Standardization of Ayurvedic Drug- *Nyctanthes arbor-tristis, Hippophae salicifolia, Ocimum tenuiflorum* and *Reinwardtia indica* and Combined Herb-Herb

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Abstract

The herbal medicines have reached extensive acceptability as therapeutic agents for various clinical diseases due to global demand. Therefore, standardization is the essential and initial step to drug development. It is for the establishment of consistent biological activity, a consistent chemical profile and biomarker identification. It improves the safety and efficacy of herbal medicine to provide the best herbal medicine to society and increase popularity rather than non-standardized extracts. In addition, it is essential to practice or maintain a quality assurance program for the production and manufacturing of herbal medicine that includes the basis of organoleptic characters and photomicrographs, physicochemical, proximate analysis phytochemical evaluation and quality control analysis and order to assess the quality of drugs, based on the concentration of their active principles. WHO has provided specific guidelines for the assessment of the safety, efficacy and quality of herbal drugs as a prerequisite for global harmonization and of utmost importance. In the present study, the herbal extracts were cleaned, dried in the shade and powdered by passing through the sieve as per the method described in the standard protocol. An overview covering various techniques employed in the extraction and characterization of *Nyctanthes arbortristis, Hippophae salicifolia, Ocimum tenuiflorum and Reinwardtia indica*, standardization is reported in this study. The obtained data would be very significant for future clinical aspects, as the bioactive molecules present in the extracts may exhibit synergistic effect with other bioactive compound and show a better therapeutic value. Thus, this study provides standardized and therapeutically potential data of active polyherbal formulations for the different ailments.

Introduction

In recent years, standardization is the essential parameter to assess the quality of any type of drugs and more important for the quality assessment of herbal formulations to justify their acceptability in the modern medical system. It plays a crucial role to assess the quality, purity, safety and efficacy of some drugs including herbal medicine. Standardization is more important in the drug discovery area due to lack of scientific evidence such as pharmacognostic evaluation, phytochemical study, and pharmacological evaluation of the polyherbal formulation. Nowadays attention has been directed towards the utilization of Ayurvedic plants in the prevention and management of clinical diseases so that the trend of using herbal medicines has increased in a tremendous mode in the last decade [1]. Consequently, the World Health Organization (WHO) has taken a broader step in the clinical system and also in phytotherapy. Sponsorship and encouragement of studies substantiating parameters of related to standardization of herbal medicines of Ayurveda and ancient system are under principal consideration of WHO for new drug discovery [2].

Ayurvedic practitioners have developed several herbal medicines by using single herb or herb-herb combinations or as a polyherbal formulation, which were used in ancient traditional medical practice for many years. These medicines offer several options to modify the progress and symptoms of various diseases [3-6]. Globally, both types of

commercial and non-commercial single herbal medicine or polyhedral formulations have been developed, but many polyherbal formulations lack scientific evidence such as pharmacognostic, phytochemical and pharmacological evaluation [4].

Nyctanthes arbor-tristis (*N. arbor-tristis*) Linn. is known as Parijat belonging to the Oleaceae family. The therapeutic utility of *N. arbor-tristis* has been described in various classical texts of Ayurveda for the management of various mental and physical disorders. In Sushruta Sutra Sthana; the yoga prepared out of *N. arbor-tristis*, along with other drugs is used for a good sleep, for the management of diabetes, stomach disorders, epilepsy and many more [3-6]. *N. arbor-tristis* is used in combination with other drugs for the management of ksharaagada and also for the purification of poisons. According to Sushruta Uttar Tantra, *N. arbor-tristisis* was also given for the management of various diseases [7-11].

The word Hippophae (Latin word) 'Hippo' meaning horse and 'Phaos' means 'shine', and *Hippophae salicifolia* known as Sea buckthorn (SBT). It was used as a medicinal plant in Tibet as early as 900 AD. *H. salicifolia* have has been used as a herbal medicine for many years globally not only as therapeutic but also as prophylactic and health promotive agents. Recently, sea buckthorn Elaeagnaceae, a unique and valuable plant has gained worldwide attention, mainly for its medicinal, nutritional potential and its edible fruit which is rich in vitamins, often made into jam. In Greece, *H. salicifolia* leaves and twigs were used to feed animals, which resulted in weight gain and a shining coat, especially in horses. It has a rich history of use in treating numerous medical conditions. Many of its pharmacological effects have been recorded in classics such as SibuYidian from the Tang Dynasty and Jing Zhu Ben Cao from the Qing Dynasty [12-14].

Ocimum tenuiflorum, also known as, Vishnu Priya, holy basil, or tulsi (also spelt Tulasi), is an Indian aromatic plant and belongs to the family Lamiaceae, and cultivated throughout the Southeast Asian tropics. Tulsi (Sanskrit:-Surasa) has been used for thousands of years in Ayurveda for its diverse healing properties.). From ancient times, Tulsi extracts were used as ayurvedic remedies for a variety of clinical ailments. It is mentioned in the Charaka Samhita, an ancient Ayurvedic text in Sutrasthan for skin disorders [15], in Shwashara Mahakashaya [16], for Nadisweda in Vatakapha disorders (Neuro-muscular disorders) [17], in Harita Varga for Hikka (Hiccough), Kasa (Cough), Vishavikara (Toxins), Swas (Asthma), Parshvashula (Pain in Chest) and Durgandhanashaka (for fragrance) [18]. In Vimanasthanatulsi has been described for the treatment of worn disorders and Panchakarma [19, 20], Katuskandha [21].

Reinwardtia indica Dumort. (*R. indica* Dumort) is also known as Basanti and belong to as flax family [22]. The medicinal power of *R. indica* mainly depends on phytochemical constituents that have great pharmacological significance. *R. indica* extract is widely used by local communities for different purposes such as for tongue wash, to bring about an increase in - lactation period, a remedy against skin diseases, as an anti-infection agent etc. [23-26] as well as in the development of several polyherbal formulations.

The selection of these four herbs was based upon its activity like an antidepressant, antioxidant and anti-inflammatory. This study reports on the standardization of polyherbal formulation, based on organoleptic assessment, photomicrographs, physicochemical properties, proximate examination phytochemical assessment and quality control investigation.

Materials and methods
Plant Material

Leaves of *N. arbor-tristis, O. tenuiflorum* and *R. indica* were collected from the forests of India. Fruits of *H. salicifolia* was collected from Himachal Pradesh (Leh & Ladakh). These plant extracts were identified, authenticated and voucher specimens of the plants have been deposited (Accession No.: SH-2010, LH-2008, SH-2008 & SH-2008) in the herbarium for further reference.

Fresh plant materials of these plants were taken for the medicinal purpose used for microscopic study. The collected materials of the plants were washed with water and dried in the shade at room temperature and sieved. The dried parts were grinded to a coarse powder. The powdered drug was stored in an airtight and light-resistant container for the study.

Chemicals

All the chemicals (Toluene: Ethyl acetate, Petroleum ether, silica gel, Chloroform, Ethanol, Methanol, NaOH, HCl, HNO₃ and Nutrient agar, Salmonella Shigella Agar (SSA) and Potato Dextrose Agar (PDA) Media etc. used, were of analytical grade and were obtained from E. Merck Limited India and Hi-Media Laboratories, Mumbai, India.

Organoleptic evaluation

Various sensory parameters of the plant material (such as colour, odour, size, shape, and taste) were studied by organoleptic evaluation [27].

Pharmacognostic studies

Pharmacognostic analysis for each sample were carried out according to the standard procedural methods.

Sample preparation

Each sample was preserved in a fixative solution. The fixative used was FAA (5 ml Formalin + 5 ml Acetic acid + 90 ml 70% Ethyl alcohol). The materials were left in the FAA for more than 48 hours. Dehydration process was followed by a graded series of tertiary-butyl alcohol as per the schedule by Sass 1940 [28]. After dehydration, paraffin infiltration was carried out till the super-saturation of tertiary-butyl alcohol was achieved. Following supersaturation, the materials were transferred to pure paraffin wax two times and the materials were cast into paraffin blocks.

Sectioning

The embedded specimens were sectioned with the help of a rotary microtome. The thickness of the section was 10 to 12 μm which were then/further stained with toluidine blue as per the method by O'Brein et al. [29].

Photomicrographs

The transverse section was photomicrographs using Zeiss AXIO trinocular microscope attached to Zeiss Axio Cam camera under bright field. Descriptive terms of the anatomical features are as given in the standard anatomy books [30].

Physico-chemical evaluation

The Physico-chemical evaluation of prepared sections/samples of *N. arbor-tristis, H. salicifolia, O. tenuiflorum* and *R. indica and a mixture of the herb* were done by testing loss of weight on drying at 105^{0}C, total ash, acid insoluble ash and water-soluble ash.

Preparation of samples

The fresh fruit of *H. salicifolia,* leaf of *N. arbor-tristis* and root of *R. indica* and whole plant of *O. tenuiflorum* were washed, air-dried in 25ºC for three days in the absence of sunlight and grinded into a coarse powder. These samples were used for Physico-Chemical studies and proximate analysis [31].

Loss on drying at 105°C/Moisture content

To determine the weight loss in drying, 10 g of each herbal extract was placed in a tarred evaporating dish after accurately weighing it. The extract was then dried at 105°C for 5 hrs and weighed. After drying, tarred evaporating dish was cooled in desiccators for 30 mins and then weight was taken.

$$\text{The \% of Loss on drying at 105° C} = \frac{\text{The difference in weight after heating}}{\text{Weight of the sample taken}} \times 100$$

2.0 g of the powdered extract was incinerated in a tarred silica dish at a temperature not exceeding 450°C until free carbon was left, then cooled and final weight was taken. The percentage of ash was calculated concerning the weight of the sample.

$$\text{The \% of Total Ash} = \frac{\text{Weight of ash obtained}}{\text{Weight of sample taken}} \times 100$$

Estimation of Acid Insoluble Ash

The ash obtained as the above method was boiled for 5 mins with 25 ml of dilute hydrochloric acid and collected the insoluble matter on an ash-less filter paper (Whatman 41), washed with hot water and ignited to constant weight. The percentage of acid-insoluble ash concerning the air-dried drug was calculated.

$$\text{The \% of Acid Insoluble Ash} = \frac{\text{Weight of Acid Insoluble residue}}{\text{Weight of the sample taken}} \times 100$$

Estimation of Water-soluble ash:

The incinerated ash of the sample was dissolved in 25 ml of dilute hydrochloric acid and made up to 50 ml with water and boiled. The suspension was filtered with the help of the Whatman filter paper and the residue was collected. It was kept in a weighed silica crucible and maintained in a muffle furnace for 6 hrs at 450-650ºc. The crucible was taken out and cooled at room temperature and

weighed. The percentage of ash obtained was thus calculated.

The % of Water − soluble ash

$$= \frac{\text{Weight of ash}}{\text{Weight of the sample taken}} \times 100$$

Estimation of Sulphated Ash:

The substance was ignited with concentrated sulphuric acid, which decomposed and oxidized organic matter, leaving a residue of inorganic sulphates. A large silica crucible was ignited to constant weight and approximately 5 g of the sample was weighed, and moistened by adding sulfuric acid. This was then heated gently at first and more strongly later so that volatile matter could be removed. Further, it was again ignited more strongly to remove the cool carbon, remoistened with sulphuric acid and then re-ignite to achieve constant weight. Finally, the percentage of sulphated ash was calculated.

The % of sulphated ash

$$= \frac{\text{Weight of ash}}{\text{Weight of the sample taken}} \times 100$$

Determination of Extractable Matter in alcohol (Cold Maceration Method)

5 g of samples were macerated with 100 ml of alcohol in a stoppered flask with frequent shaking during the first 6 hrs and allowed to stand for 18 hrs. It was filtered after 24 hrs. 25 ml of the filtrate was evaporated in a tarred dish at 105°C and weighed. Alcohol soluble extractive values were calculated. The experiment was repeated twice, and the average value was taken [32].

The % of alcohol − soluble extractive

$$= \frac{\text{Weight of extract} \times 4}{\text{Weight of the sample taken}} \times 100$$

Determination of Extractable Matter in water (Cold Maceration Method)

About 5.0 g of coarsely powdered air-dried material was accurately weighed in a glass stopper conical flask and macerated with 100 ml of distilled water specified for the plant material for 6 hrs with frequent shaking, then allowed to stand for 18 hrs. It was then filtered instantly, taking care not to lose any solvent. The extracted matter was dried at 105°C for 6 hrs, cooled in desiccators for 30 mins and then weighed. The percentage of the extractable matter was calculated.

The % of water − soluble extractive

$$= \frac{\text{Weight of extract} \times 4}{\text{Weight of sample taken}} \times 100$$

Proximate analysis
Estimation of fibers

The fiber content was estimated by the method of Raghuramulu et al. [33]. About 5 g of moisture and the fat-free sample were weighed into a 500 ml beaker and 200 ml of boiling 0.255 N (1.25% W/V) Sulphuric acid was added. The mixture was boiled for 30 mins, but the volume was kept constant by the addition of water at frequent intervals. Finally, the mixture was filtered through a muslin cloth and residue was washed with hot water until it was free from acid. The material was then transferred to the same beaker, and 200 ml of boiling 0.313 N (1.25%) NaOH was added. After boiling for 30 mins (keeping the volume constant as before) the mixture was filtered through a muslin cloth. The residue was washed with hot water until it was free from alkali, followed by washing with some alcohol and ether. It was then transferred to a crucial, dried overnight at 800-100°C and weighed (We). The crucial was heated in a muffle furnace at 600°C for 2-3 hours; then it was cooled and weighed again (Wa). The difference in the weights (We-Wa) represents the weight of fiber.

$$\text{Fiber content} = \frac{\{100 − (\text{moisture} + \text{fat})\} \times (\text{We} − \text{Wa})}{\text{Weight of sample taken}}$$

Estimation of Total Carbohydrate (Anthrone method)

The total carbohydrate content was estimated by the method of Hedge et al. [34]. 100 mg of the sample was weighed into a boiling tube, hydrolyzed by keeping it in a boiling water bath for three hours with 5.0 ml of 2.5 N HCl and cooled at room temperature. Then, the material was neutralized with solid sodium carbonate until effervesce was seen and the final volume made to 100 ml. This was centrifuged, at 5000 rpm for 10 min and the supernatant was collected. From that, 0.2 to 1.0 ml was taken and the standard was prepared from the working standard. 1.0 ml of water serves as a blank made up the volume to 1.0 ml in all the tubes with distilled water, then added 4.0 ml of anthrone reagent, heated for eight minutes in a boiling water bath, cooled rapidly and read the green to dark green colour at 630 nm. The carbohydrate content of the sample was compared with the standard curve.

Estimation of protein (Modified Lowry's Method)

The total protein content was estimated by the method from Raghuramulu et al. [33]. 5 g extracts was kept 3 separate tubes with 50 ml of water by overnight cold percolation method. In separate tubes, 0.5 ml of each; extract, blank and standard, were taken in duplicate and 0.5 ml of alkaline copper reagent was added, mixed and allowed to stand undisturbed for 10 minutes. Then 2 ml of phenol reagent was added to each tube; mixed immediately and placed at room temperature for 5 mins and absorbance of samples and standards were taken at 615 nm against a blank. The protein content of the sample was calculated by comparing with the standard curve.

Estimation of Fat

5 g of extract was placed in a soxhlet fitted with a condenser. 90 ml of petroleum ether (boiling point 40- 60°C) was taken in a 150 ml round bottom flask and boiled for 6 hrs. The extract was taken in a pre-weighed conical flask, and petroleum ether was evaporated on a water bath. The traces of petroleum ether were removed using a vacuum pump [35].

Phytochemical test

Phytochemical analysis for each sample's crude extract and her-herb combination were carried out according to the standard procedure methods [36, 37].

Preparation of Hydroalcoholic extracts

The dried powder of plant parts was exclusively removed by hydroalcoholic cold permeation technique. 10 g of dried powder of each plant were taken in 100 ml of oil ether in a cone-shaped jar, applied with cotton fleece stopper and after that kept on a turning shaker at 120 rpm for 24 hrs. After 24 hrs, it was seived through eight layers of muslin fabric, centrifuged and the supernatant was collected and air-dried under decreased strain to acquire the dried buildup. Oil ether was dissipated from the powder. This dry powder was then taken exclusively in 100 ml of every dissolvable, for example, ethanol (ET), 75% ET, half ET, 25% ET and water and after that it was kept on a revolving shaker at 120 rpm for 24 hrs. At that point, the system pursued was equivalent to above, and the deposits were weighed to get the extractive yield of

the considerable number of concentrates and were put away in hermetically sealed containers at 4°C [38].

The phytochemical studies were performed for testing the different chemical groups present in the drug. For this, 10% (w/v) solution of extract was taken, unless otherwise mentioned in the respective individual test. General screening of various extracts of the plant material was carried out for the qualitative determination of the groups of organic compounds present in them as per Sethi 1966 [39].

Test for Alkaloids

Dragendorff's test: For this test, 2 g of alcoholic and aqueous extract of the drug were dissolved in 5 ml of distilled water, followed by the addition of 2 M hydrochloric acid until an acid reaction occurs, then 1 ml of Dragendorff's reagent added. Subsequently, an orange or orange-red precipitate was produced immediately.

Test for Flavonoids

Shinoda's test: In a test tube containing 2 g of alcoholic extract of the drug, 5-10 drops of dilute Hydrochloric acid as added, followed by the addition of a small piece of magnesium. In the presence of flavonoids, a pink, reddish-pink or brown color was produced.

Test for Triterpenoids

Liebermann-Burchard's test: For this 2 ml of acetic anhydride solution was added to 1 ml of petroleum ether extract of the drug in chloroform followed by 1 ml of conc. Sulphuric acid. A violet ring was formed indicating the presence of triterpenoids.

Test for Resins

Dissolved the extract was dissolved in acetone and solution was poured into distilled water. Turbidity indicated the presence of resins.

Test for Saponins

In a test tube containing about 2 g extracts of the drug was added a drop of sodium bicarbonate solution, followed by shaking the mixture vigorously and left for 3 minutes. Honeycomb like forth was formed.

Test for Steroids

Liebermann-Burchard's test: In this test, 2 ml of acetic anhydride solution was added to 1 ml of petroleum ether extract of the drug in chloroform followed by the addition of 1 ml of Conc. Sulphuric

acid. A greenish color was developed, which turned to blue.

Salkowski Reaction: For this test, 1 ml of conc. Sulphuric acid was added to 2 ml of chloroform extract of the drug carefully, from the side of the test tube. The red color was produced in the chloroform layer.

Test for Tannins
Added a few drops of 5% $FeCl_3$ solution in few grams of extract and a green color indicated the presence of gallotannins while brown color tannins.

Test for Starch
0.015 g of Iodine and 0.075 g of Potassium Iodide were dissolved in 5 ml of distilled water and few grams of extract of the drug was added. The blue color was produced.

Test for Glycosides
For the detection of glycoside in an extract sample, on paper spray, solution No. 1 (0.5 % aqueous sol. of Sodium metaperiodate) was sprayed & waited for 10 minutes. After that spray solution No. 2 [0.5 % Benzidine (w/v) was sprayed in solution of Ethanol – acetic Acid (4:1)], Presence of glycoside was detected by formation of white spot with a blue background.

Estimation of Total Phenolics
Many natural antioxidants are polyphenolics in nature e.g. Flavonoids, Chalcones, Aurones and Phytoalexins. These polyphenolic compounds exhibited anti-inflammatory, antioxidant and analgesic activity. Hence, the amount of total phenol present in the air-dried plant, as well as hydroalcoholic extracts, were estimated using Folin-Ciocalteau reagent [40].

Detection of numerous Microbial masses in Herbs
The plant material and plant extract obtained were subjected to microbial analysis. A total 1 g of the sample was taken, and 99 ml of sterile distilled water was added for preparing the serial dilution. The samples in the flask were kept in a mechanical shaker for a few minutes to obtain uniform suspension of microorganisms and diluted to 1:100 or 10^{-2}. From that 1 ml of the 10^{-2} dilution was transferred to 9 ml of sterilized distilled water. This was 1:1000 or 10^{-3}. This procedure was repeated up to 10^{-6} dilution. 0.1 ml of serially diluted samples

were inoculated into the sterile plate containing Nutrient agar, Salmonella Shigella Agar (SSA) and Potato Dextrose Agar (PDA) Medium by spread plate method. Nutrient Agar (NA) and SSA plates were incubated at 37°C for 24 hours, and PDA plates were incubated at room temperature for 3-5 days. The bacterial and fungal colonies were counted using a colony counter. *Salmonella* sp*p*, *Shigella* spp can be counted using SS Agar medium. *Enterobacter* spp. were identified by Enterobacteriaceae kits.

Test for heavy metal toxicity
Sample collection
The samples were cleaned and dried under shade. The dried samples were grinded to powder form in an agate mortar-pestle, were labeled and stored in pre-cleaned polyethylene bottles for further analysis.

Calibration of instruments:
More than three working standard solutions of analyses were prepared, covering the concentration range as recommended by the manufacturer of the instrument for the elements to be determined. Before the analysis of samples, the instruments were calibrated with a prepared working standard solution. The calibration curves were obtained for concentration vs absorbance and were statically analyzed.

Cadmium, Lead and Arsenic analysis (Flame AAS/Graphite Furnace):
The digested sample was subjected to analysis of Cadmium, Lead and Arsenic by AAS flame/graphite Furnace with specific instrumental conditions as given by instruments manufacturer. The solutions were introduced into flame. The mean of the three readings was recorded against the standard calibration curve obtained from concentration vs absorbance.

After calibrating the instrument with prepared working standard, 10 ml of digested sample was pipette out to a specific container of Mercury Hydride system analyzer followed by adding 10 ml 1.5 % of HCl as diluents for each sample and blank. The digested samples were run through the reaction flask containing reluctant (3% $NaBH_4$ in 1 % of NaOH) to quartz cell without heating against the calibration curve obtained from concentration vs absorbance.

Fluorescence analysis

Fluorescence is an essential parameter of pharmacognostical evaluation, exhibited by various chemical constituents present in plant material and convert nonfluorescent into fluorescent derivatives by reagents. It was observed under ordinary and ultraviolet light according to the procedure of Kokoshi et al. [41]. For this, 10 mg of the extract of each plant was taken in a glass slide and treated with various reagents for the presence of their fluorescence characteristics under the ultra-violet lamp at 254 nm and 366 nm.

High-performance Thin Layer Chromatography (HPTLC)

HPTLC analysis was carried out by Sethi 1996 [39]. In this technique, 5µl each of the extract was applied on a precoated silica gel 60 F254 on aluminium plates to a bandwidth of 8mm using Linomat 5 TLC applicator. The plates were developed in toluene-ethyl acetate (9:1) and the developed plates were visualized and scanned by TLC scanner 4 Win CATS software version 1.4.6.2002 under UV 254, 366. After derivatization in vanillin-sulphuric acid was sprayed as reagent at 620 nm, R_f colour of the spots and densitometry scan were recorded. HPTLC plates of hydraulic extracts of plants separate mobile phases viz. Ethylacetate: Dichloromethane: Formic acid: Acetic acid: Water (10:2.5:1:1:0.5) were examined. Rf value, numbers of peaks, peak area and the peak height of plants extract in separate mobile phases viz. n-Ethylacetate: Dichloromethane: Formic acid: Acetic acid: Water (10:2.5:1:1:0.5) were also analyzed under 254 nm and 366 nm respectively. Further studies was done by the help of standards for quantitative estimation and identification of the ingredients peak. Present HPTLC fingerprinting data can help in authentication and identification of formulation in the performed solvent system and extract.

Statistical analysis

Statistical analysis was done by using GraphPad Prism Version 5.02. One-way analysis of variance (ANOVA), with Tukeypost-test, was used for Statistical analysis of collected data. A probability value of $p<0.05$ was considered significant, and $P<0.01$ was considered highly significant. All the data are expressed as mean ± SD (standard deviation).

Results

The organoleptic properties of herbs showed the colour revealed was light brown for leaves of *N. arbor-tristis, O. tenuiflorum* and *R. indica*, yellow for fruits of *H. salicifolia*, have a characteristic odour, bitter taste and moderately fine texture and combined herb-herb color was yellowish-brown. Pharmacognosy is the study of medicinal plants, produced from natural sources and analysis of their biological, chemical, biochemical, and physical properties, or it is the study of crude drugs based on their shape, size, color, and texture and cut surface morphology.

Microscopic features of entire Transverse Section of the Leaf of *N. arbor-tristis*.
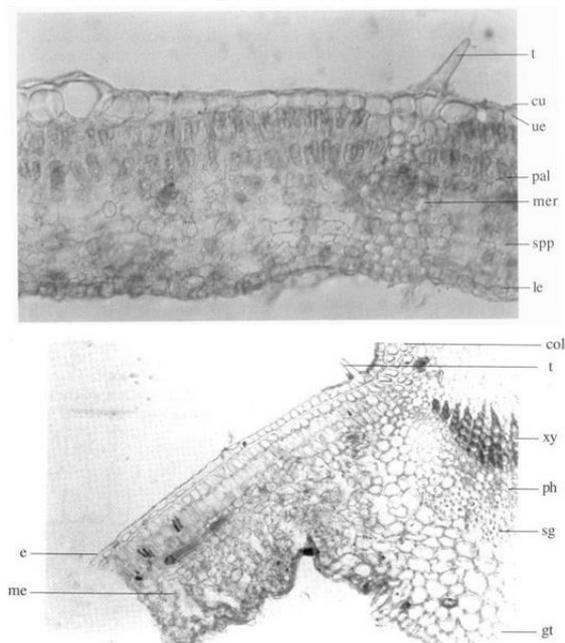

Figure 1: Microscopy of *N. arbor-tristis* leaf. Figure 1a represents microscopy of the leaf TS through midrib where col=collenchyma; e=epidermis; gt=ground tissue; me=mesophyll; ph=pholem; sg=starch grains; t=trichome; xy=xylem. Figure 1b represents microscopy of the leaf TS through lamina where cu=cuticle; le=lower epidermis; mer=meristele; pal=palisade; spp=spongy parenchyma; t=trichome; ue=upper epidermis.

The transverse section of leaf passing through the mid-rib convexly projects on the lower side, and slightly grooved with a shallow central elevation on the upper side.

Leaf lamina demonstrated an epidermis of digressively stretched cells on the two surfaces, bigger on the upper surface, secured by striated fingernail skin, mesophyll separated into 2 or 3 layers of palisade cells, 5 to 7 layers of approximately masterminded, fairly isodiametric supple parenchyma, rosette gems of calcium oxalate present in a couple of cells, stomata more on the lower surface, anisocytic where anomocytic type likewise occur on the two surfaces (**Figure 1a** & **Figure 1b**).

Midrib: show a single layered epidermis, 2 or 3 layered collenchyma on both surfaces, 4 or 5 layered parenchyma, mostly devoid of chloroplasts, central zone occupied by the vascular bundles differentiated into xylem towards ventral side and phloem towards dorsal side, phloem consisting of sieve tubes, companion cells and phloem parenchyma, xylem consisting of radial rows of vessels with xylem parenchyma.

The total thickness of the leaf ranges from 158.08–295.36 µm, the mean value being 248.46 µm. The thickness of mesophyll ranges from 110.24–249.60 µm with the mean as 197.91 µm. The ratio of height to width of upper epidermal cells speaks for their barrel-like shape. The height vis-à-vis width ratio of the lower epidermal cells reveals that the cells are more or less square shaped.

Petiole: In the transverse section of the petiole leaf of *N. arbour-tristis* have a specific feature, there are two projections adjacent to the ventral groove, epidermis single-layered, cells cubical covered by a thick cuticle, inner walls of epidermal cells adjoining the cortex much thickened, hairs absent. Hypodermis layer composed of collenchyma 2 or 3 layered and a broad zone of more or less rounded parenchyma cells present with intercellular spaces. It has a few rosette crystals of calcium oxalate. Resin canals are also present on the dorsal side of each vascular bundle except in the vascular bundles occurring projecting arms. In this section, vascular bundles 5 to 7 in number (**Figure 2a** & **Figure 2b**).

Microscopic features of entire Transverse Section of the Fruit of *H. salicifolia:*

The TS of the fleshy tissues of the fruit or hypanthium reveals various structures. Vascular tissues are oriented longitudinally through the central portion of the fruit flesh. Viewed cross-sectional (**Figure 3**), the vascular tissues are surrounded by large parenchyma cells with round,

oval, and teardrop shapes and contain large quantities of red pigments, which are likely carotenoids. Long narrow cells shaped like sclereid tissues, and containing green pigments, are found on either side of the vascular and storage tissues. These groups alternated around the middle circumference of the mesocarp.
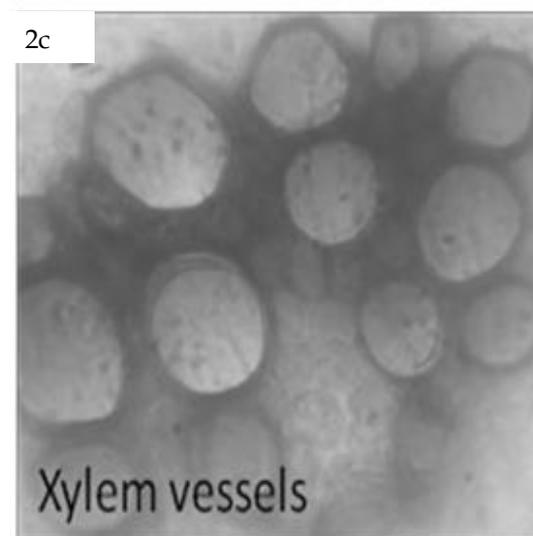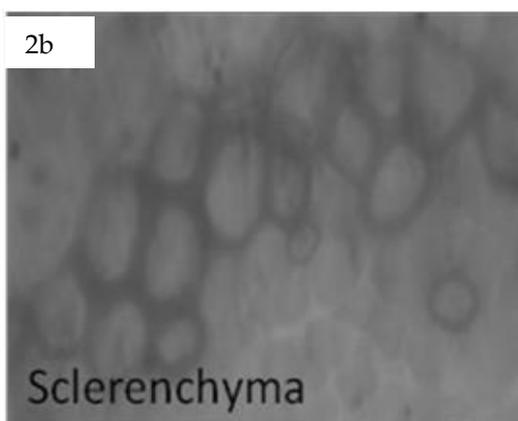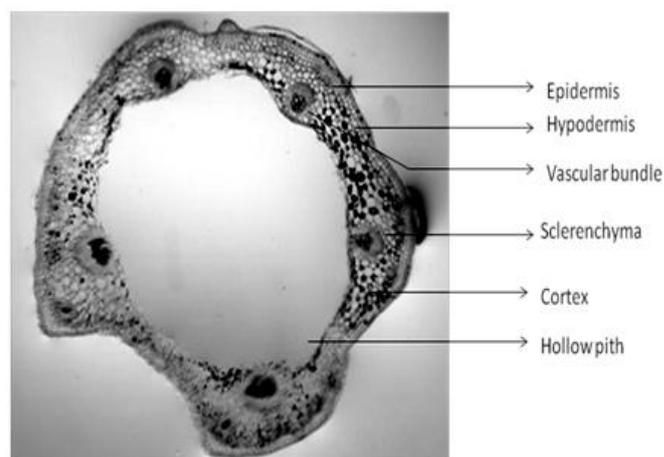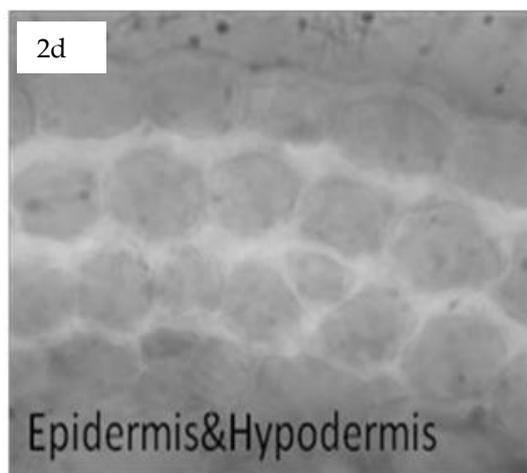
Figure 2: The transverse section of the petiole leaf of *N. arbour-tristis*. Figure 2a represents transverse section of petiole, 2b, 2c 2d represents enlarged portion of petiole
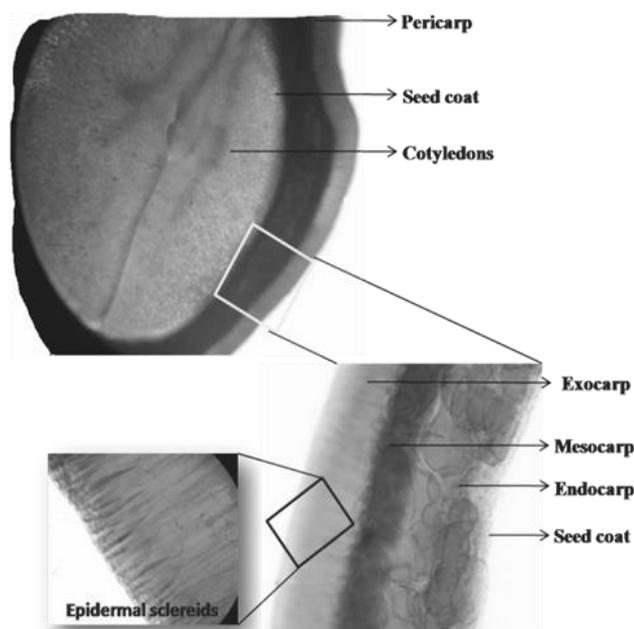

Figure 3: Transverse section of fruit from *H. salcifolia*

## Microscopic features of the entire Transverse Section of the leave of *O. tenuiflorum.*

Leaf: Transverse section of the leaf had a pot shape midrib and a flimsy lamina with uneven lower epidermis appended at the sidelong sides of its upper side leaving a curved focal dorsal. Midrib comprises of an emanating curve of xylem and phloem. Both upper and lower epidermis demonstrated straightforward, covering, uniseriate trichomes just as sessile short-stalked, glandular trichomes. Powder of the air-dried leaves of this plant was seen under the magnifying instrument. The various glandular basic trichomes of the normal length of 101 μm were found.

Petiole: indicates to some degree cordate framework, comprising of single-layered epidermis made out of flimsy walled, oval cells having various covering and glandular trichomes; covering trichomes multicellular 1-8 celled long, rarely somewhat reflexed at tip; glandular trichomes short, sessile with 1-2 celled stalk and 2-8 celled inflatable formed head, estimating 22-27 in diameterr; epidermis pursued by 1 or 2 layers and 2 or 3 layers of meager walled, stretched, parenchyma cells towards upper and lower surfaces individually; three vascular groups arranged midway, center one bigger than the other two; xylem encompassed by phloem.

Midrib: epidermis, trichomes and vascular bundles similar to those of petiole except cortical layers reduced towards the apical region.

Lamina: epidermis and trichomes similar to those of petiole; both monocytic and diacytic type of stomata present on both surfaces, slightly raised above the level of epidermis; palisade single layered followed by 4-6 layers of closely packed spongy parenchyma with chloroplast and oleo-resin (**Figure 4a** & **Figure 4b**).
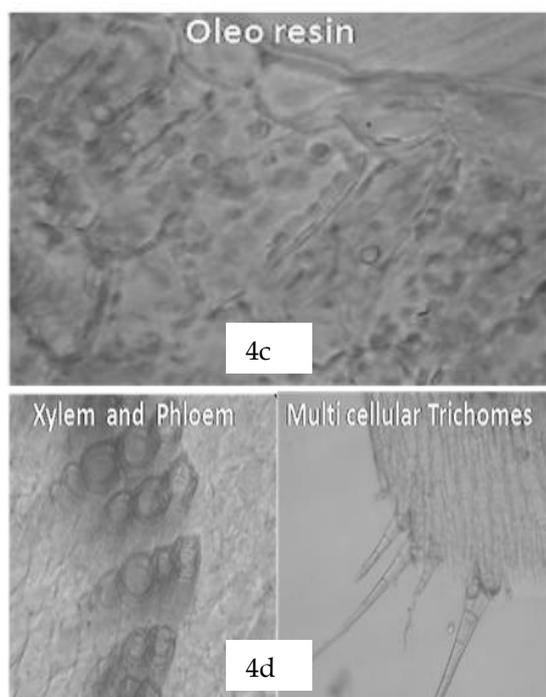

4a

4b
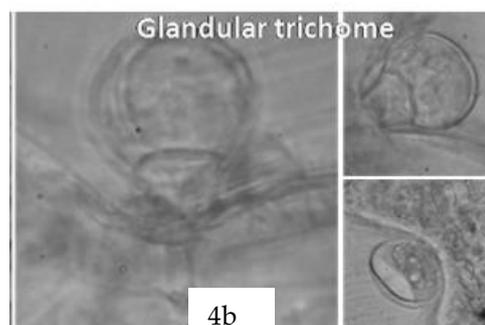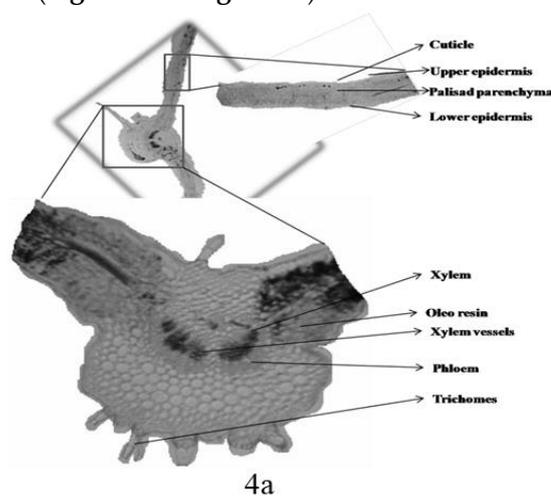
Figure 4: Transverse section of leaf from *O. tenuiflorum*. **Figure 4a** represents transverse section of leaf from O. tenuiflorum and **Figure 4b, 4c, 4d** represents enlarged section of leaf.

The physicochemical characteristics of the extracts were determined as per WHO guidelines [34]. The physico-chemical estimation of the drug is an important parameter in adulteration or improper handling of drugs [35]. It can serve as a valuable source of information and provide appropriate standards to establish the quality of plant material for further study. Equally important in the evaluation of the loss on drying, ash value, water soluble ash, sulphated ash, acid insoluble ash value and water-soluble extractive value and alcohol soluble extractive value determination. The low value of moisture content could prevent bacteria, fungal or yeast growth [40, 41, 42]. This value varies within fairly wide limits and is, therefore, an important parameter for the purpose of evaluation of crude drugs [43]. Therefore, percentage on the loss on drying, ash value, water-soluble ash, sulphated ash, acid insoluble ash value and water soluble extractive value and alcohol soluble extractive value calculated. Results for the physicochemical parameters are given in **Table 1**. Ash of any organic material contains non-volatile inorganic components. The ash content indicates that the seed, is rich in mineral elements. Controlled incineration of crud drugs result in an ash residue composed of inorganic matter such as metallic salts and/or silica. Therefore, the kind of care that must be taken in the plant drug is important. Total ash value was noted in (**Table 1**). The result showed that the negligible amount of acid-insoluble siliceous matter is present in these extracts. Percentage of total ash value, water-soluble ash, sulphated ash, acid insoluble ash in these extracts accessed falls within the margins as per. The Ayurvedic Pharmacopoeia of India, (2001), which states that the total ash, water-soluble ash, sulphated ash acid insoluble ash content of the samples tested should in a limited range[44].The water-soluble extractive value indicates the presence of sugar, acids and inorganic compounds, **Table 1**. Result reveals that the sugar, acids and inorganic compounds were presented with normal range in these extract. The alcohol-soluble extractive values indicated the presence of polar constituents like phenols, alkaloids, steroids, glycosides and flavonoids **Table 1**. The alcohol-soluble extractive content of these extracts falls within the margins as per The Ayurvedic Pharmacopoeia of India (2001), which states that the alcohol-soluble extractive content of the samples tested should be more than 3% [44, 45].

Table 1: The Physicochemical analysis of *N. arbor-tristis, H. salicifolia, O. tenuiflorum and R. indica*.

Physicochemical parameters	N. arbor-tristis	H. salicifolia	O. tenuiflorum	R. indica	Combined herbs
Loss on drying	4.59±0.05	9.87±0.09	4.87 ±0.04	4.64±0.05	5.98±0.05
Ash value	19.64±0.07	10.35±0.03	8.61±0.06	5.38 ±0.05	10.99±0.05
Water soluble ash	9.29±1.6	4.64±0.7	3.76±0.4	2.12±0.5	4.95±0.25
Sulphated ash acid	10.82±1.0	3.67±0.6	2.64±0.2	2.09±0.3	4.80±0.52
Insoluble ash value	2.11±0.9	2.09±0.6	1.53±0.3	1.11±0.1	1.71±0.47
Water soluble extractive	4.69±0.3	15.26±0.4	8.52±0.3	8.31±0.3	9.19±0.32
Alcohol soluble extractive	6.38±0.03	6.27±0.02	10.29±0.3	3.64±0.01	6.64±0.09

Value are Mean ±SD for 5 different preparations

Table 2: Proximate composition of N. arbor-tristis, H. salicifolia, O. tenuiflorum and R. indica.

Proximate parameters	N. arbor-tristis % W/W	H. salicifolia % W/W	O. tenuiflorum % W/W	R. indica % W/W	Combined herbs
Carbohydrate	9.48±1.96	3.6±0.53	24.71±1.49	29.71±1.85	16.87±1.62
Protein	15.02 ±0.96	26.31±2.02	5.01±0.09	12.01±2.52	11.68±1.39
Fat	2.10±0.36	2.03±0.02	3.26±0.56	2.16±0.23	2.38±0.29
Crude fiber	15.03±0.02	14.11±0.36	13.20±0.29	16.19±0.69	14.63±0.34

Values were expressed as mean ± S.D for 5 different preparations

Table 3: Phytochemical analysis of *N. arbor-tristis, H. salicifolia, O. tenuiflorum and R. indica*.

Parameters	N. arbor-tristis	H. salicifolia	O. tenuiflorum	R. indica	Combined herb
Alkaloids	(+)	(+)	(+)	(-)	(+++)
Flavonoids	(-)	(+)	(+)	(+)	(+++)
Terpenoids	(+)	(+)	(+)	(+)	(++++)
Resins	(+)	(+)	(+)	(+)	(++++)
Saponins	(+)	(+)	(-)	(+)	(+++)
Steroids	(+)	(-)	(+)	(+)	(+++)
Tannins	(+)	(+)	(+)	(-)	(+++)
Starch	(+)	(-)	(+)	(+)	(+++)
Glycosdes	(+)	(+)	(+)	(+)	(++++)
Total Phenolics	(+)	(+)	(+)	(+)	(++++)

Next parameter, proximate analysis reveals the nutritive content of each extract and combined extract. The proximate analysis revealed that *H. salicifolia* is a very good natural source of protein and all calculated values showed in (**Table 2**). In the Proximate analysis, the combined herbal extract possesses the nutritive content of four herbs and found the study presence of carbohydrate, protein, fat and crude fiber. According to the results of the energy values which were based on the carbohydrate, fat and fiber content in these extracts were high. Thus, it may be used as an alternative for food as well as medicinal. These results correlate with Kocchar et al. [46].

Preliminary phytochemical analysis of polyherbal formulation is of significant importance as scientist need to understand the change upon extraction of different portion of four different medicinal showed the presence of alkaloids, glycoside, flavonoids, phenol, steroids, saponins, tannins, terpenoids and these results are presented in **Table 3**. The investigation showed that *N. arbor-tristis* contains phenolic compounds, resins, tannins, starch, glycosides and alkaloids. Flavonoids were absent. The *H. salicifolia* results revealed the presence of carbohydrate, amino acids, alkaloids, flavonoids, phenolic compounds and terpenoids present in the extracts whereas Starch and Steroids were absent. The results reveal the presence of

medicinally active constituents like tannins, alkaloid, terpenoids, steroids and Flavnois, Phlobatannins, Glycosides in the leaves of *O. tenuiflorum* while saponins were absent in this plant. In *R. indica*, active constituents like steroids and Flavnois, Phlobatannins, Glycosides were present except terpenoids, tannins and alkaloid. The phytochemical analysis revealed that combined herb shows the strong presence of flavonoids, phenolic compounds, alkaloids and tannin. These values are in accordance with the results obtained by Mowl et al. and Iwalokun et al. [47, 48].

Quality Control Analysis: Numerous *Microbial Load in Herbs-* The plant material and plant extract obtained was subjected to microbial analysis and result reveals that the level of total microbial count and total yeast and mold count were not more than 1000 CFU/mL in the extracts of *N. arbor-tristis, H. salicifolia, O. tenuiflorum, and R. indica* and combined herb. The values were found to be within the limit of WHO standards. Also, results showed absence of various pathogens like *Salmonella*, *E.coli*, *S. aureus* and *Pseudomonas* (**Table 4**).

Heavy metal toxicity evaluation in the extracts of polyherbal formulation: The growth of medicinal plants not only need nutrients for normal plant growth, but also can selectively uptake and accumulate some trace elements which are good and may also be toxic for human health if there not within the limits.

Table 4. Microbiological analysis of *N. arbor-tristis, H. salicifolia, O. tenuiflorum and R. indica*. NMT=Not More Than, A=N. arbor-tristis, B=H. salicifolia, C=O. tenuiflorum, D=R. indica, E=Combined herb

Plant Extracts	Total microbial count cfu/mL	Total yeast and mold count cfu/mL	Pathogens			
			Salmonella	*E.coli*	*S. aureus*	*Pseudomonas*
A	NMT 1000	NMT 1000	Absent	Absent	Absent	Absent
B	NMT 1000	NMT 1000	Absent	Absent	Absent	Absent
C	NMT 1000	NMT 1000	Absent	Absent	Absent	Absent
D	NMT 1000	NMT 1000	Absent	Absent	Absent	Absent
E	NMT 1000	NMT 1000	Absent	Absent	Absent	Absent

Table 5. Heavy metal content of *N. arbor-tristis, H. salicifolia, O. tenuiflorum and R. indica*. A=N. arbor-tristis, B=H. salicifolia, C=O. tenuiflorum, D=R. indica, E=Combined herb

Plant Extracts	Heavy Metals (Specification-(ppm/ml))				
	Lead (> 10)	Cadmium(>0.3)	Arsenic (>5)	Mercury(>0.5)	Chromium (>10)
A	0.036	0.017	0.054	0.0010	0.024
B	0.042	0.019	0.051	0.0011	0.027
C	0.039	0.018	0.052	0.0013	0.028
D	0.049	0.015	0.059	0.0011	0.019
E	0.041	0.017	0.054	0.0011	0.024

Table 6: Inorganic elements of *N. arbor-tristis, H. salicifolia, O. tenuiflorum* and *R. indica*. Note: A=*N. arbor-tristis*, B=*H. salicifolia*, C=*O. tenuiflorum*, D=*R. indica*, E=Combined herb; Al=Aluminum, Co=Copper, Ca=Calcium, Fe=Iron, Mg=Magnesium, K=Potassium, Na=Sodium, Mn=Manganese, Zn=Zinc, Ni=Nickel

Plant Extracts	Inorganic compound									
	Al	Co	Ca	Fe	Mg	K	Na	Mn	Zn	Ni
A	32.03	0.153	0.26	4.69	0.049	0.184	0.226	0.099	0.578	0.453
B	35.78	0.192	0.22	4.703	0.043	0.192	0.217	0.094	0.679	0.441
C	36.23	0.157	0.23	4.56	0.049	0.189	0.239	0.089	0.745	0.563
D	34.15	0.195	0.29	4.26	0.042	0.163	0.215	0.062	0.697	0.438
E	34.54	0.174	0.25	4.55	0.045	0.182	0.224	0.086	0.674	0.473

The results revealed the presence of heavy metal and inorganic compounds in the extracts of *N. arbor-tristis, H. salicifolia, O. tenuiflorum* and *R. indica* and also in combined herb and the values were found to be within the limit of WHO standards (**Table 5** and **Table 6**, respectively). Heavy metal and inorganic elements are present within the permissible limits.

Fluorescent analysis

In the present study dried powder extract of each plant treated with various chemical reagents (several solvents and chemicals) showed characteristic fluorescence at 254 nm and 366 nm wavelength as shown in **Table 7a**, **Table 7b**, **Table 7c**, **Table 7d** and **Table 7e** .

High performance thin layer chromatography (HPTLC) of hydroalcoholic extracts of *H. salicifolia, N. arbor-tristis, O. tenuiflorum, R. indica*

TLC was done for the separation of different active constituents which are present in the extracts of *H. salicifolia, N. arbor-tristis* and *O.tenuiflorum.* The developed spots was visualized at various nm. In **Figure 5a** from 1 to 3 track of the plate was of leaves extract and the fourth track was of standard nyctanthoside. From the picture obtained at 254 nm assumed that standard nyctanthoside has R_f 0.47 in track 4 and similarly, obtained purple band from track 1 to 3 match with track 4 (**Figure 5a**).
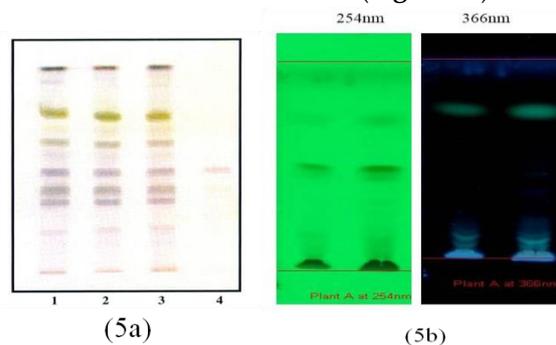

(5a)   (5b)

Figure 5a & 5b: TLC profile of test solution of *N. arbor-tristis* leaf. In Figure 5a, 1-3:test solution, 4: *N. arbor-tristis* standard. Figure 5b represents photo documentation report of hydro-alcoholic extract of *N. arbor-tristis* at 254 nm and 366 nm.

Table 7a: Fluorescence analysis of *N. arbor-tristis* leaf

Treatment	Visible light	Under UV light	
		Short wavelength (254 nm)	**The long wavelength (365 nm)**
Powder	Brown	Light brown	Green
Powder + Methanol	Brown	Light brown	Yellowish green
Powder + 70% ethanol	Brown	Light brown	Green
Powder + Pet. ether	Light brown	Light green	Green
Powder + 50% H_2SO_4	Brown	Greenish brown	Brownish
Powder + 50% HCl	Dark Brown	Green	Green black
Powder + 1N NaOH (aq.)	Light brown	Dark brown	Brownish black
Powder + 1N NaOH (alc.)	Light brown	Dark green	Greenish black
Powder + 50% HNO_3	Brown	Light brown	Light green
Powder + 5% KOH	Brown	Purplish green	Dark purplish green
Powder + Ammonia	Brown	Green	Black
Powder + Picric acid	Yellowish brown	Green	Dark brown

Table 7b: Fluorescence analysis of *H. salicifolia* fruit

Treatment	Visible light	Under UV light	
		Short wavelength (254 nm)	**The long wavelength (365 nm)**
Powder	yellow	Light yellow	Yellowish brown
Powder + Methanol	yellow	Light yellow	Yellow
Powder + 70% ethanol	yellow	Light yellow	Yellow
Powder + Pet. ether	Light yellow	Light yellow	Yellow
Powder + 50% H_2SO_4	Light yellow	Greenish yellow	Yellow
Powder + 50% HCl	Dark yellow	Greenish yellow	Colourless
Powder +1N NaOH (aq.)	Light yellow	Green fluorescence	Yellowish brown
Powder + 1N NaOH (alc.)	Light yellow	Brownish yellow	Light greenish yellow
Powder + 50% HNO_3	yellow	Greenish yellow	Yellow
Powder + 5% KOH	yellow	Brown	Greenish yellow
Powder + Ammonia	yellow	Light yellow	Yellow
Powder + Picric acid	Yellowish brown	Brownish yellow	Light greenish yellow

Table 7c: Fluorescence analysis of *O. tenuiflorum* leaf

Treatment	Visible light	Under UV light	
		Short wavelength (254 nm)	**The long wavelength (365 nm)**
Powder	Brown		Dark brown
Powder + Methanol	Brown	Green	Black
Powder + 70% ethanol	Yellowish brown	Green	Black
Powder + Pet. ether	Brown	Green	Black
Powder + 50% H_2SO_4	Yellowish brown	Green	Black
Powder + 50% HCl	Brown	Green	Black
Powder + 1N NaOH (aq.)	Yellowish brown	Green	Black
Powder + 1N NaOH (alc.)	Brown	Green	Black
Powder + 50% HNO_3	Yellowish brown	Green	Black
Powder + 5% KOH	Brown	Green	Dark brown
Powder + Ammonia	Yellowish brown	Green	Black
Powder + Picric acid	Brown	Green	Black

Table 7d: Fluorescence analysis of *combined herb-herb*

Treatment	Visible light	Under UV light	
		Short wavelength (254 nm)	The long wavelength (365 nm)
Powder	Brown	Brown	Brown
Powder + Methanol	Brown	Brown	Brown
Powder + 70% ethanol	Yellowish Brown	Brown	Brown
Powder + Pet. ether	Dark brown	Light brown	Light Brown
Powder + 50% H$_2$SO$_4$	Dark brown	Light brown	Light Brown
Powder + 50% HCl	Brown	Yellowish green	Dark Red
Powder + 1N NaOH (aq.)	Brown	Yellowish green	Dark Brown
Powder + 1N NaOH (alc.)	Green	Green	Reddish orange
Powder + 50% HNO$_3$	Green	Yellowish brown	Orange red
Powder + 5% KOH	Brown	Reddish brown	Reddish brown
Powder + Ammonia	Yellowish brown	Light orange	Light orange
Powder + Picric acid	Brown	Yellowish brown	Orange

Table 7e: Fluorescence Analysis of *R. indica* leaf

Treatment	Visible light	Under UV light	
		Short wavelength (254 nm)	The long wavelength(365 nm)
Powder	Yellow	Brown	Yellow
Powder + Methanol	Brown	Greenish brown	Brownish
Powder + 70% ethanol	light Brown	Green	Green black
Powder + Pet. ether	Light brown	Dark brown	Brownish black
Powder + 50% H$_2$SO$_4$	Brown	Dark green	Greenish black
Powder + 50% HCl	Brown	Light brown	Light green
Powder + 1N NaOH (aq.)	Brown	Purplish green	Dark purplish green
Powder + 1N NaOH (alc.)	Green	Green	Brownish green
Powder + 50% HNO$_3$	Green	Green	Pale yellow
Powder + 5% KOH	Brown	Green	Black
Powder + Ammonia	Yellowish brown	Green	Dark brown
Powder + Picric acid	Brown	Green	Black

Table 8: R$_f$ value and color of peak of fruit extract and standard Nyctanthic acid in Toluene:Ethyl acetate (8.0:2.0) at 366 nm

S.No	R$_f$ value	Colour of the band
1.	0.17	Pink
2.	0.32	Blue
3.	0.37	Brown
4.	0.47	Purple (Nyctanthic acid)
5.	0.57	Grey
6.	0.63	Green
7.	0.70	Blue
8.	0.77	Blue

(5c)   (5d)

254 nm   366 nm

(5e)

Further, the sample track (track 1, 2, 3) and standard track (track 4) were scanned at 254 nm showed the same R$_f$ value 0.47 for nyctanthoside in both the track (**Table 8**). Finally, this nyctanthoside band in all this track which came at 0.47 R$_f$ were scanned at 366 nm. The spectral pattern for nyctanthoside in extract matched with the standard track. Thus the presence of nyctanthoside was confirmed by overlaying the UV spectra at 366 nm and 254 nm (**Figure 5b**)
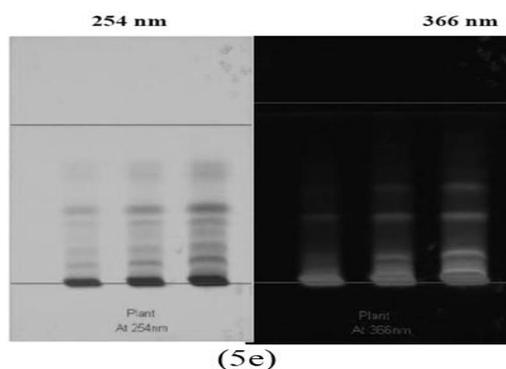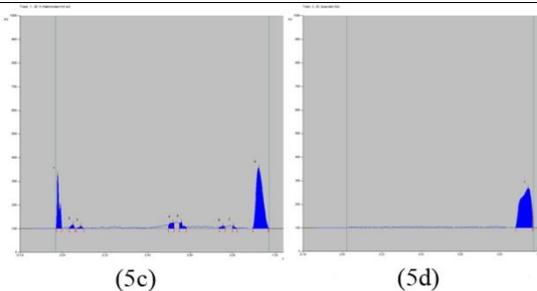
Figure 5c, **5d** & **5e**: HPTLC profile of hydroalcoholic of *H. salicifolia* with standard Quercetin as developed in Ethylacetate: Dichloromethane: Formic acid: Acetic acid: Water (10:2.5:1:1:0.5). Figure 5c represents fruit extract of *H. salicifolia* and Figure 5d represents Quercetin standard. Figure 5e represents photo documentation report of hydro-alcoholic extract of *H. salicifolia* at 254 nm and at 366 nm. FE=fruit extract; ST=Standard.

Table 9: R$_f$ value and peak area of Fruit extract and standard qurecetin in Ethylacetate: Dichloromethane: Formic acid : Acetic acid : Water (10:2.5:1:1:0.5) at 366 nm.

R$_f$ (Max)	Area(AU)
-0.01	2256.7
0.06	247.9
0.10	142.1
0.53	494.5
0.57	426.9
0.77	195.8
0.82	127.4
0.94	7453.1 (FE)
0.96	8189.5(Qurecetin standard)

The extracts of dried powder of fruits of *H. salicifolia* was subjected to HPTLC analysis and illustrated the number of active compounds. The quercetin content determined by HPTLC method in fruits of *H. salicifolia* reported in (**Table 9**) and showed 4 peaks at 254 nm and 8 peaks at 366 nm. At 366 nm, *H. salicifolia* (HA) extract showed 8 peaks and R$_f$ value matches with Quercetin standard, so this confirms the presence of Quercetin in the extract which is a flavonoid (**Figure 5c, 5d, 5e**).

The HPTLC fingerprints of hydroalcoholic extracts of *O. tenuiflorum* showed 6 peaks at 254nm (**Table 10a**) and 9 peak at 366 nm (**Table 10b**) (Shown in **Figure 6**). HPTLC fingerprint of *O. tenuiflorum* shows six different peaks at maximum R$_f$ values of 0.08, 0.32, 0.46, 0.54, 0.61 and 0.94. The peak at R$_f$ value of 0.46 is noticed to have an area of about 26650.2, which is the highest among the other peaks obtained and similar to eugenol.

HPTLC fingerprint of *R. Indica* points out eight different peaks at starting R$_f$ values of -0.02, 0.14, 0.19, 0.26, 0.35, 0.43, 0.58 and 0.74 (**Table 11** and **Figure 7**). Among those peaks, the peak with R$_f$ value of 0.58, exhibits a larger area of 4786.3. The extract shows the presence of saponin in hydro-alcoholic extract of *R. indica* at R$_f$:0.69.
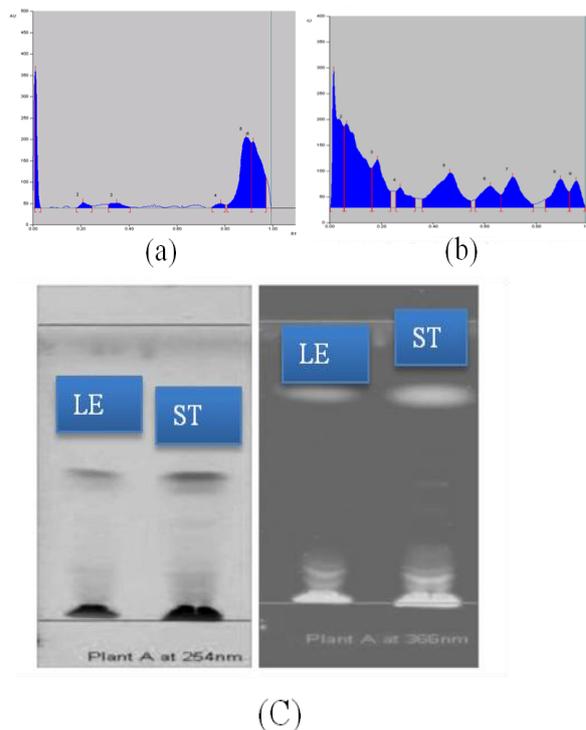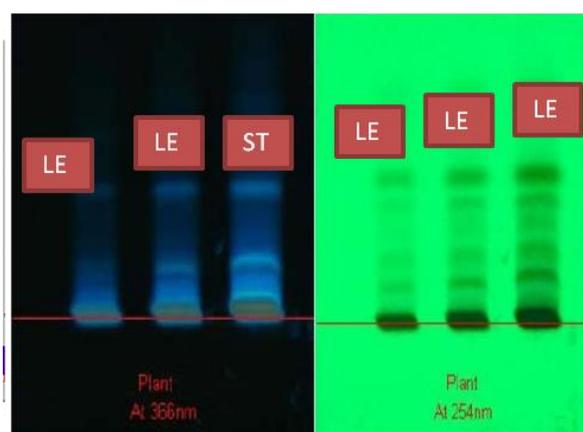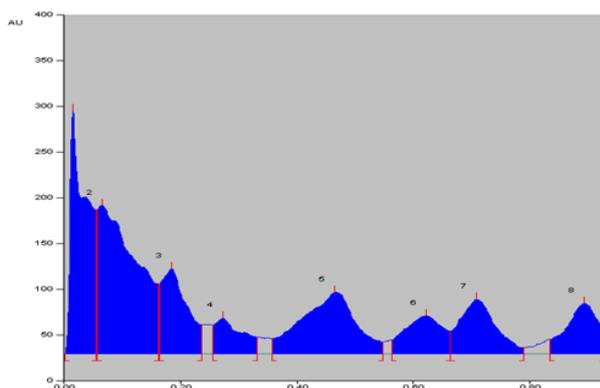

Figure 6: HPTLC profile of extract of *Ocimum tenuiflorum* with standard eugnol. (a) profil at showed 6 peak at 254nm ;(b) profil at showed 9 peak at 366 nm and (c)Photo documentation report of hydro-alcoholic extract at 254nm and at 366nm LE –leaf extract;ST-Standard.

Table 10a. R$_f$ value, No. of Peaks, peak area and height of hydroalcholic extract of *O. tenuiflorum* in Toluene: Ethyl acetate at 254 nm.

Peak	Start R$_f$	Start Height	Max R$_f$	Max Height	Height %	End R$_f$	End Height	Area	Area %
1	0.01	302.6	0.01	324.0	47.62	0.03	0.6	2776.3	17.53
2	0.18	0.2	0.21	12.7	1.87	0.25	4.7	357.8	2.26
3	0.31	8.5	0.35	11.7	1.72	0.41	2.2	557.8	3.52
4	0.75	2.2	0.78	10.4	1.53	0.80	6.7	326.6	2.06
5	0.81	7.1	0.89	166.0	24.39	0.91	152.6	6422.5	40.55
6	0.91	152.9	0.92	155.6	22.87	0.97	71.1	5398.9	34.08

Table 10b. R$_f$ value, No. of Peaks, peak area and height of hydroalcholic extract of O. tenuiflorum in Toluene: Ethyl acetate at 366 nm.

Peak	Start R$_f$	Start Height	Max R$_f$	Max Height	Height %	End R$_f$	End Height	Area	Area %
1	0.00	0.2	0.02	265.5	31.82	0.06	157.6	6275.7	18.02
2	0.06	157.9	0.07	162.0	19.41	0.16	76.6	9120.9	26.19
3	0.16	76.7	0.18	92.8	11.12	0.24	31.6	3444.2	9.89
4	0.26	31.4	0.27	39.2	4.69	0.33	18.3	1545.7	4.44
5	0.36	16.3	0.47	67.7	8.12	0.55	12.6	5161.8	14.82
6	0.56	14.7	0.62	41.4	4.97	0.66	24.9	2258.7	6.49
7	0.66	24.9	0.71	59.5	7.13	0.79	6.5	3032.2	8.71
8	0.83	15.9	0.89	55.1	6.60	0.93	31.1	2453.6	7.05
9	0.93	31.4	0.95	51.2	6.14	0.99	1.6	1527.8	4.39

Table 11: R_f value, No. of Peaks, peak area and height of hydroalcholic extract of *R. indica* in Toluene: Ethyl acetate at 254nm

Peak	Start R_f	Start Height	Max R_f	Max Height	Height %	End R_f	End Height	Area	Area %
1	0.00	0.2	0.02	265.5	31.82	0.06	157.6	6275.7	18.02
2	0.06	157.9	0.07	162.0	19.41	0.16	76.6	9120.9	26.19
3	0.16	76.7	0.18	92.8	11.12	0.24	31.6	3444.2	9.89
4	0.26	31.4	0.27	39.2	4.69	0.33	18.3	1545.7	4.44
5	0.36	16.3	0.47	67.7	8.12	0.55	12.6	5161.8	14.82
6	0.56	14.7	0.62	41.4	4.97	0.66	24.9	2258.7	6.49
7	0.66	24.9	0.71	59.5	7.13	0.79	6.5	3032.2	8.71
8	0.83	15.9	0.89	55.1	6.60	0.93	31.1	2453.6	7.05

Figure 7: HPTLC profile of hydroalcoholic of *R. indica* with standard saponin. Photo documentation report of hydro-alcoholic extract of *R. indica* at 254 nm and at 366 nm LE; Leaf extract; ST-Standard.

Discussion

In the current period, there is a need for logical assessment of natural formulation for better treatment of the clinical issues in a clinical manner. The bioactive compound of every, one of the four restorative plants exhibits inside homegrown concentrate and in joined herb-herb (polyherbal definition) that may result in all the more dominant for mental issue treatment. A few reports demonstrated that the utilization of a poly-natural plan has the greatest useful power when contrasted with a single herb. Yet different definitions couldn't be valuable because of the absence of legitimate institutionalization. In India, Ayurvedic Pharmacopeia is not progressively sufficient to guarantee the quality and virtue of plant extracts' use as a herbal drug. Since the concentrates from assembling points are not in a condition that could impact appropriately. Along these lines, for the experimentally demonstrated, customary medications and natural plans are to be institutionalized for guaranteeing and defending the best quality, immaculateness, and genuineness of the homegrown medications convert into powerful medication [49]. Along these lines, institutionalization is the easiest and least expensive approach to the investigation of homegrown medication through synthetic, different strategies, morphological, minute examination and afterward thin layer chromatography examination as well as initiating to assigning the right ID of the source plant extract [48].

As in the present investigation there is no comparable work accessible on these restoratively strong plant extricate and consolidated herb-herb. This examination work was embraced to mastermind the norms profile for building up its credibility. In this way, the results of the above discoveries will fill in, as a promising hotspot for setting down the pharmacopeia principles profile of *N. arbortristis*, *H. salicifolia*, *O. tenuiflorum* and *R. indica* and consolidated herb-herb for future investigations and research for treatment of mental malady.

Conclusion

The present study was taken up in the view to standardize these herbals in accordance with WHO norms and standard laboratory procedures.

Formulations were investigated for their organoleptic characters, physicochemical parameters, HPLC analysis and phytochemical parameters etc. The heavy metal quantity was also found within the standard limit as given by the regulatory authorities Hence, the pharmacognostic parameter of the plant extracts will be helpful in further preclinical and clinical study. The presence of phytochemicals also indicated that it can be used to develop the new lead Phyto-molecule in the treatment and management of lifestyle disease or disorder. The finding of this study can be used for evaluating the quality and purity of these plants as a polyherbal formulation for clinical application.

Author contributions
All authors have equal contributions. All authors read and approved the final manuscript.

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