Research Article

Evaluation of anti-asthmatic activity of *Neuracanthus sphaerostachyus* Dalz. leaves extract

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Abstract

Objective: *Neuracanthus sphaerostachyus* has been used traditionally to cure skin diseases, cough, and asthma in western ghat. Extensive review of literature and evidences indicating the utility of this plant in the treatment of asthma prompted us to investigate the anti-asthmatic activity of the plant in different experimental screening methods. **Materials and Methods:** In vitro & in-vivo anti-asthmatic activity of the methanolic and aqueous extract of *Neuracanthus sphaerostachyus* leaves was investigated using various experimental models. In-vivo studies like compound 48/80 induced systemic anaphylaxis and In-vitro studies like compound 48/80 induced mast cell degranulation, Milk induced leucocytosis & eosinophilia was evaluated at 125, 250, and 500 mg/kg, orally doses. **Results:** Methanolic and aqueous extract of the drug showed a significant bronchodilation, anti-histaminic, mast cell stabilizing, and anti-cholinergic activity in their respective evaluation parameters dose-dependently. Ketotifen fumarate (10 µg/ml), Dexamethasone (50 mg/kg) and Disodium chromoglycate (10 mg/kg) were used as a standard control. **Conclusion:** The present study concluded that methanolic and aqueous extract of *Neuracanthus sphaerostachyus* has significant anti-asthmatic potential benefit.

Keywords: *Neuracanthus sphaerostachyus*, compound 48/80, mast cell degranulation, leukocytosis, eosinophilia

Introduction

Asthma is a chronic inflammatory disorder with narrowing of bronchial airways (Limbasiya, 2012). World health organization stated asthma as a chronic disease with episodes of the airways and can be viewed as a syndrome (WHO, 1991). Asthma involves bronchial tubes and shows wheezing, shortness of breath and coughing. It is an allergic reaction which induces inflammation leading to narrowing of the airways and causes spasm and difficulty in breathing. Asthma can be seen in both children and adults in developed as well as developing countries. Allergic patients are more prone to develop asthma. Asthma may involve various inflammatory cells like eosinophils, macrophages, mast cells, epithelial cells, and activated lymphocytes. Inflammatory cells go on recruit various cytokines, adhesion molecules, and other mediators. Inflammation may progress to an acute, sub-acute or chronic phase which produces airway remodeling, an increase in vascular permeability as well as increases in mucus secretion. It alters airway structure reversibly or permanently (Limbasiya, 2012).

Herbal medicines are the backbone of about 75 to 80% of the world, especially in developing countries. It has better access to primary health care with good acceptability, more compatible with the human body and, less side effects. While focusing on the biological activities of plants during the last decade, it shows the presence of plenty of compounds with anti-asthmatic potential (Singh et al., 2014).

Neuracanthus sphaerostachyus Dalz known as Pincushion plant due to its floral structure and commonly known as Putliyo (Hindi), Golgonda (Marathi) and Ganthera – Gandharo (Gujarati). It is native to Indian regions and widely distributed in the Western Ghats (Goa), Deccan and

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throughout Gujarat (Khare, 2007). This plant is traditionally used in different areas of the Western Ghats. The mixture of ash of the whole plant with jaggery or honey is used for 2 - 3 times a day orally to cure cough and asthma (Punjani and Kumar, 2002). Root paste is applied to ringworm. *Neuracanthus sphaerostachyus* shows the presence of vanillic acid, syringic acid, melilotic acid and, 6-OH luteolin (Daniel and Sabnis, 1987).

The scientific literature survey reveals no report on the pharmacological investigation of *Neuracanthus sphaerostachyus* leaves prompted us to evaluate the anti-asthmatic property of leaf extracts.

Materials and methods

Collection and authentication of plant

Neuracanthus sphaerostachyus Dalz. Leaves were collected from Girnar forest region of Junagadh, Gujarat. Plant material was authenticated by NISCAIR-CSIR (National Institute of Science Communication and Information Resources), New Delhi (NISCAIR/RHMD/Consult/2016/2987-14).

Extraction of plant material

Extractive values of crude drugs were used to determine the number of active constituents extracted with solvents from a given amount of medicinal plant material. The successive extraction was carried out in soxhlet apparatus with a known quantity of powder in different organic solvents like Hexane, chloroform, methanol and then water. After exhaustive extraction, the solvent was filtered and concentrated under reduced pressure at 50-55°C (Indian Pharmacopoeia, 1966).

Chemicals

Ketotifen and Disodium chromoglycate were procured from Chemdyes Corporation, Rajkot. Dexamethasone was obtained from Restech Pharmaceuticals, Ahemdabad.

Animals

Female Wistar rats (150-200 g) and Swiss albino mice (25-30 g) were used for the study. The animals were kept in polypropylene cages and maintained at a temperature of 26 ± 2°C. Animals were fed with diet provided by Pranav agro industries Ltd., Sangli. All the animal experiments were conducted in accordance with the guidelines of the CPCSEA (Reg. No. 1846/PO/RE/s/16/CPCSEA), a guide for the care and use of laboratory animals. The animals were acclimatized for 10 d under standard husbandry conditions as Relative humidity 45 - 55%, and 12 h light and dark cycle (CPCSEA, 2003).

Acute toxicity study

Female Wistar rats of 150 – 200 g and Swiss albino mice of 25-30 g body weight were selected to find out the acute toxicity study of methanolic and aqueous extract of *Neuracanthus sphaerostachyus* leaves. The dose of 2000 mg/kg was selected on the basis of Up and Down Procedure (UDP) as per OECD Guideline No.425. All animals were observed for 24 h to detect autonomic or behavioral changes in responses to the extracts. Then the Mortality in each group was observed for 14 d (OECD, 2001). The methanolic and aqueous extracts of leaves of *Neuracanthus sphaerostachyus* were found to be nontoxic at a dose of 1500 mg/kg, orally. Hence, LD cut off value of methanolic and aqueous extract were fixed as 1500 mg/kg. Therefore $1/10^{th}$, $1/6^{th}$, $1/3^{rd}$ of the LD_{50} cut off value that was approximately 150, 250 and 500 mg/kg were selected as screening dose for anti-asthmatic activity.

Assessment of *in vitro* anti-asthmatic activity

Compound 48/80 induced degranulation of rat mesenteric mast cells

Normal saline containing 5 units/ml of heparin was injected in the peritoneal cavity of male rats lightly anesthetized with ether. After a gentle abdominal massage, peritoneal fluid containing mast cell was collected in centrifuge tubes placed over ice. Peritoneal fluid of 5 rats was collected and centrifuged at 2000 rpm for 5 min. The supernatant solution was discarded and the cells were washed twice with saline and re-suspended in 1 ml of saline. (MENS- Methanolic extract of *Neuracanthus sphaerostachyus* leaves, AENS – Aqueous extract of *Neuracanthus sphaerostachyus* leaves)

Test tube I: Normal control, 0.1 ml peritoneal fluid

Test tube II: Disease control, 0.1 ml peritoneal fluid + 0.1 ml compound 48/80.

Test tube III: Standard control, 0.1 ml peritoneal fluid + 0.1 ml compound 48/80 + 0.1 ml Ketotifen fumarate (10 µg/ml)

Test tube IV: 0.1 ml peritoneal fluid + 0.1 ml compound 48/80 + MENS (0.5 mg/ml)

Test tube V: 0.1 ml peritoneal fluid + 0.1 ml compound 48/80 + MENS (1 mg/ml)

Test tube VI: 0.1 ml peritoneal fluid + 0.1 ml compound 48/80 + MENS (2 mg/ml)

Test tube VII: 0.1 ml peritoneal fluid + 0.1 ml compound 48/80 + AENS (0.5 mg/ml)

Test tube VIII: 0.1 ml peritoneal fluid + 0.1 ml compound 48/80 + AENS (1 mg/ml)

Test tube IX: 0.1 ml peritoneal fluid + 0.1 ml compound 48/80 + AENS (2 mg/ml)

Each test tube was incubated for 15 min at 37°C with peritoneal fluid and respective drug treatment. Compound 48/80 (0.1 µg/ml) was added to each test tube except test

tube no. I. After further incubation for 10 min at 37°C, the cells were stained with 0.1 % toluidine blue solution made in distilled water and examined under the high power of light microscope. Same procedure was repeated in triplicate manner. % degranulation of the mast cells in the different test tubes treated with different doses of methanolic and aqueous extracts was calculated by counting the number of degranulated mast cells from total of at least 100 mast cells counted under microscope and from it percentage protection against degranulation was calculated. Each piece was observed under high power light microscope (Vogel, 2008).

% Protection of mast cell from total of at least 100 mast cells will be counted.

% Protection of mast cells=Total mast cells-Degranulated mast cells

Milk induced leucocytosis and eosinophilia

Swiss albino mice (female) of 25-30 gm were selected and randomly divided into nine groups of six each. All samples including suspensions, solutions of drugs and plant extracts were freshly prepared. The extracts were used as a suspension in 0.5% v/v Tween 80 in normal saline (0.9%) and administered orally. Blood samples were collected from retro orbital plexus under light ether anesthesia and different groups were assigned as described below:

Group I : Normal control, Distilled water
Group II: Disease control, Boiled cooled milk (4 ml/kg, s.c.)

Group III: Standard control, Dexamethasone (50 mg/kg i.p.)

Group IV: Boiled, cooled milk + MENS (150 mg/kg)

Group V: Boiled, cooled milk + MENS (250 mg/kg)

Group VI: Boiled, cooled milk + MENS (500 mg/kg)

Group VII: Boiled, cooled milk + AENS (150 mg/kg)

Group VIII: Boiled, cooled milk + AENS (250 mg/kg)

Group IX: Boiled, cooled milk + AENS (500 mg/kg)

Treatment drugs were administered orally 1 h before milk injection. Total leucocyte and eosinophil count was performed in each group before and after 24 hours of milk injection. Total and differential count was performed in high power light microscope (Vogel, 2008; Taur and Patil, 2012; Mali and Dhake, 2011; Limbasiya, 2012).

Total leucocyte and eosinophil count was measured before and 24 hours after milk injection.

Assessment of *in vivo* anti-asthmatic activity

Compound 48/80 induced systemic anaphylaxis

The study was conducted by using swiss albino mice. Compound 48/80 was administered by intraperitoneal route to the albino mice which causes mortality. The mice of different 9 groups were pretreated with respective different doses of various plant extract by oral route and the standard drug, Disodium chromoglycate by intraperitoneal route. After one hour of the treatment compound 48/80 was given by intraperitoneal route.

Group I : Normal control, saline (1 ml/kg)
Group II : Disease control, Compound 48/80 (8 mg/kg)

Group III: Standard control, Disodium chromoglycate (10 mg/kg)
Group IV : Methanolic extract of leaves of *Neuracanthus sphaerostachyus* (150 mg/kg)
Group V: Methanolic extract of leaves of *Neuracanthus sphaerostachyus* (250 mg/kg)

Group VI: Methanolic extract of leaves of *Neuracanthus sphaerostachyus* (500 mg/kg)

Group VII: Aqueous extract of leaves of *Neuracanthus sphaerostachyus* (150 mg/kg)

Group VIII: Aqueous extract of leaves of *Neuracanthus sphaerostachyus* (250 mg/kg)

Group IX: Aqueous extract of leaves of *Neuracanthus sphaerostachyus* (500 mg/kg)

The mortality was observed after 1 hour of anaphylactic shock. Percentage of mortality was calculated by using the following formula (Chitme, 2010, Subhashini et al., 2013, Mehta, 2008, Das and Chauhan 2013).

Percentage of mortality= (No.of dead mice/Total No. of mice) ×100

Statistical analysis

All values are presented as mean ± SEM of six animals. Differences between means were assessed by one-way analysis of variance (ANOVA) followed by Dunnett's test. $P<0.05$ was considered significant.

Results

Compound 48/80 induced degranulation of rat mesenteric mast cells

MENS was significantly effective in inhibiting compound 48/80 induced degranulation of rat mesenteric mast cells at 2 mg/ml dose with76.26 % inhibition of degranulation. Standard anti-asthmatic drug ketotifen fumarate showed the maximum inhibition of degranulation with 82.46% and compared with disease control (Table 1).

Milk induced leucocytosis and eosinophilia

The effects of extracts of MENS and AENS on milk induced leucocytosis and eosinophilia are shown in table 2 and 3. The methanolic extract at 500 mg/kg dose showed a

Table 1. Effect of MENS and AENS on compound 48/80 induced degranulation of rat mesenteric mast cells

Treatment	Concentration (mg/ml)	% Mast cell degranulation	% Inhibition of degranulation
Normal control	-	2.03±0.256	-
Disease control	-	72.83±0.546	-
Standard control	0.01	12.362±0.245***	82.46
MENS	0.5	28.25±1.023*	61.23
MENS	1	22.56±0.356*	64.43
MENS	2	17.03±0.589**	76.26
AENS	0.5	33.56±0.128*	56.74
AENS	1	26.28±0.652*	61.29
AENS	2	20.46±0.458**	72.56

Values were expressed as mean ± SEM. N=6 in a group; *p<0.05, **p<0.01, ***p<0.001; Vs Disease control. Data are analysed by using one way ANOVA followed by Dunnett's test.

Table 2. Effect of MENS and AENS on milk induced leukocytosis in mice

Treatment	Concentration (mg/kg)	Difference in total leucocyte count (cu/mm)
Normal control	-	78±11.25
Disease control	-	4456±218.12
Standard control	50	2108±158.06***
MENS	150	4123±128.24
MENS	250	3624±246.42*
MENS	500	2621±214.24**
AENS	150	4223±198.26
AENS	250	3841±126.84*
AENS	500	2832±196.08**

Values were expressed as mean ± SEM. N=6 in a group; *p<0.05, **p<0.01, ***p<0.001; Vs Disease control. Data are analysed by using one way ANOVA followed by Dunnett's test.

Table 3. Effect of MENS and AENS on milk induced eosinophilia in mice

Treatment	Concentration (mg/kg)	Difference in eosinophil count (cu/mm)
Normal control	-	34±3.21
Disease control	-	148±8.36
Standard control	50	56±2.36***
MENS	150	110±5.34
MENS	250	91±6.32*
MENS	500	62±4.28**
AENS	150	118±6.26
AENS	250	96±4.48*
AENS	500	64±2.64**

Values were expressed as mean ± SEM. N=6 in a group; *p<0.05, **p<0.01, ***p<0.001; Vs Disease control. Data are analysed by using one way ANOVA followed by Dunnett's test.

maximum anti-asthmatic effect (Table 2 and 3). The anti-asthmatic effect of the MENS was more potent and significant, compared to AENS.

Compound 48/80 induced systemic anaphylaxis

Compound 48/80 showed 100% mortality in mice. Standard control – DSCG showed 83% protection from mortality. Treatment with MENS 250, 500 mg/kg and AENS 250, 500 mg/kg showed protection dose dependently against compound 48/80 induced mortality. A significant protection (67%) was observed at 500 mg/kg of MENS treatment (Table 4).

Discussion

Mast cell degranulation is an important parameter in the initiation of many immediate responses following to exposure with kinds of allergens. Degranulated mast cells allow to release mediators of inflammation like histamine, leukotrienes, platelet activating factors (PAFs) with chemotactic factors for eosinophils, neutrophils etc (Limbasiya, 2012). Compound 48/80 (10 µg/ml) produced significant disruption of mast cells which was significantly inhibited in a dose-dependent manner by pretreatment with the methanolic and aqueous extracts of *Neuracanthus*

Table 4. Compound 48/80 induced systemic anaphylaxis

Sr. No.	Treatment	Dose (mg/kg)	% Mortality
I	Normal control	-	-
II	Disease control	8	100
III	Standard control	10	17***
IV	MENS	150	100
V	MENS	250	50**
VI	MENS	500	33***
VII	AENS	150	100
VIII	AENS	250	83
IX	AENS	500	50**

Values are expressed as mean ± SEM. N=6 in a group; *p<0.05, **p<0.01, ***p<0.001 Vs Disease control. Data are analysed by using one way ANOVA. Mortality (%) within 1 h following compound 48/80 injection was represented as the number of dead mice ÷ total number of mice × 100.

sphaerostachyus in 0.5, 1 and 2 mg/kg doses. The results were comparable to the reference standard ketotifen (10 µg/ml). Thus, it indicates that MENS has significant mast cell stabilizing activity.

Leukocyte recruitment releases the inflammatory mediators such as cytokines, histamine and other asthmatic inflammatory cells promoting ongoing inflammation. Biopsies taken from asthmatics exhibits eosinophil as the most characteristic inflammatory cell in the respiratory tract and reside in submucosal and epithelial layers. Eosinophil count to more than 4% of total leukocyte is an abnormal increase and termed as eosinophilia. Most cases of asthma involve increase in the eosinophil count. Eosinophil is a critical contributing parameter involving bronchial mucosa with allergic inflammation to the late asthmatic reaction producing hyper-secretion of mucus and bronchial congestion. Eosinophil plays role as an inflammatory cell during later phases in the development of allergic asthma which secretes mediators such as tumor necrosis factor (TNF-α), eosinophil cationic protein, neurotoxins derived from eosinophil and prostaglandins resulting in epithelial shedding, bronchospasm, and allergic inflammation in the respiratory tract.

Milk induced leukocytosis and eosinophilia in mice mimics the condition of stress induced asthma and it helps to evaluate such clinical findings (Mali and Dhake, 2011). It has been exhibited that parental administration of milk leading to increasing leukocyte and eosinophil count significantly after 24 hours of administration. Among both these extracts, 250 and 500 mg/kg dose has shown significant activity as compared to standard Dexamethasone (50 mg/kg) in a dose-dependent manner.

Systemic anaphylaxis involves allergic reactions to occur and may exhibit fatal consequences. The process can be demonstrated by challenging mice with compound 48/80. Untreated mice may show 100% mortality. It involves the chain of reaction with decreased cyclic AMP production and histamine release due to the influx of Ca^{+2} (Kimata et al., 2000). However, pre-treatment with both the methanolic and aqueous extracts of leaves of *Neuracanthus sphaerostachyus* showed significant protection up to 67% with a low mortality rate at 500 mg/kg dose.

Conclusion

The present study establishes acute toxicity study, in vitro & in vivo anti-asthmatic screening for methanolic and aqueous extracts of leaves of *Neuracanthus sphaerostachyus.* The anti-asthmatic activity of *Neuracanthus sphaerostachyus* is might be due to the presence of Flavonoid (6-OH Luteolin) and other various potential phytoconstituents like Phenolic compounds / Tannins, Steroids and, Triterpenoids (Daniel and Sabnis, 1987). Methanolic extract shows significant anti-asthmatic activity at a dose of the 500mg/kg. Further investigations are required to extrapolate active component of the extract and to establish the mechanism of action.

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Conflicts of interest

The authors declared that they have no conflicts of interest.

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