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RESEARCH ARTICLE

LC-TOF-MS/MS-Based Phytochemical Profiling and Antibacterial Activity of *Nephelium lappaceum* Leaf Extracts

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Abstract Medicinal plants are a valuable source of pharmaceuticals, as their phytochemical constituents exhibit diverse biological activities with fewer side effects and lower possibility of resistance development compared to synthetic drugs. This study investigated the antibacterial properties of methanolic and chloroform-methanol-water (CMA) extracts from the leaves of *Nephelium lappaceum* L. from three cultivars - R191, AG28, and GB44 against *Staphylococcus aureus* and *Escherichia coli,* using agar well diffusion method. The methanolic extracts exhibited significantly higher antibacterial activity compared to CMA extracts. To tentatively identify metabolites potentially associated with the observed antibacterial activity, the methanolic extracts were analysed using LC-TOF-MS/MS techniques. The Liquid Chromatography-Time-of-Flight Mass Spectrometry/Mass Spectrometry (LC-TOF-MS/MS) analysis tentatively identified three, ten, and nine compounds in R191, AG28, and GB44 respectively, which were subsequently categorized as flavonoids, tryptophan alkaloids, and triterpenoids. Two compounds, Procyanidin B2, and Quercetin were detected in all extracts. These findings highlight the potential of *N. lappaceum* leaf extracts as a source of natural antibacterial agents and warrant further *in vivo* studies for the development of novel antibacterial drugs.

Keywords: Rambutan cultivars, Natural antibacterial agents, Secondary metabolites, *Staphylococcus aureus, Escherichia coli*

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Introduction

Nephelium lappaceum L., commonly known as rambutan, is a tropical fruit-bearing plant belonging to the Sapindaceae family. While the fruit is widely consumed for its sweet and edible aril, other parts of the plant, particularly the leaves, have been traditionally utilized for their medicinal properties, including antimicrobial, anti-inflammatory, and analgesic effects [1]. Despite its ethnopharmacological relevance, the phytochemical composition and antibacterial activity of *N. lappaceum* leaf extracts remain relatively unexplored [2,3].

Plant-derived natural products have gained increasing attention as potential alternatives to synthetic drugs due to their diverse bioactive compounds, reduced side effects, and lower likelihood of inducing antimicrobial resistance (AMR) [4]. This is particularly important in light of the growing global threat of AMR, which has significantly reduced the effectiveness of conventional antibiotics and increased morbidity, mortality, and healthcare burden [5].

Among clinically significant pathogens, *Staphylococcus aureus* and *Escherichia coli* are frequently implicated in a wide range of infections and are known for their multidrug resistance capabilities [6]. These bacteria represent Gram-positive and Gram-negative groups, respectively, and their structural differences play a crucial role in determining their susceptibility to antibacterial agents. Therefore, exploring plant-based antibacterial agents against these pathogens is of considerable scientific and clinical interest. Previous studies have reported that *N. lappaceum* contains various phytochemical constituents, including phenolic compounds such as flavonoids and alkaloids, which exhibit antimicrobial activity [7]. However, most studies have focused on specific plant parts or single cultivars, and there is limited information on the comparative phytochemical profiling and antibacterial activity of leaf extracts across different cultivars.

Despite these findings, comparative studies evaluating the antibacterial activity and phytochemical profiles of different Malaysian *N. lappaceum* leaf cultivars are still limited. Scarce information is available on whether cultivar-dependent differences in antibacterial activity are associated with differences in phytochemical composition as revealed by Liquid Chromatography-Time-of-Flight Mass Spectrometry/Mass Spectrometry (LC-TOF-MS/MS)-based metabolite profiling. Addressing these gaps may improve our understanding of the phytochemical diversity of *N. lappaceum* and facilitate the identification of metabolites potentially associated with antibacterial activity. Advances in metabolomics, particularly LC-TOF-MS/MS, have enabled comprehensive profiling of bioactive compounds in plant extracts. These analytical techniques facilitate the identification of complex phytochemical compositions and provide insights into compounds potentially responsible for biological activities [9,10].

Therefore, this study aims to evaluate the antibacterial activity of methanolic and chloroform-methanol-water (CMA) leaf extracts of *N. lappaceum* cultivars (R191, AG28, and GB44) against *S. aureus* and *E. coli*, and to characterize their phytochemical composition using LC-TOF-MS/MS. The findings of this study are expected to contribute to a better understanding of the phytochemical diversity and antibacterial potential of *N. lappaceum* as a natural source of antibacterial agents.

Materials and Methods

Sample Collection and Preparation

Leaves from three *N. lappaceum* L. cultivars, namely Anak Sekolah (R191), Mutiara Merah (AG28), and Mutiara Wangi (GB44), were collected from Bukit Kayu Hitam, Kedah, Malaysia (6.4883°N, 100.4822°E). The collected leaves were thoroughly washed with distilled water to remove debris and dried in an oven at 45 °C until a constant weight was achieved. The dried samples were then ground into a fine powder using a mechanical blender and stored in airtight containers until further use.

Preparation of Plant Extracts

Both methanolic and CMA extracts were initially prepared for antibacterial screening. Following antibacterial evaluation, only the methanolic extracts were subjected to LC-TOF-MS/MS analysis because they consistently exhibited higher antibacterial activity and were therefore considered more suitable for metabolite profiling.

Methanolic Extracts

Methanolic extracts were prepared using the maceration method as previously described [8]. Briefly, 20 g of powdered leaf samples from each cultivar were immersed in 300 mL of 100 % methanol (solid-to-solvent ratio of 1:15, w/v) in a conical flask. The mixtures were incubated in a shaking incubator at 37 °C and 220 rpm for three days. Following incubation, the extracts were filtered through Whatman No. 1 filter paper to remove plant residues. The filtrates were then concentrated using a rotary vacuum evaporator at 40 °C to remove the solvent, yielding crude methanolic extracts.

Chloroform-Methanol-Water (CMA) Extracts

CMA extracts were prepared using a sonication-assisted extraction method. Briefly, 20 g of powdered leaf samples were mixed with a solvent system consisting of chloroform, methanol, and distilled water (120:120:60 mL), maintaining a solid-to-solvent ratio of 1:15 (w/v). The mixture was initially subjected to shaking at 4 °C for 10 minutes, followed by sonication in an ultrasonic bath at room temperature for 15 minutes. Subsequently, the extracts were centrifuged twice at 5000 rpm for 30 minutes at 4 °C. The combined supernatant obtained after centrifugation was collected, filtered, and freeze-dried. The aqueous and organic phases were not separated for independent analyses in the present study, following previously reported protocols [11,9].

Determination of Antibacterial Activity

Preparation of Bacterial Suspension

Bacterial inoculum of *S. aureus* and *E. coli* were prepared by selecting well-isolated colonies from freshly cultured agar plates. The colonies were transferred into sterile saline solution and vortexed to obtain a homogeneous suspension. The turbidity of the bacterial suspension was adjusted to match a 0.5 McFarland standard, corresponding to approximately 1.5×10^8 CFU/mL [12].

Preparation of Bacteria Agar Plate

Mueller–Hinton agar (MHA) was prepared by dissolving 15.2 g of MHA powder in 400 mL of distilled water. The medium was sterilized by autoclaving at 121 °C for 15 minutes. Approximately 25 mL of the sterilized medium was poured into sterile Petri dishes and allowed to solidify at room temperature. The standardized bacterial suspension was then uniformly spread onto the agar surface using a sterile cotton swab [13].

Agar Well Diffusion

Four wells were aseptically created on each inoculated agar plate. Three wells were filled with 100 µL of (20% (w/v) concentration) plant extract, while the fourth well served as a negative control containing 100 µL of 10% (v/v) dimethyl sulfoxide (DMSO). A standard erythromycin disc (10 µg/disc) was used as the positive control. Erythromycin was selected as the positive control because it is a clinically established antibiotic with activity against Gram-positive bacteria and has been widely used in agar diffusion assays.

The plates were refrigerated for 30 minutes to allow diffusion of the extracts, followed by incubation at 37 °C for 24 hours. After incubation, the diameter of the inhibition zones surrounding each well was measured in millimetres, including the well diameter. All assays were performed in triplicates.

Phytochemical Characterization by Liquid Chromatography-Time-of-Flight Mass Spectrometry/Mass Spectrometry (LC-TOF-MS/MS)

Phytochemical analysis of the methanolic extracts was performed using an UltiMate 3000 UHPLC system (Dionex) equipped with a Thermo Scientific C18 column (Acclaim™ Polar Advantage II, 3 × 150 mm, 3 µm particle size). A volume of 20 µL of filtered extract was injected into the system.

The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (100% acetonitrile). A gradient elution was performed over 22 minutes at a flow rate of 0.4 mL/min and a column temperature of 40°C, with the following conditions: 5% B (0–3 min), 80% B (3–10 min), 80% B (10–15 min), and 5% B (15–22 min).

Mass spectrometric detection was carried out using an electrospray ionization (ESI) source coupled to a high-resolution MicroTOF QIII mass spectrometer (Bruker Daltonics) operated in positive ion mode. The instrument parameters were set as follows: capillary voltage of 4500 V, nebulizer pressure of 2.0 bar, drying gas flow rate of 8 L/min, and drying temperature of 300°C. Compounds within a mass-to-charge ratio (m/z) range of 50–1500 were detected.

Data acquisition and analysis were performed using Compass Data Analysis software (Bruker Daltonik GmbH). Compound annotation was performed based on accurate mass measurements and MS/MS fragmentation patterns using reference databases including MetFrag, METLIN, MassBank, MoNA, NIST14, and HMDB. Therefore, all metabolites reported in this study should be considered as tentatively identified compounds [14,9].

Data Analysis

All experimental data were expressed as mean ± standard deviation (SD) from triplicate measurements. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to determine significant differences between groups. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

Results and Discussion

Methanolic and CMA leaf extracts of *N. lappaceum L.* show antibacterial activity against *S. aureus* and *E. coli*

The leaf extracts of three *N. lappaceum* cultivars (R191, AG28, and GB44) exhibited varying degrees of antibacterial activity against *S. aureus* and *E. coli*, as evidenced by the formation of inhibition zones (Table 1; Figure 1–4). For *S. aureus*, the average zone of inhibition was slightly higher for methanolic extracts (22.1 mm) compared to CMA extracts (21.6 mm) (Table 1), indicating only a marginal difference between the two extraction methods. In contrast, a more pronounced solvent-dependent effect was observed for *E. coli*, where methanolic extracts produced a higher average inhibition zone (20.8 mm) than CMA extracts (18.3 mm). This trend is clearly illustrated in Figure 1–4, where larger inhibition zones are observed for methanolic extracts across all cultivars. Statistical analysis further revealed significant differences between methanolic and CMA extracts across all cultivars against *E. coli*.

Among the tested extracts, methanolic R191 demonstrated the highest antibacterial activity against *S. aureus*, whereas methanolic GB44 exhibited the strongest inhibitory effect against *E. coli* (Table 1; Figure 1–4). These findings suggest that methanol is a more effective solvent for extracting antibacterial compounds, particularly those active against Gram-negative bacteria. Based on these observations, all methanolic leaf extracts were selected for subsequent MS-based metabolomic analysis.

All extracts demonstrated measurable antibacterial activity against both *S. aureus* and *E. coli*, although the extent of inhibition varied depending on the extraction solvent and bacterial species. The differences observed between methanolic and CMA extracts (Figure 1–4) indicate that solvent polarity plays a critical role in the extraction efficiency of antibacterial phytochemicals. The antibacterial activity of *N. lappaceum* leaf extracts observed in this study is consistent with previous findings [15].

The variation in antibacterial susceptibility between the two bacterial species can be attributed to differences in their cell wall structures. Gram-positive bacteria such as *S. aureus* lack an outer membrane, which facilitates the penetration of bioactive compounds into the cell. In contrast, Gram-negative bacteria such as *E. coli* possess an outer membrane that acts as a permeability barrier, thereby limiting the entry of certain antimicrobial agents [16].This structural distinction may contribute to the differences in antibacterial activity observed between the two organisms.

Significantly, all extracts exhibited higher antibacterial activity against *E. coli* compared to the positive control (erythromycin) under the conditions tested, as shown in Figure 1–4, suggesting that rambutan leaf extracts may serve as a promising source of antibacterial agents. The superior activity of methanolic extracts is consistent with previous reports indicating that methanol is more efficient in extracting phenolic compounds, which are known to contribute significantly to antibacterial activity [17,15]. However, this observation should be interpreted with caution because the plant extracts and the antibiotic differed in composition, concentration, and diffusion characteristics. Therefore, the present results should not be interpreted as evidence that the extracts are inherently more potent than erythromycin.

Table 1. Diameter of zone of inhibition (mm) of methanolic and CMA leaf extracts of *N. lappaceum* L. (R191, AG28 and GB44) against *S. aureus* and *E. coli* at 20% (w/v) concentration. Results for extracts were the mean values of triplicate assays. NA indicates no inhibition observed. Data followed by different letters are significantly different at $p<0.05$.

Diameter of zone of inhibition (mm)	Growth Inhibition on *S. aureus* (Mean ± SD)		Growth Inhibition on *E. coli* (Mean ± SD)	
Cultivars \ Solvent	Methanol	CMA	Methanol	CMA
R191	23.0 ± 0.0	21.0 ± 1.0	21.0 ± 0.0[a]	19.3 ± 0.6[b]
AG28	22.7 ± 0.6	21.0 ± 0.0	18.7 ± 0.6[a]	19.7 ± 0.6[b]
GB44	20.7 ± 0.6	22.7 ± 0.6	22.7 ± 1.2[a]	16.0 ± 1.7[b]
Average	22.1	21.6	20.8	18.3
Positive control (Erythromycin 10 µg/disc)	23.7 ± 1.5	24.0 ± 1.0	15.3 ± 0.6	15.0 ± 0.0
Negative control (10% v/v DMSO)	0.00	0.00	0.00	0.00

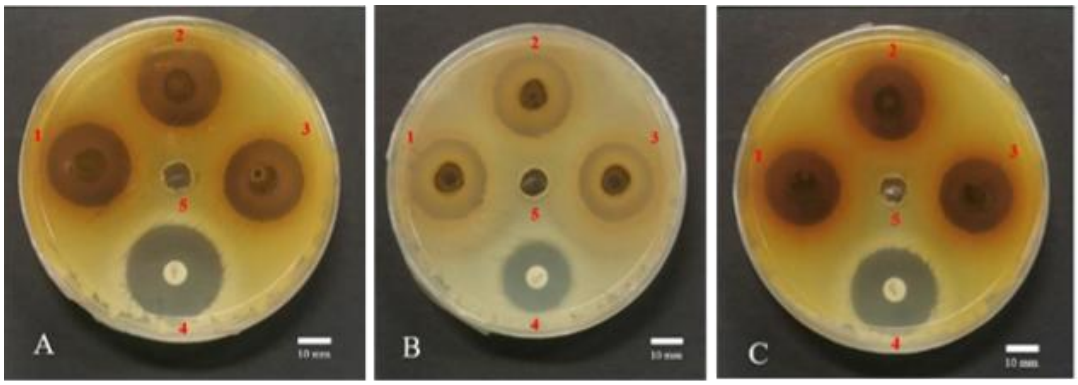


Figure 1. The inhibition zone of three methanolic leaf extracts of *N. lappaceum* L. **A.** R191 **B.** AG28 **C.** GB44 at 20% (w/v) concentration against *S. aureus*. (1-3) represent the extracts; (4) represent the positive control, erythromycin 10 µg/disc; (5) represent the negative control, 10% v/v DMSO. Scale bar: 10 mm

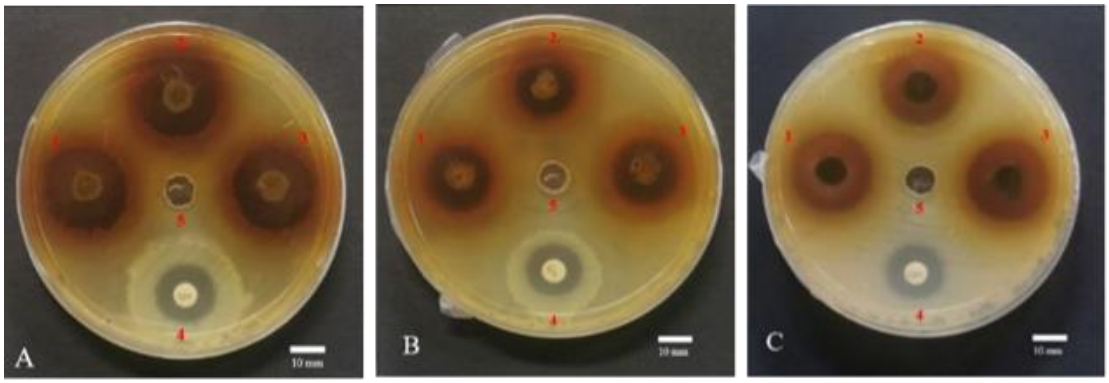


Figure 2. The inhibition zone of three methanolic leaf extracts of *N. lappaceum* L. **A.** R191 **B.** AG28 **C.** GB44 at 20% (w/v) concentration against *E. coli*. (1-3) represent the extracts; (4) represent the positive control, erythromycin 10 µg/disc; (5) represent the negative control, 10% v/v DMSO. Scale bar: 10 mm

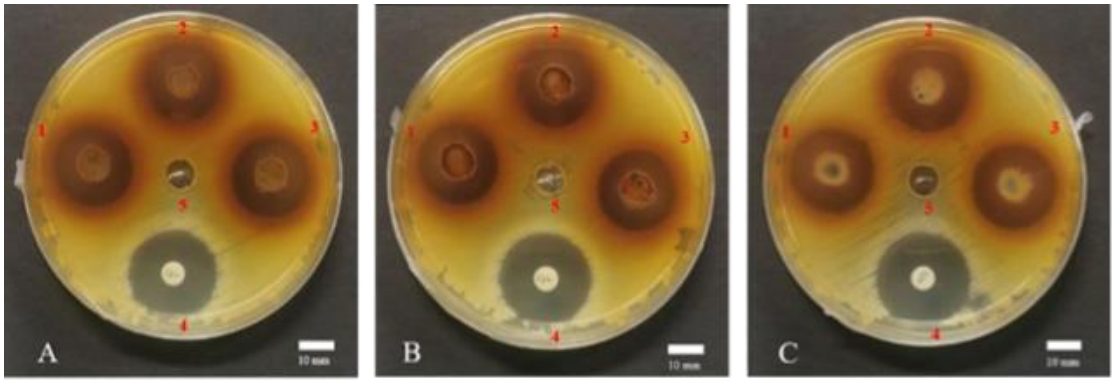


Figure 3. The inhibition zone of three CMA leaf extracts of *N. lappaceum* L. **A.** R191 **B**. AG28 **C.** GB44 at 20% (w/v) concentration against *S. aureus*. (1-3) represent the extracts; (4) represent the positive control, erythromycin 10 µg/disc; (5) represent the negative control, 10% v/v DMSO. Scale bar: 10 mm

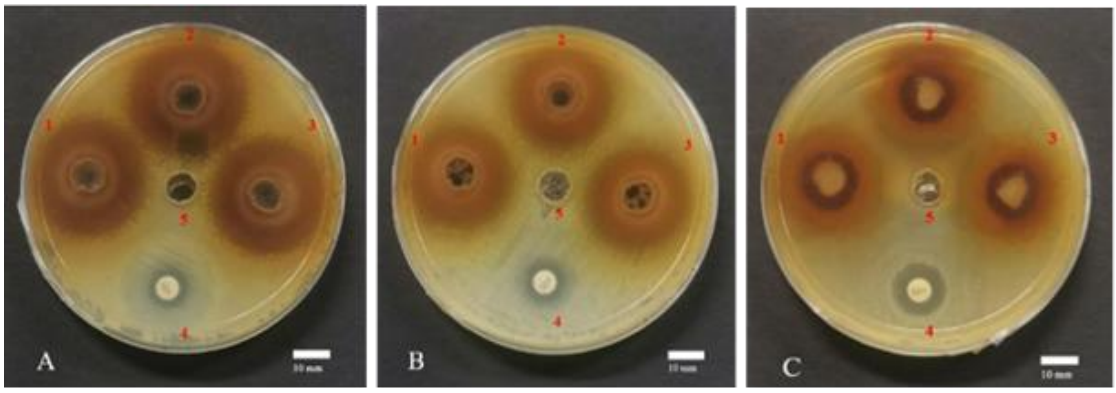


Figure 4. The inhibition zone of three CMA leaf extracts of *N. lappaceum* L. **A.** R191 **B.** AG28 **C.** GB44 at 20% (w/v) concentration against *E. coli*. (1-3) represent the extracts; (4) represent the positive control, erythromycin 10 µg/disc; (5) represent the negative control, 10% v/v DMSO. Scale bar: 10 mm

LC-TOF-MS/MS analysis tentatively identified three, ten, and nine compounds in R191, AG28, and GB44 methanolic leaf extracts of *N. lappaceum L*. respectively

The untargeted screening and identification of phytochemicals in the methanolic leaf extracts of *N. lappaceum* L. cultivars (R191, AG28, and GB44) were carried out using LC-TOF-MS/MS analysis. The detected compounds were tentatively identified based on retention time, precursor ions, ion type, and MS/MS fragmentation patterns, with reference to established databases including MetFrag, METLIN, MassBank, MoNA, NIST14 and HMDB. Tables 2–4 summarize the retention time, precursor ions, ion type, fragmentation data, molecular formula, superclass, class, and biosynthetic pathways of the identified compounds.

A total of three, ten, and nine compounds were tentatively identified in methanolic extracts of R191, AG28, and GB44, respectively. The higher number of compounds detected in AG28 and GB44 suggests greater chemical diversity compared to R191. The observed differences in phytochemical profiles among the three *N. lappaceum* cultivars are likely attributable to both genetic and environmental factors. Genetic variation among cultivars can influence the expression of genes involved in secondary metabolite biosynthesis, resulting in cultivar-specific accumulation of flavonoids, alkaloids and triterpenoids [18]. In addition, environmental conditions such as light intensity, temperature, rainfall, soil composition and nutrient availability are known to affect the production of plant secondary metabolites [19]. Although all leaf samples were collected from the same geographical area, microenvironmental variation and differences in physiological maturity may also contribute to the observed metabolite diversity. Therefore, the greater number of metabolites detected in AG28 and GB44 compared with R191 may reflect inherent cultivar-specific metabolic characteristics rather than differences in extraction or analytical procedures [20]. In methanolic extracts of R191 leaf extracts, three compounds—procyanidin B2, (+)-catechin, and quercetin—were identified, all of which belong to the flavonoid subclass of phenolic compounds (Table 2). This indicates that the phytochemical profile of R191 is dominated by flavonoids.

Interestingly, a greater number of detected metabolites did not necessarily correspond to stronger antibacterial activity. Although AG28 and GB44 contained more diverse phytochemical profiles than R191, the R191 methanolic extract exhibited the highest antibacterial activity against *S. aureus*. This observation suggests that antibacterial efficacy is influenced not only by metabolite diversity but also by the relative abundance, bioactivity and synergistic interactions of specific antibacterial constituents. Similar observations have been reported in medicinal plants, where biological activity is more closely associated with the composition and concentration of key metabolites than with the total number of compounds detected [21,10].

In contrast, methanolic AG28 leaf extracts exhibited a more diverse phytochemical composition, with ten compounds tentatively identified (Table 3). Among these, quercetin, procyanidin B2, tiliroside, quercitrin, (–)-epicatechin and spiraeoside were classified as flavonoids, while 10S-hydroxypheophorbide A and pheophorbide A were categorized as tryptophan alkaloids. Additionally, oleanolic acid and α-hederin were identified as triterpenoids. Qualitatively, phenolic compounds were the dominant class in AG28 extracts.

Similarly, nine compounds were tentatively identified in methanolic GB44 leaf extracts (Table 4). Flavonoids—including quercetin, procyanidin B2, tiliroside, quercitrin, (–)-epicatechin and rutin—constituted the major class of compounds. In addition, 10S-hydroxypheophorbide A and pheophorbide A were identified as alkaloids, while α-hederin was classified as a triterpenoid. Overall, phenolics were the predominant class in GB44 extracts, followed by alkaloids and saponins.

Table 2. LC-TOF-MS/MS data-positive ion mode for phytochemical identification in methanolic leaf extracts of *N. lappaceum* L. (R191).

Compound name	Molecular formula	Super class	Class	Biosynthesis pathway	Retention time (min)	Precursor ion (m/z)	Ion type	MS/MS (m/z) fragment ions	Mass error (%)
Procyanidin B2	$C_{30}H_{26}O_{12}$	Flavonoids	Proanthocyanins	Shikimates and Phenylpropanoids	8.5	579.15	M+H	139.0394, 291.0869, 579.1484	-0.0651
(+)-Catechin	$C_{15}H_{14}O_6$	Flavonoids	Flavan-3-ols	Shikimates and Phenylpropanoids	8.5	291.086	M+H	139.0396	-0.065
Quercetin	$C_{15}H_{10}O_7$	Flavonoids	Flavonols	Shikimates and Phenylpropanoids	9.5	303.05	M+H	291.0869, 785.0821 129.0550, 303.0510, 303.0524	-0.2975

Table 3. LC-TOF-MS/MS data-positive ion mode for phytochemical identification in methanolic leaf extracts of *N. lappaceum* L. (AG28).

Compound name	Molecular formula	Super class	Class	Biosynthesis pathway	Retention time (min)	Precursor ion (m/z)	Ion type	MS/MS (m/z) fragment ion	Mass error (%)
Quercetin	$C_{15}H_{10}O_7$	Flavonoids	Flavonols	Shikimates and Phenylpropanoids	9.6	303.05	M+H	147.0442, 303.0020, 303.0046	-0.0637
10*S*-Hydroxypheophorbide A	$C_{35}H_{36}N_4O_6$	Tryptophan alkaloids	Pheophorbides	Alkaloids	16.9	609.272	M+H	591.1578, 609.1644, 609.166	-0.0685
Oleanolic acid	$C_{30}H_{48}O_3$	Triterpenoids	Oleanane triterpenoids	Terpenoids	13.8	439.357	M+H- H2O	189.1297, 191.1445, 203.1443	-0.076
Procyanidin B2	$C_{30}H_{26}O_{12}$	Flavonoids	Proanthocyanins	Shikimates and Phenylpropanoids	8.4	579.15	M+H	127.0260, 163.0214, 579.0705	-0.0651
Tiliroside; Tribuloside; [(2*R*,3*S*,4*S*,5*R*,6*S*)-6-[5,7-dihydroxy-2-(4-hydroxyphenyl)-4-oxochromen-3-yl]oxy- 3,4,5-trihydroxyoxan- 2-yl]methyl (*E*)-3-(4- hydroxyphenyl)prop-2- enoate	$C_{30}H_{26}O_{13}$	Flavonoids	Flavonols	Shikimates and Phenylpropanoids	9.1	595.145	M+H	303.0034, 304.0058, 465.0337	-0.0641

Quercitrin; Quercetin 3-O-alpha- rhamnopyranoside	$C_{21}H_{20}O_{11}$	Flavonoids	Flavonols	Shikimates and Phenylpropanoids	9.6	449.11	M+H	303.0044, 303.0052, 304.0062	-0.061
(-)-Epicatechin	$C_{15}H_{14}O_6$	Flavonoids	Flavan-3-ols	Shikimates and Phenylpropanoids	8.6	291.087	M+H	123.0271, 139.0196, 291.0421	-0.0647
alpha-Hederin	$C_{41}H_{66}O_{12}$	Triterpenoids	Oleanane triterpenoids	Terpenoids	10	751.463	M+H	287.0095, 455.2818, 751.3486	-0.0662
Spiraeoside	$C_{21}H_{20}O_{12}$	Flavonoids	Flavonols	Shikimates and Phenylpropanoids	9.1	465.103	M+H	303.0034, 303.0038, 465.0337	-0.0603
Pheophorbide A	$C_{35}H_{36}N_4O_5$	Tryptophan alkaloids	Carbazole alkaloids	Alkaloids	17.3	593.269	M+H	533.1611, 593.1696, 593.1714	-0.071

Table 4. LC-TOF-MS/MS data-positive ion mode for phytochemical identification in methanolic leaf extracts of *N. lappaceum* L. (GB44).

Compound name	Molecular formula	Super class	Class	Pathway	Retention time (min)	Precursor ion (m/z)	Ion type	MS/MS (m/z) fragment ion	Mass error (%)
alpha-Hederin	$C_{41}H_{66}O_{12}$	Triterpenoids	Oleanane triterpenoids	Terpenoids	11.8	751.463	M+H	593.2687,	-0.0662
Tiliroside; Tribuloside; [(2*R*,3*S*,4*S*,5*R*,6*S*)-6-[5,7-dihydroxy-2-(4-hydroxyphenyl)-4-oxochromen-3-yl]oxy-3,4,5-trihydroxyoxan-2-yl]methyl (*E*)-3-(4-hydroxyphenyl)prop-2-enoate	$C_{30}H_{26}O_{13}$	Flavonoids	Flavonols	Shikimates and Phenylpropanoids	10.5	595.144	M+H	594.2713, 751.4525 147.0432, 287.0522,	-0.0642
Quercetin; 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen- 4-one	$C_{15}H_{10}O_7$	Flavonoids	Flavones and Flavonols	Shikimates and Phenylpropanoids	9.6	303.05	M+H	593.2695 147.0644,	-0.0637
Procyanidin B2	$C_{30}H_{26}O_{12}$	Flavonoids	Proanthocyanins	Shikimates and Phenylpropanoids	8.4	579.15	M+H	303.0473, 303.0486 127.0387, 287.0530, 579.146	-0.0651

Pheophorbide A	C35H36N4O5	Tryptophan alkaloids	Carbazole alkaloids	Alkaloids	10	593.269	M+H	287.0521, 309.0984, 593.2715	-0.071
Quercitrin	C21H20O11	Flavonoids	Flavonols	Shikimates and Phenylpropanoids	9.6	449.108	M+H	303.0481, 303.0485, 304.0513	-0.0614
10*S*-Hydroxypheophorbide A	C35H36N4O6	Tryptophan alkaloids	Pheophorbides	Alkaloids	16.9	609.272	M+H	279.2256, 609.2573, 609.2592	-0.0686
(-)-Epicatechin	C15H14O6	Flavonoids	Flavan-3-ols	Shikimates and Phenylpropanoids	8.6	291.087	M+H	123.0436, 139.0377, 291.0646	-0.0647
Rutin	C27H30O16	Flavonoids	Flavonols	Shikimates and Phenylpropanoids	10.2	611.161	M+H	147.0434, 303.0484, 611.1349	-0.0594

Across all three cultivars, a total of 12 distinct compounds were tentatively identified, with flavonoids representing the most abundant class of secondary metabolites, followed by tryptophan alkaloids and triterpenoids. This highlights the phytochemical richness of *N. lappaceum* leaf extracts and suggests that flavonoids may contribute substantially to the antibacterial activity observed in the present study because they were consistently detected across all cultivars and have previously been reported as major antibacterial constituents of rambutan leaves [1,3].

Flavonoids were the predominant metabolites detected in all methanolic extracts, with procyanidin B2 and quercetin consistently identified in the three cultivars. Their occurrence is consistent with previous phytochemical studies of *N. lappaceum* leaves, which reported flavonoids as the principal phenolic constituents responsible for antibacterial activity [1]. Flavonoids exert antibacterial effects through multiple mechanisms, including disruption of bacterial membrane integrity, increased membrane permeability, inhibition of nucleic acid synthesis, interference with energy metabolism, and inhibition of biofilm formation. Therefore, the higher antibacterial activity observed for the methanolic extracts is likely associated with the efficient extraction of these polar phenolic compounds [7,1,22].

Besides the common flavonoids detected in all cultivars, cultivar-specific metabolites such as (+)-catechin, spiraeoside and rutin were also identified. Rutin is a well-characterised flavonoid widely reported to possess multiple biological activities, including antioxidant and anti-inflammatory properties [23]. Although these metabolites have individually been reported to possess antibacterial activity, the present results do not indicate that any single compound is solely responsible for the antibacterial activity observed. Instead, the activity is more likely attributable to the combined effects of multiple flavonoids present in the extracts [24,25,21,9].

Tiliroside, quercitrin and (-)-epicatechin were detected only in AG28 and GB44 extracts. (–)-Epicatechin is a well-recognised bioactive flavanol with diverse biological activities and has been extensively studied in plant-derived phytochemicals [26]. Previous studies have demonstrated that these flavonoids inhibit both Gram-positive and Gram-negative bacteria by increasing membrane permeability and interfering with essential bacterial metabolic pathways [25,20]. Their simultaneous occurrence in AG28 and GB44 may therefore contribute to antibacterial activity through additive or synergistic interactions. Pheophorbide A and 10S-hydroxypheophorbide A were detected only in AG28 and GB44 extracts but were absent from R191, which exhibited the strongest antibacterial activity against *S. aureus*. This observation suggests that these metabolites are unlikely to be the principal antibacterial constituents but may contribute synergistically with flavonoids [25,22].

Oleanolic acid and α-hederin were identified only in AG28 and GB44 extracts. Although both compounds have been reported to exhibit antibacterial activity, their restricted occurrence among cultivars suggests that they are unlikely to account for the antibacterial activity observed in all extracts. Instead, these triterpenoids may complement the antibacterial effects of flavonoids through synergistic interactions [27,28,29].

Overall, the LC-TOF-MS/MS analysis indicates that flavonoids are the metabolites most consistently associated with the antibacterial activity of *N. lappaceum* leaf extracts. Notably, quercetin and procyanidin B2 were detected in all cultivars despite differences in phytochemical diversity, suggesting that these compounds may contribute substantially to the antibacterial effects. Nevertheless, the antibacterial activity is more likely to result from synergistic interactions among multiple phytochemicals rather than the action of a single metabolite [30,31,32].

The antibacterial activity observed in the present study is consistent with previous reports describing the antibacterial potential of *N. lappaceum* leaf extracts against *S. aureus* and *E. coli* [4,26]. However, unlike earlier studies that primarily focused on crude extract activity or total phenolic content, the present work further characterised the metabolite composition of three Malaysian cultivars using LC-TOF-MS/MS. The predominance of flavonoids identified in this study provides additional evidence supporting their contribution to the antibacterial properties of *N. lappaceum* leaves [3,21].

Limitations of the Study

A limitation of the present study is that detailed sampling information, including plant age, phenological stage, collection date, sampling time, voucher specimen information, the number of individual plants sampled, the number of leaves collected per cultivar, and the biological replication strategy, were not recorded during sample collection. Future studies should incorporate standardized botanical authentication, comprehensive sampling documentation, and biological replication to improve the

reproducibility and interpretation of phytochemical and antibacterial analyses.

In addition, although the present study demonstrated the antibacterial potential of *N. lappaceum* leaf extracts using the agar well diffusion assay, the antibacterial efficacy was evaluated only based on inhibition zone diameters. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays were not performed, which limits the quantitative assessment of the antibacterial potency of the extracts. Future studies should incorporate in vivo evaluation to further validate the antibacterial efficacy and therapeutic potential of *N. lappaceum* leaf extracts.

Furthermore, the phytochemicals identified by LC-TOF-MS/MS were tentatively assigned based on accurate mass measurements and fragmentation patterns without confirmation using authentic reference standards. Therefore, the relationship between the detected metabolites and the observed antibacterial activity should be interpreted with caution.

Although representative TIC/BPC chromatograms are not available, metabolite annotation was based on high-resolution accurate mass measurements, MS/MS fragmentation patterns, and cross-validation using multiple public metabolite databases (MetFrag, METLIN, MassBank, MoNA, NIST14, and HMDB). Therefore, the reported compounds should be regarded as tentative identifications, consistent with the Metabolomics Standards Initiative (MSI) Level 2 annotation.

Conclusions

In conclusion, the present study demonstrated that methanolic and CMA leaf extracts of *N. lappaceum* cultivars (R191, AG28, and GB44) exhibited significant antibacterial activity against *S. aureus* and *E. coli*. Among the extraction methods, methanolic extracts consistently showed higher antibacterial activity, indicating that methanol is a more effective solvent for extracting bioactive compounds, particularly those active against Gram-negative bacteria.

Qualitative LC-TOF-MS/MS analysis revealed a diverse phytochemical composition across the cultivars, with flavonoids identified as the predominant class of secondary metabolites, followed by tryptophan alkaloids and triterpenoids. Key compounds such as procyanidin B2, quercetin, (+)-catechin, tiliroside, and quercitrin may contribute to the antibacterial activity observed. The presence of multiple bioactive compounds suggests that the antibacterial effects of *N. lappaceum* leaf extracts are likely due to synergistic interactions rather than the action of a single compound.

Overall, this study provides comparative insights into the antibacterial potential and phytochemical diversity of three Malaysian *N. lappaceum* cultivars, contributing to the growing body of knowledge on plant-derived antibacterial agents.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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