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AN ANTI-INFLAMMATORY AGENT FROM *TYLOPHORA INDICA* (ASCLEPEADACEAE) LEAVES

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Tylophora indica, a branching indigenous climber, is a major medicinal plant having various activities like anti-asthmatic, hepato protective, anti-tumour and anti-inflammatory etc.. Tylophorin was extracted, isolated and characterized by using the techniques like TLC,HPTLCand IR along with the standard reference compound of tylophorin.The isolated tylophorin was evaluated for anti-inflammatory activity by using HRBC *in vitro* method with standard compound DFS. 10 mg/ml concentration of tylophorin has given significantly good result (45.14%) among all the concentration used ,which suggested its usefulness in the herbal preparations to combat the inflammatory disease.

Keywords: Anti-inflammatory activity; DFS; Leaves extract ; *Tylophora indica*; Tylophorin.

Introduction

Inflammation is a condition when a body reacts to any infection, injury or irritation, environmental changes or malignancy, but sometimes some internal inflammation can result giving rise to fever and other discomforts. The inflammatory response involves the activation of white blood cells that start releasing some chemicals such as cytokines and prostaglandin[1]. Prostaglandins are produced within the body's cells by the enzyme cyclo oxygenase (COX). There are two COX enzymes, COX-1 and COX-2,produce prostaglandins that promote inflammation, pain, and fever. However, only COX-1 produces prostaglandins that support platelets and protect the stomach. Nonsteroidal anti-inflammatory drugs (NSAIDs) block the COX enzymes and reduce prostaglandins throughout the body. As a consequence, ongoing inflammation, pain, and fever are reduced.Anti-inflammatory refers to the property of a substance or treatment that reduces inflammation. Despite the progress made in the medical science chronic inflammation is still considered as major health problem thus this area need new drugs and further research. Many herbs have been evaluated for the anti-inflammatory activity including *Tylophora indica,* which is a perrienial plant belongs to family Asclepeadaceae. The plant has been explored biotechnologically as well as phytochemically[2-11]. This climber plant is reported to have many pharmacological important activity[12]. Anti-inflammatory activity of different plant alkaloids has been reviewed extensively[13]. These natural products are most common in flowering plants, and usually in the Papaveraceae (poppies), Papilonaceae (lupins), Ranunculaceae (aconites), and Solanaceae (tobacco and potatoes) families[14]. Isoquinoline, indole and diterpene alkaloids were the alkaloids that were extensively examined by many scientist.They were effective on different assays including carrageenin-induced paw oedema, adjuvant-induced arthritis and acetic acid induced vascular permeability tests [15,16].

Tylophora alkaloids originate from various plants of the Asclepiadaceae family, are native of India and Southeast Asia[17]. They have antitumor[18-22], anti-inflammatory[23], anti-arthritis[24] and anti-lupus activity *in vivo*[25]. Tylophorin from *Ficus septica* from china has been evaluated for anti-inflammatory activity,70% anti-inflammatory activity was reported in this case at the concentration of 3-10 µg/ml[26]. Some scientist have carried out the study of anti-inflammatory effect of *Tylophora indica* plant extract on carrageenin induced paw oedema

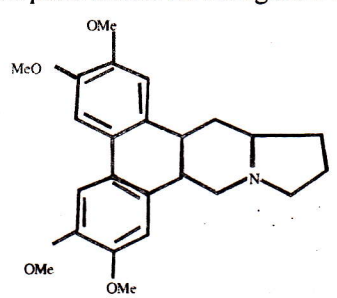

Fig.1. Stucture of Tylophorin alkaloid

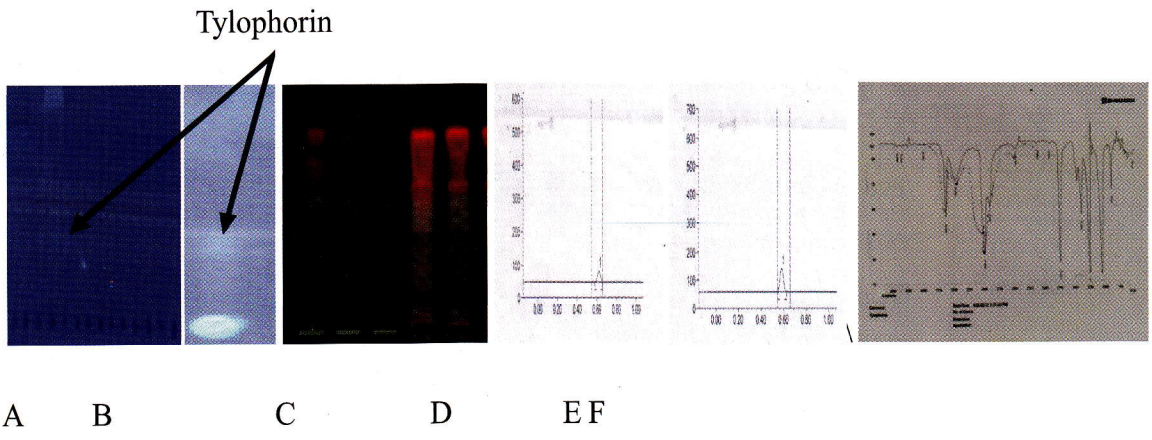

Fig.2. A -Preparative Thin Layer Chromatography (at 254 nm) ,B- Standard of Tylophorin (254nm),C-Fingerprinting of High Performance Liquid Chromatography of methanol: ethanol (8:2) extract of Tylophora indica leaves,D-HPTLC Chromatogram of methanol: ethanol (8:2) extract of Tylophora indica leaves, E-Standard, F-Infra Red spectral studies of isolated Tylophorin with standard.

model. They have worked on ethanolic extract of the plant, but did not isolated the bioactive compound, responsible for the anti-inflammatory activity[22], so it was highly essential to find the Indian source of tylophorin. To fulfill this need Tylophora indica leaves from Maharashtra region was examines for this activity.Hence, in the present study,the isolation,identification and characterization of tylophorin were carried out qualitatively from Tylophora indica by using different techniques such as Thin Layer Chromatography (TLC),Preparative Thin Layer Chromatography (PTLC),High Performance Liquid Chromstography (HPTLC) and Infra Red Spectral studies (IR)along with the standard reference compound of tylophorin .The isolated and characterized compound was used for anti-inflammatory assay.

Material and Methods

The experimental plant of Tylophora indica was procured from different areas of Mumbai and maintained in Haffkine Institute campus till full flourish growth was achieved. The mature leaves (20g) of climber plant were collected in the month of April. The fresh paste was prepared and then subjected for cold extraction for 48 hours at room temperature with solvent methanol: ethanol (8:2). The extract was then decanted into petri plates and the extract was allowed to evaporate and dried, which was now ready for further experimental work.

Qualitative method-The Thin Layer Chromatography (TLC)wasusedtoidentifythetylophorinalkaloidwiththat of the reference compound. TLC chamber was saturated withsolventsystemToluene:Diethylamine:Ethylacetate (14:2:2)for15minpriortouse.0.1mmthicksilicadelplates were used as stationary phase and Toluene: Diethylamine: Ethyl acetate (14:2:2) was used as mobile phase. The confirmation of alkaloid was done by spraying the developed chromatogram with Dragendorf's reagent which gave the brick red colored five spot. The tylophorin was further confirmed by running TLC plate with standard tylophorin (Allexis Co. New Delhi). A very small amount of methanol was poured into the dried extract in the Petri plates and this was further used for TLC analysis. Further confirmation of presence of tylophorin was carried out by using Co-TLC along with standard tylophorin (Rf value 0.59). When developed plates were sprayed with Dragendorf's reagent brick red color was suggest the presence of alkaloid. The developed chromatograms were observed in UV light at 254nm (Fig. 2), which gave bright yellow color. The further confirmation of tylophorin was done by using HPTLC and IR spectral studies with reference compound of the same (Fig.2).

Preparative Thin Layer Chromatography (PTLC) -The isolation of tylophorine was carried out by Preparative TLC method. After being separated out on the TLC plate, tylophorine was purified by scraping off the silica from the plate and dissolving it in ethanol. This filtrate was then collected and allowed to evaporate ,which left behind pale yellow crystal of tylophorine. This was done repeatedly two times to procure the pure compound.The compound was then subjected for anti-inflammatory activity by using the mentioned method.

High Performance Liquid Chromatography (HPTLC) Analysis - HPTLC analysis was carried out in Anchrom Test Lab Pvt. Ltd using silica gel plates (60F254 Manufacturer E. MERCK KGaA), Sample application was carried out on CAMAG Linomat 5 Instrument (CAMAG Linomat 5 "Linomat5_080222" S/N 080222). Inert gas was used as spray gas. Sample solvent type was methanol.

Table 1. The anti-inflammatory % activity of tylophorin from *Tylophora indica* leaves and comparison with DFS a standard compound. SD ± mean of three replicates significant p value p>0.01 (Chi square method)

Concentrations mg/ml	% Haemolysis (DFS)	% DFS Activity	%Haemolysis (Tylophorin)	% Tylophorin activity
Positive (+) control				
0.001	89.20 ±0.016	10.79±0.017	85.09±0.052	14.90±0.096
0.01	87.04±0.056	12.96±0.005	84.23±0.063	15.76±0.056
0.1	77.32±0.051	22.67±0.009	80.99±0.025	19.06±0.082
1.0	76.67±0.071	23.26±0.078	71.70±0.067	28.29±0.008
10	75.37±0.059	24.62±0.019	54.85±0.012	45.14±0.051

$$\%\text{Hemolysis} = \frac{+\text{ve control OD} - \text{sample OD}}{+\text{ve control OD}} \times 100$$

$$\%\ \text{activity} = 100 - \%\text{hemolysis}$$

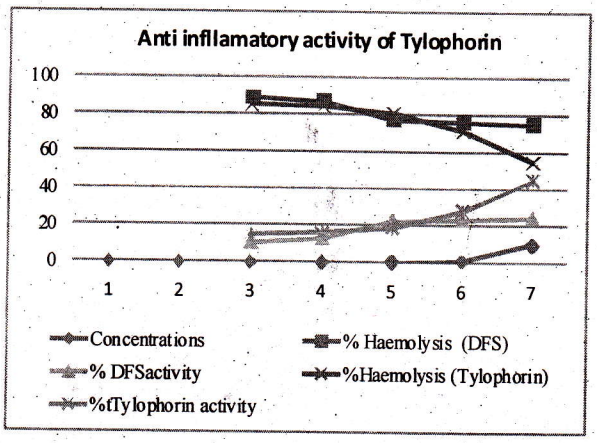

Fig.3. The graphical presentation of anti-inflammatory activity of tylophorin as compared to standard compound DFS.

Dosage speed was 150nl/s and syringe size was 100µl and the analysis wave length was 430 nm. Toluene: Ethyl acetate: Diethyl amine (14:2:2) was used as mobile phase.

Anti-inflammatory test of tylophorine: The extraction, isolation, and characterization of tylophorin alkaloid from *Tylophora indica* leaves were carried out and it was evaluated for anti inflammatory activity using the HRBC mambrane method.

Principle : The lysosomal enzyme is produced during the process of inflammation which in turn create a variety of disorder. The extra cellular activity of this enzyme is said to be related to the acute or chronic inflammation. The non-steroidal drugs Diclo Fenac Sodium (DFS) acts either by inhibiting these lysosomal enzyme or by stabilizing the lysosomal membrane.

The hyposaline used causes the hemolysis of RBC as it's concentration increased. This causes the membrane components to leak out. Which is then measured spectrophotometrically at 560nm. Increased concentration of hyposaline increases the OD (as increased concentration of hemolysis increases the extent of hemolysis), DFS (standard compound) stabilizes the membrane, thereby reducing the hemolysis. With increment in the concentration of DFS membrane is stabilized and membrane components are prevented from leaking. Thus, as the concentration.of DFS increases the OD decreases thereby decreasing the effect of hypo tonicity caused by hypo saline.

%Hemolysis= (OD of drug treated sample/OD of control)*100

The ability of the test sample to stabilize the membrane or to prevent hemolysis is compared with that of standard.

Anti-inflammatory activity of Tylophorin :

Protocol - Step 1- RBC solution

- Alsevers solution (3ml) + Blood (3ml).
- Centrifuge at 3000 rpm for 10min.
- Discard the supernatant and wash the pallet with iso-saline thrice.
- Dilute 1ml of pallet with 9ml of iso-saline *i.e.* 10% v/v.

STEP-2 : Sample (Tylophorine & DFS Dilutions)

The dilutions (10mg/ml, 1mg/ml,0.1mg/ml, 0.01mg/ml, 0.001mg/ml) of Tylophorin and DFS (Standard compound) were made using DMSO separately.All the samples for further study were prepared (0.5ml sample + 1 ml Phosphate buffer + 2 ml Hyposaline +0.5ml RBC Solution) separately. Here positive control (1ml Phosphate buffer + 2ml Hyposaline +0.5 ml RBC solution+ 0.5ml DMSO) and Blank (2ml Phosphate buffer + 4ml Hyposaline +2ml DMSO) were used.

STEP 3: All the samples were prepared as stated earlier and kept for 30 min at 37°C following by centrifugation at 300rpm for 20 min. After the completion of the process the OD of the supernatant were taken at 560 nm separately.On the completion ofthe experiment % hemolysis and % anti inlammatory activity of samples were calculated by using their respective formula.

The process of making Alsevers solution, Phosphate Buffer, Hyposaline and Isosalineare as follows.

Alsevers solution (Add Dextrose - 1gm,Sodium citrate - 0.4gm,Citric acid - 0.025gm,Sodium chloride - 0.21gm and dissolve in distilled water to make up the volume up to 50ml).

Phosphate buffer (A-0.2 M of NaH_2PO_4Dissolve 4.792gm of NaH_2PO_4 in 200ml of distilled water,B-0.2 M of Na_2HPO_4 Dissolve 5.678gm of Na_2HPO_4 in 200ml of distilled water and mix 32ml of 'A' with 168ml of 'B'and make C solution.Take 150ml of 'C' adjust the pH to 7.5 and make up the volume to 200ml using distilled water).

Hyposaline (Dissolve 0.9gm of NaCl in 250ml of distilled water).

Isosaline (Dissolve 2.125gm of NaCl in 250ml of distilled water).

Results and Discussion

Tylophorin from *Tylophora indica* leaves extract exhibit the significant anti-inflammatory effect as compared to DFS (Table 1, Fig. 2).

The main objective of the study was to find out the Indian herbal source for anti-inflammatory activity. The potentiality of *T. indica* as anti-inflammatory agent was confirmed singnificantly. Tylophorine was extracted from *T. indica* leaves by using metanol: ethanol (8:2) solvent at room temperature. The TLC chromatogram were developed with standard compound of tylophorin in Toluene: Di ethylamine: Ethyl acetate(14:2:2) mobile phase.On spraying with Dragendorf's reagent, brick red color appeared, which confirm the presence of tylophorin alkaloid (Rf value 0.59) qualitatively. For Further confirmation of presence of tylophorin HPTLC and IR spectral studies was done..The cyto toxicity of methanolic extract of *T. indica* leaves has been evaluated by MTT assay and LC_{50} 30mg/ml was obtained in this experiment[4]. The anti-inflammatory activity of tylophorine was compared with standard anti inflammatory drug DFS. From the graph (Fig.2) it is clear that the activity of tylophorine at the correspondent dilution was far more better than the DFS. Increase in concentration of tylophorine gradually brings down hemolysis activity denoted by OD. When compared with standard DFS (24.62%) ,tylophorin has given better results(45.14%) at the concentration of 10mg/ml. From this study, it can be concluded that the tylophorine of *T. indica* of Maharashtra origin acts as an anti-inflammatory agent significantly and in future it might be used in the pharma industries. Asclepeadaceae was evaluated for anti-inflammatory activity[27]. It was established that phenanthro indolizidine alkaloid inhibit the activation of COX-II promoter activity[22].The alpha metoxy functional group of tylophorin is involved in anti-inflammatory activity[28] and hence it acts as an anti-inflammatory agent.In this experiment scientists have reported around 70% inhibition at 9-10μM (activity at 393mg/ml;Mol.wt.of tylophorin is 393.48) concentration .In this case tylophorin exhibited potent suppression of nitric oxide production, which is twenty times lower than our results (10mg/ml:45.14%).The study showed the enthusiastic and considerable results, now it can be said that the Indian herbal source of tylophorin is much more efficient to combat inflammatory disorder.

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