Assessment of Anti-İnflammatory Activities and Antioxidant Properties of Methanolic Extract of '*Sideritis montana*'

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Abstract
In this study, methanolic extract of *Sideritis montana* was investigated on total phenolic and flavonoid content, antioxidant capacities, scavenging activities of hydrogen peroxide and hydroxyl radicals and DPPH free radical. In addition, the anti-inflammatory effect of the plant extract was studied. The extract of *S.montana* showed that equivalents of phenolic content (157.29 ± 3.71 mg GAE/g DW), flavonoid content (403.99 ± 0.92 mg QUE/g DW) and total antioxidant capacities as (154.47 ± 4.12 mM α-tocopherol acetate / g dry weight).Also, the amount of extract supplying 50% inhibition of DPPH was assayed (58.48 ± 1.30 mg.L^{-1}). *S.montana* was more effective than BHT, which is a synthetic antioxidant in hydrogen peroxide scavenging. But, when the hydroxyl radicals were scavenged the situation was exactly opposite. *S.montana* plant extract was able to inhibit heat-induced protein denaturation, but this activity was lower than that of diclofenac sodium used as a positive control. It has been determined that Sideritis montana methanol extract with high antioxidant activity also has anti-inflammatory activity.

Keywords: *Sideritis montana*; antioxidant; DPPH; anti-inflammatory

1. Introduction

All of aerobic organisms have a defence system against non-free radical hydrogen peroxide (H_2O_2), superoxide anion radicals (O_2^-•) and hydroxyl radicals (•OH), which are called reactive oxygen species (ROS).These organisms also defend themselves against the singlet oxygen ($^1O_2$) with antioxidant defence system. ROS is balanced by antioxidant defence system which plays an important role versus oxidative damage [1].
Medicinal plants contain chemical component which show powerful antioxidant activity and they can be used as a safe source in industrial foods, crops and products. Recently, there has been great interest in biochemistry of plants because they have many protective compounds in the treatment of many present days diseases like cardiovascular, aging and other chronic problems [2-3].
The *Lamiaceae* is a plant family within that some species of them have potential therapeutic activity [4]. The genus *Sideritis*, a member of the *Lamiaceae* family, commonly known as "mountain tea" in Turkey. Traditional usages of these plants as tea and herbal drug form are currently available in Turkey where the genus *Sideritis* contains 46 species, 31 of which are endemic [5].
Inflammation is the natural defense response initiated by tissues against any kind of living or lifeless foreign influence or internal/external damage that the can occur. The triggering of inflammation can occur for many reasons, including infectious (gram-positive and gram-negative bacteria, viruses, fungi, etc.) or non-infectious (trauma, burns, foreign bodies, ischemia, etc.) reasons. Against these stimuli,

tissues produce a series of coherent cellular, humoral, and vascular responses that are well controlled. The main goal in this response is to eliminate the cause leading to inflammation, alleviate its effect and/or assist the tissue repair process [6-7].
Free radicals cause inflammation by creating oxidative stress especially in ROS (reactive oxygen species) cells. Phagocytic cells containing mononuclear cells (macrophages and leukocytes) and polymorphonuclear leukocytes (neutrophils, eosinophils), which play an important role in the host defense mechanism, produce excessive amounts of ROS. In addition to taking part in defense, this overproduced ROS disrupts cellular functions and causes cell and tissue damage leading to the development of inflammatory conditions [8].
Neutralization of ROS by antioxidants may alleviate inflammation. With the help of these antioxidant molecules, cells also protect against infection by inhibiting protein denaturation agents and protecting against membrane lysis [9].
Considering the involvement of ROS in the inflammatory processes, it can be understood that antioxidant compounds also have potential anti-inflammatory effects [10]. Plants have antioxidant, antimicrobial and anti-inflammatory effects because of phenolic compounds and flavonoid contents in their structure [9].
Protein denaturation is one of the well-known causes of inflammation. In some arthritis diseases, the production of autoantigens results from protein denaturation [11].
In recent years, anti-inflammatory properties of extracts of many plants (*Semecarpus anacardium, Osbeckia parvifolia, Myxopyrum smilacifolium Blume,* etc.) have been determined using the protein denaturation method, which is a simple and applicable method[12-14].

2. Materials and methods

2.1. Preparation of the extract

Air-dried *S.montana* plant material (100g) was shred and extracted with methanol using a Soxhlet apparatus for approximately 4 hours [15]. Thereafter, the extract was drained and evaporated to dryness in vacuo at 45 ^{o}C. Extract was lyophilised and stored in the dark at +4 ^{o}C until tested filtered.

2.2. Determination of total phenolic content (TPC)

Using Folin-Ciocalteau reagent and spectrophotometric method, the (TPC) value of plant extract was determined[16]. After, sample solution (500µL) and 11,0 mL distilled water was mixed, undiluted FCR (250µL) was added. At the end of 3 minutes, sodium carbonate (750µL, 2%) was mixed. The sample absorbance was measured 760 nm after 2 h incubation at 25 ^{o}C. TPC was determined comparing with a standart curve of gallic acid at 0.5-100 mg.L^{-1} and the result was denoted as milligram of gallic acid equivalents per gram dry weight.

2.3. Determination of total flavonoid content (TFC)

The total flavonoid content (TFC) was performed by spectrophotometric method [17]. The sample solution in methanol (1,0 mL) was mixed with aluminum chloride %2 (1,0 mL) which is prepared in half the volume of methanol and glacial acetic acid. After solution mixture was incubated at room temperature for 10 minutes, the absorbance was measured at 364 nm. A calibration curve was performed for standart quercetin at 0.5-50 mg.L^{-1} and the total flavonoid content was denoted as milligram of quercetin equivalents per gram dry weight.

2.4. Determination of total antioxidant capacity (TAC)

This metod is based on the formation of green-colored complex of phosphate/Mo(V) in acidic pH [18]. Sample solution (0.2 mL) containing a reducing species methanol was combined in an Eppendorf tube with reagent solution (2,0 mL) which is including 4 mM ammonium molybdate, 28 mM sodium phosphate and 0.6 M sulfuric acid. The mixtures was incubated in in 95 ^{o}C for 90 minutes. The solutions had cooled at room temperature before the absorbance was measured at 695 nm against a blank solution. TAC was standardized against α-tocopherol acetate and denoted as mM α-tocopherol acetate equivalents per gram dry weight.

2.5. DPPH radical scavenging activity

The antioxidant activity of plant extract was described by using 1,1-diphhenyl-2-picryhydrazyl (DPPH) radical with some modifications [19]. The different concentration of sample solution in methanol (0.1 mL) was added to 3.9 mL of methanolic solution of DPPH (0.0025 g / 100 mL methanol). The mixture was incubated 30 minutes at room temperature and the absorbance was measured at 515 nm against

blank solution which was formed methanol. Scavenging activity of the DPPH radicals of each solution was calculated as DPPH inhibition (%) according to equation 2.1.

$$\text{DPPH Inhibition (\%)} = [\ (A_{control} - A_{sample}) / A_{control}\ ] \times 100 \quad [2.1]$$

Where, A_{sample} is the absorbance in the methanolic extracts of plant and $A_{control}$ is the absorbance of the control. Antioxidant activities of plant extract was denoted as IC_{50} which was defined as the amount of extract supplying 50% inhibition of DPPH.

2.6. Hydrogen peroxide scavenging activity

Hydrogen peroxide (H_2O_2) scavenging activity of the methanolic extract was carried out following the procedure of Ruch et al. [20]. Solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Methanolic extract was added to H_2O_2 solution (0.6 mL, 40 mM) also that incubation in room temperature for 10 minutes. The absorbance value of the reaction mixtures were recorded at 230 nm. Buffer solution without H_2O_2 was used as a blank. The percentage of H_2O_2 scavenging rate was calculated with respect to the equation 2.1.

2.7. Hydroxyl radicals radical scavenging activity

The scavenging activity of hydroxyl radicals was studied according to the method of Fenton reactions. [21]. Reaction mixture contained in, 100 µL 3,0 mM deoxyribose, 100 µL 1,0 mM $FeCl_3$, 100 µL 1,0 mM EDTA, 100 µL 1,0 mM ascorbic acid, 100 µL 1,0 mM H_2O_2 and 500 µL 20 mM phosphate buffer and 2,0 mL of extract at various concentrations or positive controls such as BHT. Reaction mixture was incubated at 37 ^{o}C for 60 minutes. Afterwards 1,0 mL 1% thiobarbituric acid (TBA) and 1,0 mL 2.8% trichloroacetic acid (TCA) were added in mixture and was boiled for 30 minutes. The absorbance was measured as a pink malondialdehyde-TBA chromagen at 532 nm. The percentage of hydroxyl radicals scavenging activity was calculated with respect to the equation 2.1.

2.8.Evaluation of in vitro anti-inflammatory activity

Anti-inflammatory activity of the plant extract was determined by protein denaturation method using bovine serum albumin (BSA) [22]. 2850 µL of BSA (0.2%) was added to the solutions at different concentrations of plant extract dissolved in methanol and incubated for 15 minutes at room temperature in the dark. The test tubes were then incubated for more 4 minutes in 72 °C water bath. The tubes were removed from the water bath and cooled down at room temperature for 20 minutes. The reaction mixture containing methanol and BSA(0.2%) solution was used as control. The absorbance value of the reaction mixtures were recorded at 660 nm against a blank solution. The inhibition values of plant extract and diclofenac sodium used as positive control were calculated using equation 2.2.

$$\text{Inhibition (\%)} = 100\text{-}\ [\ (A_{control} - A_{sample}) \times 100 / A_{control}\ ] \quad [2.2]$$

Where, A_{sample} is the absorbance in the methanolic extracts of plant and $A_{control}$ is the absorbance of the control. Inhibition (%) values of plant extract and positive control were plotted and IC_{50} values were determined.

3. Results and discussion

3.1. Total phenolic and flavonoid content

The phenolic and flavonoid compounds show high antioxidant activity [23]. This situation causes to the determination of both total phenolic and total flavonoid content in the same study. Besides, several investigation suggest that plants including high phenolic and flavonoid content can be source of powerful antioxidants. High polar solvent methanol is used for preparation of *Sideritis* extract. The phenolic compounds which are very important components due to their scavenging ability of reactive oxygen species because of their hydroxyl groups, contain one or more hydroxyl groups attached to an aromatic pheniyl ring. [24]. The TPC was determined compairing with a calibration curve of gallic acid at at 0.5-100 $mg.L^{-1}$ (R^2=0.994). The result of phenolic content which is expressed in milligram of gallic acid equivalents / gram dry weight i.e. mg GAE/g DW, was given Table 1. The methanolic extract of *S.montana* had got (157.29 ± 3.71 mg GAE/g DW). The other hand, flavonoids are show that powerful antioxidant activities [25-26]. A calibration curve was performed for standard quercetin (R^2=0.998) and the result of flavonoid content was stated as milligram of quercetin equivalents / gram dry weight i.e. mg QUE/g DW. Referring to Table 1, *S.montana* showed a high flavonoid content as

(403.99 ± 0.92 mg QUE/g DW). These results indicated that flavonoid equivalent quercetin higher than phenolic equivalent gallic acid in *S. montana.*

Table 1. Total phenolic content (TPC), Total flavonoid content (TFC), Total antioxidant capacity (TAC) for *Sideritis montana*

Extract	TPC (mg gallic acid g^{-1} dry weight)	TFC (mg quercetin g^{-1} dry weight)	TAC (mM α-tocopherol acetate g^{-1} dry weight)
Sideritis montana	157.29 ± 3.71	403.99 ± 0.92	154.47 ± 4.12

Values are mean of triplicates ± SD

3.2. Total antioxidant capacity

TAC of the extract from *S.montana* was utilized by phosphomolybdenum method. And the result was presented in Table 1. The basic of the metod is based on the formation of green-colored complex of phosphate/Mo(V) in acidic pH with the reduction of acidic Mo(VI) to Mo(V). TAC was determined compairing with a calibration curve of α-tocopherol acetate at 100- 500 $mg.L^{-1}$ (R^2=0,996) and result was stated as mM α-tocopherol acetate / gram dry weight. Methanolic extract showed that total antioxidant capacity as (154.47 ± 4.12 mM α-tocopherol acetate / g dry weight).

3.3. Scavenging activity of DPPH

The antioxidants react with the DPPH molecule which contains a stable free radical. The free radical scavenging abilities of *S.montana* plant extract was analysed by using DPPH. It was studied at concentration between 2.5 $mg.L^{-1}$ and 160 $mg.L^{-1}$. The observable inhibition percentage was seen as 70.20% at maximum concentration. Antioxidant avtivities of *S.montana* was expressed as IC_{50} in Table 2. The amount of extract supplying 50% inhibition of DPPH was asseyed (58.48 ± 1.30 $mg.L^{-1}$). The synthetic antioxidant buthylated hyroxytoluene (BHT) was used to make compare with *S.montana.* BHT was showed that IC_{50} amount is (61.52 ± 1.87 $mg.L^{-1}$). Lower IC_{50} reflects better protective action against DPPH radical. *S.montana* plant extract more scavenger for DPPH radical than synthetic antioxidant BHT.

Table 2 Scavenging activity ROS and DPPH free radical of the methanolic extract *Sideritis montana*

Sample	Hydrogen peroxide H_2O_2 IC_{50} ($mg.mL^{-1}$)	Hydroxyl radicals (•OH) IC_{50} ($\mu g.mL^{-1}$)	IC_{50} for DPPH scavenging ($mg.L^{-1}$)
Sideritis montana	25.05 ± 1.46	124.75 ± 3.04	58.48 ± 1.30
BHT	36.85 ± 1.52	43.45 ± 1.02	61.52 ± 1.87

Values are mean of triplicates ± SD

3.4. Scavenging activity of hydrogen peroxide

Hydrogen peroxide is produced by some kind of oxidases such as superoxide dismutase and does not exhibit any radical character. The hydrogen peroxide scavenging activities of the methanolic extract of *S.montana* and positive control were shown in Table 2. IC_{50}, a value showing 50% inhibition of radicals, can be used in the assesment of protective roles of the plant extracts against radicals mentioned above sentences[12]. *S.montana* (25.05 ± 1.46 $mg.mL^{-1}$) showed a better scavenging effect

than BHT (36.85 ± 1.52 mg.mL^{-1}) that synthetic antioxidant.

3.5. Scavenging activity of hydroxyl radicals

Hydoxyl radical is the most reactive free radicals in biological cells, which causes lipid oxidation and huge biological damage [27-28]. The plant extract showed maximum inhibition of 58% in the working concentration range. The scavenging activity of hydroxyl radicals of plant extract and positive control presented to Table 2. Maximum scavenging effect was shown by BHT. The IC_{50} of BHT (43.45 ± 1.02 µg.mL^{-1}), was substantially lower than the IC_{50} of *S.montana* (124.75 ± 3.04 µg.mL^{-1}), suggesting that BHT was stronger hydroxyl radical scavenger.

Table 3 In vitro anti-inflammatory effect of *Sideritis montana* extract and positive control

Sample	**Concentration (µg.mL^{-1})**	**% Inhibition**
Sideritis montana	100	43.78 ± 2.45
Diclofenac sodium	100	92.80 ± 4.50

Values are mean of triplicates ± SD

3.6. In vitro anti-inflammatory assay

In the present study, the anti-inflammatory activity of *S.montana* extract was determined by measuring its activity of inhibition of protein denaturation, which is a simple and feasible method. According to the results of our study, *S.montana* extract was found to be effective in inhibiting albumin denaturation at different concentrations. Referring to Table 3, while *S.montana* gave 43.78 ± 2.45% inhibition at 100 µg.mL^{-1} concentration, the maximum inhibition value of standard diclofenac sodium was calculated to be 92.81 ± 4.50% at the same concentration value.

S.montana plant extract was able to inhibit heat-induced protein denaturation, but this activity was lower than that of diclofenac sodium used as a positive control. It has been determined that Sideritis montana methanol extract with high antioxidant activity also has anti-inflammatory activity

It has been determined that the activity of inhibition of albumin denaturation of the plant extract is also increased depending on dosage as is the case with diclofenac sodium, which is a standard anti-inflammatory drug.

4. Conclusions

Medicinal plants contain chemical component which show powerful antioxidant activity and they can be used as a safe source in industrial foods, crops and products. In this study, the parameters investigated in terms of antioxidant chemical contents of *Sideritis Montana* extract were tested *in vitro* conditions. In addition, the anti-inflammatory effect of the plant extract was studied.

In the study by Kar et al., it has been shown that the fact that plant extracts are rich in flavonoid, tannin, saponin, terpenoid, and phenolic compound content — in other words, their antioxidant capacity is high — may be responsible for *in vitro* anti-inflammatory activity[29].

Phenolic compounds, flavonoids, tannins and saponins have the ability to bind cations and other biomolecules and can protect proteins against denaturation. It has also been shown in the study that *S.montana*, which has high phenolic content, can inhibit protein denaturation.

Further work is required, including isolation and purification of chemical compounds, so that the plants are home to invaluable sources having naturally anti-inflammatory and antioxidant chemicals.

The result of this study indicate that methanolic extracts of *S.montana* can be used as a medicinal purposes for antioxidants and anti-inflammatory qualities. By reason of in order to use these valuable *S.montana* species in pharmaceutical products and food, their cultivation and conservation are of great importance.

5. References

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