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Anti-Inflammatory Activity of Polysaccharide Fraction of *Curcuma longa* Extract (NR-INF-02)

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Abstract: The aim of the study was to investigate the safety and anti-inflammatory effects of polysaccharide fraction (F1) of *Curcuma longa* extract (NR-INF-02) in classical rodent models of inflammation. F1 was evaluated for its acute oral toxicity and found to be safe upto 5000 mg/kg body weight in rats. The anti-inflammatory activity of F1 was evaluated in acute (carrageenan - induced paw edema; xylene - induced ear edema) and chronic (cotton pellet - induced granuloma) models of inflammation. The results of the study demonstrated that F1 significantly ($p \leq 0.05$) inhibited carrageenan-induced paw edema at 1 h and 3 h at doses of 11.25, 22.5 and 45 mg/kg body weight in rats. Also, F1 at doses of 15.75, 31.5 and 63 mg/kg significantly inhibited the xylene induced ear edema in mice. In a chronic model, F1 at 11.25, 22.5 and 45 mg/kg doses produced significant reduction of wet and dry weights of cotton pellets in rats. Overall results indicated that F1 of NR-INF-02 significantly attenuated acute and chronic inflammation in rodent models. This study emphasizes on the importance of *Curcuma longa* polysaccharide's role in acute and chronic inflammation.

Keywords: Anti-inflammatory, *Curcuma longa*, NR-INF-02, Polysaccharide fraction.

1. INTRODUCTION

Inflammation is generally life preserving which arises in any tissue in response to trauma, infection, toxic stimuli and culminates in tissue repair, recovery and healing. However, if the targeted repair and destruction of harmful stimuli are not phased properly, inflammation persists and leads to diseases and disorders. Inflammation is reported to play a major role in the pathogenesis of several diseases like osteoarthritis, multiple sclerosis, Crohn's disease, and atherosclerosis and also diseases of infectious origin viz., *Helicobacter pylori* gastritis, filariasis, hepatitis C, tuberculosis etc., [1]. In view of role of inflammation in several diseases and disorders, there is an inevitable need for anti-inflammatory therapies.

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A variety of anti-inflammatory agents are available, including the most widely used non-steroidal and steroidal anti-inflammatory drugs. As these drugs are associated with serious adverse effects, research for the development of safe and effective anti-inflammatory therapies is still ongoing [2]. Meanwhile, with the rising interests in herbal substances, extensive research to investigate the anti-inflammatory properties of natural substances is also growing at a faster pace [3].

One such herb that has a huge anti-inflammatory potential is *Curcuma longa*. *C. longa* (turmeric) belonging to the family *Zingiberaceae* is a perennial herb, cultivated in Southeast Asia [4]. *C. longa* consists of carbohydrates (69.4% of total mass), curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin), and essential oil [5]. Curcuminoids especially curcumin is considered as a key active constituent of *C. longa* and was extensively researched for its anti-inflammatory activity. A great body of evidence in the available literature indicates the anti-inflammatory activity

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of curcuminoids via down-regulation of cyclooxygenase-2 (COX-2), lipoxygenase, inducible nitric oxide synthase (iNOS), and by inhibition of tumor necrosis factor-alpha (TNF-α), inflammatory cytokines, monocyte chemoattractant protein (MCP) etc. [6]. In addition, turmerones are also reported for their anti-inflammatory effects in both acute and chronic models of inflammation [7, 8]. However, only few reports on the pharmacological activities such as anti-tumor, anti-diabetic, antioxidant, anti-depressant and immune modulatory of polar fractions that constitute majorly polysaccharides of *C. longa* are available [9-12]. While, curcuminoids and turmerones were explored and established for their anti-inflammatory potentials, the polar extracts or polysaccharides containing extracts of *C. longa* were seldom reported for anti-inflammatory activity. In view of the biological activities of polysaccharides of *C. longa*, NR-INF-02 a formulation that is a combination of turmeric oil and aqueous extract of *Curcuma longa* standardized to contain polysaccharides (>10 % w/w) was developed and investigated for its anti-inflammatory activity.

In vitro studies on NR-INF-02 revealed anti-inflammatory activity by inhibiting prostaglandins and interleukins [13]. While, *in vivo* studies on NR-INF-02 also reinstated the *in vitro* findings by exhibiting anti-inflammatory activity in both acute (Carrageenan and xylene) and chronic (cotton pellet granuloma) animal models of inflammation [14]. In addition, the findings of randomized placebo controlled clinical study on NR-INF-02 are also in conformity with the preclinical studies. The clinical trial on NR-INF-02 in osteoarthritis subjects revealed that this formulation is effective in the management of painful knee osteoarthritis [15].

NR-INF-02 was fractionated into polysaccharide rich fraction and polysaccharide free fraction and both the fractions were tested for anti-inflammatory activity *in vitro* and the polysaccharide rich fraction was found to have remarkable activity against inflammation [13]. Hence, this fraction with the highest activity was considered as active polysaccharide fraction (F1).

To further elucidate whether the active polysaccharide fraction of NR-INF-02 contributes for the anti-inflammatory activity *in vivo*, the present study was undertaken. The current study evaluated anti-inflammatory activities of active polysaccharide fraction of NR-INF-02 (F1) in acute and chronic inflammatory models. In order to ascertain the safety of F1, an acute oral toxicity study in rats, was also conducted.

2. MATERIAL AND METHODS

The studies on acute oral toxicity and the anti-inflammatory efficacy studies were performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment and Forests, Government of India. The studies were approved by the Institutional Animal Ethics Committee, R&D Centre, Natural Remedies, Bangalore, India.

2.1. Test Substance

The preparation of the test substance (F1) has been described in our earlier report [13]. In brief, NR-INF-02 was dissolved in water and added to 5 volumes of ethanol. The above contents were centrifuged at 2000 rpm for 20 min. The precipitate obtained after centrifugation was stirred with 5 volumes of ethanol at room temperature for 10 min and filtered. The retentate obtained after filtration was dried under vacuum at < 70°C to obtain polysaccharide fraction (F1).

2.2. Acute Oral Toxicity Study

Female nulliparous and non-pregnant albino Wistar rats (8 - 10 weeks old) were accommodated in individual polypropylene cage with stainless steel grill top and clean bedding. Animals were provided free access to rodent feed pellets (M/s Amrut Laboratory Animal Feeds, India) and UV purified water *ad libitum* and were housed under standard laboratory conditions of 12 h/12 h light/ dark cycle at 25±2^{o}C with 30-70 % relative humidity.

2.2.1. Experimental Procedure

The study was conducted as per the Organisation for Economic Cooperation and Development (OECD) guideline for the testing of chemicals (Test guideline No. 420), acute oral toxicity - fixed dose procedure, adopted on 17 December 2001 [16]. F1 in demineralized water was orally administered in one animal at 5000 mg/kg dose level in the sighting study and four animals at 5000 mg/kg in the main study. The experimental animals were

fasted overnight before dosing. On the day of dosing, all the animals were observed for mortality and clinical signs for first 10 min, 30 min, 1 h, 2 h, 4 h and 6 h after dosing and, thereafter, twice daily for mortality and once a day for clinical signs, for 14 days. The body weights of animals were recorded individually before dosing and at weekly intervals thereafter. Changes in the skin, fur, eyes and mucous membrane; respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behavior pattern, if any, were recorded. Particular attention was directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. And at termination of the study, rats were sacrificed for complete gross pathology examination.

2.3. Anti-Inflammatory Activity

The anti-inflammatory studies were performed on albino Wistar rats (150-180 g) of either sex or male Swiss albino mice (20-30 g) bred at Central Animal Facility, Research and Development Centre, Natural Remedies, Bangalore. Animals were provided free access to rodent feed pellets (M/s Amrut Laboratory Animal Feeds, India) and UV purified water *ad libitum* and were housed under standard laboratory conditions of 12 h/12 h light/ dark cycle at 25 ± 2^0C with 30-70 % relative humidity.

2.3.1. Drugs and Chemicals

The following drugs and chemicals were purchased and used: Diclofenac sodium (Ankur Drugs and Pharma, India), dexamethasone (Cadila Healthcare, India), xylene (Ranbaxy Fine Chemicals, India) λ -carrageenan (Sigma Life Science, Switzerland) and carboxymethyl cellulose (CMC) sodium salt (HiMedia, India).

2.3.2. Carrageenan-Induced Paw Edema in Rats

Male and female Wistar rats were administered with CMC (0.5 %; 10 ml/kg), diclofenac sodium (10 mg/kg), F1 (11.25, 22.5 and 45 mg/kg), respectively to individual groups of six rats each orally. Diclofenac sodium was used as a positive control. Acute paw edema was produced by injecting 0.1 ml of λ - carrageenan [prepared as 1% solution in normal saline solution (0.9% w/v NaCl)] into the sub plantar region of the right hind paw of rats [17] 60 min after administration of vehicle/ diclofenac/test substance. The paw volume was measured by the volume displacement method using a plethysmometer (PanLab, Spain) just before (0 h), and on hourly intervals (1, 2, 3 and 4 h) following injection of carrageenan. The increase in the paw volume at hourly intervals was recorded. Accordingly, the percent increase in paw volume was calculated.

2.3.3 Xylene-induced Ear Edema in Mice

The xylene-induced ear edema test was performed as described by Atta and Alkofahi [18]. Male Swiss albino mice were randomly allocated to five groups of six animals each and were administered orally with vehicle (0.5 % CMC; 10 ml/kg), diclofenac sodium (50 mg/kg), and various doses of F1 (15.75, 31.5 and 63 mg/kg) respectively 1 h prior to the xylene application. Xylene (50 µl) was applied to the anterior and posterior surfaces of the right ear, while left ear was considered as control. After 4 h of xylene application, mice were sacrificed, both the ears were dissected and ear discs of 6 mm diameter were punched out and weighed. The average weight difference between the right and left ear was taken as the measure for inflammatory response. The percent inhibition of ear edema between the F1/ diclofenac treated groups and vehicle treated group was computed as follows:

Percent inhibition (%) = (Rc-Lc) - (Rt-Lt)/(Rc - Lc)*100

Where Rc and Lc represent mean weights of right and left ear discs of vehicle treated group, while Rt and Lt represent mean weights of right and left ear discs of Diclofenac/ F1 treated groups respectively.

2.3.4. Cotton Pellet-induced Granuloma in Rats

Cotton pellet-induced granuloma test was performed as described by D' Arcy *et al.* [19]. Rats of either sex were randomly allocated to five groups (n = 6) and were anesthetized. Granuloma formation was induced by subcutaneous implantation of sterile cotton pellets weighing 10 mg each in the axilla and groin regions of the anesthetized rats. Post cotton pellet implantation, rats of five groups were orally administered vehicle (0.5% CMC; 10 ml/kg) or dexamethasone (0.5 mg/kg) or F1 (11.25, 22.5 and 45 mg/kg) once daily for seven

consecutive days respectively. On the eighth day, rats were sacrificed and the cotton pellets covered by the granulomatous tissue were meticulously excised, weighed and dried in hot air oven at 60°C for 24 h.

2.4. Statistical Analysis

All the values were expressed as mean ± SEM. Data were analyzed using one way ANOVA followed by post-hoc Dunnett's test. If error variance was found to be heterogenous, logarithmic transformation of raw data was performed and analyzed accordingly. Values of $p \leq 0.05$ were considered statistically significant.

3. RESULTS

3.1. Acute oral Toxicity

Animals treated at the dose level of 5000 mg/kg body weight in sighting and main study survived throughout the study period and did not show any major adverse clinical signs. Occurrence of loose stools for transient period was observed only on the day of dosing in two animals (one animal of sighting study and one animal of main study) (Table **1**). Body weight in treated animals after 7 and 14 days of treatment was found to be normal (Table **2**). On necropsy, no major gross pathological changes were observed in any of the treated rats. Based on the findings of this study, F1 was found to be safe up to 5000 mg/kg body weight after oral administration as a single dose to female albino Wistar rats.

3.2. Carrageenan-induced Paw Edema in Rats

The percent increase in paw swelling/edema caused by 1% carrageenan injection is presented in (Fig. **1**). The paw edema in rats of carrageenan control group increased along with the time course up to 3 h and peak edema was observed at 3 h. The standard drug, diclofenac sodium (10 mg/kg) showed significant ($p \leq 0.05$) reduction in the paw edema volume at 1 h, 2 h and 3 h as compared to

Table 1. Summary of clinical symptoms, mortality and gross pathology observed in rats receiving single dose of F1.

Study	Animal number	Dose (mg/kg)	Symptoms	Time (onset-recovery)	Mortality (death/total)	Gross pathology findings
Sighting (n=1)	1	5000	Loose stools	3 h, 4 h post treatment – 6 h	0/1	NAD
Main (n=4)	2	5000	None	-	0/1	NAD
	3	5000	Loose stools	5 h post treatment – 6 h	0/1	NAD
	4	5000	None	-	0/1	NAD
	5	5000	None	-	0/1	NAD

h: hour.

NAD: No abnormality detected.

n= number of animals.

Table 2. Effect of F1 on body weight of rats.

Study	Animal number	Dose (mg/kg)	Body weight (g)		
			Day 0	Day 7	Day 14
Sighting (n=1)	1	5000	156	215	237
Main (n=4)	2	5000	165	192	215
	3	5000	160	183	201
	4	5000	164	204	215
	5	5000	176	200	224

n= number of animals.

the carrageenan control group. Similarly, F1 at all the dose levels significantly ($p \leq 0.05$) reduced the paw edema volume at 1 h, 2 h and 3 h after carrageenan injection as compared with the carrageenan control group.

3.3. Xylene-induced Ear Edema in Mice

The average weights of ear edema and percentage inhibition of edema are presented in Fig. **2** and Table **3** respectively. Xylene demonstrated evident increase in the weight of the right ear when compared to left ear of the vehicle control group. However, standard drug diclofenac, F1 administration at all dose levels significantly ($p \leq 0.05$) decreased edema induced by xylene.

3.4. Cotton Pellet-induced Granuloma in Rats

The mean wet and dry weights of cotton pellets are summarized in Table **4**. The vehicle control group showed marked inflammatory response evident from the increase in the wet and dry weights of the pellets. Dexamethasone and F1 at all the

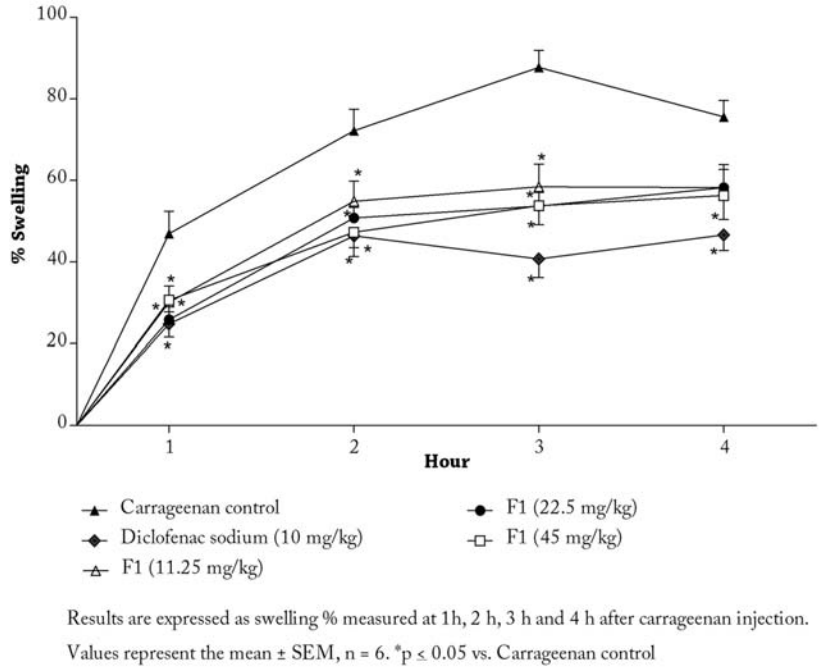

Fig. (1). Anti-inflammatory effect of F1 on carrageenan-induced paw edema in rats.

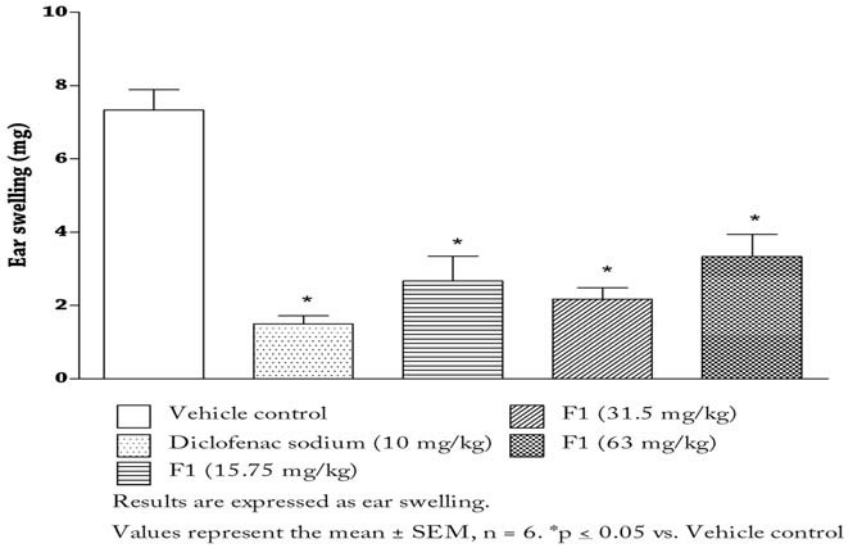

Fig. (2). Anti-inflammatory effect of F1 on xylene-induced ear edema in mice.

dose levels showed significant ($p \leq 0.05$) reduction in the wet weights and dry weights of the cotton pellets, as compared to the vehicle control.

Table 3. Percentage anti-inflammatory activity of F1 on xylene-induced ear edema in mice.

Treatment	Percentage inhibition (%)
Vehicle Control (10 ml/kg)	-
Diclofenac sodium (50 mg/kg)	79.54
F1 (15.75 mg/kg)	63.57
F1 (31.5 mg/kg)	70.39
F1 (63 mg/kg)	54.57

Table 4. Anti-inflammatory effect of F1 on cotton pellet-induced granuloma in rats.

Treatment	Weight of cotton pellets (mg) (wet)	Weight of cotton pellets (mg) (dry)
Vehicle Control (10 ml/kg)	171.79±5.14	39.83±1.64
Dexamethasone (0.5 mg/kg)	95.92±2.47*	20.79±0.53*
F1 (11.25 mg/kg)	144.50±2.44*	34.50±0.88*
F1 (22.5 mg/kg)	146.00±3.62*	33.29±0.62*
F1 (45 mg/kg)	151.29±2.52*	34.54±0.54*

Results are expressed as dry and wet weights of cotton pellets.

Values represent the mean ± SEM, n = 6.

*$p \leq 0.05$ vs. Vehicle control.

4. DISCUSSION

Inflammation is a typical host response to harmful stimuli. Nevertheless, inflammation dysfunction contributes to a range of acute and chronic inflammatory diseases which are a major cause of morbidity and mortality in the world [1]. These insights into inflammation provide an understanding that effective and safe anti-inflammatory agents are vital in the treatment of inflammatory conditions. Although synthetic drugs available in plethora, are associated with adverse / toxic effects and as a consequence of this a clear shift in the interest of researchers and the people towards herbal or plant therapies for the management of inflammatory disorders. The fact that global market for use of herbal drugs in the treatment of inflammatory diseases constitutes 83% worldwide provides evidence of inclination for plant-based anti-inflammatory agents [3]. Since ancient times a number of medicinal plants are being used for the treatment of inflammatory disorders. In the recent times, research has been centered on a wide range of phytoconstituents including phenolics, alkaloids, and terpenoids demonstrating their role in the modulation of inflammatory responses. Besides these, there are numerous phytoprinciples that are still unexplored for their anti-inflammatory potentials. Hence, investigating the potential activity of the new bioactives that could be beneficial to mankind becomes an important goal of research in biomedical sciences [20]. In view of the aforesaid, a preparation of *Curcuma longa* with negligible amount of curcuminoids and rich in polysaccharides, NR-INF-02 has been formulated and was investigated for its activity against inflammation.

This novel preparation NR-INF-02 was tested *in vitro* and *in vivo* for its anti-inflammatory activity. NR-INF-02 demonstrated strong inhibition on LPS stimulated PGE_2 and IL-12 production by macrophages and also showed mild inhibitory effects on NO and IL-6 production *in vitro* [13]. In *in vivo* models of acute and chronic inflammation, NR-INF-02 showed marked effects in suppressing the inflammation [14]. Additionally, a separate *in vitro* study on polysaccharide rich (F1) and polysaccharide free fractions (F2) of NR-INF-02 revealed that fraction with polysaccharide is effective against inflammation and is considered as active fraction of NR-INF-02 [13]. In order to further elucidate anti-inflammatory activities of active fraction of NR-INF-02 (F1), *in vivo* and to clearly understand if polysaccharides contribute for anti-inflammatory activity of NR-INF-02, the current study was undertaken. In addition safety of the F1 was tested in acute oral toxicity study in rats.

The acute oral toxicity study conducted as per the experimental protocol recommended by OECD revealed that F1 did not cause mortality or major pathological changes up to dose level of 5000 mg/kg. Although there were few instances of loose stools immediately after exposure, these signs were limited to only brief periods and all treated rats survived till termination of the study with normal body weight gain. Therefore, F1 can be categorized as "unclassified" according to the Globally Harmonized System.

Inflammation is complex and diverse ranging from acute to chronic inflammatory process. Acute inflammation is characterized by cardinal signs such as redness, swelling, pain, and heat and any disruption in the checkpoints will hamper natural resolution of acute inflammation which progresses to chronic inflammation characterized by excessive leukocyte infiltration, tissue damage and loss of function. Uncontrolled chronic inflammation is associated with cardiovascular, gastrointestinal, nervous system pathologies etc., [21]. Hence therapeutic agent that shows activity against acute and chronic inflammation would be ideal and beneficial. In the present study we have investigated the protective effect of F1 on acute (carrageenan-induced paw edema and xylene-induced ear edema) and chronic (cotton pellet-induced granuloma) inflammatory models.

In order to study the effect of F1 on acute inflammation, carrageenan-induced paw edema and xylene-induced ear edema models were employed. These are animal models widely used to test the protective effects of synthetic/natural products against acute inflammation [22, 23]. The paw edema induced by carrageenan is characterized as biphasic event with release of various inflammatory mediators in two phases. The initial phase of edema is induced by histamine, bradykinin and serotonin on vascular permeability. Second phase of accelerated edema begins at the end of first hour and persists through third hour and is characterized by release of prostaglandins, interleukin beta, Tumor Necrosis Factor alpha etc [24, 25]. Recently, it has been reported that overproduction of nitric oxide (NO) in the initial phase and inflammatory prostaglandin such as PGE_2 also play a key pathophysiological role in inducing paw edema by carrageenan [26]. In the present study, paw edema was maximum at third hour and such peaking at third hour is also noticed in several published studies [27]. Moreover F1 at all dose levels significantly inhibited development of paw edema in the initial and the second phase. Similar effect was observed in NR-INF-02 [14]. The reduction in paw edema can be correlated to the inhibitory effects of F1 on inflammatory mediators PGE_2 and IL-12 secretion as reported by Chandrasekaran *et al.* [13] *in vitro*. IL-12 is said to have inhibitory activity on NO [28]. Thus dual inhibition of PGE_2 directly and NO production indirectly by inhibition of IL-12 possibly might have attenuated carrageenan -induced edema.

Xylene-induced ear edema model reflects the edematization during the early stages of acute inflammation. Xylene in mice induces neurogenous edema, which is partially associated with substance P, an undecapeptide of central and peripheral nervous system. In the periphery, release of substance P from sensory neurons directly causes vasodilatation and plasma extravasations and indirectly causes release of inflammatory mediators causing swelling of ear in mice [29]. Substance P is also reported to upstream expression of cyclooxygenase-2 and prostaglandin E_2 through JAK-STAT pathways [30]. The significant inhibitory effects of F1 at all dose levels on xylene-induced edema in the present study are likely indication of their anti-inflammatory effects against acute inflammation. Such similar effects were shown by NR-INF-02 [14]. The suppression of edema induced by xylene is plausible either by antagonizing effects of substance P or inhibiting the inflammatory mediators released by substance P. Though there is no enough evidence of F1 antagonizing or inhibiting substance P, the *in vitro* studies demonstrated inhibitory effects on PGE_2 [13], an inflammatory mediator released indirectly by substance P. Hence, F1 might have shown protection against edema induced by xylene by inhibiting PGE_2.

Chronic inflammation is a condition characterized by continued inflammatory response, tissue destruction and is suggested to have a serious role in several diseases including osteoarthritis, diabetes, cardiovascular, autoimmune etc [1]. The current study used cotton pellet granuloma model, a widely employed model to evaluate effects of test substance on transudative, exudative and proliferative components of chronic inflammation [31]. The implanted wet weights of cotton pellets correlates with the transudative and exudative phases of chronic inflammation and dry weights correlate with the proliferative phase of chronic inflammation [19]. F1 notably decreased wet and dry cotton pellet weights. The primary effects of F1 on chronic inflammation may be associated with inhibitory effects on proinflammatory cytokines (IL-12) as observed in *in vitro* studies [13]. IL-12 is a heterodimeric cytokine that induces cytokines production, primarily of interferon gamma which

plays a key role in chronic inflammation [32, 33]. However, the direct effects on monocytes and macrophages need to be elucidated.

It is important to note that a substantial body of evidence indicates that plant polysaccharides have significant effects on immune functions and inflammation. Plant polysaccharides, such as *Radix astragali*, *Lentinus edodes*, *Agaricus blazei Muri*, *Ginkgo biloba*, *Viola odorata L*., *Malva pusilla* Smith, *Caesalpinia ferrea*, *Pholiota nameko* and *Azadirachta indica* have been shown to possess anti-inflammatory effects [34-37]. Furthermore, the evidence from studies on the animals and humans suggests that the oral glucans, arabinogalactans and other polysaccharides are well tolerated in toxicity studies and have immunomodulatory and anti-inflammatory activities [38]. Hence, it is apparent that polysaccharides are bioavailable, safe and have activity against inflammation. In the present study, F1 (polysaccharide fraction of NR-INF-02) was found to be safe and revealed potent anti-inflammatory activity suggesting that plausibly the arabinogalactans/ukonans are the phytoactives responsible for the anti-inflammatory activity of F1. The findings of the current *in vivo* study are in conformity to *in vitro* study results in which the polysaccharide rich fraction of NR-INF-02 (F1) demonstrated anti-inflammatory activity. In conclusion, the present study revealed that the polysaccharides of *Curcuma longa* contributed to the observed anti-inflammatory activity of NR-INF- 02".

LIST OF ABBREVIATIONS

ANOVA = Analysis of variance

CMC = Carboxymethyl cellulose

COX-2 = Cyclooxygenase-2

F1 = Active polysaccharide fraction of NR-INF-02

IL-12 = Interleukin-12

IL-6 = Interleukin-6

iNOS = Inducible nitric oxide synthase

JAK-STAT = Janus kinase and signal transducer and activator of transcription

LPS = Lipopolysaccharides

MCP = Monocyte chemoattractant protein

NaCl = Sodium chloride

NO = Nitric oxide

NR-INF-02 = A polysaccharide rich extract prepared from rhizome of *Curcuma longa*

PGE_2 = Prostaglandin E_2

Rpm = Revolutions per minute

SEM = Standard error of mean

TNF-α = Tumor necrosis factor-alpha

UV = Ultraviolet

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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