Formulation and Evaluation of Novel Herbal Aerosol for Arthritis

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Abstract

Arthritis is the most common cause of disability in adults all over the world, limiting everyday activities for billions of people. Herbs have been paid more attention since they have very little side effects. *Cardiospermum halicacabum* Linn. (Sapindaceae) and *Vitexnegundo* Linn. (Verbenaceae) were used in traditional medicine for the treatment of arthritis. The present investigation was undertaken to evaluate anti-arthritic activity of novel herbal aerosol spray formulation containing methanol leaf extracts of *Cardiospermum halicacabum* (MLECH) and *Vitexnegundo* (MLEVN). A novel topical aerosol spray was formulated using MLECH and MLEVN (2% w/v each) and their quality control, stability and efficacy studies was carried out as per United States Pharmacopoeia (USP), International Conference on Harmonization (ICH) and Organization for Economic Co-Operation and Development (OECD) guidelines respectively. CFA induced arthritis method was employed for the evaluation of anti-arthritic activity. Assessment of body weight, biochemical parameters, hematological parameters, serum biomarker levels, photographic, radiographic analysis and histopathological investigations were carried out as a supportive evidence for the anti-arthritic efficacy of the developed novel herbal aerosol spray formulation. The results of the quality control test complied with USP standards and the stability study clearly revealed that the formulated topical herbal aerosol spray was found to be stable. Significant ($p < 0.01$) alterations in rat paw volume, body weight, biochemical, hematological, serum biomarker levels and reduction in inflammation and hyperplasia of synovium of formulation treated rats were observed compared with arthritic rats. Study concluded that the formulated novel herbal aerosol spray exhibited good anti-arthritic activity.

Keywords: CFA induced arthritis; Rheumatoid Factor; TNF α ; herbal aerosol

Introduction

Arthritis is a disease condition that affects the musculoskeletal system, causing painful inflammation and stiffness around joints. In Greek “arthro” means joint and “itis” means inflammation and so arthritis is a form of joint disorder that involves inflammation of joints. Among the 100 different forms of arthritis, the most common forms are osteoarthritis, Rheumatoid Arthritis (RA), psoriatic arthritis and related autoimmune disease,

septic arthritis caused mainly by joint infection. There is no specific drug available for the treatment of arthritis and hence pharmaceutical companies continuing their research for the discovery and development of a drug specifically for RA. However, steroidal, non-steroidal anti-inflammatory, Disease Modifying Antirheumatic Drugs (DMARDs) and immunosuppressant drugs are being most commonly prescribed for the treatment of RA. Though the goal of these drugs is to relieve pain and to decrease joint inflammation, to prevent joint destruction and to restore function of disabled joints, these drugs are known to produce adverse side effects [1]. Most notable adverse effects produced by NSAIDs are gastric erosions, ulceration, bleeding, perforation [2] and acute renal failure [3]. Serious and fatal liver toxicity was reported for DMARDs [4]. Therefore, reducing side effects should be considered while designing a formulation with improved therapeutics for RA. The Siddha and Ayurvedic systems of treatment are being increasingly recognized as an alternate approach to arthritic treatment. Besides, investigation of the efficacy of plant based drugs used in the traditional medicine has been paid more attention since they are cheap, readily available and have very little side effects. In view of this fact, an attempt was made to develop an alternative or traditional medicine for the treatment of arthritis by the herbs used in traditional practice. Since CH and VN were most commonly used in traditional practice for the treatment of arthritis, they were selected for the study.

Cardiospermum Halicacabum Linn (CH) is a climber belongs to the family Sapindaceae locally known as balloon vine and called as “modakathon” in Tamil. “Modaku” means crippling joint pain; “thon” means remedy. It is a deciduous, branched, herbaceous climber distributed throughout the plains of India. CH reported to contain arachidic acid, apigenin, apigenin-7-O-glucuronide, chysioeriol-7-O-glucuronide, luteolin, luteolin-7-O-glucuronide, saponin, quebrachitol, proanthocyanidin, beta sitosterol and stigmasterol [5]. Irulas in Thanjavur, Tamilnadu, India, provided evidence for the use of this plant as a traditional remedy that has been used for centuries to treat RA and it is still used by some locals to treat RA in Asian [6] communities, substantiated by many researchers [7-10]. Anti-inflammatory properties of CH have

been researched [11], but no clinical trials have been reported so far. Experimental pharmacological studies of CH have shown antioxidant activity [12] analgesic and vasodepressant activities [13], antipyretic activity [14], Antimalarial activity [15], diuretic activity [16], anti-diarrheal activity [17], anti-diabetic activity [18] and anti-ulcer activity [19].

Vitexnegundo Linn (VN) (Verbenaceae), grows gregariously in wastelands and is also planted as a hedge-plant. It was reported for the presence phytochemical constituents, flavonoids such as casticin, orientin, isoorientin, luteolin, lutecin-7-O-glucoside, corymbosin; gardenins A and B [20]. VN has been documented for potent anti-inflammatory [21], anti-arthritis [22], anticonvulsant [23], hepatoprotective [24], laxative [25] and bronchial relaxant activities [26]. Though all parts of CH and VN are used, leaves are majorly used for treating arthritis in traditional medicine [27] and hence a dosage form was designed using the leaf extracts of the plants.

The existing topical formulations available have the disadvantages viz. difficulty in applying the formulation to the respective site, staining cloth due to greasy nature, more chance of contamination and more wastage due to inaccuracy of dose. The designed herbal aerosol spray formulation have the advantages viz. ease of administration, cause no contamination, no clean up procedure after using the product, protection of active constituents from oxygen and moisture, minimum pain and irritation experience during application, minimum side effects as it is a herbal medicine, stable as the constituents are enclosed in a pressurized container, rapid onset of action, accuracy of the administered dose, no first pass effect and GIT degradation and can be delivered directly on to the affected area. Further, exhaustive work on CH and VN has been done for its biological activity and mechanism of action through systemic application. But there is no report on preclinical studies of CH and VN for its anti-arthritis activity by topical application. Thus, keeping all this in view, a topical herbal aerosol spray was developed using MLECH and MLEVN and evaluated for anti-arthritis activity.

Materials and Methods

Plant Materials and Chemicals

Both the plants CH and VN were collected in November 2010 from hilly area of Palakkad district, Kerala, India. Plants were authenticated by GVS. Murthy, Director of Botanical survey of India, Coimbatore, India and The Voucher specimens were deposited at KMCH College of Pharmacy, Coimbatore. Apigenin, carrageenan, Complete Freund's adjuvant (CFA), Diclofenac sodium and luteolin purchased from Sigma Aldrich, USA was used for study. Solvents were procured from Qualigens, India. Aerosol aluminum containers fitted with valve assembly and propellants were procured from Aspire industries, Mumbai, India.

Preparation of Extracts

Fresh mature leaves of CH and VN was cleaned and then shade dried at room temperature ($32 \pm 20^\circ\text{C}$) for 10 days, pulverized to coarse powder, passed through a # 40 mesh sieve and extracted separately with methanol at 660°C in a soxhlet apparatus for

72h. Each of the solvent extracts was filtered and concentrated separately under reduced pressure with IKA Rotary evaporator (Model No RN 10 digital V, ILMAC Germany) at 400°C .

Preparation of Topical Herbal Aerosol Spray Formulation

Aerosol dosage form was formulated using, the product concentrate (2% w/v of extract each of *Cardiospermum halicacabum* and *Vitexnegundo* containing 0.02% w/v of sodium benzoate and the propellant (mixture of propane and butane). The product concentrate was quantitatively packed to the cold sterilized aerosol container, the valve assembly was inserted into the container and crimped using semi automatic aerosol filling machine (Twin Tech, India). Then the propellant was filled into the container using automatic aerosol filling machine. Packed containers passed through a quality check for leakage followed by labeling and stamping of batch number and manufacturing date.

Quality Control of Topical Herbal Aerosol Spray Formulation

The quality control tests viz. flammability test, physico-chemical characterization, performance and biological evaluation for the finished topical herbal aerosol spray formulation were evaluated as per the procedure described in Lachman et al [28] and USP [29].

Stability Studies of Topical Herbal Aerosol Spray Formulation

The stability study for the topical herbal aerosol spray formulation was done as per ICH guidelines in a stability chamber for a period of 6 months. The selected topical herbal aerosol spray formulation consisting of 2% of each MECH and MEVN were loaded in a humidity chamber ($300 \times 300 \times 300$ mm, $15-60^\circ\text{C}$, Technico, India) at $25^\circ\text{C} \pm 2^\circ\text{C}/60\% \text{ RH} \pm 5\% \text{ RH}$, $32^\circ\text{C} \pm 2^\circ\text{C}/60\% \text{ RH} \pm 5\% \text{ RH}$ and $40^\circ\text{C} \pm 2^\circ\text{C}/75\% \text{ RH} \pm 5\% \text{ RH}$. Samples were withdrawn at an initial, first, second, third and sixth months and evaluated for change in color, odor, weight, pH, density, viscosity, net content, leakage test, microbial load and sterility test and the observations were recorded in Table 2.

Animals

Wistar strains rats of either sex were obtained from the animal house of Kovai medical center research and educational trust, Coimbatore, India. The animals were kept in polypropylene cages at a temperature of $25 \pm 2^\circ\text{C}$, humidity (50 ± 5) RH and 12 h light and dark cycles. They were fed with standard laboratory animal diet and water *ad libitum*. Animals were acclimatized to laboratory conditions before the test. Experiments were designed and conducted in accordance with ethical norms approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) and Institutional Animal Ethical Committee (KMCRET/DST/01/2011, dt.16/07/2011).

Anti-Arthritic Activity

Efficacy of the topical herbal aerosol spray formulation was

studied by CFA induced arthritis model [30] in rats through oral administration and topical application. The rats were divided into six groups, each consisting of six animals. Group 1 received (1 ml) 1% w/v of Sodium Lauryl Sulphate (SLS) was considered as normal control. Arthritis was induced to group 2 to 6 by injecting a 0.1 ml (0.1% w/v) suspension of killed *Mycobacterium tuberculosis bacteria* (Genei, Bangalore) homogenized in liquid paraffin into the left hind foot in the subplantar region of rats. Groups (2 to 6) administered with CFA were allowed to develop arthritis for 21 days. During the experimental period, body weight of the animals and the rat paw volume of control and treatment groups were measured on 4th, 8th, 14th and 21st day by using digital Vernier caliper (Miteto yodigimatic caliper, Japan).

The overnight fasted animals were anaesthetized with ketamine (20 mg/kg, i.p) followed by withdrawal of blood samples from retro-orbital sinus and the collected blood samples were evaluated for hematological parameters. A portion of the blood samples were centrifuged at 10000 rpm for 10 min. The separated serum was analyzed for C-reactive protein (CRP) and Rheumatoid Factor (RF). Photographic and radiographic analysis was carried out using ALLANS fully computerized X-ray instrument (AXD-100i, Mumbai, India) for the confirmation of development of arthritis.

After the confirmation of arthritis development, Diclofenac sodium 10 mg/kg (p.o.) and the combined doses of MLECH and MLEVN (400 mg/kg each) (p.o.) were administered orally from 22 to 42 days to Group 3 (served as reference standard for oral route) and Group 4 respectively. Topical Diclofenac Diethylamine

aerosol spray (8 mg/day) and topical herbal aerosol spray formulation (64 mg/day) were sprayed continuously for 3 sec from 22 to 42 days to the left knee joint region of Group 5 (served as reference standard for topical route) and Group 6 respectively. During the treatment period, the body weight of the animals and the rat paw volume of control and treatments were measured on 25th, 29th, 35th and 42nd day by using digital Vernier caliper (Miteto yodigimatic caliper, Japan). At the end of 42nd day the pain test score of the animals were recorded visually³¹. The overnight fasted animals were anaesthetized with ketamine (20 mg/kg, i.p) followed by withdrawal of blood samples from retro-orbital sinus and the collected blood samples were evaluated for hematological parameters [32]. A portion of the blood samples were centrifuged at 10000 rpm for 10 min. The separated serum was analyzed for urea, uric acid and serum biomarkers viz. CRP, RF (Omega diagnostics Limited, Scotland, UK), TNF α (ELISA Kit, Gen-Probe, France), IL-1 β and IL6 (ELISA Kit, R&D systems, USA). Biochemical investigations were carried out in a Autoanalyzer (Photometer 5010 V5+, Robert Riely, Berlin) using Piramal healthcare limited reagent kit.

Photographic and radiographic analysis was carried out at the end of the arthritis experiment using ALLANS fully computerized X-ray instrument (AXD-100i, Mumbai, India) for the assessment of reduction in arthritis (Figures 1A and 1B). The animals were sacrificed by cervical dislocation and the organs thymus, spleen and bone joints of ankle joint were isolated, weighed and [33] observed for histopathological changes under microscope and digital images were acquired [Figures 1A and 1B].

Figure 1B: Histopathological sections of ankle joint of (d) MLECH and MLEVN (400+400 mg/kg) treated rats (p.o.) (e) diclofenacdiethylamine topical aerosol spray treated rats (f) topical herbal aerosol spray formulation treated rats under 10 X and 40 X magnification.

Statistical Analysis

The results are expressed as the mean $\pm$ SEM. The significance of the difference was evaluated by one-way ANOVA followed by Dunnett's test. Data were considered statistically significant if $p < 0.05$.

Results

Quality Control Test for Topical Herbal Aerosol Spray Formulation

In flammability test the flame extended for 6 cm after spraying the formulation in open flame and flame extension test confirms that the product is not extremely flammable. The results of physico chemical characterization viz. vapour pressure, density, pH, viscosity, compatibility of product concentrates with

propellants, discharge rate, spray pattern, Net content, particle size, leakage test and spray angle complied with the standards of USP [Table 1]. No edema or redness of skin was observed in rabbits after topical administration of the aerosol spray formulation.

Stability Studies of Topical Herbal Aerosol Spray Formulation

There is no change in color, odour, pH, density, viscosity, net content and leakage of the topical herbal aerosol spray formulation were examined at 0,1,2,3 and 6 months [Table 2]. Thus the results of the study clearly revealed that the formulated topical aerosol is found to be stable.

Table 1: Quality Control test for topical herbal aerosol spray formulation

S.No		Parameters	Value	Units
01	Flammability	Flame Extension	6	cm
02	Physico chemical characteristics	Vapour Pressure	160	psig
		Density	0.851	g
		pH	4.20-4.33	--
		Viscosity	0.695	centipoise
		Compatibility of product concentrates with propellants	No turbidity was observed	
03	Performance	Aerosol valve discharge rate	0.284	g/3 sec
		Spray pattern	6	cm
		Net content	84	g
		Leakage test	No leakage was observed	
		Particle size range	13 -60	µm
		Total number of discharges per container	98 puff	3 sec for each puff
		Spray angle	21	°
04	Biological Evaluation	Skin irritation test	No edema or redness of skin observed	

Table 2: Stability studies of topical herbal aerosol spray formulation

S.N	Parameters	Topical herbal aerosol spray formulation containing 2% w/v of each MECH and MEVN															
		Storage condition															Study Time period
		Months					Months					Months					
		0	1	2	3	6	0	1	2	3	6	0	1	2	3	6	
1	Color	No change in color					No change in color					No change in color					Short term Six Months
2	Odour	No change in odour					No change in odour					No change in odour					
3	pH	4.22	4.22	4.22	4.22	4.21	4.22	4.20	4.20	4.19	4.18	4.22	4.16	4.10	4.08	4.02	
4	Density (g/cm³)	0.851	0.851	0.851	0.851	0.851	0.851	0.851	0.851	0.851	0.851	0.851	0.848	0.836	0.830	0.822	
5	Viscosity (centipoise)	0.695	0.695	0.695	0.695	0.695	0.695	0.694	0.692	0.690	0.688	0.695	0.674	0.638	0.614	0.596	
6	Net content (g)	84	84	84	84	84	84	84	84	84	84	84	84	84	84	84	
7	Leakage test	No leakage observed					No leakage observed					No leakage observed					
8	Microbial load (Bacteria & Fungi)	No microbial growth was observed at 24, 48 and 72 h					No microbial growth was observed at 24, 48 and 72 h					No microbial growth was observed at 24, 48 and 72 h					
9	Sterility test	No microbial growth was observed at 24, 48 and 72 h					No microbial growth was observed at 24, 48 and 72 h					No microbial growth was observed at 24, 48 and 72 h					

Anti-Arthritic Activity

Body Weight

The average gain/reduction in body weight was observed for all the groups after induction of arthritis in rats. Reduction in body weight was observed for arthritic control group whereas gain in body weight was observed in Diclofenac sodium treated (p.o.), MLECH and MLEVN treated groups, Diclofenac aerosol spray treated groups and topical herbal aerosol spray formulation treated groups, when compared with the normal group [Table 3].

Paw Volume

Alteration in rat paw volume was recorded on 25th, 29th, 35th and 42nd days in Table 4. The arthritic control groups showed signs of arthritis development as seen by increase in paw volume. Significant ($p < 0.01$) reduction in rat paw volume was observed in Diclofenac sodium (p.o.), MLECH and MLEVN (p.o.), Diclofenac aerosol spray and herbal aerosol spray formulation treated groups, on 21st day after CFA induction [Table 4].

Table 3: Effect of MLECH, MLEVN and formulations on body weight changes in CFA Induced arthritic rats.

Groups	Initial body weight (g)	Body wt after 22 days of CFA induction	Body wt after treatment 25 th day	Body wt after treatment 29 th day	Body wt after treatment 35 th day	Body wt after treatment 42 nd day	Weight gain (g)
Normal Control (1% w/v SLS)	146.26 ± 1.47	174.16 ± 1.53	171.50 ± 0.84	175.66 ± 0.88	182.00 ± 1.03	194.16 ± 0.98	47.91 ± 1.78
Arthritic control	143.45 ± 1.22	158.30 ± 1.25	160.50 ± 1.25	161.66 ± 1.08	163.16 ± 0.94	164.33 ± 1.17	20.88 ± 2.15
Diclofenac sodium 10 mg/kg (p.o.)	148.53 ± 0.68	160.66 ± 0.84 ^b	170.00 ± 0.96 ^b	173.33 ± 1.62 ^b	180.5 ± 0.56 ^b	186.33 ± 0.61 ^b	38.30 ± 1.08 ^b
MLECH 400 mg/kg & MLEVN 400 mg/kg (p.o.)	146.26 ± 1.47	155.00 ± 1.06 ^b	169.50 ± 0.42 ^b	172.83 ± 0.30 ^b	174.5 ± 1.11 ^b	184.33 ± 0.91 ^b	37.4 ± 1.54 ^b
Diclofenac topical aerosol spray (8 mg/day)	144.43 ± 1.81	154.50 ± 1.28 ^b	174.33 ± 1.66 ^b	177.83 ± 1.49 ^b	188.33 ± 0.88 ^b	193.50 ± 0.61 ^b	51.6 ± 1.14 ^b
Topical herbal aerosol spray formulation (64 mg/day)	143.16 ± 1.01	154.60 ± 1.17 ^b	171.00 ± 0.73 ^b	176.80 ± 1.42 ^b	184.16 ± 1.60 ^b	187.00 ± 1.13 ^b	42.56 ± 2.01 ^b

Data provided as mean ± SEM (n=6); b $P < 0.01$ treated groups vs. arthritic control

Table 4: Evaluation of anti-arthritic activity of MLECH, MLEVN and formulations in CFA induced arthritic rats

Groups	Dose	Rat paw volume (mm)					
		Before treatment			After treatment		
		Initial	After 21 days	25 th day	29 th day	35 th day	42 nd day
Normal control	1% w/v SLS (5 ml/kg)	4.91±0.19	5.26± 0.23	4.90 ± 0.31	5.25±0.17	5.39 ± 0.13	5.52 ± 0.16
Arthritic control	1% w/v SLS (5 ml/kg)	4.98± 0.30	10.48± 0.26	10.75 ± 0.10	10.26 ± 0.08	10.41 ± 0.07	10.42 ± 0.10
Diclofenac sodium (p.o.)	10 mg/kg	5.33 ± 0.16	10.48± 0.22	9.36 ± 0.07 ^b	8.93 ± 0.09 ^b	7.71 ± 0.26	6.91 ± 0.32 ^b
MLECH + MLEVN	400 + 400 mg/kg	4.94± 0.17	10.62± 0.33	9.66 ± 0.07 ^b	8.04 ± 0.21 ^b	7.59 ± 0.18 ^b	7.30 ± 0.16 ^b
Diclofenac topical aerosol spray	8 mg/day	5.05± 0.14	10.41± 0.21	9.72 ± 0.06 ^b	8.84 ± 0.15 ^b	7.71 ± 0.15 ^b	7.55 ± 0.17 ^b
Topical herbal aerosol spray formulation	64 mg/day	4.87± 0.18	10.39± 0.25	9.98 ± 0.15 ^a	7.91 ± 0.34 ^b	7.87 ± 0.16 ^b	6.91 ± 0.24 ^b

Data provided as mean ± SEM (n=6).

^ap < 0.05, treatment groups Vs arthritic control

^bp < 0.01, treatment groups Vs arthritic control

Arthritic Score

The severity of arthritis was assessed by visual arthritic scoring systems described by Laird et al [31]. The arthritic test scores were assigned as shown in Table 5 revealed that the pain associated with CFA induced arthritis was significantly decreased in Diclofenac, MLECH & MLEVN and topical herbal aerosol spray formulation treated groups. [Table 5]

Hematological Parameters

Rats treated with Diclofenac sodium (p.o.), MLECH and MLEVN, Diclofenac aerosol spray and herbal aerosol spray formulation elicited decrease in WBC count, ESR and increase in Hb count and RBC count, when compared with arthritic control groups [Table 6].

Biochemical Parameters

Significant ($p < 0.01$) decrease in urea and uric acid concentration was observed in Diclofenac sodium treated (p.o.), MLECH and MLEVN treated groups, Diclofenac aerosol spray treated groups and herbal aerosol spray formulation treated groups, when compared with the arthritic control group [Table 6].

Serum Biomarkers

In Vitro Determination of CRP

The serum of the tested animal groups before treatment showed agglutination and no agglutination was observed for the serum of groups treated with Diclofenac sodium (p.o.), MLECH and MLEVN (p.o.), Diclofenac topical aerosol spray and topical herbal aerosol spray formulation.

In Vitro Determination of Rheumatic Factor

RF is the most useful prognostic marker for the diagnosis of rheumatoid arthritis, agglutination is used because greater sensitivity and simplicity. No agglutination was observed in the serum of the tested animal groups the treatment of Diclofenac sodium (p.o.), MLECH and MLEVN (p.o.), Diclofenac topical aerosol spray and topical herbal aerosol spray formulation.

In Vitro Determination of TNF- α

TNF α level in arthritic induced rats was found to be 44.90 pg/ml. The TNF α level of Diclofenac treated (p.o.), MLECH and MLEVN treated, Diclofenac topical aerosol spray treated and topical herbal aerosol spray formulation treated groups found to be less than the arthritic induced rats. Thus this study revealed that Diclofenac treated (p.o.), MLECH and MLEVN treated, Diclofenac topical spray treated and topical herbal aerosol spray formulation treated groups reduced the levels of TNF α [Table 7].

Table 5: Alterations in various pains test scores in CFA Induced arthritis in rats

Groups	Pain test		Mobility score	Stance score
	Extension	Flexion		
Arthritic control	9.66 $\pm$ 0.21	8.50 $\pm$ 0.34	1.16 $\pm$ 0.16	1.33 $\pm$ 0.21
Diclofenac 10 mg/kg p.o.	4.33 $\pm$ 0.21 ^b	3.83 $\pm$ 0.16 ^b	2.83 $\pm$ 0.16 ^b	3.16 $\pm$ 0.16 ^b
MLECH 400 + MLEVN 400 p.o.	6.16 $\pm$ 0.16 ^b	4.16 $\pm$ 0.16 ^b	2.16 $\pm$ 0.16 ^a	2.50 $\pm$ 0.22 ^b
Diclofenac topical aerosol spray (8 mg/day)	4.50 $\pm$ 0.22 ^b	3.50 $\pm$ 0.34 ^b	3.00 $\pm$ 0.36 ^b	2.83 $\pm$ 0.16 ^b
Topical herbal aerosol spray formulation (64 mg/day)	5.50 $\pm$ 0.22 ^b	4.33 $\pm$ 0.21 ^b	2.33 $\pm$ 0.33 ^a	2.10 $\pm$ 0.16 ^b

Data provided as mean $\pm$ SEM (n=6);
^a $P < 0.05$ treated groups vs. arthritic control;
^b $P < 0.01$ treated groups vs. arthritic control

Table 6: Effect of MLECH, MLEVN and formulations on hematological and biochemical parameters in CFA induced arthritic rats

Groups	Hb (mg %)		WBC ($\times 10^3/\text{mm}^3$)		RBC ($\times 10^6/\text{mm}^3$)		ESR (mm/h)		Before treatment		After treatment	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment	Urea (mg/dl)	Uric acid (mg/dl)	Urea (mg/dl)	Uric acid (mg/dl)
Normal control	15.56 $\pm$ 0.27	16.13 $\pm$ 0.15	6.61 $\pm$ 0.27	6.78 $\pm$ 0.06	9.71 $\pm$ 0.22	10.26 $\pm$ 0.15	4.83 $\pm$ 1.47	5.00 $\pm$ 0.63	16.16 $\pm$ 0.91	2.66 $\pm$ 0.09	16.60 $\pm$ 0.91	2.86 $\pm$ 0.07
Arthritic control	10.9 $\pm$ 0.17 ^a	10.26 $\pm$ 0.10	11.78 $\pm$ 0.32 ^a	10.2 $\pm$ 0.2	7.66 $\pm$ 0.30 ^a	5.67 $\pm$ 0.20	36.16 $\pm$ 1.19 ^a	40.33 $\pm$ 1.43	44.35 $\pm$ 1.50 ^a	6.96 $\pm$ 0.16 ^a	44.8 $\pm$ 3.62	7.15 $\pm$ 0.13
Diclofenac sodium (10 mg/kg)	11.01 $\pm$ 0.03 ^a	15.91 $\pm$ 0.21 ^b	11.95 $\pm$ 0.35 ^a	7.53 $\pm$ 0.17 ^b	7.23 $\pm$ 0.19 ^a	9.01 $\pm$ 0.23 ^b	33.5 $\pm$ 1.17 ^a	13.66 $\pm$ 1.11 ^b	43.75 $\pm$ 1.49 ^a	7.20 $\pm$ 0.11 ^a	25.93 $\pm$ 1.25 ^b	3.43 $\pm$ 0.16 ^b

MLECH 400 mg/kg & MLEVN 400 mg/kg	10.73±0.16 ^a	15.48±0.26 ^b	12.68±0.47 ^a	8.76±0.06 ^b	7.71±0.22 ^a	9.20±0.12 ^b	34.33±1.28 ^a	28.66±1.08 ^b	43.88±1.41 ^a	7.13±0.16 ^a	32.56±1.79 ^b	4.46±0.10 ^b
Diclofenac topical aerosol spray 8 mg/day	11.43±0.20 ^a	15.43±0.19 ^b	11.65±0.29 ^a	7.81±0.07 ^b	7.32±0.18 ^a	9.23±0.19 ^b	35.33±1.64 ^a	24.83±0.79 ^b	43.75±0.65 ^a	7.35±0.13 ^b	27.25±0.60 ^b	3.36±0.10 ^b
Topical herbal aerosol spray formulation 64 mg/day	11.41±0.21 ^a	15.11±0.66 ^b	11.86±0.37 ^a	8.26±0.04 ^b	7.48±0.07 ^a	9.81±0.25 ^b	36.33±0.61 ^a	22.66±0.66 ^b	44.25±1.31 ^a	6.90±0.12 ^a	31.16±0.84 ^b	4.41±0.09 ^b

Data provided as mean ± SEM (n=6);

^ap<0.01 arthritic control Vs normal control^bp<0.01 treated groups Vs arthritic control**Table 7:** *In vitro* determination of serum TNFα and interleukin levels in CFA induced arthritic rats

Groups	TNFα (pg/ml)	Interleukin levels (pico g/ml of serum)	
		IL-1β	IL-6
Normal control	1.36 ± 0.23	1.38 ± 0.24	19.58 ± 1.14
Arthritic control	44.90 ± 2.36 ^a	67.70 ± 1.09 ^a	728.61 ± 31.99 ^a
Diclofenac sodium 10 mg/kg (p.o.)	2.36 ± 0.45 ^b	23.34 ± 1.34 ^b	323.33 ± 16.55 ^b
MLECH 400 mg/kg + MLEVN 400 mg/kg (p.o.)	29.04 ± 0.63 ^b	45.11 ± 1.08 ^b	512.72 ± 24.87 ^b
Diclofenac topical aerosol spray (8 mg/day)	6.28 ± 0.71 ^b	20.99±0.60 ^b	313.95±14.66 ^b
Topical herbal aerosol spray formulation (64 mg/day)	19.79 ± 0.81 ^b	36.55 ± 1.25 ^b	428.12 ± 10.17 ^b

All the values are mean ± SEM (n=6).

^ap<0.01 arthritic control Vs normal control^bp<0.01, treatment groups Vs arthritic control

In Vitro Determination Of IL 1β And IL 6

Cytokines, IL 1β and IL 6 play a key role in mediating both acute and chronic inflammation and hence *In vitro* determination of IL-1β and IL-6 was determined for the serum of all the groups used in the arthritic experiment (Table 7). The cytokine levels was found to be increased in arthritic control, which was significantly (p<0.01) reduced in Diclofenac sodium treated (p.o.), MLECH and MLEVN treated groups, Diclofenac aerosol spray treated groups and herbal aerosol spray formulation treated groups.

Photographic and Radiographic Analysis

Representative photographs and radiographs (Figures 1A and 1B) of tarsotibial joint swelling of the left hind paws of rats from different groups were taken on 42nd day. The soft tissue swelling around the joints observed in the arthritic control rats was significantly reduced in the Diclofenac sodium treated (p.o.), MLECH and MLEVN treated, Diclofenac topical aerosol spray treated and topical herbal aerosol spray formulation treated groups. [Figures 2A and 2B]

Spleen and Thymus Weight

The weights of spleen and thymus recorded after sacrificing

the rats on 42nd day. Significant (p<0.01) reduction of spleen and thymus weight was observed for the Diclofenac sodium treated (p.o.), MLECH and MLEVN treated groups, Diclofenac aerosol spray treated groups and herbal aerosol spray formulation treated groups when compared with the arthritic control group.

Histopathological Studies

Histological examination of normal specimen of joint showed normal joint space, normal adjacent soft tissue and synovium and cartilage. CFA induced specimen of joint, showed adjacent soft tissue and synovial hyperplasia with dense inflammation in the soft tissue around the joint. Diclofenac (p.o.) treated specimen joint exhibited occasional inflammatory cells & hyperplasia of the synovium when compared with CFA treated groups. Specimen of joint, orally treated with MLECH, MLEVN showed mild inflammation & hyperplasia of the synovium when compared with CFA treated groups and Diclofenac (p.o.) treated groups. Specimen of joint of Diclofenac topical aerosol spray treated groups showed moderate inflammation & hyperplasia of the synovium. Specimen of joint of topical herbal aerosol spray formulation treated groups showed occasional inflammatory cells and hyperplasia of the synovium when compared with CFA and Diclofenac aerosol spray treated groups.

Normal control (a)

Normal control showing no soft
Tissue swelling around the joints (a)

CFA Control (b)

CFA Control showing soft tissue swelling
around the joints (b) CFA and diclofenac
Treated (p.o) (c) CFA and diclofenac
Treated (p.o) (c)

Figure 2A: (a) Photographic and radiographic analysis of normal rats. (b) Photographic and radiographic analysis of the same left hind paw taken 21 days after CFA injection (c) Photographic and radiographic analysis of the same left paw of diclofenac treated (p.o.) taken 21 days after CFA injection

Discussion

Cardiospermum halicacabum and *Vitexnegundo* individually reported to produce beneficial effects on RA [34-35]. The phytoconstituents, luteolin and apigenin were reported to be present in leaf extract of CH and VN and they were isolated by Srinivas et al [5] and Achari et al [20]. Further these flavonoid aglycones reported to be present in these plants can penetrate the human skin [36] and exhibited in vivo anti-inflammatory activity via topical and oral routes of administration [37]. The above findings, prompted us to formulate the topical aerosol spray using MLECH and MLEVN for the treatment of arthritis. Aluminium containers were used for packing the aerosol formulation as they withstand pressure up to 180 psig, temperature up to 130°F, less cost, light weight, compatible with the active ingredients of the formulation, greater resistance to corrosion and it is good for light sensitive drugs, Mixture of hydrocarbons viz. propane and butane

was used as propellant as they are less toxic, less reactive, less susceptible to hydrolysis and their environmental acceptance. After filling the product concentrate the containers were heated to 130°F and tested for leakage and labeled.

Quality control test performed for topical herbal aerosol spray formulation complied with USP standards and the stability study performed for 6 months for the formulated topical herbal aerosol spray was found to be stable and complied with ICH guidelines.

In our earlier acute oral toxicity study, carried out separately for MLECH and MLEVN was found to be safe upto a dose of 2000 mg/kg and hence 1/20th, 1/10th and 1/5th dose of 2000 mg/kg viz. 100, 200, 400 mg/kg were used for anti-arthritis activity study. It was also observed from our earlier study, that the combination (100 + 100, 200 + 200, 400 + 400 mg/kg) of MLECH and MLEVN showed significant inhibition of inflammation in a dose dependent manner than the individually administered

CFA and MLECH + MLEVN treated (p.o) (d)

CFA and MLECH + MLEVN treated (p.o) showing no soft tissue swelling around the joints (d)

CFA and diclofenac aerosol treated (e)

CFA and diclofenac aerosol treated showing no soft tissue swelling around the joints (d) (e)

CFA and herbal aerosol formulation treated (f)

CFA and herbal aerosol formulation treated showing no soft tissue swelling around the joints (d) (f)

Figure 2B: (d) Photographic and radiographic analysis of the same left hind paw of MLECH and MLEVN treated (p.o.) taken 21 days after CFA injection (e) Photographic and radiographic analysis of the same left hind paw of diclofenac topical aerosol spray treated taken 21 days after CFA injection (f) Photograph and radiographic analysis of the same left hind paw of Topical herbal aerosol spray formulation taken 21 days after CFA injection.

doses of MLECH (100, 200, 400 mg/kg) and MLEVN (100, 200, 400 mg/kg). Based on these observations, each 400 mg/kg dose of MLECH and MLEVN was used for anti-arthritis study.

CFA-induced arthritis is the most widely used chronic test model which is associated with an immune-mediated inflammatory reaction and rat is unique in developing polyarthritis after CFA treatment and hence they were used for the study [38]. In first phase, irritant nature of the adjuvant induces inflammation and hence measurement of 3 to 5 day effect of treatment represents the efficacy on inflammation of primary lesions such as edema and soft-tissue thickening. The second phase, reduction in body weight due to immunogenic responses [39].

In the present study, administration of Diclofenac sodium, MLECH and MLEVN (400 +400 mg/kg, p.o.), topical Diclofenac

topical aerosol spray and topical herbal aerosol spray formulation in rats significantly reduced the progression of RA when compared with arthritic rats. Significant alterations in flexion pain test score, mobility score and stance score was observed for all the treated group of rats when compared with arthritic control rats. This alteration of arthritic test scores support the anti-arthritis activity of the MLECH & MLEVN and topical herbal aerosol spray formulation.

Information related to the pathology of arthritis was obtained during the study from body weight changes and hematological parameters. Restoration of body weight of rats was observed after treatment of MLECH and MLEVN and topical herbal aerosol spray formulation. Decrease in body weight during inflammation in arthritic control group was reported [40] due to deficient absorption of nutrients through intestine. It was reported that

treatment of anti-inflammatory drugs normalizes the process of absorption and hence the restoration of body weight in MLECH, MLEVN and Diclofenac sodium treated groups may involve improvement of intestinal absorption of nutrients and reduction in the distress caused by the severity of arthritis. It has been reported that a moderate rise in the WBC count occurs in arthritic conditions due to an IL-1B mediated rise in the respective colony stimulating factors. The present study reveals that MLECH, MLEVN and Diclofenac sodium treatments tend to normalize the WBC count. Characteristic hematological alterations such as increased Hb count and decreased ESR by MLECH and MLEVN support the anti-arthritic effect. It is proposed that the reduction in the Hb count during arthritis results from reduced erythropoietin levels, a decreased response of the bone marrow erythropoietin and premature destruction of red blood cells and increase in ESR is due to formation of endogenous protein, globulin and fibrinogen [41]. Increase in serum levels of CRP, RF37 TNF- α , IL1 β and IL6 are characteristic feature of RA [42] and hence, in vitro determination of serum biomarkers such as CRP, RF, TNF- α , IL1 β and IL6 were performed for the arthritic control and all the treated groups. Significant reduction of serum biomarker levels in treated rats when compared with arthritic control rats support the anti-arthritic activity.

Radiological observations of the rats provided the evidence for the inhibition of arthritis after treatment of Diclofenac sodium p.o, MLECH and MLEVN (400 +400 mg/kg, p.o.), topical Diclofenac topical aerosol spray and topical herbal aerosol spray formulation.

Synovial Inflammation and hyperplasia is considered to be the important pathological condition of rheumatoid arthritis [43]. Histological examination of joint showed significant reduction of inflammation in treated group of rats when compared with arthritic control rats.

Significant reduction in thymus and spleen weight of rats after treatment with MLECH & MLEVN and topical herbal aerosol spray formulation support the anti-arthritic activity.

Conclusion

Thus from our study results, we conclude that the developed topical herbal aerosol spray formulation elicited anti-arthritic activity which may be due to the presence of luteolin and apigenin in MLECH and MLEVN respectively.

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Declaration of interest

The authors report no declaration of interest.

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