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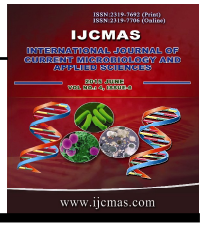

Original Research Article

Anti-inflammatory Effect of *Kalanchoe crenata* Extract in Mice with Experimentally Induced Inflammation

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A B S T R A C T

Most of the non-steroidal anti-inflammatory drugs (NSAIDs) like indomethacin cause some form of damage to liver while giving protection against inflammation. The anti-inflammatory effect of leaf extract of *Kalanchoecrenata*in different solvents with that of indomethacin on mice upon induction of inflammation with formalin was observed. Leaf extracts of *K. crenata* possess anti-inflammatory activity against the inflammation induced by inflammatory agent, formalin on mice paw. Ethanolic extract of the leaves shows dose dependent effect in compared to indomethacin. Biochemical tests carried out with the mice blood after injecting mice paw with formalin and simultaneously administering extract decreases inducible nitric oxide synthase (iNOS) but increases the activity of the antioxidant enzymes super oxide dismutase (SOD), Glutathione peroxidase (GPx), Glutathione reductase (GSR) and catalase. Acid phosphatase activity in mice liver homogenate shows no abnormal liver function.

Introduction

Plant extract according to Ayurvedic system (Sharma, 2009; Dev, 1999) has anti-inflammatory and antiarthritic activity. Phenolics and tannins are important since the role of free radicals in inflammation cascade is well known and phenolicsare established to have free radical scavenging activity. For antimicrobial activity (Cowan, 1999) flavonoids, tannins, alkaloids, tarpenoids and phenolic compounds are responsible. *K. crenata* leaf extract is also used for the treatment of ailments such as ear ache, head ache, inflammation, convulsion, palpitation, small pox, general disability (Dimo *et al.*, 2006) etc. It is also used for the treatment of diabetes mellitus and has also cardio vascular effects (Nguelefack *et al.*, 2004).

This study aims to observe the anti-

inflammatory effect of *Kalanchoecrenata* on mice by artificially inducing inflammation in the hind limb of mice with formalin and also to compare the anti-inflammatory activity of the extract and a non-steroidal anti-inflammatory drug, indomethacin. The experiments have been designed to determine the activity of the antioxidant enzymes (Guzik *et al.,* 2003; Quinlan *et al.,* 1994; Winterbourn, 2008) and iNOS (Dedonand Tannenbaum, 2004) in mice blood after administering the extract and also to determine whether the extract has any toxic effect by estimating the activity of liver acid phosphatase.

Materials & Methods

All the chemicals were purchased from SRL, India. Indomethacin (generic name and brand name Inmecin, 25mg) was purchased from Replica Remedies Pvt. Ltd, India. All other reagents used for the experiment were of Analytical grade. Paw volume was measured by slide callipers.

The plant leaves were collected from *K. crenata* plant in the medicinal plant garden maintained by the Department of Botany, Lady Brabourne College, Kolkata. The leaves were washed thoroughly with distilled water and then with ethanol. Leaves were smashed in mortar by pestle with definite volumes (5 g of leaves in 5 ml solvent) of ethyl alcohol, butanol, and water respectively to prepare ethanolic, butanolic, aqueous extract. The supernatant of centrifugation at 2500 rpm was taken as the final extract for each of the solvents for administration. The three different doses of the leaf extract were prepared having concentrations 1 g/ mL, 2.5 g/ mL and 5g/ mL for each of absolute ethanol, butanol and distilled water.

Female albino mice (Charles Foster strain) weighing 80–100 g purchased from animal house of Indian Institute of Chemical Biology, Kolkata were housed in cages at 27±2°C, 55 % humidity and a 12 : 12 h light-dark cycle at departmental animal house of Lady Brabourne College, Kolkata. They were fed with standard laboratory food (Hindustan Lever Foods, Bangalore, India) and provided with water *ad libitum*.

The investigation conforms to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). It was also approved by the Institutional Animal Ethics Committee, University of Calcutta (Registration no. 885/ac/05/CPCSEA), registered under "Committee for the Purpose of Control and Supervision of Experiments on Laboratory Animals" (CPCSEA), Ministry of Environment and Forests, Government of India.

For the study of the anti-inflammatory effect of the leaf extract, mice were divided into4 groups each having 5 animals.

Group I served as control received only water (mice C)

Group II served as second type of controlfor inflammatory agent formalin (mice F),

Group III served as the test group animals for leaf extractand formalin (mice LE),

Group IV served as the test group animals for indomethacin and formalin(mice IN).

After injecting and measuring the paw volume of mice the 1% formalin was standardized as the potent inflammatory agent and used for the experiment.

Before injection of formalin, the average diameter (Vo) of the right hind paw (Dimo *et al.,* 2003) of each mice was calculated

with Vernier slide calliper method. Paw diameter (Vt) was obtained for each mice at 30, 60, and 120 mins after the administration of formalin as well as extract and indomethacin treatment.

The %inhibition of inflammation was calculated using the formula (Dimo *et al.*, 2003):

% inhibition of inflammation=

$$\frac{(Vt - Vo)\ \text{control} - (Vt - Vo)\ \text{treated}}{(Vt - Vo)\ \text{control}} \times 100$$

Group II (mice F) - The hind paw of each mouse was injected with 0.04 ml of 1% formalin.

Group III (mice LE) - The hind paw of each mice was injected with 0.04 ml of 1% formalin. Simultaneously mice were orally administered with 0.1ml of the extract.

3 different doses of three different types of extracts (ethanolic, butanolic, and aqueous) of the leaves were prepared of 1 g/ml, 2.5 g/ml and 5 g/ml.

Group IV (mice IN) were orally administered with indomethacin (Moscou, 2009) (25mg/ml).

Statistical analysis

The data was analysed using standard formulae (Goon, 1975). The results were expressed as mean ± S.E.M of 5 mice.

After the treatment of 3 consecutive days mice were sacrificed on the 4th day using mild chloroform anaesthesia. Blood was collected by cardiac puncture for biochemical analysis. Livers were excised and immediately kept on ice for enzyme assay.

The SOD assay (Marklund and Marklund, 1974; Nandi and Chaterjee, 1988) was performed from mice blood with pyrogallol (2.6 mM in 10 mMHCl) in presence of 1 mM EDTA and 0.1 M phosphate buffer (pH=8.5). After the addition of enzyme increase in reading at 420 nm by auto oxidation of pyrogallol will be inhibited.50% of inhibition of increase in reading within 2 mins is counted as 1 unit of enzyme per 3 ml of assay mixture.

The assay of Glutathione peroxidase (Gunzler *et al.*, 1974) was performed with sodium azide (1mM), NADPH (0.007 gm/ml), glutathione (40 mg/ml) and hydrogen peroxide in presence of 0.1 M phosphate buffer (pH 7). The enzyme was directly added in cuvette and following 30 sec the decrease in reading at 340 nm was noted.

The enzyme Glutathione reductase was assayed (Bregelues *et al.*, 1983) directly by adding the source to the cuvette containing 0.1M Phosphate buffer (pH=7), 0.2MKCl, 80 mMEDTA, NADPH (20mg/ml) and distilled water. Following 30 sec the decrease in reading at 340 nm was noted.

The enzyme Catalase was added directly in cuvette containing 1 mM sodium azide, 30 mM hydrogen peroxide, 50 mMphosphate buffer (pH=7) and distilled water(Chance and Machley, 1955). Following 15 sec the reading at 240 nm was noted.

The substrate for the enzyme iNOS is 0.5 mM arginine. In this assay (Nins *et al.*, 1996) also the enzyme was added directly in cuvette containing 0.1MMgCl_2, 100 mMNa^+/K^+f errocyanide solution and HEPES buffer (0.1M pH=7.4) along with substrate. Following 3 sec the reading at 420 nm was noted.

Liver was homogenized in ice-cold Na

acetate acetic acid buffer by a homogenizer and homogenate was poured into centrifuge tube and were kept immediately in ice. After centrifugation at 2500 rpm for 10 mins supernatant was collected and used for the enzymatic assay (de Duve *et al.,* 1955) of acid phosphatase in liver. The substrate p-nitrophenyl phosphate (5mM) was incubated with the enzyme in presence of sodium acetate-acetic acid buffer (0.1M, pH=4.5-5). The reaction was stopped with 1N NaOH. Reading was taken at 415 nm after 10-15 mins.

The protein concentration in blood & liver were measured (Lowry et al, 1951) by Folin Lowry method at 660 nm.

Results and Discussion

Anti-inflammatory studies: Formalin induced paw edema showed that among the 3 concentrations of formalin (0.25%, 0.5% and 1%) both 0.5% and 1% formalin induced inflammation. The effect was more in case of 1% solution.

The comparison of the paw diameters of mice after administration of extracts of *K. crenata* on paw edema induced by formalin in mice at 30, 60 and 120 min in different solvents is given in the Table 1. The photograph of the paws, before and after treatment is given in figure 1(A), 1(B) and 1(C).

Comparative study of the % of inhibition of inflammation of different extracts (ethanolic, butanolic, aqueous extracts) of the leaves of *K. crenata* shows that ethanolic extract is the most effective among the three.The effects were comparable for ethanolic and butanolic extracts but as butanol is more toxic than ethanol for further studies ethanolic extract was used (Table 1A).

The % inhibition of inflammation at different doses of ethanolic extract of *K. crenata* leaves at different time showed 62% inhibition at the highest dose compared to the other two doses (25% and 37% at highest time point of other two doses respectively) (Table 1A).

The comparative study of the anti-inflammatory activity of the ethanolic extract and the nonsteroidal anti-inflammatory drug indomethacin shows that the highest dose of ethanolic extract (5g/ml of extract of leaves) is more effective (62.18% inhibition of inflammation) than the indomethacin (41.01% inhibition of inflammation) at 60 mins.

Just before the biochemical analysis *i.e.* before sacrificing the mice the paw diameter of the mice of the respective groups were taken and the readings is summarised in Table 1B. The comparisons of the estimation of SOD, catalase, GPx, Glutathione reductase, iNOS and acid phosphatase is given in the figure 2.

The results of this study indicate that the leaf extracts of *K. crenata* possess anti-inflammatory activity against the inflammation induced by inflammatory agent, formalin on mice paw.

Comparative study of the % of inhibition of inflammation of different extracts (ethanolic, butanolic, aqueous extracts) of the leaves of *K. crenata* shows that ethanolic extract is the most effective among the three, though the effect of the butanolic extract is comparable with that of the ethanolic extract. But as butanol is more toxic than ethanol, for the study of the dose dependent activity of the extract, ethanol was used.

From the result it is also evident that the ethanolic extract of the leaves of *K. crenata*

shows dose dependent effect and this anti-inflammatory activity of the extract increase with dose- higher doses are more effective.

Table.1A Comparison of the effect of different doses of ethanolic extract at different time in paw diameter in mice

Doses	Total increase in paw diameter (mm) (Mean±SEM)		
	30 minutes	60 minutes	120minutes
Dose 1 (1 g/ml)	4.18±0.05	3.59±0.47	3.63±0.44
Dose 2 (2.5 g/ml)	4.18±0.05	3.308±0.74	3.29±0.75
Dose 3 (5 g/ml)	4.18±0.05	2.70±0.50	2.69±0.48

Table.1B Paw diameter (mm) of the mice of four groups before sacrifice

Treatment	Paw volume (Mean ±SEM)
Control(Water)	1.796±.06
Control(Extract)	1.778±.38
Control(formalin)	4.18±.05
Test(extract+formalin)	2.52±.46

a)n=5 in each group

Fig.1(A) Normal mice C paw; **(B)** Mice F paw after injecting only formalin; **(C)** Mice LE paw 1hour after injecting formalin & administration of extract

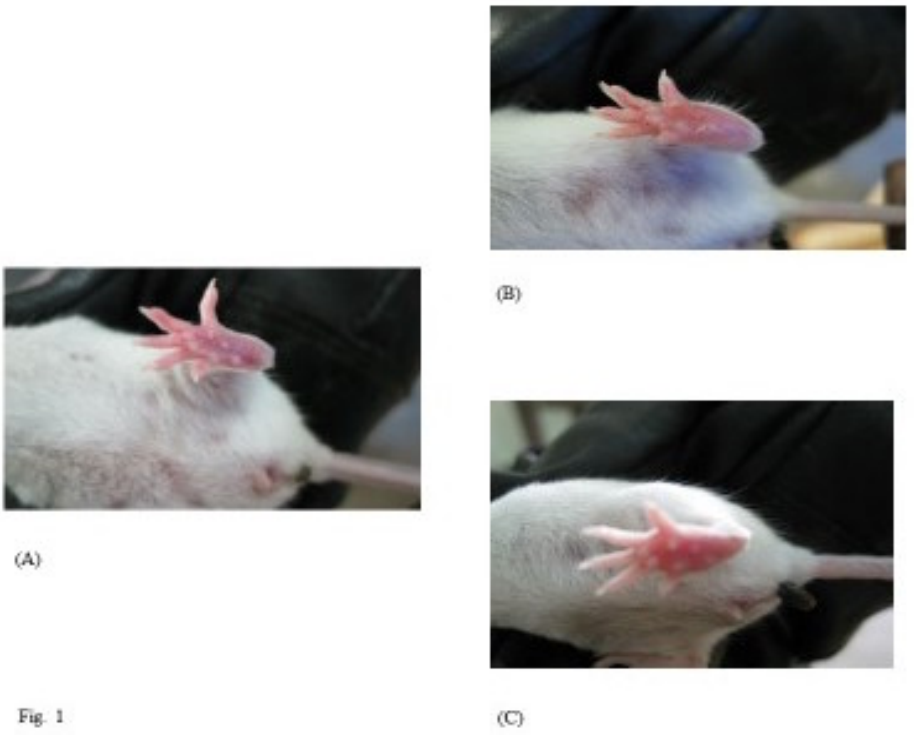

Fig. 1

Fig.2(A) Comparison of the Catalase and iNOS activity in blood of different groups of mice. Each value ±SEM for 5 mice in each group. $p<0.001$ significantly different from control group; **(B)** Comparison of the Glutathione reductase, Glutathione peroxidase and SOD activity in blood of different groups of mice. Each value ±SEM for 5 mice in each group. $p<0.001$ significantly different from control group; **(C)** Comparison of the Acid phosphatase activity in blood of different groups of mice. Each value ± SEM for 5 mice in each group.$p<0.001$ significantly different from control group

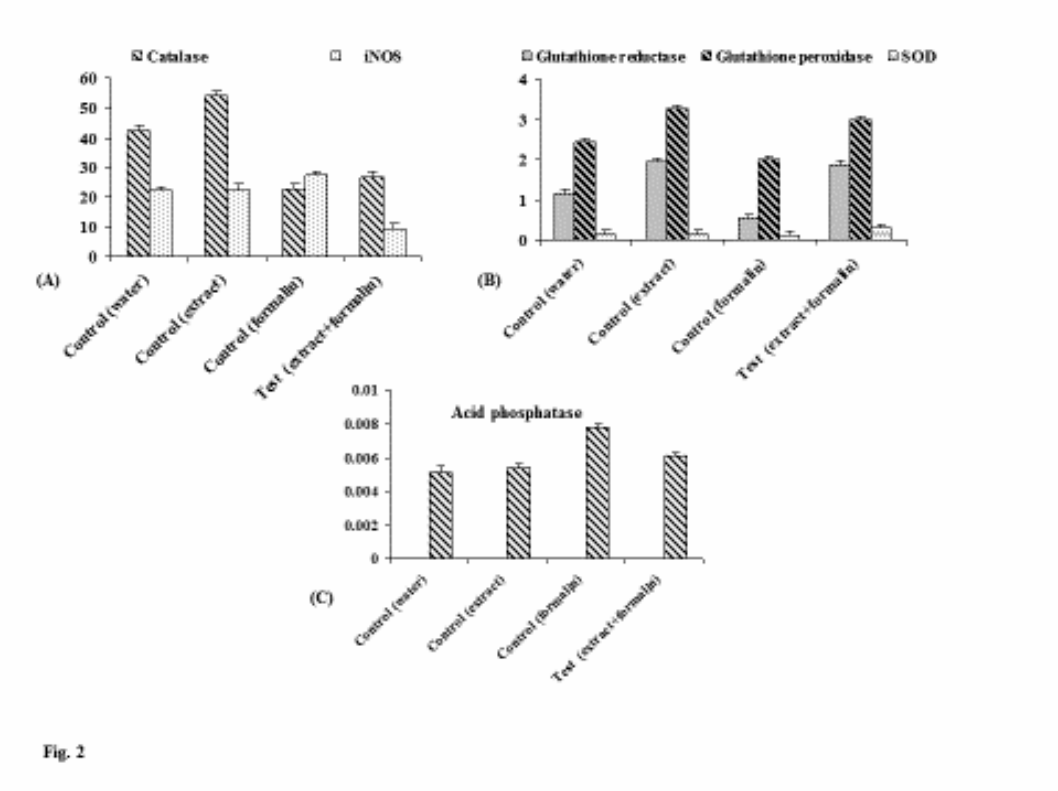

Fig. 2

The comparative aspect of the anti-inflammatory activity of the ethanolic extract with that of indomethacin proved that ethanolic extract has higher anti-inflammatory effect than indomethacin.

In order to establish the anti-inflammatory activity of the extract biochemical tests were carried in mice blood. This study shows that after injecting mice paw with formalin and simultaneously administering extract increases the activity of the antioxidant enzymes SOD, catalase, Glutathione peroxidase and Glutathione reductase (Fig. 2A and B). On the other hand after injecting the mice paw with formalin the activity of these enzymes decreases than that of the mice C. This is due to the fact that on injecting inflammatory agent excess reactive oxygen species are generated causing oxidative stress which is very harmful. The overproduction of ROS overwhelms the capacity of endogenous antioxidant enzyme system. But administration of the extract increases the antioxidant enzyme activity which neutralizes the free radicals generated.

On the other hand the biochemical analysis shows that the administration of the extract simultaneously with the injection of formalin decreases the activity of iNOS

(Fig. 2A) and the activity of this enzyme increases in mice F. Administration of extract overcomes the situation by decreasing the activity of iNOS.

Acid phosphatase activity in mice liver homogenate shows that the activity of the enzyme decreases (Fig. 2C) in case of the mice LE than that of the mice F. The acid phosphatase activity in liver homogenate of mice LE and the mice C are comparable which indicates that the extract has no toxic effect.

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