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Original Research Article

Antiallergic polyphenols from *Citharexylum spinosum*

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ABSTRACT

Three polyphenolic compounds, namely 1,3,6-tri-O-galloyl-β-D-glucopyranose (TGG) (**1**), methyl gallate (**2**) and hydroxyquinol (**3**) have been isolated from the aerial parts of fiddlewood (*Citharexylum spinosum* L.). The structures of the isolated compounds have been characterized on the basis of spectroscopic methods in addition to comparison with literature data. These compounds along with their methanol extract were tested to evaluate their anti-allergic activity through inhibition of β-hexosaminidase enzyme. It was noticed that all the tested compounds and methanol extract have good inhibition for β-hexosaminidase release without affecting the cell viability where the production of β-hexosaminidase was decreased to about 69%, 60% and 56% by compounds (**1**), (**2**) and (**3**), respectively. The anti-allergic activity of fiddlewood has been reported for the first time in the present report.

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1. Introduction

Citharexylum spinosum L. is a species of flowering plants in the *Verbenaceae* family, that is native to the south of United States, the Caribbean, Guyana, Suriname, and Venezuela. The common names of this plant include Florida fiddlewood and Spiny fiddlewood. It is also cultivated in Egypt as a street tree and prescribed as a popular ornamental drug in many tropical and subtropical regions of the world (Lawrence, 1951; Marwa et al., 2014).

The plant has been used in folk medicine as a potent diuretic, antipyretic, antiarthritic remedy and in the treatment of liver disorders (Marwa et al., 2014). In addition, it possesses a wide range of pharmacological actions such as the antibacterial activity against multidrug-resistant uropathogens, which are the microorganisms capable of causing the urinary tract disease (Bag et al., 2013). Moreover, it is a useful remedy against various diseases, e.g. the decoctions from its young twigs and barks are recommended to treat thrush in babies and colds, respectively (Acevedo-Rodríguez, 1996; Shalaby and Bahgat, 2003; Mar and Pripdeevech, 2014). The leaves are used as a potential source of anthelmintic, antiallergic and antiarthritic drugs (Balaázs et al., 2006; Lans, 2007).

Biological screening of the 70% aqueous ethanolic extract of *C. spinosum* revealed significant antiulcer, antihypertensive and hepatoprotective effects (Khalifa et al., 2002). The chloroformic extract of the plant was found to have potent nephroprotective and antioxidant properties in carbon tetrachloride (CCl_4; 20% in olive oil, 2 mL/kg body weight) treated Sprague-Dawley male rats (Khan and Siddique, 2012). Recently, the leaves of *C. spinosum* showed important pharmacological activities such as anti-inflammatory, anti-pyretic, gastroprotective and anti-diabetic remedies (Marwa et al., 2016).

A survey of the literature revealed the isolation and identification of iridoid glycosides, phlomiol, 5-deoxy pulchelloside, durantoside and lamidoside from the plant of this study (Rizk, 1986). Stigmasterol and oleanolic acid in addition to cirsimaritin and cirsiliol 4'-O-β-D-glucopyranoside and other flavone glycosides were also reported from the plant (Balaázs et al., 2006).

The present study deals with the isolation and

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identification of three polyphenoilc compounds from *C. spinosum* L. Furthermore, the inhibition of β-hexosaminidase enzyme was studied for the first time for this plant.

2. Experimental

2.1. Material and methods

2.1.1. Plant material

The aerial parts of *C. spinosum* L. (*Verbenaceae*) were collected in May 2009 from Faculty of Agriculture Garden, Assiut University, Assiut City, Egypt. The collected parts were dried in the shade in the absence of sunlight. The plant was identified and authenticated by Prof. Dr. A. Fayed, Professor of Plant Taxonomy, Faculty of Science, Assiut University. A voucher specimen (Ci-17) was deposited at the Department of Pharmacognosy, Faculty of Pharmacy Assiut University, Assiut, Egypt.

2.1.2. Extraction and isolation

Air-dried aerial parts of *C. spinosum* L. (2 Kg) were subjected to extraction using 5 L of MeOH at room temperature. The solvents were combined and filtered through filter paper (Advantec MFS Incorporated). The solvent was removed under reduced pressure at 40 ºC to yield the methanol extract (640 g), which was partitioned between distilled water, chloroform, ethyl acetate and *n*-butanol (5 L each) to give the chloroform fraction (220 g), ethyl acetate fraction (90 g), *n*-butanol fraction (88 g) and the rest aqueous fraction (210 g). The ethyl acetate fraction was in turn partitioned between (methanol-water 90%) and *n*-hexane to give 90% methanol fraction (52 g) and *n*-hexane fraction (19 g). The *n*-butanol fraction (88 g) was sub-fractionated on Diaion HP-20 column using water and methanol (25%, 50%, 75% and 100%) (2 L of each).

The fraction of 50% methanol-eluted (28.7 g) was further separated by chromatography on an ODS column (80×200 mm) using six concentrations of methanol: H_2O (20, 40, 50, 60, 70 and 90% v/v; elution volume: 1.5 L of each) to give six corresponding fractions and the fraction eluted with 60% methanol (5.2 g) was further fractionated using silica gel column chromatography and eluted stepwise with some mixtures of chloroform-methanol (ratios of 9: 1, 6: 1, 4: 1, 3: 1 and 1:1, v/v). In the next step, 200 mL of each fraction was collected and concentrated to give five corresponding fractions (I-V). 160 mg from the fraction II was further fractionated by preparative HPLC (ODS column: TOSO, 18 mm×250 mm, particle size: 5 µm, flow-rate: 3 mL/min) equipped with UV detector (210 nm), the mobile phase was 20% CH_3OH in H_2O which resulted in elution of compound (**1**). The preparative HPLC conditions were also used to separate components of fraction III giving compound (**2**) and compound (**3**) (Fig. 1). The three characterized compounds were as follows.

1,3,6-tri-O-galloyl-β-D-glucopyranose (TGG) (1); Obtained as a yellow amorphous powder (34 mg). ^{1}H NMR (400, MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): Table 1. FAB MS at *m/z*: (635) $[M-H]^-$ $C_{27}H_{24}O_{18}$.

Methyl gallate (2); Obtained as a yellow amorphous powder (13 mg). ^{1}H NMR (400, MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): Table 1. EI-MS at *m/z*: (184) $C_8H_8O_5$.

Hydroxyquinol (3); Obtained as a colorless solid (11 mg). ^{1}H NMR (400, MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): Table 1. EI-MS at *m/z*: (126) $C_6H_6O_3$.

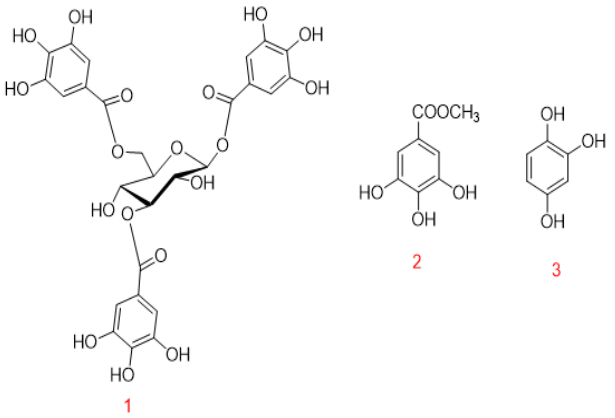

Fig. 1. Structure of the characterized compounds (**1**-**3**).

2.2. Apparatus

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were measured on a JEOL GS×400 spectrometer in CD_3OD. Reversed-phase high-performance chromatography experiments were undertaken on ODS columns (particle size: 5 µm, TOSO, 18×250 mm) RP-23 (5 µm; Waters). Diaion HP-20 (Mitsubishi, Tokyo, Japan), silica gel (63-210 µm; Kanto Kagaku) and ODS (63-212 µm; Wako Pure Chemical, Tokyo, Japan) were used for open column chromatography. Thin-layer chromatography (TLC) was carried out on silica gel (SiO_2, 60-100 mesh; Wako Pure Chemical) 60 F_{254} and RP-18 F_{254S} (Merck). Structural assignments were based on spectra resulting from one or more of the NMR experiments; ^{1}H-, ^{13}C NMR, HMQC and HMBC. Fast Atom Bombardment (FAB) experiment was carried out on JEOL Mstation and electron impact mass experiment was operated on JEOL JMS SX-102A mass spectrometer.

2.3. Procedure: RBL-2H3 Cell Line Assay

RBL-2H3 cells are the tumor analog of mast cells, which after being sensitized with mouse monoclonal IgE or ionophore A23187 respond by releasing inflammatory mediators such as β-hexosaminidase (Qianqian et al., 2015; Taehun et al., 2015). As a result, the sample is considered to have anti-allergic activity if it can inhibit mast cells degranulation and produce a significant reduction in β-hexosaminidase release.

Table 1

^{1}H (CD_3OD, 400 MHz) and ^{13}C (CD_3OD, 100 MHz) NMR assignments for compounds (**1**-**3**).

	Compound 1			**Compound 2**			**Compound 3**	
No.	^{1}H NMR	^{13}C NMR	No.	^{1}H NMR	^{13}C NMR	No.	^{1}H NMR	^{13}C NMR
Glc								
1	5.75 (1H, d, *J*=8.2 Hz)	95.86	1	-	121.40	1	-	134.84
2	3.71 (1H, dd, *J*=9.3, 8.2 Hz)	72.61	2,6	7.03 (2H, s)	110.00	2	-	145.29
3	5.2 (1H, t, *J*=9.3 Hz)	78.89	3,5	-	146.47	3	6.90 (1H, d, *J*=4.1 Hz)	114.67
4	3.72 (1H, t, *J*=9.3 Hz)	69.69	4	-	139.74	4	-	146.38
5	3.79 (1H, ddd, *J*=9.3, 4.8, 1.7 Hz)	77.61				5	6.80 (1H, dd, *J*=8.2, 4.1 Hz)	116.55
6	6a: 4.37 (1H, dd, *J*=12.0, 4.8 Hz) 6b: 4.52 (1H, dd, *J*=12.0, 1.7 Hz)	64.20				6	6.73 (1H, d, *J*=8.2 Hz)	118.92
1-Gall			C=O	-	166.12			
1	-	121.23	OCH_3	3.80 (3H, s)	52.27			
2,6	7.08 (2H, s)	110.36						
3,5	-	146.49						
4	-	139.91						
C=O	-	168.10						
3-Gall								
1	-	121.58						
2,6	7.06 (2H, s)	110.55						
3,5	-	146.52						
4	-	140.48						
C=O	-	168.20						
6-Gall								
1	-	120.44						
2,6	7.02 (2H, s)	110.17						
3,5	-	146.42						
4	-	139.79						
C=O	-	168.81						

Firstly, a cell viability assay (using MTT) was performed to ensure that the activity of the sample at the used concentration was related to the inhibition of histamine release rather than to the cytotoxicity of the RBL-2H3 cells. The cell viability assay was done as follows: RBL-2H3 cells (100 µL, 1×105 cells/well) were cultured with EMEM in a 96-well plate for 24 h. Then, 1 µL/well and 1 µL of DMSO (as control) were added. After 24 h incubation in a CO_2 incubator at 37 ºC, MTT (10 µL, 5 mg/mL in PBS) was added to each well, and the plate was incubated for another 4 h. The medium was then removed, acidic *iso*-propanol (100 µL) containing HCl (0.04 N) reagent was added to each well, the plate was incubated overnight at room temperature, and the absorbance was read at 570 nm using a microplate reader. The anti-allergy assay was determined as described by (Yun et al., 2010), with minor modification, as follows: RBL-2H3 cells (1×106 cells/well) were inoculated with EMEM in a 96-well plate for 48 h, then EMEM medium was replaced by tyroid buffer with the composition; [100 µL, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2.6H_2O$, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 5.6 mM glucose, 0.1% BSA, pH 7.2/well], after preparing tyroid medium, sample (1 µL/well) was added, and the plate was incubated for 30 min in CO_2 incubator at 37 ºC. A23187 (Calcimycin, Calcium Ionophore, Antibiotic A23187) (10 µg/mL, 2 µL/well) was added after removal of the sample and the addition of new tyroid buffer (100 µL/well). After 30 min incubation, 50 µL from each well was collected and transferred to another 96-well plate. An equal volume of substrate solution (l mM), *p*-nitrophenyl-N-acetyl-β-glucosaminide, was added to each well, and the plate was left at room temperature on the shaker for 1 h. Finally, the reaction

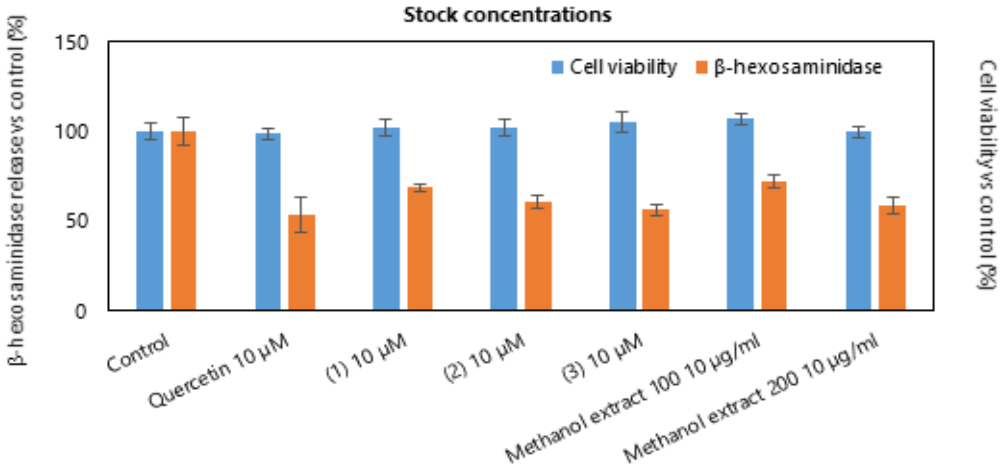

Fig. 2. Cell viability and effects of the isolated compounds (1-3) and methanol extract on ionophore A23187-stimulated β-hexosaminidase release from RBL-2H3 basophilic leukemia cells, all the values are the mean ± SD (n=3).

was terminated by adding 100 µL of stopping buffer (Na_2CO_3, 100 mM, pH=10). The stopping buffer is a solution used to terminate the peroxidase reaction for ELISA applications. The absorbance was finally measured at 405 nm using a microplate reader. The statistical difference between the control and each sample was determined by student's t-test.

3. Results and Discussion

3.1. Identification of compounds

The aerial parts of *C. spinosum* L. were extracted with methanol and then fractionated with $CHCl_3$, EtOAc, *n*-BuOH and H_2O. By using combined chromatographic separation of the *n*-BuOH fraction, three polyphenolic compounds (**1**), (**2**) and (**3**) (Fig. 1) were isolated.

Investigation of ^{1}H NMR spectrum of compound (**1**) (Table 1) concluded the presence of three galloyl moieties attached to one glucose unit which was obvious from the presence of three sets of proton singlet signals at δ_H=7.08, 7.06 and 7.02 (each 2H,s) and an anomeric proton signal at δ_H=5.75 (1H, d, *J*=8.2 Hz). Moreover, the ^{13}C NMR spectrum (Table 1) confirmed the presence of three galloyl moieties and one glucose moiety. The configuration at the glucose C-1 position was concluded to be β on the basis of the *J*-value (8.2 Hz) of the anomeric proton signal at δ_H=5.75. The site of attachments of each galloyl moiety was confirmed to be at C-1, C-3 and C-6 of the glucose unit from the ^{1}H-, ^{13}C NMR downfield shifts at these sites in addition to the 2D NMR correlations including both HMQC and HMBC experiments. On the basis of the previously mentioned data and by comparison with literature data (Ka-Wing et al., 2009), compound (**1**) was identified as 1,3,6-tri-O-galloyl-β-D-glucopyranose (TGG).

The ^{1}H NMR spectrum of compound (**2**) (Table 1) exhibited the characteristic signals of methyl gallate by the appearance of a signal at δ_H=7.03 (2H, s, H-2,6) in addition to a signal at δ_H=3.80 (3H, s, OCH_3).The ^{13}C-NMR spectrum (Table 1) revealed the presence of six carbon signals comprising C=O signal at δ_c=166.12 (s) indicating the presence of a carbonyl ester, one oxygenated aromatic signal at δ_c=139.74 (C-4), two symmetrical oxygenated aromatic carbon signals at δ_c =146.47 (C-3 and C-5), one quaternary aromatic carbon signal at δ_c=121.40 (C-1), two symmetrical aromatic methane carbon signals at δ_c=110.00 (C-2 and C-6) and one carbon signal at δ_c=52.27 (OCH_3). Thus, compound (**2**) was identified as methyl gallate by comparison of these data with those reported in the literature (Mohd Nazrul Hisham et al., 2011).

The ^{1}H NMR spectrum of compound (**3**) (Table 1) exhibited the pattern of a tri-substituted benzene ring where the presence of three aromatic proton signals at δ_H=6.90 (1H, d, *J*=4.1 Hz), 6.80 (1H, dd, *J*=8.2, 4.1 Hz) and at δ_H=6.73 (1H, d, *J*=8.2 Hz), confirming this pattern.

The ^{13}C NMR spectrum (Table 1) revealed the presence of six carbon signals from which, three oxygenated aromatic carbon signals at δ_c=134.84, 145.29 and 146.38 (C-1, C-2 and C-4 respectively), confirmed the previous suggestion, and hence, compound **3** can be identified as hydroxyquinol by comparison of these data with those reported in the literature (Ferraroni et al., 2005).

3.2. RBL-2H3 Cell Line Assay

After establishing the structures, compounds (**1**-**3**) were investigated using RBL-2H3 Cell Line Assay. It was noticed that all tested compounds and methanol extract exhibited inhibition for β-hexosaminidase release without affecting the cell viability. Compound (**3**) exhibited the highest inhibition activity (44%), while compounds (**2**) and (**1**) respectively exhibited (40%) and (32%) for β-hexosaminidase activity at a final concentration of 20 µM the same as the positive control quercetin. For the methanol extract, it exhibited about

Table 2
Effects of the methanol extract and the isolated compounds on β-hexosaminidase production[a].

Compound	β-Hexosaminidase production(%)	Cell Viability(%)
Control	100.00±8.055	100.00±4.40
Positive control (quercetin)[b]	53.19±9.82	98.78±3.17
1	68.85±2.12	102.15±4.72
2	60.54±3.68	102.28±4.45
3	56.09±2.87	104.88±5.79
Methanol extract (100 µg/mL)	72.40±4.67	107.03±3.15
Methanol extract (200 µg/mL)	58.65±3.76	99.43±2.65

[a]The results are expressed as mean values±SD (n=3).
[b]Final concentration of the compounds and the positive control (quercetin) was 10 µM.

28% inhibition activity in 100 µg/mL, but after increasing the concentration to 200 µg/mL, the inhibition activity was increased to about 41% but with a little effect on cell viability (Table 2, Fig. 2).

4. Concluding remarks

Three polyphenolic compounds have been isolated from the aerial parts of *C. spinosum* L. These compounds in addition to the methanol extract were tested to examine their ant-allergic activity through inhibition of β-hexosaminidase enzyme. The isolated compounds (**1**-**3**) exhibited inhibition activities of 31, 40 and 44 %, respectively without affecting the cell viability and hence, compound (**3**) (hydroxyquinol) is the best candidate to be considered as an anti-allergic compound. These results suggest the potential use of *C. spinosum* L. as a good and safe ant-allergic source. Therefore, it could have a supportive role in the pharmaceutical field towards the development of new drugs.

Conflict of interest

The author declares that there is no conflict of interest.

Supplementary material

Spectroscopic data of compounds **1**, **2** and **3** are available as electronic supplementary material. The online version of this article contains supplementary material, which is available to all researchers free of charge.

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