Research Article

Evaluation of acute toxicity and anti-asthmatic activity of *Phyllanthus niruri* L. leaves extracts

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Abstract

Objective: Evaluation of Acute toxicity and Anti-asthmatic activity of the ethyl acetate extract from the leaves of *Phyllanthus niruri* (L.) were studied as per OECD guideline 423. **Materials and Method:** Anti -asthmatic activity of the extract was investigated against compound 48/80 and egg albumin induced mast cell degranulation. Percentage mast cell degranulation was calculated at different concentration level i.e.1, 10, 100 and 1000µg/mL against compound 48/80 and at 200mg/kg body weight of dose P.O. against egg albumin. **Results**: The leaf extracts showed significant protection of rat mesenteric mast cells from disruption caused by compound 48/80 and egg albumin. Significant dose dependent effect was observed in percentage mast cell degranulation at different dose level of compound 48/80 and egg albumin in comparison to negative control at 30.88 ± 0.50 and at 38.26 ± 1.27 mast cell degranulation was observed at 1000µg/mL and 200 mg/kg body dose level of the leaf extracts. **Conclusion**: The present study revealed that the *Phyllanthus niruri* L. leaf extracts of the ethyl acetate fraction has significant anti-asthmatic activity against compound 48/80 -induced mast cell degranulation as well as that induced by egg albumin in comparision to that produced by Ketotifen fumarate. There was no lethal and toxic reactions found in the tested animals.

***Keywords*:** Acute oral toxicity, compound 48/80, egg albumin, mast cell degranulation, Ketotifen fumarate

Introduction

Asthma is a common chronic inflammatory disease of the airways characterized by variable and recurring symptoms, reversible airflow obstruction, and bronchospasm (Yawn, 2008). It may be caused by various factors like allergens, drugs, respiratory infection, dust, cold air, exercise, emotions, occupational stimuli, chemicals, histamine, etc. (Sagar and Sahoo, 2012). It is thought to be caused by a combination of Genetic factor, Environmental factors (like tobacco, hygiene hypothesis, volatile organic compounds, phthalates), Gene–environment interactions, Exacerbation, Socioeconomic factors (Fanta, 2009). The prevalence of asthma has increased significantly since the 1970s. As of 2010, 300 million people were affected worldwide. In 2009 asthma caused 250,000 deaths globally (Global Strategy for Asthma Management and Prevention, 2010). As per a report, there has been an alarming increase in number of diseases and disorders caused by synthetic drugs prompting a switch over to herbal medicine (Bhatt, Upadhyay, Upadhyay, Soni and patel, 2013). The common elicit of asthma such as airway irritants like tobacco smoke, air pollution, allergens, respiratory infections, stress, mold and termites and it is caused through genetic and environmental factors also the asthma attack begins, when the allergen inhaled through respiration and it binds to the IgE antibodies on mast cell in the lung and the mast cell produced histamine, leucotrienes (Sangilimuthu et al., 2015). These biologically active mediators cause the smooth muscle cells or bronchial contract. This cause narrowing the luman of the bronchi due to the accumulation of eosinophils. The repeated attacks or accumulation of eosinophils causes bronchial damage in the lung. In the late stage of the asthma attacks is difficult to breathing due to the smooth muscle cells in the bronchi constrict, and the airway become inflamed and swollen. Asthma is typified by airway eosinophilia, edema, mucus hypersecretion, bronchial epithelial injury and hyperactivity. Disease pathogenesis includes contributions from several cell types including airway epithelial cells, eosinophils, macrophages, dendritic cells, T-helper type 2 (Th2) cells, IgE-secreting B cells and mast cells, as well as

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changes in bronco alveolar lavage fluid (Herrick and Bottomly, 2003). Nearly 300 million people suffered from asthma in worldwide and 2, 50, 000 annual deaths were recorded due to asthma. The prevalence rate of asthma in the last five years was gradually increased. The present treatment methods and resources for asthma have low effectiveness and related to adverse effects subjected to fulfilment (Salib et al., 2003). In view of the above Plants are major sources of natural medicines for human being saving their life from various diseases with their bioactive constituents. However, the systematic evidence sustaining the worth of herbal treatment is imperfect. Few preventive mechanisms maintain the efficacy of herbal remedies in the treatment of asthma. Existing systematic confirmation has not yet established the strength of their accepted role in the treatment of asthma (Szelyni and Brune, 2002). The present article demonstrates the effects of ethyl acetate extracts from the leaves of *Phyllanthus niruri* (L.) in asthma and evaluates its acute toxicity to afford a reasonable point of view of scientific information on herbal therapy and isolated bioactive compounds.

Materials and methods

Collection of plant material

P. niruri (L.) was collected from Burdwan district of West Bengal and authenticated by Dr. Sharmistha Gupta Scientist West Bengal State Council of Science & Technology, Department of Science & Technology, Government of West Bengal. The fresh leaves were cleaned thoroughly with water and dried by shade for three weeks and powdered using grinder.

Preparation of extract

Approximately 150 g of the leaf powder was taken and was successively extracted using 350 ml volumes of solvents of increasing polarity (petroleum ether, ethyl acetate, ethanol and water) for 3 days of period using percolator. Extracts were evaporated and, residue was collected. Total residue was weighed and stored in an air tight container.

Phytochemical screening

The ethyl acetate extracts of *P. niruri* was subjected to qualitative analysis for the various phytoconstituents like alkaloids, carbohydrates, glycosides, phytosterols, saponins, tannins, proteins, amino acids and flavonoids. Phytochemical examinations were carried out as per the standard methods (Tiwari et al., 2011).

1. Test for Reducing Sugars: The aqueous ethanol extract (0.5 g in 5 ml of water) was added to boiling Fehling's solution (A and B) in a test tube. The solution was observed for a colour reaction.

2. Test for Anthroquinone: To 0.5 g of the extract was boiled with 10 ml of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for color changes.

3. Test for Saponins: To 0.5 g of extract was added 5 ml of distilled water in a test tube. The solution was shaken vigorously and the mixture is observed for a stable persistent froth. The frothing was mixed with 3 drops of Olive Oil and shaken vigorously after which it was observed for the formation of an emulsion.

4. Test for Cardiac Glycosides: To 0.5 g of extract diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

5. Test for Terpenoids: To 0.5 g each of the extract was added 2 ml of chloroform. Concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids.

6. Test for Tannins: About 0.5 g of the extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue -black colouration.

7. Test for Flavonoids: Three methods were used to test for flavonoids. First, dilute ammonia (5ml) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1 ml) was added. Second, a few drops of 1% aluminium solution were added to a portion of the filtrate. Third, a portion of the extract was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. In all the cases, a yellow coloration indicating the presence of flavonoids was observed.

8. Test for Alkaloids: Drug solution along with few drops of Mayer's reagent (potassium mercuric iodide), formation of creamy-white precipitant.

Drugs and chemicals

Compound 48/80 was purchased from Sigma Aldrich, ketotifen fumarate and toluidine blue were procured from local market along with egg albumin for the study. Histamine solution was freshly prepared in normal saline (NaCl, 8.5 g/l). All the other chemicals were of analytical grade. Chemicals were also obtained from Merck (Mumbai, India), Ranbaxy (New Delhi, India) and Qualigens

(Mumbai, India).

Experimental Animals

Healthy Swiss albino mice (25-30g) of either sex were selected for acute oral toxicity study and Wistar albino rats (180-210g) were taken to evaluate anti -asthmatic activity. All the experimental animals were fed on commercial pellet diet (Amrut, Pranav Agro Industries Ltd, India). They were group housed under standard conditions of temperature (22±2^{0}C), relative humidity (60±5%) and 12:12 light/dark cycle. They were divided in groups of six animals each. The saline fed group served as control and one group was treated with a standard drug. Before experimentation, the animals were kept on fast for 24 h but water was given ad libitum. During experiments, animals were also observed for any alteration in their general behavior. All the experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC), Sigma Institute of Pharmacy (SIP/IAEC/03/2012-13). All the experiments and the care of the laboratory animals were conducted according to current ethical guidelines by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India, New Delhi.

Acute Oral Toxicity Study

The acute toxicity study was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD), revised draft guidelines no. 423, received from Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Govt. of India (OECD, 2002)**.** Vehicle (0.5% carboxymethyl cellulose; CMC) (Group I) was used as control and group.

Anti-asthmatic activity of ethyl acetate extract on Compound 48/80 induced Mast Cell degranulation

Six rats were taken for the study. Normal saline containing 5units/ml of heparin was injected in the peritoneal cavity of rat lightly anaesthetized with ether. After a gentle abdominal message, the peritoneal fluid containing mast cell was collected in centrifuge tubes placed over ice. Peritoneal fluid of 6 rats were collected and centrifuged at 2000rpm for 5 min. Supernatant solution was discarded and the cells were washed twice with saline and re-suspended in 1 ml of saline (Parmar et al., 2010). 7 samples were prepared in different test tubes for each rat's peritoneal fluid. In the below manner 6 sets of such 7 samples were prepared.

Test tube no. 1: 0.1ml peritoneal fluid

Test tube no. 2: 0.1ml peritoneal fluid + 0.1ml compound 48/80

Test tube no. 3: 0.1ml peritoneal fluid + 0.1ml compound 48/80 + 0.1ml of 10µl/ml of ketotifen fumarate

Test tube no. 4: 0.1ml peritoneal fluid + 0.1ml compound 48/80 + 0.1ml of test agent in saline (1µl/ml of extracts)

Test tube no. 5: 0.1ml peritoneal fluid + 0.1ml compound 48/80 + 0.1ml of test agent in saline (10µl/ml of extracts)

Test tube no. 6: 0.1ml peritoneal fluid + 0.1ml compound 48/80 + 0.1ml of test agent in saline (100µl/ml of extracts)

Test tube no. 7: 0.1ml peritoneal fluid + 0.1ml compound 48/80 + 0.1ml of test agent in saline (1000µl/ml of extracts).

Each test tube was incubated for 15 minutes at 37°C with peritoneal fluid and respective drug treatment. The aliquots were carefully spread over glass slides and the mast cells were stained with 1% toluidine blue. Then slides were dried in air and mast cells were counted using randomly selected high power objective fields (X450). The percent degranulation of the mast cells was calculated by following formula.

%inhibition of Mast Cell Degranulation = [1- Number of degranulated mast cells / Total number of mast cells] × 100

Anti-asthmatic activity of ethyl acetate extract on Egg albumin induced allergy condition due to Mast Cell degranulation

A method described by Norton (1954) was used. The rats were sensitized by 0.1 ml of 1% w/v egg albumin solution intraperitoneally as well as subcutaneously on the first, third, fifth and twelfth day of first egg albumin administration. The animals were administered drug as per the following schedule from sixth day to twelfth day.

Group I : Control (0.5 % sodium CMC)

Group II : Prednisolone (2 mg/kg. p.o.)

Group III : Ethyl acetate extract of *P. niruri* (100 mg/kg)

Group IV : Ethyl acetate extract of *P. niruri* (200 mg/kg)

Group V : Aqueous extract of *P. niruri* (100 mg/kg)

Group VI : Aqueous extract of *P. niruri* (200 mg/kg)

On Day 12, animals were again exposed to 0.05 ml of 1% egg albumin. The animals were sacrificed with ether on the twelfth day after one hour of oral administration. Pieces of mesentery with connecting lobes of fat and blood vessels were rapidly dissected out and placed in Ringer-Locke solution (NaCl 0.9%, KCl 0.042%, CaCl2 0.024%, NaHCO3 0.015% and dextrose 0.1%) in different Petri dishes with labels and then treated with 0.1 ml of 1% w/v solution of egg albumin for 20 min. All these processes were done at room temperature. Then all these mesenteries were transferred in separately labeled Petri dishes containing 10% formaldehyde solution for 24 h to stop further physiological changes in mast cell structure. Then these

mesenteries were stained with 0.1% toluidene blue for 10 min. Then these mesenteries were wash with 95% alcohol to remove traces of water and then with xylene. Usually five pieces of mesentery were used for each concentration of drug. Each piece was observed under microscope with 45× and 10× magnification and 100 cells were counted and from it, percentage protection from degranulation was calculated (Patel et al., 2011).

Statistical analysis

The results were expressed as mean values ± S.E.M. (standard error of mean). The significance was evaluated by one way ANOVA, followed by Tukey's multiple comparison test. The results were considered statistically significant when $P < 0.05$.

Results and discussion

Phytochemical screening

Ethyl acetate leaf extracts revealed the presence of reducing sugars, anthraquinones, saponins, terpenoids, alkaloids, flavonoids, and tannins, cardiac glycosides were found to be absent.

Acute Toxicity Study

During the acute oral toxicity study, the animals did not show any signs of toxicity and mortality at 2000 mg/kg single dose any sign of toxicity and mortality at 2000mg/kg single dose administration of ethyl acetate leaf extracts of *P. niruri*. The body weight was not significantly increased or decreased and results were shown in table 1 and figure 1.

Anti-Asthmatic Activity of Ethyl Acetate extract from the leaves of *Phyllanthus niruri* (L.) On Compound 48/80 Induced Mast Cell Degranulation

In normal control group 01.01 ± 0.28 % mast cell degranulation was observed. Compound 48/80 added to the Negative control showed 85.26 ± 0.50 % mast cell degranulation. Pre incubation of rat peritoneal cells with the Positive control at a concentration of 10µg/ml for 15 minutes showed 28.82±0.27% mast cell degranulation. Prior incubation of rat peritoneal mast cells with test drug (Ethyl acetate leaf extracts of *P. niruri)* at 1 µg/ml, 10 µg/ml, 100 µg/ml, 1000 µg/ml for 15 minutes showed in significant reduction of % mast cell degranulation in a dose dependent manner 57.00 ±0.63, 44.60 ±1.15, 39.00 ± 0.33, 30.88 ± 0.50 respectively, in comparision to reference standard drug, the result is depicted in table 2.

Table 1. Effect of Ethyl acetate extracts of *Phyllanthus niruri* (L.) on body weight of mice at 2,000 mg/kg body weight

Product	Dose	Mean Body Weight (G)		
		0 Day	7th Day	14th Day
0.5% Carboxymethyl Cellulose (CMC)	2000 mg/Kg	26.23±0.96	26.43± 0.96	27.03±0.99
Ethyl acetate extract from the leaves of *Phyllanthus niruri* (L.)	2000 mg/Kg	26.38±0.98	26.73±0.97	27.44±0.74

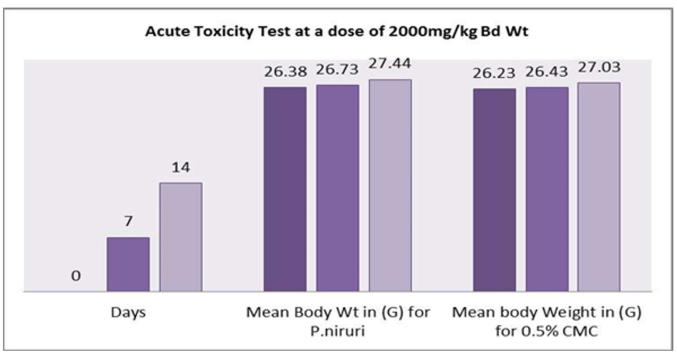

Figure 1. Acute toxicity study of extract

Table 2. Effect of Ethyl acetate extracts of *Phyllanthus niruri* (L.) on % Mast cell degranulation (Compound 48/80)

Treatment groups	Concentration	% Mast cell degranulation
Normal Control		01.01 ± 0.28
Negative Control treated with Compound 48/80	-	85.26 ± 0.50
Positive control (Ketotifen fumarate) (Standard)	10µg/ml	28.82 ± 0.27**
Test-1(Leaf Extract)	1µg/ml	57.00 ± 0.63**
Test-2(Leaf Extract)	10µg/ml	44.60 ± 1.15**
Test-3(Leaf Extract)	100µg/ml	39.00 ± 0.33**
Test-4(Leaf Extract)	1000µg/ml	30.88 ± 0.50**

All values are represented as Mean ±S.E.M, Where n= 6; Significance at the level of ** $p<0.001$ as compared to Negative Control

Anti-Asthmatic Activity of Ethyl Acetate extract from the leaves of *Phyllanthus niruri* (L.) On Egg albumin induced Degranulation of Mast Cell

As compared to control group, the ethyl acetate extracts from the leaves of ***Phyllanthus niruri* (L.)** demonstrated a dose-dependent significant mast cells protection at doses of (100 and 200 mg/kg, p.o.) where the intact mast cell percentage varied from 48.87±1.66 and 60.23±1.27 and disrupted mast cells percentage varied from 53.18±1.66 and

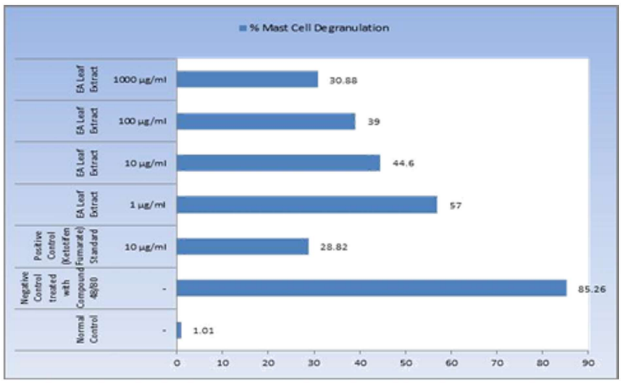

Figure 2. Effect of Ethyl acetate extracts of *Phyllanthus niruri* (L.) on percent Mast cell degranulation

Table 3. Effect of Ethyl acetate extracts of *Phyllanthus niruri* (L.) on % Mast cell degranulation (egg albumin)*

Treatment	Dose (mg/kg) P.O	Mast Cells (Intact Cell %)	Mast Cells (Disrupted Cells %)
Control	--	26.18 ±1.10	75.02 ±1.10
Prednisolone	002**	67.33 ±1.51	33.66±1.51
Ethyl Acetate Extract	100	48.87 ±1.66	53.18 ± 1.66
Ethyl Acetate Extract	200	60.23 ±1.27	38.26 ± 1.27

*Values are expressed as mean ± SEM (n=5)
**Intraperitoneal Route
***p<0.01, compared with control (One Way ANOVA followed by Dunnett Multiple Comparisons Test)

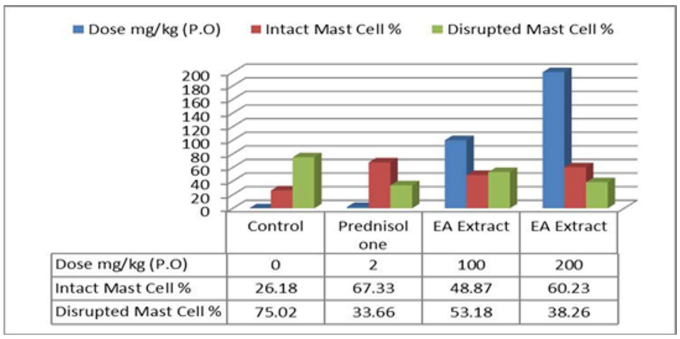

Figure 3. Effect of Ethyl acetate extracts of *Phyllanthus niruri* (L.) on egg albumin induced percent Mast cell degranulation

38.26 ±1.27 ($p < 0.01$) as shown in table 2, Whereas prednisolone (2 mg/kg, i.p.) had similar effect with ($p < 0.01$).

Conclusion

From the above results it can be concluded that the ethyl acetate extracts from the leaves of *Phyllanthus niruri* (L.) have promising results in tackling asthma in rats. Moreover the extracts gave positive results for the presence of Reducing Sugars, Anthraquinone, Saponins, Cardiac glycosides, Terpenoids, Tannins, Flavonoids, Alkaloids with toxicity within the limits of consideration, so these extracts may be assumed safe to use possessing mast cell stabilisation properties. These extracts can be useful in the treatment of asthma in higher organisms.

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