Research Article

A BIOANALYTICAL AND MULTIDISCIPLINARY APPROACH FOR QUALITY ASSESSMENT OF *SUFOOF - E - ZIABETES – DULABI*: A POPULAR TRADITIONAL UNANI ANTIDIABETIC MEDICINE

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ABSTRACT

Sufoof - e - Ziabetes - Dulabi (*SZD*) is an Unani formulation, used for diabetes and renal disorders since ancient times. In the present work, quality of the raw material and the final product *SZD* was assessed by modern bioanalytical techniques. Quality of the ingredients of *SZD* was evaluated prior to its preparation. Values of proximate analysis for the raw materials used were found to be within the permissible pharmacopoeial limits. *SZD* was prepared as per the National Formulary of Unani Medicine (NFUM). *SZD* was evaluated for its preliminary phytochemicals and reducing sugar content. A simple, rapid, accurate and sensitive HPTLC method was developed for the estimation of β-sitosterol, a pharmacologically active marker. HPTLC method was validated as per ICH guidelines and the content of β-sitosterol in *SZD*, its ingredients and stability samples were estimated. As a safety parameter, acute toxicity studies of *SZD* was carried out on Albino Swiss mice. No evident toxicity was observed up to observation period of 14 days. The obtained values of physical, chemical and biological parameters for *SZD* evaluated using scientific methods can be adopted to lay down new pharmacopoeial standards to be followed in its preparation with batch to batch consistency.

Keywords: Standardization, *Sufoof - e - Ziabetes - Dulabi,* β – sitosterol, HPTLC.

INTRODUCTION

In the past decade, there has been renewed attention and interest in the use of traditional medicine (Ayurveda, Naturopathy, Unani, Siddha, and Homeopathy) and Yoga globally. Under the parasol of traditional medicinal systems, the Unani system of medicine is also gaining global acceptance due to the amazing clinical efficiency of the formulations[1].

Tibb-e-Unani (Unani medicine) claims to possess many safe and effective single drugs and compound formulations of herbal, animal and metal origin which are used to cure a wide range of diseases[2]. Unani compound preparations are commonly used in the four forms viz. solid (*Habb, Qurs, Sufoof, Kushta* etc.), semi solid (*Majoon, Laooq, Marham, Zimaad* etc.), liquid (*Sheera, Rooh, Sharbat, Tila* etc.) and gaseous (*Bakhoor, Inkibaab, Ghalia* etc.)[3].

Although such Unani medicines have been used since ancient times, there exists mere documented evidence regarding their standardization and quality control[1].

Quality assurance of traditional Unani formulations relies upon good manufacturing practices with adequate batch to batch analysis and standardized method of preparation. There are reports on the standardization of some of the herbal preparations widely used in Unani System of medicine, using various modern techniques[1, 4].

Sufoof - e - Ziabetes - Dulabi (*SZD*) is a polyherbal Unani solid preparation widely prescribed for *Ziabetus sadiq* (diabetes mellitus) and *Zof-e-kulya* (weakness of the kidney). According to the NFUM[5], formula composition of *SZD* (table 1) includes six medicinal herbs with *Gil-e-Armani* (a type of soil used in many Unani preparations) and *Qand Safaid* (sugar).

Table 1: Formula composition for *SZD*

Ingredients		Quantity
Unani name	**Botanical identity**	
Gular	Stem bark of *Ficus racemosa* L.	2 parts
Gulnar Farsi	Flowers of *Punica granatum* L.	1 part
Dana Anar Shireen	Seeds of *Punica granatum* L.	1 part
Maghz-e-Tukhm-e-Anba	Seeds of *Mangifera indica* L.	1 part
Amla	Pericarp of dried fruits of *Phyllanthus emblica* Gaertn.	1 part
Kishneez Khushk	Fruits of *Coriandrum sativum* L.	1 part
Gil-e-Armani	Soil	1 part
Qand Safaid	Sugar	1 part

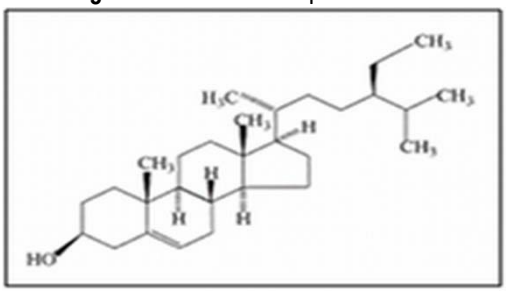
Due to lack of modern pharmacopoeial standards for *SZD*, the medicine may have undesired quality and lack consistency in different batches. Hence there is a need for standardization of *SZD* using modern bioanalytical techniques.

In the present work, *SZD* (In-House) was prepared using standardized raw materials as per NFUM[5]. Quality of herbal raw materials and the finished product *SZD* was assessed in terms of respective ash values, loss on drying and foreign matter as per standard pharmacopoeial procedure[6]. *SZD* was subjected to Preliminary phytochemical evaluation[7] and its reducing sugar content was estimated as per standard methods[8].

β-sitosterol (figure 1), a phytosterol reported in all the ingredients of *SZD*[9-13] [except *Amla*], exhibits hyperlipidemic[14], cholesterol lowering[15], anticancer[16], immunomodulatory[17] and antidiabetic[18] properties. Though there are reports on estimation of β-sitosterol

from single herbal drugs and polyherbal formulations using HPLC and HPTLC[19-22] but β-sitosterol has not yet been quantified from *SZD* and its ingredients using HPTLC (except from *Maghz-e-Tukhm-e-Anba*[20]).

Figure 1: Structure of β-sitosterol



A simple, sensitive and rapid HPTLC method was developed and validated as per ICH guidelines[23] for estimation of β-sitosterol from *SZD* and its ingredients. Effect of storage on the content of β-sitosterol in *SZD* samples stored at different storage period was evaluated using HPTLC. The acute oral toxicity of aqueous slurry of *SZD* was evaluated in Albino Swiss mice using fixed dose procedure[24].

MATERIALS AND METHODS

Plant materials

Raw materials used for the preparation of *SZD* were procured from Ratan Gandhi Shop, Mumbai, India and authenticated by Dr. Sunita Shailajan, Ramnarain Ruia College, Mumbai. Materials were dried in oven at 45°C, powdered and sieved through an 85-mesh (BSS) sieve. All the raw materials were stored in an air tight container at ambient temperature prior to *SZD* preparation.

Standard and reagents

The organic solvents and chemicals of analytical grade were procured from Qualigens Fine Chemicals, Mumbai, India. Standard β-sitosterol (98% purity) was procured from Sigma-Aldrich Chemie (Steinheim, Germany).

Proximate analysis of raw materials

Standard pharmacopoeial methods[6] were followed to assess the quality of *SZD* and its herbal ingredients in terms of proximate parameters like ash values (total ash, acid insoluble ash and water soluble ash), loss on drying and foreign matter.

Preparation of *SZD*

Traditional formula composition of *SZD* is listed in (Table 1). Herbal ingredients of *SZD* (complying pharmacopoeial quality and quantity) were taken and mixed thoroughly. *Gil-e- Armani* and *Qand Safaid* were added to the herbal mixture, mixed well and stored in air tight container till analysis. Inorganic content of *Gil-e-Armani* was qualitatively evaluated as per standard tests[7], prior to preparation of *SZD*.

Preliminary phytochemical and physicochemical evaluation

Phytoconstituents in *SZD* were evaluated by performing preliminary phytochemical tests for flavonoids, essential oils, tannins, glycosides, alkaloids and resins as per standard methods[7]. Anthrone method[8] was used to determine reducing sugar content of *SZD*.

Chromatographic evaluation

HPTLC conditions

From the stock of HPTLC plates pre-coated with silica gel 60 F_{254} (E. Merck) of 0.2 mm thickness with aluminum sheet support, appropriate size of plates were cut and used for chromatographic separation. Samples were spotted using Camag Linomat IV sample applicator (Camag, Switzerland) equipped with syringe (Hamilton, 100.0 µL). Plates were developed in a glass twin trough chamber (Camag) pre-saturated with mobile phase. The developed plates were scanned with Camag TLC Scanner II conjugated with Cats III software. The ambient temperature was maintained at 20 ± 2°C. Sample solution (10.0 µL) was applied in triplicates to the HPTLC plate. The plates were derivatized with 10 % methanolic sulphuric acid and scanned at 366 nm for detection of β-sitosterol. Camag Reprostar 3 system was used for photo documentation.

Preparation of standard solutions of β-sitosterol

Stock solution of β-sitosterol (1000.0 µg/mL) was prepared in methanol. Aliquots of 10.0 µg/mL-80.0 µg/mL were prepared from the stock solution for calibration curve of β-sitosterol. Three quality control samples (15.0 µg/mL, 27.5 µg/mL and 65.0 µg/mL) were prepared for method validation in terms of system suitability, precision, accuracy and ruggedness.

Extraction of Phytoconstituents from SZD and its ingredients

Extraction of phytoconstituents from *SZD* was optimized to achieve good fingerprinting and to resolve the marker compound β-sitosterol efficiently. Different extraction factors, including concentration of solvent, sample–solvent ratio and extraction time were tested and optimized. Finally, one gram each of *SZD* and its herbal ingredients were subjected to vigorous extraction using 10.0 mL of methanol. Mixture was vortexed for 1-2 minutes and kept standing overnight at room temperature. Next day it was filtered through Whatmann filter paper No. 41 (E. Merck, Mumbai, India), the filtrates were re-filtered through nylon microfilter (0.45 µm, Millipore) and used for HPTLC analysis.

Solvent system

Each matrix [single plant based drug or formulation (polyherbal or herbo-mineral etc.)] is unique and hence conditions have to be selected to optimize an accurate method in presence of other chemical constituents. Detection and separation of marker compounds was a

daunting task from polyherbal formulation like *SZD* in which six plant ingredients along with soil and sugar have been added. Hence, different solvent systems were tried (from published and unpublished reports on separation of β-sitosterol) and finally solvent system consisting of toluene: ethyl acetate: methanol: glacial acetic acid (8: 1: 5: 0.3, v/v/v/v) was used to resolve and quantify β-sitosterol from the matrix of *SZD* and its herbal ingredients.

Method validation

ICH guidelines[22] were followed for the validation of the developed analytical method for estimation of β-sitosterol from *SZD* and its herbal ingredients. Various parameters assessed during the course of validation were:

Specificity

Specificity was affirmed by analyzing standard compound with sample. The band of β-sitosterol from samples was confirmed by comparing its R_f at three different levels (start, middle, and end positions of the bands) with that from standard.

System Suitability and repeatability

System suitability was checked by scanning seven spots of β-sitosterol (5.0 µg/mL, n = 7) and further expressed as relative standard deviation (% RSD). The repeatability of the method was affirmed by scanning five spots of β-sitosterol (5.0 µg/mL) and expressed as % RSD.

Inter-day and Intra-day precision

Variability of the method was studied by analyzing quality control samples of β-sitosterol on the same day (intra-day precision, n = 3) and on three different days (inter-day precision, n = 3). The results were expressed as % RSD.

Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOQ) and limit of quantitation (LOQ) for the developed method was determined by spotting progressively low concentrations of the standard solution of β-sitosterol. Limit of detection (LOD) and limit of quantitation (LOQ) were established at a signal to noise ratio of 3:1 and 10:1 respectively.

Linearity

Linearity (calibration curve) was obtained by analyzing working standard solutions of β-sitosterol at eight different concentration levels (10.0 µg/mL, 20.0 µg/mL, 30.0 µg/mL, 40.0 µg/mL, 50.0 µg/mL, 60.0 µg/mL, 70.0 µg/mL and 80.0 µg/mL). The peak areas and their respective concentration for β-sitosterol were subjected to regression analysis for evaluating the correlation coefficient (r^2) and regression equation (y = mx + c) by the least square method.[23]

Estimation of β-sitosterol from SZD and its ingredients

Samples (10.0 µL) were applied in triplicate to HPTLC plates. Plates were developed in mobile phase, air dried and derivatized with 10 % methanolic sulphuric acid for detection of β-sitosterol. Plates were scanned under fluorescence (366 nm) and respective area under curve for β-sitosterol was recorded. Peaks of β-sitosterol were assigned according to the resolution factor of authentic standard and samples under optimized chromatographic condition. Amount of β-sitosterol in *SZD* and its herbal ingredients was calculated using the regression equation.

Recovery

Accuracy of the method was assessed by performing recovery studies at three different levels (10 %, 25 % and 50 %). Appropriate concentrations of β-sitosterol were spiked into *SZD* matrix and analyzed. The percent recoveries at each level were calculated to determine average percent recovery.

Ruggedness

Ruggedness of the method was assessed by deliberately incorporating the small variations in the optimized chromatographic condition. Effect of change in analyst, change in mobile phase composition [toluene: ethyl acetate: methanol: glacial acetic acid (8.1: 1.1: 5.1:0.4 and 7.9: 0.9: 4.9: 0.2 v/v/v/v) and change in spotting volume (9.0 µL and 11.0 µL) on the response and R_f of quality control samples were observed.

Method application

The developed HPTLC method was applied further to study the stability of *SZD* samples stored at different storage period (stored at room temperature) in terms of their respective β-sitosterol content.

Safety evaluation

Animals

Albino Swiss mice of either sex weighing 18-22 g were procured from Haffkine Biopharmaceuticals, Mumbai. All animals were housed in polypropylene cages under standard experimental conditions with 26 ± 2^0C ambient temperature and 12 h light-dark cycle. The animals were fed standard pellet diet (Amrut laboratory animal feed, India) and were provided water *ad libitum*. This study was approved by the Institutional Animal Ethics committee (CPSEA/315).

Acute toxicity study

In order to evaluate safety of *SZD*, acute toxicity study (fixed dose procedure, OECD guide lines No. 420)[24] was conducted on healthy mice. Mice of either sex (three females and three males) received aqueous slurry of *SZD* (2.0 g/kg body weight) orally by gavage. A separate group (control) of six mice (three male and three female) received water. The animals were observed for toxic symptoms continuously for the first 4 h after dosing. Finally, the number of survivors was noted after 24 h. These animals were then maintained for further 13 days (total 14 days) with observations made daily for change in body weight, food and water intake.

RESULTS AND DISCUSSION

Quality assurance is an integral part of all systems of medicine to ensure the quality medicament. Existence of the need to scientifically evaluate quality parameters which can be adopted by the pharmaceutical industries is well supported by the published reports on the standardization of traditional formulations[1-4, 25].

In current work, modern methods for quality evaluation of the raw materials as well as the finished product of *SZD* were approached. The results for proximate analysis of herbal raw materials for parameters like ash values (total ash, acid insoluble ash and water soluble ash), loss on drying and foreign matter are represented in table 2. These values were in compliance with the limits documented in the Pharmacopoeia.[26] *Gil-e-Armani* and *Qand Safaid* were subjected to quality evaluation tests prior to the preparation of *SZD*. Inorganic content of *Gil-e-Armani* is shown in table 3.

Table 2: Proximate analysis of raw materials and *SZD*, % Mean (n=6) ± SD

Sample	Total ash	Acid insoluble ash	Water soluble ash	Loss on drying	Foreign matter
Gular	2.50 ± 0.288	1.516 ± 0.028	1.783 ± 0.028	9.966 ± 0.557	0.8733 ± 0.0929
Gulnar Farsi	4.50 ± 4.333	0.246 ± 0.011	2.041 ± 0.071	8.586 ± 0.594	Not observed
Dana Anar Shireen	3.411 ± 0.057	0.533 ± 0.057	1.459 ± 0.006	7.860 ± 0.472	0.7200 ± 0.0600
Maghz-e-Tukhm-e-Anba	3.373 ± 0.057	0.563 ± 0.005	1.522 ± 0.038	7.816 ± 1.901	0.6433 ± 0.1721
Amla	3.450 ± 0.005	1.049 ± 0.010	0.382 ± 0.004	7.860 ± 0.472	0.1040 ± 0.0205
Kishneez Khushk	3.663 ± 0.005	1.220 ± 0.005	3.966 ± 0.057	2.533 ± 0.057	Not observed
SZD	3.373 ± 0.057	2.339 ± 0.008	2.227 ± 0.060	9.988 ± 0.595	Not applicable

Table 3: Qualitative evaluation of *Gil-e-Armani* for its inorganic content

Inorganic component	Results
Sulphate	+
Chloride	+
Carbonate	+
Nitrate	-
Sodium	+

(+) indicates presence while (-) indicates absence

Standard Operating Procedure (SOP) for the preparation of *SZD* as per NFUM[5] is documented in the current work. *SZD* was found to be enriched with essential oils, tannins, glycosides, flavonoids and alkaloids as per the preliminary phytochemical tests. Presence of these major secondary metabolites in *SZD* may attribute to its therapeutic efficacy and assures its traditional use. Similar results for preliminary phytochemicals have been reported for some popular traditional formulations used in India[1-4, 24]. Reducing sugar content of *SZD*, estimated using Anthrone method was 0.38 ± 0.003 %.

Table 4: β – sitosterol content in ingredients of *SZD*

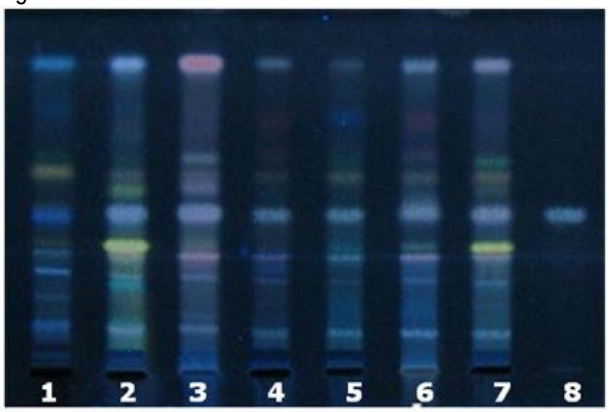
Ingredients	β – sitosterol content (mg/g) [Mean (n=3) ± S. D.]
Gular	0.626 ± 0.0076
Gulnar Farsi	1.186 ± 0.0175
Dana Anar Shireen	1.603 ± 0.0155
Maghz-e-Tukhm-e-Anba	0.44 ± 0.0069
Amla	0.583 ± 0.0111
Kishneez Khushk	1.535 ± 0.0199

In the present work, β-sitosterol content in *SZD* and its herbal ingredients was estimated using HPTLC for the first time (table 4 and table 5) although estimation of β-sitosterol from herbal raw materials and formulations is common. Among the various solvent systems tried, mixture containing toluene: ethyl acetate: methanol: glacial acetic acid (8: 1: 5: 0.3 v/v/v/v) resulted in proper resolution of β-sitosterol (R_f = 0.49) from the plant matrix which enabled its quantification using HPTLC (figure 2).

Table 5: Stability study of *SZD*

Storage period (Months)	β – sitosterol content (mg/g) [Mean (n=3) ± S. D.]
0	1.057 ± 0.0188
1	1.055 ± 0.0156
2	1.057 ± 0.0102
3	1.056 ± 0.0058

Figure 2: HPTLC Detection of β-sitosterol from *SZD* and its ingredients at 366 nm



HPTLC fingerprints from methanolic extracts of *SZD* and its ingredients; 1: *Gular*, 2: *Gulnar farsi*, 3: *Dana Anar Shireen*, 4: *Maghz-e-Tukhm-e-Anba*, 5: *Amla*, 6: *Kishneez Khushk*, 7: SZD, 8: β-sitosterol

Phytochemical fingerprints (through chromatographic techniques) of finished product and its ingredients are quite often used by the pharmaceutical industry to examine the source of the drug substance and the method of preparation. Such fingerprints are strongly recommended for the purpose of quality control of herbal medicines, since they represent the "chemical integrities"

of herbal medicines. Phytochemical finger prints for *SZD* and its ingredients are represented in figure 2. Bands related to the ingredients of *SZD* were found in the HPTLC fingerprint pattern of *SZD.*

The method was validated as per the current norms of ICH guidelines and results are presented in (Table 6). Method was found to be precise (% RSD value were < 2 % for Intra-day and Inter-day precision) (table 7), sensitive (LOD and LOQ were 2.0 µg/mL and 4.0 µg/mL respectively), rugged (% difference were within the range of ± 5 % for ruggedness parameters) and yielded 94.12 ± 1.6527 % recovery of β-sitosterol (table 8) from complex matrix of formulation. The content of β-sitosterol in *SZD* will provide a baseline data to trace the variations in quality of *SZD* (batch-to-batch variations) manufactured by different manufacturers. Phytochemical finger prints of stability samples are represented in figure 3.

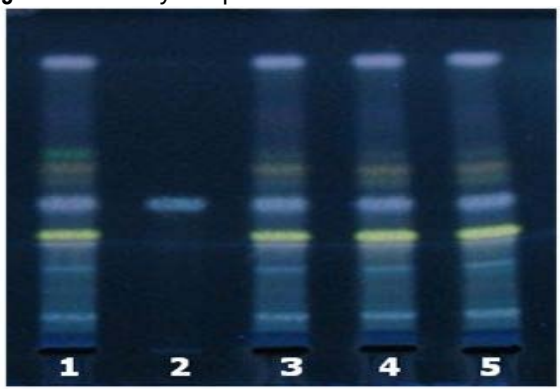
Table 6: Method validation parameters for β – sitosterol

Parameters	Results
System suitability (% RSD, n = 5)	1.72
Instrumental precision (% RSD, n = 7)	1.54
Linear Working Range (µg/mL)	10.0 to 80.0
Regression equation	y = 28.89x - 107
Correlation coefficient (r^2)	0.995
LOD (µg/mL)	2.0
LOQ (µg/mL)	4.0
Specificity	Specific
Ruggedness	Rugged

Table 7: Precision studies for β – sitosterol

Concentration (µg/mL)	Intra-day (% RSD)	Inter-day (% RSD)
15.0	0.07	0.83
27.5	0.12	0.96
65.0	0.17	1.09

Figure 3: Stability samples of *SZD* on HPTLC at 366 nm



Stability samples of *SZD* stored at different storage conditions; 1: 0 month sample, 2: β-sitosterol, 3: 1 month sample, 4: 2 month sample, 5: 3 month sample

Stability samples of *SZD* stored at different storage periods (0 month, 1 month, 2 month and 3 month) were subjected to HPTLC evaluation to estimate respective β-sitosterol content. Variation within the acceptance criteria for β-sitosterol content in stability samples (table 5) indicated that, *SZD* is stable for at least three months of storage conditions. The results of stability studies are supported by the frequent references in Unani pharmaceutical methods regarding use of *Sufoof*.

Table 8: Recovery studies for β – sitosterol from *SZD*

Amount of β – sitosterol			Recovery (%)	Accuracy (average % recovery)
Present in *SZD* (µg/mL)	Added in *SZD* (µg/mL)	Amount found (µg/mL)		
52.83	0	50.13	94.89	94.12 ± 1.6527
52.83	5.283	54.11	93.11	
52.83	13.21	61.04	92.43	
52.83	26.42	76.12	96.05	

In acute toxicity studies, no significant change in body weight, food intake and water intake of the animals was observed compared to animals of control group and no mortality was recorded too. Thus, at the doses empirically used in traditional medicine, the formulation, at least its aqueous slurry (2.0 g/kg body weight of animals), could be considered with a wide margin of safety for oral use. Since toxicity in humans cannot always be entirely extrapolated from animal studies, clinical evaluation should be performed to precisely define the safe dosage.

Such reproducible modern techniques can make the traditional Unani medicines more acceptable in the local and global market. Thus rationally designed, carefully standardized, synergistic traditional formulations and botanical drug products with robust scientific evidence can be used as an alternative to modern medicine.

CONCLUSION

Results of the present study can be used to characterize the samples in industry to check their uniformity. The obtained values of physical, chemical and biological parameters for *SZD* can be adopted to lay down new pharmacopoeial standards to be followed in its preparation with batch to batch consistency. A routine use of such scientific techniques will lead to standardization of the Unani medicine to a certain extent and would definitely help in building confidence in use of these products.

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