

Protective Effect of High Molecular Weight Protein Sub-fraction of *Calotropis procera* Latex in Monoarthritic Rats

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Submitted: 25-03-2015

Revised: 16-06-2015

Published: 11-05-2016

ABSTRACT

Background: Proteins present in the latex of *Calotropis procera* have been shown to produce anti-inflammatory effect and to afford protection in various disease models. **Objectives:** To determine the efficacy of high molecular weight protein sub-fraction (LP_{PI}) of latex of *C. procera* in ameliorating joint inflammation and hyperalgesia in a preclinical model of arthritis. **Materials and Methods:** Monoarthritis was induced in rats by intra-articular injection of Freund's complete adjuvant (FCA) and the effect of two doses of LP_{PI} (5 and 25 mg/kg) and diclofenac (5 mg/kg) was evaluated on joint swelling, stair climbing ability, motility, and dorsal flexion pain on day 3. The rats were sacrificed on day 3 to measure tissue levels of reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS). Evaluation of joint histology was also made. **Results:** Intra-articular injection of FCA produced joint swelling and difficulty in stair climbing ability, motility, and pain on flexion of the joint as revealed by scores obtained for these functional parameters. LP_{PI} produced a dose-dependent decrease in joint swelling and improved joint functions. Arthritic rats also revealed altered oxidative homeostasis where joint tissue GSH levels were decreased and TBARS levels were increased as compared to normal rats. The levels of these oxidative stress markers were near normal in arthritic rats treated with LP_{PI}. Moreover, treatment with LP_{PI} also maintained the structural integrity of the joint. The protective effect of LP_{PI} was comparable to the standard anti-inflammatory drug, diclofenac. **Conclusion:** The findings of the present study show that LP_{PI} fraction comprising high molecular weight proteins could be used for the alleviation of arthritic symptoms.

Key words: Arthritis, hyperalgesia, inflammation, latex proteins, oxidative stress

SUMMARY

- High molecular weight protein sub-fraction of latex of *Calotropis procera* (LP_{PI}) reduced joint swelling and hyperalgesia in arthritic rats
- LP_{PI} produced a significant improvement in stair climbing ability and motility in arthritic rats

- LP_{PI} normalized the levels of oxidative stress markers in the arthritic joints
- Treatment with LP_{PI} reduced neutrophil influx and edema in the arthritic joints

High molecular weight protein sub-fraction (LP_{PI}) of Latex of *Calotropis procera*



Calotropis procera

High molecular weight protein sub-fraction (LP_{PI}) isolated from fresh latex



FCA-induced monoarthritis

→

→

➤ Reduces inflammation

➤ Improves joint functions

➤ Ameliorate hyperalgesia and oxidative stress

➤ Preserves tissue architecture

Abbreviations used: FCA: Freund's complete adjuvant, GSH: Glutathione, TBARS: Thiobarbituric acid reactive substances, TBA: Thiobarbituric acid, MDA: Malondialdehyde, LP_{PI}: Latex protein fraction PI.

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DOI: 10.4103/0973-1296.182151

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INTRODUCTION

Arthritis is an inflammatory condition of the joints associated with pain and functional limitations that affect the quality of life. The goal of treatment in this condition is to provide symptomatic relief and to arrest the further progression of disease. The drug therapy for arthritis includes the long-term use of nonsteroidal anti-inflammatory drugs and steroids, both of which are known to produce serious side effects.^[1] In view of this, plants have been considered as a safer alternative source of medicinal agents that have been shown to provide relief both in experimental and clinical set up.^[2] According to WHO, the primary health care need of a large proportion of world population is being met through the use of herbal preparations.^[3] Hence, there is a need to explore such preparations having better efficacy and safety profiles though their standardization remains a big challenge. Studies carried out in *in vivo* models provide a scientific basis for the clinical use of plant-derived preparations and validate their traditional use.

Calotropis procera (Ait) R. Br. is a perennial shrub that grows in wild and belongs to the family Apocynaceae. This plant is found throughout the tropics of Asia and Africa and has been widely used in various traditional medicinal systems.^[4] In last two decades, a number of *in vivo* and *in vitro* studies have been carried out that provide scientific evidence for the medicinal properties of *C. procera*.^[5-8] This plant yields large quantities

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Cite this article as: Chaudhary P, Ramos MV, Vasconcelos Md, Kumar VL. Protective effect of high molecular weight protein sub-fraction of *Calotropis procera* Latex in Monoarthritic Rats. Phcog Mag 2016;12:S147-51.

of milky white latex synthesized by specialized laticifer cells that is a tremendous source of various biologically active metabolites, enzymes, natural polymers, defense proteins, and nonprotein constituents.^[9-12] It also contains rubber (poly-isoprene) responsible for the undesirable effects produced by the latex. Recently, proteins separated from the remaining constituents of the latex have been found to exhibit potent anti-inflammatory and anti-hyperalgesic properties in the rodent model of arthritis.^[13] Further segregation of these proteins by ion-exchange chromatography provided a sub-fraction as peak I (LP_{PI}) comprising high molecular weight proteins that have been shown to alleviate edema formation and neutrophilic influx in acute inflammation.^[14,15] The present study was carried out to evaluate the efficacy of these proteins in ameliorating joint inflammation and dysfunction in a rat model of monoarthritis.

MATERIALS AND METHODS

Animals

Wistar albino rats of either sex, weighing 150–180 g, were used for the study. The animals were kept in the departmental animal house under ambient environmental conditions with free access to food and water. The study was conducted in accordance with the guidelines of Institutional Animal Ethics Committee.

Reagents and drugs

Freund's complete adjuvant (FCA) and diclofenac were purchased from Sigma-Aldrich Co., St. Louis, MO, USA, and Biochem Pharmaceutical Industries Ltd., New Delhi, India, respectively. All other chemicals were of analytical grade and of highest purity commercially available.

Plant material and preparation of high molecular weight protein fraction

The plant *C. procera* was identified by a taxonomist and a voucher specimen has been retained in Prisco Bezerra Herbarium of the Universidade Federal do Ceará, Brazil. The fresh latex collected in distilled water (1:1, v/v) was centrifuged to separate the rubber-like material and the supernatant was dialyzed using a membrane having a cut-off value of 8000 Da. The nondialyzable fraction comprising proteins was freeze dried and subjected to ion-exchange chromatography where three peaks (LP_{PI}, LP_{PII}, and LP_{PIII}) were obtained.^[15] The peak LP_{PI} revealed the presence of high molecular weight proteins by sodium dodecyl sulfate- polyacrylamide gel electrophoresis and was used to carry out the present study.

Experimental design

To study the joint function-related parameters, the overnight fasted rats were trained to climb a staircase (steps at 5, 10, and 15 cm) having water at 2nd step and food at 3rd step for around 1 week. The trained rats were randomly divided into five groups ($n = 6$): Group I served as normal control (NC), Group II served as FCA control (FC) whereas arthritic rats in Groups III and IV received LP_{PI} at 5 and 25 mg/kg doses (LP_{PI}-5 and LP_{PI}-25), respectively, and Group V received standard anti-inflammatory drug diclofenac at 5 mg/kg dose (D5). All the drug solutions were prepared in normal saline. The standard anti-inflammatory drug diclofenac was given orally 1 h before whereas LP_{PI} was given intravenously 30 min before the induction of arthritis and then daily till the end of the study.

Induction and assessment of joint inflammation

Monoarticular arthritis in rats was induced by injecting 0.1 mL of 0.1% FCA into the intra-articular space of right ankle joint according to the method described by Butler *et al.*^[16] Joint inflammation was induced

within 24 h and reached its peak on day 3 as described earlier.^[13] Joint diameter was measured just before the adjuvant challenge (day 0) and daily till the time of peak inflammation (day 3) by using micrometer screw gauge. The extent of inflammation was determined by subtracting the joint diameter of day 0 from day 3 and expressed in mm. After evaluating all the joint function-related parameters on day 3, the animals were sacrificed and the joint tissue was preserved at –80°C to measure the oxidative stress markers. The effect of these proteins was also evaluated on joint histology in a separate set of experiment.

Stair climbing ability

The ability of trained arthritic rats to climb the staircase was scored as: 0 - if the rats did not climb, 1 - if the rats climbed to step one, 2 - if the rats climbed up to step two, and 3 - if the rats climbed all the three steps.^[13]

Motility

The arthritic rats were allowed to move freely on the floor for 5 min and their motility pattern was scored as: 0 - if the rats avoided touching the floor, 1 - if rats walked with difficulty with toes touching the floor, and 2 - if rat walked normally.^[13]

Dorsal flexion pain

The ankle joint of rat was flexed dorsally until the toe touched the anterior part of the leg. The leg was flexed five times at an interval of 5 s and squeaking and leg withdrawal with each flexion was counted. A score of 0 was given if there was no squeaking and no leg withdrawal, 1, if either squeaking or leg withdrawal was present, or 2, if both squeaking and leg withdrawal were present.^[13]

Estimation of tissue glutathione level

Reduced glutathione reacts with 5-5-dithiobis 2-nitrobenzoic acid at alkaline pH to form thionitrobenzoic acid, a yellow-colored compound that gives absorbance at 412 nm. The joint tissue was homogenized in 5% trichloroacetic acid and centrifuged at 3000 rpm for 10 min. The supernatant was used to evaluate the tissue glutathione (GSH) levels according to the method of Ellman.^[17] The standard curve was prepared by using various concentrations of reduced glutathione. Tissue GSH level was determined from the standard curve and expressed as µg/g tissue.

Estimation of tissue thiobarbituric acid reactive substances level

Thiobarbituric acid (TBA) reacts with lipid peroxides and aldehydes (malondialdehyde [MDA], N-aldehyde, and α , β aldehyde) to form colored chemical compound that gives absorbance at 532 nm. The joint tissue was homogenized in 0.15 M KCl and the homogenate was used to evaluate the tissue MDA levels according to the method of Ohkawa *et al.*^[18] The standard curve was prepared by using various concentrations of 1, 1, 3, 3-tetraethoxypropane. Tissue MDA level was determined from the standard curve and expressed as nM/g tissue.

Histological analysis

The arthritic joint was removed and fixed in 1% formaldehyde in saline. The fixed joints were decalcified in ethylenediaminetetraacetic acid, processed for paraffin embedding, and sectioned. The mounted slides were stained with eosin and hematoxylin to examine the inflammatory changes.^[19]

Statistical analysis

Increase in joint diameter and oxidative stress parameters are presented as mean \pm standard error of mean and one-way ANOVA followed by

post hoc test (least significant difference) was used to compare the treated groups with FC group. The joint functional parameters, i.e., stair climbing ability, motility, and dorsal flexion pain are expressed as median score, and Fisher's exact test was used to compare the treated groups with FC group. The statistical analysis was carried out by SPSS program version 17 (SPSS Inc. Chicago) and STATA version 11 (Stata Corp LP, College Station, Texas). The statistical significance was considered at $P < 0.05$.

RESULTS

Effect of latex protein fraction PI on joint inflammation

The intra-articular injection of FCA in rat ankle joint produced a peak inflammatory response on day 3 with increase in joint diameter of 4.04 ± 0.17 mm. However, injection of 0.1 mL normal saline produced a marginal increase in joint diameter of 0.12 ± 0.01 mm on day 3. Treatment with LP_{PI} produced a dose-dependent inhibition of joint swelling, and increase in diameter was 2.81 ± 0.12 and 1.97 ± 0.13 mm in LP_{PI}-5 and LP_{PI}-25 groups, respectively (30% and 51% inhibition). The inhibitory effect of LP_{PI}-25 was found comparable to that of D5 group where the increase in joint diameter was 1.90 ± 0.20 mm (53% inhibition) [Figure 1].

Effect of latex protein fraction PI on stair climbing ability

The rats in FC group experienced difficulty in climbing the staircase, and a median score of 0 was obtained in this group in comparison with median score of 3 in NC group on day 3. Treatment with LP_{PI} improved the stair climbing ability of the arthritic rats, and median scores of 2.5 and 3 were obtained in LP_{PI}-5 and LP_{PI}-25 groups, respectively. The effect of LP_{PI}-25 was found comparable to that of standard anti-inflammatory drug diclofenac, where the median score was 3 [Table 1].

Effect of latex protein fraction PI on motility

The intra-articular injection of FCA affected the motility of rats, and a median score of 0 was obtained in FC group as compared with NC group having median score of 2. Treatment with LP_{PI} at 5 and 25 mg/kg doses improved the motility of arthritic rats, and median scores of 1.5 and 2 were obtained on day 3 at the two doses [Table 1].

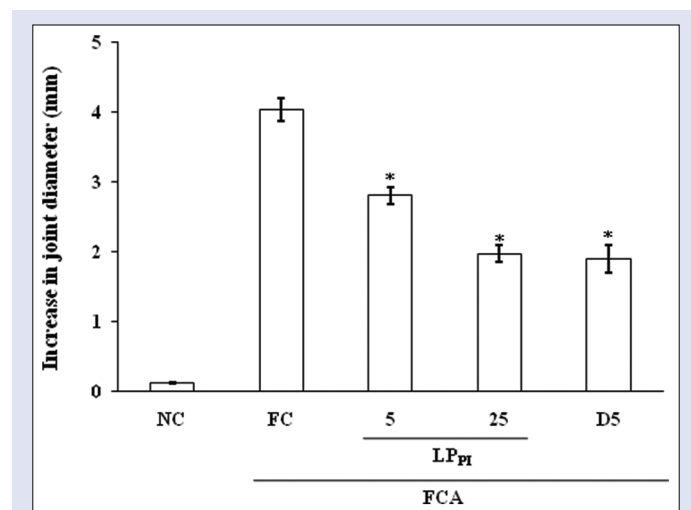


Figure 1: Effect of LP_{PI} on increase in joint diameter in monoarthritic rats. $n = 6$. * $P < 0.05$ versus Freund's complete adjuvant control group, ANOVA followed by least significant difference *post hoc* test

Effect of latex protein fraction PI on dorsal flexion pain

The inflamed joints of arthritic rats were flexed and scored to assess the effect of LP_{PI} on hyperalgesia. The injection of FCA lowered the threshold of pain in FC group, and a median score of 9 was obtained on day 3. Treatment with LP_{PI} afforded protection against pain, and median scores of 6 and 5 were obtained in LP_{PI}-5 and LP_{PI}-25 groups, respectively [Table 1].

Effect of latex protein fraction PI on tissue glutathione level

Intra-articular injection of FCA produced a marked decrease in tissue GSH level (83.33 ± 18.16 $\mu\text{g/g}$) in FC group in comparison with NC group (279.16 ± 26.74 $\mu\text{g/g}$ tissue). Treatment with LP_{PI} increased the tissue levels of GSH and in LP_{PI}-5 and LP_{PI}-25 groups, the tissue GSH levels were 184.37 ± 8.80 and 187.50 ± 43.69 $\mu\text{g/g}$ tissue, respectively [Figure 2a].

Effect of latex protein fraction PI on tissue thiobarbituric acid reactive substances level

The injection of FCA produced a marked increase in TBA reactive substances (TBARS) level (291.66 ± 54.67 nM/g tissue) as compared with NC group (104.16 ± 14.28 nM/g tissue). Treatment with LP_{PI} dose dependently decreased the tissue TBARS levels to 135.00 ± 22.32 and 95.00 ± 18.16 nM/g tissue in LP_{PI}-5 and LP_{PI}-25 groups, respectively. The TBARS level in D5 group was 105.83 ± 31.31 nM/g tissue [Figure 2b].

Effect of latex protein fraction PI on joint histology

The intra-articular injection of FCA in rat joint produced peak inflammation in the joint on day 3, which is characterized by neutrophilic infiltration and tissue edema. Treatment with LP_{PI} maintained joint tissue integrity and its effect was comparable to that of diclofenac [Figure 3].

DISCUSSION

Preparations derived from different parts of plants have been used for medicinal purpose from time immemorial. *C. procera* is a wild growing plant that has been shown to possess numerous medicinal properties and it has been used in the traditional medicinal system for treating various diseases.^[4] The plant produces latex when it gets injured and this latex has also been shown to afford protection in several disease models.^[20-23] Recently, it has been shown that the latex of this plant comprises both nonprotein and protein constituents that inhibit inflammation induced in an animal model.^[5,6] The proteins present in latex have been separated into three fractions by ion-exchange chromatography, and the fraction comprising high molecular weight proteins (LP_{PI}) has been demonstrated to possess anti-inflammatory property.^[15] The present study was aimed to evaluate the protective effect of LP_{PI} against inflammatory hyperalgesia, functional disability, and oxidative stress in monoarthritic

Table 1: Effect of LP_{PI} on joint functions

Groups	Joint functions test		
	Stair climbing ability (median score)	Motility (median score)	Dorsal flexion pain (median score)
NC	3	2	0
FC	0	0	9
LP _{PI} 5	2.5	1.5	6*
LP _{PI} 25	3*	2*	5*
D5	3*	2*	5*

Values given are median scores ($n=6$). * $P < 0.05$ versus FC group, Fisher's exact test. NC: Normal control; FC: Freund's complete adjuvant control group; LP_{PI}: Latex protein fraction PI

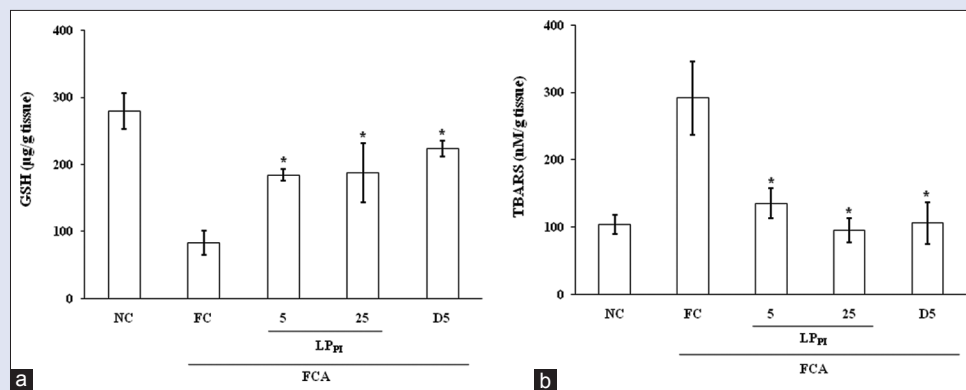


Figure 2: Effect of LP_{pi} on the levels of glutathione (a) and thiobarbituric acid reactive substances (b) in joint tissue of monoarthritic rats. $n = 6$. * $P < 0.05$ versus Freund's complete adjuvant control group, ANOVA followed by least significant difference *post hoc* test

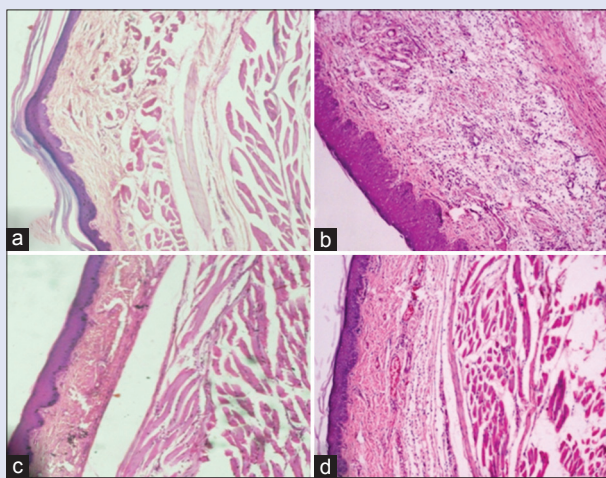


Figure 3: Effect of LP_{pi} on joint histology in arthritic rats. Normal control (a), Freund's complete adjuvant control (b), Diclofenac 5 mg/kg (c) and LP_{pi}-25 mg/kg (d) ($X = 100$)

rat. Upon intra-articular injection, FCA induced inflammation of the joint as evident by swelling, redness, and pain. Treatment with LP_{pi} suppressed the joint inflammation and decreased the joint swelling in a dose-dependent manner. Recently, this fraction has been shown to inhibit edema formation in the rat model of acute inflammation.^[14]

As inflammation and associated pain are characteristic features of the arthritic joint that affect daily activity, the present study also evaluated the effect of LP_{pi} on joint functions. Compared to arthritic rats, LP_{pi} treated rats were able to climb and move like normal rats and their pain threshold was also elevated as revealed by dorsal flexion pain test. In this regard, the present study shows that the beneficial effect of LP in arthritis could be due to the proteins present in its sub-fraction LP_{pi}. Further, it is interesting to note that the proteins derived from the latex inhibit the release of tumor necrosis factor alpha (TNF- α), prostaglandin E₂, and inflammatory response elicited by other mediators, which have a significant role in pain perception.^[24] Earlier LP has been reported to exhibit anti-nociceptive property that has been found to be comparable to that of morphine.^[20]

It is well established that oxidative homeostasis gets altered in inflammatory conditions and free radicals generated in the swollen joints produce damage to the cellular components such as proteins,

DNA, and membrane lipids. Unchecked production of free radicals leads to disease progression and destruction of cartilage in arthritic conditions.^[25] In the present study, FCA injection into the joint increased the levels of TBARS, a marker of lipid peroxidation, along with reduction in the level of GSH, an intracellular antioxidant. Treatment with LP_{pi} and diclofenac maintained the tissue levels of these oxidative stress markers in monoarthritic rats. Several reports on the antioxidant property of proteins derived from latex, roots, and explant culture of *C. procera* are available.^[8,22,26] Besides, antioxidants have also been shown to ameliorate arthritic dysfunction by scavenging free radicals.^[27]

Histological analysis of the arthritic joints revealed that the FCA-induced inflammation was associated with intense neutrophil infiltration and tissue edema. The protection afforded by LP_{pi} was also evident from a marked reduction in neutrophil infiltration as observed in treated groups. The protective effect of LP_{pi} was found to be better than that of diclofenac. The anti-infiltrative and anti-edematogenic property of latex proteins have been well established in animal models.^[14,15] The activated neutrophils are known to contribute to arthritic conditions by the release of inflammatory mediators, oxygen radicals, and tissue degrading enzymes. In addition, neutrophils synthesize eicosanoids and pro-inflammatory cytokines that eventually degrade the matrix components.^[28] The inhibitory effect of latex proteins has been demonstrated on the expression of iNOS, COX-2, TNF- α , and interleukin (IL-1 β) in hamster model of oral mucositis.^[21]

Thus, the findings of present study indicate that LP_{pi} has a therapeutic potential in the treatment of arthritis.

Acknowledgments

Part of the study has been supported by grants from National Council for Research and Development (CNPq), Brazil. This study is part of the consortium Molecular Biotechnology of Plant Latex. The Senior Research Fellowship to PC from DBT, New Delhi, is acknowledged.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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