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Pain and Inflammation Management: Part-I Pre-clinical Study of a Topical Ayurvedic Cream called HerboCare or HerboJoint™.

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ABSTRACT

Background: The properties of a system of essential oils derived from GRAS (Generally Recognized as Safe) medicinal plants were studied by drawing upon the wisdom of Ayurveda. The objective was to address muscle relaxation, stiffness, swelling and inflammation of human subjects.

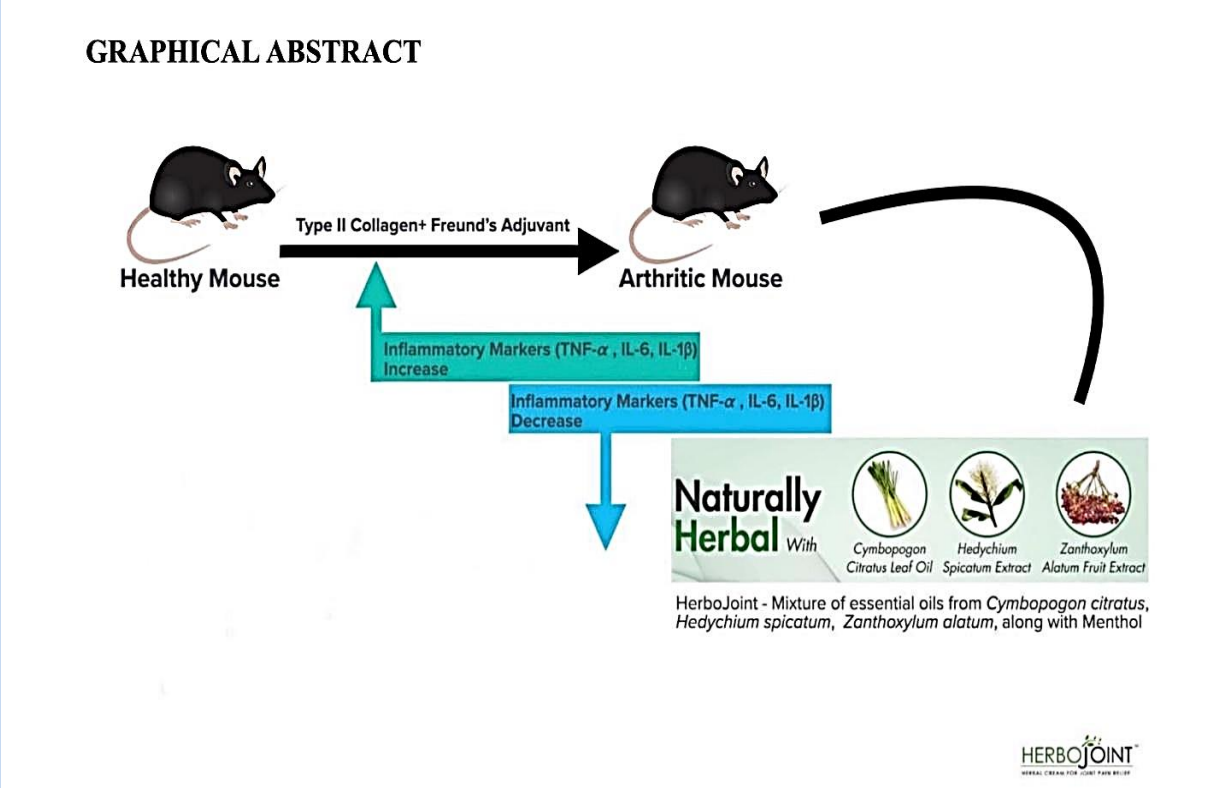
Objective: A novel formulation is reported here, namely Kattrna or Lemongrass (CC-*Cymbopogon citratus*) (3.0%), Sati (HS-*Hedychium spicatum*) (1.0%) and Tumuru (ZA- *Zanthoxylum alatum*) oil (1.0%) along with USP grade Menthol (which can also be derived from *Mentha)* (1 to 10%). This formulation, called HerboCare or HerboJoint™, was patented.

Methods: Its composition was investigated via HPLC and GC-MS analysis by comparing it with identifiable marker components. Cell line studies were performed followed by a Type-II collagen-induced arthritic mice model investigation over a 21-day period of blood plasma of before vs. after treatment for the inflammatory markers.

Results: The synergistic action of the selected essential oils and Menthol was demonstrated to reduce the inflammatory cytokines, including TNF-α, IL-6, and IL-1β, in the blood plasma of the treated arthritic mice.

Conclusion: It is believed to provide significant relief against inflammation. Therefore, formulations were subsequently prepared in a non-greasy oil-in-water emulsion cream base for applying topically around the affected joint and other areas for a human clinical investigation.

Keywords: Joint Pain; Essential Oils; Menthol; Inflammatory Cytokines; Pre-Clinical Study



INTRODUCTION:

Joint pain has emerged as one of the most traumatizing and debilitating conditions for elderly people worldwide. If left untreated, it can eventualize into almost incurable chronic ailments such as Osteoarthritis (OA) and Rheumatoid Arthritis (RA) referred to respectively as "Sandhigatavata" [1] and "Amavata" [2] in the ancient Indian scriptures of Ayurveda or herbal medicines. Apart from OA and RA, other ailments resulting from joint pain are gout caused by crystal deposition in the joint, bursitis caused by overuse of the hip, knee, elbow, or shoulder joint, and tendinitis or inflammation of the tendons [3]. OA is typically a bone joint disorder arising out of progressive degeneration of cartilage due to wear and tear accompanied by the consequent low-grade inflammatory response [4]. Aging-associated cartilage degeneration and severe joint pain are well-established [5]. Despite unprecedented advances in medical science equipped with state-of-the-art diagnostic technologies, joint pains are yet to achieve the status of being completely curable. All treatments to date are symptomatic and based on providing relief to the patient.

Under the circumstances, there has been no definitive strategy for the cure or long-term relief from joint pains and symptom-based treatment has been the best possible alternative to alleviate the chronic or acute pain experienced by affected individuals. An effective preventive measure to curb or minimize arthritis-induced joint pain involves the consumption of a Mediterranean diet rich in fish, fruits, and vegetables with ample sources of antioxidants [6][7], whereas very few actual treatment options exist. Almost every therapeutic strategy for more serious joint pain issues such as RA involves the use of non-steroidal anti-inflammatory agents (NSAIDs) like aspirin and ibuprofen [6][7], disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate and hydroxychloroquine [8], and biologics such as infliximab (a TNF blocker) [9]. All similar strategies are employed for the management of OA, which is accompanied by a low-grade inflammatory response. In most cases, oral analgesics such as acetaminophen and antidepressants such as duloxetine are prescribed by clinicians for temporary alleviation from pain and restricted movement [10][11]. In more severe cases, intraarticular glucocorticoid injections are administered. However, all these approaches are not without side effects such as gastritis and peptic ulcers.

Ayurvedic medicines fortified with the goodness of herbs and rich in antioxidants as well as other protective principles had been in vogue for centuries, much before the advent of modern therapeutics. Accordingly, herbal formulations made from the traditional knowledge of Ayurveda had also been employed in treating joint pains with promising results [12][13]. However, in most cases, the success of these formulations has been largely driven by the synergistic effect of multiple active components present in the herbs [14][15]. The present study reports the invention of a novel anti-inflammatory herbal formulation for effective anti-inflammatory efficacy. It is made from essential oils of GRAS (Generally Recognized As Safe) enlisted medicinal plants, namely Lemongrass (*Cymbopogon citratus*) leaf oil (3.0%), Sati (*Hedychium spicatum*) extract (1.0%), Tumuru (*Zanthoxylum alatum*) fruit extract (1.0%), and Menthol (*Mentha arvensis*) (1.3%). These formulations were screened in an animal model of collagen-II-induced arthritis in mice (16). Furthermore, cell culture and ELISA studies were conducted to exhibit the anti-inflammatory efficacy of these essential oils.

MATERIALS AND METHODS

HerboCare or HerboJoint™ Cream: HerboJoint™ (or HerboCare) was manufactured by Bordoloi Biotech India Pvt. Ltd. (BBIPL) Assam, India. These formulations used essential oils from the herbs Lemongrass leaf oil (CC-*Cymbopogon citratus*) (3.0%), Sati extract (HS-*Hedychium spicatum*) (1.0%), Tumuru fruit extract (ZA-*Zanthoxylum alatum*) oil (1.0%) along with USP grade Menthol crystal (M- Mentha arvensis contains Menthol) (1.3 %). These were evaluated individually as well as in combinations thereof. Then, a non-greasy oil-in-water emulsion cream was prepared. [Bordoloi and Saini, US Patent, 2019] [16]. Its efficacy in relieving joint pain was first investigated and confirmed in a mouse model where arthritis was artificially induced with type-II collagen [17][18].

It is important to mention that initially the formulation was developed using *Zanthoxylum armatum*, however, later, because of the availability, quality, and consistency, the formulation was developed with *Zanthoxylum alatum*. Linalool was a major component in *Zanthoxylum alatum*, which was detected in both alatum and armatum in similar proportions.

Culture Cell Line and Animal Model Studies: The RAW 264.7 cell lines were obtained from the American Type Culture Collection. Cells were cultured in DMEM high

glucose medium supplemented with 10% FBS (all the experiments were performed using the same FBS batch) and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) , in an atmosphere of 5% CO2 and 95% humidity at 37°C. Cells were passaged after reaching 90% confluence, detached with cell scraper after trypsinization using TrypLE Express Enzyme (Gibco), and subcultured in a 1:6 ratio in T-75 flasks. All cell culture equipment (flasks, pipettes, etc.) used in this study were from the same batch. To avoid differences between culture techniques, only one-person cultured cells.

In vitro assessment of Essential Oil(s) Mediated Inhibition of TNFα in Macrophage Cell Line: The RAW264.7 cells were cultured and grown in a 12-well plate (5 x 104 cells/well) and incubated for 24 hrs. These cultured cells were pretreated with different essential oil(s) at a final concentration of 100 µg/ml in 0.5% DMSO. After 30 min, lipopolysaccharide (LPS) (100 ng/ml) was added to induce the activation of the inflammatory response [19]. Following 24 hours of LPS stimulation, the culture medium was collected and the secreted TNFα was quantified using ELISA Kit (R & D systems).

Assessment of Essential Oil(s) Mediated Inhibition of TNFα, IL-6, and IL-1β in Mouse Arthritis Model representative of joint pain: Arthritis was artificially induced in mice using type-II collagen [17][18]. Briefly, Type-II collagen was dissolved in 10 mM acetic acid to prepare a 3 mg/ml solution. Then 1 ml of this solution along with 1 ml of Complete Freund adjuvant was mixed and 50 µl of this was injected intra-dermally at the tail vein into a set of mice each weighing 20-25 gm. If required, a booster dose was injected after a gap of 21 days at the same site. Induction of arthritis was assessed by enumeration of levels of the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β.

Measurement of Levels of Inflammatory Cytokines: Pro-inflammatory cytokines TNF-α, IL-6, and IL-1β were evaluated as markers of inflammatory response. The plasma from the blood samples were refrigerated overnight in plastic tubes, at which time aliquots were prepared and stored at −80°C. The cytokines were then measured using ELISA kit (R & D systems). The lower limits of detection in each case were set at 1.7 pg/ml [5].

Compositional Analysis of HerboJoint™ (or HerboCare) using HPLC and GC-MS: To assess the compositional analysis of the formulation, the amounts of the essential oils present in the cream were extracted and analyzed. The analysis comprised of identifying the unique component found in a given oil for quantitation. The components were selected such that each one was unique for a given oil, and thus could be detected in a blend of the oils without interference. The analytical methods were a combination of GC-MS and HPLC. These techniques were used to quantify the essential oils and Menthol using a key marker chemical present in each of them [20].

Composition analysis of HerboCare or HerboJoint™ using GC: Briefly, 100 mg of each oil was mixed in 1 ml of HPLC grade acetonitrile to prepare a mother stock for each. The working stock was made by dissolving 5 µl of the mother stock in 1 ml HPLC grade acetonitrile. Samples in aliquots of 1 µl were subsequently subjected to GC-MS analysis using Agilent GC column (SH-Rtx-5, Thickness: 0.25µm, Length: 30.0 m, Diameter:0.25 mm) with a flame ionization detector (FID) and nitrogen as a carrier gas at a flow rate of 0.46 mL/min. Temperatures of the injector and detector were respectively set at 200°C and 280°C, respectively. The oven temperature gradually increased from 45°C to 280°C at a ramp rate of

3°C/min and finally maintained under isothermal conditions for 22 min. The peak area was calculated from the FID signal using the GC HP-Chemstation software (Agilent Technologies, Santa Clara, CA, USA). To carry out GC-MS analysis, the standard control blend of the three neat oils (CC, HS, ZA) and menthol was made at the target ratio of 3.0 g, 1.0 g, 1.0 g, and 1.3 g by weight, respectively and was subsequently diluted in acetonitrile at a concentration of 20 mg/ml. Helium was used as carrier gas at a flow rate of 1 mL/min with an electron ionization system as the detector and an ionization energy of 70 eV was used. A scan rate of 0.6 s (cycle time: 0.2 s) was applied, covering

a mass range from 35 to 600 amu. Identification of key components of the essential oils were carried out based on mass spectra and retention indices of library compounds obtained under exactly similar conditions employing a two-dimensional search algorithm [27]. Area % signal of each of the four key markers corresponding to the three essential oils and Menthol were calculated from the chromatogram and data corresponding to a concentration of 10 mg /ml were interpreted simply by taking 50% of the signal values. The HerboCare or HerboJoint™ formulation was also prepared in acetonitrile in a concentration of 10 mg/ml and analyzed in a similar way. Each of the four components were then quantitated by taking the ratio of the signal to the standard control blend signal.

Composition analysis of HerboCare or HerboJoint™ using HPLC: HPLC analysis was performed at 25 ± 1°C using 10 µl- 30 µl sample solutions filtered through 0.45-micron Whatman's syringe filter and subsequently analyzed using a Shimadzu system with a LC-20AT Prominence Liquid Chromatography set-up. The stock solution and test solution of oil: Menthol mix as well as other preparations to be analyzed were prepared according to Table 1. Separation was achieved using Phenomenex RP C18 column with a gradient mobile phase of acetonitrile in water (40% to 100%) at a flow rate of 1 ml/min. The eluate was monitored at 210 nm. A calibration curve was made using the reference compounds Methyl Cinnamate, Linalool, Citral or Geranial, Myrcene, Limonene, Menthol. Alongside, 20 mg/ml solution of pure Menthol and the Menthol equivalent of 10 mg/ml HerboCare or HerboJoint™ composition was mixed in HPLC grade acetonitrile-water. The Menthol present in the composition was extracted and analyzed. When the oil-in-water emulsion cream was subsequently developed, it was extracted into the solvent phase after sonication and centrifugation and analyzed in the same manner.

Table 1: Preparation of Oil and Cream Solution for HPLC Analyses

Oil Menthol Mix LG:ZA:HS:Menthol:: 3:1:1:1.3 % w/v (stock solution)	300 mg LG + 100 mg ZA + 100 mg HS + 130 mg Menthol was dissolved in acetonitrile and 10 mg of stock solution was weighed out and dissolved in 1 ml of HPLC grade acetonitrile and the final volume was made in such a way so as to give final concentration as LG:ZA:HS: Menthol = 3:1:1:1.3 w/w
HerboJoint™ (or HerboCare) cream	10 mg in 1000 µl of HPLC grade Methanol: Acetonitrile mixture was prepared
Oil stock solution	100 mg of each of LG, ZA and HS oil was weighed and dissolved separately in 1 ml of HPLC grade acetonitrile
HPLC solution preparation	For ZA and HS oil, 4 µl of oil was taken and dissolved in 1 ml of HPLC grade acetonitrile. For LG oil, 8 µl of oil was taken from the stock solution and dissolved in 1 ml HPLC grade acetonitrile

Statistical Analyses: All data reported were expressed as the means ± standard deviation (SD) of at least 3 independent measurements for each experiment.

RESULTS

Assessment of Composition of the Formulation: The purity or consistency of the essential oils was ascertained in the labs as developed earlier, using IR, NMR, HPLC, and GC-MS [16]. Therefore, a control blend of the three oils along with Menthol was prepared to find the effective concentration of the marker compounds in the blend as compared to those of the corresponding pure oils determined individually. Subsequently, the control blend was used as a reference to assess the composition of the HerboJoint™ (or HerboCare) formulation with respect to the three available EOs as well as Menthol. First, the key markers based on which the comparative assessment would be made were identified by employing HPLC.

Subsequently, these were used in GC-MS to quantify and compare the actual vs predicted amounts of the three essential oils along with Menthol (Table 2). In Lemongrass (CC oil), Neral and Geranial were identified as key markers. The presence of 29.3 % Neral and 37.4 % in Geranial represented 100% pure Lemongrass oil. Similarly, the amounts of Methyl Cinnamate and epi-curzerenone were detected to be 17.1% and 29.7% in pure ZA oil and HS oil respectively (Table 2).

Table 2: Key Component Markers in Essential Oils for Method Development

Name of Essential Oil	Key Markers Used for GC/MS Based Quality Assurance Methods
Cymbopogon citratus (Lemongrass Oil)	Geranial (37.4 %)
Hedychium spicatum (Sati Oil)	epi-Curgerenone (29.7 %)
Zanthoxylum armatum (Tumuru Oil)	Methyl Cinnamate (17.1 %)

Depending on the evaluated concentrations of these key markers, the quality of the HerboCare or HerboJoint™ formulation was assessed by GC-MS. As stated already, the components were analyzed at a concentration of 20 mg/ml due to a lack of adequate detection sensitivity at 10 mg/ml, and the detected peak area was halved to get an estimate of the corresponding components at 10 mg/ml. CC oil was added at 3 parts per 6.3 parts of the control blend. Neral and Geranial cumulatively yielded a concentration of 18.5 %. Both ZA oil and HS oil were added at a concentration of 1 part per 6.3 parts and yielded a concentration of 1.3% and 2.1% in the control blend respectively. 12.9% Menthol was recovered as per GC-MS peak corresponding to 1.3 parts per 6.3 parts added Menthol in the control blend.

It is worthwhile to mention that 1.25% to 16% Menthol has been recommended as per 21 CFR Part 348 external analgesic drug product for OTC human use, while US Federal Register 21CFR Part 348.12 counter irritant active ingredient (Menthol 1.25% to 16%) recommended to be used as cooling pain relief or penetrating pain relief for arthritis treatment and pain alleviation.

The data from the control blend was subsequently used to find concentrations of the components present in the HerboCare or HerboJoint™ cream by preparing a 10 mg/ml solution of the cream in acetonitrile. The evaluated results as read from the signals are presented in Table 3.

Table 3: GC-MS Quantification of Key Markers of Individual Oils, alone and in Combination, for the Cream

Cream Formula Components (CC/ZA/HS/M)	Key Marker Components of Individual Oils and Blend					Key Marker Calculated at 10 mg/ml: for Control Blend CC/ZA/HS/M= 3/1/1/1.3 % W/W
	Geranial % W/W	Methyl Cinnamate % W/W	Epi-Curzere-none % W/W	Menthol % W/W	Key-Marker in Control Blend CC/ZA/HS/M = 3/1/1/1.3 At 20 mg/ml % W/W	
CymbopoganCitratus **(or Lemongrass)**	37.4 ± 0.9 %	--	--	--	20.9 ± 0.4 %	10.5 ± 0.4 %
ZanthoxylumAlatum	--	17.1 ± 0.5 %	--	--	2.6 ± 0.1 %	1.3 ± 0.1 %
Hedychium Spicatum	--	--	29.7 ± 0.9 %	--	4.2 ± 0.1 %	2.1 ± 0.1 %
Menthol (USP Grade)				100.0	25.7 ± 0.7 %	12.9 ± 0.7 %

An effective recovery of the three essential oils and Menthol based on the standard markers chosen was obtained in the HerboCare or HerboJoint™ formulation and subsequently in the cream. As compared to 18.5% of evaluated CC oil, 17.6% was recovered in a similar concentration of HerboCare or HerboJoint™ similarly, a 1.17% recovery was obtained as compared to 1.3% for ZA oil, and 2.06% recovery was obtained with respect to 2.1% of HS oil. Similarly, Menthol was detected. Finally, the data obtained from GC-MS was also confirmed by HPLC (Table 4). Similar to GC-MS the purity profile obtained from HPLC also corroborated well with that predicted by the theoretical distribution reflected in the control oil-Menthol blend.

Table 4: GC/MS Based Quantification of Key Markers in HerboJoint™ (or HerboCare) Cream

	Components in HerboCare or HerboJoint™ Cream	Theoretical In HerboCare or HerboJoint™ (Weight %)	Key Markers in Standard Control Blend at 10 mg/ml (Weight %)	Key Markers in Actually Analyzed HerboCare or HerboJoint™ Cream at 10 mg/ml (Weight %)	HerboCare or HerboJoint™ Components Determined Based on Standard Control and Cream Data; Wt %
1	*Cymbopogon Citratus* Oil	3.0 %	18.5 ± 0.4	17.60±0.29	2.85 ± 0.07 %
2	*Zanthoxylum Alatum Oil*	1.0 %	1.3 ± 0.1	1.17 ± 0.02	0.88 ± 0.02 %
3	*Hedychium Spicatum* Oil	1.0 %	2.1 ± 0.1	2.06 ± 0.04	0.98 ± 0.02 %
4	Menthol	1.3 %	12.9 ± 0.7	15.86 ± 0.38	1.23 ± 0.03 %

All results expressed in ±S.D. of three independent experiment sets.

The results suggested very good corroboration between the two methods and established the relative composition of the three essential oils as well as the Menthol used in the formulation.

Anti-inflammatory Potential of Essential Oils on Macrophage Cell Line: ***T****he* efficacy of essential oils to prevent the induction of inflammatory response was assessed on macrophage RAW 264.7 cell lines (Figure 1). LPS-induced inflammatory response was confirmed by a

significant increase in the secretion of TNF-α (1.1 pg./ml of protein), while essential oils reversed this inflammatory response. Cymbopogon citratus (CC)-derived oil exhibited the highest potential to curb the inflammatory response with an almost three times decrease in the level of TNF-α. The other two essential oils from *Zanthoxylum alatum* (ZA) and *Hedychium spicatum* (HS) as well as Menthol resulted in more than a 50% reduction in the levels of TNF-α. Overall, these essential oils as well as Menthol exhibited potent anti-inflammatory potential.

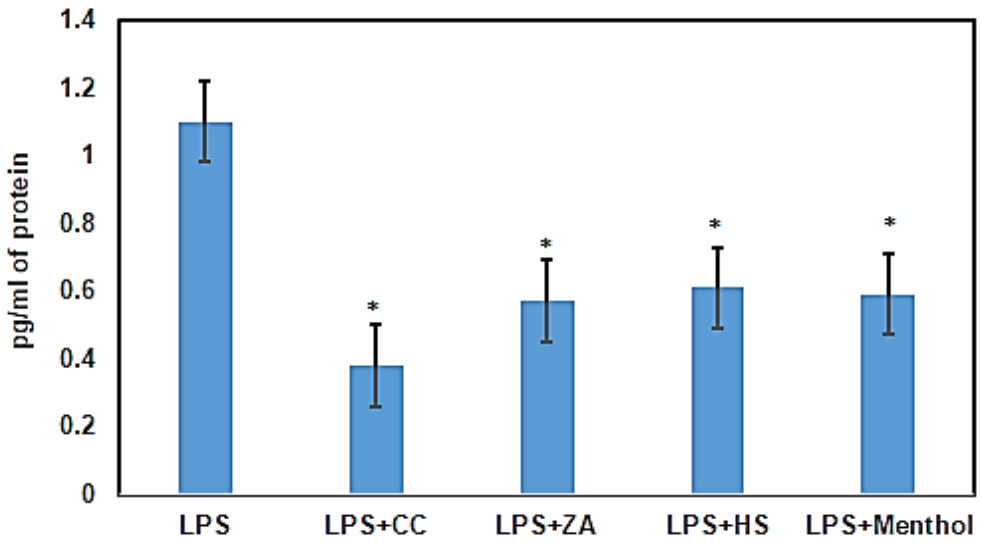


Figure 1: Anti-inflammatory effect of essential oils-the primary herbojoint[tm] (or herbocare) constituents in macrophage cell line

The effect of the three constituent essential oils CC = *Cymbopogon citratus;* HS = *Hedychium spicatum*; ZA = *Zanthoxylum alatum* and Menthol were ascertained individually on TNF-α secretion by LPS induced RAW 264.7 cell line. Error bars represent standard deviation from five independent sets of experiments and asterisk denote statistically significant data over the control group of cells.

Anti-Inflammatory Potential of Essential Oils in the Synergistic Oil Blend in an Arthritic Mouse Model: Detection of cytokines is a robust and authentic method for assessment of inflammatory response during joint pain and inflammation [21][22][23]. We evaluated the potent anti-inflammatory efficacy of this herbal formulation on three inflammatory markers TNF-α, IL-6, and IL-1β in an arthritis mouse model. Here arthritis was induced by type-I collagen [collagen-induced arthritis (CIA)] (Figure 2). Induction of arthritis in mice was confirmed through observed changes in the activity of the experimental mice set including slow movement, loss of cage riding ability, dragging feet to walk, and redness of the paw. Additionally, the elicitation of inflammatory response was confirmed by the significantly elevated levels of all three marker cytokines. The level of TNF-α was elevated by 2.5 times (Figure 2A) whereas those of IL-6 and IL-1β both rose by about 2.4 times. As evident from Figures 2A, 2B, and 2C, the application oil blends in combination (CC+ZA, CC+HS, CC+ZA+HS) as a droplet on the collagen-treated mice skin brought down the levels of all three inflammatory cytokines almost to the basal levels indicating that the mixture of oils acted synergistically to progressively inhibit elicitation of inflammatory response in the mouse model and the anti-

inflammatory potential was highest when the three oils were used together.

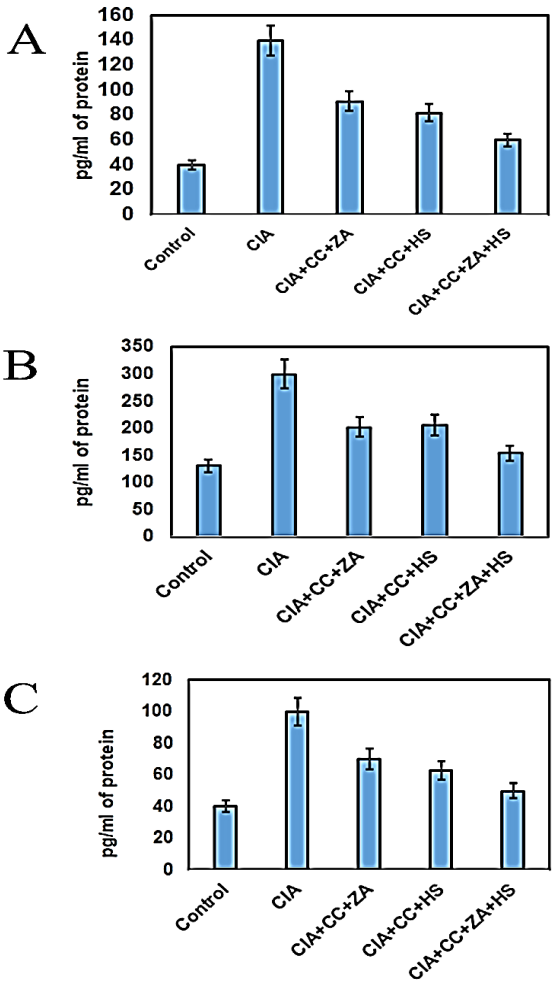


Figure 2: Anti-Inflammatory Effect of Essential Oils-the Primary HerboCare or HerboJoint™ Constituents in an Arthritic Mice Model:

The effect of the three essential oils in combination was investigated over a period of 21 consecutive dates in reducing inflammatory response in a mice model where arthritis was induced by type I collagen (CIA). Levels of TNF-α (Figure 2A), IL-6 (Figure 2B), and IL-1β (Figure 2C) were assessed in the arthritic mice model after administration of CC+ZA, CC+HS, and CC+ZA+HS respectively. Standard error bars were drawn from the data of 8 mice in each set.

DISCUSSION

Use of herbal alternatives for the treatment of a plethora of joint pains was popular in the past [24][25] and has recently gained prominence due to the high cost and associated toxic effects of conventional drugs like methotrexate and NSAIDs [26][27]. However, several key medicinal plants like *Tripterygium wilfordii Hook F, Boswellia serrate*, *Aristolochia bractaeta, Boerhaavia diffusa*, *Lantana camara*, *Piper longum*, etc. have been demonstrated and identified to possess strong anti-inflammatory effects and have shown promising results in alleviating joint pain. The synergistic efficacy of selected botanical formulations had been reported in the literature [28][29][30]. In addition, many of these botanical formulations also have allied benefits of anti-carcinogenic, hepatoprotective, antidiabetic, and anti-viral activities. Researchers have been constantly trying to optimize the delivery modules for maximizing bioavailability [31].

This study demonstrated the efficacy of a novel herbal formulation. This synergistic oil blend was made from essential oils of GRAS (Generally Recognized As Safe) enlisted medicinal plants, namely Lemongrass (*Cymbopogon citratus*, CC) leaf oil (3.0%), Sati (*Hedychium spicatum*, HS) extract (1.0%), Tumuru (*Zanthoxylum alatum*, ZA) fruit extract (1.0%), and Menthol (*Mentha arvensis*) (1.3%). It was screened in an animal model of collagen II-induced arthritis in mice for demonstrating efficacy.

The anti-inflammatory efficacy of the essential oils was evaluated on the lipopolysaccharide (LPS)-induced inflammatory response in macrophage-derived RAW 264.7, which are major mediators of inflammatory response in a mammalian system (Figure 1). Essential oil(s) significantly inhibited these inflammatory responses, while *Cymbopogon citratus* (CC) exhibited the maximum inhibitory potential with an almost three times decrease in the level of TNF-α. The other two essential oils, *Zanthoxylum alatum* (ZA) and *Hedychium spicatum* (HS), and Menthol induced more than 50% reduction in the levels of TNF-α. Taken together, these results indicated the strong anti-inflammatory potential of the essential oils as well as that of Menthol.

Chronic debilitating joint pain ultimately leads to different forms of arthritis. Cartilage degeneration during inflammation and arthritis is accompanied by the localization of a battery of pro-inflammatory cytokines and hydrolytic enzymes such as matrix metalloproteinases (MMPs), especially in the affected synovial fluids [21]. Therefore, the detection of these cytokines is a robust and authentic method for the assessment of inflammatory response during joint pain and inflammation [22][23]. Following the induction of collagen-induced joint pain and arthritis in the mice model, visible changes in their diverse movement activities including slow movement, loss of cage riding ability, dragging feet to walk, and redness of paw were observed. Moreover, the increase in inflammatory response was confirmed by the significantly elevated levels of all three cytokine markers following the induction of collagen.

The anti-inflammatory efficacy of the synergistic oils blended with Menthol used in HerboCare or HerboJoint™ was evaluated on three predominant inflammatory markers TNF-α, IL-6, and IL-1β (Figure 2). Elevated levels of TNF-α, IL-6, and IL-1β were observed following tail-vein injection of collagen (Figure 2). These oil blends were CC+ZA, CC+HS, and CC+ZA+HS, in combination with a fixed concentration of Menthol. These droplets were applied to the collagen-treated mice. The anti-inflammatory potential was highest when the three oils in conjunction with Menthol were used together. These brought down the levels of all three inflammatory cytokines almost to the basal levels indicating that the mixture of oils acted synergistically.

This led to strategizing the formulation of HerboCare or HerboJoint™ with the synergistic blend of these three essential oils and Menthol. It was subsequently prepared in an oil-in-water emulsion cream base for topical applications for anti-inflammatory efficacy.

CONCLUSION

The anti-inflammatory efficacy of the synergistic oils blended with Menthol used in HerboCare or HerboJoint™ was evaluated on three predominant inflammatory markers TNF-α, IL-6, and IL-1β (Figure 2). Elevated levels of TNF-α, IL-6, and IL-1β were observed following tail-vein injection of collagen (Figure 2). These oil blends were CC+ZA, CC+HS, and CC+ZA+HS, in combination with a fixed concentration of Menthol. These droplets were applied to the collagen-treated mice. The anti-inflammatory potential was highest when the three oils in conjunction with Menthol were used together. These brought down the levels of all three inflammatory cytokines almost to the basal levels indicating that the mixture of oils acted synergistically.

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List of abbreviations: IICB: Indian Institute of Chemical Biology; NIPER: National Institute of Pharmaceutical Education and Research; CSIR: Council of Scientific & Industrial Research; CC: *Cymbopogon citratus*; HS: *Hedychium spicatum*; ZA: *Zanthoxylum alatum*; RA: Rheumatoid Arthritis; OA: Osteoarthritis; DMARDs: Disease-Modifying Anti-Rheumatic Drugs; NSAIDs: Non-Steroidal Anti-Inflammatory Agents, GRAS: Generally Recognized as Safe

Competing interests: Authors listed under Bordoloi Biotech LLC were responsible to prepare the Ayurvedic oils' emulsion cream samples. The rest of the authors carrying out the pre-clinical studies declare that they had no competing or financial interests.

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