The comparative study of the antioxidant activity of aqueous and hydroalcoholic extracts of an Iranian endemic species *Rhabdosciadium aucheri* Boiss.

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Abstract

Rhabdosciadium aucheri (Apiaceae) is a perennial herbaceous species, which is endemic to W, S and C Iran. In the present study aqueous, ethanolic and methanolic extracts were obtained from aerial parts of the specimens collected from Hamadan province, using both Maceration and Soxhlet methods. Due to the direct relationship between total phenolic and flavonoid contents and the plant antioxidant activity, the aim of the present investigation was the comparative study of total amount of each parameter extracted by aqueous and hydroalcoholic solvents. The results demonstrated that, the highest amount of phenolic and flavonoid compounds extracted by ethanolic solvents using Maceration technique were 37.28 ± 1.03 mg GAE/gr GW and 22.68 ± 0.63 mg QE/gr DW respectively. These results were significant at $P< 0.05$. Moreover, 72.17 ± 17.10 % was the highest free radical inhibition percentage for methanolic extracts. However, the differences were not considered as significant. The results also showed that using hydroalcoholic solvents are more powerful compared to aqueous solvents in the extraction of phenolic and flavonoid contents and consequently their antioxidant activity.

Keywords: antioxidants, ethanol extract, methanol extract, total phenol, total flavonoid

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Introduction

Apiaceae (Umbelliferae), with approximately 450 genera and over 3700 species worldwide, is one of the most important families of the flowering plants (Davis et al., 1982; Downie et al., 2000; Duran et al., 2010). Iran with over 100 genera and almost 400 species has a wide diversity of Apiaceae members (Amiri and Joharchi, 2016). Some members of the family are aromatic plants, that are important for food, flavoring, and medicinal purposes (Saleem, 2010; Christova-Bagdassarian et al., 2013a; Christova-Bagdassarian et al., 2013b; Christova-Bagdassarian et al., 2014; Amiri and Joharchi, 2016). This family is one of the most important sources of phenolic compounds that are directly related to the plant antioxidant activity (Martins et al., 2016; Mazandarani et al., 2012). Antioxidants are known as natural products of plant sources which have the capability to decline oxidative stress (Ghribia et al., 2014; Lefahal et al., 2018). Phenolic components from natural sources could

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be taken into consideration as radical scavengers. Therefore, as mentioned, this family with high levels of phenolic contents could be used as a source of natural antioxidants (Senthilkumar et al., 2013; Martins et al., 2016; Lefahal et al., 2018). The genus *Rhabdosciadium* Boiss. (Apiaceae) comprises eight herbaceous species in the world (Akalın and Akpulat, 2012; Lyskov et al., 2017; Firat and Güzel, 2019), three of which being endemic to Iran (Davis et al., 1982; Davis et al., 1988). These species include *R. strausii* Hausskn. ex Bornm., *R. petiolare* Boiss. Hausskn. ex Boiss., and *R. aucheri* Boiss. The other five species i.e., *R. hizanense*, *R. oligocarpum* (Post ex Boiss.), *R. microcalycinum*, and *R. urusakii* grow in Turkey (Duran et al., 2010; Akalın and Akpulat, 2012; Firat and Güzel, 2019; Lyskov et al., 2017). The genus could be distinguished from other related genera by their leafless rush-like branches and single-fruited umbellules (Akalın and Akpulat, 2012). *Rhabdosciadium aucheri* is an endemic species distributed in west and south of Iran (Davis et al., 1982). It differs from two other Iranian species by its flattened leaves with ovate-oblong segments, mericarps 10–15 mm and short-conical stilopodium embedded in corky pericarp (Davis et al., 1982; Akalın and Akpulat, 2012). Recently, essential oil composition of this species has been investigated by several authors (Eghtesadi et al., 2006; Kalvandi et al., 2013). Due to the lack of investigation on the antioxidant activity of aqueous and hydroalcoholic extracts of *R. aucheri*, the present study aimed to compare different solvents to find out the most reliable solvent suitable for extraction of total phenolic and flavonoid compounds, which are definitely related to the plant antioxidant activity.

Materials and Methods

Plant material and aqueous and hydroalcoholic extracts

Dried aerial parts of the species *Rhabdosciadium aucheri*, collected from Hamadan Province during July-August 2018, were used in this study (Table 1).

Aqueous, ethanolic, and methanolic extraction were performed through maceration method. For preparation process, first, 10 g of specimen's stem and leaf powder were added to 200 ml water, 200 ml oethanol (80:20 v/v), and 200 ml methanol (80:20 v/v). The mixture was shaken at 40 ℃ and 100 rpm, during 24 hrs. Then, the mixture was filtered using Whatman filter paper and the plant residue was removed.

Soxhlet technique was also used for methanolic extraction. For this purpose, 10 g of plant powder were added to 250 ml methanol (80:20, v/v). The process of extraction was carried out for around three to four hrs. Rotary evaporator was used to remove methanol solvent from mixture at 45-50 ℃, 40 rpm, at a pressure of 170 bar (Heidolph Laborata Rotary Evaporator, WB eco, Germany) and then lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA). The lyophilized extract was re-dissolved in water.

Evaluation of Total Phenolic Content (TPC)

Total phenolic contents were distinguished using Folin-Ciocalteau assay. For this purpose, 0.5 ml of Folin-Ciocalteu's phenol reagent was added to 0.5 ml of each aqueous, ethanol, and methanol extract and shaken. Thereafter, 0.5 ml of 10% sodium carbonate solution was added to the mixture. After incubation at room temperature, the absorbance against prepared reagent blank was determined at 765 nm with a Cary 60 UV-VIS spectrophotometer (Thermo, Waltham, MA, USA). Gallic acid was used as a standard for calibration curve and data of total phenolic contents were expressed as Gallic acid equivalents (µg GAE/mg).

Evaluation of Total Flavonoid Content (TFC)

Total flavonoid contents were evaluated using aluminum (III) chloride colorimetric assay. Firstly, 0.4 ml AlCl3 (25 gr/L) was added to 0.03 ml, 0.06 ml, and 0.09 ml of aqueous, ethanol, and

Table 1
Geographical properties of the collection sites of *R. aucheri* samples

Location	Longitude	Latitude	Altitude (m)
Hamadan-Asadabad	34° 48' 59"	48° 11' 22"	2485
Hamadan-Alvand Mountain	34° 47' 42"	48° 21' 00"	1854

methanol extract, respectively. Then, 0.5 ml of sodium acetate (100 gr/L) was added to the solution. After that, 4 ml of dd water was added. After incubation for 15 minutes at room temperature, the reaction procedure was completed. The absorbance was measured against prepared reagent blank at 430 nm with a Cary 60 UV-VIS spectrophotometer (Thermo, Waltham, MA, USA). Quercetin was used as a standard for calibration curve and the data of total flavonoid contents were expressed as Quercetin equivalents (µg QE/mg).

Evaluation of the antioxidant activity

Antioxidant activity of the specimen was carried out using 1,1-diphenly-2-picrylhydrazyl (DPPH) method. This method is used as an assay radical scavenging activity (Yassa et al., 2008). For this reason, different volumes of specimens including 0.03 ml, 0.05 ml, and 0.07 ml were added methanol to obtain 0.1 ml solution. To each concentration, 0.3 ml DPPH solution (1 mM) was added. All samples were added methanol to obtain 1.5 ml solution. The absorbance was measured after incubation at room temperature in the dark for 20 minutes, at 517 nm and the radical scavenging activity was calculated. The inhibition percentage was measured by the following equation:

$$\text{Inhibition } \% = \frac{\text{blank absorption-sample absorption}}{\text{blank absorption}} \times 100.$$

All tests were carried out in triple replicates and the concentration of the extract essential for scavenging 50% of DPPH free radical (i.e., IC50) was calculated.

Statistical Analysis

All the experiments for determination of total phenolics, total flavonoids, and antioxidant properties using DPPH were conducted in triplicates. The values were expressed as the mean ± standard deviation (SD). The statistical analysis of the results was performed by SPSS, ver. 25 (2017) and Excel software. Analysis of variance and significance of difference among means were tested by nonparametric Kruskal-Wallis (one-way ANOVA nonparametric test). The differences at $p < 0.05$ were considered to be significant.

Table 2
The statistical results of Kruskal-Wallis test

	TPC	TFC	DPPH
Kruskal-Wallis H	9.359	10.385	1.359
df	3	3	3
Asymp. Sig	0.025	0.016	0.715

symp. Sig: the p value based on chi-square approximation, TPC: Total Phenolic Content, TFC: Total Flavonoid Content

Table 3
The mean ranks of the characters in different methods of extraction using Kruskal-Wallis test

Extraction methods	Mean Rank TPC	Mean Rank TFC	Mean Rank of Inhibition%
Aqueous ext.	3.33	2	6.33
Ethanolic ext.	11	11	7
Methanolic ext.	8	8	8
Methanolic ext. Soxhlet	3.67	5	4.67

TPC: Total Phenolic Content, TFC: Total Flavonoid Content

Results

In the present study, Kolmogorov-Smirnov statistical test was used firstly and data normality assumption was rejected. Therefore, Kruskal-Wallis nonparametric test (one-way ANOVA on ranks) was used to study the statistical significance of the mean differences. Unlike ANOVA, this method does not assume the normal distribution of residuals (Table 2). According to the Table 2, both total flavonoid content (0.016) and total phenolic content (0.025) presented statistically significant differences. Since, the differences at $p \leq 0.05$ are regarded as significant, only DPPH inhibition percentage did not show significant difference (0.715). In Kruskal-Wallis test the comparison between means is performed via the comparison of their ranks. Therefore, the mean rank was evaluated precisely for each character using four types of extraction (Table 3).

In this study, antioxidant activity of the endemic species, *Rhabdosciadium aucheri,* was investigated for the first time. Three different polar solvents (aqueous, ethanol, and methanol) were used for the extraction using maceration

Table 4
The comparison of total phenols, total flavonoids, inhibition percentage, and IC50 value among different types of extraction

Extraction methods	Total phenols mg GAE/g DW	Total flavonoids mg QE/g DW	Inhibition %	IC50 mg
Aqueous ext.	25.57 ± 1.23	9.58 ± 0.36	61.90 ± 12.24	0.47
Ethanolic ext.	37.28 ± 1.03	22.68 ± 0.63	66.79 ± 22.61	0.26
Methanolic ext.	32.83 ± 0.65	20.00 ± 0.26	72.17 ± 17.10	0.19
Methanolic ext. Soxhlet	26.26 ± 0.94	17.60 ± 0.42	53.93 ± 25.18	0.25

Each value is shown as mean ± standard deviation (SD) (n=3); means are significantly different at p≤ 0.05.

method to find the best result. Moreover, two different methods (maceration and Soxhlet) were compared with each other using the methanolic solvent. The investigation of total percentage of products obtained from using methanolic extract, Soxhlet, and maceration methods, as well as ethanolic and aqueous extracts demonstrated that the highest percentage of the condensed extract s belonged to Soxhlet method of extraction (1.55%). However, there was no significant difference between various solvents using maceration method (methanolic extract (9.6%), ethanolic extract (9%), and aqueous extract (9.5%)). In fact, these percentages were not statistically significant. Moreover, the amount of each character (total phenolic and flavonoid contents, inhibition percentage, and IC50) was evaluated using different types of extraction and the findings are presented in Table 4.

Total phenolic contents

The comparative results of total phenolic contents are shown in Fig. I and Table 4. The ethanolic extract were found to contain the highest amount of phenols in contrast with other solvents. The total phenolic contents for ethanolic extract were 37.28 ± 1.03 mg GAE/g DW while the lowest amount of total phenol was allocated to aqueous extract, which was 25.57 ± 1.23 mg GAE/g DW. The difference between means was significant at p≤0.05.

Total flavonoid contents

The comparative results of total flavonoid contents are shown in Fig. II and Table 4. The results revealed that the highest amount of total flavonoid was dedicated to ethanolic extracts (22.68 ± 0.63 mg QE/gr DW) while the lowest total flavonoid contents were obtained from aqueous extracts (9.58 ± 0.36 mg QE/gr DW). The difference between means of extracted flavonoid contents was significant at p≤0.05.

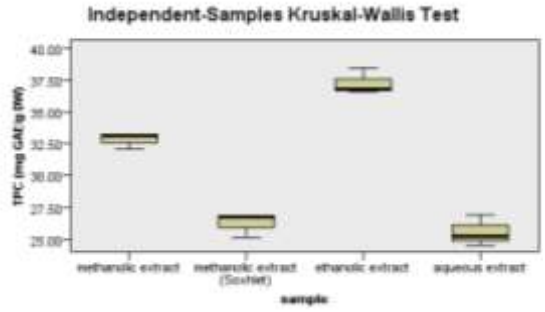

Fig. I. Comparison of the total phenolic contents (TPC) using different methods of extraction

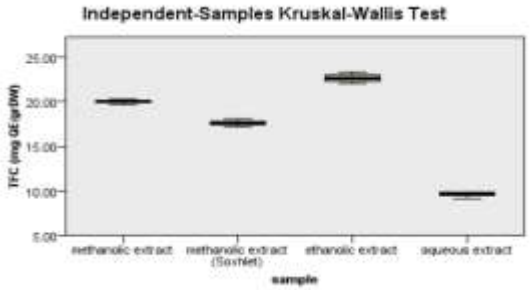

Fig. II. Comparison of total flavonoid contents (TFC) using different types of extraction

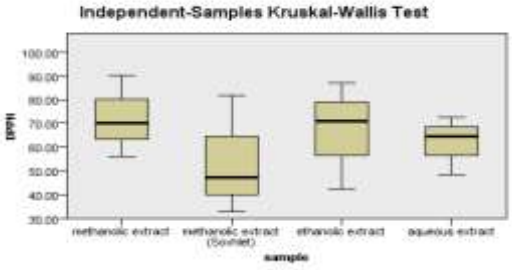

Fig. III. Comparison of inhibition percentages among different extraction types using DPPH assay

Antioxidant activity using DPPH assay

DPPH free radical scavenging activity of aqueous and hydroalcoholic extracts is shown in Fig. III and

Table 5
Pairwise comparison of extraction methods for both total phenolic and total flavonoid contents (p<0.05 shows statistically significant).

Pairwise Comparisons Extraction Methods	Sig. TPC	Sig. TFC
Aqueous extract - Ethanolic extract	0.009	0.002
Aqueous extract - Methanolic extract	0.113	0.042
Aqueous extract - Methanolic extract. Soxhlet	0.910	0.308
Methanolic extract - Methanolic extract. Soxhlet	0.141	0.308
Ethanolic extxtract - Methanolic extract. Soxhlet	0.013	0.042
Ethanolic extract - Methanolic extract	0.308	0.308

Table 5. The results showed that the highest amount of inhibition percentage was for methanolic extracts using maceration method (72.17 ± 17.10 %). On the other hand, the lowest percentage was related to the methanol extracted by Soxhlet method (53.93 ± 25.18%). Therefore, maceration method was far more effective compared with Soxhlet technique, regarding its highest DPPH scavenging effect. However, the differences in DPPH inhibition percentage was not statistically significant based on the results of the statistical analyses (Table 2). Therefore, these results could not be considered as reliable.

Moreover, the IC50 value of the extracts was determined. A low IC50 value shows high antioxidant activity. The IC50 value of methanolic extracts, using maceration method, was the lowest among other extracts, which confirms its highest antioxidant activity and consequently, good DPPH radical scavenging property among other extracts (Table 4, Fig. III).

Pairwise comparison of extraction methods

The pairwise comparison of each extraction method on the amount of phenolic contents showed a significant difference between aqueous and ethanolic extracts (P=0.009) as well as ethanolic and Soxhlet methanolic extracts (P=0.013) in terms of the amount of total phenolic contents. Likewise, the pairwise comparison of each extraction method on the amount of total flavonoid contents showed a significant difference between aqueous and ethanolic extracts (P=0.002), aqueous, and methanolic extracts (P=0.042) and also ethanolic and Soxhlet methanolic extracts (P=0.042) (Table 5). According to the data obtained from Table 2, since there was no significant difference between DPPH inhibition percentage ranks, the comparison between methods was undoubtedly impossible.

Discussion

According to our results, the volume of total phenolic and flavonoid contents in the extracts varied based on the solvent type used. The results of total flavonoid contents conformed to those of (Ahmed et al., 2011; Khazai et al., 2011; Köroglu et al., 2012; Mileski et al., 2014; Kaska and Mammadov, 2019) performed on the Apiaceae family. It has also been established that both total phenolic and flavonoid contents were at the highest level in the polar organic extracts of *R. aucheri* compared to polar aqueous extracts. Kaska and Mammadov (2019) in their study on *Echinophora tournefortii* Jaub. and Spach found that methanolic extract possessed the highest total phenolic content while this solvent was not suitable enough for the extraction of the highest flavonoid contents.

Based on our study, aqueous extracts possessed the highest level of IC50 value, which represents its lowest antioxidant activity. Similar to our findings, Ahmed et al. (2011) found that the antioxidant activity of the species *Prangos* Lindl. (Apiaceae) altered based on the solvents used. Their results showed that, ethanolic extracts compared to aqueous extracts had the highest antioxidant activity using both DPPH and TBA tests.

Several authors are of the opinion that the antioxidant activity is directly related to the plant phenolic and flavonoid contents (Jung et al., 2003; Matejić et al., 2015; Al-Dabbas, 2017; Pirbalouti et

al., 2013). The results of the present study also corroborate those findings; but the difference is that, no significant difference was observed between two organic solvents, i.e. ethanol and methanol, in the power of phenolic and flavonoid extraction. Based on the graphs, the highest amount of phenols and flavonoids were obtained in the ethanolic solvent, followed by the methanolic extract with slight changes. Also, the highest antioxidant activity observed in both ethanolic and methanolic solvents was almost the same through maceration method. However, based on the graphs, the IC50 of methanolic extract was slightly lower compared with the ethanolic extract. Our results conform to Matejić et al. (2015), who found that two different solvents (methanol and ethyl-acetate) possessed high antioxidant property, but methanol had better DPPH scavenging effect compared to ethyl-acetate. However, the differences were not statistically significant and complementary studies are required to find out the exact correlation between different methods of extraction and antioxidant activity.

Conclusion

In general, the results of the present study showed that the polar organic solvents are much more suitable compared to the polar aqueous solvents for extraction of phenolic and flavonoid compounds as well as their antioxidant activity.

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