

Partitioning Phytochemicals in *Orthosiphon aristatus* Extract with Antioxidant and Antibacterial Properties

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Abstract: *Orthosiphon aristatus* has been used as a traditional remedy for many diseases, mainly due to its remarkable bioactive phytochemicals. The phytochemicals were extracted with ultrasound energy and then partitioned into individual fractions according to solvent polarity. Two partition techniques, namely liquid-liquid extraction (LLE) and column chromatography (CC), were applied to prepare the fractions from the crude extract. The individual fractions were then subject to LC-MS/MS analysis for compound screening. Rosmarinic acid was the most intense peak and mostly partitioned into ethyl acetate (EA) through LLE, and CC-D from column chromatography. It was also one of the dominant phytochemicals, besides other polar compounds like octadecadienoic acid and its derivatives in radical quenching. However, the non-polar fractions (LLE-EA and CC-B), which contained polymethoxylated flavones, were more effective against the growth of Gram-positive bacteria (*Streptococcus agalactiae* and *Staphylococcus aureus*). The Gram-negative bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) were more susceptible to polar fractions (LLE-AQ and CC-F). Therefore, the polar fractions showed higher antiradical activity, whereas the non-polar fractions appeared to have higher antibacterial activity. Interestingly, *O. aristatus* extract and its fractions were also better performance than ampicillin in inhibiting bacterial growth, especially *E. coli*.

Keywords: *Orthosiphon aristatus*; rosmarinic acid; antioxidant; antibacterial; liquid-liquid extraction; column chromatography.

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1. Introduction

Herbal plants have been widely used as a traditional and complementary remedy for many illnesses. They are commonly applied as a decoction or powdered extracts after processing. *Orthosiphon aristatus* or its synonym name *O. stamineus* is locally known as ‘misai kucing’ in Malaysia or ‘kumis kucing’ in Indonesia. Such a name is given for the plant because of its terminal appearance of flowers mimicking cat’s whiskers. It is a perennial shrub belonging to the Lamiaceae family. This herbal plant has been traditionally used as ethnomedicine by local folks in Southeast Asia. The plant leaves are usually prepared as herbal tea to treat rheumatism, diabetes, urinary lithiasis, edema, eruptive fever, influenza, hepatitis, jaundice, and hypertension [1]. Nowadays, *O. aristatus* has been formulated into various commercial products such as tea sachets and health supplements in the market. Researchers applied compost to increase the yield and phytochemicals of the plant [2].

The therapeutic effects of herbal plants are mostly contributed by active compounds such as alkaloids, glycosides, tannins, essential oils, resins, phenols, and flavonoids [3]. Previous studies reported more than 20 phenolic compounds from *O. stamineus* [1,4]. The phenolic compounds that have been scientifically proven for their pharmacological properties include caffeic acid, rosmarinic acid, sinensetin, eupatorium, and polymethoxylated flavones. The reported biological activities were antioxidant [5], antibacterial [6], antifungal [7], antidiabetic [8], anti-inflammatory [9], antimutagenic [10], antiviral [11] and antiarthritic [12]. Sinensetin showed the anticancer property by inhibiting protein tyrosine phosphatase 1B, which is a major negative regulator of insulin and leptin signaling [13,14]. The plant extracts showed promising antimicrobial activity against multidrug-resistant microbes [15] and bacteria from post-operative wounds [16]. *O. aristatus* has also been recommended for its potential in healing complicated diabetic wounds [17]. Gram-negative bacilli were isolated more frequently than Gram-positive cocci in the diabetic wound. The most common isolate was *Pseudomonas sp.*, followed by *Escherichia coli* and Methicillin Sensitive *Staphylococcus aureus* [18]. The other microorganisms included *Streptococcus pyogenes*, *Klebsiella spp.*, *Acinetobacter spp.*, Methicillin-Resistant *Staphylococcus aureus*, *Proteus mirabilis*, *Citrobacter spp.*, and *Enterococcus spp.* [18].

In the present study, liquid-liquid extraction and column chromatography were used to partition the crude extract of *O. aristatus* into individual fractions according to solvent polarity. The phytochemicals in the fractions were analyzed using a high throughput LC-MS/MS system. The biological properties such as antiradical and antibacterial activities of the fractions were also compared in relation to the phytochemical profile. The colorimetric antiradical assay was used to investigate the role of phytochemicals in inhibiting radical generation. In the antibacterial assay, two Gram-positive (*S. aureus* and *S. agalactia*) and two Gram-negative (*P. aeruginosa* and *E. coli*) bacteria were chosen. This is the first study to relate the group of phytochemicals in the plant fractions to biological activities.

2. Materials and Methods

2.1. Chemicals and reagents.

The ground (≤ 0.5 cm) and dried raw material of *O. aristatus* (leaves and stems) were purchased from Fieda Resources Sdn. Bhd. (Selangor, Malaysia). The dispersive solid-phase extraction kits (dSPE) were sourced from Phenomenex (California, USA). The kits consisted of a mixture of graphitized carbon black (2.5 mg), magnesium sulfate (150 mg), and primary/secondary amine (25 mg) that were used to clean up plant crude extract. Silica 60 (0.063-0.200 mm) was purchased from Macherey-Nagel (Duren, Germany). Acid wash sea sand and glass wool were obtained from R&M Chemicals (Selangor, Malaysia).

Analytical grades of ethanol, methanol, dimethyl sulfoxide (DMSO), ethyl acetate (EA), and ascorbic acid were bought from Merck (Darmstadt, Germany). DPPH (2,2-diphenyl-1-picrylhydrazyl), butylated hydroxyanisole ($\geq 98.5\%$), ascorbic acid (99%), and ampicillin (95%) were purchased from Sigma-Aldrich (St. Louis, USA). 18.2 M Ω -cm water was produced from Barnstead NANOpure Diamond water purification system (Thermo, Waltham, MA).

Luria-Bertani (LB) and Muller-Hinton (MH) broths were sourced from Pronadisa (Madrid, Spain), whereas granulated agar-agar was obtained from Merck (Darmstadt, Germany). KWIK-STIK bacterial strains were purchased from Microbiologics (Minnesota, USA). The strains consisted of two Gram-positive bacteria; *Streptococcus agalactiae* (ATCC

12403) and *Staphylococcus aureus* (ATCC 11632), and two Gram-negative bacteria; *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 10536).

2.2. Ultrasonic-assisted extraction.

The procedures of ultrasonic-assisted extraction were modified based on the method described by Ho *et al.* [19]. One gram of *O. aristatus* was soaked in 20 mL of 70% ethanol overnight in a 125 mL conical flask and then extracted in an ultrasonic bath (Daihan Scientific, South Korea, 50 kHz, 400W) for 20 min. The supernatant was harvested by centrifugation at 12,000 rpm for 15 min and concentrated using a rotary evaporator (Heidolph, Laborota 4003, Germany). The concentrated supernatant was further dried in the oven at 60 °C until complete dryness. The dried crude extract was stored at -4°C before analysis.

2.3. Sample clean-up.

The crude extract (1 g) was dissolved in methanol (10 mL) and poured into a dSPE tube containing clean-up salts. Vigorous shaking was carried out for 1 min and then centrifuged at 12,000 rpm for 15 min. The supernatant was dried in an oven at 60°C until complete dryness and stored at -4°C before analysis.

2.4. Liquid-liquid extraction.

Liquid-liquid extraction (LLE) was subsequently carried out by dissolving cleaned crude extract (1 g) into water (20 mL) and partitioned vigorously by ethyl acetate (20 mL) in a separating funnel (250 mL). After extraction, the mixture was left vertically in the funnel to form two separated layers. The top layer was withdrawn, and fresh ethyl acetate (20 mL) was added to the remaining aqueous layer for extraction again. The process was repeated thrice, and the collected ethyl acetate was combined for drying. After drying, the weights of both aqueous (LLE-AQ) and ethyl acetate (LLE-EA) fractions were recorded.

2.5. Normal phase column chromatography.

The cleaned crude extract was also partitioned using normal phase column chromatography. An empty glass column with 2 x 14 cm dimension was packed with silica prepared in slurry and homogeneously loaded into the column. The cleaned crude extract (1 g) was reconstituted in ethyl acetate and loaded onto the column. Ethyl acetate (50 mL) was slowly added to elute compounds from the column. The eluent-containing compounds were collected into individual tubes (5 mL for each tube). The elution was continued by using the second and third solvent systems of ethyl acetate: methanol (50%:50%) and methanol (100%), respectively, to increase solvent polarity. A total of 30 tubes was collected and dried before HPLC analysis. Tubes with similar HPLC profiles were combined to form column chromatographic fractions (CC-A, CC-B, CC-C, CC-D, CC-E, and CC-F).

2.6. Antiradical assay.

The antiradical capacity of samples was analyzed using DPPH assay according to the procedures described by Pownall *et al.* [20]. Methanolic DPPH reagent (100 µM) was freshly prepared during analysis. Samples (2 mg/mL) were diluted in serial dilution in 96 well microplates. Each well was filled with sample (100 µL) and DPPH reagent (100 µL). The

microplates were then incubated at 30 °C for 30 min in the dark place. The absorbance of samples was recorded using a microplate reader (Gen5™, BioTek, USA) at 517 nm. The radical scavenging of samples can be determined using Eq. (1), where A_s is the sample absorbance and A_c is control absorbance. Ascorbic acid was used as a positive control. All experiments were conducted in triplicate unless otherwise stated.

$$\text{Scavenging activity (\%)} = \frac{A_c - A_s}{A_c} \times 100 \quad \text{Eq. (1)}$$

2.7. Antibacterial assay.

The bacterial culture was prepared before the antibacterial assay. One colony from the stock was aseptically swapped and transferred into sterilized LB agar plates. The plates were then incubated at 37 °C for 18 hours. The bacterial colony from the plates was then sub-cultured aseptically to 100 mL sterilized LB broth in conical flasks. Each flask containing cultured bacteria was incubated at 37 °C for 24 hours. The cultured bacteria suspension was diluted until achieving approximately 2×10^5 CFU/mL before the antibacterial assay.

The antibacterial capacity of samples was determined using a microdilution susceptibility assay. Samples were dissolved in DMSO and diluted in a serial concentration ranging from 0.313 to 2 mg/mL. Sample (100 µL) and cultured bacterial suspension (100 µL) with approximately 2×10^5 CFU/mL were put into 96 well flat-bottom microplates. The optical density was measured at 600 nm using a microplate reader (Gen5™, BioTek, USA) after 20 hours of incubation at 37 °C. Ampicillin was used as a positive control.

2.8. Liquid chromatography-tandem mass spectrometer.

Samples were analyzed by liquid chromatography (Dionex Corporation Ultimate 3000; Sunnyvale, CA) tandem mass spectrometer (AB SCIEX QSTAR Elite; Foster City, CA). The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). The separation was conducted in a gradient elution; 0–10 min, 10%B; 10–12 min, 10–90%B; 12–20 min, 90%B; 20–22 min, 90–10%B and 22–30 min, 10%B. A C18 reversed-phase Acquity column (1.7 mm, 2.1 x 150 mm) was used for compound separation with 0.15 mL/min flow rate. Samples were filtered with 0.22 µm nylon filters, and the injection volume was 5 µL. The mass spectrometer was used to identify compounds with the mass range of m/z 100-1000 at the negative ion mode. Nitrogen gas was used for nebulizing (40 psi) and curtain gas (25 psi). Collision gas was set at 3, and the accumulation time was 1 s. The ion spray voltage was –4500 V. The declustering potential was 40 V, and the focusing potential was set at 300 V.

3. Results and Discussion

3.1. Extraction and fractionation.

The herbal extract is often used to develop herbal products in the market. The functionality of the products is strongly dependent upon the phytochemicals in the herbal extract. In line with the finding of Li *et al.* [21], 70% of aqueous ethanol was suggested as the extraction solvent and could produce more bioactive constituents. In the present study, the crude extract of *O. aristatus* was prepared using ultrasound-assisted extraction. The yield of crude extract was 8.3 %w/w. It was sticky and dark in color. Therefore, dSPE was used to remove plant pigments and polysaccharides as an impurity from the crude extract. The cleaned

crude extract was 7.8 %w/w. The removal of impurity did not wash out phytochemicals, as shown in Fig. 1. On the other hand, sample clean-up had increased the concentration of phytochemicals. In particular, the peak intensity at the retention time of 17.3 min. Based on the mass fragmentation, the compound exhibited the product's ion of m/z 197, 179, and 161. Hence, the peak was rosmarinic acid, which was reported as the major phytochemical in *O. aristatus* [22].

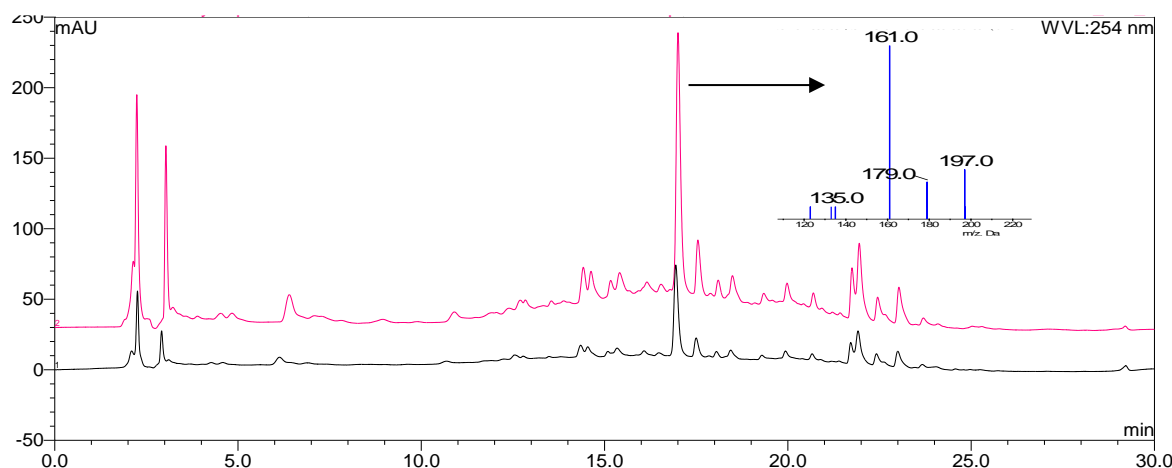


Figure 1. Chromatograms of crude extract (black line) and cleaned extract (red line) after washing with dispersive solid-phase extraction salts. The mass fragments of the intense peak at 17.1 min belong to rosmarinic acid.

The cleaned crude extract was further partitioned into fractions according to solvent polarity. Two fractionation techniques, namely liquid-liquid extraction and column chromatography, were used to partition crude extract. The weights of fractions recovered from both fractionation techniques are presented in Table 1. The weights of collected fractions explained that *O. aristatus* extract consisted mostly of semi-polar and polar compounds. The table shows the recovery of liquid-liquid extraction was 87.5% %w/w, but only 65.5 %w/w for column chromatography. Some compounds might strongly bind on the surface of silica gel in the packed column, and they could be washed out by water.

Table 1. Weights of individual fractions collected from the partition techniques of liquid-liquid extraction and column chromatography using the crude extract *O. aristatus*.

Partition technique	Fraction	Weight (mg)	Weight (%w/w)
Liquid-liquid extraction	LLE-EA	65.70 ± 7.49	5.8 ± 0.6
	LLE-AQ	918.90 ± 59.26	81.5 ± 4.9
Column chromatography	CC-A	2.30 ± 0.56	0.3 ± 0.1
	CC-B	64.30 ± 0.91	7.9 ± 0.1
	CC-C	30.13 ± 1.05	3.7 ± 0.1
	CC-D	76.73 ± 1.97	9.4 ± 0.2
	CC-E	56.03 ± 0.56	6.9 ± 0.1
	CC-F	302.30 ± 0.96	37.2 ± 0.1

Liquid-liquid extraction partitioned most phytochemicals, which were detected at 254 nm in the organic phase of ethyl acetate (Fig. 1). The most intense peak of rosmarinic acid was also mostly partitioned in ethyl acetate, about 16 times higher than in water. The observation was better than the findings of Aydi *et al.* [23], who reported that the solubility of rosmarinic acid in ethyl acetate was about 2.5 to 3.5 times higher than in water at the temperature ranged from 20 to 40 °C. The column chromatography technique fractionated the crude extract into 6 individual fractions (Fig. 2). In a normal phase column chromatography, non-polar compounds would elute out from the silica packed column. Consequently, polar compounds would desorb

from the surface of the absorbent with the use of methanol. Hence, the chromatograms of fractions A to F show the detection of compounds with increasing polarity.

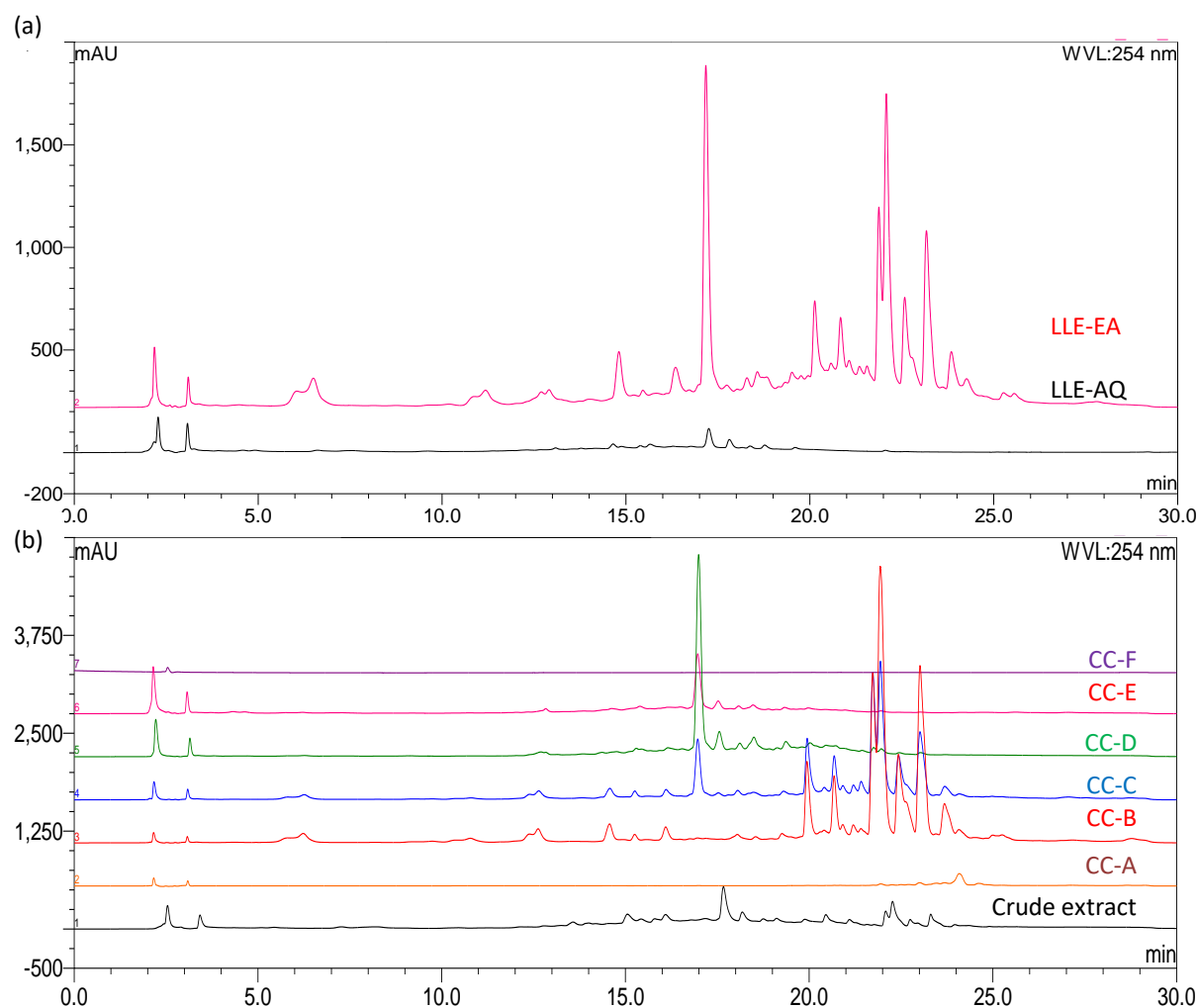


Figure 2. Chromatograms of individual fractions prepared from partition techniques of (a) liquid-liquid extraction and (b) column chromatography.

3.2. Antioxidant capacity of plant fractions.

The fractionation techniques had partitioned phytochemicals in the crude extract into individual fractions according to solvent polarity. The phytochemicals in the plant fractions played an important role in inhibiting free radicals generated by DPPH reagent. The progress of inhibition can be seen in a concentration-dependent manner (Fig. 3). The radical scavenging activities of samples increased with the increase of sample concentrations. The increment was slowed down after the sample concentration achieved more than 0.5 mg/mL. The crude extract and LLE-AQ appeared to have better inhibitory curves against free radicals. The maximum inhibition was 76% for crude extract and about 71% for LLE-AQ at 2 mg/mL. LLE-AQ consisted mostly of non-chromophore compounds for detection at 254 nm (Fig. 2). The mass screening found the presence of 9-oxo-octadecadienoic acid (m/z 293/236/221), oxodihydroxy-octadecenoic acid (m/z 327/229), trihydroxy-octadecenoic acid (m/z 329/229/211), and polyhydrolic alcohols. These metabolites had been shown to exhibit antioxidant responses [24,25]. On the other hand, both non-polar CC-A and CC-C fractions showed the lowest inhibitory at 45%. Mostly, non-polar compounds were mostly accumulated in fraction CC-B. Its antioxidant capacity (higher EC_{50}) was lower than that of fraction CC-D with high

rosmarinic acid concentration (Fig. 2). Fraction CC-C was the fraction that showed a non-polar and polar compounds mixture. The inhibitory action of CC-C was leveraged and resulted in lower antioxidant capacity. The observation explains that the antiradical capacity of *O. aristatus* could be mainly contributed by polar compounds under the synergistic effects [26]. The antioxidant capacity of fractions did not proportionally relate to compound polarity but was more likely to attribute to functional groups of compounds [27]. Phenolic compounds were likely to have stronger antioxidant activity than flavonoids [28]. Methoxyl and hydroxyl groups of phenolics could promote antioxidant activities. The explanation was supported by the results in Fig. 3. Table 2 shows the antiradical capacity of samples at 50 % inhibition. Rosmarinic acid was one of the key phytochemicals contributing to antiradical properties, even though Han Jie *et al.* [29] highlighted that sinensetin contributed to the plant antioxidant activity. Nevertheless, the antiradical capacity of *O. aristatus* was still 4 to 6 times lower than standard chemicals of butylated hydroxyanisole and ascorbic acid.

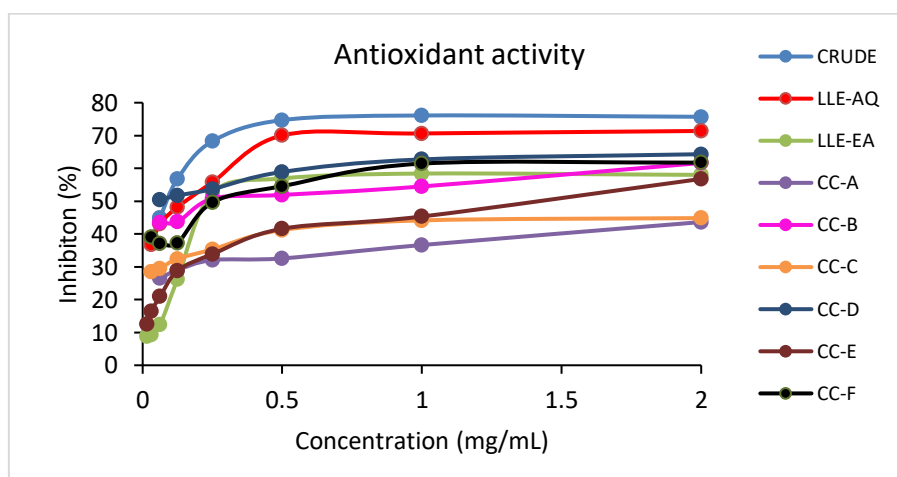


Figure 3. Antiradical activities of crude extract and its individual fractions prepared from liquid-liquid extraction and column chromatography for *Orthosiphon aristatus* where —●—, crude extract; —●—, LLE-AQ; —●—, LLE-EA; —●—, CC-A; —●—, CC-B; —●—, CC-C; —●—, CC-D; —●—, CC-E and —●—, CC-F.

Table 2. Effective concentration to inhibit 50% free radicals (EC₅₀) using standards, crude extract and its individual fractions.

Fraction	EC ₅₀ (mg/mL)	Rosmarinic acid (mg/g)
Butylated hydroxyanisole	0.018	na
Ascorbic acid	0.012	na
Crude extract	0.074	36.72
LLE-EA	0.275	729.08
LLE-AQ	0.124	45.38
CC-A	3.273	0.00
CC-B	0.436	0.00
CC-C	4.971	18.30
CC-D	0.071	36.09
CC-E	1.312	11.24
CC-F	0.161	0.00

na: not application

3.3. Antibacterial action of plant fractions.

The antibacterial activity of samples was carried out using a microdilution susceptibility assay. Fig. 4 shows that the antibacterial activity was increased with the increase of sample concentration. The EC₅₀ was less than 0.0313 mg/mL, which was the lowest concentration of samples used in this study. Therefore, the inhibitory activities at 2 mg/mL are tabulated in Table 3. The results indicated that the performance of all samples was better than that of

positive control, ampicillin, in inhibiting bacterial growth. *O. aristatus* appeared to be a good bacterial inhibitor, especially against Gram-negative bacteria. In the present study, *E. coli* was the most susceptible bacterium under the treatment of *O. aristatus* samples.

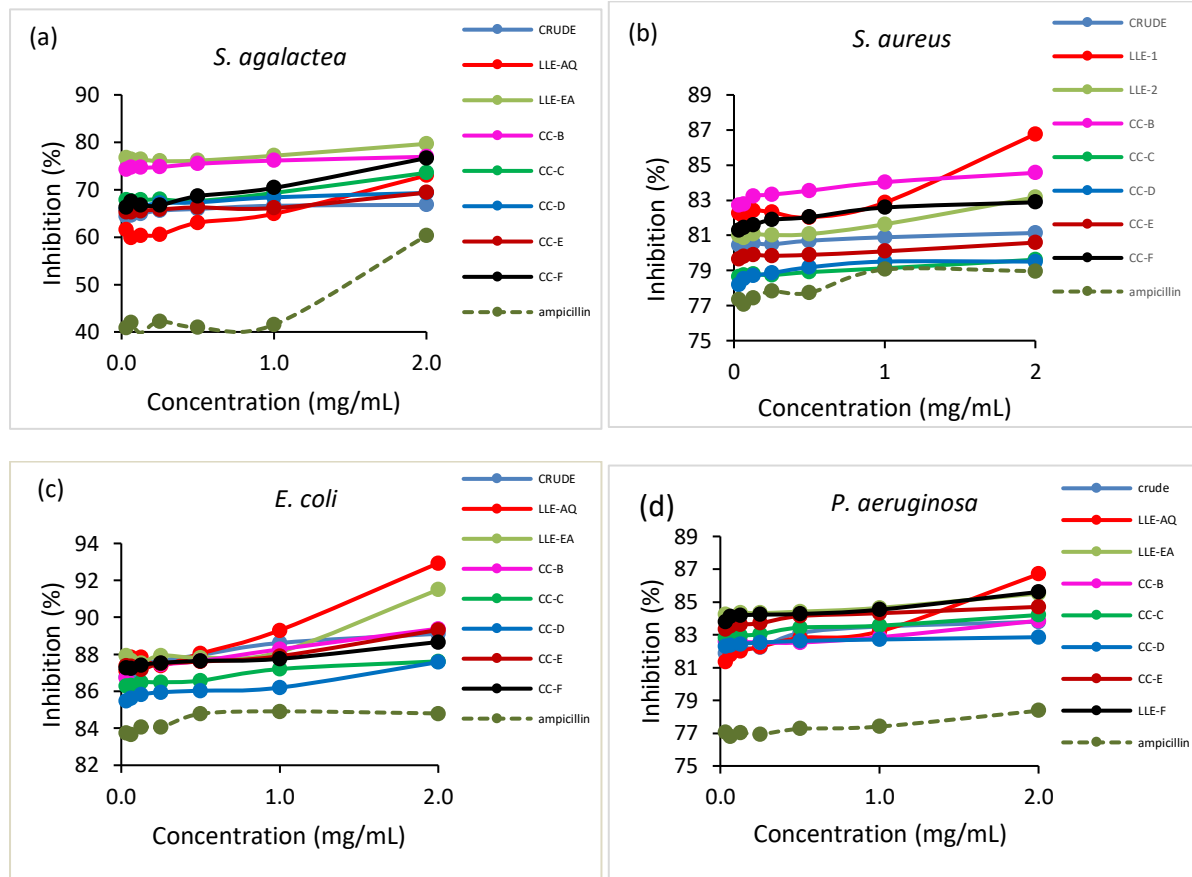


Figure 4. The antibacterial activities of *O. aristatus* extract and its fractions against two Gram positive bacteria; (a) *S. agalactia*, (b) *S. aureus* and two Gram negative bacteria; (c) *E. coli* and (d) *P. aeruginosa*, where - - ● - -, ampicillin; —●—, crude extract; —●—, LLE-EA; —●—, LLE-AQ; —●—, CC-B; —●—, CC-C; —●—, CC-D; —●—, CC-E and —●—, CC-F.

Table 3. Antibacterial activity of different fractions partitioned from *O. aristatus* crude extract.

Sample	Inhibition (%) at 2 mg/mL			
	<i>S. agalactea</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
Ampicillin	60.3	79.0	84.8	78.4
Crude extract	66.8	81.1	89.1	83.8
LLE-AQ	73.0	86.8	92.9	86.7
LLE-EA	79.7	83.2	91.5	85.5
CC-B	76.9	84.6	89.4	83.9
CC-C	73.6	79.6	87.6	84.2
CC-D	69.3	79.5	87.6	82.9
CC-E	69.4	80.6	89.3	84.7
CC-F	76.7	82.9	88.7	85.6

Previous studies conducted by Kong *et al.* [30] showed that the aqueous extract of *O. stamineus* could modulate the immune response of nematodes against *S. aureus* and methicillin-resistant *S. aureus* infection. Studies also reported the antimicrobial activity of *O. aristatus* leaf extract against food-borne bacteria [6] and pathogenic oral bacteria [31]. According to Ho *et al.* [6] and Akuwoah *et al.* [32], rosmarinic acid in the *O. aristatus* extract contributed to the antibacterial action. The present study revealed that non-polar fractions like LLE-EA and CC-B exhibited higher antibacterial activity. In particular, CC-B showed better performance in inhibiting gram-positive bacteria. The compounds detected in CC-B were

mostly polyhydroxymethoxylated flavones such as tetra- and penta-methoxylated flavones (sinensetin). On the other hand, fractions containing rosmarinic acid-like CC-C, CC-D, and CC-E were found to be less effective in antibacterial activities among the samples. Therefore, fractionation allows having a better understanding of bioactive components that contribute to the antibacterial action. Rosmarinic acid was not the key compound to inhibit bacterial growth; other compounds in the fractions of LLE-EA and CC-B were more likely to be potent bacteriostatic agents in *O. aristatus*.

4. Conclusions

Liquid-liquid extraction and column chromatography had successfully fractionated the crude extract into individual fractions according to solvent polarity. Functional groups of compounds could be the dominant factor scavenging free radicals. Polar fractions appeared to contribute to the free radical scavenging activity, whereas non-polar fractions seemed superior in inhibiting bacterial growth, especially *E. coli*. Interestingly, the crude extract and its fractions showed higher antibacterial activity than ampicillin.

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Conflicts of Interest

The authors declare no conflict of interest.

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