# INVESTIGATION OF PHYSICOCHEMICAL PROPERTIES, PHYTOCHEMICAL CONSTITUENTS, FLUORESCENCE BEHAVIOR, EXTRACTION METHODS, AND TLC/HPTLC ANALYSIS OF LEAVES FROM AMARANTHUS CRUENTUS: A COMPREHENSIVE STUDY

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**Abstract**

The aim of the study to investigate the Physicochemical analysis, Fluorescence analysis, Extraction, Phytochemical screening, Thin-layer chromatography (TLC) and High Performance thin-layer chromatography (HPTLC) of leaves of *Amaranthus cruentus (A. cruentus)*. Physicochemical analysis, including the determination of various parameters such as loss on drying, swelling index, foaming index, total ash, acid-insoluble ash, water-soluble ash, extractive value, and fluorescence analysis was conducted on the powdered leaves. To extract the bioactive compounds, a Continuous hot Soxhlet apparatus was employed using solvents with increasing polarity, namely petroleum ether, chloroform, and ethyl acetate. Phytochemical screening was then performed on these extracts to identify the presence of various phytoconstituents. Thin-layer chromatography (TLC) and High-performance thin layer chromatography (HPTLC) were carried out exclusively on the ethyl acetate extract. The solvent system used for TLC and HPTLC was a mixture of toluene and ethyl acetate in a ratio of 7:3. HPTLC was performed with two marker compounds, Gallic acid, and Rutin, as these were the predominant phytoconstituents identified in the ethyl acetate extract. For the quantitative HPTLC analysis, a precoated silica gel 60 F254 plate was utilized, and UV detection was conducted at a wavelength of 254nm. This allowed for the precise determination of the amounts of Gallic acid and Rutin present in the ethyl acetate extract. The Physicochemical evaluation of powder indicated value of loss on drying was found 5.1%, swelling index value was 0.23%, the foaming index less than 100, total ash value was 11.86%, acid-insoluble ash value was 2.56%, water soluble ash value was 3.33% and pet. ether extractive value was 1.45%, chloroform extractive value was 1.21% and ethyl-acetate extractive value was 1.36%. In UV and visible light, many fluorescent colors were seen during fluorescence analysis. Pet. ether extraction value was 3.04%, chloroform extraction value was 3.41% and ethyl acetate extraction value was 3.64%. The preliminary phytochemical analysis of various extracts revealed the presence of alkaloids, carbohydrates, glycosides, amino acids, proteins, steroids, tannins and phenolics, saponins, flavonoids, fats, and oils. Additionally, TLC was performed on the ethyl acetate extract, resulting in the identification of seven unknown phytoconstituents. To further investigate the presence of specific compounds, HPTLC technique was employed. This technique enabled the detection and quantification of gallic acid and rutin. The Rf (retention factor) values obtained for gallic acid and rutin were 0.05 and 0.88, respectively. The amounts of gallic acid and rutin were determined to be 958.69 µg/100 mg and 107.65 µg/100 mg, respectively. These findings provide valuable insights into the composition and quantity of these compounds in the analyzed extract. The present physicochemical parameters, fluorescence, extraction, phytochemical, TLC and HPTLC study results could help in standardization, identification, and in carrying out further research on *A. cruentus* leaf-based herbal drugs.

**Keywords:** Physicochemical parameters, fluorescence value, extraction value, phytochemical studies, TLC, HPTLC

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## 1. Introductions

Medicinal plants are known to contain diverse bioactive compounds that exhibit significant pharmacological properties. Extracts and fractions derived from these plants have been used for the treatment of various ailments since ancient times [1]. The utilization of herbal medicine has experienced a surge in popularity and significance in contemporary times, owing to the extensive chemical diversity that they provide in both developed and developing nations. The increasing demand for herbal drugs has led to a greater focus on guaranteeing their quality, safety, and effectiveness [2]. Herbal remedies are significant in the management of health and the prevention of illnesses. Herbal medicines have become a popular choice for treating and managing human diseases due to their accessibility and low toxicity, particularly in light of the growing demand for safer pharmaceuticals [3].

*A. cruentus*, commonly known as red amaranth or blood amaranth, is a plant species that belongs to the Amaranthaceae family. The leaves of *A. cruentus*, commonly known as purple amaranth or red amaranth, are an important part of the plant and have various culinary uses. The leaves are generally large, lanceolate or ovate in shape, and have a dark green to reddish-purple color, depending on the variety. They are characterized by a smooth or slightly wavy texture and may have a glossy appearance. The leaves of *A. cruentus* are edible and have a mild, earthy flavor. The leaf shape can vary slightly depending on the variety. The Nutritional Value of Amaranth leaves are highly nutritious and packed with essential vitamins and minerals. They are a good source of vitamin A, vitamin K, vitamin C and folate. They also contain minerals like calcium, iron, and potassium.

They are commonly used as a leafy green vegetable in many cuisines, including African, Asian, and Caribbean cuisines. They are known for their high protein content and are a good source of antioxidants. These leaves are often considered a nutritious and healthy addition to a balanced diet.

In addition to their culinary uses, the leaves of *A. cruentus* are also used in traditional medicine in some cultures.

They are believed to have various health benefits, including anti-inflammatory and antimicrobial properties.

The peptides generated during incomplete digestion can display a range of activities such as opiate-like, immunomodulatory, antimicrobial, antioxidant, antithrombotic, antihypertensive, and hypocholesterolemia effects, contingent on the arrangement of amino acids. Several bioactive peptides that have been identified exhibit multifunctionality and are capable of exerting multiple effects [4–7].

*A. cruentus* leaves, besides being a nutritious food source, have also been studied for their potential pharmacological activities. Here are some of the reported pharmacological properties and activities associated with *A. cruentus* leaves. *A. cruentus* leaves possess antioxidant properties due to the presence of bioactive compounds such as phenolic compounds, flavonoids, and carotenoids. Antioxidants help neutralize harmful free radicals in the body, protecting cells from oxidative damage.

Polyphenols are a class of phytoconstituents that are commonly present in a variety of plant-based sources such as grains, fruits, bark, roots, stems, leaves, vegetables, and flower [8]. Polyphenols such as gallic acid and rutin are prominent constituents of medicinal plants, including *A. cruentus*, and exhibit diverse pharmacological properties that are recognized for their advantageous impact on human health. Phenolic acids of plant metabolites, including Gallic acid and its derivatives, are extensively distributed throughout the plant kingdom.

Gallic acid has been found to have many good effects, such as anti-inflammatory, anti-cancer, and protective benefits. This compound has been reported to have therapeutic activities in gastrointestinal, neuropsychological, metabolic and cardiovascular disorders, which make it a significant option to be used as therapeutic agent or dietary supplement. This renders it an important applicant for employment as a therapeutic agent or dietary supplement.

Rutin is a flavonoid that can be found in various plant sources, including *A. cruentus*. It has been studied for its pharmacological properties and potential health benefits. Here are some of the reported pharmacological actions of rutin. Rutin exhibits strong antioxidant properties, helping to scavenge free radicals and protect cells from oxidative stress and damage. It can also enhance the activity of other antioxidants in the body. Studies have demonstrated that Rutin exhibits anti-inflammatory properties through the inhibition of diverse inflammatory pathways and the reduction

of pro-inflammatory molecules. This action can help alleviate inflammation-related conditions.
High-Performance Thin-Layer Chromatography is a very sophisticated instrumental method that makes full capabilities of thin-layer chromatography's. Numerous advantages of the method include automation, scanning, complete optimization, the selective detection concept, minimal sample preparation, and hyphenation. It has shown to be an easy, reliable, and efficient way to get chromatographic data for complicated mixes of medicines, natural products, clinical samples, and foods [9].

## 2. Materials and Methods

### 2.1 Authentication and Collection of Plant

The leaves of *A. cruentus L*. were collected from the Department of Botany, School of Science IFTM University Lodhipur Rajput Moradabad of Uttar Pradesh. The Plant and leaves material were certified as *A. cruentus* L. (Family-Amaranthaceae) and taxonomically recognized by Dr. Sunita Garg, Former Chief Scientist and Head of Raw Material Herbarium and Museum, Delhi (RHMD), CSIR-NIScPR.

### 2.2 Chemicals

All the chemicals used in this study were of analytically grade and purchased from central drug house (New Delhi).

### 2.3 Equipment and apparatus

The following instruments were employed in the study-Soxhlet apparatus, crucible, hot plate, Whatman filter paper No 42 (125 mm), condenser, mortar and pestle, plastic bottle, volumetric flask, Hot air oven, electronic balance, separatory funnel, metal stand, pipette, conical flask, measuring cylinder, test tube, moisture disc.

### 2.4 Sample collection and preparation

Leaves were collected and meticulously cleansed by sequentially rinsing them with tap water and distilled water to effectively eliminate any surface impurities. Subsequently, the leaves were subjected to shade sir dried for 5 days. For additional physiochemical investigation, the dried sample was crushed into coarse particles using a mortar and pestle and stored in an airtight container.

### 2.5 Physicochemical Parameters

According to the procedures outlined in WHO recommendations, the physicochemical properties of Leaves from *A. cruentus L*. were determined.

#### 2.5.1 Loss on drying

A quantity of 3 grams of powder was accurately weighed and transferred into a clean and sterile porcelain dish. The sample was kept in hot air oven at temperature of 105°C for 1 hour The ceramic plate was extracted from the oven and subsequently, the mass of the substance was re-evaluated [10]. The loss on drying can be calculated by the formula 1.

$$\%\ \mathbf{LOD} = \frac{\mathrm{Xi}-\mathrm{Xf}}{\mathrm{Xi}} \times 100 \quad \textbf{.....(1)}$$

Where, Xi is initial weight. Xf is final weight

#### 2.5.2 Swelling Index

1 gram of powder was precisely measured and added to a graduated cylinder containing 10 mL of water. The recorded data pertains to the original volume of powder present within the graduated cylinder. Subsequently, the concoction was subjected to rigorous agitation at 10-minute intervals for a duration of 60 minutes, followed by a 24-hour incubation period at ambient temperature. Following a 24-hour period, the quantity of powder was assessed for volume and subsequently utilized to determine the swelling index [11]. The formula 2 is used to determine the sweeling index.

$$\%\ \mathbf{Swelling\ Index} = \frac{100\ (\mathrm{Xf}-\mathrm{Xi})}{\mathrm{Xi}} \times 100\textbf{.....(2)}$$

Where, Xf = final volume after hydration, mL, Xi = initial volume of mucilage in graduated cylinder (mL)

#### 2.5.3 Foaming Index

Approximately 1 gram of plant material was ground into a coarse powder, precisely weighed, and subsequently subjected to moderate boiling for a duration of 30 minutes. The sample was transferred into a 100 ml volumetric flask after undergoing cooling and filtration. The detection solution was dispensed into 10 millilitre aliquots and subsequently standardized by the addition of water to achieve a final volume of 10 millilitres in each respective tube. The tubes were stoppered and subjected to a lengthwise shaking motion for a

duration of 15 seconds, with a frequency of two shakes per second. The samples were permitted to remain undisturbed for a duration of 15 minutes, following which the foam's vertical dimension was quantified [13]. The formula 3 is used to determine of foaming index.

$$\textbf{Foaming index} = \frac{1000}{\text{A}} \textbf{.....(3)}$$

Where, A = the volume in ml of the decoction used for preparing the dilution in the tube where foaming to a height of 1 cm is observed.

**2.5.4 Total Ash Value**

3 grams of leaf powder were placed in a previously-weighed silica crucible. At 450 degrees Celsius, material was incinerated with the aid of a muffle furnace until almost no vapours remained. The dish was subjected to a heating process until complete combustion of carbonaceous matter occurred. The dish was allowed to cool down to room temperature, following which the percentage of total ash was determined. The Total Ash value can be calculated by the formula 4.

$$\textbf{Average} = \frac{\text{Difference of sample}}{\text{Total number of sapmle}} \times 100$$

$$\%\ \textbf{of Ash} = \frac{\text{Average}}{\text{Weight of Sample taken}} \times 100 \textbf{.....(4)}$$

**2.5.5 Acid-Insoluble Ash Value**

The ash sample were a boiling process with 25 milliliters of 5% hydrochloric acid for a duration of 5 minutes. The ashless filter paper was utilized to gather the insoluble material. The sample were a neutralization process using hot water, followed by ignition and subsequent cooling in desiccators prior to being weighed. Percentage of acid insoluble ash was calculated by formula 5.

$$\textbf{Average} = \frac{\text{Difference of sample}}{\text{Total number of sapmle}} \times 100$$

$$\%\ \textbf{of Acid insoluble ash} = \frac{\text{Average}}{\text{Weight of Sample taken}} \times 100 \textbf{.....(5)}$$

**2.5.6 Water-Soluble Ash Value**

The procedure involves boiling the ash in 25 ml of water for a duration of 5 minutes. The insoluble residue is then collected either on an ashless filter paper or in a Gooch crucible. The residue is washed with hot water and subsequently ignited for a period of 15 minutes at a temperature that does not exceed 450 ºC. To determine the water-soluble ash, it is necessary to subtract the weight of the insoluble matter from the weight of the ash. The resulting difference in weight represents the water-soluble ash [12]. The water-soluble ash value can be calculated by the formula 6.

**Average** = Differences of sample / Total no. of sample

$$\%\ \textbf{of water soluble ash} = \frac{\text{Average}}{\text{Weight of Sample taken}} \times 100 \textbf{.....(6)}$$

**2.5.7 Determination of Extractive value**

A maceration process was employed to extract the dry powdered plant material of *A. cruentus* leaves using pet. ether, chloroform, and ethyl acetate. A quantity of 2 grams of plant material that had been coarsely powdered was measured using a weighing bottle and subsequently transferred into a 250 ml conical flask that had been dried beforehand. Subsequently, the flask was filled with distinct solvents, each measuring 30ml. The flasks were sealed with cork stoppers and allowed to rest undisturbed at ambient temperature for a duration of 24 hours, with periodic agitation. The filtrates were transferred into a cylindrical container with a volume of 50 milliliters. Once the filtrate was obtained, it was subsequently transferred into petri plates that had been previously weighed. The extracted material was concentrated to dryness by retaining the filtrate to allow for the complete evaporation of solvents [13]. The extractive value in percentage was calculated by using formula (7).

$$\textbf{Extractive value}\ (\%) = \frac{\text{weight of dried extract}}{\text{weight of plant materials}} \times 100\text{……..(7)}$$

**2.6 Fluorescence Analysis**

The mentioned substances include Picric acid, 1 N H2SO4, 5% Fecl3, 5% Iodine, Glacial acetic acid, 1 N NaOH (Aqueous), 1N NaOH (Alcoholic), Methanol, 1N HCl, Conc. $HNO_3$, Ammonia, and $HNO_3$ + Ammonia. Fluorescence analysis was conducted on extracts comprising of distilled water, Toluene, Pet. Ether, Chloroform, Ethyl Acetate, Lead acetate, and Benzene (1 ml). The observation of colour variations in day light, short UV light (254 nm), and long UV light (365 nm) was utilized for this purpose [14,15]. The experiment involved the combination of a small quantity of desiccated leaf powder with newly formulated reagents and solvents, and subsequent observation of the resulting color under daylight, short-wavelength ultraviolet light (254 nm), and long-wavelength ultraviolet light (365 nm).

**2.7 Extraction Methods**

The experiment involved the placement of 50gm of powdered leaves in a Soxhlet apparatus, followed by sequential extraction using pet. ether, chloroform, and ethyl acetate. The extracts were subjected to drying using a water bath [16]. The extraction yield can be calculated by the formula 8.

$$\%\ \textbf{of Extraction yield}\ = \frac{\text{Amount (g) of the dry crude extract obtained}}{\text{Amount (g) of the dry sample used}} \times 100\text{……..(8)}$$

**2.8 Phytochemicals Screening**

Standard methods were employed to conduct phytochemical analyses on all of the extracts.

**2.8.1 Detection of Alkaloid**

**2.8.1.1 Dragendroff's Test:** The filtrates were treated with Dragendroff's reagent, which consist of Potassium Bismuth Iodide solution. The occurrence of a red precipitate following this reaction serves as an indicator for the presence of alkaloids.

**2.8.1.2 Hager's Test:** The filtrates were treatment with Hager's reagent, which is a solution of saturated picric acid. The confirmation of the presence of alkaloids was achieved through the observation of a yellow-colored precipitate formation [17].

**2.8.2 Detection of Carbohydrates**

**2.8.2.1 Fehling's Test:** The filtrates were subjected to hydrolysis through the use of diluted hydrochloric acid. This was followed by neutralization with an alkali and subsequent heating with Fehling's A and B solutions. The presence of reducing sugars may be determined by the presence of a red precipitate.

**2.8.2.2 Benedict's test:** The filtrates were treatment with Benedict's reagent and were subjected to gentle heating. The presence of reducing sugars can be indicated by the formation of an orange-red precipitate. [18].

**2.8.3 Detection of Glycosides**

**2.8.3.1 Legal's Test:** The samples were treatment with sodium nitroprusside in the presence of pyridine and sodium hydroxide. The observation of a colour change from pink to blood red is indicative of the presence of cardiac glycosides.

**2.8.3.2 Modified Borntrager's Test:** The samples were treatment with a solution of Ferric Chloride and were subsequently subjected to immersion in water at boiling temperature for a duration of approximately 5 minutes The concoction was subjected to cooling and subsequently extracted with equivalent quantities of benzene. The layer of benzene was isolated and subjected to treatment with a solution of ammonia. The presence of anthranol glycosides can be inferred by the formation of a rose-pink color in the ammoniacal layer [19].

**2.8.4 Detection of Amino-acids**

**2.8.4.1 Ninhydrin Test:** The extract was boiled for a short period of time with 0.25% w/v Ninhydrin reagent added. The development of blue color suggests the presence of an amino acid.

**2.8.4.2 Xanthoproteic Test:** The extracts were treated with a few drops of concentrated nitric acid. The development of a yellow tint shows the presence of proteins [20].

**2.8.5 Detection of Proteins**

**2.8.5.1 Millon's test:** In the Millon test, 3 ml T.S. is combined with 5 ml of Million's reagent. White is used for the presentation. A red brick if you don't

provide the ppt. makes a solution that is crimson in colour and dissolves.

**2.8.5.2 Biuret's test:** Take 3 ml T.S., add 4 % NaOH and few drops of 1 % $CuSO_4$ Solution. It appears violet or pink color [21].

**2.8.6 Detection of Steroids**

**2.8.6.1 Salkowski's Test:** The samples were treatment with chloroform and subsequent filtration. The filtrates were treatment with a small quantity of concentrated sulphuric acid, followed by agitation and subsequent settling. Appearance of golden yellow is indicative of the presence of triterpenes.

**2.8.6.2 Liebermann Burchard test:** The samples were treatment with chloroform followed by filtration. The filtrates were treatment with a small quantity of acetic anhydride, followed by boiling and subsequent cooling. Concentrated sulfuric acid was introduced. The appearance of a brown ring at the interface signifies the presence of phytosterols [23].

**2.8.7 Detection of Tannins and Phenolics**

**2.8.7.1 Gelatin Test:** A 1% solution of gelatin, which included sodium chloride, was introduced to the sample. The amount of a white precipitate denotes the presence of tannins.

**2.8.7.2 Ferric Chloride Test:** The samples were treatment with a solution of ferric chloride, consisting of 3-4 drops. The observation of a bluish-black color suggests the presence of phenolic compounds [22].

**2.8.8 Detection of Saponins**

**2.8.8.1 Froth Test:** The extracts were diluted using distilled water to a volume of 20 milliliters and subjected to agitation in a graduated cylinder for a duration of 15 minutes. The detection of saponins can be incidental from the formation of a foam layer measuring 1 cm in thickness.

**2.8.8.2 Foam Test:** A quantity of 0.5 grammes of extract was agitated with 2 milliliters of water. The persistence of foam for a duration of 10 minutes is indicative of the existence of saponins [23].

**2.8.9 Detection of Flavonoids**

**2.8.9.1 Alkaline Reagent Test:** The samples were subjected to treatment with a small quantity of sodium hydroxide solution. The presence of flavonoids can be inferred by the development of a intense yellow color, which is reversible upon the introduction of a diluted acidic solution.

**2.8.9.2 Lead acetate Test:** The samples were subjected to treatment with a small quantity of lead acetate solution. The emergence of a yellow-colored precipitate denotes the existence of flavonoids [26].

**2.8.10 Detection of Fats and Oils**

**2.8. 10.1 Solubility test**: In solubility test oils are soluble in ether, chloroform and benzene but insoluble in 90% ethanol and in water [24].

**2.8.10.2 Saponification test:** To acquire 10ml of oils, evaporate the extract. Add 25ml of 10% NaOH to oils. 30 minutes of boiling in a water bath, then cooling. Add the $Na_2So_4$ solution amount. As it develops, soap rises to the top. $H_2SO_4$ is added, then the filtrate is evaporated. Gather the glycerol-containing residue. With ethanol, dissolve the residue. Add a few drops of 1% $CuSo_4$ and 10% NaOH solution to the ethanolic solution. There is a clear blue solution present [25].

**2.6 Thin-layer chromatography (TLC)**

The TLC analysis of the ethyl acetate leaves extract of *A. cruentus* was optimized using a variety of solvent systems, but ultimately the solvent system listed below, shown in Table 1, was used. The formula 8 below was used to calculate the $R_f$ value of various spots or solutes.

$$\textbf{Retention factor} = \frac{\textbf{Distance travelled by solute}}{\textbf{Distance travelled by the solvent front}} \quad \textbf{.....(8)}$$

**Table** 1- TLC and HPTLC solvent system for Ethyl Acetate Extract e of *A. cruentus.*

| Leaves | Solvent | Ratio | Detection |
|---|---|---|---|
| *A. cruentus* | Toluene: Ethyl Acetate | 7:3 | Day Light |

**2.6.1 HPTLC Analysis for quantitative estimation of Gallic Acid and Rutin**

Authors also wanted to find out how much amount of gallic acid and rutin are present in Ethyl acetate Leave Extract of *A. cruentus* for this we used HPTLC technique because HPTLC is simple and cheaper technique as compared of other techniques.

### 2.6.2 Preparation of Standard Solution

Standard solutions with a concentration of 100 g/ml were generated by individually mixing standard amounts of gallic acid and/or rutin, each of which weighed 1 mg. This resulted in the production of the standard solutions.

### 2.6.3 Preparation of test solution

100 mg Ethyl Acetate Extract extract of *A. cruantus* was added in 10 mL ethanol and shaken for ten minutes and filtered. Filtrate was used for HPTLC analysis.

### 2.6.4 Validation of the method

The analytical technique that was developed was validated in accordance with the recommendations provided by ICH for factors such as linearity, specificity, accuracy, precision, limit of detection, limit of quantification, and robustness [26].

## 3. Result

### 3.1 Physiochemical studies

*A. cruentus* powder was preliminary evaluated by determining physical constants like loss on drying, swelling index, Foaming index, Ash value and Extractive value in the table 2.

**Table 2-** Physiochemical studies results of leaves powder of *A. cruentus*

| Parameters | Mean ± SEM (%w/w) n=3 |
|---|---|
| Loss on drying | 5.1±0.96 |
| Swelling Index | 0.23±0.05 |
| Foaming Index | Less than 100 |
| Total Ash | 11.86±1.72 |
| Acid-insoluble Ash value | 2.56±0.49 |
| Water soluble Ash value | 3.33±0.57 |
| Petroleum Ether soluble Extractive value | 1.45±0.38 |
| Chloroform soluble Extractive value | 1.21±0.20 |
| Ethyl Acetate soluble Extractive value | 1.36±0.07 |

### 3.2 Fluorescence analysis

The fluorescence color varies depending on the substance being studied. When a nonfluorescent substance is combined with fluorescent impurities, the complex may exhibit fluorescence. As a result, it is helpful in identifying the substitutes and adulterants that are present. Leaves powder was treated with different reagents and observed showed following results in the table 3.

**Table 3-** Fluorescence analysis result of leaves powder of *A. cruentus*

| S. No | Drug Powdered | Day light | UV 254 nm | UV 365 nm |
|---|---|---|---|---|
| 1. | Powder as such | Purple | Purple | Light purple |
| 2. | Drug Powder + Picric acid | Dark red | Brown | Light brown |
| 3. | Drug Powder + 1 N $H_2SO_4$ | Dark purple | Light purple | Light purple |
| 4. | Drug Powder + 5% $Fecl_3$ | Brown | Purple | Purple |
| 5. | Drug Powder + 5% Iodine | Brown | Purple | Light purple |
| 6. | Drug Powder + Glacial acetic acid | Light brown | Purple | Purple |
| 7. | Drug Powder + 1 N NaOH (Aqueous) | Light purple | Dark purple | Light purple |
| 8. | Drug Powder + 1 N NaOH (Alcoholic) | Light purple | Purple | Light purple |
| 9. | Drug Powder + Methanol | Light purple | Light purple | Brown |
| 10. | Drug Powder + 1 N HCl | Brownish purple | Light purple | Light purple |
| 11. | Drug Powder + conc. $HNO_3$ | Brown | Light purple | Light purple |
| 12. | Drug Powder + Ammonia | Brownish purple | Dark purple | Light purple |
| 13. | Drug Powder + $HNO_3$ + Ammonia | Brownish purple | Light purple | Light purple |
| 14. | Drug Powder + Distilled water | Dark purple | Light purple | Light purple |
| 15. | Drug Powder + Toluene | Light yellow | Light yellow | Light purple |
| 16. | Drug Powder + Pet. Ether (60-80%) | Light purple | Light purple | Fluorescent purple |
| 17. | Drug Powder + Chloroform | Light yellow | Light purple | Light brown |
| 18. | Drug Powder + Ethyl Acetate Extract | Yellowish purple | Light purple | Light purple |
| 19. | Drug Powder + Lead acetate | White | White | Purplish white |
| 20. | Drug Powder + Benzen | Light brown | Light purple | Purple |

### 3.3 Determination of Extraction value

The Extraction value of Powder of *A. cruentus* leaves with different solvents like pet. ether, chloroform and ethyl acetate are shown as in table 4.

**Table 4 –** Result of extractive value in different solvents

| Sr. No | Parameters | Extraction value |
|---|---|---|
| 1. | Pet. Ether | 3.04±0.02 |
| 2. | Chloroform | 3.41±0.11 |
| 3. | Ethyl acetate | 3.64±0.33 |

### 3.4 Preliminary phytochemical study

Preliminary phytochemical analysis results of petroleum ether, chloroform and Ethyl-acetate Extract of the leaves showed presence of Alkaloids, Carbohydrates, Glycosides, Amino-acids, Proteins, Steroids, Tannins and Phenolics, Saponins, Flavonoids and Fats and Oils represented in Table 5.

**Table 5.** Preliminary phytochemical analysis of various leaves powder of *A. cruentus*

| Phytoconstituents | Method | Pet. Ether Extract | Chloroform Extract | Ethyl-acetate Extract |
|---|---|---|---|---|
| Alkaloids | Dragendroff's test | _ | + | + |
| | Hager's test | _ | + | + |
| Carbohydrates | Fehling's test | _ | + | _ |
| | Benedict's test | _ | + | _ |
| Glycosides | Legal's test | _ | _ | + |
| | Modified Borntrager's test | _ | _ | + |
| Amino-acids | Ninhydrin test | _ | + | + |
| | Xanthoproteic test | _ | + | + |
| Proteins | Millon's test | _ | + | + |
| | Biuret test | _ | + | + |
| Steroids | Salkowski test | + | + | + |
| | Liebermann's test | + | + | + |
| Tannins and Phenolics | Gelatin test | _ | + | + |
| | Ferric chloride test | _ | + | + |
| Saponins | Froth test | + | + | + |
| | Foam test | + | + | + |
| Flavonoids | Alkaline reagent test | + | + | + |
| | Lead acetate test | + | + | + |
| Fats and Oils | Solubility test | + | _ | _ |
| | Saponification test | + | _ | _ |

### 3.5 Thin layer chromatography

TLC technique was mainly used for qualitative analysis of extract. Findings of TLC analysis of the Ethyl Acetate Extract *of A. cruentus* are presented in the table 6 and figure 1.

**Table 6-** Number of spots and $R_f$ values at wavelength $UV_{254}$ nm of Ethyl Acetate Extract of *A. cruentus* leaves

| Sr. No | Spot No | Rf Value |
|---|---|---|
| 1 | 1 | 0.05 |
| 2 | 2 | 0.08 |
| 3 | 3 | 0.10 |
| 4 | 4 | 0.60 |
| 5 | 5 | 0.72 |
| 6 | 6 | 0.90 |
| 7 | 7 | 0.922 |

### 3.6 HPTLC Analysis for Quantitative analysis of gallic acid and Rutin

The chromatogram and HPTLC fingerprint of the *A. cruentus* ethyl acetate leaf extract revealed the presence of gallic acid and rutin (Figure 2, Figure 3). When the plates were scanned at 254 nm, the Rf values of the gallic acid and rutin of ethyl acetate leaves extract of *A. cruentus* were equal to the

standard at 0.05 and 0.88 (Figures 4 and 5). Rf value of each spot listed in table 6. For both, the correlation coefficient value was 0.997. The ethanolic leaf extract of *A. cruentus* contained 958.69 and 107.65 g/100 mg, respectively, of gallic acid and rutin. Table 7 shows the quantity of spots and Rf values for the ethyl acetate extract of *A. cruentus* leaves at wavelength $UV_{254}$ nm.

**Table. 7-** Number of spots and $R_f$ values at wavelength $UV_{254}$ nm of Ethyl Acetate Extract of *A. cruentus* leaves

| No of Spot | Standard $R_f$ | *A. cruentus* $R_f$ |
|---|---|---|
| 1 | **0.05 (Gallic Acid)** | **0.05 (Gallic Acid)** |
| 2 | **0.88 (Rutin)** | **0.26 (Unknown)** |
| 3 | | **0.30 (Unknown)** |
| 4 | | **0.58 (Unknown)** |
| 5 | | **0.75 (Unknown)** |
| 6 | | **0.80 (Unknown)** |
| 7 | | **0.88 (Rutin)** |

**HPTLC**

**Discussion**

According to the Indian Pharmacopoeia and WHO recommendations for the quality control of herbal medications, physicochemical parameters such as water-soluble ash, LOD, swelling index, total ash, acid-insoluble ash, foaming index, and extractive values were observed. As per common practice, the coarse powder made from the leaves of *A. cruentus* was submitted to numerous systemic physicochemical evolutions. The average of three results (Table 2) allowed for the observation of all physicochemical standards values.

The analysis of fluorescence for leaf powder was performed. With the help of several chemical agents, the powder was suspended. By contrasting the color development in day light and UV (254 and 366 nm) light shown in Tables 3.

The leaves were crushed into a coarse powder and shade dried. Various solvents with increasing polarity, including petroleum ether, chloroform, and ethyl acetate, were used to extract the coarse powder using the continuous hot percolation technique in a Soxhlet apparatus. The solvent was removed from the extracts while under decreased pressure, and the residue was then dried in a vacuum (Table 4).

To identify the phytoconstituents, all extracts were preliminary phytochemical screening utilizing high-quality chemical reagents. All of the extracts contained phytosterols, alkaloids (in chloroform and ethyl acetate), glycosides (in ethyl acetate), saponins (present in all extracts), phenolic compounds (in chloroform and ethyl acetate), and flavonoids (present in all extracts), according to the results of the phytochemical tests. The majority of the phytoconstituents were contained in the ethyl acetate extracts compared to petroleum ether and chloroform extracts. The early phytochemical data were provided in (Table 5). Ethyl acetate extracts were selected for TLC and HPTLC examination based on the preliminary phytochemical research.

As a result, the chromatographic parameters were chosen to provide an analytical fingerprint with comprehensive chemical composition information, acceptable resolution, and a manageable analysis time [22]. On silica gel HPTLC plates, several ratios of toluene and ethyl acetate were tested as the mobile phase based on hit and trial, and the ratio of 7:3 (V/V) provided satisfactory resolution. To ensure accurate, precise, and repeatable Rf values, symmetrical peak shapes, and improved resolution for both drugs, several chromatographic conditions were optimized. This included adjusting the sample application rate and volume, sample application positions, chamber saturation time, total run length, and distance between tracks. These optimizations were implemented to achieve more reliable and consistent results in the chromatographic analysis. For each metabolite in both samples, the acquired chromatographic results showed well-defined and distinct peaks at the appropriate wavelengths. Figures 2 and 3 show a typical chromatogram of *A. cruentus* at 254 nm. The HPTLC plate, scanned at 254 nm, clearly showed gallic acid and rutin. Rutin ($R_f$ 0.88) and standard gallic acid ($R_f$ 0.05) displayed a single peak in the HPTLC chromatogram shown in Figures 4 and 5. Figures 6 and 7 show the calibration curves for gallic acid and rutin, which were created by graphing the concentrations of the two substances against the areas of their respective peaks. Figures 8 and 9

show the 3D graph of conventional gallic acid and rutin.

Some of the most significant polyphenols, such as gallic acid and rutin, are found in a number of medicinal plants, such as *A. cruentus*, and have a broad range of pharmacological action.

**Figure.1** Pictogram of developed TLC plate in Iodine chamber

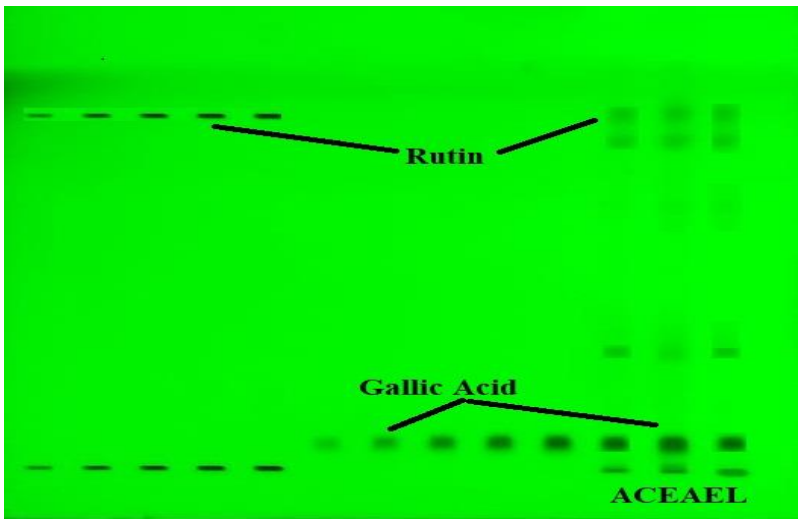


**Figure 2:** HPTLC fingerprint of ethyl acetate extract of *A. cruentus* leaves at 254nm.

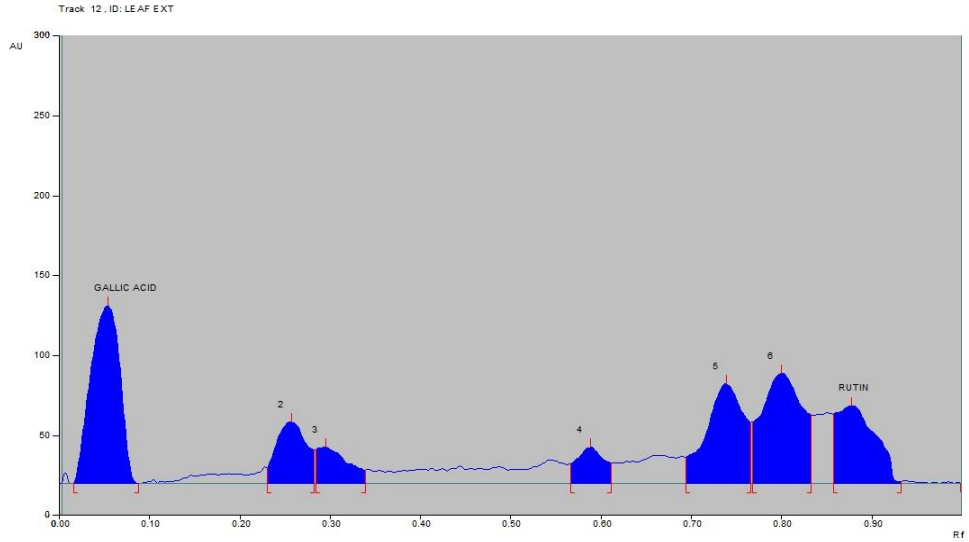


**Figure 3**. HPTLC chromatogram of ACEA Extract at a 254 nm

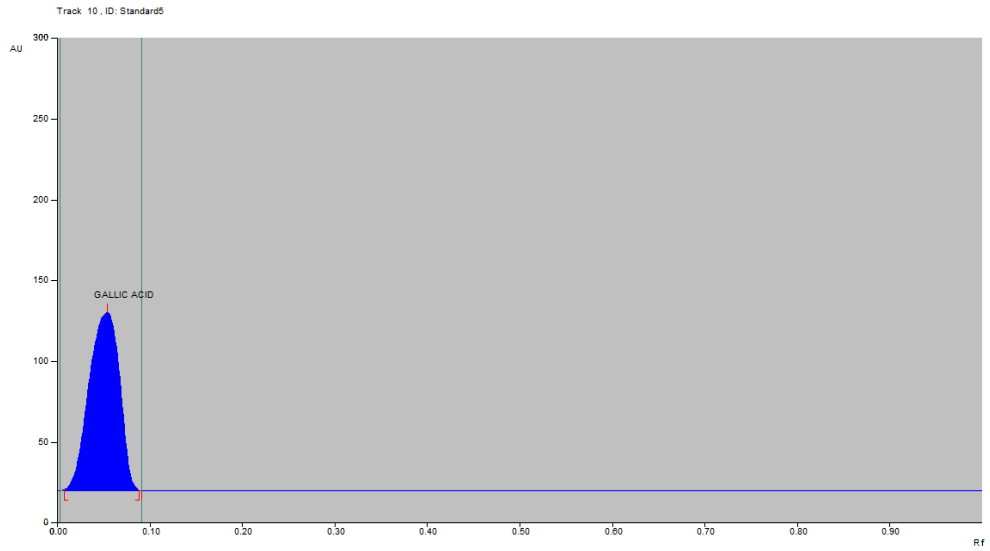


**Figure 4**. HPTLC chromatogram of gallic acid at a 254 nm

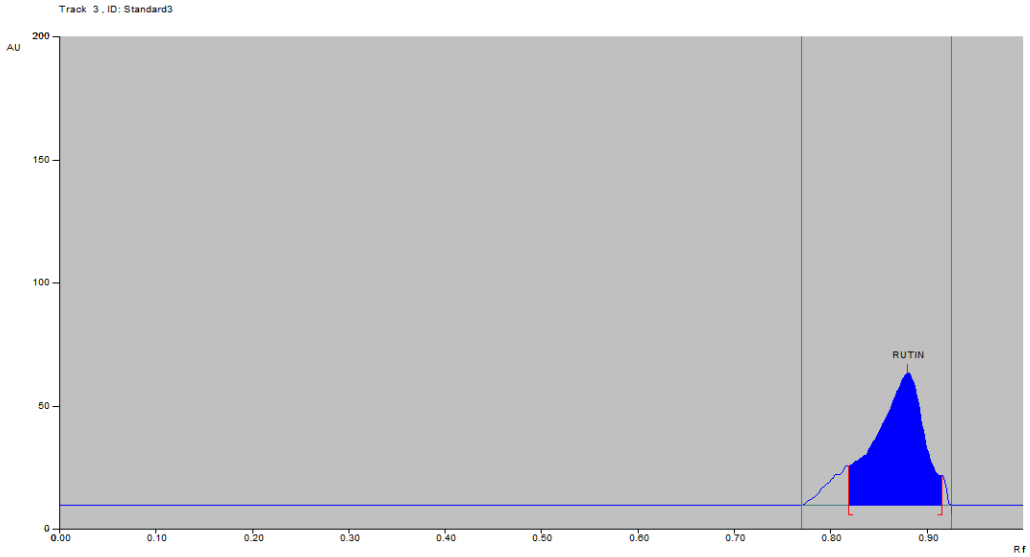


**Figure 5**. HPTLC chromatogram of Rutin at a 254 nm

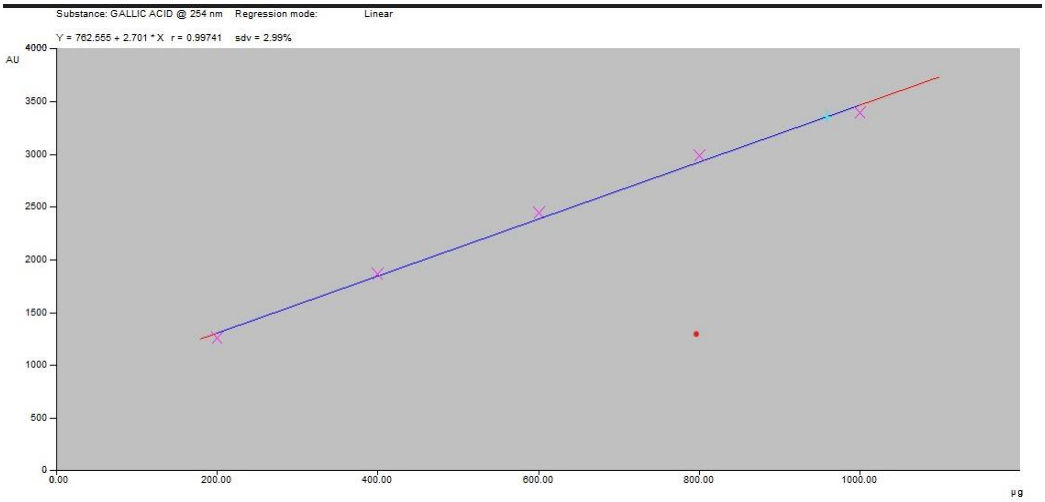


**Figure 6**. The calibration curve of gallic acid prepared by plotting conc. of gallic acid versus peak area

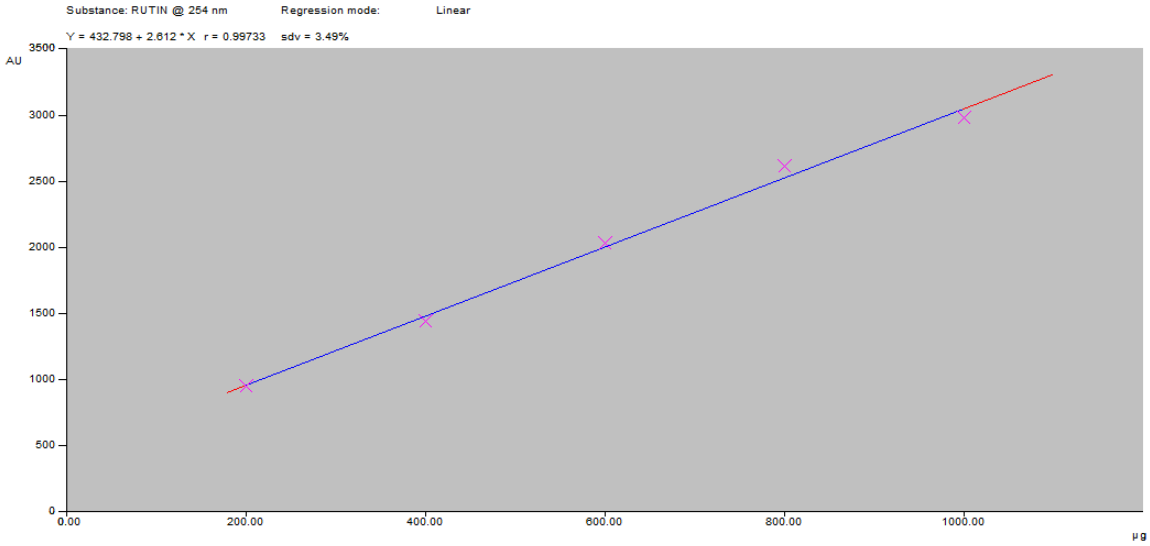


**Figure 7**. The calibration curve of gallic acid prepared by plotting conc. of Rutin versus peak area

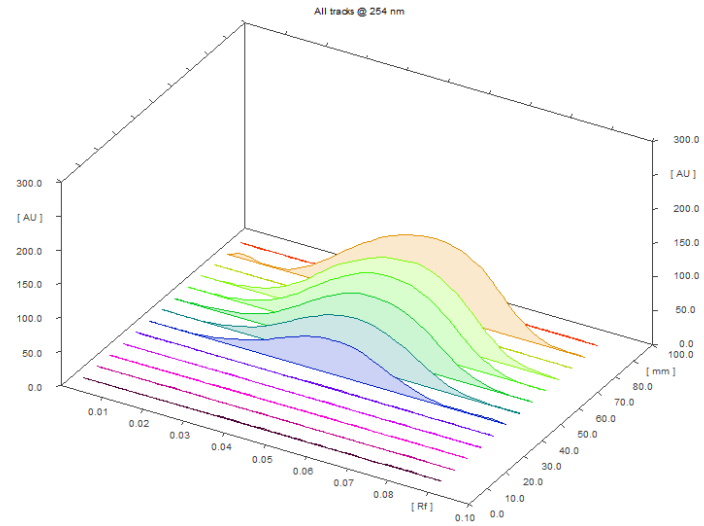


**Figure 8.** 3D Graph of Gallic acid at 254 nm

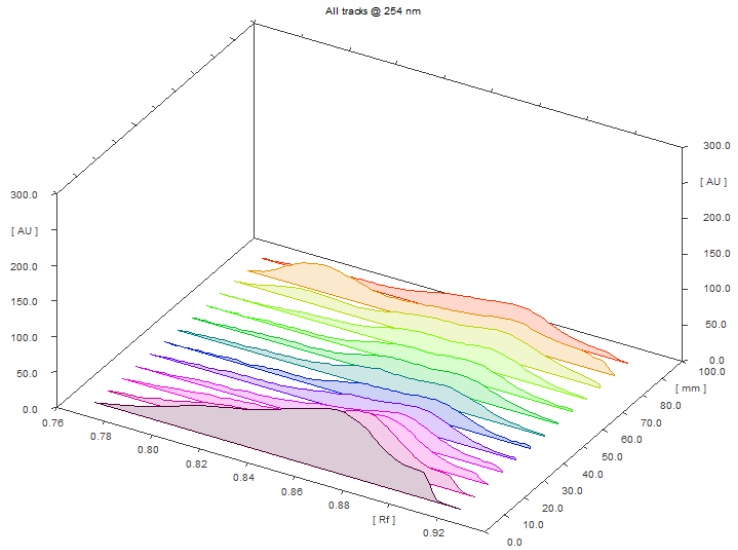


**Figure 9.** 3D Graph of Rutin at 254 nm

## Conclusion

The current Pharmacognostical data showed that people were aware of the characteristics and name of the plant *A. cruentus.* The qualitative and quantitative indicators displayed the crucial plant data. These investigations demonstrated the existence of several significant bioactive substances and demonstrated the potential therapeutic use of plant leaves. These details will also help to distinguish *A. cruentus* from the closely related other species since the plant is a morphologically varied species. These findings may help in the standardization, identification, and further study of medications made from *A. cruentus* leaves that are included in both traditional and contemporary pharmacopoeia.

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