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Original Article

HPTLC Fingerprinting and Anti-asthmatic Activity of Roots of Two Different Sources of Bharangi

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ABSTRACT

The present research work aims at developing HPTLC fingerprints of two sources of Bharangi *Clerodendrum serratum* (Linn.) and *Clerodendrum indicum* (Linn.), along with in vivo anti-asthmatic evaluation in OVA-induced Wistar rat model. Air dried roots of both plants were subjected for extraction by maceration followed by soxhlet using ethanol (80%). Further a HPTLC fingerprints was developed for the quantification of Oleanolic acid and Stigma sterol to distinguish both the plants. The anti-asthmatic activity of *Clerodendrum serratum* and *Clerodenrum indicum* was evaluated in Ovalbumin induced Wistar rat model and inflammatory parameters like absolute eosinophil count in BALF, total leukocyte count in BALF, absolute eosinophil count in the Blood, IgE antibodies in serum along with the histopathological changes of lungs were studied. HPTLC fingerprinting showed the presence of Oleanolic acid in *Clerodendrum serratum* and it was found to be absent in *Clerodendrum indicum* whereas Stigmasterol was found to be present in *Clerodendrum indicum* and absent in *Clerodendrum serratum*. In *in vivo* anti-asthmatic activity, test drugs have shown significant decrease in inflammatory parameters such as Absolute eosinophil count in Blood, Absolute eosinophil count in BALF, Total leukocyte count in the BALF and Concentration of IgE antibodies. Among Extract treated groups CSE1 and CSE2 showed good results with $p<0.0001$ when compared with asthmatic group. All the studied parameters clearly conclude that both these plants with controversial botanical identity can be distinguished based on their physiochemical and HPTLC fingerprint profiles. The results suggest that the hydroalcoholic extracts of both the plants significantly possess anti-asthmatic activity.

Key words: Bharangi, *Clerodendrum serratum, Clerodendrum indicum*, HPTLC, Anti-asthmatic activity.

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INTRODUCTION

Traditional herbal drugs with various phytoconstituents and properties have been used as medicines for the treatment of a wide range of diseases from ancient times. These medicines have been considered to be intrinsically safe, due to their natural occurrence, efficacy and less side effects.[1] The special status for botanical medicines is due to their complex composition and the resulting challenges for analytical methodologies and activity test. Now a days a shift has been taking place from classical herbal drugs to phyto-pharmaceuticals, which defined as labeled or standardized extracts.[2] A number of Indian medicinal plants are using for treating various disease conditions. They may be tonics, antimalarial, antipyretics, aphrodisiacs, expectorants, hepatoprotectives, antirheumatics, diuretics etc. However, proper methodologies for the research and development are the need of the day for tapping the full therapeutic potentials of plants.[3]

HPTLC is a powerful modern analytical method for qualitative, quantitative standardization and simultaneous assay of several components in a multi-component formulation.[4] It has been investigated for Authentication of various species of plant along with evaluation of stability and consistency.[5] In this regard, this project was selected to develop the HPTLC fingerprint and quality control parameters for *Clerodendrum serratum* and *Clerodendrum indicum* belonging to Verbenaceae family, which are commonly known as Bharangi. It is one important herb used in many formulations in Ayurveda for various ailments. These plants are used clinically in treatment of bronchitis, asthma, fevers, blood disease, tumors, inflammations, burning sensation, epilepsy, malaria, ulcer and wounds. Leaves are used in fever and hiccough. Root bark contains mainly sapogenins, while leaves contain flavonoids and phenolic acids.[6]

Asthma is a chronic inflammatory condition associated with repeated wheezing, breathlessness, chest tightness and coughing.[7] At present, it is managed by using beta-2 agonist, anticholinergics, methylxanthines, mast cell stabilizers, leukotrine antagonist, glucocorticoids, Anti-IgE antibody like omalizumab and need to be used for prolonged time. Since from ancient days people have found the relief from the disease by using natural products, the natural drugs used in the treatments of asthma are enumerated as *Ephedra, Vasaka, Licorice, Coleus forshosli, Tylophoraindica, Ginkobilobo, Shinpi* and *Nyctanthesarbotristis* Linn.[8] *Clerodendrum serratum* and *Clerodendrum indicum* are used as anti-asthamatic agents in traditional system of medicine under the name Bharangi. The proposed research work is adopted to provide beneficial information regarding the standardization and *in vivo* anti asthamatic activity of the two species of Bharangi.

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MATERIALS AND METHODS

Chemicals and Reagents

The solvents used for HPTLC analysis such as chloroform, formic acid, toluene, methanol were purchased from Merck, Mumbai. Stigmasterol was procured from Sigma–Aldrich, Bangalore. All other reagents used were of laboratory grade.

Collection of Plant material

The plants of *Clerodendrum serratum* was collected from the local areas of kanburgi, Belagavi and its botanical identification was confirmed from RMRC-ICMR, Belagavi, (Karnataka), India. The herbarium specimens of the plants have been deposited at RMRC-ICMR with accession number RMRC-1286. *Clerodendrum indicum* was procured and authenthified by Dr. Madhava Chetty, Professor at Sri Venkateshwara University, Tirupati, Andhra Pradesh. The voucher specimens of the species have been deposited in RMRC and preserved with accession numbers RMRC-992 and SVU-1169.

Processing and Extraction of plant material

The roots of the collected plants were washed, shade dried and powdered then subjected to maceration with ethanol (80%) for 24 hr. The marc was then subjected to successive hot continuous extraction (soxhlet) with ethanol. The extract was filtered and concentrated in Rota evaporator at 50°C.[60]

Pharmacognostic Evaluation[9]

Dry powdered roots were subjected for various quality control parameters like Extractive value, ash value, swelling index and foaming index by following standard procedures. Hydro alcoholic extracts of both the species were subjected to phytochemical screening for analyzing the presence of secondary metabolites.

HPTLC Fingerprinting analysis[10,11]

Preparation of standard and sample solutions

The stock solutions of standard Stigmasterol and Oleanolic acid were prepared by dissolving 5mg of each in 5ml of methanol (1.0mg/ml) separately. The above solution 1ml was taken and further diluted with 10ml of methanol (0.1mg/ml) this stock solution was used to make calibration curves of Stigmasterol and Oleanolic acid. Sample Solutions of the extracts of *Clerodendrum serratum and Clerodendrum indicum* were prepared by dissolving 500mg of each of the extracts in 5 ml of methanol.

Chromatographic conditions

Precoated TLC silica gel Aluminium plates 60 F_{254} (20X10cm, 250µm thickness, Merck, Darmstadt, Germany) were used for chromatographic analysis. The sample solution were applied on the plates in the form of bands of 8mm width with the help of Hamilton syringe(100µl) using CAMAG Linomat V (Camag, Muttenz, Switzerland) sample applicator and were controlled by WinCATS software 1.4.4. Plates were developed in 20 x 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). A TLC scanner IV was used for scanning the HPTLC plates. Two different aliquots of solution of the extracts (10.0, 20.0µl) and that of standard (2.0, 5.0µl) were applied on 10 x 10 cm HPTLC plates for the generation of fingerprint profile.

For analysis with Stigmasterol, the mobile phase consisted of toluene: ethyl acetate: formic acid in the ratio of 14.5: 3.5: 0.1 and for the analysis with Oleanolic acid the mobile phase consisting of toluene: ethyl acetate: formic acid in the ratio of 16: 4: 0.1 was used per plate. The optimized chamber saturation time for mobile phase was 10 min at room temperature (25 ± 2°C) at relative humidity of 60 % ± 5 RH. The plates were developed and scanned within 10 min using densitometric scanner IV in the remission mode at 254, 366 and 540nm. The quantitative analysis was carried out by comparing the retention time and peak area of the standards and that of the sample extracts.

Pharmacological investigation

Animal selection

Albino Wistar rats weighing 100-120 gm were used in the experiment and female albino wistar rats weighing 100-120gm used for acute toxicity. The rats were kept on *ad libitum* feed and water. After fifteen days of acclimatization period, they were randomly selected for different experimental groups. Ethical clearance was obtained from Institutional Animal Ethics Committee (IAEC) Resolution No. KLECOP/IAEC/Res.17-31/08/2013.

Acute oral toxicity study and dose selection

The acute oral toxicity study was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD), revised draft guidelines 423. According to Acute Oral Toxicity study, hydro-alcoholic *Clerodendrum serratum* and *Clerodendrum indicum* extract did not show any significant toxic effect till 2000mg/kg dose. Thus, in the present study, the anti-asthmatic effect of *Clerodendrum serratum* and *Clerodendrum indicum* extracts was evaluated against Ovalbumin induced asthmatic rats with the dose of 400mg/kg (1/5 of LD_{50}) and 200mg/kg(1/10 of LD_{50}) for each extract.

Induction of Asthma[12]

Asthma was induced in rats by i.p administration of alum precipitated OVA and Ovalbumin exposure in histamine chamber.

Sensitization: In this stage animals are sensitized with the alum precipitated Ovalbumin allergen through the intra peritoneal route of administration.

Challenge: To the sensitized animals the Ovalbumin allergen is administered through the aerosol form at 1% concentration in PBS solution from 14th day to 35th day. Then the dose is increased and aerosol of ovalbumin was administered from the 36th day to 42nd day at 2% concentration.

Animal groupings and treatment

Animals were randomly divided into six groups (6 rats/group). Asthma is induced by ovalbumin (OVA). Group 1 received only Phosphate Buffer saline which served as a normal control (untreated), group 2 OVA-aerosols, groups 3 and 4 received different doses of *Clerodendrum serratum* extracts (200 and 400 mg/kg p.o.). Group 5 and 6 received different doses of *Clerodendrum indicum* extracts (200 and 400 mg/kg p.o.). (Table 1)

Table 1: Animal groupings for *in vivo* anti-asthmatic study.

Group type	Group	Description
Normal	Group 1	PBS (1 ml P.O) for 14 – 42nd day
Asthma	Group 2	Only OVA Aerosol
Asthma+CSE1	Group 3	CSE (200mg/kg P.O) + OVA Aerosol
Asthma +CSE2	Group 4	CSE (400mg/kg P.O) + OVA Aerosol
Asthma +CIE1	Group 5	CIE (200mg/kg P.O) + OVA Aerosol
Asthma +CIE2	Group 5	CIE (400mg/kg P.O) + OVA Aerosol

Evaluation parameters

On the 43rd day animals were anaesthetized, blood was collected through retro-orbital puncture and subjected for biochemical studies, serum is separated for IgE estimation, lungs are separated for the Histopathology studies and BALF is collected from trachea by using catheter.

Eiosinophil count in blood:

Blood was allowed to coagulate for 20 min and then centrifuged at 2000 rpm for 15 min. The serum was separated and used for estimations of eiosinophis.

Estimation of eiosinophil's and WBC in BALF

Bronchiolar lavaging was done with the normal saline 7ml normal saline was flushed in to bronchi by using feeding needle. Cells in the Lavage treated with ACK buffer which kills RBC and WBC cells was collected by centrifuging at 5000rpm for 10 min they were stored in 20%foetal bovine serum in RPMI solution. WBC cells was counted by using WBC diluting fluid. By using total leukocyte count and differential leukocyte count, absolute eosinophil count was done by using indirect method.

IgE Estimation

The concentration of IgE in serum was determined using sandwich ELISA kits according to the manufacturer's instruction. The absorbance was measured at 450 nm using a micro plate reader.

Histo-pathological evaluation of lungs

To obtain information about the histological changes in lungs was observed by performing Histopathology of Lungs in normal, diseased, CSE1, CSE2, CIE2, CIE2 group's animals. Lungs are embedded in paraffin, sectioned and stained with hematoxylin and eosin.[12,13] Pathological changes in lungs was observed and reported.

Statistical analysis

Results were expressed as Mean ± S.D., where n= 6. Differences among data were determined using one way ANOVA followed by Tukey's multiple comparison test (Graph Pad Prism software, version 5.01). p<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Pharmacognostic evaluation

The dried powdered roots of both the plants species were evaluated for pharmacognostic evaluation and the results for each parameter is summarized in Table 2. The preliminary phytochemical studies revealed the presence of major chemical constituents in ethanol extracts of both the plants such as alkaloids, flavonoids, terpenoids and tannins. Anthraquinone glycosides were found to be absent in both the plants. Whereas cardiac glycosides were found to be present in *Clerodendrum serratum* and absent in *Clerodendrum indicum*. Saponins were found to be present in *Clerodendrum serratum* and absent in *Clerodendrum indicum*.

Table 2: Pharmacognostic evaluation parameters.

Parameters	*Clerodendrum serratum* (%w/w)	*Clerodendrum indicum* (%w/w)
Alcohol soluble extractive value	11.35±0.46	8.12±0.58
Water soluble extractive value	6.062±0.38	1.08±0.45
Total ash value	13.65±0.12	15.2±0.15
Water soluble ash value	0.874±0.28	0.884±0.28
Acid insoluble ash value	7.9±0.08	8.9±0.14
Loss on drying	12.681±0.21	10.11±0.45
Foaming index	125.35±9.08	<100
Swelling index	5.20±0.53	5.62±0.57

HPTLC Fingerprinting

HPTLC fingerprint patterns have been evolved for extracts of *Clerodendrum serratum* and *Clerodendrum indicum* as shown in Figure 1 and Figure 2 respectively. The R_f value of Oleanolic acid matched with the R_f value of *Clerodendrum serratum* extract which was about 0.29. Oleanolic acid was found to be absent in extracts of *Clerodendrum indicum*. Whereas, the R_f value of stigmasterol matched with the R_f value of extract of *Clerodendrum indicum* which was about 0.34. Stigmasterol was found to be absent in extracts of *Clerodendrum serratum*.

Quantification of Oleanolic acid and Stigmasterol

The optimized chromatographic conditions were applied for the quantification of markers in the herbal extracts. The HPTLC analysis revealed the presence of 0.24% w/w of Oleanolic acid in *Clerodendrum serratum* extract. Whereas, the quantity of Stigmasterol in *Clerodendrum indicum* was found to be 1% w/w.

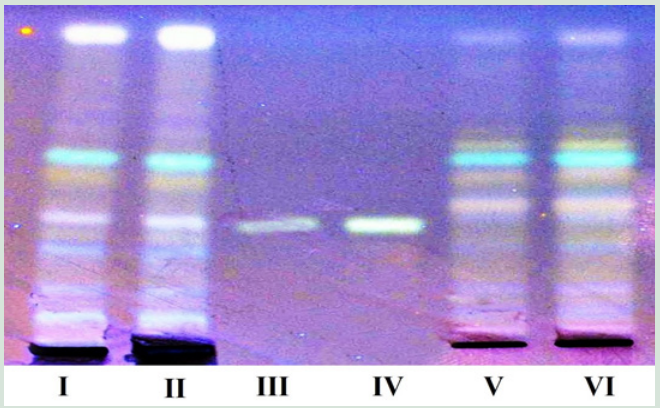

Figure 1: HPTLC fingerprint of *Clerodendrum serratum* (I, II) and *Clerodendrum indicum* (V, VI) with Oleanolic acid (III, IV).

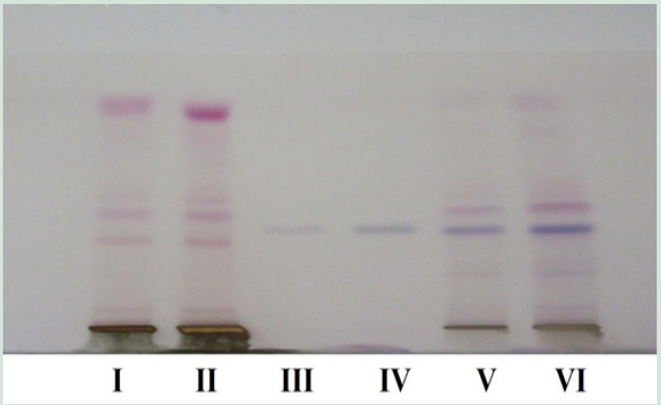

Figure 2: HPTLC fingerprint of *Clerodendrum serratum* (I, II) and *Clerodendrum indicum* (V, VI) with Stigmasterol (III, IV).

In vivo Anti-asthmatic activity

Evaluation of biochemical parameters

Eosinophil Count in BLOOD:

Eosinophil count was significantly (P<0.0001) increased in Asthma group when compared to normal group. From Figure 3(A) it is evident that Eosinophil count was decreased significantly in the treatment groups (ASTHMA+CSE1, ASTHMA+CSE2, ASTHMA+CIE1 and ASTHMA+CIE2) when compared to the Asthma group, however there was a less significant result with ASTHMA+CIE2 group but there was marked decrease in the eosinophil count in comparison to Asthma group (Table 3).

WBC count and Eiosinophil count in BALF

The WBC count and Eiosinophil count was significantly (P<0.0001) increased in the Asthma group in comparison to the normal group. The treatment group (ASTHMA+CSE1, ASTHMA+CSE2, ASTHMA+CIE1 and ASTHMA+CIE2) showed significant decrease in the WBC count as well as in eiosinophil count when compared to the Asthma group. However, a less significant result were seen with ASTHMA+CIE2 group. From Figure 3 (B-C) it can be seen that a ASTHMA+CSE2 group showed good significance among all other treatment groups (Table 4 and 5).

IgE antibody concentration in blood serum

A significant increase of IgE concentration was observed in the Asthma group in comparison to the normal group. Consequently, a significant decrease in the IgE concentration was observed in the treatment groups (ASTHMA+CSE1, ASTHMA+CSE2, ASTHMA+CIE1 and ASTHMA+CIE2). However, there was a less significant result with ASTHMA+CSE2 group but there was marked decrease in the IgE concentration in comparison to Asthma group. The results are depicted in Figure 3(D) (Table 6).

Histopathology of lungs

The Histopathological changes of the normal, Asthma and treated group are shown in Figure 4 and 5. The Ovalbumin exposed group showed hyperplasia of the bronchial walls and also eosinophil infiltration in the lungs. The damage in the treatment (ASTHMA+CSE1, ASTHMA+CSE2, ASTHMA+CIE1 and ASTHMA+CIE2) groups was markedly attenuated in comparison to the Asthma group.

Table 3: Effect of *Clerodendrum serratum* and *Clerodendrum indicum* root extract on AEC of blood in control and experimental rats.

Animals	Absolute Eosinophil Count (cells/µl)					
	Normal	Asthma	Asthma+CSE1 (200mg/kg)	Asthma+CSE2 (400mg/kg)	Asthma+CIE1 (200mg/kg)	Asthma+CIE2 (400mg/kg)
1	50	375	50	125	212	250
2	50	350	175	150	175	195
3	50	360	125	225	215	215
4	75	325	75	175	186	245
5	60	320	125	50	150	235
6	59	350	150	350	195	255
Mean±SEM	57.33±4.01	346.7±8.53#	116.7±19.0***	179.2±41.54***	188.8±9.94***	232.5±9.46**

#P<0.0001 when compared with normal, ***P<0.0001 when compared with Diseased, **P<0.001 when compared with Diseased

Table 4: Effect of *Clerodendrum serratum* and *Clerodendrum indicum* root extract on Total Leukocyte Count in BALF in control and experimental rats.

Animals	Total Leucocyte Count (cells/µl)					
	Normal	Asthma	Asthma+CSE1 (200mg/kg)	Asthma+CSE2 (400mg/kg)	Asthma+CIE1 (200mg/kg)	Asthma+CIE2 (400mg/kg)
1	1300	10000	3450	2000	3900	5150
2	1150	11300	4100	2650	3300	4650
3	2800	10150	4850	2900	3650	4400
4	1550	9900	3250	2800	4200	3950
5	1400	7650	3750	3650	3350	4450
6	1100	11800	3800	3100	3450	5750
Mean±SEM	1550±634.0	10133±1441#	3867±230.5***	2850±221.4***	3642±143.4***	4725±259.4***

#P<0.0001 when compared with normal, ***P<0.0001 when compared with Diseased, **P<0.001 when compared with Diseased

Table 5: Effect of *Clerodendrum serratum* and *Clerodendrum indicum* root extract on Absolute Eosinophil Count in BALF in control and experimental rats.

Animals	Absolute Eosinophil Count in Balf (cells/µl)					
	Normal	Asthma	Asthma+CSE1 (200mg/kg)	Asthma+CSE2 (400mg/kg)	Asthma+CIE1 (200mg/kg)	Asthma+CIE2 (400mg/kg)
1	52	400	138	80	156	206
2	46	452	164	106	132	186
3	112	406	194	116	146	176
4	62	396	130	112	168	158
5	56	306	150	146	134	178
6	44	472	152	124	138	230
Mean±SEM	62.00±25.36	405.3±57.63#	154.7±9.12***	114.0±8.85***	145.7±5.73***	189.0±10.38***

#*P*<0.0001 when compared with normal, ***P*<0.0001 when compared with Diseased, **P*<0.001 when compared with Diseased

Table 6: Effect of *Clerodendrum serratum* and *Clerodendrum indicum* root extract on Absolute Eosinophil Count in BALF in control and experimental rats.

Animals	Immunoglobulin E Concentration in Serum (ng/ml)					
	Normal	Asthma	Asthma+CSE1 (200mg/kg)	Asthma+CSE2 (400mg/kg)	Asthma+CIE1 (200mg/kg)	Asthma+CIE2 (400mg/kg)
1	56.34	96.52	23.47	46.54	36.19	33.68
2	55.72	93.46	22.01	50.37	39.94	35.28
3	53.01	101.94	24.02	53.15	37.23	38.97
4	54.95	90.89	44.74	51.89	39.04	37.99
5	59.33	108.96	47.45	54.26	36.67	40.36
6	52.03	120.50	27.36	55.44	35.98	36.26
Mean±SEM	55.24±2.59	102.0±11.11#	31.51±4.68***	51.95±1.3***	37.51±0.66***	37.09±1.01***

#*P*<0.0001 when compared with normal, ***P*<0.0001 when compared with Diseased, **P*<0.001 when compared with Diseased

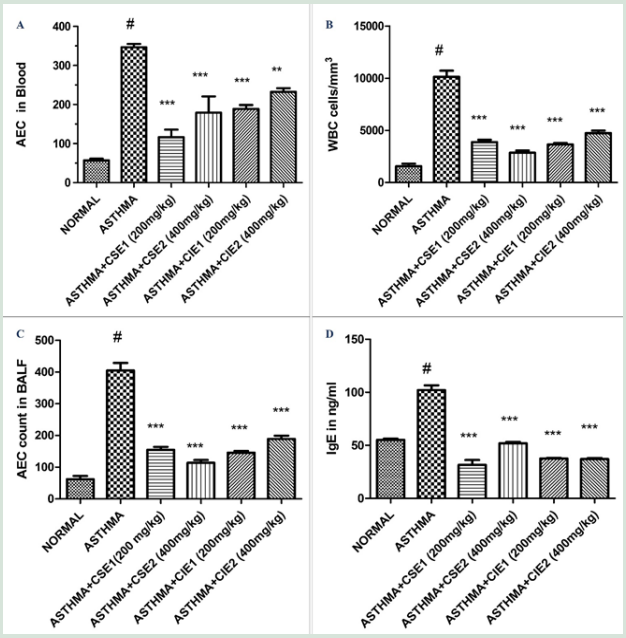

Figure 3: Graphical representation of A) Effect of CSE and CIE on Absolute Eosinophil count in blood, B) Effect of CSE and CIE on Leucocyte count in BALF, C) Effect of CSE and CIE on Absolute Eosinophil count in BALF and D) Effect of CSE and CIE on IgE Concentration in Blood Serum.

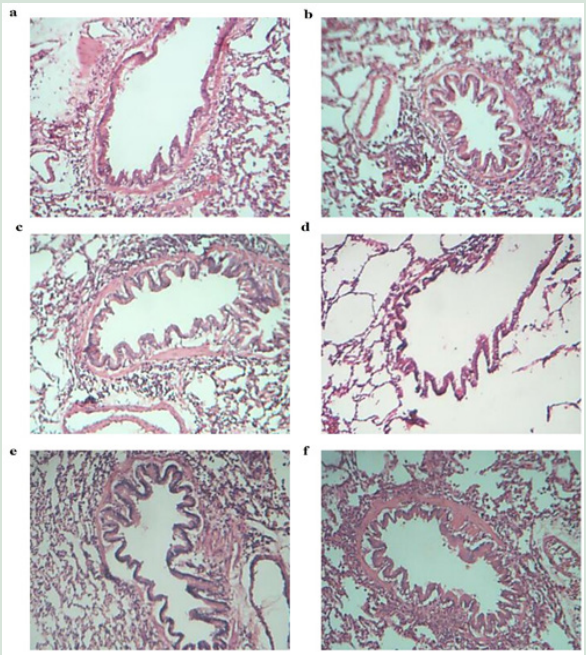

Figure 4: Histopathological changes in Bronchiole in a) Normal group, b) Asthmatic group, c) ASTHMA+CSE1 Group, d) ASTHMA+CSE2 Group, e) ASTHMA+CIE1 Group, f) ASTHMA+CIE1 Group.

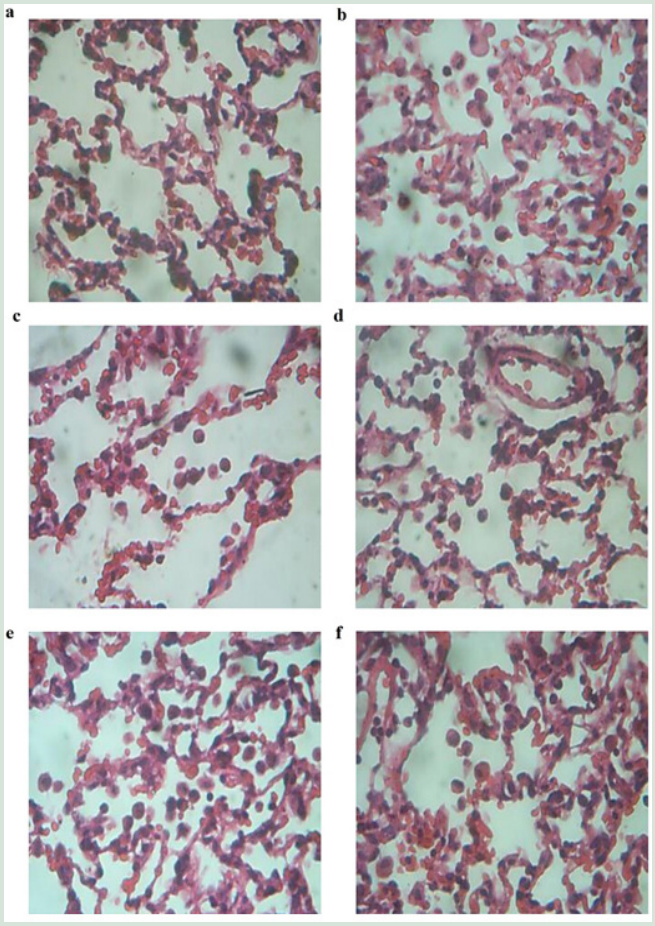

Figure 5: Histopathological changes in Alveolar cells in a) Normal group, b) Asthmatic group, c) ASTHMA+CSE1 Group, d) ASTHMA+CSE2 Group, e) ASTHMA+CIE1 Group, f) ASTHMA+CIE1 Group.

CONCLUSION

In the present research work the correct identification and authentication of two sources of Bharangi has been reported. The HPTLC fingerprinting analysis revealed that marker compound Oleanolic acid was found to be present in *Clerodendrum serratum* and absent in *Clerodendrum indicum* and Stigmasterol was found to be present in *Clerodendrum indicum* and absent in *Clerodendrum serratum*. Both these plants with controversial botanical identity can be clearly distinguished based on their physiochemical and HPTLC fingerprint profiles. An attempt was made in the present study to evaluate the effects of *Clerodendrum serratum* and *Clerodendrum indicum* on airway inflammation and remodelling were investigated. *Clerodendrum serratum* and *Clerodendrum indicum* reduces infiltration leukocytes and eosinophils in BALF. Also influx of eosinophils and plasma cells in lung tissue were decreased, hyperplasia of bronchioles were markedly reduced compared to asthma group76. Anti-OVA IgE antibody levels were reduced in serum of OVA sensitised and challenged rats treated with of *Clerodendrum serratum* and *Clerodendrum indicum* root extract. The results of present study suggest that the hydroalcoholic extracts of *Clerodendrum serratum* and *Clerodendrum indicum* significantly attenuated the airway inflammation induced by ovalbumin hence the plants possess anti-asthmatic activity.

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CONFLICT OF INTEREST

The authors declare no conflict of Interest.

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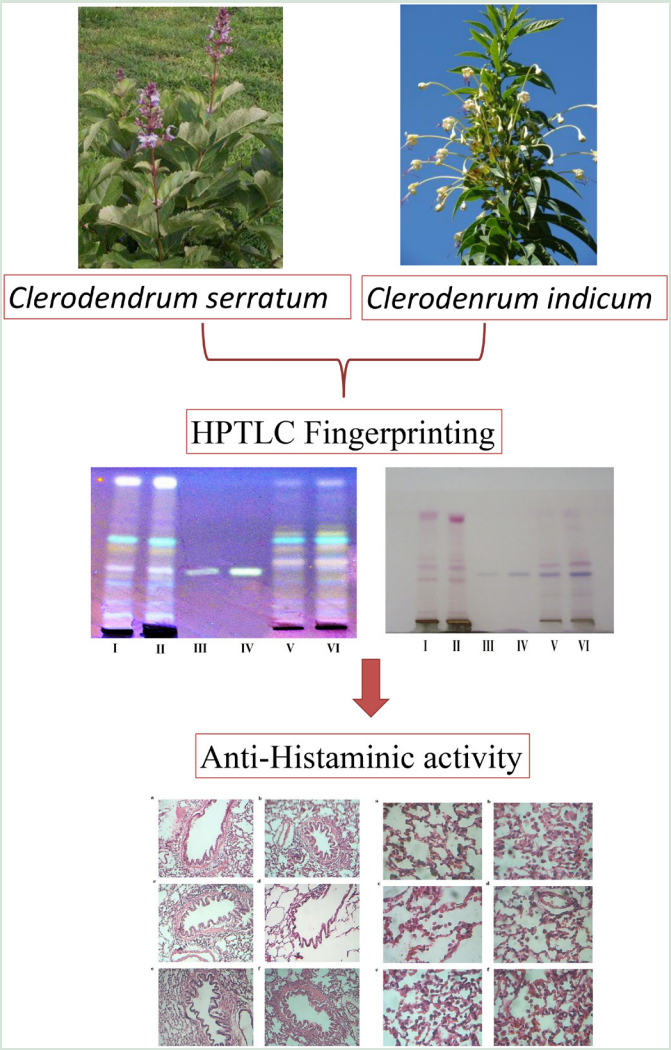
GRAPHICAL ABSTRACT



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SUMMARY

In the present research work HPTLC fingerprints of two sources of Bharangi *Clerodendrum serratum* (Linn.) and *Clerodendrum indicum* (Linn.), along with *In vivo* anti-asthmatic evaluation in OVA-induced Wistar rat model has been carried out. Further a HPTLC fingerprints was developed for the quantification of Oleanolic acid and Stigmasterol to distinguish both the plants. The anti-asthmatic activity of *Clerodendrum serratum* and *Clerodenrum indicum* was evaluated in Ova albumin induced Wistar rat model and inflammatory parameters like absolute eosinophil count in BALF, total leukocyte count in BALF, absolute eosinophil count in the Blood, IgE antibodies in serum along with the histopathological changes of lungs were studied. HPTLC fingerprinting showed the presence of Oleanolic acid in *Clerodendrum serratum* and it was found to be absent in *Clerodendrum indicum* whereas Stigmasterol was found to be present in *Clerodendrum indicum* and absent in *Clerodendrum serratum*. In *in-vivo* anti-asthmatic activity, test drugs have shown significant decrease in inflammatory parameters such as Absolute eosinophil count in Blood, Absolute eosinophil count in BALF, Total leukocyte count in the BALF and Concentration of IgE antibodies. Among Extract treated groups CSE1 and CSE2 showed good results with $p<0.0001$ when compared with asthmatic group. The results suggest that the hydroalcoholic extracts of both the plants significantly possessanti-asthmatic activity.

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