Original article

Inhibitory effect of *Calotropis gigantea* extract on Ovalbumin-induced airway inflammation and Arachidonic acid induced inflammation in a murine model of asthma

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ARTICLE INFO

ABSTRACT

The root of *Calotropis gigantea* has been reported as a traditional folkloric medicine in treatment of asthma in the Indian literature. Root contain α-and β-amyrin are reported to possess anti-lipoxygenase activity. Present study was undertaken to investigate the effect of methanolic extract of root of *Calotropis gigantea* (Linn.) R.Br. (CG) on ovalbumin induced asthma and arachidonic acid induced paw edema in rats. In ovalbumin induced asthma, rats were sensitized and challenged with ovalbumin (OVA). The effect of CG at 100, 200, 400 mg/kg, p.o. on inflammatory cell count, level of nitric oxide and total protein in bronchalveolar lavage (BAL) fluid, lung antioxidant enzymes (LPO, GSH, SOD, Catalase) and histopathological changes were observed. Change in paw edema volume was measured in arachidonic acid induced paw edema model. CG at 200, 400 mg/kg, p.o. showed significant inhibition of eosinophil, neutrophil and lymphocyte and total leukocyte count in bronchalveolar lavage (BAL) fluid ($p < 0.05$). In BAL fluid, CG significantly reduced the nitric oxide and total protein levels ($p < 0.05$). CG significantly restored the levels of GSH, SOD and LPO in lungs ($p < 0.01$). CG at doses of 200, 400 mg/kg significantly inhibited OVA induced histological changes ($p < 0.01$). CG significantly reduced the arachidonic acid induced paw edema volume ($p < 0.05$). These results suggest that CG may prove to be potential therapeutic drug for treating asthma owing to its anti-inflammatory, anti-lipoxygenase and antioxidant activities.

1. Introduction

The prevalence of asthma is rapidly increasing around the world, especially in young children, and it has become a significant cause of morbidity and mortality in developed countries [1]. Asthma is chronic respiratory disease characterized by reversible airway obstruction, increased mucus production, infiltration of eosinophils and nonspecific airway hyper-responsiveness [2]. Asthma causes different phenotypes and varies with age, gender, and ethnic groups. Inhaled pollutants including allergens, viruses, bacteria, fungi, tobacco smoke and ozone enhances the risk of developing asthma [3, 4]. OVA-induced asthma has been recognized as a disease that results from chronic airway inflammation characteristically associated with infiltration of lymphocytes, eosinophils, and neutrophils into bronchial lumen. The levels of reactive nitrogen and oxygen species are also increased [5].

An increasing number of clinical and experimental evidence suggest that ROS plays important role in the pathogenesis of airway inflammation [6, 7]. Hence by inhibiting oxidative stress we may be able to treat asthmatic condition.

Various medicinal plants are used for treatment of asthmatic patients. Calotropis gigantea (Linn.) R.Br. (Asclepiadaceae), commonly known as milkweed or swallow-wort, is found chiefly in wasteland throughout India [8]. It has been reported as a traditional folkloric medicine in treatment of asthma in the Indian

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Vipin Bulani et al. / Int J Cur Bio Med Sci. 2011; 1(2): 19 – 25.

20

literature [9]. Traditionally, the root of *Calotropis gigantea* is used in treatment of leprosy, asthma, bronchitis, and expectorant [10]. Root of CG contains α-amyrin, β-amyrin, taraxasterol, β-sitosterol, stigmasterol [11, 12], α-and β-amyrin are reported to possess anti-lipoxygenase activity by inhibiting 5-HETE [13]. Calotropis gigantea reported free radical scavenging [14, 15], procoagulation activity [16], antidiarrheal [17], anticonvulsant [18, 19], analgesic [20], pregnancy interceptive [21], anticancer [22], immunomodulatory [23], wound healing activity [24], anti-inflammatory [25, 26, 27, 28], hepatoprotective [29, 15] and Anti-diabetic [30].

Hence, taking into consideration the traditional claims and reported activities, the present study was planned to investigate the effect of CG on OVA induced airway inflammation and arachidonic acid induced inflammation in murine model of asthma, as no work is done in this direction and to check possible role of plant in asthma.

The inhibitory effects of CG extract on inflammation were compared with doses of 1mg/kg, i.p. dexamethasone [31], 10 mg/kg, i.p. Indomethacin [32] and 10 mg/kg, i.p. montelukast [33].

2. Materials and methods
2.1. Animals

Male Wistar rats weighing 200 to 250 g were used for study and were kept in animal house at 24 ± 2°C with relative humidity 44-56 % along with light and dark cycles of 12 h respectively. Animals were provided with standard diet and water ad libitum. Laboratory animal handling and experimental procedures were performed in accordance with the guidelines of CPCSEA and experimental protocol was approved by Institutional Animal Ethics Committee (198/99/CPCSEA).

2.2. Plant material

Standardized dry methanolic extract of root of Calotropis gigantea was procure from Amsar Pvt. Ltd., Batch No. 6386, Indore (M.P.), India, along with certificate of analysis.

2.3. Experimental design
2.3.1. Acute toxicity study [34]

The acute toxicity study for methanolic extract of root of Calotropis gigantea (Linn.) R.Br. was performed using rats. The animals were fasted overnight prior to the experiment and maintained under standard conditions. CG was found safe up to dose of 2000 mg/kg, p.o.

2.3.2. Sensitization and challenge with antigen [35]

Wistar rats (200 to 250 g) were divided into six groups (n = 6) viz. NS, S, DEXA, CG-100, CG-200 and CG-400. The animals, except in the non-sensetized group (NS), were sensitized by an intra-peritoneal injection of 1 ml alum precipitate antigen containing 20 μg of ovalbumin (Central Drug House Pvt. Ltd., India) and 8 mg of alum suspended in 0.9% sodium chloride solution. A booster injection of this alum-ovalbumin mixture was given 7 days later. Non-sensitized (NS) animals were injected with alum only. Seven days after (15 day) second injection, the animals were exposed to aerosolized ovalbumin (1%) for 30 min into a closed plexiglass chamber. The 'S' group served as a sensitized control and received distilled water 10 ml/kg p.o., DEXA group received

dexamethasone (1mg/kg, i.p.) and CG-100, CG-200,CG-400 received Calotropis gigantea 100, 200, 400 mg/kg, p.o., respectively, 5 hr before the antigen challenge. Bronchoalveolar lavage (BAL) fluid was collected by lavaging the lungs with 2 aliquots of 5 ml of 0.9% sodium chloride solution. Total recovery volume per rat was approximately 8 ml. The total cell count in the bronchoalveolar lavage was calculated using a hemocytometer. For the differential white cell count, BAL fluid was centrifuged at 1500 rpm for 10 mins using a Remi refrigerated centrifuge, supernatant liquid was discarded and cellular pellets were resuspended in 100 μl of PBS for differential count using Leishmans stain [36].

2.3.3. Lung tissue histopathology

For histological evaluation of the lung tissue, the lung was isolated and immersed in formalin and embedded in paraffin wax. Sections of lung tissue were cut (5 μm thickness), mounted on glass slides and stained with hematoxylin and eosin (H & E, 400×) to assess the lung histopathology. Asthmatic lung injury was graded from 0 (normal) to 4 (severe) in each of the following: Infiltration of leucocytes (infiltration of eosinophils, neutrophils), type of inflammatory exudates (catarrhal and mucoid material present in the bronchiolar epithelium), status of bronchi (constriction of the secondary bronchus and some of the tertiary bronchi.), perivascular status of lung blood vessels (infiltration of mononuclear cells around the lung blood vessels), integrity of alveoli (focal alveolar emphysema, and hemorrhages) and activation of alveolar macrophages.

2.3.4. Lung antioxidant enzyme assay (Estimation of MDA, GSH, SOD, and CAT).

Whole lung samples were dissected out and washed immediately with ice cold saline to remove as much blood as possible. Lung homogenates (5% w/v) were prepared in cold 50 mM Tris buffer (pH 7.4) using Remi homogenizer. The unbroken cells and cell debris were removed by centrifugation at 3000 rpm for 10 min using a Remi refrigerated centrifuge. The supernatant was used for the estimation of GSH [37], malondialdehyde (MDA) [38], superoxide dismutase (SOD) [39] and catalase [40] levels.

2.3.5. Nitric oxide and Total Protein analysis.

The pulmonary production of nitric oxide in the BAL fluid was spectrophotometrically determined by assaying BAL fluid for nitrite using the Griess reagent (1% sulfanilic acid, 0.1% N-1 naphthylethylenediamine dihydrochloride, 5% phosphoric acid). Absorbance was measured at 550 nm and nitrite concentration was determined using sodium nitrite as a standard [41]. Total protein was estimated according to the manufacturer's instructions (Biolab diagnostic Pvt. Ltd., India).

2.3.5. Measurement of lung wet/dry (W/D) weight

The pulmonary edema was determined by calculating the wet/dry weight ratio of lung tissues. The whole lung was excised and immediately weighed using a precision balance to obtain the "wet" weight then re-weighed after being dried at 80°C for 72 h to obtain the "dry" weight. The wet/dry ratio was calculated by dividing wet weight by dry weight.

2.3.6. Arachidonic acid induced rat paw edema [32]

Wistar rats (200 to 250 g) were divided into six groups (n=6). Paw edema was induced by subplantar injection of 0.1 ml 0.5%

Vipin Bulani et al. / Int J Cur Bio Med Sci. 2011; 1(2): 19 – 25.

21

arachidonic acid dissolved in carbonate buffer, pH 8.5 into the right hind paw. Indomethacin (10 mg/kg, i.p., cycloxygenase inhibitor) and montelukast (10 mg/kg, i.p., lipoxygenase inhibitor) as reference standards and methanolic extract of Calotropis gigantea (100, 200, 400 mg/kg, p.o.) was administered 30 min before arachidonic acid injection. Percentage inhibition of paw edema volume was measured by a plethysmograph (UGO Basile 7140, Italy) immediately after arachidonic acid injection at 30, 60, 90 and 120 min.
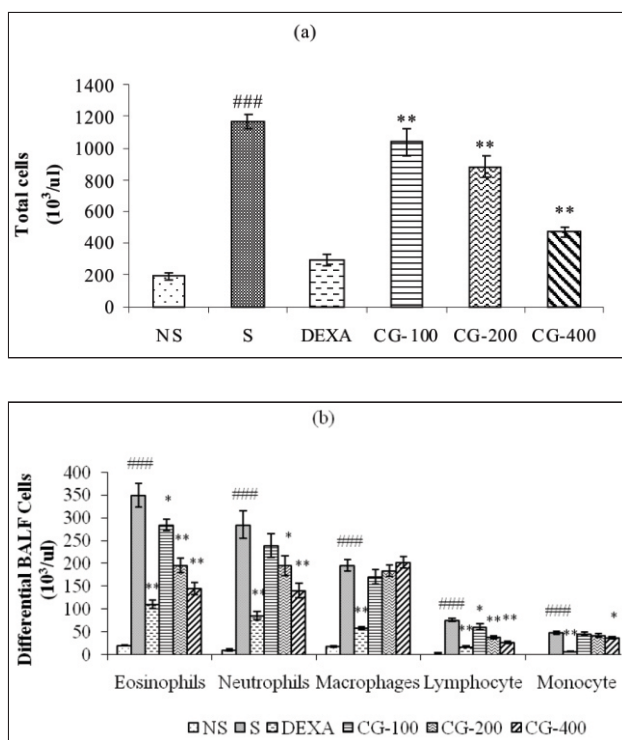
3. Statistical analysis

The results were expressed as Mean ± SEM and statistically analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test, with level of significance set at p<0.05.

4.1. Effect of CG on inflammatory cell counts in BAL fluid.

The total leukocyte, eosinophil, neutrophil, macrophage, lymphocyte and monocyte count was significantly (p<0.001) increased in the OVA sensitized group when compared with non-sensitized group (Fig.1). Dexamethasone (1 mg/kg, i.p.) showed significant (p<0.01) suppressive effect on the total leukocyte, eosinophil, neutrophil, macrophage, lymphocyte and monocyte count in the BAL fluid as compared to the sensitized group. CG extract significantly inhibited (p<0.01) the total leukocytes, eosinophilia and lymphocytes at 200, 400 mg/kg (Fig. 1), where as it significantly reduced (p<0.05) the neutrophil count at the dose of 200, 400 mg/kg. CG extract did not produce any alteration in the macrophage count, while at 400 mg/kg p.o., it showed significant inhibition (p<0.05) of monocytes.

Fig.1. Effect of CG on the recruitment of inflammatory cells in BAL fluid obtained from OVA-induced asthma model in rat.

Statistical analysis done by ANOVA followed by Dunnett's test. Data are expressed as Mean ± S.E.M, n=6, Total cells (a) and Differential cells (b) in BAL fluid., NS = Non-sensitized received 8 mg alum in 1 ml, i.p., S = Sensitized group received ovalbumin 20 µg + 8 mg alum in 1 ml, i.p., DEXA = Dexamethasone 1mg/kg, i.p., CG = Methanolic extract of Calotropis gigantea 100, 200, 400 mg/kg, p.o.,

###p<0.001 compared with non-sensitized; *p<0.05 and **p<0.01 compared with sensitized.

4.2. Effect of CG on histopathological changes in lung tissue.
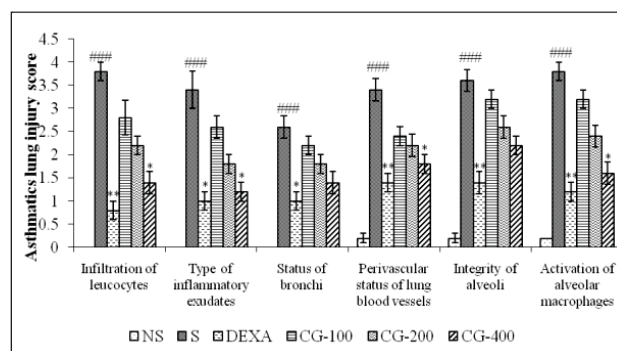
Statistical analysis done by using Nonparametric Kruskal Wallis followed by Dunn's test. Data are expressed as Mean ± S.E.M, n=6, NS = Non-sensitized received 8 mg alum in 1 ml, i.p., S = Sensitized received ovalbumin 20 µg + 8 mg alum in 1 ml, i.p., DEXA = Dexamethasone 1mg/kg, i.p., CG = Methanolic extract of Calotropis gigantea 100, 200, 400 mg/kg, p.o.,

###p<0.001 compared with non-sensitized; *p<0.05 and **p<0.01 compared with sensitized.

Fig.3. Effect of CG on OVA- induced histopathological changes in lung tissue.

Vipin Bulani et al. / Int J Cur Bio Med Sci. 2011; 1(2): 19 – 25.

22
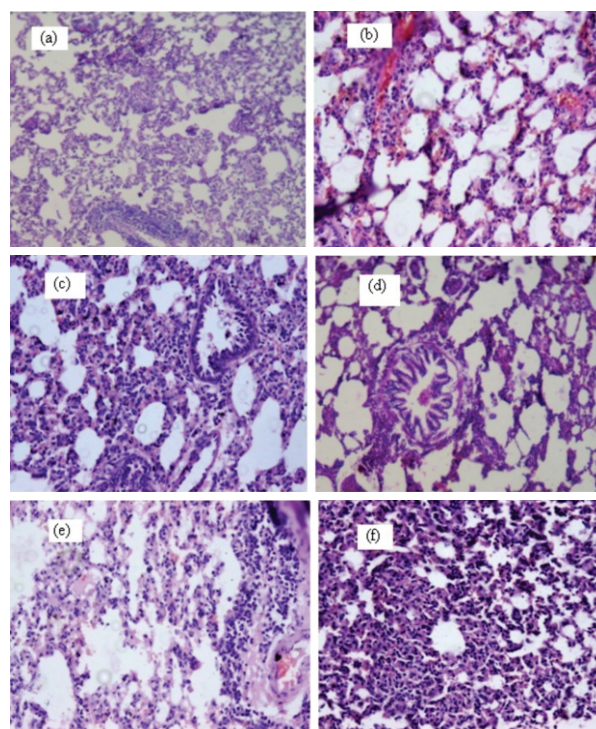
The lung section shown that (a) non-sensitized received 8 mg alum in 1 ml, i.p., (b) sensitized received ovalbumin 20 μg + 8 mg alum in 1 ml, i.p., (c) Dexamethasone 1 mg/kg, i.p., (d), (e), (f) received methanolic extract of Calotropis gigantea 100, 200, 400 mg/kg p.o. respectively.

4.3. Effect of CG on LPO, GSH, SOD, and CAT level in lung tissue.

Ovalbumin significantly (p<0.001) increased the level of LPO and decreased the level of GSH, SOD and CAT in the OVA sensitized group when compared with non-sensitized group (Table.1). Dexamethasone (1 mg/kg i.p.) did not shown any effect on GSH, SOD and CAT levels but it significantly decreased (p<0.01) the level of LPO as compared to the sensitized group. Sensitized group showed significant increase in LPO level of the lung tissue, while animals treated with CG 200, 400 mg/kg significant reduced (p<0.05) the LPO level. Treatment with CG (200, 400 mg/kg) significantly restored (p<0.05) the level of GSH and SOD but did not show any effect on catalase when compared with sensitized group.

Table.1. Effect of CG on lung antioxidant status

Groups	Antioxidant status			
NS	LPO	SOD	GSH	Catalase
NS	8.92±0.45	95.58 ±9.23	345.67 ± 31.34	11.45 ± 0.42
S	19.62 ± 0.76###	54.44 ± 4.46###	204.24 ± 19.48###	5.98±0.46###
DEXA	13.43±0.29**	82.32±7.37	215.83 ± 17.622	6.4 ± 0.23
CG-100	17.85 ± 0.94	67.42 ± 4.99	48.43 ± 26.54	6.59 ± 0.45
CG-200	16.92 ± 0.90*	79.78 ± 4.52*	298.67 ± 23.12	7.12 ± 0.59
CG-400	14.67 ± 0.57**	90.85 ± 7.66**	334.52 ± 28.23**	7.7 ± 0.78

Statistical analysis done by ANOVA followed by Dunnett's test. Data are expressed in Mean ± SEM., NS = non-sensitized received 8 mg alum in 1 ml, i.p., S = sensitized group received ovalbumin 20 μg + 8 mg alum in 1 ml, i.p., DEXA = dexamethasone 1 mg/kg, i.p., CG = methanolic extract of Calotropis gigantea 100, 200, 400 mg/kg, p.o., LPO as nM of MDA/gm of tissue, SOD as units/gm of tissue, GSH is expressed as μg of GSH/gm of tissue, Catalase as μM of H2O2 consumed/gm of tissue.

###p<0.001 compared with non-sensitized; *p<0.05 and **p<0.01 compared with sensitized.

4.4. Effect of CG on nitric oxide and total protein level in BAL fluid

The nitric oxide and total protein level in the BAL fluid was significantly (p<0.001) increased in the sensitized group as compared to the non-sensitized group (Table.2). The nitric oxide and total protein level was significantly decreased (p<0.05) by CG at 100, 200 and 400 mg/kg when compared with the sensitized group. Dexamethasone showed significant reduction in nitric oxide and total protein level (p<0.01) when compared with the sensitized group.

Table.2. Effect of CG on nitric oxide and total protein level in BAL fluid.

Groups	Nitrite (μMol/lit)	Total protein (Gms/dl)
NS	19 ± 1.2	0.98 ± 0.04
S	36 ± 2.6###	3.46 ± 0.044###
DEXA	21 ± 1.8**	1.89 ± 0.07**
CG-100	34 ± 2.1*	3.31 ± 0.082
CG-200	27 ± 1.7**	3.12 ± 0.13*
CG-200	23±2.0**	2.87 ± 0.14**

Statistical analysis done by ANOVA followed by Dunnett's test. Data expressed as Mean ± S.E.M, n=6, NS = Non-sensitized received 8 mg alum in 1 ml, i.p., S = sensitized group received ovalbumin 20 μg + 8 mg alum in 1 ml, i.p., DEXA = dexamethasone 1 mg/kg, i.p., CG = methanolic extract of Calotropis gigantea 100, 200, 400 mg/kg, p.o.,

###p<0.001 compared with non-sensitized; *p<0.05 and **p<0.01 compared with sensitized.

4.5. Effect of CG on lung wet-to-dry weight ratio.

Wet/dry weight ratio was higher in the sensitized group when compared with the non-sensitized group. Pretreatment with CG (200 and 400 mg/kg) significantly reduced (p<0.05) the wet/dry weight ratio which was increased during the OVA induced asthma (Table.3).

Table.3. Effect of CG on lung wet-to-dry (W/D) weight ratio in asthma and chronic

Groups	Lung (W/D) ratio
NS	2.96 ± 0.07
S	4.48 ± 0.09###
DEXA	3.21 ± 0.08**
CG-100	4.15± 0.33
CG-200	3.74 ± 0.07*
CG-200	3.42± 0.13**

Statistical analysis done by ANOVA followed by Dunnett's test. Data are expressed as Mean ± S.E.M, n=6, NS = Non-sensitized received 8 mg alum in 1 ml, i.p., S = Sensitized group received ovalbumin 20 μg + 8 mg alum in 1 ml, i.p., DEXA = Dexamethasone 1 mg/kg, i.p., CG = Methanolic extract of Calotropis gigantea 100, 200, 400 mg/kg, p.o.,

###p<0.001 compared with non-sensitized; *p<0.05 and **p<0.01 compared with sensitized.

23

4.6. Effect of CG on paw edema volume in arachidonic acid induced paw edema in rats.

Arachidonic acid increased the paw edema volume in the control group which was significantly reduced (p<0.01) in the all the other drug treated groups except the indomethacin treated group (Table). CG at dose of 200, 400 mg/kg showed 42.59%, 46.29% inhibition where as montelukast showed 61.11 % inhibition of the paw edema.

Table.4. Effect of CG on Percentage Inhibition in arachidonic acid induced paw edema in rats.

Groups	Percentage Inhibition of Paw Edema			
	30 min	60 min	90 min	120 min
INDO	12.50	5.10	3.70	5.55
MONTE	62.50	56.89	59.25	61.11
CG-100	15.62	20.68	22.22	22.22
CG-200	25.00	34.48	37.03	42.59
CG-400	37.50	41.37	44.44	46.29

Where, n=6, INDO = Indomethacin 10mg/kg i.p., MONTE = Montelukast 10mg/kg i.p., CG = Methanolic extract of Calotropis gigantea 100, 200, 400 mg/kg, p.o.

5. Discussion

In the present study, CG significantly inhibited the characteristics of airway inflammation, including infiltration of inflammatory cells such as lymphocytes, eosinophils, and neutrophils. In addition, CG decreased the activity of reactive oxygen and nitrogen species (ROS/RNS) in OVA-induced airway inflammation reaction.

Asthma is a chronic inflammatory airway disease in which multiple complex pathways are involved. Because of its chronic nature, long term medications are required for therapy. Although, there are numerous conventional medicines available, they are unable to prove satisfactory because, they are unable to block all mechanisms that are responsible for causing asthma, low efficacy, various adverse effects, desensitization of receptor and compliance issue. OVA-induced asthma results from chronic airway inflammation characteristically associated with the infiltration of macrophages, lymphocytes, mast cell, neutrophils and eosinophils into the bronchial lumen [4, 5].

Inflammatory cells recruited to asthmatic airways have an exceptional capability to produce ROS. At the site of inflammation, multiple inflammatory cells including eosinophils, neutrophils and macrophages are capable of generating ROS, which can participate in the development of a variety of diseases, including allergic asthma [42, 43]. Such ROS may contribute to tissue injury and inflammatory reactions. The present results showed that there was ROS generation in BAL fluid of the OVA-sensitized group, which was probably due to the recruited inflammatory cells. The increased ROS generation was substantially reduced by CG.

At physiological concentration, NO functions as vasodilator, neurotransmitter and immune regulator [44]. Excess of NO can react with superoxide radicals leading to formation of harmful peroxynitrite radicals which are responsible for protein nitration, DNA strand breakage and guanine nitation. NO is produced by a variety of cells within the respiratory tract, including not only the inflammatory cells but epithelial cells also [45]. It is generally believed that NO produced by iNOS is associated with pro-inflammatory and damaging effects [46]. All this leads to cytotoxic effect and mutagenesis [47]. In this study, CG extract significantly reduced the production of NO in BAL fluid and thus avoiding the damaging effect of excess NO production.

Leukotrienes are potent pro-inflammatory mediators in the pathogenesis of asthma. Antagonists of LTs agent are beneficial in patients with asthma. Arachidonic acid induced paw edema in rats is an in vivo model to distinguish between cyclooxygenase and lipoxygenase inhibitors [48]. Subplantar injection of arachidonic acid produced significant edema as early as after 30 min and reached a peak at 75 min. It is well known that rat paw edema induced by arachidonic acid is more sensitive to the LOX inhibitor that to the COX inhibitors [48, 32]. The rat paw edema induced by arachidonic acid is perceptibly reduced by inhibitors of arachidonic acid metabolism and by corticosteroids and is insensitive to selective cyclooxygenase inhibitors [49].

The present study showed that intraperitoneal administration of indomethacin did not block edema formation, but edema was inhibited by montelukast and CG. This result indicates that CG causes the inhibition of lipoxygenase pathway of arachidonate metabolism.

Therefore, it can be concluded that Calotropis gigantea may have similar mechanism of action as that of dexamethasone along with antioxidant and anti-lipoxygenase effect may be because of presence of α-amyrin and β-amyrin.

6. Conclusion

The results obtained in the present study suggest that CG may prove to be potential therapeutic drug for treating asthma owing to its anti-inflammatory, anti-lipoxygenase and antioxidant activity. Further detailed experimentation with regards to isolation, purification, mechanisms and pharmacological screening of the active principles in methanolic extract of root of Calotropis gigantea needs to be done.

Acknowledgements

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Vipin Bulani et al. / Int J Cur Bio Med Sci. 2011; 1(2): 19 – 25.

24

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