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ORIGINAL ARTICLE

Free Radical Scavenging, α-Glucosidase Inhibitory and Anti-Inflammatory Constituents from Indian Sedges, *Cyperus scariosus* R.Br and *Cyperus rotundus* L.

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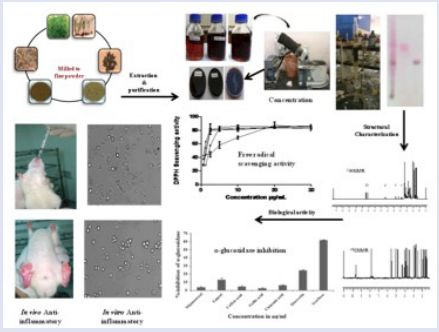
ABSTRACT

Background: *Cyperus scariosus* R. Br and *Cyperus rotundus* L are widely used in ayurvedic preparation for the treatment of diabetes and other diseases. The early literature ,so far, does not indicate the presence of any bioactive principle isolated from these plants. **Objective:** To identify free radical scavenging, anti-diabetic and anti- inflammatory principles from these two species. **Materials and Methods:** The bioassay guided fractionation and isolation of active constituents was done by chromatographic techniques. They also evaluated their anti-oxidant activity by DPPH and ABTS. The anti-diabetic activity was screened by α- glucosidase and α- amylase assays.Also, the further evaluation of *in vitro* anti-inflammatory activity using THP-1 monocytic cells and *in vivo* anti- inflammatory activity, was confirmed by carrageenan induced rat paw edema as model. **Results:** The activity guided isolation led to isolation of twelve compounds Which are: Stigmasterol[1], β- sitosterol[2], Lupeol[3], Gallic acid[4], Quercetin[5], β- amyrin[6], Oleanolic acid[7], β- amyrin acetate[8], 4- hydroxyl butyl cinnamate[9], 4- hydroxyl cinnamic acid[10], Caffeic acid,[11] and Kaempferol[12] respectively. Among the isolates, the compounds 4 and 5 displayed potent radical scavenging activity with an IC_{50} values of 0.43 and 0.067 µg/ml. The compounds 4, 5 and 10 showed significant anti-diabetic activities. while lupeol[3] showed potent IL-1 β activity inhibition in THP-1 monocytic cells and also displayed significant ($p<0.0025$) *in vivo* anti-inflammatory activity. **Conclusion:** Inbrief, we isolated twelve compounds from both the species. Collectively, our results suggested that aromatic compounds showed good anti-oxidant and anti-diabetic activities.

Key words: Alpha amylase, alpha glucosidase, antioxidant activity, IL-1β inflammation, *Cyperus scariosus* and *Cyperus rotundus*.

SUMMARY

- The study investigates the free radical scavenging, α-glucosidase inhibitory and anti-inflammatory effects of constituents isolated from Indian sedges viz. *C. scariosus* and *C. rotundus*. The results indicated that phenolic compounds displayed potent fee radical scavenging activty and alpha-glucosidase inhibition activity. While terpene constituent, Lupeol[3] showed good IL-1 β activity inhibition in THP-1 monocytic cells and also displayed significant ($p<0.0025$) *in vivo* anti inflammatory activity in carrageenan induced rat paw edema. However, further studies are required to know the exact molecular mechanism.



Abbreviations used: DPPH: 2,2- Diphenyl-1-1-picryl hydrazyl, ABTS: 2,2-Azinobis-3-ethylbenzo thiazoline-6-sulfonic acid, THP-1: Human leukaemia monocytic cell line, IL-1β: Interleukin-1β, IC_{50}- Inhibitory concentration 50%.

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INTRODUCTION

Human body has an antioxidant defence system towards free radical, but over production of free radicals cause imbalance condition between the free radical and antioxidant defence in the body that leads to several diseases including inflammation, aging and diabetes.[1] In the case of diabetes, alphaglucosidase is an enzyme that catalyzes the decomposition of disaccharides to glucose for the absorption into the blood stream, in small intestine. Therefore, alphaglucosidase inhibitors could regulate abnormally high stages of plasma glucose after carbohydrate ingestion.[2] Presently, synthetic α- glucosidase inhibitors such as acarbose has been accepted for clinical use in the treatment of type-2 diabetes.[3] On the other hand, a number of biological agents have been developed to treat inflammation, including agents that reduce the activity of specific cytokines or their receptors, block lymphocyte trafficking into tissues, prevent the binding of monocyte- lymphocyte to stimulatory molecules.[4] Many of the existing synthetic anti-diabetic and anti-inflammatory drugs can cause several side effects such as flatulence, diarrhoea, cardiovascular diseases, gastrointestinal, and urinary tract infections.[5,6]

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In recent years, search for novel therapeutic agents and approaches for diabetes and inflammation is still an active research field stimulated by the discovery of new biological targets, and by the possibility of obtaining new bioactive compounds with multiple biological activities. The past decade has witnessed an increasing interest in search of plant based lead compounds for the development of new pharmaceuticals.[7] In this regard, plants of the cyperaceae family which comprises a group of more than 5000 different species, distributed in tropical and subtropical areas of the world have attracted the scientific interest because of their wide range of biological activities such as anti-oxidant, antidiabetic, anticancer, antibacterial, antiviral, anti-inflammatory, anti-allergic, and vasodilatory actions.[7-13] Among the plants of Cyperaceae family, *Cyperus scariosus* R. Br and *Cyperus rotundus* L, are prominent species which are extensively used in the traditional systems of medicine for the treatment of various ailments. Herbal mixtures of both the species are used to enhance microbial protein synthesis in the rumen of buffaloes. These mixtures are anti-cancer, anti-diabetic, anti-inflammatory activity, suppresseage formation and prevent wrinkles, protein oxidation, antino-ciceptive and, anti-hyperglycemic activities.[14-17] In spite of the various pharmacological uses of *C. scariosus and C. rotundus* extracts, little is known about their chemical constituents. Previous studies on this species have resulted in the isolation of sesquiterpenes, flavonoids, triterpenoids, and steroids.[18-20] Our continuing efforts are directed towards identification of bio active compounds from the Indian medicinal flora. Recently, we reported morphological and chemoprofiling comparisons of various solvent extracts of these two species *C. scariosus* and *C. rotundus* using analytical techniques.[21] Consequently, in line with this interest, the current study reports on the presence of free radical scavenging, alpha glucosidase inhibitory and anti-inflammatory activity constituents. These results are reported for the first time in the crude extracts of Indian sedges. Based on these results, we also discussed structure activity relationship of the isolates.

MATERIALS AND METHODS

General procedure

The solvents used were all of analytical grade and purchased from local distributor. The thin layer chromatography was performed on precoated silica gel glass plate 60 F_{254}. Visualization was performed using chemical spray reagent solution, followed by charring on hotplate. Column chromatography was carried out using silicagel 60-120, 100-200 and 230-400 mesh (quingdao Marine chemical, China). Melting points were recorded on a fisher scientific melting point apparatus. FT-IR spectra was recorded on Thermo Nicolet Nexus 670 FT-IR spectrometer (Thermo fisher).^{1}HNMR and[13] CNMR spectra were recorded on Bruker spectrometer. ESI-MS was measured on LC-MS-Trap-SL- instrument. DPPH, ABTS, Piroxicam, phorbol-12-Myristate-13- acetate (PMA) were purchased from sigma aldrich.

Plant material collection, identification and extraction

The whole plants of *C. scariosus* were collected from the Sri Satyadeva nursery, Kadiyam, East Godavari district, Andhrapradesh and *C. rotundus* plants were collected from rice fields. These plants were taxonomically identified by Dr. A. Prasada Rao, Taxonomist, KLUniversity, Vaddeswaram, Guntur, Andhra Pradesh, India. The voucher specimens were deposited at KLUniversity, botanical garden, voucher specimen number (KLU-1250 and KLU-1251) for further use. The rhizomes of these herbs were separated, washed, shade dried and grounded to fine powder. The air dried and powdered rhizomes of *C. scariosus* (5 kg) and *C. rotundus* (5 kg), were sequentially extracted with hexane, chloroform, and methanol by cold maceration for 72 hours. Following extraction, the solvents from each extract were concentrated under reduced pressure to the dryness in a rotary vaccum evaporator.

Phytochemical chemical investigation of rhizomes of *C. scariosus*

The crude hexane extract of *C. scariosus* (10 gm) was subjected to silica gel column chromatography and eluted successively with hexane: ethyl acetate (100:0, 90:10, 80:20, 70:30, 60:40 and 50:50) mixture in the increasing order of polarity. The column eluted fractions were collected and concentrated. The purity of eluted fractions were analyzed by TLC (solvent system hexane: EtOAc 90:10) and fractions with similar R_f value were combined to get six major fractions (*Viz*: F_1, F_2, F_3, F_4, F_5 and F_6). The repeated purification of resultant fractions led to the isolation of two compounds [1 and 2]. Similarly, 10 gm's chloroform extract of *C. scariosus* was subjected to column chromatography on a silica gel column, and eluted with a step wise gradient of chloroform: acetone. Initially, the column was eluted with plain chloroform. Then ,the column was eluted with chloroform: acetone, (100:0, 90:10, 80:20, 70:30, 60:40 and 50:50) mixture in the increasing order of polarity. The collected fractions purity were analyzed by TLC (mobile phase chloroform: acetone 70:30). These fractions with similar TLC patterns were combined to give six major fractions (*Viz*: F_1, F_2, F_3, F_4, F_5 and F_6),and the repeated purification of resultant fractions led to the isolation of compound **3** in addition to the compounds **1** and **2**. Likewise, the methanol extract (10 gm) of *C. scariosus* was loaded on a silica gel column and elution was carried out with a step wise gradient of chloroform: methanol. Initially the column was eluted with plain chloroform, then it was eluted with chloroform: methanol, (100:0, 98:02, 96:04, 95:05 and 90:10) mixture in the increasing order of polarity. All these column fractions were analyzed by TLC (silica gel 60 F_{254}, chloroform: methanol 90:10) and fractions with similar TLC patterns were combined to give five major fractions (*viz*: F_1, F_2, F_3, F_4 and F_5). The extensive chromatographic purification of resultant fractions led to the isolation of two compounds.[4 and 5]

Phytochemical chemical investigation of rhizomes of *C. rotundus*

The crude hexane extract of *C. rotundus* (10 gm) was loaded on silica gel column and eluted successively with hexane: ethyl acetate (100:0, 98:02, 96:04, 95:05, 94:06 and 90:10) mixture in the increasing order of polarity. All these column fractions were analyzed by TLC (silica gel 60 F_{254}, hexane: EtOAc (90:10) and fractions with similar TLC patterns were combined to give six major fractions (*viz*: F_1, F_2, F_3, F_4, F_5 and F_6). The repeated purification of resultant fractions led to the isolation of three compounds **6**, **7** and **8** in addition to the compounds (**1** and **2**). Similarly, the crude chloroform extract (10 gm) of *C. rotundus* elution was carried out with a step wise gradient of hexane: acetone. Initially, the column was eluted with plain hexane, then it was eluted with hexane: acetone, (100:0, 98:02, 96:04, 95:05 and 90:10) mixture in the increasing order of polarity. The collected fractions banding pattern were analyzed by TLC (mobile phase hexane: acetone 90:10). These fractions with similar TLC patterns were combined to give four major fractions (*viz* : F_1, F_2, F_3 and F_4), which yielded compounds **9** and **10** after repeated purifications. Later, methanol extract (10 gm) of *C. rotundus* was carried out on a silica gel column with a step wise gradient elution of chloroform: methanol. Initially the column was eluted with plain chloroform. The column was, then, eluted with chloroform: methanol, (100:0, 98:02, 96:04, 95:05 and 90:10) mixture in the increasing order of polarity. The column fractions were analyzed by TLC (silica gel 60 F_{254}, chloroform: methanol 90:10) and fractions with similar TLC patterns were combined to give five major fractions (*Viz:* F_1, F_2, F_3, F_4 and F_5). The repeated purifications of resultant fractions led to the isolation of compounds.[11 and 12]

Determination of free radicals scavenging antioxidant potentials

DPPH radical scavenging activity

The scavenging of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined as per the standard protocols.[22] Briefly, in a 96 - well micro plate reaction, mixture was prepared with 25 µl compound (1 mg/ml DMSO), 100 µl of 0.1M Tris - HCl buffer (pH7.4), 125 µl DPPH solutions (0.5 mM in methanol) and incubated in dark for 30 minutes. DPPH decolourization was recorded spectrophotometrically (BioTek synergy4 multi-mode micro plate reader, BioTek Instruments, Inc Winooski, VT, USA) at 517 nm. The Percentages of DPPH scavenging and IC_{50} values were calculated.

% inhibition = [(Absorbance control-Absorbance test)/Absorbance control] x 100.

[IC_{50}] The values were calculated using linear regression analysis. The IC_{50} values signify the concentration of sample, which is appropriate in scavenging 50% of the DPPH free radicals.

ABTS radical scavenging activity

The ABTS free radical scavenging activity was performed as per the standard protocols.[22] The 100 ml stock solution of ABTS (0.5 mM) was prepared by an addition of 1 ml potassium persulfate (6.8 mM PBS, pH 8.0) and stored in dark for 24 hrs. In a 96-well micro plate, 10 µl of compound was mixed with 190 µl of ABTS. After 15 mins of incubation in dark, decolorized ABTS absorbance was measured spectrophotometrically at 734 nm.

% of inhibition] = [(Absorbance control-Absorbance test)/Absorbance control] x 100.

Intestinal α-glucosidase inhibition assay

The inhibition of rat intestinal α-glucosidase enzyme was determined as per the standard protocols.[23] 20µL of compound (1 mg/ml DMSO) was incubated with 50 µl of crude intestinal α-glucosidase for 5 mins and then with 50 µl of substrate 5 mM p-nitro phenyl-α–D-glucopyranosideand the absorbance was measured at 405 nm. The percentage of enzyme inhibition was calculated by applying the formula mentioned above.

Pancreatic α-amylase inhibition assay

The inhibition of a- amylase enzyme was determined as per the standard protocols.[23] 40 µl of compound was reconstituted in 100 µl phosphate buffer (20 mM, p^H 6.8), containing 6.7 mM sodium chloride in 2 ml eppendorf tubes, and incubated with 200 µl porcine pancreatic α-amylase for 10 minutes. Add 100 µl of soluble potato starch solution (0.5%), and exactly after 10 minutes, incubation 400 µl of DNS (Di nitro salicylic acid) color reagent was added. The closed tubes were placed in water bath (85-90°C) for 10 minutes to develop color and were cooled. Then, reaction mixture (50 µl) was diluted with 175 µl of distilled water in a 96 - well micro plate and absorbance (540 nm) was read spectrophotometrically. Percentage of inhibition was calculated by applying above formula.

IN VITRO SCREENING OF ANTI-INFLAMMATORY ACTIVITY

Cells and culture

Human acute monocytic leukaemia cell line (THP-1) was procured from National Centre of Cell Sciences (NCCS, Pune, India). The cell line was cultured in Roswell Park Memorial Institute Medium 1640 (RPMI, Sigma, USA). The medium was supplemented with 10% (v/v) heat inactivated fetal bovine serum (Sigma, USA), 1% 100 U/ml penicillin (Sigma, USA) and 1% 100 U/ml streptomycin (Sigma, USA) in 25-cm2 culture flask and incubated at 37°C in an atmosphere of 5% CO2. The THP-1 monocytes were treated with PMA (Phorbol- 12 - myristate-13-acetate) in 24 well plates. After 48 hr stimulation, medium was removed, washed twice with PBS and phase contrast images were taken by light microscopy to study the monocyte to macrophage adherence and transformation.

Enzyme-linked immunosorbent assay for cytokine analysis (ELISA)

The THP-1 monocytes were seeded at a density of 2 x 10^5 cells/ ml in 24 well plates in triplicates. After 48 hrs of stimulation of cells with phorbol 12-myristate 13-acetate (PMA) in the presence or absence of compounds, supernatant was harvested from the cell culture plates into 1.5 ml eppendorf tubes and centrifuged to remove the cell debris. The cell free supernatants were transferred into fresh 1.5 ml eppendorf tubes and transferred them immediately into -80°C until use. On the next day of analysis, samples were carefully thawed and used for the analysis of IL-1β levels using Human Ready-SET-Go ELISA kit (eBiosciences, San Diego, CA, USA) following the manufacturer's instructions.[24] Briefly, 100 µl of cell free supernants of test sample, standard, and control was added to 1% BSA- blocked ELISA plate that had been coated overnight with 100 µl/ well anti- murine IL-1β. Following sample addition, 100 µl/well biotin conjugated IL-1β antibody was added, and then plates were incubated at room temperature for 3hrs. Following detecting antibody addition, 100 µl/well (Horse reddish peroxidise) HRP–conjugated avidin was added and incubated at room temperature for 30-45 mins, then aspirated and washed 5-7 times with 250 µl/ well of wash buffer with soaking for 5 mins during each wash step. After that 100 µl of TMB (Tetra Methyl Benzidine) substrate solution was added to each well and incubated plate at room temperature for 15 mins for the colour development. Following substrate addition, 50 µl of 2N H_2SO_4 was added to each well to stop the reaction. The absorbance measurements were read at 450 nm with a Tecan M 200 multimode reader. The concentrations of cytokines released were obtained and the percentage inhibition of cytokines production was calculated as compared to control conditions.

Evaluation of *in vivo* anti-inflammatory activity

Experimental animals

The male wistar rats weighing between 130 - 150 gm were used for the experiments. They were kept in polypropylene cages under standard laboratory conditions (12:12 hr light/dark) cycle at 24°C. The rats were provided with commercial rat diet (NIN, Hyderabad) and water *adlibitum*. The experiments were conducted after obtaining approval from Institutional Animal Ethical Committee of IICT. The animals were quarantined and acclimatized to laboratory conditions for 7 days prior to study initiation, and they were also observed for general health and suitability for testing during this period.

Carrageenan induced rat paw edema

The anti-inflammatory activity of the test compounds was evaluated in wistar rats employing the standard method.[25] The animals were fasted overnight and were divided into control, standard, and different test groups. The test compounds were administered by oral route as gum acacia suspension (2% w/v) at the dose of 100 mg/kg. The animals in the standard group, received indomethacin at the dose of 10 mg/kg by oral route, and rats in the control group received the vehicle solution without test compounds. One hour after test drugs administration, rats in all the groups were administered with 0.1 ml of 1% carrageenan in the sub plantar region of right hind paw. The paw volumes were measured before and after 3 hrs after the administration of carrageenan using

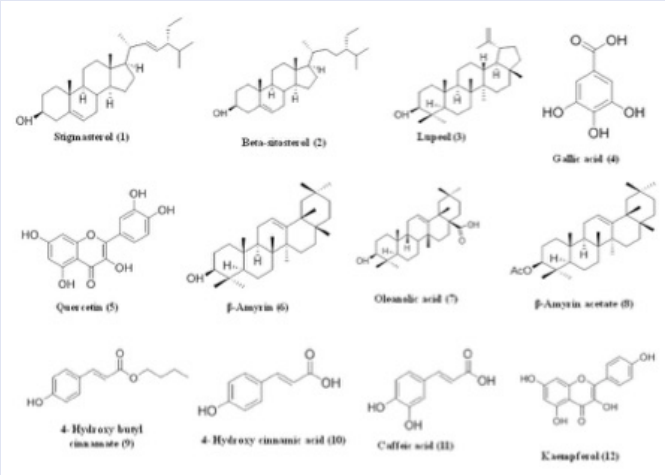

Figure 1: Isolated compounds in rhizomes of *Cyperus scariosus* R.Br and *Cyperus rotundus* L.

digital plethysmometer (Ugo Basile, Italy). The percentage inhibition of paw volume for treated groups was calculated by comparing with mean paw volume of control group.

Percentage of edema inhibition=1- Vt / Vc × 100

Where V_t = Volume of paw edema in drug treated groups

V_c= Volume of paw edema in control groups

RESULTS AND DISCUSSION

Isolation of compounds and structure elucidation

The hexane, chloroform and methanol extracts of *C. scariosus* and *C. rotundus* were chromatographed on silica gel (100-200 mesh) , and extensive purification of the resultant fractions resulted in the isolation of twelve compounds[1-12] The structures of the isolates were established by the interpretation of the NMR and Mass spectral data. After comparing their physicochemical and spectrometric data with those reported in the literature, they were identified as known compounds and confirmed as: Stigmasterol[1,26] β- sitosterol[2,27] Lupeol[3,26] Gallic acid[4,28] Quercetin[5,29] β- amyrin[6,30] Oleanolic acid[7,31] β- amyrin acetate[8,30] 4- hydroxy butyl cinnamate[9,32] 4- hydroxy cinnamic acid[10,33] Caffeic acid[11,34] and kaempferol[12,35] Structures of these compounds are shown in [Figure 1].

Spectral data:

Stigmasterol (1): White solid (200 mg), mp 174-176ºC, EIMS: (*m/z*) 412 [M+], IR (KBr) ν_{max} cm^{-1}: 3424, 2936, 2867, 1640, 1464 cm^{-1}. ^{1} HNMR [300 MHz, $CDCl_3$]: δ 5.35 [t, *J* = 6.1Hz, 1H], 5.14 [m, 1H], 4.98 [m, 1H], 3.52 [m, 1H], 2.52 - 2.08 [m,5H], 1.98 - 1.92[m, 3H], 1.03 [3H, s], 1.51 [m,1H], 1.52 [m, 2H], 1.32 - 1.40 [m, 3H], 1.18 [m, 2H], 1.14 [m, 2H], 1.01 [s, 3H], 1.01 [s, 3H], 1.02 m, 1H], 0.96 [m, 1H], 0.91 [d, *J* = 6.2 Hz, 3H], 0.83 [t, *J* = 7.2 Hz, 3H), 0.82 (d, *J* = 6.6 Hz, 3H), 0.80 (d, *J* = 6.6 Hz, 3H), 0.71 [s, 3H]. ^{13}C NMR [75 MHz, $CDCl_3$]: δ 140.71 [C-5], 138.2 [C-22], 72.1[C-3], 121.7 [C-6],56.8 [C-14], 56.2 [C-17], 51.1 [C-24], 29.6 [C-25], 42.4 [C-13], 42.6, 40.4, 39.8, 37.5, 36.4, 32.3, 32.1 [C-2], 31.8 [C-7, C-8], 20.9 [C-21], 29.3 [C-16], 18.9 [C-28], 24.4 [C-15], 20.9 [C-21], 21.5 [C-11], 19.3 [C-27], 21.7 [C-19], 12.2 [C-29], 40.6 [C-18].

β -sitosterol (2): White amorphous powder (150 mg), mp 134-135 ºC, **EIMS**: (*m/z*) 414 [M+], **IR (KBr) ν_{max} cm^{-1}**: 3424, 2930, 2852, 1724, 1463, 1378, 1271, 1059, 1023, 963 and 802 cm^{-1}. ^{1}**H NMR** ($CDCl_3$, 300 MHz): δ 5.36 (1H, d, *J* = 6.4Hz, H-6), 3.53 (1H, m, H-3), 1.01, 0.68 (3H, s, H-19 and H-18), 0.83 (3H, d, *J* = 6.4 Hz, H-21), 0.81 (3H, d, *J* = 6.4 Hz, H-29) and 0.85 (3H, t, *J* = 7.1 Hz, H-26). ^{13}**C NMR** ($CDCl_3$, 75MHz): δ 37.5 (C-1), 31.9 (C-2), 72.0 (C-3), 42.5 (C-4),140.9 (C-5),121.9 (C-6), 32.1 (C-7), 32.1 (C-8), 50.3 (C-9), 36.7 (C-10), 21.3 (C-11), 39.9 (C-12), 42.6 (C-13), 56.9 (C-14), 26.3 (C-15), 28.5 (C-16), 56.3 (C-17), 36.3 (C-18), 19.2 (C-19), 34.2 (C-20), 26.3 (C-21), 36.2 (C-22), 46.1 (C-23), 23.3 (C-24), 12.2 (C-25), 29.4 (C-26), 20.1 (C-27), 19.6 (C-28), 12.0 (C-29).

Lupeol (3): Pale yellow powder (100 mg) mp 215-216ºC, **EIMS**: (*m/z*) 426 $[M^+]^+$, **IR (KBr) νmax cm-1**: 3308, 2924, 1465, 1379, 1042 and 880 cm^{-1}. ^{1}**H NMR** (200 MHz, $CDCl_3$): δ 4.69 and 4.56 (each 1H, s, H-29), 3.16 (1H, dd, H-3), 2.35 and 1.49 (each 1H,m,21a), 1.67 (1H,t, H-15a), 1.71 (3H, s, H-30), 1.61 (2H, d, H-12A, 1b), 1.67 (1H, t, H-13),1.50 (1H, q, H-2b), 1.42 (1H, m, H-22a), 1.41 (2H, m, H-7), 1.50 (1H, q, H-6B), 1.38 (1H, t, H-16b), 1.39 (1H, m, H-21B), 1.25 (1H, q, H-11B), 1.28 (1H, m, H-22b), 1.71 (3H, s, H-26),1.00 (3H, s, H-23), 0.94 (3H, s, H-27), 0.92 (s, 3H), 0.80, 0.79 and 0.73 (each 3H, H-25, 28, 29) and 0.66 (1H, d, H-5). ^{13}**C NMR** (75 MHz, $CDCl_3$): δ 150.9 (C-20), 109.3 (C-29), 79.0 (C-3), 55.3 (C-5), 50.4 (C-9), 48.0 (C-18), 48.3 (C-19), 25.1 (C-12), 42.8 (C-14), 40.8 (C-8), 40.0 (C-22), 38.8 (C-4), 38.7 (C-1), 38.0 (C-13), 37.1 (C-10), 35.6 (C-16), 34.3 (C-7), 29.1 (C-21), 28.0 (C-23), 27.4 (C-15), 27.4 (C-2), 25.1 (C-12), 28.0 (C-23), 27.4 (C-15), 20.9 (C-11), 19.3 (C-30), 18.3 (C-6), 18.0 (C-28), 15.9 (C-25), 16.1 (C-26), 15.3 (C-24) and 14.6 (C-27).

Gallic acid (4): White amorphous solid (20 mg), **EIMS** (negative mode) *m/z* 169 [M-H], mp 260ºC . **IR (KBr)** ν_{max} cm^{-1}: 3369, 3286, 1711, 1620, 1540, 1308 and 1024 cm^{-1}. ^{1}**H NMR** (300 MHz, CD_3OD): δ 7.02 (1H, d,J =0.8). ^{13}**C NMR** (75 MHz, CD_3OD): δ 120.4 (C-1), 109.2 (C-2), 144.3 (C-3), 17.6 (C-4), 144.3 (C-5), 109.2 (C-6) and 169.1 (C-7).

Quercetin (5): Pale yellow powder (20 mg), mp 300°C, **ESI-MS** (negative mode) *m/z* 301 [M-H]. **IR (KBr)**ν_{max} cm^{-1}: 3407, 3318, 1667, 1610, 1521, 1382, 1168, 1131, 1013 and 821 cm^{-1}. ^{1}**H NMR** (300MHz, CD_3OD): δ 6.08 (1H, d, *J* =2.07), 6.29 (1H, d, *J* =2.07Hz), 7.64 (1H, d, *J* =2.2Hz), 6.78 (1H, d, *J* =8.4Hz), 7.54 (1H, dd, *J* = 2.2 & 8.4Hz). ^{13}**C NMR** (75MHz, CD_3OD**):** δ 148.2 (C-2), 137.2 (C-3), 177.5 (C-4), 162.6 (C-5), 99.4 (C-6), 165.7 (C-7),94.6 (C-8),158.4 (C-9),104.7 (C-10), 124.3 (C-11), 116.1 (C-12),146.3 (C-13),150.3 (C-14), 116.6 (C-15) and 121.8 (C-16).

β-Amyrin (6): White amorphous powder (8 mg), mp 189-191^{0}C, **IR (KBr)** ν_{max} cm^{-1}: 3536 and 1619. **ESIMS**: *m/z* 426 $[M^+]^+$. ^{1}**H NMR** (300 MHz, $CDCl_3$): 5.26 (d, *J* =6.0Hz, 1H), 3.28 (dd, *J* =4.9,10.8Hz, 1H), 2.12-1.86 (m, 2H), 1.84-1.69 (m, 4H),1.66-1.48 (m, 11H) overlapped with each other, 1.46-1.28 (m, 7H, overlapped), 1.06 (s,3H), 1.0 (s,3H), 0.98 (s, 2×3H), 0.96 (s, 3H), 0.86 (s, 3H), 0.78 (s, 3H), 0.75 (s,3H). ^{13}**CNMR** ($CDCl_3$, 75MHz): δ 138.6, 116.8, 78.0 54.2, 46.8, 48.2, 42.6, 42.4, 43.0, 38.6, 38.4, 37.2, 36.4, 36.2, 34.5, 28.6, 28.4, 27.3, 26.0, 22.4, 22.0, 18.4, 17.6, 16.4, 15.8, 15.2 and 14.8.

Oleanolic acid (7): White amorphous powder (5 mg), **ESIMs**: *m/z* 456 $[M^+]$. **IR (KBr)** ν_{max} cm^{-1}: 3423, 2922, 2851, 1691, 1463, 1383 and 1182 cm^{-1}. ^{1}**HNMR** ($CDCl_3$, 300 MH_Z): δ 1.02 (1H,m), 1.57 (1H,m), 3.24 (1H,dd, *J* = 10.5Hz, CH-3), 0.88 (1H,m), 1.58 (1H,m), 1.39 (1H,m), 1.53 (1H,m), 1.36 (1H,m), 1.71 (1H,m), 1.96 (1H,m), 5.28 (1H,s), 1.72 (1H,m), 2.19 (1H,m), 2.12 (1H,m), 1.96 (1H,m), 2.83 (1H,dd, *J* =13.8, 4.5Hz), 1.83 (1H,m), 1.32 (1H,m), 1.46 (1H,m), 1.23 (1H,m), 1.82 (1H,m), 2.04 (1H,m), 1.00 (3H,s), 0.79 (3H,s), 0.93 (3H,s), 0.77 (3H,s), 1.15 (3H,s), 1.15 (3H,s), 0.92 (3H,s) and 0.95 (3H,s). ^{13}**CNMR (**75 MH_Z, $CDCl_3$): δ 15.3 (C-25), 15.4 (C-24), 17.1 (C-26), 18.3 (C-6), 22.9 (C-11), 23.6 (C-30), 23.4 (C-16), 25.9 (C-27), 27.2 (C-2), 28.1 (C-23), 28.4 (C-15), 30.7 (C-20), 32.4 (C-22), 32.6 (C-29), 33.1 (C-7), 33.8 (C-21), 37.1 (C-10), 38.4 (C-1), 38.8 (C-4), 39.3 (C-8), 41.0 (C-18), 41.6 (C-14), 45.9 (C-19), 46.7 (C-17), 47.6 (C-9), 55.2 (C-5), 79.6 (C-3), 122.6 (C-12), 143.6 (C-13), 182.9 (C-28).

β-Amyrin acetate (8): colourless needles (6 mg), **IR (KBr)** ν_{max} cm^{-1}: 3490, 2986, 1606, 1482 and 1066 cm^{-1}. The **EI-MS**: *m/z* 468 [M^+]. ^{1}**HNMR** ($CDCl_3$, 300 MH_Z): δ 5.18 (t, *J* = 3.5 Hz, 1H), 4.50 (t, *J* = 8.0 Hz), 2.05 (S, 3H), 1.64 (m), 1.88 (m), 0.84 (m), 1.53 (m), 1.58 (m), 1.80 (m), 1.76 (m), 1.98 (m), 2.31 (m), 1.93 (m), 1.60 (m), 1.32 (m), 1.08 (m), 0.88 (s), 0.96 (s), 0.86 (s), 0.97 (s), 1.13 (s), 0.83 (s), 0.88 (s), 0.87 (s). ^{13}**CNMR** ($CDCl_3$, 75 MHz): δ 171.1 (C-31),145.2 (C-13), 121.6 (C-12), 81.0 (C-3),55.2 (C-5), 47.6 (C-9), 47.2 (C-18), 46.8 (C-19), 39.8 (C-4), 38.3 (C-1), 37.7 (C-8), 34.7 (C-21), 33.4(C-29), 31 (C-20), 26.9 (C-16), 26.1 (C-15), 23.4 (C-11), 21.4 (C-32), 18.3 (C-6), 16.8 (C-26), 16.7 (C-25) and 15.6 (C-24).

[4-] hydroxy butyl cinnamate (9): white amorphous powder (5 mg), **IR (KBr)** ν_{max} cm^{-1}: IR 3388, 2921, 1675 cm^{-1}. Mass *m/z* 220 [M^+]. ^{1}**HNMR** ($CDCl_3$, 300 MH_Z): δ 6.84 (1H, d, *J* = 8.6), 7.4 (1H, d, *J* =8.6 Hz), 7.63 (1H, d, *J* =15.6Hz), 6.30 91H, d, *J* =15.6Hz), 4.18 (2H,t, *J* = 6.7Hz), 1.69 (2H, m), 0.88 (3H, t, *J* = 6.9Hz). The ^{13}**CNMR** ($CDCl_3$, 75 MH_Z): δ 127.4 (C-1), 115.6 (C-2), 129.9 (C-3), 165.9 (C-4), 129.9 (C-5), 115.8 (C-6), 156.3 (C-7), 167.4 (C-9), 64.64 (C-10), 25.98 (C-11), 22.68 (C-12) and 14.1 (C-13).

[4-] hydroxy cinnamic acid (10)**:** white solid (6 mg), **IR (KBr)** ν_{max} cm^{-1}: 3371, 2929, 1672 cm^{-1}. Mass *m/z* 163 [M-H], The ^{1}**H NMR** (CD_3OD), 300 MHz): δ 6.84 (1H, d, *J* = 8.4Hz), 7.43 (1H, d, *J* = 8.4Hz), 7.64 (1H, d, *J* =16.05Hz), 6.26 (1H, d, *J* =16.05Hz). The ^{13}**CNMR** (CD_3OD, 75MHz): δ 114.2 (C-1), 115.2 (C-2), 129.7 (C-3), 159.0 (C-4), 159.0 (C-4), 129.7 (C-5), 115.2 (C-6), 145.4 (C-7), 125.7 (C-8) and 169.5 (C-9).

Caffeic acid (11): yellow solid (10 mg), mp 137-140°C, **ESI-MS m/z** 179 [M-H]. IR (KBr) ν_{max} cm^{-1}: 3407, 3229, 2923, 1645 and 1449 cm^{-1}. ^{1}**H NMR** (CD_3OD, 300 MHz): δ 6.97 (1H, d, *J* =1.8), 6.73 (1H, d, *J* = 8.1Hz), 6.85 (1H, dd, *J* =1.8 & 8.1Hz), 6.12 (1H, d, *J* =15.8Hz), 7.48 (1H, *J* =15.8Hz). ^{13}**CNMR** ($CDCl_3$, 75MHz): δ 128.0 (C-1), 115.2 (C-2), 145.9 (C-3), 146.5 (C-4), 117.2 (C-5), 123.2 (C-6), 144.9 (C-7), 116.5 (C-7), 116.5 (C-8), 171.5 (C-9).

Kaempferol (12): Yellow powder (5 mg), mp 286°C. **ESI-MS**: m/z 285 [M-H]. ^{1}**HNMR** (DMSO-d_6, 300 MHz): δ 6.27, 6.46 (1H, *J* =2.1Hz), 8.0 (1H, d, *J* = 8.7Hz), 6.99 (1H, d, *J* =8.7Hz). ^{13}**CNMR** (DMSO-d_6, 75MHz): δ145.9 (C-2), 135.5 (C-3), 175.5 (C-4), 156.4 (C-5), 99.06 (C-6), 167.3 (C-7), 93.8 (C-8), 160.5 (C-9), 101.5 (C-10), 121.7 (1'), 129.1 (2'), 115.4 (3'), 159.2 (4'), 115.4 (5'), 129.1 (6').

BIOLOGICAL ACTIVITIES OF ISOLATED COMPOUNDS

DPPH and ABTS radical scavenging activity

In present days, oxidative stress leads to several diseases in human beings such as atherosclerosis, diabetes, cancer, inflammation, cancer, alzheimer's, and parkinson's.[36] The oxidative stress is mainly caused by free radicals. These species include both oxygen and non oxygen radicals. The oxygen radical include peroxyl, superoxide anion, hydroxyl, peroxy nitrile, and nitric oxide radical. The non oxygen radical includes H_2O_2, hypochlorous acid, and singlet oxygen. These free radicals (ROS) produced by biochemical reaction in human body cause the structural and functional changes in human metabolism. The various plant extracts and different types of secondary metabolites have been shown not only as free radical scavenging and antioxidant activities. This characteristic hydroxyl functional group scavenge free radicals, and prevent the oxidation of biological molecules by converting the more ROS by donating hydrogen atom into inactive species. Therefore, antioxidant and free radical scavenging constituents are more useful in the prevention and treatment of oxidative stress induced disorders and diseases. In our study, all these isolates were evaluated for their DPPH (1, 1-diphenyl-2-picrylhydrazyl) and $ABTS^{\cdot+}$ [2, 2'- azino - bis (3-ethyl benzothiazoline-6-sulfonic acid)] free radical scavenging activities. The values obtained for antioxidant assays were represented as percentages of radical scavenging activities at various concentrations. IC_{50} values denote the concentration of sample required to scavenge 50% DPPH radicals [Figure 2a]. In our experiment, we used a well known anti oxidant ascorbic acid as standard. As shown in [Table 1], kaempferol[12] caffeicacid[11] quercetine,[5] and gallic acid[4] were displayed potent DPPH scavenging activities with IC_{50} values of 3.85, 1.24, 1.1 and 0.43 µg/ml, respectively. While the remaining compounds, stigmasterol[1], sitosterol[2] and lupeol[3] have not shown DPPH inhibition at 25 µg/ml. The pattern and potentials in decreasing order of DPPH scavenging activity was observed as follows: **gallic acid > quercetin > caffeic acid > kaempferol**. Similarly, all these compounds were evaluated for ABTS free radical scavenging activity using trolox as standard. The results represents the percentage inhibition of ABTS at 10 µg/ml [Table 1 and Figure 2b]. The analysis of the results indicated that quercetin[5], kaempferol[12], cinnemic acid[10], caffeic

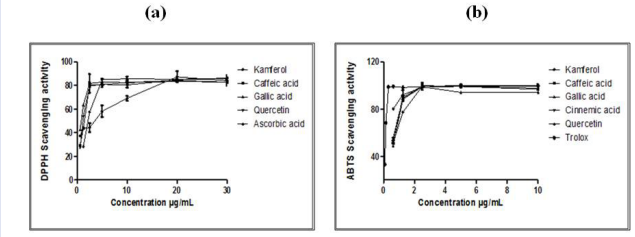

Figure 2: Concentration dependent (a) DPPH and (b) ABTS free radical scavenging pattern of compounds isolated from *C. scariosus* and *C. rotundus*.

Table 1: Concentration required for scavenging DPPH and ABTS free radicals by 50% (IC_{50}).

Sample	% inhibition of DPPH at 25 µg/ml	DPPH IC_{50} µg/ml	% inhibition of ABTS at 10 µg/ml	ABTS IC_{50} µg/ml
Stigmasterol	NA	--	NA	--
Lupeol	NA	--	NA	--
Kaempferol	87.8 ± 0.8	3.85 ± 0.4	97.61 ± 1.1	0.45 ± 0.05
Caffeic acid	84.3 ± 0.4	1.24 ± 0.1	96.72 ± 0.9	0.48 ± 0.2
Gallic acid	83.3 ± 1.0	0.43 ± 0.1	94.13 ± 2.0	0.56 ± 0.01
4- hydroxy cinnemic acid	22.4 ± 2.3	--	99.4 ± 0.5	0.45 ± 0.01
Quercetin	85.6 ± 1.2	1.1 ± 0.2	98.21 ± 0.2	0.067 ± 0.01
β- sitosterol	NA	--	34.83 ± 2ç.0	--
Ascorbic acid	84.2 ± 0.6	2.17 ± 0.2		
Trolox			93.64 ± 0.9	0.1± 0.01

Note: NA= Not active, Values represent mean ± SD (n=3)

acid,[11] and gallic acid[4] displayed potent ABTS scavenging activities with IC_{50} values of 0.067, 0.45, 0.45, 0.48, and 0.56 µg/ml, respectively. The order of the inhibitory potentials was oberved as follows: **quercetin > kaempferol = cinnemic acid>caffeic acid > gallic acid**. However, the compounds stigmasterol[1], sitosterol[2] and lupeol[3] has not shown ABTS inhibition at 10 µg/ml. These observations revealed the fact that that presence phenolic hydroxyl groups positively affect compounds radical scavenging potential, and absence of phenolic hydroxyl group drastically reduced its DPPH and ABTS radical scavenging capacity. The phenolic compounds are being major contributors to the antioxidants behaviour of plants and the antioxidant activity of phenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen.[37] Furthermore, free radical scavenging and antioxidant activity of phenols mainly depends on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenol molecules. This characteristic of hydroxyl functional group scavenge free radicals and prevent the oxidation of biological molecules by converting the more ROS by donating hydrogen atom into inactive species.[38] Therefore, antioxidant and free radical scavenging constituents are more useful in the prevention and treatment of oxidative stress induced disorders and diseases.

α- glucosidase and α- amylase inhibitory activities

C. scariosus and *C. rotundus* are widely used in ayurvedic preparation for the treatment of diabetes and other diseases. However, there has been relatively little information pertaining to its active constituents and their alpha-glucosidase activities. Thus, in this study, we have tested all the compounds isolated from *C. scariosus* and *C. rotundus* for α-glucosidase inhibition activity using a standard protocols. In our experiment, we used a well known anti diabetic drug acarbose as standard. Our results represent that percentage inhibition of a- glucosidase at 20 µg/ml [Table 2 and Figure 3a]. Among all the compounds quercetin[5] and lupeol[3] showed [24.7%] and [13.1%] inhibition of alpha glucosidase activity at 20 µg/ml, respectively, whereas as the other compounds showed mild activity. Similary, we also measured α- amylase inhibitory potentials of all the isolates. The results represent the percentage inhibition of α-amylase at 40 µg/ml [Table 2 and Figure 3b]. The results indicated that gallic acid[4] and 4- hydroxy cinnemic acid[10] showed alpha amylase activity with the 31.2% and 23.5% inhibitory potentials. Comparing our experimental results revealed the fact that phenolics and flavanoids have shown antidiabetic activity. The results from earliar studies suggest that flavanoids and phenolics isolated from several medicinal plants could have potential to treat type-II diabetes mellitus.[39] These compounds are capable not only for oxidative stress but also of inhibiting carbohydrate hydrolyzing enzymes to prevent hyperglycemia.[40]

Table 2: Percentage inhibition of a-glucosidase and a- amylase activities

Sample	% inhibition of AGI at 20 µg/ml	% inhibition of alpha amylase at 40 µg/ml
Stigmasterol	4.0 ± 0.7	12.3 ± 1.3
Lupeol	13.1 ± 1.9	07.3 ± 1.3
Kaempferol	NA	05.4 ± 0.8
Caffeic acid	4.8 ± 1.0	09.7 ± 0.5
Gallic acid	2.7 ± 0.9	31.2 ± 0.2
4- hydroxy cinnemic acid	6.3 ± 0.7	23.5 ± 1.5
Quercetin	24.7 ± 1.0	05.6 ± 0.6
β- sitosterol	NA	10.2 ± 1.0
Acarbose	61.6 ± 0.4	40.0 ± 1.0

Note: NA= Not active, Values represent mean ± SD (n=3)

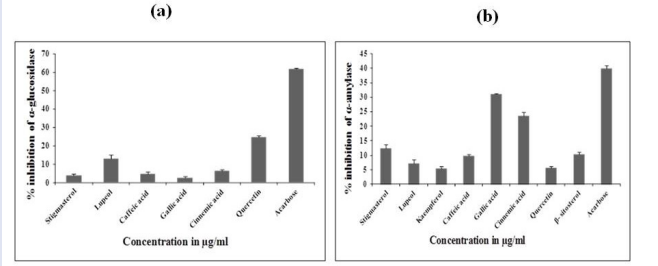

Figure 3: (a). α- glucosidase (b) α- amylase inhibitory potential of isolated compounds.

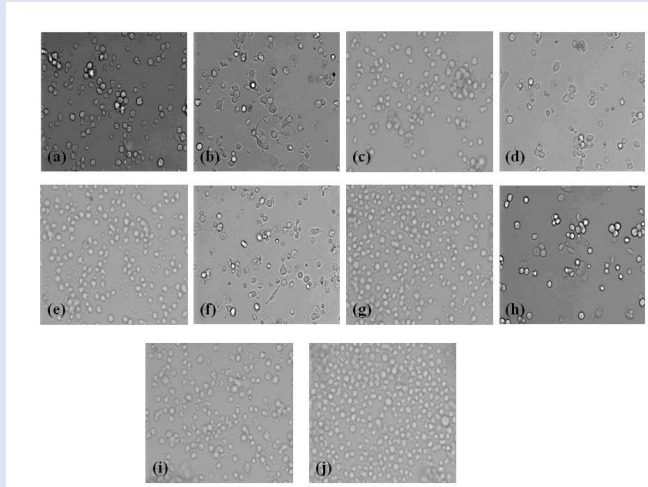

Figure 4: Phase contrast images of control, standard and test compounds in Thp-1 cells captured by light microscopy. (a). THP-1 monocytic cells. (b). THP-1 cells treated with PMA to induce monocyte to macrophage differentiation. (c). THP-1 cells treated with stigmasterol without PMA. (d). THP-1 cells + PMA with stigmasterol. (e). THP-1 cells treated with sitosterol without PMA. (f). THP-1 cells + PMA with sitosterol. (g). THP-1 cells treated with lupeol without PMA. (h). THP-1 cells + PMA with lupeol. (i). THP-1 cells treated with piroxicam (standard) without PMA. (j). THP-1 cells + PMA with piroxicam.

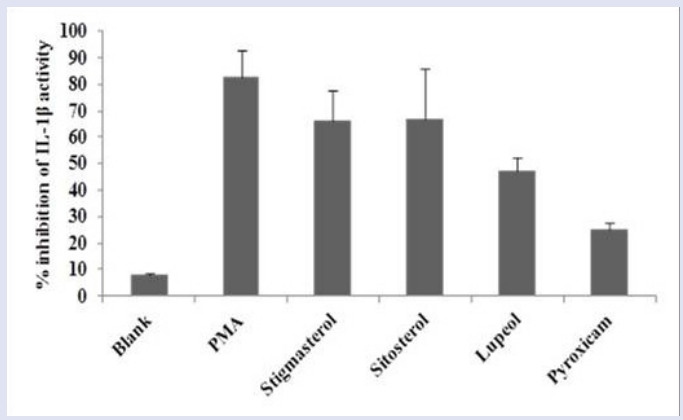

Figure 5: Represents % inhibition of IL-1β activity in ThP-1 monocytic cells treated with stigmasterol, sitosterol and lupeol.

In vitro anti-inflammatory screening of compounds 1, 2 and 3 using PMA- induced production of IL-1β model

Measurement of IL-1β production by ELISA

During acute or chronic inflammation excessive IL-1β is produced as pro inflammatory cytokine, which resuts in the systemic inflammatory response syndrome, severe tissue damage, and septic shock. The effects of stigamasterol[1], β- sitosterol,[2] and lupeol[3] isolated from *C. scariosus* on modulating the production levels of IL-1 β, was tested by stimulating human monocytic cell line THP-1, using PMA, and the results were compared with standard drug piroxicam.The THP-1 monocytic cells were treated with 100 ng/ml PMA in the presence or absence of isolated compounds and standard piroxicam 10 μM/ml. After 48 hours PMA stimulation, the monocytes differetiated into macrophages and cytokines released into the media. Following stimulation period, supernatant was collected and the respective wells washed twice with phosphate buffer saline (PBS), and phase contrast images were taken by light microscopy. The phage contrast micrographs of control cells and treated cells are given in [Figure 4]. The cells and nuclei of the control cells are normal with clear-cut membranes, while nuclei are elliptical. Whereas, the cells stimulated with PMA, the monocytes differentiated into macrophages, and some of the phagocytic changes observed in the cultured media. Whereas, the cells treated with test compounds stigamasterol,[1] β- sitosterol[2] and lupeol[3] are showing distorted membranes and nucleus with blebs. After morphological changes observation, from the collected supernatant we estimated IL-1 β levels by using sandwich ELISA as per the manufacturers instructions. Among the three tested compounds lupeol exhibited 47% promising result by down regulating levels of IL-1β, whereas stigamasterol **(1)**, β- sitosterol[2] were found

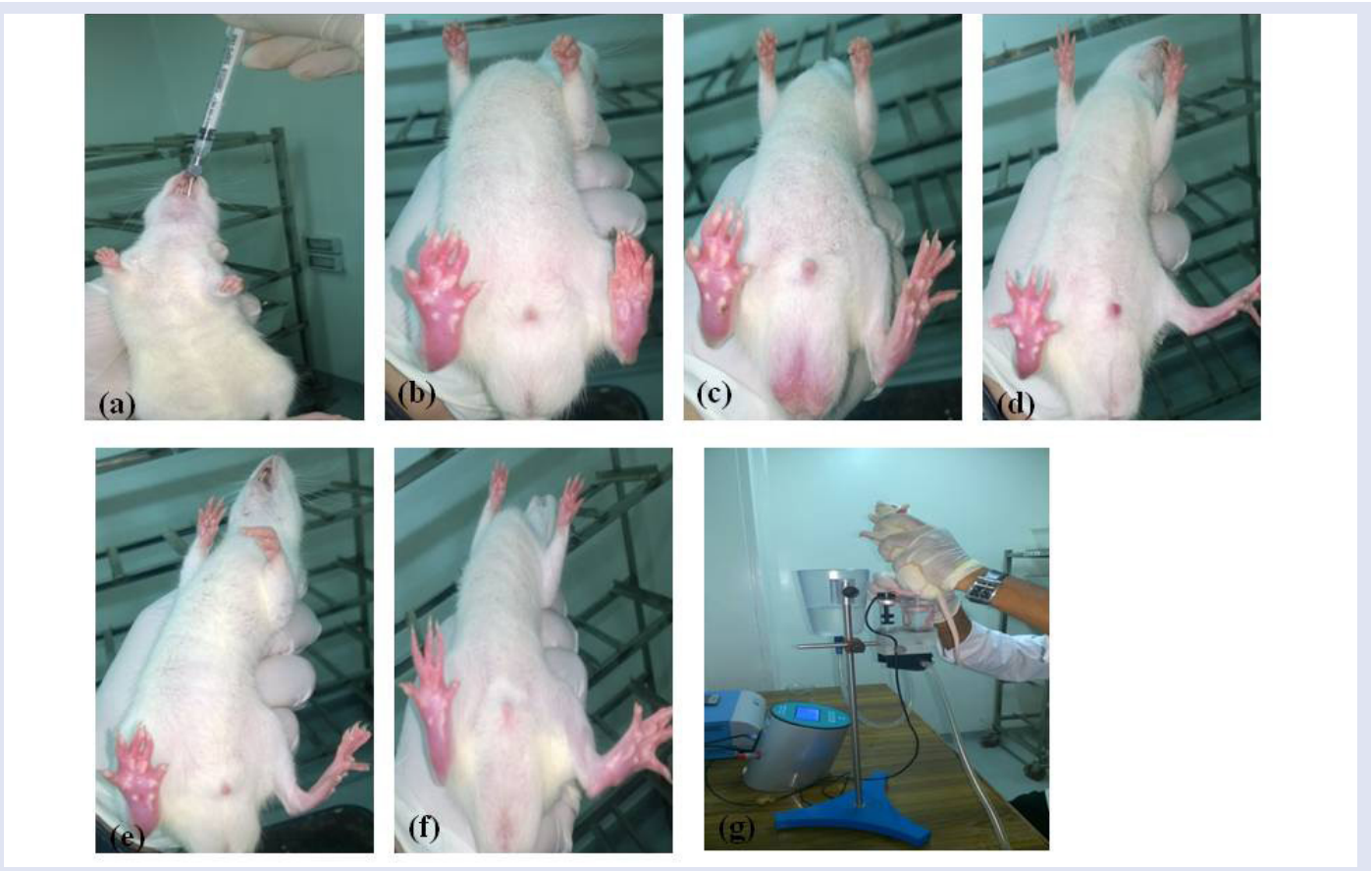

Figure 6: *In vivo* anti-inflammatory activity in rats. (a). Oral feeding (b). Treated with carrageenan (c). Treated with stigmasterol (d). Treated with sitosterol (e). Treated with lupeol. (f). Treated with indomethacin (g). Measurement of paw volume by using Plethysmometer.

Table 3: Effect of compounds **1**,**2** and **3** of *C. scariosus* on carrageenan-induced paw edema in rats.

Groups	Dose (mg/kg)	Paw volume (0hr)	Paw volume (3hr)	Paw volume difference	% inhibition at 3hr
Carrageenan (Control)	--	1.205 ± 0.012	2.545 ± 0.03	1.34 ± 0.033	--
Stigmasterol	100	1.17 ± 0.044	2.635 ± 0.044	1.46 ± 0.026	0.0 ± 0.0
β- sitosterol	100	1.18 ± 0.040	2.54 ± 0.099	1.36 ± 0.063	2.42 ± 1.59
Lupeol	100	1.23 ± 0.008	2.41 ± 0.014	1.18 ± 0.007(a)	11.94 ± 0.52(a)
Indomethacin (Standard)	10	1.18 ± 0.018	1.92 ± 0.0359	0.73 ± 0.035(b)	44.9 ± 2.66(b)

Note: Values represent mean ± SD (n=4). Unpaired 't' test with welch's correction was applied for Statistical analysis. [a]$p < 0.0025$ and [b]$p < 0.0001$, when compared with paw volume of control group at the end of 3hr.

to be equally active and showed mild activity in inhibition of IL-1β as compared to lupeol [Figure 5]. The three compounds activity results were compared with standard drug piroxicam (10 μM), which exhibited 75% inhibition of IL-1β concentration. Around 53 % of IL-1β inhibition was observed with 10 μM/ml treatment of lupeol.[3] Our results revealed that among the three compounds, lupeol has shown to decrease the generation of proinflammatory cytokine IL-1β. The earlier studies showed that phytosterols and triterpenoids decrease the generation of proinflammatory cytokines such as tumour necrosis factor-α and interleukin-1β in lipopolysaccharide in treating macrophages.[41,42]

In vivo anti - inflammatory activity of compounds 1, 2 and 3

In traditional medicine, rhizomes of *C. scariosus* were used to treat inflammation. Till today there is no scientific proof for justification of traditional use of rhizomes in treatment of inflammation. Hence, the present study focussed to study anti-inflammatory activity of compounds[1-3] isolated from *C. scariosus*.They were studied for *in vivo* anti inflammatory activity by using carrageenan-induced rat paw edema as the method. The Carrageenan is a strong chemical used for the release of inflammatory and pro inflammatory mediators (prostaglandins, leukotrienes, histamine, bradykinin, TNF-a, etc). To test the anti inflammatory activity, male wistar rats were divided into three groups such as control, standard, and different test groups. The percentage reduction in paw volume after treatment with respective test compounds was considered as anti- inflammatory activity. It was found that stigmasterol 1 and β- sitosterol[2] did not display anti- inflammatory activity. However, lupeol[3] reduced significantly ($p<0.0025$) the paw volume, when compared with the control group of animals [Figure 6 and Table 3]. Therefore, it is inferred that the lupeol[3] has anti-inflammatory effect in rats at a dose of 100 mg/kg body weight with marked inhibition 12% inflammation at the third hour. Comparing these results with earlier studies, lupeol and its derivatives were shown to exhibit higher anti-inflammatory activity than commonly used non steroidal anti-inflammatory drug indomethacin in rat and mouse models of inflammation.[43]

CONCLUSION

In this study, we investigated anti-oxidant, anti-diabetic, and anti-inflammatory constituents from the Indian sedges which resulted in the isolation of twelve compounds. Biological activities of these isolates revealed that aromatic compounds gallic acid[4] quercetin[5] caffeic acid[11], and kaempferol[12] showed potent antioxidant activity and anti-diabetic activity. While lupeol[3] showed potent IL-1 β activity inhibition in THP-1 monocytic cells, and also displayed significant ($p < 0.0025$) *in vivo* anti-inflammatory activity in carrageenan induced rat paw edema.

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Conflicts of interest

There are no conflicts of interest.

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