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Bioactivity-guided isolation of two sesquiterpenes with potential anti-fungal activity from *Citrullus colocynthis* L. (Schard)

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Abstract
Fungal infection is one of the most frequent and widely distributed skin diseases of human, pets and livestock. Different pharmacological treatments have been recommended, but recently the use of some natural plant products has been emerged since they are safer to human than the chemical antifungal compounds.
Phytochemical investigation of aerial parts of *C. colocynthis* resulted in isolation of two new sesquiterpineMS-6 (Germacr-3-ol-8-en-6,12-oxy-15-oic acid) and MS-2 (5,6-[4,butyl-1,3-dioxino]-7-ene-oxecine) along with several known compounds. The two compounds have shown potent anti-fungal activity evaluated by using of Cup-Plate agar diffusion method. These compounds may provide a chemical moiety for more potent analogues.

Keywords: Sesquiterpene, *Citrullus colocynthis*, anti-fungal

1. Introduction
Over the last decade there has been a growing interest in natural products as a source of bioactive compounds which have potential role for development of some novel therapeutic agents. [1] The wide variety of chemical structures of sesquiterpene is matched by a diversity of biological activities, such as cytotoxic, anti-tumorigenic, anti-bacterial, anti-fungal [2] and antioxidant properties. [3]
Fungal infection is one of the most frequent skin diseases of human, pets and livestock, which is widely distributed all over the world with various degrees [4]. Fungus invades the superficial keratin of the skin, and the infection remains limited to this layer. [5] Fungal cell wall also contains manna's, which can inhibit the body immune response and reduce keratinocyte proliferation, resulting in a decreased rate of sloughing and chronic state of infection. [6] Different treatments have been recommended for control fungal infections. In general, pharmacological treatment option includes antifungal agents, but recently the use of some natural plant products has been emerged to inhibit the causative organisms. The antimicrobial and antitoxin properties of some plants, herbs, and their components have been documented since the late 19th century [4], They are safe to human and the ecosystem than the chemical antifungal compounds, and can easily be used by the public who used them for thousands of years to enhance flavor and aroma of foods as well as its economic value [4].
Citrullus colocynthis (L.) Schrad (family Cucurbitaceae), is well known from ancient times, Greeks and Romans. [7] It occupies the vast area extending from the west coast of northern Africa (Senegambia, Morocco and the Cape Verde islands), eastward through the Sahara, Egypt, Arabia, Persia, Balochistan and through India, as far as the Coromandel coast and Ceylon, touching northward the Mediterranean and Caspian seas. At the Red sea, near Kosseir, it occurs in immense quantities. [8]. *C. colocynthis* having anti-inflammatory activity, [9] activity against Gram-negative, Gram-positive bacteria and various Candida spp. [10] also showed significant reduction in blood sugar level. [11]

2. Materials and Methods
2.1 General
In this experiment methanolic extract (Leaves and seeds) of *C. colocynthis* was subjected to normal phase column chromatography for separation of compounds. Various fractions have been collected and purified to get the crystals of pure compounds. The chemical structure of the compounds were elucidated with the help of 500/125 MHz NMR using 1D and 2D spectral methods viz. 1H and 13C NMR, ESIMS and FABMS aided by IR spectroscopy and then

confirmed by mass spectroscopic analysis. Extracts and isolated compounds were evaluated for anti-fungal activity using the cup-plate agar diffusion method.

2.2 Plant material

Leaves and seeds of *C. colocynthis* L. were collected from North Kordofan state and Salha village South Oumdorman. The plant was identified and authenticated. Voucher specimens were deposited at the Herbarium of the Medicinal and Aromatic Plant Research Institute (MAPRI).

2.3 Micro organisms

Six species of standard fungi were used in this study (*Aspergillus Niger*, *Candida albicans, Trichophyton rubrum*, *Microsporum canis*, *Microsporum furfur* and *Trichophyton violaceum*). The organisms were supplied from National Public Health Laboratory and Microbiology lab (Central Research-Khartoum-Sudan).

2.4 Extraction

The coarsely powdered plant material (50 g) were successively extracted with sufficient quantities of petroleum ether, chloroform, methanol and water by Soxhlet apparatus. In each step the extract was filtered by Whatman filter paper and evaporated under reduced pressure (petroleum ether, chloroform and methanol extracts) or by freeze dryer for water extract, then the marc was air-dried, repacked and extracted again. The extracts were dried and stored at room temperature.

2.5 Chromatographic separation from methanol Extract

A portion (30 g) of this extract was fractionation a normal phase silica gel column eluted with petroleum ether-chloroform and chloroform–ethyl acetate mixtures of increasing polarity to give fractions 1–60. Various fractions were collected separately and matched by TLC to check homogeneity. Similar fractions (having same Rf values) were combined and crystallized. The pure compounds (MS-6 and MS-2) were obtained by purification of fraction No. 58 - which obtained by eluting of column by 80% methanol - over the preparative TLC plates in butanol: acetic acid: water (40: 10: 50) as solvent system.

2.6 Anti-fungal activity

2.6.1 Preparation of compounds

Tested compounds (2 mg) was dissolved in sterile distill water, and the volumes were completed to 20 ml (conc. 100mg/ml). The solutions were stored in Refrigerator.

2.6.2 Preparation of the test Organisms

The fungal cultures were maintained on Sabouraud dextrose agar, incubated at 25 ^{0}C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspension in 100 of sterile normal saline, and the suspension were stored in the refrigerator until used.

2.6.3 Preparation of the media

The Potato dextrose agar (PDA) was used for routine isolation and maintenance of the whole fungus in this study, and water agar agar 20% was used for single spore cultures of the beginning of the study.

(PDA) was prepared by adding 200 gm of peeled potato to 500 ml of boiled distilled water and boiled for 20-30 min, then 17 gm of agar were added to suspension till dissolved and then 20 gm of dextrose added. The media was autoclaved at 121 ^{0}C for 2 mints.

2.6.4 The Cup-Plate agar diffusion method

This method was adopted according to Kavanagh (1972) to assess the antifungal activity of compounds. [12]

One ml of standardized fungal suspension 10^8-10^9 C.F.U were through mixed with 100 ml of molten sterile PDA agar which was maintained at 45 ^{0}C. 20ml aliquots of the inoculated PDA agar were distributed into sterile Petri-dishes.

The agar was left to set in each of these plates 4 cups (100mm in diameter) were cut using a sterile cork borer (No.4) and agar discs were removed. Alternate cups were filled with 0.1ml samples using automatic micro litter pipette, and allowed to diffuse at room temperature for two hours. The plate then incubated in the upright position at 37 ^{0}C for 18 hours. Two replicates were carried out for each compound against the test organisms. Simultaneously negative control involving the addition of distil water instead of the compound were carried out separately. After incubation the diameters of resultant separated growth inhibition zones were measured, averaged and the mean values were tabulated.

3. Results and Discussion

3.1 Structure Elucidation of Compound MS-6

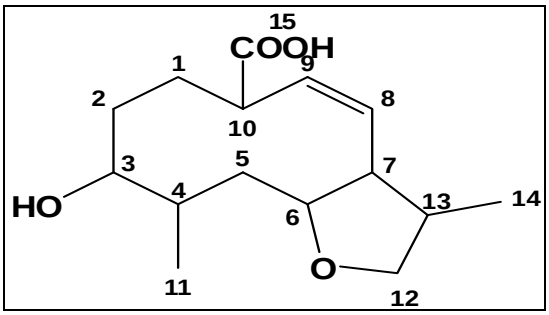
Compound MS-6was obtained as transparent powder. The UV spectrum showed a band, characteristic for conjugated benzene moiety at λmax = 275 nm.

^{1}H-NMR spectrum revealed the presence of protons signals at oxygenated methine region at δ 3.71, δ 3.62 and δ 3.33 assigned to H_3, H_6 and H_{12} protons respectively. Two signals at δ 7.75 and δ 7.65 due to olefinic protons are assigned to H_9 and H_8. Two multiplets signals at δ 1.62 and δ 1.45 are ascribed to methylated methine H_{13} and H_4 protons respectively. ^{1}H-NMR data also showed multiplets signals attributed to methylene protons at δ 1.80, δ 1.92 and δ 1.31 are assigned to H_1, H_2 and H_5 respectively. Two doublets signal at δ 0.98 and δ 0.95 ascribed to methyl H_{14} and H_{11} protons.

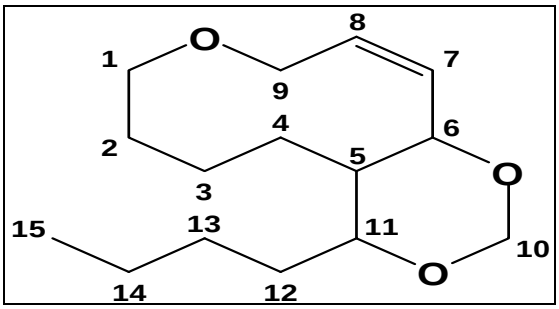
^{13}C-NMR data revealed clear signals attributed to carbonyl carbon at δ 180.10 is assigned to C_{15}, two carbons contributed in the double bonds at δ 132.44 and 129.89 are assigned to C_9 and C_8 respectively. The^{13}C-NMR data also showed intense signals attributed to eight methine signals at δ 62.15, δ 73,46 (attached to oxygen), δ 132.44, δ 129.88 (olefenic carbon), δ 69.13 (attached to carbonyl group), δ 30.76, δ 40.19 and δ 33.08 are assigned to C_3, C_6, C_9, C_8, C_{10}, C_7, C_{13} and C_4 respectively, four methylene signals at δ 72.02 (attached to oxygen), δ 31.46, δ 24.04 and δ 23.76 are assigned to C_1, C_5 and C_2 respectively, two methyl groups at δ 11.43 and δ 14.46 assigned to C_{11} and C_{14} respectively.

The ^{1}H-^{1}H Cosy spectrum of MS-6 revealed correlations between H_2 with H_3, H_4 with H_{11} and H_8 with H_9. The HMBC spectrum of MS-6 exhibited interactions of H_{11} (δ 0.95) with C_2 (δ23.76) by four bonds correlation. H_5 (δ 1.31) with C_4 (δ 30.76) by two bonds correlation, H_6 (δ 3.62) with C_3 (δ 62.15) and H_3 (δ 3.71) with C_6 (δ 73.46) by four bond correlation (W-coupling). In HSQC spectrum of MS-6, C_6 at δ 73.46 interact with H_6 at 3.62; C_3 at δ 62.15 with H_3 at δ 3.71; C_2 at δ 23.76 with H_2 at δ 1.92; C_{14} at δ 14.4 with H_{14} at δ 0.98 and C_4 at δ 30.76 with H_4 at δ 1.45.

On the bases of these evidences the structure of MS-6 has been established as Germacr-3-ol-8-en-6,12-oxy-15-oic acid ($C_{15}H_{24}O_4$).



Germacr-3-ol-8-en-6, 12-oxy-15-oic acid



5, 6-[4, butyl-1, 3-dioxino]-7-ene-oxecine

Table 1: ^{1}H and ^{13}C NMR Spectral Data of Compound MS-6, (500 MHz, MeOD):

Position	Proton	δ ^{1}H (Multiplicity, *J* in Hz)	δ ^{13}C
1	H_1	1.80	31.64
2	H_2	1.92	23.67
3	H_3	3.71	62.15
4	H_4	1.45	30.76
5	H_5	1.31	24.04
6	H_6	3.62	73.46
7	H_7	1.18	40.19
8	H_8	7.65	129.89
9	H_9	7.75	132.44
10	H_{10}	2.05	69.13
11	H_{11}	0.95	11.43
12	H_{12}	3.33	72.02
13	H_{13}	1.62	33.08
14	H14	0.98	14.46
15	-	-	180.02

Table 2: ^{1}H and ^{13}C NMR Spectral Data of Compound MS-2, (500 MHz, MeOD):

Position	Proton	δ ^{1}H (Multiplicity, *J* in Hz)	δ ^{13}C
1	H_1	3.33	60.71
2	H_2	1.3	31.55
3	H_3	1.0	28.95
4	H_4	1.0	29.24
5	H_5	1.9	38.67
6	H_6	4.64	72.32
7	H_7	7.73	130.91
8	H_8	7.64	128.35
9	H_9	3.59	62.88
10	H_{10}	3.68	67.61
11	H_{11}	4.80	71.98
12	H_{12}	1.4	30.11
13	H_{13}	1.4	22.51
14	H_{14}	1.4	22.22
15	H_{15}	0.98	12.92

3.2 Structure Elucidation of Compound MS-2

Compound MS-2was obtained as white powder. The U.V spectrum of MS-2 showed peak at λ_{max}329 nm.

^{1}H-NMR spectrum revealed the presence of protons signals at oxygenated methine region at δ 4.80 and δ 4.84 assigned to H_{11} and H_6 protons respectively. Two signals at δ 7.73 and δ 7.64 due to olefinic protons are assigned to H_7 and H_8. Three signals at oxygenated methylene region at δ 3.68, δ 3.59 and δ 3.33 assigned to H_{10}, H_9 and H_1 protons respectively. ^{1}H-NMR data also showed six multiplets signals attributed to methylene protons at δ from 1.4 to 1.0 are assigned to H_2, H_3, H_4, H_{12}, H_{13} and H_{14}. One methine signal at δ 1.9 assigned to H_5 and one signal at δ 0.92 ascribed to methyl H_{15} protons.

^{13}C-NMR data revealed clear two carbons contributed in the double bonds at δ 130.90 and 128.35 are assigned to C_8 and C_7 respectively. The^{13}C-NMR data also showed intense signals attributed to three methine signals at δ 72.32, δ 71.98 (attached to oxygen) and δ 38.67 are assigned to C_6, C_{11} and C_5 respectively, nine methylene signals at δ 67.61, δ 62.88, 60.71 (attached to oxygen), δ 31.55, δ 30.11, δ29.24, δ28.95, δ 22.51 and δ 22.22 are assigned to C_{10}, C_9, C_1,C_2,C_{12},C_4,C_3,C_{13} and C_{14} respectively, one methyl groups at δ 12.9 assigned to C_{15}.

The ^{1}H-^{1}H Cosy spectrum of MS-2 revealed correlations between H_7 with H_8. The HMBC spectrum of MS-2 exhibited interactions of H_{15} (δ 0.92) with C_{13} (δ 22.52) and C_{12} (δ 30.11) by three and four bonds correlations respectively. H_2 (δ 1.3) with C_4 (δ 29.24) by three bonds correlation. In HSQC spectrum of MS-2, C_{12} at δ 30.11 interact with H_{11} at 1.4.

On the bases of these evidences the structure of MS-2 has been established as 5, 6-[4, butyl-1,3-dioxino]-7-ene-oxecine ($C_{15}H_{26}O_3$).

3.3 Anti-fungal activity of two compounds (MS-6 and MS-2)

The two compounds exhibited a very high antifungal activity against all organisms under investigation (MS-6 having higher activity than MS-2 against most fungi) Table No-3. So the two compounds may serve as potent antifungal treatment after further safety studies or as analogue to more active compound(s).

Table 3: Antifungal activity of MS-6 and MS-2:

Fungus	Sensitivity (MDIZ)	
	MS-2	MS-6
Trichophyton rubrum	30	28
Microsporum Furfur	31	29
Microsporum canis	28	26
Trichophyton violaceum	30	27
Aspergillus Niger	16	17
Candida albicans	30	33

- MDIZ: Mean Diameter of Inhibition Zone (mm), 15mm= Sensitive, <15=Resistant or no inhibition.
- Concentration used is 10mg/ml.

4. Conclusion

Two promising sesquiterpenes (Germacr-3-ol-8-en-6, 12-oxy-15-oic acid and 5, 6-[4, butyl-1,3-dioxino]-7-ene-oxecine) from *Citrullus colocynthis* (L.) Schrad, had been isolated and characterized with potent anti-fungal activity.

5. Acknowledgment

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