BIOACTIVE COMPOUNDS FROM *APAMA CORYMBOSA*

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ABSTRACT

Apama corymbosa (Griff)Willd. (Syn. *Thottea corymbosa* Griff) known as akar julong bukit, akar surai (Malay) is a shrub found in the forests of Malaysia and Sumatra. Preliminary studies on *Apama tomentosa* showed that the plant contained Aristolochic acid, Aristololactam, steroid and fatty acids. Aristolochic acid has been reported to increase phagocytosis of leucocytes and exhibit tumour inhibitory activity. The roots of *T.corymbosa* collected from Ayer Hitam Forest Reserve, Puchong were extracted by conventional method using petroleum ether,chloroform and methanol. In this paper we will discuss the isolation and purification of the toxic crude from chloroform extract. Brine Shrimp Lethallity Bioassay gave LC_{50}~200 ppm. Separation and isolation of the components were carried out using chromatographic methods(CC,PTLC,FCC,SPE). Structural identifications were achieved by spectroscopic methods i.e. GC-MS,IR,UV,^{1}H- and ^{13}C-NMR. As a result three major known compounds isolated were identified as Ariskanin-A, Ariskanin-D and Cepharadione-A.

INTRODUCTION

Apama corymbosa is a shrub found in the forests of Malaysia and Sumatra.In herbal medicinal practice, the pounded leaves are applied to the gums or to the tooth cavity in order to relieve toothaches. The root mixed together with *Myristica fragrans* nuts make a protective post-partum medicine. This plant can also be used as an analgesic , antiasthmatic preparation, antifertility, impotent and also for the treatment of snakebite. The pharmacological properties of several components from several *Aristolochia* species have motivated studies on two species of the related genus *Apama* of Malaysia.

In pharmacological studies of several Aristolochiaceae species from India and China they were found to contain bioactive compounds. The roots of *Aristolochia indica* Linn yielded phenanthrene derivatives (Pakrashi et al. 1977) and a sesquiterpene which posseses antifertility activity (Pakrashi and Shaha 1977) besides being known as a source of aristolochic acid(1a), a tumour-inhibitory principle. In China, *Aristolochia debilis* known as Qing Mu Xiang has a pungent and bitter taste and a cold property, acting on the lung and stomach channels. The decoction and extract of this herb has a direct constrictive action on blood vessels and inhibitory action on the heart (Wang 1983). Aristolochic acids A and C (Aristolochic IIIa), 9-hydroxyaristolochic acid I and 9-methoxyaristolochic acid I, aristolochic acids

II,IV,IVa, aristolochic acid methyl ester and debilic acid have been isolated from the root of *A. debilis*. Recently it was found that acid aristolochic I and II were reported to produce interstitial nephritis (Hashimoto et al. 1999). The alkaloids magnoflorine, cyclanoline, tentrandrine and allantonin were also isolated from the root part (Rucher et al. 1985).

Although *A.corymbosa* is widely used in herbal medicine locally, but to date no study on any biological properties has ever been reported.

MATERIALS AND METHODS

UV spectra were recorded with a Shimadzu UV-160 Spectrophotometer, in ethanol solution using 1 cm Quartz cells.

Mass spectra (MS) were obtained on Hewlett Packard 5989A spectrometer operating at 70 eV and attached to a VG-display digispec data acquisition system computer.

Proton and Carbon-13 Nuclear Magnetic Resonance(^{1}H and ^{13}C-NMR) spectra were measured in $CDCl_3$ on a JEOL FX 400(400 MHz) spectrometer and the chemical shifts are expressed as δ values(ppm) downfield from TMS as an internal standard.

For column chromatography either silica gel 60[230-400 Mesh ASTM(Merck) and 70-230 Mesh ASTM(Merck)]was used. Thin layer chromatography and preparative thin layer chromatography utilizes layers of silica gel 60 GF254 and silica gel 60 PF254, 0.25 mm and 0.50 mm thick respectively, spread on glass plates. Merck pre-coated silica gel GF254 plates were also used. TLC spots were visualised under ultravoilet light(254 nm and 366 nm) and by spraying the plates with 10% H_2SO_4 or Dragendorff reagent.

Sample preparation. The air-dried roots of *Apama corymbosa* voucher no. AZ6681 collected from Ayer Hitam Forest Reserve, Puchong were cut into pieces and ground to produce 1.35kg sample. A specimen was deposited in the Herbarium of UKM,Bangi (UKMB)

Extraction of sample. The sample was consecutively soaked in petroleum ether, chloroform and methanol and the extracts evaporated to give total crude extracts.

Phytochemical screening. 25g Air-dried powder of the root was extracted with methanol . Evaporation of the solvent yielded brown crude extract.The extract was tested for the presence of terpenoid,steroid and alkaloid.

Brine Shrimp Lethallity Bioassay. The BLSB has been developed(McLaughlin,1991) as a simple, fast and inexpensive bioassay in the search for bioactive compounds from plant extracts. Each fraction of the extract was tested for their toxicity against *Artemia salina* Leach and LC_{50} values were estimated using a simple computer program.
Extraction and Isolation of components. The chloroform extract(9.18g) was separated by dry flash column chromatography with silica gel 60(230-400 mesh) of MERCK and hexane ,ethylacetate and methanol used as solvents. The methanol fractions were combined and further fractionated by colum chromatography(silica gel

230-400 mesh) using hexane-ethylacetate as eluent. The fraction from hexane:ethylacetate(4:6) showed four major spots and three minor spots on Tlc (ACB3, 0.80g). This was again separated by micro- column chromatography resulting in three major components, isolated and labelled as AC3 (61.3 mg). AC4 (25 mg) and AC6 (25 mg), respectively together with four other components in trace quantities.

Ariskanin-A Yellow needles,AC3 (61.3mg). Tlc(silica gel) with solvent system hexane:EtOAc,8:2, Rf 0.9. UV λmax.(nm): 256,272,295,372. IR $\nu_{max.}$(cm^{-1}): 1714, 1584, 1513, 1448, 1342, 1235. MS m/z 341[M^+, $C_{18}H_{15}NO_6$)], 295 [$M-NO_2$]$^+$,280 [$M-NO_2$-Me]$^+$,264 [$M-NO_2$-OMe]$^+$, 252, 237, 222, 194, 179, 166, 150, 111, 98, 87, 69, 59.
^{1}H-NMR,δ(ppm): 9.66 (1H,d,H-5), 8.33 (1H,s,H-9), 7.97 (1H,dd,H-8),7.89 (1H,s,H-2), 7.80 (1H,tdH-6),7.70 (1H,td,H-7),4.10 (3H,s,3-OCH_3), 3.98 (3H,s,4-OCH_3), 3.88 (3H,s,$OCOCH_3$).

Ariskanin-D Yellow amorphous,AC4(25mg). Tlc(silica gel) with solvent system hexane:EtOAc,8:2, Rf 0.88. UV λmax(nm):257,308,399. MS m/z 357 [M^+,$C_{18}H_{15}NO_7$], 311,298 [M-CO] , 277, 260, 248, 219, 190, , 163, 138, 125, 95, 81, 55
^{1}H-NMR,δ(ppm): 8.99 (1H,d,J=8 Hz,H-5), 8.86 (1H,s,H-9),7.88 (1H,s,H-2), 7.75 (1H,t,H-6), 7.13 (1H,d,J=8 Hz,H-7) , 6.40 (1H,s,OH), 4.06 (3H,s,8-OMe), 3.88 (3H,s,4-OMe), 3.84 (3H,s,1-COOMe)
^{13}C-NMR, δ(ppm): 167.3 (C=O), 156.8 (C5), 148.3 (C9), 146.0 (C8), 145.7, 131.1 (C4), 130.9 (C10), 126.3, 126.0, 120.9, 120.2, 119.9, 118.9, 116.9, 107.7, 60.8 (4-OMe), 55.9 (8-OMe), 52.1 (OCOMe).

Cepharadione-A Orange needles, AC6 (25 mg). Tlc(silica gel) with solvent system EtOAc: MeOH,9:1, Rf 0.82. GC,t_R=45.87 min. MS m/z 305[M^+, $C_{18}H_{11}NO_4$], 277 [M- CO] $^+$, 260, 248, 163

^{1}H-NMR,δ(ppm): 8.97 (1H,m,H11), 8.12 (1H,s,H3), 7.87 (1H,m,H8), 7.67 (2H,m,H-9,10), 7.48 (1H,s,H7), 6.44 (3H,s,OCH_2O), 3.84 (3H,s,NCH_3).

RESULT AND DISCUSSION

Phytochemical screening showed that the root of *A.corymbosa* contains terpenoid, steroid and alkaloid. Brine Shrimp Lethallity Bioassay showed that the chloroform extract(ACB) was slightly toxic with LC_{50}~200 ppm. The Tlc profile of ACB indicated the presence of six major components. ACB was then fractionated by dry flash column chromatography. The hexane:ethylacetate (4:6) fraction (ACB3) was further separated using column and microcolumn chromatography. Three major compounds were subsequently isolated and labelled as AC3 ,AC4 and AC6 respectively. The physical and spectroscopic data of the compounds are in accordance with literature values.

AC3 was isolated by column chromatography of fraction ACB3 using hexane;ethyl acetate(4:6) as eluent. Tlc on silica gel using solvent system hexane:ethyl acetate (8:2) gave R_f 0.9. The UV spectrum of AC3 indicated bathochomic shifts at 256,272,295 and 372 nm. Its mass spectrum gave a molecular weight of 341 corresponding to a

molecular formula of $C_{18}H_{15}NO_6$. The presence of m/z 295 fragmentation peak indicated a loss of NO_2 while m/z 280 indicated a further loss of Me and m/z 264 indicated a loss of OMe.

^{1}H-NMR spectra (Table 1) of AC3 showed the presence of 15 protons consisting of two aromatic methoxyl groups at δ 4.10 (3-OCH_3) dan δ 3.98 (4-OCH_3). The signals for carbomethoxy group ($OCOCH_3$) appeared at δ 3.88 ppm. The ^{13}C-NMR spectrum showed the presence of 18 carbons in the skeleton, resonating at δ 167.6 (CO), 151.6 (C5), 149.5 (C9), 146.1 (C8), 131.1 (C4), 130.4 (C10), 128.3, 127.3, 126.2, 124.9, 116.8, 116.3, 60.3 (OMe-C3), 56.7 (OMe-C4) and 52.1 ($OCOCH_3$). Four coupling aromatic proton were indicated at δ 7.71, 7.80, 7.97 and 9.65 belonging to H7, H6, H8 and H5 respectively. Down field signal at δ 9.65 (H5) is characteristic of proton at C5 in Aristolochic acid ring. Two proton singlet at δ 7.87 and 8.32 belonging to H2 and H9. Based on the above spectral and published data (Wu,T.S., et al., 1994), we propose AC3 as Ariskanin-A.

Table 1 ^{1}H-NMR spectral data for AC3 and AC4 (400 MHz,$CDCl_3$)

Proton	AC3 δ(ppm)	J(Hz)	AC4 δ(ppm)	J(Hz)
H-2	7.89,1H,s		7.88,1H,s	
H-5	9.66,1H,dd	8.0,1.8	8.99,1H,dd	8.0,1.8
H-6	7.80,1H,td	8.0,1.6	7.75,1H,t	8.0,1.6
H-7	7.71,1H,td	8.0,1.8	7.13,1H,d	8.0,1.8
H-8	7.97,1H,dd	8.0,1.8	-	
H-9	8.32,1H,s		8.86,1H,s	
8-OCH3	-		4.06,3H,s	
3-OCH_3	4.10,3H,s		-	
4-OCH_3	3.98,3H,s		3.88,3H,s	
1-$OCOCH_3$	3.88,3H,s		3.84,3H,s	
3-OH	-		6.40,1H,s	

AC4, yellow amorphous solid isolated from the hexane :ethyl acetate(4:6) fraction from column chromatography of ACB3. Gas chromatogram showed one peak at t_R=33.6 min. Tlc on silica gel using hexane: ethyl acetate(8:2) gave R_f 0.88. The UV spectral data of AC4 indicated three absorption peaks at 257nm (conjugated carbonyl group),309 nm(n→π* for ketone group) and 399 nm.

Its mass spectrum gave a molecular weight of 357[M^+] corresponding to a molecular formula $C_{18}H_{15}NO_7$. The presence of m/z 277 indicated the loss of a methoxy group.

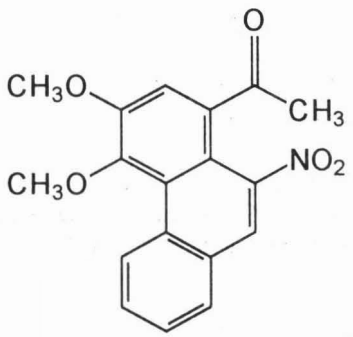
^{1}H- NMR spectral (Table 1) of AC4 showed the presence of two methoxy protons at δ 4.06 ppm(8-OMe) and δ3.88 ppm(4-OMe). δ6.40 indicated the presence of a hydroxy group(OH). Other important signals are δ8.99 ppm(H-5), δ8.86(H-9), 7.88(H-2) ,δ7.75(H-6), and δ7.13(H-7).[13] C- NMR spectrum showed the presence of 18 carbons which could be interpreted in detail with COSY 2D-NMR.

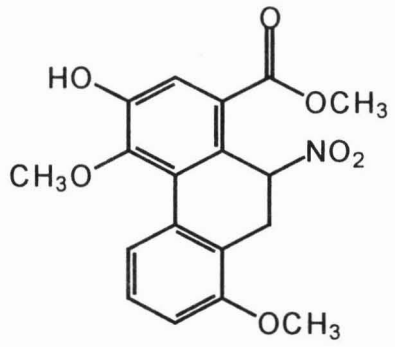
From the above spectroscopic data and comparison with literature data, we suggest AC4 to be Ariskanin-D **2** previously reported by Wu et al.(1994).

AC6 (25mg) was isolated by column chromatography using ethyl acetate 100% as eluent. It was the third major component isolated from ACB4 fraction. AC6 isolated as orange crystal which gave Rf 0.82 on Tlc with ethyl acetate: methanol (9: 1). When visualised under UV at 366 nm, it showed highly flourescent orange spot. The colour change when sprayed with Dragendorff Reagent revealed an alkaloid.Gas chromatography of AC6 showed one peak at t_R=45.87 min. ^{1}H-NMR indicated six protons in the aromatic region. A downfield singlet at δ 8.12 ppm can be assigned to H3 at ring A of oxoaporphine. A second singlet at δ 7.48 ppm was assigned to H7 in ring C. Two protons in methylene dioxyl group were identified as a singlet at δ 6.44, those of C1 and C2 in the substituted ring A. Other protons gave multiplets at δ 8.97 (1H,m) (H11), 7.87(1H,m) (H8), 7.67(2H,m) (H9,10) and one singlet for 3H at δ 3.84 belonging to methyl group attached to Nitrogen atom (N-CH3). Table 2 showed the ^{1}H-NMR data of AC6 compared to Cepharadione-A. The various proton-proton connectivities can be investigated using the most common 2D NMR experiment i.e. ^{1}H-^{1}H COSY. Protons in aromatic region showed interaction clearly. The proton at C11 (H11) had a strong correlation with the one at C-9,10 (H-9,10), while the proton at C8 (H8) also had correlation with H-9,10. All the protons identified are aromatic proton in ring D of aporphine nucleus. Cepharadione-A was reported occuring in Aristolochiaceae especially in *Aristolochia* sp.

Table 2 ^{1}H-NMR data for AC4 in comparison with Cepharadione-A **3**

Proton	**AC6**	**Cepharadione-A**
H-3	8.12,1H,s	7.98,1H,s
H-7	7.48,1H,s	7.91,1H,s
H-8	7.87,1H,m	8.10,1H,m
H-9,10	7.67,2H,m	7.71,2H,m
NCH3	3.84,3H,s	3.84,3H,s
H-11	8.97,1H,m	8.96,1H,m
-OCH2O-	6.44,2H,s	6.57,2H,s





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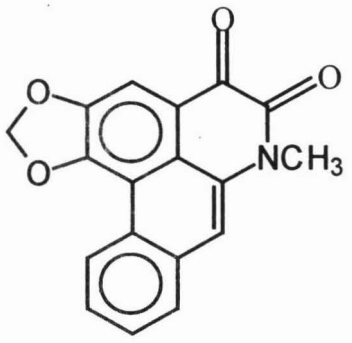

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