



LC-MS/MS Analysis, Antioxidant Properties and Inhibition Effect on Some Important Metabolic Enzymes of *Nicotiana rustica* L

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ABSTRACT

Being widely used since its discovery, tobacco contains many chemical substances including nitric oxide, hydrocarbons, aldehydes, phenols, quinone and semiquinone radicals, and causes the formation of oxygen-induced free radicals directly or indirectly. Therefore, it is important to know the oxidant/antioxidant properties, phenolic content of *Nicotiana rustica* Linn., and the effect studies on Paraoxonase (PON 1) and Acetylcholinesterase (AChE) which are important metabolic enzymes. In the study, the effect of extracts (water and methanol) of *Nicotiana rustica* Linn on PON 1 and AChE enzyme activity was examined. Water extract of *Nicotiana rustica* Linn Maras Powder with walnut, (MPWW) and methanol extract Maras Powder with *Nicotiana rustica*, (MPNR), Maras Powder with walnut, (MPWW) and Maras Powder with copper cauldron, (MPCC) had an inhibitory effect on AChE (IC₅₀ values in the range of 0.104-0.203 mg ml⁻¹). Also water extract MPCC and methanol extract MPNR, MPWW and MPCC showed an inhibition effect on PON 1 (IC₅₀ values 0.013-0.225 mg ml⁻¹). MPNR water extract showed the highest activity with 26% DPPH radical scavenging activity. MPCC water extract showed a 13% ABTS radical scavenging activity.

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Nicotiana rustica L'nin Bazı Önemli Metabolik Enzimleri Üzerindeki İnhibisyon Etkisi, LC-MS / MS Analizi, Antioksidan Özellikleri

ÖZET

Nitrik oksit, hidrokarbonlar, aldehidler, fenollar, kinon ve semikinon radikalleri gibi pek çok kimyasal maddeyi bünyesinde barındıran ve doğrudan ya da dolaylı olarak oksijen kaynaklı serbest radikal oluşumuna yol açan tütün keşfedilmesinden günümüze kadar geçen zaman içinde yaygın olarak kullanılmaktadır. Bu yüzden dolayı *Nicotiana rustica* L'nin fenolik içeriğinin, antioksidan/oksidan özelliklerinin bilinmesi ve önemli metabolik enzim olan Paraoksonaz (PON 1) ve Asetilkolinesteraz (AChE) üzerine etki çalışmaları önem arz etmektedir. Çalışmada *Nicotiana rustica* L'nin su ve metanol ektresinin, PON 1 ve AChE enzim aktivitesi üzerine etkisine bakılmış. *Nicotiana rustica* Linn'in su ektresi ceviz ile karıştırılmış maraş otu, (MPWW) ve metanol ektresi meşe ile karıştırılmış maraş otu (MPNR), ceviz ile karıştırılmış maraş otu (MPWW) ve bakır kazanlarında nemlendirilmiş maraş otu (MPCC) AChE üzerine inhibisyon etkisi (IC₅₀ değerleri 0.104-0.203 mg ml⁻¹ aralığında) gösterirken su ektresi MPCC ve metanol ektresi MPNR, MPWW ve MPCC ise PON 1 üzerine inhibisyon etkisi göstermiştir (IC₅₀ değerleri 0.013-0.25 mg ml⁻¹). En yüksek radikal giderme aktivitesini MPNR su ektresi % 26 oranında DPPH radikal giderme aktivitesi gösterirken, MPCC su ektresi % 13 oranında ABTS radikal giderme

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INTRODUCTION

Tobacco has been used in various ways since its discovery until today. Although it is mostly used by burning in the form of cigarettes today, it is also widely used in the world, directly chewing smokeless or orally. Studies have reported that most smokeless tobacco products contain substances known to be harmful such as nicotine, cadmium, and specific nitrosamines (Benowitz, 1988; Kurtul, 2005). Tobacco, which contains many chemical substances such as nitric oxide, hydrocarbons, aldehydes, phenols, quinone and semiquinone radicals, and causes the formation of oxygen-induced free radicals directly or indirectly, has been widely used since its discovery (Church, 1985). It is used as a pleasurable substance because of the nicotine alkaloid, which is found in tobacco leaves and consists of organic nitrogenous substances. Leaves of some species of *Nicotiana* genus such as *Nicotiana tobaccum* L. and *Nicotiana rustica* L. are used in different ways all over the world. Although there are approximately 65 species included in the *Nicotiana* genus, only *Nicotiana tobaccum* and *Nicotiana rustica* are used in the production of tobacco products. The main tobacco products produced for cigarettes, cigars, tobacco, pipe, snuff, shredded tobacco and chewing tobacco in the world (Davis, 1982).

Since the *Nicotiana* plant is grown in various regions, different culture forms have been formed depending on the environmental conditions. Therefore, different species or varieties were defined (Davis and Nielson, 1999). The dried leaves of *Nicotiana rustica* L. are used to make cigarettes, tobacco and smokeless tobacco called Maraş grass (Kurtul et al., 2005; Kurtul and Gökpınar, 2012). "*Nicotiana rustica* L" is used under different names in many regions of the world. In Bangladesh "bit", India and Southeast Asia, "Gutkin" and also Turkey, "Maraş powder" is known by name. In addition, *Nicotiana rustica* L., known as mouth grass, mad tobacco or Hasankeyf tobacco in Turkey, is called Turkish tobacco, East Indian tobacco or Aztec tobacco in some regions. In the Southeastern Anatolia regions of Turkey, especially around Kahramanmaraş and Gaziantep, the powder obtained from the tobacco leaf called "Maraş Herb" is used through the oral mucosa (Özkul, 1997; Erenmemişoğlu 1999). Before using *Nicotiana rustica* L., the leaves are pulverized, then the ash obtained from vine, oak rod or walnut is mixed in certain proportions, crushed and slightly moistened. It is known that the ash mixed during the preparation of tobacco creates an alkaline environment and facilitates its absorption from the oral mucosa. Approximately one teaspoon of Maraş grass is

moistened slightly and wrapped in cigarette paper, then placed inside the lower lip and used by sucking or chewing. This process is done throughout the day depending on the habit of the individual. Some individuals even sleep by placing Maraş grass in their mouths (Erenmemişoğlu, 1991). Due to the N-nitrosamines (DNAs) contained in tobacco, it can cause pancreatic, esophagus, lung, and oral cavity cancers in users. In addition, the heart and vascular system are negatively affected by smokeless tobacco use. There are also components in tobacco smoke that are reported to have anti-oxidative properties other than nicotine. (Nair et al., 1989; Chen and Loo, 1995; Lapenna et al., 1995; Kamisaki et al., 1997; Nilsson, 1998; Weitberg and Corvese, 1999). The studies show that PON1 inhibits oxidation of high-density lipoprotein (HDL) and low-density lipoprotein (LDL). It suggests that it has antioxidant properties as it prevents the lipid peroxide (Senti et al. 2003). Serum PON1 activities and levels are also affected by acute phase proteins, smoking, and diet. Several studies have been reported showing that PON1 levels are lower in smokers than in non-smokers. It has been reported that smoking plays a role an important for coronary heart disease (CHD). (Mackness et al., 2002; Aviram and Rosenblat, 2004; Işık et al., 2007; Huen et al., 2009).

The free oxygen radical (FOR) level is also normally kept in balance by neutralizing the body's antioxidant defense systems. If this balance is destroyed in favour of FOR, destructive reactions occur in molecules such as protein, lipid, nucleic acid. This condition, called "oxidative stress", ultimately leads to tissue damage (Çalkoğlu, 2002; Atlan, 2006). Studies have shown that acetylcholinesterase (AChE) inhibitors used in the symptomatic treatment of Alzheimer's disease are used to protect cells from oxidative stress. It has also been emphasized that some AChE inhibitors play a role in increasing angiogenesis in cardiovascular patients. In order to prevent the occurrence of cardiovascular disease, it is important to know the important metabolic enzymes that provide protection by reducing oxidative stress in serum and tissues. PON 1 (Paraoxonase 1), one of these enzymes, inhibits the development of atherosclerosis by preventing the oxidation of lipoproteins (Kakinuma et al., 2010; Işık et al., 2017; Işık, 2020).

The aim of the study was to determine the phenolic content of *Nicotiana rustica* L., by LS-MS / MS method. The radical removal and metal reduction activities of MPRN, MPWW and MPCC aqueous and methanol extracts and effect on the important metabolic

enzymes such as AChE and PON 1 were investigated.

MATERIAL and METHOD

General Procedure

BHT, BHA, Trolox, trichloroacetic acid (TCA), ABTS, CuCl₂, neocuprin, ammonium acetate (NH₄Ac), DPPH, Potassium ferricyanide (K₃Fe(CN)₆), potassium persulfate (K₂S₂O₈) were used for antioxidant studies and all solvents and chemical compounds were provided from E. Merck. LC-MS/MS analyses of 25 phenolic compounds were conducted using a Nexera model Shimadzu UHPLC connected to a tandem MS device. SIL-30AC autosampler, DGU-20A3R degasser, CTO-10ASVP column oven and LC-30AD dual pumps are used in the execution of chromatographic processes. The column temperature determined as 40°C in chromatographic separation was performed on a C18 intersil ODS-4 (3.0 mm x 100 mm, 2 µm) analytical column. The flow rate of the elution gradient generated from mobile phase A (Water, 0.1% Formic acid) and mobile phase B (Methanol, 0.1% Formic acid) was set at a flow rate of 0.3 mL/min and an injection volume of 2 µL.

Herbal Material

Maraş grass, '*Nicotiana rustica* Linn, is a smokeless tobacco variety widely used in the Eastern Mediterranean region of Turkey. The leaves of this tobacco plant are pulverized and mixed with oak and walnut ash and kept in copper cauldrons slightly moistened and used for different purposes (Aral et al 2006). In this study, the powder form of *Nicotiana rustica* Linn plant leaves (MPNR) mixed with oak (MPWW) or mixed with walnut (MPCC) was kept in a copper cauldron with a slight moistening.

Infusion Extraction

Extractions obtained with water, methanol and ethanol solvents were used for LC-MS / MS studies. Solvent was added onto *N. rustica* (solute/solvent ratio; 1/10) and mixed in a flask with a closed mouth for 24 hours on a shaker. For water extraction, 5 g of *N. rustica* samples were milled to a fine powder in a mill and mixed with 50 mL of 30 °C water by magnetic stirrer for 15 minutes. Then Extract Whatman No. It was filtered with 1 sheet of paper. For ethanol extraction, 5 g of *N. rustica* samples were ground to a fine powder in a mill and mixed with 50 mL of ethanol. The residue was repeated under the same circumstances until the extraction solvent became colourless. The extracts obtained were filtered with Whatman No. 1 paper. Both aqueous and ethanolic extracts were placed in falcon tubes and stored at -20 °C until use. LC-MS / MS instrument was used for bioactive component analysis in *N. rustica* extracts. Validation studies and analysis of the method

developed using 16 phenolic substances; It was made in Harran University Central Research Laboratory. It is possible to classify this phenolic compound common in plant materials as flavonoids, flavonoid glycosides, hydroxycinnamic and hydroxybenzoic acids. Analysis of phenolic compounds by LC-MS / MS was carried out using a Nexera model Shimadzu HPLC coupled to a dual MS device. Liquid chromatograph LC-30AD is equipped with double pump, DGU-20A3R degasser, KTO 10AS vp column oven and SIL-30AC autosampler. Chromatographic separation was executed with the C18 reverse-phase analytical column Inertsil ODS-4 (150 mm × 4.6 mm, 3 µm). Column temperature was kept constant at 40°C. The elution gradient was formed with mobile phase A (water, 5mM ammonium formate and 0.1% formic acid) and mobile phase B (methanol, 5mM ammonium formate and 0.1% formic acid). Gradient program was applied t (min) according to the following values of B solvent. B%: (0.40), (20.90), (23.99), (24.40), (29.40). Solvent flow rate was applied at 0.5 mL / min and the injection volume was set to 4µL. Detection of MS was done using a mass spectrometer equipped with Shimadzu LC-MS 8040 model triple, quadrupole and ESI source operation in both positive and negative ionization modes. Calculations were made by obtaining LC-MS / MS data with Lab Solutions software (Shimadzu, Kyoto, Japan). Multiple reaction tracking (MRM) mode was used to measure analysis. Three applications were made for each compound analysis in the experiment. The first analysis was done for quantitative results. The second and third analysis were made for confirmation. Optimum Electrospray Ionization (ESI) parameters; 350 ° C interface temperature, 250 ° C DL temperature, 400 ° C heatsink temperature, 3L / min. Nebullizer gas flow and 15 L / min. determined as the drying gas flow (Köksal et al., 2017). When calculating LC-MS / MS analysis results, it was calculated by considering the total dry matter ratio. In addition, the standard deviation (±) value of the results was determined with the formula = Analyte value result * U value / 100 (Taban et al., 2013; Agar et al., 2015).

Evaluation of Antioxidant Activities

Metal reducing power assay

FRAP Iron Ions (Fe³⁺) Reduction Analysis the reducing power of *N. rustica* extracts was carried out with a modified version of Oyaizu method. (Oyaizu, 1986, Elmastaş et al., 2006; Necip and Işık, 2019). 2.5 mL of 1% potassium ferricyanide [K₃Fe (CN)₆] and phosphate buffer (0.2 M, pH 6.6) were added to solutions of *N. rustica* extracts (10, 20, 40 µg mL⁻¹) in 1 mL of extraction solvent. The mixtures were then incubated at 50 ° C for 20 minutes. After incubation, FeCl₃ (0.25 mL, 0.1%) and trichloroacetic acid (2.5 mL, 10%) were added to each mixture and centrifuged (10 minutes,

3000 rpm). The absorbance values of the mixture at 700 nm were recorded and increased absorbance indicates increased reduction capacity.

The Cuprak method based on the reduction of Cu (II) -Nc to Cu (I) -Nc chelate was applied. 1 mL of CuCl₂ (0.01 M) solution and 1 mL of ammonium acetate (NH₄Ac) buffer solution was added to the test tube to 1 mL of neocuprin (2,9-dimethyl-1,10-phenanthroline; 7.5 × 10⁻³ M alcohol) and mixed by vortex. Then extracts of different concentrations (10, 20, 40 µL) were added and the total volume was adjusted to 4 mL with water. After 30 minutes incubation at room temperature, the absorbance was recorded at 450 nm and increasing absorbance indicates an increased capacity of Cu⁺⁺ ion degradation (Apak et al., 2004).

Radical scavenging activity (DPPH and ABTS assay)

DPPH scavenging activity of *N. rustica* extracts and standard antioxidants was performed by the Blois method (Blois, 1958). 0.1 mM DPPH[·] solution was prepared in ethanol, and 1 mL of this solution was taken from 10, 20, 40 mL of stock solutions at different concentrations to make up 3 mL of ethanol and added to the sample solution. 0.1 mM solution of DPPH[·] in ethanol was prepared and 1 mL of this solution was made up to a final volume of 3 mL. These solutions were vortexed and then incubated in the dark for 30 minutes, their absorbances measured at 517 nm. Here, low absorbance values indicate high radical scavenging activity.

It was made by the colour change method, which indicates that the dark blue / green coloured ABTS^{·+} radical has lost its radical characteristic as a result of chemical reaction with antioxidants (Re et al., 1999). The ABTS^{·+} radical was obtained by mixing the ABTS^{·+} solution prepared with 2 mmolL⁻¹ H₂O and 2.45 mmolL⁻¹ of potassium persulfate (K₂S₂O₈) solution at a ratio of 1: 2 and incubating for 14 hours in the dark and at room temperature. Before using the ABTS^{·+} radical, the ABTS^{·+} solution was diluted with sodium phosphate buffer (0.1 mol L⁻¹, pH 7.4) to obtain an absorbance of 0.750 ± 0.025. Then 10, 20, 40 µL of the stock solutions of the *N. rustica* extracts were added to the phosphate buffer until its volume became 3mL, then 1 mL of ABTS^{·+} solution was added and vortexed. These solutions were incubated, 30 minutes in the dark, and their absorbance was measured at 734 nm. Here, low absorbance values indicate high radical scavenging activity. The radical scavenging activity was calculated using the following equation 1.

$$\text{Radical scavenging activity (\%)} = \frac{\text{Absorbance of the control} - \text{Absorbance of the sample}}{\text{Absorbance of the control}} \times 100$$

Determination of Enzyme Activities

In the study, the inhibitory effect of *N. rustica* extracts

on AChE and hPON1 enzyme at various concentrations was investigated. IC₅₀ values of each extract for these enzymes were determined. The inhibitory effects of water and methanol extracts on AChE enzymes were tested by Ellman's method. Briefly, the reaction containing 50 µl of 5,5'-dithio-bis (2-nitro-benzoic) acid (DTNB), 100 µl Tris-HCl buffer (1 M, pH 8.0) and 50 µl AChE (5.32x10⁻³ U) the solution was incubated for 15 minutes at 30 ° C with mixing (Ellman et al., 1961). Finally, the reaction was started by adding 50 µl of substrate (acetylthiocholine iodide). Enzymatic activity was recorded at a wavelength of 412 nm. hPON1 activity was measured using paraoxon (diethyl p-nitrophenyl phosphate) as a substrate. Approximately 1 mL of reaction mixture contains 1 mM CaCl₂ and 50 mM glycine / NaOH (pH 10.5). hPON1 activity is based on measurement of p-nitrophenol (1 mM) at 412 nm (Türkeş et al., 2014; Demir et al., 2017).

RESULTS and DISCUSSION

Antioxidant properties of secondary metabolites in plants is an important parameter in terms of pharmacokinetics. Functional compounds have the potential to generate less reactive phenoxyl radicals, indicating that they have an antioxidant free radical scavenging mechanism (Perry et al., 2000). This antioxidant mechanism can be described as the ability to give hydrogen atoms from hydroxyl groups; Therefore, phenoxyl radicals gain resonance stability and a stable molecule is formed. The antioxidant effects of phenolics have been investigated on the prevention of age-related degenerative brain disorders, coronary diseases and cancer (Perry et al., 2000; Wolf, 2005; Seyoum et al., 2006). In this study, antioxidant activity of *N. rustica* extracts was compared with standard antioxidants (BHT, BHA and Trolox).

The scavenging capacity of DPPH radicals has been observed with a decrease in absorbance as a result of induction by antioxidants. They make the antioxidant molecules inactive radical by giving hydrogen proton to DPPH radical. As a result of this reaction, low absorbance is obtained. Therefore, DPPH as a substrate is often used to evaluate the antioxidant activity of samples or molecules (Duh et al., 1999; Chang et al., 2002). Results showed a significant decrease in the concentration of DPPH radical due to the radical scavenging capacity of both standards and *N. rustica* extracts. MPRN water extract of *N. Rustica* showed stronger DPPH removal activity than the methanol extract. Scavenging effect of *N. rustica* Maras Powder with *Nicotiana rustica*, (MPNR) extract and standards at the same concentration (0.05 mg ml⁻¹) on DPPH radical, Trolox (58%)> BHA (47%)> MPNR water extract (26%)> BHT (22%) decreased in the order. As a result of these results, *N. rustica* MPNR water extract has shown a significant effect on free

radical removal. The ABTS^{•+} radical scavenging of *N. rustica* extracts was measured and compared against standard antioxidants such as BHA, BHT and Trolox. In the method, antioxidants oxidize the ABTS^{•+} dark radical, resulting in a reduction of the dark colour. The color change produced by this reaction is used as a parameter for measuring the antioxidant potential (Gülçin, 2012). Methanol extracts and water extracts

of *N. rustica* extracts showed similar activities and the results are given in Table 1. In a study, DPPH radical scavenging activities of four different extracts obtained from the roots of *Nicotiana tabacum* L were investigated. It was determined that these extracts have radical scavenging activity in the range of 30-88 % (Al-Lahham et al 2020).

Table 1. DPPH and ABTS removal activity of *Nicotiana rustica* Linn
 Tablo 1. *Nicotiana rustica* Linn'in DPPH ve ABTS uzaklaştırma aktivitesi

Samples	DPPH ^a assay (0.05 mg ml ⁻¹)	ABTS ^a assay (0.2 mg ml ⁻¹)	AChE ^b IC ₅₀ (mg ml ⁻¹)	PON 1 ^b IC ₅₀ (mg ml ⁻¹)
BHA	47.031 ± 2.321	98.910 ± 3.068	-	-
BHT	21.729 ± 1.317	79.551 ± 2.786	-	-
Trolox	57.818 ± 3.066	94.361 ± 2.908	-	-
MPNR ^c water extract	26.695 ± 1.873	5.051 ± 0.023	N.D	N.D
MPWW ^d water extract	5.034 ± 0.345	4.849 ± 0.103	0.104	ND
MPCC ^e water extract	4.508 ± 0.293	13.096 ± 0.187	N.D	0.225
MPNR ^c methanol extract	6.898 ± 0.367	11.132 ± 0.148	0.203	0.025
MPWW ^d methanol extract	3.814 ± 0.086	4.755 ± 0.102	0.167	0.013
MPCC ^e methanol extract	6.254 ± 0.402	12.099 ± 0.304	0.123	0.165

^aValues are expressed as percent radical scavenging activity

^bEffect of *Nicotiana rustica* Linn on AChE and PON 1 Enzyme Activity

^cMaras Powder with *Nicotiana rustica*, (MPNR)

^dMaras Powder with walnut, (MPWW)

^eMaras Powder with copper cauldron, (MPCC)

It is reduced to Cu (I) by the reaction of Cu (II) with antioxidants and transferring electrons (Apak et al., 2004). As a result of this reaction, a colour change is observed and the spectrum is formed. Based on the spectrum given by Cu (II) at 450 nm, the absorbance values at 450 nm as a result of the reaction of *N. rustica* extracts and standard antioxidants with Cu (II) were recorded. Both methanol and water extracts of *N. rustica* extracts showed low activity compared to standard antioxidants and the results are given in Table 2.

Antioxidant activity of bioactive substances; prevention of radical chain reaction, binding ion catalysts of transition metals, separating peroxides, prevention of continuous hydrogen separation, metal

reducing capacity and radical scavenging such as based on a variety of mechanisms (Diplock, 1997; Gulcin et al., 2002). It is the reduction of Fe³⁺ (ferricyanide) complex to Fe²⁺ form, which is an important parameter in the determination of antioxidant activity. Therefore, the Prussian blue formation of Fe²⁺ can be monitored by measuring at 700 nm (Meir et al., 1995). In this study, the yellow color of the solutions prepared for the activity turns into various shades of green and blue depending on the reducing power of the antioxidant samples. Table 3 shows the reducing power of *N. rustica* extracts compared to the standard antioxidants BHT, BHA and Trolox. It has been observed that methanol extracts have more activity than water extracts.

Table 2. Metal reducing activity (Frap and Cuprac assay) of *Nicotiana rustica* Linn
 Tablo 2. *Nicotiana rustica* Linn'in metal indirgeme aktivitesi (Frap ve Cuprac testi)

Samples	Frap ^a assay (0.2 mg mL ⁻¹)	Cuprac ^a assay (0.2 mg mL ⁻¹)
BHA	0.748 ± 0.042	0.487 ± 0.065
BHT	0.521 ± 0.033	0.563 ± 0.072
Trolox	0.537 ± 0.028	0.423 ± 0.048
MPNR ^b water extract	0.120 ± 0.014	0.149 ± 0.022
MPWW ^c water extract	0.130 ± 0.036	0.158 ± 0.017
MPCC ^d water extract	0.140 ± 0.019	0.124 ± 0.011
MPNR ^b methanol extract	0.184 ± 0.034	0.267 ± 0.058
MPWW ^c methanol extract	0.165 ± 0.027	0.221 ± 0.072
MPCC ^d methanol extract	0.158 ± 0.053	0.178 ± 0.013

^aValues are expressed as absorbance. High absorbance indicates high reduction capacity.

^bMaras Powder with *Nicotiana rustica*, (MPNR)

^cMaras Powder with walnut, (MPWW)

^dMaras Powder with copper cauldron, (MPCC)

Table 3. Analytical parameters of the standard mixture for LC-MS/MS analysis method.

Tablo 3. LC-MS / MS analiz yöntemi için standart karışımın analitik parametreleri.

Standards	Mainion (m/z) ^a	RSD % ^b	LOD/LOQ (µg L ⁻¹) ^c	Recovery (%)	U ^d	RT ^e	R ² ^e	Equation
Quercetin	300.90	0.0136	22.5/25.7	100.130	0.0298	6.091	0.999	Y=(13.7831)X+(-146.951)
Acetohydroxamic Acid	43.01	0.0082	2.8/8.2	100.075	0.0167	1.986	0.999	Y = (150.982)X + (23.1833)
Catechin hydrate	152.95	0.0236	8.2/11.4	0.99404	0.0523	4.958	0.999	Y = (79.2933)X + (-2406.22)
Vanillic Acid	166.90	0.0062	125.5/142.2	100.093	0.0208	6.026	0.997	Y = (48.0522)X + (-876.904)
Resveratrol	227.0	0.0131	9.0/13.6	0.9985	0.0265	5.713	0.997	Y = (46.4361)X + (-1314.61)
Fumaric Acid	115.00	0.0047	25.2/31.3	0.99748	0.0116	3.674	0.998	Y = (20.2986)X + (-762.592)
Gallic acid	168.85	0.0136	0.90/1.6	100.004	0.0288	4.134	0.999	Y = (65.3835)X + (-2699.84)
Caffeic Acid	178.95	0.0137	6.3/10.7	100.917	0.0254	5.283	0.996	Y = (124.785)X + (-487.132)
Floridzin Dihydrate	436.5	0.0564	61.0/207.0	10.001	0.1272	5.646	0.998	Y = (33.4069)X + (-1396.90)
Oleuropein	377.123	0.0694	0.05/1.0	0.997	0.1364	5.643	0.998	Y = (25.9240)X + (-558.916)
4-Hydroxy Cinamic Acid	222.95	0.0139	8.7/16.1	100.164	0.0281	5.738	0.995	Y = (13.1516)X + (717.421)
Elagic Acid	301.00	0.0856	0.101/0.333	100.232	0.154	5.895	0.999	Y = (5.25903)X + (-1167.31)
Myricetin	317.00	0.0079	55.4/59.6	0.99982	0.0148	5.858	0.999	Y = (37.0934)X + (2684.23)
Protocatechuic acid	152.95	0.0129	30.3/35.4	101.070	0.0243	4.96	0.997	Y=(307.55)X + (31226.26)
Silymarin	482.00	0.0138	0.5/1.2	0.9984	0.0302	5.987	0.995	Y = (31.9969)X + (-1823.79)
2-Hydroxy 1,4Naphthaquinone	206.5	0.0121	0.5/1.5	0.9989	0.0256	6.58	0.997	Y = (203.469)X + (29033.1)
Pyrocatechol	109.00	0.0134	22.7/28.6	0.9998	0.0258	6.42	0.998	Y=(32.87)X + (15856)
Naringenin	270.95	0.0205	5.4/6.4	0.99883	0.0521	6.104	0.995	Y = (317.241)X + (33733.3)
Luteolin	284.75	0.0057	0.5/2.5	100.772	0.0174	6.19	0.997	Y = (34.6668)X + (3721.79)
Kemferol	284.75	0.0144	206.6/214.3	0.99971	0.0209	6.288	0.999	Y = (2.63905)X + (-206.494)
Curcumin	369.30	0.0598	0.1/0.75	10.121	0.1023	6.516	0.996	Y = (227.706)X + (-10111.1)
Thymoquinone	165.08	0.0766	1.5/4.5	0.9986	0.1482	6.632	0.999	Y = (60.4553)X + (2285.92)
Alizarin	239.05	0.035	65.2/77.5	0.9667	0.0794	6.800	0.998	Y = (3.97487)X + (1614.23)
4-Hydroxybenzoic Acid	136.95	0.0154	30.5/40.25	0.99662	0.0426	6.130	0.998	Y = (735.804)X + (-498.102)
Salicylic acid	136.95	0.0124	4.2/7.6	100.989	0.0258	6.104	0.999	Y = (746.369)X + (6072.41)

^a Main ion (m/z): Molecular ions of standard compounds (m / z ratio), ^b RSD: Relative standard deviation, ^c LOD/LOQ (µg/L): Determination limit / Detection limit, ^d U (%):Relative standard uncertainty at 95% confidence level (k=2), ^e RT: Retention time, ^f R²: Determination coefficient

Contained in the standard mixture; Result of the LC-MS / MS validation study of 25 phenolic compounds are given in Table 1. The phenolic compound composition of *N.rustica* analyzed by tandem mass / charge based liquid chromatographic (TDS) method was evaluated with the phenolic compounds introduced as standard. As seen in Table 1, it has been determined that the amount of vanillic acid and fumaric acid are relatively high compared to the others. LC-MS / MS results of TDS different extractions are given in Table 4. According to these results; Out of a total of 25 phenolic compounds, 16 could be detected in different extracts of TDS. 15 phenolic compounds in ethanol extract; 12 phenolic compounds in methanol extract; 15 phenolic compounds in ethanol / water (1/1) extract; 11 phenolic compounds were detected in the water extract and 13 phenolic compounds in the hydrolyzed extract. According to the results in Table 4; major phenolic compounds of TDS; It was concluded that there are pyrocatechol, vanillic acid and fumaric acid. It is also understood that 4-Hydroxycinamic acid, quercetin and caffeic acid are major secondary components. It has been determined that the phenolic content of TDS is

rich in diversity but poor in quantity.

The over activity of the acetylcholinesterase (AChE) enzyme, increases the hydrolysis of the neurotransmitter acetylcholine in the cholinergic system, leading to the development of Alzheimer's disease (AD). AChE inhibitors used in the treatment of AD are known to protect cells from oxidative damage. It has also been emphasized that some AChE inhibitors used for this disease play a role in the increase of angiogenesis in cardiovascular patients (Kakinuma et al., 2010; Işık et al., 2017; Işık, 2019).

PON1 is an antioxidant enzyme that protects cells against the harmful effects of free radicals (Aviram and Rosenblat, 2004; Işık et al., 2015). Due to its antioxidant properties, PON1 significantly reduces lipid peroxide formation during LDL oxidation and protects HDL from oxidation by lipid peroxidation (Nishio and Watanabe, 1997; Işık et al., 2019). PON1 enzyme activities in smokers have been reported in studies. (Kurtul et al., 2014; Haj Mouhamed et al., 2010). This study found that *Nicotiana rustica* reduced plasma PON 1 activity.

Table 4 Analytical parameters of MPRN^a,MPWW^b and MPCC^c for LC-MS/MS analysis method.
 Tablo 4 LC-MS / MS analiz yöntemi için MPRN^a,MPWW^b and MPCC^c nin analitik parametreleri.

	MPRN ^a	MPWW ^b	MPCC ^c
Quercetin	N.D*	N.D*	N.D*
Acetohydroxamic acid	54	32.94	34.75
Catechin hydrate	1.62	0.94	1.19
Vanillic acid	4546.43	3251.39	2808.09
Resveratrol	16.2	1.28	23.12
Fumaric acid	800.53	1163.43	1029.45
Gallic acid	N.D*	N.D*	N.D*
Caffeic acid	10.01	8	5.63
Phloridzin dyhrate	6.43	5.3	1.5
Oleuropein	N.D*	N.D*	N.D*
Hydroxycinnamic acid	0.52	0.31	0.27
Ellagic acid	1.53	4.35	N.D*
Myricetin	N.D*	N.D*	N.D*
Protocatechuic acid	N.D*	N.D*	N.D*
Silymarin	N.D*	N.D*	N.D*
2 Hyroxy 1,4-naphthquinone	N.D*	N.D*	N.D*
Bütein	27.47	N.D*	N.D*
Naringenin	N.D*	N.D*	N.D*
luteolin	N.D*	N.D*	N.D*
Kaempferol	N.D*	N.D*	N.D*
Curmin	N.D*	N.D*	N.D*
Thymoquinone	N.D*	N.D*	N.D*
Alizarin	47.28	50.28	7.26
Hydroxybenzoic acid	17.87	16.87	5.47
Salicylic acid	1.3	7.85	16.74

N.D* Not Detection

^aMaras Powder with *Nicotiana rustica*, (MPNR)

^bMaras Powder with walnut, (MPWW)

^cMaras Powder with copper cauldron, (MPCC)

The water extract with methanol extract MPRN, MPWW and MPCC and water extract the MPWW showed an inhibitory effect on AChE and the results are given in Table 2 (IC₅₀: in the range 0.104-0.203 mg / ml). MPCC water extract and methanol extract of MPRN, MPWW and MPCC showed an inhibition effect on PON 1 (IC₅₀: in the range 0.013-0.225 mg / ml). While water extract of MPWW shows the highest inhibitory effect on AChE (IC₅₀: 0.104), methanol extract MPWW shows the highest inhibitory effect on PON 1. (IC₅₀: 0.013). Many studies have shown that phenolic and flavonoid compounds have anti-acetylcholinesterase activity.

In the study, ellagic acid has been shown to act as a potent tyrosinase and acetylcholinesterase inhibitor (Fan et al., 2008). Phenolic compounds have neuroprotective effects and may play an important role in the pathogenesis of AD (Feng et al., 2009; Neagu et al., 2015; Işık, 2019). Donepezil is known to have AChE inhibitory activity approximately 10000 times stronger than ellagic acid (Jha et al., 2018). Looking at the LS-MS / MS content analysis, this study experimental results are thought to have an inhibitory effect on

AChE and PON 1 due to the presence of ellagic acid and other phenolic acids in *Nicotiana rustica* Linn.

As a result, many phenolic compounds were found in *Nicotiana rustica* L. content and it was determined that it had moderate antioxidant activity. It was determined that especially methanol extract of this plant has inhibitory effect on AChE and PON 1. Due to the inhibitory effect of the methanolic extract of this plant on PON 1, their use may pose a risk for the development of atherosclerosis.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

Author's Contributions

The contribution of the authors is equal.

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