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PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITIES OF LEAF EXTRACTS OF FIVE *NEPHELIUM LAPPACEUM* CULTIVARS FROM MALAYSIA

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Abstract

Nephelium lappaceum is a tropical tree that is widely distributed in Malaysia and Indonesia. Rambutan fruit is nutritious and rich in vitamins, minerals, and beneficial compounds that offer many health benefits. The research on the potential of *N. lappaceum* for pharmaceutical use is underestimated. This study aims to determine the phytochemical constituents and unravel the potential of leaf extracts for antibacterial activity. Mature leaves of five cultivars of *N. lappaceum* (R191, R3, S1, AG28, and GB44) were extracted using two different solvents (methanol and water) and these extracts were subjected to phytochemical screening. The effects of crude leaf extract on antimicrobial activity against the Gram-positive bacteria, *Staphylococcus aureus* dan the Gram-negative bacteria, *Escherichia coli* were conducted using the agar well diffusion method. The methanol extraction method recorded a higher yield than the water extraction method in all tested cultivars with AG 28 recording the highest percentage of 28.2%. Phytochemical screening showed the presence of coumarins, flavonoids, phenols, saponins, and tannins in both methanolic and water leaf extracts. Alkaloids were only present in the water extracts except for R191, while no detection of anthocyanin presence in both methanolic and water extracts. Analysis of antibacterial activity based on the zone of inhibition growth for methanolic and water extracts of five different cultivars showed significant antibacterial effects ($p<0.05$) with methanolic extract being the most effective against *S. aureus* and *E. coli* with the highest inhibition zones of 39 (±0.3) mm and 29 (±0.6) mm, respectively. The minimum inhibitory concentration (MIC) value was 1.25 % w/v for R3, AG28, and GB44 in methanolic extracts against *E. coli* and the lowest MIC (1.25% w/v) was recorded for AG28 in methanolic extracts against *S. aureus*. Based on the phytochemicals available in the extracts, flavonoids, phenols, and coumarin may be responsible for the antibacterial activity in this study.

Key words: Phytochemicals; Cultivars; Leaf extracts; Antibacterial activity.

Introduction

Rambutan (*Nephelium lappaceum* L.) is a plant belonging to the family of Sapindaceae. The fruit is edible and popular in Southeast Asian countries, particularly in Malaysia. Rambutan has been used by indigenous people to cure various ailments. Decoction of the roots of rambutan was used to treat fever, while the leaves can be applied as a poultice to relieve headache, hair treatment and as an antidote for diarrhea (Morton, 1987; Sulistiyaningsih *et al*., 2018). The ethnomedicinal practices have raised the interest in exploring medicinal plants in modern medicine for more effective and safer treatments (Sukmandari *et al*., 2017).

Different species and parts of the plants contain different chemical structures and therapeutic properties. Recently, the search for bioactive compounds from plants with antimicrobial agents is highly needed due to the high resistance of pathogenic microorganisms to available antibiotics, which leads to multiple drug resistance (MDR) (Gupta & Birdi, 2017; Seukep *et al*., 2020). For example, Methicillin resistance in *S. aureus* causes high fatality, prolonged hospital stays and high medical costs (Carvalho *et al*., 2010; Khan *et al*., 2017; Guo *et al*., 2020). Therefore, identifying the phytochemicals in plant extracts could be a potential alternative as resistance-modifying agents.

Many studies have reported the phytochemicals and medicinal values of *N. lappaceum*. For example, the flesh of the rambutan fruit and peel extracts possessed antimicrobial, antioxidant, and anti-cancer effects (Khonkarn *et al*., 2010; Nethaji *et al*., 2015; Manrroy *et al*., 2020; Perumal *et al*., 2021; Jantapaso *et al*., 2022). However, studies on comparative phytochemicals in different rambutan cultivars are scarce. Therefore, this study aimed (1) to screen and compare phytochemical compounds in the methanolic and water extracts of five different cultivars of rambutan grown in Malaysia, namely *Anak Sekolah* (R191), *Gula Batu* (R3), Seedless (S1), *Mutiara Merah* (AG28) and *Mutiara Wangi* (GB44), (2) to evaluate the antibacterial activity of the extracts against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria.

Material and Methods

Plant materials: The mature leaves of five cultivars of *N. lappaceum* (R191, R3, S1, AG28 and GB44) were collected from Malaysian Agricultural Research and Development Institute (MARDI) in Sintok, Kedah (6.4883° N, 100.4822° E) and Dengkil, Selangor (2.8669°N, 101.6954°E). The plant specimens were identified by Dr. Farah Alia Nordin, preserved using the standard herbarium technique of Bridson and Foreman (1998) and deposited in the Herbarium of School of Biological Sciences, Universiti Sains Malaysia (USMP) (Thiers, 2021) with accession number of USM11914 - USM11918.

Phytochemical screening

Leaf extraction for phytochemical screening: Leaf samples from each cultivar were dried in the oven at 45°C for 72 hours and ground using a mechanical blender. The leaves were extracted following the maceration process using two types of solvents: methanol and water. A 7.5 g of the leaf powder was soaked in 150 ml of each solvent to create a 5% suspension (w/v) and allow to stand for five days at room temperature, with intermittent shaking. Then, the suspensions were filtered using filter paper (Whatman No. 1). The filtrates were then used in the phytochemical screening.

Phytochemical screening of the crude extract: The qualitative analyses were conducted to screen alkaloids, anthocyanin, coumarins, flavonoids, phenols, saponins, and tannins following the standard procedure by Kokate (1994).

Test for alkaloids: 2 ml of Mayer's reagent was inserted into a test tube containing 2 ml of extract and shaken vigorously. The alkaloid presence was detected by the formation of greenish colour or creamy precipitation.

Test for anthocyanin: 1 ml of 2 N NaOH was added to 0.2 ml of leave extract and heated for five minutes at 100°C. The presence of anthocyanin was indicated by the appearance of a bluish-green colour.

Test for coumarins: 1 ml of 10 % NaOH was added to 1 ml of leaf extract. The presence of coumarins was detected by the formation of a yellow colour.

Test for flavonoids: 1 ml of 2N NaOH was added to 2 ml of leaf extract. The presence of flavonoids is shown by the yellow colour formation.

Test for phenols: 1 ml of leaf extract was mixed with 2 ml of distilled water and a few drops of 10% $FeCl_3$. The presence of phenol was detected by the formation of a blue or green colour.

Test for saponins: Distilled water was added to the leaf extract with a ratio of 1:1. The reaction mixture was shaken well and held for 15 minutes. The presence of saponin was indicated by the formation of foam.

Test for tannins: 2 ml of 5% $FeCl_3$ was added to 1 ml of leaf extract. The presence of tannin was detected by the formation of a dark blue or greenish-black colour.

Antibacterial activity

Leaf extraction for antibacterial assay: 20 g of each tested leaf powder was soaked in 180 ml distilled water and heated for 30 minutes at 90°C before being incubated overnight at 37°C and 150 rpm in a shaking incubator (Xu *et al*., 2008). Similarly, 10 g powder of plant material were mixed separately with methanol (9:1) followed by incubation overnight at 37°C and 150 rpm. The liquid extracts were filtered (Whatman No. 1) to separate the liquid extract from the solid residue before being concentrated using a rotary evaporator. The concentrated methanolic extracts were dissolved in 10% Dimethyl sulfoxide (DMSO). Meanwhile, water extracts were dissolved in distilled water (Gurnani *et al*., 2016). All extracts had a final concentration of 20% w/v.

Determination of extraction yield: The extraction yield was calculated following Felhi *et al*., 2017.

$$\text{Yield (\%)} = [X_1 * 100] / X_0$$

X_1 = the weight of the extract after the solvent has evaporated
X_0 = the dry weight of the plant powder before extraction.

Preparation of inoculum and antibacterial assay: The antimicrobial effects of leaf extracts were tested against *S. aureus* and *E. coli*. The bacteria were cultured overnight in Mueller Hinton broth (MHB) in a rotary shaker (INFORS HT, Switzerland) at 180 rpm, 37°C. *S. aureus* and *E. coli* were obtained from Microbiology Laboratory, School of Biological Sciences, Universiti Sains Malaysia (5.3558°N, 100.3012°E). The antibacterial activities of various solvent extracts were tested using the agar well diffusion method, as described by Daoud *et al*., 2019. In the centre of a sterile petri dish, 1 ml of fresh bacterial culture was pipetted. Muller Hinton Agar (MHA) or molten-cooled Muller Hinton Agar (MHA) for bacteria strains was put onto the petri dish holding the inoculum and thoroughly mixed. Following solidification, wells were drilled into agar plates containing inoculums with a sterile pipette tip (6 mm in diameter). Then, 100 µl of each extract (20% w/v) was poured into the respective wells (Gonelimali *et al*., 2018). The plates were chilled for 30 minutes to allow the extracts to fully diffuse into the agar. The plates were then incubated for 18 hours at 37°C. After the incubation time, the zone of inhibition including the diameter of the well was measured. DMSO (10 %) was used as a negative control for methanolic extract and distilled water was used as a negative control for water extract.

Determination of the minimum inhibitory concentrations (MIC): All tested antimicrobial activities were exhibited at a concentration 20% (w/v) in methanolic and water extracts. Therefore, 20% concentration was manipulated using a two-fold serial dilution to obtain different concentrations of 10%, 5%, 2.5% and 1.25% to determine the MIC of *N. lappaceum* crude leaf extracts. 1 ml of each inoculum was pipetted into sterile petri dishes before MHB was added and mixed. 100 µl of different concentrations of extract (10, 5, 2.5, and 1.25%) was added to the respective wells. Plates were chilled for 30 minutes and incubated at 37°C for 18 hours. MIC was detected at the lowest concentration of the extract at which inhibited the growth of the bacteria.

Statistical analysis

The data represent the mean of three replicates ± standard error (SE). One-way ANOVA test was used to analyse the effect of concentration on the zone of inhibition in tested bacteria. Then, post-hoc test was performed by Tukey's analysis using SPSS version 26.0. At a P-value ≤ 0.05, differences between means were considered significant.

Results

Extraction yield: Methanolic extracts showed a higher extraction yield in all tested cultivars compared to water extracts except for S1 showed equal yield between methanolic and water extracts. The highest extraction yield recorded for methanolic extract is in cultivar AG28 with 28.2%, followed by GB44 with 22.3%, R3, R191 and S1 with 18.4%, 13.8%, and 12.5% respectively. Meanwhile, the extraction yield recorded for water extract is highest in cultivars AG 28 with 16.7%, followed by S1, GB44, R191 and R3 with 12.5%, 11.45%, 9.75%, and 7.65% respectively. Cultivars AG28 recorded the highest extraction yield among the other cultivars in methanolic and water extracts (Fig. 1).

Qualitative phytochemical screening: The determination of phytochemical components showed the availability of coumarins, flavonoids, phenols, saponins and tannins in the methanolic and water extracts of all tested cultivars. Anthocyanin was not detected in leaf methanolic and water extracts. Four cultivars (R3, AG28, GB44 and S1) showed the presence of alkaloids in water extracts while the alkaloids were absent in R191 water extract (Table 1).

Antibacterial activity in methanolic extracts against *S. aureus* and *E. coli*: The antibacterial activity of methanolic extracts of R191, R3, S1, AG28 and GB44 at a concentration of 20%, 10%, 5%, 2.5% and 1.25% against *S. aureus* and *E. coli* have been evaluated. The result revealed that the methanolic extracts of selected cultivars are efficiently suppressing the growth of bacteria with variable potency at different concentrations (Fig. 2 and Fig. 4). Both methanolic and water extracts in all cultivars tested showed the highest inhibition zone against *S. aureus* and *E. coli* at the concentration 20% (w/v) (Fig. 3 and Fig. 5). Methanolic extracts of R191 showed the maximum zone of inhibition against *S. aureus* (38.6±0.3 mm) (Fig. 2 and Fig. 3A.), while AG 28 and R191 showed the maximum zone of inhibition against *E. coli*, with 28±0.6 mm and 28±0.3 mm, respectively (Fig. 4 and Fig. 5A and 5D).

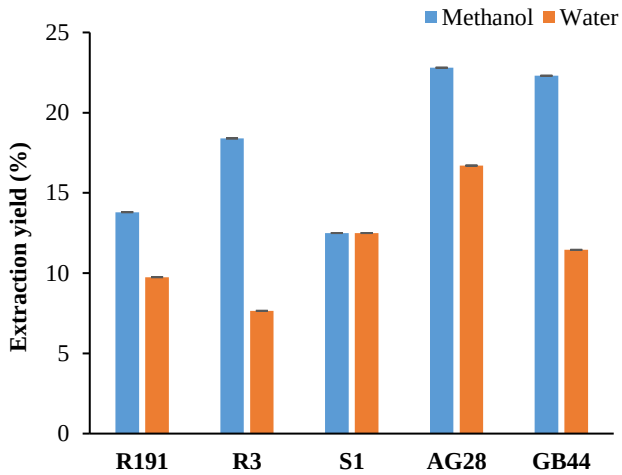

Fig. 1. Extraction yield (%) of five *N. lappaceum* cultivars in methanolic and water solvents. Data are expressed as mean ± Standard error (SE), n=3, $p<0.05$.

Antibacterial activity in water extracts against *S. aureus* and *E. coli*: The antibacterial activity of water extracts at different concentrations recorded a smaller inhibition zone compared to methanolic extracts. The inhibition zone showed efficient results against *S. aureus* at concentrations of 20% and 10% (v/w), while in *E. coli* at only 20% (w/v) (Fig. 6 and Fig. 7). There was no inhibition detected in 5% (v/w) and below against both *S. aureus* and *E. coli*. The highest inhibition zone was recorded in GB 44 with 17.0 (±0.0) mm in both *S. aureus* and *E. coli* (Fig. 8 and Fig. 9).

Minimum inhibitory concentrations (MIC): The antimicrobial activities of *S. aureus* and *E. coli* increased relative to the concentration of plant extracts. The variations of MIC in plant extracts against *S. aureus* and *E. coli* are shown in Table 2. The lowest MIC values (1.25%) were recorded by methanolic extracts of R 3, AG 28, and GB 44 against *E. coli*, and the lowest MIC value (1.25%) was recorded by AG 28 methanolic extract against *S. aureus*.

Table 1a. List of phytochemicals profiles in the methanolic (A.) and water (B.) crude leaf extracts of five *N. lappaceum* cultivars.

Phytochemicals	A					B				
	R 3	AG 28	GB 44	R 191	S 1	R 3	AG 28	GB 44	R 191	S 1
Alkaloids	-	-	-	-	-	+	+	+	-	+
Anthocyanin	-	-	-	-	-	-	-	-	-	-
Coumarins	+	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+
Phenols	+	+	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+	+	+

+ Present – Absent

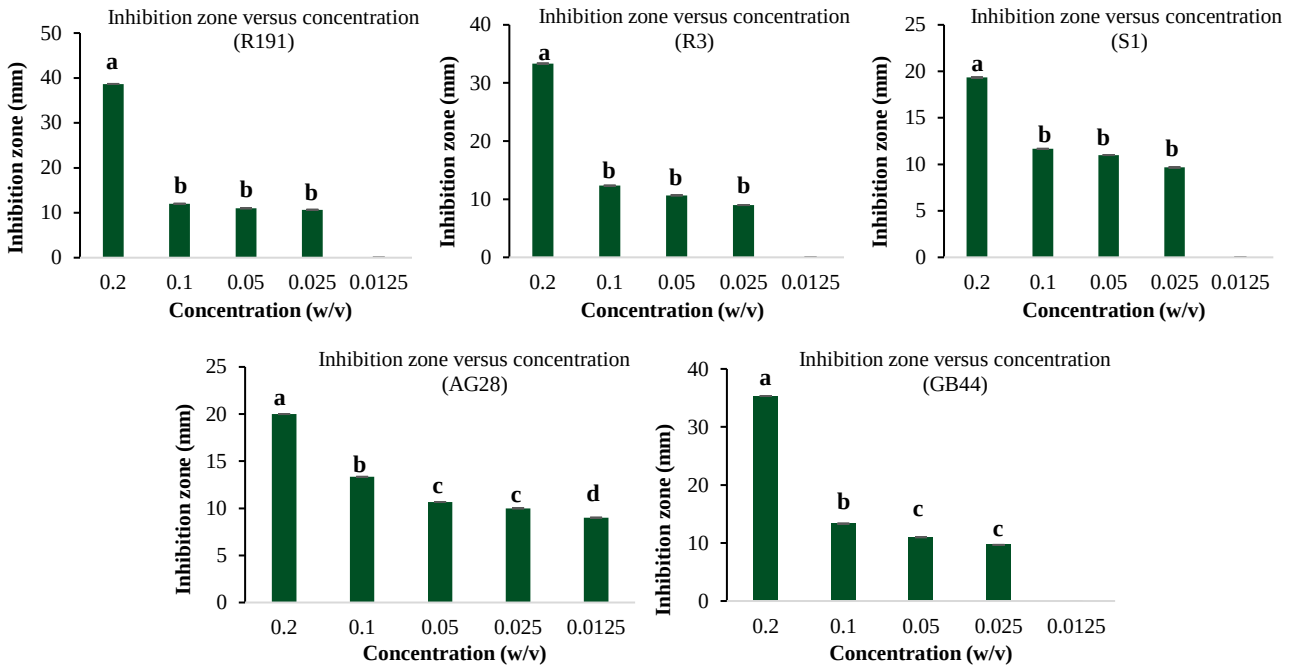

Fig. 2. Effects of leaf R191, R3, S1, AG28 and GB44 methanolic extracts on *S. aureus* at different concentrations. Values represent the means of triplicate readings (n=3), and bars represent the standard error (SE).

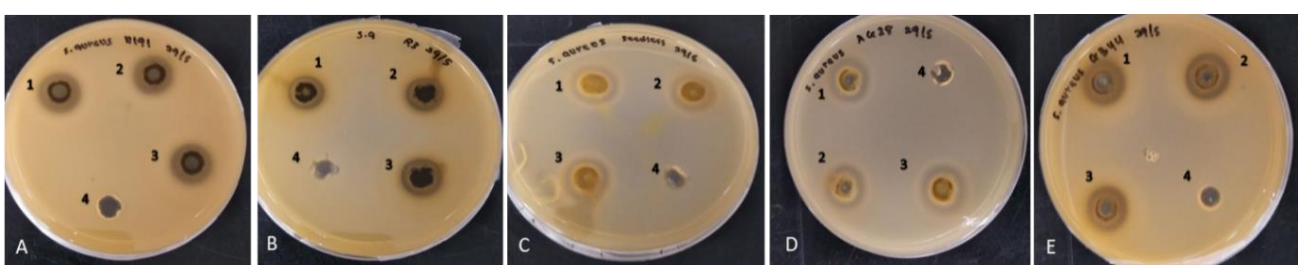

Fig. 3. The inhibition zone of methanolic extracts of five *N. lappaceum* cultivars. **A.** R191 **B.** R3 **C.** S1 **D.** AG28 **E.** GB44 against *S. aureus* at concentration of 20% (w/v). **1**,**2**,**3** represent the replicates, and **4** represents control (10% w/v DMSO).

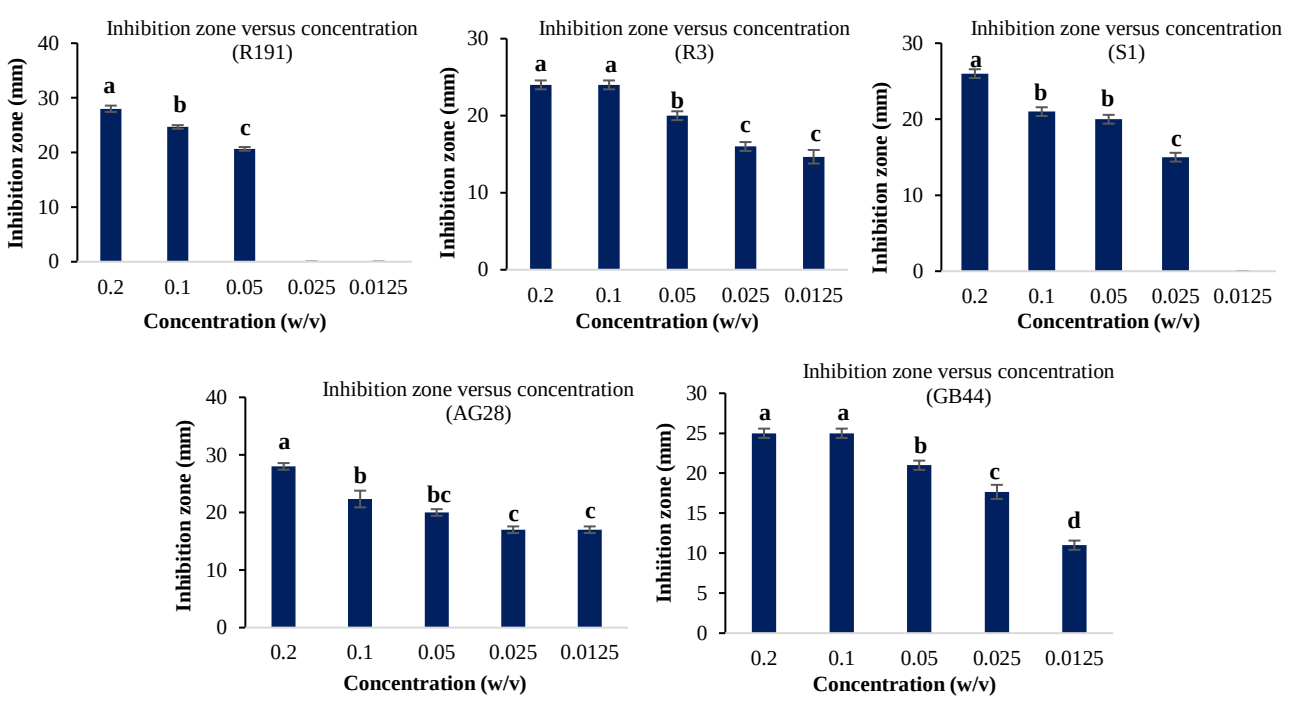

Fig. 4. Effects of leaf R191, R3, S1, AG28 and GB44 methanolic extracts on *E. coli* at different concentrations. Values represent the means of triplicate readings (n=3), and bars represent the standard error (SE).

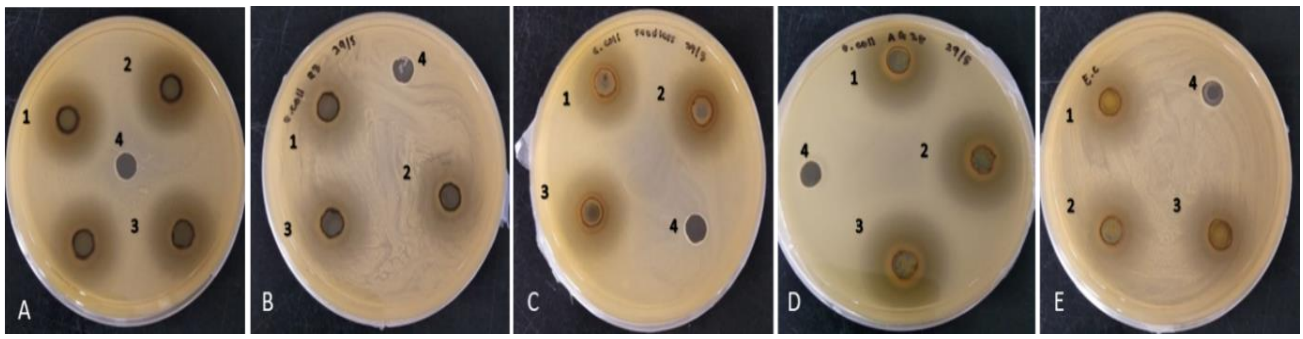

Fig. 5. The inhibition zone of methanolic extracts of five *N. lappaceum* cultivars. **A.** R191 **B.** R3 **C.** S1 **D.** AG28 **E.** GB44 against *E. coli* at concentration of 20% (w/v). 1,2,3 represent the replicates, and 4 represents control (10% w/v DMSO).

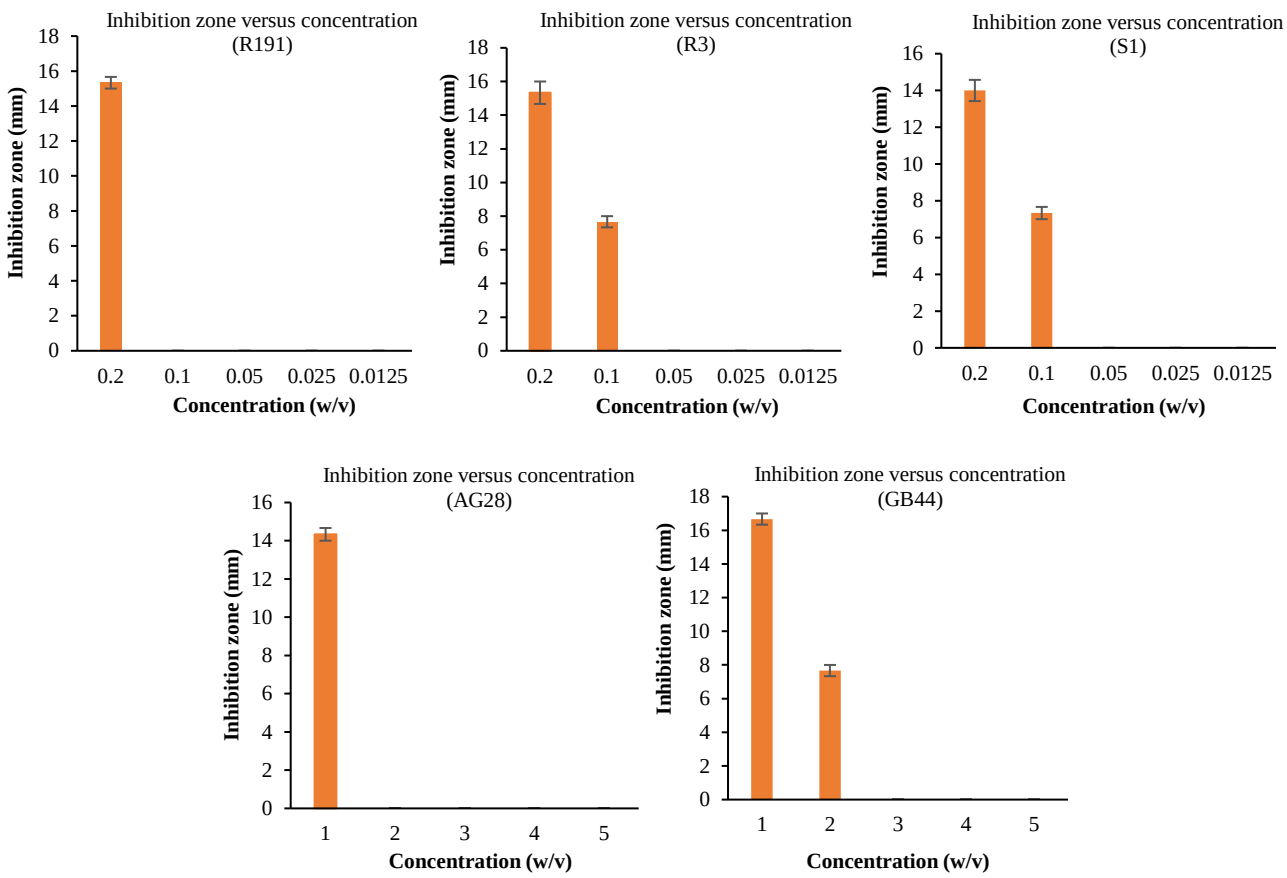

Fig. 6. Effects of leaf R191, R3, S1, AG28 and GB44 water extracts on *S. aureus* at different concentrations. Values represent the means of triplicate readings (n=3), and bars represent the standard error (SE).

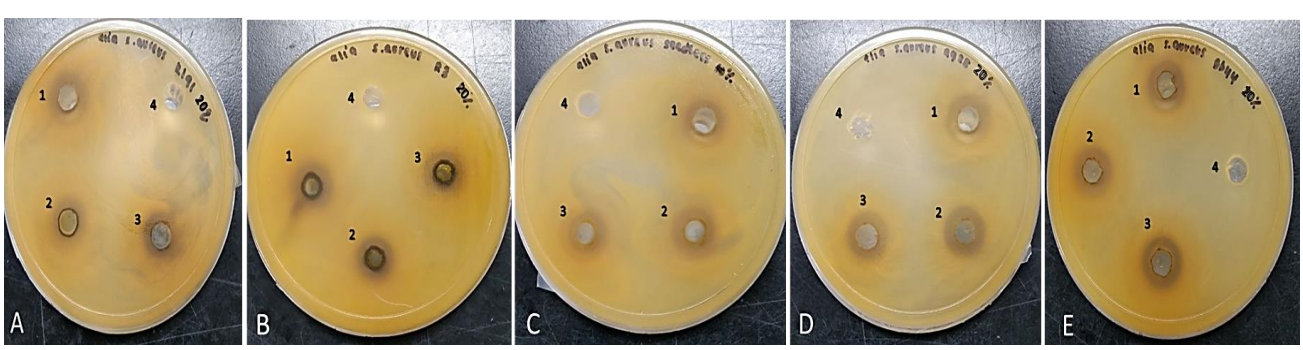

Fig. 7. The inhibition zone of water extracts of five *N. lappaceum* cultivars. **A.** R191 **B.** R3 **C.** S1 **D.** AG28 **E.** GB44 against *S. aureus* at concentration of 20% (w/v). 1,2,3 represent the replicates, and 4 represents control (10% w/v DMSO).

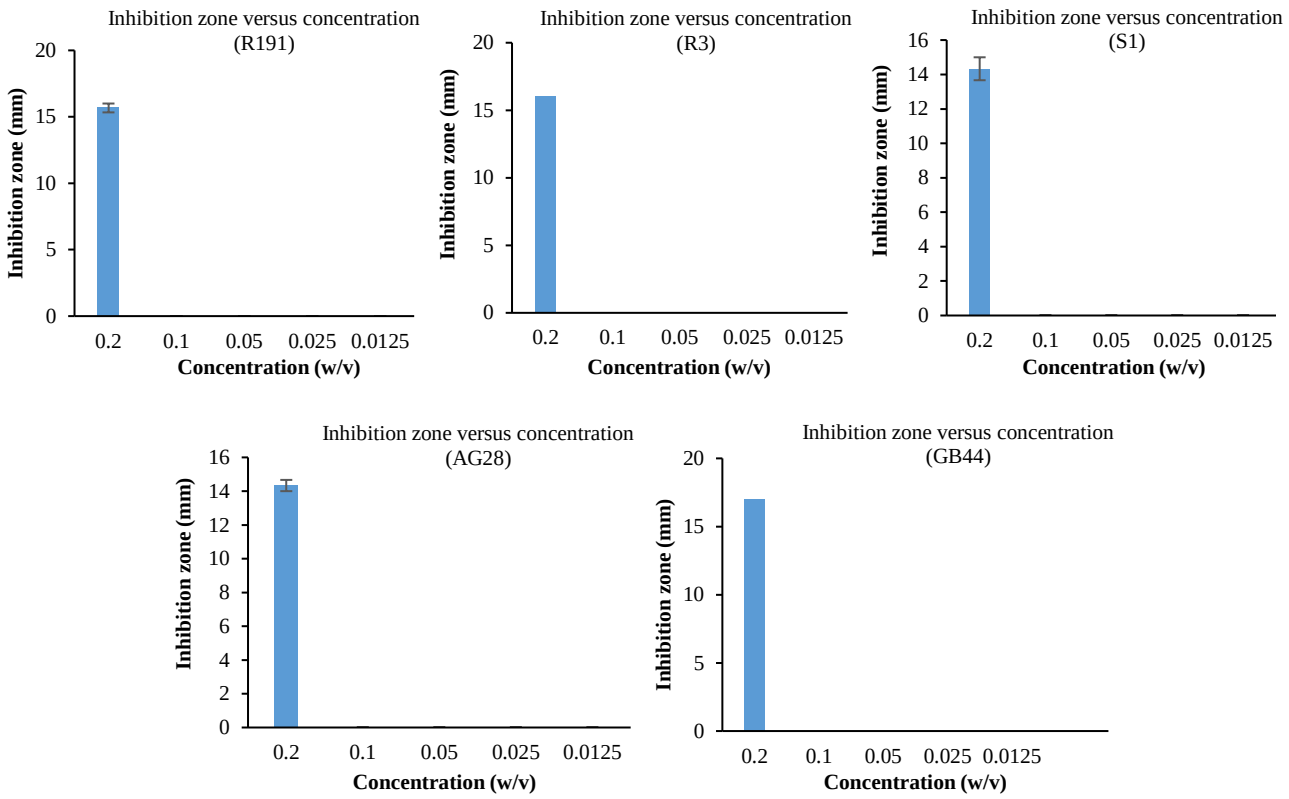

Fig. 8. Effects of leaf R191, R3, S1, AG28 and GB44 water extracts on *E. coli* at different concentrations. Values represent the means of triplicate readings (n=3), and bars represent the standard error (SE).

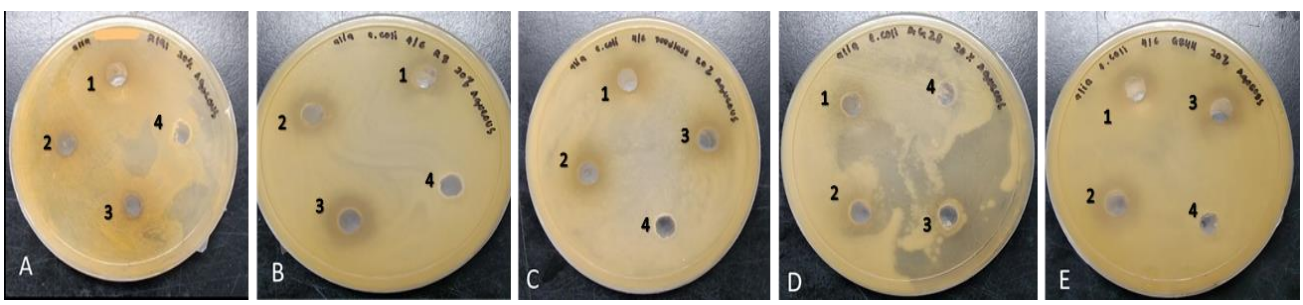

Fig. 9. The inhibition zone of water extracts of five *N. lappaceum* cultivars. **A.** R191 **B.** R3 **C.** S1 **D.** AG28 **E.** GB44 against *E. coli* at concentration of 20% (w/v). 1,2,3 represent the replicates, and 4 represents control (10% w/v DMSO).

Table 2. The Minimum inhibitory concentration (MIC) of five *N. lappaceum* cultivars extracts against S. aureus and *E. coli*.

	Minimum inhibitory concentration (MIC) (% w/v)*			
Cultivars	***S. aureus***	**Water**	***E. coli***	**Water**
	Methanol		**Methanol**	
R 191	2.5	20	5	20
R 3	2.5	10	1.25	20
S 1	2.5	10	2.5	20
AG 28	1.25	20	1.25	20
GB 44	2.5	10	1.25	20

*Values are means of n=3

Discussion

This study used the phytochemical screening method to perform secondary metabolite profiling on five *rambutan* cultivars from Malaysia. This study found that all five rambutan cultivar's water and methanolic extracts contained potential medicinal properties from coumarins, flavonoids, phenols, saponins, and tannins. It also revealed similar phytochemicals found in the leaf of different cultivars of *N. lappaceum*. This study's results align with most comparative studies on the phytochemical compounds in different plant cultivars (González-Gómez *et al*., 2010; Ndhlala *et al*., 2014; Oszmiański *et al*., 2017).

The solvents used in the extraction will have a different polarity that may influence the yield of the plant extract (Nawaz et al., 2020). Our result clearly shows the methanol solvent yielded the highest extraction rate compared to the water. Similar results were also recorded in a study using the same methanol extract approach (Truong *et al*., 2019). This could be due to the phytochemicals and other components are more soluble in methanol than water.

Different phytochemicals were detected in *N. lappaceum* extracts in different solvents (Table 1). Both methanolic and water leaf extracts in five cultivars

showed the presence of coumarins, flavonoids, phenols, saponins, and tannins supporting the ethnomedicinal use of *N. lappaceum*. Alkaloids were detected in water extracts of all cultivars except for R191, in contrast to methanolic extracts. The alkaloids were not detected in R191 because of natural variations that influence the number of metabolic compounds in plants (Bazargani *et al*., 2021). Furthermore, different cultivars possess different compositions of metabolites (Mena *et al*., 2011). The polarity of the solvent used to extract biomolecules affects the rate of extraction (Altemimi *et al*., 2017). The result demonstrated that water is more suitable for extracting alkaloids from *N. lappaceum* leaf as the polarity of the solute is similar to the solvent. The list of compounds also may differ depending on the methods of extraction and plant parts used (Ahmed *et al*., 2020).

The inhibition zone showed antibacterial activity in methanolic and water leaf extracts against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. The efficient antibacterial activity indicates a broad spectrum of potential antibiotic activity even for Gram-negative bacteria which have different cell wall structures. The size of the inhibition zone was relative to the concentration of the extracts supporting the previous finding that antimicrobial activities have a direct relation to increasing the concentration of the extracts (% w/v) (Bhalodia & Shukla, 2011). The methanolic extracts exhibited larger inhibition zones against *S. aureus* and *E. coli* than water extracts with variable potency at different concentrations. Data from previous studies also reported methanolic extracts exhibited stronger antibacterial activities than water extracts (Shittu *et al*., 2006; Mudzengi *et al*., 2017; Ibrahim & Kebede, 2020). Rambutan leaf extracts showed higher potency against *S. aureus* than *E. coli* in this study due to the Gram-negative bacteria (*E. coli*) having an outer layer surrounding their cell walls that contains lipopolysaccharide. This layer acts as a barrier to many substances, including antibiotics (Ebbensgaard *et al*., 2018). R191 recorded the highest inhibition zone in 20 (% w/v) concentration against *S. aureus* and *E. coli*, indicating this cultivar has potential as an antibacterial supplementation. R191 extracts were rich in bioactive compounds that showed antibacterial activities. The activity does not only depend on the presence of the compounds, but other factors also influence the activity of R191 towards both bacteria, like the concentration of the metabolites as well as the interaction between other components (Dzotam *et al*., 2016).

MIC represents the lowest concentration of the extracts to inhibit the growth of bacteria. In this study, the methanolic extracts recorded lower MIC values than most of the corresponding water extracts indicating methanolic extracts have higher antimicrobial activity (Kinsalin *et al*., 2014; Ibrahim& Kebede, 2020). Most of the methanolic extracts in this study required a low MIC value to inhibit Gram-negative bacteria indicating that *E. coli* was more sensitive than *S. aureus*. This finding is in agreement with earlier reports by Elisha *et al*., 2017.

The presence of flavonoids, phenols, saponins, tannins and coumarins in *N. lappaceum* leaf extracts indicates its bioactive components in plants. Earlier studies on the phytochemical constituents in plants reported various medicinal roles that showed therapeutic effects (Xie e*t al*., 2014; Lin *et al*., 2016; Ahmed *et al*., 2020). However, the effectiveness of the bioactive compound depends on their chemical composition, concentrations as well as interaction with other components (Dzotam *et al*., 2016). Flavonoids and phenols are compounds that involve in antibacterial activity and various other biological activities (Xie *et al*., 2014; Lin *et al*., 2016). Saponins could reduce the body's cholesterol level and improve cardiovascular health (Moghimipour & Handali 2015). Tannins are polyphenolic compounds which have specific chemical and physical properties that give a medicinal benefit (Ahmed *et al*., 2020). Coumarins contain biological properties which can be used as antimicrobial, antioxidants, anti-HIV and anticancer agents (Witaicenis *et al*., 2014; Al-Majedy *et al*., 2017; Liu *et al*., 2020). Therefore, in this study, the effectiveness of *N. lappaceum* leaf extracts against *S. aureus* and *E. coli* might be due to flavonoids, phenols and coumarin contents in the extracts.

Conclusion

Phytochemical constituents of *N. lappaceum* are almost similar between the cultivars. Leaf methanolic and water extracts showed inhibition against *S. aureus* and *E. coli* with different potency at different concentrations. The high sensitivity of *E. coli* towards leaf methanolic extracts indicate great potential to be used as pharmaceutical ingredients. Further investigation is needed to determine the specific bioactive compound responsible for the antibacterial activities.

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