International Journal of Pharmacy and Biological Sciences-IJPBS™ (2020) 10 (3): 256-270
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | OA Journal | MCI Approved | Index Copernicus

A Pre-Clinical Study of *Glycyrrhiza Glabra* Linn. For Treatment of Male Sexual Disorders Using Pharmacognostical, Chromatography (HPTLC and HPLC) and Spectroscopy (UV-Visible and FTIR) Chemical Analysis

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Received: 18 Mar 2020 / Accepted: 16 Apr 2020 / Published online: 01 Jul 2020
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Abstract
An Ayurvedic formulation prepared from dried root powder of *Glycyrrhiza glabra* Linn. is standardized through pharmacognostical and phytochemical pre-clinical studies for treatment of male sexual disorders. While total ash value was 6.27%, acid insoluble ash was 0.17% and water soluble ash was 4.1%. Among elements, Nitrogen was found present. Flavonoids, carbohydrates, glycosides, tannin and saponin were found present in both alcoholic and aqueous extracts. The results also showed high concentrations of flavonoidic compounds (27.40 µg Quercetin equivalent / mg) and phenolic content (29.50 µg Gallic acid equivalent / mg) in alcoholic as compared to aqueous extract. HPTLC analysis at 280 and 360 nm indicated the presence of Ellagic acid in both these extracts. Similarly, HPLC analysis at 276 nm showed elution of five compounds in aqueous and ten compounds in alcoholic extract whose analysis confirmed the presence of Ellagic acid in both extracts. UV-Visible spectroscopy scanning showed peaks at 315, 265.5 and 194.5 nm in aqueous and at 896, 315, 271 and 211 nm in alcoholic extract. FTIR analysis indicated presence of C-H stretching, Aryl -CH_3 in-phase bending, -CH_3 in-phase bending, Aryl out-phase stretching and Aryl 5 adjacent C-H wagging functional groups in aqueous and (N)-CH_3 in-phase bending, Tertiary butyl-CH_3 in-phase bending, (S)-CH_3 in-phase bending, (S)-CH_3 in-phase bending and -NO_2 in-phase stretching functional groups in alcoholic extract suggesting the presence of phenolic & alkaloidal group 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, Chromatography, *Glycyrrhiza Glabra* Linn., Pre-clinical, Spectroscopy.

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1. INTRODUCTION
Several medicinal 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. In fact, plant derived medicines have primarily

become the first line of defence in maintaining health and combating diseases. [1] Chemical principles obtained from natural sources have become much simpler and contributed significantly to the development of new drugs from medicinal plants. [2] 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.

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. The plant attains a maximum height up to 2 m. The underground stem grows horizontally up to 2 m length, highly branched consisting of short tap root with large number of rhizomes. The diameter of the root varies from 0.75 to 2.5 cm, grey-brown exterior and yellow interior. Externally, it is longitudinally wrinkled with patches of cork. It has a characteristic pleasant sweet taste. Flowering & fruiting is from August to February. 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. 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. Glycyrrhizic acid is not a glycoside since it yields on hydrolysis one molecule of Glycyrrhetic acid and two molecules of Glycuronic acid but no sugar. Glycuronic acid is, however, very closely related to the hexose sugars, and Glycyrrhetic acid has a haemolytic action like that of the saponins. Liqourice also contains glucose (up to 3.8 per cent), sucrose (2.4 to 6.5 percent), bitter principles, resins, mannite, asparagines (2 to 4 percent) 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. [3-7] After obtaining satisfactory results in the spermatogenic, antioxidant, rejuvenator and toxicity studies in the animal models, *Glycyrrhiza glabra* Linn. was taken up for this pre-clinical study before evaluating its spermatogenetic therapeutic efficacy in clinical trial on male human subjects. [8-14]

The herbs are natural materials and their constituents may vary due to differing geographical locations, climatic conditions, environmental hazards, harvesting methods and collection protocols. Such factors make it difficult to standardize or reproduce the quality of the end product. Hence, this preclinical study focuses on the standardization and quality control of different extracts of the research drug (*Glycyrrhiza glabra* 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 & METHODS

The roots of *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). [15]

2.1. Pharmacognostical analysis

2.1.1. Macroscopic and microscopic study of powder

The roots of the plant were carefully 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 using this fine root powder mounted in glycerine which was 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 a properly calibrated pH meter 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. [16] 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 What man 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. [17-18] 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. [17-19]

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. [5, 20]

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). [21]

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: The initial proportion of solvent A was 15 % which was gradually increased to 25% over 12 minutes and retained at this level up to 20 minutes. It was again brought back to 15% at 22 minutes and kept at this level up to 30 minutes during this experiment.

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. [21]

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 analysed by HATR sampling accessory through ZnSe plate 45. [22]

3. RESULTS

3.1. Pharmacognostical analysis

3.1.1. Macroscopic and microscopic study of powder

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 1. 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.

The *Yashthimadhu* powder looks shiny brown in colour (figure 1) and its microscopic analysis shown in figure 2 shows the various components which include the Scleramatous type of parenchyma cells in the groups, cells containing pigment, vessel with sceleriform orientation, pitted vessel, Crystal Sheath and Cork cells.

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 6.27% (w/w), containing Acid Insoluble Ash of 0.17% and Water soluble ash of 4.1%. While the moisture content was 8.8%, the pH value was found to be 5.9.

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.

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.

3.2. Chemical analysis

3.2.1. Continuous extraction of research formulation

The Extractive value of *Yashthimadhu* Root (in % w/w) was evaluated as 0.167, 3.49, 0.135, 0.955, 12.22 and 6.21 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 Ethyl Acetate extract, only flavonoids were present. Chloroform extract showed flavonoids and glycosides, whereas the Acetone extract indicated the presence of flavonoids, tannin and carbohydrates. The Alcoholic and aqueous extracts demonstrated the presence of flavonoids, carbohydrate, glycosides, tannin and saponin; whereas alkaloids were also observed in the aqueous extract.

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.

The Total Flavonoid Content (TFC) expressed as µg Quercetin equivalent/mg of extract was found to be 27.40 and 11.45 for the Alcoholic extract and Aqueous extract respectively. The Total Phenol Content (TPC) was found to be 29.50 and 13.94 µg Gallic acid equivalent/mg of extract in case of Alcoholic extract and Aqueous extract respectively. The Alcoholic extract shows more Quercetin equivalent and Gallic acid equivalent content than its aqueous 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.

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.

The *Rf* values scanned at 280 and 360 nm wavelength showed that sl. no. 1, 3-9 & 13 of aqueous and alcoholic extracts of Yashtimadhu root are quite similar. This observation exhibited nine common compounds in both the extracts, scanned at different wave lengths. It was also observed that standard phenolic compound Ellagic acid (*Rf* value 0.59) was common in both the extracts at sl. no. 10 at both wavelengths.

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.

The HPLC chromatograms of the aqueous and alcoholic extracts of Yashtimadhu root on the basis of the elution of the peaks at 276 nm wavelength showed five common RT values. In the aqueous extract five compounds were found which exactly matched with RT values observed in alcoholic extract where total ten compounds were found.

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 8 and the comparative analysis of the absorbance observed is detailed in table 6.

UV spectrum of the aqueous extract revealed the presence of three peaks at 315, 265.5 and 194.5 nm, corresponding to three different compounds whereas alcoholic extract showed four peaks at 896, 315, 271 and 211 nm, corresponding to four different compounds. Only one peak at 315 nm was 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 analyzed and the possible functional groups present in both the extracts are shown in table 7.

Aqueous and alcoholic extract of Yashthimadhu exhibited different characteristic bands representing various functional groups like CH stretching, Aryl CH_3 in phase stretching, (N)-CH_3 and (S)-CH_3 in phase bending, NO_2 in phase stretching, etc. These result indicated presence of phenolic & alkaloidal group of compounds, which would be further validated by different chromatographic analysis.

Table 1: Results of fluorescence analysis (observed colours)

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

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

Table 3: Phytochemical Constituents in *Yashthimadhu* 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]

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		0.03	0.02
2			0.06	2			
3		0.11	0.10	3		0.11	0.10
4		0.14	0.14	4		0.14	0.14
5		0.18	0.18	5		0.18	0.18
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		0.37	0.36
9		0.48	0.47	9		0.51	0.51

10		0.50	10		0.55	0.55
11	Ellagic acid	0.59	11	Ellagic acid		0.60
12			12			0.64
13	0.73	0.74	13		0.73	0.73

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

Sl. No.	Standard	RT value of Aqueous extract	Sl. No.	Standard	RT value of Alcoholic extract
1		3.094	1		2.962
2		7.698	2		7.689
3		8.130	3		8.124
4		8.476	4		8.454
5			5		9.432
6	Ellagic acid	9.762	6	Ellagic acid	9.732
7			7		9.873
8			8		10.091
9			9		10.679
10			10		11.342

Table 6: Comparative analysis of UV-Visible spectrum data

Wavelength	Absorbance of Aqueous Extract	Wavelength	Absorbance of Alcoholic Extract
		896.0	0.074
315.0	0.165	315.0	0.618
265.5	0.261	271.0	0.795
194.5	0.793	211.0	1.480

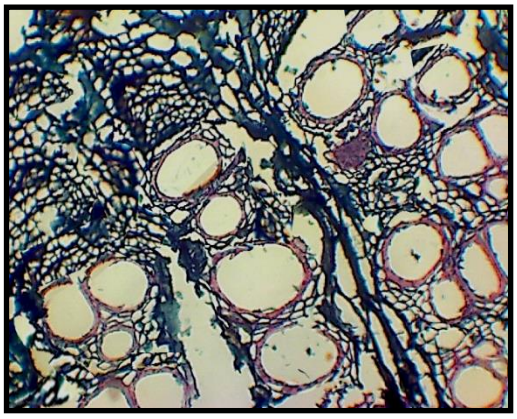
Table 7: Comparative analysis of FT-IR data

Aqueous Extract		Alcoholic Extract	
Wavenumber (cm^{-1})	Possible functional group	Wavenumber (cm^{-1})	Possible functional group
2929.7	C-H stretching	1416.3	(N)-CH_3 in-phase bending
1387.9	Aryl -CH_3 in-phase bending	1365.2	Tertiary butyl-CH_3 in-phase bending
1369.0	-CH_3 in-phase bending	1333.0	(S)-CH_3 in-phase bending
1232.5	Aryl out-phase stretching	1308.3	(S)-CH_3 in-phase bending
756.9	Aryl 5 adjacent C-H wagging	1279.9	-NO_2 in-phase stretching

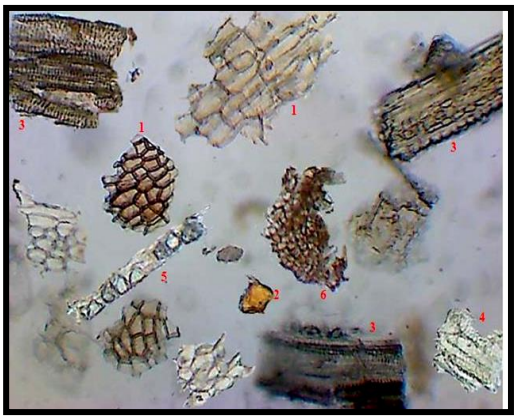
Roots of *Glycyrrhiza glabra* Linn.

Powder of *Glycyrrhiza glabra* Linn. roots

Figure 1: *Glycyrrhiza glabra* Linn. roots and powder

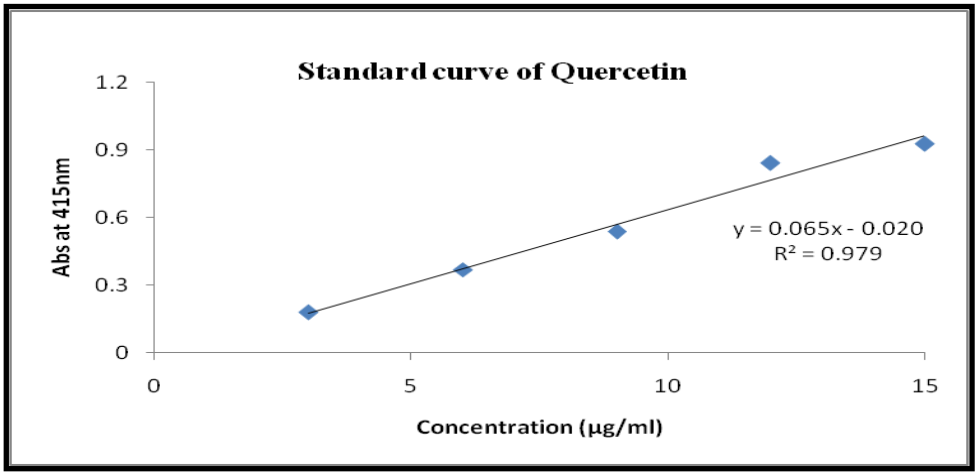


T.S. of *Glycyrrhiza glabra* Linn. Root

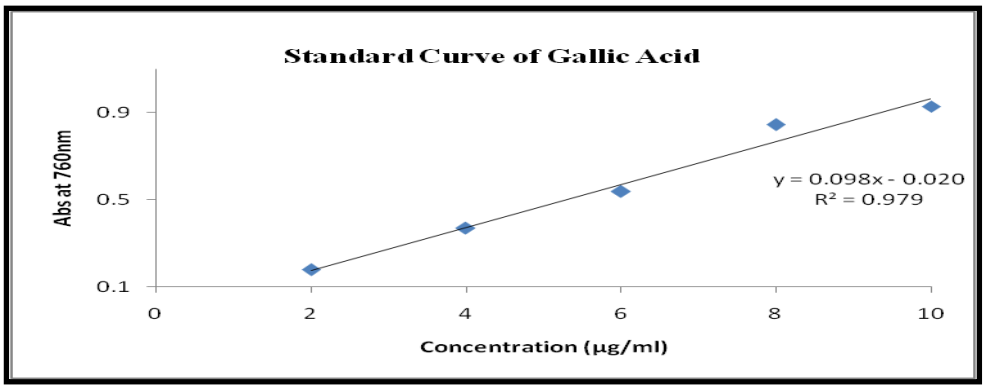


Microscopic study of *Glycyrrhiza glabra* Linn. Root powder: 1. Scleramatous type of parenchyma cells in the groups 2. Cell containing pigment 3. Vessel with sceleriform orientation 4. Pitted Vessel 5. Crystal Sheath 6. Cork cell.

Figure 2: Microscopic analysis of *Glycyrrhiza glabra* Linn. root & its powder



Standard curve of Quercetin



Standard curve of Gallic Acid

Figure 3: Standard curves of Quercetin & Gallic Acid

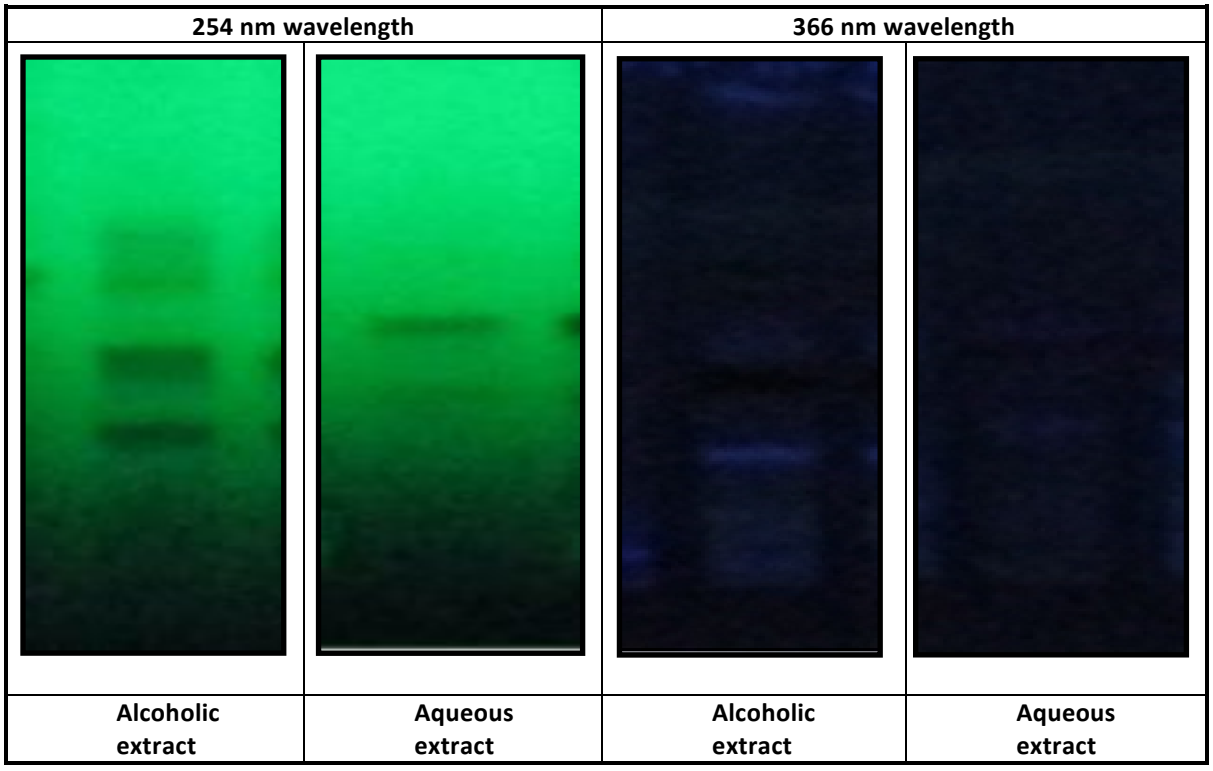
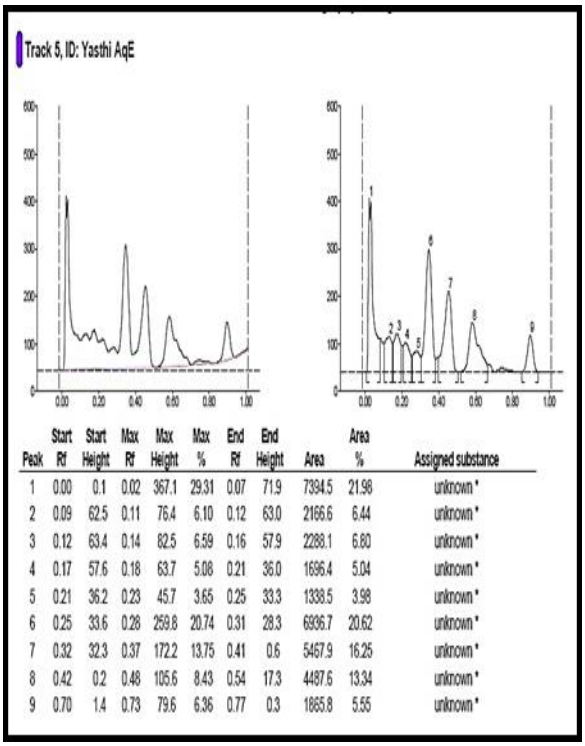
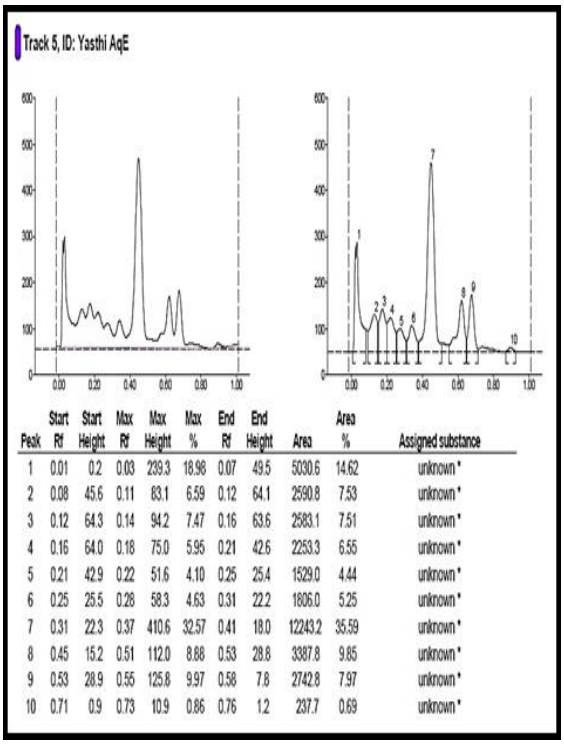


Figure 4: HPTLC plates visualised at 254 and 366 nm wavelengths



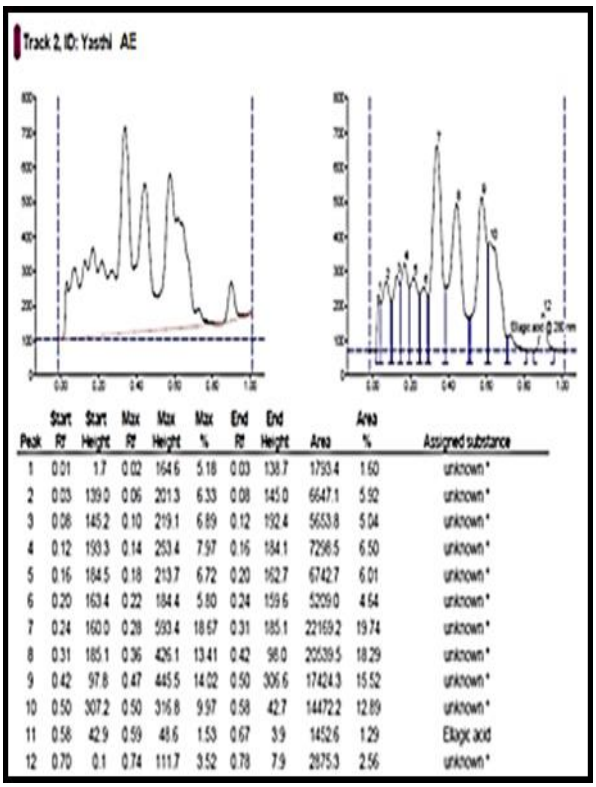
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.00	0.1	0.02	367.1	29.31	0.07	71.9	7394.5	21.98	unknown *
2	0.09	62.5	0.11	76.4	6.10	0.12	63.0	2166.6	6.44	unknown *
3	0.12	63.4	0.14	82.5	6.59	0.16	57.9	2286.1	6.80	unknown *
4	0.17	57.6	0.18	63.7	5.08	0.21	36.0	1696.4	5.04	unknown *
5	0.21	36.2	0.23	45.7	3.65	0.25	33.3	1338.5	3.98	unknown *
6	0.25	33.6	0.28	259.8	20.74	0.31	28.3	6936.7	20.62	unknown *
7	0.32	32.3	0.37	172.2	13.75	0.41	0.6	5457.9	16.25	unknown *
8	0.42	0.2	0.48	105.6	8.43	0.54	17.3	4487.6	13.34	unknown *
9	0.70	1.4	0.73	79.6	6.36	0.77	0.3	1865.8	5.55	unknown *



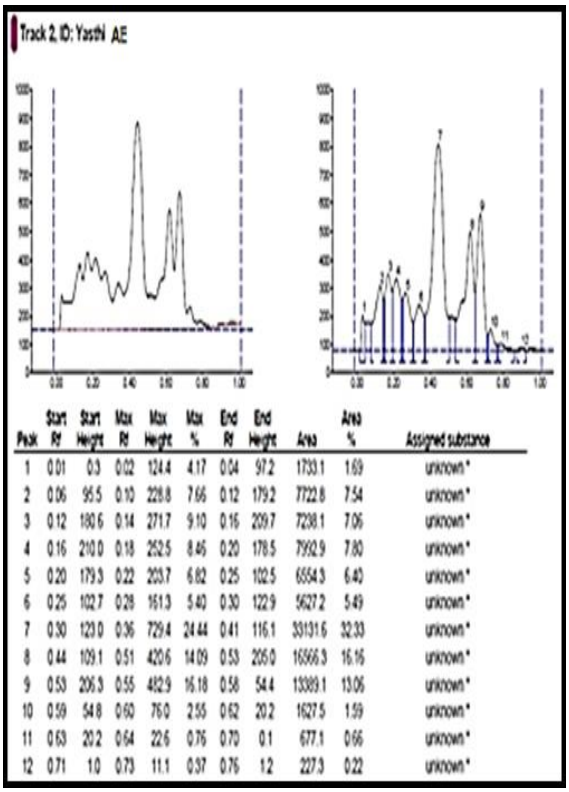
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	0.2	0.03	239.3	18.98	0.07	49.5	5030.6	14.62	unknown *
2	0.08	45.6	0.11	83.1	6.59	0.12	64.1	2590.8	7.53	unknown *
3	0.12	64.3	0.14	94.2	7.47	0.16	63.6	2583.1	7.51	unknown *
4	0.16	64.0	0.18	75.0	5.95	0.21	42.6	2253.3	6.55	unknown *
5	0.21	42.9	0.22	51.6	4.10	0.25	25.4	1529.0	4.44	unknown *
6	0.25	25.5	0.28	58.3	4.63	0.31	22.2	1806.0	5.25	unknown *
7	0.31	22.3	0.37	410.6	32.57	0.41	18.0	12243.2	35.59	unknown *
8	0.45	15.2	0.51	112.0	8.88	0.53	28.8	3387.8	9.85	unknown *
9	0.53	28.9	0.55	125.8	9.97	0.58	7.8	2742.8	7.97	unknown *
10	0.71	0.9	0.73	10.9	0.86	0.76	1.2	237.7	0.69	unknown *

280 nm wavelength **360 nm wavelength**

Figure 5: Chromatographs of aqueous extract during HPTLC study



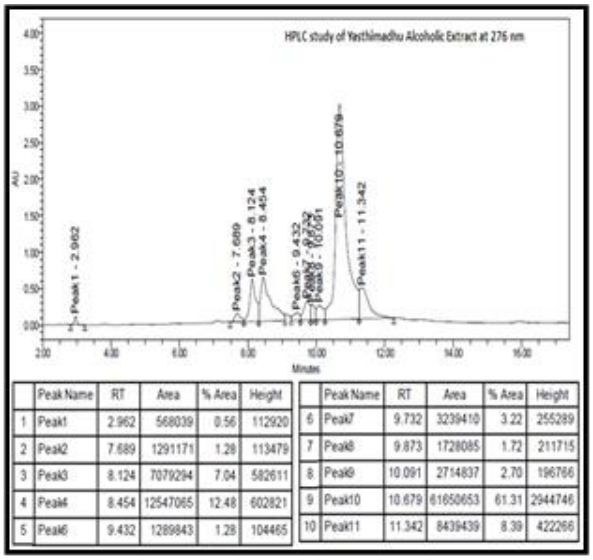
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	1.7	0.02	164.6	5.18	0.03	138.7	1793.4	1.60	unknown *
2	0.03	139.0	0.06	201.3	6.33	0.08	145.0	6647.1	5.92	unknown *
3	0.08	145.2	0.10	219.1	6.89	0.12	102.4	5653.8	5.04	unknown *
4	0.12	150.3	0.14	253.4	7.97	0.16	184.1	7296.5	6.50	unknown *
5	0.16	184.5	0.18	213.7	6.72	0.20	162.7	6742.7	6.01	unknown *
6	0.20	163.4	0.22	184.4	5.80	0.24	159.6	5229.0	4.64	unknown *
7	0.24	160.0	0.28	593.4	18.67	0.31	185.1	22169.2	19.74	unknown *
8	0.31	185.1	0.36	426.1	13.41	0.42	98.0	20539.5	18.29	unknown *
9	0.42	97.8	0.47	445.5	14.02	0.50	306.6	17424.3	15.52	unknown *
10	0.50	307.2	0.50	316.8	9.97	0.58	42.7	14472.2	12.89	unknown *
11	0.58	42.9	0.59	48.6	1.53	0.67	3.9	1452.6	1.29	Ellagic acid
12	0.70	0.1	0.74	111.7	3.52	0.78	7.9	2875.3	2.56	unknown *



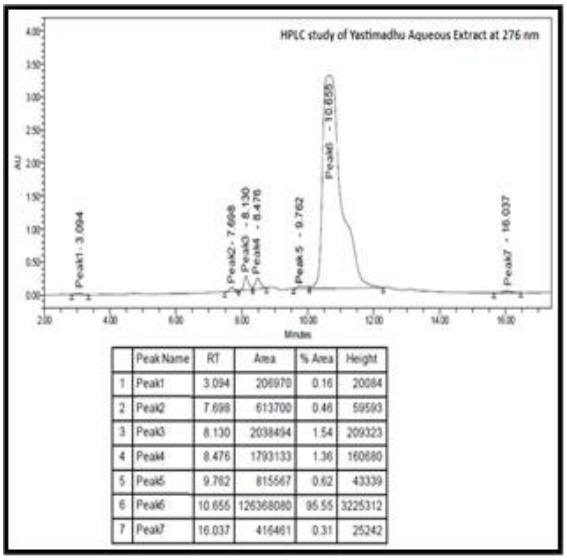
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	0.3	0.02	124.4	4.17	0.04	97.2	1733.1	1.69	unknown *
2	0.06	95.5	0.10	228.8	7.66	0.12	179.2	7722.8	7.54	unknown *
3	0.12	180.6	0.14	271.7	9.10	0.16	209.7	7238.1	7.06	unknown *
4	0.16	210.0	0.18	252.5	8.46	0.20	178.5	7992.9	7.80	unknown *
5	0.20	179.3	0.22	203.7	6.82	0.25	102.5	6554.3	6.40	unknown *
6	0.25	102.7	0.28	161.3	5.40	0.30	122.9	5627.2	5.49	unknown *
7	0.30	123.0	0.36	729.4	24.44	0.41	116.1	33131.6	32.33	unknown *
8	0.44	109.1	0.51	420.6	14.09	0.53	205.0	16566.3	16.16	unknown *
9	0.53	206.3	0.55	482.9	16.18	0.58	54.4	13389.1	13.06	unknown *
10	0.59	54.8	0.60	76.0	2.55	0.62	20.2	1627.5	1.59	unknown *
11	0.63	20.2	0.64	22.6	0.76	0.70	0.1	677.1	0.66	unknown *
12	0.71	1.0	0.73	11.1	0.37	0.76	1.2	227.3	0.22	unknown *

280 nm wavelength **360 nm wavelength**

Figure 6: Chromatographs of alcoholic extract during HPTLC study



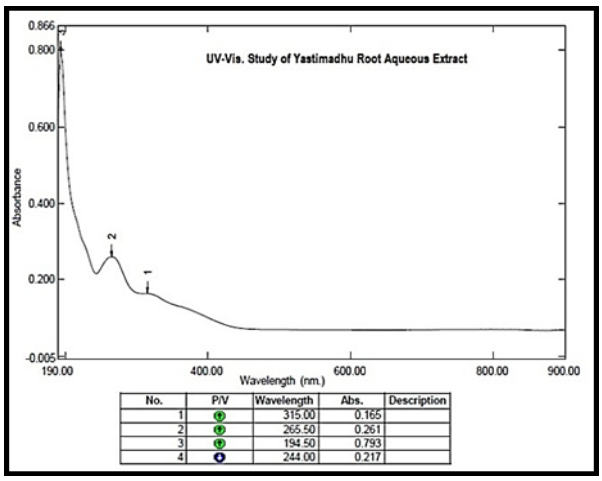
	Peak Name	RT	Area	% Area	Height
1	Peak1	2.962	568039	0.56	112920
2	Peak2	7.689	1291171	1.28	113479
3	Peak3	8.124	7079294	7.04	582611
4	Peak4	8.454	12547065	12.48	602821
5	Peak6	9.432	1289843	1.28	104465
6	Peak7	9.732	3239410	3.22	255289
7	Peak8	9.873	1728085	1.72	211715
8	Peak9	10.091	2714837	2.70	196766
9	Peak10	10.679	61650653	61.31	2944746
10	Peak11	11.342	8439439	8.39	422266



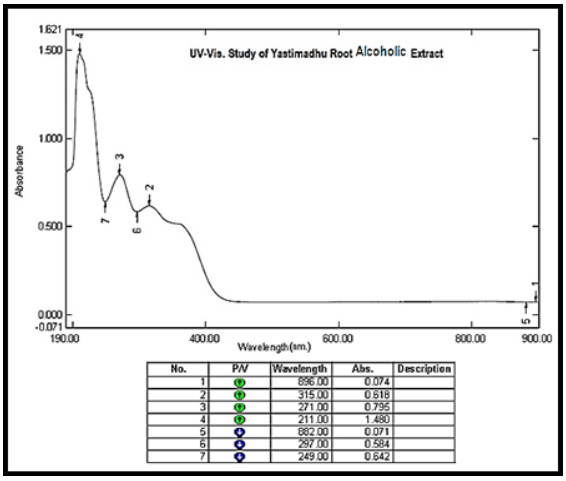
	Peak Name	RT	Area	% Area	Height
1	Peak1	3.094	206970	0.16	20084
2	Peak2	7.698	613700	0.46	59593
3	Peak3	8.130	2038494	1.54	209323
4	Peak4	8.476	1793133	1.36	160680
5	Peak5	9.762	815567	0.62	43339
6	Peak6	10.655	126368080	95.55	3225312
7	Peak7	16.037	416461	0.31	25242

Alcoholic Extract **Aqueous Extract**

Figure 7: HPLC Chromatographs at 276 nm



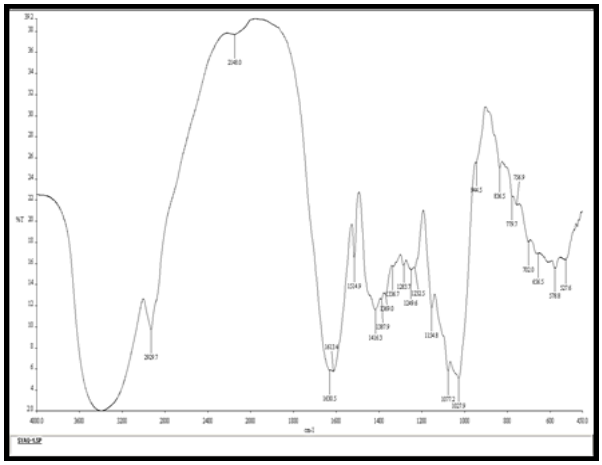
No.	P/V	Wavelength	Abs.	Description
1	▲	315.00	0.165	
2	▲	265.50	0.261	
3	▲	194.50	0.793	
4	▼	244.00	0.217	

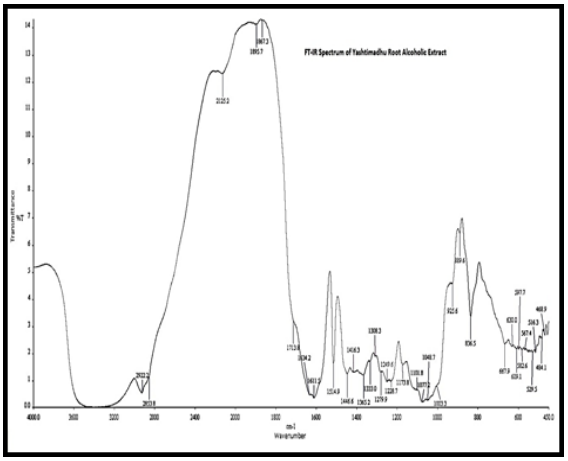


No.	P/V	Wavelength	Abs.	Description
1	▲	896.00	0.074	
2	▲	315.00	0.618	
3	▲	271.00	0.795	
4	▲	211.00	1.480	
5	▼	882.00	0.071	
6	▼	297.00	0.584	
7	▼	249.00	0.642	

Aqueous Extract **Alcoholic Extract**

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





Aqueous Extract **Alcoholic Extract**

Figure 9: FT-IR spectrum of Aqueous & Alcoholic extract of research drug

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 Ayurvedic drugs are generally seen as safe and free from side effects. Many pharmaceutical companies are now concentrating on manufacturing of quality Ayurvĕdic phyto-pharmaceutical products while ensuring the 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. [2] 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 Yashthimadhu (*Glycyrrhiza glabra* Linn.) extends to the treatment of peptic ulcer, hepatitis C, pulmonary and skin diseases, although clinical and experimental studies suggest that it has several other useful pharmacological properties such as anti-inflammatory, antiviral, antimicrobial, anti-oxidative, anticancer, immunomodulatory, hepato-protective and cardio-protective activities.

A large number of components have been isolated f rom liquorice, including triterpenes and saponins, flavonoids, isoflavonoids and chalcones, with glycyrrhizic acid normally being considered to be the main biologically active component. The hypocholesterolaemic and hypoglycemic activities of *Glycyrrhiza glabra* Linn. have been reported due to the presence of Isoflavan derivatives glabridin, hisplaglabridin A hisplaglabridin B and 4' O-methyl glabridin, which were reported to provide protection against oxidative stress.

While macroscopic examination indicated brown outside and yellow inside colour, sweet smell, rough surface and longitudinal small scales, about 12-16 inches' length and 1 inch diameter of roots, its powder looks shiny brown in colour. The microscopic analysis of powder showed the presence of Scleramatous type of parenchyma cells in groups, cells containing pigment, vessel with sceleriform orientation, pitted vessel, Crystal Sheath and Cork cells.

The total Ash value was 6.27% w/w which primarily consisted of water soluble ash (4.1%). The moisture content in the research formulation was found to be 8.8 % w/w while the pH value of 5.9 indicated its acidic nature. The extractive value of alcoholic and aqueous research formulation was found as 12.22% and 6.21% w/w while preliminary phytochemical analysis revealed the presence of flavonoids, carbohydrate, glycosides, tannin and saponin in both extracts. The results also showed high concentrations of flavonoidic compounds (27.40 µg Quercetin equivalent/ mg of extract) and phenolic content (29.50 µg Gallic acid equivalent / mg of extract) in the alcoholic extract as compared to 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 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 five different compounds in aqueous extract while ten compounds were observed in the alcoholic extract. Further analysis with database of standard compounds confirmed the presence of Ellagic acid at RT 9.75 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 315, 265.5 and 194.5 nm, corresponding to three different compounds whereas alcoholic extract showed four peaks at 896, 315, 271 and 211 nm, corresponding to four different compounds. Out of these observed peaks, only one peak at 315 nm was found 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 2929.7, 1387.9, 1369.0, 1232.5 and 756.9 cm^{-1} indicating the presence of the functional groups C-H stretching, Aryl -CH_3 in-phase bending, -CH_3 in-phase bending, Aryl out-phase stretching and Aryl 5 adjacent C-H wagging respectively. At the same time, the alcoholic extract revealed characteristic peaks at 1416.3, 1365.2, 1333.0, 1308.3 and 1279.9 cm^{-1} indicating the presence of (N)-CH_3 in-phase bending, Tertiary butyl-CH_3 in-phase bending, (S)-CH_3 in-phase bending, (S)-CH_3 in-phase bending and -NO_2 in-phase 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 phenolic & alkaloidal group of compounds.

5. CONCLUSION

Pharmacognostical analysis indicates high concentration of flavonoids, carbohydrates, glycosides, tannin and saponin in both extracts. Higher concentration of flavonoidic compounds and phenolic content was observed in the alcoholic extract than the aqueous extract. Spectroscopic and chromatographic examination using UV-Visible, HPTLC and HPLC analysis indicated the presence of Ellagic acid in both these extracts. FTIR analysis indicated the presence of phenolic & alkaloidal group 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.

ACKNOWLEDGEMENTS

This research work was supported by grants from the Department of Science & Technology, Government of West Bengal (project sanctioned vide memo No: 940 (Sanc.) /ST/P/S&T/9G-11/2016 dated 10.1.2017) and the authors kindly acknowledge the same. The authors would also like to thank the project scientists and other staff members of Dravyaguna Laboratory of IPGAER Kolkata who extended their kind cooperation during this study.

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