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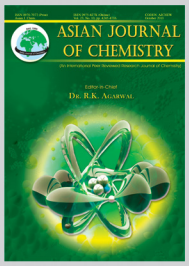

Free Radical Scavenging Effect of *Artocarpus kemando* and *Artocarpus odoratissimus*: Structure-Activity Relationship of Flavonoid Derivatives

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Antioxidant activities of extracts from *Artocarpus kemando* and *Artocarpus odoratissimus* and their pure chemical constituents (**1-5**) were investigated for their DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activities. The reduction of DPPH by the extracts from *Artocarpus kemando* gave promising results in the assays indicating that the extracts of *Artocarpus kemando* have high ability to act as free radical scavengers. Two compounds which are artosimmin and artomandin gave good activities while cycloartobiloxanthone indicated moderate activity. Hence, *Artocarpus kemando* may be considered a good source of natural antioxidant. However, all the extracts of *Artocarpus odoratissimus* exhibited weak inhibition activity (IC_{50} > 120 µg/mL) against DPPH. This paper describes the structure-activity relationships of the compounds tested.

Key Words: *Artocarpus kemando*, *Artocarpus odoratissimus*, Antioxidant, Free radical, Structure-activity, Flavonoids.

INTRODUCTION

Flavonoids are natural polyphenolic compounds in plants and are important for human health[1]. The different level of oxidation at the C ring of the basic benzopyrone structure differentiates the flavonoids among their various classes[2]. Energy produced due to the transfer of unpaired electrons between atoms hence producing free radicals are harmful to human cells[3]. To maintain a healthy life, inhibition of free radical activity is necessary. Due to the rejection of some synthetically produced antioxidants (BHA and BHT) by consumers, the demand for plant-originated natural antioxidants which are more stable and have better antioxidant properties are needed[4]. Artocarpus species have been reported to have a wide range of pharmacological activities[5-11]. This research concentrates on the antioxidant potential of the extracts of *Artocarpus kemando* and *Artocarpus odoratissimus*. Structure-activity relationships of cycloartobiloxanthone (**1**), artoindonesianin C (**2**), artomandin (**3**), artonol B (4) and artosimmin (**5**) isolated from the stem bark of these two plant species are reported here.

EXPERIMENTAL

The stem bark of *Artocarpus kemando* and *Artocarpus odoratissimus* were collected in May 2007 from Sri Aman, Sarawak, Malaysia. The specimens was identified by Dr. Rusea Go from Department Biology, Faculty of Science, UPM, Serdang, Selangor in Malaysia.

IR spectra were recorded on either potassium bromide (KBr) disks on a Perkin-Elmer Fourier Transform Infrared (FT-IR) Spectrum BX or Perkin-Elmer 100 series spetrophotometer. UV spectra were recorded on a Shimadzu UV-160A, UV-visible recording spectrophotometer. Mass spectral data were obtained using either a Shimadzu GCMS-QP5050A spectrometer or Kent Mass Spectrometry Service, UK, using EI and CI modes. NMR spectra were recorded with a JOEL JNM EX-400 FTNMR spectrometer, operating at 399.7 MHz (^{1}H, COSY, HMQC and HMBC) and 100.4 MHz (^{13}C, DEPT), respectively. $CDCl_3$, CD_3COCD_3 or CD_3OD were used as solvent. The absorbance in DPPH free radical scavenging of antioxidant assay were measured by using ELISA microplate reader (µQuant, Bio-Tek Instrument USA).

Extraction and isolation: The ground, air-dried stem bark (4.7 kg) of *Artocarpus kemando* was defatted with *n*-hexane and extracted exhaustively using ethanol, acetone and methanol at room temperature for more than 48 h. This yielded 23.3 g hexane, 50.2 g ethanol, 98.6 g acetone and 198.5 g methanol extracts. The methanol extract was dissolved in a mixture of water- acetone (1:3, 500 mL) and the soluble portion partitioned using chloroform (3 × 400 mL each time) to afford a chloroform (20.0 g) extract. Meanwhile, solvent extractions on the dried powdered stem bark of *Artocarpus odoratissimus* (3.5 kg) gave hexane (6 g), chloroform (15 g), ethyl acetate (42.5 g) and ethanol (12.8 g) extracts. The ethanol extract of *Artocarpus kemando* was chromatographed on a silica gel vacuum column chromatography using a stepwise gradient system (hexane/chloroform, chloroform/ ethyl acetate, ethyl acetate/ acetone and methanol) to give 20 fractions. Fractions with similar profile on TLC were combined to give six major fractions. Repeated column chromatography on fraction **4** (2.57 g) afforded cycloartobiloxanthone (**1**). Silica gel column chromatography followed by repeated radial chromatography of fraction **5** gave artoindonesianin C (**2**). Similar methods of purification on the acetone extract (45.0 g) of *Artocarpus kemando* gave cycloartobiloxanthone A (**3**). Radial chromatography purification on the chloroform extract (20 g) of the same plant afforded artonol B (**4**) while the ethyl acetate extract (42.5 g) of *Artocarpus odoratissimus* gave artosimmin (**5**).

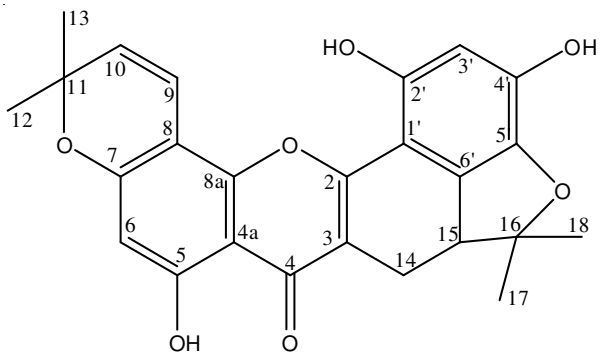
Cycloartobiloxanthone (1): Dark yellow solid; m.p. 283-285 ºC (Lit. 285-287 ºC[12]); UV (MeOH) λ_{max} (log ε) nm: 229 (4.31), 281 (4.31), 392 (4.02); IR (KBr, ν_{max}, cm^{-1}): 3434 (OH br), 2976, 2932 (C-H stretching), 1650 (C=O chelating), 1560, 1476 (C=C aromatic), 1358 (CH_3 alkane bending), 1272, 1160 (C-O); EIMS m/z (rel. int.): 434 (28) [M^+, $C_{25}H_{22}O_7$], 420 (27), 419 (100), 417 (3), 391 (3), 377 (7), 363 (1), 361 (2), 347 (4), 337 (1), 331 (1), 293 (1), 203 (6), 201, (3), 174 (3); FABMS m/z 435 [$(M + H)^+$, $C_{25}H_{23}O_7$]. ^{1}H NMR (CD_3COCD_3, 400 MHz) and ^{13}C NMR (CD_3COCD_3, 100 MHz): spectral data are in agreement with published data[12].

Artoindonesianin C (2): Yellow solid; m.p. 210-212 ºC (Lit. 209-211 ºC[13]); UV (MeOH) λ_{max} (log ε) nm: 232 (3.58), 267 (3.53), 396 (3.09); IR (KBr, ν_{max}, cm^{-1}): 3520 (OH), 2922, 2853 (C-H stretching), 1727 (C=O ester), 1646 (C=O chelating), 1591 (C=O cyclic), 1473 (C=C aromatic), 1359 (CH_3 alkane bending), 1270, 1168, 1108, 1107 (C-O br); EIMS m/z (rel. int.): 462 (18) [M^+, $C_{26}H_{22}O_8$], 449 (5), 448 (24), 447 (100), 388(6), 387 (28). ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz): spectral data are in agreement with published data[13].

Artomandin (3): Yellow solid; m.p. 288-290 ºC; UV (MeOH) λ_{max} (log ε) nm: 206 (3.89), 230 (3.91), 283 (3.93), 393 (3.64); IR (KBr, ν_{max}, cm^{-1}): 3351 (OH br), 2916, 2849 (C-H stretching), 1646 (C=O chelating), 1556, 1465 (C=C aromatic), 1348 (CH3 alkane bending), 1272, 1159, 1017 (C-O); EIMS m/z (rel. int.): 434 (28) [M^+, $C_{25}H_{22}O_7$], 420 (27), 419 (100), 417 (3), 391 (3), 377 (7), 363 (1), 361 (2), 347 (4), 337 (1), 331 (1), 293 (1), 203 (7), 201, (7), 174 (4). ^{1}H NMR ($CDCl_3$ + CD_3OD, 400 MHz): δ 13.33 (1H, s, 5-OH), δ 6.89 (1H, d, *J* = 9.6 Hz, H-9), δ 6.26 (1H, s, H-2'), δ 6.23 (1H, s, H-6), δ 5.58 (1H, d, *J* = 9.6 Hz, H-10), δ 3.40 (1H, dd, *J* = 15.6, 7.3 Hz, H-15), δ 2.40 (1H, t, *J* = 15.6 Hz, H-14b), δ 3.19 (1H, dd, *J* = 15.6, 7.3 Hz, H-14a), δ 1.67 (3H, s, H-18), δ 1.48 (3H, s, H-13), δ 1.47 (3H, s, H-12), δ 1.35 (3H, s, H-17); ^{13}C NMR ($CDCl_3$ + CD_3OD, 100 MHz): δ 180.7 (C, C-4), δ 161.4 (C, C-5), δ 160.7 (C, C-2), δ 158.9 (C, C-7), δ 151.1 (C, C-8a), δ 150.3 (C, C-4'), δ 146.0 (C, C-3'), δ 136.9 (C, C-5'), δ 132.3 (C, C-6'), δ 127.4 (CH, C-10), δ 114.9 (CH, C-9), δ 111.8 (C, C-3), δ 104.7 (C, C-4a), δ 104.2 (CH, C-2'), δ 103.8 (C, C-1'), δ 101.3 (C, C-8), δ 100.0 (CH, C-6), δ 93.9 (C, C-16), δ 78.0 (C, C-11), δ 46.6 (CH, C-15), δ 28.1 (2 × CH_3, C-12 and C-13), δ 28.0 (CH_3, C-18), δ 22.6 (CH_3, C-17), δ 19.8 (CH_2, C-14).

Artonol B (4): Fine yellow solid; m.p. 189-194 ºC (Lit. 189-196 ºC[14] and 265-267 ºC[15]); UV (MeOH) λ_{max} (log ε) nm: 233 (2.39), 278 (2.35), 359 (2.24); IR (KBr, ν_{max}, cm^{-1}): 3423 (OH), 2969, 2921, 2851 (CH stretching), 1770 (C=O acetoxyl), 1717 (C=O cyclic), 1651 (conjugated C=O), 1606, 1581, 1479 (C=C aromatic), 1361, 1109 (C-O) ; EIMS m/z (rel. int.): 420 (14) [M^+, $C_{24}H_{20}O_7$], 406 (35), 405 (100), 334 (6), 195 (10), 146 (1), 105 (1). ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR (CD_3COCD_3, 100 MHz): spectral data are in agreement with published data[14].

Artosimmin (5): Yellow solid (95 % chloroform/5 % methanol); m.p. 213-215 ºC; UV (MeOH) λ_{max} (log ε) nm: 213 (4.16), 271 (3.72), 340 (3.42); IR (KBr, ν_{max}, cm^{-1}): 3527 (OH), 2853 (C-H stretching), 1734 (C=C unsaturated), 1653 (conjugated C=O), 1598, 1567, 1513, 1488, 1447 (C=C aromatic), 1306, 1239 (C-O); EIMS m/z (rel. int.): 436 (100) [M^+, $C_{25}H_{24}O_7$], 421 (40), 393 (15), 381 (78), 365 (20), 337 (12), 325 (62), 297 (6), 254 (5), 206 (4), 191 (12), 183 (2), 165 (4), 153 (19).^{1}H NMR (CD_3COCD_3, 400 MHz) and ^{13}C NMR (CD_3COCD_3, 100 MHz): spectral data see published data[15].



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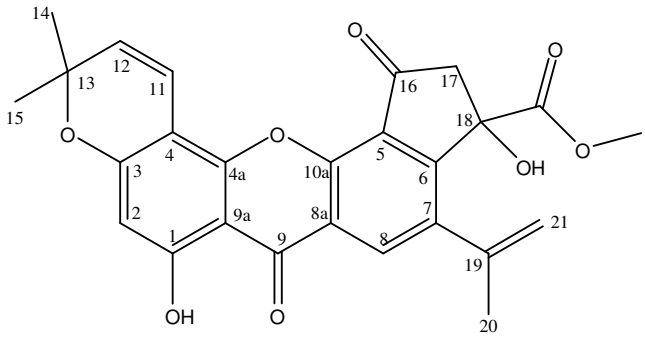

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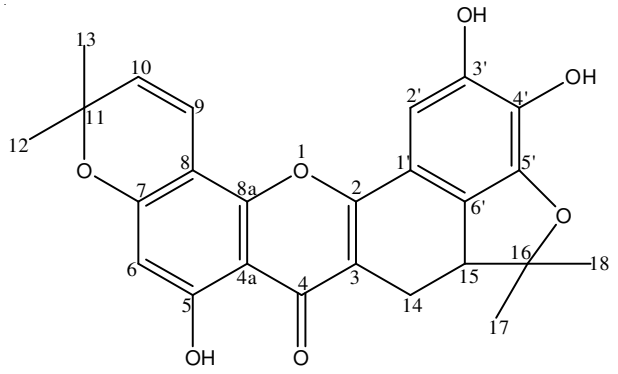

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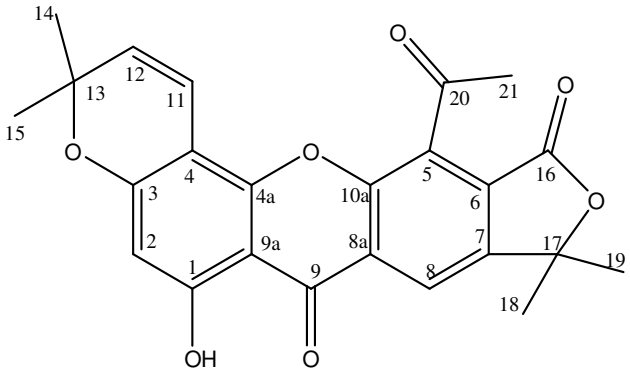

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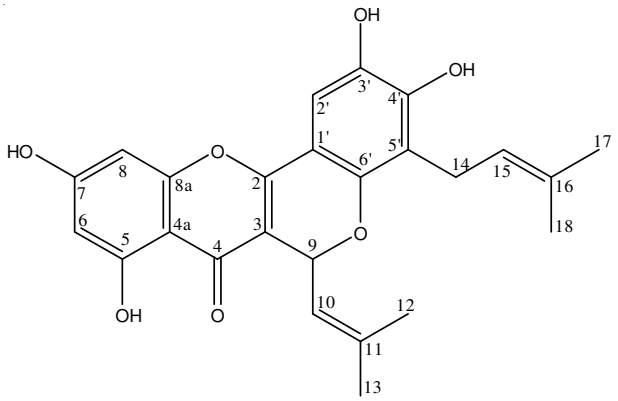

(**5**)

DPPH assay: The assay was performed according to the protocol obtained from a previous researcher with modifications by using a 96-well plate[16]. A solution of DPPH was prepared by dissolving 4 mg of DPPH (Sigma, USA) in 1 mL of methanol. The solution was kept in the dark at 4 ºC after shaking in a sonicator for 5 min. The sample was dissolved in CH_3OH to give 2 mg/mL stock solutions. Each stock solution was diluted two fold (series dilution) in micro centrifugal tubes to make seven sample solutions at concentrations 2, 4, 8, 15, 30, 60 and 120 µg/mL. The standard control used in this assay was vitamin C (Sigma, USA). The plate was wrapped with aluminium foil and kept at 37 ºC. The reaction was allowed to proceed for 0.5 h. The absorbance (OD) of each well was measured spectrophotometrically at 517 nm using an ELISA microplate reader. Each sample was assayed in triplicate. A dose response curve presented in a graph of inhibition rate *versus* concentration of samples was plotted to determine the IC_{50} value. The percentage inhibition rate of samples was calculated using the formula below:

$$\text{Inhibition rate} = \frac{\text{OD(DPPH)} - \text{OD(DPPH + Sample)}}{\text{OD(DPPH)}} \times 100\ \%$$

RESULTS AND DISCUSSION

It was observed that all the extracts of *Artocarpus odoratissimus* tested exhibited weak inhibition activity (IC_{50} > 120 µg/mL) against the DPPH radical. However, the reduction of DPPH by the various extracts from *Artocarpus kemando* gave promising results in the scavenging assay. The chloroform, ethanol and methanol extracts revealed very strong antioxidant ability in the free radical DPPH tests with extremely low IC_{50} values of 27.1, 19.2 and 23.3 µg/mL, respectively. Meanwhile, the acetone extract showed only moderate activity with an IC_{50} value of 52.2 µg/mL. This test which gave such promising results for the antioxidant inhibition capacity indicates that all the extracts of *Artocarpus kemando* have high ability to act as a free radical scavenger implying the stem bark extract to have a profound antioxidant activity. Hence, the results obtained *in vitro* experiments clearly suggest that this Artocarpus species is a natural source of antioxidants which could serve as a nutraceutical with potential applications in reducing the level of oxidative stress and produce related health benefits.

The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms (C6-C3-C6) arranged in three rings labeled A, B and C. The various classes of tricyclic flavonoid skeleton with dissimilar pattern of substitution at the A, B and C rings give different levels of oxidation. Table-2 summarizes the percentage inhibition rate (%) and scavenging concentration (µg/mL) to obtain 50 % of the maximum scavenging capacity of DPPH by several compounds from the two plants. No compound showed inhibition values which are comparable to the IC_{50} value of the positive control. However, compounds such as cycloartobiloxanthone (**1**), artomandin (**3**) and artosimmin (**5**) which consist of 2',4'-dioxygenated and 3',4'-dioxygenated rings scavenged the free radical of DPPH with inhibition rates more than 50 % at concentrations of 120 µg/mL each. Both artomandin (**3**) and artosimmin (**5**), exhibited the highest potential of scavenging effect towards the DPPH radical test with IC_{50} values of 38.0 and 32.1 µg/mL, respectively whereas, cycloartobiloxanthone (**1**) revealed a lower DPPH scavenging effect with an IC_{50} value of 87.2 µg/mL. These assay results indicate that both artomandin (**3**) and artosimmin (**5**) which are 3',4'-dioxygenated at the B-ring of the structure exhibited a stronger scavenging effect when compared to that of cycloartobiloxanthone (**1**) which has 2',4'-dioxygenated substitution. Hence, it is deduced that flavonoids with the presence of the 2',4'- and 3',4'-positions of free hydroxyl moieties at B-ring is inclined to exhibit a prominent degree of scavenging activity. Moreover, the only example of a 5'-(3-methylbut-2-enyl) pyranoflavone (**5**) type compound tested revealed a slightly higher activity than artomandin (**3**), which is without the prenyl unit at the structure. Thus, it is suggested that the 3-methylbut-2-enyl group is essential for outstanding antioxidant activity. However, artoindonesianin

TABLE-1
SCAVENGING EFFECT OF PLANT EXTRACTS ON DPPH RADICALS

Plants	Extracts	DPPH Scavenging Capacity	
		IC_{50} (μg/ mL^{-1})*	I (%)**
Artocarpus kemando	Chloroform	27.1	85.2
	Acetone	52.2	77.6
	Ethanol	19.2	88.5
	Methanol	23.3	84.5
Artocarpus odoratissimus	Chloroform	> 120	1.7
	Ethyl acetate	> 120	45.1
	Ethanol	> 120	24.1

*Concentration sufficient to obtain 50 % of the maximum scavenging antioxidant capacity. **Percentage of inhibition rate at 120 μg/mL as mean of triplicate analysis.

TABLE-2
SCAVENGING EFFECT OF **1-5** ON DPPH RADICALS

Compounds	DPPH scavenging capacity	
	IC_{50} (μg/mL^{-1})8	I (%)**
Cycloartobiloxanthone (**1**)	87.2 ± 7.3	69.9
Artoindonesianin C (**2**)	> 120	4.7
Artomandin (**3**)	38.0 ± 6.4	89.4
Artonol B (**4**)	> 120	6.2
Artosimmin (**5**)	32.1 ± 3.1	79.5
Vitamin C***	12.2	95.4

*Concentration sufficient to obtain 50 % of the maximum scavenging antioxidant capacity. **Percentage of inhibition rate at 120 μg mL^{-1} as mean of triplicate analysis. [3]Positive control.

C (2) and artonol B (4) gave weak scavenging activity with IC_{50} values of more than 90 μg/mL probably due to the modified and totally opened B-ring of the flavonoid.

Conclusion

The unsaturated 5C prenyl side chain (3-methylbut-2-enyl) at C-5' and the presence of the 2',4'- and 3',4'- positions (*ortho* or *meta*) of free hydroxyl moieties at B-ring of flavonoids derivative are highly needed for the strong free radical scavenging properties.

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