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Lysosomal enzyme inhibiting activity of flavone fraction from *Tylophora indica* leaves in arthritic rats

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ABSTRACT
Tylophora indica (Burm) Meril, (Asclepiadaceae), commonly known as Antamul possesses various pharmacological activities including anti-inflammatory activity. The present study was designed to evaluate lysosomal enzyme inhibiting activity of flavone fraction from *Tylophora indica* leaves in arthritic rats. The flavone fraction (FF) showed significant dose dependent lysosomal enzyme inhibiting activity against adjuvant-induced arthritis at 20-50 mg/kg. FF showed statistically significant inhibition of arthritic lesions ($P<0.05$) from day 18, ($p<0.025$) from day 20 and ($p<0.001$) from day 21 onwards in the adjuvant-induced arthritis studies which was comparable to the response of standard drug Indomethacin. FF may thus be able to inhibit the destructive activity of these enzymes on structural macromolecules like collagen etc. and prevent decrease in collagen levels and increase in urinary excretion levels of hydroxyproline, hexosamine, hexuronic acid etc. and synovial damage observed during arthritis.
KEY WORDS: *Tylophora indica*; synovial damage, adjuvant arthritis, lysosomal enzymes.

INTRODUCTION
Rheumatoid arthritis (RA) and other inflammatory diseases affect 2–5 % of population in various countries. The inflammatory process of RA is reportedly associated with an increase of the pro-inflammatory cytokines TNF- α and IL-1 β (1, 2, 3). Moreover, lysosomal acid hydrolases play an important role in inflammation associated with rheumatoid arthritis (4, 5) initiating several reactions and synthesis of inflammatory mediators namely, thromboxanes, prostaglandins and leukotrienes. The levels of these enzymes, which are mainly hydrolases (namely acid phosphatases, cathepsin and other collagenolytic enzymes), have been found increased in inflamed tissues including rheumatoid synovial membrane. This is why adjuvant induced arthritis in rats, showing changes in the lysosomal enzyme levels, is a commonly used pathological model for the study of arthritis (6) and is used for the evaluation of efficacy of antiinflammatory drugs (7). These enzymes cause degradation of glycosaminoglycans, glycoproteins and other structural molecules in connective tissue and cartilages in rheumatoid arthritis and solubilise insoluble collagen, thus decreasing collagen levels in synovial tissues. Consequently, urinary levels of break away fractions of collagen, for example hydroxyproline etc, are increased. Thus, drugs showing capacity to stabilise and preserve lysosomal membrane integrity should show antiinflammatory as well as synovial damage protective activity (6).

Tylophora indica (Burm) Meril, (Asclepiadaceae) commonly known as Antamul, is a small slender much branched climber and found growing normally in Uttar Pradesh, Bengal, Assam, Orissa, Himalaya and Sub Himalayas in India (8). Leaves have been reported to possess expectorant, emetic and antidysentric activities (9, 10), β –adrenergic stimulant activity (11), use in the treatment of bronchial asthma (12, 13), bronchitis and rheumatism (14) and smooth muscle relaxant activity (15). Preliminary antiinflammatory activity has also been reported for this plant (16). Since flavonoids are reported to be good anti-inflammatory agents and *Tylophora indica* leaves are reported to contain both alkaloid and flavone (17), the present study was designed to investigate the Lysosomal enzyme inhibiting activity of flavonoid fraction from the plant in arthritic rats.

MATERIALS AND METHOD
Plant material
Tylophora indica leaves were collected from medicinal garden in our institute campus in the month of February 2004. The plant material was authenticated by Dr. A.K.S. Rawat, National Botanical Research Institute (NBRI), Lucknow and voucher specimen was preserved for future reference (LWG, 224811) in the

herbarium maintained by NBRI, Lucknow. The leaves were then shade dried and powdered (moderately coarse).

Preparation of flavone fraction (FF) from Tylophora indica leaves

Powdered leaves (2 kg) were extracted with 90 % alcohol with 1 % acetic acid, the extract concentrated to a syrupy mass, and further extracted with 0.5 N Sulphuric acid till no alkaloidal reaction were shown. The acidic extract was concentrated and extracted with ether. The ether extract gave typical chemical reactions for flavones, and was then concentrated and crystallized from water and alcohol to get the flavone as a crystalline yellow compound (1gm) (17,18).

Animals

Female Wistar rats (weighing 180–200 g) were obtained from the animal house of Babu Banarasi Das (BBD) National Institute of Technology and Management, Lucknow. They were housed in polypropylene cages under standard environmental conditions and had free access to pellet diet and tap water. The animal studies were carried out vide CPCSEA approval no. 645/02/c/CPCSEA.

Induction of Adjuvant- induced arthritis

Arthritis was induced by i.d. injection of 0.05 ml of a 5 mg/ml suspension of heat killed *Mycobacterium tuberculosis* in liquid paraffin into the plantar surface of the hind paws. Animals were divided into five groups (each containing 6 animals), Group I- Normal control; Group II- Arthritic Control; Group III- Arthritic rats treated with FF 20mg/kg; Group IV- Arthritic rats treated with FF 50mg/kg; Group V- Arthritic rats treated with Indomethacin 2.5mg/kg. FF and standard were injected intra peritoneally from 11th—18th day post administration of Complete Freud's Adjuvant media (CFA). Rats developed signs of polyarthritis 8–10 days following the adjuvant injection. The clinical features of AA manifested as erythema, induration and edema, and presented in multiple-joints as follows: (a) onset: clinical signs around days 8–10; (b) early phase: progressive severity of the clinical signs over the next 7–10 days; and (c) late phase: spontaneous regression during the next 10–14 days (19).

Arthritis assessments

Rats were assessed daily for signs of arthritis between days 7 and 25 post-CFA using a standard arthritic scoring system. The maximal arthritic score per rat was set at 16 (maximum of 4 points× 4 paws). All four paws were examined and graded for severity and loci of erythema, swelling and induration using a 5-point scale: 0 = no signs of disease; 1 = signs involving the ankle/wrist; 2 = signs involving the ankle plus tarsals (proximal part of the hind paw) and/or wrist plus carpals of the forepaw; 3 = signs extending to the metatarsals or metacarpals; and 4 = severe signs involving the entire hind or fore paw (20). Paw volume was measured every other day between days 16 and 24 with a plethysmometer. Animals with pronounced arthritis were separated into five groups and treated with FF and indomethacin intraperitonealy daily for next 7 days. Mean paw volumes obtained for each day for treated groups were compared with appropriate scores of the control group.

Lysosomal enzyme inhibitory activity

Arthritis was induced and extracts administered as above. At the end of 18th day, rats were housed in the metabolic cages and their urine collected for 24 hours in beakers maintained at 0° C in ice bath. Rats were sacrificed on the 19th day by decapitating. Plasma was separated from the blood collected with EDTA. Immediately after sacrificing, liver kidney and spleen were separated and homogenised in ice cold 0.01M tris HCl buffer (pH7.4) to give 10% homogenates, which were then utilized for study of lysosomal enzyme levels (21). Urine samples were utilized for study of the breakaway components of collagen and other synovial tissues. The activities of lysosomal enzymes were investigated in liver, plasma, kidney and spleen of FF treated arthritic rats and non-arthritic rats. Liver, kidney and spleen were exposed and perfused with cold buffer saline of pH 7.4. Blood organs were taken out and homogenized and used for biochemical estimations. Blood drawn was centrifuged for 10 min at 2000 rpm and separated plasma was used for estimation of acid phosphatase, cathepsin –D, ß-glucuronidase, glycosaminoglycan and glycoprotein.

Biochemical Estimation

Acid phosphatase was measured by the method of King (22) on the basis of the action of the enzyme on disodium phenyl phosphate (substrate) to liberate phenol. Cathepsin -D was determined by the method of Etherington (23) in which, cathepsin- D liberates TCA soluble products (which can be estimated for Thyrosine content by Folins Phenol reagent) on incubation with hemoglobin. Glucuronidase was estimated by the method of Kawai and Anno (24) based on the liberation of p-nitrophenol by the action of the enzyme on the substrate p-nitrophenol ß-d-glucuronide which was then estimated by UV absorbance measurement. Glycosaminoglycan and glycoprotein degradation products were determined as Hexosamine by the method of Wagner (25) and as Hexuronic acid by

the method of Bitter and Muir (26) in both plasma and urine. Collagen was estimated as its degradation products hydroxyproline and hexuronic acid in urine by the method of Woessner (27).

Statistical analysis

The data were analysed using one way analysis of variance (ANOVA) followed by student's Newmann Keuls Test. The values are expressed as mean ± S.D.

RESULTS AND DISCUSSION

Adjuvant- induced arthritis

The results presented in figure 1, showed that FF injected intraperitonealy was capable of reducing the severity of arthritic lesions, and a statistically significant ($P<0.05$) inhibition of the severity was observed in treatment as compared to the control group. The effect was dose dependent and it showed significant inhibition of arthritic lesions ($P<0.05$) from day 18, ($p<0.025$) from day 20 and ($p<0.001$) from day 21 onwards. The fraction injected in higher doses reduced the lesions to a greater extent showing a dose dependent decrease in lesions. CFA-induced secondary inflammation mimics sub-acute RA (20, 28). Because RA is characterized by excessive immunologic activity in the synovium (29), the anti-polyarthritis effect of FF may be achieved by the potential immunomodulatory properties.

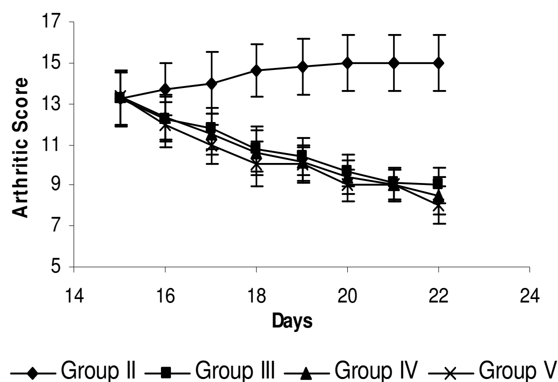

Figure 1. Effect of** flavone fraction **from Tylophora indica on Arthritic Rats.

Group I- Normal control; Group II- Arthritic Control; Group III- Arthritic rats treated with FF 20mg/kg; Group IV- Arthritic rats treated with FF 50mg/kg; Group V- Arthritic rats treated with Indomethacin 2.5mg/kg.

Lysosomal enzyme inhibitory activity

Table 1 shows the effect of various fractions on protein bound carbohydrates in plasma, measured to estimate the extent of presence of collagen degradation products. Arthritic rats (group II) showed a significantly increased level of these carbohydrates ($p<0.001$) as compared to control. Arthritic rats treated with FF (20 and 50 mg/kg) showed a significant ($p<0.05$ and $p<0.001$) reduction in these carbohydrates levels as compared to arthritic rats. Alternation in lysosomal integrity and metabolism of connective tissue are the prominent features in adjuvant arthritis which is a systemic disease. Glycoproteins, glycosaminoglycans and collagen are the major macromolecules in synovial tissues of considerable importance. Glycoproteins may be involved in maintaining the structural stability of collagen fibrils and thus stabilizing the tissue (30), and are primarily responsible for their antigenic property in tissue transplants (31). The level of glycoproteins increases in arthritic rats due to the increased connective tissue activating factor, as found in inflammatory conditions (32). Lysosomal enzymes cause degradation of glycosaminoglycans, glycoproteins and other structural molecules in connective tissue and cartilages in rheumatoid arthritis and solubilise insoluble collagen, thus decreasing collagen levels in synovial tissues (21).

Tables 2, 3 and 4 present the lysosomal enzyme levels measured in control and experimental rats treated with various doses of FF. In the case of adjuvant induced arthritis (group II), the enzymes- acid phosphatase, cathepsin D, β-glucuronidase were significantly increased ($p<0.001$) as compared to the normal rats. Group III and IV, indicating enzyme levels in FF treated animals respectively, show significant reduction in the levels of these enzymes ($p<0.001$ and $p<0.05$). Numerous animal tissues contain a group of cytoplasmic organelles called lysosomes characterized by their acid hydrolases content (33), which are extruded out into the extra cellular environment during endocytosis of the immune complexes by the leucocytes (34). Significantly increased activities of lysosomal enzymes are found in arthritic rats than control animals in extra cellular fluid due to decrease in lysosomal stability (35). This alters glycoprotein and glycosaminoglycan metabolism. They are also involved in the destruction of structural macromolecules in connective tissue and cartilage during rheumatoid arthritis by destroying proteoglycans. Treatment of arthritic rats with flavone fraction from *T. indica* leaves decreased the level of glycoproteins and

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Table1. Effect of EE and flavone fraction (FF) on protein bound carbohydrates in arthritic rats.

Groups	Hexose mg/dl	Hexosamine mg/dl	Hexuronic acid mg/dl
Group I	140.48 ± 8.42	30.10 ± 2.15	52.22 ± 5.13
Group II	200.01±7.29	54.73 ± 3.23	81.79 ± 5.12
Group III	158.95 ± 8.49 *	41.84 ± 2.02 *	64.39 ± 4.99 *
Group IV	151.26 ± 7.95 *	38.70 ± 2.36 *	53.26 ± 4.21 *
GroupV	142.26 ± 7.94 *	37.70 ± 2.36 *	53.16 ± 4.21 *

Group I- Normal control; Group II- Arthritic Control; Group III- Arthritic rats treated with FF 20mg/kg; Group IV- Arthritic rats treated with FF 50mg/kg; Group V- Arthritic rats treated with Indomethacin 2.5mg/kg. $^{+}p<0.001$ as compared to normal control, $*p<0.001$ as compared to arthritic control. (ANOVA followed by student's Newman Keuls Test).

Table 2. Effect of EE and Flavone fraction (FF) on lysosomal acid phosphatase activities in various tissues of normal and arthritic rats

Groups	Acid phosphatase (μM x 10^{-2} of Phenol)			
	Blood	Liver	Kidney	Spleen
Group I	0.15 ± 0.02	2.21 ± 0.21	1.35 ± 0.14	3.28 ± 0.15
Group II	0.28 ± 002 $^{+}$	4.44 ± 0.29 $^{+}$	2.29 ± 0.17 $^{+}$	4.56 ± 0.39 $^{+}$
Group III	0.14 ± 0.02 **	3.42 ± 0.26 **	1.79 ± 0.15 **	3.18 ± 0.22 **
Group IV	0.12 ± 0.02 **	3.03± 0.22 *	1.32 ± 0.13 **	3.00 ± 0.24 *
Group V	0.14 ± 0.01 **	3.33± 0.22 *	1.40± 0.13 **	3.19 ± 0.24 *

Group I- Normal control; Group II- Arthritic Control; Group III- Arthritic rats treated with FF 20mg/kg; Group IV- Arthritic rats treated with FF 50mg/kg; Group V- Arthritic rats treated with Indomethacin 2.5mg/kg. $^{+}p<0.001$ as compared to normal control,$*p<0.01$, $**p<0.001$ as compared to arthritic control. (ANOVA followed by student's Newman Keuls Test).

Table 3. Effect of EE and Flavone fraction (FF) on lysosomal cathepsin in various tissues of normal and arthritic rats

Groups	Cathepsin (μ M of tyrosine liberated /mg protein/min)			
	Blood	Liver	Kidney	Spleen
Group I	0.32 ± 0.03	0.17 ± 0.01	0.28 ± 0.05	0.36 ± 0.02
Group II	0.75 ± 0.06 $^{+}$	0.64 ± 0.11 $^{+}$	0.84 ± 0.08 $^{+}$	0.69 ± 0.08 $^{+}$
Group III	0.47 ± 0.04 *	0.24± 0.04 *	0.36 ± 0.04 *	0.41 ± 0.04 *
Group IV	0. 39 ± 0.02 *	0.20± 0.04 *	0.30 ± 0.02 *	0.37± 0.24 *
Group V	0. 36 ± 0.02 *	0.22± 0.04 *	0.32± 0.02 *	0.31± 0.24 *

Group I- Normal control; Group II- Arthritic Control; Group III- Arthritic rats treated with FF 20mg/kg; Group IV- Arthritic rats treated with FF 50mg/kg; Group V- Arthritic rats treated with Indomethacin 2.5mg/kg. $^{+}p<0.001$ as compared to normal control, $*p<0.001$ as compared to arthritic control. (ANOVA followed by student's Newman Keuls Test).

Table 4. Effect of EE and Flavone fraction (FF) on lysosomal β-Glucuronidase in various tissues of normal and arthritic rats

Groups	β-Glucuronidase (μM x 10^{-2} of p-nitrophenol liberated/hr/mg protein)			
	Blood	Liver	Kidney	Spleen
Group I	2.34 ± 0.13	33.86 ± 2.97	38.46 ± 3.29	31.63 ± 2.72
Group II	6.29 ± 0.59 $^{+}$	47.64 ± 3.29 $^{+}$	50.83 ± 3.15 $^{+}$	41.36 ± 2.61 $^{+}$
Group III	3.248 ± 0.44 *	33.58 ± 2.45 *	40.41 ± 2.79 *	33.59 ± 2.67 *
Group IV	3.02 ± 0.32 *	32.00 ± 3.12 *	40.00 ± 3.23 *	31.84±2.65 *
Group V	2.42 ± 0.32 *	32.57 ± 3.12 *	39.69 ± 3.23 *	32.24±2.65 *

Group I- Normal control; Group II- Arthritic Control; Group III- Arthritic rats treated with FF 20mg/kg; Group IV- Arthritic rats treated with FF 50mg/kg; Group V- Arthritic rats treated with Indomethacin 2.5mg/kg. $^{+}p<0.001$ as compared to normal control, $*p<0.001$ as compared to arthritic control. (ANOVA followed by student's Newman Keuls Test).

Table 5. Effect of EE and Flavone fraction (FF) on collagen and urinary proteoglycan degradation products in normal and arthritic rats.

Groups	Collagen (mg/g cartilage)	Hydroxyproline (mg/mg creatinine)	Hexosamine – m mg/100 mg creatinine	Glycosaminoglycans (mg/24 hrs)
Group I	421.16±6.71	2.08±0.15	74.76±1.66	4.58±0.22
Group II	297.09±4.19 +	4.41±0.23 +	129.56±4.35 +	4.95±0.13
Group III	340.76±6.50	2.09±0.20	74.07±2.41	4.55±0.2
Group IV	385.42±5.79 **	2.045±0.19 **	73.421±1.76 **	4.50± 0.22
Group V	339.42±5.79 **	2.08±0.19 **	73.21±1.76 **	4.52±0.22

*Group I- Normal control; Group II- Arthritic Control; Group III- Arthritic rats treated with FF 20mg/kg; Group IV- Arthritic rats treated with FF 50mg/kg; Group V- Arthritic rats treated with Indomethacin 2.5mg/kg. +p<0.001 as compared to normal control,*p<0.01, **p<0.001 as compared to arthritic control. (ANOVA followed by student's Newman Keuls Test).*

lysosomal enzymes, which may be due to stabilization of lysosomal membranes.

The effect of flavone fraction on collagen levels and excretion levels of collagen products in urine are given in Table 5. Group II arthritic animals showed decrease in collagen levels (p<0.001) and increase in urine degradation products (hydroxyproline, hexosamine and glycosaminoglycan). The Group III and IV animals treated with FF showed significant reversals (p<0.001) in the conditions observed in the group II arthritic animals. Collagen and proteoglycans make the extensive extra cellular matrix, which together with small number of relatively isolated cells make the substance of cartilage tissue. Collagen, the most susceptible tissue, is degraded to hydroxyproline by collagenase and other collagenolytic enzymes during inflammation due to accumulation of granulocytes, and so increased hydroxyproline levels in urine are observed during adjuvant inoculation (36, 37, 38). The destruction of cartilage in human rheumatoid arthritis was reported to be due to the enzymatic degradation of proteoglycans by lysosomal enzymes (39). An increased excretion of urinary hydroxyproline (p < 0.001) appears to indicate an alteration in the pattern of collagen metabolism due to either changes in collagen synthesis or in the rate of conversion of one form of collagen to another (40), because hydroxyproline is exclusively found in the collagen making 13-14% of the total amino acid. The increased catabolism of glycoproteins and glycosaminoglycans, which may be due to altered levels of glycohydrolases in the process of adjuvant induced arthritis, causes increased excretion of hexosamine, hexuronic acid and glycosaminoglycans (41, 42).

CONCLUSION

The flavone fraction from *Tylophora indica* leaves shows statistically significant antiarthritic action against CFA induced inflammation by preventing synovial damage through lysosomal enzymes inhibition. Further studies are needed to identify the active flavone responsible for this observed activity.

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