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Original article

Evaluation of *Cuscuta reflexa* seed essential oil on TPA-induced inflammation in mice



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ABSTRACT

Objectives: *Cuscuta reflexa* is parasitic plant of subtropical and temperate areas, commonly named as amarbel or akasvel in India. Present study is to investigate the *In-vivo* anti-inflammatory potential of *C. reflexa* seed essential oil.

Materials and methods: In this study the essential oil was hydro-distilled from dried seeds and analyzed for chemical profiling and anti-inflammatory activity. *In-vivo* activity was performed on mice inducing TPA as disease causing agent. Study of biochemical parameter and oxidative stress was performed by taking homogenate of ear pinna of mice.

Results: Chemical analysis is done by GC and GC-MS study, we found β -Bisabolene and Hexahydro farnesyl acetone as the major component as 13.2 and 14.2% respectively along with *cis*-chrysanthenyl acetate, caryophyllene oxide, carotol, caryophyllene oxide. *In-vitro* activity of cell viability was assessed using RAW 264.7 macrophages, showed no reduction in viable cell in the processed culture. *In-vivo* it significantly reduces the inflammation by inhibiting pro-inflammatory cytokines (IL-1 β , IL-6 and TNF- α).

Conclusions: *Cuscuta reflexa* seed essential oil was found effective in the management of inflammation by encountering the inflammatory mediators (cytokines).

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1. Introduction

Akashbela, devil's hair or *Cuscuta reflexa* Roxb belong to Convolvulaceae family. It is rootless climbs towards sky. This parasitic plant is commonly located at Ceylon upto an altitude of 2346 m, India, Pakistan and many other countries. Traditionally the plant has reported for many medicinal properties. Many pharmacological activities have been reported at extract level only. (Khan

et al., 2018). The essential oil of *C. reflexa* contain mainly *cis*-3-butyl-4-vinylcyclopentane (26.4%) along with limonene (5.1%) and (E)-nerolidol (9.5%) (Paudel et al., 2014). On non-volatile components, many chemical compounds have been isolated by researcher's time to time but some are commonly available as lycopene, kaemferol, cuscatalin, palmitic acid, oleic acid. The major bioactive compound in the plant are bergenin, hyperoside and amarbellin (Saini et al., 2015) and alkaloid, saponins and flavonoids in the plant which attribute role in biological activities.

Seed of *C. reflexa* has been studied for bioactivities like antispasmodic, antiviral anti-inflammatory, antibacterial antifungal, antidiabetic and antitumor (Costea and François, 2005) anthelmintic, treatment of bilious and carminative (Khan et al., 2010), diuretic activity (Sharma et al., 2009). The extract of *C. reflexa* has been reported for muscle relaxant, antioxidant, cardiostimulant, hair growth promoter, anticonvulsant and hemodynamic properties (Seru et al., 2013). According to tribal information this plant has been used to cure inflammation and cancer (Knolle and Greken, 2000). *C. reflexa* showed moderate anti-proliferative activities in HCT116 colorectal

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cell lines against while its component 1-O-p-hydroxycinnamoyldlucose showed promising activity (Riaz et al., 2017). Water extract of the plant was able to block the binding of NF- κ B to its DNA binding motifs, making it evident that the extract down regulates TNF- α and COX-2 via NF- κ B inhibition thus it has anti-inflammatory property (Suresh et al., 2011). Lots of essential oils are used from long time to reduce inflammation. *Angelica sinensis* volatile oil showed marked reduction in the inhibition of pro-inflammatory cytokines (Li et al., 2016).

Exhaustive work has been done to investigate new drug molecule, extract and oil used for relief from pain and inflammation from decades. Inflammation is a complex biological response to various stimuli hence activation of inflammatory mediators which cause emergence of pro-inflammatory cytokines. This causes damage in cells, tissue and organs. If it remains persistent may become life threatening disease like rheumatoid arthritis and cancer.

Many non-steroidal anti-inflammatory medicines have been investigated but almost all synthetic compound showed side effect. The presence of bioactive compound in plant has attracted many researchers to work. Nowadays phytochemicals are gaining attention for treatment of disease over synthetic molecule. Present work is chemical investigation and anti-inflammatory profile of *C. reflexa* essential oil followed by chemical characterization.

2. Materials and methods

2.1. Chemicals and reagents

Acetone, dimethyl sulfoxide (DMSO), Griess reagent kit, malondialdehyde, thiobarbituric acid (TBA), trichloroacetic acid (TCA), 12-O-tetradecanoylphorbol-13-acetate (TPA), and indomethacin were purchased from Sigma-Aldrich (USA), phosphate buffer saline tablet (PBS) was taken from Himedia, Mumbai and from Thermo Scientific (USA) TNF- α , IL-6 and IL-1 β biochemical kit. Every material used in present work was of analytical grade.

2.2. Plant material

The seed of *C. reflexa* were purchased in August 2018 from the local market in Lucknow, India and identified by the Scientist, Botany and Pharmacognosy, Department, CSIR-CIMAP, Lucknow. Voucher no. 774 has been deposited with Institute's herbarium.

2.3. Isolation of essential oil

The essential oil of crushed seed of *C. reflexa* (500 g) was obtained by hydro-distillation in a Clevenger-type apparatus. The yield of the essential oil was 0.05% (w/w) on a dry weight basis. The oil was dried over anhydrous sodium sulfate and stored in a sealed glass vial at low temperature until further analysis.

2.4. Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis

GC analysis of the samples was done with a Agilent gas chromatograph (7890 B), equipped with a HP-5 fused silica capillary column (5% phenyl)-polymethyl siloxane stationary phase; 30 m \times 0.32 mm internal diameter; film thickness 0.25 μ m and a flame ionization detector (FID). Hydrogen was the carrier gas at 1.7 mL min⁻¹. Oven temperature was programmed from 60 °C to 240 °C with increase of 3 °C min⁻¹ and final hold time of 2 min. Injector and detector temperatures were set at 280 °C and 290 °C, respectively. The samples (0.01 μ L, neat for essential oil and 0.1 μ L, diluted in hexane for absolute) were injected in the split mode (1:100).

Gas chromatography-Mass spectroscopy of *C. reflexa* essential oil was performed on a Clarus 680 GC interfaced with a Clarus SQ 8C Quadrupole mass spectrometer of Perkin Elmer fitted with Elite-5 MS fused-silica capillary column (30 m \times 0.25 mm i.d., film thickness 0.25 μ m). Temperature of oven was planned from 60 °C to 240 °C, at 3 °C min⁻¹ to 270 °C at 5 °C min⁻¹; Temperature of injector was 250 °C; transfer line and source temperatures were 220 °C; 0.03 μ L neat of injection size; 1:50 split ratio; Helium as a carrier gas at 1.0 mL min⁻¹; 70 eV ionization energy and 40–450 amu mass range scan.

The constituents of the essential oil were identified on the basis of retention index, determined using a homologous series of *n*-alkanes (C₇–C₃₀ hydrocarbons, Supelco Bellefonte, PA USA), To compare data of sample and literature data from MS Library search (NIST and WILEY). The relative quantity of individual chemical compounds was determined by optimizing the peak area of GC (FID response) without employing correction factor (Adams, 2007).

2.5. Experimental animals

Present study was conducted on female Swiss albino mice (18–22 g). PSIT Kanpur has Animal House Facility to bred and maintain. The animals were fed with standard rodent pellet diet and water was given to the animals. Permission and approval for animal studies was obtained from CPCSEA (Reg. No. 1273/PO/RE/S/09/CPCSEA), Government of India through the Institutional Animal Ethics Committee.

2.6. Cell viability assay

Cell viability was determined by using MTT assay. RAW 264.7 were washed by buffer (PBS), decant the solution and seeded the cell in DMEM in 96-well microplates for 24 hrs. at 8X 10⁴ densities per cell. Test samples of *C. reflexa* essential oil (CREO) 1,2 and 5% were dissolved in DMSO. After incubation of 48 h 10 μ L of MTT mg/mL in a PBS buffer were added again plates were incubated at 37 °C for 4 h., removed the medium and dissolved formazan crystals in 100 mL DMSO. ELISA plate reader from BioRad was used to determine the optical density at 540 nm. Cell viability was calculated by following equation from six replicate well.

$$\% \text{ Viability} = (\text{OD treated cells} / \text{OD control cells}) \times 100$$

2.7. In-vivo study

Swiss albino female mice (7–8 week; 25 \pm 2 g) were acclimatized in controlled environmental conditions (55 \pm 10% RH and 12 h day/ night cycle), 7 days before commencement of experiment. During experiment mice were divided into six group (n = 6). Right ear of the mouse was used for testing. Sample was applied topically followed by TPA after 30 min (Yadav et al., 2009). Experiment were performed under the guidelines of Institutional Animal Ethics Committee approved by CPCSEA, Government of India.

2.8. Acute dermal irritation test

Determination of acute dermal irritation study was performed on the swiss albino mice. 1-inch square area was shaved on (n = 6). 5% sample was applied on the shaved skin while acetone as the vehicle. Assessment of dermal vulnerability, itchiness, redness, skin irritation and skin reactions were observed at 0, 4, 24, 48, 72 h. Skin oedema and erythema were recorded on the basis of arbitrary grading scale of 1–5. For ear erythema the score was taken as 1 erythema formation, 2 very slight erythema (barely perceptible), 3 average erythema, 4 well-developed erythema, 5 seri-

ous erythema (meat redness). Similarly, weight of both the ears (left & right) were taken and calculate the difference. On last day 6 hr after treatment the animals were sacrificed by cervical dislocation method and ears of each mice were collected. With the help of 8 mm biopsy punch ears were excised, weighed and calculate the difference. Right ear part of every ear was retaining in PBS buffer (PH 7.4) till further experiment. For ear oedema, 1 no oedema, 2 very slight oedema, 3 well-defined oedema, 4 moderate oedema, 5-severe oedema (beef raise than 1 mm). Similarly, primary irritation score was assessed for all animals of the study by cumulative irritation index (Aroonrerk and Kamkaen, 2009).

2.9. TPA induced skin inflammation in mice

Present study was done as described in Kumar et al., 2018. TPA (disease control) was applied to both the ears (inner and outer side) as 2.5/ear solubilize in 20 μ l acetone. Test sample of *C. reflexa* essential oil (CREO) along with positive control was applied on the ear after 30 min of TPA application. All the test samples and positive control sample should be properly dissolved in acetone. In vehicle control group only, acetone was applied whereas in toxin group TPA was introduced only and maximum inflammation was observed.

2.10. Tissue weight measurement

Thickness of ear was measured by digital micrometer before and after induction of inflammation. After sacrificing the mice 6.5 mm diameter disc was used to remove ear by help of metal punch and weighed ear. Tissue (ear) homogenates (10% PBS) were prepared collect the supernatant and proceed for further investigations for the effect of CREO for pro-inflammatory cytokines.

2.11. Evaluation of pro-inflammatory cytokines

Previously collected mice ear was homogenized by using tissue homogenizer (Pro 200; Pro Scientific, USA) with 10% PBS and placed in cold ice and centrifuged for 15 min at 4 °C and 10,000 rpm. Supernatant fluid was collected for further experiments.

Estimation of cytokines TNF- α , IL-6 and IL-1 β were done as prescribed in the ELISA kits (Thermo Scientific, USA) (Deepika et al., 2014).

2.12. Measurement of malondialdehyde (MDA) and nitric oxide (NO) production

- (i) Lipid peroxidation assay: 200 μ l of w/v was mixed with 200 μ l of ear supernatant followed by mixing. Prepared solution was centrifuged for 5 min at 5000 rpm 200 μ l aliquot solution was taken and 0.67% TBA inequal amount was added. Boil the solution on water bath for 10 min. Absorbance was measured at 600 and 535 nm including blank with TCA and TBA
- (ii) 75 μ l of Griess reagent taken with equal amount of ear supernatant in a micro titer plate and mix well followed by incubation for 10 min at 37 °C. At 540 nm absorbance was taken and nitrite concentration was determined by standard curve plotted by sodium nitrite serial dilution method

2.13. Histopathology

Ear of animals was kept in 10% formalin solution. Each sample was treating with xylene and then fixed with paraffin. Cut into section of 5 μ m and stained with hematoxylin-eosin and examined

the image under light microscope at magnification of 10X and 40X for finding the inflammatory cellular response.

2.14. Statistical analysis

Experimental data was recorded as mean \pm standard error. One-way analysis of variance (ANOVA) was used to summarize the results. GraphPad PRISM[®] version 5.01(GraphPad Software, Inc., USA). $p < 0.05$ was considered indicative of significance.

3. Results

3.1. GC and GCMS analysis

A total of 29 chemical constituents were identified in the seed essential oil by retention index and mass spectral data (Fig. 1). The concentrations of the volatile components are presented in Table 1, according to their elution order on an HP-5 fused silica capillary column. Components of the seed essential oil were Hexahydro farnesyl acetone (14.2%), β -Bisabolene (13.7%), and Carotol (9.8%), *cis*-Chrysanthenyl acetate (4.7%), Caryophyllene oxide (4.9%), α -Asarone (3.0%), *trans*- α -Bergamotene (3.2%), (E)-caryophyllene (3.1%).

3.2. Cytotoxicity assessment

The cell viability of RAW 264.7 macrophages was evaluated. Fig. 2 shows that CREO at concentrations of 1%, 2% and 5% did not affect cell viability. There is no significant change in live cell population when compared with normal control.

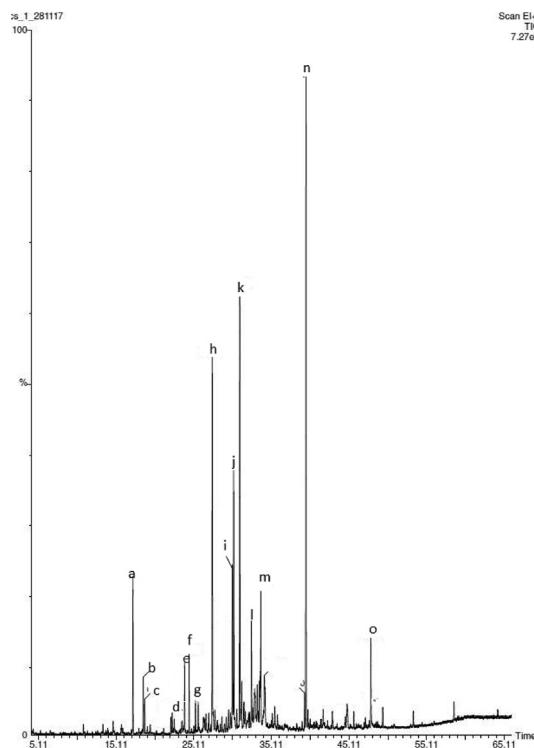


Fig. 1. Gas chromatographic profile (TIC) of *C. reflexa* essential oil as a) Cis-chrysanthenol acetate, b) *trans*-sabinyl acetate, c) carvacrol, d) (E)- β -Damasconone, e) (E)-caryophyllene, f) α -*trans*-bergamotene, g) (E)- β -farnesene, h) β -Bisabolene, i) spathlenol, j) caryophyllene oxide, k) carotol, l) Daucol, m) α -asarone, n) Hexahydrofarnesyl acetone, o) (E)-phytol.

Table 1
Essential oil composition of *Cuscuta reflexa* seed.

S.no.	Compound	RI ^a	RI ^b	Content (%)
1.	Linalool	1100	1095	0.3
2.	<i>cis</i> -Chrysanthenol	1164	1160	0.2
3.	α -Terpineol	1193	1186	0.2
4.	<i>cis</i> -Chrysanthenyl acetate	1264	1261	4.7
5.	<i>trans</i> -Sabinyl acetate	1294	1289	2.1
6.	Carvacrol	1300	1298	0.4
7.	Isolodene	1383	1374	0.7
8.	(E)- β -Damascenone	1388	1383	0.8
9.	β -Cubebene	1392	1387	0.2
10.	(E)-caryophyllene	1426	1417	3.1
11.	<i>trans</i> - α -Bergamotene	1439	1432	3.2
12.	(E)- β -Farnesene	1459	1454	0.9
13.	Germacrene D	1489	1480	1.5
14.	(E)-Methyl <i>iso</i> -eugenol	1495	1491	0.7
15.	β-Bisabolene	1512	1505	13.2
16.	δ -Amorphene	1518	1511	0.7
17.	Spathulenol	1583	1577	3.2
18.	Caryophyllene oxide	1590	1582	4.9
19.	Salvial-4(14)-en-1-one	1600	1594	0.3
20.	Carotol	1605	1594	9.8
21.	Daucol	1650	1641	2.0
22.	14-Hydroxy-9-epi-(E)-caryophyllene	1671	1668	0.4
23.	α -Asarone	1681	1675	3.0
24.	α -Bisabolol	1695	1685	0.6
25.	Hexahydrofarnesyl acetone	1846	1847	14.2
26.	(5E,9E)-Farnesyl acetone	1905	1913	0.7
27.	(E)-Phytol	2117	2112	2.0
28.	n-Tricosane	–	2300	0.9
29.	n-Pentacosane	–	2500	0.9

^a RI: experimental retention index (BP-5 capillary column); ^bRI: retention index value from literature (Adams, 2007; Andriamaharavo, 2014).

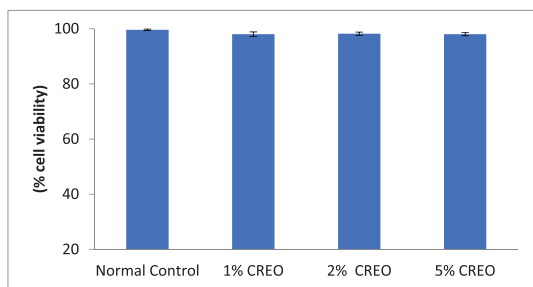


Fig. 2. Cell viability of CREO using MTT assay in RAW 264.7 Data are mean \pm SEM; n = 3 normal versus test.

3.3. In-vivo anti-inflammatory

3.3.1. Acute dermal irritation

Impact of CREO on TPA-induced oedematogenic response was noticed in TPA induced ears of mice after 6 hrs. In present study reduction in skin inflammation is shown in time dependent manner reported as 38.51, 43.64, 52.32% decreased in weight of ear was recorded. 5% sample showed marked decreased in edema as compared to the toxin control group (TPA).

3.3.2. TPA induced skin inflammation

Initially no erythema on the ear of animals was observed. After 4, 24, 48, and 72 hrs of treatment significant increase in auricle blood flow was recorded as compared to the vehicle control as 0.33 and 0.5 respectively. Ear thickness was recorded before and after application of TPA at the duration of 4 and 24 h with acetone. The vehicle and positive control group did not showed edema when compared to toxin group. Ear weight 6.5 mm discs biopsy punch are used to calculate the weight of each ear and found that there is tremendous increased in weight in TPA induced group

while reduction in positive control or also showed dose dependent decrease in the ear weight (Fig. 3).

3.3.3. Measurement of oxidative stress markers

Lipid per oxidation and nitric oxide oxidative parameters are performed. The treatment of CREO showed dose dependent reduction in the level. Both the oxidative markers have significantly decrease the TPA induced activity.

Table 2.

3.3.4. Measurement of IL-1 β , IL-6, TNF- α level in serum

After the completion of experiment the serum was collected and measure IL-6, IL-1 β , and TNF- α in mice serum by ELISA kits according to the instructions (Fig. 4). This experiment TPA induced group clearly showed increased in cytokines level whereas the concentration of 5% showed significantly reduced the in production of IL-6, TNF- α and IL1- β and these results were compared to TPA (negative) control and positive control groups (Table 3).

3.4. Histopathology

In present study the vehicle used during the experiment showed no effect in the cutaneous morphology of the ear of mice, while the TPA control group showed severe increase in the diameter of ear. Leucocyte infiltration and ear edema are observed vigorously but after the treatment with 5% CREO there is significantly reduce in the elevated interleukins Fig. 5.

4. Discussion

C. reflexa have many medicinal properties as reported in the ancient literatures its time of the flowering time is from March to April. Here we isolate the seed part and investigate for anti-inflammatory activity. (Muhammad et al., 2021). The plant seed

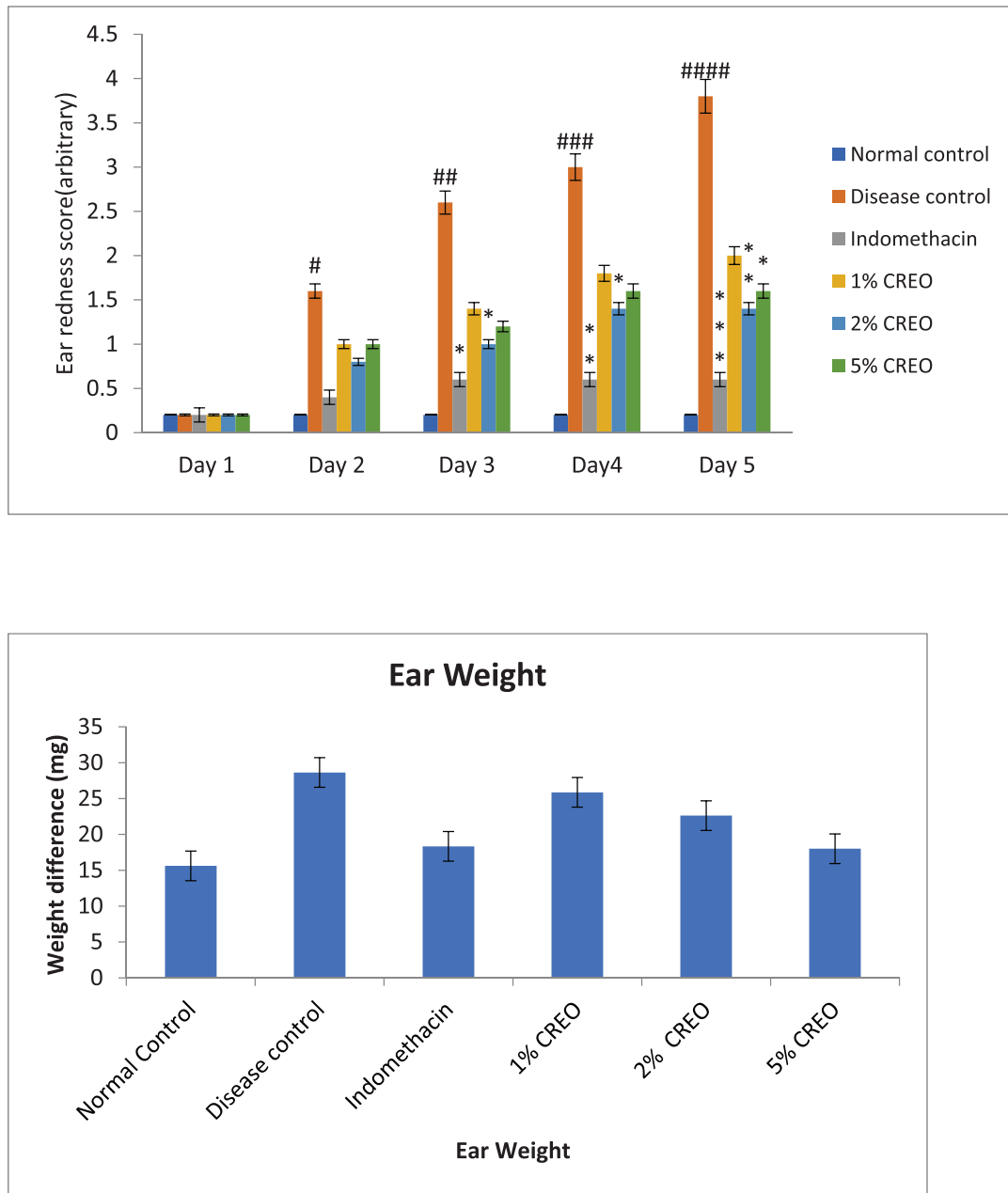


Fig. 3. Effect of CREO on TPA-induced ear inflammation. Data are mean ± SEM; n = 5 (a) Ear redness, (b) Ear weight, ANOVA; Tukey test p < 0.05.

Table 2

Effect of CREO on TPA –induced oxidative stress mediator nitric oxide (NO) and malonaldehyde (MDA).

Parameter	Normal control	Disease control	Indomethacin	1% CREO	2% CREO	5% CREO
LPO (µm/ml)	2.80 ± 0.50	20.85 ± 1.40	6.81 ± 0.60	9.81 ± 1.64	8.45 ± 1.63	6.17 ± 0.59
NO (µm/ml)	5.39 ± 0.85	32.18 ± 2.08	9.14 ± 0.84	20.64 ± 1.56	14.62 ± 1.04	7.56 ± 2.03

Data are mean ± SEM; N = 5.

#Normal versus toxin, * Toxin versus treatment (ANOVA; Tukey test), p < 0.05.

has ability to survive in dormant condition upto 20 years and regrow in favorable condition. They have no roots but its seed grows into host plant, after sometimes it destroys the host plant fully and covers large area. According to literature it destroys many fields of farmers. It is a global problem in agriculture. But it has many benefits as claimed by traditional literatures like anti-inflammatory agent used by Unani physician since long time. Tra-

ditionally this whole plant is used in muscular pain, liver diseases, depression, fever, urination disorders, mental disorders, skin diseases in Ayurvedic system of medicine (Ansari et al., 2020). One of the researchers investigated for analgesic and anti-inflammatory activities of the plant and exhibited significant anti-inflammatory effect against carrageenan induced paw edema in methanolic and aqueous fraction (Saha et al., 2016). In another

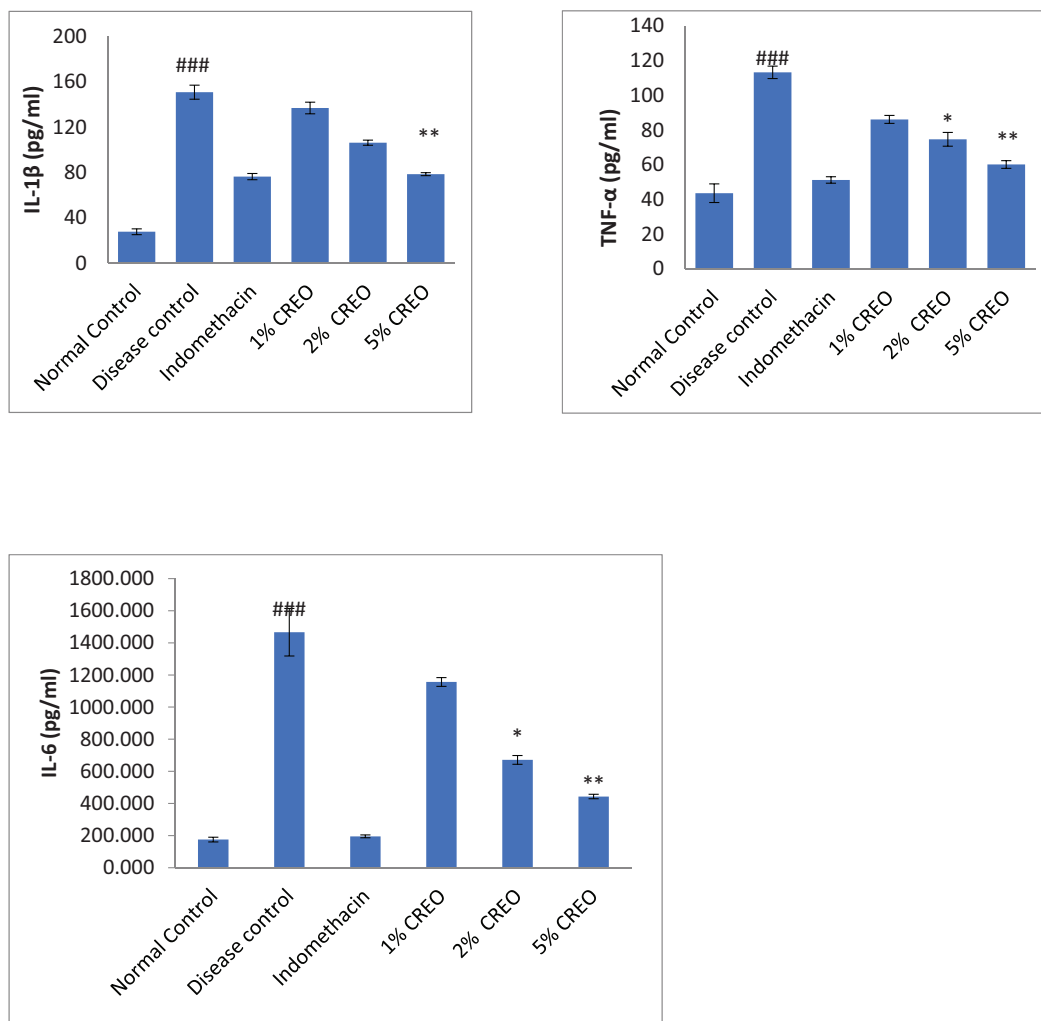


Fig. 4. Effect of CREO on inflammatory mediators in the ears tissue isolated from TPA induced inflammation. Data are mean ± SEM; n = 5 #Normal versus disease control, *Disease control versus treated, ANOVA; Tukey test p < 0.05.

Table 3
Effect of CREO on pro-inflammatory cytokines.

Parameter	Normal control	Disease control	Indomethacin	1% CREO	2% CREO	5% CREO
TNF- α	43.64 ± 5	113.34 ± 3	51.28 ± 1	86.18 ± 2	74.7 ± 3*	60.2 ± 2**
IL-1 β	27.65 ± 2	150.76 ± 6	51.27 ± 2	136.81 ± 5	106.24 ± 3	78.43 ± 1**
IL-6	175.37 ± 14	1466.08 ± 147	195.40 ± 8	1156.18 ± 27	671.18 ± 27*	443.07 ± 13**

Data are mean ± SEM; N = 5.

#Normal versus toxin, * Toxin versus treatment (ANOVA; Tukey test), p < 0.05.

study a compound terrestrosin D showed marked reduction in inflammation and fibrosis in the lungs of mice when exposed to bleomycin (Qiu et al., 2019). Methanolic and aqueous extract of *C. reflexa* was determined for anti-inflammatory activity by cargeenan and histamine induced paw edema models and found effective at the dose of 300 mg/Kg p.o (Suresh et al., 2011).

CREO at various concentrations are tested for anti-inflammatory activity. Inflammation, basic response of any type of injury is to protect the body. Excretion of inflammatory mediators like IL-1β, IL-6 and TNF-α inhibits pro-inflammatory mediator synthesis and elevates anti-inflammatory cytokines. In order to promote cascade, TPA is applied to the mouse ear which increases vascular permeability, mast cell infiltration and edema. Histamine and serotonin released by mast cells which further elevate neu-

trophil influx and vascular permeability. Arachidonic acid released from phospholipid is caused by using TPA which further release inflammatory mediators. These inhibitors are active against edema and leukocyte infiltration in present TPA model of mouse ear inflammation. CREO contain linalool, carvacrol, β-bisabolene, spathulenol, phytol and many more compounds which shows antioxidant and anticancer activities.

In present study we found CREO possess the ability to reduce inflammation by inhibiting IL-1β, IL-6 and TNF-α inhibit by stimulated monosaccharide with lipo-polysaccharide and nitric oxide. Increase in lipo-polysaccharide and involvement in redox-sensitive steps enhance nuclear factor-κβ activation that is why CREO shows anti-inflammatory action against TPA induce ear mouse model. Thus, we can say that *C. reflexa* essential oil signifi-

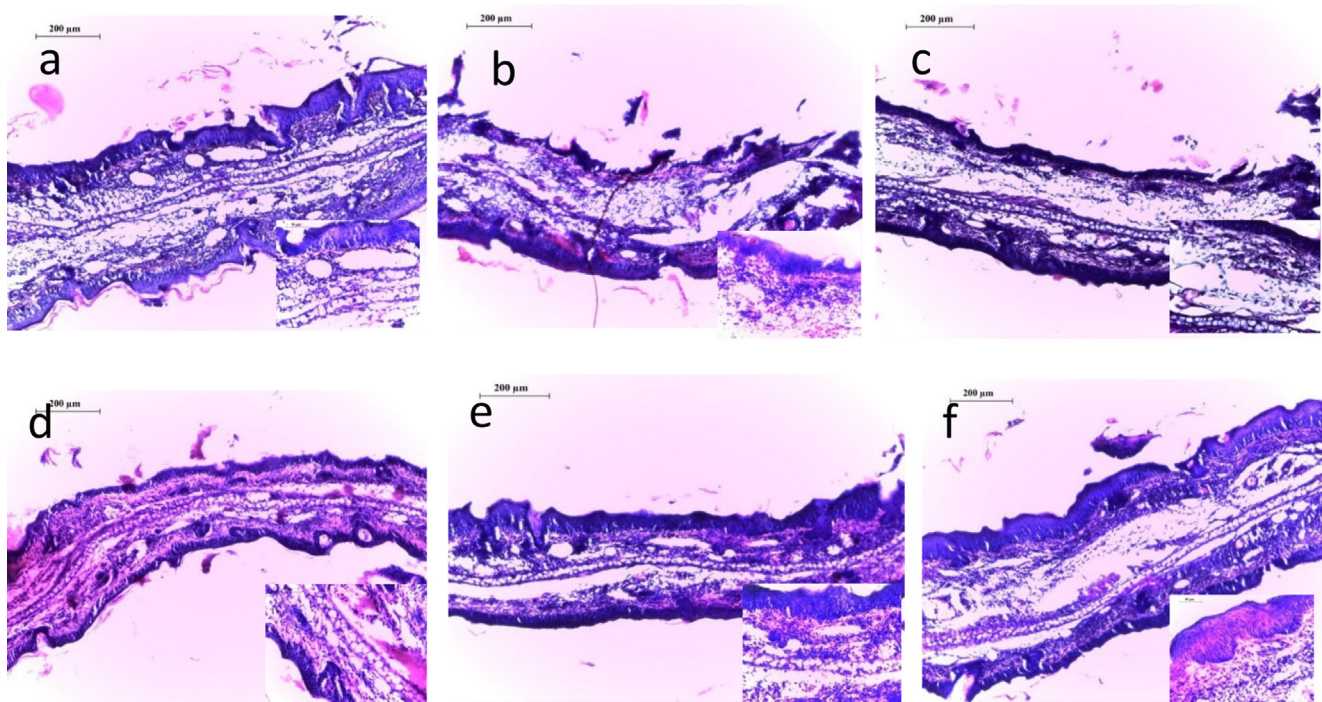


Fig. 5. Histopathological profile of ear tissues under microscopy (a) Normal control, (b) Disease control, (c) Indomethacin, (d) 1%CREO, (e) 2%CREO (f) 5%CREO.

cantly reduces inflammation by reduction in inflammatory responses against TPA in skin. By using these results one can further use the oil for preparation of pharmaceutical product for anti-inflammatory purpose.

5. Conclusion

The present study significantly demonstrates the chemical profiling of volatile oil of *C. reflexa* seed and also first time for anti-inflammatory activity and found that the essential oil of this plant is helpful in the reduction of inflammation at the concentration of 5%. This may be an important finding for patient and farmers both. As this plant is known as weed so, from this result we can say that it can be used in formulation in future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Further reading

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