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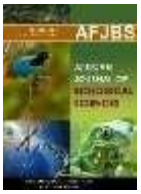

PHYSIOCHEMICAL INVESTIGATION OF UNANI FORMULATIONS WITH ANTI-INFLAMMATORY PROPERTIES

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Absctract

Habb-e-asgand, Habb-e-suranjan and Halwa Gheekwar are the Unani Formulations mainly used for various ailments like arthritis, gout, joint Pain, inflammations, anticancer, antioxidant, and immunomodulator. Unani Formulations are quite safe and effective. Although the formulations of the Unani system of medicine are more recognized, not so much scientific work has been reported so far. Many Pharmaceutical Companies manufacture the formulation for commercial supply but they fail to maintain the desired standards for it. The Unani Formulations was subjected to evaluate physiochemical parameters like Foreign Content, LOD, Ash Value, Extractive Value, Heavy Metals Estimation, Pesticide Residue, Test for Aflatoxins and were found to be under the limit and microbial content were also execute to check the presence of any hazardous substance in the formulation. The evidence produced in this study will lead to develop pharmacopeial standards of Habb-e-asgand, Habb-e-suranjan and Halwa Gheekwar which eventually assist to estimate the Standard, safety and potency of the formulation.

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1. Introduction

The utilization of herbs is making a resurgence, and herbal medicines now provide a safer alternative to synthetic drugs, which are generally considered hazardous to humans and the environment. Even though herbs have been valued for their medicinal, flavoring, and aromatic properties for a significant amount of time, synthetic items have, for the most part, exceeded the significance of herbs. On the other hand, the mindless dependency on synthetic goods has ended, and people are increasingly going back to natural products in hopes of improved safety and security[1,2].

Ahmed et al. examined into and discussed about standardizing Unani formulations in 2015. The Ayurveda, Yoga & Naturopathy, Unani System, and Homoeopathy (AYUSH) part of alternative medicinal systems in India include Unani medicine. Since decade, such techniques have been expanding with the social and cultural recommendation of the common masses. The Achilles' heel of these systems is now standardization/quality control of herbal formulations. National and international regulatory bodies have established standards for the quality control and quality assurance of herbal formulations, ensuring consistency across batches. The current research aimed to create and standardize the Habb-e-asgand, Habb-e-suranjan and Halwa Gheekwar, a pharmacopoeial Unani formulation adopted as an anti-inflammatory medication, particularly for joint and arthritic conditions. The completed items were subjected to various tests, such as physicochemical analysis, to standardize them.[3]

2. MATERIALS AND METHODS

2.1 Drugs and Chemicals Used

Habb-e-Asgand (HEA), Habb-e- Suranjan and Halwa Gheekwar was procured from the Unani local medical store of Nandurbar, Maharashtra,

0.9% sodium chloride (Hospira, Lake Forest, IL, USA), Deoxycholate citrate agar, soybean-casein digested medium (HIMEDIA), cetrimide agar (HIMEDIA), N, N, N/, N/tetramethyl p-phenylenediamine dichloride, Methanol-HPLC Grade, Hexane (Sigma-Aldrich), Acetonitrile.

2.2 Physico-chemical Analysis of Unani Formulations

2.2.1. Foreign Content

Around 100 grams of Formulation powder was evenly distributed into a thin layer and inspected with the naked eye to detect any foreign matter, which was separated and weighed to determine the percentage (%) of aged foreign content using a specific formula.[4].

$$\text{Percentage of foreign content} = \frac{\text{Weight of sample} - \text{Weight of foreign matter}}{\text{weight of sample}} \times 100$$

2.2.2 LOD

To determine the loss on drying at 105℃, 3 gm of Formulations were accurately weighed and placed in a Petri dish. The Unani formulation was then heated in an oven at 105℃ until a constant weight was reached, allowing for the calculation of the sample's percentage moisture content based on the initial weight.[5].

2.2.3 Ash Values

a) Total ash: 3 gm of Formulation were carefully weighed and incinerated in a crucible at a temperature below 45 ℃ until completely carbon free, cooled and resulting ash was then weighed three times and the percentage of ash were determined using the air-dried drug as a reference.

$$Total\ Ash = \frac{\text{Weight. of ash}}{\text{Weight. of drug}} \times 100$$

b) Acid-insoluble ash: After five minutes of boiling 25 ml of mild hydrochloric acid, the entire ash was retrieved. The insoluble residue was then gathered in a Gooch crucible, cleaned with hot water, and burned to a consistent weight. The air-dried medication was used as a reference to estimate the percentage of acid-insoluble ash.

c) Sulphated ash value: For the investigation, 1 gram of the Unani Formulation was placed in a crucible and gently ignited in a protected area to ensure thorough charring of the substance. The resulting residue was mixed with 1 mL of sulfuric acid after cooling and moistening, and then gently heated until no more white fumes were produced. It was then heated at (800 ± 25) ^{0}C until all black particles disappeared. It was allowed to cool before a few carefully placed drops of sulfuric acid were added and another cycle of ignition was performed. After cooling, the crucible

was weighed, and the procedure was repeated until there was no more than a 0.5 mg difference between two consecutive weights.[6–8].

d) Water soluble ash value: The entire amount of ash, about 1gm, was boiled for 5 minutes with 25 ml of water. The insoluble material was then collected in an Ash-Less filter paper or a Gooch crucible. After being cleaned with hot water, the ash was burnt at a low temperature to achieve a constatnt weight.[6–8]

2.2.4) Extractive Values

a) Alcohol-soluble extractive: weighed accurately 5 gm of powdered drug (Formulation) and it was placed in a closed conical flask, and add 100 ml of 90% alcohol. The flask was shaken consistently for six hours in an electrical shaker and then allow to stand overnight for maceration. After the flask was filtered, upon evaporation, the filtrate was dried thoroughly until no moisture remained. The weight of the extractive was then measured, and the percentage was calculated.

$$Alcohol - Soluble\ Extractive = \frac{\text{Wt. of extractive}}{\text{Wt. of drug}} \times 100$$

b) Water-soluble extractive: 5 grams of precisely measured powdered drug (Formulation) was placed into 100 ml of chloroform water in a sealed conical flask and consistently shaken for 6 hours using an electrical shaker. After leaving the flask overnight for maceration, then carefully filtered its contents and allowed the filter to evaporate to dryness. Taken the extractive weight and the percentage was calculated[7-9].

$$Water - Soluble\ Extractive = \frac{\text{Wt. of extractive}}{\text{Wt. of drug}} \times 100$$

2.2.5) Heavy Metals Estimation

The Unani formulations were subjected to heavy metal analysis. After drying, the sample was carefully ground into a fine powder and then underwent the microwave digestion method. A 5-gm portion of the raw sample was put in an Erlenmeyer flask, to which 20 ml of 3M HNO3 extracting solution was added. Once the flask has been placed on the magnetic stirrer, proceed to stir the mixture for a duration of 20 minutes The sample was permitted to kept overnight, and the

mixture was cautiously ignited in a water bath until the red nitrous oxide fumes ceased and then left to cool. The resultant mixture obtained was passed through a Whatman filter paper No. 42, transferred into a 50 ml polypropylene vial, and then brought to a volume of 50 ml using the extracting solution. The leftover material was suspended in a solution of HNO3 and adjusted to a volume of 50ml. We created standard solutions by diluting the original solution with 0.1 M nitric acid to test for linearity. We also prepared analytical reagent blanks. The wavelength range for the Atomic Absorption Spectrophotometer was adjusted to span from 185 to 900 nm and it was equipped with a high-intensity hollow cathode. The analysis of the sample's preferred metals involved the use of compressed air and Acetylene gas, with the Air Flow set to 17.0 (L/min), the Acetylene flow at 1.5 (L/min), and the Lamp current maintained at 15 mA. Burner Head is 10 cm. The test substance was examined against the standard for determining the concentration of the desired data. All the samples and standard solutions were tested three times to ensure accuracy. We established standard operating parameters for the elements we were working with. The HNO3 solution extracted the metals as metal nitrites, and then the responses (absorbance or peak) were plotted against the concentration of each standard solution. The heavy metals analyzed in the selected medicinal plant included Arsenic, Cadmium, Lead, Mercury, Zinc, Copper, Chromium, and Manganese.[10-12].

2.2.6) Pesticide Residues

In order to analyze the pesticide residues, we extracted 2 g of each Formulation using the Soxhlet apparatus with 150 ml of hexane. The hexane extract was purged of any residual water and oil traces. After the oil extraction, the concentrate underwent processing using a rotary evaporator at low pressure. The resulting concentrated extract was then moved to the cleansed-up column. After collecting the elute carefully, it was adjusted to a volume of 5 ml using hexane. The concentrated solution was divided into portions and then injected into a precalibrated GC machine equipped with a 63Ni electron capture detector. The column, injector, and detector were each set to temperatures of 195 ^{0}C, 200 ^{0}C, and 220 ^{0}C, respectively. The carrier gas utilized was purified nitrogen gas with a flow rate of 60 ml/min, matching the flow rate of the gas. The detection limit for the analysis of organo chlorine pesticides ranged from 0.1 to 0.5 parts per billion (ppb). We regularly utilized procedural blanks to verify and prevent cross-contamination. Our recovery studies using purified samples showed an overall recovery rate of over 80%. To

identify and quantify the compounds, we employed known amounts of external standards obtained from Sigma-Aldrich.[10-12]

2.2.7) Test for Aflatoxins (B_1, B_2, G_1, G_2)

Sample Preparation: 100 g of the sample should be finely grounded. The sample was treated with a mixture of methanol and deionized water (80:20 v/v), then agitated for 30 minutes at 120 RPM to extract aflatoxins from 50g of the sample. The solution was filtered through filter paper, and 20 ml of the filtered solution was then diluted with Phosphate Buffer Solution (PBS) in a 4:1 ratio to achieve a pH of 7.4. The diluent was centrifuge at 3400 RPM for 15 minutes and then passed through a nylon membrane filter with a pore size of 0.45µm.[13-15].

HPLC Analysis of Aflatoxins: The levels of aflatoxin in both standards and samples were analyzed using HPLC with fluorescent detection. The HPLC system consists of a Knaur pump (Germany) and a Knauer fluorescence detector (Germany), and it separated aflatoxins using a mobile phase of water, methanol, and acetonitrile in a ratio of 60:30:15 (v/v/v). The fluorescence was detected using an excitation wavelength of 365 nm and an emission wavelength of 440 nm. The retention times for Aflatoxin, with a flow rate of 1.2 ml/min, were 8-9 min for AFG2, 10.5-11.5 min for AFG1, 13-14 min for AFB2, and 16-17 min for AFB1, resulting in a total run time of 25 min. The spiked sample underwent 10 injections, yielding results for B2, B1, G2, and G1.[16]

2.3 Microbial Content Determination

One gram of solid Unani samples, such as tablets and powders, were dissolved in nine ml of sterile distilled water, and one ml of liquid formulations was suspended in nine ml of sterile distilled water. The pour plate method was used to evaluate the viability of the serial dilutions that were made. The plates were incubated for twenty-four hours at 37 ^{0}C. After placing the plate on a colony counter, the number of colony-forming units was counted. The average of the repeated measurements was used to calculate the microbial content. The media used were MacConkey agar, Cetrimide nutrient agar, Salt nutrient agar, and Nutrient agar. Sabouraud dextrose agar was added to the plate, allowed to set, and then 1 millilitre of each sample was spread out on the surface. The plates were then incubated for 72 hours at 27 ^{0}C for bacterial and fungal counts, respectively, in order to detect fungal growth in the Unani samples.[17,18].

2.3.1 Tests for Specific Microorganisms

Test for *Escherichia coli* Contamination

A sterile screw-capped container was filled with a suitable amount of powder (0.5g), 50ml of nutrient broth was added, and the mixture was cultured at 37 ^{0}C for 24 hours (enrichment culture). As directed, 0.1 ml of the enrichment culture was added to a tube holding 5 ml of MacConkey broth, and the tube was then incubated for 48 hours at 36–38 ^{0}C. Once gas and acid were found in the tube's contents, it was thought that Escherichia coli might be present.[17,18]

Test for *Salmonella spp*

100 ml of nutrient broth were added to a sterile screw-capped container holding 0.5g of the powdered formulations. The container was shaken, let stand for an hour, stirred once more, the cap was taken off, and it was incubated at 37 ^{0}C for 24 hours (enrichment culture). Following the enrichment process, 0.1ml of the culture was transferred to two separate tubes. The first tube contained 10ml of Selenite broth, while the second tube contained 10ml of Tetrathionate broth. Both tubes were then placed in an incubator at 36-38 ^{0}C for 48 hours. Afterwards, each of these cultures was used to inoculate a plate with a layer of Deoxycholate citrate agar. The plates were then placed in an incubator at 36-38℃ for 24 hours, and any colonies present were carefully examined for the potential presence of Salmonella spp[19-21].

Test for *Shigella spp*.

After adding 10g of the sample material to a pH 8.0 sterile nutritional broth and culturing it for eighteen hours at 37 ^{0}C, the bacteria were enriched. Following pre-enrichment, the culture was placed onto recently made Salmonella shigella selective differential agar plates and incubated for 48 hours at 37℃. non-lactose fermenters that build up as colourless colonies were recognized with a suitable biochemical test such as Triple Sugar Iron (TSI) agar test[19-21].

Pseudomonas aeruginosa

For the *Pseudomonas aeruginosa* (Schroter) Migula, homogenized pretreated formulation sample was taken to soybean-casein digested medium (HIMEDIA) incubated at 35-37 ^{0}C for 24-48 hrs, then set on cetrimide agar (HIMEDIA) and incubated at 35-37 ^{0}C for 24-48 hrs. The

presence of *P. aeruginosa* was verified by an oxidase test using N, N, N/, N/tetramethyl p-phenylenediamine dichloride[19-21].

Staphylococcus aureus

1g of the sample was added to peptone water in a sterile McCartney bottle, and the mixture was then incubated for 18 hours at 37°C to enrich the bacteria. By streaking the pre-enriched culture from the peptone water onto a newly made Mannitol Salt Agar (MSA) selective differential agar plate in accordance with the manufacturer's directions, the Staphylococcus aureus was isolated. After that, the plates were incubated aerobically for 24 hours at 37^{0} C. Colonies were recognized as Staphylococcus when they showed a golden yellow or colorless tint, and biochemical assays such as catalase, slide, and tube coagulase were performed to verify the presence of Staphylococcus aureus.[19-21]

3. Result and Discussion

3.1 Physico-chemical Analysis of Habb-e-asgand, Habb-esuranjan and Hawal gheekwar

Foreign Content

Herbal remedies must contain only the specified portion of the plant and neither other plant parts nor portions of the same plant. They must be completely clear of mold, insects, excrement, obvious contaminants like sand and stones, hazardous and toxic foreign objects, and chemical residues. Herbal remedies may also contain animal debris, such as insects, and "invisible" microbial pollutants that can create poisons.[35] We examine the Unai formulations and no foreign content was found. The foreign content of HEA, HES, and HG are tabulated in Table 1.

LOD

It is necessary to remove moisture from crude herbal drugs as much as possible as it is an expected component. Drying the crude medication is crucial for preservation, limiting the hydrolytic breakdown of its active ingredients, and facilitating the simple reduction of its size after it has been collected. Drug deterioration resulting from microbial development is caused by either excessive moisture or inadequate drying. As a result, the drying procedure should lower the drug's moisture content below a certain limit.[35.]

The LOD as per the pharmacopeial limit is not more than 12% and Habb-easgand, Habb-e-suranjan and Halwa gheekwar found within prescribed limit. Illustrated in table no. 1.

Ash Values

The ash values often indicate the inorganic residues that are found in herbal medications. These inorganic residues include phosphates, carbonates, and silicates. These are essential indicators that demonstrate not only the quality but also the authenticity of herbal medication. Because of this, conducting routine testing and analysis of heavy metals, particularly within the context of industry, is of the utmost importance. Toxic exposure to heavy metals may cause impairment or damage to the brain and central nervous system function, decreased levels of energy, and harm to blood composition as well as the lungs, kidneys, liver, and other essential organs.[25]

In this study we estimated Total Asha value, Acid insoluble ash value, Water soluble ash value, Sulphated ash value of Unani formulations and it was found within the prescribed limit. The Ash Value of HEA, HES, and HG are tabulated in Table 1.

Heavy Metals Estimation

Toxic metal contamination can occur accidentally or on purpose. The presence of heavy metals including mercury, lead, copper, cadmium, and arsenic in herbal treatments can be caused by a variety of factors, including pollution in the environment. As a result, there may be limited risks to the user's health that are clinically significant.[35] Heavy metal estimation was done by Atomic Absorption Spectrophotometer at wavelength range of 185 to 900 nm and it found within limit. The result of Heavy Metals Estimations of HEA, HES, and HG are tabulated in Table 1.

Pesticide Residues

Excessive pesticide residues have been associated with various health issues, which include cancer, liver and nervous system illnesses, and even blindness. The long-term consequences might lead to a decrease in the number of viable sperm and fertility, a raise in cholesterol levels, a high possibility of newborn mortality, and a number of metabolic and genetic diseases.[26]

The Pesticide residue was analysed by precalibrated GC machine equipped with a 63Ni electron capture detector and no pesticide residues was found in formulation.

Test for Aflatoxins (B_1, B_2, G_1, G_2)

Aflatoxins pose the greatest risk to human health and are responsible for the financial losses that are linked with the contamination of processed foods and feeds. The level of Aflatoxins were analyzed by HPLC at excitation wavelength of 365 nm and an emission wavelength of 440 nm and it was found that Unai formulation was free from Aflatoxins

Table 1. The physicochemical analysis of Habb-e-asgand, Habb-e-suranjan, Halwa Gheekwar

Parameters	**Habb-e-agand**	**Habb-e-suranjan**	**Halwa Gheekwar**
Foreign Content (%)	0	0	0
LOD (%)	4.89±0.84	5.16±0.14	4.71±0.04
Ash values			
Total Ash value (%)	7.2±0.20	4.1±0.10	6.96±0.03
Acid insoluble ash value (%)	1.89±0.19	1.97±0.02	1.82±0.04
Water Soluble Ash Value (%)	3.21±0.20	2.19±0.05	2.12±0.02
Sulphated Ash Value (%)	3.56±0.51	2.98±0.13	4.23±0.01
Heavy metals estimation (present)	Zinc, Copper, Manganese	Zinc, Copper, Manganese	Zinc, Manganese
Pesticide residues	Absent	Absent	Absent
Test for Aflatoxins (B_1, B_2, G_1, G_2)	Absent	Absent	Absent

The physicochemical examination of plant medications is an essential step in determining whether or not pharmaceuticals have been improperly handled or adulterated.[24]

When determining the potency and purity of various medications, the Determination of the total ash content is of utmost importance. The substance that comprises any or all of the elements described above is referred to as foreign organic matter. Other components of the organ or organs from which the medicine is generated, in addition to those mentioned components listed in the definition and description, or components for which the limit is given in the particular monograph.[29]

Microbial Content Determination

Table 2. The microbial content determinations of Uanani Formulations

Parameters	**Habb-e-agand**	**Habb-e-suranjan**	**Halwa Gheekwar**
Escherichia coli	-	-	-
*Salmonella*spp	-	-	-
Shigellaspp	-	-	-
Pseudomonas aeruginosa	-	-	-
Staphylococcus aureus	-	-	-

It is vital to develop adequate criteria for the microorganisms that may be found in herbal remedies in order to limit the hazards that are posed to the health of customers. The existence of moulds and feces-associated coliforms indicates that there is a possibility for contamination. An

analysis was done to assess the microbiological contamination of herbal medications. It was observed that all the values were below the permitted limits. [31-33]

An analysis was done to assess the microbiological contamination of herbal medications. It was observed that all the values were below the permitted limits. The microbial content determinations of Uanani Formulations are tabulated in table 2.

4. Conclusion

The quality of the product was determined through a physiochemical analysis which included assessing parameters such as Foreign Content, LOD, Total Ash Value, as well as safety parameters like Heavy metals estimation, Pesticide residues, and Test for Aflatoxins (B1, B2, G1, G2). These assessments found that Habb-e-asgand, Habb-e-suranjan, and Halwa Gheekwar were safe for utilization as they were found to be within the safety limits. The establishment of standard parameters and quality control of pharmacopoeial formulations recommended in the Unani medical system will benefit from this work. The Herbal medicine standardization increases the medication's safety and therapeutic efficacy while also potentially gaining global popularity. The drug's inherent worth, or the quantity of therapeutic elements and constituents present, the presence or absence of adulterants, etc, is clearly depicted by the standardization

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