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Antioxidant and proliferative effects of aqueous and ethanolic extracts of *Symphytum officinale* on 3T3 Swiss albino mouse fibroblast cell line

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ABSTRACT

The aim of the present study is to evaluate the antioxidant and proliferative effects of aqueous and ethanolic extracts of Symphytum officinale. Antioxidant activities of extracts were determined by 2,2- diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, superoxide radical scavenging and Folin-Ciocalteu assays. Proliferative effects of the extracts were evaluated on 3T3 Swiss albino mouse fibroblast cells by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and neutral red uptake (NRU) assays. According to the results, ethanolic extract exhibited stronger radical scavenging activity against DPPH radical (IC_{50} value of 39.97 µg/ml) in comparison with aqueous extract (IC_{50} value of 96.21 µg/ml). Similarly, superoxide radical scavenging activity of ethanolic extract (IC_{50} value of 190.76 µg/ml) was found to be higher than aqueous extract (IC_{50} value of 307.42 µg/ml). The total phenolic contents of ethanolic and aqueous extracts of S. officinale were found as 116.93 mg GAE/g and 99.49 mg GAE/g, respectively; showing that the greater amount of phenolic compounds leads to more potent radical scavenging effect. Also, the results of cell proliferation assays indicated that both extracts have proliferative activity on 3T3 Swiss albino mouse fibroblast cells. Ethanolic and aqueous extracts of S. officinale showed promising antioxidant and free radical scavenging activity and proliferative effect, thus justifying their traditional use.

Keywords: *Symphytum officinale*, antioxidant, DPPH, Folin−Ciocalteu, proliferative activity

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INTRODUCTION

Symphytum genus commonly known as comfrey, is a member of Boraginaceae family. The genus *Symphytum* is native to Europe and Western Asia and it comprises approximately 35 species [1]. The Turkish flora includes 17 *Symphytum* species and 8 of them are reported as endemic [2].

The roots and leaves of *Symphytum* species contain many active components including allantion (0.7 %), large quantities of mucilage, tannins, proteins, vitamins, mucopolysaccharides, steroidal sapponins, phenolic acids, mainly rosmarinic acid and pyrrolizidine alkaloids [3]. In dried roots of *Symphytum officinale*, the amount of total alkaloid content is 0.25 to 0.29 %, usually lower in leaves [4]. Three major components; allantoin, rosmarinic acid and polysaccharides in *Symphytum* species are regarded as a reason of pharmacological effect and clinical efficacy of this plant [5,6]. Allantoin is a cell- proliferant that promotes cell division and renewal of epidermal cells, as well as accelerates wound healing and reduces scar formation [7]. Also rosmarinic acid, is an ester of caffeic acid, demonstrates both antioxidative and antiinflammatory activity. In connection with pharmacological properties of *Symphytum* components, the plant has been reported to be useful in treating skin wounds. Several authors reported that the extract of *Symphytum* species stimulate granulation, tissue regeneration and support callus formation [8,9].

Symphytum extracts have been extensively used in folk medicine in the treatment of various diseases such as joint complaints, muscle aches, arthritis, rheumatism, gout and gastrointestinal ulcers. *Symphytum* species have also been used as a poultice, for treating burns and wounds [10,11]. In Germany, *S. officinale* has been applied against

musculoskeletal conditions since 1920 [9]. Investigators reported that, this plant species have also been used in Turkish folk medicine for a long time due to their healing properties [12].

The present study was undertaken to evaluate the antioxidant and proliferative activity of ethanolic and aqueous extracts of *S. officinale*. Proliferative effects of the extracts were evaluated on 3T3 Swiss albino mouse fibroblast cells by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and neutral red uptake (NRU) assays. Antioxidant potential of extracts were determined by microplate 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and superoxide radical scavenging assays. Due to the significant correlation between total phenolic content and antioxidant activity of plants, phenolic content of the plant extracts were determined by Folin- Ciocalteu assay.

MATERIALS AND METHODS

Collection of plant material

The plants were collected from local market in Istanbul, Turkey. The leaves of *S. officinale* were dried at room temperature and grounded into uniform powder using a grinder.

Preparation of plant extracts

Air dried plant samples were extracted by using two extraction techniques, soxhlet extraction and decoction method. The soxhlet extraction was performed two times in a soxhlet extracion system (İldam, Turkey). 25 g of plant sample was extracted with 250 ml 96 % ethanol. Decoction performed according to the method [13] with slight modification. 50 g of powdered plant sample was boiled with 1L deionized water at 100 °C for 30 minutes in the water bath. Then the extract was cooled to 40 °C and thereafter, filtered. In both extraction methods, following the extraction processes solvents were evaporated to dryness under reduced pressure using rotary evaporator (Heidolph, Laborota 4000, Germany) at 40 °C. Finally, the extracts were lyophilized with a freeze- dryer (Christ Alpha 2-4 LD plus, Germany) and stored at –20 °C until further use.

Determination of extraction yield (%)

The yield (%) from dried extracts were calculated using the following formula:

$Y_{extract} = (W_1 \times 100) / W_{2,}$

where W_1 is the weight of the extract after lyophilization of solvent and W_2 is the weight of the plant powder.

Determination of total phenolics

The amount of phenolic compounds in the extract was estimated by Folin–Ciocalteu reagent according to the method [14]. Briefly, 10 µl sample or standard (50–400 mg/L gallic acid) plus 150 µL diluted Folin– Ciocalteu reagent (1:4 reagent:water) was placed in each well of a 96-well plate, and incubated at room temperature for 3 min. Following the addition of 50 µL sodium carbonate (2:3 saturated sodium carbonate: water) and a further incubation of 2 h at room temperature, absorbance was read at 725 nm. Gallic acid was used as a standart for the calibration curve and total phenolic compounds concentration in the extract was expressed as miligrams of gallic acid equivalent per gram of dry weight (mg GAE/g) of extract. All tests were conducted in triplicate.

DPPH radical scavenging assay

The DPPH radical scavenging effect was assessed by the discoloration of methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) [15]. The assay is based on the reduction of DPPH. The color of DPPH solution turns from deep violet to yellow, when DPPH reacts with a reducing agent. DPPH (50 µL, 1 mM) solution was added to MeOH solution (200 µL) of the extract, fractions or compounds at various concentrations (25, 50, 100 µg/ml). The reaction mixture was shaken vigorously and the absorbance of remaining DPPH was measured with microplate reader (FilterMax F5, Molecular Devices, USA) at 517 nm after 30 min. The radical scavenging activity was determined by comparing the absorbance with that of blank (100%) containing only DPPH and solvent. Ascorbic acid was used as standart compound. DPPH radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

% DPPH radical scavenging activity = [(Control absorbance - sample absorbance)/Control absorbance] x 100

Samples were analyzed in triplicate. The inhibition curves were prepared and the concentration of the sample required to scavenge the 50% DPPH free radical (IC_{50}) was calculated.

Superoxide radical scavenging activity by alkaline DMSO method

Superoxide radical scavenging activities of *S. officinale* extracts were determined by alkaline dimethyl sulfoxide (DMSO) method [16] with slight modification. In this method, superoxide radical is generated by the addition of sodium hydroxide to air saturated DMSO. The generated superoxide remains stable in solution, which reduces nitro blue tetrazolium (NBT) into red formazan dye at room temperature and that can be measured at 560 nm. All the extracts and standart compound ascorbic acid at various concentrations (25, 50, 100 µg/ml) were dissolved in DMSO. To the reaction mixture containing 10 µl of NBT solution (1 mg/ml in DMSO) and 30 µl of extracts or standart compound, 100 µl of alkaline DMSO (1 ml DMSO containing, 5 mM NaOH in 0.1 ml water) was added to give a final volume of 140 µl. The absorbance was measured at 560 nm using microplate reader (FilterMax F5, Molecular Devices, USA). The percentage of superoxide radical scavenging by the extracts and standard compounds were calculated as follows:

% Superoxide radical scavenging activity = [(Test absorbance - Control absorbance)/Test absorbance] x 100

All samples were performed in triplicate and the amount of extracts needed to inhibit free radical concentration by 50 % (IC_{50}) was determined for both extracts and standart ascorbic acid.

Cell culture

3T3 Swiss albino mouse fibroblast cells were supplied from Istanbul Faculty of Medicine, Department of Biophysics. All of the cell culture media and chemical reagents were purchased from Gibco BRL. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)- F12 supplemented with 10 % foetal bovine serum (FBS), 1 % antibiotic- antimycotic solution. Cells were maintained in a humidified atmosphere of 5 % CO_2 at 37 ºC with medium changed every 2 to 3 days. The culture was continued until reaching 80 % confluency. 3T3 swiss albino mouse fibroblast cells were detached from the culture surface using 0.25% trypsin-EDTA solution.

MTT assay

The plant extracts were dissolved in DMSO and diluted with DMEM-F12 to prepare different concentrations (5, 25, 50 µg/ml) of crude extract. The final concentration of DMSO in the culture medium was 0.02 % and the control group received the same amount of DMSO. For quantitative evaluation of cell viability and proliferation, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was used, in which only viable cells can reduce MTT to insoluble purple formazan. Thus, the intensity of purple color in turns represents the number of viable cells. MTT cell proliferation kit (Roche, Germany) was performed according to the manufacturer instructions. 3T3 Swiss albino mouse fibroblast cells were cultured in 96-well plate at a density of 1 x 10^4 cell/100 µL DMEM-F12 for 24 h. Then the medium was changed to fresh medium (control) and medium supplemented with different concentrations (5, 25, 50 µg/ml) of *S. officinale* extract. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37 ºC for 72 h. At the end of the treatment period, 10 µl of MTT solution was added to each well. Following the incubation for 4 h at 37 ºC, 100 µl of solubilizing buffer (10 % SDS dissolved in 0.01 M HCl). After overnight incubation, the absorbance of each well was determined by multimode microplate reader (FilterMax F5, Molecular Devices, USA) at 595 nm [17]. Cell viability of treated samples was calculated in reference to vehicle treated control that was defined as 100 % viable. Thus, the degree of inhibition of extract treated cells is expressed as the percentage of the untreated cell control using the formula: Viability (%) = (sample absorbance/control absorbance) x 100

Neutral red uptake assay

The inhibition of cell growth was determined by neutral red uptake (NRU) assay. The living cells can only take up red dye and incorporate in lysosomes, while dead or damaged cells did not show any activity. The cells were cultured and incubated as mentioned in MTT assay. After incubation period, medium was removed and neutral red solution (50 µg/ml) was added to each well including control samples. Then the plates were incubated for further 3 h. The neutral red solution was carefully removed and rinsed with pre warmed PBS. Then, 200 µL fixative (1% w/v $CaCl_2$ in 1% v/v formalin) was added to each well and the plate was incubated at room temperature for 1 min to fix the cells. The fixative was then removed and replaced with 100 µl NR extracting solution (50 % v/v ethanol containing 1% v/v acetic acid). The plate was incubated for an additional 20 minutes at room temperature with continued shaking to solubilize the NR. The absorbance of each well was measured at 595 nm in a multimode microplate reader (FilterMax F5, Molecular Devices, USA) [18]. % proliferation was calculated using the formula: inhibition (%) = (sample absorbance/control absorbance) x 100

Statistical analysis

Samples were run in triplicate and the results were analyzed statistically using one-way ANOVA. Results were given as mean ± S.D. The data were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

DPPH radical scavenging assay

Radical scavenging activities of the *S. officinale* extracts were screened against DPPH radical which is widely used to investigate the scavenging activities of several natural compounds. As shown in Table 1 and Figure 1, the radical scavenging activities of ethanolic and aqueous extracts of *S. officinale* were calculated in terms of IC_{50} value and percentage of DPPH inhibition. The high inhibition percentage indicates strong scavenging activity. Both extracts show a gradual increase in activity with increase of concentration. The percentage of inhibition of the DPPH radical was varying from 38.72 - 85.09 % in ethanolic extract and 21.68- 52.19 % in aqueous extract. The extract that can lower the initial absorbance of DPPH solution by 50% has been chosen as the endpoint for measuring the antioxidant activity. The ethanolic extract exhibited stronger radical scavenging activity against DPPH radical (IC_{50} value of 39.97 µg/ml) in comparison with aqueous extract (IC_{50} value of 96.21 µg/ml). As seen from the results, ethanolic extract exhibited activity as strong as standard compound, ascorbic acid (IC_{50} value of 26.33 µg/ml). Thus, ethanolic extract contains much more active biocompounds than aqueous extract that were relatively strong scavengers of free radicals. Our findings are in good agreement with the previous studies that have shown that *S. officinale* extracts have significant antioxidant effect radical scavenging activity [19, 20]. Similar to the results of our study; it is reported that as the solvent is concerned the ethanolic extract exhibited a little higher inhibition of DPPH than the methanolic extract [19].

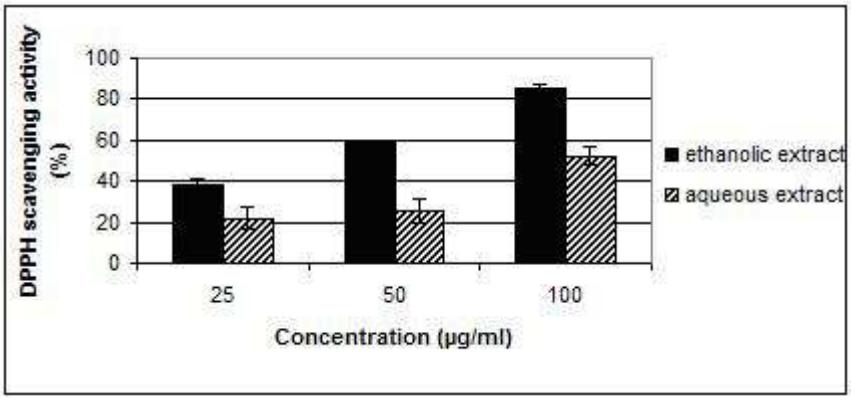

Figure 1: **DPPH scavenging activity of *Symphytum officinale* extracts**

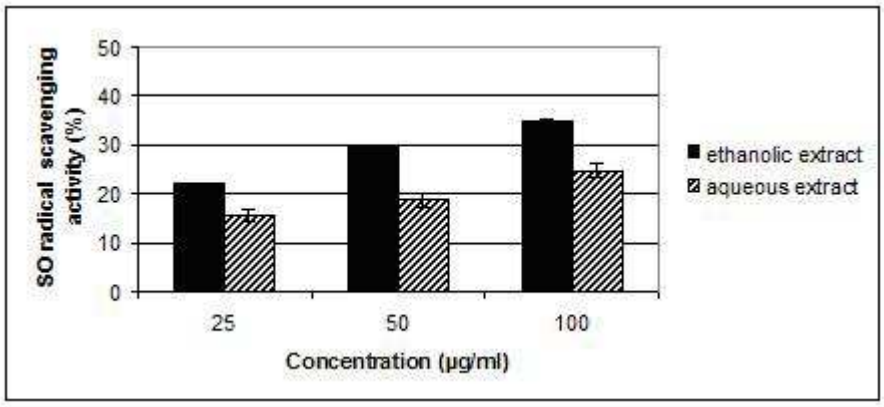

Figure 2: Superoxide (SO) radical scavenging activities of *S. officinale* extracts

Superoxide radical scavenging activity by alkaline DMSO method

The percentage of superoxide radical scavenging activities and IC_{50} values of ethanolic and aqueous extracts of *S. officinale* are presented in Figure 2 and Table 1. The maximum inhibition was found to be 34.74 % for ethanolic and 24.73 % for aqueous extract at 100 µg/ml. The superoxide radical scavenging activity of ethanolic extract was found to be better (IC_{50} of 190.76 µg/ml) than aqueous extract with (IC_{50} of 307.42 µg/ml). Superoxide anion radical is one of the strongest reactive oxygen species (ROS) among the free radicals and get converted to other harmful ROS such as hydrogen peroxide and hydroxyl radical, damaging biomolecules which results in chronic diseases. The results demonstrates that *S. officinale* extract is capable of non-enzymatically inhibiting the superoxide radical, produced in biological system, which is a precursor of many ROS and is shown to be harmful for various cellular components.

Although the enzyme superoxide dismutase possessed in aerobic and anaerobic organisms can catalyze the breakdown of superoxide radical.

Table 1: DPPH and Superoxide (SO) radical scavenging activity of *S.officinale*

Symphytum officinale extra	IC_{50} (µg/ml) (mean ± SD)	
	DPPH	SO
Ethanolic extract	39.97 ± 2.82	190.76 ± 2.27
Aqueous extract	96.21 ± 10.51	307.42 ± 1.32
Ascorbic acid	26.33 ± 0.54	25.99 ± 0.43

Determination of total phenolics

The extraction yields and the amount of total phenolic contents of *S. officinale* extracts are shown in Table 2. The total phenolic contents of ethanolic and aqueous extracts of *S. officinale* were found as 116.93 mg GAE/g and 99.49 mg GAE/g ($y = 0.001x + 1.234$; $R^2 = 0.988$) respectively (Figure 3). The antioxidant properties of plant extracts have been attributed to their phenolic contents. The amount of total polyphenols in the ethanolic and aqueous extracts of *S. officinale* were determined by Folin- Ciocalteu reagent. This assay has been extensively used to measure the total phenolics in plant materials for many years and based on electron transfer reaction and actually measures a sample's reducing capacity. Therefore, it is accepted as a routine assay for rough estimation of the antioxidant capacity of herbal samples. As seen from the results, ethanolic extract possessed higher phenolic content than the aqueous extract. The total phenolic contents in the S. *officinale* concentrated extracts were proportional to their free-radical scavenging-linked antioxidant activities. Since ethanolic extract of *S. officinale* has been found to show higher DPPH and SO radical scavenging activity than aqueous extract, direct correlation between the high phenolic content of the extract and its radical scavenging activity indicated that radical scavenging activity of *S. officinale* was arising from phenolic content of the ethanolic fraction.

Table 2: Extraction yield and total phenolic content of *S. officinale* using different extraction methods

Sample	Extraction yield (%)	Total phenolic content (mg/g)
Ethanolic extract	5.52	116.93 ± 5.69
Aqueous extract	14.4	99.49 ± 2.53

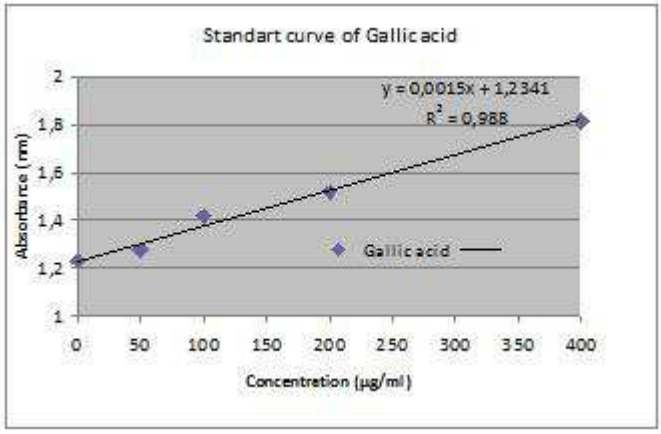

Figure 3: Standart curve of Gallic acid

Cell viability and proliferation response

Cell-based assays can be influenced by cytotoxic effects resulting in false negative results. Therefore, the effects of plant extracts on 3T3 Swiss albino mouse fibroblast cell viability were studied. For the assessment of cell survival MTT assay was used, which measures the dehydrogenase activity of viable cells by the cleavage of the tetrazolium salt to formazan in viable cells. Cell survival was estimated after 72 h treatment. The effects of *S. officinale* extracts on cell viability are presented in Table 3. The vehicle control at 0.02 % concentration did not affect the viability of 3T3 cells. Both of extracts did not exhibit cytotoxic activity against 3T3 Swiss albino mouse fibroblast cells at 5, 25 and 50 µg/ml concentrations. Despite, both extracts exhibited proliferative activity on 3T3 Swiss albino mouse fibroblast cells at applied concentrations. Phytochemical studies of *S. officinale* have identified the presence of allantoin and rosmarinic acid, tannin, mucopolysaccharides and pyrrolizidine alkaloids. It is reported that allantoin is the active ingredient, responsible of cell division initiation and of growth of the conjunctive tissue, bones, cartilages and of acceleration of wound healing [19] since the results obtained from ethanolic and aqueous extracts of *S. officinale* showed an increase in cell proliferation suggest that the component(s) especially allantoin present in extracts of *S. officinale* stimulate the proliferation of 3T3 Swiss albino mouse fibroblasts.

Table 3: The effects of *S. officinale* extracts on cell viability

Concentrations (µg/ml)	Cell Viability (%) (mean ± SD)			
	MTT Assay		Neutral Red Uptake Assay	
	Aqueous extract	Ethanolic extract	Aqueous extract	Ethanolic extract
5	107.53 ± 1.09	112.11 ± 3.57	107.29 ± 1.87	102.92 ± 2.87
25	116.22 ± 2.58	115.42 ± 2.76	118.96 ± 2.29	108.33 ± 3.70
50	115.55 ± 1.29	112.16 ± 3.34	117.29 ± 2.59	110.42 ± 3.39

Neutral red (NR) uptake assay

The NR assay has been found to be more sensitive than the MTT assay [21]. The NR assay is based on the incorporation of the supravital dye into the lysosomes of viable cells that only living cells are able to manage the active uptake of neutral red. The effects of the plant extracts on the proliferation of the 3T3 Swiss albino mouse fibroblast cells were shown in Table 3. The NR assay produced results that were in good agreement with those obtained with the MTT assay. The NR assay confirmed that cell viability in the presence of the aqueous extract was higher than the ethanolic extract.

In conclusion, the results of the present study revealed that ethanolic and aqueous extracts of *S. officinale* possessed potent free radical scavenging ability and proliferative activity. In antioxidant assays the ethanolic extract was found to be more potent radical scavenger when compared to that of aqueous extract. The antioxidant activity observed may be attributed to the presence of flavonoid content in the ethanolic extract. Also, the results of cell proliferation assays indicated that both extracts have proliferative activity on fibroblast cells. The results obtained in the present study are in agreement to a certain degree with the traditional uses of *S. officinale* such as wound healing. The antioxidant effects exerted may be attributed to wound healing effectiveness of *S. officinale*. Therefore, it is suggested that further *in vivo* studies could be performed on the wound healing properties of *S. officinale.*

CONCLUSION

Ethanolic and aqueous extracts of *S. officinale* showed promising antioxidant and free radical scavenging activity and proliferative effect on 3T3 Swiss albino mouse fibroblast cells which may be responsible and favorable for wound healing and this plant may be useful in the management of healing.

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