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Phytochemical Investigation and Pharmacological Evaluation of Indian Medicinal Plant Extract, and Characterization of Bioactive Phytoconstituents for Anti-inflammatory Activity

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Abstract: Vascularized living tissue responds to a local insult by becoming inflamed. We began looking for novel anti-inflammatory medications from indigenous sources due to the terrible adverse effects of both steroidal and non-steroidal anti-inflammatory treatments. The anti-inflammatory properties of the methanol extract of Clerodendron infortunatum Linn. (MECI) leaves were assessed in relation to rat paw edoema caused by dextran, histamine, and carrageenan. The methanol extract, at doses of 250 and 500 mg/kg body weight, had noteworthy efficacy ($p<0.01$) against every pharmacological agent examined. Phynylbutazone was used as a reference medication to compare all of these effects.

Keywords: Clerodendron infortunatum Linn, Phynylbutazone, Inflammation, Carrageenan, Histamine, Dextran, etc.

Introduction

One of the most popular sources of medicines is the biodiversity of plants. The use of traditional herbal remedies has increased recently, and many pharmaceuticals are now either directly or indirectly derived from natural sources. The secondary metabolism of the plants produces a variety of compounds, such as flavonoids, alkaloids, tannins, steroids, glycosides, phenols, fixed oils, and saponins, which are then stored plant parts like leaves, bark, flowers, seeds, fruits, and roots, giving the plants their medicinal qualities. There were notable biological and pharmacological characteristics displayed by these secondary metabolites and their derivatives. They protect the plant from microbes and serve as antioxidants, scavengers of free radicals, and agents that inhibit the proliferation of cells.

An appropriate defensive reaction to tissue damage brought on by harmful chemicals, microorganisms, or physical trauma is inflammation. It is the body's reaction to get rid of the irritant, inactivate or destroy the invasive organisms, and prepare the tissue for healing. Chemical mediators released by wounded tissue and migratory cells cause it to occur [1]. In India, traditional medicine uses drugs derived from plants to treat a wide range of illnesses. Clerodendron infortunatum Linn., a member of the Verbenaceae family, has bronchitis, asthma, fever, burning sensation, blood illness, inflammation, and epilepsy, according to [2] sources. The herb has historically been used as an anthelmintic and an antipyretic. The plant's leaves are prescribed for tumour, specific skin conditions, and scorpion stings [3]. Alkyl sterols [4] and 2, -(3, 4-dehydroxyphenyl) ethanol 1- O-"-2 rhamnopyranosyl-(163)-$-D-(4-O-caffeoyl) glycopyranoside (acteoside) [5] were found in the plant according to earlier phytochemical analysis. The goal of the current investigation was to determine whether a methanol extract of Clerodendron infortunatum Linn. leaves could reduce inflammation in rats.

Material and Method

Plant Material

Collection and Drying

Mature leaves were gathered from the Nanded district and then cleaned, dried, and stored out of direct sunlight at room temperature. In a mixer grinder, the dried leaves were reduced in size to a coarse texture. The powdered material was sieved through 60–120 mesh to remove fines and bigger particles, and the powder was used for further evaluation. The particle size difference of crude drug increases the extraction time, and fine particles can form bed and increase the extraction time.

Standardization of Leaf

The physical confirmation of crude drug contains the confirmation of identity, quality, and purity. Purity contains the absence of any unnecessary content whether inorganic or organic and quality means required concentration of the active constituents in crude drug which makes it as important drug. The following standardization parameters were evaluated to obtain the quality and purity of *Clerodendrum Infortunatum L.* Ash value, acid-insoluble and water soluble as well as sulphated ash value, alcohol and water-soluble extractive value, determination of loss on drying and foreign organic matter were performed as per the methods described in I.P. 1996.

Determination of ash value

Ash value is important in identification of quality & purity of *Clerodendrum Infortunatum L.* leaf. It mainly consists of different inorganic variables and radicals like phosphates, carbonates, silica as well as calcium, magnesium, sodium and potassium silicates calcium oxalate and carbonate, silica are present as inorganic variables which changes 'Total Ash Value.' These variables can be removed by concentrated acid treatment, known as acid insoluble ash.

Total Ash Value

Method

2 gm of completely dried powder of *Clerodendrum Infortunatum L.* leaf was weighed and added in pre weighed silica crucible and burned at room temperature not more than 450 degrees until it become free from carbon. It was determined by cooling the silica dish in desiccator. This process was performed again and again till it gives fixed weight. Total ash percentage was determined by consideration with weight of initial powder of Clerodendrum *Infortunatum L leaves.*

$$\%\, Total\ ash\ value = Wt.\ of\ total\ ash \div Wt.\ of\ crude\ drug\ taken \times 100$$

Water soluble Ash

Method

Clerodendrum Infortunatum L. powder was taken and 2gm powder was added in previously weighed crucible of silica and it was then kept at high temperature not more than 450 degrees until it become free from carbon. It was determined by cooling the silica crucible in desiccator and weighed. The same process was repeated until constant weight was identified. The ash thus obtained was further boiled in 25ml of water for about 5minutes, ash insoluble in water was collected by filtration in silica plate and washed. The content was burned for few minutes at high temperature but not more than 450 degrees. From the total ash weight of insoluble matter was deducted to get weight of water-soluble ash. Percentage was determined by considering the initial weight of crude drug.

$$\%\, Water\ soluble\ ash\ value = Wt.\ of\ total\ ash - Wt.\ of\ water\ insoluble\ ash \div Wt.\ of\ crude\ drug\ taken \times 100$$

Acid-insoluble Ash

Method

2gm of dried *Clerodendrum Infortunatum L.* powder was added in pre weighed crucible of silica and burned at high temperature, less than 450 degrees until free from carbon. It was determined by cooling the silica dish in desiccator and weighed. The same process was repeated till constant weight was obtained. The ash obtained was mixed in 25ml 2M HCL and boiled for 5 minutes. Then insoluble content was added in a silica gel crucible. Again, hot water was added and filtered, then burned and cooled in a desiccators, weight was taken. The percentage was determined by considering initial weight of *Clerodendrum Infortunatum L* leaves.

$$\%\ Acid\ insoluble\ ash\ value = Wt.of\ acid\ insoluble\ ash \div Wt.of\ crude\ drug\ taken \times 100$$

Sulphated ash value

Method

2 gm of powdered *Clerodendrum Infortunatum L. leaves* was added in previously weighed crucible. It was heated at high temperature not more than 450 degrees until it became free from carbon. It was determined by cooling the silica crucible in desiccators and weighed. The same process was repeated till constant weight was identified. The ash obtained was mixed with 1ml of H_2SO_4, heated until release of white colored fumes finished. Further ignited at $800^o \pm 25^0$ C till all black particles get disappeared. The heating was done away from direct air. The silica crucible was cooled. Again, few drops of H_2SO_4 were added and ignited. This process was done repeatedly to get constant weight.

Extractive Value Determination

The process for determination of extractive value was done as per described in Indian Pharmacopoeia (1996).

Water-soluble extractive value

Method

5gm *Clerodendrum Infortunatum L.* leaves powder was weighed was added in closed flask and kept for maceration in chloroform water (100ml) for one day with frequent shaking in for first 6 hours, then for 18hours kept aside and filter. In preweighed silica dish, 25ml of filtrate was taken and evaporated to dryness. The percentage was determined by considering initial weight of *Clerodendrum Infortunatum L. leaves.*

Calculations

If 25 ml of aqueous filtrate gives X g of residue,

Then 100 ml of filtrate will give 4X g of residue,

So 5gm of powdered *Clerodendrum Infortunatum L.* contains 4X g of water-soluble residue

Therefore, percentage of water-soluble extractive value will be 80X.

Alcohol-soluble extractive value

Method

Accurately weight 5gm of powdered *Clerodendrum Infortunatum L.* leaves was mixed with 95% ethanol (100ml) in a closed vessel. It was macerated for 24hours with occasional shaking for initial six hours. Kept aside for 18 hours and filtered carefully to avoid evaporation of ethanol. Filtrate (25ml) was evaporated in pre weighed porcelain dish, weight was calculated. The percentage of alcohol soluble extractive value was determined by considering initial weight of powdered *Clerodendrum Infortunatum L.* leaves.

Calculations

25 ml of alcohol filtrate possess about A g of residue,

So, 100 ml of filtrate contains 4A gm of residue.

Then this 100ml filtrate was prepared from 5gm of powdered of *Clerodendrum Infortunatum L.*

So 5gm of powdered *Clerodendrum Infortunatum L.* contains 4A gm of residue.

And percentage of extractive value will be 80A gm of alcohol (90%) soluble residue.

Foreign organic matter Determination

The foreign matter in crude drugs mainly contains either parts of the plant or product. Sometimes it might also contain any organism. It also indicates any mineral content which is not the medicinal plant materials like stones, soil and dust etc. Enough crude drug was spread on a thin layer of clean paper. With help of magnifying lens or by visual inspection, the content present other than the crude drug was collected and weighed.

Determination of Loss on Drying or Moisture content

The shallow glass-stoppered weighing bottle was dried and weighed. 2g crude drug was added in the bottle and closed, the weight was taken and crude drug was spread evenly to a height not more than 10mm. Then the bottle was kept in the oven for drying keeping open without the stopper. The sample was dried to constant weight. It was cooled to normal temperature in desiccator again weighed loss on drying was calculated in percent w/w (Indian Pharmacopoeia 1996).

$$\%\ Loss\ on\ Drying = Loss\ in\ weight\ of\ the\ sample \div Weight\ of\ the\ sample \times 100$$

Extraction Method

The solid extraction of drug represents a solid from solid separation. The liquid-liquid extraction is one, in which any of the two immiscible liquids are used for the extraction (Solvent extraction).

Extraction process comes to halt when the distribution of the extractive substance between miscella and drug residue reaches the value 'K', i.e. when the concentration gradient between miscella and reaches has become zero. (Mukherjee,2002).

K=Concentration of extracted substances in the miscella / Concentration of extractive substance in the drug residue

Hot continuous extraction – Soxhlation

Soxhlet extractor is the simplest way for preparation of extracts of crude drugs. Pure solvent is used in this technique. The crude drug used for extraction is kept in a 'thimble' made of cloth or cellulose in middle portion of the soxhlet apparatus. Siphon tube and a side arm both are connected to a lower portion. The solvent used for extraction is kept in the lower portion and a condenser is connected above the middle compartment.

The solvent is added in Round Bottom Flask and heated to boil to form vapors. The vapors travel through the side arm into the reflux condenser. The vapor cools there and falls onto the thimble containing the crude drug kept for extraction. The hot solvent passes through the crude drug and extraction takes place. The extract gets deposited in the lower portion of middle compartment. As the height of extract reached to the top of the siphon tube, the extract gets deposited in the middle portion passes through it and goes into the lower container i.e. round bottom flask. The same process was repeated till complete extraction of crude drug takes place.

In this technique of extraction, the extract gets collected in the lower RBF, gradually becomes concentrated. The soxhlet extraction process is very helpful for the total extraction of the crude drug with a specific solvent. Different solvents with increasing polarity can be used for the continuous total extraction, e.g., benzene, hexane, pet. Ether, chloroform, methanol, ethanol, water. The crude drug was dried when it was subjected for extraction using another solvent. The previous solvent should be removed completely and powder should be dried totally. It prevents the mixing of the previous solvent into another solvent. (Mukherjee,2002; Harbone1998)

Extraction Procedure

The dried powder of leaves used for extraction procedure was sieved through 60-120 mesh to separate fine and course powder. This course powder was utilized for further extraction. The extraction was performed by using continuous hot extraction using soxhlet apparatus till removal of all constituents takes place. The confirmation of complete extraction was done by taking a drop of extract from exit of side tube on TLC, drying and exposing to iodine vapors. If extraction is completed then it shows absence of colored spot-on TLC plate. After the complete extraction, solvent was evaporated on rotary evaporator and solvent was removed, the extract thus obtained with 95% alcoholic solvent was measured. The extract was stored in desiccator. (Mukherjee,2002)

Percentage yield of Extract

This method gives solubility of chemical constituents from sufficient quantity of crude drug when it is done by specific solvent. When extraction done with different solvents and for different crude drugs. It gives variable results about phyto-constituents. The availability of different constituents in specific solvent mainly depends upon nature of drug and solvent used for extraction. (Mukherjee, 2002)

Accurately about 500gm of powdered stem was subjected to the extraction as per the methodology described and percentage yield was calculated.

Preliminary Phytochemical screening for alcoholic extract of *Clerodendrum Infortunatum L.*

The qualitative phyto-chemical evaluation of alcoholic extract *Clerodendrum Infortunatum L.* was performed to detect different chemical constituents. The different reagents and tests were used to detect various secondary metabolites.

Tests for Carbohydrates: (Peach and Tracey, 1965; Driver,1955)

General test for Carbohydrates (Molisch's test)

Few drops of α-naphthol dissolved in alcohol were added in 2-3 ml of extract, mixed well and conc. H_2SO_4 was added from side wall of test tube. Violet colored ring formation at junction of two liquids shows that carbohydrates are present in the extract.

Benedict's test

Clerodendrum Infortunatum L. extract 2ml mixed with benedict's reagent (2ml) and mixed well. The mixture was kept in boiling H_2O bath for 4-5 minutes, mixture in test tube forms red, yellow, or green color depends upon the quantity of reducing sugar present in extract.

Fehling's test

Equal quantity of Fehling's A and Fehling's B solutions was mixed. In mixture of Fehling's solution 2ml of *Clerodendrum Infortunatum L.* extract was mixed, Shaked well. The complete mixture was boiled in water bath for 4-5 min. and color of formed precipitate was determined. It gives initially yellow and then brick red precipitate if reducing sugar present.

Barfoed's test

Barfoed's test and *Clerodendrum Infortunatum L.* extract was mixed in equal quantity and boiled in water bath for 1-2minutes. Red colored precipitate indicates availability of reducing sugars.

Tests for Non-Reducing Sugars

The *Clerodendrum Infortunatum L.* extract do not show positive tests for response to Benedict's and Fehling's test.

Tests for Proteins (Hawk et al.,1954)

Test Solution Preparation: *Clerodendrum Infortunatum L.* extract was dissolved in water to prepare clear solution**.**

Biuret Test (General Test)

To 5ml test solution, 4 % NaOH (5ml) and 1% $CuSO_4$ (few drops) was mixed. Pink/violet color formation indicates presence of proteins.

Million's Test

The test solution of *Clerodendrum Infortunatum L.* extract was mixed with double quantity of Million's reagent, presence of proteins gives formation of white colored precipitate.

Tests for Amino Acids:(Hawk, et al., 1954)

General test of amino acid Ninhydrin Test:

To equal quantity of *Clerodendrum Infortunatum L.* extract same quantity of drops Ninhydrin reagent (5%) was kept in water bath for some time and formation of purple or bluish color was observed.

Test for tryptophan

Few drops glycol –oxalic acid and conc. H_2SO_4 were added in 3ml of the *Clerodendrum Infortunatum L.* extract. The reddish violet ring formation takes place at the junction of the two layers.

Test for Tyrosine

Equal volume of *Clerodendrum Infortunatum L.* extract and Million's reagent mixed and boiled. Formation of dark red color indicates presence or absence of amino acids.

Tests for Steroids: (Hawk, et al.,1954)

Salkowski reaction

Equal volume of *Clerodendrum Infortunatum L. extract, conc.*H_2SO_4 and chloroform were mixed well. Red color appears to layer of chloroform, acid layer gives greenish yellow colored fluorescence for presence of steroids.

Liberman's test:

Equal quantity of *Clerodendrum Infortunatum L.* extract and acetic anhydride was mixed. Heated and cooled and few drops conc. H_2SO_4 was mixed and formation of blue color indicates availability of steroids.

Libermann-Burchard Test

5 ml *Clerodendrum Infortunatum L.* extract when mixed with $CHCl_3$. Acetic anhydride (2-3 ml) was added and conc. H_2SO_4 (2-3 drops) were added from side wall and formation of initially red, then blue lastly green color indicates steroids are present.

Tests for Glycosides: (Rosenthaler, 1930; Trease and Evans, 1983; Middeltone, 1956)

Preparation of Test solution: *Clerodendrum Infortunatum L.* extract was dissolved in hydro-alcoholic solution/ in alcohol.

Cardiac Glycoside test

Baljet Test

Addition of Sodium Picrate to test solution gives formation of yellow to orange color due to presence of cardiac glycoside.

For Cardenoloids-Legal's test

Alcoholic or Hydro-alcoholic test solution, pyridine (1ml) and Na-nitroprusside solution (1ml) was added, formation of pink to red color indicates presence of Cardenoloids.

Test for Deoxysugars (Killer Killani Test)

1 ml of GAA, 2ml Test Solution and a drop of $FeCl_3$ (5%) were mixed and conc. H_2SO_4 was added from sides of test tube. Formation of bluish green color to upper layer and reddish-brown color formation at the junction of two liquids shows that Deoxy sugars are present.

Liberman's test (For bufadenolids)

Equal quantity of extract and acetic anhydride was mixed and heated and cooled. When few drops of concentrated H_2SO_4 were added presence of bufadienolides gives formation of blue color.

Test for anthraquinone glycosides

Borntrager's test for O-anthraquinones

The powdered *Clerodendrum Infortunatum L.* was mixed with 10% Sulphuric acid (5ml). Boiled and filtered. Filtrate was mixed with benzene and shaken for few minutes. Isolation of benzene layer was done and mixed with 10% NH_3. Both layers were separated. Rosey pink color appears to ammoniacal layer indicates presence of O-anthraquinone glycosides.

Modified Brontrager's test (O-anthraquinone glycoside)

C-anthraquinone glycosides cannot get hydrolyzed easily it requires oxidative hydrolysis. Hydrolysis of crude drug was performed using acid and $FeCl_3$. The powder of *Clerodendrum Infortunatum L.* was mixed with dilute HCL and 5% $FeCl_3$ solution (5ml), boiled, filtered, cooled then shaken with organic solvent like chloroform or benzene. The organic solvent was separated, further mixed with ammonia solution (10%) and kept aside. The ammoniacal layer shows reddish pink color due to presence of C-anthraquinones.

Tests for Saponin Glycosides

Hemolytic Test

One drop of blood was kept on glass slide and a drop of *Clerodendrum Infortunatum L.* extract was added in it. Formation of Hemolysis was observed for presence of Saponins.

Foam test

Powder of *Clerodendrum Infortunatum L.* crude drug was mixed with water and shaken vigorously. Formation of stable foam for minimum of 30 min indicates presence of saponin glycosides.

Cyanogenic glycosides Test

Sodium Picrate test

10 % picric acid was soaked in a filter paper strip and dried and then 10% sodium carbonate was soaked in it and again dried.

The powder of *Clerodendrum Infortunatum L.* was kept in flask and small quantity of water is added, mixed, and closed. The filter paper strip soaked in picric acid and sodium carbonate was placed in the slit in cork above the powder mixture. The color of filter paper turned brick red or maroon due to presence of Cyanogenic glycosides in the crude drug.

Flavonoids Test: (Shellard, 1957; Trease and Evans, 1983)

Shinoda Test: In the *Clerodendrum Infortunatum L.* extract, addition of conc. HCL and 0.5gm Mg-turnings was done. Pink color formation indicates that flavonoids are present.

Lead acetate solution was added to *Clerodendrum Infortunatum L.* extract. Yellow colored precipate formation indicates that flavonoids are present.

When sodium hydroxide was added in increasing amount in above residue it gives yellow coloration, and it decolorizes on addition of any acid.

Alkaloids Test

The *Clerodendrum Infortunatum L.* extract was boiled in alcohol and filtered. The filtrate was used to perform different chemical tests for identification of alkaloids (Rosenthaler, 1930; Trease and Evans, 1983).

Dragendroff's Test: To the 2-3 ml filtrate, Dragendroff's reagent (1ml) was mixed. Orange brown precipitate formation indicates that alkaloids are present.

Mayer's Test: Few drops of Mayer's reagent were mixed with few ml of filtrate. Formation of buff colored precipitate shows that alkaloids are present.

Hager's test: Alcoholic extract was mixed with half volume of Hager's reagent; formation of orange brown precipitate shows presence of alkaloids.

Wagner's test: Wagner's reagent was mixed in 2-3ml filtrate. Presence of alkaloids can be confirmed by appearance of brownish red colored precipitate.

Tannins and phenolic compound test: (Finar, 1959; Trease and Evans, 1983)

$FeCl_3$ Test: *Clerodendrum Infortunatum L.* extract was mixed with 5% $FeCl_3$ solution (1ml), Tannins indicate development of greenish clack coloration.

Lead acetate solution test: *Clerodendrum Infortunatum L.* (5ml) was mixed with 10% aq. Lead solution (1ml). Presence of tannins gives formation of yellow colored precipitate.

Potassium Dichromate Test: *Clerodendrum Infortunatum L.* extract (5ml) when mixed with 10% aq. Potassium dichromate solution (1ml), yellowish brown precipitate indicates formation of tannins**.**

Bromine water test: When freshly prepared bromine water was mixed in 5 ml of *Clerodendrum Infortunatum L.* extract. Discoloration of bromine water shows presence of tannins.

Dilute of $KMnO_4$ Test: Few ml of *Clerodendrum Infortunatum L.* extract was mixed with diluted $KMnO_4$ solution. Presence of tannins gives discoloration of $KMnO_4$.

Test for mucilage: (Peach and Tracey, 1965; Driver, 1955)

The *Clerodendrum Infortunatum L.* extract was mixed to few ml of ruthenium red solution in alcohol. Pink color formation indicates that mucilage was present.

Test for Gums: (Paech and Tracey, 1965; Driver, 1955)

The *Clerodendrum Infortunatum L.* extract was hydrolyzed by addition of HCl. The testes for presence of reducing sugar i.e., Benedict's or Fehling's test were performed. Formation of red color indicates presence of gums.

Identification of constituents by High Resolution Liquid Chromatography Mass Spectroscopy (HRLCMS) technique

Method

The powdered crude drug sample of *Clerodendrum Infortunatum* L was dissolved in the mobile phase in which it was soluble (i.e., methanol). The different runs were done for the method, the result was analyzed and reported.

Report (HRLCMS)

The report included the compound table which included the list of compounds (active constituents) present in the sample as follows:

Sr. No	Rt	Mass	Abund	Name	Formula	Hits (DB)
1	1.027	169.0378	32978	2-Furoylglycine	C7 H7 N O4	10
2	7.351	476.0915		Diosmetin 7-O-beta-D-glucuronopyranoside	C22 H20 O12	7
3	7.985	460.097		Irilone 4'-glucoside	C22 H20 O11	10
4	10.418	284.0662		Prunetin	C16 H12 O5	10
5	10.885	954.4333	51277	Deltorphin A	C44 H62 N10 O10 S2	3
6	10.99	438.2724		LPA (0:0/18:0)	C21 H43 O7 P	4
7	11.345	438.2721		LPA (0:0/18:0)	C21 H43 O7 P	4
8	12.34	381.9678		Fluopicolide	C14 H8 Cl3 F3 N2 O	1
9	12.696	410.2416		LPA (0:0/16:0)	C19 H39 O7 P	9
10	12.756	536.1568		1,4-beta-D-Glucan	C18 H32 O18	8
11	13.04	410.2417		LPA (0:0/16:0)	C19 H39 O7 P	9
12	13.114	482.1737	48648	Melleolide D	C24 H31 Cl O8	6
13	14.872	568.0962	36769	Gadodiamide	C16 H26 Gd N5 O8	2
14	15.064	632.1083	33802	UDP-2,4-bis(acetamido)-2,4,6-trideoxy-beta-L-altropyranose	C19 H30 N4 O16 P2	3
15	15.614	760.1309	30631	Prodelphinidin A2 3'-gallate	C37 H28 O18	3
16	15.637	696.1183	40443	Maclurin 3-C-(2"-p-hydroxybenzoyl-6"-galloyl-glucoside)	C33 H28 O17	2
17	15.947	760.1321	70176	Prodelphinidin A2 3'-gallate	C37 H28 O18	3
18	15.96	696.1194	40761	Maclurin 3-C-(2"-p-hydroxybenzoyl-6"-galloyl-glucoside)	C33 H28 O17	2
19	16.123	478.2953		25-Acetylvulgaroside	C27 H42 O7	5
20	16.292	838.1608	52659	Beta-Alanyl-CoA	C24 H41 N8 O17 P3 S	1
21	16.368	760.1313	55138	Prodelphinidin A2 3'-gallate	C37 H28 O18	3
22	18.536	562.3759	52127	Rhodoxanthin	C40 H50 O2	5

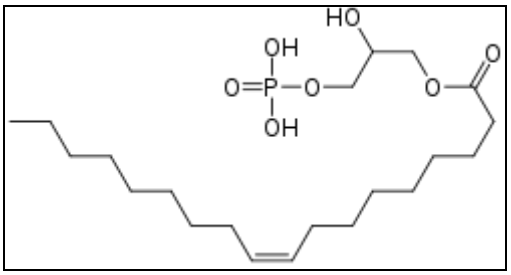
Studying the above table, the presence of LPA (Lysophosphatidic Acid) and Rhodoxanthin gave confirmation to select it for isolation. Reasons for selection of LPA are as follows:

1. They are reported for the anti-asthmatic activity previously, which is the aim of our present study.
2. The hits of constituents i.e., amount of active constituent in sample is at higher rate, which enhances the rate of success of isolation method.

Characterization by Chromatographic Studies

Lysophosphatidic Acid (LPA)

Lysophosphatidic acid (LPA), a water-soluble phospholipid, has gained significant attention in recent years since the discovery that it acts as a potent signaling molecule with wide ranging effects on many different target tissues. Because of its ability to stimulate cell proliferation, aberrant LPA-signaling has been linked to cancer in numerous ways.



Structure of Lysophosphatidic acid (LPA)

The isolation and purification for further studies was done by High Performance Thin Layer Chromatography Method (HPTLC). The isolation method was found for the LPA (Lysophosphatidic Acid).

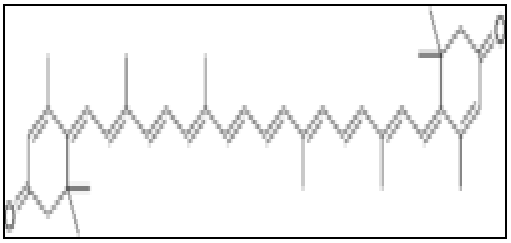
Method

1. The mobile phase used for the development was combination of 65ml chloroform, 32ml methanol, 5.5ml ammonium hydroxide.
2. Sample was applied to the pre-coated plates of HPTLC plates with application of micro-pipette and kept for air drying at room temperature for 5 minutes.
3. The plates were put into the chamber containing mobile phase for 90 minutes.
4. After HPTLC separation, the plates were removed from the chamber and dried at room temperature for 3 minutes.
5. For the trace of movement of purified compound, 0.1% 8-anilino-1-napthalene-sulfonic acid was sprayed at the marker lane of plate.
6. The sample was scraped from plates according to marker lane.
7. 2ml of 2:1 (v/v) methanol-chloroform was added to the recovered powder.
8. Vortexed and kept in ice for 1 hour and centrifuged at 3000xg at 10^{o} for 10 minutes.
9. Top layer was transferred into tube containing the organic phase and dried under nitrogen.
10. Then 1.5ml 2:1(v/v) methanol-chloroform was added into powder again for second extraction.
11. The mixture was vortexed and centrifuged at 3000xg at 10 degree for 10 minutes.
12. Top layer was transfer into tube containing the organic phase and dried under nitrogen.
13. The purified compound was separated and stored for further analysis.

Rhodoxanthin

Rhodoxanthin is a xanthophyll pigment with a purple color that is found in small quantities in a variety of plants including Taxus baccata and Lonicera morrowii. It is also found in the feathers of some birds. As a food additive

it is used under the E number E161f as a food coloring. It is not approved for use in the EU or US; however, it is approved in Australia and New Zealand.



Structure of Rhodoxanthin

The isolation and purification for further studies was done by Thin Layer Chromatography Method (TLC). The isolation method was found for the Rhodoxanthin.

Method

1. Partition TLC was performed on precoated silica gel layers type Polygram Sil-G (Macherey-Nagel).
2. Further purification was achieved by adsorption TLC on a mixed layer of $CaCO_3$, MgO and Ca $(OH)_2$ (30:6:5).
3. In both systems mixtures of petroleum benzene (100-140^{0}C) and iso-propanol were used as solvents; the ratio was varied (100:5 to 100:16; v/v) to obtain optimal separations.
4. For mass spectrometry the carotenoids were finally run on pre-coated layers of ultrapure silica gel type G-25 HR which had been prewashed with methanol.
5. After TLC the colored bands were scraped off and the pigments eluted with acetone.

Anti-Inflammatory Activity [6-12]

Animal Used

Wistar strain rats weighing 150-180 g were kept in identical lab settings with a temperature of 25–30 °C and a relative humidity of 55–65%. They were also fed commercial pellet food and had unlimited access to water. The conditions were changed every 12 hours. The University's animal ethics committee examined and authorized each of the procedures listed (ref no. 367001/C/CPCACA).

Carrageenan-Induced Rat Paw Edema

There were four groups of rats (n=6). The rats' right-hand paw was given 0.1 ml of 1% w/v carrageenan in normal saline supplanter, which resulted in acute inflammation. Using a Plethysmometer, the paw volume was assessed 0 and 3 hours after the carrageenan injection. As a saline control, group I animals were given normal saline (3 ml/kg b.w., intraperitoneal, i.p.). Group IV was given the reference medication phenylbutazone (100 mg/kg b. w., i.p.), while groups II and III were given the methanol extract of Clerodendron infortunatum (250 and 500 mg/kg b.w., i.p., respectively). The reference medication and extract were given to the animals in all groups one hour prior to the carrageenan being administered.

Mediator-Induced Inflammation

The extract's ability to reduce inflammation was assessed using phlogistic agents, such as histamine and dextran, which function as mediators of inflammation. Rats were given sub plantar injections of a newly made histamine (1 mg/ml) and dextran (1 mg/ml) solution to induce paw edoema, and the paw edoema was then quantified as previously indicated.

Result and Discussion

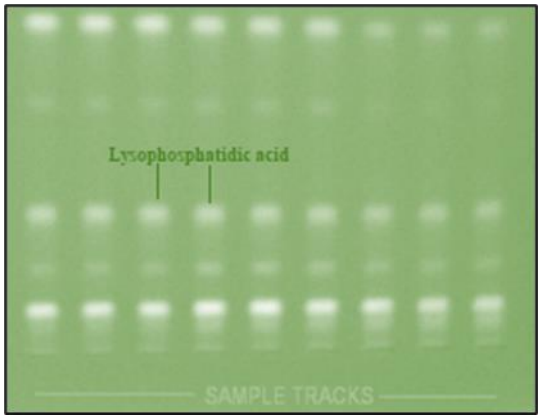
Table: Physico-chemical Parameters of leaves of *Clerodendrum Infortunatum L.*

Sr. No	Physico-chemical Parameter	*Clerodendrum Infortunatum L.*
1	Foreign Organic Matter	**<2**
2	Total Ash	**9.95% (w/w)**
3	Acid Insoluble Ash	**0.70% (w/w)**
4	Water Soluble Ash	**2.15% (w/w)**
5	Sulphated Ash Value	**0.985%**
6	Moisture Content	**3.65**
7	Extractive Values	**Water=13.50%(w/w)** **Ethanol=7.50%(w/w)** **Ether=3.65%(w/w)**
8	Foaming index	**<100**

Result: The % yield of 95% alcoholic extract of *Clerodendrum Infortunatum L.* = %w/w.

Table: Preliminary Phyto-chemical screening of *Clerodendrum Infortunatum L.* extract.

Sr. No	Name of test	*Clerodendrum Infortunatum L.* *(Present/Absent)*
1	Test for Carbohydrates	**Present**
2	Test for Proteins	**Absent**
3	Test for Phenolics	**Present**
4	Flavonoids Test	**Present**
5	Test for Glycosides	**Present**
5a	Cyanogenic Glycosides	**Absent**
5b	Tests for Anthraquinone Glycosides	**Absent**
5c	Saponin Glycosides	**Present**
5d	Cardiac Glycosides	**Absent**
6	Alkaloids Test	**Absent**
7	Test for Tannins	**Present**
8	Test for Coumarins	**Present**
9	Test for Saponins	**Present**
10	Test for Steroids	**Absent**



Images of developed HPTLC plates after development

Observations

Under 486 nm in three rhodoxanthin fractions obtained by TLC from *Clerodendrum Infortunatum*.

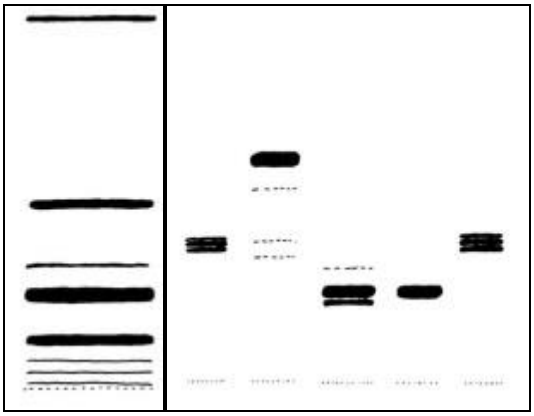


Image of TLC plate

Mass Spectra

Lysophosphatidic Acid

The mass spectrum shows a molecular ion peak at m/z 409.2344 $(M-1)^-$ which is in good agreement for the proposed structure of known lysophosphatidic acid (LPA).The mass spectra shown in figure.

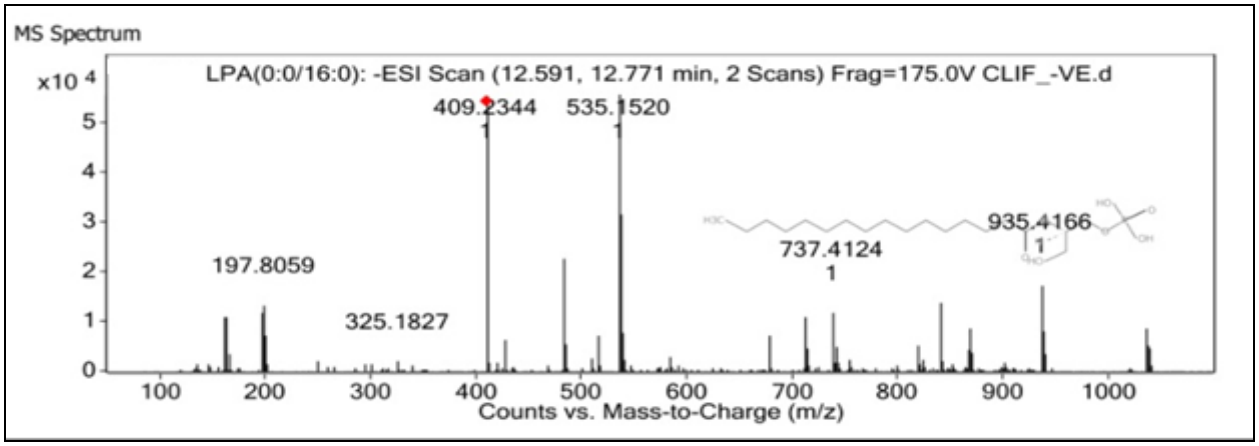
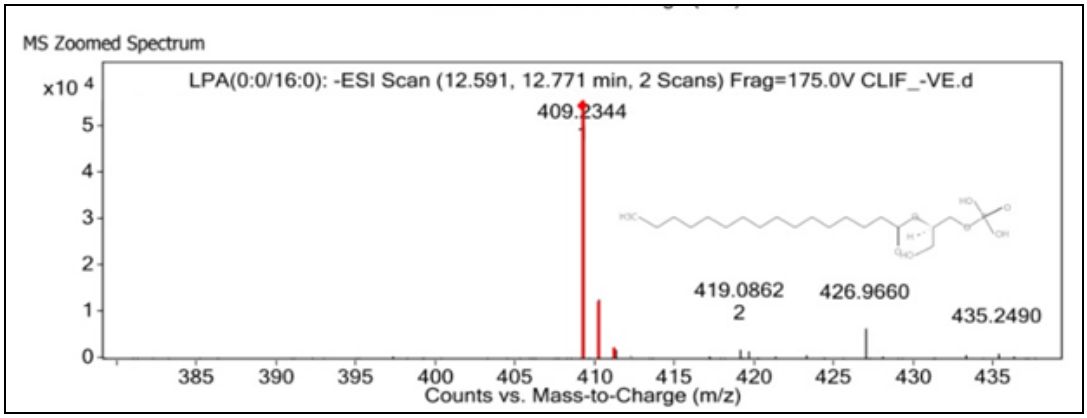
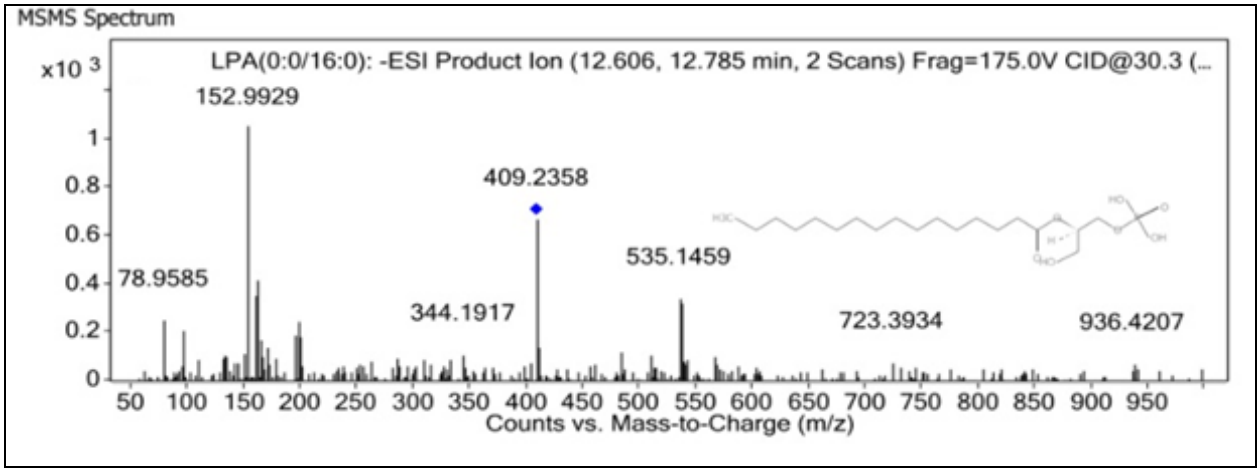


Figure: The mass spectra of Compound 1 (LPA)



MS Spectrum Peak List

m/z	Calc m/z	Diff(ppm)	z	Abund	Formula	Ion
197.8059				13526.64		
409.2344	409.2361	4.12	1	55418.56	C19 H39 O7 P	(M-H)-
410.2373	410.2395	5.43	1	12797.54	C19 H39 O7 P	(M-H)-
411.24	411.2419	4.57	1	1932.16	C19 H39 O7 P	(M-H)-
483.271			1	22958.2		
535.152			1	83451.6		
536.1548			1	22340.9		
537.1494			1	31638.47		
839.1786			1	14056.59		
935.4166			1	17502.39		



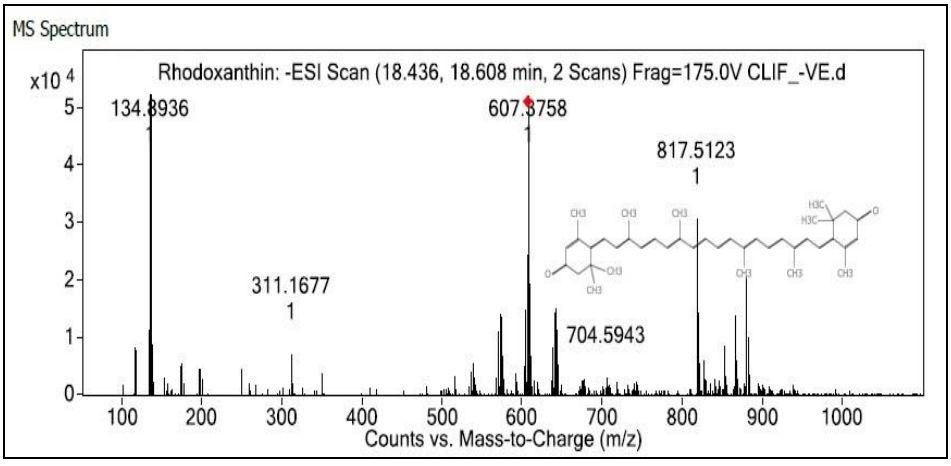
MS/MS Spectrum Peak List

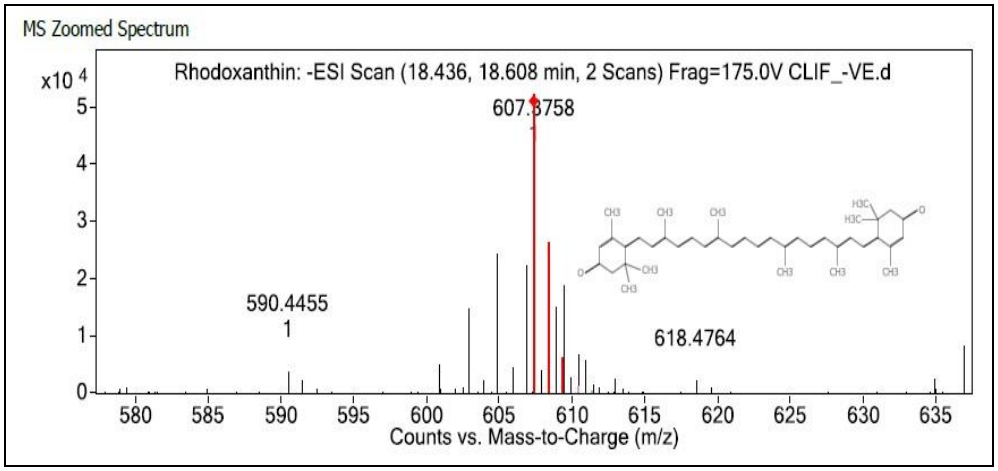
m/z	z	Abund
78.9585		250.4
96.9691		208.65
152.9929		1055.51
160.8403		351.85
162.8379		414.9
195.8098		190.4
197.8038		246.87
409.2358	1	668.27
535.1459	1	340.7
537.15	1	320.61

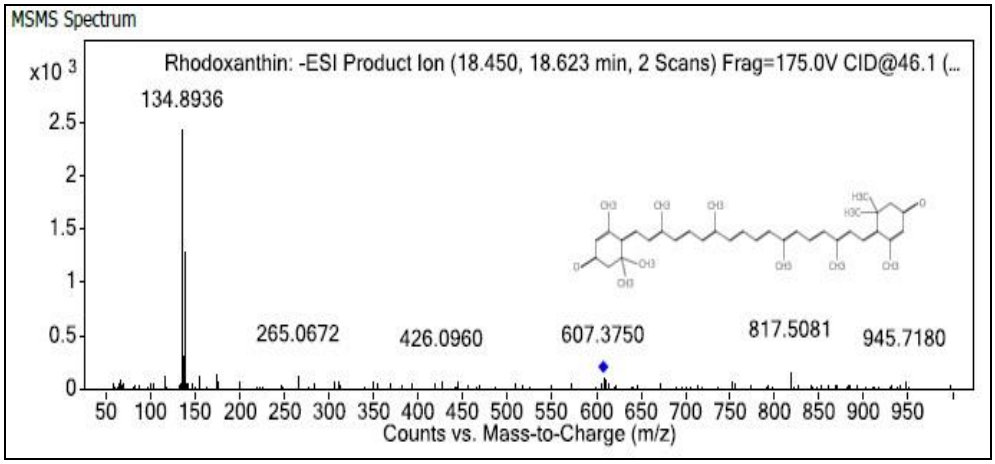
Compound Structure

Rhodoxanthin

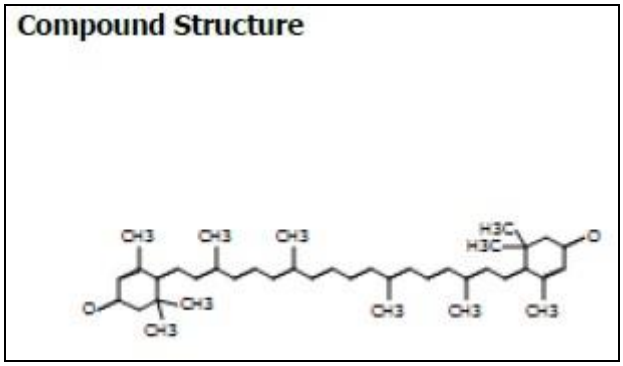
The mass spectrum shows a molecular ion peak at m/z 607.3758 (M-1)- which is in good agreement for the proposed structure of known Rhodoxanthin. The mass spectra shown in figure.







m/z	z	Abund
115.9177		135.28
134.8936	1	2440.76
135.8914	1	318.32
136.891	1	1285.52
153.8631		138.56
172.8431		142.07
265.0672		124.39
607.375	1	115.7
608.7686		102.03
817.5081		165.94



NMR Analysis

Lysophosphatidic acid

The mass $[M+H]^+$ of 409 was determined using LC-MS and 13-C NMR analysis. Compound was identified as Lysophosphatidic acid based on NMR data analysis.

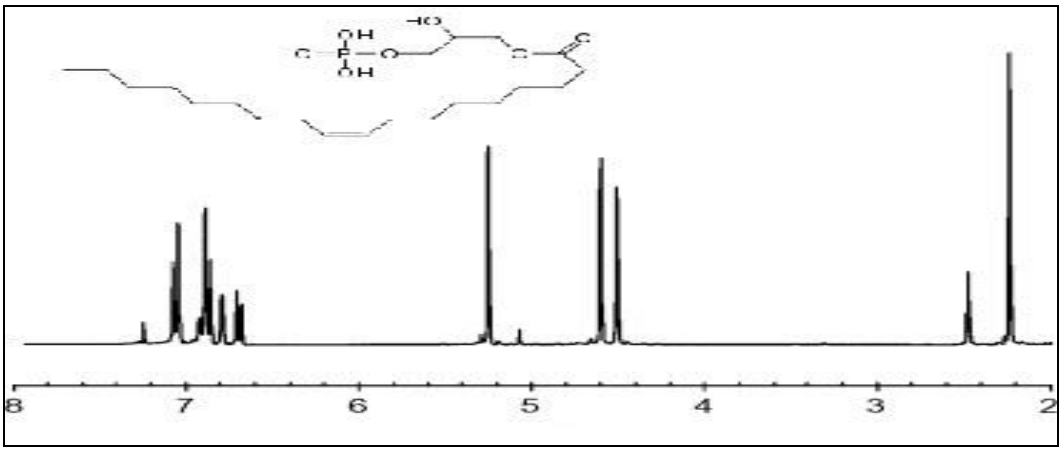


Figure: Zoomed NMR spectrum of Compound LPA

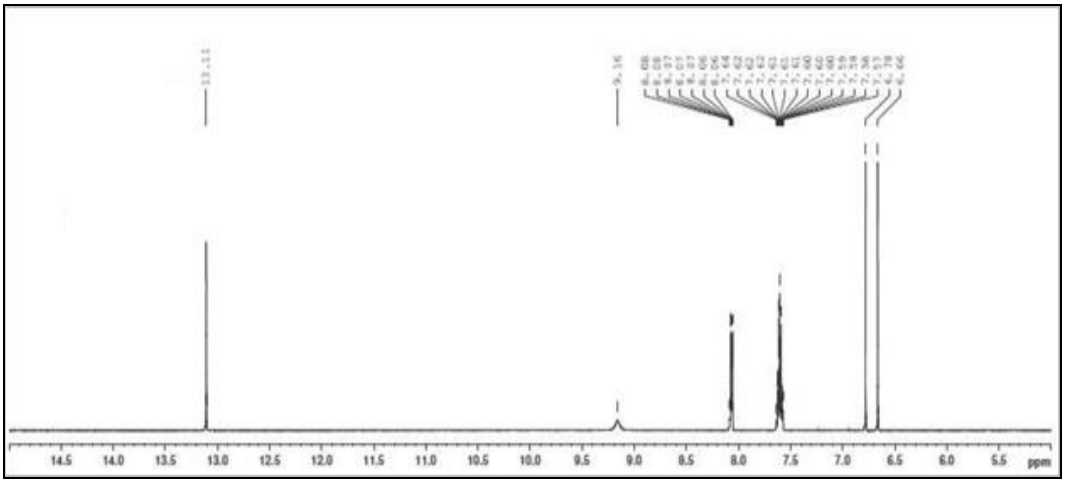


Figure: The full spectrum NMR of Compound LPA

Rhodoxanthin:

The mass $[M+H]^+$ of 607.3758 was determined using LC-MS and 13-C NMR analysis. Compound was identified as Rhodoxanthin based on NMR data analysis.

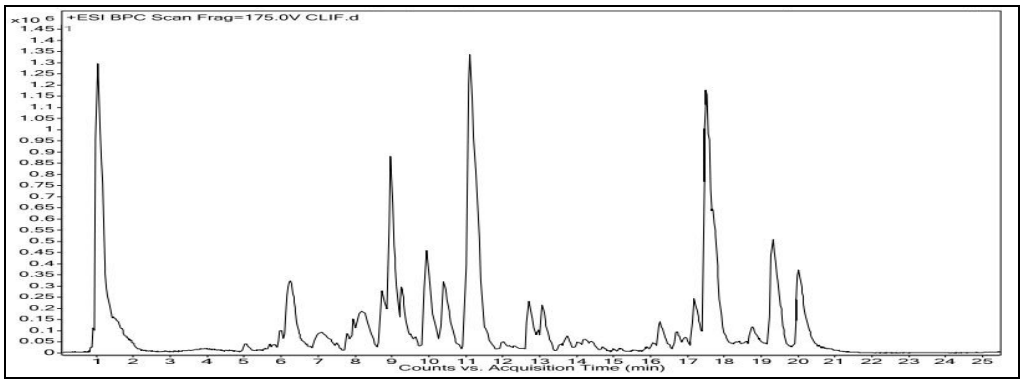
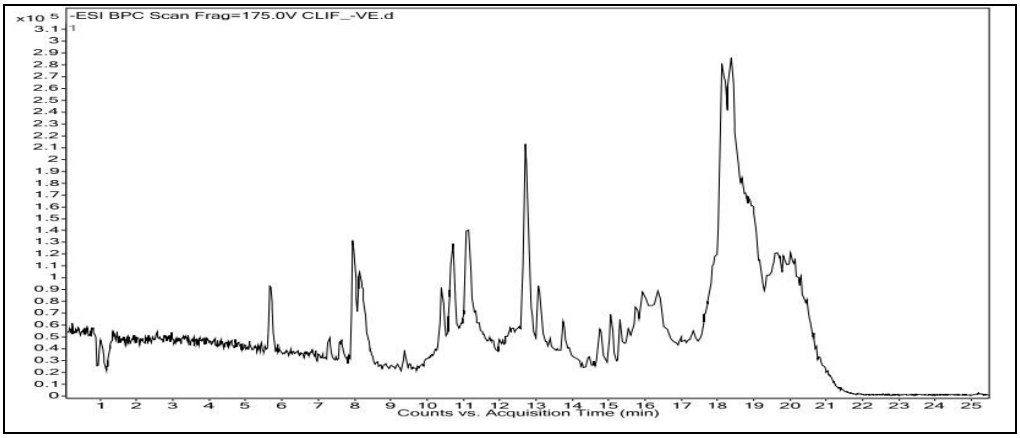


Figure: Full spectrum of Rhodoxanthin



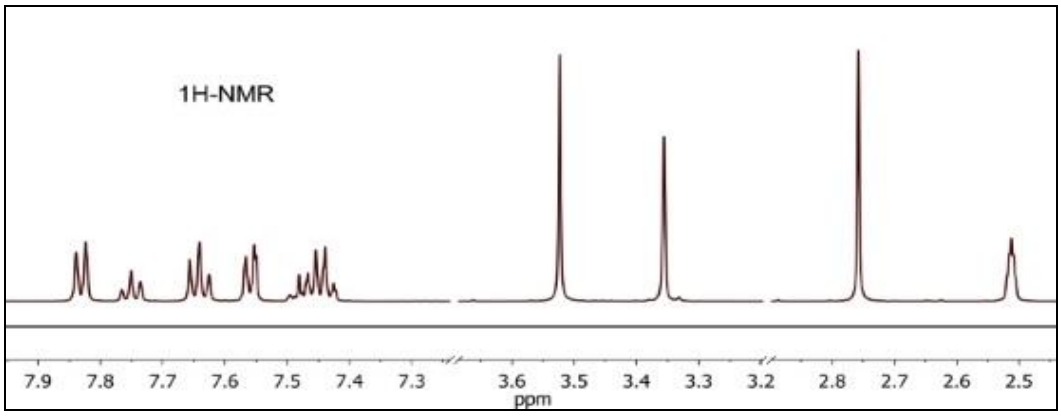


Figure: Zoomed spectrum of Rhodoxanthin

Anti-Inflammatory Activity

Table 1 displays Clerodendron infortunatum anti-inflammatory effectiveness against carrageen-induced paw edoema. The outcomes were like those of the reference medication, phenylbutazone. After three hours of extract treatment, the methanol extract of Clerodendron infortunatum showed maximum inhibition against carrageenan-induced paw edoema of 50.65 and 66.64% at the dose of 250 and 500 mg/kg body wt., respectively (Table 1), while the reference drug produced 77.30% of inhibition at the dose of 100 mg/kg body wt. The methanol extract

produced 46.86 and 59.03 percent of inhibition (Table 2) in the case of histamine-induced paw edoema at doses of 250 and 500 mg/kg body weight, respectively, while the reference medication produced 72.23 percent of inhibition. The methanol extract caused 40.66 and 58.91 percent inhibition (Table 3) at doses of 250 and 500 mg/kg body weight, respectively, in the case of dextran-induced paw edoema, while the reference medication produced 70.15 percent inhibition. The results of the study showed that the methanol extract of Clerodendron infortunatum (MECI) showed statistically significant ($p < 0.01$) inhibition of paw volume at doses of 250 and 500 mg/kg body weight, respectively, after 3 hours of carrageenan, histamine, and dextran administration. This was less than that observed with standard drug phenylbutazone ($p < 0.01$) given at a dose of 100 mg/kg body weight.

Table 1: Effect of Clerodendron infortunatum extract and phynylbuyazone on carrageenan induced paw edema in rats (n=6)

Treatment	Dose (mg/kg)	Paw volume (ml)	Percentage of inhibition	*P value
Carrageenan control		0.9067 ± 0.03591	-	-
Extract	250	0.4667 ± 0.02376	50.65 %	< 0.01
Extract	500	0.3217 ± 0.03569	66.64 %	< 0.01
Phenylbutazone	100	0.216 ± 0.01922	77.30 %	< 0.01

Table 2: Effect of Clerodendron infortunatum extract and phynylbuyazone on histamine induced paw edema in rats (n=6)

Treatment	Dose (mg/kg)	Paw volume (ml)	Percentage of inhibition	*P value
Histamine control		1.1334 ± 0.04846	-	-
Extract	250	0.6184 ± 0.04037	46.86 %	< 0.01
Extract	500	0.4817 ± 0.02570	59.03 %	< 0.01
Phenylbutazone	100	0.3334 ± 0.03822	72.23 %	< 0.01

Table 3: Effect of Clerodendron infortunatum extract and phynylbuyazone on dextran induced paw edema in rats (n=6)

Treatment	Dose (mg/kg)	Paw volume (ml)	Percentage of inhibition	*P value
Dextran control		1.2251 ± 0.03911	-	-
Extract	250	0.7434 ± 0.03130	40.66 %	< 0.01
Extract	500	0.5217 ± 0.03330	58.91 %	< 0.01
Phenylbutazone	100	0.3851 ± 0.02046	70.15 %	< 0.01

Conclusion

Evidently, carrageenan-induced edoema is frequently employed as a biphasic model in experimental inflammation research; the first phase is linked to the release of histamine, serotonin, and kinin, while the

second phase is associated with the production of prostaglandin and bradykinins. Therefore, it was investigated how the extract inhibited inflammation caused by each of these distinct mediators. The extract successfully reduced the inflammation that dextran and histamine caused. Therefore, these chemical ingredients may be the source of this plant's anti-inflammatory properties. It is well known that flavonoids have an anti-inflammatory impact and block the enzyme responsible for synthesizing prostaglandins, namely the endoperoxide enzyme. MECI has strong anti-inflammatory action, according to preliminary biological research, yet the exact mechanisms underlying these pharmacological benefits are unknown. The evaluation of the pharmacological effects found with isolated individual chemical constituents warrants additional research to improve our comprehension of the molecular mechanisms behind the MECI's anti-inflammatory activity. Nonetheless, the results of this investigation indicated that Clerodendron infortunatum methanol extracts might be utilized as a herbal treatment for inflammation. Even though MECI at a dose of 500 mg/kg body weight did not show any signs of toxicity after three hours of administration, a thorough toxicological analysis must to be carried out before using this plant extract for medicinal purposes. It is determined that, in comparison to the evaluated models, the methanol extract of Clerodendron infortunatum leaves exhibits considerable anti-inflammatory effect in a dose-dependent manner.

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