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Boswellic acids: A leukotriene inhibitor also effective through topical application in inflammatory disorders

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Abstract

Boswellic acids (BA), a natural mixture isolated from oleo gum resin of *Boswellia serrata* comprised of four major pentacyclic triterpene acids: β-boswellic acid (the most abundant), 3-acteyl-β-boswellic acid, 11-keto-β-boswellic acid, and 3-acetyl-11-keto-β-boswellic acid, is reported to be effective as anti-inflammatory, immunomodulatory, anti-tumor, anti-asthmatic and in Chron's disease. It inhibits pro-inflammatory mediators in the body, specifically leukotrienes via inhibition of 5-lipoxygenase, the key enzyme of leukotriene synthesis, is the scientifically proved mechanism for its anti-inflammatory/anti-arthritic activity. All previous work on BA for its biological activity has been done through the systemic application but no pre-clinical data reported for its anti-inflammatory activity by topical application. We here by report anti-inflammatory activity of BA through this route by applying different acute and chronic models of inflammation i.e., arachidonic acid and croton oil-induced mouse ear edema, carrageenan-induced rats paw edema and adjuvant-induced developing arthritis in rats. The results of the study revealed that the effect observed through this route is in accordance to the study conducted with the systemic route, thus establishing that BA when used through topical application is as effective as through the systemic route.

Keywords: *Boswellia serrata*; Boswellic acids; Topical application; Arachidonic acid; 5-Lipoxygenase; Inflammatory

Introduction

Boswellic acids (BA), a mixture comprised of four major pentacyclic triterpene acids: beta-boswellic acid, 3-acteyl beta boswellic acid, 11-keto-beta-boswellic acid and 3-acetyl-11-keto-beta-boswellic acid, isolated from the oleo gum resin of *Boswellia serrata* is reported to be effective as anti-inflammatory (Singh et al., 1996), immunomodulatory (Sharma et al., 1996), anti-tumor (Huang et al., 2000), anti-asthmatic (Gupta et al., 1998) and in chronic colitis (Gupta et al., 2001). Its anti-inflammatory activity has been attributed to the inhibition of 5-lipoxygenase in a selective, enzymelinked nonredox and noncompetitive manner (Ammon et al., 1991; Safayhi et al., 1992). The anti-cancer activity is mediated through triggering of apoptosis via caspase-8 pathway-dependent activation of signaling cascade (Liu et al., 2002) and topoisomerases-I and II-alpha inhibition as well (Syrovets et al., 2000). The anti-asthmatic reports on BA revealed that leukotriene and elastase enzyme inhibition might be responsible for this effect (Badria et al., 2004; Safayhi et al., 1997). Although BA acts through several mechanisms for its biological activities, but the inhibition of leukotrienes is the primary and the most scientifically proved mechanism

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for its anti-inflammatory/anti-arthritic activity (Safayhi et al., 1992).

In view of above reports, exhaustive work has been done on BA for its biological activity and mechanism of action through the systemic application but no pre-clinical data has been published for its anti-inflammatory activity by topical application.

Hence, it was thought desirable to evaluate the anti-inflammatory activity of BA through this route. The results of the present study suggest that BA when used through topical application is as effective as through the systemic route.

Materials and methods

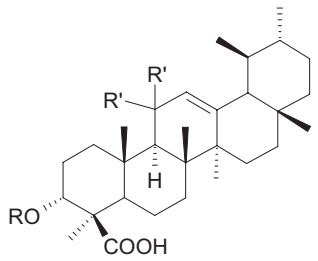
Plant material

The gum resin of the plant was collected from Tirumala hills of Andhra Pradesh, India, authenticated by Dr. B.K. Kapahi, senior botanist of the institute and a voucher specimen of the same was deposited in the herbarium section of the institute under accession no. 19921.

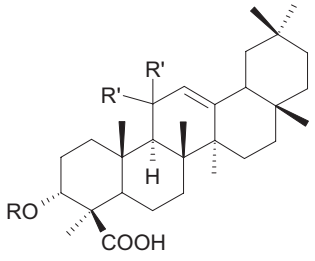
Extraction and isolation

The fraction containing BAs was prepared by extracting *B. serrata* gum resin (1 kg) successively with ethanol (95%) in a percolator and evaporated under reduced pressure on a thin film evaporator at 40 °C to obtain a thick brown residue (490 g). The total extract was stirred with 3% sodium hydroxide solution till it produced a uniform emulsion. The aqueous part was filtered and extracted with hexane: ethyl acetate (95:5) to remove the nonacidic part. The aqueous portion was then acidified with 1 N hydrochloric acid to precipitate the total organic acids. The filtered acids were washed 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 till precipitation was complete. The precipitates were dried in a vacuum oven at temperature below 50 °C to yield 280 g creamish powder of BAs. This mixture was used for biological studies.

The total acid content of the boswellia gum resin estimated by acid base titrations was found to be $93 \pm 3\%$. This total acid content yielded four major pentacyclic triterpenic acids (Fig. 1) and were identified on the basis of their ^{1}H NMR, ^{13}C NMR and mass spectral data. The percentage of individual BA in BA as estimated by HPLC are α and β-BA (29.41%), α and β-acetyl-BA (14.63%), 11-keto-β-boswellic acid (3.56%) and acetyl-11-keto-β-BA (7.35%). The data of these compounds were in agreement with that reported in literature (Pardhy and Bhattacharaya, 1978).



a. R= H, R' R' = H_2 (β - Boswellic acid)
b. R= OAc, R'R' =H_2 (Aceyl - β - Boswellic acid)
e. R=H, R'R' = O (11-keto - β - boswellic acid)
f. R= OAc, R'R' = O (Acetyl 11-keto - β - boswellic acid)



c. R=H, R' R' = H_2 (α - Boswellic acid)
d. R=OAc, R' R' = H_2 (Aceyl - α - Boswellic acid)

Fig. 1. Structure of different boswellic acids isolated from *Boswellia serrata*. Different boswellic acids are (a) β-boswellic acid, (b) aceyl-β-boswellic acid, (c) α-boswellic acid, (d) Aceyl-α-boswellic acid, (e) 11-keto-β-boswellic acid and (f) acetyl 11-keto-β-boswellic acid.

The separated BAs were subjected to analysis by reverse-phase liquid chromatography and their identity in the mixture was confirmed by LC–MS. Further, the HPLC analysis of BAs 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) was 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 boswellic acid mixture, both boswellic acid (Fig. 1a) and its acetyl derivative (Fig. 1b) 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. 1e) and its 3-acetyl derivative (Fig. 1f) were detected as single peaks. The identity of markers was also established under 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 were employed on

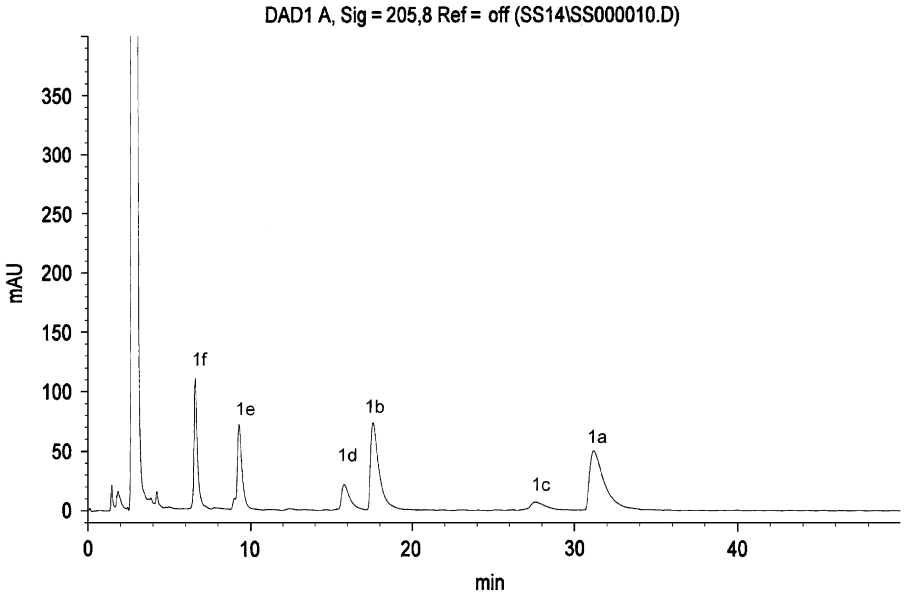


Fig. 2. LC–UV(DAD) chromatogram (210 nm), a mixture of, four marker boswellic acids. (a) β-Boswellic acid, (b) acetyl-β-boswellic acid, (c) α-boswellic acid, (d) acetyl-α-boswellic acid, (e) 11-keto-β-boswellic acid and (f) acetyl-11-keto-β-boswellic acid.

Shimadzu HPLC for the separation of marker compounds. Fig. 2 shows the LC–UV(DAD) chromatogram of four marker compounds at 210 nm.

Animals

Male Wistar rats (160–180 g) and Swiss albino mice (22–25 g) were housed at 24 ± 1 °C on a 12:12 h light and dark cycle with free access to pallet food (Ashirwad Industries, Chandigarh, India) and water. All the experiments were conducted between 10.00 and 17.00 h and were in accordance with the ethical guidelines of the International Association for Study of Pain (Zimmerman, 1983) and approved by the institutional ethics committee.

Arachidonic acid-induced mouse ear oedema

The method described by Gupta et al. (1999) with some modifications was followed. Inflammation was induced by topical application of arachidonic acid (AA) (2 mg in 20 µl of acetone) on both surfaces of the right ear of each mouse. Left ear (control) received vehicle only. BA was applied topically (1.25, 2.5 and 3.75 mg/ear as an ointment) 45 min before AA application. A total of five groups, two control and three treatment groups were used: Group I with application of AA on the right ear as vehicle control and group II that received AA + piroxicam (0.25 mg/ear ointment) as positive control group. Groups III, IV and V were used as the treatment groups with different doses of BA + AA. Inflammation was followed for 1 h and thereafter animals were sacrificed by cervical dislocation. A 8 mm section from the inflamed area of each ear was removed with a metal punch and weighed. Ear edema was calculated by subtracting the weight of the left ear (vehicle) from the right ear (treatment), and was expressed as edema weight. Inhibition percentage was expressed as a reduction in weight with respect to the control group.

Croton oil (CO)-induced mouse ear oedema

The CO assay procedure was carried out according to the method described by Tubaro et al. (1985). In brief cutaneous, inflammation was induced by the application of 20 µl of a 0.5% solution of the irritant CO in acetone. A total of five groups, two control and three treatment groups were used: Group I with application of CO on the right ear as vehicle control and group II that received CO + piroxicam (0.25 mg/ear ointment) as positive control group. Groups III, IV and V were used as the treatment groups with different doses of BA + CO. The mice were sacrificed 6 h after CO application (the peak of inflammation), and a plug of 8 mm in diameter was removed from each ear and weighed. The percent inhibition of edema formation was determined by comparing the edema of the treated group with that of the control group.

Carrageenan-induced edema in rats

Edema was induced in groups of five rats using the method of Winter et al. (1962) by injecting 100 µl of 5 mg/ml (w/v) freshly prepared carrageenan solution in

the subplanter region of the left hind paw. A total of five groups were used: Group I injected with carrageenan only and serves as vehicle control, Group II that received carrageenan + piroxicam (0.25 mg/ear ointment) and work as positive control group. Groups III, IV and V were used as the treatment groups with different doses of BA + carrageenan. Drug was applied topically in three different schedules i.e., 45 min before, 45 min before and 1 h after carrageenan injection, and 45 min before and after every hour i.e., 1, 2 and 3 h. Volume of the paw was measured with a volume differential meter model 7101, Ugo Basile (Italy) after every hour. Percent inhibition was determined as compared to the control group.

Adjuvant-induced developing arthritis in rats

The evaluation is in accordance with the method of Newbould (1963). Animals were immunized with an injection of 50 µl of 5 mg/ml (w/v) suspension of heat killed *Mycobacterium tuberculosis* (Difco) in liquid paraffin into the left hind foot in the subplantar region. Variable doses of drug application (as ointment) were started a day before the immunization to three treatment groups i.e., III, IV and V and continued till day 13. Group II received piroxicam (0.25 mg/paw) in similar manner whereas Group I received vehicle only. Paw volume was measured on day 0 and every alternate day till day 13 with volume differential meter model 7101, UGO Basile (Italy).

Preparation of topical formulation

The formulations were prepared by the following procedure. Oil phase ingredients i.e., white petrolatum, liquid paraffin, lanolin, bee wax and Tween-20 were mixed with a double blended mixer at 75 ± 5 °C. Similarly, the aqueous phase ingredients i.e., water, glycerin and lemon juice were mixed and filtered. Boric acid and ammonium carbonate were added to the above filtered aqueous phase solution at 75 ± 5 °C. The warm aqueous solution was slowly added to the oil phase with constant stirring over a water bath at 75 ± 5 °C. After complete addition of both the phases, heat source was withdrawn and stirring continued till the temperature reached to 45 ± 5 °C. BA was added slowly at this temperature with continuous stirring to make a homogenized topical preparation to be used at room temperature. Table 1 shows the constituents of investigated preparation.

Statistical analysis

The results are presented as mean ± S.E. The statistical significance of difference between the groups was obtained using analysis of variance complemented by Dunnett's test. *P*-value less than 0.05 was considered as indicative of significance, as compared to the control group.

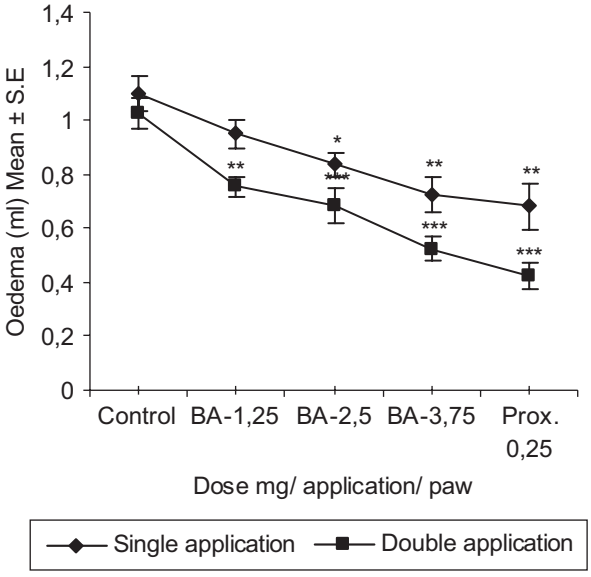


Fig. 3. Dose-dependent effect of BA on carrageenan-induced paw edema in rats. ◆, Single application i.e., drug applied 45 min before carrageenan injection. ■, Double application i.e., drug applied 45 min before and 1 h after carrageenan injection. Each value is the mean ± S.E. of five observations and represented as edema. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Table 1. Composition of formulation containing different concentrations of BA

Oil phase						Aqueous phase				
BA	White petroleum	Liquid paraffin	Lanolin	Bee wax	Tween-20	Water	Glycerine	Lemon juice	Boric acid	Ammonium carbonate
–	18.6	10.0	7.0	10.0	9.0	6.4	16.4	19	1.6	2.0
2.5	17.35	8.75	7.0	10.0	9.0	6.4	16.4	19	1.6	2.0
5.0	16.10	7.50	7.0	10.0	9.0	6.4	16.4	19	1.6	2.0
7.5	14.85	6.25	7.0	10.0	9.0	6.4	16.4	19	1.6	2.0

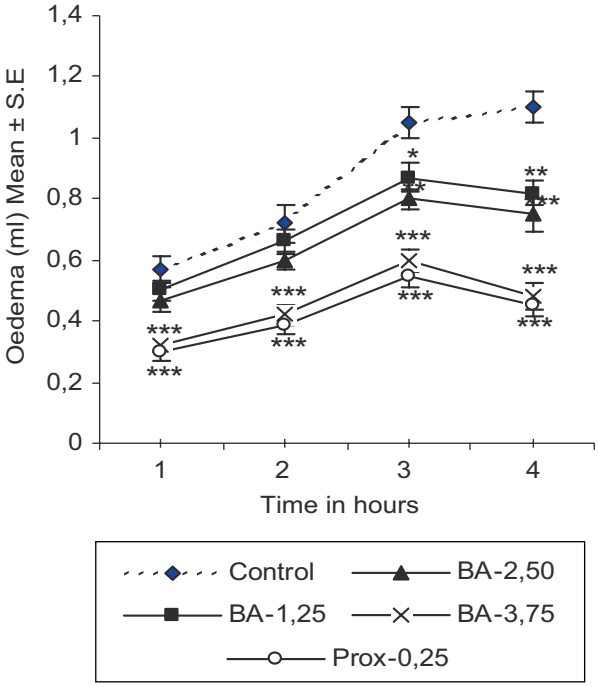


Fig. 4. Dose-dependent effect of BA on carrageenan-induced paw edema in rats after multiple applications i.e., 45 min before and after 1, 2 and 3 h. Dose applied is in mg/animal/paw. Each value is the mean ± S.E. of five observations and represented as edema. $*p<0.05$, $**p<0.01$ and $***p<0.001$.

Results

Inhibition of paw edema of rats by treatment of BA against carrageenan-induced inflammation

Fig. 3 shows that BA significantly inhibited acute paw edema evoked by carrageenan injection by topical application. The maximum phlogistic response of carrageenan was observed at 4 h after the injection in the vehicle treated animals. Data from the BA treated animals with the doses of 1.25, 2.5 and 3.75 mg/paw (single application) at different time intervals showed significant difference in the paw edema in comparison to the vehicle treated animals at the same time point. The results of the same doses with double application of BA showed better inhibition of the paw swelling. Similarly, when BA applied 45 min before and 1, 2 and 3 h after carrageenan injection the effect was highly significant than the early two conditions (Fig. 4). The reference drug piroxicam at a dose of 0.25 mg/paw showed significant inhibition in all the three conditions.

Inhibition of ear edema of mice by treatment of BA against AA and CO-induced inflammation

Results of AA and CO-induced inflammation in mice are depicted in Fig. 5. It is observed that application of

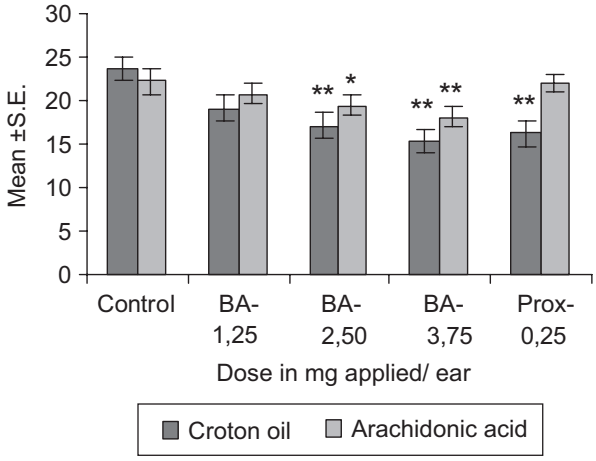


Fig. 5. Dose-related effect of BA on croton oil and arachidonic acid-induced ear inflammation. Each value is the mean ± S.E. of five observations and represents the weight of biopsy punch of 8 mm of inflamed ear. $*p<0.05$ and $**p<0.01$.

AA and CO could effectively induce mouse ear edema with a peak at 1 h with AA and 6 h with CO. Topical application of BA with the doses of 1.25, 2.5 and 3.75 mg/ear could inhibit ear edema. The inhibition was dose dependently significant at 2.5 and 3.75 mg where as it was insignificant at 1.25 mg in both the cases. Piroxicam also showed highly significant results against CO-induced edema, however, it did not show any effect against AA swelling.

Inhibition of paw edema of rats by treatment of BA against *Mycobacterium tuberculosis*-induced inflammation

Fig. 6 shows the arthritic pattern of *Mycobacterium*-induced developing arthritis in rat paw edema as well as the inhibitory effect of BA on arthritis after topical application. It can be seen in Fig. 6 that *Mycobacterium*-induced inflammation was inhibited from the day of injection and continued up to day 13. The effect was in a dose-dependent manner i.e., at 1.25 mg the effect was insignificant whereas it was significant (<0.05) at 2.5 mg and highly significant (<0.01) at 3.75 mg. Paw swelling was also significantly (<0.01) reduced with 0.25 mg/paw of piroxicam application.

Comparative anti-inflammatory (Carrageenan paw edema) and anti-arthritic (*Mycobacterium*-induced developing arthritis) efficacy of BA via systemic administration and topical application in rats

Results of the comparative efficacy of BA for anti-inflammatory and anti-arthritic potential in systemic

administration (Singh, et al., 1996) and topical application were sumrised in Table 2. The data in Table 2 demonstrate that the anti-inflammatory activity of BA both in systemic and topical application was nearly the same whereas, anti-arthritic inhibition was better in topical application at 3.75 mg dose in comparison to the 200 mg/kg dose of systemic administration.

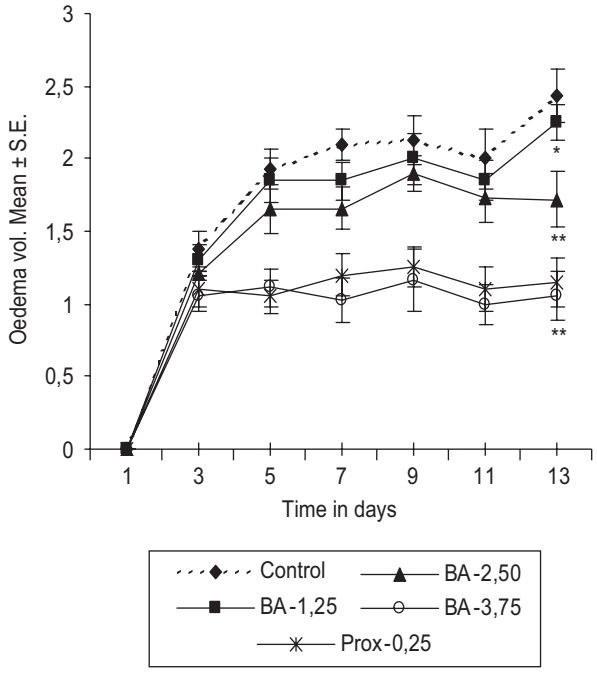


Fig. 6. Dose-related effect of paw swelling after immunization to the rats with 50 µl of 5 mg/ml solution of heat killed *Mycobacterium tuberculosis* in liquid paraffin. Paw volumes were taken with volume differential meter after every alternate day. Each value is the mean ± S.E. of five observations. Dose applied is in mg/animal/paw. *$p < 0.05$ and **$p < 0.01$.

Discussion

As Indian herbal medicines are increasingly becoming popular world wide, pharmacological evidences to understand the action of these medicines and the underlying mechanisms to support the proper and safe use of these medicines in clinic are indispensable. They are commonly prescribed alone or in combination to achieve sufficient effect in complex conditions such as rheumatoid arthritis. The pharmacological results of our current investigations revealed that BA elicited significant anti-inflammatory and anti-arthritic activity in rat and mouse models of paw and ear inflammation. Carrageenan paw inflammation is one of the most commonly used models for the investigation of new anti-inflammatory agents (Villar et al., 1987). Development of paw edema of rats induced by carrageenan is commonly correlated with the early exudative stage of inflammation, one of the important processes of inflammatory pathology (Liew and Mclnnes, 2002). The elevation of the paw volume in the first hour is due to the action of histamine and serotonin on the vascular permeability (Garcia et al., 1973). Inflammation gradually increases and attains peak within 3–4 h. This second phase could be due to the liberation and over production of prostaglandins and kinins in paw tissue, which accompanies leukocyte migration (Vane 1971). In our study, the inflammatory pattern of the rat paw evoked by carrageenan is in close accordance with the previous reports (Kochi et al., 2006), while the dose-dependent inhibition after topical application suggests that BA may act in both early and late phases of inflammation. The effect of BA increased with the increase in the frequency of BA application because the anti-inflammatory effect enhanced from single to double dose and to even every hour application (Figs. 3 and 4). The pharmacokinetic studies revealed that BA attains its peak after 30 h, if administered at a frequency of 6-h

Table 2. Comparative anti-inflammatory (carrageenan paw edema) and anti-arthritic (*Mycobacterium* induced developing arthritis) efficacy of boswellic acids through systemic and topical applications

Treatment	Anti-inflammatory (carrageenan)				Anti-arthritic (*Mycobacterium*)			
	Systemic route		Topical route		Systemic route		Topical route	
	Dose, mg/kg	% Inhibition	Dose, mg/paw	% Inhibition	Dose, mg/kg	% Inhibition	Dose, mg/paw	% Inhibition
BA	50	26*	1.25	13	50	32*	1.25	03
BA	100	39**	2.5	24*	100	42**	2.5	29*
BA	200	44**	3.75	34**	200	50**	3.75	56**
Ibuprofen	100	47**	–	–	100	49**	–	–
Piroxicam	–	–	0.25	41**	–	–	0.25	52**

For anti-inflammatory activity a single dose data of topical application is included in the table because of the comparison with single-dose systemic administration whereas, for anti-arthritic activity 13 days treatment in both the cases.Each value is the mean ± S.E. of five observations.*$p < 0.05$, **$p < 0.01$.

interval (Sharma et al., 2004). The increase in the effect from single to multiple doses could be because of the limited bioavailability of the drug for longer period due to the frequency of drug administration (Nadinic et al., 1999). The anti-inflammatory mechanism of BA is well established and is through the inhibition of the leukotrienes synthesis (Safayhi et al., 1992). Although leukotriene inhibitors did not effect the first phase of inflammation yet topical application of BA showed inhibition in the first phase.

The comparative data of BA for anti-inflammatory and anti-arthritic activity for systemic (data taken from our previous publication i.e., Singh et al., 1996) and topical application revealed that it is effective through both the routes but the effect seems better via topical application in chronic arthritic inflammation.

Topical application of AA and CO induces skin inflammation and is the appropriate models for the evaluation of anti-inflammatory agents (Hua et al., 2006). AA-induced ear edema is useful for the evaluation of lipoxygenase inhibitors (Young and Young, 1989). The results of the present study revealed that significant inhibition of AA-induced edema with different doses proves the previous results that BA is leukotriene inhibitor because only leukotriene inhibitors are effective against AA-induced inflammation. This also supports that BA is effective topically by the same mechanism as through the systemic route. However, bioavailability factor may also be involved because after topical application a large concentration of BA may be available to the target tissues whereas this may be limited with the systemic administration.

The advantages of CO-induced ear inflammation model are – its good predictive value for screening topical anti-inflammatory activity and its sensitivity to both steroidal and nonsteroidal drugs. However, this sensitivity is dependent on the time course of the response (Tubaro et al., 1985). The inhibition of inflammatory condition by BA in this model further proves the efficacy of BA mixture for topical application.

In arthritis, joint swelling and pain are the most commonly co-existing symptoms. An ideal therapeutic agent should at least possess anti-inflammatory and analgesic property. The results of *Mycobacterium*-induced developing arthritis revealed that BA showed a significant dose-dependent anti-arthritic activity after topical application proving that the effect can be achieved through this route in both acute and chronic models of inflammation. These results are in accordance with the previous results of our study (Singh et al., 1996) suggesting that BA is equally effective through topical application.

From these data, it is concluded that BA, which is already proved as a leukotriene inhibitor and is in clinical use as an anti-arthritic agent has shown similar results with topical application as with systemic administration in both acute and chronic models of inflammation. Furthermore, this may prove to be a highly beneficial to the rheumatic patients because in ointment form it can be directly applied to the target tissues for more effective results. Further work on its bioavailability through topical application in comparison to systemic administration is in progress.

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