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NEW CYTOTOXIC ANTHRAQUINONE GLUCOSIDE FROM *ATRIPLEX NUMMULARIA* L.

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ABSTRACT

Objective: the present study aims to investigate the phytochemical content of *Atriplex nummularia* and evaluate their cytotoxic activity. **Methods**: The ethyl acetate extract of *A.nummularia* was subjected to fractionation using Silica gel chromatographic column and ethyl acetate methanol eluting system. The structure of the isolated compound was elucidated using ^{1}HNMR, ^{13}CNMR, COSY, HSQC, HMBC, DEPT, FT-IR and LC-MS. Cytotoxic activity of this compound was evaluated against Caco-2, HCT, HepG-2 and MCF-7 Cell lines. **Results**: A new cytotoxic anthraquinoneglucoside was isolated for the first time from *Atriplex nummularia* L. and named 1-hydroxy-3-hydroxymethyl-6-β-D-O-glucopyranosyl-anthracene-9,10-dione. It showed significant cytotoxic activity against different cell lines. The highest activity appeared against HCT followed by MCF-7.

Key words: *Atriplex nummularia*, Anthraquinone, Cytotoxic activity, Caco-2, HepG-2, MCF-7, HCT cell lines.

1. INTRODUCTION

Genus *Atriplex* belongs to the family Chenopodiaceae (Goosefoot) and includes 225 species, *Atriplex nummularia* (old man saltbush) is a halophyte shrub that grows to an average height of 2.0 m. [1]. Various species of *Atriplex* have been used for their important medicinal values, for example; *A. semibacata* and *A. vestita* have been used as an antifungal agent and

in the treatment of bronchitis [2]. *A. inflata* and *A. parvifolia* extracts were found to be effective against Herpes simplex viral infection [3]. Several species have been evaluated for their antidiabetic effects, such as *A. halimus* [4]. In traditional medicine, a cocktail of minerals in *A. halimus* is used to benefit glycemic control in diabetic patients [5]. *A. halimus* produce the polyphenols and other bioactive substances potentially useful for medicinal properties and as natural food preservation [6].

The nonpolar extract of *A. A. lindleyi* could be medically used as antibacterial agent while its polar extract which is rich inflavonoids and flavonoid glycosides is medicinally useful as antidiabetic andantioxidant drug [7].

Two phytoecdysteroids; 20-hydroxyecdysone and polypodine B were isolated from methanol extract of the seeds of *A. nummularia* [8]. Quercetin-4′-methoxy-7-glucorhamnoside, kaempferol-4′-methoxy-3-glucorhamnoside, quercetin-6,4′-dimethoxy-3-glucorhamnoside, were isolated from *A. lentiformis* [9]. Quercetin,3′-methoxy-3-gluco-rhamnoside, Quercetin-3′-methoxy-3-galacto-rhamnoside-xyloside and Quercetin,3′-methoxy-3-galacto-rhamnoside-apioside were isolated as potent estogenic flavone glycoside from *A. semibaccata*[10].

2.MATERIALS AND METHODS

2.1.Plant Material

The aerial parts *Atriplex nummularia* L. was collected from West Northern Coast, Egypt (2010). The collected plant was kindly authenticated by Dr. Ahmed Morsy Ahmed, Prof. of plant eco-physiology, Desert Research Center, Egypt. A voucher specimen is deposited at the herbarium of Desert Research Center, Cairo, Egypt. Plant material was air-dried in shade, reduced to fine powder.

2.2.Extraction

One kg of the dried powder of the aerial parts of*Atriplex nummularia* was extracted by percolation in 70% aqueous ethanol (3L). The ethanol extract of the plant was filtered and the marc was re-percolated for three times. The combined ethanol extract was concentrated under reduced pressure at a temperature not exceeding 35^{o}C to yield 91 g of dark green semisolid total extract. The total ethanol extract was suspended in distilled water and successively extracted with diethyl ether, chloroform, ethyl acetate and n-butanol to give diethyl ether, chloroform, ethyl acetate and n-butanol fractions, respectively. Each fraction was dried over anhydrous sodium sulfate and the solvent was distilled off.

Ethyl acetate fraction (7.5 g) was applied onto silica gel column (130 g, Merck) and gradiently eluted with ethyl acetate and ethyl acetate containing increasing proportions of methanol. Similar fractions were pooled together. Each subfraction was reapplied on silica gel column and gradiently eluted with ethyl acetate containing increasing proportions of methanol. Further purification was carried out using Chromatotron (preparative, centrifugally accelerated, radial, thin-layer chromatograph) model 7924T on silica gel 60 PF_{254} containing gypsum (2mm) and eluted with ethyl acetate and ethyl acetate containing increasing proportions of methanol to afford compound An30.

2.3.Acid hydrolysis and TLC of the sugar moiety

Two mg of An30 was dissolved in 2 mL of methanol: water (1:1, v/v), mixed with one mL of 2N HCl, and refluxed at 60^{o}C for 3 h. The aglycone moiety was subsequently extracted with ethyl acetate. The aqueous phase was neutralized with silver oxide then filtered. The filtrate was used to identification of the sugar moiety [11].

2.4.Measurement of Potential cytotoxicity by SRB assay

Potential cytotoxicity of the compound was tested using the method of [12] as the follows:

- Cells were plated in 96-multiwe1l plate (104 cells/well) for 24hrs before treatment with the compound to allow attachment of cell to the wall of the plate.
- Different concentration of the compound under test (5, 12.5, 25 and 50 µg/ml) were added to the cell monolayer triplicate wells were prepared for each individual dose.
- Monolayer cells were incubated with the compound for 48hrs at 37^{o}Cand in atmosphere of 5% CO_2.
- After 48hrs, Cells were fixed, washed and stained with Sulfo-Rhodamine-B stain.
- Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer.
- Color intensity was measured in an ELISA reader.
- The relation between surviving fraction and drug conc. is plotted to get the survival curve of each tum or cell line after the specified compound.

2.5.Apparatus

UV spectra were measured on UV-VIS spectrophotometer Model: V-6301. MS: Waters AQUITY UPLC-MS, LCT Premter XE MicroMASS, Masslynx 4.1was used for measurement of the mass. FT/IR: was done using JASCO FT/IR-4100typeA. Proton (^{1}H) and

carbon 13 (^{13}C-NMR) spectra were recorded on Bruker VX500 NMR spectrometer operating at 500 and 125 MHz respectively. ^{1}H-^{13}C correlations were established by using HMQC and HMBC pulse sequences respectively. ^{1}H-^{1}H correlations were determined by double quantum filtered COSY.

3.RESULTS

3.1.Compound An30: yellow powder,UV: λ_{max} (MeOH): (nm) 224, 255, 421. FTIR cm^{-1}: 3539 (OH), 3342 (aromatic CH), 2928 (CH), 1643 (C=O), 1486, 1278.LC-MS: *m/z* 431 [M-H]$^{+}$,^{1}H NMR (500 MHz, Methanol (MeOD)δ: 7.27 (1H, d, *J* = 2.3 Hz, H-2), 7.63 (1H, d, *J* = 2.3 Hz, H-4), 7.87 (1H, dd, *J* = 7.2, 2.5 Hz, H-5), 7.85 (1H,dd, *J* = 7.4, 2.5 Hz, H-7), 7.71 (1H,dd, *J* = 7.4, 2.5 Hz, H-8), 4.61 (2H, s, CH_2), 5.41 (1H, s, OH-1), 5.17 (1H, d, J=7.3 Hz, H-1$^{\backslash}$), 3.47 (2H, m, H-2$^{\backslash}$), 3.36 (1H, m, H-3$^{\backslash}$), 3.35 (1H, m, H-4$^{\backslash}$), 3.47 (1H, m, H-5$^{\backslash}$), 3.44 (2H, m, H-6$^{\backslash}$ a), 3.34 (2H, m, H-6$^{\backslash}$ b), 2.0 (4H, br.s, 4 glu-OH). ^{13}C NMR (125 MHz, MeOD) δ: 187.5 (C-9), 182.0 (C-10), 161.6 (C-1), 158.2 (C-6), 152.2 (C-3), 135.9 (C-7), 134.7 (C-12), 132.2 (C-14), 122.4 (C-8), 120.7 (C-5), 120.6 (C-2), 120.5 (C-13), 116.0 (C-4), 115.4 (C-11), 100.4 (C-1$^{\backslash}$),77.2 (C-5$^{\backslash}$), 76.5 (C-3$^{\backslash}$), 73.2 (C-4$^{\backslash}$), 69.5 (C-2$^{\backslash}$), 62.0 (C-CH_2OH), 60.6 (C-6$^{\backslash}$). HMBC correlations arecited in table 1.

3.2. Cytotoxic activity

The prescreen of An30 in one dose (5, 12.5, 25 and 50 µg /ml) effected inhibition of the growth of the cells. The percentage inhibitions are recorded in table 2. The IC_{50} of t of An30 shown in tale 3.

The IC_{50} of the isolated compound (An30) against HCT cell line was 6.77 µg/mL (Dox=3.73 µg/ml), 11.3 µg/mL against MCF-7 cell line (Dox=2.97 µg /ml),15.6 against HepG-2 cell lines (Dox=3.73 µg /ml) and 14.5 µg/mL against Caco-2 cell lines (Dox=3.58 µg /ml).

4.DISCUSSION

4.1.Compound An30 is identified as 1-hydroxy-3-hydroxymethyl-6-β–O-D-glucopyranosyl-anthracene-9,10-dione as follows: It gave positive test for anthraquinone and positive molish's test indicating its anthraquinone glycosidic nature.HR-LCMS displayed [M-1]$^{+}$ at m/z 431.197 (calculated 431.0978) and consistent with the molecular formula $C_{21}H_{20}O_{10}$.

^{13}C NMR, DEPT and HSQC experiments displayed 21 signals assigned for 2 ketonic carbons, 2 oxygenated quaternary carbons 5 quaternary non-oxygenated carbons and 5

protonated aromatic carbons. Besides, it displayed signals of 5 methines and 2 methylenes carbons. Six of these could be assigned for the sugar moiety.

Acid hydrolysis and TLC of the sugar moiety indicated that it is β-D-glucose. β-configuration of glucose was established from the coupling constant (7.3 Hz) of the anomeric proton at δ 5.17 ppm.

COSY spectrum displayed correlation of the proton 7.27 (H-2) and 7.63 (H-4) and correlation of the protons at 7.87 (H-5) and 7.85 (H-7) which, in turn, is correlated to the proton at 7.71 (H-8) and indicating tetra substituted aromatic ring.

HMBC correlation of the proton at δ4.61 ppm (H-15) with the carbons at 120.6 (C-2) and 116.0 (C-4) confirmed positioning of the hydroxyl methyl group at position 3.The correlations of the proton at 7.87, d (H-5) with carbons at 158.2 (C-6), 135.9 (C-7), 182.0 (C-10), and 120.5 (C-13) as well as the correlation of the proton at 7.85, dd(H-7) with the carbonsat 120.7 (C-5), 158.2 (C-6), 122.4 (C-8), and 120.5 (C-13) and between the proton at 7.71, d (H-8) with the carbons at 158.2 (C-6), 135.9 (C-7), 187.5 (C-9), and 120.5 (C-13) indicated that position 6 is oxygenated. This is substantiated by the multiplicity and coupling constants of these protons. β-D-glucopyranoside is attached to position 6 based on the HMBC correlation of the anomeric proton at 5.16, d with the carbon at 158.2 ppm.

Reviewing the current literatures, nothing was traced concerning the isolation of this compound or its aglycone from any natural source. Therefore, the present study is considered as the first report for the isolation of this new anthraquinone glycoside (1-hydroxy-3-hydroxy methyl-6-β-D-glucopyranosyl-anthracene-9,10-dione) natural source.

4.2.Cytotoxic activity

The isolated compound An 30 showed a pronounced activity comparable to that of Doxorubicin against the tested four cell lines especially HCT cell line. This result is substantiated by the previous investigation of aloe-emodin, which inhibited cell proliferation and induced apoptosis in both human liver cancer cell lines (HepG2 and Hep 3B) [13]. Other reports indicated that aloe-emodin produces apoptosis and cell death through S-phase arrest via promoted p53, p21 and p27, but inhibited cyclin A, E, thymidylate synthase and Cdc25A levels [14]. Aloe-emodin produces the release of apoptosis-inducing factor, endonuclease G, pro-caspase-9 and cytochrome c from the mitochondria. Aloe-emodin inhibited HeLa cells growth [15]. The cytometric analysis indicated that HeLa cells were arrested at the G2/M

phase. This effect was accompanied by the reduction in cyclin A and CDK2, and the elevation in cyclin B1 and CDK1. ALP activity is increased by aloe-emodin treatment, and associated with the inhibition of PCNA expression. In addition, aloe-emodininhibted the expression of PKC alpha and c-myc[16]. From these results,An30 may offer a potential natural anticancer agent

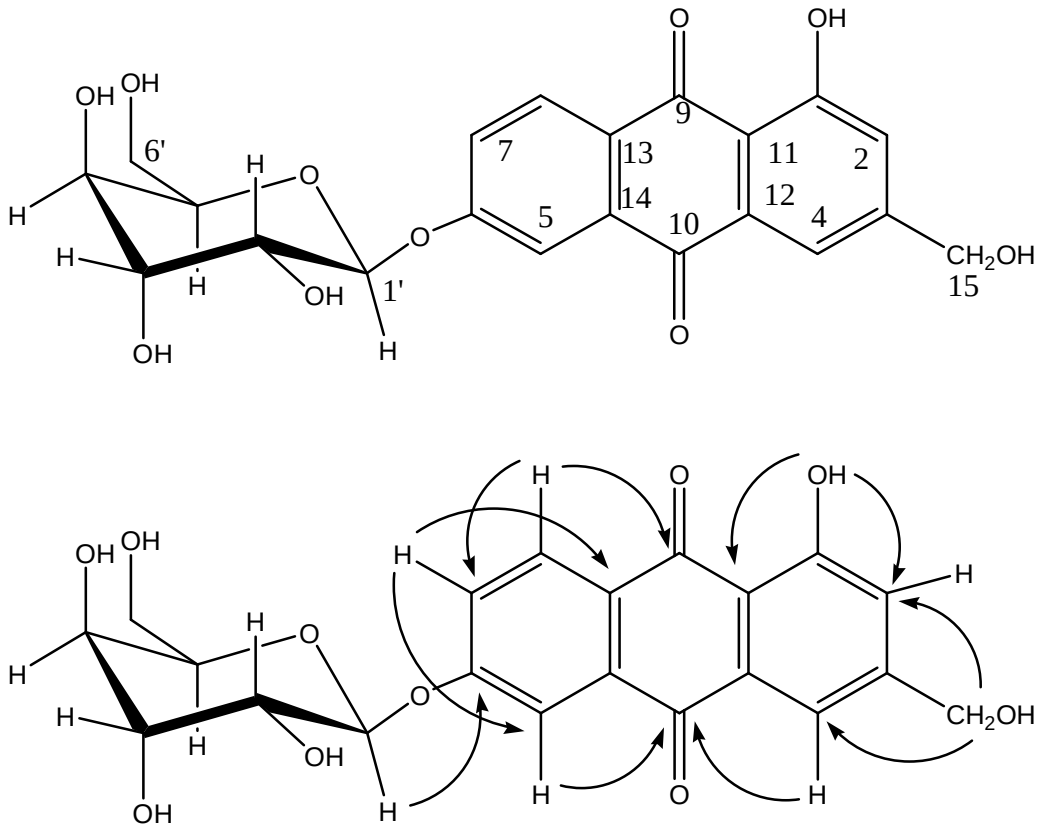

Fig. 1: Some important HMBC correlations of AN30

Table (1): Carbon chemical shift, Proton Chemical shift and HMBC Correlation of An30.

Carbon atom No.	Carbon chemical shift (Multiplicity)	Proton Chemical shift	HMBC Correlation With proton at:
1	161.6	---	2
2	120.6	7.27, d, *J* = 2.3 Hz	4, 15
3	152.2	---	2, 4
4	116.0	7.63, d, *J* = 2.3 Hz	2, 15
5	120.7	7.87, d, *J* = 2.5 Hz	7
6	158.2	---	5, 7, 8, 1’
7	135.9	7.85, dd, *J* = 7.4, 2.5 Hz	5, 8
8	122.4	7.71, d, *J* = 7.4 Hz	7
9	187.5	---	8

10	182.0	---	4,5
11	115.4	---	2
12	134.7	---	4
13	120.5	---	7,8
14	132.2	---	5,8
15 (CH_2-)	62.0	4.61, s	2,4
C1\	100.4	5.17, d, *J* = 7.3 Hz	---
C2\	69.5	3.47, m	---
C3\	76.5	3.36, m	---
C4\	73.2	3.35, m	---
C5\	77.2	3.47, m	---
C6\	60.6	3.44, m 3.34, m	---

Table 2: **Cytotoxicity of An 30 against cancer cell lines at conc. of (5, 12.5, 25 and 50 µg /ml) comparing with doxorubicin (Dox).**

Conc. (µg /ml)	Drug Cytotoxicity							
	Caco-2		HCT		HepG-2		MCF-7	
	Dox	An30	Dox	An30	Dox	An30	Dox	An30
0	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
5	0.293	0.931	0.352	0.542	0.331	0.892	0.194	0.747
12.5	0.244	0.652	0.278	0.318	0.211	0.625	0.171	0.390
25	0.2514	0.201	0.244	0.155	0.188	0.241	0.185	0.235
50	0.263	0.131	0.277	0.168	0.262	0.121	0.201	0.223

Table 3: IC_{50} of the isolated compound (An30) against cancer cell lines

	IC_{50} (µg/ml)			
	Caco-2	HCT	HepG-2	MCF-7
An30	14.5	6.77	15.6	11.3
DOX	3.58	3.73	3.73	2.77

Supporting information

^{1}H NMR and ^{1}C NMR, COSY, DEPT, FT/IR of compound **An30** are available as Supporting Information.

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Conflict of Interest: The authors declare no conflict of interest.

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