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Cellular and molecular mechanisms of anti-inflammatory effect of Aflapin: a novel *Boswellia serrata* extract

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Abstract There is significant number of evidences suggesting the anti-inflammatory properties of gum resin extracts of *Boswellia serrata* containing 3-*O*-acetyl-11-keto-β-boswellic acid (AKBA) and their promising potential as therapeutic interventions against inflammatory diseases such as osteoarthritis (OA). Unfortunately, the poor bioavailability of AKBA following oral administration might limit the anti-inflammatory efficacy of standardized *Boswellia* extract(s). To address this issue, we describe a novel composition called Aflapin, which contains *B. serrata* extract enriched in AKBA and non-volatile oil portion of *B. serrata* gum resin. Our observations show that the availability of AKBA in systemic circulation of experimental animals is increased by 51.78% in Aflapin-supplemented animals, in comparison with that of 30% AKBA standardized extract or BE-30 (5-Loxin®). Consistently, Aflapin confers better anti-inflammatory efficacy in Freund's Complete Adjuvant (FCA)-induced inflammation model of Sprague–Dawley rats. Interestingly, in comparison with BE-30, Aflapin® also provides significantly better protection from IL-1β-induced death of human primary chondrocytes and improves glycosaminoglycans production in human chondrocytes. In Tumor necrosis factor alpha (TNFα)-induced human synovial cells, the inhibitory potential of Aflapin (IC_{50} 44.736 ng/ml) on matrix metalloproteinase-3 (MMP-3) production is 14.83% better than that of BE-30 (IC_{50} 52.528 ng/ml). In summary, our observations collectively suggest that both the *Boswellia* products, BE-30 (5-Loxin®) and Aflapin, exhibit powerful anti-inflammatory efficacy and anti-arthritic potential. In particular, in comparison with BE-30, Aflapin provides more potential benefits in recovering articular cartilage damage or protection from proteolytic degradation due to inflammatory insult in arthritis such as osteoarthritis or rheumatoid arthritis.

Keywords 3-*O*-acetyl-11-keto-β-boswellic acid · Aflapin · *Boswellia serrata* · Chondrocytes · Matrix metalloproteinase-3 · Osteoarthritis

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Introduction

Boswellia serrata is a type of deciduous tree grows naturally in Indian subcontinent. For centuries, the gum resin of *Boswellia serrata* has been used for the treatment of various inflammatory diseases including arthritis [1, 2]. The pentacyclic triterpenic acids, named boswellic acids, present in the gum resin of *B. serrata* are the main constituents responsible for its anti-inflammatory property [2]. Suppression of leukotriene synthesis by inhibiting 5-lipoxygenase (5-LOX) is considered the main mechanism underlying their anti-inflammatory effect. Boswellic acids (BAs) are specific and non-redox inhibitors of 5-lipoxygenase, and they do not affect 12-lipoxygenase and cyclooxygenase

(COX) activities [2–4]. Among the known BAs, 3-*O*-acetyl-11-keto-b-boswellic acid (AKBA) possesses the most potent inhibitory activity on 5-LOX [2, 5].

A significant number of studies support that *Boswellia* extract (BE) is beneficial in patients suffering from various diseases such as bronchial asthma, Crohn's disease, and arthritis [6–8]. AKBA is a minor constituent of the *Boswellia* extract and it typically varies from 2 to 3% in the higher grade commercial material. Therefore, with an intention to generate more efficacious anti-inflammatory product, we had developed a standardized novel *Boswellia* compound comprising 30% AKBA (BE30 or 5-Loxin®) [9–11]. Earlier, in a human genome screen study we showed that BE-30 (5-Loxin®) down-regulated several important genes in TNFα-induced human microvascular endothelial cells (HMECs). These genes are directly related to inflammation, cell adhesion, and proteolysis [10]. In another study, we showed BE-30 almost completely abrogated the gene expression and activities of matrix metalloproteinase-3, -10, and -12 in TNFα-induced HMECs [11]. Interestingly, BE-30 (5-Loxin®) exhibited significantly better anti-inflammatory efficacy than the regular *Boswellia* extract containing 3% AKBA in Freund's adjuvant-induced arthritis model of rats [10, 11]. In continuation, we explored the molecular basis of anti-inflammatory properties of BE-30. BE-30 inhibits the production of pro-inflammatory cytokine TNFα and it down regulates the key modulatory proteins of 5-lipoxygenase-arachidonic acid cascade such as 5-lipoxygenase-activating protein (FLAP) and 5-lipoxygenase in LPS-induced THP-1 human monocytes. To explore further, we showed that BE-30 (5-Loxin®) down-regulated mitogen-activated protein kinase (MAPK)/NFκb activation in LPS-induced human monocytes [12]. A broad spectrum safety of BE-30 was established in acute oral, acute dermal, primary skin, and eye irritation and a 90-day sub-chronic toxicity studies conducted in various animal models [13]. Furthermore, a double-blind placebo controlled human clinical study suggests that BE30 significantly effective in improving various pain scores in osteoarthritis patients. Interestingly, the improvement in pain scores in the treated subjects is correlated with the reduction of synovial fluid MMP-3, a potent cartilage-degrading enzyme [14].

However, a series of pharmacokinetic studies conducted in human and in animal models indicate that after oral administration of Boswellia products, sufficient systemic concentration of AKBA is required for its anti-inflammatory activities [15–19]. Poor absorption through intestine, and/or extensive metabolism is the crucial factor affecting the systemic availability of AKBA and thus limiting the anti-inflammatory efficacy of Boswellia products. Therefore, attempts to achieve an increased systemic availability of AKBA to improve further the anti-inflammatory potential of boswellia product are highly desirable. With an intention to develop an improved anti-inflammatory boswellia product, we developed a novel, standardized AKBA formulation, Aflapin. This communication addresses some comparative analyses between BE-30 (5-Loxin®) and Aflapin on following issues: (1) whether Aflapin confers more bioavailable concentration of systemic AKBA after a single dose oral administration; (2) whether Aflapin exhibits more potent anti-inflammatory efficacy; and (3) whether Aflapin is more effective in alleviating the pathological conditions of osteoarthritis at the cellular and molecular levels.

Materials and methods

Description of *Boswellia* products

BE-30 (5-Loxin®) is a novel *Boswellia serrata* extract standardized to contain at least 30% 3-*O*-acetyl-11-keto-β-boswellic acid (AKBA) using a selective enrichment process (Indian patent # 205269). The process involves selective enrichment of AKBA while simultaneously suppressing the concentration of triterpene compounds that are less active and those that antagonize the activity of AKBA [9]. Aflapin is a novel synergistic composition containing *B. serrata* extract selectively enriched with AKBA and *B. serrata* non-volatile oil. The non-volatile oil was prepared using a special process (PCT application # PCT/IN2009/000505) involving selective removal of Boswellic acids followed by removing volatiles under high vacuum. The composition was standardized to contain at least 20% AKBA. Table 1 summarizes these two compounds' characteristics.

5-Lipoxygenase assay

5-LOX enzyme inhibitory activity was measured using the method described earlier [12]. Briefly, the assay mixture contained 80 mM of linoleic acid and a sufficient amount of potato 5-LOX in 50 mM phosphate buffer (pH 6.3). The reaction was initiated by addition of enzyme buffer mix to linoleic acid and the enzyme activity was monitored as the increase in absorbance at 234 nm, which is the absorption maximum of the major reaction product, 9(S)-10E, 12Z-hydroperoxy octadecadienoic acid (9[S]-HPODE) (em = 23,000/M/cm). The reaction was monitored for 2 min and the inhibitory potential of the test substances was measured by incubating various concentrations of test substances 2 min before addition of linoleic acid. Each evaluation was performed in triplicate. Percentage inhibition of enzyme activity was calculated by comparing the slope of test substances with that of control.

Table 1 Comparison of specifications between BE-30 and Aflapin

Parameter	BE-30	Aflapin
Description	Off white to cream color dry powder	Light brown to dark brown dry powder
Particle size (μm)	180	425
Alcohol solubility (%)	95	95
Bulk density (gm/cc)	0.20–0.30	0.25–0.35
Tapped density (gm/cc)	0.35–0.40	0.50–0.65
3-*O*-Acetyl-11-keto-β-boswellic acid (%)	30	20

Maintenance of animals

Wister albino rats used in experiments were procured from National Institute of Nutrition (Hyderabad, India). Animals were provided with standard rodent chow and charcoal filtered UV exposed water ad libitum. Rats were housed and maintained in pathogen-free rooms at controlled temperature (24–26°C), humidity (45–70%), and light/dark cycle (12/12 h). Animal study protocols were approved by Institutional Animal Ethics Committee (IAEC). All the studies were performed in compliance with the guidelines stipulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and OECD.

Freund's adjuvant-induced inflammation model of rat

Albino Wistar rats (10–12 weeks, 220–250 g body weight) were supplemented with BE-30 (100 mg/kg), Aflapin (100 mg/kg), or prednisolone (10 mg/kg) in 0.5% CMC for 14 days. The control group received the same volume of vehicle (i.e., 0.5% CMC). On 15th day, 50 μl of Freund's Complete Adjuvant was injected subcutaneously in the sub planter region of the left hind paw. Before and after the inoculation of adjuvant the paw volumes were measured by a water displacement plethysmometer (UgoBasile, Italy). The difference between the paw volumes recorded on 14th day after adjuvant injection and on the day of injection was calculated to estimate the inflammatory response.

TNFα ELISA

Modulation of pro-inflammatory cytokine TNFα in serum samples of FCA-induced rats supplemented with BE-30 or Aflapin was quantitatively measured by TNFα Quantikine ELISA kit (R&D Systems Inc., Minneapolis, MN). The assay was performed following the protocol provided by the vendor. Circulatory TNFα concentration was quantitatively measured using a standard curve constructed by plotting the OD versus respective known concentration of TNFα standard.

Bioavailability study

Bioavailability study was conducted in male and female Wister rats of 10 weeks old. The animal protocol number is LI-090409. The protocol was approved by Institutional Animal Ethics Committee of Laila Impex R&D Centre. Albino Wistar rats (200–210 g body weight) were distributed into two groups. Each group consisted of five animals. Either 5-Loxin or Aflapin was mixed with 0.5% CMC. Each animal of the first group was supplemented with 20 mg of BE-30 (equivalent to 6 mg AKBA). The supplementation was given as a single dose through oral gavage with a volume of 1 ml of CMC suspension. Similarly, 30 mg of Aflapin (equivalent to 6 mg AKBA) was supplemented with 1 ml bolus of 0.5% CMC suspension to each animal of the second group. Blood samples were collected from all animals just before the oral administration of either Aflapin or BE-30; and at 0.5, 1, 2, 4, 8, and 12 h after oral administration. The serum samples were deproteinated with 100 μl of 20% TCA and 1.8 ml of HPLC grade methanol. The samples were clarified at $1{,}800 \times g$ for 10 min, 4°C; and the clear supernatants were analyzed for AKBA content by using LC–MS (Series 1100, Agilent Technologies, Santa Clara, CA). The separation was done through Phenyl-Hexyl C-18 column (250 × 4.6 mm, 5 μm particles, Phenomenex, Torrance, CA) at a flow rate of 1 ml/min. The mobile phase consisted of methanol–water-glacial acetic acid (8:1:0.4, v/v/v). The limit of quantification of AKBA was 4 ng/ml.

Cell culture

Human primary Chondrocytes (HCH) was procured from Promo Cell GmbH (Heidelberg, Germany). HCH cells were cultivated in the growth medium (Promo Cell, Catalog number C-27101) supplemented with Supplement Mix (Promo Cell, Catalog number C-39635). SW 982 human Synovial Sarcoma cells were purchased from American Type Culture Collection Centre (ATCC, Manassas, VA). SW 982 cells were grown in Dulbecco's modified Eagle's red medium (DMEM) (Sigma Chemical Co., St. Louis,

MO) containing 10% fetal bovine serum (HyClone, Logan, UT) and 4.5 g/l D-glucose.

Cell proliferation Assay

Proliferation of HCH cells was studied using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. Equal number of HCH cells was plated in each well of a 96-well microplate and treated with 1 ng/ml of IL-1β in presence or absence of different concentrations of BE-30 or Aflapin for 72 h. Vehicle control cultures were maintained in DMEM containing 0.1% DMSO (v/v). After 72 h of treatment, 5 µl of MTT reagent (R&D Systems Inc., Minneapolis, MN) diluted with 45 µl of phenol-red-free and FBS-free DMEM was added to each well and plates were incubated at 37°C with 5% CO_2 for 4 h. Thereafter, 50 µl of solubilization buffer (R&D Systems) was added to each well to dissolve the formazan crystals produced by the reduction of MTT. After 24 h, the optical density was measured at 550 nm using a microplate reader (Bio Rad, Hercules, CA).

Cartilage matrix production assay

Modulation of cartilage matrix production in BE-30- or Aflapin-treated chondrocytes was assayed using Alcian Blue binding method [20]. Briefly, equal number of HCH cells was cultivated in 24-well cell culture plate. Chondrocytes were treated with 1 ng/ml of IL-1β in presence or absence of different concentrations of BE-30 or Aflapin for 72 h. Cartilage matrix production in cells was quantified by Alcian blue staining of intracellular glycosaminoglycans (GAG). Cells were stained with Alcian blue (1% in 3% acetic acid) for 30 min, washed three times for 2 min in 3% acetic acid, rinsed once with water, and solubilized in 1% SDS. The optical density was measured at 605 nm using microplate reader (Bio Rad, Hercules, CA).

MMP-3 ELISA

Equal number of SW 982 human synovial sarcoma cells (5000 cells/well) was plated in 96-well plate. Cells were treated with 10 ng/ml of TNFα in presence or absence of different concentrations of BE-30 or Aflapin for 24 h. Vehicle control cultures were received only 0.1% DMSO. After 24 h incubation, cell culture supernatant was collected. Matrix metalloproteinase (MMP-3) was quantitatively measured in the cell culture supernatant by human MMP-3 kit following the instructions provided by the vendor (R&D Systems Inc., Minneapolis, MN). Standard wells contained known concentrations of recombinant human MMP-3 and the standard curve was constructed by plotting the OD versus respective concentration of standard.

Statistical analysis

The results were presented as mean ± standard deviation. The data were analyzed by non-paired Student's t test and $P < 0.05$ was considered statistically significant.

Results

Improved anti-inflammatory efficacy of Aflapin

Previously, we demonstrated that enrichment of AKBA content up to 30% in BE-30 greatly enhanced the 5-lipoxygenase (5-LOX) inhibition and anti-inflammatory properties of BE-30 compared to the regular commercial extracts of *B. serrata* resin containing only 2–3% AKBA [12]. In this study, we describe a novel synergistic composition of AKBA-enriched *B. serrata* extract and the non-volatile oil fraction of *B. serrata* (Aflapin) significantly improves its anti-inflammatory efficacy in in vitro and in vivo experiments. Enzymatic assays in a cell free system show that Aflapin possesses 21.06% ($P = 0.0001$) more effectiveness in inhibiting 5-LOX activity in comparison with BE-30. The IC50 (mean ± SD) for 5-LOX activity of Aflapin and BE-30 are 31.71 ± 0.69 and 40.17 ± 1.84 µg/ml, respectively (Fig. 1a).

For further substantiation of anti-inflammatory activity, we evaluated the efficacy of Aflapin in a Freund's Complete Adjuvant (FCA) induced inflammation model of Sprague Dawley rats. FCA challenge in vehicle-treated control animals produced paw edema (mean ± SD) of 0.51 ± 0.05 cu. cm (cc). Whereas, the animals supplemented with 100 mg/kg of Aflapin and BE-30 showed paw edema volumes of 0.24 ± 0.05 cc ($P < 0.0001$, vs. vehicle control) and 0.32 ± 0.03 cc (vs. vehicle control), respectively. The edema volumes recorded in a standard drug, Prednisolone-treated animals were 0.24 ± 0.11 cc ($P = 0.0003$, vs. vehicle control) (Fig. 1b). Therefore, comparing with the vehicle control group, Aflapin and BE-30 provided 53.6 and 36.7% protection from FCA-induced inflammation in the rat model. In addition, it is interesting to note that the protection provided by Aflapin is significantly ($P = 0.0102$) better than BE-30 in FCA-induced inflammation.

To further strengthen the in vivo anti-inflammatory efficacy of Aflapin and BE-30, we evaluated the modulation of circulatory TNFα in serum samples of rats supplemented with BE-30 or Aflapin included in the FCA induced inflammation study. Figure 1c shows that BE-30 and Aflapin reduce serum TNFα by 38.83% ($P = 0.0446$) and 65.04% ($P = 0.0018$), respectively, when compared to vehicle-treated control animals. The standard reference drug, Prednisolone exhibited 77.67% ($P < 0.001$) decrease

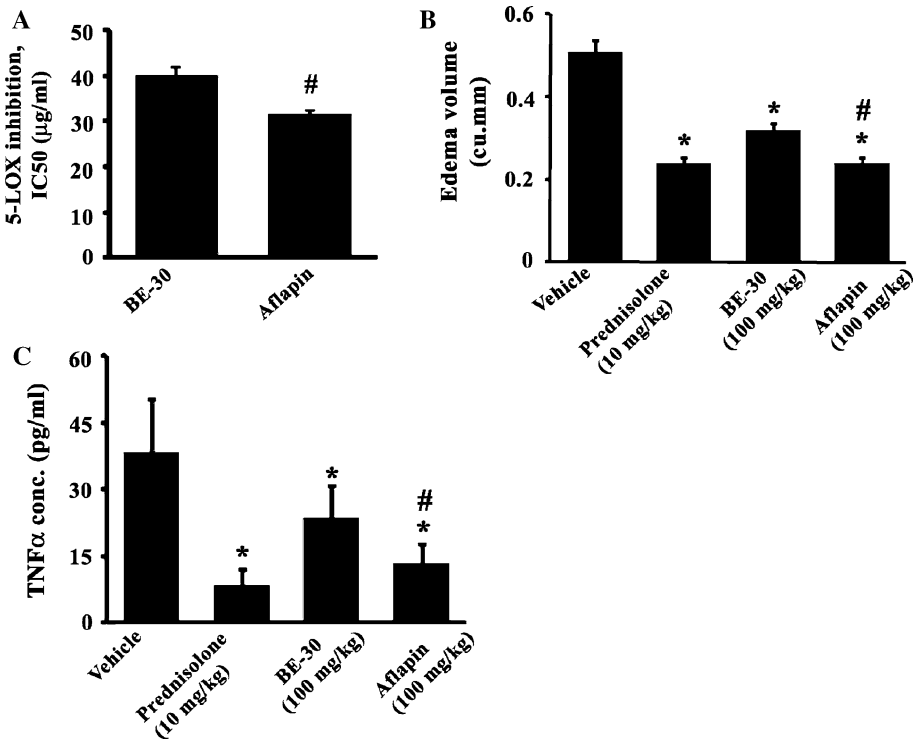

Fig. 1 Comparative analysis of anti-inflammatory properties of BE-30 and Aflapin. **a** *Bars* represent mean ± SD of IC_{50} (half maximal inhibitory concentration) of BE30 and Aflapin inhibiting 5-lipoxygenase activity in enzymatic assays ($n = 4$). **b** *Bar* diagram represents paw edema volumes (mean ± SD) of Sprague–Dawley rats ($n = 6$) in a Freund's Complete Adjuvant (FCA)-induced model of inflammation, as indicated. Animals in the vehicle-treated group received 0.5% CMC. **c** *Bar* diagram represents serum TNFα concentrations in different groups of animals ($n = 6$) included in Freund's Complete Adjuvant (FCA)-induced model of inflammation, as indicated. $*P < 0.05$ indicates significance versus vehicle control group; $\#P < 0.05$ indicates significance, BE-30 versus Aflapin

in serum TNFα in comparison with the vehicle control animals. Interestingly, Aflapin-treated group showed significantly ($P = 0.0315$) better reduction in serum TNFα concentration than that exhibited by the BE-30-treated group (Fig. 1c). These in vitro and in vivo data together suggest that Aflapin possesses significantly better anti-inflammatory efficacy than BE-30.

Aflapin provides increased bioavailability of ABKA

In a comparative assessment of bioavailability of AKBA in serum, we provided a single dose administration of 100 mg/kg body weight of BE-30 or Aflapin into Sprague–Dawley rats. Our observation indicates that AKBA is more bio-available in Aflapin-supplemented animals than BE-30. According to the plot constructed by taking serum concentration of AKBA versus time intervals after Aflapin or BE-30 administration, the area under the curve (AUC) was found 51.78% more in Aflapin-supplemented animals (AUC = 14.07), in comparison with BE-30-supplemented animals (AUC = 9.27) (Fig. 2). It is also interesting to note that in BE-30-supplemented animals the peak serum concentration (2.0 μg/ml) of AKBA reached at 1.5 h followed by a sharp decline, whereas, the Aflapin-supplemented animals showed a broader peak with a peak concentration of 2.28 μg/ml AKBA reached at 3.5 h. Overall, these data indicate that in comparison with BE-30, Aflapin provides 51.78% increase in bioavailability of AKBA in systemic circulation.

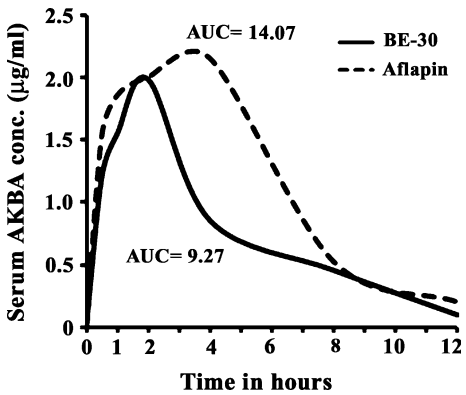

Fig. 2 Comparative bioavailability of AKBA in BE-30 and Aflapin-supplemented Sprague–Dawley rats. *Line diagram* represents mean AKBA concentration (μg/ml) in serum samples of Sprague–Dawley rats ($n = 5$) at different time points after a single dose oral administration of BE-30 and Aflapin. *AUC* area under the curve

Aflapin provides protection from IL-1β-induced death of human chondrocytes

Destruction of chondrocytes is a crucial patho-physiological feature in joints of osteoarthritis subjects. The chondrocytes are destroyed or killed under the influence of pro-inflammatory cytokine such as IL-1β, TNFα, etc. Therefore, it would be interesting to assess whether, Aflapin and BE-30 can protect pro-inflammatory cytokine-induced death of human chondrocytes. MTT incorporation-based cell proliferation experiment was conducted in IL-1β-induced model of human primary chondrocytes. Both BE-30 and Aflapin exhibited significant protection from cell death in a dose-dependent manner (Fig. 3). In comparison with the vehicle control, the proliferation index of chondrocytes was reduced by 18.42% ($P = 0.00532$) in 1 ng/ml IL-1β-treated culture and the reduced index was improved in the cultures concomitantly treated with either BE-30 or Aflapin in a dose-dependent manner. Interestingly, Aflapin provided significantly better protection than BE-30 at 0.25 μg/ml ($P = 0.0324$), 0.5 μg/ml ($P = 0.0412$), and at 1 μg/ml ($P = 0.0187$).

Aflapin improves glycosaminoglycans production in human chondrocytes

Reduced synthesis of matrix proteoglycans in chondrocytes and subsequent loss of matrix substances from articular cartilage are the key events in the early stages of destructive joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA). Pro-inflammatory cytokine, IL-1β is known to decrease proteoglycan synthesis in articular cartilage [21–23]. Glycosaminoglycans (GAGs) are the non-protein part of proteoglycans. Previously, it was found that IL-1β inhibited GAG synthesis in rat articular cartilage via nitric oxide-dependent pathway [24]. We examined whether BE-30 or Aflapin can modulate GAG synthesis in IL-1β-induced human chondrocytes. Upon incubation of chondrocytes with 1 ng/ml of human recombinant IL-1β, the intracellular GAG content was reduced by 23.08% ($P < 0.0001$) from the vehicle-treated cells. Concomitant incubation of different concentrations of BE-30 or Aflapin with IL-1β exhibited gradual recovery of intracellular GAG content in a dose-dependent manner (Fig. 4). Interestingly, Aflapin provided significantly better recovery than BE-30 at 0.05 μg/ml ($P = 0.0427$), 0.1 μg/ml ($P = 0.0310$), 0.5 μg/ml ($P = 0.0445$), and at 1 μg/ml ($P = 0.0107$).

Aflapin inhibits secretion of collagen degrading enzyme from human synovial cells under inflammatory stimulation

Under inflammatory conditions in OA, the chondrocytes and synovial cells produce large amount of matrix metalloproteinases such as MMP-3, MMP-13, etc., which degrade collagen matrix of cartilage. To evaluate whether the *Boswellia* products can down-regulate MMP-3 production in synovial cells under inflammatory condition, we

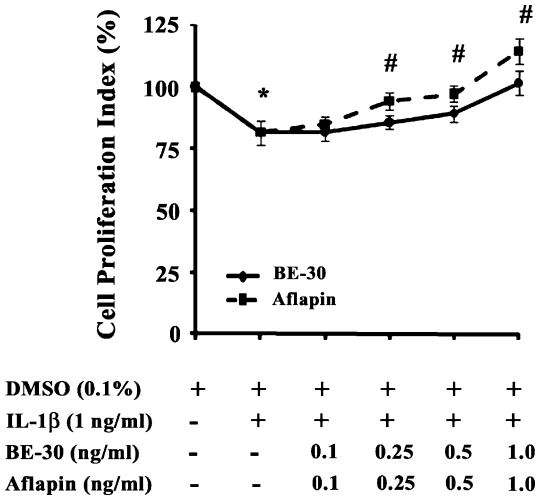

Fig. 3 Boswellia products improve proliferation of IL-1β-treated human primary chondrocytes. *Line diagram* represents cell proliferation index (mean ± SD, $n = 6$) of human primary chondrocytes treated with 1 ng/ml of IL-1β in presence or absence of different concentrations of BE-30 or Aflapin as indicated. Vehicle-treated cultures received 0.1% DMSO and the cell proliferation index of vehicle-treated cultures was considered 100%. *$P < 0.05$ indicates significance versus vehicle control group; #$P < 0.05$ indicates significance, BE-30 versus Aflapin

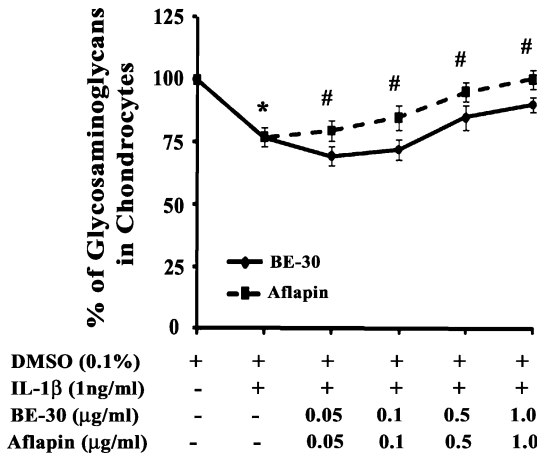

Fig. 4 BE-30 or Aflapin increase glycosaminoglycans (GAG) synthesis in IL-1β-treated human primary chondrocytes. *Line diagram* depicts percentage of GAG content (mean ± SD, $n = 6$) in human primary chondrocytes treated with 1 ng/ml of IL-1β in presence or absence of different concentrations of BE-30 or Aflapin as indicated. Vehicle-treated cultures received 0.1% DMSO and the GAG content in the vehicle-treated cells was considered 100%. *$P < 0.05$ indicates significance versus vehicle control group; #$P < 0.05$ indicates significance, BE-30 versus Aflapin

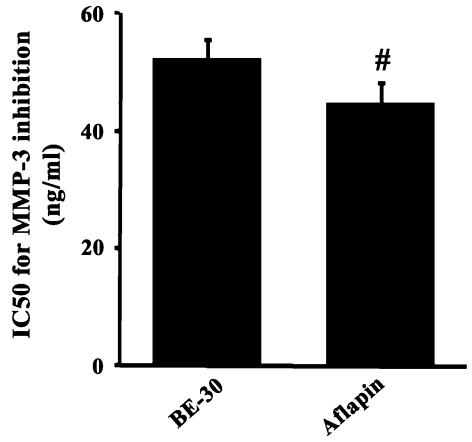

Fig. 5 BE-30 and Aflapin inhibit matrix metalloproteinase-3 secretion from TNFα-induced human primary chondrocytes. *Bar* diagram represents mean ± SD of IC_{50} (half maximal inhibitory concentration) of BE-30 and Aflapin in culture supernatants ($n = 4$) of human recombinant TNFα-induced human synovial cells. Vehicle control cultures received 0.1% DMSO. #$P < 0.05$ indicates significance, BE-30 versus Aflapin

incubated SW982 human synovial cells with TNFα in presence or absence of different concentrations of Aflapin or BE-30. We observed that upon stimulation with 10 ng/ml of human recombinant TNFα, the synovial cells produced 95% more MMP-3, compared to the vehicle-treated cells (data not shown). BE-30 and Aflapin significantly inhibited MMP-3 production in TNFα-induced human synovial cells, the half maximal inhibitory concentration (IC50s) were 52.528 and 44.736 ng/ml, respectively (Fig. 5).

Discussion

AKBA is a minor compound among the boswellic acids (BAs), but is the most active component of *B. serrata* gum resin extract for its anti-inflammatory properties via inhibiting 5-lipoxygenase, human leukocyte elastase, and the NF-kB pathway [2, 25–27]. To enhance the anti-inflammatory efficacy of *Boswellia* product, we enriched the AKBA content in the *Boswellia* extract and standardized a novel composition (BE-30 or 5-Loxin®) containing at least 30% AKBA [9]. Previously, we showed that BE-30 (5-Loxin®) exhibited significantly better anti-inflammatory efficacy than the regular *Boswellia* extract containing 3% AKBA [10, 11]. We also characterized the molecular basis of anti-inflammatory properties of BE-30 [12] and the set of genes regulated for its anti-inflammatory potential [10]. Furthermore, a double-blind placebo controlled human clinical study suggested that BE-30 was significantly effective in treatment of osteoarthritis [14]. However, one of the limiting factors of the efficacy of a drug candidate is bioavailability. It is known that because of the lipophilic nature of AKBA, it is poorly bio-available in systemic circulation after oral administration of *Boswellia* extract [19]. Therefore, to improve further the anti-inflammatory efficacy of *Boswellia* product we intended to develop a novel formulation by combining different components of *B. serrata* gum resin. Interestingly, it was found that a composition containing *B. serrata* extract enriched in AKBA and *B. serrata* non-volatile oil exhibited inhibition of 5-lipoxygenase activity in a synergistic manner (data to shown). It is also interesting that the HPLC analyses showed no detectable amount of boswellic acids present in the non-volatile oil fraction of *B. serrata*. So, addition of non-volatile oil fraction does not contribute any additional AKBA or other boswellic acids in the novel formulation (Aflapin). So, the final concentration of AKBA in the formulation is only 20%. However, in line of our hypothesis, we have found that a single dose administration of Aflapin provided 51.78% increase in bioavailability of AKBA in systemic circulation, in comparison with BE-30 (5-Loxin®). It is interesting to note that the peak serum concentration of AKBA was increased by 14% in Aflapin-supplemented animals, wherein, the AKBA content is 33.3% less than that of BE-30 (5-Loxin®). In addition, when compared to BE-30 (5-Loxin®), Aflapin® also provided longer retention of peak concentration of AKBA in systemic circulation (Fig. 2). So, from these observations we assume that the oil fraction present in Aflapin might be acting as a vehicle and might provide the basis for more bioavailable AKBA in the systemic circulation and to the target cells as well.

Earlier, we showed that BE-30 (5-Loxin®) inhibited TNFα production in LPS-induced human monocytes THP-1 cells with an IC50 of 4.61 ± 0.87 μg/ml [12]. To extend further the anti-inflammatory efficacy of the Boswellia products in in vivo animal model, we found that Aflapin and BE-30 (5-Loxin®) provided 53.6 and 36.7% protection from FCA-induced inflammation in the rat model. It is interesting to note that the same dose (100 mg/kg body weight) of Aflapin conferred 17.9% better protection ($P = 0.0102$) than BE-30 (5-Loxin®) (Fig. 1b). In addition, 65.04% ($P = 0.0018$) and 38.83% ($P = 0.0446$) reductions in serum TNFα concentration was also observed in Aflapin®- and 5-Loxin®-supplemented animals, respectively. Interestingly, Aflapin conferred 26.21% ($P = 0.0315$) better reduction in serum TNFα concentration than that of BE-30 (5-Loxin®)-treated group (Fig. 1c). These data suggest two lines of evidences; (1) both 5-Loxin® and Aflapin® have potential to be used as oral anti-TNFα therapy, and (2) Aflapin possesses significantly better anti-inflammatory potential that 5-Loxin®. It is noteworthy that 100 mg of 5-Loxin® and Aflapin® contain 30 and 20 mg of AKBA, respectively; and the non-volatile oil portion of Aflapin does not contain either AKBA or other boswellic acids.

Therefore, we assume that enhanced bioavailability of AKBA in Aflapin-supplemented animals might provide the argument in favor of higher anti-inflammatory potential of Aflapin than that of BE-30 (5-Loxin®) at the equal dose.

The chondrocytes is the only cell type found in mature cartilage and is responsible for the synthesis of collagen and proteoglycans; and maintenance of the extracellular matrix [28]. Evidences suggest that chondrocyte death may contribute to the progression of OA. Several studies have shown that OA cartilage has a higher number of apoptotic chondrocytes than does normal cartilage in animal models [29] and humans [30, 31]. The presence of increased numbers of apoptotic cells or reduction in viable chondrocytes correlates with the extent of cartilage matrix loss under inflammatory conditions in OA [30, 32]. Therefore, we were interested to assess whether the *Boswellia* products can modulate the cell proliferation of human primary chondrocytes treated with a pro-inflammatory cytokine IL-1β. Dying cells exhibit a sharp decrease in MTT reductive activity and this strategy is widely used to measure cell death [33, 34]. In MTT-based cell proliferation assay, we observed that BE-30 (5-Loxin®) and Aflapin provided significant improvements in cell proliferation in IL-1β-treated human chondrocytes. However, interestingly, Aflapin conferred significantly better protection from loss of cellular viability than does 5-Loxin® (Fig. 3).

Loss of matrix proteoglycans from articular cartilage is one of the key events in the early stages of destructive joint diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA). This leads to decreased resistance of articular cartilage against compressive load, which is followed by development of fissures and progressive destruction of articular cartilage. Interleukin-1 (IL-1) is known to decrease proteoglycan synthesis in articular cartilage [21, 23, 35]. Our observations suggest that BE-30 (5-Loxin®) and Aflapin modulate the glycosaminoglycans synthesis in IL-1β-induced human chondrocytes. Interestingly, in comparison with BE-30 (5-Loxin®), Aflapin provides significantly better protection from damaging action of IL-1β on GAG synthesis in chondrocytes.

Collagen is another important component of the cartilage extracellular matrix, providing tensile strength to the tissue [36, 37]. In arthritis, collagen degradation is initiated by collagenases such as matrix metalloproteinases (MMPs). MMPs are expressed and synthesized by different cell types present in the joint, including synovial cells and chondrocytes, in response to pro-inflammatory cytokines such as IL-1 [38, 39]. Therefore, therapeutic candidates possessing matrix metalloproteinases, such as MMP-3, MMP-13 inhibitory properties, provide potential promise to treat OA or RA. From our experiments, we observed that in comparison with BE-30 (5-Loxin®), Aflapin® provided 14.83% better efficacy in inhibiting MMP-3 production in TNFα-induced human synovial cells (Fig. 5). Consistently, Aflapin also provided significantly better efficacy than BE-30 (5-Loxin®) in inhibiting MMP-3 production in TNFα-induced human chondrocytes (data not shown, communicated in a separate manuscript). However, these observations suggest that Aflapin possesses more MMP-3 inhibitory potential than BE-30 (5-Loxin®) to provide protection from the proteolytic degradation of cartilage in inflammatory conditions of OA or RA.

In conclusion, these data together suggest that Aflapin®, an improved formulation containing AKBA enriched *Boswellia serrata* extract provides increased anti-inflammatory efficacy than 5-Loxin®. In addition, the observations also suggest that in comparison with 5-Loxin®, Aflapin® might confer more potential benefits of recovering articular cartilage damage or protection from proteolytic degradation due to inflammatory insult in arthritis.

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Competing interests The authors declare there are no competing interests.

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