

Full Length Research Paper

***Cinnamomum zeylanicum* extract inhibits proinflammatory cytokine TNF α : *in vitro* and *in vivo* studies**

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***Cinnamomum zeylanicum*, a commonly used spice and well-known medicinal plant has been reported to have anti-diabetic, anti-oxidant and anti-microbial properties. We have investigated its anti-inflammatory activity using ethanol extract obtained from bark. *In vitro* and *in vivo* experiments were performed targeting TNF- α using flow cytometry. Ethanol extract of *C. zeylanicum* showed suppression of intracellular release of TNF- α in murine neutrophils as well as leukocytes in pleural fluid. The extract was found to inhibit TNF- α gene expression in LPS-stimulated human PBMCs at 20 μ g/ml concentration. A potent anti-inflammatory activity of cinnamon extract is suggestive of its anti-arthritic activity, which could be confirmed in various models of arthritis.**

Key words: *Cinnamomum zeylanicum*, TNF- α , flow cytometry, anti-inflammatory, gene expression.

INTRODUCTION

Inflammation is a process involving multiple factors acting in a complex network. The ingress of leukocytes into the site of inflammation is crucial for the pathogenesis of inflammatory conditions. Neutrophils and macrophages are known to recruit and play pivotal roles in acute and chronic inflammation, respectively (Wajant et al., 2003). Recruited cells are activated to release many inflammatory responses, causing a change from the acute phase of inflammation. Therefore, inhibition of the cellular reactions is one of the targets that are generally used as an *in vitro* model for anti-inflammatory testing.

TNF- α is a pro-inflammatory cytokine, mainly produced by activated monocytes and macrophages. Excessive production of TNF- α is believed to underlie the progression of many serious inflammatory diseases, such as rheumatoid arthritis (RA), Crohn's disease and psoriasis.

Therefore, anti-TNF- α therapy would be a possible tool for treatment of acute and chronic inflammatory diseases (Tracey and Cerami, 1993; Graninger and Smolen, 2002). Botanical substances having TNF- α inhibitory properties could be one of the therapeutic approaches for inflammatory disorders.

Cinnamomum zeylanicum (Lauraceae), which originates from the island of Sri-Lanka (formerly called Ceylon) southeast of India, has been used for its anti-diabetic, anti-nociceptive, astringent and diuretic activities. It has been demonstrated that bark and leaves of *C. zeylanicum* contain antifungal substances (Mishra et al., 2009). Its aqueous extract possessed antioxidant properties and it could be a potential therapeutic approach for the pathologies associated with the damage due to free radicals (Hasani-Ranjbar et al., 2009). It was found to have inhibitory effects on osteoclastogenesis through the suppression of nuclear factor of activated T cells, cytoplasmic1 (NFATc1) mediated signal transduction (Tsuji-Naito, 2008). In combination with other botanical

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products, *C. zeylanicum* showed potential clinical utility in patients with allergic rhinitis (Corren et al., 2008). It exhibited insulin-mimetic action in 3T3-L1 adipocytes while inhibiting the secretion of adiponectin (Roffey et al., 2006) and decreased blood glucose levels in glucose tolerance test (GTT) (Verspohl et al., 2006). It is also reported to have a role in wound healing (Kamath et al., 2003). Additionally, it has insecticidal activity (Abdel, 2006). Present investigation was carried out to evaluate the anti-inflammatory potential of hydro alcoholic bark extract of *C. zeylanicum* (OA4-50) and its effect on Tumor necrosis factor- α (TNF- α) secretion and gene expression. In the preliminary experiments all the extracts; aqueous, 50% ethanolic, 80% ethanolic and hexanoic extracts were evaluated for TNF- α inhibitory properties. As 50% ethanolic extract showed inhibitory effect on TNF- α release from human peripheral blood mono-nuclear cells (PBMCs), it was further evaluated in acute and chronic models.

MATERIALS AND METHODS

Chemicals

Histopaque-1077 density gradient solution, sterile Phosphate Buffered Saline (PBS), Trypan blue (0.4%), RPMI-1640 medium, HEPES, Glutamine, Fetal Bovine Serum (FBS), Penicillin, Streptomycin, Dimethylsulphoxide (DMSO), Ethylene Diamine Tetra Acetic acid (EDTA), Rolipram, Lipopolysaccharide (LPS), γ -cargenan typeIV, Toluene, Ethyl acetate, Ethanol and Cinnamaldehyde were procured from Sigma-Aldrich (St. Louis, MO, USA), TNF- α enzyme-linked immunosorbent assay kit was purchased from R and D systems (Minneapolis, MN, USA), cDNA synthesis kit from Invitrogen (California, USA), SYBR Green RT-PCR reaction kit from Applied Biosystems (California USA), Phycoerythrin labeled mouse TNF- α monoclonal antibody, FACS lysing solution, permeabilizing solution and Golgi plug were obtained from BD Biosciences (California, USA). All the biological studies were done at Indian Institute of Integrative Medicine, Jammu.

Plant material and extract preparation

C. zeylanicum bark was collected from Green pharmacy (Pune), authenticated and a voucher specimen was submitted at the Botanical Survey of India, Western Circle, Pune (No. SPCIV7). Plant material was dried under shadow and extraction was carried out by cold maceration. Dried coarse powder was macerated overnight with 50% ethanol in orbital incubator shaker (Orbitek, Scigenics) at 20°C at 60 rpm. The extract was then filtered and centrifuged at 800 g for 10 min. Solution was allowed to dry by lyophilisation. The percentage yield was calculated on the basis of the dried plant material weight for testing. The extract was dissolved in vehicle and diluted to the desired concentration.

HPTLC analysis

High performance thin layer chromatography (HPTLC) characterization of the cinnamon bark extract was done. Presence of volatile oil in the extract was confirmed during preliminary phytochemical screening of the extract. The hydro-alcoholic extract was characterized by HPTLC (F₂₅₄-Merk-silica gel plates) using reported TLC method for volatile oil (Colditz, 1985). Chromatographic separation

was carried out using solvent system of toluene: ethyl acetate (9:1.5) and plates were viewed at 225 and 365 nm (Camag Multi wavelength scanner III, version IV). The spots were developed with vanillin - sulphuric acid and scanner at visible range (wavelength of 300 - 800).

Stock solution was prepared by dissolving the extract in 50% ethanol (26.27 ng/ μ l). Standard was prepared by dissolving the extract in 50 μ l, then dissolve in 10 ml of ethanol. Injection volume was 5 μ l (500 ng/spot).

Biological activity testing

Animals

Swiss albino mice and Wistar rat were obtained from the Indian institute of Integrated medicine, Jammu. They were kept in standard environmental conditions and maintained on a standard rodent diet with water given *ad libitum*.

Isolation of human peripheral blood mononuclear cells

After obtaining written informed consent, venous blood sample was obtained from healthy adult donors. Human PBMCs were isolated from heparinized blood samples using histopaque density gradient, washed and were suspended in complete RPMI-1640 medium supplemented with 10 mM HEPES, 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml of penicillin, 100 μ g/ml of streptomycin.

TNF- α inhibition in human PBMCs

Cell treatment

The stock solution of extracts (aqueous, 50% ethanolic, 80% ethanolic and hexanolic) and pure compound were prepared in DMSO and then diluted to the desired concentration in which the final maximum concentration of DMSO in the media was not more than 0.1% DMSO. Cells were seeded at a density of 10⁶ cells/ml in 96-well microtiter plate. Cells were pretreated with different extracts at 20 μ g/ml and then stimulated with LPS (1 μ g/ml). Positive controls were treated only with LPS where as in negative control wells medium containing 0.1% DMSO was added. Cells were maintained at 37°C in a humidified incubator under an atmosphere supplemented with 5% CO₂.

Quantitation of cytokines

Supernatants were collected for cytokine analysis. Cytokine levels were quantitated using enzyme-linked immunosorbent assay (ELISA) kit from R and D systems, according to the manufacturer's instructions. Supernatant (100 μ l) was added to antibody-coated polystyrene wells and incubated for 2 h. After washing, the plates were incubated with biotin-labeled anti-cytokine antibody for 2 h. The plates were washed and incubated for 20 min with a streptavidin/horseradish peroxidase conjugate.

The plates were washed and incubated with trimethylbenzidine (TMB) and peroxide, to detect the horse radish peroxidase. The reaction was stopped by the addition of 2N sulphuric acid and the absorbance read at 540 and 450 nm on a titertek Multiskan MCC/340 microplate reader (Palladino et al., 2003).

Gene expression studies

RNA isolation

Human PBMCs were washed in 6-well plates twice with 1 ml of

sterile-ice cold PBS and lysed directly with 1 ml of monophasic lysis reagent. RNA concentration and integrity were determined using Biophotometer and formaldehyde gel (Wagner, 1988).

cDNA synthesis

The mixture (20 μ l) contained 1 μ g total RNA, 1 μ l oligo(dt) primer, 2 μ l dNTP mix, 0.1 M DTT, 1 μ l RNase out, 1 μ l thermoscript RT (invitrogen) in 5X cDNA synthesis buffer. The synthesis reactions were preceded at 50°C for 60 s and 85°C for 20 min.

PCR primers

The primers were designed using complete cDNA sequence of human TNF- α gene available at NCBI (accession number NM_00594). The sequence (5' to 3') of the forward primer and reverse primer for amplification of Human TNF- α gene are A G C CCATGTTGTAGCAAACC and TGAGGTACAGGCCCTCTGAT respectively.

Real time polymerase chain reaction

Reaction mixture (50 μ l) contained 10 μ l of RNA-derived cDNA, 1 μ l each of forward primer and reverse primer with 2X of 25 μ l SYBR Green PCR master mix (ABI kit). The reactions were performed in 96-well plates in Opticon 2 real time PCR instrument. The thermal cycle conditions were as follows: 30 s each at 94, 50, 72°C for denaturation, annealing and polymerization reaction for 40 cycles, respectively.

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The C_T method of relative quantification was used to determine the fold change in expression (Wagner, 1988).

Cell viability assay

Viability of cells were determined by MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] colorimetric assay. After removing the supernatant, treated and control cells were incubated with MTT (5 mg/ml) for 4 h at 37°C and solubilized in DMSO. The amount of formazan production was determined spectrophotometrically at 545 nm.

Flow cytometric study (Prabhakar, 2002; Yin et al., 2001)

Sample preparation

OA4-50 was prepared as homogenous suspension in 0.1% gum acacia. Rolipram was used as a reference compound for comparison and validation of test model applied.

In vitro experiment: TNF- α estimation in neutrophils

Neutrophils were separated from whole blood taken from retro-orbital plexus of normal Swiss albino mice; added 2.5 μ l of protein transport inhibitors, centrifuged and histopaque was added, incubated for 10 min and then centrifuged, added fluorescence-activated cell sorting (FACS) lysing solution. After subsequent washing with PBS, acquisition was done on the flow cytometry.

LPS was added to the isolated murine neutrophils. Drug sample at different doses was added and incubated for 3 h at 37°C with 5%

CO₂ concentration. After incubation permeabilizing solution (10X) was added. Sample was thoroughly washed with PBS and spun at 900 rpm. Anti-TNF- α monoclonal antibody was added and analyzed by flow cytometer (BD LSR).

In vivo experiment: TNF- α estimation in pleural leukocytes

In vivo studies were carried out using Pleurisy model for assessment of effect of OA4-50 extract on inflammatory parameter TNF- α . Wistar rats having a weight range of 120 - 180 g were employed for the study. Drug was administered orally using metal drug feeding cannula at the concentration of 5, 10, 20 mg/kg, 1 h before injection of 0.5 ml carrageenan into the pleural cavity of rat.

After 4 h of carrageenan challenge pleural fluid (50 μ l) was taken in heparinized tubes and Golgi stop was added. Then it was treated with FACS lysing solution for the elimination of red blood cells if any. After 3 washes with PBS, 500 μ l of permeabilizing solution was added and incubation was carried out for 15 min. Then samples were incubated for 30 min after addition of anti-TNF- α monoclonal antibody and were analyzed on flow cytometer (BD-LSR) using Cellquest Pro software.

Data analysis

Data were analyzed using Excel and GraphPad Prism 5. Statistical analysis was done using One-way ANOVA complemented with Dunnett test. $P < 0.05$ was considered as indicative of significance. Percentage inhibition of TNF- α release in human PBMCs was calculated using the formula:

$$\% \text{ Inhibition} = 100 - \left[\frac{\text{TNF-}\alpha \text{ levels in test} - \text{TNF-}\alpha \text{ levels in negative control}}{\text{TNF-}\alpha \text{ levels in positive control} - \text{TNF-}\alpha \text{ levels in negative control}} \right] \times 100$$

Percentage inhibition of TNF- α expression in murine neutrophils and pleural leukocytes was calculated using the formula:

$$\% \text{ Inhibition} = 100 - (\text{Test/Control}) \times 100$$

RESULTS

Preparation of extract

Cold maceration procedure was followed to obtain hydro-alcoholic extract. Percentage yield was 80%. Extracts were light brown in colour, sparingly soluble in water and highly hygroscopic in nature.

Phytochemical analysis

Figure 1 depicts the HPTLC analysis of the HPTLC profile shows the presence of 4 peaks at RF 0.1, 0.4, 0.6 and 0.9. Three minor peaks with peak height 61.1, 13.7 and 10.4 with peak area 658.8, 158.9 and 169.7 were observed. Major component (Cinnamaldehyde) was observed at RF 0.6 with peak height and area 108.2 and 5214 respectively, which was confirmed by comparing with the HPTLC profile of standard Cinnamaldehyde. From calibration curve it was observed that concentration of major component (cinnamaldehyde) in extract was 2.3%.

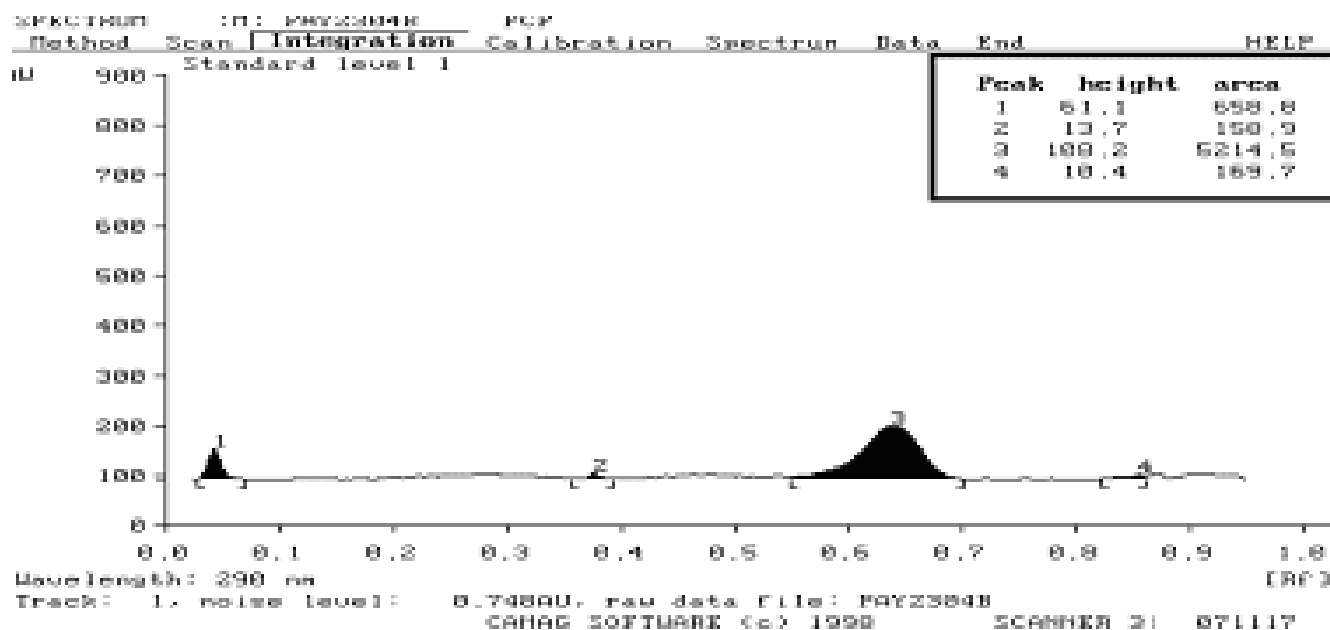


Figure 1. HPTLC analyses of hydro-alcoholic cinnamon bark extract OA4-50.

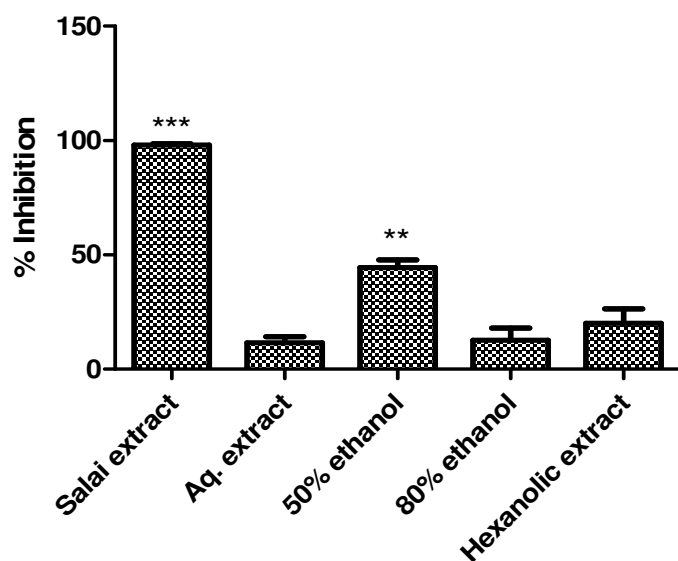


Figure 2. The effect of *C. zeylanicum* extracts on the release of TNF- α in hPBMCs.

Estimation of TNF α secretion form hPBMCs by ELISA

The extracts, including aqueous, 50% ethanolic, 80% ethanolic and hexanolic, were tested for inhibition of TNF- α secretion in human PBMCs (hPBMCs). Salai Guggul(Khan et al., 2006) was used as reference compound.

Fifty percentage ethanolic extract (OA4-50) was found

to inhibit significantly the TNF- release form human PBMCs with a maximum inhibition of 44.38% at a concentration of 20 $\mu\text{g/ml}$ (Figure 2). Salai extract, the reference compound gave a 98% inhibition in the same experimental setup.

Cell viability

Effects of OA4-50 on viability of human PBMCs and neutrophils were evaluated using MTT. The survival of cells was not significantly affected by treatment for 24, 48 or 72 h with hydro-alcoholic extracts at concentration ranging from 1 - 20 $\mu\text{g/ml}$. However, higher doses of extract (25 -100 $\mu\text{g/ml}$) decreased cell survival by over 80%. Thus, non-cytotoxic concentration of extract was used in subsequent studies.

RNA isolation from hPBMCs

RNA was isolated from human PBMCs (10^6 cells /ml), treated with LPS (1 $\mu\text{g}/\mu\text{l}$) and OA4-50 using the method described. Yield of RNA was 2 - 4 $\mu\text{g}/10^6$ hPBMCs. Integrity of RNA samples was checked on formaldehyde gel. Presence of 28s and 18s bands indicated intactness of RNA is intact. This RNA was used for cDNA synthesis.

Standardization of PCR

cDNA was amplified with TNF- α and β -actin specific primers. Reaction conditions were standardized using

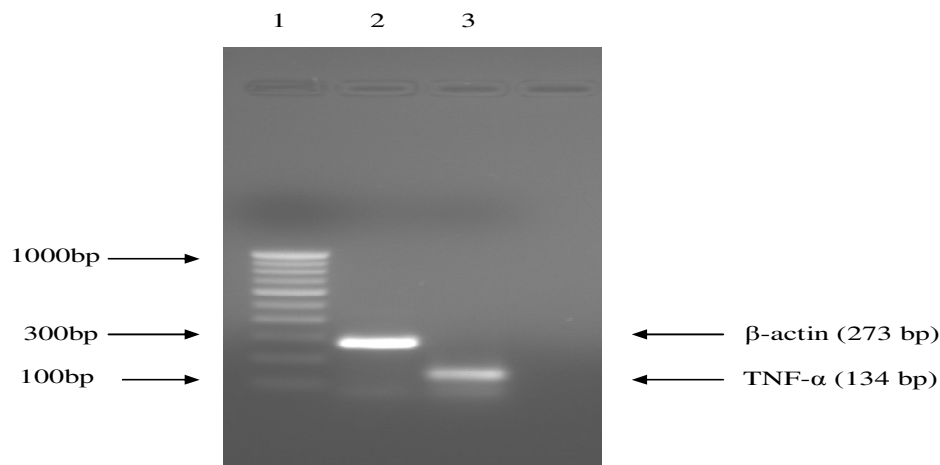


Figure 3. PCR standardization using β -actin and TNF- α primers on 2% agarose gel. Lane 1: Molecular weight marker (100 bp ladder); Lane 2: β -actin; Lane 3: TNF- α .

Table 1. Effect of cinnamon bark extract (OA4-50) on TNF- α expression in murine neutrophils.

Treatment group	Conc. (μ g/ml)	Values of gated cell			Mean \pm SE	% Inhibition
		n1	n2	N3		
OA4-50	1	2.26	2.01	2.04	2.10 \pm 0.07	19.54
	2.5	1.84	2.14	2.42	2.13 \pm 0.16	18.39
	5	1.88	2.44	1.89	2.07 \pm 0.18	20.68
	10	1.68	1.93	1.67	1.76 \pm 0.08	32.56
	20	1.3	1.38	1.48	1.38 \pm 0.05	47.12
Control		2.59	2.58	2.68	2.61 \pm 0.03	

various concentrations of primers, cDNA concentration and taq polymerase. These conditions were used further for real time PCR. Figure 3 depicts the amplification of cDNA with TNF- α primer (Lane 2) and β -actin primer (Lane 3). Desired product of sizes 134 and 273 bp were obtained without any non-specific amplification. 100 bp ladders was used as molecular weight marker (Lane 1).

Real time PCR

Real time PCR was carried out on Opticon 2 RT PCR machine using SYBR Green. Melting curve analysis was carried out at 60 to 95°C at 1°C time intervals. SYBR Green is very non specific dye and can bind to any contaminant of the reaction and gives rise to non-specific products and multiple peaks appear on melting curve. Single peak indicated pure PCR product obtained after amplification on real time PCR and absence of non-specific products. Thermal cycling conditions optimized for PCR were met for real-time PCR. Lower the Ct value—more transcript abundance and vice versa. OA4-50 was

found to inhibit TNF- α gene expression by three fold as compared to LPS+ control.

Flow cytometric study

Intracellular TNF- α level in murine Neutrophils were estimated by Flow cytometer employing anti-TNF- α monoclonal antibody; data is summarized in Table 1. The test substance (OA4) was used at different concentrations ranging from 1 to 20 μ g/ml. OA4 showed significant inhibition of intracellular expression with the maximum inhibition of 47.12% at 20 μ g/ml concentration (Figure 4). Effect of cinnamon extract (50%) on TNF- α production in carrageenan induced inflammation Model (Table 2).

Carrageenan-induced pleurisy in wistar rat was used to evaluate the effect of test compound on TNF- α expression in pleural leukocytes (Figure 5). Animals were treated with different doses of 50% ethanolic extract (5, 10 and 20 mg/kg) administered by intraperitoneal route. Carrageenan treated animals were taken as vehicle control. The test compound showed suppression of TNF- α expression at 10 and 20 mg/kg (20.94 and 44.60%).

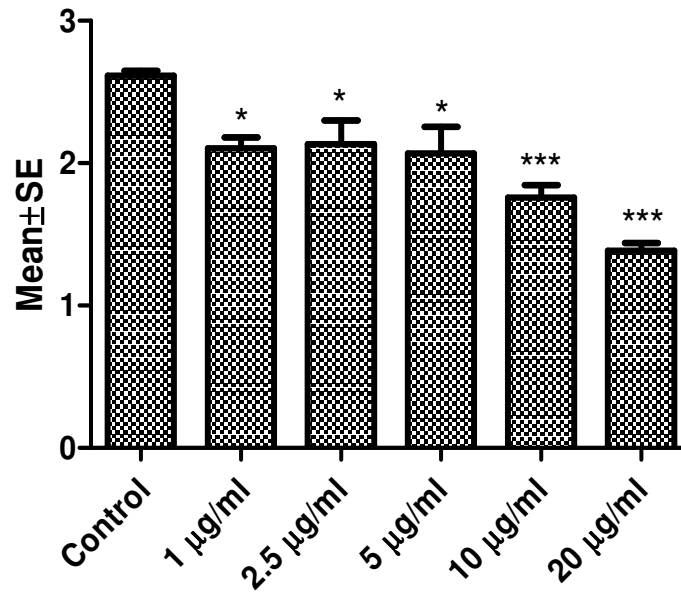


Figure 4. Effect of cinnamon bark extract (OA4-50) on TNF- α expression in murine neutrophils.

Table 2. Effect of cinnamon bark extract (OA4-50) on TNF- α expression in leukocytes from rat model of pleurisy.

Treatment group	Dose (mpk)	Values of gated cell			Mean \pm SE	% Inhibition
		n1	n2	n3		
OA4-50	5	21.85	21.7	22.27	21.94 \pm 0.17	9.71
	10	19.55	16.98	21.1	19.21 \pm 1.20	20.94
	20	13.53	13.32	13.53	13.46 \pm 0.07	44.6
Vehicle control		24.23	24.09	24.58	24.3 \pm 0.14	

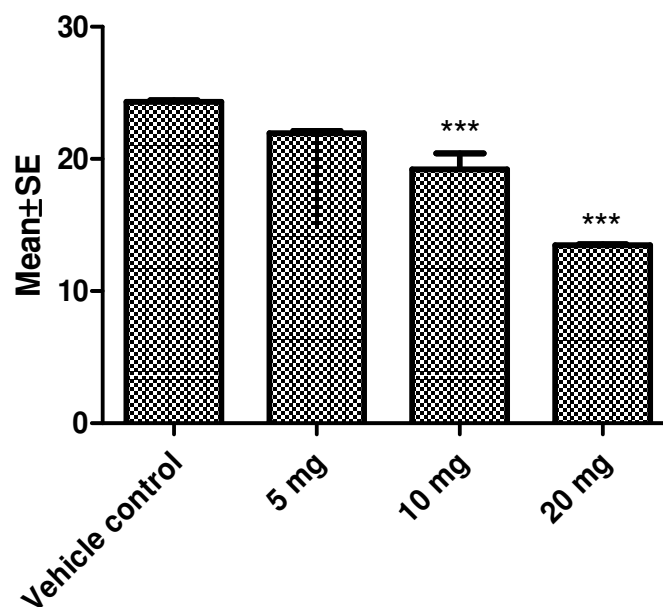


Figure 5. Effect of cinnamon bark extract (OA4-50) on TNF- α expression in leukocytes from rat model of pleurisy.

These results indicated that cinnamon bark is a potent anti-inflammatory drug.

DISCUSSION AND CONCLUSION

C. zeylanicum bark extract was found to have anti-inflammatory activity. *Cinnamomum* species including *Cinnamomum osmophloeum* (Fang et al., 2005; Rao et al., 2007; Tung et al., 2008), *Cinnamomum camphora* (Lee et al., 2006), *Cinnamomum insularimontanum* (Lin et al., 2008) and *Cinnamomum cassia* (Li et al., 2007) have also been demonstrated to have anti-inflammatory properties. The extract was tested with the standard models for both acute and chronic inflammation. For the acute inflammatory model, the *in vitro* test was performed using LPS-stimulated mice neutrophil model by Flow cytometer. It was found that the extract inhibited TNF- α and probably accounted for its anti-inflammatory effect. The inhibitory effect of the extract on TNF- α was supported by rat pleurisy model.

The present study shows that the 50% ethanol extract of cinnamon, at the highest dose (20 mg/kg) has an inhibitory effect on edema formation in carrageenan-induced rat pleurisy model. The edema induced by the injection of carrageenan into the pleural cavity of rat. Additionally, it was found to inhibit the release of TNF- α in human PBMCs. The result suggests that the inhibitory effect of the extract on edema formation is probably due to the inhibition of release of TNF- α . Therefore, the release of TNF- α after stimulation of neutrophils with LPS is a valid model system to test novel compounds for potential anti-inflammatory effects (Nazir et al., 2007). Inhibitory effect of the extract on TNF- α release from neutrophils was demonstrated in a dose-dependent manner. TNF- α plays a critical role in both acute and chronic inflammation. TNF- α facilitates inflammatory cell infiltration by promoting the adhesion of neutrophils and lymphocytes to endothelial cells. Additionally, TNF- α stimulates neutrophils to transcribe and release cytokines and chemokines biosynthesis (Khanna et al., 2007). Inhibition of TNF- α release can reduce the severity of inflammation. TNF- α is shown to accelerate both angiogenesis and matrix degradation by induction of vascular matrix metalloproteinase. Therefore, the inhibition of tissue granuloma by the extract at least in part, may be through interference with TNF- α release (Pandey et al., 2005).

It is interesting to know if the extract acted at expression level for TNF-alpha inhibition. Cinnamon (50%) inhibited the expression of TNF- α . It may inhibit either the initiation of transcription or the stability of the mRNAs encoding these molecules. It has been also shown that activation of transcription factor NF- κ B by TNF- α is required for the transcriptional activation of endothelial cell adhesion molecules. Thus, NF- κ B is believed to play an important role in the regulation of inflammatory response. NF- κ B activation was associated with the

phosphorylation and degradation of I κ B- α and the nuclear translocation of p⁶⁵ (Eigler et al., 1997). NF- κ B, a key transcription factor is implicated in the regulation of a variety of genes participating in immune and inflammatory responses. Effect of the herbal extract on transcription factor NF- κ B could be studied to understand mechanism of TNF- α expression inhibition.

C. zeylanicum has been reported to increase the hydroxyproline content in tissues, which is reduced in degenerative diseases like Osteoarthritis (OA) and thus promotes damaged cartilage repair and healing (Chakrabarti et al., 2006).

Potent anti-inflammatory activities of cinnamon extract at *in vitro* and *in vivo* experiments along with cartilage protecting activity is suggestive of its anti-inflammatory, anti-arthritis activity, which could be confirmed in various models of arthritis.

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