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Antimicrobial Flavonoids from *Artocarpus anisophyllus* Miq. *and Artocarpus lowii* King

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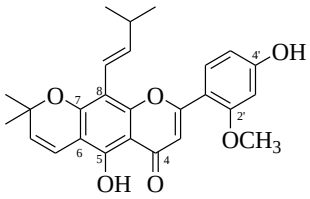
Graphical abstract



Abstract

Antimicrobial activities of flavonoids isolated from the leaves and heartwoods of *Artocarpus anisophyllus* Miq. and *Artocarpus lowii* King were evaluated. Gram positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*), Gram negative bacteria (*Escherichia coli* and *Pseudomonas putida*) and fungi (*Candida albicans* and *Candida glabrata*) were used in this study. Disc diffusion method was used as the qualitative assay while minimum inhibitory concentration and minimum microbicidal concentration methods were used as the quantitative assays. Artocarpin (8) showed strong antimicrobial activity towards all bacteria with inhibition zone diameter more than 11 mm and minimum microbicidal concentration value of 0.45 mg/mL. Isobavachalcone (3) exhibited strong antibacterial activity towards Gram positive bacteria with minimum microbicidal concentration value of 0.45 mg/mL. The findings of this study revealed that the isolated flavonoids from *A. anisophyllus* and *A. lowii* have potential to be developed as antimicrobial agents.

Keywords: Antimicrobial activity; flavonoids; *Artocarpus anisophyllus*; *Artocarpus lowii*

Abstrak

Aktiviti antimikrob sebatian flavonoid yang telah diasingkan daripada daun dan batang pokok *Artocarpus lowii* King dan *Artocarpus anisophyllus* Miq. telah dianalisa. Bakteria Gram positif (*Staphylococcus aureus*, *Bacillus cereus*), Bakteria Gram negatif (*Escherichia coli* dan *Pseudomonas putida*) dan kulat (*Candida albicans* dan *Candida glabrata*) telah digunakan dalam kajian ini. Kaedah penyebaran cakera telah digunakan sebagai analisa kualitatif manakala kepekatan minimum perencatan dan kepekatan minimum mikrobisidal digunakan sebagai ujian kuantitatif. Artocarpin (8) menunjukkan keputusan antimikrob yang kuat terhadap semua jenis bakteria dengan zon perencatan lebih daripada 11 mm dan nilai kepekatan minimum mikrobisidal adalah 0.45 mg/mL. Isobavachalkon (3) juga menunjukkan aktiviti yang kuat terhadap bakteria Gram positif dengan nilai kepekatan minimum mikrobisidal, 0.45 mg/mL. Keputusan ujikaji ini menunjukkan sebatian flavonoid yang telah diasingkan daripada *A. anisophyllus* dan *A. lowii* mempunyai potensi untuk dimajukan sebagai agen antimikrob.

Kata kunci: Aktiviti antimikrob; flavonoid; *Artocarpus anisophyllus*; *Artocarpus lowii*

1.0 INTRODUCTION

Flavonoids are the major secondary metabolites that can be found in *Artocarpus* species. *A. anisophyllus* Miq. and *A. lowii* King are two rare *Artocarpus* species that can be found in lowland forest of Malaysia. *A. anisophyllus* is known as "keledang babi" while *A. lowii* is locally known as "miku". *Artocarpus* plants are rich with phenolic compounds especially prenylated flavonoids and pyranoflavonoids. These type of flavonoids also showed interesting bioactivities including antimicrobial, antioxidant, anti-inflammatory, antipoliferative and tyrosinase inhibitory activities [1-5].

Flavonoids having various functional groups, chiral centres and ring fusions might have unexpected cell penetration, absorption or solubility that cannot be explained which make the secondary metabolites connect better with the biological targets [6]. Therefore, antimicrobial screening is necessary to increase the knowledge on the types of substituent that can contribute to better activity. Several studies on structure activity relationship of the antimicrobial flavonoids from other genus had also been reported [7-11]. Ten flavonoids from *A. anisophyllus* and *A. lowii* were investigated for antimicrobial activity which had not been reported elsewhere since both *Artocarpus* species were first investigated by our research group.

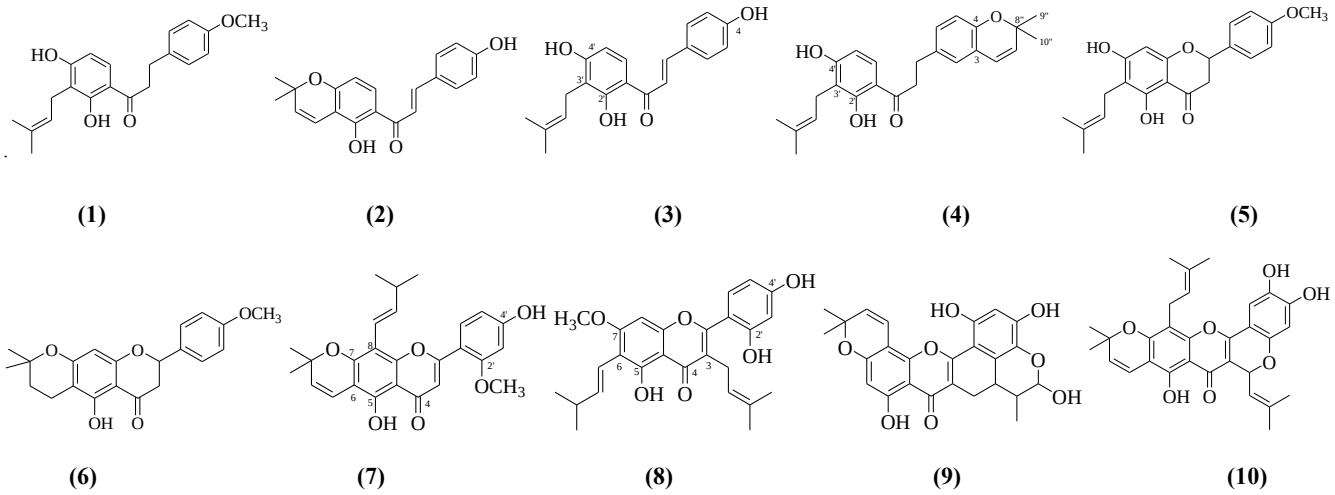


Figure 1 Flavonoids Isolated from *A. anisophyllus* Miq. and *A.lowii* King

2.0 EXPERIMENTAL

2.1 Plant Material

The leaves and heartwoods of *A. anisophyllus* were collected from Madek Kahang Forest located in Johor, Malaysia while the leaves and heartwoods of *A. lowii* were collected from Paka, Terengganu. Voucher specimen for *A. anisophyllus* (HTBP 1568) was deposited at Putrajaya Botanical Garden Herbarium while voucher specimen for *A. lowii* (AZ 7094) was deposited at Herbarium of Universiti Kebangsaan Malaysia, Bangi, Selangor.

2.2 Tested Samples

Six crude extracts of *A. anisophyllus* and *A. lowii* leaves i.e *n*-hexane crude extract (AALH, ALLH), dichloromethane crude extract *(*AALD, ALLD), ethyl acetate crude extract *(*AALE), methanol crude extract *(*ALLM) together with six crude extracts of *A. anisophyllus* and *A. lowii* heartwoods i.e *n*-hexane crude extract (AAHH, ALHH), dichloromethane crude extract *(*AAHD, ALHD), ethyl acetate crude extract *(*AAHE), methanol crude extract *(*ALHM) were tested. Ten flavonoids comprised of chalcones, flavanones, flavones and xanthones that were isolated from both *Artocarpus* species were also tested.

Figure 1 showed the structures of the isolated compounds which were spectroscopically elucidated as 2',4'-dihydroxy-4-methoxy-3'-prenyldihydrochalcone (1), 4-hydroxyonchocarpin (2), isobavachalcone (3), 2',4'-dihydroxy-3,4-(2",2"-dimethylchromeno)-3'-prenyldihydrochalcone (4), 5,7-dihydroxy-4'-methoxy-6-prenylflavanone (5), 5-hydroxy-6,7-(2,2-dimethylchromano)-4'-methoxyflavanone (6), 4',5-dihydroxy-6,7-(2,2-dimethylchromeno)-2'-methoxy-8-γ,γ-dimethylallylflavone (7), artocarpin (8), pyranocycloartobiloxanthone A (9) and cycloheterophyllin (10). Structural elucidation of (1), (2), (3), (5), (8), (9) and (10) had been reported previously [12-18] while structural elucidation of (4), (6) and (7) had not been published yet.

2.3 Microbial Strains

Four bacteria strains namely *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 29737), *Escherichia coli* (ATCC 10536), *Pseudomonas putida* (ATCC 49128) and two filamentous fungi known as *Candida albicans* (ATCC 10231) and *Candida glabrata* (ATCC 2001) were used for the antimicrobial assay.

2.4 Disc Diffusion Method

Stock solution of each flavonoid was prepared using DMSO to get 1.80 mg/mL concentration before experiment. Each disc was impregnated with 10 µL of the stock solution. Disc impregnated with DMSO served as negative control while disc with antibiotic streptomycin sulfate act as positive control for bacteria and nystatin for fungi. The discs were arranged on surface of agar plates that were previously streaked with bacterial suspension adjusted to 5% Mc Farland (150×10^6 colony/unit). The reading for inhibition zone around the disc was calculated after 24 hours incubation in millimetre (mm). Tested samples which did not show any inhibition zone were not tested for the MIC/MBC experiments.

2.5 Minimum Inhibitory Concentration (MIC)

MIC was determined using broth micro dilution method in sterilised 96-well microplates. All stock solutions were prepared using DMSO to get the final concentration 1.80 mg/mL. Sample stock solutions (100 µL) were added to wells in row A and B. Sterilised broth solution (100 µL) was then added to microwell from row B until H. Then the mixture of samples and sterilised broth at row B was transferred to each well in order to obtain a twofold serial dilution of the stock sample. The final concentrations of stock sample from A to H were 1.80, 0.90, 0.45, 0.22, 0.11, 0.06, 0.03 and 0.01 mg/mL respectively. Microbial suspensions (100 µL) in suitable growth medium were added to each well. The final volume in each well was 200 µL. Control wells were prepared with the same method using DMSO as the negative control. Streptomycin sulfate (SS) was used as positive control for bacteria while nystatin was used as positive control for fungi.

2.6 Minimum Microbicidal Concentration (MMC)

The samples that did not show any visible bacteria growth from MIC was taken (15 µL) and subcultured on the surface of nutrient agar. The sealed petri dishes were incubated for 16-20 hours. No

microbial growth observed on the agar after incubation time will be considered as the MMC.

3.0 RESULTS AND DISCUSSION

Six crude extracts as well as ten isolated flavonoids (**Figure 1)** from *A. lowii* King and *A. anisophyllus* Miq. were tested for their antimicrobial effect towards four bacteria strains namely *Basilus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas putida* and two fungi known as *Candida albicans* and *Candida glabrata*. Disc diffusion method was used as the qualitative analysis in this assay. Inhibition zone of all tested samples are given in **Table 1**. All flavonoids showed inhibitory activity towards selected bacteria and fungi. Most of the crude extracts except the leaves of *A. anisophyllus* crude extracts showed antibacterial activities with inhibition zone diameter in range of 7.5 - 12.2 mm while all crude extracts gave negative response towards fungi.

From **Table 1**, it is noticeable that the crude extracts have different modes of action and exhibited stronger biological activities against the Gram positive bacteria. Isobavachalcone (3), 4',5-dihydroxy-6,7-(2,2-dimethylchromeno)-2'-methoxy-8-γ,γ-dimethylallylflavone (7) and artocarpin (8) showed broad spectrum activity by inhibiting the growth of all bacteria and fungi with inhibition zone diameter in the range of 9.0 - 13.7 mm. 4-Hydroxyonchocarpin (2), 2',4'-dihydroxy-3,4(2",2"-dimethylchromeno)-3'-prenyldihydrochalcone (4), 5,7-dihydroxy-4'-methoxy-6-prenylflavanone (5), pyranocycloartobiloxanthone A (9) and cycloheterophyllin (10) showed inhibition activity only towards selected bacteria and fungi with inhibition zone between 7.25–13.5 mm. 2',4'-Dihydroxy-4-methoxy-3'-prenyldihydrochalcone (1) exhibited inhibition only towards bacteria with diameter of inhibition zone in the range of 9.0 – 10.75 mm while 5-hydroxy-6,7-(2,2-dimethylchromano)-4'-methoxyflavanone (6) showed no inhibition towards all microbes. Absence of inhibition zone did not necessarily mean that the compound was inactive, especially for less polar compound since they diffused more slowly into the medium culture [19]. Diffusion assay is not very suitable for natural antimicrobial compounds since their hydrophobic nature such as prenylated flavonoid prevent uniform diffusion through the agar media [20]. In order to confirm the results, all crude extracts and isolated compounds were tested for determination of their minimum inhibition concentration (MIC) and minimum microbicidal concentration (MMC). The MIC and MMC values of flavonoids tested are summarized in **Table 2**.

Marcal *et al*. (2010) had proposed classification for the antimicrobial activity of natural products as follows: strong inhibitors (< 0.1 mg/mL), moderate inhibitors (0.1-0.5 mg/mL), weak inhibitors (> 0.5-1.0 mg/mL) and inactive (> 1.0 mg/mL) [21]. **Table 2** shows that all crude extracts exhibited weak antimicrobial activities since all MMC values were equalled or more than 1.80 mg/mL. Artocarpin (8) showed moderate and broad antimicrobial activity with MMC value of 0.45 mg/mL towards all bacteria. Isobavachalcone (3) showed moderate antimicrobial activity towards Gram positive bacteria and *E. coli* (MMC value of 0.45 mg/mL) while 4-hydroxyonchocarpin (2) also showed moderate antimicrobial activity but only towards Gram positive bacteria with MMC value of 0.45 mg/mL.

Artocarpin (8) possessed hydroxyl groups at position C-2' and C-4' and two lipophilic groups at position C-3 and C-6. The presence of hydroxyl groups in flavonoids may contribute to the antimicrobial activity [8, 22]. The existence of two lipophilic groups may exhibit high affinity for proteins and acted as inhibitors of microbial enzymes by penetrating the cell membrane [23]. The lipophilic groups such as prenyl and dimethylallyl in flavonoids are also significant for antimicrobial activity [24-25]. Study conducted by Tsuchiya *et al.* (1996) showed that the presence of lipophilic group is also significant although the position is not important for antimicrobial activity [8]. Artocarpin (8) have two lipophilic groups compared to others and it showed higher potential in the antibacterial activity. The presence of lipophilic group is also the reason for isobavachalcone (3) to have higher activity compared to 4-hydroxyonchocarpin (2). In chalconoid type of structure, hydroxyl group at position C-4 in ring B is also an important requirement. When the hydroxyl group is methylated such as in 2',4'-dihydroxy-4-methoxy-3'-prenyldihydrochalcone (1), the antimicrobial activity decreased. The reduction of the double bond at α,β-position may also decrease the antimicrobial activity [10].

Table 1 Inhibition Zone of Crude Extracts and Isolated Flavonoids (1-10) Towards Bacteria and Fungi

Tested Samples	Gram positive bacteria		Gram negative bacteria		Fungi	
	B.s	*S.a*	*E.c*	*P.p*	*C.a*	*C.g*
AALH	ND	ND	ND	ND	ND	ND
AALD	ND	ND	ND	ND	ND	ND
AALE	ND	ND	ND	ND	ND	ND
AAHH	10.5 ± 0.80	8.2 ± 0.50	ND	ND	ND	ND
AAHD	9.5 ± 0.40	-	7.5 ± 1.50	8.25 ± 0.50	ND	ND
AAHE	10.0 ± 0.60	8.0 ± 0.50	11.0 ± 0.55	12.2 ± 2.62	ND	ND
ALLH	ND	ND	ND	ND	ND	ND
ALLD	9.1 ± 0.54	ND	8.3 ± 0.52	7.9 ± 0.64	ND	ND
ALLM	10.5 ± 0.45	8.9 ± 0.58	ND	ND	ND	ND
ALHH	ND	ND	ND	ND	ND	ND
ALHD	10.4 ± 0.52	7.0 ± 0.51	8.8 ± 0.46	7.5 ± 0.54	ND	ND
ALHM	ND	9.5 ± 0.42	ND	7.0 ± 0.51	ND	ND
(1)	9.25 ± 0.35	9.75 ± 2.06	10.75 ± 1.5	9.0 ± 1.41	ND	ND
(2)	ND	7.5 ± 0.48	ND	ND	ND	ND
(3)	10.1 ± 0.45	8.2 ± 0.56	9.8 ± 0.65	8.8 ± 0.52	9.0 ± 1.00	9.5 ± 1.00
(4)	ND	7.5 ± 0.57	7.25 ± 0.50	ND	ND	ND
(5)	ND	ND	9.0 ± 0.50	9.25 ± 0.50	ND	ND
(6)	ND	ND	ND	ND	ND	ND
(7)	8.2 ± 0.50	9.0 ± 0.15	8.2 ± 0.50	13.7 ± 2.98	10.7 ± 0.5	12.2 ± 3.2
(8)	11.2 ± 2.88	12.5 ±0.50	12.0 ± 2.16	13.7 ±0.60	9.5 ± 2.12	11.0 ± 2.8
(9)	13.5 ±0.55	11.5 ±0.60	ND	ND	12.5 ± 0.70	9.0 ± 1.0
(10)	10.5 ± 0.55	ND	ND	ND	11.6 ± 0.55	10.0 ± 0.0
SS	12.0 ± 0.66	15.8 ± 3.52	17.3 ± 2.94	14.1 ± 3.47	NT	NT
Nystatin	NT	NT	NT	NT	17.0 ± 1.41	18.6 ± 0.5

*All values are expressed in milimeter (mm) *NT-not tested *ND-not detected

Table 2 MIC and MMC values (mg/mL) of Crude Extracts and Flavonoids towards Bacteria and Fungi

Tested Samples	Gram positive bacteria				Gram negative bacteria				Fungi			
	B.s		*S.a*		*E.c*		*P.p*		*C.a*		*C.g*	
	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
AAHH	0.90	1.80	0.90	1.80	1.80	1.80	1.80	1.80	NT	NT	NT	NT
AAHD	0.90	1.80	0.45	1.80	0.90	1.80	0.45	1.80	NT	NT	NT	NT
AAHE	1.80	1.80	1.80	1.80	1.80	1.80	1.80	>1.80	NT	NT	NT	NT
ALLD	0.90	1.80	0.45	1.80	0.90	1.80	0.45	1.80	NT	NT	NT	NT
ALLM	0.90	1.80	0.90	1.80	1.80	1.80	1.80	>1.80	NT	NT	NT	NT
ALHD	0.90	1.80	0.45	1.80	0.90	1.80	0.45	1.80	NT	NT	NT	NT
ALHM	0.90	1.80	0.90	1.80	0.90	1.80	0.90	>1.80	NT	NT	NT	NT
(1)	0.90	1.80	0.45	0.90	1.80	1.80	1.80	1.80	NT	NT	NT	NT
(2)	0.45	0.45	0.45	0.45	0.90	0.90	0.90	0.90	>1.80	>1.80	>1.80	>1.80
(3)	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.90	1.80	1.80	1.80	1.80
(4)	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	NT	NT	NT	NT
(5)	1.80	1.80	1.80	1.80	0.90	0.90	0.90	1.80	1.80	>1.80	1.80	>1.80
(6)	1.80	>1.80	1.80	>1.80	1.80	>1.80	1.80	1.80	NT	NT	NT	NT
(7)	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	1.80	1.80	1.80	1.80
(8)	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	1.80	1.80	1.80	1.80
(9)	0.45	0.90	0.45	1.80	0.90	1.80	0.90	1.80	1.80	1.80	1.80	1.80
(10)	0.90	1.80	0.90	0.90	0.90	1.80	0.90	0.90	1.80	1.80	1.80	1.80
SS	0.0146	0.0146	0.0146	0.0146	0.0146	0.0146	0.0146	0.0146	NT	NT	NT	NT
Nystatin	NT	NT	NT	NT	NT	NT	NT	NT	0.0146	0.0146	0.0146	0.0146

*The values are expressed in mg/mL *NT-not tested

■4.0 CONCLUSION

Artocarpin (8) isolated from both *A. anisophyllus* and *A. lowii* was found to be moderately active towards all tested bacteria with MMC value of 0.45 mg/mL. Hydroxylated and prenylated flavonoids with MMC value of 0.45 mg/mL isolated from *A. anisophyllus* and *A. lowii* have the potential to be developed as an antimicrobial agents.

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