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EVALUATION OF HYDRO ALCOHOLIC EXTRACT OF *CLERODENDRUM INFORTUNATUM* LEAF FOR ANTIASTHEMATIC ACTIVITY

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ABSTRACT

The present study is directed towards the hydroalcoholic extract of Clerodendrum infortunatum leaf categorized by antiasthmatic plants. Many herbal formulations are available for asthma but scientific validation and standardization is very difficult for this formulations. Phytochemical evaluation of the crude drugs such as ash value, extractive value were carried out and complies the limit given by quality standards of medicinal plants (ICMR). The fingerprint analysis was carried out for the Clerodendrum infortunatum, and compared the R_f values with gallic acid for the confirmation. The prepared hydroalcoholic extract of Clerodendrum infortunatum was subjected to determination of physical and chemical evaluations and the results complies as per the standards. The pharmacological findings concluded that the hydroalcoholic extract of Clerodendrum serratum could reverse asthmatic condition and reduced the total and differential WBC cell count in BALF, retained protein and MDA level in lung tissue, increased intact mast cells thus preventing its degranulation and mediators release. This Polyherbal hydroalcoholic extract of Clerodendrum serratum formulation is could be a promising new therapeutic approach for the treatment of clinical asthma.The stability studies and clinical trials to be carried out in future.

KEY WORDS: *Hydro alcoholic extract, clerodendrum infortunatum, Antiasthematic activity, Finger print analysis*

INTRODUCTION

The medicinal properties of plant species have made an outstanding contribution in the origin and evolution of many traditional herbal therapies. These traditional knowledge systems have started to disappear with the passage of time due to scarcity of written documents and relatively low income in these traditions [1]. Traditional systems of medicine continue to be widely practised on many accounts. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments. Global estimates indicate that 80% of about 4 billion population cannot afford the products of the Western Pharmaceutical Industry and have to rely upon the use of traditional medicines which are mainly derived from plant material [2]. This fact is well documented in the inventory of medicinal plants, listing over 20,000 species. In spite of the overwhelming influences and our dependence on modern medicine and tremendous advances in synthetic drugs, a large segment of the

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world population still likes drugs from plants. In many of the developing countries the use of plant drugs is increasing because modern life saving drugs are beyond the reach of three quarters of the third world's population although many such countries spend 40-50% of their total wealth on drugs and health care. As a part of the strategy to reduce the financial burden on developing countries, it is obvious that an increased use of plant drugs will be followed in the future [3].

Plant material includes juices, gums, fatty oils, essential oils, and any other substances of this nature. Herbal medicines may contain excipients in addition to the active ingredients. Medicines containing plant material combined with chemically defined active substances, including chemically defined, isolated constituents of plants is not considered to be herbal medicines. Exceptionally, in some countries herbal medicines may also contain, by tradition, natural organic or inorganic active ingredients which are not of plant origin [4].

MATERIALS AND METHODS

Materials

The Marker compounds such as Piperine, Gallic acid and Bovine serum albumin all are Sigma chemicals and 1,1,3,3, Tetramethoxy propane Himedia laboratories. Methanol, Chloroform, n-Butanol, Benzene, Toluene, Isopropyl alcohol, Acetone are from Ranbaxy laboratories ltd. Mumbai.

METHODOLOGY

Processing of Herbals

All the plants were subjected into processing which includes initially, other than the plant parts (foreign matters) such as grass, sand mud, and other plant parts was removed manually to ensure the plant quality. Plant materials are cutting into the small chops; barks are cut by longitude, to ensure the drying. Shade drying method was followed to maintain the chemical constituents of the plant. All the dried chop of plants were packed separately in the cover and kept in suitable storage condition for avoiding the moisture [5].

Physiochemical Constant Determination [6-8]

Ash value

The ash remaining after ignition of medicinal plant materials is determined by three different methods which measure total ash, acid-insoluble ash and water-soluble ash

- Total ash
- Acid insoluble ash
- Water soluble ash.

The total ash value method is designed to measure the total amount of material remaining after ignition. This includes both "physiological ash", which is derived from the plant tissue itself, and "non-physiological" ash, which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plant surface.

Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.
Water-soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water. The ash of any organic material is composed of their non-volatile inorganic components controlled incineration of crude drugs results in an ash residue consisting of an inorganic material (silica).

i. Total Ash

Weighed accurately 2 g of the air dried crude drugs in tarred silica dish and incinerated at a temperature not exceeding 450°C until free from carbon then cooled and weighed. This was repeated till the constant weight was obtained. The percentage of ash with reference to the air dried drug was calculated.

ii. Acid Insoluble Ash

The ash obtained in the total ash was boiled with 25 ml of dil. Hcl for 5 minutes. The insoluble ash was collected on ash less filter paper and washed with hot water. The insoluble ash was transferred to a tarred silica crucible incinerated at the temperature 650°C until free from carbon. The crucible was cooled and weighed. The entire procedure was repeated till a constant weigh is observed. The percentage of the total ash was calculated with reference to the weight of the air dried drug.

iii. Water Soluble Ash

The ash obtained in the total ash was boiled with 25 ml of water for 5 minutes. The insoluble ash was collected on ash less filter paper and washed with hot water. The insoluble ash was transferred to a tarred silica crucible incinerated at the temperature 650°C until free from carbon. The crucible was cooled and weighed. The entire procedure was repeated till a constant weigh is observed. The percentage of the total ash was calculated with reference to the weight of the air dried drug.

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iv. Sulphated Ash: The obtained in the total ash was treated with Conc. Sulphuric acid and kept at 850°C, until free from phosphates and silicates.the crucible was cooled and packed. The entire procedure repeated until constant weight. The percentage of sulphated ash was calculated with reference to air dried drug.

Extractive Values

This method determines the amount of active constituents extracted with solvents from a given amount of medicinal plant material. It is employed for materials for which as yet no suitable chemical or biological assay exists. The extraction method involves, hot extraction and cold maceration. Different solvents like water, alcohol and ether were used.

This technique determines the amount of active constituents in a drug when extracted with solvents. The extraction of any crude drug with a particular solvent yields a solution containing different phyto constituentes. The composition of these phyto constituents in that particular solvent depends upon the nature of the drug and solvent used [9].

Types of extractive values

- Water soluble extractive
- Alcohol soluble extractive
- Ether soluble extractive
- Hexane soluble extractive
- Ether soluble extractive gives information about the drugs containing fixed oils and coloring matter presents.
- Hexane soluble extractive gives information about the drugs containing fatty materials.

i. Water Soluble Extractive

5 g of the air dried coarse drug powder taken in a Stoppard flask and macerated with 100 ml of water for 24 hours, shaking frequently during every six hours and allowed to stand for 24 hours. It was then filtered rapidly taking precaution against loss of the solvent. 25 ml of filtrate was evaporated to dryness in a tarred flat bottom dish and dried at 105°C to constant weight and weighed. The percentage of the water soluble extractive was calculated with reference to the air dried drug.

ii. Ethanol Soluble Extractive

5 g of the air dried coarse drug powder taken in a stoppered flask and macerated with 100 ml of ethanol of the specified strength, in a closed flask for 24 hours, shaking frequently during every six hours and allowed to stand for 24 hours .It was then filtered rapidly taking precaution against loss of the solvent. 25 ml of filtrate was evaporated to dryness in a tarred flat bottom dish and dried at 105°C to constant weight and weighed. The percentage of the ethanol soluble extractive was calculated with reference to the air dried drug.

Extraction of Selected Plant Materials

Clerodendrum Infortunatum leaf was subjected to 7 days maceration with methanol and water (1:1).

Recovery of Extracts

Extract was recovered by using rotary evaporator (Buchi), which is operated under reduced pressure and low temperature, in this large space is available for evaporation of solvent, and less chance to charring the constituents. All the plant extract recovered by this technique.

Qualitative Analysis of Extracts [10-15]

Phytochemical Studies: Qualitative Phytochemical tests were carried out for all prepared extracts.

A. Test for Alkaloids

To the solvent free extract, 50 mg is stirred with few ml of dilute HCl then shake well and filtered. The filtrate was used for the following tests:

1. **Dragendorff's test:** To the filtrate (0.5 ml), few drops Dragendorff's reagent was added, appearance of orange brown precipitate indicates the presence of alkaloids.
2. **Hager's test**: To the filtrate (0.5 ml), few drops Hager's reagent was added, appearance of yellow precipitate indicates the presence of alkaloids.
3. **Mayer's test:** To the few ml of filterate, a drop or two of Mayers reagent added by the side of the test tube. A white or creamy precipitate indicates the test as positive.
4. **Wagner's test**: To the few ml of filtrate, a drop or two of Wagners reagent added by the side of the test tube. A reddish-brown precipitate confirms the test as positive.

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B. Test for Glycosides

General test for glycosides: Coarsely powderd plant material 1gm was introduced in to two different beakers. To one of the beaker sulphuric acid (5 ml) was added while water (5 ml) was added to the other beaker. The two beakers were heated for 3 minutes and the contents filtered in to labeled test tubes. The filterate was made alkaline with sodium hydroxide (0.5 ml) and allowed to stand for 3 minute. The presence of reddish brown precipitates in the filterate was taken as positive for glycosides.

For detection of glycosides: 50 mg of extract is hydrolysed with concentrated hydrochloric acid for 2 hrs. On a water bath, filtered and the hydrolysate was subjected to the following test.

Borntrager's test: The filtrate (0.5 ml) was boiled with (0.5 ml) dilute H_2SO_4 and filtered. To the cold filtrate an equal volume of benzene-chloroform was added. To the separated organic layer ammonia solution (0.5 ml) was added, appearance of pink or red colour in the ammonia layer indicates the presence of glycosides

Legal's Test: 50 mg of the extract was dissolved in pyridine; sodium nitroprusside solution was added and made alkaline using 10% sodium hydroxide. Presence of glycosides is indicated by the pink color.

C. Test for the Steroidal Glycosides

Preperation of test extract solution: The extract was prepared with water and 1 volume of 10% v/v sulphuric acid solution was added. The mixture was heated on the water bath for half an hour and the hydrolyzed extract was extracted with the $CHCl_3$. The $CHCl_3$ layer was separated and concentrated. The test for steroid /phytosterol was carried out on the concentrated fraction.

D. Test for Steroids / Phytosterol

Preperation of the test extract solution: the extract was refluxed separately with alcoholic solution of pottesium hydroxide till complete saponification. The saponified extract was diluted it water and unsaponifiable matter was extracted with diethyl ether. The ethereal extract was evaporated and the residue (unsaponifiable matter) was subjected to the following test by dissolving the residue in chloroform.

1. **Salkowski reaction**: To the 0.5 ml of test sample solution 0.5 ml of chloroform and con.H_2SO_4 was added and shaken well. Appearance of red color in the chloroform layer and appearance of yellow fluorescence in the acid layer indicates the presence of steroids.
2. **Liebermann –Burchard reaction**: To the test sample solution, 1 ml of chloroform and 1 ml of acetic anhydride was added and mix well. To the above solution 2 drops of conc.H_2SO_4 was carefully added along the side of test tube. Appearance of red, then blue and finally green color indicates the presence of steroids.

E. Test for Phenolic compounds

1. **Ferric chloride test**: To the samples solution, few drops of ferric chloride solution were added. Appearance of green colour indicates the presence of phenolic compounds.
2. **Lead acetate test:** To the 0.5 ml of sample solution, 0.5 ml of 10% lead acetate solution was added. Appearance of white precipitate indicates the presence of phenolic compounds.

F. Test for Flavonoids

1. **Shinoda test:** To the sample solution (0.5 ml), 0.5 ml of 95% ethanol, few drops concentrated HCl and 0.5 g magnesium turnings were added. Appearance of pink color indicates the presence of flavonoids.
2. **Zinc test:** To the sample solution (0.5 ml), 0.5 ml of dilute HCl & zinc dust was added. Appearance of pink color indicates the presence of flavonoids.
3. **Alkaline reagent test**: To the sample solution (0.5 ml), few drops of 10% ammonium hydroxide solution was added. Appearance of yellow fluorescence indicates the presence of flavonoids.
4. **G. Test for Carbohydrates** The extract is dissolved in 5 ml of water and filtered. The filterate is subjected to the following test:

1. **Molish test:** To 2 ml of filterate, two drop of alcoholic solution of α-napthol was added, this mixture is shaken well and 1 ml of concentrated sulphuric acid was added slowly along the sides of the test tubes and allowed to stand. A violet ring indicates the the presence of carbohydrate.

2. **Fehling's test:** 1 ml of filterate was boiled on the water bath with each 1 ml of Fehling solution A and B. A red precipitate indicate the presence of sugar

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3. **Benedict's test:** To 0.5 ml of the sample solution, 0.5 ml of Benedict's reagent was added. The mixtures were heated on boiling water bath for 2 min. A characteristic red color precipitate indicates the presence of sugars.
4. **Barfoed's test**: To 1 ml of filterate, 1 ml of Barfoed's reagent was added and heated on the boiling water bath for 2 min. Red precipitate indicates the presence of sugar.

H. Test for Saponins
1 ml of the sample solution was diluted with distilled water and made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 min. 2 cm layer of foam indicates the presence of saponins.

Test for Protein /Amino acids
The extract is dissolved in 10 ml of distilled water and filtered through Whatmann No.1 filter paper and the filterate is subjected to test for protein and amino acids.

Ninhydrin test: Two drops of freshly prepared 0.2% ninhydrin reagent was added to the extract and heated. Development of blue color indicates the presence of proteins, peptides or amino acids.

Biuret test: An aliquot of 2 ml of filterate was treated with one drop of 2% copper sulphate solution. To this, 1 ml of ethanol was added, followed by excess of KOH pellets. Pink color in the ethanolic layer indicates the presence of protein.

Millon's test: to the 2 ml of filterate, few drops of millon's reagent are added. A white precipitate indicates the presence of proteins.

J. Test for Triterpenoid: Preperation of test extract solution: The test solution was prepared by dissolving the extracts in $CHCl_3$.
Salkowoski test: Few drops of concentrated H_2SO_4 was added to the test solution of the extract , the solution was shaken and on standing lower layer turns golden yellow indicating the presence of triterpenoid.

Liebermann – Burchard reaction: To the test sample solution, 1 ml of chloroform and 1 ml of acetic anhydride was added and mixed well. To the above solution 2 drops of Con.H_2SO_4 was carefully added along the side of test tube. Appearance of red, then blue and finally green color indicates the presence of steroids.

K. Test for Saponin
Preperation of sample: The solution was prepared by dissolving the extract in water.

Foam Test: The test solution was shaken vigorously. The formation of foam, which is stable for 15 min, was considered as positive for saponin.

Quantitative Analysis [16-18]
Estimation of herbal drugs by advanced chromatographic techniques which are most reliable and widely used for the estimation of herbal drugs in their formulation. They are;

- HPTLC
- HPLC
- GC

Herbal Drugs
Herbal drugs and herbal drug preparations are complex mixtures of compounds, from medicinal point of view, the drug in its entirety must be considered as the active principle. This can be create several problems for the analyst

- For many herbal drugs and extracts thereof, the extract chemical composition is not known.
- Because of natural variability, the qualitative and quantitative composition of the raw materials may vary considerably.
- The quantitative content of known active or inactive components of the drug (markers) is not sufficient as a criterion of quality.
- The presence of other substance must be established for proper identification well.
- The availability of chemical and botanical reference materials is limited in many cases because the active constituents have not yet been investigated.

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Most analytical questions can be suitably answered on the basis of chromatographic fingerprints, which best represent the complexity of the analyte. For that highest resolving power using a detector allowing positive identification the best choice. GC/MS, HPLC/DAD/MS, HPLC/NMR, such analytical tool is costly and time consuming.

HPTLC

A. HPTLC is able to provide either equally suitable results with complementary information and can help in a decision making process.

- Some of the advantage of HPTLC shall be reemphasized at this point, analysis time is comparatively short and many samples can conveniently be compared side by side on the same plate. This is particularly important for screening and selection of raw material and for process control during manufacturing.
- The fingerprint can be optimized for certain targeted compounds. even if some components migrate with the solvent front and others remain at the application position, the fingerprint always represents the sample in its entirely. Unlike column chromatography, it is not problematic if portions of the sample can be analyzed in several different samples without modification to the sample preparation step.
- The lower resolution power can be advantage, because small insignificant differences between samples due to natural variability will not be visible. It is therefore often easier to define acceptance criteria that have to be met by the sample.

The extreme flexibility of detection, the convenience of specific derivatization and the possibility of multi able detections without repeating the chromatography are particularly useful for fingerprint analyses because the chromatogram can be visualized.

- HPTLC plates are not only reported as peak data but can also be presented and communicated as images.

A. Standard Preparation

Piperine and Gallic acid

10 mg of standard Piperine and Gallic acid were dissolved in 10 ml of methanol individually and used for HPTLC quantification at a concentration of 1 mg/ml.

B. Sample Preparations

Raw material

1000 mg of raw material was extracted with 10 ml of methanol at a slight warm condition. This solution was filtered in Whatman filter paper to get a clear solution and used for HPTLC analysis at a concentration of 100 mg/ml.

Extracts

100 mg of extract was extracted with 10 ml of methanol at a slight warm condition. This solution was filtered in Whatman filter paper to get a clear solution and used for HPTLC analysis at a concentration of 10 mg/ml.

Pharmacological Study

Induction of asthma [19-22]

I. Preparation of ovalbumin (OVA) suspension for sensitization

For sensitization the ovalbumin (OVA) suspension was prepared in the concentration of 2mg/ml in phosphated buffered saline (PBS). This suspension was precipitated with aluminium hydroxide gel ($AlOH_3$) in the ratio of 1:1, which was added as an adjuvant.

II. Airway challenge

For airway allergen challenge 1% w/v OVA suspension was prepared in phosphated buffered saline (PBS) and then the animals were sensitized by exposing to the aerosol of ovalbumin with the help of a commercially available spray the output of the spray was 3ml/min. The procedure for sensitizing the animals was carried in a Perspex made histamine chamber.

III. Sensitisation procedure

The animals (180-220g) were sensitised intradermally with ovalbumin (OVA) precipitated with aluminium hydroxide gel in PBS on 0^{th} day for the short(15 day) study group. The sensitisation procedure was followed by airway challenge on 1^{st}, 3^{rd}, 5^{th}, and 7^{th} day. A last airway challenge was performed 18 hours before sacrificing the animals for 21 days.

Antiasthematic screening [23-25]

I. Total WBC and differential count

The bronchoalveolar lavage fluid (BALF) was collected and centrifuged at 170g for 10 minutes. The pellets obtained after centrifugation were resuspended in 0.5ml of PBS and a thin smear was prepared on a slide. Then the smeared slide was air dried and then stained for ten minutes using Giemsa stain. The slide was then subjected to distilled water for the purpose of de-staining. Counter staining was later

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carried out using May Grunwald stain. The differential count was carried out using a digital light microscope (Motic, Japan, Cat. No.B-1 series) at 100X magnification by oil immersion technique.

II. Estimation of Total protein from lung tissue homogenate

Estimation of protein was done by Lowry's method, which requires 4 different solutions namely: solution A, solution B, solution C, and solution D. Solution A is the mixture of sodium carbonate (2%), sodium tartarate (0.05%), and sodium hydroxide (4%). Solution B contains freshly prepared copper sulphate solution (1%). Solution C contains 9 parts of solution A mixed with 1 part of solution B and solution D contains the Folins-Ciocalteaus reagent.

Step I- 0.2, 0.4, 0.6, 0.8 ml of the supernatant was pipetted out and 1 ml of the working standard was added into a series of test tubes.

Step II- 0.1 ml and 0.2 ml of the sample extract was pipetted out into two other test tubes.

Step III- The volume was made up to 1 ml in all the test tubes with double distilled water and a test tube with 1ml of water was served as the blank.

Step IV- 5 ml of reagent C was then added to each tube including the blank. They were then mixed well and allowed to stand for 10 minutes.

Step V- 0.5 ml of reagent D was then added, mixed well and incubated at room temperature in dark for 30 minutes. Blue color was developed.

Step VI- Readings were taken at 660 nm.

Step VII- A standard graph was plotted and the amount of protein in the sample was calculated. The standard graph was drawn by plotting concentration values on X axis and optical density values on Y axis. Amount of protein was expressed as µg/gm or 100gm/ sample.

III. Estimation of malonyldialdehyde (MDA) from lung tissue.

An incubation mixture was prepared as shown in the following table

Table 2: Sequence of the dilutions to be followed for MDA estimation

Ingredients	Volume
Tissue homogenate	0.5 ml (lung)
8.1 sodium dodecyl sulphate (SDS)	0.2 ml
20% acetic acid solution (adjusted to pH 3.5 with in NaOH /0.1N HCl solution)	1.5 ml
0.8% aqueous solution of thiobarbituric acid to pH7.4 with in NaOH /0.1N HCl solution)	1.5 ml

The incubation mixture was made up to 5.0 ml with double distilled water and then heated in boiling water bath for 30 minutes. After cooling, the rate chromogen was extracted into 5ml of a mixture of n-butanol and pyridine (15:1 v/v), centrifuged at 4000 rpm for 10minutes.The organic layer was then separated and its absorbance was measured at 532nm. 1, 1, 3, 3-tetra ethoxypropane (TEP) was used as an external standard and the level of lipid peroxides was expressed in nmoles of lipid peroxidation units /mg tissue. The calibration curve for TEP was prepared by the above described procedure taking TEP as standard. Linearity was obtained over the range of 80-240 nmoles of TEP.

IV. Estimation of lung tissue Myeloperoxidase (MPO) concentration

Neutrophil sequestratiion in lung was quantified by measuring tissue myeloperoxidase activity. Tissue samples (200mg) were thawed, minced, homogenized in 20 mM phosphate buffer solution (PH 7.4), centrifuged (10,000×g, 10 mts, 4 degree Celsius) and the resulting pellet was resuspended in 50 mM phosphate buffer (PH 6.0) containing 0.5%w/v hexadecyltrimethylammonium bromide (HTAB). The suspension was subjected to four cycles of freezing and thawing and further disrupted by sonication (40 secs). The sample was then centrifuged (10,000g, 5 mt, 4 degree Celsius) and supernatant used for MPO assay.

V. Preparation of MPO assay solution;

MPO assay solution was prepared by mixing 107.6 ml of distilled water 12 ml of 0.1M sodium phosphate buffer (PH 7.0), 0.192 ml of guiacol and 0.4 ml of 0.1M hydrogenperoxide. 20 µl of lung homogenate was mixed with 980 µl of MPO assay solution. The generation of tetraguiacol was measured spectrophotometrically at 470 nm and the change of optical density per minute was calculated from the formula.

Units/ml = Δ OD/min×45.1 and expressed as U/mg of protein. One unit of enzyme is defined as the amount that consumed 1 µmol of hydrogenperoxide/min.

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Statistical Analysis

The collected data were subjected to appropriate statistical tests like one-way ANOVA (Analysis of Variance) followed by an appropriate post hoc analysis such as Bonferroni post test multiple comparison tests. P values of less than 0.01 were considered significant. The analysis was carried using Graph pad Instat software of version 4.

RESULTS AND DISCUSSION

Pharmacognostical Studies

A. Ash value

The ash value viz total ash, acid insoluble and water soluble ash and sulphated ash were performed for all the crude drugs. All the results were complies with quality standards of Indian Medicinal Plants (ICMR) and shows the considerable quality.

Table 3: Ash value of selected medicinal plants

S. No	Parameter	*Clerodendrum Infortunatum*
1.	Total Ash (w/w)	11.60
2.	Acid insoluble ash (w/w)	04.61
3.	Water soluble ash (w/w)	05.79
4.	Sulphated ash (w/w)	01.93

B. Extractive Values

The water soluble extractives and alcohol soluble extractives were performed for all the crude drugs.

Table 4: Extractive values of selected medicinal plants

S. No.	Parameters	*Clerodendrum Infortunatum*
1.	Water soluble extractive value (w/w)	01.67
2.	Alcohol soluble extractive value (w/w)	04.75

C. Extraction

The extraction was carried out for all the selected plants by maceration method using suitable solvents.

Table 5: Percentage yield of the selected medicinal plants

S.No.	Selected Plant Name	Percentage yield in w/w
1.	***Clerodendrum Infortunatum***	03.4%

Phytochemical Studies

Qualitative Phytochemical tests were carried out for all the selected plant extracts. The results reveal that carbohydrates, phytosterols are present in all the selected species, proteins, fixed oils and fats, gums and mucilages were absent in all the plants.

Table 6: Result of qualitative analysis

S.no.	Phytochemicals	*C. Infortunatum*
1	Alkaloids	+ve
2	Carbohydrates	+ve
3	Glycosides	-ve
4	Saponins	+ve
5	Proteins	-ve
6	Fixed oils & Fats	-ve
7	Phytosterols	+ve
8	Steroidal Glycosides	-ve
9	Phenolic tannins	-ve
10	Flavonoids	-ve
11	Gums and mucilages	-ve
12	Volatile oils	-ve
13	Triterpenoids	+ve

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Qualitative and Quantitative Analysis by HPTLC

Finger print analysis was carried out for *Inula racemosa by* comparing the R_f values of standard.

Fingerprint analysis of *Clerodendrum Infortunatum*

I. *Clerodendrum Infortunatum* (Raw material)

Totally 7 peaks observed in *Clerodendrum Infortunatum*, in this sixth peak with R_f value of 0.65 was matched with standard β - sitosterol (0.65) and confirmed the presence of β-sitosterol in the raw material.

II. *Clerodndrum Infortunatum*, (Extract)

Totally 7 peaks observed in *Clerodendrum Infortunatum* in this sixth peak with R_f value of 0.65 was matched with standard β-sitosterol (0.65) and confirmed the presence of β-sitosterol in the extract.

Table 7: Result of HPTLC analysis I

S.No.	Sample	Marker compounds	Standard R_f values	Sample R_f values	Amount of Marker Compound % w/w	
					Tablet	Capsule
1	*Clerodendrum Infortunatum*	Finger print	0.65	0.65	-	-

B. HPTLC standardization

Quantification of marker compound was carried out by HPTLC technique for the formulation and all the chromatographic condition.

Table 8: Fingerprint Analysis of *Clerodendrum Infortunatum*

S.No	Sample	No. of peaks	R_f values
1	*C. Infortunatum* Raw material	8	0.04, 0.16, 0.21, 0.28, 0.47, 0.65, 0.76
2	*C. Infortunatum* Extract	7	0.02, 0.21,0.29,0.32,0.46,0.65, 0.98

The results of Fingerprint analysis of *C. Infortunatum shows that* 3 common peaks were observed in raw material, extract, tablet and capsule formulation. This confirmed the presence of *C. Infortunatum* in both the formulations.

Pharmacological Screening

Result Description

1. Total and differential WBC count

The results revealed that there was a significant ($p<0.001$) reduction in the number of total and differential WBC cells in treatment groups, when compared to the ova sensitized control. When compared with the saline control, less significant ($P<0.05$) increase in WBC total count was observed in standard drug treated and hydroalcoholic extract of *Clerodendrum Infortunatum* treated groups.

2. Effect of Polyherbal formulation on quantity of lung protein.

The lung protein was estimated. The level of lung protein was significantly ($P<0.001$) less or reduced in sensitized control when compared with normal control. The lung protein level in standard drug treated group was not protected as that of hydroalcoholic extract of *Clerodendrum Infortunatum.* The lung protein was significantly ($P<0.001$) protected or not degraded in hydroalcoholic extract of *Clerodendrum Infortunatum.*

3. Effect of Polyherbal formulation on Lipid peroxidation (MDA)

When compared with the ova sensitized control, treatment with hydroalcoholic extract of *Clerodendrum Infortunatum* significantly ($p<0.001$) decreased the MDA level. The lipid peroxidation in sensitized control was higher than that of other groups. The standard drug treatment could not reduce the MDA level as that of hydroalcoholic extract of *Clerodendrum Infortunatum.* Meanwhile the test drug treatment significantly ($P<0.001$) reduced the MDA levels when compared with sensitized control.

4. Effect of Polyherbal formulation on Myeloperoxidase enzyme

When compared with ova sensitized control, treatment groups showed significant ($P<0.001$) decrease in the Myeloperoxidase enzyme activity. The standard drug treated group's MPO level was significantly lower than that of sensitized control groups.The treatment with hydroalcoholic extract of *Clerodendrum Infortunatum* significantly ($P<0.001$) reduced the MPO level in experimental groups.

5. Mast cell stabilization activity of Polyherbal formulation

The percentage of intact mast cells was significantly ($P<0.001$) increased in treatment groups when compared with the ova sensitized control. The standard drug treated groups showed more number of intact mast cell when compared with sensitized control. There was a significant ($P<0.001$) high number of intact mast cell observed when treated with hydroalcoholic extract of *Clerodendrum Infortunatum*

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V – RESULTS

Table 9: Effect of hydroalcoholic extract of *Clerodendrum Infortunatum* on WBC and differential count

Treatment	Dose	WBC Cells/mm^3
Control- I	1ml/kg	4202.68±0.521
Control-II (Sensitized control)	2mg/ml (OVA)	8912.54±0.74***
Standard drug (Ketotifen & Dexamethasone p.o)	0.072mg/kg and 0.090mg/kg	4148.181±2.23 $^{ns,###}$
hydroalcoholic extract of *Clerodendrum Infortunatum*	5mg/kg	4052.68±0.98 $^{ns,###}$

Values are mean ± SEM; n=6 number of animals in each group
***P<0.001 vs control-I
^{ns}P>0.05 vs control -I
$^{###}$P<0.001 vs sensitized control-II
One way ANOVA followed by Bonferroni multiple comparison test.

Table 10: Effect of Polyherbal formulation on Tissue protein

S.No	Treatment groups	Dose	Total tissue Protein(Mg/gm)
1	Control-I (Normal)	1ml/kg	6.148±0.1002
2	Control-II (Sensitized control)	2mg/ml	2.821±0.0031***
3	Standard drug (Ketotifen+Dexamethasone,p.o)	0.072 and 0.090mg/kg	2.941±0.0178***
4	hydroalcoholic extract of *Clerodendrum Infortunatum*	05mg/kg	4.819±0.00189$^{*,###}$

Values are mean ± SEM; n=6 number of animals in each group
***P< 0.001,*P<0.05 vs Control
$^{###}$P<0.001 vs Control
One way ANOVA followed by Bonferroni multiple comparison tests.

Table 11: Estimation of Malonyldialdehyde (MDA)

S.No	Treatment groups	Dose	MDA (nM of MDA/mg of protein×10^3)
1	Control-I (Normal)	1ml/kg	0.91±0.1290
2	Control-II (Sensitized control)	2mg/ml	1.51±0.1201***
3	Standard drug (Ketotifen+Dexamethasone,p.o)	0.072 and 0.090mg/kg	0.81±0.1131$^{**,###}$
4	hydroalcoholic extract of *Clerodendrum Infortunatum*	05mg/kg	0.74±0.00146$^{**,###}$

Values are mean ± SEM; n=6 number of animals in each group
***P< 0.001, **P< 0.01 vs control
$^{###}$P<0.001 vs ova sensitized control.
One way ANOVA followed by Bonferroni multiple comparison tests.

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Table 12: Estimation of Myeloperoxidase (MPO) concentration

S.No	Treatment groups	Dose	Myeloperoxidase (U/g of protein)
1	Control-I (Normal)	1ml/kg	0.1971±0.10211
2	Control-II (Sensitized control)	2mg/ml	0.4182±0.0414***
3	Standard drug (Ketotifen+Dexamethasone,p.o)	0.072and 0.090mg/kg	0.188±0.00324*,###
4	hydroalcoholic extract of *Clerodendrum Infortunatum*	5mg/kg	0.172±0.0330**,###

Values are mean ± SEM; n=6 number of animals in each group.
***P<0.001, **P<0.01,*P<0.05, nsP>0.05 vs control
###P<0.001 vs Sensitized control
One way ANOVA followed by Bonferroni multiple comparison tests.

Table 13: Mast cell stabilizing activity

S. No	Treatment groups	Dose	CPD. 48/80	Intact mast cells (%)
1	Control-I (Normal)	1ml/kg	100µg/ml	88.35±0.34
2	Control-II (Sensitized control)	2mg/ml	100µg/ml	18.42±0.1436***
3	Standard drug (Ketotifen+Dexamethasone,p.o)	0.072 and 0.090mg/kg	100µg/ml	84.34±0.5676###
4	hydroalcoholic extract of *Clerodendrum Infortunatum*	5mg/kg	100µg/ml	83.91±0.0.71###

Values are mean ± SEM; n=6 number of animals in each group
***P<0.001 vs control-I
###P<0.001 vs sensitized control
One way ANOVA followed by Bonferroni multiple comparison test

DISCUSSION

The antiasthematic activity of the hydroalcoholic extract of *Clerodendrum Infortunatum* was assessed and studied by standard procedure Rats were being selected for the anti-asthmatic screening activity; since experimental data suggests that most characteristics feature of human asthma including pathological change can be duplicated in rats.

This study the total and differential cell count from BALF was increased two fold in ova treated animals and less significant increase in treatment groups was observed. During asthmatic attack because of antigenic response of the allergen, it is a common phenomenon that the WBC count seems to be increased. Treatment with hydroalcoholic extract of *Clerodendrum Infortunatum* might have reduced the migration of number inflammatory cells in the BALF and hence reduced the number total and differential of leukocyte count.

Myeloperoxidase enzyme activity is an index of polymorphonuclear leukocyte accumulation. Within seconds of binding to a foreign particle neutrophils increase their oxygen consumption to 100 folds. During respiratory burst, myeloperoxidase (MPO) enzyme activity

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in neutrophils will be increased. Myeloperoxidase a constituent of neutrophil would be a marker for elevated neutrophil count. The ability of the hydroalcoholic extract of *Clerodendrum Infortunatum* in MPO activity reduction, pointed that there was a significant reduction in migration of neutrophils in to the lung tissue and hence protects the tissue from damage to inflammatory mediators.

Mast cells play a key role in the pathogenesis of asthma. It is known that the physiological stimulus for the release of inflammatory mediators from mast cells is provided by the combination of antigen with specific antibody fixed on the cell surface. This combination is believed to transiently increase the permeability of the membrane to calcium ions for the secretory process to occur.

Compound 48/80 induced secretion from mast cells share a common requirement as far as the presence of calcium is concerned. Compound 48/80 can utilize intracellular calcium store to initiate the release process even in the absence of calcium in the extracellular medium. However anaphylactic release requires the presence of calcium in the extracellular medium which moves in to the cell via calcium gates in the membrane

Although oxygen is essential for life, its transformation to ROS may provoke uncontrolled reactions. Lipids are more susceptible macromolecules and are present in the form of polyunsaturated fatty acids (PUFA). ROS attack PUFA in the cell membrane leading to a chain of chemical reactions called lipid peroxidation. During initiation; the free radicals react with fatty acid chain and release lipid free radical which reacts further with molecular oxygen to form lipid peroxyl radical. Peroxyl radicals again react with fatty acid to produce lipid free radical and this reaction is propagated. During termination process two radicals react with each other and the process comes to an end. This process of fatty acid break down produces hydrocarbon gases (ethane or pentane) and aldehydes.

SUMMARY AND CONCLUSION

In present study medicinal plants which are having scientific evidence for therapeutic efficacy were selected in less number. In this combination, bronchodilator, antihistaminic, antioxidant and anti inflammatory category of plants were used. Phytochemical evaluation of the crude drugs such as ash value, extractive value were carried out and complies the limit given by quality standards of medicinal plants.

The fingerprint analysis was carried out for the *Clerodendrum Infortunatum*, and compared the R_f values with gallic acid for the confirmation. The prepared hydroalcoholic extract of *Clerodendrum Infortunatum* was subjected to determination of physical and chemical evaluations and the results complies as per the standards. The pharmacological findings concluded that the hydroalcoholic extract of *Clerodendrum Infortunatum* could reverse asthmatic condition and reduced the total and differential WBC cell count in BALF, retained protein and MDA level in lung tissue, increased intact mast cells thus preventing its degranulation and mediators release. This Polyherbal hydroalcoholic extract of *Clerodendrum Infortunatum* formulation is could be a promising new therapeutic approach for the treatment of clinical asthma.The stability studies and clinical trials to be carried out in future.

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