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Chemical Constituents of *Euphorbia Polyacantha* Boiss. and their Immunomodulatory Properties

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Abstract: Phytochemical investigation on the stem of *Euphorbia polyacantha* Boiss. (Euphorbiaceae) afforded a new anthraquinone named as 1, 8-dihydroxy -3- (hydroxyl methyl) -5- methoxy anthracene- 9,10- dione (**1**) along with three known compounds, aloe emodin (**2**) , 3, 4, 3′–tri-*O*-methyl ellagic acid (**3**), and 3,3′,4, 4′- tetra-*O*-methyl ellagic acid (**4**). Their structures were determined on the basis of IR, 1D- and 2D- NMR spectroscopy and mass spectrometry analysis. The effect of isolated compounds on oxidative burst of neutrophils and T-cell proliferation response was studied. Compounds **3** and **1** displayed strong oxidative burst inhibitory activity with IC_{50} 2 ± 0 and 3.5 ± 1.1 µg/mL respectively, whereas they moderately inhibited the T-cell proliferation with IC_{50} 28.5 ± 1.8 and 22.5 ± 2.0 µg/mL respectively. Compound **2** was relatively inactive with IC_{50} 43.2 µg/mL in oxidative burst and >50 µg/mL in case of T-cell proliferation. This is the first report on the phytochemical profile and biological activity of *E. polyacantha*.

Keywords: *Euphorbia polyacantha;* anthraquinone; immunomodulation; oxidative burst; T-cell proliferation.

1. Introduction

Euphorbiaceae is a large family of flowering plants account approximately for 300 genera and over 5000 species [1–2]. In Sudan, there are 7 species distributed mainly in the dry savanna zones of Kordofan and Red Sea Hills. Species of *Euphorbia* have been reported for the treatment of different ailments. Latex of some species of *Euphorbia* has traditionally been used in the treatment of skin

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diseases, gonorrhea, migraines, intestinal parasites and warts [2–3]. The plants are also used as a laxative and diuretic in different parts of the world [4].

Earlier studies have shown that some *Euphorbia* species possess inflammatory, antiarthritic, antiamoebic, spasmolytic, antiviral, hepatoprotective, and antitumor, antibacterial, and antifungal activities [5–15]. Ursolic acid isolated from *E. microsciadia* showed interesting inhibitory activity against T-cell proliferation with IC_{50} 3.1± 0.47 µg/mL [16].

In Sudan, latex of *E. polyacantha* Boiss. is used for the treatment of snake bite in traditional medicine. Scanty reports were found on this species. Thus, the present work is aimed to isolate and characterize compounds from the stems of *E. polyacantha* and to evaluate the immunomodulatory properties of isolated compounds using oxidative burst of neutrophils and PHA stimulated T-cell proliferation assays.

2. Materials and Methods

2.1. Plant Material

Stems of *E. polyacantha* were collected from Arkawit, Sudan, in February 2009. The plant was identified by Dr. Maha Kordofani and a voucher specimen (No. 2009/2G) has been deposited in Botany Department Herbarium, Faculty of Science, University of Khartoum, Sudan.

2.2 Analytical Methods

The FT IR spectra were recorded on a vector 22 instrument. UV was recorded on Unicam Helos BVI 14 model No. 053925. The ^{1}H and ^{13}C NMR spectra as well as 2D-NMR spectra (COSY, HMQC, HMBC and NOESY) were recorded on a Bruker AMX 500 MHz NMR (Avance) instruments using the UNIX data solvent. EI MS spectra were recorded on a Finnigan MAT 312. FAB mass measurements were performed on Jeol JMS HX 110 mass spectrometer using glycerol as the matrix. HREI MS was carried out on Jeol JMS 600 mass spectrometer. Column chromatography was carried out on silica gel (M and N), 70–230 mesh. Thin layer chromatography (TLC) was performed on pre-coated silica gel plates (DC-Alufolien 60 F_{254} of E. Merck) and spots were located by using ceric sulphate or methanolic KOH spraying reagents. All reagents were of analytical grade.

2.3 Extraction and Isolation Procedures

The dried ground plant material (1.5 kg) was subjected to sequential extraction in hexane (5 × 3 L); CH_2Cl_2 (5 × 3 L); EtOAc (5 × 3 L) and 80% MeOH (5 × 3 L) for 5 days. Extracts were filtered, concentrated under reduced pressure, weighed to obtain 5 g (hexane), 17 g (CH_2Cl_2), 20 g (EtOAc) and 55 g (MeOH). The EtOAc extract (20 g) was loaded on a silica gel column (45 x 2 cm) and the column was eluted with EtOAc: hexane gradient mixtures. A total number of 80 fractions (50 mL each) were collected and finally 7 fractions were obtained on combining the eluates according to their similarity behaviour on TLC. Fractions 3 (2.1 g), 5 (4.4 g), and 7 (2.0 g) were separately and repeatedly chromatographed on silica gel columns (25 x 2 cm) with gradient mixtures of EtOAc: hexane to obtain compounds **3** (25.3 mg) and **4** (8.1 mg) in EtOAc: hexane (1 : 9, v/v), compounds **2** (12.2 mg) and **1** (15.7 mg) in EtOAc: hexane (2 : 8, v/v).

Compound **1**: R_f value 0.67 (solvent system; DCM: EtOAc (9.5 : 0.5 v/v)) IR (γ_{max}, KBr): 3751. (OH), 1703, 1653 (C=O), 1510 (aromatic unsaturated), 1460, 1423 (aliphatic, C-H bond deformation), 1377, 1263 (aromatic C-O strech), 1029, 802, 671 cm^{-1}. UV λmax (MeOH) (log Ɛ): 240 (1.095), 273(1.038) nm. EIMS m/z (rel. int): 300 $[M]^{+}$, 282, 265, 253, 241, 225, 213, 197, 173, 155, 139, 121 , 105 , 83, 43. $^{1}H^{-}$ and ^{13}C-NMR: See table 1.

2.4 Biological assay

2.4.1 Chemiluminescence Assay

The luminol-enhanced chemiluminescence assay was performed as described previously [17]. Different concentrations (100 µg/mL to 1 µg/mL) of pure compounds were used for assay. 25 µL modified Hank's Solution was added to each well of the 96 well flat bottom plates except of the first row. The first well received an additional quantity of HBSS++ which made up to volume 25 µL after addition the sample solution to have the desirable concentration. Samples were mixed properly by continuous pipetting and then serially diluted in a series of 6 wells. To obtain serial dilution, 25 µL from the first well was transferred to the next well, mixed and then 25 µL from this well was transferred to next well. Each sample was tested in triplicate. Twenty-five microliter of cell suspension (1×10^6/mL) or diluted blood was added into each well. The plate was incubated at 37 °C for 30 min. 25 µL zymosan-A suspension and 25 µL luminal was added to give final volume of 100 mL in each well. The plate was immediately placed into lumiometer and scanned for 50 min at 37 °C. The lumiometer was set with repeated scan mode, 50 scans with 30 sec intervals and one second point measuring time. Data for each well containing peak value, peak time of cell activity and total integral was recorded.

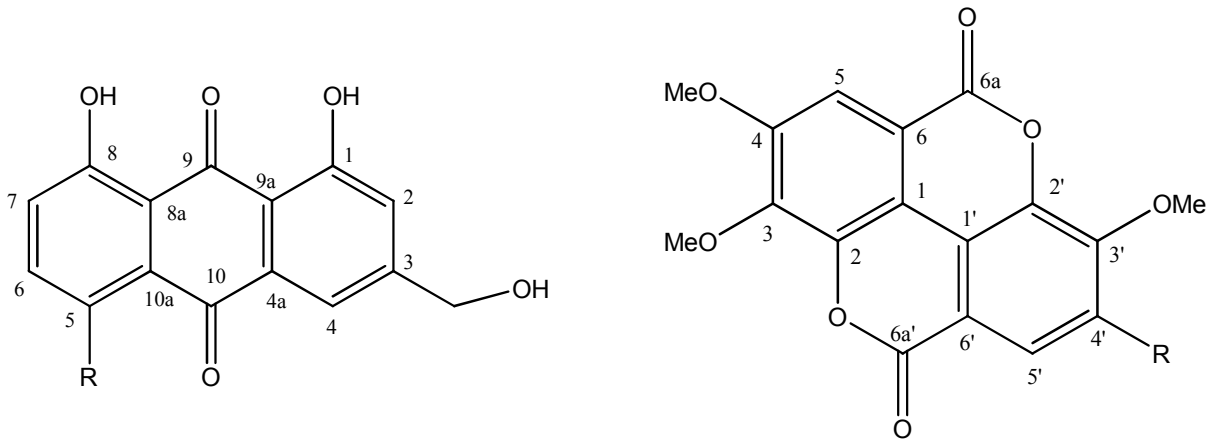
2.4.2 T-Cells Proliferation Assay

The T-cell proliferation assay was performed as described by Nielsen *et al*. [18]. In this assay isolated lymphocytes were stimulated by adding phytohemagglutinin (PHA) in culture. The rate of proliferation and survival was measured by radio-labeled thymidine incorporation method. Fresh venous blood from normal healthy volunteer was mixed with equal volume of RPMI-1640 incomplete media containing 2 mM L-glutamine. The diluted blood was then layered onto lymphocyte separation medium (LSM) and centrifuged at 400 g for 20 min at 25 ºC. The mononuclear cell buffy coat layer was removed and cells washed with incomplete RPMI-1640 and centrifuged for 10 min at 4 ºC and 300 g. The peripheral blood mononuclear cells (PBMNCs) were resuspended in supplemented RPMI-1640 with 10% fetal bovine serum (FBS). In a 96 wells round-bottomed plate, 50 µL of cell suspension (2.5×10^6 cell/mL), 50 µL of PHA with a final dilution of 5 µg/mL, 50 µL supplemented RPMI-1640 and 50 µL of test samples in a final concentration of 0.5, 5 and 50 µg/mL in triplicates were added. Plates were then incubated at 37 ºC in a humidified atmosphere of 5% CO_2 in air for 72 h. Further incubation for 18 h after the addition of thymidine [^{3}H] was done and cells were harvested using a cell harvester (Inotech Dottikon, Switzerland). Finally, proliferation level was determined by liquid scintillation counter (Beckman coulter, LS 6500, Fullerton, CA, USA).

3. Results and Discussion

3.1 Characterization of Isolated Compounds from Euphorbia polyacantha Stems

Four compounds were isolated from the ethyl acetate extract of *E. polyacantha* stems (Figure 1). The structures of the known compounds were identified by comparison of their ^{1}H- and ^{13}C-NMR and ESI mass spectra with published data as aloe emodin (**2**) [19], 3, 4, 4'-tri-*O*-methyl ellagic acid (**3**) [20] and 3,3',4,4'-tetra-*O*-methyl ellagic acid (**4**) [20].



1 R = OMe **3** R = OH

2 R = H **4** R = OMe

Figure 1. Structures of compounds **1-4**

*3.2 Characterization of Compound **1***

Compound **1** (15.7 mg) was obtained as yellow crystals and developed pink colour with KOH reagent. IR pectrum showed absorptions at 3751 (OH), 1703, 1653 (C=O), 1510 (aromatic unsaturated) cm^{-1}. UV spectrum displayed absorptions at 240 and 273 nm. EIMS showed molecular ion peak at *m/z* 300. The ^{1}H NMR spectrum (Table 1) showed a three protons singlet at δ 3.83 indicated the presence of a methoxy group. A resonance for two protons at δ 4.6 was attributed to the hydroxyl methylene protons. Four aromatic signals resonated at δ 7.23 (s), 7.30 d ($J_{3,4}$ = 8.5 Hz), 7.62 (s) and 7.90 d ($J_{4,3}$ = 8.5 Hz) were assigned to the H-7, H-3, H-5 and H-4 respectively. One phenolic proton signal at δ 12.77 was also present. The ^{13}CNMR showed signals for one methoxy carbon at δ 60.4 and one hydroxyl methylene carbon at δ 62.1, two carbonyl carbons at δ 188.82 (C-9) and 181.19 (C-10), three oxygenated aromatic carbons at δ 161.68 (C-1), 148.18 (C-5) and 158.18 (C-8) (Table 1). Structure of compound **1** was deduced by COSY and HMBC data (Figure 2). According to the above data compound **1** was assigned as 1, 8-dihydroxy -3- (hydroxyl methyl) -5- methoxy anthracene- 9,10-dione (Figure 1).

*3.3 Effect of Compounds **1**, **2** and **3** on the Oxidative Burst*

Compounds **1**, **2** and **3** have been investigated for their possible effect on the oxidative burst, ROS production of phagocytic cell (neutrophil) using the chemiluminescence (CL) technique. Results illustrated that compounds **1** and **3** showed low IC_{50} values of 3.5 and 2.0 µg/mL, respectively pinpointing their high suppressive effect whereas, compound **3** was inactive (Table 2).

*3.4 Effect of Compounds **1**, **2** and **3** on T-Cell Proliferation*

The anti-proliferation effect of compounds **1**, **2** and **3** was determined. Compounds **1** and **3** showed moderate inhibitory effect with an IC_{50} values of 22.5 and 28.5 µg/mL respectively whereas, compound **2** was relatively inactive (IC_{50} >50 µg/mL) (Table 2).

Table 1. ^{13}C and ^{1}H NMR chemical shift data (at 500 MHz in DMSO-d) of compound **1**.

Carbon No.	δ_C	δ_H (*J*, Hz)
1	161.7	-
2	120.1	7.23 s
3	152.2	-
4	115.9	7.62 s
4a	132.7	-
5	148.2	-
6	121.7	7.9 d (8.5)
7	125.1	7.32 d (8.5)
8	158.2	-
8a	115.0	-
9	180.8	-
9a	121.8	-
10	188.2	-
10a	115.9	-
5-OCH_3	60.4	3.82 s
3-$C\underline{H_2}OH$	62.1	4.59 d (5.4)
3-$CH_2O\underline{H}$	-	5.54 t (5.4)
1-OH	-	12.76 s

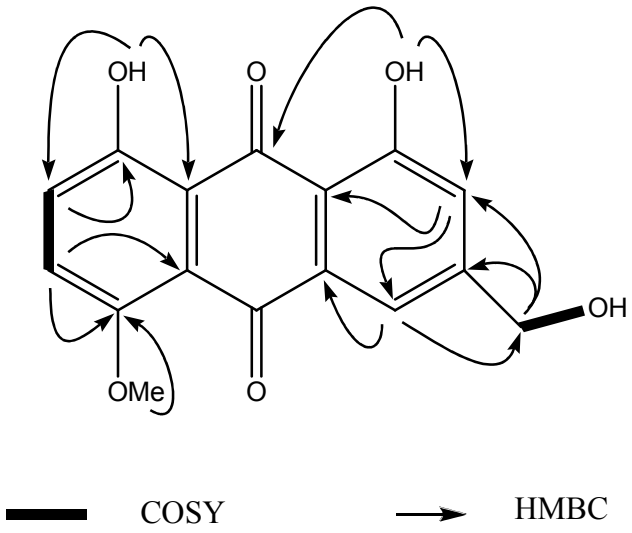


Figure 2. Key COSY and HMBC correlations of compound **1**.

4. Conclusion

This is the first phytochemical and biological study on *E. polyacantha. S*tems displayed anti-inflammatory propertise by inhibiting the cellular immune response. *In vivo* studies are highly demanded on these active compounds to understand the precise mechanism by which they exert their effects.

Table 2. Effect of isolated compounds from *Euphorbia polyacantha* stems on the the neutrophils of oxidative burst and on T-cell proliferation.

Compound	Oxidative burst on neutrophils (IC_{50} µg/mL)	T-cell proliferation (IC_{50} µg/mL)
1	3.5 ± 1.1	22.5 ± 2.0
2	43.2 ± 2.12	> 50
3	2.0 ± 0.0	28.5 ± 1.8
Ibuprofen (control)	1.2 ± 0.1	
Cyclosporine (control)		1.5 ± 0.2

Results are expressed as mean ± SD of three replicates.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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