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ORIGINAL ARTICLE

The Study of Antioxidant and Cellular Toxicity Effects of Methanol, Ethyl Acetate, Aqueous and n-Hexane Extracts of *Symphytum Kurdicum* Plant

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KEYWORDS

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ABSTRACT: This study was aimed to investigating the antioxidant and cellular toxicity of *Symphytum kurdicum*. The methanolic extracts of the aerial parts of the plant were prepared through soaking and non-polar to polar cutting of the extract by the liquid-liquid cutting method. The antioxidant effect of the samples was specified by the methods of determining the free radical scanenging 2, 2-diphenyl-1-picrylhydrazyl(DPPH), ferric reducing ability of plasma (FRAP) and the total phenolic content by folin ciocalteu method. Cellular toxicity of the samples on peripheral blood mononuclear cells (PBMC) was performed by 3-(4,5-dimethylthiazol-2-yl) and 2, 5-diphenyltetrazolium bromide (MTT). The results indicated that ethyl acetate and aqueous fractions with IC_{50} equal to 33.67 and 29.43 µg/ml, respectively, showed the highest ability in DPPH free radicals Scavenging. Moreover, in the study of ferric iron regeneration, the ethyl acetate fraction with a capability of 280.985± 14.007 mM mg^{-1} dry weight of sample showed the best regenerative effect against trolox control. The aqueous and ethyl acetate fractions had the highest total phenolic content with 150.765 ± 0.035 and 130.570 ± 0.056 (Gallic acid milligrams/ gram dry weight of sample), respectively. The results of MTT test revealed that all fractions at a concentration much higher than the effective antioxidant concentrations lacked cellular toxicity, too. Given the role of oxidative stress as a predisposing factor in diseases like diabetes, cancer, and cardiovascular disease, aqueous and ethyl acetate fractions are likely to be introduced as pharmacological supplements.

INTRODUCTION

Antioxidants are chemical compounds, or mixtures of chemical compounds that delays or prevents oxidation; for example, they deactivate the activated oxygen species from the oxidation of lipids, sugars, proteins, and nucleic acids

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through producing aldehyde, ketones, and ester compounds, and other beneficial compounds involved in the biological system [1]. These compounds reduce the incidence of various diseases such as cardiovascular disease, atherosclerosis [2, 3], central nervous system disorders [4], diabetes [5, 6], and cancer [7, 8] male infertility [9] through reducing the number of free radicals caused by the oxidative stress process. Therefore, to achieve optimal cell function and prevent the above mentioned disorders, it is necessary to control the oxidative stress process by consuming antioxidant compounds and consequently reducing free radicals [10]. According to the World Health Organization (WHO), despite advances in modern medicine, 80% of the population in developing countries tends to use herbal medicines [11]. Today, plants are regarded as natural antioxidant sources by food producers [12] and the tendency to use natural antioxidants as an additive in foods and beverages is increasing [13].

In the present research, first, the methanolic extract of aerial parts of *Symphytum kurdicum* and its non-polar to polar fractions were investigated and then the antioxidant effects of these fractions were examined by the methods of investigating free radicals DPPH inhibition and oxidized iron regeneration and total phenolic content. In the end, to investigate the safety of using this plant as an antioxidant, the cellular toxicity of its extracts on peripheral blood lymphocyte cells was examined.

Symphytum kurdicum Boiss. & Hausskn is a perennial flowering plant in the family Boraginaceae which is covered with short stiff hairs. Lower cauline leaves are ovate to oblong, membraneous, bases cordate and the margin are entire and undulate. Upper cauline leaves are ovate to oblong, petiolate. Inflorescence scorpoid cymes. Flowers nodding. Calyx campanulate, enlarged in fruit. Corolla tubular shaped, with white to cream color, 2 to 3 times longer than the calyx. Nutlets 4, ovoid, obliquely curved, tuberculate and often wrinkled [14].

MATERIALS AND METHODS

Plant collecting

The flowered aerial parts of *Symphytum kurdicum* Boiss. & Hausskn were collected from kurdistan province, Marivan city on May 2016 from an altitude of 1350-1800 meters above sea level in oak forests near Zarivar Lake and were identified by Dr. Nastaran Jalilian. A voucher speciemen (No. 9045 RANK) was deposited at herbarium of Agricultural and Natural Resources Research and Education Center in Kermanshah province.

Extraction and cutting

The dried aerial parts of the plant were milled and soaked with methanol (1:10 W/V) for 48 hours at room temperature through using shaker (150 rpm). The methanolic extract was filtered using Whatman paper (No. 1) and filtrated was concentrated at 40 ºC using the rotary evaporator instrument. methanolic extract dissolved in distilled water and further extraction was performed by N-hexane and ethyl acetate solvents (1:1 V/V) by the liquid-liquid cutting method. Three fractions were obtained at this stage, which include two fractions with organic solvent and the remaining aqueous part. The methanolic extract and three fractions were dried by freeze dryer at -60ºC and were kept at 4ºC.

The yield percentage (Y) was calculated by the following equation:

$$Y = (W1 \times 100) / W2$$

In this equation, W1 represents the dry weight of the total sample of extract or fractions of *S. kurdicum* and W2 stands for the weight of the dried plant samples.

Antioxidant activity

Investigating of phenolic content

Determining the total content of phenolic compounds is a rapid method in investigating the antioxidant effect of the studied materials [15]. The total phenolic content of the samples was specified by spectrophotometry method [16].

10 microliters of fresh storage solution of each sample (1 mg ml^{-1}) was mixed with 75 µl of folin-ciocalteu reagent (10 % v/v in distilled water) for 3 min at 23 ºC. Then, 75 µl of sodium carbonate (60 g L^{-1}) was added to the mixture, after 60 min, the sample adsorption at 725 nm was read by BioTeck plate reader (Epoch model, BioTeck Company). Different concentrations of gallic acid storage solution in methanol were used to plot the standard curve (all experiments were repeated three times).

Investigating the DPPH free radicals scavenging

The method of determining the free radical scavenging capacity of DPPH was used with a slight compared to previous reports [17, 18]. The storage solution of each sample was used at a concentration of 2 mg ml^{-1} in methanol (5, 10, 20, 30, 40µl). 150µl of fresh DPPH solution (80 µg ml^{-1}) were added to each 96-plate well containing different amounts of the sample. After mixing all the contents of the microplate, for 30 minutes, the sample absorbance was read at 517 nm through the microplate reader. 1 mg ml^{-1} Butylated hydroxytoluene (BHA) solution was used as a positive control. The inhibition percentage in each concentration was calculated by the following formula.

$$\text{Inhibition ratio (DPPH. scavenging effect) (\%)} = [(A\text{control} - A\text{sample}) / A\text{control}] \times 100$$

The inhibition curves were prepared and the line equation was obtained. The concentration required to inhibit 50% free radicals (IC50) was determine. Five replications were performed for all samples, controls, and Blanks and the mean absorption of replicates was entered in the formula.

Investigation of the antioxidant effect of Ferric Reducing Ability of Plasma (FRAP) Method

This section was conducted according to the conversion of (Fe (III)-TPTZ) complex to (Fe (II)-TPTZ) [19]. The FRAP reagent was prepared from 300 mM acetate buffer, TPTZ with 10 mM concentration and 20 mM ferrous chloride ($FeCl_3.6H_2O$) (10: 1: 1 ratio) immediately before the experiment. 20 microliters of each sample with 200 microliters of FRAP reagent were added to each 96-cell well, Mixed on a rotating shaker for 30 minutes at room temperature and the adsorption of the samples was read by means of a plate reader at the wavelength of 593 nm. The adsorption rates of different concentrations of ferrous sulfate (200, 400, 800, 1200, and 1600 mM) were used to plot the standard curve. Trolox was used as a positive control.

Investigation of the effects of cell toxicity on Peripheral Blood Mononuclear Cells (PBMCs)

1. Preparation of Cells: Environmental mononuclear cells were obtained from peripheral venous blood samples of healthy individuals. Using a centrifuge and a histopaque1077, the blood cells were separated by density and size. After washing it for two times in the sterile RPMI 1640 culture medium, the cellular precipitate obtained from this stage was mixed with an equal volume of the vital dye of trypan-blue (with a concentration of 2 mg.ml^{-1} in distilled water). After 5 minutes of treatment, live cells were counted on the Neubauer chamber. According to the mean of the results of the three counts, the cell suspension was diluted by a culture medium containing fetal calf serum (inactivated)) and penicillin-streptomycin so that the number of cells per milliliter of culture medium would be 10^6 [20].
2. Investigating cellular toxicity: In this section, the toxicity of samples obtained from the aerial parts of the plant on peripheral blood mononuclear cells was investigated. The use of MTT is one of the most common colorimetric methods in examining the toxicity of substances on cells. In this measurement, the regenerative power of tetrazolium dye is investigated by cellular enzymes and its conversion to purple formazan crystals [21].

Sterile filter with a diameter of 0.2µm was used to prepare the solutions from the studied plant. Different concentrations of each sample were prepared under sterile conditions.

150µl of cell suspension and 50µl of sterile dilutions of each sample were added to each well, and the plates were incubated at 37°C for 6 hours with CO_2 (5%).

Subsequently, 20μl of sterile MTT solution at a concentration of 0.5 mg ml^{-1} was added to each well and the heating was continued for another 3 hours. The sterile culture medium was used as a negative control. The absorption rate of the wells was recorded by the plate reader at wavelengths of 540 with reference 620 nm and the percentage of bioavailability and cytotoxicity of each extract were calculated from the following equation

Viability (%) = mean of absorbance of sample × 100/ mean of absorbance of negative control

Cytotoxicity (%) = 100 – Viability (%)

RESULTS AND DISCUSSION

Preparation of methanolic extracts and fractions

Different samples were prepared using solvents with different polarity (water, methanol, ethyl acetate, and n-hexane) in order to extract a range of different compounds from the aerial parts of *S. kurdicum*. The yield percentage of each stage of extraction or cutting is shown in Table 1.

Table 1. Yield percentage of plant fractions.

Extracts	yield percentage
methanolic	4.35
Ethyl acetate	1.05
n-hexan	8
Aqueous	5.8

Total phenolic content

The total phenolic content of the samples was specified using the standard graph of gallic acid (*R2* = 0.99) and the results are shown in Table 2 as equivalent to milligrams of gallic acid per gram dry weight of the extract.

Investigation of antioxidant effect by DPPH free radical scavenging

The results obtained in this stage are shown in Table 2 and Figure 1. As observed in the diagram, the aqueous fraction and then the ethyl acetate fraction have had the best antioxidant effect. Comparison of IC$_{50}$ concentration of the samples with positive control (BHT) revealed that aqueous and ethyl acetate extracts have a high antioxidant effect (Table 2).

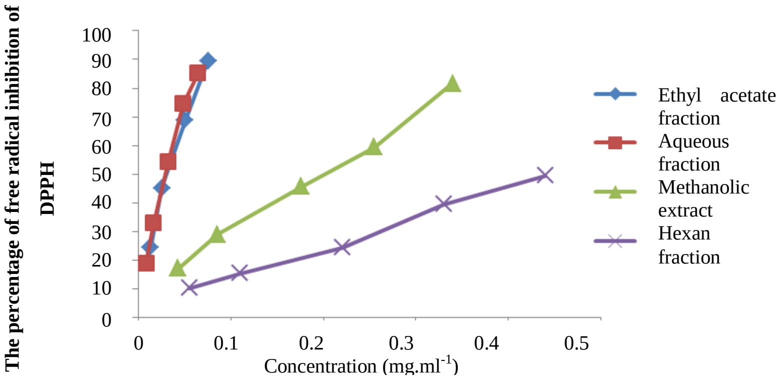

Figure 1. The percentage of free radical scavenging of DPPH by aerial fractions of the *S. kurdicum* plant.

Investigation of FRAP Antioxidant Effect

The test results of this step are also shown in Table 2. According to the results obtained in this method, the highest antioxidant effect was observed in ethyl acetate fraction.

Table 2. Total phenolic content and antioxidant effects by DPPH and FRAP methods of *S. kurdicum* plant.

Extracts	**[1] total phenolic content**	**[2]DPPH (IC50)**	**[3]FRAP Value**
Methanolic	47.956±0.027	195.85±0.020	0.061659
Ethyl acetate	130.570±0.056	33.67±0.005	0.356920
N-hexan	5.314±0.318	461.24±0.023	0.01928
Aqueous	150.765±0.035	29.43±0.020	0.08806
BHT		11.38±0.00	

1. Equivalent to milligrams of gallic acid per gram dry weight of extract.
2. Micrograms per milliliter.
3. The equivalent of gram Trolox per gram dry weight of extract.

Plants that are rich in secondary metabolites (like phenolic compounds) have a high antioxidant effect [15]. The strength of phenolic compounds to regenerate other compounds and the ability to trap free radicals make it possible to create antioxidant effects for these compounds [22]. The antioxidant compounds of medicinal herbs may play a role in their protecting effect against diseases, so that the use of natural antioxidants is inversely related to mortality from degenerative disorders [23].

Sialic acid, coumaric acid, caffeic acid, and p-Cresol are among the phenolic compounds reported in *S. kurdicum* [24]. The antioxidant role of phenolic compounds, including rosmarinic acid, has been confirmed in previous researches [25].

The amount of phenolic content in aqueous fractions (150.765 ± 0.035) and ethyl acetate (130.0570 ± 0.056) compared to methanolic extract (47.965 ± 0.027) and n-hexane fraction (5.314 ± 0.318) has significantly increased. Comparison of the antioxidant effect of the above samples by DPPH and FRAP methods (Table 2) indicates the increased antioxidant power of aqueous and ethyl acetate fractions compared to methanolic extract and n-hexane fraction. The positive relationship between total phenol content and antioxidant effect of aqueous and ethyl acetate extracts of *S. kurdicum* once again confirms the role of phenolic compounds in antioxidant effect [26, 27].

Cell toxicity

The toxicity of *S. kurdicum* plant extracts on peripheral blood mononuclear cells was investigated by MTT method (Table 3).

Table 3. Percentage of Cell viability of PBMCs.

Extracs	**Cell viability (Control)**	**Cell viability (0.2 mg ml^{-1})**	**Cell viability (0.4 mg ml^{-1})**	**Cell viability (0.8 mg ml^{-1})**	**Cell viability (1.7 mg ml^{-1})**
Methanolic	100 ±16.38	177.64±8.21	206.14±36.18	228.52±34.74	256.75±41.20
Ethyl acetate	100 ±24.97	133.18±20.00	173.18±13.96	207.99±14.10	246.07±23.49
N-hexan	100±4.96	107.84±3.97	107.35±6.42	113.23±6.79	119.11±13.27
Aqueous	100±11.32	110.25±16.18	110.25±16.18	141.07±24.45	141.03±34.05

S. officinale L plant, from the *Symphytum* family, has been used as an herbal medicine in the treatment of bone fractures, tendon injuries, joint inflammation, and wound healing for two thousand years [28]. Previous research has revealed that extracts of the species of the *Symphytum* family stimulate tissue regeneration as well as cell formation [29].

Three compounds of rosmarinic acid, allantoin, and polysaccharides have been reported to be major effective compounds in plants of the *Symphytum* family [30]. Allantoin increases cell division and consequently cell repair [31]. Moreover, MTT results in this study revealed that *S. kurdicum* plant extracts not only had no toxic effect on peripheral blood lymphocyte cells, but also it accelerated the proliferation of the studied cells (Table 3).

CONCLUSIONS

The results showed that aqueous and ethyl acetate fractions of *S. kurdicum* plant had good antioxidant effects and these effects were observed in very low concentrations and without cytotoxicity. Thus, further studies are recommended to identify the effective antioxidant compounds in the plant for use in drug design research. In addition, such compounds may be effective as antioxidant supplements in diseases such as diabetes, cancer, cardiovascular disease, as well as wound healing.

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