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A NEW IRIDOID GLUCOSIDE FROM *ANISACANTHUS VIRGULARIS* AND ITS ANTIAMOEBIC ACTIVITY

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Abstract – Phytochemical investigation of *Anisacanthus virgularis* aerial parts resulted in isolation of a new iridoid glucoside named as 7-*O*-*p*-*trans*-coumaroyl caryoptoside (**1**) together with two known iridoids, caryoptoside (**2**) and lamalbide (**3**). Their structures were elucidated by spectroscopic methods including 1D and 2D-NMR techniques. The isolated compounds together with the MeOH and aqueous extracts of the plant were investigated for their antiamoebic activity *in vitro*. The micro dilution technique was employed to investigate antiamoebic activity against *Entamoeba histolytica* HM1-SS strain. The results showed that the MeOH extract had good inhibition along 72 hours followed by the aqueous extract at concentration of 15 mg/mL. The compounds **1-3** showed good inhibition percent 72.2%, 50.1% and 52.6% after 24 hours of incubation at a concentration of 4 μM, compared to the standard antiamoebic drug, Metronidazole.

Acanthaceae family is one of the iridoid rich families, and it comprises of about 346 genera and around 4300 species.[1,2] *Anisacanthus* genus comprises about 18 species which have centers of diversity in Mexico

(8 species) and Brazil (6 species).[3] *Anisacanthus virgularis* (Salisb.) Nees is an erect shrub, native to tropical and subtropical regions of the Americas and Mexico. It can be used in the Oasis and Xeric landscape gardens as a border accent and attractor of hummingbirds.[4] The former phytochemical studies revealed the isolation of lignans, flavonoids, triterpenes and sterols from the aerial parts.[5] The biological studies on *A. virgularis* showed weak radical scavenging activity of leaves and branches using DPPH assay and ascorbic acid as a reference drug.[6] In addition, the leaves and branches extracts showed weak cytotoxic activities against different cancer cell lines.[7] In the present study, we declare the isolation and structure elucidation of a new iridoid glucoside together with two known ones (Figure 1) for the first time from *A. virgularis*. *In vitro* antiamoebic activity of the MeOH and aqueous extracts of the plant and isolated compounds were investigated against HM1-SS strain of *E. histolytica* using micro dilution method.

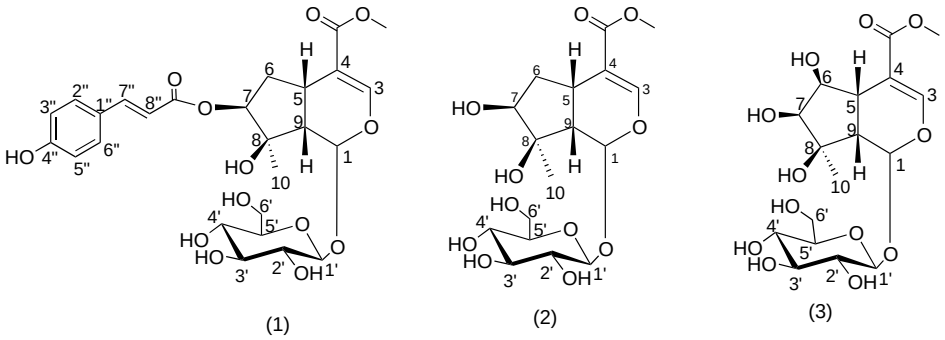

Figure 1. Iridoid glucosides isolated from *A. virgularis*

Compound **1** was obtained as colorless needles. Its molecular formula was established from the positive HRFAB-MS $[M+Na]^+$ ion peak at *m/z* 575.1737, calculated for $C_{26}H_{32}O_{13}Na$, 575.1741; Positive FAB-MS *m/z* 575 $[M+Na]^+$. The ^{1}H- and ^{13}C-NMR of **1** (Table 1) demonstrated a hemiacetal proton signal at δ_H 5.59 (d, J = 2.2 Hz), an olefinic carbon at δ_C 151.7 and 113.8 ppm with high deshielded corresponding to olefinic proton at δ_H 7.40 ppm suggesting the presence of an iridoid skeleton. The carbon resonances at δ_C 169.0 and 51.7 ppm in addition to the signal at δ_H 3.69 ppm (3H, s) verified the presence of an ester group (CO_2Me). ^{1}H-NMR also showed three-protons singlet at δ_H 1.28 ppm, could be assigned to a methyl group positioned at a tertiary carbon carrying oxygen atom and this can be regarded at C-8.[8] The downfield shifted resonance at δ_H 4.90 (dd, J = 7.3, 2.5 Hz) indicated an oxygenated methine carbon at this position, while the signals at δ_H 2.33 (ddd, J = 15.0, 9.6, 2.5 Hz) and δ_H 1.83 (dt, J = 15.0, 5.6 Hz) suggesting the presence of methylene protons at the five-membered ring of an iridoid skeleton. The position of the methylene and oxymethine protons may be at positions C-6 or C-7. This assumption was also supported by HMBC correlations (Figure 2) and by comparison of these data with those reported in literature.[9-11] The ^{13}C-NMR data of C-6 to C-10 are in agreement with those of C-6 to C-10 in caryoptoside with a hydroxy group in the β-position at C-8.[12,13]

Table 1. ^{1}H- and ^{13}C-NMR data for compound **1** (300 and 100 MHz, in CD_3OD)

Compound 1					
No.	**δ_C**	**δ_H (m, *J*, Hz)**	**No.**	**δ_C**	**δ_H (m, *J*, Hz)**
1	94.8	5.59, d (J = 2.2)	5′	78.2	3.78, m
3	151.7	7.40, brs	6′	62.8	3.90, m 3.66, m
4	113.8	-	1″	127.2	-
5	27.6	3.15, br.d, (J = 8.0)	2″	131.8	7.47, d, (J = 8.6)
6	37.1	6_a: 2.33, ddd, (J = 15.0, 9.6, 2.5) 6_b: 1.83, dt, (J = 15.0, 5.6)	3″	116.8	6.80, d, (J = 8.6)
7	81.4	4.90, dd, (J = 7.3, 2.5)	4″	161.0	-
8	79.5		5″	116.8	6.80, d, (J = 8.6)
9	49.2	2.67, dd, (J = 10.5, 2.2)	6″	131.8	7.47, d, (J = 8.6)
10	22.0	1.28	7″	146.7	7.66, d, (J = 15.9)
1′	99.9	4.64, d, (J = 7.9)	8″	115.3	6.38, d, (J = 15.9)
2′	74.6	3.15, m	9″ C=O	168.7	
3′	77.9	3.34, m	OCH_3	51.7	3.69
4′	71.6	3.24, m	$COCH_3$	169.0	-

It is noticed that there is a downfield shift (*ca*. 2.4) at C-7, indicating that the substitution at C-7 is more electron withdrawing group other than hydroxyl as in caryoptoside. The ^{1}H- and ^{13}C-NMR of **1** showed characteristic peaks of *p-trans*-coumaroyl moiety confirmed from H-7″ and H-8″ resonances at δ_H 7.66 (d, J = 15.9) and 6.38 (d, J = 15.9), respectively indicating their *trans* position. Depending on data from the ^{1}H-^{1}H-COSY, HSQC, HMBC experiments and the downfield shift of C-7, the a *p-trans*-coumaroyl moiety - is esterifying the hydroxyl group at C-7 of the caryoptoside (**2**) producing the new structure **1**. The stereochemisty at the chiral centers C-1, C-5, C-7, C-8, C-9 and C-1′ was established on the basis of the ^{1}H- and ^{13}C-NMR chemical shifts in comparison with literatures.[10,12,14,15] In addition, the relative configuration of compound **1** was established by NOESY experiment which showed correlations between H-1/H-10 and H-7/H-10 indicating their α-orientation, while correlations between H-5/H-9 were β-oriented. From the aforementioned spectroscopic data, compound **1** was identified as 7-*O*-*p*-*trans*-coumaroyl caryoptoside.

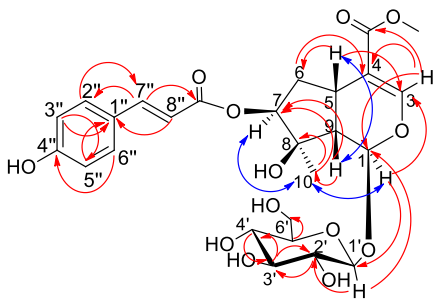

Figure 2. Key HMBC ( ) and NOESY C ( ) correlations of **1**

Compounds **1-3** together with MeOH and aqueous extracts of *A. virgularis* aerial parts were evaluated for their antiamoebic activity *in vitro*, using micro dilution method. Metronidazole, a popular drug for treatment of amebiasis was used as positive control. The effect of isolated compounds and extracts against *Entamoeba histolytica* trophozoites were recorded at 24, 48 and 72 h. The results showed that total MeOH extract exhibited good inhibition growth percent (Table 2) compared to Metronidazole, while the aqueous extract revealed moderate inhibition. On the other hand, compound **1** exhibited a high growth inhibition (Table 2) through the first 24 h compared to Metronidazole, while compounds **2** and **3** exhibited a moderate growth inhibition along the three days.

Table 2. Effect of isolated compounds and extracts of *A. virgularis* on *E. histolytica* trophozoites after 24, 48 and 72 h incubation

Sample	Concentration	Growth inhibition (%)*		
		24 h	48 h	72 h
MeOH extract of *A. virgularis*	15 mg/mL	61.12	63.89	41.67
Aqueous extract of *A. virgularis*	15 mg/mL	38.6	27.79	44.45
Compound **1**	4 µg/mL	72.23	44.50	58.33
Compound **2**	4 µg/mL	50.10	61.12	52.77
Compound **3**	4 µg/mL	52.68	30.56	33.34
Metronidazole	3 µg/mL	86.12	75.02	86.11

***** The values are mean values obtained in the assay done in triplicate.

EXPERIMENTAL

General Experimental procedures

^{1}H-NMR spectra were recorded in methanol-d_4 (Nacalai Tesque, Inc., Kyoto, Japan) with Varian Unity Plus 400 spectrometer (Palo Alto, CA, USA) operating at 100 MHz for ^{13}C and with a JEOL JNM-AL 300 spectrometer (JEOL Ltd, Tokyo, Japan) at 300 MHz for ^{1}H-NMR. FAB-MS spectra were recorded on a JMS 700N spectrometer (JEOL Ltd., Tokyo, Japan) in positive ion mode, with glycerol or *m*-nitrobenzyl alcohol, with or without NaCl, as the matrix. The optical rotation measurements were done using Jasco P-1020 polarimeter (Jasco Co. Ltd., Tokyo, Japan). IR spectra were recorded using a Jasco FT/IR-410K spectrometer (Jasco Co. Ltd., Tokyo, Japan) with a range of 400–4000 cm^{-1}. Column chromatography (CC) was performed using silica gel 60 F254 (0.2 mm, Merck), Sephadex LH-20 (25–100 µm, GE Healthcare UK Ltd., Buckinghamshire HP7 9NA, UK) and Reversed Phase C18 silica gel (Nacalai Tesque, Inc., Kyoto, Japan). TLC was performed on 0.25 mm thick, precoated silica gel 60 F254 and silica gel RP-18 F254 plates (Merck, Darmstadt, Germany). Spots were developed with 10%

H_2SO_4 in MeOH and detected by illumination under a short wavelength UV (254 nm). 1D and 2D-NMR spectra were recorded using the residual solvent signal as an internal standard on a Bruker BioSpin Gm bH 300 and 500 spectrometers, respectively (Bruker, Rheinstetten, Germany). High resolution mass spectrometry was measured using a Bruker BioApex FT mass spectrometer (Bruker, Rheinstetten, Germany). Versatile refrigerated centrifuge (Tomy tech, USA), hemocytometer (Tokyo, Japan), Biostate peptone (BD, USA).

Plant material

The fresh aerial parts of *A. virgularis* were collected from El-Orman Botanical Garden, Giza, Egypt (August 2016). Eng. Therese Labib, consultant of plant taxonomy at the Ministry of Agriculture and ex. director of El-Orman Botanical Garden, Giza, Egypt, confirmed the identification of the plant. A voucher specimen (USC-Ph-Cog-02) was deposited at the department of pharmacognosy, faculty of pharmacy, University of Sadat City, Menofia, Egypt.

Extraction and isolation

Air-dried powdered aerial parts of *A. virgularis* (1.8 kg) were extracted with 80% aqueous MeOH by maceration at room temperature till exhaustion (4 × 8L). The combined MeOH extracts were concentrated under reduced pressure at 40 °C and left to dry to afford 320 g of a yellowish green viscous residue. The dried MeOH extract was suspended in distilled water and successively partitioned between CH_2Cl_2 (3 × 5 L). Each phase was concentrated under reduced pressure separately to give CH_2Cl_2 fraction (17 g) and remaining aqueous extract (290 g). CH_2Cl_2 fraction subjected to VLC silica gel column and eluted using gradient systems of *n*-Hexane-CH_2Cl_2 and CH_2Cl_2-MeOH; fractions of 100 mL each were collected. The elutes with CH_2Cl_2-MeOH (90:10 and 85:15) were combined and subjected to silica gel column chromatography (CC) using CH_2Cl_2-MeOH gradient elution started with 100% CH_2Cl_2 to 100% MeOH to give nine sub-fractions. The sub-fraction 5 (56 mg) was subjected to Sephadex LH-20 eluted with MeOH to give 3 sub-fractions (5a, 5b and 5c). The sub-fraction 5b subjected to silica gel CC using CH_2Cl_2-MeOH gradient elution started with 100% CH_2Cl_2 to CH_2Cl_2-MeOH (80:20, v/v) to afford compound **1** (3.0 mg). The aqueous fraction was loaded on VLC silica gel column and eluted using gradient systems of CH_2Cl_2-MeOH, EtOAc-CH_2Cl_2 and EtOAc-MeOH; fractions of 100 mL each were collected. The collected fraction (4.0 g) eluted with EtOAc-MeOH 50:50 (4.0 g) subjected to silica gel column chromatography using CH_2Cl_2-MeOH in a gradient elution manner, where three sub-fractions (a, b and c) were collected. Sub-fraction b (0.7 g) subjected to Rp-18 CC using MeOH-H_2O (1:1, v/v) as an elution system to give three sub-fraction b_1, b_2 and b_3. The sub-fraction b_2 subjected to silica gel cc using CH_2Cl_2-MeOH gradient elution started with CH_2Cl_2-MeOH (90:10, v/v) to CH_2Cl_2-MeOH (70:30, v/v) to afford compound **2** (2.3 mg). The sub-fraction c (1.7 g) subjected to Rp-18 CC using MeOH-H_2O (1:1, v/v) as an elution system to give three sub-fractions C_1, C_2 and C. The sub-fraction C_1 subjected to RP-18

cc using MeOH-H_2O (1:1) as an elution system to afford compound **3** (3.0 mg).

Antiamoebic activity:

Entamoeba histolytica trophozoites culture

Trophozoites of *E. histolytica* (HM1-SS strain) obtained from Parasitology Department, Institute of Tropical medicine, Nagasaki University was grown axenically at 37 °C in BIS-33 medium.[16,17] The medium was supplemented with 15% bovine serum (Tokyo, Japan), and 5% Diamond vitamin mixture (Wako Pure Chemical Industries, USA). Cultures were maintained by sub-culturing the cells twice a week and the cells were counted using a Neubauer chamber.

Growth inhibition assay

To evaluate the effect of plant extracts and isolated compounds on *E. histolytica* growth, trophozoites were collected by centrifugation at 1500 rpm for 5 min at 4 °C. The obtained trophozoites were suspended in BI-S-33 medium and about 6×10^4 trophozoites in 1 mL of BI-S-33 medium were poured into each borosilicate tube supported with 5 mL of BIS-33 media and incubated for 72 h.[18] To a medium containing 1% (v/v) penicillin/streptomycin (Thermo Fischer scientific, Japan); 200 µL of isolated compounds and 500 µL of extracts solutions were added to each borosilicate tube in order to obtain final concentration of 4 µg/mL and 15 mg/mL, respectively. Metronidazole (Sanofi-Aventis-Egypt) was used as positive control in a final concentration of 4 µg/mL in all biological experiments, while 0.1% DMSO (Chameleon, Osaka, Japan) were used as negative control. All the plant extracts and compounds including metronidazole were dissolved in dimethyl sulfoxide (DMSO). The final solutions contained 0.1% DMSO or less, which did not affect viability.[19,20] After incubation periods, the cells were harvested by cooling and counted using a Neubauer chamber and dye exclusion test. The total number of parasites was counted, including those which had excluded the dye and cell viability was calculated as the percentage of viable cells in the samples relative to the untreated cells. Experiments were performed in triplicate.

7-*O*-*p*-*trans*-Coumaroyl caryoptoside (1): obtained as colorless needles; $[\alpha]_D^{23}$ -40.7 (c 0.3, MeOH); IR ν_{max} cm^{-1} 3432, 2920, 1695, 1634, 1538, 1513, 1442, 1290, and 1078; ^{1}H- and ^{13}C-NMR data (CD_3OD, 300 and 100 MHz), see Table 1. Positive FAB-MS *m/z* 575 $[M+Na]^+$; Positive HRFAB-MS *m/z* 575.1737 $[M+Na]^+$ (calcd for $C_{26}H_{32}O_{13}Na$, 575.1741).

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