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Research Article

DI-(2'-ETHYLHEXYL) PHTHALATE AND STIGMASTEROL WITH ANTI-INFLAMMATORY EFFECT FROM *CYPERUS ROTUNDUS* L

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ABSTRACT

Activity- guided isolation of anti-inflammatoryactive fractionsof*Cyperusrotundus* L. Family (Cyperaceae) led to isolation and identification of two known compounds (Di-(2'-ethylhexyl) phthalate and Stigmasterol.The anti-inflammatory effect was evaluated by Carrageenaninduced rat paw edema. Different chromatographic and spectroscopic techniques were used for isolation and identification of the two compounds.

Keywords: Cyperusrotundus, anti-inflammatory, Di-(2'-ethylhexyl) phthalate, stigmasterol.

1. INTRODUCTION

C. rotundus (Cyperaceae) is a perennial sedge that is distinguished by its thin long creeping thread-like squamous rhizome, which often produces long lines of small rosettes of leaves along the ground and swollen into white tubers that succulent when young, turning brown or black and fibrous with age. The tuber size is about the size of a hazelnut . Stems are thin triangular, nodeless and leafy only at the bottom. There is a simple or compound umbel of Spikelets at the ends of stems tops; the spikelets are linear, reddish-brown in colour flowered. In Asian countries, *Cyperusrotundus* rhizomesare used as traditional folk medicines for the treatment of spasms, stomach disorders, bowel disorders and inflammatory diseases[1].

In Chinese pharmacopoeia, it was described as an agent to regulate circulation, normalize menstruation, and relieve pain[2].

In Sudan the tubers of *Cyperusrotundus* L. are used in stomach disorders and bowels irritation. An infusion of the tubers is used in dyspepsia, diarrhea, dysentery, ascites, vomiting, cholera and fevers. The tubers are given in large doses as an anthelmintic. A poultice of the fresh tubers is used to cure wounds, ulcers and sores; it is also applied to the breast to promote the flow of milk. Paste is used in scorpion stings[3]. The methanolic extract of the tubers showed an anti-inflammatory effect for the treatment of inflammatory diseases mediated by over production of nitric oxide and superoxide[1]. Moreover, it showed significant antidiarrhoeal activity in castor oil induced diarrhea in mice[4]. The dried tubers of *C. rotundus* are used to treat dysmenorrhea and other menstrual irregularities. The aqueous extract of the dried tubers of *C. rotundus* has an inhibitory effect on the uterus, (uterine relaxation) in both pregnant and non-pregnant women, and relieving pain. The herb can stimulate gastric and salivary secretion. In addition, the aqueous extract of the dried tubers has antibacterial and antimalarial effects[2].

The growth and acid production of *Streptococcusmutans* were reduced by the tuber extract of *C. rotundus* - *S.mutans* is known as the causative bacteria in the formation of dental plaque and dental caries - Moreover, the same tuber extract inhibited the adherence of *S.mutans* to saliva-coated hydroxyapatite beads.

Glucosyltransferase enzyme, which synthesizes water-insoluble glucan from sucrose, was also inhibited by the tuber extract. So, these results suggested that *C. rotundus* may inhibit cariogenic properties of S. *mutans*[5].*n*-Hexane extract of the tubers of *C. rotundus* proved to be a new herbal supplement for controlling body weight because it induced a significant reduction in weight gain without affecting food consumption or inducing toxicity[6]. Also, *C. rotundus* extract has antihyperglycemic and antioxidant activities[7,8].

Cyperus species were thoroughly investigated for their secondary metabolites such as sesquiterpenes, quinones, flavonoids, saponins, alkaloids, phenolic acids, coumarins and steroids[2]. Approximately 1% of the tuber's weight consists of essential oils. The major components of the oil are α- and ß-cyperol (40 to 49%), α- and ß-cyperene (30 to 40%) and cyperone (0.3%)[2].

2. MATERIAL AND METHODS

2.1. Plant material

The plant collected from different regions in Sudan: Kordofan and Nuba Mountains (western Sudan), Khartoum (central Sudan) and Ingassana area (Blue Nile). The botanical identity of the plants material was confirmed by taxonomist and voucher specimens were deposited in the Medicinal and Aromatic Plant Research Institute Herbarium (Sudan).

2.2. Preparation of the plant extracts

The plant were dried under shade and then powdered. The powdered plant materials (200gram) were extracted two times with sufficient quantities of dichloromethane and 80% methanol at room temperature for 48 hour. The extracts were filtered using Whatman filter paper and the filtrates were concentrated under reduced pressure and stored at room temperature.the resulted extracts tested for anti inflammatory activity.

2.3. Carrageenan induced rat paw oedema

The anti-inflammatory effect was evaluated by Carrageenaninduced rat paw edema according to the method described by Sudhir et al. (9).\Each animal was marked on its right ankle in a circular manner using a non-erasable blue ink. The volume of each paw up to the ankle mark was then measured using Ugo-Basil plythesmometer using 0.45% Sodium chloride solution as a displacement fluid. The volume of the immersed paw was then read in the digital display. For each extract group of three animals were injected, each with a dose of a different extract in a dose of 2g/kg intra peritoneal. After 60 minutes each rat was then injected intra plantarly with 0.2% aqueous Carrageenan using a fine 1-ml hypodermic syringe. The control group was injected with the suspending agent solution intra peritoneally in a volume equivalent to the test volumes injected. After 60 minutes, Carrageenan was injected intra plantarly as described above.

Following Carrageenan injections, paw volumes to the marked sites were read at 1, 2 and 3 hours intervals. The volume of formed oedema was then calculated using the following formula:

$$\text{Percentage inhibition} = \frac{(Ct - Co)\ \text{control} - (Ct - Co)\ \text{treated}}{(Ct - Co)\ \text{control}} \times 100$$

Where Co is volume of oedema in control group and Ct is volume of oedema in test group. Net oedema volumes formed two hours following injection of Carrageenan were used to calculate the effect of the extracts on the induced oedema. Data were expressed as the mean ± SEM. Significant difference between the control and the treated groups was performed using Student's t-test and P values. The difference in results was considered significant when $P < 0.001$.

2.4. Samples preparation for anti inflammatory activity study

The plants extracts were suspended in water (0.25% Sodium carboxy methyl cellulose was used as suspending agent) followed by homogenization.

2. 5. Expermental animals

*Male Wistar rats weighing 200-220 g were used for anti inflammatory activity study.

The animals were obtained from the Experimental Animal Care Centre, College of Pharmacy, King Saud University, Riyadh. The animals were housed under constant temperature (22 ± 2ºC) and light/dark cycle (12/12).

2. 6. Fractionation

Ten grams from methanol extracts of the *Cyperusrotundus* were dissolved in methanol and dried. Re- dissolved again in 100 ml of distilled water, and fractioned three times with 15 ml of chloroform, ethyl acetate and n-butanol sequentially.

Chloroform, ethyl acetate and n-butanol fractions were evaporayed to dryness under reduced pressure, while water fractions were evaporayed by using freeze drier. All these fractions were tested for anti inflammatory activity.

2. 7. Isolation and structure determination of Di-(2'-ethylhexyl) phthalate and Stigmasterol

2.7.1. Isolation of compounds Cy F2-2-7 (Di-(2'-ethylhexyl) phthalate)and Cy F2-6-3(Stigmasterol)

Six fractions were obtained from elution of sephadex column (F1-F6). F2 was further purified by using preparative TLC and toluene: ethylacetate: formic acid (90:10:10) as solvent system and seven sub fractions were obtained (F2-1 to F2-7). F2-2 and F2-6 were further purified by preparative TLC by using Chloroform: ethylacetate: benzene in different ratios to obtain CyF2-2-7 and CyF2-6-3.

2.7.2. Nuclear magnetic resonance (NMR)

^{1}H- and ^{13}C-NMR spectra were carried out on the Bruker AM 500 and 700 spectrometer operating at 500 and 700 MHz (^{1}HNMR) in spectroscopic grade D_2O, MeOD and$CDCl_3$. The chemical shifts values are expressed in δ (ppm) units using (TMS) as an internal standard and the coupling constants (J) are expressed in Hertz (Hz). Standard pulse sequences were used for generating COSY, HMQC and HMBC spectra (2D experiments).

5 mg of the compound being tested was dissolved in 0.6 ml of suitable solvent and injected into the NMR instrument. The temperature was 296.1 K.

3. RESULTS AND DISCUSSION

3.1 Anti-inflammatory activity of investigated extracts

Carrageenan induced rat hind paw edema has been widely used for anti-inflammatory estimation of some drugs. The Carrageenan induced inflammatory process in the rat involves three phases: initial, second and third phases caused by the release of histamine and serotonin; bradykinin and prostaglandins, respectively (10,11).Both histamine and serotonin are characterized by the increase of vascular permeability. Prostaglandins mediate maximum vascular responses during the third phase of inflammation[12]. In this study, the *Cyperusrotundus* extracts gives a wide range of potency showed in **Table-1. The** methanolic extract of *Cyperusrotundus* (Rhizomes)showed good antiedematous effect (73.5±1.5 %) of inhibition ,indicating the good ability to combat with the inflammatory mediators. The Chloroform and water portionsof methanolic extract showed good anti inflammatory activity at a dose of 1gm/kg body weight, while n-butanol and ethyl acetate fractions werenon active at the same dose (**table-2).**

Table 1: Net oedema 2 hours after injection of Carrageenan and % inhibition by *Cyperusrotundus* extracts at doses of 2g/kg body weight

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Part extracted	Solvent	Net oedema volume (ml)	% inhibition
Rhizomes	Methanol	0.42±0.06	73.5±1.5
Leaves	Methanol	0.75±0.08	53.2±5.1
Leaves	Dichloromethane	1.6±0.1	Not active

*The volume of oedema formed in the control rats two hours after Carrageenan was 1.6±0.1 ml.
Values represent the mean±S.E.M. of three rats.

Table 2: Anti-inflammatory activity of Fractionated Methanolic extract of *Cyperusrotundus* (rhizomes) at dose of 1gm/kg body weight (i.p)

Type of fraction	Mean net oedema after 2 hour (ml)	% inhibition
Chloroform	0.47 ±0.02	71.9
Water	0.9 ±0.06	46.2
n-butanol	1.645 ± 0.02	Not active
Ethyl acetate	1.6552 ± 0.01	Not active

Net mean oedema after 2 hours = 1.675 ± 0.01

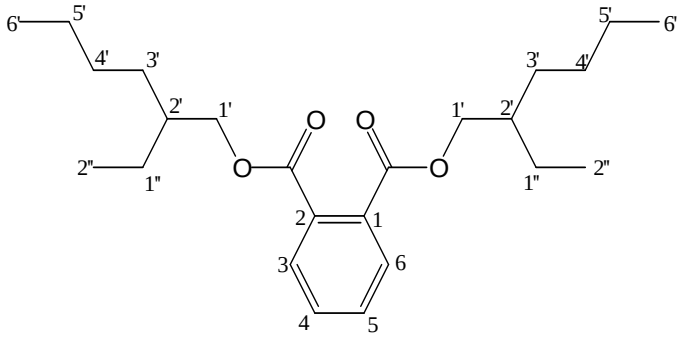
3.2. Structure Elucidation of the isolated Compounds

3.2.1. Structure Elucidation of compound Cy F2-2-7[Di-(2'-ethylhexyl) phthalate]

Compound Cy $F_{2\text{-}2\text{-}7}$was identified by ^{13}CNMR and ^{1}HNMR. Spectra showed the presence of four terminal methyls at δ 0.67 and 0.96 are assigned for $H_{6'}$,and $H_{2''}$, three downfield signals at δ 4.43, 4.29 and 3.42 are assigned for $H_{1'}$, $H_{2'}$ and $H_{3'}$, respectively. In addition, two downfield aromatic signals at δ 8.31 and 7.17 (each integrated for 2H, are assigned for $H_{3,6}$ and $H_{4,5}$ respectively).

^{13}CNMR data showed intense signals attributed to two quaternary carbons including ester carbonyl group at δ 168.9, three methine signals attributed to aromatic ring at δ 132.6, 130.5 and 128.1; five methylenes including an oxygen-bearing at δ 71.1 ($C_{1'}$), and two terminal methyl groups. Data obtained from ^{1}H and^{13}C spectrum showed that this compound has molecular formula of $C_{12}H_{19}O_4$ corresponding to MW equal to 195 which is half of the value obtained from its MS fig (1). Thus we must have twofold symmetry in the molecule with a sum formula of $C_{24}H_{38}O_4$. The higher order aromatic signals shows an AA'BB' spin system implicate an ortho di substituted benzene moiety (AA': 0.5, AB: 7.7, AB': 1.3 and BB': 7.5). Oxygentated methylene indicatd the direct attach of carbonyl group into benzene ring and two different methyl groups indicated that remaining side chain is branched octyl.

The NMR spectral data of compound Cy $F_{2\text{-}2\text{-}7}$were in good agreement with that reported for di-(2'-ethylhexyl) phthalate and. ^{13}C and ^{1}H NMR assignments of compound Cy $F_{2\text{-}2\text{-}7}$in comparison with reported data for di-(2'-ethylhexyl) phthalate are shown in Table-3[13].



Structure of Compound CyF$_{2\text{-}2\text{-}7}$[Di-(2'-ethylhexyl) phthalate]

Table 3: ^{1}H and ^{13}C NMR Assignment of Compound CyF$_{2\text{-}2\text{-}7}$ (700 MHz, MeOD) and in Comparison with the Reported Data for di-(2'-ethylhexyl) phthalate (in MeOD)[13]

Position	δ ^{1}H (Multiplicity, J in Hz)	δH (M, J in Hz) of di-(2'-ethylhexyl) phthalate (108)	δ ^{13}C	δ^{13}C of di-(2'-ethylhexyl) phthalate
1 & 2	-	-	132.6	132.5
3 & 6	8.31 (*m*)	7.53 (*m*)	130.5	130.9
4 & 5	7.17 (*m*)	7.70 (*m*)	128.1	128.7
1'	4.43	4.77 (*d*, 13.2)	71.0	68.0
2'	4.29	4.22 (*sept*)	31.6	32.2
3'	3.42 (m)	3.72 (*q*, 7.32, 6.6)	29.3	29.9
4'	1.92 (m)	1.23-130 **	29.1	28.8
5'	1.36 (m)	1.23-130 **	22.7	23.0
6'	0.90 (m)	0.91**	13.1	13.9
1''	1.66	1.23-130 **	22.2	23.7
2''	0.67	0.71	9.2	9.2
C=O	-	-	167.9	167.0

** Overlapped signals

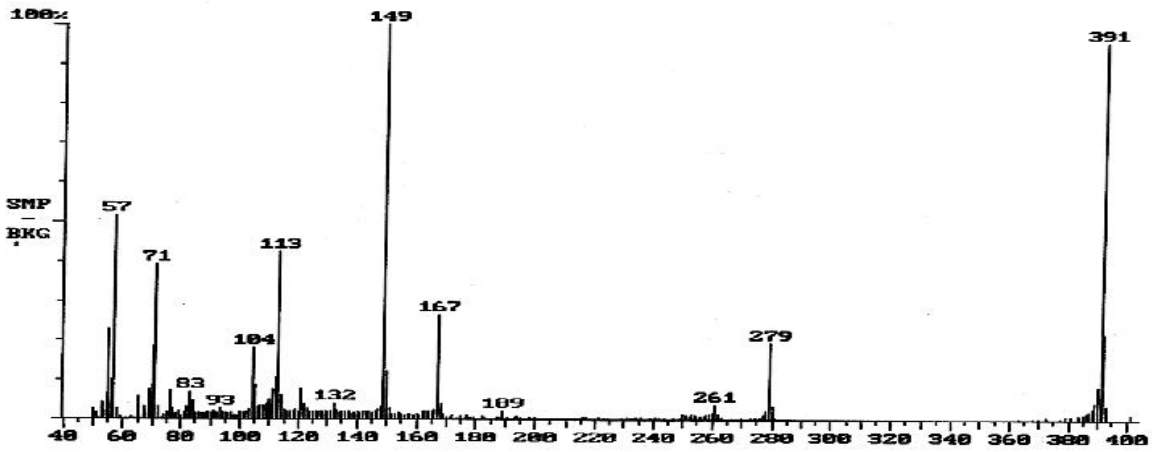


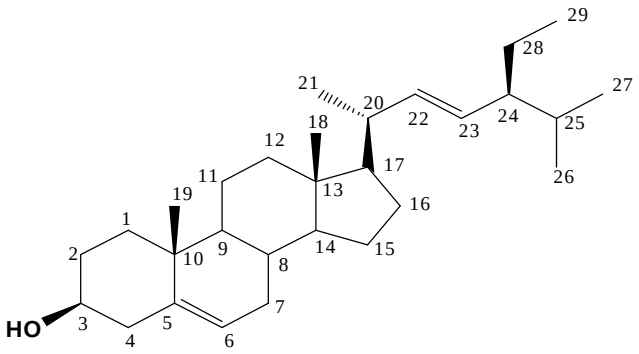
Fig. 1: Mass spectrum of compound Cy F_{2-2-7}

3.2.2 Structure Elucidation of compound CyF_{2-6-3}

Compound Cy $F_{2-6-3}$was identified by NMR (1D and 2D). The ^{1}H and ^{13}C NMR spectral data presented in Table-26 showed typical steroidal CH_3 and CH_2 signals from δ 0.73-2.40 and twenty nine carbon resonances. The ^{1}HNMR spectrum showed proton appeared as triplet of double doublet (tdd) at δ 3.55 (H_3); three olefinic protons appeared at δ 5.38 (H_6, m), δ 5.07 (H_{23}, m) and δ 5.19 (H_{22}, m); six methyl groups resonating at δ 0.73, 0.83, 0.87, 0.95, 1.04 and 1.28 ppm together with the ^{13}CNMR of six carbons at δ 12.06 (C_{18}), δ 12.27 (C_{19}), δ 19.84 (C_{21}), δ 21.23 (C_{26}), δ 18.99 (C_{27}) and δ 12.27 (C_{29}); four of the carbon signals resonated in the olefinic region at δ 140.77 (C_5), δ 138.35 (C_{22}), δ 129.28 (C_{23}) and δ 121.74 (C_6) and one oxygenated carbon resonated at δ 71.83 assigned for C_3. DEPT 135 experiment of Cy F_{2-6-3}represented nine methylene groups and seventeen methyl and/or methin groups, while DEPT 90 represented ten methine groups.

The ^{1}H-^{1}H Cosy spectrum of Cy F_{2-6-3}revealed correlations between H_3 and H_4.The HMBC spectrum of Cy F_{2-6-3}exhibited interactions of H_6 (δ 5.38) with C_4 (δ 42.22), C_8 (δ 31.67) and C_{19} (δ 36.52) by three bond correlation. H_{22} (δ 5.19) is correlated to C_{17} (δ 55.95) and C_{24} (δ 51.25) by three bonds correlation, C_{20} (δ 40.52) and C23 (δ 129.28) by two bond correlation. H_{23} (δ 5.07) interacted with C_{24} (δ51.25) by two bonds correlation and C_{20} (δ 40.52) by three bond correlation.

On the bases of these evidences the structure of Cy F_{2-6-3} has been established as Stigmasterol with molecular formula $C_{29}H_{48}O$, The identification of the Cy F_{2-6-3} was confirmed by comparing its chromatographic and spectral data (TLC and NMR) with reference sample. All of these data were in full agreement with those reported for Stigmasterol[14].



Stigmasterol

Table 4: ^{1}H and ^{13}C NMR Spectral Data of Compound $CyF_{2\text{-}6\text{-}3}$ in comparison with reported data for Stigmasterol (700MHz, $CDCl_3$)

Position	δ ^{1}H (Multiplicity, J in Hz)	δ ^{13}C	δ ^{13}C of Stigmastero[14]
1	1.9 (*m*)	37.26	37.22
2	1.32 (*m*)	29.14	31.63
3	3.55 (tdd)	71.83	71.80
4	2.28 (*m*)	42.22	42.26
5	-	140.77	140.72
6	5.38 (m)	121.74	121.71
7	1.52 (*m*)	31.90	31.87
8	1.52 (*m*)	31.67	31.87
9	0.97 (*m*)	50.13	50.10
10	-	36.52	36.48
11	1.52 (*m*)	21.08	21.07
12	1.52 (*m*)	39.68	39.66
13	-	42.31	42.26
14	1.8 (*m*)	56.87	56.85
15	1.28 (*m*)	24.37	24.34
16	1.25 (*m*)	28.94	28.91
17	0.95 (*m*)	55.95	55.93
18	1.04 (*s*)	12.06	12.03
19	0.73 (*d, 5.7*)	19.41	19.39
20	1.2 (*m*)	40.52	40.48
21	0.95 (*d*, 5.5)	19.84	21.07
22	5.19 (*m*)	138.35	138.31
23	5.07 (*m*)	129.28	129.25
24	1.6 (*m*)	51.25	51.22
25	1.28* (*m*)	28.26	31.87
26	0.83*	21.23	21.20
27	0.87*	18.99	18.96
28	1.12 (*m*)	25.42	25.39
29	1.28*(m)	12.27	12.32

* Overlapped signals.

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