



ORIGINAL ARTICLE

Essential oil from waste leaves of *Curcuma longa* L. alleviates skin inflammation

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Abstract

Background *Curcuma longa* L. is an important industrial crop used by medicinal and cosmetic industries in the world. Its leaves are a waste material after harvesting rhizomes. The aim of the study was to evaluate the chemical and pharmacological profile of essential oil from waste leaves of *Curcuma longa* (EOCl) against skin inflammation.

Methods EOCl was subjected to gas chromatography (GC) analysis for identification of essential oil constituents and its anti-inflammatory evaluation through in vitro and in vivo models.

Results Chemical fingerprinting using GC and GC–MS analysis of EOCl revealed the presence of 11 compounds, representing 90.29% of the oil, in which terpinolene (52.88%) and α -phellandrene (21.13%) are the major components. In the in vitro testing EOCl inhibited the production of pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β) in lipopolysaccharide (LPS) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in the human keratinocyte cell line (HaCaT). Topical application of EOCl produced anti-inflammatory effects by reducing ear thickness, ear weight and ameliorating the level of pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β) at protein and mRNA levels as well as regulating the overproduction of oxidative markers and restoring the histopathological damage in a TPA-induced mouse model of inflammation.

Conclusion These findings of topical anti-inflammatory properties of EOCl provide a scientific basis for medicinal use of this plant material against inflammatory disorders.

Graphical Abstract

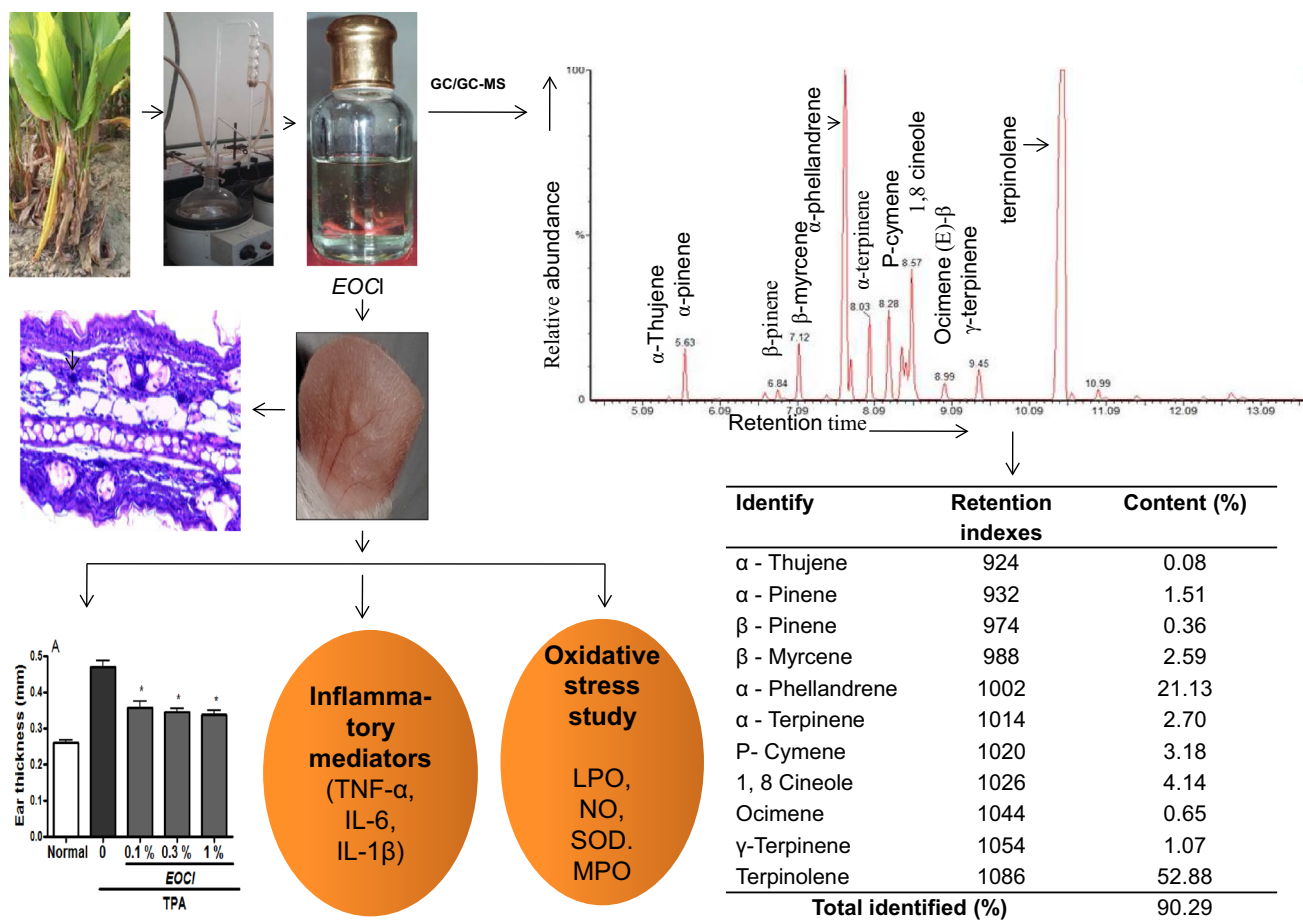
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Keywords *Curcuma longa* · Leaves · Waste · Skin · Inflammation · Oxidative stress

Introduction

Skin is the largest sensory organ in the mammalian body and plays a key role in host immunological defences (Kupper and Fuhlbrigge 2004). Inflammation is a complex and an important host defence mechanism in response to different stimuli, such as pathogens, physical injury, and chemical injury. Psoriasis, atopic dermatitis, and cancer diseases are the consequences of defensive inflammation in many parts of the organism. Cutaneous inflammation is caused by the accumulation of inflammatory cells that move to the inflammation site in association with the secretion of pro-inflammatory mediators, such as cytokines, prostaglandins, and leukotrienes (Singh et al. 2014). Pathophysiology of inflammatory skin disorders is interconnected with the changes in the level of these cytokines, reactive oxygen species (ROS), and reactive nitrogen species (Trouba et al. 2002). The inflammatory reactions of skin diseases resemble those of 12-tetradecanoylphorbol-13-acetate (TPA)-induced pleiotropic tissue reactions and ROS generation as confirmed

by substantial experimentation (Verma et al. 2006). Several non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs (SAIDS) are used for the treatment of inflammatory diseases, but these drugs show severe complications of inflammation by causing adverse and side effects. Since the critical aetiology and exacerbating mechanisms are not completely understood, it is difficult to develop a magic bullet for chronic inflammatory disorders (Kim et al. 2004). Essential oils are more advantageous as natural remedies because of their safety compared with synthetic drugs. The ability of the antioxidants in such oils to penetrate cell membranes and affect various biological activities is much higher owing to their low molecular weight (Reische et al. 1998).

Curcuma longa, commonly known as turmeric, belongs to the family Zingiberaceae, is cultivated mostly in Asia, and India accounts for more than 90% of the total output of turmeric in the world (Jain et al. 2007). It is a perennial herb with simple large leaves and rhizomes. Extracts, molecules, and essential oil of the rhizomes are of great therapeutic

importance (Jena et al. 2017). Several recent studies reported the external applications of *C. longa* rhizome extracts, molecules, and its essential oil for the treatment of skin diseases (Srivilai et al. 2017). Leaves of *C. longa* are a waste material after harvesting of the rhizome for spices and herbal medicinal products. An exploration of possible therapeutic effects along with the chemical characterization of essential oil isolated from medicinal plant waste plays a key role in converting the waste materials into therapeutic value-added products. In an attempt to convert the waste material into therapeutic value-added products, we have isolated the essential oil from waste leaves of *C. longa* after harvesting of the rhizome and investigated its chemical composition and in vitro and in vivo anti-inflammatory potential with particular reference to skin inflammation as an experimental pharmacology study. Our results revealed that the essential oil from waste leaves of *C. longa* reduces pro-inflammatory cytokine production and ameliorates skin inflammation without any toxic effect on the skin.

Materials and methods

Reagents

Sodium sulfate, acetone, hydrogen peroxide, and sodium acetate were purchased from Merck Ltd (Mumbai, India). Fetal bovine serum was a product of GIBCO (Grand Island, NY, USA). Dulbecco's Modified Eagle's Medium (DMEM), antibiotic antimycotic solution, dimethyl sulfoxide (DMSO), thiobarbituric acid, trichloroacetic acid, pyrogallol, LPS, and TPA were purchased from Sigma (St. Louis, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), sulfanilamide, phosphoric acid, haematoxylin, and eosin were purchased from Himedia (Mumbai, India). *N*-1-Naphthylethylenediamine dihydrochloride was purchased from S. D. Fine Chem. Ltd (Mumbai, India). Tetramethylbenzidine HCl was purchased from BD Biosciences (USA).

Collection of plant material and extraction of essential oil

Dried leaves of *C. longa* were collected from the experimental farms at CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, UP, India. The leaves were identified in the botany department and assigned the accession number C-051. Leaves (500 g) were hydrodistilled in a Clevenger apparatus for 3 h to afford 13.5 ml [2.7% (v/w)] of essential oil. The oil was collected, dried over anhydrous sodium sulfate and stored at 4 °C prior to analysis. The isolated essential oil was coded as EOCl to conduct a blind

experimental study (chemical and pharmacological) with particular emphasis on skin inflammation to avoid any bias.

GC and GC–MS analysis

The analysis was performed by capillary GC and GC–MS. A PerkinElmer auto system XL GC fitted with an Equity-5 column (60 m × 0.32 mm i.d., film thickness 0.25 µm) was used to identify and quantify the essential oil components of EOCl. Hydrogen was used as carrier gas at constant pressure of 10 psi. The column oven was programmed from 70 °C (hold for 2 min) to 240 °C (hold for 10 min) at the rate of 3 °C/min, injector and detector (FID) temperatures were maintained at 280 and 300 °C, respectively. The split ratio was 1:30. For GC–MS, an Elite-5 MS fused silica capillary column (30 m × 0.25 µm i.d., film thickness 0.25 µm) was used in a PerkinElmer Clarus® 680 GC interfaced with a Clarus® SQ 8C quadrupole mass spectrometer. Programmed split/splitless (PSS) injector temperature was 250 °C with a split ratio of 1:80. The column oven was programmed from 60 to 240 °C at the rate of 3 °C/min. Helium was used as carrier gas at a constant flow of 1 mL/min. Transfer line and source temperatures were 250 °C; electron impact ionization mode was used with ionization energy of 70 eV; scan duration comprised a scan time (0.39 s) and inter-scan delay (0.01 s) and mass scan range 40–400 amu. Characterization was achieved on the basis of retention time, elution order, relative retention index using a homologous series of *n*-alkanes (C6–C28 hydrocarbons, Polyscience Corp. Niles IL), coinjection with standards in GC–FID and GC–MS (Aldrich and Fluka), mass spectral library search NIST/EPA/NIH version 2.0 g and Wiley registry of mass spectral data (9th edition) and by comparing with the mass spectral literature data (Adams 2001).

In vitro study

The human keratinocyte cell line (HaCaT) was obtained from the National Centre for Cell Sciences Pune, India. Cells were cultured in DMEM supplemented with 10% fetal bovine serum with antibiotic antimycotic solution in a CO₂ incubator at 37 °C with 5% CO₂ and 90% relative humidity.

Cell toxicity assessment

A cytotoxicity study of EOCl was performed using the MTT assay as described (Sharma et al. 2012). Briefly, HaCaT cells (0.5 × 10⁶ live cells/ml) were used for the experiment. Cells were treated with EOCl (0.1, 0.3, 1%) and DMSO (1%) and incubated for 24 h at 37 °C in 5% CO₂. After incubation 20 µl of MTT solution (5 mg/ml in PBS) was added to each well and left for 4 h, followed by media removal and cell solubilization in DMSO (100 µl) for 10 min. The absorbance

was recorded at 550 nm on a microplate reader (Spectramax; Molecular Devices, USA). Cell cytotoxicity was calculated as the percentage of MTT absorption by using the following formula: percentage (%) of survival = mean experimental absorbance/mean control absorbance \times 100.

In vitro anti-inflammatory assessment

HaCaT cells (0.5×10^6 live cells/ml) were treated with EOCl at the concentration of 0.1, 0.3, 1% respectively, followed by stimulation with LPS and TPA for 16 h, separately. Pro-inflammatory mediators like tumour necrosis factor- α (TNF- α), interleukin-6 (IL-6) and interleukin- β (IL- β) in cell culture supernatant were determined by using human-specific enzyme immunoassay (EIA) kits (BD Biosciences, USA) as per the method described (Singh et al. 2014). Briefly, the ELISA plates coated with 100 μ l per well of TNF- α , IL-6 and IL-1 β capture antibody were incubated overnight at 4 $^{\circ}$ C and following this wells were blocked with 200 μ l/well assay diluents. Culture supernatant and standard (100 μ l) were added into the washed wells and incubated for 2 h at room temperature (20–25 $^{\circ}$ C). After incubation wells were washed five times with wash buffer and 100 μ l of detecting solution (detection antibody and streptavidin–horseradish–peroxidase conjugate) was added. The wells were again washed with wash buffer and 100 μ l of tetramethylbenzidine (TMB) substrate solution was added. Finally, after 30 min, 50 μ l of stop solution (2 N H₂SO₄) was added. The colour density was measured at 450 and 570 nm. Absorbance measured at 570 nm was subtracted from absorbance measured at 450 nm. The values of TNF- α , IL-6 and IL-1 β were expressed as picograms per millilitre.

In vivo study

Experimental animals

All experiments were performed using female New Zealand white rabbits (1500 \pm 250 g) and female BALB/c mice ranging in weight from 18 to 22 g. The animals' residential area was maintained at a temperature of 22 \pm 2 $^{\circ}$ C for a 12 h light/dark cycle throughout the whole the experimental period and animals were allowed to swallow only water to prevent food interaction/metabolism with drugs before starting the experiment. The aforementioned conditions were produced by following the Guidelines of Institutional Animal Ethics Committee (CIMAP/IAEC/2016-19/01 and CIMAP/IAEC/2016-19/05) approved by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Government of India (Registration no: 400/GO/ReBi/S/01/CPCSEA).

Acute dermal irritation studies

Acute dermal irritation was examined on New Zealand white rabbits ($n = 4$). Samples were spread to the shaved parts of skin at a concentration of 1% on 1 inch square area. Acetone was used as a vehicle control at the opposite side of the skin. Throughout the course of dermal vulnerability, assessment of type and severity of symptoms in the skin was ranked according to the classification of skin reaction, as was the skin irritation effect at 1, 4, 24, 48 and 72 h. Skin reactions were assessed independently for erythema and oedema, based on a 0–4 grading scale. For erythema: 0, erythema formation; 1, very slight erythema (barely perceptible); 2, well-defined erythema; 3, moderate erythema; 4, severe erythema (beef redness). For oedema: 0, no oedema; 1, very slight oedema (barely perceptible); 2, well-defined oedema (edges of area well defined by definite rising); 3, moderate oedema (raised approximately 1 mm); 4, severe oedema (raised more than 1 mm and extending beyond the area of exposure).

Primary irritation score of each experimental rabbit was calculated by summing up the scores for erythema and oedema at the test site minus the sum of erythema and oedema on the control site in the analysed period divided by the number of observations. The cumulative irritation index (CII) was calculated as the arithmetic mean of the sum of primary irritation score for all animals in the group (Aroonrerk and Kamkaen 2009).

TPA-induced skin inflammation in mice

The experiment was conducted on the basis of previously described methods (Bralley et al. 2008). TPA 2.5 μ g/ear dissolved in acetone was applied in a volume of 20 μ l to the inner and outer surface of both the ears. EOCl in acetone was applied topically after 30 min of TPA application at the doses of 0.1, 0.3 and 1%. The non-treated control group to which only TPA with solvent controls (vehicle) was applied was used as a measure of maximum inflammation and a non-TPA-treated group to which only acetone was applied was used as a normal group.

Ear oedema and tissue weight measurement

Ear thickness was measured using a digital micrometer (Aerospace Instruments) prior to and after the inflammation induction. We found that time-dependent oedematogenic response peaked at 6 h. Following completion of the experiment, mice were killed and a 6.5-mm-diameter disc was removed from each ear with a metal punch and weighed. Homogenates (10%) were prepared from the ear tissue and

processed for the preparation of post-mitochondrial supernatant (PMS) to investigate the effect of EOCl on pro-inflammatory cytokines and oxidative markers.

Quantification of pro-inflammatory cytokine

Pro-inflammatory cytokines TNF- α , IL-6 and IL- β in ear tissue homogenate were quantified using the methods established at our laboratory as described above.

Measurement of malondialdehyde (MDA) formation in lipid peroxidation

The amount of MDA formed in each sample was assayed according to the method as described earlier (Padmaja et al. 2011) by measuring the optical density (OD) of the reaction mixture of 0.8% (w/v) thiobarbituric acid with resulting supernatant of centrifuged ear homogenate and 20% (v/v) trichloroacetic acid at 535 nm.

Measurement of nitric oxide (NO) production

Nitric oxide formation was measured in tissue samples using the procedure as described earlier (Kim et al. 2008) by assaying nitrite, one of the stable end products of NO oxidation. Briefly Griess reagent [1% sulfanilamide in 5% phosphoric acid (sulfanilamide solution) and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in distilled H₂O (NED solution)] was incubated with ear homogenate for 15 min in the dark, after which the absorbance was measured at 540 nm.

Superoxide dismutase (SOD) assay

The tissue homogenate was assayed for superoxide dismutase activity by following the inhibition of pyrogallol autooxidation as described earlier (Marklund and Marklund 1974). Briefly, 100 μ l of tissue homogenate was added to Tris HCl Buffer, pH 8.5. Lastly 25 μ l of pyrogallol was added and changes in absorbance at 420 nm were recorded at 1-min intervals for 3 min. The increase in the absorbance at 420 nm after the addition of pyrogallol was inhibited by the presence of SOD.

Determination of myeloperoxidase (MPO) activity

MPO activity was determined as described earlier (Bradley et al. 1982). Briefly, the tissue homogenate (20 μ l) was added to a mixture (200 μ l) of phosphate buffered saline and hydrogen peroxide. The reaction was initiated by 20 μ l of 18.4 mM tetramethylbenzidine HCl in DMSO. This reaction was quenched by addition of 30 μ l of 1.46 M sodium acetate. The absorbance of the complete reaction mixture was measured at 620 nm.

Real-time polymerase chain reaction (RT-PCR) analysis of cytokine expression

Total RNA was isolated from ear biopsies using an RNA ladder kit (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instructions. The quality of RNA was accepted in the range of 1.9–2.1 at the ratio of absorbance A260/A280. Total RNA (1.5 μ g) was used to synthesize cDNA using the Applied Biosystems kit according to the manufacturer's protocol. We performed RT-PCR with reagents and protocols from TaqMan fast universal PCR master mix kit (2x) (Applied Biosystems, Foster City, CA, USA) and mouse TaqMan gene expression assays. The relative expressions of TNF- α , IL-6 and IL-1 β mRNA were normalized to the amount of β -actin in the same cDNA using the relative quantification method ($2^{-\Delta\Delta C_t}$ method) (Livak and Schmittgen 2001). The fold change in target gene cDNA relative to the β -actin internal control was determined by the following formula: fold change = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{t_{\text{target gene}}} - C_{t_{\beta\text{-actin}}}) - (C_{t_{\text{control}}} - C_{t_{\beta\text{-actin}}})$. Primer sequences for analysis of TNF- α , IL-6, IL-1 β and GAPDH are described in Table 1.

Histopathology

Ear samples were fixed in 10% buffered formalin solution. Each sample was rinsed with water, dehydrated with graded concentration of ethanol and embedded in paraffin, cut into 5- μ m sections and stained with haematoxylin–eosin. A representative area was selected for qualitative light microscopic analysis of the inflammatory cellular response at magnifications of 40 \times .

Statistical analysis

The statistical significance was assessed using one-way analysis of variance followed by Tukey's test. The values are expressed as mean \pm SEM (standard error of mean) and $p < 0.05$ was considered significant.

Table 1 Real-time PCR primers used in the experiment

Gene		Sequence 5' to 3'
GADPH	Forward	ATTGTCAGCAATGCATCCTG
	Reverse	ATGGACTGTGGTCATGAGCC
TNF- α	Forward	CCCTCACACTCAGATCATCTTCT
	Reverse	GCTACGACGTGGGCTACA
IL-6	Forward	TAGTCCTTCCACCCCAATTTC
	Reverse	TTGGTCCTTAGCCACTCCTTC
IL-1 β	Forward	GCAACTGTTCTGAACTCAACT
	Reverse	ATCTTTTGGGGTCCGTCAACT

Results

Composition of EOCl

EOCl has been subjected to GC analysis by using flame ionization and mass spectrometry (MS) detections. The GC–MS chromatogram of active constituents is shown in Fig. 1. The analysis by GC/MS identified 11 compounds, namely terpinolene (52.88%), α -phellandrene (21.13%), 1,8-cineole (4.14%), *p*-cymene (3.18%), α -terpinene (2.70%), β -myrcene (2.59%), α -pinene (1.51%), γ -terpinene (1.07%), ocimene (0.65%), β -pinene (0.36%) and α -thujene (0.08%). Together these represent 90.29% of the EOCl composition.

Cytotoxicity assessment

Significant changes in the percentage of live cell population were not observed ($p < 0.05$) at any concentration (0.1, 0.3, 1%) of the treatment when compared with normal cells (Fig. 2).

Effect of EOCl against LPS- and TPA-induced production of inflammatory cytokines

The production of cytokines such as interleukins and TNF- α is significantly increased to amplify the pro-inflammatory reaction. We investigated whether EOCl (0.1, 0.3 and 1%) modulates the level of inflammatory cytokines. Secreted levels of TNF- α , IL-6 and IL-1 β from LPS- and

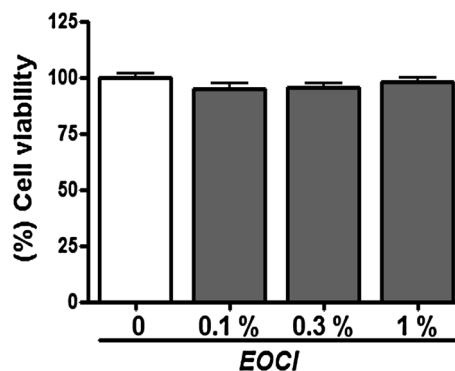


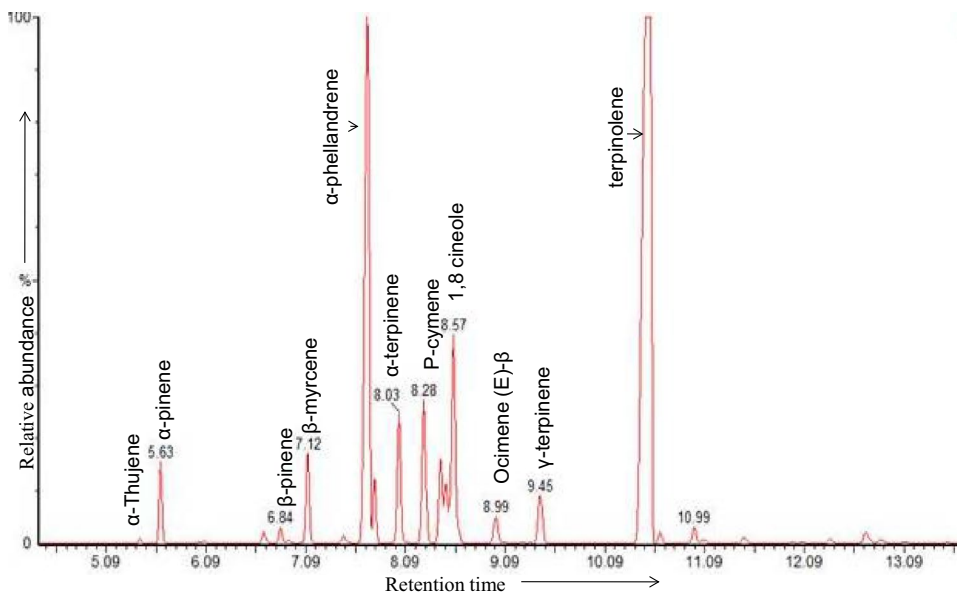
Fig. 2 Cell viability of EOCl using MTT assay in HaCaT cells. Data are mean \pm SEM; $n = 3$. Normal versus vehicle

TPA-activated HaCaT cells were significantly reduced by EOCl (Fig. 3).

Effect of EOCl on TPA-induced oedema in mouse ears

Time-dependent oedematogenic response was obtained in the TPA-induced skin inflammation model and reached its maximum after 6 h of TPA application. The ear oedema caused by TPA was dose-dependently inhibited by topical treatment. The oedema reduction caused by EOCl was 41.41, 43.62 and 60.73% at the doses of 0.1, 0.3 and 1%, respectively. The increase in weight of ear plugs was also protected by EOCl. All the treated groups showed significant inhibition effects compared with the control (TPA + vehicle group) (Fig. 4).

Fig. 1 Essential oil constituents identified in *Curcuma longa* leaves (EOCl) using gas chromatography/mass spectrometry



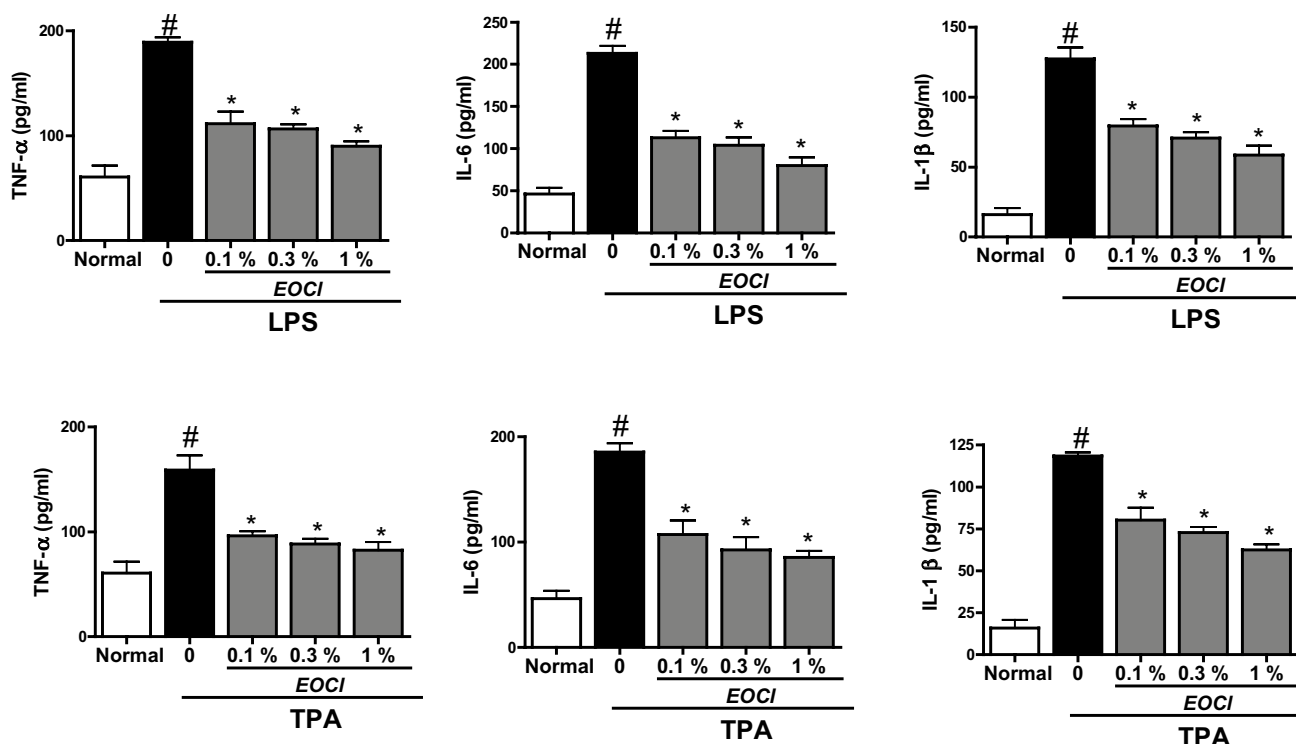


Fig. 3 Effect of EOCI on LPS- and TPA-induced inflammation in HaCaT cells. Data are mean ± SEM; n = 3. #Normal versus vehicle, *vehicle versus treatment, (ANOVA; Tukey test), p < 0.05

Fig. 4 Effect of EOCI in TPA-induced ear inflammation model. **a** Ear thickness. **b** Ear weight. Data are mean ± SEM; n = 3. *Vehicle versus treatment, #normal versus vehicle (ANOVA; Tukey test), p < 0.05

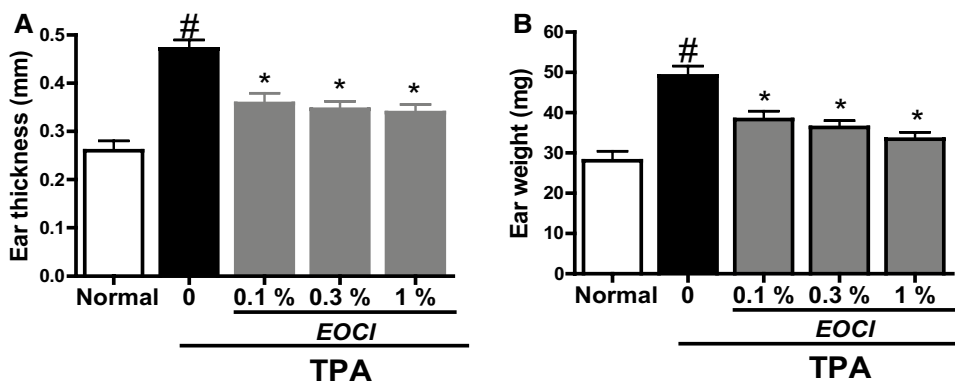


Table 2 Results of acute dermal irritation studies

Hours after treatment	Average score		Cumulative irritation index (CII)
	Control site	Treatment site	
1	0	0	0
4	0	0.25	0.06
24	0	0.5	0.12
48	0	0	0
72	0	0	0

Acute dermal irritation studies

The results obtained from acute dermal irritation studies are shown in Table 2. No oedema reactions appeared at test and control sites in all the rabbits. No higher grades for erythema were observed. A dermal irritation test was conducted to confirm safe usage in the case of substance subjected to human application.

Effect of EOCl against TPA-induced production of inflammatory cytokines

Regulation of the production of inflammatory mediators is the main procedure used to develop and test prospective remedial agents for inflammatory diseases. In this model, we clearly demonstrated that the results from ELISA of TPA-treated ear skin showed increasing production of inflammatory cytokines: EOCl (1%) significantly ($p < 0.05$) reduced the production of TNF- α , IL-6 and IL-1 β in comparison to control (TPA + vehicle group) (Table 3).

Effect of EOCl on oxidative stress markers

The activity of SOD, the major detoxifying enzyme for superoxide anion radicals, decreases while the levels of NO, MDA and MPO increased significantly in the ear tissues treated with TPA. Treatment with EOCl (0.1, 0.3 and 1%) significantly inhibited this TPA-induced suppression of SOD activity, as well as reduced the levels of NO, MDA and MPO when compared to the control (TPA + vehicle) group and their effects were dose dependent (Table 4).

Effect of EOCl on mRNA level of inflammatory cytokines

Examination of pro-inflammatory gene expression in TPA-induced skin inflammation model by RT-PCR showed the significant inhibition of mRNA expression of inflammatory cytokines (TNF- α , IL-6, IL-1 β) at the doses of EOCl (0.3 and 1%) in comparison to control (TPA + vehicle group). These results substantiate that the inhibition of production of these cytokines at protein level in the ELISA test was mediated through the transcriptional downregulation of these target genes (Fig. 5).

Histopathology

Treatment with the vehicle showed no alterations in cutaneous morphology in the hispathological analysis. Applications of TPA induced an intense increase in the ear diameter (oedema formation) and leucocyte infiltration into the dermis. Treatments of EOCl suppressed inflammatory parameters, such as oedema formation and leucocyte infiltration (Fig. 6).

Table 3 Effect of EOCl on ear homogenate content of inflammatory mediators (TNF- α , IL-6, IL-1 β) in TPA-induced ear inflammation model

Treatment	Dose	TNF- α		IL-6		IL- β	
		pg/ml	% inhibition	pg/ml	% inhibition	pg/ml	% inhibition
Normal	–	47.50 \pm 16.80	–	149.33 \pm 31.33	–	26.17 \pm 3.53	–
Vehicle	–	111.50 \pm 4.25 [#]	–	1420.17 \pm 113.16 [#]	–	132.50 \pm 6.66 [#]	–
EOCl	0.1%	79.33 \pm 5.09	28.32 \pm 7.14	1347.50 \pm 32.76	3.66 \pm 9.19	124.83 \pm 2.85	5.42 \pm 4.07
EOCl	0.3%	69.83 \pm 16.37	37.87 \pm 13.69	1277.83 \pm 98.24	8.12 \pm 12.64	119.17 \pm 3.38	9.84 \pm 2.39
EOCl	1%	58.83 \pm 3.47*	47.31 \pm 1.21	784.83 \pm 138.31*	45.52 \pm 5.07	110.17 \pm 2.91*	16.40 \pm 4.95

Data are mean \pm SEM; $n = 3$

*Vehicle versus treatment, [#]normal versus vehicle (ANOVA; Tukey test), $p < 0.05$

Table 4 Effect of EOCl on TPA-induced oxidative stress mediators malonaldehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD) and myeloperoxidase (MPO): quantification from ear tissue homogenate

Parameters studied	EOCl				
	Normal	Vehicle	0.1%	0.3%	1%
Lipid per oxidation (μ m/ml)	1.27 \pm 0.79	19.68 \pm 0.70 [#]	9.92 \pm 0.67*	8.14 \pm 0.47*	6.17 \pm 0.88*
Nitric oxide (μ m/ml)	6.33 \pm 1.36	39.78 \pm 2.25 [#]	17.73 \pm 1.26*	16.94 \pm 1.80*	14.77 \pm 2.89*
Superoxide dismutase (U/ml)	1.86 \pm 0.04	1.22 \pm 0.09 [#]	1.53 \pm 0.04*	1.55 \pm 0.03*	1.57 \pm 0.11*
Myeloperoxidase (OD)	0.17 \pm 0.01	0.65 \pm 0.03 [#]	0.32 \pm 0.04*	0.29 \pm 0.04*	0.25 \pm 0.03*

Data are mean \pm SEM; $n = 3$

*Vehicle versus treatment, [#]normal versus vehicle (ANOVA; Tukey test), $p < 0.05$

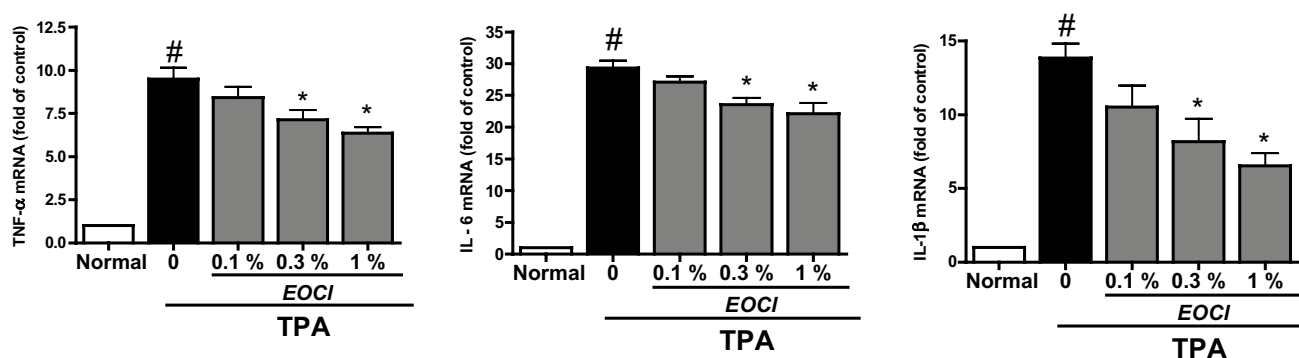


Fig. 5 Effect of EOCI on mRNA expression of inflammatory mediators (TNF- α , IL-6, IL-1 β) in ear tissue isolated from TPA-induced ear inflammation mice. Data are mean \pm SEM; $n = 3$. *Vehicle versus treatment, #normal versus vehicle (ANOVA; Tukey test), $p < 0.05$

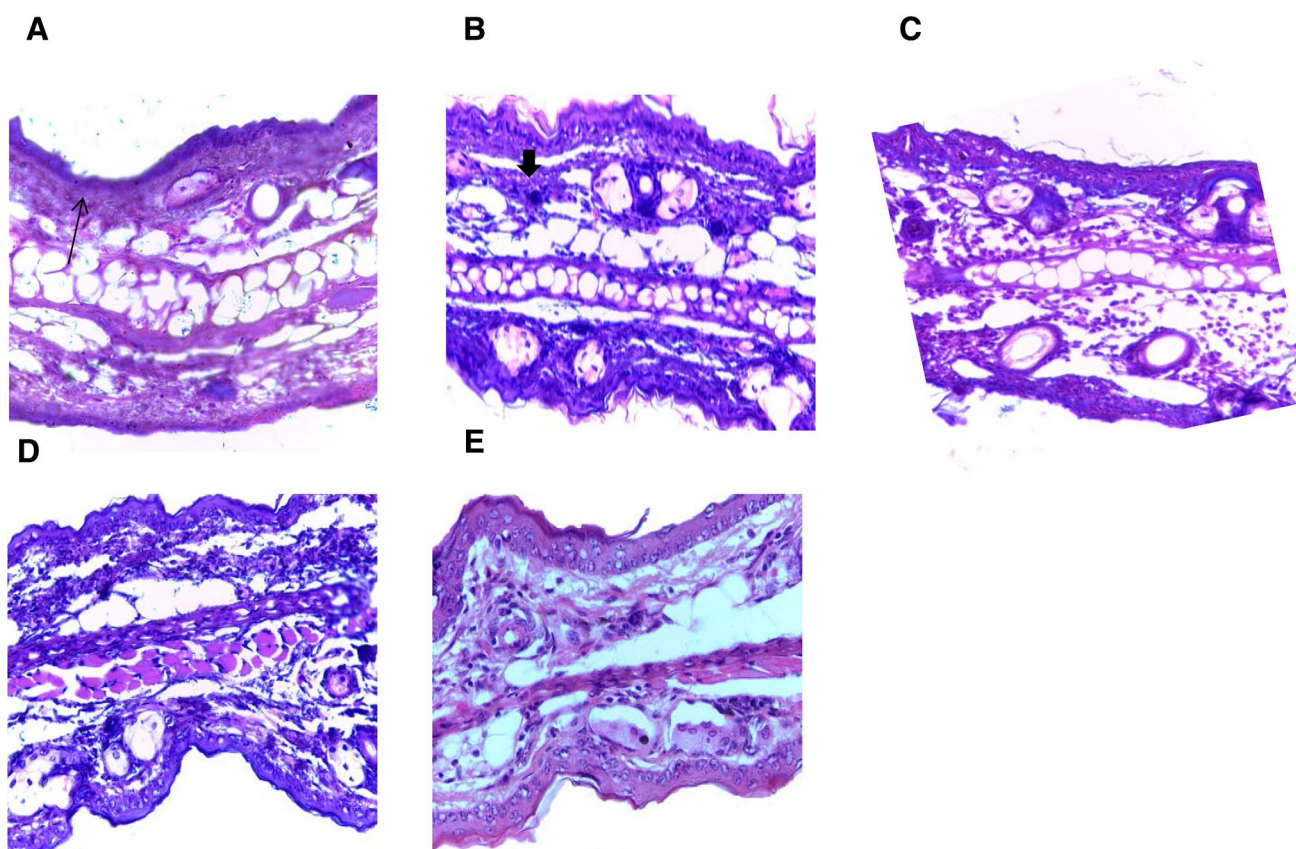


Fig. 6 Histopathological pictures of H&E-stained ear tissues excised 6 h after TPA application examined under light microscopy (magnification $\times 40$). Treatments: **a** normal, **b** vehicle, **c** EOCI 0.1%, **d** EOCI

0.3% and **e** EOCI 1.0%. Arrows indicate the epidermis and arrowheads indicate the inflammatory cells (infiltrated leucocyte). The shown sections are representative of five animals per group

Discussion

The plant of *C. longa* is harvested in the large quantities mainly in Asian to extract its rhizome for spices, food supplements and phytomedicine. Therefore, rhizome

production represents a substantial economic factor. However, during harvesting of *C. longa* whole plants, dried leaves are produced in large amounts as the major waste. An exploration of possible therapeutic effects along with the chemical characterization of essential oil isolated from medicinal plant waste plays a key role in converting

the waste materials into therapeutic value-added products (Maurya et al. 2018). In this study, an essential oil was extracted by hydrodistillation from waste leaves of *C. longa* and subjected to GC analysis using flame ionization to obtain area percentage of each constituent whereas MS was used for identification of each constituent. Chemical analysis of EOCl revealed the presence of terpinolene and α -phellandrene as major constituents and α -thujene, α -pinene, β -pinene, β -myrcene, α -terpinene, *p*-cymene, 1,8-cineole, ocimene, and γ -terpinene as minor constituents. The chemical profile of EOCl is well correlated with the previous report on the chemical composition of essential oil obtained from leaves of *C. longa* (Tripathi et al. 2002; Jena et al. 2017).

In vitro pharmacology study demonstrated that EOCl treatment significantly inhibited the production of pro-inflammatory cytokines (TNF- α , IL-6 and IL-1 β) in TPA- and LPS-induced inflammation in HaCaT cells in a dose-dependent manner without any cytotoxic effect. We have further evaluated its safety in laboratory animal models. Acute dermal test of EOCl in rabbits revealed that it is not an irritant and is safe for topical application on skin. Cutaneous inflammation is caused by the accumulation of keratinocytes that move to the inflammation site in association with the secretion of pro-inflammatory mediators, such as cytokines (Murphy et al. 2000). In many studies in vivo topical anti-inflammatory activity induced by TPA has been shown to resemble the in vitro findings of TPA-induced pro-inflammatory cytokine appearance in keratinocytes (Wilmer et al. 1994). Mouse ear oedema induced by TPA has been used as an animal model for resolving the action of anti-inflammatory agents on skin inflammation. TPA is one of the phorbol ester constituents of croton oil and its topical application caused inflammatory response that is correlated to the migration of mononuclear and polymorphonuclear leucocytes (De young et al. 1989) and induces skin inflammation through excessive production of inflammatory mediators such as TNF- α , IL-6 and IL-1 β (Chung et al. 2007). We clearly demonstrated that the ear oedema and protein levels of TNF- α , IL-6 and IL-1 β were significantly increased in TPA-treated mouse ears, and topical treatment with EOCl dose-dependently inhibited ear oedema with significant decrease in ear weight and thickness as well as protein levels of TNF- α , IL-6 and IL-1 β in mouse ears. Progressive studies confirm that oxidative stress leads to critical conditions in inflammatory skin diseases (Young et al. 2008). A single assay can not prove the potential of EOCl and hence it is necessary to use different experiments to find out the mechanism of the oxidation process. Additionally EOCl acts as an antioxidant across diverse targets. MDA is an end-product of the polyunsaturated fatty acids and esters, and quantified from the ear homogenate as an index of lipid peroxidation (Del Rio et al. 2005). The inhibitory activity of

nitric oxide (NO) production by EOCl may come from the inhibition of iNOS (nitric oxide synthase) enzyme activity and appearance of nitric oxide synthase. Superoxide dismutase (SOD) catalyzed the transformation of superoxide anion ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and oxygen (O_2) and gives the primary cellular defence against the toxicity of superoxide anion (Halliwell and Gutteridge 1990). MPO quantification evidenced the neutrophil influx and found in the azurophilic granules of neutrophils and other cells of myeloid origin (Bradley et al. 1982). These studies confirm antioxidant activity and histopathological examination evidenced the protective effect of EOCl in inflammatory skin disease. Our observations related to the anti-inflammatory effect of EOCl topical application are similar to the previous findings that essential oil and its constituents are able to reduce the severity of skin inflammation by reducing the level of pro-inflammatory mediators and oxidative stress markers (Yadav et al. 2013).

Conclusions

Chemically characterized essential oil from waste leaves of *C. longa* exhibited an anti-inflammatory action by regulating the inflammatory and oxidative stress markers. Taken together, our data suggests the suitability of EOCl to be incorporated in skin care formulations for pharmaceutical purposes with particular reference to skin inflammation.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest.

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