

Protective Effect of Proteins Derived from Seed Explant Cultures of *Calotropis procera* in a Preclinical Model of Arthritis

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Abstract

Proteins isolated from the *in vitro* cultures of callus and roots developed from the germinating seed explants of *Calotropis procera* were evaluated for their protective effect in a rat model of arthritis at 1 and 5 mg/kg doses in two independent sets of experiments comprising of respective controls. Joint swelling, functional parameters, markers of inflammation, oxidative stress, tissue histology, and cyclooxygenase-2 expression in these rats were compared with those treated with the standard anti-inflammatory drug diclofenac on day 3. Both callus and root proteins produced a dose-dependent reduction in joint swelling, and at the 5-mg/kg dose, their inhibitory effect was more pronounced (57 and 55% inhibition) than that of diclofenac (42 and 46% in two sets). Both of the protein fractions alleviated functional limitations in arthritic rats and normalized the levels of prostaglandin E₂, tumor necrosis factor- α , and other biochemical markers of inflammation and oxidative stress, maintained tissue architecture, and suppressed cyclooxygenase-2 expression compared to arthritic controls. This study demonstrates that the proteins derived from the *in vitro* culture of *C. procera* explants have potential in the treatment of arthritis.

Key words

Calotropis procera · Apocynaceae · callus · inflammation · medicinal plant · roots

Plants have served as living factories for time immemorial to meet the requirement of medicinal agents that have been used in various traditional systems for the prevention and cure of several diseases. Their demand for a medicinal purpose has been met either from wild growing or cultivated plants, and various environmental factors, methods of collection, and processing are well known to result in variability in efficacy and safety of medicinal preparations derived from them [1]. In this regard, their correct identification, documentation, and conservation are important aspects to be considered in order to have a sustainable resource [2]. Thus, growing the medicinal plants in a controlled environment is an alternative option to minimize variability, and plant tissue cultures have been used as a source of pharmacologically active secondary metabolites [3,4].

Calotropis procera Ait. R. Br., a member of the Apocynaceae family, is a wild-growing plant that has been used for the treatment of various diseases of the liver, abdomen, and those involving inflammation. Different parts of this plant, including its latex, have been shown to possess medicinal properties [5]. Both the non-

protein constituents present in the aqueous and organic extracts of latex and the proteins isolated from the latex have been reported to produce anti-inflammatory and antinociceptive effects in rodent models [6–9]. Recently, proteins isolated from the callus and roots developed from *in vitro* explants of the germinating seeds of this plant have also been shown to inhibit an acute inflammatory response [10,11]. The present study was carried out to investigate the efficacy of these proteins in a preclinical model of monoarthritis.

Monoarthritis was induced by an intra-articular injection of Freund's complete adjuvant (FCA) and the effect of callus proteins (CP) and root proteins (RP) was evaluated in two independent sets of experiments. **Fig. 1** shows that FCA produced an increase in joint diameter by 4.61 ± 0.18 and 4.40 ± 0.26 mm in two sets on day 3 against a marginal increase observed in the normal control (NC) group, where normal saline was injected into the joint. Treatment of arthritic rats with CP and RP produced a dose-dependent reduction in joint swelling at 1 and 5 mg/kg doses. These proteins have been reported earlier to inhibit edema formation, mediator release, and neutrophil migration in rat models of acute inflammation [10,11]. As functional limitations of the joint and hyperalgesia are common complaints in arthritis, stair climbing ability (SCA), motility, and dorsal flexion pain scores (DFP) were evaluated in the arthritic rats. **Table 1** shows that the arthritic rats experienced difficulty in climbing and moving (median score 0 against 3 for climbing and 2 for motility in the NC group in both sets) and also experienced pain on flexion of the inflamed limb (median DFP score 9 and 10 in sets I and II against 0 in the NC group). Both CP and RP improved joint functions and ameliorated hyperalgesia, and their effect was comparable to that of diclofenac, an anti-inflammatory drug commonly used in arthritis [12]. Such a protection has earlier been reported for proteins derived from the latex of this plant though their protein profile is distinct from CP and RP and for the dried latex and its methanol extract [10,13–15]. Like latex proteins, both CP and RP have also been reported to exhibit antinociceptive property [9,10]. Their antihyperalgesic effect observed in the present study was accompanied by the normalization of levels of oxidative stress markers and a reduction in the levels of inflammatory mediators, namely nitrite and myeloperoxidase (MPO), compared to arthritic controls (**Fig. 2**). MPO, a hemoprotein, is a well-known marker of neutrophils that participates in both primary host defense and cellular signaling. Along with nitric oxide (NO), it is well known to affect vascular events in an inflammatory response. Therapeutic strategies aiming at restoring redox homeostasis, removing MPO, and inhibiting NO generation have been considered promising tools in inhibiting an inflammatory response [16–18].

Inflammation of the joint as seen in arthritis is driven through the excessive production of prostaglandin E₂ (PGE₂) and tumor necrosis factor- α (TNF- α). Their role in the perpetuation of inflammation and pain is evident from the fact that nonsteroidal anti-inflammatory drugs (NSAIDs) and TNF- α blockers provide symptomatic relief from pain and stiffness [12,19]. In the present study, a marked increase in the level of TNF- α and PGE₂ was observed in arthritic rats at the time of peak joint swelling, a time that coincides with maximum hyperalgesia and loss of function. Treatment of arthritic rats with both CP and RP brought down the levels of these mediators (**Fig. 3**). The protection afforded by these proteins was comparable to that of diclofenac, a drug well known to exert its action by inhibiting cyclooxygenase and resultant prostaglandin synthesis [20]. The protective effect of these

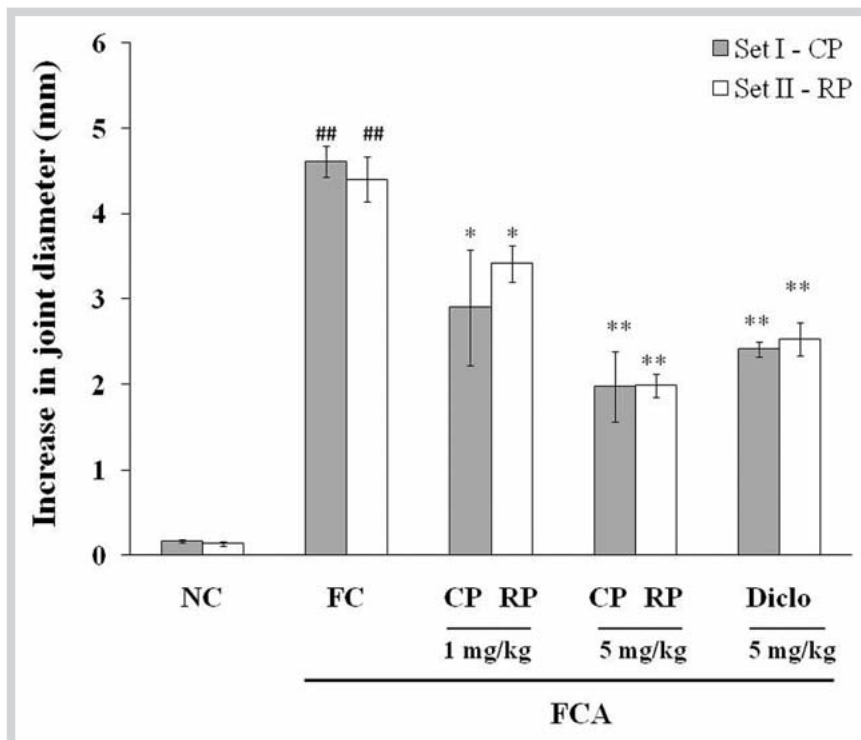


Fig. 1 Effect of CP and RP on the increase in joint diameter in monoarthritic rats on day 3. The results are given as mean \pm SEM (n = 6). ##P < 0.001 compared to NC, *p < 0.05 and **p < 0.001 compared to FC.

Treatment groups	DFP		Motility		SCA	
	Set I	Set II	Set I	Set II	Set I	Set II
NC	0	0	2	2	3	3
FC	9 [#]	10 [#]	0 [#]	0 [#]	0 [#]	0 [#]
CP/RP 1 mg/kg	5*	5*	1.5*	1.5*	2*	2*
CP/RP 5 mg/kg	3*	5*	2*	2*	3*	3*
Diclo 5 mg/kg	5*	6*	2*	2*	3*	2*

Table 1 Effect of CP and RP on functional parameters in monoarthritic rats.

Set I and Set II were treated with CP and RP, respectively. DFP: dorsal flexion pain; SCA: stair climbing ability. Values given are median scores (n = 6). #P < 0.05 compared to NC, *p < 0.05 compared to FC

proteins was also evident from the histological analysis of the joint where a marked reduction in edema and neutrophilic infiltration was observed and from immunohistochemical analysis where these proteins suppressed cyclooxygenase-2 (COX-2) expression (► Fig. 4). As COX-2 is an inducible enzyme that brings about the synthesis of PGE₂, its inhibition by CP and RP is an important protective mechanism in arthritis.

Thus, our study shows that proteins derived from the callus and roots developed from the germinating seed explants of *C. procera* have potential in the treatment of arthritis.

Materials and Methods

Seeds collected from a *C. procera* plant growing in Fortaleza, Brazil (voucher specimen No. 32663, Prisco Bezerra herbarium of the Federal University of Ceará) were germinated and the *in vitro* grown plantlets were used as a source for hypocotyl and cotyledon explants that were induced to develop into callus and roots, respectively, and freeze-dried. Proteins were obtained from the freeze-dried material by homogenizing and stirring it in 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl followed by centrifugation to obtain a soluble fraction that was dialyzed against distilled water using the membrane with a cutoff of 8000 Da and

freeze-dried. The details of the procedure and protein profile of CP and RP as revealed by two-dimensional polyacrylamide gel electrophoresis have been reported earlier [10].

Wistar rats of either sex (150–180 g) were trained to climb a staircase having three steps (5, 10, and 15 cm) for a week as described earlier [13]. The experiments were carried out as per protocol approved by the Institutional Animal Ethics Committee (645/IAEC/11 dated 30/1/2012). Joint inflammation was induced by an intra-articular injection of 0.1 ml of FCA in the left ankle joint in two sets of experiments (one each for CP and RP comprising of five groups, n = 6 per group) having independent normal and arthritic controls (NC and FC). Rats were treated with 1 and 5 mg/kg of CP in set I and RP in set II, and diclofenac (5 mg/kg; Diclo) in both sets administered intravenously and orally 30 min and 1 h before inducing arthritis, respectively, and then daily. Parameters like joint diameter, SCA, motility, and DFP were recorded before inducing arthritis and then daily for 3 days [13, 14]. Joint swelling was expressed as an increase in joint diameter in mm on day 3 while SCA was scored 1, 2, or 3, motility was scored 0, 1, or 2, and DFP was scored 0 to 10 based on squeaking and leg withdrawal when tested five times as described earlier [13]. The animals were sacrificed and the joint tissue was pre-

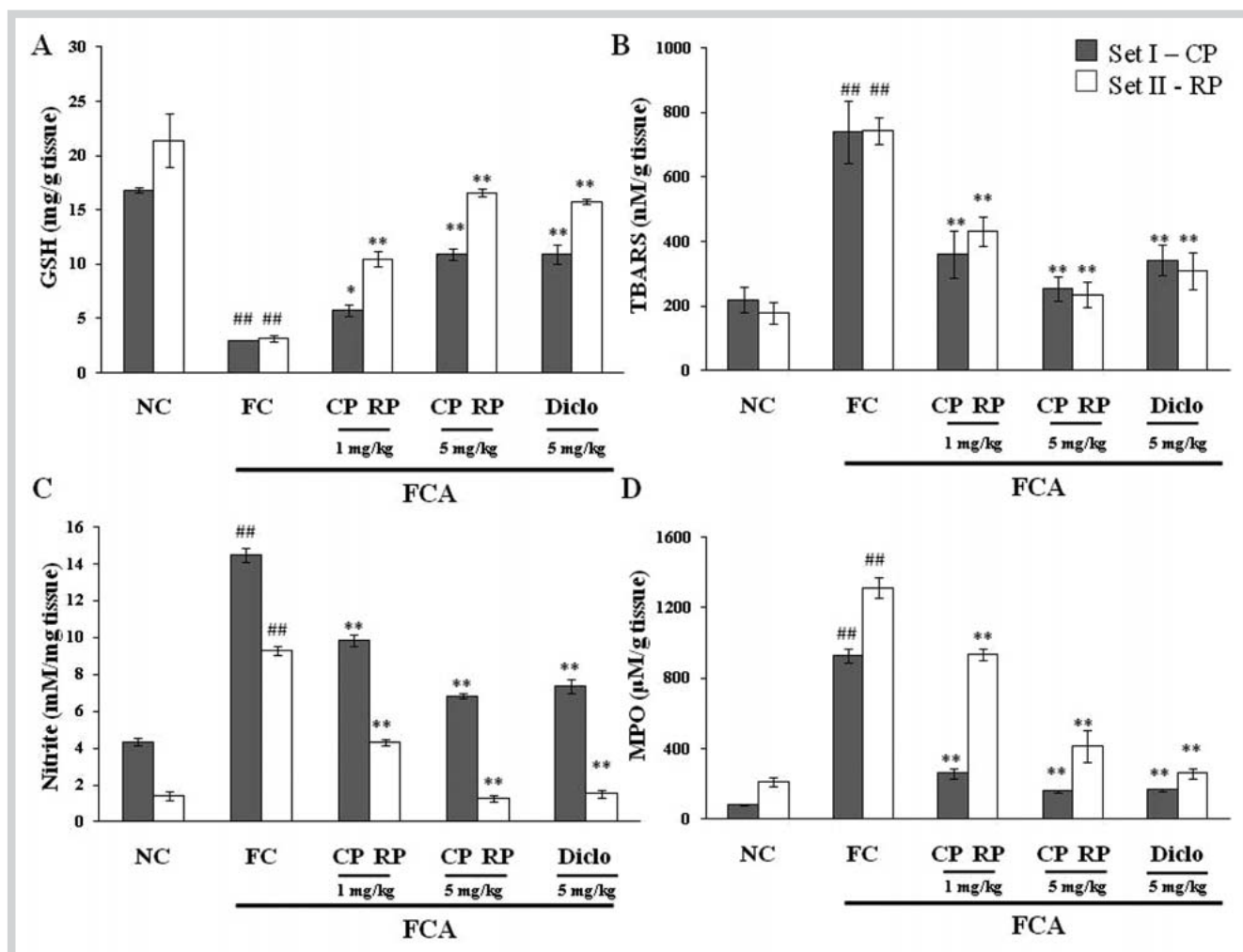


Fig. 2 Effect of CP and RP on tissue levels of GSH (A), TBARS (B), nitrite (C), and MPO (D) in monoarthritic rats on day 3. The results are given as mean \pm

SEM (n = 6). ##P < 0.001 compared to NC, *p < 0.05 and **p < 0.001 compared to FC.

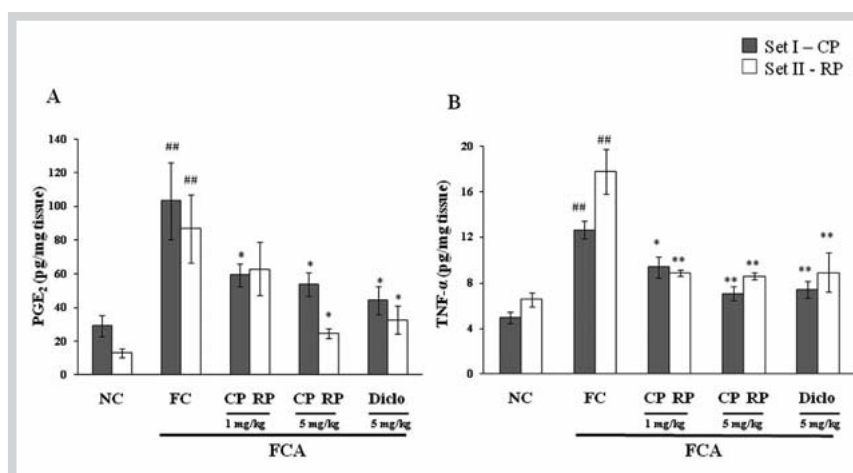


Fig. 3 Effect of CP and RP on tissue levels of PGE₂ and TNF- α in monoarthritic rats on day 3. The results are given as mean \pm SEM (n = 6). ##P < 0.001 compared to NC, *p < 0.05 and **p < 0.001 compared to FC.

served for biochemical estimations at -80°C while histological and immunohistochemical analyses for COX-2 expression were carried out in a separate set of experiments [21]. Reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS, an index of lipid peroxidation), nitrite (an index

of nitric oxide production), and MPO were measured by the methods described earlier [22–25]. The tissue levels of TNF- α and PGE₂ were measured by ELISA kits (Diacclone and Cayman Chemical Company).

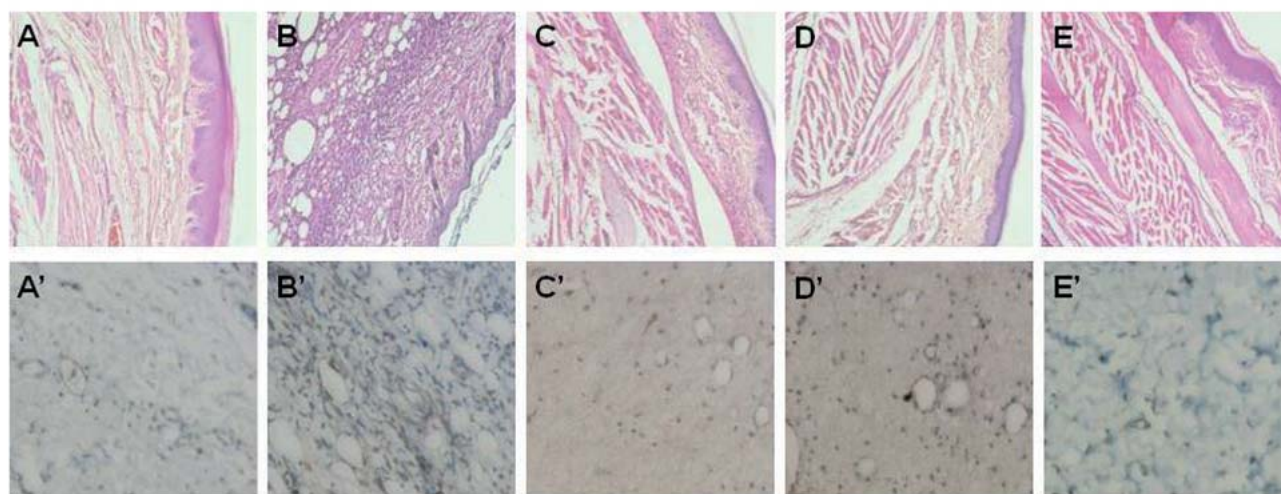


Fig. 4 Effect of CP and RP on joint tissue histology (A–E, magnification 100×) and COX-2 expression (A'–E', magnification 400×) in monoarthritic

rats on day 3. **A** and **A'**: normal control; **B** and **B'**: FCA control; **C** and **C'**: CP 5 mg/kg; **D** and **D'**: RP 5 mg/kg; **E** and **E'**: diclofenac 5 mg/kg.

Values for the increase in joint diameter and biochemical parameters are given as mean ± SEM and were analyzed by ANOVA followed by a post hoc test (LSD). Functional parameters are given as a median score and the Kruskal-Wallis test was used for comparison.

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Conflict of Interest

The authors declare no conflict of interest.

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