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Antibacterial activity of *Plectranthus amboinicus* (Lour.) Spreng extracts against pathogenic bacteria

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Abstract *Plectranthus amboinicus* (Lour.) Spreng is the most important aromatic medicinal plant and has antimicrobial activity. This study investigated the major bioactive constituents and antibacterial activity of *P. amboinicus* extracts. The results revealed that the dominant phytochemicals that might be responsible for its bioactivity are alkaloids, terpenoids, tannins, phenolics, luteolin, and verbascoside. The herbal extract showed antibacterial capacity against the *Staphylococcus aureus* (MRSA) strain PB57, with a maximum zone of inhibition. The MIC and MBC values of the extract were found to be 7.81 mg/ml and 15.63 mg/ml, respectively. However, the cytotoxicity results indicated that the extract at concentrations lower than the MIC had a cytotoxic effect on fibroblasts. These findings suggest that *P. amboinicus* extract has great potential for use as an antimicrobial agent. Therefore, the toxicity of each compound contained in the herbal crude extract should be examined further in the future.

Keywords: antibacterial activity, *Plectranthus amboinicus*, pathogenic bacteria, phytochemical analysis

1. Introduction

The emergence of infectious diseases has increased in frequency in recent decades (Jones et al., 2008). The growth-inhibitory phenomenon of antibiotics has intense evolutionary effects on bacteria, thereby leading to increased resistance (Waglechner et al., 2020), which is considered a crucial cause of morbidity and mortality worldwide (Mendelson & Matsoso, 2015). Rapid progress in resistance naturally occurs over time; hence, research and development of new therapies for treating bacterial infections are urgently needed (WHO, 2014). For thousands of years, plant-based medicines have been widely applied in folk medicine. Herbal medicines are safe, inexpensive, and effective at inhibiting the growth of pathogenic microbes and fighting several diseases (Chariandy et al., 1999; Arumugam et al., 2016; Newman et al., 2000). Plants contain numerous biologically active compounds, many of which have been shown to exhibit antimicrobial properties. They are employed as antimicrobial agents in traditional medicines and are extensively used in the pharmaceutical industry (Arumugam et al., 2016; Kumara et al., 2011). Consequently, the demand for drug discovery from herbal medicines has increased globally in this modern era, thereby affecting the exploration and exploitation of new botanical sources for their medicinal ability (Arumugam et al., 2016).

Plectranthus amboinicus (Lour.) Spreng is an aromatic herb belonging to the Lamiaceae family. This herbal plant can be found in tropical areas of Asia, Africa and Africa. This herbal medicine contains 76 volatile and 30 nonvolatile compounds with therapeutic and nutritional properties. These compounds differ in their phytochemical classes, such as monoterpenoids, diterpenoids, triterpenoids, sesquiterpenoids, phenolics, flavonoids, esters, alcohols, and aldehydes (Ashaari et al., 2020). It is commonly used in folk medicine to treat patients who have several symptoms, including asthma, constipation, headache, cough, fever, and cold and skin diseases. Indigenous people usually eat raw leaves of the herb or use it as a flavoring agent in traditional foods (Arumugam et al., 2016; Ashaari et al., 2020).

P. amboinicus was selected for this study due to its extensive use in folk medicine and its diverse range of bioactive compounds, which offer significant therapeutic potential. Additionally, the herb's wide distribution and history of medicinal use make it a valuable candidate for exploring novel compounds that could contribute to drug discovery efforts, particularly in the search for alternatives to combat antibiotic resistance. Previous studies suggested that the plant's bioactive components hold potential for discovering new, economically valuable substances, such as alkaloids, tannins, and oils (Farnsworth, 1966). Thus, this research aimed to screen for bioactive phytochemicals in *P. amboinicus* extract, assess its antibacterial activity, and evaluate its toxicity and safety implications for human fibroblasts.

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2. Materials and Methods

2.1. Preparation of the ethanol extract

Dried leaves of *P. amboinicus* were purchased from Thaiherbart. Ten grams of dried leaves of *P. amboinicus* were ground into powder, followed by extraction with 80% ethanol (100 ml). The mixture was incubated for 48 hours at room temperature. Afterwards, the mixture was filtered through Whatman filter paper (No. 1) followed by evaporation under reduced pressure in a rotary evaporator (Büchi, Konstanz, Germany) at 55°C to remove excess solvent. The dried extracts were stored in airtight containers, labeled, and kept refrigerated at -20°C for further usage.

2.2. Phytochemical analysis

Both qualitative and quantitative phytochemical evaluations of the extracts were performed via standard methods (Harborne, 1998; Khandelwa, 2008).

Alkaloids: A total of 10 mg of plant extract in 4 ml of ethanol was prepared. Dragendorff reagent was added, and a brownish-orange precipitate was taken as evidence of the presence of alkaloids.

2.2.1. Terpenoids

First, 10 mg of each plant extract was measured in 4 ml of chloroform ($CHCl_3$), and 3 ml of concentrated sulfuric acid (H_2SO_4) was carefully added. A reddish-brown color indicates the presence of terpenoids.

2.2.2. Tannins

Plant extracts (10 mg) were added to 10 ml of boiled water, and two drops of 1% $FeCl_3$ were added. A green–violet precipitate indicated the presence of tannins.

2.2.3. Total phenolic content

First, 0.5 ml of leaf extract (1 mg/ml in distilled water) was added. Afterwards, 2.5 ml of Folin-Ciocalteu reagent (10-fold diluted) and 2.0 ml of sodium carbonate (75 g/l in distilled water) were added and mixed well. The mixture was incubated in a water bath at 50°C for 5 min, after which the absorbance of the mixture was read at 760 nm via a spectrophotometer. Different concentrations of gallic acid were used to prepare a calibration curve. The results are expressed as grams of gallic acid equivalents (GAE) per kilogram of sample in dry weight (g/kg). The total phenolic content was then calculated via the following formula:

Total phenolic equivalent gallic acid (g GAE/kg) = [(y + intercept)/(slope × a)] × 1000

where y = A-B,

A = absorbance of the extract when mixed with Folin–Ciocalteu reagent and sodium carbonate

B = absorbance of the solvent of the extract when mixed with Folin–Ciocalteu reagent and sodium carbonate

a = concentration of extract **(**mg/ml**)**

Note: All the experiments were carried out in triplicate, and the data reported are the means (averages) and standard deviations (SDs)**.**

2.3. High-performance thin-layer chromatography (HPLC)

2.3.1. Luteolin

Luteolin is a flavonoid found in a wide range of plant materials and was analyzed via HPLC. One milligram/ml of the plant extracts was injected into the Agilent 1,260 Infinity 2 using an Agilent Poroshell 120 EC-C18 column (4 µm, 150x4.6 mm), with a flow rate of 1.2 ml/min. The mobile phase consisted of 2% acetic acid (A) and acetonitrile (B) with the following gradient elution procedure: 0 min, 15% B; 10 min, 20% B; 13 min, 20% B; 15 min, 25% B; 20 min, 30% B; 25 min, 30% B; and 30 min, 50% B. Last, the amount of luteolin was quantitatively estimated via comparison with the luteolin standard.

2.3.2. Verbascoside

Verbascoside or VER, also known as acteoside, has various pharmacological activities, such as antioxidant, antimicrobial, and anti-inflammatory effects (Attia et al., 2018). HPLC was performed to determine the total luteolin content. The mobile phase was composed of phosphoric buffer:acetonitrile (77:23, v/v). An Agilent 1260 Infinity 2 Phenominex column (250 mm × 4.6 mm) with a 5 µm particle size at a flow rate of 1.2 ml/min was used to separate the compounds, after which the absorbents were measured at 332 nm. A standard solution of verbascoside was used for comparison. One milligram/ml of *P. amboinicus* extract was used for an injection volume of the sample solution.

2.4. Preparation of Bacterial Strains

Seventeen pathogenic bacteria (*Salmonella* Thyphi, *Shigella flexneri*, 4 *Acinetobacter baumannii*, 4 *Escherichia coli*, 3 *Klebsiella pneumoniae*, *Enterococcus faecalis*, and 3 *Staphylococcus aureus*) were used for antibacterial activity. These bacteria were cultured on Mueller–Hinton agar (MHA) and incubated at 37°C for 24 hours. A single colony was dissolved in 0.85% normal saline solution, and the bacterial concentration was adjusted to 0.5 McFarland standards.

2.5. Antibacterial activity

Antibacterial activity was determined via a disk diffusion assay. Bacterial strains that were previously adjusted to 0.5 McFarland standards were evenly spread on Mueller–Hinton agar (MHA) agar via sterile cotton swabs under aseptic conditions. The extracts were dissolved in dimethyl sulfoxide (DMSO) to obtain a concentration of 500 mg/ml. Subsequently, 10 μl of the prepared extract was dropped onto sterile paper discs. To ensure reliable results, both positive and negative controls were included in the assay. The positive control was represented by commercial antibiotic discs, which are known to exhibit antibacterial activity. These served as a benchmark to compare the efficacy of the plant extract. The negative control consisted of 10% DMSO, which was used to demonstrate that the solvent had no inhibitory effect on bacterial growth. All the plates were subsequently incubated at 37°C for 24 hours. The inhibition zone was measured via a ruler. Tests were performed in triplicate, and the mean diameter was recorded.

2.6. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC were determined via the microtiter broth dilution method. The crude extract was first prepared in dimethyl sulfoxide (DMSO) to obtain a final concentration of 500 mg/ml. The serial dilutions from the stock solution of the extract were made via cation-adjusted Mueller–Hinton broth (CAMHB) in 96-well microplates. Subsequently, 100 µl of 0.5 McFarland standard bacterial suspension was prepared and inoculated into each well. The bacterial cultures without the extract were used as positive controls. The plate was incubated at 37 °C for 24 hours. After incubation, the concentration that completely inhibited bacterial growth was recorded as the MIC value. Then, 10 µl of the solution from each well of the MIC experiment was removed and added to an MHA plate. The lowest concentration of the extract showing no visible growth was noted as the MBC value after 24 hours of incubation at 37 °C. All tests were performed in triplicate.

2.7. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

The effect of the extract on the viability of fibroblasts was determined by measuring mitochondrial dehydrogenase activity via the MTT assay (Fotakis & Timbrell, 2006). This study was designed for examining the cytotoxic effect of the plant extract on human cells to prove its further application as the antimicrobial agents. Primary fibroblasts were chosen as a model for human cell type. In brief, fibroblasts (ATCC, Manassas, VA, USA) at a density of 1×10^4 cells/well were seeded into a 96-well plate containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were allowed to attach to the surface of a 96-well plate overnight at 37°C in a 5% CO_2 humidifier incubator. Then, the cells were treated with various concentrations of the plant extract ranging from 25, 125, 250, 500, 1,000, 1,500, and 2,000 µg/ml. The control samples were cells incubated with 0.5% DMSO in complete DMEM without the extract. After incubation for 24 hours, the supernatant was aspirated and replaced with complete DMEM, after which 20 μl of MTT solution (5 mg/ml in PBS) was added to each well. The plate was further incubated for 2 hours before the supernatant was removed. DMSO solution (100 µl) was then added to each well to dissolve the formazan crystals. The absorbance at 540 nm was measured with a microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The percentage of cell viability was calculated compared with that of the control. The concentration of the extract that caused a 50% reduction in cell viability compared with that of the control was assigned as the IC_{50}. The IC_{50} was calculated via Graph Pad Prism version 5 by plotting the percentage of cell viability versus the log concentration of the extract. Three independent experiments with triplicate wells for each condition were performed. One-way analysis of variance (ANOVA) with Tukey's comparison test was used to assess the statistically significant differences among the experimental groups.

3. Results and Discussion

3.1. Phytochemical analysis

Preliminary screening of phytochemicals in *P. amboinicus* extracts revealed the presence of alkaloids, terpenoids, and tannins (Table 1). Figure 1 presents the calibration curve of the total phenolic content. The calculation indicated that the total phenolic content was 97.67 g GAE/kg. For quantitative phytochemical analysis (Figure 2), the herbal extract contained both luteolin and verbascoside. The contents of luteolin and verbascoside from 10 mg of the extract were 0.012 ± 0.007 mg or 0.12% of the extract and 0.040 ± 0.002 mg or 0.40% of the extract, respectively.

3.2. Antibacterial activity

The antibacterial activity of the *P. amboinicus* extract against 17 pathogenic bacteria was evaluated and compared by measuring the inhibition zone through a disk diffusion approach. The extracts significantly exhibited antibacterial activities against the tested microorganisms (Table 2). The eight bacterial strains that were inhibited by the herbal extract were *A. baumannii* strain AB322 (MDR), *S. aureus* ATCC20475, *S. aureus* strain PB57 (MRSA), *E. coli* ATCC35218, *E. coli* strain PB1 (ESBL+MDR), *E. coli* strain PB30 (MDR), *Salmonella* Thyphi, and *Shigella flexneri*. The zone of greatest inhibition was found for the *S. aureus* strain PB57 (MRSA), which was 11 mm in size. The MIC and MBC values were 7.81 and 15.63 mg/ml, respectively.

Table 1 Preliminary phytochemical profile of the *P. amboinicus* extract.

	Phytochemical testing		
	Alkaloid	Terpenoids	Tannin
P. amboinicus extract	+	+	+

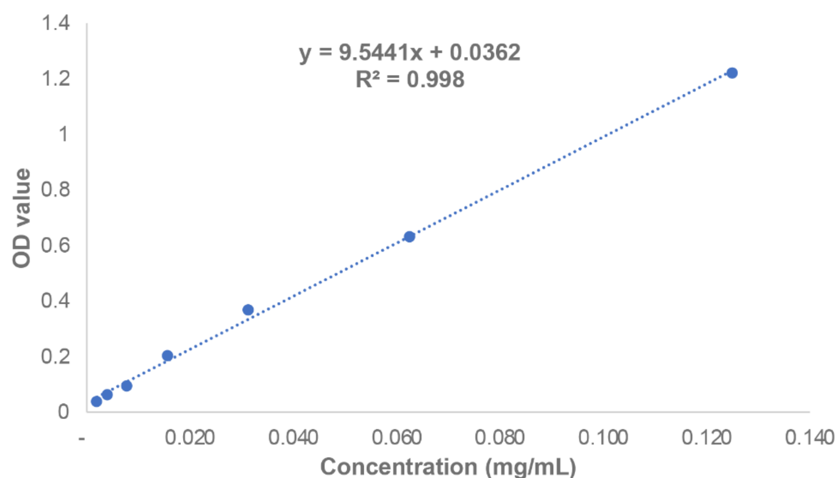

Figure 1 Calibration curve of total phenolic content.

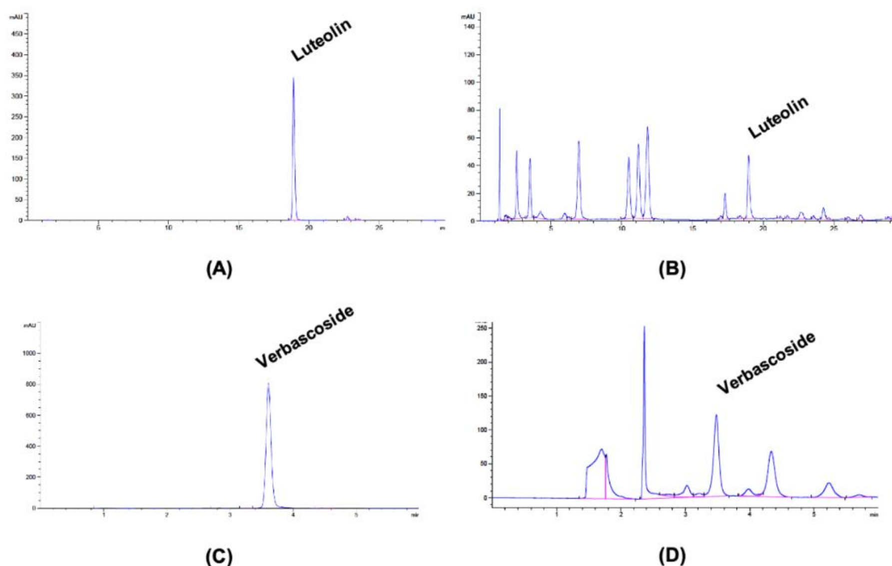

Figure 2 High-performance liquid chromatography chromatograms of standard luteolin solution (A), *P. amboinicus* extract (B), standard verbascoside solution (C) and *P. amboinicus* extract (D).

3.3. Cytotoxicity test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay)

The viability of fibroblasts after exposure to the extract for 24 hours is shown in Figure 3A. The results indicated that the extract caused a reduction in cell viability in a dose-dependent manner. Compared with the control, a significant reduction in cell viability was observed when the cells were treated with extracts ranging from 125 to 2,000 μg/ml. Compared with those in the control group, the viability of the cells treated with the extract at the highest dose, 2,000 μg/ml, was reduced to approximately 95% (approximately 5% cell viability remaining). The concentration of the extract that caused a 50% reduction in the number of viable cells (IC_{50}) was 541.2 μg/ml (Figure 3B). Compared with the IC_{50} value, the concentration of extract at the MBC (1,563 μg/ml) had a cytotoxic effect on fibroblasts after 24 hours of exposure.

Table 2 Disk diffusion results of the *P. amboinicus* extract against 17 pathogenic bacteria.

No.	Bacteria	Inhibition zone
1	*Salmonella* Thyphi	+
2	*Shigella flexneri*	+
3	*Acinetobacter baumannii* strain AB322 (MDR)	+
4	*Acinetobacter baumannii* strain AB321(MDR)	-
5	*Acinetobacter baumannii* strain AB324(XDR)	-
6	*Acinetobacter baumannii* strain AB320 (XDR)	-
7	*Escherichia coli* strain PB1 (ESBL+MDR)	+
8	*Escherichia coli* strain PB231 (ESBL+CRE)	-
9	*Escherichia coli* strain PB30 (MDR)	+
10	*Escherichia coli* ATCC35218	+
11	*Klebsiella pneumoniae* ATCC700603	-
12	*Klebsiella pneumoniae* strain PB5 (ESBL+MDR)	-
13	*Klebsiella pneumoniae* strain PB21 (ESBL+CRE)	-
14	*Enterococcus faecalis* ATCC51299	-
15	*Staphylococcus aureus* ATCC20475	+
16	*Staphylococcus aureus* strain PB36 (MRSA)	-
17	*Staphylococcus aureus* strain PB57 (MRSA)	++

Note: * - No activity (6 mm), + weak inhibition (7–10 mm.), ++ moderate/average inhibition (11–15 mm.), methicillin-resistant *Staphylococcus aureus (MRSA*), multidrug resistant (MDR), carbapenem-resistant Enterobacteriaceae (CRE), extended-spectrum beta-lactamases (ESBL), and extensively drug resistant (XDR).

Phytochemical screening was initially performed to characterize the herbal extract. This study revealed the presence of alkaloids, terpenoids, tannins, and phenolics. Several studies have reported that alkaloids are effective in treating various diseases, including malaria, diabetes, cancer, cardiac dysfunction, venereal diseases, and intestinal inflammatory disorders (Ain et al., 2016; Peng et al., 2019; Rijo et al., 2019). Terpenoids, tannins, and phenolics, which have been observed in various *Plectranthus* species, are considered probable antimicrobial and antioxidant substances (Adebooye et al., 2008; Oyedemi et al., 2011; Thaniarasu et al., 2015; Jiangseubchatveera et al., 2017). These phytochemical constituents are also similar to those in previous reports (Jones et al., 2008; Gejalakshmi et al., 2020; Anie et al., 2022; Singh et al., 2022; Lopez-Lazaro, 2009). Furthermore, the ethanolic extract was positive for luteolin and verbascoside compounds. These compounds have several biological activities, including antimicrobial, anticancer, antioxidant and anti-inflammatory effects (Lopez-Lazaro, 2009). In addition, verbascoside compounds exhibit numerous pharmacological properties, such as wound-healing, antineoplastic, and neuroprotective properties (Alipieva, 2014). This study was extended to evaluate the antibacterial activity of the extract against certain gram-positive and gram-negative pathogenic bacteria. The extract exhibited growth inhibitory activity against *A. baumannii*, two *S. aureus* strains, three *E. coli* strains, *Salmonella* Thyphi and *Shigella flexneri*. The antibacterial activity was more noticeable with gram-positive bacteria than with gram-negative bacteria. This may be due to the easy accessibility of the extracts to the cell walls of gram-positive bacteria (Gejalakshmi, 2020). However, the cytotoxic effect of the extract on fibroblasts was lower than that of the MIC. Indeed, based on the United States National Cancer Institute plant screening program, a crude extract is considered as cytotoxic effect when the IC_{50} is less than 30–40 µg/mL (Oskoueian et al., 2011). Although our crude extract met the above criteria, antimicrobial purpose of the *P. amboinicus* extracts has limited its usage as antimicrobial agent in human. Hence, further studies should be carried out to examine the toxicity of each fraction of the herbal extract to identify which fraction maintains antimicrobial activity with safety for human cells. Alternatively, chemical modification of the active constituent from *P. amboinicus* may enhance the safety of this plant for further application.

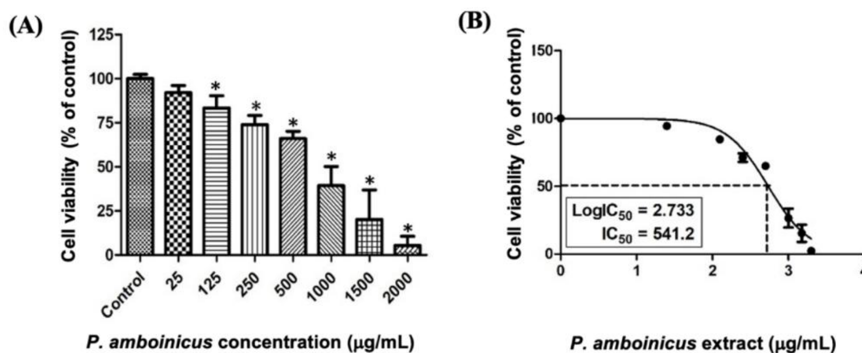

Figure 3 Effects of different herbal extract concentrations on fibroblast viability. (A) Fibroblasts were treated with the extract at 25, 125, 250, 500, 1,000, 1,500, or 2,000 µg/ml for 24 hours. The control group included cells incubated with 0.5% DMSO in medium without the extract. Then, cell viability was assessed via the MTT assay and expressed as a percentage of cell viability compared with 100% of the control. (B) The concentration of the extract that caused the reduction in cell viability to 50% was calculated via Graph Pad Prism. Data from three independent experiments are shown as the mean ±SD, n =3, *p<0.05.

4. Conclusions

The crude leaf extract of *Plectranthus amboinicus* contains a diverse array of promising phytochemicals and secondary metabolites that may interact directly with pathogenic bacteria, exhibiting notable antibacterial properties. The extract demonstrated significant antibacterial activity against eight pathogenic bacteria, with the strongest inhibition observed against *Staphylococcus aureus* strain PB57. Given the growing threat of antibiotic resistance, the discovery of alternative treatments is of critical importance. *P. amboinicus*, with its rich phytochemical profile and demonstrated antibacterial effects, holds promise as a potential source of new antibacterial agents. While these findings are promising, further research is essential to fully explore the therapeutic potential of *P. amboinicus*. Future studies should focus on isolating and characterizing individual bioactive compounds to determine their specific antibacterial mechanisms. In addition to *in vitro* testing, *in vivo* studies are necessary to evaluate the efficacy and safety of these compounds in more complex biological systems. Furthermore, structural modifications of the bioactive compounds could be explored to enhance their safety profiles and improve their therapeutic potential. Therefore, advancing this research could contribute to the development of novel therapies that address the global challenge of antibacterial resistance.

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Ethical considerations

Not applicable.

Conflict of Interest

The authors declare no conflicts of interest.

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