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A pre-clinical study for pharmacognostical and chemical standardization of aqueous and alcoholic extracts of Ayurvedic spermatogenetic formulation using spectroscopy & chromatography (including GC-MS) methods

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Abstract
An Ayurvedic formulation prepared from root powder of *Sida cordifolia* Linn. and *Glycyrrhiza glabra* Linn. is standardized through pharmacognostical and phytochemical pre-clinical studies for treatment of male sexual disorders. Flavonoids, carbohydrates, glycosides, tannin and Nitrogen were found present in both alcoholic and aqueous extracts. The results also showed high concentrations of flavonoidic compounds (80.31 µg QE/mg) in alcoholic and high phenolic content (28.60 µg GAE/mg) in aqueous extract. HPTLC analysis at 280 and 360 nm indicated presence of Ellagic acid and Gallic acid in both extracts. Similarly, HPLC analysis at 276 nm showed elution of twelve compounds & confirmed presence of Vanillin in both extracts. UV-Visible spectroscopy scanning showed peaks at 822.5, 261 and 194.5 nm in aqueous and at 896, 361, 315, 273.5 and 211 nm in alcoholic extract. FTIR analysis indicated presence of -CH_3 out-phase stretching, -NH_2 bending, (N)-CH_3 in phase bending, C=O in-phase rocking functional groups in aqueous and C=N stretching, –CH_2 bending, (N)-CH_3 in-phase bending, Aryl-CH_3 in-phase bending, C-O-C out-phase stretching and − O-H Wagging functional groups in alcoholic extract suggesting presence of phenolic, flavonidic & alkaloidal compounds possibly responsible for its antioxidant, analgesic, antipyretic, anti-inflammatory and antimicrobial properties.

Keywords: *Sida cordifolia* Linn., *Glycyrrhiza glabra* Linn., Ayurveda, pre-clinical, spectroscopy, chromatography

1. Introduction
Many therapeutic solutions have been developed over the years for treatment of male sexual disorders but many of the available allopathic cures have been associated with adverse side effects, resulting in a renewed interest in herbal cures. The traditional knowledge of Ayurvedic system of medicine which is inherently holistic in nature and its systems approach which are validated by experimental findings can serve as an innovative and powerful starting point for discovery of newer, safer and affordable medicines [1]. Plant species mentioned in these ancient textbooks may be examined afresh with modern scientific approaches for ensuring their authenticity and mechanisms evolved for their standardization through identification of key bioactive compounds and fingerprinting of phytochemical constituents [2, 3].

Sida cordifolia Linn. which belongs to the Malvaceae family is a well-known medicinal plant in Ayurvedic literature. Also known as *Bala*, it is a small, erect, annual downy shrub. It finds use as a cooling, astringent, aromatic, diuretic and tonic in Ayurveda for treatment of diseases like asthma, cough, fever, skin diseases, heart ailments, facial paralysis, muscle and joints pain, swelling, wounds, inflammation, urinary infection, lack of sexual desire and unwanted weight loss [4, 5]. Its roots and seeds contain alkaloid ephedrine, vasicinol, vasicinone, β-sitosterol and stigmasterol and N-methyl tryptophan while the leaves of *Sida cordifolia* contain small amounts of both ephedrine and pseudoephedrine. Its pharmacological actions include hypoglycemic, wound healing, anti-microbial, antioxidant, anti-inflammatory, analgesic, adaptogenic and hepato-protective activities [6, 8].

Glycyrrhiza glabra Linn. also called Liquorice root belongs to the Fabaceae family. It is a perineal herb/sub-shrub found in the subtropical and temperate zones. Its underground stems and roots are used medicinally for treatment of cough, hyperacidity, skin and ophthalmic diseases and as a tonic, rejuvenator, demulcent, expectorant, etc. [4,9,10] The chief constituent of liquorice is glycyrrhizin, which is present in the drug in the form of the potassium and calcium salts of Glycyrrhizic acid. Liqourice also contains glucose (up to 3.8 per cent), sucrose (2.4 to 6.5 per cent), bitter principles, resins, mannite, asparagines (2 to 4 per cent) and fat

(0.8 per cent). Its pharmacological activities are reported to be muscle depressant, anti-microbial, hypo-lipidaemic, anti-antherosclerotic, antiviral, hypotensive, hepato-protective, anti-exudative, spasmolytic, antidiuretic, antiulcer, anti-mutagenic, antipyretic, antioxidant, anti-inflammatory, anti-nociceptive and expectorant [11, 14].

Therefore, a novel Ayurvedic formulation having equal amount of roots of *Sida cordifolia* Linn. (Bala) and *Glycyrrhiza glabra* Linn. (Yashthimadhu). has been prepared for utilizing the synergistic effect of similar therapeutic activities of the roots of these two plants for improvement of sexual desire and associated symptoms in male human subjects. After obtaining satisfactory results in the spermatogenetic, antioxidant, rejuvenator and toxicity studies in the animal models, this research formulation consisting of root powder of *Sida cordifolia* Linn. and *Glycyrrhiza glabra* Linn. was taken up for this pre-clinical study before evaluating its therapeutic efficacy in clinical trial on male human subjects [6, 8, 15, 17].

The aim of the present study was to evaluate the chemical compounds found in the aqueous extract and the alcoholic extract of the research formulation by analysis of its macroscopic, microscopic and physical properties for ensuring quality control and standardization of the drug. In this study, the Ultraviolet and visible spectroscopy (UV-Vis.), Fourier Transform infra-red (FTIR) spectroscopy, High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid chromatography (HPLC) analysis techniques were used following the guidelines of Ayurvedic pharmacopeia and ICMR Guidelines for quality control, pharmacognostical and phytochemical studies. Comparison between the obtained results of the aqueous and alcoholic extracts has been done to highlight specific chemical constituents that may be responsible for its observed therapeutic properties.

2. Materials & Methods

The roots of *Sida cordifolia* Linn. and *Glycyrrhiza glabra* Linn. were purchased from a reputed drug supplier of Burdwan district, West Bengal and plant samples were authenticated by the Botanical Survey of India, Howrah, India. Authenticated specimens bearing numbers (REF./NO. BSI/CNH/SF/Tech./2016) and IPGAE&R/Dravyaguna/M.Gupta/07 & 08 were deposited in the herbarium museum of the department of Dravyaguna at I.P.G.A.E. &R., Kolkata for future reference. Chemical reagents such as Toluene, Formic acid, Acetonitrile, Gallic acid, Phosphoric acid, Acetic acid, Vanillin, Resorcinol and HPLC grade water were procured from Merck Specialities Pvt. Ltd and Chloroform, Ethyl Acetate, Ascorbic acid, Acetyl Salicylic acid, Catechol, Ellagic acid and Benzoic acid were purchased from Nice Chemicals Pvt. Ltd. The pharmacognostical and chemical analysis of the research formulation has been done following the protocols of drug standardization mentioned in the Ayurvedic Pharmacopoeia of India (2001) [18].

2.1 Pharmacognostical analysis

2.1.1 Macroscopic and microscopic study of powder

The roots of the plants were thoroughly washed, air-dried and pre-heated in oven before being powdered in a grinding machine to 120 # mesh particle size. The research formulation was prepared by mixing root powder of the plants and sieving it before storage in an airtight container. This fine powder was mounted in glycerine and stained with different reagents before undertaking observation under microscope to find out the characteristics of the various cell structures.

2.1.2 Physio-chemical analysis

2.1.2.1 Determination of pH value, ash value and moisture content

The pH measurement was done using the pH meter after proper calibration and standardization of the instruments and all observations were repeated three times. To determine ash values, three gm of accurately weighed powdered sample was incinerated in a Gooch crucible at a temperature of 450°C in the muffle furnace until free from carbon, cooled and weighed to ascertain the percentage of ash calculated with reference to the air dried drug. The values of total ash, acid insoluble ash and water soluble ash were calculated following the standard methods. Similarly, about 5 gm accurately weighed powdered drug was taken on a dish and its moisture content was determined using IR moisture content apparatus at 105°C.

2.1.2.2 Fluorescence analysis

Fluorescence analysis is a one of the essential parameters for assessing the quality and standardization of plant samples during pharmacognostical studies where the plant parts are examined as powder, in solution or as extracts. Although in most cases the actual substances responsible for the fluorescence properties may not been identified, the merits of simplicity and rapidity of the process make it a valuable analytical tool in the identification of plant samples and crude drugs [19]. A small quantity of dried finely powdered sample was placed on a grease free microscopic slide and 1-2 drops of freshly prepared solution are added, mixed by gently tilting the slide and waiting for 1-2 minutes. Then the slide was placed inside the UV viewer chamber and viewed in day light, short (254 nm) and long (365 nm) ultraviolet radiations. The colours observed by application of different reagents in various radiations were recorded.

2.1.2.3 Elemental analysis

Elemental analysis was performed to detect the presence of nitrogen, sulphur and halogens using routine chemical analysis techniques. A piece of metallic sodium was taken in a test tube and melted by slow heating. Then about 0.5 gm of research drug powder was added which was strongly heated for about 2 min. Twenty ml of distilled water was taken in a mortar and pastel, the red-hot test tube was broken and ground in mortar distilled water. The aqueous solution was filtered through Watman-40 filter paper and the filtrate was subjected to test for these elements.

2.2 Chemical analysis

2.2.1 Continuous extraction of research formulation

The roots of the plants were washed, air-dried and pre-heated in oven before being powdered in a grinding machine to 40# mesh particle size. Powdered dried roots ground into coarse powder were sequentially extracted with petroleum ether (60°C – 80°C), chloroform, acetone, ethanol and water using Soxhlet apparatus. These extracts were filtered using a Buckner funnel and Whatman No. 1 filter paper at room temperature and concentrated at reduced temperature & pressure using rotary evaporator. All obtained extracts were stored in refrigerator below 10°C for subsequent experiments [20, 21]. During this study, the aqueous and alcoholic (ethanol) extracts were standardized by using different types of instruments to assess the presence of chemical compounds

which could be responsible for their antimicrobial and anti-inflammatory pharmacological activities.

2.2.2 Preliminary phytochemical screening

The research extracts were subjected to preliminary phytochemical testing to detect the presence of different group of compounds such as saponins, tannins, alkaloids, flavonoids, glycosides, carbohydrates, oils and fats, proteins and amino acids following the standard methods [21, 22].

2.2.3 Determination of total phenol content and total flavonoid content

Total phenol content (TPC) was determined using the Folin-Ciocalteu reagent. To 0.5 ml aliquot of dried aqueous extract, 2.5 ml of 10 % Folin- Ciocalteu's reagent and 2 ml of 7.5% sodium carbonate were added. The absorbance was read after 30 min incubation period at room temperature at 760 nm colorimetrically. A standard calibration plot was generated at 760 nm using known different concentrations of Gallic acid (100, 200, 300, 400, and 500 µg/ml). The concentration of phenol in the test samples was calculated from the calibration plot and expressed as mg Gallic Acid Equivalents (GAE) per gm sample extract.

The Aluminium chloride [$AlCl_3$] method was used to determine the total flavonoid content (TFC). An aliquot of 0.5 ml of sample (1 mg/ml) was mixed with 1.5 ml of methanol, 0.1 ml of 1% aluminium chloride and 0.1 ml of potassium acetate solution (1 M). In the mixture, 2.8 ml of distilled water was added to bring up the total volume to 5 ml. The test solution was shaken vigorously and absorbance at 415 nm was recorded after 30 minutes of incubation. A standard calibration plot was generated at 415 nm using different concentrations of Quercetin. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg Quercetin equivalent/gm of sample [23, 24].

2.2.4 Chromatography

Chromatography refers to a class of analytical methods for separation of the components of a molecular mixture by distributing the components between two phases - a mobile phase passing over the stationary phase. The mobile phase separates the components in a mixture by adsorption and partitioning interactions with the stationary phase. In general practice, the separation is executed in chromatographic bed, in the form of a column (Column Chromatography) or on a thin layer (Thin Layer Chromatography) [25].

2.2.4.1 High performance thin layer chromatography (HPTLC)

HPTLC is an enhanced form of thin-layer chromatography (TLC). The position of any solute spot in HPTLC is characterized by its retention/retardation factor R_f. It is a fundamental qualitative value and is expressed as distance travelled by the spot / distance travelled by the solvent.

Four different methods having varying mobile phases were tried for chromatographic separation of the research drugs as detailed below:

Method-I (Toluene: Ethyl Acetate: Formic acid: Methanol = 6: 6: 1.6: 0.4)

Method-II (Chloroform: Ethyl acetate: Formic acid = 2.5: 2.0: 0.8)

Method-III (Toluene: Ethyl acetate: Formic acid: Methanol = 2: 2: 1: 2)

Method –IV (Toluene: Chloroform: Methanol: Formic acid = 7.0: 5.0: 1.5: 0.5)

Since the best separation of chemical compounds was observed in case of Method –IV as compared to the other three methods, final analysis was performed using this method having parameters as given below:

Plate: Pre-coated silica gel $60F_{254}$ plate (10cm X 10cm)

Mobile phase: Toluene: Chloroform: Methanol: Formic acid = (7.0: 5.0: 1.5: 0.5)

Wavelength: 280 nm & 360 nm

Applicator: CAMAG Linomat 5 automated TLC applicator

Scanner: CAMAG TLC scanner 3 equipped with WINCATS software

Sample concentration: 50 mg/ml

Standard concentration: 0.6 mg/ml

2.2.4.2 High Performance Liquid Chromatography (HPLC)

HPLC is a technique in analytical chemistry which is used to separate the components in a mixture, to identify each component, and to quantify each component. It relies on pumps to pass a pressurized 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. In this study, the detection and quantization was carried out using 515 HPLC pumps and 2489 UV/Visible Detectors of Waters Company while the software used was Empower.

Two methods using different mobile phases were tried for chromatographic separation of the research drugs –

Method I (binary gradient method of Acetonitrile & 0.1% Phosphoric acid in Water)

Method II (binary gradient method of Methanol & 1:25 Acetic acid in Water).

Results obtained during Method I have been discussed since better separation of compounds was observed during this analysis. The chromatographic conditions for Method I are as given below:

Column: Symmetry C18, 5µm, 4.6x250mm

Run Time: 30 minutes

Injection Volume : 20 µl

Wave length (Dual): 276 nm

Solvent A: Acetonitrile

Solvent B: 0.1% Phosphoric acid in water

Flow rate: 1.0 ml/min.

Pump Mode: Gradient

Processing Method:

Time (min.)	% A	% B
0	15	85
12	25	75
20	25	75
22	15	85
30	15	85

2.2.5 Spectroscopy

2.2.5.1 UV- Visible Spectroscopic Study

Ultraviolet and visible spectroscopy deals with recording of absorption of radiations in the ultraviolet and visible regions of the electromagnetic spectrum. The characteristics of molecules to absorb radiations under specific wavelengths were scanned in the entire range of 190 - 900 nm to find out

the elution of the compounds in different wavelengths on the basis of different peaks observed during data analysis using Shimadzu make UV-2450 model UV-Vis Spectrophotometer [25].

2.2.5.2 Fourier Transform Infrared (FTIR) spectroscopy

The FTIR spectroscopy is used for determination of presence of different functional groups. Infrared spectroscopic analysis is commonly carried out of solid samples by preparing a transparent KBr disc using 7-10 Tons of pressure. The characteristics of molecules to pass the infrared radiation under specific wave numbers were scanned in the entire range of 400 nm to 4000 nm to find out the functional groups in different wave numbers on the basis of observed peak values**.** Infrared spectroscopy is based on the fact that molecules absorb specific frequencies that are characteristic of their structure called resonant frequencies, i.e., the frequency of the absorbed radiation matches the transition energy of the bond or group that vibrates. During this study, detection & quantization was carried out using Perkin-Elmer Precisely Spectrum 100 FT-IR Spectrometer, with HATR sampling accessory ZnSe through plate 45, serial no. 80944, Hydrolic pellet press Type KP, serial no. 814, manufactured by Kimaya engineers, Thane, Maharashtra. Five mg of the lyophilized dried extract research powder was mixed with Potassium bromide (KBr) to make the mass up to 100 mg and a transparent KBr disc was prepared by giving 7-10 Ton pressure using hydraulic pellet press. The pellet of each solid sample was loaded in the FTIR spectroscope for analysis while the liquid samples were analyzed by HATR sampling accessory through ZnSe plate 45 [26].

2.2.6 GC- MS analysis

The GC-MS instrument consists of two main components. The gas chromatography portion separates different compounds in the sample into pulses of pure chemicals based on their volatility (Oregon State University 2012) by flowing an inert gas (mobile phase), which carries the sample, through a stationary phase fixed in the column (Skoog *et al*. 2007). Spectra of compounds are collected as they exit a chromatographic column by the mass spectrometer, which identifies and quantifies the chemicals according their mass-to-charge ratio (m/z). These spectra can then be stored on the computer and analysed [21].

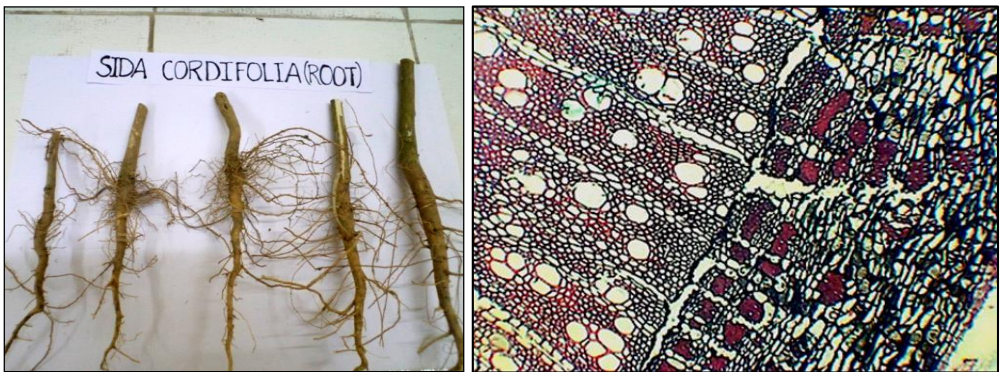
GC-MS analysis was carried out on a GC-MS Shimadzu QP2010 instrument with GCMS solution version 2.53 software. The alcoholic extract of the research formulation was analyzed with DB-5M column (30×0.25 mm). Initially oven temperature was maintained at 50º C for 2.0 min and then gradually increased up to 280º C. One µL of research sample was injected for analysis. Helium gas having 99.999% purity was used as a carrier gas as well as eluent using the flow rate of 1.0 mL/min. The sample injector temperature was maintained at 250ºC and the split ratio was 20 throughout the experiment. The ionization mass spectroscopic analysis was done with 70 eV. The mass spectra were recorded across the range 40-900 m/z for the duration of 45 minutes [25].

3. Results

3.1 Pharmacognostical analysis

3.1.1 Macroscopic and microscopic study of powder

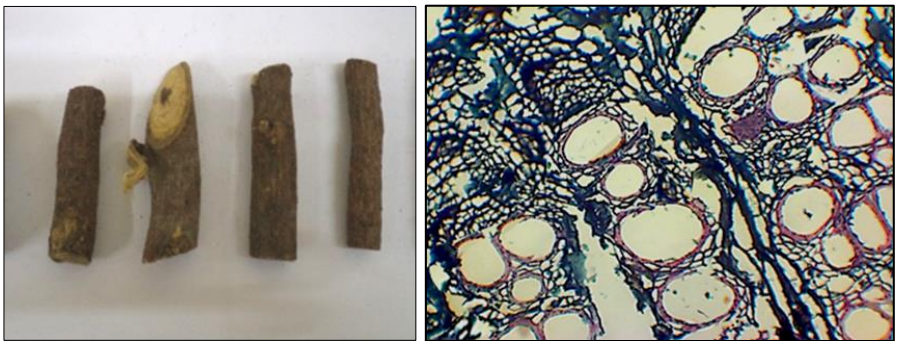
The roots of *Sida Cordifolia* Linn. are whitish yellow in colour having smooth surface, hard, about 12-16 inches long and 1 inch in diameter as shown in figure 1. The transverse section of its root also shown in figure 1 reveals a single layered epidermis while the Cortex is made up of a group of parenchymatous cells where few cells are sparsely distributed with starch grains. The Endodermis is distinct, single layered made up of polygonal thick walled cells. Secondary phloem occurs next to the cortex. This region consists of 6-8 tangential bands of thick walled phloem fibre groups alternating with thin walled phloem elements. The Vascular cambium is not very distinct. Secondary xylem contains vessels, xylem parenchyma, xylem fibres and medullary rays. Vessels many, occur in scattered groups of 2 to 4. Xylem parenchyma cells are thick walled, surround the vessels but do not form concentric rings. Xylem fibres are thick walled and more in number than xylem parenchyma. The Metaxylem are towards periphery and protoxylem are towards centre.



Roots of *Sida cordifolia* Linn. T.S. of *Sida cordifolia* Linn. Root

Fig 1: *Sida cordifolia* Linn. Roots and its Transverse Section

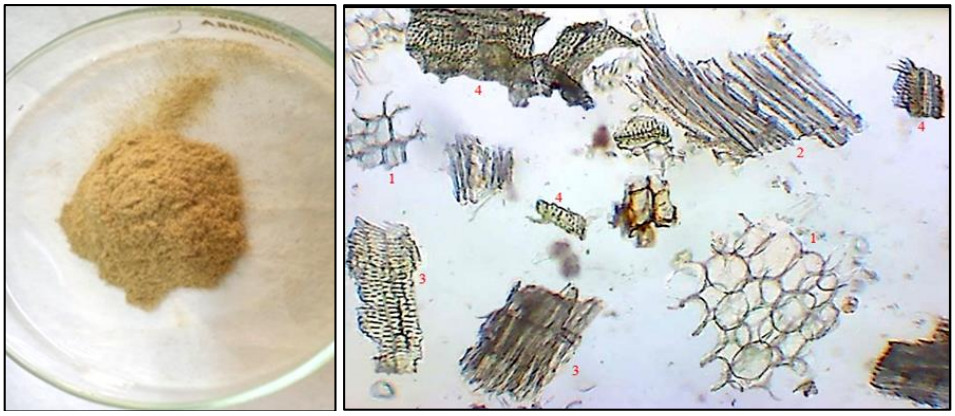
The roots of *Glycyrrhiza glabra* Linn. are brown in colour outside and yellow inside, having rough surface and longitudinal small scales, about 10-12 inches long and 1 inch in diameter with sweet smell as shown in figure 2. The transverse section of its root shown in figure 2 reveals a layer of parenchymatous epidermis while the Cortex consists of multi layered tabular cells, outer layer with reddish-brown contents, inner few layers show presence of cells having thicker, colourless walls; and secondary cortex usually of 1- 3 layers of radially arranged parenchymatous cells. The Stele or Secondary phloem is a broad band containing cells, outer lignified, radially arranged and surrounded by parenchymatous cells. The Secondary xylem distinctly radiates with medullary rays, and the Pith contains parenchymatous cells in longitudinal rows. The Stem is Dicotyledonous with secondary growth.



Roots of *Glycyrrhiza glabra* Linn. T. S. of *Glycyrrhiza glabra* Linn. Roots

Fig 2: *Glycyrrhiza glabra* Linn. Roots and its Transverse Section

The research drug powder looks light brown in colour and appears smooth in texture (figure 3). Its microscopic analysis shown in figure 3 shows the various components which include the single layer Parenchymal cells in group, Trichome overlapping with vessel, Reticulate Vessel, Vessel with parenchyma patches and Pitted vessel.



Research drug powder Microscopic analysis of research drug

Fig 3: Research drug powder and its microscopic analysis

3.1.2 Physio-chemical analysis

3.1.2.1 Determination of pH value, ash value and moisture content

The physio-chemical analysis of extract of Bala roots revealed a Total Ash value of 9.72% (w/w), containing Acid Insoluble Ash of 0.13% and Water soluble ash of 7.87%. While the moisture content was 7.4%, the pH value was found to be 5.72.

3.1.2.2 Fluorescence analysis

The results of fluorescence analysis of *Glycyrrhiza glabra* Linn. Root powder using various reagents at 254 nm and 365 nm are detailed in Table 1.

Table 1: Results of fluorescence analysis (observed colours)

Reagent	Day Light	UV 254	UV 365
1M Sodium hydroxide	Light Brown	Dark Green	Black
1% Picric acid	Light Green	Green	Dark Green
Acetic acid	Brown	Green	Black
1M Hydrochloric acid	Colourless	Light Green	Dark Green
Dil. Nitric acid	Light Brown	Light Green	Dark Green
5% Iodine	Dark Green	Dark Green	Black
5% Ferric chloride	Light Brown	Light Green	Dark Brown
Methanol	Light Brown	Light Green	Black
50% Nitric acid	Light Brown	Light Green	Dark Brown
1 M Sulphuric acid	Colourless	Colourless	Colourless
Dil. Ammonia	Brown	Light Green	Dark Brown
10% Potassium dichromate	Orange	Dark Green	Dark Brown
Sodium hydroxide in methanol	Light Green	Dark Green	Dark Brown

3.1.2.3 Elemental analysis

Chemical elements namely nitrogen, sulphur, phosphorus & halogen were tested using sodium fusion technique to detect their presence in the research drug whose findings are shown in Table 2.

Table 2: Results of elemental analysis

Sl. No.	Test	Observation	Inference
1.	Prussian-blue Test	Prussian blue or green precipitate or colour	N – Present
2.	Lead Acetate Test	No Black ppt.	S – not present
3.	Nitroprusside Test	No Violet or Purple colour	S – not present
4.	Silver nitrate Test	No ppt.	Cl, Br or I – not present
5.	Ammonium Molybdate Test	No Canary Yellow ppt	P – not present

3.2 Chemical analysis

3.2.1 Continuous extraction of research formulation

The Extractive value of research drug formulation (in % w/w) was evaluated as 0.445, 2.62, 0.124, 1.367, 1.546 and 7.912 in case of Petroleum ether, Ethyl acetate, Chloroform, Acetone, Alcohol and Aqueous extracts respectively.

3.2.2 Preliminary phytochemical screening

The results of the preliminary testing to assess the presence of various phytochemical constituents is given in table 3.

Table 3: Phytochemical Constituents in research drug formulation extracts

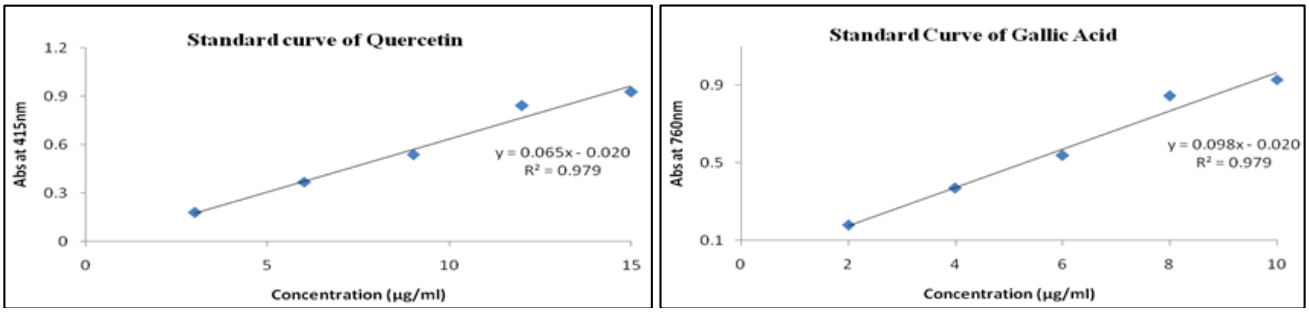
Plant Constituents Test/ Reagents used	Petroleum ether extract	Ethyl Acetate extract	Chloroform extract	Acetone extract	Alcohol extract	Aqueous extract
Alkaloids						
Mayer's reagent	–	–	–	–	–	+
Dragendroff's reagent	–	–	–	–	–	+
Flavonoids						
Shinoda test	–	+	–	+	+	–
Lead acetate test	–	–	–	–	–	+
Sodium hydroxide test	–	–	–	+	+	+
Tannins						
Ferric chloride test	–	–	–	–	+	+
Saponins						
Foam test	–	–	–	–	–	+
Carbohydrate						
Molisch's test	+	–	–	–	+	–
Fehling's test	–	–	–	+	+	+
Barfoed's test	–	–	–	–	–	+
Glycosides						
Borntrager's test	–	–	+	–	–	–
Libermann- Burchard test	+	–	+	–	+	+
Proteins & Amino acids						
Ninhydrin reagent	–	–	–	–	–	–
Fixed oils and fats	+	–	–	–	–	–
Saponification test	–	–	–	–	–	–
Spot Test	+	–	–	–	–	–

[+ → Present, – → Absent]

In Petroleum ether extract, carbohydrates, glycosides, fixed oil and fats were found to be present while in Chloroform extract, only glycosides were present. Acetone extract showed flavonoids and carbohydrates whereas the Alcoholic extract indicated presence of flavonoids, tannins, carbohydrates and glycosides. The aqueous extract showed presence of alkaloids, flavonoids, tannins, saponins, carbohydrates and glycosides.

3.2.3 Determination of total phenol content and total flavonoid content

The concentration of flavonoids in the test samples were calculated from the calibration plot using standard curve given in figure 4 and expressed as mg Quercetin equivalent of flavonoid/gm sample. Similarly, the concentration of phenols in the test samples were calculated from the calibration plot using the standard curve of Gallic Acid given in the same figure and expressed as mg Gallic acid equivalent of phenol content/gm of sample.



Standard Curve of Quercetin Standard curve of Gallic Acid

Fig 4: Standard curves of Quercetin & Gallic Acid

The Total Flavonoid Content (TFC) expressed as µg Quercetin equivalent/mg of extract was found to be 80.31 and 26.31 in case of the Alcoholic extract and Aqueous extract respectively. The Total Phenol Content (TPC) was found to be 26.50 and 28.60 µg Gallic acid equivalent/mg of extract in case of the Alcoholic extract and Aqueous extract respectively. The Alcoholic extract shows more Quercetin equivalent content than its aqueous extract, while the aqueous extract shows more Gallic acid equivalent Phenol content than its alcoholic extract.

3.2.4 Chromatography

3.2.4.1 High performance thin layer chromatography (HPTLC)

The photos of HPTLC plates visualized at 254 nm and 366 nm for both alcoholic and aqueous extract are shown in figure 5.

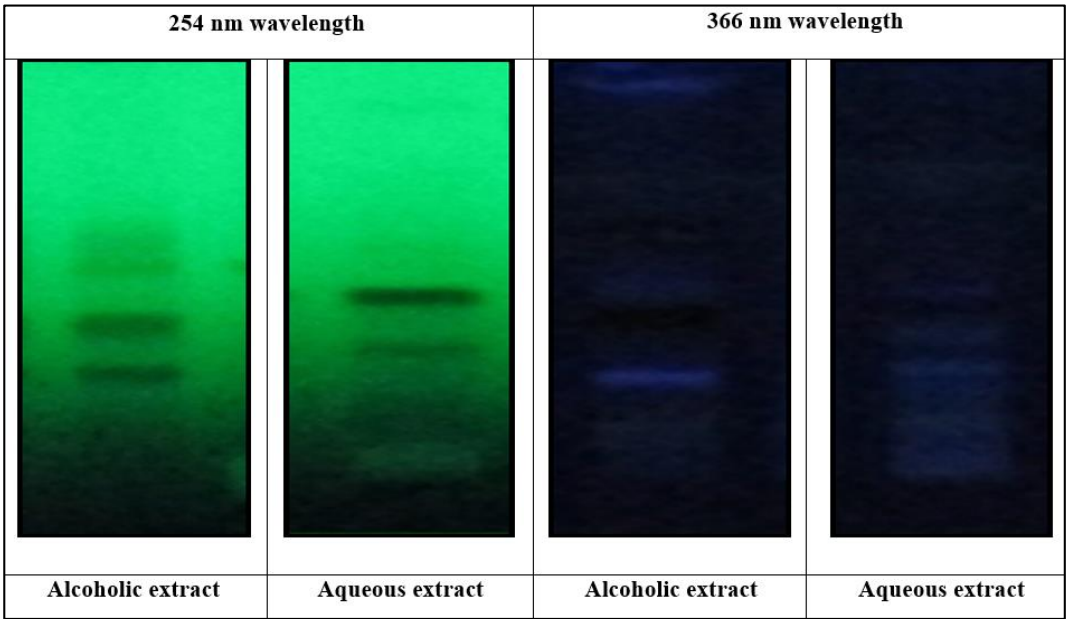
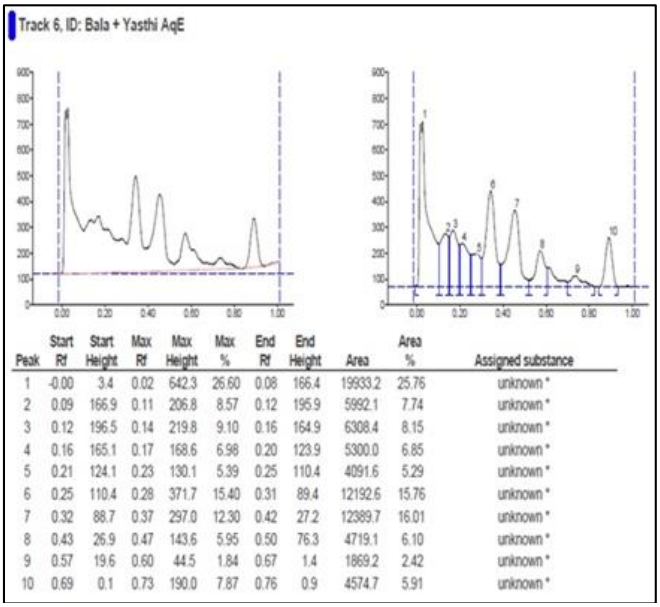


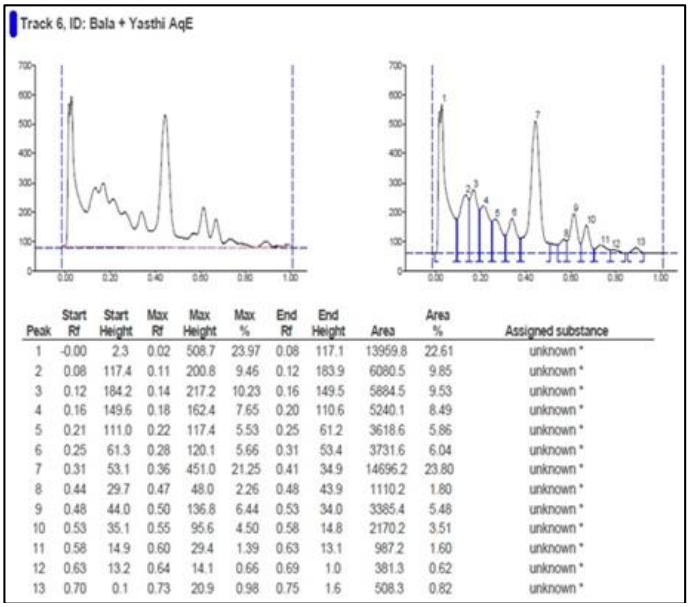
Fig 5: HPTLC plates visualised at 254 and 366 nm wavelengths

During the HPTLC study, both the research extracts were scanned at 280 nm and 360 nm and the obtained chromatographs are shown in Figures 6 and 7 and Rf values obtained from these chromatographs are shown in table 4.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.00	3.4	0.02	642.3	26.60	0.08	166.4	19933.2	25.76	unknown *
2	0.09	166.9	0.11	206.8	8.57	0.12	195.9	5992.1	7.74	unknown *
3	0.12	196.5	0.14	219.8	9.10	0.16	164.9	6308.4	8.15	unknown *
4	0.16	165.1	0.17	168.6	6.98	0.20	123.9	5300.0	6.85	unknown *
5	0.21	124.1	0.23	130.1	5.39	0.25	110.4	4091.6	5.29	unknown *
6	0.25	110.4	0.28	371.7	15.40	0.31	89.4	12192.6	15.76	unknown *
7	0.32	88.7	0.37	297.0	12.30	0.42	27.2	12389.7	16.01	unknown *
8	0.43	26.9	0.47	143.6	5.95	0.50	76.3	4719.1	6.10	unknown *
9	0.57	19.6	0.60	44.5	1.84	0.67	1.4	1869.2	2.42	unknown *
10	0.69	0.1	0.73	190.0	7.87	0.76	0.9	4574.7	5.91	unknown *

280 nm wavelength



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.00	2.3	0.02	508.7	23.97	0.08	117.1	13959.8	22.61	unknown *
2	0.08	117.4	0.11	200.8	9.46	0.12	183.9	6080.5	9.85	unknown *
3	0.12	184.2	0.14	217.2	10.23	0.16	149.5	5884.5	9.53	unknown *
4	0.16	149.6	0.18	162.4	7.65	0.20	110.6	5240.1	8.49	unknown *
5	0.21	111.0	0.22	117.4	5.53	0.25	61.2	3618.6	5.86	unknown *
6	0.25	61.3	0.28	120.1	5.66	0.31	53.4	3731.6	6.04	unknown *
7	0.31	53.1	0.36	451.0	21.25	0.41	34.9	14696.2	23.80	unknown *
8	0.44	29.7	0.47	48.0	2.26	0.48	43.9	1110.2	1.80	unknown *
9	0.48	44.0	0.50	136.8	6.44	0.53	34.0	3385.4	5.48	unknown *
10	0.53	35.1	0.55	95.6	4.50	0.58	14.8	2170.2	3.51	unknown *
11	0.58	14.9	0.60	29.4	1.39	0.63	13.1	987.2	1.60	unknown *
12	0.63	13.2	0.64	14.1	0.66	0.69	1.0	381.3	0.62	unknown *
13	0.70	0.1	0.73	20.9	0.98	0.75	1.6	508.3	0.82	unknown *

360 nm wavelength

Fig 6: Chromatographs of aqueous extract during HPTLC study

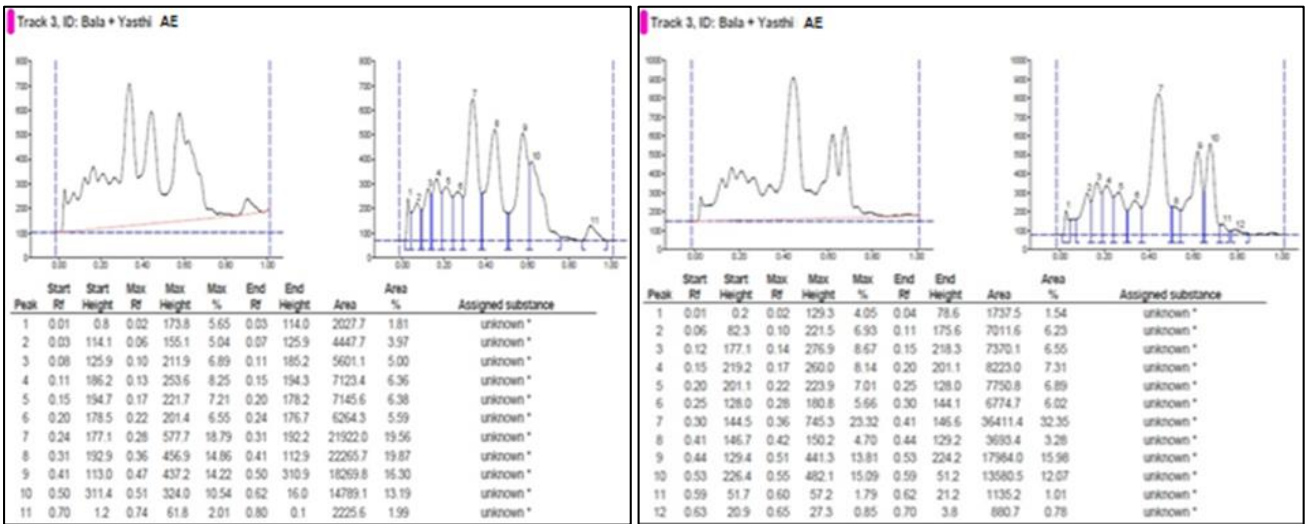


Fig 7: Chromatographs of alcoholic extract during HPTLC study

Table 4: Comparative analysis of *Rf* values of Aqueous & Alcoholic extract

Scanned at 280 nm				Scanned at 360 nm			
Sl. No.	**Standard**	***Rf* value of Aqueous Extract**	***Rf* value of Alcoholic Extract**	**Sl. No.**	**Standard**	***Rf* value of Aqueous Extract**	***Rf* value of Alcoholic Extract**
1		0.02	0.02	1		0.02	0.02
2			0.06	2			
3		0.11	0.10	3		0.11	0.10
4		0.14	0.13	4		0.14	0.14
5		0.17	0.17	5		0.18	0.17
6		0.23	0.22	6		0.22	0.22
7		0.28	0.28	7		0.28	0.28
8		0.37	0.36	8		0.36	0.36
9		0.47	0.47	9		0.47	0.42
10			0.51	10		0.50	0.51
11				11		0.55	0.56
12	Ellagic Acid	0.60		12	Ellagic Acid	0.60	0.60
13				13		0.64	0.65
14	Gallic Acid	0.73	0.74	14	Gallic Acid	0.73	

The *Rf* values scanned at 280 and 360 nm wavelength showed that compounds at sl. no. 1, 3-9, 12 & 14 of aqueous and alcoholic extract were similar. This observation expressed nine common compounds in both the extracts, scanned at different wave lengths. The comparison of the obtained *Rf* values with known standard values indicated the presence of two standard compounds namely Ellagic acid and Gallic acid in both these extracts.

3.2.4.2 High Performance Liquid Chromatography (HPLC)

The observed chromatographs of both the Alcoholic and the Aqueous extracts are shown in figure 8 while the RT values obtained and their comparison with standard compounds is detailed in table 5.

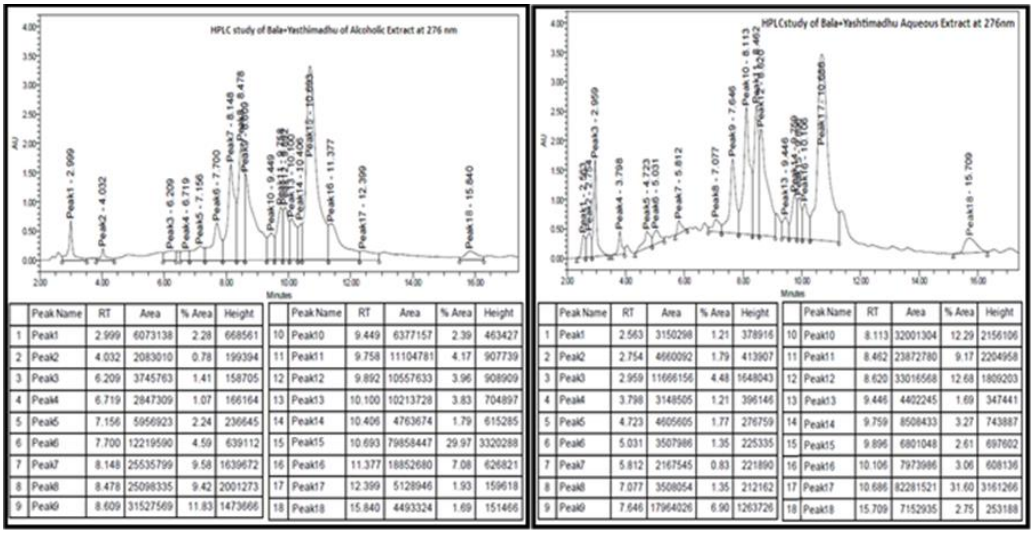


Fig 8: HPLC Chromatographs at 276 nm

Table 5: Comparative analysis of RT values of Aqueous & Alcoholic extracts at 276 nm

Sl. No.	Aqueous Extract.	Standard	Sl. No.	Alcoholic Extract	Standard
1	2.563		1	2.999	
2	2.754		2	4.032	
3	2.959		3	6.209	
4	3.798		4	6.719	
5	4.723		5	7.156	
6	5.031		6	7.700	
7	5.812		7	8.148	
8	7.077		8	8.478	
9	7.646		9	8.609	Vanillin
10	8.113		10	9.449	
11	8.462		11	9.758	
12	8.620	Vanillin	12	9.892	
13	9.446		13	10.100	
14	9.759		14	10.406	
15	9.896		15	10.693	
16	10.106		16	11.377	
17	10.686		17	12.399	
18	15.709		18	15.840	

The HPLC chromatogram of the aqueous and alcoholic extracts on the basis of the elution of the peaks at 276 nm wavelengths showed twelve common RT values. During analysis and comparison with standard database, one compound namely Vanillin having RT value of 8.62 was found common in both the extracts.

3.2.5 Spectroscopy

3.2.5.1 UV- Visible Spectroscopic Study

The results from the spectroscopic scanning of both the extracts are shown in figure 9 and the comparative analysis of the absorbance observed is detailed in table 6.

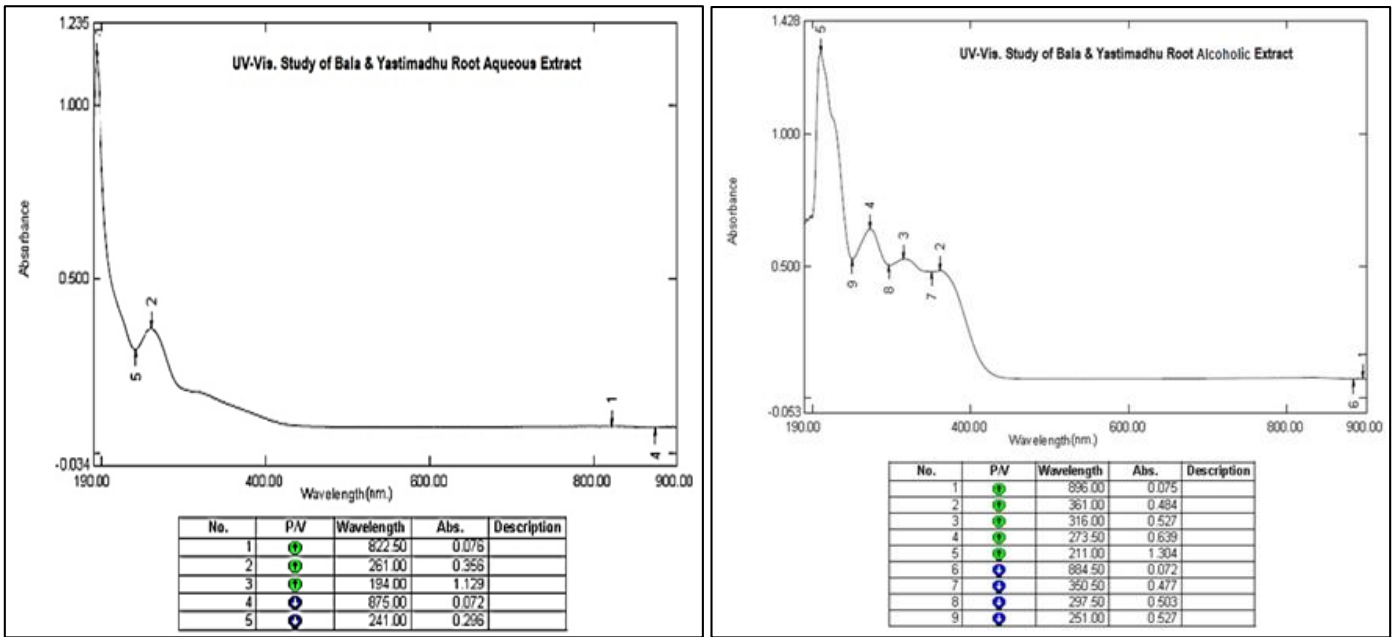


Fig 9: Results of the UV- Visible Spectroscopic Scanning Study

Table 6: Comparative analysis of UV-Visible spectrum data

Wavelength	Absorbance of Aqueous Extract	Wavelength	Absorbance of Alcoholic Extract
822.5	0.076	896.0	0.075
261.0	0.356	361.0	0.484
194.5	1.129	315.0	0.527
		273.5	0.639
		211.0	1.304

UV spectrum of the aqueous extract revealed the presence of three peaks at 822.5, 261 and 194.5 nm, corresponding to three different compounds whereas alcoholic extract showed five peaks at 896, 361, 315, 273.5 and 211 nm, corresponding to five different compounds. No common compound was found in both the extracts.

3.2.5.2 Fourier Transform Infrared (FTIR) spectroscopy

The spectrum observed during the FT-IR Spectroscopic Analysis is shown in Figure 10. The obtained data was analyzed and the possible functional groups present in both the extracts are shown in table 7.

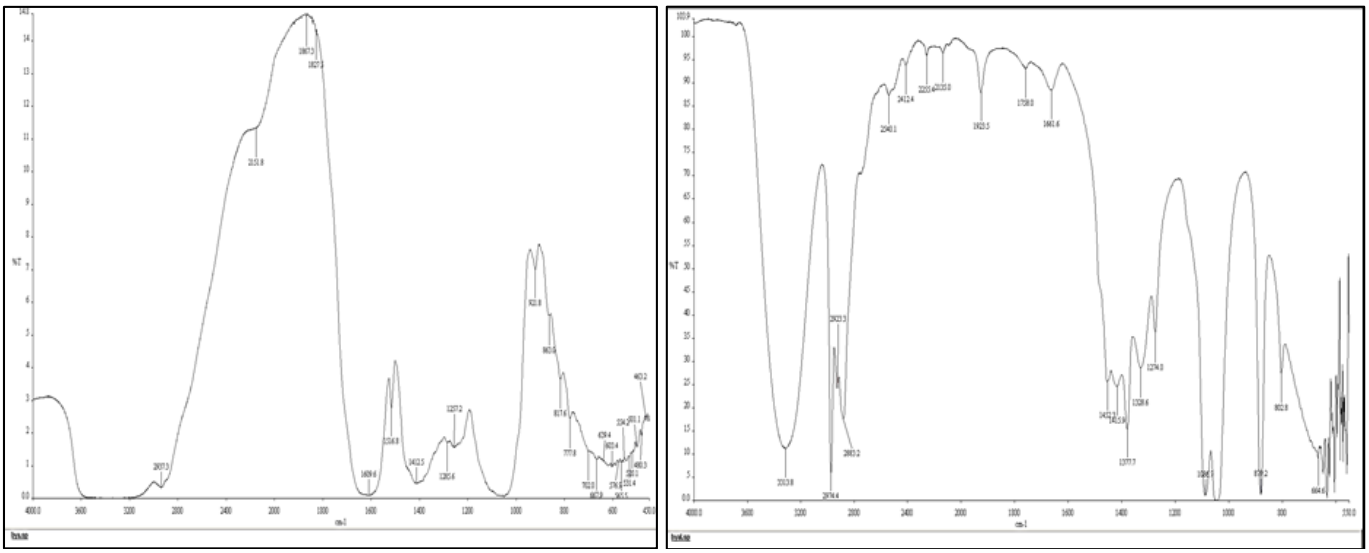


Fig 10: FT-IR spectrum of Aqueous & Alcoholic extract of research drug

Table 7: Comparative analysis of FT-IR data

Aqueous Extract		Alcoholic Extract	
Wavenumber (cm^{-1})	**Possible functional group**	**Wavenumber (cm^{-1})**	**Possible functional group**
2937.3	-CH_3 out-phase stretching	1661.6	C=N stretching
1609.6	-NH_2 bending	1452.3	–CH_2 bending
1412.5	(N)-CH_3 in phase bending	1415.9	(N)-CH_3 in-phase bending
531.4	C=O in-phase rocking	1377.7	Aryl-CH_3 in-phase bending
		1086.7	C-O-C out-phase stretching
		664.6	−O-H Wagging

Aqueous and alcoholic extracts exhibited different characteristic bands representing various functional groups like (N)-CH_3 in phase bending, -CH_3 out-phase stretching, -NH_2 bending, C=O in-phase rocking, Aryl-CH_3 in-phase bending, C=N stretching, etc. These results indicated presence of phenolic & alkaloidal group of compounds, which would be further validated by different chromatographic analysis.

3.2.6 GC-MS analysis

The GC-MS analysis of the research drug ethanol extract revealed the presence of eleven specific compounds (phytochemical constituents) that could contribute to the medicinal quality of extract as shown in figure 11. The identification of the phytochemical compounds was confirmed based on the peak area, retention time and molecular formula. The active principles with their Retention time (RT), Molecular formula and structure are presented in table 8. The first compound identified with lowest retention time (5.49 min) was Ethyl ortho-formate, whereas Phthalic acid, cyclohexylethyl 2-propyl ester was the last compound which showed the longest retention time (28.16 min) to identify.

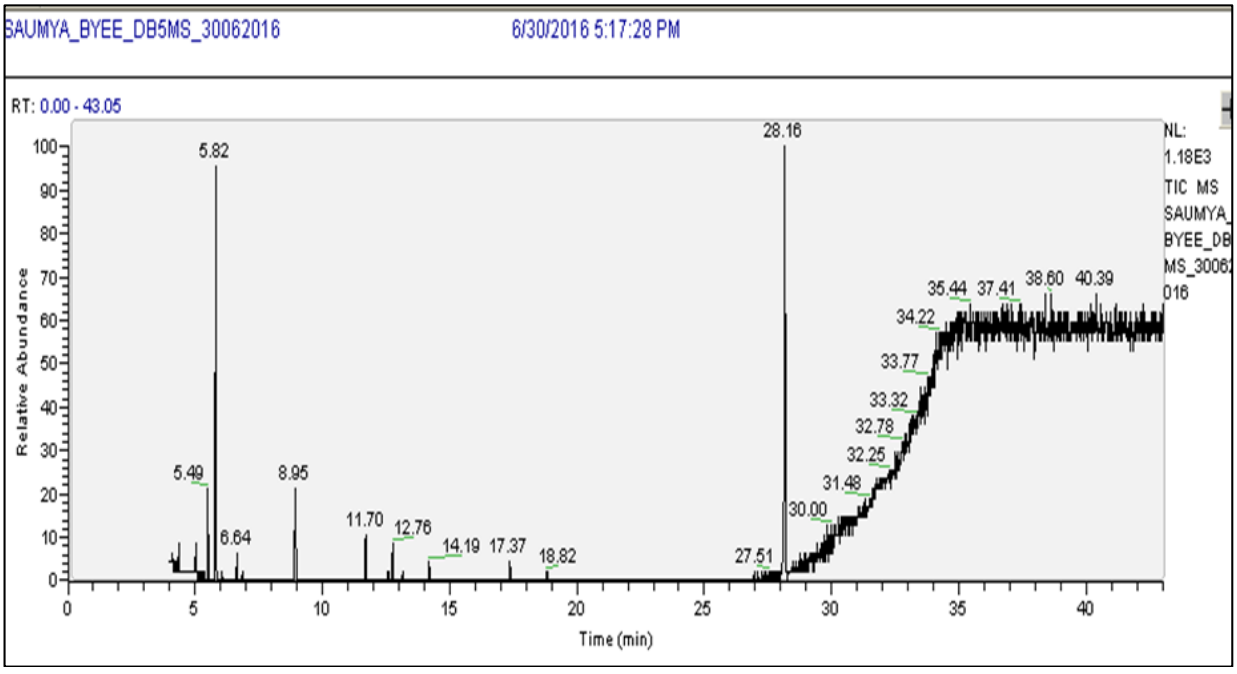
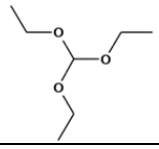
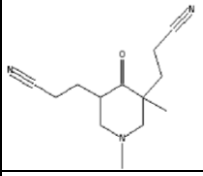
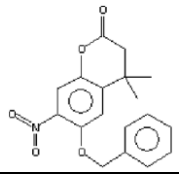
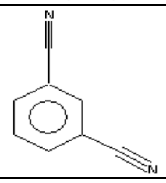
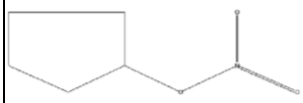
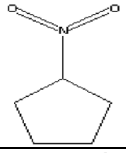
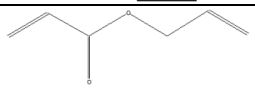
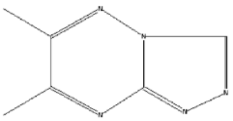
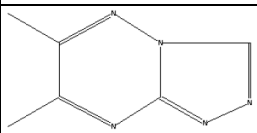
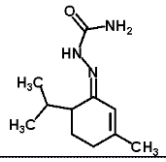
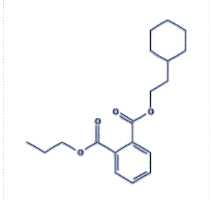


Fig 11: GC-MS Chromatogram of Alcoholic extract

Table 8: Compounds identified and their properties from GC-MS analysis

Sl. No.	RT (min)	Name of the Compound	Molecular Formula	MW	Structure	Therapeutic Uses
1.	5.49	Ethyl ortho-formate	$C_7H_{16}O_3$	148		Alkylating agent used to initiate many chemical synthesis
2.	5.82	1,3-Dimethyl-3,5-di (cyanoethyl) piperidone-4	$C_{13}H_{19}N_3O$	233		Piperidone exhibits strong antioxidant activity by electron donating mechanism
3.	6.64	Coumarin, 6-benzyloxy-3,4-dihydro-4,4-dimethyl-7-nitro	$C_{18}H_{17}NO_5$	327		It is used to treat cancer, minimize side effects caused by radiotherapy, highly effective against inflammatory response, used in high protein edema and also used as principal oral anticoagulant
4.	8.95	1,3-Benzene-di-carbonitrile	$C_8H_4N_2$	128		Have potential therapeutic benefit through anabolic activity in the muscle
5.	11.70	Cyclopentanol, nitrate	$C_5H_9NO_3$	131		It is effective in treating male impotence and erectile dysfunction through topical or intracavernosal administration to the penis
6.	12.76	Cyclopentane, nitro	$C_5H_9NO_2$	115		It acts as smooth muscle relaxant throughout the body, including the sphincter muscles of the anus and the vagina, producing a sensation of heat and excitement
7.	14.19	Acrylic acid, allyl ester	$C_6H_8O_2$	112		Acrylic acid derivatives exhibit antifungal activity
8.	17.37	6,7-Dimethyl-triazolo (4,3-b) (1,2,4)-triazine	$C_6H_7N_5$	149		1,2,4-Triazine derivatives have been found to exhibit antifungal, anti-HIV, anticancer, analgesic, antihypertensive, cardio-tonic, neuroleptic, estrogen receptor modulators and anti-parasitic properties
9.	18.82	6,7-Dimethyl-triazolo (4,3-b) (1,2,4)-triazine	$C_6H_7N_5$	149		1,2,4-Triazine derivatives have been found to exhibit antifungal, anti-HIV, anticancer, analgesic, antihypertensive, cardio-tonic, neuroleptic, estrogen receptor modulators and anti-parasitic properties
10.	27.51	p-Menth-1-en-3-one, semicarbazon	$C_{11}H_{19}N_3O$	209		Antimicrobial activity
11.	28.16	Phthalic acid, 2-cyclohexylethyl propyl ester	$C_{19}H_{26}O_4$			

4. Discussions

There has been an increase in demand for phyto-pharmaceutical products relating to the Ayurvedic system of medicine all over the world because of the fact that many allopathic drugs have adverse side effects. Majority of the medicines are now prepared from plant and animal products, minerals and metals, etc., and major pharmaceutical industries depend on plant products for the preparation of Ayurvedic medicines [1]. Biologically active compounds and chemical principles from natural sources have become much simpler and have contributed significantly to the development of new drugs from medicinal plants [2].

In order to achieve the objective of ensuring that medicinal products are of the prescribed quality, quantitative determination of some pharmacognostical parameters is very useful for setting standards for crude drugs [21]. The detection of adulteration or errors in handling of the drug depends upon the evaluation of important parameters like physical constants. The purity of the drug i.e. the presence or absence of foreign inorganic matter can be indicated by the various ash values. Different plant species would obviously have different chemical profiles after their abstract prepared in different solvents is analysed using various standard chemical and phyto-chemical techniques.

The results obtained during the macroscopic, microscopic and physiochemical analysis such as ash value, moisture content, colour, pH value and characteristic fluorescent properties could be used as standard benchmarks in the identification and authentication of plant samples for assessing their purity, quality and the presence of adulterants as per the WHO 1998 guidelines & Ayurvedic pharmacopeia for drug development.

Many medicinal plants have been mentioned in the Ayurvedic text books for enhancement of *sukra dhatu*. Among these, the rejuvenating action of Yashtimadhu (*Glycyrhiza glabra* Linn.) and Bala (*Sida cordifolia* Linn.) extends to the treatment of peptic ulcer, hepatitis C, pulmonary and skin diseases, although clinical and experimental studies suggest that they have several other useful pharmacological properties such as anti-inflammatory, antiviral, antimicrobial, anti-oxidative, anticancer, immunomodulatory, hepato-protective and cardio-protective activities.

The research drug powder looks light brown in colour and appears smooth in texture. Its microscopic analysis shows single layer Parenchymal cells in group, Trichome overlapping with vessel, Reticulate Vessel, Vessel with parenchyma patches and Pitted vessel. The total Ash value was 9.72% w/w which primarily consisted of water soluble ash (7.87%). The moisture content in the research formulation was found to be 7.4 % w/w while the pH value of 5.72 indicated its acidic nature. The extractive value of alcoholic and aqueous research formulation was found as 1.546% and 7.912% w/w while preliminary phytochemical analysis revealed the presence of flavonoids, tannins, carbohydrates and glycosides in both extracts. The results also showed high concentrations of flavonoidic compounds (80.31 µg Quercetin equivalent / mg of extract) in the alcoholic extract and high concentrations of phenolic content (28.60 µg Gallic acid equivalent / mg of extract) in the aqueous extract which could be directly responsible for its antimicrobial, anti-inflammatory and astringent properties.

The *Rf* values (distance moved by the solvent front/ distance moved by the solute) of aqueous and alcoholic extracts have been obtained by using the HPTLC Chromatography analysis. The *Rf* values scanned at 280 nm and 360 nm wavelengths showed that 9 spots of aqueous and alcoholic extracts are quite similar. The comparison of the obtained *Rf* values with known standard values indicated the presence of phenolic compound Ellagic acid and Gallic acid in both these extracts.

High Performance Liquid chromatography (HPLC) has been used to find out the retention time (RT) which depends upon the separation of compounds in the C18 column under high pressure and different solvent systems in gradient pattern of Acetonitrile and 0.1% Phosphoric acid in water for 30 minutes. The HPLC chromatogram of the aqueous and alcoholic extracts on the basis of the elution of the peaks at 276 nm wavelength showed twelve common compounds in the aqueous and alcoholic extract. Further analysis with database of standard compounds confirmed the presence of Vanillin at RT 8.62 in both the extracts. The presence of phenolic compounds in the aqueous and alcoholic extracts of the research formulation may be responsible for its pharmacological activities because these phenolic compounds are already known for their antioxidant, tonic, analgesic, antipyretic, anti-inflammatory and antimicrobial properties.

The UV-Visible spectroscopy scanning during chemical analysis of aqueous extract of the research formulation showed three peaks at 822.5, 261 and 194.5 nm, corresponding to three different compounds whereas alcoholic extract showed five peaks at 896, 361, 315, 273.5 and 211 nm, corresponding to five different compounds.

The comparative data on the peak values with wave numbers and the possible functional groups during FTIR analysis has been detailed above. The aqueous extract of the research formulation exhibited different characteristic bands at 2937.3, 1609.6, 1412.5 and 531.4 cm^{-1} indicating the presence of the functional groups -CH_3 out-phase stretching, -NH_2 bending, (N)-CH_3 in phase bending, C=O in-phase rocking respectively. At the same time, the alcoholic extract revealed characteristic peaks at 1661.6, 1452.3, 1415.9, 1377.7, 1086.7 and 664.6 cm^{-1} indicating the presence of C=N stretching, –CH_2 bending, (N)-CH_3 in-phase bending, Aryl-CH_3 in-phase bending, C-O-C out-phase stretching and − O-H Wagging functional groups respectively. It may be inferred that the aqueous and alcoholic extracts of research formulation exhibited almost similar types of functional groups and indicated the presence of phenolic & alkaloidal group of compounds.

The GC-MS analysis of the research formulation revealed the presence of eleven specific phytochemical compounds based on their retention times as detailed above. These include Ethyl ortho-formate, 1,3-Dimethyl-3,5-di (cyanoethyl) piperidone-4, Coumarin, 6-benzyloxy-3,4-dihydro-4,4-dimethyl-7-nitro, 1,3-Benzene-di-carbonitrile, Cyclopentanol nitrate, Cyclopentane nitro, Acrylic acid allyl ester, 6,7-Dimethyl-triazolo (4,3-b) (1,2,4)-triazine, 6,7-Dimethyl-triazolo (4,3-b) (1,2,4)-triazine, p-Menth-1-en-3-one, semicarbazon and Phthalic acid, 2-cyclohexylethyl propyl ester. All these phytochemical constituents have known specific therapeutic and medicinal properties as given above.

5. Conclusion

Pharmacognostical analysis indicates high concentration of flavonoids, tannins, phenolic content, carbohydrates and glycosides in both extracts. Spectroscopic and chromatographic examination using UV-Visible, HPTLC and HPLC analysis indicated the presence of Ellagic acid, Gallic acid and Vanillin in both these extracts. FTIR analysis indicated the presence of phenolic & alkaloidal group of compounds in the extracts. GC-MS analysis further suggested

the presence of eleven specific phenolic and flavonidic compounds in the extracts of the research formulation which may be responsible for its pharmacological activities because these compounds are known for their antioxidant, analgesic, antipyretic, anti-inflammatory and antimicrobial properties.

6. Acknowledgements

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