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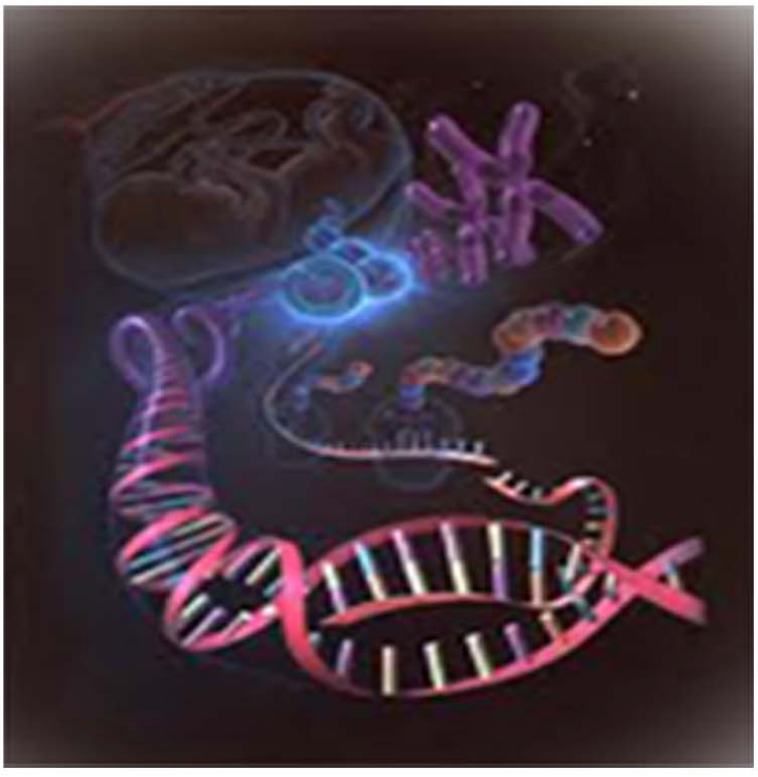

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Research Paper

ANTI-HISTAMINIC AND MAST CELL STABILIZING ACTIVITY OF A FERN-*LYGODIUM FLEXUOSUM*

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Increasing pollution has elicited various airway complications of which the Allergic asthma is one. Manifestation of allergic asthma is always associated with anaphylaxis and mast cell degranulation. In the pursuit of new herbal drugs to prevent or treat allergic asthma, *Lygodium flexuosum* was studied for its anti-histaminic and mast cell stabilizing activity. In the present study rats were sensitized by injecting horse serum and triple antigen. The ethanolic extract at 250 mg/kg and 500 mg/kg showed intact mast cells 70.67% and 72.00% while the 250 mg/kg and 500 mg/kg of aqueous extract showed 69.17% and 70.33%, respectively. Both the extracts at two different doses also showed protective activity *in vitro* passive mast cell degranulation and systemic anaphylaxis in mice. The percent protection shown by the two extracts were also noteworthy in case of compound 48/80 induced mast cell degranulation assay. To further elucidate the role of this fern in anaphylaxis and anti-histaminic principles the nitric oxide level in serum and peritoneal fluid and Histamine content were carried out in whole blood, respectively. At the end, antigen-antibody interaction was monitored which validated the beneficial effects of the climbing fern, *Lygodium flexuosum* in allergic asthma.

Keywords: *Lygodium flexuosum*, Mast cells, Compound 48/80, Anti-histaminic

INTRODUCTION

Allergic asthma is creating havoc in the present days as millions of people are exposed to filthy pollutants and dust particles. It has become a significant cause of morbidity and mortality in developed countries as well as developing countries (Braman, 2006). According to the Health Ministry's latest report released by the Indian Council of Medical Research, severe burden of Chronic Respiratory Diseases (CRDs) is emerging in India as 13 million people aged 15 years and above suffer from asthma (Sinha, 2010). In asthma and allergic diseases mast cells play an important role in defending the intruding pathogens (Prussin *et al*., 2003). IgE antibody formed in response to an allergen has a strong tendency to attach to mast cell and basophils through their surface receptors. While doing so,

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degranulation of mast cells occurs by antigen-antibody reactions which leads to a Type I allergic reactions (Chaudhary, 2006). In doing so, this in turn rapidly releases pro-inflammatory mediators such as histamine and eicosonoids (Geetha *et al*., 1981).

As an alternative medicine to cure allergic asthma herbal medicines have been used for hundreds of years (Mahajan *et al*., 2011). Particularly, the ferns had an important role in folklore medicine. These pteridophytes have been successfully used in the different systems of medicines like Ayurvedic, Unani, Homeopathic and other systems of medicines (Perumal, 1935).

Lygodium flexuosum Linn. (Schizaeaceae) commonly known as 'Bhutraj' or 'Maiden hair creeper' is an important medicinal plant. Fresh roots are used in external application for rheumatism, sprains, scabies, and eczema and cut wounds while leaves are used to treat boils (Chopra *et al*., 2002, Kirtikar *et al*., 1999). The whole plant is also known to posses Antifertility and Antibacterial activity (Gaiton *et al*., 1980). It is believed to be helpful in treating measles. *Lygodium flexuosum* (Linn) Sw, is an expectorant and used in ulcers, cut wounds and sprains (Nehete *et al*., 2011). The rhizomes, roots and leaves are ethnomedicinally useful in the treatment of Jaundice (Jain, 1991). It was reported that aqueous rhizome extract is used for gonorrhea (Benjamin *et al*., 2007).

Thus the present study was carried out to evaluate the role of *Lygodium flexuosum* in mast cell stabilizing activity as well as anti-histaminic property in experimental animal models.

MATERIALS AND METHODS

Plant Material

Collection of Plant Material and Authentication

The fern was collected in the month of August from Dapoli Tehsil of Maharashtra. The Herbarium was authenticated from Botanical Survey of India, Pune under the voucher no BSI/WC/TECH/2011/307 by P G Diwakar and submitted to APT Research Foundation, Pune.

Preparation of Plant Extract

The fronds were washed and shade dried for 48 h before extraction. The dried fronds were grinded into course powder and kept in the Soxhlet apparatus for 24 h for obtaining ethanolic and aqueous extract. The aqueous as well as ethanolic extracts obtained were concentrated in rotary evaporator under vacuum and their percent yield was determined.

Chemical and Reagents

All the chemicals and reagents used in the study were of analytical grade. Horse serum and Triple antigen (DPT) were procured from Serum Institute of India, Pune. Compound 48/80, Griess reagents, Histamine, were purchased from Sigma Aldrich (ST. Louis USA).

Animals

Swiss albino mice (18-20 g) and Wistar rats (150-170 g) were obtained from the in-house animal facility of National Toxicology Centre, Pune. They were fed with standard pellet food and water *ad libitum* and maintained in optimum temperature and relative humidity with 12 h light/dark cycle. The experimental protocols for usage of animals were approved by IAEC for National Toxicology Centre through Protocol No. 121 on 30/03/2011 and performed following the guidelines of CPCSEA.

Acute Toxicity Study

Acute toxicity study was performed according to the OECD 423 Guidelines. Female Swiss Albino

mice (18-20 g) were dosed once with 2000 mg/kg of the two extracts respectively and observed for 14 days (Goswami *et al*., 2010). No mortality and toxicity signs were seen at the end of 14 days suggesting that 2000 mg/kg of the extracts to be safe for further experimentation, of which lower 1/4th and 1/8th i.e., 500 mg/kg and 250 mg/kg were selected for the present study.

Sub Acute Toxicity Study

Sub Acute toxicity study was performed according to the OECD 407 Guidelines. The animals were dosed daily for 28 days with the two extracts, respectively and observed for clinical signs and symptoms. Body weight gain, SGPT, SGOT, Creatinine and Glucose levels were done weekly. At the end of 28 days, no mortality and toxicity signs were seen suggesting the extracts to be safe for further experimentation.

In Vivo Mast Cell Degranulation

Male Wistar rats (150-170 g) were sensitized by injecting the rats with 0.5 mL of Horse serum and 0.5 mL of triple antigen (DPT) containing 20,000 million *Bordetella pertusis* subcutaneously (S.C) for inducing active anaphylaxis (Mitra *et al*., 1999).

After sensitization rats were divided into seven groups (n = 6).

Group 1: Normal Control (unsensitized)

Group 2: Disease Control (sensitized)

Group 3: Standard Drug (10 mg/kg p.o)

Group 4: Aqueous extracts of LF (250 mg/kg)

Group 5: Aqueous extracts of LF (500 mg/kg)

Group 6: Ethanolic extracts of LF (250 mg/kg)

Group 7: Ethanolic extracts of LF (500 mg/kg)

On 14th day, two hours after the last treatment dose the rats were sacrificed and blood was withdrawn and the intestinal mesentery were dissected out. The mesenteric pieces were kept in Ringer Locke's solution at 37 ^{0}C for 30 min and then challenged with 5 % horse serum for 10 min *invitro.* The mesenteric pieces were placed in the slides, stretched and stained with 0.1% Toluidine blue. The stained mast cells were observed under microscope and the numbers of intact and degranulated mast cells were counted at 100 X magnification (Harish *et al*., 2001)

In vitro Passive Mast Cell Degranulation

The sera of the active anaphylaxis induced animals were collected and used for the passive mast cell degranulation. Briefly, 0.05 mL of serum was incubated with equal volume of antigen (Horse serum and triple antigen) , normal rat serum and Peritoneal mast cell suspension from a donor rat for 3 min at 37 ^{0}C in an eppendorf tube. The mixture was incubated for 3 min and fixed with freshly prepared 2% gluteraldehyde solution in 0.2 M Sodium Phosphate buffer. The cell mixtures were centrifuged at 300 g for 15 min at 4 ^{0}C. The cellular pellets obtained were resuspended in a minimum amount of supernatant solution and the supernatant was discarded. A 0.05 mL of the suspension was taken on a slide and a smear was made. The smear was allowed to dry and then stained with 0.1% Toluidine Blue and mast cells counted. The results were expressed as the percentage of degranulated and intact mast cells (Johansson *et al*., 1968).

Antigen-Antibody Interaction by ELISA Method

In the 1st day, 7th day and 14th day ELISA was performed to see the antigen: antibody interaction

in the sera of treated group as compared to disease control group. Subcutaneous injections of horse serum and DPT to the rats were given once at the onset and later as booster doses twice. The absorbance corresponding to the amount of allergen was assessed weekly.

Systemic Anaphylaxis in Mice

For systemic anaphylactic reaction, Compound 48/80 a known mast cell degranulator was injected intraperitoneally at a dose of 8 mg/kg and observed for 1 h for percent mortality (Chitme *et al*., 2009) One hour prior to the administration of Compound 48/80 the aqueous and the ethanolic extracts of LF were given at three doses orally at a dose of 500 and 250 mg/kg to Swiss albino mice (n = 10) and percent mortality was calculated by the formula given below by Venkatesh *et al*., 2009.

$$\frac{\text{The number of dead mice}}{\text{Total number of experimental mice}} \times 100$$

Compound 48/80 Induced Degranulation in Rats

The mice dose showing highest protection rate in the systemic anaphylaxis was converted to rat dose according to method given by Ghosh (1984). Both the aqueous and ethanolic extracts of LF were given orally whereas standard reference drug Disodium Chromoglycate (DSCG) at 10 mg/kg was injected to the rats intraperitoneally for seven days. The normal control group was given no treatment. On day 7, 2 h after the last dose treatment the rats were injected with 10 mL of 0.9% saline into the peritoneal cavity. Collections of the peritoneal fluids were done from all groups of animals after gentle lavage by Pasteur pipette into the siliconised tubes containing RPMI-1640 medium (Patel *et al*., 2011)

Peritoneal fluid containing mast cells were washed two to three times by centrifugation at a low speed (400-500 rpm). The supernatants were discarded and remaining pellets were resuspended in a small amount of media. Mast cell suspensions of different groups were then incubated with 1 mg/mL of Compound 48/80 at 37 ^{0}C for 10 min. After incubation the mast cells were stained with 0.1% Toluidine Blue and intact and degranulated mast cells were examined under microscope (Singh *et al*., 1998).

Measurement of Serum and Peritoneal Fluid Nitric Oxide Level

Standard drug disodium chromoglycate (10 mg/kg i.p) and 500 mg/kg of both aqueous and ethanolic extracts of LF were given orally to rats for the duration of 7 days prior to collection of blood. The blood was collected from retro-orbital plexus and centrifuged at 500 rpm for 5 min. After that equal volume of serum, acidic griess reagent (pH 2) and 40 mL glycine buffer (100 mM of glycine, 100 mM of NaCl and 40 mM of HCl) were added and incubated for 15 min at 37 ^{0}C. Serum from normal rats was incubated with saline and sera from positive control animals and treated (standard and test) animals were incubated with C48/80 at a concentration of 1 mg/mL (Mc Cauley *et al*., 2009).

For the measurement of rat peritoneal fluid nitric oxide level, the peritoneal fluid was collected from the animals as described before and procedures similar to serum nitric oxide were followed. After incubation the samples were centrifuged and the absorbance of the supernatant was measured at a wavelength of 546 nm and plotted on standard graph of sodium nitrate.

The standard graph of sodium nitrate was prepared from 100 mM stock solution from which concentrations of 25 mM, 12.5 mM, 6.25 mM, 3.125 mM, and 1.562 mM were made by dilution. Following this 1.5 mL of solution from each concentration were mixed with 1.5 mL of griess reagent and placed in dark for 10 min and absorbance were taken at 546 nm (Savali *et al*., 2010).

Quantification of Histamine

For measuring the histamine content in the blood Swiss albino mice were used in the study. The mice were treated with 500 mg/kg of the aqueous and ethanolic extract of LF and standard drug (DSCG) at 10 mg/kg for seven days respectively. At the end of seven days the mice were injected with a toxicant known as Compound 48/80 at the dose of 8 mg/kg except the normal control group. After 30 min, blood was collected from heart of each animal for histamine analysis. The histamine analysis was done according to the protocol described by Chitme *et al*. (2009) using spectrofluorometric method.

Statistical Analysis

The results were analyzed for statistical significance by one way ANOVA and were expressed as Mean $\pm$ SEM by using Graph pad prism 5.0 version (Dunnett's test).

RESULTS

Effect of LF on Active Anaphylaxis *In vivo*

After 14 days of sensitization, the normal unsensitized animals showed 91.15±0.75 intact mast cells while in sensitized untreated rats, the intact mast cell were 25.50±1.05. On the other hand, the sensitized standard drug Prednisolone treated groups showed 73.00±1.47 intact mast cells.

There was a significant reduction in number of degranulated mast cells in both the extract (aqueous and the ethanolic) treated rats ($p < 0.001$). The administration of the aqueous extract at 250 mg/kg showed 69.17±1.47 intact mast cells and at 500 mg/kg dose showed 70.33±1.75 intact cells. In the ethanolic extract treated groups there were 70.67±1.37 and 72.00±1.41 intact mast cells were found at 250 mg/kg and 500 mg/kg dose respectively (Figure 1).

Effect of LF on *In Vitro* Passive Mast Cell Degranulation

In the normal control group the percent degranulation of mast cell in peritoneal fluid was found to be 12.67±1.03 whereas, the disease control group (untreated sensitized group) showed 84.00±2.10 degranulation. The peritoneal fluid of the standard drug treated group when incubated with horse serum showed 26.16±1.47 degranulation. In the aqueous extract treated groups, the 250 mg/kg showed 28.66±1.75 degranulation and 500 mg/kg showed 25.00±1.41 degranulation. However, the ethanolic extract treated groups displayed 25.16±1.33 and 23.67±1.37 degranulation of mast cells at 250 mg/kg and 500 mg/kg respectively ($p<0.001$) (Figure 2).

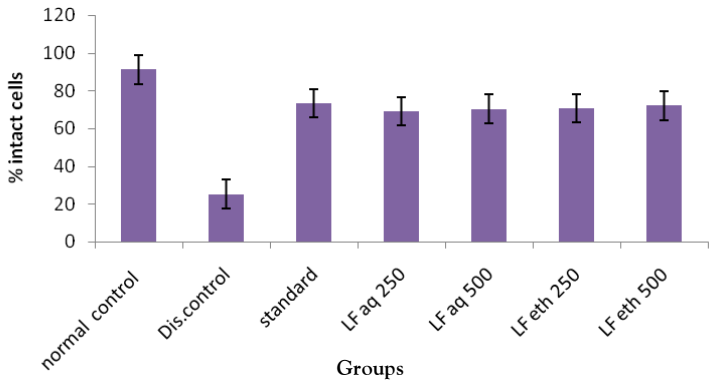
Effect of LF on Antigen-Antibody Interaction

In the ELISA the test drug treated as well as standard drug treated animals sera showed lower absorbance as compared to disease control animals' sera (Figure 3).

Effect of LF on WBC Count

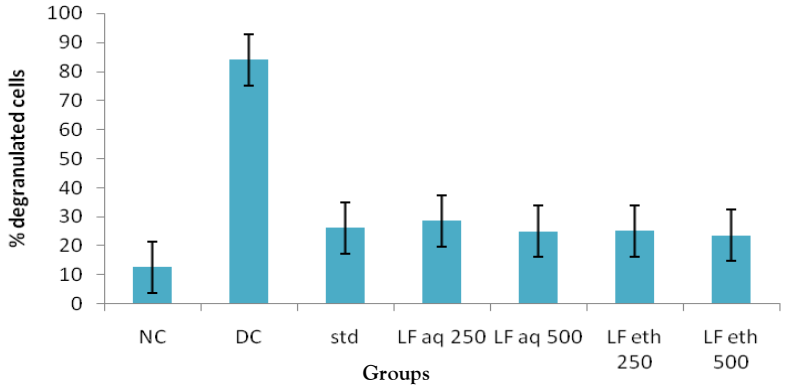
A change in the WBC count was observed in the 14 days of study period. WBC counts were found higher in case of disease control group but the standard drug and test drug treated animal

Figure 1: Effect of LF on Active Anaphylaxis *In Vivo*



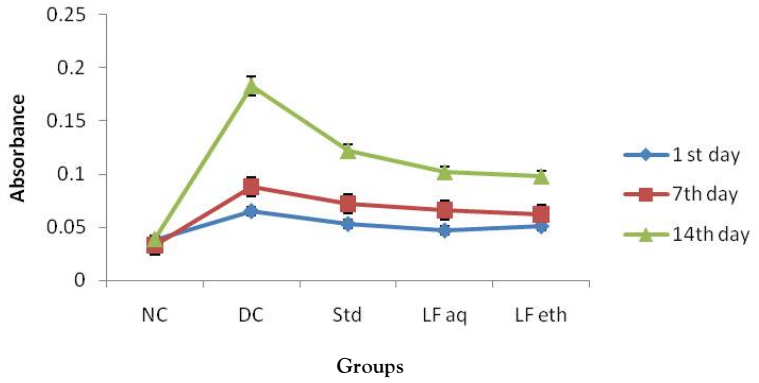
Note: The results were presented as Mean±S.E.M (n=6) data were analyzed by one way ANOVA test. All values were significant (p<0.001).

Figure 2: Effect of LF on *In Vitro* Passive Mast Cell Degranulation



Note: The results were presented as Mean±S.E.M (n=6) data were analyzed by one way ANOVA test. All values were significant (p<0.001).

Figure 3: Effect of LF on Antigen Antibody Interaction by ELISA



Note: The results were expressed as Mean±S.E.M (n=6).

showed reduction in WBC counts during 7th day and 14th day (Figure 4).

Effect of LF on Systemic Anaphylaxis

Percent mortality was monitored in the systemic anaphylaxis study in mice for 1 h after challenging with Compound 48/80. The mice treated with Standard drug Disodium Chromoglycate (DSCG) at 10 mg/kg give 60% protection rate with 40% mortality rate. The aqueous extract of LF at 250 and 500 mg/kg showed the protection of 60% and 80% with 40% and 20% mortalities respectively. Whereas, the ethanolic extract of LF at 250 and 500 mg/kg showed 70% and 80%, respectively. In the control group 100% mortality was recorded (Table 1).

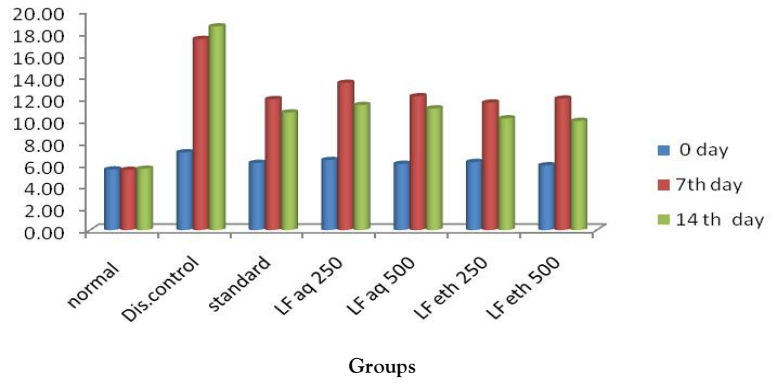
Effect of LF on Compound 48/80 Induced Mast Cell Degranulation in Rats

Treatment with standard drug DSCG at 10 mg/kg showed a 31% reduction of rat peritoneal mast cell degranulation induced by Compound 48/80. The animals treated with aqueous extract exhibited 30.83% degranulation which was 27.33% in case of ethanolic extract treated rats. The positive control animals showed approximately 91.5 % mast cell disruption. The normal negative control group showed 11.5% mast cell degranulation. All the values were statistically significant at $P < 0.001$ (Table 2).

Effect of LF on Nitric Oxide Level of Serum and Rat Peritoneal Mast Cell

Compound 48/80 is known toxicant that can

Figure 4: Effect of LF on WBC Counts in Blood



Note: The data were expressed as Mean±S.E.M (n=6)

Table 1: Effect of LF on systemic anaphylaxis

Groups	% mortality	% protection
Normal control	100%	0%
Standard Drug (DSCG)	40%	60%
LF aq. Extract (250mg/kg)	40%	60%
LF aq. Extract (500mg/kg)	20%	80%
LF eth. Extract (250mg/kg)	30%	70%
LF eth. Extract (500mg/kg)	20%	80%

Note: The results were expressed as Mean±S.E.M (n=10)

Table 2: Effect of LF on Compound 48/80 Induced Degranulation

Groups	% degranulated cells
Normal control	11.50 ± 1.33
Positive control	90.16 ± 2.48###
Standard	30.33 ± 2.07***
LF Aqueous extract	30.83 ± 1.60***
LF Ethanolic extract	27.33 ± 1.63***

Note: The results were expressed as Mean±S.E.M (n=6). Results were analyzed by one way ANOVA test. All values were statistically significant. ### $p<0.001$compared with normal control ***$p<0.001$ when compared with the disease control.

induce the release of nitric oxide from tissue. After challenging the sera of mice and peritoneal fluid containing mast cells with compound 48/80, a significant ($p<0.001$) increase in the nitric oxide level was obtained in positive control group. The standard drug treated group and as well as *Lygodium flexuosum* treated groups showed a decreased nitric oxide levels (Table 3).

Effect of LF on Blood Histamine Content

The histamine content in the blood was found to be high in the untreated control group (disease control) which was injected with compound 48/80. While the standard drug and both the test drug treated groups showed decrease levels of histamine when compared with untreated positive control group (Figure 5).

DISCUSSION

Allergic asthma is a state where the body shows abnormal response towards allergens, leading to the production of chemicals such as histamines and leukotriens (Pescatore, 2003). Anaphylaxis is induced by horse serum and triple antigen for studying the symptomatic effect of asthma in rats. The globulin fraction of the horse serum is believed to provoke the allergic reaction (Dale *et al*., 1916).

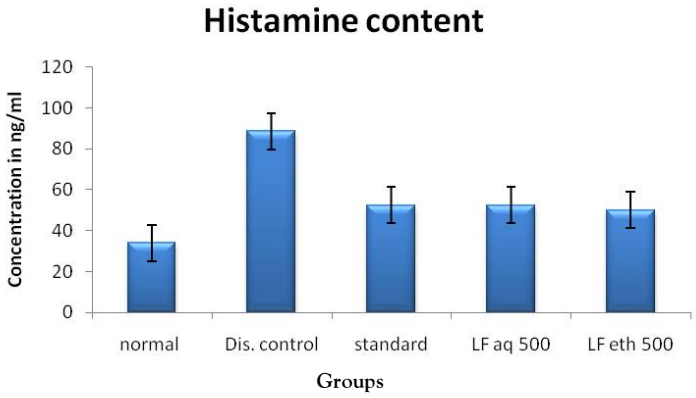
In the toxicity studies, both the extracts were found to be safe for therapeutic uses with no known side effects. In the present study, both the aqueous and ethanolic extracts obtained from *Lygodium flexuosum* (LF) were studied for anti-histaminic and mast cell stabilizing activity. Both

Table 3: Effect of LF on Nitric Oxide Level

Groups	Serum nitric oxide	Peritoneal nitric oxide
Normal control	11.62 ± 0.38	5.21 ± 0.17
Positive control	34.83 ± 0.76	18.57 ± 0.35
Standard Drug	15.33 ± 0.58***	11.72 ± 0.43***
LF aqueous extract	14.50 ± 0.56***	13.33 ± 1.53***
LF ethanolic Extract	11.83 ± 0.76***	12.00 ± 0.87***

Note: Results were expressed as Mean ± SEM. Data were analyzed by one way ANOVA test. All values were statistically significant. ***P<0.001 when compared with the disease control.

Figure 5: Effect of LF on Histamine Content In Blood



Note: Results were expressed as Mean ± SEM. Data were analyzed by one way ANOVA test. All values were statistically significant.

the extracts showed anti anaphylactic activity by reducing mast cell degranulation induced by Horse serum and triple antigen (DPT) in experimental animals. The ethanolic extract at the concentration of 500 mg/kg has shown significant protection against mast cell degranulation followed by 250 mg/kg. The aqueous extract has also showed good mast cell stabilizing potential as compared to the disease control. The results were comparable with the standard drug.

In the passive anaphylaxis the percentage of degranulation was much higher in case of disease control group but treatments with the test drugs at the two concentrations have significantly reduced the percentage of mast cell degranulation.

Systemic anaphylaxis is a process where the allergic reactions occur and results into fatal consequences. It can be produced in mice by challenging with compound 48/80 that produces 100% mortality in mice that are untreated. However, pretreatment with both the aqueous and ethanolic extract of LF showed a significant low mortality rate.

Compound 48/80, exhibits its mast cell degranulation property by acting as a calcium channel ionophore that disrupts the mast cell membranes (Chadi *et al*., 2000). In the present study both the extracts were found to have potential in inhibiting the rat peritoneal mast cell degranulation *in vitro* when incubated with Compound 48/80 at a dose of 1 µg/ml.

Besides, allergens when enter the body lead to the activation of mast cells and neutrophils which in turn generate nitric oxide as a defense mechanism. According to Mitsuhata *et al*. (1995), nitric oxide further antagonizes the effects of vasoconstriction and thereby prevents the release of chemokines. It was also reported by Chitme *et al.* that inhibition of nitric oxide synthase might have correlation with anti allergic activity.

Similarly, in the present experiment it was found that pretreatment of animals with standard drug as well as LF extracts could stabilize the mast cell membrane and thereby inhibit the production of nitric oxide synthase resulting in a decreased level of nitric oxide in both serum and peritoneal fluid.

Histamine being the prime mediator of allergic asthma was estimated in the present study. The quantification of histamine from the whole blood

of mice has shown a significant reduction in their levels in the test drug treated group. The two extracts of LF has shown activity equivalent to standard marketed drugs. These results thus confirm the antihistaminic potential of *Lygodium flexuosum*.

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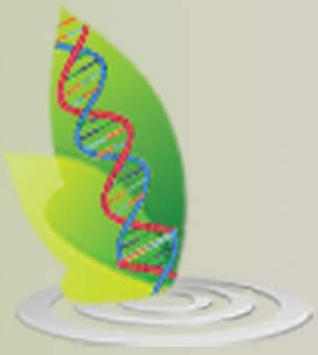

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