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Anti-inflammatory activity of flower extract of *Calendula officinalis* Linn. and its possible mechanism of action

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Calendula officinalis flower extract possessed significant anti-inflammatory activity against carrageenan and dextran-induced acute paw edema. Oral administration of 250 and 500 mg/kg body weight *Calendula* extract produced significant inhibition (50.6 and 65.9% respectively) in paw edema of animals induced by carrageenan and 41.9 and 42.4% respectively with inflammation produced by dextran. In chronic anti-inflammatory model using formalin, administration of 250 and 500 mg/kg body weight *Calendula* extract produced an inhibition of 32.9 and 62.3% respectively compared to controls. TNF-α production by macrophage culture treated with lipopolysaccharide (LPS) was found to be significantly inhibited by *Calendula* extract. Moreover, increased levels of proinflammatory cytokines IL-1β, IL-6, TNF-α and IFN-γ and acute phase protein, C- reactive protein (CRP) in mice produced by LPS injection were inhibited significantly by the extract. LPS induced cyclooxygenase-2 (Cox-2) levels in mice spleen were also found to be inhibited by extract treatment. The results showed that potent anti-inflammatory response of *C. officinalis* extract may be mediated by the inhibition of proinflammatory cytokines and Cox-2 and subsequent prostaglandin synthesis.

Keywords: Anti-inflammatory agents, *Calendula officinalis*, Cyclooxygenase-2, Proinflammatory cytokines.

Inflammation is a physiological process in response to tissue damage resulting from microbial pathogen infection, chemical irritation, and/or wounding[1]. The relation between inflammation and atherosclerosis, diabetes, cancer, arthritis and alzheimer's disease has been well substantiated[2-5]. The functioning of the immune system is finely balanced by the activities of proinflammatory and anti-inflammatory mediators or cytokines. The mediators of the inflammation such as cytokines, prostaglandins, and free radicals have direct or indirect effect on the pathophysiology of diseases. Chronic inflammation develops from unresolved symptomatic acute inflammation with or without any clinical manifestations. This may activate macrophages and lymphocytes which release inflammatory mediators and also result in excessive formation of reactive oxygen and nitrogen species that damage DNA and cell membranes. Inflammatory cells release prostaglandins with concomitant increase in the expression of key enzyme cyclooxygenase which in turn can activate several transcription factors including NF-κB[6]. Inflammation activates a variety of inflammatory cells, which induce and activate oxidant generating enzymes like NADPH oxidase, xanthine oxidase, myeloperoxidase etc., which produce superoxide anion and other reactive nitrogen species like nitric oxide through activation of inducible nitric oxide synthase (iNOS)[7].

Free radicals play major role in persistence of inflammation. During the process of inflammation, phagocytes secrete chemically reactive oxidants, radicals, and electrophilic compounds that bring about the elimination of infectious agents[8,9]. These inflammatory mediators can damage the surrounding host tissue[10,11]. Many drugs of plant origin having antioxidant activity have been reported to have anti-inflammatory activity[12,13].

Calendula officinalis Linn. (Compositae) is widely used in traditional medicines as an anti-inflammatory agent[14] and has also been reported to have anti-bacterial[15], anti-fungal[16] and anti-viral activities[17]. *In vitro* and *in vivo* evaluation of *C. officinalis* flower extract revealed that the extract possessed superoxide, hydroxyl and nitric oxide radicals, as well as 2,2-diphenyl-1-picrylhydrazyl ($DPPH^+$) and 2,2-azobis–3-ethylbenzthiazoline-6-sulphonicacid ($ABTS^+$) radicals scavenging activities and was found to inhibit lipid peroxidation[18]. The extract also significantly inhibited the superoxide radicals induced by phorbol-13-

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myristate acetate (PMA) *in vivo*. Moreover, treatment with the extract enhanced the antioxidant status in the body, as seen by increased levels of endogenous antioxidants catalase, superoxide dismutase and glutathione in animals[18]. The present study has been undertaken to evaluate the anti-inflammatory activity of *Calendula officinalis* flower extract in acute and chronic models of inflammation in mice.

Materials and Methods

Chemicals — Minimum Eagle's Medium (MEM) was purchased from Hi-Media, Mumbai, India. Foetal Calf Serum (FCS) was purchased from Biological Industries, Israel. Carrageenan was purchased from Sigma Aldrich, USA. Elisa kits for mouse IL-1β, IL-6, TNF-α and IFN-γ were purchased from Pierce Biotechnology, USA and for C-reactive protein was purchased from R&D systems, USA. Oligonucleotide primer sequences of cyclooxygenase-2 gene and GAPDH for reverse transcription-polymerase chain reaction (RT-PCR) were purchased from Maxim Biotech Inc. (San Francis Co, Calif). All the other reagents and chemicals used were of analytical reagent grade.

Cell line — L929 (Lung fibroblast) cells were purchased from National Facility for Animal Tissue and Cell Culture, Pune, India and maintained in Minimum Eagle's Medium (MEM) supplemented with 10% foetal calf serum and antibiotics.

Preparation of the flower extract — Fresh *Calendula* flower tops were used for extraction of the active components. They were collected from Government Botanical Gardens, Ooty, Nilgiris during January 2006 and were authenticated by Dr. S. Rajan, Field Botanist, Central Council for Research in Homeopathy, Ooty, India and the voucher specimen was deposited at Amala Ayurvedic Research Centre (Voucher No: Co05). Extraction was done as per homeopathic pharmacopoeia[19]. *Calendula* flowers (700 g) were extracted with 450 ml ethyl alcohol by masturation. For this, the flowers were placed in a wide mouth bottle and the alcohol was added. The jar was stoppered and sealed to prevent evaporation. It was placed in a dark room at room temperature and shaken everyday for two weeks. Clear liquid was decanted and the residue was pressed out through clean linen, which was added to the decanted liquid. Volume was made upto 1 litre with alcohol to make "mother tincture". 100 ml of this tincture of *Calendula officinalis* was evaporated to dryness in a shaker water bath at 42°C. The yield was found to be 1.1 g/100 ml. Dried extract (1 g) was dissolved in a known amount of distilled water and used for all the experiments.

Animals — Male BALB/C mice (20-25 g) were purchased from Small Animal Breeding Station, Mannuthy, Thrissur, Kerala. They were housed in well-ventilated cages and fed with normal mouse chow (Sai Durga Feeds and Food, Bangalore, India) and water *ad libitum*. All the animal experiments were done after getting approval from the Institutional Animal Ethical Committee and as per the instructions prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

Determination of anti-inflammatory activity of Calendula officinalis using carrageenan and dextran induced paw edema in mice — Freshly prepared 1% carrageenan or dextran in 0.1% carboxy methyl cellulose (0.02 ml) was injected on subplantar region of the right paw of mice to induce acute inflammation[20,21]. The animals were divided into following 8 groups of 6 animals each. Group I remained as control for carrageenan. Group II and III received 250 and 500 mg/kg body weight extract (0.1 ml in distilled water) orally by intubation gauge 1 hr before carrageenan injection and group IV, positive control received 10 mg/kg body weight diclofenac (ip). Group V remained as control for dextran model. Group VI and VII received 250 and 500 mg/kg body weight of *Calendula* extract (0.1 ml) orally by intubation gauge and group VIII (positive control) received 10 mg/kg body weight diclofenac (ip) 1 hr before dextran injection. The thickness of the paw was measured using a vernier caliper before and after carrageenan or dextran injection and thereafter at every hour upto 6 hr. The percentage of inhibition of paw thickness was calculated using the formula:

$$\% \text{ inhibition of paw thickness} = \left[\frac{(tCn - tC0 - (tTn - tT0)}{(tCn - tC0)}\right] \times 100$$

where tCn = paw thickness at particular time point of control animal; tC0 = paw thickness before induction; tTn = paw thickness at particular time point of treated animal; and tT0 = paw thickness before induction.

Determination of anti-inflammatory activity of C. officinalis using formalin induced paw edema in mice — Single dose of 0.02 ml of freshly prepared 2% formalin was used to induce chronic inflammation in mice. The animals were divided into 4 groups of 6

animals each. Group I remained as control. Group II and III received 250 and 500 mg/kg body weight extract (0.1 ml) orally and group IV received 10mg/kg body weight diclofenac (ip). Drug treatment was started 1 hr prior to formalin injection and continued for 6 consecutive days[22]. The thickness of the paw was measured using a vernier caliper every day for 6 days. The percentage inhibition was calculated using the formula, given earlier.

Determination of effect of C. officinalis on tumor necrosis factor-α (TNF-α) produced by macrophages in vitro — Administration of lipopolysaccharide (LPS) has been shown to stimulate the production of tumor necrosis factor- α in the serum and cytotoxicity of TNF-α to the lung fibroblast cell line L929 formed the basis of the bioassay for TNF-α[23, 24].

BALB/C mice (18; 3 animals/group) were grouped as follows: TNF-α was induced by injection (ip) of lipopolysaccharide (LPS). Group I- normal; Group II- normal + *C. officinalis* extract (0.1 ml) alone treated (100mg/kg body weight); Group III- normal + *C. officinalis* alone treated (250 mg/kg body weight); Group IV- control treated with lipopolysaccharide (LPS) alone (250 µg/ animal); Group V- LPS + *C. officinalis* extract treated (100mg/kg body weight); Group VI- LPS + *C. officinalis* extract treated (250 mg/ kg body weight).

Sodium caesinate (5%, 0.2 ml/ animal) was injected to animals intraperitoneally to induce macrophages. Thereafter, animals were treated with the drug orally for 5 consecutive days. On 5th day, LPS (250 µg/ animal) was injected intraperitoneally and after 6hr, all the animals were sacrificed and macrophages were collected from the peritoneal cavity after injecting 5 ml PBS followed by aspiration. Macrophages (2×10^5) were plated to each well of 96 well titre plate and incubated for 24 hr at 37°C. Non-adherent macrophages were removed after incubation and fresh medium was added and again incubated for 24 hr at 37°C in CO_2 atmosphere. The plates were centrifuged after incubation and the medium (100 µl) from each well was added to L929 cells (10^4 cells) plated in 96 well titre plate. Plates were incubated for 48 hr and at the end of incubation, the cells were fixed and stained with crystal violet and photographed and the action of TNF- α was assessed morpholoically[25].

Determination of effect of C. officinalis on proinflammatory cytokines level. — BALB/C mice (15; 3 animals/group) were grouped as follows: Group I- normal; Group II- control treated with lipopolysaccharide (LPS) (ip) alone (250 µg/ animal); Group III- LPS + *C. officinalis* extract treated (50mg/kg body weight); Group IV- LPS + *C. officinalis* extract treated (100 mg/ kg body weight); Group V- LPS + *C. officinalis* extract treated (250 mg/ kg body weight).

Drug administration (0.1 ml, oral) was started 5 days prior to LPS injection. On 6th day, LPS along with last dose of drug was given. After 6 hr of LPS injection,[26] all the animals were sacrificed, blood samples were collected and serum was separated and the levels of proinflammatory cytokines like IL-1β, IL-6, and TNF-α and IFN-γ and C- reactive protein (CRP) were measured by ELISA method.

Determination of effect of C.officinalis on expression of cyclooxygenase-2 gene — Inflammation was induced by single injection (ip) of LPS (250 µg/ animal). The animals were treated with 100 and 250 mg/ kg body weight extract for 5 days prior to LPS treatment. Control animal received LPS alone and normal were kept untreated. After 90 min of LPS induction, all the animals were sacrificed, spleens were removed and RNA was isolated by guanidinium thiocyanate method[27]. Equal amounts of RNA were reverse transcribed into cDNA using Cox-2 primers. (forward primer — 5 prime-GTGGAAAAACCTCG TCCAGA-3 prime; reverse primer — 5 prime-GATGGTGGCTGTTTTGGTA-3 prime). The cDNA was prepared from RNA by RT-PCR and was amplified under the following cycling conditions: 1 min at 94°C, 1 min at 58°C and 1 min at 72°C for 40 cycles followed by a 10 min extention at 72°C. The house keeping gene GAPDH was used as an internal standard (forward primer — 5 prime-TGCTGGCGCTGAGTACGTCGT- 3 prime; reverse primer — 3 prime- GTGGAGGAGTGGGTGTC GCTG -5 prime). Amplified PCR product was electrophoresed on a 1.8% agarose gel and stained with ethidium bromide and documented using a gel documentation system (Vilber Lourmat, France).

Statistical analysis — The results are expressed as mean ± SE. Statistical evaluation of the data was done by one-way ANOVA followed by Dunnet's test (posthoc) using InStat 3 software package.

Results

Effects of C. officinalis extract on inflammation — Anti-inflammatory activity of *C. officinalis* is given in Fig.1. Treatment with the extract significantly reduced the paw edema induced by carrageenan. The

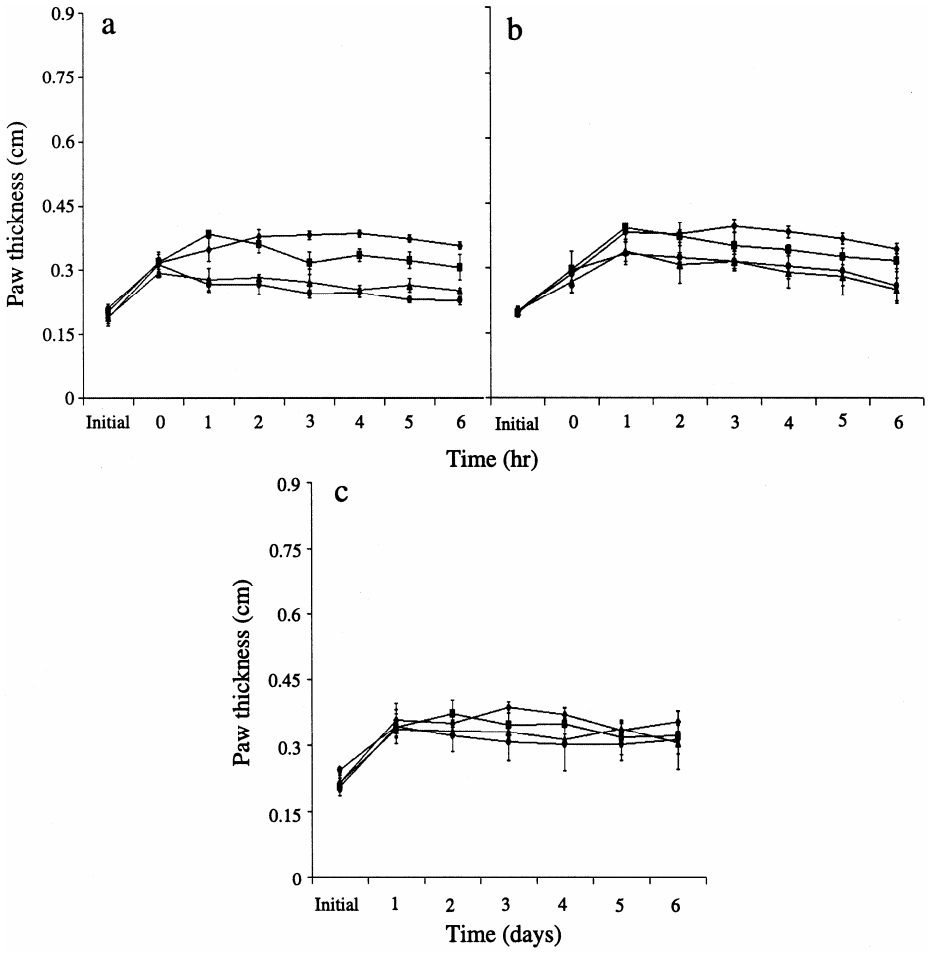

Fig. 1—Effect of *C. officinalis* extract on inflammation induced by (a) Carrageenan (b) Dextran (c) Formalin [–◆– control –■– standard diclofenac 10 mg/kg b w, –▲– 250 mg/kg b w *Calendula* extract, –●– 500 mg/kg b w *Calendula* extract]

paw edema was found to reach the peak at 3rd hour and after that it was found to decrease. Administration of the extract produced 50.6 and 65.9% inhibition in paw thickness at doses of 250 and 500 mg/kg body weight extract at 3rd hr (P<0.001). When dextran was used as an acute inflammatory agent, there was 41.9 and 42.4% inhibition of paw edema at 3rd hr in the 250 and 500 mg/kg body weight extract treated groups (P<0.001). Significant inhibition in paw edema was also seen in the treated groups in chronic inflammatory model using formalin which was 32.9 and 62.3% on third day after treatment with 250mg (P<0.05) and 500 mg/kg body weight (P<0.001) of the extract.

Effect of C.officinalis on tumor necrosis factor-α (TNF-α) production—The culture supernatant collected from macrophage culture of normal animals did not produce any change to the L929 cells (Fig. 2a). Similarly culture supernatant collected from macrophage culture of drug alone treated animals (100 and 250 mg/kg body weight) did not produce any cytotoxicity (Fig. 2e and f). When L929 cells were treated with the culture supernatant collected from the macrophage culture of LPS alone treated animals, it produced 100% cytotoxicity (Fig. 2b). When the L929 cells were treated with culture supernatant collected from LPS induced and 100 and 250 mg/kg body weight extract treated animals, minimal cytotoxicity was seen as the cell growth was in the normal pattern (Fig. 2c and d). These results indicate that *Calendula* extract can inhibit the cytotoxicity induced by LPS stimulated macrophages.

Effect of C. officinalis on proinflammatory cytokines level — There was a significant increase in the level of proinflammatory cytokines like IL-1β, IL-6, and TNF-α in the sera of LPS induced animals (Table 1). Similarly IFN-γ and C - reactive protein were also increased compared to normal animals. This

increase was found to be significantly inhibited by the administration of the *Calendula* extract.

Effect of C.officinalis on expression of Cox-2 gene—Cox-2 gene was expressed in the spleen of LPS treated animals (Fig. 3). Treatment with *Calendula* extract (250 mg/kg body weight) produced inhibition in the expression of Cox-2 gene as seen from the band intensity.

Discussion

The extract of *Calendula officinalis* flowers showed significant anti-inflammatory activity in both acute and chronic model. Acute inflammatory agents carrageenan and dextran induce inflammation through different mechanisms. Carrageenan induces edema through release of histamine, 5-hydroxy tryptamine, kinins and prostaglandins. It induces protein rich

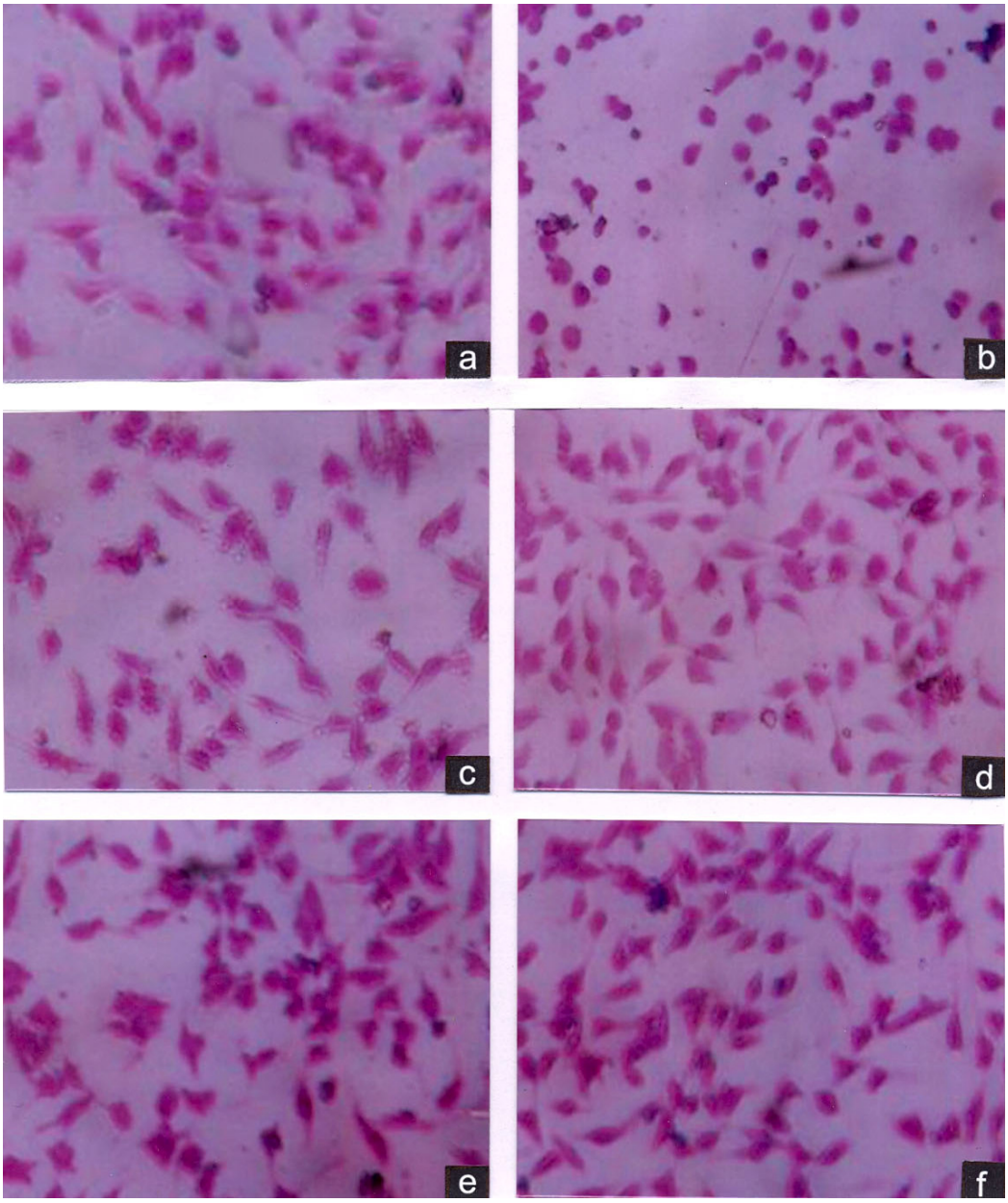

Fig. 2— Morphology of L929 cells incubated with medium from macrophage culture from (× 100) [(a) normal animals (b) LPS stimulated animals (c) LPS stimulated animals treated with 100 mg/kg b w *Calendula* extract (d) LPS stimulated animals treated with 250 mg/kg b w *Calendula* extract (e) normal animals treated with 100 mg/kg b w *Calendula* extract (f) normal animals treated with 250 mg/kg b w *Calendula* extract]

exudates with neutrophils at the site of inflammation[28-30]. Dextran mediates histamine and serotonin to the site of inflammation and the fluid accumulation is through mast cell degradation[31]. Chronic inflammatory agent formalin induced inflammation closely resembles human arthritis[32]. The results obtained with the treatment using the *C. officinalis* extract showed the anti-inflammatory activity irrespective of the induction of inflammation.

Cytokines are the key molecules that can inhibit or propagate inflammation by activating or deactivating the genes involved in cellular process[33]. The proinflammatory cytokines like IL-1, IL-6 and TNF-α can stimulate the liver to produce CRP which is increased several folds during acute inflammation. These molecules act as mediators of inflammation, persistence may lead to secondary activation of many genes involved in pathogenesis of inflammatory diseases as well as cancer[9]. Acute inflammation causes the release of IFN-γ into the circulation and mediates host inflammatory response. Treatment with *Calendula* extract lowered the IFN-γ level compared to that of control. Moreover the expression of cyclooxygenase 2, a key enzyme involved in inflammatory process was found to be inhibited by the treatment with the extract. Thus the *C. officinalis* flower extract may be exerting its anti-inflammatory activity through modulating the activity of proinflammatory cytokines as well as by inhibiting the expression of Cox-2.

Reactive oxygen species are produced in the body by endogenous and exogenous sources. The potential endogenous sources include activating inflammatory cells such as neutrophils, eosinophils, and macrophages. Activated macrophages, through "respiratory burst," elicit a rapid but transient increase in oxygen uptake that gives rise to a variety of reactive oxygen species, including superoxide anion, hydrogen peroxide, and nitric oxide[34] which can produce tissue damage and are the main culprits of cellular transformation in chronic inflammation.

Flavonoids and carotenoids are potent antioxidants at very low concentrations. The chemopreventive properties of flavonoids are generally believed to reflect their ability to scavenge endogenous ROS. By inhibiting or stimulating various signaling pathways, flavonoids at low concentration could affect cellular function[35].

Calendula officinalis has been reported to contain coumarins, flavonoids including quercetin,

Table 1— Effect of *C. officinalis* flower extract on LPS induced proinflammatory cytokines level in serum [Values are mean ± SE]

Groups	IL-1β (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)	INF-γ (pg/ml)	CRP (μg/ml)
Normal	18.12 ± 3.50	36.80 ± 5.60	34.00 ± 1.50	1958.47 ± 56.24	524.00 ± 22.80
Control (LPS alone)	99.54 ± 2.07**	335.09 ± 27.09**	646.53 ± 23.99**	2767.80 ± 52.37**	4680.00 ± 223.00**
Treated (LPS + 50 mg/kg body weight *Calendula officinalis* extract)	45.27 ± 2.44**	131.64 ± 6.53**	174.25 ± 8.27**	2555.90 ± 27.89*	1839.00 ± 54.70**
Treated (LPS+100 mg/kg body weight *Calendula officinalis* extract)	42.88 ± 1.97**	120.21 ± 2.01**	151.07 ± 11.99**	2503.39 ± 15.30**	1313.33 ±17.90**
Treated (LPS+250 mg/kg body weight *Calendula officinalis* extract)	37.82 ± 2.04**	106.38 ± 6.49**	129.01 ± 14.32**	2410.17 ± 67.07**	694.33 ± 6.50**

P values: *<0.01; **<0.001. The Control group was compared with the normal and the treated with that of control.

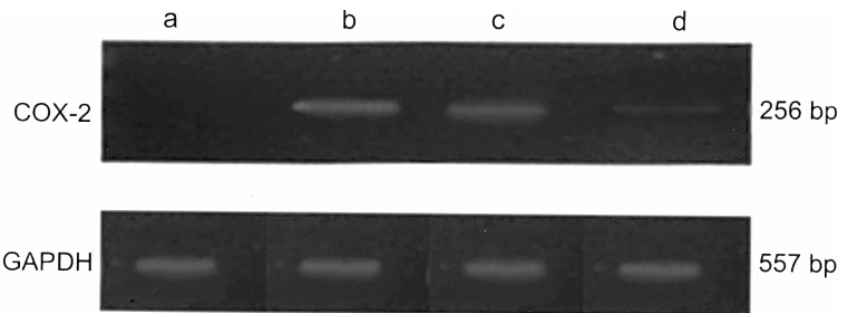

Fig. 3— Effect of *C. officinalis* extract on Cox-2 expression [(a) normal, (b) control LPS alone, (c) LPS + 100 mg/kg b w *Calendula* extract (d) LPS + 250 mg/kg b w *Calendula* extract]

protocatechuic acid etc., triterpenoids- faradiol, oleanolic acid, beta-amyrin, calenduladiol etc and the alkaloid narcissin[36]. Flowers also are rich in carotenoids of which flavoxanthin has been reported to be present at 28.5% of total carotenoids followed by luteoxanthin. Flowers are also found to contain lycopene and β-carotene[37].

The ingredients of *C. officinalis* like lutein possess chemopreventive potential[38] and β-carotene has been reported to quench reactive oxygen species[39]. Lycopene consistently reduces transcript levels of proinflammatory cytokines[40]. Hence the combined action of the active ingredients present in *C. officinalis* through their free radical scavenging and inhibition of mediators of inflammation especially cytokines and prostaglandins may be exerting the anti-inflammatory activity.

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