Methodology

5. Extraction

5.1 Successive solvent extraction

About 5 kilogram of root free corm was thoroughly washed under running water to remove adherent soil and impurities. The cut chips of the corm were shade dried by making into chips and finally powdered to mesh 60#.

The air dried corm powder was exhaustively extracted by hot percolation method (soxhlation) with different solvents of increasing order of polarity, starting with a highly nonpolar solvents viz., Petroleum ether followed by Chloroform, Ethanol (95%) and Chloroform Water.

Initially about 200 gm of corm powder was extracted with 600 ml of Pet. Ether. The extraction was continued until the solvent in the thimble became clear. After complete extraction, the extract was filtered and the solvent was distilled off using rotary vacuum flash evaporator. The obtained residue was dried in a desiccator over anhydrous sodium sulphate. The average yield, colour, odour and constituency were recorded.

The left over mark was air dried at room temperature and was similarly extracted with Chloroform, Ethanol and Water respectively. All the extracts were stored in a refrigerator for preliminary phytochemical investigation, acute toxicity studies and pharmacological screening.

5.1.2. Hydro-Alcoholic (70%) extraction

About 200 gm / batch of air dried corm powder was subjected to exhaustive extraction with about 600 ml of 70% v/v hydro alcohol in a soxhlet extractor, until the solvent in the thimble became clear. Later the solvent from the filtered extract was
evaporated off on rotary vacuum evaporator at 50°C temperature. The extract thus obtained was dried in a desiccator over sodium sulphate. Some part of the total extract was used for phytochemical investigation and rest for the *in vitro* antioxidant estimations.

### 5.1.3. Methanolic extraction

The methanolic extract was prepared by following the above procedure using methanol instead of hydroalcohol. Some part of the total extract was used for phytochemical investigation and rest for the *in vitro* and *in vivo* antioxidant activities.

The percentage of yield of the all above extracts were calculated with reference to air dried drug and the colour, odour and constituency of the each extract were noted (table No.1.).

### 5.2. Phytochemical analysis of various extract

#### 5.2.1 Preliminary phytochemical investigations:

All the extracts of corm were subjected for systematic preliminary qualitative phytochemical investigations.

1) **Tests for alkaloids:**

The various extracts were basified with ammonia and extracted with chloroform. The resulted chloroform solution was acidified with dilute hydrochloric acid. The acid layer was used for testing the alkaloids.

- *Mayer’s test* (Potassium Mercuric Iodide solution): The acid layer was treated with few drops of Mayer’s reagent. Formation of creamy white precipitate indicates the presence of alkaloids.
• **Wagner’s test** (Solution of Iodine in Potassium Iodide): The acid layer was treated with few drops of Wagner’s reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

• **Dragendorff’s test** (Solution of Potassium Bismuth Iodide): The acid layer was treated with few drops of Dragendorff’s reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

• **Hager’s test** (saturated picric acid solution): The acid layer was treated with few drops of Hager’s solution. Formation of yellow precipitate indicates the presence of alkaloids.

2) **Tests for Tannins and Phenolic compounds:**

   The test solution was prepared by dissolving extract in ethanol or aqueous ethanol solution and added few drops of the following reagents.

   • Ferric chloride test: Deep blue black colour
   • Lead acetate solution: White precipitate
   • 1% Gelatin solution: precipitation
   • Bromine water: Discoloration of bromine water.
   • Dilute iodine solution: Transient red colour
   • Potassium dichromate: red precipitate
   • Dilute potassium permanganate solution: Discoloration
   • Acetic acid solution: Red colour solution
   • One drop of NH₄OH, excess 10% AgNO₃ solution, Heated for 20 min. in boiling water bath. White precipitate observed, then dark silver mirror deposits on wall of test tube.
3) Tests for flavanoids:

The flavanoids are basically derived from parent moiety called flavones. Naturally flavonoids occur in the free form as well as glycosides along with sugars. Hence the plant extracts are hydrolyzed and subjected to flavonoidal detection.

Preparation of test solution – A small quantity of extract was added with equal volume of 2M HCL and heated in the test tube for 30-40 minutes at 110°C. Later the cooled extract was filtered, extracted with ethyl acetate. The resulted extract was concentrated, dried and subjected for flavonoid testing.

- **Shinoda test**: To the ethyl acetate fraction a few fragments of magnesium ribbon and concentrated hydrochloric acid was added. Appearance of magenta colour after few minutes indicates the presence of flavonoids.

- **Ferric chloride test**: Test solution when treated with few drops of neutral ferric chloride solution formation of violet colour indicates the presence of phenolic nucleus.

- **Zinc-Hydrochloric acid reduction test**: The test solution was treated with a pinch of zinc dust and few drops of concentrated hydrochloric acid. Formation of Magenta colour after few minutes indicates the presence of flavonoids.

- **Lead acetate test**: Few drops of lead acetate solution (10%) were added to the test solution formation of yellow precipitate indicates the presence of flavonoids.

- **Alkaline reagent test**: Test solution was treated with sodium hydroxide solution. Increase in intensity of yellow colour, which becomes colourless on addition of few drops of dilute acid indicates presence of flavanoids.
4) Tests for sterols:

The extract was dissolved in chloroform, filtered and filtrate was tested for sterols.

- *Salkowski test*: Few drops of concentrated sulphuric acid was added to the chloroform solution, shaken and allowed to stand, appearance of red colour in lower layer indicates the presence of sterols.

- *Liebermann – Burchardt’s test*: To the chloroform solution, few drops of acetic anhydride was added and mixed well. 1ml of concentrated sulphuric acid was added from the sides of the test tube, appearance of green fluorescence in chloroform layer indicates the presence of sterols.

- Sulphur test: Sulphur when added to the chloroform solution, it sank in it.

5) Tests for Glycosides:

The test solution was prepared by dissolving extract in ethanol or hydro alcohol and subjected to the following tests.

**Tests for cardiac Glycosides:**

- *Baljet’s test*: The test solution treated with sodium picrate gives yellow to orange colour.

- *Keller-Killani test for digitoxose*: The test solution treated with glacial acetic acid containing traces of ferric chloride, then sulphuric acid was added, it forms two layers. Lower layer reddish brown, upper layer turns bluish green.

- *Legal’s Test*: Test solution when treated with pyridine (made alkaline by adding sodium nitroprusside solution) gives pink to red colour.

- *Raymond’s test*: Test solution treated with dinitrobenzene in hot methonolic alkali gives violet colour.
• *Bromine water test:* Test solution was treated with bromine water giving yellow precipitate.

**Tests for anthraquinones glycosides:**

• *Borntrager’s test:* The extract was boiled with 5ml of 10% Sulphuric acid for 5 minutes. Filtered while hot and after cooling the filtrate, shake gently with equal volume of benzene. Benzene layer was separated, then treated half of its volume with solution of ammonia (10%). Allowed to separate, ammonical layer acquired rose pink colour due to the presence of anthraquinones.

• *Modified Borntrager’s test:* Hydrolysis of the extract was carried out with 5 ml of dilute hydrochloric acid and 5ml of 5% solution of ferric chloride. For hydrolyzed extract procedure was carried out as described under Borntrager’s test.

**Tests for Cyanogenetic glycosides:**

• *Grignard’s test:* Strips of sodium picrate filter paper were inserted between split cork stopper which was fitted into the tube containing a small amount of residue in water. Care was exercised not to touch the inner side of the test tube with paper strips. The content was warmed for thirty minutes. The red colour of the strips indicates the presence of cyanogenetic glycosides.

6) **Coumarin glycosides:**

Extract made alkaline, showed blue or green fluorescence under uv light.

7) **Tests for saponins:**

The test solution was prepared by dissolving extract in ethanol or aqueous ethanol solution and the following test were done.
- **Haemolysis test:** To 2 ml of 1.8% sodium chloride solution in two test tubes, 2 ml distilled water was added to one test tube and to other 2 ml of 1% extract was added. Few drops of blood was added to both test tubes and gently mixed, observed under microscope. If the haemolysis observed in the tube containing the extract it indicates the presence of saponins.

- **Foam test:** Small quantity of extract was shaken with little amount of water, if the foam produced persists for 10 minutes is the indication of presence of the saponin.

- Test for steroidal saponin: The extract was hydrolyzed with dilute sulphuric acid and extracted with chloroform. The obtained chloroform layer was tested for sterols.

- Test for triterpenoidal saponins: The extract was hydrolyzed with dilute sulphuric acid and extracted with chloroform. The obtained chloroform layer was tested for triterpenoids.

8) **Tests for triterpenoids:**

The chloroform extract was prepared and used to test the presence of triterpenoids.

- **Salkowski test:** Few drops of concentrated sulphuric acid was added to the chloroform solution, shaken and allowed to stand for some time, appearance of golden yellow color indicates the presence of triterpenoids.

- **Liebermann–Burchard test:** To the chloroform solution, few drops of acetic anhydride was added then mixed well. 1 ml concentrated sulphuric acid was added from the sides of the test tube, appearance of red ring indicates the presence of triterpenoids.
9) **Tests for Proteins:**

The test solution was prepared by dissolving extract in ethanol or aqueous ethanol solution and the following test were done.

- Millon’s test: The solution treated with Millon’s reagent. The resulted white precipitate formed turns to red precipitate and upon dissolving forms red coloured solution.

- Biuret test (general test): The solution treated with 40% sodium hydroxide and dilute copper sulphate solution gives blue colour.

- Precipitation test: The test solution gives white colloidal precipitate with 5% HgCl₂ solution, 5% copper sulphate solution, 5% lead acetate solution and with 5% ammonium sulphate.

- Xanthoprotein test: The test solution treated and boiled with concentrated nitric acid gives yellow precipitate, turns yellow upon adding NH₄OH.

10) **Tests for Amino acids:**

The test solution was prepared by dissolving extract in water or hydroalcohol and the following tests were carried-out.

- Ninhydrin test: Test solution with Ninhydrin solution gives blue colour.

11) **Tests for fats and fixed oils:**

The filter paper gets permanently gets stained with fats and oils.

12) **Tests for carbohydrates:**

Small quantities of extracts were dissolved in little amount of distilled water and filtered separately. The filtrate was used to test the presence of carbohydrates as follows.
• **Molisch’s test:** The test solution was treated with Molisch reagent (alcoholic solution of α-nepthhaol) and concentrated sulphuric acid was added along the sides of the test tube to form a layer. A reddish violet ring shows the presence of carbohydrates.

• **Fehling’s test:** The test solution was treated with equal amount of Felhing’s A (copper sulphate in distilled water) and B (potassium tartarate and sodium hydroxide in distilled water) reagents and boiled. Formation of red precipitate indicates the presence of reducing sugars.

• **Benedict’s test:** The test solution was treated with few drops of Benedict’s reagent (alkaline solution containing cupric citrate complex) and boiled on water bath. Formation of red brown precipitate indicates the presence of reducing sugars.

13) **Tests for Gum and Mucilages:**

Hydrolyze the above test solution using dilute hydrochloric acid and performed Felhing’s or Benedict’s test as mentioned earlier. Gums and mucilage are present if red colour develops. Presence of mucilage is tested with addition of ruthenium red solution to get rose to pink colour.

14) **Tests for lactones**

The test solution was prepared by dissolving extract in ethanol or aqueous ethanol solution and the following test were carried-out.

• **Legal test:** The test solution was dissolved in pyridine and a mixture of sodium nitroprusside and sodium hydroxide was added. Deep red colour indicates the presence of lactones.
• **Baljet test:** To the extract, sodium picrate solution was added. Formation of yellow colour indicates the presence of lactones.

15) **Tests for vitamins:**

  • **Vitamin A:** Dissolved a quantity equivalent to 10 to 15 units in 1ml of chloroform and added 5ml of antimony chloride solution, the formation of a transient blue colour immediately indicates the presence of vitamin A.

  • **Vitamin C:** Diluted 1ml of 2% w/v extract solution with 5ml of water and added few drops of freshly prepared 5%w/v solution of sodium nitroprusside and 2ml sodium hydroxide solution. Addition of few drops of hydrochloric acid drop wise, produces yellow colour turning blue indicates presence of vitamin A.

  • **Vitamin D:** Dissolve a quantity equivalent to about 1000 units of vitamin D in chloroform and added 10ml of antimony trichloride solution, a pinkish – red colouration at once indicates presence of vitamin D.

The results of preliminary phytochemical screening are compiled in section 6.2.1, table No.2.

**5.2.2. Flourescence analysis and colour reactions**

The powdered drug sample was observed under visible light and ultraviolet light after treatment with different reagents viz., 90% alcohol, 10% Potassium hydroxide and 10% Sulphuric acid to reveal the presence of chemical constituents with fluorescent characters.
Colour reactions of corm powder

The powder drug sample was treated with various acids and chemical reagents to unveil the presence of different chemical constituents.

The results are summerised in section 6.2.2 and table No. 3 & 4.

5.2.3. Chromatographic studies

Thin Layer Chromatography (TLC) studies were carried out to confirm the presence of different phytocostituents detected in the qualitative chemical investigation of the extracts. Much attention was given for the TLC analysis of methanolic extract which is preferred in the present work for *in vitro* and *in vivo* pharmacological screening.

TLC is accepted as a separation method for the phytocconstituents in an extract at analytical laboratory levels after suitable sorbents became available for self resolution on thin layer plates. Qualitative initial screening of the extracts is routinely performed and the presence of ubiquitous compounds such as plant sterols and certain phenolics can be ascertained at an early stage by running the appropriate standard alongside of test extract.

Separation of phytocconstituents by TLC, a mode of liquid chromatography, is effected by application of the extract as a spot or thin line onto a sorbent layer that has been applied to a backing plate. The solvent front that migrates up the plate through the sorbent by capillary action resulting in the resolution of phytocconstituents due to their differential adsorption / partition co-efficient with respect to both mobile and stationary phases. Each separated compound has same migration time but different distances.
The mobile phase may be of single solvent or mixture of solvents and the sorbents may be Silica Gel, Cellulose, Polyamide, and Alumina etc. Among these Silica Gel (type 60) is most commonly used. For the present work Silica Gel 60 GF<sub>254</sub> precoated sheets as sorbent system was employed to analyze the extracts.

The information provided by finished chromatography includes ‘Migration behavior’, given in the form of the Retardation Factor (R<sub>f</sub>) value using the formula;

\[
\text{Retardation factor (R}_f\text{)} = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}}
\]

5.2.3.1. Qualitative TLC analysis

Thin Layer Chromatography using Silica Gel 60 GF<sub>254</sub> precoated sheets as sorbent system was employed to analyze the various extracts.

1) **TLC profile of Petroleum ether extract**

On precoated and preactivated aluminium TLC plates (E-Merck.5548) of silica gel 60GF<sub>254</sub>, the extract was spotted and the plate was developed using Benzene. After the development it was observed in U V chamber under longer wavelength (365nm). There after the developed plates were sprayed with acid (10% sulphuric acid in methanol), heated at 110°C for 5 minutes again observed. The number of spots resolved and their R<sub>f</sub> values before and after the acid spray were recorded.

2) **TLC profile of 70% Hydroalcoholic extract**

The test extract spotted on the precoated plate was developed using Solvent system Toluene : Ethyl acetate (93:07) and the developed plates were observed as earlier method. R<sub>f</sub> values of the resolved spots were recorded.
3) **TLC profile of Methanolic extract for flavonoids and Coumarins**

The precoated plates spotted with the above extract was developed using solvent systems n-Butanol : Acetic acid : water (4:1:5) and 10% Acetic acid separately. The resolved spots were tested for Flavonoids and Coumarins by using suitable detecting agents and their \( R_f \) values were recorded.

4) **TLC profile of ethyl acetate fraction of Methanolic extract**

On Silica Gel precoated plates, the above fraction was spotted and the plates were developed with solvent system Benzene : Toluene : Glacial acetic acid (3:6:1) and the \( R_f \) of resolved spots were recorded.

5) **TLC profile of ethyl acetate fraction of Methanolic extract for phenolics**

On Silica Gel precoated plates, the above fraction was spotted along side of authentic markers Quercitin and Gallic acid and the plates were developed with solvent system Chloroform : Ethyl acetate : Formic acid (5:4:1). The developed plates were visualized by spraying with 2% Ferric chloride solution in ethyl alcohol.

The \( R_f \) values of the resolved compounds were recorded and compared with authentic markers viz., Quercetin and Gallic acid.

The results of all the above qualitative TLC analysis are summarized in section 6.2.3.1.

**5.2.3.2. HPTLC Finger print analysis**

A) Qualitative HPTLC Analysis of ethyl acetate fraction of Methanolic extract was carried out with solvent system Benzene : Toluene : Glacial acetic acid (3:6:1).
The samples of ethyl acetate were spotted in the form of bands on precoated and preactivated aluminium silica gel 60GF$_{254}$ p HPTLC plates (10cm x10 cm width, 0.2mm thickness-E-Merk) by means of Linomat IV automatized spray on band applicator equipped with a 100 µL syringe. The linear ascending development was carried out in Camag HPTLC twin trough chamber saturated with mobile phase consisting of Benzene : Toluene : Glacial acetic acid (3:6:1). Subsequent to the development the plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed on Camag TLC scanner III in absorbance mode at 254 nm.

The results are summarized in section 6.2.3.2, Fig.No.1 & 2, table No.5.

B) Qualitative HPTLC Analysis of ethyl acetate fraction of Methanolic extract was carried out for phenolics with solvent system Chloroform : Ethyl acetate : Formic acid (5:4:1).

After optimization of mobile phase, the solvent system consisting of Chloroform : Ethyl acetate : Formic acid (5:4:1) was selected for HPTLC finger print analysis to get the best resolution of the phenolics from ethyl acetate fraction of methanolic extract.

**Equipment:** A Camag HPTLC system equipped with a sample applicator Linomat IV Twin trough plate development chamber, Camag TLC Scanner III and Cats 4 integration software.

The ethyl acetate fraction and the markers were spotted in the form of bands on 20 cm x 10 cm width HPTLC aluminum plates precoated with silica gel 60 F$_{254}$ of
thickness 0.2 mm by means of Linomat IV automatised spray on band applicator equipped with a 100µL syringe.

**Operation parameters**

- Band width : 6 mm
- Application rate : 15 µL⁻¹
- Volume applied : 2 ml
- Space between bands : 10 mm
- Distance of plate side edge :15 mm
- Distance from the bottom of the plate : 10 mm
- Distance of development : 75.0 mm

**Development of Chromatogram**

The plates were developed in a twin trough chamber at room temperature (25 ± 2°C) at relative humidity of 60 ± 5% with the mobile system Chloroform : Ethylacetate : Formic acid (5:4:1) v/v. Ascending mode was used for development of thin layer chromatography and the development time was about 15 minutes.

**Detection and densitometric evaluation of chromatogram**

Following the development, the TLC plates were dried in a current of air with the help of an air dryer in a wooden chamber with adequate ventilation. The developed plate was observed under U V chamber at 254 nm and at 366 nm and photographed for the resolved components. There after the plate was sprayed with 2% Ferric chloride solution in ethyl alcohol. The $R_f$ values of two resolved dark greenish spots were noted and immediately scanned at 254 nm using CAMAG TLC SCANNER-III with CATS 4 software. Data of each band was recorded.
Scanning operating parameters

Mode: Absorption/Reflection

Slit dimension: 5x 0.1mm

Scanning rate: 20 mms⁻¹

Optimized wavelength: 254 nm.

The results are summarized in section 6.2.3.2, Fig.No.3,4,5a & 5b, table No.6.

For references, HPTLC finger print analysis of standards markers viz., Gallic acid and Quercetin were developed with solvent system Chloroform: Ethyl acetate: Formic acid (5:4:1). Densitometric scanning was performed and Rf values were recorded.

The results are summarized in Fig.No.6 & 7.

5.2.4. GC-MS Spectroscopic Analysis of Methanolic extract

The GC-MS analysis of methanolic extract was carried out in a GC MS Model: GCMS-QP2010 Plus. Make: SHIMADZU gas chromatograph fitted with ZB-624 30 m X 1.4 mm ID 0.25µm film thickness or equivalent column. Carrier gas was helium with a flow rate of 2.5 ml/min; column temperature initially was at 120°C for 2min. Then rose to 250°C at the rate of 10°C per minute maintained at 250°C for 20minutes; injector temperature was 240°C, detector temperature 260°C, volume injected 1µL with liquid injector of 70% ethanol extract in ethanol (1gm in 5ml ethanol). The mass spectra operating parameters were as follows: ion source temperature: 250°C, ionization potential, 70eV; solvent delay 3.0 min, program run time: 31 minutes and scan range 30-350 amu, EV voltage 3000 volts.
Structural assignments of components in the methanolic extract were based on interpretation of mass spectrometric fragmentation and confirmed by comparison of retention time through computer matching with the commercial library Wiley 07.

The results are summarized in table No.7.

5.3. Isolation and characterization Phenolic compounds

Isolation and purification of phenolics by column chromatography

About 10 gm of ethyl acetate fraction of defatted methanolic extract was column chromatographed over silica gel and elution was carried out from non-polar to polar solvents by gradient elution method with following data process:

- Adsorbent : Silica gel (100-200 mesh)
- Activation : 100°C for 1 hour.
- Length of column : 50cm
- Length of packing : 35 cm
- Inner diameter of column : 5cm
- Rate of elution : 10 to 12 drops/min.
- Volume of elute collected : 5 ml
- Type of elution : Gradient elution

Preparation of sample

The ethyl acetate fraction was dissolved in 20 ml of methanol and mixed uniformly with approximate 2 gm of silica gel (100-200 mesh) and dried at 45°C in an oven. The resulted material was transferred to the packed column.
Column packing

Prior to packing column, it was thoroughly washed with petroleum ether and allowed to get dried. About 130-150 gm of silica gel was subjected to activation at $110^0$C for 1 hour and the dry packing method was followed for column packing.

Sample loading

The petroleum ether was charged into the column up to 75% of the column length and the activated silica gel was charged into the column in small quantities successively with gentle tapping ensuring the uniform packing and avoiding entrapment of air bubbles in the packed column. A sufficient quantity of solvent was allowed to remain at the top of column and left out overnight for column stabilization.

After running the column with petroleum ether in order to remove hydrophobic impurities, a thin uniform disc of cotton pad was placed carefully on the packed column above which the sample-silica gel mixture was loaded uniformly. Finally yet another cotton pad was placed to ensure no perturbation to the surface of sample mixture during repeated loading with the varying solvent system.

Gradient elution

Gradient elution technique was followed for the resolution and elution of the compounds from the loaded sample mixture from the packed column. Gradient elution was carried out initially by chloroform followed by different proportion of chloroform and ethyl acetate like 100:10, 100:20, 100:30, 100:40, 100:50 100:60, 100:70, 100:80, 100:90, 100:100, 100:110, 100:120, 100:130, 100:140, 100:150, 100:160, 100:170, 100:180, 100:190, 100:200. The rate of elution was adjusted to 10-
15 ml/min and every time the column was run with 200 ml of solvent mixture. For all the eluted fractions TLC studies were carried using solvent system Chloroform : Ethylacetate : Formic acid (5:4:1) v/v and checked for the eluted phenolics using 2% Ferric chloride in ethyl alcohol as spraying agent.

Fractions with similar spots were pooled together and further purified by rechromatography using small columns. The eluted fractions were concentrated under reduced pressure, which on complete drying off the solvent afforded unique sample.

The results are given in table No.8.

**Characterization of phenolic compounds**

The fraction eluted with 100:100 proportion of chloroform and ethyl acetate exhibited single spot upon TLC with solvent system Chloroform : Ethyl acetate : Formic acid (5:4:1) v/v, being identified as yellow fluorescent compound under 366 nm in UV chamber and developed a bluish black colour with spraying agent Ferric chloride solution. And hence fractions with similar spots in the range of 100:100 were pooled together and concentrated under reduced pressure. Thus obtained yellowish crystalline powder was designated as (AP-1).

Similarly, the fraction eluted with 100:200 proportion of chloroform and ethyl acetate exhibited single spot upon TLC with same solvent system as above. And hence fractions with similar spots in the range of 100:200 were pooled together and concentrated under reduced pressure. The white amorphous powder obtained was designated as (AP-2).
Phytochemical tests for the isolated phenolic compound AP-1 and AP-2

2-3 mg of isolated compound AP-1 was dissolved in alcohol and following tests were conducted.

**Ferric chloride test**: Test solution when treated with few drops of neutral ferric chloride solution formation of violet colour indicated the presence of phenolic nucleus.

**Lead acetate test**: Few drops of lead acetate solution (10%) were added to the test solution formation of yellow precipitate indicated as flavonoid.

**Shinoda test**: To the test solution a few fragments of magnesium ribbon and concentrated hydrochloric acid was added. Appearance of magenta colour after few minutes indicated as flavonoid.

**Zinc-Hydrochloric acid reduction test**: The test solution was treated with a pinch of zinc dust and few drops of concentrated hydrochloric acid. Formation of Magenta colour after few minutes confirmed as flavonoid.

2-3 mg of isolated compound AP-2 was dissolved in alcohol and following tests were conducted.

**Folin-Ciocalteu test**: The test solution was treated with few drops of Folin-Ciocalteu reagent. Appearance of blue colour immediately indicated as Phenolic acid.

**Ferric chloride test**: Test solution when treated with few drops of neutral ferric chloride solution formation of violet colour indicated the presence of phenolic nucleus.

**Lead acetate test**: Few drops of lead acetate solution (10%) were added to the test solution formation of precipitate indicated as tannin.
Physical properties of isolated compounds (AP-1 and AP-2)

The eluted compound AP-1 and AP-2 were observed under normal light and in UV at 365 nm to check colour and the fluorescent nature respectively. And tested for content uniformity by TLC on Silica Gel precoated plates with solvent system Chloroform : Ethylacetate : Formic acid (5:4:1) v/v using Ferric chloride as spraying agent. The recorded R_f values were compared with authentic samples Quercetin and Gallic acid. The melting point was recorded using melting point apparatus.

Spectral characterization

The structure of isolated compounds AP-1 and AP-2 were determined by IR, \(^1\)H-NMR, Mass spectral analysis.

Instruments used

Infrared spectrum: The infrared spectrum of the isolated compounds are recorded in the range of wave number 4000 cm\(^{-1}\) to 650 cm\(^{-1}\) on FTIR 5300 JASCO.

Nuclear magnetic resonance spectrum (\(^1\)H-NMR): Nuclear magnetic resonance spectrum was recorded on BRUKER NMR Spectrophotometer (400 MHz) by dissolving the compound in MeOD and chemical shifts are expressed in \(\delta\) ppm.

Mass spectrum: The mass spectrum was recorded on LCMS 2010, SHIMADZU, JAPAN. The following method was used in running LC-MS.

HPLC conditions:

Column : C\(_{18}\)

Mobile phase : methanol : water (90:10)

Flow rate : 0.2 ml/min
Volume injected : 5 µl

UV-Visible wave number : 254 nm

**Mass Spectrum Conditions:**

Probe : ESI (Electron spray ionization)

    APCI (Atmospheric pressure chemical ionization).

Mode : Positive gives M+1 values, Negative gives M-1. In presence of halogens the values will show M and M+2 in positive, M and M-2 in negative.