Anti-inflammatory terpenoids from *Cyperus rotundus* rhizomes

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Abstract: Phytochemical investigation of the methanolic extract of *Cyperus rotundus* L. (Cyperaceae) rhizomes afforded a new norterpenoid with an unprecedented carbon skeleton, namely cyperalin A (1) and sugetriol triacetate (2). Their structures were identified by using advanced spectroscopic technique such as UV, IR, 1D (^{1}H and ^{13}C), 2D (^{1}H-^{1}H COSY, HSQC, HMBC, and NOESY) NMR, and HRESIMS as well as comparison with literature data. The isolated compounds were evaluated for their anti-inflammatory activity. Compound 1 displayed the highest inhibitory activity of PGE2, COX-2, and LOX-5 with IC_{50}s 0.22, 1.03, and 1.37 μM, respectively compared to indomethacin (IC_{50}s 0.15, 0.69, and 0.81 μM, respectively). Moreover, 2 demonstrated significant activity with IC_{50}s 0.57 (PGE2), 1.74 (COX-2) and 2.03 (LOX-5) μM.

Keywords: *Cyperus rotundus*, cyperaceae, terpenoids, cyperalin a, anti-inflammatory.

INTRODUCTION

Cyperus rotundus L. (Nutgrass, family Cyperaceae) is distributed widely in subtropical and tropical areas of the world. In the Egyptian folk medicine, its tubers are utilized as aphrodisiac, anthelmintic, diuretic, carminative, sedative, tonic, stimulant and stomachic. Also, they are used as a remedy for renal colic and dysentery (Boulos and El-Hadidi, 1984). The rhizomes have been used in Asian traditional medicine for treating diverse inflammatory diseases (Sayed *et al.,* 2007). Furthermore, the plant possessed different biological activities such as anti-oxidant, cytotoxic (Sayed *et al.,* 2008; Sayed *et al.,* 2007), antipyretic, anti-inflammatory, anti-emetic, hypotensive (Sayed *et al.,* 2001), anticonvulsant (Mayur *et al.,* 2011), anti-malarial, anti-microbial (Thebtaranonth *et al.,* 1995), anti-diarrheal (Daswani *et al.,* 2011), anti-diabetic (Sivapalan, 2013; Sayed *et al.,* 2008), hepatoprotective (Sivapalan, 2013), and insecticidal (Singh *et al.,* 2012). The former studies of *C. rotundus* led to the separation of sesquiterpenes (Kim *et al.,* 2013; Lawal and Oyedeji, 2009), flavonoids (Sayed *et al.,* 2001; Sayed *et al.,* 2007; Sayed *et al.,* 2008; Krishna and Renu, 2013), phenylpropanoids, phenolic acids (Sayed *et al.,* 2008; Zhou and Zhang, 2013; Mohamed, 2015), alkaloids (Jeong *et al.,* 2000), and saponins (Singh and Singh, 1980). Continuing the phytochemical study on *C. rotundus* rhizomes, the current work reported the separation and identification of a new norterpenoid with an unprecedented carbon skeleton: cyperalin A (1), in addition to sugetriol triacetate (2) (fig. 1). Moreover, compounds 1 and 2 were assessed for their anti-inflammatory potential, using enzyme-linked immunosorbent assay (ELISA).

MATERIAL AND METHODS

General Experimental Procedures

Perkin-Elmer 341 LC Polarimeter was utilized to record the optical rotation. JEOL 102A JMS-SX/SX spectrometer was used to get the EIMS. HRESIMS was acquired by LTQ Orbitrap. NMR spectra were measured on a Bruker DRX400 and 600. Chromatographic separations were carried out on SiO_2 60 and RP_{18}. Pre-coated plates with silica gel 60 F_{254} (0.2 mm) were used for TLC. $CHCl_3$/MeOH (9:1, S_3), *n*-hexane/EtOAc (9:1, S_2), and *n*-hexane/EtOAc (9.5:0.5, S_1) were utilized as solvent systems for developing the TLC chromatogram.

Plant material

C. rotundus rhizomes were collected in June 2014 from King Abdulaziz University campus, Jeddah, Saudi Arabia. The plant was kindly identified by Dr. Nahed Morad, Faculty of Science, King Abdulaziz University. A voucher sample (2014-CR110) was kept in the Natural Products and Alternative Medicine Department`s herbarium, King Abdulaziz University.

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Extraction and isolation

The freshly-cut rhizomes (1.2 kg) were defatted using *n*-hexane (3 × 2 L), after that they were extracted with methanol (4 × 2 L). The methanol extract had been concentrated to afford a brown residue (14.1 g). The latter was submitted to VLC using *n*-hexane: EtOAc and EtOAc: MeOH gradients to yield five fractions: CR-I to CR-V: CR-I (2.3 g, EtOAc/*n*-hexane 25:75%), CR-II (1.9 g, EtOAc/*n*-hexane 50:50%), CR-III (2.1 g, EtOAc/*n*-hexane 75:25%), CR-IV (1.8 g, EtOAc 100%), and CR-V (5.2g, MeOH 100%). The fraction CR-II (1.9 g) was subjected to SiO_2 CC (120 g × 50 × 2cm), using a gradient of *n*-hexane/EtOAc to give four sub-fractions CR-IA to CR-ID. SiO_2 CC of sub-fraction CR-IB (350 mg) using *n*-hexane/EtOAc as an eluent gave compound 1 (18 mg, colorless oil). Sub-fraction CR-ID (215mg) was separated on SiO_2 CC, using a gradient of *n*-hexane/EtOAc to obtain impure 2 which was purified by repeated RP_{18} CC eluting with MeOH/H_2O gradient to get 2 (45mg, colorless needles).

Spectral data of cyperalin A (1)

Colorless oil, R_f 0.89 [silica gel 60 F_{254} (S_2)], $[\alpha]_D$ +71.3° (*c* 0. 5, $CHCl_3$), IR (KBr): ν_{max} = 3375, 2962, 1732, 1640, 1050 cm^{-1}. NMR: see table 1. HRESIMS: *m/z* = 395.2972 (calcd. for $C_{27}H_{39}O_2$, 395.2950, $[M + H]^+$).

Anti-inflammatory activity

Isolation and of stimulation human peripheral blood mononuclear cells (PBMCs)

PBMCs were separated from the healthy donors' blood, from whom an informed consent was obtained that their blood may be utilized for scientific purposes. Separation and treatment of blood cells were performed as previously outlined (Al-Attas *et al*., 2015). The cells (1.5 x 10^6 cells/mL in supplemented DMEM) were seeded in 6-well plates. Then, they were pretreated with the tested compounds (Conc. 0.05-3.2μM) and stimulated as previously stated (Al-Attas *et al*., 2015; Virella, 1998).

COX-2 inhibitory assay

The evaluation of this mediator was carried out using ELISA kits as discussed previously (Al-Attas *et al.*, 2015; Virella, 1998).

PGE2 inhibitory assay

PGE_2 was quantified using ELISA kits as previously mentioned [32]. The assay is based on the sequential competitive binding technique in which the sample competes with horseradish peroxidase-labeled PGE_2 for a number of binding sites on monoclonal antibodies (Al-Attas *et al.,* 2015; Virella, 1998).

LOX-5 inhibitory assay

A tested compound (10 μL) in MeOH, LOX-5 (20 μL; 70 units) in 0.1 M aq, and phosphate buffer (pH 8.0) in a total volume of 160 μL were incubated for 10 min at 25°C. Then, the reaction was started by adding linoleic acid solution (10 μL; 20 μM) as a substrate, leading to the formation of (9*Z*,11*E*,13*S*)-13-hydroperoxyoctadeca-9,11-dienoate. All experiments were carried out in triplicate and analyzed with a 96-well reader (Tecan Genios microplate) as described previously (Mohamed, 2016).

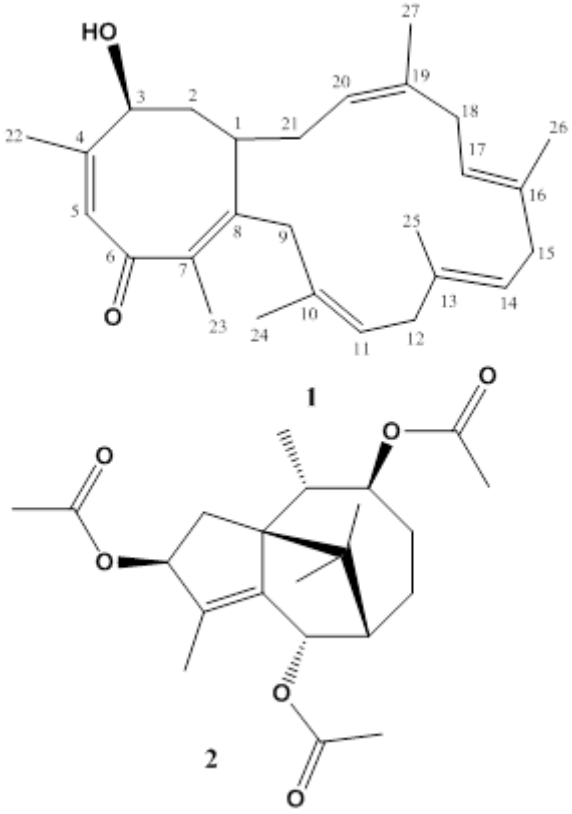

Fig. 1: Structures of the isolated compounds.

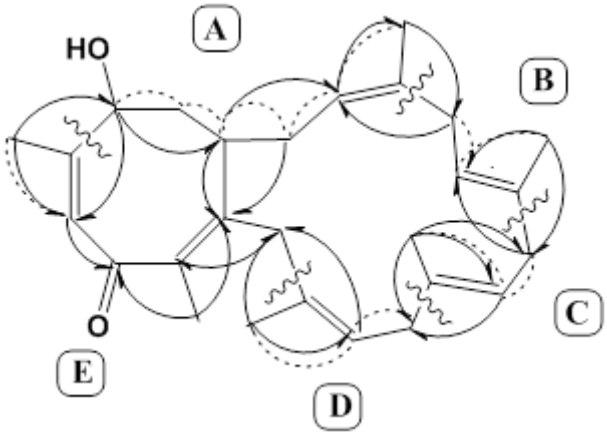

Fig. 2: Key ^1H-^1H COSY and HMBC correlations and substructures A-E of 1.

RESULTS

Compound 1 was separated as colorless oil. It had a molecular formula $C_{27}H_{38}O_2$, which was determined by HRESIMS quasi-molecular ion peak at *m/z* 395.2972 $[M + H]^+$ requiring nine degrees of unsaturation. Thus, seven of the nine double bond equivalents required by the

Table 1: NMR data of compound 1 ($CDCl_3$, 400 and 100 MHz)

No.	δ_H [mult., *J* (Hz)]	δ_H (mult.)	HMBC
1	2.13 m	43.5 (CH)	3, 8, 20, 21
2	2.53 ddd (12.8, 6.3, 5.4) 2.51 ddd (12.8, 6.8, 3.5)	41.2 (CH_2)	1, 3
3	4.10 dd (6.3, 3.5)	73.3 (CH)	1, 5
4	-	135.3 (C)	-
5	5.82 d (0.8)	126.6 (CH)	3, 6, 7
6	-	198.9 (C)	-
7	-	135.5 (C)	
8	-	163.8 (C)	-
9	2.03 m	26.5 (CH_2)	7, 10, 11, 24
10	-	131.2 (C)	-
11	5.08 tq (6.6, 1.2)	124.1 (CH)	9, 12
12	2.11 m	26.7 (CH_2)	10, 11, 14, 15
13	-	134.9 (C)	
14	5.07 tq (6.8, 0.8)	124.3 (CH)	12
15	2.06 m	39.8 (CH_2)	14, 16, 17
16	-	134.9 (C)	
17	5.09 tq (6.6, 1.1)	123.8 (CH)	18
18	1.95 m	39.6 (CH_2)	17, 19, 20
19	-	138.3 (C)	-
20	5.16 brt (5.1)	120.6 (CH)	1, 21, 27
21	2.35 m	30.7 (CH_2)	1, 8, 20
22	2.02 brs	20.4 (CH_3)	3, 4, 5
23	1.58 brs	16.2 (CH_3)	6, 7, 8, 9
24	1.58 brs	16.0 (CH_3)	9, 10, 11
25	1.57 brs	15.9 (CH_3)	12, 13, 14, 15
26	1.66 d (1.1)	25.7 (CH_3)	15, 16, 17, 18
27	1.62 brs	17.6 (CH_3)	18, 19, 20
3-OH	2.74 brs	-	-

Table 2: Results of PGE2, COX-2, and LOX-5 inhibition assays

	IC_{50} (μM)		
	PGE2	COX-2	LOX-5
Indomethacin	0.15	0.69	0.81
Compd 1	0.22	1.03	1.37
Compd 2	0.57	1.74	2.03

molecular formula was encountered by six olefinic double bonds and a carbonyl group, indicating 1 was a bicyclic molecule. The IR spectrum of 1 showed absorption bands at 3375, 2962 and 1732 cm^{-1}, corresponding to hydroxyl, C-H aliphatic and ketone carbonyl groups, respectively. DEPT, ^{13}C NMR, and HSQC spectra implied the existence of 27 carbons: 6 methyls, 6 methylenes, 7 methines, including an oxymethine at δ_C 73.3 (C-3) and five *tri*-substituted olefinic double bonds and 8 quaternary carbons one of them was assigned to a ketone carbonyl (δ_C 198.9, C-6). The ^1H NMR showed methylene proton signals at δ_H 2.53 (H-2A) and 2.51 (H-2B), which correlated with an oxymethine proton at δ_H 4.10 (H-3) and a methine proton at δ_H 2.13 (H-1) in the COSY spectrum (table 1).

Furthermore, a COSY correlation was noticed between H-1 and a methylene group at δ_H 2.35 (H-21), which showed cross peak to an olefinic proton of a *tri*-substituted double bond at δ_H 5.16 (H-20) (substructure A, fig. 2). The HMBC cross peaks of H-1/C-3, C-19, C-20 and C-21, H-3/C-1, H-2/C-1 and C-3, and H-27/C-19 and C-20 confirmed this substructure. In the COSY spectrum, a triplet quartet olefinic proton at δ_H 5.09 (H-17) correlated with a multiplet methylene group at δ_H 1.95 (H-18) and showed allylic coupling with the methyl signal at δ_H 1.66 (H-26) to give substructure B (fig. 2). They had cross peaks to the carbons, resonating at δ_C 123.8 (C-17), 39.6 (C-18), and 25.7 (C-26) in the HSQC, respectively. Substructure B was established by the HMBC cross peaks of H-26 to C-16, C-17 and C-18 and H-17 to C-18.

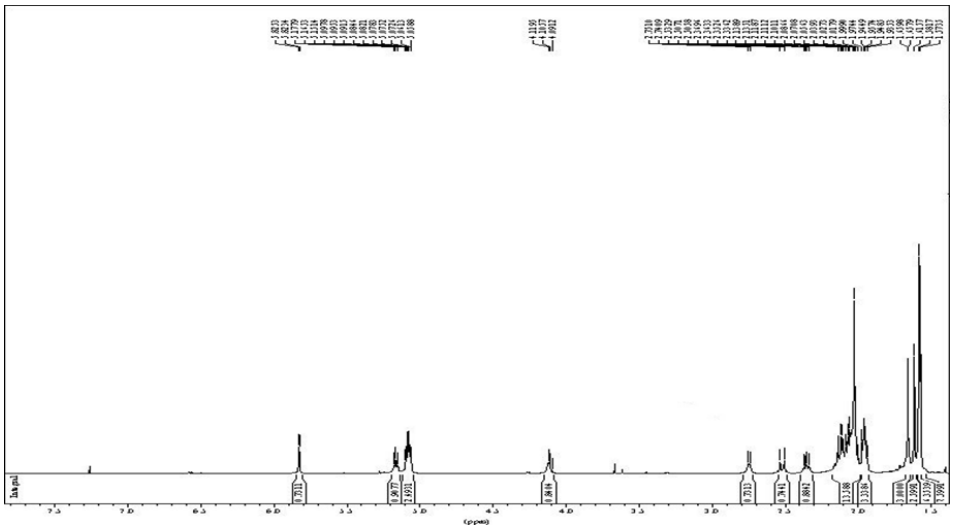

Fig. S1: ^{1}H NMR spectrum of compound 1 (400 MHz, $CDCl_3$).

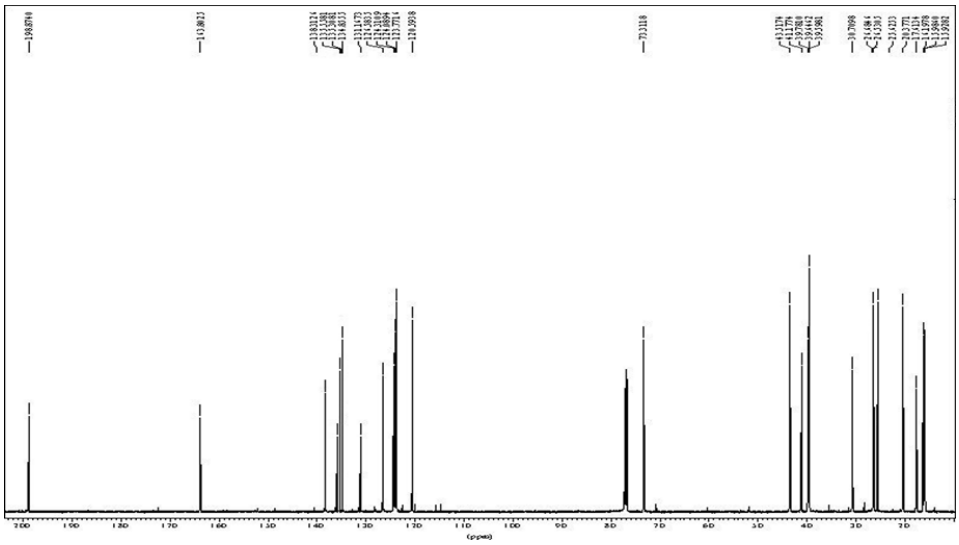

Fig. S2: ^{13}C NMR spectrum of compound 1 (100MHz, $CDCl_3$).

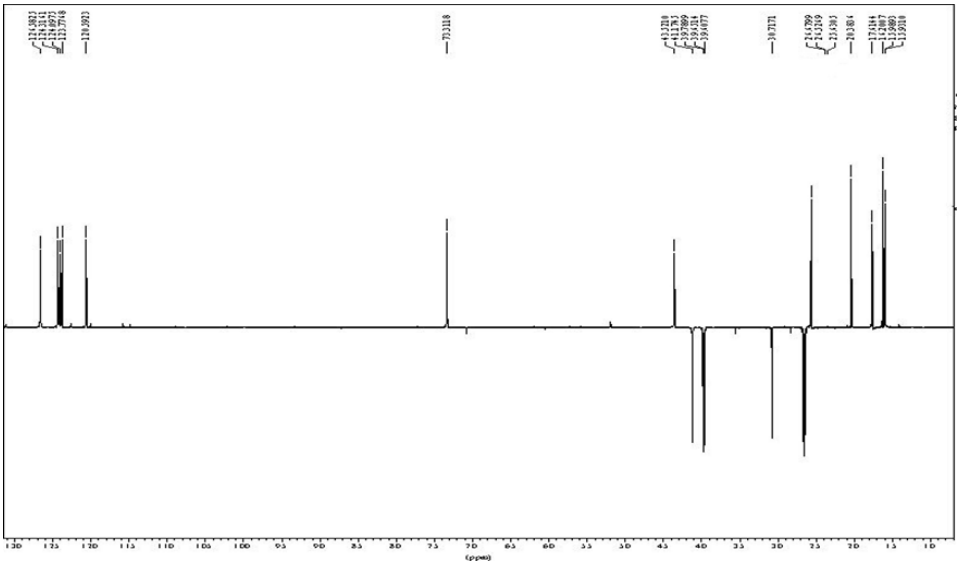

Fig. S3: DEPT ^{13}C NMR spectrum of compound 1.

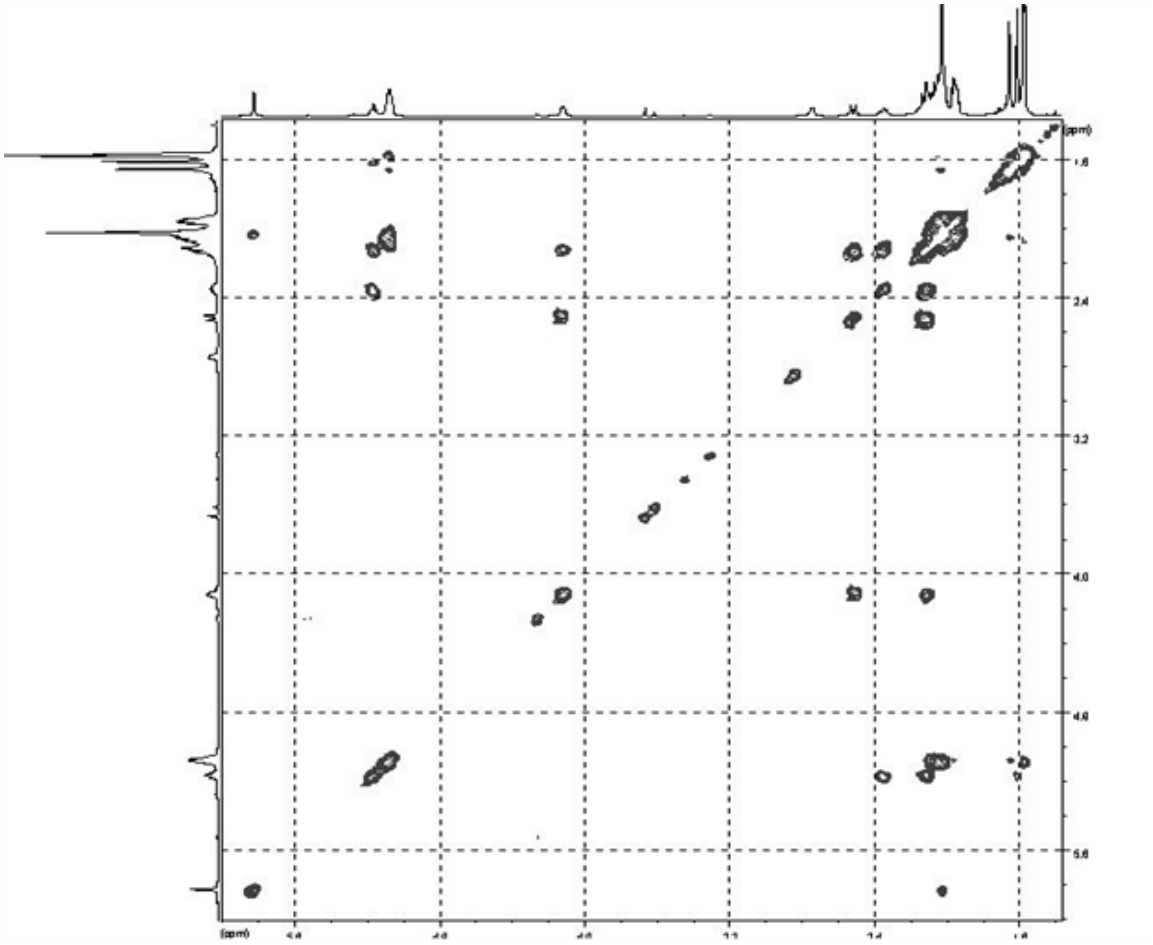

Fig. S4: ^{1}H-^{1}H COSY spectrum of compound 1.

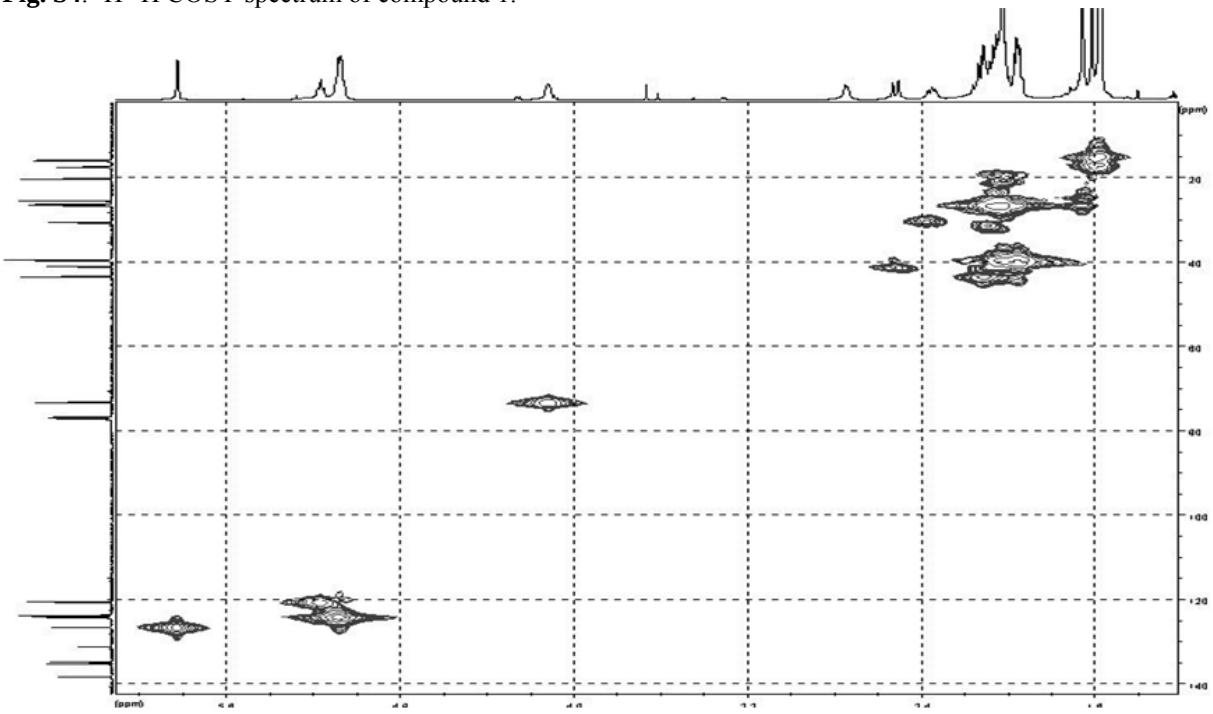

Fig. S5: HSQC spectrum of compound 1.

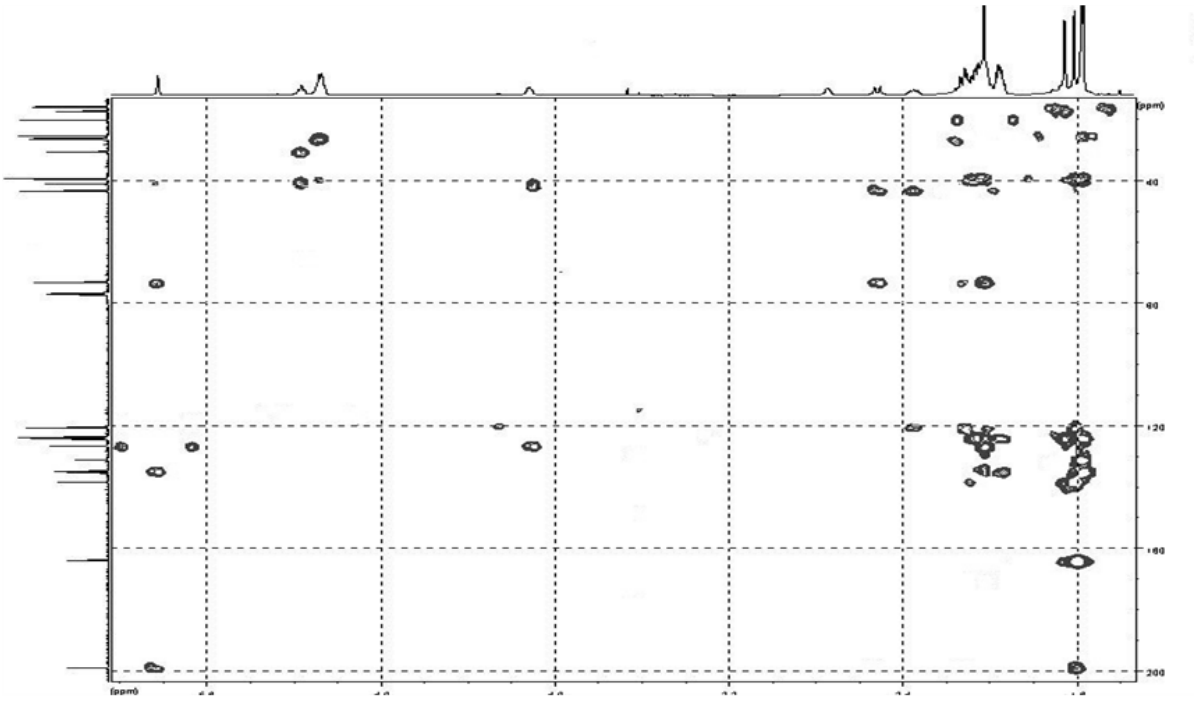

Fig. S6: HMBC spectrum of compound 1.

The HMBC cross peaks of H-18/C-20 and C-19 and H-27/C-18 assured the linkage of substructures A and B. Additional spin system, consisting of a methylene group (δ_H 2.06, H-15), an olefinic proton (δ_H 5.07, H-14), and a methyl group (δ_H 1.57, H-25) was observed in the COSY spectrum (substructure C). This was assured by the HMBC correlations. The connection of substructure C to B was ensured by the HMBC correlations of H-26/C-15 and H-15/C-16 and C-17. The COSY correlations from H-11/H-12 and H-24 and the cross peaks of H-12 and H-24/C-10 and C-11 and H-11/C-12 in HMBC supported the assignment of substructure D. The HMBC correlations of H-14 and H-25 to C-12 established the connectivity between substructures D and C at C-13. Moreover, the ^1H and ^{13}C spectra showed methylene group at δ_H 2.03 (H-9)/δ_C 26.5 (C-9), *tri*-substituted double bond at δ_H 5.82 (H-5)/δ_C 126.6 (C-5), 2.02 (H-22)/20.4 (C-22) and 135.3 (C-4), and *tetra*-substituted double bond at δ_H 1.58 (H-23)/16.2 (C-23), 135.5 (C-7), and 163.8 (C-8). The ^{13}C NMR spectrum showed a signal at δ_C 198.9 (C-6), indicating the presence of a carbonyl ketone and this was further assured by the IR band at 1732 cm^{-1}. The cross peaks of H-9/C-7, H-23/C-6, C-7, and C-8, H-22/C-4 and C-5 in HMBC gave CH_2-C=C-CO-CH=C-CH_3 moiety (substructure E). The connectivity of substructure E to D at C-10 was evident by the HMBC correlations of H-11/C-9 and H-9/C-11 and C-10. Substructures A and E were linked on the basis of the HMBC correlations from H-3/C-5 and H-22/C-3. The formation of a bicyclic structure at C_1-C_8 in 1 was assured by the HMBC correlations of H-1 and H-21 to C-8. The *Z* geometry of the olefinic bonds was assigned by comparison of *J* values and chemical shifts with literature (Khedr *et al.,* 2016; Mohamed *et al.,* 2014; Elkhayat *et al.,* 2013). This was ensured by the observed NOESY correlations of H-5/H-11, H-14, H-17, H-20, and H-22, H-20/H-5 and H-27, H-11/H-20 and H-24, H-14/H-5 and H-25, and H-17/H-5, H-20, and H-26. The NOESY correlation of H-1 to H-3 as well as their small *J* values indicated their α-orientation. Thus, 1 was unequivocally defined and named cyperalin A. it is noteworthy that this is the first report for the isolation of a norterpenoid with this carbon skeleton from nature.

Sugetriol triacetate (2) was characterized by analyzing the spectroscopic data and comparing them with literature (Sayed *et al.,* 2001, Hikino *et al.,* 1967). The inhibitory activities of 1 and 2 were assessed towards COX-2, PGE_2, and LOX-5, using enzyme-linked immunosorbent assay (table 2). Interestingly, 1 displayed the highest inhibitory activity against PGE2, COX-2, and LOX-5 with IC_{50}s 0.22, 1.03, and 1.37 μM, respectively compared to indomethacin (IC_{50}s 0.15, 0.69 and 0.81 μM, respectively). In addition, 2 demonstrated significant activity with IC_{50}s 0.57 (PGE2), 1.74 (COX-2), and 2.03 (LOX-5) μM.

DISCUSSION

Inflammation is a defense reaction of the body and a local response of living tissues to injury aimed at eliminating or limiting the spread of an injurious agent (Patgiri *et al.,*

2014). The utilization of medicinal plantsor their active metabolites is becoming a progressively attractive aspect for treating diverse inflammatory disorders (Vitor *et al.,* 2009). The anti-inflammatory capacities of various medicinal plants can be attributed to the presence of various substances: triterpenoids, flavonoids, tannins, alkaloids, saponins and anthraquinones which act as inhibitors of pro-inflammatory mediators and molecular targets in inflammatory responses (Mohamed *et al.,* 2014; Soumaya *et al.,* 2013). The anti-inflammatory potential of the MeOH extract of *C. rotundus* was attributed to the inhibition of prostaglandins, serotonin, and histamine synthesis (Sayed *et al.,* 2001). It is noteworthy to report that sugetriol triacetate (2) was tested here for the first time for its anti-inflammatory activity. It was reported that terpenoids have shown anti-inflammatory activities through inhibition of lipooxygenases and cyclooxygenases, which are responsible for eicosanoids generation. Thus, they reduce the inflammatory mediators' concentrations as leukotrienes and prostaglandins. Also, they prohibited the activation of NF-κB target genes, as those for the cytokines, IL-1α, IL-1β, IL-8, IL-6 and TNF (Al-Attas *et al*., 2015; Virella, 1998).

CONCLUSIONS

A new norterpenoid, namely cyperalin A (1) and sugetriol triacetate (2) were separated from *C. rotundus* rhizomes. Their structural elucidation was achieved with the aid of extensive spectroscopic techniques. They possessed a significant anti-inflammatory activity. The obtained results supported and justified the traditional uses of *C. rotundus* as a wealthy source of bioactive molecules to treat various inflammatory diseases. Moreover, the extract`s constituents may alleviate rheumatism and represent an additional advantage of repressing inflammatory response initiated by tissue injury when the rhizomes are utilized in folk treatment of wounds and arthritis.

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