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Evaluation of possible antioxidant activities of barberry solid formulation, a selected formulation from Traditional Persian Medicine (TPM) via various procedures

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ABSTRACT

The purpose of this study is to reformulate a solid traditional Ghors-e- zereshk (barberry traditional tablet) from Traditional Persian Medicine (TPM) and to assess the antioxidant effect and determining the total phenol and flavonoids contents of water and hydroalcoholic extract of mentioed formulation. The antioxidant effects of water and hydroalcoholic extracts of barberry solid formulation were measured by three methods: DPPH radical scavenging, nitric oxide free radical scavenging and Ferric Reducing Antioxidant Power Assay (FRAP) reducing method. The total phenol and flavonoid content were measured by Folin-Ciocalteu and aluminum chloride colorimetric methods. For standardization ashes' test also used. The hydro-alcoholic extract has more phenolic compounds (31.46±.13 mg GAE⁄g of dry matter) than the water extract and the water extract has more flavonoid compounds (6.38±. 25 mg Quercetin ⁄g of dry matter) than the hydro-alcoholic extract. The result of DPPH radical scavenging shows that the hydro-alcoholic extract has more effect than the water extract. FRAP value indicates that the hydro-alcoholic extract has more reductive activity than the water extract. The study of NO radical scavenging represents water extract 74.61±2.95 %, hydro-alcoholic extract 61.72±1.79 % thus, water and hydro-alcoholic extracts are powerful antioxidants and have hepatoprotective effects. Other relative biological effects, for example, the protective effects through oxidative stress can also be considered.

Keywords: *barberry solid formulation; Antioxidant activity; DPPH; NO scavenging; FRAP; Hepatoprotective; phenol and flavonoids contents.*

1. INTRODUCTION

The liver is an important organ, and it is involved with totally the biochemical paths related to growth, fight against disease, nutrient supply, energy creation, and reproduction. Due to its functions, the liver is a key target for toxicity created by drugs, chemical materials, xenobiotics, and oxidative stress [1]**.** Genus *Berberis* is intrinsic to areas of Asia, Africa, North America, Europe, and South America. Iran is the major producer of *B. vulgaris* fruit in the world [2,3]. There are different kinds of alkaloids such as berbamine, berberine and berberrubine in this plant [4]. Berberine has different activities, including antioxidant, anti-inflammatory, hypoglycemic [5,6], antidiabetic, antiobesity [7] antihyperlipidemic and antihypertensive activities [4]. *B. vulgaris* L. has been traditionally used for the treatment of fever, hyperlipidemia, bleeding, hyperglycemia, and liver disease in Traditional Persian Medicine [8,9]. The genus *Cichorium* (Asteraceae) involves six species and is native to areas of Asia and Europe. *Cichorium intybus* L. is an erect fairly woody perennial herb [10]. *C. intybus* is used for the treatment of jaundice, diarrhea, fever, and gallstones in traditional medicine. The studies on rats have been reported that *C. intybus* has anti-hepatotoxic, hyperglycemic, anti-bacterial, anti-ulcerogenic, anti-diabetic and anti-inflammatory effects [11]. *Cucumis sativus* L. (Cucumber) fruits have been used for treating constipation and aid indigestion. Seeds of *C. sativus* are anti-fever, cooling, antidiabetic, Antiulcer, tonic, diuretic, demulcent and anthelmintic in Traditional Persian Medicine. In animal study *C. sativus* fruit extract has a hypoglycemic effect in diabets [12]. *Portulaca oleracea* L. from Portulacaceae family is a herbaceous plant extensively spread all over the world. It contains high biologically effective compounds high contents of a variety of phytoconstituents and many nutrients like cardiac glycosides, alkaloids, omega-3 fatty acids, flavonoids, coumarins, anthraquinone, free oxalic acids and protein [13]. Many biological activities reported for *P. oleracea* like antibacterial, antifungal, analgesic, anti-inflammatory [14], anti-fertility [15], muscle relaxant [16] and wound healing properties [17]. *Glycyrrhiza glabra* L. is a common plant of Asian areas and Mediterranean. Licorice and Sweetwood are other famous names. It has extensive root system and its soft, fibrous main taproot with the bright yellow interior is used for therapeutic purpose [18]. *G. glabra* root has antibacterial, anti-inflammatory, antiviral [19], anti-Helicobacter pylori [20], hypocholesterolemic, protective role against oxidative stress [21] and hypoglycemic effects [22]. It is used to treat liver diseases and is a major component of polyherbal formulations for the cure of hepatotoxicity in traditional medicine [23]. The aim of this study was to assess the possible antioxidant activities of barberry solid formulation as a traditional preparation which consists of 5 parts of *B. vulgaris*, 2 parts of *C. sativus* seed, 2 part of *P. oleracea* seed, 2 part of *C. intybus* seed and 1.5 part of *G. glabra* and 25% rose-water as binder.

2. MATERIALS AND METHODS

2.1. Chemicals. Ferric chloride (anhydrous), quercetin, ethanol, methanol, diethyl ether, chloroform, petroleum ether, ethyl acetate, n-Butanol, H2SO4, formic acid, and silica gel TLC plate were purchased from Merck chemical company (Germany).

Sodium nitroprusside dehydrate was purchased from Sigma-Aldrich chemical company (USA) and 1-diphenyl-2-picrylhydrazyl (DPPH), tripyridyl-s-triazine (TPTZ) and Griess reagent were purchased from Fluka chemical company (Switzerland). All chemicals and reagents used were analytical grades.

2.2. Plant material.

The plant materials deposited in the herbarium of Pharmacognosy (Voucher No. 1048 for *Berberis vulgaris* L., 1047 for *Cucumis sativus* L., 1052 for *Portulaca oleracea* L., 1046 for *Cichorium intybus* L.and 1128 for *Glycyrrhiza glabra* L., Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

2.3. Extraction.

Dried total botanical powder (150 g) and dried parts powder (30 g) were extracted with ethanol and water (70:30) and water. The solvent was removed under vacuum in a rotary evaporator with a low temperature, and afterward in freeze dryer pending dryness.

2.4. Antioxidant assays.

2.4.1. DPPH scavenging assay.

1-diphenyl-2-picryl-hydrazil (DPPH) solution (100mmole) was prepared freshly in methanol. The activity was measured in different concentrations of each extract ranging from 6.25-3200 µg⁄mL. Control wells contain (200 µL DPPH solution of and 20µL methanol), blank wells contain (20 µL of each extract and 200 µL methanol) and sample wells contain (200 µL DPPH solution and 20 µL of each extract). The prepared dilutions were dispersed in 96 well plates and incubated at 37°C for 30 min. The absorbance was measured at 492 nm in a micro plate reader. Quercetin was control in this test. The percentage of DPPH radical scavenging activity was calculated in the following way [24,25]:

Equation 1.

Inhibition%=100− [(Sample absorption- Blank absorption)/ Control absorption] ×100

The end with CurveExpert Professional 1.6.5 software DPPH radical scavenging activity charts was plotted and IC_{50} was calculated.

2.4.2. NO scavenging assay.

Nitric oxide scavenging test is based on producing nitrite ion. Control wells contain (50 µL of Sodium nitroprusside and 50µL methanol), blank wells contain (50 µL of each extract (200 µg/ml)) and sample wells contain (50 µL Sodium nitroprusside and 50µL of each extract (200 µg/ml)). The 96 well plate incubated at 27°C for 150 min. then 100 µL of Griess reagent was added to each well except blank wells. Absorption was readen at 542 nm in a micro plate reader. The nitric oxide scavenging ratio was calculated in the following way [24,26]:

Equation 2.

NO scavenging(%)=[(Sample absorption- Blank absorption)/ Control absorption] ×100

2.4.3. Ferric reducing ability.

FRAP measured by investigating the $Fe^{3+} \rightarrow Fe^{2+}$ transformation conforming to the method of Strain and Benzie. In the presence of antioxidants, purple ferric-tripyridyltriazine complex reduced to its yellow ferrous form [27]. The activity was measured in different concentrations of each extract ranging from 6.25-3200 µg⁄mL. Control wells contain (180 µL FRAP reagent and 20 µL methanol) and sample wells contain (180 µL FRAP reagent and 20 µL of each extract). the 96-well plates incubated at 37 °C for 15 min. The absorbance of the sample was read against control at 593 nm. The percentage of FRAP Value was calculated in the following way [28,29]:

Equation 3.

FRAP Value =100− [(control absorption / Sample absorption) ×100]

With CurveExpert Professional 1.6.5 software Ferric reducing ability charts were plotted and IC_{50} was calculated.

2.5. Phytochemical analysis.

2.5.1. Ash content analysis.

2.5.1.1.Total ash.

Approximately 4 g sample of the solid formulation was heated in a ceramic crucible at 500°C about 5hrs in a muffle furnace until black ash was transformed into white ash. After cooling, the ash was weighted. The ash was then moisturized by a limited drops of distilled water, mixed with 2 ml concentrated hydrochloric acid, and then dried with periodic heating on a hot plate in a fume chamber at 150°C. The mixture was then filtered with the ashless paper. The percentage of total ash was calculated in the following way [30].

Equation 4.

Total ash(%) = $((M_2-M_1) \times 100)/M_0$

M_0=sample weight M_1= ceramic crucible weight M_2 = ceramic crucible and ash weight

2.5.1.2. Acid_insoluble ash.

Total ash by 25 ml of dilute hydrochloric acid was boiled about 5 minutes on heater, insoluble material was filtered on an ashless filter paper, afterward was washed by hot water until the acid state was neutralized. The percentage of acid-insoluble ash was calculated in the following way [30].

Equation 5.

Acid_insoluble ash(%) = $((M_2-M_1) \times 100)/M_0$

M_0=sample weight M_1= ceramic crucible weight M_2 = ceramic crucible and ash weight

2.5.1.3. Water_soluble ash.

Total ash by 25 ml of water was boiled about 5 minutes on heater, insoluble material was filtered on an ashless filter paper, afterward was washed by hot water. The percentage of water-soluble ash was calculated in the following way [30].

Equation 6.

Water_ soluble ash (%) = $((M_2-M_1) \times 100)/M_0$

M_0=sample weight M_1= ceramic crucible weight M_2 = ceramic crucible and ash weight

2.5.2. Heavy Metals Investigation.

The presence of heavy metals connects with pollution inland of agriculture; therefore determining the count of Cadmium, Arsenic and Lead is very important. The contents of Lead, Arsenic, and Cadmium determined by atomic absorption spectrophotometry [30].

2.5.3. Total phenolic content.

The total phenolic content of total water and hydroalcoholic extracts was determined by Folin_ Ciocalteu's reagent (FCR). This method was using gallic acid as a reference compound. 500 µl of total water and hydroalcoholic extracts solutions (0.5 mg/ml) was mixed with 5 ml of the FCR and 4 ml of sodium carbonate (1 mol/ml). The mixture was shaken exhaustively after 15 min the absorbance at 765 nm was

determined against a blank that contained all reagents without the samples or the gallic acid at the same conditions. All determinations were carried out in triplicates. The total phenolic content was expressed as the number of equivalents of gallic acid (GAE) [29,31].

2.5.3. Total flavonoid content.

The flavonoids content was determined by the aluminum chloride method and the reference compound was quercetin. 3 ml of total water and hydroalcoholic extracts solutions (0.5 mg/mL) were mixed with 3 ml of 2% aluminum trichloride in methanol. After 15 min the absorption was determined at 415 nm. Blank was methanol. The absorption of the standard quercetin solution (20-100 µg/mL) in methanol was measured under the same conditions [29,31].

2.5.4. Microbial Control.

Total microbial count, *E.coli*, *Salmonella*, and *Pseudomonas aerujinosa* were assayed. The total microbial count determined by plate count method. For determinating *E. coli* 1 g sample in lactose broth was transferred to 100 ml of Mac Conkey broth than was incubated for 24 hours at 45°C. For determinating *Salmonella spp.* the suspension of the sample was incubated for 24 hours at 37°C, then 10 ml sample was transferred to 100 ml of tetrathionate bile and brilliant green broth than was incubated for 24 hours at 42°C. And for determinating *Pseudomonas aeruginosa* 1g of the sample was mixed with 100 ml of soybean-casein digest medium and was incubated for 24 hours at 35°C, then a subculture on a cetrimide agar plate was ready and was incubated for 24 hours at 37 °C [30].

3. RESULTS

3.1. Results of extraction efficiency.

The percentages of extraction efficiency (yield) were listed in below table. According to the results of table 1, the lowest percentages of extraction efficiency is related to *Portulaca oleracea* EtOH extract (5.53%) and the highest percentages of extraction efficiency is related to *Berberis vulgaris* aqueous extract (11.56%). In general, the percentages of extraction efficiency by aqueous solvent is more than by hydroalcholic solvent. Total EtOH extraction efficiency is (7.56%) and total aqueous extraction efficiency is (8.6%).

Table 1. Extraction efficiency.

Extracts	Extraction efficiency (%)
Total EtOH ext.	7.56%
Total H_2O ext.	8.6%
Berberis vulgaris EtOH ext.	10.16%
Berberis vulgaris H_2O ext.	11.56%
Cichorium intybus EtOH ext.	5.46%
Cichorium intybus H_2O ext.	6.83%
Cucumis sativus EtOH ext.	5.9%
Cucumis sativus H2O ext.	7.16%
Portulaca oleracea EtOH ext.	5.53%
Portulaca oleracea H2O ext	6.96%

3.2. Results of antioxidant tests.

Results of FRAP test, NO test and DPPH test were showed in table 2. According to the FRAP values, *Berberis vulgaris* EtOH extract (236.58±3.4 µg/ml) and *Cichorium intybus* EtOH extract (867.53±4.6 µg/ml) have antioxidant effects. Percentages of NO Scavenging showed that all the extracts have antioxidant effects. *Berberis vulgaris* H_2O extract has the highest percentages of NO Scavenging (80.07±2.44 %), so it has a powerful antioxidant effect. Percentages of NO Scavenging for total EtOH extract is (61.72±1.79%) and percentages of NO Scavenging for total H_2O extract is (74.61±2.95%). In general, the percentages of NO Scavenging for Total H_2O extract and *Berberis vulgaris* H_2O extract are more than Total EtOH extract and *Berberis vulgaris* EtOH extract. Results of DPPH scavenging activity showed that *Cichorium intybus* EtOH extract has an antioxidant effect (394.24±2.14 µg/ml).

Table 2. Results of antioxidant tests.

Extracts	FRAP value(µg/ml)±SD	NO Scavenging (%) (200µg / ml)±SD	IC_{50} (µg/ml) DPPH scavenging activity±SD
Total EtOH ext.	1003.14±5.19	61.72±1.79	1852.84±13.91
Total H_2O ext.	1190.69±29.58	74.61±2.95	2895.06±24.53
Berberis vulgaris EtOH ext.	236.58±3.4	55.47±2.95	3041.18±51.13
Berberis vulgaris H_2O ext.	906.03±5.97	80.07±2.44	1073.88±13.70
Cichorium intybus EtOH ext.	867.53±4.6	57.42±2.03	394.24±2.14
Cichorium intybus H_2O ext.	1658.57±14.41	52.34±2.44	17233.73±104.69
Cucumis sativus EtOH ext.	1411.11±24.11	71.09±2.44	3527.60±31.47
Cucumis sativus H2O ext.	3380.98±13.58	66.41±2.44	24631.49±128.93
Portulaca oleracea EtOH ext.	Not Detected	51.17±2.44	7989.97±237.96
Portulaca oleracea H2O ext.	Not Detected	66.02±1.79	19747.79±185.03
Quercetin	16.11±.25	55.08±2.34	78.97 ± .95

3.3. Results of phytochemical.

3.3.1. Results of total phenolic and flavonoid content.

According to the results were showed in the table 3, total EtOH extract (31.46±.13 mg GAE/g of dry matter) has more total phenolic content than total H_2O extract (26.84±.47 mg GAE/g of dry matter) and total H_2O extract (6.38±.25 mg Quercetin/g of dry matter) has more total flavonoid content than total EtOH extract (5.26±.16 mg Quercetin/g of dry matter).

Table 3. Total phenolic and flavonoid content

extracts	Total phenolic content± SD (mg GAE/g of dry matter)	Total flavonoid content ±SD (mg Quercetin/g of dry matter)
Total EtOH ext.	31.46±.13	5.26±.16
Total H2O ext.	26.84±.47	6.38±.25

3.3.2. Ash content

Due to the results of ash content in the table 4, dissoluble in acid ash is low percentage (5.4%) so there is not cheating by adding silica materials or soil for increasing plants weight. The percentage of total ash content is (8.2%) and the percentage of soluble in water ash content is (2%).

Table 4. Ash content.

Ash type	Total ash content	Dissoluble in acid ash	soluble in water ash
Ash content (%)	8.2%	5.4%	2%

3.3.3. Results of Heavy Metals Assay.

Heavy Metals have the property of accumulating in body tissues and causing poisoning. The standard limit for Pb, Cd and As is 10000 ppb, 300 ppb and 4000 ppb respectively [30].

Table 5. Heavy Metals assay

Heavy metals	Pb	Cd	As
Standard limit (ppb) [30]	10000	300	4000
Sample results (ppb)	199	15	<20

3.3.4. Results of Microbial Control.

The amount of heavy Metals in the sample is lower than the standard limit , there are 199 ppb of Pb, 15 ppb of Cd and less than 20 ppb of As in the sample (table 5). *Salmonella, E.coli* and *Pseudomonas aeruginosa* should not be detected in oral herbal formulations. The standard limit for Total bacterial count in oral herbal formulations is 10^5 cfu (table 6).

In the sample *Salmonella, E.coli* and *Pseudomonas aeruginosa* were not detected and total bacterial count is lower than standard limit (10^4 cfu).

Table 6. Results of Microbial Control.

experiments	results	Standard limit [30]
Total bacterial count	10^4	10^5
Salmonella in gram	Not Detected	Not Detected
E.coli in gram	Not Detected	Not Detected
Pseudomonas aeruginosa in gram	Not Detected	Not Detected

4. CONCLUSIONS

In recent years, there is a growing interest in the use of herbal medicines instead of synthetic drugs, to manage a variety of disorders such as liver diseases [32]. Results of researches shows that *B.vulgaris* extract can decrease liver damage [33] and barberry supplementation significantly decreased the levels of total cholesterol, triglyceride, and low-density lipoprotein cholesterol [34]. In other studies, *B.vulgaris* extract effectively inhibited lipid peroxidation, protein carbonyls formation and significantly normalized the antioxidant enzymes such as GPx, CAT and SOD [35] and suppressed the increase in levels of MDA, ALT and AST [36]. Barberry solid formulation is a hepatoprotective formulation in Traditional Persian Medicine. In this study according to the results of microbial control, heavy metal assay and ash content this formulation is standard.

The hydro-alcoholic extract has more phenolic compounds (31.46±.13 mg GAE⁄g of dry matter) than the water extract and the water extraction has more flavonoid compounds (6.38±. 25 mg Quercetin ⁄g of dry matter) than the hydro-alcoholic extract. The result of DPPH radical scavenging shows that the hydro-alcoholic extract has more effect than the water extract. FRAP value indicates that the hydro-alcoholic extract has more reductive activity than the water extract. The study of NO radical scavenging represents water extraction 74.61±2.95 %, hydro-alcoholic extract 61.72±1.79 % thus Water and hydro-alcoholic extracts are powerful antioxidants and have hepatoprotective effect.

The antioxidant activity may be due to the inhibition of the formation of radicals or scavenging of the formed radical. These results concluded that barberry solid formulation has hepatoprotective effects against oxidative stress and liver dysfunction. The findings thus establish the potential medicinal value of the plants used in indigenous systems of medicines in Iran and also initiate further detailed investigations on the components of this formulation in order to justify their use in polyherbal formulations prescribed in the treatment of liver disorders.

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6. ACKNOWLEDGEMENTS

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