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Phytochemical Screening and Antibacterial Activity of *Pimeleodendron griffithianum*

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Pimeleodendron griffithianum is one of the species in the Euphorbiaceae family. The family consists of species with excellent research potential that are believed to possess therapeutic properties or exert beneficial pharmacological effects on the animal body. Since the chemical constituents and biological activity of this species are not widely studied and reported, this research has been conducted to determine the phytochemical content and antibacterial activity of the polar, medium, and non-polar fractions from the stembark of this plant. The stembark of *Pimeleodendron griffithianum* was collected from Pos Kuala Mu, Sungai Siput, Perak. The sample was cleaned, cut, and ground into powder before extraction. The extract was brought to separation and fractionation using liquid-liquid extraction (LLE) to obtain polar, medium, and non-polar fractions. The fractions were then subjected to a phytochemical screening test to identify alkaloids, terpenoids, steroids, flavonoids, saponins, and tannins. All the fractions were also tested for antibacterial activity using the Disc Diffusion Method against Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative bacteria (*Klebsiella pneumoniae* and *Escherichia coli*). The phytochemical results indicated the presence of terpenoids in all fractions, flavonoids in non-polar and medium polar fractions, steroids in non-polar fractions, saponin in medium polar fractions, and tannin in polar fractions. As for the antibacterial activity, the medium polarity fraction exhibited significant inhibition against *Bacillus subtilis* and *Escherichia coli* with values of 10 and 14 mm diameter of zone inhibition, respectively. The non-polar fraction showed inhibition of growth in both gram-negative and positive bacteria.

Keywords: *Bacillus subtilis*; *Escherichia coli; Pimeleodendron griffithianum*

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Euphorbiaceae is among the large flowering plant families with a wide variety of vegetative forms, some of which are plants of great importance. This family, as traditionally delimited, is one of the most prominent families, composed of five subfamilies, 49 tribes, over 317 genera, and about 8000 species [1]. The Euphorbiaceae family occurs mainly in the tropics, with most species can be found in the Indo-Malayan region and tropical America. This complex family implies a lot of research potential [2]. Research has shown that some Euphorbiaceae are potent medicinal plants, and their extracts have been isolated and patented as modern drugs [3]. Many Euphorbiaceae plant concoctions, fresh latex, and teas are used in alternative medicine. *E. tirucalli* is known for its curative features against diseases like warts, cancer, gonorrhea, arthritis, asthma, cough, earache, neuralgia, rheumatism, toothache, swellings, tumors, and others. Some researchers have similarly but independently reviewed a variety of Euphorbiaceae-based phyto-chemicals, including alkaloids, phenols, flavonoids, saponins, tannins, and essential oils, and described their origins, characteristics, and therapeutic uses [4,5].

Pimeleodendron is a small genus in the Euphorbiaceae family. This genus comprises five species, which are *P. amboinicum, P. griffithianum, P. macrocarpum, P. zoanthogyne, and P. dispersum* [6]. Some of the species in this genus are economically important since they provide food, medicine, or varnish for local people. In eastern Peninsular Malaysia, *P. amboinicum* furnishes edible seeds, which taste like hazelnuts; its bark is used as a purgative; the juices of the leaves are used in a mixture that cleans the mouths of children and acts as a gentle laxative (adults can eat the leaves without purging), and the latex serves as varnish. People in the Solomon Islands drink an infusion of the bark of *P. amboinicum* as a remedy for fever [6]. In Indonesia, *P. griffithianum* is used as a traditional medicine [7], and the fruit of *P. griffithianum* is used for seasoning [6].

Pimeleodendron griffithianum is distributed in Thailand, Peninsular Malaysia, Sumatra, Borneo, and the Philippines. This plant is usually found in wet forests, secondary forests, pole forests, semi-swamps, along logging roads, or on hill slopes [8]. The local

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names of this plant are nuah mambur, kelampai sitak, mampulut, njulir, perah ikan, and tampang. Research has been done and reported on the *Euphorbiaceae*, but the study on the *Pimeleodendron* is limited as it has not been widely studied and reported. Only one compound has been reported from *P. griffithianum* [8]. The antibacterial activity of this species had also not been reported. A report was published on the antifungal activity of the methanol extract against *Gloeophyllum trabeum* and *Pycnoporus sanguineus* (brown-rot fungus and white-rot fungus) by Kawamura, F. et al. in 2010 [9]. Hence, the study on this species is carried out in order to determine the phytochemical content and evaluate the antibacterial activity.

EXPERIMENTAL

Sample Preparation

The fresh stem bark of *P. griffithianum* was collected from Pos Kuala Mu, Sungai Siput, Perak. The stem bark was cleaned, chopped into smaller pieces, and dried under shade at room temperature in order to remove moisture. Then the sample was ground to a coarse powder using a mechanical blender.

The powder was extracted using acetone via the maceration method. The solvent was evaporated, obtaining a crude extract. The crude obtained was subjected to decantation and evaporation of methanol-petroleum ether to minimize the tannin content and was then used for further fractionation [8]. The crude was separated into fractions based on non-polar, medium, and polar compounds. As mentioned below, a series of separation methods were carried out to obtain these fractions using liquid-liquid extractions (LLE) and thin-layer chromatography (TLC).

For LLE, the crude was initially dissolved in the first solvent. The second liquid solvent was added, immiscible or partially miscible, with the solution, and the solutes were distributed between the two phases. The desired component to be extracted during the process was transferred into the extract phase (second solvent). The leftover components from the solution remained in the raffinate. A few series of extractions were taking place in this separation process. The phase solvents used as the first solvent and second solvents were as follows: (chloroform: methanol) (hexane: ethyl acetate) (methanol: hexane). For LLE, the crude was initially dissolved in the first solvent. The second liquid solvent was added, immiscible or partially miscible, with the solution, and the solutes were distributed between the two phases. The desired component to be extracted during the process was transferred into the extract phase (second solvent). The leftover components from the solution remained in the raffinate. A few series of extractions were taking place in this separation process. The phase solvents used as the first and second solvents were as follows: chloroform: methanol; hexane: ethyl acetate; methanol: hexane.).

The crude was separated into three fractions (non-polar, medium, and polar) from the LLE process and examined using the TLC. The chromatography solvent was made by putting hexane, chloroform, ethyl acetate, and methanol to increase polarity [8].

Phytochemical Screening

A phytochemical screening test was carried out for all fractions to identify the presence of alkaloids, terpenoids, steroids, flavonoids, saponins, and tannins in the fractions. The tests were conducted according to the following method [10].

Mayer's Test for Alkaloid Determination

Two millilitres (2 mL) of the crude extract were mixed with a few drops of concentrated hydrochloric acid and a few drops of Mayer's reagent in a glass tube. A yellow precipitate indicated the presence of alkaloids.

Salkowski's Test for Terpenoids

A 5 mL extract was dissolved in 2 mL chloroform, and 3 mL concentrated sulphuric acid (H_2SO_4) was carefully added to form a layer. A reddish-brown coloration indicated the presence of terpenoids.

Steroids Test

In a test tube, 2 mL of the extract was added along with 3–5 drops of acetic acid and stirred to allow mixing. Then, 1-2 drops of concentrated sulfuric acid were added to the mixture by slowly dripping them down the test tube wall. The presence of steroids was evident by the purple coloration.

Flavonoids Test

A piece of magnesium ribbon was dropped into a test tube containing 2 mL of extract, followed by a few drops of concentrated hydrochloric acid. The solution was allowed to mix and settle for 10 minutes. Yellow substance formation proved the presence of flavonoids.

Froth Test (Saponin Determination)

1 mL of the extract was added to distilled water in a test tube. The solution was shaken and allowed to mix. The formation of an emulsion indicated the presence of saponins.

Tannins Test

Two millilitres of the extract were mixed with 3–5 drops of 5 % ferric chloride in a test tube. The appearance of the blue-black solution showed the presence of tannins.

Antibacterial Activity

This antibacterial activity was conducted on three

fractions: polar, medium, and non-polar. Four different extract concentrations were prepared for each fraction: 40 %, 60 %, 80 %, and 100 %. The solvent used for the sample preparation were methanol, acetone, and hexane for polar, medium, and non-polar fractions. These solvents were also used as the negative control, while the antibiotic gentamycin was used as the positive control. Mueller Hinton Agar, nutrient agar, and nutrient broth were prepared as the agar and broth medium for the bacterial culture. Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative bacteria (*Klebsiella pneumoniae* and *Escherichia coli*) were used for the test as the pathogens. The methods used for the antibacterial activity using the disc diffusion technique were described by [11, 12, 13].

The disc diffusion test is a method to determine the sensitivity of bacteria to a specific antimicrobial agent; thus, the large zone of inhibition surrounding the agent-containing disc on the solid media showed the bacterial responses toward the agent. The zone of inhibition size was interpreted by referring to Table 1, and the bacterial response was reported either to be resistant, susceptible, or intermediate towards the extract. Figure 1 shows how to measure the zone of inhibition diameter for bacteria.

IC_{50} Calculation

The simulated estimate of the IC_{50} value was to plot an x-y graph and connect the data with a straight line (linear regression). The IC_{50} value was then measured using the formula given below:

$$IC_{50} = \frac{(0.5 - B)}{A}$$

Where; A: x-intercept (gradient of the graph)

B: c-intercept of the plotted graph

Table 1. The standard measurement of the zone inhibition for a bacterial response towards the extract.

Bacteria response	Diameter of zone of inhibition
Resistant	10 mm or less
Intermediate susceptible	11 mm – 15 mm
susceptible	16 mm or more

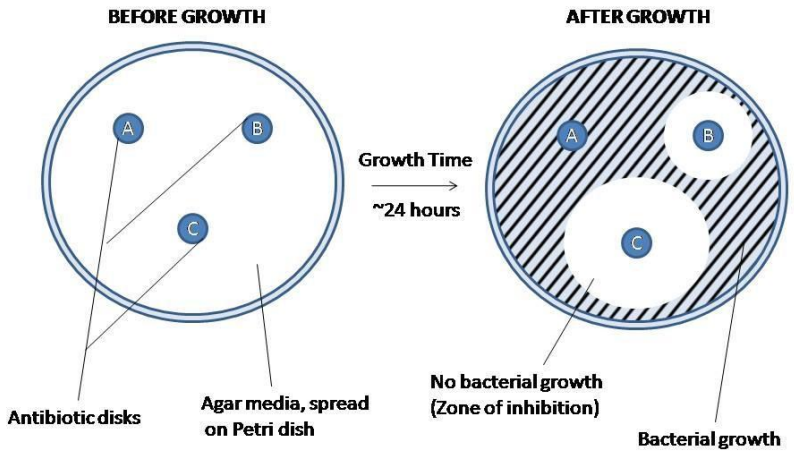

Figure 1. Description of how to measure the zone of inhibition diameter for bacteria.

Table 2. Phytochemical screening of three different fractions.

Chemical compound	Results		
	Non-Polar Fraction	Medium Polar Fraction	Polar Fraction
Alkaloids	-	-	-
Terpenoids	+	+	+
Steroids	+	-	-
Flavonoids	+	+	-
Saponins	-	+	-
Tannins	-	-	+

Note: (+): presence, (-): absence

RESULTS AND DISCUSSION

Phytochemical Screening

This test was performed to determine the presence of alkaloids, terpenoids, steroids, flavonoids, saponins, and tannins. The phytochemical analysis of the results indicated the presence of terpenoids, steroids, flavonoids, saponins, and tannins and the absence of alkaloids. Table 2 shows the result of the phytochemical screening of three different fractions.

Table 2 shows the absence of alkaloids in all fractions due to the absence of a yellow precipitate in the solution after Mayer's reagent was added. Some of the species in the Euphorbiaceae family revealed the presence of terpenoids, steroids, flavonoids, saponins, tannins, and the absence of alkaloids in specific genera. Alkaloids and their derivatives have been reported to act as potent poisons and show anti-inflammatory, anti-malarial, antimicrobial, cytotoxic, antispasmodic, and pharmacological effects [14].

For terpenoids, the formation of a reddish-brown precipitate at the interface of two layers between chloroform and sulfuric acid solution indicates the presence of terpenoids. It showed that terpenoids were present in all fractions. Terpenoids are plants' most common and structurally diverse group of secondary metabolites. They play an important role in interactions between plants and insects, plants and pathogens, and plants and other plants [14]. A Pentacyclic triterpenoid (acetyl aleuritolic acid) was reported to be present in *P. griffithianum* [8]. Diterpenes and ent-abietanes have been reported to be contained in *Eurphorbia pubescens Vahl* and *Jatropha podagrica Hook* (Euphorbiaceae family). The antibacterial properties of this compound were also observed [1].

The steroids were present in the non-polar fraction, as shown by the purple color's appearance when a small amount of extract was treated with concentrated sulfuric acid and acetic anhydride. Steroids derived from plants are known to have a cardiotonic effect and possess antibacterial and insecticidal properties. They are often used in medicines due to their well-known biological activities [16]. *Euphorbia segetalis* is in the family Euphorbiaceae. It has been shown to kill bacteria because it has coumarins and steroids [1].

Flavonoids were present in non-polar and medium polar fractions. This indicated a change in color to yellow when concentrated hydrochloric acid was added to the magnesium strip in the extract. Flavonoids have antimicrobial activity, probably due to their ability to complex with extracellular and soluble proteins and bacterial cell walls [17].

Saponins were present in the medium polar fraction, while tannins were in the polar fraction. Previous research [17] shows saponins have antibiotic and antimicrobial activity. These properties contained in the saponins were due to their ability to cause leakage of proteins and certain enzymes from the cell. As for tannins, they are known to have antibacterial, antitumor, and antiviral activities. They work by precipitating microbial protein, thus making nutritional protein unavailable to them [16].

Euphorbia hirta and *Euphorbia heterophylla* are also members of the Euphorbiaceae family. There have been reports of alkaloids, flavonoids, saponins, and tannins in these species. These phytochemicals have also been attributed to having antibacterial properties [1].

Antibacterial Activity

Antibacterial activity was performed using the disc-diffusion technique and observing the potential effectiveness based on the zone of inhibition on bacterial culture plates. The larger the zone of inhibition, the more bacteria showed susceptibility to the extract. The

extracts of three different polarities were tested against four pathogenic microbes: *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae*, and *Escherichia coli*. The samples were first tested at 100 % concentration for each bacterium. Then, the bacteria that showed inhibition on the fraction were tested with other concentrations: 40 %, 60 %, and 80 %.

Figure 2 shows the response of the gram-positive bacteria toward 100 % concentration of polar, non-polar, and medium polar extracts. In comparison, Figure 3 shows the reaction of the gram-negative bacteria toward 100 % concentration for the extracts. It displayed no inhibition zone for all bacteria (gram-positive and gram-negative) against 100 % polar extract. Thus, it showed that all bacteria were resistant to the polar extract, where the diameter of the inhibition zone was less than 10 mm [11, 12, 13].

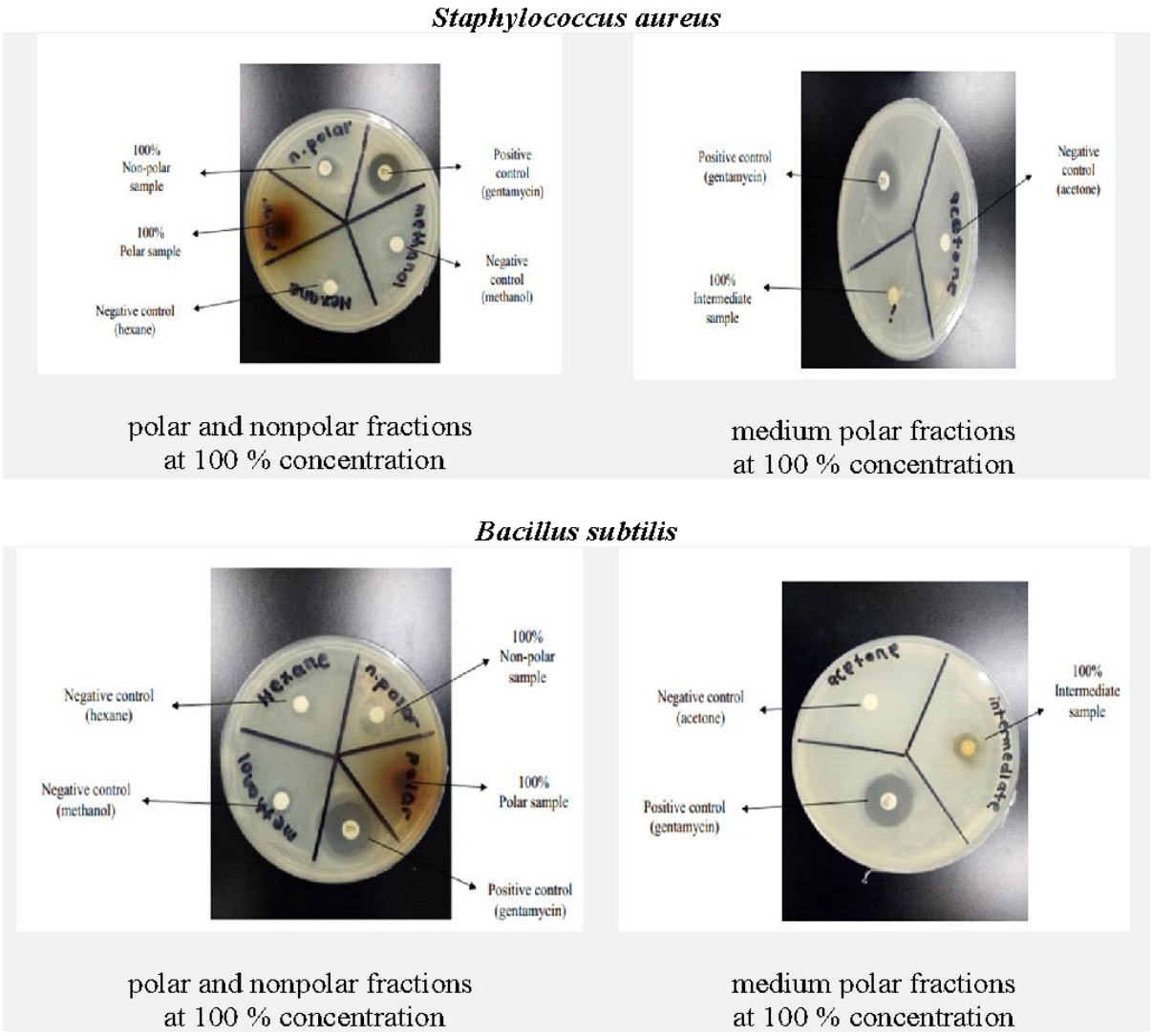

Figure 2. *Staphylococcus aureus*, and *Bacillus subtilis* response against 100 % concentration polar, non-polar and medium polar extract.

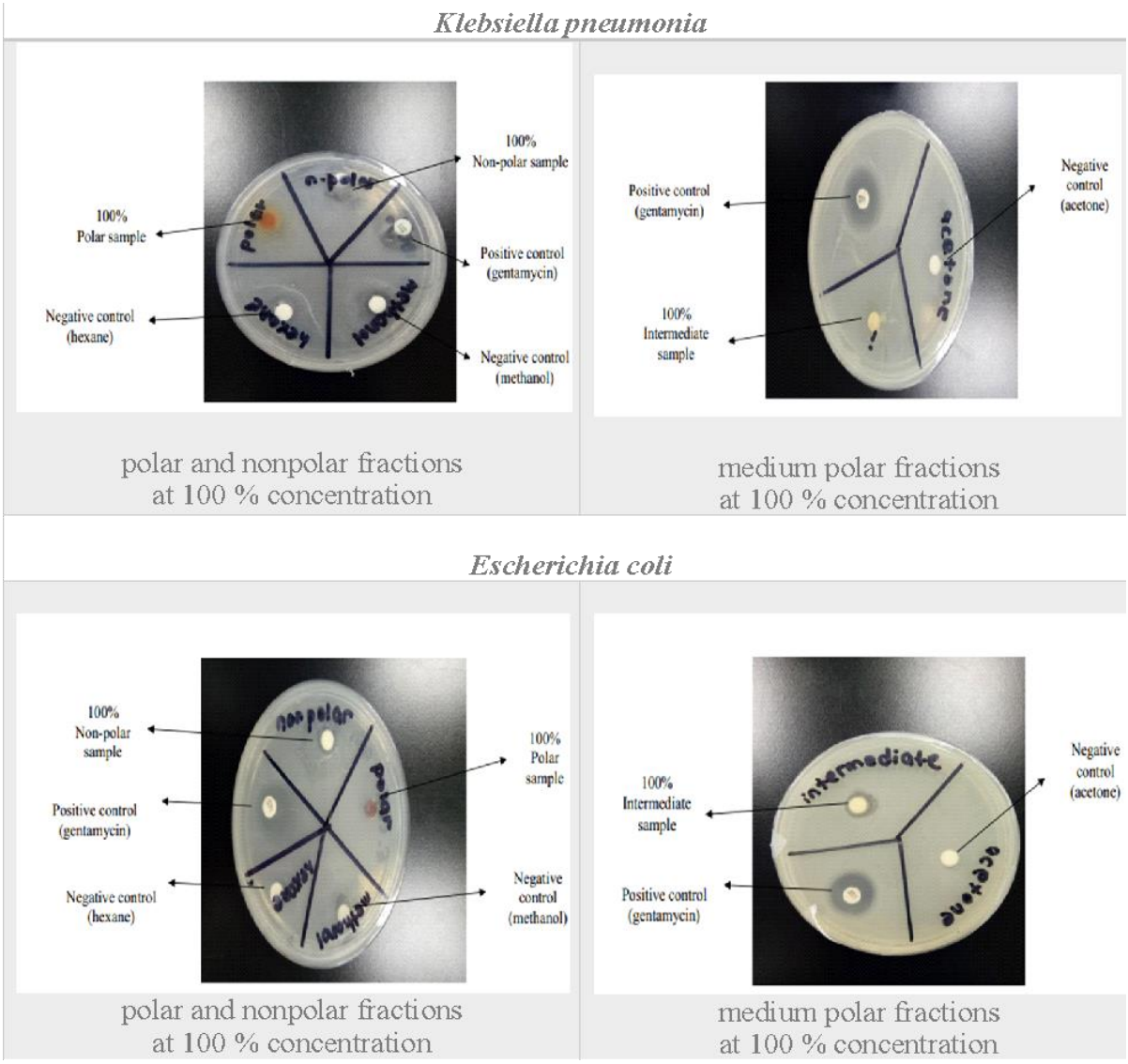

Figure 3. *Klebsiella pneumoniae* and *Escherichia coli* response against 100 % concentration polar, non-polar and medium polar extract.

Table 3. The bacterial response towards 40 %, 60 %, 80 %, and 100 % concentrations non-polar.

Bacterial pathogens	**Diameter of inhibition zone (mm)**					
	100%	80%	60%	40%	Positive control	Negative control
Gram Positive						
S. aureus	10	10	9	9	20	-
B. subtilis	15	11	9	7	21	-
Gram Negative						
K. pneumoniae	13	9	11	13	20	-
E. coli	11	11	11	14	21	-

Noted: bacterial response range (resistant: ≤ 10 mm, intermediate: 11-15 mm, susceptible: ≥ 16 mm); '-' indicate no inhibition.

Since no inhibition zone was observed at 100 % concentration for polar extract, a further antibacterial assay was carried out for other concentrations only for non-polar and medium polar fractions. Tables 3 and 4 display the bacterial response toward 40 %, 60 %, 80 %, and 100 % of non-polar and medium polar fractions, respectively.

Table 3 shows the intermediate susceptibility response of Gram-negative *K. pneumoniae*, *E. coli*, and gram-positive *B. subtilis* towards 100 % by having an inhibition zone of 13, 11, and 15 mm, respectively. At the same time, gram-positive *S. aureus* response showed resistance with the diameter of the inhibition zone 10 mm. With the extract concentrations at 80 %, the bacterial response of *K. pneumoniae* and *S. aureus* were resistant by showing an inhibition zone of 10 and 9 mm, respectively. The *B. subtilis* (gram-positive) and *E. coli* (gram-negative) showed similar bacterial responses towards extract in the susceptible intermediate range, indicating an inhibition zone diameter of 11 mm. Both gram-positive bacteria responded as resistant at 60 % and 40 % extract concentration by showing an inhibition zone of less than 10 mm. The gram-negative bacteria (*K. pneumoniae* and *E. coli*) response was intermediate susceptible with 60 % and 40 % concentrations. The inhibition zone showed an 11-14 mm diameter.

From the results obtained, Gram-positive bacteria (*S. aureus and B. subtilis*) showed a smaller zone of inhibition as the concentration of the fraction decreased. Bacteria became resistant as the extract concentration decreased, indicating that the extract's strength depended on the concentration. For gram-negative (*E. coli*), it showed that as the fraction's concentration decreased, the extract's zone of inhibition was increased. The lessening of concentration can increase the potential of the non-polar extract as an antibacterial agent due to synergistic effects beyond their influences. The non-polar extract may have a synergistic effect by stopping the next steps in the extraction process from interfering with the solvent. It is assumed that the bacterial response is not directly related to the changes in concentration of the non-polar extract. Antibacterial activities are influenced by many factors, including the extraction technique, potential interactions with other ingredients, the presence of secondary metabolites, the type of solvent used, the test microorganism, the environment, and the plant's climate. These factors include the extract's concentration, environment, and climate [19].

Table 4 displays the resistant susceptibility response of Gram-positive *S. aureus* and gram-negative *K. pneumoniae* towards all concentrations of medium polar extract by showing no inhibition zone. At the 100% concentration, both *B. subtilis* and *E. coli* bacteria responded similarly, with an inhibition zone of 12 mm. For Gram-positive bacteria *B. subtilis*, the outcome of the bacterial response towards 80 %, 60 %, and 40 % of the extract were similar, indicating resistant susceptibility where the zone inhibition was 9, 10, and 9 mm, respectively. *E. coli* were susceptible when the concentration was 40%, intermediate when it was 60%, and resistant when it was 80%, as shown by an inhibition zone of 18, 14, and 9 mm, respectively.

The results for the medium polar extract showed that as the concentration of the extract decreased, gram-positive bacteria (*B. subtilis*) showed less zone inhibition. In contrast, gram-negative *E. coli* showed that, as the concentration decreased, the zone of inhibition increased. The factor responsible for the high susceptibility of the bacteria toward the extract may be the presence of secondary plant metabolites in the sample. The composition of the cell envelopes of gram-negative and gram-positive bacteria may influence the type of response [20]. Antibacterial activity varied with the different extracts, as they showed different anti-bacterial activities on an organism. This may be due to the distribution of various antibacterial substances. [21].

The validity of the result was confirmed by having no inhibition zone on the negative control disc that contained the solvent. On the other hand, all the samples showed an inhibition zone around the positive control disc with an antibiotic (gentamycin).

Table 4. Bacteria response towards 40 %, 60 %, 80 %, and 100 % concentration of medium polar.

Bacterial pathogens	**Diameter of inhibition zone (mm)**					
	100%	80%	60%	40%	Positive control	Negative control
Gram Positive						
S. aureus	-	-	-	-	20	-
B. subtilis	12	9	10	9	21	-
Gram Negative						
K. pneumonia	-	-	-	-	20	-
E. coli	12	9	14	18	21	-

Noted: bacteria response range (resistant: ≤ 10 mm, intermediate: 11-15 mm, susceptible: ≥ 16 mm); '-' indicate no inhibition.

Table 5. The IC_{50} value of bacterial response towards non-polar and medium polar.

Pathogen	IC_{50} value (ppm)	
	Non-polar	**Medium polar**
E. coli	3.38	3.10
K. pneumonia	3.38	-
S. aureus	4.00	-
B. subtilis	3.38	3.75

IC_{50} Value for Active Antibacterial Assays

The IC_{50} is the concentration of an inhibitor at which the response (or binding) is reduced by half. It measures how well a substance stops a particular biological or biochemical process from happening. Table 5 shows the IC_{50} value of bacterial response toward non-polar and medium polar extracts. The calculation of the IC_{50} value was done using the formula given in the procedure. Smaller values of IC_{50} indicate better antibacterial activity.

Table 5 shows that for non-polar extracts, *E. coli, K. pneumoniae,* and *B. subtilis* have the same IC_{50} value of 3.38 ppm. While *S. aureus* response toward non-polar extract was the highest, the value displayed was 4.00 ppm. For the medium polar fractions, *B. subtilis* presented 3.75 ppm, while *E. coli* indicated the lowest value by showing 3.10 ppm. In medium polar, no IC_{50} values were observed for K. pneumoniae and S. aureus. Table 5 shows that the *E. coli* response toward the medium polar fraction has better antibacterial activity than the *S. aureus* response toward the non-polar fraction. For *E. coli*, the medium fraction is likely to be the best inhibitor, while for *S. aureus*, the non-polar fraction is likely to be the worst.

CONCLUSION

This study evaluated the phytochemical content and antibacterial activity in the polar, medium, and non-polar fractions of the stembark extract of *P. griffithianum.* The present study showed that terpenoids were present in all fractions, steroids were present only in non-polar fractions, flavonoids were present in non-polar and medium polar fractions, saponins were present in medium fractions, and tannin was present only in polar fractions. At the same time, alkaloids were absent in all fractions. Meanwhile, for antibacterial activity, no inhibition zone was shown on the polar fraction; the medium fraction only showed inhibition on *B. subtilis* and *E. coli.* The non-polar fraction showed inhibition against all bacteria. Thus, *P. griffithianum* is recommended as a promising source of plant-based antibacterial agents.

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