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Research Article

HABITAT INFLUENCE ON THE METABOLITE COMPOSITION OF *Aglaomorpha propinqua* AND ITS BIOLOGICAL ACTIVITY STUDIES

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ABSTRACT

In this study, we explore the phytochemical components of *Aglaomorpha propinqua,* a plant of the Polypodiaceae family, and their potential antibacterial effects on various pathogenic bacteria. Plant rhizomes from lithophytic and epiphytic habitats were selected for extraction using ethanol and water as solvents. The soxhlet method yielded 30.37% and 27.716% extracts in ethanol for epiphytic and lithophytic plants, respectively. Interestingly, unlike higher plants, this fern did not display many metabolites. Compared to the lithophytic plants grown at the same site and tested under similar conditions, higher zone of inhibition (ZOI) values were reported in epiphytic plants. The results were almost identical for the lithophytic plants (higher values of 9-10 mm and lower values of 6-9 mm). Evaluation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) provided additional support for antibacterial potency. Both epiphytic and lithophytic plants exhibited low MIC values, especially when interacting with *K. pneumonia*.

Keywords: *Aglaomorpha propinqua*, epiphytic, lithophytic, MBC, MIC, phytochemical screening

INTRODUCTION

Identifying novel compounds and understanding their apparent therapeutic effects in the human body requires a thorough understanding of the chemical constituents of plants. Many biologically active substances, such as terpenoids, alkaloids, flavonoids, tannins, and phenolic compounds, are found in plants (Abeysinghe *et al.*, 2021; Mousavi *et al.*, 2018; Murali *et al.*, 2021; Olayinka *et al.*, 2021). The chemistry of such classes is representative of activities that can be expected from members. Pteridophytes (ferns and their allies) belong to the class of non-flowering plants, the studies of which are overshadowed by the study of flowering and higher plants. However, they have a long evolutionary history. Pteridophytes are one of the oldest groups of plants in the world (Praveen & Pandey, 2020). These are seedless vascular plants. Pteridophytes fall between non-tracheophytes and higher spermatophytes. During the Carboniferous period, pteridophytes were the dominant vegetation of the earth. Fern-like plants developed seeds, which probably made up half of the fern-like foliage in carboniferous forests, and later gave rise to flowering plants during that period. Globally, there are about 12,000 species of pteridophytes (Green *et al.*, 1992), with approximately 97% being ferns, and the remaining 3% classified as fern allies or lycophytes. These 12,000 named species of pteridophytes are distributed worldwide, and the variety found among them is staggering in its array of forms, textures, and even colors (Hoshizaki & Moran, 2001). Ferns are highly successful and can be found in virtually any habitat where flowering plants are found, unlike fern allies, which are a relict group.

12,000 species of pteridophytes are distributed throughout the moist and shady places of the world and are more prevalent in equatorial regions (Christenhusz *et al.*, 2011). Ferns and fern allies are an integral part of world vegetation and are found in various types of ecological habitats such as terrestrial, epiphytic, aquatic, and lithophytic habitats (Ojha & Niroula, 2021). They are also being used as an important source of foodstuffs, such as *Diplazium esculentum*, *D. maximus*, *Dryopteris cochleata*, etc. Many species of pteridophytes are used in ornamental and horticultural practices, including *Nephrolepis cordifolia*, *Huperzia squarrosa*, *Adiantum* spp. etc. In addition, *Angiopteris helferiana*, *Aleuritopteris bicolor, Drynaria propinqua*, *Tectaria coudanta,* etc. have different traditional medicinal values in different parts of the world (Upreti *et al.*, 2009).

Nepal is a beautiful landlocked country neighbouring China to the north side and India on the other three sides. Geographically, it features a diverse landscape with snow-covered Himalayas in the north, transitioning to lush, tropical fertile plains in the south. Nepal ranks 11th in Asia and 25th in the world for species diversity, with 2 % world flowering plants, 3 % herbal plants, 4 % mammals, and 9 % birds (Shrestha & Khadgi, 2019). A total of 30 plants from 24 families and 29 genera were utilized for firewood, fodder, food, medicine, and rituals. Among these, 12 species of medicinal plants were specifically used for gastrointestinal disorders and ear, nose, and throat (ENT) problems (Bhattarai, 2018). Due to climatic and geographical variations, alpine and temperate plants of the Himalayas offer better possibilities of having the most significant quantities of bioactive compounds. The vegetation in Nepal is dominated by ferns, which are abundant in humid and shady forests. In Nepal, these plants are discovered in various ecological

habitats namely lithophytic, epiphytic, terrestrial, hanging club mosses, climbers, tree ferns, and hydrophytes within different climatic zones. However, some species occur in multiple habitats (Rajbhandary, 2016). Despite being a small country, Nepal has a diverse ecosystem with extreme variability and microclimates created by its mountainous habitats. A total of 583 pteridophytes taxa (550 species and 33 subspecies) belonging to 99 genera in 32 families have been reported in Nepal (Kandel, 2020).

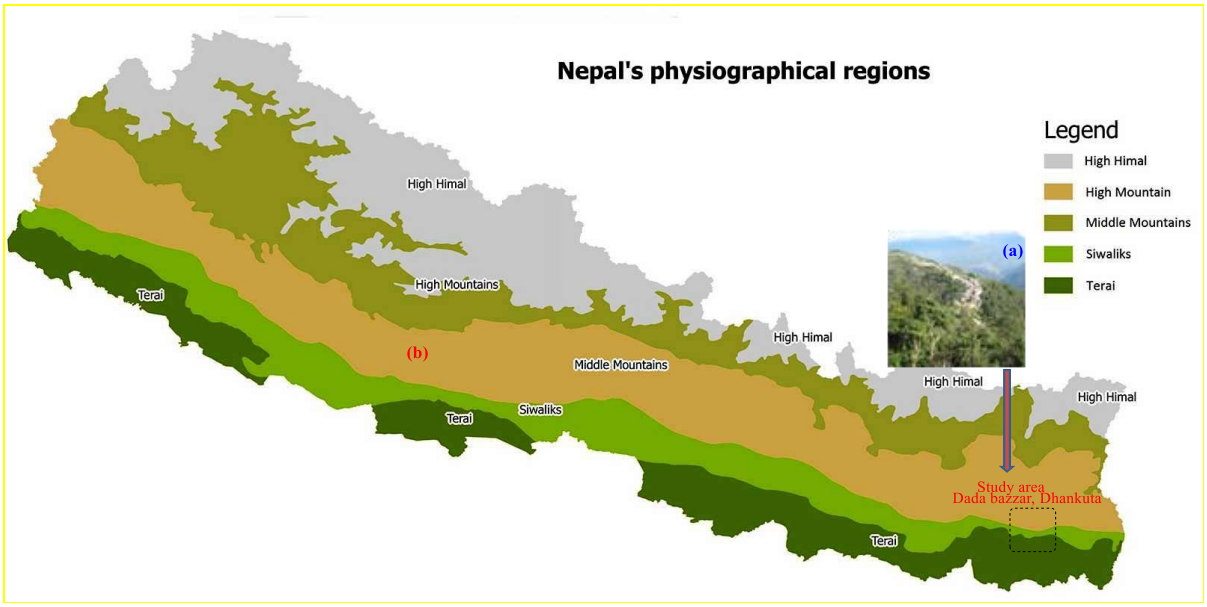

Figure 1. Study area shown in the map of Nepal (Sources: MONTEROSA, 2024; HMT, 2024)

Figure 2. *Aglaomorpha propinqua* a) epiphyte b) lithophyte

As stated by the World Health Organization (WHO), medicinal plants are the primary source of a wide range of therapeutic drugs. Since ancient times, various plant species, including fruits, vegetables, spices, and medicinal herbs, have been used to treat various diseases. Although synthetic pharmaceuticals are widely available and have been very successful in treating various disorders, some people still use traditional folk medicines because they are less hazardous. These remedies are rich in various compounds, particularly secondary metabolites derived from plants, which include phytochemicals such as flavonoids, phenolic compounds, glycosides, tannins, terpenoids,saponins, and other endogenous metabolites (Abdelwahab *et al.*, 2010; Shan *et al.*, 2007). Studies have demonstrated that these compounds exhibit a range of therapeutic properties such as antibacterial, analgesic, antitumor, anticancer, anti-inflammatory, antiviral, and many other activities (Miliauskas *et al.*, 2004; Shan *et al.*, 2007). We conducted phytochemical screening of both epiphytic and lithophytic varieties of *Aglaomorpha propinqua* to determine their essential pharmaceutical activities. Three different extraction techniques, soxhlet, autoclave, and maceration, were used to make the plant extract. Some physicochemical parameters of the plant were also analyzed to ensure the influence of habitat on plant composition. The experiment has been designed to observe antibacterial action against some human pathogenic bacteria. Their antibacterial activities were precisely determined by performing MIC/MBC studies.

EXPERIMENTAL

Materials and reagents

Analytical reagent (AR) grade chemicals and reagents were used in this study. The chemicals used included HCl, H_2SO_4, NH_4OH, $CuSO_4$, $FeCl_3$, I_2, $Pb(CH_3COO)_2$, $HgCl_2$, KI, NaOH, and Na_2CO_3. Distilled ethanol was used as the solvent for extraction and antibacterial tests. Double distilled water was used throughout the laboratory procedure. Clinical strains of pathogenic bacteria were obtained from the microbiology laboratory of Koshi Zonal Hospital, Biratnagar, Nepal. All apparatus used were washed thoroughly, rinsed with double distilled water, and completely dried before use.

Collection and identification of the plant

The plant sample was collected from Sagurigadhi rural municipality, Dada bazaar, Dhankuta, Nepal, in January 2021 (Figure 1). It was authenticated by the Department of Botany at the Mahendra Morang Adarsh Multiple Campus, Biratnagar. Rhizomes of *Aglaomorpha propinqua* were collected from two different habitats and stored separately (Figure 2). The first type was a parasitic plant found on trees (epiphytic), and another type was found on rock (lithophytic). The rhizomes of the plant were carefully cleaned with tap water and distilled water to remove dust and impurities.

Drying

The cleaned rhizomes were cut into fine pieces with scissors and knives. They were left for shade drying for about 1 month. The dried rhizomes were crushed into a fine powder using an electric grinder machine. Afterward, they were dried for two more days at 37 ºC to remove absorbed moisture before extraction.

Extraction

In the present study, three extraction methods were used: maceration, autoclave, and soxhlet extraction, using ethanol and water as solvents to produce a crude extract.

Maceration: A 5 g powdered plant extract (epiphytic and lithophytic) was placed in two separate conical flasks and 50 mL ethanol was added to each. With occasional stirring (several times a day), the extraction process was left for seven days. Afterwards, the contents were shaken and filtered through Whatman filter paper, which took nearly 8 h for complete filtration. Finally, the extracts were concentrated, and phytochemical tests were performed.

Autoclave extraction: For lithophyte and epiphyte extraction, 5 g of the sample was added to 50 mL of double distilled water. The mouth of the flask was covered with a lid (prepared manually using cotton, bandage, and thread). The top face of the conical flask was wrapped in aluminum foil and autoclaved. The flasks were then allowed to cool and subjected to a slow filtration process for concentration, and the tests were performed.

Soxhlet extraction: In this method, a thimble of 20-25 g of dried plant powder was extracted at 40 ºC for approximately 8 h in 250 ml ethanol using a heating mantle. Phytochemical screening was conducted on a small portion of the extract, whereas evaporation was conducted on the remainder of the extract to obtain the solid extract. The dried samples were tested for antibacterial activity.

PHYSICOCHEMICAL ANALYSIS

Physicochemical parameters such as the total ash content, acid insoluble ash, and mass loss during drying were determined following the standard methods of the WHO (WHO, 2011).

PHYTOCHEMICAL SCREENING

Secondary metabolites were qualitatively screened using standard phytochemical methods. The following chemical tests were used to identify and confirm these compounds (Alqethami & Aldhebiani, 2021; Guragain *et al.*, 2020; Ly *et al.*, 2021; Prasathkumar *et al.*, 2021; Shaikh & Patil, 2020; Sousa *et al.*, 2021).

Test for alkaloids

Mayer's Test:

The prominent gelatinous white precipitate produced by adding a few drops of Mayer's reagent (aqueous solution of K_2HgI_4) to the acidified plant extract confirmed the presence of alkaloids.

Wagner's test:

A reddish-brown precipitate of potassium alkaloid produced by adding a drop or two of Wagner's reagent to the extract can also confirm the presence of alkaloids. Here the nitrogen of the alkaloid covalently links with the potassium of the reagent and produces a complex precipitate of the potassium alkaloid.

Test for coumarins:

Coumarins were detected by adding a small volume of alcoholic NaOH to the plant extract which produced a yellow colour.

Test for saponin (froth flotation test):

A small volume of water was added to the plant extract taken in a test tube and the mixture was stirred continuously. The formation of froths on the upper part of the test tube indicated the presence of saponin.

Test for tannins:

A small amount of 5% $FeCl_3$ solution was added to the extract, a black precipitate was formed, which confirmed the presence of tannins.

Test for Flavonoids:

Lead acetate [$Pb(CH_3COO)_2$] solution with the plant extract produced an intense yellow color indicating the presence of flavonoids.

Test of glucoside:
The addition of a few drops of conc. H_2SO_4 to the plant extracts turned black coloration, which indicated the presence of glucosides.

Test for glycoside (killer killiani test):
A (1 mL) glacial acetic acid solution was added to the plant extract and gradually a few drops of $FeCl_3$ solution were added followed by acidification with conc. H_2SO_4. The formation of a green/blue precipitate indicated the presence of glycosides.

Test for emodins:
To the 2 mL of plant extract, 2 mL of NH_4OH and 3 mL of benzene were added. A red coloration indicated the presence of emodins.

Test for terpenoids:
Two mL $CHCl_3$ was added to 5 mL plant extract, left for evaporation in a water bath and 3 mL conc. H_2SO_4 was then added and the mixture was boiled. The grey colored solution indicated the presence of terpenoids.

Test for protein (Biuret Test):
Five percent NaOH solution was added to the 2 mL plant extract, followed by adding a drop or two copper sulphate solution. The appearance of blue coloration confirmed the presence of protein.

Test for steroids (Hesse's response):
The addition of 2 mL $CHCl_3$, and 2 mL conc. H_2SO_4 to 5 mL of extract turned the solution to a pink/red color in the lower chloroform layer, indicating the presence of steroids.

Test for carbohydrates (Fehling's Test):
5 mL of distilled water was added to the 2 mL of plant extract and then filtered. Few drops of dil. HCl was added to hydrolyze the filtrate and neutralized by alkali. Fehling's solution was added to it and boiled for 10 min. The development of red precipitate in the mixture confirmed the presence of reducing sugars.

Antibacterial Activity Study
The antibacterial activity of crude plant extracts was assayed by the standard Kirby–Bauer disc diffusion method using Muller–Hinton agar media (Bauer *et al.*, 1966; Phuyal *et al.*, 2019). Several well-isolated colonies of fresh cultures of pathogenic bacteria such as *E. coli, K. pneumonia, Enterococci spp.*, and *P. aeruginosa* were collected from the microbiology laboratory of Koshi Zonal Hospital, Biratnagar, Nepal. The bacteria were first inoculated in 5 mL nutrient broth and incubated for 2 h at 37 ºC until visible growth was observed. The broth was applied to a nutrient agar medium in Petri dishes using a stick swab. The previously seeded bacterial culture was affixed with well-sterilized paper discs of 5 mm diameter (Whatman no. 1) that were loaded with test chemicals at different concentrations in ethanol (Rolinson & Russell, 1972). Afterwards, the Petri plates were then incubated at 37 ºC for 24 h, and the diameter of the zone of inhibition around each disc was measured using an antibiogram zone measuring scale (Mohamed *et al.*, 2010).

As both epiphytic and lithophytic plant extracts showed remarkable antibacterial activity, they were tested *in vitro* for MIC and MBC measurements. The MIC denotes the minimum inhibitory concentration of a compound needed to inhibit or stop the proliferation of bacteria. MBC is another simultaneously observable parameter that represents the minimum bactericidal concentration of a compound, at which the compound can completely kill the bacteria in the sample. MBC is almost always higher than MIC, with slight differences in concentration. Precision in the assessment of antibacterial activity, therefore, can be obtained using MIC and MBC.

RESULTS AND DISCUSSION

Physicochemical Analysis and Percentage Yield
Table 1 presents the physicochemical parameters (% total ash, % acid insoluble ash, and % moisture) of epiphytes and lithophytes. The distinct physicochemical properties and percentage yield reflect the influence of habitat on plant physiology and metabolite composition. Lithophytes are adapted to soil contact and gain higher mineral content from the soil, while epiphytes retain moisture and accumulate secondary metabolites. It was found that lithophytes had higher total ash content, and epiphytes had higher acid insoluble ash and moisture contents. The percentage yield refers to the % amount of extract obtained from the known weight of the powder sample of the plant. A high % yield was obtained via soxhlet extraction of the plants of both habitats (epiphyte: 30.37% and lithophyte: 27.716%) (Table 2). In a similar study, Sharma (2017) reported a much lower percentage yield of *Drynaria propinqua* (synonym of *Agloamorpha propinqua*), of 10.4 % (Sharma, 2017). In this case, the nature of the solvent may not be a crucial factor because both solvents (methanol and ethanol) are former members of the alcohol family. This variation may be attributed to the extraction technique. Sharma soaked a sample for 72 h in methanol and studied the concentrated extracts. The discussed work provides no information on the nature of plant (epiphytic or lithophytic). Environmental factors may also affect nature and the composition of secondary metabolites.

Table 1. Physicochemical analysis of epiphyte and lithophyte plant

Parameters	Quantity	Epiphyte	Lithophyte
Total Ash	2 grams	3.5%	4%
Acid insoluble ash	2 grams	1.25%	1.15%
Loss on drying	2 grams	10.5%	9%

Table 2. Percentage yield of both epiphytic and lithophytic plant by soxhlet process

Name of plant	Quantity	Part used	Extraction method	solvent	% yield
Epiphytic plant	25 grams	rhizome	soxhlet	ethanol	30.37%
Lithophytic plant	25 grams	rhizome	soxhlet	ethanol	27.716%

Phytochemical Screening

Table 3 illustrates the findings of the phytochemical tests. The presence of tannins was observed in all tests. Glycosides were detected in both lithophytic and epiphytic plants using soxhlet and autoclave extraction techniques. These two techniques were used at temperatures higher than those of maceration process. The maceration technique revealed the presence of tannins in both plants, but the absence of glycosides. Ethanol was used for both soxhlet and maceration extractions. Water was used as the solvent in the autoclave extraction method. The extracts from both techniques showed different results. Glucosides were detected in epiphytic plants using the first two techniques, but not in lithophytic plants. Both soxhlet and autoclave extraction techniques were used to extract tannin. Both lithophytic and epiphytic plants produced saponins in the autoclave process, but only the lithophytic plant produced saponins in the soxhlet process.

Table 3. Phytochemical results of *Aglaomorpha propinqua*

S. No.	Phytochemical tests	Soxhlet extract		Autoclave extract		Ethanol solvent	
		Lithophyte	Epiphyte	Lithophyte	Epiphyte	Lithophyte	Epiphyte
1.	Test for alkaloids						
	a.Mayer's test	-	-	-	-	-	-
	b. Wagner's test	-	-	-	-	-	-
2.	Test for coumarins	-	-	-	-	+	-
3.	Test for saponin	+	+	+	+	-	-
4.	Test for tannin	+	+	+	+	+	+
5.	Test for flavonoids	-	-	-	-	-	+
6.	Test for reducing sugar	-	-	-	+	-	-
7.	Test for Glucoside	-	+	-	-	-	+
8.	Test for glycoside	+	+	+	+	-	-
9.	Test for anthraquinone	-	-	-	-	-	-
10.	Test for emodins	-	-	-	-	-	-
11.	Test for phlobatonins	-	-	-	-	-	-
12.	Test for terpenoids	-	-	-	-	-	-
13.	Test for protein	-	-	-	-	-	-
14.	Test for steroid	-	-	-	-	-	-
15.	Test for emodol	-	+	-	+	-	-
16.	Test for anthraquinone	-	-	-	-	-	-

A lesser amount of research has been done on ferns in terms of their metabolites and properties. Angiosperms are more widely distributed and exhibit greater diversity than pteridophytes do. In addition to their use as ornamental plants and food sources, many ferns have medicinal importance. Ferns are phylogenetic bridges between the lower and higher classes of plants. Many secondary metabolites in ferns have not been found in other plants (Cao *et al.*, 2017). The observation showed higher numbers of metabolites in the epiphytic plant extracts than in the lithophytic extracts. The autoclave extraction technique resulted in the highest number, followed by soxhlet extraction, although the difference between them was small.

Autoclave is an indispensable instrument, even in basic laboratories, because it sterilizes instruments and samples. However, its use in the extraction of phytochemicals from plant powders is seldom known. Its work resembles hectic subcritical water extraction (except for the inert atmosphere requirement). Water can readily dissolve polar compounds compared with non-polar compounds. This was true, but only at lower temperatures. As the temperature increased, the polarity of water decreased with a marked decrease in the dielectric constant from 79 to 35, as the temperature increased from 25 to 200 °C. This water now has a dielectric constant close to that of organic solvents, such as methanol (35) and ethanol (24), so the capability to solvate organic compounds increases. Furthermore, as the temperature increased, the surface tension and viscosity of water decreased. This enhances the diffusion rate and improves the mass transfer rate during the extraction process. As a result, higher

extraction yields were obtained at higher temperatures (Cvetanović *et al.*, 2015). This extraction method is known as subcritical water extraction. It is one of the green approaches of extracting bioactive components from plants' powder.

Antibacterial Activity

The antibacterial potency of the extracts was assessed against four different pathogenic bacteria: *E. coli*, *P. aeruginosa, Enterococci* spp., and *K. pneumonia*. Activity was measured using a zone measuring scale in millimeters. A higher zone of inhibition (ZOI) indicates a greater level of activity. The antibacterial activity of the extract was significant compared to that of an earlier study (Sharma, 2017), which showed no activity against *E. coli*, *S. aureus*, *B. subtilis*, and *S. typhi*. In this assessment, all the samples showed some activities compared to the lithophytic plant, and the epiphytic plant showed better activity. The antibacterial ZOI values were directly proportional to extract concentration. The highest ZOI was observed against *K. pneumonia* in both lithophytic and epiphytic plants (Table 4, Figure 3, and Figure 4).

Table 4. Antibacterial activity of *Aglaomorpha propinqua* rhizome extracts

	Diameter of the zone of inhibition (mm)							
	E coli		*K pneumonia*		*Enterococci spp*		*P aeruginosa*	
Concentration (mg/mL)	100	50	100	50	100	50	100	50
Epiphytic	11	8	14	12	11	7	11	10
Lithophytic	10	6	10	6	10	9	9	7

Table 5. Minimunm inhibitory concentration and minimum bactericidal concentration

	E. coli		*K. pneumonia*		*Enterococci spp*		*P. aeruginosa*	
Concentration (mg/mL)	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Epiphytic	1.562	3.125	0.781	1.56	3.125	6.25	1.56	6.25
Lithophytic	3.125	6.25	3.125	6.25	3.125	6.25	3.125	6.25

The habitat and environmental factors play a crucial role in determining the metabolomic profile of plants. The habitats directly affect the biosynthesis of secondary metabolites responsible for antibacterial properties. Plant metabolites in the same genus and species vary to a certain extent. The variations depend on primary factors, such as climate, nutrition, and hostility to plants, as well as secondary factors, such as extraction method, extraction duration, and extraction temperature. Giri and colleagues recently conducted a green synthesis of silver nanoparticles using tea leaves collected from 86, 1700, and 2000 m elevations in eastern Nepal (Chandra *et al.*, 2020). Interestingly, homogeneous and smallest-sized silver nanoparticles were obtained from a synthesis that used tea from 1700 m. This study indicates differences in the composition of phytochemicals and so biological functions offered by the same plant at different locations. Moreover, the search for novel compounds or activities should not be hindered by earlier studies until the differentiating factors fade. The MIC and MBC values of the extracts are reported in Table 5. The better antibacterial potency of the epiphytic plants was supported by the MIC and MBC studies too. The lowest MIC and MBC values are reported for *K. pneumonia* in both cases.

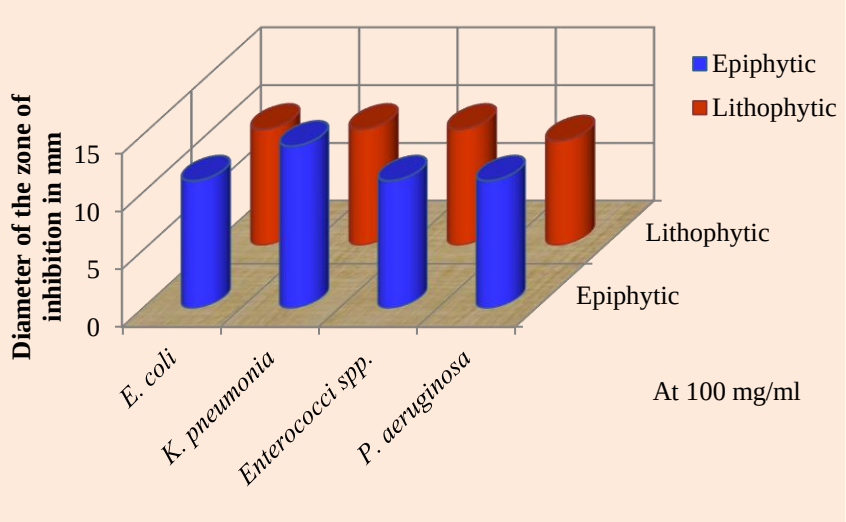

Figure 3. Antibacterial activity of plant extracts at higher concentration (100 mg/mL)

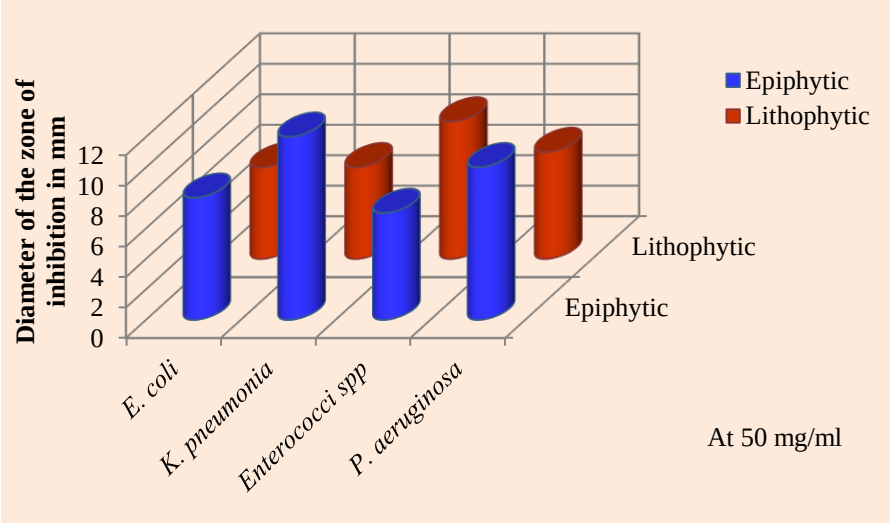

Figure 4. Antibacterial activity of plant extracts at lower concentration (50 mg/mL)

CONCLUSIONS

Phytochemical screening, antibacterial activity test, minimum inhibitory concentration, and minimum bacterial concentration of *Aglaomorpha propinqua* were performed. Various phytochemicals, including saponin, tannin, glucoside, glycoside, emodol, reducing sugar, coumarin, and flavonoid were reported. Three extraction processes were used using two solvents (water and ethanol). Phytochemical tests revealed more phytochemicals in soxhlet and autoclave extraction than in the maceration process. In our study, we found that epiphytes contain more phytochemicals than lithophytes. In the antibacterial test, the epiphytic plant showed higher antibacterial activity than the lithophytic plant. Epiphytic plants at both concentrations showed a higher zone of inhibition against *K. pneumonia*. The value of MBC and MIC of the epiphytic plant is higher than the lithophytic plant.

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AUTHOR CONTRIBUTIONS

NKC: Writing original draft, conceptualization, laboratory work, investigation, data curation, formal analysis; PR: Laboratory work, investigation; BG: analyzing data, review; SB: Writing review & editing, AB: Writing review & editing.

COMPETING INTEREST

There are no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

All the data are included in the manuscript and are available for the readers.

ABBREVIATIONS

MHA: Mueller Hinton Agar; MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactricidal Concentration; ZOI: Zone of Inhibition; ENT: Ear Nose and Throat

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