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Phytochemical Screening and Antibacterial Activity of the Selected Species of Mangroves

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ABSTRACT

As a productive ecosystem component, mangroves provide critical services to humankind. Mangroves may be a source of antibacterial compounds that could be used as a natural treatment for managing bacterial infections. This study determined the phytochemical constituents of leaf extracts of three selected mangrove species *Avicennia marina* (Bungalon), *Rhizophora apiculata* (Bakhaw), and *Sonneratia alba* (Pagatpat), using the Test-tube Method and antibacterial sensitivity using an Agar Well-Diffusion Method found in the coastal areas of Talisay City, Negros Occidental, Philippines. The results showed that the three species of mangroves leaf methanolic extracts exhibit susceptibility to antibacterial properties against *S. aureus*; it was observed that *S. alba* has the highest inhibitory effect on *S. aureus* among the three selected mangrove species. There was a significant difference between the positive control (Linezolid 30 mcg) and the methanolic crude extract of *R. apiculata* (MD = 27.97, $p < 0.01$), *A. marina* (MD = 24.37, $p < 0.01$), and *S. alba* (MD = 23.09, $p < 0.01$). On the contrary, *A. marina* and *S. alba* did not differ significantly with $p = 0.172$; this means that the antibacterial activity of methanolic crude extracts of *A. marina* and *S. alba* are comparable but significantly differ with the positive control (Linezolid 30 mcg). The qualitative phytochemical analyses of the three species of mangroves' leaf methanolic extracts showed flavonoids, phenols, saponins, and tannins. Terpenoids were present only on *R. apiculata.* Phytochemical analysis suggests that mangrove leaves have the potential to be used as a natural pharmaceutical component, such as an antibacterial gel.

Keywords — methanolic extract, mangrove, antibacterial sensitivity, phytochemical analysis, *avincennia marina, sonneratia alba, rhizophora apiculata*

INTRODUCTION

The Philippines has one of the longest coastlines in the world, and it has many diverse mangroves due to its geographical location and tropical climate. It also belongs to the Top 15 countries with the most significant number of mangroves (Long & Giri, 2011). Mangroves are trees and shrubs that grow in the intertidal zone in over 118 countries worldwide between 5º N and 5º S latitudes (Sharma, 2018). Mangroves grow in saline and brackish water environment due to their morphological and physiological adaptation, including prop roots wriggling out of the mud, which could resist tidal waves. Mangrove forests are a highly productive ecosystem component, specifically marine and coastal ecosystems that provide critical services to humans and other biological organisms. Mangrove forests maintain biodiversity in coastal areas.

The mangrove forest encompasses diverse fauna, including fishes, crabs, shrimps, mollusks, and other invertebrates. Mangroves also serve as coastal protection, flood regulation, wildlife habitats, nutrient supply and generation, and erosion control (Primavera, 2004). Also, mangrove ecosystems are becoming popular for their eco-tourism activities, which give additional compensation and sustain coastal communities (Acanto, 2016).

Apart from its environmental and economic importance, several mangrove species are getting attention nowadays due to potential sources of bioactive compounds, which have commercial applications in the field of ethno-pharmaceutical research (Nebula et al., 2013). Local communities have historically used mangroves for medicinal purposes, yet potential sources for novel biological materials, such as antibacterial and antifungal compounds, remain mostly undiscovered.

Phytochemical compounds are essential in different products, especially in pharmaceuticals. Phytochemical compounds are secondary metabolites that have diverse functions. These are used in human therapy, medicine, agriculture, and research (Ukuri et al., 2019). Also, in vitro studies shows that inhibitory effects in some types of microorganisms have been demonstrated by several phytochemicals that belong to several classes (Cowan, 1999). This revelation may suggest the importance of plants such as mangroves as an essential source of phytochemicals that can be used as an alternative treatment for some diseases.

Commercial antibacterial medications are expensive, especially in rural areas. Some antibiotics are not readily available in some areas of the country. Levy (1991) says that funds are scarce in developing countries such as the Philippines.

Thus, it is often impossible to obtain more expensive, effective antibiotics, which are critical in treatment. Because of unequal access to universal healthcare, rural communities often rely on alternative medicine, like herbal remedies, to treat bacterial infections. Some cultural communities also used some parts of trees and herbs found in their area as first aid treatment for infection and diseases.

The vast coastline of Negros Occidental is home to several species of mangroves, which play a vital role in the marine ecosystem and eventually support the livelihood of local people. In the Philippines, efforts have been made by the local government unit and residents to increase the number of species of mangroves along its coastline. The typical mangrove trees found in its coastal areas are the *Avicennia marina* (Bungalon)*, Rhizophora apiculata* (Bakhaw), and *Sonneratia alba* (Pagatpat) (Pingcale et al., 2017). Awareness of these mangrove species in the locality could improve the protection and conservation status in the area. A healthy ecosystem, such as mangrove forests, provides livelihood to many coastal communities. Natural and herbal remedies are also readily available to them.

OBJECTIVE OF THE STUDY

The study was conducted to investigate the presence of phytochemicals present in the leaves and the antibacterial activity in three different mangrove species found in the coastal communities of Talisay City, Negros Occidental, namely: *Avicennia marina*, *Rhizophora apiculata*, and *Sonneratia alba*. The study could be utilized to determine the potential use of leaf extract of the mangroves in antibacterial gel found in the coastal areas of the Philippines. Also, the leaf extract could be used as a baseline for further utilization of selected mangrove leaf extracts for antibacterial susceptibility of other pathogenic microorganisms.

MATERIALS AND METHODS

Collection and Preparation of Sample

Fresh mature green leaves of *A. marina*, *R. apiculate*, and *S. alba* were collected in the morning throughout the low tide from Barangay Catabla Baybay, Barangay Zone 2, Barangay Zone 5, Barangay Zone 15, Barangay Zone 16 and Barangay Bubog in Talisay City, Negros Occidental, Philippines. The mangrove samples were authenticated and certified through their leaf shape, fruits, leaf color, and flower by the Provincial Environment and Natural Resources and the Bureau of Plant Industry in Bacolod City, Negros Occidental.

The collected samples were packed in a polyethylene bag with their corresponding labels. The leaves were washed using tap water, rinsed with distilled water, and air-dried until dry. The air-dried samples were packed in a polyethylene bag and brought to Herbanext Laboratory for processing. Each sample was oven-dried to remove the excess moisture until it was crisp, then pulverized using mortar and pestle and weighed using a digital balance.

The extraction of the sample followed the established protocol by Jacoeb & Suptijah (2013). About 50g of each pulverized sample of *A. marina*, *R. apiculata*, and *S. alba* were soaked with 250ml of methanol using 500ml Erlenmeyer flask and covered with aluminum foil to avoid contaminants and were left for 48 hours at ambient temperature.

The mixtures were filtered in vacuo using the Buchner funnel. The solvent in the filtrate was separated from the mangrove leaves crude extract using a rotary evaporator at 64.7 °C and 760 torr in a medium rotation; this was done until all the solvent had been evaporated. After separation, the crude extracts were stored in dark aluminum containers and stored in the refrigerator at 4 °C.

Phytochemical Screening

The crude extracts of three selected mangrove leaves were screened for various phytochemicals (saponins, glycosides, tannins, flavonoids, phenol, alkaloids, and terpenoids) through standard qualitative tests or test-tube method based on the protocol established by Acanto et al. (2022), Acanto & Cuaderes (2021), especially in the countryside. With its favorable characteristics, this macrofungi shows potential to be utilized in the field of natural products. The study aims to ascertain the antimicrobial effects and determine the active components present in the ethanolic extracts. The extract's antibacterial activity was determined using Kirby-Bauer Disk Diffusion Assay, antifungal susceptibility through Poisoned Food Method, and minimum inhibitory concentration (MICSurya and Hari (2017) & Aguinaldo et al. (2004).

Test for Alkaloids. About 1 ml of *A. marina* extract was mixed with 0.2 ml of 1% HCl in a 10 ml test tube using a pipette. Then, 1 ml of Mayer's reagent was added. Any yellow precipitate or turbidity indicates the presence of alkaloids. The process was repeated for *R. apiculata* and *S. alba* extracts.

Test for Flavonoids. Using a pipette, about 1 ml of *A. marina* extract was mixed with a few fragments of magnesium ribbon and concentrated hydrochloric acid in a 10 ml test tube. The appearance of pink or magenta-red color or yellow precipitate indicates the presence of flavonoids. The process was repeated for *R. apiculata* and *S. alba* extracts.

Test for Glycosides. Using a pipette, about 1 ml of *A. marina* extract was mixed with 2 ml of glacial acetic acid containing 1-2 drops of ferric chloride solution in a 10 ml test tube. The mixture then flowed into another test tube containing 2 ml of concentrated sulfuric acid. The appearance of the brown ring stipulates the presence of glycosides. The process was repeated for *R. apiculata* and *S. alba* extracts.

Test for Phenols. Using a pipette, about 1 ml of *A. marina* extract was mixed with 2 ml of ferric chloride solution using a 10 ml test tube. The appearance of green, blue, and white indicates phenols' presence. The process was repeated for *R. apiculata* and *S. alba* extracts.

Test for Saponins. Using a pipette, about 1 ml of *A. marina* extract was watered-down with 20 ml of distilled water in a 25 ml test tube and shaken vigorously. The formation of stable foam indicates the presence of saponins. The process was repeated for *R. apiculata* and *S. alba* extracts.

Test for Tannins. Using a pipette, about 1 ml of *A. marina* extract was watered-down with 20 ml of distilled water in a 25 ml test tube and heated using an alcohol lamp. Then a few drops of 0.1% ferric chloride are added. The appearance of a brownish-green or blue-black color indicates the presence of tannins. The process was repeated for *R. apiculata* and *S. alba* extracts.

Test for Terpenoids. About 1 ml of *A. marina* extract was mixed with 2 ml chloroform in a test tube using a pipette. Then 3 ml of sulfuric acid was carefully added. The appearance of a reddish-brown or pinkish-brown ring/color indicates the presence of terpenoids. The process was repeated for *R. apiculata* and *S. alba* extracts.

Antibacterial Activity

Staphylococcus aureus was obtained from Herbanext Laboratories in Barangay Taluc, Bago City. This was used as the test organism for the antibacterial activity in the study via the agar-well diffusion method. The *S. aureus* was prepared using 5ml NaCl-peptone buffer, and 0.5 McFarland turbidity standard was observed to secure ~1x10^8 CFU/ml; this was confirmed by determining bacterial count by plating. The Mueller Hinton Agar (MHA) was prepared using 19g of MHA dissolved entirely in 500 mL of distilled water in an Erlenmeyer flask covered with a cotton plug and aluminum foil. The MHA mixture was then autoclaved at 121 °C for 15 minutes; enough MHA solution was poured to cover the entire surface of sterile Petri dishes while observing the right thickness (not too thin and not too thick); the agar plates were left to set at room temperature to cool and solidify, ready for inoculation.

Using an aseptic technique, the standardized inoculum was swabbed on the surface of Mueller Hinton Agar (MHA) using a sterile cotton-tip swab; this was done by dipping the cotton swab in the inoculum and streaking the cotton swab from the topmost portion until the bottom portion of agar surface from edge to edge of Petri dish repeating it several times in different directions to ensure that the inoculum was spread evenly into the agar surface. The inoculated MHA dishes were covered and stacked upside down to prevent condensation on the surface cover. This was done in a laminar flow hood.

A cork borer (about 6.0 mm hole-sized) was sterilized by dipping in 70% ethyl alcohol, passed through the flame of a Bunsen burner, and was used to make a well on the agar surface. Four agar wells were made in each agar plate, equidistant from each other. About 30 μL of each mangrove extract (*R. apiculate*, *A. marina*, and *S. alba* and the control (Linezolid 30 mcg) were placed in one of each agar wells on each agar plates and allowed to pre-dried for 15 minutes before incubation. Each treatment has three replicates and was conducted in three trials.

The plates were incubated for 24 hours at 37 °C in an incubator and evaluated for antimicrobial activity. The measurement across the growth inhibition zones was measured in millimeters (mm) using a vernier caliper. In measuring the zone of inhibition, a zero tip was placed at the center of the hole of the well and measured from the center to the edge of the area with zero growth; the zone of inhibition was determined through color difference in the agar surface. If the inhibition zone overlaps the other, measure the inhibition zone's radius, then multiply by two to get the diameter (Hudzicki, 2009). Table 1 presents the Zone Diameter Interpretative Table based on Cheesebrough's (2005) zone diameter interpretative standard for human pathogens. This was used to determine the antibacterial susceptibility of the mangrove leaf extracts.

Table 1. Zone of Inhibition Diameter Interpretative Table

Verbal Interpretation	Zone of Inhibition
Susceptible	15mm ≤ above
Intermediate	13mm – 14mm
Resistant	≤ 12mm

Statistical Analyses

Mean and standard deviations (SD) were used as statistical tools for the descriptive analysis of data specific to determine which among the experimental treatments (methanolic crude extracts of *R. apiculata*, *A. marina*, and *S. alba* can inhibit the growth of *S. aureus.* One-way Analysis of Variance (One-way ANOVA) and Fisher's Least Significant Difference (LSD) for post hoc analysis were used for the inferential analysis of data, specifically in comparing the means (in terms of zone of inhibition) of experimental treatments with the positive control.

RESULTS AND DISCUSSION

Phytochemical Screening of the Leaves of *R. apiculate*, *A. marina*, and *S. alba*

Table 2 shows the phytochemical screening – the presence or absence of secondary metabolites present in the methanolic crude extracts of *R. apiculata*, *A. marina*, and *S. alba* using the Test Tube Method.

Flavonoids, phenols, saponins, and tannins were present in the methanolic crude extracts of *R. apiculata*, *A. marina*, and *S. alba*. In contrast, glycosides and alkaloids were both absent or may be present but in minute amounts in the three species of mangroves. Terpenoids were the only bioactive compounds present in *R. apiculata* but not found in both *A. marina* or *S. alba*. When extracted from mangrove species, these secondary metabolites are essential sources of compounds necessary for developing novel drugs against many diseases that humans are currently fighting (Acanto, 2020; Saxena et al., 2013). Thus, the presence of flavonoids, phenols, terpenoids, and tannins in the methanolic crude extracts of the three selected mangrove species are important secondary metabolites that can inhibit the growth of microorganisms and shows a potential antibiotic property.

Table 2. Qualitative Phytochemical Screening of *R. apiculata (Bakhaw)*, *A. marina (Bungalon)*, and *S. alba (Pagatpat)*

Phytochemical Constituents	Bakhaw *(R. apiculata)*	*Bungalon (A. marina)*	*Pagatpat (S. alba)*
Flavonoids	+	+	+
Phenols	+	+	+
Saponins	+	+	+
Terpenoids	+	-	-
Tannins	+	+	+
Glycosides	-	-	-
Alkaloids	-	-	-

Note: Positive (+) indicates the presence of a phytochemical constituent; (-) indicates the absence of a phytochemical constituent, or (-) denotes compounds that may be present in relatively smaller amounts.

Flavonoids present in three species of mangroves are considered polyphenolic compounds abundant in nature, found explicitly in fruits, vegetables, and beverages humans consume daily, like tea and coffee. The flavonoids have played a significant role in the victorious medical nursing of classical times, and their use has been determined to date (Ullah et al., 2020 & Panche et al., 2016). It has broad biological and pharmacological activities and has been reported to exert multiple antimicrobial, anti-inflammatory, anti-tumor, and cytotoxicity activities. Flavonoids are best known for their capacity to act as powerful antioxidants that can protect the human body from free radicals (Kumar & Pandey, 2013). Catechins and luteolins are the two best-known antioxidants, better than other antioxidants such as vitamins C and E and β-carotene. Flavonoids protect biological systems by a capacity to transfer electrons free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce alpha-tocopherol radicals, and inhibit oxidases (Iqbal et al., 2019; Tiwari et al., 2001). The most numerous and structurally diverse among plant secondary metabolites are phenolic compounds. Phenols present in the three species of mangroves are considered the most precise class of bioactive compounds. As secondary metabolites, they have an essential role as defense compounds and exhibit several properties beneficial to humans. Their antioxidant properties protect humans against free-radical-mediated diseases (Wu et al., 2008; Ukachukwu et al., 2013). The presence of flavonoids and phenols in the leaves of three species of mangroves suggests that their leaves could be utilized as a component of food supplements to provide antioxidant compounds.

Terpenoids unique to *R. apiculata* possess medicinal properties such as antimicrobial, antimalarial, and anti-carcinogenic. Most terpenoids have a multi-cyclic structure that differs from each with unique functional groups and carbon skeletons. Many terpenoids are the main constituents of essential oils, according to the measurable quantity of isoprene units, such as diterpenes, monoterpenoids, and triterpenes, among others (Saxena et al., 2013). This result suggests that *R. apiculata* has a potential for ethno-pharmaceutical use in the management of certain bacterial and viral infections. With this, further studies must be conducted to establish the potential use of leaves of *R. apiculata* as alternative medicine, especially as antimalaria.

Tannins are essential plant-derived secondary metabolites and water-soluble polyphenols usually used in tanning animal skins and textile dyes. In Asian natural healing, tannin-containing plant extracts are antiseptic, antidiarrheal, antioxidant, anti-inflammatory, diuretics, astringents, and hemostatic pharmaceuticals. Tannins clarify wines and antioxidants found in fruit juices in the food processing industry. The biological action of tannin-containing plant extracts becomes increasingly crucial for the new lead compounds to develop novel pharmaceutical products (Abioye et al., 2013; Thirunavukkarasu et al., 2018; Sowunmi & Afolayan, 2015; Tiwari et al., 2001; Saxena et al., 2013). With this, further studies may be conducted to observe the potential use of leaves of three species of mangroves as herbal medicine for diarrhea and wound healing, especially in rural coastal areas. The phytochemical results also suggest that the leaf extract of the three selected mangrove species could be used to develop natural products such as soap and hand sanitizer, especially with the advent of new infectious diseases that affect the world today.

Antibacterial Activity of Selected Species of Mangrove against *S. aureus*.

Table 3 presents the sensitivity testing of *S. aureus* to the methanolic crude extracts of *A. marina, R. apiculata*, and *S. alba* using the Agar Well Diffusion method. The antibacterial assay is crucial to test and screen the inhibitory effects of methanolic crude extract of three mangrove species against *S. aureus* before establishing what product can be produced based on the result.

The methanolic crude extracts of three species of mangroves, namely: *R. apiculata*, *A. marina*, and *S. alba*, showed a positive response and hindered the growth of *S. aureus* with the average ranging from 15.82 mm as a minimum to 20.70 mm as a maximum zone of inhibition and interpreted as "susceptible." Linezolid (30 mcg) was used as a positive control in this study with a 43.79 mm (SD= 1.27) inhibition zone. The table also shows the mean diameter of the zone of inhibition of the three species of mangroves, *S. alba* has 20.70 mm (SD= 1.33), *A. marina* has 19.42 mm (SD= 0.74), and *R. apiculata* has a 15.82 mm (SD= 0.68) zone of inhibition, respectively. All mangrove leaf extract is susceptible to *S. aureus*. The mean shows that *S. alba* leaf extract has the most significant growth inhibitory effect among the three species of mangroves. This suggests that *S. alba* leaves could have the potential as an alternative medicine for bacterial infection, especially in rural and coastal areas where access to commercially available drugs is difficult, if not expensive. The results further show that the methanolic extract of mangrove leaves of the three selected mangrove species shows antibiotic properties that could be a potential for the development of ethno-pharmaceutical products that could help people in the community be aware of the medicinal properties of mangroves around their communities for the management of infectious diseases, especially in areas where access to healthcare is challenging and expensive. Using natural remedies will help ease the financial burden of people in rural communities, especially those without access to healthcare professionals.

Saad et al. (2012) confirmed that the methanolic leaf extract of *S. alba* appeared to be the most effective extract against *S. aureus*. In the study conducted by Thamizharasan and Saravanan (2016), the methanolic crude extract of *Rhizophora* species significantly differed from nine other species of mangroves being studied and showed maximum activity with a 15 mm zone of inhibition against test pathogens. This result is in line with the study conducted by Saad et al. (2012), which revealed that the methanolic leaf extracts of *S. alba* be an effective extract against *S. aureus. A. marina* leaf extract could also be used to treat bacterial infection. They can be used to discover new bioactive natural products that can be used as a potential source that may control microbial infections (Sharief & Rao, 2014). On the contrary, *R. apiculata* methanolic extracts exhibited excellent antimicrobial activity against human clinical pathogens (Ramalingam et al., 2018). Jadhav and Jadhav (2012) proved that methanol extracts have high antimicrobial activity than their ethanol counterpart, and cold methanol is an excellent solvent for extracting the antimicrobial compounds from extracts.

*S. aureus' s*usceptibility to the three mangrove species' methanolic crude extracts may be due to flavonoids, phenols, terpenoids, and tannins in the extract. These phytochemicals can inhibit the microorganisms' growth (Gowri & Vasantha, 2010; Shahla et al., 2010).

Results also showed a highly significant difference in the mean zone of inhibition between and among the experimental groups, $F_{(3,8)}= 444.37$, $p= 0.00$. Furthermore, the results also revealed that there was a significant difference between the positive control (30mg Linezolid) and the three mangrove species during the post hoc analysis; thus, this suggests that though leaf extracts of selected mangrove species have exhibited susceptibility to *S. aureus*, a synthetic antibiotic such as Linezolid is still more effective. Perry and Jarvis (2001) indicate that Linezolid is an effective agent against staphylococci such as *S. aureus* and effective in methicillin-resistant strains, glycopeptides, and other antibacterial agents. However, the results also present that the antibacterial susceptibility of *A. marina* and *S. alba* were comparable with each other with a mean difference of 1.28, $p= 0.17$; this implies that the antibacterial susceptibility against *S. aureus* of the two mangrove species could have the same effect.

The study of Behera et al. (2014) evaluated the antibacterial potential of mangrove species against nine human pathogenic bacteria, and the result was compared to amphoxylin as a standard antibiotic. It was found that the methanolic extract of *A. marina* showed moderate antibacterial activity against pathogenic bacteria compared to standard antibiotic drug resistance. Thus, the result of this study suggests that *A. marina* and *S. alba* could be used to manage bacterial infections, specifically bacterial skin infections caused by *S. aureus* in rural areas, especially in coastal communities that have no access to healthcare specifically for first aid treatments of bacterial infections.

Table 3. *Antibacterial Activity of Selected Species of Mangrove against S. aureus.*

Samples	Zone of Inhibition (mm)		
	Mean	SD	Interpretation
*R. apiculata***	15.82	0.68	Susceptible
*A. marina***[a]	19.42	0.74	Susceptible
S. alba **[a]	20.70	1.33	Susceptible
Linezolid (30mg)**	43.79	1.27	Susceptible

**$F_{(3,8)}$= 444.37, p=0.00; [a]p>0.05

Note:		
	Susceptible (S)	15mm ≤ above
	Intermediate (I)	13mm – 14mm
	Resistant (R)	≤ 12mm

CONCLUSION

The following conclusions were drawn from the results of the study: (1) *R. apiculata* (Bakhaw), *A. marina* (Bungalon), and *S. alba* (Pagatpat) contained flavonoids, phenols, saponins, terpenoids (*R. apiculata* – Bakhaw), and tannins. Therefore, the leaves of the three mangrove species could potentially develop ethno-pharmaceutical products and be used for natural product development to help communities in rural areas. (2) The methanolic crude extracts of *R. apiculata* (Bakhaw), *A. marina* (Bungalon), *S. alba* (Pagatpat) were susceptible to *S. aureus.* It was also found that *S. alba* is the most effective extract to inhibit the growth of *S. aureus* among the three mangrove leaf extracts.

RECOMMENDATION

Researchers and pharmaceutical companies may use the study's results to thoroughly investigate the potential of mangroves as antibacterial agents and explore other parts of mangroves, such as the roots, leaves, bark, and flowers, for more effective antibacterial drugs. They may develop other antibacterial products like disinfectant gel. The study only uses one test pathogen; thus, it is also essential to test the bacterial inhibitory effect of these three mangrove species leaf extracts, such as in *Pseudomonas sp.* and *E. coli,* to determine further its viability as an antibacterial agent.

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