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ANTI-INFLAMMATORY EFFECT OF AN AYURVEDA POLYHERBAL TOPICAL APPLICATION IN EXPERIMENTAL ARTHRITIS

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ABSTRACT

Background: Topical application of herbs is one of the recommended treatment modalities for Osteoarthritis (OA) in Ayurveda (Indian system of medicine). The current study intended to evaluate the anti-inflammatory effect of a polyherbal powder used as upanaha (poultice) for OA by in vitro and in vivo techniques. **Materials and methods:** The polyherbal formulation Upanahachoornam (UC) was sourced from Vaidyaratnam Oushadhasala (P) Ltd., Thrissur, India. Changes in the secretion of TNF-α and NO and expression of Cox-2 genes were evaluated by semi quantitative PCR activity to establish anti-inflammatory action in vitro. Macrophages and connective tissue of mice were used as media for the former two experiments and only macrophages for the latter. In vivo anti–inflammatory activity was evaluated by TPA induced ear oedema in Swiss Albino mice (n=24), divided into 4 groups as Group I – saline treatment, Group II – Indomethacin treatment, and Groups III and IV treated with 30% and 60% of UC respectively. **Results:** In the in vitro study, UC at 1000 µg/ml and 500 µg/ml upregulated the COX-2 level by 0.08 and 0.03 folds respectively as compared to control. Release of TNF-α, and NO in LPS-induced RAW cells were significantly inhibited in a dose dependent manner. The TPA induced ear oedema significantly reduced in Groups III and IV ($F=1250$, $p<0.001$) **Conclusion:** The current study demonstrates the safety and anti-inflammatory activity of a polyherbal formulation Upanahachoornam as a topical application. This indicates the potential of select herbs in managing degenerative conditions like OA.

Keywords: polyherbal, upanaha, Indomethacin, TNF-α, Cox-2

INTRODUCTION

Chronic degenerative conditions like Osteoarthritis (OA) have a negative impact on the quality of life of an individual and more often than not require long term treatment and management [1]. Worldwide, OA is regarded as the 11th most frequent cause of disability and in India, a prevalence of 17 to 60.6% has been reported [2,3]. Inflammation is an integral part of the disease process in OA necessitating the use of non-steroidal anti-inflammatory drugs (NSAIDs) frequently to manage the symptoms [4,5]. Prolonged use of NSAIDs however can adversely affect the liver, kidney, gut, skin and cardiovascular system and hence, has to be prescribed with extreme caution [6]. As the disease affects the ageing population, co-morbidities like hypertension, diabetes mellitus, cardiac disease etc. also prevent optimal prescription of oral medication. Hence safer and effective topical applications need to be explored as alternatives. Ayurveda (Indian system of medicine) emphasises three major lines of treatment viz, internal medications (*antahparimarjana*), topical applications (*bahirparimarjana*) and surgical/para-surgical procedures (*shastrapranidhana*). Osteoarthritis is regarded as *sandhigatavata*, a chronic degenerative condition associated with ageing. Among the different modalities of managing this disease, topical application of herbs in the form of paste (*kalka*), powder (*churna*), oil (*taila*) are widely prescribed [7]. Though there are few studies that have explored the potential of Ayurveda interventions in OA, most of them are restricted to evaluating the effect of oral medication. Herbs like *Tribulusterrestris* Linn. *(gokshura), Tinosporacordifolia* Hook F & Thoms. *(guduchi), Zingiberofficinale* Rosc. *(shunti), Emblicaofficinalis* Gaertn. *(amalaki), Boswelliaserrata* Roxh. *(shallaki), Withaniasomnifera*Linn. *(ashwagandha)* have been demonstrated to be safe and effective in ameliorating OA symptoms. These herbs have shown activity equivalent to that of standard care drugs like glucosamine sulphate [8-11].

Upanahachoornam (UC) tested in the current study was sourced from VaidyaratnamOusadhasala, (P) Ltd., Thrissur, India. It is a polyherbal formulation, referenced from *Ashtangahridaya*, commonly prescribed for topical application in *sandhigatavata*[12]. The powder is usually mixed in hot water and applied over the joint region as a poultice. It consists of herbs *Acoruscalamus* Linn. (*vacha*), *Anethumsowa*Roxb. (*shatahwa*), *Cedrusdeodara*Roxb. Ex.lamb (*devadaru*), *Pluchealanceolata* DC Heirn /Alpania galangal Lance. (*rasna*), *Ricinuscommunis* Linn. (*eranda), Nardostachysjatamansi* DC Heirn (*jata*), Rock salt (*saindhavalavana*), grains like *Sesamumindica* Linn. (*tila), Brassica campestris* Linn. (*sarshapa*), *Dolichusbiflorus* Linn. (*kulattha*), *Phaseolusmungo*Linn. (*mudga*), *Phaseolus vulgaris* Linn. (*masha*), *Oryzasativa* Linn. (*shashtika*), *Panicumsumatrense*-Roth ex Roem. &Schult. (*shyamaka), Aquillariaagallocha*Roxh (*agaru*), *Santalum alba* Linn. (*hima*), *Coleus vettiveroides* KC Jacob. (*ambu*), *Sausserrialappa* CB Cl (*gada*), *Vettiveriazizinoides* Linn. (*usheera*). A cursory review of topical herbal therapies for OA details Arnica gel, capsicum extract gel, comfrey extract gel and an adhesive patch of Chinese

herbal mixture [13]. An in vitro study of oral and topical application of boswellic acid showed reduction in cartilage loss, synovitis and osteophyte formation [14]. Though topical applications are greatly used in Ayurveda for the management of OA, minimal data is currently available to demonstrate its effectiveness and also indicating the molecular and cellular mechanisms of their action.

With this background, the anti-inflammatory effect of UCwas tested in rats induced with arthritis along with evaluating its activity on the expression of inflammatory markers.

MATERIALS AND METHODS:

The polyherbal formulation UC was sourced from Vaidyaratnam Oushadhasala (P) Ltd., Thrissur, India, Batch no. 3843, manufactured in March 2015. The in vitro and in vivo study was conducted at Radiant Research Services Pvt. Ltd, Bangalore, India, following their Standard Operating Procedure.

1.1 In vitro studies: The test systems used for in vitro evaluation were RAW 264.7 (macrophages of mouse) and L929 (connective tissue of mouse). The test product UC was stored under normal room temperature, coded as RR2706 in the records.

1.1.1 Preparation of Test Doses: For studies, each weighed UC were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

1.1.2 Determination of Cytotoxicity in Raw and L929 cell lines [15]

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of UC was added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^o C in 5% CO_2 atmosphere. After 24 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^o C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated and concentration of UC needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

2.1.3 In vitro TNF-α inhibitory activity of extracts [16]

Step I: Induction of TNF-α in RAW cells: RAW cells seeded in to 6 well culture dishes at a cell population 1.5 to 2x10^5 cells/ml in DMEM with 10% FBS. After 24 h, the cells were treated with known non-toxic concentration of test extracts along with 1µg/ml of lipopolysaccharide (LPS) and incubated at 37^{0}C with 5% $CO_2$for 4 h. After

incubation, the cell supernatant was collected, centrifuged, separated and stored at -20^0 C till use.

Step II: Estimation of TNF-α in cell supernatant by bioassay: L929 monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using MEM medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of diluted samples from the step I were added to the cells in quadruplicate wells. The cultures were then incubated at 37°C for 24 h in 5% CO_2 atmosphere. After 24h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilise the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm and the percentage cell viability was calculated. The cell viability is direct indication of inhibitory properties of extracts against LPS induced TNF production in RAW cells.

2.1.4Nitrite determination (Nitric oxide inhibition): RAW cells were treated with LPS and test samples as described above and incubated for 24 h and conditioned media collected were used for nitrite determination. Determination of nitrite as a biomarker for NO was carried out. In brief, equal volume (50 µL) of 0.1% N-1-napthylethylenediamine dihydrochloride prepared in water, 1% sulfanilamide prepared in 5% phosphoric acid and cell culture media were mixed in flat bottom 96-well plate incubated for 10–15 min. Colored end product was measured at 530 nm. Percentage of nitric oxide inhibition was calculated over LPS control

2.1.5 RT-PCR Procedure: The mRNA expression levels of COX 2 carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, the RAW cells were cultured in 60 mm petridish and maintained in DMEM medium for 48 hrs. The DMEM medium was supplemented with FBS and Amphotericin. To the dish was added the required concentration of Test sample (1000 and 500 µg/ml) and incubated for 24 hr. Total cellular RNA was isolated from the untreated (control) and treated cells using Tri Reagent according to manufacturer's protocol. cDNA was synthesized from total isolated RNA by reverse transcriptase kit according to manufacturer's instructions. Then 50µl of the reaction mixture was subjected to PCR for amplification of COX 2 cDNAs using specifically designed primers procured from Eurofins India, as an internal control, the house keeping gene GAPDH was co-amplified with each reaction.

2.2 In vivo study: The in vivo study was performed in Swiss Albino mice (n=24) by TPA induced ear oedema. The study was approved by committee for the purpose of control and supervision of experiments on animals (CPCSEA) and Institutional Animal Ethics Committee (IAEC) with registration number 1803/PO/RcBiS/2015/CPCSEA. The mice were randomly grouped into 4 groups with 6 mice in each, weighing 25 to 30 gm accommodated in standard polycarbonate cages under standard laboratory conditions. The animals were acclimatised using laboratory con-

ditions for 5 days, 7 days and 9 days respectively for step I, step II and step III respectively after veterinary examination. Only the animals without any physical signs of illness were selected. The mode of application of the UC was topical. The animals were allocated as: Group I was Control group received normal saline only, Group II was treatment group treated with STD (Indomethacin), Group III was Treatment group treated with UC (30% of UC) and Group IV was treatment group treated with UC (60% of UC). The left ear was considered as contra lateral control and received only saline solution. Inflammation was induced to the ear of mice by applying TPA (12-O-tetradecanoyl-phorbol-13-acetate) (2.5 µg/ear) and Xylene (20 µL/ear) on the inner and outer surfaces of the right ear and left undisturbed for 30 mins. The ear oedema was evaluated at 0, 30, 60, 120, 180, 240 and 300 mins after TPA application. After 5 hours of the treatment, mice were subjected to CO_2 euthanasia and sacrificed by cervical dislocation and each ear (8mm diameter) biopsies was done and weighed immediately. Oedema was measured in terms of weight difference of the ears and the ear thickness was measured before and after induction of the inflammatory response using a digital micrometer.

RESULTS

3.1 In vitro study: UC showed moderate cytotoxicity, on the basis of CTC_{50} value the concentration of the UC in RAW cell line. The cytotoxicity activity against the RAW cells and L929 cells increased with increasing dose (Tables 1-2). The test product showed dose dependent anti-inflammatory activity with moderate TNF-alpha inhibition, nitric oxide inhibition properties (Tables 3-4). In gene expression study, UC showed regulation on the levels of COX-2 expression. UC i.e. 1000 µg/ml and 500 µg/ml up regulate the COX-2 level by 0.08 fold and 0.03 as compare to control (Figure 1).

3.2 In vivo study: UC decreased the TPA induced ear oedema during the observation period when compared to control group ($F=1250$, $p<0.001$) (Figure 2). The inhibition was found to be 52.3 and 61.9 % at 300 mins, respectively (Table 5).

DISCUSSION

Osteoarthritis is a joint disorder associated with chronic inflammation. Targeting the inflammation is often the initial step for effective management of the condition. In the current study the anti-inflammatory potential of an Ayurveda polyherbal formulation used for topical application was evaluated. Both in vitro and in vivo experimental protocols were followed and significant effects were obtained. Considering that oral medications have their limitations, there is scope for exploring the potential of herbal topical applications.

Different herbs and formulations have been studied for their anti-inflammatory effect in animal models and clinical settings; though, mostly for Rheumatoid arthritis (RA) [17]. The mode of administration in most of the studies on OA has been oral. To the best of our knowledge, there are very few studies that have looked in to the utilitarian aspect of topical applications for inflammatory conditions

like OA. The latest recommendations too indicate towards the positive outcomes of topical applications [18]. The mechanism of action by which these provide analgesia is proposed to be similar to oral medications, albeit with a better safety profile [1]. Topical NSAIDs have produced fewer systemic adverse events and also shown effect in OA as per a Cochrane review [19]. Though even in Ayurveda pharmacopoeia, topical applications are widely used for *sandhigatavata* vis-à-vis OA, there are very few studies that have delved into the mechanisms by which these medications work. Singh and colleagues reported the anti-inflammatory activity of Boswellic acids when applied topically in models of acute and chronic inflammation [20]. Another in vitro study of a polyherbal formulation containing *Z. officinalis*, *T. cordifolia*, *E.officinalis* and *B.serrata* on human knee cartilage has shown significant reduction of breakdown products glycosaminoglycan and aggrecan[21]. The chondroprotective effect and regenerative ability of *Cissusquandrangularis* Linn. (*asthishrinkala*) has also been demonstrated in human chondrocytes. The effect is attributed to the inhibition of the p38MAPK signalling pathway by the upregulation of *survivin*[22]. A polyherbal compound BV-9238 containing *W. somnifera*, *B. serrata*, *Z. officinale* and *C. longa* inhibited TNF-α and nitric oxide (NO) production in RAW cell lines [23]. Curcumin, by virtue of itsanti-apoptotic and anti-catabolic effects on IL-1beta-stimulated articular chondrocytes, is also considered as a potent remedy in OA [24]. It also inhibits COX-2 activity [25].

In clinical settings too, herbal topical applications have shown reduction in pain and stiffness, and improvement in mobility. A cream containing *Trigonellafoenum-graceu* Linn. (fenugreek), *Curcuma longa* Linn. (turmeric), coconut oil, castor oil, capsaicin, *Menthasylvestris* Linn. (menthol) and other ingredients reduced pain and stiffness in hand and knee OA. The activity of the cream though decayed at onset, sustained for longer duration when compared with other topical applications [26]. A meta-analyses of topical herbal applications reported that trials of boswellia and curcumin mixture and *B.serrata* alone, reduced pain and improved movement [27]. Another herbal ointment containing *Cinnamomumverum*Presl. (cinnamon), *G. officinalis*, *saghez* (resin of tree *Pistacialentiscus*) and sesame oil decreased pain, morning stiffness and limited motion in patients of knee OA; its effect were comparable with Salicylate ointment [28]. Topical ginger (*Z. officinalis*) compress and pack reduced pain and fatigue and improved functionality and satisfaction in OA patients [29]. Herbal compounds are hypothesized to modulate several inflammation triggering factors, pathway mediators and conditions, thereby offering relief in such conditions [30].
Upanahachoornam showed a significant anti-inflammatory activity, comparable to that of standard analgesic Indomethacin. Increased concentration did not seem to offer better benefit. The in vitro studies helped to understand the mechanism of action of the polyherbal formulation. The role of inflammatory cytokines in the pathogenesis of OA is seemingly very clear. TNF-α and IL-1β are promi-

nently involved, the former driving the inflammatory process and the latter contributing to cartilage destruction [31]. Cartilage breakdown is also promoted by NO, though its beneficial effects are also proposed [32]. Increase in COX-2 levels is also observed in inflammation, leading to efforts to curb its expression [33]. It is also proposed that anti-cytokine therapy may be a potential Disease-modifying OA drugs [34-36]. UC too inhibited TNF-α and NO and COX-2 expression and hence we may infer that it has the potential to reduce destruction in OA.

Herbs have been used since times immemorial for their medicinal properties, especially in India which has its own indigenous healthcare system Ayurveda. We have here reported the anti-inflammatory activity of an Ayurveda polyherbal formulation *Upanahachoornam*, as a topical application in Osteoarthritis. This may have far reaching implications in better management of this degenerative condition.

Table 1: Cytotoxic properties of UC against RAW cell line

Test Conc. (%)	% Cytotoxicity	CTC_{50} (µg/ml)
1000	44.91±1.2	
500	43.59±1.8	
250	30.73±0.5	>1000
125	27.22±2.9	
62.5	15.24±1.3	

Table 2: Cytotoxic properties of UC against L929 cell line

Test Conc. (%)	**% Cytotoxicity**	**CTC_{50} (µg/ml)**
1000	43.83±1.1	
500	36.62±2.5	
250	26.19±1.2	>1000
125	16.71±1.8	
62.5	7.05±1.2	

Table 3: Anti-inflammatory effect of UC in RAW cells against LPS induced TNF production

Sl. No	**Material**	**Concentration tested**	**% TNF inhibition**
1	UC	500 µg/ml	48.2±1.9
2	UC	250 µg/ml	25.6±1.0
3	Dexamethasone	200 µM	67.3±1.7

Table 4: Anti-inflammatory effect of UC in RAW cells against LPS induced Nitric oxide production.

Sl. No	Concentration tested	% NO inhibition
1	500 µg/ml	14.8±0.39
2	250 µg/ml	9.5±0.13

Table 5: Effect of UC on TPA induced ear edema in mice

GROUPS	Ear thickness (mm)						
	0 mins	30mins	60mins	120mins	240mins	300mins	% Inhibition
Group I	0.19	0.31	0.37	0.40	0.41	0.40	-
Group II	0.24	0.34	0.32	0.29	0.27	0.26	90.4
Group III	0.23	0.37	0.37	0.35	0.34	0.33	52.3

Figure legends

Figure 1: RT-PCR profile of COX-2 gene amplified from *Upanahachoornam* treated RAW cell line.

Figure 2: Densitometric analysis of gene transcripts. The relative level of COX 2 gene expression is normalized to GAPDH. Values shown depict arbitrary units

Figure 3: Effect of *Upanahachoornam* on TPA induced ear edema in mice.

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