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Lysosomal Enzyme Inhibiting Activity of Alkaloidal Fraction from *Tylophora indica* Leaves in Arthritic rats

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Abstract – Ethanolic extract (50% v/v) and alkaloid fraction of *Tylophora indica* leaves were examined for lysosomal enzyme inhibitory activity in adjuvant-induced arthritic rats. The alkaloid fraction 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, which was comparable to the response of standard drug Indomethacin. The ethanolic extract was less significant than the alkaloidal fraction in inhibition of arthritis. Alkaloid fraction showed significant ($p < 0.001$) inhibitory effect on the lysosomal enzyme activities in adjuvant-induced arthritic rats. It also significantly prevented decrease in collagen levels and synovial damage observed during arthritis and also inhibited increase in urinary excretion levels of collagen degradation products like hydroxyproline, hexosamine, hexuronic acid, etc. Both ethanolic extract as well as the alkaloid fraction, however, did not show any significant activity in normal nonarthritic rats. The ethanolic extract and the alkaloid fraction may thus be able to inhibit the progress of inflammation and inhibit the destructive activity of lysosomal enzymes on structural macromolecules like collagen etc. in the synovial capsule in joints during arthritic states. They may thus prevent synovial damage observed during arthritis.
Keywords – *Tylophora indica,* tylophorine, 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 (Fleischmann, *et al.,* 2004, Dayer, *et al*., 2003, Shin, *et al.,* 2003). Moreover, lysosomal acid hydrolases play an important role in inflammation associated with rheumatoid arthritis (Safina, *et al.,* 1992a, Safina, *et al.,* 1992b) initiating several degradation reactions, apart from other 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 inflammed 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 (Lewis A.J., 1985) and is used for the evaluation of efficacy of antiinflammatory drugs (Billingham, 1983). 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.

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. It shows a stout rootstock, which is cordlike and knotty and fissured longitudinally, with ovate, orbicular and cordate leaves, and flowers as umbels of 2 - 3 flowers each with outer side greenish yellow and inner side purple (*Wealth of India,*1969). Leaves have been reported to posses expectorant, emetic and antidysentric activity (Kirtikar and Basu, 1933, Ratnagiriswami, *et al.,* 1935), β-adrenergic stimulant activity

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(Arora, *et al.,* 1973) and smooth muscle relaxant activity (Dhananjayan, *et al.,* 1975). The leaves have been reported to be used in bronchial asthma (Shivpuri, *et al*., 1968, Shivpuri, *et al.,* 1969), bronchitis and rheumatism (Chopra, *et al*., 1958). Preliminary antiinflammatory activity has been reported for the plant (Gopalkrishnan, *et al*., 1979).

The leaves are reported to contain up to 0.2 - 0.3% alkaloids, the content being rather stable over various seasons. Tylophorine and Tylophorinine have been isolated earlier and their structure has also been elucidated by Govindachari, *et al.,* (1954). Three other alkaloids have been reported from the plant (Rao, *et al.,* 1971). The present study was designed to investigate the antiinflammatory effect of ethanolic extract and alkaloidal fractions on adjuvant-induced arthritis and ability to inhibit release of lysosomal enzymes and prevent synovial cartilage damage in arthritis.

Experimental

Plant Collection and extraction – *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. Rawat, A.K.S., National Botanical Research Institute, Lucknow and voucher specimen was preserved for future reference (LWG, 224811) in the herbarium. The leaves were then shade dried and then powdered (moderately coarse).

50% ethanolic extract (**EE**) was prepared by taking 3 kg powdered (moderately coarse) leaves and then extracting by continuous solvent extraction method.

Preparation of Alkaloidal fraction – Extraction and fractionation of alkaloidal fractions was done as performed earlier by Govindachari, *et al*. (1954). Briefly, powdered (moderately coarse) leaves (3.2 kg) were extracted with methanol containing 0.5% acetic acid. The extract was then concentrated to a syrupy dark brownish mass under reduced pressure and then extracted with 0.5 N H_2SO_4 till it gave negative alkaloidal reactions. The acidic portion was then concentrated, washed with ethyl acetate, made alkaline with NH_4OH (pH 8.4) and finally extracted with chloroform, and concentrated under reduced pressure to yield alkaloidal fraction (3.5 g) which was further resolved into a single alkaloidal component fraction (AF-A) by column chromatography. One gram of the concentrated fraction was dissolved in 30 ml of chloroform and passed over an alumina column and eluted with Ether: Chloroform (1 : 1). Nine 50 ml portions of elute (fractions) were collected with Ether: Chloroform (1 : 1) of which fractions 1 - 4 were collected as alkaloidal fraction **AF-A**, on the basis of complementary TLC and qualitative HPTLC done for ethanolic extract (EE), and then for alkaloidal fraction AF-A), on Silica gel GF_{254} HPTLC 0.2 mm plates (SD Fine) using Chloroform: Acetone: Diethyl amine (5 : 4 : 1) as the mobile phase.

Animals – Wistar rats (weighing 180 - 200 g) obtained from the animal house of our institute were used. They were housed in polypropylene cages under standard environmental conditions and had free access to pellet diet and tap water.

Induction of Adjuvant-induced arthritis and measurement of polyarthritic lesions – 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 six groups (each containing 6 animals), Group I- Normal control; Group II- Arthritic Control; Group III- Arthritic rats treated with AF-A 20 mg/kg; Group IV- Arthritic rats treated with AF-A 100 mg/kg; Group V-Arthritic rats treated with Indomethacin 2.5 mg/kg. Group VI- Arthritic rats treated with EE 100 mg/kg. AF-A, EE and standard were injected intraperitoneally from 15 th - 22 ndth day post administration of Complete Freud's Adjuvant media (CFA). Rats developed signs of polyarthritis 8 - 10 days following the adjuvant injection. (Newbould, 1963). 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 (Moudgil, *et al.* 1997).

Lysosomal enzyme inhibitory activity – Arthritis was induced and the extracts were administered as above. At the end of 22nd day, rats were housed in the metabolic cages and their urine was collected for 24 hours in beakers maintained at 0 °C in ice bath. Rats were sacrificed on the 23rd day by decapitating. Plasma was separated from the blood collected with EDTA. Immediately after sacrificing, liver, kidney, and spleen were separated and homogenized in ice-cold 0.01 M tris HCl buffer (pH 7.4) to give 10% homogenates, which were then utilized for study of lysosomal enzyme levels (Geetha, *et al.,* 1999). 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 from AF-A- and EE- treated arthritic rats and non-arthritic rats. Liver, kidney and spleen were exposed and perfused with cold buffer saline of pH 7.4. 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, β-glucuronidase, glycosaminoglycan and glycoprotein.

Biochemical Estimations – Acid phosphatase was measured by the method of King (1965) on the basis of the action of the enzyme on disodium phenylphosphate (substrate) to liberate phenol. Cathepsin -D was determined by the method of Etherington (1972) in which, cathepsin-D liberates TCA soluble products (which can be estimated for Tyrosine content by Folins Phenol reagent) on incubation with hemoglobin. Glucuronidase was estimated by the method of Kawai and Anno (1971) based on the liberation of *p*-nitrophenol by the action of the enzyme on the substrate *p*-nitrophenol β-d-glucuronide, which was then estimated by UV absorbance measurement. Glycosaminoglycan and glycoprotein degradation products were determined as Hexosamine by the method of Wagner (1979) and as Hexuronic acid by the method of Bitter and Muir (1962) in both plasma and urine. Collagen was estimated as its degradation products hydroxyproline and hexuronic acid in urine by the method of Woessner (1961).

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 & Discussion

The fraction AF-A was analysed by chemical and spectroscopical methods and shown to be containing a single major constituent by HPTLC (Fig. 1) and giving positive alkaloidal reactions with Dragendroff's reagent with the UV λ_{max} being 255, 290, 340 and 352 nm being similar to the data reported earlier for Tylophorine reported by Govindachari, *et al.* (1954).

The results presented in Fig. 2, show that ethanolic extract EE and alkaloidal fraction AF-A injected intraperitonealy significant reduced the severity of arthritic lesions produced by Complete Freud's adjuvant (CFA) media in treated group as compared to the control group in a dose dependent manner, ($p < 0.05$) from day

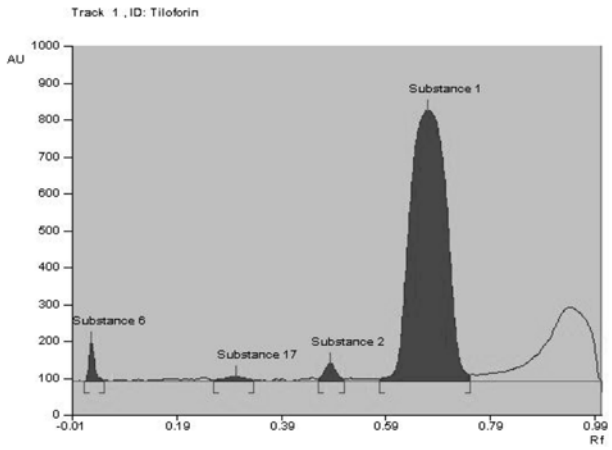

Fig. 1. HPTLC chromatogram of alkaloidal fraction (AF-A) of Tylophora *indica* at 200 nm.

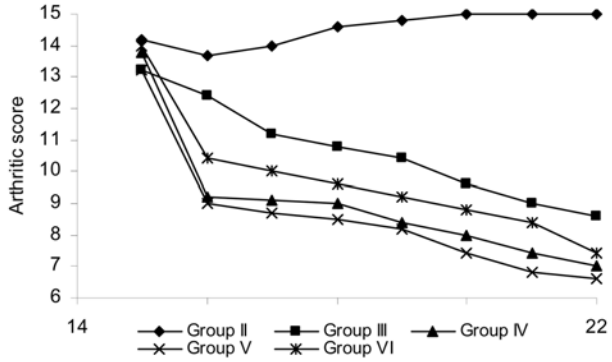

Fig. 2. Antipolyarthritic effect of EE, AF-A fractions from *Tylophora indica* in Complete Freud's Adjuvant (CFA) induced athritic rats (arthritic score).

Group I- Non Arthritic saline treated rats, Group II- Arthritic rats, Group III- Arthritic rats treated with AF-A 20 mg/kg, Group IV- Arthritic rats treated with AF-A 100 mg/kg, Group V- Arthritic rats treated with Indomethacin 2.5 mg/kg, Group VI- Arthritic rats treated with EE.

18, ($p < 0.025$) from day 20 and ($p < 0.001$) from day 21 onwards for AF-A treated groups. CFA-induced secondary inflammation mimics sub-acute RA (Moudgil, *et al.*, 1997, Lathigra, *et al.*, 1988). Because RA is characterized by excessive immunologic activity in the synovium (Firestein, 2004), the anti-polyarthritis effect of AF-A may be due to immunomodulatory effect. EE was less significant than AF-A producing ($p < 0.05$) inhibition from day 19 to 21.

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 AF-A (20 and 100 mg/

Table 1. Effect of EE and alkaloidal fraction 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	150.26 ± 7.94*	40.70 ± 2.30*	55.26 ± 4.28*
Group IV	149.26 ± 7.95*	38.70 ± 2.24*	53.26 ± 4.17*
GroupV	142.26 ± 7.90*	37.70 ± 2.20*	53.16 ± 4.24*
Group VI	158.95 ± 8.49*	41.84 ± 2.02*	64.39 ± 4.45*

Group I- Normal control; Group II- Arthritic Control; Group III- Arthritic rats treated with AF-A 20 mg/kg; Group IV- Arthritic rats treated with AF-A 100 mg/kg; Group V- Arthritic rats treated with Indomethacin 2.5 mg/kg, Group VI Arthritic rats treated with EE 100 mg/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).

kg) showed a significant ($p < 0.05$ and $p < 0.001$) reduction in these carbohydrates levels as compared to nontreated arthritic rats. Group VI rats treated with EE showed similar results though lesser than AF-A. Alteration 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 (Jackson, *et al*., 1968), and are primarily responsible for their antigenic property in tissue transplants (Robert, *et al*., 1968). The level of glycoproteins increases in arthritic rats due to the increased connective tissue activating factor, as found in inflammatory conditions (Caster, *et al*., 1985). 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 (Geetha, *et al.,* 1999).

Tables 2, 3 and 4 present the lysosomal enzyme levels measured in control and experimental rats treated with various fractions. 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 AF-A treated animals show significant reduction in the levels of these enzymes ($p < 0.001$ and $p < 0.05$ respectively). Group VI, EE- treated animals showed significantly similar results though lesser than AF-A. Numerous animal tissues contain a group of cytoplasmic organelles called lysosomes characterized by their acid hydrolases

Table 2. Effect of EE and alkaloidal fraction on lysosomal acid phosphatase activities in various tissues of normal and arthritic rats

Groups	Acid phosphatase (iM 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 ± 0.02^{+}	4.44 ± 0.29^{+}	2.29 ± 0.17^{+}	4.56 ± 0.39^{+}
Group III	0.148 ± 0.05**	3.33 ± 0.28*	1.62 ± 0.19**	3.19± 0.22*
Group IV	0.12 ± 0.03**	3.03 ± 0.20*	1.32 ± 0.13**	3.00 ± 0.21*
Group V	0.14 ± 0.01**	3.33 ± 0.24*	1.40 ± 0.12**	3.19 ± 0.28*
Group VI	0.14 ± 0.02**	3.42 ± 0.26**	1.79 ± 0.15**	3.18 ± 0.27**

Group I- Normal control; Group II- Arthritic Control; Group III- Arthritic rats treated with AF-A 20 mg/kg; Group IV- Arthritic rats treated with AF-A 100 mg/kg; Group V- Arthritic rats treated with Indomethacin 2.5 mg/kg, Group VI Arthritic rats treated with EE 100 mg/kg. $^{+}p < 0.001$ as compared to normal control, *$p < 0.01$ and **$p < 0.001$ as compared to arthritic control. (ANOVA followed by student's Newman Keuls Test).

Table 3. Effect of EE and alkaloidal fraction on lysosomal cathepsin in various tissues of normal and arthritic rats

Groups	Cathepsin (m 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	0.42 ± 0.05*	0.28 ± 0.06*	0.36 ± 0.02*	0.41 ± 0.21*
Group IV	0.39 ± 0.08*	0.20 ± 0.04*	0.30 ± 0.04*	0.37 ± 0.24*
Group V	0.36 ± 0.02*	0.22 ± 0.09*	0.32 ± 0.06*	0.31 ± 0.29*
Group VI	0.47 ± 0.04 *	0.24 ± 0.02*	0.36 ± 0.01*	0.41 ± 0.09*

Group I- Normal control; Group II- Arthritic Control; Group III- Arthritic rats treated with AF-A 20 mg/kg; Group IV- Arthritic rats treated with AF-A 100 mg/kg; Group V- Arthritic rats treated with Indomethacin 2.5 mg/kg, Group VI Arthritic rats treated with EE 100 mg/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 alkaloidal fraction on lysosomal β-Glucuronidase in various tissues of normal and arthritic rats

Groups	β-Glucuronidase (μM × 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 ± 2.64^{+}
Group III	3.288 ± 0.31*	32.57 ± 3.12*	40.69 ± 3.23*	32.24 ± 2.65*
Group IV	3.02 ± 0.32*	32.00 ± 3.14*	40.00 ± 3.28*	31.84 ± 2.69*
Group V	2.42 ± 0.39*	32.57 ± 3.19*	39.69 ± 3.21*	32.24 ± 2.61*
Group VI	3.248 ± 0.44*	33.58 ± 2.45*	40.41 ± 2.79*	33.59 ± 2.67*

Group I- Normal control; Group II- Arthritic Control; Group III- Arthritic rats treated with AF-A 20 mg/kg; Group IV- Arthritic rats treated with AF-A 100 mg/kg; Group V- Arthritic rats treated with Indomethacin 2.5 mg/kg, Group VI Arthritic rats treated with EE 100 mg/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 alkaloidal fraction on collagen and urinary proteoglycan degradation products in normal and arthritic rats

Groups	Collagen (mg/g cartilage)	Hydroxyproline (mg/mg creatinine)	Hexosamine 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	380.42 ± 5.81**	2.095 ± 0.22**	73.821 ± 1.76**	4.59 ± 0.22
Group IV	385.42 ± 5.73**	2.045 ± 0.19**	73.421 ± 1.79**	4.50 ± 0.29
Group V	339.42 ± 5.77**	2.08 ± 0.24**	73.21 ± 1.70**	4.52 ± 0.19
Group VI	340.76 ± 6.50	2.09 ± 0.20	74.07 ± 2.41	4.55 ± 0.2

Group I- Normal control; Group II- Arthritic Control; Group III- Arthritic rats treated with AF-A 20 mg/kg; Group IV- Arthritic rats treated with AF-A 100 mg/kg; Group V- Arthritic rats treated with Indomethacin 2.5 mg/kg, Group VI Arthritic rats treated with EE 100 mg/kg. $^{+}p < 0.001$ as compared to normal control, $*p < 0.01$ and $**p < 0.001$ as compared to arthritic control. (ANOVA followed by student's Newman Keuls Test).

content (Novikoff, 1961), which are extruded out into the extra cellular environment during endocytosis of the immune complexes by the leucocytes (Oronsky, *et al.,* 1973). Significantly increased activities of lysosomal enzymes in extra cellular fluid are found in arthritic rats than control animals due to decrease in lysosomal stability (Weissman, 1967). 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 alkaloidal fraction from *T. indica* leaves decreased the level of glycoproteins and lysosomal enzymes, which may be due to stabilization of lysosomal membranes.

The effect of AF-A 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 collagen degradation products (hydroxyproline, hexosamine and glycosaminoglycan). The Group III and IV animals treated with AF-A showed significant reversals ($p < 0.001$) in the conditions observed in the group II arthritic animals. Group VI EE treated animals also showed significant results, though lesser than AF-A. 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 (Kubersampath, *et al*., 1978a, Kubersampath, *et al.,* 1978b, Wirl, 1977). The destruction of cartilage in human rheumatoid arthritis was reported to be due to the enzymatic degradation of proteoglycans by lysosomal enzymes (Krane, 1974). 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 (Prockop, 1964), 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 (Carevic, *et al* .,1988, Subrata, *et al.,* 1994).

Conclusion

The AF-A fraction from *Tylophora indica* leaves shows statistically significant antiarthritic action against CFA-induced inflammation by preventing synovial damage through lysosomal enzymes inhibition while the ethanolic extract was slightly less significant. The ethanolic extract and the alkaloid fraction may thus be able to inhibit the progress of inflammation and inhibit the destructive activity of lysosomal enzymes on structural macromolecules like collagen etc. in the synovial capsule during arthritic states. They may thus prevent synovial damage observed during arthritis.

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