Topical Anti-Inflammatory Activity of Essential Oils of *Alpinia calcarata* Rosc., Its Main Constituents, and Possible Mechanism of Action

Madhuvanthi Chandrakanthan,1,2 Shiroma M. Handunnetti,2 Galbada Sirimal Arachchige Premakumara,3 and Selvaluxmy Kathirgamanathar1

1Industrial Technology Institute, Colombo-07, Sri Lanka
2Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Colombo-03, Sri Lanka
3Faculty of Nursing, University of Colombo, Sri Jayewardenepura, Thalapathpitiya, Nugegoda, Sri Lanka

Correspondence should be addressed to Madhuvanthi Chandrakanthan; ibmbbphd13008@stu.cmb.ac.lk

Received 4 February 2020; Accepted 10 March 2020; Published 28 April 2020

1. Introduction

Inflammation is a defending process exhibited by organisms against noxious stimuli, marked by an intensified blood influx to the affected tissue resulting in pain, heat, swelling, redness, and loss of function of the affected part [1, 2]. Referred commonly as “double-edged sword,” it aids in the elimination of pathogens, whereas uncontrolled inflammation could lead to tissue injury and neoplastic transformation [3]. Further, inflammation-related acute and chronic diseases are accompanied by pain which subjugates the quality of life and overall productivity [4]. Macrophages, the remarkably plastic cells of the immune system, get activated in the inflammatory process, thereby producing proinflammatory mediators such as nitric oxide (NO), PGE2 (prostaglandin E2), COX 1 and 2 (cyclooxygenase 1 and 2), reactive oxygen species (ROS), and cytokines [5]. Skin acts as the primary interface between the body and the external environment and provides the first line of defence against disease-causing pathogens and traumatic injury [6]. In addition, as a physical barrier [7], the skin has many active immune defence mechanisms. A breach in the immunological balance can lead to acute and chronic inflammatory skin diseases such as psoriasis and allergic contact dermatitis [8]. In this condition, topical treatments of skin diseases have combined benefits that include simplicity in...
application, escaping of hepatic first-pass metabolism, attaining maximum efficacy with less drug dosage, easy termination of drug if needed, site-specific drug delivery, high adherence, and risks associated with oral or intravenous administration [9, 10]. Further, topical anti-inflammatory agents can inhibit the variety of factors and mediators of inflammation such as expression of cytokines, growth factors, adhesion molecules, nuclear factor-κB (NF-κB), nitric oxide, and prostanooids [11]. TPA-induced skin inflammation is a widely used model to study the anti-inflammatory effects of natural and chemically synthesized drugs. High levels of inflammatory cytokines and reactive oxygen species are proposed to contribute to the pathophysiological mechanisms associated with TPA-induced cutaneous inflammation [12]. Conventional nonsteroidal anti-inflammatory drugs (NSAIDs) ameliorate the inflammation by suppressing the mediators and also act as local analgesics [13]. However, evidences suggest that the long-term use of NSAIDs may induce gastrointestinal ulcers, bleeding, and renal disorders leading to the exploration of less or noninvasive therapeutics which could aid in the treatment strategy of the acute and chronic inflammatory diseases [14].

*Alpinia calcarata* Rosc. (*Zingiberaceae*) is a widespread perennial plant throughout the tropical and subtropical Asian countries including Sri Lanka, India, Bangladesh, Thailand, and Malaysia [15, 16]. Rhizomes have been commonly used in Sri Lankan and Indian traditional medicine to treat chronic inflammatory diseases such as rheumatism and asthma [17]. Studies on the hot water, ethanolic extract, and oil extract of rhizome exhibited potent anti-inflammatory activity in carrageenan-induced mice models [18–20]. Previous findings reported that extracts of AC had antimicrobial, antifungal, antihelmintic, antinociceptive, antioxidant, aphrodisiac, gastroprotective, and antidiabetic properties [21]. Earlier, researchers have reported the chemical composition of ACEO grown in Sri Lanka to be rich in oxygenated monoterpenes with 1,8-cineole like the major constituent of rhizome and leaf EOs [22]. But this study lacks to give the detailed profile of volatile constituents from flowering AC grown in Sri Lanka. Similar supporting reports have been documented with ACEOs from germplasms in South India [23–26]. Further, the main constituents 1,8-cineole (CIN) and α-terpineol (TPN) have been known to act as anti-inflammatory agents in vivo [27, 28]; its topical anti-inflammatory effect and mechanism of action for skin diseases such as atopic dermatitis were never reported. Taking this into account, we postulated that ACEO which is rich in monoterpenes like 1,8-cineole and α-terpineol could be effective in preventing TPA-induced acute skin inflammation in mice and inhibit inflammatory mediators in vitro. With the existing knowledge from literatures, this is the first study that reports the detailed profile of volatile constituents, topical anti-inflammatory activity, and in vitro mechanism of action of AC. To test this possibility, we have studied the effects of ACEO and main constituents on the TPA-induced cutaneous inflammation. In order to determine ACEOs mechanism in vitro, RAW 264.7 cells induced with LPS have been used to measure the NO, ROS, cytokines, COX, and PGE2. In addition, the cytotoxicity of the biologically active ACEOs was also evaluated on macrophages, intestinal epithelial cells, human hepatocytes, and keratinocytes in order to assess the effect if targeted for oral or topical application and further gauge the therapeutic edge over the existing drugs.

2. Materials and Methods

2.1. Plant Material. Whole plants of *A. calcarata* were collected from the Western province of Sri Lanka in 2015 during the flowering season. The plants were authenticated by N. P. T. Gunawardena, and voucher specimens were deposited at National Herbarium, Peradeniya, Sri Lanka (Voucher Specimen Number: 6/01/H/03).

2.2. Chemicals. Luminol (3-aminophthalhydrazide), HBSS (Hank’s balanced salt solution), zymosan A (*Saccharomyces cerevisiae* origin), DMSO (dimethylsulphoxide), aspirin (acetylsalicylic acid), indomethacin, diclofenac, dexamethasone, NMMA (Nω-methyl-L-arginine acetate salt), PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide), NADH (β-nicotinamide adenine dinucleotide), 12-O-tetradecanoylphorbol-13-acetate (TPA), PMS (phenazine methosulfate), formaldehyde (37%), LPS (lipopolysaccharides (*Escherichia coli* origin)), Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl tetrazolium bromide (MTT), NBT (nitrotetrazolium blue chloride), H2O2 (hydrogen peroxide solution), sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride (NED. 2 HCl), sodium nitroprusside dihydrate (SNP), 1,8-cineole, and α-terpineol were procured from Sigma (USA). Murine macrophages (RAW 264.7), human hepatocellular carcinoma (HepG2), human keratinocytes (HaCaT), and rat intestinal epithelial cells (IEC-6) were procured from National Centre for Cell Sciences, Pune, India. TNF-α, IL-1β, and IL-6 assay kits were purchased from Becton Dickinson (BD), USA. COX activity assay kit was purchased from Abcam (USA).

2.3. Animals. Eight-week-old male and female Swiss albino mice (18–22 g) from a colony maintained at the in vivo Testing Facility, Central Institute of Medicinal and Aromatic Plants, India, were used for the experiments. The mice were housed under standardized conditions (25 ± 2°C), 12 h light/12 h dark cycle, and fed with animal pellets and water ad libitum. The protocol (CIMAP/IAEC 2016-19/05) was duly approved by the Institutional Animal Ethics Committee (IAEC).

2.4. Extraction of ACEOs. The whole plant was washed, and the rhizome and leaves were chopped separately. Each part (450 g) was separately hydrodistilled for 4 h using 500 mL distilled water in a Cleveger-type apparatus to obtain the essential oils. After decanting, oil samples were dried with anhydrous Na2SO4 and stored at 4°C prior to analysis. The
with LPS (1 μg/mL) cells were treated
2.7.1. Nitric Oxide and iNOS Production Inhibition and
2.7. In Vitro Anti-Inflammatory Assays
(0.32–1.25 μg/mL) for 4h followed by removal of culture supernatant containing ACEO and added LPS containing culture media. After 20 h, the concentration of nitrite, the stable product of NO, was quantified in the culture supernatant by Griess reagents (1% sulfanilamide and 0.1% NED·HCl) as described previously [30]. The amount of nitrite in the sample was calculated from a sodium nitrite standard curve, and the absorbance was measured by Synergy HTX multimode reader (BioTek Instruments, United States). In an attempt to determine the involvement of iNOS in NO production inhibition by ACEOs, RAW 264.7 cells were induced with LPS for 12 h prior to treatment with ACEOs for 24 h. In vitro stimulation of cells to an inflammatory state prior to treatment with drug enables the synthesis of intracellular iNOS and accumulation of high levels with corresponding enhanced synthesis and secretion of NO [31]. L-NMMA was used as a specific inhibitor of iNOS enzyme activity (positive control). The supernatants were removed and assayed for nitrite using the Griess assay as described above. In a separate experiment, the free radical nitrite scavenging ability of ACEOs was estimated by generating a NO production system with SNP (10 mM) and phosphate buffer (pH 7.4), followed by the addition of Griess reagent, and the absorbance was measured. PTIO, a synthetic nitrite scavenger, was used as a positive control.

2.7.2. Measurement of Intracellular ROS Production. The inhibition of intracellular ROS production by ACEOs was quantified through chemiluminescence as described by Koko et al. [32]. Briefly, RAW 264.7 cells (1 × 10⁵ cells/well) were suspended in HBSS with Ca²⁺ and Mg²⁺ (pH 7.4) and 5% CO₂. Incubation of cells with culture medium, not exceeding 0.5% DMSO in content for 24 h at 37°C and 5% CO₂, incubation at 37°C for 30 min in the thermostated chamber of the BioTek Synergy™ multimode reader. The production of ROS was initiated by the addition of opsonized zymosan A followed by 25 μL luminol, and the volume was adjusted to 200 μL with HBSS++. The results were monitored as relative luminescence units (RLUs) with peak and total integral values set with repeated scans at 30 s intervals for 1 h.

2.7.3. Superoxide Radical Scavenging Activity. Superoxide anion scavenging activity was measured in sodium phosphate buffer (100 mM, pH = 7.4) containing NBT solution (150 μM), NADH solution (468 μM), and different concentrations (1.56–50 μg/mL) of ACEOs. The reaction was started with the addition of PMS (60 μM) to the mixture followed by incubation at 25°C for 5 min and measurement of the absorbance at 560 nm. Compared with the optical density (OD) with no test sample added, the reduction of the absorbance was quantified as the superoxide scavenging activity.

2.7.4. Measurement of Cytokine Production. Secreted cytokine levels were evaluated by incubating RAW 264.7 cells induced with 1 μg/mL of LPS and treated with ACEOs at 0.5, 5, and 50 μg/mL for 4 h. The modulatory activity of ACEO...
on the LPS-induced production of TNF-α, IL-1β, and IL-6 was quantified through EIA (BD, USA) using the culture supernatants of RAW 264.7 cells collected after 24 h of incubation. Dexamethasone at 10 μM was used as the positive control.

2.7.5. Measurement of PGE2 Levels. RAW 264.7 cells were induced with LPS 1 μg/mL together with ACEOs at 0.5, 5, and 50 μg/mL for 4 h. After 4 h, ACEO-containing media was removed and replaced with LPS-containing media and incubated for 24 h. After 24 h, culture supernatant was collected for ELISA quantification of PGE2 using PGE2 assay kits (ParameterTM; R&D Systems, MN, USA). z™}he PGE2 standard and the RD5-39 in the kits were used to construct a standard curve. Culture medium (100 μL) was mixed with 50 μL of primary antibody solution and PGE2 conjugate and incubated for 2 h at room temperature with continuous shaking. Wells were then washed using 400 μL of washing buffer followed by addition of colour reagent (200 μL), and 30 min later, stop solution (50 μL) was added. Absorbance was measured at 450/570 nm using a BioTek Synergy HTX multimode reader. Indomethacin was used as the positive control.

2.7.6. In Vitro COX Inhibition Assay. The efficacy of ACEOs to inhibit ovine COX-1 and COX-2 was determined using an enzyme immunoassay (EIA) kit (catalog no. 560101; Cayman Chemical Co., Ann Arbor, MI, USA). COX catalyzes the first step in the biosynthesis of arachidonic acid (AA) to PGH₂. PGF₂α, produced from PGH₂ by reduction with stannous chloride, was measured by EIA (ACE™ competitive EIA, Cayman Chemical, Ann Arbor, MI, USA). Briefly, to a series of supplied reaction buffer solutions (160 μL 0.1 M Tris-HCl (pH 8.0) containing 5 mM EDTA and 2 mM phenol) with either COX-1 or COX-2 (10 μL) enzyme in the presence of hemoglobin (10 μL), 10 μL of various concentrations of ACEO (0.5, 5, and 50 μg/mL) were added. These solutions were incubated for 5 min at 37°C, and subsequently, 10 μL AA solution (100 μM) was added. Further, it was incubated for 2 min at 37°C. The COX reaction was stopped by the addition of 30 μL of stannous chloride and incubated for 5 min at room temperature. Prostaglandins, PGF₂α, produced were quantified by ELISA. This assay is based on the competition between PGs and a PG-acetylcholinesterase conjugate (PG tracer) for a limited amount of PG antiserum. Standard and samples were mixed with PG screening AChE tracer and PG antiserum and was incubated for 18 h at room temperature. After incubation, the plate was washed to remove any unbound reagent, followed by addition of Ellman’s reagent (200 μL), and the mixture was incubated for 60 min at room temperature (until the absorbance of Bowell is in the range of 0.3–1.0 A.U.). The product of this enzymatic reaction develops a yellow colour that absorbs at 412 nm. The intensity of this colour is proportional to the amount of PG tracer bound to the well, which is inversely proportional to the amount of PGs present in the sample. Percent inhibition was calculated by the comparison of the compounds treated to the various control incubations.

2.7.7. Assay for Membrane Stabilization. Fresh whole human blood (2 mL) was collected from a healthy volunteer and was washed three times with normal saline and constituted as 40% v/v suspension with normal saline as described by Sadique et al. [33]. The reaction mixture consisted of ACEOs (1.56–50 μg/mL) or dexamethasone (10 μM) and 20 μL of 40% RBCs suspension, considering only RBCs as controls. The reaction mixture (in triplicate) after incubation in a water bath (54°C for 25 min) was centrifuged at 2500 rpm for 5 min, and the absorbance of the supernatants was taken at 560 nm. Percent membrane stabilization activity was derived mathematically. The ethical approval for collection of blood from human donors was obtained from Research Ethics Committee, Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Sri Lanka.

2.8. Topical Anti-Inflammatory Activity

2.8.1. TPA-Induced Skin Inflammation. Acute inflammation in the ear pinna was induced by instilling 20 μL of TPA dissolved in acetone (2.5 μg/ear). Mice were divided into 9 groups with 6 animals per group. TPA (2.5 μg/ear) dissolved in 20 μL of acetone was applied to the inner and outer surfaces of mouse ears with the aid of a micropipette [34]. Treatments, ACEOs (5, 1, 0.5, and 0.2%), CIN (2.5, 0.5, and 0.1%), and TPN (10, 2, and 0.4%) or indomethacin (0.5 mg/ear) was applied after the TPA induction. Four h later, the thickness of the ear was measured using a digital screw gauge. Six h after the treatment, the animals were euthanized for the collection of tissue. Ear biopsies (5 mm diameter punches) were weighed, homogenized in PBS (pH 7.5) with 1 mM EDTA, and centrifuged (10,000 × g for 15 min) for the collection of supernatant which were used for the quantification of various cytokines.

2.8.2. Histopathological Analysis of Mouse Ear Tissue. For the assessment of skin inflammation, biopsies from control and treated ears of mice in each treatment group were collected and fixed in 4% formaldehyde (0.1 M phosphate buffer, pH 7.4). Subsequently, the tissues were dehydrated, blocked in paraffin, and serially sliced at a thickness and epidermal thickness (μm). The measurements of ear thickness and epidermal thickness (μm) were acquired by the Image Processing software of Leica Microsystems.

2.8.3. Assay for Myeloperoxidase Enzyme Activity in Ear Tissue. MPO activity was quantified according to the method proposed by Pulli et al. [35]. Briefly, the homogenate was added with a mixture containing 80 mM-PBS (pH 5.4), 0.22 M-PBS (pH 5.4), and 0.017% hydrogen peroxide. The
reaction was initiated by the addition of 20 μL of 18.4 mM TMB in dimethylsulphoxide. The plate was incubated at 37°C for 4 min followed by the addition of 1.46 M sodium acetate (pH 3.0) to stop the reaction. The absorbance at 620 nm was measured using a plate reader to determine the enzyme activity. MPO activity was expressed as absorbance of TPA-treated ear tissue homogenate/absorbance of ACEO-treated ear tissue homogenate.

2.8.4. Formalin-Induced Nociception and Edema in Mice. The procedure described by Lee and Jeong [36] was followed. Briefly, nociception was induced by injecting 20 μL of 2.5% formalin in 0.9% saline in the subplantar region of the right hind paw. Mice (n = 6, per group) were pretreated topically with ACEOs (5%, 1%, and 0.1%), CIN (2.5, 0.5, and 0.1%), TPN (10, 2, and 0.4%), 1% diclofenac cream, and vehicle for 1 h prior to injecting formalin. These mice were individually placed in a transparent glass chamber for observation. The amount of time spent licking and flinching of the injected paw was indicative of pain. The number of flinches and lickings after injection of formalin was counted during 0 to 5 min (early phase) and 20 to 30 min (late phase). Antinociception was considered as a statistically substantial reduction in the time spent in licking and flinching of the injected paw in comparison with the control group. Edema was estimated by measuring the paw volume before and after 4 h of formalin injection using plethysmometer (IITC, Life Scientific Instruments, Woodland Hills, CA, USA). The percentage reduction in paw volume by ACEOs was calculated in comparison with the formalin-treated group.

2.9. Statistical Analyses. The raw data were analyzed by t-tests and one-way ANOVA (analysis of variance) followed by Tukey’s test and Dunnett’s comparison test, where applicable using GraphPad Prism version 5.0 (GraphPad Prism Software Inc., San Diego, CA, USA). Differences were considered to be statistically significant when \( p < 0.05 \).

3. Results and Discussion

3.1. Analysis of Essential Oil. The yield of essential oil from rhizome and leaf was 9 and 12 mL/kg, respectively. Results are the summary of three batches of analysis. Essential oils are complex mixtures consisting of various compounds. Each of these compounds contributes to the beneficial or adverse activity of the essential oil. For this reason, it is necessary to elucidate the complete composition of an essential oil when investigating the viability of a specific application [37]. GC-MS equipped with a capillary column is the most popular technique used to analyze the chemical ingredients of an essential oil. The list of compounds in ACEO from rhizome and leaf is presented in Table 1 and Figures 1(a) and 1(b). Previous study on the volatile oils of AC grown in Sri Lanka reports 3.60% and 0.42% of oil yield from dry rhizome and fresh leaves, respectively [22]. Earlier reports of rhizome EO from germplasms collected from India showed variation ranging from 0.29% to 0.96% in rhizome and 0.26% to 0.69% in aerial leafy shoots on a dry weight basis [38]. All the previous studies were done on either dry or fresh rhizome and aerial parts, and the information on the harvest stage was not mentioned. In our study, oils were collected from flowering plants. Fresh rhizome constituted mainly of oxygenated monoterpenes (71.28%), followed by monoterpenes (9.64%), sesquiterpenes (5.4%), and oxygenated sesquiterpenes (0.15%). The major compounds identified were 1,8-cineole (38.45%), α-terpineol (11.62%), and fenchyl acetate (10.73%). The compounds in leaf oil were also occupied by the same order, and the major compounds were 1,8-cineole (31.08%), α-terpineol (11.62%), and camphor (10%). The observations made in the current study gave a detailed profile of the essential oils from rhizome and leaf and also support the previous reports from different origins on the EO content of AC marked with 1,8-cineole as the major component independent of the flowering or condition of the plants [39]. The most important chemical feature of the oils obtained from flowering stages of AC was the presence of a higher percentage of oxygenated monoterpenes, mainly 1,8-cineole and fenchyl acetate which were of industrial interest in terms of bioactivity.

3.2. Cytotoxicity and Proliferative Inhibition Effects of ACEOs. Figures 2(a) and 2(b) show the effect of ACEOs on RAW 264.7, HaCaT, HepG2, and IEC-6 cell viability and proliferative ability. Treatment with 100 μg/mL caused a reduction in cell viability in all the above cell lines. Therefore, ACEOs were considered at 50 μg/mL and below for further assays. Considering that intestinal epithelial cells and keratinocytes are the first point of contact when drugs are used orally or applied topically and virtually, all NSAIDs that have been used extensively are linked to at least rare cases of clinically apparent drug-induced liver injury [40]. Hence, the need to analyze the cytotoxicity becomes a necessity to identify safe and bioactive concentrations to mammalian cells for oral and topical curative applications. At 100 μg/mL, RAW 264.7, IEC-6, and HepG2 cells have less than 80% viability (Figure 2(a)). However, HaCaT cells were not affected at 100 μg/mL, which gives an interesting niche, suggesting a potential safe application of these ACEOs in topical therapeutics. Further, the proliferative inhibition efficacy of ACEOs was tested in order to explore the efficacy for prolonged inflammatory diseases, since chronic inflammation leads to neoplastic transformation of cells with the aid of proinflammatory markers [41]. Recent evidences on the TNF-α and IL-6-induced tumour progression of inflammatory cells [42] urge the search for anti-inflammatory drugs with tumour inhibition potential. In the present study, ACEOs exhibited inhibition of proliferation in a concentration-dependent manner in all the cell lines tested (Figure 2(b)). Among the 4 cell lines tested, HaCaT cells are the least affected with 23 ± 4.23 and 45 ± 3.45 percentage inhibition of proliferation at 50 μg/mL for RO and LO, respectively.

3.3. Inhibition of NO Release, Indirect iNOS Activation, and Free Radical Nitrite Scavenging of ACEOs. ACEOs were tested for anti-inflammatory activity by the effect of them in
| Compounds                        | Rhizome       | Leaf          |          |          |          |
|---------------------------------|---------------|---------------|----------|----------|----------|
|                                 | RT  | RSI | Area | RT  | RSI | Area |
| **Monoterpenes (M)**            |     |     |      |     |     |       |
| **α-Pinene**                    | 5.29 | 926 | 0.07 | 5.32 | 900 | 0.06  |
| **β-Pinene**                    | —   | —   | —    | 13.23 | 958 | 0.68  |
| **O-cymene**                    | 6.09 | 961 | 0.82 | 6.12 | 967 | 0.35  |
| **D-limonene**                  | 4.58 | 908 | 5.52 | 4.60 | 904 | 5.74  |
| **Pinocarvone**                 | 15.04 | 834 | 0.23 | —    | —   | —     |
| **Myrtenal**                    | 17.15 | 909 | 0.65 | 17.17 | 860 | 0.04  |
| **γ-terpinene**                 | 5.51 | 929 | 0.39 | 5.54 | 917 | 0.59  |
| **α-Ocimene**                   | 5.66 | 929 | 0.26 | 5.68 | 933 | 0.70  |
| **Terpinolene**                 | 5.72 | 919 | 0.38 | 5.76 | 866 | 0.68  |
| **α-Copaene**                   | 14.27 | 824 | 0.21 | 14.30 | 851 | 0.04  |
| **(+)-3-Carene**                | 5.01 | 830 | 0.28 | 30.14 | 806 | 0.88  |
| **Fenchone**                    | —   | —   | —    | 11.5  | 852 | 10    |
| **Pinocarveol**                 | 15.08 | 834 | 0.27 | —    | —   | —     |
| **α-Terpinyl acetate**          | 5.72 | 919 | 0.56 | —    | —   | —     |
| **Total (M)**                   | 9.64 |      |       | 19.76 |      |       |
| **Oxygenated monoterpenes (OM)**|     |     |      |     |     |       |
| **Eucalyptol (1,8-cineole)**    | 4.76 | 948 | 38.45 | 4.76 | 954 | 31.08 |
| **α-Terpineol**                 | 19.03 | 838 | 11.62 | 19.95 | 881 | 10.31 |
| **Camphor**                     | 12.49 | 823 | 7.29 | 12.52 | 959 | 10.0  |
| **Boronyl acetate**             | 15.57 | 933 | 0.38 | 15.59 | 930 | 0.51  |
| **Terpinen-4-ol**               | 16.49 | 916 | 1.68 | 16.50 | 907 | 1.09  |
| **Fenchol**                     | 15.87 | 944 | 0.51 | —    | —   | —     |
| **Fenchyl acetate**             | 11.81 | 955 | 10.73 | 11.81 | 866 | 0.39  |
| **Linalool**                    | 18.28 | 863 | 0.62 | 18.32 | 866 | 0.39  |
| **Total (OM)**                  | 71.28 |      |       | 53.51 |      |       |
| **Sesquiterpenes (S)**          |     |     |      |     |     |       |
| **Carotol**                     | 30.50 | 923 | 3.36 | 30.50 | 924 | 6.53  |
| **Caryophyllene oxide**         | 29.09 | 911 | 0.17 | 22.67 | 894 | 0.35  |
| **α-Bisabolene**                | 33.99 | 831 | 0.05 | 18.51 | 886 | 0.61  |
| **α-Famesene**                  | —    | —   | —    | 18.95 | 931 | 1.67  |
| **β-Sesquiphellandrene**        | 34.95 | 826 | —    | 34.59 | 826 | 0.25  |
| **α-Himachalene**               | —    | —   | —    | 35.34 | 893 | 0.55  |
| **β-Nerolidol**                 | —    | —   | —    | 18.12 | 866 | 0.6   |
| **Cubedol**                     | 32.43 | 852 | 0.15 | 32.43 | 852 | 0.15  |
| **10-epi-Elemol**               | —    | —   | —    | 32.34 | 836 | 3.22  |
| **B-calarene**                  | 21.85 | 836 | 1.51 | 21.86 | 859 | 7.53  |
| **D-guaiene**                   | 20.28 | 916 | 0.14 | —    | —   | —     |
| **Total (S)**                   | 5.4  |      |       | 21.46 |      |       |
| **Aliphatic alcohol**           |     |     |      |     |     |       |
| **2-Heptanol**                  | 7.31 | 918 | 0.11 | 7.35 | 899 | 0.12  |
| **6-epi-Shyobunol**             | 31.18 | 802 | 1.13 | 28.57 | 797 | 1.82  |
| **Total**                       | 1.24 |      |       | 1.94  |      |       |
| **Others**                      |     |     |      |     |     |       |
| **Phytol**                      | —    | —   | 40.98 | 899 | 0.88  |
| **Daucol**                      | 36.43 | 827 | 0.12 | —    | —   | —     |
| **Trans-α-bergamotene**         | 38.08 | 899 | 0.09 | 38.07 | 874 | 0.52  |
| **cis-p-Mentha-1 (7),8-dien-2-ol**| 24.82 | 846 | 0.07 | 19.60 | 792 | 0.14  |
| **2-Butanone, 4-phenyl-**       | —    | —   | —    | 6.71  | 841 | 0.45  |
| **2-Propenoic acid, 3-phenyl-, methyl ester**| 32.24 | 936 | 5.43 | 32.24 | 936 | 0.35  |
| **Methyl 9,11-octadecadienoate**| 37.75 | 826 | —    | 19.47 | 797 | 0.51  |
| **Cholestan-3-ol, 2-methylene, (3α,5α)**| 36.93 | 830 | 0.1  | 35.55 | 816 |      |
| **Octaethylene glycol monododecyl ether**| 43.48 | 788 | 0.12 | 39.59 | 755 |      |
| **2-Methyl-4-(2,6,6-trimethylcyclohex-1-enyl)but-2-en-1-ol**| 14.61 | 808 | 0.48 | 37.38 | 811 | 0.27  |
| **5-Azulenemethanol**           | 32.59 | 911 | 0.42 | 38.70 | 801 | 0.11  |
| **1H-benzocycloheptene,2,4a,5,6,7-Octahydro-α,α,3,8-tetramethyl- [3S-(3α,3β,5α)]**| 20.84 | 847 | 0.10 | —    | —   | —     |
| **(-)-Myrtenol**                | 23.21 | 921 | 0.23 | 23.22 | 872 | 0.07  |
LPS-induced NO production in murine macrophages. Figure 3 shows the concentration-dependent response of ACEOs towards the production of NO, indirect iNOS, and nitrite scavenging. LPS-induced RAW 264.7 cells produce large amounts of NO and the stable product, nitrite, which was measured by the colourimetric Griess assay. ACEOs exhibited significant inhibition of NO production at all concentrations tested in a concentration-dependent manner. EO from rhizome showed maximum inhibition of 85% at 50 μg/mL, whereas the leaf oil showed 81% inhibition. L-NMMA was used as a positive control at a concentration of 250 μM, and it exhibited NO inhibition of 87% when

| Compounds                                                                 | Rhizome | Leaf |
|---------------------------------------------------------------------------|---------|------|
| Benzenepropanol, a-methyl-β-nitro-(R^∗, R^∗)-(+-+)                          | 10.85   | 0.32 |
| 3,7-Cyclodecadiene-1-methanol, a,a,a,8-tetramethyl-[s-(Z,Z)]               | 32.12   | 0.26 |
| 1-Heptatriacotanol                                                         | 34.84   | 0.03 |
| Cyclohexene-1-methyl-4-(1-methylethylidene)                               | 4.24    | 0.08 |
| **Total**                                                                 | **7.85**| **2.7**|
| **Overall total**                                                          | **95.41**| **99.37**|

(a) GC-MS profile of rhizome (a) and leaf (b) essential oils of AC.
RAW 264.7 cells were treated with LPS for 24 h. NO is a short-lived signaling molecule that plays an important role as an immunoregulatory mediator [43]. High NO levels cause a variety of pathophysiological processes including inflammation and carcinogenesis [44]. In addition, regulation of the iNOS-mediated release of NO from macrophages is considered as one of the strategies to develop therapeutics against various inflammatory diseases such as rheumatoid arthritis [45]. To investigate the involvement of iNOS in the mechanism of inhibition of NO production by ACEOs, RAW cells were treated with LPS for 12 h prior to the treatment with ACEOs, which showed a reduced NO production at the rate of 57.5% and 46.25% for rhizome and leaf, respectively, at 50 µg/mL. It also showed a
Table 2: Intracellular ROS inhibition, superoxide anion scavenging, and inhibition of heat-induced hemolysis by ACEOs.

| Treatment | Concentration (μg/mL) | RLU ± SEM | Inhibition of intracellular ROS (%)** | Superoxide inhibition (%)* | Inhibition of heat-induced hemolysis (%)** |
|-----------|-----------------------|----------|--------------------------------------|---------------------------|------------------------------------------|
| Normal    | 298.53 ± 104.76       |          |                                      |                           |                                          |
| Control   | 3587.56 ± 123.56#     |          |                                      |                           |                                          |
| RO        | 50                    | 568.59 ± 20.67*** | 84.16 ± 5.56                        | 13.45 ± 3.45             | 72.12 ± 0.25                            |
|           | 25                    | 1305.76 ± 54.76** | 63.51 ± 7.34                        | 10.54 ± 6.56             | 56.34 ± 0.45                            |
|           | 12.5                  | 2135.98 ± 15.78*  | 40.46 ± 5.87                        | 8.45 ± 7.34              | 34.87 ± 0.76                            |
|           | 6.25                  | 2582.45 ± 30.98*  | 28.09 ± 6.34                        | 6.44 ± 3.89              | 22.56 ± 1.22                            |
|           | 3.12                  | 2902.23 ± 45.76** | 18.65 ± 4.34                        | 2.56 ± 6.77              | 15.67 ± 2.34                            |
|           | 1.56                  | 3276.67 ± 46.32** | 8.67 ± 6.56                         | 2.31 ± 1.45              | 12.45 ± 0.23                            |
| LO        | 50                    | 452.76 ± 67.23*** | 87.32 ± 2.31                        | 12.12 ± 3.24             | 83.32 ± 0.34                            |
|           | 25                    | 1056.34 ± 59.56** | 70.56 ± 1.12                        | 12.34 ± 1.11             | 67.23 ± 0.76                            |
|           | 12.5                  | 1809.67 ± 23.34** | 49.56 ± 2.45                        | 2.46 ± 6.57              | 54.09 ± 2.23                            |
|           | 6.25                  | 2598.43 ± 67.23*  | 27.57 ± 4.56                        | 2.11 ± 1.35              | 24.76 ± 0.34                            |
|           | 3.12                  | 2765.34 ± 25.67** | 22.91 ± 2.23                        | 2.56 ± 2.23              | 14.65 ± 0.23                            |
|           | 1.56                  | 3015.56 ± 34.87** | 15.98 ± 3.45                        | 2.61 ± 2.45              | 10.23 ± 0.45                            |
| Dexamethasone 5 µM | 777.45 ± 34.56* | 78.33 ± 2.56 | 2.56 ± 1.77 | 91.77 ± 0.56 |

Data are mean ± SEM (n = 6). # p < 0.05, significantly different from the normal group; * p < 0.05, ** p < 0.01, and *** p < 0.005, significantly different from the control group. RO: rhizome EO; LO: leaf EO.

Figure 4: Effects of ACEOs on LPS-induced cytokine production in murine macrophages. TNF-α levels in supernatant (a), IL-1β levels in supernatant (b) and IL-6 levels in supernatant (c). N: normal; LPS: LPS-treated cells; RO: rhizome essential oil; LO: leaf essential oil; LPS + DEX: LPS along with dexamethasone at 10 μM. The bars represent the mean ± SEM (n = 6). Symbols **, *** and **** represent a significant difference p < 0.05, p < 0.01, and p < 0.001, between LPS+ and EO-treated cells. Symbol # represents a significant difference p < 0.05 between normal and LPS + cells.
concentration-dependent inhibition that further confirms
the iNOS enzyme-mediated inhibition of NO production
which facilitates the inhibition of iNOS gene expression at
the genome transcriptional level. Further, ACEOs were
tested for the scavenging activity of nitrite generated by SNP
mediation that exhibited the moderate scavenging activity
from rhizome (46.5%) and leaf (34.8%). This adds to the
effect of ACEOs on NO inhibition which is by the inhibition
of enzyme (iNOS) and not by mere scavenging activity.

3.4. Inhibition of Intracellular ROS Production and Superoxide
Scavenging Activity by ACEOs. ACEOs were studied for the
inhibition of oxidative burst induced by opsonized zymosan

| Class                  | PGF2α (pg/mL) | Percentage of inhibition |
|-----------------------|---------------|--------------------------|
| Background tube       | 2.25 ± 0.75   |                          |
| COX-1 100% initial activity tube | 221 ± 14.5 |                          |
| Rhizome EO            |               |                          |
| 0.5 μg/mL             | 178 ± 4.5     | 19.45                    |
| 5 μg/mL               | 158 ± 2.23    | 28.5                     |
| 50 μg/mL              | 52 ± 5.23     | 76.47                    |
| Leaf EO               |               |                          |
| 0.5 μg/mL             | 198 ± 5.45    | 10.4                     |
| 5 μg/mL               | 161 ± 2.56    | 27.14                    |
| 50 μg/mL              | 76 ± 2.12     | 65.61                    |
| Indomethacin          |               |                          |
| 10 μg/mL              | 22 ± 3.45     | 90                       |

| Class                  | PGF2α (pg/mL) | Percentage of inhibition (%) |
|-----------------------|---------------|------------------------------|
| Background tube       | 0.75 ± 0.1    |                            |
| COX-2 100% initial activity tube | 154 ± 4.5 |                            |
| Rhizome EO            |               |                            |
| 0.5 μg/mL             | 121 ± 4.5     | 21.4                        |
| 5 μg/mL               | 98 ± 1.23     | 36.3                        |
| 50 μg/mL              | 22 ± 2.56     | 85.7                        |
| Leaf EO               |               |                            |
| 0.5 μg/mL             | 145 ± 2.78    | 5.84                        |
| 5 μg/mL               | 111 ± 1.54    | 27.92                       |
| 50 μg/mL              | 46 ± 1.12     | 70.12                       |
| NS-398                | 4 ± 2.21      | 97.4                        |

Figure 5: Effect of ACEOs on PGE2 production. C: normal control; LPS: LPS-treated cells; RO: rhizome essential oil; LO: leaf essential oil; LPS + IM: LPS along with indomethacin at 10 μg/mL. The bars represent the mean ± SEM (n = 6). Symbol “∗∗∗∗” represents a significant difference p < 0.001, between control and LPS-treated cells, and comparing LPS+ and EO-treated cells.

Table 3: Effect of ACEOs on COX-1 enzyme activity.

Table 4: Effect of ACEOs on COX-2 enzyme activity.
(OPZ) by a luminol-dependent chemiluminescence assay. Induction by OPZ caused the activation of NADPH oxi-
dative complex which results in the production of ROS and 
this is mainly expressed in phagocytic cells of the immune 
system [46]. Prolonged local inflammatory reaction triggers 
the production of excessive amounts of ROS, and since 
macrophages have the highest burst capacity among the 
antigen-presenting cells, this harmful massive burst further 
triggerstheimmuneactivationofproinflammatorycytokine 
synthesis and results in tissue damage [47]. In the present 
study,thepotentinhibitoryactivitywasobservedfortheleaf 
EO (87% inhibition) and both the ACEOs exhibited a 
concentration-dependent inhibition of ROS production, as 
shown in Table 2. OPZ triggers both interferon-
and 
complement receptor activation producing superoxide an-
ions that are spontaneously converted to halide ions by the 
oxidative enzymes [48] aggravating the oxidative burst. 
Luminol has its ability to enter the cell, which reacts with 
intracellular $\text{HOCl}^{-}$ exhibiting the ability of ACEOs in the 
inhibition of oxidative burst. In a separate experiment, 
superoxide anions were generated by incubating PMS with 
NADH, and the free radical $\text{O}_2^-$ scavenging ability was 
studied in a quest to identify the extracellular superoxide 
scavenging activity of ACEOs. Interesting observation was 
obtained with the less inhibition pattern of 10–13% for the 
higher concentrations of ACEOs tested (Table 2). Earlier 
reports also indicated the essential oils as moderate scav-
engers of free radicals [49].

### Figure 6: Effects of ACEOs on TPA-induced ear edema

Inhibition of TPA-induced ear edema by topical application of EO (5, 1, and 0.25%) was analyzed by measuring changes in ear thickness in millimeters (a), changes in ear weight in grams (b), and percentage inhibition of myeloperoxidase activity in tissue homogenates (c). N: normal; C: TPA-treated control group; RO: rhizome essential oil; LO: leaf essential oil; IMN: indomethacin at 0.5 mg/ear. Symbols “∗∗∗∗” represent a significant difference $p < 0.001$ between TPA treatment and EO-treated animals. Symbol “#” represents a significant difference $p < 0.05$ between normal and TPA-treated groups.

3.5. **Inhibition of Cytokine Secretion by ACEOs.** Figures 4(a)–4(c) show the amount of cytokines quantified in the culture medium of RAW 264.7 cells treated with several concentrations of ACEOs prior to LPS induction for 3 h and further incubated for 24 h with LPS. Pretreatment of RAW 264.7 cells with ACEOs markedly decreased the production of TNF-$\alpha$, IL-1$\beta$, and IL-6 when compared to the LPS-treated cells. Macrophages react to the LPS by...
recognizing the threat using the Toll-like receptor complex (TLR-4) which results in the intensified production of NO, ROS, and cytokines [50]. Intensified production of cytokines in the activated macrophages of the inflammatory process results in tissuedamage as seen in chronic inflammatory diseases [51]. Treatment with ACEOs significantly inhibited the production of cytokines by LPS ($p < 0.05$). Interestingly, ACEOs also decreased the proliferation of macrophages along with the inhibition of cytokine induction which makes it an effectual lead for the preparation of formulations for chronic disorders [52].

3.6. Effect of ACEOs on PGE2 Production. PGE2 is an eicosanoid lipid mediator produced when arachidonic acid is released from the plasma membrane by phospholipases and metabolized by two cyclooxygenases (COX 1 and COX 2) and three specific isomerases. NSAIDs act on COX enzymes, thus reducing the generation of PGE2. This, downstream pathways in the COX enzymes are responsible for response of PGE2 and are more specific targets in the treatment of inflammation and pain [53]. The effects of ACEO on the LPS-induced release of PGE2 from RAW 264.7 cells were studied. As PGE2 is one of the most important inflammatory mediators, the cells were pretreated with ACEOs for 2h followed by incubation with $1 \mu g/mL$ of LPS. After 24h of LPS treatment, the PGE2 contents in the culture medium were detected. The LPS-induced PGE2 secretion level was inhibited by treatment with the ACEOs at all the concentrations examined, and the maximum inhibition was observed at a concentration of $50 \mu g/mL$ for both oils. Indomethacin (IMN) was used as a positive control. Diverse studies have proven that the expression of COX-2 is largely determined by transcriptional activation [54, 55]. NF-κB, which is a mammalian transcription factor that regulates several genes and important in immunity and inflammation, can be triggered by LPS and other proinflammatory cytokines. In macrophages, NF-κB binds to COX-2 promoter and plays a role in LPS-mediated induction of COX-2. In addition, binding of CCAAT-
enhancer-binding proteins (C/EBPs), c-AMP response element binding proteins (CREBs), and c-Jun to the COX-2 promoter ameliorates its transcriptional activation [56]. The present study is confined to the understanding of in vitro enzyme inhibitory activity of COX-1 and COX-2 and the production of PGE2; thence, it may be significant to understand the effect of ACEOs at the transcriptional activation level involving the NF-κB, C/EBP, CREB, and c-Jun proteins.

3.7. In Vitro COX Enzyme Inhibition by ACEOs. COX-1 and COX-2 catalyze the biosynthesis of PGH2 from the AA substrate. The COX-1 inhibition results in certain unsuitable side effects, whereas COX-2 inhibition provides therapeutic effects in pain, inflammation, cancer, Alzheimer’s disease, and Parkinson disease [57]. Therefore, the present study aimed at examining the COX-1 and COX-2 inhibitory activity of ACEO on purified enzymes as a mechanism of anti-inflammatory action. The oils showed inhibitory effects on COX-1 and COX-2 in a concentration-dependent manner (Tables 3 and 4). Furthermore, the concentrations that inhibited COX-2 had no effect on COX-1. The decrease in PGE2 production after ACEO treatment corresponded with the decrease in COX activity in vitro (COX-1 and COX-2), particularly COX-2. EOs of Illicium anisatum constituted mainly of 1,8-cineole and demonstrated its ability for inhibiting NO and PGE2 production in LPS-stimulated RAW 264.7 cells, along with the decrease in iNOS and COX-2 expression [58]. Further, Beer et al. [59] have studied the inhibitory effect of 1,8-cineole on COX 1 and 2 activity and declared that 1,8 cineole is a potent and selective COX 2 blocker. Abundance of 1,8-cineole in ACEOs makes it an important candidate for its inhibitory activity.

3.8. Inhibition of Heat-Induced Hemolysis by ACEOs. Reports on the mechanism of action of NSAIDs on anti-inflammatory reaction are suggested to be exerted by stabilization of lysosomal membranes, which suppress the release of tissue-destroying enzymes which have been implemented in the pathogenesis of rheumatoid arthritis [60]. Protection against hypotonicity or heat-induced hemolysis by the red blood cell (RBC) membrane system is widely employed in the testing of drugs for anti-inflammatory activity [61]. Table 2 shows the percentage inhibition of RBC membrane lysis by the treatment of ACEOs. EO of leaf exhibited 83.32 ± 0.34% inhibition of RBC membrane lysis in closer rate to the dexamethasone (91.77 ± 0.56), commonly used drug in the autoimmune hemolytic anaemia [62] at 5 μM.

Figure 8: Effects of ACEOs on TPA-induced cytokine production. IL-1β levels in homogenate (a), IL-6 levels in homogenate (b), and TNF-α levels in homogenate (c). N: normal; TPA: TPA-treated control group; RO: rhizome essential oil (5, 1, and 0.25%); LO: leaf essential oil (5, 1, and 0.25%); IMN: indomethacin at 0.5 mg/ear. The bars represent the mean ± SEM (n = 6). All groups showed, p < 0.01, significantly reduced cytokine level than the control group.
3.9. Topical Anti-Inflammatory Activity

3.9.1. Effects of ACEOs on TPA-Induced Skin Inflammation.
Local application of TPA induces cutaneous inflammation and epidermal hyperplasia. Further, it stimulates infiltration of inflammatory cells, which releases large amounts of inflammatory mediators such as PGE2 and cytokines (TNF-α, IL-1β, and IL-6) [63]. In the current study, the therapeutic effect of ACEOs and its main constituents on TPA-induced skin inflammation was examined in a dose-dependent manner. Ear thickness was measured prior to the application of TPA. The concentrations of ACEO and compounds for testing were determined by doing a skin irritability test (data not shown). ACEO, CIN, and TPN presented significantly similar and effective anti-inflammatory activity in the experimental animal model used, which induced a strong dose-dependent edema inhibition (Figures 6(a) and 6(b) and Figures 7(a) and 7(b)). In particular, CIN showed a strong and similar edema inhibitory activity (29%) at 2.5% with indomethacin (25%) at 0.5 mg/ear. The mouse ear weight was reduced by 25% after indomethacin treatment, similar to rhizome EO (25%) and leaf EO (24.2%). TPN induced 23.4% edema reduction at 10% and 17% at 2% concentration. In summary, CIN and TPN which are the major compounds of AC presented a potent anti-inflammatory activity. To the best of our knowledge, it is the first report to demonstrate the topical anti-inflammatory activity of ACEO and also the compound TPN. Mounting evidences of the role of neutrophil enzyme, myeloperoxidase, in promoting oxidative stress in inflammatory pathologies and in many chronic inflammatory diseases such as atherosclerosis, glomerulonephritis, multiple sclerosis, rheumatoid arthritis, asthma, and cystic fibrosis makes it an important biochemical marker and a therapeutic target in antirheumatic drug development [64]. TPA-induced ear tissue homogenates showed high levels of MPO activity, and this activity was significantly reduced by the treatment with serial doses of ACEOs and compounds (Figures 6(c) and 7(c)). Further, the
topical application also reduced the TNF-α, IL-1β, and IL-6 levels significantly in comparison with the TPA-induced group (Figures 8(a)–8(c)). In all the cases, rhizome oil exhibited more potential than the leaf oil ($p < 0.05$). Suggestive evidences on 1,8-cineole and monoterpene-rich EOs on reducing airway inflammation, MPO activity, and decreased cytokine levels portray the importance and efficacy of this topical study for therapeutic development [65]. Held et al. [28] have studied the oral anti-inflammatory effect of 1,8-cineole in various animal models, but the mechanism by which it exerts the activity was not clear. Our studies on the topical anti-inflammatory effect of CIN and TPN have explored the cytokine profile (Figures 9(a)–9(c)) and inhibition of MPO activity. From the results of this investigation, valuable research references can be provided for the clinical medicine or pharmaceutical application in the future.

### 3.9.2. Histopathological Analysis of Mouse Ear Tissue

Further investigation on the H & E-stained ear biopsies from TPA-induced animals showed substantial increase in the ear thickness with vivid indication of edema, epidermal hyperplasia, and considerable neutrophil infiltration in the dermis associated with disruption in connective tissue. By comparison, 5% of ACEOs, CIN at 5%, and TPN at 10% treatment reduced ear thickness and affiliated pathological indicators to an extent comparable to the positive control, indomethacin, as shown in Figures 10 and 11. These results directly exemplify the effects of ACEO and major compounds in the amelioration of TPA-induced contact dermatitis.

#### 3.9.3. Effects of ACEOs on Formalin-Induced Pain and Edema

Acute and chronic inflammatory diseases are associated with neuralgic as well as inflammatory pain [66]. Since topical drug formulations of NSAIDs are amongst the most frequently administered over-the-counter (OTC) drugs, the introduction of a new preparation of this group requires any comparison of its anti-inflammatory and analgesic efficacy to the corresponding standard [67]. 2.5% formalin injection to the mice paw shows a biphasic response and exhibits behavioral pain by flinching and licking of the affected paw [68]. The present study was carried out to test the analgesic and antiedema effect of ACEOs in a dose-dependent manner. Results of the present study indicate distinctly the topical analgesic effect of ACEOs by suppressing the pain induced by inflammation in the second...
Data are mean ± SEM. The numbers are frequencies (freq.) of flinching and total time (seconds; sec.) spent licking the formalin-injected paw. *p < 0.05, compared with the formalin-treated group (n = 6); **p < 0.05 compared with the normal group. Baseline values of paw volume represent preinjection diameters of paws. *p < 0.05, compared with the formalin-treated group (n = 6) (ANOVA with Tukey’s test). RO: rhizome EO; LO: leaf EO.

Table 5: The effect of rhizome and leaf EOs on formalin-induced pain behavior and edema in mice at different concentrations.

| Group            | Phase I response                        | Phase II response                        | Paw volume (mL)                      |
|------------------|-----------------------------------------|-----------------------------------------|-------------------------------------|
|                  | Flinching (freq.) | Licking (sec.) | Flinching (freq.) | Licking (sec.) | Baseline | 4 h after injection |
| Normal           | 2 ± 2                     | 0                   | 2 ± 2                     | 0                   | 0.16 ± 0.2 | —                    |
| Formalin-2.5%    | 20 ± 3.45#               | 72 ± 6.78#          | 112 ± 10.67#             | 225 ± 13.14#        | 0.18 ± 0.4 | 0.32 ± 0.02*         |
| RO-5%            | 6 ± 0.44                  | 22 ± 3.24           | 35 ± 5.33                | 102 ± 5.67          | 0.16 ± 0.2 | 0.20 ± 0.02*         |
| RO-1%            | 10 ± 0.23                 | 34 ± 2.56           | 54 ± 2.34                | 156 ± 3.45          | 0.18 ± 0.2 | 0.23 ± 0.03*         |
| RO-0.25%         | 18 ± 0.45                 | 45 ± 1.24           | 82 ± 4.78                | 198 ± 4.35          | 0.16 ± 0.4 | 0.27 ± 0.02*         |
| LO-5%            | 3 ± 0.45                  | 18 ± 2.45           | 28 ± 5.23                | 76 ± 5.67           | 0.17 ± 0.2 | 0.18 ± 0.02*         |
| LO-1%            | 8 ± 0.34                  | 32 ± 2.23           | 36 ± 2.76                | 112 ± 6.89          | 0.16 ± 0.2 | 0.21 ± 0.04*         |
| LO-0.25%         | 14 ± 0.56                 | 40 ± 3.23           | 62 ± 5.45                | 154 ± 4.78          | 0.18 ± 0.4 | 0.24 ± 0.02*         |
| Diclofenac-1%    | 2 ± 0.21                  | 12 ± 1.24           | 22 ± 2.34                | 54 ± 1.34           | 0.16 ± 0.2 | 0.17 ± 0.02*         |

Table 6: The effect of 1,8-cineole and α-terpineol on formalin-induced pain behavior and edema in mice at different concentrations.

| Group            | Phase I response                        | Phase II response                        | Paw volume (mL)                      |
|------------------|-----------------------------------------|-----------------------------------------|-------------------------------------|
|                  | Flinching (freq.) | Licking (sec.) | Flinching (freq.) | Licking (sec.) | Baseline | 4 h after injection |
| Normal           | 2 ± 2                     | 0                   | 2 ± 2                     | 0                   | 0.16 ± 0.2 | —                    |
| Formalin-2.5%    | 20 ± 3.45#               | 72 ± 6.78#          | 112 ± 10.67#             | 225 ± 13.14#        | 0.18 ± 0.4 | 0.32 ± 0.02*         |
| CIN-2.5%         | 8 ± 0.42                  | 12 ± 1.21           | 15 ± 1.22                | 87 ± 2.56           | 0.16 ± 0.2 | 0.22 ± 0.02*         |
| CIN-0.5%         | 16 ± 0.32                 | 36 ± 1.56           | 24 ± 1.45                | 108 ± 2.45          | 0.17 ± 0.2 | 0.27 ± 0.02*         |
| CIN-0.1%         | 18 ± 0.56                 | 45 ± 2.24           | 52 ± 3.46                | 178 ± 2.55          | 0.16 ± 0.2 | 0.29 ± 0.02*         |
| TPN-10%          | 13 ± 0.22                 | 21 ± 2.56           | 18 ± 4.67                | 96 ± 6.78           | 0.17 ± 0.2 | 0.24 ± 0.02*         |
| TPN-2%           | 18 ± 0.12                 | 35 ± 2.89           | 46 ± 3.56                | 122 ± 5.45          | 0.16 ± 0.2 | 0.21 ± 0.02*         |
| TPN-0.4%         | 21 ± 0.14                 | 49 ± 1.23           | 72 ± 1.34                | 164 ± 7.4           | 0.16 ± 0.4 | 0.29 ± 0.02*         |
| Diclofenac-1%    | 2 ± 0.12                  | 12 ± 3.24           | 22 ± 4.56                | 54 ± 3.23           | 0.16 ± 0.2 | 0.29 ± 0.02*         |

Data are mean ± SEM. The numbers are frequencies (freq.) of flinching and total time (seconds; sec.) spent licking the formalin-injected paw. *p < 0.05, compared with the formalin-treated group (n = 6); **p < 0.05 compared with the normal group. Baseline values of paw volume represent preinjection diameters of paws. *p < 0.05, compared with the formalin-treated group (n = 6) (ANOVA with Tukey’s test). CIN: 1,8-cineole; TPN: α-terpineol.
phase by significantly reducing the number of lickings and flinching (Table 5). EO of leaf showed significantly greater activity (66%) in reducing the number of flinches and lickings compared to the rhizome oil (52%) \((p < 0.05)\). Analgesic efficacy of aromatic plants and EOs is attributed to the major constituents (Table 6), and the synergism between such chemical constituents is always the main concern of pharmaceutical industries in the interest of pain management [69].

4. Conclusion

The composition of essential oils collected was rich in oxygenated monoterpenes and its major compounds were 1,8-cineole, \(\alpha\)-Terpineol, and fenchyl acetate in rhizome, whereas leaf oil showcased the richness of camphor together with 1,8-cineole and \(\alpha\)-Terpineol. Further, ACEOs and main constituents ameliorate the production of inflammatory mediators such as nitric oxide, ROS, cytokines, and prostanoids \textit{in vitro} and alleviate the edema and pain when applied topically in mice model of inflammation and pain. Further, ACEOs decrement the ontogeny of rat intestinal epithelial cells, human keratinocytes, and hepatocytes without affecting their viability. Hence, ACEOs featured the potential to be formulated into a more safe and sound alternative therapy for acute and chronic inflammatory maladies.

Abbreviations

AC: Alpinia calcarata
EO: Essential oil
ACEOs: \textit{Alpinia calcarata} essential oils
RO: Rhizome oil
LO: Leaf oil.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Acknowledgments

The authors gratefully thank Dr. Ajit Kumar Shasany, Dr. Anirban Pal, and Dr. Anil Kumar Maurya from CSIR-Central Institute of Medicinal and Aromatic Plants, India, for providing scientific facilities and technical support. The work was supported by the grant of National Research Council of Sri Lanka (Grant no: 12-081).

Supplementary Materials

Graphical abstract. (Supplementary Materials)

References

[1] J. I. Majno, \textit{Cells, Tissues, and Disease}, Oxford University Press, New York, NY, USA, 2004.
[2] S. L. Robbins, V. Kumar, A. K. Abbas, and J. C. Aster, \textit{Basic Pathology}, Saunders, Philadelphia, PA, USA, 8th edition, 2007.
[3] T. Hagemann, F. R. Balkwill, and T. Lawrence, "Inflammation and cancer: a double-edged sword," \textit{Cancer Cell}, vol. 12, no. 4, 2007.
[4] F. Marchand, M. Perretti, and S. B. McMahon, "Role of the immune system in chronic pain," \textit{Nature Reviews Neurosci.}, vol. 6, no. 7, pp. 521–532, 2005.
[5] D. M. Mosser and J. P. Edwards, "Exploring the full spectrum of macrophage activation," \textit{Nature Reviews Immunology}, vol. 8, no. 12, pp. 958–969, 2008.
[6] F. Abdallah, L. Mijouin, and C. Pichon, "Skin immune landscape: inside and outside the organism," \textit{Mediators of Inflammation}, vol. 2017, Article ID 5095293, 17 pages, 2017.
[7] T. S. Kupper and R. C. Fuhlbrigge, "Immune surveillance in the skin: mechanisms and clinical consequences," \textit{Nature Reviews Immunology}, vol. 4, no. 3, 2004.
[8] F. O. Nestle, P. Di Meglio, J. Z. Qin, and B. J. Nickoloff, "Skin immune sentinels in health and disease," \textit{Nature Reviews Immunology}, vol. 9, no. 10, pp. 679–691, 2009.
[9] H. Sigmundsdottir Hekla, "Improving topical treatments for skin diseases,"\textit{ Trends in Pharmacological Sciences}, vol. 31, no. 6, pp. 239–245, 2010.
[10] A. S. Panwar, N. Upadhyay, M. Bairagi, S. Gujar, G. N. Darwhekar, and D. K. Jain, "Emulgel: a review," \textit{Asian Journal of Pharmacy and Life Science}, vol. 1, no. 3, pp. 333–343, 2011.
[11] L. Uva, D. Miguel, C. Pinheiro et al., "Mechanisms of action of topical corticosteroids in psoriasis," \textit{International Journal of Endocrinology}, vol. 2012, Article ID 561018, 16 pages, 2012.
[12] K. J. Trouba, H. K. Hamadeh, R. P. Amin, and D. R. Germolec, "Oxidative stress and its role in skin disease," \textit{Antioxidants and Redox Signaling}, vol. 4, no. 4, 2002.
[13] J. N. Cashman, "The mechanisms of action of NSAIDs in analgesia," \textit{Drugs}, vol. 52, no. 5, pp. 13–23, 1996.
[14] A. Aminoshariae, J. C. Kulild, and M. Donaldson, "Short-term use of nonsteroidal anti-inflammatory drugs and adverse effects: an updated systematic review," \textit{Journal of the American Dental Association}, vol. 147, no. 2, pp. 98–110, 2016.
[15] D. Jayaweera, \textit{Medicinal Plants (Indigenous and Exotic) Used in Ceylon}, National Science Council of Sri Lanka, Colombo, Sri Lanka, 1982.
[16] L. Asolkar, K. Kakkar, and O. Chakra, \textit{Second Supplement to Glossary of Indian Medicinal Plants with Active Principles}, Council of Scientific and Industrial Research, New Delhi, India, 1992.
[17] C. Basanayake, \textit{Traditional Treatment in Sri Lanka for Chronic Arthritis}, Council of Scientific and Industrial Research, New Delhi, India, 1998.
[18] L. S. R. Arawwawala, L. D. A. M. Arawwawala, and W. D. Ratnasooriya, "Antinociceptive activities of aqueous and ethanolic extracts of \textit{Alpinia calcarata} rhizomes in rats," \textit{Journal of Ethnopharmacology}, vol. 95, no. 2-3, pp. 311–316, 2004.
[19] L. D. A. M. Arawwawala, L. S. R. Arawwawala, and W. D. Ratnasooriya, \textit{Alpinia calcarata roscoe: a potent antiinflammatory agent}, \textit{Journal of Ethnopharmacology}, vol. 139, no. 3, pp. 889–892, 2012.
[20] M. Rahman, A. Rahman, M. A. Hashem, M. Ullah, S. Afroz, and V. Chaudhary, "Anti-inflammatory, analgesic and GC-
Evidence-Based Complementary and Alternative Medicine

MS analysis of essential oil of Alpinia calcarata rhizome,” *International Journal of Pharma and Bio Sciences*, vol. 3, no. 4, 2012.

[21] S. Ghosh and L. Rangan, “Alpinia: the gold mine of future therapeutics,” *Biotech*, vol. 3, no. 3, 2013.

[22] L. S. R. Arambawela, A. Kumaratunge, M. Arawwawela, N. L. Owen, and L. Du, “Volatile oils of Alpinia calcarata rosco. grown in Sri Lanka,” *Journal of Essential Oil Research*, vol. 17, no. 2, pp. 124–125, 2005.

[23] P. N. Kaul, B. R. Rajeswara Rao, K. Singh, A. K. Bhattacharya, G. R. Mallavarapu, and S. Ramesh, “Volatile constituents of essential oils isolated from different parts of Alpinia calcarata rosco.” *Journal of Essential Oil Research*, vol. 17, no. 7–9, 2005.

[24] A. Tewari, A. K. Pant, C. S. Mathela, N. Mengi, E. Kohl, and H. J. Bestmann, “Volatile constituents of Alpinia calcarata rosco,” *Journal of Essential Oil Research*, vol. 11, no. 6, pp. 739–741, 1999.

[25] M. Nazrul Islam Bhuiyan, “Volatile constituents of essential oils isolated from different parts of Alpinia calcarata rosco,” *African Journal of Plant Science*, vol. 5, 2011.

[26] A. P. Raina and Z. Abraham, “Chemical composition of essential oils obtained from plant parts of Alpinia calcarata rosco.(lesser galangal) germplasm from south India,” *Journal of Essential Oil Research*, vol. 27, no. 3, pp. 238–243, 2015.

[27] M. G. B. De Oliveira, R. B. Marques, M. F. Santana et al., “α-terpineol reduces mechanical hypernociception and inflammatory response,” *Basic and Clinical Pharmacology and Toxicology*, vol. 111, pp. 120–125, 2012.

[28] S. Held, P. Schieberle, and V. Somoza, “Characterization of α-terpineol as an anti-inflammatory component of orange juice by in vitro studies using oral buccal cells,” *Journal of Agricultural and Food Chemistry*, vol. 55, no. 20, 2007.

[29] G. J. Yu, C. I. Whan, K. G. Young et al., “Anti-inflammatory potential of saponins derived from cultured wild ginseng roots in lipopolysaccharide-stimulated raw 264.7 macrophages,” *International Journal of Molecular Medicine*, vol. 35, no. 6, 2015.

[30] P. N. Yadav, Z. Liu, and M. M. Rafi, “A diarylheptanoid from lesser galangal (Alpinia officinarum) inhibits proinflammatory mediators via inhibition of mitogen-activated protein kinase, p44/42, and transcription factor nuclear factor-kB,” *Journal of Pharmacology and Experimental Therapeutics*, vol. 305, no. 3, 2003.

[31] A. K. Kiemer, C. Müller, and A. M. Vollmar, “Inhibition of LPS-induced nitric oxide and TNF-α production by a-lipoic acid in rat kupffer cells and in raw 264.7 murine macrophages,” *Immunology and Cell Biology*, vol. 80, no. 6, 2002.

[32] W. S. Koko, M. A. Mesaik, S. Youssaf, M. Galal, and M. I. Choudhary, “In vitro immunomodulating properties of selected Sudanese medicinal plants,” *Journal of Ethnopharmacology*, vol. 118, no. 1, pp. 26–34, 2008.

[33] B. E. Sadique and W. A. Al Robahs, “The bioactivity of certain medicinal plants on the stabilization of RBC membrane system,” *Fitoterapia*, vol. 60, pp. 525–532, 1989.

[34] H. Lim, H. Park, and P. K. Hyun, “Inhibition of contact dermatitis in animal models and suppression of proinflammatory gene expression by topically applied flavonoid, wogonin,” *Archives of Pharmacal Research*, vol. 27, no. 4, 2004.

[35] B. Pulli, M. Ali, R. Forghan et al., “Measuring myeloperoxidase activity in biological samples,” *PLoS One*, vol. 8, no. 7, 2013.

[36] I. O. Lee and Y. S. Jeong, “Effects of different concentrations of formalin on paw edema and pain behaviors in rats,” *Journal of Korean Medical Science*, vol. 17, no. 1, 2002.

[37] E. R. Adlard, *Handbook of Essential Oils Science, Technology and Applications*, CRC Press, Boca Raton, FL, USA, 2010.

[38] A. P. Raina and Z. Abraham, “Essential oil profiling of Alpinia species from Southern India,” *Indian Journal of Experimental Biology*, vol. 55, 2017.

[39] P. N. Kaul, B. R. R. Rao, K. Singh, A. K. Bhattacharya, G. R. Mallavarapu, and S. Ramesh, “Volatile constituents of essential oils isolated from different parts of Alpinia calcarata rosco,” *Journal of Essential Oil Research*, vol. 17, 2005.

[40] S. Verma and N. Kaplowitz, “Diagnosis, management and prevention of drug-induced liver injury,” *Gut*, vol. 58, no. 11, 2009.

[41] Z. Li, Z. Zheng, J. Ruan, Z. Li, and C. M. Tseng, “Chronic inflammation links cancer and parkinson’s disease,” *Frontiers in Aging Neuroscience*, vol. 8, no. 126, 2016.

[42] L. F. Mager, M. H. C. Wasmer, T. T. Rau, and P. Krebs, “Cytokine-induced modulation of colorectal cancer,” *Frontiers in Oncology*, vol. 6, 2016.

[43] A. J. Farrell, D. R. Blake, R. M. Palmer, and S. Moncada, “Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases,” *Annals of the Rheumatic Diseases*, vol. 51, no. 11, 1992.

[44] D. Fukumura, S. Kashiwagi, and R. K. Jain, “The role of nitric oxide in tumour progression,” *Nature Reviews Cancer*, vol. 6, no. 7, 2006.

[45] C. Nathan and Q. Xie, “Nitric oxide synthases: roles, tolls, and controls,” *Cell*, vol. 78, pp. 915–918, 2017.

[46] K. Makni-Maalej, M. Chiandotto, M. Hurtado-Nedelec et al., “Zymosan induces NADPH oxidase activation in human neutrophils by inducing the phosphorylation of p47phox and the activation of rac2: involvement of protein tyrosines kinases, pi3kinase, PKC, ERK1/2 and P38 mapkinase,” *Biochemical Pharmacology*, vol. 85, no. 1, pp. 92–100, 2013.

[47] S. Reuter, S. C. Gupta, M. M. Chaturvedi, and B. B. Aggarwal, “Oxidative stress, inflammation, and cancer: how are they linked?” *Free Radical Biology and Medicine*, vol. 49, no. 11, 2010.

[48] L. F. Marchi, R. Sesti-Costa, M. D. C. Ignacchiti, S. Chedraoui-Silva, and B. Mantovani, “In vitro activation of mouse neutrophils by recombinant human interferon-gamma: increased phagocytosis and release of reactive oxygen species and proinflammatory cytokines,” *International Immunopharmacology*, vol. 18, no. 2, 2014.

[49] S. Thusoo, S. Gupta, R. Sudan et al., “Antioxidant activity of essential oil and extracts of Valeriana jatamansi roots,” *BioMed Research International*, vol. 2014, Article ID 614187, 4 pages, 2014.

[50] S. Akira and K. Takeda, “Toll-like receptor signalling,” *Nature Reviews Immunology*, vol. 4, pp. 499–511, 2004.

[51] M. De Miguel, D. C. Kraychete, and R. J. Meyer Nascimento, “Chronic pain: cytokines, lymphocytes and chemokines,” *Inflammation & Allergy-Drug Targets*, vol. 13, no. 5, 2014.

[52] A. F. Valledor, L. Arpa, T. E. Sánchez et al., “IFN-[gamma]-mediated inhibition of MAPK phosphatase expression results in prolonged MAPK activity in response to M-CSF and inhibition of proliferation,” *Blood*, vol. 112, no. 8, 2008.

[53] L. Xu and B. S. Croix, “Improving VEGF-targeted therapies through inhibition of COX-2/PGE 2 signaling,” *Molecular and Cellular Oncology*, vol. 1, no. 4–6, 2014.
[54] J. R. Mestre, D. E. Rivadeneira, P. J. Mackrell et al., “Overlapping CRE and e-box promoter elements can independently regulate COX-2 gene transcription in macrophages,” *FEBS Letters*, vol. 496, no. 2, 2001.

[55] Y.-J. Kang, B. A. Wingerd, T. Arakawa, and W. L. Smith, “Cyclooxygenase-2 gene transcription in a macrophage model of inflammation,” *The Journal of Immunology*, vol. 177, no. 11, 2006.

[56] J. K. Srivastava, M. Pandey, and S. Gupta, “Chamomile, a novel and selective COX-2 inhibitor with anti-inflammatory activity,” *Life Sciences*, vol. 85, no. 19, 2009.

[57] B. Everts, P. Währborg, and T. Hedner, “COX-2-specific inhibitor—the emergence of a new class of analgesic and anti-inflammatory drugs,” *Clinical Rheumatology*, vol. 19, no. 5, 2000.

[58] J. Y. Kim, S.-S. Kim, T.-H. Oh et al., “Chemical composition, antioxidant, anti-elastase, and anti-inflammatory activities of *Illicium anisatum* essential oil,” *Acta Pharmacutica*, vol. 59, no. 3, 2009.

[59] A. M. Beer, P. Zagorchev, D. M. Filipova, and J. Lukanov, “Effects of 1,8-cineole on the activity of cyclooxygenase and cyclooxygenase 1 and cyclooxygenase 2 isoforms,” *Natural Products Chemistry & Research*, vol. 5, no. 8–11, 2017.

[60] D. A. Lewis and E. H. Day, “Biochemical factors in the action of steroids on diseased joints in rheumatoid arthritis,” *Annals of the Rheumatic Diseases*, vol. 31, no. 5, 1972.

[61] C. A. Anosike, O. Obidoa, and L. U. S. Ezeanyika, “Membrane stabilization as a mechanism of the anti-inflammatory activity of methanol extract of garden egg (*Solanum aethiopicum)*,” *DARU Journal of Pharmaceutical Sciences*, vol. 20, p. 76, 2012.

[62] A. Zanella and W. Barcellini, “Treatment of autoimmune hemolytic anemias,” *Haematologica*, vol. 99, no. 10, 2014.

[63] D. H. Kuo, Y. S. Lai, C. Y. Lo, A. C. Cheng, H. Wu, and M. H. Pan, “Inhibitory effect of magnolol on TPA-induced skin inflammation and tumor promotion in mice,” *Journal of Agricultural and Food Chemistry*, vol. 58, no. 9, 2010.

[64] B. Chassaing, J. D. Aitken, M. Malleshappa, and M. Vijay-Kumar, “Dextran sulfate sodium (DSS)-induced colitis in mice,” *Current Protocols in Immunology*, vol. 104, no. 1, p. 5859, 2001.

[65] L. V. Forbes, T. Sjögren, F. Auchere et al., “Potent reversible inhibition of myeloperoxidase by aromatic hydroxamates,” *Free Radical Biology and Medicine*, vol. 65, 2013.

[66] T. Neogi, A. Guermazi, F. Roemer et al., “Association of joint inflammation with pain sensitization in knee osteoarthritis: the multicenter osteoarthritis study,” *Arthritis and Rheumatology*, vol. 68, no. 3, 2016.

[67] A. J. Alonso-Castro, M. A. Zavala-Sánchez, J. Pérez-Ramos, E. Sánchez-Mendoza, and S. Pérez-Gutiérrez, “Antinociceptive and anti-arthritic effects of kramecyne,” *Life Sciences*, vol. 121, pp. 70–77, 2015.

[68] J. Damas and J. F. Liégeois, “The inflammatory reaction induced by formalin in the rat paw,” *Naunyn-Schmiedeberg’s Archives of Pharmacology*, vol. 359, 1999.

[69] R. de Cássia Da Silveira E Sá, L. N. Andrade, and D. P. De Sousa, “A review on anti-inflammatory activity of monoterpens,” *Molecules*, vol. 18, no. 1, 2013.