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Phenolic Characterization and *in vitro* Biological Activities of *Ranunculus Cornutus* DC.

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Abstract
In this study, *Ranunculus cornutus* DC. (Evlimemedotu) content analysis and some biological activities were investigated. For this purpose, methanol and water extracts of the plant were taken and their phenolic analyzes were determined by LC-MS-MS. Total antioxidant method, FRAP, CUPRAC, iron chelation method, DPPH and ABTS methods were used for antioxidant studies. According to LC-MS-MS results, the highest amounts of phenolic substances were determined as fumaric acid (3261.43 µg/L), vanillic acid (1217.70 µg/L) and ferulic acid (769.40 µg/L), respectively. According to the antioxidant results, it was determined that water and methanol extracts exhibited lower activity than standard antioxidants. It can be said that the reason for this is the amount of phenolic they contain. However, it was observed that the methanol extract generally had better antioxidant activity than the water extract. Finally, according to the DNA study results, it was determined that water and methanol extracts alone did not have a negative effect on the stable structure of DNA. Since there are no studies with *R. cornutus* DC., it is thought that this study will make a very important contribution to the literature and provide important data for future studies.

1. Introduction

Ranunculaceae family, which has approximately 50 genera and 2000 species in the world, has about 85 species in our country. 15 of these species are endemic [1]. *Ranunculus* species, which are annual or perennial herbaceous, rarely shrub-like or climbing plants, are also known by the names of "buttercup, hemlock and evlimemedotu" among the people [2]. These plants are used in the treatment of various diseases among people in many parts of our country. It is used in the treatment of diseases such as constipation, rheumatic diseases, hemorrhoids, edema, abscess, healing of wounds and jaundice [1], [3], [4].

Several compounds isolated from Ranunculus species have previously undergone clinical trials. Some are used in the treatment of various conditions such as cancer, cardiac dysfunctions, severe hemostasis, and various types of inflammation [5]–[7]. Numerous reports have been made of the traditional use of members of the Ranunculaceae family in the treatment of various oxidative stress-related diseases. Despite this fact, as far as we know, studies on the antioxidant properties of these plants are limited [8]–[11].

It has been reported by various studies that oxidative stress, which occurs as a result of excessive accumulation of reactive oxygen species in the organism, causes various diseases [12], [13]. In recent years, there have been various studies on the interaction of compounds with DNA. It is known that DNA damage occurs as a result of excessive accumulation of free radicals in the organism [14]–[16]. For this reason, it is important whether the DNA remains stable as a result of the interaction of plants with plasmid DNA.

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In our literature search, no study on the biological activities of *R. Cornutus* (RC) was found. Therefore, in this study, phenolic content analysis was performed by preparing methanol and water extracts of RC. The total antioxidant method according to thiocyanate method, ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity (CUPRAC), iron chelation activity, 1,1-diphenyl-2-picrylhydrazil (DPPH), 2,2'-azino -bis(3-ethylbenzothiazoline-6-sulfonate) methods were used for the antioxidant analysis of extracts. PBR322 plasmid DNA was used to determine the interaction of extracts on DNA. Since no previous studies on this plant have been found, this study will make a very important contribution to the literature and will be a reference for future studies.

2. Material and Method

2.1. Plant material

R. cornutus DC. was collected from Eastern Anatolia, Muş, Çöğürlü Village and the species were determined by Murat Kurşat from the Biology Department of the Faculty of Arts and Sciences at Bitlis University. Chemical processes were carried out in the laboratories of Muş Alparslan University.

2.2. Preparation of Extracts

After the plants were collected during the vegetation period, they were left to dry. After species determination, water (RcWtr) and methanol (RcMetOH) extracts were obtained. For extracts, approximately 50 g of plant samples were dissolved in 300 mL of solvent. The extracts prepared with the help of the Soxhlet Extraction Device were filtered with the help of filter paper and lyophilized in the Laboratories of the Chemistry Department of Muş Alparslan University. It was stored in colored bottles at +4 ºC until analysis.

2.3. Phenolic Compound Analysis by LC-MS/MS

Detection of phenolic compounds was performed with Agilent 6460 Triple Quadrupole LC-MS/MS (Liquid Chromatography-Tandem Mass/Mass Spectrometer, Agilent Technologies) equipped with a Zorbax SB-C18 (4.6x100mm; 3.5 Micron) column. Analysis mode is multiple reaction monitoring mode (MRM). The mobile phase was filtered with a 0.45 µm Millipore membrane filter before use. The total run time was 7.0 minutes. The injection volume for sample extracts was 5 µl. Mobile phase A consisted of 0.1% (v/v) formic acid in water (solvent A) and mobile phase B consisted of 0.1% formic acid in acetonitrile (solvent B). LC-MS/MS analyzes were performed in the laboratories of the Eastern Anatolia Advanced Technologies Research and Application Center (DAYTAM).

2.4. Total Antioxidant Activity Assay

According to the thiocyanate method [17], the volumes of the extracts at 15µl, 30µl and 45µl concentrations were made up to 500 µl with phosphate buffer and 500 µl of linoleic acid was added to each. The extracts were incubated at 37 °C. Every eight hours, 20 µl of the extract was taken and placed in test tubes containing ethanol. 20 µl of Fe^{2+} and SCN^- solutions were added to each. The absorbance of the samples at 500 nm was read on a Microvolume Spectrophotometer (Thermo Scientific Multiscan Go).

2.5. FRAP Assay

Distilled water was used to make the volumes of the extracts of different concentrations (15, 30 and 45 µg/ml) to 200 µl. 500 µl of phosphate buffer and potassium ferricyanide [$K_3Fe(CN)_6$] were added to the extracts. After 20 min incubation at 50ºC, 500 µl of trichloroacetic acid (TCA) was added. The same amount of distilled water and 100 µl of $FeCl_3$ were added to the 500 µl mixture taken from the upper phase, and the absorbance values at 700 nm were recorded [18].

2.6. CUPRAC Assay

For the test, extracts of different concentrations and standard antioxidants were placed in the sample cups and their volumes were completed to 1 ml with distilled water. After adding 250 µl of $CuCl_2$ solution, ethanolic neocuprine and acetate buffer, they were incubated at room temperature for 30 minutes. Finally, absorbances at 450 nm were recorded [19].

2.7. Metal Chelating Ability

Iron chelating activities of extracts and standard antioxidants (BHA, BHT and Ascorbic acid) were performed according to the previous method [20]. For this process, 2 mM solution containing 100 µL of $FeCl_2.4H_2O$ and 70 µL of distilled water was added to 50 µL of solution containing samples at concentrations of 15, 30 and 45 µg/ml. The total volume was made up to 1 ml by adding distilled ethanol. The reaction was initiated by adding 50 µL of 5 mM ferrozine solution. After mixing the solution

in a vortex, it was incubated at room temperature for 10 minutes. After incubation, the absorbance of the solutions at 562 nm was recorded using a UV-Vis spectrophotometer.

2.8. DPPH Radical Scavenging Assay

The DPPH free radical scavenging capacities of the samples were determined according to the method of Blois [21]. Samples (15, 30 and 45 µg/µl) were taken into test tubes and their total volume was made up to 600 µl with ethyl alcohol. After adding 200 µl of DPPH• solution on them, they were incubated for 30 minutes. Finally, their absorbances at 517 nm were recorded.

2.9. ABTS Radical Scavenging Assay

According to the method of Re et al., the ABTS solution was first diluted with phosphate buffer (pH 7.4) to obtain an absorbance of 0.750 ± 0.025 at 734 nm. Then, 15, 30, and 45 µL of stock solutions were taken and their volumes were made up to 100 µL with distilled water. 1 mL of $ABTS^{\bullet+}$ solution prepared on them was added and vortexed. Radical scavenging activity was measured at 734 nm [22].

2.10. Plasmid DNA Interaction Assay

The possible effect of *R. cornutus* DC. on DNA was determined by agarose gel electrophoresis method using pBR322 plasmid DNA [23]. For this purpose, the main stock materials were prepared by dissolving 10 mg of the substance sample in 1 ml of DMSO. These stocks were diluted to use substances with concentrations of 1, 0.5 and 0.25 mg/ml. Amounts to 16 PCR tubes, respectively; 1. pBR322 DNA (50 ng) 10 µl, Hydrogen peroxide (H_2O_2) 5 µl, 3. DMSO 10 µl and 4. *R. cornutus* DC. It was prepared to be completed to 25 µl in total with concentrates. 5 µl of deionized distilled water was used to make up the tubes without adding H_2O_2 to 25 µl. The effect of each sample on DNA was determined by agarose gel electrophoresis. PCR tubes were incubated in the dark (24 hours at 37 ºC). After incubation, 5 µl of the DNA mixture was mixed with loading buffer and loaded onto a 1% agarose gel. Then electrophoresis was performed in TBE buffer at 40 V for 2-3 hours. After electrophoresis, the gels were stained with ethidium bromide and the gel photographs were viewed with the aid of the CemiDoc XRS BIORAD imaging device.

3. Results and Discussion

3.1. Phenolic compound analysis

The phenolic compound composition of *R. cornutus* DC. analyzed by LC-MS/MS method was evaluated with the phenolic compounds introduced as standard. As seen in Table 1, fumaric acid (3261.43 µg/L), vanillic acid (1217.70 µg/L), ferulic acid (769.40 µg/L), caffeic acid (509.10 µg/L), The amount of chlorogenic acid (311.90 µg/L) and p-Coumaric Acid (136.60 µg/L) were higher than the others (Sinapic Acid, Cyanidin-3-O-Glucoside, Quinic Acid, Rosmarinic Acid, etc.).

In the literature search, no study was found on the phenolic contents of *R. cornutus*. However, there are studies on phenolic substance analyses of different *Ranunculus* species. In a study conducted by Bhatti et al. in 2015, the presence of caffeic acids (0.017%) in the methanol extract of the *R. arvensis* plant was shown. In addition, it was determined that there was less caffeic acid (0.008%) in the water extract compared to the methanol extract [24]. In another study; The presence of quercetin-7-O-glucoside and rutin in R. peltatus extracts was determined [25]. It has been reported by Noor et al. that *R. repens* contains many flavonoids and phenolics [26]. In another study conducted by Campos et al in 1996, the phenolic profiles of pollen belonging to the *R. sardous* Crantz species were examined. It has been stated that all pollens analyzed contain flavonol glycosides, usually quercetin, kaempferol, herbacetin or isorhamnetin derivatives, and some contain myricetin, tricetin, luteolin and 3-O-methylquercetin. Significant levels of phenolic acid derivatives were also detected in some pollen [27]. In the study conducted by Wang et al., it was reported that the extract of the *R. japonicus* species contains Chaphthoside, Apigenin-7-O- β-D-glucoside, Yangonin, Luteolin-7-O- β-D-glucoside and Berberine [28]. In a study by Deghima et al, it was reported that *R. macrophyllus* ethyl acetate extract contains high amounts of gallic acid, dihydroxybenzoic acid and hesperidin [29].

Table 1. Quantitative determination of phenolic compound content of *R. cornutus* DC by LC-MS/MS method

Phenolic Compounds	RT	Concentration (µg L^{-1})
4-OH-Benzoic Acid	11,269	33,8778
Apigenin	14,033	ND
Ellagic acid	12,073	ND
Epigallocatechin Galate	11,518	ND
Epicatechin	11,233	ND
Ferulic Acid	12,571	769,4048
Fumaric Acid	3,597	3261,4255
Galanjin	15,672	ND
Gallic acid	5,451	ND
Hesperidin	11,937	26,9232
İsorhamnetin	14,160	ND
Caffeic Acid	11,571	509,1006
Catechin	11,174	ND
Keracyanin Chloride	10,385	0,5261
Quinic Acid	2,390	36,3593
Chlorogenic Acid	10,896	311,8981
Chrysin	15,586	ND
Quercetin	13,494	ND
Curcumin	15,682	ND
Luteolin	13,423	ND
Myricetin	12,753	ND
Naringenin	14,062	ND
Naringin	11,909	ND
Peonidin-3-O-Glucoside	11,039	9,3682
Pyrogalol	6,570	ND
p-Coumaric Acid	12,312	136,5990
Resveratrol	13,107	10,6104
Rosmarinic Acid	12,520	35,0895
Sinapic Acid	12,367	50,1630
Syringic Acid	11,657	ND
Cyanidin-3-O-Glucoside	10,706	38,8923
Taxifolin	12,391	ND
Vanillic Acid	11,877	1217,7027
Vanillin	12,617	11,3878
Vitexin	11,736	ND

ND: not detected, RT: Retention time

3.2. Antioxidant Activity

Six different tests were used to determine the antioxidant properties of RCWtr and RCmetOH. FRAP and CUPRAC results were calculated as µg TE/ml and other test results were calculated by taking the IC50 value (Table 2).

Ferric thiocyanate method was used for the total antioxidant activity measurements of the extracts. For this purpose, the lipid peroxide removal percentages of the extracts were calculated using the equation below.

According to the total antioxidant results, it was observed that standard antioxidants scavenge lipid peroxides better, however, the activities of the samples generally increased depending on the increase in concentration. In addition, it was determined that water extract (IC50:106,79) exhibited stronger activity than methanol extract (IC50:146,41). The lipid peroxide removal powers of the samples were respectively as follows: BHT (IC50: 24.96) > BHA (IC50: 26.13) > AA (IC50: 46.72) > RcWtr (IC50: 106.79) > RcMetOH (IC50: 146.41).

Iron ions (Fe^{3+}) reduction analysis (FRAP) can be defined as an electron transfer. It can be defined as an electron transfer-based assay that measures the reducers (antioxidants) present in a sample based on a spectrophotometric redox reaction [30]. According to the FRAP results, it was observed that BHA, BHT and AA used as standard were stronger reducing agents than extracts. According to

the table, BHA (90.34 µg TE/ml) showed the best effect, while AA (38.71 µg TE/ml), BHT (18.16 µg TE/ml), RcMetOH (6.01 µg TE/ml) and RcWtr (5.03 µg TE/ml) followed.

$$\text{Lipid peroxidation scavenging } (\%) = 100 - (\frac{As}{Ac} x100) \quad (1)$$

Table 2. Antioxidant activity results from extracts and standard antioxidants (BHA, BHT and AA)

Samples	Total antioxidant activity (IC50)	FRAP (µg TE/ml)	CUPRAC (µg TE/ml)	Metal chelating (IC50)	DPPH (IC50)	ABTS (IC50)
Rc-Wtr	106.79±0.12	5.03±0.01	6.21±0.01	128.2±0.77	152.67±0.06	155.23±0.22
Rc-MetOH	146.41±0.04	6.01±0.02	7.88±0.01	124.1±1.98	121.24±0.02	125.43±0.15
BHA	26.13±0.02	90.34±0.01	101.47±0.03	38.29±0.81	20.14±0.01	18.29±0.08
BHT	24.96±0.02	18.16±0.01	72.81±0.04	38.1±0.43	26.86±0.04	24.65±0.02
AA	46.72±0.01	38.71±0.02	21.33±0.01	32.41±0.91	19.4±0.01	20.19±0.03

TE: Trolox equivalent

According to the results of the Kuprak method, it was observed that BHA, BHT and AA reduced Cu^{+2} ions better than the extracts. According to the table, BHA (101.47 µg TE/ml) exhibits the best effect, while BHA (72.81 µg TE/ml), AA (21.33 µg TE/ml), RcMetOH (7.88 µg TE/ml) and RcWtr (6.21 µg TE/ml) followed.

The metal chelating activity results were in line with the FRAP results. The results showed that BHA, BHT and AA chelated iron ions more strongly than extracts. According to Table 2, AA (IC50: 32.41) showed the best effect, while RcWtr (IC50: 128.2) showed the lowest activity.

The antiradical activities of the extracts and standard antioxidants were investigated by two different methods and it was observed that the results supported each other. According to the DPPH radical scavenging results, it was observed that BHA, BHT and AA scavenged radicals more powerfully than the extracts. According to the table, AA (IC50: 19.4) showed the best effect, while BHA (IC50: 20.14), BHT (IC50: 26.86), RcMetOH (IC50: 121.24) and RcWtr (IC50: 152.67) followed. Similarly, BHA, BHT, and AA were found to scavenge $ABTS^{\bullet+}$ radicals more strongly than extracts. According to the table, AA (IC50: 20.19) showed the best effect, while RcWtr (IC50: 155.23) showed the lowest activity.

In the literature, no study was found on the antioxidant properties of the *Rc* DC. However, there are various studies on different species of the same family. In a study, it was determined that *R. macrophyllus* ethyl acetate extract had better antioxidant capacity than chloroform, hexane and water extracts. It has also been reported that the ethyl acetate extract exhibits stronger performance than the standard antioxidants of BHA and quercetin [29]. In another study, it was stated that *R. repens* methanol extract showed the best antioxidant activity [31].

Shadid et al. [32] reported that *R. sceleratus* ethyl acetate extract reduced iron ions most strongly, while Say et al. [33] reported that *R. constantinopolitanus* methanol extract reduced Cu^{+2} ions better than hexane, ethyl acetate, water and trolox extracts.

When metal chelating activities of different extracts were examined in previous studies, it was observed that some extracts formed strong chelates with ferrous metal, while some extracts had very weak activities [34], [35].

According to the results of DPPH and ABTS radical scavenging activity of n-hexane, ethyl acetate, methanol and water extracts prepared from *R. marginatus* and *R. sprunerianus* species, it was observed that methanol extract performed stronger than other extracts [34]. In another study, it was stated that *R. sceleratus* ethyl acetate extract scavenged DPPH radicals better than other extracts [35]. Solanki et al. [35] reported that R. sceleratus ethanol extract scavenges ABTS radicals better than chloroform extract.

While this study has similar aspects to previous studies, it also has different aspects. This situation is thought to be caused by the difference in

of factors such as different species, climatic conditions, different solvents, phenolic content, etc.

3.3. Interaction with plasmid DNA

pBR322 plasmid DNA was used to determine the interaction of the extracts with DNA (Figure 1).

DNA can be found in different forms in organisms, and these forms are shaped according to the genetic character of the organisms. When any molecule interacts with this DNA, it can cause these forms to degrade or turn into each other. The double-stranded superhelix structure of DNA is known as form I. After one of the strands forming the Form I structure is broken, Form II, which moves more slowly in the agarose gel and has a looser structure than Form I, occurs. In addition, after the other strand is broken, the DNA becomes linear and this structure is known as Form III [36].

According to the results of the study, it was observed that H_2O_2 and H_2O_2+DMSO disrupted Form I, making it more unstable. It was observed that water and methanol extracts alone did not have a negative effect on the stable structure of DNA and kept it in Form I and Form II structures. In addition, it was determined that the increase in concentration did not have a significant effect on DNA, however, a decrease in the Form III structure occurred with the increase in concentration in the water extract. When the activities of water and methanol extracts to reduce the harmful effects of peroxide on DNA were compared, it was determined that the water extract was more effective. When water extract was used together with peroxide, it was determined that DNA remained in Form I and Form II structures, but methanol extract did not show a significant effect.

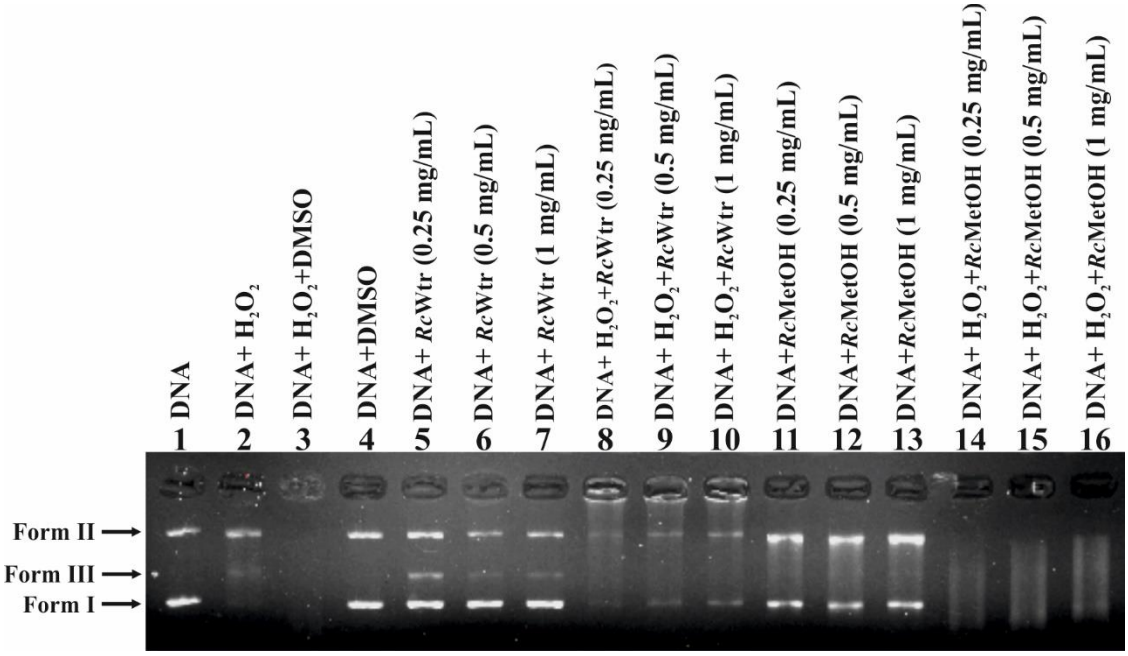

Figure 1. Comparison of the effects of *R. cornutus* DC. extracts on DNA

Studies on the plasmid DNA interaction of *Ranunculus* species are very limited. In a study, it was reported that the ethanol extract of *R. japonicus* Thunb was able to inhibit the replication of Hepatitis B Virus (HBV) in more than one step and showed inhibitory effects, especially on CCC DNA formation and capsid formation. This indicates that *R. japonicus* Thunb as a source of antiviral agents against HBV specifically targets viral CCC DNA, which is the molecular basis of HBV chronic infection [37].

4. Conclusion and Suggestions

In this study, the content and biological activities of *R. cornutus* grown in Muş province were investigated. In the literature review, no study was found about the biological activities of this plant species. For this reason, after the secondary metabolite content of the plant was determined by LC-MS-MS, different methods were used to determine in vitro antioxidant activities. The results were compared with the standard antioxidants (BHA, BHT and AA). Finally, pBR322 plasmid DNA was used to determine the effects of the plant on DNA. When the phenolic content results were examined, it was determined that

the plant contained fumaric acid, vanillic acid, ferulic acid, caffeic acid, chlorogenic acid and p-Coumaric Acid metabolites more than the others, but overall the total phenolic content was at an average value. When the antioxidant results were evaluated, it was seen that the water and methanol extracts of the plant exhibited lower activity than the standard antioxidants. It can be said that the reason for this is related to the amount of phenolics they contain. However, it was observed that the methanol extract generally had better antioxidant activity than the water extract. According to the results of the effects of the extracts on DNA, it was determined that the water extract was more effective. When water extract was used together with peroxide, it was determined that DNA remained in Form I and Form II structures, but methanol extract did not show a significant effect. Considering all the results, we believe that this study will make very important contributions to the literature and will shed light on the studies to be done with this plant.

Acknowledgment

Conflict of Interest Statement

There is no conflict of interest between the authors.

Statement of Research and Publication Ethics

The study has complied with research and publication ethics

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