

Research Article

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Protective Effect of Various Extracts of *Allium hirtifolium* and *Satureja khuzestanica* Plants on AAPH-Induced Oxidative Hemolysis

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Abstract

Background: *Allium hirtifolium* and *Satureja khuzestanica* are the Iranian endemic plants and proper candidates for antioxidant studies. This study investigated the antioxidant, anti-hemolytic properties, and phytochemical composition in different extracts of *A. hirtifolium* and *S. khuzestanica*.

Methods: Hydroalcoholic, methanolic, and n-hexane extracts of *A. hirtifolium* and *S. khuzestanica* plants were prepared using soaking and ultrasonic methods. Different plant extracts were evaluated for the presence of secondary metabolites using standard methods based on colorimetric analysis. The antioxidant properties of the compounds were measured by the ferric reducing ability of power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods. Finally, the anti-hemolytic effects of the extracts were investigated using the 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) methods.

Results: Phytochemical analysis indicated the presence of flavonoids, phenolic acids, glycosides, steroids, and terpenoids in *S. khuzestanica*, as well as amino acid compounds, flavonoids, saponins, glycosides, and phenols in *A. hirtifolium*. FRAP assay showed that the methanolic extract of *S. khuzestanica* and the n-hexane extract of *A. hirtifolium* have the highest total antioxidant activity. The results of the DPPH assay also demonstrated that the minimum IC₅₀ was related to the hydroalcoholic extract of *S. khuzestanica* (18.58 µg/mL) and the n-hexane extract of *A. hirtifolium* (87.95 µg/mL). In general, the extracts of both plants could reduce the percentage of AAPH-induced hemolysis while being significant only in some concentrations ($P < 0.001$).

Conclusion: Overall, *A. hirtifolium* and *S. khuzestanica* can be used as a herbal supplement in the human diet due to their anti-hemolytic effects.

Keywords: *Satureja khuzestanica*, *Allium hirtifolium*, Antioxidant, Anti-hemolytic

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Introduction

Oxidative stress is the situation in which there is an impairment in the balance between free radicals and antioxidant defenses. Following oxidative stress, reactive oxygen species (ROS) and reactive nitrogen species can damage cellular biomolecules such as protein, lipids, and DNA and lead to disease progression (1,2). In contrast, endogenous and exogenous antioxidant defenses have been developed for the protection of biological systems from the distractive effects of free radicals. Endogenous antioxidants are primary antioxidant enzymes (3,4). The usage of exogenous antioxidants, which are often derived from plant sources, can be an effective strategy for increasing the level of intracellular antioxidants. According to the World Health Organization report, the use of medicinal herbs or herbal supplements for sustaining or upgrading health has been developed in recent years (5).

These herbs can prevent oxidative stress-related diseases through their antioxidative ability and radical scavenging activity (6).

Satureja khuzestanica Jamzad, which is commonly known as Jamzad, is an aromatic herb of the Lamiaceae family that is growing in the south of Lorestan Province in southwestern Iran (7). This herbal medicine is applied as a dental analgesic and an oral antiseptic agent in traditional medicine. In addition, various studies have investigated its different pharmacological effects such as antibacterial (8), antifungal (9), antioxidant (10), anti-diabetic (11), and anti-inflammatory properties (12), which are often related to carvacrol components (13-15).

Allium hirtifolium Boiss is an Iranian endemic plant that belongs to the Alliaceae family and contains saponins, sapogenins, flavonoids (e.g., quercetin and kaempferol), and sulphur compounds (e.g., diallyl thiosulfinate or



allicin which is responsible for the remarkable potentials of this plant). Although the main traditional usage of *A. hirtifolium* is spicing different foods, antibacterial, antifungal, antiviral (16), hepatoprotective (17), anticancer properties (18), some studies have reported wound healing activity (19) and immunomodulatory effect (20-22).

Due to phenolic and flavonoid compounds of *S. khuzestanica* and *A. hirtifolium*, it seems that they may be the proper supplement for oxidative damages. This study aimed to investigate the antioxidant and anti-hemolytic properties of different extracts of *S. khuzestanica* and *A. hirtifolium*.

Materials and Methods

Ethical Considerations

The animal experiments were approved by the Ethics Committee of Hamadan University of Medical Science (HUMS), Hamadan, Iran (with the ethical code of ID: IR.UMSHA.REC.1397.488) in accordance with the guideline of the Research Ethics Committee of the Health and Medical Education, Iran (2019) and the Helsinki Protocol (Helsinki, Finland, 1975).

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), and 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Plant Materials: Collection and Extraction Procedure

The *S. khuzestanica* and *A. hirtifolium* plants were collected from Lorestan and Hamadan provinces, Iran, respectively. These plants were identified by the herbarium section of HUMS, Hamadan, Iran. Hydroalcoholic, methanolic, and n-hexane extracts were prepared by soaking, ultrasonic, and heating methods. Briefly, 25 g of the shade-dried plants were crushed, extracted with methanol-water (50/50 v/v %), methanol, and n-hexane, and then separately shaken (72 hours at 37°C) and filtered through a Whatman No.1 filter paper. Finally, the filtered solutions were concentrated and kept at 4°C.

Phytochemical Screening

The phytochemical analyses were conducted on different extracts using the standard techniques to identify secondary metabolites such as alkaloids, flavonoids, saponins, tannins, steroids, terpenoids, proteins, amino acids, and glycosides.

DPPH Radical Scavenging Assay

Overall, 0.25 mL of the extract was mixed with 0.75 mL of the methanol solution of DPPH (0.1 mM) and incubated at room temperature for 30 minutes in darkness. Then, its optimum absorbance was recorded at 517 nm using a microplate reader (Synergy HTX, BioTek, USA). The

percentage of free radical scavenging capacity (RSC) was calculated as follows:

$$REC(\%) = \frac{(\text{absorbance of blank} - \text{absorbance of sample})}{\text{absorbance of blank}} \times 100$$

It should be noted that the IC_{50} index was computed against different concentrations of ascorbic acid (vitamin C) as the standard.

Measurement of the Total Antioxidant Capacity

Ferric reducing antioxidant power (FRAP) is based on the ability of the extracts to reduce Fe (III) to Fe (II) in the presence of TPTZ. The complex from Fe (II) and TPTZ reactions has a maximum absorbance at 593 nm. The aqueous solution of known Fe II concentration was used for calibration in the range of 7-125 µg/mL. Different concentrations of the extract were added to 0.25 mL of the FRAP solution (1 volume of 20 mM FeCl₃, 10 volumes of 300 mM acetate buffer, and 1 volume of 10 mM TPTZ in 40 mM HCL) and allowed to react for 40 minutes at room temperature, and eventually, the absorbance was measured at 593 nm. The results were reported as nmol Fe²⁺/mg of the sample using the ferric chloride standard curve: $y = 1.6723x + 0.1261$, $r^2 = 0.998$.

Measurement of Anti-hemolytic Activity

The blood samples were obtained from male Wistar rats and centrifuged at 3000 rpm for 10 minutes. Subsequently, 10% v/v erythrocyte suspension was prepared by red blood cell (RBCs) pellets in phosphate buffer (pH = 7.4). The 0.1% Triton X-100 solution was used for complete hemolysis, and phosphate buffer and vitamin C were considered as negative and positive controls, respectively. For the assessment of the hemolysis ability of *S. khuzestanica* and *A. hirtifolium*, RBC suspension was incubated with different concentrations of the extracts and AAPH (50 mM) at 37°C for 6 hours. The optimum absorbance was measured at 540 nm. Ultimately, the hemolysis percentage was calculated as follows:

$$\text{Hemolysis}(\%) = \frac{\text{absorbance of samples combined with AAPH}}{\text{absorbance of 0.1\% Triton X-100 solution}} \times 100$$

Statistical Analysis

Data were presented as the mean ± SD and analyzed using SPSS software (version 16) by the analysis of variance (ANOVA) and Tukey's post hoc test. The value of $P < 0.05$ was considered to be statistically significant.

Results

Phytochemical Screening

Based on the results (Table 1), amino acids, flavonoids, saponins, glycosides, and phenols were found in the *A. hirtifolium* extract. Further, flavonoids, phenolic acid, glycosides, steroids, and terpenoids were observed in the *S. khuzestanica* extract. These results showed that the

Table 1. Phytochemical Screening of the Secondary Metabolites of *Satureja khuzestanica* and *Allium hirtifolium* Extracts

Phytochemical Constituents	<i>Satureja khuzestanica</i>			<i>Allium hirtifolium</i>		
	HA	Met	Hex	HA	Met	Hex
Flavonoids	+++	++	-	++	++	-
Phenols	++	+	-	++	++	-
Alkaloids	-	-	-	-	-	-
Proteins	-	-	-	-	-	-
Terpenoids	++	++	-	+	+	-
Steroids	++	++	-	+	+	-
Saponin	+	+	-	+++	+++	-
Amino acids	-	-	-	+	+	-
Tannins	-	-	-	+	+	-
Glycosides	+	+++	-	+	++	-

Note. Strongly positive: +++; Moderately positive: ++; Slightly positive (+) and negative (-); HE: Hydroalcoholic; ME: Methanolic and nHE: n-hexane extracts.

hydroalcoholic extract of *S. khuzestanica* and *A. hirtifolium* has more phenolic, flavonoid, and terpenoid compounds compared to other extracts.

Measurement of Total Antioxidant Capacity

As shown in Figure 1, the n-hexane extract of *A. hirtifolium* has the most antioxidant power while its hydroalcoholic extract has the lowest value compared to other extracts. Based on these results, the antioxidant potential of the n-hexane extract of *A. hirtifolium* is 32.6% and 61.5% higher than that of methanolic and hydroalcoholic extracts respectively. In addition, the methanolic and n-hexane extracts of *S. khuzestanica* demonstrated the highest and lowest antioxidant activity, respectively.

DPPH Radical Scavenging Assay

The IC_{50} index for methanolic, hexane, and hydroalcoholic extracts of *S. khuzestanica* was determined as 22.674, 89.70, and 19.537, respectively. Furthermore, this index was

calculated 279.95, 100.7857, and 114.35 for the methanolic, hexane, and hydroalcoholic extracts of *A. hirtifolium*, respectively. The reported IC_{50} levels for hydroalcoholic and methanolic extracts were 27.53% and 15.86% less than vitamin C, respectively. In contrast, the amount of the IC_{50} for the n-hexane extract of *S. khuzestanica* was 330.63% higher than vitamin C (Table 2).

Measurement of Anti-hemolytic Activity

AAPH (Figure 2) could increase RBC hemolysis compared to the negative control group (PBS, $P < 0.01$). On the other hand, vitamin C (100 $\mu\text{g/mL}$) could significantly reduce the AAPH hemolytic effects (75.78%). The minimum and maximum anti-hemolytic effects of hydroalcoholic, methanolic, and n-hexane of the *S. Khuzestanica* extract were estimated to be 54.4-69.8, 48.4-74.7, and 29.2-58.9%, respectively. Regarding the minimum applied concentration for each extract, it seems that the anti-hemolytic activity of the hydroalcoholic extract is higher

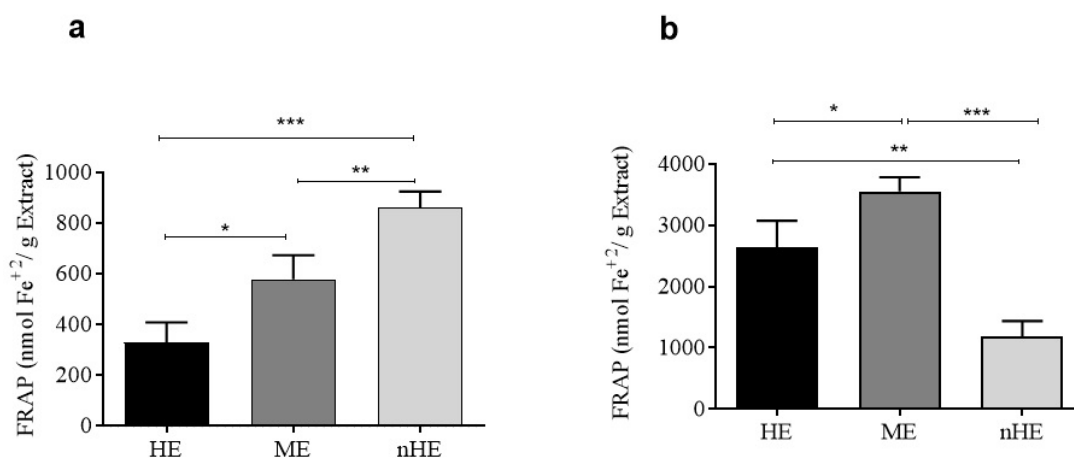


Figure 1. Total Antioxidant Capacity of *Satureja khuzestanica* (a) and *Allium hirtifolium* Extract (b). Note. Data are the average of triplicate experiments and presented as the mean $\pm$ SD. Significant differences are defined as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. SD: Standard deviation; HE: Hydroalcoholic, ME: Methanolic and nHE: n-hexane extracts, FRAP: Ferric reducing antioxidant power.

Table 2. Free Radical Scavenging Activity of *Allium hirtifolium* and *Satureja khuzestanica* and Ascorbic Acid (Vitamin C)

Herbal Medicine	Type of the Extract	Concentration (µg/mL)	IC ₅₀
<i>Allium hirtifolium</i>	Hydroalcoholic extract	37.5-82.5	270.33-299.07
	Methanolic extract	37.5-82.5	109.25-117.56
	n-hexane extract	37.5-82.5	93.47-100.78
<i>Satureja khuzestanica</i>	Hydroalcoholic extract	7.5-37.5	18.03-22.02
	Methanolic extract	7.5-37.5	21.56-24.08
	n-hexane extract	37.5-82.5	85.03-96.56
Ascorbic acid	–	10-100	18.13-33.52

Note. IC₅₀: Half maximal inhibitory concentration.

than that of the other two extracts. On the other hand, the anti-hemolytic activity of the *A. hirtifolium* hydroalcoholic extract in the concentration range of 62.5-1000 µg/mL was estimated to be equivalent to 163-216.2% of vitamin C.

Discussion

In biological systems, the production of free radicals and ROS is unavoidable, and the body can partially neutralize their harmful effects through intrinsic antioxidant defense

mechanisms. Antioxidants protect tissues against the destructive effects of free radicals and can inhibit or delay the oxidation process. These compounds are divided into synthetic and natural categories (23). Among these, natural antioxidants are often secondary metabolites such as flavonoid and phenolic acid compounds, which are responsible for plant sustenance under adverse environments (24).

In this study, the phytochemical screening of secondary

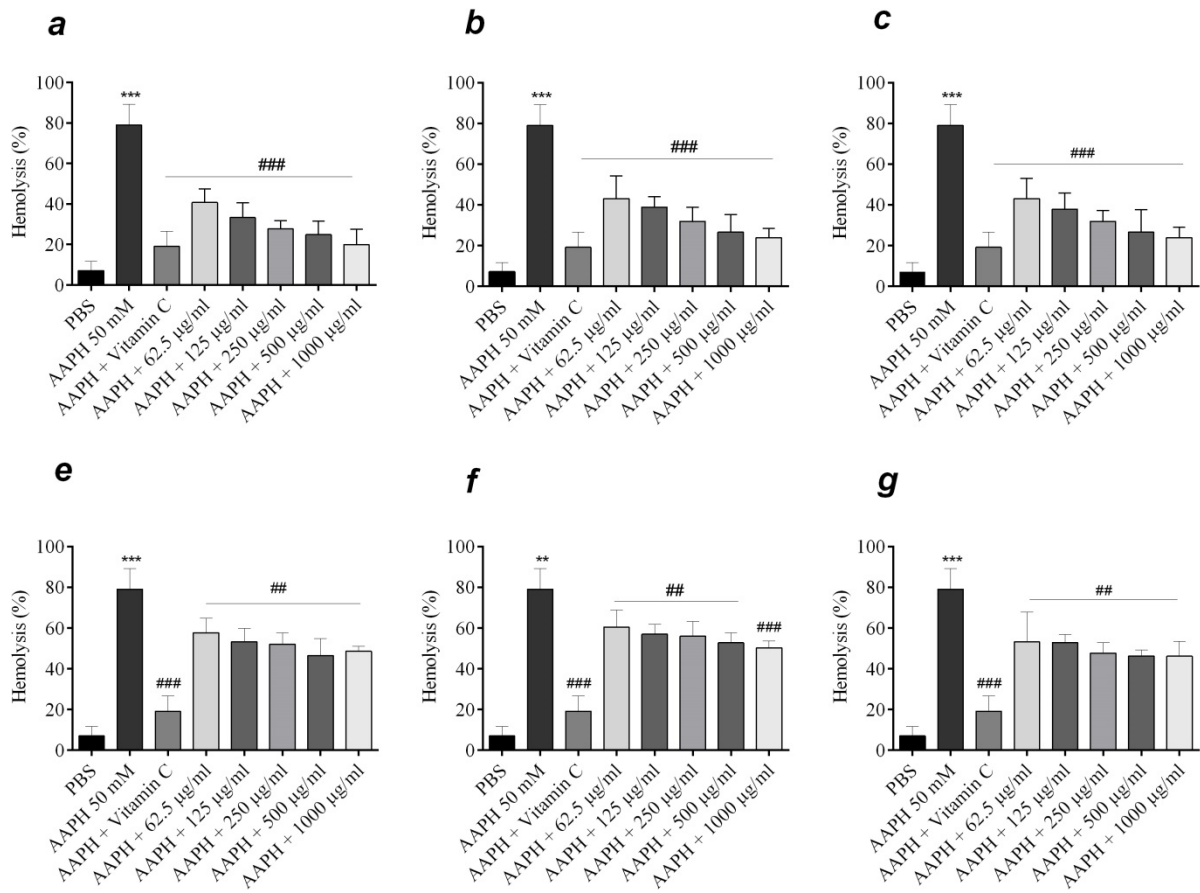


Figure 2. The Effect of Various Extracts of *Allium hirtifolium* and *Satureja khuzestanica* Plants on AAPH-induced Oxidative Hemolysis: (a) methanolic, (b) hydroalcoholic, (c) n-hexane of *S. khuzestanica* extracts, (d) methanolic, (e) hydroalcoholic, and (f) n-hexane of *A. hirtifolium* Extracts. Note. Data are the average of triplicate experiments and presented as the mean ± SD. Significant differences as *** $P < 0.001$ compared to PBS and ** $P < 0.01$, *** $P < 0.001$ in comparison to the AAPH group. AAPH: 2,2'-Azobis(2-amidinopropane) dihydrochloride, PBS: Phosphate-buffered saline; SD: Standard deviation.

metabolites proved the presence of flavonoids, phenolic acids, glycosides, steroids, and terpenoids in various extracts of *S. khuzestanica* (more properly in the methanolic extract), which is in line with previous studies (9,25). Previously, Golparvar et al (9) reported that the major components of the *S. khuzestanica* essence were carvacrol (69.62%), γ -terpinene (9.25%), and p-cymene (8.36%). Likewise, Mahboubi et al indicated that the *S. khuzestanica* essential oil has more phenolic content while the ethanol extract had higher flavonoid contents (25). Additionally, the presence of amino acids, flavonoids, saponins, glycosides, and phenols was confirmed in different extracts of *A. hirtifolium*, especially in the hydroalcoholic extract (26). However, it was shown that the collected *A. hirtifolium* species from different regions of Iran have different total phenolic contents, and the highest amount belongs to the Sahneh region (8.4 mg GAE/g sample). It seems that the phytochemical properties of these plants may be related to the geographical location (27).

In this study, two different biochemical assays, including FRAP and DPPH experiments, were used to determine the antioxidant properties of the extracts. Our DPPH findings revealed that the hydroalcoholic extract of the *S. khuzestanica* and n-hexane of *A. hirtifolium* has the lowest IC_{50} (18.58 and 93.47 μ g/mL, respectively), indicating their remarkable antioxidant properties. In addition, our result indicated that the lowest anti-radical property is related to the hydroalcoholic extract of *A. hirtifolium* and the n-hexane extract of *S. khuzestanica* (IC_{50} ; 270.33 and 85.03 μ g/mL, respectively). Additionally, the n-hexane extract of *A. hirtifolium* and the hydroalcoholic extract of *S. khuzestanica* had the highest total antioxidant capacity, which may be related to their phenolic and polyphenolic constituents. In this regard, Mahboubi et al (28) showed that the methanolic extract of *S. khuzestanica* has the highest antioxidant activity with the lowest IC_{50} (40 μ g/mL) for its aqueous extract (80 μ g/mL) and the essential oil (95 μ g/mL). This discrepancy could be related to the accumulation of secondary metabolites such as phenol and flavonoid in this plant. Phenolic compounds are good electron donors via their hydroxyl groups and exhibit free radical inhibition (29). Therefore, due to the existence of phenol and flavonoids in the hydroalcoholic and methanolic extracts of *S. khuzestanica* and the hydroalcoholic extracts of *A. hirtifolium*, their antioxidant properties are extremely higher compared to the n-hexane extract. Previously, the antioxidant activity of the methanolic extract of the Sahneh (Iran) population of *A. hirtifolium* was reported to be higher in comparison with other similar species (IC_{50} : 60.9 ± 4.7 mg/mL). However, *A. hirtifolium* belonging to Nahavand (Iran) had the lowest level of antioxidant activity (IC_{50} 267.2 ± 21 mg/mL), which was determined by DPPH and FRAP. In this respect, Pirbalouti et al (30) reported that the ethanolic extract of *A. hirtifolium* from Dasht-e Laleh areas has the highest phenolic compounds

and thus the highest antioxidant activity compared to the population of Khaki and Samsami (IC_{50} : 1.90 ± 0.31 mg/mL). The different antioxidant activity of *S. khuzestanica* and *A. hirtifolium* may be related to different kinds of extraction and their methods, and/or their geographical locations.

AAPH, as a water-soluble radical, decreases the level of glutathione. Glutathione depletion can induce the oxidation of cellular membranes and subsequently, the hemolysis of the cells. The pretreatment of RBCs with antioxidant compounds can reduce these destructive effects. Therefore, this *in-vitro* model can be considered as a suitable laboratory method for evaluating the ability of new protective compounds through free radical scavenging. In this study, *S. khuzestanica* and *A. hirtifolium* extracts can reduce hemolysis caused by AAPH. The protective effect of these plants may be due to phenolic and flavonoid components such as kaempferol and carvacrol which is moderate AAPH-induced intracellular glutathione depletion (31-33). It was previously shown that flavonoids have free radical scavenger and antioxidant properties (34). Flavonoids as polyphenolic compounds have anti-hemolytic properties and prevent the lysis of RBCs in a dose-dependent manner (35). For instance, Cacciatore et al concluded that kaempferol, as a flavonoid of allium species, can inhibit RBC hemolysis (26%) at the dose of 10 μ g/mL (36).

Conclusion

Generally, the *S. khuzestanica* and *A. hirtifolium* extract could prevent AAPH-induced oxidative hemolysis. This feature could be due to the phenol and flavonoid content of these plants and their inhibitory effects on free radical generation. Therefore, our findings can offer a new application for *S. khuzestanica* and *A. hirtifolium* plants as herbal supplements in the human diet.

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Authors' Contributions

NMM helped in performing the experimental parts and drafting the manuscript as his Pharm. D thesis. ANA conceived and supervised the research and edited the manuscript. DD was the cosupervisor of the thesis.

Conflict of Interests

The authors declare that there is no conflict of interests and are alone responsible for the accuracy and the integrity of the paper content.

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