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Safety Evaluation and Standardization of a Cardioprotective Unani Polyherbal Formulation with Modern Techniques

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ABSTRACT

Background: The use of herbal products and traditional medicines are increasing globally day by day in health care system especially in various chronic lifestyle and non-infectious diseases. Therefore, it is necessary that everything that used by human being as medicine must be safe and genuine to avoid their adverse effects. Sharbat Badranjboya(SB) is traditional Unani syrup formulated as a cardio tonic for the treatment of *Zof-e-Qalb* (weakness of the heart), *Khafqan* (palpitation), and *Twahush Sodawi* (Insanity). It is a liquid polyherbal preparation (syrup) of 10 medicinal plants. ***Methods:*** The parameters were adopted for the standardization and safety evaluation are physic chemical and phytochemical parameters, thin layer chromatography (TLC), microbialload, aflatoxins, heavy metals, and pesticidal residues revealing specific identities for the particular drug and to evaluate pharmacopoeia l standards. ***Results and Conclusion*:** Phytochemical, physicochemical, chromatographic and safety profile of the formulation was established. Findings of the present work can be used as a reference standard by quality control/ assurance laboratory of a pharmaceutical firm in order to have a proper quality check over its preparation and processing.

INTRODUCTION

Unani System of Medicine is a rich authentic source of natural medicine which keeps thousands of different single as well as compound formulations inside it for various cardiovascular diseases. Many drugs from this list has been tested scientifically and showed pleasing results while a long list still exists which needs proper screening for evaluation of their efficacy and safety at modern parameters and to provide the good therapeutic agents and some potent natural origin drugs for cardiovascular elements and to build up a healthy society. This dream will come true only when the crude materials will be used in its pure and genuine form. It is a fact that every natural drug has its own life span; a period in which all of its chemical components are present in correct proportion. When the drug crosses this period, it exhausts and reduces its therapeutic effects slightly or completely. But unfortunately many available Unani drugs in open market are adulterated or replaced with other cheap material or incorrectly collected from unusual sources which are the major reason behind low efficacy of Unani drugs. The contamination with cheap unwanted materials or earthy substances when cross permissible limits they become harmful and even causes danger for consumer's life. So it is worthy that plant materials should be collected from authentic sources and identified not only based on Unani literatures or ethno botanical description but all parameters of standardization and identification of plant material should also be involved to find correct and genuine plant. Unani medication has fewer or no side effect in comparison to Western medications prescribed for myocardial infarction. Along with side effects, allopathic medicines are very costly and it is hard for many people to buy and use them on regularly basis. Unani medicines from ancient time till now gain attention worldwide due to their significant role in many aspect of healthcare including cardiovascular disorders. In spite of great advances of allopathic medicine, traditional medicines including Unani and Ayurveda are still the primary form of treating diseases of majority of people in developing countries including India; even among those to whom western medicine is available. Also some people believe in adjuvant therapy from Unani medicines along with Western medicines. There are thousands of plant based formulations and single medicines used in Unani System of medicine for the treatment of coronary heart diseases and other cardiac disorders. Most of these medicines have not been scientifically investigated and there is little scientific data supporting their therapeutic use. Therefore; the present study was undertaken to standardize the Sharbat Badranjboya for their identity and safety by all possible modern methods. This formulation comprises of leaves of Nepeta hindostana (Barg Badranjboya), seeds of N.

hindostana, seeds of Cichorium intybus (Tukhm Kasni), Ocimum gratissimum (Faranjmushk), Leaves of Borago officinalis (Barg Gaozaban), root of Glycyrrhiza glabra (Mulethi), Foeniculum vulgare (Sonf), Polypodium vulgare (Bisfayij), Rosa damascene (Gul Surkh) and Malus domestica (Seb) in varying quantities.

Collection and authentication of ingredients of the formulation

The ingredients of test formulation "sharbat Badranjboya"Were procured from Dawakhana Tibbiya College AMU Aligarh except the Badranjboya (Nepeta hindostana) which was directly collected from Forest Research Institute of Dehradun and Shankar Nagar Village of Balrampur District, Uttar Pradesh. Badranjboya was cultivated at herbal garden of Department of Ilmul Advia as well as the lawn of SS Hall South AMU. All the drugs were identified in the Pharmacognosy section of Department of Ilmul Advia Ajmal Khan Tibbiya College, AMU Aligarh. The drugs samples were submitted to Mawalid-e-Salasa Museum of the department after identification, for future reference bearing the voucher No SC- 0261/21 (Badranjboya), SC-0262/21 (Gul Surkh), SC-0263/21 (Tukhm Kasni), SC-0264/21 (Faranjmushk), SC0265/21 (Badiyan), SC-0266/21 (Aslu s Sus), SC-0267/21 (Bisfayij), SC-0268/21 (Seb), SC-0269/21 (Gaozaban).

Preparation of Safoof (Powder)

The test drugs were cleaned from the earthy material, and dried in sunlight then powdered in electrical grinder. There after the powdered sample was passed through sieve no. 80 to confirm its fineness and uniformity of particle size. The sample was then subjected to physicochemical and phytochemical studies to determine various constants. The powder was also evaluated for the presence of microbial load, pesticides residue, aflatoxins estimation and heavy metal from Delhi Test House, Azadpur, Delhi-110033 (INDIA) [QR-0302, Report No.2097210813IM4014, Sample Date:13/08/21, Date of Report:19/08/21].

Evaluation of Organoleptic Characteristics

The organoleptic characters of all single ingredients of the test formulation and the compound powdered drug were evaluated based on the method described by (Anonyms, 1998)[1] Organoleptic evaluation refers to determination of the powder drug by its color, odor, taste and texture etc.

Physicochemical Studies

The physicochemical study included the determination of successive extractive values of the test drugs in different solvents, alcohol and water soluble contents, moisture content, ash values, loss of weight on drying, pH values and bulk density.

Successive Extractive Values

The successive extractive values of the test drug in different organic solvents viz. petroleum ether, diethyl ether, chloroform, acetone, alcohol and distilled water were determined by soxhlet method (hot method) using a soxhlet's apparatus. The heat was applied for six hours for each solvent on water bath and/or heating mantle. The extracts were evaporated on water bath and after evaporation of the solvents; the extractive values were determined with reference to the weight of air dried powdered drug. The procedures was repeated three times and the mean value for each extract was calculated (Anonymous, 1998).[1]

Water and Alcohol Soluble Contents

Two sample of 5 gram of the air dried powdered drug was taken with 100 ml of distilled water and 100 ml ethanol separately in a glass stoppered conical flask for 24 hours. The mixture was carefully shaken frequently for 6 hours and then allowed standing for 18 hours. It was filtered and filtrate was evaporated to dryness on a water bath. The residue was dried at 105 °C to constant weight, cooled in desiccator for 30 minutes and weighed. The percentage of water soluble matter was calculated with reference to the amount of air dried drug. The percentage of alcohol soluble matter was determined as above (Afaq, 1994; Anonymous, 1998).[1,2]

Loss on Drying (Gravimetric determination) and Total Solid Content

LOD is the loss in weight in % (w/w) resulting from water and volatile matter of any kind that can be driven off under specified conditions. 10 gram accurately weighed sample was taken, uniformly spread to obtain thin layer of powder in previously dried and tarred shallow petridish. It was kept in an oven and dried at a regulated temperature of 105 °C; cooled in a desiccator and weighed. The process was repeated many times till two consecutive weights were found constant. The percentage of loss in weight was calculated with respect to initial weight in mg per gram. Total solid content was determined by the formula: Total solid (%) = 100-Moisture (%) (Anonymous, 1998; Jenkins et.al, 2008; Bhargava et.al, 2013).[1,3,4]

Moisture Content (Azeotropic method)

The toluene distillation method (Dean and Stark Method) was used for the determination of moisture content. 10 gram of drug was taken in a thoroughly cleaned flask of the apparatus and about 40 ml of distilled toluene was added to cover the sample completely. Immediately the receiving tube was also filled with toluene, pouring it through top of condenser. Distillation was carried out for one hour (initially at the rate of 2 drop/second until most of the water passes over then the rate was increased at 4 drop/second). After the receiving tube came to room temperature, the volume of water collected in lower layer of graduated receiver was noted and the percentage of moisture calculated with reference to the weight of the air dried drug taken for the process. This process was repeated three times with both toluene and xylene solutions (Anonymous, 1998 and Jenkins et.al, 2008). [1,3]

Determination of Ash Values

The ash remaining after the ignition of herbal materials is determined by three different methods which measures total ash, acid insoluble ash and water soluble ash. Total ash measures the total amount of material remaining after ignition. This includes both physiological ash (from the plant tissue itself) and non-physiological ash (sand and soil etc.). In acid insoluble ash, the amount of silica is measured. Water soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water. An ash value is useful in the determination of purity and authenticity of crude drug (Afaq, 1994; Anonymous, 1998).[2,1]

(i) Total Ash

2 gram of accurately weighed air dried drug was spread in an even layer and incinerated in a previously ignited and tarred silica crucible at a temperature not exceeding 450 ˚C in a muffle furnace until became white indicating the absence of carbon, cooled in desiccator and weighed. The percentage of ash was calculated by subtracting the weight of crucible from the weight of crucible + ash (mg/gm.). The percentage of total ash was calculated with reference to the weight of dried drug taken (Anonymous, 1998 and Jenkins et. al, 2008).[1,3]

(ii) Water Soluble Ash

To the crucible containing the total ash, 25ml distilled water was added and boiled for 5 minutes. The insoluble matter collected on an ashless filter paper; (Whatman No. 42), was

washed with hot water and ignited in crucible for 15 minutes at a temperature not more than 450˚C; the weight of insoluble ash was subtracted from the weight of total ash, giving the weight of water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried drug taken (Afaq, 1994; Anonymous, 1998 and Jenkins et .al, 2008).[2,1,3]

(iii) Acid Insoluble Ash

The total ash was boiled gently with 25 ml of 2M hydrochloric acid for 5 min. The insoluble matter collected on ashless filter paper (Whatman No. 42), was washed with hot water until it became neutral. The filter paper containing insoluble material was transferred into the crucible and ignited at a temperature not exceeding 450˚C till constant weight was achieved. The percentage of acid-insoluble ash was calculated with reference to the weight of air dried drug taken (Anonymous, 1998; Jenkins et. al, 2008).[1,3]

pH Value

The pharmaceutical compounds are acids, bases or salts. They play an important role in drug stability and its therapeutic activity, therefore; the determination of pH of drug is an important tool. Determination of pH was carried out by digital pH meter. The instrument was standardized by using buffer solution of 4.0, 7.0, and 9.20 to ascertain the accuracy of the instrument prior to the experiment. The pH value of 1% and 10% aqueous solution of powdered drug solution was measured.

The pH value of 1% aqueous solution

An accurately weighed 1 gram powdered drug was dissolved in distilled water and the volume was adjusted accurately to 100 ml in a conical flask then allowed to stand overnight. Then it was filtered and the pH of 1% solution was measured with pH meter at room temperature until two successive reading agreed within +0.02 units (Anonymous, 1987; Jenkins et. al, 2008).[1,3]

The pH value of 10% aqueous solution

An accurately weighed 10 gram powdered drug was dissolved in distilled water and the volume was adjusted accurately to 100 ml in a conical flask and allowed to stand overnight. It was filtered and the pH of 10% solution was measured with pH meter at room temperature

until two successive reading agree within +0.02 units (Anonymous, 1987; Jenkins et .al, 2008).[1,3]

Bulk Density

The bulk density of a powder is the ratio of the mass of an untapped powder sample and its volume including the contribution of the inter particulates void volume. The bulking properties of a powder are dependent upon the preparation, treatment and storage of the sample, i.e. how it was handled. The slighter disturbance may lead to change in bulk density thus; it is very difficult to measure it with good reproducibility (Anonymous, 1987)[5]. (i) Poured Bulk Density It was determined by pouring 50 gram of sample drug into a graduated cylinder and the volume occupied by the drug was measured. (ii) Tapped Bulk Density The tapped density is an increased bulk density attained after mechanically tapping a graduated measuring cylinder or vessel containing the powder sample. It was determined by measuring the volume occupied by the sample of known mass (50 gram) into a graduated cylinder after subjected to prescribed amplitude and frequency of tapping over a prescribed period of time (15 minutes) using digital tapped densitometer. The poured and tapped bulk density arc expressed in gm/ml here ml and cm arc equivalent volume (Anonymous, 1987)[5].

Bulk Density= Weight of the powder drug (gm.) / Volume (cm3 or ml).

Determination of Swelling Index

The swelling index is the volume in ml taken up by the swelling of 1g of herbal material under specified conditions (Anonymous, 1998)[1]. Accurately weighed fine powdered drug was taken into a 25-ml glass-stoppered measuring cylinder which has an internal diameter of about 16mm, marked with 0.2-ml divisions from 0 to 25 ml in an upward direction. 25 ml of distilled water was added and the mixture was shaken thoroughly every 10 minutes for 1 hour. Then the mixture was allowed to stand for 3 hours at room temperature. The volume occupied by the sample drug in ml was measured. Simultaneously three determinations were done and mean was calculated (Anonymous 1998)[1].

Determination of Foaming Index

Some herbal drugs contain saponins that can cause persistent foam when an aqueous decoction is shaken. This foaming ability of an aqueous decoction is measured in terms of foaming index. 1 g of accurately weighted coarse drug powder was transferred to 500-ml

conical flask containing 100ml boiling water. A moderate boiling was maintained for 30 minutes then cooled and filtered into a 100-ml volumetric flask. Sufficient amount of water was added through the filter to dilute the volume. The decoction was poured into 10 stoppered test-tubes (height 16cm, diameter 16mm) in successive portion of 1ml, 2ml, 3ml, up to 10ml and volume of the liquid was adjusted in each tube with distilled water to 10 ml. The tubes were shaken in a lengthwise motion for 15 seconds, two shake per second. Allowed to stand for 15 minutes and height of foam was measured and calculated with the formula: 1000/a Where a = the volume in ml of decoction used for preparing the dilution in the tube where foaming to a height of 1cm is observed. If the height of the foam in every tube is less than 1 cm, the index is less than 100.If the height of the foam is more than 1cm, the index is over 1000. In this case the test should be repeated (Anonymous, 1998)[1].

Determination of Refractive Index

When a ray of light passes from a less dense to a high denser medium, it is bent or "refracted" towards the normal. Refractive index is the ratio of the velocity of light of a specified wavelength in air to its velocity in the examined substance. When the principle of measurement is used it may be defined as the site of the angle of incidence divided by the sine of the angle of refraction (George wypych, 2014).[6] The refractive index was determined by the Abbe Refractometer (Bosch and Lomb), in which the angle measured is the critical angle for total reflection between glass of high refractive index and the substance to be examined. By this means and by selecting a particular wave length of light, at which to make the measurement, it is possible to calibrate the instrument directly in term of Refractive index. It is emergent beam that is viewed in the instrument and the critical angle is indicated by edge of dark part of the field of view. The instrument was adjusted in this way so that half of view was dark while the material is on the prism and the demarcation line of the dark and illuminated field should be as sharp as possible. The prism was cleaned carefully with alcohol and then with ether. Few drops of sample were placed on prism and closed firmly by tightening the screw head. It allowed standing for a few minutes before the reading is made so that the sample and instrument will be at the same temperature. Alidade was moved backward and forward until the field of vision is divided into a light and dark portion. The line dividing these portions is the "border line," and, as a rule, will not be a sharp line but a band of color. The colors are eliminated by rotating the screw head of the compensator until a sharp, colorless line is obtained. The border line was adjusted so that it falls on the point of intersection of the cross hairs and the direct refractive index was noted. A second reading was

also taken a few minutes later to assure that temperature equilibrium has been attained (Jenkins et. al, 1967; Novak et.al, 2018).[3,7]

Determination of Specific Gravity

Specific gravity means relative density. The specific gravity of a liquid is the relative weight of that liquid compared to an equal volume of water at specific temperature (Stauffer et.al, 2008).[8] A specific gravity bottle was carefully filled with the material and weighed. The weight of the material was determined by subtracting the weight of the specific gravity bottle from total weight. The weight of an equal volume of distilled water was similarly determined by taking the water in same bottle and subtracting it from the total weight of the bottle. Specific gravity was determined by dividing the weight of the material by the weight of distilled water according to the following formula (Anonymous 1998).[1]

$$\textbf{Specific gravity} = \frac{weight\ of\ SG\ bottle with\ substances - weight\ of\ empty\ bottle}{weight\ of\ SG\ bottle\ with\ water - weight\ of\ empty\ bottle}$$

Determination of viscosity

Viscosity of any fluid means, measure of its resistance to flow. It was measured at room temperature by filling the viscometer, previously washed with chromic acid E and five times with distilled water with the liquid being examined through tube L to slightly above the mark G, using a long pipette to minimize wetting the tube above the mark. Tube was placed vertically and the volume of liquid was adjusted so that the bottom of the meniscus set at the mark G. The rubber tube was attached to E end of the tube and the liquid was sucked 5mm above the point E. After releasing the suction, the time required for the bottom of the meniscus to fall from the top edge of mark E to the top edge of mark F was measured (Anonymous, 2008).[9]

Phytochemical Analysis

Qualitative analysis The qualitative screening of powdered crude drugs was carried out using the following standard procedures (Debiyi, 1978; Trease and Evans, 1983; Afaq, 1994; Anonymous, 1992; Mukherjee, 2002; Anonymous, 2008).[10,11,2,12,13 ,9]

One gram of the chloroform, ethanol and aqueous extracts of the ingredients of Sharbat Badranjboya was dissolved in 100 ml of its own mother solvents to obtain a stock of

concentration 1% (w/v). The extracts thus obtained were subjected to preliminary phytochemical screening.

Test for Anthraquinones

10-ml of benzene was added in 6 g of the sample powder in a conical flask and soaked for 10 minutes and then filtered. Further 10ml of 10% ammonia solution was added to the filtrate and shaken vigorously for 30 seconds. The pink, violet, or red color indicated the presence of anthraquinones in the ammonia phase.

Tests for Flavonoids Shinoda Test

Pieces of magnesium ribbon and concentrated Hcl were mixed with aqueous crude plant extract after few minutes, the pink color was observed to ensure the presence of flavonoid. Alkaline Reagent Test 2ml of 2.0% NaOH mixture was mixed with aqueous plant crude extract; concentrated yellow color was produced, which became colorless after adding 2 drops of diluted acid to mixture. This result showed the presence of flavonoids.

Test for Alkaloids

(i) Dragendroff Test: A drop of Dragendroff's reagent (potassium bismuth iodide solution) to the extract was added. The brown precipitate was observed. **(ii) Hager's test:** Few drops of Finger's reagent (Appendix) were added in 1 ml of alcoholic test solution. The presence of yellow color precipitate was an indication of the presence of alkaloids.

Test for Carbohydrate

Fehling's Test: 1 ml Fehling's A (deep blue aqueous solution of copper (II) sulfate) and 1 ml Fehling's B solutions (colorless solution of aqueous potassium sodium tartrate) was mixed and boiled for one minute. On adding equal volume of test solution and heating in boiling water bath for 5-10 min and observed for a yellow, then brick red precipitate.

Tests for Glycosides

Hydrolysis of extract A minimum quantity of the extracts was hydrolyzed with hydrochloric acid for few minutes on water bath and the hydrolysate is subjected to the Borntrager's test. **Borntrager's test** Hydrolysate was treated with chloroform and the chloroform layer was separated. To this, equal quantity of dilute ammonia solution was added. Color changes in the ammonical layer showed the presence of glycoside.

Test for Cardiac Glycosides

Keller-Kiliani Test: A solution of glacial acetic acid (4.0ml) with 1 drop of 2.0% FeCl3 mixture was mixed with the 10ml aqueous plant extract and 1ml H2SO4 concentrated. A brown ring formation between the layers was observed to entity of cardiac steroidal glycosides.

Salkowski's Test: 2ml concentrated H2SO4 was added to the whole aqueous plant crude extract. A reddish brown color was observed for the presence of steroidal aglycone part of the glycoside. **Test for Tannin Lead acetate solution test**: When few drops of lead acetate solution were added to 2 ml of extract a white precipitate was observed.

Ferric chloride solution test: Ferric chloride solution was added in the aqueous extract of the drug. A bluish-black color, which disappeared on addition of dilute sulphuric acid followed by a yellowish brown precipitate, showed the presence of tannin.

Tests for Phyto Steroids: Small quantity of extract was dissolved in 5 ml of chloroform separately. The above obtained chloroform solutions were subjected to Salkowski and Liebermann- Burchard tests.

Salkowski test: To the 1 ml of above prepared chloroform solution, few drops of concentrated sulphuric acid were added. Formation of brown ring indicated the presence of phytosterols. **Liebermann-Burchard test:** The above prepared chloroform solution was treated with few drops of concentrated sulphuric acid followed by 1 ml of acetic anhydride solution. A bluish green color solution was observed for the presence of phytosterols.

Test for Proteins

Biuret's test: 5-8 drops of 10 W/V Sodium hydroxide solution was added to I ml hot extract of test drug followed by I or 2 drops of 3 % W/V copper sulphate solution. A red or violet color was obtained.

Test for Amino Acids (Ninhydrin Test): The 3 ml test solution and 3 drops 5% Ninhydrin solution were heated in boiling water bath for 10 min. observed for purple or bluish color.

Test for Starch 0.015 g of Iodine and 0.015 g of Potassium Iodide was added in 5 ml of distilled water; 2 ml of iodine solution formed was added to 2 ml of aqueous test solution, the presence of blue color was observed.

Test for Phenol (Ferric chloride Test): Ferric chloride solution was added in 2 ml of ethanolic or aqueous test solution. Blue or green color indicated the presence of phenols.

Libermann's Test: 2m1 of ethanolic or aqueous test solution was dissolved with 0.5 ml of 70 % H2SO4 followed by the addition of few drops of aqueous sodium nitrite solution (0.5%). Red color on dilution indicated the presence of phenols.

Test for Terpenoids 2 ml of chloroform was added with the 5 ml aqueous plant extract and evaporated on the water bath and then boiled with 3 ml of concentrated H2SO4. A grey color formed which showed the entity of terpenoids.

Thin Layer Chromatography (TLC)

All the extracts of Sharbat Badranjboya were subjected to thin layer chromatographic studies, to determine the probable number of compounds present. Preparation of the plates The precoated TLC plates made up of silica gel G (Layer thickness 0.20-0.25 mm) as an adsorbent were activated in an oven for 30 minutes at 110°C. Test samples (1mg/ml of all extracts in respective solvents) were applied in the form of bands using Linomat IV applicator.

Development of solvent system: A number of solvent systems were tried in order to get maximum separation on plate. After development of plates, they were air-dried and numbers of spots were noted & Rf values were calculated by the following formula: Rf Value = Distance travelled by the spot / Distance travelled by the solvent. Spots were visualized by spraying with various spraying reagents to find different compounds present in the extract (Stahl, 1969; Mukherjee, 2002 and Anonymous, 2007).[14,13,15]

Safety Studies

Microbiological determination tests: Total viable aerobic count (TVC): For detection of the antibacterial activity of the test drug, the total viable aerobic count (TVC) of the test drug was carried out, determined, as specified in the test procedure, using following methods:

Pre-treatment of the test drug: Depending on the nature of the compound sample used, it was dissolved using a suitable method and any antimicrobial property present in the sample was eliminated by dilution or neutralization. Buffered Sodium Chloride-Peptone Solution, pH 7.0 (MM1275-500G Himedia Labs, Mumbai, India) was used for dilute the test sample.

Plate Count for bacteria: 1 ml of the pre-treated test sample was added to about 15 ml of the liquefied casein-soybean digest agar in a petridish of 90 mm diameter at a temperature not exceeding 45 0C. Alternatively the test sample was spread on the surface of the solidified medium. Two dishes were prepared with the same dilution, they were inverted and incubated at 30-350C for 48-72 hrs, unless a more reliable count was obtained in a short period of time. The number of colonies so formed was counted and the results were calculated using the plates with the largest number of colonies, up to a maximum of 300.

Plate Count for fungi: 1 ml of the pre-treated test sample was added to about 15 ml of the liquefied Sabouraud glucose agar with antibiotics in a petridish of 90 mm diameter at a temperature not exceeding 450C. Alternatively the test sample was spread on the surface of the solidified medium. Two dishes were prepared with the same dilution; they were inverted and incubated at 20-250C for 5 days, unless a more reliable count was obtained in a short period of time. The number of colonies so formed was counted and the results were calculated using the plates with not more than 100 colonies.

Heavy Metals Determination: Heavy metals including Lead, Mercury, Arsenic and Cadmium were determined in the test sample using Atomic Absorption Spectroscopy (AAS).

Aflatoxin Estimation: The test for the determination of aflatoxins B1, B2, G1 and G2 was carried out using LC-MS/MS. 2 gm of test drug was blended at high speed with 20 ml of 60% acetonitrile/water for two minutes. The blended sample was centrifuged for ten minutes using 1600 rpm (av.), supernatant was retained and diluted with 2 ml of filtrate with 48 ml of phosphate buffered saline (PBS, pH 7.4) to give a solvent concentration of 2.5% or less; methanol/water was prepared by taking 2 ml of sample and diluted with 14 ml of PBS (pH 7.4) to give a solvent concentration of 10% or less. The sample diluent was passed through the immunoaffinity column at a flow rate of 5 ml/ min. The column was then washed by passing 20 ml of distilled water through the column at the flow rate of approximately 5 ml/ min and dried by rapidly passing air through the column. 1.5 ml of distilled water was added to the sample elute. 500 µl of sample was injected onto the LCMS-MS (LCPerkin, MS Applied Bio System, Model No.2000, Mobile Phase). A- Water 100%, B-ACN 100%, Column oven temperature = 30, Column ZORBAX Rx c18, narrow base 2.1×150 mm - 5 micron, Flow = 0.750 ml). The aflatoxin concentration was quantified by comparing sample peak heights or areas to the total aflatoxin standard (R-Biopharm) (Lohar, 2007).[16]

Pesticidal Residue Estimation: The test for the assessment of specific pesticide residues like Organochloride compounds, Organophosphorous compounds and Pyrethroids compound was conducted using GC-MS/MS (Ramkrishanan et al., 2015)[17].

OBSERVATION AND RESULTS

Table-1: Organoleptic Characters of powder of Sharbat Badranjboya

S.No	Parameters	Result
1	Color	Reddish brown
2	Appearance	Coarse
3	Odor	Rose scent
4	Taste	Sweet

Table 2: Physicochemical parameters

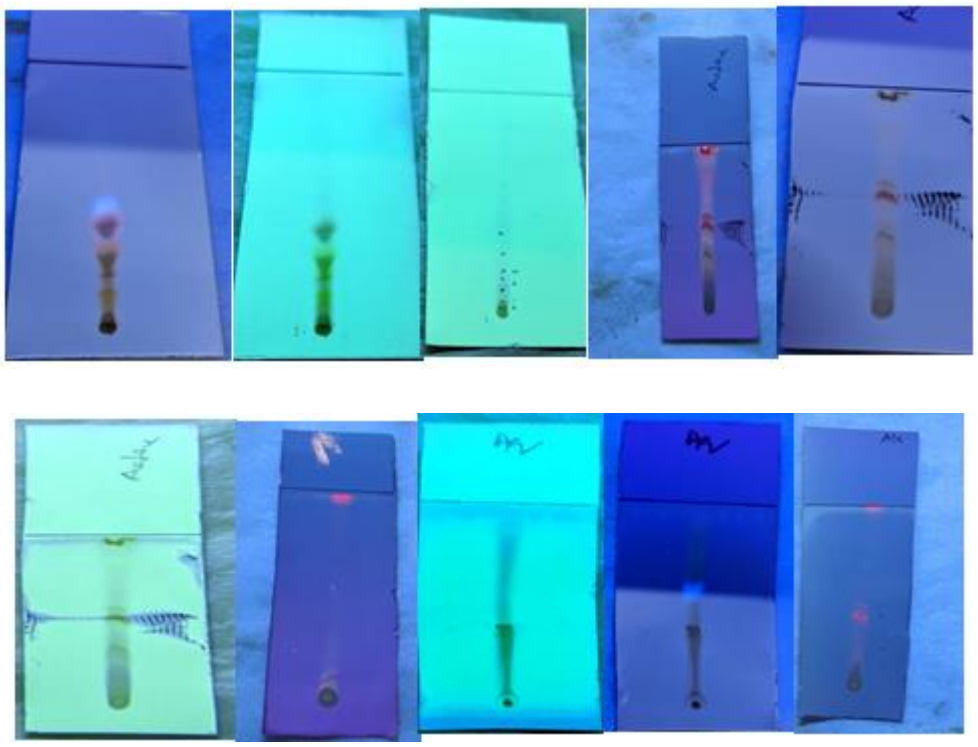
S.No	Parameters	Results
1	Successive Extractive values	Petroleum ether 0.5±0.0 Di ethyl ether 1.5±0.0 Chloroform 1.83±0.83 Acetone 2.33±0.16 Alcohol 6.16±0.33 Aqueous 11±0.5
2	Solubility	Water Soluble Content 7.22±0.02 Alcohol Soluble Content3.94±0.006
3	Loss of weight on drying at 105^0C	9.53±0.14
4	Moisture Content through toluene distillation method	9±0.0
5	Ash Value	Total Ash 4.44±0.24 Acid Insoluble Ash 0.36±0.003 Water Soluble Ash 1.92±0.014
6	pH Value	1% Aqueous Solution 6.76±0.033 10% Aqueous Solution 6.26±0.033
7	Bulk Density	Poured Density 0.45±0.01 Tapped Density 0.54±0.01
8	Swelling Index	2.66±0.16
9	Foaming Index	<100±0.0
10	Refractive Index	8.46±0.31
11	Specific Gravity	0.301±0.009
12	Viscosity	Kinematic Viscosity (stokes) 1.4156 Dynamic Viscosity (Pascal second)1.6010

Table-03: Qualitative Analysis of the Phytochemicals Present in the Sharbat Badranjboya Powder

S.No.	Chemical Constituents	Test/Reagents	Result
1	**Anthraquinones**	Benzene and Ammonia Test	–
2	**Flavonoids**	Shinoda Test	+
		Alkaline Reagent Test	+
3	**Alkaloids**	Dragendroff Test	+
		Hager's test	+
4	**Carbohydrate**	Fehling's Test	+
5	**Glycosides**	Borntrager's test	+
	Cardiac Glycosides	Keller-Kiliani Test	–
		Salkowski's Test	+
6	**Tannin**	Lead acetate solution test	+
		Ferric chloride solution test	+
7	**Phyto Steroids**	Salkowski test	+
		Liebermann-Burchard test	–
8	**Proteins**	Biuret's test	+
9	**Amino Acids**	Ninhydrin Test	+
10	**Starch**	Iodine Test	+
11	**Phenol**	Ferric chloride Test	+
		Libermann's Test	+
12	**Terpenoids**	Salkowski's Test	+

Table-04: Thin Layer Chromatography (TLC) of Sharbat Badranjboya

Extract	Solvent System	Treatment	No. of Spots	R_f Value & Color of Spots
Petroleum ether	Petroleum ether: ethyl acetate(24:1)	UV Short	4	0.034, 0.068, 0.120,0.189 (Yellowish)
		UV Long	7	0.051, 0.068,(Green) 0.103, 0.155, 0.189, 0.258, 0.344 (Yellow)
Diethyl ether	Petroleum ether: Ethanol (19:1)	UV Short	6	0.046, 0.062, 0.093, 0.171,0.218,0.312 (Green)
		UV Long	8	0.046, 0.109, (Brown) 0.234, 0.281,(Greenish Yellow) 0.347, 0.406 , 0.468, 0.531 (Green)
Acetone	Chloroform: Methanol (9:1)	UV Short	9	0.019. 0.076, 0.192 (Green), 0.288, 0.326, 0.443, 0.50,0.75,0.90 (Brownish)
		UV Long	6	0.057, 0.173, 0.269 (Brown), 0.346, 0.519, 0.923 (Pink)
Ethanol	Chloroform : Acetone (5:1)	UV Short	2	0.240, 0.375 (Green)
		UV Long	4	0.107, 0.196, 0.375 (Brownish), 0.982 (Green)
Aqueous	n-Butanol :Acetic Acid: Water (5:1:4)	UV Short	5	0.078, 0.313, 0.411, 0.666, 0.823 (Brown)
		UV Long	6	0.058, 0.137, 0.352, 0.431, 0.568, 0.784 (Brown)



TLC Plate of different extracts in both UV Short and Long

Table 05: Microbial Load in Sharbat Badranjboya Powder

S.No	Test	Result	Permissible Limit
1	Total Bacterial Count	1500	Not more than $1x10^5$cfu/gm
2	Total Yeast & Moul	160	Not more than 1 $x10^3$ cfu/gm

Table 06: Test for specific pathogens in Sharbta Badranjboya Powder

S. No	Pathogens (/gm)	Result (gm)	Permissible limit as per API
1	Escherichia coli	Absent	Absent
2	Salmonella	Absent	Absent
3	Staphylococcus aureus	Absent	Absent
4	Pseudomonas aeruginosa	Absent	Absent

Table 07: Heavy metals in Sharbta Badranjboya Powder

S. No	Heavy Metals	Result	Permissible limit	Method
1	Lead (Pb)	7.0 ppm	Not more than 10ppm	AAS
2	Mercury (Hg)	Not detected(0.5ppm)	Not more than 1ppm	AAS
3	Arsenic (As)	Not detected(1.25ppm)	Not more than 3ppm	AAS
4	Cadmium (Cd)	Not detected(0.25ppm)	Not more than 0.3ppm	AAS

AAS = Atomic Absorption spectroscopy

Table 08: Aflatoxins in Sharbat Badranjboya Powder

S. No	Aflatoxins	Result	Permissible limit	Method
1	Aflatoxin B1	Not detected	Not more than 0.5ppm	LCMSMS
2	Aflatoxin G1	Not detected	Not more than 0.5ppm	LCMSMS
3	Aflatoxin B2	Not detected	Not more than 0.1ppm	LCMSMS
4	Aflatoxin G1	Not detected	Not more than 0.1ppm	LCMSMS

ppm= parts per million

LCMS/MS = Liquid Chromatography Mass Spectrometry

Table 09: Pesticidal residue in Sharbat Badranjboya

S. No	Pesticide Residue (mg/kg)	Resul (mg/kg)	LOQ (mg/kg)	Permissible limit (mg/kg)	Method
1	Alachlor	Not Detected	0.02	0.02	GCMSMS
2	Aldrin &Dieldrin	Not Detected	0.04	0.05	GCMSMS
3	Azinophos-methyl	Not Detected	0.04	1.0	GCMSMS
4	Bromopropylate	Not Detected	0.08	3.0	GCMSMS
5	Chlordane	Not Detected	0.04	0.05	GCMSMS
6	Chlorfenvinphos	Not Detected	0.04	0.5	GCMSMS
7	Chlorpyrifos	Not Detected	0.04	0.2	GCMSMS
8	Chlorpyrifos-methyl	Not Detected	0.04	0.1	GCMSMS
9	Cypermethrin	Not Detected	0.10	1.0	GCMSMS
10	DDT (Sum of pp-DDT, pp-DDE and pp-TDE)	Not Detected	0.04	1.0	GCMSMS

11	Deltamethrin	Not Detected	0.10	0.5	GCMSMS
12	Diazinon	Not Detected	0.04	0.5	GCMSMS
13	Dichlorvos	Not Detected	0.04	1.0	GCMSMS
14	Dithiocarbamates	Not Detected	0.1	2.0	UV-VIS Spectrophotometry
15	Endosulfan (Sum of Isomer and Endosulfan sulphate)	Not Detected	0.04	3.0	GCMSMS
16	Endrin	Not Detected	0.04	0.05	GCMSMS
17	Ethion	Not Detected	0.04	2.0	GCMSMS
18	Fenitrothion	Not Detected	0.04	0.5	GCMSMS
19	Fenvalerate	Not Detected	0.10	1.5	GCMSMS
20	Fonofos	Not Detected	0.04	0.05	GCMSMS
21	Heptachlor (Sum of Heptacholar& Heptachlor epoxide)	Not Detected	0.04	0.05	GCMSMS
22	Hexachlorobenzene	Not Detected	0.04	0.1	GCMSMS
23	Hexachlorocyclohexane isomer (other than ý)	Not Detected	0.04	0.3	GCMSMS
24	Lindane (ý-Hexachlorocylohexane	Not Detected	0.04	0.6	GCMSMS
25	Malathion	Not Detected	0.04	1.0	GCMSMS
26	Methidathion	Not Detected	0.04	0.2	GCMSMS
27	Parathion	Not Detected	0.04	0.5	GCMSMS
28	Parathion Methyl	Not Detected	0.04	0.2	GCMSMS
29	Permethrin	Not Detected	0.04	1.0	GCMSMS
30	Phosalone	Not Detected	0.04	0.1	LCMSMS
31	Piperonylbutoxide	Not Detected	0.04	3.0	LCMSMS
32	Primiphos Methy	Not Detected	0.04	4.0	LCMSMS
33	Pyrethrins	Not Detected	0.10	3.0	GCMSMS
34	Quintozen (Sum of Quintozene, pentachloroaniline and methyl pentachlorophenyl sulphide)	Not Detected	0.10	1.0	LCMSMS

DDT = Dichloro diphenyl trichloroethane

DDE = Dichloro diphenyl dichloroethylene

GCMS/MS = Gas Chromatography Mass Spectrometry

LCMS/MS = Liquid Chromatography Mass Spectrometry

DISCUSSION

Unani System of Medicine is a rich authentic source of natural medicine which keeps thousands of different single as well as compound formulations inside it for various cardiovascular diseases. Many drugs from this list has been tested scientifically and showed pleasing results while a long list still exists which needs proper screening for evaluation of their efficacy and safety at modern parameters and to provide the good therapeutic agents and some potent natural origin drugs for cardiovascular elements and to build up a healthy society. This dream will come true only when the crude materials will be used in its pure and genuine form. It is a fact that every natural drug has its own life span; a period in which all of its chemical components are present in correct proportion. When the drug crosses this period, it exhausts and reduces its therapeutic effects slightly or completely. But unfortunately many available Unani drugs in open market are adulterated or replaced with other cheap material or incorrectly collected from unusual sources which are the major reason behind low efficacy of Unani drugs. The contamination with cheap unwanted materials or earthy substances when cross permissible limits they become harmful and even causes danger for consumers' life. So it is worthy that plant materials should be collected from authentic sources and identifiednot only based on Unani literatures or ethnobotanical description but all parameters of standardization and identification of plant material should also be involved to find correct and genuine plant.The quality assurance and purity of plant origin drugs is mandatory for its safety and efficacy. An authentic and genuine crude drug sample is very important for its therapeutic uses. Since; efficacy of the drugs depends upon its physical and chemical properties: therefore, determination of physicochemical parameters to ascertain the quality of drugs is necessary before subjecting them to pharmacological studies. So various physicochemical and phytochemical parameters were adopted for the test sample for the first time. The plants are considered as biosynthetic laboratories for secondary metabolites which contribute to the therapeutic effects. Phytochemicals or chemicals present in plants play important roles from their growth and development to protection from harmful agents such as insects and microbes as well as stressful events such as ultraviolet (UV) radiation and extreme temperatures. They also attract beneficial birds and insects that promote pollination, germination, and seed dispersal. Phytochemicals provide colors to plants and an array of

flavors both pleasant and unpleasant when consumed. They are unique to specific plants and parts of plants, and they usually increase in abundance during stressful events. Phytochemicals also provide health benefits when consumed. They consist of nutrients essential for optimal health e.g., proteins, carbohydrates, vitamins, and minerals and other chemicals e.g., phenolic acids, flavonoids, and other phenolics (Watson RR, 2017).[18] Phytochemicals are naturally present in the plants may vary, not only from plant to plant but also among different samples of the same species. Some phytochemicals like alkaloids, flavonoids, terpenoids and tannins have property of precipitating proteins. Their presence may help in identifying the phytochemicals responsible for therapeutic effect which will further establish by scientific revalidation of drug being used in specific diseases. The phytochemical analysis of the chemical constituents present in the drug revealed that Sharbat Badranjboya contains alkaloids, carbohydrates, flavonoids, glycosides, proteins, phenols, sterols, saponins, and resins. Thin layer chromatography is one of the important parameters used for determining the identity and adulteration or for judging the quality of the drugs. It is applied for standardization of single as well as compound formulations. The various compounds present in the drugs got separated, depending on the affinity of mobile and stationary phases. If the drug is adulterated with other compounds it may increase the number of spots, on the other hand the exhausted or deteriorated drug may lose the component or change its chemical character, resulting in the less number of spots or appearance of new spots with different Rf value. Keeping this in mind, TLC studies of various extracts of test drug obtained in different solvent systems were noted in day light and UV light, The Rf values were used as a tool for the standardization of drugs, and these finger prints were used for identification. Sharbat Badranjboya as such has not been studied so far on physicochemical and phytochemical parameters in order to determine its quality standards. Therefore the findings of the present study could be served as constants for standard and also as reference for further studies.

CONCLUSION

The drug under study was subjected to physicochemical analysis, which is helpful in establishing the standard along with the other parameters such as microscopic study. Heavy metal analysis, aflatoxins contamination, pesticide residue analysis was done and found absent as reported in the present investigation besides the presence of microbial load was found within the permissible limits of WHO guidelines and TLC fingerprinting further confirmed the identity, purity and quality of the drug sample and revealed the presence of

phytochemicals and it is evident that plants having therapeutic values usually contain diverse groups of secondary metabolite. Therefore, these findings may be used as the standards for identity, purity and quality. The results of safety study revealed the presence of Microbial load but within permissible limit, which was unable to produce any toxicity. Heavy metals, Pesticide residue and aflatoxins were found absent in the test drug, indicating that the crude drug is free from toxicity and can be used safely without any side effects. So, it can be concluded that the drug is safe and can be explored for its therapeutic effectiveness.

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