SUPPLEMENTARY MATERIAL

Antibacterial and antioxidant activity of naphthofuranquinones from the twigs of tropical mangrove *Avicennia officinalis*

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Abstract:
Mangrove plants are endowed with various biologically active compounds which have potent antibacterial and antioxidant properties. In present study, a bioactivity-guided fractionation for antibacterial and antioxidant active metabolites from the twigs of *Avicennia officinalis* collected from Kuala Selangor Nature Park, Selangor, Malaysia gave 13 major fractions. The antibacterial activity of *A. officinalis* fractions using well-diffusion showed strong selectivity on the Gram-positive bacteria (*Staphylococcus epidermidis*, *S. aureus* and *Bacillus subtilis*) with minimum inhibition concentration (MIC) values of 0.156-5.00 mg/mL. However, no antibacterial activities were observed on the Gram-negative bacteria (*Vibrio cholera*, *Enterobacter cloacae* and *Escherichia coli*). The active antibacterial fractions were further isolated using several chromatographic techniques to give two naphthofuranquinones, namely, avicenol C (**1**) and stenocarpoquinone B (**2**). Meanwhile, the antioxidant activity of *A. officinalis* fractions were evaluated using DPPH radical scavenging assay exhibited low antioxidant activities. Molecular structure of the naphthofuranquinones was elucidated using 1D and 2D NMR spectroscopy.

Keywords: *Avicennia officinalis*; Avicenniaceae; naphthofuranquinones; NMR; antibacterial; DPPH radical scavenging

Experimental:

General Experimental Procedures:

Structure determination of the isolated compounds was based on NMR and MS spectroscopy data. One dimensional NMR (1D NMR) data consisted of ^{1}H and ^{13}C NMR spectra captured using Bruker (^{1}H 400 MHz, ^{13}C 100.5 MHz, IMB, Universiti Malaysia Terengganu) and was confirmed by two dimensional NMR (2D NMR) spectra such as COSY, HMQC or HSQC and HMBC as well as comparisons with the literature. A pure sample was dissolved in 500 µL of chloroform-*d* (Sigma-Aldrich, Malaysia) and transferred into 5 mm Norell NMR tube (Sigma-Aldrich, USA). The spectra were then processed with MestReNova-9.0 (MNova) 2.8 (Mestrelab Research, S.L, Santiago de Compostela, Spain).

Plant materials:

The twigs of *Avicennia officinalis* were collected from Kuala Selangor Nature Park, Selangor, Malaysia. The specimen was deposited in the Universiti Malaysia Terengganu herbarium with the voucher specimen code UMT-2.

Extraction and Fractionation*:*

The dried powdered twigs (2 kg) of *A. officinalis* was macerated in methanol overnight (8 L/extraction, 3x) and the methanol extract was concentrated under vacuum using a rotary evaporator (Büchi, Switzerland), yielding 30.5428 g. The methanol extract was partitioned by liquid-liquid extraction (90%H_2O + 10%MeOH: EtOAc, 1:1, 3x) and concentrated using a rotary evaporator to give a crude ethyl acetate extract weighing 10.9758 g. Thin layer chromatography (TLC) silica gel 60 F_{254} (Merck KGaA, Germany) was carried on the ethyl acetate extract of *A. officinalis*. The ethyl acetate crude extract was dissolved in ethyl acetate and mixed with 20 g of silica gel 60 GF234 (70-230 mesh ASTM). The mixture was completely dried using rotatory evaporator prior to fractionation work.

Chromatography techniques of Compounds Fractionation and Purification:

The total crude extract was dissolved in methanol, mixed with silica gel 60 (70-230 mesh ASTM) (Merck, art 9385) then fractionated by vacuum liquid chromatography (VLC) through stack-gradient elution commencing with 100% hexane to 100% ethyl acetate, followed by 100% ethyl acetate to 30% ethyl acetate and 70% methanol. A glass chamber with dimensions of (d=100.5, h=100) mm was packed using silica gel 60 GF_{254} (Merck, art. 7747) and silica particle size of 20-45 µm was used. The fraction collection volume was

collected yielding 24 fractions. Each sub-fraction was concentrated using a vacuum rotary evaporator and TLC was carried out to monitor the separation. Sub-fractions with the similar profile were pooled together to give 13 major fractions. The fractions were tested for antibacterial and antioxidant activity and further isolation work was carried out on the active fractions using radial chromatography. The glass plate of radial chromatography was coated with silica gel 60 PF_{254} gypsum (Merck, art. 7749) to isolate and purify the active metabolites. Compound **1** (8.8 mg, 0.08%) and **2** (10.8 mg, 0.10%) were obtained after purification on fractions F7 and F8, respectively.

Determination of Antibacterial Activity:

Preparation of nutrient agar and Mueller-Hinton agar:

28 g of nutrient agar powder was weighed and added into 1 L of distilled water; 35 g of Mueller-Hinton agar powder was added into 1 L of distilled water. Both media was then heated and autoclaved at 121 ºC for 20 minutes. The screw cap was left loosely for the autoclave. About 20 mL of agar medium was poured into each plate and let to be cool. The agar plates were sealed with parafilm and stored upside-down in the refrigerator for further use. Mueller-Hinton agar plates must be used within a week.

Bacteria culture:

Six Gram-positive bacteria- *Staphylococcus epidermidis, S. aureus, Bacillus subtilis* and Gram-negative bacteria- *Vibrio cholera, Enterobacter cloacae, Escherichia coli* (from glycerol stock) were obtained from Microbiology Laboratory, School of Fundamental Science, Universiti Malaysia Terengganu. All bacteria were clinically isolated at the Pathology Department, Sultanah Nur Zahirah Hospital, Kuala Terengganu, Terengganu, Malaysia. Each bacterium was streaked using inoculation loop on a labeled agar plate and incubated for more than 20 hours. A single colony of bacterium was selected and streaked using inoculation loop to another agar plate for the sub-culture of bacteria.

Agar well diffusion and minimum inhibitory concentration (MIC):

An antibacterial activity of ethyl acetate crude extract from the twigs of *A. officinalis* was conducted using agar well-diffusion method as described (Balouiri et al. 2016). Prior to assay, wells on Mueller Hinton agar plate were created using 8 mm sterile cork borer. Then, six bacteria- *S. epidermidis, S. aureus, B. subtilis, V. cholera, E. cloacae* and *E. coli* which were first adjusted of their concentration at optical density (O.D) 0.5 were swabbed on the

Mueller-Hinton agar plates. A series of eight concentrations of each selected fractions diluted in DMSO was prepared with 2-fold dilutions ranging from 5 mg/mL to 0 mg/mL. 50 µL of the serial dilutions was pipetted into the agar well accordingly. Oxytetracycline (OT) antibiotic disc (30 µg) (CT0041B, Thermo Scientific Oxoid, United Kingdom) was placed in the middle of the agar plate as a positive control. All plates were incubated for 24 hours at 37 ^{o}C to allow the test extract to diffuse into the agar medium. Three replicates were done for each fraction to obtain average values. Then, the inhibition zone diameter (IZD) was measured to the nearest millimeters. Minimum inhibitory concentration (MIC) was taken as the lowest concentration of the fraction that shows the inhibition zone.

Determination of Antioxidant Activity:

The antioxidant assay was conducted to evaluate the potential of selected fractions of *A. officinalis* according to method described by Suvik and Effendy (2016). Approximately 2.3659 mg of DPPH powder (D9132, Sigma Aldrich, United States) was weighed and dissolved in 100 mL of 100% methanol to obtain a stock solution. A series of eight concentrations of fractions F5-F10 was diluted in DMSO with 2-fold dilutions ranging from 0-1.0 mg/mL. 20 µL of each serial dilution was pipetted into respective wells of the 96-well plate and the 0 mg/mL dilution was used as a blank. 200 µL of DPPH solution was added to each well containing the fractions, except the blank. The plate was incubated in dark at room temperature for 30 minutes. Then, the absorbance was read at 520 nm using a multiwell scanning spectrophotometer (Variouskan, USA). Quercetin was used as positive control. The concentrations of fractions require to achieve 50% inhibition concentration of DPPH radical (IC_{50}) was determined from the linear regression curve. All determinations were performed in triplicates to obtain a mean value. The radical scavenging activity will be calculated by the following formula:

$$\text{Inhibition percentage (IP)} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100\%$$

Where A_0 = Absorption of blank sample A_1 = Absorption of tested fractions mixed with DPPH

Statistical Analysis:

All data were presented as mean $\pm$ SD and statistically analyzed with One-Way ANOVA in

the comparison between selected fractions using statistical analyses software PRISM Ver. 5. Data are significantly different at $p < 0.05$.

Table S1. Average inhibition zone diameter (IZD, mm) ± S.D. of fractions F5-F10 from the twigs of *A. officinalis* a different concentration values of 0-5 mg/mL against Gram-positive bacteria.

Fractions	Concentration values (mg/mL)								OT	MIC
	5.00	**2.50**	**1.25**	**0.625**	**0.312**	**0.156**	**0.078**	**0**		
	Staphylococcus epidermidis									
5	20.3 ± 0.6	16.7 ± 0.6	12.3 ± 0.6	10.0 ± 0	0.0	0.0	0.0	0.0	25.7 ± 1.2	0.625
6	23.0 ± 1.0	19.0 ± 1.0	15.7 ± 0.6	11.3 ± 1.5	0.0	0.0	0.0	0.0	27.3 ± 0.6	0.625
7	21.3 ± 0.6	18.3 ± 1.5	15.7 ± 2.5	13.0 ± 3.0	11.3 ± 1.5	0.0	0.0	0.0	27.0 ± 0	0.312
8	24.7 ± 0.6	21.7 ± 1.5	19.0 ± 1.0	16.0 ± 1.0	11.3 ± 1.5	0.0	0.0	0.0	27.7 ± 0.6	0.312
9	21.3 ± 1.2	19.3 ± 0.6	17.3 ± 0.6	15.0 ± 1.0	13.3 ± 1.2	10.0 ± 0	0.0	0.0	26.3 ± 2.1	0.156
10	20.0 ± 1.0	17.7 ± 1.2	15.7 ± 1.2	13.0 ± 0	10.7 ± 0.6	0.0	0.0	0.0	27.0 ± 1.0	0.312
	Bacillus subtilis									
5	18.0 ± 2.0	14.0 ± 1,0	10.3 ± 0.6	0.0	0.0	0.0	0.0	0.0	26.0 ± 0	1.25
6	17.7 ± 1.5	14.3 ± 2.3	12.7 ± 2.1	10.7 ± 1.2	0.0	0.0	0.0	0.0	26.0 ± 0	0.625
7	17.0 ± 2.6	15.3 ± 2.3	11.7 ± 2.9	0.0	0.0	0.0	0.0	0.0	26.3 ±0.6	1.25
8	20.7 ± 0.6	18.0 ± 0	15.7 ± 0.6	13.3 ± 1.2	12.0 ± 1.0	10.3 ± 0.6	0.0	0.0	26.0 ± 1.0	0.156
9	17.0 ± 2.0	15.0 ± 2.0	13.3 ± 2.1	11.3 ± 1.5	0.0	0.0	0.0	0.0	26.7 ± 0.6	0.625
10	16.3 ± 1.2	13.7 ± 1.2	12.3 ± 0.6	10.7 ± 0.6	0.0	0.0	0.0	0.0	26.7 ± 1.0	0.625
	S. aureus									
5	15.7 ± 1.2	12.3 ± 0.6	10.0 ± 0	0.0	0.0	0.0	0.0	0.0	32.3 ± 1.2	1.25
6	11.0 ± 1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	32.0 ± 1.0	5.00
7	21.3 ± 0.6	15.7 ± 1.2	13.0 ± 1.0	10.0 ± 1.0	0.0	0.0	0.0	0.0	29.7 ± 0.6	0.625
8	17.7 ± 2.1	13.3 ± 1.5	0.0	0.0	0.0	0.0	0.0	0.0	32.0 ± 1.0	2.50
9	12.3 ± 0.6	10.3 ± 0.6	0.0	0.0	0.0	0.0	0.0	0.0	32.0 ± 1.7	2.50
10	10.3 ± 0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	31.7 ± 0	5.00

Note: The antibacterial assay was done in triplicates using agar well-diffusion technique and oxytetracycline (OT) antibiotic disc (30 µg) used as positive control. The minimum inhibitory concentration (MIC) was determined in each assay plate.

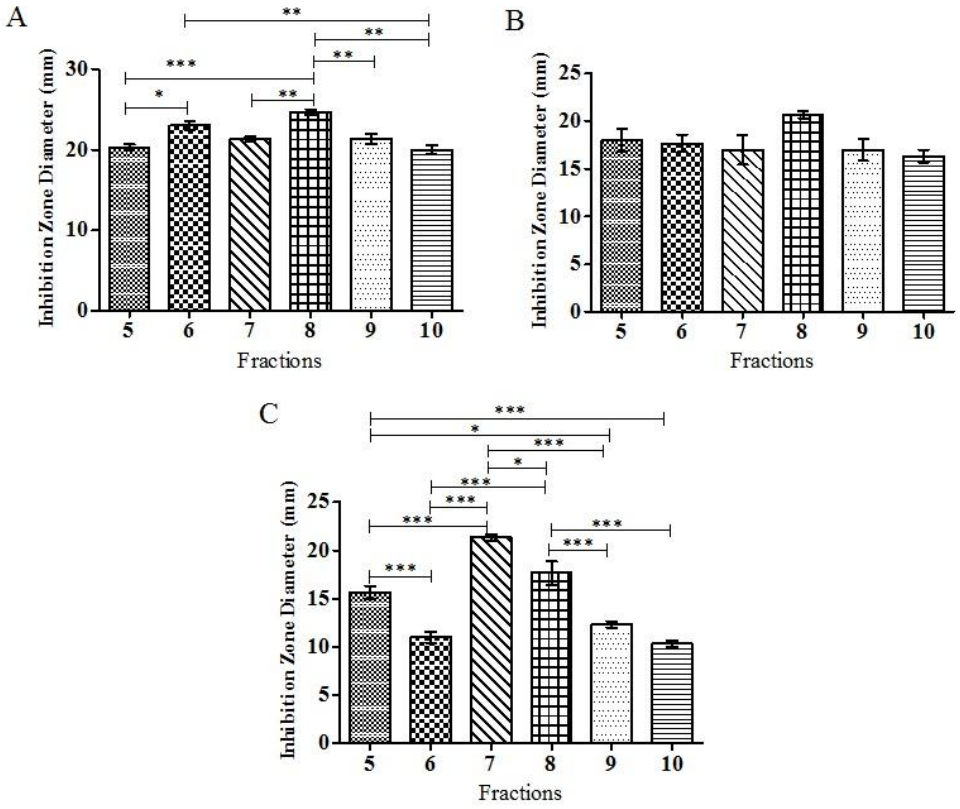

Figure S1. Antibacterial activity of fractions F5-F10 from the twigs of *A. officinalis* on the Gram-positive bacteria; (A) *S. epidermidis*; (B) *B. subtilis*; (C) *S. aureus* at 5 mg/mL. Data are represented as mean of inhibition diameter zone (IZD) ± S.D (n=3).

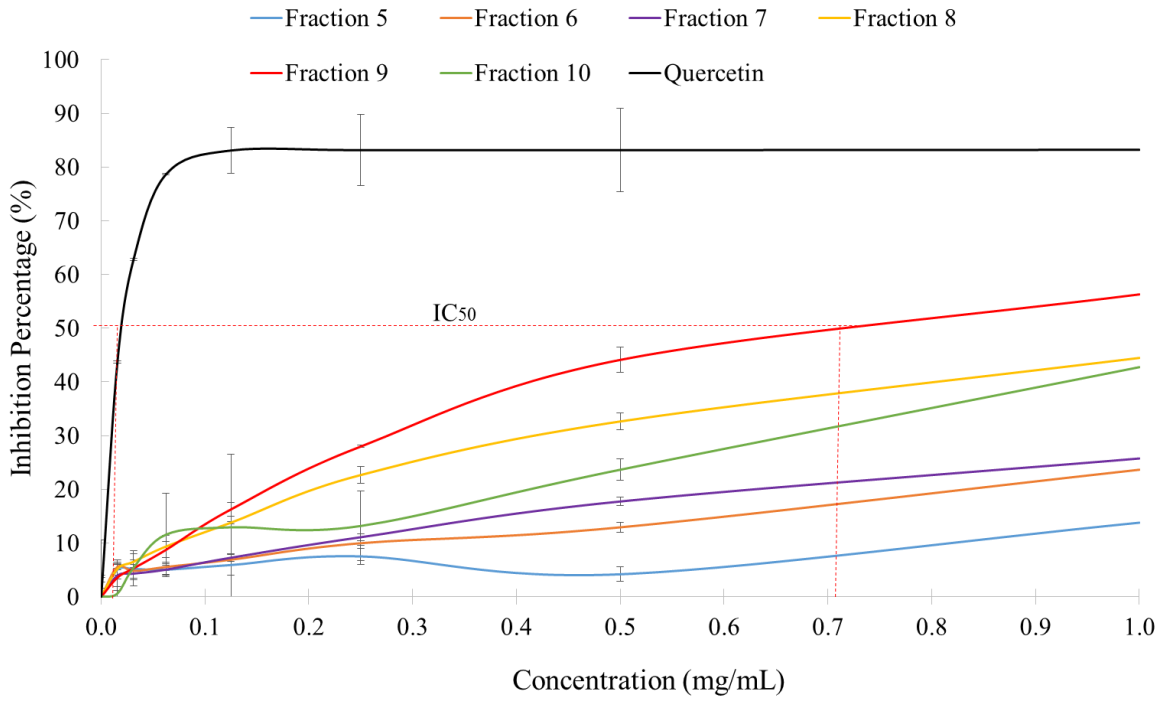

Figure S2. Antioxidant activity of fractions F5-F10 from the twigs of *A. officinalis* with different concentration values of 0-1.0 mg/mL. The antioxidant activity was done in triplicates using DPPH free radical scavenging assay.

References

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