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Amelioration of inflammatory responses by Chlorogenic acid via suppression of pro-inflammatory mediators

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ABSTRACT

The premise of the present investigation was to evaluate the detailed anti-inflammatory properties of chlorogenic acid (CGA) and to delineate the possible mode of action. To explore the anti-inflammatory profile, we evaluated the effect of CGA on TNF-alpha expression (*in vitro*); carrageenan induced rat paw edema and carrageenan induce pleurisy (*in vivo*). In our studies, CGA significantly inhibited the TNF-alpha expression, paw edema and antioxidant enzymes in livers of rats in pre-treatment schedule but failed to exert any effect when administered 1 h after carrageenan injection. CGA was also found to be safe, as confirmed by the results of acute toxicity studies and MTT assay. CGA also caused reduction in total leucocytes count most probably by inhibiting neutrophils, but could not alter mononuclear cells count in carrageenan induced pleurisy. Inhibition of exudation was evidenced by the less exudate formation in CGA treated animals, which may be due to decrease in vascular permeability which was further confirmed in acetic acid induced vascular permeability model in mice where significant decrease in vascular permeability was observed. CGA was highly effective in reducing the arachidonic acid metabolites, nitric oxide and pro-inflammatory cytokines production in a dose dependent manner and in some conditions effect observed was almost comparable to ibuprofen. Result of the present investigation shows the anti-inflammatory effects of CGS in different models.

Key words: Chlorogenic acid, pro-inflammatory cytokines, nitric oxide, PGE2, LTB4.

INTRODUCTION

Inflammation is a series of well coordinated dynamic events that depend on sequential arrival of inflammatory mediators to the site of inflammation, and its initiation, maintenance and shutting down whenever required is a tightly regulated process, which keeps the inflammation under control. However, under certain circumstances, inflammation goes unchecked and results in cellular damage. The pathological consequences of inflammation are well known and documented which includes reddening, swelling and migration of immune cells to the area of injury. There are many inflammatory mediators which participate in this response such as metabolites of arachidonic acid (prostaglandins and leukotrienes) and cytokines (Surh et al 2001) and it is well demonstrated that these can elicit the production of tissue-degrading enzymes, a mechanism eventually involved in tissue destruction and loss of articular function (Abramson, 2002) in autoimmune diseases such as rheumatoid arthritis (RA). Thus the inhibition of these mediators can be a useful approach for the amelioration of inflammatory diseases. In treating inflammatory diseases, medical doctors have been commonly prescribing non steroidal anti-inflammatory drugs (NSAIDs) and immunosuppressant's, however, some of them can cause serious adverse effects such as gastric mucosal damage, water and salt retention and even, possibly, carcinomas (Suleyman et al 2001). Thus, alternative agents with less severe side effects are required, and

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botanical products are important candidates (Wonga et al, 2008). CGA is a phenolic natural product isolated from the aerial parts of *Bidens pilosa* and has been explored for its antioxidant and anti-carcinogenic effects. More recent reports show that CGA also has antifungal effect (Ma et al, 2001), analgesic, antipyretic and of more importance, anti-inflammatory activities (Santos et al, 2006) as well. Especially, regarding its anti-inflammatory activity, the researchers determined such activity in an experimental model of carrageenan induced paw edema in rats, where CGA was shown to be effective when administered before carrageenan injection, however no one has ever demonstrated the effect of CGA on carrageenan induced edema when given after carrageenan injection (later phase of inflammation) and levels of antioxidant enzymes in this model. To the best of author's knowledge, it has never been claimed anywhere whether CGA has any effect on leucocytes migration, vascular permeability and pro-inflammatory cytokines in pleurisy model. Moreover, the effect of CGA on arachidonic acid metabolites (PGE2) is controversial, therefore present study was designed to answer some unexplored anti-inflammatory properties of CGA and mode of relevant action has been clarified.

MATERIALS AND METHODS

Preparation of test material

CGA was isolated from aqueous fraction of *Bidens pilosa* by the method as described elsewhere (Jassbi AR et al 2003) and its NMR and LC-MS spectral data was compared with those in the literature, which confirmed that the isolated compound was CGA. Chemical structure of CGA is given in Fig. 1.

Fig. 1

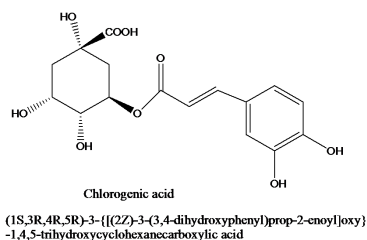


Fig. 1. Structure of Chlorogenic acid.

Animals

Experiments were performed on Swiss albino mice (weighing 20-25 g each) and male Wistar rats (weighing 130-150 g each) after obtaining the clearance from Institutional Animals Ethics Committee. All the animals were kept in standard cages and maintained under standard laboratory conditions (temperature $22 \pm 2^{\circ}\text{C}$ with 12 h light/ 12 h dark cycle) with free access to pellet food (Lipton India Ltd) and water ad libitum throughout the study. According to ethical regulations on animal research, all animals used in experimental work received humane care. Test material prepared as a homogenized suspension in distilled water and administered orally by gavage to the experimental animals for the duration of experiment unless otherwise specified.

Chemicals and Drugs used

- Ibuprofen, Lipopolysaccharide, Carrageenan, Rolipram (Sigma Chemical CO, USA).
- PGE2, LTB4, NO, TNF-alpha and IL-1beta ELISA kits (R&D Systems).
- PE labeled anti-mouse TNF-alpha monoclonal antibody, Facs permeabilizing solution, Golgi plug (BD Biosciences)
- All other reagents used were of analytical grade.

Flowcytometric analysis of LPS induced TNF-alpha production in murine neutrophils (*in vitro*)

Peripheral blood was collected from healthy mice and neutrophils were separated by histopaque-1077 gradient centrifugation following the method as described elsewhere (Bueno C et al 2002). The viability of neutrophils was tested by trypan blue exclusion test and was found to be greater than 95%. Neutrophils samples (5×10^9 cells/ml) were then incubated with different doses (2.5, 5, 10, 20 and 40 $\mu\text{g/ml}$) of CGA in the presence of LPS (10 ng/ml) for 2 h in CO_2 incubator at 37°C to see its dose related effect on intracellular TNF-alpha expression as compared to control (LPS alone). After incubation, neutrophils were permeabilized using facs permeabilizing solution and following washing in PBS incubated with PE labeled anti-mouse TNF-alpha monoclonal antibody in dark for 30 min. After final washing in PBS, samples were acquired directly on flowcytometer (BD-LSR II) using cell quest pro software. Rolipram was used as standard drug as it has been shown to reveal optimum response at the dose of 100 $\mu\text{g/ml}$ during our previous studies in this model (Chauhan et al, 2008).

Cell viability

Cell viability was assessed by using a MTT-based colorimetric assay. Neutrophils (5×10^9 cells/ml) diluted with Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal calf serum (FCS) were pipetted into 96 well microtiter plates, and were incubated overnight at 37°C and 5% CO_2 . The cells were exposed to various concentrations (2.5, 5, 10, 20 and 40 $\mu\text{g/ml}$) of CGA for 3 h under the same conditions of incubation. MTT solution dissolved in PBS was added to the wells at 1 mg/ml final concentration. After carefully aspirating the medium, 100 μl of DMSO were added for the dissolution of formazan crystals. The absorbance of each well was then read at 520 nm using a microplate reader.

Vascular permeability in mice

Mice were injected with 0.2% solution of evans blue dye (0.25% w/v in normal saline) intravenously after 30 min of oral administration of CGA and ibuprofen. 15 minutes later the mice were injected intraperitoneally (i.p) (1 ml/100 g of body weight) with freshly prepared 0.6% acetic acid (v/v) in normal saline. After 30 min of acetic acid injection the peritoneal cavities were washed with 5 ml of heparinised sterile normal saline and centrifuged ($3000 \times g$) for 10 min (Whittle, 1964). Absorbance of the

supernatant was measured at 610 nm using a spectrophotometer (Uvikon-810, Kontron Instruments, Switzerland).

Carrageenan induced edema (pre and post treatment)

The anti-inflammatory effect was evaluated by carrageenan induced rat paw edema test (Winter et al, 1962). Edema was induced by injection of 1% suspension of carrageenan (100 μ l) in 0.9% sterile saline solution into the right plantar region of the rat. CGA (2.5, 5, 10, 20 and 40 mg/kg) or standard drug (Ibuprofen-100 mg/kg), were administrated orally 1 h before (pre-treatment) or 1 h after (post-treatment) injection of carrageenan. The paw volumes (up to the tibiotarsal articulation) were estimated by plethysmography after 4 h of carrageenan injection and edema is reported as the difference between the initial and final paw volumes.

Measurement of antioxidant enzymes

For the measurement of antioxidant enzymes, experimental animals from carrageenan induced edema model were sacrificed and liver was isolated. The whole liver tissue was rinsed in ice-cold normal saline, and immediately placed in cold normal saline one time their volume and homogenized at 4°C. Then the homogenate was centrifuged at 12,000 rpm for 5 min. The supernatant was obtained and stored in the refrigerator at -20°C for the antioxidant enzymes activity assays (Tsong-Chun L et al 2007). The following biochemical parameters were analyzed to detect the antioxidant activities of CGA by the methods given below. Superoxide dismutase (SOD) enzyme activity was determined according to the method of Misra and Fridovich 1972, at room temperature. Glutathione peroxidase (GPx) and Glutathione reductase (GRx) enzyme activities were determined as described elsewhere (Flohe L et al 1984; Carlberg I et al 1985) at 37°C.

Carrageenan induced pleurisy (*in vivo*)

Following the procedure of Meacock and Kitchen 1979, pleurisy was induced in the rats by injecting 0.5 ml of carrageenan (1%, w/v in sterilized normal saline) into the pleural cavity. Carrageenan injection and drug administration schedule is given in Fig. 2. After 24 h of carrageenan injection, rats were anesthetized with anesthetic ether, chest was carefully opened and pleural fluid collected. Total leucocytes count were performed in a Neubauer chamber after diluting the pleural fluid with Turk solution (1:200) and cytopspin preparations of pleural wash were stained with May-Grunwald Giemsa for the differential leucocytes count, which was performed under an oil immersion objective.

Fig. 2

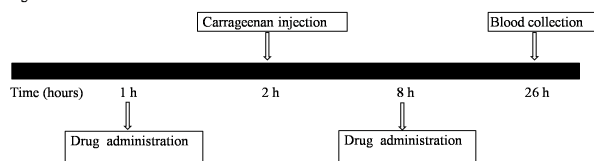


Fig. 2. Schematic representation of carrageenan injection and drug administration schedule in carrageenan induced pleurisy model.

The production of PGE₂, LTB₄ and nitrite/nitrate concentrations were measured in the cells free supernatant of pleural fluid by a standard sandwich ELISA procedure to assess the pharmaceutical effect on inflammatory mediator's quantity *in vivo* model. The assay for PGE₂ and LTB₄ was performed with manufacturer's instructions of kits from R&D systems. Nitrite/nitrate production (an indicator of NO synthesis) was measured in the exudate as previously described (Da Motta et al, 1994). Briefly, the nitrate in the exudate was first reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and NADPH (160 μ M) at room temperature for 3 h. The nitrite concentration in the samples was then measured by the Griess reaction, by adding 100 μ l of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H₂O and 1% sulfanilamide in 5% concentrated H₂PO₄; vol. 1:1) to 100 μ l samples. Absorbance results were assessed using an ELISA microplate reader set (Meglun, USA).

Measurement of cytokines

Samples of exudates obtained from the control and treated animals were collected and immediately prepared for the analysis of cytokines (TNF-alpha and IL-1beta) levels. In this protocol, commercially available kits were used with monoclonal specific antibodies for each cytokine. The cytokine levels were measured by ELISA kits according to the manufacturer's instructions.

Statistical analysis

The results were subjected to statistical analysis using ANOVA with post Bonferroni test and expressed as the Mean \pm S.E.M. ***p<0.001; **p<0.01; *p<0.05.

RESULTS

CGA ameliorated TNF-alpha expression in murine neutrophils

TNF-alpha is a major pro-inflammatory cytokine, the amelioration of which has been shown to have suppressive effect on inflammatory disorders in many conditions. As can be seen in Fig. 3, CGA produced highly significant and dose dependent inhibition of intracellular expression of TNF-alpha in murine neutrophils as assessed by flowcytometry. Neutrophils samples incubated with the graded doses (2.5, 5, 10, 20 and 40 μ g/ml) of CGA produced varied levels of inhibition with the most significant effect being obtained at the higher doses of 20 and 40 μ g/ml, an effect almost comparable to standard drug-rolipram. Rolipram at 100 μ g/ml produced 71.94% (p<0.001) inhibition of TNF-alpha expression.

Cell viability

To determine the cytotoxicity of CGA, and to rule out the possibility that inhibitory effect observed during *in vitro* studies was due to the cytotoxic effects of CGA, MTT assay was carried out and it was observed that CGA did not cause any toxicity as assessed by the evaluation of cells viability after 3 h incubation with CGA at different doses (Fig. 4).

Fig. 3

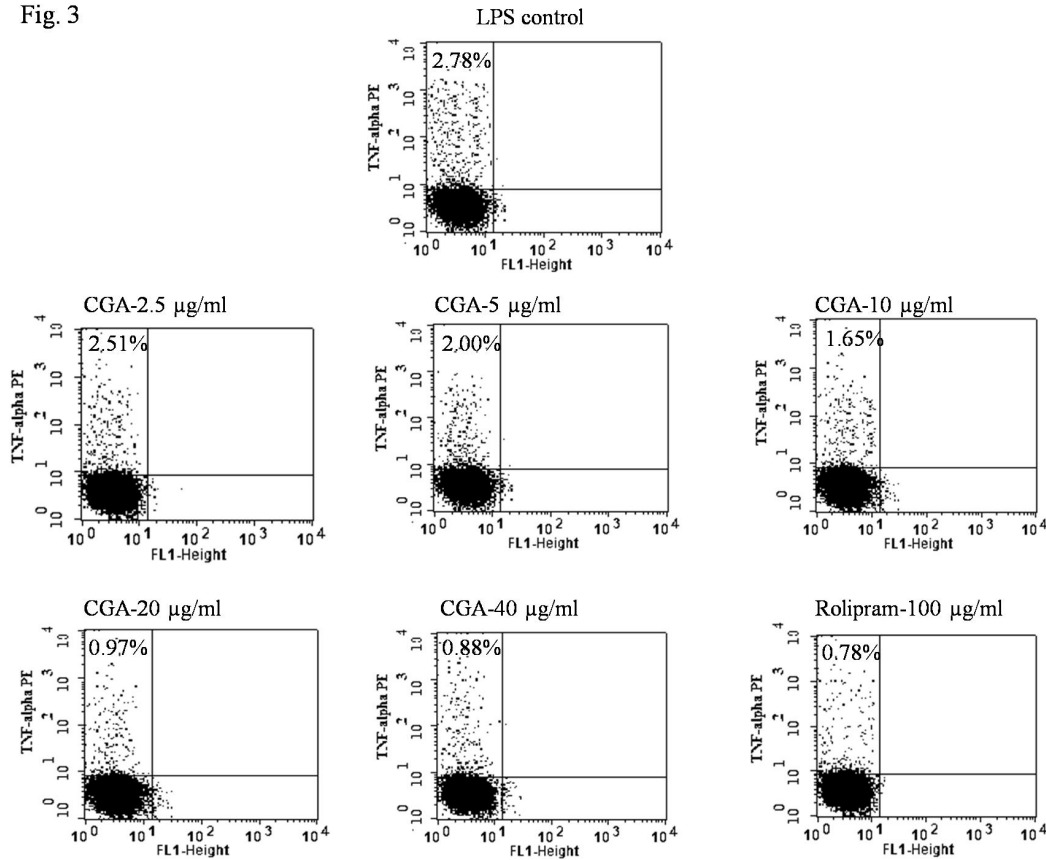


Fig. 3. Flowcytometric evaluation of the effect of CGA (2.5-40 µg/ml) and rolipram (100 µg/ml) on LPS induced intracellular TNF-alpha expression in murine neutrophils (*in vitro*). Flowcytometric pictures are representatives of three individual experiments. CGA-Chlorogenic acid.

Fig. 4

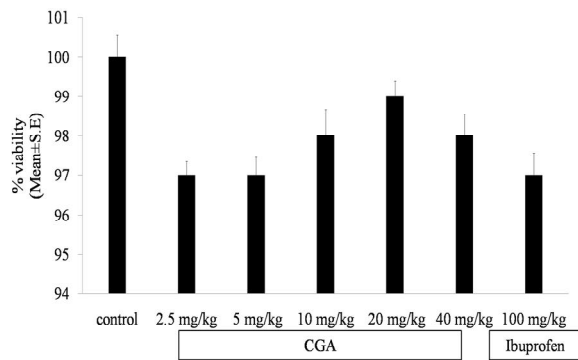


Fig. 4. Effect of CGA on cell viability as assessed by MTT assay. Results are represented as mean±S.E.M with n=6 in each group. Statistical test employed is ANOVA followed by post Bonferroni test. CGA-Chlorogenic acid.

Vascular permeability in mice

CGA when administered orally at 2.5-40 mg/kg caused highly significant decrease in vascular permeability as assessed by the dye concentration (Table 1). However it was very interesting to see that at the dose of 40 mg/kg effect exhibited by CGA was higher than ibuprofen. Ibuprofen produced 38.46% (p<0.001)

inhibition whereas CGA at 40 mg/kg caused 39.74% (p<0.001) decrease in vascular permeability, which suggests that decrease of vascular permeability may be one of the mechanisms by which CGA exerts its anti-inflammatory effects.

CGA inhibited carrageenan induced rat paw edema in pre-treatment schedule

As can be seen in Table 2, oral administration of CGA to animals 1 h before the carrageenan injection (pre-treatment) inhibited the inflammation as assessed by the paw edema measurement using plethysmometer, however failed to modify any changes in paw edema during post-treatment schedule. Similar results were obtained in group of animals administered ibuprofen at 100 mg/kg.

CGA increased antioxidant enzymes

At the fourth hour following the intrapleural injection of carrageenan in experimental animals, liver tissues were also analyzed for biochemical parameters such as, SOD, GPx and GRx activities (Table 3). It was observed that oral administration of CGA significantly increased the antioxidant enzymes levels in the liver tissues of groups of animals treated at graded doses after carrageenan injection into the pleural cavity. However ibuprofen resulted in most significant increase of antioxidant enzymes at the dose of 100 mg/kg. Ibuprofen increased SOD from 31.78±2.87 to

Fig. 5

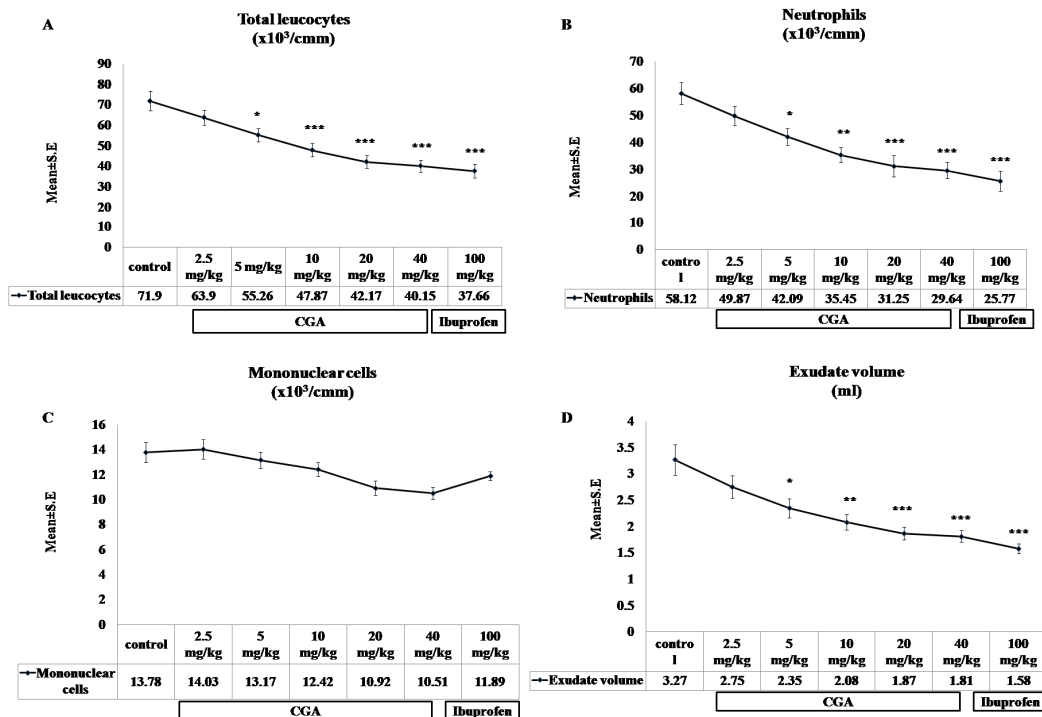


Fig. 5. Effect of graded doses (2.5-40 mg/kg/body weight) of CGA and ibuprofen (100 mg/kg/ body weight) on total leucocytes count (Fig. 5A), neutrophils count (Fig. 5B), mononuclear cells count (Fig. 5C) and exudate volume (Fig. 5D) in carrageenan induced pleurisy. Results are represented as mean±S.E.M with n=6 in each group. *p* value; ****p*<0.001; ***p*<0.01; **p*<0.05; Statistical test employed is ANOVA followed by post Bonferroni test. CGA-Chlorogenic acid.

55.65±3.44 (*p*<0.001), GPx increased from 2.12±0.18 to 3.87±0.18 (*p*<0.001) and GRx increased from 0.097±0.005 to 0.155±0.004 (*p*<0.001).

Quantification of leucocytes migration and exudation

Fig. 5 shows the effect of CGA on inhibition of leucocytes migration and exudation induced by injection of carrageenan into the pleural cavity. It can be seen that total leucocytes and neutrophils count were significantly suppressed in a dose dependent manner with the most significant effect being obtained at the higher doses of 20 and 40 mg/kg respectively as compared to control. However CGA was ineffective in decreasing mononuclear cells count at any dose administered. Similar results were obtained by ibuprofen. Both CGA and ibuprofen were found very effective in inhibiting the exudate volume.

CGA suppressed arachidonic acid metabolites (PGE2 and LTB4) and nitric oxide

In the acute model of inflammation induced by carrageenan injection into the pleural cavity, we observed highly elevated levels of PGE2 and LTB4. However group of animals treated with CGA at the doses ranged from 2.5-40 mg/kg, showed highly significant inhibition of PGE2 (421.41±39.48, *p*<0.001, Fig. 6A) and LTB4 (338.99±20.01, *p*<0.001, Fig. 6B) at the dose of 20 mg/kg. Group of animals treated with ibuprofen also showed highly significant inhibition of PGE2 and LTB4 at the dose of 100 mg/kg. It can be seen in Fig. 6C that oral treatment with CGA 1 h

before and 6 h after the carrageenan injection also significantly attenuated the NO levels. At the higher dose levels of 20 and 40 mg/kg, CGA demonstrated the effect almost comparable to the standard drug-ibuprofen, which shows CGA to be a potent anti-inflammatory agent.

Suppression of pro-inflammatory cytokines (TNF-alpha and IL-1beta)

The pro-inflammatory cytokines, TNF-alpha and IL-1beta were measured in the supernatant of pleural fluid by ELISA kits according to the manufacturer's instructions. As can be seen in Fig. 7A and 7B, the levels of TNF-alpha and IL-1beta in pleural fluid supernatants of CGA treated groups were significantly and dose dependently reduced in comparison with that in the pleural fluid supernatants of control group. However only at the higher dose of 20 and 40 mg/kg CGA showed the effect comparable to ibuprofen.

DISCUSSION

In the present study, we investigated the effects of CGA on various pro-inflammatory mediators such as arachidonic acid metabolites, nitric oxide and pro-inflammatory cytokines in experimental animals.

Today, there are number of inflammatory disorders that cannot be cured, however efforts to develop safer and more effective treatments that are based on an improved understanding of the role of inflammatory mediators and use of natural products

Fig. 6

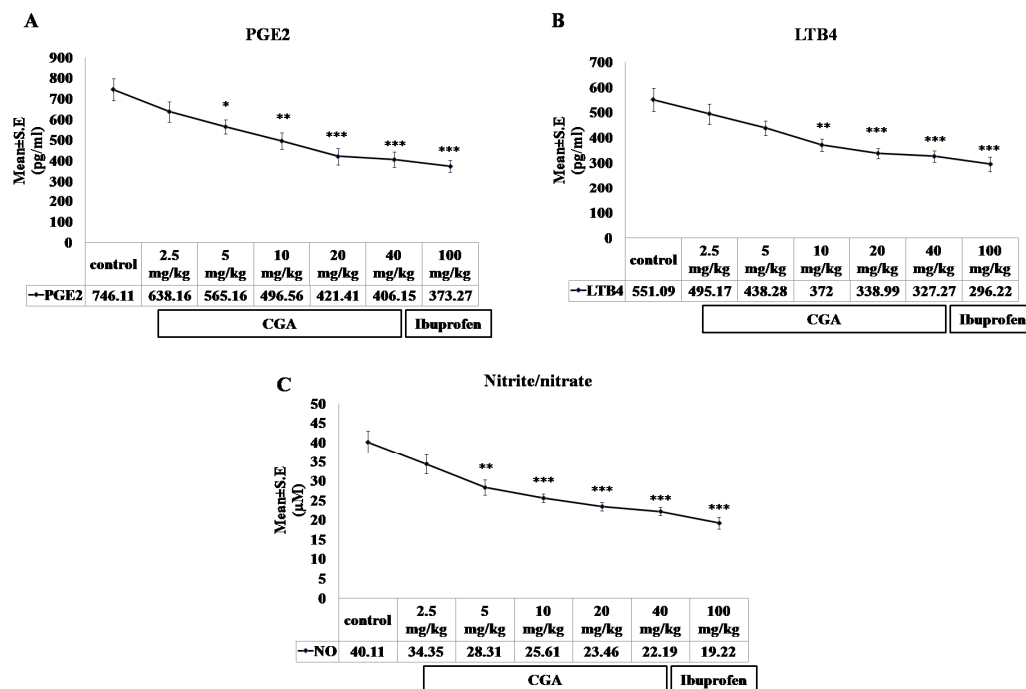


Fig. 6. Effect of graded doses (2.5-40 mg/kg/body weight) of CGA and ibuprofen (100 mg/kg/ body weight) on PGE2 (Fig. 6A), LTB4 (Fig. 6B) and nitrite/nitrate (Fig. 6C) levels in cells free supernatant of pleural fluid. Results are represented as mean±S.E.M with n=6 in each group. *p* value; ****p*<0.001; ***p*<0.01; **p*<0.05; Statistical test employed is ANOVA followed by post Bonferroni test. CGA-Chlorogenic acid.

are beginning to bear fruit (Ernest et al, 2001). Our Institute is actively engaged in plant based drug discovery and in our efforts to discover and develop effective and safer treatment for inflammatory disorders; we isolated and studied the anti-inflammatory potential of CGA. In the past two decades, a number of botanical derived medicines have been developed as anti-inflammatory agents but only a few of them have been studied with the goal of elucidating the molecular mechanisms of their actions. To address this issue, we firstly evaluated the safety, cytotoxicity and anti-inflammatory activity of CGA, and then determined the molecular mechanisms relevant to these actions, focusing on several key targets in inflammation, including arachidonic acid metabolites, nitric oxide, and pro-inflammatory cytokines. CGA administered at the dose of 300 to 2000 mg/kg caused no mortality or any abnormal behavior in experimental animals, which is indicative of the safety of CGA even at higher doses. Next we examined the cytotoxicity of CGA using MTT assay in order to assess if the anti-inflammatory activity of CGA reported in literature so far is due to killing effects on cells and perhaps, we are the first to demonstrate that CGA do not cause any cytotoxicity since in our studies, the viability of cells incubated with CGA was more than 95% and comparable to control cells (cells alone). Neutrophils play a very important role in the cellular pathology of inflammatory diseases. Accumulated neutrophils are activated and induce tissue injury through secretion of harmful mediators such as lysosomal enzymes and cytokines (Henson et al, 1987). LPS, a potent pro-inflammatory factor stimulates neutrophils to synthesize

or release harmful mediators-cytotoxic contents, pro-inflammatory cytokines such as TNF-alpha and prolongs the functional lifespan of neutrophils, leading to the exacerbation of inflammation in the local tissue (Liu et al, 2005), therefore it has been postulated that any kind of agent, that is able to block the LPS triggered TNF-alpha production in neutrophils, may act as promising anti-inflammatory agent in many diseases (Lee et al, 2009).

Table 1. Effect of graded doses (2.5-40 mg/kg/body weight) of CGA and ibuprofen (100 mg/kg/ body weight) on vascular permeability.

Groups	Concentration (mg/kg)	Dye concentration (Mean±S.E)	% inhibition (against control)
Control	-	0.78±0.05	-
CGA	2.5	0.69±0.04	11.53%
CGA	5	0.61±0.03 *	21.79%
CGA	10	0.56±0.04 **	28.20%
CGA	20	0.50±0.04 ***	35.89%
CGA	40	0.47±0.05 ***	39.74%
Ibuprofen	100	0.48±0.03 ****	38.46%

Results are represented as mean±S.E.M with n=6 in each group. *p* value; ****p*<0.001; ***p*<0.01; **p*<0.05; Statistical test employed is ANOVA followed by post Bonferroni test. CGA-Chlorogenic acid.

Our result shows that CGA exhibited highly significant inhibition of TNF-alpha expression which is indicative of anti-inflammatory

properties of CGA. In the carrageenan induced rat paw edema model, CGA and ibuprofen were not only found to be effective in reducing the edema formations but SOD, GPx and GRx levels as well during pre-treatment schedule but in post-treatment studies both CGA and ibuprofen failed to inhibit the edema formation at any dose administered, therefore we did not test the effect on antioxidant enzymes in this schedule. These results are suggestive of NSAIDs type action of CGA, since NSAIDs may not be effective in reducing the inflammation in the later phase of such edema formation (Boughton-Smith et al, 1993). Leucocytes migration and exudation represents two very important events of inflammation and their inhibition is believed to be a holistic approach for the amelioration of inflammation. In healthy conditions, the pleural fluid contains only a small number of cells, rarely exceeding 100 cells/ μ l and consisting mainly of lymphocytes (Light, 1990) however, carrageenan induced pleurisy is associated with the exudation and infiltration of leucocytes such as neutrophils, or mononuclear cells in various proportions.

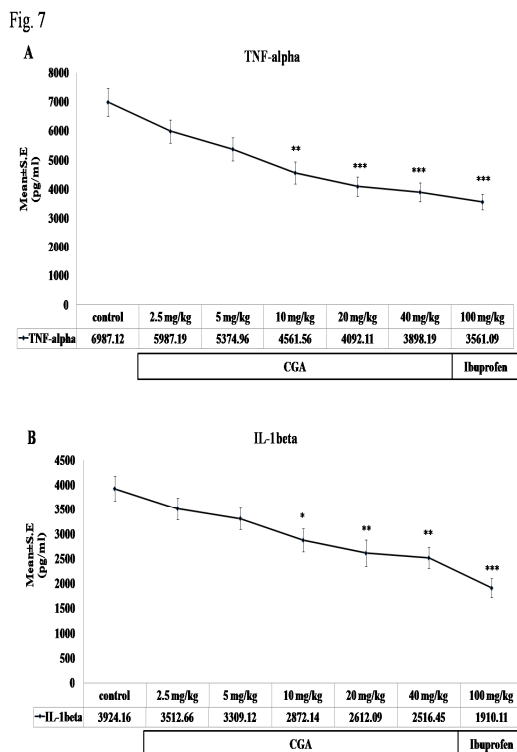


Fig. 7. Effect of graded doses (2.5-40 mg/kg/body weight) of CGA and ibuprofen (100 mg/kg/ body weight) on TNF-alpha (Fig. 7A) and IL-1beta (Fig. 7B) levels in cells free supernatant of pleural fluid. Results are represented as mean \pm S.E.M with n=6 in each group. *p* value; ****p*<0.001; ***p*<0.01; **p*<0.05; Statistical test employed is ANOVA followed by post Bonferroni test. CGA-Chlorogenic acid.

We observed that carrageenan injection into the pleural cavity resulted in exudation (3.39 ml in control animals [Mean \pm S.E]), whereas oral administration with both CGA and ibuprofen were highly effective in decreasing exudate volume in pleural cavity which may be due to the suppressive effects of CGA on vascular permeability, since CGA also decreased the acetic acid induced vascular permeability in mice. Decreased total leucocytes count as compared to control may be explained by the highly

significant inhibition of neutrophils as they comprise about 80% of total leucocytes count in this model. Neutrophils are the first line of defense against any pathogen; however in several inflammatory diseases like rheumatoid arthritis, they become a potential cause of tissue damage by the production of tissue degrading enzymes, oxygen and nitrogen species and metalloproteinase. In this study, we demonstrate for the first time that CGA has a potent capacity to reduce the migration of neutrophils at the site of inflammation. Many inflammatory conditions are associated with the production of comparatively higher amounts of arachidonic acid metabolites (PGE2 and LTB4) and nitric oxide (NO). The increase in the formation of PGE2 and LTB4 corresponds to the release of arachidonic acid from membrane phospholipids and the up-regulation of cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzymes. PGE2 is synthesized in substantial amounts at sites of inflammation where it acts as a potent vasodilator and synergistically with other mediators such as histamine and bradykinin causes an increase in vascular permeability and production of pro-inflammatory cytokines (Davies et al, 1984), while, LTB4 is a strong chemoattractant for neutrophils. We observed highly significant inhibition of PGE2 and LTB4 which is possibly one of the mechanisms of anti-inflammatory actions of CGA. In the present study, we also observed decreased NO production, which might be responsible for decreased production of PGE2 at the site of inflammation, as NO maintains the carrageenan evoked inflammatory response by activation of COX pathway (Salvemini et al, 1993) and in the absence of sufficient activation signals by NO, COX pathway might not have been activated which in turn would have suppressed the PGE2 production and might have resulted in subsequent inhibition of inflammation. Since NO activity is also an indirect marker of activated leucocytes and is implicated in cell migration and exudation (Dutra et al, 2009), the low NO levels following the CGA treatment could also be explained by the inhibition of leucocytes influx. Our results are different from those reported by other groups.

Table 2. Effect of graded doses (2.5-40 mg/kg/body weight) of CGA and ibuprofen (100 mg/kg/ body weight) on carrageenan induced edema (pre and post-treatment schedule)

Groups	Concentration (mg/kg)	Pre-treatment		Post-treatment	
		Mean \pm S.E	% inhibition	Mean \pm S.E	% inhibition
Control	-	1.45 \pm 0.06	-	1.65 \pm 0.09	-
CGA	2.5	1.29 \pm 0.08	11.03%	1.65 \pm 0.07	0.00%
CGA	5	1.05 \pm 0.08**	27.58%	1.55 \pm 0.03	6.06%
CGA	10	0.92 \pm 0.07***	36.55%	1.44 \pm 0.06	12.72%
CGA	20	0.79 \pm 0.04***	45.51%	1.36 \pm 0.06	17.57%
CGA	40	0.76 \pm 0.06***	47.58%	1.31 \pm 0.08	20.60%
Ibuprofen	100	0.71 \pm 0.03***	51.02%	1.27 \pm 0.08	23.03%

Results are represented as mean \pm S.E.M with n=6 in each group. *p* value; ****p*<0.001; ***p*<0.01; **p*<0.05; Statistical test employed is ANOVA followed by post Bonferroni test. CGA-Chlorogenic acid.

In the year 2006, Jin et al reported that CGA was unable to lower PGE2 levels in RAW264.7 mouse macrophages stimulated with LPS, and in 1992, Cunha et al reported that CGA was ineffective in inhibiting PGE2 synthesis by LPS-stimulated J774 macrophages. However, the difference in the observations can be explained by the experimental models used, different experimental conditions and differences in doses administered. There are several evidences which support the hypothesis that some pro-inflammatory cytokines are highly engaged in the priming of neutrophils and activation of arachidonic acid metabolites (Thomson et al, 2003). To address this question, whether CGA have any effect on expression of pro-inflammatory cytokines, we next examined the effect of CGA on TNF-alpha and IL-1beta levels in cells free supernatant of pleural fluid as they are believed to be involved in extension of inflammatory process and found that CGA significantly and dose dependently inhibited the cytokines level and interestingly, inhibition of TNF-alpha was almost comparable to ibuprofen, which indicates the potency of CGA. Pro-inflammatory cytokines are considered as the backbone of inflammatory reactions; especially TNF-alpha seems to be on top of the cascade. It increases the level of IL-1beta and of the granulocyte monocytes-colony stimulating factor (GM-CSF) and down-regulation of TNF-alpha could down-regulate the production of IL-1, IL-6, IL-8 and GM-CSF. In addition, it is also responsible for the accumulation of neutrophils at the site of inflammation (Beck G et al 1986) and up-regulates the expression of COX-2 and inducible nitric oxide synthase (iNOS), two potent pro-inflammatory enzymes that lead to increased synthesis of PGE2 and NO (Goldring et al, 2004).

Table 3. Effect of graded doses (2.5-40 mg/kg/body weight) of CGA and ibuprofen (100 mg/kg/ body weight) on antioxidant enzymes in carrageenan induced edema model.

Table 3

Groups	Concentration (mg/kg)	SOD (U/mg protein)	GPx (U/mg protein)	GRx (U/mg protein)
Control	-	31.78±2.87	2.12±0.18	0.097±0.005
CGA	2.5	34.31±2.00	2.34±0.16	0.111±0.005
CGA	5	39.09±1.88 *	2.56±0.11 *	0.126±0.003 *
CGA	10	41.77±2.09 **	2.98±0.12 **	0.141±0.004 **
CGA	20	47.17±2.18 ***	3.21±0.13 ***	0.149±0.005 ***
CGA	40	51.11±2.17 ***	3.66±0.16 ***	0.154±0.005 ***
Ibuprofen	100	55.65±3.44 ***	3.87±0.18 ***	0.155±0.004 ***

Results are represented as mean±S.E.M with n=6 in each group. *p* value; ****p*<0.001; ***p*<0.01; **p*<0.05; Statistical test employed is ANOVA followed by post Bonferroni test. CGA-Chlorogenic acid.

CONCLUSION

Taken together, it can be concluded that CGA is a potent anti-inflammatory agent the mode of action of which is related to TNF-alpha inhibition causing suppression of arachidonic acid metabolites & nitric oxide and subsequent inhibition of inflammation.

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