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Inflammopharmacology

ORIGINAL ARTICLE

Phenolic rich *Cocos nucifera* inflorescence extract ameliorates inflammatory responses in LPS-stimulated RAW264.7 macrophages and toxin-induced murine models

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Abstract

Anti-inflammatory and antinociceptive effects of the acetone extract of *Cocos nucifera* (CnAE), an important ingredient in several traditional drugs, have been studied using different in vitro and in vivo models. CnAE did not show any observable toxicity in RAW264.7 macrophages by MTT assay. The calorimetric analysis (total COX, 5-LOX, MPO, iNOS and NO), ELISA (IL-1β, IL-6, TNF-α and PGE_2) and qRT-PCR (IL-1β, IL-6, TNF-α and NF-κB) were performed in LPS-induced RAW264.7 macrophages. Phosphorylation of NF-κBp65 and IκB was determined by western blotting. CnAE (100 µg/mL) remarkably inhibited total COX (68.67%) and 5-LOX (63.67%) activities, and subsequent release of iNOS, NO and PGE_2 ($p \leq 0.05$) in RAW264.7 cells treated with LPS. ELISA showed CnAE markedly decreased the level of pro-inflammatory cytokines IL-1β ($p \leq 0.001$), IL-6 ($p \leq 0.001$) and TNF-α ($p \leq 0.001$) in LPS treated RAW264.7 cells. CnAE (100 µg/mL) also significantly down-regulated the mRNA expressions of pro-inflammatory cytokines (IL-1β, $p \leq 0.05$; IL-6, $p \leq 0.01$ and TNF-α, $p \leq 0.001$) and NF-κB ($p \leq 0.001$) against LPS-induction. Moreover, LPS-induced phosphorylation of IκB-α and NF-κB p65 was significantly inhibited by CnAE (100 µg/mL). In vivo anti-inflammatory studies showed that CnAE (400 mg/kg) significantly inhibited carrageenan-induced acute paw oedema (59.81%, $p \leq 0.001$) and formalin-induced chronic paw oedema (52.90%, $p \leq 0.001$) in mice. CnAE at a dose of 400 mg/kg also showed a significant anti-nociceptive effect on acetic acid-induced writhing (48.21%, $p \leq 0.001$) and Eddy's hot plate methods. These findings suggest that CnAE has significant anti-inflammatory and anti-nociceptive properties, mainly attributed to the inhibition of NF-κB/IκB signalling cascade.

Keywords *Cocos nucifera* L. inflorescence · Acetone extract · Anti-inflammatory activity · Anti-nociceptive activity · RAW264.7 macrophages · Swiss albino mice

Abbreviations

ANOVA	Analysis of variance
CD14	Cluster of differentiation 14
CnAE	*Cocos nucifera* acetone extract
COX	Cyclooxygenase
DCFDA	2′,7′-Dichlorofluorescin diacetate
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
IL	Interleukin
IκB	Inhibitor kappa B
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin receptor-associated kinase
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MD2	Myeloid differentiation 2
MPO	Myeloperoxide
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOX	Nicotinamide adenine dinucleotide phosphate oxidase

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OECD	Organisation for economic co-operation and development
PCR	Polymerase chain reaction
PGE2	Prostaglandin 2
qRT	Quantitative real time
SD	Standard deviation
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
TRAF6	TNF receptor associated factor 6
UV	Ultra violet

Introduction

Inflammation is one of the major causes of morbidity and mortality throughout the world and is involved in a host of different infectious and non-infectious diseases (Bhatia, et al. 2009). The inflammatory response is coordinated by a large range of mediators that form complex regulatory networks. The acute inflammatory response is a complex, but highly coordinated mechanism involving molecular, cellular and physiological alterations (Chen et al. 2018). Macrophages play a critical role, serving as an essential interface between innate and adaptive immunity during inflammatory responses. Many of the recent works demonstrated that LPS-induction up-regulates the level of cellular pro-inflammatory mediators (such as COX-2, 5-LOX, NO and PGE_2) and pro-inflammatory cytokines (such as IL-1β, IL-6 and TNF-α) through NF-κB signaling pathway (Lawrence et al. 2001; Parajuli et al. 2012; Badshah et al. 2016). As a first step, LPS could bind to LPS-binding proteins (LBP) in plasma, and this LPB-LPS complex has affinity for the MD2 and the CD14 that together connect to the TLR4. The binding of LPS to TLR4 leads to the activation of NF-κB through the recruitment and activation of MyD88, IRAK, TRAF-6 as well as NOX, and ultimately triggers the release of pro-inflammatory mediators and cytokines (Akira 2003; Park et al. 2004).

Cocos nucifera L. is an important member of the monocotyledonous family Arecaceae, commonly known as the Coconut tree. It is originally from Southeast Asia (Malaysia, Indonesia, and the Philippines) and the islands between the Indian and Pacific Oceans (Lima et al. 2015). *C. nucifera* is ascribed to possess diverse medicinal, culinary and commercial applications. In India, the fresh juice of inflorescence is traditionally used to treat diarrhoea, dysentery, diabetes, haemoptysis, strangury, leprosy and general debility (Vaidyaratnam 1994; Renjith et al. 2013). In Ayurveda, the inflorescence is used to treat menorrhagia (Bhandary et al. 1995; Padumadasa et al. 2016) and backache (Renjith et al. 2013). Also, it is the main ingredient of *Thengin Pookkuladi Lehyam*, an ayurvedic rejuvenating nutraceutical for post-natal convalescence (Rajith et al. 2009). The sweet watery sap that drips from the immature inflorescence is used as a coolant, aperient and aphrodisiac (Vaidyaratnam 1994). In addition, several pharmacological studies of various extracts, fractions, and isolated compounds from the different parts of *C. nucifera*, reported a wide range of biological activities (Salil et al. 2001; Esquenazi et al. 2002; Loki and Rajamohan 2003; Rinaldi et al. 2009; Al-Adhroey et al. 2011; Pal et al. 2011; Preetha et al. 2013). To evaluate the therapeutic claim of *C. nucifera* inflorescence in Ayurveda, the present study was carried out with a view to examine its ameliorative effect on in vitro and in vivo inflammatory models.

Materials and methods

Drugs and chemicals

Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco, Thermo Fisher Scientific, USA. Lipopolysaccharide (*Escherichia coli*, serotype 0111:B4), MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; M5655); DCFDA (2′,7′-dichlorofluorescin diacetate) and primers (forward and reverse, IL-1β, IL-6, TNF-α, NF-κB and GAPDH) were purchased from Sigma–Aldrich, USA. Verso cDNA synthesis kit and Dynamo flash SYBR Green RT-PCR Kit were purchased from Thermo Fisher Scientific Inc., USA. ELISA kit for IL-1β, IL-6, TNF-α and PGE2 were purchased from Cayman Chemicals, USA. Antibodies (pIκBα and pNF-κB p65) were obtained from Cell Signaling Technology, USA. Biochemical reagents were purchased from Merck, India. Tissue culture plates and flasks were purchased from Nunc, Thermo Fisher Scientific, USA. All other chemicals, solvents and reagents used were of analytical grade.

Collection of plant material and preparation of extract

The un-opened flowering inflorescence of *Cocos nucifera* (Cn) was collected from Kallara Village (8.7591° N, 76.9462° E) of the Western Ghats region of Thiruvananthapuram District, Kerala State, India. The freshly collected inflorescence (950 g) was crushed and extracted with acetone at room temperature for 48 h with occasional stirring. The supernatant liquid was then decanted and filtered. The process was repeated four times. The extracts were combined, distilled and concentrated under reduced pressure using a rotary vacuum evaporator (IKA® RV10, Germany) to remove the residual water and solvent. The extract (CnAE) was then stored at 4 °C until further use.

Experimental animals

Male Swiss albino mice (25–30 g) were selected for the study, housed in poly acrylic cages (two animals per cage), and maintained under standard laboratory conditions (temperature 24–28 °C, relative humidity 60–70% and 12 h day/night rhythm). They were fed commercial rat feed (Sai Durga Feeds and Foods, Bangaluru, India) and boiled water ad libitum. Experiments were done according to OECD guidelines, after getting the approval of the Institute's Animal Ethics Committee (IAEC; Reg. no. 149/199/CPCSEA), Amala Cancer Research Centre, India.

Phytochemical analysis

The phytochemical analysis of CnAE was carried out with standard protocols (Harborne 1998; Trease and Evans 2002). Total phenolic (Lachman et al. 2000) and total flavonoid (Chang et al. 2002) content were also determined.

HPTLC fingerprint

TLC silica gel 60 F_{254} aluminum plate (Merck, India) was used for the HPTLC fingerprinting. 10 mg of CnAE was dissolved in 1 mL methanol. The sample (10 µL) was applied on TLC plate (5 × 10 cm) using Linomat V Sample Applicator and CAMAG Linomat 5 instrument. After spotting and drying, the TLC plate was developed up to 90 mm in a twin trough glass chamber using chloroform–methanol (8:2) as the mobile phase. Then the plate was kept in photodocumentation chamber and was scanned at 254 (short UV), 366 (long UV) and 580 nm using TLC Scanner 3 equipped with WinCats Software. The plate was then derivatised using anisaldehyde sulphuric acid reagent, heated at 110 °C for 10 min and scanned densitometrically at 580 nm. The chromatograms and the percentage composition of the spots were noted.

Cell culture

RAW264.7 macrophages were obtained from National Centre for Cell Sciences (NCCS), India. The cells were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin G and 100 µg/mL streptomycin and maintained at 37 °C in an atmosphere of 5% CO_2. Cells were allowed to grow to 90–95% confluence, at which point they were washed with phosphate-buffered saline (PBS, pH 7.4). For cell viability assay, RAW264.7 macrophages were grown to 60% confluency, followed by addition of different concentrations of CnAE (6.25–100 µg/mL) and incubated at 37 °C for 24 h. For anti-inflammatory studies, RAW264.7 macrophages were grown to 60% confluency, followed by activation with 1 µL lipopolysaccharide (1 µg/mL) and treated with different concentrations (25, 50 and 100 µg/mL) of CnAE and diclofenac sodium. After 24 h of incubation, the whole cell lysates were used to determine anti-inflammatory activity. Briefly, the treated cells were collected with ice-cold PBS and extracted by lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 10 µg/mL leupeptin, 10 µg/mL aprotinin) on ice for 15 min. The insoluble matter was removed by centrifugation at 12,000×*g* for 15 min at 4 °C, and the supernatant fraction (whole cell lysate) was collected for further analysis (Intayoung et al. 2016).

MTT assay

After 24 h incubation, the medium containing samples were removed and 30 µL of reconstituted MTT solution (0.5 mg/mL) was added to all the test and control wells and further incubated at 37 °C for 4 h. After incubation, MTT solution was removed and formazan crystals in each well were dissolved in 100 µL DMSO into a colored solution. Absorbance of the formazan solution was quantified by an ELISA microplate reader (Erba LisaScan II, Germany) at 570 nm (Talarico et al. 2004). The optical density of the formazan formed in the control (untreated) cells was considered to represent 100% viability.

Cell viability assay by microscopy

After 24 h incubation the entire plate was observed in an inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD Camera, Japan) and microscopic observations were recorded as images. Observable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Total cyclooxygenase activity

The cell lysate was incubated in Tris–HCl buffer (pH 8), glutathione 5 mM/L, and hemoglobin 5 mM/L for 1 min at 25 °C. The reaction was initiated by the addition of arachidonic acid 200 mM/L and terminated after 20 min incubation at 37 °C, by the addition of 10% trichloroacetic acid in 1 N hydrochloric acid. After the centrifugal separation and the addition of 1% thiobarbiturate, activity was determined by reading absorbance at 632 nm (Walker and Gierse 2010).

5-Lipoxygenase activity

Briefly, the reaction mixture (2 mL) contained Tris–HCl buffer (pH 7.4), 50 µL of cell lysate, and sodium linoleate (200 µL). The 5-LOX activity was monitored as an increase

of absorbance at 234 nm, which reflects the formation of 5-hydroxyeicosatetraenoic acid (Axelrod et al. 1981).

Myeloperoxidase activity

Cell lysate was homogenized in a solution containing 50 mM potassium phosphate buffer and 0.57% hexadecyltrimethylammonium bromide (HTAB), the samples were centrifuged at 2000×*g* for 30 min at 4 °C, and supernatant was assayed for MPO activity. MPO in the sample was activated by the addition of 50 mM phosphate buffer (pH 6) containing 1.67 mg/mL guaiacol and 0.0005% H_2O_2. The change in absorbance at 460 nm was measured. MPO activity was presented as units per mL of cell lysate. One unit of MPO activity was defined as that degrading 1 µM of peroxide per minute at 25 °C (Suzuki et al. 1983).

Inducible nitric oxide synthase activity

Cell lysate was homogenized in 2 mL of HEPES buffer. The assay system contained substrates 0.1 mL L-arginine, 0.1 mL manganese chloride, 0.1 mL 30 µg dithiothreitol (DTT), 0.1 mL NADPH, 0.1 mL tetrahydropterin, 0.1 mL oxygenated haemoglobin and 0.1 mL enzyme (sample). Increase in absorbance was recorded at 401 nm (Salter et al. 1991).

Estimation of cellular nitrite level

To 0.5 mL of cell lysate, 0.1 mL of sulphosalicylic acid was added and vortexed well for 30 min. The samples were then centrifuged at 5000 rpm for 15 min. The protein-free supernatant was used for the estimation of nitrite levels. To 200 µL of the supernatant, 30 µL of 10% NaOH was added, followed by 300 µL of Tris–HCl buffer and mixed well. To this, 530 µL of Griess reagent was added and incubated in the dark for 10–15 min, and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained (Bryan and Grisham 2007).

Evaluation of intracellular ROS production

The ROS scavenging activity of CnAE was measured by the oxidant-sensing probe 2′,7′-dichlorofluorescin diacetate (DCFDA) (Kim et al. 2011). The cells were seeded on a 96-well plate at a density of 5000–10,000 cells per well. After 24 h of seeding (at 40–70% density), the cells were treated with LPS (1 µg/mL) for 1 h followed by the addition of CnAE (100 µg/mL) and incubated for 24 h in a CO_2 incubator. The cells were washed with PBS and added with 50 µL of DCFDA (100 µM DCFDA was diluted in DMEM + 1% FBS) and incubated for 30 min (at 37 °C, 5% CO_2) in the dark. Excess dye was washed with PBS and the DCF fluorescence was measured at an excitation wavelength of 470 nm and emission wavelength of 635 nm (Qubit 3.0, Life technologies, USA) and expressed in arbitrary units. Fluorescence was imaged in Olympus CKX41 with Optika Pro5 CCD Camera, Japan.

ELISA of cytokines and PGE_2

The production of TNF-α, IL-1β, IL-6 and PGE_2 in cell-free culture supernatants was determined using a commercial ELISA kit, according to the manufacturer's protocol.

RNA extraction and qRT-PCR

Total RNA was isolated from RAW264.7 macrophages using TRIzol reagent (Ambion, Life Technologies, USA). Total RNA was then treated with RNase-free Dnase I (Sigma Aldrich, USA), quantified by measuring the absorbance at 260 and 280 nm and stored at − 80 °C until analysis. cDNA was synthesized using Verso cDNA synthesis kit according to the manufacturer's protocol. The mRNA levels of various genes were evaluated by quantitative real time polymerase chain reaction (qRT-PCR, StepOne System, Applied Biosystems, USA) using a Dynamo flash SYBR Green RT-PCR Kit. Specific primers used to measure gene expression at mRNA levels were as follows; for IL-1β F5′-ACC TGT CCT GTG TAA ATG AAA GAC G-3′ and R5′-TTG GTA TTG CTT GGG ATC C-3′; for IL-6 F5′-GGT ACA TCC TCG ACG GCA TCT-3′ and R5′-GAG GAT ACC ACT CCC AAC AGA CC-3′; for TNF-α F5′-CCC AGG CAG TCA GAT CAT CTT C-3′ and R5′-AGC TGC CCC TCA GCT TGA-3′; for NF-κB F5′-CCT AGC TTT CTC TGA ACT GCA AA-3′ and R5′-GGG TCA GAG GCC AAT AGA GA-3′ and for GAPDH F5′-AAT GCA TCC TGC ACC ACC AAC TGC-3′ and R5′-GGA GGC CAT GTA GGC CAT GAG GTC-3′. The PCR amplification was performed for 40 repetitive thermal cycles with SYBR green (95 °C for 10 s, 60 °C 20 s, and 72 °C for 15 s, followed extenson at 72 °C for 10 min). The mRNA expression profiles were normalised with respect to GAPDH. Fold increase of each gene was calculated using $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). Each sample was assessed in triplicate.

Determination of pIκB-α and pNF-κB p65 expression

RAW264.7 cells were pre-treated with LPS (1 µg/mL) for 1 h and then incubated with CnAE (100 µg/mL) for 24 h at 37 °C. After incubation the cells were collected in PBS and lysed in an ice-cold lysis buffer. The concentration of proteins in each sample was measured using the Bradford protein assay. During separation in gel electrophoresis, 30 µg of protein mixture from each sample was loaded in the wells of

12% polyacrylamide gels. Once the electrophoresis was finished, the proteins in the gels were transferred onto Western blot nitrocellulose membranes for 2 h at 50 volts. After the transfer, both sides of the membranes were blocked with 5% of skim milk diluted in PBS-Tween 20 (PBST) for 1 h. The membranes were then washed with PBST three times and incubated with diluted antibodies (pIκBα and pNF-κB p65) overnight. After three consecutive washings, the membranes were incubated with secondary antibodies conjugated with peroxidase for 1 h and a half, and the protein bands were revealed in a dark room (Ngabire et al. 2018).

Eddy's hot plate test

Male Swiss albino mice were divided into five groups of six animals each. Distilled water was administered orally to Group 1 and Group 2 (standard control group) received sodium salicylate (100 mg/kg) p.o. Groups 3, 4 and 5 received 100, 200, and 400 mg/kg b.w. of CnAE p.o., respectively. Mice were placed on Eddy's hot plate (thermal stimulus) maintained at 55 ± 10 °C for 0, 15, 30, 60, 90 and 120 min after drug administration. The time taken by animals to lick the fore or hind limb or jump off the plate was taken as the reaction time (sec); 15 s cut off was used to prevent tissue damage (Turner 1965).

Acetic acid-induced writhing

Male Swiss albino mice were divided into five groups (6 per group), fasted overnight, but were allowed free access to water. All the groups received i.p., 0.5% aqueous solution of acetic acid. Distilled water was administered orally to Group 1 and Group 2 (standard control group) received acetyl salicylic acid (aspirin) 100 mg/kg b.w. p.o. Groups 3, 4 and 5 received 100, 200, and 400 mg/kg b.w. of CnAE p.o., respectively. All these treatments were performed 20 min prior to the administration of acetic acid. The number of writhes per animal was recorded during the 20 min period, beginning 5 min after the injection of acetic acid (Koster et al. 1959). Percentage inhibition was calculated using the following formula:

$$\text{Percentage inhibition} = [(W_c - W_t) \times 100]/W_c$$

where, W_c = No. of writhes in control group, W_t = No. of writhes in treatment groups.

Carrageenan-induced acute paw oedema

Male Swiss albino mice were divided into five groups (6 per group), fasted overnight, but were allowed free access to water. Acute inflammation was produced in all animals by sub-plantar injection of 20 µl of a freshly prepared 1% suspension of carrageenan in carboxy methyl cellulose on the right hind paw. Distilled water was administered orally to Group 1 and standard anti-inflammatory drug diclofenac (10 mg/kg) was given orally to the Group 2. CnAE (100, 200, and 400 mg/kg, respectively) was administered orally using oral gavage tube to Groups 3, 4, and 5. All these treatments were performed 1 h prior to carrageenan injection. Paw thickness of all the animals were measured before carrageenan injection using Plethysmometer and it was taken as initial paw thickness. After 3 h of oedema induction, paw thickness was again measured and noted as final paw thickness. The increase in paw thickness and percentage inhibition were calculated (Winter et al. 1962). Percentage inhibition of paw oedema was calculated using the following formula:

$$\text{Percentage inhibition} = [(PV_c - PV_t) \times 100]/PV_c$$

where, PV_c = Paw volume in control group, PV_t = Paw volume in treatment groups.

Formalin-induced chronic paw oedema

Male Swiss albino mice were divided into five groups (6 per group), fasted overnight, but were allowed free access to water. 20 µl of freshly prepared 2% formalin was injected to right hind paw of all animals to induce chronic inflammation. Distilled water was administered orally to group 1 and the standard anti-inflammatory drug diclofenac (10 mg/kg) was given orally to group 2. CnAE (100, 200, and 400 mg/kg, respectively) was administered orally using oral gavage tube to groups 3, 4 and 5. All of these treatments were performed 1 h prior to formalin injection. The standard drug and extract administration were continued once daily for 6 consecutive days. The paw thickness was measured using Plethysmometer before and 6 days after formalin injection. The increase in paw thickness and percentage inhibition were calculated (Ajith and Janardhanan 2001). Percentage inhibition of paw oedema was calculated using the following formula:

$$\text{Percentage inhibition} = [(PV_c - PV_t) \times 100]/PV_c$$

where, PV_c = Paw volume in control group, PV_t = Paw volume in treatment groups.

Statistical analysis

Each experimental data were expressed as mean ± standard deviation of 3/6 values. The in vitro results were analysed by one-way ANOVA followed by Tukey's multiple comparison tests and two-way ANOVA followed by Bonferroni multiple comparisons, and in vivo results were analysed by one-way ANOVA followed by Dunnett's multiple comparison test using Graph Pad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA). $p \leq 0.0001$;

$p \leq 0.001$; $p \leq 0.01$ and $p \leq 0.05$ were considered to be statistically significant.

Results

Phytochemical analysis and HPTLC fingerprint

The yield of the acetone extract (CnAE) was 7.68%. Phytochemical screening showed the presence of terpenoids, steroids, phenolics, flavonoids, coumarins, saponins and carbohydrates. The total phenolic content of CnAE was found to be 222.61 µg GAE/g dry extract with reference to the gallic acid standard curve ($y = 0.023x + 0.119$; $R^2 = 0.996$). The flavonoid content of CnAE was found to be 120.83 µg QE/g dry extract with reference to the quercetin standard curve ($y = 0.012x + 0.236$; $R^2 = 0.977$).

The HPTLC fingerprint of CnAE showed the presence of 13 peaks scanned at 366 nm and 580 nm with R_f range 0.06–0.94 (Fig. 1). The R_f values obtained are 0.01, 0.06, 0.14, 0.17, 0.23, 0.31, 0.35, 0.40, 0.51, 0.57, 0.64, 0.71 and 0.86 (Table 1). The major peaks appeared at R_f 0.01(13.4%), 0.57 (10.11%), 0.64 (11.46%) and 0.71 (39.96%).

MTT assay

Cell viability assay by MTT revealed that CnAE did not show any toxicity in RAW264.7 macrophages (Fig. 2). Cells showed > 82% viability even at the higher concentration tested (100 µg/mL). So sample concentrations of 25, 50 and 100 µg/mL were used for further in vitro pharmacological investigations.

Cell viability by microscopy

After 24 h incubation with CnAE, morphology of RAW264.7 macrophages were examined under inverted phase contrast microscope and no significant changes were observed in the morphology of the cells (Fig. 3).

Effect of CnAE on total COX activity

RAW264.7 macrophages treated with LPS showed an increased level of total COX activity. Cells initially treated with CnAE at different concentrations (25, 50 and 100 µg/mL) showed a dose-dependent decrease in the level of total COX activity. The maximum inhibition (68.67%) in total COX activity was observed at a concentration of 100 µg/mL (Fig. 4).

Effect of CnAE on 5-LOX activity

RAW264.7 macrophages treated with LPS showed an increased level of 5-LOX activity. Cells initially treated with CnAE at different concentrations (25, 50 and 100 µg/mL) showed a dose-dependent decrease in the level of 5-LOX

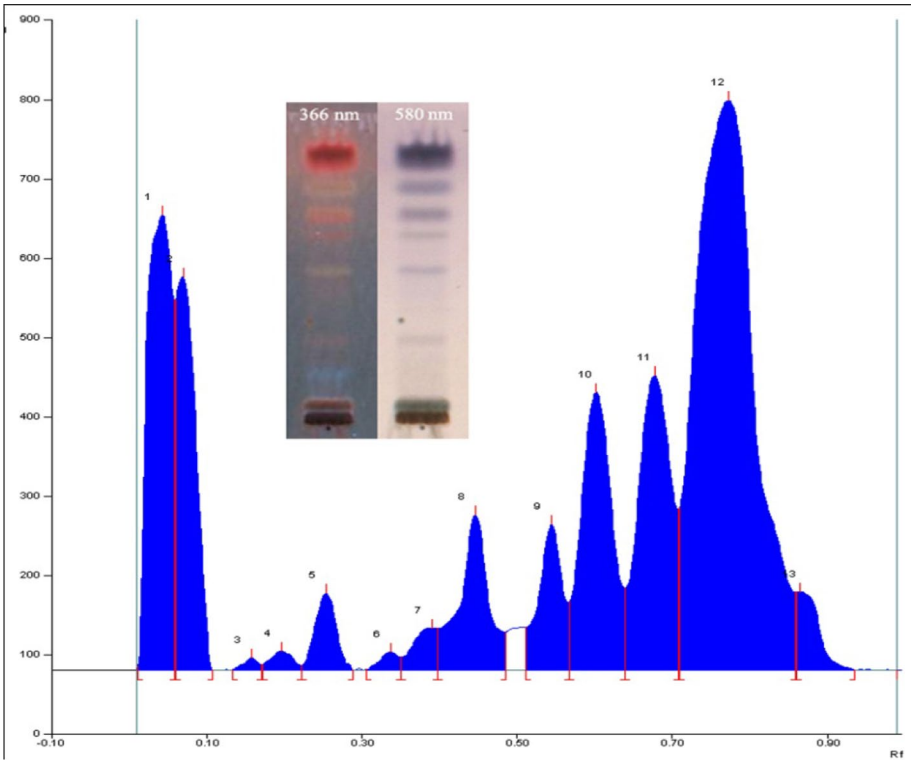


Fig. 1 HPTLC fingerprint of CnAE scanned at 366 and 580 nm showed 13 peaks representing major chemical constituents. Mobile phase: chloroform/methanol (8:2). The plates were derivatised with anisaldehyde sulphuric acid reagent

Table 1 HPTLC chromatogram peak data of CnAE including percentage composition of each peak scanned at 366 and 580 nm

Peak	Start R_f	Start height	Max R_f	Max height	Max (%)	End R_f	End height	Area	Area (%)
1	0.01	4.8	0.05	573.6	17.92	0.06	66.8	17402.0	13.4
2	0.06	467.3	0.07	496.1	15.49	0.11	0.4	11968.5	9.21
3	0.14	0.4	0.16	16.0	0.50	0.17	7.1	283.2	0.22
4	0.17	7.3	0.20	24.4	0.76	0.22	5.9	671.3	0.52
5	0.23	6.2	0.26	97.3	3.04	0.29	0.6	2532.7	1.95
6	0.31	1.0	0.34	22.6	0.71	0.35	16.3	519.4	0.40
7	0.35	16.3	0.39	53.3	1.67	0.40	52.7	1553.4	1.20
8	0.40	52.8	0.45	196.1	6.13	0.49	48.1	6974.4	5.37
9	0.51	53.7	0.55	183.6	5.74	0.57	35.2	5356.4	4.12
10	0.57	86.1	0.60	350.2	10.94	0.64	03.7	13127.2	10.11
11	0.64	103.9	0.68	371.3	11.60	0.71	03.3	14889.8	11.46
12	0.71	204.3	0.78	717.9	22.42	0.86	38.2	51896.6	39.96
13	0.86	98.3	0.87	99.1	3.10	0.94	0.10	2706.4	2.08

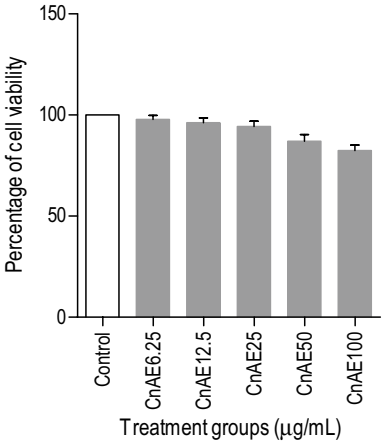


Fig. 2 CnAE showed > 82% cell viability in RAW264.7 cells. Cell viability assay of CnAE (6.25, 12.5, 25, 50 and 100 µg/mL) in RAW264.7 cells by MTT revealed > 82% viability even at 100 µg/mL after 24 h incubation at 37 °C. Values are mean ± SD ($n = 3$)

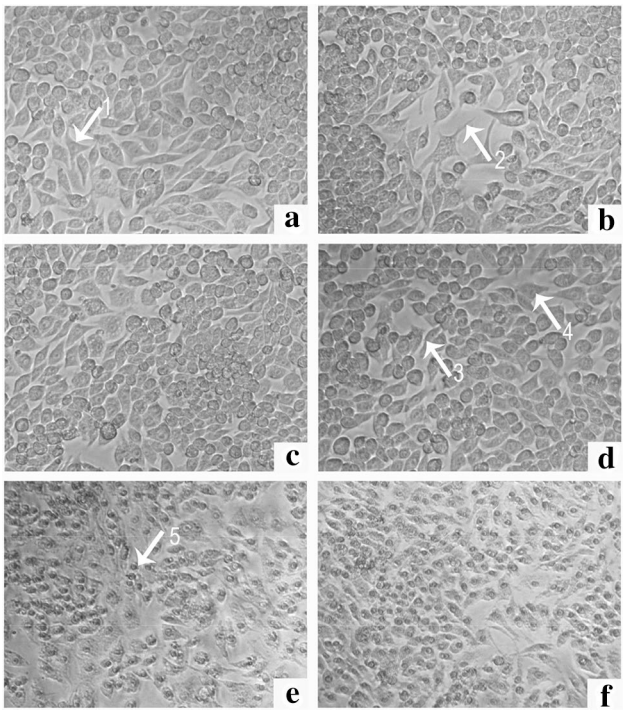


Fig. 3 Photomicrographs of MTT assay of CnAE treated RAW264.7 cells showed no significant changes in the morphology. Cells were observed under inverted phase contrast microscope after 24 h incubation at 37 °C (100× magnification). **a** Normal control group; **b** cells treated with 6.25 µg/mL of CnAE; **c** cells treated with 12.5 µg/mL of CnAE; **d** cells treated with 25 µg/mL of CnAE; **e** cells treated with 50 µg/mL of CnAE; **f** cells treated with 100 µg/mL of CnAE (Arrows 1 control cell; 2 echinoid spike; 3 cell shrinkage; 4 membrane blebbing and 5 condensed nuclei)

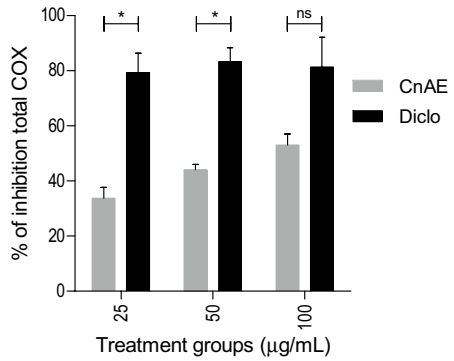
activity. The maximum inhibition (63.67%) in 5-LOX activity was observed at a concentration of 100 µg/mL (Fig. 5).

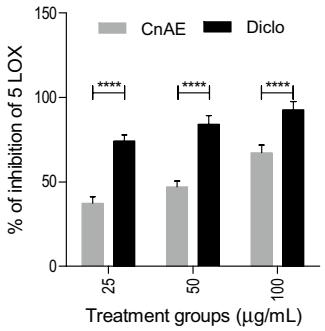
Effect of CnAE on MPO activity

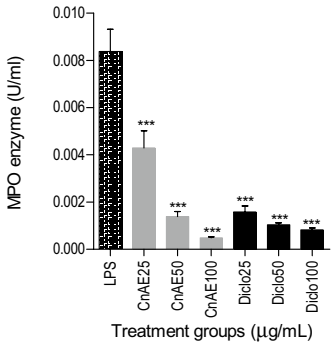
LPS treatment on RAW264.7 cells significantly increased the MPO activity, while cells treated with CnAE (25, 50 and 100 µg/mL) and diclofenac significantly inhibited the MPO production in a dose-dependent manner. Concentration of CnAE at 100 µg/mL showed activity better than that of diclofenac standard (Fig. 6).

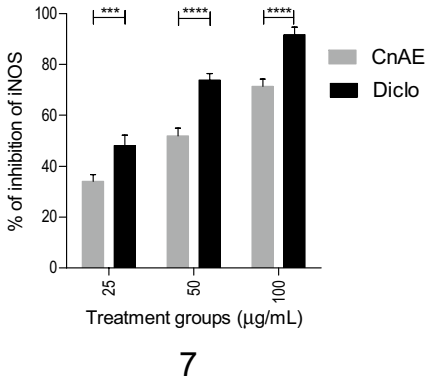
Effect of CnAE on iNOS activity

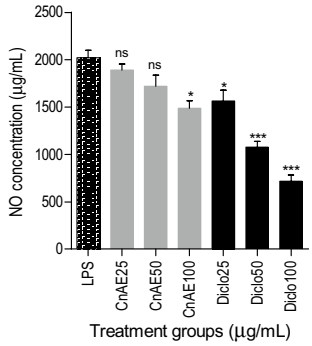
CnAE (25, 50 and 100 µg/mL) and diclofenac significantly inhibited the iNOS production in RAW264.7 cells when compared to the LPS control group. CnAE at a concentration of 100 µg/mL showed 71.35% inhibition in LPS-induced iNOS production (Fig. 7).











Figs. 4–8 CnAE showed modulatory effect on total COX, 5-LOX, MPO, iNOS and NO activities in LPS-induced RAW264.7 cells. Cells were pre-treated with LPS (1 µg/mL) for 1 h and then incubated with CnAE and diclofenac sodium at varying concentrations (25, 50 and 100 µg/mL) for 24 h at 37 °C. Values are mean ± SD ($n = 3$). Two-way ANOVA followed by Bonferroni multiple comparisons were used to analyse total COX, 5-LOX and iNOS, and one-way ANOVA followed by Tukey's multiple comparison tests were used to analyse MPO and NO. ns = $p > 0.05$, ****$p \leq 0.0001$; ***$p \leq 0.001$ and *$p \leq 0.05$. **4** The total COX level of cell lysate was significantly decreased by CnAE at 25 µg/mL ($p \leq 0.05$) and 50 µg/mL ($p \leq 0.05$) and is compared with respective concentration of drug standard diclofenac sodium. **5** The 5 LOX level of cell lysate was significantly decreased by CnAE at 25 µg/mL ($p \leq 0.0001$), 50 µg/mL ($p \leq 0.0001$) and 100 µg/mL ($p \leq 0.0001$) and is compared with respective concentration of diclofenac sodium. **6** There is a significant ($p \leq 0.001$) decrease in concentration of MPO in cell lysate by CnAE (25, 50 and 100 µg/mL) when compared with LPS control. **7** The iNOS level of cell lysate was significantly decreased by CnAE at 25 µg/mL ($p \leq 0.001$), 50 µg/mL ($p \leq 0.0001$) and 100 µg/mL ($p \leq 0.0001$) and is compared with respective concentration of diclofenac sodium. **8** There is a significant ($p \leq 0.05$) decrease in NO concentration by CnAE at 100 µg/mL when compared with LPS control

Effect of CnAE on nitrite level

Induction of RAW264.7 cells with LPS significantly increased the nitric oxide production, compared with that of control group. CnAE (25, 50 and 100 µg/mL) significantly decreased the nitrite accumulation in a dose-dependent manner (Fig. 8).

Effect of CnAE on ROS production

The effect of CnAE on intracellular ROS production was analysed by DCFDA method. ROS produced in RAW264.7 cell supernatant was imaged using fluorescent microscope (Fig. 9a, b) and is measured by flourimeter (Fig. 9c). The LPS stimulation increases the ROS level in RAW 264.7 macrophages and a significant reduction in ROS level was observed in CnAE (100 µg/mL) treated group.

ELISA of cytokines and PGE_2

LPS-induced RAW264.7 macrophages exhibited significant increase in IL-1β, IL-6 and TNF-α production when compared with the control group. CnAE at a dose of 100 µg/mL exhibited a significant inhibition in IL-1β, IL-6 and TNF-α production when compared with that of LPS control (Fig. 10a–c).

PGE_2 level in the culture medium of cell treated with LPS also showed an obvious increase than that of the control group. RAW264.7 cells pretreated with CnAE significantly decreased the level of PGE2 in a dose-dependent manner (Fig. 10d).

Effect of CnAE on cytokines and NF-κB gene expression

The relative gene expression levels of the pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α were analyzed (Fig. 11a–c). Expression of IL-1β, IL-6 and TNF-α cytokines were up-regulated (1.7, 2.07 and 2.43-fold increase, respectively) upon LPS-injection in contrast to control group. Pre-treatment with CnAE inhibited the elevation of all the tested pro-inflammatory cytokines in a dose-dependent manner. CnAE at a concentration of 100 µg/mL exhibited a significant inhibition. The mRNA expression of NF-κB were markedly increased upon LPS-challenge (2.77-fold), whereas pre-treatment with CnAE significantly inhibited the production in a dose-dependent manner (Fig. 11d). Drug standard diclofenac also significantly decreased the expression level of pro-inflammatory cytokines and NF-κB.

pIκB-α and pNF-κB p65 analysis

To investigate the effect of CnAE on the regulation of NF-κB pathway, the pIκB-α and pNF-κB p65 were examined using Western blotting method. As shown in Fig. 12a, b phosphorylation of IκB-α and NF-κB p65 were significantly inhibited by CnAE (100 µg/mL). LPS markedly induced phosphorylation of IkB-α and NF-κB p65 in RAW264.7 cells.

Eddy's hot plate test

CnAE produced a highly significant increase in the latency of response in mice to hot plate thermal stimulation when compared to the control group (Fig. 13a–f). Maximum protection was produced at 90 min by CnAE at a dose of 400 mg/kg (Fig. 13e). Sodium salicylate (100 mg/kg) showed significant increase in the latency of response at 120 min (Fig. 13f).

Acetic acid-induced writhing

Intraperitoneal injection of acetic acid produced 56.0 ± 3.0 writhes in the control group, 20 min after injection. Treatment of mice with CnAE (400 mg/kg) produced a significant ($p \leq 0.001$) inhibition (48.21%) in acetic acid induced abdominal writhes, when compared to the control group. Acetyl salicylic acid (100 mg/kg) produced 53.88% inhibition in writhes (Fig. 14).

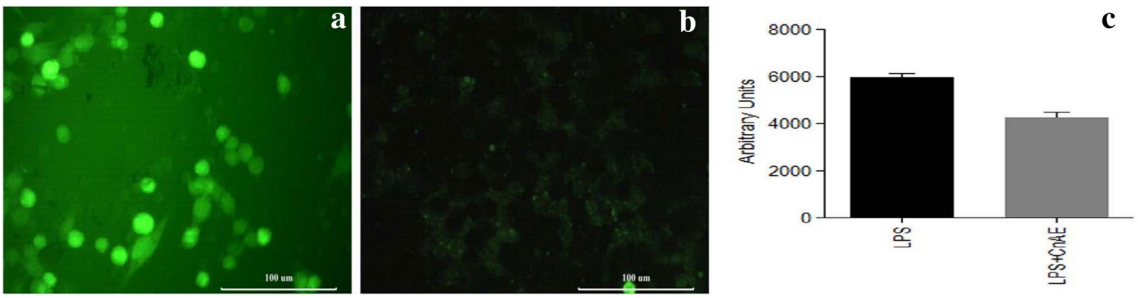


Fig. 9 CnAE inhibited ROS generation in LPS-induced RAW264.7 cells. ROS scavenging activity of CnAE (100 µg/mL) was analysed by DCFDA method. **a**. LPS, **b**. LPS + CnAE and **c**. Expression of fluorescence in arbitrary units. Fluorescence was measured using a fluorimeter, excitation at 470 nm and emission at 635 nm. Values are mean ± SD ($n = 3$)

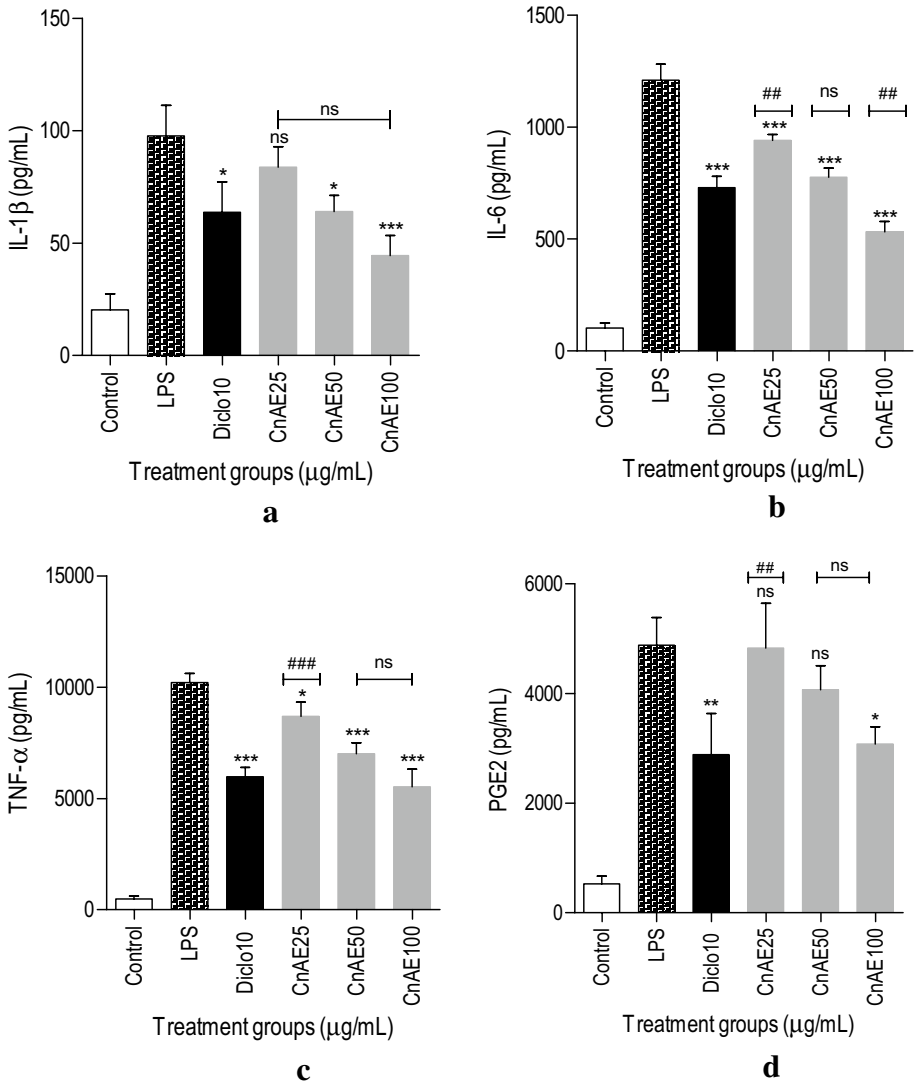


Fig. 10 **a**–**d** CnAE regulates production of IL-1β, IL-6, TNF-α and PGE_2 in LPS-induced RAW264.7 cells. Modulatory effect of CnAE on IL-1β, IL-6, TNF-α and PGE_2 production in LPS-induced RAW264.7 cells were determined by ELISA. Cells were pre-treated with LPS (1 µg/mL) for 1 h and then incubated with CnAE (25, 50 and 100 µg/mL) and drug standard diclofenac sodium (10 µg/mL) for 24 h at 37 °C. Values are mean ± SD ($n = 3$). One-way ANOVA followed by Tukey's multiple comparison tests were used to analyse the data. ns = $p > 0.05$, $p \leq 0.05$ (*, #), $p \leq 0.01$ (**, ##) and $p \leq 0.001$ (***, ###). **a** IL-1β production was significantly decreased by CnAE at 50 µg/mL ($p \leq 0.05$) and 100 µg/mL ($p \leq 0.001$) when compared with LPS control. **b** IL-6 production was significantly decreased by CnAE at 25 µg/mL ($p \leq 0.001$), 50 µg/mL ($p \leq 0.001$) and 100 µg/mL ($p \leq 0.001$) when compared with LPS control. **c** TNF-α production was significantly decreased by CnAE at 25 µg/mL ($p \leq 0.05$), 50 µg/mL ($p \leq 0.001$) and 100 µg/mL ($p \leq 0.001$) when compared with LPS control. **d** PGE_2 production was significantly decreased by CnAE at 100 µg/mL ($p \leq 0.05$) when compared with LPS control

Carrageenan-induced acute paw oedema

The concentration of 400 mg/kg of CnAE showed significant decrease in oedema volume (59.81%) 3 h after carrageenan injection in mice (Fig. 15). The results were compared with diclofenac (10 mg/kg), which showed 65.42% inhibition of oedema volume.

Formalin-induced chronic paw oedema

CnAE at doses of 200 and 400 mg/kg significantly inhibited the formalin-induced chronic paw oedema in mice, 50.32 and 52.90%, respectively after 6 days treatment. The group treated with diclofenac (10 mg/kg) showed 59.35% inhibition (Fig. 16).

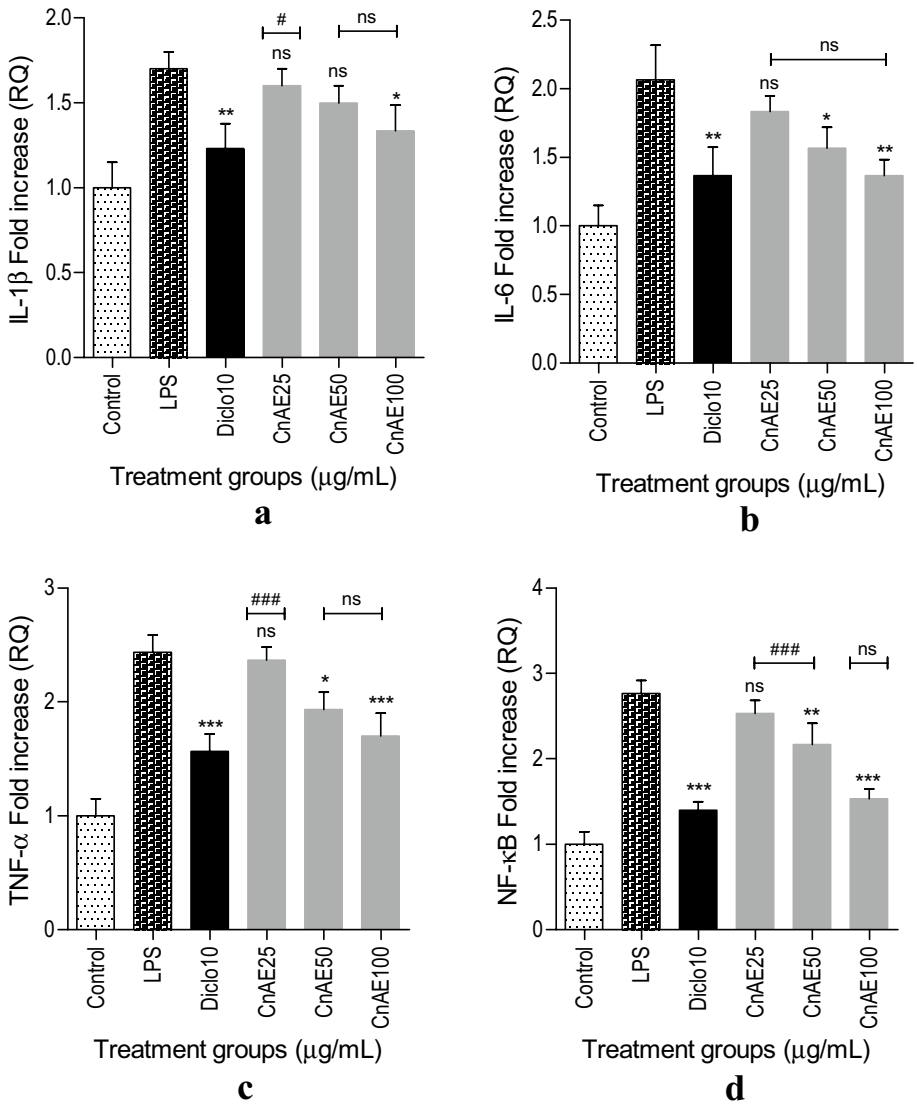


Fig. 11 **a**–**d** CnAE exerts modulatory effect on IL-1β, IL-6, TNF-α and NF-κB expression in LPS-induced RAW264.7 cells. Cells were pre-treated with LPS (1 µg/mL) for 1 h and then incubated with CnAE (25, 50 and 100 µg/mL) and diclofenac sodium (10 µg/mL) for 24 h at 37 °C. Total RNA was isolated and subjected to qRT-PCR for IL-1β, IL-6, TNF-α and NF-κB levels. Values are mean ± SD ($n = 3$). One-way ANOVA followed by Tukey's multiple comparison tests were used to analyse the data. ns = $p > 0.05$, $p \leq 0.05$ (*, #), $p \leq 0.01$ (**, ##) and $p \leq 0.001$ (***, ###). **a** IL-1β expression was significantly decreased by CnAE at 100 µg/mL ($p \leq 0.05$) when compared with LPS control. **b** IL-6 expression significantly decreased by CnAE at 50 µg/mL ($p \leq 0.05$) and 100 µg/mL ($p \leq 0.01$) when compared with LPS control. **c** TNF-α expression was significantly decreased by CnAE at 50 µg/mL ($p \leq 0.05$) and 100 µg/mL ($p \leq 0.001$) when compared with LPS control. **d** NF-κB expression was significantly decreased by CnAE at 50 µg/mL ($p \leq 0.01$) and 100 µg/mL ($p \leq 0.001$) when compared with LPS control

Discussion

Modern approaches to the treatment of inflammation and associated diseases rely on the inhibition of these pro-inflammatory mediator productions and of mechanisms that initiate the inflammatory responses (Lawrence et al. 2001). The currently available repertoire of approved anti-inflammatory agents mainly consists of steroidal and non-steroidal anti-inflammatory drugs. However several discussions have been raised about its side effects. Thus, the discovery of new anti-inflammatory compounds with improved therapeutic safety and efficacy is still a challenge to scientists in academia and industry. Phytotherapy generally assumes that a synergy of all ingredients (multi-component) of the plants will bring about the maximum of therapeutic efficacy (multi-targeting) with less toxic side effects (Ulrich-Merzenich et al. 2010; Pushpangadan et al. 2015). Polyphenols interact with the cells mainly through receptors or enzymes involved in signal transduction. Several research findings have shown that different polyphenols modulate the activity of arachidonic acid metabolizing enzymes such as cyclooxygenase (COX), lipoxygenase (LOX), and nitric oxide synthase (NOS) (Cheon et al. 2000; Hong et al. 2001). Ruiz and Haller (2006) found that polyphenols inhibit kinases by inhibiting their phosphorylation or ubiquitination and therefore prevent the subsequent degradation of IkB. This prevents nuclear translocation of NF-κB. Further, inhibition of the interaction of NF-κB subunits with target DNA has also been proposed as a mode of action of anti-inflammatory drug. Prevention of NF-κB nuclear translocation and interaction with DNA further prevent the production of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) and enzymes (iNOS, LOX and COX).

Cyclooxygenases (COXs) catalyzing arachidonic acid (AA) pathway leads to the generation of prostanoids such as prostacyclins (PGI2), prostaglandins (PGs), and

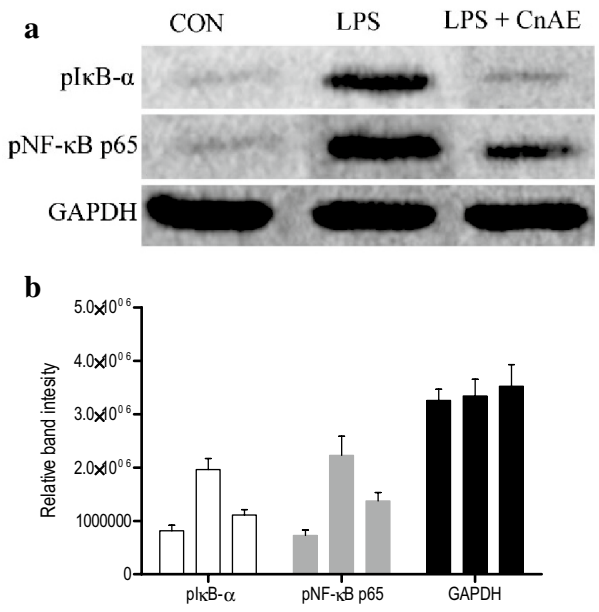


Fig. 12 **a** CnAE inhibited phosphorylation of IκB-α and NF-κB p65 in LPS-induced RAW264.7 cells. Cells were pre-treated with LPS (1 µg/mL) for 1 h and then incubated with CnAE (100 µg/mL) for 24 h at 37 °C. Cell lysates were prepared and subjected to western blotting using pIkB-α and pNF-κB p65 antibodies. Group CON (normal control), Group LPS (toxin control) and Group LPS + CnAE. GAPDH (internal control). **b** Relative band intensity of IκB-α, NF-κB p65 and GAPDH (internal control) in western blots. Bar 1 in each group represents CON, Bar 2 LPS and Bar 3 LPS + CnAE

thromboxanes (TXBs), which have been shown to be the most potent inflammatory mediators. COX-1 is expressed constitutively in most tissues and COX-2 is induced by stimuli such as bacterial LPS and cytokines (Chandrasekharan and Simmons 2004; Mendes et al. 2012). PGE_2 is a major product of COX activity and may contribute to the pathogenesis of inflammatory, autoimmune, and neoplastic diseases (Choi and Hwang 2004). 5-LOX catalyzing AA pathway results in the generation of leukotrienes (LTs) which are also actively involved in inflammatory diseases (Bishayee and Khuda-Bukhsh 2013). Therefore, the down-regulation of COXs and 5-LOX is very important for the treatment of inflammatory and associated diseases. In the present study, treatment with CnAE and diclofenac (25, 50, and 100 µg/mL) significantly reduced the total COX and 5-LOX activities in a dose-dependent manner. ELISA of PGE_2 showed, LPS-induction markedly increased the PGE_2 production, which was significantly suppressed by CnAE ($p \leq 0.05$). This result suggests that the antinociceptive activity of CnAE on mice may be partially mediated through down-regulation of the COX/PGE_2 signalling pathway.

The haem enzyme myeloperoxidase (MPO) abundantly expressed in polymorphonuclear neutrophils and macrophages, is released into extracellular fluid in the setting of inflammatory process (Lau and Baldus 2006). It uses superoxide and hydrogen peroxide to catalyse the generation of antibacterial hypochlorous acid and free radicals (Meotti et al. 2011). Our results showed that LPS exposure dramatically increased the MPO activity in RAW264.7 macrophages. However, this increase was apparently inhibited by CnAE in a dose-dependent manner. CnAE at a dose of 100 µg/mL (0.000482 U/mL) greatly inhibited the MPO activity than that of diclofenac sodium (100 µg/mL, 0.000822 U/mL).

The excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) may cause tissue injury that may lead to the inflammatory process (Willcox et al. 2004). Since activated macrophages release excessive amount of ROS during inflammation, DCFDA analysis of intracellular ROS level was carried out. LPS-stimulated RAW 264.7 cells demonstrated that pretreatment with CnAE (100 µg/mL) significantly attenuated the LPS-induced excessive ROS generation. LPS-induced macrophages also transcriptionally express the iNOS, which is responsible for the prolonged and profound production of NO. Therefore, inhibition of excess production of NO by controlling iNOS may exert anti-inflammatory effects. In the present study, induction of RAW264.7 macrophages with LPS enhanced the production of iNOS and thereby NO, where as in CnAE and diclofenac treated groups a significant reduction was observed in a dose-dependent manner.

Pro-inflammatory cytokines are critical in LPS-induced inflammation, it plays a major role in initiation and propagation of inflammatory pathogenesis (Omata et al. 2008; Rossol et al. 2011). ELISA revealed that CnAE significantly inhibited the production of IL-1β, IL-6 and TNF-α in LPS-induced RAW264.7 macrophages in a dose-dependent manner, which is further quantified by RT-PCR. Expression of IL-1β, IL-6 and TNF-α cytokines was up-regulated (1.7, 2.07 and 2.43-fold increase, respectively) upon LPS-induction in contrast to the control group. Treatment with CnAE prevented the elevation of all the tested proinflammatory cytokines in a dose-dependent manner. To further validate the above findings, qRT-PCR analysis of NF-κB was carried out. The mRNA expression level of NF-κB was significantly augmented (2.77-fold increase) upon LPS exposure, which was in concert with higher level of cytokines (IL-1β, IL-6 and TNF-α) measured in the respective treatment groups, whereas CnAE treatment inhibited the production of NF-κB dose dependently.

Lipopolysaccharide stimulate the canonical pathway by activating the inhibitor κB (IκB) kinase (IKK) and phosphorylates IκB-α. Phosphorylated IκB-α is subsequently ubiquitinated and degraded by the 26S proteasome, which results in NF-κB nuclear translocation from the cytoplasmic NF-κB-IκBα complex. NF- κB further induces the expression of inflammatory genes, such as IL-1β, IL-6, TNF-α,

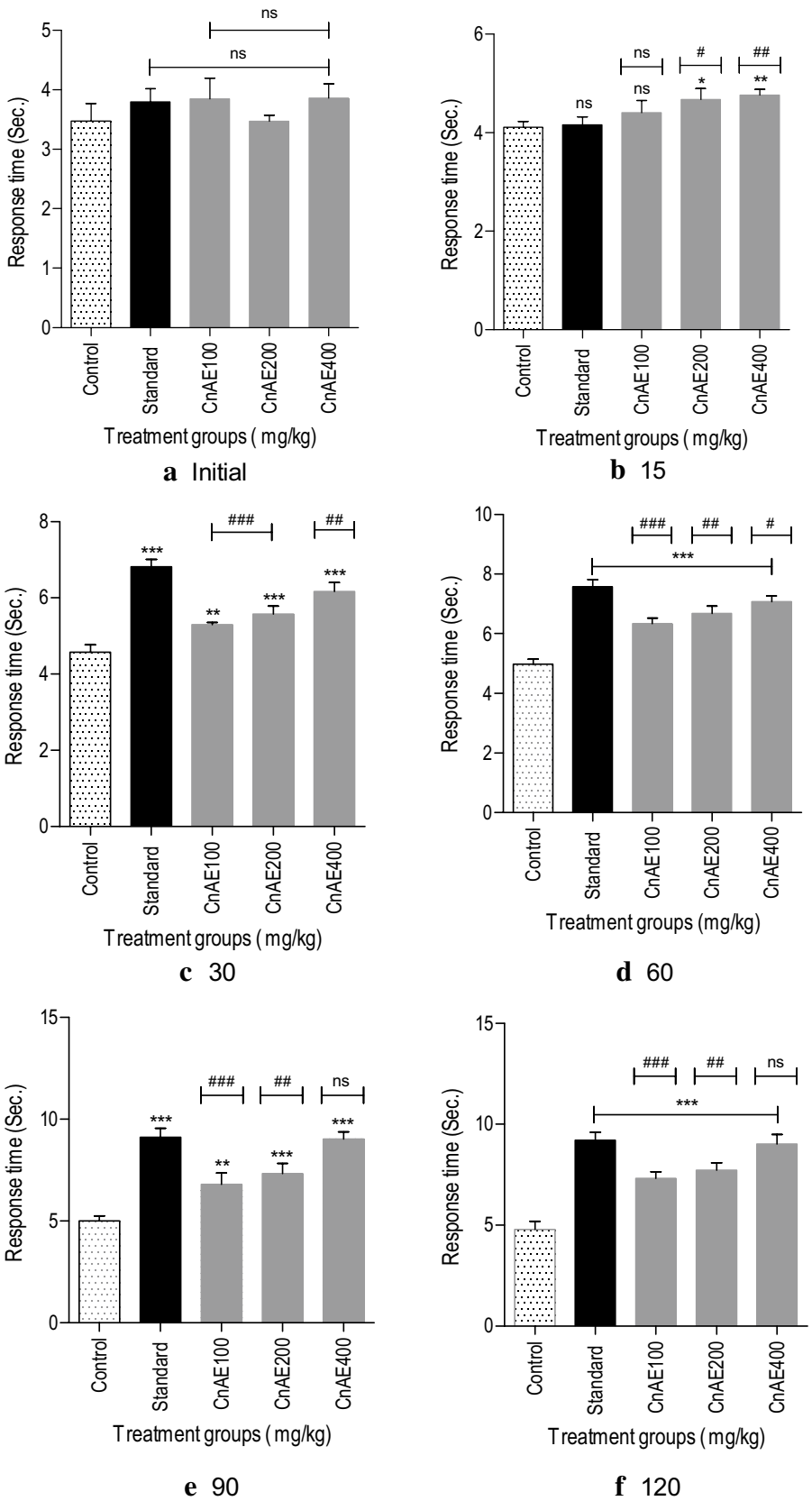


Fig. 13 **a**–**f** CnAE ameliorates hot plate induced nociception in Swiss albino mice. CnAE 100, 200 and 400 mg/kg concentrations were used for the study. Sodium salicylate (100 mg/kg) was used as drug standard. **a** initial; **b** 15 min; **c** 30 min; **d** 60 min; **e** 90 min and **f** 120 min. Values are mean ± SD ($n = 6$). One-way ANOVA followed by Dunnett's multiple comparison test were used to analyse the data. ns = $p > 0.05$, $p \leq 0.05$ (*, #), $p \leq 0.01$ (**, ##) and $p \leq 0.001$ (***, ###). In 90th minute mice treated with CnAE (400 mg/kg) showed significant ($p \leq 0.001$) increase in the response time when compared with positive control

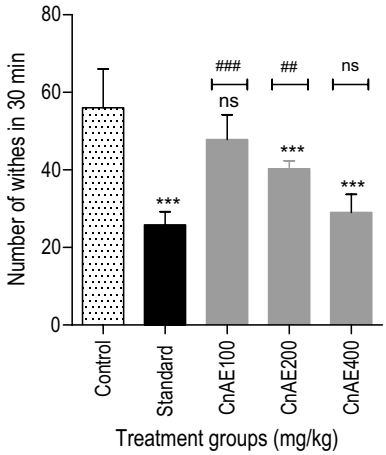


Fig. 14 CnAE ameliorates nociception in acetic acid induced Swiss albino mice. Acetic acid induced writhing in mice model was used for the study. CnAE (100, 200 and 400 mg/kg) and acetyl salicylic acid (drug standard, 100 mg/kg) was used for the assay. Values are mean ± SD ($n = 6$). One-way ANOVA followed by Dunnett's multiple comparison test were used to analyse the data. ns = $p > 0.05$, $p \leq 0.01$ (##) and $p \leq 0.001$ (***, ###). CnAE at concentrations of 200 and 400 mg/kg showed significant ($p \leq 0.001$) decrease in number of writhes when compared with positive control

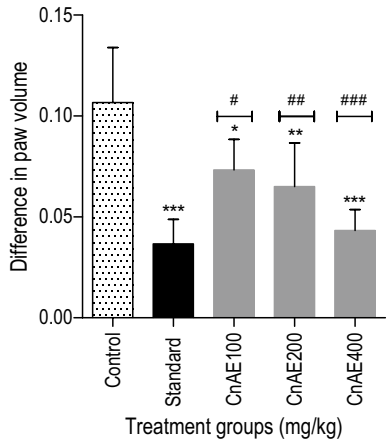


Fig. 15 CnAE attenuates acute inflammation in Swiss albino mice. CnAE (100, 200 and 400 mg/kg) showed anti-inflammatory effect against carrageenan-induced acute paw oedema in mice model. Diclofenac sodium (10 mg/kg) was used as drug standard. Values are mean ± SD ($n = 6$). One-way ANOVA followed by Dunnett's multiple comparison test were used to analyse the data. $p \leq 0.05$ (*, #), $p \leq 0.01$ (**, ##) and $p \leq 0.001$ (***, ###). CnAE at concentrations of 100, 200 and 400 mg/kg showed significant ($p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively) decrease in paw volume when compared with positive control

iNOS and COX-2 (Tian et al. 2005). Our findings indicated that CnAE (100 µg/mL) has the potential to inhibit the LPS-induced phosphorylation of IκBα as well as NF-κB p65 nuclear translocation. These results from western blotting clearly demonstrated that CnAE significantly suppress

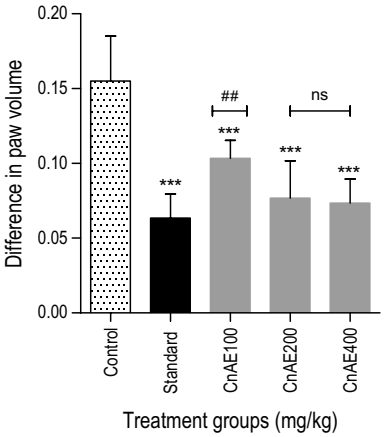


Fig. 16 CnAE attenuates chronic inflammation in Swiss albino mice. CnAE (100, 200 and 400 mg/kg) showed anti-inflammatory effect against formalin-induced chronic paw oedema in mice model. Diclofenac sodium (10 mg/kg) was used as drug standard. Values are mean ± SD ($n = 6$). One-way ANOVA followed by Dunnett's multiple comparison test were used to analyse the data. ns = $p > 0.05$, $p \leq 0.01$ (##) and $p \leq 0.001$ (***). CnAE at concentrations of 100, 200 and 400 mg/kg showed significant ($p \leq 0.001$) decrease in paw volume when compared with positive control

LPS-induced NF-κB stimulation. From quantitative qRT-PCR analysis our data indicated that CnAE significantly suppressed their expression of pro-inflammatory mediators and cytokines in LPS-induced RAW264.7 macrophages. ELISA results also confirmed the inhibitory effect of CnAE on the production of PGE_2 and cytokines from the LPS treated RAW 264.7 cells. Based on the findings, it is inferred that CnAE exhibits anti-inflammatory activity through the suppression of NF-κB/IκBα signalling, probably resulting in the reduced productions of pro-inflammatory cytokines and mediators.

To extend these results in in vivo we investigated therapeutic efficacy and safety profile of CnAE in different in vivo antinociceptive and anti-inflammatory models. The antinociceptive activity of CnAE was assessed using in vivo models like acetic acid induced writhing and Eddy's hot plate test in Swiss albino mice. The pain is generated by producing localized inflammatory response due to release of free arachidonic acid from membrane phospholipids, specifically via COX-2 mediated production of PGE_2. The level of 5-LOX products may also increase in peritoneal fluids (Khan et al. 2010). The acetic acid writhing test is widely used to study the peripheral antinociceptive activity of drugs in vivo (Collier et al. 1968). The hot plate test postulates the assessment of centrally mediated analgesic effect (response latencies). The hot plate test is usually used in conjunction with the writhing test to distinguish central nervous system effects from peripheral ones (Srinivasan et al. 2003). It was observed that CnAE showed potent inhibitory effect against these two models tested, in a dose-dependent manner. The

percentage of inhibition at highest dosage tested (400 mg/kg) is 48.21% in acetic acid induced writhing. In Eddy's hot plate method, maximum latency was observed at 90th minute at a dose of 400 mg/kg. These findings suggest that CnAE have both peripheral and centrally acting analgesic effect.

The CnAE extract was then screened for its anti-inflammatory activity using animal models like carrageenan-induced acute paw oedema and formalin-induced chronic paw oedema. The carrageenan test is highly sensitive to NSAIDs, and has long been accepted as a useful phlogistic tool for investigating new drug therapies (Spector and Willoughby 1963; Whiteley and Dalrymple 1998). It is a biphasic event, first phase occurs within 1 h of injection due to the trauma of injection and the release of histamine and serotonin, and the second phase of oedema is due to the release of prostaglandins (Vinegar et al. 1969; Crunkhorn and Meacock 1971). Formalin-induced chronic inflammation in hind-paw of mice have generally been used as a classic method to detect the efficacy of antiinflammatory drugs, because marked chronic inflammation is evoked by aponeurotic formalin injection (Akindele and Adeyemi 2007; Kim et al. 2010). In both acute and chronic models, CnAE showed good inhibition in a dose-dependent manner. Thus from the results of the present investigation, it may be inferred that the inhibitory effect of CnAE in all the animal models may be due to the inhibition of NF-κB, mainly responsible for the production of proinflammatory mediators and cytokines. Thus, it is assumed that the polyphenols in CnAE may act either individually or may synergistically act against LPS-induced oxidative stress and inflammation.

Conclusion

Cocos nucifera inflorescence extract, a constituent in traditional medicines such as Ayurveda showed marked antiinflammatory and antinociceptive in both in vitro and in vivo models. CnAE acts via inhibiting the activation of NF-κB/IκB signalling pathway and attenuation of oxidative/nitrosative stress. Thus the study provides experimental and molecular mechanistic evidence that clearly justifies the traditional use of *Cocos nucifera* inflorescence. Further investigations are warranted with pure isolated components.

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Author contributions MAC, TPI, HK, RKS, VG, PP designed study; MAC, TPI performed research; TPI, MAC, HK, RKS, VG analyzed data; TPI, MAC, VG wrote the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the Institution. Experiments were conducted after getting the approval of the Institute's Animal Ethics Committee (IAEC; Reg. No. 149/199/CPCSEA), Amala Cancer Research Centre, Thrissur, Kerala, India.

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