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STANDARDIZATION OF THE HYDRO-ALCOHOLIC EXTRACT OF AYURVEDIC VAGINAL FORMULATION (NA) BY USING CHROMATOGRAPHY (HPTLC, HPLC) AND SPECTROSCOPY (UV-VIS & FTIR & GC-MS) METHODS

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ABSTRACT: The research herbal formulation prepared by mixing dried stem barks of *Azadirachta indica* A. Juss. and *Saraca asoca* Roxb. in equal amounts is standardized through pharmacognostic and phytochemical studies for assessing its efficacy in the treatment of leucorrhoea/vaginitis/ excessive white discharge. Pharmacognostical analysis revealed total ash value of 9.43% having 1.89% acid insoluble ash, 4.62 pH and 7.70% moisture content and presence of nitrogen and halogens. Flavonoids, tannins, carbohydrates, glycosides, saponins and fixed oils were found present in the hydro-alcoholic extracts. The results also showed a high concentration of flavonoids compounds (43.37 µg quercetin equivalent/mg) and high phenolic content (101.22 µg gallic acid equivalent/mg). UV-Visible spectroscopy scanning showed a prominent peak at 277 nm in the hydro-alcoholic extract. HPTLC analysis at 254 nm, 277 nm, and 330 nm indicated the presence of tannic acid, gallic acid, quercetin and catechol. Similarly, HPLC analysis at 277 nm suggested the presence of gallic acid, tannic acid and catechol. FTIR analysis exhibited the presence of N-H stretching, C-H stretching, N-H bending, N-O asymmetric stretching, C-C stretching, C-H rocking, C-N stretching, C-O stretching, C-H wagging, C-N stretching, C-Cl stretching, C-H bending and C-Br stretching functional groups suggesting the presence of amines/amides, alkenes, nitro group, aromatics, alcohol/ carboxylic acids/esters/ethers, alkyl halides, and aliphatic/ aromatic amines. Eluted chemicals during GC-MS revealed the presence of nimbiol; 13-Methoxypodocarpa-8, 11,13-trien-3-one; Podocarpa-8, 11, 13-triene, 14-isopropyl-13-methoxy; 8-Chloro-5-quinolinecarboxylic acid; and 7-Chloro-2-cyclohexyl-4[3H]quinazolinone which has antimicrobial, antibacterial, anti-inflammatory, antifungal and analgesic properties validating the efficacy of the research formulation.

INTRODUCTION: Standardization using objective parameters ensures that products are reliable in terms of quality, efficacy, performance, and safety. Hence, every single medicinal herb needs to be quality checked to ascertain that it conforms to quality requirement and delivers the properties consistently.

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The chemistry of plants involves the presence of therapeutically important constituents since the active principles extracted from the plants are purified for therapeutic utility due to their selective pharmacological activity. In order to meet the new thrust of inquisitiveness, standardization of herbals is mandatory and, therefore, the quality control of

herbal crude drugs and their constituents is of great importance in the modern system of medicine [1]. The research Ayurvedic vaginal herbal formulation has been prepared by adding equal amounts of the dried parts of the stem bark of *Azadirachta indica* A. Juss. and *Saraca asoca* Roxb. since 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 & hemostatic and useful for arresting excessive abnormal vaginal discharge [2, 3, 4]. This is a new herbal vaginal formulation which has not been evaluated in the form of vaginal tablets till now although it is likely to exhibit sustained and significant antimicrobial action due to the synergetic effect of the phenolic and flavonoids compounds present in this research drug and the pharmacological properties of its constituent herbs.

Nimba (*Azadirachta indica* A. Juss.), the versatile medicinal plant belonging to Meliaceae family, is the unique source of various types of compounds having diverse chemical structures. Every part of the tree has been used as traditional medicine for against various human ailments from antiquity. The importance of the nimba tree has been recognized by the US National Academy of Sciences, which published a report in 1992 titled 'Nimba – a tree for solving global problems'. The medicinal utilities have been described especially for leaf, fruit, and bark. Nimba oil and the stem bark and leaf extracts have been therapeutically used in Ayurveda to control leprosy, intestinal helminthiasis, respiratory disorders, blood morbidity, biliary afflictions, itching, various skin diseases, burning sensations and phthisis and also as a general health promoter.

Its fruits and seeds are the sources of nimba oil. The isoprenoids include diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and csecomeliacins such as nimbin, salanin and azadirachtin. The non-isoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds, *etc*. It is typically grown in tropical and semi-tropical regions. While Azadirachtin has exhibited pesticide and anti-feedant activities, nimbidin, a major crude bitter principle extracted from the oil of seed kernels of this plant is having several biological activities. All parts of nimba tree are used as anthelmintic, anti-fungal, anti-diabetic, anti-bacterial, antiviral, contraceptive and sedative.

Ashoka (*Saraca asoca* Roxb.) of Leguminosae family has been very widely used in Indian system from times immemorial for the treatment of uterine, genital and other reproductive disorders in women, ailments of the urogenital tract, fever, and pain, *etc*. Charak has mentioned its properties in the ancient Ayurvedic textbook Charak Samhita under Vedanasthapan (analgesic, antipyretic and anti-inflammatory) category. It is an evergreen tree found almost throughout India up to an altitude of 750 m. This plant is medium-sized and about 30 feet high. Leaves are compound paripinnate, leaflets 4-6 pairs, oblong to lanceolate, glabrous, acute and short petiole. The stem bark is channeled, the outer surface is rough with warty protuberances, brown in color and the inner surface of the bark is smooth, reddish-brown. Flowers are orange or orange-yellow, eventually turning vermillion, a fragment in dense axillary corymbs. Fruits (Pods) are flat. Linear- oblong, leathery, 10-25 cm. long, seeds are 4-8 in numbers, 1.5 inches long ellipsoid-oblong, compressed. Flowering is in spring and fruiting in autumn seasons.

The stem bark of this plant in the Indian system of medicine has been used for its bitter, astringent, sweet, refrigerant, anthelmintic, styptic, stomachic, febrifuge, demulcent properties. It has a stimulating effect on the endometrium and ovarian tissue. It is useful in dyspepsia, fever, biliousness, burning sensation, colic dysentery, internal bleeding, hemorrhoids, ulcers, menorrhagia, uterine fibroid, and leucorrhoea. Flowers are also used in the burning sensation, hemorrhagic dysentery, and inflammation. Seeds are used as a diuretic and in vesical calculi diseases. The stem bark contains tannin, catechol, sterol, and organic calcium compounds, an essential oil, hematoxylin, a keto-sterol, crystalline glycoside constituents, a saponin and an organic iron compound, leucocynadin, quercetin. The oleic acid, stearic acid, palmitic acids are found in seeds. The pharmacological activities of stem bark are uterogenic, antibacterial, oxytocic, antitumor, anticancer, antiprogestational.

The stem bark of this tree is rich in tannins, flavonoids, steroids, volatile oil, glycosides, and various steroidal glycosides. Leaves contain various carbohydrates, tannins, gallic acid, and egallic acid. Flowers are rich in saracasin, saracadin, waxy substances, proteins, carbohydrates, and steroids. Seeds of this plant contain various fatty acids like oleic, linoleic, palmitic and stearic acid. Ashoka has cooling properties. Its pain-relieving action can help relieve painful dysmenorrhea, swelling, and pain at any site of the body. It is very commonly used in females to regularize hormones and menstrual cycles to improve the strength and stamina in young females having menstrual irregularities such as dysmenorrhea and leucorrhea.

The main aim of this research study was to evaluate the pharmacognostic, chemical, spectroscopy (UV-Visible & FT-IR spectroscopy) and chromato-graphy (HPTLC, HPLC and GC-MS Chromatography) characteristics before proceeding to prepare the formulation and standardization of the Ayurvedic vaginal tablet which will be prescribed to female patients having symptoms like vaginitis or leucorrhea (abnormal excessive vaginal discharge) during the clinical study. In this study, the hydro-alcoholic (70:30) extract of the research formulation having equal amounts of power of stem bark of *Saraca asoca* Roxb. and *Azadirachta indica* A. Juss was standardized using different types of instruments to ascertain the presence of chemical compounds which are responsible for its antimicrobial and anti-inflammatory pharmacological activities for curing excessive abnormal vaginal discharge following the guidelines of Ayurvedic pharmacopeia and ICMR.

MATERIALS AND METHODS: The stem barks of *Saraca asoca* Roxb. and *Azadirachta indica* A. Juss. were purchased from the 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/09 & IPGAE&R/ Dravyaguna/M.Gupta/10 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 M/s Merck Specialities Pvt. Ltd., and chloroform, ethyl acetate, ascorbic acid, acetylsalicylic acid, catechol, ellagic acid, and benzoic acid were purchased from M/s Nice Chemicals Pvt. Ltd. The pharmacognostic and chemical analysis of the research formulation has been done following the protocols of drug standardization mentioned in the Ayurvedic Pharmacopoeia of India (2001) [5].

Pharmacognostical Analysis:

Macroscopic and Microscopic Study of Powder: The stem barks of both the plants were thoroughly washed, air-dried and pre-heated in the 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 a microscope (Dewinter, Italy) to find out the characteristics of the various cell structures.

Physio-Chemical Analysis:

A) 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 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; the residue was then 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.

B) Fluorescence Analysis: Fluorescence analysis is one of the essential parameters for assessing the quality and standardization of plant samples during pharmacognostic studies where the plant parts are examined 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. 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 min. 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.

C) 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 pastel, the red-hot test tube was broken and ground in mortar distilled water. The aqueous solution was filtered through Whatman-40 filter paper and the filtrate was subjected to test for these elements.

Chemical Analysis:

A) Continuous Extraction of Research Formulation: The stem barks of both the plants were washed, air-dried and pre-heated in the 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. The hydroalcoholic extract was also prepared using the Soxhlet apparatus in the ratio of 70:30. These extracts were filtered using a Buckner funnel and Whatman-1 filter paper at room temperature and concentrated at reduced temperature and pressure using rotary evaporator.

All obtained extracts were stored in a refrigerator below 8 °C for subsequent experiments [6]. During this study, the hydro-alcoholic extract was standardized by using different types of instruments to assess the presence of chemical compounds which could be responsible for the antimicrobial and anti-inflammatory pharmacological activities required for curing the excessive abnormal vaginal discharge.

B) Preliminary Phytochemical Screening: The research extracts obtained from Soxhlet apparatus were subjected to preliminary phytochemical testing to detect the presence of a different chemical group of compounds such as saponins, tannins, alkaloids, flavonoids, glycosides, carbohydrates, oils and fats, proteins and amino acids following the standard methods.

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

The aluminum chloride [$AlCl_3$] method was used to determine the total flavonoid content (TFC). An aliquot of 0.5 ml of extract 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 5.0 ml. The test solution was shaken vigorously and absorbance at 415 nm was recorded after 30 min of incubation. A standard calibration plot was generated at 415 nm using different and known concentrations of quercetin. The concentrations of flavonoid in test samples were calculated from the calibration plot and expressed as mg quercetin equivalent/gm of sample [7, 8].

D) Chromatography: Chromatography is the general name of a class of analytical methods for separation of the components of a molecular mixture by distributing the components between

two phases - a mobile phase passing over the stationary phase. The mobile phase separates the components in a mixture by adsorption and partitioning interactions with the stationary phase. In general practice, the separation is executed in chromatographic bed, in the form of a column (Column Chromatography) or on a thin layer (Thin Layer Chromatography). Analysis of pharmaceutical and natural compounds and newer drugs is commonly used in all the stages of drug discovery and development process.

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 expressed as distance traveled by the spot/distance traveled by the solvent.

Pre-coated silica gel $60F_{254}$ plate (10cm × 10cm) was used along with CAMAG Linomat 5 automated TLC applicator and CAMAG TLC scanner 3 equipped with WINCATS software. 100 mg/ml of research drug sample concentration was analyzed at 254, 277 & 330 nm wavelengths using toluene, ethyl acetate, formic acid & methanol in the ratio of 6:6:1.6:0.4, where the best separation of chemical compounds was observed after trying many other ratios.

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 were carried out using 515 HPLC pumps and 2489 UV/Visible Detectors of Waters Company while the software used was Empower. Symmetry C18, 5µm, 4.6 × 250 mm columns were used with 20 µl injection volume and 20 min run time at 1.0 ml/min flow rate. Acetonitrile & water in the ratio of 40: 60 was used as the mobile phase for conducting the analysis at 277 & 360 nm wavelengths.

E) Spectroscopy:

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 radiation under specific wavelengths were scanned in the entire range of 190-1100 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.

Fourier Transform Infrared (FTIR) Spectroscopy: The FTIR spectroscopy is used in analytical chemistry for determination of the presence of different functional groups; such as hydroxyl group, carboxyl group, *etc.* The IR-spectroscopic analysis is commonly carried out of solid samples, by preparing a transparent KBr disc by giving (7-10) Tons pressure. Liquid samples may also be studied using different sample accessories. The characteristics of molecules to pass infra-red radiations under specific wave-number were scanned in the range of 400 to 4000 nm to find out the functional groups in different wave-numbers on the basis of different peak values through data analysis.

Lyophilized dried powder of hydro-alcoholic extract of the research drug was used for FTIR analysis. 5 mg of dried extract powder was mixed with KBr to make the mass up to 100 mg and a transparent KBr disc was prepared by giving 7-10 Tons pressure using hydraulic pellet press. The pellet of each solid sample was loaded in FTIR spectroscopy (Perkin-Elmer Precisely Spectrum 100 FTIR Spectrometer), with a scan range of 400 to 4000 cm^{-1}.

The liquid samples were analyzed by H-ATR sampling accessory through ZnSe plate 45. [9]

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 by flowing an inert gas (mobile phase), which carries the sample, through a stationary phase fixed in column [10]. 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.

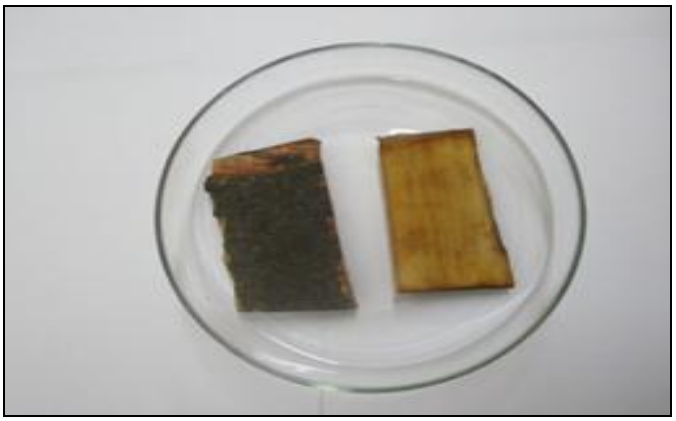
The GC-MS analysis was carried out on a PolarisQ Series paired with reliable TRACE GC Ultra instrument from M/s Thermo Electron Corporation at the Bose Institute, Kolkata using GCMS solution version 2.53 software. The alcoholic extract of the research formulation was analyzed with DB-5M column (30×0.25 mm). Initially, the oven temperature was maintained at 50 ºC for 2.0 min and then gradually increased up to 280 ºC. One µl of the 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 250 ºC 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 min [11].

RESULTS:

Pharmacognostical Analysis :

Macroscopic and Microscopic Study of Powder: During the macroscopic examination, the stem bark of *Azadirachta indica* A. Juss. (Nimba) was noticed to be 1.5 cm thick, blackish-brown color with longitudinal cracks in the rough hard coarse outer surface, light brown in color inner side as shown in **Fig. 1**, while its powder was deep brown in color, rough in texture, and has a bitter taste and pungent smell. The stem bark of *Saraca asoca* Roxb. (Ashoka) was dark brown in color, rough surface 1/2 inch in diameter and the inner side of the bark was yellow in color **Fig. 2** whereas its powder was deep brown-reddish in color and have an astringent taste.

FIG. 1: STEM BARK OF *AZADIRACHTA INDICA*



F IG. 2: STEM BARK OF*SARACA ASOCA*

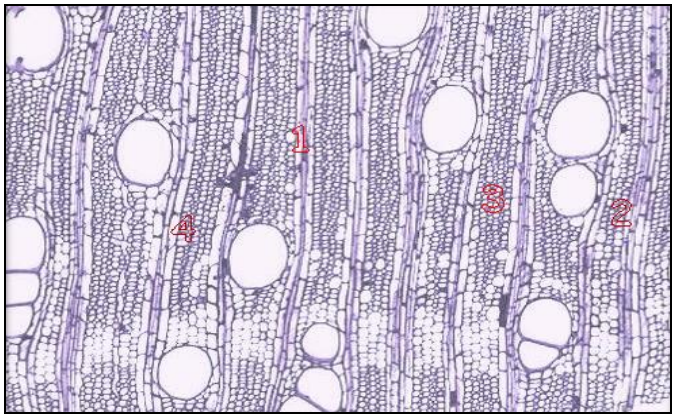


FIG. 3: MICROSCOPIC EXAMINATION OF T.S. OF STEM BARK OF *A. INDICA*. 1) Vacuoles; 2) Parenchymatous cells; 3) Medullary rays and 4) Xylem vessels

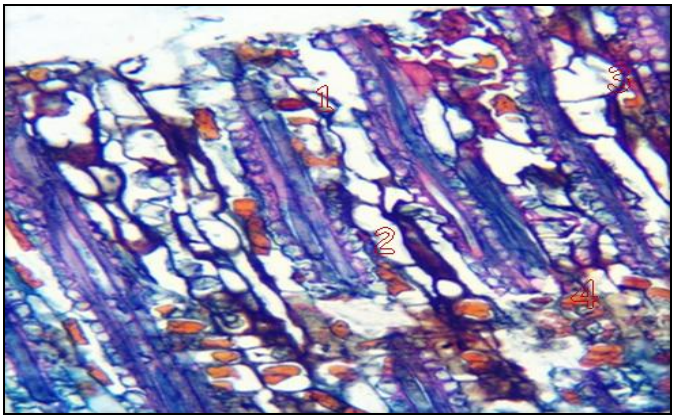


FIG. 4: MICROSCOPIC EXAMINATION OF T. S. OF STEM BARK OF *S. ASOCA*. 1) Vacuoles; 2) Parenchymatous cells; 3) Medullary rays and 4) Tannin containing blastocytes

The microscopic examination of the transverse sections of the stem bark of both these plants shown in **Fig. 3** and **4** revealed the presence of vacuoles, parenchymatous cells, medullary rays, and xylem vessels.

The macroscopic examination of powder of the research formulation indicated that it was light brown in colour having a bitter and astringent taste as shown in **Fig. 5**. The microscopic analysis indicated the presence of stone cells, oil containing granules, parenchyma patches containing tannin, etc. as shown in **Fig. 6**.

FIG. 5: MACROSCOPIC VIEW OF RESEARCH POWDER

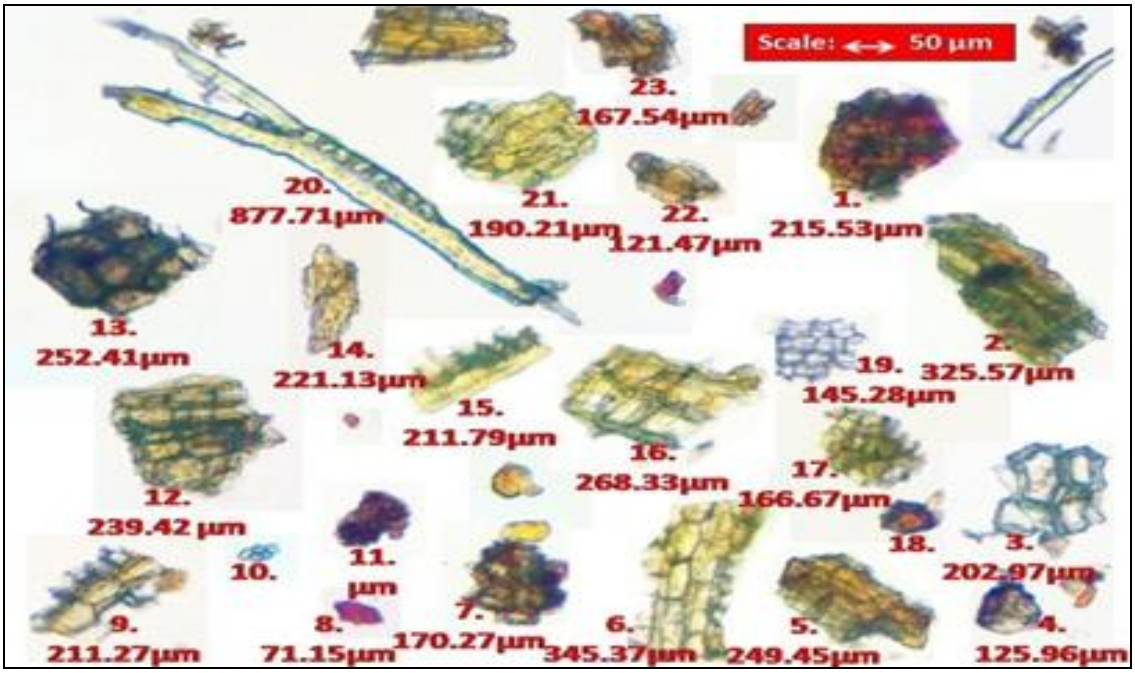


FIG. 6: MICROSCOPIC EXAMINATION OF RESEARCH POWDER. 1) Surface view of parenchymatous cells containing tannin; 2) Chlorophyll containing parenchymatous tissue; 3) Group of stone cells; 4) Surface view of stone cell; 5) Fragment of parenchyma; 6) Fragment of reticulate xylem; 7) Tannin containing stone cell; 8) Tannin pigment; 9) Fragment of phloem parenchyma; 10) Starch grain; 11) Sclerides; 12) Oil containing parenchyma cell; 13) Stone cells; 14) Oil containing granule; 15) Fragment of xylem (spiral); 16) Parenchymatous tissue; 17) Chlorophyll rich parenchymatous cell; 18) Lignified stone cell; 19) Cork fragment; 20) Fragment of Xylem fiber; 21) Epidermal cells; 22) and 23) Tannin containing stone cells.

Physio-Chemical Analysis:

A) 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)	9.43
Acid insoluble Ash (in % w/w)	1.89
Water soluble Ash (in % w/w)	7.65
pH value	4.62
Moisture Content (in % w/w)	7.70
Extractive value of hydro-alcoholic extract (in % w/w)	8.17

B) Fluorescence Analysis: The findings of the fluorescence analysis have been summarized in **Table 2** below.

C) Elemental Analysis: The results of the elemental analysis are shown in **Table 3** below. During this study, Nitrogen and Halogen elements were found present in the research formulation.

Chemical Analysis:

A) Continuous Extraction of Research Formulation: The values obtained during sequential extraction of the research drug are highlighted in **Table 4**.

TABLE 2: RESULTS OF FLUORESCENCE ANALYSIS

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

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

TABLE 4: EXTRACTIVE VALUES OF RESEARCH FORMULATION

Solvent	Petroleum-Ether	Ethyl acetate	Chloroform	Acetone	Alcohol	Aqueous	Hydro-alcoholic
Extractive value (in % w/w)	0.532	0.857	3.201	1.751	3.748	2.91	8.17

B) 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 hydro-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

Plant Constituents Test/ Reagents used	Petroleum ether extract	Ethyl acetate extract	Chloroform extract	Acetone extract	Alcohol extract	Aqueous extract	Hydroalcoholic extract
Alkaloids							
Mayer's reagent	-	-	-	-	-	-	+
Dragendroff's reagent	-	-	-	-	-	-	+
Waner'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	-	-	-	-	-	+	++
Proteins and Amino acids							
Ninhydrin reagent	-	-	-	-	-	-	-
Fixed oils and fats							
Saponification test	++	++	+	-	-	-	-
Spot test	+++	++	+	-	-	-	-
Liebermann-Burchard test							
Steroid test	-	+	-	-	-	-	-

+ → Present, - → Absent

C) Determination of Total Phenol Content (TPC) and Total Flavonoid Content (TFC): 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

Test Parameters	Hydro-alcoholic Extract
Flavonoid content (µg quercetin equivalent/mg of extract) following the standard curve (R^2=0.999)	43.37 µg/mg
Phenol content (µg gallic acid equivalent/mg of extract) following the standard curve (R^2=0.997)	101.22 µg/mg

D) Chromatography:

High Performance Thin Layer Chromatography (HPTLC): The visualization of plate for Method I is shown in **Fig. 7** while the comparison of R_f values obtained during the HPTLC analysis is shown in **Table 7**. The obtained chromatographs at 254, 277 and 330 nm are shown in **Fig. 8**, **9** and **10**.

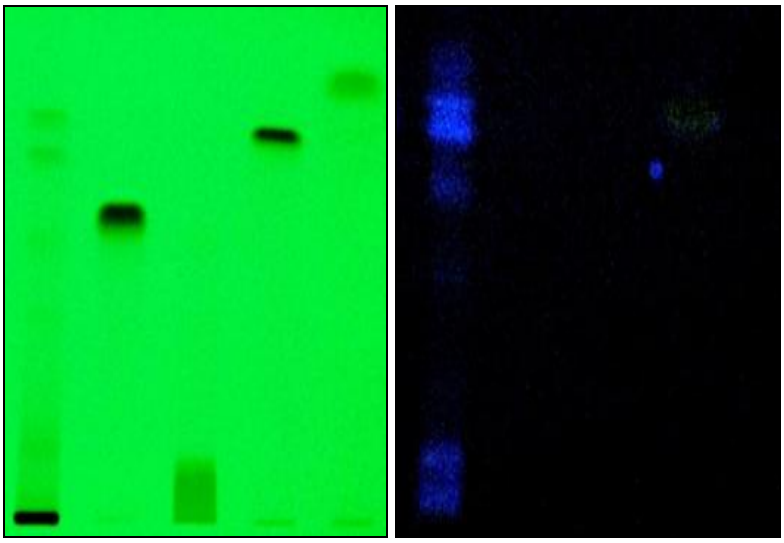
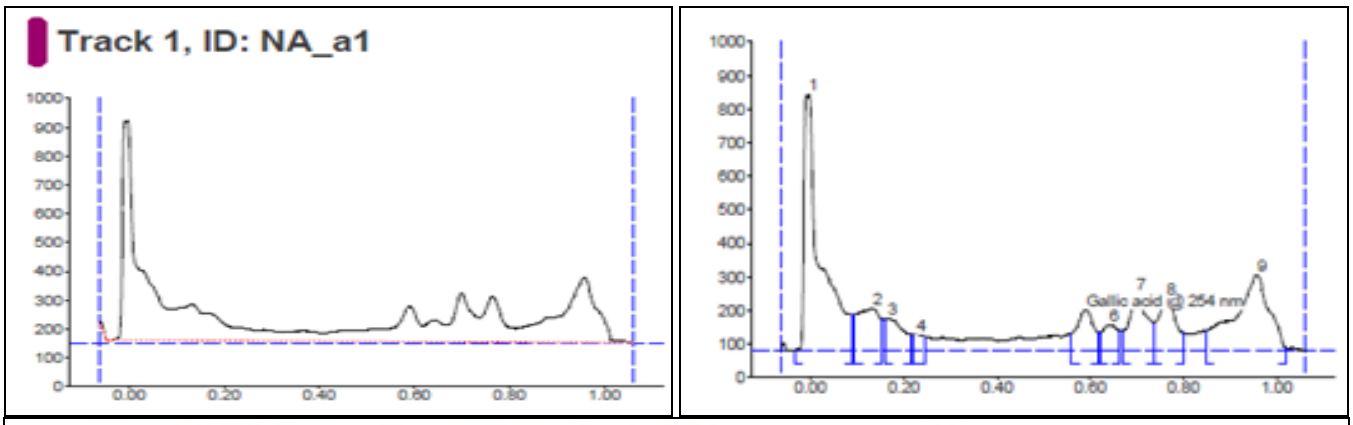
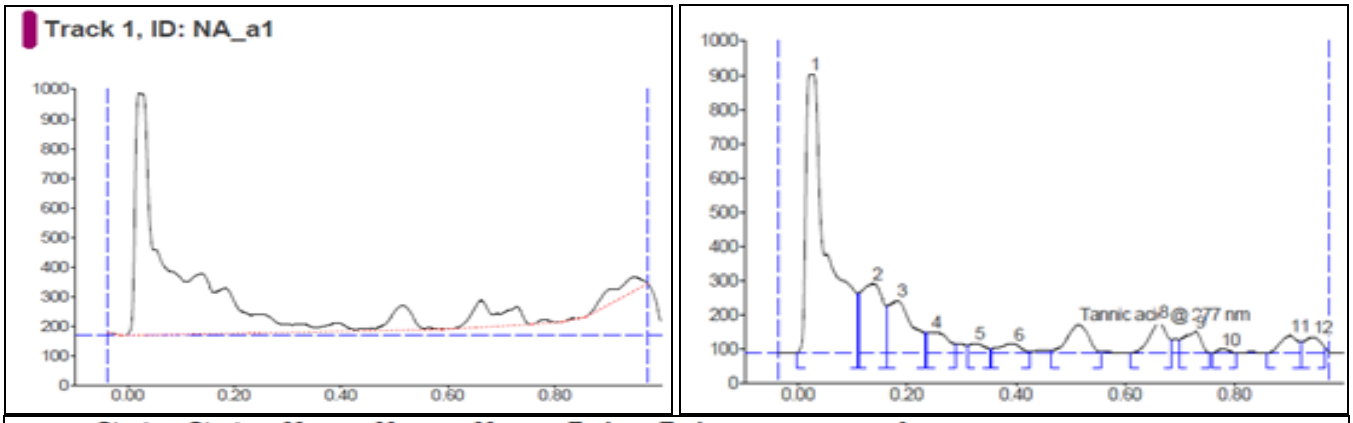


FIG. 7: VISUALIZATION OF PLATE FOR METHOD I. Tr 1 = Hydro-alcoholic Extract of group sample, Tr 2 = Gallic acid standard; Tr 3 = Tannic acid standard, Tr 4 = Quercetin standard; Tr 5 = Catechol standard



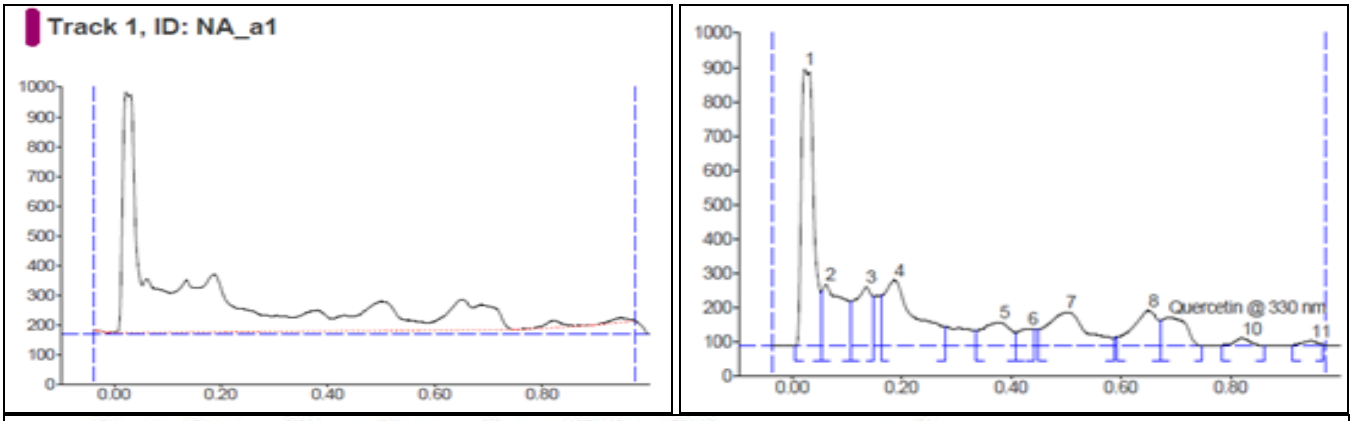
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.03	0.6	-0.00	766.5	42.79	0.09	106.9	24553.9	36.16	unknown *
2	0.09	107.8	0.13	127.2	7.10	0.15	94.2	5495.0	8.09	unknown *
3	0.16	92.6	0.17	97.1	5.42	0.22	44.9	3515.1	5.18	unknown *
4	0.22	45.5	0.23	48.5	2.71	0.25	41.2	1055.1	1.55	unknown *
5	0.56	50.5	0.59	123.0	6.87	0.62	55.1	4157.8	6.12	Gallic acid
6	0.62	56.1	0.64	76.6	4.27	0.66	59.4	2315.2	3.41	unknown *
7	0.67	59.4	0.70	170.8	9.54	0.74	82.5	5903.3	8.69	unknown *
8	0.74	82.6	0.76	157.5	8.79	0.80	53.2	5377.1	7.92	unknown *
9	0.85	58.7	0.96	224.2	12.51	1.02	7.5	15525.9	22.87	unknown *

FIG. 8: CHROMATOGRAPH AT 254 nm WAVELENGTH



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.00	0.4	0.02	815.1	49.80	0.11	176.0	30199.6	50.67	unknown *
2	0.11	176.3	0.14	203.2	12.41	0.16	139.9	7849.6	13.17	unknown *
3	0.16	140.2	0.18	153.6	9.38	0.23	60.0	6270.5	10.52	unknown *
4	0.24	60.6	0.25	60.7	3.71	0.29	27.5	2254.1	3.78	unknown *
5	0.31	25.4	0.32	28.4	1.74	0.35	14.7	845.6	1.42	unknown *
6	0.35	14.8	0.40	27.9	1.70	0.43	5.9	1119.0	1.88	unknown *
7	0.46	7.9	0.52	82.9	5.06	0.56	4.1	3101.7	5.20	Tannic acid
8	0.61	0.0	0.66	90.4	5.52	0.69	40.6	2790.0	4.68	unknown *
9	0.70	40.5	0.73	62.4	3.81	0.76	0.3	1892.4	3.17	unknown *
10	0.76	1.4	0.78	12.8	0.78	0.80	2.3	272.5	0.46	unknown *
11	0.86	0.0	0.90	51.2	3.13	0.92	34.0	1590.7	2.67	unknown *
12	0.92	34.1	0.95	48.3	2.95	0.97	16.3	1419.4	2.38	unknown *

FIG. 9: CHROMATOGRAPH AT 277 nm WAVELENGTH



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.01	2.1	0.02	806.6	44.83	0.05	157.6	17130.9	28.56	unknown *
2	0.05	160.5	0.06	179.7	9.99	0.11	131.7	6598.7	11.00	unknown *
3	0.11	132.0	0.14	173.0	9.62	0.15	147.2	5106.5	8.51	unknown *
4	0.16	148.5	0.19	192.3	10.69	0.28	54.9	10528.6	17.56	unknown *
5	0.34	45.6	0.38	69.4	3.86	0.41	40.7	3374.2	5.63	unknown *
6	0.41	40.7	0.43	52.1	2.89	0.44	49.4	1396.5	2.33	unknown *
7	0.45	49.8	0.50	98.0	5.45	0.59	25.5	6753.8	11.26	unknown *
8	0.59	27.0	0.65	103.6	5.76	0.67	73.8	4462.3	7.44	unknown *
9	0.67	74.3	0.69	84.5	4.70	0.75	0.1	3441.7	5.74	Quercetin
10	0.79	2.8	0.82	24.1	1.34	0.86	1.1	714.4	1.19	unknown *
11	0.91	2.9	0.95	16.0	0.89	0.97	5.0	464.7	0.77	unknown *

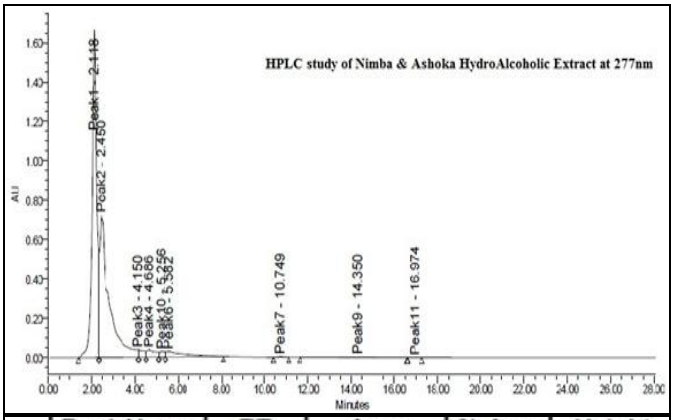
FIG. 10: CHROMATOGRAPH AT 330 nm WAVELENGTH

TABLE 7: COMPARATIVE ANALYSIS OF R_f VALUES

S. no.	Standards	R_f value of the extract Scanned at 254 nm	R_f value of extract Scanned at 277 nm	R_f value of the extract Scanned at 330 nm
1		0.13	0.14	0.14
2		0.17	0.18	
3		0.23	0.23	
4	Tannic acid (0.55)		0.52	
5	Gallic acid (0.59)	0.59	0.58	
6	Quercetin (0.71)	0.70		0.69
7	Catechol (0.78)	0.76		0.76
8		0.96	0.95	0.95

The R_f values scanned at 254, 277 and 330 nm wavelength showed that at s. no. 4, 5, 6 & 7 of hydro-alcoholic extract of NA (a sample) is quite similar to standard tannic acid, gallic acid, and quercetin respectively shown in comparative analysis in **Table 7**.

High Performance Liquid Chromatography (HPLC): The chromatogram of hydro-alcoholic extracts of research formulation obtained during HPLC analysis using Method-II-acetonitrile: water (40: 60) at 277 nm wavelengths are shown in Figure 11 below while the comparative position of RT values is detailed in **Table 8**. The RT values at 277 nm wavelength showed that s. no. 5, 6 & 7 of the hydro-alcoholic extract are quite similar to standard gallic acid, tannic acid, and catechol respectively.



	Peak Name	RT	Area	% Area	Height
1	Peak1	2.118	22814440	45.33	1663653
2	Peak2	2.450	23256249	46.21	722344
3	Peak3	4.150	739401	1.47	36605
4	Peak4	4.686	1000340	1.99	35866
5	Peak5	4.927			
6	Peak10	5.256	509173	1.01	28703
7	Peak6	5.582	1683037	3.34	28270
8	Peak7	10.749	60993	0.12	3787
9	Peak8	11.850			
10	Peak9	14.350	246405	0.49	1459
11	Peak11	16.974	15979	0.03	796

FIG. 11: HPLC CHROMATOGRAM AT 277 nm WAVELENGTH

TABLE 8: COMPARATIVE ANALYSIS OF RT VALUES OF EXTRACT WITH STANDARDS

S. no.	RT values at 277 nm	RT values of Standard
1	2.118	
2	2.345	
3	4.150	
4	4.686	
5	5.256	Gallic acid (5.399)
6	5.582	Tannic acid (5.705)
7	10.749	Catechol (10.894)
8	14.350	
9	16.974	

E) Spectroscopy:

UV- Visible Spectroscopic Study: The obtained results during Ultraviolet-Visible spectroscopy are shown in **Fig. 12** and **Table 9**.

TABLE 9: UV-VISIBLE SPECTROSCOPIC ANALYSIS

Wavelength	Absorbance of hydro-alcoholic extract
1013.0	0.063
277.0	1.833
238.0	3.956

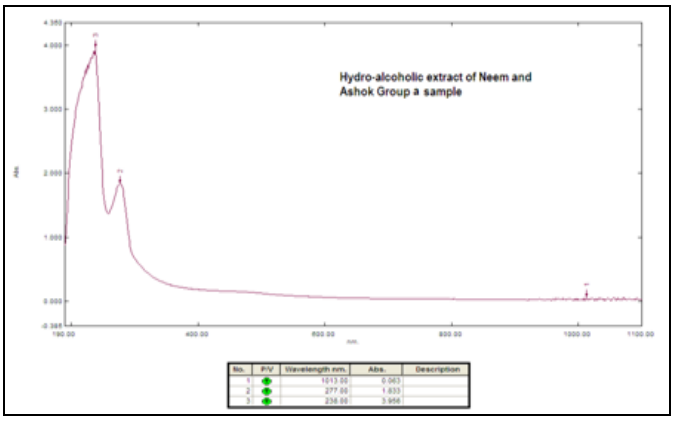


FIG. 12: UV- VISIBLE SPECTROSCOPIC SCANNING OF EXTRACT

Fourier Transform Infrared (FTIR) Spectroscopy: The FTIR spectrum of the Hydro-alcoholic extract is shown in **Fig. 13** while the location of observed peaks along with their wave numbers representing a possible functional group of compounds is elaborated in **Table 10**.

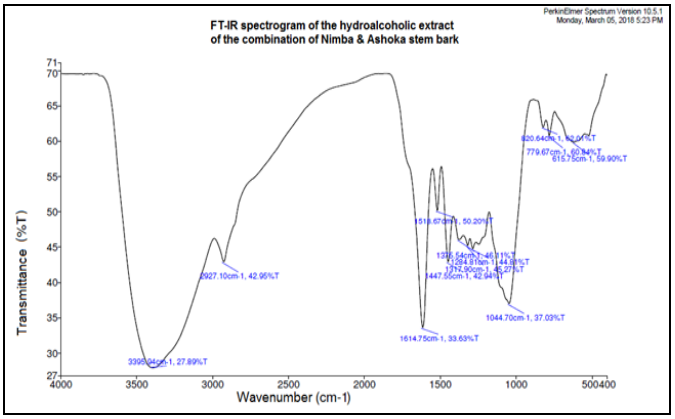


FIG. 13: FTIR FINGERPRINTING OF HYDRO-ALCOHOLIC EXTRACT

TABLE 10: WAVE NUMBERS AND POSSIBLE FUNCTIONAL GROUPS REPRESENTING THE OBSERVED PEAKS OF SEPARATED COMPOUNDS IN FTIR ANALYSIS

Peak no.	Transmittance (%T)	Wavenumber (cm^{-1})	Types of Vibration	Possible Functional Group
1	27.89	3395.94 (m)	N-H stretch	1^o, 2^o amines, amides
2	42.95	2927.10 (m)	C-H stretch	alkenes
3.	33.63	1614.75 (m)	N-H bend	1^o amines
4	50.20	1518.67	N-O asymmetric stretch	Nitro compounds
5	42.94	1447.55	C-C stretch(in-ring)	Aromatics

6	46.11	1375.54	C-H rock	alkenes
7	45.27	1317.90	C-N stretch	Aromatic amines
			C-O stretch	Alcohols, carboxylic acids, esters, ethers
8	44.81	1284.81	C-H wag(-CH_2X)	Alkyl halides
9	37.03	1044.70	C-N stretch	Aliphatic amines
10	62.01	820.64	C-Cl stretch	Alkyl halides
11	60.84	779.67	C-Cl stretch	Alkyl halides
12	59.90	615.75	-C≡C-H: C-H bend	Alkynes
			C-Br stretch	Alkyl halides

m=medium; w=weak; s=strong; n=narrow; b=broad; sh=sharp.

GC-MS Analysis: The results of GC-MS study of the research formulation are shown as a chromatogram in **Fig. 14** while the chemical compounds identified, their chemical structure and properties are detailed in **Table 11**.

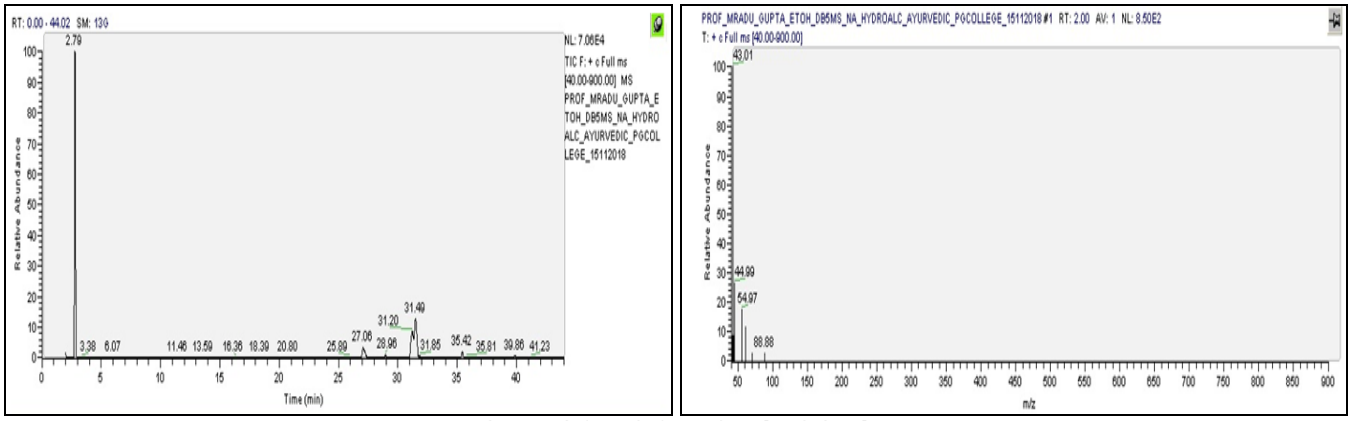
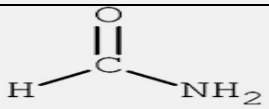
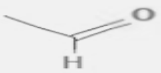
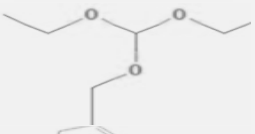
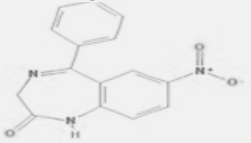
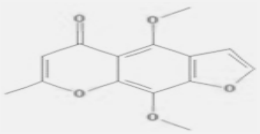
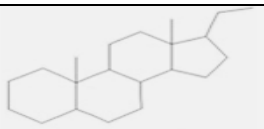
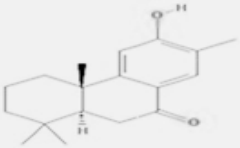
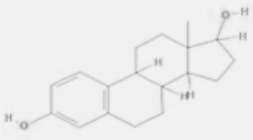
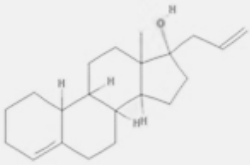
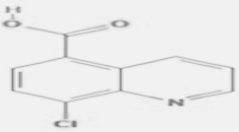
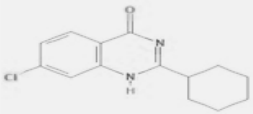


FIG. 14: GC-MS CHROMATOGRAM

TABLE 11: RETENTION TIMES (R.T.) AND POSSIBLE CHEMICAL COMPOUNDS RELATED THERETO OBSERVED DURING GC-MS ANALYSIS

S. no.	Retention Time (R T)	Chemical name, Chemical Formula& Molecular weight	Chemical structure	Pharmacological activities
1	2.26	Carbamaldehyde, Formamide CH_3NO (45)		It is a chemical feedstock for the manufacture of sulfa drugs, other pharmaceuticals, herbicides, pesticides and the manufacture of hydrocyanic acid
2	2.79	Acetaldehyde $C_6H_{14}O_2$(118)		It is used in tanning, preserving and embalming and as a germicide, fungicide, and insecticide for plants and vegetables
3	6.07	Ethyl orthoformate $C_7H_{16}O_3$(148)		It is used in the preparation of N-(2-triethoxysilylpropyl) formamide and N-methyl-3-phenylbicyclo heptan-2-amine hydrochloride and in the synthesis of iso-flavone
4	13.59	3H-pyrazol-3-one $C_{15}H_{11}N_3O_3$(281)		It is used for the treatment of insomnia and for epileptic spasms in infants (West's syndrome). It has a role as an anticonvulsant, an antispasmodic drug, a GABA modulator, a sedative, and a drug metabolite
5	18.39	Phthalic acid, Cyclobutyl tridecyl ester $C_{25}H_{38}O_4$(402)		It is an aromatic dicarboxylic acid which is used as anhydride to produce dyes, perfumes, saccharin, phthalates, and many other useful products
6	27.06	6-Acetyl-4methyl-2-oxo-2H-chromen-7-yl acetate $C_{14}H_{12}O_5$(260)		It is used in common cold, flu, allergies, or other breathing illnesses such as sinusitis&bronchitis. Antihistamines help relieve watery eyes, itchy eyes/nose/throat, runny nose, and sneezing

7	28.59	1,3,5-Cycloheptatriene,2, 4-dihexyl-7,7-dimethyl $C_{21}H_{36}$(288)		It is used in dyspnea, kidney stones and cardiovascular disorders
8	28.96	Nimbiol $C_{18}H_{24}O_2$(272)		It is used to treat mild to moderate pain caused due to sprains and strains of joints and muscles and acute pain associated with osteoarthritis and Menstruation.
9	28.96	13Methoxypodocarpa-8,11,13-trien-3-one $C_{18}H_{24}O_2$(272)		It is used to treat mild to moderate pain caused due to sprains and strains of joints and muscles and acute pain associated with Osteoarthritis and Menstruation.
10	30.53	Podocarpa-8,11,13-triene, 14-isopropyl-13-methoxy $C_{21}H_{32}O$ (300)		It is used to temporarily relieve symptoms caused by the common cold, flu, allergies or other breathing illnesses such as sinusitis& bronchitis. It is also used for the treatment of recurrent and threatened miscarriage and to prevent premature labor
11	34.73	8-Chloro-5-quinolinecarboxylic acid $C_{10}H_6ClNO_2$(207)		It shows pronounced antimicrobial activity
12	41.23	7-Chloro-2-cyclohexyl-4[3H]quinazolinone $C_{14}H_{15}ClN_2O$ (262)		It is used to temporarily treat symptoms caused by the common cold, flu, allergies, *etc.* Dextromethorphan is also a cough suppressant

DISCUSSION: Vaginitis is an inflammation of the vagina that can result in discharge, itching, and pain due to changes 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. 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 cases [12, 13].

The efficacy of traditional herbal medicines is a result of the characteristics of a complex mixture of chemical compounds present in the herbs. Therefore, the use of the chemical fingerprints of specific chemical compounds can help in proper identification and quality control of herbal medicines. In fact, the interdisciplinary nature of the quality control of herbal medicines implies that biochemistry, molecular biology, and cell biology are invaluable in establishing quantifiable and reproducible assays. Chemical fingerprints might be linked to these biological assays to provide assurance of efficacy and consistency. Hence, research efforts to validate the relationships between chromatographic fingerprints and efficacy of the herbal medicines are urgent requirements [14, 15]. 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 Nimba (*Azadirachta indica* A. Juss.) and Ashoka (*Saraca asoca* 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, color, 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 light brown in colour, rough hard surface, about 12-16-inch-long and 1 inch in diameter for Nimba bark and dark brown in colour, rough surface more than 10-12 inches long and 1/2 inch in diameter and yellow coloured inner side of bark for Ashoka, the microscopic analysis showed the presence of parenchyma cells containing tannins, sclerites and stone cells in the powder.

The total ash value was 9.43% w/w which was relatively high possibly due to the presence of carbonate, sulfate & silicate compounds. The moisture content in the research formulation was found to be 7.70% w/w while the pH value of 4.62 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 hydro-alcoholic research formulation was found as 8.17% w/w while preliminary phytochemical analysis revealed the presence of flavonoids, tannins, and carbohydrates. The results also showed a high concentration of flavonoids compounds (43.37 µg quercetin equivalent/mg of extract) and high phenolic content (101.22 µg gallic acid equivalent/mg of extract) in the hydro-alcoholic extract which could be directly responsible for their antimicrobial and anti-leucorrhoea properties.

The UV-Visible spectroscopy scanning during the chemical analysis of the hydro-alcoholic extract of the research formulation showed two clear sharp peaks at 238 and 277 nm which indicated the presence of two main compounds having high concentration *i.e.*, 3.956 & 1.833 respectively in the research drug.

The R_f values (distance moved by the solvent front/ distance moved by the solute) of hydro-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: ethyl acetate: formic acid: methanol = (6.0: 6.0: 1.6: 0.4) when scanning was done at 254 nm, 277 nm, and 330 nm wavelengths. During analysis at 254 nm, 7 spots were found in the hydro-alcoholic extract of the research formulation which showed the presence of three standard chemical compounds, namely gallic acid, quercetin and catechol at R_f values of 0.59, 0.70 and 0.76 respectively. Similarly, chromatographic examination at 277 nm wavelength revealed the presence of phenolic compounds like tannic acid and gallic acid in the hydro-alcoholic extract having R_f values of 0.52 and 0.58. Similar analysis at 330 nm wavelength revealed the presence of quercetin and catechol at R_f values of 0.69 and 0.76 respectively.

High Performance Liquid Chromatography (HPLC) has been used to find out the retention time (RT) which depends upon the separation of compounds in the C-18 column under high pressure and different solvent systems in isocratic system of acetonitrile and water (in 40:60 ratio) for 30 min. HPLC analysis at 277 nm showed the elution of 9 compounds at different retention times in the hydro-alcoholic extracts. The obtained RT values of these eluted chemical compounds in the hydro-alcoholic extract were compared with those of the standard phenolic compounds indicating the presence of a maximum three standard compounds. Further analysis confirmed the presence of gallic acid at RT 5.399, tannic acid standard at RT 5.705 and catechol standard at RT 10.894 in the hydro-alcoholic extract. The presence of phenolic compounds in 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 wavenumbers and the possible functional groups during the FT-IR analysis of the hydro-alcoholic extract of the research formulation are presented in **Table 10**. The extract exhibited different characteristic bands at 3395.94, 2927.10, 1614.75, 1518.67, 1447.55, 1375.54, 1317.90, 1284.81, 1044.70, 820.64, 779.67 and 615.75 cm^{-1} indicating the presence of the functional groups N-H stretching, C-H stretching, N-H bending, N-O asymmetric stretching, C-C stretching (in-ring), C-H rocking, C-N stretching, C-O stretching, C-H wagging ($-CH_2X$), C-N stretching, C-Cl stretching and C-Br stretching respectively. It may be inferred

that the extract of research formulation exhibited almost similar types of functional groups on the basis of range of wavenumbers and suggesting the presence of amines/amides, alkenes, nitro group, aromatics, alcohol/ carboxylic acids/ esters/ ethers, alkyl halides and aliphatic/ aromatic amines groups of compounds in the extracts.

The spectrum of GC-MS analysis of a hydro-alcoholic extract of the research formulation revealed the presence of twenty 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. One of the prominent chemical compounds was nimbiol ($C_{18}H_{24}O_2$) eluted at RT 28.96 which is reported as a potent anti-inflammatory and antimicrobial agent along with its important role in the treatment of acute pain associated with osteoarthritis and menstruation. It is also used to treat mild to moderate pain caused due to sprains and strains of joints and muscles.

Another important compound was Podocarpa-8,11,13-triene, 14-isopropyl-13-methoxy ($C_{21}H_{32}O$) eluted at RT 30.53 which is used in the treatment of recurrent and threatened miscarriage and to prevent premature labor. The third isolated compound, 8-Chloro-5-quinolinecarboxylic acid ($C_{10}H_6ClNO_2$), is reported as a potent antimicrobial agent; while the fourth compound 7-Chloro-2-cyclohexyl-4[3H]quinazolinone ($C_{14}H_{15}ClN_2O$) is used to relieve symptoms caused by the common cold, flu, allergies or other breathing illnesses (such as sinusitis, bronchitis) as an antihistaminic agent.

CONCLUSION: Pharmacognostic analysis indicates a high concentration of flavonoids compounds such as flavonoids, glycosides and saponins as well as high phenolic content in the hydro-alcoholic extract. Spectroscopic and chromatographic examination using UV-Visible, HPTLC and HPLC analysis indicated the presence of tannic acid, gallic acid, quercetin and catechol along with the likely presence of amines/amides, alkenes, aromatics, alcohol/carboxylic acids/esters/ ethers, alkyl halides and aliphatic/ aromatic amines. Similarly, GC-MS analysis revealed the presence of specific compounds such as nimbiol, 8-Chloro-5-quinoline carboxylic acid and 7-Chloro-2-cyclohexyl-4[3H] quinazolinone, which have antimicrobial, antibacterial, anti-inflammatory, antifungal and analgesic properties validating the efficacy of the research formulation. The overall analysis indicated that the research formulation is having potent antimicrobial, antibacterial, anti-inflammatory, analgesic, antihistaminic and anti-fungal properties as revealed by its isolated chemical compounds. Hence, this research formulation is likely to be an effective therapeutic remedy for the treatment of leucorrhea and vaginitis on account of its abovementioned properties.

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