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Evaluation of Anti-Inflammatory Activity of *Tylophora asthmatica*

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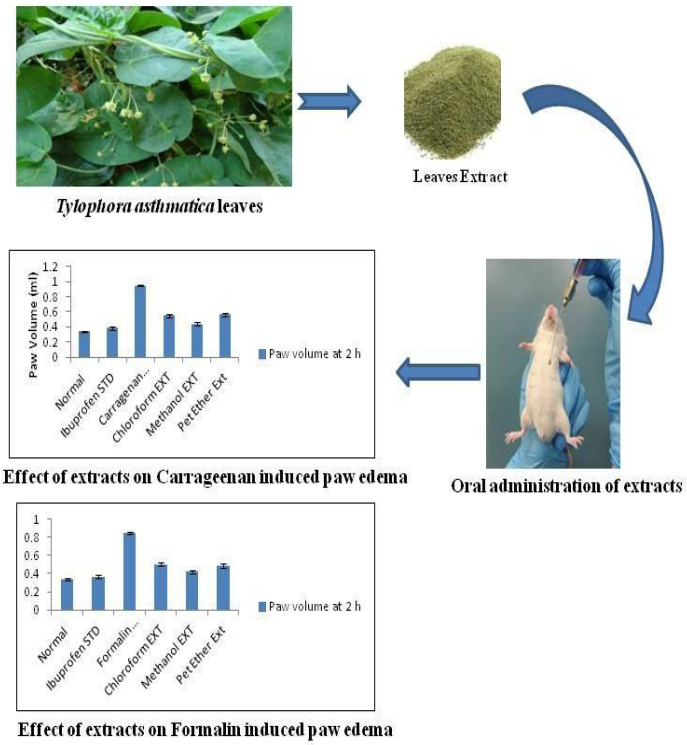
Abstract

In this study, the anti-inflammatory activity of leaves of the *Tylophora asthmatica* (*T. asthmatica)* was assessed by using carrageenan induced and formalin induced paw edema model. Different extracts such as petroleum ether, chloroform and methanolic extract of the leaves of *T. asthmatica* were prepared using soxhlet method. Further, the extracts were identified for various phytochemical constituents and percentage yield of extract was calculated. The anti-inflammatory activity of extracts was determined by using different dosages of 400 mg/kg in Wistar rats. The percentage inhibition of paw edema was determined using plethysmometer. The methanolic extract of *T. asthmatica* treated rats showed significantly higher anti-inflammatory activity as compared to rats treated with petroleum ether and chloroform extracts. The results clearly indicated that the leaves of *T. asthmatica* have potent anti-inflammatory activity and it could be further used for the development of novel formulations for the management of inflammatory diseases.

Keywords Anti-inflammatory; carrageenan; formalin; ibuprofen; *Tylophora asthmatica*

Graphical Abstract



Introduction

Inflammation is a part of complex biological response of body tissues to harmful stimuli such as

pathogens, damages cells or irritants and is a protective response involving immune cells, blood vessels and molecular mediators [1]. It is a pathophysiological process by which a living tissue responds to injury and involves vascular compartments and the connective tissues [2].The inflammatory response is triggered through two phases (a) acute (b) chronic and each of these are apparently mediated by a different mechanism [3]. These immune responses which involved in acute inflammation are divided into types: vascular and cellular inflammation. The responses which occur in micro-vascular level, normally appears in few minutes following tissue injury or microbial infection in the presence of other inflammatory stimuli named vascular events [4]. The process of inflammation is brought about by vascular as well as cellular events, the former appears to contribute maximum for the pathogenesis of acute inflammation. This complex phenomenon involves endogenous chemical mediators such as histamine, 5-hydroxytryptamine, various chemotactic factors, bradykinin, leukotrienes and prostaglandins [5].

The occurrence of these processes is rapid and eventually will lead to vasodilatation and subsequently makes the vessels more permeable. This process results in entry of inflammatory mediators and formation of interstitial edema. The function of inflammation is to eliminate the initial cause of cell injury, clears out necrotic cells and tissues damaged from the original insult and the inflammatory process and to initiates the tissue repair. The classical signs of acute inflammation are pain, heat, redness, swelling and loss of function. Inflammation is a generic response, and therefore it is considered as a mechanism of innate immunity, as compared to adaptive community, which is specific for each pathogen [6].

Currently there are various chemical drugs, which are used for the treatment of inflammation under the name of NSAIDs (non-steroidal anti-inflammatory drugs). These drugs are used to relieve the symptoms of pain, stiffness, swelling, heat and redness, which are the prominent symptoms of inflammation. The NSAID drugs include traditional drugs such as paracetamol, ketoprofen, ibuprofen, aspirin, naproxen, diclofenac and etodolac etc. and cyclooxygenase-2 inhibitors like celecoxib. These drugs are associated with several side effects which can be serious, including gastro-intestinal diseases and cardiovascular complications. In US, every year around 100,000 hospitalizations and 17,000 deaths are recorded due to the

consumption of NSAIDs [7]. Gunter 2016 performed meta-analysis of current literature to determine the risk of cardiac disorders with the use of COX-2 inhibitors [8]. The results clearly indicated that refecoxib, a COX-2 inhibitor, enhances the risk of myocardial infarction and stroke. In another study by Wallace 2000, it is described that the acid present in gastric tract triggers the pathogenesis of NSAID-induced ulcers and bleeding, by interfering with homeostasis and inactivation of growth factors, important in mucosal defense and repair [9]. Moreover, NSAIDs are consumed in a higher dose multiple times in a day to attain a required therapeutic level. Therefore, an alternative approach is immediately required, which not only minimizes the side effects but also decreases the dose of drug.

Since ancient times plants have been explored to treat different diseases due to their multiple properties, minimal side effects, accessibility, availability, inherited practice, economic feasibility, and perceived efficacy. There are various plants which showed anti-inflammatory activity such as green tea, curcumin, *agele marmelos*, *moringa oliefera*, *cassia fistula*, *emblica officinalis* and *hibiscus rosa* etc [10]. *T. asthmatica* is a large class of natural products and is found in planes, hilly slopes and forests. The plant grows in the area with lesser rainfall. *T. asthmatica* grows in wide range of well drained soil and prefer scanty localities. About 60 species are found in tropical, sub-tropical Asia, Africa and Australia and about 35 species are reported from China. However, this plant has been explored very less for its anti-inflammatory activity's. It contains 0.46% of alkaloids such as tylophorine, tylophorinine, tylophorinidine, septicine, sterols, flavonoids, wax, resins and tannins [11]. The major constituent is tylophorine, which is responsible for a strong anti-inflammatory effect [12]. *T. asthmatica* is traditionally used as a folk remedy in certain regions of India for the treatment of bronchial asthma, inflammation, allergy, bronchitis and dermatitis. It has antitumor, immunomodulatory, antioxidant, antiasthmatic, muscle relaxant properties [13]. In this study, the anti-inflammatory activity of *T. asthmatica* in the inflammation induced Wistar rats was assessed using carrageenan and formalin induced rat models. To explore the activity, different extracts were prepared and anti-inflammatory response of the extracts was determined after identifying various chemical constituents. It is expected that the findings could open the new dimension in alternative therapeutics in the management of inflammation in future.

Materials and Methods

Materials

All the solvents and chemicals such as ethyl acetate, potassium mercuric iodide, potassium bismuth iodide, dilute hydrochloric, picric acid, fehling's solution A, fehling's solution B, sodium hydroxide, petroleum ether, gelatin, sodium chloride, lead acetate, concentrate nitric acid, ninhydrin, copper acetate, glacial acetic acid, ammonia solution, magnesium turnings and ethanol etc. were purchased from S.D. Fine-Chem Ltd., Mumbai, India and Loba Chemi Limited, Mumbai, India. Carrageenan and formalin were purchased from S.D. Fine-Chem. Ltd, Mumbai, Ibuprofen standard drug was purchased from local supplier of Abott Pharma, Mumbai, India.

Collection of plant Tylophora asthmatica

T. asthmatica leaves were collected from Botanical garden of G.H.G Khalsa College of Pharmacy, Gurusar Sadhar, Ludhiana (Punjab). The leaves were washed with water and air dried at 35-45˚C for a week and coarsely powered.

Animals

Wistar rats (150-250g) were used for the study. Animals were grouped and housed in separate cages for not more than 5 animals. All rats were fed with pelleted diet and water *ad libitum.* The experimental protocols were approved by Institutional Animal Ethical Committee (IAEC) and care of animals was taken as per guidelines of CPCSEA (1801/PO/Re/S/15/CPCSEA), Department of Animal Welfare, and Government of India.

Methodology

Preparation of extract

To prepare the extracts, 100g leaves of *T. asthmatica* were washed with water to remove the adhering dirt and then dried in shade and coarsely powdered. The powdered material was subjected to extraction with petroleum ether, chloroform and methanol using soxhlet apparatus until extracts were obtained. The extracts were placed in desiccator to remove all the moisture [14]. These extracts were further used for different studies.

Phytochemical screening of extracts of Tylophora asthmatica

The obtained extracts by using petroleum ether, chloroform and methnol were filtered using whattman filter paper no. 41 and evaporated on water bath and finally dried in vacuum. Further, the extracts were screened for various classes of phytoconstituents such as alkaloids, glycosides, carbohydrates, proteins, amino acids, saponins, flavanoids etc. by following their respective qualitative tests (Table 1). The percentage yields of all three extracts were also calculated [15].

In-vivo studies

To determine the anti-inflammatory effect of the developed extracts of *T. asthmatica*, carrageenan and formalin induced rat models were used. The animals were divided into five groups with five animals in each group in both models as given below:

Group 1– Normal control (vehicle treated group)

Group 2– Standard drug (Ibuprofen 40 mg/kg) was administered intraperitonally

Group 3 – Petroleum ether extract (400 mg/kg) was administered orally

Group 4 – Chloroform extract (400 mg/kg) was administered orally

Group 5 – Methanol extract (400 mg/kg) was administered orally

To prepare the carrageenan suspension, 1% suspension was prepared by sprinkling 100 mg of carrageenan powder in 10 ml of saline (0.9% w/v NaCl) solution and set aside and soaked for 1 hr. A homogenous suspension was then obtained by thorough mixing with magnetic stirrer [16].

Assessment of Carrageenan induced Paw edema

All the animals were treated with the plant extracts according to their above given respective, groups. After 30 min of the above

treatment an inflammatory edema was introduced in the right hind paw by injecting 0.1 ml Carrageenan (1%) in the planter tissue of the paw of all animals. Paw edema was assessed by determining the volume of paw by using plethysmometer (Ugo basil, 7140, Italy) immediately after carrageenan was injected and for 6 hr at hourly intervals subsequently. A mark was made on right hind paw just below the tibia-tarsal junction so that every time the same length of paw was dipped into the column of the plethysmometer up to that particular mark to ensure a constant paw volume. For each animal, edema was expressed as increase in paw volume (ml) after CAR injection relative to pre-injection value [17]. The relative increase in the paw volume was measured in control, standard and treated groups at different time internal 0 min, 30 min, 60 min, 120 min after carrageenan injection. The percentage inhibition of paw edema was calculated by using the following formulas:

Percentage of edema inhibition = (Vc-Vt/Vc) × 100 Vc – Volume of paw edema in control group

Vt – Volume of paw edema in treated group

Assessment of Formalin Induced Paw Edema

Similarly, in formalin induced paw edema model, rats were treated with extracts according to respective groups and after 30 min of drug administration 0.1 ml of 10% v/v solution of formalin was injected into the sub-planar region of the right hind paw of each rat. The paw volume will be measured by using plethysmometer (Ugo basil, 7140, Italy**)** immediately after injection and again at 30, 60, 120 min interval using following formula:

Percentage of edema inhibition = (Vc-Vt/Vc) × 100 Vc – Vol of paw edema in control

Vt – Volume of paw edema in treated group

Data Analysis

Results of *in vivo* anti-inflammatory activity were reported as mean ± SD (n=3), and the difference between the groups in animal studies were tested using one way ANOVA followed by Tukey's multiple comparison post hoc test was applied for paw edema model and data analysis tool in Microsoft Excel and the results were reported significant at $p<0.05$.

RESULTS

Development of different extracts of leaves of T. asthmatica

The three extracts were developed using petroleum ether, methanol and chloroform and further their percentage yield was observed and results showed that methanol extract has maximum yield of 16.25% as compared to petroleum ether and chloroform extract, which indicated that the extraction favors the highly polar solvent (Table 2).

Determination of Phytoconstitutents of T. asthmatica

The different qualitative tests were carried out to estimate the presence of chemical constituents in the developed extracts. The results of tests confirmed that all the three extracts have different constituents such as alkaloids, carbohydrates, amino acids, steroids, glycosides, tannins, flavonoids and phenolic compounds.

***In vivo* anti-inflammatory studies**

Table 1: Qualitative tests used for the estimation of chemical constituents

Chemical Constituent	Qualitative Test
Alkaloids	Dragendorff's test
Carbohydrates	Fehling's test
Glycosides	Keller-Killani test
Proteins	Ninhydrin test
Amino acids	Millon's test
Saponins	Froth test and foam test
Flavanoids	Lead subacetate test

Table 2: Determination of percentage of yields of various extracts of T. *asthmatica*

Extract	Yield % w/w
Petroleum ether	2.50%
Chloroform	2.65%
Methanol	16.25%

The injection of both the carrageenan / formalin resulted in an acute inflammation of right hind paw of the rats within the first 30 min. The rats were treated with the Standard drug Ibuprofen and different extracts of *T. asthmatica* at 400 mg/kg. It was observed that all the extracts showed decrease in inflammation in the carrageenan-induced paw edema/ formalin induced-paw edema in rats, but significant decrease in paw volume is observed in case of methanolic extract in carrageenan induced paw edema rats and in formalin induced paw edema rats at different time points. At 60 min and 120 min methanolic extract of *T. asthmatica* showed significant reduction in paw edema and comparable results to standard group whereas petroleum ether extract (400 mg/kg) and chloroform extract at 120 min showed moderate action on paw inflammation as compared to standard. This observation signified that all test compounds have anti-inflammatory properties comparable to Standard drug. But methanol extract of *T. asthmatica* showed significant high effect and decreased paw inflammation efficiently (Table 3 and 4; Figure 1, 2, 3 and 4; Figure 5, 6, 7 and 8).

Table 3: Determination of anti-inflammatory activity by carrageenan induced rat paw edema model

S. No.	Groups (n=5)	Initial paw vol. at 0 min(ml)	Mean Changes in paw vol. (ml) at different intervals		
			30 min	60 min	120 min
1	Vehicle Control	0.3±0.008	0.3±0.008	0.3±0.008	0.3±0.008
2	Ibuprofen (Standard)	0.4±0.011	0.4±0.021	0.4±0.02	0.3±0.021
3	Petroleum ether extract	0.6±0.033	0.6±0.041	0.5±0.03	0.5±0.024
4	Chloroform extract	0.6±0.037	0.6±0.04	0.5±0.014	0.5±0.022
5	Methanol extract	0.5±0.014*	0.4±0.048*	0.4±0.022*	0.4±0.018*

Each value is the mean ±SEM of all estimations. Only statistically significant outcomes at $p<0.05$* have been reported. Analyzed by one way ANOVA test followed by Tukey's multiple comparison post hoc test.

Table 4: Determination of anti-inflammatory activity of formalin induced rat paw edema

S. No.	Groups (n=5)	Initial paw vol. at 0 min(ml)	Mean Changes in paw vol. (ml) at different intervals		
			different intervals	60 min	120 min
1	Vehicle Control	0.3±0.008	0.3±0.008	0.3±0.008	0.3±0.008
2	Ibuprofen (Standard)	0.3±0.011	0.3±0.021	0.3±0.02	0.3±0.021
3	Petroleum ether extract	0.5±0.033	0.5±0.0419	0.5±0.03	0.4±0.024
4	Chloroform extract	0.5±0.0378	0.5±0.04	0.5±0.014	0.5±0.022
5	Methanol extract	0.4±0.014*	0.4±0.048*	0.4±0.022*	0.4±0.018*

Each value is the mean ±SEM of all estimations. Only statistically significant outcomes at $p<0.05$* have been reported. Analyzed by one way ANOVA test followed by Tukey's multiple comparison posthoc test.

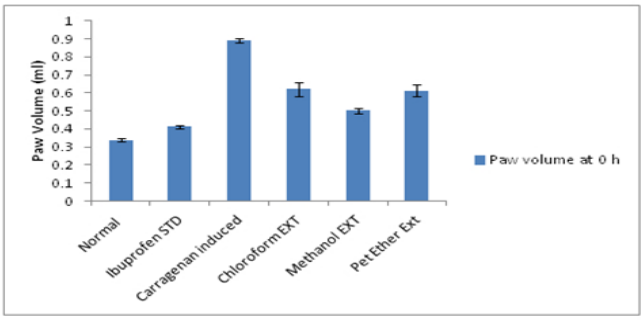


Figure 1: Determination of anti-inflammatory activity of different extracts of *T. asthmatica* in carrageenan induced paw edema rats at 0 min

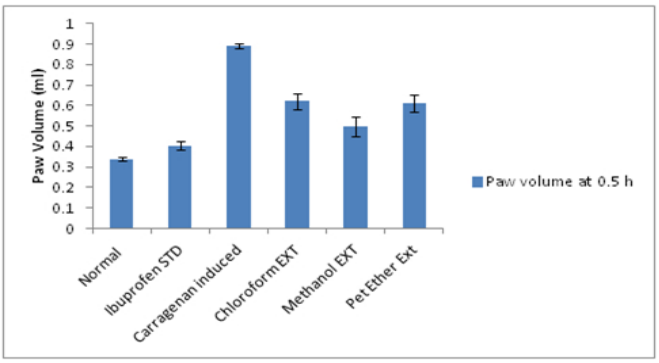


Figure 2: Determination of anti-inflammatory activity of different extracts of *T. asthmatica* in carrageenan induced paw edema rats at 30 min

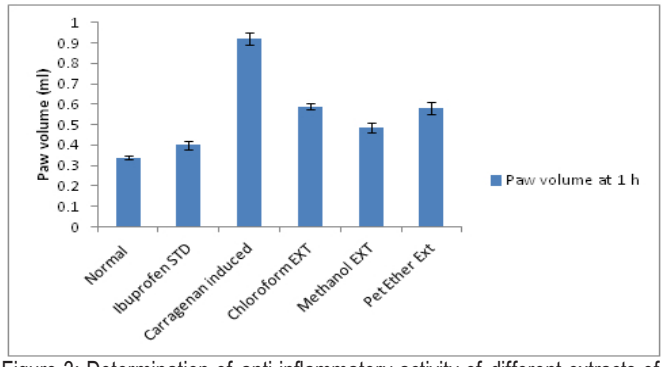


Figure 3: Determination of anti-inflammatory activity of different extracts of *T. asthmatica* in carrageenan induced paw edema rats at 60 min

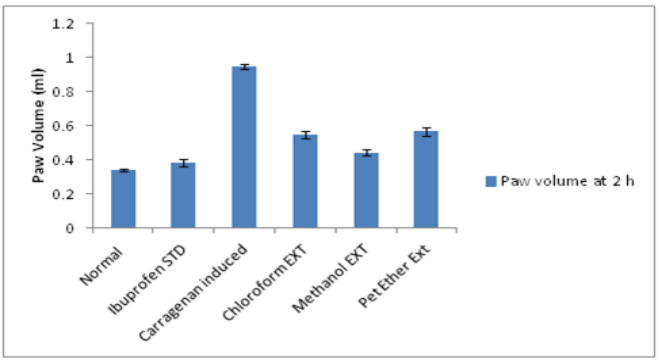


Figure 4: Determination of anti-inflammatory activity of different extracts of *T. asthmatica* in carrageenan induced paw edema rats at 120 min

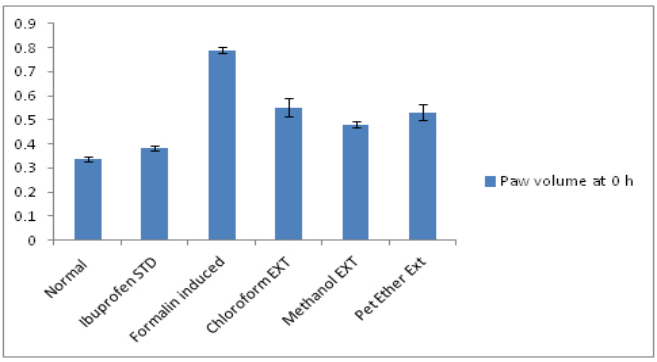


Figure 5: Determination of anti-inflammatory activity of different extracts of *T. asthmatica* in formalin induced paw edema rats at 0 min

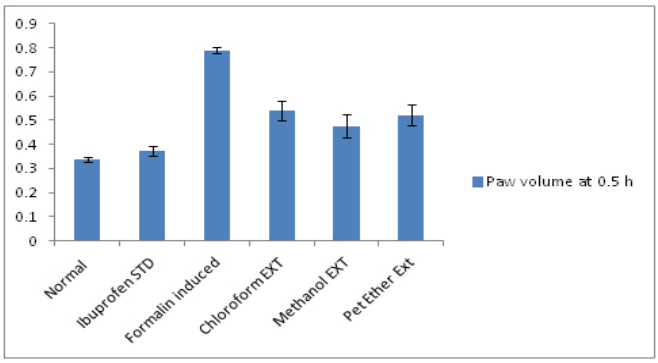


Figure 6: Determination of anti-inflammatory activity of different extracts of *T. asthmatica* in formalin induced paw edema rats at 30 min

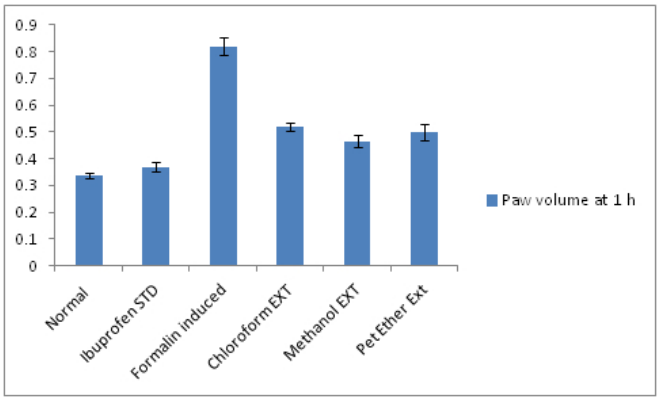


Figure 7: Determination of anti-inflammatory activity of different extracts of *T. asthmatica* in formalin induced paw edema rats at 60 min

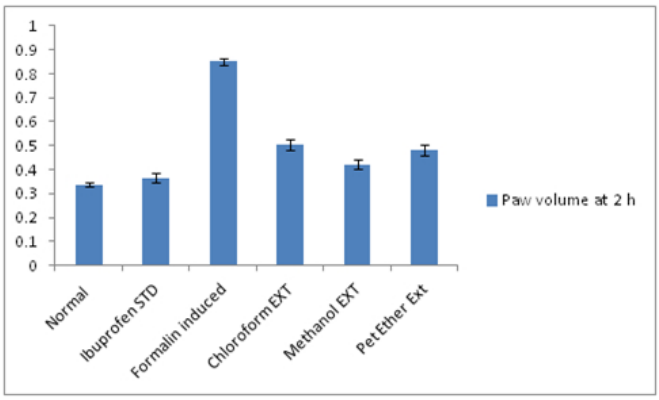


Figure 8: Determination of anti-inflammatory activity of different extracts of *T. asthmatica* in formalin induced paw edema rats at 0 min

Discussion

Inflammation is a universally identified problem and considered as the vital cause of co-morbidity across the people. At acute level, it acts as a defensive response of the human body against incursion of pathogens, which is usually represented by an order of actions such as heat, redness, swelling, pain and loss of function. In normal conditions, autonomic defensive effect of inflammatory cascade and efficiency of tissue destruction can be balanced. However, in chronic conditions inflammation may lead to different states of diseases such as rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and many more [2].

Currently for the management of inflammatory conditions, NSAIDS are the most trusted and first choice of drug among the population, but these are associated with numerous side effects ranging from gastric problems, kidney toxicity to cardiac problems [7]. To minimize the repeated use of synthetic drugs, the interest in the use of medicinal plants is growing tremendously because herbal plant based products have minimal side effects and are considered as healthier replacements for the treatment of various diseases. Moreover, the plants products are cost effective, biocompatible and provide multimode of actions and can be used to cure different diseases [18]. Therefore, the development of investigation of new activities of plant compounds remains to be the subject of interest among researchers. From past few decades, due to the rising number of infectious and inflammatory diseases, the research for the herbal drugs with anti-inflammatory and anti-oxidant activity has extremely increased.

T. asthmatica is known for its anti-allergic, anti-fungal, anti-diabetic and anti-asthmatic activity. It is infused with various active constituents such as 0.46% of alkaloids viz Tylophorine, tylophorinine, tylophorinidine, septicine, isotylocrebrine, tylophoricine, sterols, flavanoids, wax, resins and tannins [11]. The main constituent present is the tylophorine, which is responsible for inflammatory activity. It has been used traditionally for the treatment of bronchial asthma and jaundice. It also has antitumor, immuno-modulatory, anti-oxidant, antiasthmatic and muscle relaxant activities. In the northern parts of the India, its leaves and roots are considered as effective remedy for the treatment of jaundice and liver disorders. It is also used by the people for the treatment of inflammatory bronchitis, allergy and dermatitis. Moreover, it is also reported to have laxative, expectorant, purgative, stimulant, emetic and cathartic properties. From many times, it is also used for treating cold, hay fever and arthritis. Therefore, its various activities clearly indicated that *T. asthmatica* is a golden plant and could also be used to treat acute to chronic inflammatory diseases [13]. So, to investigate inflammatory prosperities of the plant, extracts were prepared using chloroform, petroleum ether and methanol. The results clearly indicated that methanol extract was able to provide maximum yield among all with value of 16.25% w/v. Further, to identify the purity of the extracts, different tests were conducted to check the presence of chemical constituents. Preliminary qualitative phyto-chemical screening of petroleum ether extract revealed the presence of amino acid, glycosides, flavonoids, fats and steroids and chloroform extract showed the presence of alkaloids, carbohydrates, amino acids, steroids, glycosides, tannins, flavonoids and phenolic compounds. Maximum diversity of constituents were present in methanol extract i.e. alkaloids, carbohydrates, amino acids, steroids, tannins and phenolic compounds, flavonoids and glycosides. The inflammatory activity of the petroleum ether, chloroform and methanol extract were carried out using carrageenan and formalin induced rat paw edema models in Wistar rats. Carrageenan induced paw edema model is a sensitive and reproducible method to identify the anti-inflammatory activity of a compound. Carrageenan is an inflammation inducer and responsible for the occurrence of acute as well as chronic inflammatory responses. Due to this it could be used for investigating anti-inflammatory activity of orally administered drugs. In the carrageenan induced paw edema, the first response leads to edema followed by exudation of fluids, plasma proteins and sensitization of primary sensory neurons, which is an essential event of the inflammatory pain [19]. In humans, the sensitization of the sensory neurons usually leads to clinical problems such a hyperalgesia (increased response to painful stimulus) and allodynia (pain evoked due to non-noxious stimuli [20]. On the other hand, formalin induced model is also a suitable procedure to induce paw edema and also involves biphasic events [21]. So, for this study both models were used to assess the anti-inflammatory activity of the develop extracts. The results showed that the methanolic extract was able to inhibit the inflammation events more effectively as compared to the petroleum ether and chloroform extract. In the process of inflammation, one of the major biochemical events that occurred is the changes in the metabolism of connective tissues. Due to which the constituents of connective tissue such as muco-polysaccharides, glycoproteins, hydroxyl proline and sialic acid get altered and their level increases. Pre-liminary investigation showed that the plant extract has large flavonoids, which are responsible for having diuretic, laxative, antispasmodic, antihypertensive and anti-inflammatory activity. Mostly, the inflammatory drugs showed anti-inflammatory activity by inhibiting the release of enzymes which stabilize the lysosomal membrane, which is a main event in the process of inflammation [22]. In this study, it was clearly observed that methanolic extract was able to showed maximum anti-inflammatory activity as compared to the petroleum ether and chloroform extract. It is assumed that it could be due to the inhibition of lysosomal enzymes and stabilizing the membrane. Moreover, the anti-inflammatory action could also be due to the presence of phenanthroindolizidine alkaloids which suppresses the lipopolysaccharides and interferon induced nitric oxide production in cells [23]. Therefore, the findings indicated that the *T. asthmatica* leaves have potent anti-inflammatory activity.

Conclusion

T. asthmatica plant has been used as popular remedy for asthma,

diabetes and allergy. In the present study, the anti-inflammatory activity of various extracts of leaves of *T. asthmatica* was explored along with its phytochemical screening. The developed extracts showed the presence of alkaloids, glycosides and flavonoids. Amongst all the extracts methanol extract showed the maximum percentage of yield. Moreover, the anti-inflammatory activity of various extracts of *T. asthmatica* was carried out using carrageenan and formalin induced rat paw edema models in Wistar rats. The results indicated that the methanolic extract of the leaves of *T. asthmatica* showed maximum anti-inflammatory activity equivalent to Standard drug Ibuprofen and it could be used in development of novel phytotherapeutics and formulations for the treatment of inflammatory diseases.

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Declaration of Interest

The authors declare no conflict of interest.

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