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Efficacy and Safety of Aflapin®, a Novel *Boswellia Serrata* Extract, in the Treatment of Osteoarthritis of the Knee: A Short-Term 30-Day Randomized, Double-Blind, Placebo-Controlled Clinical Study

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ABSTRACT

Background and Objective: Aflapin®, also known as AprèsFlex® was developed as an enhanced bioavailable extract of *Boswellia serrata* gum resin, standardized to 20% 3-O-acetyl-11-keto-β-boswellic acid. This randomized, double-blind, placebo-controlled clinical trial confirms the efficacy of Aflapin in ameliorating the symptoms of osteoarthritis (OA) of the knee.
Methods: Based on the inclusion/exclusion criteria of the American College of Rheumatology, seventy subjects were recruited and randomized into Placebo (n = 35) and Aflapin (n = 35) groups. Subjects received either 100 mg Aflapin or a placebo for 30 days. All subjects were evaluated for pain and physical function using the standard tools i.e., Visual Analog Scale (VAS), Lequesne Functional Index (LFI), and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) at the baseline (Day 0), 5, and 30 days of treatment. Additionally, several inflammatory and cartilage biomarkers, including matrix metalloproteinase-3 (MMP-3), tumor necrosis factor-α (TNFα), high-sensitive C-reactive protein (hsCRP), Cartilage Oligomeric Matrix Protein (COMP), and collagen type II cleavage (C2C) were evaluated. Total blood chemistry analyses were conducted to affirm the safety of Aflapin.
Results: Sixty-seven subjects completed the study. Aflapin conferred significant improvements in pain scores as early as five days of treatment. Post-trial, VAS, LFI, WOMAC pain, WOMAC stiffness, WOMAC function, and total WOMAC scores decreased in the Aflapin group by 45%, 40.9%, 44.4%, 66.3%, 44.4%, and 48%, respectively. Aflapin supplementation also reduced circulating MMP-3, TNFα, hsCRP, and C2C.
Conclusion: This investigation affirms that Aflapin is clinically efficacious, fast-acting, and safe in the management of osteoarthritis. No significant adverse effects were observed.

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Aflapin; *Boswellia serrata*; cartilage; knee pain alleviation; osteoarthritis

Introduction

Osteoarthritis (OA) is a chronic degenerative musculoskeletal disorder characterized by structural changes including loss of articular cartilage, subchondral bone changes, development of osteophytes, and synovial inflammation. Worldwide estimates indicate that 9.6% men and 18% women of ≥ 60 years have symptomatic osteoarthritis (1). Although there is variation in the prevalence of OA among different countries, it was reported that there were 303.1 million cases of hip and knee OA worldwide in 2017 (including nearly 15 million new cases reported that year), with a global age-standardized point prevalence of 3754.2 (95% uncertainty interval (UI), 162.6–202.4 per 100,000) (2). In India, the prevalence of osteoarthritis in the elderly (60–79 years) population ranges between 14 and 47% (2). In the USA alone, OA affects 32.5 million adults (3). In osteoarthritis, mechanical factors can directly elevate pro-inflammatory cytokines and proteolytic enzymes, resulting in the degradation of matrix substances in articular cartilage. The complex interplay between these factors contributes to the natural history of the disease, making affected joints progressively more painful and arthritic (4). Globally, the prevalence and economic impact of osteoarthritis is high, causing a significant burden on society through disability, comorbid disease, and expensive therapies (5).

Gum resin extracts of *Boswellia serrata*, commonly known as Indian Frankincense, have been widely used in traditional and Ayurvedic medicine for centuries owing to their potent anti-inflammatory properties. Boswellia has been traditionally used to treat various inflammatory conditions, such as inflammatory bowel disease, asthma, allergies, as well as osteoarthritis and pain (6–12). Major constituents of *Boswellia serrata* gum resin are monoterpenes, diterpenes, triterpenes, and tetracyclic and pentacyclic triterpene acids, or boswellic acids (BAs). Six major boswellic acids have demonstrated the anti-inflammatory activities of the Boswellia gum resin, i.e., keto-β-boswellic acid (KBA),

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3-O-acetyl-11-keto-β-boswellic acid (AKBA), α-boswellic acid (α-BA), β-boswellic acid (β-BA), 3-O-acetyl-α-boswellic acid (α-ABA), and 3-O-acetyl-β-boswellic acid (β-ABA) (13). Among these BAs, AKBA is the most potent anti-inflammatory agent, and thus the most clinically relevant constituent of *B. serrata* gum resin (14).

Aflapin® is a novel synergistic composition derived from *Boswellia serrata* gum resin. Series of *in vitro* and *in vivo* observations have indicated that Aflapin® inhibits inflammatory cytokine-induced-matrix metalloproteinase-3 (MMP-3) production and provides protection in recovering cartilage ground substance, i.e., glycosaminoglycans (GAG) in primary human chondrocytes (15). Earlier, Sengupta et al. demonstrated the efficacy, benefits, and tolerability of Aflapin® in a 90-day placebo-controlled clinical study conducted in knee osteoarthritis subjects (age 40–70 years), in which Aflapin® conferred significant efficacy in pain reduction and improving the quality of life (8). Subsequently, an independent placebo-controlled clinical study conducted on subjects with osteoarthritis of knee demonstrated that Aflapin® provides significant relief from osteoarthritis symptoms in as early as five days of supplementation (9).

The objective of the present clinical study was to affirm and validate the short-term efficacy of Aflapin in the management of clinical symptoms of osteoarthritis of the knee. Furthermore, for the first time, this study assessed several inflammatory biomarkers, tumor necrosis factor-α (TNFα), MMP-3, high-sensitive C-reactive protein (hsCRP), and Cartilage Oligomeric Matrix Protein (COMP) and collagen type II cleavage (C2C), key biomarkers of cartilage metabolism in serum, to elucidate the molecular basis of the efficacy of Aflapin in supporting joint health.

Materials and methods

Study material

A novel standardized *Boswellia serrata* oleo-gum-resin extract, standardized to 20% AKBA (commercially known as Aflapin® and AprèsFlex®, batch no. F20010006), the most potent anti-inflammatory Boswellia composition was investigated in this study.

Study design

This study was conducted at two independent sites: Meher Hospital and Pujitha Hospital, Vijayawada, Andhra Pradesh, India, from August 2011 to May 2012; and monitored by Maanav Health Foundation, Vadodara, Gujarat, India. The study protocol was approved by the Independent Ethics Committee (IEC, Approval Number: MHF/EC/OU11/137) of Maanav Health Foundation (Vadodara, Gujarat, India). The trial was registered in the Clinical Trial Registry of India (CTRI/2011/07/001912). A brief overview of the clinical study protocol is provided in Figure 1. Study volunteers reviewed and signed the IEC approved consent form before the initiation of the investigation. Out of 111 screened osteoarthritis subjects, a total of 70 healthy subjects were selected according to inclusion/exclusion criteria (Table 1). Subjects, who were otherwise healthy, were aged 40 years or older and had a diagnosis of osteoarthritis as per the American College of Rheumatology classification criteria (16). The subjects were randomized and assigned to either Aflapin or a matching placebo.

Subject recruitment

The subject recruitment process and the study design were as follows: Screening visit occurred 7 days prior to the first dose of the investigational product (Day 0). The principal investigator discussed with each subject about the nature of the study, its requirements, and restrictions as per inclusion-exclusion criteria (Table 1). Written informed consent was obtained prior to performing any protocol-specific procedures.

Randomization, blinding, and intervention

Seventy subjects were randomly allocated to receive either placebo (n = 35) or Aflapin (n = 35) using a computer-generated block randomization process. The subjects in the Aflapin group received one 50 mg capsule of Aflapin b.i.d. (100 mg/d), whereas the subjects in the Placebo group received identical placebo capsules having similar organoleptic properties. Each subject completed a questionnaire, providing details regarding demographics, medical history, nutritional status, and prior or concomitant medication at the baseline evaluation and during each follow-up evaluation on days 5 and 30. The study investigators were blinded; the randomization codes were secured and confidential. The clinical trial pharmacist and the statistician ensured that the treatment codes remained confidential. Active or placebo capsules, compliance cards, instructions, and follow-up evaluations dates were provided to all the subjects at the baseline visit.

Outcome measures

Pain and function score evaluations

Change in pain and physical function scores was measured using the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score at the end of the intervention from the baseline and served as the primary outcome measure of the study. The WOMAC score on a scale of 0 to 100 normalized units measured joint pain, stiffness, and physical function in OA subjects (17). The total index consists of 24 questions: a pain subscale or WOMAC-A consisting of 5 questions; the WOMAC-B subscale for measuring stiffness, consisting of 2 questions; and WOMAC-C subscale for physical function, consisting of 17 questions. The WOMAC assessments were performed at baseline and on days 5 and 30 of supplementation.

Secondary efficacy outcome measures included improvements in the Visual Analog Scale (VAS) (18), and Lequesne Functional Index (LFI) (19) scores. These scores are widely accepted measures for pain and the severity of OA.

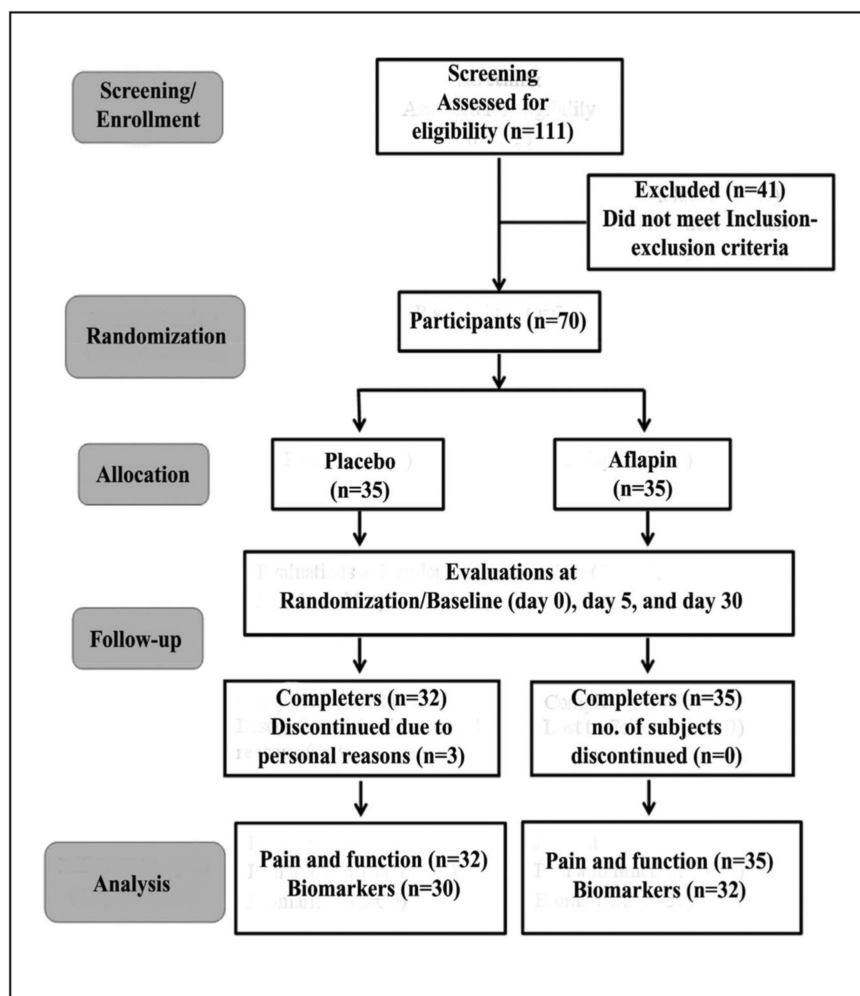

Figure 1. A CONSORT diagram presents the participant flow and different steps of the trial.

Table 1. Inclusion-exclusion criteria.

Inclusion Criteria	Exclusion Criteria
Male and female subjects of 40–70 years of age.	History of underlying inflammatory arthropathy or severe rheumatoid arthritis (RA), or Systemic Lupus Erythematosis (SLE).
Unilateral or bilateral OA of the knee for more than 3 months.	Hyperuricemia (men > 7 mg/dL, women > 6 mg/dL) and/or past history of gout.
Radiographic evidence (X-ray diagnosis) during screening.	Recent injury in the area affected by OA of the knee (past 4 months) and expectation of surgery in the next 4 months.
VAS score during the most painful knee movement between 40–70 mm after 5 days of withdrawal of usual medication.	Hypersensitivity to NSAIDs, abnormal liver or kidney function tests, history of peptic ulceration and upper GI hemorrhage, congestive heart failure, hypertension, hyperkalemia.
Lequesne's functional index score greater than 7 points after 5 days of withdrawal of usual medication.	Major abnormal findings on complete blood count, history of coagulopathies, hematological or neurological disorders.
Ability to walk.	BMI < 18, or BMI > 30 kg/m^2
Females of childbearing potential must agree to use an approved form of birth control and have a negative pregnancy test result.	Pregnant, breastfeeding, or planning to become pregnant during the study.
Availability of the duration of the entire study period.	Intra-articular corticosteroid injections within the last 3 months
	Abnormal Liver or kidney function tests.
	Use of concomitant prohibited medication other than ibuprofen
	High alcohol intake (>2 standard drinks per day)
	Use of other natural health products, including glucosamine and chondroitin, one month before and during the study.

The VAS and LFI scores were measured at baseline and on days 5, and 30 of supplementation.

Serum biomarkers

TNFα, hsCRP, and MMP-3

Pro-inflammatory modulators such as TNFα, hsCRP, and a cartilage degrading enzyme, Matrix Metalloproteinase-3 (MMP-3) in serum samples were quantitatively measured by ultra-sensitive ELISA method (R&D Systems, Minneapolis, MN, USA). ELISAs were performed according to the manufacturer's protocol. The specific immune reaction was detected by substrate solution, and the color development was read in a microplate reader (Bio-Rad, Hercules, CA, USA). For each assay, a 7-point linear standard curve was generated by plotting the absorbance vs concentration of the respective analyte. The sensitivities of TNFα, hsCRP, and MMP-3 detection ELISA kits were 0.106 pg/mL, 10 pg/mL and 9 pg/mL, respectively.

COMP and C2C

Key markers of cartilage metabolism, including Cartilage Oligomeric Matrix Protein (COMP) and Collagen Type II Cleavage (C2C) were quantitatively measured in serum samples by using an ultra-sensitive ELISA method. COMP Quantikine ELISA kit was purchased from R&D Systems (Minneapolis, MN); C2C ELISA kit was purchased from IBEX Pharmaceuticals Inc. (Montreal, Quebec, Canada). Assays were performed following the protocols provided by the manufacturers. The specific immune reaction was detected by substrate solution, and the color development was measured using a microplate reader (x-Mark™, Bio-Rad, Hercules, CA, USA). For each assay, a standard curve was generated by plotting the optical density at the respective known concentration of the analyte molecule. The sensitivities of COMP and C2C detection ELISA kits were 10 pg/mL and 10 ng/mL, respectively.

Two serum samples in the Placebo group and three samples in the Aflapin group were found hemolyzed. These serum samples were not included in the biomarker evaluations. Therefore, 30 and 32 serum samples in the Placebo and Aflapin groups, respectively, were tested and analyzed in biomarker measurements.

Safety measures

Hematological and biochemical evaluations

For assessment of safety of Aflapin, several parameters were evaluated in serum, urine, and whole blood samples of all subjects at each visit of the study. Serum biochemistry parameters included albumin, alkaline phosphatase, total bilirubin, cholesterol, creatinine, creatine kinase-n-acetyl cysteine, glucose, high-density lipoprotein, low-density lipoprotein, potassium, serum glutamic oxaloacetate transaminase, serum glutamate pyruvate transaminase, triglycerides, and urea. Hematology parameters included total count and differential blood count, erythrocyte sedimentation rate (ESR), hemoglobin, platelet count, mean corpuscular volume, and mean corpuscular hemoglobin. The test parameters in urine analyses include specific gravity, pH, albumin, bile salts, bile pigment, glucose, red blood cells, and ketone bodies. Serum biochemical and hematological parameters were measured using an automated analyzer (HumaStar 300, Human, Wiesbaden, Germany) and a hematological counter (Humacount, Human, Wiesbaden, Germany). The urine analysis was carried out using UroColor™10 Dip Sticks and Urometer 600 (Standard Diagnostics, Kyonggi-do, Korea) and by sediment analysis under a light microscope (model DC3-163, National Optical & Scientific Instruments, Inc., Schertz, TX).

Rescue medication: The principal investigator, a practicing physician and researcher recommended Ibuprofen in case of knee pain. The subjects were instructed not to take rescue medicine at least three days before each evaluation. No other pain-relieving interventions were allowed during the study period.

Statistical analysis

The data are presented as mean ± SD. Detailed statistical analyses were performed using SPSS software (SPSS v20, SPSS Inc. Chicogo, IL) to evaluate the efficacy of Aflapin in comparison with the Placebo group in terms of improvement in pain and physical function scores at baseline and on days 5 and 30 of treatment; and serum biomarkers at baseline and on day 30 of treatment. The significance of the effects of the study supplement was evaluated using a t-test. Results with $p < 0.05$ are considered statistically significant.

The sample size was calculated using power analysis. For power calculations, the estimates for the variability of 11.9 VAS score and assumed effect size of 15.2 (23.5 in treatment minus 8.3 in placebo) were considered. The estimates were based on observations from an earlier placebo-controlled study on Aflapin conducted in subjects with OA (9). A group size of 30 would provide more than 90% power to detect the treatment-related changes. Considering 10–20% dropouts, 35 subjects were allocated in each group.

Results

Baseline characteristics

Following recruitment and randomization, the subjects enrolled in this clinical investigation were provided either Aflapin 100 mg/day (n = 35) or placebo (n = 35) over a period of 30 consecutive days. A total of sixty-seven participants completed the study: thirty-two subjects (24 females, 50.63 ± 7.72 years; 8 males, 55.38 ± 3.89 years) in the placebo group; thirty-five subjects (26 females, 50.08 ± 8.24 years; 9 males, 56.00 ± 8.00 years) in the Aflapin group. Table 2 summarizes the comparative analyses on demographic variables, baseline characteristics, and baseline outcome measures (VAS, LFI, total WOMAC and its subscale scores viz. pain, function, and stiffness). Demographic variables and disease-related baseline parameters of the two groups did not differ significantly (Table 2).

Table 2. Demographic data and baseline characteristics of the subjects who completed the study.

Characteristics	Placebo (n = 32)	Aflapin 100 mg/day (n = 35)	*p* value (Aflapin vs Placebo)
Sex (male/female; n)	8/24	9/26	–
Age (years)	51.81 ± 7.21	51.60 ± 8.48	0.9121
Body weight (kg)	62.31 ± 11.41	61.24 ± 8.02	0.6615
Body mass index (kg/m^2)	25.35 ± 3.17	25.25 ± 3.11	0.8947
Visual analog score	54.46 ± 5.64	53.29 ± 6.24	0.4220
Lequesne's Functional Index	11.61 ± 2.25	11.84 ± 2.29	0.6761
WOMAC score			
Total WOMAC	1050.00 ± 211.75	1037.14 ± 202.68	0.8008
Pain subscale	42.97 ± 7.92	43.86 ± 8.58	0.6620
Stiffness subscale	32.81 ± 13.38	29.64 ± 10.09	0.2751
Function subscale	45.27 ± 9.6	44.62 ± 9.0	0.7776

P values were obtained by unpaired t-test (two-tailed, α = 0.05), Aflapin vs placebo.

Table 3. Unpaired t-test analyses for comparison of the scores obtained from the placebo and Aflapin supplemented subjects.

Parameter and treatment	Baseline mean ± SD	Day-30 mean ± SD	*p* value (vs baseline)	*p* value (vs placebo)
Visual analog scale score				
Placebo (n = 32)	54.5 ± 5.64	52.9 ± 12.14	< 0.4241	NA
Aflapin 100 mg/day (n = 35)	53.3 ± 6.24	29.3 ± 10.77	< 0.0001	<. 0001
Lequesne Functional Index				
Placebo (n = 32)	11.6 ± 2.25	11.9 ± 3.32	0.4753	NA
Aflapin 100 mg/day (n = 35)	11.8 ± 2.29	7.00 ± 2.63	< 0.0001	< 0.0001
WOMAC Total				
Placebo (n = 32)	1050.00 ± 211.75	1014.84 ± 328.48	0.80008	NA
Aflapin 100 mg/day (n = 35)	1037.14 ± 202.68	539.29 ± 230.03	< 0.0001	< 0.0001
WOMAC pain subscale				
Placebo (n = 32)	43.0 ± 7.92	39.2 ± 12.71	0.0613	NA
Aflapin 100 mg/day (n = 35)	43.9 ± 8.58	19.4 ± 11.17	< 0.0001	< 0.0001
WOMAC stiffness subscale				
Placebo (n = 32)	32.8 ± 13.38	28.5 ± 17.46	0.1552	NA
Aflapin 100 mg/day (n = 35)	29.6 ± 10.09	10.0 ± 8.99	< 0.0001	< 0.0001
WOMAC function subscale				
Placebo (n = 32)	45.3 ± 9.60	44.8 ± 14.34	0.7864	NA
Aflapin 100 mg/day (n = 35)	44.6 ± 9.00	24.8 ± 10.00	< 0.0001	< 0.0001

NA, not applicable; WOMAC, Western Ontario and McMaster Universities Osteoarthritis Index. *P* values were obtained from within the group' and 'Between the group' comparisons by unpaired t-test (two-tailed, α = 0.05).

Clinical efficacy

The normalized pain and function scores were subjected to statistical analysis using SPSS software. Unpaired t-test analysis was applied to compare Aflapin vs placebo, and ANOVA followed by Tukey's multiple comparison analysis was used. The data are summarized in Table 3.

Significant reductions in pain and function scores were observed on day 5 of supplementation of Aflapin (100 mg/day) compared to baseline and to placebo (Figure 2). Significant reductions in all the pain scores were observed in the Aflapin group on day 30 of supplementation when compared with the placebo group (Figure 2). Supplementation of Aflapin for 30 days conferred 44.6%, 41.2%, 50.5%, 64.9%, 44.6%, and 46.86% reductions in VAS, LFI, WOMAC pain, WOMAC stiffness, WOMAC function, and total WOMAC scores, respectively, when compared with those obtained with placebo. Significant ($p < 0.05$) reductions in all pain scores were also observed in the Aflapin group as early as 5 days of supplementation, when compared with placebo. Aflapin supplementation showed 23.9%, 22.4%, 24.8%, 47.6%, 21.2%, and 23.58% better reduction than placebo in VAS, LFI, WOMAC pain, WOMAC stiffness, WOMAC function, and total WOMAC scores, respectively on day 5 of supplementation. Compared to the placebo group, the reductions in WOMAC scores were significant after 5 days of supplementation (Figure 2). Aflapin supplementation for 30 consecutive days afforded highly significant ($p < 0.0001$) reductions in all the pain scores and exhibited 45.0%, 40.9%, 44.4%, 66.3%, 44.4%, and 48% reductions in VAS, LFI, WOMAC pain, WOMAC stiffness, WOMAC function, and total WOMAC scores, respectively, as compared with their baseline values (Figure 2).

Serum biomarkers

Supplementation of Aflapin resulted in 59.05% ($p < 0.001$; day 30: 1.97 ± 1.11 pg/mL vs baseline: 4.82 ± 2.37 pg/mL) reduction in baseline serum TNFα concentration. In contrast, 37.24% ($p < 0.001$; day 30: 2.85 ± 1.73 pg/mL vs baseline: 4.55 ± 1.85 pg/mL) reduction in baseline serum TNFα was observed in the placebo group. At the end of the study, the reduction in serum TNFα in the Aflapin group was also significantly greater ($p < 0.05$) as compared to the placebo (Table 4).

Intra and inter-group comparison analyses show that the changes in other biomarkers were not significant at the end of the study. Effects of Aflapin supplementation on hsCRP, MMP-3, COMP, and C2C at post-intervention are summarized in Table 4.

The Aflapin supplemented group showed 16.92% (day 30: 18.0 ± 8.7 ng/mL vs baseline: 21.7 ± 16.0 ng/mL) and 20.06% (day 30: 2.73 ± 2.07 µg/mL vs baseline: 3.42 ± 2.56 µg/mL) reductions in serum MMP-3 and hsCRP, respectively,

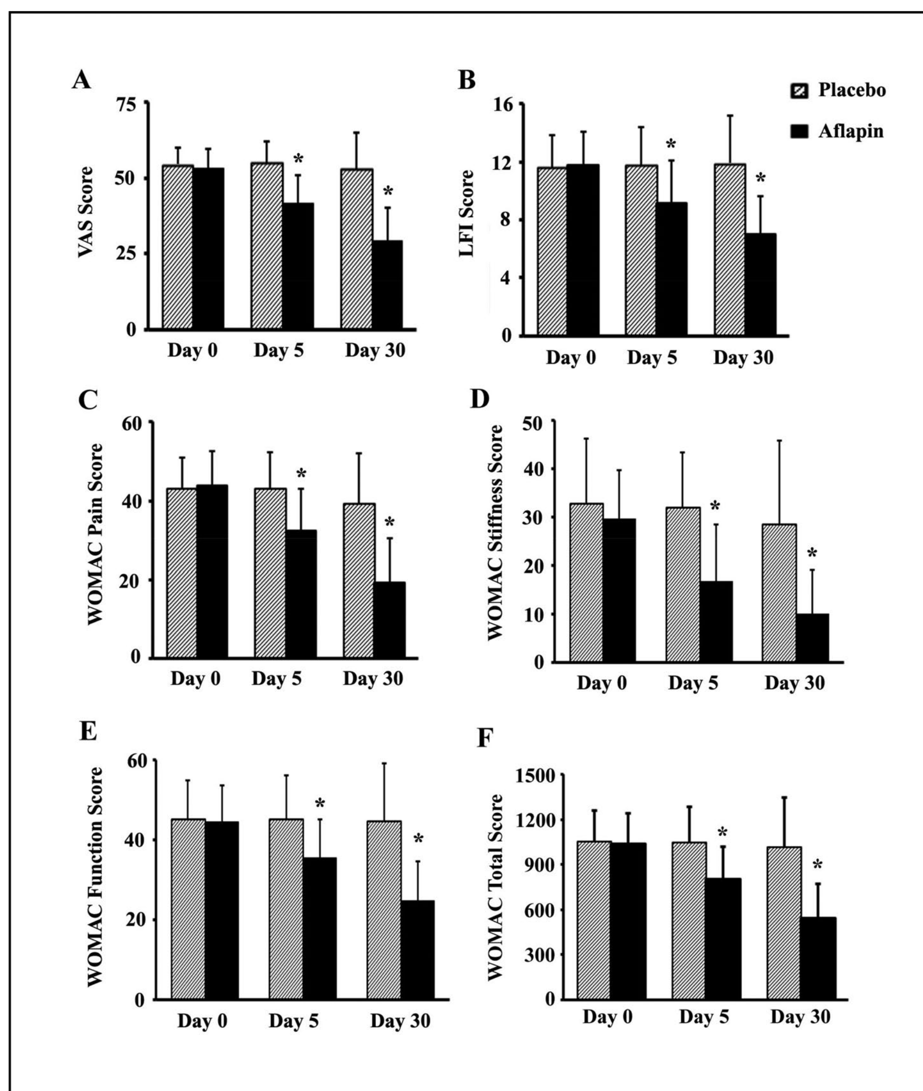

Figure 2. Pain, Function and Stiffness scores. Presented are the mean scores for Visual Analog Scale **(A)**, Lequesne Functional Index **(B)**, WOMAC-pain **(C)**, WOMAC-stiffness **(D)**, WOMAC-function **(E)**, and total WOMAC **(F)** scores in Placebo and 100 mg/day Aflapin groups at different time points day 0, day 5 and day 30. Each bar represents mean ± SD. * indicates $p < 0.05$, the mean scores in the treatment groups were compared with placebo at particular visit using unpaired t-test.

at the end of the study. In contrast, the placebo showed only 3.69% and 0.55% reductions from baseline in serum MMP-3 and hsCRP, respectively (Table 4).

In the Aflapin group, serum COMP was increased by 2.49% (day 30: 268.1 ± 87.6 ng/mL; vs baseline: 261.6 ± 88.4 ng/mL); in contrast, placebo showed 5.05% increase (day 30: 287.6 ± 130.2 ng/mL; vs baseline: 273.7 ± 127.4 ng/mL), at the end of the study (Table 4).

Post-trial, in Aflapin-supplemented subjects, serum C2C level was reduced by 1.05% (day 30: 309.2 ± 69.3 ng/mL vs baseline: 312.5 ± 48.6 ng/mL); whereas, the placebo group had a 2.67% increase (day 30: 315.9 ± 58.8 ng/mL vs baseline: 307.7 ± 54.4 ng/mL), from baseline (Table 4).

Biochemical evaluations

As a part of the safety evaluation, laboratory tests were performed to evaluate hematological parameters and different biochemical parameters in serum and urine. The repeated measure ANOVA was used to compare the values at different time points over the 30 days period as compared to the baseline. Unpaired and paired t-tests were used to analyze the inter-group and intragroup comparisons, respectively. Statistical analyses of these parameters did not indicate any significant changes. Although minor changes were observed in some of the parameters, they remained within the normal laboratory range. Similarly, no significant changes in hematological and urinary parameters were observed in the active treatment group when compared to those in the placebo (data not shown).

Consumption of rescue medication

Subjects who expressed pain were provided rescue medication by a study physician, and the same was recorded. Total consumption of rescue medication in the placebo group was

Table 4. Comparative analyses of biomarkers in placebo and Aflapin supplemented subjects.

Parameter and treatment	Baseline (mean ± SD)	Day-30 (mean ± SD)	% change at Day-30 from Baseline	p-value (vs baseline)	p-value (vs placebo)
TNFα (pg/ml)					
Placebo (n = 30)	4.55 ± 1.85	2.85 ± 1.73	−37.24	< 0.001	NA
Aflapin 100 mg/day (n = 32)	4.82 ± 2.37	1.97 ± 1.11	−59.05	< 0.001	<0.05
MMP3 (ng/ml)					
Placebo (n = 30)	19.6 ± 11.4	18.8 ± 12.7	−3.69	>0.05	NA
Aflapin 100 mg/day (n = 32)	21.7 ± 16.0	18.0 ± 8.7	−16.92	>0.05	>0.05
hs-CRP (µg/ml)					
Placebo (n = 30)	3.25 ± 1.93	3.23 ± 2.14	−0.55	>0.05	NA
Aflapin 100 mg/day (n = 32)	3.42 ± 2.56	2.73 ± 2.07	−20.06	>0.05	>0.05
COMP (ng/ml)					
Placebo (n = 30)	273.7 ± 127.4	287.6 ± 130.2	5.05	>0.05	NA
Aflapin 100 mg/day (n = 32)	261.6 ± 88.4	268.1 ± 87.6	2.49	>0.05	>0.05
C2C (ng/ml)					
Placebo (n = 30)	307.7 ± 54.4	315.9 ± 58.8	2.67	>0.05	NA
Aflapin 100 mg/day (n = 32)	312.5 ± 48.6	309.2 ± 69.3	−1.05	>0.05	>0.05

NA = not applicable; Intragroup (vs baseline) and Intergroup (vs placebo) statistics was done unpaired t-test. P < 0.05 was considered as statistically significant.

Table 5. Adverse events recorded during the intervention.

Adverse events	Placebo (n = 32) n (%)	Aflapin (n = 35) n (%)
Backache	0 (0.0)	3 (8.57)
Bilateral wheeze	1 (3.13)	0 (0.0)
Cough and headache	0 (0.0)	1 (2.86)
Fever	0 (0.0)	2 (5.71)
Gastritis	1 (3.13)	0 (0.0)
Headache	3 (9.38)	2 (5.71)
Headache and constipation	1 (3.13)	0 (0.0)
Insomnia	1 (3.13)	0 (0.0)
Nausea	1 (3.13)	0 (0.0)
Shoulder pain -Right	1 (3.13)	0 (0.0)
Stomachache	0 (0.0)	1 (2.86)
Weakness and headache	1 (3.13)	0 (0.0)
Number of subjects with at least one adverse event	10 (31.25)	6 (17.14)

higher when compared to the Aflapin group. During the study period, only 7 ibuprofen tablets were consumed by the subjects in the Aflapin group; in comparison, the subjects in the placebo group consumed 26 tablets.

Adverse events and dropouts

Three subjects dropped out from the placebo group, whereas there were no dropouts in the Aflapin group. Of these three dropouts, two subjects could not attend the final evaluation due to personal reasons, and one subject was non-compliant with the study supplement regimen. None of the dropped-out subjects experienced adverse events. No serious adverse events were reported during the course of 30-day study. However, a few subjects reported minor adverse events, such as ankle pain, back pain, cough, fever, and headache. These events were incidental and evenly distributed between the Placebo- and Aflapin-supplemented groups. Ten subjects (31.25%) in the Placebo group and six subjects (17.14%) in the Aflapin group reported at least one minor adverse event during the study (Table 5).

Discussion

Boswellic acids (BAs) present in the gum resin of *B. serrata* are known for their anti-inflammatory properties. BAs are specific non-redox inhibitors of 5-Lipooxygenase and suppress leukotriene synthesis. Among the known BAs, 3-O-acetyl-11-keto-β-boswellic acid (AKBA) is the most potent inhibitor of 5-LOX activity (14). A variety of preparations of *B. serrata* gum resin extracts (BSE), standardized to different percentages of the major boswellic acids, have been shown to alleviate the clinical symptoms of OA at a dose range of 339 to 3600 mg/day (20–23). These standardized preparations contained either naturally occurring or enriched concentrations of total BAs. Aflapin is a patented, bioavailability enhanced, synergistic Boswellia composition standardized to 20% AKBA (15). Poor bioavailability of BAs due to their hydrophobic nature and low water solubility limit the efficacy of BSE when ingested orally (24). In this context, due to improved bioavailability, it is expected that a lower dose of Aflapin could yield enhanced therapeutic benefits compared to the standard Boswellia preparations.

An earlier independent double-blind clinical study demonstrated that Aflapin provided significant improvements in clinical symptoms of OA in comparison with another Boswellia composition, 5-LOXIN® (8). Further, Vishal et al. demonstrated the clinical efficacy and early onset of action of Aflapin in an independent, double-blind, placebo-controlled clinical study conducted on subjects with knee OA (9). With this study, we intended to confirm early efficacy of Aflapin on knee OA symptoms. We therefore conducted an independent double-blind placebo-controlled study on subjects with mild to moderate OA of the knee. The subjects attended the outpatient departments of two orthopedic clinics in Vijayawada, Andhra Pradesh. Significant pain relief and improvements in physical ability measured by VAS, WOMAC, and LFI scores in Aflapin-supplemented subjects, in comparison with the placebo were considered as the outcome measures of the study. We also evaluated the modulation of inflammatory markers TNFα, hsCRP, MMP-3, and the markers of cartilage metabolism, e.g., COMP C2C as secondary measures.

Aflapin is a potent, selective 5-LOX enzyme inhibitor (15). A series of assays conducted in cellular models demonstrated that Aflapin suppressed key inflammatory markers, including TNFα, ICAM-1, and MMP-3 (8, 15). These modulators are crucial for the onset of joint inflammation and are contributory factors in the pathophysiology of OA (25–28). Furthermore, Aflapin improves cell viability in IL-1β treated human chondrocytes and protects against the damaging effects of IL-1β on glycosaminoglycans (GAG) synthesis in human chondrocytes (15). Taken together, these observations suggest that Aflapin reduces joint inflammation and inflammation-induced cartilage breakdown and thus contributes in maintaining healthy joint.

The present study demonstrates the efficacy of Aflapin in alleviating symptoms and clinical manifestations of osteoarthritis, including pain and joint stiffness, and in improving physical function in osteoarthritic subjects (Table 3, Figure 2). Pain, stiffness of joints, reduced joint mobility, and enhanced physical discomforts are the major clinical manifestations of OA (29, 30). To assess the improvements in the treatment group, we compared the baseline data of various parameters with those obtained on days 5 and 30 of supplementation. Statistical analyses revealed that compared to placebo, Aflapin supplementation significantly improved all clinical OA scores, including VAS, LFI, WOMAC pain, WOMAC stiffness, WOMAC function, and total WOMAC scores following 5 days of supplementation (Figure 2). These findings are well corroborated with earlier observations demonstrated by Vishal et al. (9). Of note, lower consumption of rescue medication in the Aflapin group compared with the Placebo group further suggests the efficacy of Aflapin. Taken together, these observations clearly confirm that Aflapin supplementation provides fast and significant pain relief, improvement in physical ability, and quality of life in OA subjects.

Elevated levels of pro-inflammatory cytokines like TNFα and/or its soluble receptors TNF-sR1, TNF-sR2, Interleukin-6 (IL-6), and MMP-3 in the circulatory system, along with the increased amounts of cytokines and MMPs in synovial compartments, have been observed in osteoarthritic patients (26, 29). Taken together, these observations support that osteoarthritis is not only a localized condition but appears to have a systemic inflammatory component. Interestingly, it is worth noting that our observations demonstrate that Aflapin reduced the levels of serum TNFα and hsCRP by 59.05% and 20.06% from baseline, respectively. We also observed a 37.24% reduction in TNFα from baseline in placebo, presumably due to the higher consumption of the rescue medication by the subjects in this group during the study. At the end of the study, reduction of baseline TNFα in the Aflapin group was significant compared to baseline, suggesting that Aflapin could protect against inflammatory damage to synovial tissue in osteoarthritis.

In the present study, Aflapin reduced serum MMP-3 compared to baseline, whereas the MMP-3 level in the Placebo group was virtually unchanged (Table 4). This finding corroborates earlier *in vitro* data showing Aflapin reduces MMP-3 production. Consumption of Aflapin may thus prevent proteolytic damage to cartilage tissue and thereby slow the progression of osteoarthritis.

Cartilage Oligomeric Matrix Protein (COMP) is a non-collagen protein of the articular cartilage matrix. Chondrocytes and synovial cells synthesize COMP following activation by cytokines. It is a critical marker of cartilage degeneration in OA progression (30, 31). Our observations indicate that Aflapin supplementation for 30 days resulted in moderate reduction of COMP level in circulation when compared with the placebo. Type II collagen is the major structural protein of cartilage. Fragments derived from collagen degradation i.e., Collagen type II cleavage (C2C), have been investigated as a potential marker for remodeling cartilage pathologies in OA (32, 33). Thirty days of Aflapin supplementation reduced serum C2C level. In contrast, the serum C2C level was increased in the placebo group, which is an indicative of progression in cartilage degradation (32, 33). These observations suggest that Aflapin supplementation provides chondro-protection via inhibition of catabolic damage to cartilage tissue, and thus helps to maintain normal joint health, which collectively strengthens the basis of anti-osteoarthritic efficacy of Aflapin. Overall, the mechanisms of action of Aflapin in alleviating the knee OA symptoms and cartilage protection are schematically presented in Figure 3.

Aflapin-supplemented subjects did not show any major changes in the hematological parameters, serum biochemical parameters, or in urine analysis compared to placebo. In addition, no serious adverse events were reported by the subjects in the Aflapin-supplemented group. Together, these observations support that Aflapin is a safe therapeutic intervention for pain management and other clinical manifestations of osteoarthritis in humans.

The potential strength of the present investigation is the demonstrated impact of novel Boswellia serrata extract Aflapin (AprèsFlex) on inflammatory biomarkers and cartilage degradation products, and its correlation to

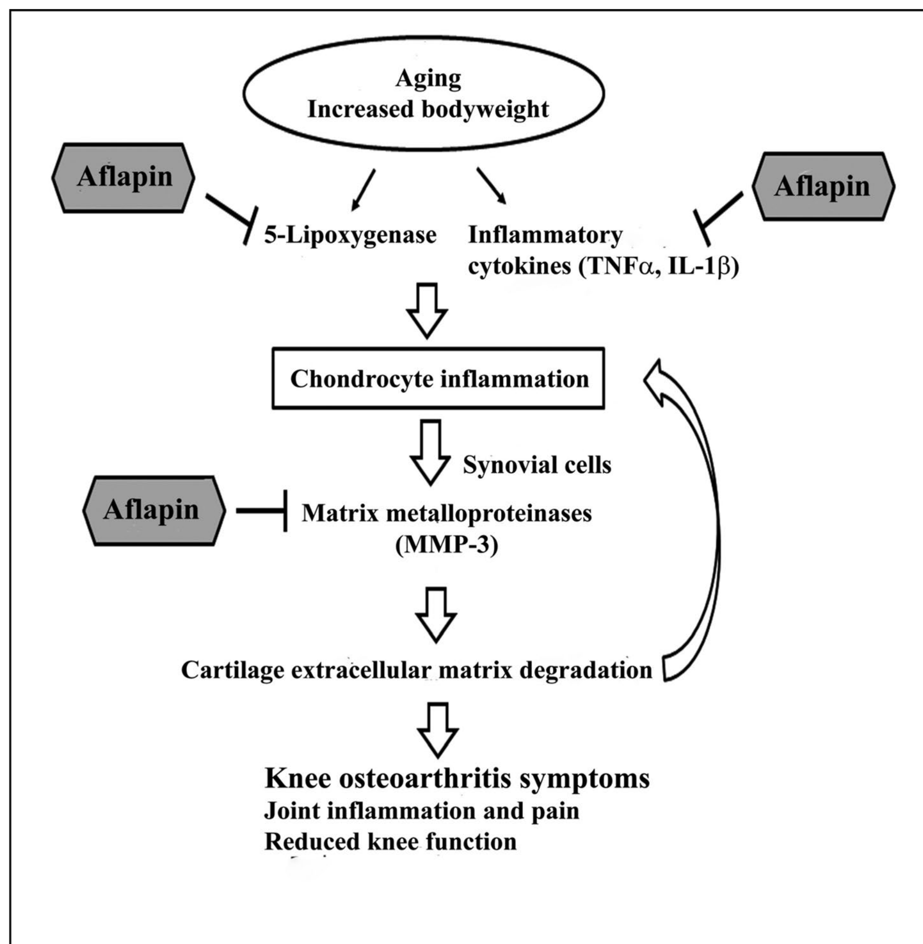

Figure 3. A schematic presentation shows the mechanisms of action of Aflapin mitigating the symptoms of osteoarthritis of knee.

symptomatic relief from knee OA. For the first time, we demonstrate that Aflapin supplementation helps in ameliorating pro-inflammatory status via reduction of TNFα, hsCRP, and protects against degradation of cartilage tissue by inhibiting MMP-3. Also, the present study reestablishes the rapid onset of efficacy of Aflapin in attenuating the clinical symptoms of OA, as well as its broad safety in humans. Although, Aflapin supplementation resulted in promising improvements in the cartilage biomarkers of the participants, the changes were not significant. A longer duration of clinical trial with larger group size would result in significant improvements in the OA progression markers. Therefore, a larger, longer clinical study of Aflapin is thus warranted.

Abbreviations

AKBA 3-O-acetyl-11-keto-beta-boswellic acid
ANOVA analysis of variance
BMI Body Mass Index
COMP Cartilage Oligomeric Matrix Protein
C2C Collagen type II cleavage
ELISA enzyme-linked immunosorbent assay
LFI Lequesne Functional Index
hsCRP high sensitive C-reactive protein
IEC Institutional Ethics Committee
MMP-3 matrix metalloproteinase-3
OA osteoarthritis
TNFα tumor necrosis factor-alpha
VAS visual analog scale
WOMAC Western Ontario and McMaster Universities Osteoarthritis Index

Disclosure statement

The authors declare that there is no conflict of interest to conduct the study and publishing the research finding.

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Research Paper

A Double Blind, Randomized, Placebo Controlled Clinical Study Evaluates the Early Efficacy of Aflapin® in Subjects with Osteoarthritis of Knee

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Abstract

Aflapin® is a novel synergistic composition derived from *Boswellia serrata* gum resin (Indian Patent Application No. 2229/CHE/2008). Aflapin is more efficacious as an anti-inflammatory agent compared to the existing *Boswellia* products, 5-Loxin® and traditional 65% Boswellia extract. A 30-day, double-blind, randomized, placebo-controlled study was conducted to validate the efficacy of Aflapin® in the management of clinical symptoms of osteoarthritis (OA) of the knee (Clinical trial registration number: ISRCTN69643551). Sixty eligible OA subjects selected through screening were included in the study. The subjects received either 100 mg (n=30) of Aflapin® or placebo (n=30) daily for 30 days. Each subject was evaluated for pain and physical functions by using the standard tools (visual analog scale, Lequesne's Functional Index, and Western Ontario and McMaster Universities Osteoarthritis Index) at the baseline (day 0), and at days 5, 15 and 30. A series of biochemical tests in serum, urine and hematological parameters established the safety of Aflapin. The observations suggest that Aflapin conferred clinically and statistically significant improvements in pain scores and physical function scores in OA subjects. Aflapin provided significant improvements in pain score and functional ability in as early as 5 days of treatment. In conclusion, our observations suggest that Aflapin is a safe, fast acting and effective alternative intervention in the management of OA.

Key words: Aflapin, Clinical study, *Boswellia serrata*, Osteoarthritis, Visual Analog Scale

Introduction

Osteoarthritis (OA) is a degenerative joint disorder of articular cartilage and is the most common type of arthritis in elderly persons. In OA, breakdown of cartilage and synovial proliferation result in pain and stiffness of joints. [1-3]. It has been estimated that OA affects more than 27 million people in the United States alone and is the leading cause of physical disability and impaired quality of life in elderly worldwide [4]. Unfortunately, till today there is no proper therapeutic intervention available to treat OA. Currently, acetaminophen and non-steroidal anti-inflammatory drugs (NSAIDs) including cyclo-oxygenase II inhibitors are used for relief of pain and stiffness [5,6]. Although, these pharmaceutical agents reduce both pain and improve physical functions temporarily without healing the cartilage and subchondral damage, long term usage of NSAIDs is associated with enhanced risk for renal insufficiency, gastrointestinal bleeding, hypertension and congestive heart failure [6-8]. Because of the high incidence

of adverse events associated with NSAID therapy, effective and safer alternative treatments for the management of OA pain are highly desirable.

In recent years, the gum resin extracted from the ancient herb, *Boswellia serrata* has gained considerable attention as a potent anti-inflammatory, anti-arthritic and analgesic agent [9,10]. 3-O-Acetyl-11-keto-beta-boswellic acid (AKBA) is the most active compound of *Boswellia* extract and is a potent inhibitor of 5-lipoxygenase (5-LOX), a key enzyme in the biosynthesis of leukotrienes from arachidonic acid in the cellular inflammatory cascade [11,12]. A number of independent clinical studies support the anti-inflammatory and anti-arthritic properties of *Boswellia* extracts [13-16].

Aflapin® is a novel synergistic composition derived from *Boswellia serrata* gum resin (PCT/IN2009/000505) [17-19]. Interestingly, the oral bioavailability of AKBA from Aflapin was found to be significantly higher in comparison with that of commercially available *Boswellia* extracts [17]. Aflapin exhibited enhanced 5-lipoxygenase inhibition in enzyme based *in vitro* assay and Matrix Metalloproteinase 3 (MMP3) inhibition in pro-inflammatory cytokine induced human primary chondrocytes. In a comparative analysis, various *in vitro* and *in vivo* studies have established that in comparison with regular *Boswellia* extracts Aflapin possesses more powerful anti-inflammatory efficacy and exhibits better recovery of glycosaminoglycans (GAG) in pro-inflammatory cytokine induced human chondrocytes. [17]. Furthermore, safety studies conducted according to Organization for Economic Co-operation and Development (OECD) guidelines manifested the overall safety of Aflapin in animal models [18].

In a 90-day placebo controlled clinical study the anti-arthritic efficacy of Aflapin was evaluated in OA subjects. Aflapin demonstrated a significant reduction in pain and improvement in the quality of life in OA subjects [19]. Supplementation of 100 mg Aflapin/day conferred significant improvements in pain scores and physical function. These observations led us to substantiate the anti-OA efficacy of Aflapin in a second independent clinical study. We conducted an independent double blind placebo controlled trial in a different set of subjects with OA. This study design was intended to evaluate, (i) the anti-OA efficacy of Aflapin and (ii) to assess whether Aflapin supplementation can provide fast relief from clinical symptoms of OA. The present communication describes the anti-OA efficacy of Aflapin, which substantiates the earlier observation; and demonstrates that Aflapin provides significant pain relief in subjects with OA in as early as 5 days of treatment.

Materials and Methods

Study material

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 by selective removal of Boswellic acids followed by removing volatiles under high vacuum (PCT application # PCT/IN2009/000505). This composition was standardized to contain at least 20% AKBA.

Research design

This randomized, double-blind, placebo controlled trial was conducted during August 2009 to December 2009. The study protocol was approved by the Institutional Review Board (IRB) of Alluri Sitarama Raju Academy of Medical Sciences (ASRAM), Eluru, Andhra Pradesh, India (Clinical Trial Registration No. ISRCTN69643551).

Subjects

One hundred and fifty two patients of either gender were selected for screening. They were between 40 and 80 years of age, and had been suffering from unilateral or bilateral OA of the knee according to the criteria of the American College of Rheumatology [20] for more than 3 months. After the use of usual medications had ceased for 7 days, the visual analog scale (VAS) score that assessed pain during the most painful knee movement had to be more than 40, and Lequesne's functional index [21] had to be over 7 points. Participants had to be able to walk and give both verbal and written information regarding the study. Signed informed consent was obtained prior to entry. Exclusion criteria included an underlying inflammatory arthropathy, hyperuricemia, expectation of surgery in the near future, recent injury in the area affected by OA of the knee, intra-articular corticosteroid injections within the last 3 months, hypersensitivity to NSAIDs, abnormal liver or kidney function tests, major abnormal finding on complete blood count, history of coagulopathies, history of peptic ulceration and upper GI hemorrhage, uncontrolled hypertension, congestive heart failure, hyperkalemia, pregnancy, lactation and malignant tumors.

Randomization and treatment

A total of 60 subjects with symptoms of mild to moderate OA were selected and recruited into the study. Each subject was randomly assigned to the treatment group or placebo group using a randomization table generated using validated computer software CODE; IDV, Gauting, Germany. The ran-

domization codes were secured confidential by the clinical trial pharmacist and statistician. Thirty subjects were allocated each into placebo and Aflapin groups. The subjects in Aflapin group received 50 mg of encapsulated Aflapin® twice daily, whereas, the subjects in the placebo group received two capsules having similar organoleptic properties including weight, taste, color, odor and feel. Each subject filled a questionnaire, providing details regarding demographics, medical history and nutritional status, at the baseline evaluation and during each follow-up evaluation on days 5, 15 and 30.

Assessments

Functional disability was assessed at baseline and at all follow-up visits (days 5, 15 and 30) by the investigators. Pain, stiffness and physical function were assessed using Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) [22], LFI [21] and VAS [23] scores. The pain, stiffness and function subscales of the WOMAC were normalized to a scale of 0 to 100 units [24]. Analyses of these end-points were based upon the time-weighted average change from baseline over 30 days.

For assessment of safety of Aflapin®, several parameters were evaluated in serum, urine and whole blood of all subjects at each visit of the study duration (Table 1). Serum biochemical parameters and hematological parameters were measured using an automated analyzer (HumaStar 300) and a hematological counter (Humacount, Human, Wiesbaden, Germany). The urine analysis was carried out using UroColor™10 Dip Sticks and Urometer 600 (Standard Diagnostics, Kyonggi-do, Korea) and by sediment analysis using microscopy.

Rescue medication

Subjects were prescribed 400 mg ibuprofen tablets (maximum 400 mg thrice daily; total 1,200 mg) as rescue analgesia during the study based on pain intensity reported to the study physician by some subjects. Those subjects were advised not to take the rescue medicine at least 3 days before each evaluation. No other pain relieving interventions were allowed during the study period.

Statistical analysis

Detailed statistical analyses were performed using SAS software to evaluate the efficacy of Aflapin in comparison with the placebo group in terms of improvement in pain and physical function scores at baseline and on days 5, 15 and 30 of treatment. Wilcoxon's signed-rank test was used for inter group and intra-group comparisons of pain scores. Pair-wise changes were examined by carrying out least significant difference (LSD) test for all possible pairs. The significance of the effects of the treatment groups was compared by using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Results with $P<0.05$ are considered statistically significant. This is a two-arm (Aflapin and placebo), randomized, double-blind, placebo-controlled, single-centre trial conducted over 30 days. The trial's primary objective was to validate the efficacy of Aflapin on reduction of pain, joint stiffness and improvement in physical function in subjects with osteoarthritis of knee.

Table 1: Parameters tested in Serum, urine and whole blood samples

Biochemical Parameters
Blood sugar
Alkaline phosphatase
SGOT
SGPT
Total Bilirubin
Direct Bilirubin
CK Nac
Creatinine
Total Protein
Triglycerides
Cholesterol
HDL, LDL
Urea
Hematology
Total Leukocyte count
Total RBC count
Hemoglobin %
Mean Corpuscular volume (MCV)
Mean corpuscular hemoglobin (MCH)
Mean corpuscular hemoglobin Concentration
Platelet count
Differential count (DC)
Urine Analysis
Blood
Bilirubin, Urobilinogen
Ketone
Protein
Nitrite
Glucose
pH, Specific gravity
Leucocytes
Pus cells, Epithelial cells, Crystals

Results

Baseline characteristics

The subjects were randomly distributed into two groups and the descriptive statistics comparing demographic variables, baseline disease characteristics and baseline outcome measures (LFI, VAS, WOMAC pain, function and stiffness sub-scores) are provided in **Table 2**. The demographic variables, disease-related and baseline outcome parameters of two groups, one receiving Aflapin® 100 mg/day (n=30) and the other receiving placebo (n=29) did not differ significantly at baseline.

Table 2: Characteristics of patients in study groups.

Characteristics	Placebo (n = 29)	100 mg/day Aflapin® (n = 30)
Sex (male/female; n)	11/18	11/19
Age (years)	55.3 ± 8.8	53.2 ± 6.5
Body weight (kg)	59.7 ± 10.5	61.9 ± 10.9
Body mass index (kg/m2)	24.9 ± 2.6	25.7 ± 3.3
Visual analog score	47.6 ± 9.7	48.0 ± 6.0
Lequesne's Functional Index	12.5 ± 3.4	12.8 ± 3.7
WOMAC score		
Pain subscale	45.9 ± 10.5	47.8 ± 12.4
Stiffness subscale	37.5 ± 14.9	38.8 ± 13.3
Function subscale	40.6 ± 9.5	41.1 ± 11.8

Clinical efficacy

The data regarding the normalized pain and function scores are summarized in **Table 3**. At the end of the study, significant reductions in pain and function scores were observed in treatment group supplemented with 100 mg/day of Aflapin when compared to either baseline or placebo.

Significant ($p<0.05$) reduction in all the pain scores was observed in the Aflapin group by day 30, when compared to the placebo group. In comparison with placebo, supplementation of Aflapin for 30 days conferred 37.6, 32.0, 40.1, 41.3 and 38.8 percent reductions in VAS, LFI, WOMAC pain, WOMAC stiffness and WOMAC function scores, respectively. Interestingly, significant ($p<0.05$) reductions in VAS and LFI scores were also observed in Aflapin group over placebo by day 5. Aflapin supplementation showed 14.8 and 16.3 percent better reduction in VAS and LFI scores respectively over placebo by 5th day. Compared to the placebo group, the reductions in WOMAC scores were not significant after 5 days of treatment. Aflapin supplementation for 30 days afforded highly significant ($p<0.001$) reductions in all the pain scores exhibiting 49.1, 34.4, 49.5, 48.4 and 45.2 percent reduction, in VAS, LFI, WOMAC pain, WOMAC stiffness and WOMAC function scores, respectively, when compared to the baseline. However, significant ($p<0.05$) reductions were observed in VAS, WOMAC pain and WOMAC function scores in placebo group when compared to the base line and the magnitude of the reductions are 17.6, 12.0 and 9.24 percent respectively; which are small in comparison with those of the Aflapin group (**figure 2**).

Table 3. Normalized pain and function scores.

Parameter and treatment	Baseline mean ± SD	Day-30 mean ± SD	p value (vs. baseline)	p value (vs. placebo)
Visual analogue scale score				
Placebo (n=29)	47.6 ± 9.7	39.3 ± 9.5	<0.0001	NA
Aflapin 100 mg/day (n=30)	48.0 ± 6.0	24.5 ± 11.9	<0.0001	<0.0001
Lequesne's Functional Index				
Placebo (n=29)	12.5 ± 3.4	12.4 ± 2.6	0.7646	NA
Aflapin 100 mg/day (n=30)	12.8 ± 3.7	8.4 ± 3.8	<0.0001	<0.0001
WOMAC pain subscale				
Placebo (n=29)	45.9 ± 10.5	40.3 ± 11.4	0.001	NA
Aflapin 100 mg/day (n=30)	47.8 ± 12.4	24.2 ± 12.0	<0.0001	<0.0001
WOMAC stiffness subscale				
Placebo (n=29)	37.5 ± 14.9	34.1 ± 15.6	0.2024	NA
Aflapin 100 mg/day (n=30)	38.8 ± 13.3	20.0 ± 15.6	<0.0001	0.0014
WOMAC function subscale				
Placebo (n=29)	40.6 ± 9.5	36.8 ± 11.5	0.0029	NA
Aflapin 100 mg/day (n=30)	41.1 ± 11.8	22.5 ± 11.1	<0.0001	<0.0001

NA, not applicable; WOMAC, Western Ontario and McMaster Universities Osteoarthritis Index.

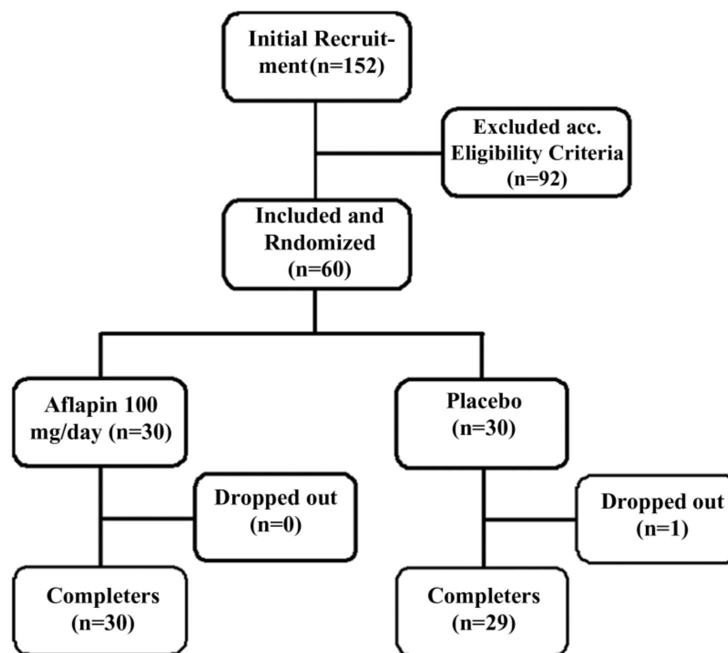

Figure 1: Flow chart of the subjects who participated in the clinical trial. Evaluations of physical activity and pain scores, serum biochemistry, hematology, urine biochemistry and pro-inflammatory biomarkers were done at baseline (day 0) and on days 5, 15 and 30 during follow up.

Figure 2: Pain, Function and stiffness scores. Presented are the mean scores for (a) visual analog scale, (b) Lequesne's Functional Index, (c) Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC)-pain, (d) WOMAC-stiffness, and (e) WOMAC-function in placebo and 100 mg/day Aflapin® groups at different time points day 0, day 5, day 15 and day 30. Each bar represents mean ± standard deviation. In comparison with placebo the mean scores in the treatment groups were tested for significance using Wilcoxon's rank-sum test; * $p < 0.05$ and # $p < 0.01$.

Biochemical evaluations

As a part of the safety evaluation, laboratory tests were performed for assessment of different biochemical parameters (in serum and urine) and hematological parameters. The repeated measure ANOVA was used to compare the values at different evaluations over the 30 days period with those of baseline. Statistical analyses of these parameters did not indicate any significant changes. Although minor changes were observed in some of the parameters, they remained within the normal laboratory range. Similarly, no significant changes in hematological and urinary parameters were observed in the active treatment groups when compared to the placebo (data not shown).

Adverse Events and Dropouts

During the course of the 30-day study, no major adverse events were reported. However, nausea and headache were reported as minor adverse events by two subjects during the study; one each from placebo and Aflapin supplemented groups.

One subject from placebo groups was dropped out from the study due to un-availability for the follow up evaluations.

Discussion

The primary objective of conducting the present study was to substantiate the observation that Aflapin, a novel *Boswellia* extract reduces clinical symptoms of osteoarthritis, pain, physical discomfort. *Boswellia serrata* is an ancient Indian medicinal plant, and the gum resin of this plant has long been known for anti-inflammatory, anti-arthritic and analgesic properties [9,10]. Earlier studies indicate that 3-O-Acetyl-11-keto-beta-boswellic acid (AKBA) is the most active principle present in the Boswellia extracts, which mainly contributes the anti-inflammatory activities of this herbal extract by inhibiting 5-lipoxygenase activity [11,12].

To date, the anti-inflammatory and anti-arthritic efficacy of different forms of Boswellia extracts have been established in various models either *in vitro* or *in vivo* or in clinical studies [13-16,19,25-30]. However, studies indicate that upon oral administration, Boswellia extracts exhibit poor intestinal absorption of AKBA and poor bioavailability which limits its anti-inflammatory efficacy [31,32]. Aflapin is a novel synergistic composition, which contains *B. serrata* extract enriched to 20% AKBA and *B. serrata* non-volatile oil (PCT/IN2009/000505). In a recent communication Sengupta et al [17] have reported that Aflapin® provides 51.78% more bioavailable concentration of systemic AKBA after a single dose oral administration in comparison with 30% AKBA enriched Boswellia extract (5-Loxin®). In corroboration, it was observed in a recent double blind placebo controlled study that Aflapin provides significantly better improvements in clinical symptoms in OA subjects when compared with 30% AKBA enriched Boswellia extract (5-Loxin®) [19]. The present 30-day double blind, placebo controlled clinical study was designed with two approaches; (i) to reassess the anti-arthritic efficacy of Aflapin and (ii) to evaluate the early onset of action of Aflapin in pain reduction and improvement of physical function in OA subjects.

The present study demonstrates the potential of Aflapin in alleviating pain, joint stiffness and improving physical functions in OA subjects (Figure 2). Pain, stiffness of joints, reduced joint movement and physical discomfort are the major clinical manifestations of OA [24,29,30]. In comparison with the placebo, at the end of the study, the Aflapin supplemented group showed statistically significant improvements in all pain scores including VAS, LFI, WOMAC pain, WOMAC stiffness and WOMAC function scores (Figure 2). Aflapin provided significant reductions in pain scores of VAS and LFI in as early as 5 days. Whereas, in the previous study Aflapin demonstrated significant relief from joint pain and physical discomfort in OA subjects after 7 days of treatment [19]. Together, these findings clearly suggest that Aflapin confers quick and significant pain relief, improvement in physical ability and quality of life in OA subjects.

Therapeutic efficacy and fast action of Aflapine can be attributed to its role in intervening the cellular and molecular mechanisms associated with the pathologic processes of OA. Earlier we have demonstrated multiple beneficial effects of Aflapin over 5-Loxin; (1), better anti-inflammatory efficacy of Aflapin through inhibiting 5-lipoxygenase enzyme activity, and inhibiting TNFα production; (2), provides significant protection from damaging action of IL-1β by increasing chondrocytes proliferation and increasing synthesis of cartilage matrix substances such as collagen and glycosaminoglycans in human primary chondrocytes; (3), Aflapin also inhibits MMP3 production in TNFα induced human chondrocytes [17].

Overall, the data demonstrate the efficacy of Aflapin in pain management, improving physical function, quality of life and joint health. Presumably, the pleotropic beneficial effects of Aflapin might provide potential anti-osteoarthritic efficacy, which helps improving joint health in OA subjects [17,19,25].

In corroboration with the previous studies [19,25], the present investigation does not show any major changes in the hematological parameters, serum biochemical parameters and in urine analysis in

Aflapin supplemented subjects in comparison with placebo. In addition, no major adverse effect has been reported by the subjects included in Aflapin group. Taken together, these observations further demonstrate and substantiate the anti-osteoarthritic potential of Aflapin.

Conclusion

In summary, the present study validates the potential anti-OA efficacy and safety of Aflapin. In addition the present study also establishes the fast onset of therapeutic action of Aflapin® in OA subjects. Aflapin significantly improves joint function and relieves pain at as early as 5 days of treatment. This study bears potential promise in favor of Aflapin as a useful alternative therapeutic strategy for the management of OA in humans.

Abbreviations

AKBA: 3-O-acetyl-11-keto-beta-boswellic acid; ANOVA: analysis of variance; ASRAM: Alluri Sitarama Raju Academy of Medical Sciences; BMI: Body Mass Index; LFI: Lequesne's Functional Index; NSAID: nonsteroidal anti-inflammatory drug; NU: normalized units; OA: osteoarthritis; VAS: visual analog scale; WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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Research Paper

Comparative Efficacy and Tolerability of 5-Loxin® and Aflapin® Against Osteoarthritis of the Knee: A Double Blind, Randomized, Placebo Controlled Clinical Study

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Abstract

Aflapin® is a novel synergistic composition derived from *Boswellia serrata* gum resin (Indian Patent Application No. 2229/CHE/2008). Aflapin is significantly better as an anti-inflammatory agent compared to the *Boswellia* extracts presently available in the market. A 90-day, double-blind, randomized, placebo-controlled study was conducted to evaluate the comparative efficacy and tolerability of 5-Loxin® and Aflapin® in the treatment of osteoarthritis (OA) of the knee (Clinical trial registration number: ISRCTN80793440). Sixty OA subjects were included in the study. The subjects received either 100 mg (n=20) of 5-Loxin® or 100 mg (n=20) of Aflapin® or a placebo (n=20) daily for 90 days. Each patient was evaluated for pain and physical functions by using the standard tools (visual analog scale, Lequesne's Functional Index, and Western Ontario and McMaster Universities Osteoarthritis Index) at the baseline (day 0), and at days 7, 30, 60 and 90. A battery of biochemical parameters in serum, urine and hematological parameters in citrated whole blood were performed to assess the safety of 5-Loxin® and Aflapin® in OA subjects. Fifty seven subjects completed the study. At the end of the study, both 5-Loxin® and Aflapin conferred clinically and statistically significant improvements in pain scores and physical function scores in OA subjects. Interestingly, significant improvements in pain score and functional ability were recorded as early as 7 days after initiation of the study in the treatment group supplemented with 100 mg Aflapin. Corroborating the improvements in pain scores in treatment groups, our *in vitro* studies provide evidences that Aflapin® is capable of inhibiting cartilage degrading enzyme MMP-3 and has the potential to regulate the inflammatory response by inhibiting ICAM-1. Aflapin® and 5-Loxin® reduce pain and improve physical functions significantly in OA subjects. Aflapin exhibited better efficacy compared to 5-Loxin®. In comparison with placebo, the safety parameters were almost unchanged in the treatment groups. Hence both 5-Loxin® and Aflapin® are safe for human consumption.

Key words: Aflapin®, 5-Loxin®, *Boswellia serrata*, anti-inflammation, osteoarthritis and clinical study.

Introduction

Osteoarthritis (OA) is the commonest form of arthritic disease, characterized by articular cartilage degradation with an accompanying peri-articular bone response [1,2]. OA affects nearly 21 million people in the USA, accounting for 25% of visits to primary care physicians. It is estimated that 80% of the population will have radiographic evidence of OA by age 65 years, although only 60% of those will be symptomatic [3]. Clinical manifestations of OA of the knee include pain in and around the joint, stiffness of the joint, crepitation on motion and limited joint motion, among others [4]. Current recommendations for managing OA focus on relieving pain and stiffness and improving physical function as important goals of therapy [5,6]. Currently available medication regimens for most cases include nonopioid analgesics such as acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs) including cyclo-oxygenase II inhibitors. These pharmaceutical agents can reduce both pain and inflammation quite effectively, but long term use of NSAIDs has been found to associate with enhanced risk for gastrointestinal bleeding, hypertension, congestive heart failure and renal insufficiency, among other adverse effects [7-9]. Because of the high incidence of adverse events associated with both nonselective and cyclo-oxygenase II selective NSAID therapy, effective and safer alternative treatments for OA are urgently needed. In recent years, the gum resin extracted from the ancient herb, *Boswellia serrata* has gained lot of attention as a potent anti-inflammatory, anti-arthritic and analgesic agent [10,11]. 3-O-acetyl-11-keto-beta-boswellic acid (AKBA) is the most active component of *Boswellia* extract and has been demonstrated to be a potent inhibitor of 5-lipoxygenase (5-LOX), a key enzyme in the biosynthesis of leukotrienes from arachidonic acid in the cellular inflammatory cascade [12,13]. 5-Loxin® is a novel *B. serrata* extract enriched to 30% AKBA (US Patent publication no.: 2004/0073060A1). Affimatrix gene chip analysis demonstrated that 5-Loxin® can potently inhibit tumor necrosis factor α (TNFα) induced gene expression of matrix metalloproteinases (MMPs), adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1); and mediators of apoptosis in human microvascular endothelial cells [14, 15]. Cell based *in vitro* studies suggest that 5-Loxin® can inhibit pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-1β [16]. In the carrageenan-induced inflammation model, 5-Loxin® treatment yielded significant improvement in paw inflammation in albino Wistar rats. 5-Loxin® also exhibited significant Anti-arhtritic efficacy in FCA induced model of Sprague-Dawley rats [14, 15]. Extensive acute and dose dependent subchronic safety studies on rats demonstrated that 5-Loxin® is safe even at dose levels 2,000 to 3,000 times higher than the human equivalence dose [17]. In addition, 5-Loxin® was found to be non genotoxic as per the standard AMES bacterial reverse mutation assay, chromosomal aberration test in Chinese hamster cells and mouse peripheral blood micronucleus assay [18-21] The efficacy and tolerability of 5-Loxin® was assessed in a previous double blind placebo controlled clinical study. The supplementation of 5-Loxin® was well tolerated and its efficacy against osteoarthritis was found to be statistically significant. The dose dependent efficacy of 5-Loxin® was assessed against pain, joint stiffness, mobility and a cartilage degrading enzyme MMP-3 in OA subjects [22]. Aflapin® is a novel synergistic composition derived from *Boswellia serrata* gum resin (Indian Patent Application No. 2229/CHE/2008). Interestingly it was found that the oral bioavailability of AKBA from Aflapin® was better compared to that of 5-Loxin®. Aflapin exhibited better 5-lipoxygenase inhibitory activity and MMP-3 inhibition. Various *in vitro* and *In vivo* studies were performed to compare efficacy of Aflapin and 5-Loxin®. These studies proved Aflapin to be more efficacious compared to 5-Loxin® (to be presented in a separate communication). The broad spectrum safety of Aflapin was tested using a battery of safety studies conducted according to OECD guidelines and it was found to be safe [23]. Although a significant number of clinical study reports support the anti-inflammatory and anti-arthritic properties of *Boswellia* extract [24-27], no human clinical studies were done to prove the efficacy and tolerability of Aflapin in osteoarthritis. Hence in the present clinical study we sought to evaluate the comparative efficacy and tolerability of 5-Loxin® and Aflapin® in the treatment of OA of the knee.

Materials and Methods

Study materials

BE-30 (5-Loxin®) is a novel *Boswellia serrata* extract standardized to contain at least 30 percent 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. 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.

Study design

This trial was performed at Alluri Sitarama Raju Academy of Medical Sciences (ASRAM), Eluru, Andhra Pradesh, India from July 2008 to December 2008 (clinical trial registration number: ISRCTN80793440). The study protocol was evaluated and approved by the ASRAM Institutional Review Board (IRB). An overview of the clinical study is provided in **Figure 1**. Briefly, 186 subjects out of 283 attending the orthopaedic outpatient department of the ASRAM hospital were selected in the first phase of the screening procedure, based on the signs, symptoms and radiological changes consistent with OA. A total of 60 subjects suffering for more than 3 months with medial tibio-femoral OA were selected using inclusion/exclusion criteria summarized in **Table 1**. All subjects signed the IRB approved consent form. Subjects, who were otherwise healthy, were aged 40 years or older and had a diagnosis of OA, fulfilling the American College of Rheumatology classification criteria [4]. After recruitment, the subjects were randomly distributed into three groups. The demographic data and baseline characteristics are summarized in **Table 2**.

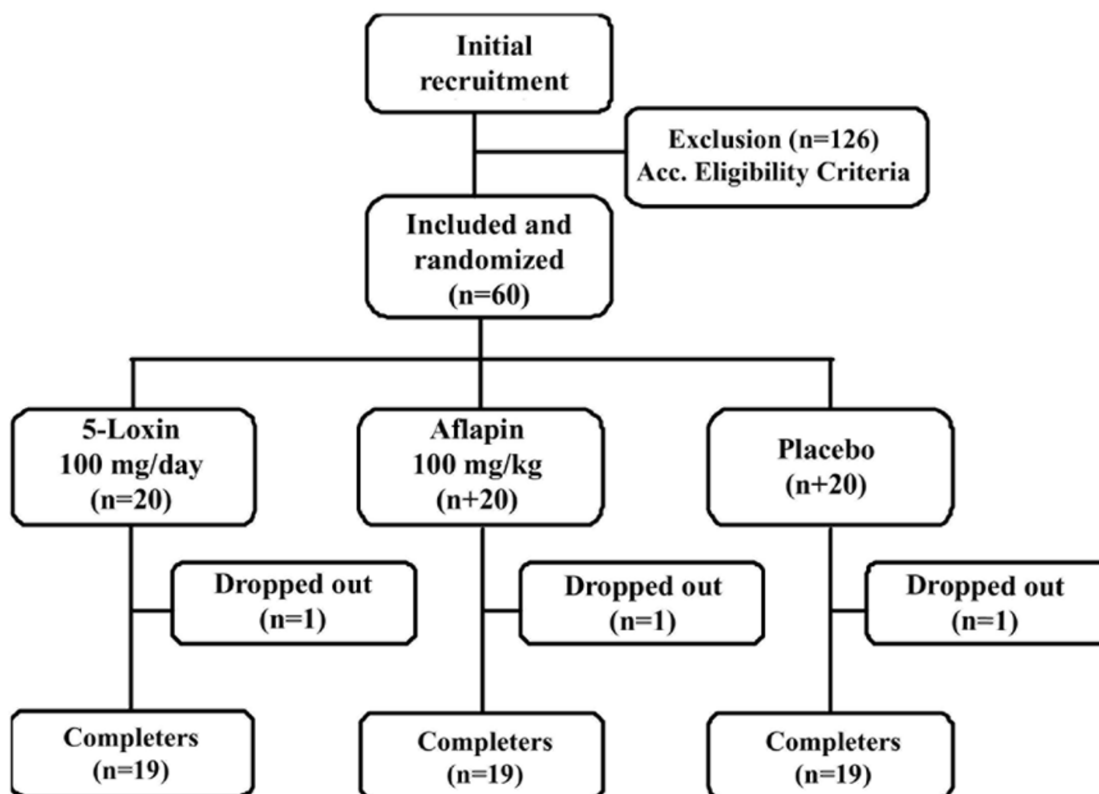

Figure 1: Flow chart of the subjects who participated in the clinical trial. Evaluations of physical activity and pain scores, serum biochemistry, hematology, and urine biochemistry were done at baseline (day 0) and on days 7, 30, 60 and 90 during follow up.

Table 1: Inclusion/exclusion criteria

Criteria	Details
Inclusion	Subjects must understand risks and benefits of the protocol and be able to give informed consent
	Male and female subjects aged 40 to 80 years
	Females of child-bearing potential must agree to use an approved form of birth control and to have a negative pregnancy test result
	Unilateral or bilateral osteoarthritis of the knee for more than 3 months
	Visual analogue scale score during the most painful knee movement between 40 and 70 mm after 7 days of withdrawal of usual medication
	Lequesne's Functional Index score greater than 7 points after 7 days of withdrawal of usual medication
	Ability to walk
	Availability for the duration of the entire study period
Exclusion	History of underlying inflammatory arthropathy or severe rheumatoid arthritis
	Hyperuricaemia (>440 µmol/l) and/or past history of gout
	Recent injury in the area affected by osteoarthritis of the knee (past 4 months) and expectation of surgery in the next 4 months
	Intra-articular corticosteroid injections within the preceding 3 months
	Hypersensitivity to nonsteroidal anti-inflammatory drugs, abnormal liver or kidney function tests, history of peptic ulceration and upper gastrointestinal hemorrhage, congestive heart failure, hypertension, cancer, hyperkalaemia
	Major abnormal findings on complete blood count, history of coagulopathies, hematological or neurological disorders
	High alcohol intake (>2 standard drinks per day)
	Pregnant, breastfeeding, or planning to become pregnant during the study
	Use of concomitant prohibited medication other than ibuprofen
	Obesity (body mass index > 30 kg/m2)

Table 2: Demographic data and baseline characteristics of the subjects

Characteristics	Placebo (n = 19)	100 mg/day 5-Loxin® (n = 19)	100 mg/day Aflapin® (n = 19)
Sex (male/female; n)	9/10	3/16	7/12
Age (years)	52.4 ± 7.5	51.6 ± 9.9	53.2 ± 7.9
Body weight (kg)	62.4 ± 14.9	57.7 ± 10.5	59.1 ± 7.4
Body mass index (kg/m2)	25.3 ± 4.4	25.1 ± 3.8	25.2 ± 3.0
Visual analog score	47.7 ± 6.5	48.2 ± 6.1	47.7 ± 7.3
Lequesne's Functional Index	12.3 ± 2.8	12.4 ± 2.6	12.0 ± 2.4
WOMAC scores			
Pain subscale	44.7 ± 11.5	46.1 ± 7.6	45.0 ± 13.3
Stiffness subscale	39.5 ± 11.2	39.5 ± 11.2	39.5 ± 13.3
Function subscale	42.0 ± 10.3	43.1 ± 7.8	42.0 ± 8.4

Before study enrollment, subjects were required to be taking an NSAID at prescription strength for at least 30 days or acetaminophen 1,200 to 4,000 mg/day on a regular basis (at least 25 of the preceding 30 days) with a history of therapeutic benefit. Eligibility requires subjects to meet specific flare criteria upon medication washout. At screening, subjects had to demonstrate a visual analog scale (VAS) score between 40 and 70 mm during the most painful knee movement, and Lequesne's Functional Index (LFI) score greater than 7 points after 7-day withdrawal of usual medication.

A total of 60 selected subjects with symptoms of moderate to mild OA were recruited into the study. Each subject was randomly assigned to a treatment group using a randomization table generated using validated computer software CODE; IDV, Gauting, Germany. The clinical trial pharmacist and statistician ensured that treatment codes remained confidential. The subjects were distributed into three groups: placebo (n=20); 5-Loxin® group, in which subjects received 50 mg encapsulated 5-Loxin® twice daily (n=20); and Aflapin group, in which subjects received 50 mg encapsulated Aflapin® twice daily (n=20). Subjects in the placebo group received two capsules of similar color, taste and appearance but filled with suitable exicipient. Each subject completed a questionnaire, providing details regarding demographics, medical history and nutritional status, at the baseline evaluation and during the follow-up evaluations on days 7, 30, 60 and 90. At the baseline evaluation, and at each visit during the 90-day follow up period, all

subjects were assessed for pain and physical function using validated pain scores. Various parameters of serum biochemistry, hematology and urine analysis were carried out on each evaluation day. Safety was monitored by clinical and laboratory assessments conducted during the study visits and subject-reported adverse experiences.

Functional disability and pain score evaluation

Functional disability was assessed by the investigators at baseline and on each follow-up visit (days 7, 30, 60 and 90). Questionnaire-based assessment of pain, stiffness and physical function were done using the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) index [28], LFI [29] and VAS [30]. The WOMAC index produces scores for three subscales: pain, stiffness and physical function. The pain, stiffness and function subscales of the WOMAC were normalized to a scale of 0 to 100 units (NU) [31]. The pain subscale was the average of the first five questions of WOMAC and measured using the NU scale from 0 ('no pain') to 100 ('extreme pain') for each question. The stiffness subscale was the average of questions 6 and 7, measured using the NU scale from 0 ('no stiffness') to 100 ('extreme stiffness') for each question. The physical function subscale was the average of questions 8 through 24 of the WOMAC and measured by NU scale from 0 ('no difficulty') to 100 ('extreme difficulty') for each question. Analyses of these end-points were based upon the time-weighted average change from baseline over 90 days.

Hematological and biochemical evaluations

For assessment of safety of 5-Loxin® and Aflapin®, several parameters were evaluated in serum, urine and whole blood of all subjects at each visit of the study duration. Serum biochemical parameters and hematological parameters were measured using an automated analyzer (HumaStar 300) and a hematological counter (Humacount, Human, Wiesbaden, Germany). The urine analysis was carried out using UroColor™10 Dip Sticks and Urometer 600 (Standard Diagnostics, Kyonggi-do, Korea) and by sediment analysis using microscopy.

In vitro studies to identify mechanisms of actions of Aflapin: Effect on expression of ICAM-1 and MMP3

Adhesion molecule (ICAM-1) expression on endothelial cells: 20,000 Endothelial cell (HDMEC, Lonza Inc., USA) per well in quadruplicate wells were treated with medium, vehicle, TNFα (20ng/ml), TNFα (20ng/ml) with 5-Loxin® or Aflapin® (4μg/ml each) for 24 hour then ICAM-1 ELISA was performed on fixed cells of these wells as per our established protocol [32].

Effect on secretion of MMP3 in TNFα induced human chondrocyte: Human primary Chondrocytes (HCH) was procured from Promo Cell GmbH (Heidelberg, Germany). HCH cells were cultivated in the growth medium (Ready-to-use; Promo Cell, Catalog number C-27101) supplemented with Supplement Mix (Promo Cell, Catalog number C-39635). Equal number of HCH cells was plated in each well of 96-well cell culture plate. Cells were treated with 5 ng/ml of TNFα in presence or absence of different concentrations of 5-Loxin® or Aflapin for 24h. Vehicle control cultures received 0.01% DMSO (v/v). MMP-3 was quantitatively measured in the cell culture supernatant by human MMP-3 EIA kit (R&D Systems, USA) following manufacturer's instructions.

Rescue medication

Subjects were prescribed ibuprofen 400 mg tablets (maximum 400 mg thrice daily; total 1,200 mg) as rescue analgesia during the study based on pain intensity reported to the study physician by the patient. However, the subjects were instructed not to take medicine at least 3 days before each evaluation. No other pain relieving interventions were allowed during the study period.

Statistical analysis

Detailed statistical analyses were performed using SAS software to evaluate the efficacy of 5-Loxin® and Aflapin® in comparison with the placebo group in terms of improvement in pain and physical function scores at baseline and on days 7, 30, 60 and 90 of treatment and serum MMP-3 levels at baseline and on day 90 of treatment. Pair-wise changes were examined by carrying out a least significant difference test for all possible pairs. The significance of the effects of the treatment groups was compared by using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Results with $P<0.05$ are considered statistically significant. This is a three-arm (5-Loxin®, Aflapin® and placebo), randomized, double-blind, placebo-controlled, single-centre trial conducted over 90 days. The trial's primary objective was to determine the effects of 5-Loxin® and Aflapin® on pain, physical function and joint stiffness. For power calculations, the estimates for variability and assumed mean changes for each treatment group were based on results from previous placebo-controlled studies of celecoxib, etoricoxib and rofecoxib conducted in subjects with OA [33-36]. We believe that an intervention that gives an average improvement of mean change ± 1 standard deviation,

rather than mean change alone will provide results of greater significance [37]. Our trial is designed to have more than 80% power to detect a situation in which either active drug dosage yields an improvement to at least mean change ± 0.9 standard deviation, under a conservative assumption, and we tested differences between groups in mean improvement using ANOVA (α=0.05, two-sided). With 20 subjects per group, we would have a 93% chance of observing at least one example of any side effect occurring in 10% or more of the patient population at a specific dosage.

Results

Baseline characteristics

Descriptive statistics comparing demographic variables, baseline disease characteristics and baseline outcome measures (LFI, VAS, WOMAC pain, function and stiffness sub-scores) are provided in Table 2. Overall, the treatment groups receiving 5-Loxin® 100 mg/day, n=19, Aflapin® 100 mg/day, n=19 and placebo n=19, were similar with respect to age, Body Mass Index and pain severity (Table 2). The subjects were randomly distributed into three groups.

Clinical efficacy

We compared the scores between the treatment groups obtained at day 90. Both the treatments with 5-Loxin® and Aflapin® conferred clinically and statistically significant improvements in pain scores and physical ability scores in OA subjects between baseline and day 90 (Table 3). Tukey's multiple comparison test revealed statistically significant improvements by 31.6% (*P*=0.006), 30.3% (P=0.009) and 42.2% (p=0.006) in VAS, WOMAC pain, and WOMAC stiffness scores, respectively, in the 100 mg 5-Loxin® treated group in comparison with the placebo group (Table 3). Improvements by 18.35% (*P*=0.060) and 21.25% (*P*=0.078) in LFI and WOMAC functional ability scores, respectively were also achieved in the 5-Loxin® group (Table 3). In comparison with the placebo group, the Aflapin® 100 mg treated group also exhibited statistically significant improvements in all parameters tested (Table 3). The Aflapin group showed improvements by 47.3% (*P*<0.0001), 35.8% (*P*=0.0004), 61.7% (*P*<0.0001), 60.1% (*P*=0.0001) and 49.4% (*P*=0.0001) in VAS, LFI, WOMAC pain, WOMAC stiffness and WOMAC functional ability scores, respectively. It is worth noting that both 5-Loxin® and Aflapin® treatment groups exhibited improvement in pain scores and physical ability scores as early as 7 days after the start of treatment, and these indices continued to improve throughout the 90 days of treatment (**Figure 2**). After 7 days, the 5-Loxin® treatment group exhibited 8.09% (*P*=0.002), 8.68% (*P*=0.031) and 8.35% (*p*=0.015) reductions in VAS, WOMAC pain and WOMAC function respectively, compared with the corresponding baseline scores. After 7 days, the Aflapin treatment group exhibited 12.8% (*P*=0.0004), 9.17% (*P*=0.003), 11.78% (*P*=0.012), 18.48% (P=0.012) and 10.24% (*p*=0.005) reductions in VAS, LFI WOMAC pain, WOMAC stiffness and WOMAC function scores respectively, compared to the corresponding baseline scores (Figure 2).

Table 3: Student's *t*-test (paired) analyses for comparison of the scores obtained from the Aflapin and 5-Loxin groups at day 90

	n	Baseline		Day 90		95% CI (versus placebo)	p
		Mean	SD	Mean	SD		
Visual analogue scale score							
Placebo	19	47.7	6.5	38.3	9.0	34.0, 42.7	0.0013
5-Loxin 100 mg/day	19	48.2	6.1	26.2	16.5	18.2, 34.1	<0.0001
Aflapin 100 mg/day	19	47.7	7.3	20.2	12.3	14.2, 26.1	<0.0001
Lequesne's Functional Index							
Placebo	19	12.3	2.8	10.9	3.0	9.4, 12.3	0.0496
5-Loxin 100 mg/day	19	12.4	2.6	8.9	3.7	7.1, 10.7	<0.0001
Aflapin 100 mg/day	19	12.0	2.4	7.0	2.6	7.1, 9.6	<0.0001
WOMAC pain subscale							
Placebo	19	44.7	11.5	36.3	10.5	31.2, 41.4	0.0021
5-Loxin 100 mg/day	19	46.1	7.6	25.3	17.2	17.0, 33.6	<0.0001
Aflapin 100 mg/day	19	45.0	13.3	13.9	8.3	10.0, 17.9	<0.0001
WOMAC stiffness subscale							
Placebo	19	39.5	11.2	29.6	9.5	25.0, 34.2 p	0.0059
5-Loxin 100 mg/day	19	39.5	11.2	17.1	16.8	9.0, 25.2	0.0001
Aflapin 100 mg/day	19	39.5	13.3	11.8	12.8	5.7, 18.0	<0.0001

	n	Baseline		Day 90		95% CI (versus placebo)	p
		Mean	SD	Mean	SD		
WOMAC function subscale							
Placebo	19	42.0	10.3	32.0	10.8	26.8, 37.2	0.0025
5-Loxin 100 mg/day	19	43.1	7.8	25.2	15.0	17.9, 32.4	<0.0001
Aflapin 100 mg/day	19	42.0	8.4	16.2	8.1	12.3, 20,1	<0.0001

CI, confidence interval; WOMAC, Western Ontario and McMaster Universities Osteoarthritis Index.

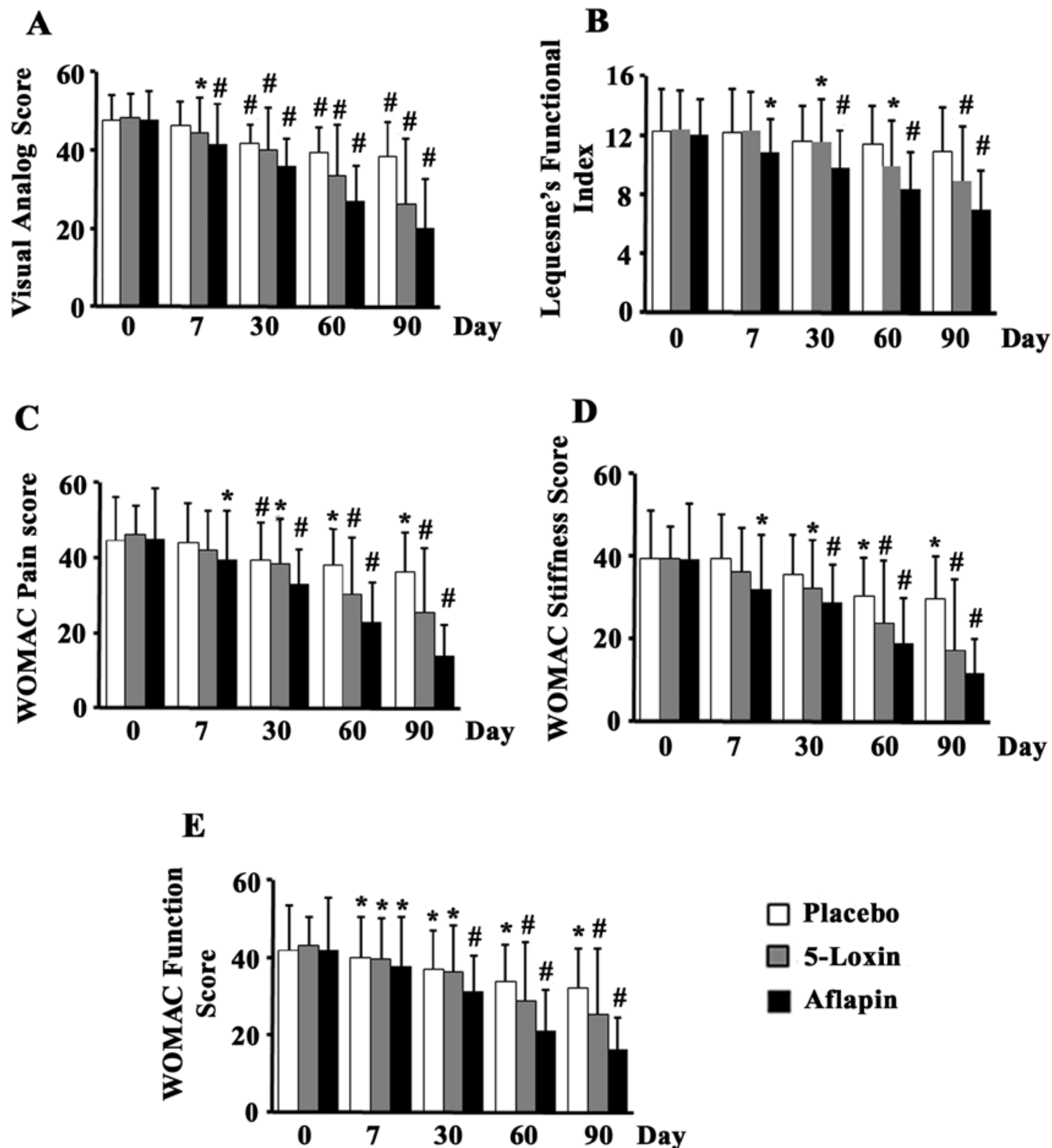

Figure 2: Bar diagrams represent the mean scores of (a) visual analog scale (VAS) (**a**); Lequesne's Functional Index (LFI) (**b**); Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC)-pain (**c**); WOMAC-stiffness (**d**); and WOMAC-function (**e**) in placebo, 100 mg/ day 5-Loxin® and 100 mg/day Aflapin® groups, respectively. 1 to 5, represent days of evaluations such as day 0, day 7, day 30, day 60 and day 90, respectively. Each bar represents mean ± standard deviation. In comparison with corresponding baseline data, the change in scores in the treatment groups was tested for significance using Tukey's multiple comparison test; * p<0.05; ** p<0.005.

Aflapin inhibits secretion of MMP-3 in TNFα-induced human primary chondrocytes

In OA, the loss of collagen from articular cartilage is proportional to the disease severity (38). Under the influence of pro-inflammatory cytokines, increased production and secretion of collagenases such as MMP-3, MMP-13 is the crucial event for enhanced collagen degradation in OA [39]. Therefore, we sought to evaluate whether 5-Loxin and Aflapin can modulate MMP-3 secretion in TNFα, a potent pro-inflammatory cytokine induced human primary chondrocytes. **Figure 3** shows a steep increase in MMP-3 secretion in TNFα-induced chondrocytes and dose-dependent inhibition of MMP-3 secretion in 5-Loxin and Aflapin treated cultures. Interestingly, we observed, Aflapin (IC_{50} at 18.5 μg/ml) provided (41.36%) better efficacy than 5-Loxin (IC_{50} at 31.71 μg/ml) in inhibiting MMP-3 secretion from TNFα-induced human chondrocytes.

Aflapin inhibits ICAM-1 expression in activated endothelial cells

OA is a degenerative joint disorder. However, there are migrations of inflammatory cells in the synovial fluid. Adhesion molecule expression on endothelial cells helps in the diapedesis of these cells. Therefore, in order to determine whether 5-Loxin® and Aflapin® treatments can ameliorate the ICAM-1 expression, we evaluated the ICAM levels on HDMEC. **Figure 4** depicts that 5-Loxin® and Aflapin® significantly reduce TNFα induced ICAM-1 expression (p<0.01, student t-test). Interestingly, Aflapin® shows more capability to reduce ICAM-1 secretion than that of 5-Loxin®.

Biochemical evaluations

As a part of the safety evaluation, laboratory tests were performed to evaluate different biochemical parameters (serum and urine) and hematological parameters. The significance of the differences between baseline and 90 days was tested by using repeated measures ANOVA. The f ratio is considered significant if $P<0.05$. Although minor changes were observed in some of the parameters, they remained within the normal laboratory range. Statistical analyses of these parameters did not indicate any significant changes. Similarly, no significant changes in hematological and urinary parameters were observed in the active treatment groups when compared to the placebo (data not shown). These findings further demonstrate the safety of 5-Loxin® and Aflapin® in humans.

Adverse Events and Dropouts

During the course of the 90-day study, no major adverse events were reported. However, acidity was reported as a minor adverse event by two subjects during the study; one each from placebo and Aflapin supplemented groups, respectively.

Three subjects one from each placebo, 5-Loxin® and Aflapin® supplemented groups were dropped out from the study due to their un-availability during the entire study period.

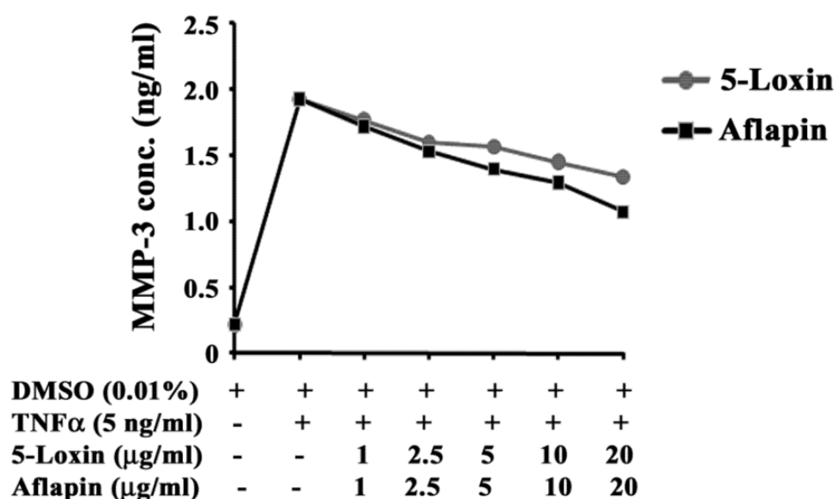

Figure 3: Aflapin and 5-Loxin inhibit matrix metalloproteinase-3 secretion from TNFα-induced human primary chondrocytes. Line diagram represents MMP-3 concentrations in the culture supernatants of chondrocytes treated with 5 ng/ml of human recombinant TNFα in presence or absence of different doses of either 5-Loxin or Aflapin as indicated. Vehicle control cultures received 0.01% DMSO. Each data point represents the mean of quadruplicate wells.

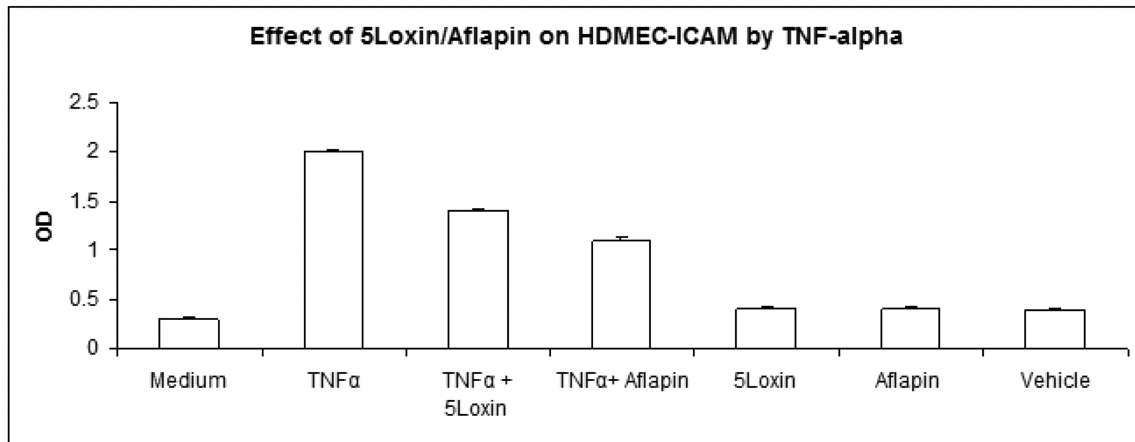

Figure 4: Aflapin and 5-Loxin inhibit TNFα-induced ICAM-1 expression on human dermal microvascular endothelial cells (HDMEC). Bar diagrams represent the ICAM-1 expression on HDMEC treated with 20ng/ml of human recombinant TNF-α in presence or absence of either 5-Loxin (4μg/ml) or Aflapin (4μg/ml) as indicated. Vehicle control cultures received 0.01% DMSO. Each experiment is done in quadruplicate wells. The results are expressed as the mean±SD of five experiments in quadruplicate wells. 5-Loxin and Aflapin significantly inhibits ICAM-1 expression induced by TNF-α (p<.01, student t-test).

Discussion

This is the first clinical study to evaluate the efficacy of Aflapin® in OA subjects. Aflapin is a novel synergistic composition comprising AKBA enriched *B. serrata* extract and non acidic gum extract of *B. serrata*. In a battery of preclinical studies designed in *in vitro* cellular models and *in vivo* animal models, Aflapin exhibited significantly better anti-inflammatory activities in comparison with 5-Loxin® (Data to be presented in a separate communication). 5-Loxin® is a *Boswellia serrata* extract standardized to 30% AKBA. Its multidirectional activities related to anti-inflammatory efficacies obtained in appropriate cellular, animal models and in human subjects have established that 5-Loxin® is a potent dietary supplement for the management of inflammatory diseases such as osteoarthritis [14-22]. In a series of experiments designed in *in vitro* cellular and *in vivo* animal models, Aflapin showed significantly better efficacy in comparison with 5-Loxin®. In addition, Aflapin exhibited better AKBA bioavailability than 5-Loxin® in Wistar rat model. Broad spectrum safety of Aflapin was also established in a battery of acute and sub-acute toxicity studies in rat and rabbits. These findings altogether motivated us to evaluate efficacy of Aflapin in comparison with 5-Loxin® against osteoarthritis in human subjects. In the present 90-day clinical study, we assessed the efficacy and tolerability of Aflapin in comparison with 5-Loxin® in OA subjects. Pain, stiffness of joints, reduced joint movement and physical disability are the major clinical manifestations of OA [1,40,41]. Our study demonstrates that Aflapin potentially improves pain, joint stiffness and physical function in OA subjects (**Figure 2**). In order to check improvements in the treatment groups, we compared the data for all parameters between the baseline and day 90. Paired *t*-test revealed that both treatment groups showed statistically significant improvements in all parameters.

Compared to the placebo, 5-Loxin® supplementation for 90 days, significantly reduced VAS, WOMAC-pain, WOMAC-stiffness (**Table 3**), which are consistent with our previous observations [22]. Whereas, Aflapin supplementation for 90 days, resulted in significant reduction in all pain scores tested in comparison with placebo. These findings suggest that Aflapin has better therapeutic efficacy against OA compared to 5-Loxin®. We observed that, in comparison with baseline, there were downward trends in VAS score and WOMAC scores in the placebo group. We believe that this might be partly attributable to the placebo effect [42,43] manifested while administering the questionnaires to placebo subjects and partly due to the consumption of ibuprofen as rescue medication by more subjects in the placebo group during the study. It is noteworthy that 5-Loxin® possesses significant efficacy in lowering VAS score by 8.09% (P=0.022), WOMAC pain score by 8.68% (0.031) and WOMAC function score by 8.35% (P<0.015) in OA subjects as early as 7 days after the initiation of treatment. In comparison, Aflapin showed significant reduction in all the pain scores assessed including VAS score by 12.8% (P=0.0004), LFI score by 9.17% (P=0.003), WOMAC pain score by 11.78% (P=0.012), WOMAC stiffness score by 18.48% (P=0.012) and

WOMAC function score by 10.24% (*P*=0.005) (**Figure 2**). These findings therefore indicate that 5-Loxin® and Aflapin® confers prompt and significant pain relief, improvement in physical ability and quality of life in OA subjects. However, Aflapin showed better reduction in all the tested pain scores and hence can be considered superior to 5-Loxin®.

Pathogenesis of osteoarthritis is a complex process. These include mechano-transduction, the interplay between metalloproteases (MMP3, MMP13), protease inhibitors and cytokines on cartilage degradation and mechanisms of cartilage repair [40,44,45]. MMP-3 is over-expressed in OA and cause degeneration of cartilage tissue [44,45]. Cytokines act via autocrine and endocrine functions to alter cartilage homeostasis. Interleukin-1 (IL-1) and TNF-α are perhaps the best characterized cytokines for cartilage degradation (46,47). They are synthesized by chondrocytes and FLS. These cytokines act in various ways in the pathogenesis of OA such as inhibition of synthesis of type 2 (articular) cartilage and activation of catabolic metalloproteases including MMP-3 which plays a critical role in cartilage degradation [44,45]. A role of synovitis in OA can't be disregarded either. It is a well established clinical observation that pain and swelling in OA improves for months following intra-articular corticosteroid injection. In addition, histologic studies suggest that localized inflammatory changes characterized by foci of inflammatory cells occur in up to 50% of OA patients [48]. In this study to find out possible mechanism of actions of Aflapin we carried out *in vitro* studies to evaluate whether Aflapin can inhibit metalloprotease secretion or influence the inflammatory component of osteoarthritis. We observed: (1) Aflapin inhibits TNFα induced MMP-3 secretion in chondrocytes; (2) Aflapin inhibits TNFα induced expression of ICAM-1 in endothelial cells.

Overall, the foregoing data together demonstrates the better ability of Aflapin compared with 5-Loxin® in terms of reducing the pain, improving physical function, quality of life and joint health. Presumably these improvements might occur through down regulation of cartilage degrading enzymes such as MMP-3 in OA subjects. The present study also demonstrates no major changes in the hematological parameters, serum biochemical parameters and in urine analysis in the treatment groups compared to placebo. In addition, no major adverse effect was reported by the subjects in the treatment groups. Taken together, these observations further demonstrate that 5-Loxin® and Aflapin® are potentially safe in the treatment of OA in humans and more specifically Aflapin® is more efficacious in the management of osteoarthritis than 5-Loxin®.

Conclusion

In summary, the present study provides the evidence in support of the potential efficacy and tolerability of 5-Loxin® and Aflapin® in subjects with OA; 5-Loxin® and Aflapin significantly improved joint function. Aflapin exhibited better therapeutic efficacy over 5-Loxin® at 100 mg/day; it reduces pain rapidly, as early as after 1 week of treatment. Furthermore, *in vitro* studies also provide evidences that compared to 5-Loxin, Aflapin is capable of inhibiting cartilage degrading enzyme MMP-3 and has the potential to regulate the inflammatory component in by inhibiting ICAM-1. Most importantly, we have observed that 5-Loxin® and Aflapin® are safe for human consumption, even for long term supplementation. 5-Loxin® and Aflapin® are promising alternative therapeutic options, that may be used as nutritional supplements for management of OA.

Authors' contributions

KS contributed to the design of the project and data analysis, and was primarily responsible for writing the manuscript. KVA contributed to the design of the project, patient recruitment and management, and data collection. ARS and AM worked with subjects to obtain informed consent, conducted clinical evaluations, took samples and evaluated therapeutic response of 5-Loxin® and Aflapin®. TG contributed as the study coordinator and helped to review the manuscript. KVSS and DD helped in clinical data analysis. SMR helped in designing and executing the mechanisms of action studies. SPR helped in designing the study, conducting data analysis and writing the manuscript.

Abbreviations

AKBA: 3-O-acetyl-11-keto-beta-boswellic acid; ANOVA: analysis of variance; ASRAM: Alluri Sitarama Raju Academy of Medical Sciences; BMI: Body Mass Index; ELISA: enzyme-linked immunosorbent assay; LFI: Lequesne's Functional Index; MMP: matrix metalloproteinase; NSAID: nonsteroidal anti-inflammatory drug; NU: normalized units; OA: osteoarthritis; VAS: visual analog scale; WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index.

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Conflict of Interest

The authors have declared that no conflict of interest exists.

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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×*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

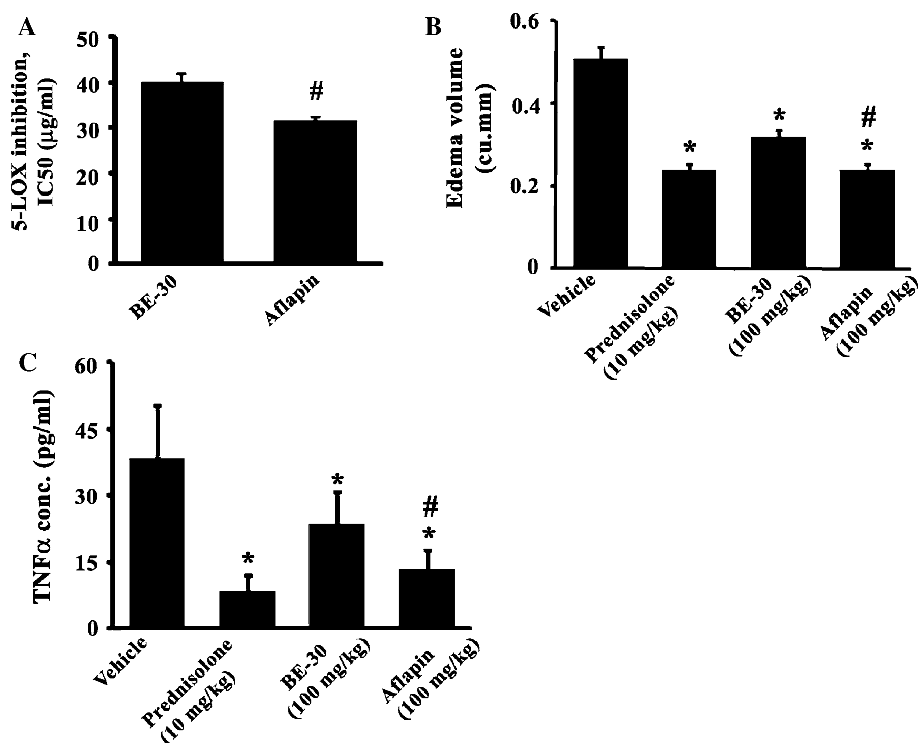

Fig. 1 Comparative analysis of anti-inflammatory properties of BE-30 and Aflapin. **a** *Bars* represent mean ± SD of IC_{50} (half maximal inhibitory concentration) of BE30 and Aflapin inhibiting 5-lipoxygenase activity in enzymatic assays ($n = 4$). **b** *Bar* diagram represents paw edema volumes (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.

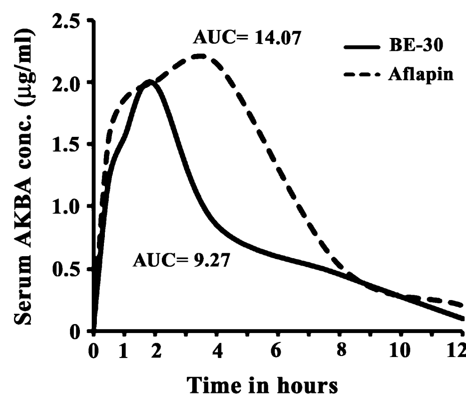

Fig. 2 Comparative bioavailability of AKBA in BE-30 and Aflapin-supplemented Sprague–Dawley rats. *Line diagram* represents mean AKBA concentration (µg/ml) in serum samples of Sprague–Dawley rats ($n = 5$) at different time points after a single dose oral administration of BE-30 and Aflapin. *AUC* area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

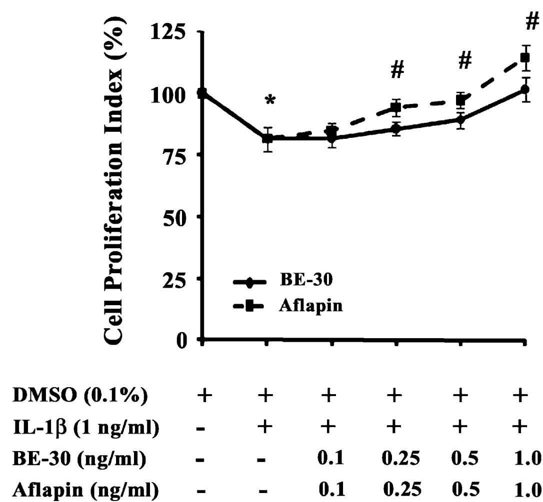

Fig. 3 Boswellia products improve proliferation of IL-1β-treated human primary chondrocytes. *Line diagram* represents cell proliferation index (mean ± SD, $n = 6$) of human primary chondrocytes treated with 1 ng/ml of IL-1β in presence or absence of different concentrations of BE-30 or Aflapin as indicated. 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

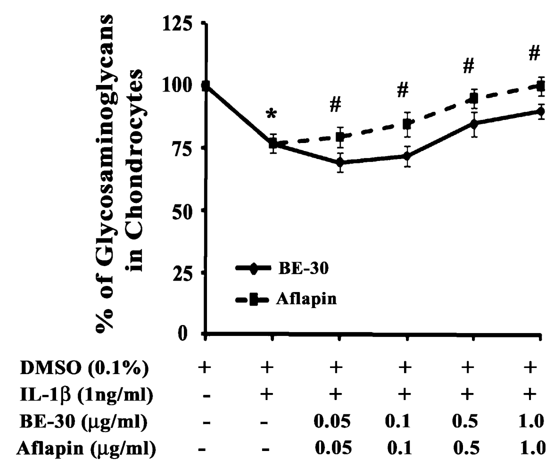

Fig. 4 BE-30 or Aflapin increase glycosaminoglycans (GAG) synthesis in IL-1β-treated human primary chondrocytes. *Line diagram* depicts percentage of GAG content (mean ± SD, $n = 6$) in human primary chondrocytes treated with 1 ng/ml of IL-1β in presence or absence of different concentrations of BE-30 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

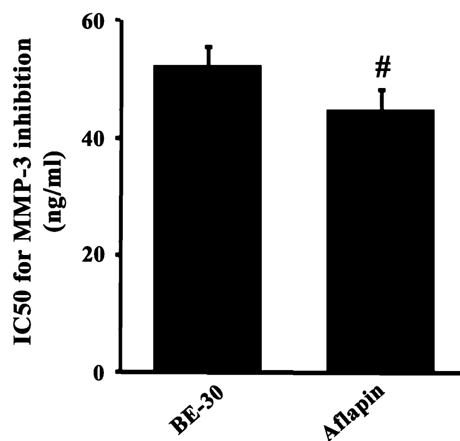

Fig. 5 BE-30 and Aflapin inhibit matrix metalloproteinase-3 secretion from TNFα-induced human primary chondrocytes. *Bar* diagram represents mean ± SD of IC_{50} (half maximal inhibitory concentration) of BE-30 and Aflapin in culture supernatants ($n = 4$) of human recombinant TNFα-induced human synovial 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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