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Original Article

Physicochemical Characterization, Standardization and *In vitro* Determination of Radical Scavenging Activity of Zereshk-e-Saghir, A Traditional Preparation, and Its Ingredients

Zarrin Sarhadynejad[1], Abbas Pardakhty[2], Ali Mandegary[3,4], Suleiman Afsharypuor[5], Fariba Sharififar[6]*
[1]Pharmaceutics Research Center, Institute of Neuropharmacology, Department of Pharmacognosy, School of Pharmacy, Kerman University of Medical Sciences, Haft-Bagh Blvd , Kerman, IRAN.
[2]Pharmaceutics Research Center, Institute of Neuropharmacology, Department of Pharmaceutics, School of Pharmacy, Haft-Bagh Blvd., Kerman, IRAN.
[3]Department of Pharmacology & Toxicology, School of Pharmacy, Kerman University of Medical Sciences, Kerman, IRAN.
[4]Gastroenterology and Hepatology Research Center, Afzalipour's Hospital, Imam Highway, Kerman, IRAN.
[5]Department of Pharmacognosy, Faculty of Pharmacy, Isfahan University of Medical Sciences, Hezar-Jerib Ave., Isfahan, IRAN.
[6]Herbal and Traditional Medicines Research Center, Department of pharmacognosy, Haft-Bagh Blvd., Kerman, IRAN.

ABSTRACT

Objective: Quality control and standardization of traditional preparations ensure their safe, pure and efficient prescription. Zereshk-e-Saghir (ZES), a folk remedy, has been recommended as a hepatoprotective agent in Traditional Persian Medicine (TPM). This study aimed at the evaluation of the physicochemical and phytochemical characterization, standardization, and *in vitro* antioxidant capacity determination of ZES and its ingredients. **Method:** Some qualitative and quantitative controls were performed like ash value, moisture content, extractable matter, phytochemical screening, TLC fingerprint and microbial contamination. Total phenolic and emodin contents of ZES were measured by spectrophotometric method to standardize this preparation. Finally in vitro radical scavenging activity of ZES and its ingredients were determined using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay. **Result:** Regarding the physicochemical evaluation, the amount of each parameter (ash value, moisture content, and extractable value) was determined as documented characteristics for ZES components. Phytochemical screening of ZES ingredients indicated the presence of tannins in all the samples and the presence of alkaloids, steroids and terpenoids in a majority of the samples. Meanwhile, ZES exhibited no pathogenic contamination. The emodin and total phenolic contents (TPC) of ZES were reported 457.0 µg emodin/100g and 381.5 mg gallic acid equivalents (GAE)/100 g of ZES, respectively. Also TPC ranged from 3607.4 to 27.9 mg GAE/ 100 g of ZES ingredients. Meanwhile, the results demonstrated that ZES could scavenge DPPH radicals (IC_{50}value: 266.4 µg/ ml). **Conclusion:** The obtained results on quality control and standardization of ZES could be proposed as a monograph having standard characteristics.

Key words: DPPH Assay, Physichochemical Characterization, Quality Control, Standardization, Traditional Medicine.

Correspondence :

Fariba Sharififar

Herbal and Traditional Medicines Research Center, Department of pharmacognosy, Haft-Bagh Blvd, Kerman, P.O. Box 7616911319, IRAN.

Phone no/Fax: +98-34-31325020/+98-34-31325003,

Email: fa.sharififar@gmail.com, fsharififar@kmu.ac.ir

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INTRODUCTION

Traditional Persian medicine (TPM) has an ancient background. Prevention and treatment are two important principles in this health care system. Moreover pharmacotherapy is one of the outstanding ways for health maintenance in this system.[1]

Over the past two decades, herbal medicines especially with traditional origins have been the center of attention in both developed and developing countries. Also pharmaceutical companies are interested in research and development of these drugs. Therefore assessment of quality, efficacy and safety of herbal medicines are so important.[2-4]

Zereshk-e-Saghir (ZES), a traditional preparation, is recommended in TPM as a hepatoprotective agent.[1] This preparation is a polyherbal formulation with a mixture of seven plants. The main part of ZES is fruits of *Berberis vulgaris* L. (Berberidaceae) known as Zereshk in Iran, and the name of ZES has essentially been derived from the Persian name of this plant. Some studies on ingredients of ZES have shown radical scavenging potential,[5] and hepatoprotective effects of these plants.[6] Moreover, some major compounds like emodin,[7] alkaloids,[8] phenolics, anthocyanins,[9] tannins, terpens[10] have been reported in these plants too.

The hepatoprotective effect of ZES on CCl_4-induced hepatotoxicty in animal model was evaluated recently.[11] So the aim of this study was the evaluation of quality and quantity controls of ZES and its standardization. The studied parameters were ash values, water and volatile matters, extractive matters, and microbial contamination. Also the standardization of ZES was carried out by estimation of its emodin and total phenolic content. Finally, the determination of *in vitro* antioxidant activities of ZES and its ingredients was performed using DPPH assay.

MATERIALS AND METHODS

Chemicals

2, 2-diphenyl-1-picrylhydrazyl, folin–Ciocalteu, dragendorff, and mayer were purchased from Sigma-Aldrich, Germany. Hydrogen chloride, sulfuric acid, ammonium hydroxide, diethyl ether, chloroform, methanol, lead (II) acetate, iron (III) chloride, magnesium fillings, acetic anhydride, formic acid, and sodium carbonate were acquired from Merck Co., Germany. Ethyl acetate, Petroleum ether, and toluene were provided

by Dr. Mojallali Chem. Lab, Iran. Gallic acid, rutin, quercetin, kaempferol, aloin, and emodin were obtained from Carl Roth Co., Germany.

Collecting herbal samples

The date and location of collecting herbal samples were from April to August in the year 2013 and farms in Kerman province (30.3°N, and 57.0°E), Iran, respectively. All the herbal samples were authenticated by a botanist and kept in the Herbarium of Pharmacognosy Department, Kerman University of Medical Sciences, Kerman, Iran, with a specific voucher specimen (Figure 1).

The entire procedure of ZES preparation was based on the traditional source[1] and according to the recent study.[11]

Physicochemical characterization

Determination of ash values and water and volatile content

Measuring of ash values (total ash, and acid-insoluble ash) and water and volatile content (using gravimetric method) of ZES ingredients was exactly performed by WHO recommended producers.[2]

Extractable matters

Aqueous extract

Aqueous extracts were obtained using maceration method. The plant samples were pulverized, passed through a sieve (Mesh no.10), and macerated with distilled water (1: 6) threefold during 24 hours at room temperature. Then the collected extracts were dried with a freeze dryer (Eyela FD-81, Japan). The quantity of the extractable matter was expressed as g/ 100g of the air-dried herbal samples.

Hydroalcoholic extract

The herbal samples were prepared as mentioned above and extracted with 80% aqueous methanol (1: 5) in an ultrasonic bath (Sonorex Digital 10 P, Iran.) threefold each time for 60 minutes, 30°C and 50 RPM. Each obtained extract was concentrated using a rotary evaporator (EyelaN-1200 B, Japan). Finally the concentrated extracts were dried in an oven at less than 40°C for 48 hours. The data were presented as g/ 100g of the air-dried herbal samples.

Phytochemical characterization

Secondary metabolite screening

The ingredients of ZES were tested for the presence of phytochemicals like alkaloids, tannins (Lead acetate and ferric chloride test), flavonoids (Lead acetate and Shinoda test), saponins (Frothing test), steroids (Liebermann-burchard test), and terpenoids (Salkowski test) in accordance with ordinary methods.[3,12]

Thin layer chromatography fingerprints

By using thin layer chromatography (TLC) method, known constituents are identified in plants.[13] In this research, ZES and its ingredients were screened by TLC. TLC silica gel 60 F_{254} was used as a chromatogram. The methanol extract of the samples and standards (100 µg/ ml) were spotted on chromatograms and placed into pre-saturated chambers with a mobile phase. The mobile phases were used as follows. Formic acid: ethyl acetate: light petroleum (1:25:75, v/v/v) for detection of emodin;[14] toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2, v/v/v/v) for detection of gallic acid;[15] and chloroform: methanol: formic acid (85:15:1, v/v/v) for detection of quercetin, kaempferol, and aloin.[16] The plates were observed at 254 and 366 nm.

Determination of microbial contamination

Microbial contamination of the raw herbal samples and finished product was examined by total viable count using pour-plate method in accordance with British Pharmacopeia.[17] The total count was expressed as a colony count unit (CFU)/g of the samples. The samples were also examined for the presence of some pathogens like *Escherichia coli* and *Salmonella*.[2] The biochemical tests were also done for the confirmation of these pathogen presences.

Measurement of ZES emodin-content based on the spectrophotometric method

According to the TLC chromatogram of ZES, the presence of emodin was confirmed. For measuring of ZES emodin content, at first, an absorbance spectrum of emodin was provided, and the wavelength of maximum absorbance was determined at 435nm.[18] So the calibration curve of emodin was prepared threefold using different serial dilutions (0.625, 1.25, 2.5, 5, 10, 20, 30, 40 and 50 µg/ml), and ZES emodin content was equivalent to µg emodin/ 100g of ZES as mean± SD.

Total phenolic content determination of ZES and its ingredients

Folin-Ciocalteu method was used to measure total phenolic content (TPC)[4] of course with slight modifications. Briefly, 500 µl of water diluted folin-ciocalteu reagent (1:10) was added to 100 µl of the sample extracts, and then 400 µl of sodium carbonate aqueous solution (7.5%w/w) was added. Next, this mixture was incubated at room temperature for 30 min in a dark place. The absorbance of the blue color was measured at 765 nm with a spectrophotometer (Synergy HTX, USA) versus a methanol blank. Later a standard curve of gallic acid solution in methanol (50, 100, 200, 400, 500,600 and 700 µg/ml) was used to quantify mg of gallic acid equivalents (GAE)/ 100g of the dried samples.

In vitro evaluation of radical scavenging activity of ZES and its ingredients

This evaluation was performed using DPPH assay with slight modification.[19] Briefly, 150 µl of the methanol solution of DPPH (0.004% (w/v)) was added to 50 µl of the methanol extract samples in different concentrations, and then the absorbance was measured at 517 nm (A_1). The following equation was finally used for determination of inhibition percentage (I %).

$I\ \% = [A_{0-(A_1-A)}] / A_0 \times 100$,

where (A_0) was the absorbance of the methanol solution of DPPH as a control, and (A) was the absorbance of each sample in methanol as a blank.

All the measurements were carried out during 40 minutes (with 10 minutes intervals), each one was done triplicates, and the results were expressed as mean± SD.

Data analysis

The Microsoft Office Excel 2007 and SPSS software (version 18.0) were used for statistical analyses. Probit analysis test and one way analysis of variance (ANOVA) followed by post hoc Tukey test ($p< 0.05$ was regarded as significant difference) were done for calculating IC_{50} and analyzing data, respectively. All the measurements were performed triplicates and presented as mean± SD.

RESULTS

Physicochemical characterization

Ash values, water and volatile contents of ZES ingredients

The results of ash values (total and acid-insoluble ash) and moisture contents each of ZES ingredients are shown in Table 1.

Extractable matters

Figure 2 shows the yield of extractable matters of ZES components using water and 80% aqueous methanol.

Phytochemical characterization

Secondary metabolites screening

The results of phytochemical screening of ZES ingredients are shown in Table 2.

TLC fingerprints

Screening of ZES methanol extract by TLC detected gallic acid and emodin Figure 3A and 3E. Fingerprints of each plant are presented in Figure 3B, C and D. The presence of quercetin, kaempferol, gallic acid, aloin and emodin was confirmed in these extracts.

Determination of microbial contamination

The results of total viable count of ZES and its ingredients were shown as bacteria and fungi colonies /g of the samples, respectively: *B. vulgaris*: 2.5×10^3, 0.1×10^3; *C.intybus*: 30×10^3, no fungal growth; *P. oleracea*: 1×10^3, 0.5×10^3;*C.sativus*: 45×10^3, no fungal growth; *R. damascena*: 2.4×10^3, no fungal growth; *R. palmatum*: 40×10^3, no fungal growth; *N. jatamansi*: 2.7×10^3, 1×10^3; and ZES: 1×10^3, 0.5×10^3.All of the samples exhibited no contamination of the tested pathogens, *E. coli* and *Salmonella*.

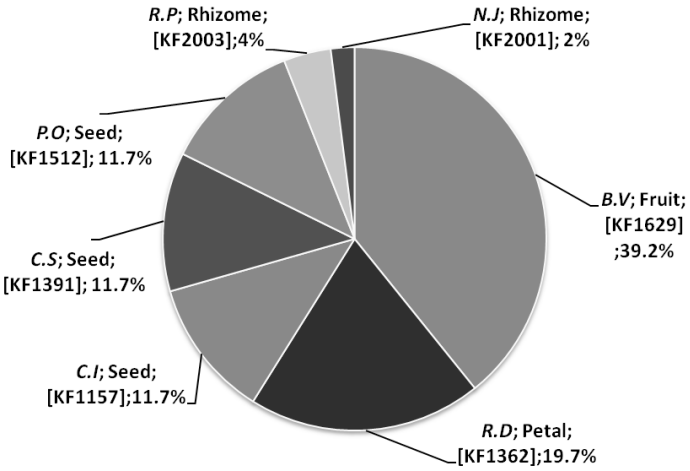

Figure 1: Characterization of ZES. The used part, voucher specimen [], and amount of ingredients (w/ w %) are shown, respectively.

B.V: Berberis vulgaris L.(Berberidaceae); *R.D: Rosa damascena Mill* (Rosaceae); *C.I: Cichorium intybus* L. (Asteraceae); *C.S: Cucumis sativus* L. (Cucurbitaceae); *P.O: Portulaca oleracea* L. (Portulacaceae); *R.P: Rheum palmatum* L.(Polygonaceae); and *N.J: Nardostachys jatamansi* DC.(Valerianaceae).

Table1: Ash values and moisture contents of ZES ingredients

Sample name	Ash value % (g/ 100g of the dried sample)		Loss on drying (mg/g of the sample)
	Total ash	Acid-insoluble ash	
B. vulgaris	3.5± 0.3	2.0± 0.4	206.3± 1.3
C. intybus	13.6± 0.1	2.4± 0.2	65.2± 1.5
P. oleracea	6.4± 0.5	0.2± 0.0	51.1± 2.9
C. sativus	11.2± 0.6	3.4± 0.1	46.8± 1.1
R. damascena	6.5± 0.0	1.7± 0.2	63.4± 5.4
R. palmatum	2.4± 0.0	0.5± 0.2	56.7± 0.2
N. jatamansi	19.5± 0.3	12.4± 0.3	58.9± 0.2

Each experiment was done in triplicates, and the results were expressed as mean± SD.

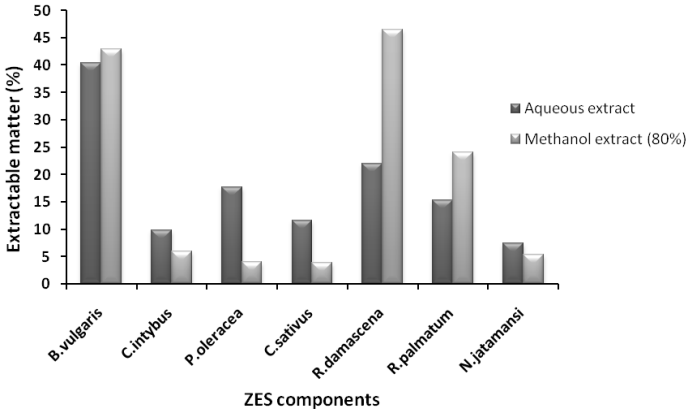

Figure 2: Yield of extractable matters of ZES components using water and 80%methanol. The results were presented as g of extract/100g of the dried samples.

Table 2: Phytochemical screening of ZES ingredients

Sample name	Alkaloids	Tannins (LA/ FC)	Flavonoids (LA/ Sh)	Saponins	steroids	Terpenoids	
B. vulgaris	-	+/ +	+/+	-	-	-	
C. intybus	+	+/ -	+/ -	-	+	+	
P. oleracea	+	+/ -	-/ -	-	+	+	
C. sativus	+	+/ -	-/ -	+	+	+	
R. damascena	-	+/ +	+/ +	-	+	+	
R. palmatum	+	+/ +	-/ -	-	+	+	
N. jatamansi	-	+/ -	-/ -	-	+	+	

+: Positive; -: negative; LA: Lead acetate test; FC: Ferric chloride test; and Sh: Shinoda test

Table 3: Total phenolic content and antioxidant capacity of ZES and its ingredients

Sample name	Total phenolic content (mg GAE/ 100g of the dried samples)	IC_{50} (µg/ ml)
B. vulgaris	2108.8± 0.8	289.7± 0.2
C. intybus	57.1± 0.1	60.7± 0.1
P. oleracea	29.3± 0.0	381.4± 0.2
C. sativus	27.9± 0.1	796.8± 0.1
R. damascena	3607.4± 12.0	57.2± 0.1
R. palmatum	982.6± 15.9	19.6± 0.1
N. jatamansi	49.8± 0.0	316.1± 0.3
ZES	381.5± 0.9	266.4± 0.0

Each experiment was done in triplicates, and the results were expressed as mean± SD. GAE: Gallic acid equivalent; IC_{50}: Concentration with 50% inhibition; and ZES: Zereshk-e-Saghir

Table 4: DPPH inhibition activity of ZES and its ingredients after 40 minutes

Sample name	Concentration (µg/ ml)	DPPH inhibition (%, at time 40 min)
B. vulgaris	100	25.3 ± 1.9
C. intybus	100	68.3 ± 1.0
P. oleracea	200	19.0 ± 3.7
C. sativus	500	46.3 ± 3.3
R. damascena	100	71.0 ± 5.6
R. palmatum	100	83.0 ± 0.9
N. jatamansi	200	31.0 ± 4.0
ZES	100	24.0 ± 4.0

Each experiment was done in triplicates, and the results were expressed as mean± SD. ZES: Zereshk-e-Saghir

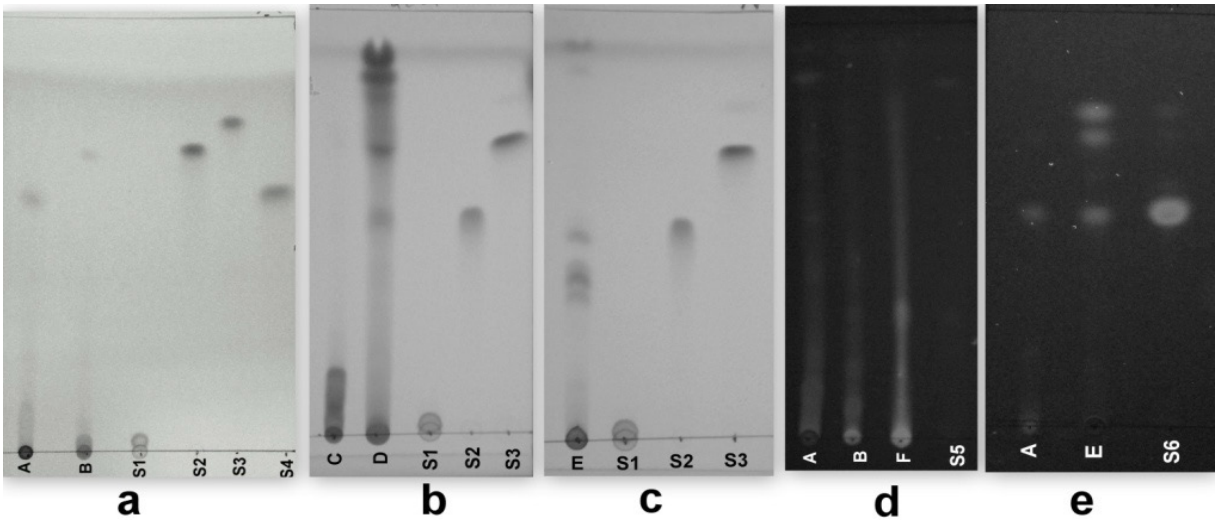

Figure 3: TLC chromatograms of ZES and its ingredients. Toluene: ethyl acetate: formic acid: methanol was used as a mobile phase for TLC plates which are shown in parts (a) and (b). The mobile phase for (c) and (d) plates was chloroform: methanol: formic acid. Emodin was detected with formic acid: ethyl acetate: light petroleum as a mobile phase that is shown in part (e). All the spots on the plates were observed at 254 and 366 nm.

Standards: S1: Rutin; S2: Quercetin; S3: Kaempferol; S4: Gallic acid; S5: Aloin; and S6: Emodin

Samples: A: ZES; B: *P. oleracea*; C:*C. intybus*; D: *N. jatamansi*; E: *R. palmatum*; F: *C. sativus*; and ZES: Zereshk-e-Saghir

Measurement of ZES emodin-content based on the spectrophotometric method

The standard curve of emodin, with the equation on a chart $Y= 0.028x+ 0.001$ and $R^2= 0.998$, was used for the calibration curve and determination of ZES emodin content (457.0± 2.8 µg/100g of ZES).

Total phenolic content determination of ZES and its ingredients

Table 3 shows the results of TPC measurement of each sample (mg of GAE/100 g of the dried samples). The standard curve of gallic acid, with the equation on a chart $Y= 0.0007x– 0.0319$ and $R^2= 0.9872$, was used for the calibration curve.

In vitro evaluation of radical scavenging activity of ZES and its ingredients

Radical scavenging activity of ZES and its components was measured by DPPH assay. IC_{50} (concentration with 50% inhibition) values are expressed as µg/ml of samples in Table 3. The antioxidant capacity of samples was measured during 40 minutes after incubation. Although no significant difference was observed between the mean values of the inhibition percentage of the extracts (100 µg/ml) 40 minutes after incubation compared to those of at the zero time, *R. palmatum* significantly inhibited DPPH radicals after 40 minutes (82.9± 0.9) in comparison to its zero time (68.2± 0.4) and 10 minutes after incubation (77.3± 0.4). See Figure 4. Also the DPPH inhibition activity of ZES and its ingredients is shown after 40 minutes (Table 4).

DISCUSSION

Some parameters like ash value, extractable matter, moisture content, preliminary phytoscreening and TLC fingerprint of ingredients are used for quality control purpose. Moreover, determination of major compounds would be a good way for standardization of herbal medicines.[20,21] Therefore, the above mentioned methods were used in this study.

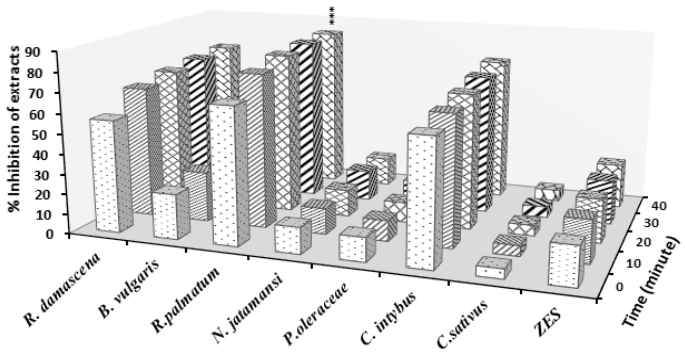

Figure 4: DPPH inhibition activity of ZES and its ingredients (100µg/ml) in different intervals are shown during 40 minutes. ZES: Zereshk-e-Saghir.

*** $p < 0.0001$ in comparison to the values at time 0 and 10 minutes after incubation.

In physicochemical evaluation, the results demonstrated that the maximum and minimum percentage of the acid-insoluble ash was related to rhizome of *N. jatamansi* (12.4± 0.3) and seeds of *P. oleracea* (0.2± 0.0), respectively. This index demonstrated sand and silica content of the samples showing their purity. The moisture content determination showed that fruits of *B. vulgaris* contained the highest amount of moisture (206.3± 1.3 mg/g sample). Perhaps it was the reason underlying the use of completely dried extract of the fruits instead of the fresh forms as recommended in the traditional recipe. This recommendation may hydrolyze active constituents less. In measuring of the extractable matters, in most of the samples, the aqueous extracts quantities were more compared to the quantities of hydroalcoholic extracts.

In another study the presence of flavonoids and phenolics has been demonstrated to have free radical scavenging activities.[22] Additionally, our results on preliminary screening of samples were confirmed the presence of flavonoids in *R. damascena*, *B. vulgaris* and *C. intybus*. Moreover, the presence of alkaloids and tannins was confirmed in some of the samples in this study. In other studies the presence of alkaloids and tannins have shown antimicrobial and antitumor activities.[23] Furthermore, in our study, terpenoids and steroids also were detected in all samples except one. Plants with terpenoids exhibited different biological effects like organoprotective activities.[24]

Different studies on some of the ZES ingredients have confirmed the antioxidant and hepatoprotective effects too. Phenolics are also famous for their antioxidant and radical scavenging activities.[6,25,26] Therefore the presence of diverse secondary metabolites and phenolics in ZES seems to be a suitable candidate as a hepatoprotective agent for human which is in accordance with the traditional uses.[11]

As stated in British Pharmacopoeia for oral administration, the microbial contamination of these pathogens like *E. coli* and *Salmonella* must be negative which was true for ZES and each of its ingredients. Also in our study, the total aerobic microbial count was reported not more than $10^5$for bacteria and 10^4 for fungi colonies per 1 g of the samples.[17]

Herbal preparations almost include a variety of constituents that are indicators for therapeutic and non-therapeutic activities. Standardization is emphatically recommended for quality control of a finished herbal product.[27] In the present research measurement of TPC and emodin contents was used for standardization of the final preparation by UV-spectrophotometric method, which can also be implemented for ZES future reproduction.

CONCLUSION

Zershk-e-Saghir (ZES) has been used in traditional Persian medicine (TPM) as a hepatoprotective medicine. In this study ZES was evaluated and standardized for the first time using qualitative and quantitative parameters which could be generally comprehensive, but suitable alternative ways. The obtained results concerning the quality controls and standardization of ZES could be proposed as an efficient monograph with reproducible and diagnostic characteristics. All these findings encourage us to use standardized ZES preparation for different therapeutic purposes in future.

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CONFLICT OF INTEREST

Authors declare no conflict of interests. This research was supported by the Deputy of Research, Kerman University of Medical Sciences (Grant No.: 91/482 to FSh) and a part of Ph.D. thesis to ZS.

ABBREVIATION USED

ZES: Zereshk-e-Saghir; **TPM:**Traditional Persian Medicine; **GAE:** Gallic acid equivalents; **TPC:** Total phenolic contents; **DPPH:2,** 2-Diphenyl-1-picrylhydrazyl; **TLC:** Thin layer chromatography; **CFU:** Colony count unit.

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