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Boswellic acids and glucosamine show synergistic effect in preclinical anti-inflammatory study in rats

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Abstract—The present study revealed the synergistic effect of boswellic acid mixture (BA) and glucosamine for anti-inflammatory and anti-arthritic activities in rats. Two studies were conducted, that is, acute anti-inflammatory by carrageenan edema and chronic anti-arthritic by *Mycobacterium*-induced developing arthritis. Five groups of animals were included in each of the study: the vehicle control, positive control (ibuprofen 100 mg/kg), boswellic acids (250 mg/kg), glucosamine (250 mg/kg) and a combination of boswellic acids (125 mg/kg) and glucosamine (125 mg/kg). BA when administered at 250 mg/kg in rats, carrageenan-induced paw edema and *Mycobacterium*-induced developing arthritis were significantly inhibited. In comparison to boswellic acids, glucosamine when administered at 250 mg/kg showed a mild effect in carrageenan-induced edema and moderate inhibition of paw swelling against developing arthritis. Although the combination of boswellic acids and glucosamine did not affect the acute inflammation to a greater extent yet a significant anti-arthritic activity was observed in rats. In conclusion, a synergistic effect was observed in chronic inflammatory conditions when two chemical entities were administered in combination in preclinical study.

The first line of clinical treatment for the inflammatory disorders is non-steroidal anti-inflammatory drugs (NSAIDs) but steroidal agents and immunosuppressants are also used.[1] NSAIDs are known to have potent activity and their long-term administration is required in chronic disorders such as arthritis. Unfortunately, the prolong use of these chemicals may have deleterious effects on some of the vital organs of the body viz., gastrointestinal tract, kidney, liver, central nervous system, and immune system.[2–4] According to one of the reports NSAIDs such as ibuprofen and aspirin kill about 16,500 Americans every year and send 103,000 to the hospital with gastrointestinal bleeding.[5] Although, NSAIDs with specific COX-2 inhibitory action have fewer gastrointestinal adverse effects in comparison to other NSAIDs,[6] but the recent findings of an elevated incidence of myocardial infraction after long-term use of rofecoxib questioned the safety of these drugs.[7] Therefore, the use of natural products and traditional medicines having favorable therapeutic effects but fewer adverse effects is gaining more attention in chronic ailments.

Boswellic acids are a mixture of triterpenic acids obtained from the oleo gum resin of *Boswellia serrata* and known for its effectiveness in the treatment of chronic inflammatory diseases including peritumor edema. BA have extensively been studied for a number of activities including anti-inflammatory, immunomodulatory, anti-tumor, and inflammatory bowel disease.[8–12] It belongs to NSAID class of agents but with a different mechanism of action, that is, the inhibition of 5-lipoxygenase, the key enzyme of leukotriene synthesis[13] (Figs. 1 and 2).

The fraction containing BA was prepared by extracting *B. serrata* gum resin (1 kg) successively with 3, 2, and 1.5 L of ethanol (95%) in a percolator to give three extracts. All the three extracts were combined and evaporated under reduced pressure at 40 °C to obtain a thick brown residue (490 g). The total extract was stirred with 6 L of 3% sodium hydroxide solution until it produced a uniform emulsion. The aqueous part was

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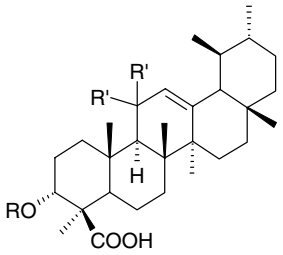
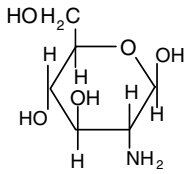


Boswellic acids (Figure 1)



Glucosamine (Figure 2)

Boswellic acids (36% activity)

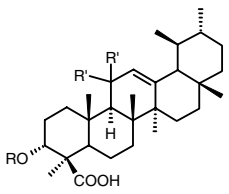
\+ → **Synergistic antiarthritic activity** (55% activity)

Glucosamine (21% activity)

Figures 1 and 2. Structure of natural boswellic acids and glucosamine.

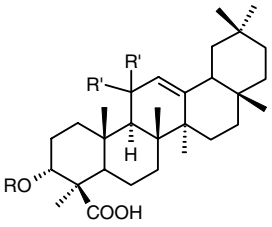
filtered through a fine cloth and extracted with 1×3 L of hexane/ethyl acetate (95:5) to remove the non-acidic part. The aqueous portion was then acidified with 1 N hydrochloric acid to precipitate the total organic acids. The filtered acids were washed several times with distilled water to remove final traces of hydrochloric acid. The crude mixture of acids was redissolved in 3% sodium hydroxide solution and whole process was repeated until precipitation was complete. The precipitates after washing were dried in a vacuum oven at temperature below 50 °C to yield 280 g creamish powder of BA. This dried mixture was directly used for biological studies.

The total acid content of BA as estimated by acid base titrations was found to be $93 \pm 3\%$. The total acid mixture yielded four major pentacyclic triterpenic acids (Figs. 3 and 4) on separation by column chromatography over silica gel (60–120 mesh). The compounds were eluted in hexane with increasing proportions of ethyl acetate. The fractions exhibiting similar patterns on TLC were pooled together and evaporated to dryness under vacuo to give residues. The residues were then taken in suitable organic solvents to yield the crystals of four different boswellic acids. Finally the compounds were identified on the basis of their ^{1}H NMR, ^{13}C NMR and mass spectral data. The data of these compounds were in agreement with that reported in the literature.[14]



1. **R= H, R' R' = H_2** (β - Boswellic acid)
3. **R= OAc, R' R' =H_2** (Acetyl- β - Boswellic acid)
5. **R=H, R' R' = O** (11-keto - β - boswellic acid)
6. **R= OAc, R' R' = O** (Acetyl 11-keto - β - boswellic acid)

Figure 3. Structure of β isomers of natural boswellic acids.



2. **R=H, R' R' = H_2** (α - Boswellic acid)
4. **R=OAc, R' R' = H_2 (Acetyl - a - Boswellic acid)**

Figure 4. Structure of α isomers of natural boswellic acids.

The natural BA mixture and their individual markers were subjected to analysis by reverse phase liquid chromatography (HPLC) and their identity in the mixture was confirmed by LC–MS.

HPLC analysis of BA was performed on a Shimadzu LC-10_{AT} system. RP-18 (5 μm, 250 mm × 4 mm) Merck column and a mobile phase consisting of acetonitrile/water/acetic acid (99:1:0.01) were used during the analysis. An isocratic flow of 0.5 ml/min with 30 °C column temperature was employed to achieve separation efficiency and for peak detection diode array detector was operated at 210 nm. In HPLC chromatogram of natural boswellic acid mixture both α and β boswellic acids (Figs. 3 and 4, Structures **1** and **2**) and their acetyl derivatives (Figs. 3 and 4, Structures **3** and **4**) were detected as twin peaks comprising of mixtures of α and β isomers (a mixture physically inseparable by normal column chromatography) in the ratio of 37:63 and 22:78, respectively, while 11-keto-β-boswellic acid (Fig. 3, Structure **5**) and its 3-acetyl derivative (Fig. 3, Structure **6**) were detected as single peaks. The identity of markers was also established by LC–ESI-MS in the negative mode of ionization which gave $[M+CH_3COOH]^-$ molecular ion peaks. The LC–ESI-MS experiments were performed on an Agilent 1100 series HPLC coupled to Esquire 3000 Brucker Daltonics Mass Spectrometer. The LC conditions for LC–MS were same as those employed on Shimadzu HPLC for the separation of marker

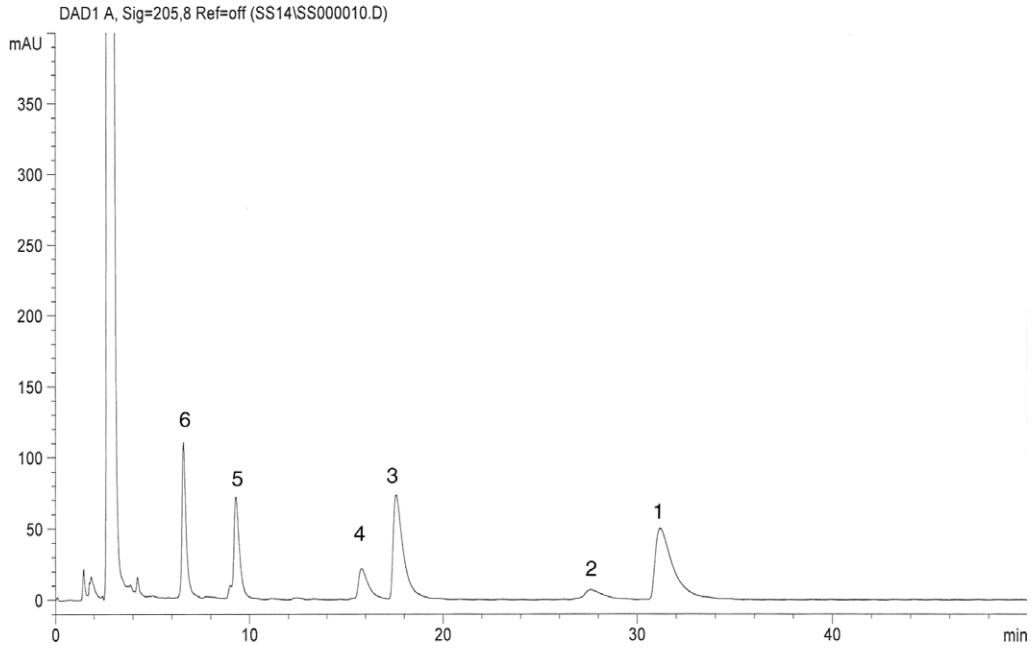


Figure 5. LC–UV(DAD) chromatogram (210 nm) of four marker BAs. **1**, β-boswellic acid; **2**, α-boswellic acid; **3**, acetyl-β-boswellic acid; **4**, acetyl-α-boswellic acid; **5**, 11-keto-β-boswellic acid; and **6**, acetyl-11-keto-β-boswellic acid.

compounds. Figure 5 shows the LC–UV(DAD) chromatogram of four combinations of marker compounds at 210 nm, whereas Figure 6 shows the LC–UV(DAD) chromatogram of a sample wherein the presence of all the four major BAs in the total acid mixture prepared from *B. serrata* gum resin has been observed. The percentage of individual boswellic acids in BA as estimated by HPLC is α and β-boswellic acids (29.41%), α and β-acetyl-boswellic acids (14.63%), 11-keto-β-boswellic acid (3.56%), and acetyl-11-keto-β-boswellic acids (7.35%). Glucosamine (2-amino-2-deoxy-alpha-D-glucose) which occurs naturally in the human body, plays a key role in the construction of cartilage—the tough connective tissue that cushions the joints. It provides strength, flexibility, and elasticity to cartilage and connective tissue by stimulating the production of glycosaminoglycans, molecules that hold joint tissue together. It is an amino polysaccharide and the essential component of mucopolysaccharides and chitin.[15] Glucosamine as well as its acetylated derivative, *N*-acetylglucosamine, are readily synthesized in the body from glucose. Because of its high concentration in joint tissues, it is believed that glucosamine supplements and provides symptomatic relief for chronic inflammatory disorders. Number of clinical

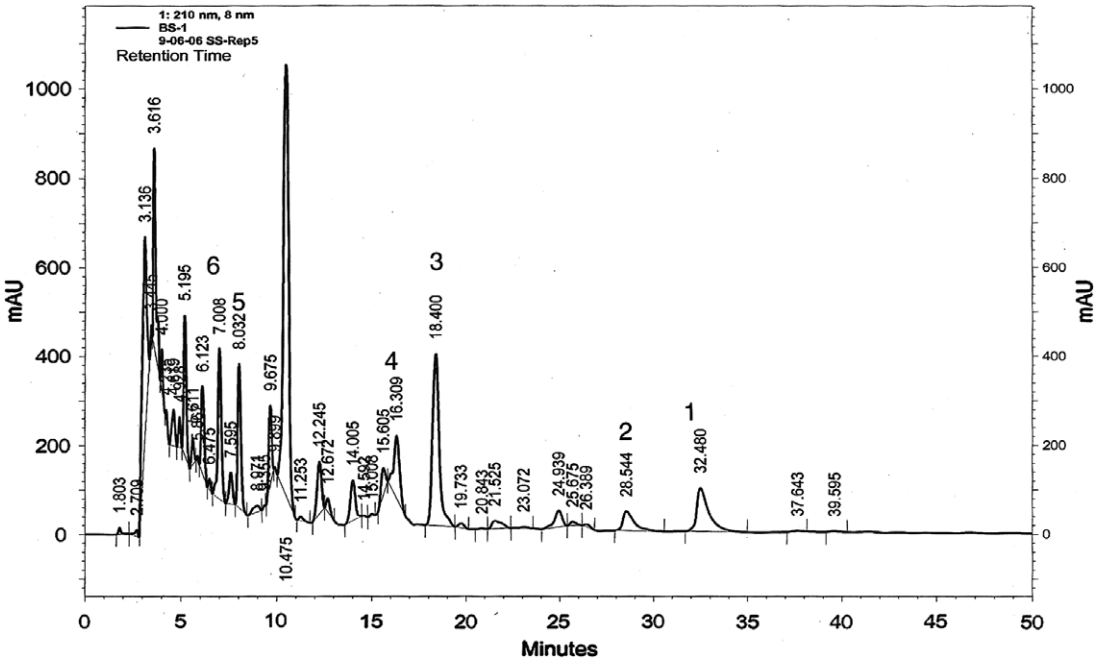


Figure 6. LC–UV(DAD) chromatogram (210 nm) of a sample wherein presence of four boswellic acids has been observed. **1**, β-boswellic acid; **2**, α-boswellic acid; **3**, acetyl-β-boswellic acid; **4**, acetyl-α-boswellic acid; **5**, 11-keto-β-boswellic acid; and **6**, acetyl-11-keto-β-boswellic acid.

trials has proved this hypothesis, and a large number of glucosamine products have been used extensively in combination with other therapeutic agents for the management of osteoarthritic and rheumatic pains.[16]

Results of the experiments are expressed as means ± standard error (SE). The statistical significance of the difference between groups was evaluated by applying Student's t test. Significant differences were set at $P < 0.05$.

As a part of our biological study with boswellic acids and glucosamine for the evaluation of anti-inflammatory[17] and anti-arthritic[18] efficacy, alone or in combination, carrageenan-induced paw edema and *Mycobacterium*-induced developing arthritic models in rats were applied. Five groups of rats were included in each of the study: the vehicle control, positive control (ibuprofen 100 mg/kg), boswellic acids (250 mg/kg), glucosamine (250 mg/kg), and combination of boswellic acids (125 mg/kg) and glucosamine (125 mg/kg), a total of 250 mg. The development of the edema induced by carrageenan has been described as a biphasic event.[19] The first phase of the inflammatory response is mediated by histamine and serotonin, whereas the second phase is mediated by kinins and presumably by prostaglandins. Edema induced by carrageenan is highly sensitive to NSAIDs and has been accepted as a useful indicator for identifying the new anti-inflammatory molecules.[20] This model reliably predicts the anti-inflammatory efficacy of NSAIDs in the later phase, that is, the prostaglandin phase. Paw edema was induced in the left hind paw of the Wistar rats (140–160 g) by the intradermal injection of 100 µl of 10 mg/ml of carrageenan solution prepared in normal saline[17] and the level of inflammation was quantified by measuring the volume of the paw, using a plethysmometer (model 7101, Ugo Basile Co., Italy). The test compounds were prepared as fine homogenized suspensions in 2% gum acacia and administered to the animals 45 min before the carrageenan injection.

Figure 7 summarizes the results of acute anti-inflammatory effects of boswellic acids and glucosamine alone or in combination against carrageenan-induced edema in rats. The paw volume of the rats after the carrageenan injection increased approximately 23.4% compared to the saline treated limb indicating that a carrageenan injection induced the inflammation. In addition, this test model was confirmed to be effective for evaluating acute inflammation and the anti-inflammatory effect, because the paw edema in the positive control group administered with ibuprofen was significantly reduced (about 55.64%) in comparison to the control group.[21]

Although the anti-inflammatory effect of boswellic acids at 250 mg/kg po was less than that of the positive control ibuprofen but significant. At 3 h and 4 h the inhibition was 28.57% and 25.80%, respectively. Glucosamine on the contrary at 250 mg/kg po reduced inflammation moderately (15.17% and 16.12% after 3 h and 4 h, respectively). The results of this study are in accordance with the already reported anti-inflammatory study of boswellic acids.[22] The rate of inhibition of edema was slightly more in the group administered with combination of boswellic acids (125 mg) and glucosamine (125 mg) in comparison to only glucosamine group (19.64% and 17.74% after 3 h and 4 h, respectively). However, this difference was not statistically significant indicating that glucosamine did not show significant synergism with boswellic acids in acute pedal edema induced by carrageenan.

The model of rat adjuvant-induced arthritis is well characterized and has been extensively used to study the impact of anti-arthritic drugs.[23] Arthritic process was induced in rats with the inoculation of Freund's adjuvant containing heat killed *Mycobacterium tuberculosis* in paraffin oil.[18] Drug administration was started a day before the immunization and continued until day 13. Anti-arthritic activity was assessed by quantifying the level of inflammation of the paw using a plethysmometer in comparison to the control group. The clinical characteristics of the *Mycobacterium*-induced arthritis are swelling and erythema, evident on day 1 in the injected paw, swelling progresses up to day 7 and begins to decline through day 8. A secondary, chronic phase of inflammation in which involvement of immune system takes place begins after eighth day of adjuvant injection. During this stage the animals

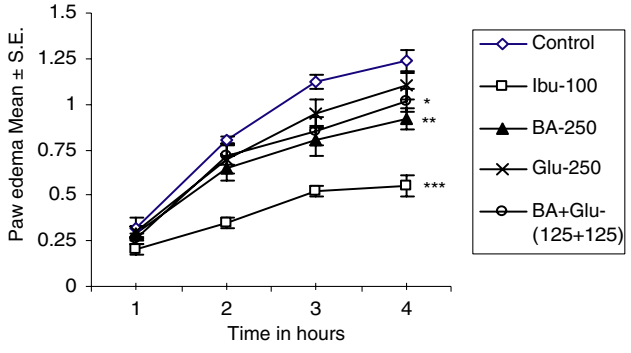


Figure 7. Anti-inflammatory effect induced by carrageenan in rats. Each value represents the means ± SE of five observations and level of significance was determined by Student's t-test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

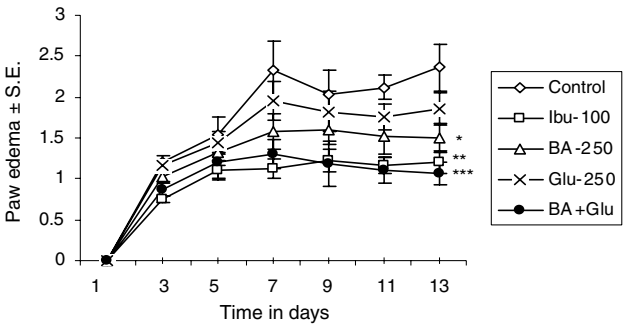


Figure 8. Graphic representation depicting the course of anti-arthritic effect of various treatments in *Mycobacterium*-induced developing arthritis in rats. Each value represents the means ± SE of five observations and level of significance was determined by Student's t-test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

exhibited renewed swelling in the injected paw, a progressive swelling in the phalangeal and tarsal joints of uninjected paw with the appearance of tail nodules and ear patches.

Figure 8 describes the results of anti-arthritic effect of boswellic acids and glucosamine either alone or in combination. Ibuprofen, a known NSAID, inhibited both the inflammatory phases, that is, primary (first to seventh day) and secondary or immunological phase (nine day onwards) significantly, whereas boswellic acids and glucosamine did not inhibit the initial paw swelling significantly. The arthritic swelling of BA group in the later phase was significantly inhibited (36.44%, P-value < 0.01) in comparison to the control group but glucosamine group remained insignificant in this phase also (21.61%). However, BA and glucosamine combination (125 mg/kg each) produced a highly significant reduction in the rate of edema formation throughout the course of inflammation (55%, P-value < 0.001 on day 13).

The synergistic effect of boswellic acids and glucosamine may not be due to any interaction in the chemical entities in the biological system, however, due to different mechanisms of action it may be possible that their effect on two different targets might be the reason of the synergism. BA are known inhibitor of 5-lipoxygenase products including 5-hydroxyeicosatetraenoic acid (5HETE) and leukotriene B_4 (LTB_4)[13] which are implicated in the progression of the diseases like arthritis, Chrohn's disease, ulcerative colitis, asthma, etc.[24] Glucosamine, an amino sugar, on the other hand, provides strength, flexibility, and elasticity to cartilage and connective tissue by stimulating the production of glycosaminoglycan molecules that hold joint tissue together and also provide 'shock absorbing' properties.[25] Although glucosamine does not appear to be effective in inhibiting either cyclooxygenase/lipooxygenase or proteolytic enzymes involved in inflammation, yet it is important for its ability to synthesize proteoglycans needed for the stabilization of cell membranes and the production of intracellular ground substance.[26]

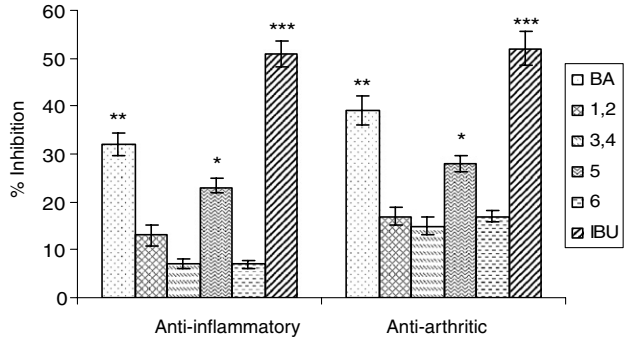


Figure 9. Inhibitory anti-inflammatory and anti-arthritic effect of BA, four pure isolates (250 mg/kg po), and ibuprofen (100 mg/kg po) in rats. Each value represents the means ± SE of five observations and level of significance was determined by Student's t-test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ **1**, β-boswellic acid; **2**, α-boswellic acid; **3**, acetyl-β-boswellic acid; **4**, acetyl-α-boswellic acid; **5**, 11-keto-β-boswellic acid; and **6**, acetyl-11-keto-β-boswellic acid.

The individual molecules of boswellic acids in the mixture viz., α and β-boswellic acids, α and β-acetyl-boswellic acids, 11-keto-β-boswellic acid, and acetyl-11-keto-β-boswellic acid were also evaluated for their anti-inflammatory and anti-arthritic activity against carrageenan and *Mycobacterium*-induced inflammation. Results of the study revealed that none of the pure molecules exhibited better activity than the crude mixture of BA. Although, 11-keto-β-boswellic acid showed significant activity in both the test models but it was less than the mixture (Fig. 9).

Keeping in view these results, it was considered important to focus our study on BA mixture in combination with glucosamine than any other pure molecules, so as to develop a synergistic therapeutic for the management of chronic inflammation.

In conclusion, BA and glucosamine in combination have an anti-inflammatory effect on acute and chronic inflammations. Although no significant synergistic effect appeared in acute inflammation, a highly significant synergistic anti-arthritic effect was observed in chronic model of inflammation in rats. These results suggest that BA which is known for its anti-inflammatory/anti-arthritic activity and is also in clinical use may prove much better for its efficacy as an anti-arthritic agent in combination with glucosamine.

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17. Carrageenan edema was induced in groups of five rats. Briefly 100 μl of 10 mg/ml (w/v) freshly prepared carrageenan solution was injected into the subplanter region of the left hind paw of rats 45 min after the drug administration. The contralateral paw was injected with equal volume of normal saline. Volume of the paw was measured immediately and after every hour of the carrageenan injection with a volume differential meter model 7101, Ugo Basile (Italy). The % protection was calculated in comparison to the control group. One group was given a standard drug ibuprofen for comparison and authentication of the experiment.
18. Arthritis was induced by immunizing the rats with an injection of 50 μl of 5 mg/ml (w/v) suspension of heat killed *Mycobacterium tuberculosis* in liquid paraffin into the left hind foot in the subplantar region. Drug administration was started a day before the immunization and continued until day 13. Volume of the paw was measured on alternate days until the duration of the experiment and percent change in the paw volume was calculated in comparison to control group.
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