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SPECTROSCOPY (UV-VISIBLE & FTIR) AND CHROMATOGRAPHY (HPTLC & HPLC) CHEMICAL ANALYSIS OF THE AQUEOUS AND ALCOHOLIC EXTRACTS OF *SIDA CORDIFOLIA* LINN: A PRECLINICAL STUDY FOR MALE SEXUAL DISORDERS

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ABSTRACT

An Ayurvedic formulation prepared from dried root powder of *Sida cordifolia* Linn. is standardized through pharmacognostical and phytochemical pre-clinical studies for treatment of male sexual disorders. While total ash value was 8.0%, acid insoluble ash was 0.5% and water soluble ash was 5.41%. Among elements, Nitrogen was found present. Flavonoids and carbohydrates were found present in both alcoholic and aqueous extracts. The results also showed high concentration of flavonoidic compounds (15.03 µg Quercetin equivalent / mg of extract) in the alcoholic extract and high phenolic content (23.26 µg Gallic acid equivalent / mg of extract) in the aqueous extract. HPTLC analysis at 280 and 360 nm indicated the presence of Ellagic acid and Gallic acid in both these extracts. Similarly, HPLC analysis at 276 nm showed elution of 4 compounds whose analysis confirmed the presence of Tannic acid, Benzoic acid and Quercetin in aqueous extract. UV-Visible spectroscopy scanning showed peaks at 896, 862.5 and 195.5 nm in aqueous and at 896, 862.5, 419.5 and 209.5 nm in alcoholic extract. FTIR analysis indicated presence of N-CH_3 out-phase bending, N-CH_3 in-phase stretching, Aryl -CH_3 in-phase stretching and C-O stretching functional groups in aqueous and -NH_2 Stretching, -CH_2 in-phase stretching, (N)-CH_3 in-phase stretching, -CH_3 in-phase bending, Aryl-N stretching and N-O stretching functional groups in alcoholic extract suggesting the presence of amides, aldehydes, alkaloids and phenolic groups of compounds in the extracts. The presence of phenolic and flavonidic compounds in research formulation extracts may be responsible for its antioxidant, analgesic, antipyretic, anti-inflammatory and antimicrobial properties.

KEYWORDS: Ayurveda, Spectroscopy, Chromatography, *Sida cordifolia* Linn., pre-clinical.

1. INTRODUCTION

Several therapeutic solutions have been explored for male sexual disorders but many of the available allopathic cures have been found associated with adverse side effects, prompting the quest for herbal cures. The traditional knowledge of Ayurvedic system of medicine with its holistic and systems approach supported by experimental base can serve as an innovative and powerful discovery engine for newer, safer and affordable medicines. Plant species mentioned in these ancient texts may be explored with modern scientific approaches for ensuring their authenticity and standardization through identification of key bioactive compounds and fingerprinting of phytochemical constituents.

Sida cordifolia Linn. belonging to the Malvaceae family is one of the most useful medicinal plants in Ayurvedic literature. Also known as *Bala*, it is a small, erect, annual downy shrub. The leaves of the plant are chordate-oblong or ovate-oblong and fruits with a pair of awns on each carpel. The tap root of the plant is odourless with slightly bitter taste and grayish yellow colour which constitute a cluster 5-15 cm long with few lateral roots of smaller size. It has been used as a cooling, astringent, aromatic, diuretic and tonic in Ayurvedic system of medicine 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.[1-5] 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.[6-7] After obtaining satisfactory results in the spermatogenic, antioxidant, rejuvenator and toxicity studies in the animal models, *Sida cordifolia* Linn. was taken up for this pre-clinical

study before evaluating its therapeutic efficacy in clinical trial on male human subjects.[8-9]

The preclinical study focuses on the quality control and standardization of the different extracts of research drug (*Sida cordifolia* Linn.) following the guidelines of Ayurvedic Pharmacopeia and ICMR Guidelines for quality control, pharmacognostical and phytochemical studies. Since it has been noticed that most Ayurvedic medicines are prepared in the aqueous/ alcoholic extract or decoction form, hence comparative study of the aqueous and the alcoholic extracts of research drug was undertaken to find out similar or specific chemical compounds in each extract which can validate their therapeutic efficacy. Apart from examination of its macroscopic, microscopic and physical properties, the study also evaluates the pharmacognostical attributes of the research drug formulation through Ultraviolet and visible spectroscopy (UV-Vis.) and Fourier transform infra-red (FTIR) spectroscopy, High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid chromatography (HPLC) analysis which can be valuable benchmarks for establishing the quality and ensuring benchmarking of parameters for identification and standardization of drugs.

2. MATERIALS AND METHODS

The roots of *Sida cordifolia* Linn. were purchased from crude drug supplier of Katwa Chowrasta, Burdwan district 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).[1]

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 (Dewinter, Italy) 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 gms 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 gms 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 such, in their powdered form, in solution or as extracts. Although in most cases the actual substances responsible for the fluorescence properties have 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. [10] 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, sulfur 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 and stored in an airtight container. 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. [11] 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 required for curing the excessive abnormal vaginal discharge.

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.[12-13]

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 Aluminum 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% aluminum 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 and known 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 (Baba and Malik 2015, Chang *et al.* 2002).[14-15]

2.2.4. Chromatography

Chromatography is the general name of 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). [16] Analysis of pharmaceutical and natural compounds and newer drugs is commonly used in all the stages of drug discovery and development process.

2.2.4.1. High performance thin layer chromatography (HPTLC)

HPTLC is an enhanced form of thin-layer chromatography (TLC). A number of enhancements can be made to the basic method of TLC to automate the different steps, to increase the resolution achieved and to allow more accurate quantitative measurements. 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 time : 30 minutes

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.[16]

2.2.5.2. Fourier Transform Infrared (FTIR) spectroscopy

The FTIR spectroscopy is used for determination of presence of different functional groups such as hydroxyl group, carboxyl group, etc. 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. These absorptions are 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, mfg. 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 (Sharma 2011). [17]

3. RESULTS

3.1. Pharmacognostical analysis

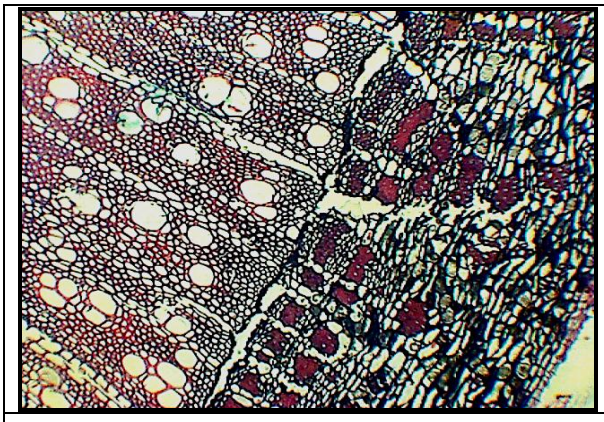
3.1.1. Macroscopic and microscopic study of powder

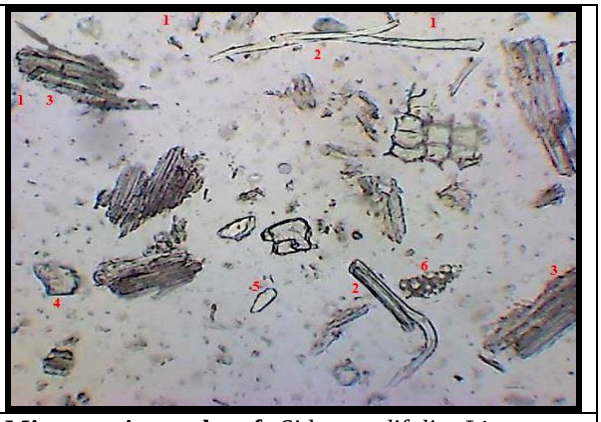
The roots of *Sida Cordifolia* Linn. are whitish yellow in colour having smooth surface, hard, about 12-16 inches long and 1 inch in diameter as shown in figure 1. The transverse section of its root shown in figure 2 reveals a single layered epidermis while the Cortex is made up of a group of parenchymatous 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.

The *Bala* powder looks light brown in colour (figure 1) and its microscopic analysis shown in figure 2 shows the various components which include the starch granules, Trichomes, Vessels with sceleriform orientation with parenchyma patches, Pitted Vessel, Crystal and Cork cells.

Roots of *Sida cordifolia* Linn.	**Powder of roots of *Sida cordifolia* Linn.**

Figure 1: *Sida cordifolia* Linn. roots and powder.





T.S of *Sida cordifolia* Linn. root	**Microscopic study of *Sida cordifolia* Linn. root powder: 1. Starch Granules; 2. Trichome, part of trichome; 3. Vessel with sceleriform orientation with parenchyma patches; 4. Pitted Vessel; 5. Crystal; 6. Cork cell.**

Figure 2: Microscopic analysis of *Sida cordifolia* Linn. root & its powder.

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 8.0% (w/w), containing Acid Insoluble Ash of 0.5% and Water soluble ash of 5.41%. While the moisture content was 4.9%, the pH value was found to be 5.8.

3.1.2.2. Fluorescence analysis

The results of fluorescence analysis of *Sida cordifolia* 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 Yellow	Colourless	Colourless
1% Picric acid	Light Green	Light Green	Colourless
Acetic acid	Colourless	Light Green	Black
1M Hydrochloric acid	Colourless	Colourless	Colourless
Dil. Nitric acid	Colourless	Light Green	Black
5% Iodine	Brown	Dark Green	Dark Brown
5% Ferric chloride	Brown	Green	Dark Brown
Methanol	Colourless	Colourless	Colourless
50% Nitric acid	Brown	Light Green	Dark Green
1 M Sulphuric acid	Colourless	Colourless	Colourless
Dil. Ammonia	Colourless	Colourless	Colourless
10% Potassium dichromate	Orange	Dark Green	Dark Brown
Sodium hydroxide in methanol	Colourless	Colourless	Colourless

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 *Bala* Root (in % w/w) was evaluated as 0.504, 0.515, 0.557, 0.855, 2.662 and 1.64 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. In Petroleum ether extract, 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 the presence of flavonoids and carbohydrates. The aqueous extract demonstrated the presence of alkaloids, flavonoids, carbohydrate, glycosides, tannin and saponin.

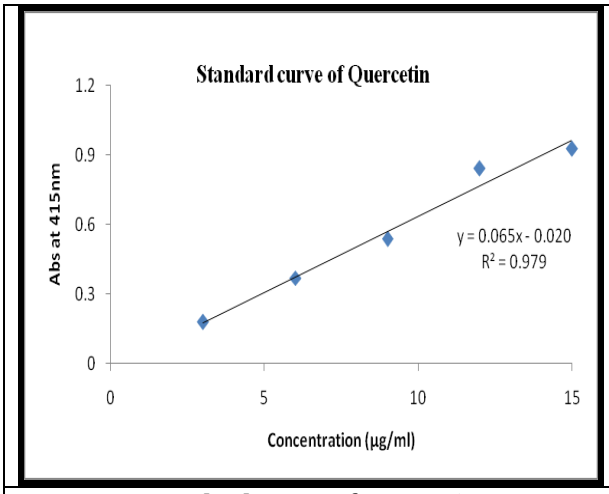
Table 3: Phytochemical Constituents in Bala root extracts.

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]

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 3 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

Figure 3: Standard curves of Quercetin & Gallic Acid.

The Total Flavonoid Content (TFC) expressed as µg Quercetin equivalent/mg of extract was found to be 15.03 and 6.62 in case of the Alcoholic extract and Aqueous extract respectively. The Total Phenol Content (TPC) was found to be 18.70 and 23.26 µg Gallic acid equivalent/mg of extract in case of the Alcoholic extract and Aqueous extract respectively. The Alcoholic extract of Bala shows more Quercetin equivalent content than its aqueous extract, while Bala 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 4.

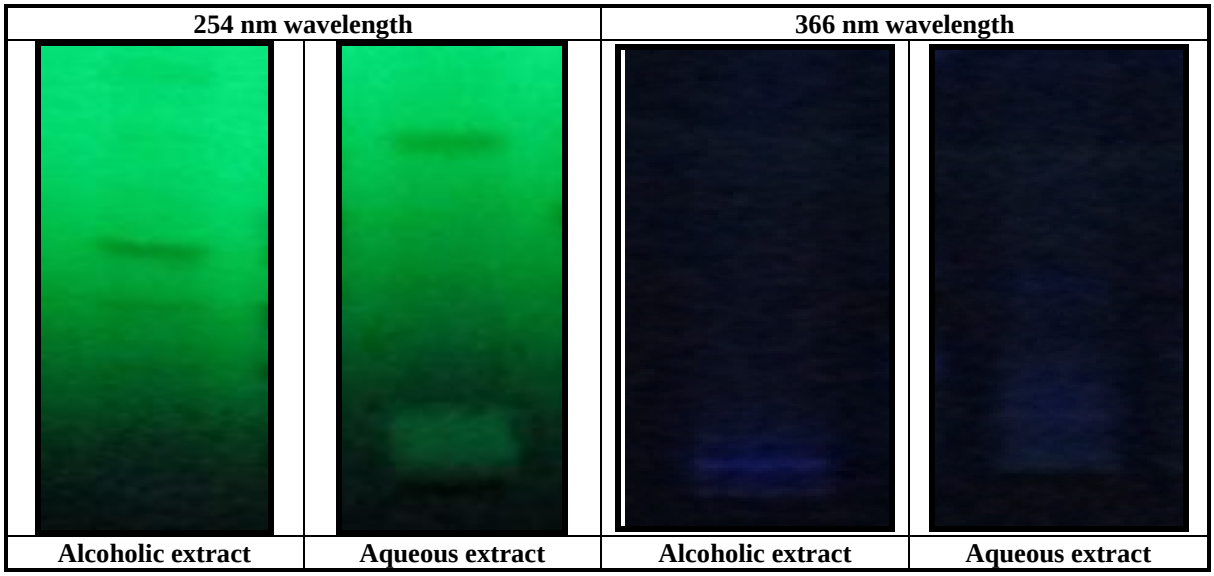
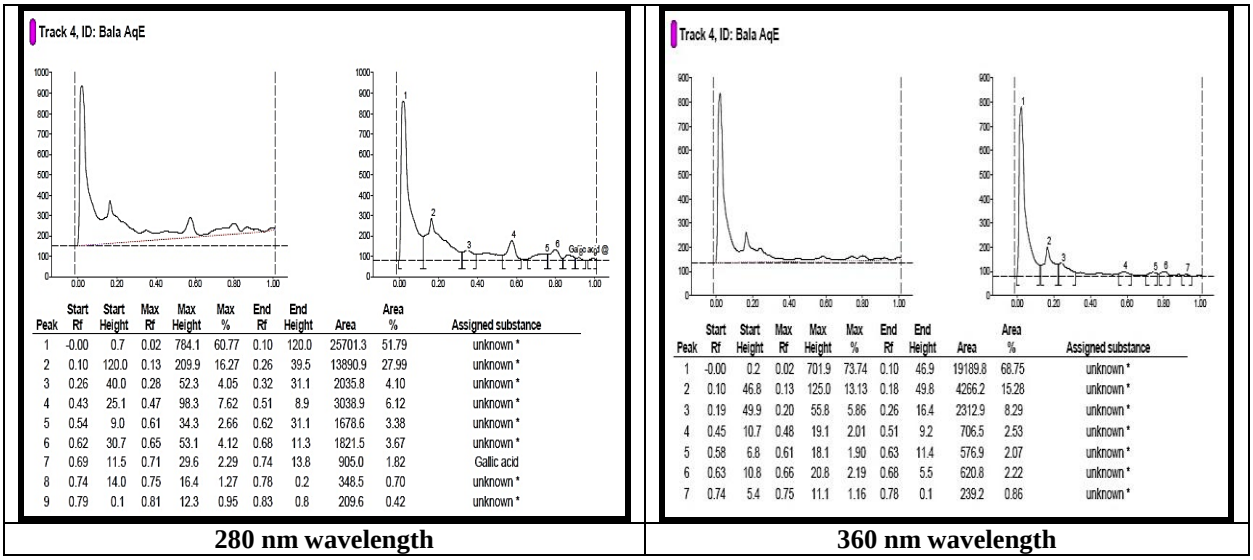


Fig. 4: HPTLC plates visualised at 254 and 366 nm wavelengths.

During the HPTLC study, both the research extracts were scanned at 280 nm and 360 nm and the obtained chromatographs are shown in Figures 5 and 6 and Rf values obtained from these chromatographs are shown in table 4.

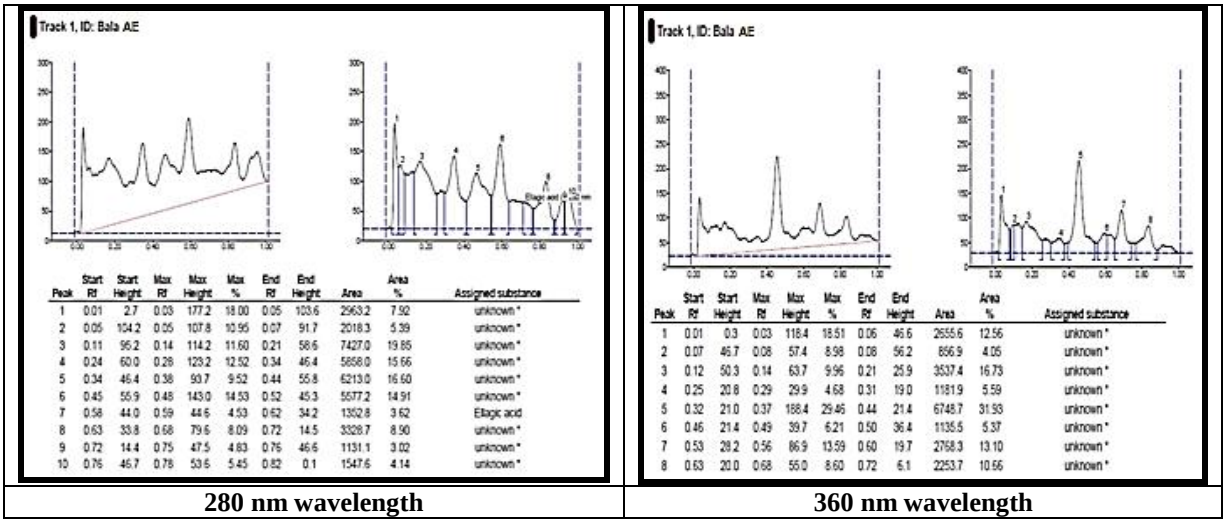


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.00	0.7	0.02	784.1	60.77	0.10	120.0	25701.3	51.79	unknown *
2	0.10	120.0	0.13	209.9	16.27	0.26	39.5	13890.9	27.99	unknown *
3	0.26	40.0	0.28	52.3	4.05	0.32	31.1	2035.8	4.10	unknown *
4	0.43	25.1	0.47	98.3	7.62	0.51	8.9	3038.9	6.12	unknown *
5	0.54	9.0	0.61	34.3	2.66	0.62	31.1	1678.6	3.38	unknown *
6	0.62	30.7	0.65	53.1	4.12	0.68	11.3	1821.5	3.67	unknown *
7	0.69	11.5	0.71	29.6	2.29	0.74	13.8	905.0	1.82	Gallic acid
8	0.74	14.0	0.75	16.4	1.27	0.78	0.2	348.5	0.70	unknown *
9	0.79	0.1	0.81	12.3	0.95	0.83	0.8	209.6	0.42	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.00	0.2	0.02	701.9	73.74	0.10	46.9	19189.8	68.75	unknown *
2	0.10	46.8	0.13	125.0	13.13	0.18	49.8	4266.2	15.28	unknown *
3	0.19	49.9	0.20	55.8	5.86	0.26	16.4	2312.9	8.29	unknown *
4	0.45	10.7	0.48	19.1	2.01	0.51	9.2	706.5	2.53	unknown *
5	0.58	6.8	0.61	18.1	1.90	0.63	11.4	576.9	2.07	unknown *
6	0.63	10.8	0.66	20.8	2.19	0.68	5.5	620.8	2.22	unknown *
7	0.74	5.4	0.75	11.1	1.16	0.78	0.1	239.2	0.86	unknown *

280 nm wavelength | **360 nm wavelength**

Figure 5: Chromatographs of aqueous extract during HPTLC study.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	2.7	0.03	177.2	18.00	0.05	103.6	2963.2	7.92	unknown *
2	0.05	104.2	0.05	107.8	10.95	0.07	91.7	2018.3	5.39	unknown *
3	0.11	95.2	0.14	114.2	11.60	0.21	58.6	7427.0	19.85	unknown *
4	0.24	60.0	0.28	123.2	12.52	0.34	46.4	5858.0	15.66	unknown *
5	0.34	46.4	0.38	93.7	9.52	0.44	55.8	6213.0	16.60	unknown *
6	0.45	55.9	0.48	143.0	14.53	0.52	45.3	5577.2	14.91	unknown *
7	0.58	44.0	0.59	44.6	4.53	0.62	34.2	1352.8	3.62	Ellagic acid
8	0.63	33.8	0.68	79.6	8.09	0.72	14.5	3328.7	8.90	unknown *
9	0.72	14.4	0.75	47.5	4.83	0.76	46.6	1131.1	3.02	unknown *
10	0.76	46.7	0.78	53.6	5.45	0.82	0.1	1547.6	4.14	unknown *

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	0.3	0.03	118.4	18.51	0.06	46.6	2655.6	12.56	unknown *
2	0.07	46.7	0.08	57.4	8.98	0.08	56.2	856.9	4.05	unknown *
3	0.12	50.3	0.14	63.7	9.96	0.21	25.9	3537.4	16.73	unknown *
4	0.25	20.8	0.29	29.9	4.68	0.31	19.0	1181.9	5.59	unknown *
5	0.32	21.0	0.37	188.4	29.46	0.44	21.4	6748.7	31.93	unknown *
6	0.46	21.4	0.49	39.7	6.21	0.50	36.4	1135.5	5.37	unknown *
7	0.53	28.2	0.56	86.9	13.59	0.60	19.7	2768.3	13.10	unknown *
8	0.63	20.0	0.68	55.0	8.60	0.72	6.1	2253.7	10.66	unknown *

280 nm wavelength | **360 nm wavelength**

Figure 6: Chromatographs of alcoholic extract during HPTLC study.

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

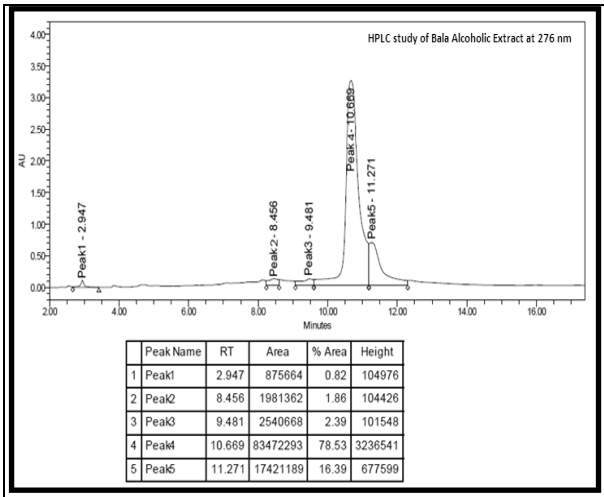
Scanned at 280 nm				Scanned at 360 nm			
Sl. No.	**Standard**	***Rf* value of Aqueous Extract**	***Rf* value of Alcoholic Extract**	**Sl. No.**	**Standard**	***Rf* value of Aqueous Extract**	***Rf* value of Alcoholic Extract**
1		0.02	0.03	1		0.02	0.03
2			0.06	2			0.08
3		0.13	0.14	3		0.13	0.14
4		0.28	0.28	4		0.20	
5			0.38	5			0.29
6		0.47	0.48	6			0.37
7	Ellagic acid	0.61	0.59	7		0.48	0.49
8		0.65	0.68	8			0.56
9	Gallic acid	0.71		9	Ellagic acid	0.61	
10		0.75	0.75	10	Gallic acid	0.66	0.68
11			0.78	11		0.75	
12		0.81					

The *Rf* values scanned at 280 nm wavelength showed that at sl. no. 1, 3-4, 6-8 and 10 of aqueous and alcoholic extracts of Bala root are quite similar and at 360 nm wavelength, sl. no. 1, 3, 7 and 10 of aqueous and alcoholic extracts showed similar *Rf* values. All similar *Rf* values (sl. no. 3, 7 and 10) scanned at 360 nm for both extracts, were matched with their corresponding *Rf* values at 280 nm with sl. no. 3, 6 and 8. This observation

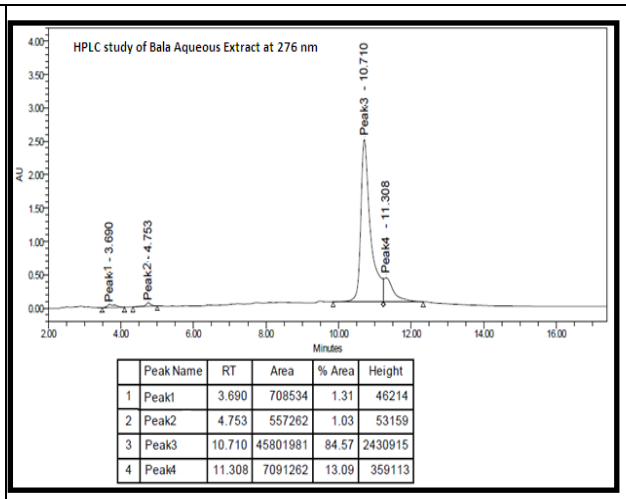
expressed three common *Rf* value in both extracts scanned at different wavelengths. The comparison of the obtained Rf values with known standard values also seemed to indicate the presence of two standard compounds namely Ellagic acid and Gallic acid in both these extracts.

3.2.4.2. High Performance Liquid Chromatography (HPLC)

The observed chromatographs of both the Alcoholic and the Aqueous extracts are shown in figure 7 while the RT values obtained and their comparison with standard compounds is detailed in table 5.



	Peak Name	RT	Area	% Area	Height
1	Peak1	2.947	875664	0.82	104976
2	Peak2	8.456	1981362	1.86	104426
3	Peak3	9.481	2540668	2.39	101548
4	Peak4	10.669	83472293	78.53	3236541
5	Peak5	11.271	17421189	16.39	677599



	Peak Name	RT	Area	% Area	Height
1	Peak1	3.690	708534	1.31	46214
2	Peak2	4.753	557262	1.03	53159
3	Peak3	10.710	45801981	84.57	2430915
4	Peak4	11.308	7091262	13.09	359113

Alcoholic Extract **Aqueous Extract**

Figure 7: HPLC Chromatographs at 276 nm.

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

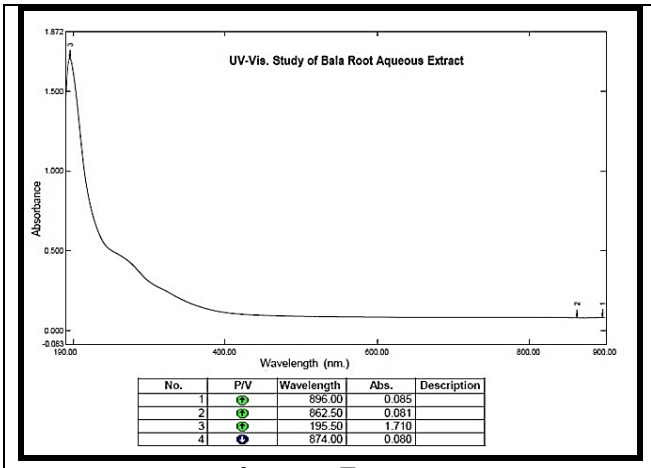
	RT Values			RT Values
Sl. No.	**Aqueous Extract**	**Standard**	**Sl. No.**	**Alcoholic Extract**
1	3.690		1	2.947
2	4.753	Tannic acid	2	
3	10.710	Benzoic acid	3	
4	11.308	Quercetin	4	

The HPLC chromatogram of the aqueous and alcoholic extracts of Bala root on the basis of the elution of the peaks at 276 nm wavelengths showed no common RT values. In aqueous extract four different compounds were observed but in the alcoholic extract only one compound was found.

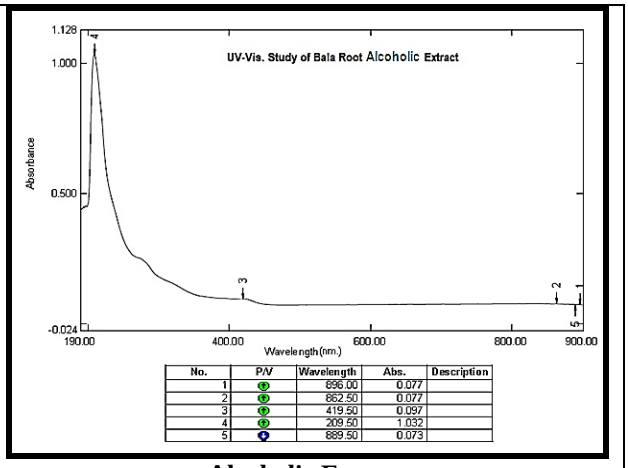
2.2.5. Spectroscopy

2.2.5.1. UV- Visible Spectroscopic Study

The results from the spectroscopic scanning of both the extracts are shown in figure 8 and the comparative analysis of the absorbance observed is detailed in table 6.



No.	P/V	Wavelength	Abs.	Description
1		896.00	0.085	
2		862.50	0.081	
3		195.50	1.710	
4		874.00	0.080	



No.	P/V	Wavelength	Abs.	Description
1		896.00	0.077	
2		862.50	0.077	
3		419.50	0.097	
4		209.50	1.032	
5		889.50	0.073	

Aqueous Extract **Alcoholic Extract**

Figure 8: Results of the UV- Visible Spectroscopic Scanning Study.

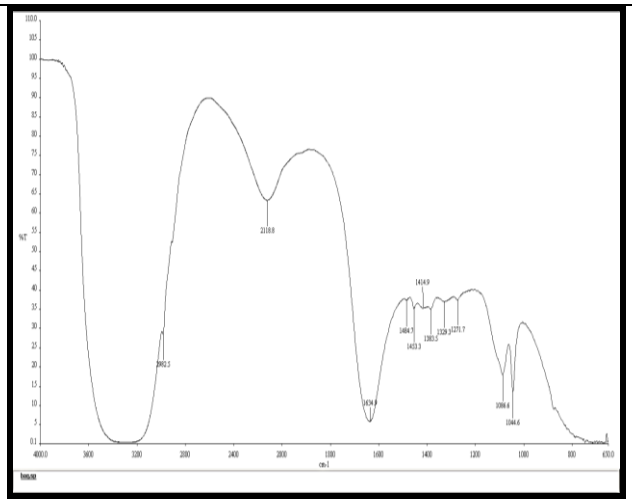
Table 6: Comparative analysis of UV-Visible spectrum data.

Wavelength	Absorbance of Aqueous Extract	Wavelength	Absorbance of Alcoholic Extract
896.0	0.085	896.0	0.077
862.5	0.081	862.5	0.077
195.5	1.710	419.5	0.097
		209.5	1.032

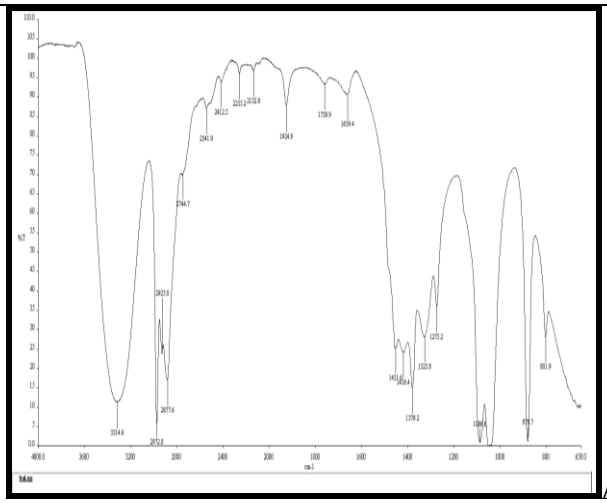
UV spectrum of the aqueous extract revealed the presence of three peaks at 896, 862.5 and 195.5 nm, corresponding to three different compounds whereas alcoholic extract showed four peaks at 896, 862.5, 419.5 and 209.5 nm, corresponding to four different compounds. Only two peaks at 896 and 862.5 nm were found similar 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 9. The obtained data was analysed and the possible functional groups present in both the extracts are shown in table 7.



Aqueous Extract



Alcoholic Extract

Figure 9: 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**
1484.7	N-CH_3 out-phase bending	3314.6	-NH_2 Stretching
1414.9	N-CH_3 in-phase stretching	2877.6	-CH_2 in-phase stretching
1383.5	Aryl -CH_3 in-phase stretching	1418.4	(N)-CH_3 in-phase stretching
1086.6	C-O stretching	1378.2	-CH_3 in-phase bending
		1275.2	Aryl-N stretching
		801.9	N-O stretching

Aqueous and alcoholic extracts exhibited different characteristic bands representing various functional groups like N-CH_3 bending, Aryl CH_3 in phase stretching, C-O stretching, NH_2 and CH_2 stretching, Aryl N and N-O group stretching etc. These results indicated presence of phenolic & alkaloidal group of compounds, which would be further validated by different chromatographic analysis.

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. Many pharmaceutical companies are now concentrating on manufacturing of Ayurvĕdic Phytopharmaceutical products using GMP (Good Manufacturing Practices) which is part of a quality system covering the manufacture and testing of pharmaceutical drugs, active pharmaceutical ingredients and products, ensuring

Quality Assurance and standardization. In order to achieve the objective of ensuring that medicinal products are of the prescribed quality, quantitative determination of some phamacognostical parameters is very useful for setting standards for crude drugs.[18] 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 Bala (*Sida cordifolia* Linn.) extends to the nervous, circulatory, and urinary systems. It has a diuretic effect, is useful in urinary problems and is also used in inflammations, bleeding disorders being cooling and astringent.

While macroscopic examination indicated whitish yellow colour, smooth texture, hard, about 12-16 inches' length and 1 inch diameter of roots, its powder looks light brown in colour. The microscopic analysis of powder showed the presence of starch granules, Trichomes, Vessels with sceleriform orientation with parenchyma patches, Pitted Vessel, Crystal and Cork cells.

The total Ash value was 8.0% w/w which primarily consisted of water soluble ash (5.41%). The moisture content in the research formulation was found to be 4.9 % w/w while the pH value of 5.8 indicated its acidic nature which is one of the important parameters for preparing the vaginal tablet for the treatment of vaginitis. The extractive value of alcoholic and aqueous research formulation was found as 2.662% and 1.64% w/w while preliminary phytochemical analysis revealed the presence of flavonoids and carbohydrates in both extracts. The results also showed high concentration of flavonoidic compounds (15.03 µg Quercetin equivalent / mg of extract) in the alcoholic extract and high phenolic content (23.26 µg Gallic acid equivalent / mg of extract) in the aqueous extract which could be directly responsible for their antimicrobial, anti-leucorrhoeal and astringent properties.

The R_f 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 done at 280 nm and 360 nm wavelengths. The *Rf* values scanned at 280 nm wavelength showed that 7 spots of aqueous and alcoholic extracts of Bala root are quite similar and at 360 nm wavelength, 4 spots of both extracts showed similar *Rf* values. Matching of these *Rf* values indicated three common *Rf* values in both extracts scanned at different wavelengths. The comparison of the obtained Rf values with known standard values also seemed to indicate the presence of two standard compounds namely 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 of Bala root on the basis of the elution of the peaks at 276 nm wavelengths showed four different compounds in aqueous extract while only one compound was observed in the alcoholic extract. Further analysis with database of standard compounds confirmed the presence of Tannic acid, Benzoic acid and Quercetin at RT 4.753, 10710 and 11.308 respectively in the aqueous extract. The presence of phenolic compounds in aqueous extract 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 896, 862.5 and 195.5 nm, corresponding to three different compounds whereas alcoholic extract showed four peaks at 896, 862.5, 419.5 and 209.5 nm, corresponding to four different compounds. Out of these observed peaks, two peaks at 896 and 862.5 nm were found to be similar in both the extracts.

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 1484.7 cm-1, 1414.9 cm-1, 1383.5 cm-1 and 1086.6 cm-1 indicating the presence of the functional groups N-CH3 out-phase bending, N-CH3 in-phase stretching, Aryl -CH3 in-phase stretching and C-O stretching respectively. At the same time, the alcoholic extract revealed characteristic peaks at 3314.6 cm-1, 2877.6 cm-1, 1418.4 cm-1, 1378.2 cm-1, 1275.2 cm-1 and 801.9 cm-1 indicating the presence of -NH2 Stretching, -CH2 in-phase stretching, (N)-CH3 in-phase stretching, -CH3 in-phase bending, Aryl-N stretching

and N-O stretching 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 amides, aldehydes, alkaloids and phenolic groups of compounds in the extracts.

5. CONCLUSION

Pharmacognostical analysis indicates high concentration of flavonoids and carbohydrates in both extracts. Higher concentration of flavonoidic compounds was observed in the alcoholic extract while high phenolic content was noticed in the aqueous extract. Spectroscopic and chromatographic examination using UV-Visible, HPTLC and HPLC analysis indicated the presence of Ellagic acid and Gallic acid in both these extracts, and Tannic acid, Benzoic acid and Quercetin in the aqueous extract. FTIR analysis indicated the presence of amides, aldehydes, alkaloids and phenolic groups of compounds in the extracts. The presence of phenolic and flavonidic compounds in the extracts of the research formulation may be responsible for its pharmacological activities because these compounds are known for their antioxidant, analgesic, antipyretic, anti-inflammatory and antimicrobial properties.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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