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EXTRACTION, SCREENING AND ESTIMATION OF PHYTOCHEMICAL CONSTITUENTS IN CHROMOLAENA ODORATA BY USING UV, HPLC

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ABSTRACT

This research work aims at the extraction, screening and estimation of various phytochemical constituents of Chromolaena odorata (Family: Asteraceae) using High Performance Liquid Chromatography (HPLC) and UltraViolet Spectroscopy. Chromolaena odorata is a traditional medicinal plant with prominent wound healing properties. Several parts of this herb are used to treat wounds, burns and skin infections. It has also been shown to possess anticancer, anti diabetic, anti hepatotoxic, anti inflammatory, antimicrobial and antioxidant properties. The present study is conducted to investigate the phytochemicals responsible for its different medicinal properties. Different solvents like Ethanol, Methanol and water have been used for extraction according to their polarity. Screening studies were applied to the crude extract. Glycosides, Terpenoids, Alkaloids, Flavonoids, Tannins, Saponins, and Phenolic compounds have been identified in the plant. Quantitative estimation results confirmed the presence of high amounts of various antioxidants in the Chromolaena odorata plant which are responsible for its numerous medicinal properties. The assay method for the quantification of Rutin in methanolic extract and aqueous extract successfully estimated the amount of Rutin. The retention time of standard Rutin is 2.3 minutes. The retention time of Rutin in selected samples was found to be 2.3 which matches with standard Rt value respectively.

INTRODUCTION

Chromolaena odorata (Family: Asteraceae) with synonym Eupatorium odoratum is a traditional medicinal plant that is widely used for its wound healing property. In particular, the several parts of this herb have been used to treat wounds, burns, and skin infections.[1] It is on the World Health Organization's List of Essential Medicines.[2]

Chromolaena odorata is a tropical and subtropical species of flowering shrub in the family Asteraceae. It is native to the Americas, from Florida and Texas in the United States south through Mexico and the Caribbean[3][4] to SouthAmerica.[5] It has been introduced to tropical Asia, West Africa, and parts of Australia.[6][7][8]

Common names include Siam weed, rouge plant, Christmas bush, jack in the box,[9] devil weed, common floss flower, rompesaragüey (in Spanish) etc.[10] According to a report by the World Health Organization, 80 % of the population in developing countries depend on traditional medicine for their primary health care, and 85 % of traditional medicine is derived from plant extracts [11] Phytoconstituents are non-nutrient active plant chemical compounds or bioactive compounds and are responsible for

protecting the plant against infections, infestations, or predation by microbes, pests, pathogens, or predators. Some are responsible for colour, aroma, and other organoleptic properties. Phytoconstituents are synthesized in plants through primary and secondary metabolic pathways and many of them may be grouped as active drug constituents and inert non drug constituents. A wide range of active components are discovered and they have been divided into 16 main or more groups and the most important of them are alkaloids, terpenoids, phenols and phenolic glycosides, coumarins and their glycosides, anthraquinones and their glycosides, flavones and flavonoid glycosides or heterosides, mucilage and gums, tannins, volatile oils, saponins, cardioactive glycosides, cyanogenic glycosides, etc. Other relevant active constituents in plants, such as vitamins, minerals, amino acids, carbohydrates and fibres, some sugars, organic acids, lipids, and antibiotics, are essential nutrients.[12]

HPLC: it is formerly referred to as high-pressure liquid chromatography, is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurised liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column.

UV/ Visible Spectroscopy: It is routinely used in analytical chemistry for the quantitative determination of diverse analytes or sample, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules. Spectroscopic analysis is commonly carried out in solutions but solids and gases may also be studied.

EXPERIMENT:

Anti Oxidant Activity:

Assay:

The extract were prepared in a concentration of 0.1, 0.025% using methanol.

1.Test Concentration in Methanol – 0.025%

2.Test concentration in methanol - 0.1%

Estimation of Free Radical Activity by DPPH Method:

Procedure : 3ml of test concentration extract of each concentration is mixed with 1ml of the 0.3 mM DDPH(Di phenyl 1 picrylhydrazyl) in test concentrations in test tubes are incubated in dark place at room temperature for 30 min and observe absorbance at 570nm using uv spectrometry. The methanol used as pure solvent. The percentage of inhibition concentration of the first test concentration is 36.9% ,where as the inhibition for the second test concentration is 39%. The effect of Anti-oxidant capacity is due to the presence of rutin in the methanol extract and the activity is prominent because of presence of rutin in the methanolic extract.

Instrumentation: TechcompUV2301 with HITACHI software model of UV/Visible Spectrophotometer was used. Cyberlab Company's electronic balance and Borosil glassware comprising Soxhlet apparatus, round bottomed flasks, beakers, test tubes, measuring cylinders and pipettes are utilised.

Materials:

Chemicals and Reagents: Hexane (LR Grade), ethyl acetate (AR grade), Methanol(HPLC grade), concentrated Sulfuric Acid (LR Grade), Acetyl Chloride (AR Grade), Acetic anhydride (AR Grade) , Zinc Chloride (AR Grade), ChloroSulfonic Acid (AR Grade) , Chloroform (AR Grade), Hager's reagent (Saturated solution of picric acid (LR Grade), Mayer's reagent (Potassium mercuric iodide solution (LR Grade) , Dragendroff's reagent (Potassium bismuth iodide)(AR Grade), Wagner's Reagent (solution of iodide in potassium iodide(AR Grade), Fehling's A and B solutions (LR Grade) Molisch's reagent , Benedict's reagent (AR Grade) and Barfoed's reagent(LR Grade) are provided by Thermos Fisher Scientific India Pvt. Ltd.

Preparation of reagent:

Bromocresol green solution: solution was prepared by heating 69.8 mg bromocresol green with 3 ml of 2N NaOH and 5 ml distilled water until completely dissolved and

the solution was diluted to 1000 ml with distilled water.

Phosphate buffer solution (pH 4.7): buffer solution was prepared by adjusting the pH of 2 M sodium phosphate (71.6 g Na2HPO4 in 1 L distilled water) to 4.7 with 0.2 M citric acid (42.02 g citric acid in 1 L distilled water).

Folin- Ciocalteu's (FC) reagent: 10ml of Folin- Ciocalteu's solution was dissolved in 90 ml of double distilled water.

Iron (III) chloride solution: 500mg of ferric chloride was weighed and was dissolved in 100ml of distilled water.

Potassium Hexacyanoferrate(III)solution : 500mg of potassium hexacyanoferrate was weighed and was dissolved in 100ml of distilled water.

Collection of plant materials:

The leaves of the plant Chromolaena odorata were collected. The collected leaves were cleaned and were shade dried. After complete drying, the leaves were powdered and were preserved in an airtight container and were used when required.

SOXHLET EXTRACTION:

Ethanol extraction: Sample was subjected to ethanol extraction until the solvent decolorizes in the Soxhlet extractor. After completion of extraction, solvent was evaporated to get crude extract. Then crude extract was diluted and subjected to preliminary tests.

Methanol extraction: Sample was subjected to methanolic extraction until the solvent decolorizes in the Soxhlet extractor. After completion of extraction, solvent was evaporated to get crude extract. Then crude extract was diluted and subjected to preliminary tests.

Aqueous extraction: Sample was further subjected to water extraction until the solvent was decolorized in the Soxhlet extractor. After completion of extraction, solvent was evaporated to get crude extract. Then crude extract was diluted and subjected to preliminary tests.

Hexane extract: sample was subjected to hexane extraction until the solvent decolorizes in the Soxhlet extractor. After completion of extraction, solvent was evaporated to get crude extract. Then crude extract was diluted and subjected to preliminary tests.

Preliminary phytochemical screening:

1. Test for phytosterols:

Salkowski Test: Few drops of concentrated sulphuric acid are added to the plant extract and shaken. On standing, lower layer turns red in colour.

LibermannBurchard's Test: To the extract, few drops of acetic anhydride are added and mixed well. 1 ml of concentrated sulphuric acid is added from the sides of test tube, a reddish-brown ring is formed at the junction of two layers.

2. Tests for Tannins:

Diluted small quantities of extracts separately with distilled water and subjected to: **Ferric chloride test:** Extract with ferric chloride solution gave blue colour.

Gelatin test: Extract with gelatin solution gave white precipitate.

Lead acetate test: Extract and lead acetate gave yellow precipitate.

3. Test for Saponins:

Foam Test: Small amount of extract is shaken with little quantity of water, the foam produced persists for 10 minutes. It confirms the presence of saponins.

4. Tests for Terpenoids:

5ml of plant extract was mixed in 2ml of chloroform and conc. Sulphuric acid was added to form a layer. A reddish-brown coloration of the interface was formed indicating the presence of terpenoids.

5. Tests for Alkaloids:

Mayer's Test: The acid layer when mixed with Mayer's reagent (Potassium mercuric iodide solution), gives creamy white precipitate.

Dragendroff's Test: The acid layer with few drops of Dragendroff's reagent (Potassium bismuth iodide) gives orange precipitate.

Wagner's Test: The acid layer when mixed with few drops of Wagner's reagent (solution of iodide in potassium iodide) gives reddish brown precipitate.

Hager's Test: The acid layer when mixed with few drops of Hager's reagent (Saturated

solution of picric acid) gives yellow precipitate.

6. Tests for Carbohydrates:

Fehling's Test: The extract when heated with Fehling's A and B solutions gives an orange red precipitate showing the presence of reducing sugar.

Molischs's Test: The extract is treated with Molisch's reagent (alpha naphthol in 95% ethanol) and conc. hydrochloric acid along the sides of the test tube, a violet ring shows the presence of carbohydrate.

Benedict's test: The extract with Benedict's reagent (copper sulphate, sodium carbonate in water) gives red colour. Indicates the presence of sugar.

Barfoed's Test: To the extract, added barfoed's reagent (copper acetate in water and glacial acetic acid. Gives red colour.

7. Test for Flavonoids:

Shinoda Test: The extract solution with few fragments of magnesium ribbon and concentrated hydrochloric acid produced magenta colour after few minutes.

Ferric chloride test: Alcoholic solution of extract reacts with freshly prepared ferric chloride solution. Gave blackish green colour.

Lead Acetate Test: Alcoholic solution of extract reacts with 10% lead acetate solution and gave yellow precipitate.

8. Test for Glycosides:

The extracts were separately hydrolysed with dil. Hydrochloric acid for few hours in a water bath and then subjected to:

Libermann Burchad's test: Extract is taken with chloroform in a dry test tube and few drops of glacial acetic acid and few drops of conc. sulphuric acid at the sides of the test tube. A red colour at the junction of two layers and the upper layers showed green colour.

9. Test for Phenolic Compounds:

Ferric chloride test: Treat the extract with ferric chloride solution then blue color appears if hydrolysable tannins are present and green color appears if condensed tannins are present.

Gelatin test: To the test solution, add 1% gelatin solution containing 10% Nacl, and then precipitate is formed.

10. Test for proteins and amino acids:

Dissolved small quantities of extracts separately with few ml of distilled water and then subjected to:

ninhydrin test: Extract with ninhydrin (ninhydrin with alpha amino acid) and ammonium, heat, gave violet colour.

Sodium bicarbonate test: Extract with sodium bicarbonate solution gave brisk effervesces.

11.Cardiac glycosides (Keller-killani test):

Extract was dissolved in 2 ml of chloroform and sulphuric acid ,thereby formation of brown ring at interphase appeared which confirms the presence of cardiac glycosides.

12.Phlobatannins:

Extract 0.5g was dissolved in distilled water and filtered. The filtrate was boiled with 2% hydrochloric acid. Formation of red precipitate indicated the presence of phlobatannins.

13. Anthraquinones (Born Tragers test):

0.5g of plant extract taken into a dry test tube containing chloroform, shaken for 5 min and filtered. Equal volume of 10% ammonia solution was added. Pink violet or red colour in the ammonical layer indicated positive results.

QUANTITATIVE ANALYSIS:

1. Quantitative Estimation of Alkaloids:

0.5g of plant extract has been taken and added 20% acetic acid (i.e,20ml) and then kept as such for 4 hours. Filtered the solution, evaporated by using a water bath, added ammonia and filtered. Weighed the crude extract.

% of alkaloids = weight of residue \ sample weight x 100.

2. Determination of total flavonoid content:

1ml of plant extract was diluted with 0.5ml of distilled water .Added 150 ul of sodium nitrite 5% (0.5ml).Incubated for 5 min and then 0.5ml of aluminium chloride solution (10%) was added and allowed to stand for 6 min. 2 ml of NaOH 4% solution was added to make up to 5.5ml with distilled water. shake well and left for 15 min at room temperature. The absorbance was measured at 510nm.pink colour shows the presence of flavonoids.

3. Quantitative Estimation of Phenolic Compounds: The total phenolics content in

different solvent extracts was determined with the Folin- Ciocalteu's reagent (FCR). In the procedure, 1 ml of extract was mixed with 500ul of diluted Folin's phenol reagent and 2.5 ml of sodium carbonate solution was added. The mixture was shaken well and incubated in dark conditions for 40 min for development of colour. After incubation, absorbance at 725 nm has been recorded.

4.Quantitative estimation of terpenoids:

0.1g of plant extract, 9 ml of ethanol and 10 ml chloroform have been taken. separated the ether layer and evaporated ether. Weight was noted and estimated the percentage.

5. Quantitative estimation of saponins :

0.5 ml of plant extract was added to 0.5 ml of distilled water. 0.5ml of vanillin reagent . 2.5 ml of 72% sulphuric acid were added and mixed well. Solution kept in a water bath at 60c for 10 min. After 10 min, cooled in ice water. Absorbance at 544 nm has been noted.

6.Quantitative estimation of glycosides :

0.5 of extract and 5 ml sodium hydroxide solution are taken. Boiled in Water bath for 5 min. cooled it and colour was observed at 510 nm.

7. Quantitative estimation of tannins :

Method 1: 0.5 ml sample extract and 1ml butanol,1ml dil.Hcl are taken and added 0.5 ml ferric reagent . boiled gently for 10 minutes . cooled it and observed absorbance at 510 nm.

Method 2 : 1 ml of extract and 7.5 ml distilled water are taken. Added 0.5 ml Folin-phenol reagent. 1 ml of sodium carbonate (35%).Kept for 30 min at room temperature . absorbance at 725 nm has been recorded.

Standard solution of the drug : For analysis, Rutin 10mg of the standard is weighed and is dissolved in 10ml of the diluents and sonicated for one min to dissolve the sample completely. Then it is filtered through 0.2micron meter ultipor filter paper to get a concentration of 1000µg/ml. Further required concentrations were prepared from 1000µg/ml solution by proper dilution.

Preparation of mobile phase: A mixture of Methanol, acetonitrile and water in the ratio 50:49:1 was measured accurately. The solution was sonicated till the solvents mixed completely. Then it was filtered through 0.45µm nylon membrane filter paper . The final filtrate solution was used as a mobile phase for the estimation of Rutin.

Preparation of sample solution:

Taken 0.5gm of sample and 10 ml of Methanol. The solution was sonicated until the solvents mixed completely and then filtered it.Then injected the sample into Hplc by sucking the sample with injection.

Repeated the same process for all samples.

HPLC Estimation of Rutin:

Detection wavelength

The UV absorption spectrum of the diluted solution of the Rutin was recorded on a UV spectrophotometer.

The peak of maximum absorbance was observed at a wavelength of 254nm.

Choice of stationary phase

Preliminary development trials have been performed with columns of different configurations from different manufacturers. Finally a peak with proper separation from solvent front and other sample excipients was succeeded using Zodiac C18 column (100 X 4.6 mm, 5µm) column.

Selection of the mobile phase: In order to get sharp peaks with baseline separation from interfering peaks carried out a number of experiments by varying the composition of solvents and mobile phase flow rate. To have an ideal separation of the drug under isocratic conditions, mixtures of solvents like methanol, water and acetonitrile with and without different buffers in different combinations were tested as mobile phase. A mixture of Acetonitrile and Water in the ratio of 50:50(v/v) was proved to be the most suitable of all the combinations, since the chromatographic peak obtained was better defined and resolved and almost free from tailing.

Flow rate : Flow rate of the mobile phase was 1.1ml/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents. It was found from the experiments,

that 1.1ml/min flow rate was ideal for the successful elution of the analysis .

Assay sample preparation: From the prepared formulation solution, 20µl of the sample was injected in to HPLC system, peak area response of the prepared formulation solution was used for the assay of Rutin in the prepared solution. %assay of the method was calculated by considering peak area response of the formulation solution and substituting peak area value in the regression equation. HPLC method was used for the estimation of Rutin in pharmaceutical formulations. Specificity was studied by comparing the standard, formulation and blank chromatograms in the method. Standard chromatogram shows peak at retention time of 1.00min and blank chromatogram doesn't have any peaks. Hence the method was found to be specific. Blank chromatogram was given.In the method, system suitability was determined at concentration of 50µg/ml. The retention time was found to be 1.00 min. The number of Theoretical plates was found to be 2562 and tailing factor was found to be 1.28. The chromatogram obeys the system's suitable acceptance limit.

Standard retention value: 2.5667

Table: Standard method conditions for the analysis of Rutin:

S No	Parameter	Condition
1	Elution	Isocratic
2	Mobile phase	Methanol , acetonitrile and water 50:49:1 (v/v)
3	Stationary Phase	Zodiac C18 column
4	Wavelength	370nm
5	Mobile phase flow rate	1.1ml/min
6	Sample volume	20µ
7	Run time	30.0min

RESULTS AND DISCUSSION

S.No	Compound	Ethyl Acetate	Methanol extract	water extract
1	**Alkaloids** Hagners Dragendorff's	Present	Absent	Absent
2	**Carbohydrates** Molisch's test Fehling's test Barfoed's test Benedict's test	Absent Absent Absent Absent	Absent Absent Absent Absent	Absent Absent Absent Absent
3	**Saponins** Foam test	Present	Present	Absent
4	**Phytosterols** Libermannburchard's	Absent	Absent	Absent
5	**Terpenoids**	Absent	Present	Absent
6	**Glycosides** Libermannburchard's	Present	Present	Absent
7	**ProteinsandAminoacids** Ninhydrin Sodium carbonate	Absent Absent	Absent Absent	Absent Absent

8	**Flavonoids** Ferric chloride Lead acetate Shinda's	Present Absent Absent	Absent Present Absent	Absent Present Absent
9	**Cardiac Glycosides**	Absent	Present	Absent
10	**Phenolic compounds** Ferric chloride Gelatin	 Absent Absent	 Absent Absent	 Absent Absent
11	**Phlobatannins**	Absent	Absent	Absent
12	**Tannins** Ferric chloride Gelatin Lead Acetate	Absent Absent Absent	Absent Absent Absent	Absent Absent Absent

ESTIMATION TABLES:

Table: Estimation of ethyl acetate extract

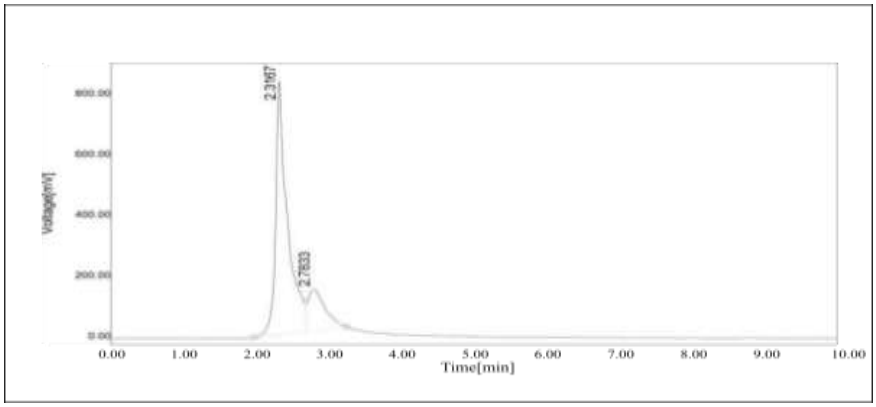
S.N0	Sample Name	Average Absorbance
1	Glycosides	4.87
2	Saponins	0.0106
3	Flavonoids	2.855

Table: Estimation of methanol extract

S.N0	Sample Name	Average Absorbance	%Estimation
1	Glycosides	3.19	3.19
2	Saponins	2.052	2.052
3	Flavonoids	4.79	15.07

Table : Estimation of water extract

S.N0	Sample Name	Average Absorbance	%Estimation
1	Tannins	5.308	195.25
2	Flavonoids	2.603	8.0



HPLC Chromatogram

No.	Name	RT[min]	Area[mV*s]	Area%			Resolution
1.	Rutin	2.3167	9506.7754	80.65	655.4	1.7421	0.0000
2	Rutin	2.7833	2280.9277	19.35	913.8	2.5618	1.2781
Sum			11787.7031				

CONCLUSION:

The chromolaena odorata plant is considered having the most promising medicinal properties for the prevention and treatment of many diseases like wound healing. The present study is conducted to investigate the phytochemicals responsible for its different medicinal properties. Different solvents have been used for extraction of the Chromolaena odorata plant according to their polarity. Ethanol, Methanol and water solvents are used for extraction and further screening studies were applied to the crude extract. Glycosides, Terpenoids, Alkaloids, Flavonoids, Tannins, saponins, and Phenolic compounds have been identified in the plant. Quantitative estimation results confirm the high amounts of various antioxidants present in the Chromolaena odorata plant which confirms the presence of various antioxidant chemicals responsible for its high medicinal properties.

The assay method for the quantification of rutin in methanolic extract and aqueous extract successfully estimated the amount of rutin. The retention time of standards rutin were found to be 2.3 minutes. The retention time of rutin in selected samples was found to be 2.3, which are matching with standard Rt values respectively.

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