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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.

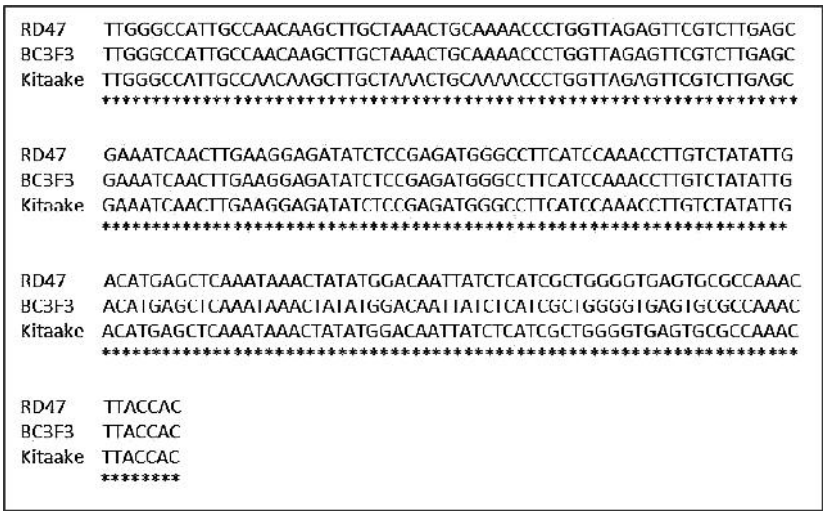

Fig. 1 Multiple sequence alignment of *XIK1* sequences in RD47 and improved BB-resistant BC_3F_3 progenies compared with the japonica cultivar Kitaake (CLUSTAL O 1.2.4).

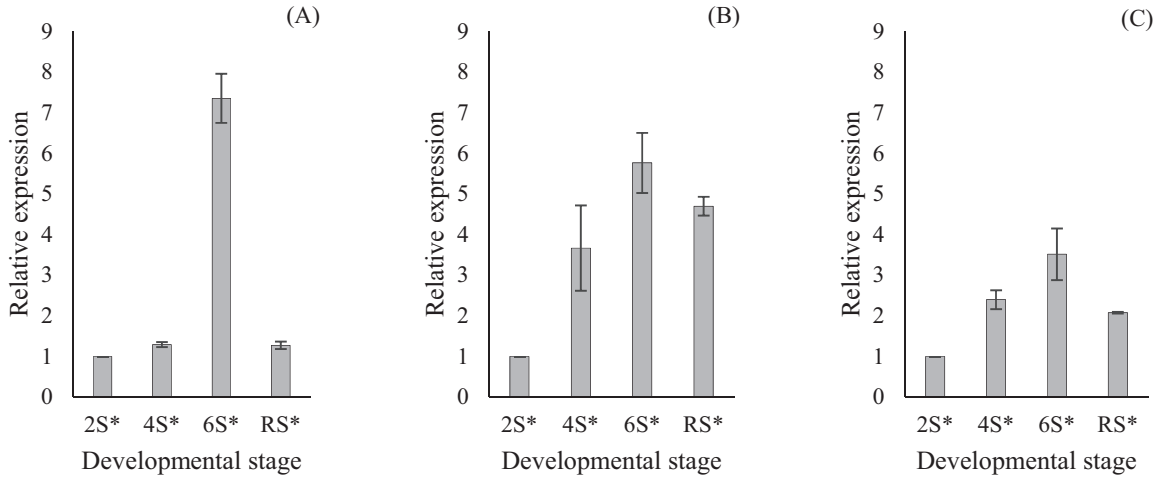

Fig. 2 Relative expression of *XIK1* in: (A) RD47; (B) BC_3F_3 progenies; (C) relative expression of *Xa21* in BC_3F_3 progenies in various growth stages, where 2S, 4S, 6S and RS represent the 2-leaf, 4-leaf, 6-leaf, and reproductive stages, respectively and asterisks indicate significant differences 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

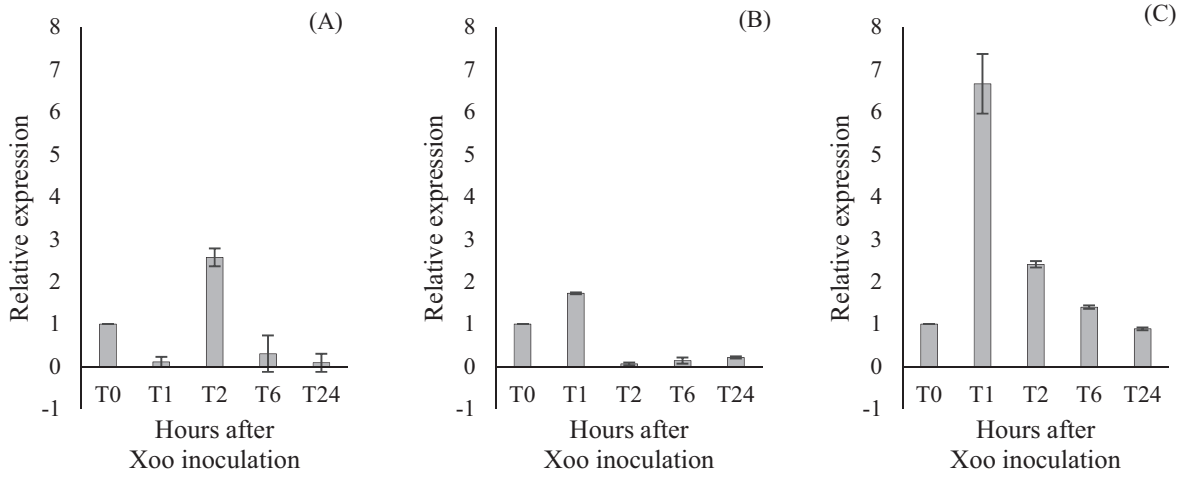

Fig. 3 Relative expression of *XIK1* in: (A) RD47; (B) BC_3F_3 lines; (C) relative expression of *Xa21* in BC_3F_3 lines after *Xoo* inoculation, where 0 hr, 1 hr, 2 hr, 6 hr and 24 hr post inoculation are presented as T0, T1, T2, T6, and T24, respectively and error bars indicate SD of the data analyzed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

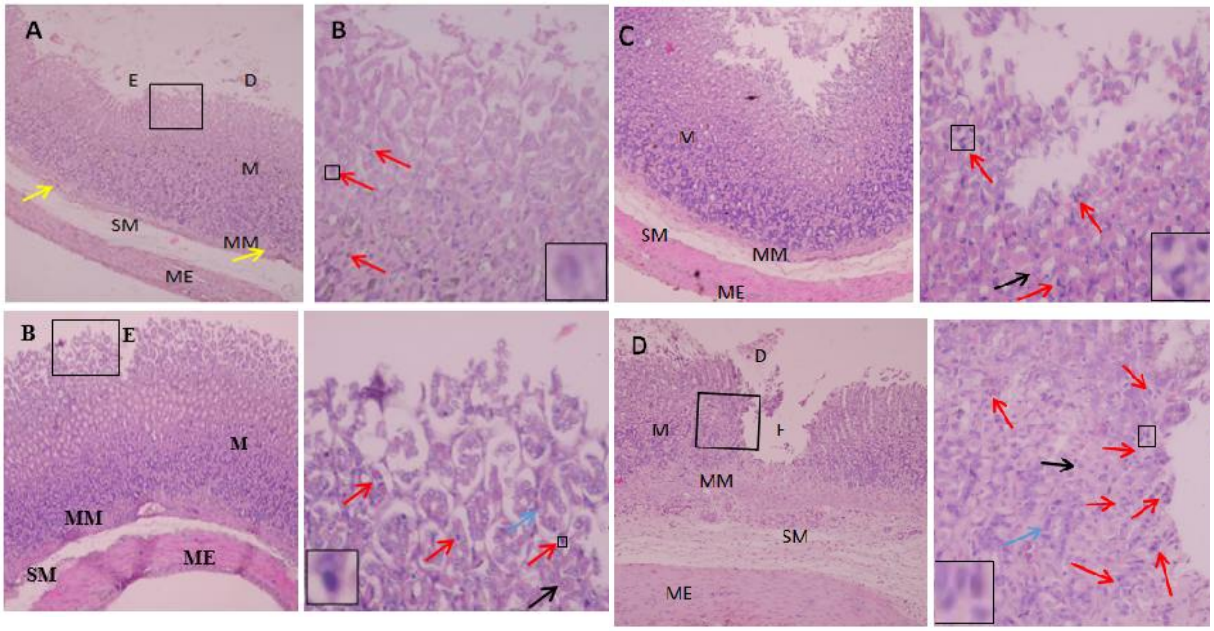

Figure 1. Histopathology of gastric mucosa. (A). Gold Standard group; (B). *P. longfolia* extract from Indonesia therapy; (C). *P. longifolia* extract from The Philippines, and (D) IBD condition.

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.

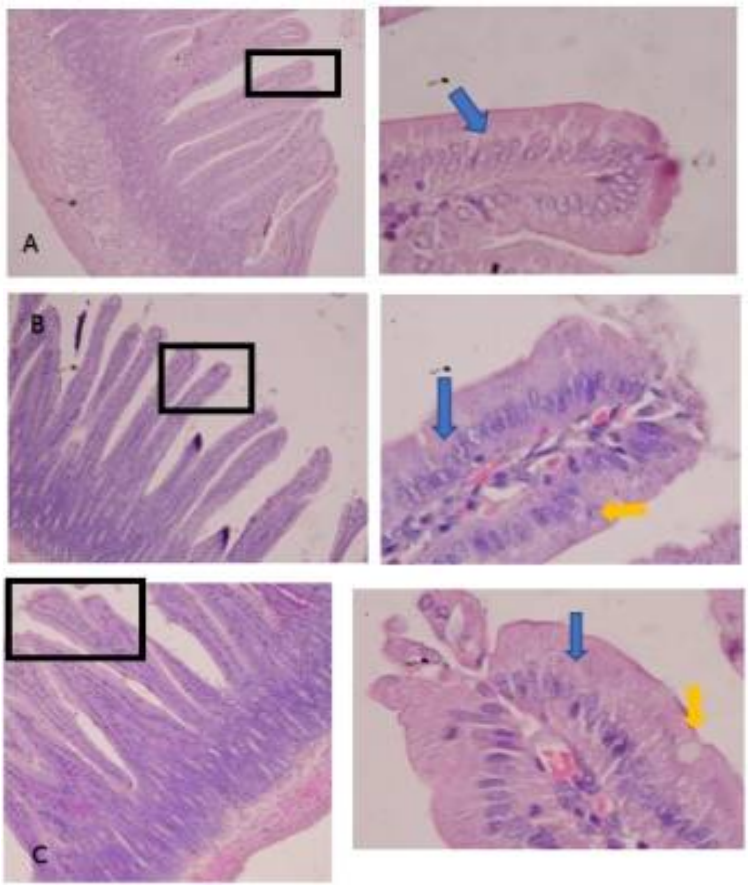

Figure 2. Histopathology of duodenum. (A). Gold Standard group; (B). P. longifolia extract from Indonesia therapy; (C). P. longifolia extract from Philippines.

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).

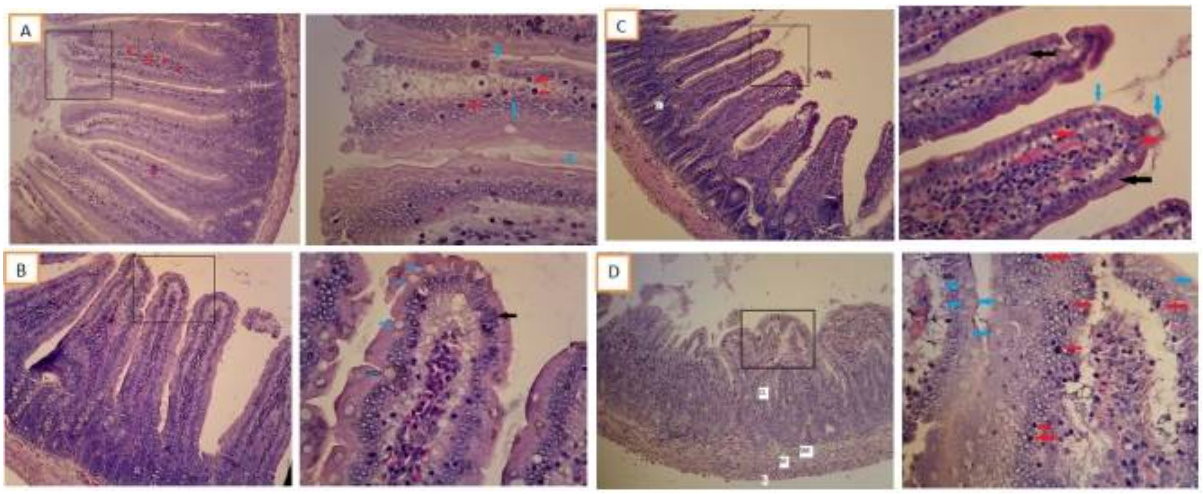

Figure 3. Histopathology of jejunum; (A). Gold Standard group; (B). *P. longifolia* extract from Indonesia therapy; (C). *P. longifolia* extract from Philippines, and (D) IBD condition.

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].

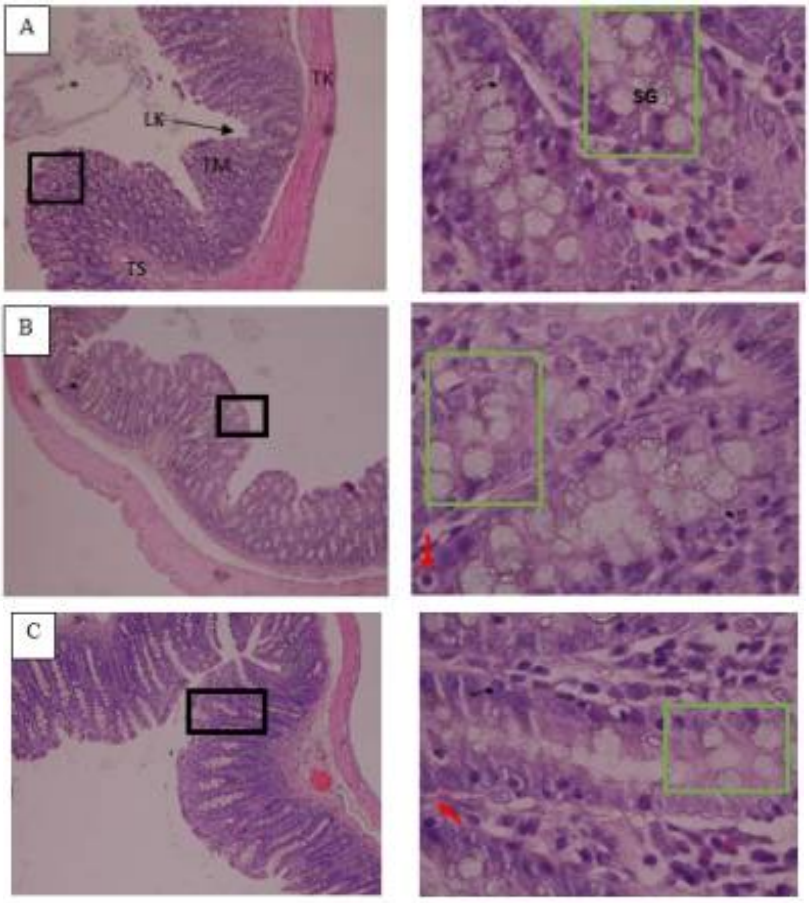

Figure 4. Histopathology of colon. A. Gold Standard group; B. EEPL from Indonesia group; C. EEPL from The Philippines group.

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)	√	-	-	-	√	√	√	√

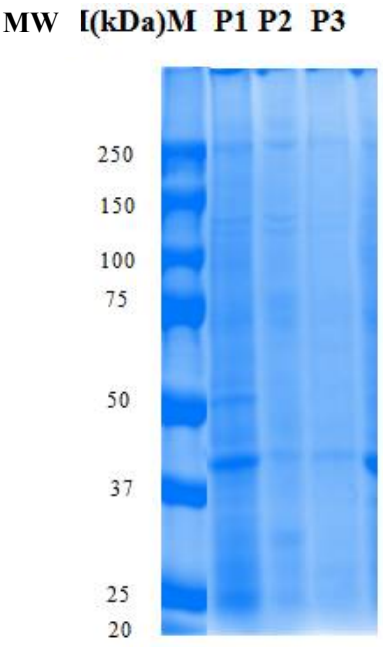

Figure 5. Protein bands of duodenum (SDS PAGE 12%).
Legend: M = marker; P1 = Sulfasalazine group; P2 = EEPL from Indonesia group; P3 = EEPL from Philippines

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)	√	√	√	-	√	√	√

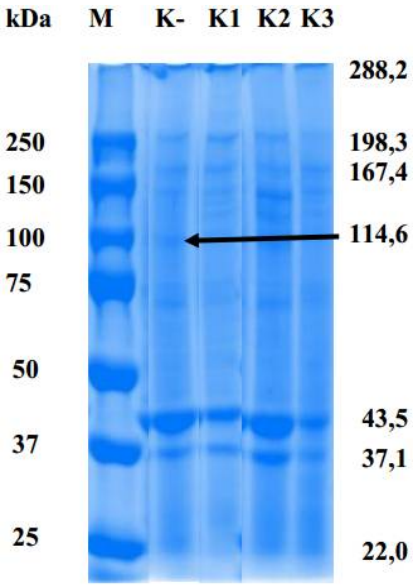

Figure 6. Protein bands of colon (SDS PAGE 12%).
Legend: M = marker; K- = negative control; K1 = Sulfasalazine group; K2 = EEPL from Indonesia group; K3 = EEPL from Philippines

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].

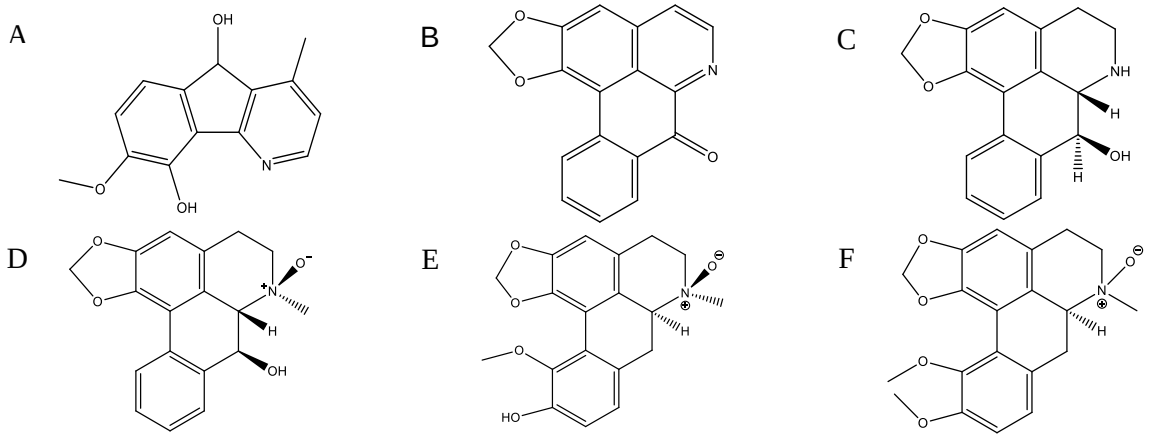

Figure 1. Structure of alkaloid compounds. A) Polylongine, B) Liriodenine, C) Noroliveroline, D) Oliveroline β-N-oxide, E) N-methylnandingerine-β-N-oxide, F) O-methylbulbocapnine-N-oxide.

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).

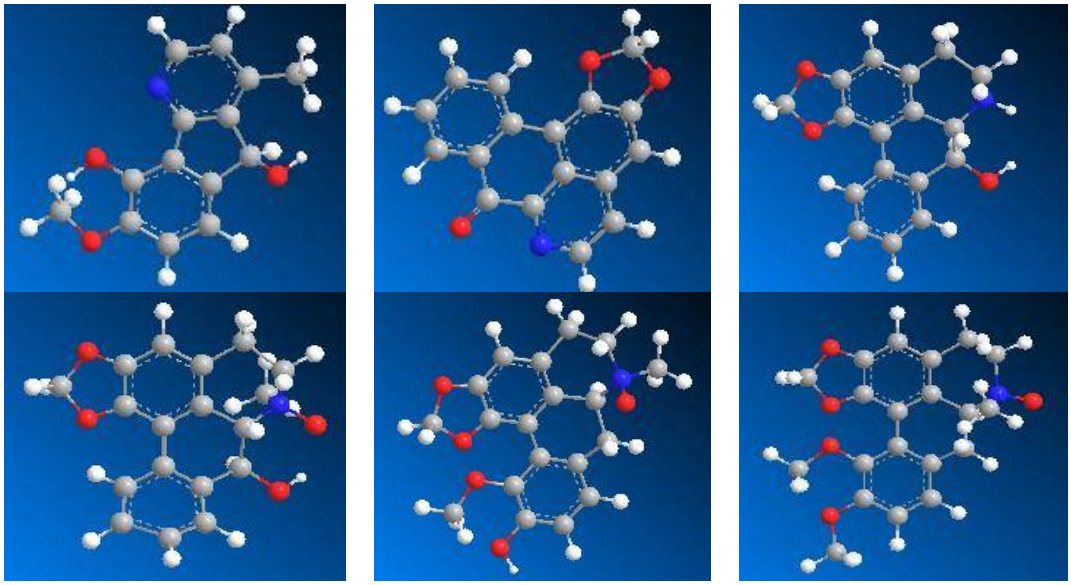

Figure 2. The 3D structure of Alkaloids. A) Polylongine, B) Liriodenine, C) Noroliveroline, D) Oliveroline β-N-oxide, E) N-methylnandingerine-β-N-oxide, F) O-methylbulbocapnine-N-oxide.

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).

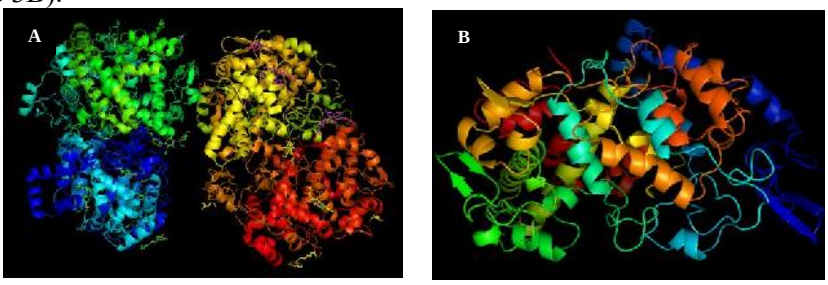

Figure 3. A) The 3D of COX-2 protein, B) The 3D chain A structure of COX-2 protein.

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.

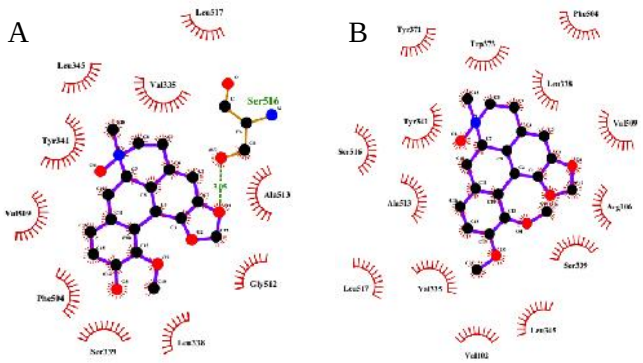

Figure 4. Visualize the results of LigPlot+. A) N-methylnandingerine-β-N-oxide, B) O-methylbulbocapnine-N-oxide.

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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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.

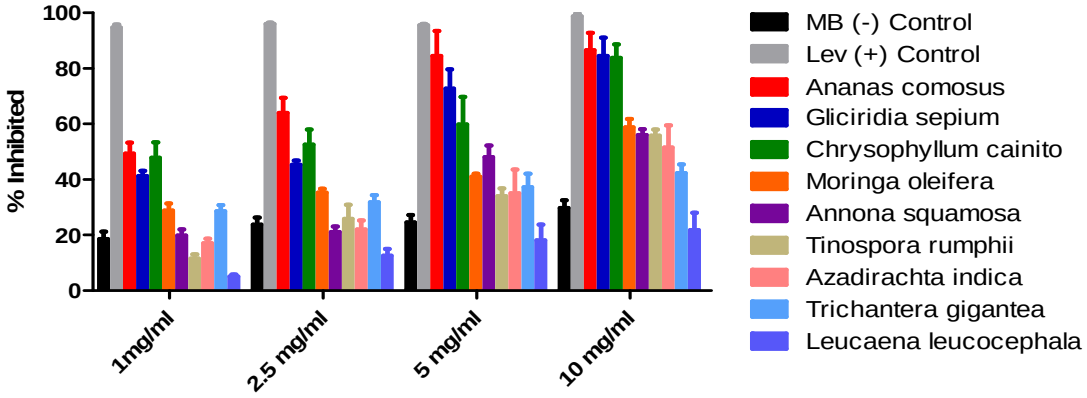

Figure 1. Inhibition percentage of nine aqueous leaves extract on *Oesophagostomum dentatum* larvae using larval migration assay.

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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Analysis of Doodles and Listening Comprehension of College Students

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Abstract
Since the discovery that doodling aids to concentration (Andrade, 2009), several studies have been conducted to further ascertain its effects on listening, ranging from its benefits on retrieval strategies, visual recall, learning content from an educational video, among others. While these studies, made excellent follow-ups on Andrade's research, theirs delved mostly on low level comprehension, i.e., recall and recognition. Another attempted to assess the effects of doodling on learning performance. However, it was not specified in their study what particular area of learning was measured. Hence, employing true experimental design particularly the pre-test/post-test control group design, the present study aimed to find the effects of doodling on the listening comprehension of students particularly on literal, interpretative, critical and creative levels. Descriptive and appropriate inferential statistics were used in analyzing the performances of the respondents in the pre-test and post-test, revealing that the students who were exposed to doodling have improved on all levels of listening comprehension and those who were not exposed also improved on literal, interpretative, and creative levels but not in critical. Through the exposure of the students to doodling activities, they produced mainly extraneous doodles or doodles which had no connection with the listening passage, followed by meta-cognitive doodles which reflected that they attempted track their understanding of the passage. The findings of the study further showed that the listening comprehension skills of the students exposed to doodling are significantly higher than those that are not exposed to doodling along literal level. However, no significant differences are found along all other comprehension levels. Finally, the results of the statistical computation showed that the profile of the students (both those who were exposed to doodling and those who were not) were not significantly related to their levels of listening comprehension. It is concluded that doodling can be an aid in improving listening comprehension skills particularly in the literal level but may not contribute much in higher levels. Hence, the researcher recommends the use of doodling as an intervention strategy to aid in improving literal comprehension skills.

Keywords: doodles, doodling, listening, listening comprehension, TOEFL iBT™

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Introduction

Listening is a complex process through which the auditory input undergoes decoding process (Yenkimaleki & Van Heuven, 2016) whereby the auditory stimulus is reconstructed mentally by the receiver (Poelmans, 2003). Such complexity is what caused listening to be seen as a cornerstone competence in language classrooms and more importantly in the workplace (Nair, Li Koo, & Abu Bakar, 2013).

Rivers (1981) as cited by Ghoneim, (2013) added that developing listening skills is important especially that adults spend their time 40% to 50% listening, 25-30% speaking, 11-16% reading, and only about 9% writing. As mentioned by Sadighi and Zare (2006), Nunan (1998) indicated that

> listening is the basic skill in language learning. Without listening skill, learners will never learn to communicate effectively. In fact, over 50% of the time that students spend functioning in a foreign language will be devoted to listening…. (p. 1)

However, listening is a widely neglected skill because of insufficient pedagogical development and teacher training (Mendelsohn, 1998). According to Sadighi and Zare (2006), listening has been a neglected skill in second language acquisition, research, teaching, and assessment for a long time and that the importance of it in the language learning has only been recognized relatively recently. The listening skill has suffered such neglect in the field of research and has not been fully investigated in foreign language acquisition mainly because listening processes are difficult to explore (Chamot, 2005) due to its nature as a covert activity.

Klein (1996) further explained that this neglect resulted largely from two factors. First, listening as well as reading is not a highly visible skill and is not easily assessed, unlike speaking and writing. Thus the sending part of the communication process receives more attention from researchers than the receiving part. Second, very few are willing to improve their listening skills, which suggestively resulted from an incomplete understanding of the process itself. Furthermore, Miller (2009) asserted that comprehending lectures is not a passive listening activity, which means that it requires much effort to master. Because of these limitations, the investigation of the mental operations involved in listening has been difficult and challenging for research.

Despite the abundance of concepts and literature about listening and its importance, past researchers have not been giving ample attention towards listening comprehension, and yet the importance of it in L2 teaching and teaching is highly recognized (Yenkimaleki & Van Heuven, 2016) adding that exposure to the target language and proper training would automatically develop language listening comprehension skills (Clement, 2007). Cross (2009) added that errors in decoding hugely affects the quality of ones understanding of the listening text. Hence, understanding these errors may promote enhanced listening comprehension.

With the recent change in the educational system of the Philippines, the Commission on Higher Education (CHED) issued the memorandum no. 20 s. 2013 stipulating changes on the general education subjects offered in tertiary institutions. One of the objectives of these change was to equip students with intellectual competencies possessing "higher levels of comprehension." Furthermore, the new subject *Purposive Communication* aimed to enhance students' five major skills (listening, writing, reading, speaking and viewing) so that at the end of the course, the "students should be able to listen, comprehend, critique, and respond to live or recorded

conversations…" Hence with the new curriculum, new and more advanced strategies to strengthen students' skills are also expected to be introduced.

As such, different strategies have been utilized to measure the performances of different respondents in various listening comprehension researches. Yenkimaleki and Van Heuven (2016) experimented on the effects of explicit teaching of prosodic features on the development of listening comprehension. Specifically, the study answered the question of whether the awareness training of prosodic features would lead to developing the global listening comprehension in message perception for student interpreter trainees. As resulted from the study, awareness training of prosodic feature contributed to the development of the listening comprehension of interpreter trainees.

Moreover, Hemmati and Ghaderi (2014) delved away from the listeners perspective but on the given questions and analyzed whether these could contribute to the development of listening comprehension. Thus they experimented on the effects of four formats of multiple-choice questions (MCQs) on the listening comprehension of EFL learners. The study implied that to measure the listening comprehension of learners, test questions must be properly constructed regardless of the format used adding that full question preview format, answer option preview format, and question preview format could have a facilitative effect on the listening comprehension.

Similarly, the study Gowhary, Pourhalashi, Jamalinesari, and Azizifar (2015) suggested that using captions had a significant effect to the students' listening comprehension concluding that captioning videos could be helpful in overcoming their listening difficulties. Similarly, Amaluddin, Salasiah and Mardiah (2018) indicated the use of audio-visual aids through the use of metacognitive strategies can significangly improve the students' listening comprehension. Paguirigan (2010) conducted a study, a rather different approach, on the effects of background music on the listening comprehension of selected pupils. Surprisingly, the study found out that the listening comprehension of those who had background music is much higher compared to those had not.

Despite the complexity of studying listening and listening comprehension, Gloria's (1996) study as affirmed by the more recent studies of Carrell et al., (2004), Lin (2006), and Amini Asl and Khierzadeh (2016) revealed another way to improve listening study skill was to employ note-taking, an act that resembled some of its characteristics with doodling particularly in scribbling, only that in note-taking, one writes important words.

Since the discovery of Andrade (2009) that doodling aids concentration, several researchers have embarked on conducting more studies about and begun discovering other benefits of doodling (Aellig et al., 2009; Chan, 2012; Qutub, 2013; Singh & Kashyap, 2014; Tadayon & Afhami, 2016; and Boggs et al., 2017). Quite impressive, these researches have taken extra efforts to prove their hypotheses.

Singh and Kashyap (2014) examined the benefits of doodling on retrieval strategies, i.e., recall and recognition. The results indicated that recall fell under the favor of recognition in retrieving memory. The study suggested that recall was harder than recognition because of the extensive effort in remembering the learning event. Moreover, the results pointed out that doodling percentage did not affect the retrieval efficiency rather it was doodling itself which provided benefits for memory retrieval. Contrary to previous discoveries, however, the study of Chan (2012) indicated that although doodling served as a tool to aid in concentration as well as auditory recall,

it did not elicit positive effects on visual recall. The study explained further that such negative effect was

because the doodlers struggled in having divided visual processing resources implying that when multitasking is engaged in similar primary modality, negative effects could occur in the amount of information processed and retained.

Boggs et al. (2017) affirmed the results of Chan's (2012) study having found out that doodling was not advantageous in increasing recall performance despite the avoidance of daydreaming. This, however, is explained by the variables in the study, where they classified the doodlers under different conditions: structured doodling for those who shaded shapes and unstructured doodling for those who were free to draw anything on their empty papers. Surprisingly, those under the structured doodling had significantly higher scores than those under the unstructured doodling. This was due to extensive mental processes they had to undergo, i.e., deciding what to doodle, reproducing the mental image to the paper and simultaneously attending to the audio recording. Furthermore, Aellig et al., (2009) explored the relationship of attention span and doodling with the ability to learn content from an educational video. Their study suggested that free doodling and the ability to learn contents from an educational video had no significant relationship.

Despite these negativities discussed and effected from these researches, more studies believe that doodling proved to be beneficial. Wammes et al. (2016) pointed out that drawing as an aid to remembering could have a significant effect on the improvement of memory. Moreover, Tadayon and Afhami (2016) assessed the effects of doodling on the learning performance of high school students. With the employment of pre-test and post-test, their study found out that those who doodled outperformed those who did not. Such a result draws the implication that doodling is, in fact, beneficial particularly in learning.

Much of these studies about doodling covered only the effects of it on the recall, a lower order thinking skill and learning, a very broad concept. The study of Andrade (2009) regarding the effect of doodling on the concentration of the respondents, where she found out that doodling was indeed effective in aiding to concentration, only discusses the first level of cognition, i.e., remembering. If one wants to utilize one's cognition fully, higher levels must be covered. This, however, reflects how difficult it would be to measure listening.

While several strategies have been devised and experimented on to aid listening comprehension, the relationship between doodling – one that has received attention to many researchers in cognitive psychology – and listening comprehension has not been explored yet in the world of research. What is missing in the above mentioned studies, however, is their consideration on the doodles of their respondents. The discussion of results purely concentrated on the act of doodling and its effects to the performances of the respondents but none of those studies presented the doodles and analyzed whether they may have helped or not.

On the search for doodle classification, most literatures only focused on the psychological meanings and classifications of doodles. But recent studies have been conducted to describe the doodles of students and their possible effects to their performances (De Leon et al., 2019; McCartney et al., 2005; & Lister et al. 2004).

Although, these studies were conducted among programming students and the doodles which they classified were highly related to programming, the classifications that they were able to generate could also be applied to the other fields that employs doodling on tests and activities.

Lister et al. (2004) in particular was the first to categorize doodles based on their students' annotations of the tests. It was found out that if a student carefully traces through the code, the likelihood of getting the correct answer is high. In contrast, not doodling only leads to the correct answer 50% of the time.

Moreover, MacCartney et al. (2005) conducted another study and utilized the categories developed by Lister et al. However, in the analysis of their results, several issues surfaced in relation to the questions and the doodles of the students. Hence to resolve the issue, four disjoint categories, reclassifying each question, were created: *Blank, Some Tracing, Elimination, or Others*. Such categories were based on their observation that that tracing and process of elimination are recognizable strategies, and, with Blank, cover 89% of the observations. They then found out that the most effective strategy is tracing and elimination which includes *alternate answer* and *ruled out.*

From the studies presented above, the study of De Leon et al. (2019) may have provided a better way of facilitating the analysis of multiple types of doodles. Although their groupings are loosely based from McCartney et al. (2004) and are applied to programming tests, the definitions they provided possess applicability in the present study. Since the respondents of the said studies were programming students, many of the generated categories of doodles may not apply to the respondents of the present study, especially, that the present study focuses on communication major students. But the classifications of De Leon et al. provides better perspective on analyzing the doodles of the students. The generality of the definitions have also paved inclusion of doodles that are produced in tests whether they be programming or not. Hence the present study adapted these classifications in the analysis of doodles of the respondents. The figure below presents these new categories and their descriptions.

Name	Description
Clarifications	Consisted of all corrections or additional instructions from the teacher.
Meta-cognitive processes	Doodles that imply that students are tracking their own learning or progress.
Trace	The Trace category was comprised of Computation, Practice Code, Number, Synchronized Trace, Odd Trace, and Trace.
Extraneous Marks	Markings that appear meaningless or irrelevant to the activity.

Figure 1. Categorization of doodles as developed by De Leon et al. (2019)

From these categories, they found out that those who doodled (37%) scored between 91 and 100 out of a possible 100. From the majority (62%) who did opt to doodle, only 15% of them had similar scores with those who doodled, and the rest scored less than 90. They then concluded that not doodling at all does not necessarily mean a student is not doing well since there were those who did not doodle but managed to get high or similar scores to those who doodled. This may also mean that might already have a good understanding of the exercises given them and that they have

no need for the extra mental aids.

Grounded on the above studies, the study attempted to venture on a more in-depth analysis of the effects of doodling on the listening comprehension of students. Furthermore, the study considered the act of doodling, the doodles produced and the listening activities in the analysis of the results as well as in the discussion of the causes that might have assisted in the improvement of students' listening comprehension. Hence, the study also adapted several variables from these mentioned studies to further provide knowledge to the world as to why many researchers believe that doodling improves listening as well as to identify what particular level of comprehension doodling could be helpful.

Statement of the Problem

Generally, the study was conducted to determine the effects of doodling on the listening comprehension of college students.

Specifically, it sought to answer the following problems:

1. What is the profile of the respondents in terms of:
   a. sex;
   b. age;
   c. first language;
   d. verbal reasoning score in the entrance examination;
   e. learning styles?
2. What is the listening comprehension level of the college students exposed to doodling activities and those not exposed to doodling activities both in their pre-test and post-test in the following:
   a. literal;
   b. interpretative;
   c. critical, and
   d. creative?
3. What are the types of doodles produced by the students while listening to recorded conversations and lectures?
4. Is there a significant difference between the pre-test and post-test mean scores of the students exposed to doodling?
5. Is there a significant difference between the pre-test and post-test mean scores of the students who are not exposed to doodling?
6. Is there a significant difference between the level of listening comprehension of the college students exposed to doodling and those that are not?
7. Is there a significant relationship between the profile variables and the level of listening comprehension of the college students not exposed to doodling and those that are exposed to doodling activities?

Null Hypotheses

The following hypotheses were tested at 0.05 level of significance:

1. There is no significant difference between the pretest and posttest mean scores of the students exposed to doodling.

2. There is no significant difference between the pretest and posttest mean scores of the students who are not exposed to doodling.

3. There is no significant difference between the level of listening comprehension of the college students exposed to doodling and those that are not.

4. There is no significant relationship between the profile variables and the level of listening comprehension of the college students not exposed to doodling and those that are exposed to doodling activities

Methodology

Research Design

The study utilized a true experimental design particularly the Pretest-Posttest Control Group Design because of three (3) factors that were duly considered: Randomization, Treatment, and Control. Firstly, in randomization, all participants were given an equal chance of being assigned into any group in the experiment. This also entails that the randomization process should result to equivalence between the two groups. Since the aim is to observe the effect of doodling on the listening comprehension of the students, this study involved two groups: those that were exposed to doodling (experimental group) and those that were not (control group). Secondly, a treatment was applied to the experimental group. Instead of listening only to the recordings, the experimental group doodled as they listen. Finally, control was used to avoid external influences on the outcome of the study. Thus, the experiment was conducted with both of the groups listening to the same recorded lectures and conversations at the same time, only that the experimental group performed an added task. The conduct of the experiment was done as illustrated in the following diagram:

Students exposed to doodling	O1	X	O2
Students not exposed to doodling	O1		O2

As shown in the diagram, the participants were grouped accordingly and are separated by the solid line. This solid line represents randomization, thus, the participants in both groups were subjected to random assignment. Moreover, as can be gleaned from the diagram, the initial measurements of the dependent variable or the pre-tests for both groups were administered at the same time. O1 symbolizes the performance of the respective groups in the pre-test. Whereas, the X denotes doodling as the treatment in the study. Finally, O2 represents the final measurement of the dependent variable which is the performance of the participants in both groups in the post-test.

Participants

The respondents of the study comprised of 38 Bachelor of Science in Development Communication students from the Tarlac Agricultural University in the second semester of academic year 2018-2019. This number of students is the total population of those that take communication and language courses in the said university. Randomization was employed to give each student an equal chance of being assigned to any of the groups.

Prior to conducting the experiment, the researcher determined first the profile of the respondents. And from that profile, the researcher utilized their final grade in the subject Purposive Communication which they have taken in the previous semester as the identifier for the randomization. The participants were sorted according to their grades from highest to lowest. Those, whose ranks were odd number, were assigned to the experimental group and those, whose

ranks were even number, were also assigned to the control group. This was done to ensure that each group would have an equal number of participants and also homogeneous performances. Hence, the groups had 19 participants each.

Data Gathering Instruments

Survey Questionnaire was used to gather the profile of the respondents in terms of sex, age, first language, verbal reasoning score in the entrance examination and final grade in Purposive Communication including the **Memletics Learning Styles Questionnaire** to identify the participants' preferred learning styles. The reliability test result of this instrument using Cronbach's alpha is 0.81 (Moenikia & Zahed-Babelan, 2010), suggesting that the internal consistency or reliability of the questionnaire is high. To get the participants' initial and final listening comprehension scores, the researcher utilized the listening comprehension questions adapted from **TOEFL iBT™**. The listening section of the TOEFL iBT test measures students' ability to understand conversations and lectures in English. The questions focus on identifying the main idea and supporting details, recognizing the speaker's attitude and purpose, and making inferences. Permissions from Educational Testing Services (ETS), the developer of TOEFL iBT™, to use the tests in this study have been duly acquired.

Data Gathering Procedure

The respondents were given the survey questionnaires and Memletic® Learning Styles Inventory to get their profile and learning styles. The data gathered for the profile of the respondents, particularly their grade in Purposive Communication were used as the basis for the randomization.

Upon the assignment of participants in the control and experimental groups through randomization, the researcher then commenced on the conduct of the experiment. The date and time of the experiment are the same to prevent the time element to impose influence on the outcome of the study.

The pre-test was first administered to assess the initial listening comprehension abilities of the students. Then, for three weeks, the respondents were asked to listen to different recordings, one recording per session. The activities were conducted twice a week, thus six (6) sessions for three weeks. The control group was asked to listen to the recording and do nothing else; while the experimental group was given blank sheets of paper and pencils. They were instructed that they can use the pen and paper should they feel bored. At the end of each session, comprehension questions was administered to the participants.

At the conclusion of the experiment, the researcher administered the post-test among the participants. The post-test was just the same with the pre-test, but the order of the items was disarranged. Finally, the doodles of the respondents were gathered and analyzed carefully to determine their classification and to ascertain how these doodles may have affected their performances in the listening activities.

Statistical Treatment

To answer the underlying problems posed by the study, fundamental statistical operations were utilized. Problem 1 sought to determine the profile of the respondents, hence, frequency counts and percentages were utilized. Problem 2 looked for the types of doodles which the students produced while listening to recorded conversations and lectures, hence, to facilitate analysis of the

doodles, the researcher adapted the taxonomy of De Leon (2019) to classify the doodles and utilized frequency counts and percentages to assist in the discussion. For Problem 3, the levels of the listening comprehension of the students were determined based on the levels of comprehension taxonomy developed by Al-Musalli (2001). Per level of comprehension, i.e. literal, interpretative, critical and creative, the researcher tallied the scores of the participants and computed their mean. Then, the percentage of the mean score for that particular level was computed against the total number of items classified under that level of comprehension. To answer problems 4 and 5, paired t-test was used to compute for any significant differences between the pretest and posttest mean scores of the students exposed to doodling and those that are not. For problem 6, independent sample t-test was used to compute for any significant differences between the levels of comprehension of the students exposed to doodling and those that are not. And for Problem 7, Pearson Product Moment Correlation Coefficient were used to determine the correlation between level of listening comprehension and the profile variables Age and Language ability score in the entrance examination; Point-biserial for Sex; and Contingency Co-efficient for First Language variable.

Results And Discussion

On the Profile of the Respondents

Table 1. Profile of the Respondents

Profile Variable	Category	College Students Not Exposed to Doodling Activities n=19		College Students Exposed to Doodling Activities n=19	
		F	Percent (%)	f	Percent (%)
Sex	Male	5	26.3	4	21.1
	Female	**14**	**73.7**	**15**	**78.9**
Age	20 years old and below	**16**	**84.2**	**14**	**73.7**
	21-30 years old	1	5.3	4	21.1
	31 and above	2	10.6	1	5.3
First Language	Ilocano	5	26.3	3	15.8
	Kapampangan	1	5.3	1	5.3
	Pangasinan	1	5.3	-	-
	Filipino	**12**	**63.2**	**15**	**78.9**
	English	-	-	-	-
Verbal Reasoning Score	Below 70	-	-	2	10.6
	71-79	1	5.3	1	5.3
	80-89	**18**	**94.7**	**15**	**78.9**
	90-95	-	-	-	5.3
	96-100	-	-	-	-
Learning styles	Visual	2	10.52	2	10.52
	Aural	2	10.52	2	10.52
	Verbal	4	21.05	5	26.31
	Physical	5	26.31	4	21.05
	Logical	1	5.26	0	0
	Social	**7**	**36.84**	6	31.58
	Solitary	6	31.58	**7**	**36.84**

Sex. Table 1 shows that the participants were equally distributed to both groups with 19 individuals each. From the students who were not exposed to doodling, 14 or 73.5% are females and 5 or 26.3% are males. While the students who were exposed to doodling were composed of 15 or 78.9% females and 4 or 21.1% males. These data only shows that the development communication program of Tarlac Agricultural University is dominated by females.

Age. In can be gleaned in table 1 that majority of the participants in both groups are 20 years old and below with 16 or 84.2% students not exposed to doodling and 15 or 78.9% exposed to

doodling. This distribution may mean that the respondents are mostly fresh graduates from high school. However, it is also noticeable that there are those whose ages do not align with the expected age for college students. Based on the interview made by the researcher, some of these students have stopped after graduating from high school and some have started college educations but, due to financial insufficiency, have stopped for some time and now are returning.

First Language (L1). The data on table 1 reveals that the majority of the participants in both groups have learned Filipino language before learning any other languages with the distribution of 12 or 63.2% for those that are not exposed to doodling and 15 or 78.9% from those that were exposed, followed by Ilocano with 5 or 26.3% and 15 or 789% from the students that were not exposed to doodling and those that were exposed, respectively. None of the respondents has the English language as their L1.

Verbal Reasoning Score. Table 1 also shows that majority of the respondents have scores ranging from 80-89 with the distribution of 18 or 94.7% from the students who were not exposed to doodling and 15 or 78.9% from the student who were exposed. This means that the students have above average verbal reasoning abilities. According to Adeyemi (2017), verbal reasoning involves making meaning based on the information given, going beyond that information to better understanding and applying verbal skills to new learning. Hence, this result of the study means that the respondents are generally aligned to the program they enrolled in because of their scores in the verbal reasoning test. Development Communication program requires that students must have good communication skills, chiefly because this program would lead them to public and even mass communication. Hence, higher language ability is an advantage. Furthermore, Adeyemi concluded in his study that there is a significant relationship between students' knowledge of verbal reasoning and performance, which means that the deeper knowledge a student has of verbal reasoning, the higher achievement he would accomplish.

Learning Styles. As shown in table 1, majority of the students that were not exposed to doodling are social learners which means that they prefer to learn in groups or with other people. On the other hand, majority of the students who were exposed to doodling are solitary which means that they prefer to work alone or use self-study. The Logical Learning style has the least possessor (one or 3.70%) which means that only one prefers the use of logic, reasoning, and systems in learning. This result means that the development communication students have multiple learning preferences as they acquire knowledge.

This particular result of the study affirms the study of Moenikia and Zahed-Babelan (2010) saying that those students with social learning styles have suitable performance in language learning. Moreover, Banner and Rayner (2000) mentioned that successful people with social personalities are more communicative and better learners of new words. Thus, based on these mentioned studies and the results of the present study, the learning styles that are possessed by the Development Communication students suit what the course commonly necessitates, that is, working with people and communicating with them.

On the Listening Comprehension Levels Of The Students Exposed To Doodling And Those That Are Not Both In Their Pre-Test And Post-Test

Literal. Table 2 presents the listening comprehension levels of the students exposed to doodling activities and those that were not both in their pre-test and post-test performances along

literal level. It can be gleaned from the table that majority (52.6%) of the students who were not exposed to doodling had "low" levels in literal comprehension as resulted from the pre-test. Similarly, majority (47.4%) of those that were exposed to doodling had "low" levels in literal comprehension before the conduct of the experiment.

Table 2. Listening Comprehension Level of the College Students Not Exposed to Doodling and Students Exposed to Doodling Activities both in their Pre-test and Post-test along Literal Level

Comprehension Level	Skill Level	College Students Not Exposed to Doodling		College Students Exposed to Doodling	
		Pre-test	**Post-test**	**Pre-test**	**Post-test**
		Percent (%)	Percent (%)	Percent (%)	Percent (%)
Literal	Very High	-	10.5	-	5.3
	High	5.3	-	10.5	10.5
	Average	31.6	**31.6**	36.8	**63.2**
	Low	**52.6**	**31.6**	**47.4**	21.1
	Very Low	10.5	26.3	5.3	-
	Mean Score (DE)	**5.95 (Low)**	**5.95 (Low)**	**6.32 (Average)**	**7.84 (Average)**

DE-descriptive equivalent

In totality, however, the pre-test mean score (5.95) of the students that were not exposed to doodling falls under low level while the mean score of those that were exposed to doodling falls under average level along literal comprehension. This indicates that, in both groups, the majority were slightly proficient in recognizing or recalling of main ideas, details, sequences, and comprehending the gist of what is said.

The table also reveals that, at the conclusion of the experiment, the students that were not exposed to doodling had two (2) majorities (31.6%): those at average level and those at low level. It can be noticed that the number of respondents who had average level in the pre-test is the same with those in the post-test which means that the number of those who were previously average did not improve at all. Whilst the number of those who possessed low levels at the pre-test, which previously was the majority (52.6%), decreased to just 31.6%. This decrease could have been attributed positively but considering the increase in the number of students possessing very low levels from 10.5% to 26.3%, this may indicate that there was not much of improvement at all. This is backed up by the mean score 5.95, which means that that although there were changes in the distribution of levels, in totality, the group mean score did not change, hence, their levels did not improve.

For the students who were exposed to doodling activities, the table reveals that their exposure may have affected their performance positively, in which they performed better in their post-test than in their pre-test. This result can be seen via the increase in the number of respondents with improved comprehension levels. Previously, the majority possessed "low" levels of literal comprehension skills, but after the exposure to doodling, majority of them incurred higher level of

comprehension skill, thus, earning "average" level. This means that they have improved their skills in literal comprehension and from slightly proficient, they became fairly proficient.

This particular result of the study is supported by Andrade (2009) who pioneered the study on doodling and listening. In her study, two groups were also utilized in a single session experiment with one group allowed to doodle while listening and the other deprived of any tasks while listening. After five minutes, the participant were asked to write names and places that they could remember especially those that were attending the party. Results show that those who doodled remembered 29% more than those who did not. She then concluded that the experiment resulted to positive effects on the exposed because through doodling the participants may have been kept awake, thus forcing them to utilize executive resources and that doodling may have reduced daydreaming. Literal comprehension as defined in the present study covered much on remembering details, thus, the results in the study of Andrade (2009) are affirmed by the present.

Furthermore, this particular result of the study also agrees with the study of Wammes et al. (2016) who found out that drawing improves memory by encouraging a seamless integration of semantic, visual, and motor aspects of a memory trace. Hence, the doodles which the students produced during the experiment may also be considered as a factor in improving their memory since in those activities, the participants may have adapted in using their schemata (Zeng, 2007) and connecting it with the listening passage, thus producing an image that reflects their learning.

By performing this process over a period of time, the students seemed to have trained themselves to remember details with such strategy. This particular result occurred because doodling may have maintained arousal while consuming a limited amount of available cognitive resources as opposed to daydreaming, which consumes a high amount of cognitive resources (Boggs, Cohen, & Marchand, 2017).

Interpretative. As shown in table 3, majority or 57.9% of the students who were not exposed to doodling had "very low" levels in interpretative comprehension as resulted from the pre-test. While of those who were exposed to doodling, majority or 47.4% had "low" levels. Hence, prior to the conduct of the experiment the students exposed to doodling activities had higher levels of interpretative comprehension than those who were not. In totality, however, the mean scores of both groups say otherwise as both groups incurred respective means scores that fall under low level: 2.31 for the students that were not exposed to doodling and 2. 68 for those that were exposed.

This means that in terms of interpretative comprehension skills, the students in the control group or those that were not exposed to doodling were not proficient in inferring information from the speaker or the social setting, while the students who were exposed were a step higher, which is slightly proficient.

Table 3. Listening Comprehension Level of the College Students Not Exposed to Doodling and Students Exposed to Doodling Activities both in their Pre-test and Post-test along Interpretative Level

Comprehension Level	Skill Level	College Students Not Exposed to Doodling		College Students Exposed to Doodling	
		Pre-test	**Post-test**	**Pre-test**	**Post-test**
		Percent (%)	Percent (%)	Percent (%)	Percent (%)
Interpretative	Very High	-	-	-	-
	High	-	10.5	-	21.1
	Average	-	10.5	10.5	**36.8**
	Low	42.1	**47.4**	**47.4**	21.1
	Very Low	**57.9**	31.6	42.1	21.1
	Mean Score (DE)	**2.31 (Low)**	**3.37 (Low)**	**2.68 (Low)**	**4.58 (Average)**

DE-descriptive equivalent

It can also be seen on the table that both groups have made improvements on this particular levels of comprehension as reflected on their post-test performances. Among the students who were not exposed to doodling, the majority (47.4%) now have low levels, while among the students who were exposed to doodling, the majority (36.8%) possess average levels. In totality, the mean scores of both groups showed a difference of improvements as seen in their new levels. The students that were not exposed to doodling scored an average of 3.37, which falls under low level while those that were exposed scored an average of 4.58, which falls under average level.

It can be inferred from this data that doodling may have been assistive among those who were exposed to doodling as doing so did not distract them in inferring information from the passage. But in contrast, as reflected from improvement of the levels of those that were not exposed, doodling may also not have contributed much since the levels of those that were not exposed have also improved. Additionally, had the students exposed to doodling improved by surpassing two or more levels, it would have been possible to conclude that doodling was more effective than non-doodling, but this is not the case as both groups stepped up with just one level only. And although both groups have improved, the level of the students who were exposed to doodling is still higher. This is also supported by the mean score of both groups

Few possible reasons may be attributed to this specific result of the study. First, the high percentage of extraneous doodles produced by the students during the experiment may have caused the lack of effectiveness of their inferring skills. Although it was observable that the students exerted efforts on producing meta-cognitive doodles, which would have been helpful to them if properly executed and substantially surpassing the extraneous ones, their exposure may not have sufficiently helped them improve or their doodles may have not represented the information from the listening passage that can only be understood when inferred. Second, Andrade (2009), in the same study where she asserted that doodling aids to concentration, noted that "tests of memory or

attention often use a second task to selectively block a particular mental process, and if that process happened to be important for the main cognitive task, it would result in performance being impaired due to this competition for cognitive resources". Hence, in this study, the respondents may have had a hard time choosing which to focus on because of competing modalities and that one particular mental process may have been blocked.

Critical. As shown in table 4, majority of the students who were exposed and those that were not exposed to doodling activities had "very low" levels in critical comprehension before the conduct of the experiment (52.6% and 63.2%, respectively). This means that prior to the conduct of the experiment, majority in both groups were not proficient in critical comprehension or, more specifically, in making assumptions, conclusions, and even evaluation. However, when computed in totality, both groups had mean scores of that fall under low level (1.11 for those that were not exposed and 1.42 for those that were exposed to doodling). Hence, when described in totality, both groups are slightly proficient.

Table 4. Listening Comprehension Level of the College Students Not Exposed to Doodling and Students Exposed to Doodling Activities both in their Pre-test and Post-test along Critical Level

Comprehension Level	Skill Level	College Students Not Exposed to Doodling		College Students Exposed to Doodling	
		Pre-test	**Post-test**	**Pre-test**	**Post-test**
		Percent (%)	Percent (%)	Percent (%)	Percent (%)
Critical	Very High	-	-	-	10.5
	High	-	10.5	-	5.3
	Average	5.3	15.8	10.5	21.1
	Low	31.6	15.8	36.8	**31.6**
	Very Low	**63.2**	**57.9**	**52.6**	**31.6**
	Mean Score (DE)	**1.11 (Low)**	**1.53 (Low)**	**1.42 (Low)**	**2.21 (Average)**

DE-descriptive equivalent

It can also be observed that both groups have somehow improved on their comprehension skills as shown by the decrease in the percentage of those that had very low levels in the post-test and the increase in the percentages of higher levels. However, for the students that were not exposed to doodling, the decline in the percentage of participants with very low levels may not have contributed much to the improvement of the whole group's performance as the majority still possessed very low level of critical comprehension skills after taking the post-test. The whole group's mean score (1.53) also did not show much of improvements as the level is still low.

Furthermore, majority of the students that were exposed to doodling previously possessed "very low" level of critical comprehension (52.6%) but after the exposure, the group performed better in the post-test. As presented, their post-test scores indicate that there are new two (2) majorities, both with 31.6% in low and very low levels. Thus, this result suggests that they became

"slightly proficient' in critical comprehension or making appropriate judgments about the message of speaker's personality, making assumptions, drawing conclusions, making evaluation. In totality, the groups mean score (2.21) also indicate an improvement as it falls under average level.

This result of the study is supported by Tadayon and Afhami (2016) in terms of the effect of artistic activities. As cited in their study, artistic activities can lead to academic achievement (Eisner, 1998). Further, in terms of educational performance, effective function of working memory is a vital element wherein when the working memory is active, concentration is also active. Hence as resulted in their study and in the present study, artistic activities (such as doodling in this study) may have improved learning and comprehension by improving working memory and concentration.

Moreover, Andrade (2009) asserted that doodling aids to concentration, hence, through doodling, the concentration of the students was improved and they were able to critically listen to recorded conversations and lectures in the tests as well as during the experiment.

Creative. As shown in table 5, majority the students exposed to doodling (47.4%) and also majority of those that were not (42.1%) possessed very low levels of creative comprehension. This means initially they were not proficient in handling verbal and non-verbal communicative strategies, identifying hesitation and making appropriate responses. In totality, the pre-test mean scores of the students not exposed to doodling (.95) fall under low level and the pre-test mean scores of students exposed to doodling (.74) fall under very low level.

Table 5. Listening Comprehension Level of the College Students Not Exposed to Doodling and Students Exposed to Doodling Activities both in their Pre-test and Post-test along Creative Level

Comprehension Level	Skill Level	College Students Not Exposed to Doodling		College Students Exposed to Doodling	
		Pre-test	**Post-test**	**Pre-test**	**Post-test**
		Percent (%)	Percent (%)	Percent (%)	Percent (%)
Creative	Very High	-	5.3	-	10.5
	High	5.3	26.3	-	21.1
	Average	26.3	**31.6**	21.1	21.1
	Low	26.3	10.5	31.6	**31.6**
	Very Low	**42.1**	26.3	**47.4**	15.8
	Mean Score (DE)	**.95 (Low)**	**1.74 (Average)**	**.74 (Very Low)**	**1.79 (Average)**

DE-descriptive equivalent

Moreover, the conduct of the experiment may have facilitated improvement of listening comprehension skills among all the participants of this study as shown by the increase in the number of students possessing higher levels. For example, for the students who were not exposed to doodling, majority possessed very low levels as reflected from their pre-test performances but after the experiment, their post-test performances reveal that the majority has earned average

levels. Hence, despite being deprived of any treatments, the group still managed to improve their creative comprehension skills. This is also attested by their post-test mean score (1.74) which fall under average.

Similarly, those students that were exposed to doodling improved on their creative listening comprehension skills. It can be noticed from the table that in the pre-test, nobody in the group possessed high and very high levels and the majority rested at very low but after being exposed to doodling, 10.5% had very high and 21.1% had high levels and the majority possessed low levels as reflected in their post-test performances. In totality, the group performance also improved as their post-test mean score resulted to 1.79 which fall under average, a level higher than their pre-test performance.

This particular result of the study affirms the claim of Tadayon and Afhami (2016) that doodling improves learning. This means that with the aid of doodling, the students, who were exposed to doing it, were able to improve their learning, specifically, in terms of listening comprehension. Furthermore, this particular result of the study is supported by the study of Wammes et al. (2016) which concluded that drawing improves memory by encouraging a seamless integration of semantic, visual, and motor aspects of a memory trace. The respondents did not only improve in remembering details but in the higher levels as well. Hence, from this particular result of the study, doodling is beneficial to students.

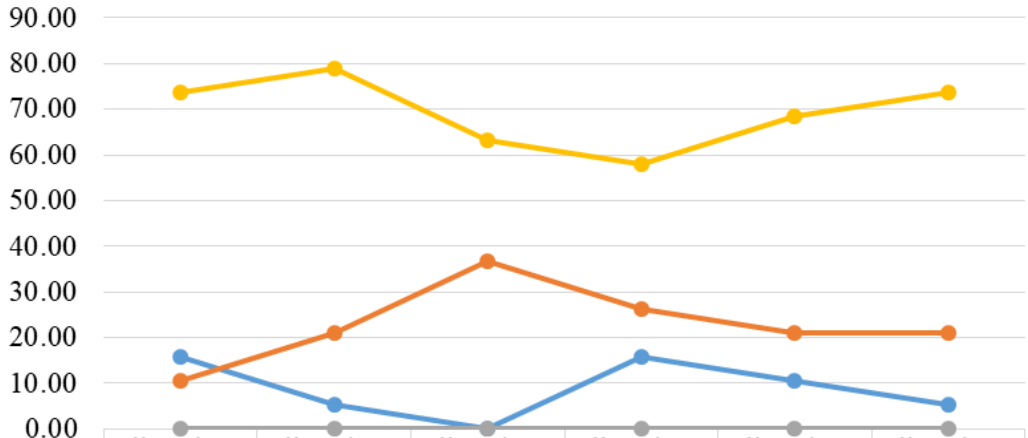
The preceding results revealed that the students of both conditions made improvements on specific levels of listening comprehension. Additionally, the statistical analysis presented in the following sections were purposefully conducted to ensure the validity of the results. Furthermore, the following sections also aimed at examining further the extent of the effect of doodling on specific levels of listening comprehension as well as to provide answers on whether doodling can be accepted as an aid to improving listening comprehension or not.

On the Types of Doodles Produced by the Students Exposed to Doodling Activities

Doodles are commonly defined as aimless scribbles to relieve boredom but this study adapted the definition of Brown (2010) which described doodles as "markings [that] help a person think." Hence, this section presents how the doodles of the students helped them understand the listening passage. This section also includes the analysis of the doodles produced by the students exposed to doodling activities during the experiment. For six (6) meetings, the students were given pieces of empty letter size papers where they asked to doodle as they listened to the recordings. The doodles were then collected and subjected to analysis. Doodles in this study are classified based on the taxonomy of De Leon et al. (2019), which as stated, leads towards finding the relationship of doodles and the achievement of students.

Since the TOEFL iBT™ was designed mainly for college students, ETS covered various disciplines in their listening comprehension tests. As surfaced on the analysis, the respondents made various doodles, some of which are related to the passage, some are about the subject which they are attending, and some are far from the topic.

attending, and some are far from the topic.



	listening activity 1	listening activity 2	listening activity 3	listening activity 4	listening activity 5	listening activity 6
clarification	15.79	5.26	0.00	15.79	10.53	5.26
meta-cognitive	10.53	21.05	36.84	26.32	21.05	21.05
trace	0.00	0.00	0.00	0.00	0.00	0.00
extraneous	73.68	78.95	63.16	57.89	68.42	73.68

Figure 6. Percentage trend of the types of doodle produced by the students across six (6) listening activities.

From the percentages of the types of doodles produced by the respondents, Figure 6 presents a clear picture of the progress of the respondents across all six listening activities. It is visible in the figure that the extraneous types of doodles are those that have prevailed among the doodles of the respondents throughout the experiment. It can also be gleaned from the table that, at the beginning of the experiment, the number of extraneous doodles are distant from the all other types. Although extraneous types of doodles were never surpassed by the other types, it is also evident that there is improvement in other types, particularly clarification and meta-cognitive. For example, in listening activity 1, the respondents needed clarifications first before using meta-cognition. However, starting from activity 2 onwards, the respondents have produced more meta-cognitive types than clarification. This may have happened due to the similarities of the activities. The first three activities were recorded lectures whilst the remaining three activities were conversations. Hence, this explains the ascent of meta-cognitive types of doodles that the respondents produced because, in those activities, the students may have found their way of doodling and understanding a recorded lecture simultaneously. Another is that the students may have already realized the role of their doodles in their comprehension. This particular result of the study may also imply that the students are tracking their learning and aware of understanding the recordings.

Moreover, the figure shows that when the type of recording was changed, i.e. the recorded lectures which were used previously were changed to conversation, the number of clarification types increased while the number of meta-cognitive types decreased. This may imply that the students may be surprised with the change hence their doodles of meta-cognitive types declined

and they needed more effort for clarifications. Furthermore, upon adjustment with the new type of activity, they may have found their way in combining multiple modalities.

This result of the study agrees with Brown (2010) that in order for information to be truly integrated, at least two of the major modalities must be integrated or one modality must be incorporated with a strong emotional experience. Moreover, Petrovsky (2009, cited by Qutub, 2012) added that visual symbols can replace speech as indirect forms of conscious expression, where in this study, the doodles of the students are representative of their understanding since they would not want to speak while trying to understand a listening passage.

On the Difference between the Pre-Test and Post-Test Mean Scores of the Students Exposed to Doodling

Table 12 presents the differences between the pre-test and post-test mean scores of the students exposed to doodling along the different listening comprehension levels. The statistical computation reveals that all listening comprehension levels of the students exposed to doodling based on their t – computed values (literal with -3.683; interpretative with -3.473; critical with -2.395; and creative with -4.472) had associated significant values (literal with .002; interpretative with .003; critical with; & creative with .000) lower than the 0.05 level of significance. Hence, for all levels, the null hypothesis is rejected. This means that there is a significant difference between pre-test and post-test mean scores of the students exposed to doodling along all comprehension levels. Moreover, the data shows that along literal, interpretative and creative level, the difference is highly significant at .01 significance value.

According to Smallwood et al. (2007), as cited by Andrade (2009), doodling made a positive effect to the listener's recall ability, implying it may have facilitated in reducing daydreaming because another undemanding task has been employed. Hence, their study supports the results of the present study that doodling could be an aid to listening. In the case of the present study, doodling is also beneficial as it aided in improving the listening comprehension of the students. As shown in table 12, the comprehension levels of the students who were exposed to doodling increased.

Table 12. Difference between the Pre-test and Post-test Mean Scores of the Students Exposed to Doodling

Comprehension Level along:	Score	Mean	Mean Difference	t	Sig.
Literal	Pre-test	6.3158	-1.5263	**-3.683****	**.002**
	Post-test	7.8421			
Interpretative	Pre-test	2.6842	-1.8947	**-3.473****	**.003**
	Post-test	4.5789			
Critical	Pre-test	1.4211	-0.7894	**-2.395***	**.028**
	Post-test	2.2105			
Creative	Pre-test	.7368	-1.0527	**-4.472****	**.000**
	Post-test	1.7895			

**Highly Significant at 0.01, *Significant at 0.05

The study of Zeng (2007) also supports this particular result of the study particularly in the mental strategies performed by the students as they listened while doodling. In their study, they emphasized the mental processes which a person is involved in listening. Specifically this processes involve top-down and bottom-up. Although it is not measured in this study, the extent of top-down and bottom-up processing of the students may have also be reinforced by their doodles. For example, in the analysis of their doodles, it was found out that the metacognitive doodles increases as the students gets their way to understanding the lecture- or conversation-type of listening passage. Hence, the from their stored knowledge or schemata, the students were able to relate the content of the listening passage and thus their doodle output resulted to meta-cognition, whereby their doodles reflect what is happening in the listening passage and what they understand about it.

Furthermore, although more extraneous doodles were produced, it only reflects that he students when get bored consistently kept their minds active. This leads to another possible reason as to why they were able to improve their levels, that is, it is the act of doodling itself that has influenced the students to focus on what they were listening to. This has been studied overtime when Adrade (2009) pioneered the effect of doodling to concentration. Although her study was the first to bravely attempt to reduced mind wandering by day-dreaming, the results paved way for the a hallmark discovery, in which later researchers ventured on affirming and even enhancing the claim. Schott (2011) supported her claim, which also supports the results of the present study, by emphasizing that doodling helps stabilize arousal and helps a person increase watchfulness, hence, the increased in the level of students' listening comprehension may be attributed as a result of the exposure to doodling.

On the Difference between the Pre-Test and Post-Test Mean Scores of the Students Not Exposed to Doodling

Table 13 shows that along literal, interpretative, and critical levels of listening comprehension, the t-computed values (literal with .000; interpretative with -2.088; & critical with -1.17) had associated significance values (literal with 1.000; interpretative with .51; & creative with .279) lower than the 0.05 level of significance. Hence, the null hypothesis is accepted for these three levels. This indicates that the pre-test and post-test mean scores of the students not exposed to doodling are not significantly different.

However, along creative level, the t – computed value of -2.535 had an associated significant value of .021 which is lower than 0.05 level of significance. Hence, for this particular level, the null hypothesis is rejected. This means that the pre-test and post-test mean score of the students not exposed to doodling along creative level are significantly different. This further means that their post-test mean score in creative level is significantly higher than their pre-test mean score in the same level.

Table 13. Difference between the Pre-test and Post-test Mean Scores of the Students Not Exposed to Doodling

Comprehension Level along:	Score	Mean Level	Mean Difference	t	Sig.
Literal level	Pre-test	5.9474	0	.000[ns]	1.000
	Post-test	5.9474			
Interpretive level	Pre-test	2.3158	-1.0526	-2.088[ns]	.051
	Post-test	3.3684			
Critical level	Pre-test	1.1053	-0.421	-1.117[ns]	.279
	Post-test	1.5263			
Creative level	Pre-test	.9474	-0.7894	**-2.535***	**.021**
	Post-test	1.7368			

[ns]Not Significant; *Significant at .05

The results presented in this section are rather stimulating because, from the series of listening test experienced by the respondents, the skills in comprehending literal, interpretative, critical information did see any improvements. It is to be understood then listening comprehension skills might not necessarily be improved even when the listener himself thinks he is concentrating. This is explained in the study of Smallwood & Schooler (2006) claiming that while the listeners think that they are concentrating on the listening passage, their brain engages in daydreaming and other task-unrelated thoughts (TUTs) especially when the listener starts to feel boredom and attempts to relieve it. Hence, upon attempting to relieve boredom by daydreaming, the listener is being led away from the details of the passage.

It is also very apparent in the results that only in creative comprehension where the students

improved as reflected in their post-test scores. This result implies that although the respondents maintained their levels in those three levels, they improved significantly in creative level, i.e. "handling verbal and non-verbal communicative strategies, identifying hesitation and prop words, and making appropriate response" (Al-Musalli, 2001). This is supported the study of Anderson (1977, cited by Yenkimaleki & Van Heuven, 2016) that their knoweledge in acknowledging hesitations and determine their comprehension which involves the activation of their schema. The listeners attempt to relate the details of the listening passage with the schemata and see the connection for better understanding. Thus the schema is activated as soon as the listeners find various parts of the listening passage familiar to them.

On the Difference between the Levels of Listening Comprehension of the College Students Exposed To Doodling and Those That Are Not

As reflected in Table 14, the findings revealed that in the literal level, the resulting t-computed value, which is 2.192, had the significance value of .035 below the 0.05 level of significance. Hence, the null hypothesis is rejected. This means further that the level of listening comprehension of the college students exposed to doodling is significantly higher than those who were not in terms of literal comprehension. However, the t-computed values of interpretative (2.018), critical (1.513), and creative levels (.127) had corresponding significance values (interpretative with .51; critical with .139; & creative with .900) higher than the 0.05 level of significance. Hence, the null hypothesis is accepted. This means further that there is no significant difference between the level of listening comprehension of college students exposed to doodling and those who were not along interpretative, critical, and creative levels.

Table 14. Difference between the Level of Listening Comprehension of the College Students Exposed to Doodling and those that are Not Exposed to Doodling Activities

Comprehension Level along:	Group	Mean	Mean Difference	t	Sig.
Literal	Students exposed to doodling	7.8421	1.8947	**2.192***	**.035**
	Students not exposed to doodling	5.9474			
Interpretive	Students exposed to doodling	4.5789	1.2105	2.018^{ns}	.051
	Students not exposed to doodling	3.3684			
Critical	Students exposed to doodling	2.2105	0.6842	1.513^{ns}	.139
	Students not exposed to doodling	1.5263			
Creative	Students exposed to doodling	1.7895	0.05270	.127^{ns}	.900
	Students not exposed to doodling	1.7368			

^{ns}Not Significant *Significant at 0.05

These findings only illustrate that based on the taxonomy of Al-Musalli (2001) doodling may have aided the students in improving their skills in recognizing or recalling main ideas, understanding the gist of what was said, identifying relevant points and rejecting irrelevant points.

Thus, the results of present study affirm and reason with the conclusions of mentioned studies about the effect of doodling on listening.

For example, Andrade (2009) published her study, "What does Doodling do?" – the first published study about doodling and listening – and revealed her discovery that doodling aided to concentration. In her study, she found out that those who doodled remembered more names than those who did not, hence, recall ability. Thus, the present study is in coherence with the results of Andrade's that doodling aids to recalling or remembering of details and as referenced to the taxonomy of Al-Musalli (2001), main ideas.

Furthermore, the present study agrees with Tadayon and Afhami (2016) that doodling improves learning. Their study assessed the effects of doodling on learning performance and found out that those who doodled outperformed those who did not. Such a result drew the implication that doodling was beneficial particularly in learning. However, what lacks in their study is the non-identification on what area of learning was measured. Hence, the present study drove its focused on the comprehension levels and attempted to find which of these areas would receive a positive impact of doodling. As a result, only in literal comprehension was doodling beneficial and not in higher comprehension levels, i.e., interpretative, critical, and creative.

However, the study conducted by Boggs et al. (2017), a supposed "replication and extension" of Andrade's (2009), arrived at a rather different and contradicting result. Their study focused on employing structured and unstructured doodling, and note-taking to see whether doodling can have beneficial effects on comprehension. As it turned out, doodling, whether it be structured or unstructured, did not make any significant effects on the performance of the respondents while note-taking did. They then concluded that doodling may not be advantageous at all in increasing recall performance because they found no support that doodling improved the recall ability of their participants. The results of the present study reasons with Boggs's et al. about the effect of doodling on the recall performance of the respondents since the former found out that the effects of doodling are significant in this level of comprehension.

Initially, the present study posed an ambition to see whether doodling can also aid in higher levels of comprehension, but as the findings revealed, the effects of doodling to these higher levels of listening comprehension, i.e. interpretative, critical, and creative, are no better than just listening. Hence, the results of this study adds, to the literature of the effects of doodling, the limitation of its effects on listening comprehension, that is, doodling can be used to aid in literal comprehension and may not be more beneficial in improving skills in higher levels of comprehension than not doodling at all.

On the Relationship Between the Profile Variables and the Listening Comprehension Levels of the College Students Not Exposed To Doodling and Those That Are Exposed To Doodling Activities

As for the students who were not exposed to doodling, the result of the statistical computations, which is presented in Table 15, revealed that the significance values across all profile variables are higher than the 0.05 level of significance from the variables were tested. Hence, the null hypothesis is rejected. It therefore means that there is no significant relationship between the profile of the students not exposed to doodling activities and their listening comprehension levels. Further, this suggests that their profile is not significantly associated with their performance wherein their profile had no influence on their levels.

Table 15. Relationship between the Profile variables and the Level of Listening Comprehension of the College Students that are Not Exposed to Doodling Activities

Profile	Listening Comprehension							
	Literal Level		Interpretative Level		Critical Level		Creative Level	
	r	Sig.	R	Sig.	r	Sig.	r	Sig.
Sex	-.092	.708	-.359	.132	.010	.966	.098	.690
Age	-.040	.872	-.001	.996	.019	.937	-.219	.368
First Language	.152	.534	-.066	.789	-.122	.619	.335	.161
Verbal Reasoning score in the entrance examination	-.001	.998	.144	.558	.007	.976	-.159	.515

As for the students who were exposed to doodling activities, the result of the statistical computation shown in Table 16 does not differ much from those that were not exposed as their computed significance values were also higher than the 0.05 level of significance. Thus, across all profile variables, the null hypothesis is rejected. This indicates that none of the profile variables is found to be significantly correlated on the level of listening comprehension of the college students are exposed to doodling activities.

Table 16. Relationship between the Profile variables and the Level of Listening Comprehension of the College Students Exposed to Doodling Activities

Profile	Listening Comprehension							
	Literal Level		Interpretative Level		Critical Level		Creative Level	
	r	Sig.	R	Sig.	r	Sig.	r	Sig.
Sex	.258	.287	-.006	.979	.175	.473	.188	.441
Age	-.066	.790	-.364	.126	-.139	.569	-.241	.321
First Language	.166	.496	-.198	.418	.116	.635	.192	.432
Verbal Reasoning score in the entrance examination	.102	.679	-.051	.837	.035	.885	.150	.539

Moreover, since their performance in the listening comprehension test came about a result after the student's exposure and non-exposure to doodling activities, the findings also imply that the profile variables did not affect the rate of effectiveness of the intervention strategy. This means that despite the application of treatment to the experimental group, their performance was still a result of the length of their exposure and type of stimuli or the listening passages which both groups were exposed to during the experiment.

Conclusions

Based on the salient findings of the study, the following conclusions were drawn.

1. The Bachelor of Science in Development Communication in Tarlac Agricultural University is dominated by females. The typical language first learned by the respondents is Filipino. In the entrance examination, majority of the respondents had above average scores (80-89) in verbal reasoning. They also vary in their way of learning and are dominant in one or more than one learning styles.
2. The students who were exposed to doodling improved on all levels of listening comprehension. The students who were not exposed to doodling improve only on literal, interpretative, and creative comprehension but not in critical.
3. The students exposed to doodling activities produced various doodles as they participated in the listening activities. Most of these doodles are extraneous, or doodles that have no connection with listening passage at all. This majority was followed by meta-cognitive doodles which reflects the efforts of the students to track their understanding of the passage.
4. Doodling can be beneficial in improving listening comprehension as evidenced by the increase of the number of respondents possessing improved levels of listening comprehension skills.
5. Doodling may significantly aid in improving literal comprehension but its benefit on the improvement of interpretative, critical, and creative comprehension levels may not be significant.
6. The students' age, sex, first language, and verbal reasoning score in the entrance examination are not significantly associated with their performance in the listening comprehension tests. Hence, the positive effect of doodling is not profile sensitive.

Pedagogical Implications

While most strategies that have been devised to improve listening comprehension have worked out in the past, EFL and ESL teachers continue to search for more and newer strategies to use. Based on the the results of the present study and the previous studies, doodling can be considered a new strategy to help struggling students comprehend listening transcripts better. As established, concentration is enhanced when doodling is performed (Andrade, 2010). With narrower direction, where doodling aids in improving literal comprehension, teachers and even students may opt to allow doodling when targeting foundational areas of comprehension.

As Smallwood et al. (2007) put it, one's environment, in order to have meaning, must coordinate with one's internal representations. Doodling introduces a way that would strengthen this relationship as it gradually decreases day-dreaming by attempting to forbid the entry of task-unrelated thoughts to one's mind. With the consistent exposure of the students to doodling while listening, they developed a strategy to learn better coordination and prevent too much entry of task-unrelated thoughts. Hence, better coordination happen led to better comprehension.

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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

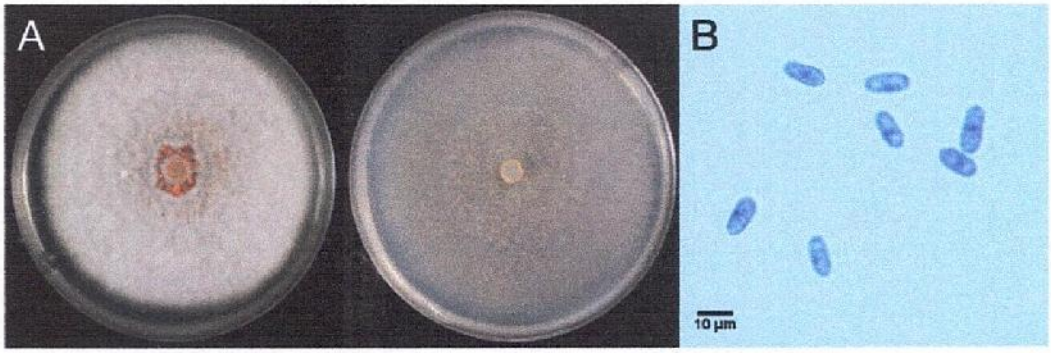

Figure 1. (A) Colony morphology of *C. gloeosporioides sensu lato* isolate MG12 and its (B) spores under 40X magnification.

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.

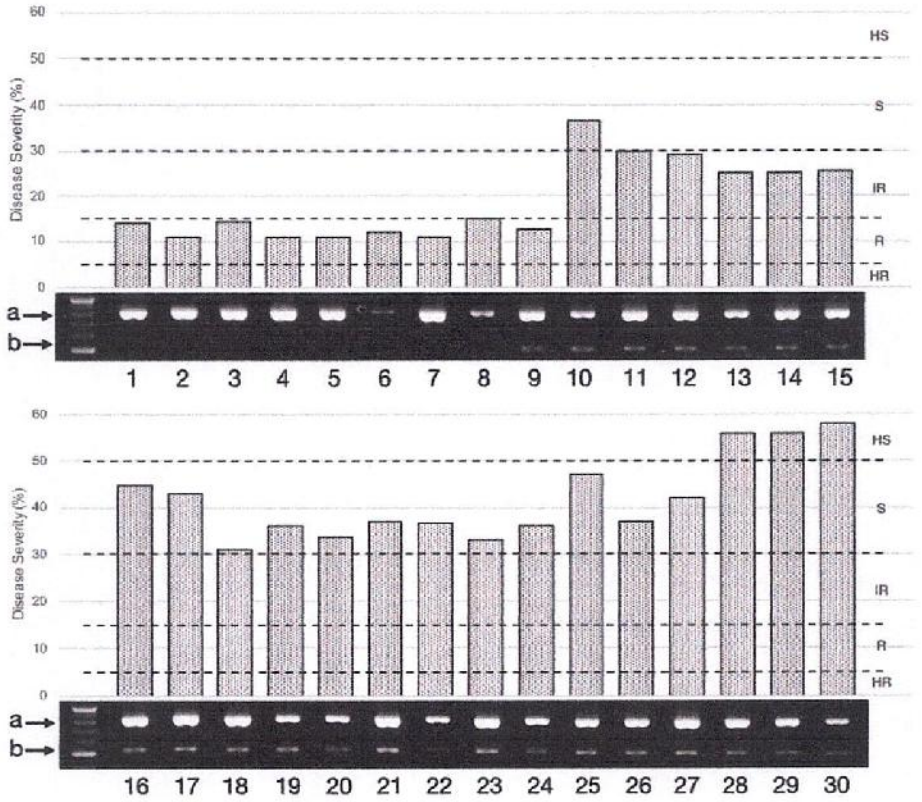

Figure 3. Association of anthracnose resistance phenotypes (% disease severity) with the banding patterns from the SNP 21881933 derived AS-PCR assay of different mango accessions. The upper band (a) is the amplification of the *β-1,3-GLU2* fragment and serves as a positive control. The lower band (b) 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.

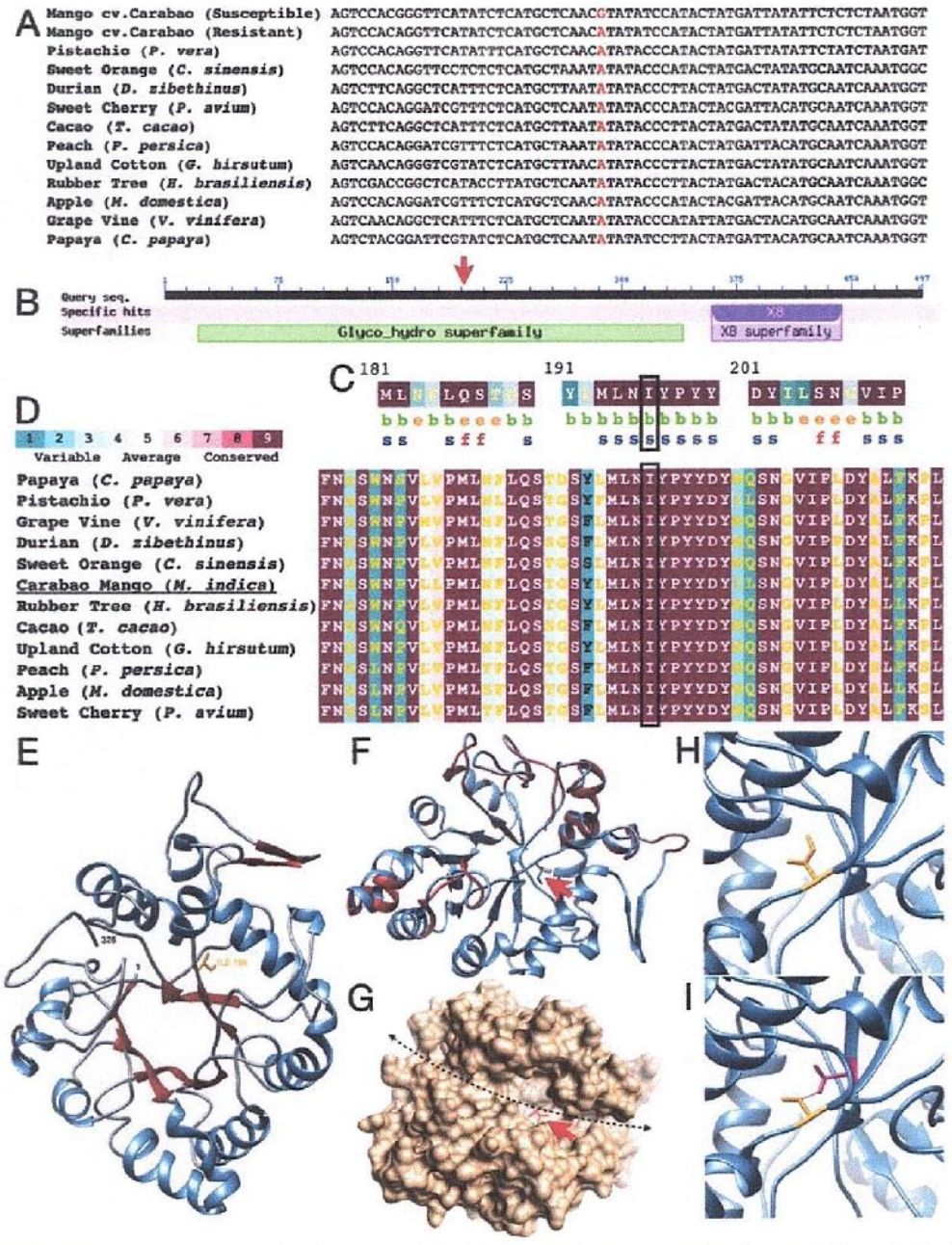

Figure 1. SNP 21881933 is a A > G transition in mango *β-1,3-GLU2* leading to an Isoleucine to Valine substitution. (A) MSA showing the SNP 21881933 site at position 6:17813013 (indicated in red) in mango *β-1,3-GLU2* and its homologs. The adenine [A] base is conserved between mango, apple, cacao and 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.

6. FUNDING

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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.

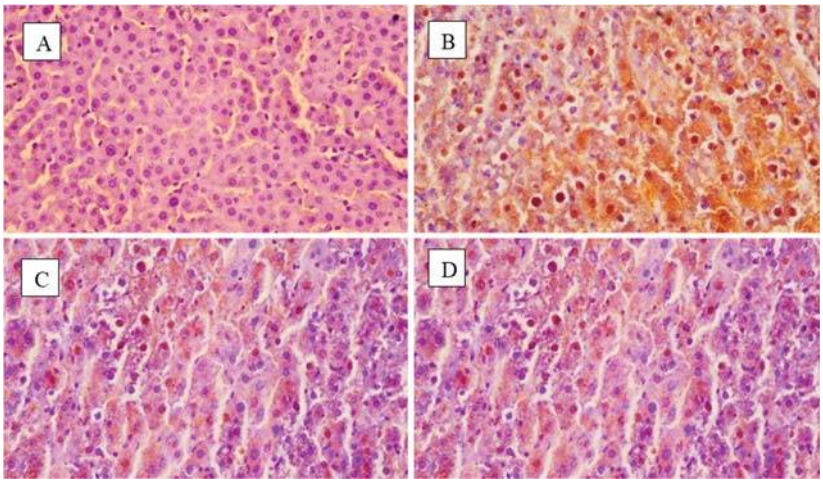


FIGURE 1. The results of the examination of Hsp70 expression by immunohistochemical staining on liver cancer tissue are shown in brown in the cell nucleus. (a) mice that did not get *Azadirachta indica* leaf extract treatment (control); (b) rats receiving 5 mg/kgBW of sorafenib treatment and (c) rats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

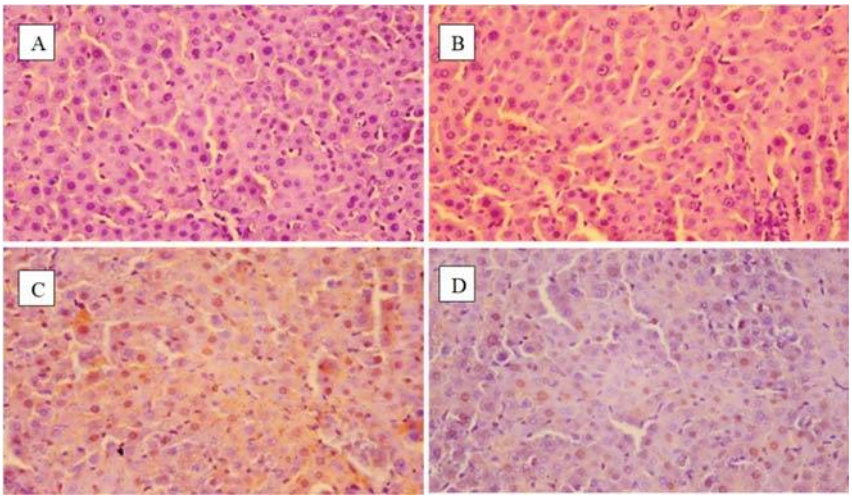


FIGURE 2. The results of the examination of AIF expression by immunohistochemical staining on liver cancer tissue are shown in brown in the cell nucleus. (a) mice that did not get *Azadirachta indica* leaf extract treatment (control); (b) rats receiving 5 mg/kgBW of sorafenib treatment and (c) rats receiving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

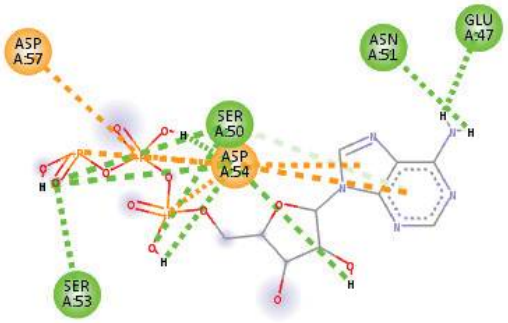
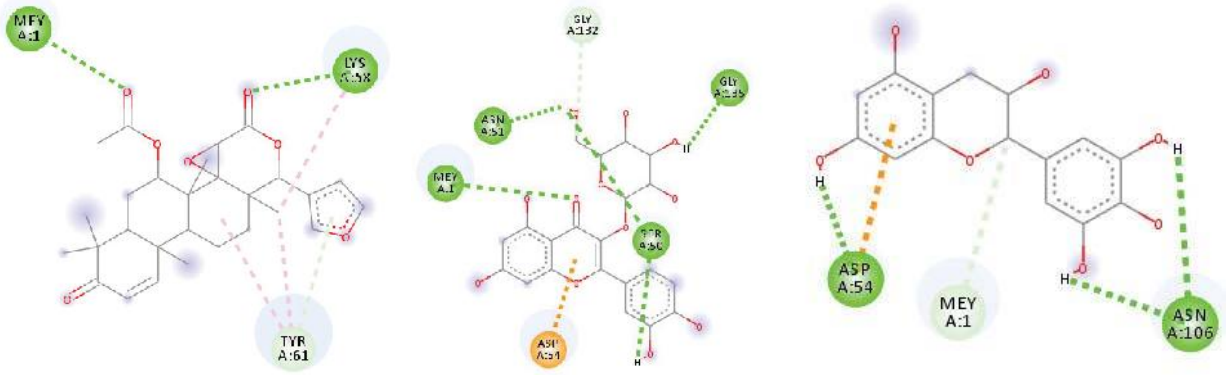


FIGURE 3. Visualization of the Interaction between Native Ligands and Target Proteins



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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A Sequential Explanatory Study on the Mental Health of Filipino Youth Living with Human Immunodeficiency Virus (HIV)

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Research studies confirm that individuals with chronic illnesses, such as Human Immunodeficiency Virus (HIV), are at risk for developing multiple mental health conditions. In the Philippines, despite the alarming increase in HIV cases among young people, research investigating their mental health remains limited. Hence, this study was conducted to explore the mental health of Filipino youth living with HIV (YLHIV). A mixed-method approach was utilized, particularly the sequential explanatory research design. In the quantitative phase of the study, 50 Filipino YLHIV participated and 10 among them underwent the phenomenological interview format (qualitative phase). The results found that 72% of the participants had mild to severe depression and 44% reported thoughts of suicide. Further, three themes that characterized their mental health emerged from descriptive phenomenology, i.e., (a) disruptive thoughts, (b) depressive mood, and (c) deteriorative behavior. These findings may serve as a basis for government officials and other advocacy groups in developing mental health programs for YLHIV. Comprehensive and accessible mental health services for this population are highly recommended.

Keywords: mental health, human immunodeficiency virus (HIV), youth living with HIV (YLHIV), suicide, depression.

At the end of 2021, the World Health Organization (WHO) reported that 38.4 million people worldwide were living with Human Immunodeficiency Virus (HIV), which makes the disease a major global public health concern. Of all the HIV cases globally, adolescents and young people represent a growing proportion of affected individuals. In 2020 alone, more than 400,000 young people were newly infected by HIV (UNICEF, 2021).

Young people with a chronic illness like HIV are also at risk of developing mental health conditions such as depression and anxiety disorders compared to the general population. This is supported by a

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quantitative study conducted in the United States showing that 17.5% of youth living with HIV had psychological symptoms of anxiety and depression (Brown et al., 2015), compared with only 2.1% and 6.7%, respectively, in the general population (Center for Behavioral Health Statistics and Quality, 2017). Similarly, a study in Mozambique indicated that adolescents with HIV had higher scores of depression, post-traumatic stress disorder, anxiety, and alcohol and drug abuse, than youth without HIV (Gennaro et al., 2022). In Thailand, Chantaratin et al. (2022) found that about 20% of young people with HIV had significant levels of depression and anxiety. Further, Kamau et al. (2012) indicated that 49% of the YLHIV participants in Kenya reported having at least one clinical diagnosis, such as anxiety disorder or major depressive disorder.

In response to the reported mental health issues of YLHIV, several countries have developed psychosocial programs for this population. Bhana et al. (2020), upon review of 16 studies on YLHIV, observed that the most often used interventions to improve the mental health of YLHIV included family-strengthening approaches, caregiver-adolescent relationships, problem-solving, and communication. In Tanzania, Njau et al. (2022) developed rather comprehensive interventions to address the depression of young people with HIV, including psycho-education, behavioural activation, cognitive restructuring, mood monitoring, and problem solving techniques.

In the Philippines, the Department of Health reported that amidst the COVID-19 pandemic, an estimated 3.6 million Filipinos faced mental health issues such as depression, substance use disorders, and bipolar disorder (University Research Co., 2021). However, despite the growing cases of HIV among Filipino youth, the literature investigating their mental health remains limited. Hence, this sequential explanatory study was conducted.

METHOD

Participants

The present study used a sequential explanatory design which consisted of two distinct phases: quantitative followed by qualitative phase. In the first phase, the researcher collected and analyzed quantitative data from 50 Filipino youth living with HIV. The participants' ages ranged from 18-30 years, with 80% identified as male, 18% married or partnered, and 84% reported living with their families. Then, the collection and analysis of qualitative data from 10 selected YLHIV who participated in the phenomenological interview were conducted in the second phase.

Prior to data gathering, all procedures performed in the present study that involved human participants were approved by the Ethics Review

Committee of the University of Santo Tomas, Manila, Philippines, with Protocol Number (G-2018-PN031).

Measures

The researcher used the following instruments to collect data for this study: *Mental Health Inventory* (MHI-38); *Beck Depression Inventory* (BDI); *Adult Suicidal Ideation Questionnaire*; and *Interview Protocol.* Each instrument is described below:

Mental Health Inventory (MHI-38). MHI-38 is a 38-item self-report tool measuring psychological distress and well-being (Veit & Ware, 1983). The MHI measures six subscales: anxiety, depression, loss of behavioral/ emotional control, general positive affect, emotional ties, and life satisfaction. Sample items of MHI are as follows: "How often did you become nervous or jumpy when faced with excitement or unexpected situations during the past month?" and "How happy, satisfied, or pleased have you been with your personal life during the past month?" As regards the psychometric properties of the MHI, Veit and Ware (1983) tested the instrument with 5089 persons aged 13–69 yrs. The results indicated that MHI had .93 Cronbach alpha, whereas its abbreviated version was found to be .82. To confirm the reliability of the instrument, a confirmatory analysis was conducted by Heubeck & Neill (2000) with adolescents who were literate in the English language and showed an internal consistency of .90.

Beck Depression Inventory (BDI). The BDI is a self-report rating inventory consisting of 21 items designed to measure symptoms of depression, such as mood, guilt, suicidal ideas, loss of appetite, pessimism, and sense of failure, among others. The *Beck Depression Inventory* has acceptable psychometric properties; its internal consistency ranges from .73 to .92 with a mean of .86 (Beck et al., 1988). The instrument has high internal consistency with alpha coefficients of .86 for psychiatric populations and .81 for non-psychiatric populations (Beck et al., 1988).

Adult Suicidal Ideation Questionnaire (ASIQ). The ASIQ consists of 25 items designed to measure the individual's level of suicidal ideation which provides valuable information about the mental health of the individual (Reynolds, 1991). The items of ASIQ are rated on a 7-point scale that measures how frequently the individual thought of committing suicide within the past month. Higher scores in this instrument are indicative of greater suicidal ideation. ASIQ includes items such as "I thought it would be better if I was not alive," and "I thought that if I had a chance, I would kill myself." In terms of the validation and standardization process, the scale was administered to more than 2,000 individuals in college, community, and psychiatric settings. The internal

consistency reliability of the scale was alpha reliability coefficient of .96 (community sample), .96 (college student sample), and .96 (psychiatric sample) (Reynolds, 1991). The scale also has a high test-retest reliability coefficient of .95 (Reynolds, 1999).

These instruments were administered in person at Pinoy Plus Advocacy Pilipinas, Inc., a pioneer support group dedicated to the welfare of people living with HIV in the Philippines. The participants finished the instruments in an average duration of 30-40 minutes.

Interview Protocol

To have a deeper understanding and corroboration of the quantitative data on the participants' mental health, the researcher employed a qualitative approach, specifically the phenomenological research design. This design focuses on describing a particular phenomenon as accurately as possible (Groenewald, 2004). Using said design, the researcher accurately described the lived experiences of YLHIV concerning their mental health. Ten YLHIV recruited from Pinoy Plus Advocacy Pilipinas Inc. participated in the phenomenological interview. Moreover, the researcher developed a semi-structured interview guide to uncover their lived experiences in relation to mental health. Particularly, the interview guide aimed to reveal their experiences with depression, anxiety, suicide, and other mental health issues. Specifically, the questions included items on the emotional, social, and psychological well-being of the participants such as "How do you view your emotional well-being now as HIV+", "Have you ever experienced long-term sadness because of your condition"? If yes, how long was it?

Data Analysis

The data were then analyzed quantitatively and qualitatively. In the quantitative phase of the study, descriptive statistics were employed to analyze the mental health profile of the participants. For the qualitative phase of the study, the researcher used Colaizzi's (1978) seven-step method to analyze the data. The process included: (1) familiarizing with the data by reading the participants accounts; (2) pulling out significant statements from the participants' accounts; (3) formulating meaningful units from the significant statements; (4) categorizing the meaningful units into clusters of themes; (5) developing a full and inclusive description of the phenomenon by incorporating all the themes produced at step 4; (6) condensing the exhaustive description down to a short, dense statement that captures just those aspects deemed to be essential to the structure of the phenomenon; and (7) returning the fundamental structure statement to all participants.

RESULTS

Quantitative Results

Table 1 shows the comprehensive profile of the participants in terms of their mental health.

Table 1

Mental Health Profile of Filipino Youth Living with HIV

Variable	Frequency	%
Mental Health Index		
High	21	42%
Low	29	58%
Psychological Distress		
High	31	62%
Low	19	38%
Psychological Well-being		
High	22	44%
Low	28	56%
BDI (Depression)		
Normal	14	28%
Mild	10	20%
Moderate	12	24%
Severe	14	28%
ASIQ (Suicidal Ideation)		
High	22	44%
Low	28	56%

N=50

Favorable results that indicate greater psychological well-being were outnumbered on every component measure. Forty-two percent (42%) of YLHIV scored as mentally healthy, while 58% scored low–an indication that more than half of the respondents had a negative state of mental health. Further, a specific result was obtained from the two global scales, whereas 62% scored high in psychological distress and only 38% reported low scores. Forty-four percent of the participants reflected positive psychological well-being when more than half (56%) suffered psychological distress. Only 28% of the respondents had normal levels of depression, whereas roughly 72% ranged from mild to severe levels. Lastly, based on the *Adult Suicidal Ideation Questionnaire*, 44% scored high, whereas 56% obtained low scores, which reflects that many of these adolescents have expressed suicidal ideation.

Qualitative Results

Through careful analysis vis-à-vis transcendental and eidetic reduction of the narratives of select youth living with HIV, this study

afforded the emergence of three themes namely: (a) disruptive thoughts, (b) depressive mood, and (c) deteriorative behavior.

Disruptive Thoughts Accommodating the fact that HIV infected them was very disturbing for the participants. They were besieged by several irrational thoughts, such as overestimation of danger and illogical interpretation of their diagnosis. Particularly, they were terrified by the thoughts of dying at a young age and were worried about their future. As verbalized by the participants:

> That time, I kept on thinking that I was dying, that there is no treatment for HIV. I was worried for my family because I am the breadwinner" (Participant 2).

> I thought I was dying. I was thinking what will happen to the dreams I have for my family. Moreover, I was bothered on how to tell my parents about my health condition (Participant 10).

Besides entertaining irrational thoughts, the participants were likewise disturbed by anxious thoughts as manifested by their fear of being rejected and discriminated against by family, friends, and romantic partner and in the workplace. As expressed by the participants:

> How can I work if I have this illness? They might trace it through medical examination (Participant 1).

> I am afraid to form a romantic relationship because I might transmit the virus. I don't want my partner to get sick because of me (Participant 3).

Cognizant of their present health condition, the participants were also bombarded with suicidal thoughts as evidenced by their death wish, thinking of killing themselves, and thoughts that other people are better off without them.

> I thought that instead of dying from the opportunistic infections of this virus, I wish I would not wake up the next day (Participant 1).

The irrational and anxious thoughts of the participants were entrenched from certain triggers in their environment, particularly the lack of available information about HIV. They articulated that they had limited information about the illness and the information they did have was mostly misconceptions about its transmission, treatment, and prevention. This wrong notion triggered the fear of being rejected and discriminated against in the workplace.

This finding supports the claim of Cournos et al. (2005) and Dorrell et al. (2008) that after learning about their HIV status, most PLHIV had personal concerns like the fear of death and uncertainties about the future. Additionally, the anxious thoughts of the participants were expressed by their fear of rejection and discrimination. The findings converged with the study of Jena (2014) in one wellness clinic in South Africa, indicating that adolescents living with HIV showed anxiety. These anxious thoughts were fueled by a lack of accurate information about their health condition. Moreover, youth living with HIV were bothered with suicidal thoughts and death wishes. According to Badiee et al. (2011), suicidal thought is common among people with HIV as compared to the general population. Alarmingly, suicide rates have been reported at elevated levels in this population (Carrico, 2010). This suicidal tendency is triggered by the burden that accompanies the long-lasting implication of being HIV positive (Kalichman et al., 2000). Further, stigma, discrimination, low self-esteem, and lack of social support are other factors directly associated with suicidal thoughts and behaviors of people living with HIV (Casale et al., 2019; Wang et al., 2018).

Depressive Mood Learning that they were infected by HIV was not easy for the participants. They were blasted with the emotional turmoil that intruded on their daily activities at home, in school, and even in the workplace. Notably, the participants articulated their experience of emotional distress on the first few months of living with the illness. They were in a deep state of agony and disbelief. As verbalized by the participants:

> I felt like I was going crazy that time. Some of my friends told me not to stress myself, but it stressed me a lot. From time to time, it sank in. I really didn't know what to do (Participant 1).

> It felt like I was blown up when they told me about the result. I even tried to ask for a second opinion because I couldn't believe it. Gosh, I was extremely terrified at that time. 'I'm certain my parents will kill me,' I uttered (Participant 2).

The other participants experienced persistent feelings of sadness. They verbalized feelings of aloneness and loneliness especially the undisclosed YLHIV. Generally, they felt miserable because of their health condition. The following are some of the verbalizations of the participants:

> After knowing the HIV test result, I kept it for a long time. When I was with my parent, I pretended to be OK, but in reality, I felt so sad most of the time" (Participant 7).
> "I felt lonely; I had difficulty coping with loneliness. I couldn't do the things that I previously enjoyed; I lost interest in almost everything" (Participant 6).

Likewise, the participants conveyed their experience of hopelessness as they struggled with HIV. They were uncertain about their future and expressed pessimism about life in general.

> I have a lot of dreams in life, dreams for my family, but all these dreams are shattered because of my illness. I felt so hopeless (Participant 4).

The indices of depressive mood were prompted by discrimination. One participant shared that he experienced discrimination from his own family. He said:

> After telling my parents about my condition, I noticed that they avoided using the utensils we used to share like drinking glass, spoon, and fork. I even shared a room with my brother before, but now, they asked him to move out of my room (Participant 1).

Another factor that incited the depressive mood of the participants was non-disclosure. They reported having difficulty disclosing their health condition due to the stigma associated with the illness.

This finding concurs with the report of UNAIDS (2018) that people with HIV have a higher risk of developing mental health conditions like depressive symptoms. The said report supports the claim that medical conditions like HIV could serve as a major source of stress that negatively affects a person's mental health (US Department of Health and Human Services, 2020). In South Africa, young people with HIV reported mental health difficulties of depression and stigma (Toska et al., 2019). In a cross-sectional study conducted in Jamaica, youth with HIV ages 15- 25 years were found to have high rates of stress (64%) and depression (63%) (Brown & Morgan, 2013). The depressive symptoms of YLHIV are triggered by factors such as discrimination, non-disclosure, and lack of social support. This particular result is aligned with the findings that living alone and having poor social support were significantly associated with depression (Amare et al., 2017; Bhatia & Munjal, 2014).

Deteriorative Behavior The participants also struggled with behavior that impaired their physical and social well-being. After knowing their HIV status, they started engaging in several deteriorative behaviors like losing

interest. Particularly, the participants started to lose interest in their work, refused to go school, and disengaged themselves with activities they previously enjoyed. As uttered by the participants:

> I came to the point that in almost two months, I did not go to work. I lost my willingness to work" (Participant 5).

> Before, I loved going to the gym. I usually spent an hour or two twice or three times a week. But now, I don't go to the gym anymore (Participant 6).

Moreover, the participants experienced self-neglect while living with a chronic illness. They disregarded the regular intake of food and ate on an irregular schedule.

> What happened to me was I skipped some meals in a day. There was even a time that I ate only once each day. I had difficulty getting up to do the usual (Participant 9).

Some participants even engrossed themselves in dangerous vices like substance use as a form of avoidant coping mechanism.

> The time that I learned about my HIV status, I started smoking cigarettes, drinking alcoholic beverages, and even tried taking marijuana. I felt so devastated. That's why I didn't care about my health anymore (Participant 1).

For people living with HIV, participating in the usual social interactions was challenging. The participants reported that they experienced withdrawal from other people, such as their friends and workmates.

> Actually, I started to avoid mingling with my friends. I was afraid that every time I was with them, they might discover my health condition (Participant 10).

Loss of interest and self-neglect were triggered by internalized stigma, specifically the thought of dying at a young age. This negative notion about the illness fueled their unwillingness to perform their usual tasks. Meanwhile, engaging in dangerous vices was entrenched in denial of HIV status. They refused to accept their diagnosis; hence, they engaged in avoidant coping mechanisms. Further, the participants' experiences of discrimination from family and friends provoked social withdrawal. They detached themselves from social activities because they were repudiated by their own family.

YLHIV displayed behaviors that harm their physical health, such as smoking cigarettes, drinking alcohol, and using marijuana (Brown & Morgan, 2018). These behaviors are coping mechanisms of YLHIV in dealing with psychological distress brought about by their illness (Duko et al., 2019). This suggests a significant link between psychological distress and substance use among young people infected by HIV. In terms of social withdrawal, the British HIV Association and British Association for Sexual Health (2018) reported that social isolation is one of the top lists of unmet social needs among YLHIV in the United Kingdom. They withdraw from other people out of fear of being stigmatized and discriminated (Dejman et al., 2015; Wallack & Brotman, 2012).

DISCUSSION

The quantitative findings imply that YLHIV struggle with mental health conditions such as anxiety, depression, and loss of behavioral/emotional control. This supports the survey conducted by Youth Stop Aids (n.d.) in America, wherein 85% of young people reported their mental health is worse and poorer than non-HIV peers. As stated by Dow et al. (2020), YLHIV with unaddressed/unattended mental health problems are indeed increasing. The Youth group alone had a high prevalence of mental health issues (Newman et al., 2021), but those who live with HIV are frequently diagnosed with mental health disorders (Pokhrel, 2019). Further, they often experience problems in emotional, psychological, and behavioral aspects at higher rates than the general population (Mellins & Malee, 2013). Moreover, this quantitative finding is also aligned with the themes that emerged from the qualitative phase of the present study. The participants reported that they experienced anxiety as manifested by their irrational fears, worries, and intrusive thoughts caused by the misconceptions they held about HIV.

BDI results showed that 52% of the respondents reported mild to severe levels of depression, which is higher than the general population. This is supported by the study of Brown et al. (2015), showing that 17.5% of youth living with HIV had psychological symptoms of depression compared with only 6.7% in the general population (Center for Behavioral Health Statistics and Quality, 2017). That is similar to the findings of Girma et al. (2021) which showed a 30.2% prevalence of depression in Ethiopia, and that of Fawzi et al. (2016) which revealed a 26% prevalence in Rwanda. Additionally, Benton (2019) found that depression simultaneously existed with HIV among the population of youth, which then, as a result, worsened the negative state of their mental health. Further, depression is one of the themes that emerged from the phenomenological interviews conducted in the present study. As reported

by the participants, they experienced long-term sadness, feelings of guilt, and hopelessness.

Based on the *Adult Suicidal Ideation Questionnaire* result, 44% scored high, while 56% obtained low results. Though low scorers are more than half, 44% is still a large portion of the sample, which reflects that many of them were suicidal or have been thinking of ending their lives. As stated by Tsegay & Ayano (2020), people with HIV have a high prevalence of suicidal attempts and ideation, and the worst is, it was linked with high risk of complete suicide. Suicide is a serious cause of death globally, but YLHIV's rate of suicidality proved to be higher than the general population (Wonde et al., 2018). They also found a magnitude of 27.1% HIV patients who have suicidal ideation, and 16.9% had attempted self-annihilation. A systematic review further unveiled that 26.9% of HIV patients have reported suicidal ideation, 22.2% had planned the deed, 20% harmed themselves, 23.1% has been thinking to end their lives, 14% desired death (Catalan et al., 2011), and 24.38% had suicidal ideation in their lifetime (Tsegay & Ayano, 2020). Consistently, qualitative data from the current study indicated that the participants experienced suicidal ideation in the form of death wish, thinking of harming or killing one's self, and thinking that others would be better off without them.

Globally, studies consistently indicate that mental health of YLHIV is poor. They have a high prevalence of depression and are suicidal. The percentage from the present study shows it is even higher in the Philippines. Such a result reveals that their population is at high risk of having mental health issues, and this may be a manifestation of a systemic problem. Stigma from society and the self, social rejections, prejudice, and disinformation worsens their situation. In the Philippines, Alibudbud (2022) reiterated that gender-based discrimination, lack of sex education and communication negatively affect their mental health. With this, HIV infection turns out to be a problem that does not only start and end on the person alone and the infection attained from several mediums. Poor mental health also reflects their unhealthy environment and negative roles played/contributed by the social system.

The present study only focused on describing the mental health status of YLHIV using a mixed-research approach. The result does not establish the causal relationship of HIV diagnosis and mental health. Other limitations of the study include small sample size and the reliance of self-report for measurement. Hence, it is suggested that additional research should be conducted to better assess the mental health of youth living with HIV.

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CURRENT STATUS ON THE HEALTHCARE WASTE MANAGEMENT OF SELECTED HOSPITALS IN THE PHILIPPINES: AN ASSESSMENT

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Abstract

Healthcare waste management has been more critical during the outbreak of the Covid-19 pandemic. Healthcare waste (HCW) not only poses serious environmental and human health risks, but it can also cause death. The problem of how to manage HCW is extremely important to prevent widespread disease transmission and environmental degradation. A descriptive survey study assessing the implementation of the healthcare waste management on storage, pre-treatment, collection and disposal of all private and public hospitals in the Municipality of Camiling, Tarlac, Philippines was determined. A mixed method research was adopted by conducting semi-structured interviews with the Head of the Waste Management Division, Pollution Control Officers, Sanitary Inspector, and other employees involved in waste disposal in private and public hospitals, clinics, and rural health units. A survey was used as a quantitative tool for data collection from respondents involved in healthcare waste management. Data were gathered using questionnaires and interviews with respondents and key informants, respectively. Data were analyzed and interpreted using frequency count and percentage. Findings revealed that 80% of the respondents used a color coding scheme while 20% used labeling as means of waste segregation. It also showed that 60% of both private and public hospitals has conformed to the waste management standards set by the Department of Health (DOH), Republic Act 9003, and World Health Organization 2009. Moreover, a clinic has conformed only to the DOH standards in terms of segregation, pre-treatment, storage, and disposal of wastes generated. However, the rural healthcare unit used labeling in segregating its wastes instead of a color coding scheme. In addition, segregation of waste was done only in its T and B DOTS and Laboratory. Further, hospital wastes were mixed with municipal wastes and disposed of in a dumpsite. Treated hazardous and infectious wastes were disposed of using burial method. The system of handling, storage, treatment, and disposal of the hazardous wastes of the healthcare units was different from each other.

Keywords: Hospital waste, waste segregation, healthcare waste management, solid and hazardous waste, waste disposal and treatment

INTRODUCTION

The management of hospitals and healthcare units has the responsibility of ensuring that hospital wastes have no adverse health and environmental consequences in their handling, storage, treatment, and disposal. Improper management of healthcare waste aids in the transmission of diseases even the Covid-19 virus. Hospital wastes or biomedical wastes consist of solid, liquid, sharps, genotoxic, pharmaceutical, infectious, chemical, risk, and non-risk. and laboratory wastes that are potentially infectious and dangerous to health care, sanitation workers, patients who are regularly exposed to these wastes, the people who will also be exposed to it, and the environment (soil, air, water) when improperly handled (Das et al., 2021, Gassemi et al., 2016. Hossain et al., 2011, and Patwary et al., 2011, and Rao, 2008). These wastes have to be properly managed to protect public health otherwise they can pose serious risks of disease transmission to waste pickers, waste workers, patients, and the public in general upon exposure to infectious agents (Das, et al., 2021). Moreover, its causing disease is not only

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the problem but if it contains hazardous wastes or radioactive wastes, it cannot be mixed with other non-hazardous solid wastes in the sanitary landfill. Proper waste management practices, safety measures for waste workers, and sanitation are crucial strategies for combating further spread of infectious diseases and landfill issues (Das et al., 2021).

On the other hand, not all hospital wastes are dangerous because there are wastes generated from its offices, cafeteria, and patient cares which does not pose a threat to landfills and public health.

With this environmental concern, government agencies such as DOH and DENR have set quality standards as well as regulations and programs so that occupational hazards of the health care workers will be avoided; the use of toxic chemicals and mixtures will be regulated; and solid and hazardous waste disposal will be managed from generation to final disposal.

The waste management programs of both public and private hospitals, clinics, and rural health units in Camiling Tarlac were determined to validate if they are conforming to the standard rules set by DOH in terms of waste management and the RA 9003 or the Ecological Solid Waste Management Act of 2000. Following the rules set indicates their ability and active responsiveness in ensuring the health and welfare of the community as well as performing their social responsibility in preserving the integrity of the environment. Some of the problems identified were mostly due to non – segregation processes, unsecured dumpsites, and landfill. Moreover, the poor implementation of some healthcare institutions regarding waste management systems was observed. This will most likely result in land degradation and a threat to human health.

On the other hand, some of the most common problems identified are inadequate waste management, lack of awareness about health hazards, insufficient financial and human resources, and poor control of waste disposal. To be able to minimize these consequences, proper implementation of rules and policies should be practiced by the management of hospitals and health care units. A framework for healthcare waste management should always consider health and occupational safety. Hence, this study on assessing the implementation of waste management in the healthcare sectors was conducted to determine the practices and conformity to ambient standards of public and private hospitals and other healthcare units in the Municipality of Camiling from generation, segregation, handling, storage, treatment, and disposal of their wastes. Moreover, the solid and hazardous wastes generated by the hospitals, clinics, and health care units were identified. The waste management programs implemented were described and further assessed on their level of conformity to the guidelines set out by the Department of Health.

The data generated from this study can be used for policy formulation of the Local Government Unit in reviewing the prevailing policies, mechanisms, programs, and facilities on segregating, storing, handling, disposing, and treating hazardous and infectious hospital wastes. Findings can also provide salient information to the local concerned authority to identify numerous issues in healthcare waste management and find opportunities to devise systems and the best solution to each.

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METHODS AND PROCEDURES

This study was carried out in a descriptive survey research design to describe the waste management practices of the premier health care units in Camiling, Tarlac, Philippines. The Head of the Waste Management Division, Pollution Control Officers, Sanitary Inspector, and other employees involved in the waste disposal of 2 private hospitals, 1 private clinic, and 1 rural health unit. To protect the reputation of these premiere healthcare providers and ensure the confidentiality of the information divulged to the researchers, the identity name was not mentioned throughout the discussion. Instead, an alternative nomenclature was used namely: Private hospitals 1 and 2, public hospital, clinic, and health center.

Questionnaires were used to determine the waste management disposal practices of the private and public hospitals, clinics, and health care units while structured interview guides for the key informants to triangulate the information provided by the respondents. The premier private and public hospitals and health care units in Camiling, Tarlac were identified. Permission to administer the questionnaires and conduct interviews with employees involved in healthcare waste management from the Heads and/or owners of the healthcare units was secured. The questionnaire was given to each of the personnel of the said institution and they were further interviewed to validate the data gathered. The gathered data were analyzed through the use of quantitative analysis. The quantitative data were tabulated and analyzed using descriptive statistics such as frequency counts, mean, and percentages.

RESULTS AND DISCUSSION

Solid Infectious and Hazardous Hospital Wastes

Data on the healthcare solid and hazardous wastes generated by the hospitals and other health care providers in Camiling, Tarlac are shown in Table 1.

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Table 1: Summary of solid infectious and hazardous wastes generated by the hospitals and health care units

Hospitals and Health Care Units	Solid Wastes Generated	Infectious or Hazardous Wastes Generated
Clinic	• Papers • Cartons • Plastics • Cans • Styropor containers	• Sharps • Blood • Chemical waste from the laboratory • Cotton swabs • Used facemasks • Used bandages • Used tubing IV • Specimen container of blood and fluids
Public Health Unit	• Glass • Slides • Papers • Cartoons • Used cans • Styropor	• Used syringes • Blood • Urinals • Blood lancets • Phlegm • Cotton swabs • Facemasks
Private hospital 1	• Empty medical bottles • Empty tetra pack containers • IVF container • Plastics, can, soft drinks, straws, wrapper, styropor containers • Waste from the offices	• Disposal materials • Used for collection of body fluid • Dressing bandages • Used folly catheters • Used cotton falls • Used gloves • Used facemasks
Private hospital 2	• Waste from the offices-paper, cans, cartoon styropor containers	• Empty vials • Sharps • Needles • Used gloves • Used cotton, pharmaceutical waste • Facemask • Radioactive waste
Public hospital	• Paper products • bottles • Packaging materials • Waste from offices	• Pharmaceutical waste • pathological waste • radioactive waste • sharps • chemical waste • used foley catheters • used blood product bags or tubing • used gloves • specimen container of blood and fluids • used suction tubes • cotton applicator soaked with blood • body fluids from dressing of infected wound and post operative cases • waste from isolation room • Facemask

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Table 1 shows that the solid wastes in the three hospitals, one public healthcare center, and one clinic are mostly similar. Most solid wastes were generated from their offices. Hazardous wastes on the other hand are mostly similar among the healthcare providers except for the presence of radioactive wastes in private hospital 2 and public hospital. Its presence can be attributed to the great number of patients either as out-patients or in-patients in these two hospitals. Many patients may mean different medical cases that will be needing low-level to high-level radioactive wastes. The influx of patients in public hospital is high due to cheaper medical expenses. However, premier private hospitals are also preferred due to the availability of comfortable facilities, sensitive and state-of-the-art diagnostic tests, and updated and advanced medical equipment (Al-Balushi et al., 2017 Meddedu et al., 2020).

In the Philippines, the Department of Health has set a standard color coding scheme for the disposal of healthcare waste. This coding scheme will be used in the health care facility as follows: Black for non-infectious dry waste, Green for non-infectious wet waste, Yellow for infectious and pathological waste, Yellow with Black Band for chemical waste including heavy metals, Orange for radioactive waste, and red for sharps and pressurized containers (Joson, 2012). The use of a color coding scheme as means to segregate hospital wastes is depicted in the table below.

Table 2: The use of color coding as a means of waste segregation of the health care units in the Municipality of Camiling

Use color coding scheme for waste segregation	Frequency (n=5)	Percentage (%)
Yes	4	80
No	1	20

Table 2 shows that the majority (80%) of the respondents were using a color coding scheme for waste segregation. These are private hospitals 1 and 2, public hospital, and clinic. However, 20% (Health Care Center) was using labeling instead of the color coding scheme in waste segregation. This color coding of the containers of the hospital wastes was set by DOH that is aligned with the UNEP/WHO 2009).

The conformity of these health care units to the standard rules set by DOH and according to RA 9003 is shown in Table 3.

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Table 3: Summary of color coding scheme as means of waste segregation of the Hospitals and Healthcare Units in compliance to the Department of Health and RA 9003

Color Code	DOH Standards		Hospitals and Health Care Units					
			Clinic	Public Health Center	Private Hospital 1	Private Hospital 1	Public Hospital	
For non-infectious dry waste or biodegradable waste				---				
Black	✓		✓		✓	✓	✓	
Green								
Yellow								
For noninfectious wet waste or non-biodegradable waste				---				
Black								
Green	✓		✓		✓	✓	✓	
Red								
For infectious waste and pathological waste								
Green								
Yellow	✓		✓	x	✓	✓	✓	
Blue								
For chemical waste including those with heavy metal								
Yellow with black band	✓		x	x	x	✓	✓	
Red								
Green								
For radioactive waste								
Green								
Black								
Orange	✓		x	x	✓	✓	✓	
For sharps and pressurized container								
Red (puncture proof container)	✓		✓	x	✓	✓	✓	
Yellow								
Black								

The result in Table 3 reveals that 80% of the health care units were using black color coding in segregating their non-infectious dry solid wastes or biodegradable wastes; green for non-infectious wet solid waste or non-biodegradable wastes; and yellow for infectious and pathological wastes. The public health center is the only unit that did not use color coding. However, in terms of segregating chemical waste including heavy metals, 60% (Private Hospital 2 and Public Hospital) were using black bands as indicators. For segregating radioactive wastes, 60% (Private Hospitals 1& 2 and Public Hospital) were using the orange indicator while 80% of the healthcare providers were using red puncture-proof containers.

The color coding scheme was set by DOH. Results show that 80% have conformed to DOH standards and RA 9003 in using a color coding scheme in segregating hospital infectious and non-infectious wastes. Only 20% had not used such a scheme because according to them, labeling is their means of waste segregation.

The provision of using a black band for chemical waste with heavy metals has not been followed by Private Hospital 2 and the Clinic (40%) because according to them they have not

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used heavy metals in their hospital/clinic. The provision for segregating wastes with radionuclides was not also followed by Dr. John Iglesia Clinic due to the non-usage of such chemicals

Segregation

Table 4: Segregation system of hospital wastes of the five healthcare Units in Camiling, Tarlac

Health Care Units (Hospitals, Health Center and Clinic)	Waste Segregation System
Public Hospital	– The hospital was implementing RA 9003 in full and was using color coding
Private Hospital 1	– The hospital was implementing RA 9003 in full and was using color coding
Private Hospital 2	– The hospital was implementing RA 9003 in full and was using color coding
Public Healthcare Center	– The center was implementing RA 9003 in full and was using labeling instead of color coding – Only Laboratory and the T and B DOTS sections were observing waste segregation – Infectious and non-infectious wastes were separated
Clinic	– The clinic was implementing RA 9003 in full and was using color coding

Data in Table 4 reveals how the 5 healthcare units segregate their wastes. All units except the Public Healthcare Center exercised full implementation of RA 9003 or known as the Ecological Solid Waste Management Act of 2000 and adhered to DOH standards in waste segregation.

Segregation of hospital wastes in Public Hospital is done by separating the different types of wastes and placed in corresponding bins. Containers are properly marked as compostable waste, non-compostable table waste, infectious waste, chemical waste, pharmaceutical waste, pathological waste, radioactive waste, sharp waste and pressurized waste.

The Private Hospitals 1 and 2 have the same way of segregating their wastes through color coding scheme. The black container is for non-infectious dry waste, green bag is for non-infectious wet waste, yellow bag is for infectious and pathological waste, orange container is for radioactive waste and red container is for the sharps and pressurized container. The green plastic lined bin is for the biodegradable waste which includes empty cartoons, empty medicine boxes, and kitchen waste, left over foods, newspapers, papers, and vegetable peelings and fruit skins. Black plastic lined bin is for inorganic waste. Each room has different containers for the various kinds of waste. The segregation of waste done at the Salvador General Hospital is in compliance to the DOH standards and RA 9003.

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The Public Health Center did not fully implement waste segregation. The laboratory room and the T and B DOTS are the only rooms that implement waste segregation. Waste segregation was through the labeling of the trash can. The infectious waste and general waste were separated.

Segregation of wastes in the Clinic is done through a color coding scheme. The color yellow container is for infectious waste, the black container was for non-infectious dry waste or inorganic waste, the red container is for sharp waste and the green container is for biodegradable waste. Each room has its trash can. The Clinic adhered the RA 9003. The institution used it as their guide in segregating waste.

Storage

On-site storage is the beginning of waste disposal because unkept waste or simple dumps are sources of nuisance, flies, smells, and other hazards (Takele, 2009). Infectious and pathological wastes however need to be treated while storing them before disposing of it properly.

Table 5: Storage of hospital wastes of the five health care units in Camiling, Tarlac

Health Care Units (Hospitals, Health Center and Clinic)	Storage System
Public Hospital	– Had Health Care Waste Management System (HCWMS)
Private Hospital 1	– Had Material Recovery Facility (MRF) for solid wastes – Solid wastes were stored for not more than 2 days
Private Hospital 2	– Had Material Recovery Facility for solid waste – Had concrete vault for temporarily storing hazardous and infectious wastes
Public Healthcare Center	– Stored infectious wastes in the drum for 1 year before disposal – Pre-treated hazardous and infectious wastes while reused and recycled solid wastes
Dr. John Iglesia Clinic	– Stored solid wastes for not more than 2 days and collected by Municipal truck every Tuesday – Available and visible waste containers

Table 5 shows that Public Hospital followed the Health Care Waste Management System (HCWM) of their institution. This is a systematic activity of the administration that provides policy on segregation at source, transport, storage, transfer, processing, treatment, and disposal of health care waste that does not harm the environment. This system is also in compliance with the DOH Standards.

Private Hospital 1 had its own Material Recovery Facility (MRF) which they use to temporarily store their waste – residual, recyclables and treated infectious waste. The wastes were stored

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for not more than 2 days before disposing to the dump site.

Private Hospital 2 had its own Material Recovery Facility which they use to temporarily store their waste for the proper segregation and inspection of solid waste. They also use concrete vaults to momentarily store the treated hazardous and infectious waste for the security of the people and the environment before finally disposing of it in San Clemente, Tarlac.

The infectious or hazardous wastes generated by Public Healthcare Center are stored in a big drum. These wastes underwent pre-treatment before storing. It takes a year before the drum is buried in Camiling cemetery. The drum should be full before they bury it.

Pre-treatment

Table 6: Pre-treatment of hospital wastes of the five healthcare Units in Camiling, Tarlac

Health Care Units (Hospitals, Health Center and Clinic)	Pre-treatment System
Public Hospital	– The hospital was using autoclaving machine to disinfect infectious and hazardous wastes
Private Hospital 1	– The hospital was using antiseptic reagents for infectious and hazardous wastes and soaked before burying – The hospital was using septic tanks for infectious wastes
Private Hospital 2	– The hospital was storing pathological wastes in a secured bottle and was using formalin to preserve it before placing it in concrete vault – The hospital was using Lysol to disinfect hazardous wastes – The hospital was using needle burner for needle and syringes
Public Healthcare Center	– The center was using safety box for used syringe before disposal to drums – The center was using chlorine to disinfect
Clinic	– The clinic had no pre-treatment system

Table 6 reveals that the Public Hospital pre-treated its infectious and hazardous wastes through autoclaving the infectious wastes.

The Private Hospital 1 however was using Chlorox and Syndex for the pre-treatment of infectious and hazardous wastes. These are soaked for 1 week before they bury it. They also were using autoclaves to disinfect the containers of these wastes before disposing of them. The laboratory room has a septic tank for hazardous and infectious waste. A needle destroyer is used before disposing of the syringes and needles. The pathological wastes of Private Hospital 2 are stored in a secured bottle with formalin to preserve them before they are put in a concrete vault and finally bury it. Infectious and hazardous wastes undergo different treatment processes

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before disposal. In disposing of blood and other pathological waste, they often use Lysol to disinfect or lessen and kill harmful bacteria while sharps like needles are burned in the needle burner. The Public Healthcare Center uses a safety box for the syringes which were chlorinated before disposing it to drum. All other infectious and hazardous wastes were pre-treated with chlorine before disposing of them. The clinic on the other hand had no pre-treatment system for its hazardous and infectious wastes.

Collection and Disposal

Collection is the removal of refuse from collection points to the final disposal site. It is the most expensive as compared with other operation and management procedures, because it demands special vehicles, experienced people to manage, more manpower, hand tools, and more funds for fuel, salary, maintenance, gathering or picking up of solid waste from the various sources, taking the collected wastes to the location where it is emptied, and unloading of the collection vehicle (Takele, 2009).

Table 7: Collection and disposal of hospital wastes of the five health care units in Camiling, Tarlac

Health Care Units (Hospitals, Health Center and Clinic)	Collection and Disposal System
Public Hospital	– Solid wastes were collected daily – Waste bags were labeled – Residual wastes were hauled using PEO dump truck in landfills at Matubog dumpsite – Infectious and hazardous wastes were transported using closed van
Private Hospital1	– Solid wastes were collected every morning and disposed at the sanitary landfills at Matubog dumpsite – Infectious wastes were disposed in burial pits
Private Hospital 2	– Solid wastes were collected in black bag and disposed at the sanitary landfill in Matubog dumpsite every day – Hazardous and infectious wastes underwent pre-treatment and collected using yellow bag; transported in a closed van and disposed in San Clemente, Tarlac.
Public Healthcare Center	– Solid wastes were collected everyday, collected by the municipal dump truck and disposed at Matubog dumpsite
Clinic	– Solid wastes were collected by on-site waste collectors and collected by municipal dump truck every Tuesday morning – Infectious and hazardous waste were pre-treated and dump at the back of the clinic

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Collection of waste in Public Hospital is done in a manner that prevents damage to the container. It is collected daily or as frequently as required. No bags are removed unless they are labeled with their point of production (hospital ward and department) and contents.

Collection of waste from the room is done every morning and afternoon. The solid wastes are collected by the municipal garbage collector truck. These are transported and disposed of in the Matubog dumpsite of Camiling while the infectious and hazardous wastes are disposed in burial pits.

Collection of waste in Private Hospital 2 is done using a black container for the general non-biodegradable waste which is collected every day at 8 am by the municipal garbage collector truck of Camiling and disposed it to Matubog dumpsite. The collection of garbage in each room in the hospital is made 3x a day or as needed. Yellow container or infectious and hazardous waste like body parts is collected every morning if ever the patient will not claim it. Their pathological wastes are stored in a secured bottle with formalin to preserve them before they are put in a concrete vault and finally bury it. A closed van is used to transport hazardous and infectious wastes in the disposal area at San Clemente, Tarlac. The municipal garbage collector truck of Camiling is used to transport all the solid wastes they generate in the Matubog dumpsite.

The general solid wastes in Public Healthcare Center are collected every morning by the municipal garbage truck. These are disposed of in Matubog dumpsite at Camiling, Tarlac.

The waste collector collects the waste in the room once a day every afternoon at the Clinic. The general wastes are collected by the municipal garbage truck every Tuesday morning. The syringes and other infectious and hazardous wastes are treated first before putting on a safety box (biohazard). All the hazardous and infectious wastes are buried behind the clinic.

Reuse and Recycle

Not all solid wastes are disposed. There are hospital wastes that are reused and recycled. The different ways of the health care units in Camiling are shown and described below.

Table 8: Reuse and recycle of hospital wastes of the five health care units in Camiling, Tarlac

Health Care Units (Hospitals, Health Center and Clinic)	Reuse and Recycle System
Public Hospital	- Practiced recycling
Private Hospital 1	- Practiced recycling - Treated material before reusing it
Private Hospital 2	- Did not practice recycling - Treated material before reusing it
Public Healthcare Center	- Did not practice recycling - Treated material before reusing it
Clinic	- Did not practice recycling - Treated material before reusing it

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The Public Hospital practiced recycling. Solid wastes like plastic and glass, syringes, cartons, and vials are cleaned and transformed into decorations.

Private Hospital 1 treats the materials before reusing them. Wastes that were reused are gloves with no punctures or tears; those that were strong enough to be autoclaved; bonnets used during surgeries; left-over sutures at the operating room or delivery room; breakable bottles used with CTT; rubber-tubing used with suction machines; and CTT drainage. The hospital also recycles materials such as newspapers, cartons, empty water bottles, and IV plastic bottles. These recyclable wastes are sold by the maintenance personnel to junkshops.

Supplies used by Private Hospital 2 are disposable for the safety of people around including patients except their medical instruments used. Medical instruments went through a process of treatment with the use of autoclave machines before they are reused. Recycling of waste is not practiced in Public Healthcare Center. Materials being reused are treated and autoclaved before reusing them. The Clinic did not practice recycling waste generated. Some of the materials were put into the autoclave for treatment before reusing it.

Strategies in Implementing Waste Management Program

The Public Hospital followed the Health Care Waste Management System or HCWM of its institution. This is the systematic administration of activities that provide for segregation at source, segregated transportation, storage, transfer, processing, treatment, and disposal of healthcare waste that do not harm the environment. This complies with the DOH Standards.

The Private Hospital 1 Waste Management Program focused on source reduction by proper waste segregation, recycling, treatment, and residual disposal. Health Care Waste Minimization centered on how to reduce waste. This was done through reduction at source, which involved complete elimination of waste or lessening the waste generated. Reuse, recycling, and segregation of waste using the color coding scheme were done. To reduce waste at source the hospital purchased/selected supplies that were less wasteful and less hazardous. They used less hazardous methods in cleaning. To make their waste management program effective, they launched a massive educational and communication program for their staff. Periodic monitoring and evaluation of the program is done.

No strategies or specific program in support of waste management program was provided by 60% of the healthcare provider (Private Hospital 2, Public Healthcare Center, and the Clinic. But their segregation, storage, collection, and disposal of their wastes show that they adhere to the RA 9003 and DOH standards.

CONCLUSIONS

The adherence of the healthcare providers in Camiling, Tarlac to RA 9003 or the Ecological Solid Waste Management Act of 2000 and the DOH standards had fully prevented human health deterioration in the community but not land degradation. Their responsibility of ensuring that there is no adverse health in their management of healthcare wastes was because they consider first the health and occupational safety of the people.

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Proper collection and disposal of solid wastes in the health care units have greatly helped in the control of insects, rodents, and filth bone diseases and prevented fire-cause hazards by instantaneous combustion in the dumpsite. Proper treatment and disposal however of hazardous and infectious wastes have prevented the short and long-term irreversible health risks.

The off-site disposal of general residuals of the health care units in the Matubog dumpsite was along the stream of the household and commercial wastes of the Municipality. The infectious and hazardous wastes were treated and disposed of properly. However, burial as means of disposing of infectious wastes is not sustainable and the lack of a common disposal system of infectious and hazardous wastes was practiced by the hospitals.

Between private and public health care units, the private hospitals were more conscientious in implementing their waste management program. The limited resources, facilities, and manpower while catering to more number of patients may be the reasons for the poor implementation of public hospitals in private hospitals.

RECOMMENDATIONS

Big or small institutions should have waste management written policies for the proper disposal of healthcare waste and strictly follow them. The Camiling Health Center should have concrete rules and regulations regarding healthcare waste management. Each unit should have a waste minimization written policy with specific goals, objectives, and timeliness to have a successful and sustainable waste management program.

The health care owners/administrators should not only consider burial ways of disposing of infectious wastes. Less permeable material should cover the burial pits to avoid seepage of liquid infectious wastes to the groundwater table and other run-offs. They should also consider other treatment methods such as radiation technology, encapsulation, inertization, etc. aside from the usual chlorination process that they employ to ensure the minimization of infection or widespread disease.

The Municipality of Camiling should allocate financial resources for the proper collection of hospital wastes. They have to ensure that hospital waste bags are properly labeled before collecting by their dump trucks. Healthcare wastes should be separately collected and disposed of from the residuals generated by the community. The Municipality should design and build a sanitary landfill, unlike the open dumping at Brgy. Matubog. Relocation of the dumpsite is worth reconsidering.

Government hospitals should allocate bigger funds for their waste management program. A stricter policy should be drafted and followed. It should also be part of their mission and goals. It should be a way of life for the healthcare providers. They have to be competitive with the private hospitals in implementing waste minimization programs.

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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+

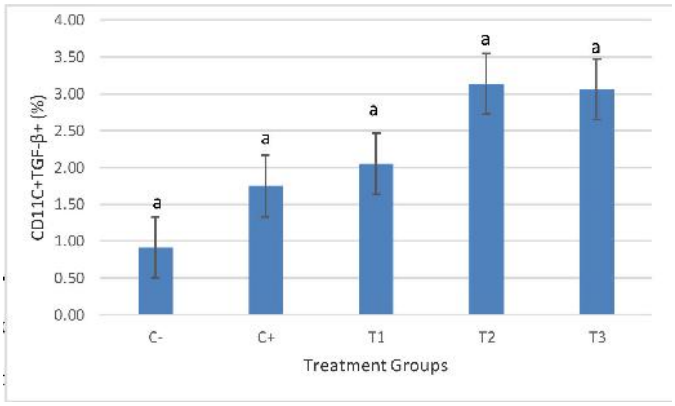

Figure 1. Flow cytometry analysis shows that giving LAB showed an increase in the relative number of CD11c+TGF-β+ cells ($p < 0.05$), but was not significantly different between treatments. There was an increase in the number when compared with the positive control. The highest average increase was seen 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.

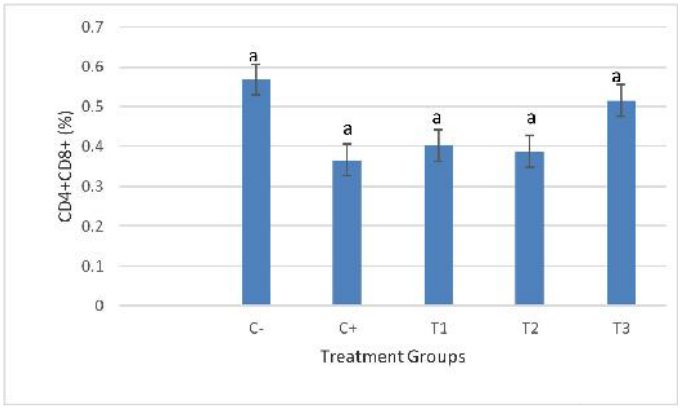

Figure 2. Treatment with LAB showed an increase in the relative number of CD4+CD8+ cells for all treatments, which did not differ significantly ($p < 0.05$). The treatment groups are: C- (healthy mice), C+ (mice induced with AFB1), and treatment groups, T1, T2, and T3 in which mice were administered 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

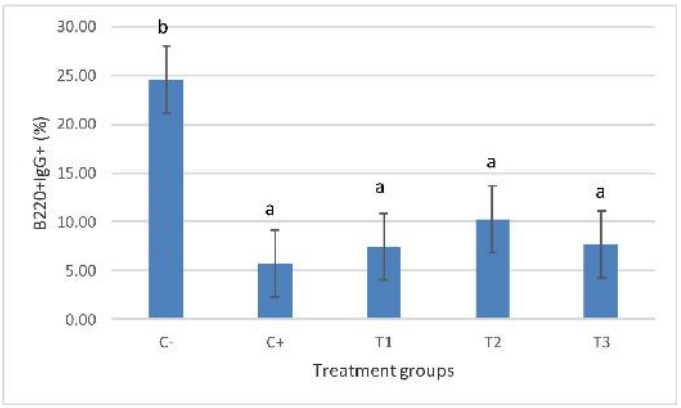

Figure 3. Treatment with LAB showed an increase in the relative number of CD4+CD8+ cells for all treatments, which did not differ significantly ($p < 0.05$). The treatment groups are: C- (healthy mice), C+ (mice induced with AFB1), and treatment groups, T1, T2, and T3 in which mice were administered 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].

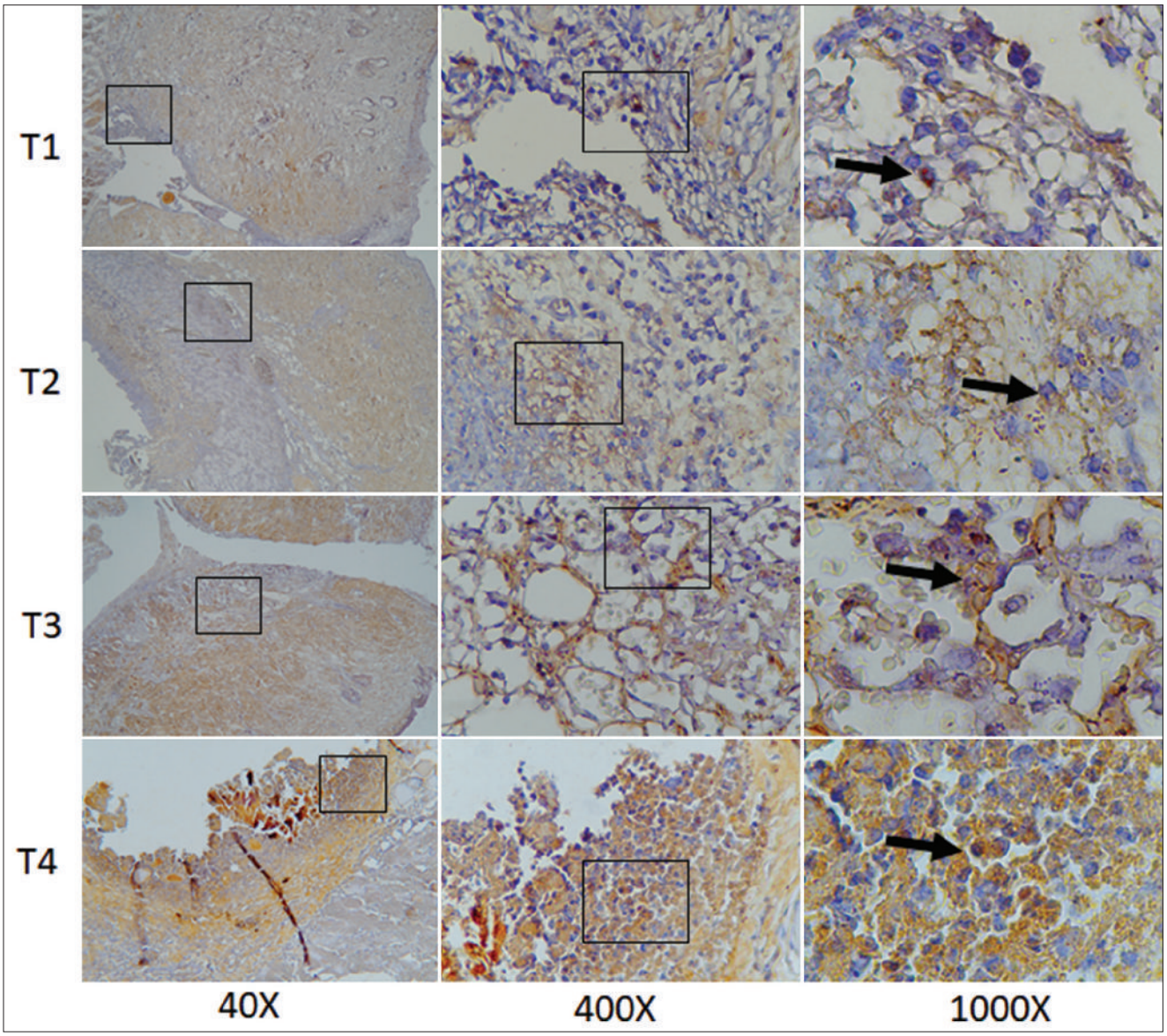

Figure 1: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against transforming growth factor-β1. (T1) positive control treated with a commercial wound healing agent; (T2) negative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

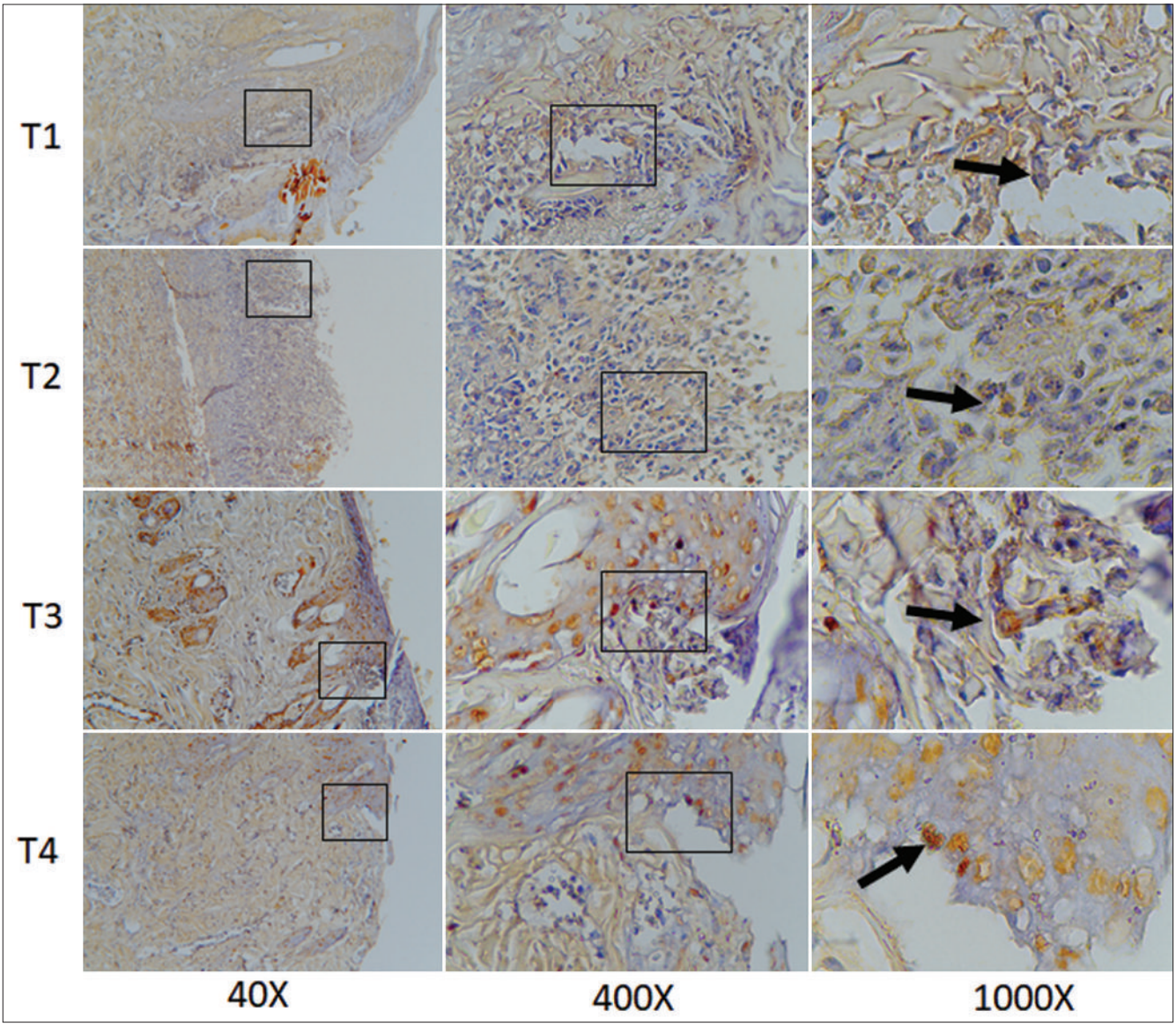

Figure 2: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against Smad3. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated with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

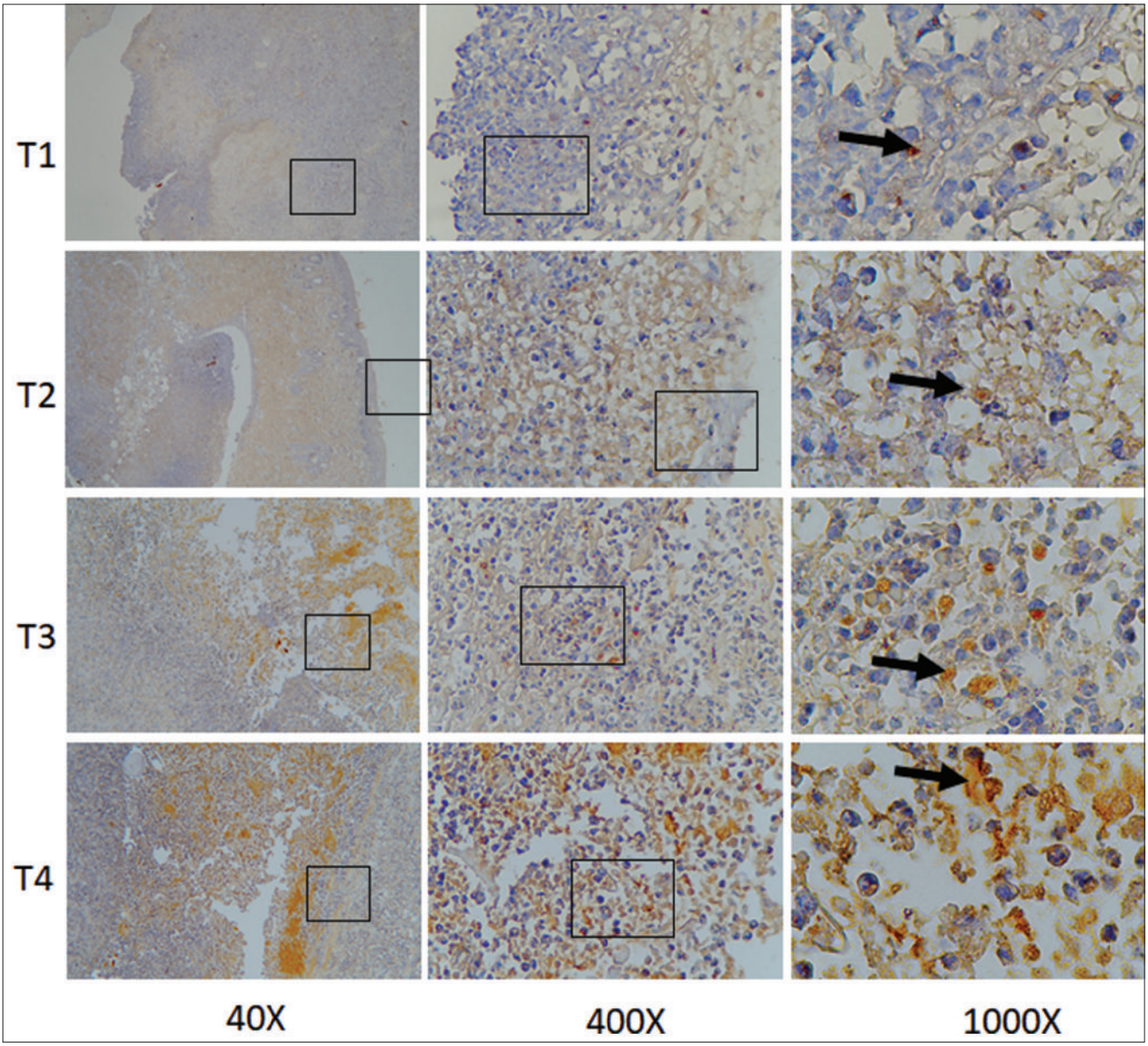

Figure 3: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against β-catenin. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated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

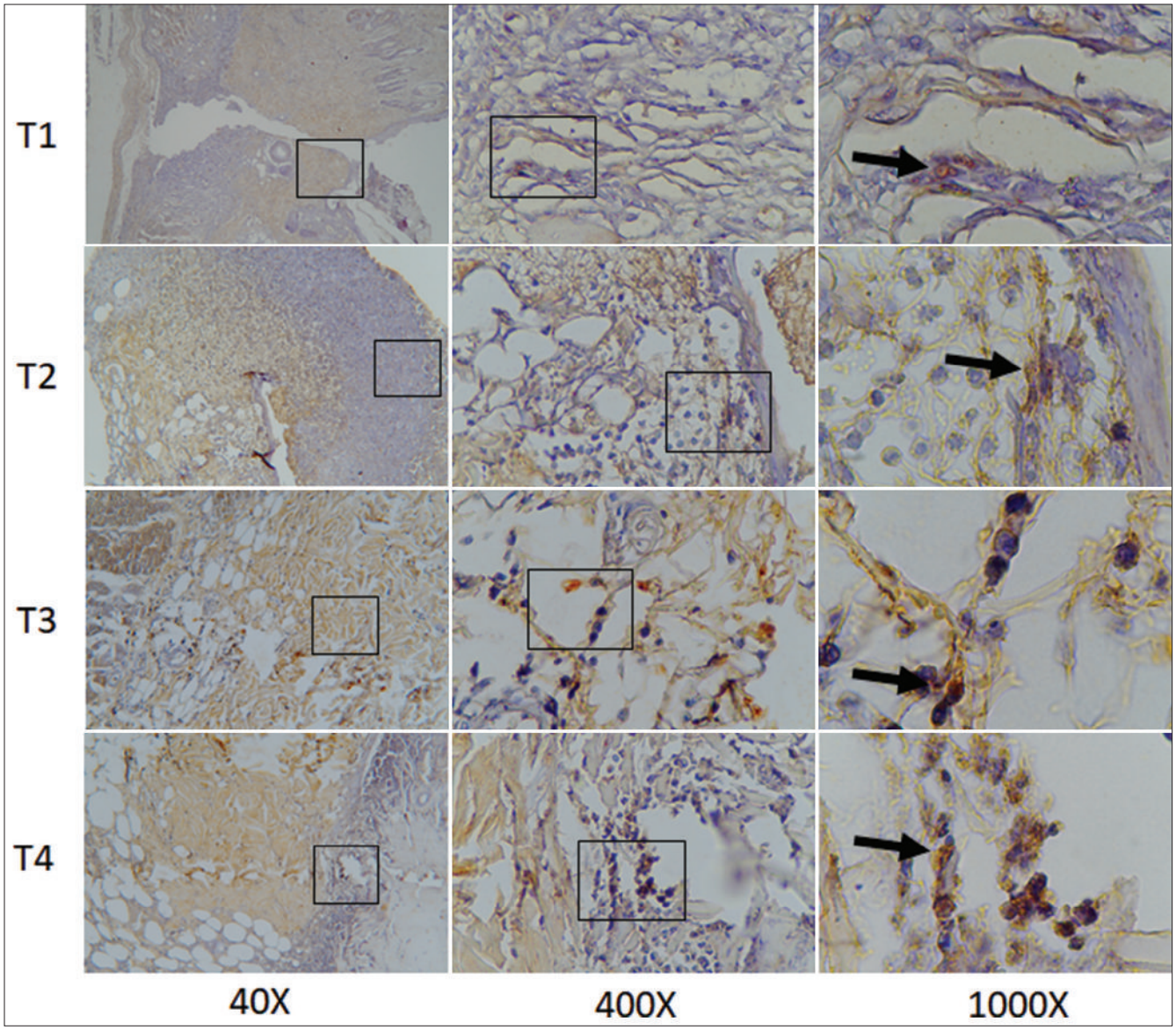

Figure 4: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against LGR-6. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated with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

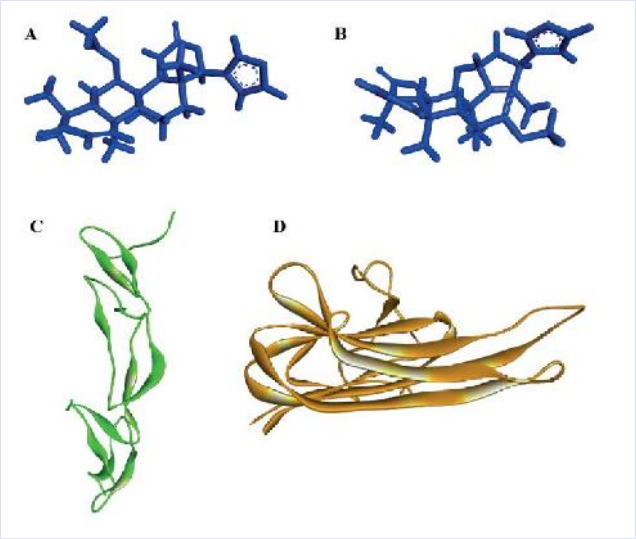

Figure 1: The chemical compound from neem: A) Gedunin (CID: 12004512); B) Nimbolide (CID: 12313376); C) death receptor 5 (DR5) (PDB ID: 1D4V); D) tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (PDB ID: 1D4V).

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]

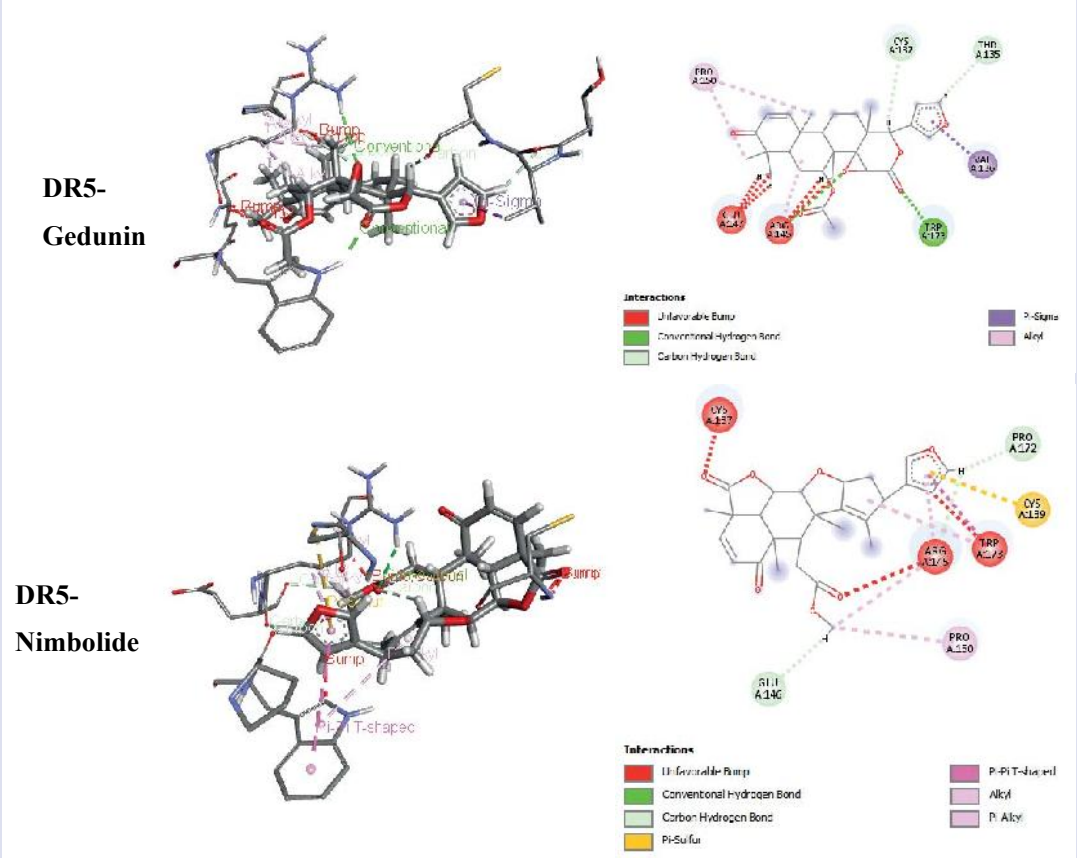

Figure 2: DR5 binding with gedunin and nimbolide in 2D diagram ligand interaction.

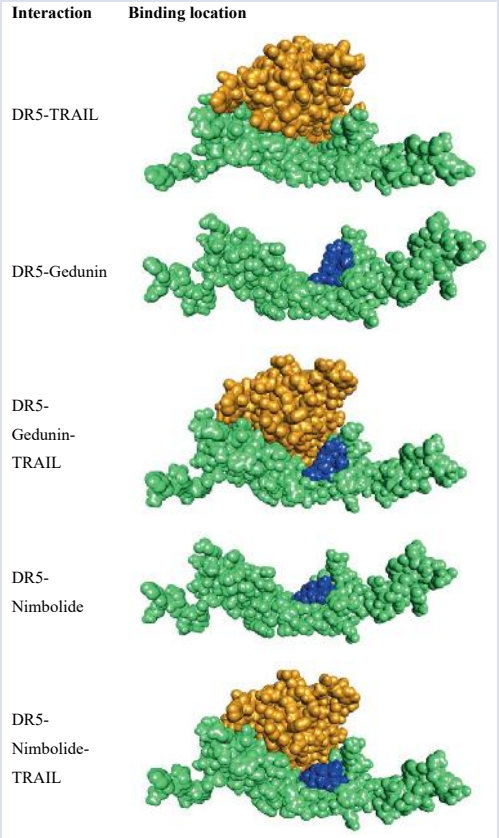

Figure 3: Binding location and interaction DR5 and ligands (DR5: green; TRAIL (native ligand): yellow; ligand: blue).

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.

ACKNOWLEDGEMENTS

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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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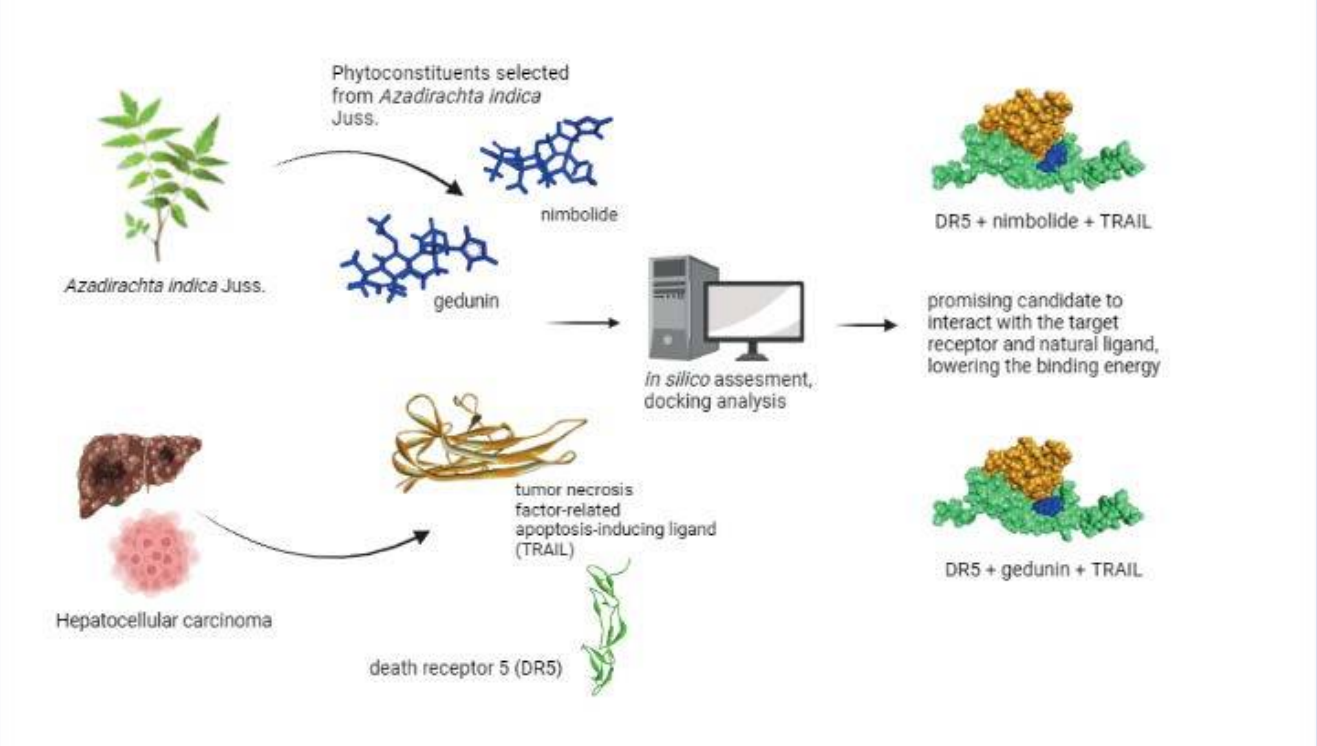
GRAPHICAL ABSTRACT



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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

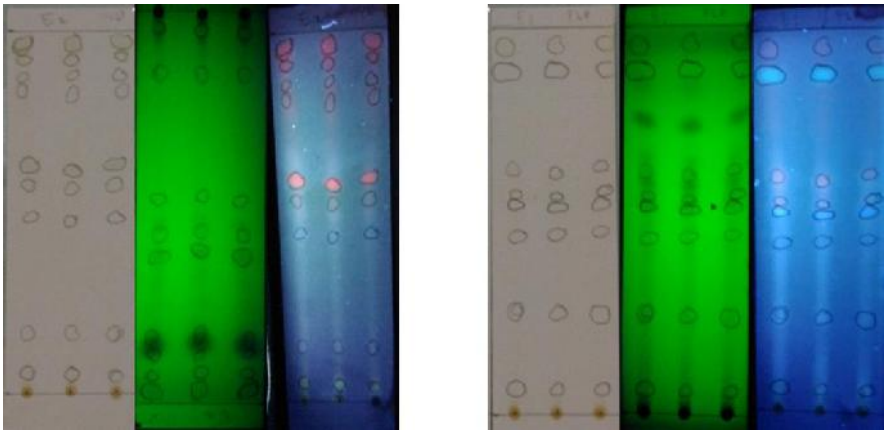
Thin-layer chromatography separation was a separation process based on the distribution of active compounds in two phases, the mobile phase (consist of a mixture of solutions that have a similar polarity with the compound to be separated) and the stationary phase was a silica gel coated plate (usually 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].

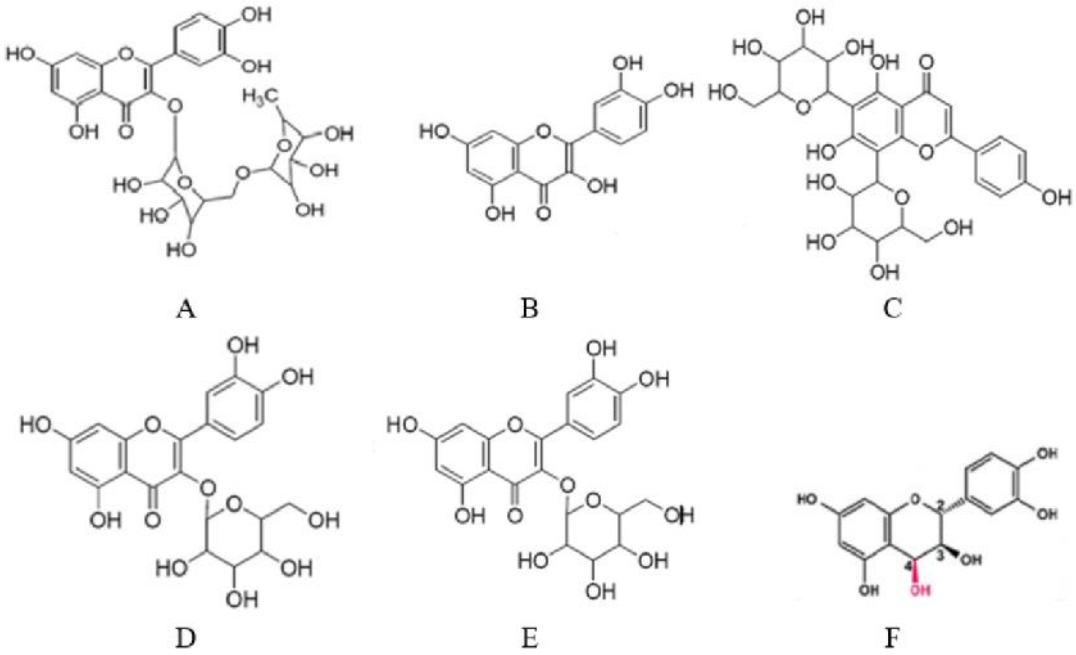


Figure 2. Structure of Flavonoid Compound. (A) Rutin, (B) Quercetin, (C) Vicenin-2, (D) Quercetin-3-O-glucoside, (E) Quercetin-O-O-galloyl hexoside, (F) Leucocyanidin-(+)2,3-3,4-cis-3,4,5,7,3',4'-hexahydroxyl flavan.

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.

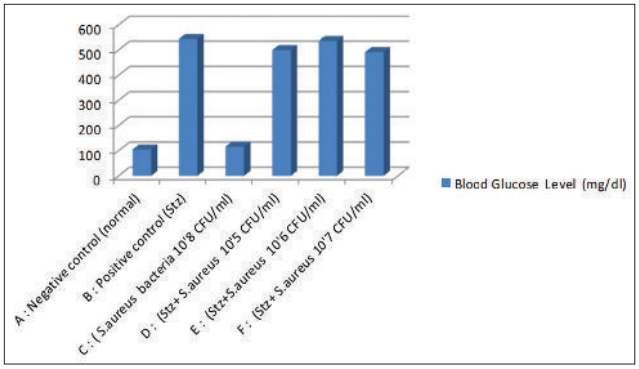

Figure-1: Blood glucose level.

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

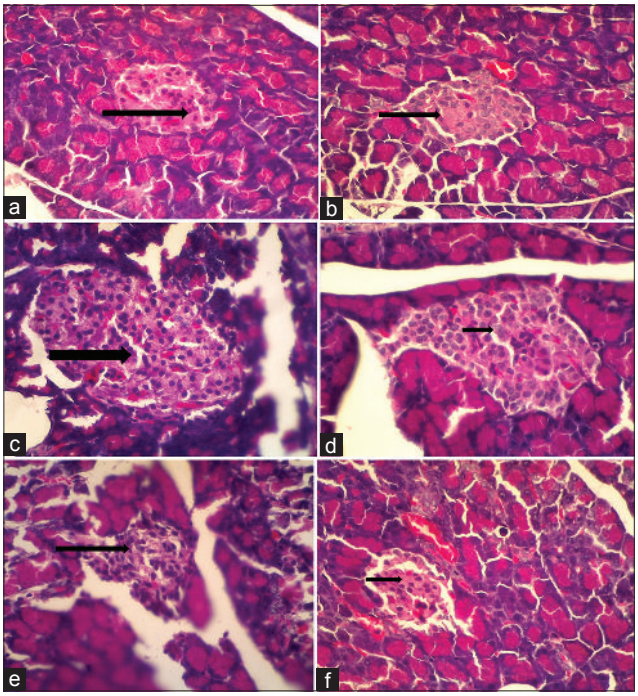

Figure-2: Images with magnifications 400×: Negative control (a) showing pancreatic beta-cell nucleus appears clear and spread evenly (arrow). Positive control diabetes (b) showed that the pancreatic beta-cell nucleus seemed to shrink even invisible and only seen cytoplasm (arrow). Positive control bacteria 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].

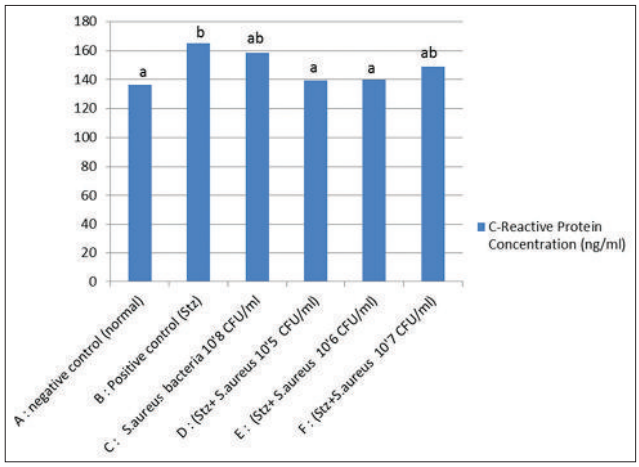

Figure-3: Level of C-reactive protein. Superscript letters are significantly different from one another based on analysis of variance with honestly significant difference tests ($p<0.05$).

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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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

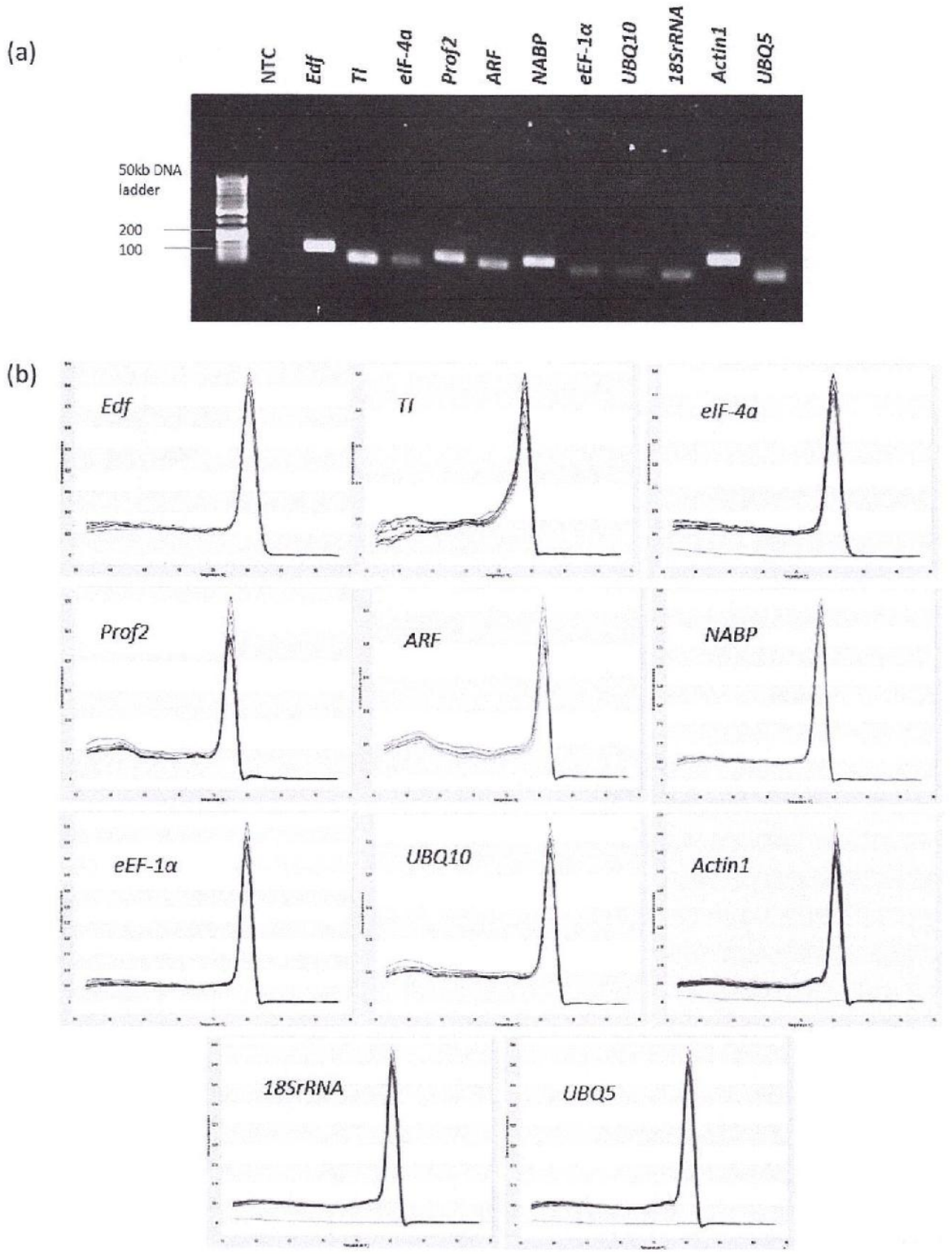

Fig. 1. Specificity of gene amplification products tested on the 6-leaf stage rice cultivar RD47 at 0 h after *Xoo* inoculation. (a) Gel migration of qRT-PCR products. A single DNA amplicon of the projected size is shown for each gene tested in this study. Agarose gel (2%). NTC-Non-Template Control; 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

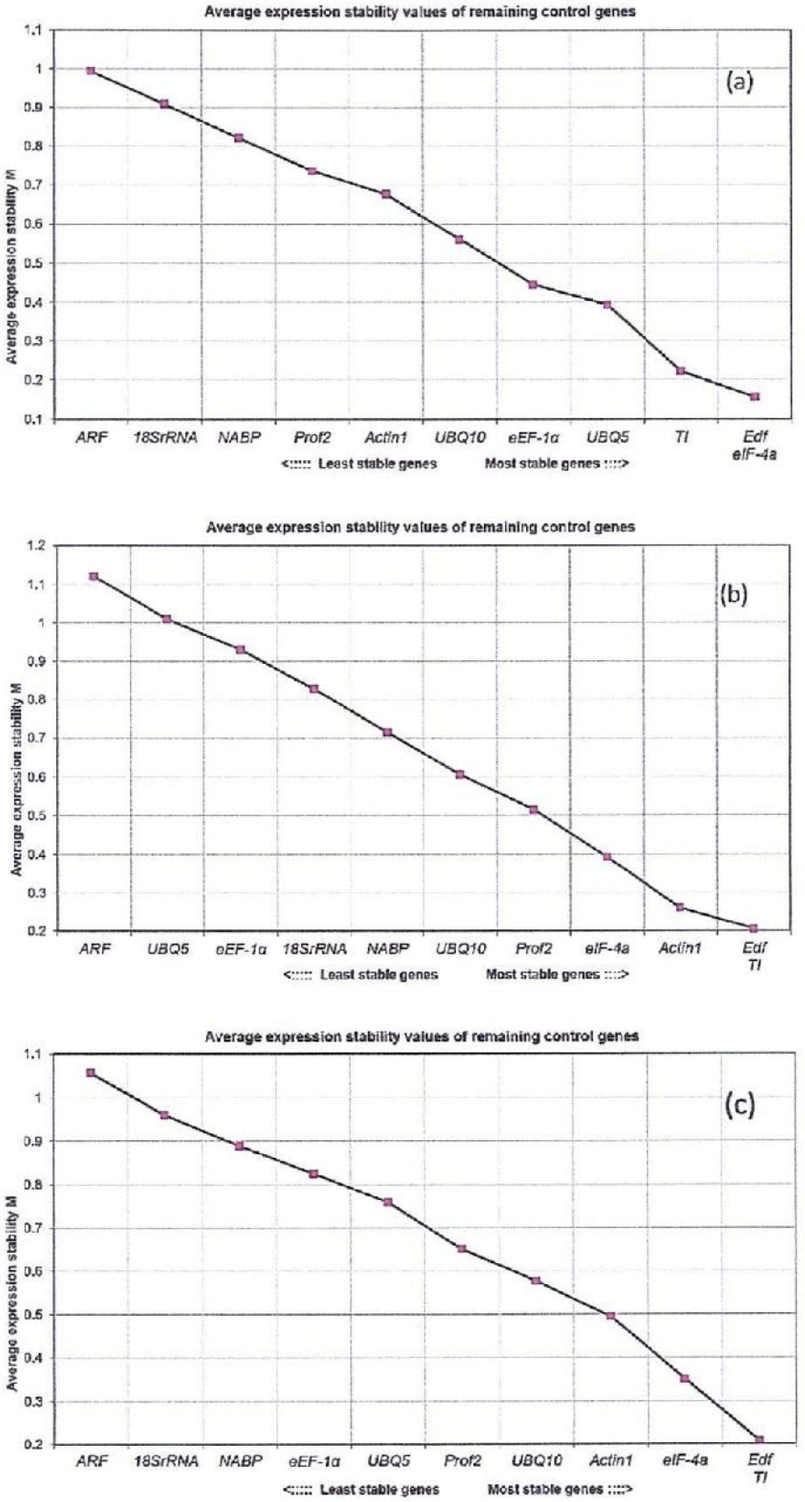

Fig. 2. Expression stability and ranking of reference genes by geNorm: (a) RD47; (b) BC_3F_3 (*Xa21-Xa21*) progeny; and (c) all samples. Gene(s) with lower average expression stability M denotes more stable expression.

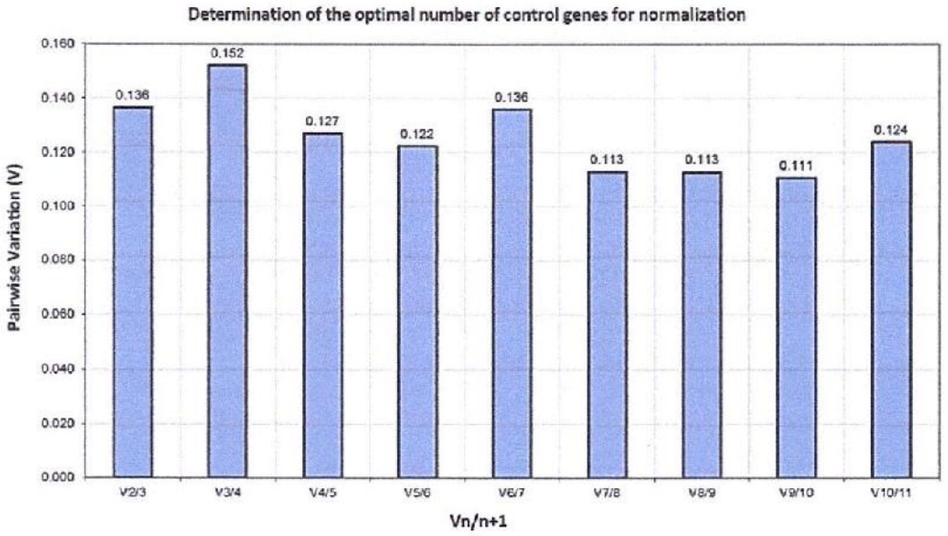

Fig. 3. Determination of the optimal number of control genes for accurate normalization by geNorm pairwise variation analysis. Pairwise variation analysis (Vn/n + 1) between the normalization factors NFn, including the n most stable reference genes, and NFn + 1, corresponding to the stepwise inclusion 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

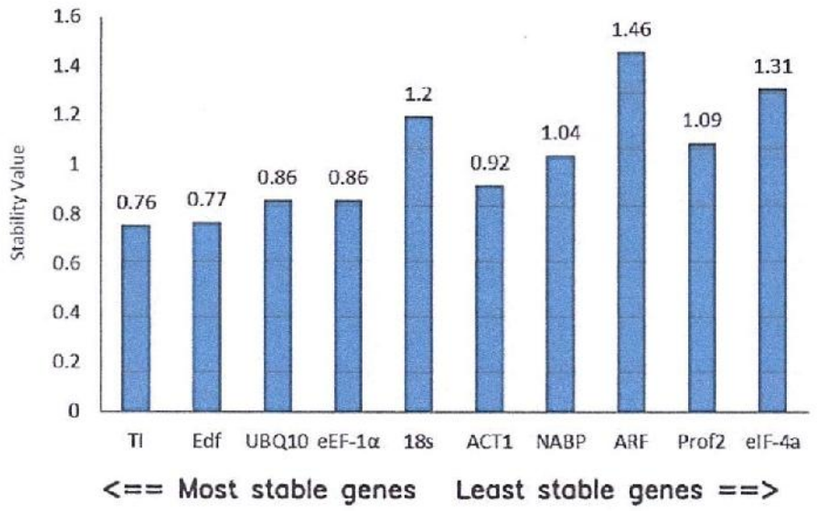

Fig. 4. Expression stability and ranking of candidate reference genes as determined by Delta-Ct method. Low gene stability values denote more stable expression.

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

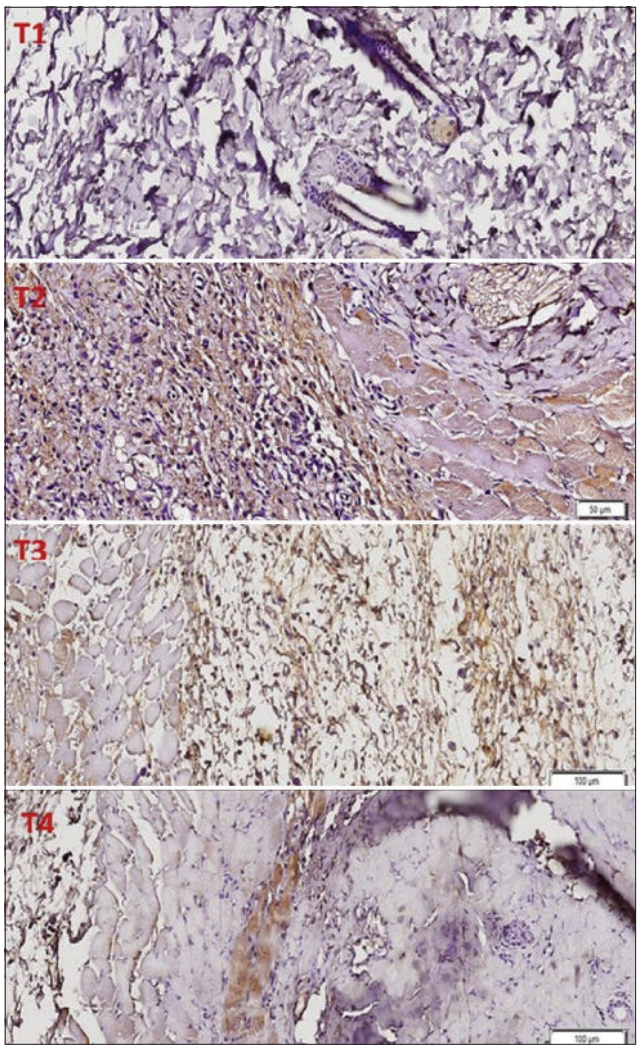

Figure-1: The expression of interleukin-1β on treatment rats.

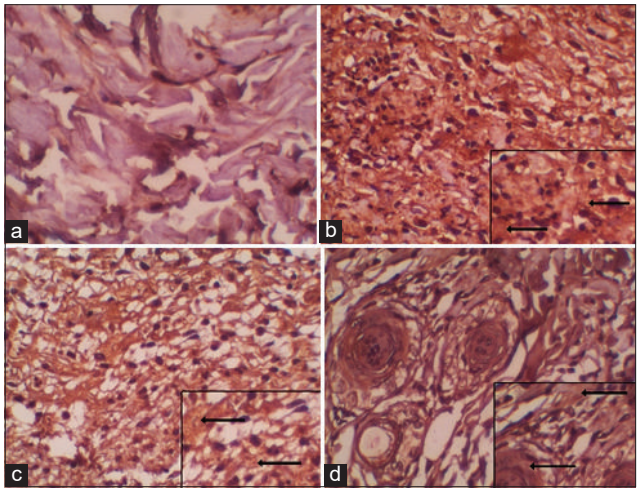

Figure-2: The expression of interleukin-6 on treatment rats: (a) (T1) Positive control, (b) (T2) negative control, (c) (T3) wounds treated with Gliricidia sepium from Indonesia, and (d) (T4) wounds treated with Gliricidia sepium from the Philippines (400×).

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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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).

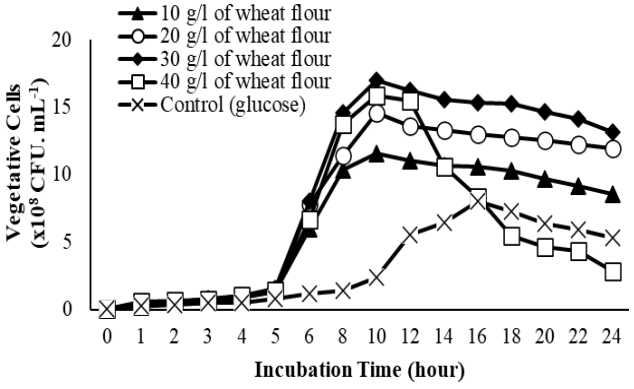

Figure 1. Vegetative cell density of *B. megaterium* BM1 under different doses of wheat flour

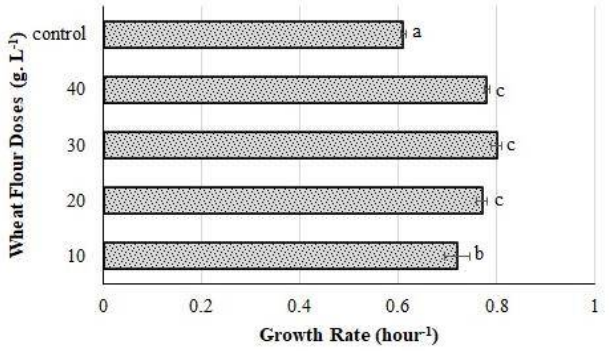

Figure 2. Maximum vegetative cell growth rate of *B. megaterium* BM1 under different doses of wheat flour. Mean data with different alphabets are significantly different at a level of $p<0.05$).

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.

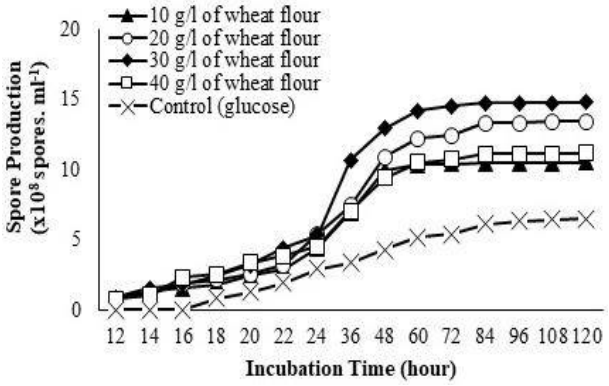

Figure 3. Spore production of *B. megaterium* BM1 under different doses of wheat flour

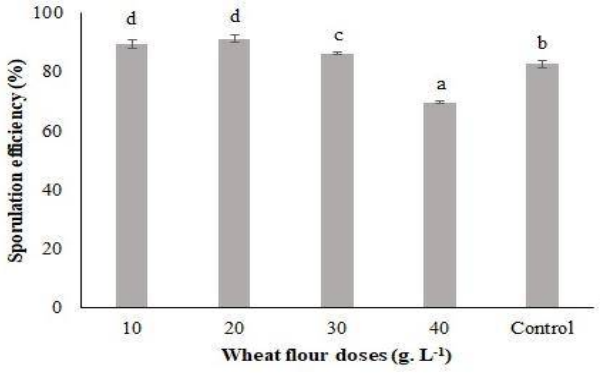

Figure 4. Sporulation efficiency of *B. megaterium* BM1 under different doses of wheat flour. Mean data with different alphabets are significantly different at a level of $p<0.05$).

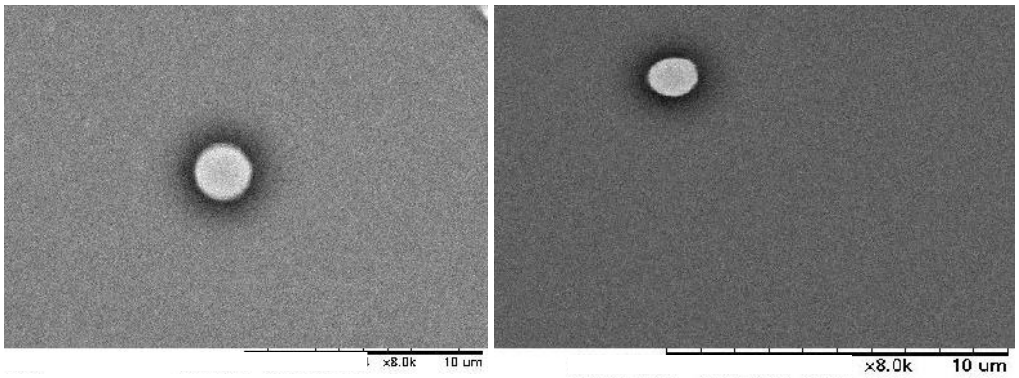

Figure 5. Morphological of *B. megaterium* spores under different carbon sources; A) control (glucose); B) Wheat flour at a dose of 30 g. L^{-1} (x8,000 magnification)

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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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].

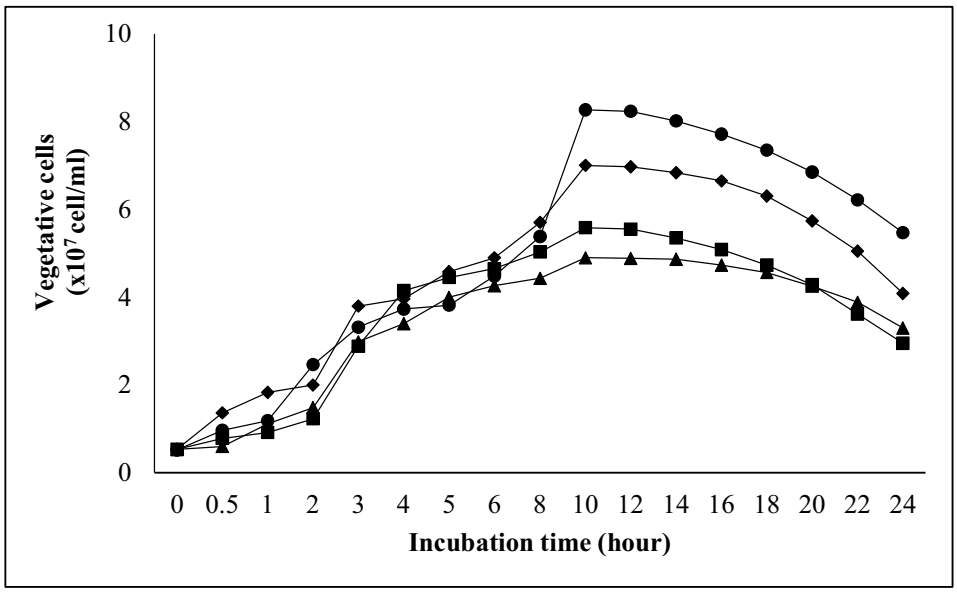

Figure 1. Time Series of vegetative cells production of *B. megaterium* under different carbon doses of tapioca flour (▲: 5 g/L; ■: 10 g/L; ●: 15 g/L; ◆: 20 g/L)

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).

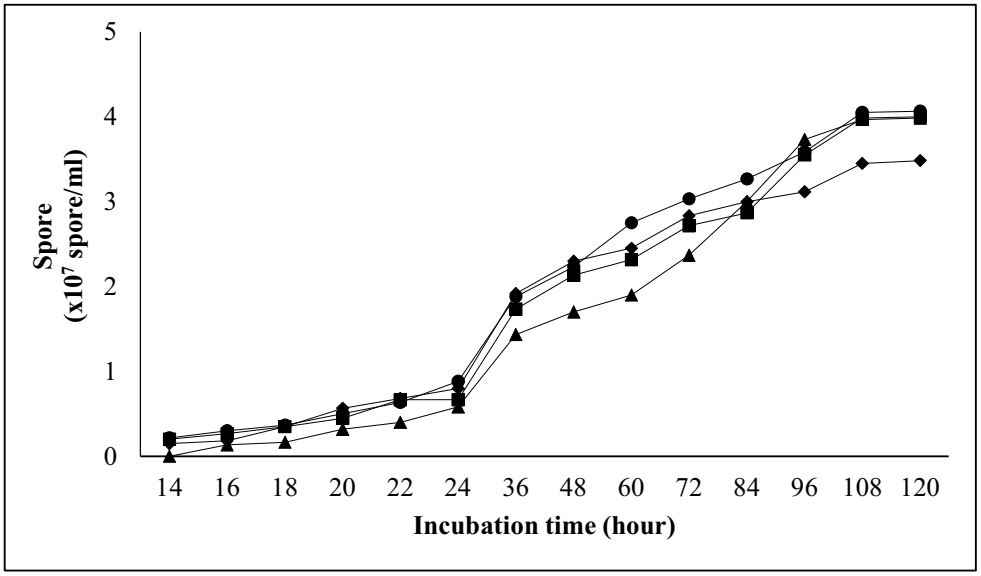

Figure 2. Time series of spores production of *B. megaterium* under different carbon doses of tapioca flour (▲: 5 g/L; ■ : 10 g/L; ● : 15 g/L; ♦ : 20 g/L)

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.

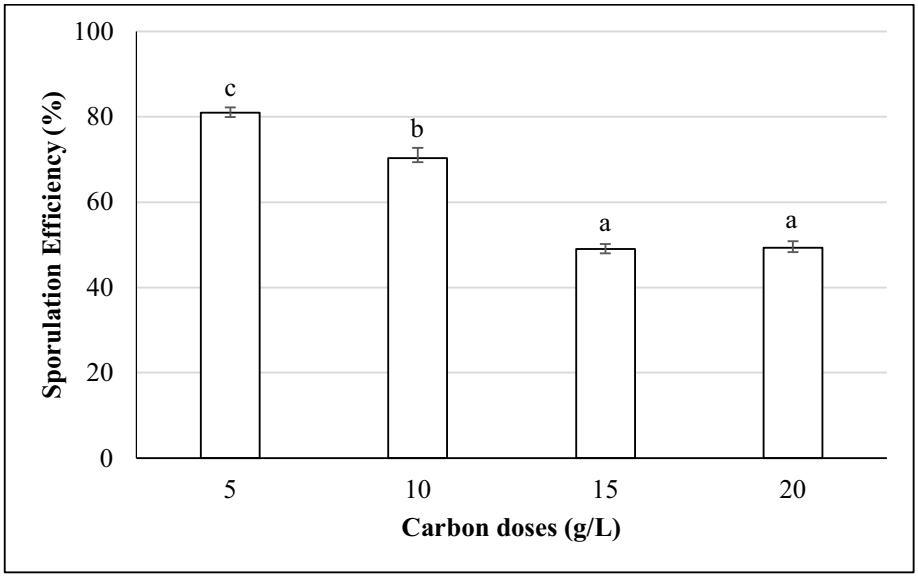

Figure 3. Sporulation efficiency of *B. megaterium* under different carbon doses of tapioca flour

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.

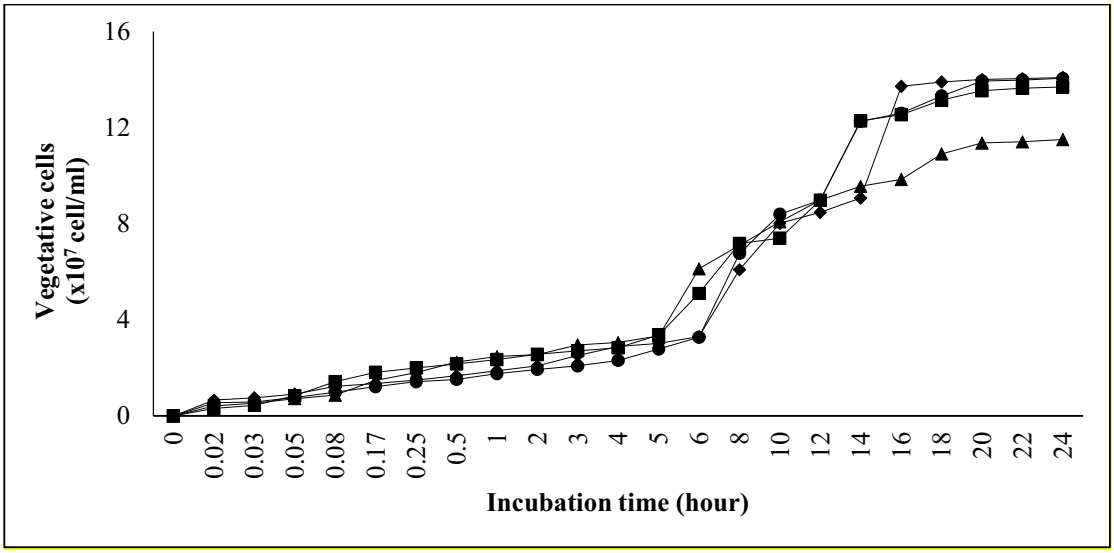

Figure 4. Time series of spore germination of *B. megaterium* under different carbon doses of tapioca flour (▲: 5 g/L; ■ : 10 g/L; ● : 15 g/L; ♦ : 20 g/L)

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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Modified Graph-theoretic Clustering Algorithm for Mining International Linkages of Philippine Higher Education Institutions

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***Abstract*—Graph-theoretic clustering either uses limited neighborhood or construction of a minimum spanning tree to aid the clustering process. The latter is challenged by the need to identify and consequently eliminate inconsistent edges to achieve final clusters, detect outliers and partition substantially. This work focused on mining the data of the International Linkages of Philippine Higher Education Institutions by employing a modified graph-theoretic clustering algorithm with which the Prim's Minimum Spanning Tree algorithm was used to construct a minimum spanning tree for the internationalization dataset infusing the properties of a small world network. Such properties are invoked by the computation of local clustering coefficient for the data elements in the limited neighborhood of data points established using the von Neumann Neighborhood. The overall result of the cluster validation using the Silhouette Index with a score of .69 indicates that there is an acceptable structure found in the clustering result – hence, a potential of the modified MST-based clustering algorithm. The Silhouette per cluster with .75 being the least score means that each cluster derived for r=5 by the von Neumann Neighborhood has a strong clustering structure.**

***Keywords*—MST-based clustering; Small World Network; von Neumann Neighborhood; internationalization; Prim's MST**

I. Introduction

Internationalization and partnership development undertakings pave way to establish identity in the international arena. As such, data in the field of internationalization as mirrored by students and international partnerships established by education institutions is growing to be a good interest of researches [1]–[4]. This is since the rate of internationalization increases with the unhindered channels of communications and affordable travel expenses. Universities seek to seize the opportunities from global partnerships and foster relationships with other organizations or institutions. Internationalization is also described to transform into mainstream strategy in higher educations and is increasingly seen as adding value to the life of universities through improving their quality [5]. The definition of internationalization being the process of integrating international, intercultural, or global dimensions into the purpose, functions or delivery of post-secondary education [6] is by common knowledge, the most frequently cited and widely accepted.

Meanwhile, methods and techniques in data mining allow analysis of very large datasets (i.e. big data) to extract and discover previously unknown structures and relations out of huge amount of details [7] for the purpose of knowledge extraction. As such, clustering in the data mining arena aims to establish high intra-cluster and low inter-cluster similarity in data. The high intra-cluster similarity should be based on the derived measurement from the data while the low inter-cluster similarity should maintain that elements in the different clusters should have maximum distance. These are intended to achieve beneficial knowledge from the data [8] for decision making and strategizing. Among different types of clustering, the most conventional distinction is whether the set of clusters is hierarchical or partitional [9] where hierarchical is a set of nested clusters while partitional clustering divides the set of data objects into non-overlapping clusters such that each object is in exactly a single cluster [10]. However in the real world, clusters come in arbitrary shapes, varied densities and unbalanced sizes that is why there is no universal clustering method which can deal with all problems [11].

Since most clustering algorithms' performance is affected by the shape and size of the detectable clusters [12], the requirement of an *a priori* knowledge about the actual number of clusters and the setting of a threshold to obtain adequate clustering results; a number of modifications to the clustering algorithms have emerged and are being explored to cope with said problems. Among which are graph-theoretic or graph-based clustering algorithms where data is represented in an undirected graph denoted as G={V,E} where the set of all data points is V and the set of connections between two distinct data objects (i.e. edges or links are contained in E. This is associated with a distance measure resulting to a connected subgraph or clusters. The use of Minimum Spanning Tree (MST) is one of said methods which either uses the Prim's [13]–[15] or Kruskal's [16], [17]. An MST is constructed for the whole data with a threshold value being set along with a number of steps to terminate the process to form clusters resulting from removing an inconsistent edge whose value is greater than the threshold value. However, this strategy is constrained by the identification and elimination of the inconsistent edge [17], detection of outliers [18], as well as insufficiently evidenced partitioning–hence, having the same weaknesses as other clustering methods that are based on distance measures [19].

This work aims to perform data mining in the data of the international linkages of Philippine Higher Education Institutions (PHEIs) using a proposed modified Prim's MST-based clustering algorithm producing a minimum spanning tree for the dataset infusing the computation of local clustering coefficient for the data points in the limited neighborhood generated by von Neumann Neighborhood.

This paper is organized as follows. Section II presents the conceptual framework of the modified Prim's MST-based clustering algorithm invoking the properties of the small-world network of graph theory. It also highlights the preparation of the International Linkages data. Section III includes the results of the simulation and the cluster validation. Section IV highlights the conclusions and future works of the study.

II. Modified Prim's MST-based Clustering Algorithm

Clustering can be used on many problems as it is helpful to seek and see relationships. It aims to congregate into clusters unlabeled data elements with high similarity based on a measure obtained solely from the data itself [20]. The distance measure defines the radius of membership depending on the type of data on hand. A good cluster is associated with high clustering value in terms of distance so the selection of distance metric is essential in clustering [21] while another clustering algorithm approach is to represent a target data set as a weighted undirected graph [20].

A. Prim's MST-based Clustering Algorithm

Prim's MST Algorithm uses a distance function to specify the closeness of data objects to establish the weight between them by choosing an arbitrary point to the next adjacent point of minimum weight. For clustering, an edge inconsistency measure is defined to identify an inconsistent edge to be removed to partition the whole dataset into clusters. Prim's MST is modified for efficient construction of spanning tree based on the k-nearest neighbor search mechanism during which a new edge weight is defined to maximize the intra-cluster similarity and minimize the inter-cluster similarity [13]. The algorithm can be used for a complete graph while using Fibonacci Heap [19], [22].

In this work, the traditional Prim's MST Algorithm for clustering defined by [18] as shown in Fig. 1 is modified by infusing the local neighborhood search by the von Neumann Neighborhood in order to facilitate the computation of the local clustering coefficients of the data elements in said neighborhood.

Higher clustering coefficient indicates the robustness on an average shortest path between any pair of nodes [23]–[25]. As such, small world networks [26] have the properties of having a small mean of shortest path length and high clustering coefficient. The Local Clustering Coefficient (LCC) quantifies the closeness of the neighbors of a vertex in becoming a clique. A concept in graph theory, LCC is basically computed as the number of triangles connected to a vertex over the number of triples around a given vertex. It is the probability that duos of neighbors of a vertex are connected by an immediate connection – the value is $0 \leq LCC \leq 1$. Thus,

$$LCC = \frac{number\ of\ connected\ neighbors}{number\ of\ neighbors} \quad (1)$$

Meanwhile, the von Neumann Neighborhood is one of the most commonly used types of neighborhood for cellular automata of two dimensions [27]. It is also used in pattern generation [28] and operations research [29] as it has been proven to have better performance than other topologies to further improve the quality of local search [30]. It can be extended by taking the set of data objects at Manhattan distance r where $r > 1$ which yields a result of a diamond-shaped region – hence, the neighborhood of data objects. The two-dimensional square lattice is composed of the central cell and the four adjacent cells around achieved through traversing North, East, West and South (NEWS) derived at a Manhattan distance 1. The number of neighbors (i.e. cells) in 2-dimensional by von Neumann Neighborhood of the cellular evolutionary algorithm for range r is defined as:

$$2r(r+1)+1 \quad (2)$$

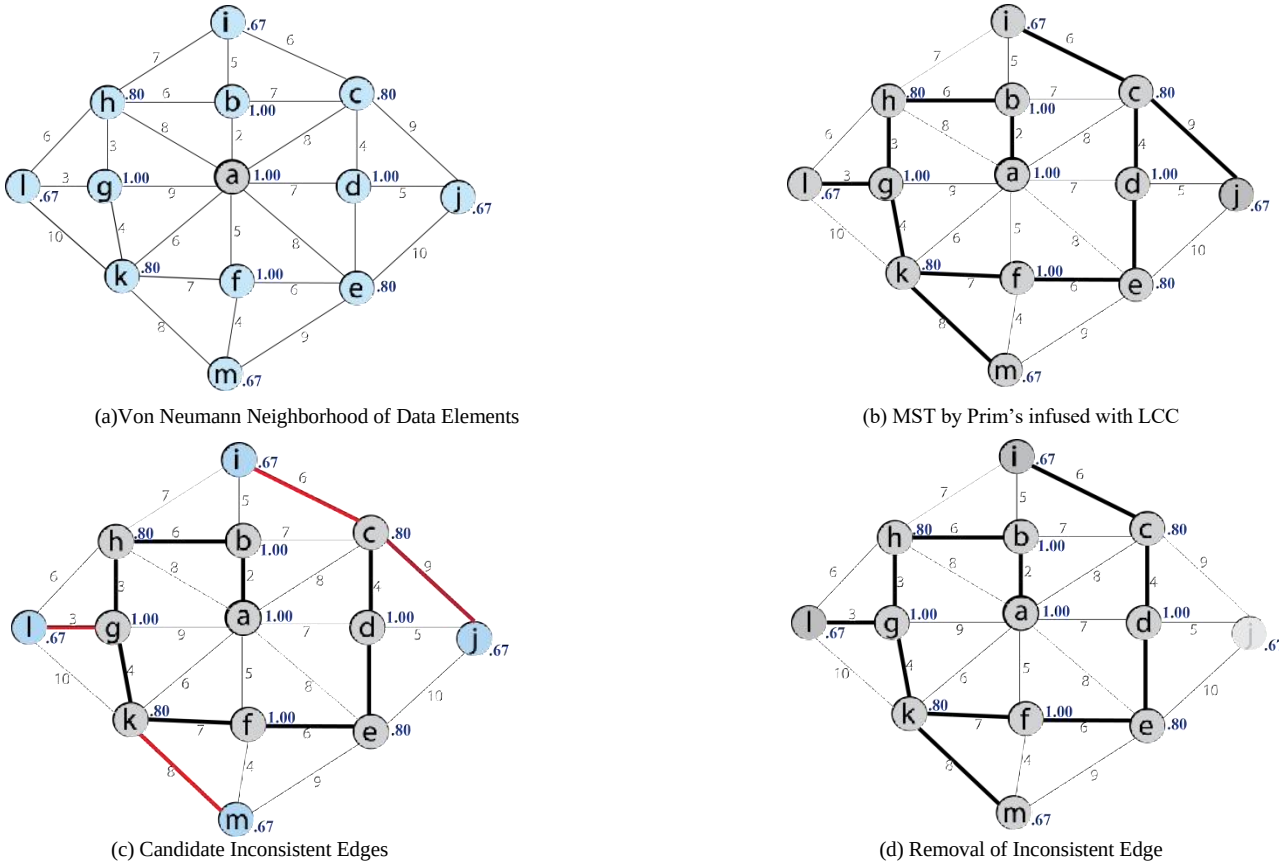
As such, the modified Prim's MST-based Clustering Algorithm establishes the adjacency of the data facilitated by the suitable cellular automaton, the von Neumann Neighborhood which simulates the establishment of neighborhood. This preludes the computation of local efficiency or local clustering coefficient. Thus, the modified Prim's MST construction for clustering is defined by (u, v, LCC(v), d(u,v)) such that u is the initial data point and v is the terminal data point.

While the traditional Prim's MST considers only the next minimum distance d(u,v) between data u in the MST being built T and the adjacent data point v in V; the modified algorithm initially considers the LCC of the adjacent data point LCC(v) to ensure a high clustering coefficient for the whole cluster – hence, pursuing clusters of density. As Prim grows the MST one edge at a time, it should be noted that the next candidate edge or connection of data point must respect the partition or cut of the set of points in the minimum spanning tree T and V to avoid having a cycle.

Pseudocode for PMST for Clustering

```
procedure MST Clustering (V: set of data points v )
construct a fully connected graph G of V such that
the
   edge weights are the distances between data
points
construct  Prim's minimum spanning tree T of G
maintain disjoint sets V and T
select minimum d(v,u) where v∈VT and u∈V
check for cycle
find all inconsistent edges of T
remove inconsistent edges to get a set of connected
    components
define the connected components as clusters
```

Fig. 1. Prim's Minimum Spanning Tree for Clustering.



(a)Von Neumann Neighborhood of Data Elements

(b) MST by Prim's infused with LCC

(c) Candidate Inconsistent Edges

(d) Removal of Inconsistent Edge

Fig. 2. Prim's Minmum Spanning Tree Construction for a Local Neighborhood Established using Von Neumann.

Being the data element having the least LCC(v) and maximum distance d is the criterion set for identifying the inconsistent edge. Data elements l, j, k and m in Fig. 2(a) are all LCC=.67 – hence, their distances to the data points in the MST were considered as indicated in Fig. 2(b). As such, d(c,j)=9 indicated in Fig. 2(c) as the connection with the greatest distance is the inconsistent edge. The algorithm will herein iterate and continue on the other data points of the data set. The resulting MST must have N-1 edges for N number of data points without cycle – hence, the cluster as seen in Fig. 2(d).

B. Data Cleaning and Preparation

The PHEI International Linkages data contains the actual and essential records for the international linkages of Philippine Higher Education Institutions. It is consist of partnerships entered by PHEIs with foreign universities and/or organizations transpiring into different internationalization activities including student exchange, faculty exchange, academic collaboration, research collaboration and other activities across different disciplines. The dataset is summarized in Table I.

An integral part of the data mining process is the data to which knowledge discovery is applied. The International Linkages data contains instances of inconsistencies, incompleteness and variations in the essence of data mining. As such, entries or values were simplified and prepared such that the proposed algorithm is able to process it. In the original data, the field for partnership form has duplicative entries and no defined options. A particular discipline is mentioned in several groups with each specific to a particular partnership. A similar case can be observed with an area of partnership (e.g. Faculty Exchange) being included and specific in a number of partnerships. Hence, in Table II are the disintegrated attributes rooted from the form of linkages attribute of the original data.

TABLE I. LIST OF PHEI LINKAGES DATASET FEATURES AND DESCRIPTION

Name	***Definition***	***Example***
country	where foreign university or organization partnered is located	Indonesia
continent	where country of foreign university or organization partnered is located	Asia
phei	the Philippine Higher Education Institution (e.g. SUC, HEI)	TAU
partner	name foreign university or organization partnered	CRRU
p_form	form of partnership	Bilateral
p_area	area of internationalization activities	Faculty Exchange
p_discipline	field of discipline covered by the partnership	Education
d_signed	date when partnership was signed	02/06
p_year	year when partnership was signed	2017
p_status	if active or inactive	Active

TABLE II. CONTENT RELATED FEATURE OF ATTRIBUTES DISINTEGRATED FROM FIELD

Name	***Definition***
p_type	Bilateral; Multilateral
p_area	Faculty Exchange; Student Exchange; Research(er) Exchange/Collaboration; Academic Collaboration; Joint Publication
p_discipline	Accounting, Arts, Education, Fashion and Textiles, Social Studies

The conversion of the textual values was necessary since most instances are texts and multiple values are specific to one entry. The data cleaning and preparation executed is where each distinct group is coded. For instance, in the area of partnership terms, the PHEI can either use its own nomenclature but certainly, it may also use the terms of reference by the prospect foreign partner university or organization. Hence, all attributes were coded and assigned a numerical value to discretize the data so that the clustering algorithm will be able to process it.

III. RESULTS AND DISCUSSION

The cluster analysis of the data on international linkages of PHEIs aimed to gain valuable insights of the data to see what groups the data elements belong to while having the modified clustering algorithm to define instances with similar properties as a group. Data may come into mix type in the real world such that one attribute may be expressed in ration and others in terms of categorical that adjustment may be hard in terms of the algorithm because some specific algorithms can only be applicable to certain types of data. There may be a need for some data transformation or preprocessing to do so that the algorithm will work. Data cleaning and preprocessing was an integral part of the data mining process to make adjustments and the data be made suitable with the proposed algorithm as it cleaned and prepared the data for the algorithm to be able to process it.

A. Simulation

The algorithm was implemented through the following Pseudocode in Fig. 3 and simulated on the discretized Internationalization data set.

```
Pseudocode for Modified Prim's MST-based Clustering with
Local Efficiency

procedure MST Clustering (V: set of data points v )
Remove all redundant data
set arbitrary data point
get arbitrary data point's Neighborhood
generate connection for each data point in neighborhood;
set LCC for each data point in neighborhood
construct  Prim's minimum spanning tree T of G
maintain disjoint sets V and T
set data point with least LCC and maximum distance as
    inconsistent edge
remove inconsistent edges to get a set of connected
    components
define the connected components as clusters
```

Fig. 3. Modified Prim's MST-based Clustering with Local Efficiency.

The International Linkages data set is composed of 12 attributes with 748 instances. With a random value r=5, seven clusters were generated. The attributes with only at most 2 possible values were not used for the experiment.

Two attributes (e.g. continent, phei) were used to define an instance-hence to illustrate, data point (x, y) defines one data element by its value on attributes continent and phei as x and y, respectively. The neighborhood of said data points determined by NEWS was derived with the nearest higher value in x for north, nearest lower value in x for south, nearest higher value in y for east, and nearest lower value in y for south until the prescribed number of neighbors of the arbitrarily chosen value through von Neumann's Neighborhood is derived.

An observation on the result of the presented data mining procedure is that the generation of edge or connection between the data points to form the neighborhood impacts the processing time of the algorithm. The complexity of this part of the modified algorithm is also challenged when the data points are not linear. The choice of value for r also is also critical as a minimum choice will produce more clusters which impact the inter-cluster separation.

B. Cluster Validation

As there is no optimal clustering algorithm [31], it is necessary to evaluate the generated clusters of the mining process on the International Data. One approach is an internal validation with which the concentration is the partitioned data such that the compactness and separation of the clusters are measured. The Silhouette index [32] is where the silhouettes show which objects lie well within their partition and which are somewhere between clusters. The silhouettes herein were formed basically by knowing the clusters or partitions generated by the modified clustering algorithm and the distance between the data points–hence, a data point i's distance to other points within the cluster it belongs to and to other data points in other clusters.

The average distance a(i) of a data point i to all other objects in the cluster it belongs to is computed in the same manner that the average distance b(i) to other objects in other clusters is also derived. Hence, the silhouette score is derived as:

$$s(i) = \frac{b(i)-a(i)}{\max\{a(i),b(i)} \quad (3)$$

The Silhouette index is chosen for the validation of the resulting clusters of the proposed graph-theoretic clustering algorithm in order to observe how well the algorithm partitioned the data set [33]. The focus is also on the quality of the clustering structure being measured only using information or feature intrinsic to the data set [34]. Another salient point in choosing the Silhouette index for cluster validity is because it measures attributes taken from the data, itself and the clusters found [35]. The silhouette scores ranging from -1 <= s(i) <= 1 can be interpreted in Table III.

The validation on the clustering result generated by the modified graph-theoretic clustering algorithm infused with small-world network structure based on the Silhouette score is presented in Table IV which presents the silhouette score of the clustering result. The average intra-cluster distance was derived

from calculating the distance of a random point (x, y) in a cluster towards all other data points in the same cluster to which it belongs to. Inter-cluster distance is the distance of this (x, y) towards the other data points in other clusters.

TABLE III. SILHOUETTE SCORE INTERPRETATION

Range	***Description***	***Interpretation***
0.71 – 1.00	Strong	A strong structure has been found.
0.51 – 0.70	Reasonable	Reasonable structure has been found.
0.26 – 0.50	Weak	Structure is weak and could be artificial.
≤ 0.25	Not Substantial	No substantial structure has been found.

TABLE IV. SILHOUTTE VALIDATION ON CLUSTERING RESULT

Cluster ID	***Average Intra-Cluster Distance***	***Average Inter-Cluster Distance***	***Silhouette Score***
1	2.57	14.65	0.81
2	2.46	9.65	0.75
3	3.09	15.82	0.80
4	3.20	19.83	0.84
5	2.86	16.64	0.83
6	2.70	15.82	0.83
7	-	15.11	0.00

The average Silhouette score derived as 0.69 indicates an acceptable structure was found which is also manifested in the scores of all the clusters which derived scores not lower than 0.75 which means that each cluster has a strong structure except for Cluster 7 which has only one (1) data point – hence, silhouette score is 0. Such constraint is present to prevent the number of groups from significantly increasing [36]. Consequently, when a clustering result is interpreted based on Table III, the clustering is acceptable when the score is at least 0.50 [37].

IV. CONCLUSION

This work performed data mining in the international linkages of Philippine Higher Education Institutions (PHEIs) data using a proposed modified Prim's MST-based clustering algorithm producing a minimum spanning tree for the data set infusing the computation of local clustering coefficient for the data points in the limited neighborhood generated by von Neumann's Neighborhood.

An integral part of this work was the preparation of the raw data to achieve the dataset that is ready for processing by the modified Prim's MST-based clustering algorithm. The numerical attributes of the International Linkages dataset were used for the clustering to work on similarity on a particular parameter.

The results of the study show that there is an acceptable structure found in the clustering result with silhouette score 0.69 and 0.75 being the least score for the 6 out of 7 clusters derived for r=5 of the von Neumann Neighborhood.

However, the algorithm is still bound by the *a priori* input value of r which dictates the number of possible neighbors in one cluster for the von Neumann Neighborhood. As such the optimum number of clusters and most ideal value of r for a particular size of data are interesting.

Also for future works, the interest is also centered on the cluster validation utilizing external validity indices particularly those which works or are specific to graph-theoretic clustering algorithms. The data can also be refined more and subjected to clustering process to compare the performance of the traditional and the modified clustering algorithm.

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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

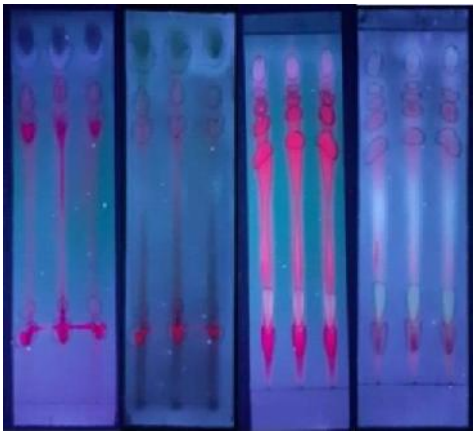
mixture of compounds through suitable chromatographic techniques. In the present study, flavonoids, saponin, tannin and terpenoid are confirmed to be present.

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)

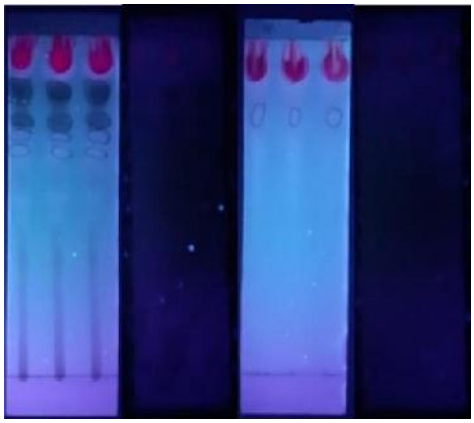
Figure 1. Photograph of TLC of flavonoid (a) Indonesian (b) Indonesian (after spray) (c) Philippines (d) Philippines (after spray).



(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

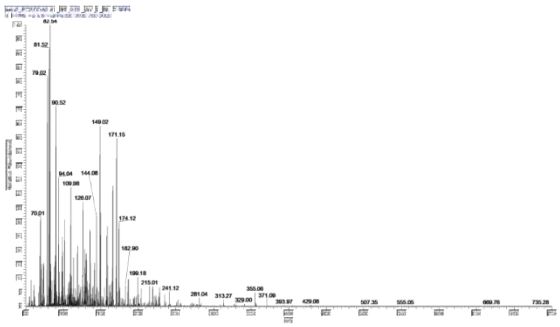


Figure 5. LC-MS chromatogram of ethanolic extract of *Azadirachta indica* varian Indonesia leaves.

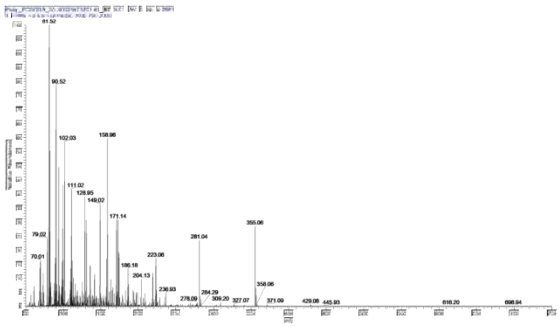


Figure 6. LC-MS chromatogram of ethanolic extract of *Azadirachta indica* varian Philippines leaves.

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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