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Research Article

Antioxidant and antimicrobial activities of methanol extracts from *Adonis paryadrica* (Ranunculaceae) – a critically endangered endemic species growing in the Turkish flora

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Abstract: This study was conducted to determine the antioxidant and antimicrobial activities of methanol extract obtained from flower, leaf and root sections of endemic *Adonis paryadrica* (Boiss.) Kandemir & Aytaç stat. nova. naturally growing in the Turkish flora. The most efficient total phenolic compounds and flavonoid contents were obtained from leaf extract at 21.24 mg GAEs (gallic acid equivalent)/g dw and 54.97 mg REs (rutin equivalent)/g dw, respectively. Among the three different sections of this plant, leaf extracts showed the highest Cupric Reducing Antioxidant Power (CUPRAC) effect with 80.28 µmol TEs (trolox equivalent)/g dw. From the three different sections, the methanol extract of the leaf parts demonstrated strong antibacterial activity against *Bacillus subtilis* with a 16.1 mm zone diameter. These valuable and current findings from these precious plants, which constitute natural resources in terms of biodiversity, contribute innovative information to the literature on endemic plant species.

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1. INTRODUCTION

Plants have been preferred as one of the main sources of natural remedies for centuries, and still today, the new compounds of these valuable organisms continue to be documented as natural sources (Egamberdieva and Tiezzi, 2019). Secondary metabolites are the basis of these natural resources. Ranunculaceae family with a wide distribution in the world contributes to these natural resources pool with its secondary compounds such as alkaloids, glycosides derivatives, saponins, and steroids, including bufadienolides and cardenolides (Hao *et al*., 2017; Kuroda *et al*., 2018). The genus of Adonis L., belonging to the Ranunculaceae family, is represented by approximately 40 species in the world. This genus has 9 species and one subspecies in Turkey. Among these 10 taxa, only *Adonis paryadrica* (Boiss.) Kandemir & Aytaç stat. nova. is a rare endemic species for Turkish flora. This species has been collected in 1858 by Tchihatcheff Pierre from Giresun, Turkey for the first time. After a long break, this species was investigated within the scope of biodiversity study by the Ministry of Agriculture and Forestry of the Republic of Turkey in 2018. Within the scope of the project, the presence

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of the species was confirmed in six locations of Şebinkarahisar-Alucra districts in Giresun and one location in Erzincan Munzur Mountains. The distance between locations with semi-acidic soil characteristics is approximately 20 km from the bird's eye view. The taxon is very insufficient in terms of the number of individuals in all of the determined locations and is stuck in a narrow area. Many limiting factors such as grazing pressure, erosion, road construction activities and insect attacks have encountered in some areas where the species lives (Ministry of Agriculture and Forestry, 2018). For all these reasons, it has been reported that the species is endangered and should be evaluated in the Critically Endangered (CR)" (criteria B2 a b (i, iii) of IUCN 2010) category (IUCN, 2010; Kandemir *et al*., 2019).

There are a limited number of studies on phytochemical components of this genus in the literature (Mohadjerani *et al*., 2014; Kuroda *et al*., 2018; Ucuncu *et al*., 2020). Although a study has been reported on the phytochemical and biological activity of ethanol extracts of *A. paryadrica*, however, there is no comprehensive study in the literature on the biological activities of extracts obtained with other solvents up to the best of our knowledge. The aim of this study was to determine the (i) antioxidant activity, (ii) total phenolic compounds, (iii) total flavonoid contents and (v) antimicrobial activity of the methanol extracts of this valuable rare natural resource as well as the ongoing in situ and ex situ conservation studies. All of these obtained results will shed light on the evaluation of bioactive phytochemical constituents of *A. paryadrica* as medically.

2. MATERIAL and METHODS

2.1. Electrochemical Method

All electrochemical measurements were obtained using an electrochemical analyzer i.e. Vertex®One (Ivium) device which includes electrode cell stand. This electrode cell stand consists of a reference (Ag/AgCl; BASi, MF-2052), a counter (platinum wire; BASi, MW-1032) and a working electrodes (glassy carbon electrode (GCE); BASi MF-2012). In order to make the indicator electrode clean, smooth, polishing the surface of GCE with aluminum silica was applied before each measurement. The electrochemical method as square wave stripping voltammetry (SWSV) was used to determine the amount of antioxidants found in the root, stem and leaves of the *A. paryadrica* on GCE. The operating conditions of SWSV were selected as pulse amplitude of 60 mV, frequency of 100 Hz, step potential of 5 mV, accumulation time of 30 s and accumulation potential of 0 mV. For the supporting electrolyte solution, the Britton-Robinson buffer solution at pH 6.0 was used to collect all SWSV data. pH of solutions was adjusted with a Mettler Toledo brand pH meter with an accuracy of ± 0.05. No pre-purification was applied to the samples. The samples of *A. paryadrica* were prepared for spectrophotometric analysis and used directly.

2.2. Reagents

The analytical standard of rutin was purchased from Aldrich-Sigma. Stock solution for rutin was prepared daily at a concentration of 500 mg/L. Britton Robinson (BR) buffer solution preferred as support electrolyte was prepared with 0.4 M of acetic acid, ortho-phosphoric and boric acid. To adjust the BR buffer solution to pH 6.0, 2.0 M NaOH or 2.0 M HCl solutions were used. Distilled water was used in the whole experimental process.

2.3. Spectrophotometric Methods

The antioxidant activities of the methanolic extracts from plants were expressed as mg trolox equivalent (TEs)/g extract. Details of the spectrophotometric methods can be found in supplementary file (Zengin *et al*., 2015a; Zengin *et al*., 2015b; Apak *et al*., 2006; Kocak *et al*., 2010).

2.4. Preparation of the extracts of *Adonis paryadrica*

Air-dried samples of the aerial parts (2 g) of plants were extracted with 50 ml of methanol for 30 min in a sonication bath at 30 °C. The extracts were filtered and then concentrated under reduced pressure. All samples were stored at –20 °C before using for experiments.

2.5. Activity Test

2.5.1. *Microbial strains*

MeOH extracts of flower, leaf and root parts of the *A. paryadrica* were individually tested against six Gram-positive, seven Gram-negative bacteria and a fungus. Detailed information about the strains of bacteria and fungus were given in the supplementary file.

2.5.2. *Disc diffusion assay*

The assay was performed by following the protocols of the Clinical and Laboratory Standards Institute (2014) and The European Committee on Antimicrobial Susceptibility Testing (2013). Details of the disc diffusion analyses were also specified in the supplementary file.

2.5.3. *Microdilution assay*

Minimum Inhibitory Concentration (MIC) was tested for four Gram-positive and two Gram-negative bacteria. MeOH extracts of flower part of the *A. paryadrica* were individually tested against only three Gram-positive bacteria. For this purpose, the flower part of this species was individually tested against four Gram-positive bacteria and two Gram-negative bacteria. Detailed information about the MIC values were given in the supplementary file.

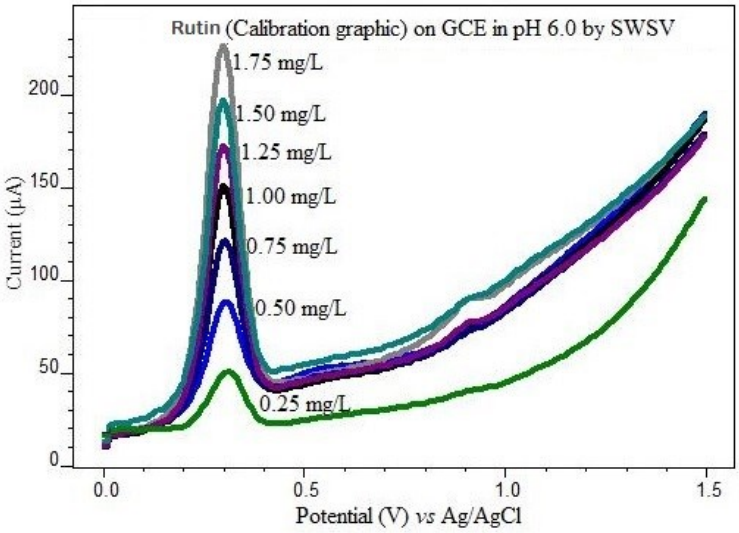
2.6. Statistical Analysis

All spectrophotometric and electrochemical tests were performed in triplicate and the results were expressed as mean with standard deviation (mean ± SD). The electrochemical analyses of antioxidant capacity of *A. paryadrica* samples were carried out by calibration method performed under the optimum condition for analytical standard i.e. rutin. Statistical significance between data was determined by using Tukey's honestly significant difference post hoc test with $\alpha = 0.05$ and ANOVA (one-way analysis of variance) test. Statistical calculations were carried out by using SPSS v. 22.0 software.

3. RESULTS and DISCUSSION

Square wave stripping voltammetry (SWSV) is generally one of the most preferred electrochemical methods for sensitive, selective, cheap and fast analysis of substances that are electroactive types such as phenolic compounds, flavonoids, vitamins, drugs and pesticides (Demir and İnam, 2014; Demir, 2019; Yıldırım *et al*., 2020; Demir and Silah, 2020; Inam *et al*., 2020; Demir *et al*., 2021). Therefore, firstly, SWSV experimental conditions were selected for the rutin, which is one of the standard antioxidants. The operating conditions of SWSV were preferred as pulse amplitude of 60 mV, frequency of 100 Hz, step potential of 5 mV, accumulation time of 30 s, accumulation potential of 0 mV and BR buffer solution at pH 6.0 on GCE. Two oxidation peaks were obtained at 320 mV and 910 mV for rutin by SWSV on GCE in pH 6.0 BR buffer solutions (Figure 1).

Figure 1. Calibration graphic of rutin by SWSV on GCE.

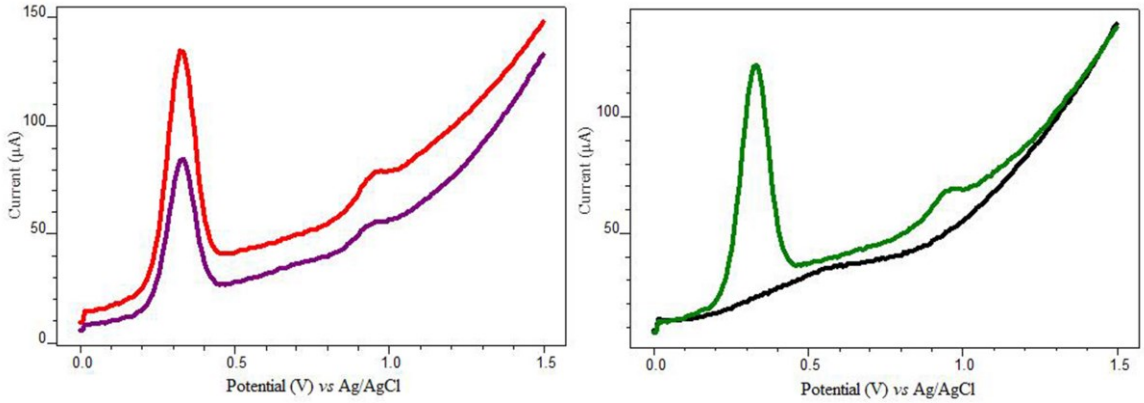


Then, under these conditions, based on the anodic peak at approximately 350 mV, calibration was created with standard addition between 0.25 mg/L and 1.75 mg/L by SWSV method. Linear calibration equation for the rutin agent was obtained by plotting peak signal on SWS voltammograms versus concentrations. The obtained equation on the linear calibration graphics for the rutin is as follows:

$$I_p\ (\mu A) = 105.15\ C\ (mg/L) + 8.\ 247\ R2 = 0.9961 \text{ for the rutin}$$

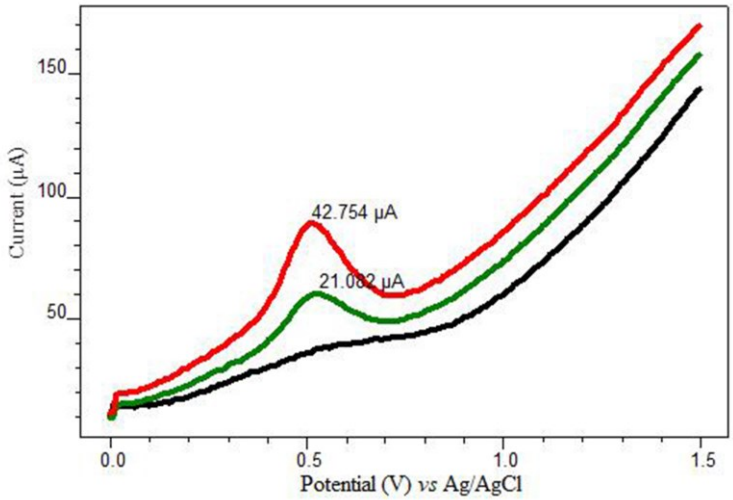
Under the conditions set for standard antioxidant substance, SWS voltammograms of real samples such as for the stem and leaves of the *A. paryadrica* were obtained on GCE in pH 6.0 BR buffer solutions (Figure 2a and 2b).

Figure 2. SWS voltammograms for the a) Flower b) Leaf sample on GCE in pH 6.0 BR buffer solutions.



Two oxidation peaks were obtained at 350 mV and 675 mV of real samples on the glassy carbon electrode (GCE) under SWSV test conditions. The peak potential values obtained for these two samples are almost identical to the peaks exhibited by rutin. Therefore, the amount of antioxidants contained in these two samples can be easily calculated in terms of rutin equivalent. Here, the first anodic peak which is high intensity peak and well-defined at approximately 350 mV was referenced. As a result of three replicate measurements for the 0.05 ml samples, average peak currents for flower samples were found to be 62.29 ± 2.78 µA, while this value was calculated as 95.88 ± 3.56 µA for leaf samples. The total antioxidant capacity (TAC) for the 1 g extract of flower and leaf samples as rutin equivalent were found as 257.0 mg/L and 416.5 mg/L, respectively. The peak obtained for root samples differs from the peak potential values obtained for flower and leaf (Figure 3).

Figure 3. SWS voltammograms for the root sample on GCE in pH 6.0 BR buffer solutions.



The main reason of this difference is dominance of phenolic compound in the root samples that is different from the rutin. However, since the peak potential of the phenolic compounds is between 0.3 V and 0.5 V, it is possible to give the total amount of antioxidants in the root sample as rutin equivalent. According to the results of three replicates for the 0.05 ml extract sample, the peak flow value was found to be 21.425 ± 0.672 µA. The TAC in root sample was calculated as rutin equivalent of 88.29 ± 2.77 mg/L (Table 1).

Table 1. Total antioxidant amounts in equivalent rutin in plant samples by SWSV.

Method	Samples	Total antioxidant capacity Equivalent rutin
SWSV	Flower	257.0 ± 11.45^{b} mg/l
	Root	88.29 ± 2.77^{c} mg/l
	Leaf	416.5 ± 14.67^{a} mg/l

Antioxidant quantification of the endemic plant *Draba cemileae* (Karaer) in leaf, root and stem samples were carried out with the electrochemical method by Cuce *et al*. (2021). The electrochemical measurements are compatible with the data obtained by spectrophotometric-based methods such as traditional Cupric Reducing Antioxidant Power (CUPRAC) reducing, Ferric Reducing Antioxidant Power (FRAP) reducing, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging and Ferrous ion chelating (Cuce *et al*., 2021). Moreover, electrochemical methods have been successfully performed to determine the total antioxidants in many products such as fruit juices, coffee samples and many different foodstuffs (Yıldırım *et al*., 2020; Demir *et al*., 2021; Öztürk *et al*., 2021). Therefore, the antioxidant capacities of *A. paryadrica* were investigated in detail by electroanalytical methods, which is a fast, inexpensive, simple, accurate and reliable new antioxidant method.

3.1. Spectrophotometric Methods

3.1.1. *Chemical composition*

The yields of the MeOH extracts obtained from the flower, leaves and roots of *A. paryadrica* were shown in Table 2. The highest yield percentage was obtained from the flower extract with 18.76% followed by the root (16.59%) and leaf (13.80) extracts, respectively.

The spectrophotometric analysis data were also given in Table 2. Leaf parts were found to be the richest in terms of total phenolic substances (21.24 mg GAEs/g) and total flavonoid content (54.97 mg REs/g). The root parts were found poorest in terms of both phenolics (18.59

mg GAEs/g), and flavonoids (0.54 mg REs/g) than other parts. Statistical analyzes showed that the phenolic profiles of flower and root extracts were not significantly different from each other ($p < 0.05$). In addition, when the flavonoid profiles were compared statistically, it was determined that the flower, leaf and root extracts were significantly different from each other ($p < 0.05$).

Table 2. Extraction yield, total phenolic and flavonoid contents of the methanol extracts from *A. paryadrica.*

Samples	Yield (%)	Total phenolics (mg GAEs/g extract)	Total flavonoids (mg REs/g extract)
Flower	18.76	19.17 ± 0.23^{b}	32.42 ± 0.01^{b}
Root	16.59	18.59 ± 0.37^{b}	0.54 ± 0.02^{c}
Leaf	13.80	21.24 ± 0.14^{a}	54.97 ± 0.51^{a}

Within each column, means sharing the different superscripts show comparison between the extracts by Tukey's test at $p < 0.05$. GAEs and REs, gallic acid and rutin equivalents, respectively

Evaluation of the biological activities of valuable natural metabolites of plants is one of the most important research areas today and there are many studies carried out in this field (Cüce *et al*., 2017, 2019; Cüce and Basançelebi, 2021; Sarikurkcu *et al*., 2021). The different techniques and methods used in these studies or the different extraction techniques and solvents for the same species can be shown among the reasons for the results obtained from the studies (Jahanban-Esfahlan *et al*., 2019; Ucuncu *et al*., 2020; Sarikurkcu *et al*., 2020). Due to this situation, the researchers preferred ethanol extracts for *A. paryadrica* in previous studies and reported that they obtained similar results in terms of total phenolic contents with the data in our study (Ucuncu *et al*., 2020). The strength of this study is that the percentage yield and total flavonoid content values were not given in the previous study on *A. paryadrica.*

3.1.2. *Antioxidant activity*

The data regarding the antioxidant activity potentials of the extracts are summarized in Table 3. The extracts differed in terms of radical scavenging capacities. DPPH radical scavenging capacity order of extracts were leaf>flower>root and ABTS radical scavenging capacity order of extracts were root>leaf>flower (Table 3). Based on these data, the highest DPPH radical scavenging effect was found to be 38.26 mg/ml in leaf extracts, while the most effective ABTS radical scavenging effect in root extracts was calculated as 44.78 mg/ml. The DPPH radical scavenging capacity of root extracts (12.58 mg/ml) created a statistically significant negative difference from others. ABTS radical scavenging capacities of the leaf and flower extract were determined to be 39.71 and 33.70 mg/ml, respectively, which is an indicator of the statistical difference between them ($p < 0.05$).

Similar scenarios were experienced in terms of the CUPRAC and FRAP reducing powers of the extracts. While the leaf extract gave the most effective CUPRAC reducing power activity with 80.28 mg/ml, the root extracts had 45.05 mg/ml FRAP reducing power. In terms of CUPRAC reducing power activity, leaf extracts were followed by flower (66.54 mg/ml) and root (49.59 mg/ml) extracts, respectively. On the other hand, in terms of FRAP reducing power activity, root extracts were followed by leaf with 42.11 mg/ml and flower (34.17 mg/ml) extracts, respectively (Table 3).

Table 3. Radical scavenging activities of methanolic extracts from *A. paryadrica*.

Samples	DPPH radical (TEs/g extract)	ABTS cation radical (TEs/g extract)	CUPRAC reducing (TEs/g extract)	FRAP reducing (TEs/g extract)
Flower	30.94 ± 0.11^{b}	33.70 ± 0.20^{c}	66.54 ± 0.25^{b}	34.17 ± 0.53^{c}
Root	12.58 ± 0.19^{c}	44.78 ± 0.01^{a}	49.59 ± 0.41^{c}	45.05 ± 0.01^{a}
Leaf	38.26 ± 0.03^{a}	39.71 ± 0.14^{b}	80.28 ± 0.25^{a}	42.11 ± 0.24^{b}

Within each column, means sharing the different superscripts show comparison between the extracts by Tukey's test at $p < 0.05$. TEs, trolox equivalents

Antioxidant studies of species belonging to the genus Adonis are limited in the literature (Mohadjerani *et al*., 2014; Ucuncu *et al*., 2020; Guo *et al*., 2022). In antioxidant studies of *A. paryadrica* with only ethanol extracts, researchers used different parts of the plant and obtained effective results on the gallic acid equivalent (Ucuncu *et al*., 2020). The data obtained from this study also showed that the difference in the extract had an effect on the antioxidant properties of the Trolox equivalent.

3.2. Antimicrobial Activity

According to the results of the agar disc diffusion test, some bacteria were inhibited by the flower and leaf extracts. The root extract of the plant and negative control (20% DMSO) did not show any antimicrobial activity against bacteria. The inhibition zones against the tested bacteria ranged from 8.21 to 16.1 mm. The highest inhibition zone was obtained from the leaf extract against *B. subtilis* with 16.1 mm (MIC = 62.5 µg/mL). Among the bacteria whose inhibition value was obtained, E. faecalis had the lowest value with a zone diameter of 8.2 mm (MIC = 125 µg/mL). Flower extract was effective as antibacterial against S. aureus and *B. subtilis* and S. faecalis with 9.2 mm, 15.2 mm and 10.2 mm inhibition zone, respectively. MIC values for the mentioned these bacterial species were calculated as 125, 31.25 and 62.5 125 µg/mL, respectively. None of the flower leaf and root extracts showed any inhibition effect on *C. albicans*. Methanolic extracts of *A. paryadrica* demonstrate antimicrobial activity against two Gram-negative and four Gram-positive bacteria according to the disc diffusion assay (Table 4).

Diameter of inhibition zone including disc diameter of 6 mm by the agar dics diffusion method at a concentration of 10 µL of extract/disc. Ofloxacin (10 µg/disc) (OFX), netilmicin (30 µg/disc) (NET30), sulbactam (30 µg) + cefoperazone (75 µg); (105 µg per disc) (SCF) were used as reference antibiotics. Dimethyl sulfoxide (DMSO) (20%) was used as negative control (N.C.). MIC (minimal inhibition concentration) was calculated as µg/ml. The values are the average ± standard deviation of three determinations ($p < 0.05$). - = not detected. Values with different letter(s) in the same line(s) were significantly different ($p < 0.05$)

Due to the fact that microbial contamination is one of the most threatening elements of today, research is focused on this subject. Researchers carry out antimicrobial studies on each pathogenic microorganism against the risks of future epidemics, and herbal phytochemicals come first as a source (Tepe *et al*., 2005; Rios and Recio, 2005; Cüce and Basançelebi, 2021). In the study conducted on the antibacterial effects of ethanol extracts of *A. paryadrica*, the researchers obtained the highest zone diameter of 14 mm from the flower extract on *Yersina pseudotuberculosis* (Ucuncu *et al*., 2020). These researchers generally reported that flower extracts showed more effective antibacterial activity on different bacterial species.

On the other hand, the calculation of the highest inhibition zone on *B. subtilis* with 16.1 mm reveals the difference in the present study. According to these results, the leaf extract of *A. paryadrica* can be said more effective than other parts in terms of the antibacterial activity.

Table 4. Zones of growth inhibition (mm) showing antimicrobial activity of *A. paryadrica*

Bacteria	Disc Diffusion (mm)			MIC (µg/mL)			N.C.	Standard Antibiotic Discs		
	Flower	Leaf	Root	Flower	Leaf	Root		OFX	NET30	SCF30
Gram Negative Bacteria										
K. pneumoniae	-	8.5 ± 0.2^{d}	-	-	125	-	-	16.7 ± 0.6^{c}	18.1 ± 0.7^{b}	21.6 ± 0.8^{a}
E. coli	-	-	-	-	-	-	-	26.6 ± 1.1^{b}	16.6 ± 0.8^{c}	29.0 ± 0.5^{a}
S. marcescens	-	-	-	-	-	-	-	26.6 ± 0.7^{b}	20.0 ± 0.6^{c}	28.4 ± 0.9^{a}
S. typhimurium	-	-	-	-	-	-	-	26.3 ± 0.2^{b}	19.1 ± 1.5^{c}	28.2 ± 0.0^{a}
P. aeruginosa	-	-	-	-	-	-	-	27.8 ± 0.1^{a}	20.6 ± 0.3^{b}	27.4 ± 0.5^{a}
P. vulgaris	-	9.3 ± 0.3^{c}	-	-	31.25	-	-	29.1 ± 0.6^{a}	16.6 ± 0.8^{b}	29.2 ± 0.9^{a}
S. enterica	-	-	-	-	-	-	-	28.7 ± 0.5^{a}	27.4 ± 0.2^{a}	19.5 ± 0.7^{b}
Gram Positive Bacteria										
S. aureus	9.2 ± 0.2^{d}	9.1 ± 0.2^{d}	-	125	125	-	-	29.1 ± 1.0^{a}	20.6 ± 0.5^{c}	25.1 ± 0.6^{b}
B. subtilis	15.2 ± 0.3^{d}	16.11 ± 0.3^{c}	-	31.25	62.5	-	-	30.4 ± 1.4^{a}	29.3 ± 1.5^{a}	28.5 ± 1.5^{b}
S. epidermidis	-	-	-	-	-	-	-	27.5 ± 1.5^{b}	26.2 ± 1.1^{b}	31.7 ± 0.0^{a}
B. thrungiensis	-	-	-	-	-	-	-	25.4 ± 0.5^{b}	23.6 ± 1.0^{c}	28.6 ± 0.8^{a}
E. faecalis	-	8.21 ± 0.1^{d}	-	-	125	-	-	19.3 ± 0.5^{b}	21.2 ± 1.3^{a}	14.3 ± 0.7^{c}
S. faecalis	10.2 ± 0.2^{d}	11.03 ± 0.3^{c}	-	62.5	62.5	-	-	19.9 ± 0.5^{a}	16.6 ± 0.0^{b}	19.9 ± 0.7^{a}
Fungi										
C. albicans	-	-	-	-	-	-	-	20.5 ± 0.5^{b}	19.8 ± 0.6^{c}	18.7 ± 0.0^{d}

4. CONCLUSION

In this study, innovative and traditional antioxidant detection methods were applied to flower, leaf and root methanol extracts of *A. paryadrica*, an important endemic plant, and effective results were obtained. In terms of total antioxidant capacity, electrochemical and spectrophotometric antioxidant analysis methods have generally shown that the leaf parts of this plant are much more effective. Leaf extracts were found to be more effective than the other parts in terms of antimicrobial analyzes on Gram-negative and Gram-positive bacterial species and a fungus. This study is important in terms of revealing the pharmaceutical properties of different extracts obtained from medicinal and aromatic plants grown in our country and have medicinal value. It is envisaged that these studies will constitute a model for similar studies to be carried out in the near future.

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Declaration of Conflicting Interests and Ethics

The author declares no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author.

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Publisher's note

Correction to: *International Journal of Secondary Metabolite,* (2022), https://doi.org/10.21448/ijsm.1071234. The family name in the title of the article has been changed to "Ranunculaceae". The "Asteraceae" family name in the title was incorrect. The original article has been corrected.

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