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# An In Vitro Investigation of the Biological Activity of Alim, *Melanolepis multiglandulosa, Euphorbiaceae* Leaf Crude Extract for Wound Healing

**Denilyn J. Lazo*[1,2], Adeltrudes B. Caburian[1]**
[1]School of Advanced Studies, Saint Louis University, Baguio City, Philippines
[2] Department of Pharmacy, University of the Assumption, Pampanga, Philippines

*Corresponding Author Email: denilynjaramillalazo@gmail.com

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**Abstract**. Wounds are a significant issue in the Philippines due to the increasing cases of diabetes, a growing elderly population, and antimicrobial resistance. This situation has led to a high burden of care for infected wounds, diabetic foot ulcers, and burns within local hospitals. Wound healing is a vital physiological process that reestablishes the integrity of damaged tissue after an injury. Alim, *M. multiglandulosa* leaves, which are not well known for their medicinal benefits, were collected from Bacnotan, La Union, authenticated, dried, and extracted with absolute ethanol, then filtered through rotary evaporation. The crude extracts were investigated for biological activities in vitro. In disc diffusion and resazurin assays against *S. aureus*, 100% and 75%w/v exhibited antibacterial activity. It also prevented protein denaturation (87.46%w/v) with an $IC_{50}$ (half-maximal inhibitory concentration) of $2.70 \times 10^{21}$ g/mL using the protein denaturation inhibition assay, and it provided 87.71%w/v protection of RBC in 1g/mL using the HRBC (human red blood cell) membrane stabilization technique, signifying anti-inflammatory. In DPPH (1,1- diphenyl-2, picrylhydrazyl) radical scavenging, there was 79.05% and 95.88% antioxidant activity at 0.0625 g/mL and 1g/mL, respectively, with an $IC_{50}$ value of 0.907 mg/mL. A substantial correlation was found between TFC (total flavonoid content, 1.78 mg QUE/g extract) and TPC (total phenolic content, 357.08 mg GAE/g) and ferric reducing antioxidant power (FRAP) assay showed values of 9.48 and 12.33 µM $FeSO_4$/g extract in 200 and 400 µL/mL extract, respectively, indicating good antioxidant activity. Additionally, it contains sterols, triterpenes, flavonoids, alkaloids, saponins, tannins, and polyphenols, which are responsible for its biological activities. *M. multiglandulosa* demonstrated its value as a plant-derived substitute for wound healing. Further studies are needed for isolating bioactive compounds for recommended drug preparation, in vivo evaluation, and efficacy determination in clinical trials.

**Keywords:** Antibacterial; Anti-inflammatory; Antioxidant; Wound healing property.

## 1.0 Introduction

In the Philippines, where injuries are among the leading causes of morbidity and mortality, a systematic epidemiological review covering the years 2011 to 2018 recorded a total of 668,179 injury cases, with open wounds constituting 37.56% of all cases. These cases lead to higher rates of chronic wounds, mainly due to a growing geriatric population and an increased prevalence of diabetes, which saw about 4.3 million cases in adults in 2021. It highlights that the injury burden could be better managed with more resources and improved healthcare

facilities, particularly in areas with high population density (Sumalapao et al., 2020). Diabetic foot ulcers comprise 16-20% of yearly emergency room visits at the Philippine General Hospital (Philippines Wound Care Market Statistics & Forecast 2032, n. d.). These numbers underscore the need for effective and accessible wound care measures nationwide. A study conducted at the University of the Philippines- Philippine General Hospital (UP-PGH) Burn Center found a significant prevalence of multidrug-resistant organisms in burn wound infections. This observation highlights the critical need for novel antimicrobial drugs to effectively treat wound infections (Abesamis & Cruz, 2019). The increasing incidence of antibiotic-resistant bacteria complicates wound care, prompting the development of new treatment drugs that target wound healing. Wound healing is a vital physiological process that restores the integrity of damaged tissues after an injury. The complex process consists of several continuous phases: hemostasis, inflammation, proliferation, and remodeling. Disruptions in any of these phases, such as those caused by infection, chronic diseases, or inadequate healthcare, can result in delayed healing, chronic wounds, and an increased risk of complications (Guo & DiPietro, 2010). It includes hypoxia, ischemia, and reperfusion damage (Wallace et al., 2023). Additionally, it may be aggravated by a local pressure gradient, tissue edema, collagen synthesis modulation, and infection. Chronic non-healing wounds, like diabetic foot ulcers, venous leg ulcers, and pressure ulcers, are severe when they present and indicate an imperative need for intervention (Nunan et al., 2014).

This case may lead to developing novel drugs from plants with bioactive components since they have long been used in traditional Filipino medicine. One such plant is the Alim tree, *Melanolepis multiglandulosa, Euphorbiaceae,* indigenous to the Philippines. The tree, which requires just direct sunlight and minimum irrigation, has been shown to have antiviral, antioxidant, and antihyperglycaemic qualities. The leaves, composed of alkaloids, flavonoids, saponins, tannins, and petals, are traditionally used (NPDC, 2023). In addition to the study of Liu Hongshan, *Melanolepis multiglandulosa* (Reinw.) Reichb. f. et Zoll. (*Euphorbiaceae*) found that methanolic extracts of the plant's roots contained friedelin, oleanolic acid, olean-12-en-3β,28-diol, β-amyrin acetate, 6β-hydroxystigmast4-en-3-one, stigmast-4-en-3-one, stigmast-4,22-dien-3-one, and 5α-stig to treat insect bites and inflammatory symptoms. It was traditionally used to alleviate inflammation by applying the leaves to the affected area. This provided relevant information that it may have benefits.

Furthermore, only a few studies have recognized this plant for its antiviral, antioxidant, and antihyperglycaemic activities. It has also been acknowledged as a source of biodiesel and a standard synthesis of seed oil methyl esters for nonmedical uses (Knothe et al., 2017). As a result, this study evaluated the wound healing activity related to the antibacterial, anti-inflammatory, and antioxidant properties of Alim, *Melanolepis multiglandulosa* leaf extract, potentially linked to various phytochemicals discovered in prior research. Additionally, the confirmation of its phytochemical constituents was screened. These findings may contribute to developing plant-derived wound healing products that are both effective and culturally acceptable in the Philippines.

## 2.0 Methodology

### 2.1 Research Design

The study utilized an experimental research design based on Em (2024), a scientific method for determining cause-and-effect correlations between variables. In this study, different concentrations of Alim crude extract were examined in vitro for antibacterial, anti-inflammatory, and antioxidant properties.

### 2.2 Research Locale

The study was conducted in the Philippines, specifically in Purok 2, San Martin Bacnotan, La Union, for plant collection, and at Don Mariano Marcos Memorial State University-North La Union Campus for both plant authentication (College of Agroforestry and Forestry) and in vitro tests (College of Veterinary Medicine-Laboratory).

### 2.3 Research Materials

The materials used include equipment and glassware, such as a blender, centrifuge, drying oven, incubator, pH meter, UV-vis spectrophotometer, water bath, 96-well plate, and centrifuge tubes. The reference drugs and chemical reagents are Alsever solution, aluminum chloride hexahydrate, deionized water, diclofenac, distilled water, egg albumin powder, Folin-Ciocalteu reagent, FRAP reagents (sodium acetate buffer, TPTZ solution, HCl solution, ferric chloride), gallic acid, gentamicin, human red blood cells, ibuprofen, isosaline, L-ascorbic acid, potassium acetate, quercetin, resazurin dye, sodium carbonate solution, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) reagent, and 95% ethanol.

**2.4 Research Procedure**

The plant collection was adapted from Razmavar (2014), in which collected plants were cleansed, disinfected, rinsed, and dried before being pulverized into powder. The powder was soaked in ethanol for 7 days, then filtered and extracted. The ethanolic extract was collected and evaporated with a rotary evaporator to obtain crude extracts. The crude extract underwent phytochemical and in vitro testing. Alim crude extracts were diluted with distilled water to achieve various concentrations (25%, 50%, 75%, 100% w/v).

In the phytochemical screening, the crude extract was analyzed for bioactive compounds, including triterpenes, sterols, flavonoids, alkaloids, saponins, tannins, and polyphenols, following Trease and Evans (2002) published methods. The following tests were conducted: (a) Liebermann-Buccard test for unsaturated sterols and triterpenes, (b) Shinoda's test for flavonoids, (c) Mayer's test for alkaloids, (d) Frothing test for saponins, (e) Ferric chloride test for tannins, and (f) Ferric chloride test for polyphenols.

The Razmavar et al. (2014) method was used to evaluate antibacterial activity using the Kirby-Bauer disc diffusion test. Before being applied to the bacterial lawn in *Staphylococcus aureus*, all discs are thoroughly soaked in Alim extracts (25%, 50%, 75%, and 100% w/v), distilled water (negative control), and gentamicin (positive control) and dried. The incubation period is 24 hours at 35°C ± 2°C. The diameter of the inhibition zone (IZ) around the discs is measured to determine antibacterial activity. The test is performed three times. The average zone of inhibition diameters (mm) obtained from the leaf extract are used to calculate antibacterial activity.

The resazurin assay uses resazurin dye, a colorimetric assay based on redox, which determines the cellular metabolic reduction that causes the least degree of cell toxicity. A 96-well microtiter plate was filled with *S. aureus* cultivated in Muller Hinton broth (MHB) and Alim crude extracts (1:1 volume/volume). 50 µL of *S. aureus* was mixed with 50 µL of Alim crude extracts (100%, 75%, 50%, 25% w/v) and gentamicin. The microtiter plates were covered and incubated at 37 °C for 24 hours. After incubation, all wells were inoculated with 30 µL of resazurin dye, and the plates were placed in the incubator for 2 hours to observe the color created (Mohammed et al., 2020). Adapted from Sarker et al. (2007), shows the control and test groups on a 96-well plate; pink shows growth, while blue indicates growth inhibition.

The anti-inflammatory activity was evaluated in vitro by inhibiting the denaturation of egg albumin (protein) (Kumarasinghe et al., 2018). The absorbance of the reaction mixture (Alim extracts as treatment and ibuprofen as the positive control) was measured at 280 nm using a proper UV/Vis spectrophotometer, with distilled water as the blank. Each test was repeated three times, and the average absorbance was recorded. The percentage of protein inhibition was calculated using the following formula:

Percentage inhibition = [(absorbance of control - absorbance of test sample)/absorbance of control] x 100

The crude extract/positive control concentration for 50% inhibition (IC50) is calculated by plotting percentage inhibition against concentration.

The addition of the anti-inflammatory effect of Alim crude extracts was studied using an in vitro HRBC (human red blood cell) membrane stabilization technique adopted by Chowdhury et al. (2014). The collected blood was diluted with an equal volume of Alsever solution (dextrose 2%, sodium citrate 0.8%, citric acid 0.05%, sodium chloride 0.42%, and distilled water 100 mL) and centrifuged with isosmotic saline. To 1 mL of the HRBC suspension, an equal volume of treatment control in four concentrations (1, 0.75, 0.5, and 0.25 g/mL) was added. All assay mixtures were centrifuged after being incubated for 30 minutes at 37°C. The hemoglobin content of the supernatant solution was measured using a UV/Vis spectrophotometer at 560 nm. The values were calculated as follows:

(a) percentage hemolysis = (OD of test / OD of control) x 100, and,
(b) Percentage protection = [(100 - OD of test) / OD of control] x 100.

The "OD of the test" indicates the optical density or absorbance of the test sample, whereas the "OD of control" represents the optical density or absorbance of the negative control. Alsever's solution with blood was the negative control and did not contain diclofenac or Alim crude extract.

The total flavonoid content is identified using the aluminum chloride colorimetric method. 0.5 mL of the extract solution (0.5 mg/mL) is mixed with 0.1 mL of 10% aluminum chloride hexahydrate, 0.1 mL of 1 mol/L potassium acetate, and 2.8 mL of deionized water. After 40 minutes of incubation at room temperature, the blank, standards, and samples are transferred to cuvettes, and the reaction mixture's absorbance at 415 nm is measured against a blank with a UV-VIS spectrophotometer. Quercetin is used as a reference molecule at 10 concentrations ranging from 10 to 100 µg/mL. Results are given as mg/g quercetin equivalents (mg QuE/g). Each sample is measured three times, and the mean value is used (Kaptaner İğci & Aytaç, 2020).

The total phenolic content is determined by the Folin-Ciocalteu technique. Gallic acid is the reference ingredient to develop a standard curve (10 different concentrations ranging from 10 to 100 µg/mL). 0.5 mL of extracts (1 mg/mL) is mixed with 2.5 mL of diluted Folin-Ciocalteu reagent (1:10) and 2 mL of sodium carbonate solution (7.5% w/v) and left to stand at 45°C for 15 minutes. Blanks, standards, and samples are transferred to cuvettes and measured with a UV-VIS spectrophotometer at 765 nm. Each sample is measured three times, and a mean value is obtained. The data is expressed in milligrams of gallic acid equivalents per gram (mg GAE/g) (Kaptaner İğci & Aytaç, 2020).

The DPPH radical scavenging test was performed with 1,1-diphenyl-2-picrylhydrazyl (DPPH). The crude extracts were produced in methanol at six concentrations (0.0625, 0.125, 0.25, 0.5, 0.75, and 1 g/ml). L-ascorbic acid, a standard antioxidant, was produced in the exact amounts. Transfer 1 ml of each crude extract to a clean test tube. Add 0.5 ml of 0.1 mM DPPH in methanol. The mixture is shaken and left to stand in the dark at room temperature for 15 minutes. Blank solutions with 2.5 mL crude extract and 1 mL methanol were used as a baseline. The negative control was 2.5 ml of DPPH solution and 1 ml of methanol, whereas the positive control was L-ascorbic acid at the same concentrations as the crude extracts. After a dark incubation at room temperature, absorbance was measured at 517 nm using a spectrophotometer. This is repeated three times. The DPPH radical scavenging activity is determined using the equation (Guchu, 2020):

% Radical scavenging activity = [(Ac-As)/Ac]x100, where As is the sample absorbance and Ac is the control absorbance.

The crude extracts' half maximum inhibitory concentration (IC50) is calculated by plotting the percentage of DPPH free radical inhibition against extract concentration.

The ferric-reducing antioxidant power (FRAP) assay used the Benzie and Strain (1996) method. FRAP reagent was freshly produced by mixing solutions 'a', 'b', and 'c'. 300 mM sodium acetate buffer, pH=3.6 (a), 10 mM TPTZ solution in 40 mM HCl solution (b), and 20 mM ferric chloride ($FeCl_3$) solution (c) in a 10:1:1 (v/v/v) ratio. The reaction was done in the dark for 30 minutes. In the test, 0.1 mL of plant extracts, positive control ascorbic acid (50, 100, 200, and 400 µg/mL), and $FeSO_4$ (329.16, 658.3, 1316.6, and 2633.3 mM) were mixed with 2.9 mL of FRAP reagent separately. A blank (control) containing 0.1 mL of pure water was used. All samples were made in triplicate, vortexed for 1 minute, then incubated in the dark for 30 minutes at 37 degrees Celsius. The increase in absorbance of the reaction mixture was measured for each sample using a UV-visible spectrophotometer at 593 nm. The results were compared to ascorbic acid as a positive reference, and $FeSO_4$ was utilized to calibrate. FRAP activity was calculated regarding ferrous equivalent (FE) in mM.

**2.5 Data Gathering Procedure**

This study used specific steps to identify phytochemical constituents and potential biological activities. These include handpicking and cleaning the leaves of Alim (*Melanolepis multiglandulosa*) before plant authentication. It was used for plant extraction by maceration (ethanol), followed by passing through a rotary evaporator to produce concentrated, crude extracts. Subsequently, various 25%, 50%, 75%, and 100% w/v concentrations were prepared for in vitro testing to compare their biological activities, including antibacterial, anti-inflammatory, and antioxidant activities, which are typically recognized in established literature for potential wound healing. This is done to identify the concentration that yields the highest biological activity. In addition, total flavonoid and phenolic content were determined, which contributed to antioxidant capacity.

**2.6 Data Analysis**

The results are presented as means derived from triplicate measurements. Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS), version 25. Analysis of Variance (ANOVA) and Tukey's

Honest Significant Difference (HSD) test were utilized for data that followed a normal distribution and demonstrated homogeneity of variance. In cases where the variance was not homogeneous, the Kruskal-Wallis test was employed. Statistical significance was established at $p \leq 0.05$.

### 2.7 Ethical Considerations

This research study followed ethical guidelines. The study has been approved by the Saint Louis University Research Ethics Committee, protocol number SLU-REC 2024-272, adhering to the significant protocols, which involved no human participation. Thus, no informed consent was required. Wildlife Gratuitous Permit No. 2025-001 from the Department of Natural Resources and Barangay certification was secured for plant collecting, as plants are not considered wildlife, to ensure environmental compliance and the sustainable use of wildlife resources. The protocol for the in vitro tests and laboratory rules was strictly followed.

## 3.0 Results and Discussion

### 3.1 Determination of Phytochemical Screening

The crude leaf extract of Alim was analyzed phytochemically to determine the presence of biologically active substances. The results are presented in Table 1.

**Table 1.** *Phytochemical constituents present in Alim, Melanolepis multiglandulosa, Leaf Crude Extract*

| Plant Constituents | Test Results |
|---|---|
| Triterpenes | + |
| Sterols | + |
| Flavonoids | + |
| Alkaloids | + |
| Saponins | + |
| Tannins | + |
| Polyphenols | + |

According to Agra et al. (2015), triterpenes have been reported to significantly affect the modulation of reactive oxygen species (ROS) production at the wound site, and, as such, they assist in tissue repair. Further, triterpenes enhance cell migration, promote proliferation, and induce collagen synthesis. Despite the broad research on triterpene pharmacological action, knowledge of its action on controlling cell types involved in the healing process, like keratinocytes and fibroblasts, is still limited. Research by Lukić et al. (2021) also showed that sterols can accelerate wound healing and decrease inflammatory bowel disease symptoms in mouse models. Among these compounds, flavonoids are distinguished by their powerful antioxidant properties that protect the cells against oxidative stress, thus helping wound healing. The study of Zulkefli et al. (2023) also contained anti-inflammatory characteristics that can further enhance the healing process. Fetse et al., in a 2014 study, showed that the alkaloidal extract obtained exhibited significant wound healing activity, reflected in its enhanced rate of wound contraction and thereby a reduction in the period of epithelisation. Also, the antibacterial action of the crude total alkaloid is an integral part of its therapeutic action. Saponin compounds also showed antimicrobial activity, which may be helpful in the prevention of infection in wounds. Polyphenols are known for their pro-inflammatory effects, which can be highly useful for attracting inflammatory cells to the place of inflammation, thus accelerating the healing process and allowing the proliferation and migration of endothelial cells and fibroblasts. As per the previous study by Su et al. (2016), total tannin extract significantly enhanced wound healing with comparable efficacy to that of Bactroban.

The combined effect of these phytochemicals indicates that the crude leaf extract from the Alim tree could be a potential source of natural compounds in developing topical formulations designed to enhance wound healing. Through scientific evidence, this study confirms the traditional use of Alim in folklore medicine as a wound healer by its phytochemical composition and potential therapeutic uses.

### 3.2 Determination of Antibacterial Activity

The results of the antibacterial activity of Alim crude extract against *Staphylococcus aureus* in terms of zone of inhibition in millimeters and color change are presented in this study (see Table 2). This was conducted at 24 hours of incubation, and the measurements are provided for each concentration of Alim leaf crude extract (100%, 75%, 50%, and 25%) and the control group. The Alim leaf crude extracts, with 100% and 75% concentrations, showed less resistant activity against *Staphylococcus aureus* than gentamicin, a positive control. Additionally, there were significant differences in antibacterial activity between the Alim crude extracts and the control antibiotic, as

indicated by a p-value of $1.86 \times 10^{20}$ ($p > 0.05$). According to several studies, extracts from different species of Euphorbia have shown antibacterial and antifungal activities against various microorganisms, including *S. aureus* (Kirbag et al., 2013). The use of plant extracts from the family *Euphorbiaceae* in traditional medicine is regarded as very common, and research has evaluated these extracts in Akwa Ibom State, Nigeria, on in vitro antimicrobial activity against *S. aureus* (Uduak & Kola, n.d.).

**Table 2.** *Antibacterial activity in terms of zone of inhibition (mm) and color change against S. aureus*

| Control/Treatment Groups | Zone of Inhibition (average mm) | Colour Change |
|---|---|---|
| 100% leaf crude extract | 9.3 | Blue |
| 75% leaf crude extract | 8.3 | Blue |
| 50% leaf crude extract | 0 | Pink (dark) |
| 20% leaf crude extract | 0 | Pink (dark) |
| Positive (gentamicin) | 32 | Blue |
| Negative (distilled water) | 0 | Pink |

The color changes developed in the resazurin assay represented the metabolic activity of bacteria in response to the control groups and crude extracts (see Table 2). The color change to blue is used as a visual marker in determining the minimum inhibitory concentration of the crude extract against the tested bacterial strains. A color change from blue to pink portrays the presence of living cells, and the remaining blue wells depict substances that kill bacteria. The negative control changed color to pink, meaning there was significant microbial growth, whereas 100% and 75% concentrations and the positive control remained blue. This now serves as an indicator of the antibacterial activity of the Alim leaf crude extract. Consistent with the findings of Teh et al. (2017), the resazurin assay proved to be a straightforward, reliable, and reasonable method for preliminary screening to evaluate the antibacterial properties of fly larval extract. Quite clearly, confirming these results has shown that the *L. cuprina, S. peregrina*, and *M. domestica* extracts of the fly larvae indeed show their antibacterial activities against *MRSA*, with *L. cuprina* showing the maximum antibacterial activity against gram-positive bacteria like *S. aureus* and *MRSA* and gram-negative bacteria like *P. aeruginosa and E. coli*. Further, Madushan et al. (2021) showed that the reduction of the resazurin dye approach occurs due to an increased microbial load in milk and a decrease in oxygen amount, as well as through an oxidation-reduction process. Also, the color of resazurin dye changes with the oxidation-reduction activities occurring in milk caused by microbial activity.

### 3.3 Determination of Anti-Inflammatory Activity

The albumin denaturation inhibition assay remains one of the most common methods to assess various compounds' anti-inflammatory and protein-stabilizing activities. Generally, the extent of inhibition of albumin denaturation is taken as an index of a drug's therapeutic efficiency since protein denaturation is one mechanism by which inflammation and tissue damage occur.

**Table 3**. *Anti-inflammatory activity in terms of inhibition of albumin denaturation activity (%), protection (%), and hemolysis (%)*

| | Inhibition of albumin denaturation activity (%) | Protection (%); Haemolysis (%) | |
|---|---|---|---|
| Concentrations (g/ml) | Alim crude extract | Positive Control | Alim crude extract |
| 0.25 | 86.37 | 79.97; 20.03 | 71.08; 28.92 |
| 0.50 | 86.71 | 83.55; 16.45 | 82.86; 17.14 |
| 0.75 | 87.13 | 86.86; 13.14 | 86.09; 13.91 |
| 1 | 87.46 | 89.98; 10.02 | 87.71; 12.29 |

**Note:** The denaturation of albumin, an activity of the ibuprofen, a positive control (0.01 g/mL), is 94.27%.

In Table 3, ibuprofen exhibited a high inhibition rate of 94.27%, which assures one of its high effectiveness as an NSAID; this supports highly described pharmacological features of ibuprofen, whose primary mechanism may be in the inhibition of the COX enzymes to limit the synthesis of pro-inflammatory mediators of inflammation, as identified by Ngo & Bajaj, 2024. In contrast, Alim crude extract has varying inhibition percentages, at 87.46% for its highest concentration. This reflects that Alim has good anti-inflammatory activity, although somewhat less than that of ibuprofen. Even at lower concentrations such as 75% (0.75 g/ml), 50% (0.50 g/ml), and 25% (0.25 g/ml), its inhibition rates are pretty significant, which suggests substantial anti-inflammatory activity for the extract at a reduced dosage. These data show that the inhibition, across the different concentrations of the Alim extract, is relatively stable, with percent inhibition fluctuations on a tiny scale. This could mean that the active constituents present in the extract may have a strong inherent capacity to inhibit albumin denaturation and that this is independent of concentration. The inhibition decreases modestly from 100% to 25% concentration, from 87.46% to 86.37%. This further implies that, whereas the extract is effective, its effectiveness is not as vital in terms

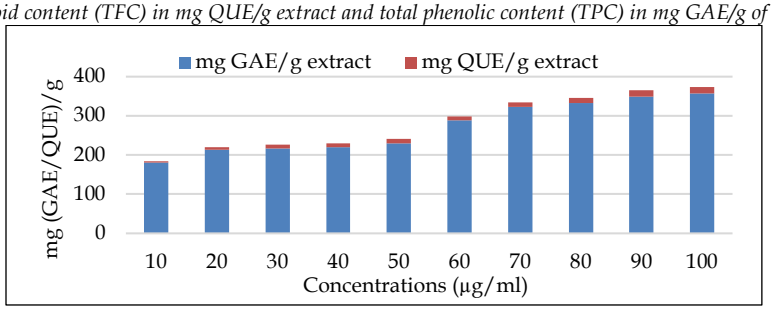
of concentration as is noted in synthetic NSAIDs like ibuprofen. The $IC_{50}$ value of $2.70 \times 10^{21}$ g/ml is considerably low; hence, the alim crude extract possesses a high inhibitory activity. The anti-inflammatory activities of Alim extract can be attributed to its diverse phytochemical profile, including flavonoids and tannins, which are known for playing critical roles in modulating inflammatory pathways. For instance, flavonoids have been on record to inhibit the formation of pro-inflammatory cytokines and reduce oxidative stress, thus further potentiating their anti-inflammatory effects (Beg et al., 2011). However, this is made evident in the statistical analysis. The treatment and control groups show no significant difference, with a p-value of 0.406 ($p>0.05$).

One of the general methods of assessing the anti-inflammatory potential of various extracts involves their capability for protecting red blood cells from lysis, induced either by hypotonic solution or heat. This assay works based on the principle that the stabilization of the HRBC membrane has an approximate similarity in effect with the stabilization of lysosomal membranes, which is vital in preventing an inflammatory response. The data indicated that the crude extracts and standard diclofenac sodium significantly prevented the occurrence of hemolysis since the percentage of hemolysis decreases with increased concentration of the extract (see Table 4). Furthermore, it is observed that the maximum percentage protection for all crude extracts was 87.71% at the highest concentration of 100%, and that of diclofenac sodium is 89.98%. While both extracts and diclofenac sodium offered significant protection, extracts showed marginally lower efficacy than the standard drug at the highest concentration. Similarly, as in the above test, the statistical analysis showed no significant difference among the groups with a p-value of 0.317 ($p > 0.05$). This implies that while the extracts possess anti-inflammatory properties, they may not be effective compared to diclofenac sodium, a known nonsteroidal anti-inflammatory drug. This is similar to how Chowdhury et al. (2014) stated that the methanolic extract of leaves of *Gardenia coronaria* at a concentration range of 100 µg/mL to 300 µg/mL protects human erythrocyte membranes against lysis induced by hypotonic solution. At 100 µg/mL, the extract inhibited 24.38% of RBC (red blood cell) hemolysis compared to 38.84% produced by NSAID (aspirin) at 100 µg/mL. This leaf extract can inhibit HRBC hemolysis in a significant and dose-dependent manner. Moreover, the anti-inflammatory test induced by heat showed that the crude ethanolic extract of *P. chaba* at 500 µg/ml and positive control ASA (aspirin) at 500 µg/ml exert 52.67% and 78% inhibition, respectively. In contrast, the hypotonicity-induced anti-inflammatory test revealed 35.67% and 59% inhibition of RBC hemolysis, respectively. At the lower dose of 100 µg/ml, the crude ethanolic extract of *P. chaba* exhibited almost identical activity to that of the reference standard (Yesmin et al., 2020).

**3.4 Determination of Antioxidant Activity with Total Flavonoid and Total Phenolic Content**

The results evidence that as the concentration of Alim crude extract increases, the total flavonoid content (TFC) increases (see Figure 1). TFCs ranged from 3.83 mg QUE/g extract at 10 µg/mL to 16.85 mg QUE/g extract at 100 µg/mL. This is normal for bioactive compounds as they accumulate in plant extracts with increased extract concentration. Flavonoids are well-recognized antioxidants; the higher the concentration, the better the antioxidant activity. The standard deviation values for each concentration are small, proving that the measurements are reliable and the assay is consistent. Minimum and maximum SD values range from 0.03 mg QUE/g extract (10 µg/mL) to 1.78 mg QUE/g extract (90 µg/mL).

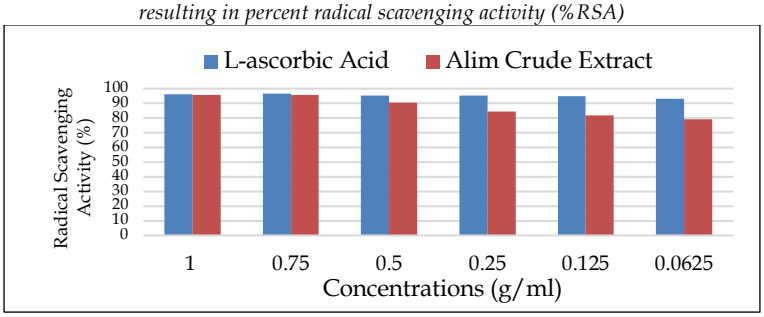
**Figure 1.** *Total flavonoid content (TFC) in mg QUE/g extract and total phenolic content (TPC) in mg GAE/g of the alim crude extracts*




It is clear from the results obtained that total phenolic content (TPC) increases with the increase in the concentration of Alim crude extract (see Figure 1). This suggests a dependent relationship on concentration, whereby high concentrations ensure high levels of phenolic compounds. The values given for mg GAE/g extract illustrate that TPC values increased steadily from 180.13 mg GAE/g (18,013 mg GAE/100 g) at the lowest concentration of 10 µg/mL to 357.08 mg GAE/g (35,708 mg GAE/100 g) at the highest concentration of 100 µg/mL. The standard deviation differences among the varying concentrations were considerably low; these were

reliable measurements, as the standard deviation for a concentration of 100 mg was only 0.17, indicating the stability of phenolic content in repeated trials. All the Alim leaf crude extracts are classified as high (>500 mg GAE/100 g), as identified by Dasgupta et al. (2021).

**Figure 2.** *Comparison of L-ascorbic acid and alim crude extract at various concentrations in terms of their antioxidant activity resulting in percent radical scavenging activity (%RSA)*




In Figure 2, results showed that both L-ascorbic acid and Alim crude extract exhibited potent radical scavenging activity, one of the key properties for their potential antioxidant activity. According to the previous study by Lalhminghlui and Jagetia (2018), the neutralization of free radicals contributes to the decrease in oxidative stress, which is related to various health disorders such as cancer, cardiovascular disease, and neurological illnesses. L-ascorbic acid demonstrated remarkable radical-scavenging activities at all tested concentrations, from 93.09% at the lowest (0.0625 g/ml) to 96.42% at the highest (1 g/ml). This agrees with the established view that ascorbic acid is a potent antioxidant molecule capable of effectively absorbing free radicals, thus reducing oxidative damage to biomolecules (Padayatty et al., 2003). Similarly, the Alim crude extract showed good radical scavenging activity, which ranged from 79.05% at 0.0625 g/ml to 95.88% at 1 g/ml. Though slightly lower than L-ascorbic acid, it still has remarkable antioxidant potential, mainly when higher concentrations are used. Results show a strong concentration-dependent relationship in L-ascorbic acid and Alim crude extract, as with higher concentration, there is increased radical scavenging activity in the effects of many antioxidants. The pattern might also indicate that more significant extract concentrations may provide more protection against oxidative stress. Therefore, the high inhibitory activity was justified by the 0.000907 g/ml (0.907 mg/ml) $IC_{50}$ value of the alim crude extract. This is classified as very high ($IC_{50}$ < 20 mg/mL) antioxidant activity (Dasgupta et al., 2021). The antioxidant activities of Alim crude extract could be attributed to its rich phytochemical composition, which includes flavonoids, phenolic compounds, and other bioactive secondary metabolites, which are well-known for their antioxidant activity. Flavonoids, mainly kaempferol and quercetin in most plant extracts, have been described to efficiently scavenge free radicals, as identified by Szerlauth et al. (2019).

The higher the TPC, the higher the antioxidant activity measured by DPPH, because the phenolic compounds are well-known free radical scavengers. Flavonoids are a subclass of phenolic compounds. Reported to be effective antioxidants. The higher the TFC, the higher the DPPH scavenging activity is usually. Flavonoids contain functional groups, the hydroxyl group interacting with the free radicals, contributing to the reduction of DPPH (Chavan et al., 2013). As also stated in the study of Liao et al. (2018), there is a strong correlation between TPC, TFC, and antioxidant activities. This suggests that the phenolic compounds are the major components responsible for the antioxidant behavior of *O. corymbosa*.

**Table 4.** *Antioxidant capacity (µM $FeSO_4$/g extract) of L-ascorbic acid and alim crude extract at various concentrations using FRAP Assay*

| | **Ascorbic Acid** | **Alim Crude Extract** |
|---|---|---|
| **Concentrations (µg/ml, %)** | **µM $FeSO_4$/g extract** | |
| 200, 20 | 9.48 | 12.33 |
| 400, 40 | 24.24 | 62.33 |

In contrast to the ferric reducing antioxidant potential (FRAP) values of the crude extracts of Alim and ascorbic acid, it is clear that ascorbic acid had better antioxidant activity in all the tested concentrations (see Table 4). This falls to the fact that ascorbic acid is a known antioxidant and one of the strongest acids used for assay comparison in most antioxidant analyses. On the other hand, the Alim crude extracts showed an increased reducing power at higher concentrations, as they contain compounds with antioxidant properties; the activity is not so pronounced as that of ascorbic acid. The evaluation of the results indicated no statistically significant differences among the groups under study, with a p-value of 0.448 (p>0.05). Antioxidant capacity in the study of Dasgupta et al. (2021)

was classified based on FRAP values, for which both 20% (200 µg/ml) and 40% (400 µg/ml) of Alim crude extracts are very low (<50 µmol $FeSO_4$/g FW). The standard fruit extracts reported in this study; most fruits (10 out of 15) had very low antioxidant capacity, with FRAP values ranging from 4.2 to 47.6 µmol $FeSO_4$/g extract, where 6 out of 10 common fruits (4.2-11.1 µmol $FeSO_4$/g extract) are lower than the 40% Alim crude extract. Moreover, 5 out of 10 (4.2 to 7.3 µmol $FeSO_4$/g extract) are lower than the 20% alim crude extract. Some studies reported further that most fruits, according to Silva and Sirasa (2016), are far below FRAP values. Alim crude extracts are somewhat comparable to other plant extracts regarding antioxidant capacity. Moreover, one possible reason for this deviation could be the loss of phytochemicals at elevated temperatures of 60°C, as the authors have used the preparation of fruit extracts at 60°C. Though cornelian cherry is rich in ascorbic acid, anthocyanin, and phenolic compounds, Pantelidis et al. (2006) have pointed out that their antioxidant capability, determined by the FRAP test, was low. Miean and Mohamed (2001) noticed that the phenolic amount decreased considerably above 60°C temperature. At higher temperatures, some phenolics may break down or integrate with other constituents of plants. Since this study was conducted by controlling all the limiting factors, the results obtained are compatible with the correct situation of the extracts. These results highlight that concentration becomes crucial in evidencing antioxidant capacity and that higher concentrations of Alim crude extracts may be needed for significant antioxidant action.

## 4.0 Conclusion

The crude extract of Alim, *Melanolepis multiglandulosa,* leaves contained triterpenes, sterols, flavonoids, alkaloids, saponins, tannins, and polyphenols. The 100% w/v crude extract demonstrated the highest antibacterial activity; as the quantity of the plant extract increases, bioactive components correlate with enhanced antibacterial activity. It also significantly inhibited albumin denaturation, indicating anti-inflammatory activity and high red blood cell protection. It also indicated a significant relationship between total flavonoid content (TFC) and total phenolic content (TPC) regarding its antioxidant properties; as these values increase, the antioxidant activity improves due to phenolic compounds. These have been described as possessing antibacterial, anti-inflammatory, and antioxidant properties, demonstrating positive benefits for wound healing. The potential for developing various wound-healing formulations for further clinical testing and quality product evaluation is suggested to be explored.

## 5.0 Contribution of Authors

The authors indicate equal contribution to each section. The authors reviewed and approved the final work.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## 7.0 Conflict of Interest

The authors declare no conflicts of interest regarding the publication of this paper.


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