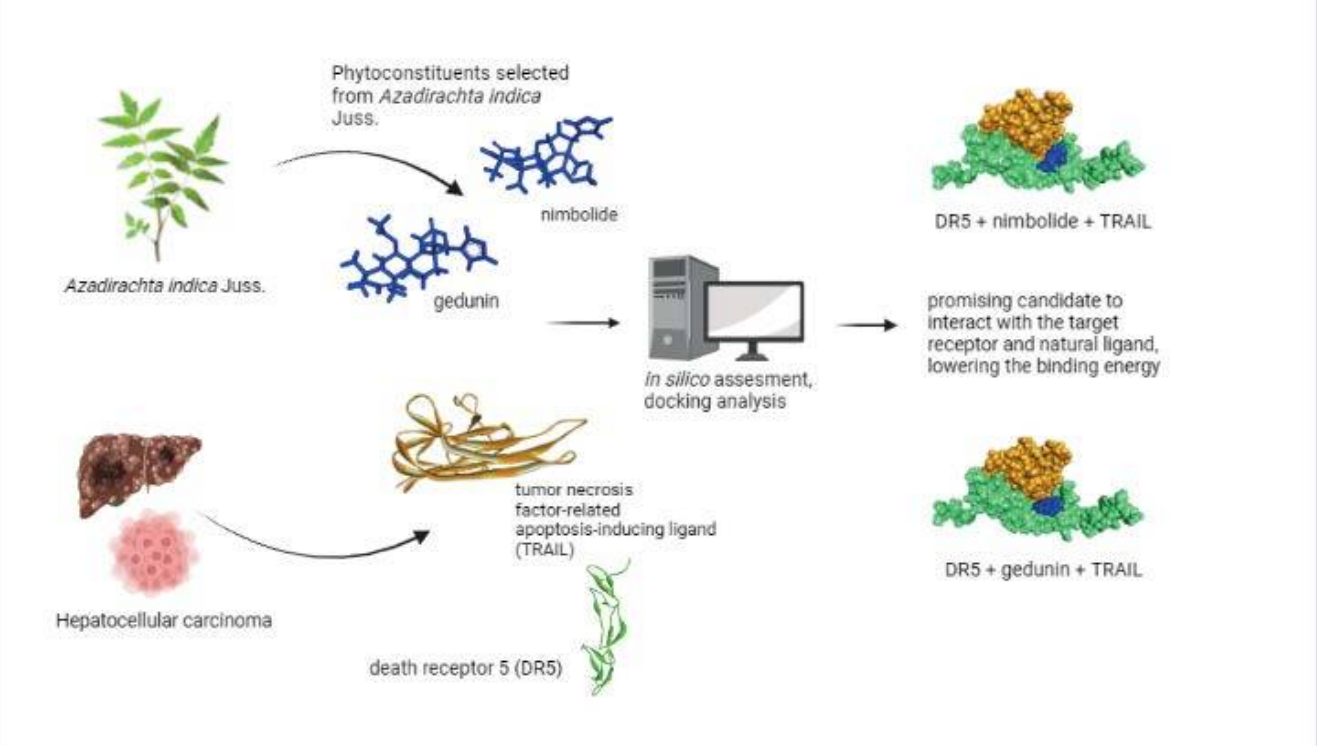
GRAPHICAL ABSTRACT



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Potentials of kawayang tinik (*Bambusa blumeana*) as new source antimicrobial agents

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Abstract

In this time where health is priority and surges of microbial resistance is highly observed within the society, discovering new, effective and sustainable sources of potential pharmacologic products is highly significant. The study explored the antimicrobial potentials of the different parts of *Bambusa blumeana* (*kawayang tinik*), a common Philippine bamboo species, against selected pathogenic bacterial and fungal species utilizing minimum inhibitory concentration via agar well diffusion method. Results of the study showed that extracts of *B. blumeana*, specifically the leaf, rhizome, roots, inner and outer culms, are capable of inhibiting microbial growth at 0.06 to 0.98 mg/ml concentrations. Specifically, the aqueous outer culm extract of *B. blumeana* proved to be most effective in inhibiting the growth of *Pasteurella multocida* at 0.24 mg/ml while *Staphylococcus aureus* and *Escherichia coli* were most susceptible to ethanolic outer culm extracts at 0.06 mg/ml and 0.12 mg/ml respectively. *Bacillus subtilis*, on the other hand, was observed to be the most sensitive to ethanolic root extracts at 0.06 mg/ml. Furthermore, *Aspergillus niger* was observed to be susceptible to ethanolic rhizome extract (0.24 mg/ml) while the ethanolic leaf, roots, inner and outer culms were equally effective in inhibiting *Penicillium chrysogenum* at 0.98 mg/ml extract concentration. Phytochemical testing further revealed the presence of phenols and flavonoids in the different parts of the bamboo species which further support its potential as a new source of pharmaceutical biocompounds.

Keywords

Antimicrobial, *Bambusa blumeana*, bamboo

Introduction

Rising cases of antibiotic resistance urges scientific communities to look for new and sustainable sources of drugs. Plants, on the other hand, are known for their ecological as well pharmaceutical potentials and was proven by sustained and effective use of ethnobotanical remedies around the world. Moreover, approved pharmaceutical products are mostly obtained from phytocompounds found in plants and plant products.

One common bamboo species found in the Philippines, particularly in Central Luzon, is *Bambusa blumeana* (*kawayang tinik*). It is frequently utilized for making furniture, native homes and is also consumed as food although, some studies have mentioned its use for the treatment of respiratory symptoms, kidney stones and Dengue fever by some ethnic communities in Luzon, Philippines (1).

Despite the commonality of the said bamboo species, limited studies, both local and international, have been conducted detailing its potential as a pharmaceutical source. On the other hand, other bamboo species thriving in other Southeast Asian countries have been established to contain phytocompounds with potential antimicrobial properties (2-4). Hence, this study was done to determine the potential of *B. blumeana* as source antimicrobial biocompounds. Specifically, the study aimed at exploring the presence and differences in antimicrobial property of the different parts of *B. blumeana* against *Penicillium multocida*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Aspergillus niger* and *Penicillium chrysogenum*. Moreover, identification of total phenolic and flavonoid contents in various *B. blumeana* extracts were also explored and correlated.

Materials and Methods

Plant Material Preparation

Plant parts of *B. blumeana* (*kawayang tinik*) were first identified and collected from the Bamboo Forest Park of Tarlac Agricultural University in Tarlac, Philippines. Plant parts including the leaves, roots, rhizomes, inner culms and outer culms were used and utilized in the study. Selected plant parts are afterwards cleaned, air dried, powderized, stored in sterile, dark containers and kept in the refrigerator with temperature regulation at 4 °C until use (5 -7). Extraction, purification and concentration of plant materials are done thereafter. Ethanolic extraction was done by macerating the individual powderized plant materials with 95% ethanol using 1:4 plant material to solvent ratio for 48 hrs under room temperature (2, 8, 9). Aqueous extraction, on the other hand, was made by mixing 100 g of plant materials in 400 ml of distilled water, boiled for about 15 min at 80 °C then cooled and macerated for 24 hrs under room temperature (5, 8). The aliquots are then strained via Whatman filter paper No. 1 and the filtrates are subjected to rotary evaporation. In making the stock solutions for antimicrobial testing, individual plant extracts are reformulated to 1 mg/ml and are sterilized with sterile millipore filter syringe (Whatman® at 0.22 millipore size) (10). The sterile plant extracts are then stored in sterile, dark containers and kept in refrigerator with temperature regulation of 4 °C (11).

Minimum Inhibitory Concentration Determination

The MIC value was performed by doing combination of the classic two- fold dilution method and agar well diffusion method (12). The same methodology was employed by various studies which validated the efficiency and effectivity of the method employed in the study (13-15). Bacterial MIC determination was facilitated by preparing sterile Himedia® nutrient agar swabbed aseptically with 100 µl of selected bacterial species initially maintained at Himedia® Nutrient Broth (NB) and further adjusted to 0.5 McFarland standards at (1.0 x 106) CFU/ml (12, 16). After drying the plates for 15 min, wells are created by punching the previously swabbed agar plates with sterile cork borer. 100 µl individual plant extracts with various concentrations were then aseptically poured in the wells. Afterwards, the prepared plates are incubated for 24 hrs at 37 °C.

Fungal MIC, on the other hand, was done by placing a 3- mm mycelium disk culture (*A. niger* and *P. chrysogenum*) at the center of a sterile, solidified Sabouraud® agar plate wherein various kawayang tinik extracts were placed on even distances around the fungal disk. The seeded plates were afterwards incubated at 72 to 96 hrs at room temperature. The interpretation of MIC for bacterial and fungi species were based on the European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI). These standards rely on the formation of optically clear inhibition zones thus, *B. blumeana* extracts which displayed optically clear zones of inhibition, both on the initial and validation testing, were included in the results of the study and those that display hazy or unclear zones are tagged at not determined. Furthermore, the extracts with the least concentration level that exhibited optically clear ZOI was treated as the MIC (17).

Qualitative Phytochemical Testing

The various *B. blumeana* ethanolic extracts was brought to Saint Louis University Natural Science Research Institute (NSRI) in Baguio City and the University of Sto. Tomas Analytical Services Laboratory in Manila, Philippines for total phenolic and flavonoid determination. The total phenolic content of ethanolic extracts was determined by diluting 100 µl of the plant extract with 3 ml of analytical grade water then mix with 0.5 ml of Folin – Ciocalteau reagent. After 3 min, 2 ml of 20% Na2CO3 (w/v) solution was added and were mixed thoroughly. The absorbance was measured at 750 nm against the blank using a spectrometer. A standard solution was prepared using gallic acid monohydrate at 15.63 µg/ml to 500 µg/ml (r2= 0.99), and the linear regression equation was obtained to determine the total phenolic content of the plant extract (18).

Flavonoid content determination in ethanolic extracts, on the other hand, were taken by mixing 500 µl of the plant extract with 2.5 ml of analytical grade water and 150 µl of 5% $NaNO_2$ solution. After 6 min, 300 µl of 10% AlCl3 ·6H2O solution was added. One (1) ml of 1M NaOH solution was added after 5 min and the mixture was brought to 5 ml using analytical grade water and mixed well. The absorbance was measured immediately at 510 nm. A standard solution was prepared using quercetin (r2= 0.99), and the linear regression equation was obtained to determine the total flavonoid content of the plant extracts (19).

Total phenol contents in aqueous extracts were evaluated by treating the aliquots with Folin-Ciocalteau reagent and Na_2CO_3 with increasing concentrations of Gallic acid used as standard. The aliquots were allowed to stand at room temperature for 90 min and the absorbance was measured at 750 nm wavelength utilizing a microplate reader. Total flavonoid of aqueous extracts, on the other hand, were calculated through the addition of equal volumes of extracts and 2% $AlCl_3$ in wells of a 96-well plat whereby absorbance was measured at 420 nm using a microplate reader after 1hr at room temperature (20).

Results and Discussion

Minimum Inhibitory Concentration (MIC) of Different Bambusa blumeana (Kawayang tinik) Extracts

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial agent that will inhibit the visible growth of a microorganism after an incubation period (21). Lower MIC values can further be interpreted as lower concentration of drug or extract needed to inhibit a certain bacterial or fungal strain. Hence, lower concentrations or MIC values mean greater bacterial susceptibility or sensitivity against the antibacterial agent being tested. The minimum inhibitory concentration (MIC) values of the different extracts of *B. blumeana* tested against several microbial species were reflected in Table 1. The variations in MIC values reveals varying susceptibility of the selected bacterial and fungal species against the ethanolic and aqueous extracts of *B. blumeana*.

Table 1. Minimum inhibitory concentration (MIC) of different *B. blumeana* extracts against several microbial species

PLANT EXTRACTS (mg/ml)	BACTERIAL SPECIES				FUNGAL SPECIES	
	P. multocida	*S. aureus*	*E. coli*	*B. subtilis*	*A. niger*	*P. chrysogenum*
AQUEOUS						
Leaf	0.49	62.5	1.95	3.91	3.91	15.63
Rhizome	nd	nd	nd	nd	1.95	3.91
Roots	0.98	3.91	7.81	nd	nd	1.95
Inner Culm	0.98	nd	3.91	15.63	nd	1.95
Outer Culm	0.24	nd	nd	nd	0.47	7.81
ETHANOLIC						
Leaf	nd	0.98	0.98	0.24	0.98	0.98
Rhizome	3.91	0.98	0.98	3.91	0.24	1.95
Roots	7.81	3.91	3.91	0.06	0.98	0.98
Inner Culm	0.49	0.49	0.49	0.98	0.98	0.98
Outer Culm	0.98	0.06	0.06	0.12	0.98	0.98

It is also revealed in Table 1 that the aqueous outer culms extract of *B. blumeana* exhibited the lowest MIC value against *P. multocida* (0.24 mg/ml) while *S. aureus* and *E. coli* were mostly susceptible to ethanolic outer culm extracts (0.06 mg/ml and 0.12 mg/ml respectively). *B. subtilis*, on the other hand, was observed to be most sensitive to ethanolic root extract at 0.06 mg/ml.

The table further revealed that selected fungal species are also susceptible to most *B. blumeana* extracts. *A. niger* and *P. chrysogenum* were also found to be more susceptible to ethanolic extracts compared to their aqueous counterparts such that ethanolic rhizome extract exhibited lower MIC value (0.24 mg/ml) against *A. niger* while ethanolic leaf, roots, inner and outer culm extract were equally effective in inhibiting *P. chrysogenum* at 0.98 mg/ml.

The antimicrobial potential of bamboo species could be attributed to various phytocompounds previously observed in the different parts of the plant. For instance, the compound apigenin, luteolin and p - coumaric acid are found in the leaf, inner and outer culms and root part of *P. pubescence* (8). Apigenin works by inhibiting cellular metabolism (22) while luteolin has the ability to destroy bacterial cell membrane (23). P- coumaric acid, on the other hand, inhibits bacterial DNA function and also disrupts bacterial cell membrane (24). Other studies also observed the presence of tannins, cardiac glycoside, terpenoids, saponins and steroids in *B. blumeana* and other bamboo species (5, 25, 26). These phytochemicals are known for having innate antimicrobial effects via various mechanisms.

Several studies also document the potentials of other bamboo species as antimicrobials. For instance, it was found out that different plant parts of *P. pubescence*, a Japanese bamboo, were effective against *S. aureus* and its ethanolic outer culm extract can express 98 – 100% inhibition rate and has a MIC value of 0.4 to 1.6 mg/ml (8). The outer culms of bamboo species from *Gramine* family were also noted to display antimicrobial potentials when tested against several microorganisms using water extraction and agar diffusion method. Accordingly, its MIC value against *S. aureus*, *E. coli*, *B. subtilis* and *A. niger* were 4.9 mg/ml, 5.3 mg/ml, 6.4 mg/ml and 4.9 mg/ml respectively (27). Additionally, ethanolic leaf extracts of *D. strictus* were also noted to be effective against *E. coli*, *S. aureus* and *B. subtilis* at 0.5 to 1.0 mg/ml (28). N- hexane, chloroform and ethyl acetate leaf extracts of *B. vulgaris*, on the other hand, was observed to inhibit *E. coli* and *S. aureus* at less than 2.5 mg/ ml MIC concentration (29).

Bamboo plants were also noted to be effective in inhibiting the growth of some fungal species. N- hexane, chloroform and ethyl acetate leaf extracts of *B. vulgaris* was observed to inhibit *A. niger* and *V. alboatrum* at less than 2.5 mg/ ml (30) while *D. strictus* ethanolic leaf extract was noted to be effective against *A niger*, *P. chrysogenum*, *F. moneliforme* and A. *flavus* at 0.5 to 1.0 mg/ml (28). Aqueous leaf extracts of *P. pubescence* were also observed to effectively inhibit *P. grisea*, causative agent for rice blast disease, at 0. 5 – 1.0 mg/ml concentration (24) while aqueous shavings of bamboo coming from the *Gramine* family also manifested effective inhibition against *A. niger* at 4.9 (+/-0.2) mg/ ml MIC concentration (27).

It can be observed that the MIC values exhibited by the *B. blumeana* extracts were comparable and even lower compared to the studies previously mentioned. This may further validate the antimicrobial potentials of *B. blumeana* (*kawayang tinik*) plant extracts. Moreover, it can also be observed that lower MIC values were obtained from bamboo plant parts that are not usually being studied such as the rhizome, root, outer culm and inner culm. These results may further support the evidences of the potential presence of antimicrobial activity on *B. blumeana* (*kawayang tinik*) not only on the leaf part but all other bamboo plant parts as well.

Determination of the Quantitative Total Phenolics and Flavonoid Content of Bambusa blumeana (Kawayang tinik) Extracts

Presented in Table 2 are the results of the total phenolic and flavonoid content determination of plant extracts derived from the different parts of *B. blumeana* (*kawayang tinik*). It was observed that all extracts are positive for the

presence of phenols and flavonoids at varying levels of concentration. Phenols and flavonoids possess innate antimicrobial properties through various mechanism such as inhibition of nucleic acid synthesis and cytoplasmic membrane function, disruption of metabolism and inhibition of oxidative phosphorylation cycle (30 - 33).

Table 2. Total phenolic (TPC) and total flavonoid content (TFC) of different *Bambusa blumeana* extracts

EXTRACTS	TOTAL PHENOLIC CONTENT (TP) ug/ml GAE	TOTAL FLAVONOID CONTENT (TF) ugQE/ml
ETHANOLIC		
Leaves	141.00	247.60
Rhizome	154. 57	440.00
Roots	140.00	156.00
Inner Culm	57.85	176.80
Outer Culm	131.29	48.80
AQUEOUS		
Leaves	1509.10	667.10
Rhizome	216.82	169.40
Roots	894.41	259.80
Inner Culm	488.67	177.30
Outer Culm	510.71	218.30

For phenolic content determination in ethanolic extracts, rhizome exhibited the highest phenol concentration (154.57 ug/ml GAE) followed by leaf (141 ug/ml GAE), root (140 ug/ml GAE) and outer culm (131. 29 ug/ml GAE) extracts. The ethanolic inner culm extracts on the contrary, shown the least amount of phenolic compound (57. 85 ug/ml GAE). Aqueous plant extracts such as the aqueous leaf extracts obtained the highest phenol concentration at 1509.10 ug/ml GAE followed by the aqueous root extracts (894.41 ug/ml GAE), outer culm (510. 71 ug/ml GAE), inner culm (488.67 ug/ml GAE) and rhizome extracts (216.82ug/ml GAE) respectively.

Flavonoid content in ethanolic extracts were also evaluated and results shows that rhizome extracts exhibited the highest flavonoid concentration (440 ug/ml GAE) followed by the ethanolic extracts of leaves (247.60 ug/ml GAE), inner culm extracts (176.80 ug/ml GAE) and root (156.00 ug/ml GAE). The ethanolic outer culm extracts exhibited the lowest total flavonoid content at 48. 80 ug/ml GAE, on the contrary. In terms of the aqueous extracts, results showed that leaf extracts contain the highest total flavonoid content at 677. 10 ug/ml GAE followed by the root extracts (259. 80 ug/ml GAE), outer culm extracts (218. 30 ug/ml GAE), inner culm (177. 30 ug/ml GAE) and aqueous rhizome extracts (169. 40 ug/ml GAE).

It is evident in the results of the TPC and TFC that majority of the aqueous extracts exhibited higher total phenolics and flavonoid concentration except for rhizome extracts which elicited higher TPC and TFC in ethanolic extraction and inner culm extracts for total flavonoid content. These observations seem to contradict the previous MIC results obtained wherein ethanolic extracts exhibited lower inhibitory values compared to aqueous extracts. This could be explained by the fact that plants are natural sources of abundant numbers of phytochemicals and bionutrients (32) and contain more than 5000 classes of phytochemical that were already discovered but not yet fully studied (33). Since only phenolics and flavonoids were measured in the study, the better performance of ethanolic extracts in the previous MIC determination could be associated with the presence of other phytochemicals of *B. blumeana*. Local studies have also documented the presence various phytocompounds in *B. blumeana* such as alkaloids, sterols, triterpenes and tannins (25, 26) which also has innate antimicrobial properties. Therefore, the antimicrobial potential of *B. blumeana* extracts could not only be associated with the amounts of flavonoids and phenolic contents alone and but rather with the totality of phytochemicals present in the extracts. This further implies the need for additional studies to fully harness and understand the antimicrobial potential of *B. blumeana*.

Differences in assay results and phytochemical testing may further be attributed to the endointeractions of individual phytochemicals (34) such that individual biomolecules in plants may either potentiate or interfere with the biological activities of phytocompounds and works by either interfering with the bioavailability of other phytocompounds, interloping with cellular transport processes, activation of pro-drugs or deactivation of active compounds to inactive metabolites, multi-target effects or inhibition of binding to target proteins (35). Thus, recommendations to further purify extracts to determine the active components responsible for the antimicrobial potentials especially in *B. blumeana* (*kawayang tinik*) is highly suggested.

Furthermore, looking at the solvents used in the study, phenolic compounds and flavonoids are better extracted in polar solvents (36). Therefore, given that water is more polar than ethanol, phenolic compounds as well as flavonoid could be better extracted using aqueous rather than ethanolic extraction. This and the above- mentioned conditions and rationale provide justification on the higher TPC and TFC values of the aqueous extracts than ethanolic extracts.

Conclusion

All findings of the study suggest *B. blumeana* as a potential source of phytocompounds for drug development. Its extracts manifested varying antimicrobial activities against selected microorganisms and its minimum inhibitory concentration is noted to range from 0.06 to 0.98 mg/ml which is comparable or even lower compared to previously studied bamboo species. Moreover, *B. blumeana* extracts also showed to contain phenols and flavonoids which are phytocompounds with known innate antimicrobial potentials.

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Compliance with ethical standards

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Improved cellular immunity and increased insulin in streptozotosin-induced mice using ethanol coriander (*Coriandrum sativum*) extract

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ABSTRACT:

An increase in blood sugar levels is an indication of diabetes mellitus (DM). DM is a metabolic disease characterized by disorders of carbohydrate and fat metabolism due to impaired activity, insulin secretion or both. Alternatively, the body's inability to secrete insulin. The purpose of this study was to determine the effect of coriander extract (Coriandrum sativum) on mice induced by streptozotocin (STZ) on the relative number of CD11b and insulin-IL6 cells. The study used Balb-C strain mice, aged 2.5-3 months, with five (5) treatment groups which were given STZ at a dose of 145mg/kg BW on the 8th day IP (Intraperitoneal) after adaptation for seven days, then add coriander extract with successive doses of 25 (T1), 50 (T2), and 100mg/kg BW (T3), negative control (healthy) (T4) and positive control (STZ) (T5) through the administration of intraperitoneal gastric tube. Blood Glucose is measured on the 13th day. Herbs are given on days 14-28. On the 29th day, the mice. An increase in blood sugar levels is an indication of diabetes mellitus (DM). DM is a metabolic disease characterized by disorders of carbohydrate and fat metabolism due to impaired activity, insulin secretion or both Alternatively, the body's inability to secrete insulin. The purpose of this study was to determine the effect of coriander extract (Coriandrum sativum) on mice induced by streptozotocin (STZ) on the relative number of CD11b and insulin-IL6 cells. The study used Balb-C strain mice, aged 2.5-3 months, with five (5) treatment groups which were given STZ at a dose of 145mg/kg BW on the 8th day IP (Intraperitoneal) after adaptation for seven days, then add coriander extract with successive doses of 25 (T1), 50 (T2), and 100mg/kg BW (T3), negative control (healthy) (T4) and positive control (STZ) (T5) through the administration of intraperitoneal gastric tube. Blood Glucose is measured on the 13th day. Herbs are given on days 14-28. On the 29th day, the mice were sacrificed Furthermore, the pancreas was taken for insulin examination, and the spleen was checked with the relative number of CD11b-IL6 and T CD4 cells using a flow cytometer. Data were quantitatively analyzed using the One Way ANOVA test ($p \leq 0.05$). The results showed that doses of coriander in T1 treatment causes an increase in insulin levels, decreased blood sugar levels, weight gain, proliferation and activation of CD4+ T cells, and decrease inflammation (CD11b cell-IL6) on the tissue. This research concludes that extracts of coriander can reduce Hyperglycemia in mice by modulation of cellular immunity and increased levels of insulin

KEYWORDS: herbs, insulin, inflammation, hyperglycemia, free radicals, Cellular.

INTRODUCTION:

Diabetes mellitus (DM) is a metabolic disease characterized by impaired metabolism of carbohydrates, and fats due to interference with activity, insulin secretion, or both (Federico *et al*., 2016; Hajiaghaalipour *et al*., 2015). DM sufferers will experience an increase from 171 million (2.8%) to 366 million (4, 4%) until 2020, and tend to experience an increase in prevalence across countries (Wild *et al*., 2004)). DM is associated with high-risk chronic diseases such as heart attacks, blindness, and kidney failure (Sharma *et al*., 2016; Hackett and Jacques, 2009). Diabetes affects the decrease in effector immune cells and regulator T cells.

The inability of β-pancreatic cells to produce insulin result in regulatory disturbances from glucose uptake signals, namely GLUT-4 protein, which causes cells to secrete proinflammatory cytokines such as TNF-α, and Il-6 which cause tissue damage (Hajiaghaalipour *et al*., 2015). DM causes an imbalance of microbiota in the intestine that will increase the number of opportunistic pathogens and oxidative stress (Tai *et al.*, 2015).

Management of DM to date uses anti-diabetes drugs and insulin to maintain quality of life and prevent ongoing damage due to impaired circulation of blood vessels. The use of anti-diabetes drugs continuously and for a long time will affect the damage to the liver and kidneys. Patients tend to choose herbal medicines that have fewer side effects, as a functional food to prevent and treat diabetes and its complications. In this study, Coriander seeds (*Coriandrum sativum L.)* will be tested on its effect of diabetes mellitus particularly on cellular immunity and the production of insulin.

MATERIAL AND METHODS:

This research used 20 mice Balb-C strain, a model of experimental animals in testing hyperglycemia. The mice were given streptozotosin before treatment of different levels of coriander seed extract. There were five (5) treatment groups and 4 replications each. The use and maintenance of experimental animals have received an ethic-worthy certificate no 1109-KEP-UB from Brawijaya University.

The Coriander Fruit Extraction Method Using Ethanol as a Solvent:

Coriander seeds were mashed using a blender until smooth into powder and were sieved using a 200 mesh sieve. 100 grams of coriander powder was mixed in 1000 ml of 96% ethanol solvent, stirred and soaked for 24 hours. The extract was filtered to separate the precipitate (pulp) and the supernatant (solution), it was repeated 3 times. The liquid extract is evaporated into a rotary evaporator at 50°C and dried using an oven at a temperature of 40°C to produce a viscous extract. The method of Sogara *et al.* (2014) was adapted with modification.

Treatment on Experimental Animals:

The mice were kept and adapted to temperature, the area and their feed for 7 days. The study used Balb-C strain mice, aged 2.5-3 months, with five (5) treatment groups which were given STZ at a dose of 145mg/kg bw on the 8th day intra-peritoneally (IP), then coriander extract was added with successive doses of 25 (T1), 50 (T2) and 100mg/kg bw (T3), negative control (healthy) (T4) and positive control (STZ) (T5) through the administration of intraperitoneally. Glucose level measurements were performed before administration of STZ and after administration of streptozotosin on days 13.18 and 23, and 28 in all treatment groups. Weighing was done on days 14, 18, 23 and 28 to determine whether there was an effect of weight loss in mice after administration of STZ and coriander extract.

Giving of Streptozotosin and Measurement of Blood Sugar Levels:

Mice were injected with a single intraperitoneal dose of Streptozotocin (STZ) (145mg/kg bw) (Biolegend®) initially dissolved in 0.01 M sodium citrate, with pH 4.5 (Wang-Fischer and Tina, 2018). Blood glucose levels were measured after 5 days of STZ injection. Mice with fasting blood glucose levels of more than 220mg/dl were considered positive for diabetes as showed by Deepa and Anuradha (2011) and Furman (2015). Blood sugar was measured using a Glucostick digital blood glucose level (Gluco-Dr.®) device on the 5th day after it was administered with STZ (Qosimah *et al*., 2019).

Necropsy of Animal:

Necropsy was carried on the 29th day of the experiment where the spleens and pancreas were collected for further observation and study.

Flowcytometer Procedure:

The spleen and pancreas of mice were observed using flowcytometer to calculate the relative cell percentage of effector cells and insulin. The procedure of Rachmawati and Rifa'i, (2014) was adapted. The antibodies used recorded were: CD4 FITC, Insulin per Cp, and CD11 by FITC-IL6PEcy5.

Data analysis:

Diabetes data collected were quantitative: blood sugar levels, relative amounts (CD11b cell-IL6, insulin and CD4 + T cells). The data were analyzed using the One-Way Analysis of Variance (ANOVA) test with a 95% confidence level to determine the effect of the treatment of coriander extract treatment on mice induced by STZ.

RESULTS:

Measurement of Blood Sugar Levels:

Blood sugar levels in all treatment groups before being induced with STZ showed normal (average range of 103-156mg/dl). Observation of sugar levels after STZ induced on the 13th day, followed by administration of coriander extracts on the 19th, 24th and 28th days of which there was a decrease in sugar levels in a row namely T1 treatment (327.5; 190.5; 177.5; 138.5) mg/dl, while the treatment of T2 and T3 decreases in sugar levels were found only at the last administration of the extract that is the 28th day namely T2 (362; 244.5; 248.5; 186.25)mg/dl and T3 (446; 322.25; 295.5; 178.8) mg / dl. The positive control group (STZ) showed an increase in blood sugar levels (436.75; 405; 326; 307.75) mg/dl while the negative control did not have an increase in blood sugar levels or normal blood sugar levels respectively (96.5; 120,75; 124,25; 126,25) mg/dl.

Weight Measurement:

The results of weight measurements of all treatment groups when given therapy for 14 days and observed on days 14, 19, 24 and 28 showed that in the T1 and negative control groups there was an increase in body weight while in T2, T3 and positive control there was a decrease in body weight (table 1).

Table 1: Observation of body weight in the treatment after administration of STZ and coriander extract

The treatments	Observation of Average Weight (gram)					
	Early prior to treatment (Day 8)	After Giving STZ (Day 13)	After administration of Coriander extract therapy			
			Day 14	Day 19	Day 24	Day 28
T1	20	20,5	21,5	22	22,5	22.75
T2	23	18,5	24,75	22,25	20,5	20,25
T3	20	22	19,25	18,5	18,25	16,5
Positive control	20	18,5	20,25	19,75	18,5	17
Negative control	23	24,5	25,5	25,75	26	26,25

The Relative Amount of Insulin:

The relative amount of insulin in the negative (healthy) control group was higher than the positive control group (STZ-induced mice). In the therapy group there was an increase in the relative amount of insulin in mice in the T1 and T2 groups that were not significantly different from the negative controls (Figure 1). A decrease in the relative amount of insulin was found in the positive control and T3 treatment found both showed no significant effect. This shows an increase in the number of insulin occur in the coriander extract with a small dose, 25mg/kg body weight.

Figure 1: Notation a, b shows the difference between treatment groups (P<0.05) using the One way ANOVA test followed by the tukey test

Relative amount of $CD4^+$ T cells:

The results showed that the activation and proliferation of the adaptive cellular immune system CD4 + T cells in mice in the negative (healthy) control group was higher than the positive control. The group of mice T1 treatment had an increase in CD4 + T cells that were not significantly different from negative control, while the decrease in the number of relative CD4 + T cells occurred in group T2, T3 which was not significantly different from positive control treatments. This suggests that activation of CD4 + T cells occurs at doses lower coriander extract is 25 mg / kg bw (Figure 2).

Figure 2: Notation a, b showed a significant difference between treatment groups on CD4 + T cells (P<0,05)

Relatif number of CD11b cell-IL6:

The results showed that the relative number of macrophage cells that produce proinflammatory cytokines (CD11b cell-IL6) of the positive control (STZ-induced mice only) higher compared with negative control group (healthy or normal). The treatment group were given extracts of coriander therapy showed a decrease in CD11b cell-IL6 significantly in the treatment of T1, T3 T2 and while the treatment is still an increase in the number of relatively CD11b cell-IL6 were not significantly different from the positive control (Figure 3). In the group of T1, coriander dose given is able to repair the pancreas through increasing insulin levels, decreased blood sugar levels, weight gain, proliferation and activation of CD4 + T cells and decrease inflammation, CD11b cell-IL6 on the tissue.

Figure 3: Notation a, b shows a significant difference between treatment groups with respect to the relative number of CD11b cell-IL6 (P<0.05)

DISCUSSSION:

Weight Gain:

Weight gain was only found in the T1 treatment while T2 and T3 treatments occurred for weight loss. Weight gain at T1 is due to an improvement in the amount of insulin to help maintain carbohydrate metabolism. This study is in accordance with that carried out by Schaschkow *et al*., 2016, that insulin therapy can increase body weight in female rats with diabetes Lewis strain. Weight loss in mice after being induced with STZ and coriander extract in T2 and T3 treatments according to research conducted by Tian *et al*., 2010 which states that STZ induction with a dose of 115mg/kg bw in mice causes an increase in fasting blood glucose levels and feed-drinking intakes but lose weight. There was a tendency for weight loss in coriander groups with doses of 50 and 100mg/kg bw because of coriander content, namely linalool which has an appetizer effect (decreases appetite) (Güler *et al*., 2005).

STZ induction causes hyperglycemia. DM is a chronic condition characterized by relative or absolute deficiency of insulin, which leads to hyperglycemia due to pancreatic β-cell damage (Damasceno *et al*., 2014). B cells experience necrosis due to excessive free radicals in the form of glucose levels in the blood (oxygen radicals or nitric oxide) thereby stimulating proinflammatory cytokine cells (Gvazava *et al*., 2018). STZ is toxic to pancreatic β cells, which causes disruption of insulin production (Wong-Fischer and Tina, 2018), followed by weight loss (Gvazava *et al*., 2018). Free radicals will mediate activation of the signal transduction cascade and transcription factors that cause increased expression of specific genes that cause tissue damage and diabetes complications. Hyperglycemia causes a decrease in the activity of antioxidant enzymes that play a role in neutralizing free radicals and decreasing the degree of inflammation. Intraperitoneal STZ is often done to induce diabetes because of the 60% success rate compared to aloxan and hypercaloric (20%) (Frederico *et al*., 2016). STZ is a natural antibiotic, produced by Streptomyces achromogenes actinomycetes. STZ causes a decrease in ATP, thus causing damage to β cells. STZ also causes impaired glucose homeostasis (oxygen consumption and glucose oxidation) and inhibition of biosynthesis and insulin secretion, not directly and directly affecting glucose transport or phosphorylation by glucokinase (Gvazava *et al*., 2018).

Relative number of Insulin:

Coriander effect can be used as insulin therapy because it can reduce the condition of hyperglycemia, through increased glucose and insulin metabolism (Chizoba, 2015). The coriander extract content β-carotene acts as an immune modulator, by neutralizing and eliminating singlet oxygen or free radical products. This compound can also be broken down enzymatically by lipoxygenase to prevent radical oxidation and photo-oxidation (Kawata *et al*., 2018). Quercetin secretes insulin and inhibits intestinal starch digestion and hepatic glucose production, increases glucose absorption in skeletal muscle, and protects against pancreatic damage (Hossain *et al*., 2016). This is the same as research conducted by Federico *at al*., 2016, that coriander extract can increase insulin levels.

Relative number of CD4+ T cells:

Type 1 DM (T1DM) causes infiltration and accumulation of leukocytes and T lymphocytes around the pancreatic islets, causing a massive destruction of beta cell insulin production found in the positive control treatment group. MHC class II molecules will bind and present antigen peptides in the form of STZ to CD4 T cells via dendritic cells which will then go to the pancreatic lymph nodes for processing (Kakoola *et al*., 2014; Wang-Fischer and Tina, 2018). T cell activation is mediated by the signaling molecules CD80, CD86 and CD28 originating from dendritic cells (Wangchuk *et al*, 2018). Activation of Th (T helper) lymphocyte cells will differentiate into Th1, Th17 and Th2. Th1 cells lead to the activation of phagocyte cells for the release of inflammatory cytokines that cause cell damage in metabolic organs such as fat tissue, liver, muscle and pancreas (Xia *et al*., 2017) and Th2 cells lead to differentiation of Th2 cells into plasma cells that will produce antibodies besides the production of anti-inflammatory cytokines. In the T1 and negative control treatments an increase in $CD4^+$ T cells was significantly different from the T2, T3 and positive control treatments. An increase in $CD4^+$ T cells is likely to produce anti-inflammatory cytokines that affect tissue repair through a decrease in proinflammatory cytokines. According to Al-Snafi, 2013, that ethanol extract of coriander seeds contained flavonoids such as caffeic acid, chlorogenic, quercetin and routine. Flavonoids function as anti-diabetics through the following mechanisms: improvement in the function of pancreatic β cells and insulin through a decrease in proinflammatory cytokines IL1-β and IL-6; increased presence of antioxidants through reduction of free radicals and lipid peroxidation; regulation of carbohydrate metabolism (decreased gluconeogenesis and increased glycogenesis) and increased insulin sensitivity (Vinayagam and Xu, 2015).

Relative number of CD11b cell-IL6:

The positive control group showed an increase in the relative number of CD11b cell-IL6, which was not significantly different from the T3 group, whereas in the negative control group and the T1 and T2 treatments showed a decrease in CD11b cell-IL6. Type 1 diabetes mellitus (T1D) is an autoimmune disease characterized by beta cell destruction, associated with cellular infiltration and inflammatory responses on the island of Langerhans. The cellular components of this infiltrate include monocytes, macrophages, $CD4^+$ and $CD8^+$ T cells, and the balance between Th1 and Th2 cells is very important in the pathogenesis of this disease and against antigens and produces inflammatory mediators such as cyclooxygenase-2 (COX2), protein nitric oxide synthase 2 (NOS2)), free radical nitric oxide (NO), and proinflammatory cytokines such as tumor necrosis factor-alpha (TNFA) and Il-6 (Interleukin 6) that affect the incidence of diseases such as diabetes (Kawata *et al*., 2018), secreted by T cells and macrophages to stimulate the immune response during inflammation associated with insulin resistance (Gomes, 2017) so that the number of CD11b cell-IL6 increases in diabetes.

Decreasing the relative amount of macrophages (CD11b), which produce Il-6 according to research conducted by Chizoba, 2015, that coriander extract contains polyphenols and essential fatty acids such as linalool, a-pinene, limonene, and camphene, can inhibit macrophages to produce nitric oxide and pro-inflammatory cytokines TNF-α (Nair *et al*., 2012; Bhat and Kumar, 2014). Decreasing free radicals such as nitrix oxide and hydrogen peroxidase can reduce tissue damage, namely the pancreas. The mechanism of free radical reduction due to polyphenols works by capturing free radicals and increasing antioxidant enzymes and modulation of enzymes involved in glucose metabolism, increased function of pancreatic β cells to produce insulin, and anti-inflammatory. Polyphenols, especially flavonoids, phenolic acids and tannins can inhibit α-glucosidase, interact with glucose absorption from the intestine by inhibiting glucose transporters that are dependent on Na +, SGLT1 and SGLT2, regulate the main pathways of carbohydrate metabolism and liver glucose homeostasis including glycolysis, glycogenesis, and gluconeogenesis, will usually be disturbed in diabetes. Ferulic acid, a hydroxycinnamic acid derivative, effectively suppresses blood glucose by increasing glucokinase activity and glycogen production in the liver and increasing plasma insulin levels in diabetic rats (Bahadoran *et al*., 2013).

CONCLUSIONS:

Coriander seed ethanol extract serves as a therapy for the hyperglycemia condition that leads to diabetes through an increase in the relative amount of insulin, a decrease in blood sugar levels, cellular immune activation of $CD4^+$ T cells and a decrease in inflammation.

DECLARATION OF CONFLICT OF INTEREST:

There was no conflict of interest between the research team.

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AUTHOR'S CONTRIBUTIONS:

All teams colalaborate to do research, write and analize data.

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Separation of Flavonoids in The Extract *Polyalthia longifolia* (Sonn.) Thw. Leaves from Indonesia and The Philippines

Separation of Flavonoids in The Extract *Polyalthia longifolia* (Sonn.) Thw. Leaves from Indonesia and The Philippines

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Abstract. *Polyalthia longifolia* (Sonn,) Thw. is a plant that has many benefits on health because every part of this plant contains secondary metabolites. Flavonoid class is one of the secondary metabolite that contained in the leaves. Flavonoids has pharmacological potencies or therapeutic effects. The objective of this research was to observe how many compound of flavonoid from the ethanolic extract *Polyalthia longifolia* (Sonn.) Thw. leaves, that obtained from Indonesia and The Philippines by using Thin Layer Chromatograpy and Liquid Chromatography-Mass Spectroscopy. Two leaves powder of *Polyalthia longifolia* (Sonn.) Thw. from Indonesia and The Philippines were extracted by using 70% of ethanol, respectively. The dry extract was obtained by rotary evaporation. The flavonoids of both dry extracts that obtained were analyzed by phytochemical assay. The separating of flavonoid class of both extract were analyzed by TLC and LC-MS. Based on the phytochemical screening, both of the ethanol extract of *Polyalthia longifolia* (Sonn.) Thw. from Indonesia and The Philippines showed that positive flavonoids. The result of TLC separation contained of flavonoids based on the yellowish-green, yellow, green, until blue spots under UV exposure. LC-MS separation showed that the both of extracts revealed the presence of flavonoids including to flavonols and cyanidine, with the similar pattern of LC-MS, respectively. The presence of flavonoids were quercetin with RT 5,39 and 5,05, respectively, and molecular weight m/z = 302,5 – 303,5. Rutin showed the single peak with RT 2,61 and 2,62, respectively, and molecular weight m/z = 610,5 – 611,5. Then, leucocyanidin with RT 5,16 and 5,15, and molecular weight m/z = 306,5 – 307,5. Beside that both of extracts also contained analogues compound. In conclusion, the both of ethanolic extract of *Polyalthia longifolia* (Sonn.) Thw. contain flavonoid class such as rutin, quercetin and analogues compounds.

1. Introduction

Glodokan tiang or *Polyalthia longifolia* (Sonn,) Thw. (Family Annonaceae) is a plant that is widely spreaded in tropical and sub-tropical countries in South Asia [1] and Southeast Asia, including Indonesia and the Philippines. This plant has potential as herbal medicines such as antiplasmodial [2], anti-inflammatory [3], antimicrobial [4], antifever, anti-diabetes and anti-hypertension. In addition, this plant is used as ornamental trees that effectively reduce noise pollution [4].

Previous studies reported that this plant contains alkaloids, sesquiterpenes, diterpenoids, flavonoids, and saponins [5]. Based on [6], stem bark also contains proantocyanidin. The content of this secondary metabolite which provides benefits to this plant. Several types of flavonoid compounds are detected in the leaves of this plant, such as quercetin and rutin, and some analog compounds [7]. Flavonoids are a large group of antioxidant compounds named as polyphenols consisting of anthocyanidins, biflavones, catechins, flavanones, flavones, and flavonoids. Quercetin is included in the flavonol compound group, which is distributed with quercetin and glycoside content of approximately 60-75% of the total flavonoids. Flavonoids consist of aromatic compound with antioxidant activity. This activity will prevent body tissue damage on the degenerative disease patient.

Based on the benefit of flavonoid compound, this study will separate the flavonoid compounds from ethanol extract *Polyalthia longifolia* (Sonn.) Thw. leaves from Indonesia and the Philippines. Then, both of extract obtained will analyzed its flavonoid by using Thin layer Chromatography (TLC) and would be confirmed by Liquid Chromatography Mass Spectroscopy (LC-MS).

2. Materials and Methods

2.1. Plant material

The leaves of the plants, especially Indonesia variant, were collected from area Malang, Indonesia at August until September. The dried leaves from The Philippines were deposited at Institute Biosains, University of Brawijaya, Malang, Indonesia. Based on the taxonomical identification, the leaves that obtained were Polyalthia longifolia (Sonn.) Thwaites.

2.2. Sample preparation and extraction

The leaves were air dried and powdered. The extracts of both of powder leaves were prepared by maceration using 70% ethanol for 3 days with occasional stirring, after that the filtrates were obtained by filtration using Whattman filter No. 1, respectively. The ethanol was evaporated by rotary vacuum evaporator at 50 °C to obtain the dry extract. The dry extract were stored at 4°C until use.

2.3. Identification of flavonoids compound

One milliliter of extract 5 % was diluted in the 50% hot methanol 2 mL. Then, it added some magnesium powder and 4-5 drops of HCl. Identification of the flavonoid in the both of extracts were showed red-orange solution.

2.4. Identification of flavonoids by Thin Layer Chromatography (TLC)

The dry extracts were dissolved in 70% ethanol (10 mg/mL), three mobile phases were prepared in the chamber that consist of (I) Toluene : Ethyl Acetate : Formic Acid (5 : 4 : 0,2); (II) ethyl acetate : methanol : water (5 : 1 : 5); (III) n-Butanol : Acetic acid glacial : water (5 : 1: 4), and those were saturated in 30 min. Thin layer chromatography was performed according [5], with modifications. On 3 x 10 cm plates pre-coated with silica gel G, the samples of extract were spotted on the lower left of the TLC plat. Then, the plats were run one dimensionally in the mobile phases at room temperature. Identification of the flavonoids in the extracts was done under UV light after the application of ammonia.

2.5. Identification of Flavonoids by Liquid Chromatography-Mass Spectroscopy (LC-MS)

PL ethanol extract samples were analyzed using LC-MS to obtain qualitative data. The results of LC-MS obtained by chromatogram and peak molecular weight of compounds contained in both extracts. The operational conditions of LC-MS equipment were adjusted for running. The column used was Hypersil Gold (50 mm x 2.1 mm x 1.9 µm). UHPLC brand ACELLA Thermo Scientific type 1250. Elution was carried out with mobile phase A consisting of 0.1% formic acid in aquabidest and mobile phase B consisting of 0.1% formic acid in acetonitrile, with linear gradient and flow rate of 300 µL/minute. The column was controlled at 30 °C and the autosampler compartment was set for C16. Mass Spectroscopy was used MS/MS Quadrupole Mass Spectrometer TSQ QUANTUM ACCESS MAX from Thermo Finnigan with ESI (Electrospray Ionization). The ionization source controlled by

TSQ Tune software which was operated by Negative ionization mode. The determination of targeted compounds was carried out by the SRM (Selected Reaction Monitoring) method.

3. Results and Discussion

3.1. Sample preparation

The leaves were air dried and powdered to get high surface area, that it can be increasing the powder contact with solvent. Extraction was done through maceration or soaking methods using 70% ethanol. The maceration method was chosen because of the easy and simple tools used. This method can maximize solvent contact and materials and can be used to extract substances that are not heat resistant. The extracts of Indonesian and Filipino PL extract obtained were 26,80 % and 23 %.

3.2. Identification of flavonoids

Identification of flavonoids was done by addition of Hydrochloric acid (HCl) solution and magnesium (Mg) powder to produce a red-orange solution. Based on this analysis, the two ethanol extracts of the leaves of *Polyalthia longifolia* (Sonn.) Thw. contain flavonoids by producing a yellowish-orange solution after adding Mg powder and HCl. The ethanol extract of *Polyalthia longifolia* leaves from two countries contain flavonoids which were have biological activities such as antimicrobial, antiradical and have cytotoxic activity against cancer cells [7].

3.3. Thin Layer Chromatography analysis

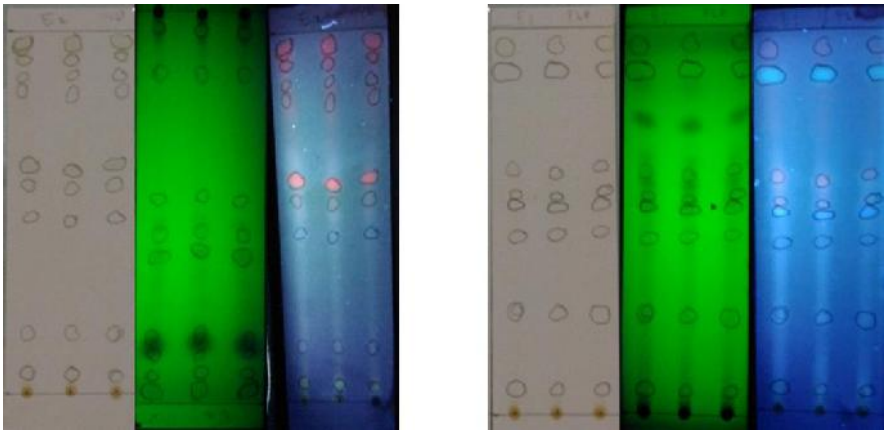
Thin-layer chromatography separation was a separation process based on the distribution of active compounds in two phases, the mobile phase (consist of a mixture of solutions that have a similar polarity with the compound to be separated) and the stationary phase was a silica gel coated plate (usually gives luminescence under UV light). The best separation product was showed by spots without tailing and no overlapping spots. Separation of flavonoid compounds using the TLC method was carried out with 3 mobile phases or eluent, and produced spots which can be luminescence under UV (ultraviolet) lamps at wavelengths of 254 nm and 366 nm. Table 1 showed the TLC results of the flavonoid compounds from the ethanol extract of leaves of *Polyalthia longifolia* (Sonn.) Thw from Indonesia and the Philippines.

Table 1. TLC Separation of Flavonoids Compound.

Source Plant	Mobile Phases	Spots	Assumption of Flavonoid spots	Rf Number	Spot Appearance under UV
Indonesia	Ethyl Acetate : Methanol : Water (5 : 1 : 5)	11	7	0.05; 0.07; 0.37; 0.49; 0.58; 0.67; 0.80	Green; Yellow; Yellowish orange; Yellowish green
The Philippines		7	4	0.04; 0.56; 0.73; 0.81	Green; Orange; Yellow; Dark Green under UV_{254}
Indonesia	Toluene : Ethyl Acetate : Formic Acid (5 : 4 : 1)	12	7	0.05; 0.08; 0.56; 0.81; 0.82; 0.86; 0.88	Yellowish green; Green; Yellow; Yellowish orange; Dark Green under UV_{254}
The Philippines		12	7	0.06; 0.26; 0.58; 0.66; 0.75; 0.85; 0.89	Yellowish green; Yellow; Yellowish orange;

					Dark green under UV_{254}
Indonesia	n-Butanol : Acetic Acid Glacial : Water (4 :1 : 5)	6	2	0.33; 0.76	Greenish; Yellowish green
The Philippines		7	4	0.10; 0.51; 0.77; 0.82	Dark green; Violet; Yellow

Based on previous study, flavonoids on the TLC system would show yellowish green [5], dark chocolate under UV_{254} [10], yellow and blue [8]. Besides that, positive flavonoid showed the fluorescence spot yellow, green, and blue [9]. The first eluent (EA: MeOH: W-5: 1: 5) was a polar solvent and it was capable of producing yellowish-green stains under UV_{366}. Retention factor (Rf) from *P. longifolia* leaves ethanol extract from Indonesia and the Philippines were 0.67 and 0.73, respectively, which were assumed to be rutin and analogs. According to Sampath and Vasanthin [5] flavonoid rutin, as standard compound, showed Rf 0.676 with a yellowish-green color under UV lamps. Secondary eluent (Toluene: EA: Acid Format-5: 4: 1) was a polar mobile phase and produces 12 spots from both of *P. longifolia* leaves ethanol extract, Indonesia and the Philippines. The result of this study was flavonoids spots showed on *P. longifolia* leaves from the Philippines, that showed spot at Rf 0.66 with a dark color under UV_{254} light. According to [10], flavonoid of quercetin from *P. longifolia* plants showed at Rf 0.62 with dark brown spot under UV_{254} light. The third eluent (nButOH: AAG: W-4: 1: 5) was a polar solvent that was able to provide good separation of polar compounds in the TLC. According to [8] separation by TLC using this mobile phase would show a clear separation between O-glycosides and C-glycoside flavones that were not hydrolyzed (mid low Rf) and aglycone (high Rf), while the expected color was light brown, bright yellow, and yellow-green. The result of separating using the third eluent showed yellow and green spots that suspected as flavonoids. Figure 1 shows the best separating of the second mobile phase.



(A) (B)

Figure 1. Separating pattern of flavonoid by TLC using Toluene: Ethyl Acetate : Formic Acid (5:4:1). (A) *P.longifolia* from Indonesia, (B) *P. longifolia* from the Philippines.

3.4. Liquid Chromatography-Mass Spectroscopy analysis

Liquid Chromatography-Mass Spectroscopy (LC-MS) was advanced procedure to analysis a compound that confirmed from TLC result. In the LC-MS system, the separated compound, from LC, would go into MS system, then identified based on molecular mass. The molecular mass of flavonoids compound would be identified by ESI/MS, then showed as fragments with m/z (M^+). There were six target compounds that detected by LC-MS analysis. **Table 2** showed the flavonoid compounds and also analog compounds that was contained in the *Polyalthia longifolia* leaves ethanol extract from Indonesia and The Philippines based on LC-MS separation.

Table 2. Flavonoid compounds from *Polyalthia longifolia* leaves ethanol extract from Indonesia and The Philippines based on LC-MS.

Prediction compound	Source plant	M^+	Retention time (RT)
Quercetin ($C_{15}H_{10}O_7$)	Indonesia	302,5 – 303,5	5,39
	The Philippines		5,05
Rutin ($C_{27}H_{30}O_{15}$)	Indonesia	610,5 – 611,5	2,61
	The Philippines		2,62
Vicenin-2 ($C_{27}H_{30}O_{15}$)	Indonesia	595,5 – 596,5	2,80
	The Philippines		2,80
Quercetin-3-O-glucoside ($C_{12}H_{20}O_{12}$)	Indonesia	464,5 – 465,5	2,69
	The Philippines		4,88
Quercetin-O-O-galloyl hexoside ($C_{30}H_{30}O_{16}$)	Indonesia	614,l5 – 615,5	5,57
	The Philippines		5,57
Leucocyanidin-(+)2,3-3,4-cis-3,4,5,7,3',4'-hexahydroxyl flavan ($C_{15}H_{14}O_7$)	Indonesia	306,5 – 307,5	5,16
	The Philippines		5,15

Based on LC-MS identification, the extract of *P.longifolia* from Indonesia and the Philippines founded quercetin. Based on the table, quercetin was found at m/z 302,5-303,5 as $[M+H]^+$ and rutin at m/z 610,5 – 611,5 as $[M+H]^+$. The similar compounds were characterized too from both of extracts. This study showed Vicenin 2, one of 4 analogue compounds of flavonoids at m/z 595,5 – 596,5 $[M+H]^+$. Quercetin derivative vicenin 2 was showed at m/z 594,8 (M) which usually following molecular peaks at m/z 611,7 $[M+O^+]$, the addition of oxygen ion, and m/z 649,2 with addition three water molecules, $[M+3H_2O]$. Quercetin-3-O-glucoside with m/z 464,5 – 465,5 as [M+H] and Quercetin-O-O-galloyl hexoside with m/z 614,5 – 615,5 as molecular ion, were detected. The molecular ion of Quercetin-O-O-galloyl hexoside was identified as M-31. That was mean MS spectra of those compounds was obtained another fragments which referred as additional of formic acid, elimination of CO_2, elimination of hexoside, and additional of acetate ion [10]. Leucocyanidin-(+)2,3-3,4-cis-3,4,5,7,3',4'-hexahydroxyl flavan was another compound that inculinding to the flavonoid class. Based on the LC-MS study, it was observed at m/z 306,5 – 307,5 as molecular ion [M+H]. **Figure 2** showed the structure of flavonoid compounds.

LC-MS analysis usually revealed the identity of compounds based on fragmentation behavior with process of protonation which throughout dehydration, loss or additional of some functional groups, and C-ring fission. Pattern of fragmentation was associated with the applied collision energy. If the collision energy is less than main fragment in the MS, the spectra produced was $(M + H)^+$. However, by enhancing collision energy, a complete fragmentation of the protonated molecule can be obtained [10].

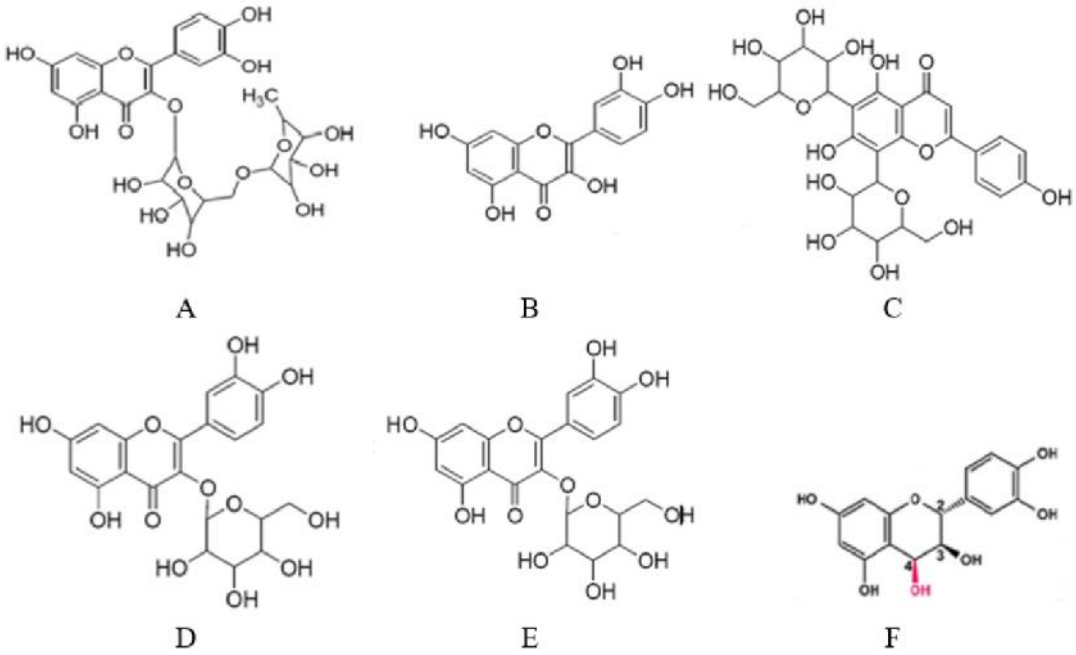


Figure 2. Structure of Flavonoid Compound. (A) Rutin, (B) Quercetin, (C) Vicenin-2, (D) Quercetin-3-O-glucoside, (E) Quercetin-O-O-galloyl hexoside, (F) Leucocyanidin-(+)2,3-3,4-cis-3,4,5,7,3',4'-hexahydroxyl flavan.

4. Conclusion

In conclusion, the ethanol extracts of *Polyalthia longifolia* (Sonn.) Thwaites. from Indonesia and the Philippines revealed the present of flavonoid compounds. Based on LC-MS analysis, the both of extracts contain of quercetin, rutin, and the analogue compounds such as Vicenin-2, Quercetin-3-O-glucoside, Quercetin-O-O-galloyl hexoside, and Leucocyanidin-(+)2,3-3,4-cis-3,4,5,7,3',4'-hexahydroxyl flavan.

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RESEARCH ARTICLE
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Diabetes sepsis on Wistar rat strain (*Rattus norvegicus*) induced by streptozotocin and bacteria *Staphylococcus aureus*

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Abstract

Background and Aim: Sepsis is characterized by loss of control of the inflammatory response, which can be triggered by various microorganisms and toxic secretions. The mortality rate increases due to impaired endothelial function caused dysfunctional organ systems. Diabetes is closely related to sepsis. The study aimed to determine the method of using animal models of sepsis diabetes through a combination of streptozotocin (STZ) and *Staphylococcus aureus* infection based on biological marker parameters.

Materials and Methods: A total of 30 male Wistar rats of 2.5-3 months old weighing approximately 150-250 g body weight (BW) divided into six treatment groups with five replications per group were used in the study. Treatment A was negative control (healthy rats) and Treatment B was the positive control (with diabetes) where rats were given STZ dose at 45 mg/kg BW on day 8 intraperitoneally (IP). The blood glucose was measured on day 10, Treatment C was a positive control (bacteria), rats inoculated with *S. aureus* with a concentration of 10^8 CFU/mL on day 8 given IP and observed sepsis conditions on day 10^{th}. Treatment group (D, E, and F): Rats given STZ dose at 45 mg/kg BW on day 8^{th} by IP and measured blood glucose on day 10^{th}, then inoculated with *S. aureus* with different concentrations of 10^5 CFU/mL, 10^6 CFU/mL, and 10^7 CFU/mL on the 10^{th} day, respectively, and were later observed the condition of sepsis on day 12^{th}. Data on diabetes bacteremia were quantitative used blood glucose levels, the bacterial count, and C-reactive protein (CRP) and were analyzed using the one-way analysis of variance test with a confidence level of 95%. Physical examination (temperature and respiration) is qualitative.

Results: Physical examination showed that all treatments had a normal temperature, an increased pulse in Groups D, E, and F and a decrease in respiratory rate in the treatment of E and F, the bacteria found in the vital organs in all groups, and CRP levels were not significantly different at all.

Conclusion: Animal model of diabetes sepsis can be observed through a combination of pancreas damage, and respiration, the bacteria in the vital organs.

Keywords: animal model, diabetes, inflammation, sepsis.

Introduction

Sepsis is a condition that damages the body, characterized by systemic activation of the inflammatory pathway and coagulation in response to microbial infections in ordinarily sterile parts of the body [1], and is often exacerbated by a number of conditions for metabolic disorders including type 1 and 2 diabetes mellitus (DM) [2]. Sepsis is characterized by loss of control of the inflammatory response, which can be triggered by various microorganisms and toxic secretions. The prevalence of sepsis in dogs showed 89 individuals (78%) had dysfunction in one or more organ systems, and 57 individual (50%) dogs showed multiple organ dysfunction organs. The mortality rate increases the number of dysfunctional organ systems [3]. In animal models of type 2 diabetes are known that the inflammation induced by more severe sepsis compared with no diabetes. Animal with sepsis diabetes also experiences an increasing number of bacterial infection and dysfunction in the expression of inflammatory cytokines and immune cells. The literature study on obese and diabetes models (given a high-fat diet) has a higher mortality rate when challenged with *Staphylococcus aureus* compared with no infection [1]. Increased mortality in animal models of diabetes occurs after 72 h associated with persistent bacteremia and reticuloendothelial microbial presence [4]. Diabetes that does not heal can cause complications such as neuropathy, vasculopathy, retinopathy, immune defects, and sepsis [5]. DM is considered a state of immunosuppression. Diabetes patients are very susceptible to endothelial dysfunction during

sepsis. A recent study showed that E-selectin, leukocyte adhesion molecules dissolved, vascular cell adhesion molecule 1, intercellular adhesion molecule 1, vascular endothelial growth factor, and increased significantly in diabetes patients compared with patients without diabetes during the most severe sepsis stage. Some research suggests that patients with diabetes showed a clear activation of multiple pathways endothelium during sepsis, especially during severe sepsis. These molecules play a role in the inflammatory response during sepsis [4]. This suggests that patients with diabetes showed a clear activation of multiple pathways endothelium during sepsis, especially during severe sepsis [5]. Treatment of diabetes with a microbial infection is still unresolved, so the mortality rate is still high. This is estimated because the incidence of the disease is difficult to detect.

Animal models of diabetes and sepsis have developed their own to create a system that can be reproduced for studying the pathogenesis, preliminary testing of the potential therapeutic agent but animal models of diabetes bacteremia yet. Until now, the existing animal models are diabetes accompanied by foot infection or gangrene or diabetes foot [5], whereas there are no diabetes animal models with *S. aureus* infection.

The study aimed to determine the method of using animal models of sepsis diabetes through a combination of streptozotocin (STZ) and *Staphylococcus aureus* infection based on biological marker parameters.

Materials and Methods

Ethical approval

Maintenance and handling of Wistar rat animals in a laboratory were based on the letter of ethics no. 937-KEP-UB from Biosains, Brawijaya University.

Research methods

This research is of true experimental laboratory post-control only design, which created an animal model of diabetes sepsis using a combination of STZ and *S. aureus*.

Location and time of research

This research was conducted at the Pharmacology Laboratory of the Medical Faculty and Veterinary Medicine of Brawijaya University, Indonesia.

Sample and population

The study sample used white rats stain Wistar used for diabetes sepsis STZ and *S. aureus* administered intraperitoneally (IP) in a completely randomized design.

Experimental design

This study used male rat (body weight [BW] 150-250 g). The rat was previously adapted for 7 days. In this study consisted of six treatment groups, namely: Treatment A (negative control): Five normal/healthy rat, Treatment B (diabetes): Five rats were given STZ dose at 45 mg/kg BW on day 8th IP and measured blood glucose on the 10th day, Treatment C (bacteria): Five rats were inoculated with *S. aureus* with a concentration of 10^8 CFU/mL on the 8th day and observed sepsis conditions on the 10th day, Treatment D: Five rats given STZ dose at 45 mg/kg BW on day 8th IP and measured blood glucose on day 10, then inoculated with *S. aureus* with a concentration of 10^5 CFU/mL on day 10th, and observed the condition of sepsis on the 12th day, Treatment E: Five rats given STZ dose at 45 mg/kg BW on day 8th IP and measured blood glucose on day 10, then inoculated with *S. aureus* with a concentration of 10^6 CFU/mL on day 10, and observed the condition of sepsis on the 12th day, and Treatment F: Five rats given STZ dose at 45 mg/kg BW on day 8 IP and measured blood glucose on day 10, then inoculated with *S. aureus* with a concentration of 10^7 CFU/mL on day 10, and observed the condition of sepsis on the 12th day.

Induction of an animal model of type 1 diabetes rats

STZ (Cat. No. 41910012-4 [714 992], bioWORLD Dublin, Dublin, OH) 32.5 mg was dissolved in (50 mM, 0.1M, pH 4.5) buffer citrate to a final concentration of 32.5 mg/mL and preserved in a frozen condition before use. Animal treatment adapted in cages for 7 days, after the treatment of diabetes control and treatment (D, E, and F) was fasted overnight (6-8 h). The rats were further injected with a single dose of STZ via intraperitoneal route (45mg/kg BB) and blood glucose levels were measured 2 days after STZ injection. Rats with fasting blood glucose >270 mg/dl were considered diabetes positive. [6,7]. Blood sugar was measured using digital blood glucose level Glucostick (Gluco-Dr®) device.

Bacterial culture

S. aureus bacteria were obtained from the Microbiology of FK Universitas Brawijaya. *S. aureus* bacterial identification using mannitol salt to be positive, catalase test positive, and Gram stain showed Gram-positive, cocci-shaped, and grape-clustered bacteria. The test bacteria have been resistant to several antibiotics, namely amoxicillin, vancomycin, cefoxitin, ceftriaxone, and penicillin.

Bacterial seeding was carried out by taking 10 colonies of *S. aureus* bacteria then cultured in Nutrient Broth media (Merck Millipore, Boston, USA) at 37°C for 24 h, then measuring optical density (OD) using a spectrophotometer. The results obtained with similar bacteria concentration of 0.1 OD 10^8 CFU/mL and then made appropriate dilution for treatment [8].

Preparation of animal model of sepsis

Wistar strain male rats were inoculated with 10^8 CFU/mL of *S. aureus* through asepsis IP, and the bacterial dose was 2 mL per tail. Clinical signs, weight, and survival rates were monitored daily for 3 days after infection [8].

Necropsy of an animal model

Necropsy begins with the administration of anesthesia using ketamine at a dose of 2 mg/kg bw

(body weight) via intramuscular route per rat. After disinfection with 70% alcohol, surgical procedure was done to extract blood, liver, heart and kidney. The numbers of *S. aureus* bacteria in the collected organs were then examined.

CRP test

Rat blood sample was taken after 3 days of infection and stored inside a 3 ml vacutainer. The blood sample was then frozen for 1 h at room temperature and centrifuged at 4000xg, 4 °C, 15 min to obtain serum. The serum was further analyzed for CRP test using a commercial kit (Life Diagnostics, West Chester, AS) [8].

Histopathological examination

Sample from pancreas was collected on the 12th day, fixed in formaldehyde at 10% with phosphate buffer (pH-7.4) and then post-fixed for 24 h, dehydrated, and embedded in paraffin. Sections, with 3-4 mm of thickness, were cut with a microtome [9].

Statistical analysis

The diabetes sepsis data that were analyzed include clinical symptoms (BW, temperature, pulse, respiration), CRP examination and the number of bacteria present in vital organs (kidneys, liver and heart). The clinical signs were analyzed descriptively. Quantitative of blood glucose and CRP levels were then analyzed using the one-way analysis of variance test with a confidence level of 95% to determine the difference in the effect of treatment on making animal models of sepsis diabetes.

Results and Discussion

Animal model of type 1 diabetes rats

The results showed that in the treatment of diabetes and diabetes sepsis showed blood glucose >270 mg/dL, whereas in normal treatment and sepsis that was not suspected STZ showed normal blood sugar levels (<120 mg/dl) (Figure-1). Blood sugar levels increase due to STZ induction. STZ is a β-cytotoxic drug, an antimicrobial agent and has also been used as an alkylating agent for acupuncture. STZ can cause pancreatic β-cell necrosis. The incidence of diabetes depends on animal species; the dose and route of administration from STZ are severe diabetes (blood glucose to 200/300 mg/dL) and mild diabetes (120-200/300 mg/dL). The pancreas can regenerate through the proliferation and neogenesis. Remodeling of the pancreas caused by increased replication and cell apoptosis on day 13-day 17. Under physiological conditions, the pancreas maintains glucose homeostasis [10,11]. Weight loss was only shown in STZ-induced rat while healthy rat and bacteria-induced rat did not show weight loss. Weight loss was significantly higher in the D and E treatment groups than in Groups B and F (unpublished data). This is in accordance with the research [8], which showed that rats induced by *S. aureus* bacteria concentrations of 4.5×10^4-4.5×10^9 CFU/mL showed no weight loss.

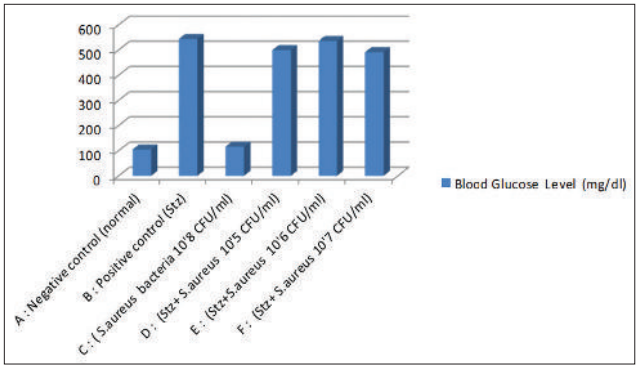

Figure-1: Blood glucose level.

According to the research conducted by Reis *et al*. [9], which showed that male Wistar rats induced by STZ at a dose of 65 mg/BW intravenous showed weight loss. The absence of insulin that serves to regulate the metabolism of sugars through the breakdown of sugars into simple molecules which are then distributed to the cells causes very high levels of glucose in the blood called as hyperglycemia. The body cannot use sugar as an energy source and stores extra glucose as fat, resulting in weight loss.

STZ causes an increase in free radicals that serve to destroy the pancreatic β-cells. This result is quite interesting to observe that diabetes rats characterized by pancreatic β damage and turned out to be clinical symptoms that appeared normal. The results showed that in the diabetes control group (B) and all diabetes and bacterial treatments (D, E, and F) showed pancreatic beta-cell nuclei shrinking and even disappearing, only cytoplasm was seen so that the Langerhans island cell density was lower or less [9] than in the group healthy (A) and a positive control bacteria (C) showing pancreatic beta-cell nucleus appear clear and evenly so that a higher density of pancreatic cells (Figure-2).

STZ works by inhibiting the enzyme activity of free radicals, thereby increasing the formation of superoxide radicals, and nitric oxide turn produces reactive oxygen species (ROS) or oxidative stress which can cause oxidative damage to cellular components (lipids, DNA, and proteins) and trigger the activation of signaling pathways and disrupt standard repair mechanism. STZ enters the tissue through glucose transport, GLUT2 in the plasma membrane, and then, it will go to the pancreas and affect other organs such as the liver and kidneys. When the pancreatic β-cell is destroyed, insulin secretion decreases so that blood glucose increases and is not controlled in the blood. ROS can increase oxidative stress through increased production of p21 and reduced insulin messenger RNA cytosolic adenosine triphosphate and calcium flux in the cytosol and mitochondria [12].

The existence of bacteria in vital organs

In this study, animal model was used to induce a combination of diabetes and sepsis. On bacterial examination, bacterial infections are found in vital

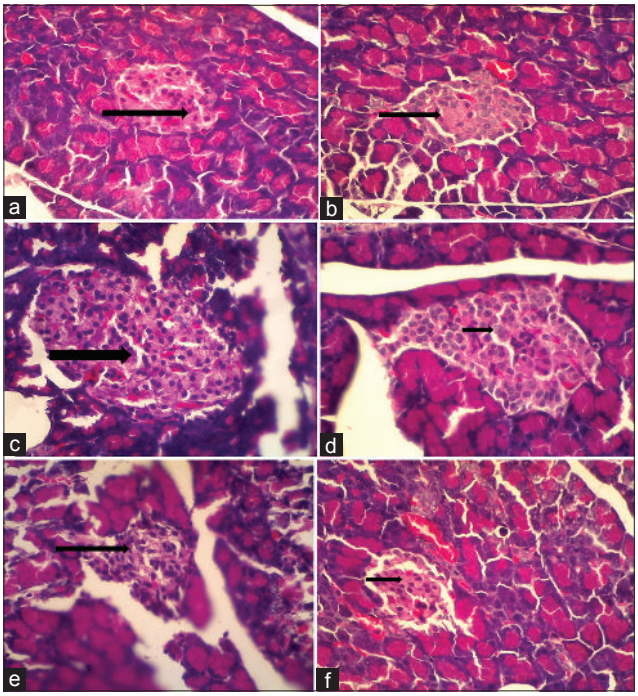

Figure-2: Images with magnifications 400×: Negative control (a) showing pancreatic beta-cell nucleus appears clear and spread evenly (arrow). Positive control diabetes (b) showed that the pancreatic beta-cell nucleus seemed to shrink even invisible and only seen cytoplasm (arrow). Positive control bacteria with a concentration of 10^8 CFU/mL of *Staphylococcus aureus* (c) showing pancreatic beta-cell nucleus appear clear and spread evenly (arrow). Treatment diabetes and bacteria *S. aureus* with a concentration of 10^5 CFU/mL (d) showed that the pancreatic beta-cell nucleus seemed to shrink even invisible and only seen cytoplasm (arrow). Treatment diabetes and bacteria *S. aureus* with a concentration of 10^6 CFU/mL (e) showed that the pancreatic beta-cell seemed to shrink (arrow). Treatment diabetes and bacteria *S. aureus* with a concentration of 10^7 CFU/mL (f) showed that the pancreatic beta-cell seemed to shrink (arrow).

organs in all treatments because bacteria would enter the blood vessels and would spread through lymphatic vessels to organs and cause multiple organ damage to death. Bacterial infections that enter the body cause hemodynamic changes that interfere with microcirculatory and cellular disorders resulting in the development of various organ dysfunctions and death. The bacteria were induced by IP in rats showed an increased pulse in all treatments (above 450 beats/min), respiration decreased only in the treatment Groups E and F (below 130 beats/min) while the temperature in normal conditions for all treatments (36.07-37.32°C). This is supported by research conducted by Popov and Paplov [13], which indicates that there are differences in the animal model of sepsis between positive and Gram-negative bacteria. Sepsis animal model was caused by the administration of gram-positive bacteria (intravenously), low hemodynamic appearance, and changes in lung disorder were observed. Whereas gram-negative bacteria causes hemodynamic shock and acute respiratory disorder.

S. aureus bacteria can replicate in the blood and colonize multiple organs and cause fatal sepsis [14]. Bacteria in the organs of diabetes rats are controlled by genes that encode toxins and protease enzymes that cause tissue damage [15]. Furthermore, there are genes related to virulence factors such as adhesion molecules, capsule polysaccharides, siderophore, and metabolic and transport systems of amino acids and carbohydrates that support the severity of endocarditis. Diabetes rats can interfere immunity which can accelerate the infection. According to Popov and Paplov [13], the development of sepsis animal models using rodents depends on the type of bacteria, route of administration, bacterial dose, and frequency of administration. The higher dose of the bacteria with a direct route into the blood vessel then will be more severe clinical symptoms.

Diabetes rat would be at high-risk exposure to pathogens and disease will be more severe. According to Mai *et al*. [16] states that high-fat feed-induced rat is at high risk of increasing the number of bacteria, decreasing the T-cell immune system to eliminate bacteria, and increasing pro-inflammatory and anti-inflammatory cytokines compared to normal mice. The study showed the death of rats in positive diabetes control and *S. aureus* bacteria concentration of 10^8 CFU/mL on day 2 after bacterial induction. The results of this study are different from those conducted by Wu *et al*. [8] which showed rat died after being induced with *S. aureus* with successive concentrations ranging from 4.5×10^7 to 4.5×10^9 CFU/mL intravenously which were observed 7 days post-infection. Death in diabetic rats is due to the impaired immune system through decreased production and function of inflammatory cytokines, loss of phagocytic function, and body antioxidant production [17]. The high concentration of *S. aureus* induced in rat resulted in increased inflammation. Bacteria that enter IP will activate macrophages to do phagocytosis so that there will be an increase in the production of free radicals and inflammatory cytokines. However, death was not found in all diabetes treatments and *S. aureus* bacteria from concentrations of 10^5-10^7 CFU/mL. The high sugar levels and bacterial induction did not affect the clinical symptoms of rat even though bacteria were found in vital organs. When viewed from the results of the study, diabetes control rat had an average blood sugar level higher at 544 mg/dl compared to diabetes and bacterial groups.

CRP test

CRP can be found in vertebrates (humans, mice, and rats) and invertebrate animals [18]. CRP is an acute inflammation produced in the liver as a result of responses to phagocytic cells that are affected by proinflammatory cytokines, namely interleukin (IL)-1, IL-6, and tumor necrosis factor-α. CRP will appear after 6-8 h after the initial infection and peak at 36-50 h thereafter. CRP is part of the ligand-binding plasma calcium-dependent family. The mechanism

of action of CRP is that it binds to phosphocholine residues and then causes membrane damage and cell apoptosis. CRP will activate the classic path complement, C1q protein will then activate C3 and end with membrane damage [19].

The results showed that rat in the group of D, E, and F (diabetes rats with a concentration of *S. aureus* bacteria consecutively 10^5 CFU/mL, 10^6 CFU/mL, and 10^7 CFU/mL) was significantly different from control diabetes but not significantly different from control *S. aureus* bacteria concentration of 10^8 CFU/mL (Figure-3) and healthy control. This shows that CRP levels increase only in diabetes conditions, whereas in diabetes with diverse high concentrations of bacteria does not show an increase in CRP levels. The results are consistent with the research conducted by Dimitrov *et al*. [20], which showed that increased CRP could be detected in animals that have decreased in inflammation and an increase in high carbohydrate levels. CRP test demonstrates that the presence of acute inflammation such as in humans is less prominent in rats as a result of induction of bacteria does not activate the complement pathway [21]. This study contradicts the results of human studies, which showed that serum CRP levels were high in conditions of bacterial sepsis compared to healthy humans [22]. CRP in the rat is not specific to indicate an acute inflammatory reaction. Levels of CRP concentrations in rats would increase the basal metabolic condition that is approximately 300-500 mg/l, 100 times higher than in humans [18]. The biological effect indicates that the average of CRP level in healthy rat lower than that of all the treatment of diabetes and bacteria and also bacterial induction courses. This shows that the induction of diabetes and bacteria can trigger inflammation. At the time of entry of the antigen in the body, there will cause inflammatory cells out of the blood vessels leading to the injured area or damaged tissue, causing the release of inflammatory mediators to clear pathogens and wound healing agent [23].

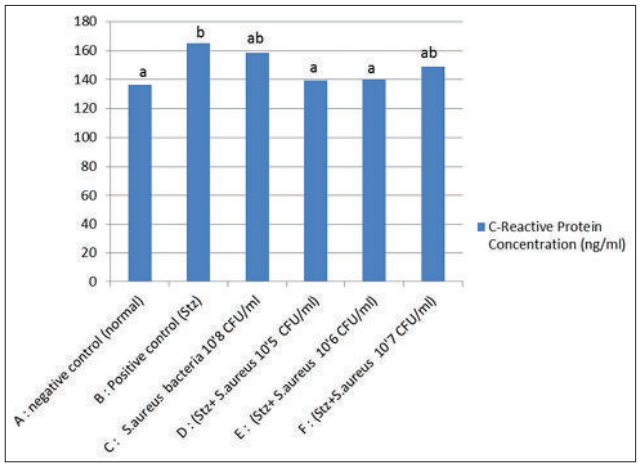

Figure-3: Level of C-reactive protein. Superscript letters are significantly different from one another based on analysis of variance with honestly significant difference tests ($p<0.05$).

CRP in the rat cannot activate complement which mediates inflammation except ligand-specific CRP mediated by C-polysaccharide from bacterium *Streptococcus pneumoniae* [18], but it can induce inflammation, pro-oxidants, and pro-coagulation through a pathway to increase macrophage activation [20]. Biomarkers of diabetes sepsis in Wistar rats can be seen from the weight loss, increased blood sugar, pancreatic cell damage, increased pulse, and decreased respiration, and the bacteria found in the vital organs.

Conclusion

Biomarkers of animal models of sepsis diabetes using Wistar rats through a combination of weight loss increased blood sugar levels and pancreatic cell damage, increased pulse and decreased respiration, and found bacteria in vital organs in all treatments.

Authors' Contributions

DQ was responsible for controlling the course of studies, culturing of bacteria, and also analyzing data. DEA did CRP test; MAGB and AA did the analysis. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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RESEARCH ARTICLE

Effect of Citrus acidity on profile of fatty acid in Virgin Coconut Oil (VCO)

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ABSTRACT:
Indonesia is one of the countries that have the highest coconut production in the world in 2016. High coconut production is widely used for health, one of which is to the manufacture of VCO. VCO contains high fatty acids, especially lauric acid, which functions as a functional health food. The production of VCO can be produced through cold, heat, enzymatic, and fermentation methods, but the use of oranges for the production of VCO still have not yet existed. The purpose of the study was to determine the effect of adding variations of citrus juice with a concentration of 1% to the profile of fatty acids in VCO (Virgin coconut oil). The method used a cold modification method, namely coconut milk blended and then separated from the coconut milk and added with various treatments. This study used five types citrus of treatment with a concentration of 1% namely limau (A), lemon (B), lime (C), orange aforer (D) and without treatment (E) and then analyzed on the fatty acid profile using the GCMS (gas chromatography-mass spectrometry) method. Data is qualitative and analyzed descriptively. The results show that effect of variation of orange juice using a 1% concentration on fatty acid profiles following SNI 2008 standards, APCC and Codex except for linoleic acid below the standard <1% and myristic acid above the standard > 21% and also no growth of bacteria and fungi. Conclusion: orange variations of a concentration of 1% can be used to produce VCO with fatty acid profiles accordance with the standard.

KEYWORDS: fatty acids, oranges, cold method, VCO, coconut

INTRODUCTION:
Coconut is very well developed in Indonesia as an archipelago with a long coastline reaching 81,000 km, which is estimated to have the largest coconut tree area in the world, which is around 3.1 million hectares (Witono *et al.*, 2007). In 2016, Indonesia ranked first in the world's most abundant oil producing up to 18.3 tons (Katadata, 2018a). In 2010, exports of coconut and coir Indonesia reached 850 thousand tons and had increased in 2015 to double to 1.7 million tons.

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It is estimated that Indonesia's coconut exports have increased by around 15.14 percent per year (Katadata, 2018b).

Virgin coconut oils are oils obtained from fresh and old coconuts without refining, heating and nor through purification with chemicals (Elfianus, 2008). VCO is colorless with a distinctive coconut flavor and fresh aroma and longer shelf life (Raghvendra and Raghvarao, 2010). Coconut products, especially coconut oil (VCO) is widely used in most industries such as for food, pharmaceuticals, and cosmetics. It has many advantages contain of medium chain fatty acids (MCFA), especially lauric acid which has a molecular size smaller than the fatty acid chain length (LCFA) (C> 14) so that it is easily absorbed by the body which allows providing a fast source of energy and is not stored as fat in the body

(Asiah *et al*., 2018). VCO also function as an antimicrobial (Prapun et al., 2016), and antioxidants (Arumugam *et al*., 2014). So that VCO becomes popular and is widely consumed as a functional food for health

VCO produced by cold processing which must involve the preservation of the natural components of oil according to the Asian Pacific Coconut Community (APCC), Codex, The Philippines National standards (PNS), Bureau of Product Standards (BPS) -2004 (Satheesh and Prasad, 2014).

On the contrary to research conducted by Abdurahman *et al*. (2011) that the production of VCO using the centrifuge method with a speed of 5000 and 8000 RPM produced a fatty acid profile that was not by the standards of APCC and codex. Other studies had shown that the production of VCO using a fermentation process using the bacteria *Lactobacillus acidophilus* produces fatty acids oleic acid according to standard except on the terms of reference of the Codex and APCC (Satheesh and Prasad, 2012). Research on the production process of VCO using a cold method added to the acidity of oranges has not yet existed.

MATERIALS AND METHODS:

The study used fresh old coconut which was still equipped with coconut shells and coir obtained from the traditional market in Blitar, Indonesia. Coconut is taken from the shell and coconut fiber. The study is an experimental design to determine the effect of citrus juice with a variety that is a lemon (*Citrus limon* (L) (A), nipis (*Citrus aurantifolia*) (B), orange *Afourer Morocco* (C), limau (*Citrus amblycarpa*) (D) using a 1% concentration and without treatment (E) of the fatty acid profile.

The Production of VCO:

Old coconut is then shredded, coconut added with water and blender. Then allowed to stand about 2-3 hours until the coconut milk cream rose to the top — coconut cream mixed with an orange 1% concentration and allowed to stand for one day to appear three parts layer (cream, VCO, and water). Furthermore, VCO tested the growth of bacteria and fatty acid profile (Modification Maradesa *et al*., 2014; Qosimah and Istiana, 2017).

Calculation of Total Fatty Acids:

The calculation of VCO to The fatty acid profile was tested using the gas chromatography-mass spectrometry (GC-MS) method (Caligiani *et al*., 2016).

The Calculation of Total Bacteria:

VCO is grown using streak method on Media Plate count to calculate the total number of bacteria subsequent bacterial colonies are counted after incubation for 48 hours (Arachchi *et al*., 2016) as colony-forming units (CFU) per mL.

The Calculation of Total Fungi:

The product of VCO is streaked in to Subarroad Dextrose Agar, incubated in 37 ℃ for 24 hours.

Data analysis:

Data were analyzed descriptively to show the fatty acid profile and growth of bacteria.

RESULTS:

The profile of Fatty Acid:

The results showed that the administration of orange variations of 1% concentration to produce VCO fatty acid accordance with the standard codex Stan 210-1999, SNI 7381-2008 and APCC, 2006 namely caprylic acid (C8), capric acid (C10), lauric acid (C12), palmitic acid (C16), stearic acid (C18:0), and oleic acid (C18:1) except Linolenic acid (C18:2) is below the standard (less than 1.00%) and the profile of myristic acid (C14) is above the standard (more than 21%).

Table 1. The profile of fatty acid profile on all treatments

Fatty Acid Profile (%)	Codex Stan 210-1999 and SNI7381-2008	Lemon	Nipis	Afourer	Limau	No addictive	APCC
C8, Caprylic acid	**4,6-10,00**	6,1	5,9	5,8	5,6	6,3	5,00-10,00
C10, Capric Acid	**5,0-8,0**	6,1	5,8	5,9	5,8	**6,6**	4,5-8,00
C 12, Lauric Acid	**45,10-52,20**	46,6	48,7	47,3	46,6	44,6	43,00-53,00
C 14, Myristic Acid	**16,80-21,00**	**23,6**	22,3	22,8	24,0	23,4	16,00-21,00
C16, Palmitic Acid	**7,50-10,20**	9,3	8,7	9,4	9,3	**9,6**	7,5-10,00
C 18:0, Stearic Acid	**2,00-4,00**	2,4	2,4	2,4	2,5	**2,7**	2,00-4,00
C18:1, Oleic acid	**5,00-10,00**	6,2	5,5	5,2	5,2	**5,7**	5,00-10,00
C18:2, Linoleic acid	**1,00-2,50**	0,4	0,6	0,4	0,4	**0,4**	1,00-2,50

The Total Amount of Bacteria and Fungi:

The VCO produced in all treatments found no bacterial

and fungi growth.

DISCUSSION:

Production of Virgin Coconut Oil (VCO) from Cocos nucifera, Linn through the demulsification (breakdown of emulsions) of coconut milk into water and oil. Coconut oil is dispersed as small spherical droplets in a layer of water in the continuous phase which can not be separated easily (Abdurahman *et al*., 2011). This study used orange as a demulsifier. The addition of variation of orange concentration of 1% was based on previous research, Qosimah and Istiana (2017) which showed that the results of rendemen VCO using orange a concentration of 1% higher than a concentration of 3%, and organoleptic qualities well as the color clear, foggy, smelling and flavor of coconut. Coconut oil is the most stable oils are highly saturated, and less than 10% unsaturated fatty acids (Syukur *et al*., 2017).

The profile of Fatty Acid:

Based on the previous study conducted by Abdurahman *et al*. (2011) showed that VCO using only cold method produce lauric origin below the standard that is less than 45.1%. Short chain fatty acids such as C8: 0, C10: 0 and C12: 0 are the main components in VCO. The data are shown in table 1. These results are similar to the research conducted by Arumugam *et al*. (2014), coconut oil fatty acid results based on the Cold Squeeze method contain C12 lauric acid (49%), C8 caprylic acid (8%), capric acid C10 (10%), C14 (17%) myristic acid, C18 stearic acid (2%) and C16 palmitic acid (7.8%) except linoleic acid (C18: 2) which has a lower value than (<1.0).

Free fatty acids (FFA) are naturally present in low amounts in all vegetable oils. During extraction and storage, additional FFA can be formed by hydrolysis reactions with residual water in oil. Hydrolysis can occur through a chemical or enzymatic mechanism. Lipase enzyme hydrolysis can be found in orange fruits (Okino-Delgado and Fleuri, 2014). Lipase acts to hydrolyze triglycerides into glycerol and free fatty acids (Ferreira-Dias *et al*., 2013; Shahidi and Wasundara, 2002). Excess enzymes can shorten fat breaking time, produce high VCO rendering quality and can be done at low temperatures (Prapun *et al*., 2016). High levels of FFA is not desirable because of their unpleasant taste (Dayrit *et al*., 2007). VCO of the hot or cold method has a low free fatty acid content (Srivastava *et al*., 2013). Free fatty acid (Free Fatty Acid) is one of the parameters of oil damage due to the hydrolysis process by the interaction with water and lipase activity (Witono *et al*., 2007). So the lower the free fatty acids indicate, the better the quality of oil produced.

The fatty acid profiles produced in VCO depend on the location of coconut growth and the genetic variation of coconut (Syukur *et al*., 2017). Blitar is a coastal area that is a potential for the growth of coconut trees and is on of center in East Java, Indonesia with a land area of about 18986 hectares (Tents and Kumaunang, 2018). The most important qualifications of VCO must be free of water to avoid rancidity, high antioxidant content, vitamins, and lauric acid. The fatty acid molecules found in coconut oil are unique and have different properties from other fats. They are called medium chain fatty acids (MCFA), namely C6-C12 (Dayrit, 2014). MCFA is the highest lauric acid (45-53%) of total fatty acids. Lauric acid is similar to breast milk and has nutraceutical benefits accordingly. These fatty acids, unlike long chain fatty acids are not stored in adipose tissue and do not need to be transported by chylomicrons (Raghvendra and Raghvarao, 2010). MCFA is metabolized quickly and cannot be stored as adipose tissue. This is because MCFA is transported directly in the portal vein system thereby reducing storage of fat deposits and causing high energy expenditure (Gunasekaran *et al*., 2017). The orange fruits may also contain protease enzymes such as those in the peel (Chitturi *et al*., 2013). The highest oil yield can be obtained from the assistant of the protease enzyme besides the amount of unsaturated fatty acids which is higher than the oil extracted by fermentation techniques and thermal cycling (Prapun *et al*., 2016).

Results of high peroxidation of unsaturated fatty acids can cause rancidity during storage VCO (Srivastava *et al*., 2013). Polyunsaturated fatty acids are essential fatty acids, which cannot be synthesized in the body so they must be obtained from food intake. Coconut oil is one of the readily available vegetable oils, consisting of saturated fatty acids (92%) and medium chain fatty acids (MCFA) (Nireeksha *et al*., 2018).

The Total Amount of Bacteria:

This is by the SNI 7381-2008 standard which shows that the requirement for the number of bacteria in VCO is less than 0.5 CFU / ml. VCO production using fermentation method causes the bacteria will ferment coconut milk which can separate coconut oil with water within 24-48 hours. But there is the possibility of contamination with microorganisms because coconut milk is a rich source of protein, carbohydrates, and moisture which can attract microorganisms that would likely to damage the coconut milk which results in the production of low-quality VCO (generally yellow) (Satheesh and Prasad, 2014).

CONCLUSIONS:

The results show that VCO uses oranges on the fatty acid profile by the codex coconut oil standard, SNI 7381-2008, and APCC except for the standard linoleic acid which is less than 1% and no bacteria in it. The results of lauric acid in VCO using nipis was higher than

all treatments.

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AUTHORS' CONTRIBUTIONS:

DQS conducts research; MAGB, AUL, AKA, IAA and DQS perform data analysis and articles.

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Validation of suitable reference genes for normalization of quantitative reverse transcriptase- polymerase chain reaction in rice infected by *Xanthomonas oryzae* pv. *oryzae*

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Xanthomonas

ABSTRACT

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a costly disease in rice that threatens global rice production. Gene expression analysis by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) allows the study of the underlying mechanisms of both BB pathogenesis and resistance. In relative quantification, reference genes are often used to normalize the results to remove technical variations allowing the determination of true biological changes in a pilot experiment. However, variations in the expression of these reference genes can lead to erroneous and unreliable results. Thus, choosing the most stable reference genes for any specific experimental condition is of utmost importance in qRT-PCR experiments. Here, we used geNorm, NormFinder, Bestkeeper, Delta-Ct and RefFinder programs and/or methods to analyze the stability of the expression of eleven candidate reference genes namely: *18S ribosomal RNA* (*18S rRNA*), *Actin-1* (*ACT1*), *ADP-Ribosylation Factor* (*ARF*), *Endothelial differentiation factor* (*Edf*), *eukaryotic Elongation Factor-1α* (*eEF-1α*), *eukaryotic Initiation Factor-4a* (*eIF-4a*), *Profilin 2* (*Prof2*), *Nucleic Acid Binding Protein* (*NABP*), *Triosephosphate Isomerase* (*TI*), *Ubiquitin 5* (*UBQ5*) and *Ubiquitin 10* (*UBQ10*) in cDNA samples from BB-susceptible and Xa21-mediated resistant rice cultivars collected at various times after *Xoo* inoculation. Under our experimental conditions, *Edf* and *TI* were the most stable reference genes while the common housekeeping genes *18S rRNA*, and *UBQ5* were among the least stable genes. Though using either *Edf* or *TI* as internal control is adequate for gene expression analysis, we suggest using both genes to normalize the data of qRT-PCR assays for rice subjected to *Xoo* inoculation.

1. Introduction

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a threatening rice disease responsible for the large percentage of yield reduction in all rice growing areas. Its symptom includes a vascular wilt at the seedling stage, a leaf blight, and unfilled panicles in mature plants which resulted from the invasion of the vascular system by *Xoo* bacteria (Mew, 1987). At the molecular level, *Xoo* secretes transcription activator-like (TAL) effectors which invade and highjack the host cells by activating the transcription of genes that enhance plant susceptibility and support bacterial virulence (Boch and Bonas, 2010; Römer et al., 2010). To date, more than 30 BB resistance genes have been identified in *Oryza sativa* and its closely related species. Among them, *Xa21* has probably been the most commonly used for rice variety improvement as it provides high level and broad-spectrum BB resistance (Nguyen et al., 2018; Singh et al., 2001; Zhang et al., 2006). *Xa21* codes for a plasma membrane receptor which recognizes the tyrosine-sulfated protein RaxX and triggers the Xa21-mediated immunity (Pruitt et al., 2015). It is known that this response involves Xa21-binding proteins (Chen et al., 2010; Park et al., 2010; Wang et al., 2007) as well as the direct interaction of a cleaved Xa21 subunit with the WRKY62 transcription factor (Park and Ronald, 2012; Peng et al., 2008), nonetheless, the precise mechanisms of the resistance are still not yet completely elucidated.

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Studying the variations in the expression of candidate genes provides perspectives of the mechanisms of plant responses to BB. The quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) remains a tool of choice to quantify mRNA expression and thus confirm the involvement of various candidate genes in a specific cellular response due to the sensitivity, precision and reproducibility of its results (Derveaux et al., 2010; Hellemans et al., 2007). However, the veracity and reliability of qRT-PCR results are essentially altered by the variations between samples, RNA quality and quantity, and the different reverse transcription and PCR efficiencies (Bustin et al., 2009; Derveaux et al., 2010). To cope with this variability, expression levels of the genes of interest (GOIs) are usually normalized against a stably and uniformly expressed reference genes according to the $\Delta\Delta CT$ method in order to provide reliable relative expression data (Rao et al., 2013). Though, in this method, the selection of reference genes is critical since variations in their expression can completely overturn the final data especially when the variations in GOI expression are restrained. The expression of the reference genes used in qRT-PCR analysis should remain stable across all experimental samples and under different conditions (Derveaux et al., 2010; Huggett et al., 2005). Yet, previous reports on the stability of the usual reference genes has shown that no universally stable reference gene could be found, thus implicating that reference genes' stability in qRT-PCR experiments needs to be validated for specific and suitable experimental conditions and purposes (Kozera and Rapacz, 2013; Laurent et al., 2008).

Several software and programs have been established to evaluate the stability of the reference genes expression. Among the most commonly used are the geNorm (Vandesompele et al., 2002a), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) algorithms, and the Delta-Ct method (Silver et al., 2006) and RefFinder system (Xie et al., 2012). Using these tools, various studies on rice have highlighted that proper validation of reference genes is essential to determine their stability and thus recommending the suitability of each reference gene for various experiments in rice. For example, *Ubiquitin 5* (*UBQ5*), *Ubiquitin-conjugating enzyme E2* (*UBC-E2*), *Endothelial differentiation factor* (*Edf*) and *eukaryotic Elongation Factor-1α* (*EF-1α*) were found to be suitable for growth and development, environmental conditions, and organ-specific gene expression analyses (Auler et al., 2017; Narsai et al., 2010; Wang et al., 2016). For hormonal and abiotic treatments and stresses, *18S ribosomal RNA* (*18S rRNA*), *25S ribosomal RNA* (*25S rRNA*), *Ubiquitin 10* (*UBQ10*) and *Ubiquitin conjugating enzyme* (*UBC*) were the most stable reference genes (Almas and Kamrodi, 2018; Jain et al., 2006; Kim et al., 2003; Moraes de Freitas et al., 2015) while for a few biotic stress like blast infection *18S rRNA, Actin, and 40S 27a ribosomal subunit (40S 27a)* were found to be the most suitable reference genes (Bevitori et al., 2014; Che Omar et al., 2016). To our knowledge, no resources are currently available on the suitable reference genes for gene expression analysis involving *Xoo* inoculation experiments. Hence, in this paper, we analyzed 11 candidate reference genes in terms of their expression stability in both BB-susceptible rice cultivar RD47 and its improved BB-resistant progenies BC_3F_3 (*Xa21/Xa21*) (Sagun, Suachaowna et al., unpublished) at different times post *Xoo* inoculation.

2. Materials and methods

2.1. Plant material and growth conditions

Rice (*Oryza sativa* L. ssp. *indica*) cultivars RD47 and IRBB21 were provided by the Bureau of Rice Research and Development, Phitsanulok, Thailand. The *Xa21* gene originated from the wild species *O. longistaminata* was transferred through wide hybridization in IR24, resulting in the near-isogenic line, IRBB21. In tests for disease resistance, IRBB21 has been reported to be resistant to many *Xoo* strains from the Philippines and India (Khush et al., 1990). Rice cultivar RD47, an elite Thai cultivar, was derived from three-line cross between Suphanburi 1 and IR64 then with CNT86074–25–9-1 at Chainat Rice Research Center, Thailand (the Rice Department, Ministry of Agriculture and Cooperatives, Thailand).

The *Xa21* gene from IRBB21 was introgressed in RD47 through backcross breeding and Marker Assisted Selection (data not shown) until homozygous-*Xa21* BC_3F_3 lines were obtained.

2.2. Xoo isolation and inoculation test

Since Thailand biosafety regulations limit the import of living microorganisms especially those causing diseases in major commodities like rice, the authors made use of the local strains of *Xoo* in this study and validated them through PCR using specific universal primers.

BB infected leaves were collected from paddy fields in Phitsanulok province and *Xoo* was isolated on nutrient agar (peptone-bovine-agar). The isolated bacteria, xoo16PK002, was identified as *Xoo* through PCR assays using *Xoo* specific primers TXT (Sakthivel et al., 2001) and *Xoo* specific primers Xoo80 (Lu et al., 2014). Furthermore, preliminary pathogenicity tests on 60 days old rice plants were done and had shown that RD47 plants were rather susceptible to highly susceptible to xoo16PK002 with clear BB lesions lengths (LL) ranging 25-29 cm ± 1.28 at 21 days after inoculation, whereas RD47's near isogenic lines BC_3F_3 and the IRBB21 cultivar were more resistant to the *Xoo* strain with LL ranging from 5 to 9 cm ± 0.26 (Sagun, Suochawna, Puttasem et al., unpublished). For the infection experiments presented here, the *Xoo* isolate xoo16PK002 was re-streaked and incubated at 28 °C for 48 h. A *Xoo* inoculum (OD600 of 0.2) was prepared and used to inoculate 60-day-old plants according to the clipping method (Kauffman, 1973). Mock (water) inoculation was used as a control. Samples corresponding to 5 cm of the leaves directly below the inoculation sites were collected at 0, 2, and 24 h post inoculation, respectively, and leaf samples were frozen in liquid nitrogen immediately.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from each 100 mg leaf sample using RNAprep Pure Kit (Tiangen Biotech LTD., China) following manufacturer's instructions. Total RNAs were quantified using Synergy H1 microplate reader (Biotek, USA) and their integrity was assessed through agarose gel electrophoresis. The qScript™ XLT cDNA synthesis kit (QuantaBio, USA) was used to reverse transcribe 1 μg of total RNA templates in order to synthesize first strand cDNAs according to manufacturer's protocol.

2.4. Quantitative Real-time PCR

For qPCR analyses, the fast SYBR Green Master Mix (Quantabio, USA) was used to prepare 20 μl qRT-PCR reactions containing 1 μl of the cDNA templates and 0.5 μM of each primer pairs. Technical triplicates and No Template Controls (NTCs) were run through the Eco48 Real-Time PCR system (PCRmax, UK) for 35 cycles (95 °C for 10s, 60 °C for 10s and 72 °C for 20s) followed by a melting curve analysis. Ct values were determined using the Eco™ 48 Study Software installed in the Eco48 Real-Time PCR system. The Eco™ 48 Study software allows for normalized data combination from multiple Eco 48 experiments into a single analysis, and supports standard curve analysis and relative quantification experiments, which were used in this study. The comparative Ct method was used to transform generated Ct values in relative quantities with the highest relative quantity for each gene set up to 1.0. All data were subjected to analysis using geNorm v3.4 (Vandesompele et al., 2002a), NormFinder v20 (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), Delta-Ct method (Silver et al., 2006) and RefFinder (Xie et al., 2012) programs.

Table 1
List of candidate reference genes validated in this study.

Candidate reference gene	Primer sequence	Reference
Endothelial differentiation factor(Edf)	5′-TCCGAACCAGCAGATCATCG-3′	Wang et al., 2016
LOC_Os08g27850	5′-GCATGGTATCAAAAGACCCAGC-3′	
Triosephosphate Isomerase (TI)	5′-CGACATCATCAACTCCGCCAC-3′	Wang et al., 2016
LOC_Os01g05490	5′-CCTCTTCAGACATCTTCCCACG-3′	
eukaryotic Initiation Factor-4a (eIF-4a)	5′-TTGTGCTGGATGAAGCTGATG-3′	Wang et al., 2016
LOC_Os03g08020	5′-GGAAGGAGCTGGAAGATATCATAGA-3′	
Profilin-2 (Prof2)	5′-CCAACTGGTCTTTTCCTTGGG-3′	Wang et al., 2016
LOC_Os06g05880	5′-GGGGTCATCGGCTCATCATAG-3′	
ADP-ribosylation factor (ARF)	5′-ATGAAAGGAAGACATGGCGG-3′	Wang et al., 2016
LOC_Os05g41060	5′-TGGTGGTGGAAGCTAAAGAGC-3′	
Nucleic acid binding protein (NABP)	5′-GGAATGTGGACGGTGACACT-3′	Narsai et al., 2010
LOC_Os03g25980.1	5′-TCAAAATAGAGTCCAGTAGATTTGTCA-3′	
eukaryotic Elongation Factor-1a (eEF-1α)	5′-TTTCACTCTTGGTGTGAAGCAGAT-3′	Jain et al., 2006
LOC_Os03g08020	5′-GACTTCCTTCACGATTTCATCGTAA-3′	
Ubiquitin 10 (UBQ10)	5′-TGGTCAGTAATCAGCCAGTTTGG-3′	Jain et al., 2006
LOC_Os02g06640	5′-GCACGACAAATACTTGAGGAAGAG-3′	
Actin-1 (ACT1)	5′-CTTCATAGGAATGGAAGCTGCGGGTA-3′	Narsai et al., 2010
LOC_Os05g36290.1	5′-CGACGACCTTGATCTTCATGCTGCTA-3′	
18S ribosomal RNA (18SrRNA)	5′-CTACGTCCCTGCCCTTTGTACA-3′	Jain et al., 2006
Locus ID: AK059783	5′-ACACTTCACCGGACCATTCAA-3′	
Ubiquitin-5 (UBQ5)	5′-CCAGTACCTCAGCCATGG A-3′	Jain et al., 2006
Locus ID: AK061988	5′-GGACAGAATGATTAGGGATC-3′	

Gene names and all their details are presented the way they are reflected in the reference cited.

3. Results

3.1. qRT-PCR of candidate reference genes

A set of 11 candidate reference genes and their specific primers was selected from previous studies on reference gene validation in rice (Table 1). The expression levels of these genes were measured by qRT-PCR in 8 different samples corresponding to the leaves from the BB susceptible RD47 (no *Xa21*) and its BB resistant progeny BC_3F_3 (homozygous *Xa21*) collected at 0, 2 and 24 h after *Xoo* inoculation and 2 h after mock inoculation. For all the tested candidate genes, NTCs showed no amplification, and the sample melting curve analysis generated single peaks indicating that a specific PCR product for each gene was amplified. Moreover, electrophoresis of qRT-PCR products showed a single band of the expected size for each candidate gene (Fig. 1). After confirming the specificity of the qRT-PCR primers, the Ct values were determined for each technical triplicate in all samples. The mean Ct values (Supplemental Table 1) were then transformed into relative quantity values, which was later used in the geNorm and NormFinder programs by fixing the highest relative quantity for each candidate gene to 1 and using the comparative Ct method. For Bestkeeeper, Delta-Ct and RefFinder analyses, the raw Ct values were used.

3.2. Stability of candidate reference genes using geNorm analysis

The geNorm software (Vandesompele et al., 2002a) uses the principle that the expression ratio of perfect reference genes should remain constant across different experimental treatments. It determines the gene expression stability measure (M) of reference genes as well as the average pairwise variation for that gene as compared to other tested reference genes. Stepwise exclusion of the gene with the highest M value allows to select the two most stable genes. The two most stable genes are determined by sequentially removing the least stable gene with the highest M value (Vandesompele et al., 2002a). Initially, analysis of the samples from the BB susceptible RD47 (Fig. 2a) and the BB resistant BC_3F_3 plants (Fig. 2b) was undertaken separately. For the RD47 samples, *Edf* and *eIF-4a* had the lowest M values, hence, they were the most stable reference genes, and followed by *TI*. Meanwhile, *ARF*, and *18S rRNA* yielded the highest M values and were the least stable genes (Fig. 2a). In the BC_3F_3 samples, *Edf* and *TI* were the most stable genes while *eIF-4a* was ranked fourth. Meanwhile, *ARF*, *UBQ5*, and *18S rRNA* were among the three least stable reference genes (Fig. 2b). Finally, when all the samples were analyzed together for the expression stability of the candidate genes (Fig. 2c), *Edf* and *TI* were the most stable genes, followed by *eIF-4a* while *ARF*, *18S rRNA*, and *UBQ5* were still among the least stable genes.

3.3. Determination of the optimal number of reference genes by geNorm

Although most published studies on gene expression suggest a single internal control for qRT-PCR normalization, it is also known that increasing the number of reference genes results in more reliable and more accurate data (Jain et al., 2018; Vandesompele et al., 2002b; Zhao et al., 2016). However, there is a trade-off between accuracy and practical considerations when selecting reference genes to be used. Pairwise variation (V) analysis calculates between two sequential normalization factors containing an increasing number of reference genes; geNorm also provides the tool in generating the optimal reference genes to be used. The program suggests that if the value of V is below the 0.15 cut-off value, the last added reference gene may not need to be included for the data normalization. In this study, the V2/3 value of 0.136 indicates that the third most stable reference gene, which is *eIF-4a*, is not required and thus, the use of the two most stable reference genes, *Edf* and *TI*, is already optimal for accurate normalization (Fig. 3).

3.4. Stability of candidate reference genes using NormFinder analysis

To validate the results of the geNorm analysis, we also assessed the expression stability of the candidate genes in our samples with the NormFinder software. NormFinder directly calculates for each gene a stability value based on its inter- and intragroup variations of expression which can prevent the selection of co-regulated genes (Andersen et al., 2004). As the NormFinder software is limited to only 10 genes for analysis, the top 10-ranked stable genes from the geNorm analysis were used for the calculation. Results of the analysis showed that *Edf* and *TI*, with the lowest individual stability values of 0.036 and 0.059, respectively, were again selected as the most stable genes with a combined stability value of 0.029. The reference gene *Edf*, in particular, was deemed to be the best reference gene as its variation values for both intra- and inter- groups were lowest while *TI* still showed more variation than *eIF-4a* in the RD47 group (Table 2). The *UBQ5* and *18S rRNA* genes, respectively, were ranked 8th and 10th among the 10 tested

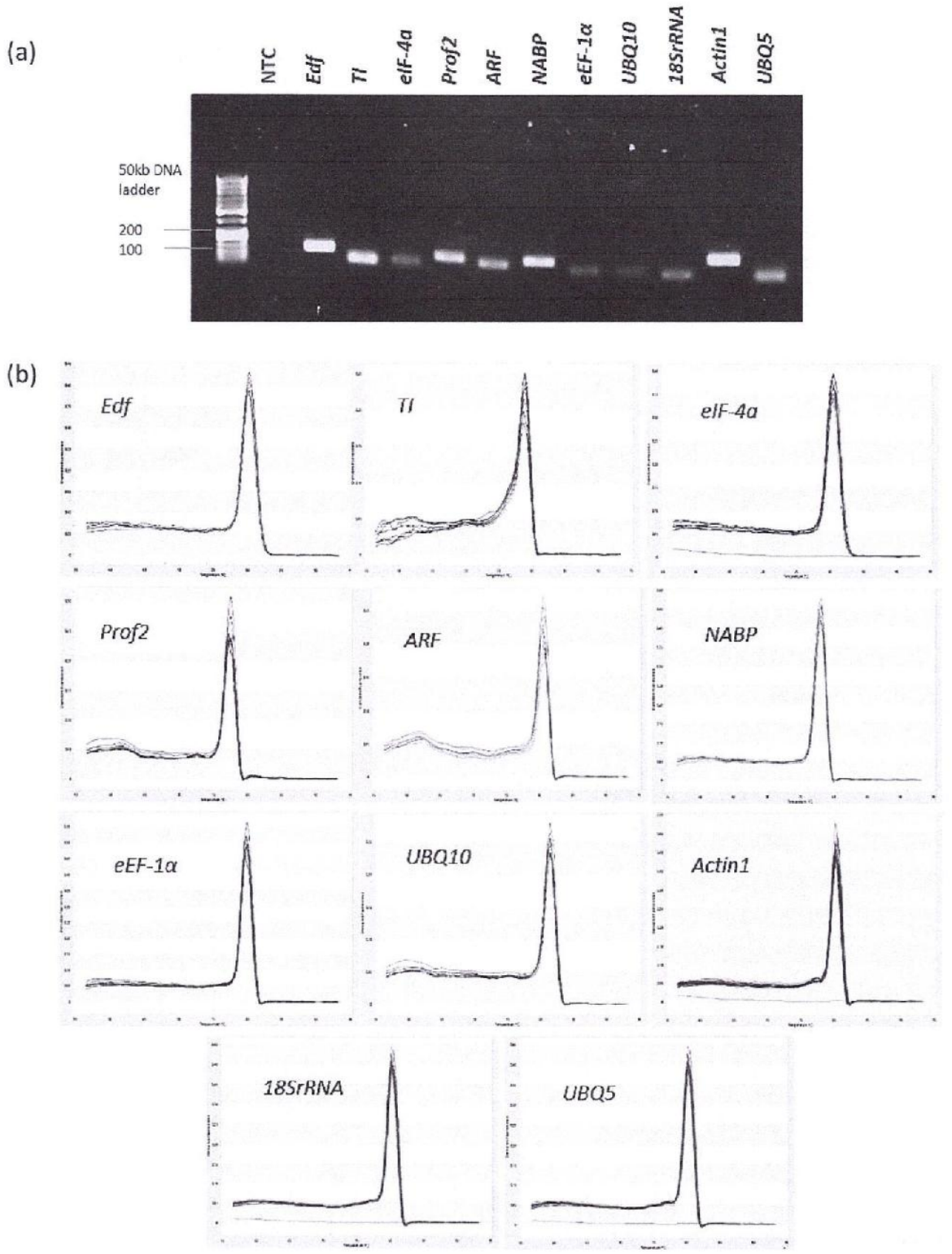

Fig. 1. Specificity of gene amplification products tested on the 6-leaf stage rice cultivar RD47 at 0 h after *Xoo* inoculation. (a) Gel migration of qRT-PCR products. A single DNA amplicon of the projected size is shown for each gene tested in this study. Agarose gel (2%). NTC-Non-Template Control; and (b) Dissociation curves of qRT-PCR products for all candidate reference genes validated in this study. Analysis of dissociation curves, also called melt curves, was used to assess whether the candidate reference genes produced single, specific products. The single peaks of dissociation curves in each candidate reference gene represented a pure, single

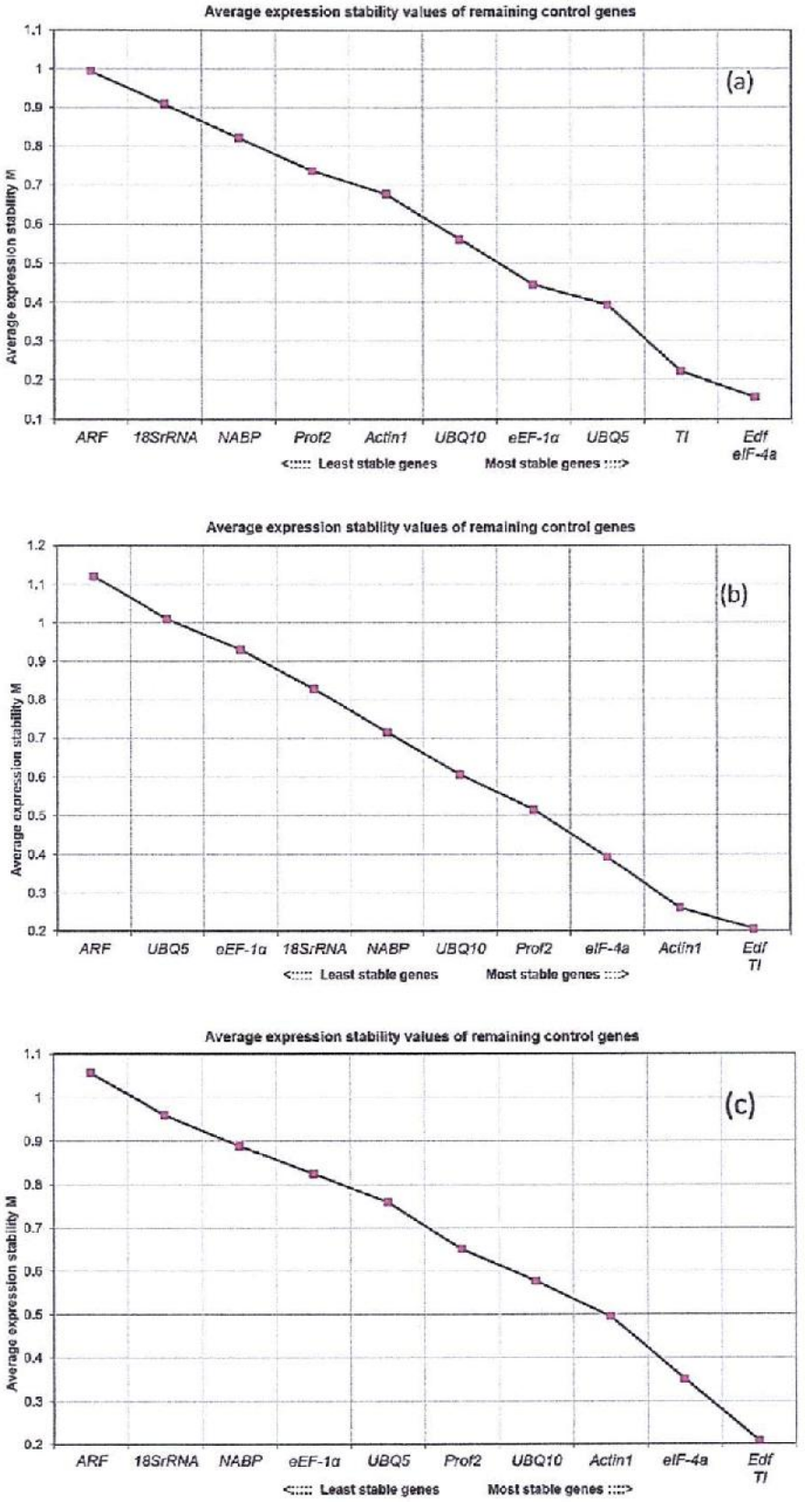

Fig. 2. Expression stability and ranking of reference genes by geNorm: (a) RD47; (b) BC_3F_3 (*Xa21-Xa21*) progeny; and (c) all samples. Gene(s) with lower average expression stability M denotes more stable expression.

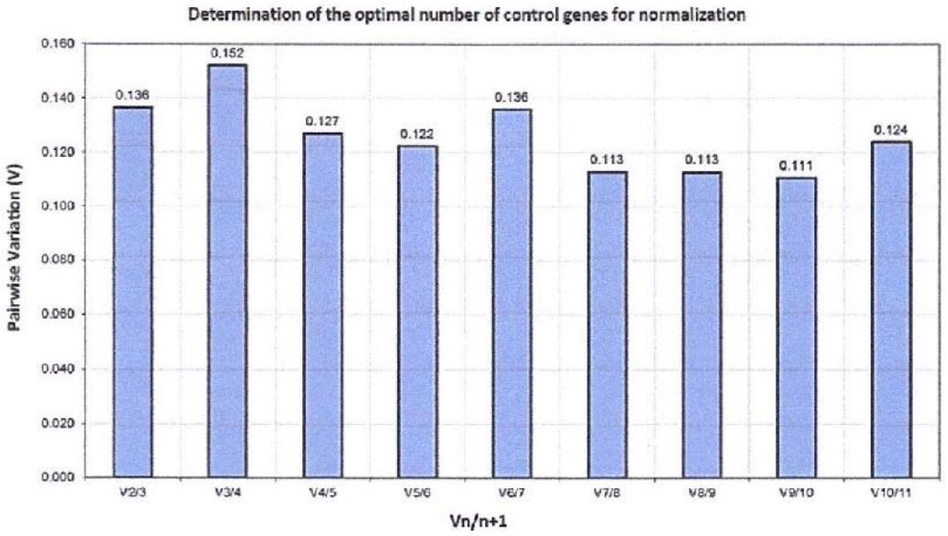

Fig. 3. Determination of the optimal number of control genes for accurate normalization by geNorm pairwise variation analysis. Pairwise variation analysis (Vn/n + 1) between the normalization factors NFn, including the n most stable reference genes, and NFn + 1, corresponding to the stepwise inclusion of the next most stable reference gene, were calculated. A large variation means the last added reference gene has a significant effect and should be included while Vn/n + 1 with a value below the 0.15 cutoff indicates n as the optimal number of reference genes.

Table 2
NormFinder analysis of top 10-ranked stable reference genes from the geNorm analysis. Gene(s) with lower stability value denotes more stable expression.

Candidate reference gene	Stability value	Intra-group variation		Inter-group variation
		RD47	BC_3F_3	
Edf	0.031	0.003	0.005	0.022
TI	0.048	0.015	0.005	0.032
eIF-4a	0.072	0.003	0.054	0.169
ACT1	0.184	0.319	0.029	0.061
UBQ10	0.208	0.135	0.217	0.037
Prof2	0.289	0.386	0.286	0.130
eEF-1α	0.322	0.280	0.575	0.297
UBQ5	0.326	0.242	0.659	0.178
NABP	0.339	0.396	0.530	0.033
18S rRNA	0.390	0.625	0.595	0.178
Best gene				*Edf*
Stability Value for Best Gene				0.031
Best combination of two genes				*Edf and TI*
Stability value for best combination of two genes				0.029

candidate reference genes indicating very low expression stability.

3.5. Stability of candidate reference genes using BestKeeper analysis

The BestKeeper software determines the best suited standards of reference genes and combines them into an index. The index is used to decide whether reference genes are differentially expressed under an applied treatment. All data processing for this software is based on crossing points and determines the optimal housekeeping genes employing the pair-wise correlation analysis of all pairs of candidate genes and calculates the geometric mean of the 'best' suited ones (Pfaffl et al., 2004). In this study, though *18S rRNA* and *ARF* were among the least stable reference genes selected by geNorm and NormFinder, the BestKeeper software identified and placed these two genes in rank 1 and 2 as the most stable genes since they have the lowest variation compared to the other genes as determined by their standard deviation (SD) of the crossing point values (CV) at 0.58 and 0.59, respectively. On the other hand, *Prof2* and *eEF1α* were among the least stable reference genes with SD values of 1.39 and 1.78, respectively (Table 3). For BestKeeper analysis, any SD values higher than 1 is considered unstable.

Table 3
Crossing point data of candidate reference genes by BestKeeper. Results of data analysis were taken from raw Ct values of RD47 and BC_3F_3 rice samples under 0, 2 and 24 h post inoculation, respectively, including mock inoculation.

	18S	*ARF*	*UBQ10*	*NABP*	*TI*	*Edf*	*UBQ5*	*ACT1*	*eEF4α*	*Prof2*	*eIF1α*
N	24	24	24	24	24	24	8	24	24	24	24
Geo mean [CP]	9.19	17.35	18.66	27.59	20.14	20.4	22.35	23	23.64	22.7	23.03
AR mean [CP]	9.21	17.37	18.69	27.62	20.18	20.4	22.39	23.1	23.69	22.8	23.12
Min [CP]	8.33	15.91	17.21	26.05	18.16	18.2	20.28	20.2	21.21	19.7	20.1
Max [CP]	10.51	18.24	20.58	29.59	21.92	22.2	25.33	25	26.12	25.1	26.63
Std dev [+/− CP]	0.58	0.59	0.89	0.97	1.09	1.12	1.22	1.26	1.28	1.39	1.78
CV [% CP]	6.3	3.39	4.77	3.49	5.43	5.47	5.44	5.46	5.41	6.09	7.7
Min [x-fold]	−1.81	−2.72	−2.74	−2.92	−3.95	−4.56	−4.18	−7.13	−5.42	−8.25	−7.6
Max [x-fold]	2.5	1.85	3.79	3.99	3.43	3.52	7.89	4.01	5.57	5.13	12.16
Std dev [+/− x-fold]	1.5	1.5	1.85	1.95	2.14	2.17	2.33	2.4	2.43	2.62	3.43

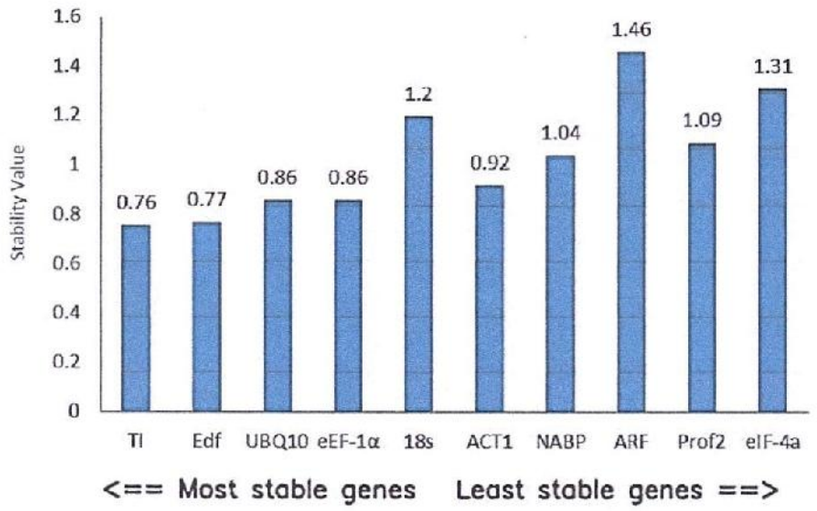

Fig. 4. Expression stability and ranking of candidate reference genes as determined by Delta-Ct method. Low gene stability values denote more stable expression.

3.6. Stability of candidate reference genes using the Delta-Ct (ΔCt) method

The ΔCt method compares relative expression of gene pairs within each sample to identify useful housekeeping genes. If the calculated value between the two tested genes remains constant in different RNA samples, it means that the expression of either both genes are stable among those samples, and if the ΔCt changes, then either one or both genes are inconsistently expressed (Silver et al., 2006). In our results, *TI* (0.77) and *Edf* (0.772) were identified as the most stable reference genes, the same genes identified by geNorm and NormFinder, while *ARF* (1.272) and *eEF1α* (1.487) were among the least stable genes (Fig. 4).

3.7. Stability of candidate reference genes using RefFinder analysis

To come up with a more comprehensive report on the expression stability of reference genes relative to our experimental conditions, we subjected our data series for RefFinder analysis to pull out a general recommendation. RefFinder is a web-based comprehensive tool developed to evaluate and screen reference genes from a widespread experimental dataset. It integrates the currently available major computational programs such as geNorm, Normfinder, BestKeeper, and the comparative Delta-Ct method. The system compares and ranks the tested candidate reference genes based on assigned appropriate weight of each individual gene and calculated geometric mean of their weights for the overall final ranking.

Results of the analysis showed that *TI* and *Edf* were among the most stably reference genes since they have the lowest stability values at 1.778 and 1.861, respectively. These two genes were consistent in all validation analyses except for BestKeeper. The *18S rRNA* (5.335) and *ARF* (7.183) which were the most stable genes determined by BestKeeper were ranked 6th and 8th, respectively. *Prof2, UBQ5,* and eEF1α were identified as the least stable reference genes with gene stability values of 7.364, 7.483, and 9.685, respectively (Table 4).

Table 4
Ranking of candidate reference gene expression stability by RefFinder.

Candidate reference gene	geNorm	NormFinder	BestKeeper	Delta Ct	RefFinder	Rank
TI	0.208	0.048	1.09	0.76	1.5	1
Edf	0.208	0.031	1.12	0.77	2.21	2
UBQ10	0.576	0.208	0.89	0.86	3.66	3
eEF-1α	0.351	0.322	1.28	0.86	4.12	4
18S rRNA	0.962	0.39	0.58	1.2	4.9	5
ACT1	0.494	0.184	1.26	0.92	5.14	6
NABP	0.891	0.339	0.97	1.04	5.63	7
ARF	1.057	N/A*	0.59	1.46	6.69	8
Prof2	0.65	0.289	1.39	1.09	7.17	9
UBQ5	0.76	0.326	1.22	1.2	7.48	10
eIF-4a	0.827	0.072	1.78	1.31	8.97	11

* N/A - not applicable.

4. Discussion

Earlier studies reported that the expression stability of various reference genes in rice vary under different experimental conditions and emphasized the need for a proper validation of the stability of reference genes for any gene expression analysis to come up with accurate and reliable results. The geNorm method was the first released algorithm in evaluating the stability of the expression of candidate reference genes and has established itself as the golden standard with more than 10,000 citations to date. However, as the expression stability value M for a gene generated from geNorm is dependent upon the other tested genes, concern has been raised about the possible selection of co-regulated genes instead of the stable ones. In this case, the NormFinder software (Andersen et al., 2004) uses a different approach which can cope with this problem. Thus, it is not rare that both of these methods alongside with BestKeeper, Delta-Ct method and RefFinder programs are usually used together to determine the best reference genes for a specific gene expression assay (Auler et al., 2017; Bevitori et al., 2014; Wang et al., 2016). In several studies, the genes selected by these algorithms are often slightly different even in most cases, the most stable reference genes selected by one program still belongs to the relatively stable genes category in the other analysis. In our study, *Edf* and *TI* were found to be the best reference genes across all approaches except for Bestkeeper thereby suggesting that their expressions are stable under *Xoo* inoculation.

Traditional reference genes like the *Actin1* (*ACT1*), *eEF-1α, β-tubulin, UBQ10, UBC-E2, UBQ5, 18S rRNA,* and *25S rRNA* were commonly used as internal controls in various experiments in rice focusing on growth and development stages, different tissue samples, and various treatments due to their recognized stability. Among these reference genes, *UBC* and *UBQ10* were found to be the most stable in rice plant responses to heavy metal stress (Almas and Kamrodi, 2018); *UBC* was the

most stable in rice treated with different nitrogen levels (Benemann et al., 2017); *18S rRNA* was the most suitable reference gene under various growth stages of etiolated seedlings, different cultivars, and various times after UV-irradiation treatment compared to *glyceraldehyde-3-phosphate dehydrogenase, actin,* and *tubulin* (Kim et al., 2003). However, other studies have also stressed that the transcription levels of these traditional reference genes may change depending on the plant developmental processes, environmental conditions and treatment sets, and expression differences of the genes of interest could be attributed to the expression variation of inappropriate reference genes (Gutierrez et al., 2008a; Gutierrez et al., 2008b; Wang et al., 2016). In the report of Jain et al. (2006), *UBQ5* and *eEF-1α* were found to be the most stable whereas *18S rRNA* and *UBQ10* were among the least stable in these commonly used reference genes when analyzed across all their samples and in developmental series. Li et al. (2009) also analyzed the stability of these commonly used reference genes during rice seed development and found out that *eIF-4a* and *ACT1* were the most suitable reference genes while again *18S and 25S rRNAs* were among the least stable in almost all the tested samples from two rice varieties at different developmental stages, and a total of 6 reference genes was optimal for qPCR calibration using most of their tissue groups.

As no holistic stable reference gene could be found among the traditionally used housekeeping genes, significant efforts have been created to find novel and more stable reference genes (Jain, 2009; Narsai et al., 2010). With the increasing number of analyses of large sets of microarray data, *Edf* and *TI* were selected as potential reference genes for rice gene expression analysis. In a recent article, the expression stabilities of *Edf, TI,* and other novel reference genes in rice were compared to those of the more traditional ones like *UBQ5* for 22 different experimental conditions (Wang et al., 2016). The results of these experiments showed that novel reference genes were globally more stable and *Edf* and *TI* were often among the most stable genes. Our study revealed similar results for the *Xoo* inoculation condition as *Edf* and *TI* were also found to be the best reference genes while the traditional housekeeping genes *18S rRNA* and *UBQ5* including *ARF* and *eEF1-α* were among the least stable genes. Though *18S rRNA* has probably been the most frequently used reference genes for gene expression analyses, increasing number of reports that it can have very low expression stability under blast infection and drought tolerance (Bevitori et al., 2014) and in different developmental and environmental conditions (Jain et al., 2006; Li et al., 2009). The present study for two different rice cultivars under *Xoo* inoculation also showed that *18S rRNA* was the least stable and therefore, should only be used as an internal control with the highest caution. Moreover, in the report of Wang et al. (2016), the stability of *Edf* and *TI* were found under hormone treatments including salicylic acid (SA), which plays an important signaling role in the activation of various plant defense responses following pathogen attacks as highlighted in the report of Dempsey et al. (2010). And since the expression of *Edf* and *TI* were found to be stable under SA treatment, these findings indicate why the expression of these two genes under *Xoo* inoculation were also stable as presented in this study.

5. Conclusion

In our study, *Edf* and *TI* were found to be the most stably expressed among all reference genes validated, thus should be suitable internal controls for the normalization of gene expression analysis in rice inoculated with *Xoo*. While the use of either of the two genes as internal control is adequate for gene expression analysis, using both genes as presented in this study, is suggested to produce more accurate and reliable results. Besides, due to the variability of the TAL effectors between *Xoo* strains, confirming the stability of these reference genes when using a new *Xoo* strain would also be advisable.

Declaration of Competing Interest

All authors declare that they have no conflict of interest and all ideas reflected in this manuscript have been agreed upon.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plgene.2019.100217.

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RESEARCH ARTICLE
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Wound healing properties of *Gliricidia sepium* leaves from Indonesia and the Philippines in rats (*Rattus norvegicus*)

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Abstract

Background and Aim: *Gliricidia sepium* is a medium-sized leguminous plant found widely in tropical to subtropical areas. It has been used as a medicinal ingredient and in rodenticides by local communities in both Indonesia and the Philippines. This study aimed to investigate the wound healing effects of an ointment containing *G. sepium* leaves on inflammatory cells using a rat model. We also determined its effect on the expression of interleukin (IL) 6 and IL-1β.

Materials and Methods: We used 16 Wistar male rats aged approximately 2 months and weighing 150-200 g. They were divided into four treatment groups (T1, positive control; T2, negative control; T3, wounds treated with *G. sepium* from Indonesia; and T4, wounds treated with *G. sepium* from the Philippines), and the ointment therapies were applied to wounds for 3 days. Hematoxylin and eosin staining was performed to examine the inflammatory cells microscopically. IL-1β and IL-6 expression were observed immunohistochemically.

Results: *G. sepium* leaves significantly ($p<0.05$) decreased the number of inflammatory cells, and the expression of IL-1β and IL-6 in the group treated with Indonesian *G. sepium* leaves was higher than that in the group treated with *G. sepium* leaves from the Philippines. The leaves contain flavonoids, saponins, and tannins, which act as anti-inflammatory agents to enhance the wound healing process.

Conclusion: Our findings suggest that *G. sepium* leaves from both the Philippines and Indonesia possess wound healing properties.

Keywords: flavonoid, *Gliricidia sepium* leaves, herbal plants, saponin, tannin, wound healing.

Introduction

Injuries occur because of pathological processes caused by internal or external factors that affect specific organs and result in structural and functional damage. Causes of injuries include sharp objects, blunt objects, changes in temperature, chemicals, electric shock, and animal bites [1]. Incision wounds are injuries that occur from cutting with a sharp instrument, such as a scalpel, during surgery. Clean and aseptic wounds are usually closed by sutures [2]. Wound healing occurs when damaged tissue is replaced by new tissue through the processes of regeneration and repair and is divided into four phases: Hemostasis, inflammation, proliferation, and remodeling [3]. Wound healing is a complex process that involves both local and systemic cellular and biochemical responses. The process of tissue repair occurs in the inflammatory phase, where the amount of released inflammatory mediators, such as interleukin (IL) 1β, IL-6, transforming growth factor-β, and tumor necrosis factor-α, is increased [4]. These cytokines act as pro-inflammatory factors that are produced in response to tissue damage, macrophage migration, and the production of other pro-inflammatory cytokines [5].

Gliricidia sepium is a leguminous plant that grows quickly in dry areas of Indonesia and the Philippines and is found widely in tropical to subtropical areas [6]. *G. sepium* is known as "gamal" in Indonesia and "kakawate" in the Philippines [6]. There have been some studies of its active substances, including flavonoids, saponins, tannins, alkaloids, polyphenols, hydroxyl acid, and coumarin [7]. Some studies have reported that *G. sepium* leaves possess anti-inflammatory properties, particularly their flavonoids, which can reduce pain and bleeding, while others have proved their antibacterial and antioxidant properties [8].

We performed this study to further determine the efficacy of *G. sepium* leaves as a wound healing agent based on the evidence of decreased inflammatory cells as well as decreased expression of IL-β and IL-6.

Materials and Methods

Ethical approval

The use of animal models in this study was approved by the Brawijaya University Research Ethics Committee (No. 1004-KEP-UB).

Study period and location

The study was conducted from May to October 2020 at the Animal Disease and Diagnostic Laboratory, Faculty of Veterinary Medicine, Brawijaya University, Malang, Indonesia.

Animal preparation

We used male Wistar rats (*Rattus norvegicus*) aged approximately 2 months and weighing 150-200 g in our study. The study design was completely randomized, and the rats were divided into four treatment groups comprising four rats per group as follows: T1, positive control, treated with a commercial wound healing agent; T2, negative control; T3, wounds treated with *G. sepium* from Indonesia; and T4, wounds treated with *G. sepium* from the Philippines. The rats were anesthetized with an intramuscular injection of ketamine (10 mg/kg body weight). The back of the rats were shaved and disinfected with 70% alcohol. A 2 cm incision was made subcutaneously in the median portion of the dorsal vertebrae using a scalpel blade. The wound was sewn using silk thread (½ 35 mm) in a simple continuous pattern. Then, the rats were returned to individual cages based on their treatment group.

G. sepium ointment preparation and injury treatment

G. sepium leaves from Indonesia and the Philippines were collected and then identified in the Plant Taxonomy Laboratory of the Biology Department at Brawijaya University. The leaves from the Philippines were transported after obtaining an appropriate permit. All leaves were dried, ground into a powder, and made into an ointment by adding a hydrocarbon-based Vaseline ointment base. The ointment was applied to the wounds for 3 days in the designated treatment groups.

Histopathology preparation and inflammatory cell count

After each group had received the appropriate treatment for 3 days, the rats were sacrificed, and the skin tissue was retrieved. The histopathological examination was conducted based on the previous methods [9], and the inflammatory cells were observed and counted microscopically following staining with hematoxylin and eosin.

Measurement of IL-1β and IL-6 expression

An immunohistochemistry technique was performed to analyze IL-1β and IL-6 expression based on the previous methods [9]. We used an ImmunoRatio software (available online: http://imtmicroscope.uta.fi/immunoratio/) to observe and analyze the expression of IL-1β and IL-6 by calculating the percentage of the affected area.

Statistical analysis

Statistical analyses were conducted using SPSS software version 14.0 (IBM, USA). The data were analyzed with one-way analysis of variance (ANOVA) and a Tukey test with α=0.05 to determine differences between the treatment groups.

Results

Effect of ointment containing *G. sepium* leaves on inflammatory cells

The macroscopic observation of wound healing in rats differed among the treatment groups. In the positive control group, the wound had not closed and appeared to be in the inflammatory phase, which generally occurs in 2-4 days. In the groups that received ointment containing *G. sepium* leaves from either Indonesia or the Philippines, the incision wound began to close or was completely closed on day 3 (Table-1).

Effect of ointment containing *G. sepium* leaves on IL-1β expression

The expression of IL-1β in the positive control (T1) group was 41.28±9, and this level was used as an indicator of IL-1β expression in normal rats (Table-2).

Table-1: The number of inflammatory cells in wounds treated with *Gliricidia sepium.*

Groups*	Inflammatory cells expression	Decreasing of inflammatory cells expression (%)
T1 (+)	6.80±3.89[a]	-
T2 (−)	76.32±36.81[b]	69.52
T3 (Indon)	15.40±7.92[a]	79.82
T4 (Phil)	10.20±8.34[a]	86.63

*(T1) positive control, (T2) negative control, (T3) wounds treated with *Gliricidia sepium* from Indonesia, and (T4) wounds treated with *Gliricidia sepium* from the Philippines

Table-2: The expression of IL-1β.

Groups*	IL-1β expression	Declining of IL-1β (%)
T1 (+)	41.28±9.28%[a]	-
T2 (−)	75.54±11.19%[c]	-
T3 (Indon)	48.68±8.20%[b]	35.55%
T4 (Phil)	28.10±7.35%[a]	62.80%

*(T1) positive control, (T2) negative control, (T3) wounds treated with *Gliricidia sepium* from Indonesia, and (T4) wounds treated with *Gliricidia sepium* from the Philippines. IL=Interleukin

Table-3: The expression of IL-6.

Groups*	IL-6 expression	Declining of IL-1β (%)
T1	24.16±2.12[a]	-
T2	96.86±1.04[d]	-
T3	70.36±1.35[c]	27.35
T4	60.52±2.27[b]	37.58

*(T1) positive control, (T2) negative control, (T3) wounds treated with *Gliricidia sepium* from Indonesia, and (T4) wounds treated with *Gliricidia sepium* from the Philippines. IL=Interleukin

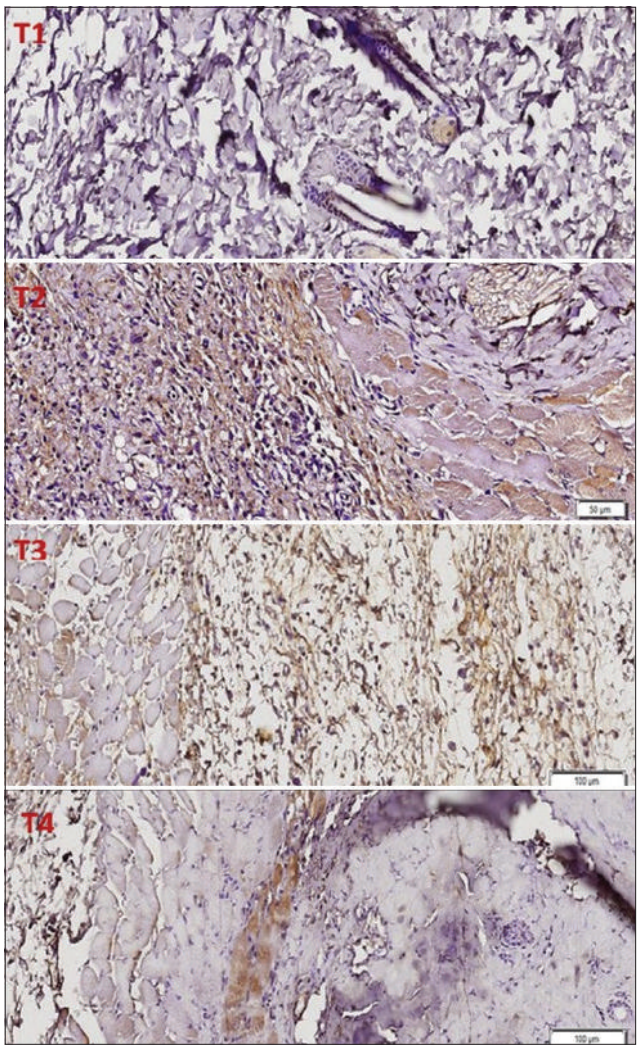

Figure-1: The expression of interleukin-1β on treatment rats.

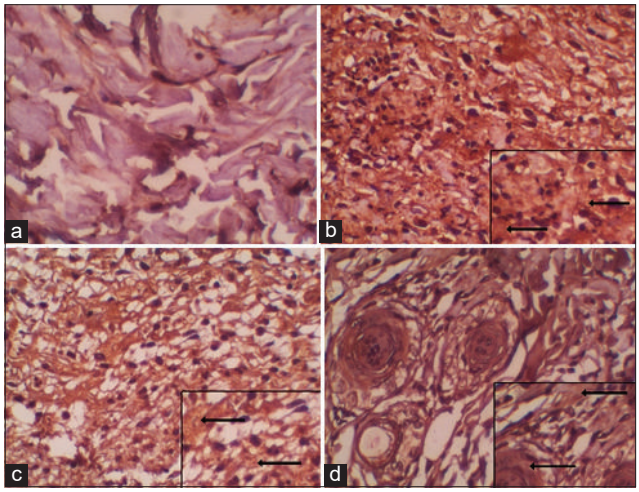

Figure-2: The expression of interleukin-6 on treatment rats: (a) (T1) Positive control, (b) (T2) negative control, (c) (T3) wounds treated with Gliricidia sepium from Indonesia, and (d) (T4) wounds treated with Gliricidia sepium from the Philippines (400×).

Effect ointment containing *G. sepium* leaves on IL-6 expression

The results of the one-way ANOVA showed that the administration of ointment containing *G. sepium* leaves from Indonesia (T3) and from the Philippines (T4) significantly reduced the expression of IL-6 ($p<0.05$) compared with the positive control group (T1) (Table-3).

Discussion

The number of inflammatory cells in the negative control group was the highest and was significantly higher than the positive control and the treatment groups, which indicated that tissue damage had occurred and the inflammatory phase was prolonged compared with the other groups. Macrophages and neutrophils increase tissue damage and increase the phagocytosis of foreign objects. The damaged cells release cytokines as chemotactic factors for inflammatory cells to induce an inflammatory response. Chemotactic factors cause macrophages, lymphocytes, and polymorphonuclear leukocytes (PMNs) to migrate to the wound area [10]. The lowest number of inflammatory cells was observed in the positive control group (T1), which was treated with a commercial wound healing agent. The number of inflammatory cells in the treated groups (T3 and T4) was comparable with T1, meaning *G. sepium* leaves possessed healing properties, as with the commercial preparation. The wounds treated with *G. sepium* leaves from Indonesia showed a decrease in inflammatory cells of 79.82%, while wounds treated with *G. sepium* from the Philippines (T4) showed a decrease of 86.63%. Both therapies showed a significant difference ($p<0.05$) compared with the positive control (T1).

G. sepium leaves contain flavonoids, saponins, tannins, and alkaloids that exert anti-inflammatory properties by inhibiting the activity of the enzymes cyclooxygenase (COX) and lipoxygenase to prevent the release of the histamine during inflammation [11]. Flavonoids also inhibit the accumulation of leukocytes in the inflammatory area, reduce the number of immobilized leukocytes, and inhibit the release of histamine from mast cells. (41.28±9) different cellular mechanism are responsible for anti-inflammation, antimicrobial, and antioxidant which inhibit antioxidant reactions by free radicals and provide nutrients to the skin [12].

Under normal skin conditions, the cytokine IL-1β is expressed at low levels in the epidermis of the skin [13]. IL-1β stimulates monocytes and macrophages to produce higher levels of other cytokines that can trigger nuclear factors, such as activators of gene transcription, and trigger an enzyme pathway that turns on prostaglandin activation [14]. IL-1β induces the endothelial excretion of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 so that inflammatory cells can be identified, which infiltrate the injured area [15]. In the positive control group (T1), the average expression of IL-1β showed a significant difference compared to negative control. Increased expression of IL-1β is observed in inflammation caused by incision wounds. IL-1β activates monocytes and PMNs and

can also stimulate inflammation [16]. IL-1β increases the migration of PMNs and monocyte/macrophages to endothelial cells and stimulates the production of prostaglandins and the release of lysosomal enzymes. The continuous production of pro-inflammatory cytokines prolongs the inflammatory phase and the wound healing time [15]. Groups T3 and T4, which were receiving *G. sepium* treatments, showed decreased IL-1β expression of 35.55% and 62.80%, respectively. Furthermore, both treatment groups showed a significant decrease in IL-1β expression when compared with the positive control group ($p<0.05$). The decrease in IL-1β expression in the T4 group was higher than in the T3 group (Figure-1).

G. sepium leaves from both Indonesia and the Philippines contain active ingredients, such as flavonoids, saponins, tannins, and alkaloids, that act as antioxidants and anti-inflammatory factors and could inhibit the activity of COX and lipoxygenase and stimulate macrophages to produce growth factors and cytokines to accelerate the wound healing process in the proliferation phase. The results of T1 and T3 were comparable and significantly different from the negative control. *G. sepium* leaves from the Philippines had a better healing effect that was significantly different from *G. sepium* leaves from Indonesia.

The average level of IL-6 expression in the negative control group was 96.86±1.04, and this value was used as an indicator of IL-6 expression in normal rats. Normally, the expression of IL-6 in serum is very high, and it increases in pathological conditions, such as inflammation [17]. The highest IL-6 level was obtained in the negative control group (T2) as the result of the inflammatory response due to injury. IL-6 is a cytokine that causes an acute inflammatory response and plays an essential role in the pathogenesis of inflammatory diseases [18]. It also activates macrophages to produce growth factors needed in the proliferative phase of the wound healing process.

The IL-6 expression levels in the groups treated with *G. sepium* leaves from Indonesia and from the Philippines were significantly different ($p<0.05$). The highest decrease in IL-6 expression was observed in the positive control (T1) followed by the ointment therapy with *G. sepium* from the Philippines and then the ointment therapy with *G. sepium* from Indonesia. This was thought to be due to the higher saponin and tannin content in *G. sepium* leaves from the Philippines. IL-6 causes macrophages to follow the migration of neutrophils to wounds after 48-72 h, and they become the predominant cells after the 3rd day of injury. Macrophages also play a major role in producing various growth factors required by fibroblasts to produce extracellular matrix in the process of neovascularization. Thus, the presence of macrophages is crucial for wound healing [19]. The flavonoid content is also believed to be beneficial in the wound healing process, and the presence of biosynthetic phase barriers inhibits the production of growth factors and cytokines, such as IL-6, by macrophages, thereby accelerating the phase of proliferation and wound healing [20]. The inflammatory phase begins immediately after the injury until the 5th post-injury day. In inflammatory conditions, various mediators of endothelial derivatives and complement factors attract leukocytes to the endothelial wall. These leukocytes are no longer able to move freely and stimulate neutrophil degranulation. Saponins and tannins can inhibit neutrophil degranulation and reduce the release of arachidonic acid by neutrophils, thereby reducing inflammation [21].

Conclusion

Our findings have shown that ointment therapy with *G. sepium* leaves from the Philippines to improve wound healing was superior to ointment therapy with *G. sepium* leaves from Indonesia. This was based on decreased levels of inflammatory cells and decreased expression of IL-1β and IL-6 compared with the negative control treatment. Future analysis of the components of *G. sepium* is necessary to prove its efficacy in wound healing.

Authors' Contributions

AA, DKW, FSP, and WR designed the research experiments, data analysis, and writing the manuscript for publications. KMO, NAA, and WR conducted the laboratory works as well as results analysis. MAGB conducted data analysis and proofread the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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RESEARCH ARTICLE

Antimycotic potential of Kawayang tinik against pathogenic fungal species

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ABSTRACT

The importance of discovering and obtaining new, natural and sustainable sources of potential drugs have been the focus of scientific communities due to the emergence of increasing cases of microbial resistance, one of the biggest health threats in our society today. This study aimed to determine the antimycotic potential of *Bambusa blumeana* (kawayang tinik) specifically its leaf, rhizome, root, inner culm and outer culm extracts using the agar well diffusion assay. Results of the study revealed that all kawayang tinik extracts produced statistically equal size zone of inhibition (ZOI) against *Aspergillus niger* at 1 mg/ ml concentration while the ethanolic root and leaf extracts showed larger ZOI against *Penicillium chrysogenum* compared to other kawayang tinik extracts. Furthermore, the results of the antifungal assay showed comparable activity of kawayang tinik extracts to Fluconazole, a pharmaceutically approved antifungal drug, at 1 mg/ml concentration. Phytochemical studies further revealed the presence of alkaloids, tannins, phenols, sterols, triterpenes and flavonoids in its different parts which may support its potential antimycotic properties.

Introduction

The Center of Disease and Prevention (CDC) and World Health Organization (WHO) considers antibiotic resistance, in both bacterial and fungal species, as one of the biggest public health threats today (1, 2). Accordingly, widespread occurrences of antibiotic resistance were recorded among 500000 people across several countries including Asia. It is due to this fact that scientific communities are gearing towards drug discovery especially those coming from natural, highly available and sustainable resources.

Plants have been known for ages to contain bioactive compounds that possessed disease - fighting and ailment - preventing capabilities. In fact, the use of plants as ethnobotanical – based remedies and medication for various illnesses have been a long-dated tradition not only in the Philippines, but in other countries as well (3-5). Moreover, most of the pharmaceutical products being sold in the market today were obtained and patterned from bioactive compounds found in plants and plant products.

The bamboo plant was traditionally utilized to make home furniture and its shoots were usually obtained for food. However, secondary to technological advances in phytochemical screening and testing, several bamboo species are now being studied and observed as potential sources of new pharmacotherapeutic products. Leaves and shoot extracts from bamboo species such as *Bambusa balbacooa*, *Bambusa bambos*, *Dendrocalamus hamiltonii*, *Bambusa vulgaris* and *Bambusa vulgaris* exhibited potential antimicrobial properties against several pathogenic microorganisms, bacterial and fungal alike (6-14). Leaf extracts of *Dendrocalamus strictus* were also cited to show antimycotic potentials against pathogenic fungal strains such as *Aspergillus niger*, *Penicillium chrysogenum*, *Aspergillus flavus* and *Fusarium moniliforme* (15). It was further observed that the antimicrobial activities in bamboo plants can be detected in the entirety of the plant, specifically in the branches, roots, knots, inner culms and rhizomes and not only on the leaf and shoot parts which are the most common plant parts used in antimicrobial studies involving bamboo plants (16).

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The *Bambusa blumeana*, also known as spiny bamboo or thorny bamboo, is a species of bamboo occurring in tropical Asia and is abundant in the Philippines. This bamboo species is known locally as 'kawayang tinik' and ethnobotanically known as 'kawayan siitan' for Ilocanos and 'kawayang batakan' for Bisayans. Although widely available in the community and also tagged as the top economically important bamboo species in the Philippines (17), very few research studies and literatures, both local and international, have been published providing information on its biological activities especially with regard to its potential antimycotic properties.

With various evidences citing the potential biological activity in several bamboo plants against pathogenic fungal species, the present study determined the innate antimycotic potentials of *B. blumeana* (kawayang tinik). Specifically, the study explored the following:

1. Presence of potential antifungal activity in *Bambusa blumeana* (kawayang tinik) against *Aspergillus niger* and *Penicillium chrysogenum*.
2. Differences in the antifungal properties of the ethanolic and aqueous extracts of *Bambusa blumeana* (kawayang tinik) in terms of its different plant parts including the leaf, rhizomes, roots and inner and outer culms.
3. Identified different phytocompounds in various *B. blumeana* (kawayang tinik) extracts.

Materials and Methods

Selection, Gathering and Preparation of Plant Extracts

Plant parts such as leaves, rhizomes, roots, inner and outer culms were identified and gathered from locally – grown *B. blumeana* (kawayang tinik) plants found at Tarlac Agricultural University (TAU) Bamboo Forest Park located in Sitio Calao, Mayantoc, Tarlac. Collected parts were initially washed and cleaned with tap water to remove majority of the dirt and debris while distilled water was used for the final washing. Clean plant specimens were afterwards air dried and powdered separately using electric blender and corn miller. Individual powderized plant parts were placed in clean, zip – locked containers, labelled and stored in the refrigerator with temperature regulation at 4 ^{0}C until further use (14, 18, 19).

Powdered plant parts for ethanolic extraction were then mixed and macerated for 48 hrs (12, 16, 20) at room temperature using 95% ethanol utilizing 1: 4 extract to solvent ratio to produce ethanolic extracts. As for the preparation of aqueous extracts, 400 ml of distilled water was added with 100 gm of individual powdered dry plant parts (leaves, roots, rhizomes, inner and outer culms). Afterwards the individual aliquots were boiled at 80 ^{0}C for at least 15 min. cooled and allowed to macerate for 24 hrs (16, 21, 22).

Frequent agitation of the solutions was observed during the course of the maceration period to further facilitate the extraction of potential bioactive compounds (20). After maceration, the aqueous and ethanolic solutions were filtered using Whatman filter paper No. 1. The filtrates were exposed thereafter to rotary evaporation to remove the solvents and concentrate the extracts.

Stock solutions were then formulated to 1mg/ml concentration and sterility- proofed through filtration of individual plant extracts using sterile millipore filter syringe (Whatman® at 0.22 millipore size) connected to a sterile syringe and test tube (23). The filtered sterile extracts contained in sterile tubes are then stored in the refrigerator with temperature regulation at 4 ^{0}C until needed for antimicrobial assay (24).

Antifungal Assay

Pure cultures of pathogenic fungi (*Aspergillus niger* and *Penicillium chrysogenum*) from the College of Arts and Sciences of Benguet State University located La Trinidad, Benguet were used to test the antifungal property of different kawayang tinik extracts.

Agar well diffusion method was utilized to determine the biological activity of *B. blumeana* (kawayang tinik) against *A. niger* and *P. chrysogenum*. In this method, a 3 mm diameter fungal or mycelium disk was placed at the center of the petri dish while various kawayang tinik extracts were placed in wells distributed in even distances around the fungal disk culture (Fig. 1). Fluconazole 1 mg/ml was used as positive control while sterile water and 95% ethanol were used as negative controls. After 72 hrs of room incubation, antifungal activity was determined by measuring the inhibition zones formed between the extracts and the mycelium disk. The zone of inhibition (ZOI) is measured by obtaining the distance of mycelium growth from the disk towards the individual kawayang tinik extracts as indicated by the blue line (Fig. 1). As shown by various studies, mycelium or fungal species grown on

Fig. 1. Antifungal assay using agar-well diffusion method.

laboratory agar tend to deviate from extracts containing antimycotic compounds, thus, minimal growth between the disk and the wells containing the individual extracts may possibly correlate to presence of bioactive compounds responsible for antifungal mechanisms (25-28). Additionally, five

replications were made per treatment per fungal species.

Phytochemical testing

Individual kawayang tinik plant extracts are subjected to qualitative phytochemical screening to determine the presence or absence of various phytocompounds using prescribed techniques and procedures (10, 29, 30). The presence of alkaloids was tested using different reagents (Mayer, Wagner, Bouchardat and Valser) while sterols and triterpenes were tested using the Lieberman's Buchard Test. The presence of flavonoids were otherwise confirmed using the Salkowski Test and Bate- Smith Metcalf test while the presence of cardiac glycosides were tested using the Keller- Killiani Test. Froth test was utilized to observed for the presence of saponin, gelatin test and ferric chloride test for the presence of tannins and phenolic compounds, Borntrager Test for the presence of antraquinone and Guignard Test for the possible presence of cyanogenic glycosides. The results of the phytochemical testing is presented in Table 3.

Table 1. Variations in the antifungal property of *Bambusa blumeana (*kawayang tinik) against *Aspergillus niger*

Extracts (1 mg/ml)	Zone of Inhibition (mm)
Ethanolic Roots	1.13a
Ethanolic Inner culm	1.04ab
Ethanolic Leaves	0.98ab
Ethanolic Outer culm	0.75ab
Fluconazole	0.55ab
Aqueous Outer culm	0.47ab
Aqueous Inner culm	0.41ab
Aqueous Rhizomes	0.22ab
Ethanolic Rhizomes	0.20b
Aqueous Roots	0.10b
Aqueous Leaves	0.09b
Water	0c
Fluconazole	0c
F-computed value = 3.74, F-critical value = 1.94, Probability = .0004	

(0.09 mm) extracts of *B. blumeana* (kawayang tinik) also showed lesser statistically comparable inhibitory activities against *A. niger*. It was further observed that the above- mentioned extracts are statistically comparable to Fluconazole, a pharmaceutically-approved medication and antifungal agent, at 1 mg/ml concentration. This would imply that the extracts of *B. blumeana* have the same inhibitory effect as the standard antifungal drug Fluconazole at 1 mg/ml drug concentration (Fig. 2).

Table 3. Phytochemical analysis of different *Bambusa blumeana* (kawayang tinik) extracts

Bioactive Compounds	Plant Extracts									
	Aqueous					Ethanolic				
	Leaf	Rhizome	Roots	Inner Culm	Outer Culm	Leaf	Rhizome	Roots	Inner Culm	Outer Culm
Alkaloid	+	+	+	+	+	+	+	+	+	+
Sterol and Triterpenes	+	-	-	+	+	+	-	-	+	+
Flavonoid	+	-	-	+	+	+	-	-	+	-
Cardiac glycoside	-	-	-	-	-	-	-	-	-	-
Saponins	-	-	-	-	-	-	-	-	-	-
Tannins and Phenols	+	+	+	+	+	+	+	+	+	+
Anthraquinone	-	-	-	-	-	-	-	-	-	-
Cyanogenic Glycoside	-	-	-	-	-	-	-	-	-	-

Results and Discussion

One-way Analysis of Variance (ANOVA) was used to determine the variation in the antifungal property of the various plant extracts derived from the different parts of *B. blumeana* (kawayang tinik).

Variations in the Antifungal Property of Bambusa blumeana (kawayang tinik) against Aspergillus niger

Table 1 presents the variations in the antimycotic potentials of *B. blumeana* (kawayang tinik) against *A. niger*. Results showed that there are highly significant variations among the *B. blumeana* (kawayang tinik) extracts against *A. niger* in terms of plant parts and the extraction solvents used since the F- computed value (3.74) is higher than the F- critical value (1.94) and the probability is less than 0.05.

The table further shows that in terms of the specific extracts derived from *B. blumeana*, the ethanolic root extract exhibited the highest comparable mean zone of inhibition (ZOI) at 1.13 mm against *A. niger*. This is followed by the extracts of ethanolic inner culm (1.04 mm), ethanolic leaves (0.98 mm), ethanolic outer culm (0.75 mm), aqueous outer culm (0.47 mm) and aqueous inner culm (0.41 mm). On the other hand, the ethanolic rhizome (0.20 mm), aqueous root (0.10 mm) and aqueous leaves

Various phytocompounds were observed to be present in different species of bamboo which could possibly explain the ZOI formed by the extracts

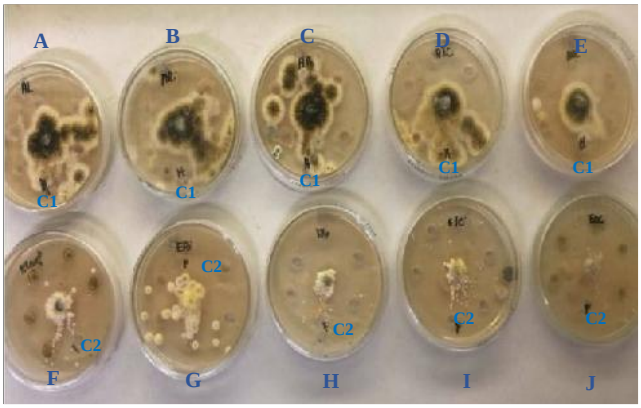

Fig. 2. *Aspergillus niger* growth on plates planted with various kawayang tinik extracts: **(A)** aqueous leaf, **(B)** aqueous rhizome **(C)** aqueous root, **(D)** aqueous inner culm, **(E)** aqueous outer culm, **(F)** ethanolic leaf, **(G)** ethanolic rhizome **(H)** ethanolic root, **(I)** ethanolic inner culm, **(J)** ethanolic outer culm, **(C1)** water and **(C2)** Fluconazole at 1 mg/ml.

against *A. niger*. For instance, apigenin, luteolin and p- coumaric acid were discovered in the different parts of *P. pubescence*, specifically in its leaf, root, rhizome and culms (16). These phytocompounds were noted to have the same antimicrobial mechanisms as that of the fluconazole, a pharmaceutically – approved antifungal drug. Fluconazole as an antifungal agent exhibits its action by increasing fungal cell wall permeability and disrupting the uptake of essential nutrients needed by the cell leading to eventual death (28). Through the application of chemotaxonomy principles which states that plants that are taxonomically related contains the same biochemical compositions (31, 32), we could consider the possibility that kawayang tinik extracts may contain the same phytocompounds hence, justifying its potential antifungal property and the ZOI produced by its individual extracts against *A. niger*. The antimycotic potentials of bamboo species could further be claimed through the identification of another antifungal protein known called Dendrocin (33). Its mechanism of action includes the formation of membrane channels, degradation of polymers in fungal cell wall or disruption of cellular ribosomes (34).

In terms of extraction solutions used in the study, it was observed that majority of ethanolic *B. blumeana* extracts exhibited better antifungal potential compared to aqueous extracts although statistical comparability is noted among these extracts at 1 mg/ml concentration. This could be associated with the polarity of the solvents used to extract the potential phytocompounds present in the different plant parts of *B. blumeana*. Water is a pure polar solvent thus, can only attract or extract polar compounds while ethanol is less polar than water hence, it can possibly attract both polar and non-polar compounds (35). The greater variety of phytocompounds which may be extracted via ethanolic extraction may provide explanation on the better inhibitory performance of ethanolic extracts compared to the aqueous extracts of kawayang tinik. Moreover, when plant sources are homogenized or extracted using the same protocols, the polarity of the solvent is the main factor to be considered (36, 37).

Variations in the Antifungal Property of Bambusa blumeana (kawayang tinik) against Penicillium chrysogenum

Table 2 presents the variations in the antifungal potential of *B. blumeana* (kawayang tinik) against *P. chrysogenum*. Results of the analysis revealed high significant variations in the antifungal potential of different *B. blumeana* (kawayang tinik) extracts against *P. chrysogenum* since the computed F- value (20.21) is higher than the F – critical value (1.94) and the probability is lower than 0.05.

Statistical analysis further revealed that the ethanolic root and leaf extracts of *B. blumeana* exhibited the highest statistically comparable antifungal potential against *P. chrysogenum* both with mean ZOI of 1.59 mm. These were followed by kawayang tinik extracts of ethanolic inner culm (0.48 mm), ethanolic rhizome (0.41 mm), ethanolic outer culm (0.12 mm), aqueous inner culm (0.06 mm) and aqueous rhizome (0.03 mm) which are comparable or having equal antifungal effects against P. chrysogenum at 1 mg/ml concentration. On the contrary, the aqueous extracts of leaves, roots and outer culm did not exhibit any effects against *P. chrysogenum* exhibiting mean ZOI of 0.00 mm (Fig. 3).

Table 2. Variations in the antifungal property of *Bambusa blumeana (kawayang tinik)* against *Penicillium chrysogenum*

Extracts (1 mg/ml)	Zone of Inhibition (mm)
Ethanolic Roots	1.59 a
Ethanolic Leaves	1.59 a
Ethanolic Inner culm	0.48 b
Ethanolic Rhizomes	0.41 b
Ethanolic Outer culm	0.12 b
Aqueous Inner culm	0.06 b
Aqueous Rhizomes	0.03 b
Aqueous Leaves	0 c
Aqueous Roots	0 c
Aqueous Outer culm	0 c
Water	0 c
Fluconazole	0 c
F-computed value = 20.21, F-critical value = 1.94. Probability = 1.75E-17=.0000	

Note: Means followed by the same letter/s are not significantly different at 5% level

Fig. 3. *Penicillium chrysogenum* growth on plates planted with various *kawayang tinik* extracts: **(A)** aqueous outer culm, **(B)** aqueous leaf **(C)** aqueous root, **(D)** aqueous rhizome, **(E)** aqueous inner culm, **(F)** ethanolic leaf, **(G)** ethanolic inner culm **(H)** ethanolic root, **(I)** ethanolic rhizome, **(J)** ethanolic outer culm, **(C1)** water and **(C2)** Fluconazole at 1 mg/ml.

As observed in the study, ethanolic extracts produced higher mean ZOI compared to the aqueous extracts of inner culm, rhizome, leaves, roots and outer culms that exhibited mean ZOI of 0.06 mm, 0.03 mm and 0.00 mm respectively. This result could still be associated to the polarity of extraction solutions used in the study whereby ethanol being less polar could harness both polar and non- polar compounds while water, a pure polar solvent, could only harness polar phytocompounds (37). Since ethanolic extraction could harness a wider variety of phytocompounds, better inhibitory potentials could be observed compared to aqueous extracts. Moreover, the better inhibitory performance of the ethanolic extracts could also indicate the possibility of greater proportions or amount of non- polar phytocomponents residing in the different plant parts of kawayang tinik especially that aqueous counterparts does not exhibit any inhibitory effects against *P. chrysogenum* (i.e. aqueous roots, leaves and outer culms producing mean ZOI of 0.00 mm).

The findings mentioned may also be correlated a previous study result wherein it was observed that phytocomponents from the leaf extracts of bamboo species *Phyllostachys* and *Moso* bamboo are not water soluble (38). However, the presence of polar phytocompounds in the different parts of *B. blumeana* could not be totally eliminated from the current study since aqueous extracts also exhibited zones of inhibitions against the previously discussed fungal species. It could be a possibility that the either *P. chrysogenum* is resistant to the polar compounds present in the different extracts of kawayang tinik or the concentration of extracts which is 1 mg/ml is inadequate to show inhibitory action against the said fungal species. Therefore, additional and more advanced studies should be conducted to fully determine the various phytocomponents present in the different parts of *B. blumeana* and how these phytocompounds relates to the antifungal potentials of kawayang tinik as well as devise further experimentations catering to different concentrations of extracts against fungal species.

The results of the study also coincide with previous findings citing that various phytocomponents were present in the different parts of the bamboo species *P. pubescence* (16). Specifically, apigenin and tricin derivatives were noted to be abundant in the root and leaf part of the said bamboo species. Antifungal mechanisms of these identified phytocompounds were noted to be fungal membrane and metabolic disruption (38, 39) the same inhibitory action of fluconazole, a standard antifungal drug (28).

Currently, there are no standard medical treatment of choice for the management of penicilliosis, however, antibiotics like amphotericin B, itraconazole, or fluconazole were reported to be effective against the said organism. The various ranges of inhibition zones exhibited by the ethanolic extracts of *B. blumeana* may indicate potential effectiveness of the said bamboo species in inhibiting the fungi *P. chrysogenum*, especially that the ethanolic root and leaf extracts exhibited more significant results compared to the standard antibiotic treatment Fluconazole at 1 mg/ml concentration. Moreover, other *B. blumeana* plant parts such as rhizomes and culms also exhibited potential antifungal activities as evidenced by the inhibition zones formed which further highlights the potential of the said bamboo species as source of potential antifungal drug against *P. chrysogenum*.

Phytochemical Testing

Table 3 shows the results of the phytochemical test involving different extracts of kawayang tinik. As shown, it is evident that all of the extracts contain bioactive compounds such as alkaloid, tannins and phenols while majority of the extracts also contains sterols and flavonoids. On the other hand, all of the extracts were screened negative for the presence of cardiac glycoside, saponins, anthraquinone and cyanogenic glycoside.

The presence of various phytochemicals in the ethanolic and aqueous extracts and their innate abilities to promote microbial inhibition may possibly verify the antimycotic potentials of kawayang tinik extracts. Alkaloid as a phytocompound exerts its antimicrobial mechanism via multitarget action such as outer membrane or cytoplasmic membrane disruption, cellular respiratory inhibition and nucleic acid synthesis or cell division inhibition (39, 40). Flavonoid, on the other hand, is able to promote inhibition of nucleic acid synthesis and disrupt the energy metabolism in pathogenic microorganisms (39). Tannins are also able to induce inhibition of bacterial and fungal enzymatic activity via direct interference on the microorganism's metabolism while triterpenes and sterols work by increasing the tolerance and resistance of plants to pathogen attacks, although the exact pathways and mechanisms are not yet known (41, 42).

Conclusion

In line with the objectives of the study, analysis and results, the following conclusions and implications were made:

1. *B. blumeana* (kawayang tinik) extracts showed varying ranges of antimycotic activity against *A. niger* and *P. chrysogenum.* Thus, kawayang tinik can be a potential source of natural pharmacotherapeutic products against these pathogenic fungal species.
2. Ethanolic extracts of roots and inner culm exhibited the highest mean zone of inhibition (ZOI) against *A. niger* while ethanolic root and leaf extracts exhibited better inhibitory potentials against *P. chrysogenum*.
3. *B. blumeana* (kawayang tinik) extracts contain phytocompounds such as alkaloid, sterols, flavonoids, tannins and phenol. Hence, further studies are needed to specifically harness the compounds producing antifungal effects.

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Conflict of interests

Author do not have any conflict of interests to declare.

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Application of wheat flour (*Triticum aestivum*) on spore density and sporulation efficiency of *Bacillus megaterium* isolated from *Litopenaeus vannamei* gastrointestinal tract

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Abstract. *Mahariawan IMD, Kusuma WE, Yuniarti A, Beltran MAG, Hariati AM. 2021. Application of wheat flour (*Triticum aestivum*) on spore density and sporulation efficiency of Bacillus megaterium isolated from* Litopenaeus vannamei *gastrointestinal tract. Biodiversitas 22: 3709-3715. Bacillus megaterium* is frequently used in fish farming, such as *white shrimp (*Litopenaeus vannamei*)* pond, which can produce spores with high stability in its implementation. Currently, spore production still requires the availability of high-cost carbon sources. The objective of this research was to evaluate the effect of different wheat flour doses on spore density and sporulation efficiency of *B. megaterium* BM1. In flasks, 50 mL of each test medium was treated with different doses of wheat (10, 20, 30 and 40 g. L^{-1}, respectively) and glucose was used as a control. Each treatment was inoculated with *B. megaterium* BM1 (2.6 x 10^8 CFU. mL^{-1}) and incubated in a shaker incubator (120 rpm) at 37 $^{\circ}$C for 120 hours. The results showed that the highest vegetative cell concentration (17 x 10^8 CFU. mL^{-1}), growth rate (0.8 $hour^{-1}$) and spore (14.7 x 10^8 spores. mL^{-1}) were found in the wheat flour dose of 30 g. L^{-1}. Furthermore, the highest sporulation efficiency was achieved at 20 g. L^{-1} of wheat (91.30%) and germination should be done at a dose less than 40 g. L^{-1}. The size of the spores was 1.35-1.39 μm. Thus, 30 g. L^{-1} of wheat flour is a potential dose to produce spore for probiotic candidates.

Keywords: Affordable carbon source, *Bacillus megaterium*, bacterial growth, spore density, sporulation efficiency

INTRODUCTION

Nowadays, the application of aquaculture biotechnology is one of the efforts to increase fish farming production (Ayoola and Idowu 2008; Danish et al. 2017; Rathore et al. 2019). Probiotics are potential biotechnology products from living microorganisms that are mostly used in the aquaculture industry (Sahu et al. 2008; Verma and Gupta 2015). The advantages of probiotic bacteria in extracellular enzyme secretion (Lee et al. 2012; Eshaghzadeh et al. 2014) and antibacterial compound production (Gozari et al. 2016; Prabhurajeshwar and Chandrakanth 2019; Silva et al. 2020) are important indicators to be continuously developed.

Bacillus is a genus of bacteria that is widely applied as a probiotic in fish and shrimp farming (Buruiana et al. 2014; Swapna et al. 2015; Won et al. 2020). One species of this genus that has been confirmed to have a positive impact on successful aquatic organisms in culture is *B. megaterium* (Li et al. 2009; Aftabuddin et al. 2013). This bacterial species has the ability to sporulate (Grage et al. 2017; Stancu 2020). Some of the benefits in spore as a probiotic candidate are surviving in acid pH at 2–2.5 (Ananthanarayanan and Dubhashi 2016), heat stability, and storage in dry form (Cutting 2011). From these benefits, bacteria in the form of spores show good stability when applied as probiotics (Bader et al. 2012; Lakshmi et al. 2017).

Production of *B. megaterium* spores involves media composed of various components. One of the media components that presented an important role in initiating the sporulation is the carbon source (Mazmira et al. 2012; Gauvry et al. 2016; Rai and Tewari 2016). Commonly available commercial carbon sources are glucose, lactate (de Vries et al. 2005), and sucrose (Hassan et al. 2014) which provide a high price. The alternative of inexpensive carbon sources that could be used as a substrate to produce spore comes from agro-industrial products (Khardziani et al. 2017a). Wheat flour is an agricultural product that is relatively cheap and easy to find. The utilization of organic carbon from wheat flour is one way to reduce the spore production cost. A previous study underlined that the implementation of carbon sources with inappropriate doses can result in less spore production (<10^5 spores. mL^{-1}) (Senthilkumar et al. 2011). Thus, the use of wheat flour dosage needs to be further evaluated to obtain the optimal dose in producing vegetative cells and spores of *B. megaterium* BM1 isolated from GI tract of white shrimp.

Therefore, this study aimed to evaluate the effect of different wheat flour doses on spore density and sporulation efficiency of *B. megaterium* BM1.

MATERIALS AND METHODS

Preparation of *B. megaterium* BM1 culture

The isolate of *B. megaterium* BM1 which isolated from gastrointestinal tract of white shrimp was cultured in Nutrient Agar (NA) with adding sodium chloride 2% (w/v) at 30°C for 24 hours. The re-culture of *B. megaterium* BM1 isolates was conducted by inoculating the bacterial colonies that have grown on solid media using a loop needle into Nutrient Broth (NB) media. Furthermore, the incubation of the broth culture was in an incubator shaker (120 rpm) at 37°C for 18 hours as a stock culture.

Composition of tested media

The test media in this study consisted of wheat flour, ammonium chloride (NH_4Cl) and mineral salts. The doses of wheat flour used were 10, 20, 30 and 40 g. L^{-1}, respectively. In contrast, the NH_4Cl dose added was adjusted to each wheat flour (*T. aestivum*) dose to obtain a C:N ratio of 5:1. The composition of the test media that has been determined was dissolved by distilled water and enriched with several minerals such as $CaCO_3$ 0.3 g. L^{-1}, $MgSO_4$. $7H_2O$ 0.00033 g. L^{-1}, $MnSO_4$. H_2O 0.12 g. L^{-1}, $FeSO_4$. $7H_2O$ 0.084 g. L^{-1}, and $CaCl_2$. $2H_2O$ 0.09 g. L^{-1}. The control treatment used was glucose as a carbon source with the same C:N ratio and minerals as the wheat flour treatment. All of these treatments were repeated 3 times.

Cultivation condition

Fifty (50) ml of test media was put into each 100-ml-Erlenmeyer flask according to the treatment and sterilized using an autoclave. The stock culture of *B. megaterium* BM1 was inoculated into the test medium as much as 1% (v/v). The initial density obtained from the bacterial culture stock was 2.6 x 10^8 CFU. mL^{-1}. Fermentation of all treatments was carried out in a shaker incubator at a speed of 120 rpm for 24 hours for the vegetative phase and was continued to the sporulation phase for 120 hours.

Counting of vegetative cell, vegetative cell growth rate, spore production, sporulation efficiency and spore germination

The vegetative cells and spores were counted microscopically with the Neubauer chamber. Shape of the cells became a key to determine distinguish both of them. The vegetative cells are rod-shaped and spores are round-shaped. The special treatment was conducted before counting spores by heating them at 80°C for 15 minutes. To assist the calculation of vegetative cells and spores, samples were serially diluted. The measurement of sporulation efficiency was carried out using the highest spore and vegetative data in the predetermined period which referred to the formula from Monteiro et al. (2014). Furthermore, the ability of spores to germinate was done by planting the spore samples onto the germination medium (NB). Spore germination was observed in an incubation time of 24 hours.

Spore visualization on Scanning Electron Microscopy (SEM)

The highest spores that have been harvested from wheat flour with the optimal dose were visualized by morphology using a Scanning Electron Microscopy (SEM). This shape and size of the spore from the optimal dose of wheat will be compared to the spore that was grown in glucose media. The spore samples were separated between the supernatant and the pellets by centrifugation process at a rate of 1,000 x g using 4°C of temperature in 5 minutes. Afterward, the sample was conducted through incubation with 2.5% of glutaraldehyde in 1.5 hours. The pellets collected from the last stage of centrifugation were followed by gradual dehydration. Visualization of spores was observed in SEM TM 3,000. Spores were measured to determine the size of the spores produced from each treatment.

Statistical analysis test

The results obtained of whole parameters in this study were tested statistically by one-way ANOVA through SPSS 20 software. The differences of all treatments can be seen from DMRT Test.

RESULTS AND DISCUSSION

Vegetative cell density and growth rate of *B. megaterium* BM1

All doses of wheat showed a higher vegetative cell production than the control treatment (glucose) (Figure 1). Even at wheat flour doses of 20, 30, and 40 g. L^{-1} indicated that the vegetative cell density was two times greater than the control treatment. In this study, the highest of the vegetative cell was obtained at 30 g. L^{-1} of wheat flour with a density of 17 x 10^8 CFU. mL^{-1}. The growth rate of the vegetative cells of *B. megaterium* BM1 (Figure 2) in all wheat flour concentrations exhibited a higher value than that of the control treatment (glucose). The average growth rate of *B. megaterium* BM1 grown on wheat flour with a dose greater than 10 g. L^{-1} had a value above 0.75 $hour^{-1}$ and indicated no significant difference ($p>0.05$).

Table 1. Vegetative cell densities of germinated *B. megaterium* BM1 spores (x10^8 CFU. mL^{-1})

Incubation (hour)	Wheat Flour Doses (g. L^{-1})				
	10	20	30	40	Control
8	3.43±0.03^b	4.64±0.04^c	4.67±0.07^c	2.42±0.04^a	2.47±0.02^a
16	7.65±0.13^b	8.00±0.20^b	8.93±0.25^c	6.03±0.15^a	6.13±0.21^a
24	9.46±0.06^b	9.97±0.21^c	10.13±0.42^c	8.56±0.09^a	8.59±0.23^a

Note: The different notation between the numbers indicated significant difference ($p<0.05$)

Spore production of *B. megaterium* BM1

The emergence of spores in this study occurred at 12 hours of incubation for all wheat flour treatments. However, in the control (glucose) treatment, spores first appeared at 18 hours after inoculation (Figure 3). The density of spores continues to increase in line with the decreasing density of vegetative cells. After the initial spores were formed, the number of spores gradually increased up to 84 hours. For all dosages of wheat flour, the highest spore density was reached at hour 84. In contrast, the control treatment reached peak spore production at hour 96. Furthermore, the spores indicated the stationary phase until the end of the sporulation time. The highest spore density occurred at a dose of 30 g. L^{-1} of wheat flour with a value of 14.7 x 10^8 spores. mL^{-1}. This result revealed 233% greater than that of the control treatment (glucose). The trends as true for the vegetative cells in all wheat flour concentrations.

Sporulation efficiency and spore germination of *B. megaterium* BM1

In terms of the highest sporulation efficiency, can be seen from Figure 4 that there is no statistical difference in the dosage of wheat flour at 10 and 20 g. L^{-1} with a value of more than 90%.

In addition, the spores of *B. megaterium* BM1 that germinated in all treatments increased within 8 hours of the incubation period (Table. 1). The highest vegetative cell density from germination was obtained in the treatment of wheat at a dose of 30 g. L^{-1} (10.13 x 10^8 CFU. mL^{-1}). These results suggested that the spore regrowth from this dose had a value 117% higher than that of the control treatment (glucose).

Visualization of *B. megaterium* BM1 spores

Visualization of *B. megaterium* BM1 spores grown on optimal media (wheat flour at a dose of 30 g. L^{-1}) compared to commercial media (glucose) was aimed to determine the morphology and size of the spores obtained. The results revealed that there were no significant differences in the shape in each of the media tested (Figure 5). The results of spore size in this study were grown in wheat flour at a dose of 30 g. L^{-1} and glucose were 1.35 – 1.39 μm and glucose 1.09 – 1.47 μm) respectively.

Discussion

Microbes require a culture medium consisting of various components to grow, one of them is a carbon source. The results of this study recorded that the implementation of organic carbon sources derived from wheat flour under different doses had a significant effect on the production of vegetative cells. The availability of carbon as nutrition at the appropriate concentration can affect the maximum bacterial growth in the incubation period (Sarudu et al. 2015; Allen and Waclaw 2018). The distinction in time and density achieved by *B. megaterium* BM1 between the wheat flour and control (glucose) treatment was influenced by the different substrate types. Mikkelsen et al. (2009) and Nur et al. (2015) noted that the utilization of different carbon sources in culture media would affect bacterial growth. Moreover, another factor of carbon concentration in different carbon sources also influences vegetative cell growth (Molina-Ramírez et al. 2017). The carbon source available in the environment was absorbed by bacteria cells that function as the substrate of metabolic tissue, then was broken down to supply amino acids and several components that compose the cell (Wang et al. 2019). After reaching the maximum density, the vegetative cells of *B. megaterium* deceased at 12 hours or more. This condition was caused by the availability of nutrients in the culture media gradually decreased. In consequence, the nutrient uptake by vegetative cells would slow down and impacted starvation (Biselli et al. 2020).

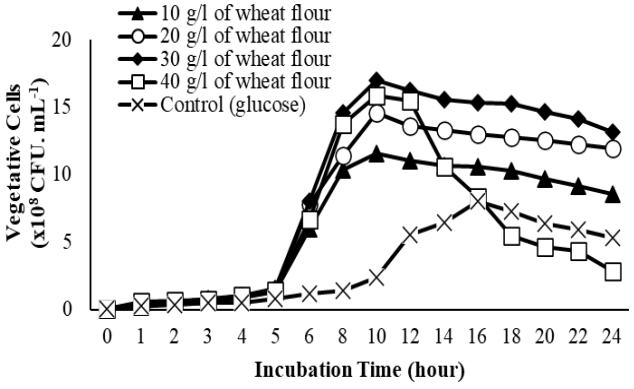

Figure 1. Vegetative cell density of *B. megaterium* BM1 under different doses of wheat flour

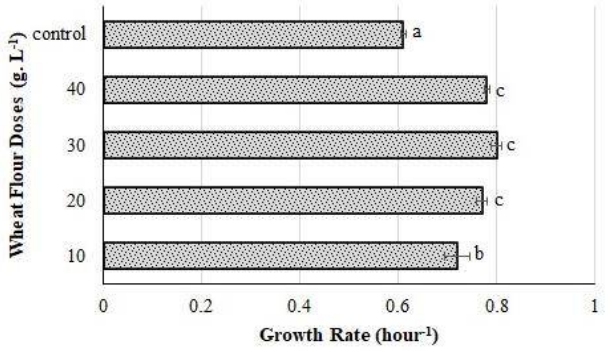

Figure 2. Maximum vegetative cell growth rate of *B. megaterium* BM1 under different doses of wheat flour. Mean data with different alphabets are significantly different at a level of $p<0.05$).

The maximum growth rate of vegetative cells was directly comparable to the highest density of vegetative cells obtained. The growth rate had different values depending on the growth phase of the bacteria. The bacterial growth rate actually began to decline when the culture conditions entered the stationary phase (Hall et al. 2013). Furthermore, physiologically, there was an increase in the size of *B. subtilis* cells in line with the growth rate especially in cell length, while the width was constant (Sauls et al. 2019). In this study, the dosage of wheat flour presented an important indicator in influencing the growth rate of vegetative cells. It can be seen that the doses of 30 g. L^{-1} and 40 g. L^{-1} of wheat are very close in value. The availability of nutrients in the environment at the right dose could modulate vegetative cells to carry out the macronutrient absorption process in the culture medium. Vadia and Levin (2015) explained that the nutrients available in the culture media would be catabolized by bacteria for biosynthetic process. Metabolites produced through central carbon metabolism could serve as intracellular signals that activated effector proteins in modulating cell cycle development.

The process of vegetative transformation into spores is known as sporulation. The difference in time of spore formation was initiated by nutritional factors in the culture media which began to decline after passing through the stationary phase. The stress condition of vegetative cells formed self-defense against nutrient depletion in the environment. The carbon source of wheat exhibited a higher spore density compared to the control treatment. The utilization of wheat flour in this study showed a good substrate for producing *B. megaterium* BM1 spores. Other studies that utilize local materials such as molasses and soy flour could produce spores at 5.90 x 10^8 spores. mL^{-1} (Salazar-Magallon et al. 2015). Apart from being influenced by the type of carbon sources, the concentration of the carbon sources used as a growth medium also played important role in the expression of the capacity for spore formation in the genus *Bacillus* (Khardziani et al. 2017b).

Certainly, the concentration of the various carbon sources showed a different number of spores obtained. For instance, the results of this study explained that the highest dosage of wheat flour (40 g/L) resulted in a lower spore production of *B. megaterium* BM1 than the doses of 30 g. L^{-1} and 20 g. L^{-1}. This result was in line with the research by Posada-Uribe et al. (2015) who showed that the use of a carbon dose (2 g. L^{-1}) could produce spores of 5.1 x 10^8 CFU. mL^{-1} while at a carbon dose (>11 g. L^{-1}) did not generate spores of *B. subtilis*. Jackson and Bothast (1990) stated that giving high carbon concentration up to 40.8 g. L^{-1} inhibited sporulation. According to Kang et al. (1992), at 200 g. L^{-1} of carbon, the bacteria unable to generate the spores. In terms of the results of this study, the application of lower (10 g. L^{-1}) and higher (40 g. L^{-1}) concentrations of wheat were revealed at the same time resulted in close spore production values. This condition was suspected by the uptake of available nutrients by bacteria at different carbon concentrations. At the lowest carbon dose, it has not been able to provide high vegetative cells density that would be spore candidates. In addition, the administration of the highest carbon dose also impacted the sporulation. The implementation of high glucose levels could inhibit the sporulation by repressing the *spo0A* gene transcription (Monteiro et al. 2005). The inhibition of the sporulation was known as the pathway of carbon metabolism (Gauvry et al. 2016). Thus, the carbon concentration should be employed in optimal conditions. In consequence, the environment did not experience a shortage or excess carbon source that could prevent the number of spore production.

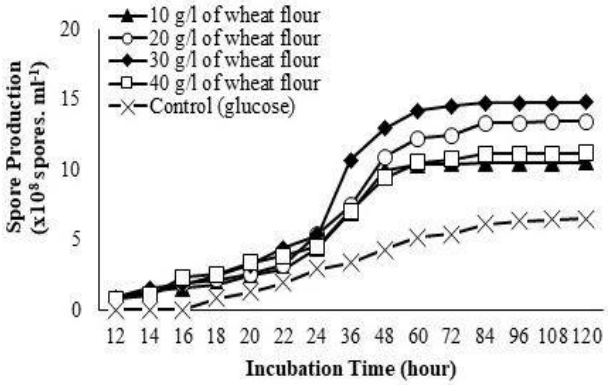

Figure 3. Spore production of *B. megaterium* BM1 under different doses of wheat flour

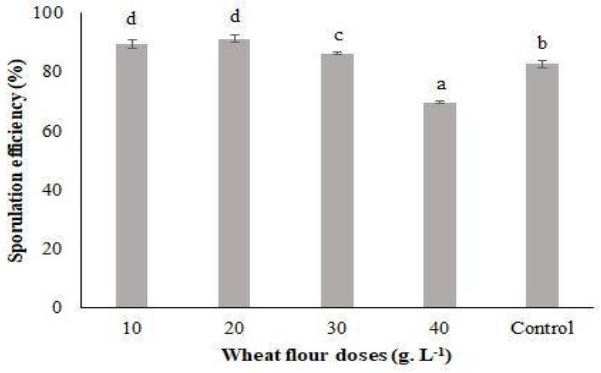

Figure 4. Sporulation efficiency of *B. megaterium* BM1 under different doses of wheat flour. Mean data with different alphabets are significantly different at a level of $p<0.05$).

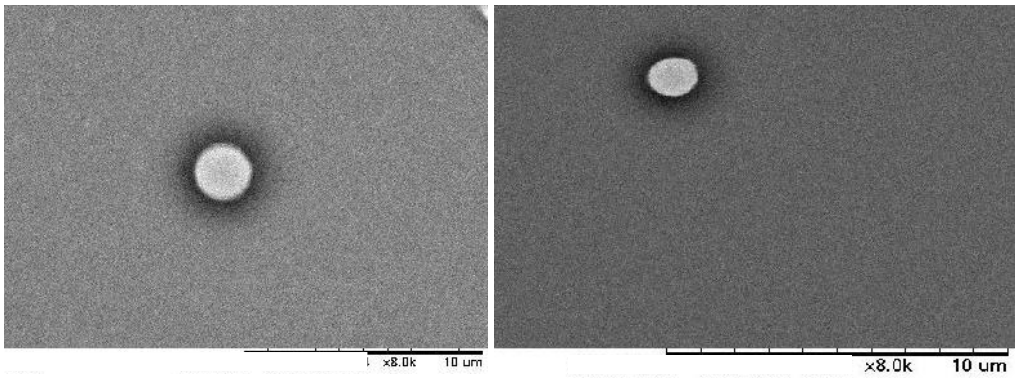

Figure 5. Morphological of *B. megaterium* spores under different carbon sources; A) control (glucose); B) Wheat flour at a dose of 30 g. L^{-1} (x8,000 magnification)

Sporulation efficiency at doses of flour more than 20 g. L^{-1} showed a decreasing value. Even the highest dose of wheat (40 g. L^{-1}) showed a lower sporulation efficiency than the control treatment. This condition explained that the high production of vegetative cells and spores was not supported by the high sporulation efficiency obtained. Sporulation efficiency could be achieved with a high percentage when supported by spore availability (Stojanović et al. 2019) and high growth rates (Carvalho et al. 2010). Several factors that influence spore production in increasing efficiency were carbon, nitrogen, and other macronutrients. The carbon and nitrogen sources played an important role in metabolic synthesis by bacteria to initiate sporulation (Anderson and Jayaraman, 2003). The highest sporulation efficiency of *B. megaterium* BM1 in this study had a value of 15% greater than the sporulation efficiency of *B. subtilis* AWS133 grown on DSM media that researched by Serio et al. (2006). In addition, the concentration of carbon sources (glucose) of 0.8–2 g. L^{-1} in the culture media resulted in the sporulation efficiency of *B. subtilis* at 50–90% (Posada-Uribe et al. 2015). Hence, it could be concluded that the sporulation efficiency really depended on the *Bacillus* strain used and the nutritional components in the culture media (Roy et al. 2015).

In general, the higher the carbon doses given, the higher the vegetative cell production obtained. On the other hand, in this study, the dose of wheat flour at 30 g. L^{-1} exhibited a bacterial density higher than at the dose of 40 g. L^{-1}. This condition explained that the high dose of wheat as a carbon source impacted vegetative cells that could not absorb the total of carbon available in culture media optimally. In line with these results, Thomas et al. (2014) also revealed that bacterial growth on excess carbon sources could reduce the survival of *S. aureus* when it entered the stationary phase. Moreover, the availability of excess carbon in bacteria caused metabolic stress which can inhibit bacterial growth (Zhang et al. 2020). Interestingly, excess carbon (glucose) levels not only inhibited the respiration process but also encouraged the transfer of electron to alternate acceptors (Thomas et al. 2014).

The number of spores germinated into vegetative cells showed that wheat flour with a dose of less than 40 g. L^{-1} was the best treatment. If the dose of wheat flour given was more than that, it would show lower germination than the control treatment. Germination is a process of returning spores to vegetative cells induced by the availability of new nutrients (carbon, protein, and nucleosides) (Setlow 2003; Krawczyk et al. 2017). Apart from being influenced by nutrient availability, the germination process was also initiated by the presence of non-nutritional factors such as Ca^{2+}-DPA, cationic surfactants, salts, and lysozymes (Setlow 2003). The occurrence of spore germination through releasing of DPA depended on the degradation of the spore cortex layer (Francis and Sorg 2016). Some of the variable conditions that affected sporulation were nutritional composition, temperature, spore preparation method, salt concentration, and oxygen availability (Bressuire-Isoard et al. 2018). Thus, the results showed that the spore quality from different doses of wheat flour at the time of sporulation led to differences in the density of vegetative cells from the germinated spores.

The spore size in this study had a larger size than the size of *B. subtilis* spores (0.8 to 1.2 μm) studied by Ricca and Cutting (2003). The difference in size can be caused by the different *Bacillus* species used. In addition, the cause of differences in spore size can be tested by sequencing analysis and gene expression of the strains used (Carrera et al. 2007). Although not many studies have shown the effect of differences in spore size and structure on the quality of *Bacillus* spores, this study showed that lower spore size grown in wheat flour at a dose of 30 g. L^{-1} resulted in a higher spore count than glucose.

In conclusion, the application of wheat flour can be a potential carbon source to produce *B. megaterium* BM1 spores with the highest production at the dosage of 30 g. L^{-1} and sporulation efficiency established less than 40 g. L^{-1}.

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Determining the Quality and Quantity of Bioethanol Production using Golden Shower (*Cassica fistula*) Fruit

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Abstract:

Depletion of fossil fuel is one of the main reasons why the bioethanol has become popular. It is a renewable energy source. In order to meet the great demand of bioethanol, it is best that the bioethanol production is from cheap raw materials. Since the golden shower fruit is not being utilized and is considered as waste material, hence, this study was conducted to make use of the large volume of the residue as feedstock to test its potential for bioethanol extraction.The main goal of this study is to obtain the most volume of bioethanol from the golden shower fruit liquid residue by the factors, days of fermentation (3, 5, and 7 days) and sugar concentration (15, 20 and 25 brix) of the liquid residue. Also, part of the study is to compute the cost of production in extracting bioethanol from the golden shower fruit. Each treatment was replicated three (3) times. The Two-Factorial Analysis of Variance (ANOVA) of the Complete Randomized Design (CRD) was used to analyze the treatments. Treatments means were compared using the Duncan's Multiple Range Test (DMRT).

Keywords: Bioethanol, Extraction, Fermentation, Renewable Energy, Waste.

Introduction:

In recent years, one of the serious problems that our country as well as the world has been facing is the energy crisis. Furthermore, the demand for fossil fuels has increased over the past few decades.

Bioethanol, which is considered one of the renewable energy sources, production became popular because of the rapid decrease of the fossil fuels. Because of this, exhaust gases of bio ethanol are much cleaner combustion that fossil fuels. Bioethanol production can lead to healthy environment because of the lower emission of air pollutants as well as the carbon neutral. Its production may also lead to free sulfur and aromatic. Sugar feedstock from starch is the main ingredient in the production of bioethanol globally. She also stated that out of the total production, 61% is accounted to sugar crops while the remaining 39% is derived from starch as feedstock. In the Philippines, sugarcane is the main component for the production of ethanol while in the United States; corn is considered the main component in their bioethanol production. Nevertheless, considering the increasing demand for human food, using this feedstock for bioethanol production may be a competitor for human and livestock consumption.

In order to meet the great demand of bioethanol, it is best that the bioethanol production is from cheap raw materials. In this case, production of bioethanol should be derived from cheap raw materials such as agricultural wastes, fruit wastes, vegetable wastes, municipal and industrial wastes.

Materials that contain sugar such molasses, sugarcane (cane juice or cane syrup), cereal crops, sugar beet and sweet sorghum and other materials that contain sugar are fermented to produce bioethanol. Increasing focus on using the lignocellulosic biomass became the result of the development in the biotechnology (1). In these studies, the lignocellulosic biomass is used in the production of liquid fuels and other chemical that is used in bioethanol production. Many biomass substrates have high content of cellulose and hemicellulose and have been enumerated to be of great potential for bioethanol production. The pretreatment step of the raw materials is the main challenge in the conversion of ethanol from biomass. In the pretreatment step, the structure of

the ligocellulosic complex needs to be degraded (2). The steps that need to be done in the pretreatment are the removal of lignin, the partial or total hydrolysis of the hemicellulose, the decrease in the fraction of crystalline cellulose, and subsequently, the hydrolysis step. In the hydrolysis step, in order to obtain glucose that is converted into ethanol by microorganisms, the cellulose undergoes enzymatic hydrolysis (3). Eventually, ethanol is the result of the conversion of the sugars that is released during the hydrolysis of hemicellulose (4). Industrially, two processes can be used in the hydrolysis and fermentation of the pretreated materials; the separate hydrolysis and fermentation (SHF). Hydrolysis or fermentation can also be done in one single step as identified to be simultaneous saccharification and fermentation (SSF) (5). There are species that have been reported that has the ability to directly ferment cellulose into ethanol. These species are *Neurospora, Monilia, Paecilomyces and Fusarium sp* (6).

A flowering plant in the family Fabaceae is the golden shower or the Cassia fistula. The golden shower tree is a medium-sized tree which grows to 10-20 meters tall and is fast growing tree. Since the fruit of golden shower is considered as waste material, hence, this study was conducted to make use of the large volume of the residue as feedstock to test its potential for bioethanol extraction.

The objective of the study is to obtain bioethanol from golden shower fruit residues. Specifically, it aims to determine the volume of ethanol produced based on the duration of fermentation and sugar content of the feedstock, and to determine the cost of bioethanol production from golden shower fruit.

Materials and Methods:

The flow diagram in the production of bioethanol from golden shower fruit is shown in Fig. 1. The following is the process used in the production of bioethanol from golden shower fruit.

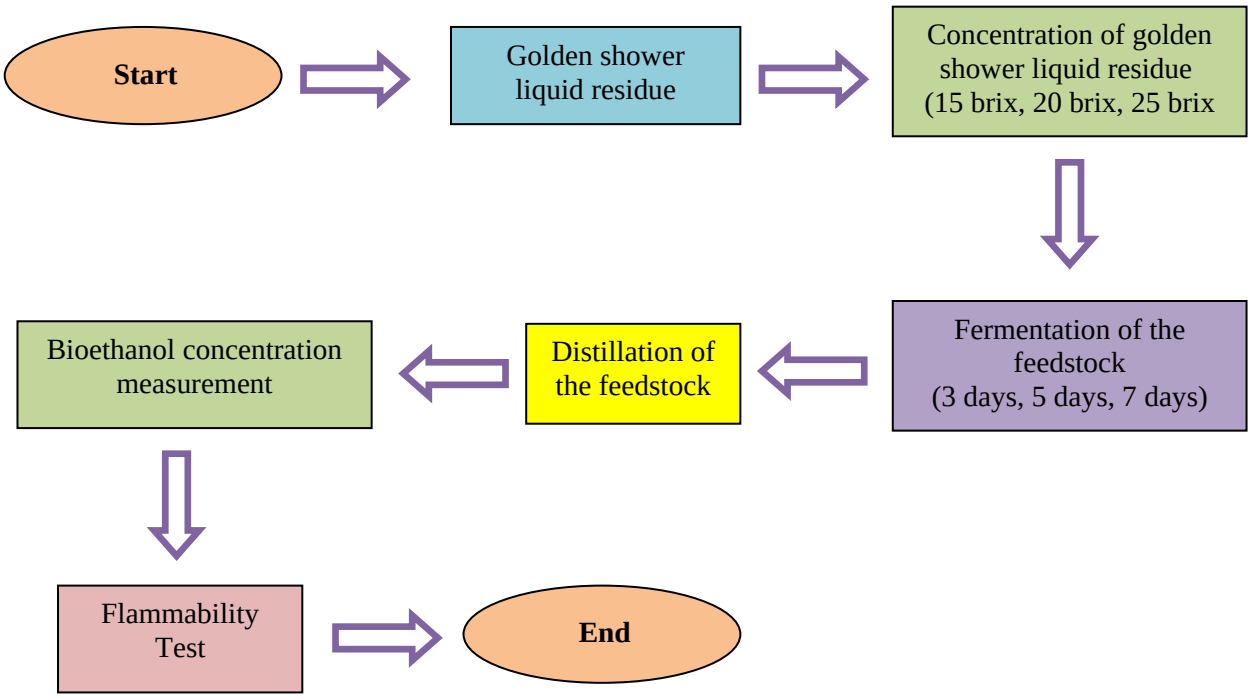

Figure 1. Flow diagram of the production of bioethanol from golden shower fruit.

Collection of Raw Materials

The golden shower fruit was collected from a farm in Camiling, Tarlac. Only the matured fruits are collected. The collected golden shower fruit were chopped using a multi-crop chopper available in the University. Twenty – five kilograms (25 kg) of chopped golden shower fruit with enough amount of water were boiled in a container (Fig. 2) for 6 hours. The boiled golden shower fruit was placed in a screen and poured with water to remove the gelatinous texture from the impurities. The poured water was collected and served as the liquid residue for the study.

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Figure 2. Golden shower fruit was boiled with enough amount of water.

Concentration Process

The initial sugar content of the golden shower fruit residue was measured using the refractometer. The refractometer was calibrated first by putting few drops of distilled water on the prism. The screw calibration was turned to adjust it to zero reading.

After calibrating the refractometer, a few drops of a sample from the liquid residue was placed on the prism and the sugar content was obtained and recorded. If the sugar content is below the desired sugar content, the golden shower fruit residue was heated until the desired sugar content of the feedstock was obtained.

The golden shower fruit liquid residue was divided into three parts. Each part was allowed to boil. The first batch of golden shower fruit liquid residue was boiled for 1.5 hours (1 hour and 30 minutes) to obtain the 15 brix. Wood and biomass stove was used as the source of heat. The golden shower fruit liquid residue was stirred continuously and the concentration of sugar was checked every 30 minutes using the refractometer.

The second part underwent through the same process, boiled and stirred continuously, until 20 brix was obtained. Also, sugar content was monitored at 30 minutes interval. After 2.5 hours, sugar content of 20 brix was obtained.

The third part was also boiled and stirred continuously until the sugar content reached 25 brix. Also, the sugar content was obtained every 30 minutes while boiling. After 3 hours, sugar content of 25 brix reading was obtained from the refractometer.

Fermentation

After attaining the desired sugar content in each part, the feedstock was set aside to cool down. For each sugar content level, nine liters of feedstock were obtained. One liter of the feedstock was measured (using the graduated cylinder) was placed in container.

Meanwhile, the optimum weight of yeast per liter of feedstock is 1.5 g/L. Yeast was weighed using the electronic scale. The yeast weighing 1.5 grams was dissolved in warm water and mixed continuously until bubbles appeared. The bubbles in the mixture indicate that the yeast is already active (7). In every container, a mixture of 1.5 grams of yeast was transferred into the liter of feedstock. An airlock using the hose and water seal (Fig. 3) were connected to the container to avoid the entry of air in the feedstock during the fermentation process. Absence of air should be done in the fermentation process to be able to produce bioethanol, otherwise, ethanoic acid will be produced (8).

Figure 3. Airlock of the feedstock during the fermentation process.

The samples were placed in a shaded area. For each sugar content level, three liters were subjected 3 days of fermentation, other 3 liters to 5 days of fermentation, the remaining 3 liters were fermented for 7 days. The fermentation performance was monitored by weighing the fermentation bottles every 6 hours of fermentation. The weight loss is due to the product of fermentation, ethanol and carbon dioxide (9).

Distillation

In the extraction of bioethanol, distillation of the fermented feedstock took place. A reflux distiller was used to distill the feedstock. The sugar concentration of the fermented broth was checked in the refractometer before fermentation. In the fermentation process, the sugar is broken down with the aid of the yeast enzyme zymase. The gas that bubbles into the air is the carbon dioxide while the alcohol in the mixture with the water is the ethanol.

On the third day of fermentation, the feed stocks were placed in a reflux distiller and heated using the electric stove. The hose for the condenser of the distiller was connected to a tap water with a flow rate of 600 ml/min. The water flow rate was measured by filling a container with a tap water

within a minute. The amount of water was measured using a graduated cylinder. Too much flow of water in the condenser made it hard to reach a higher temperature to produce bioethanol. The desired temperature range for bioethanol production is 78 ºC to 98 ºC. A thermostat is connected to the distiller to determine the temperature. After 20 minutes of heating, 78 ºC was obtained and first drop of distillate was obtained. The distillation process was stopped when no distillate was collected. The collected distillates were measured using the graduated cylinder. The obtained volume was recorded. The same procedure was done for the 5 days and 7 days fermentation.

Concentration of Ethanol

The concentration of ethanol of the distillates was measured using the hydrometer. The collected distillates for each treatment were placed in a graduated cylinder. The hydrometer was allowed to float on the distillate as shown in Fig. 4. The concentration of the bioethanol was read and then recorded.

Figure 4. Concentration of ethanol determination using the hydrometer.

Flammability Test

To test the flammability of the obtained distillates, few drops of distillates from each sample were placed on the floor and lit using a lighter.

Discussion:

After the collection of the golden shower liquid residue, the sugar content was determined. The sugar content of the samples will determine the ability of the samples to be converted to bioethanol. The obtained initial sugar content of the liquid residue was 3 brix. Fig. 5 shows the initial brix reading.

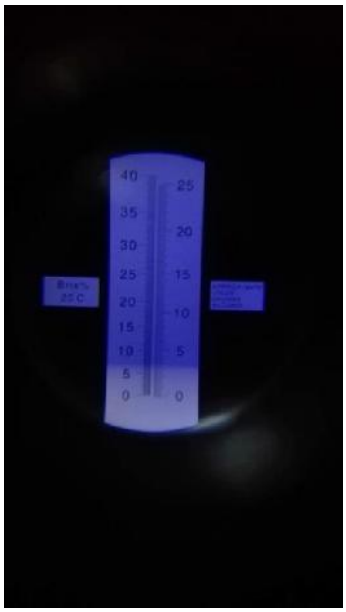

Figure 5. Initial brix reading using the refractometer.

The first batch of liquid residue was concentrated for 1.5 hours. After 60 minutes of boiling and continuous stirring, the sugar concentration of the liquid residue became 10 brix. The golden shower fruit liquid residue became 15 brix after another 90 minutes of continuous stirring. The second batch of liquid residue was concentrated for 2.5 hours. After 1 hour of the same procedure, the sugar content reading became 14 brix. After 2 hours and 30 minutes passed, the concentration of the sugar became 20 brix. The last batch of liquid residue was concentrated for 3 hours with the same procedure. After 150 minutes, the concentration of sugar in the broth became 18 brix. The sugar concentration became 25 brix after 180 minutes.

During fermentation, the weight of the samples was decreasing. The decrease in weight of the samples indicated active fermentation of the yeast. The sugar concentration of the feed stocks was read using the refractometer. The difference of the sugar content reading before and after fermentation shows the alcohol converted during the fermentation process.

Based from the results, the average extracted bioethanol from the samples is 77.96 mL. Treatment T1A3 (25 brix x 7 days) gave the highest extracted bioethanol from the fermented feedstock with an amount of 139.33 mL while treatment T3A1 (15 brix x 3 days) gave the least extracted bioethanol from the fermented feedstock with an amount of 41.33 mL. The results obtained are similar to the study conducted stating that the

ethanol content increased with increasing fermentation time in both 27 ºC and 32 ºC, though, the increase was higher at 32 ºC. The highest ethanol content was obtained on day 5 in temperature at 32 ºC (10).

Table 1 shows the obtained bioethanol using the reflux distiller using the combination of different days of fermentation and different sugar concentration. Analysis of Variance revealed significant differences on the extracted amount of bioethanol from different treatments. T1A3 and T3A1 were significantly different to all treatments. All other treatments were not significantly different from each other.

Table 1. Extracted bioethanol (mL) from the feedstock.

Treatment	R1	R2	R3	Total	Mean	
T1A1 (25 brix, 3 days)	70.0	65.0	72.0	207.0	69.00	d
T1A2 (25 brix, 5 days)	92.0	87.0	85.0	264.0	88.00	bc
T1A3 (25 brix, 7 days)	123	143	152.0	418.0	139.33	a
T2A1 (20 brix, 3 days)	65.0	60.0	69.0	194.0	64.67	ef
T2A2 (20 brix, 5 days)	75.0	72.0	80.0	227.0	75.67	cd
T2A3 (20 brix, 7days)	90.0	110	97.0	297.0	99.00	b
T3A1 (15 brix, 3 days)	40.0	45.0	39.0	124.0	41.33	h
T3A2 (15 brix, 5 days)	50.0	62.0	55.0	167.0	55.67	fg
T3A3 (15 brix, 7 days)	65.0	72.0	70.0	207.0	69.00	de
Grand total				**2105.0**		

Note: Means with the same letter are not significantly different at 1% level by DMRT.

Table 2 shows that the different level of sugar content has significant effect on the bioethanol produced. The treatment using 25 brix has the largest amount of bioethanol produced followed by 20 brix and 15 brix has the least amount of bioethanol produced. Also, the days of fermentation affect significantly on the amount of bioethanol extracted. Treatments under 7 days fermentation produced the largest amount of bioethanol. It is followed by 5 days fermentation and 3 days fermentation.

Table 2. Mean amount of bioethanol produced in mL.

Treatment	A1	A2	A3	Total	Mean	
T1	207.0	264.0	418.0	889.0	296.3	a
T2	194.0	227.0	297.0	718.0	239.3	b
T3	124.0	167.0	207.0	498.0	166.0	c
Total	525.0	658.0	922.0			

Note: Means with the same letter are not significantly different at 1% level by DMRT.

The concentration of the extracted ethanol was measured using a hydrometer. The results of concentration ranges from 95% - 97% due to the efficiency of the reflux distiller used in the study.

The bioethanol produced was lit using the lighter. The flame produced by the samples was blue which indicates complete combustion process. Figure 6 shows the flame produced from the extracted bioethanol.

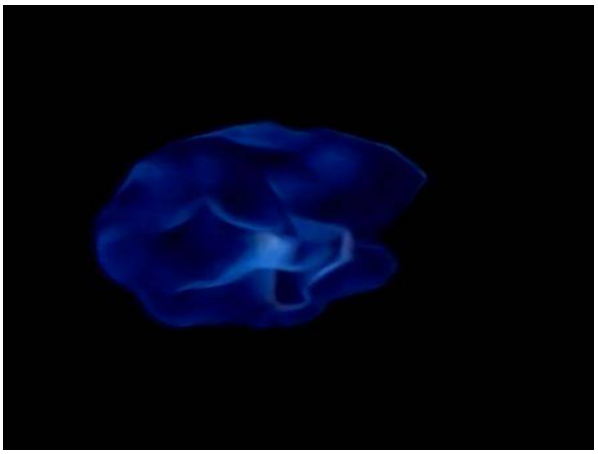

Figure 6. The flame produced by the extracted bioethanol.

The materials used in the extraction of bioethanol from golden shower fruit and the cost of production of bioethanol from golden shower fruit entailed an amount of Php50.89 in every liter of bioethanol obtained.

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Author's declaration:

- Conflicts of Interest: None.
- I hereby confirm that all the Figures and Tables in the manuscript are mine. Besides, the Figures and images, which are not mine, have been given the permission for re-publication attached with the manuscript.

- Ethical Clearance: The project was approved by the local ethical committee in Tarlac Agricultural University.

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تحديد نوعية وكمية انتاج الايثانول الحيوي باستخدام فاكهة الدش الذهبي (ناسور كاسيا)

إيمي ليزبيث ج.ريكو

كلية الهندسة والتكنولوجيا، تارلاك، الجامعة الزراعية، الفلبين

الخلاصة:

يعد استنفاد الوقود الأحفوري أحد الأسباب الرئيسة وراء انتشار الإيثانول الحيوي حيث إنه مصدر للطاقة المتجددة. ومن أجل تلبية الطلب الكبير على الإيثانول الحيوي ، فمن الأفضل أن يكون إنتاجه من مواد خام رخيصة. لكن بالنظر لعدم استخدام فاكهة الدش الذهبية بل اعتبارها مادة نفايات ، فقد أجريت هذه الدراسة للاستفادة من الحجم الكبير من المخلفات كمادة وسيطة لاختبار امكانيتها لاستخراج الإيثانول الحيوي.الهدف الرئيسي من هذه الدراسة هو الحصول على أكبر حجم من الإيثانول الحيوي من بقايا سائلة فاكهة الدش الذهبي حسب العوامل وأيام التخمير (3 و 5 و 7 أيام) وتركيز السكر (15 و 20 و 25 بريكس). ان جزء من الدراسة كذلك هو حساب تكلفة الإنتاج في استخراج الإيثانول الحيوي من هذه الفاكهة.تم تكرار كل علاج ثلاث (3) مرات عن طريق استخدام التحليل ثنائي العوامل للتباين للتصميم العشوائي الكامل لتحليل المعالجات. وقورنت العلاجات باستخدام اختبار دنكان متعدد النطاق.

الكلمات المفتاحية: الايثانول الحيوي، الاستخراج، التخمير، الطاقة المتجددة، النفايات.

Effect of different carbon doses of tapioca (*Manihot esculenta*) flour on vegetative cells and spore production of *Bacillus megaterium*

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Effect of different carbon doses of tapioca (*Manihot esculenta*) flour on vegetative cells and spore production of *Bacillus megaterium*

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Abstract. *Bacillus megaterium* is a spore forming bacteria that is mostly used as a probiotic in aquaculture. Spore formation for probiotic production using carbon source is costly. This research evaluated the effect of different carbon doses of tapioca (*Manihot esculenta)* flour on vegetative cells, spore production, sporulation efficiency and spore germination of *B. megaterium*. Experiments were carried out in Aquaculture Laboratory, University of Brawijaya. In flasks, 50 ml of growth media were used and were inoculated with 1% of *B. megaterium* (2.6 x 10^8 cells/ml). The cultures were mixed (120 rpm) and incubated at 37 ^{0}C for 120 hours with 4 different treatments of carbon doses (5, 10, 15 and 20 g/L, respectively). The results showed that the highest vegetative cells (8.4 x 10^7 cell/ml) and spore production (4.1 x 10^7 spore/ml) of *B. megaterium* were found at 15 g/L carbon dose. However, it was not followed by high sporulation efficiency (only 49.01%). The high spore germination was observed in more than 5 g/L carbon dose. Thus, 5-15 g/L carbon doses of tapioca flour could positively enhance vegetative cells, spore production, sporulation efficiency and spore germination of *B. megaterium* and could be used as a potential source of probiotics in aquaculture.

1. Introduction

Bacillus is a genus that commonly used as a probiotic in the aquaculture industry [1,2]. Several advantages of using it for the aquatic animal are supporting digestive fish function through an essential enzyme production [3], reducing ammonia and nitrite concentration [4] and improving immune system and health status of fish [5]. On the other hand, in aquaculture practice, there are some problems of probiotic in the vegetative cell such as inactivated bacteria in an acid environment (stomach) [6] and stressed in limited of nutrition [7]. The impact of this case is bacterial cell damage [8] consequently it will not work optimally in the digestive tract of an organism. One of the solutions to repair the problems is by using spore bacillus as probiotic [9]. The spore is dormant, resistant to lack of nutrition, heat temperature and organic chemicals [10,11]. The spores can survive in a long time even hundreds of years [12]. One of *Bacillus* species that produce spore is *B. megaterium* which has exosporium as a special characteristic in the outer membrane [13].

Production of *B. megaterium* spore requires medium that consisted of carbon source. Glucose and lactate as commonly commercial carbon source but they have costly [14,15]. Alternative carbon source that could be used in medium to produce spore comes from local farming product such as tubers and beans. Tapioca (*Manihot esculenta*) flour is a carbon source with the composition of organic carbon more than 40% based on experimental study (data unpublished). In Indonesia, tapioca flour is cheap, easily found and contained high carbon content. Development of low-cost medium is needed to reduce the cost of spore production which can be used as a probiotic in fish farming. Thus, this study evaluated the effect of different carbon levels of tapioca flour on vegetative cells, spore production, sporulation efficiency and spore germination of *B. megaterium*.

2. Materials and methods

2.1 Strain of bacteria

B. megaterium used in this study was isolated from shrimp pond in Tuban East Java which had confirmed by molecular analysis 16SrRNA.

2.2 Culture Media

Tapioca flour was used as the carbon source and ammonium chloride (NH_4Cl) served as nitrogen source. The doses of the carbon were divided by four treatments (5, 10, 15 and 20 grams per liter of distilled water (Aquadest®), respectively), while the nitrogen doses were adjusted to the carbon doses to achieve C:N ratio 5:1. Culture media consisted of tapioca flour and ammonium chloride based on treatments were dissolved with Aquadest® and enhanced with some minerals such as $CaCO_3$ (0.3 g), $MgSO_4$ (0.00033), $MnSO_4$ (0.12 g), $FeSO_4$. (0.084 g), $CaCl_2$ (0.09 g) [16], with little modifications. All treatments were replicated three times.

2.3 Inoculum preparation

Bacteria from the frozen stock were inoculated by a loop needle to a 100 mL shake flask containing 20 mL of nutrient broth as an inoculum medium. The culture was incubated in rotary shaker at 37 ^{0}C and 120 rpm for 18 hours. Bacterial cells were counted as an initial density that would be the stock culture.

2.4 Cultivation condition

The research used 100-ml-erlenmeyer flasks with 50 ml medium and were inoculated 1% (v/v) stock culture of *B. megaterium* (2.6×10^8 cells. ml^{-1}) in each flask. The culture conditions were similar to inoculum preparation in rotary shaker (at 37 ^{0}C, 120 rpm) for 24 hours of vegetative cells and continued to 120 hours for sporulation. In continuum, the spore germinations were carried out in the 100-ml-erlenmeyer flasks with 50 ml nutrient broth medium and were inoculated for high spore production in all treatments. The desired density of spore germination in each of treatments was 10^6 cfu/ml.

2.5 Determination of vegetative cells, spore count, sporulation efficiency and spore germination

Characteristics of vegetative cells and spore were observed and determined by their shapes. Vegetative cell has a rod-shaped and spore has a circle one. They were diluted with Aquadest® for sterilization to simplify counting before counting in the Neubauer chamber. Sporulation efficiency (%) was determined based on the ratio of maximum spore production and vegetative cells [17]. Spore germination was calculated by the number of spores that have changed to the vegetative cell.

2.6 Statistical analysis

All data were analyzed by one-way ANOVA in statistical software. The differences of all treatments were tested using Duncan's Multiple Range Test ($P<0.05$).

3. Result and discussion

3.1 Vegetative Cells of B. megaterium

Vegetative cells production of *B. megaterium* cultured at different carbon (tapioca flour) levels are presented in Figure 1.

The highest vegetative cells were obtained at carbon dose 15 g/L with a density of 8.4 x 10^7 cells/ml. Overall, result shows that the higher carbon doses, the higher vegetative cells produced. However, at 20 g/L carbon dose, the peak of production was reached of vegetative cells and were found lower than carbon dose 15 g/L. This condition is caused by the amount of carbon available in the environment exceeds the limits of the bacterial ability to use carbon as a nutrient that supports their lives. In giving too much carbon, the bacteria could not utilize and absorb all the carbon available optimally, although incubation time before 10 hours has the highest density when given 20 g/L carbon doses. Carbon is a limiting factor for bacterial growth [18,19]. The main principle of carbon as a limiting factor occurs when the bacterial growth rate increases with an increasing C/N ratio [20]. Excessive of acetate concentration as the carbon source in the environment causes unbalanced carbon metabolism, therefore, it can inhibit the bacterial cellular growth [21].

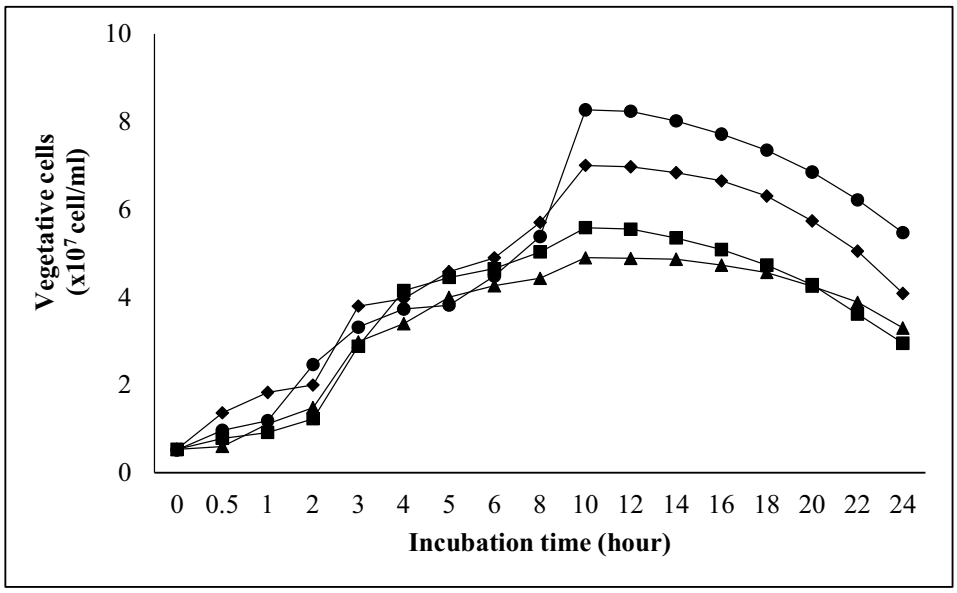

Figure 1. Time Series of vegetative cells production of *B. megaterium* under different carbon doses of tapioca flour (▲: 5 g/L; ■: 10 g/L; ●: 15 g/L; ◆: 20 g/L)

In all treatments, the highest vegetative cell was found at the incubation time of 10 hours, then the stationary phase occurred until the 12 hours. Afterwards, a phase of decreased vegetative cells occurred until 24 hours of incubation time. Vegetative cells decline is caused by the longer incubation time affect the availability of the nutrients in the culture media decreases. Nutrients derived from carbon source (tapioca flour) and nitrogen source (ammonium chloride) are used as food sources for the lives of these bacteria. Bacteria require carbon as constituents of cells and energy source while the nitrogen as the main element in amino acids and protein [22]. Decreasing of vegetative cells is influenced by inappropriate culture media such as lack of nutrients, pH, oxygen and temperature [23]. Apart from cell death, this research reveals that a declining vegetative cell population is also indicated by the formation of *Bacillus* spores to survive in the environmental conditions that are less supportive of life.

3.2 Spore production of B. megaterium

Spore-forming bacteria can change themselves from vegetative cells to spores when an unfavourable environmental condition (nutrients decline) [24]. The production of *B. megaterium* spores in the culture media with different carbon doses are presented in Figure 2.

B. megaterium spore initially appeared 14 hours of incubation in almost all treatments of carbon doses of tapioca flour. The beginning of the spore formation was after the growth of vegetative cell of *B. megaterium* reached a peak. Initial spores continued to increase in line with a decrease of vegetative cell density [25]. At 14 – 24 hours of incubation, the spores in all treatments increased gradually and reached a stationary phase at 120 hours. The highest spore production was with dose level of 15 g/L dose (4.1 x 10^7 spore/ml) and showed almost similar results when given lower dose of 5-10 g/L carbon dose. On the other hand, 20 g/L carbon dose showed a lower number of spores compared to other treatments (3.5 x 10^7 spore/ml).

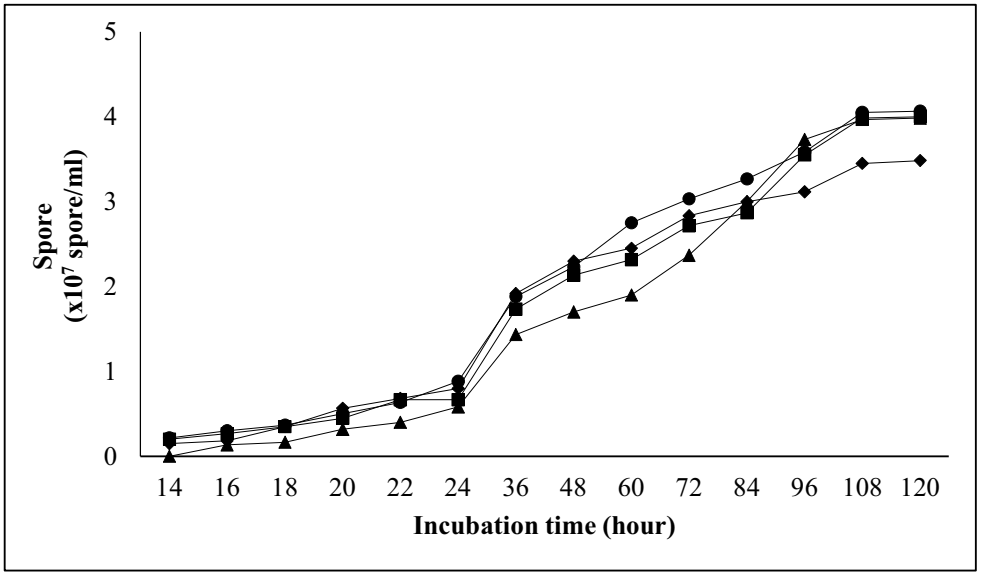

Figure 2. Time series of spores production of *B. megaterium* under different carbon doses of tapioca flour (▲: 5 g/L; ■ : 10 g/L; ● : 15 g/L; ♦ : 20 g/L)

The high vegetative cell was not an indicator of the high spore production. Given a carbon dose of 15 g/L, the vegetative cells count was 8.4 x 10^7 cell/ml but the spore production was recorded only at 4.1 x 10^7 spore/ml. Increasing amount of carbon doses affects the amount of carbon available in the culture media that impacted to bacterial lives. Other studies concurred with the recent result that the higher carbon doses given, the lower the spores produced. Glucose concentration as a carbon source showed that glucose with a dose of 3.5 g/L produces 4.3 x 10^9 spores/ml. Contrastingly, a dose of 20 g/L carbon produced 3.4 x 10^9 spores/ml. This condition inhibited the induction of several enzymes involved in the sporulation process [26]. Likewise, *Trichoderma harzianum* when given carbon source (glucose) 30 g/L, smaller spores count was recorded at 1.6 x 10^9 spore [27]. This case indicated that the dose of carbon depends on bacterial strain and carbon source that used in spore production.

3.3 Sporulation efficiency of B. megaterium

Sporulation efficiency is the percentage maximum vegetative cell that converts the highest spore production [17]. The sporulation efficiency of *B. megaterium* was seen in Figure 3.

The sporulation efficiency of *B. megaterium* was affected by carbon dose of tapioca flour. The highest of sporulation efficiency of *B. megaterium* was showed in the media with the carbon dose of 5 g/L with 80.97% sporulation efficiency. However, results showed no significant differences when

given with a higher dose of 15 and 20 g/L carbon levels. The high counts of vegetative cells and spores did not follow a high sporulation efficiency. Increasing the sporulation efficiency of *B. coagulans* can be expected through the addition of carbohydrates, mineral salts, pH in culture media [28]. In addition, the concentration of glucose as a carbon source should be reduced because it can decrease the results of sporulation efficiency [17]. In fact, the usage of low glucose content (2.0 g/L) increases spore production between 0.51 x 10^9 cfu/ml and 1.87 x 10^9 cfu/ml with sporulation efficiency 50.7% and 93.2%, respectively [29]. Another carbon source (acetate) only requires ≤ 0.04% to increase the sporulation efficiency of *Saccharomyces cerevisiae* [30]. The results shown by some of these researches indicated that each carbon source has an optimal dose to achieve the highest sporulation efficiency.

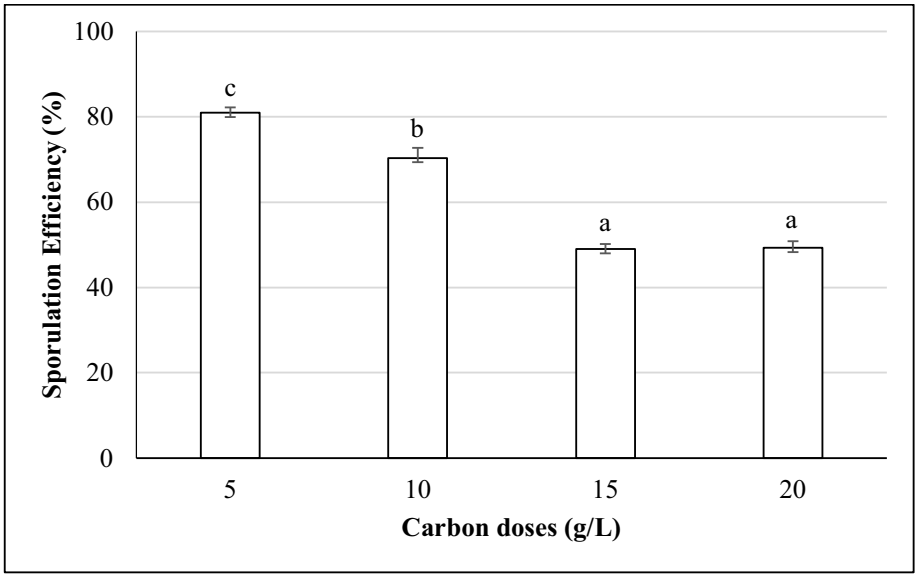

Figure 3. Sporulation efficiency of *B. megaterium* under different carbon doses of tapioca flour

3.4 Germination of B. megaterium

Germination process is the proliferation of spore to vegetative cell when the environmental supporting condition ensues [31]. The spore germination of *B. megaterium* was presented in Figure 4.

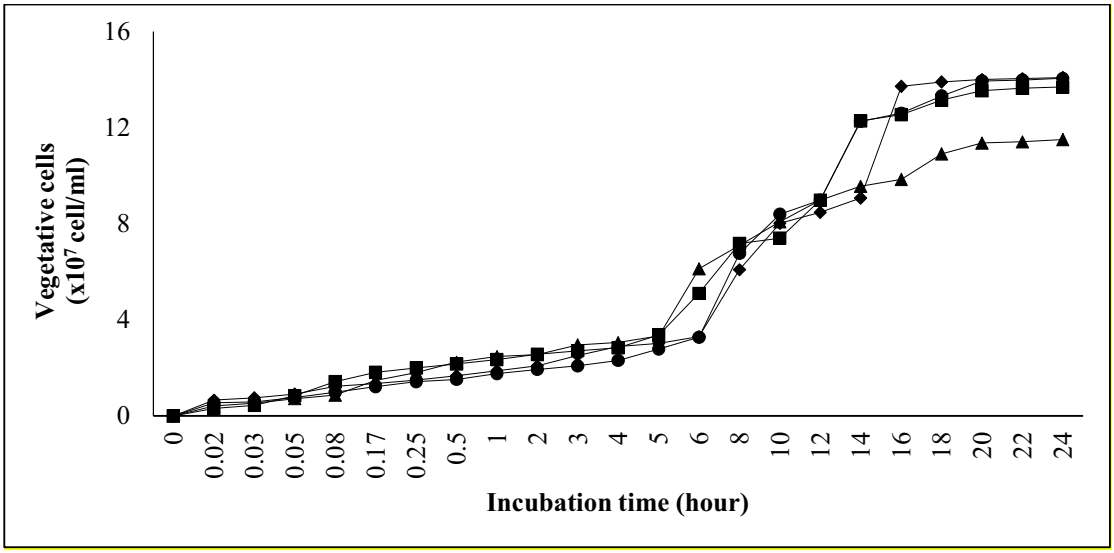

Figure 4. Time series of spore germination of *B. megaterium* under different carbon doses of tapioca flour (▲: 5 g/L; ■ : 10 g/L; ● : 15 g/L; ♦ : 20 g/L)

In this study, the spore germination of *B. megaterium* was recorded at five (5) hours since there was no significant difference between the different treatment means. The highest vegetative cell from spore germination was found more than 5 g/L of carbon dose. The entire treatments achieved 100% spore germination before 0.5 hours in the nutrient broth medium due to the presence of new nutrients that stimulated bacteria cell division. Other reasons from some studies showed that spore germination influenced by the heat activation of spore. The percentage spore germination reached 100% after 0.5 hours when heat activation of spore at 65 ^{0}C [32] different to this study did not use heat activation. Another study showed that *B. subtilis* produce lower spore germination at 52.9%, than when spore was not heated treatment with 94% spore germination rate [31]. The difference of this germination percentage was influenced by concentration, density of spore, incubation temperature, heat activation and oxygen [33].

4. Conclusion

Carbon doses of tapioca flour affected the vegetative cells, spore production, sporulation efficiency and germination of *B. megaterium*. The highest vegetative cells (8.4 x 10^7 cell/ml) and spore production (4.1 x 10^7 spore/ml) of *B. megaterium* were found in the carbon dose of 15 g/L with sporulation efficiency 49.01%. The spore germination optimally was observed in more than 5 g/L of carbon dose.

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Phytoconstituents Investigation on the Ethanolic Extract of *Azadirachta indica* var. Indonesian and Philippines

Phytoconstituents Investigation on the Ethanolic Extract of *Azadirachta indica* var. Indonesian and Philippines

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Abstract. In the present study, phytochemical screening of ethanolic extracts of *Azadirachta indica* leaves from Indonesia and the Philippines revealed the presence of different phytoconstituents. Preliminary qualitative chemical test, TLC and LC-MS were used. TLC for all the extracts showed bands in long UV 366 for presence of flavonoid, tannin, saponin, terpenoid. As a result of LC-MS analysis of ethanolic extract *Azadirachta indica* leaves from Indonesia and Philippines, 10 compounds from Indonesian varian and 7 compounds from the Philippines varian were detected using m/z value. In conclusion, phytochemical screening based on TLC and LC-MS/MS show diverse bioactive compounds in ethanolic extract *Azadirachta indica* leaves from Indonesia and the Philippines. These can be effective approach for selecting best quality of varian leaves and planting area.

1. Introduction

Characterize natural product for new drug discovery has been concerned lately. Some of herbal products prove to offer synthetic drug substance. There are several factors that influence the quality of the herbs. Variation, environment conditions, storage, processing can be those influence quality factors of the herbs. Characterization compounds of herbs extract can be a standard procedure to find out the quality of the herbs.

Azadirachta indica (Neem) leaves are native of dry areas (Rojas-Sandoval *et al.*, 2014). It was naturally distributed in Thailand, Malaysia, Philippines and Indonesia and has become one of the most widespread trees in tropical and subtropical areas. Neem plant as medicinal plant is reported to have antifungal (Lloyd *et al*., 2005), hepatoprotective (Pingale, 2010), anti-inflammatory (Jagadeesh *et a*l., 2014), anthelmintic (Beltran *et al.*, 2019), anti-cancer, insecticidal. The chemical constituents of *Azadirachta indica* leaves have a very important role in medicinal applications and it is believed due to its biologically active components.

In this present study, analytical method TLC and LC-MS was undertaken for identifying phytoconstituent. Thin layer Chromatography (TLC) is a very commonly used technique for identifying compounds, is a method of analysis in which the stationary phase, a finely divided solid, is spread as a thin layer on a rigid supporting plate; and the mobile phase, a liquid is allowed to migrate across the surface of the plate (Gennaro, 2000). This analytical tool is used because of its simplicity, speed of separation, cost effectiveness and high sensitivity.

LC-MS/MS are used for characterization and quantitation of herbal medicines because full characterization of these product. The advantages of LC-MS/MS are its high sensitivity and high-throughput to confirm the identity of the components in complex herbal extract, along with the detection and identification of unknown and unexpected compounds (Krug *et al*., 2008).

The aim of this research study was to assess the bioactive components present in the ethanolic extract of *Azadirachta indica* leaves varian Indonesia and Philippines using phytochemical screening and chromatographic analysis. So the result could be developed and applied to the pharmaceutical production and quality control of botanical product.

2. Material and Methods

2.1. Collection and Authentication of Plant Material

Fresh *Azadirachta indica* leaves were collected from Indonesia (Madura) dan Philippines (Camiling). The plant specimens were authenticated by Laboratorium of Plant Taxonomy, Universitas Brawijaya. Number identification of these plant is 0238/UN10.F09.42/03/2018.

2.2. Preparation of Plant Extract

The leaves were cleaned by washing with running water and shade dried and the milled to pass through 100-mesh sieve. The leaf powder was extracted by maceration for three days with 80% ethanol at room temperature. The extracts were concentrated at 45C using Rotary vacuum evaporator to yield 80% hydroethanolic fraction as brownish green viscous residue. The concentrated extracts were keep in refrigerator at 4°C until further use.

2.3. Preliminary Phytochemical Screening

Test for the presence and absence of phytochemical compounds using standard methods involves the addition of an appropriate chemical agent to all the extract in a test tube and shaken. The different qualitative chemical test were performed for establishing profile of given extract for its chemical composition. Phytochemical screening of ethanolic extract of *Azadirachta indica* varian Indonesia and Philippines were carried out for alkaloids, flavonoid, saponin, tannin, terpenoid.

2.3.1. Alkaloid. 500 µL of ethanolic extract of *Azadirachta indica* varian Indonesia and Philippines of 10,000 ppm put in a test tube, then added 0.5 mL 2% hydrochloric acid. The solution was divided into three tubes. Tube 1 solution was added 0.5 mL acid solution dilute as a comparison, tube 2 was added 2-3 drops of Dragendorff's reagent, and tube 3 was added 2-3 drops of Mayer's reagents. If tube 2 was formed orange precipitation and tube 3 was formed yellowish precipitation. It indicated the presence of alkaloids.

2.3.2. Flavonoid. 500 µL of ethanolic extract of *Azadirachta indica* var. Indonesia and Philippines of 10,000 ppm put in a test tube, then added 1-2 ml hot methanol 50%. The solution was mixed by Magnesium and 4-5 drops of hydrochloric acid concentrate. Formation of red or orange colour indicated the presence of flavonoid.

2.3.3. Saponin. 500 µL of ethanolic extract of *Azadirachta indica* var. Indonesia and Philippines of 10,000 ppm was diluted with 10 ml distilled water and this was shaken 1 minute. Formation of bubble was added 2 drops of HCl 1 N. The stable bubble indicated the presence of saponin.

2.3.4. Tannin. 500 µL of ethanolic extract of *Azadirachta indica* var. Indonesia and Philippines of 10,000 ppm was diluted with 1-2 ml distilled water and this was added 2 drops $FeCl_3$ solution.. Formation of green blackish colour indicated the presence of tannin.

2.3.5. Terpenoid. 500 µL of ethanolic extract of *Azadirachta indica* var. Indonesia and Philippines of 10,000 ppm was diluted with 0,5 ml chloroform and this was added 0,5 ml anhydrous acetic acid. Then 1-2 ml

concentrated sulphuric acid. The appearance of reddish brown or violet ring indicated the presence of terpenoids.

2.4. Thin Layer Chromatography

The presence of number of phytoconstituents flavonoids, saponins, tannins, terpenoid in ethanolic extract of *Azadirachta indica* var Indonesia and Philippines, which further became the basis for the phytochemical investigations through TLC on analytical plates over silica gel (TLC grade; Macherey-Nagel; Germany). The plates were dried in hot air oven at 110°C for 30 mins and then stored in a dry atmosphere and used whenever required. Samples were prepared by diluting the crude extracts of ethanol with respective solvent and then applied usually 1-10µl volumes to the origins of a TLC plate 1 cm above its bottom with the help of capillary tubes. After the application of the sample on the plate the plates were kept in TLC glass chamber (solvent saturated) than mobile phase was allowed to move through adsorbent phase up to top of the plate. The developed TLC plates were air dried. They were later spray with different spraying reagent. Rf were observed under long UV 366 nm. Calculation of Rf value is done with this formula:

$$Rf = \frac{migration\ distance\ of\ substance}{migration\ distance\ of\ solvent\ front}$$

2.5. LC-MS Screening

LC-MS screening was performed using LC-MS Thermo Scientific Dionex Ultimate 3000 RSL Cnano with micro flow meter in Lembaga Sentral Ilmu Hayati Universitas Brawijaya, Malang, equipped with Electrospray ionization (ESI). The LC-MS analytical data were optimized using a background subtraction technique of chromatography with the Analyst version: Compound Discoverer with mzCLoud MS/MS Library. The principle of this method is to reduce background. Data containing more real m/z were observed. Each compound was then identified from reference compounds based molecular weight of the structure.

3. Result and Discussion

3.1. Phytochemical Screening

The presence and absence of the phytochemical in *Azadirachta indica* var. Indonesia and Philippines are listed in the table 1. Our result revealed that flavonoid, tannin, saponin, terpenoids are present in ethanolic extract of *Azadirachta indica* var. Indonesia and Philippines.

Table 1. Result of phytochemical screening.

Constituents	Test performed	Ethanolic extract	
		Indonesia	Philippines
Alkaloid	Dragendorff's Test	-	-
	Mayer’s Test	-	-
Flavonoid	Hydrochloric acid Test	+	+
Saponin	Froth Test	+	+
Tannin	Ferric chloride Test	+	+
Terpenoids	Salkowski's Test	+	+

3.2. Thin Layer Chromatography Profiling

TLC profiling of all extracts gives result the presence of number of phytochemicals. Various phytochemicals gives different Rf values. Different Rf values of the compound reflect an idea about their polarity. Mixture of solvents can be used for separation of pure compound from plant extract. The presence of any significant bioactive natural product indicates the necessity of separation of the compound from the

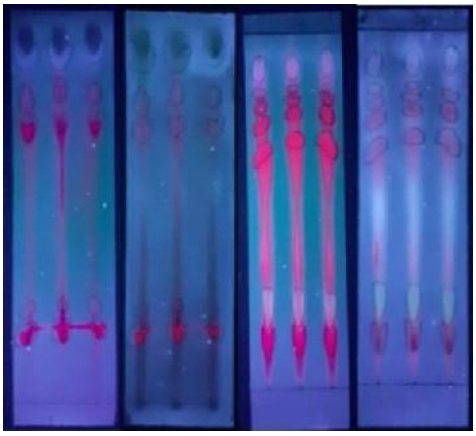
mixture of compounds through suitable chromatographic techniques. In the present study, flavonoids, saponin, tannin and terpenoid are confirmed to be present.

Table 2. Phytochemical analysis of *Azadirachta indica* leaves from Indonesia and the Philippines by Thin Layer Chromatography (TLC).

Chemical Name	Solvent System	Extract Neem Leaves	Rf Values	Spray Reagent
Flavonoid	ethyl acetate: methanol: aqua (5:1:5	Indonesia	0.03, 0.15	Ammonia
		The Philippines	0.06, 0.77	
Saponin	chloroform: methanol: aqua (13:7:2)	Indonesia	0.26, 0.87	Sulphuric acid 10%
		The Philippines	0.22, 0.85, 0.90	
Terpenoid	toluene: ethyl acetate (7:3)	Indonesia	0.19, 0.49	Liebermann burchard
		The Philippines	0.27, 0.32	
Tanin	n-butanol: acetic acid glacial: Aqua (2:0,5:1,1)	Indonesia	0.27	$FeCl_3$ 1%
		The Philippines	0.23	

(a) (b) (c) (d)

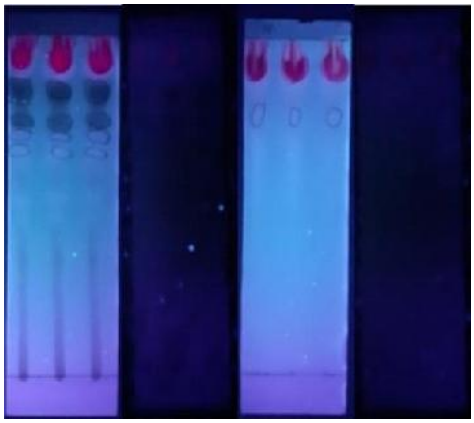
Figure 1. Photograph of TLC of flavonoid (a) Indonesian (b) Indonesian (after spray) (c) Philippines (d) Philippines (after spray).



(a) (b) (c) (d)

Figure 2. Photograph of TLC of saponin (a) Indonesian (b) Indonesian (after spray) (c) Philippines (d) Philippines (after spray).

Figure 3. Photograph of TLC of terpenoid (a) Indonesian (b) Indonesian (after spray) (c) Philippines (d) Philippines (after spray).



(a) (b) (c) (d)

Figure 4. Photograph of TLC of tanin (a) Indonesian (b) Indonesian (after spray) (c) Philippines (d) Philippines (after spray).

3.3. LC-MS Screening

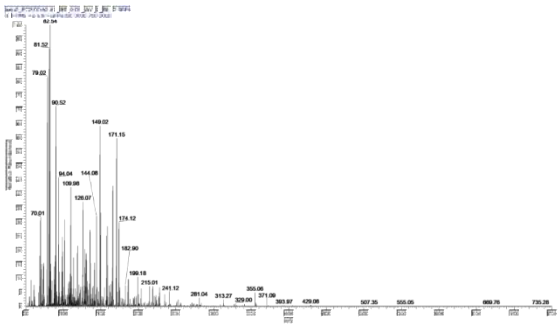


Figure 5. LC-MS chromatogram of ethanolic extract of *Azadirachta indica* varian Indonesia leaves.

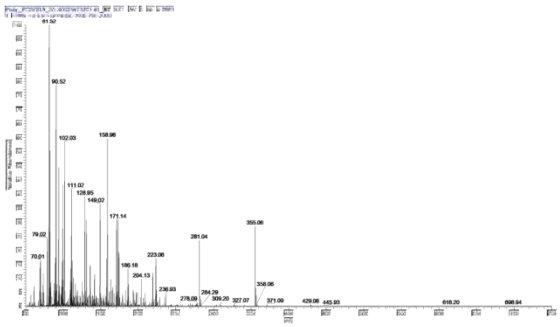


Figure 6. LC-MS chromatogram of ethanolic extract of *Azadirachta indica* varian Philippines leaves.

Table 3. Bioactive compounds in ethanolic extract of *Azadirachta indica* varian Indonesia leaves.

No.	Name of Compounds	Group	Molecular Formula	Molecular Weight	[M+HJ]+ (m/z) observed
1.	Betulin	Terpenoid	$C_{30}H_{50}O_2$	442.728	442
2.	Ginsenoside		$C_{42}H_{72}O_{13}$	785.025	785
3.	Caryophyllene oxide		$C_{15}H_{24}O$	220.356	220
4.	Soyasaponin I	Saponin	$C_{48}H_{78}O_{18}$	943.134	943
5.	Ecgonine	Alkaloid	$C_9H_{15}NO_3$	185.223	185
6.	Scutellarin	Flavonoid	$C_{21}H_{18}O_{12}$	462.363	462
7.	Epicatechin		$C_{15}H_{14}O_6$	290.271	290
8.	Icariin		$C_{33}H_{40}O_{15}$	676.668	676
9.	Sesamolin		$C_{20}H_{18}O_7$	370.357	370
10.	Lupeol		$C_{30}H_{50}O$	426.729	426

Table 4. Bioactive compounds in 80% ethanolic extract of *Azadirachta indica* varian Philippines leaves.

No.	Name of Compounds	Group	Molecular Formula	Molecular Weight	[M+HJ]+ (m/z) observed
1.	Betulin	Terpenoid	$C_{30}H_{50}O_2$	442.728	442
2.	Caryophyllene oxide		$C_{15}H_{24}O$	220.356	220
3.	Andrographolide		$C_{20}H_{30}O_5$	350.455	350
4.	10-Deacetylbaccatin III		$C_{29}H_{36}O_{10}$	544.597	544
5.	3-Acetyl-11-keto-β-boswellic acid		$C_{32}H_{48}O_5$	512.731	512
6.	O-chloroacetylcarbamoylfumagillol		$C_{19}H_{28}ClNO_6$	401.884	401
7.	Rutin	Flavonoid	$C_{27}H_{30}O_{16}$	610.521	610

On the basis of the LC-MS the known compounds, 10 compounds (4 terpenoids, 1 alkaloid, 2 flavonoids, 2 lignins, 1 saponin) were identified from 80% ethanolic extract of Azadirachta indica varian Indonesia leaves and 7 compounds (6 terpenoids, 1 flavonoid) were identified from 80% ethanolic extract of Azadirachta indica varian Philippines leaves. Betulin and caryophyllene oxide were identified in both varian.

4. Conclusion

In the present study, ethanolic extract of *Azadirachta indica* var. Indonesia and Philippines showed the presence of bioactive compound such as flavonoids, terpenoids, saponins, tannin. This study also leads to the further research in the way of isolation and identification of the active compound from the leave of *Azadirachta indica* var. Indonesia and Philippines using chromatographic and spectroscopic techniques.

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