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ANTIOXIDANT, ANTI-HAEMOLYTIC ACTIVITY, VITAMIN AND ELEMENT PROFILE OF *RHAMNUS CORNIFOLIUS*

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ABSTRACT. The antioxidant, antiradical, and anti-hemolytic activities of the methanol extract of *Rhamnus cornifolius* leaves were investigated, along with the determination of element and vitamin levels in the dried plant material. Element analyses were determined by ICP-OES dry ashing, vitamins by HPLC, vitamin C, total phenolic, flavonoid, antioxidant capacity, $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and anti-haemolytic activity spectrophotometrically. Total phenolic, flavonoid and antioxidant capacities of the plant were measured as 4.482 ± 0.159 mg GA/g, 27.420 ± 2.551 mg QE/g and 41.795 ± 18.506 mM AA/g, while retinol, α-tocopherol, phylloquinone and ascorbic acid as 0.2167 ± 0.019, 4.313 ± 0.685, 0.5622 ± 0.09 µmol/kg and 403.631 ± 8.682 mg 100/g. IC_{50} values of $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and anti-haemolytic assays were determined as 33.974 ± 1.918 (BHT = IC_{50} 26.979 ± 1.116), 58.197 ± 1.826 (trolox = IC_{50} 27.854 ± 1.352) and 68.51 ± 5.33 (trolox = IC_{50} 44.040 ± 0.290) µg/mL, respectively. According to the results of this study, it was determined that the vitamin and element content of the plant was at high level, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ tests showed a promising antioxidant power. These findings represent the first report in the literature, and the plant's composition was evaluated as a basis for future in vivo studies.

Keywords: *Rhamnus cornifolius, methanol extract, antioxidant, element, vitamin, anti-haemolytic activity*

INTRODUCTION

There are many definitions of medicinal and aromatic plants in the literature. According to the Food and Agriculture Organisation of the United Nations (FAO), medicinal and aromatic plants are defined as plants that provide

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medicine to people to prevent diseases, maintain health and treat ailments [1]. At least 8000 phenolic substances of different amounts and qualities have been identified so far in the structure of plants [2]. These compounds play an important role in destroying or inhibiting free radicals. Thus, they increase the body's resistance to cell damage and prevent unwanted oxidation products [3].

Vitamins are compounds that are essential for performing some special cellular events other than proteins, lipids and carbohydrates which are macronutrients and are needed at trace levels in the organism [4]. Vitamins have been found to have important roles in reactions involving free radicals, electron transport and membrane biochemical strengthening [5]. Fat-soluble vitamins A, D, E and K do not need to be taken frequently when they are stored in the liver and fat [6]. However, for this, it is necessary to take these vitamin elements necessary for tissues with appropriate nutrition.

Dietary minerals, which are not included in the term vitamins but support health, are essential nutrients for the body [7]. Functioning as catalytic cofactors of enzymes, the roles and functions of these elements are extremely diverse and vital. They are useful for plant growth, development and yield [8]. They interact directly with free radical formation and free radical scavengers. Maintaining the integrity of the cell membrane depends on a protection or repair mechanism that can neutralise oxidative reactions [9]. Even minor deficiencies in vitamins and minerals can lead to serious health issues, though they often go unnoticed until they become severe [7]. Preventing this unfavourable situation is possible with an appropriate diet.

Hakkari Province, located in the Eastern Anatolia Region of Turkey, has different ecological and climatic characteristics, high mountains and a wide range of endemic plant diversity as a result of the plant flora spread over a very large area. *Rhamnus Cornifolius Boiss.& Hoh.* belongs to the *Rhamnaceae* family and is generally distributed in Northern Iraq, South-Western Iran and Hakkari Province in Turkey. *R. cornifolius* is one of 22 *Rhamnus* species growing naturally in Turkey [10].

What makes these plant species valuable are the yellow coloured dyes contained in their fruits. These colouring substances are flavonoids and partly anthroquinones. Anthroquinones are organic compounds found in some plants. They are used as dyes, pigments and also for medicinal purposes. The plant has been traditionally used for its antioxidant and anti-inflammatory effects in the treatment of constipation related to liver diseases [11]. Numerous studies, both past and ongoing, have investigated the potential of plant-derived phytochemicals in the prevention of diseases such as cardiovascular disorders, cancer, and osteoporosis. [12,13].

The aim of this study was to investigate the antioxidant, antiradical properties, biolements and vitamin content of the methanol extract of the leaf part of *R. cornifolius* growing in Hakkari province of Turkey, which has a rich flora,

by various methods. For this purpose, total phenolic, flavonoid and antioxidant capacity of the plant and vitamin A, E, K and C contents were determined. $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and phenylhydrazine radical scavenging capacity and element levels were also determined.

RESULTS AND DISCUSSION

Total antioxidant capacity, total phenolic and total flavonoid content, $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and anti-haemolytic activity levels were measured to determine the antioxidant properties of the methanol extract of *R. cornifolius* leaves. In addition, the levels of vitamins A, E, K and C and elements (K, Na, Ba, V, Ti, Cr, Cu, Sr, As, Se, Cd, Pb, Mo, Fe, Mn, Al, Zn, Co) of *R. cornifolius* were determined and all results are shown in Table 1 and Table 2.

Table 1. Vitamins A, E, K and C, total phenolic and flavonoid content, total antioxidant capacity, element (Ba, V, Ti, Cr, Cu, Sr, As, Se, Cd, Pb, Mo, Fe, Mn, Al, Zn, Co, K and Na) levels of *R. cornifolius*.

Parameters	***Rhamnus cornifolius*** ($\bar{X}$ ± **SEM)**
Retinol (µmol/kg)	0.2167 ± 0.0190
α-tocopherol (µmol/kg)	4.3130 ± 0.6850
Phylloquinone (µmol/kg)	0.5622 ± 0.0900
Vitamin C (mg 100/g)	403.6310 ± 8.6820
Total phenolic content (mg GAE/g)	4.4820 ± 0.1590
Total flavonoid content (mg QE/g)	27.4200 ± 2.5510
Total antioxidant capacity (mM A.A/g)	41.7950 ± 18.5060
Ba (µmol/kg)	18.6250 ± 0.6090
V (µmol/kg)	5.8710 ± 0.0740
Ti (µmol/kg)	19.3550 ± 0.4620
Cr (µmol/kg)	3.5240 ± 0.6650
Cu (µmol/kg)	9.1210 ± 0.4550
Sr (mmol/kg)	0.0443 ± 0.0015
As (µmol/kg)	0.5240 ± 0.0550
Se (µmol/kg)	1.0650 ± 0.0360
Cd (µmol/kg)	0.0720 ± 0.0058
Pb (µmol/kg)	0.4490 ± 0.1600
Mo (µmol/kg)	0.0448 ± 0.0200
Fe (mmol/kg)	0.6850 ± 0.0132
Mn (µmol/kg)	142.8090 ± 4.3980
Al (µmol/kg)	6.6964 ± 0.3500
Zn (µmol/kg)	24.6830 ± 1.6380
Co (µmol/kg)	17.5250 ± 0.0125
K (mmol/kg)	3.0010 ± 0.0041
Na (mmol/kg)	1.8940 ± 0.1540

Values are expressed as mean ± standard error of the mean ($\bar{X}$ ± SEM).

Table 2. Values of % inhibition and IC_{50} (µg/mL) in methanol extracts of leaf of *R. cornifolius* compared with a positive controls.

	Control	% Inhibition $\overline{X}$ ± SEM	IC_{50} (µg/mL) $\overline{X}$ ± SEM
DPPH$^{\bullet}$		65.339 ± 0.443	33.974 ± 1.918
		74.792 ± 0.764	26.979 ± 1.116
	BHT		
ABTS$^{\bullet+}$		89.333 ± 2.571	58.197 ± 1.826
		95.493 ± 0.849	27.854 ± 1.352
	Trolox		
$PhNHNH_2$		71.560 ± 2.390	68.510 ± 5.330
	Trolox	58.220 ± 0.310	44.040 ± 0.290

Values are expressed as mean ± standard error of the mean ($\overline{X}$ ± SEM). Samples were carried out in triplicate. DPPH$^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl; ABTS$^{\bullet+}$: (2,2'-azinobis (3--ethylbenzothiazoline-6-sulfonic acid)); $PhNHNH_2$: Phenylhydrazine.
DPPH: $y=6E\text{-}05x^2+0.1286x+45.514$; ABTS: $y=-0.0002x^2+0.5555x+16.444$;
$PhNHNH_2$: $y=-15.18\ln(x)+114.52$

Total phenolic, flavonoid, antioxidant capacity and vitamin C content of the plant methanol extract were calculated with the help of standard graphs and shown in Figure 1.

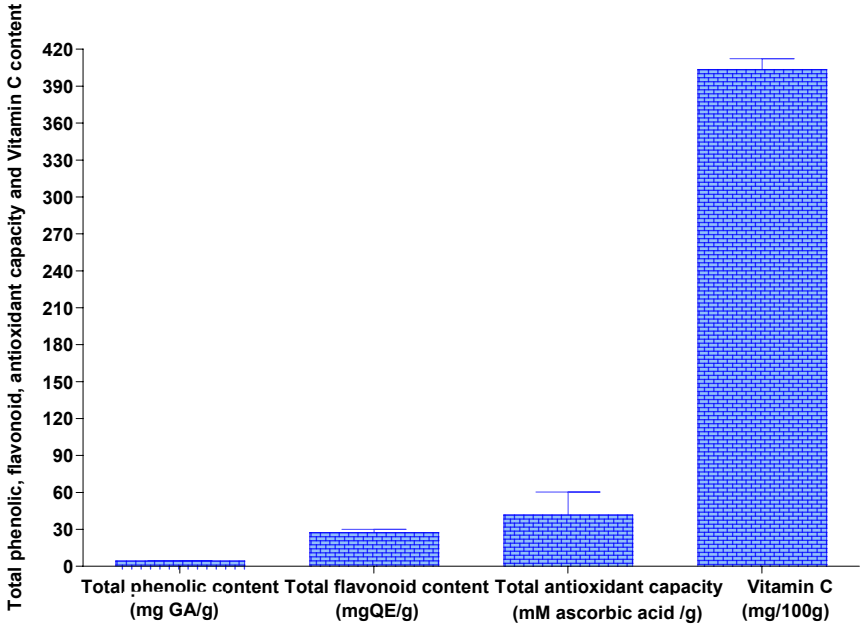

Figure 1. Total phenolic, flavonoid, total antioxidant capacity and vitamin C content of *R. cornifolius.*

Comparison with positive controls (BHT and trolox) was performed to determine the scavenging of radicals ($DPPH^{\bullet}$, $ABTS^{\bullet+}$) and haemolysis ($PhNHNH_2$) of the plant methanol extract. The % inhibition, % haemolysis and IC_{50} values calculated after $DPPH^{\bullet}$/BHT, $ABTS^{\bullet+}$/trolox and $PhNHNH_2$/trolox comparisons were determined and shown in Figure 2, Figure 3.

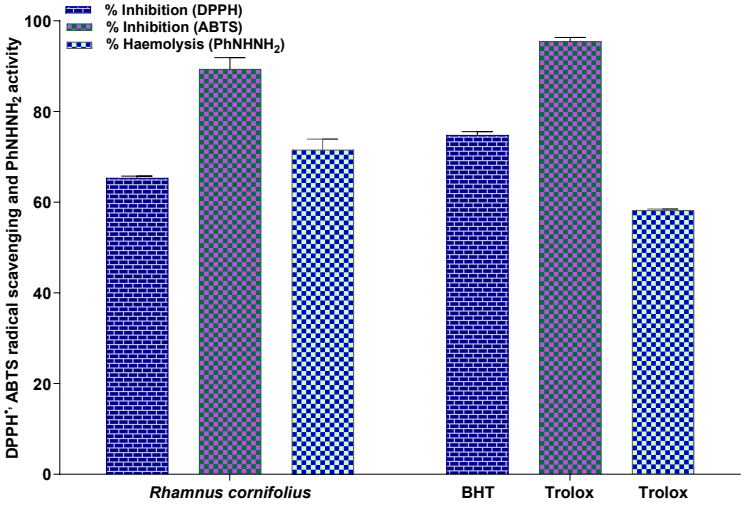

Figure 2. % inhibition values of BHT and Trolox used in comparison with DPPH, ABTS and anti-hemolytic activity of *R. cornifolius*

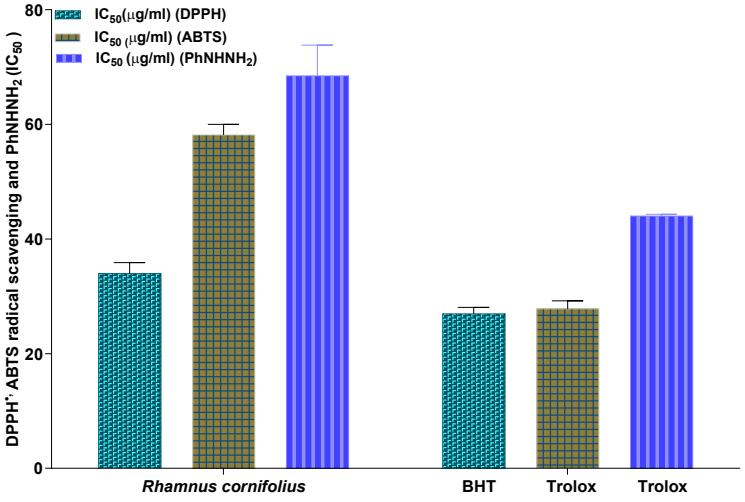

Figure 3. IC_{50} values of BHT and Trolox used in comparison with DPPH, ABTS and anti-hemolytic activity of *R. cornifolius*

The element content of the dry leaves of *R. cornifolius* was determined and all the determined amounts are expressed in Figure 4, Figure 5 and Figure 6.

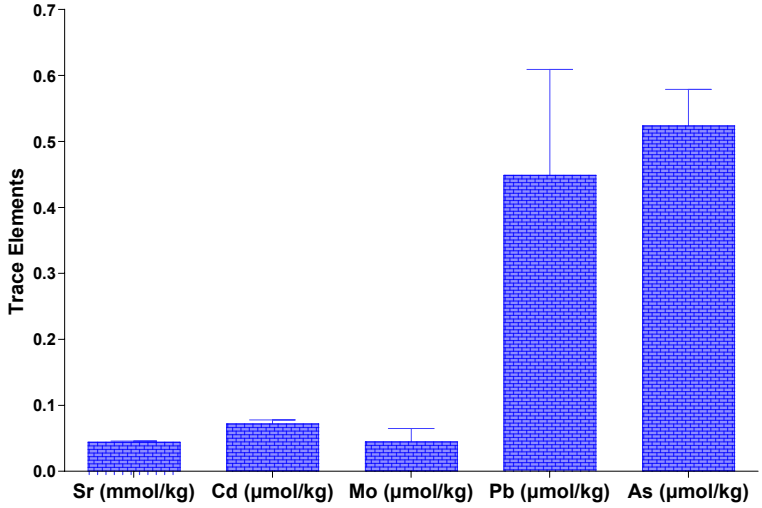

Figure 4. Trace element (Sr, Cd, Mo, Pb, As) levels of dry leaf parts of *R. cornifolius.*

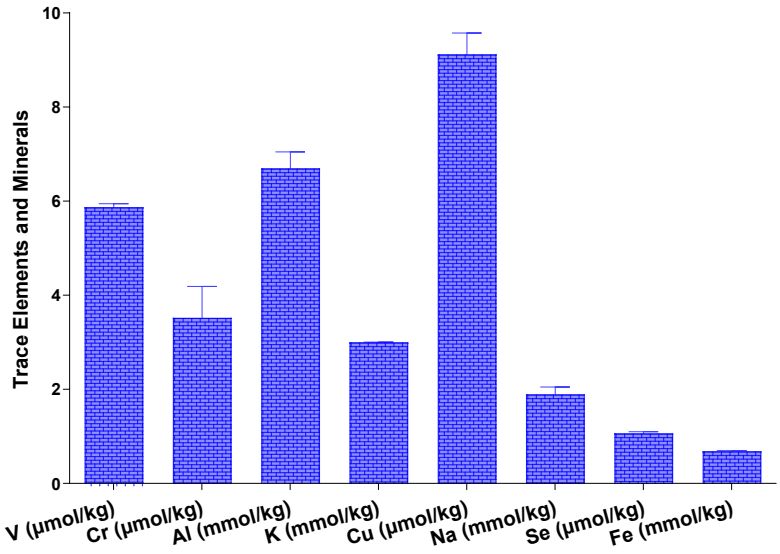

Figure 5. Element (V, Cr, Al, Cu, Se, Fe, K and Na) levels of *R. cornifolius* ry leaf parts.

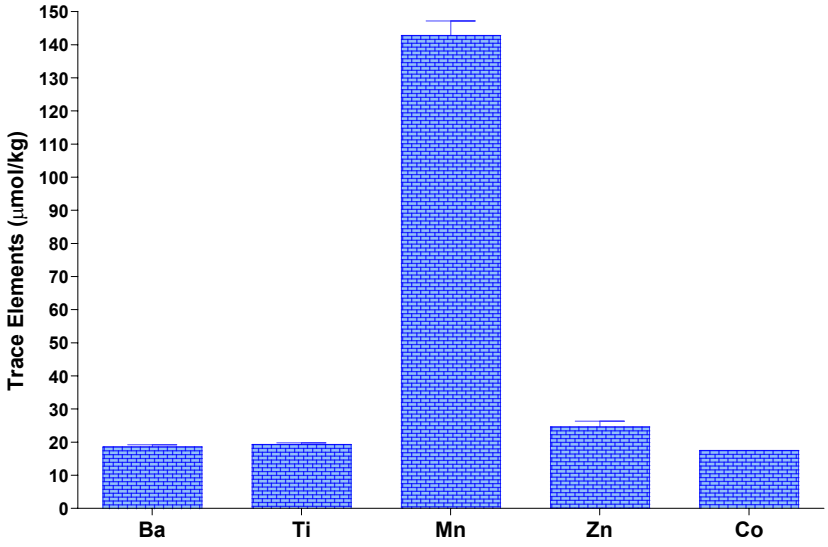

Figure 6. Element (Ba, Ti, Mn, Zn, Co) levels of *R. cornifolius* dry leaf parts.

Since vitamins are not produced in appropriate amounts by bodily metabolic processes, they must be taken in the diet at the necessary and sufficient level [7]. According to the World Health Organisation (WHO) data, 2 billion people face micronutrient deficiency. This deficiency partly includes vitamins A, D and various B vitamins and essential minerals [14]. Vitamin C, which is included in the subgroup of vitamins, shows antioxidant properties by minimising the damage caused by free radicals to cells. On the other hand, this vitamin, which is a common dietary supplement, is also a prerequisite for the production of collagen, the conversion of iron into a form that is easily absorbed in the intestines and the synthesis of the hormone serotonin, which is secreted for the regular functioning of the immune system [6]. The antioxidant properties of α-tocopherol, the most common form of vitamin E, are well known. This property is mainly due to the accepted idea that it terminates the chain reactions resulting from peroxyl radicals caused by free radicals [15]. Recent studies on the effect of vitamin K in combination with vitamin C on anti-cancer activity are of great interest [16]. The K_1 form of this vitamin, phylloquinone, is found in green leafy vegetables.

In this study, the amounts of vitamins A, E, K and C in the dried leaves of *R. cornifolius* were determined. The level of vitamin C included in the study was 403.631 ± 8.682 mg 100/g. Vitamins A, E and K levels were determined as 0.2167 ± 0.019 µmol/kg, 4.313 ± 0.685 µmol/kg and 0.5622 ± 0.09

µmol/kg, respectively and all values are given in Table 1. No studies on vitamins A, E and K of both *Rhamnaceae* family and *R. cornifolius* species were found. This is the first report of these vitamin values for *R. cornifolius* and therefore it is considered to be an important reference for future studies.

On the other hand, the values of vitamin C in the methanol extract of the leaves of *R. frangula* and *R. kurdica*, two species close to *R. cornifolius* in previous studies, were 85/63 mg 100/g, 5.937 ± 5.53 mg 100/g and 14.06 ± 1.07 µg 100/mg, respectively [17,18]. When the vitamin C content of *R. cornifolius*, *R. frangula* and *R. kurdica* were compared, it was found that the vitamin C value was higher in *R. cornifolius*. Considering the vitamin values in *R. cornifolius* as a whole, the presence of vitamins A, E and K is important and the high vitamin C value shows that this plant is a good source of vitamins.

Flavonoids, phenolic acids and phenolic compounds, even in the polyphenol class, are the most frequently encountered groups in plant contents [19]. Flavonoids are of particular interest due to their multiple roles in plants and their effects on human health [20,21]. Phenolic compounds are powerful and natural antioxidants that play an important role in destroying or inhibiting free radicals. Thus, they increase the body's resistance to cell damage and prevent lipid peroxidation [3,22].

In this study, total phenolic, total flavonoid content and total antioxidant capacity of *R. cornifolius* were determined. Total phenolic content was calculated from gallic acid standard curve graph, total flavonoid content was calculated using quercetin curve and antioxidant capacity was calculated according to ascorbic acid standard graph. Total phenolic, flavonoid and antioxidant amounts were found to be 4.482 ± 0.159 mg GAE/g, 27.420 ± 2.551 mg QE/g and 41.795 ± 18.506 mM AA/g, respectively and the results are shown in Table 1. When the literature was examined, the total phenolic value in the methanol extract of *R. alaternus* leaves, which is close to the plant, contains 77.8 mg GAE/g and this value is higher than the value observed in our study [23]. In another study, TPC value in 60% EtOH extract of *R. prinoides* stems was determined as 228.21 ± 13 mg GAE/g [24].

There is a balance between the presence of minerals and trace elements and metabolic reactions. The energy balance necessary for the survival of any organism (plant or human) is largely determined by the values of these minerals and trace elements [25,26]. Trace elements form complex structures with proteins, enzymes and carbohydrates to participate in biochemical reactions. Antioxidant enzymes require elements such as copper, zinc, iron and selenium for optimum catalytic activity [27,28].

The daily amount of trace elements required for the body is between 15-80 µg. Despite the small amount of trace elements in the human body, they are essential for the continuity of many important physiological functions

in the organism. They function as cofactors in the structure of some enzymes, participate in the structure of bone, teeth and many organic substances, maintain acid-base balance in metabolic reactions, transport processes of gases during immunity and respiration, participate in membrane structures and regulate the functional pathways of neurons [29,30].

In this study, when the element level of *R. cornifolius* plant was analysed, it was determined that it had the content and shown in Table 1, 18.625 ± 0.609 μmol/kg (5.7401 ± 2.5416 mg/kg) Ba, 5.871 ± 0.074 μmol/kg (0.2991 ± 0.0038 mg/kg) V, 19.355 ± 0.462 μmol/kg (0.9275 ± 0.0221 mg/kg) Ti, 3.524 ± 0.665 μmol/kg (0.1832 ± 0.0346 mg/kg) Cr, 9.121 ± 0.455 μmol/kg (0.5796 ± 0.0289 mg/kg) Cu, 0.0443 ± 0.0015 mmol/kg (0.0039 ± 0.0001 mg/kg) Sr, 0.524 ± 0.055 μmol/kg (0.0393 ± 0.0041 mg/kg) As, 1.065 ± 0.036 μmol/kg (0.0841 ± 0.0028 mg/kg) Se, 0.072 ± 0.0058 μmol/kg (0.0081 ± 0.0007 mg/kg) Cd, 0.449 ± 0.160 μmol/kg (0.0930 ± 0.0332 mg/kg) Pb, 0.0448 ± 0.02 μmol /kg (0.0043 ± 0.0019 mg/kg) Mo, 0.685 ± 0.0132 mmol/kg (38.2552 ± 0.7372 mg/kg) Fe, 142.809 ± 4.398 μmol/kg (7.8456 ± 0.2416 mg/kg) Mn, 6.6964 ± 0.350 μmol/kg (0.1628 ± 0.0085 mg/kg) Al, 24.683 ± 1.638 μmol/kg (1.6135 ± 0.1071 mg/kg) Zn, 17.525 ± 0.0125 μmol/kg (1.0328 ± 0.0007 mg/kg) Co, 3.001 ± 0.00412 mmol/kg (117.3451 ± 0.1611 mg/kg) K ve 1.894 ± 0.154 mmol/kg (43.5427 ± 3.5404 mg/kg) Na.

When the elemental and mineral levels of *R. cornifolius* were compared, it was determined that K > Na > Fe > Sr > Mn > Zn > Ti > Ba > Co > Cu > Al > V > Cr > Se > As > Pb > Cd > Mo. Considering these results, it was determined that besides macro elements such as K and Na, the most critical and important trace elements such as Fe, Mn, Zn, Co, Cu, V, Cr, Se and Mo were also present. When the literature was examined, some elemental analyses of *Rhamnus prinoides*, which is close to the plant we used in the study, were found as follows. The results were 129.90 ± 1.572 mg/kg K, 2.60 ± 0.520 mg/kg Na, 28.311 ± 1.263 mg/kg Cu, 10.267 ± 0.467 mg/kg Mn, 1.487 ± 0.0231 mg/kg Cr, 0.022 ± 0.0076 mg/kg Cd, 8.1 ± 1.153 mg/kg Fe and 10.177 ± 0.0643 mg/kg Zn [31]. When *R. cornifolius* and *R. prinoides* plants were compared, it was determined that *R. cornifolius* had rich and different elemental content, although the elemental values of *R. prinoides* were higher.

$DPPH^{\bullet}$ and $ABTS^{\bullet+}$ are common methods based on spectrophotometric determination used to evaluate the antioxidant potential of foods and phenolics in vitro [32]. In these methods, the reduction that occurs between a radical and a substance that can give hydrogen (H^+) atom results in a decrease in absorbance. This result emphasises the relationship between antioxidant and free radical. The total antioxidant capacity of the methanol extract prepared to evaluate the antioxidant activity of *R. cornifolius* was determined by these methods. One of the most important signs in these methods is IC_{50} value.

IC_{50} (mg/mL) measurement was evaluated by analysing the relationship between antioxidants and radical concentration and spectrophotometric monitoring of their absorbance [33].

The scavenging power of *R. cornifolius* methanol extract against $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals was compared with synthetic antioxidants BHT and trolox. The highest % inhibition value for $DPPH^{\bullet}$ radical was found to be 65.339 ± 0.443 and 74.792 ± 0.764 for BHT and shown in Table 2. IC_{50}, decreaseing with 50% the initial DPPH concentration. Lower IC_{50} values indicatee higher antioxidant potential. [34]. IC_{50} value of 33.974 ± 1.918 μg/mL for *R. cornifolius* and 26.979 ± 1.116 μg /mL for BHT were recorded and given in Table 2. From these results, it was found that the % inhibition value of the plant was low compared to BHT, but the IC_{50} value was high and in this case it was less effective than BHT in scavenging $DPPH^{\bullet}$ radical. Methanol extract of the plant showed significant antioxidant activity by $DPPH^{\bullet}$ method. In previous studies, the IC_{50} values for $DPPH^{\bullet}$ were 21.04 ± 1.35 and 11.82 ± 1.06 μg/mL and for BHT were 19.50 ± 1.06 and 19.30 ± 1.06 μg/mL in *R. kurdica* and *R. pallasi subsp.sintenisii* plants, respectively [17,11].

In our study, the scavenging capacity of the methanol extract of *R. cornifolius* on $ABTS^{\bullet+}$, a stable radical, was determined and compared with trolox, a water-soluble analogue of vitamin E. The highest % inhibition value of the plant on $ABTS^{\bullet+}$ radical was found to be 89.333 ± 2.571 and 95.493 ± 0.849 for trolox. The lower the IC_{50} value, the higher the radical reducing activity. The IC_{50} value was calculated and the values of 50% inhibition of $ABTS^{\bullet+}$ radical were measured as 58.197 ± 1.826 μg/mL for the plant and 27.854 ± 1.352 μg/mL for trolox and all results are shown in Table 2. Trolox is a powerful synthetic antioxidant. Although the plant showed a lower percentage of inhibition, the IC_{50} value was higher than trolox, which can be concluded that it is less effective in scavenging $ABTS^{\bullet+}$ radical.

In previous studies, the $ABTS^{\bullet+}$ radical scavenging activity of *R. prinoides*, a species close to the plant we studied, was compared with BHT and trolox. IC_{50} values in these results were measured as 0.596 ± 0.005 μg/mL for the plant, 0.059 ± 0.003 μg/mL and 0.041 ± 0.002 μg/mL for BHT and trolox, respectively [24]. Although the plant extract was high in terms of IC_{50} values against synthetic antioxidants BHT and trolox, it can be said that polyphenols containing active H^{+} atom donors in the plant exhibit strong antioxidant properties in capturing these radicals.

Phenylhydrazine (PHZ) is used as an anaemia inducing agent in laboratory animals [35]. The toxic effect of PHZ for the body is associated with its effect on red blood cells (RBC). The main function of RBCs is to carry oxygen to cells. PHZ causes anaemia by decreasing the number of RBCs and consequently the haemoglobin level and packed cell volume (PVC) [36].

In this study, anti-haemolytic activity values were determined for both the methanol extract of the plant leaf and trolox, which was used for comparison. For this, the highest inhibition values were measured as 71.56 ± 2.39 for phenylhydrazine and 58.22 ± 0.31 for trolox. On the other hand, the IC50 values for phenylhydrazine and trolox were measured as 68.51 ± 5.33 µg/mL and 58.22 ± 0.31 µg/mL, respectively, and these values are shown in Table 2.In terms of anti-haemolytic activity, *R. cornifolius* extract showed weak values against trolox. However, the molecular diversity of antioxidant substances may always prevent the formation of a positive or linear relationship. Therefore, different methods should be used to make accurate judgements about antioxidant capacities in plants. In previous studies, a slight increase was found in the extracts of the leaves of *R. alaternus*, a neighbouring plant, at a concentration of 50 mg/mL [37]. When the plants were compared, it was concluded that *R. cornifolius* showed higher haemolytic power.

CONCLUSIONS

In this study, it is important that the results analysed in the methanol extract of the leaves of *R. cornifolius* are the first and in this respect, it is important to be included in the literature. On the other hand, the determination of vitamin A, E and K values only in this study in the *Rhamnaceae* family, which contains a very large number of species, has a different importance. The variety and richness of macromineral (K and Na) and micromineral (Fe, Mn, Zn, Co, Cu, V, Cr, Se and Mo) content, which are necessary to maintain optimal health and growth, indicate that it will be consumed as food. In this study, it was concluded that vitamin C (ascorbic acid), total phenolic content, flavonoid content and total antioxidant potential of the plant were at sufficient level and these results of the plant will be recorded and will constitute a reference for further studies. Our analysis of the plant extract's antiradical properties revealed its effectiveness in countering radical attacks, with $ABTS^{\bullet+}$ and DPPH• assays confirming strong antioxidant capacity (IC_{50} =µg/mL). These results comprehensively demonstrated that *R. cornifolius* is a good source of natural polyphenols, provides important parameters for further studies and will be a potential candidate for future in vivo studies.

EXPERIMENTAL SECTION

Plant collection location and preparation

R. cornifolius Boiss. Hoh: Turkey. C9 Hakkari: Yüksekova, Oremar region, Sat mountains, Herduav locality, 1320 m, 37°22'41", 44°10'08", 7 July 2018, *M. Fırat* & *A. Bakır*. Species identification of the plant was made

by Dr. Mehmet FIRAT and specimen vouchers were deposited to the herbarium with the code number 34042 (VHLF).

The leaf and characteristic appearance of *R. cornifolius* are given in Figure 7. The leaf part of the plant was used in the study. The flowering period was selected as the most effective time to obtain the maximum yield of the plant leaf. The leaves of the plant were dried in a cool place without light for three weeks. After drying, the leaves were ground to a fine powder in a plant grinder and stored in glass bottles for analyses.

(a) *(b)*

Figure 7. Characteristic images of *R. cornifolius*. a) general view of the plant, b) leaf view of the plant

Preparation and analysis of methanol extract of plant leaves

For the preparation of MeOH extract of the plant extract, the method of Cai *et al.* [38] was modified and adapted to this study. An appropriate amount (20 g) of dry plant leaves, previously pulverised in a plant grinder and stored in glass bottles, was weighed and transferred to a coloured bottle and MeOH at 80% concentration was added. The plant leaf, which was kept away from light, was subjected to extraction at 30˚C for 36 h in a water bath with stirrer. Then centrifuged at 4500 rpm for 15 min and filtered with suitable filter paper (Whatman No. 1). MeOH in the filtrate was extracted under reduced pressure at 40˚C using a rotary evaporator and removed. Finally, the crude extracts were lyophilised in a -65˚C refrigerator until dry and stored in the dark at +4˚C for further analysis. The yield of methanol extracts obtained from the leaf of *R. cornifolius* was measured as 28.97% according to the formula below.

$$\%\ yield = \frac{\text{Weight of the crude (g)}}{\text{Weight of the dried } R.cornifolius \text{ powder used for extraction (g)}}$$

Determination of element content

The element content of *R. cornifolius* leaves was determined by dry digestion method [39]. According to this method, 1 g of *R. cornifolius* leaf samples were placed in porcelain crucibles and 2 ml of ethanol+sulfuric acid was added. For the results to be reliable, 3 replicates were performed. Initially, the muffle furnace was set at 250˚C and gradually increased (by 50˚C per hour) to 550˚C.

The purpose of doing this is to heat the samples in a controlled manner and not to damage the elements in them. The samples made in porcelain crucibles were placed in the muffle furnace, which reached a temperature of 250˚C. When the oven temperature reached 550˚C, the samples were trapped in the muffle furnace until the next day. 5 mL HCl was added to the cooled samples. After the samples were filtered with blue band filter paper, they were removed from the muffle furnace, subjected to a series of procedures and prepared for analysis using Inductively coupled plasma (ICP) and optical emission spectrometry (OES) [Brand of the device: thermo scientific; model: ICAP6300DOU] and ınductively coupled plasma (ICP) and mass spectrometry (MS) [Brand of the device: Thermo scientific; model: X II series].

Determination of total phenolic content

The total phenolic content in the extract of *R. cornifolius* was measured spectrophotometrically after some modifications in the FCR method [40]. To the leaf extract samples of the plant prepared by dilution with MeOH, 300 µl of 2% sodium carbonate, 100 µl of FCR marker were added and incubated at room temperature for 2 hours. The absorbance of the samples was read at 765 nm. A standard curve was prepared using different concentrations of gallic acid solutions. Results were reported in milligrams of gallic acid equivalent (GAE) per gram of dried extract (mg GAE/g).

Determination of total flavanoid content

Flavonoid content of *R. cornifolius* extract was determined using aluminium nitrate method [41,42]. First, 500 µL of the diluted methanol extract stock solution was taken. Then, 100 µL of CH_3COOK, 100 µL of $Al(NO_3)_3$, and 4600 µL of ethanol were added sequentially. The solutions were then vortexed and incubated at room temperature for 45 minutes. Finally, the absorbances were read against the control sample at 415 nm wavelength. Flavonoid concentration was calculated by plotting quercetin (QE) and the total flavonoid content of the samples was reported as quercetin equivalent (mg QE/g).

Determination of total antioxidant capacity

The aim is based on the reduction of acidic Mo-VI to Mo-V by a series of reactions to form the green coloured phosphate/Mo(V) compound at acidic pH [43]. Different concentrations of the plant MeOH extract were prepared. 200 µL of these samples were taken and 0.2 L of marker solution [600 mM H_2SO_4 + 28 mM Na_2HPO_4 + 4 mM $(NH_4)_2MoO_4$)] was added, followed by 2 h in a water bath previously set at 100˚C. The samples were removed from the water bath and allowed to stand at room temperature. The samples were read against the control sample at 695 nm wavelength. Total antioxidant capacity was calculated by drawing ascorbic acid standard graph and total antioxidant capacity of the samples was given as mM ascorbic acid/g.

Determination of DPPH$^{\bullet}$ radical scavenging activity

In the study, free radical scavenging activities of MeOH extracts of the plant were determined using DPPH$^{\bullet}$ [44,45]. In order to determine the free radical scavenging activity of the prepared extracts by using DPPH 'total antioxidant potential' measurement method, 2 mg/mL stock extract solution was diluted with MeOH and prepared in different concentrations. DPPH$^{\bullet}$ system follows a stable radical generating procedure. In order to determine the scavenging activity of this radical, 4000 µl of 0,04 mg/mL DPPH$^{\bullet}$ solution was added to the solutions of different concentrations prepared by dilution with MeOH and incubated at appropriate temperature for 45 min. Absorbance readings were made at 517 nm. Absorbance values of the prepared samples were expressed against the control. BHT, a synthetic antioxidant, was used as a control against the sample. Antiradical activity was determined from the formula below [46].

$$\text{Inhibition (\%)} = \left\{ \frac{A_{\text{Blank}} - A_{\text{Sample}}}{A_{\text{Blank}}} \right\} X\ 100$$

Using the above % inhibition formula, the plant extraction concentrations were calculated as IC_{50} (The micromolar concentration required to inhibit half of the radical formed in any given situation is µg/mL.) [47,48].

Determination of ABTS$^{\bullet+}$ radical scavenging activity

ABTS$^{\bullet+}$ radical is produced by the reaction of ABTS$^{\bullet+}$ with an oxidising species. Sodium persulfate was used in our study. Firstly, ABTS$^{\bullet+}$ (0.002 M) and $K_2S_2O_8$ (2.45 mM) solutions were mixed to prepare the reactive radical. This mixture was incubated in a dark place for 13-16 hours. 1800 µL of ABTS$^{\bullet+}$

reagent was added to each of the test samples (200 µL) and measured at 734 nm after 2 h in the dark room. Plant methanol extract was carried out by modifying the method described by [49,50]. Trolox was used as synthetic antioxidant. The active power of $ABTS^{\bullet+}$, a redox radical, was calculated from the following formula [51].

$$\text{Inhibition (\%)} = \left\{ \frac{A_{Blank} - A_{Sample}}{A_{Blank}} \right\} X\ 100$$

Haematological parameter analysis

The radical scavenging activity of *R. cornifolius* leaf extract after haemolysis of erythrocytes with phenylhydrazine (PHZ) was determined according to this method [52]. Briefly, 1 mL PHZ, 0.1 mL 20% PCV, 1.850 mL buffer were added to samples prepared from different concentrations of methanol extract of *R. cornifolius* leaves. 37˚C for 1 hour and then centrifuged at 4000 rpm for 10 minutes. After transferring the supernatant to other tubes, the absorbance at 540 nm was read against the control sample. The results were compared with trolox.

Determining vitamin C content

Vitamin C content in *R. cornifolius* dry leaves was determined by spectrophotometric device at 521 nm wavelength. The first step in the determination of vitamin C content was to add 2 mL of HPO_3 and 0.5% oxalic acid to 1 g samples (3 replicates) and centrifuge at 5000 rpm for 5 min. After centrifugation, 2000 µL of the filtrate was taken, 1-2 drops of thiourea and 500 µL of 2,4-dinitrophenylhydrazine (2,4-DNFH) were added and taken in a water bath at 100˚C. After the water bath, sulfuric acid solution was added slowly. The tubes prepared at room temperature were vortexed for 5 min. Absorbic acid concentrations of the samples were calculated using the calibration graph obtained [53,54].

Vitamin A, E and K analyses

Standard solution and calibration

Retinol, α-tocopherol and phylloquinone stock solutions were prepared at 500 µg/mL. To prepare the standard solution, the stock solutions were diluted with methanol accordingly. Linear regression analysis of the peak area versus standard solution concentrations was used to calculate the calibration.

Vitamin extraction process

The amounts of retinol, α-tocopherol and phylloquinone in *R. cornifolius* leaves were determined by modifying the studies of [55,56]. Weighed 4000 mg of dried and shade ground plant leaf samples were extracted with n-hexane and EtOH. BHT (0.02%) was added to the prepared samples, mixed at the appropriate time and then kept in the dark for 30 hours. Then centrifuged at +4˚C and 5000 rpm for 15 min.

The clear solution in the resulting phase was filtered using filter paper and 500 µl of n-hexane was added. Finally, it was left to dry under nitrogen gas (at 37˚C). Immediately after the drying process was completed, the remaining part was dissolved in 250 µL of MeOH+C_4H_8O (tetrahydrofuran) (98%) and made ready for analysis.

Chromatographic conditions

Vitamin retinol, α-tocopherol and phylloquinone analyses were performed on a GI Science C_{18} reversed phase high performance liquid chromatography column (250 x 4.6 mm ID), MeOH (80 ml) + tetrahydrofuran (20 ml) mobile phase, 1500 µL/min flow rate at 24˚C. HPLC - applications were performed for retinol (325 nm), α-tocopherol (290 nm) and phylloquinone (248 nm) in 0.1 mL volumes in dark coloured vials in a tray autosampler (-10˚C) using a PDA array detector. Chromatographic values of vitamins are shown in figure 8. Also chromatographic analysis measurements were performed by isocratic elution (40˚C), a separation technique of HPLC.

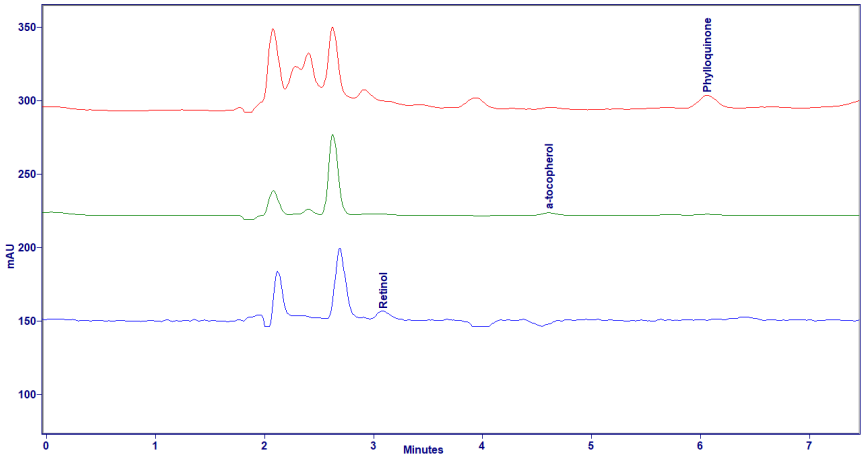

Figure 8. HPLC chromatogram of retinol, α-tocopherol, and phylloquinone at 325, 290, and 248 nm

Statistics data

The data of the measurement results are expressed as mean and mean standard error (X ± SEM). Group graphs are also expressed in the same way (X ± SEM). Nonlinear regression analysis was used to find the IC_{50} measurement data. The measurements are presented in three replicates.

Running head: ANTIOXIDANT, ANTI-HAEMOLYTIC ACTIVITY OF *R. CORNIFOLIUS*

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