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Efficacy of boswellic acid on lysosomal acid hydrolases, lipid peroxidation and anti-oxidant status in gouty arthritic mice

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ABSTRACT

Objective: To evaluate the efficacy of boswellic acid against monosodium urate crystal-induced inflammation in mice. **Methods:** The mice were divided into four experimental groups. Group I served as control; mice in group II were injected with monosodium urate crystal; group III consisted of monosodium urate crystal-induced mice who were treated with boswellic acid (30 mg/kg/b.w.); group IV comprised monosodium urate crystal-induced mice who were treated with indomethacin (3 mg/kg/b.w.). Paw volume and levels/activities of lysosomal enzymes, lipid peroxidation, anti-oxidant status and inflammatory mediator TNF-α were determined in control and monosodium urate crystal-induced mice. In addition, the levels of β-glucuronidase and lactate dehydrogenase were also measured in monosodium urate crystal-incubated polymorphonuclear leucocytes (PMNL) *in vitro*. **Results:** The activities of lysosomal enzymes, lipid peroxidation, and tumour necrosis factor-α levels and paw volume were increased significantly in monosodium urate crystal-induced mice, whereas the activities of antioxidant status were in turn decreased. However, these changes were modulated to near normal levels upon boswellic acid administration. *In vitro*, boswellic acid reduced the level of β-glucuronidase and lactate dehydrogenase in monosodium urate crystal-incubated PMNL in concentration dependent manner when compared with control cells. **Conclusions:** The results obtained in this study further strengthen the anti-inflammatory/antiarthritic effect of boswellic acid, which was already well established by several investigators.

1. Introduction

Gouty arthritis is a metabolic disease manifestated by an increase in serum urate concentration and deposits of monosodium urate crystals with intense pain, reddening and swelling in joints[1]. Gouty arthritis is the most common form of arthritis seen in general practice in adults, with a prevalence of about 1.4%. The deposition of monosodium urate ctystals in synovium and cartilage can promote chronic inflammation that may lead to the damage to bone, cartilage, and other joint tissues such as tophaceous destruction or degenerative joint disease[2]. The primary pathologic hallmark of gout is neutrophil influx into the joint fluid. Neutrophils accumulate in both the joint fluid and the synovial membrane, where a small fraction of these cells actively phagocytose monosodium urate crystals resulting in membranolysis, generation of oxygen derived free radicals and the release of lysosomal enzymes, prostaglandin E2, leukotrienes and interleukin-1[3]. Gout has become more common and more clinically complex in recent years, particularly in older subjects. In addition, men with gout have an increased risk of death as a result of an elevated risk of cardiovascular diseases, particularly coronary heart diseases[4]. Nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids and colchicine have been used for treatment of crystal-induced inflammation for many years. However, although these agents are generally effective, they also present serious side effects such as gastrointestinal toxicity, renal toxicity, or gastrointestinal bleeding.

Recently, herbal medicines have been receiving attention as an alternative medicine and health supplement. Crude drugs prepared from plant materials are traditionally used and their active principles have been extensively studied from various view points. In particular, it was reported that boswellic acid, a mixture of triterpenic acids obtained from the oleo gum resin of *Boswellia serrata* has extensively been studied for a number of activities including anti-

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inflammatory, immunomodulatory, anti-tumor activities, and inflammatory bowel disease. It also belongs to a non-steroidal anti-inflammatory class of drugs with a different mechanism of action rather than those of the common NSAIDs[5]. Its anti-inflammatory properties were proved by inhibiting 5-lipoxygenase, human leukocyte elastase and the nuclear factor-_B pathway, without exerting the adverse effects known for steroids[6]. Among the six most important derivatives of boswellic acids, KBA and AKBA are the most potent inhibitors of 5-lipoxygenase[7]. Even though, several investigators reported the anti-inflammatory effect of boswellic acids. The scientific data supporting the use of boswellic acid in gouty arthritis are not available, therefore the present study was aimed to evaluate the efficacy of boswellic acid on monosodium urate crystal inflammation in mice which is an experimental model for gouty arthritis.

2. Materials and methods

2.1. Animals

Swiss albino mice, (25-30 g), of either sex were obtained from Tamil Nadu Veterinary College, Chennai, India. They were acclimatized for a week in a light and temperature -controlled room with a 12 h dark-light cycle and fed with commercial pelleted feed from Hindustan Lever Ltd. (Mumbai, India) and water was freely available. The animals were treated and cared for in accordance with the guidelines recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, Ministry of Culture, Chennai. The experimental protocol was approved by our departmental ethics committee.

2.2. Drugs

The commercially available boswellic acid (pentacyclic triterpenoid acid mixture ($\alpha+\beta$) isolated from gum resin of *Boswellia serrata* Roxb., Family: Burseraceae, a fine white crystalline powder, >95% purity by HPLC, Lot no: T7P011) was purchased from Natural Remedies Ltd., Bangalore, India and stored at -20 ℃. Indomethacin was purchased from Tamil Nadu Dadha Pharmaceuticals Ltd., Chennai, India. A homogenous suspension of boswellic acid and indomethacin was made with 0.5% carboxy methyl cellulose in phosphate buffered saline. Fresh solution was prepared before each experiment. All other reagents used were standard laboratory reagents of analytical grade and were purchased locally.

2.2.1. Dosage

Based on our preliminary studies with different dosages (10 mg, 20 mg, 30 mg) of this boswellic acid, it was found that 30 mg/kg b.w. dosage produced significant anti-inflammatory effect by reducing paw swelling in monosodium urate crystal-induced animals. Hence, 30 mg/kg b.w. dosage was considered for this study. The dosage of standard drug indomethacin (3 mg/kg b.w.) used in this study was selected based on our previous reports[8,9].

2.3. Synthesis of monosodium urate crystals

About 4 g of uric acid was dissolved and heated in 800 mL H_2O with NaOH (9 mL/0.5 N), adjusted to pH 8.9 at 60 ℃; cooled over night in a cold room; washed and dried. Needle-like crystals were recovered and were suspended in sterile saline (20 mg/mL)[8].

2.4. Monosodium urate crystal-induced inflammation in mice

The mice were divided into four groups with six animals in each group. Group I served as a control group. In group II, inflammation was induced by intradermal injection of 0.2 mL (4 mg) of monosodium urate crystal suspension into the right foot pad[8]. Group III comprised monosodium crystal-induced mice who were treated with boswellic acid (30 mg/kg b.w., i.p.) and group IV consisted of monosodium crystal-induced mice who were treated with indomethacin (3 mg/kg b.w., i.p.). Boswellic acid and indomethacin were suspended in 0.5% carboxy methyl cellulose in phosphate buffered saline and administered intraperitoneally, 1 h before the monosodium urate crystal injection and which was repeated for 3 more days on a daily basis.

2.4.1. Assessment of inflammation

The inflammation was quantified by measuring the thickness of the paw with a vernier scale at different intervals for 3 days. At the end of the experimental period (72 h), the mice were killed by cervical decapitation. Blood from each animal was collected for serum separation. The liver and spleen were immediately dissected out and homogenized in ice-cold (0.01 M), Tris HCL buffer (pH 7.4) to give a 10% homogenate. The tissue homogenate of spleen, liver and serum was used for assaying the lysosomal enzymes, lipid peroxidation, antioxidant status and inflammatory mediator tumour necrosis factor- α.

2.4.2. Effect of boswellic acid and indomethacin on lysosomal enzymes

The activity of acid phosphatase was assayed by the method of King[10]. β-glucuronidase was determined by the method of Kawai and Anno[11] and β-galactosidase by the method of Rosenblit[12]. The method of Marhun[13] was followed for the determination of N-acetyl glucosaminidase and the protein content was measured by the method of Lowry *et al*[14].

2.4.3. Effect of boswellic acid and indomethacin on lipid peroxidation and antioxidant status

Lipid peroxidation in plasma was estimated by the method of Ledwozy *et al*[15]. Spleen and liver lipid peroxidation was carried out by the procedure of Hogberg *et al*[16] using thiobarbituric acid as the colouring agent. Malonaldehyde (MDA) produced during peroxidation of lipids served as an index of lipid peroxidation. MDA reacts with TBA to generate a colour product, which absorbs at 532 nm.

Superoxide dismutase (SOD) activity in spleen and liver was determined by the method of Marklund and Marklund[17]. The degree of inhibition of the auto-oxidation

of pyrogallol at an alkaline pH by SOD was used as a measure of the enzyme activity. Catalase and glutathione peroxidase activities in spleen and liver were estimated by the method of Sinha[18] and Rotruck *et al*[19]. The activity of catalase was expressed as μg of H_2O_2 consumed/mn/mg protein. Glutathione peroxidase was expressed as μg of glutathione utilized /min/mg/protein.

2.4.4. Effect of boswellic acid and indomethacin on TNF-α production

TNF-α levels in plasma, liver and spleen of control and monosodium urate crystal-induced mice were determined by enzyme-linked immunosorbent assay (ELISA, Cayman Chemicals Company, USA), according to the manufacturer's instruction.

2.5. In vitro studies (monosodium urate crystal-PMNL cell interaction)

Human polymorphonuclear leucocytes (PMNL) cell suspension (3×10^6/mL) was pre-incubated at 37 ℃ for 20 min with boswellic acid (50/100 μg/mL) or indomethacin (10 μg/mL) before addition of monosodium urate crystals (1 mg/mL). After incubation for a further 30 min at 37 ℃, the cell suspension was removed and centrifuged at 1 500 × g at 40 ℃ for 20 min. The resulting cell free supernatant was assayed for the released activities of β-glucuronidase and lactate dehydrogenase. Appropriate control experiments were performed by measuring the release of enzymes tested in the untreated specimens and those incubated for 30 min at 37 ℃ without drug. In all instances, the experiments were carried out in triplicate.

Lactate dehydrogenase, a cytoplasmic enzyme was assayed by the method of King[20]. β-Glucuronidase, an enzyme present in azurophilic granules was measured by the method of Kawai and Anno[11]. Enzyme released was expressed as a percentage of maximal enzyme release after disruption of the cells with Triton X-100. Specific enzyme activity was expressed as units/mg of protein.

2.6. Statistical analysis

Results were expressed as mean ± SD and a statistical analysis was performed using ANOVA, to determine the significant differences between the groups, followed by Student's Newman-Keul's test. $P<0.05$ implied significance.

3. Results

3.1. Effect of boswellic acid and indomethacin on the activities of lysosomal enzymes in monosodium urate crystal-induced mice

Table 1 summarized the activity of 6-shogaol on lysosomal enzymes in the plasma, liver and spleen of control and experimental animals. The activity of acid phosphatase, β-glucuronidase, N-acetyl glucosaminidase and β-galactosidase were found to be significantly augmented in plasma, liver and spleen of monosodium urate crystal-induced mice when compared with that of control mice. However, boswellic acid reduced these enzyme activities near to the control animals.

Table 1
Effect of boswellic acid and indomethacin on the activities of lysosomal enzymes in monosodium urate crystal-induced mice (mean±SD).

Groups	Acid phosphatase (A)			β-Glucuronidase (B)			N-acetyl glucosaminidase (B)			β-Galactosidase (B)		
	Serum	Liver	Spleen	Serum	Liver	Spleen	Serum	Liver	Spleen	Serum	Liver	Spleen
Group I	0.23±0.10	2.14±0.14	2.18±0.19	2.23±0.10	21.30±0.68	23.30±1.40	1.35±0.33	22.80±1.54	19.80±1.03	2.13±0.15	9.53±0.38	5.18±019
Group II	0.80±0.17^{a*}	4.96±0.47^{a*}	4.16±0.21^{a*}	5.63±0.34^{a*}	34.00±1.05^{a*}	38.70±0.82^{a*}	3.38±0.30^{a*}	44.08±1.16^{a*}	37.08±2.22^{a*}	4.63±0.34^{a*}	18.78±0.51^{a*}	12.00±0.34^{a*}
Group III	0.33±0.12^{a*b*}	2.66±0.53^{a*b*}	2.96±0.28^{a*b*}	2.78±0.50^{a*b*}	24.16±0.60^{a*b*}	26.80±2.11^{a*b*}	1.83±0.35^{a*b*}	26.60±2.21^{a*b*}	22.16±1.51^{a*b*}	2.55±0.55^{a*b*}	11.08±0.80^{a*b*}	6.30±0.17^{a*b*}
Group IV	0.30±0.12^{a*b*}	2.45±0.37^{a*b*}	2.86±0.25^{a*b*}	2.80±0.48^{a*b*}	23.66±1.29^{a*b*}	27.00±2.81^{a*b*}	1.58±0.36^{a*b*}	25.66±2.16^{a*b*}	21.83±2.46^{a*b*}	2.63±0.49^{a*b*}	11.61±0.93^{a*b*}	6.28±0.19^{a*b*}

a: Group I *vs* Groups II, III, and IV; b: Group II *vs* Group III and IV; *: $P<0.05$; A: μ moles × 10^{-2} of phenol; B: μ moles × 10^{-2} of p- nitro phenol liberated /h/mg protein.

Table 2
Effect of boswellic acid and indomethacin on lipid peroxidation in monosodium urate crystal-induced mice (mean±SD).

Parameters	Group I	Group II	Group III	Group IV
Plasma (A)	4.23±0.30	6.48±0.23^{a*}	4.83±0.35^{a*b*}	5.10±0.29^{a*b*}
Liver (B)	2.03±0.25	3.73±0.19^{a*}	2.35±0.28^{b*}	2.48±0.27^{b*}
Spleen (B)	3.08±0.26	4.73±0.19^{a*}	3.78±0.18^{a*b*}	3.86±0.24^{a*b*}

a: Group I *vs* Groups II, III, and IV; b: Group II *vs* Group III and IV; *: $P<0.05$; A: nanomoles of malonaldehyde formed/mg/dL; B: nanomoles of malonaldehyde formed/mg protein.

Table 3
Effect of boswellic acid and indomethacin on enzymic antioxidant status in monosodium urate crystal-induced mice (mean±SD).

Groups	Superoxide dismutase (A)		Glutathione peroxidase (B)		Catalase (C)	
	Liver	Spleen	Liver	Spleen	Liver	Spleen
Group I	4.26±0.10	3.37±0.14	6.36±0.21	5.29±0.10	14.26±0.37	10.69±0.41
Group II	1.85±0.10^{a*}	2.14±0.12^{a*}	4.18±0.14^{a*}	3.65±0.11^{a*}	8.26±0.28^{a*}	6.15±0.42^{a*}
Group III	3.81±3.70^{a*b*}	3.02±0.12^{b*}	5.40±0.21^{a*b*}	4.65±0.10^{b*}	12.31±0.39^{a*b*}	9.08±0.65^{a*b*}
Group IV	3.90±0.11^{a*b*}	3.22±8.21^{a*b*}	5.93±0.22^{a*b*}	5.05±0.11^{b*}	13.56±0.32^{a*b*}	9.73±0.51^{a*b*}

a: Group I *vs* Groups II, III, and IV; b: Group II *vs* Group III and IV; *: $P<0.05$; A: units/mg protein; B: μg of GSH utilized / min/mg protein; C: μmol of H_2O_2 consumed /min/mg protein.

3.2. Effect of boswellic acid and indomethacin on lipid peroxidation in monosodium urate crystal-induced mice

Table 2 depicted the effect of boswellic acid on lipid peroxidation in plasma, liver and spleen of control and experimental mice. Lipid peroxide level in plasma, liver and spleen was increased significantly in monosodium urate crystal-induced mice as compared with untreated control group. Treatment of boswellic acid to monosodium urate crystal-induced mice modulated the above changes by regulating the lipid peroxide level to nearly that of normal levels.

3.3. Effect of boswellic acid and indomethacin on enzymic antioxidant status in monosodium urate crystal-induced mice

Table 3 showed the effect of boswellic acid on the enzymic antioxidant levels in liver, and spleen of control and experimental mice. The activities of enzymic antioxidants were significantly reduced in monosodium urate crystal-induced mice when compared with control mice. Treatment of boswellic acid significantly increased the enzymic antioxidant levels in monosodium urate crystal-induced mice considerably, which indicated its antiperoxidative action.

3.4. Effect of boswellic acid and indomethacin on monosodium urate crystal-induced paw oedema in mice

The effect of boswellic acid and indomethacin on paw oedema induced by monosodium urate crystals was reported in Figure 1. The paw volume of monosodium urate crystal-induced mice revealed an increase in ankle diameter as shown in Figure 1, whereas boswellic acid treatment showed maximum inhibition of the paw oedema in monosodium urate crystal-induced mice.

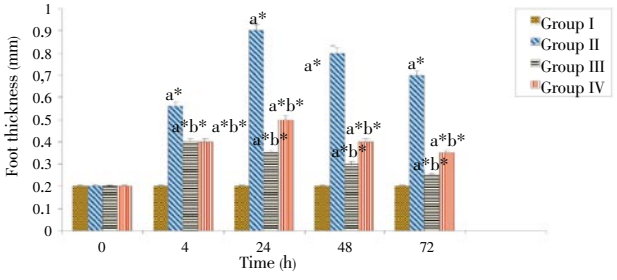

Figure 1. Effect of boswellic acid and indomethacin on monosodium urate crystal-induced paw oedema in mice.
Values are expressed as mean±SD of six animals. a: Group I *vs* Groups II, III, and IV; b: Group II *vs* Group III and IV; *: $P<0.05$.

3.5. Effect of boswellic acid and indomethacin on TNF-alpha production in monosodium urate crystal-induced mice

The level of tumour necrosis factor-α in the monosodium urate crystal-induced mice was significantly elevated in the serum, liver and spleen as shown in Figure 2. However, the elevated levels of tumour necrosis factor-α were found to be diminish in boswellic acid treated monosodium urate crystal-induced mice.

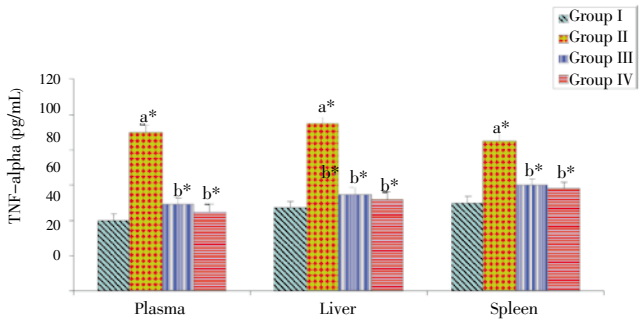

Figure 2. Effect of boswellic acid and indomethacin on TNF-alpha production in monosodium urate crystal-induced mice.
Values are expressed as mean±SD of six animals. a: Group I *vs* Groups II, III, and IV; b: Group II *vs* Group III and IV; *: $P<0.05$.

3.6. Effect of boswellic acid and indomethacin on enzyme leakage from the PMNL cells upon incubation with monosodium urate crystals

Figure 3 depicted the effect of boswellic acid and indomethacin on enzyme leakage from the PMNL cells upon incubation with monosodium urate crystals. The pretreatment of boswellic acid significantly suppressed the β-glucuronidase (lysosomal) and lactate dehydrogenase (cytoplasmic) enzyme release from the PMNL cells incubated with monosodium urate crystals as compared with untreated PMNL cells incubated with monosodium urate crystals at the dose dependent manner.

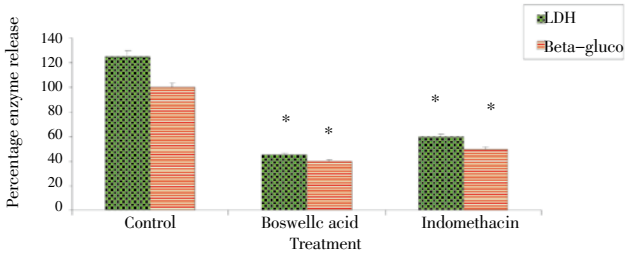

Figure 3. Effect of boswellic acid and indomethacin on enzyme leakage from the PMNL cells upon incubation with monosodium urate crystals.
Values are expressed as mean±SD of six animals. *: $P<0.05$ comparing with the control.

4. Discussion

Gout is an acute rheumatic disorder that results an excess of uric acid (urate) in the body (hyperuricemia) leading to the formation of monosodium urate crystals in various tissues. Experimentally, we have reproduced it by injecting a known amount of urate crystals in mice joints and analysed the paw edema, lysosomal enzymes, lipid peroxidation, anti-oxidant status and inflammatory mediator TNF-α. A characteristic feature of crystal-induced arthritis is the rapid accumulation of polymorphonuclear cells (PMNs) that is associated with the ability of the crystals to induce inflammatory mediators including TNF[21]. In the present study, paw volume and lysosomal enzymes were found to be increased in monosodium crystal-induced mice. Changes in lysosomal enzyme activities may result in impairment of

phagocytic and endocytic activities, inadequate extracellular matrix turnover, and remodeling, which suggest that lysosomal enzyme activities might be involved in the pathogenesis of autoimmune diseases. The increased free radicals produced by neutrophils react with the lipid bilayer of intracellular organelles including lysosomes, which destabilizes lysosomal membranes and results in the rupture of lysosomes. It was reported that exposure of mammalian cells to oxidant stress causes early lysosomal rupture followed by apoptosis or necrosis of the cell[22]. Since, extracellular release of lysosomal enzymes may be crucial to the pathogenesis of tissue injury and inflammation[8], it is likely that a reduction in the release of such enzymes would prove beneficial. The reduction in the paw edema and lysosomal enzyme activities after boswellic acid treatment suggests that its anti-inflammatory effect was associated with significant reduction of total leukocytes migration as well as lymphocytes and monocytes/macrophages migration from the blood into the synovial cavity. It has been well established that boswellic acids acts as a leukotriene LTB4 inhibitor[5] and reduces the infiltration of leucocytes into an inflammation site[23].

The inflammatory response to monosodium urate crystals is characterized by the recruitment of large numbers of activated neutrophils into the joint, resulting in the production of high levels of superoxide by the neutrophil NADPH oxidase, a phenomenon commonly known as the "respiratory burst"[24]. Therefore, inhibition of superoxide production represents an altered target of acute inflammatory response[25]. The increased lipid peroxide level observed in monosodium urate crystal-induced mice in our present study (group II) also suggests the role of free radicals during phagocytosis of immune complexes in gouty arthritis. The result of the present study indicates that the antioxidant defense system is compromised in monosodium urate crystal-induced mice as evidenced by increased lipid peroxidation concentration and decreased activity of antioxidant enzymes, which in turn falls in line with our earlier reports[26]. The treatment with boswellic acid normalized the levels of lipid peroxidation and antioxidant status in monosodium urate crystal-induced inflamed mice. This effect clearly reflects the anti-peroxidative effect of boswellic acid similar to the other pentacyclic triterpenes such as ursolic acid[27] and lutein[28].

Monocytes and macrophages are partners in the orchestration of acute gout. Monocytes/macrophage differentiation may influence the inflammatory response to monosodium urate crystal precipitation in joint fluids[29]. Interactions of monosodium urate crystals with monocytes lead to the induction of number of proinflammatory cytokines, such as IL-6, and TNF-alpha[30]. TNF-alpha released by monocytes can activate vascular endothelial cell expression of E-selectin, intracellular adhesion molecule 1 ($ICAM^{-1}$), and vascular cell adhesion molecule 1 ($VACM^{-1}$), which leads to secondary neutrophil recruitment to sites of crystal deposition and the amplification of inflammatory response[31-39]. Recently, it has been reported that boswellic acids act as inhibitors of expression of NF-_B-dependent genes including cytokines[40]. This argument was supported in our study by the depleted tumour necrosis factor- α level in boswellic acid treated monosodium urate crystal-induced mice when compared with monosodium urate crystal-induced mice.

Monosodium urate crystal-promoted inflammation is manifested by massive infiltration of neutrophils into the joints, which leads to tissue damage[3]. The interaction of monosodium urate crystals with human neutrophils leads to the production of mediators of inflammation, such as lysosomal enzymes, reactive oxygen species, prostaglandin E2 and chemokines that promote vasodilation, erythema, and the pain associated with acute gout attack[41]. Human polymorphonuclear leukocytes (PMNLs) play a fundamental role in the pathogenesis of many inflammatory diseases. The pro-inflammatory activity of PMNLs is exerted in part through the release of preformed mediators, including several lysosomal enzymes. In the present study, human polymorphonuclear leucocyte (PMNL) cells, significantly released β-glucuronidase and lactate dehydrogenase upon exposure to monosodium urate crystals. Boswellic acid treatment suppressed the enzyme release from the PMNL cells incubated with monosodium urate crystals, by its anti-inflammatory effect, which has already been established[23].

In conclusion, the results obtained in this study further strengthen the anti-inflammatory/antiarthritic effect of boswellic acid, which was already well established by several investigators. This study may have significant impact to consider boswellic acid as a useful tool for the treatment of acute gouty arthritis. Further studies are in underway to investigate the exact mechanism of action of boswellic acid against gouty arthritis.

Conflict of interest statement

We declare that we have no conflict of interest.

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