Cytotoxic and antimicrobial activities of *Emex spinosa* (L.) Campd. extract

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Abstract: The current research was designed to evaluate the phytochemical contents, cytotoxic and antimicrobial activity of *Emex spinosa* extracts. The different plant extracts and Aloe-emodin glucoside were screened using the colorimetric MTT method (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl- tetrazolium bromide) assay to test their in vitro cytotoxic activity against HepG2, MCF-7, Caco-2 and HCT. The clinically used anticancer drug doxorubicin was used as standard for comparative purposes. Anthraquinones (Aloe-emodin-*O*-glucoside, Emodin and nataloin (1, 2, 8-trihydroxy, 6-methyl, 10-anthrone-C-glucoside) together with β-sistosterol and β-sitisterol-*O*-β-D-glucoside were isolated from *Emex spinosa.* Aloe-emodin glucoside together with four fractions from this plant were evaluated for their anticancer and antimicrobial activities. Aloe-emodin glucoside showed anticancer activity against HCT, HepG-2, MCF-7 and Caco-2 cell lines. The total ethanol extract of *E. spinosa* and diethyl ether, chloroform, ethyl acetate and butanol fractions shown antibacterial activity against *Staphylococcus aureus, Streptococcus pyogenes* and *Bacillus subtilis.*

Keywords: Anthraquinones, Aloe-emodin glucoside, cytotoxic activity, HCT, HepG-2, MCF-7, Caco-2 cell lines.

INTRODUCTION

Emex spinosa (L.) Campd. (Polygonaceae); is an annual stout herb with spreading stems and sweet roots, common in winter. *E. spinosa* is one of the important medicinal plants used to relief dyspepsia; stimulate appetite, and as a remedy for stomach disorders and to relief colic. It is believed to be purgative and diuretic (Watt and Breyer-Brandwjik, 1962). The boiled leaves of the plant are used by African tribes for the cure of dyspepsia and biliousness and to stimulate appetite (Mossa *el al.,* 1987). In a previous study, oral administration of *E. spinosa* extract in doses up 4000 mg/kg did not produce any symptoms of acute toxicity (Soliman *et al.*, 2012). Its phytochemical investigation revealed the presence of alkaloides, anthraquinones, coumarins and flavonoids (Rizk, 1986). Moreover, chrysophenol, physcion, stigmasterol and campesterol were isolated from the plant (Abdel-Fattah *et al.,* 1990). A New α-methylanthraquinone glucoside, laccaic acid *8-O*-glucoside, six known anthraquinones have been isolated from *E. spinosa* inaddition to two known flavonol glycosides (Hawas *et al.,* 2006).

The aqueous leaves ethanol extract (70%) of *E. spinosa* exhibited free radical scavenging action against 2,2-diphenyl-2-picrylhydrazyl (DPPH). Its concentration of 50% inhibition was 20.73 µg/ml. Luteolin and rutin isolated from this plant, showed strong scavenging activity (Emam *et al.,* 2010).

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E. spinosa contains substantial amounts of omega-3 fatty acids primarily, and omega-6 fatty acids mainly, suggesting that those leaves could become an important nutritional and pharmacological source of fatty acids suitable for health promotion and disease prevention (Freije *et al.,* 2013).

MATERIALS AND METHODS

Plant Material

Emex spinosa was collected from the wild plants growing at Al Arish area, Egypt (2009). The collected plant was kindly authenticated by Prof. Dr. Abd El Naser El-Gifri, Prof. of Taxonomy, Salman Bin Abdulaziz University, KSA. A voucher specimen has been deposited in the Pharmacognosy department, Salman Bin Abdulaziz University. The collected plant was dried under shade and then grinded to fine powder.

Extraction

One kg of the dried powder of *Emex spinosa* (aerial parts and roots) was extracted by percolation in 70% aqueous ethanol with occasional shaking for 72h. The ethanol extract of the plant was filtered and the marc was re-percolated for three times. Total ethanol extract was concentrated under reduced pressure at a temperature not exceeding 35^{o}C to yield a dry extract of 82g. Total ethanol extract was suspended in distilled water and extracted successively with diethyl ether, chloroform,

ethyl acetate and n-butanol to give E1, E2, E3 and E4 fractions, respectively. Each fraction was dried over anhydrous sodium sulfate and the solvent was distilled off. Chloroform and ethyl acetate fractions were combined together and applied on silica gel for column chromatography (350 g) and gradiently eluted with chloroform containing increasing proportions of methanol. Similar fractions were pooled together to obtained 5 groups. Each group was reapplied on silica gel column and gradiently eluted with chloroform containing increasing proportions of methanol. Further purification was carried out using Sephadex LH-20 columns to offer compounds 1-5.

Evaluation of cellular cytotoxicity

The total plant extract and the fractions (E_1, E_2, E_3 and E_4) as well as the isolated Aloe-emodin glucoside (AEG) were initially screened for their *in vitro* cytotoxic activity using the colorimetric MTT (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl- tetrazolium bromide) assay against HepG2, MCF-7, Caco-2 and HCT cell lines at concentration of 20 µg/ml. Doxorubicin was used as standard for comparative purposes. Stock solutions of the investigated compounds were prepared in dimethylsulfoxide (DMSO). The cell lines were grown in RPMI-1640 medium supplemented with 10% calf serum. For growth assays; cells were suspended in the medium at a density of 103–105 cells/well, seeded onto 96-well plates (200 µl/well), and incubated at 37°C in a humidified 5% CO_2 for 24 h. After that, the medium in test wells was changed to new culture medium containing the required concentrations of the tested samples, while the cell medium in control wells was changed to new culture medium containing an equivalent volume of solvent. After incubation at 37°C in a humidified 5% CO_2 atmosphere for 3 days, 100 µl of MTT (0.5 µg/ml) in serum free medium was added to each well and incubated at 37°C for an additional 4 h. Then, 200 µl of DMSO was added to each well and mixed thoroughly to dissolve the resulting formazan product. The cell viability was evaluated by measuring the optical density at 544 nm using a Microelisa Reader. The percentage of cell growth inhibition was calculated as follows:

$$\% \text{ inhibition } \frac{\text{Mean oD control-Mean oD test}}{\text{Mean oD contro}} \times 100\%$$

The dose-response relationships of the compounds effecting ≥ 50% inhibition in one dose (20 µg/ml) prescreening for each cell line were measured using concentrations of 10, 0.1 and 0.01 µg/ml, and the concentration causing 50% cell growth inhibition (IC_{50}) was calculated.

Antimicrobial activity

Test organisms

The microorganisms used for the experiments were obtained from the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt.

Gram-positive bacteria

Staphylococcus aureus (ATCC 25923), *Streptococcus pyogenes* (ATCC 19615), *Bacillus subtilis* (ATCC 9372) and *Micrococcus luteus* (NRRL B-4375).

Gram-negative bacteria

Escherichia coli (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (MTCC 618) and *Enterobacter aerogenes* (ATCC 13048)

Antibacterial testing

In vitro antibacterial activity of the total ethanol extract and the isolated fractions was studied against some Gram-negative and Gram-positive bacteria by the agar well diffusion method (Nair *et al.*, 2005). All the bacterial strains were grown and maintained on nutrient agar slants at 4°C. The total ethanol extract, fractions and the isolated compound were dissolved in dimethylsulfoxide (DMSO) to obtain 100, 50 and 10 mg/mL, respectively. The extracts were filtered over Whatman No 1 filter paper. The filtrates were sterilized by membrane filtration using 0.45 µm pore size filters DMSO was taken as a negative control. 100 µl of bacterial cultures species (incubated for 12-16 h) were mixed in molten Mueller Hinton Agar medium (Merck) and poured in pre-sterilized petri plates. A cork borer (6 mm diameter) was used to punch wells in the solidified medium and filled with 50 µl of extracts and control compound. The plates were incubated at 37°C for 24 h and the diameter of the zone of inhibition produced by each agent was measured in mm. Each sample was assayed in triplicate and the mean values were observed. The antibacterial activity was interpreted from the size of the diameter of zone of inhibition measured to the nearest mm as observed from the clear zones surrounding the wells.

Determination of minimum inhibitory concentration (MIC)

The MIC values of the isolated fractions: E1, E2, E3 and E4 were determined by agar well diffusion method against the *S. pyogenes*. Concentrations of 10, 7.5, 5.0, 2.5, 1.0 and 0.5 mg/ml of E1, E2, E3 and E4 fractions were prepared separately. The lowest concentration that inhibited visible growth of the test organisms on the agar plate after 24 h incubation at 37°C was identified as the MIC.

RESULTS

Isolated compounds

Compound 1: amorphous powder; ^{1}H NMR (500 MHz, Pyridine): δ 5.36 (1H, d, *J* = 6.0 Hz, H-6), 3.54 (1H, m, H-3), 2.27 (2H, s, H-4), 2.04 (2H, d, *J* = 2.2, H-7), 1.87 (1H, s, H-25), 1.68 (1H,s, H-20), 1.60 (2H, d, *J* = 9, (3H, s, H-19), 0.92 (3H, d, *J* = 6.4 Hz, H-21), 0.89 (3H, d, *J* = 6.0 Hz, H-29), 0.83 (3H, d, *J* = 6.0 Hz, H-26), 0.82 (3H, d, *J* = 6.0 Hz, H-27), 0.61 (3H, s, H-18). ^{13}C-NMR (125

MHz, pyridine): δ 140.76 (C-5), 121.74 (C-6), 103.1 71.77 (C-3), 56.76 (C-14), 56.04 (C-17), 51.2 (C-9), 45.8 (C-24), 42.3 (C-14), 40.5 (C-4), 39.77 (C-12) , 37.25 (C-10), 36.51 (C-1), 33.94 (C-20), 31.9(C-22), 29.7 (C-7), 29.31 (C-8), 28.94 (C-25), 26.04 (C-23), 25.43 (C-15), 24.31 (C-16), 23.8 (C-28), 21.08 (C-26), 21.08 (C-27), 19.84 (C-21), 19.41 (C-19), 12.31 (C-29), 12.2 (C-18).

*Compound 2***:** amorphous powder; ^{1}H-NMR (500 Mz, Pyridine): 5.33 (1H, s, H-6), 5.11 (1H, s, anomeric proton, H-1$^{\backslash}$), 3.11 (1H, m, H-3) 2.03-3.71 (3H, m, proton of sugar moiety), 1.28 (3H, s, H-19), 1.0 (3H, dd, *J* = 6.5, 2.5 Hz, H-21), 0.97 (3H, t, *J* =7.1 Hz, H-29), 0.89 (3H, s, H-26), 0.89 (3H, s, H-27), and 0.87 (3H, s, H-18); ^{13}C-NMR (125 Mz, pyridine): δ 148.9 (C-5), 121.91 (C-6), 103.1 (C- 1$^{\backslash}$), 79.1 (C-3), 78.99 (C-5$^{\backslash}$), 78.6 (C-3$^{\backslash}$), 75.8 (C-2$^{\backslash}$), 72.1 (C-4$^{\backslash}$), 57.3 (C-14), 56.7 (C-17), 50.8 (C-9), 46.5 (C-24), 42.4 (C-14), 39.9 (C-4), 39.8 (C-12) , 37.6 (C-10), 36.87 (C-1), 34.67 (C-20), 33.1(C-22), 32.66 (C-7), 32.53 (C-8), 29.9 (C-25), 26.1 (C-23), 25.6 (C-15), 25.2 (C-16), 23.8 (C-28), 21.6 (C-26), 21.6 (C-27), 19.3 (C-21), 19.2 (C-19), 12.3 (C-29), 12.1 (C-18).

Compound 3: Orange- yellow needle, ^{1}H NMR (125 MHz, DMSO): δ 7.49 (1H, d, *J* = 2.4 H-4), 7.22 (1H, d, *J* = 2.5, H-7), 7.18 (1H, d, *J* = 2.4, H-2), 6.75 (1H, *J* = 2.5, H-5), 2.40 (3H, s, CH3)., protons at δ 12.04 and 12.06 ppm are assignable to hydroxy protons at the positions 1 and 8 respectively. ^{13}C NMR (125 MHz, DMSO): 186.3 (C-9), 181.9 (C-10), 163.8 (C-1), 161.2 (C-8), 147.9 (C-3), 137.2 (C-12), 132.5 (C-6), 121.1 (C-11), 119.2(C-2), 115.6 (C-5), 115.4 (C-4), 106.4 (C-7).

*Compound 4***:** yellow powder, ^{1}H NMR (500 MHz, dimethyl sulphoxide (DMSO, d6) δ: 6.78 (1H, d, *J* = 2.3 Hz, H-2), 7.19 (1H, d, *J* = 2.3 Hz, H-4), 7.28 (1H, dd, *J* = 7.2, 2.5 Hz, H-5), 7.46 (1H, dd, *J* = 7.5, 8.1 Hz H-6), 7.02 (1H,dd, *J* = 7.4, 2.5 Hz, H-7), 4.85 (2H, s, CH_2), 5.0 (1H, s, OH-1), 5.16 (1H, s, H-1$^{\backslash}$), 3.47 (2H, s, H-2$^{\backslash}$), 3.36 (1H, s, H-3$^{\backslash}$), 3.35 (1H, s, H-4$^{\backslash}$), 3.47 (1H, s, H-5$^{\backslash}$), 3.45 (2H, s, H-6$^{\backslash}$), 2.0 (4H, s, 4 glu-OH). ^{13}C NMR (125 MHz, DMSOd6) δ: 187.56 (C-9), 182.1 (C-10), 161.6 (C-8), 158.2 (C-1), 152.2 (C-3), 135.9 (C-12), 134.7 (C-6), 132.2 (C-13), 122.4 (C-11), 120.9 (C-5), 120.76 (C-2), 20.6 (C-4), 115.9 (C-7), 115.4 (C-14), 100.1 (C-1$^{\backslash}$),75.9 (C-5$^{\backslash}$), 69.2 (C-4$^{\backslash}$), 68.8 (C-CH_2OH), 67.5 (C-2$^{\backslash}$), 74.1 (C-3$^{\backslash}$), 65.2 (C-6$^{\backslash}$).

*Compound 5***:** yellow powder, ^{1}H NMR (500 MHz, dimethyl sulphoxide (DMSO, d6) δ: 7.42 (1H, d, H-3), 6.57 (1H, s, H-5), 6 (1H, d, H-4), 7 (1H, m, H-6), 2.1 (3H, s, CH_3-6), 5.0 (1H, s, OH-1), 4.94 (1H, s, H-1$^{\backslash}$), 4.71 (2H, s, H-2$^{\backslash}$), 3.73 (1H, s, H-3$^{\backslash}$), 4.3 (1H, s, H-4$^{\backslash}$), 4.91 (1H, s, H-5$^{\backslash}$), 4.3 (2H, s, H-6$^{\backslash}$). ^{13}C NMR (125 MHz, DMSO d6) : δ 186.8 (C-9),160.3 (C-8), 157.5 (C-1), 134.7 (C-12), 134.6 (C-14), 134 (C-6), 123.6 (C-2),122.2 (C-3), 122 (C-4), 117.3 (C-5), 116.9 (C-7), 112.7 (C-11), 112.7 (C-13), 103.1 (C-1$^{\backslash}$)**,** 77.5 (C-5$^{\backslash}$), 75.6 (C-3$^{\backslash}$), 73.6 (C-4$^{\backslash}$), 69.7 (C-2$^{\backslash}$), 60.8 (C-6$^{\backslash}$), 54.6 (C-10), 30.6 (-CH_3).

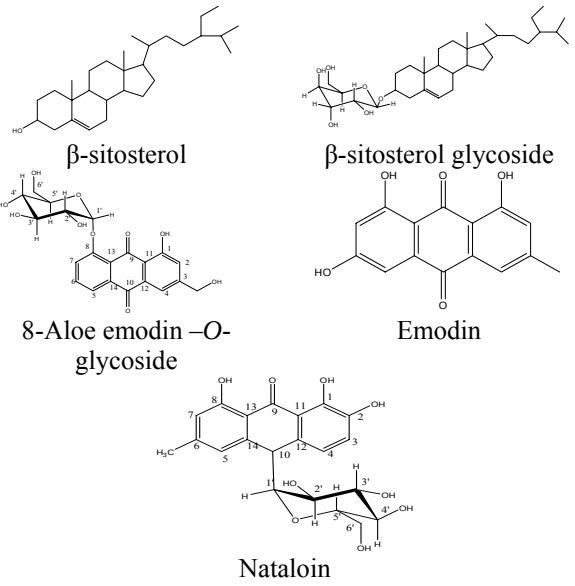

Fig. 1: Isolated compounds from *Emex spinosa*

Antimicrobial activity

The total ethanol extract of *E. spinosa*, fractions and the isolated compound were investigated for their antimicrobial potentiality against 8 clinically important bacterial strains. Results are cited in table 1. The total extract and the fractions showed activity against the tested gram-positive bacteria; *Streptococcus pyogenes* > *Staphylococcus aureus* > *Bacillus subtilis* but no activity against *Micrococcus luteus* and gram-negative bacteria was observed. MIC was determined for each fraction (table 2). The most effective fractions were E3 (MIC=1 mg/ml) > E2 (MIC= 2.5 mg/ml) > E1 = E4 (MIC= 5 mg/ml). The isolated compound (AEG) didn't show any antibacterial activity suggesting that the activity of the plant is attributed to the other components of the extract. Details of the result are shown in tables 1 and 2. The current work has shown that *E. spinosa* is a potential source of gram-positive antimicrobial agents.

Cytotoxic activity

The prescreen of the total extract, fractions and AEG in one dose (20 µg/ml) effected inhibition of the growth of the cells. The percentage inhibitions are recorded in table 3. AEG effected inhibition > 50% in all tested cell lines (HepG2=76%, MCF-7=69%, Caco-2=62% and HCT=59%). The IC_{50} of the different samples are shown in tale 4.

The IC_{50} of the isolated compound (AEG) against HCT cell line was 6.3 µg/mL (Dox=3.73 µg/ml) > 10.1 µg/mL against MCF-7 cell line (Dox=2.97 µg /ml) > 16.4 against Hepg-2 cell lines (Dox=3.73 µg /ml) > and 16.7 µg/mL against Caco-2 cell lines (Dox=3.58 µg /ml). The results also indicated IC_{50} values for fractions of total

Table 1: The antibacterial effect of the total ethanol extract of *E. spinosa* and its fractions

Microorganisms	*Diameters of the inhibition zone (mm)						
	Total extract	E1	E2	E3	E4	AEG	DMSO (Control)
S. aureus	16	20	21	21	19	-	-
S. pyogenes	28	38	41	42	38	-	-
B. subtilis	13	18	19	21	18	-	-
M. luteus	-	-	-	-	-	-	-
E. coli	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	-
K. pneumonia	-	-	-	-	-	-	-
E. aerogenes	-	-	-	-	-	-	-

*Values are mean of three replicates, Cork borer diameter = 6 mm, (-) = No inhibition

ethanol extract (E_1, E_2, E_3 and E_4) ranging from 32-45 µg/mL against all tested cell lines.

DISCUSSION

Five compounds were isolated from *E. spinosa*. Compounds were identified examining their ^{1}H-NMR, ^{13}C-NMR and as well comparison with the published data. Acid hydrolysis and TLC of the sugar part (ethyl acetate - methanol - acetic acid - water (65:15:10:10) revealed that: compounds 2 and 4 contain glucose. From this data compound (1) was identified as β-sitosterol also by comparing with published data (Maridass & Ramesh, 2010).

Table 2: Determination of MIC (mg/ml) value of fractions of *E. spinosa* against *S. pyogenes.*

Fraction / Test organism	E1	E2	E3	E4
S. pyogenes	5	2.5	1	5

E1= Diethyl ether extract, E2= Chloroform extract, E3= Ethyl acetate extract, E4=Butanol extract.

Table 3: percentage Inhibition of cell growth by extracts and isolated compound against cancer cell lines at conc. of 20 µg/ml.

Extracts	IC_{50} (µg/ml)			
	Caco-2	HCT	Hepg-2	MCF-7
E_1	21	25	26	23
E_2	22	23	26	23
E_3	22	23	24	21
E_4	21	20	25	18
AEG	62	76	59	69
Dox	74	74	80	80

From these data compound (2) was identified as β-sitosterol-*O*-β-D-glucoside by comparing our data with published by (Maridass & Ramesh, 2010).

From the given data ^{1}H NMR, ^{13}C NMR, HMBC and HMQC spectra, compound (3) was identified as emodin (1,6,8-trihydroxy-3-methylanthracene-9,10(4aH,9aH)-dione) (Singh *et al.,* 2011). From obtained spectral data; ^{1}HNMR,^{13}C NMR, HMBC, HMQC and COSY as well as comparison with the published data, compound (4) was identified as Aloe-emodin glucoside (Anand *et al.,* 2010). From obtained spectral data and ^{1}H NMR,^{13}C NMR, HMBC, HMQC and COSY as well as comparison with the published data, compound (5) was identified as nataloin (1,2,8-trihydroxy, 6-methyl, 10 - anthrone -C-glycoside), this compound was isolated for the first time from this plant.

Table 4: IC_{50} of extracts and isolated compound against cancer cell lines

Extracts	IC_{50} (µg/ml)			
	Caco-2	HCT	Hepg-2	MCF-7
E_1	37.5	34	32	45
E_2	34.5	37.5	32	36
E_3	37.5	37.5	34	39
E_4	34.5	37.5	34	39
AEG	16.7	6.3	16.4	10.1
Dox	3.58	3.73	3.73	2.97

AEG: Aloe-emodin glucoside

Antimicrobial activity

The tested extract and fractions showed varying results against the microbial strains. The total ethanol extract and fractions showed antimicrobial activity against most of the tested gram positive bacteria, except *M. luteus*. This observation supports the earlier reports that plant extracts are more active against Gram-positive bacteria than Gram-negative bacteria (Rabe and Van Staden, 1997; Vlietinck *et al.,* 1995). Zone diameters were found roughly the same for each fraction. In addition there was no antimicrobial activity of the extract and fractions against all gram negative bacteria.

The isolated compound (AEG) did not show any antibacterial activity against all the tested bacteria, suggesting that the activity of the plant is attributed to the

other components of the extract. The current work indicates that *E. spinosa* is a potential source of gram-positive antimicrobial agents especially the ethyl acetate extract, which needs more extensive investigation.

Cytotoxic activity

The isolated compound Aloe-emodin glucoside seems to be the active cytotoxic principle of *E. spinosa*, showing 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 the aglycon, Aloe-emodin, which inhibited cell proliferation and induced apoptosis in both human liver cancer cell lines (Hep G2 and Hep 3B) Kuo *et al.*, (2002). 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 Chiu et al. (2009). Aloe-emodin produces the release of apoptosis-inducing factor, endonuclease G, pro-caspase-9 and cytochrome c from the mitochondria. Guo et al., (2007) found that Aloe-emodin inhibited HeLa cells growth. 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-emodin inhibted the expression of PKC alpha and c-myc. The same results were reported by Ahirwar & Jain (2011). From these results aloe-emodin glucoside may offer a potential natural anticancer agent.

CONCLUSION

In conclusion, it has been observed that Aloe-emodin glucoside, isolated from *E. spinosa* showed anticancer activity against tested cell lines.

ACKNOWLEDGMENT

The authors extend their appreciation to the Deanship of Scientific Research at Salman Bin Abdulaziz University, Al-Kharj, KSA, for the work through the research project No. 5/Ph/1432.

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