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ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICALS OF ETHANOLIC EXTRACT OF LOCAL *ORTHOSIPHON ARISTATUS* LEAVES

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Abstract

The interest in drug discovery from plant sources has been the focus for many researchers due to the increasing emergence of drug-resistant microbial strains. This study investigated the antibacterial activity of *Orthosiphon aristatus* leaves and their phytochemical compounds against gram-positive (*Streptococcus mutans, Staphylococcus epidermidis*, and *Propionibacterium acnes*) and gram-negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*). The antimicrobial activity of the methanolic extract of *Orthosiphon aristatus* leaves was determined by using well diffusion assay, minimum inhibitory (MIC) and minimum bactericidal concentration (MBC). The extract showed inhibition against *S. epidermidis*, *S. mutans, P. acnes* and *K. pneumoniae*, ranging from 9 mm to 30 mm. *E. coli* was not inhibited. The MIC and MBC values for *S. mutans* were 7.81 mg/ml, *S. epidermidis* were 125 mg/ml, *P. acnes* were 3.91 mg/ml, and *K. pneumoniae* were 62.5 mg/ml. Data analysis showed that *S. mutans*, *P. acnes*, and *K. pneumoniae* had no significant mean difference between the plant extract and the corresponding antibiotic, while *S. epidermidis* showed statistical significance. Qualitative phytochemical tests showed the presence of glycosides, flavonoids, alkaloids, saponins, tannins, phenolics, and terpenoids in the extract. The findings indicate *Orthosiphon aristatus* as a promising antimicrobial agent.

Keywords: *Orthosiphon aristatus*, Antimicrobial Activity, Phytochemical Compounds, Gram-positive, Gram-negative

Introduction

Microorganisms exist in every nook and corner of the world. Though not necessarily pathogenic, microorganisms can pose a problem to human health. This problem becomes a more pressing matter when recent trends show an increased resistance of bacteria against several antibiotics. *Streptococcus mutans* is a gram-positive facultative anaerobic coccus and normal flora in the mouth. It causes a common infection for dental carriers due to its ability to form a biofilm that would later form dental plaque if not regularly cleaned (1). World Health Organization stated dental caries is the most common non-communicable disease worldwide (2), while the Centers for Disease Control and Prevention reported that caries prevalence among children were 17%, adolescents were 57%, while 9 in 10 adults were affected by caries (3). There was an increase in dental caries in Europe from 9000 BC to 1850 AD (4). The biofilm is a thick layer of microorganisms that develops a colony and sticks to a surface while covering the microbe in a slime layer for protection. The organisms in the nutrient-poor zones contribute to drug resistance in the nutrient and oxygen gradient (5). The existence of antibiotic-resistant genes, such as the tet gene in tetracycline (6) or the *mefA, ermB*, and *ermTR* genes in erythromycin resistance, is another pathway for antibiotic resistance (7). *S. mutans* showed an increased frequency of antibiotic resistance compared to other oral streptococci (8). *Staphylococcus epidermidis* is a gram-positive coccus. Although typically regarded as commensals of the human skin, *S. epidermidis* has also emerged as one of the most critical human infections acquired via health care settings. Due to the increasingly common usage of medical devices and implants, especially catheters, *S. epidermidis* is the leading suspect in catheter-related infections (9). *S. epidermidis'* virulence mechanism stems from its ability to form a biofilm, allowing them to adhere deeply to medical devices and implants (10). Approximately 50% of the catheters inserted into patients

are infected (11). 60% of the organisms responsible are *S. epidermidis. S. epidermidis'* antimicrobial resistance is attributed to its biofilm and *mecA* gene that encodes penicillin-binding protein 2a (PBP 2a) (12). The *mecA* gene could be transferred to other staphylococci, causing more staphylococci to resist beta- lactam antibiotics. Recent trends show *S. epidermidis* becoming more resistant to erythromycin, methicillin, and rifampicin (12, 13). *Propionibacterium acnes* is an anaerobic gram-positive bacillus. It is a human skin commensal that can cause acne. According to the Global Burden of Disease Survey, acne vulgaris is the third most common dermatologic illness and ranks eighth among the world's ten most common diseases (14). *P. acnes* antibiotic resistance is mostly caused by chromosomal mutations, and erythromycin and clindamycin resistance have been linked to point mutations in the genes encoding the 23S subunit of ribosomal RNA. On the other hand, the tetracycline resistance gene has been linked to a mutation in the 16S rRNA (15). Recent reports show *P. acnes* gaining immunity against macrolide antibiotics (16, 17). *P. acnes* develops Tn5432, a *Corynebacterium* transposon carrying the erm(X) resistance gene, which mediates the development of Macrolide-Lincosamide-Streptogramin (MLS) resistance. (15). Aside from gram-positive organisms, gram-negative organisms are also a threat to human health. *Escherichia coli* is a gram-negative bacillus that has been proven to cause worldwide outbreaks via food and is the leading cause of urinary tract infection (UTI) (18). *E. coli* possesses several virulence factors that enable them to cause UTIs easily (19). Women are generally more easily infected due to the proximity of their urethra and anus and their shorter urethra, which makes access to the bladder easier (20). It is estimated that 40% of women would contract UTI at least once in their lifetimes (21). According to a report, extended-spectrum β -lactamase-producing gram-negative bacteria, such as *E. coli*, are becoming more and more medication resistant (22). Drug resistance in gram-negative bacteria is caused by their outer membrane. Most hydrophilic antibiotics, like β-lactams, penetrate gram-negative bacteria's outer membrane via porins, whereas hydrophobic antibiotics diffuse their way through the membrane to reach their targets. The alterations or modifications of the outer membrane, such as porin mutations or a change in the hydrophilic structure, are what causes drug resistance (23). *Klebsiella pneumoniae* are gram-negative bacteria that cause a wide array of nosocomial infections accounting for 10% of all nosocomial infections (24). The capsule surrounding the bacteria contributes the most to its virulence factor by evading opsonophagocytosis and serum killing from the host along with its polysaccharides outer coat, fimbriae, and siderophores (25). A study detected an increase in *K. pneumoniae* infections in a hospital between 2006 to 2020, as high as 30% (26). The possible rise is brought about by *K. pneumoniae's* growing resistance to seven different medicines, particularly ciprofloxacin, and imipenem. Many antibiotics are used to treat them, which in turn causes antimicrobial resistance. An alternative is searched to treat them, which includes herbal treatment.

Research for alternative antibacterial agents from natural plants are becoming increasingly popular, especially after it has been proven that some plants contain broad-spectrum antimicrobial activity (27). One of these researched plants is a typical Southeast Asian plant called the Java tea plant. Java tea, also known as *Orthosiphon aristatus* or *Misai Kucing* in local dialects, is a plant native to southern China, Southeast Asia, tropical Queensland, and the Indian Subcontinent that is primarily valued for its medicinal benefits in traditional folk medicine (28). The plant typically stands 75 cm tall and has a quadrangular stem that has a faint purple tint. The globose leaves are lanceolate with serrated margins and are grouped in pairs. The flowers that grow along terminal racemes are either white or purple (29). *O. aristatus* has been widely known for its numerous benefits in traditional medicine, including anticancer (30, 31), anti-inflammatory (32), antioxidant (33), diuretic and anti-lithic (34). *O. aristatus* also possesses bioactive compounds that exhibit antimicrobial properties. Alkaloids, flavonoids, and terpenes are examples of secondary metabolites derived from crude plant extracts that may also function as antimicrobials and resistance modifiers by binding to protein domains and altering interactions between proteins (35). A clear advantage of plants instead of synthetic antibiotics is that they could also affect the main events in the pathogenesis, reducing the risk of the bacteria developing resistance to them (35). Thus, this study aims to investigate the phytochemical compounds and the antimicrobial efficacy of methanolic *O. aristatus* leaves extract against *S. mutans*, *S. epidermidis*, *P. acnes, E. coli*, and *K. pneumoniae.*

Materials and Methods

Sample collection and extraction

Healthy leaves of *O. aristatus* were collected from a local nursery in Shah Alam, Selangor, Malaysia (3.04867, 101.51353). The leaves were cleaned with distilled water, air-dried, and powdered with a mechanical blender. It was kept in an air-tight container at 4°C when not in use. The extraction was done using the maceration technique according to Ashraf et al. (33) with some modifications. 200g of the powdered leaves were macerated in 2000 ml of absolute methanol for 48 hours with periodic shaking. The solution was first filtered with a muslin cloth sieve and then again with Whatman No. 1 filter paper before the extract was collected in a Scott bottle and concentrated until dry with a rotary evaporator under reduced pressure of 200 mbar at 40°C. 15000 mg of methanolic extract of *O. aristatus* leaves were weighed and added to 15 ml of DMSO to obtain a concentration of 1000 mg/ml.

Bacterial culture

The stock cultures *Streptococcus mutans* (ATCC 25175), *Staphylococcus epidermidis* (ATCC 12228), *Propionibacterium acnes* (ATCC 6919), *Escherichia coli* (ATCC 25922), and *Klebsiella pneumoniae* (ATCC 13883) were obtained from the Centre for Medical Laboratory

Technology Studies, Universiti Teknologi MARA, Puncak Alam. Before experimental use, the cultures were subcultured onto 10% sheep blood agar and nutrient agar. *E. coli, K. pneumoniae,* and *S. epidermidis* were incubated overnight at 37°C, *S. mutans* were incubated in a CO_2 condition at 37°C for 48 hours, and *P. acnes* were incubated in a candle jar for up to 7 days.

Antimicrobial susceptibility test (AST)

The AST on each bacterial species was performed by well diffusion agar following the method by Rubab et al. (36) with modifications. Inoculum preparation was done by suspending 3-5 colonies of each bacterial species in Muller-Hinton broth and incubating at 37°C. The turbidity of the suspensions was compared with the 0.5 McFarland standards, which was equal to 1.5 X 10^8 CFU/ml microbial load. The well agar diffusion technique was used for the AST of the methanolic extract of *O. aristatus* leaves against *S. mutans, S. epidermidis, P. acnes, K. pneumoniae,* and *E. coli.* Lawn cultures were made on a Muller-Hinton agar plate for all bacterial species. Chloramphenicol (30 µg) was used as a positive control for gram-positive bacteria, gentamicin (10 µg) for *E. coli* and amikacin (30 µg) for *K. pneumoniae*. Ten per cent of DMSO was used as a negative control. Wells were formed using a sterile cork borer before the methanolic extract of O*. aristatus* leaves, the positive and negative controls were added to them. Then, the plates were incubated at 37°C overnight for *K. pneumoniae, E. coli,* and *S. epidermidis*, 48 hours in CO_2 for *S. mutans*, and seven days in a candle jar for *P. acnes.*

Minimum inhibitory concentration (MIC)

MIC is the lowest concentration of an antimicrobial agent that inhibits the visible growth of bacteria. It can be used to determine the antimicrobial activity of a test agent against a specific bacterial species. The minimum inhibitory concentration (MIC) was performed by the micro broth dilution method according to the Clinical and Laboratory Standard Institute (CLSI) with slight modification (37). One thousand µl of the methanolic extract of *O. aristatus* leaves was added to well 1 and 50 µl to well 2-10 of a 96-microtitreplate. Two-fold dilution serial dilution was performed for well 1- 10. Bacterial suspension that achieved exponential phase was adjusted to meet 0.5 McFarland standards. The suspension was diluted by adding 200 µl of suspension to 19.8 ml sterile Muller-Hinton broth to reach a final concentration of 5 x 10^5 CFU/ml. Fifty µl of the adjusted suspension was dispensed to wells 1-10. The last two wells act as positive and negative growth control, which contain Muller-Hinton broth with organisms and sterile Muller-Hinton broth, respectively. The plates were incubated for 24 hours. The presence of turbidity or the formation of pallets at the bottom of the wells confirmed bacterial growth. MIC endpoint is well with the lowest concentration inhibiting bacterial growth.

Minimum bactericidal concentration (MBC)

MBC is the lowest concentration of an antimicrobial agent that kills a specific bacterial species. An aliquot from every twelve wells was streaked onto Muller-Hinton agar plates. The plates were then incubated. The MBC was defined as the concentration at which 99.9% of the bacteria were dead (37).

Qualitative phytochemical analysis

The qualitative analysis is based on the visual colour change reaction in response to the presence of a specific phytochemical component. The methanolic extract of *O. aristatus* leaves was tested for glycosides, flavonoids, terpenoids, alkaloids, phenolics, saponins, tannins, and anthraquinone using the previous standard method with slight modification (38, 39). Fehling's test for glycosides was done by mixing equal amounts of Fehling A and Fehling B solutions with 2 ml of methanolic extract of *O. aristatus* leaves and were heated in a water bath. The presence of a red precipitate indicates positive glycosides. Alkaline reagent test was done to determine flavonoids. Two ml of sodium hydroxide solution was mixed with 2 ml of methanolic extract of *O. aristatus* leaves. A yellow or red colour indicates the presence of flavonoids. Salkowski test was done to determine presence of terpenoids. Two ml of chloroform were dropped to 5 ml of methanolic extract of *O. aristatus* leaves before adding 3 ml of sulfuric acid. The formation of a reddish-brown layer between the two solutions indicates the existence of terpenoids. Wagner test was used for alkaloids determination by adding 4 drops of Wagner reagent to 2 ml of methanolic extract of *O. aristatus* leaves. A reddish-brown precipitate indicates the presence of alkaloids. Ferric chloride was used to determine phenolics. About 50 mg of the methanolic extract of *O. aristatus* leaves were dissolved in distilled water. Twenty drops of 5% ferric chloride solution were added to the mixture and mixed well. Any colour change to purple, red, green, or blue indicates the positive phenolic compounds. Borntrager's test was used to detect anthraquinones. About 0.2 ml of crude extract was added to 10 ml of benzene before being shaken together and filtered. The filtrate was added to a 10% ammonia solution. The appearance of pink, red, or violet colour in the ammonical (lower) phase indicates a positive reaction. Saponins were detected using the foam test by adding 3 ml of distilled water was added to 2 ml of plant extract. The mixture was shaken vigorously to obtain a stable foam. The formation of stable, persistent foam on the upper layer indicates a positive reaction. Finally, tannins were tested by dropping 1 ml of extract to 3 drops of 5% ferric chloride. The formation of a greenish- black precipitate indicates a positive reaction.

Statistical analysis

The result of the antimicrobial test was analysed using SPSS software and expressed as mean ± standard deviation (SD). An Independent t-test was used to establish the significant difference in the means between the zone of inhibition of plant extract against each bacterial species and its corresponding standard antibiotic. The acceptance level of significance for the results was $p < 0.05$.

Results

Orthosiphon aristatus leaves crude extract

The crude extract obtained was greenish-black with a viscous, sticky consistency (Figure 1). About 7.62% of yield extract was obtained from the extraction process with 200 g of powdered *O. aristatus* leaves and 15.23 g of extract. The following formula calculated the percentage yield of crude extract:

Percentage yield = (Extract weight / Powdered plant weight) x 100

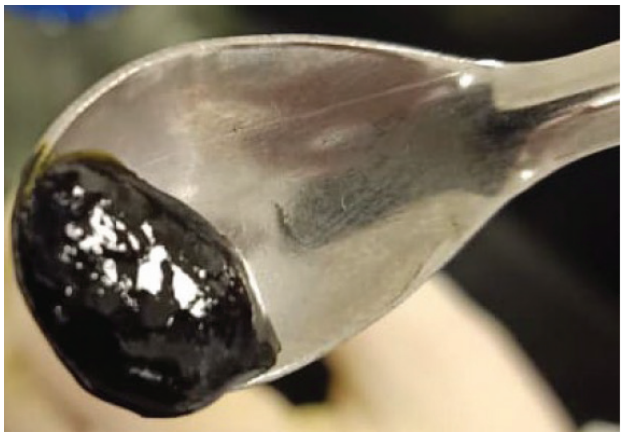

Figure 1: Crude extract of *O. aristatus* leaves.

Antimicrobial susceptibility test

Table 1: The mean of inhibition zones (mm) for the methanolic extract of *O. aristatus* leaves against selected bacteria

Tested bacteria	Methanol extract (1000 mg/ml)	Positive control	Negative control[d]
S. mutans	16.00 ± 1.00	31.00 ± 1.00[a]	0.0 ± 0.0
S. epidermidis	18.67 ± 1.15	20.67 ± 0.57[a]	0.0 ± 0.0
P. acnes	30.33 ± 0.57	43.00 ± 0.57[a]	0.0 ± 0.0
E. coli	0.0 ± 0.0	23.66 ± 1.52[b]	0.0 ± 0.0
K. pneumoniae	9.70 ± 1.53	29.67 ± 0.57[c]	0.0 ± 0.0

Note: [a]Chloramphenicol (30 µg) [b]Gentamicin (10 µg) [c]Amikacin (30 µg) [d]10% DMSO.

Values are represented as the mean ± S.D of three replicates.

The result of AST in this study showed that *S. mutans* is moderately inhibited by methanolic *O. aristatus* leaves extract with a zone of inhibition of 16 ± 1 mm, *S. epidermidis* with an inhibition zone of 18.67 ± 1.15 mm, *P. acnes* with an inhibition zone of 30.33 ± 0.57 mm while *E. coli* is completely resistant to the extract, with a 0 mm inhibition zone. Meanwhile, *K. pneumoniae* was moderately inhibited with an inhibition zone of 9.7 ± 1.53 mm.

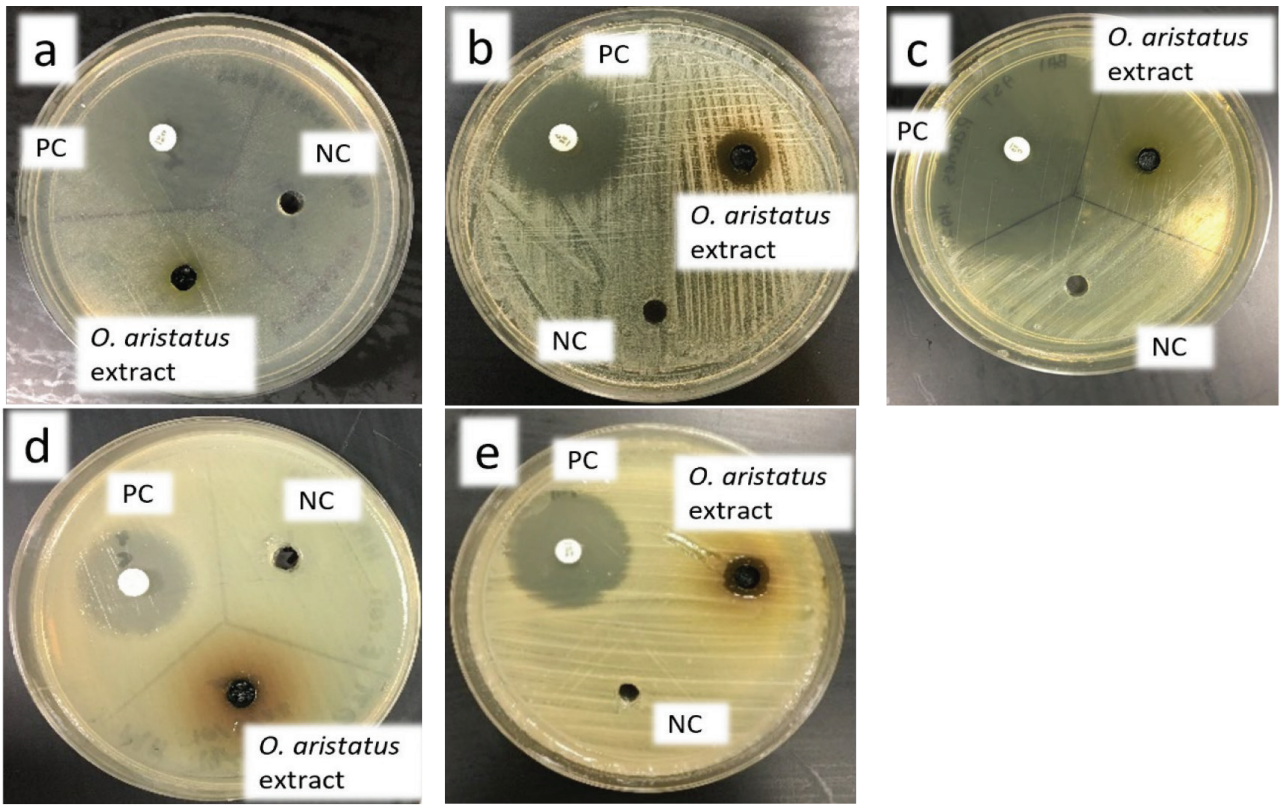

Figure 2: Growth inhibition of (a) *S. mutans,* (b) *S. epidermidis*, (c) *P. acnes*, (d) *E. coli* and (e) *K. pneumoniae* against *O. aristatus* extract, standard commercial antibiotic, and 10% DMSO. A positive control (PC) = indicates the bacterium's corresponding standard commercial antibiotic, negative control (NC) = indicates 10% DMSO.

Determination of minimum inhibition concentration and minimum bactericidal concentration

The MIC concentrations used in this study ranged from 1.96 mg/ml to 1000 mg/ml. The lowest concentration of methanolic *O. aristatus* leaves extract required to inhibit *S. mutans* was 7.81 mg/ml, followed by *S. epidermidis* (125 mg/ml) and *K. pneumoniae* (62.5 mg/ml). The methanolic extract of *O. aristatus* leaves showed bactericidal effects against all selected bacteria. The MBC value for *S. mutans* was 7.81 mg/ml, *S. epidermidis* was 125 mg/ml and *K. pneumoniae* was 62.5 mg/ml (Table 2). The MIC and MBC concentrations showed the same values for each bacterial species.

Table 2: The MIC and MBC values of the methanolic extract of *O. aristatus* leaves

Selected bacteria	MIC (mg/ml)	MBC (mg/ml)
S. mutans	7.81	7.81
S. epidermidis	125	125
P. acnes	3.91	3.91
K. pneumoniae	62.5	62.5

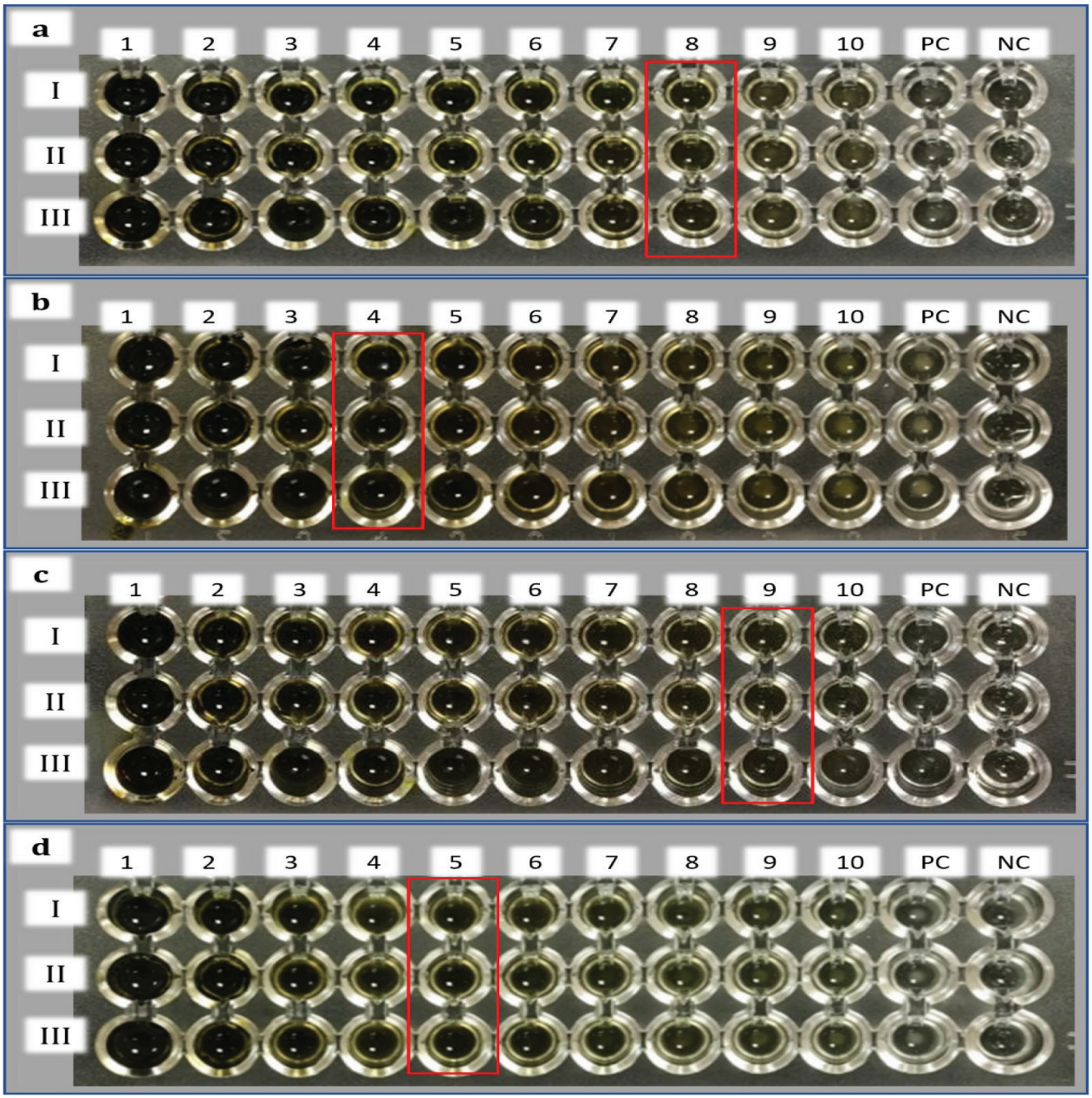

Figure 3: MIC result of (a) *S. mutans*, (b) *S. epidermidis*, (c) *P. acnes* and (d) *K. pneumoniae* against methanolic *O. aristatus* leaves extract in triplicates (I, II, III). Well 1 to 10 are two-fold dilutions of samples. PC = positive control, NC = negative control, wells in red box represent the MIC.

Qualitative phytochemical analysis

Table 3: Phytochemical screening of *O. aristatus* leaves extract

Bioactive compounds	Result
Glycosides	Present
Flavonoids	Present
Alkaloids	Present
Saponins	Present
Tannins	Present
Phenolics	Present
Terpenoids	Present
Anthraquinones	Absent

Results for qualitative phytochemical screening of *O. aristatus* leaves extract were based on the colour change or precipitation, shown in Table 3. The result showed that the methanolic *O. aristatus* leaves extract contained glycosides, flavonoids, alkaloids, saponins, tannins, phenolics, and terpenoids. However, anthraquinones were not detected.

Data analysis

An independent t-test was performed to compare the inhibition zone between the methanolic extract of *O. aristatus* leaves against the corresponding standard antibiotic for each bacterial species. P-value < 0.05 was used to set the significance level with a 95% Confidence Interval. *S. mutans*, *P. acnes,* and *K. pneumoniae* showed no significant difference in mean between the plant extract and the corresponding standard antibiotic (p = < 0.001; 95% CI: -17.27,-12.33), (p = < 0.001; 95% CI: -14.62,-12.0) and (p = < 0.001; 95% CI: -22.62,-17.38) respectively (Table 4). *S. epidermidis* showed statistical significance (p = 0.055), with the standard antibiotic having a higher mean than the methanolic extract (Table 4).

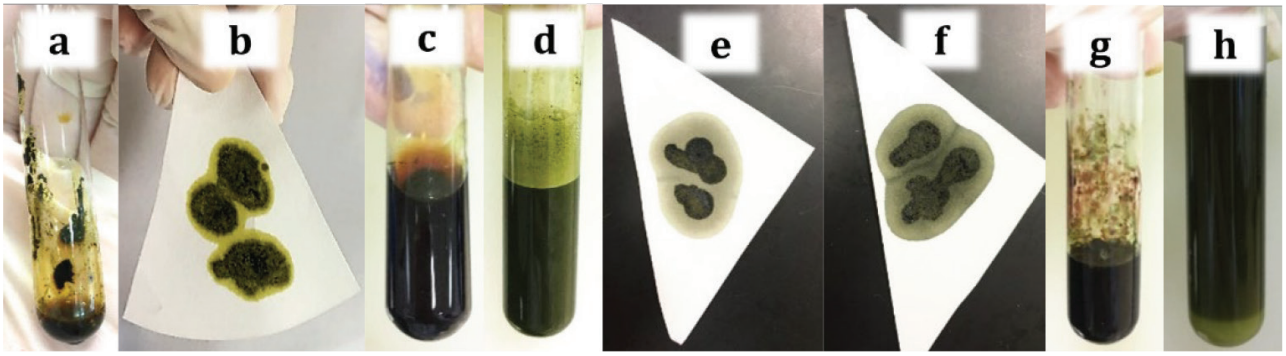

Figure 4: Phytochemical screening results methanolic *O. aris*tatus leaves extract for (a) glycosides, (b) flavonoids, (c) alkaloids, (d) saponins, (e) tannins, (f) phenolics, (g) terpenoids and (h) anthraquinones.

Table 4: Independent t-test of comparing inhibition zone between plant extract and the corresponding standard antibiotic for each tested bacterium

Tested bacteria	Mean difference (95% CI*)	t-test (df)	P-value**
S. mutans	-15.0 (-17.27, -12.33)	-18.371 (4)	< 0.001
S epidermidis	-2.0 (-4.07, 0.07)	-2.68 (4)	0.055
P. acnes	-13.33 (-14.62, -12.02)	-28.284 (4)	< 0.001
K. pneumoniae	-20.0 (-22.62, -17.38)	-21.21 (4)	< 0.01

*95% Confidence Interval

**P-value < 0.05

Discussion

Plants contain various compounds with diverse structures unique to themselves; thus, choosing the most suitable extraction method and solvent is crucial to preserving these bioactive compounds. The selection of the solvent and approach depends on the target compounds and the different parts of the studied plant. Different parts of the plants contain different phytochemical compounds due to plant matrices (40). The difficulty of choosing the best solvent is attributed to other substances that might affect the solubility of the compound of interest; thus, different solvents offer the best yields of different compounds (41). Because plant materials contain large amounts of polar compounds, which are soluble in highly polar solvents like methanol, water, and ethanol, polar solvents are preferred to non-polar solvents for bioactive compound extraction (42). Comparing methanol and water, Ho et al. (43) highlighted methanol as the best extraction solvent for phenolic compounds in *O. aristatus* leaves. However, Mohamed@Mahmood et al. (44) reported that the addition of water in an organic solvent increases the extraction yield in *O. aristatus* extract. Adding water as a

polar solvent to alcohol as a less polar solvent can help to better extract both polar and non-polar components in the sample. Other than that, the extraction method also gives out different extraction yields. A study stated that between maceration, ultrasound (80kHz), and microwave (150W), maceration is proven to have the highest yield of active ingredients, with the differences between the three methods being statistically significant ($p < 0.05$) (45). In recent years, supercritical fluid extraction (SFE) and ultrasound-assisted extraction (UAE) are among the most popular advanced techniques used for obtaining higher yields, shorter extraction time and less solvent consumption compared to the conventional method (46). The plant material used in this study was dried in the shade; however, plant materials can be used in either dried or fresh forms. Studies have shown that dried or fresh plant samples affect the phytochemical compound yield due to thermostability of those compounds (47, 48). Fresh plant samples deteriorate faster than dried samples (49). The drying technique could also affect phytochemical compounds. It is preferable to use air-dried samples as they will lose less antibacterial capabilities, and the heat-sensitive compound can be preserved (48). In this study, the antimicrobial activity of *O. aristatus* was evaluated by the inhibition made by the methanolic plant extract against *S. mutans*, *S. epidermidis*, *P. acnes*, *E. coli* and *K. pneumoniae*. The result revealed that *O. aristatus* was potentially effective in suppressing bacterial growth opportunistic normal flora with variable potency with a more significant potential against gram-positive bacteria than gram-negative bacteria. Gram-positive and gram-negative bacteria have a cell wall from peptidoglycan; however, due to its unique structure, the cell wall of gram-negative bacteria contributes more to drug resistance than gram-positive bacteria. The cell wall of gram-positive bacteria are mainly composed of multiple layers of peptidoglycan. In contrast, the cell wall of gram-negative bacteria is made up of an outer membrane composed of lipoproteins, phospholipids, porin channels, lipopolysaccharides (LPS), and several layers of peptidoglycan (50). The result of AST in this study showed that *S. mutans* is moderately inhibited by methanolic *O. aristatus* leaves extract with a zone of inhibition of 16 ± 1 mm. A previous study had purported that the zone of inhibition of *S. mutans* inhibited by *O. aristatus* extracts with a concentration of 50%, 25%, 12.5%, 6.25%, and 3.125% were 11.50 ± 0.38 mm, 10.78 ± 0.22 mm, 9.90 ± 0.13 mm, 9.10 ± 0.08 mm, and 8.25 ± 0.25 mm (51). Other than that, *S. epidermidis* is also moderately inhibited by the plant extract, with a zone of inhibition of 18.67 ± 1.15 mm, which is in line with a previous study that reported 25.04% inhibition of aqueous *O. aristatus* extract and 80.21% of ethanolic *O. aristatus* extract against *S. epidermidis* (52). The result of this study also shows that the methanolic *O. aristatus* leaves extract also inhibits *P. acnes* with an inhibition zone of 30.33 ± 0.57 mm. Aside from methanol, a study showed that *O. aristatus* extract from various solvents, including distilled water, ethanol, dichloromethane, and hexane are also effective in inhibiting *P. acnes*, with ethanol giving the largest inhibition zone (52). Additionally, Khalisha et al. (53) showed that ethanolic *O. aristatus* extract could inhibit *P. acnes* even as low as 1%, giving an inhibition zone of 7.37 ± 0.28 mm. The antimicrobial activity of the plant extract suggests that *E. coli* is entirely resistant to the plant. This result is in accordance with the study by Mangali (54) and Hussain et al. (30) that obtained zero inhibition against *E. coli* by *O. aristatus* extract. However, the extraction method might contribute to this resistance as Mangali (54) had shown that using *O. aristatus* nanoparticles had better potential in inhibiting *E. coli* than traditional methanolic extraction. A previous report showed that *E. coli* is particularly resistant to various herbal remedies (55). On the other hand, the AST result of this study shows that *K. pneumoniae* is moderately inhibited by methanolic *O. aristatus* leaves extract, with an inhibition zone of 9.7 ± 1.53 mm. A previous study reported a 15.8 mm zone of inhibition of *K. pneumoniae* against ethanolic *O. aristatus* extract (56). The MIC and MBC can determine an antimicrobial agent's potential bacteriostatic and bactericidal activities. If the MBC/MIC ratio is 1, the effect of the antimicrobial agent is bactericidal. However, if the MBC/MIC ratio is more than 2, it is bacteriostatic (57). In this study, *O. aristatus* extract showed potential bactericidal activities against *S. mutans, S. epidermidis, P. acnes and K. pneumoniae*. Based on the data analysis of this study, for all bacterial species, the standard antibiotics have a larger inhibition zone than the methanolic *O. aristatus* leaves extract. A possible explanation for this occurrence is the low yield of bioactive compounds in the extract that contribute to antimicrobial activity. The bacterial inhibition in methanolic *O. aristatus* leaves extract indicates the presence of antimicrobial compounds in the extract. Phytochemical compounds are responsible for those antimicrobial properties. Phytochemical constituents play an active role in various activities such as antimicrobial, antioxidant, and anticancer (58). Many bioactive compounds in plants display antimicrobial properties through different biological mechanisms (59). Terpenoids are derivatives of terpenes that differ in functional groups. Terpenoids have a low molecular weight of ≤500 g/mol and can act as adjuvants and exhibit synergic effects to disrupt biofilm formation via combination therapy (60). It has been proven that terpenoids have different antimicrobial activities based on their hydrogen bonding parameters, with cases of smaller terpenoids possessing greater activities (60). Flavonoids are classified based on their biosynthetic origin. Flavonoids play a prominent role in a plant's life, providing colour in flowers or promoting protection and photosynthesis in leaves (61). Flavonoids show antimicrobial effects via efflux pump inhibition of bacterial cell wall (62), biofilm inhibition (63) inhibition of nucleic acid synthesis (64) cell lysis and disruption of the cytoplasmic membrane (65). Alkaloids have been researched thoroughly for their biological activities. Their most notable characteristic is forming hydrogen bonds with enzymes, receptors, and proteins due to a proton accepting nitrogen atoms and one or more protons donating amine hydrogen atoms (66). The antimicrobial mechanism of alkaloids was found to be

different between each class. It can be summarized by their ability to inhibit nucleic acid and protein synthesis (67), disrupt biofilm formation (68), damage cell membranes causing electrolyte leakage and inhibit bacterial metabolism (69). Saponins are glycosides characterised by their surfactant properties, forming foams when dissolved in water. Saponins produce antimicrobial properties by attacking the biofilm formation via structural changes in biological macromolecules and binding to surface sterols in the cell membrane to rupture the structure (70). Saponins in various plant extracts can produce a synergistic effect when associated with antibiotics, potentially leading to new options for treatment amidst the emergence of drug-resistant bacteria (71). Tannins are a type of polyphenol that exhibit antimicrobial properties via their anti-biofilm mechanism rather than a direct antibacterial effect. Tannins can pass through a bacteria's cell membrane and interfere with bacterial metabolism (72) and the synthesis of extracellular polysaccharides (73). Based on the data analysis of this study for all bacterial species (Table 4), the standard antibiotics have a larger inhibition zone than the methanolic extract of *O. aristatus* leaves. A possible explanation for this occurrence is the low yield of phytochemical compounds in the extract that contribute to antimicrobial activity (74).

Conclusion

The present antimicrobial study of methanolic extract of *O. aristatus* leaves exhibits variable antimicrobial activity against gram-positive *S. mutans, S. epidermidis* and *P. acnes* and gram-negative *E. coli* and *K. pneumoniae* bacteria. The *O. aristatus* leaves extract was more effective against gram-positive bacteria. The extract inhibited all gram-positive bacteria. For gram-negative, *E. coli* was resistant, while *K. pneumoniae* was susceptible. The alkaloids, glycosides, tannins, terpenoids, saponins, phenolics, and flavonoids present in the methanolic extract of *O. aristatus* leaves are responsible for the biological control of infectious agents in plants. Based on the findings, the *O. aristatus* leaves have the potential to be developed as an alternative natural antimicrobial agent for the current synthetic antibiotics. Further research, such as quantitative phytochemical analysis using high-performance liquid chromatography or spectrophotometry, is needed to isolate and quantify the phytochemical compounds of the plant.

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Competing interests

The authors declare that they have no competing interests.

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