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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.

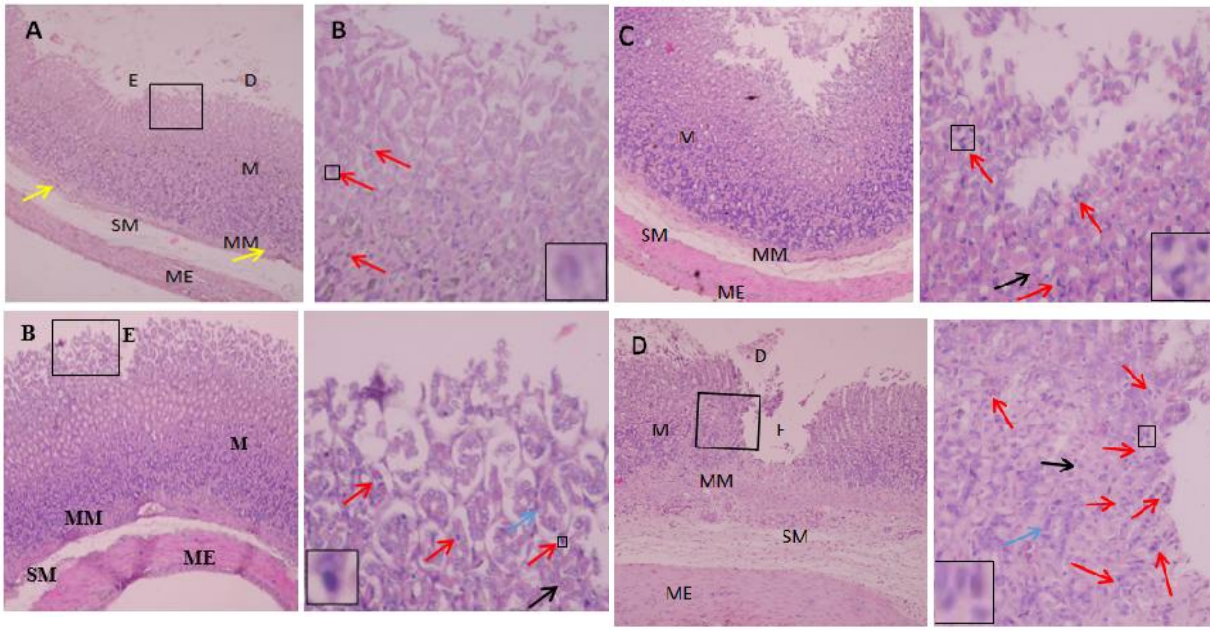

Figure 1. Histopathology of gastric mucosa. (A). Gold Standard group; (B). *P. longfolia* extract from Indonesia therapy; (C). *P. longifolia* extract from The Philippines, and (D) IBD condition.

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.

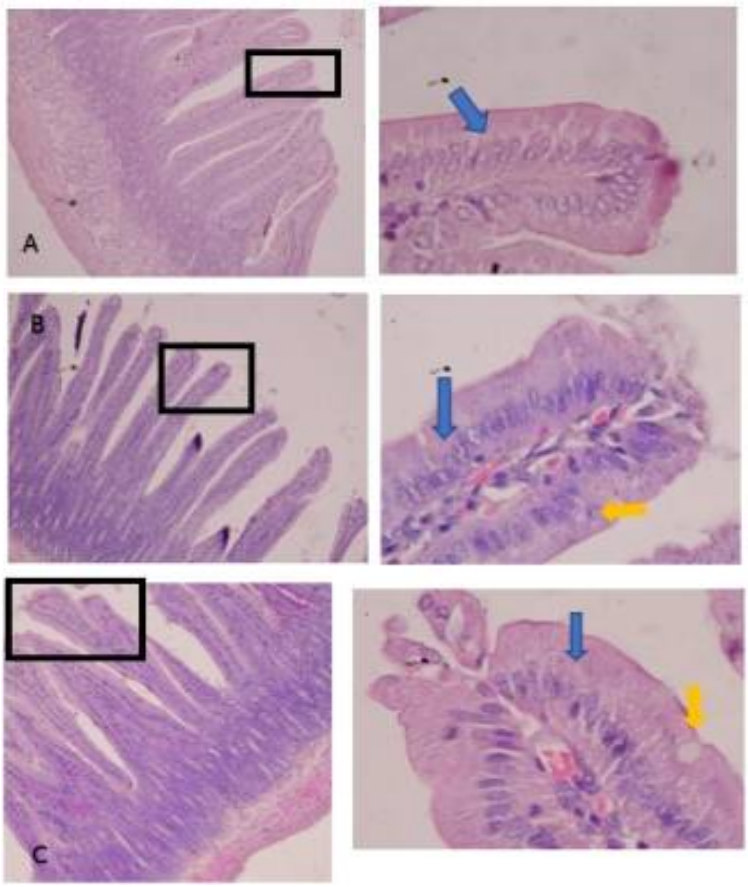

Figure 2. Histopathology of duodenum. (A). Gold Standard group; (B). P. longifolia extract from Indonesia therapy; (C). P. longifolia extract from Philippines.

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).

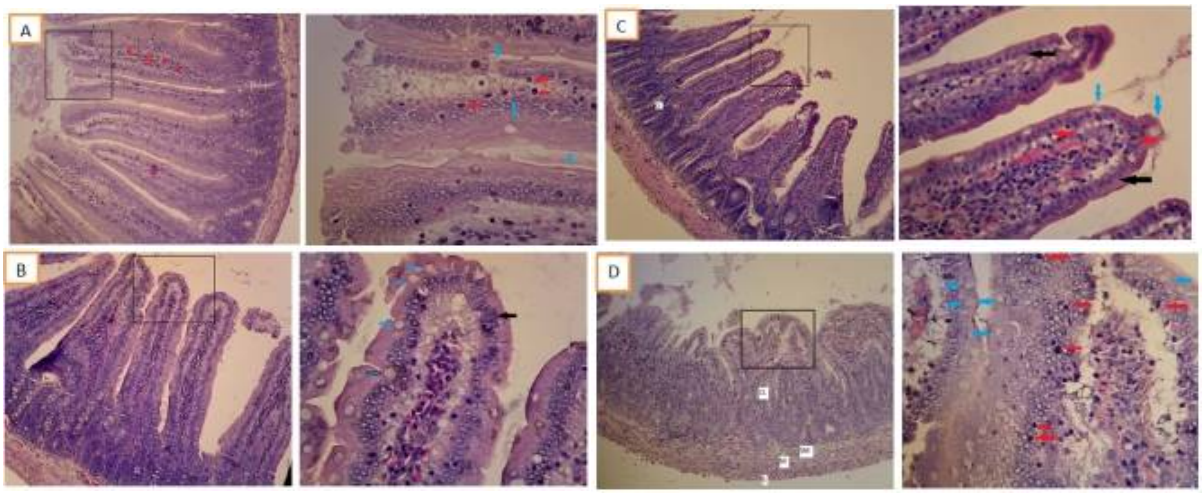

Figure 3. Histopathology of jejunum; (A). Gold Standard group; (B). *P. longifolia* extract from Indonesia therapy; (C). *P. longifolia* extract from Philippines, and (D) IBD condition.

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].

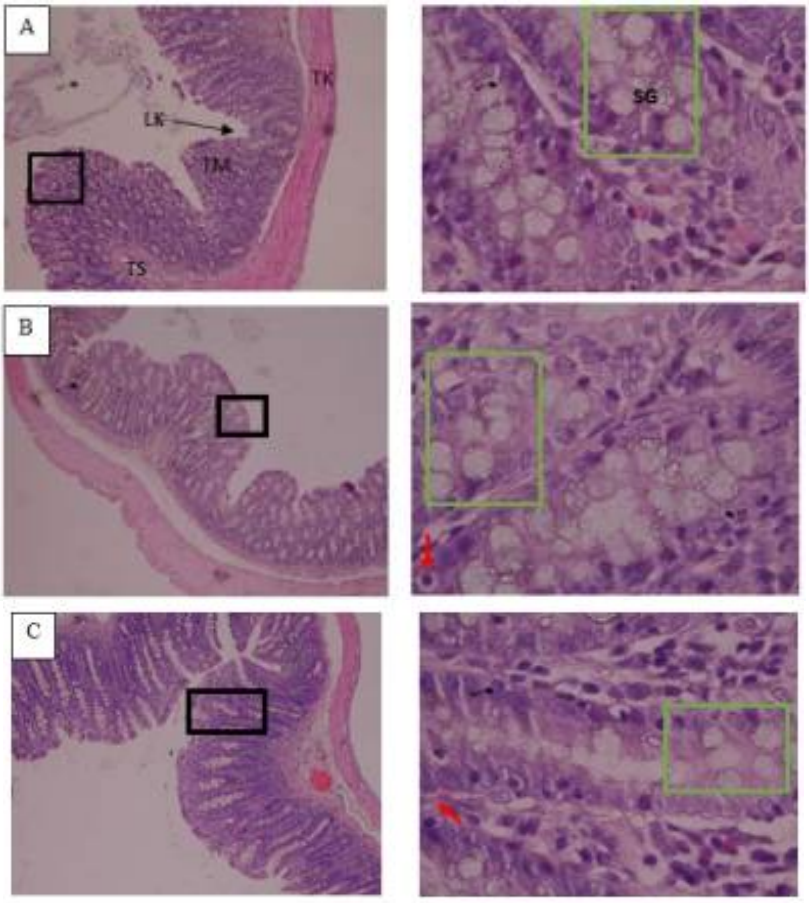

Figure 4. Histopathology of colon. A. Gold Standard group; B. EEPL from Indonesia group; C. EEPL from The Philippines group.

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)	√	-	-	-	√	√	√	√

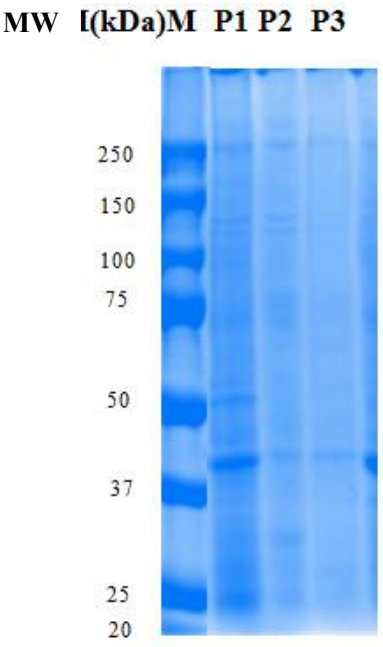

Figure 5. Protein bands of duodenum (SDS PAGE 12%).
Legend: M = marker; P1 = Sulfasalazine group; P2 = EEPL from Indonesia group; P3 = EEPL from Philippines

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)	√	√	√	-	√	√	√

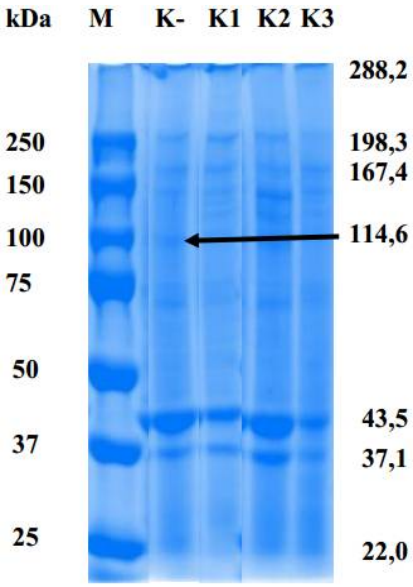

Figure 6. Protein bands of colon (SDS PAGE 12%).
Legend: M = marker; K- = negative control; K1 = Sulfasalazine group; K2 = EEPL from Indonesia group; K3 = EEPL from Philippines

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.

References

[1] Jo R Am L and Ceballos R. 2013 *Altern Integr Med* **2** 1–6.
[2] Ekor M. 2014 *Front Pharmacol* **4** 1–10
[3] Msomi N Z and Simelane M B 2019 *Herbal Medicine* **11** 215–271
[4] Sampath M and Vasanthi M 2013 *Int J Pharm Pharm Sci* **5** 336–41
[5] Peng J, Zheng T, Li X, Liang Y, Wang L, and Huang Y 2019 *Front Pharmacol* **10** 1–15.

[6] Vezza T, Rodríguez-nogales A, Algieri F, Utrilla MP, Rodriguez-cabezas ME, and Galvez J 2016 *Nutrients* **8** 1–22
[7] Aulanni'am, Rosdiana A, and Rahmah NL 2011 *J Life Sciences* **4** 57–64
[8] Shofia V, Aulanni'am A, and Prasetyawan S 2015 *Int J ChemTech Res* **8** 640–644
[9] Nagarjun S, Dhadde SB, Veerapur VP, Thippeswamy BS, and Chandakavathe BN 2017 *Biomed Pharmacother Elsevier Masson* **89** 1061–6
[10] Indonesian Endocrinology Association (PERKENI). 2011. National Consensus for IBD therapy in Indonesia. PERKENI. pp.1-42 (Write in Bahasa Indonesia)
[11] Sharma R, Mandal S, Gp R, Gupta N, and Dp S 2016 *International Journal of Drug Development & Research* **3** 351–359
[12] Sampath M 2013 *J Appl Pharm Sci* **3** 148–52
[13] Sashidhara K V., Singh SP, Srivastava A, and Puri A 2011 *Nat Prod Res* **25** 918–26
[14] Obaid AK, Salbiah S, Abdul S, Bakri YM, Nafiah MA, Awang K 2018 *J Pharm Sci Res* **10** 2718–21
[15] Balqis U, Hanafiah M, Januari C, Salim MN, Aisyah S, Fahrimal Y 2015 *Med Vet* **9** 1999–2002
[16] Craven A, Derubertis R, December R, October A 2008 *Gastroenterology* **92** 1998–2008
[17] Saxena A, Kaur K, Hegde S, Kalekhan FM, Baliga MS, Fayad R 2014 *J Tradit Complement Med* **4** 203–217
[18] Lichtenberger LM, JJ Romero, and EJ Dial 2007 *Br J Pharmacol* **150** 913–919
[19] Triantafilidis, J.K.A., C. Triantafyllidi and A. Papalios 2016 *Annals of Gastroenterology* **22** 268-281
[20] Lahav, J 1993 *Thrombospondin* (CRC Press-London)
[21] Nivetha A and V. Mohanasrinivisan 2018, *Research Journal of Pharmacy and Technology*, **11** 1778-1783

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].

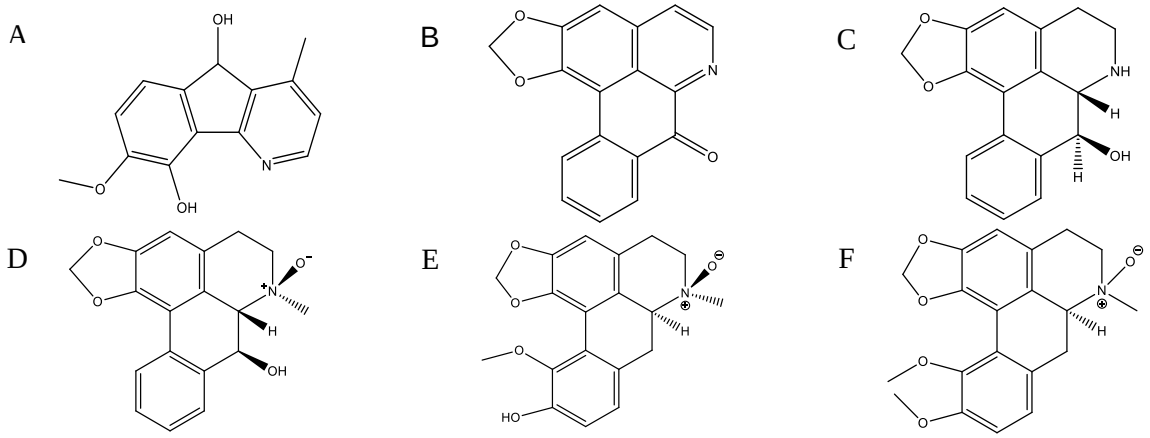

Figure 1. Structure of alkaloid compounds. A) Polylongine, B) Liriodenine, C) Noroliveroline, D) Oliveroline β-N-oxide, E) N-methylnandingerine-β-N-oxide, F) O-methylbulbocapnine-N-oxide.

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).

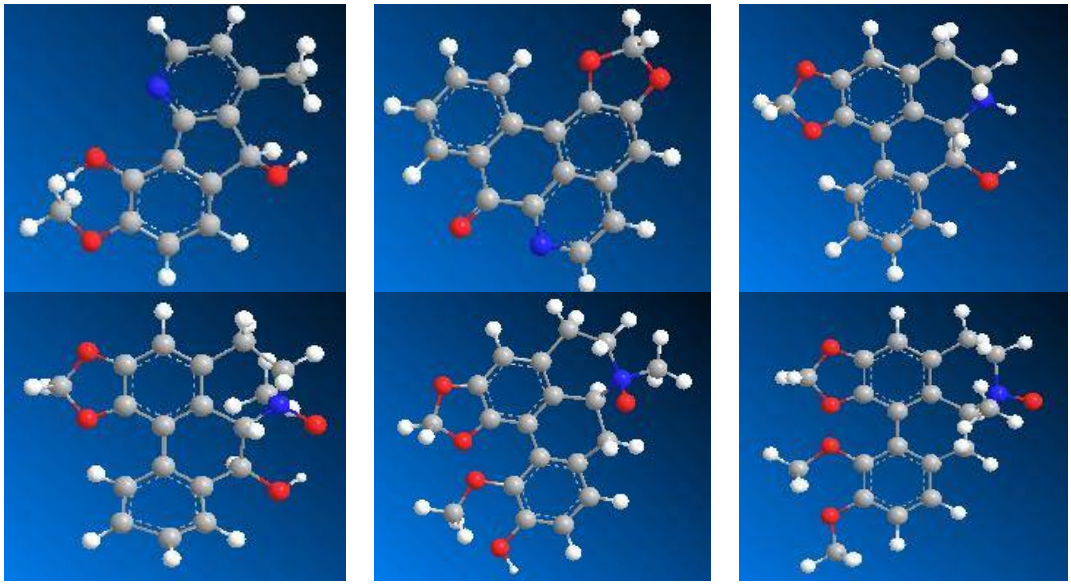

Figure 2. The 3D structure of Alkaloids. A) Polylongine, B) Liriodenine, C) Noroliveroline, D) Oliveroline β-N-oxide, E) N-methylnandingerine-β-N-oxide, F) O-methylbulbocapnine-N-oxide.

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).

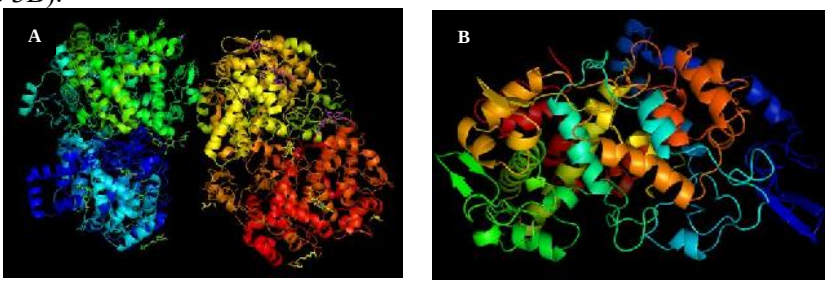

Figure 3. A) The 3D of COX-2 protein, B) The 3D chain A structure of COX-2 protein.

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.

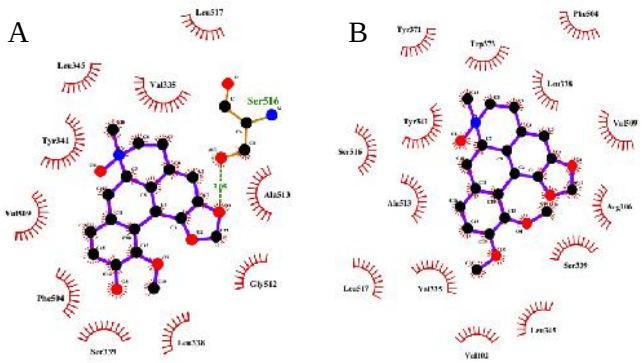

Figure 4. Visualize the results of LigPlot+. A) N-methylnandingerine-β-N-oxide, B) O-methylbulbocapnine-N-oxide.

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.

References

[1] Thenmozhi M and Sivaraj R 2010 Phytochemical Analysis and Antimicrobial Activity of Polyalthia longifoia *Int. J. Pharma Bio Sci.* **1** (3) 1–7

[2] Sampath M and Vasanthi M 2013 'Isolation, Structure Elucidation of Flavonoids From Polyalthia longifolia (Sonn.) Thaiwaites and Evaluation of Antibacterial, Antioxidant and Anticancer Potential', *Int. J. Pharm. Pharm. Sci.*, **5** (1) 336–341

[3] C. Lavanya, B. G. Rao, and D. Ramadevi 2018 Phytochemical and Pharmacological Studies on Polyalthia longifolia', *Int. J. Pharm. Sci. Res.* **3** (4) 1–7

[4] W. Liu, Y. Dongxue, N. Li, H. Xiaogai, W. Dongmei, D. Li, and J. Liu, 'Influence of Environmental Factors on the Active Substance Production and Antioxidant Activity in Potentilla fruticosa L . and Its Quality Assessment', *Nature*, no. July, pp. 1–18, 2016.

[5] A. L. Souto, J. F. Tavares, M. Sobral, M. De Fátima, F. Melo, P. F. De Athayde-filho, J. Maria, and B. Filho, 'Anti-Inflammatory Activity of Alkaloids: An Update from 2000 to 2010', *Molecules*, **16**, pp. 8515–8534, 2011.

[6] J. Peng, T. Zheng, X. Li, Y. Liang, L. Wang, Y. Huang, and L. Wang 2019 Plant-Derived Alkaloids : The Promising Disease-Modifying Agents for Inflammatory Bowel Disease *Front. Pharmacol.* **10** 1–15

[7] D. D. Agistia, H. Purnomo, M. Tegar, and A. E. Nugroho, 'Interaction Between Active Compounds From Aegle marmelos Correa as Anti Inflammation Agent With COX-1 and COX-2 Receptor', *Tradit. Med. J.* **18** (2) 80–87, 2013.

[8] G. C. Krisnamurti and F. Fatchiyah, 'Interaction of acetaminophen and caffeine towards cyclooxygenase-2 (COX-2) in inhibition of prostaglandin (PGH 2) synthesis Interaction of acetaminophen and caffeine towards cyclooxygenase-2 (COX-2) in inhibition of prostaglandin', in *IOP Conf. Series: Journal of Physics: Conf. Series*, 2019 **2**, pp. 1–6.

[9] V. Shofia, A. Aulanni'am, and S. Prasetyawan, 'In Silico Study of Apigenin as COX-2 Inhibitor and In vivo Studies of Apigenin contained in Methanolic Extract of of Atactodea striata for decreasing Malondialdehyde Levels On Inflammatory Bowel Disease Rats', *Int. J. ChemTech Res.* **8** (6) 640–644, 2015.

[10] I. de la C. Chaco´ and A. R. G. Lez-Esquinca, 'Liriodenine Alkaloid in Annona Diversifolia During Early Development', *Nat. Prod. Res.*, **21** (1) 42–49, 2012.

[11] C. N. Loumpangou, L. Justin, C. Bonazaba, A. Murphy, E. Ndinga, A. D. Empfani, Y. Mahmout, and J. Moutsabote, 'Dosage and evaluation of the antiradical activity of alkaloids of the root barks of Oulotricha Le Thomas : isolation of Liriodenine', *Int. J. Herb. Med.* **6** (6) 96–100, 2018.

[12] A. K. Obaid, S. Salbiah, S. Abdul, Y. M. Bakri, M. A. Nafiah, K. Awang, S. A. Aowda, N. M. Hassan, Y. Z. H. Hashim, and F. Abdullah, 'Antioxidative and cytotoxic activities of crude and isolated compounds of P . Lateriflora (bl .) King', *J. Pharm. Sci. Res.* **10** (11) 2718–2721, 2018.

[13] N. P. L. Laksmiani, N. L. P. V. Paramita, and I. M. A. . Wirasuta, 'In Vitro and In silico Antioxidant Activity of Purified Fractions From Purple Sweet Potato Ethanolic Extract', *Int. J. Pharm. Pharm. Sci.*, **8** (8) 177–181, 2016.

[14] A. Yanuar, I. Pratiwi, and R. R. Syahdi, 'In silico Activity Analysis of Saponins and 2 , 5-Piperazinedione from Marine Organism against Murine Double Minute-2 Inhibitor and Procaspase-3 Activator', *J. Young Pharm.* **10** (2) 16–19, 2018.

[15] S. R. Lestari, B. Lukiati, S. N. Arifah, A. R. Nurul, and A. Gofur, 'Medicinal Uses of Single Garlic in Hyperlipidemia by Fatty Acid Synthase Enzyme Inhibitory : Molecular Docking', in *IOP Conference Series: Earth and Environmental Science PAPER* 2019 **276** 1–8

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.

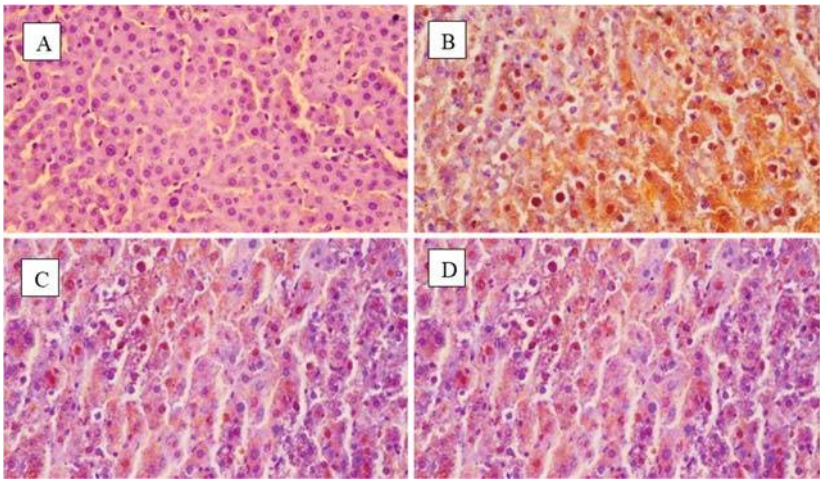


FIGURE 1. The results of the examination of Hsp70 expression by immunohistochemical staining on liver cancer tissue are shown in brown in the cell nucleus. (a) mice that did not get *Azadirachta indica* leaf extract treatment (control); (b) rats receiving 5 mg/kgBW of sorafenib treatment and (c) rats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

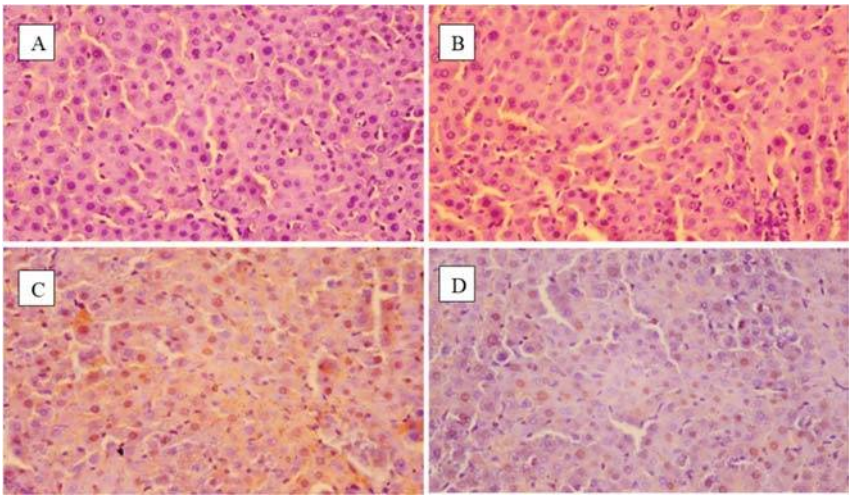


FIGURE 2. The results of the examination of AIF expression by immunohistochemical staining on liver cancer tissue are shown in brown in the cell nucleus. (a) mice that did not get *Azadirachta indica* leaf extract treatment (control); (b) rats receiving 5 mg/kgBW of sorafenib treatment and (c) rats receiving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

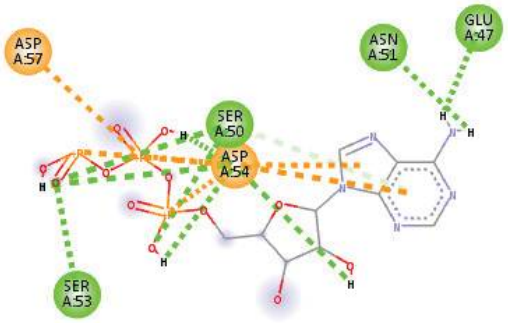
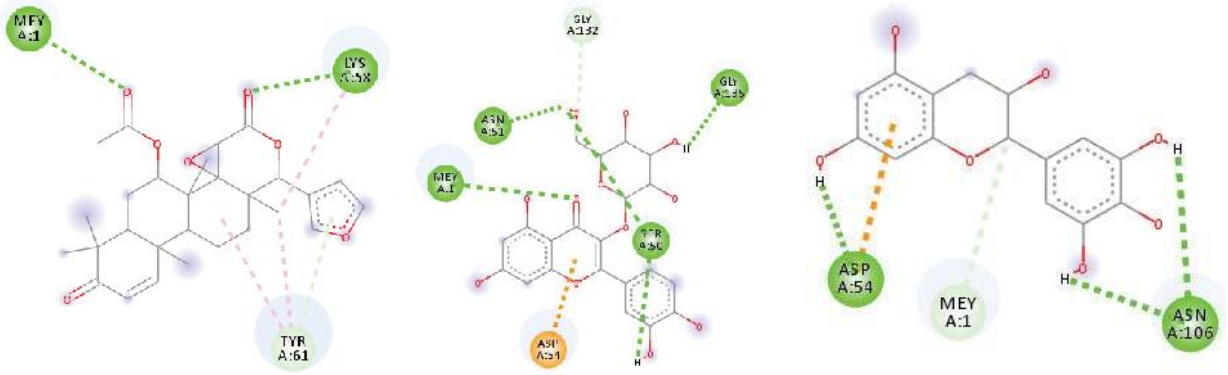


FIGURE 3. Visualization of the Interaction between Native Ligands and Target Proteins



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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REFERENCES

1. J.M. Llovet, R.K. Kelley, Villanueva. Nat Rev Dis Primers 7, 6 (2021).
2. J. Balogh, Victor, Burroughs, H. S., Boktour G., Saharia M, Li A., Ghobrial X., Monsour, H. P. Journal of hepatocellular carcinoma, 3, 41–53. (2016).
3. R. Tolba, T. Kraus, C. Liedtke, M. Schwarz, & R. Weiskirchen. Lab. Anim. 49, 59–69. (2015).
4. D. Scholten, J. Trebicka, C. Liedtke, & R. Weiskirchen. Lab. Anim. 49, 4–11. (2015)
5. K. Nurgali, R. T. Jagoe, & R. Abalo. Front. Pharmacol, 9, 245. (2018)
6. J. Escalante, R. M. McQuade, V. Stojanovska, & K. Nurgali. Maturitas, 105, 23–29. (2017) J. Sophia, J. Kowshik, A. Dwivedi, *Cell Death Dis* **9,** 1087 (2018).
7. T. R. Govindachari, G. Suresh, G. Gopalakrishnan, B. Banumathy, S. Masilamani, *Phytoparasitica.* **26**, 109–116. (1998)
8. C. Vergallo, E. Panzarini, L. Dini, *Pure Appl. Chem*. **91**, 1631-1640 (2019).
9. S. Chaudhary, R. K. Kanwar, A. Sehgal, D. M. Cahill, C. J. Barrow, R. Sehgal, J. R. Kanwar, *Front. Plant Sci*. **8**:610 (2017)
10. M. P. Rodrigues, A. L. Astoreca, A. A. Oliveira, L. A. Salvato, G. L. Biscoto, L. Keller, C. Rosa, L. R. Cavaglieri, M. I. Azevedo, M. I., K. M. Keller, *Toxins **11***, 579. (2019)..
11. M. J. Mitchell, S. L. Smith, S. Johnson, E. D. Morgan, *Arch. Insect Biochem. Physiol.* ***35***, 199–209. (1997).
12. R. Paul, M. Prasad, N. K. Sah, *Cancer Biol Ther **12***, 467–476 (2011).
13. M. Kashif, Y. Hwang, W. J., G. Kim, *Iran. J. Pharm. Sci.* **18**, 846–859 (2019).
14. M. Bonora and P. Pinton P, *Front. Oncol.* **4**:302 (2014).
15. L. Dalla Via, A. N. García-Argáez, M. Martínez-Vázquez, S. Grancara, P. Martinis, A. Toninello, *Curr. Pharm. Des.*, ***20***, 223–244 (2014)
16. S. Cregan, V. Dawson, R. Slack, *Oncogene* **23,** 2785–2796 (2004).
17. M. Bonora, M. Wieckowski, C. Chinopoulos, *Oncogene* **34,** 1475–1486 (2015).
18. A. Saleh, S. Srinivasula, L. Balkir, *Nat Cell Biol* **2,** 476–483 (2000).
19. H. Beere, B. Wolf, K. Cain, *Nat Cell Biol* **2,** 469–475 (2000).
20. L. Ravagnan, S. Gurbuxani, S. Susin. *Nat Cell Biol.* **3,** 839–843 (2001).
21. O. Trott and A.J. Olson, *J. Comput. Chem*., **3**, 455–461 (2010).
22. K. Sargsyan, C. Grauffel, C. Lim, *J. Chem. Theory Comput*., **13**, 1518–1524 (2017).
23. E. A. Coutsias and M. J. Wester, *J. Comput. Chem*., **40**, 1496–1508 (2019).

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.

REFERENCES

Alibudbud, R. (2022). Social and psychological factors influencing HIV sexual risk behaviors among young adult Filipino Men who have sex with men

(MSM) in Metro Manila. *Sexual Health & Compulsivity, 29*(1-2), 38-55. https://doi.org/10.1080/26929953.2022.2056555

Amare, T., Getinet, W., Shumet, S., & Asrat, B. (2018). Prevalence and associated factors of depression among PLHIV in Ethiopia: Systematic review and meta-analysis. *AIDS Research and Treatment.* https://doi.org/10.1155/2018/5462959.

Andersen, L., Kagee, A., Cleirigh, C., Safren, S., & Joska, J. (2015). Understanding the experience and manifestation of depression in people living HIV/AIDS in South Africa. *AIDS Care, 27* (1). 59-62. https://www.ncbi.nlm.nih.gov/pubmed/25303372.

Badiee, J., Moore, D., Atkinson, H., Vaida, F., Gerard, M., Duarte, N., Franklin, D., Gouaux, B., McCutchan, A., Heaton, R., Mcarthur, J., Morgello, S., Simpson, D., Collier, A., Marra, C., Gelman, B., Clifford, D., & Grant, I. (2011). Lifetime suicidal ideation and attempt is common among HIV+ individuals. *Journal of Affective Disorders, 136* (3). 993-999. https://doi.org/10.1016/j.jad.2011.06.044.

Beck, A. T., Steer, R.A., & Garbin, M.G. (1988) Psychometric properties of the Beck Depression Inventory: Twenty-five years of evaluation. *Clinical Psychology Review, 8* (1), 77-100. https://doi.org/10.1016/0272-7358(88)90050-5.

Bekker, L. G., Johnson, L., Wallace, M., & Hosek, S. (2015). Building our youth for the future. *Journal of the International AIDS Society, 18*, 20027. https://doi.org/10.7448/IAS.18.2.20027

Benton, T. D., Ng, W., Leung, D., Canetti, A., & (follow APA style) Karnik, N. (2019). Depression among youth living with HIV/AIDS. *Child and Adolescent Psychiatric Clinics of North America, 28*(3), 447-459. https://doi.org/10.1016/j.chc.2019.02.014

Bhana, A., Mellins, C.A.,Petersen, I., Alicea, S., Myeza, N., Holst, H., Abrams,, E., John, S., Chhagan, M., Nestadt, D., Leu, C., & McKay, M. (2013). The VUKA family program: Piloting a family-based psychosocial intervention to promote health and mental health among HIV infected early adolescents in South Africa. *AIDS Care, 26* (1), 1-11. https://doi.org/10.1080/09540121.2013.806770

Bhatia, M. S., & Munjal, S. (2014). Prevalence of depression in people living with HIV/AIDS undergoing ART and factors associated with it. *Journal of Clinical and Diagnostic Research: JCDR, 8*(10), WC01–WC4. https://doi.org/10.7860/JCDR/2014/7725.4927.

British HIV Association and British Association for Sexual Health. (2018). *Loneliness and isolation top list of unmet social needs among people living with HIV in the UK.* http://www.aidsmap.com/news/apr-2018/loneliness-and-isolation-top-list-unmet-social-needs-among-people-living-hiv-uk.

Brown, T., & Morgan, K. (2013). Psychological distress and substance abuse in Jamaican youths living with HIV/AIDS. *West Indian Medical Journal, 62* (4). 341-345. https://doi.org/10.7727/wimj.2013.024.

Brown, L.K., Whiteley, L., Harper, G.W., Nichols, S., & Nieves, A. (2015). Psychological symptoms among 2032 youth living with HIV:

A multisite study. *AIDS Patient Care and STDs, 29* (4). https://doi.org/10.1089%2Fapc.2014.0113

Cain, R., Jackson, R., Prentice, T., Collins, E., Mill, J., & Barlow, K. (2013). The experience of HIV diagnosis among aboriginal people living with HIV/AIDS and depression. *Qualitative Health Research, 23* (6). 815-824.https://doi.org/10.1177%2F1049732313482525.

Carrico, A.W. (2010). Elevated suicide rate among HIV positive persons despite benefits of antiretroviral therapy: Implications for a stress and coping model of suicide. *The American Journal of Psychiatry, 167* (2). https://doi.org/10.1176/appi.ajp.2009.09111565.

Casale, M., Boyes, M., Pantelic, M., Toska, E., & Cluver, L. (2019). Suicidal thoughts and behavior among South African adolescents living with HIV: Can social support buffer the impact of stigma?. *Journal of Affective Disorders, 245* (15). 82-90. https://doi.org/10.1016/j.jad.2018.10.102.

Catalan, J., Harding, R., Sibley, E., Clucas, C., Croome, N., & Sherr, L. (2011,). HIV infection and mental health: Suicidal behaviour – Systematic review. *Psychology, Health & Medicine, 16*(5), 588-611. https://doi.org/10.1080/13548506.2011.582125

Center for Behavioral Health Statistics and Quality. (2017). *2016 National survey on drug use and health: Methodological summary and definitions*. Rockville, MD: Substance Abuse and Mental Health Services Administration.Centers for Disease Control and Prevention. (2013). HIV among youth in the US. https://www.cdc.gov/ vitalsigns/hivamongyouth/index.html

Ciesla, J. A., & Roberts, J. E. (2001,). Meta-analysis of the relationship between HIV infection and risk for depressive disorders. *The American Journal of Psychiatry, 158*, 725–730. https://doi.org/10.1176/appi.ajp.158.5.725

Chantaratin S., Trimetha, K., Werarak, P., Lapphra, K., Maleesatharn, A., Rungmaitree, S., Wittawatmongkol, O., Phongsamart, W., Kongstan, N., Khumcha, B., & Chokephaibulkit, K. (2022). Depression and anxiety in youth and young adults living with HIV: Frequency and associated factors in Thai setting. *Journal of the International Association of Providers of AIDS Care, 21* (1-12). https://doi.org/10.1177%2F23259582221101811

Cournos, F., Walnber, M., & Horwath, E. (2005). *Psychiatric care and antiretrovirals for second level care.* Mental Health and HIV/AIDS Series. Geneva: World Health Organization.

Dejman, M., Ardakani, H., Malekafzali, B., Moradi, G., Gouya, M., Shushtari, J…Mohraz, M. (2015). Psychological, social, and familial problems of people living with HIV/AIDS in Iran: a qualitative study. *International Journal of Preventive Medicine. 6* (126). https://doi.org/10.4103/2008-7802.172540.

Department of Health. (2022). HIV/AIDS and Art Registry of the Philippines. https://doh.gov.ph/sites/default/files/statistics/EB_HARP_June_AIDSreg2022.pdf

Dorrell, J., Earle, S., Katz, J., & Reveley, S. (2008). Growing up with HIV: The experiences of young people living with HIV since birth in the UK. Sage Publications.

Dow D. E., Mmbaga, B. T., Gallis, J. A., Turner, E. L., Gandhi, M., Cunningham, C. K., & O'Donnell, K. E. (2020,). A group-based mental health intervention for young people living with HIV in Tanzania: Results of

a pilot individually randomized group treatment trial. *BMC Public Health, 20*(1358). https://doi.org/10.1186/s12889-020-09380-3

Duko, B., Ayalew, M., & Ayano, G. (2019). The prevalence of alcohol use disorders among people living with HIV/AIDS: a systematic review and meta-analysis. *Substance Abuse Treatment Prevention Policy, 14*(1). https://doi.org/10.1186/s13011-019-0240-3.

Elsayed, H., O'Connor, C., Leyritana, K., Salvana, E., & Cox, S. E. (2021,). Depression, nutrition, and adherence to antiretroviral therapy in men who have sex with men in Manila, Philippines. *Frontiers in Public Health, 9*(644438). https://doi.org/10.3389/fpubh.2021.644438

Fawzi, M., Ng, L., Kanyanganzi, F., Kirk, C., Bizimana, J., Cyamatare, F., Mushashi, C., Kim, T., Kayiteshonga, Y., Binagwaho, A., & Betancourt, T. S. (2016,). Mental health and antiretroviral adherence among youth living with HIV in Rwanda. *Pediatrics, 138*(4). https://doi.org/10.1542/peds.2015-3235

Frontline AIDS Organization. (2020). *Supporting mental health of adolescents living with or affected by HIV.* https://frontlineaids.org/resources/supporting-mental-health-of-adolescents-living-with-or-affected-by-hiv/

Gennaro, F., Marotta, C., Saracino, A., Occa, E., & Putoto, G. (2022). Mental health needs of adolescents with HIV in Africa. *Correspondence, 9* (6). https://doi.org/10.1016/S2352-3018 (22)00130-8

Gentz, S. G., Romano, I. C., Arias, R. M., Zeng, C., & Casares, M. R. (2018). Mental health among adolescents living with HIV in Namibia: The role of poverty, orphanhood and social support. *AIDS Care, 30*(2), 83-91. https://doi.org/10.1080/09540121.2018.1469727

Girma, D., Assegid, S., & Gezahegn, Y. (2021) Depression and associated factors among HIV-positive youths attending antiretroviral therapy clinics in Jimma town, southwest Ethiopia. *PLoS ONE 16*(1). https://doi.org/10.1371/journal.pone.0244879

Grainger, C. (2017). Understanding disclosure behaviors in HIV-positive young people. *Journal of Infection Prevention, 18* (1). 35-39. https://doi.org/10.1177/1757177416680871.

Heubeck, B. G., & Neill, J. T. (2000). Internal validity and reliability of the 30 item Mental Health Inventory for Australian Adolescents. *Psychological Reports, 87,* 431-440.

Ivancova, N.V., Creswell, J.W., & Stick, S.L. (2006). Using mixed-methods sequential explanatory design: From theory to practice. *Field Methods, 18* (1), 3-20. http://dx.doi.org/10.1177/1525822X05282260.

Jena, P. P. (2014). Exploring the lived experiences of adolescents living vertically acquired HIV. (Unpublished doctoral dissertation, University of South Africa).

Kalichman, S., Heckman, T., Kochman, A., Sikkema, K., & Bergholte, J. (2000). Depression and thoughts of suicide among middle-aged and older persons living with HIV-AIDS. *Psychiatric Services, 51* (7). https://dx.doi.org/ 10.1176/appi.ps.51.7.903.

Kamau, J.W., Kuria, W., Mathai, M., Atwolli, L., & Kangethe, R. (2012). Psychiatric morbidity among HIV- infected children and adolescents in a resource- poor Kenyan urban community. *AIDS Care, 24* (7). https://dx.doi.org/ 10.1080/09540121.2011.644234.

Kim, M., Mazenga, A., Yu, X., Devandra, A., Nguyen, C., Ahmed, S., Kazembe, P., & Sharp, C. (2015). Factors associated with depression among adolescents living with HIV in Malawi. *BioMedCentral Psychiatry, 15* (264), https://doi.org/10.1186/s12888-015-0649-9.

Kimera, E., Vindevogel, S., Kintu, M., Rubaihayo, J., De Maeyer, J., Reynaert, D., Engelen, A., Nuwaha, F., & Bilsen, J. (2020). *BMC Public Health, 20* (79). https://doi.org/10.1186/s12889-020-8198-7.

Leserman, J. 2008. Role of depression, stress, and trauma in HIV disease progression. *Psychosomatic Medicine* 70(5): 539-45. https://doi.org/10.1097/PSY.0b013e3181777a5f.

Mellins, C. A., & Malee, K. M. (2013). Understanding the mental health of youth living with perinatal HIV infection: Lessons learned and current challenges. *Journal of the International AIDS Society, 16*(1). https://doi.org/10.7448/IAS.16.1.18593

Newman, P., Prabhu, S., Akkakanjanasupar, P. & Tepjan, S. (2021). HIV and mental health among young people in low-resource contexts in Southeast Asia: A qualitative investigation. *Global Public Health: An International Journal for Research, Policy, and Practice, 17* (7), 1200-1214. https://doi.org/10.1080/17441692.2021.1924822

Njau, T., Ngakongwa, F., Sunguya, B., Kaaya, S., & Fekadu, A. (2022). Development of a psychological intervention to improve depressive symptoms and enhance adherence to antiretroviral therapy among adolescents and young people living with HIV in Dar es Salaam Tanzania. *Healthcare 2022, 10* (12). 1-15. https://doi.org/10.3390/healthcare10122491

Pokhrel, K. N., Pokhrel, K. G., Sharma, V. D., Poudel, K. C., Neupane, S. R., Mlunde, L. B., & Jimba, M. (2019, August). Mental health disorders and substance use among people living with HIV in Nepal: Their influence on non-adherence to anti-retroviral therapy. *AIDS Care, 31*(8), 923-931 http://doi.org/10.1080/09540121.2019.1587365.

Reynolds, W.M. (1991). *Adult Suicidal Ideation Questionnaire professional manual.* Odessa, FL: Psychological Assessment Resources, Inc.

Tsegay, L., & Ayano, G. (2020). The prevalence of suicidal ideation and attempt among young people with HIV/AIDS: A systematic review and meta-analysis. *Psychiatric Quarterly, 91*, 291–1304. https://doi.org/10.1007/s11126-020-09851-1

Toska, E., Cluver, L., Orkin, M., Bains, A., Sherr, L., Berezin, M., & Gulaid, L. (2019). Screening and supporting through schools: educational experiences and needs of adolescents living with HIV in a South African cohort. *BMC Public Health, 19* (272). https://doi.org/10.1186/s12889-019-6580-0.

UNAIDS. (2018). *Better integration of mental health and HIV services needed.* https://www.unaids.org/en/resources/presscentre/featurestories/2018/october/mental-health-and-hiv-services.

UNAIDS. (2010). Young people most at risk of HIV. https://www.hivpolicy.org/Library/HPP001861.pdf

UNICEF. (2021). HIV and AIDS in adolescents. https://data.unicef.org/topic/adolescents/hiv-aids/

University Research Co. (2021). *Mental health on the move in the Philippines – Meet the lusog-isip app.* https://www.urc-chs.com/news/mental-health-on-the-move-in-the-philippines-meet-the-lusog-isip-app/

U.S. Department of Health and Human Services. (2017). *HIV and mental health.* https://aidsinfo.nih.gov/understanding-hiv-aids/fact-sheets/27/92/hiv-and-mental-health

U.S. Department of Health and Human Services. (2020). *HIV and mental health.* https://aidsinfo.nih.gov/understanding-hiv-aids/fact-sheets/27/92/hiv-and-mental-health.

Veit, C. T., & Ware, J. E. (1983). The structure of psychological distress and well-being in general populations. *Journal of Consulting and Clinical Psychology, 51*(5), 730-742. http://dx.doi.org/10.1037/0022-006X.51.5.730.

Wallach, I., & Brotman, S. (2012). Ageing with HIV/AIDS: a scoping study among people aged 50 and older living in Quebec. *Ageing and Society, 33* (7). 1212-1242. https://doi.org/10.1017/S0144686X12000529.

Wang, W., Xiao, C., Yao, X., Yang, Y., Yan, H., & Li, S. (2018). Psychosocial and suicidal ideation among people living with HIV/AIDS: A cross-sectional study in Nanjing, China. *Plos One, 13* (2). R https://doi.org/10.1371/journal.pone.0192940.

Wonde, M., Mulat, H., Birhanu, A., Biru, A., Kassew, T., & Shumet, S. (2019,) The magnitude of suicidal ideation, attempts and associated factors of HIV positive youth attending ART follow ups at St. Paul's hospital Millennium Medical College and St. Peter's specialized hospital, Addis Ababa, Ethiopia, 2018. *PLoS ONE 14*(11). https://doi.org/10.1371/journal.pone.0224371

World Health Organization. (2022). HIV/AIDS. https://www.who.int/data/gho/data/themes/hiv-aids

World Health Organization (WHO). (2018). HIV and youth. http://www.who.int/maternal_child_adolescent/topics/adolescence/hiv/en/

Youth Stop Aids (n.d.). We conducted research into mental health and young people living with HIV globally. This is what they told us. https://youthstopaids.org/mental-health/

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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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References

1. Al-Balushi S. and Khan F.R. (2017) Factors Influencing the Preference of Private Hospitals to Public Hospital in Oman. International Journal of Management, Innovation & Entrepreneurial Research EISSN: 2395-7662, Vol. 3. No. 2 pp. 67-78
2. Das, A.K., Islam, N., Billah, M., Sarker, A. (2021). Covid-19 pandemic and healthcare solid waste management strategy - A mini-review. Science of the the Total Environment Vol. 778, 146220
3. DOH Health Care Waste Management Manual 2020. 4th Edition. Wash in Health Care Facilities. https://www.washinhcf.org
4. Ghassemi, M.K. and Yussuf, R.B.M. (2016) Advantages and Disadvantages of healthcare waste treatment and disposal alternatives: Malaysian Scenario. Pol. J. Environ. Stud. Vol 25, No. 1 17-25
5. Hossain, M.S., Santhanam, A., Norulaina NAN, et al. (2011) Clinical solid waste management practices and its impact on human health and environment-A review. Waste Management 31: 754-766.
6. Mededdu, M., Pulina M., and Scuderi, R. (2020). Public and private healthcare services: What drives the choice? Socio-Economic Planning Sciences, Vol. 70
7. Patwary, M.A., O'Hare, W.T., Sarjer, M.H. (2011) Assessment of occupational and environmental safety associated with medical waste disposal in developing countries: A qualitative approach. Safety Science 49:1200-1207
8. Rao, P.H**.** (2008). Waste management & research: The journal of the international solid wastes and public cleansing association**.** Waste Management Research Journal. 2008 Jun; 26(3):297-303
9. Takele T. (2009). Lecture on solid and hazardous waste. University of Gondar in collaboration to the Ministry of Health, Ethiopia and USAID.

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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+

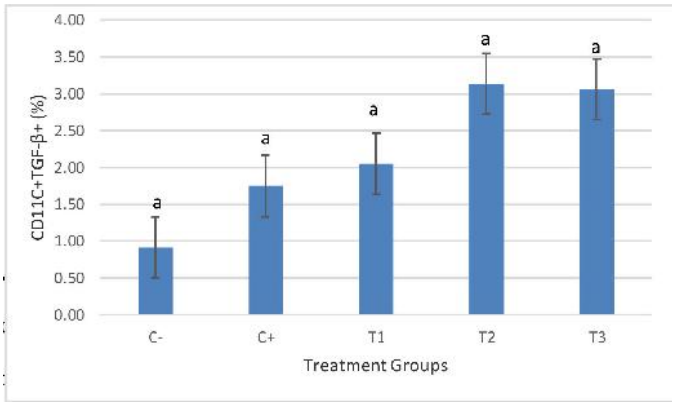

Figure 1. Flow cytometry analysis shows that giving LAB showed an increase in the relative number of CD11c+TGF-β+ cells ($p < 0.05$), but was not significantly different between treatments. There was an increase in the number when compared with the positive control. The highest average increase was seen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

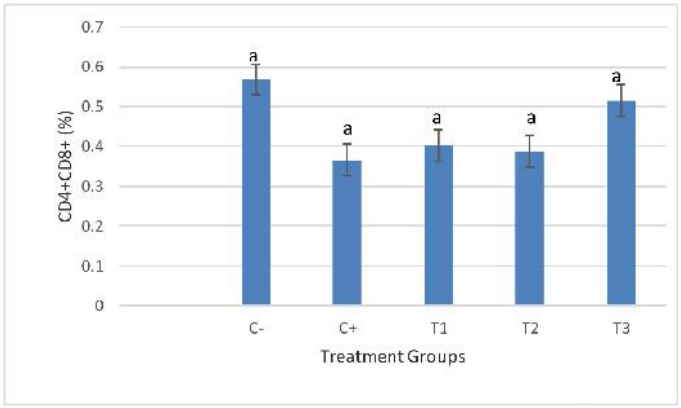

Figure 2. Treatment with LAB showed an increase in the relative number of CD4+CD8+ cells for all treatments, which did not differ significantly ($p < 0.05$). The treatment groups are: C- (healthy mice), C+ (mice induced with AFB1), and treatment groups, T1, T2, and T3 in which mice were administered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

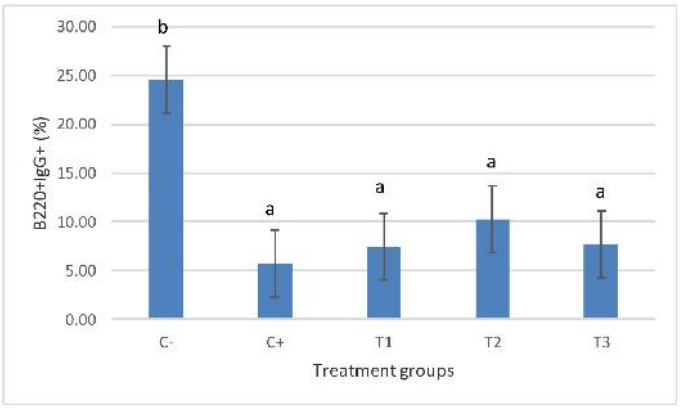

Figure 3. Treatment with LAB showed an increase in the relative number of CD4+CD8+ cells for all treatments, which did not differ significantly ($p < 0.05$). The treatment groups are: C- (healthy mice), C+ (mice induced with AFB1), and treatment groups, T1, T2, and T3 in which mice were administered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

REFERENCES

Abbès S, Salah-Abbès JB, Jebali R, Younes RB, Oueslati R. Interaction of aflatoxin B1 and fumonisin B1 in mice causes immunotoxicity and oxidative stress: Possible protective role using lactic acid bacteria. J Immunotoxicol, 2015; 13(1):46–54.

Adilah ZN, Liew WPP, Redzwan SM, Amin I. Effect of high protein diet and probiotic *Lactobacillus casei* Shirota supplementation in aflatoxin B1-induced rats. Biomed Res Int, 2018; 2018:9568351.

Ardiana O, Rifa'i M. The effect of dexamethasone treatment to humoral immunity in BALB / C mice models. J Biotropika, 2015; 3(3): 112–6.

Azad MAK, Sarker M, Wan D. Immunomodulatory effects of probiotics on cytokine profiles. Biomed Res Int, 2018; 2018:8063647.

Braat H, Van Den Brande J, Van Tol E, Hommes D, Peppelenbosch M, Van Deventer S. *Lactobacillus rhamnosus* induces peripheral hyporesponsiveness in stimulated CD4+ T cells via modulation of dendritic cell function. Am J Clin Nutr, 2004; 80(6):1618–25.

Bravo M, Combes T, Martinez FO, Cerrato R, Rey J, Garcia-Jimenez W, Fernandez-Llario P, Risco D, Gutierrez-Merino J. Lactobacilli isolated from wild boar (Sus scrofa) Antagonize Mycobacterium bovis Bacille Calmette-Guerin (BCG) in a species-dependent manner. Front Microbiol, 2019; 10:1663.

Chen CHC, Tsai YCC. Effects of multistrain lactic acid bacteria with probiotic properties on enhancements of IgA, IgG levels and anti-*Salmonella typhimurium* invasion activity. 2011; (34).

Gaudino SJ, Kumar P. Cross-talk between antigen presenting cells and t cells impacts intestinal homeostasis, bacterial infections, and tumorigenesis. Front Immunol, 2019; 10:360.

Hamidi A, Mirnejad R, Yahaghi E, Behnod V, Mirhosseini A, Amani S, Sattari S, Darian EK. The aflatoxin B1 isolating potential of two lactic acid bacteria. Asian Pac J Trop Biomed, 2013; 3(9):732–6.

Hayati M, Herman H, Rezano A. The effect of probiotic Lactobacillus casei supplementation on the secretory immunoglobulin A level in the saliva of wistar rats. Bali Med J, 2018; 7(3):727–31.

Hussain AF, Sulaiman GM, Dheeb BI, Hashim AJ, Alrahman ESA, Seddiq SH, Khashman BM. Histopathological changes and expression of transforming growth factor beta (TGF-β3) in mice exposed to gliotoxin. J King Saud Univ – Sci, 2020; 32(1):716–25.

Ibrahim AAE. Vitamin A downregulating Bcl-2 and TGF-α expression during colon cancer in AFB1-induced female rats. J Nat Sci Res, 2013; 3(5):67–84.

Møller GOD, Freire L, Rosim RE, Margalho LP, Balthazar CE, Franco LT, Sant'Ana AD, Corassin CH, Rattray FP, de Oliveira CAF. Effect of lactic acid bacteria strains on the growth and aflatoxin production potential of Aspergillus parasiticus, and their ability to bind aflatoxin B1, ochratoxin A, and zearalenone in vitro. Front Microbiol, 2021; 12:655386.

Mohamadzadeh M, Olson S, Kalina W V., Ruthel G, Demmin GL, Warfield KL, Bavari S, Klaenhammer TR. *Lactobacilli* active human dendritic cells that skew T cells toward T helper 1 polarization. Proc Natl Acad Sci USA, 2005; 102(8):2880–5.

Mohammadi A, Mehrzad J, Mahmoudi M, Schneider M. Environmentally relevant level of aflatoxin B1 dysregulates human dendritic cells through signaling on key toll-like receptors. Int J Toxicol, 2014; 33(3):175–86.

Muñoz R, Arena M, Silva J, González S. Inhibition of mycotoxinproducing *Aspergillus nomius* VSC 23 By Lactic Acid Bacteria and *Saccharomyces cerevisiae*. Brazilian J Microbiol, 2010; 41:1019–26.

Murugesan GR, Ledoux DR, Naehrer K, Berthiller F, Applegate TJ, Grenier B, Phillips TD, Schatzmayr G. Prevalence and effects of mycotoxins on poultry health and performance, and recent development in mycotoxin counteracting strategies. Poult Sci, 2015; 94(6):1298–315.

Nazarizadeh, H and Pourreza J. Evaluation of three mycotoxin binders to prevent the adverse effects of aflatoxin B1 in growing broilers. J Appl Anim Res, 2019; 47(1):135–9.

Niderkorn V, Morgavi DP, Aboab B, Lemaire M, Boudra H. Cell wall component and mycotoxin moieties involved in the binding of fumonisin B1 and B2 by lactic acid bacteria. J Appl Microbiol, 2009; 106(3):977–85.

Oswald IP, Marin DE, Bouhet S, Pinton P, Taranu I, Accensi F. Immunotoxicological risk of mycotoxins for domestic animals. Food Addit Contam, 2005; 22(4):354–60.

Perczak A, Goliński P, Bryła M, Waśkiewicz A. The efficiency of lactic acid bacteria against pathogenic fungi and mycotoxins. Arh Hig Rada Toksikol, 2018; 69(1):32–45.

Perdigón G, Fuller R, Raya R. Lactic acid bacteria and their effect on the immune system. Curr Issues Intest Microbiol, 2001; 2(1):27–42.

Pierron A, Alassane-Kpembi I, Oswald IP. Impact of mycotoxin on immune response and consequences for pig health. Anim Nutr, 2016; 2(2):63–8.

Qian G, Tang L, Wang F, Guo X, Massey ME, Williams JH, Philips TD, Wang J. Physiologically based toxicokinetics of serum aflatoxin B1-lysine adduct in F344 rats. Toxicology, 2013; 303:147–51.

Rohmawati E, Rifa'i M. Ethanol extracts of propolis (Eep) against lymphocyte activation cells in healthy mice (*Mus Musculus*) Balb / C. J Biotropika, 2014; 2(4):203.

Sadiq FA, Yan B, Tian F, Zhao J, Zhang H, Chen W. Lactic acid bacteria as antifungal and anti-mycotoxigenic agents: a comprehensive review. Compr Rev Food Sci Food Saf, 2019; 18(5):1403–36.

Tabari G, Kermanshahi H, Golian A, Heravi R. In vitro binding potentials of bentonite, yeast cell wall and lactic acid bacteria for aflatoxin B1 and ochratoxin A. Iran J Toxicol, 2018; 12(2):7–13.

Tomková I, Ševčíková Z, Levkut M, Revajová V, Čonková E, Laciaková A, Lenhardt L. Effect of aflatoxin B1 on CD3 T cells and alkaline phosphatase in the intestine of mice. Mycopathologia, 2002; 154(1):15–9.

Tran VN, Viktorová J, Rum T. Mycotoxins: biotransformation and bioavailability assessment using Caco-2 cell monolayer. Toxins, 2020; 12(10):628.

Vinderola G, Ritieni A. Role of probiotics against mycotoxins and their deleterious effects. J Food Res, 2014; 4(1):10.

Wells JM. Immunomodulatory mechanisms of lactobacilli. Microb Cell Fact, 2011; 10(Suppl. 1):1–15.

Wold AE. Immune effects of probiotics. Scand J Nutr, 2001; 45(2):76–85.

Zain ME. Impact of mycotoxins on humans and animals. J Saudi Chem Soc, 2011; 15(2):129–44.

Zimmermann C, Machado A, Cadoná F, Jaques J, Schlemmer K, Lautert C, Da cruz I, Zanette RA, Leal DBR, Santurio J. In-vitro cytotoxicity of aflatoxin B1 to broiler lymphocytes of broiler chickens. Brazilian J Poult Sci, 2014; 16(3):307–12.

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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].

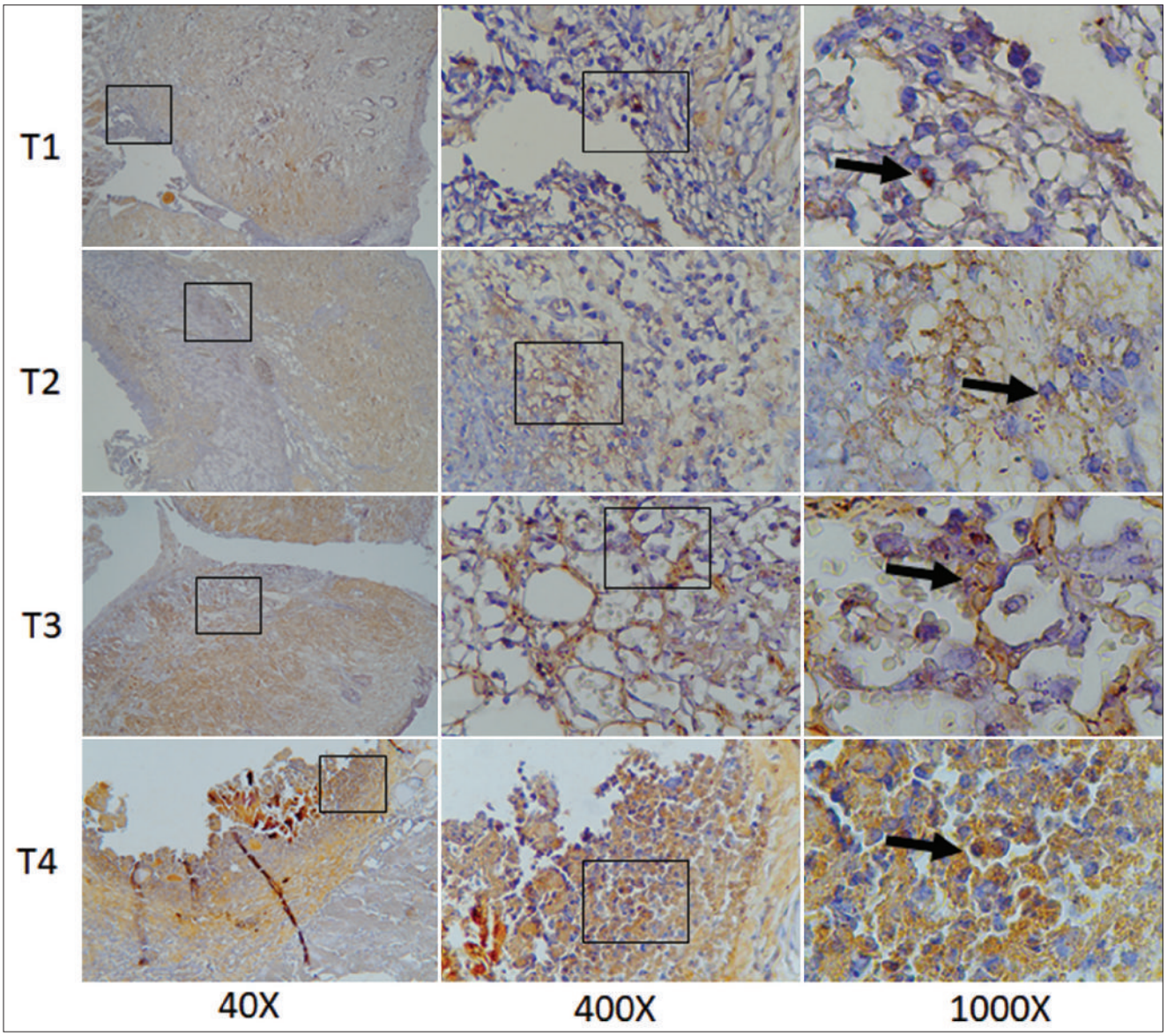

Figure 1: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against transforming growth factor-β1. (T1) positive control treated with a commercial wound healing agent; (T2) negative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

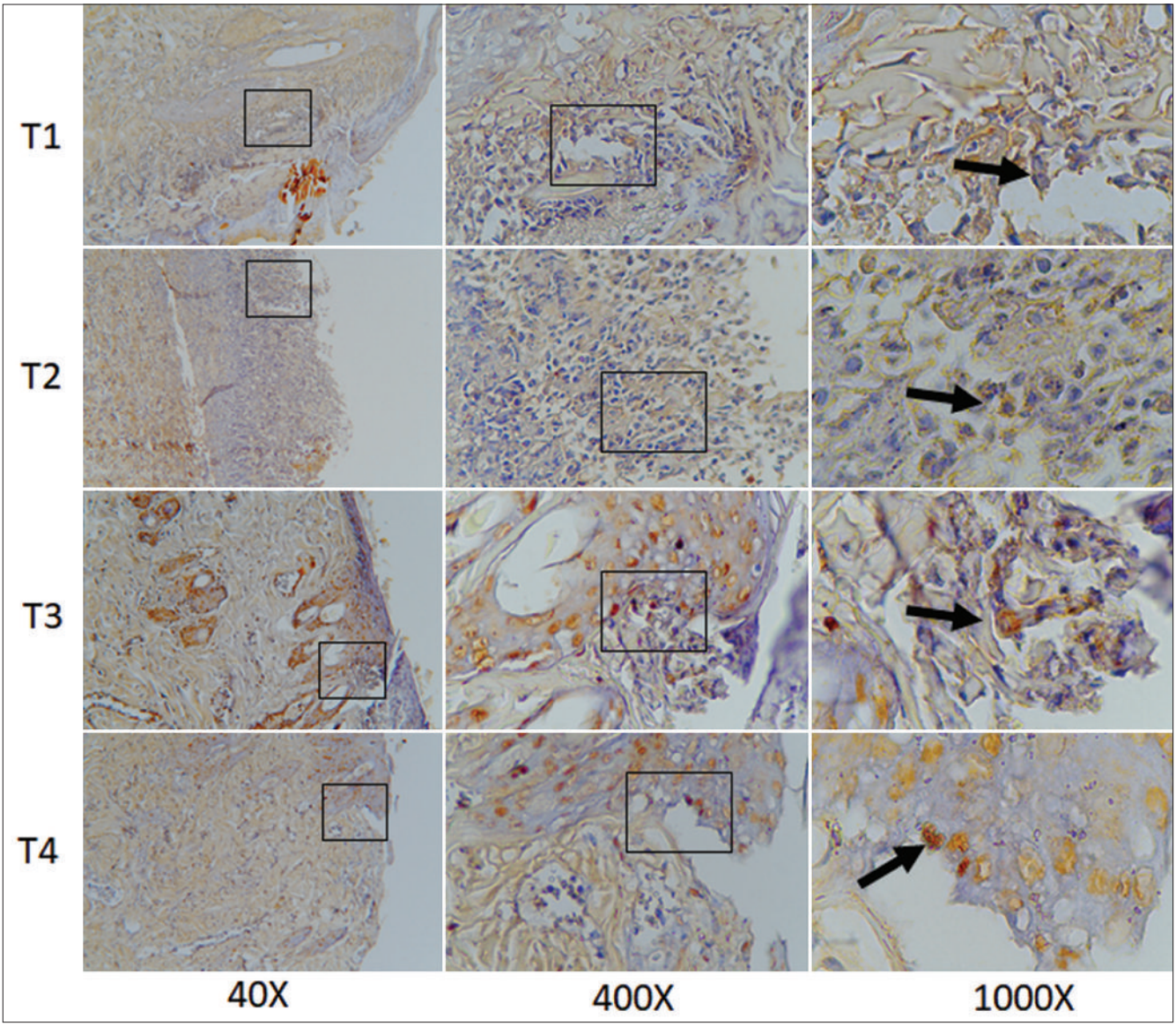

Figure 2: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against Smad3. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated with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

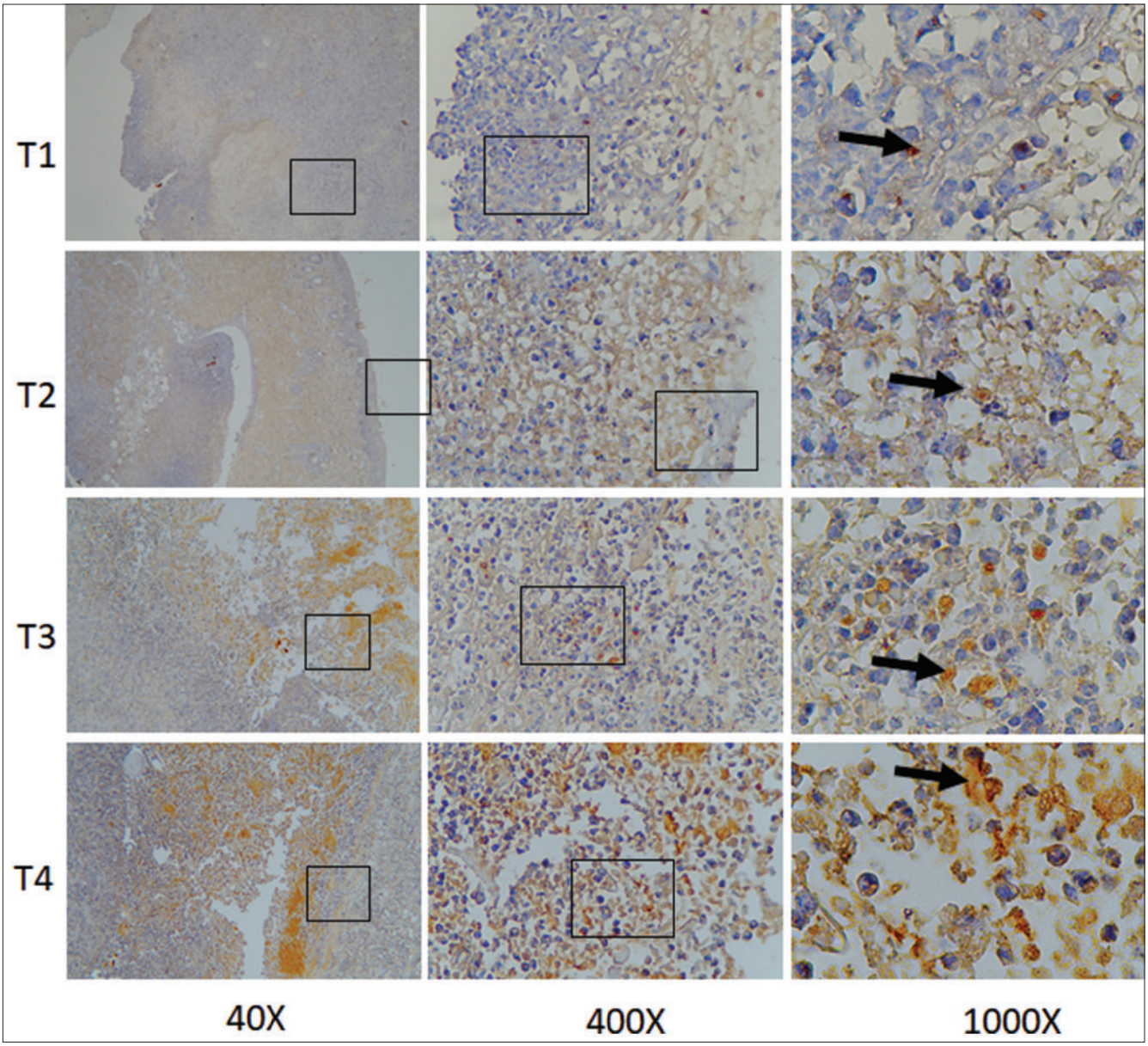

Figure 3: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against β-catenin. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated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

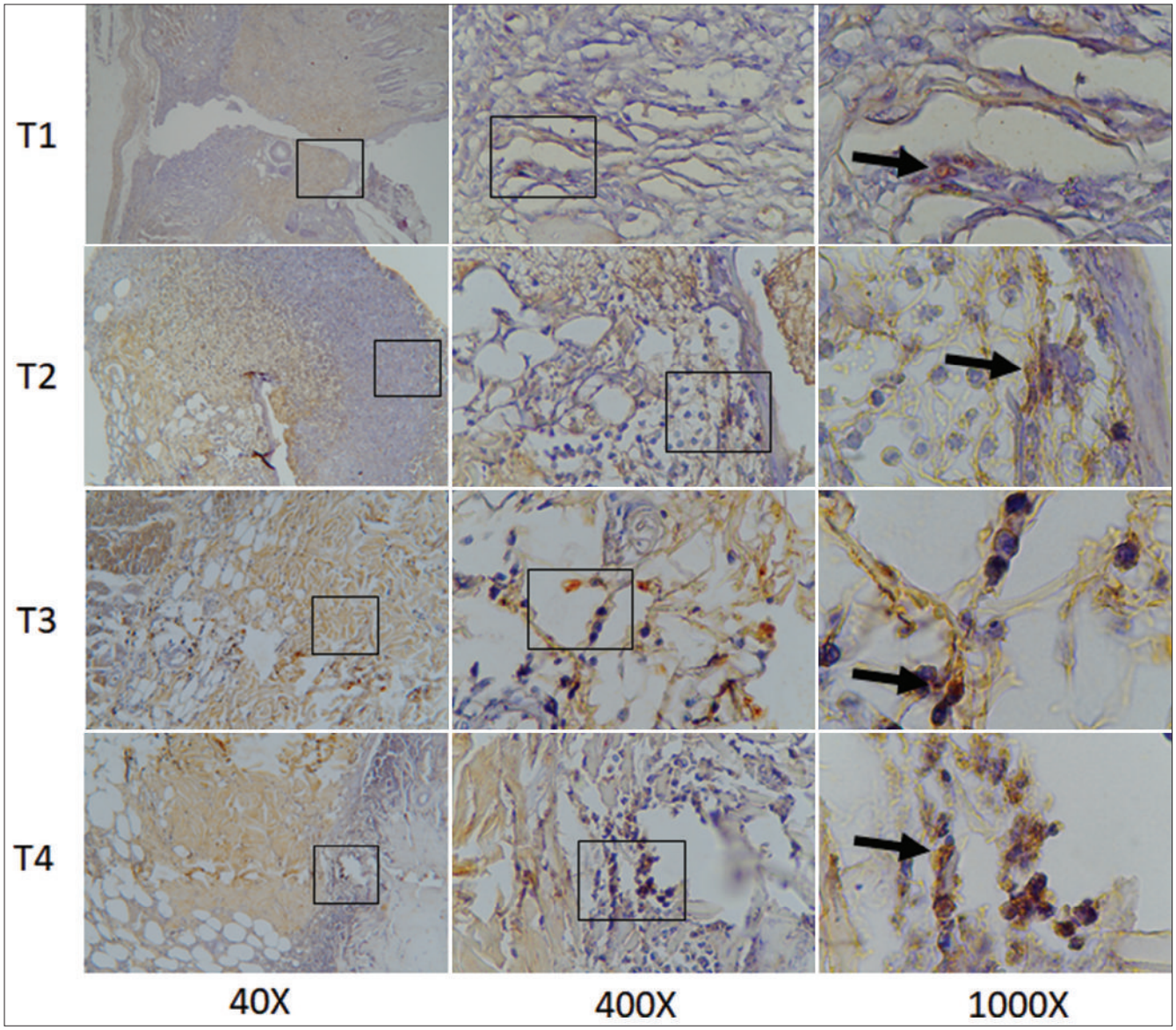

Figure 4: Histological sections of the wound on the 3rd day after wounding in rats were stained either by immunohistochemistry or counterstained with Haematoxylin with antibodies against LGR-6. (T1) positive control treated with a commercial wound healing agent; (T2) negative control; (T3) treated with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

References

1. Gonzalez AC, Costa TF, Andrade ZA, Medrado AR. Wound healing – A literature review. An Bras Dermatol. 2016;91(5):614-20. https://doi.org/10.1590/abd1806-4841.20164741
   PMid:27828635
2. Coalson E, Bishop E, Liu W, Feng Y, Spezia M, Liu B, *et al*. Stem cell therapy for chronic skin wounds in the era of personalized medicine: From bench to bedside. Genes Dis. 2019;6(4):342-58. https://doi.org/10.1016/j.gendis.2019.09.008
   PMid:31832514
3. Cañedo-Dorantes L, Cañedo-Ayala M. Skin Acute wound healing: A comprehensive review. Int J Inflam. 2019;2019:3706315. https://doi.org/10.1155/2019/3706315
   PMid:31275545
4. Rodrigues M, Kosaric N, Bonham CA, Gurtner GC. Wound healing: A cellular perspective. Physiol Rev. 2019;99(1):665-706. https://doi.org/10.1152/physrev.00067.2017
   PMid:30475656
5. Sorg H, Tilkorn DJ, Hager S, Hauser J, Mirastschijski U. Skin wound healing: An update on the current knowledge and concepts. Eur Surg Res. 2017;58(1-2):81-94. https://doi.org/10.1159/000454919
   PMid:27974711
6. Teng M, Huang Y, Zhang H. Application of stems cells in wound healing – An update. Wound Repair Regen. 2014;22(2):151-60. https://doi.org/10.1111/wrr.12152
   PMid:24635168
7. Watt FM, Collins CA. Role of beta-catenin in epidermal stem cell expansion, lineage selection, and cancer. Cold Spring Harb Symp Quant Biol. 2008;73:503-12. https://doi.org/10.1101/sqb.2008.73.011
   PMid:19022747
8. Sakaki-Yumoto M, Katsuno Y, Derynck R. TGF-β family signaling in stem cells. Biochim Biophys Acta. 2013;1830(2):2280-96. https://doi.org/10.1016/j.bbagen.2012.08.008
   PMid:22959078
9. Shi X, DiRenzo D, Guo LW, Franco SR, Wang B, Seedial S, *et al*. TGF-β/Smad3 stimulates stem cell/developmental gene expression and vascular smooth muscle cell de-differentiation. PLoS One. 2014;9(4):e93995. https://doi.org/10.1371/journal.pone.0093995
   PMid:24718260
10. Ojeh N, Pastar I, Tomic-Canic M, Stojadinovic O. Stem cells in skin regeneration, wound healing, and their clinical applications. Int J Mol Sci. 2015;16(10):25476-501. https://doi.org/10.3390/ijms161025476
    PMid:26512657
11. Lichtenberger B, Mastrogiannaki M, Watt F. Epidermal β-catenin activation remodels the dermis via paracrine signalling to distinct fibroblast lineages. Nat Commun 2016;7:10537. https://

doi.org/10.1038/ncomms10537
PMid:26837596

12. Wang PH, Huang BS, Horng HC, Yeh CC, Chen YJ. Wound healing. J Chin Med Assoc. 2018;81(2):94-101. https://doi.org/10.1016/J.JCMA.2017.11.002
PMid:29169897
13. Yang R, Liu F, Wang J, Chen X, Xie J, Xiong K. Epidermal stem cells in wound healing and their clinical applications. Stem Cell Res Ther. 2019;10(1):229. https://doi.org/10.1186/s13287-019-1312-z
PMid:31358069
14. Park SR, Lee JH, Kim PH. Smad3 and Smad4 mediate transforming growth factor-beta1-induced IgA expression in murine B lymphocytes. Eur J Immunol. 2001;31(6):1706-15. https://doi.org/10.1002/1521-4141(200106)31:6<1706:aid-immu1706>3.0.co;2-z
PMid:11385614
15. Jian H, Shen X, Liu I, Semenov M, He X, Wang XF. Smad3-dependent nuclear translocation of beta-catenin is required for TGF-beta1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells. Genes Dev. 2006;20(6):666-74. https://doi.org/10.1101/gad.1388806
PMid:16543220
16. Penn JW, Grobbelaar AO, Rolfe KJ. The role of the TGF-β family in wound healing, burns and scarring: A review. Int J Burns Trauma. 2012;2(1):18-28. PMid:3415964
17. Walton KL, Johnson KE, Harrison CA. Targeting TGF-β mediated SMAD signaling for the prevention of fibrosis. Front Pharmacol. 2017;8:461. https://doi.org/10.3389/fphar.2017.00461
PMid:28769795
18. Cheon SS, Wei Q, Gurung A, Youn A, Bright T, Poon R, *et al*. Beta-catenin regulates wound size and mediates the effect of TGF-beta in cutaneous healing. FASEB J. 2006;20(6):692-701. https://doi.org/10.1096/fj.05-4759com
PMid:16581977
19. Liao XH, Nguyen H. Epidermal expression of Lgr6 is dependent on nerve endings and Schwann cells. Exp Dermatol. 2014;23(3):195-8. https://doi.org/10.1111/exd.12340
PMid:24499442
20. Whyte JL, Smith AA, Liu B, Manzano WR, Evans ND, Dhamdhere GR, *et al*. Augmenting endogenous Wnt signaling improves skin wound healing. PLoS One. 2013;8(10):e76883. https://doi.org/10.1371/journal.pone.0076883
PMid:24204695
21. Oyebode O, Kandala NB, Chilton PJ, Lilford RJ. Use of traditional medicine in middle-income countries: A WHO-SAGE study. Health Policy Plan. 2016;31(8):984-91. https://doi.org/10.1093/heapol/czw022
PMid:27033366
22. Ang AM, Enot MM, Baltazar GJ, Alinapon CV, Buncales EO, Barbosa GB. Antioxidant and cytotoxic activity of the leaf ethanolic extracts of *Tithonia diversifolia* and *Gliricidia sepium* from Bukidnon, Philippines. AJBLS. 2019;8(1):8-15. https://doi.org/10.5530/ajbls.2019.8.2
23. Molina-Botero IC, Montoya-Flores MD, Zavala-Escalante LM, Barahona-Rosales R, Arango J, Ku-Vera JC. Effects of long-term diet supplementation with *Gliricidia sepium* foliage mixed with *Enterolobium cyclocarpum* pods on enteric methane, apparent digestibility, and rumen microbial population in crossbred heifers1. J Anim Sci. 2019;97(4):1619-33. https://doi.org/10.1093/jas/skz067
PMid:30785622
24. Aulanni'am A, Ora KM, Ariandini NA, Wuragil DK, Permata FS, Riawan W, *et al*. Wound healing properties of *Gliricidia sepium* leaves from Indonesia and the Philippines in rats (*Rattus norvegicus*). Vet World. 2021;14(3):820-4. https://doi.org/10.14202/vetworld.2021.820-824
PMid:33935433
25. Carandang RR, Buemio KC, Lopez A. The wound healing action of kakawati gel from *Gliricidia sepium* (Jacques) Steudel (Family *Fabaceae*). IJPTP. 2015;6(4):2642-9.
26. Kim SW, Roh J, Park CS. Immunohistochemistry for pathologists: Protocols, pitfalls, and tips. J Pathol Transl Med. 2016;50(6):411-8. https://doi.org/10.4132/jptm.2016.08.08
PMid:27809448
27. Blanpain C, Fuchs E. Epidermal homeostasis: A balancing act of stem cells in the skin. Nat Rev Mol Cell Biol. 2009;10(3):207-17. https://doi.org/10.1038/nrm2636
PMid:19209183
28. Han G, Li F, Singh TP, Wolf P, Wang XJ. The pro-inflammatory role of TGFβ1: A paradox? Int J Biol Sci. 2012;8(2):228-35. https://doi.org/10.1006/excr.2000.493010.7150/ijbs.8.228
PMid:22253566
29. Ferrari G, Cook BD, Terushkin V, Pintucci G, Mignatti P. Transforming growth factor-beta 1 (TGF-beta1) induces angiogenesis through vascular endothelial growth factor (VEGF)-mediated apoptosis. J Cell Physiol. 2009;219(2):449-58. https://doi.org/10.1002/jcp.21706
PMid:19180561
30. Ghosh D, McGrail DJ, Dawson MR. TGF-β1 Pretreatment improves the function of mesenchymal stem cells in the wound bed. Front Dev Biol. 2017;5:28. https://doi.org/10.3389/fcell.2017.00028
PMid:28421182
31. Mori Y, Chen SJ, Varga J. Modulation of endogenous Smad expression in normal skin fibroblasts by transforming growth factor-beta. Exp Cell Res. 2000;258(2):374-83. https://doi.org/10.1006/excr.2000.4930
PMid:10896788
32. Owens P, Han G, Li AG, Wang XJ. The role of Smads in skin development. J Invest Dermatol. 2008;128(4):783-90. https://doi.org/10.1038/sj.jid.5700969
PMid:18337711
33. Nakerakanti S, Trojanowska M. The role of TGF-β receptors in fibrosis. Open Rheumatol J. 2012;6:156-62. https://doi.org/10.2174/1874312901206010156
PMid:22802914
34. Li Y, Zhang J, Yue J, Gou X, Wu X. Epidermal stem cells in skin wound healing. Adv Wound Care (New Rochelle). 2017;6(9):297-307. https://doi.org/10.1089/wound.2017.0728
PMid:28894637
35. Ku AT, Shaver TM, Rao AS, Howard JM, Rodriguez CN, Miao Q, *et al*. TCF7L1 promotes skin tumorigenesis independently of β-catenin through induction of LCN2. Elife. 2017;6:e23242. https://doi.org/10.7554/eLife.23242
PMid:28467300
36. Kretzschmar K, Weber C, Driskell RR, Calonje E, Watt FM. Compartmentalized epidermal activation of β-catenin differentially affects lineage reprogramming and underlies tumor heterogeneity. Cell Rep. 2016;14(2):269-81. https://doi.org/10.1016/j.celrep.2015.12.04
PMid:26771241
37. Romero N, Areche C, Cubides-Cárdenas J, Escobar N, García-Beltrán O, Simirgiotis MJ, *et al*. *In vitro* anthelmintic evaluation of *Gliricidia sepium*, *Leucaena leucocephala*, and *Pithecellobium dulce*: Fingerprint analysis of extracts by UHPLC-orbitrap mass spectrometry. Molecules. 2020;25(13):3002. https://doi.org/10.3390/molecules25133002
PMid:32630065

38. Zhang Y, Guo L, Lu X, Cheng C, Sun S, Li W, *et al*. Characterization of Lgr6+ cells as an enriched population of hair cell progenitors compared to Lgr5+ cells for hair cell generation in the neonatal mouse cochlea. Front Mol Neurosci. 2018;11:147. https://doi.org/10.3389/fnmol.2018.00147
PMid:29867341

39. Kim JY, Suh W. Stem cell therapy for dermal wound healing. Int J Stem Cells. 2010;3(1):29-31. https://doi.org/10.15283/ijsc.2010.3.1.29
PMid:24855538

40. Saputro ID, Rizaliyana S, Noverta DA. The effect of allogenic freeze-dried platelet-rich plasma in increasing the number of fibroblasts and neovascularization in wound healing. Ann Med Surg. 2022;73:103217. https://doi.org/10.1016/j.amsu.2021.103217
PMid:35079361

41. Husen SA, Syadzha MF, Setyawan MF, Pudjiastuti P, Ansori AN, Susilo RJ, *et al*. Evaluation of the combination of *Sargassum duplicatum*, *Sargassum ilicifolium*, *Abelmoschus esculentus*, and *Garcinia mangostana* extracts for open wound healing in diabetic mice. Syst Rev Pharm. 2020;11(9):888-92. https://doi.org/10.31838/srp.2020.9.129

42. Husen SA, Setyawan MF, Syadzha MF, Susilo RJ, Hayaza S, Ansori AN, *et al*. A novel therapeutic effects of *Sargassum ilicifolium* alginate and okra (*Abelmoschus esculentus*) pods extracts on open wound healing process in diabetic mice. Res J Pharm Technol. 2020;13(6):2764-70. https://doi.org/10.5958/0974-360X.2020.00491.6

43. Puspitaningrum MS, Rahmadhani D, Rizqianti Y, Ridwan RD, Ansori AN, Fadholly A, *et al*. Freeze-dried epigallocatechin-3-gallate and stem-cells from human exfoliated deciduous-teeth scaffold as the biocompatible anti-relapse material post-orthodontic treatment: A review. Biochem Cell Arch. 2020;20:2935-42.

44. Fadholly A, Ansori AN, Proboningrat A, Kusala MK, Putri N, Pertiwi VR, *et al*. An investigation on the *Euphoria longan* (Lour.) Steud Seeds in Wound Healing in *Rattus norvegicus*. Indian Vet J. 2020;97(2):26-9.

45. Budi AC, Hamid IS, Legowo D. Tekelan leaves (*Chromolaena odorata*) infusion and 10% Povidone-iodine on incision wound healing process of mice (*Mus musculus*) infected with *Staphylococcus aureus*. World Vet J. 2021;11(1):60-5. https://dx.doi.org/10.54203/scil.2021.wvj8

46. Wulandari PA, Ilmi ZN, Husen SA, Winarni D, Alamsjah MA, Awang K, *et al*. Wound healing and antioxidant evaluations of alginate from *Sargassum ilicifolium* and *Mangosteen* rind combination extracts on diabetic mice model. Appl Sci. 2021;11(10):4651. https://doi.org/10.3390/app11104651

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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	

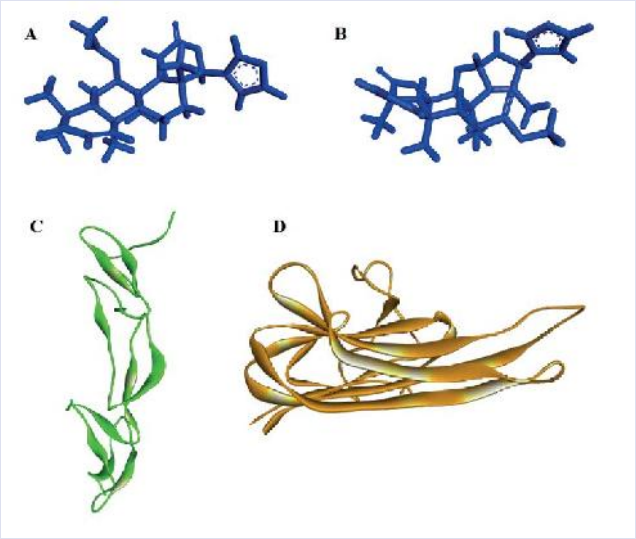

Figure 1: The chemical compound from neem: A) Gedunin (CID: 12004512); B) Nimbolide (CID: 12313376); C) death receptor 5 (DR5) (PDB ID: 1D4V); D) tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (PDB ID: 1D4V).

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]

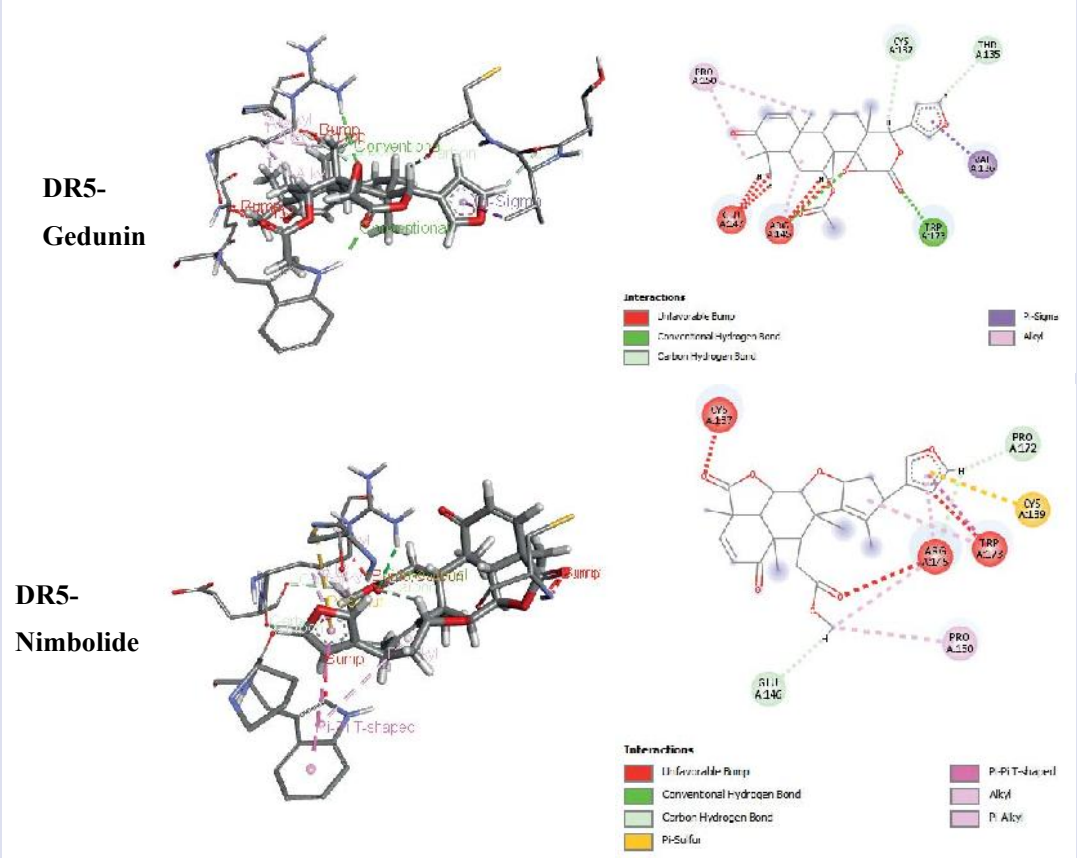

Figure 2: DR5 binding with gedunin and nimbolide in 2D diagram ligand interaction.

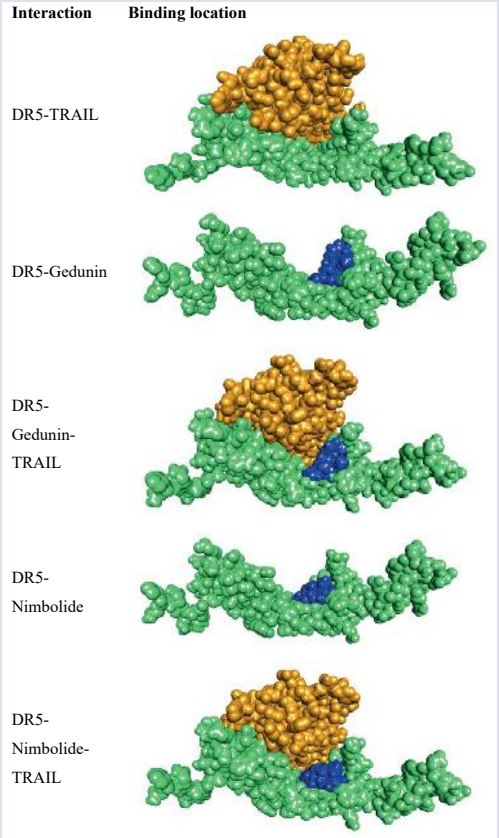

Figure 3: Binding location and interaction DR5 and ligands (DR5: green; TRAIL (native ligand): yellow; ligand: blue).

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

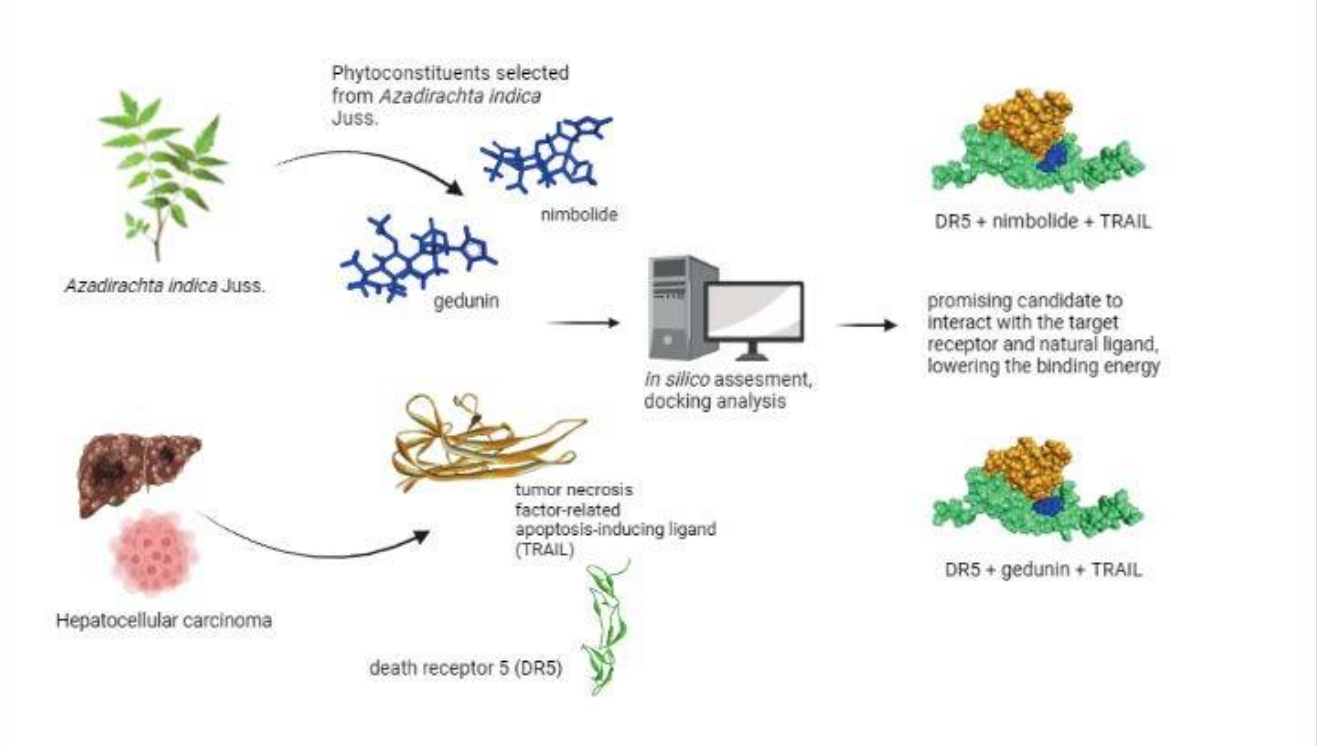
Caspases: Cysteine-aspartic proteases; CID: PubChem Compound Identifier; CRDs: Concatenated cysteine-rich domains; DD: Death domain; DISC: Death-inducing signaling complex; DR: Death receptor; DR5: Death receptor 5; HCC: Hepatocellular carcinoma; HCV: Hepatitis B or C viruses; PDB: Protein Data Bank;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

REFERENCES

1. Llovet JM, Kelley RK, Villanueva A. Hepatocellular carcinoma. Nat Rev Dis Prim. 2021;7.
2. Llovet JM, Zucman-Rossi J, Pikarsky E. Hepatocellular carcinoma. Nat Rev Dis Prim. 2016;2:1-23.
3. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. Nat Rev Cancer. 2006;6(9):674-87.
4. Mu X, Español-Suñer R, Mederacke I. Hepatocellular carcinoma originates from hepatocytes and not from the progenitor/biliary compartment. J Clin Invest. 2015;125(10):3891-903.
5. Pistritto G, Trisciuoglio D, Ceci C, Alessia Garufi, D'Orazi G. Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies. Aging (Albany NY). 2016;8(4):603.
6. Wang B, Wang L, Kothambawala T. IGM-8444 as a potent agonistic Death Receptor 5 (DR5) IgM antibody: Induction of tumor cytotoxicity, combination with chemotherapy and *in vitro* safety profile. 2020;38:3595.
7. Elrod HA, Fan S, Muller S. Analysis of Death Receptor 5 and Caspase-8 Expression in Primary and Metastatic Head and Neck Squamous Cell Carcinoma and Their Prognostic Impact. PLoS One. 2010;5(8):e12178.
8. Shlyakhtina Y, Pavet V, Gronemeyer H. Dual role of DR5 in death and survival signaling leads to TRAIL resistance in cancer cells. Cell Death Dis. 2017;8(8):e3025.
9. Trivedi R, Mishra DP. Trailing TRAIL Resistance: Novel Targets for TRAIL Sensitization in Cancer Cells. Front Oncol. 2015;5.
10. Min K, Woo SM, Shahriyar SA, Kwon TK. Elucidation for modulation of death receptor (DR) 5 to strengthen apoptotic signals in cancer cells. Arch Pharm Res. 2019;42(1):88-100.
11. Pfeffer CM, Singh ATK. Apoptosis: A Target for Anticancer Therapy. Int J Mol Sci. 2018;19(2):448.
12. Iman M, Taheri M, Bahari Z. The anti-cancer properties of neem (*Azadirachta indica*) through its antioxidant activity in the liver; its pharmaceutics and toxic dosage forms; a literature review. J Complement Integr Med. 2021.
13. Raissa R, Safitri A, Beltran MAG, Aulanni'Am A. Phytoconstituents Investigation on the Ethanolic Extract of *Azadirachta indica* var. Indonesian and Philippines. J Phys Conf Ser. 2019;1374:012018.
14. Gupta SC, Reuter S, Phromnoi K. Nimbolide Sensitizes Human Colon Cancer Cells to TRAIL through Reactive Oxygen Species- and ERK-dependent Up-regulation of Death Receptors, p53, and Bax. J Biol Chem. 2011;286(2):1134.
15. Rout KK, Mishra SK. Development of a Sensitive HPTLC Method for Quantification of Nimbolide in *Azadirachta indica* and Its Dosage Form. J Chromatogr Sci. 2014;52(9):1089-94.
16. Wang Q, Zhang L, Yuan X. The Relationship between the Bcl-2/Bax Proteins and the Mitochondria-Mediated Apoptosis Pathway in the Differentiation of Adipose-Derived Stromal Cells into Neurons. PLoS One. 2016;11(10):e0163327.
17. Spradlin JN, Hu X, Ward CC. Harnessing the anti-cancer natural product nimbolide for targeted protein degradation. Nat Chem Biol. 2019;15(7):747-55.
18. Hao F, Kumar S, Yadav N, Chandra D. Neem components as potential agents for cancer prevention and treatment. Biochim Biophys Acta. 2014;1846(1):247.
19. Bodduluru LN, Kasala ER, Thota N, Barua CC, Sistla R. Chemopreventive and therapeutic effects of nimbolide in cancer: The underlying mechanisms. Toxicol Vitr. 2014;28(5):1026-35.
20. Braga TM, Rocha L, Chung TY. Biological Activities of Gedunin-A Limonoid from the *Meliaceae* Family. Mol. 2020;25(3):493.
21. Uddin SJ, Nahar L, Shilpi JA. Gedunin, a limonoid from Xylocarpus granatum, inhibits the growth of CaCo-2 colon cancer cell line *In Vitro*. Phyther Res. 2007;21(8):757-61.
22. Kamath SG, Chen N, Xiong Y. Gedunin, a novel natural substance, inhibits ovarian cancer cell proliferation. Int J Gynecol Cancer. 2009;19(9):e15.

23. Tharmarajah L, Samarakoon SR, Ediriweera MK. *In Vitro* Anticancer Effect of Gedunin on Human Teratocarcinomal (NTERA-2) Cancer Stem-Like Cells. Biomed Res Int. 2017.
24. Subramani R, Gonzalez E, Arumugam A. Nimbolide inhibits pancreatic cancer growth and metastasis through ROS-mediated apoptosis and inhibition of epithelial-to-mesenchymal transition. Sci. 2016;6:1-12.
25. Ritchie DW, Kozakov D, Vajda S. Accelerating and focusing protein–protein docking correlations using multi-dimensional rotational FFT generating functions. Bioinformatics. 2008;24(17):1865.
26. Duan G, Ji C, Zhang JZH. Developing an effective polarizable bond method for small molecules with application to optimized molecular docking. RSC Adv. 2020;10:15530-40.
27. Lieberman J. Cytotoxic Lymphocytes. Encycl Immunobiol. 2016;3:363-73.
28. Stöhr D, Jeltsch A, Rehm M. TRAIL receptor signaling: From the basics of canonical signal transduction toward its entanglement with ER stress and the unfolded protein response. Int Rev Cell Mol Biol. 2020;351:57-99.
29. Mongkolsapaya J, Grimes JM, Chen N. Structure of the TRAIL-DR5 complex reveals mechanisms conferring specificity in apoptotic initiation. Nat Struct Biol. 1999;6(11):1048-53.
30. Tang W, Wang W, Zhang Y, Liu S, Liu Y, Zheng D. TRAIL receptor mediates inflammatory cytokine release in an NF-κB-dependent manner. Cell Res. 2009;19(6):758-67.
31. Cha SS, Sung BJ, Kim YA. Crystal Structure of TRAIL-DR5 Complex Identifies a Critical Role of the Unique Frame Insertion in Conferring Recognition Specificity. J Biol Chem. 2000;275(40):31171.
32. Mobley C, Zarrinpar A. Molecular and Cellular Basis of Liver Failure. Transplant Liver Third Ed. 2015:40-57.
33. Sahai R, Bhattacharjee A, Shukla VN. Gedunin isolated from the mangrove plant Xylocarpus granatum exerts its anti-proliferative activity in ovarian cancer cells through G2/M-phase arrest and oxidative stress-mediated intrinsic apoptosis. Apoptosis. 2020;25(7-8):481-99.
34. Pantsar T, Poso A. Binding Affinity *via* Docking: Fact and Fiction. Mol A J Synth Chem Nat Prod Chem. 2018;23(8):1899.
35. Wang S, El-Deiry WS. TRAIL and apoptosis induction by TNF-family death receptors. Oncogene. 2003;22(53):8628-33.

GRAPHICAL ABSTRACT



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

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Abstract

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

Keywords

Antimicrobial, *Bambusa blumeana*, bamboo

Introduction

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

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

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

Materials and Methods

Plant Material Preparation

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

Minimum Inhibitory Concentration Determination

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

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

Qualitative Phytochemical Testing

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

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

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

Results and Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Conclusion

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

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Compliance with ethical standards

Conflict of interest: Author do not have any conflict of interests to declare.

Ethical issues: None

References

1. Tantengco OAG, Condes MLC, Estadilla HHT, Ragragio EM. Ethnobotanical survey of medicinal plants used by Ayta communities in Dinalupihan, Bataan, Philippines. Pharmacogn J. 2018;10 (5):859-70. https://doi.org/10.5530/pj.2018.5.145
2. Kalita C, Ganguly M, Devi A. Evaluation of antioxidant capacity and antimicrobial properties of ethnic Bambuseae species and identification of the active components. International Journal of Pharmaceutical and Biological Achieves. 2016;3(1):61-71.
3. Nguyen PT, Xuan TD, Ha PT, Tu anh TT, Khanh TD. Inhibitory effects of bamboo leaf on the growth of *Pyricularia grisea* fungus. Molecular Diversity Preservation International: Agriculture [Internet]. 2018 [cited 2018 Nov 30];8(92):1-8. https://doi.org/10.3390/agriculture8070092
4. Mahunu GK, Zhang H, Tibiru M, Qiya A, Xiaoyun Y, Zhao ZL. Bamboo leaf flavonoid enhances the control effect of *Pichia caribbica* against *Penicillium expansum* growth and Patulin accumulation in apples. Postharvest Biology and Technology [Internet]. 2018 [cited 30 June 2019];141:1-7. https://doi.org/10.1016/j.postharvbio.2018.03.005
5. Wasnik DD, Tumane PM. Antibacterial activity of *Bambusa bambose* L. against multiple drug resistant (MDR) bacteria isolated from clinical specimen. International Journal of Pharmaceutical Sciences. 2014;25(1):215-18.
6. Ambika K, Rajagopal B. *In vitro* antimicrobial and antiproliferative activity of *Bambusa vulgaris*. International Journal of Pharmacy and Pharmaceutical Research. 2017;9(1):10-22.
7. Chuah EL, Zakaria ZA, Suhaili Z, Abu bakar S, Desa MNM. Antimicrobial activities of plant extracts against Methicillin – Susceptible and Methicillin – Resistant *Staphylococcus aureus*. Journal of Microbiology Research [Internet]. 2014 [cited 30 June 2018]; 4 (1):6-13. https://doi.org/10.5923/j.microbiology.20140401.02
8. Tanaka A, Zhu Q, Tan H, Horiba H, Ohnuki K, Mori Y et al. Biological activities and phytochemical profiles of extracts from different parts of Bamboo (*Phyllostachys pubescens*). Molecules [Internet]. 2014 [cited 30 June 2018];1(19):8238-60. https://doi.org/10.3390/molecules19068238
9. Selvamohan T, Ramadas V, Shibila S, Kishore S. Antimicrobial activity of selected medicinal plants against some selected human pathogenic bacteria. Pelagia Research Library Advances in Applied Science Research. 2012;3(5):3374-81.
10. Sule IO, Agbabiaka TO. Antibacterial effect of some plant extracts on selected Enterobacteriaceae. Ethnobotanical Leaflets. 2008;1 (2):1035-42.
11. Lakshmanan K, Arumungam A, Mani R. Phytochemical screening and *in – vitro* antimicrobial activity of *Vitex negundo* L. var. *purpurascens* Sivar and Mold Against Pathogenic Microorganisms. Drug Invention Today. 2012;4(12):667.
12. Balouiri M, Sadiki M, Ibnsouda SK. Methods for *In Vitro* Evaluating Antimicrobial Activity: A Review. Journal of Pharmaceutical Analysis. 2016;(6):71-79. https://doi.org/10.1016/j.jpha.2015.11.005
13. Islam MA, Alam MM, Choudhury ME, Kobayashi N, Ahmed MU. Determination of minimum inhibitory concentration (MIC) of cloxacillin for selected isolates of Methicillin- Resistant *Staphylococcus aureus* (MRSA) with their antibiogram. Bangladesh Journal of Veterinary Medicine [Internet]. 2008 [Cited 24 Nov 2019];6 (1):121-26. https://doi.org/10.3329/bjvm.v6i1.1350v
14. Scorzoni L, Benaducci T, Ameida AMF, Silva DHS, Da Silva BV, Gianinni MJSM. The use of standard methodology for the determination of antifungal activity of natural products against medical yeast *Candida* sp. and *Cryptococcus* sp. Brazillian Journal of Microbiology [Internet]. 2007 [cited 30 June 2018];38(1):391-97. https://doi.org/10.1590/S1517-83822007000300001
15. Magaldi S, Mata- essayag S, Hartung de capriles C, Perez C, Colella MT, Olaizola C et al. Well diffusion for antifungal susceptibility testing. Int J Infect Dis [Internet]. 2004 [cited 30 June 2018];1(8):39-45. https://doi.org/10.1016/j.ijid.2003.03.002
16. Lakshmanan K, Arumungam A, Mani R. Phytochemical screening and *in – vitro* antimicrobial activity of *Vitex negundo* L. var. *purpurascens* Sivar. and Mold against pathogenic microorganisms. Drug Invention Today. 2012;4(12):667.
17. Alastruey-Izquierdo A, Melhem M, Bonfietti L, Rodriguez-Tudela J. Susceptibility Test for Fungi: Clinical and Laboratorial Correlations in Medical Mycology. Rev Inst Med Trop Sao Paulo. 2015;57 (19): 57–64. https://doi.org/10.1590/s0036-46652015000700011
18. Kaur C, Kapoor HC. Anti-oxidant activity and total phenolic content of some Asian vegetables. International Journal of Food Science and Technology. 2002; (37): 153-61. https://doi.org/10.1046/j.1365-2621.2002.00552.x
19. Dewanto V, Wu X, Adom KK, Liu RH. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. Journal of Agricultural and Food Chemistry. 2002;50(10):3010-14. https://doi.org/10.1021/jf0115589
20. Gajula D, Verghese M, Boateng J, Walker LT, Shackelford L, Mentreddy SR, Cedric S. Determination of Total Phenolics, Flavonoids and Antioxidant and Chemopreventive Potential of Basil (*Ocimum basilicum* L. and *Ocimum tenuiflorum* L.). International Journal of Cancer Research. 2009;5:130-43. https://dx.doi.org/10.3923/ijcr.2009.130.143
21. Andrews JM. Determination of Minimum Inhibitory Concentrations. Journal of Antimicrobial Chemotherapy. 2001;49(6):5-16. https://doi.org/10.1093/jac/48.suppl_1.5
22. Sen P, Sahu PK, Haldar R, Kumar Sahu K, Prasad P, Roy A. Apigenin Naturally Occurring Flavonoids: Occurrence and Bioactivity. Pharmaceutical and Biosciences Journal. 2016; 4(6): 56-68. http://dx.doi.org/10.20510/ukjpb/4/i6/134666
23. Wang Q, Xie M. Antibacterial activity and mechanism of luteolin on *Staphylococcus aureus*. National Center for Biotechnology Information. 2010;50(9):1180-84.
24. Lou Z, Wang H, Ra S, Sun J, Ma C, Li J. P-Coumaric Acid Kills Bacteria through Dual Damage Mechanisms. Food Control. 2012; 25 (2): 550-54. https://doi.org/10.1016/j.foodcont.2011.11.022
25. Valentino MJG, Ganado LS, Ganado MR, Undan JR. Phytochemical screening and bio assay of the anti-microbial activity of three species of bamboo in Nueva Ecija, Philippines. Advances in Environmental Biology. 2015;9(24):389-96.
26. Austria KC, Waing KGW, Valentino MJ. Anti-oxidant and antibacterial potentials of *Bambusa blumeana* JA and JHP Schultes and *Bambusa vulgaris* Schrad. ex Wendl. International Journal of Biology, Pharmacy and Allied Sciences. 2017;6(11):2175-88.
27. Zhang JJ, Liu M, Li Y, Zhou T, Xu DP. Nutritional values and biological activities of bamboo shoots and leaves. International Journal of Food and Nutrition Safety. 2016;7(2):98-108.
28. Wangawar SN, Shendarkar GR, Shelke DP, Daswad AK, Pohare JG, Roge AB. Phytochemical screening and antimicrobial activity of *Dendrocalamus strictus* leaves extracts. World Journal of Pharmaceutical Research. 2017; 6(4):1029-41. https://doi.org/10.20959/wjpr20174-8128

29. Owolabi MS, Lajide Preliminary Phytochemical screening and antimicrobial activity of crude extracts of *Bambusa vulgaris* Schrad. ex J.C. Wendl. (Poaceae) from Southwestern Nigeria. American Journal of Essential Oils and Natural Products. 2015;3 (1):42-45.

30. Cushnie T, Lamb JA. Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents [Internet]. 2005 [cited 30 June 2018];26(5):343-56. http://dx.doi.org/10.1016/j.ijantimicag.2005.09.002

31. Wafa N, Sofiane G, Mouhamed K. The antioxidant and antimicrobial activities of flavonoids and tannins extracted from *Phlomis bovei* De Noé. European Journal of Experimental Biology. 2016;6 (3):55-61.

32. Saxena M, Nema R, Jyoti S, Dharmendra S, Abishek G. Phytochemistry of Medicinal Plants. Journal of Pharmacognosy and Phytochemistry. 2013;1(6):168-82.

33. Huang Y, Xiao D, Burton-Freeman BM, Edirisinghe I. Chemical changes of bioactive phytochemicals during thermal processing. Reference Module in Food Science. 2015. https://doi.org/10.1016/B978-0-08-100596-5.03055-9

34. Lila MA, Raskin I. Health related interactions of phytochemicals. Journal of Food Science. 2006;70(1):20-27. https://doi.org/10.1111/j.1365-2621.2005.tb09054.x

35. Efferth T, Koch E. Complex interactions between phytochemicals. The multi-target therapeutic concept of phytotherapy. Current Drug Targets. 2011;12(1):122-32. https://doi.org/10.2174/138945011793591626

36. Sultana B, Anwar F, Ashraf M. Effect of extraction solvent or technique on the antioxidant activity of selected medicinal plant extracts. Molecules. 2009;14(6):2167-80. https://doi.org/10.3390/molecules14062167

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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.

REFERENCES:

1. Al-Snafi, A.E. A review on chemical constituents and pharmacological activities of Coriandrum sativum. OSR Journal of Pharmacy. 2013; 6(7): 17-42
2. Bahadoran Z, Mirmiran P, Azizi F. Dietary polyphenols as potential nutraceuticals in management of diabetes: a review. Journal of Diabetes and Metabolic Disorders. 2013; 12:43
3. Bhat SP, Kumar WRA. Oriandrum Sativum on Pain and Inflammation. JRPC. 2014; 4(4): 939-945
4. Chizoba EF. A Comprehensive Review on Coriander and its Medicinal properties. International Journal of Scientific Research and Reviews. 2015; 4(2): 28 – 50
5. Damasceno DC, Netto AO, Iessi I., Gallego F, Corvin, SB, Dallaqua, B, Sinzato YK, Bueno A, Calderon MP, Rudge MVC. Streptozotocin-Induced Diabetes Models: Pathophysiological Mechanisms and Fetal Outcomes. Biomed Res Int. 2014; 819065.
6. Deepa B, Anuradha CV. Antioxidant Potential of *Coriandrum sativum* L Seed Extract. Indian Journal of Experimental Biology. 2011; 4930: 38
7. Frederico EHFF, Cardoso, A.L.B.D., Guimarães, C.A.S., Neves, R.F., Sá-Caputo, D.C., Moreira-Marconi, E., Dionello, C.F., Morel, D., Paineiras-Domingos, L.L., Costa-Cavalcanti, R.G., Sousa-Gonçalves, C.R., Arnóbio, A and Bernardo-Filho, M. 2016. Possible Benefits of the Coriandrum sativum in the Management of Diabetes in Animal Model: A Systematic Review Herb Med
8. Furman BL. Streptozotocin-Induced Diabetic Models in Mice and Rats. Current Protocols in Pharmacology. 2015; 5.47.1-5.47.20
9. Gomes KB. IL-6 and type 1 diabetes mellitus: T cell responses and increase in IL-6 receptor surface expression. Ann Transl Med. 2017; 5(1): 16

10. Güler T, Ertaş ON, Çiftç M, Dalkılıç B. The effect of coriander seed (Coriandrum sativum L.) as diet ingredient on the performance of Japanese quail. South African Journal of Animal Science. 2005; 35 (4)
11. Gvazava IG, Rogovaya OS, Borisov M., Vorotelyak EA, Vasiliev AV. Pathogenesis of Type 1 Diabetes Mellitus and Rodent Experimental Models. Acta Naturae. 2018; 10(1): 24–33.
12. Hackett E, Jacques N. Tipe 2 Diabetes Pathophysiology and Clinical Features. Clinical Pharmacist. 2009; 1
13. Hossain K., Dayem AA, Han J, Yin Y, Kim K, Saha SK, Gwang-Mo Y, Choi, HY, Ssang-Goo Cho. Molecular Mechanisms of the Anti-Obesity and Anti-Diabetic Properties of Flavonoids. International Journal of Molecular Sciences. 2016; 17, 569
14. Hajiaghaalipour MF, Khalilpourfarshbafi M, Arya A. Modulation of Glucose Transporter Protein by Dietary Flavonoids in Type 2 Diabetes. International Journal of Biological Sciences. 2015; 11(5): 508-524.
15. Kakoolaa DN, Curcio-Brinta A, Lenchika N, Gerling IC. Molecular pathway alterations in CD4 T-cell of non obesediabetic (NOD) mice in the pre insulitis phase of autoimmune diabetes. Results in Immunology. 2014; 4. 30-45
16. Kawata A, Yukio M, Seiji S and Seiichiro F. Anti-inflammatory Activity of β-Carotene, Lycopene and Tri-n-butylborane, a Scavenger of Reactive Oxygen Species. In vivo. 2018; 32: 255-264
17. Nair V, Singh S, Gupta YK. Evaluation of disease modifying activity of Coriandrum sativum in experimental models. Indian J Med Res. 2012; 135(2): 240–245.
18. Qosimah D, Dhita EA, Ma AGB, Aulanni'am A. Diabetes sepsis on Wistar rat strain (Rattus norvegicus) induced by streptozotocin and bacteria *Staphylococcus aureus*. Veterinary World. 2019; 12(6): 849-854.
19. Rachmawati I, M. Rifa'i. In Vitro Immunomodulatory Activity of Aqueous Extract of Moringa oleifera Lam. Leaf to the CD4 +, CD8+ and B220+ Cells in Mus musculus. J. Exp. Life Sci. 2014; 4:1
20. Schaschkow A, Mura C, Dal S, Langlois A, Seyfritz E, Sookhareea C, Bietiger W., Peronet C, Jeandidier N, Pinget M, Sigrist S, Maillard E. Impact of the Type of Continuous Insulin Administration on Metabolism in a Diabetic Rat Model. Journal of Diabetes Research. 2016; Article ID 8310516, 10 pages.
21. Sharma B, Joshi SC, Jasuja ND, Singh SK, Gaurav S. Evaluation of Anti-Diabetic and Hepatoprotectivity of *Coriandrum Sativum* In Alloxan Induced Experimental Animals: A Histopathology Study. International Journal of Pharmaceutical Sciences And Research. 2016; 7(11): 4510-4515
22. Sogara PPU, Fatimawali, Bodhi W. Pengaruh Ekstrak Etanol Buah Ketumbar (*Coriandrum sativum* L.) Terhadap Penurunan Kadar Gula Darah Tikus Putih Yang Diinduksi Aloksan. Pharmacon Jurnal Ilmiah Farmasi – UNSRAT. 2014; 3: 3
23. Tai N, Wong FS, Wen L. The role of gut microbiota in the development of type 1, obesity and type 2 diabetes mellitus. Rev Endocr Metab Disord. 2015; 16(1): 55–65
24. Tian HL, Xu ZV, Wei LS, Zhao RT. Correlations between blood glucose level and diabetes signs in streptozotocin-induced diabetic mice. Global Journal of Pharmacology. 2010; 4(3): 111-116
25. Vinayagam R, Xu B. Antidiabetic properties of dietary flavonoids: a cellular mechanism review. Nutr Metab (Lond). 2015; 12: 60.
26. Xia C, Rao X, Zhong J. Role of T Lymphocytes in Type 2 Diabetes and Diabetes-Associated Inflammation. J Diabetes Res. 2017: 6494795.
27. Wangchuk P, Apte SH, Smout MJ, Groves PL, Loukas A, Doolan DL. Defined Small Molecules Produced by Himalayan Medicinal Plants Display Immunomodulatory Properties. Int J Mol Sci. 2018; 19(11): 3490.
28. Wang-Fische Y and Tina G. Improving the Reliability and Utility of Streptozotocin-Induced Rat Diabetic Model. Research Article. Journal of Diabetes Research. 2018, Article ID 8054073, 14 pages
29. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care. 2004; 27(5):1047-53.

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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

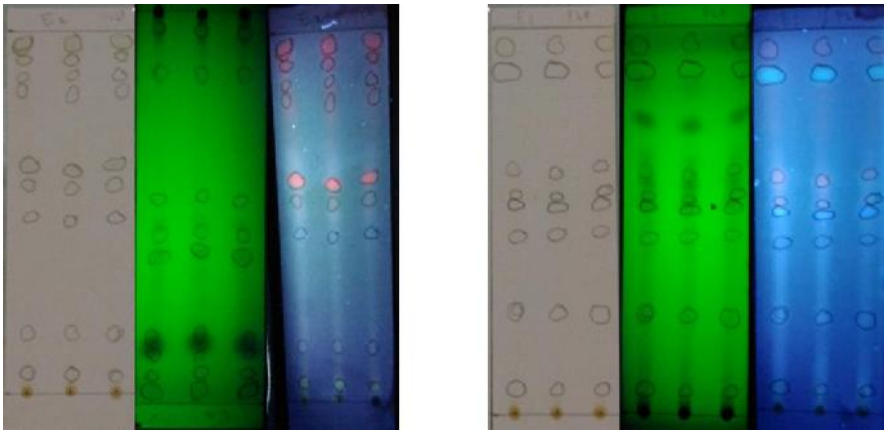
Thin-layer chromatography separation was a separation process based on the distribution of active compounds in two phases, the mobile phase (consist of a mixture of solutions that have a similar polarity with the compound to be separated) and the stationary phase was a silica gel coated plate (usually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

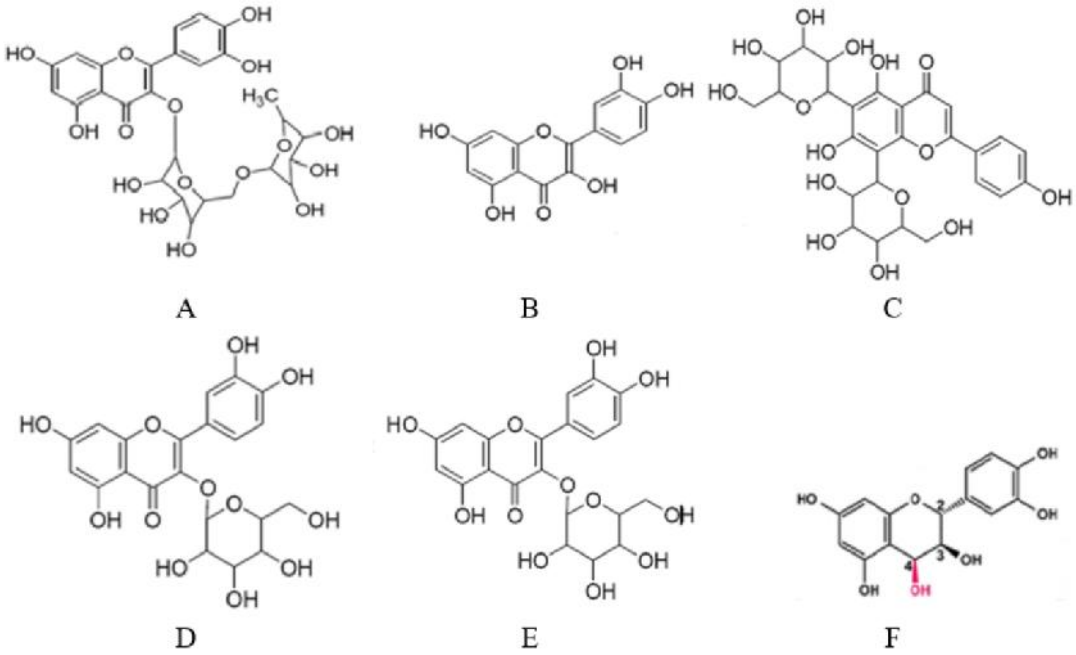


Figure 2. Structure of Flavonoid Compound. (A) Rutin, (B) Quercetin, (C) Vicenin-2, (D) Quercetin-3-O-glucoside, (E) Quercetin-O-O-galloyl hexoside, (F) Leucocyanidin-(+)2,3-3,4-cis-3,4,5,7,3',4'-hexahydroxyl flavan.

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.

References

[1] Katkar K V, Suthar A C and Chauhan V S 2010 The chemistry, pharmacologic, and therapeutic applications of P. longifolia *Pharmacognosy Reviews* **4** (7) 62-68
[2] Annan K, Ekuadzi E, Asare C, Sarpong K, Pistorius D, Oberer L, Gyan B A and Ofori M 2015 *Phytochemistry Letters* **11** 28-31
[3] Vezza T, Rodriguez-Nogales A, Algieri F, Utrilla M P, Cabezaz M E R and Galvez J 2016 Flavonoid in Inflammatory Bowel Disease : A Review *Nutrients* **8** 1-22
[4] Ghosh A, Dash B K, Chatterjee S K and Chandra G 2008 Antibacterials Potentiality and Phytochemichal Analysis of Mature Leaves of Polyalthia longifolia (Magnoliales : Annonaceae) *South Pac. J. Nat. Sci.* **26** 68-72
[5] Sampath M and Vasanthi M 2013 Isolation, Structural Elucidation Of Flavonoids From Polyalthia Longifolia (Sonn.) Thawaites And Evaluation Of Antibacterial, Antioxidant And Anticancer Potential *Int J Pharm Pharm Sci* **5** (1) 336-41
[6] Agrawal S and Misra K 1979 Proantocyanidin From Polyalthia longifolia Stem Bark *Current Science* **48** 141-143
[7] Doshi G M, Nalawade V V, Mukadam A S, Chaskar P K, Zine S P and Somani R R 2016 Elucidation of Flavonoids from Carissa congesta, Polyalthia longifolia, and Benincasa hispida Plant Extracts by Hyphenated Technique of Liquid Chromatography-mass Spectroscopy *Phcog. Res.* **8** 281-286
[8] Harborne J B 1987 *Metode Fitokimia Cara Modern Menganalisis Tumbuhan* Terjemahan Kosasih Padmawinata dan Iwang Soediro (Bandung: Penerbit ITB)

[9] Wagner H and Bladt S 1996 *Plant Drug Analysis:A Thin Layer Chromatography Atlas, Second Edition* pp 359, 362, 364 (New York: Springer)
[10] Gaurav, Doshi M and Une H D 2015 High Performance Thin Layer Chromatography and High Performance Liquid Chromatography determination of Quercetin from Polyalthia longifolia leaves *Free Radicals and Antioxidants* **2**

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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.

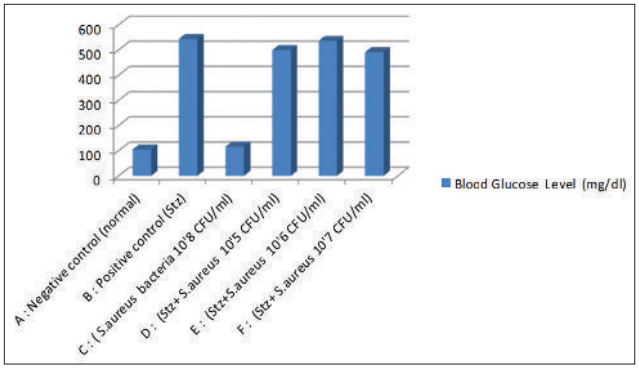

Figure-1: Blood glucose level.

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

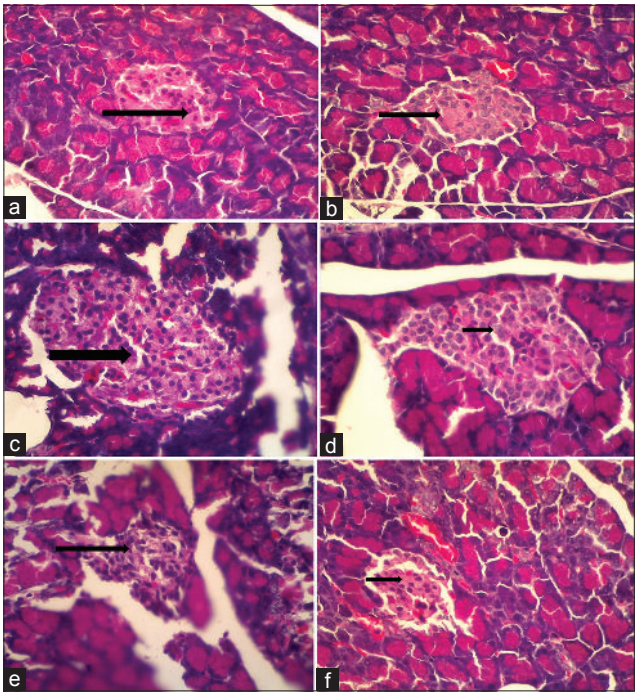

Figure-2: Images with magnifications 400×: Negative control (a) showing pancreatic beta-cell nucleus appears clear and spread evenly (arrow). Positive control diabetes (b) showed that the pancreatic beta-cell nucleus seemed to shrink even invisible and only seen cytoplasm (arrow). Positive control bacteria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

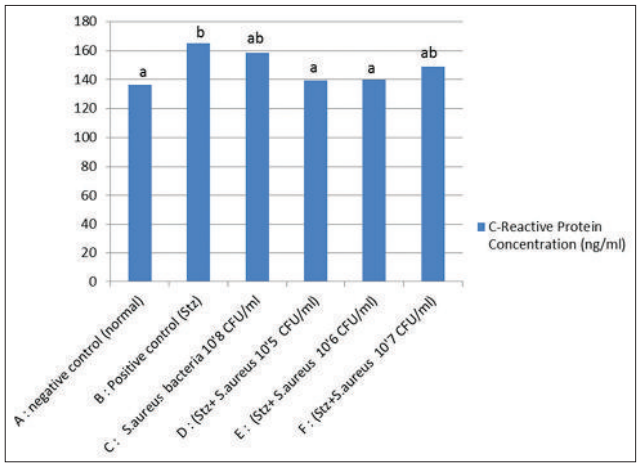

Figure-3: Level of C-reactive protein. Superscript letters are significantly different from one another based on analysis of variance with honestly significant difference tests ($p<0.05$).

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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References

1. Fox-Robichaud, A., Mai, S., Khan, M. and Patricia, L. (2012) Experimental Sepsis Model. Available from: http://www.intechopen.com/books/sepsis-an-ongoing-andsignificant- Challenge. Last accessed on 09-12-2018.
2. Osuchowski, M.F, Florin, L.C., Elizabeth, S., Corneliu, S., Robert, S. and Daniel, G.R. (2010) Untreated Type 1 diabetes increases sepsis-induced mortality without inducing a pre-lethal cytokine response. *Shock*, 34(4): 369-376.
3. Kenney, E.M. (2010) Association between outcome and organ system dysfunction in dogs with sepsis: 114 cases (2003-2007). *J. Am. Vet. Med. Assoc*., 236(1): 83-87.
4. Schuetz, P., Pedro, C.A. and Athan, I.S.H. (2011a) Diabetes and sepsis: Preclinical findings and clinical relevance. *Diabetes Care*, 34(3): 771-778.
5. Rastogi, A. and Anil, B. (2016) Diabetes foot infection: An Indian scenario. *J. Foot Ankle Surg. (Asia-Pacific)*, 3(2): 71-79.
6. Dask, A.K., Mishra, J. and Dask, D.K. (2014) Antidiabetes activity and modulation of antioxidant status by *Ocimum*

americanum in streptozotocin-induced diabetes rats. *Eur. Sci. J*., 10(6): 168-177.
7. Singh, M.P. and Pathak, K. (2015) Animal models for biological screening of anti-diabetic drugs: An overview. *Eur. J. Exp. Biol*., 5(5): 37-48.
8. Wu, D., Shusheng, Z., Shijing, H. and Bao, L. (2017) Inflammatory responses and histopathological changes in a mouse model of *S. aureus* induced bloodstream infections. *J. Infect. Dev. Ctries*., 11(4): 294-305.
9. Reis, C.P., Figueiredo, I.V., Rui, A.C, Jone, J., Nunes, P., Soares, A.F., Silva, C.F., Ribeiro, A.J., Veiga, F.J., Damge, C., Cabrita, A.N.M. and Neufeld, R.J. (2008) Toxicological assessment of orally delivered nanoparticulate insulin. *Nanotoxicology*, 2(4): 205-217.
10. Damasceno, D.C., Netto, A.O., Iessi, I.L., Gallego, F.Q., Corvino, S.B., Dallaqua, B., Sinzato, Y.K., Bueno, A., Calderon, I.M.P. and Rudge, M.V.C. (2014) Review article streptozotocin-induced diabetes models: Pathophysiological mechanisms and fetal outcomes. *BioMed Res. Int*., 2014: 1-11.
11. Hasibuan, M.S., Yasni, S., Bintang, M. and Ranti, A.S. (2016) Antihyperglycemic activity of *Piper crocatum* leaves and *Cinnamomum burmanii* bark mixture extract in streptozotocin-induced diabetes rats. *J. Math. Fund. Sci*., 48(2): 178-191.
12. Aslam, F., Iqbal, S., Nasir, M., Anjum, A.A., Swan, P. and Sweazea, K (2017) Evaluation of white sesame seed oil on glucose control and biomarkers of hepatic, cardiac, and renal functions in male Sprague-Dawley rats with chemically induced diabetes. *J. Med. Food*, 20(5): 448-457.
13. Popov, D. and Paplov, G. (2007) Sepsis models in experimental animals. *Trakia J. Sci*., 11(1): 13-23.
14. Santana, S., Lenzi-Almeida, K.C., Fernandes-Santos, C., Paes-De-Almeida, E. and Aguiar-Alves, F. (2016) Rat infection by methicillin-resistant *S. aureus* from different colonization sites in humans resulting in diffusion to multiple organs. *J. Clin. Exp. Pathol*., 6(2016): 283.
15. Hanses, F., Roux, C., Dunman, P.M., Salzberger, B. and Lee, J.C. (2014) *S. aureus* gene expression in a rat model of infective endocarditis. *Genome Med*., 6(10): 93.
16. Mai, S., Khan, M., Liaw, P. and Fox-Robichaud, A. (2007) Experimental Sepsis Model. An Ongoing and Significant Challenge. Available from: http://www.intechopen.com/books/sepsis-an-ongoing-andsignificant-challenge. Last accessed on 10-12-2018.
17. Li-Tzu, Y., Yi-Ping, C., Shyi-Jou, C., Chin-Chen, C. and Huey-Kang, S. (2014) Diabetes animal models with infectious diseases: Focus on the dysfunction of the immune system. *J. Diabetes Metab.*, 5(8): 417.
18. Padilla, N.B., Bleeker, W.K., Lubbers, Y., Rigters, G.M.M., Mierlo, G.J.V., Daha, M.R. and Hack, C.E. (2007) Rat C-reactive protein activates the autologous complement system. *Immunology*, 109(4): 564-571.
19. Lelubre, C., Anselin, S., Boudjeltia, K.Z., Biston, P and Piagnerelli, M. (2013) Interpretation of C-reactive protein concentrations in critically ill patients. *BioMed Res. Int*., 2013: Article ID 124021.
20. Dimitrov, I., Kamenov, V., Angelova, P. and Petrov, M. (2014) Concentration of C-reactive protein in white adipose tissue, liver and blood serum of male Wistar rats. *Trakia J. Sci.*, 12(1): 29-33.
21. Rittirsch, D., Marco, H. and Peter, A.W. (2007) The disconnect between animal models of sepsis and human sepsis. *J. Leukoc. Biol*., 81(1): 137-143.
22. Menon, M.S., Marwah, S., Mehta, M. and Dipak, A.D. (2014) Diagnostic accuracy of CRP in immunocompromised patients with sepsis in intensive care units. *Natl. J. Physiol. Pharm. Pharmacol*., 5(3): 166-169.
23. Schnoor, M., Alcaide, P., Mathieu-Benoit V. and Buul, J.D.V. (2016) Recruitment of immune cells into inflamed tissues: Consequences for endothelial barrier integrity and tissue functionality. *Mediat. Inflamm*., 2016: 1-24.

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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.

REFERENCES:

1. Abdurahman NH, Khoo CG, Azhari NH. Production of Virgin Coconut Oil (VCO) by Centrifugation Method. International Conference of Chemical Engineering and Industrial Biotechnology in conjunction with 25th Symposium of Malaysian Chemical Engineers (ICCEIB –SOMChE). 2011.
2. Arachchi LACNL, Gunathilake KDP, Prasadi VPN. Shelf Life and Quality Evaluation of Deep Frozen Coconut Cream, Coconut Scrapings, and Coconut Slices. Cord. 2016; 32: 1.
3. Asiah N, Cempaka L, T Maulidini T. Sensory Evaluation And Yield Value of VCO Produced By Various Culture. Journal of Agricultural Science And Agriculture Engineering. 2018; 36-44
4. Caligiani A, Nocetti M, Lolli V, Marseglia A, PallaG. Development of a Quantitative GC–MS Method for the Detection of Cyclopropane Fatty Acids in Cheese as New Molecular Markers for Parmigiano Reggiano Authentication. J. Agric Food Chem. 2016; 64: 20
5. Chitturi S, Talatam VG Vuppu S. Studies on protein content, protease activity, antioxidants potential, melanin composition, glucosinolate and pectin constitution with brief statistical analysis in some medicinally significant fruit peels. Der Pharmacia Lettre. 2013; 5 (1):13-23
6. Dayrit FM, Buenafe ORM, Chainani ET, De Vera IMS, Dimzon ILD, Gonzales EG, Santos JER. Standards for Essential Composition and Quality Factors of Commercial Virgin Coconut Oil and its Differentiation from RBD Coconut Oil and Copra Oil. Philippine Journal of Science. 2007; 136 (2): 119-129
7. Dayrit FM. Lauric Acid is a Medium-Chain Fatty Acid; Coconut Oil is a Medium-Chain Triglyceride. Philippine Journal of Science. 2014; 143 (2): 157-166.
8. FAO. Codex Alimentarius Stan 210-1999. Standard for Named Vegetable Oils. Available from: URL: http://www.fao.org/fao-who-codexalimentarius/sh-proxy/it/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCODEX%2BSTAN%2B210-1999%252FCXS_210e.pdf
9. Ferreira-Dias S, Sandoval G, Plou F, Valero F. The potential use of lipases in the production of fatty acid derivatives for the food and nutraceutical industries. Electronic Journal of Biotechnology. 2013; 16 : 3
10. Gunasekaran R, Shaker MR, Mohd-Zin SW, Abdullah A, Ahmad-Annuar A, Abdul-Aziz NM. Maternal intake of dietary virgin coconut oil modifies essential fatty acids and causes low body weight and spiky fur in mice. BMC Complementary and Alternative Medicine. 2017; 17: 79
11. Katadata. Indonesia, negara penghasil kelapa terbesar di dunia. Available from: URL: https://databoks.katadata.co.id/datapublish/2017/01/06/indonesia-negara-produsen-kelapa-terbesar-di-dunia.
12. Katadata. Produksi dan ekspor kelapa Indonesia meningkat. Available from: URL: https://databoks.katadata.co.id/datapublish/2017/01/09/2010-2015-produksi-dan-ekspor-kelapa-indonesia-meningkat. Accessed on 15 March 2019
13. Nireeksha, Hegde MN, Kumari S. Fatty Acids and Oral Health: A Review. Research Journal of Pharmaceutical, Biological, and Chemical Sciences. 2018; 9: 1080.
14. Prapun R, Cheetangdee N, Udomrati. Characterization of Virgin coconut oil (VCO) recovered by different techniques and fruit maturities. International Food Research Journal. 2016; 23: 2117-2124.
15. Okino-Delgado CH, Leuri LF. Obtaining lipases from byproducts of orange juice processing. Short communication. Food Chemistry. 2014; 163: 103-107.
16. Qosimah D, Istiana S. Prosiding Temu Nasional. Inovasi, Pengelolaan, Pemanfaatan dan Festival Sumberdaya Genetik Lokal. 2017; 751-754
17. Raghavendra SN, Raghavarao KSMS. Effect of different treatments for the destabilization of coconut milk emulsion. Journal of Food Engineering. 2010; 97:341–347
18. Satheesh N, Prasad NBL. Production of Virgin Coconut Oil from Dry and Wet Methods of Induced Fermentation and its Characterization. European Journal of Lipid Science and Technology. 2012; 44:2
19. Satheesh N, Prasad NBL. Production of virgin coconut oil by induced fermentation with Lactobacillus Plantarum NDRI strain 184: Original Scientific Paper. Croatian Journal of Food Technology, Biotechnology, and Nutrition. 2014; 9:37-42
20. SNI (Indonesia National Standard) 7381. 2008. Minyak Kelapa Virgin (VCO). Available from: URL:https://kupdf.net/download/sni-7381-2008-minyak-kelapa-virgin-vco_59b4024edc0d600b0c568edc_pd
21. Srivastava Y, Semwal AD, Sharma GK. Studies on storage stability of hot extracted (HEVCO) and cold extracted virgin coconut oil (CEVCO) in the different flexible and rigid packaging system. International Food Research Journal. 2013; 20(4): 1971-1976
22. Syukur S, Syafrizayanti, Zulaiha S, Ismet M, Fachrial E. Virgin Coconut Oil Increase High-Density Lipoprotein (LDL), Lower Triglyceride And Fatty Acids Profile (C6-C18) In Blood Serum of Mus musculus. Research Journal of Pharmaceutical, Biological, and Chemical Sciences. 2017; 8: 1077.
23. Tenda E.T, Kumaunang J. Keragaman Fenotipik Kelapa Dalam di Kabupaten Pacitan, Tulung Agung dan Lumajang, Jawa Timur Phenotypic Variation of Tall Coconut in Pacitan, Tulung Agung and Lumajang, East Java Elsje T. Tenda dan Jeanette Kumaunang. Available from: URL:http://ejurnal.litbang.pertanian.go.id/index.php/palma/article/download/8357/7159.
24. Witono Y, Aulanni'am, Subagio,A, Widjanarko SB. Enzymatic Extraction of Virgin Coconut Oil Using Protease from Biduri Plant (Calotropis gigantea). AGRITECH. 2007; 27: 3

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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

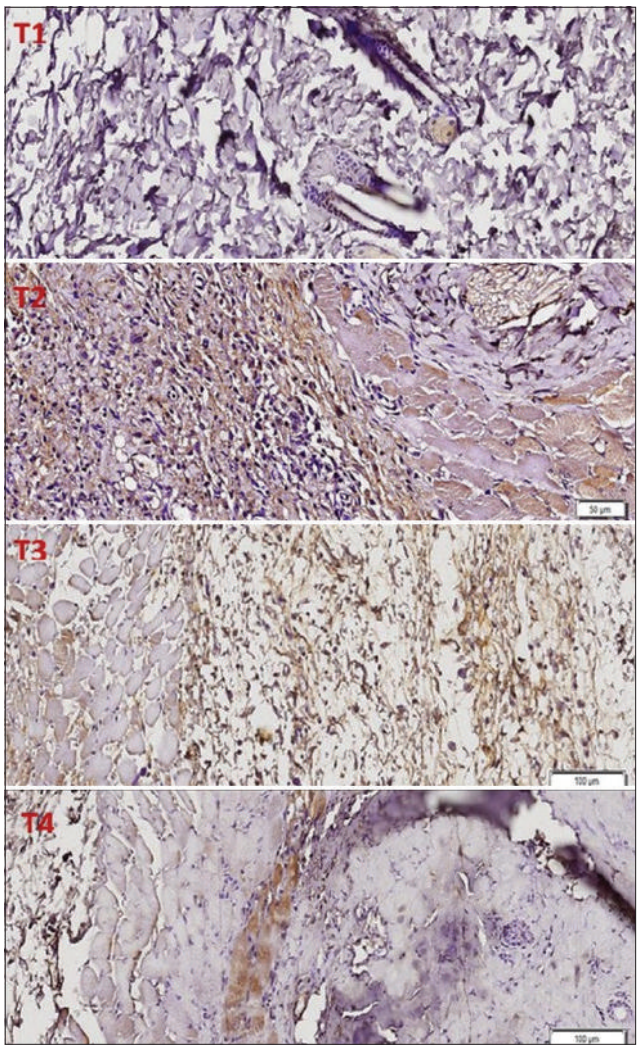

Figure-1: The expression of interleukin-1β on treatment rats.

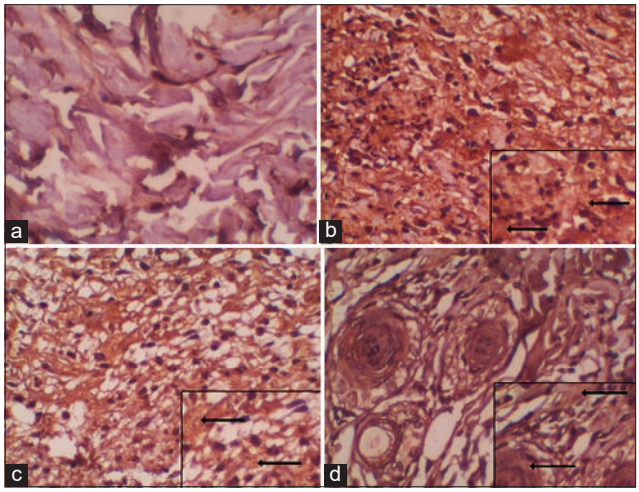

Figure-2: The expression of interleukin-6 on treatment rats: (a) (T1) Positive control, (b) (T2) negative control, (c) (T3) wounds treated with Gliricidia sepium from Indonesia, and (d) (T4) wounds treated with Gliricidia sepium from the Philippines (400×).

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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References

1. Potter, P.A. and Perry, A.G. (2005) Fundamental Nursing: Concept, Process and Practise. 1st ed., Vol. 1. Write in Bahasa Indonesia, Jakarta.
2. Briant, A.R. and Nix, P.D. (2007) Acute and Chronic Wounds Current Management Concepts. 3rd ed. Mosby, St Louis, Missouri.
3. Mackay, D. and Miller, A.L. (2003) Nutritional support for

wound healing. *Altern. Med. Rev*., 8(4): 359-377.
4. Lopez-Castejon, G. and David, B. (2011) Understanding the mechanism of IL-1β secretion. *Cytokine Growth Factor Rev*., 22(4): 189-195.
5. Patel, H.C., Boutin, H. and Allan, S.M. (2003) Interleukin-1 in the brain mechanism of action in acute neurodegeneration. *Ann. N. Y. Acad. Sci*., 992(1): 39-47.
6. Anonymous (20191) *Gliricidia sepium:* Crop Protection Compedium: Invasive Species Compedium [available online]: https://www.cabi.org/isc/datasheet/25380. Retrieved on 19-03-2021.
7. Smith, O.B. and Van Houtert, M.F.J. (1987) The feeding value of *Gliricidia sepium*. *World Anim. Rev*., 62: 57-68.
8. Andriyani, R. (2017) Insecticides properties of *Giliciridia sepium*, Jacq. From Pringsewu culture toward white fleas (*Planococcus minor*, Maskell) (*Hemiptera*: *Pseudococcidae*) Cocoa. Biology Department, Faculty of Science Lambung Mangkurat University (Write in Bahasa Indonesia).
9. Mescher, A.L. (2016) Junquiera's Basic Histology Text and Atlas. 14th ed. McGraw Hill Education, New York.
10. Williams, J.W. and dan Moores, A. (2009) BSAVA Manual of Canine and Feline Wound Management and Reconstruction. BSAVA, London.
11. Riansyah, Y. and dan Ratu Choesrina, L.M. (2015) Anti-inflammatory Activity of Ethanolic Extract of *Ipomoea batatas* L. Lank Leaves on Male Wistar Rats. Department of Pharmacy, Faculty of Sciences, Bandung Islamic University (Write in Bahasa Indonesia), Bandung.
12. Calzada, F. and Bautista, E. (2020) Review: Plants used for the treatment of diarrhoea from Mexican flora with amoebicidal and giadicidal activity, and their phytochemical constituents. *J. Ethnopharmacol*., 253(4): 112676.
13. De-Rodriguez, D.J., Garcia-Hernandez, L.G., Guzman, N.E.R., Moreno-Jimenez, M.R., Rodriguez-Garcia, R., Diaz-Jimenez, M.L.V., Flores-Lopez, M.L., Villareal-Quintanilla, J.A., Ramoz, F.M.P. and Carillo-Lomell, D.A. (2017) Hypoglycemic and anti-inflammmatory effect of *Psacalium paucicapatium* corms infusion. *Ind. Crop. Prod*., 107: 482-488.
14. Maiti, S., Nazmeen, A., Medda, N., Patra, R. and Gosh, T.K. (2019) Flavonoid green tea against oxidant stress and inflammation with related human diseases. *Clin. Nutr. Exp*., 24: 1-14.
15. Abbas, A.K., Licthman, A.H. and Pillai, S. (2007) Cellular and Molecular Immunology. 6th ed. Saunders Elsevier, USA. p19-39, 262.
16. Lie, Y., Guo, Y., Weng, X., Yang, L., Wang, Y., Chen, Y., Zhang, D., Li, Q., Liu, L., Kan, X., Chen, X., Zhu, X., Kmoniekova, E. and Zidek, Z. (2015) Flavonoid castitin and crysosphenol D from *Artemisia annua* L inhibit inflammation *in vitro* and *in vivo*. *Toxicol. Appl. Pharmacol*., 286(3): 151-158.
17. Tahir, Z. (2013), The Effect of Epidural Multimodal Analgesic of 0.125% Bupivakain and Intravenous of 40 mg Parecoxib 40Mg to Level Ratio of Interleukin-6 and Interleukin-10 with Pain Intensity on Gynecology Laparotomy [Thesis]. Graduate School of Biomedic, Hasanuddin University (Write in Bahasa Indonesia).
18. Velnar, T., Bailey, T. and Smrkolj, V. (2009) The wound healing process: An overview of the cellular and molecular mechanisms. *J. Int. Med. Res*., 37(5): 1528-1542.
19. Lee, D., Park, J., Choi, J., Jang, H. and Seol, J. (2020) Anti-inflammatory effect of natural flavonoids diosmection in IL-4 and LPS induced macrophage activation and atopic dermatitis model. *Int. Immunopharmacol*., 89(Pt A): 107046.
20. Mawarti, H. (2018) The Flavonoid Effect as Wound Healing Time of Grade II Burns on Rats Faculty of Medical Sciences, Darul Ulum University, Jombang (Write in Bahasa Indonesia), Jombang.
21. Nasir-Deen, A.Y., Boakye, Y.D., Osafo, N., Agyre, C., Boamah, D., Boamah, V.E. and Agyei, E.K. (2020) Anti-inflammatory and wound healing properties of methanol leaf extract of *Physalis augulata* L. *S. Afr. J. Bot*., 133: 124-131.

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Enhanced Manhattan-based Clustering using Fuzzy C-Means Algorithm for High Dimensional Datasets

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***Abstract*—The problem of mining a high dimensional data includes a high computational cost, a high dimensional dataset composed of thousands of attribute and or instances. The efficiency of an algorithm, specifically, its speed is oftentimes sacrificed when this kind of dataset is supplied to the algorithm. Fuzzy C-Means algorithm is one which suffers from this problem. This clustering algorithm requires high computational resources as it processes whether low or high dimensional data. Netflix data rating, small round blue cell tumors (SRBCTs) and Colon Cancer (52,308, and 2,000 of attributes and 1500, 83 and 62 of instances respectively) dataset were identified as a high dimensional dataset. As such, the Manhattan distance measure employing the trigonometric function was used to enhance the fuzzy c-means algorithm. Results show an increase on the efficiency of processing large amount of data using the Netflix ,Colon cancer and SRCBT an (39,296, 38,952 and 85,774 milliseconds to complete the different clusters, respectively) average of 54,674 milliseconds while Manhattan distance measure took an average of (36,858, 36,501 and 82,86 milliseconds, respectively) 52,703 milliseconds for the entire dataset to cluster. On the other hand, the enhanced Manhattan distance measure took (33,216, 32,368 and 81,125 milliseconds, respectively) 48,903 seconds on clustering the datasets. Given the said result, the enhanced Manhattan distance measure is 11% more efficient compared to Euclidean distance measure and 7% more efficient than the Manhattan distance measure respectively.**

***Keywords*— fuzzy C-Means; high dimensional dataset; Manhattan distance; clustering.**

I. INTRODUCTION

The high dimensional dataset is common nowadays due to the colossal amount of information being gathered electronically by varying information systems. Movies, medical health record, and agricultural dataset can be observed to be as high dimensional dataset. Duplication of records, multiple attributes and thousands number of records were categorized as high dimensional datasets, and most of the data mining algorithms suffer low accuracy and high computational cost in processing when a high dimensional dataset was supplied [1]. This high dimensional dataset can also be observed to know what this dataset shows and implies.

A common technique to observe this dataset is using clustering. Clustering splits a large amount of data and performs grouping considering the similarities of the individual data supplied [2]. However, several clustering algorithms suffer from high computational cost and one of which is the Fuzzy C-Means algorithm.

Fuzzy C-Means also suffers from its accuracy and speed when a dataset contains high dimension or not [3], [4]. The study aims to enhance the Fuzzy C-Means algorithm by changing the distance measure to solve the weakness of the said algorithm. Manhattan distance measure was used since it is also ideal when applied to high dimensional dataset [5]. The trigonometric approach was utilized to the said distance measure since the accuracy of the Manhattan distance measure suffers when centroid and points are connected diagonally [6], [7].

Data mining procedures will also be used to prepare the actual dataset for mining. The computational cost will be observed by testing the algorithm with different distance measures (Euclidean, Manhattan and Enhanced Manhattan) and three different high dimensional datasets (Netflix Movie Rating, Colon Cancer and SRCBT) which will lead on what specific distance measure is faster when applied to the said algorithm.

II. MATERIALS AND METHOD

To investigate the performance of the modified algorithm, Knowledge Discovery Model were used proposed by [8] consisting the step of data selection, data pre-processing, transformation, and data mining. Figure 1 shows the actual process of KDD.

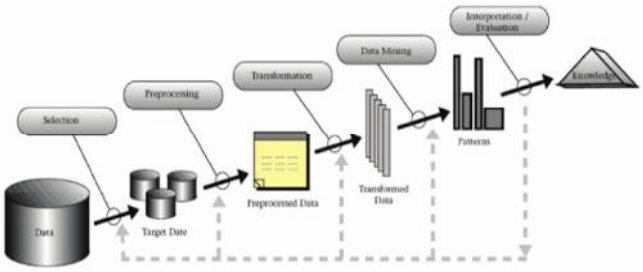

Fig.1 the Knowledge Discover Model

With the KDD model, the dataset should be ideal to be processed from the part of the selection to the step of data mining.

A. Data Selection

Selection of the actual dataset for clustering was done by searching for the appropriate dataset that has high dimensionality. High dimensional datasets are the ones who have multiple fields and thousands of records [1]. The high dimensionality of data is also when dataset features are greater than the number of instances [9]. The Netflix movie rating, small round blue cell tumors (SRBCTs) and Colon Cancer dataset are also categorized as high dimensional considering these definitions. Table 1 shows the number of features of the said datasets.

TABLE I
THE FEATURES AND INSTANCES OF THE DATASETS

Dataset	Features	Instances
Netflix Movie	5	1,500
Colon Cancer	2000	62
SRCBT	2308	83

B. Pre-Processing

The pre-processing technique was also done to prepare the dataset that will be used. This technique reduces the dimensionality of the dataset [10], [11]. The dataset was merged into a file and field were also observed to identify the process needed to be done to reduce its dimensionality. The term discretization technique describes another advantage of this step. In this part, the equal frequency binning was used. This step converts the text into a numeric value. Each instance in the dataset that has the same value are considered as one and converted to a similar numeric value [12]. In Table II, the values of the feature, genre, were discretized to fit the algorithm.

TABLE II
A PORTION OF THE MOVIE DATASET WITH ITS GENRE

No	Movie Title	Genre	Discretised Value
1	Cat Run 2 (2014)	Action	1
2	He Who Dares (2014)	Action	1
3	How to Train Your Dragon 2 (2014)	Action|Adventure| Animation	2
4	Hercules (2014)	Action|Adventure	3
5	Falcon Rising (2014)	Action|Adventure	3
6	Land Ho! (2014)	Adventure|Comedy| Documentary|Mystery	4
8	Seventh Son (2014)	Adventure|Children |Sci-Fi	6

In this process, the field, genre, was discretized to be applicable with clustering. The same process was done for the two remaining datasets (Colon Cancer and SRCBT). The feature class was converted into a numeric value.

C. Transformation

Making the dataset suitable for knowledge discovery requires the dataset to be transformed. The dataset for Netflix movie rating is composed of several tables (Movie, Rating, and Tags) that are connected via Primary Key (PK) and a Foreign Key (FK). A foreign key is several techniques can do a specific property of dataset, which is described by the implementation of the primary key to another data table [13] and merging this dataset. One technique for combining this data table for preparation for data mining is union. The union is the process of identifying the intersection of two or more data table with their PK and FK[14]. Hence, the researcher created a tool for merging the data table into a single dataset concerning the primary key and foreign key.

For the two remaining datasets, features were already normalized aside from the pre-processing technique. Observation of the actual content of the dataset was also needed to be observed thoroughly to see how these datasets were constructed such that the enhanced algorithm can process it. Based on the pre-processing and transformation techniques, the following portions of the datasets of Netflix Movie Rating, Colon Cancer and SRCBT had been derived.

TABLE III
NETFLIX MOVIE DATASET

Rating	UserID	Time Stamp	Genre	MovieID
2.5	53930	1393064439	30	22306
2.5	87813	1387131563	30	22306
3	137200	1398867354	30	22306
4.5	13494	1421295240	114	23623
4	15720	1426647292	114	23623

TABLE IV
COLON CANCER DATASET

FTR1	FTR1	FTR1	FTR to 2000	Class
88.23	39.67	67.83	28.7	2
82.24	85.03	152.2	16.77	1
76.97	224.62	31.23	15.16	2
74.53	67.71	48.34	16.09	1
54.56	223.36	73.1	31.81	2
33.2	91.85	5.88	21.88	1
98.54	54.62	30.54	24.45	2

TABLE V
SRCBT DATASET

FTR1	FTR1	FTR1	FTR to 2308	Class
0.143	0.888	0.068	0.108	2
0.085	0.324	0.635	0.271	1
0.193	0.39	0.378	0.107	3
0.159	0.248	1.164	0.224	4

D. Data Mining

Clustering algorithm will be enforced in this study by using the Fuzzy C-means algorithm. This tool can be used to address its problem on clustering high dimensional datasets. Figure 2 shows the actual process of how Fuzzy C-Means Clustering works.

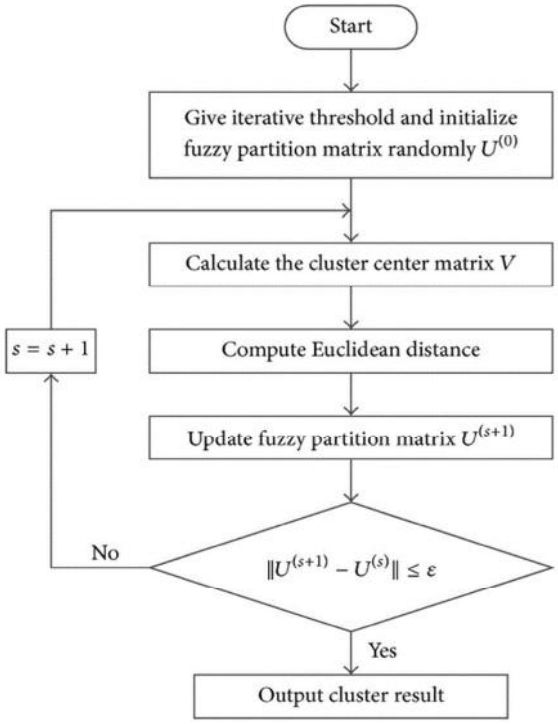

Fig. 2 The actual process of clustering using Fuzzy C-means algorithm

The first step is that Fuzzy C-Means selects the number of cluster and membership functions ranging from zero to one. The calculation of the actual centroid with the corresponding parameter follows. The computation of the actual centroid plays a vital role in creating the clusters[15]. This will identify how many iterations will be done. The third step is to date the actual cluster with the specific distance measure and lastly, validate the result. The iterations take place until convergence is achieved [16]. With this given process of Fuzzy C-Means algorithm, changing the distance measure can improve the performance of the said algorithm.

E. Manhattan Distance Measure

Providing a result with lesser computational cost can be achieved using different strategies. Observing the distance measure used by the algorithm and its performance can be a basis in identifying what distance measure is applicable for the high dimensional dataset. The Manhattan distance measure is commonly used when the point that is generated was vertically or horizontally connected. Selecting an appropriate distance measure plays a vital role in providing a good set of clusters [17]. The study also shows that Manhattan distance measure is more accurate in the calculating distance when the dataset is high dimensional compared to other distance measures [18]. Table VI shows the side by side comparison of several distance measure.

TABLE VI
COMPARISON OF SEVERAL DISTANCE MEASURE.

Distance Measure	Benefits	Drawbacks
Euclidean	Easy to Implement and Test	Results are greatly influenced by variables that have the largest value. Does not work well for Image data, Document Classification
Manhattan	Easily generalized to a higher dimension	Does not work well for image data and document classification
Cosine	Handles both continuous and categorical variables	Does not work well for nominal data
Jaccard Index	Handles both continuous and categorical variables	Does not work well for nominal data

The use of the Manhattan Distance Measure will allow the algorithm to speed up its processing time, although Manhattan distance measure has a problem needed to be addressed.

F. Euclidean Distance Measure

On the other hand by default Euclidean distance measure were used in Fuzzy C-Means, it produces a more accurate result but higher computational cost [19], this is the main reason why the algorithm needed to be improved with the proposed modification conceptualized.

G. Enhancement of the Distance measure

A weakness of the Manhattan distance measure is in terms of clustering points that are connected diagonally. Fig. 3 shows the actual points connecting to the centroid diagonally.

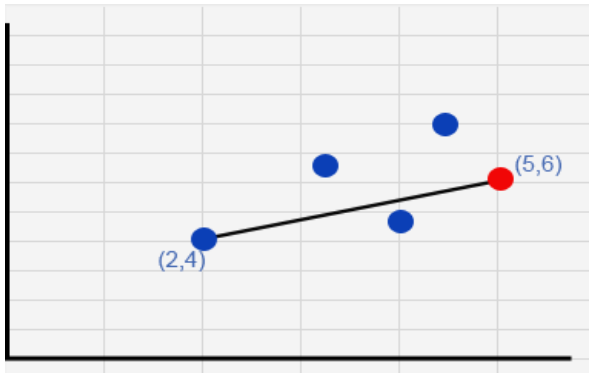

Fig. 3 Centroid and data point are connected diagonally

Employing trigonometric function specifically COSINE Equation 2 was tested in order to check the speed of the said

distance measure when applied to the Fuzzy C-Means algorithm.

$$cosine(\emptyset) = \frac{\text{adjacent}}{hupothenuse} \quad (2)$$

Where adjacent (next to) is to the angle θ and Hypotenuse is the long line, equation 3 shows the actual solution to address the problem of Manhattan Distance.

$$d = \sum \frac{|(x3-x2)+ (y_2-y_3)|}{cosine(\emptyset)} \quad (3)$$

Where y_3 is the point of intersection from the created imaginary line and y_2 is the y-coordinate of the centroids. The difference of y_3 and y_2 will be divided to $cosine(\emptyset)$. $Theta\ (\emptyset)$ is used since the actual angle is not yet solved. To calculate the actual distance the following, steps were considered.

Step 1. *Create an imaginary line to form a right triangle*

Step 2. *Identify the point of intersection*

Step 3. *Compute the Distance of the Imaginary line using Manhattan. Given that (x2=5,x3=5) and (y2=6,y3=4)*

(5-5)+(6-4)= 2

Step 4. *Compute for the distance*

2/Cosine(53.60)=3.61

The given steps in calculating the actual distance of the centroid to the dataset points may lead to higher accuracy for the Fuzzy C-Means Algorithm when supplied.

H. Fuzzy C-Means

To further test the algorithm, the steps for the distance measure were invoked by the enhanced Manhattan distance measure. By default, Fuzzy C-Means uses Euclidean distance. Fig. 4 shows the actual process of clustering the dataset using the enhanced Manhattan distance.

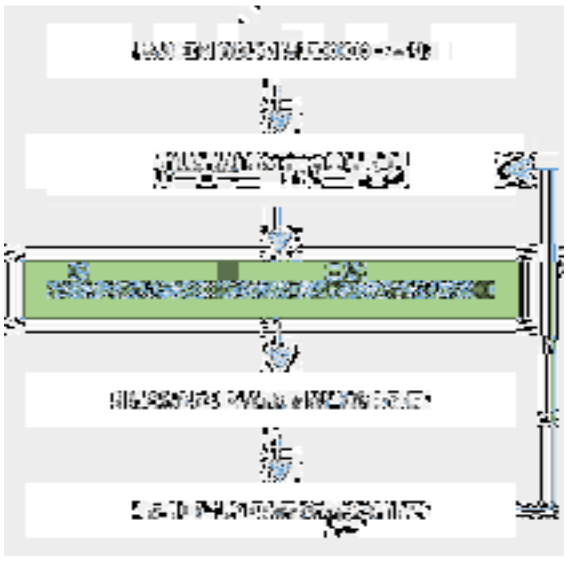

Fig. 4 The actual process of clustering using Fuzzy c-means algorithm.

The following pseudo-code was used to implement the Modified Manhattan distance measure over Fuzzy C-Means Algorithm.

Start
Required Array of Points and Centroid
Declare Distance

For counter=0; to LengthofPoints step 2

If Centroid is equal to Points
Get the absolute difference of points and the centroid
Else
Get the absolute difference of centroid and Imaginary line
Divide the absolute difference to $cosine(\emptyset)$

EndIf

Update distance by adding the difference
Iterate to each column and pair it with (x,y) format and do the calculation for the distance.
End

I. Evaluation

To validate the performance of the said modified algorithm, the duration to complete the process of clustering using Fuzzy C-Means with different distance measures were compared, and the starting points of the program were tracked. The differences were calculated to identify how many milliseconds were needed to complete the actual clustering process. The following pseudo code was used to evaluate the performance of Fuzzy C-Means on applying the three distance measures and three high dimensional datasets.

Start
Get Start time in milliseconds
Declare Threshold=1, iteration=0
While Threshold is not equal to 0
Update value of iteration +1
Assign new center
End While
Elapsed time = end time - start time
End

The process of Fuzzy C-Means clustering stops when the convergence is reached. This means that when the threshold becomes zero, the actual clustering process is finished on clustering [16] and as prescribed by the algorithm threshold use was zero. Tracking the execution time of the program can now be observed along with the behavior of the algorithm when different distance measures and different datasets with high dimensions was applied.

III. Results and Discussion

With the procedure of pre-processing and transformation, the dataset Netflix Movie composed of 4 attributes with 1,500 instances, Colon Cancer having 2000 features and 65 instances and SRCBT 2308 features and 83 instances are

now ready for clustering and comparison of the actual speed of the modified algorithm to the standard Fuzzy C-Means Algorithm. The algorithm was tested by computing the actual time elapsed when the clustering processes were simulated. Table VI showed the actual result of the algorithm when Euclidean and Enhanced Manhattan distance measures were used.

TABLE VII
RESULT OF THE ALGORITHM IN (MS), WHEN EUCLIDEAN AND ENHANCED MANHATTAN IS USED

Dataset	Clusters	Euclidean	Enhanced Manhattan
Netflix Movie	10	39296	33216
Cancer	4	38952	32368
SRCBT	3	85774	81125

Observing the actual result, the Enhanced Manhattan distance measure outperformed the Euclidean distance Measure. To further investigate, the Manhattan distance measure was also used to compare the actual results as shown in Table VIII.

TABLE VIII
RESULT OF THE ALGORITHM IN (MS), WHEN MANHATTAN AND ENHANCED MANHATTAN IS USED

Dataset	Clusters	Manhattan	Enhanced Manhattan
Netflix Movie	10	36858	33216
Cancer	4	36501	32368
SRCBT	3	82860	81125

With the dataset supplied to the Manhattan distance and enhanced Manhattan distance, the result shows that the actual modification decreases the processing time for clustering the three datasets. Comparison of the actual result for clustering using Fuzzy C-Means with the three distance measure is indicated in Figure 5 and 6. The behavior of the algorithm varies on the dataset supplied, especially when it comes to high dimensions.

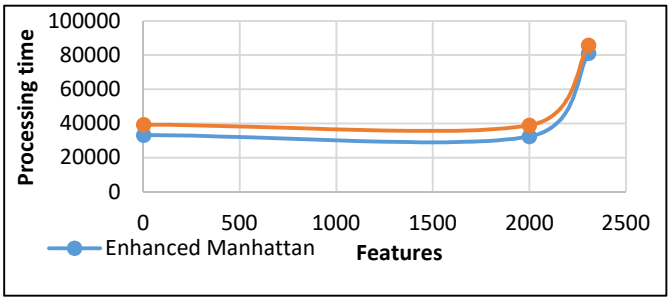

Fig. 5 The comparison of the processing time of Euclidean and Enhanced Manhattan against the Dataset Features.

The trend of the three distance measure plotted along with the number of features and to its processing time showed an improvement when the Enhanced Manhattan Distance measure was supplied. This indicates that the modification can now be applied to the algorithm to increase its speed on clustering high dimensional datasets.

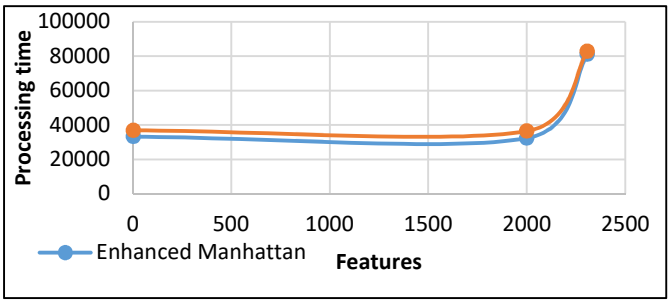

Fig. 6 The comparison of the processing time of Manhattan and Enhanced Manhattan against the Dataset Features.

The trend of the three distance measure plotted along with the number of features and to its processing time showed an improvement when the Enhanced Manhattan Distance measure was supplied. This indicates that the modification can now be applied to the algorithm to increase its speed on clustering high dimensional datasets.

IV. CONCLUSION

Fuzzy C-means algorithm is an algorithm that suffers from high computational cost when a high dimensional dataset is applied. One way to address the said problem is by invoking the distance measure used. In this study, an Enhanced Manhattan-based clustering was used employing trigonometric function to address the issue of Manhattan distance measure.

Results show that an increase in the efficiency in terms of speed of the said algorithm can be observed when using the enhanced Manhattan distance measure. Euclidean distance measure shows that clustering the three datasets such as Netflix Movie Rating, Colon Cancer, and SRBT has a (39,296, 38,952 and 85,774 milliseconds to complete the different clusters, respectively) average of 54,674 milliseconds while Manhattan distance measure took an average of (36,858, 36,501 and 82,86 milliseconds, respectively) 52,703 milliseconds for the entire dataset to cluster. On the other hand, the enhanced Manhattan distance measure took (33,216, 32,368 and 81,125 milliseconds, respectively) 48,903 seconds on clustering the datasets.

Given the said result, the enhanced Manhattan distance measure is 11% more efficient compared to Euclidean distance measure and 7% more efficient than the Manhattan distance measure respectively. While the efficiency increases for the said algorithm, it needs further observation on the behavior of the algorithm in clustering a standard type of dataset. Accuracy also needs to be studied in applying this modified algorithm. Other factors can also be considered to increase the efficiency of the said algorithm.

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REFERENCES

[1] N. Raksha and R. Alankar, "Detection of fuzzy duplicates in high dimensional datasets," *2016 Int. Conf. Adv. Comput. Commun. Informatics, ICACCI, 2016*, pp. 1423–1428, 2016.

[2] Y. G. Jung, M. S. Kang, and J. Heo, "Clustering performance comparison using K- means and expectation maximization algorithms," *Biotechnol. Biotechnol. Equip. ISSN1310-2818*, vol. 2818, no. October 2015.

[3] Z. Cebeci and F. Yildiz, "Comparison of K-Means and Fuzzy C-Means Algorithms on Different Cluster Structures," *J. Agric. Informatics*, vol. 6, no. 3, pp. 13–23, 2015.

[4] R. Winkler, F. Klawonn, and R. Kruse, "Problems of Fuzzy c-Means Clustering and Similar Algorithms with High Dimensional Data Sets," *Challenges Interface Data Anal. Comput. Sci. Optim.*, pp. 1–8, 2012.

[5] S. Pandit and S. Gupta, "A Comparative Study On Distance Measuring," *Int. J. Res. Comput. Sci.*, vol. 2, no. 1, pp. 29–31, 2011.

[6] T. K. Mohana, V. Lalitha, L. Kusuma, N. Rahul, and M. Mohan, "Various Distance Metric Methods for Query Based Image Retrieval," vol. 7, no. 3, pp. 5818–5821, 2017.

[7] M. Khan and T. Shah, "A copyright protection using watermarking scheme based on nonlinear permutation and its quality metrics," *Neural Comput. Appl.*, vol. 26, no. 4, pp. 845–855, 2014.

[8] U. Fayyad, G. Piatetsky-Shapiro, and P. Smyth, "From Data Mining to Knowledge Discovery in," vol. 17, no. 3, pp. 37–54, 1996.

[9] J. Zhang and M. Pan, "A high-dimension two-sample test for the mean using a cluster," *Comput. Stat. Data Anal.*, vol. 97, pp. 87–97, 2016.

[10] L. Zhou, "Preprocessing Method before Data Compression of Cloud Platform," pp. 1223–1227, 2017.

[11] M. A. Chaudhari, P. M. Phadatare, P. S. Kudale, R. B. Mohite, and R. P. Petare, "Preprocessing of High Dimensional Dataset for Developing Expert IR System," pp. 417–421, 2015.

[12] Z. Marzuki and F. Ahmad, "Data Mining Discretization Methods and Performances Data Mining Discretization Methods and Performances," no. December, pp. 3–6, 2014.

[13] N. A. Mian and N. A. Zafar, "Key Analysis of Normalization Process using Formal Techniques in DBRE," 2010.

[14] C. Ordonez, "Data Set Preprocessing and Transformation in a Database System," vol. 15, no. 4, pp. 1–19, 2011.

[15] Z. Wang, N. Zhao, W. Wang, R. Tang, and S. Li, "A Fault Diagnosis Approach for Gas Turbine Exhaust Gas Temperature Based on Fuzzy C-Means Clustering and Support Vector Machine," *Math. Probl. Eng.*, vol. 2015, pp. 1–11, 2015.

[16] N. Grover, "A study of various Fuzzy Clustering Algorithms," *Int. J. Eng. Res.*, vol. 5013, no. 3, pp. 177–181, 2014.

[17] L. H. Son, "Generalized picture distance measure and applications to picture fuzzy clustering," *Appl. Soft Comput. J.*, pp. 1–12, 2016.

[18] C. C. Aggarwal, A. Hinneburg, and D. A. Keim, "On the Surprising Behavior of Distance Metrics in High Dimensional Space," pp. 420–434, 2001.

[19] A. Fahad *et al.*, "A Survey of Clustering Algorithms for Big Data : Taxonomy & Empirical Analysis," 2014.

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RESEARCH ARTICLE

Antimycotic potential of Kawayang tinik against pathogenic fungal species

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ABSTRACT

The importance of discovering and obtaining new, natural and sustainable sources of potential drugs have been the focus of scientific communities due to the emergence of increasing cases of microbial resistance, one of the biggest health threats in our society today. This study aimed to determine the antimycotic potential of *Bambusa blumeana* (kawayang tinik) specifically its leaf, rhizome, root, inner culm and outer culm extracts using the agar well diffusion assay. Results of the study revealed that all kawayang tinik extracts produced statistically equal size zone of inhibition (ZOI) against *Aspergillus niger* at 1 mg/ ml concentration while the ethanolic root and leaf extracts showed larger ZOI against *Penicillium chrysogenum* compared to other kawayang tinik extracts. Furthermore, the results of the antifungal assay showed comparable activity of kawayang tinik extracts to Fluconazole, a pharmaceutically approved antifungal drug, at 1 mg/ml concentration. Phytochemical studies further revealed the presence of alkaloids, tannins, phenols, sterols, triterpenes and flavonoids in its different parts which may support its potential antimycotic properties.

Introduction

The Center of Disease and Prevention (CDC) and World Health Organization (WHO) considers antibiotic resistance, in both bacterial and fungal species, as one of the biggest public health threats today (1, 2). Accordingly, widespread occurrences of antibiotic resistance were recorded among 500000 people across several countries including Asia. It is due to this fact that scientific communities are gearing towards drug discovery especially those coming from natural, highly available and sustainable resources.

Plants have been known for ages to contain bioactive compounds that possessed disease - fighting and ailment - preventing capabilities. In fact, the use of plants as ethnobotanical – based remedies and medication for various illnesses have been a long-dated tradition not only in the Philippines, but in other countries as well (3-5). Moreover, most of the pharmaceutical products being sold in the market today were obtained and patterned from bioactive compounds found in plants and plant products.

The bamboo plant was traditionally utilized to make home furniture and its shoots were usually obtained for food. However, secondary to technological advances in phytochemical screening and testing, several bamboo species are now being studied and observed as potential sources of new pharmacotherapeutic products. Leaves and shoot extracts from bamboo species such as *Bambusa balbacooa*, *Bambusa bambos*, *Dendrocalamus hamiltonii*, *Bambusa vulgaris* and *Bambusa vulgaris* exhibited potential antimicrobial properties against several pathogenic microorganisms, bacterial and fungal alike (6-14). Leaf extracts of *Dendrocalamus strictus* were also cited to show antimycotic potentials against pathogenic fungal strains such as *Aspergillus niger*, *Penicillium chrysogenum*, *Aspergillus flavus* and *Fusarium moniliforme* (15). It was further observed that the antimicrobial activities in bamboo plants can be detected in the entirety of the plant, specifically in the branches, roots, knots, inner culms and rhizomes and not only on the leaf and shoot parts which are the most common plant parts used in antimicrobial studies involving bamboo plants (16).

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The *Bambusa blumeana*, also known as spiny bamboo or thorny bamboo, is a species of bamboo occurring in tropical Asia and is abundant in the Philippines. This bamboo species is known locally as 'kawayang tinik' and ethnobotanically known as 'kawayan siitan' for Ilocanos and 'kawayang batakan' for Bisayans. Although widely available in the community and also tagged as the top economically important bamboo species in the Philippines (17), very few research studies and literatures, both local and international, have been published providing information on its biological activities especially with regard to its potential antimycotic properties.

With various evidences citing the potential biological activity in several bamboo plants against pathogenic fungal species, the present study determined the innate antimycotic potentials of *B. blumeana* (kawayang tinik). Specifically, the study explored the following:

1. Presence of potential antifungal activity in *Bambusa blumeana* (kawayang tinik) against *Aspergillus niger* and *Penicillium chrysogenum*.
2. Differences in the antifungal properties of the ethanolic and aqueous extracts of *Bambusa blumeana* (kawayang tinik) in terms of its different plant parts including the leaf, rhizomes, roots and inner and outer culms.
3. Identified different phytocompounds in various *B. blumeana* (kawayang tinik) extracts.

Materials and Methods

Selection, Gathering and Preparation of Plant Extracts

Plant parts such as leaves, rhizomes, roots, inner and outer culms were identified and gathered from locally – grown *B. blumeana* (kawayang tinik) plants found at Tarlac Agricultural University (TAU) Bamboo Forest Park located in Sitio Calao, Mayantoc, Tarlac. Collected parts were initially washed and cleaned with tap water to remove majority of the dirt and debris while distilled water was used for the final washing. Clean plant specimens were afterwards air dried and powdered separately using electric blender and corn miller. Individual powderized plant parts were placed in clean, zip – locked containers, labelled and stored in the refrigerator with temperature regulation at 4 ^{0}C until further use (14, 18, 19).

Powdered plant parts for ethanolic extraction were then mixed and macerated for 48 hrs (12, 16, 20) at room temperature using 95% ethanol utilizing 1: 4 extract to solvent ratio to produce ethanolic extracts. As for the preparation of aqueous extracts, 400 ml of distilled water was added with 100 gm of individual powdered dry plant parts (leaves, roots, rhizomes, inner and outer culms). Afterwards the individual aliquots were boiled at 80 ^{0}C for at least 15 min. cooled and allowed to macerate for 24 hrs (16, 21, 22).

Frequent agitation of the solutions was observed during the course of the maceration period to further facilitate the extraction of potential bioactive compounds (20). After maceration, the aqueous and ethanolic solutions were filtered using Whatman filter paper No. 1. The filtrates were exposed thereafter to rotary evaporation to remove the solvents and concentrate the extracts.

Stock solutions were then formulated to 1mg/ml concentration and sterility- proofed through filtration of individual plant extracts using sterile millipore filter syringe (Whatman® at 0.22 millipore size) connected to a sterile syringe and test tube (23). The filtered sterile extracts contained in sterile tubes are then stored in the refrigerator with temperature regulation at 4 ^{0}C until needed for antimicrobial assay (24).

Antifungal Assay

Pure cultures of pathogenic fungi (*Aspergillus niger* and *Penicillium chrysogenum*) from the College of Arts and Sciences of Benguet State University located La Trinidad, Benguet were used to test the antifungal property of different kawayang tinik extracts.

Agar well diffusion method was utilized to determine the biological activity of *B. blumeana* (kawayang tinik) against *A. niger* and *P. chrysogenum*. In this method, a 3 mm diameter fungal or mycelium disk was placed at the center of the petri dish while various kawayang tinik extracts were placed in wells distributed in even distances around the fungal disk culture (Fig. 1). Fluconazole 1 mg/ml was used as positive control while sterile water and 95% ethanol were used as negative controls. After 72 hrs of room incubation, antifungal activity was determined by measuring the inhibition zones formed between the extracts and the mycelium disk. The zone of inhibition (ZOI) is measured by obtaining the distance of mycelium growth from the disk towards the individual kawayang tinik extracts as indicated by the blue line (Fig. 1). As shown by various studies, mycelium or fungal species grown on

Fig. 1. Antifungal assay using agar-well diffusion method.

laboratory agar tend to deviate from extracts containing antimycotic compounds, thus, minimal growth between the disk and the wells containing the individual extracts may possibly correlate to presence of bioactive compounds responsible for antifungal mechanisms (25-28). Additionally, five

replications were made per treatment per fungal species.

Phytochemical testing

Individual kawayang tinik plant extracts are subjected to qualitative phytochemical screening to determine the presence or absence of various phytocompounds using prescribed techniques and procedures (10, 29, 30). The presence of alkaloids was tested using different reagents (Mayer, Wagner, Bouchardat and Valser) while sterols and triterpenes were tested using the Lieberman's Buchard Test. The presence of flavonoids were otherwise confirmed using the Salkowski Test and Bate- Smith Metcalf test while the presence of cardiac glycosides were tested using the Keller- Killiani Test. Froth test was utilized to observed for the presence of saponin, gelatin test and ferric chloride test for the presence of tannins and phenolic compounds, Borntrager Test for the presence of antraquinone and Guignard Test for the possible presence of cyanogenic glycosides. The results of the phytochemical testing is presented in Table 3.

Table 1. Variations in the antifungal property of *Bambusa blumeana (*kawayang tinik) against *Aspergillus niger*

Extracts (1 mg/ml)	Zone of Inhibition (mm)
Ethanolic Roots	1.13a
Ethanolic Inner culm	1.04ab
Ethanolic Leaves	0.98ab
Ethanolic Outer culm	0.75ab
Fluconazole	0.55ab
Aqueous Outer culm	0.47ab
Aqueous Inner culm	0.41ab
Aqueous Rhizomes	0.22ab
Ethanolic Rhizomes	0.20b
Aqueous Roots	0.10b
Aqueous Leaves	0.09b
Water	0c
Fluconazole	0c
F-computed value = 3.74, F-critical value = 1.94, Probability = .0004	

(0.09 mm) extracts of *B. blumeana* (kawayang tinik) also showed lesser statistically comparable inhibitory activities against *A. niger*. It was further observed that the above- mentioned extracts are statistically comparable to Fluconazole, a pharmaceutically-approved medication and antifungal agent, at 1 mg/ml concentration. This would imply that the extracts of *B. blumeana* have the same inhibitory effect as the standard antifungal drug Fluconazole at 1 mg/ml drug concentration (Fig. 2).

Table 3. Phytochemical analysis of different *Bambusa blumeana* (kawayang tinik) extracts

Bioactive Compounds	Plant Extracts									
	Aqueous					Ethanolic				
	Leaf	Rhizome	Roots	Inner Culm	Outer Culm	Leaf	Rhizome	Roots	Inner Culm	Outer Culm
Alkaloid	+	+	+	+	+	+	+	+	+	+
Sterol and Triterpenes	+	-	-	+	+	+	-	-	+	+
Flavonoid	+	-	-	+	+	+	-	-	+	-
Cardiac glycoside	-	-	-	-	-	-	-	-	-	-
Saponins	-	-	-	-	-	-	-	-	-	-
Tannins and Phenols	+	+	+	+	+	+	+	+	+	+
Anthraquinone	-	-	-	-	-	-	-	-	-	-
Cyanogenic Glycoside	-	-	-	-	-	-	-	-	-	-

Results and Discussion

One-way Analysis of Variance (ANOVA) was used to determine the variation in the antifungal property of the various plant extracts derived from the different parts of *B. blumeana* (kawayang tinik).

Variations in the Antifungal Property of Bambusa blumeana (kawayang tinik) against Aspergillus niger

Table 1 presents the variations in the antimycotic potentials of *B. blumeana* (kawayang tinik) against *A. niger*. Results showed that there are highly significant variations among the *B. blumeana* (kawayang tinik) extracts against *A. niger* in terms of plant parts and the extraction solvents used since the F- computed value (3.74) is higher than the F- critical value (1.94) and the probability is less than 0.05.

The table further shows that in terms of the specific extracts derived from *B. blumeana*, the ethanolic root extract exhibited the highest comparable mean zone of inhibition (ZOI) at 1.13 mm against *A. niger*. This is followed by the extracts of ethanolic inner culm (1.04 mm), ethanolic leaves (0.98 mm), ethanolic outer culm (0.75 mm), aqueous outer culm (0.47 mm) and aqueous inner culm (0.41 mm). On the other hand, the ethanolic rhizome (0.20 mm), aqueous root (0.10 mm) and aqueous leaves

Various phytocompounds were observed to be present in different species of bamboo which could possibly explain the ZOI formed by the extracts

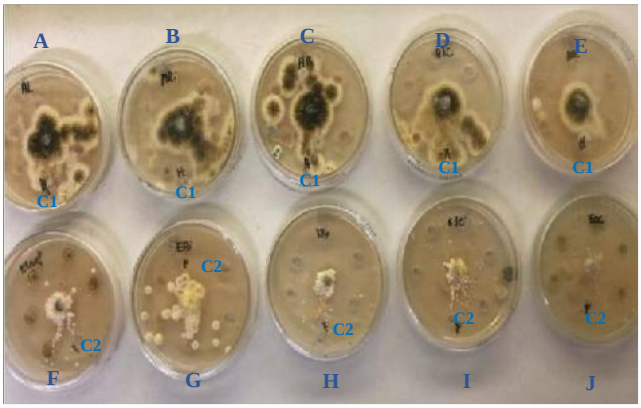

Fig. 2. *Aspergillus niger* growth on plates planted with various kawayang tinik extracts: **(A)** aqueous leaf, **(B)** aqueous rhizome **(C)** aqueous root, **(D)** aqueous inner culm, **(E)** aqueous outer culm, **(F)** ethanolic leaf, **(G)** ethanolic rhizome **(H)** ethanolic root, **(I)** ethanolic inner culm, **(J)** ethanolic outer culm, **(C1)** water and **(C2)** Fluconazole at 1 mg/ml.

against *A. niger*. For instance, apigenin, luteolin and p- coumaric acid were discovered in the different parts of *P. pubescence*, specifically in its leaf, root, rhizome and culms (16). These phytocompounds were noted to have the same antimicrobial mechanisms as that of the fluconazole, a pharmaceutically – approved antifungal drug. Fluconazole as an antifungal agent exhibits its action by increasing fungal cell wall permeability and disrupting the uptake of essential nutrients needed by the cell leading to eventual death (28). Through the application of chemotaxonomy principles which states that plants that are taxonomically related contains the same biochemical compositions (31, 32), we could consider the possibility that kawayang tinik extracts may contain the same phytocompounds hence, justifying its potential antifungal property and the ZOI produced by its individual extracts against *A. niger*. The antimycotic potentials of bamboo species could further be claimed through the identification of another antifungal protein known called Dendrocin (33). Its mechanism of action includes the formation of membrane channels, degradation of polymers in fungal cell wall or disruption of cellular ribosomes (34).

In terms of extraction solutions used in the study, it was observed that majority of ethanolic *B. blumeana* extracts exhibited better antifungal potential compared to aqueous extracts although statistical comparability is noted among these extracts at 1 mg/ml concentration. This could be associated with the polarity of the solvents used to extract the potential phytocompounds present in the different plant parts of *B. blumeana*. Water is a pure polar solvent thus, can only attract or extract polar compounds while ethanol is less polar than water hence, it can possibly attract both polar and non-polar compounds (35). The greater variety of phytocompounds which may be extracted via ethanolic extraction may provide explanation on the better inhibitory performance of ethanolic extracts compared to the aqueous extracts of kawayang tinik. Moreover, when plant sources are homogenized or extracted using the same protocols, the polarity of the solvent is the main factor to be considered (36, 37).

Variations in the Antifungal Property of Bambusa blumeana (kawayang tinik) against Penicillium chrysogenum

Table 2 presents the variations in the antifungal potential of *B. blumeana* (kawayang tinik) against *P. chrysogenum*. Results of the analysis revealed high significant variations in the antifungal potential of different *B. blumeana* (kawayang tinik) extracts against *P. chrysogenum* since the computed F- value (20.21) is higher than the F – critical value (1.94) and the probability is lower than 0.05.

Statistical analysis further revealed that the ethanolic root and leaf extracts of *B. blumeana* exhibited the highest statistically comparable antifungal potential against *P. chrysogenum* both with mean ZOI of 1.59 mm. These were followed by kawayang tinik extracts of ethanolic inner culm (0.48 mm), ethanolic rhizome (0.41 mm), ethanolic outer culm (0.12 mm), aqueous inner culm (0.06 mm) and aqueous rhizome (0.03 mm) which are comparable or having equal antifungal effects against P. chrysogenum at 1 mg/ml concentration. On the contrary, the aqueous extracts of leaves, roots and outer culm did not exhibit any effects against *P. chrysogenum* exhibiting mean ZOI of 0.00 mm (Fig. 3).

Table 2. Variations in the antifungal property of *Bambusa blumeana (kawayang tinik)* against *Penicillium chrysogenum*

Extracts (1 mg/ml)	Zone of Inhibition (mm)
Ethanolic Roots	1.59 a
Ethanolic Leaves	1.59 a
Ethanolic Inner culm	0.48 b
Ethanolic Rhizomes	0.41 b
Ethanolic Outer culm	0.12 b
Aqueous Inner culm	0.06 b
Aqueous Rhizomes	0.03 b
Aqueous Leaves	0 c
Aqueous Roots	0 c
Aqueous Outer culm	0 c
Water	0 c
Fluconazole	0 c
F-computed value = 20.21, F-critical value = 1.94. Probability = 1.75E-17=.0000	

Note: Means followed by the same letter/s are not significantly different at 5% level

Fig. 3. *Penicillium chrysogenum* growth on plates planted with various *kawayang tinik* extracts: **(A)** aqueous outer culm, **(B)** aqueous leaf **(C)** aqueous root, **(D)** aqueous rhizome, **(E)** aqueous inner culm, **(F)** ethanolic leaf, **(G)** ethanolic inner culm **(H)** ethanolic root, **(I)** ethanolic rhizome, **(J)** ethanolic outer culm, **(C1)** water and **(C2)** Fluconazole at 1 mg/ml.

As observed in the study, ethanolic extracts produced higher mean ZOI compared to the aqueous extracts of inner culm, rhizome, leaves, roots and outer culms that exhibited mean ZOI of 0.06 mm, 0.03 mm and 0.00 mm respectively. This result could still be associated to the polarity of extraction solutions used in the study whereby ethanol being less polar could harness both polar and non- polar compounds while water, a pure polar solvent, could only harness polar phytocompounds (37). Since ethanolic extraction could harness a wider variety of phytocompounds, better inhibitory potentials could be observed compared to aqueous extracts. Moreover, the better inhibitory performance of the ethanolic extracts could also indicate the possibility of greater proportions or amount of non- polar phytocomponents residing in the different plant parts of kawayang tinik especially that aqueous counterparts does not exhibit any inhibitory effects against *P. chrysogenum* (i.e. aqueous roots, leaves and outer culms producing mean ZOI of 0.00 mm).

The findings mentioned may also be correlated a previous study result wherein it was observed that phytocomponents from the leaf extracts of bamboo species *Phyllostachys* and *Moso* bamboo are not water soluble (38). However, the presence of polar phytocompounds in the different parts of *B. blumeana* could not be totally eliminated from the current study since aqueous extracts also exhibited zones of inhibitions against the previously discussed fungal species. It could be a possibility that the either *P. chrysogenum* is resistant to the polar compounds present in the different extracts of kawayang tinik or the concentration of extracts which is 1 mg/ml is inadequate to show inhibitory action against the said fungal species. Therefore, additional and more advanced studies should be conducted to fully determine the various phytocomponents present in the different parts of *B. blumeana* and how these phytocompounds relates to the antifungal potentials of kawayang tinik as well as devise further experimentations catering to different concentrations of extracts against fungal species.

The results of the study also coincide with previous findings citing that various phytocomponents were present in the different parts of the bamboo species *P. pubescence* (16). Specifically, apigenin and tricin derivatives were noted to be abundant in the root and leaf part of the said bamboo species. Antifungal mechanisms of these identified phytocompounds were noted to be fungal membrane and metabolic disruption (38, 39) the same inhibitory action of fluconazole, a standard antifungal drug (28).

Currently, there are no standard medical treatment of choice for the management of penicilliosis, however, antibiotics like amphotericin B, itraconazole, or fluconazole were reported to be effective against the said organism. The various ranges of inhibition zones exhibited by the ethanolic extracts of *B. blumeana* may indicate potential effectiveness of the said bamboo species in inhibiting the fungi *P. chrysogenum*, especially that the ethanolic root and leaf extracts exhibited more significant results compared to the standard antibiotic treatment Fluconazole at 1 mg/ml concentration. Moreover, other *B. blumeana* plant parts such as rhizomes and culms also exhibited potential antifungal activities as evidenced by the inhibition zones formed which further highlights the potential of the said bamboo species as source of potential antifungal drug against *P. chrysogenum*.

Phytochemical Testing

Table 3 shows the results of the phytochemical test involving different extracts of kawayang tinik. As shown, it is evident that all of the extracts contain bioactive compounds such as alkaloid, tannins and phenols while majority of the extracts also contains sterols and flavonoids. On the other hand, all of the extracts were screened negative for the presence of cardiac glycoside, saponins, anthraquinone and cyanogenic glycoside.

The presence of various phytochemicals in the ethanolic and aqueous extracts and their innate abilities to promote microbial inhibition may possibly verify the antimycotic potentials of kawayang tinik extracts. Alkaloid as a phytocompound exerts its antimicrobial mechanism via multitarget action such as outer membrane or cytoplasmic membrane disruption, cellular respiratory inhibition and nucleic acid synthesis or cell division inhibition (39, 40). Flavonoid, on the other hand, is able to promote inhibition of nucleic acid synthesis and disrupt the energy metabolism in pathogenic microorganisms (39). Tannins are also able to induce inhibition of bacterial and fungal enzymatic activity via direct interference on the microorganism's metabolism while triterpenes and sterols work by increasing the tolerance and resistance of plants to pathogen attacks, although the exact pathways and mechanisms are not yet known (41, 42).

Conclusion

In line with the objectives of the study, analysis and results, the following conclusions and implications were made:

1. *B. blumeana* (kawayang tinik) extracts showed varying ranges of antimycotic activity against *A. niger* and *P. chrysogenum.* Thus, kawayang tinik can be a potential source of natural pharmacotherapeutic products against these pathogenic fungal species.
2. Ethanolic extracts of roots and inner culm exhibited the highest mean zone of inhibition (ZOI) against *A. niger* while ethanolic root and leaf extracts exhibited better inhibitory potentials against *P. chrysogenum*.
3. *B. blumeana* (kawayang tinik) extracts contain phytocompounds such as alkaloid, sterols, flavonoids, tannins and phenol. Hence, further studies are needed to specifically harness the compounds producing antifungal effects.

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Conflict of interests

Author do not have any conflict of interests to declare.

References

1. Center of Disease Control and Prevention (CDC). Antibiotic/Antimivrobial resistance (AR/AMR) [Internet]. CDC 24/7; 2020 July 20 [cited 2009 Jan 8]. Available from: https://www.cdc.gov/drugresistance/index.html
2. World Health Organization (WHO). WHO list of bacteria for which new antibiotics are urgently needed [Internet]. WHO Online; 2017 February 27 [cited 2018 Oct 14]. Available from: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

3. Stuart GU. Philippine medicinal plant: kawayang tinik [Internet]. Philippine Medicinal Plant; 2018 January [cited 2018 Nov 30]. Available from: http://www.stuartxchange.org/Kauayan.html

4. Tantengco OAG, Condes MLC, Estadilla HHT, Ragragio EM. Ethnobotanical survey of medicinal plants used by Ayta communities in Dinalupihan, Bataan, Philippines. Pharmacogn J. 2018; 10(5): 859-70. https://doi.org/10.5530/pj.2018.5.145

5. Shukla R, Sumit G, Sajal S, Dwivedi PK, Mishra A. The medicinal importance of bamboo. International J Biopharm Phytochem Research. 2012;1(1):9-15.

6. Kalita C, Ganguly M, Devi A. Evaluation of antioxidant capacity and antimicrobial properties of ethnic Bambuseae species and identification of the active components. International Journal of Pharmaceutical and Biological Achieves. 2016.3(1):61-71.

7. Nguyen PT, Xuan TD, Ha PT, Tu anh TT, Khanh TD. Inhibitory effects of bamboo leaf on the growth of *Pyricularia grisea* fungus. Molecular Diversity Preservation International: Agriculture [Internet]. 2018 [cited 2018 Nov 30];8(92):1-8. https://doi.org/10.3390/agriculture8070092

8. Mahunu GK, Zhang H, Tibiru M, Qiya A, Xiaoyun Y, Zhao ZL. Bamboo leaf flavonoid enhances the control effect of *Pichia caribbica* against *Penicillium expansum* growth and Patulin accumulation in apples. Postharvest Biology and Technology [Internet]. 2018 [cited 2019 June 30];141:1-7. https://doi.org/10.1016/j.postharvbio.2018.03.005

9. Patil RN, Rothe SP. Antifungal activity of some fodder plants leaf extract. International Advanced Research Journal in Science, Engineering and Technology. 2016; 3(11):205-08.

10. Valentino MJG, Ganado LS, Ganado MR, Undan JR. Phytochemical screening and bio assay of the anti-microbial activity of three species of bamboo in Nueva Ecija, Philippines. Advances in Environmental Biology. 2015;9(24):389-96.

11. Darma R, Purnamasari M, Agustina D, Pramudito TE, Sugiharti M, Suwanto A. A strong antifungal-producing bacteria from bamboo powder for biocontrol of *Sclerotium rolfsii* in melon (*Cucumis melo* var. *amanta*). J Plant Pathol Microbiol [Internet]. 2016 [cited 2019 June 18];7(2):1-7. https://doi.org/10.4172/2157-7471.1000334

12. Shen X, Zheng D, Gao J, Hou C. Isolation and evaluation of endophytic fungi with antimicrobial ability from *Phyllostachys edulis*. Bangladesh Journal of Pharmacology [Internet]. 2012 [cited 2019 Jan 27];7(1):249-57. https://doi.org/10.3329/bjp.v7i4.12068

13. Owolabi MS, Lajide L. Preliminary Phytochemical screening and antimicrobial activity of crude extracts of *Bambusa vulgaris* Schrad. ex J. C. Wendl. (Poaceae) from Southwestern Nigeria. American Journal of Essential Oils and Natural Products. 2015;3(1):42-45.

14. Wasnik DD, Tumane PM. Antibacterial activity of *Bambusa bambose* L. against multiple drug resistant (MDR) bacteria isolated from clinical specimen. International Journal of Pharmaceutical Sciences. 2014;25(1):215-18.

15. Wangawar SN, Shendarkar GR, Shelke DP, Daswad AK, Pohare JG, Roge AB. Phytochemical screening and antimicrobial activity of *Dendrocalamus strictus* leaves extracts. World Journal of Pharmaceutical Research. 2017;6(4):1029-41. https://doi.org/10.20959/wjpr20174-8128

16. Tanaka A, Zhu Q, Tan H, Horiba H, Ohnuki K, Mori Y et al. Biological activities and phytochemical profiles of extracts from different parts of Bamboo (*Phyllostachys pubescens*). Molecules [Internet]. 2014 [cited 2018 June 30];1(19):8238-60. https://doi.org/10.3390/molecules19068238

17. Roxas CA. Bamboo research in the Philippines. In: Rao AN, Ramanatha Rao V, (ed). Proceedings of Training Course cum workshop Bamboo - Conservation, Diversity, Ecogeography, Germplasm, Resource Utilization and Taxonomy; 2015 May 10; Yunnan, China. International Plant Genetic Resources Institute. ISBN: 92-9043-414-7.

18. Ambika K, Rajagopal B. *In vitro* antimicrobial and antiproliferative activity of *Bambusa vulgaris*. International Journal of Pharmacy and Pharmaceutical Research. 2017;9(1):10-22.

19. Chuah EL, Zakaria ZA, Suhaili Z, Abu bakar S and Desa MNM. Antimicrobial activities of plant extracts against *Methicillin* – Susceptible and Methicillin – Resistant *Staphylococcus aureus*. Journal of Microbiology Research [Internet]. 2014 [cited 2018 June 30];4(1):6-13. https://doi.org/10.5923/j.microbiology.20140401.02

20. Selvamohan T, Ramadas V, Shibila S, Kishore S. Antimicrobial activity of selected medicinal plants against some selected human pathogenic bacteria. Pelagia Research Library Advances in Applied Science Research. 2012;3(5):3374-81.

21. Austria KC, Waing KGW, Valentino MJ. Anti-oxidant and antibacterial potentials of *Bambusa blumeana* J. A. and J. H. P Schultes and *Bambusa vulgaris* Schrad.ex Wendl. International Journal of Biology, Pharmacy and Allied Sciences. 2017;6(11):2175-88.

22. Kootstra AMJ, Huurman S. Extraction of steviol glycosides from fresh Stevia using acidified water: comparison to hot water extraction including purification. Application Center for Renewable Resources - Wageningen Plant Research. 2017;1:7-22.

23. Sule IO, Agbabiaka TO. Antibacterial effect of some plant extracts on selected Enterobacteriaceae. Ethnobotanical Leaflets. 2008;1(2):1035-42.

24. Lakshmanan K, Arumungam A, Mani R. Phytochemical screening and *in – vitro* antimicrobial activity of *Vitex negundo* L. var. *purpurascens* Sivar. and Mold Against Pathogenic Microorganisms. Drug Invention Today. 2012;4(12):667.

25. Islam MA, Alam MM, Choudhury ME, Kobayashi N, Ahmed MU. Determination of minimum inhibitory concentration (MIC) of cloxacillin for selected isolates of Methicillin- resistant *Staphylococcus aureus* (MRSA) with their antibiogram. Bangladesh Journal of Veterinary Medicine [Internet]. 2008 [Cited 2019 Nov 24];6(1):121-26. https://doi.org/10.3329/bjvm.v6i1.1350v

26. Magaldi S, Mata- essayag S, Hartung de capriles C, Perez C, Colella MT, Olaizola C et al. Well diffusion for antifungal susceptibility testing. Int J Infect Dis [Internet]. 2004 [cited 2018 June 30];1(8):39-45. https://doi.org/10.1016/j.ijid.2003.03.002

27. Scorzoni L, Benaducci T, Ameida AMF, Silva DHS, Da silva bolzani V, Gianinni MJSM. The use of standard methodology for the determination of antifungal activity of natural products against medical yeast *Candida* sp and *Cryptococcus* sp. Brazillian Journal of Microbiology [Internet]. 2007 [cited 2018 June 30];38(1):391-97. https://doi.org/10.1590/S1517-83822007000300001

28. Dash AK, Elmquist WF. Fluconazole. Analytical profile of drug substances and excipients [Internet]. 2001 [cited 2019 Sept 16];27:67-113. https://doi.org/10.1016/S1075-6280(01)27005-0

29. Coffie GY, Antwi-Boasiako C, Darkwa NA. Phytochemical constituents of the leaves of three bamboo (Poaceae) species in Ghana. Journal of Pharmacognosy and Phytochemistry. 2014;2(6):34-38.

30. Auwal MS, Saka S, Mairiga IA, Sanda KA, Shuaibu A, Ibrahim A. Preliminary phytochemical and elemental analysis of aqueous and fractionated pos extracts of *Acacia nilotica* (Thorn mimosa). Veterinary Research Forum. 2014;5(2):95-100.

31. Singh R. Chemotaxonomy: a tool for plant classification. Journal of Medical Plant Studies. 2016;4(2):90-93.

32. Hao DC, Gu XJ, Xiao PG. 3 - Taxus medicinal resources: a comprehensive study. Medicinal Plants: Chemistry, Biology and Omics [Internet]. 2015 [cited 2018 June 30];12(2):97-136. https://doi.org/10.1016/j.chmed.2020.03.002

33. Zhang JJ, Liu M, Li Y, Zhou T, Xu DP. Nutritional values and biological activities of bamboo shoots and leaves. International Journal of Food and Nutrition Safety. 2016; 7(2):98-108.

34. Wang HX, Ng TB. Dendrocin, a distinctive antifungal protein from bamboo shoots. Biochemical and Biophysical Research Communications [Internet]. Extending the benefits of antifungal proteins from plants. [Internet]. 2003 [cited 2019 Jul 30];307(3):750-55. http://dx.doi.org/10.1016/S0006-291X(03)01229-4

35. Zumdahl SS, Decoste DJ. Introductory Chemistry. Cengage Learning, Inc. United States of America; 2009. p. 477.

36. Truong DH, Nguyen DH, Ta NTA, Bui AV, Do TH, Nguyen HC. Evaluation of the use of different solvents for phytochemical constituents, antioxidants and *in vitro* anti-inflammatory activities of *Severinia buxifolia*. Journal of Food Quality [Internet]. 2019 [cited 2019 Jul 16]; 1(1):1-9. https://doi.org/10.1155/2019/8178294

37. Pandey A, Tripathi S. Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. Journal of Pharmacognosy and Phytochemistry. 2014;2(5):115-19.

38. Toan NP, Xuan TD, Thu ha PT, Tu anh TT, Khanh TD. Inhibitory effects of bamboo leaf on the growth of *Pyricularia grisea* fungus. Plant Disease Control and Agriculture [Internet]. 2018 [cited 2019 June 30];8(7):92. https://doi.org/10.3390/agriculture8070092

39. Cushnie T, Lamb JA. Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents [Internet]. 2005 [cited 2018 June 30];26(5):343-56. http://dx.doi.org/10.1016/j.ijantimicag.2005.09.002

40. Lee H, Woo ER, Lee DG. Apigenin induces cell shrinkage in *Candida albicans* by membrane perturbation. FEMS Yeast Research [Internet]. 2018 [cited 2019 Ju 19];18(1):1-9. https://doi.org/10.1093/femsyr/foy003

41. Marutescu L, Popa M, Saviuc C, Lazar V, Carmen Chifiriuc M. 8- Botanical pesticides with viricidal, bactericidal and fungicidal activity. In: Grumezescu AM, (ed). New Pesticides and Soil Sensors. Oxford, UK: Academic Press. 2017. p. 311–35. https://doi.org/10.1016/B978-0-12-804299-1.00009-6

42. Wafa N, Sofiane G, Mouhamed K. The antioxidant and antimicrobial activities of flavonoids and tannins extracted from Phlomis bovei De Noé. European Journal of Experimental Biology. 2016;6(3):55-61.

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

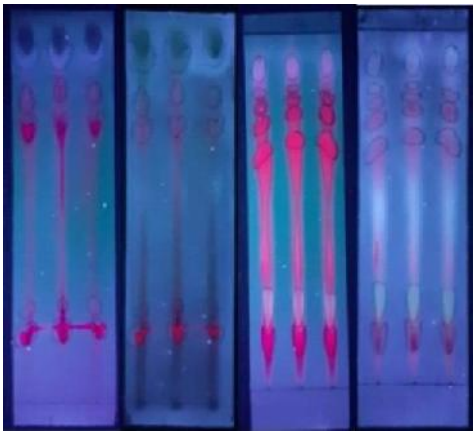
mixture of compounds through suitable chromatographic techniques. In the present study, flavonoids, saponin, tannin and terpenoid are confirmed to be present.

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)

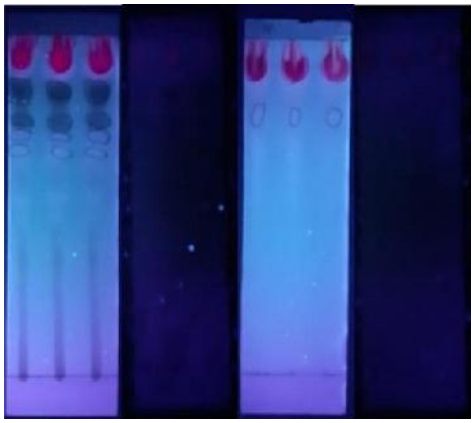
Figure 1. Photograph of TLC of flavonoid (a) Indonesian (b) Indonesian (after spray) (c) Philippines (d) Philippines (after spray).



(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

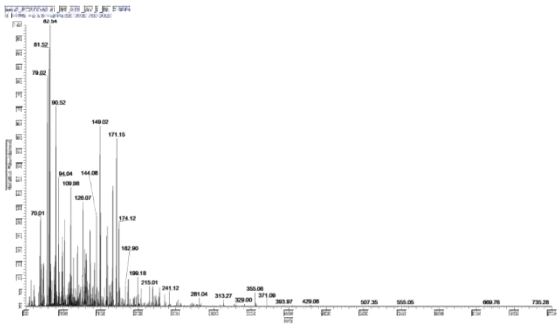


Figure 5. LC-MS chromatogram of ethanolic extract of *Azadirachta indica* varian Indonesia leaves.

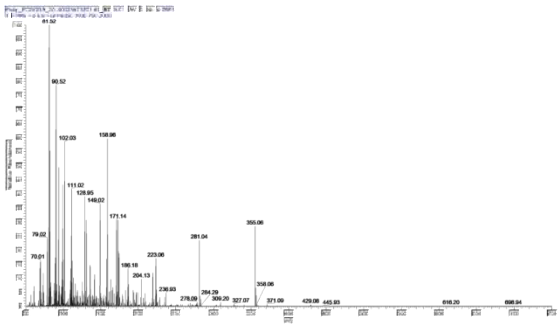


Figure 6. LC-MS chromatogram of ethanolic extract of *Azadirachta indica* varian Philippines leaves.

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.

Reference

[1] Beltran M A G, Robertson A and Martin R J 2019 The Effects of Plant Leaves Variants from The Philippines on Infective *Oesophagostomum dentatum* Larvae., Veterinary Biomedical and Clinical Journal 1

[2] Gennaro A R 2000 *Remington: The Science and Practice of Pharmacy*, 20th ed, Vol. II, Mack Publishing Company, Pennsylvania, 1016

[3] Jagadeesh K, Srinivas K and Revankar S P 2014 Anti Inflammatory Effect of Azadirachta Indica (Neem) In Albino Rats-An Experimental Study IOSR Journal of Pharmacy 4

[4] Krug D, Zurek G, Revermann O, Vos M, Velicer G J and Muller R 2008 Discovery the hidden secondary metabolome of Myxococcus xanthus: A study of intraspecific diversity *Appl. Environ. Microbiol.* **74** 3058-68

[5] Lloyd A C C, Menon T and Umamaheshwari K 2005 Anticandidal activity of Azadirachta indica, Indian Journal of Pharmacology **37**

[6] Pingale S S 2010 Hepatoprotection Study of Leaves Powder of *Azadirachta Indica* A. Juss., International Journal of Pharmaceutical Sciences Review and Research

[7] Rojas-Sandoval J and Acevedo-Rodriguez P 2014 *Azadirachta indica* (neem tree) Invasive Species Compendium (Wallingford, UK: CAB International)