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PHARMACOGNOSTIC EVALUATION OF *SUFOOF - E - HAZIM*

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ABSTRACT: *Sufoof - e -Hazim* (SEH), Unani formulation with high therapeutic efficacy, has been reported to cure indigestion and flatulence. The formulation is available with different manufacturers but scientific data on its pharmacognosy is lacking. Hence, the current research work is an attempt to standardize the formulation and establish quality control parameters in terms of its phytochemical and chromatographic evaluation. SEH was prepared as per National Formulary of Unani Medicine (NFUM). As a rapid method for detection of adulteration, powder microscopy was carried out for the formulation along with its individual ingredients in order to locate persistant and discernable characters belonging to the individual ingredients from the formulation. Phytochemical evaluation was carried out along with estimation of reducing sugars and crude fibre content in the formulation. Further, a simple, rapid, accurate and sensitive HPTLC method was developed for the simultaneous estimation of pharmacologically active markers, betasitosterol and lupeol. The method was validated as per ICH guidelines for the parameters such as linearity, intra and interday precision, accuracy, recovery *etc*. The content of markers was calculated from the formulation as well as from the individual ingredients. Further, acute oral toxicity studies of SEH were carried out on albino Swiss mice following OECD guidelines no. 420 (fixed dose procedure). SEH was found to be safe and no mortality or abnormal behavioural changes was observed. This scientific data can be adopted to lay down new Pharmacopoeial standards to be followed in the preparation of SEH with increased and reproducible batch to batch consistency.

INTRODUCTION: Standardization of traditional medicines is requisite for its acceptance globally and to obtain effective drug with proven efficacy. Under the parasol of traditional medicine system, the Unani system of medicine is one of the oldest systems of medicine gaining global acceptance due to its clinical efficiency [1].

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Unani compound preparations are commonly used in four forms *viz*. solids (Habb, Qurs, Sufoof, Kushta *etc*.), semi-solids (Majoon, Laooq, Marham, Zimaad *etc*.), liquid (Sheera, Rooh, Sharbat, Tila *etc*.) and gaseous (Bakhoor, Inkibaab, Ghalia *etc*.)[2].

Although Unani formulations have been used since ancient times, there is limited data available regarding their standardization and quality control parameter. Hence, Standardization of herbal formulation in terms of raw materials, manufacturing practices and composition is important to ensure quality and optimum levels of active principles for their bio-potency [3].

According to Unani system of medicine, Sufoof is a fine powder form of medicinal preparation made from a definite mixture of plants, animal and mineral sources [2] and Hazim means 'Digestion'. Sufoof-e-hazim (SEH) hence is a polyherbal Unani formulation reported for its therapeutic activity in curing indigestion, flatulence in the stomach and is also known to be a good appetizer.

Due to lack of modern pharmacopoeial standards, the prepared medicine can potentially have undesired quality and enormous variation of consistency in different batches. Hence, there is need for standardization using modern bio-analytical techniques [4]. In the current research work, Sufoof-e-Hazim (In-House) was prepared using standardized raw materials as per NFUM (National Formulary of Unani Medicine) [5].

In-house formulation was prepared in the Herbal Research Laboratory of Ramnarain Ruia College, Mumbai. Preliminary phytochemical evaluation, powder microscopy, reducing sugar content and crude fiber content of sufoof-e-hazim was evaluated as per standard methods [6, 7].

A simple, sensitive and rapid HPTLC method was developed and validated as per ICH guidelines for estimation of therapeutically potent biomarkes such as betasitosterol and lupeol from Sufoof-e-hazim and its ingredients. Further, safety of the formulation was established in albino swiss mice following OECD guidelines no. 420. **Table 1** illustrates the composition of SEH as per NFUM. The formulation is composed of nine ingredients out of which six are plants whereas three are Salts.

TABLE 1: TABLE OF INGREDIENTS AS PER NFUM

S. no.	Ingredients		Quantity	Voucher No.
	Unani names	Botanical identity		
1	Filfil Siyah	*Piper nigrum* Linn.	5 g	HRL/AUTH/2016/003
2	Nankhwah	*Trachyspermum ammi* Linn.	5 g	HRL/AUTH/2016/004
3	Namak-e-sang	Rock salt	5 g	--
4	Jawakhar	salt of Tar-tar	5 g	--
5	Zeera Safaid	*Cuminum cyminum* Linn.	5 g	HRL/AUTH/2016/005
6	Badiyan	*Foeniculum vulgare* Mill.	5 g	HRL/AUTH/2016/006
7	Kishneez Khushk	*Vitis vinefera* Linn.	5 g	HRL/AUTH/2016/007
8	Amla	*Emblica officinalis* Gaertn.	5 g	ARI, 2008; AUTH 08- 65
9	Namak Siyah	Black salt	5 g	--

MATERIALS AND METHOD:

Plant Material: Raw materials used for the preparation of *Sufoof- e-hazim* were procured from Dadar pharmacy, Mumbai, India and authenticated by Dr. Sunita Shailajan, Ramnarain Ruia College, Mumbai and at Agharkar Research Institute, Pune

Materials were dried in oven at 37 °C, powdered and sieved through an 85-mesh (BSS) sieve. All raw materials were stored in an air tight container at an ambient temperature prior to preparation.

Standards and Reagents: All solvents and chemicals were of analytical grade and were procured from Merck Specialities Pvt., Ltd., Mumbai, India. Standard betasitosterol (95% purity, lot no.T9G076) and lupeol (94% purity, L5632, lot no. SLBB2734V) were procured from Sigma-Aldrich Chemie (Steinheim, Germany).

Preparation of *Sufoof-e-hazim*: Traditional formula composition of Sufoof-e-hazim is listed in **Table 1**. Herbal ingredients of Sufoof-e-hazim (complying pharmacopoeial quality and quantity) were mixed thoroughly and stored in air tight container till analysis.

Preliminary Phytochemical and Physico-Chemical Evaluation: Phytoconstituents in *Sufoof-e-hazim* were evaluated by performing preliminary phytochemical tests for flavonoids, essential oils, tannins, glycosides, alkaloids and resins as per standard methods [6] DNSA method [8] was used to determine reducing sugar content of Sufoof-e-hazim. Crude fibre content in the formulation was also estimated following standard methods.

Chromatographic Evaluation: HPTLC Instrumentation and Operating Conditions: Chromatographic separation was achieved on TLC plates pre-coated with silica gel $60F_{254}$. Samples were spotted using the CAMAG Linomat 5 sample spotter (CAMAG Muttenz, Switzerland) equipped with syringe (Hamilton, 100µL). Plates were developed up to 85 mm in a glass twin trough

chamber (CAMAG) pre-saturated with toluene: methanol (8:1, v/v) mobile phase for 20 min. Post development, the plate was air dried and derivatization was carried out by dipping it in 10% methanolic sulphuric acid reagent. Densitometric scanning was performed using CAMAG TLC Scanner 4 at 366 nm and CAMAG ‑Reprostar 3 was used for photo documentation.

Quantitative evaluation of the plate was carried out in the fluorescence mode at 366 nm with slit width of 6 mm × 0.45 mm, scanning speed of 20 mm/s and data resolution set at 100 µm /step.

Preparation of Standard Solutions: Stock solutions of both the standards (1000 µg/ mL) were individually prepared by dissolving 10 mg of accurately weighed standards in small amount of methanol and making the volume up to 10 mL in a standard volumetric flask. The stock solutions were further diluted for the preparation of working solutions.

Quantitation of Betasitosterol and Lupeol: Extraction of phytoconstituents from Sufoof-e-hazim was optimized to resolve the marker compound efficiently. Different extraction factors, including concentration of solvent, sample-solvent ratio and extraction time were tested and optimized. Finally, 1.0 g each of Sufoof-e-hazim and its ingredients were subjected to extraction using 10.0 mL of ethanol. Mixture was vortexed for 1-2 min and kept standing overnight at room temperature.

Next day it was filtered through Whatman filter paper no. 41 (E. Merck, Mumbai, India). The filtrates were used for HPTLC analysis.

Method Validation: The developed HPTLC method for estimation of betasitosterol, and lupeol was validated as per ICH guidelines for the parameters like sensitivity, linearity, precision, recovery, specificity and ruggedness.

Specificity and Sensitivity: Specificity of the method was confirmed by comparing the bands of the sample solutions with that of the respective reference standards in terms of R_f and color in fluorescence mode. Sensitivity of the method was determined with respect to limit of detection (LOD, S/N of 3:1) and limit of quantification (LOQ, S/N of 10:1).

Preparation of Calibration Curve and Quality Control Samples: For constructing the calibration curve, appropriate dilutions were prepared from the stock solutions. The working standards in the range of 5 - 60 µg/ mL and 5 - 75 µg/mL for beta-sitosterol and lupeol, respectively, were used to obtain a seven point linear calibration curve. Further, quality control samples were prepared and analyzed for precision, accuracy and ruggedness studies.

Repeatability and Precision: The repeatability of the method was affirmed by analysing 5 µg/ mL of both the markers on a HPTLC plate (n = 5) and expressed as % RSD. Precision were assessed by measurement of intra and inter-day variation. The result was expressed as % RSD.

Accuracy and Ruggedness: The accuracy of the method was assessed by spiking the QC samples in plant matrix and calculating the percent recovery for each marker. Ruggedness was assessed by deliberately incorporating small variations like change of analyst, mobile phase and change in spotting volume like in the optimized chroma-tographic conditions. Response and R_f of QC samples were observed. Results were expressed in terms of percent mean difference.

Assay: The content of two markers from SEH was determined by applying the samples (10 µL) along with pure standards.

Estimation of the markers: The quantity of the markers was calculated using the regression equation obtained from the regression analysis of the calibration curve.

Statistical Analysis: The statistical analysis of the results obtained was done using Microsoft Excel 2007.

Safety Evaluation: Safety study of Sufoof-e-hazim was conducted in albino swiss mice following OECD guidelines no. 420 (fixed dose procedure). The mice were fasted overnight for 12 h and administered with the extract (2.0 g/kg body weight) orally. The animals were observed individually during the first 30 min for all reflexes, periodically during the first 48 h with special attention given during the first 4 h (short-term toxicity) and daily thereafter for a total of 14 days

(long-term toxicity) for any alteration from general behaviour and clinical symptoms like alteration of skin and fur texture, ptosis, excessive salivation, breathing problems, diarrhoea *etc*. Daily body weight, food and water intake record was also maintained.

RESULTS: Quality assurance is an integral part of all systems of medicine to ensure quality medicament [9]. Thus, there is an urgent need to evaluate parameters that can be adopted by the pharmaceutical industries for quality assessment of traditional preparations. Different scientific approaches have been reported for standardization of traditional Unani preparations. In this research work, standardization of SEH was carried out in terms of its physicochemical, phytochemical and safety profile.

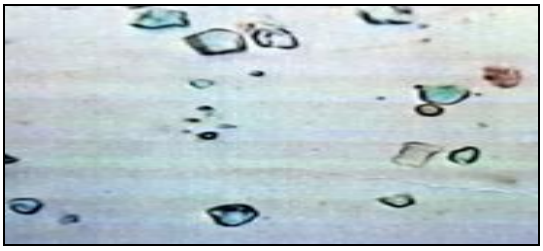
Physicochemical and Phytochemical Profile of *Sufoof-e-hazim*: Reducing sugar and the crude fiber content of SEH was found to be 6.00 ± 0.322% (5.034% - 6.966%) and 3.95 ± 0.037% (3.839 % - 4.061%) respectively. Limits for both the parameters have been prescribed as per standard statistical methods as no monographs are available for the same. In preliminary phyto-chemical evaluation; the ethanolic extract of SEH showed the presence of flavonoids, glycosides, alkaloids and essential oils while resins and tannins were not qualitatively detected.

Microscopic Evaluation: Microscopic characters from the powder can be used as a measure of authenticity for the formulation. The **Fig. 1 (a, b, c** and **d)** illustrate the characters specific to an ingredient which were observed in the formulation. Thus, these characters can be used as anatomical markers in evaluating the quality of raw materials and help in establishing the identity and purity of the formulation. Trichomes, a characteristic of *Piper nigrum*, warty trichomes of *Trachyspermum ammi etc*. were observed from the formulation. Confirmation of the presence starch grains was carried out by staining with dil. Lugol's iodine.

TRICHOMES OF *PIPER NIGRUM*

WARTY TRICHOME OF *TRACHYSPERMUM AMMI*

ENDOSPERM WITH MICROROSETTE OF CALCIUM OXALATE CRYSTALS OF *CUMINUM CYNIMUM*



OIL GLOBULES AND CA – OXALATE CRYSTALS OF *FOENICULUM VULGARE*

FIBRES FROM DRIED FRUITS OF *VITIS VINIFERA*

STARCH GRAINS OF *EMBLICA OFFICINALIS*

FIG. 1: POWDER MICROSCOPY

Chromatographic Characterization of S*ufoof-e-hazim*: The separation of betasitosterol and lupeol was achieved from ethanolic extract of the sample on TLC plates using mobile phase composition of toluene: methanol (8:1, v/v). The R_f values of betasitosterol and lupeol were found to be 0.48 ± 0.02 and 0.59 ± 0.02 respectively. Optimized chromatographic conditions are summarized in **Table 2**. The method was developed and validated in terms of specificity, linearity, repeatability, precision, accuracy, recovery and ruggedness as per ICH guidelines [10]. Details of the validation parameters have been summarized in **Table 3**.

TABLE 2: OPTIMIZED CHROMATOGRAPHIC CONDITIONS

Parameters	Specifications
Stationary Phase	Merck silica gel 60 F254 pre-coated TLC plates
Sample Applicator	Camag Linomat 5
Development distance	85 mm
Band length	7 mm
Spotting volume	10 µL
Derivatization	10% methanolic sulphuric acid reagent
Densitometric scanner	Camag scanner 4
Software	WinCATS planar chromatography manager software version 1.4.7
Lamp, wavelength	Mercury, 366 nm
Photo documentation	Camag Reprostar 3

TABLE 3: SUMMARY OF VALIDATION PARAMETERS

Parameters	beta-sitosterol	Lupeol
R_f	0.48	0.59
LOD and LOQ (µg mL^{-1})	5 and 15	15 and 45
Linear Range (µg mL^{-1})	15-35	45-105
System Suitability (% CV)	1.6344	1.5781
Intraday Precision (% CV)	0.50	0.89
Interday Precision (% CV)	0.93	1.15
Recovery	99.32%	98.62%
Regression equation	y=65.74x+91.0	y=21.75x+211.4

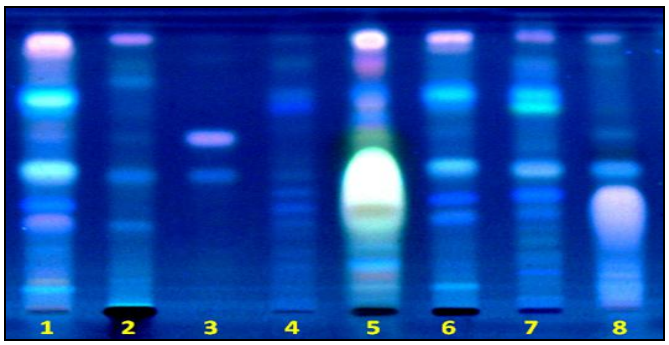


FIG. 2: PHOTODOCUMENTATION AT 366 nm (AFTER DERIVATIZATION)

Track Details:
Track 1: Zeera safaid (*Cuminum cyminum*)
Track 2: Amla (*Emblica officinalis*)
Track 3: Betasitosterol and Lupeol (20 ppm)
Track 4: Filfil Siyah (*Piper nigrum*)
Track 5: Nankhwah (*Trachyspermum ammi*)
Track 6: Kishneez khushk (*Vitis vinefera*)
Track 7: Badiyan (*Foeniculum vulgare*)
Track 8: Sufoof-e-Hazim

Developed chromatographic plate was derivatized in 10% methanolic sulphuric acid reagent. Reproducible results were obtained with dipping technique in a glass chamber filled with reagent instead of spraying. The derivatized plate was air dried and kept in oven for 5 - 7 min at 110 °C before densitometrically scanning the plate at 366 nm. **Fig. 2** and **3** showing the plate photo and 3D overlay at 366 nm respectively.

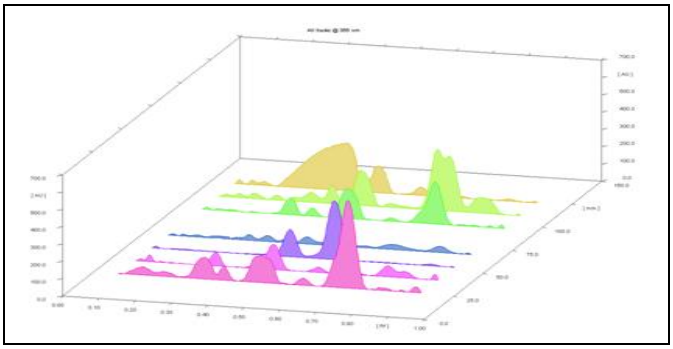


FIG. 3: 3D-OVERLAY AT 366 nm

Using the regression equation content of betasitosterol and lupeol in SEH was quantitated. Identification of these two phytoconstituents was confirmed by comparing the R_f, overlay and colour of band with that of the standard. The content was found to be 0.082 ± 0.0017 and 0.052 ± 0.0120 mg/g in SEH respectively summarized in **Table 4**.

TABLE 4: CONTENT OF BETASITOSTEROL, LUPEOL IN THE FORMULATION

	Betasitosterol	Lupeol
	Concentration (mg/g) Mean ± SD, n=3	
Zeera safaid *(Cuminum cyminum)*	0.124 ± 0.0021	0.038 ± 0.0016
Amla *(Emblica officinalis)*	0.078 ± 0.0011	0.025 ± 0.0005
Filfil Siyah *(Piper nigrum)*	0.010 ± 0.0006	-
Kishneez khushk *(Vitis vinefera)*	0.100 ± 0.0009	0.010 ± 0.0014
Badiyan *(Foeniculum vulgare)*	0.110 ± 0.0010	-
Sufoof-e-hazim	0.082 ± 0.0017	0.052 ± 0.0120

Safety Evaluation: The ethanolic extract of the formulation was found to be safe as it showed no abnormal fluctuation in body weights and food and water intake of the animals. Clinical symptoms of toxicity were also found to be absent during observation period and no mortality was recorded. The safety study of SEH revealed that it can be considered safe with a wide margin for oral use.

DISCUSSION: The current research work is an attempt in standardizing unani formulation *Sufoof-e-hazim*. For chromatographic separation of beta-sitosterol and lupeol from SEH and its ingredients, different solvent systems were tried. Finally a mobile phase reported by our group for chromatographic characterization of some medicinally important plants was employed [11, 12, 13, 14]. This demonstrates the reproducibility and application of a validated method to other complex polyherbal formulations. This is the first attempt for quantitation of betasitosterol and lupeol from SEH.

Betasitosterol **Fig. 4**, a phytosterol reported in all the ingredients of SEH exhibits hyperlipidemic, cholesterol lowering, anticancer and antidiabetic properties [15]. Similarly, Lupeol **Fig. 5** a triterpene also known as Fagarsterol reported in SEH, possesses beneficial effects as a therapeutic and preventive agent for a range of disorders. It is a well known chemopreventive agent for the treatment of inflammation and cancer [16]. Hence, the presence of betasitosterol and lupeol in the formulation can also be used as a rationale for future studies. Studies have proved similar formulations such as SZD as potent antidiabetic agents too [9]. Hence, the generated data can be used to analyse other therapeutic potentials of SEH based on the content and presence of bioactive markers.

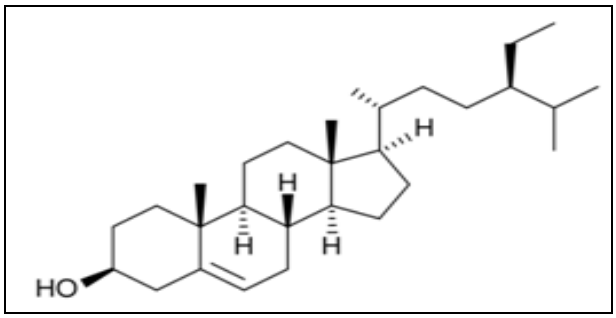


FIG. 4: STRUCTURE OF BETASITOSTEROL

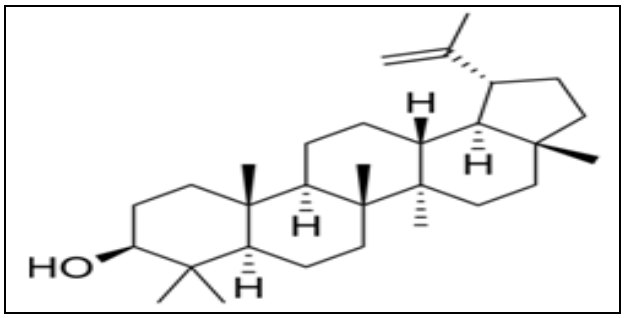


FIG. 5: STRUCTURE OF LUPEOL

CONCLUSION: The need for ensuring the quality control of formulations by the use of modern bioanalytical techniques has been emphasized by WHO [17]. The current research work focuses on standardization of Unani formulation *Sufoof-e-Hazim* by analyzing various parameters like phytochemical evaluation, powder microscopy, reducing sugar content and crude fiber content, quantitation of therapeutically important bio-markers betasitosterol and lupeol and safety evaluation (acute oral toxicity) of the formulation. The results can be used to analyze marketed samples in order to check their uniformity. The obtained values can also be used as base line in industries and manufacturing units in order to prevent adulteration and to ensure quality. A routine use of such scientific techniques will lead to standardization of the Unani medicine to a certain extent and would help in building confidence in use of these traditional products.

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