IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
May 2017 Vol.:9, Issue:2

Pharmacognostic and Chemical Standardization of Vaginal Herbal Formulation Extracts Using Spectroscopy (UV-VIS & FTIR) and Chromatography (HPTLC, HPLC & GC-MS) Methods

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Mradu Gupta*, Nandita Karmakar, Saswati Sasmal

Institute of Post Graduate Ayurvedic Education and Research, 294/3/1, A. P. C. Road, Kolkata, India – 700009

Submission: 2 May 2017
Accepted: 7 May 2017
Published: 25 May 2017

Keywords: Ayurveda, vaginal herbal, spectroscopy, chromatography

ABSTRACT

A vaginal herbal formulation prepared by mixing dried stem barks of *Ficus glomerata* Roxb. and *Symplocos racemosa* Roxb. in equal amounts is standardized through pharmacognostical and phytochemical studies as a cure for leucorrhoea/vaginitis. While total ash value was 12.30%, acid insoluble ash was 0.77% and water soluble ash was 10.66%. Nitrogen and sulfur elements were found present. Alkaloids, tannins, flavonoids and carbohydrates were found present in alcoholic and aqueous extracts. The results also showed high concentration of flavonoidic compounds (74.76 µg Quercetin equivalent/mg) in the alcoholic extract and high phenolic content (225.67 µg Gallic acid equivalent/mg) in the aqueous extract. UV-Visible spectroscopy scanning showed peaks at 277 & 197 nm in aqueous and at 279 & 213 nm in alcoholic extract. HPTLC analysis at 280 and 360 nm indicated the presence of pyrogallol and quercetin aqueous & alcoholic extract. Similarly, HPLC analysis at 272 and 360 nm showed elution of 4-5 compounds at different retention times whose analysis confirmed the presence of Gallic acid in aqueous and Resorcinol in alcoholic extract. FTIR analysis indicated presence of C-H stretching, carbonyl C=O stretching, C=C stretching, C=C stretching, CH_2 bending, (CH_3) symmetric deformation, C-O broad stretching, C-N group and O-H (H-bonded) stretching functional groups suggesting the presence of amides, aldehydes, alcohol, carboxylic acids and phenols. Eluted chemicals during GC-MS revealed elution of 1,3-Dimethyl-3,5-di(cyanoethyl)piperidone-4, 1,3-Dimethyl-4-amino-4,5(1H)-dihydro-1,2,4-triazol-5-one,Cyclopentanolnitrate and 6,7-Dimethyl-triazolo(4,3-b)(1,2,4)-triazine, which have antimicrobial, antibacterial, anti-inflammatory, antifungal and analgesic properties.

1. INTRODUCTION

Plants have been used globally since ancient times as a valuable and safe natural source of medicines and as agents of therapeutic utility. Most herbal plants possess pharmacological principles which make them useful as curative for numerous ailments. According to the World Health Organization (WHO) reports, 70-80% of the world population confides in traditional medicine for primary healthcare. From the inception of civilization, human beings have relied on plants that could meet their basic necessities such as food, shelter, clothing, fuel and health. Of all the uses ascribed to plants, their curative abilities played an important part in the lives of primitive societies as plants comprised their sole source for healing ailments. The sacred knowledge about the healing powers of plants was initially passed down orally through generations but later as civilizations grew written records were prepared for the benefit of the population.

In the Ayurvedic system of Indian medicine, three types of medicines have been prescribed, either as single drug or as combination of drugs, for prevention and treatment of diseases – those of plant origin, mineral origin or animal origin. The research Ayurvedic vaginal herbal formulation has been newly prepared by adding equal amounts of dried parts of the stem bark of *Ficus glomerata* Roxb. and *Symplocos racemosa* Roxb because these two plants have been used since ancient times in the Ayurvedic system of medicine and elaborated in ancient texts such as Charak Samhita (Chikitsa Sthanam) as an astringent, anti-inflammatory & haemostatic and useful for arresting excessive abnormal vaginal discharge (Shastri 1988, Sharma 1995, Sharma *et al.* 2001). This is a new herbal formulation which has not been evaluated till now although it is likely to exhibit sustained and significant antimicrobial action due to the synergetic effect of the phenolic and flavonoidic compounds present in this research drug and the pharmacological properties of its constituent herbs.

This research formulation contains the plant *Ficus glomerata* Roxb. or Cluster Fig which belongs to the Moraceae family. It is a moderate sized spreading lactiferous tree without much prominent aerial roots found throughout India whose fruits are eaten by villagers. Its leaves are dark green, ovate or elliptical while the fruits contain 2-5 cm diameter sub-globose & smooth receptacles. The fruits are orange & dull reddish when ripe and having a pleasant smell. The stem bark is 0.5-1.8 cm. thick, grayish-green in color and having an uneven soft surface (Sharma 1995, Sharma *et al.* 2001). On rubbing it, white papery flakes come out from the outer surface; the inner surface is light brown, fracture fibrous and mucilaginous taste.

The stem bark, fruits, leaves and latex of this plant have been used since ancient times as mentioned in the Ayurvedic text book for treatment of dysentery, diarrhoea, toothache, stomachache, vaginal disorders, menorrhagia, haemoptysis, diabetes, piles and glandular swelling, etc. The roots of the plant are used in dysentery, pectoral complications, and diabetes, and also applied in inflammatory glandular enlargement, mumps, and hydrophobia. The latex is externally applied on wounds to decrease inflammation, pain and edema and to promote healing. The phytochemical compounds isolated from the stem bark are leucocyanidin-3-o-B-glucopyranoside, leucopelarogonidin-3-O-a-L-rhamnopyranoside, B-sitosterol, stigmasterol, tetracyclic triterpene-gluanol acetate and tiglic acid. The reported pharmacological properties of the different plant parts are hypoglycemic, antiulcer, antioxidant, wound-healing, anti-inflammatory, anti-diarrhoeal, antibacterial, antifungal, antipyretic and antidiuretic (Joshi and Mohini 2008).

Symplocos racemosa Roxb. known as Lodhra belonging to the Symplocaceae family is found distributed throughout North Eastern India up to 2,500 ft. elevation. It is a small evergreen tree with stem up to 6 m in height and 15 cm in diameter. Its stem bark is useful in bowel complaints such as diarrhea& dysentery, in dropsy, eye disease, liver complaints, wound healing, excessive vaginal discharge, menstrual problems, fevers, ulcers, scorpion-string, etc. The bark is often employed in the preparation of plasters and is reported to promote maturation or resolution of boils, stagnant tumors and other malignant growths. A decoction of the bark or wood is used as gargle for giving firmness to spongy and bleeding gums and relaxed uvula. The phytochemical investigation of the n-butanol soluble fraction of the bark of stem of *Symplocos racemosa* Roxb yielded two phenolic glycosides of salirepin series namely symplocuronic acid and sympocemoside while salirepin has also been isolated from this plant (Ahmad *et al.* 2003). The alcohol extract of stem bark indicated the presence of carbohydrates, glycosides, saponins, terpenoids & alkaloids while its ether extract indicated the presence of glycosides, phytosterol and steroids. The prominent pharmacological activities of its stem bark are antibacterial, anti-inflammatory, antiulcer, antitumor, antimicrobial and antioxidant (Devmurari 2010).

The objective of this study is to provide a standardized quality Ayurvedic herbal vaginal formulation in the form of vaginal tablet for treatment of vaginitis or leucorrhea in order to arrest abnormal excessive vaginal discharge. Therefore, the pharmacognostical, chemical, spectroscopy (UV- Visible spectroscopy & FTIR) and chromatography (HPTLC, HPLC and

GC-MS) analysis were done before proceeding to prepare the Ayurvedic vaginal tablet. The pharmacognostical and phytochemical studies following the guidelines of Ayurvedic pharmacopeia and ICMR Guidelines were conducted for standardization of the herbal drug. In this study, the aqueous and alcoholic (ethanol) extracts of the research formulation were standardized by using different types of instruments to ascertain the presence of chemical compounds which responsible for its antimicrobial and anti-inflammatory pharmacological activities for curing excessive abnormal vaginal discharge.

2. MATERIALS & METHODS

The stem barks of *Symplocos racemosa* Roxb. and *Ficus glomerata* Roxb. 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 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).

2.1. Pharmacognostical analysis

2.1.1. Macroscopic and microscopic study of powder

The stem barks of both the plants were thoroughly washed, air-dried and preheated in oven before being powdered in a grinding machine to 120 # mesh particle size. The research formulation was prepared by mixing equal amounts of stem bark powder of both 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. Physiochemical 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, 3 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 (*Denston*, 1946). 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 daylight, short (254 nm) and long (365 nm) ultraviolet radiations. The colors 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. 20 ml of distilled water was taken in a mortar and pestle, 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 barks of both the plants were washed, air-dried and preheated in oven before being powdered in a grinding machine to 40# mesh particle size and stored in an airtight container. Powdered dried barks of the plants were mixed in an equal ratio and this coarse powder was 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 and pressure using rotary evaporator. All obtained extracts were stored in refrigerator below 10°C for subsequent experiments (Furniss *et al.* 1989). 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 chemical groups of compounds such as saponins, tannins, alkaloids, flavonoids, glycosides, carbohydrates, oils and fats, proteins and amino acids following the standard methods.

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 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).

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 through 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). 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 traveled by the spot/distance traveled 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 = 5:4:1)

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, its parameters are given below and results are discussed later.

Plate : Precoated 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 quantitation were 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 used for chromatographic separation of the research drugs – Method I (binary gradient method of Acetonitrile & 0.1% Phosphoric acid in Water) and 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, 5 µm, 4.6x250 mm

Run Time : 30 minutes

Injection Volume : 20 µl

Wavelength (Dual) : 272 nm & 360 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.

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 wavenumbers were scanned in the entire range of 400 nm to 4000 nm to find out the functional groups in different wavenumbers 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, Hydraulic pellet press Type KP, serial no. 814, Mfg. by Kimaya engineers, Thane, Maharashtra. 5 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).

2.2.5.3. 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 to their mass-to-charge ratio (m/z). These spectra can then be stored on the computer and analyzed (Oregon State University 2012).

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 280ºC. 1 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 (Eltayeib and Ismaeel 2014).

3. RESULTS

3.1. Pharmacognostical analysis

3.1.1. Macroscopic and microscopic study of powder

During macroscopic examination, the stem bark of *Ficus glomerata* Roxb. was noticed to be 0.5 cm thick & very hard having outer surface rough & coarse having exfoliating plaques and grayish brown in color while the inner surface was reddish brown in color as shown in fig. 1. Its powder was deep brown in color and having sweet to astringent taste.

The stem bark of Lodhra (*Symplocos racemosa* Roxb) was thin, glabrous, about 0.2 cm width golden brown in color having transverse cracks found all over the surface while the inner surface was dark brown in color with rough surface (fig. 2). The taste of the powder was slightly sweetish at the beginning and bitter later.

The macroscopic examination of powder of the research formulation indicated that it was light brown in color having more fibers and astringent taste as shown in fig. 3. The microscopic analysis indicated the presence of starch granules, trichomes, vessel overlap with parenchyma patches, pitted vessels, crystals and parenchyma patches containing tannin as shown in fig. 4.

Fig. 1: Stem Bark of *Ficus glomerata*

Fig. 2: Stem Bark of *Symplocos racemosa*

Fig. 3: Macroscopic view of research powder

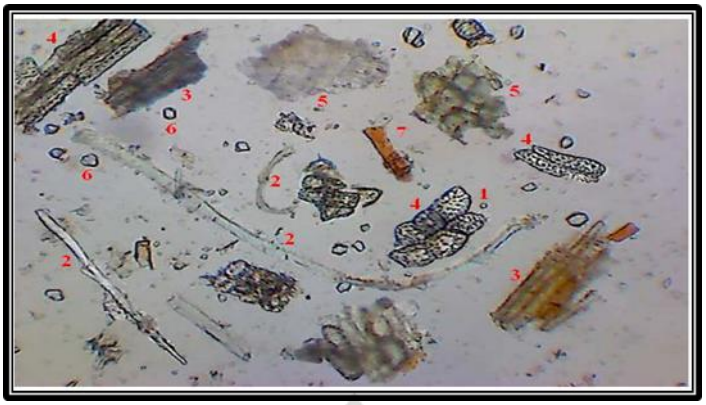


Fig. 4: Microscopic examination of research powder - 1. Starch Granules, 2. Trichome, part of trichome, 3. Vessel overlap with parenchyma patches, 4. Pitted Vessel, 5. Parenchyma patches, 6. Crystal, 7. Parenchyma patches containing tannin.

3.1.2. Physiochemical analysis

3.1.2.1. Determination of pH value, ash value and moisture content

The obtained results have been shown in table 1 below.

Table 1: Results of the basic physio-chemical study

Parameter	Value
Total Ash value (in % w/w)	12.30
Acid insoluble Ash (in % w/w)	0.77
Water soluble Ash (in % w/w)	10.66
pH value	5.29
Moisture Content (in % w/w)	8.2

3.1.2.2. Fluorescence analysis

The findings of the fluorescence analysis have been summarized in table 2 below.

Table 2: Results of fluorescence analysis

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

3.1.2.3. Elemental analysis

The results of the elemental analysis are shown in table 3 below. During this study, Nitrogen and Sulphur elements were found present in the research formulation.

Table 3: Results of elemental analysis

Test	Observation	Inference
Prussian-blue test	Prussian blue colour is found	Nitrogen is present
Lead Acetate test	Black ppt is found	Sulfur is present
Nitroprusside test	No violet or purple colour	Sulphur is not present
Silver nitrate test	No ppt.	Cl, Br or I- not present
Ammonium Molybdate test	No canary yellow ppt.	Phosphorus is not present

3.2. Chemical analysis

3.2.1. Continuous extraction of research formulation

The values obtained during sequential extraction of the research drug are highlighted in table 4.

Table 4: Extractive values of research formulation

Solvent	Petroleum-Ether	Ethyl acetate	Chloroform	Acetone	Alcohol	Aqueous
Extractive Value (in % w/w)	0.846	0.926	0.165	1.25	1.70	1.64

3.2.2. Preliminary phytochemical screening

The outcome of the preliminary phytochemical analysis highlighting the presence of major chemical compounds in the various extracts is shown in table 5. The alkaloids, flavonoids, tannins and carbohydrates were found to be present in both the aqueous and alcoholic extracts while saponins were found present only in the aqueous extract. The fixed oil and fats were found to be present in petroleum ether, ethyl acetate and chloroform extracts.

Table 5: Results showing the presence of various phytochemical 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						
Liebermann- Burchard test	–	–	–	–	–	–
	–	–	–	–	–	–
Proteins & Amino acids						
Ninhydrin reagent	–	–	–	–	–	–
Fixed oils and fats						
Saponification test	+	+	+	–	–	–
Spot Test	+	+	–	–	–	–

3.2.3. Determination of total phenol content and total flavonoid content

The total flavonoid content (TFC) and total phenol content (TPC) was calculated from the absorbance calibration curve generated with different concentrations of Quercetin and Gallic acid respectively which is shown in Table 6.

Table 6: Estimation of Total Flavonoid Content and Total Phenol Content

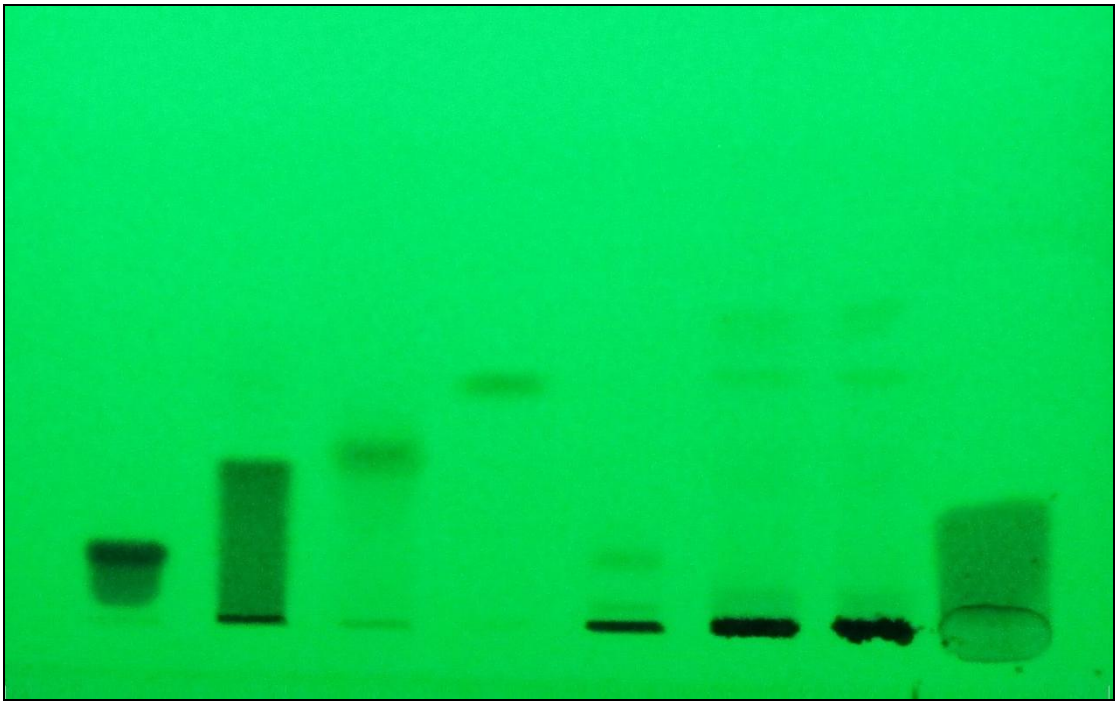
	Alcoholic Extract	Aqueous Extract
Flavonoid content (µg Quercetin equivalent/mg of extract) following the standard curve (R^2 =0.999)	74.76	59.14
Phenol content (µg Gallic acid equivalent/mg of extract) following the standard curve (R^2 =0.997)	220.00	225.67

3.2.4. Chromatography

3.2.4.1. High performance thin layer chromatography (HPTLC)

The visualization of plate for Method IV is shown in figure 5 while the comparison of Rf values obtained during the HPTLC analysis are shown in table 7.

Figure 5: Visualization of plate during HPTLC analysis



Track 1 Track 2 Track 3 Track 4 Track 5 Track 6 Track 7 Track 8

Visualized at 254nm wavelength

Track 1 = Standard Gallic Acid solution

Track 2 = Standard Quercetin solution

Track 3 = Standard Pyrogallol solution

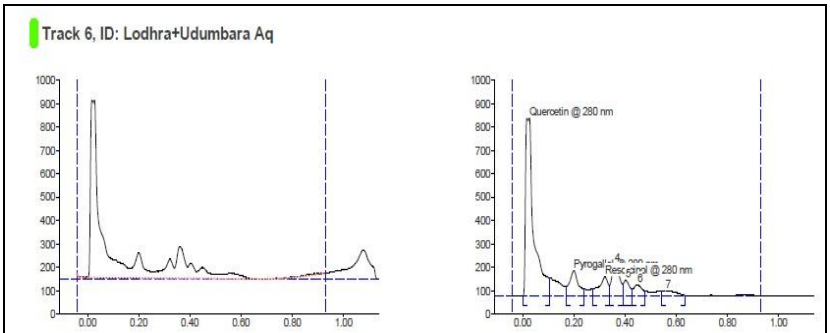
Track 4 = Standard Resorcinol solution

Track 5 = Standard Tannic Acid solution

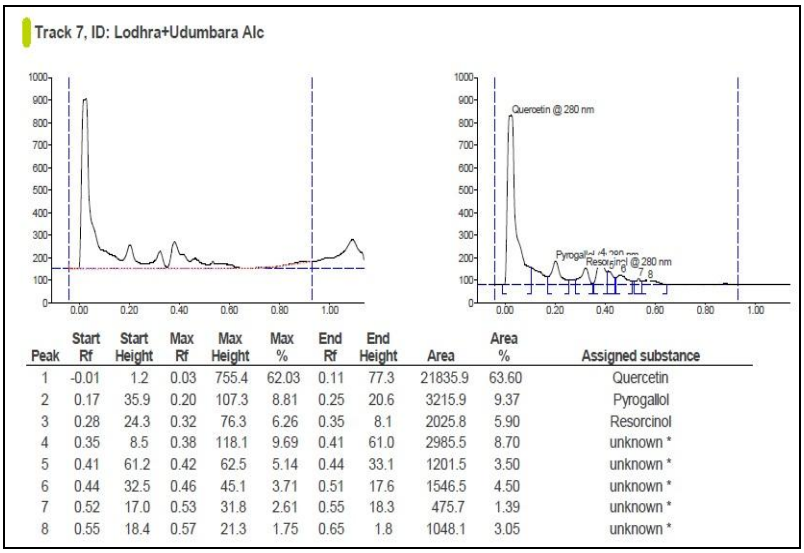
Track 6 = Aqueous extract of research formulation

Track 7 = Alcoholic extract of research formulation

Track 8 = Standard Ellagic Acid solution



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.00	0.4	0.03	760.6	62.19	0.11	75.7	20095.4	61.11	Quercetin
2	0.17	39.4	0.20	107.7	8.80	0.24	29.7	3182.5	9.68	Pyrogallol
3	0.28	29.6	0.32	81.3	6.65	0.34	43.5	2364.8	7.19	Resorcinol
4	0.34	44.4	0.36	136.9	11.19	0.39	49.8	3566.6	10.85	unknown *
5	0.39	50.8	0.40	66.7	5.45	0.43	30.2	1273.8	3.87	unknown *
6	0.43	30.6	0.45	47.1	3.85	0.48	20.5	1346.5	4.09	unknown *
7	0.54	19.3	0.56	22.7	1.86	0.63	1.6	1055.6	3.21	unknown *



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.01	1.2	0.03	755.4	62.03	0.11	77.3	21835.9	63.60	Quercetin
2	0.17	35.9	0.20	107.3	8.81	0.25	20.6	3215.9	9.37	Pyrogallol
3	0.28	24.3	0.32	76.3	6.26	0.35	8.1	2025.8	5.90	Resorcinol
4	0.35	8.5	0.38	118.1	9.69	0.41	61.0	2985.5	8.70	unknown *
5	0.41	61.2	0.42	62.5	5.14	0.44	33.1	1201.5	3.50	unknown *
6	0.44	32.5	0.46	45.1	3.71	0.51	17.6	1546.5	4.50	unknown *
7	0.52	17.0	0.53	31.8	2.61	0.55	18.3	475.7	1.39	unknown *
8	0.55	18.4	0.57	21.3	1.75	0.65	1.8	1048.1	3.05	unknown *

Table 7: Comparative analysis of *Rf* values of aqueous & alcoholic extract

Rf value at 280 nm			Serial no.	*Rf* value at 360 nm		
Standard	**Aqueous extract**	**Alcoholic extract**		**Standard**	**Aqueous extract**	**Alcoholic extract**
	0.08	0.08	1			0.09
Quercetin	0.18	0.18	2	Quercetin	0.17	0.17
Pyrogallol	0.23	0.22	3	Pyrogallol	0.23	0.21
Resorcinol	0.30	0.30	4	Resorcinol		
	0.39	0.37	5			
	0.46	0.45	6		0.47	
	0.50		7		0.50	
	0.54	0.53	8			
		0.90	9			
	0.94		10			
	0.97		11			

3.2.4.2. High Performance Liquid Chromatography (HPLC)

The chromatogram of aqueous and alcoholic extracts of research formulation obtained during HPLC analysis at 272 nm and 360 nm wavelengths is shown in figures 6 and 7 while the comparative position of RT values is detailed in table 8.

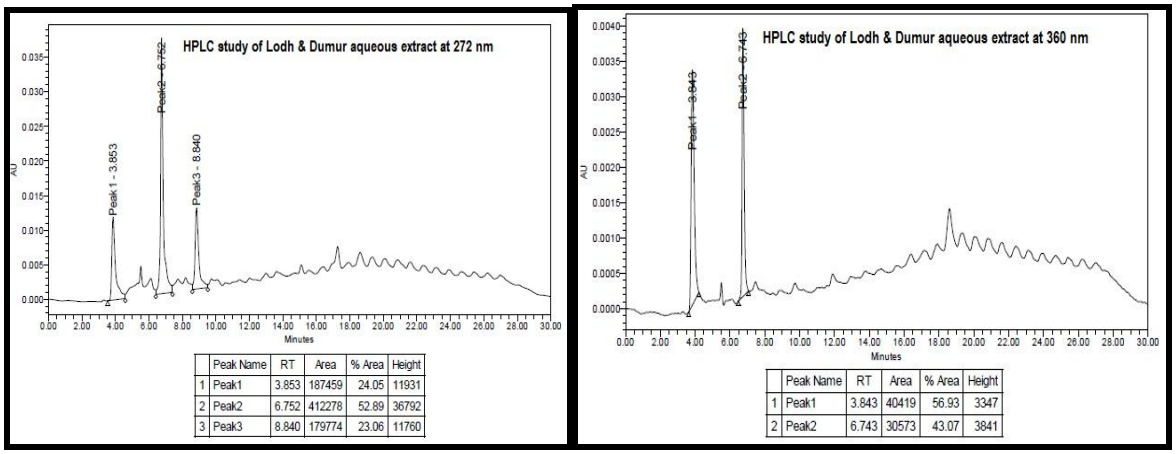
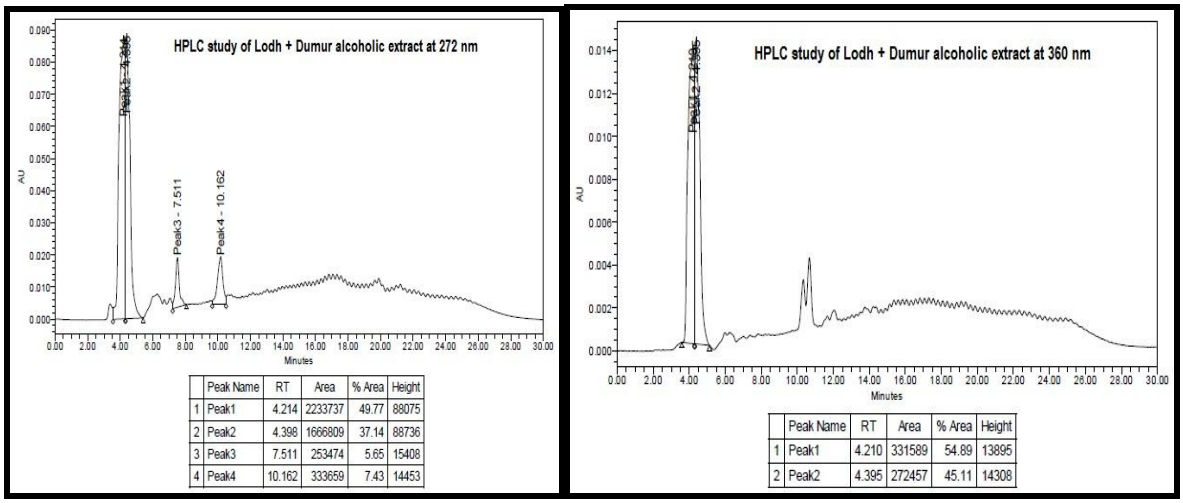


Figure 6: HPLC chromatogram of aqueous extract at 272 nm and 360 nm wavelength



	Peak Name	RT	Area	% Area	Height
1	Peak1	4.214	2233737	49.77	88075
2	Peak2	4.398	1666809	37.14	88736
3	Peak3	7.511	253474	5.65	15408
4	Peak4	10.162	333659	7.43	14453

	Peak Name	RT	Area	% Area	Height
1	Peak1	4.210	331589	54.89	13895
2	Peak2	4.395	272457	45.11	14308

Figure 7: HPLC chromatogram of alcoholic extract at 272 nm and 360 nm wavelength

Table 8: Comparative analysis of RT values of aqueous & alcoholic extracts

RT value at 272 nm			Sr. No.	RT value at 360 nm		
Standard	**Aqueous extract**	**Alcoholic extract**		**Standard**	**Aqueous extract**	**Alcoholic extract**
Gallic acid	3.853		1	Gallic acid	3.843	
		4.214	2			4.210
		4.398	3			4.395
	6.752		4		6.743	
Resorcinol		7.511	5			
	8.840		6			
		10.162	7			

3.2.5. Spectroscopy

3.2.5.1. UV- Visible Spectroscopic Study

The obtained results during Ultraviolet – Visible spectroscopy are shown in figure 8 and table 9.

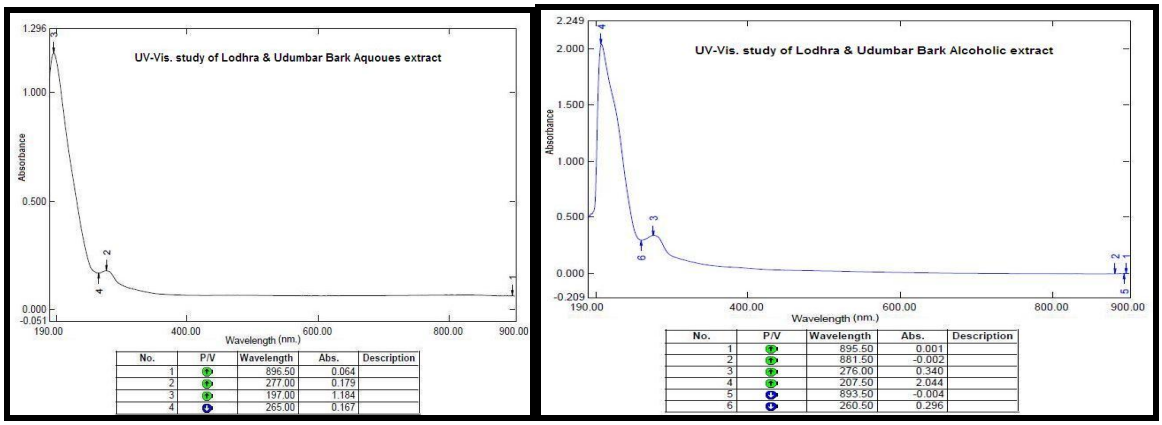


Figure 8: UV- Visible spectroscopic scanning of aqueous & alcoholic extracts

Table 9: Comparative analysis of UV-Visible spectroscopy data

Wavelength	Absorbance of aqueous extract	Wavelength	Absorbance of alcoholic extract
277.0	0.179		
		276.0	0.340
		207.5	2.044
197.0	1.184		

3.2.5.2. Fourier Transform Infrared (FTIR) spectroscopy

The FTIR spectrograms of the aqueous and alcoholic extracts are shown in figures 9 and 10 while the location of observed peaks along with their wave numbers representing possible functional group of compounds is elaborated in table 10.

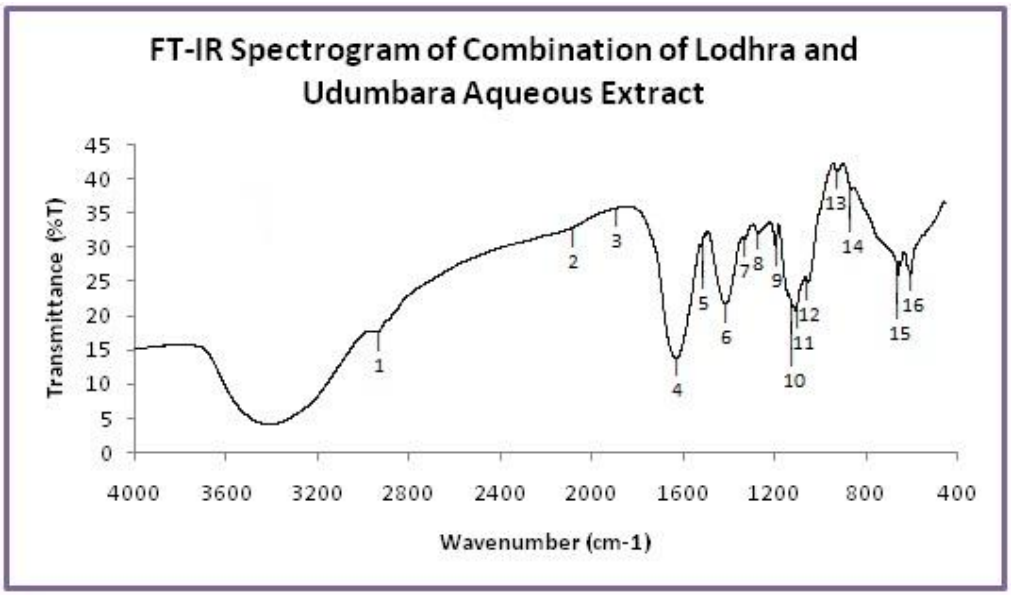


Figure 9: FTIR fingerprinting of aqueous extract

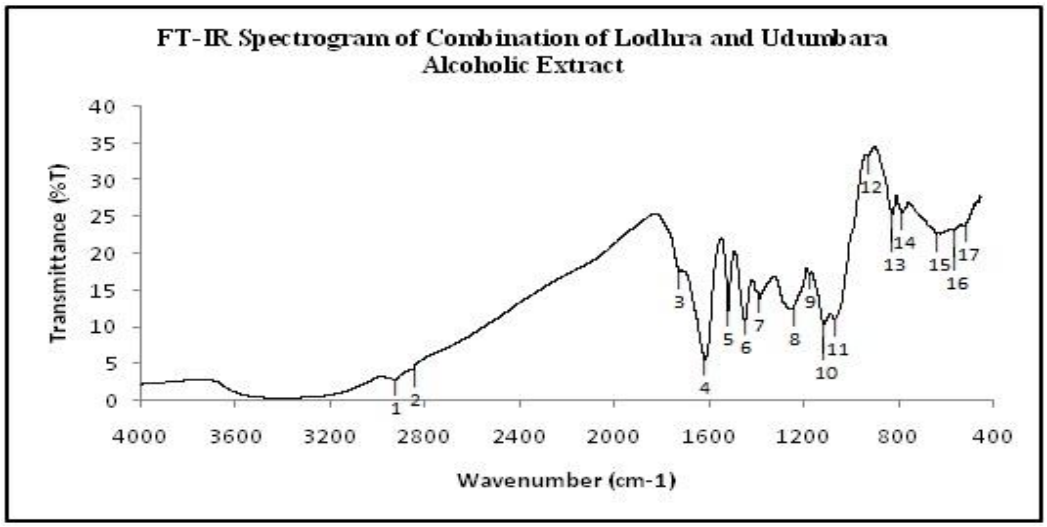


Figure 10: FTIR fingerprinting of alcoholic extract

Table 10: Wave numbers and possible functional groups representing the observed peaks of separated compounds in FTIR analysis

Aqueous Extract			**Alcoholic Extract**	
Wavenumber (cm^{-1})	**Possible Functional Group**	**Sr. no.**	**Wavenumber (cm^{-1})**	**Possible Functional Group**
2941.1	CH_2 Out of phase stretching	1		
		2	2925.9	-
		3	2853.8	C-H Stretching
2121.4	Si-H Stretching	4		
1827.5	Carbonyl C=O Stretching Vibration	5		
		6	1721.4	Carbonyl (C=O) stretching
1626.7	C=C Stretching	7		
		8	1613.4	Ring Stretching
1518.7	**C=C stretching**	**9**	**1518.7**	**C=C Stretching**
		10	1446.6	CH_2 Deformation
1412.5	COO^{-} Symmetric Stretching	11		
		12	1384.1	(CH_3) Symmetric Deformation
1325.4	(CH)- Deformation	13		
1270.4	C=O Stretching	14		
		15	1249.6	C=S Stretching
1194.6	P-O-C Stretching	16		
		17	1173.8	PO_4^{2-} Out-Phase-Stretching
1124.5	C-O Stretching, C-C Stretching	18		
		19	1113.2	SO_3 Out-of-Phase Stretching
1103.6	C-O Stretching, C-C Stretching	20		
		21	1067.7	SO_3 in-Phase Stretching
1048.7	C-C-OH Deformation	22		
		23	925.6	Cyclohexane ring Vibrations
921.8	CH_2 Wagging	24		
861.1	CH_2 Wagging	25		

		26	821.3	-
		27	781.6	-
658.4	-	28		
		29	637.5	C-H Bend
601.5	-	30		
		31	565.5	-
		32	520.1	S-S Stretching

3.2.5.3. GC- MS analysis

The results of GC-MS study of the research formulation are shown as a Chromatogram in figure 11 while the chemical compounds identified, their chemical structure and properties are detailed in table 11.

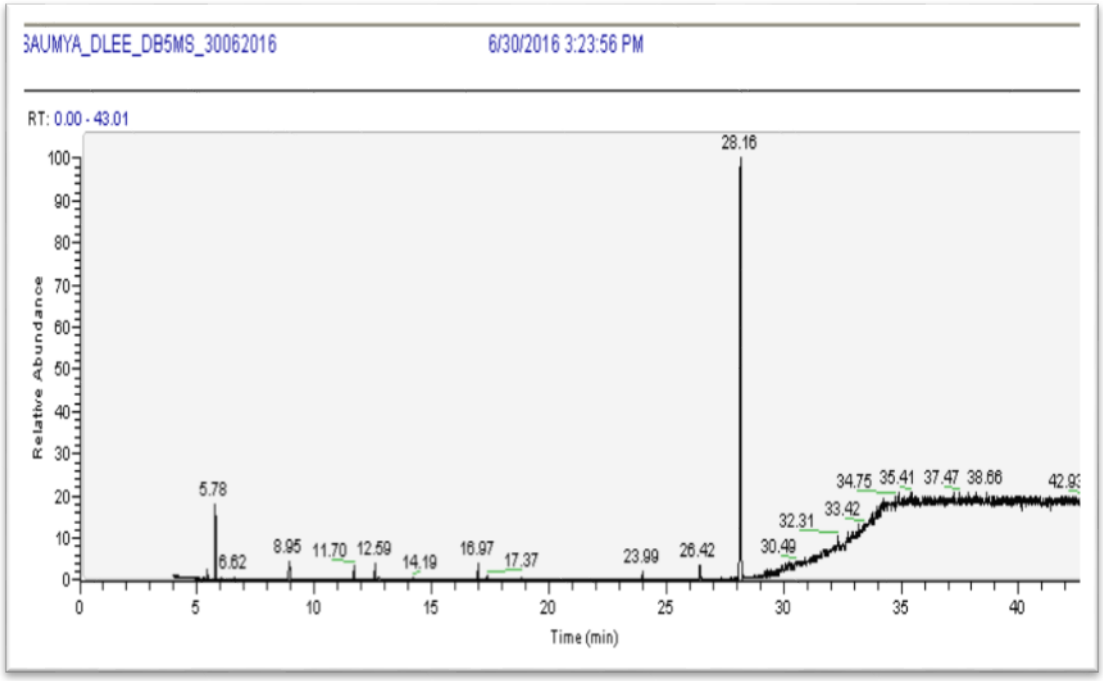
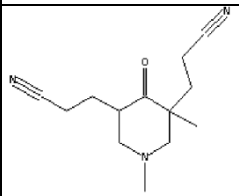
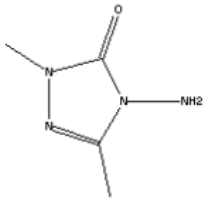
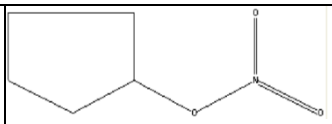
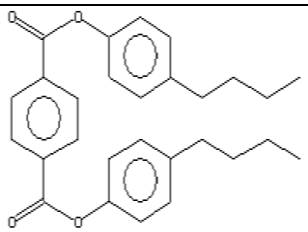
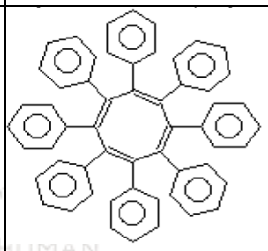
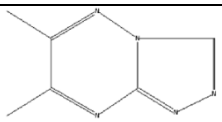
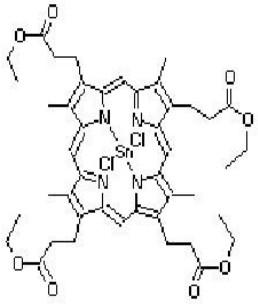
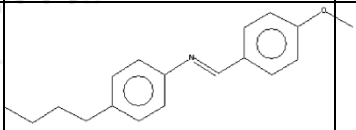
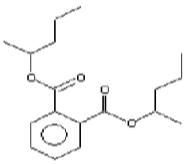


Figure 11: GC-MS Chromatogram

Table 11: Compounds identified and their properties obtained from GC-MS analysis

Sl. no.	RT	Name of the Compound	Molecular Formula	M.W.	Structure	Reported as
1	5.78	1,3-Dimethyl-3,5-di(cyanoethyl) piperidone-4	$C_{13}H_{19}N_3O$	233		It acts as a stabilizing agent in polymers. Reported as a potent corticotrophin-releasing factor-1 (CRF-1) receptor antagonist. Also described as a potent GABA inhibitor with selectivity towards insect versus mammalian receptors. Also used as has been recently reported as a potent antibacterial agent with a very broad spectrum
2	6.62	1,6-Heptadiyne	C_7H_8	92		
3	8.95	1,3-Dimethyl-4-amino-4,5 (1H)-dihydro-1,2,4-triazol-5-one	$C_4H_8N_4O$	128		The derivatives of the group with their amino derivatives are known as promising antimicrobial agents and they are being also screened for their antioxidant activities.

4	11.70	Cyclopentanol, nitrate	$C_5H_9NO_3$	131		It is useful as anti-inflammatory agents and the method of meliorating inflammation in mammals.
5	12.59	1,4-Benzene dicarboxylic acid, bis(4-butyl phenyl) ester	$C_{28}H_{30}O_4$	430		
6	16.97	1,3,5,7-Cyclooctatetraene, 1,2,3,4,5,6,7,8-octaphenyl-	$C_{56}H_{40}$	712		The derivative of cyclo-octate traene group has been used to construct artificial muscles.
7	17.37	6,7-Dimethyl-triazolo(4,3-b)(1,2,4)-triazine	$C_6H_7N_5$	149		These compounds have been found to exhibit the variety of biological applications such as antifungal, anti-HIV, anti-cancer, anti-inflammatory, analgesic and antihypertensive, cardiotonic, neuroleptic, nootropic, antihistaminic, tuberculostatic, antiviral, antiprotozoal,

						estrogen receptor modulators, antimalarial, cyclin-dependent kinase inhibitors, antimicrobial and anti-parasitic
8	23.99	Tetraethyl 2,7,12,17-tetramethyl-21H,23H-porphine-3,8,13,18-tetrapropionate tin(IY) dichloride	$C_{44}H_{52}Cl_2N_4O_8Sn$	954		
9	26.42	Benzenamine, 4-butyl-N-[(4-methoxyphenyl)methylene]-	$C_{18}H_{21}N_5O$	267		
10	28.16	Phthalic acid, bis (2-pentyl) ester	$C_{18}H_{26}O_4$	306		For container contamination

DISCUSSION

The World Health Organization estimated that there are 333 million new cases of curable vulvovaginal infections per year. A study in India has shown that the prevalence of reproductive tract infections was 37.0% based on symptoms and 36.7% by laboratory investigations, including 31% candidiasis, 3% gonorrhea, 2% trichomoniasis and 45% BV (Puri *et al.* 2009, Gupte *et al.* 2009). Vaginitis is an inflammation of the vagina that can result in discharge, itching and pain. The cause is usually a change in the normal balance of vaginal

bacteria or an infection. Leucorrhoea refers to the medical condition where excessive abnormal thick and sticky white or yellowish discharge occurs from the vagina accompanied with inflammation & associated with symptoms like itching, burning sensation and pain followed by body ache and tiredness.

Many herbal plants and their combinations in the nature of Ayurvedic drugs have been prescribed for oral administration and external application in the Ayurvedic text for the treatment of vaginitis or leucorrhoea. The aim of the present study was to standardize the new vaginal herbal formulation before preparing the herbal vaginal tablet by mixing the equal parts of stem barks of *Ficus glomerata* Roxb. and *Symplocos racemosa* Roxb. because both these plants are having antimicrobial and astringent properties.

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. While macroscopic examination indicated brown color, smooth texture, pungent sweet smell and astringent taste, microscopic analysis showed the presence of parenchyma cells, crystals, tracheids and fibers in the powder. The total Ash value was 12.30 % w/w which was relative high possibly due to presence of carbonate, sulfate & silicate compounds. The moisture content in the research formulation was found to be 8.2 % w/w while the pH value of 5.29 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 1.70 and 1.64 % w/w while preliminary phytochemical analysis revealed the presence of alkaloids, flavonoids, tannins and carbohydrates in both extracts. The results also showed high concentration of flavonoidic compounds (74.76 µg Quercetin equivalent/mg of extract) in the alcoholic extract and high phenolic content (225.67 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 UV-Visible spectroscopy scanning during chemical analysis of aqueous and alcoholic extracts of the research formulation showed two peaks at 277nm & 197nm in the aqueous extract and two peaks at 279 and 213 nm in case of the alcoholic extract which indicated the presence of two main compounds, out of which one compound may be same due to

separation of these compounds within very close wavelengths and high concentration shown in the alcoholic extract.

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 in four different types of solvent systems and maximum separation of compounds was found in the solvent system Toluene: Chloroform: Methanol: Formic acid = (7.0: 5.0: 1.5: 0.5) when scanning was done at 280 nm and 360 nm wavelengths. During analysis at 280 nm, 10 spots were found in the aqueous extract and 8 spots in the alcoholic extract of the research formulation which showed the presence of two standard chemical compounds, pyrogallol and quercetin at R_f values of 0.08 and 0.18 respectively. Similarly, chromatographic examination at 360 nm wavelength revealed the presence of phenolic compounds like pyrogallol in the aqueous extract and quercetin in the alcoholic extract at similar R_f values.

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. HPLC analysis at 272 nm and 360 nm showed the elution of 4 to 5 compounds at different retention times in the aqueous and alcoholic extracts. The obtained RT values of these eluted chemical compounds in the aqueous and alcoholic extract were compared with those of the standard phenolic compounds indicating the presence of maximum three standard compounds. Further analysis confirmed the presence of Gallic acid at RT 3.853 in the aqueous extract at both wavelengths while Resorcinol standard compound was found present in the alcoholic extract at RT 7.511. The presence of phenolic compounds in both 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 comparative data on the peak values with wave numbers and the possible functional groups during FTIR analysis of the two extracts of the research formulation are presented in table. The aqueous extract exhibited different characteristic bands at 2941 cm^{-1}, 2121.4 cm^{-1}, 1827 cm^{-1}, 1626 cm^{-1}, 1518 cm^{-1}, 1412 cm^{-1}, 1325 cm^{-1}, 1270 cm^{-1}, 1194 cm^{-1}, 1124 cm^{-1} and 1103 cm^{-1} indicating the presence of the functional groups C-H stretching, C≡C group, carbonyl C=O stretching vibration, C=C stretching, C=C stretching, C-H bending, CH-deformation, C-O stretching, C-O stretching, C-O stretching and C-C stretching respectively.

At the same time, the alcoholic extract revealed characteristic peaks at 2853 cm^{-1}, 1721 cm^{-1}, 1613 cm^{-1}, 1518 cm^{-1}, 1446 cm^{-1}, 1384 cm^{-1}, 1249 cm^{-1}, 1173 cm^{-1} and 1113 cm^{-1} indicating the presence of C-H stretching, carbonyl C=O stretching, C=C stretching, C=C stretching, CH_2 bending, (CH_3) symmetric deformation, C-O broad stretching, C-N group and O-H (H-bonded) stretching functional groups respectively. It may be inferred that the aqueous and alcoholic extracts of research formulation exhibited almost similar types of functional groups on the basis of range of wave numbers and indicated the presence of amides, aldehydes, alcohol, carboxylic acids and phenolic groups of compounds in the extracts.

The spectrum of GC-MS analysis of an alcoholic extract of the research formulation revealed the presence of nine chemical compounds on the basis of retention times which are directly characteristic of certain compounds. Most of these separated chemical compounds have been reported for their pharmacological activities on the basis of their molecular formula and chemical structure that could contribute to the medicinal quality of the extract. While the main eluted chemical compound of the research formulation was 1,3-Dimethyl-3,5-di (cyanoethyl)piperidone-4 which is reported as a potent antibacterial agent, another compound 1,3-Dimethyl-4-amino-4,5(1H)-dihydro-1,2,4-triazol-5-one is directly related to antimicrobial agents and antioxidant activities. Cyclopentanol nitrate is reported for the presence of its anti-inflammatory agents and for ameliorating inflammation in mammals, whereas 6,7-Dimethyl-triazolo(4,3-b)(1,2,4)-triazine exhibited a variety of biological applications such as antifungal, anti-HIV, anti-cancer, anti-inflammatory, analgesic and antihypertensive, cardio-tonic, neuroleptic, nootropic, anti-histaminergic, tuberculostatic, antiviral, antiprotozoal, estrogen receptor modulators, antimalarial, cyclin-dependent kinase inhibitors, antimicrobial and antiparasitic activities. The overall analysis indicated that the research formulation is having potent antibacterial, antimicrobial, anti-inflammatory, analgesic and antipyretic properties as revealed by its isolated chemical compounds.

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 Indian Council of Medical Research (Sanction letter No. 59/1/2011/BMS/TRM, dated 12.12.2013), Government of India and the

authors are grateful for this support. We are thankful to the Botanical survey of India, Howrah, Kolkata for identification and other scientific help during the research work.

REFERENCES

1. Ahmad VU, Abbasi MA, Hussain H, Akhtar MN, Farooq U, Fatima N and Choudhary MI. Phenolic glycosides from *Symplocos racemosa*: natural inhibitors of phosphodiesterase I. Phytochemistry, 2003; 63(2): 217-220.
2. Ayurvedic Pharmacopoeia of India, Ministry of Health & Family Welfare, Government of India, 2001.
3. Baba SA and Malik SA. Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii*. Journal of Taibah University for Science, 2015; 9: 449-454.
4. Chang C, Yang M, Wen H and Chern J. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Anal. 2002; 10: 178–182.
5. Denston TC. A textbook of Pharmacognosy. Sir Isaac Pitman & Sons Ltd. London, 1946.
6. Devmurari VP. Antibacterial Evaluation and Phytochemical Screening of S*ymplocos racemosa* Roxb. International Journal of Pharm Tech Research, 2010; 2(2): 1359-1363.
7. Eltayeib A A and Ismaeel H U. Extraction of *Cyperus rotundus* Rhizomes Oil, Identification of Chemical Constituents and Evaluation of Antimicrobial Activity of the Oil in North Kordofan State. International Journal of Advanced Research in Chemical Science, 2014; 1(9):18-29.
8. Furniss BS, Hannaford AJ, Smith PWG and Tatchell AR. Vogel's Textbook of Practical Organic Chemistry, 5th Edition, Addison Wesley Longman Inc. 1989, 1205.
9. Gupte P, Patil S and Pawaskar R. News and Filler: Vulvovaginal hygiene and care, Indian Journal of Sexually Transmitted Diseases and AIDS, 2009; 30 (2): 130-13.
10. Joshi U and Mohini U. Evaluation of Antioxidant Activity of Aqueous Extract Bark of *Ficus glomerata*. Research Journal of Pharmacy and Technology, 2008; 1(4): 537-538.
11. Oregon State University, GC-MS – How does it work? Unsolved Mysteries of Human Health, 2012. http://unsolvedmysteries.oregonstate.edu/MS_05.
12. Puri KJ, Madan A and Bajaj K. Incidence of various causes of vaginal discharge among sexually active females in age group 20-40 years. Indian J Dermatol Venereol Leprol 2003; 69:122-125.
13. Sharma P. Cinnamic acid derivatives: A new chapter of various pharmacological activities. J. Chem. Pharm. Res, 2011; 3(2): 403-423.
14. Sharma PC, Yelne MB and Dennis TJ. Database on medicinal plants used in Ayurveda, Central Council for Research in Ayurveda & Siddha, Department of Indian system of medicine, Govt. of India, New Delhi, 2001, vol. 3, 76-87, 166.
15. Sharma PV. Introduction to Dravyaguna (Indian Pharmacology), vol. 2, Chaukhamba Orientalia, Varanasi, India, 1995, pp. 616, 666.
16. Shastri SN. Charak Samhita (Chikitsa Sthanam), Chapter 3, Chaukhamba Bharati Academy, Varanasi, India, 1988, p. 47.
17. Skoog D A, Crouch SR and Holler FJ. Principles of instrumental analysis. Edition 6th. Belmont, CA, United Kingdom, Thomson Brooks/Cole, 2007.