Vol.64: e21210249, 2021
https://doi.org/10.1590/1678-4324-2021210249
ISSN 1678-4324 Online Edition

Article - Biological and Applied Sciences

The Influence of Different Extraction Methods/Solvents on Composition, Biological Activities and ADMET Predictions of Phenolics in *Tribulus terrestris*

Duygu Taşkın[1*]
https://orcid.org/0000-0002-5279-0900

Beyza Nur Yılmaz[2]
https://orcid.org/0000-0003-4316-020X

Turgut Taşkın[2]
https://orcid.org/0000-0001-8475-6478

Gülden Zehra Omurtag[3]
https://orcid.org/ 0000-0002-2018-9619

[1]University of Health Sciences, Faculty of Pharmacy, Department of Analytical Chemistry, Istanbul, Turkey; [2]Marmara University, Faculty of Pharmacy, Department of Pharmacognosy, Istanbul, Turkey; [3]Istanbul Medipol University, School of Pharmacy, Department of Pharmaceutical Toxicology, Istanbul, Turkey.

Editor-in-Chief: Alexandre Rasi Aoki
Associate Editor: Jane Manfron Budel

Received: 2021.04.17; Accepted: 2021.07.02.

*Correspondence: duygu.taskin@sbu.edu.tr; Tel.: +90-506 3069982 (D.T.).

HIGHLIGHTS

- The effects of different extraction methods/solvents on the plant's biological activity
- The effect of various extraction methods/solvents on the chemical components of the plant
- Biological activities of different extracts from the plant
- Analysis of major phenolic compounds and in-silico ADMET prediction

Abstract: *Tribulus terrestris* is a plant that has medical importance in treating diseases such as cancer, urinary tract infections, kidney stones, rheumatism, and hypertension. The aim of this study is to examine the effects of extraction methods/solvents on the biological activity and chemical content of the plant and then to determine ADMET predictions of phenolics that were analysed quantitatively using HPLC-DAD in the plant. Maceration methanol (IC_{50}:0.277 mg/mL) and chloroform (0.263 mM$FeSO_4$/mg extract) extracts were found to have the strongest DPPH radical scavenging and iron (III) ion reducing activity, respectively. It was determined that Soxhlet methanol (0.0225 mMTE/mg extract) and Soxhlet chloroform (1.360 mMTE/mg extract) extracts exhibited stronger radical cation ($ABTS^{\cdot+}$) scavenging and cupric ion reducing activity compared to other extracts, respectively. It was found that ultrasonic bath methanol extracts showed the highest anti-urease (21.47%) and calcium oxalate anti-crystallization (71.54%, 80.52%) activity. It was also found that Soxhlet methanol extract (66.763%) has strong acetylcholinesterase enzyme inhibition capacity compared to other extracts. Pyrocatechol, vanillic acid, vanillin, rutin and rosmarinic acid were analysed by HPLC-DAD in the plant. Moreover, it was determined that methanol extracts obtained using soxhlet,

ultrasonic bath, and maceration contained the highest amount of rutin, pyrocatechol, and rutin, respectively. ADMET predictions of phenolics show that these compounds are easily absorbed and do not have toxic effects, suggesting that this species may be used as a natural medicinal and nutritional source in the future after detailed analysis tests.

Keywords: antioxidant; enzyme inhibition; anti-crystallization; HPLC-DAD.

INTRODUCTION

The *Tribulus* genus is a member of the Zygophyllaceae family. There are known 25 species of this genus in the world [1]. *Tribulus terrestris* grows in Southern Europe, Africa, Asia, Australia, and. *T. terrestris* is known as puncturevine, goat head, yellow vine, bull's head, and devil's horn [2,3]. Used medicinally by people in many regions, including Chinese traditional medicine, *Tribulus terrestris* is a medically important plant. It is known that it is traditionally used in the treatment of some diseases such as cancer, urinary tract infections, kidney stones, rheumatism, and hypertension [4]. Pharmacological studies on *T. terrestris* have shown the presence of steroidal saponins such as diosgenin and protodioscin, alkaloids, flavonoids, and glycosides. The proportions of these substances differ according to the parts of the plant used and the way the extract is obtained. The infusion extract obtained from the fruit of the plant is used in the treatment of gout, as well as for coughing and asthma. The plant's leaves have been used as a vasoconstrictor and blood coagulant, diuretic, aphrodisiac, depurative and tonic [1,4-6]. Free radicals are defined as highly reactive and unstable molecules with unpaired electrons in their final trajectories. They are formed because of various natural reactions in living things and different factors entering the organism from outside (some drugs, radiation, smoking, etc). Reactive nitrogen (RON) and reactive oxygen species (ROS) are formed because of many reactions in humans, as in other living things that require oxygen to live. This oxygen-rich ROS causes oxidative damage to biological molecules such as protein, lipid, amino acid, and DNA in the organism. There are endogenous and exogenous antioxidants for occurred free radicals. Thus, there is a balance in the body. However, this balance is disrupted by several factors such as stress, smoking, air pollution, and radiation; as a result, it causes diseases such as cancer, cardiovascular disease, immune system disease, cataracts, diabetes, kidney, and liver diseases. Therefore, the need for natural exogenous antioxidants with low side effects and high effectiveness is increasing [7-9]. *Helicobacter pylori* is a gram negative, motile, spirally microaerophilic bacterium that colonizes the lower gastric mucosa. *H. pylori* is the main cause of gastritis, gastric and duodenal ulcers, and is one of the most important factors in the formation of gastric adenocarcinoma [10,11]. Alzheimer's disease is a neurodegenerative disorder characterized by physical and cognitive dysfunction. Alzheimer's is one of the biggest causes of dementia. It has clinical diagnoses such as memory loss, aphasia, apraxia, agnosia, disorientation, and depression. Various mechanisms such as the oxidative stress hypothesis, plaque formation caused by β-amyloid protein aggregation, and the formation of neurofibrillary tangles because the hyperphosphorylation of the tau protein are proposed as the pathophysiology of the disease. Current treatments aim at regulating these mechanisms and minumizing the symptoms. However, uncertainties in the causes and treatment of the disease limit the solutions. For this reason, studies on Alzheimer's disease maintain their importance today [12,13]. Stone formation in urinary tract and kidneys is a worldwide disease. The stone formation process is characterized by crystal nucleation and aggregation. Calcium oxalate dihydrate (COD) and calcium oxalate monohydrate (COM) crystals found in normal urine play a role in this formation with the supersaturation of urine. COM crystals have an especially high affinity for renal tubularity. Treatment methods are aimed at preventing these crystal formations, as well as the nucleation and aggregation processes [14-16]. The extraction procedure is crucial for the correct measurement, separation, and recovery of phenolics. Extraction is often influenced by sample size, particle, nature, solvent type and extraction techniques [17]. Traditional methods for recovering phenolics from solid materials include Soxhlet and maceration. The Soxhlet process is usually carried out at 90 degrees Celsius for several hours, whereas maceration is carried out at room temperature for days. These procedures are straightforward, involve minimal equipment, and yield sufficiently high phenolic extraction rates [18,19]. The extraction of chemical and inorganic chemicals from solid matrices using liquid solvents is made easier by ultrasonic radiation with frequencies greater than 20 kHz. A sample is combined with an appropriate solvent and sonicated for a set amount of time at a controlled temperature. Wave frequency and ultrasonic wave dispersion affect extract recovery in addition to sonication time, temperature, and solvent choice. To extract phenolics from plant materials, ultrasound is employed in both static and dynamic modes. A static system is a closed-vessel extraction in which there is no continuous solvent transport. Fresh solvent is continuously supplied in dynamic extraction, allowing for efficient analyte adsorption and effective transport from the

extraction vessel. The heat associated with sonication prevents any thermo-labile chemicals from being degraded by continuous transfer of extracted analytes. Ultrasound-assisted extraction is one of the most basic and affordable extraction technologies available, and it can be used quickly in a wide range of solvents for large-scale preparations suitable for industrial use [20, 21]. Predicting the pharmacokinetic properties of pharmaceutical molecules increases the likelihood of reaching the target faster and more securely. The word ADMET stands for absorption, distribution, metabolism, excretion, and toxicity. These criteria define the pharmaceutical activities of drug candidates [22]. In this study, in order to examine the effects of extraction methods on biological activities and phytochemicals and to investigate the potential of the plant to become a pharmaceutical product, (I) different extracts were obtained using different extraction methods, (II) in vitro biological activities of the extracts were examined, (III) the phenolic compounds of the extracts showing strong biological activity were analyzed qualitatively and quantitatively by HPLC-DAD, (IV) ADMET predictions of the compounds obtained from the plant were determined.

MATERIAL AND METHODS

Preparation of *T. terrestris* extracts

The *T. terrestris* root parts were collected in Turkey. The identity of the plant was confirmed by Dr. Ahmet Doğan. The identified *T. terrestris* is registered in the herbarium with the code as MARE-19161 in Marmara University. The plant sample was dried at room temperature. The dried numunes were powdered with an herb grinder (Renas, RBT1250). In presented study, three different extraction methods (maceration, soxhlet and ultrasonic bath) were used for extraction of the plants. The methanol, chloroform, and petroleum ether were used to extract 30 g of plant powder in three extraction methods. The extracts were concentrated using a rotary evaporator at low pressure and temperature at the end of the extraction process. The raw extracts were kept in the refrigerator at +4 °C until biological activity began.

FRAP assay: By mixing 190 µL of FRAP reagent with 10 µL extract, the absorbance increase against the reference, prepared by adding distilled water instead of the extract, was measured at 593 nm in the 4th minute. By comparing the absorbance values of the extracts at 593 nm with the values of the calibration chart prepared with $FeSO_4.7H_2O$, the FRAP value was expressed as mM Fe^{2+}/mg extract [23]. The FRAP values of the extracts were compared with the butylated hydroxytoluene (BHT) compound used as a standard.

ABTS assay: 40 µL of extracts prepared at concentrations of 0.5-5 mg/mL were taken, 3960 µL $ABTS^{\bullet+}$ working solution was added, and the resulting reduction in color was measured spectrophotometrically at 734 nm against distilled water at 6 minutes. A control was prepared under the same conditions by using 40 µL of distilled water instead of the sample. The assay results of the samples were given as mM TroloxE/mg extract [24]. Ascorbic acid was used as the standard compound in this experiment.

DPPH assay: From the extracts prepared at concentrations of 0.5-5 mg/mL, 10 µL of each was taken and 240 µL of 0.1 mM DPPH solution was added. The extracts and standard solutions (ascorbic acid) to which DPPH solution were added were vortexed for 1 minute and then kept at room conditions darkness for 30 minutes. Absorbances were measured against the reference using a microplate reader at 517 nm. Results are expressed as IC_{50} values (mg/mL) [25].

CUPRAC assay: In this assay, 60 µL each of copper (II) solution, neocuproin solution and ammonium acetate buffer (1 M) were mixed. 10 µL ethanol and 60 µL of extract were added and shake the solution. The solutions were kept in room conditions with their mouth closed for 60 minutes. At the end of this period, absorbance values at 450 nm were measured against the reference solution that does not contain a sample [26]. CUPRAC results are expressed as mM TroloxE/mg extract. The CUPRAC values of the extracts were compared with the butylated hydroxyanisole (BHA) compound used as a standard.

Inhibition potential of AChE: 20 µL of samples prepared at different concentrations and 20 µL of AChE enzyme solution were added to 40 µL 0.1 M phosphate buffer solution (pH = 8). This solution was incubated at 25 °C for 10 minutes. After incubation, 100 µL of DTNB reagent and 20 µL of AcI (substrate) were added. The same procedure was applied to galantamine, which is used as a standard. The yellow-colored 5-thio-2-nitrobenzoic acid anion formed as a result of the reaction of thiocholine with DTNB, which is released by the hydrolysis of the substrates (AcI), was spectrophotometrically monitored at a wavelength of 412 nm. The inhibition of acetylcholinesterase of extracts was evaluated according to the Ellman method using a 96-well microplate reader. The findings from this study were given as percent acetylcholinesterase enzyme inhibition [27].

Urease assay: 100 µL of the working solution was taken, and 500 µL of urease enzyme was added to it and kept in an incubator at 37 °C for 30 minutes. Later, 1100 µL of urea was added to this mixture and it was

kept in the incubator at 37 ° C for 30 minutes. R_1 (1% phenol, 0.005% sodium nitroprusside) and R_2 (0.5% NaOH, 0.1% Sodium hypochlorite) reagents were added to the mixture taken from the incubator, and the mixture was kept at 37 ºC in the incubator for 2 hours. The absorbance of the mixture was read against the reference at 635 nm. The same procedure was applied to thiourea, which is used as a standard.The anti-urease activity of the extracts was evaluated using the indophenol method. The findings from this study were given as percent urease enzyme inhibition [28].

Calcium oxalate anti-crystallization activity: Nucleation assay: In this assay, solutions of sodium oxalate and calcium chloride were prepared in Tris HCl (0.05 M, pH 6.5) buffer containing 0.15 M NaCl for the formation of calcium oxalate crystals. Three mL of each of the prepared solutions were taken and 1 mL of extract was added on them and this mixture was vortexed for 30 seconds. The mixture was then incubated at 37 ± 0.1 °C for 30 minutes based on human body temperature. The absorbance of the samples was measured at 620 nm [28]. **Aggregation assay:** To prevent the growth of the calcium oxalate monohydrate crystal, different concentrations of plant extracts were added to the crystal we formed, and the absorbance of the mixtures was measured spectrophotometrically at 620 nm, as stated in the relevant literature in the literature [28]. The characterization of the crystals was done according to the same literature information.

Analysis of phenolic compounds: Content analysis of the methanol extracts was carried out using an HPLC-DAD instrument (Agilent 1260 Infinity). In this study, a C18 reverse-phase Nova-Pak (3.9 mm × 150 mm inner diameter, 5 µm) analytical column was used for separation. The column temperature was kept at 30 °C. The chromatography mobile phases were water (0.05% formic acid) and (B) acetonitrile (0.05% formic acid). The gradient elution step was used: the mobile phase B was increased from 0% to 20% in 5 minutes, 40% in 10.00 min, 50% in 20.00 min, 60% 30.00 min, 90% B 40.00 min and 45.00 min, 20%. Re-equilibration of the column was made within 10 minutes that enabled coloumn equilibration. The injection volume was settled as 20 µL. Authentic standards of five compounds were used to develop the analytical method. 10 mL stock solutions were prepared at 1000 µg/mL concentration. Standard solutions were prepared using several different solvents and it was determined that the most suitable solvent was methanol for stock solutions and mobile phase A for standard solutions. However, since rutin molecules did not dissolve in phase A, standard solutions of this molecule were prepared in a solvent mixture of methanol: water (2:1 v/v). Before injecting all standards, a 0.45 µm injector tip was filtered through the filter and 20 µL of it was injected into the HPLC system. In this study, 5 phenolic compounds were determined in methanol extracts of *T. terrestris*. The LOD and LOQ values of the method reported in this study were dependent on the calibration curve generated from five measurements. LOD and LOQ values were calculated according to the following equations (Table 1):

LOD=Mean+3×SD (Standard deviation)

LOQ= Mean+10×SD (Standard deviation)

Table 1. Quantitative determination of five phenolic compounds using HPLC-DAD.

Compounds	Regression equation	R^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Pyrocatechol	Y=113.1x-648.6	0.9881	20-100	1.55	4.70
Vanillic acid	Y=308.8x-98.34	0.9962	4-20	0.25	0.76
Vanillin	Y=420.3x+22.18	0.9757	2-10	0.02	0.06
Rutin	Y=38.59x-116.5	0.9998	50-250	1.42	4.74
Rosmarinic acid	Y=410.1x-453.2	0.9873	4-20	0.8	2.6

ADMET Prediction: Anticipating the pharmacokinetic properties of pharmaceutical molecules increases the likelihood of reaching the target faster and with greater reliability. A(absorption) D (distribution) M (metabolism) E (excretion), T(toxicity) is an approach that offers parameters about the placement of a compound into a living organism. The pkCSM was used to profile compounds' PK characteristics, including absorption, distribution, metabolism, excretion, and toxicity (ADMET). pkCSM, a free online web server (http://structure.bioc.cam.ac.uk/pkcsm) was used to predict the ADMET properties of the analyzed compounds from *T. terrestris*. The 2D polar surface area (PSA 2D, a main predictor of fractional absorption) and the lipophilicity levels in the form of atom-based LogP are two significant chemical descriptors that correlate well with PK characteristics. Molecular polar surface area (PSA) is used for the prediction of drug transport properties. PSA is defined as the sum of surfaces of polar atoms in a molecule. Molecular lipophilicity potential (MLP) is useful property to help explain various molecular ADME characteristics. PSA

and MLP were calculated using Molinspiration cheminformatics (www.molinspiration.com/cgi-bin/properties)[22,29]. Drug absorption is influenced by a variety of parameters, including membrane permeability (as measured by the Caco-2 colon cancer cell line), intestinal absorption, skin permeability, and P-glycoprotein substrate or inhibitor levels. The CYP models for substrate or inhibitor are used to predict metabolism. The entire clearance model and renal OCT2 substrate are used to predict excretion. AMES toxicity, hERG inhibition, hepatotoxicity, and skin sensitivity are all used to predict drug toxicity. In this study, 5 phenolic compounds in methanol extracts were calculated in terms of these parameters and their standard ranges were tested for suitability [30].

Statistical analysis: Graphpad Prism 5 program was used to evaluate the data obtained from this study. Statistical differences between study groups were analyzed using ANOVA and Tukey's multiple comparison test and p values less than 0.05.

RESULTS

Antioxidant activity of the extracts: The DPPH radical scavenging activities of the extracts obtained from plants were determined using DPPH method. The antioxidant activities of the extracts and ascorbic acid used as a standard were evaluated by comparing their IC_{50} values. Methanol extracts from all extraction methods had stronger DPPH radical scavenging potential than the others. When we compared the effect of extraction methods with the DPPH method, maceration methanol extract showed higher radical scavenging activity (IC_{50}:0.277 mg/mL) compared with the others. It was also found that all the extracts showed lower DPPH free radical scavenging activity than the ascorbic acid (IC_{50}: 0.0028 mg/mL). Radical cation ($ABTS^{\cdot+}$) scavenging activity is based on the spectrophotometric measurement of the decrease in color because of the decrease of the ABTS cation radical, which is a blue-green durable compound. As this method is suitable for both hydrophilic and lipophilic compounds, it is frequently used for measuring the activity of plant extracts [24]. Methanol extracts from all extraction methods exhibited the highest ABTS radical scavenging activity. Soxhlet methanol extract (0.225 mMTE/mg extract) had the highest TEAC value. In addition, it was found that soxhlet and ultrasonic bath petroleum ether extract did not have ABTS radical scavenging activity. The iron (III) ion reducing power of plants is very important in evaluating their antioxidant potential. The iron reduction power is based on the reduction of the herbal extract Fe^{3+} to Fe^{2+} and measured spectrometrically at 593 nm. In this method, high absorbance indicates high iron reduction potential [23]. For this purpose, in this study, the antioxidant power of iron (III) ion reduction of different extracts obtained from the plant was examined comparatively. It was determined that maceration chloroform (0.263 mM$FeSO_4$/mg extract) and ultrasonic bath extracts (0.259 mM$FeSO_4$/mg extract) had stronger iron (III) ion reducing power than other extracts. All extracts obtained from different extraction methods were found to have lower FRAP values than BHT compound (1.10 mM$FeSO_4$/mg extract). When the methods/solvents were compared, it was determined that the maceration chloroform method was the most suitable method for FRAP activity. The copper (II) ion-reducing antioxidant capacity of different extracts prepared from the plant was evaluated by the CUPRAC method. According to the chloroform extracts' results, soxhlet (1.36 mMTE/mg extract), maceration (1.277 mMTE/mg extract), and ultrasonic bath (0.951 mMTE/mg extract) methods exhibited the strongest copper (II) ion-reducing antioxidant capacity. These findings show that maceration and soxhlet chloroform extracts have copper (II) ion-reducing potential close to the BHA compound (1.83 mMTE/mg extract). When the methods/solvents were compared, it was determined that the maceration and soxhlet chloroform methods were the most suitable method for CUPRAC activity (Table 2).

Table 2. Effects of extraction/solvents techniques on antioxidant activities of different extracts obtained from the plant (n=3).

Extracts	DPPH (IC_{50}: mg/mL)			FRAP (mM$FeSO_4$/mg extract)			CUPRAC (mMTE/mg extract)			ABTS/TEAC (mMTE/mg extract)		
	Ultrasonic bath	Maceration	Soxhlet	Ultrasonic bath	Maceration	Soxhlet	Ultrasonic bath	Maceration	Soxhlet	Ultrasonic bath	Maceration	Soxhlet
Petroleum ether	0.724±0.419[a]	0.48±0.2436[a]	0.529±0.407[a]	0.227±0.0203[a]	0.117±0.0169[a]	0.216±0.0148[a]	0.643±0.0193[a]	0.034±0.7705[a]	0.995±0.0321[a]	NA	0.0032±0.0013[a]	NA
Chloroform	0.556±0.376[b]	0.712±0.406[b]	2.367±1.539[b]	0.259±0.0303[a]	0.263±0.0319[b]	0.169±0.0316[b]	0.951±0.0268[b]	1.277±0.0150[b]	1.36±0.0237[b]	0.0037±0.0008[a]	0.0094±0.0009[b]	0.0209±0.0078[a]
Methanol	0.36±0.232[c]	0.277±0.145[c]	0.372±0.189[c]	0.055±0.0146[c]	0.005±0.0057[c]	0.008±0.0006[c]	0.602±0.0116[a]	0.671±0.025[c]	0.708±0.0119[c]	0.0185±0.0012[b]	0.0216±0.0009[c]	0.0225±0.0051[a]
AA		0.0028±0.0004									0.132±0,001	
BHA								1.83±0.0002				
BHT					1.10±0.001							

NA: no activity; BHA (Butylated hydroxyanisole): pozitive control for CUPRAC assay; BHT (Butylated Hydroxytoluene): positive control for FRAP assay; Ascorbic acid: pozitive control for DPPH and ABTS assays; TE: Trolox equivalent; Values are mean of triplicate determination (n =3) ±standard deviation; [a]P <0.05 compared with the positive control, [b]P <0.01 compared with positive control, [c]P <0.001 compared with positive control.

Enzyme inhibitory activity: The inhibition percentage of urease enzyme of different extracts at 12.5 µg/mL concentration obtained from *T.terrestris* was determined by the indophenol method [28]. It was determined that the ultrasonic bath methanol (21.47%), maceration chloroform (19.22%) and Soxhlet methanol (18.84%) extracts showed stronger anti-urease activity than other extracts. When the three methods were compared, it was found that the most powerful anti-urease activity was in the methanol extract prepared with an ultrasonic bath. In this study, it was found that all extracts obtained by different extraction methods had lower urease enzyme inhibition potential than the thiourea (70.05%) at 12.5 mg concentration (Table 3). The acetylcholinesterase enzyme inhibition potential of the plant's different extracts and galantamine compound at 200 µg/mL concentration were analyzed comparatively using the Ellman method [27] and are shown in Table 3. It was found that the enzyme inhibition potential of methanol extracts obtained using the soxhlet (66.76%), maceration (60.84%) and ultrasonic bath (50.94%) methods was higher compared to other extracts in this study. According to the findings obtained in this experiment, it was observed that all the extracts had a lower percentage of enzyme inhibition than the galantamine compound (85.29%). Hence, soxhlet methanol extract had the strongest enzyme inhibition potential.

Table 3. The effect of extraction /solvent techniques on the enzyme inhibition potential of different plant extracts (n=3).

	Urease inhibition (%) (12.5 µg/mL)			Acetylcholinesterase inhibition (%) (200 µg/mL)		
	Ultrasonic bath	Maceration	Soxhlet	Ultrasonic bath	Maceration	Soxhlet
Petroleum ether	14.99±2.7321[a]	4.34±2.8053[a]	15.18±4.228[a]	30.472±7.6575[a]	22.286±4.0598[a]	49.3±1.9217[a]
Chloroform	15.73±1.1453[a]	19.22±1.908[b]	15.77±5.112[a]	11.496±5.4245[b]	14.608±3.1555[b]	34.973±6.3418[b]
Methanol	21.47±1.0112[b]	9.58±2.7603[c]	18.84±1.784[a]	50.941±2.7496[c]	60.843±1.0542[c]	66.763±3.0125[c]
Thiourea		70.05±0.007				
Galantamine					85.289±0.06	

Values are mean of triplicate determination (n =3) ±standard deviation; [a]P <0.05 compared with the positive control, [b]P <0.01 compared with positive control,
[c]P <0.001 compared with positive control

Crystallization assay: In many studies, it has been stated that phenolic compounds with antioxidant potential or extracts containing these compounds have the ability to prevent kidney stone [31]. Hence, the presented study, the calcium oxalate (CaOx) crystallization inhibitory of methanol extracts, prepared with different extraction techniques and having antioxidant potential, was examined and the data obtained are showed in Table 4. Ultrasonic bath methanol extracts were effective to prevent nucleation of CaOx crystals as 5 mg/mL (71.54%), 2.5 mg/mL (63.48) and 1.25 mg/mL (55.81%) respectively. It was determined that the maceration methanol extract did not have the potential to inhibit the nucleation of calcium oxalate crystals at all concentrations. In addition, it was determined that the 1.25 mg/mL (9.55%) concentration methanol extract obtained with the soxhlet method showed a low percentage nucleation value. The potential of the methanol extract obtained from the ultrasonic bath method to prevent the nucleation of calcium oxalate crystals was determined to be close to the potassium citrate compound that was used as a standard. The results of the aggregation assay showed that the ultrasonic bath methanol extract of 5 mg/mL (80.52%), 2.5 mg/mL (74.89%) and 1.25 mg/mL (45.74%) respectively had stronger CaOx crystallization inhibitory effect than soxhlet and maceration methanol extracts. In the aggregation experiment, the CaOx crystallization inhibitory of potassium citrate at respective 5 mg/mL (98.41%), 2.5 mg/mL (72.73%) and 1.25 mg/mL (57.72%) concentrations were found to be close to the activity of methanol extract obtained by ultrasonic bath. The findings obtained from this study showed that the ultrasonic bath methanol extract has significant anti-crystallization activity. To our knowledge, no studies have been conducted on the plant's calcium oxalate anti-crystallization activity. In this study, for the first time, the effect of different extraction methods/solvents on the calcium oxalate anti-crystallization activity of the plant's aerial parts was examined in detail.

Table 4. Crystallization experiment results of methanol extracts from *Tribulus terrestris*

	Nucleation (%)				Aggregation (%)			
Concentration (mg/mL)	Maceration	Soxhlet	Ultrasonic bath	Potassium citrat	Maceration	Soxhlet	Ultrasonic bath	Potassium citrat
5	NA*	NA	71.54	97.94	78.21	70.71	80.52	98.41
2.5	NA	NA	63.48	64.23	44.88	48.49	74.89	72.73
1.25	NA	9.55	55.81	56.12	33.33	NA	45.74	57.72

*NA: no activity.

Light microscope observation: To confirm the crystallization results, we followed the presence of crystal nucleation and aggregation using an optical microscope (Figure 1-2). During the nucleation stage, crystals' number in the control (without extract) was high. It was found that different concentrations of methanol extracts prepared by both maceration and soxhlet methods did not cause any decrease in the number of crystals. It was also observed that the crystals aggregate over time. However, after adding methanol extracts obtained by ultrasonic bath at different concentrations (especially 5 mg/mL), crystals' number decreased compared to the control sample (Figure.1). Also, in the aggregation experiment, the control sample showed the presence of more and larger clusters. All three methods of methanol extracts caused a decrease in the number and size of aggregation compared to the control. When we compared the methods, it was found that 5 mg/mL concentration of methanol extract prepared by the ultrasonic method had a stronger aggregation inhibiting effect compared to other extracts. It was also determined that methanol extract at 5 mg/mL concentration prepared by the ultrasonic method caused a significant decrease in crystal number and size compared to the other extracts (Figure.2). In the present study, the CaOx crystallization inhibitory of the extracts were evaluated by both light microscopy and spectroscopic methods, and the ultrasonic bath methanol extract had a significant activity potential in both methods.

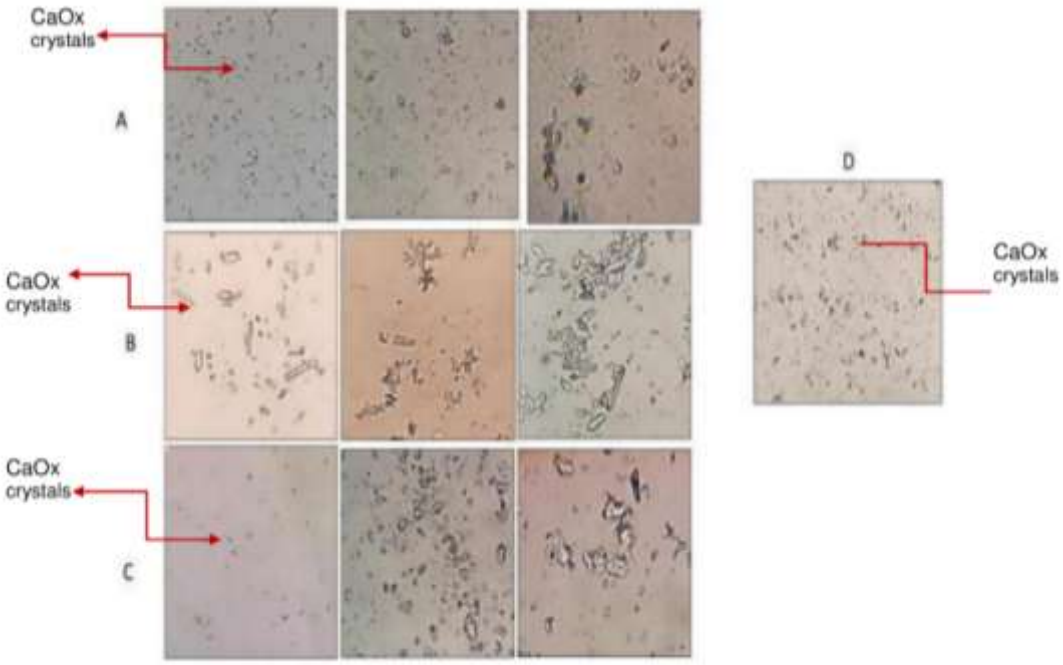


Figure 1. Calcium oxalate crystals in nucleation stages (at a concentration of 1.25; 2.5; 5 mg/mL): A: maceration methanol extract, B: Soxhlet methanol extract, C: ultrasonic bath methanol extract, D: absence of inhibitor.

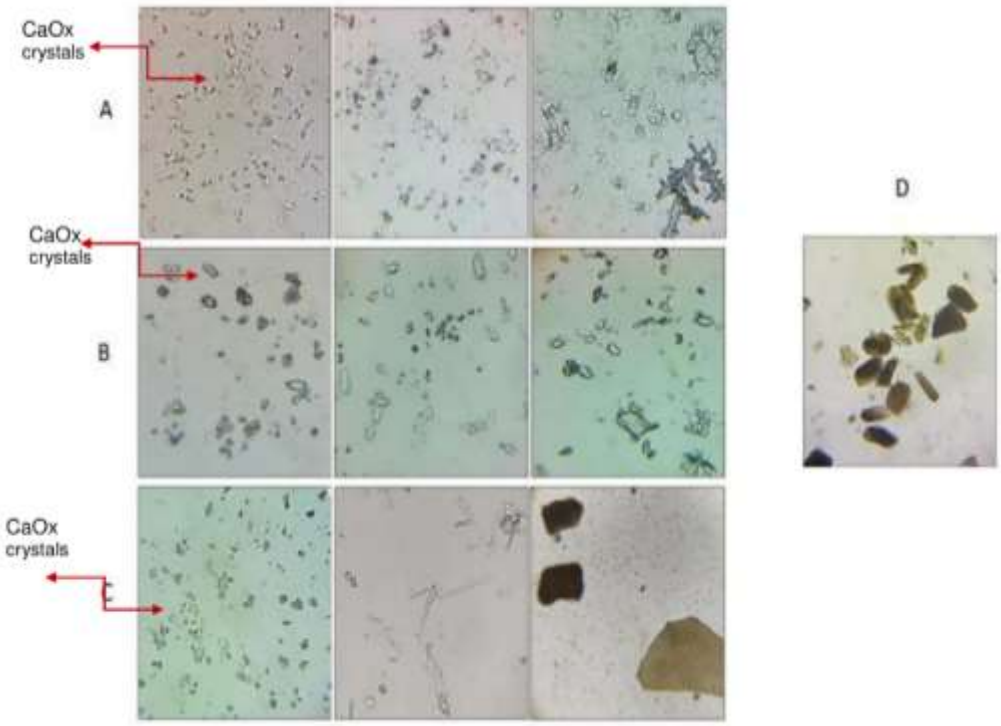


Figure 2. Calcium oxalate crystals in aggregation stages: (at a concentration of 1.25; 2.5; 5 mg/mL): A: maceration methanol extract, B: Soxhlet methanol extract, C: ultrasonic bath methanol extract, D: absence of inhibitor.

Quantitation of phenolic compounds in *T. terrestris* by HPLC-DAD

For centuries, medicinal plants or their extracts have been used to treat many diseases. Studies have revealed that the compounds responsible for the biological activities of plants arise from the secondary metabolites (phenolic acids, flavonoids etc.) Phenolic compounds are the most commonly occurring groups of compounds among the secondary metabolites found in plants. Numerous studies have revealed that these compounds have antioxidant, anti-inflammatory, anti-cancer, anticholinesterase, and antimicrobial effects. The extraction procedure is crucial for the correct measurement, recovery, and separation of phenolics. Extraction is often influenced by sample particle size, extraction techniques and solvent type [17]. Therefore, the effects of different extraction methods (soxhlet, maceration, ultrasonic bath) on the separation and number of phenolic compounds in *T. terrestris* were determined in this study. Phenolic compounds of three methanol extracts from different extraction methods were determined qualitatively and quantitatively using the HPLC-DAD technique. Thus, the effect of extraction methods on the analysis of phenolic compounds in *T. terrestris* has been revealed. Linear regression analysis was performed to establish the relationship between peak area and concentration. We created calibration curves for different standards in four different ranges (20-100 µg/mL for pyrocatechol; 4-20 µg/mL for vanillic acid and rosmarinic acid; 2-10 µg/mL for vanillin; 50-250 µg/mL for rutin). Quantitative investigation of phytochemicals in methanol extracts of different extraction techniques indicated that pyrocatechol (2.179 µg analyte/mg extract in ultrasonic bath), vanillic acid (0.181, 0.260, and 0.178 µg analyte/mg extract in ultrasonic bath, soxhlet, maceration, respectively), vanillin (0.028, 0.007, and 0.110 µg analyte/mg extract in ultrasonic bath, soxhlet, maceration, respectively), rutin (1.581, 4.391, and 2.638 µg analyte/ mg extract in ultrasonic bath, soxhlet, maceration, respectively), rosmarinic acid (0.400 µg analyte/ mg extract in maceration) were present in methanol extracts. Additionally, pyrocatechol and rosmarinic acid were not detected in the soxhlet, maceration, ultrasonic bath extraction techniques, respectively. In this study, it was determined that the vanillic acid and rutin compounds were mostly extracted into the extract by the soxhlet method. Moreover, the maceration technique was determined to be the most suitable method for the extraction of vanillin compound. The data obtained from this study revealed that extraction methods are important in analyzing phenolic compounds in parallel with the literature. It is thought that the number of phenolic compounds extracted can vary depending on the method of extraction, and therefore different methods should be compared in plant studies (Table 5, Figure 3).

Table 5. Phenolic compounds content in methanol extracts from different extraction techniques (µg/mg extracts).

Compounds	Ultrasonic Bath (Average amount ± SD)	Soxhlet (Average amount ± SD)	Maceration (Average amount ± SD)
Pyrocatechol	**2.179 ±0.006**	ND	ND
Vanillic acid	0.181±0.002	0.260±0.004	0.178±0.002
Vanillin	0.028±0.002	0.007±0.001	0.110±0.002
Rutin	1.581±0.029	**4.391±0.021**	**2.638±0.044**
Rosmarinic acid	ND	ND	0.400±0.001

ND: no detected

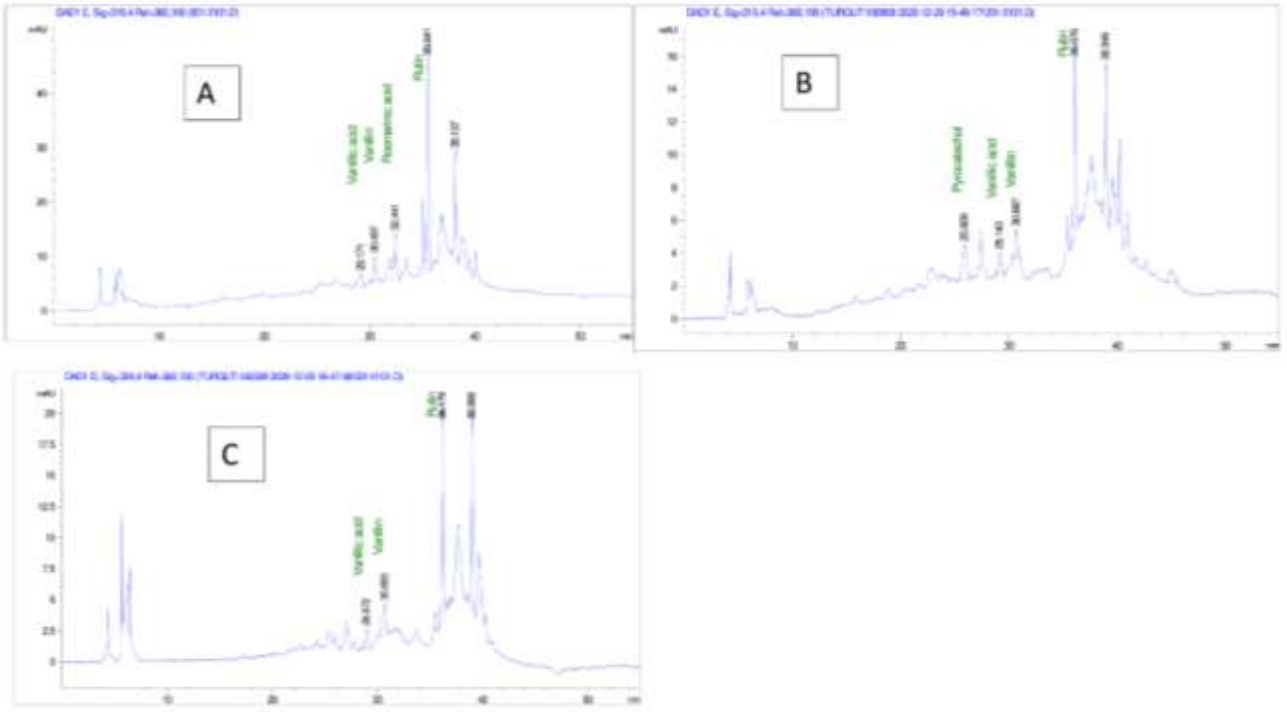


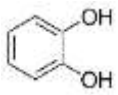
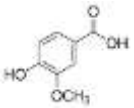
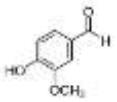
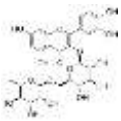
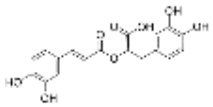
Figure 3. The HPLC-DAD chromatogram of different extracts from plant. A: Maceration-methanol, B: Ultrasonic baht-methanol; C: Soxhlet- methanol.

In silico ADMET profiling of phenolic compounds from plant

The pharmacokinetics of compounds were predicted by the parameters of absorption, distribution, metabolism, excretion, and toxicity as shown in Table 6. The Caco-2 permeability values of pyrocatechol and vanillin were predicted to be high among the compounds analysed. All of compounds were predicted to have high absorbed the intestinal absorption (human) except rutin. It is estimated that all the compounds detected because of HPLC analysis cannot pass through the skin. All the compounds were predicted not to have P-glycoprotein substrate and inhibitory effects. Vanillic acide and vanillin had a low volume of distribution. Rutin and rosmarinic acide were predicted to be poorly distributed to the blood-brain barrier and are also unable to penetrate the Central Nervous System. It is estimated that p450 enzymes, mostly found in the liver, do not metabolize the analysed compounds. It is also predicted that the same molecules are not substrates for this enzyme. It is estimated that none of the analysis's compounds are a substrate for organic cation transport protein. Not all the compounds analyzed are predicted to have mutagenic effects. None of the compounds were predicted to have the potential to inhibit hERG I. However, rutin has hERG II inhibitory effects. All compounds were predicted not to have a hepatotoxicity effect, or minnow toxicity. Pyrocatechol is predicted to display skin sensitization. When the log P values of all compounds except rutin are examined, it is estimated that they are lipophilic (Table 7). As a result, the high Caco-2 permeability of pyrocatechol and vanillin compounds, as well as the high intestinal absorption (human) of all compounds (except rutin) revealed that the analyzed compounds had good absorption properties. The vanillic acid, vanillin and rutin compounds were predicted to exhibit low distribution properties. It is estimated that p450 enzymes, mostly found in the liver, do not metabolize the analyzed compounds. Not all compounds analyzed are predicted to have

mutagenic, hepatotoxicity and minnow toxicity effects. The ADMET predictions of phenolic compounds show that these compounds are well absorbed and do not have toxic effects, suggesting that this species may be used as a natural medicinal and nutritional source in the future after detailed analyses. According to our present knowledge, no studies have been reported regarding the ADMET profiling of phenolic compounds in the plant. In this study, the compounds that may be responsible for the plant's activity were analyzed and the ADMET properties of these phenolic compounds were examined in detail for the first time.

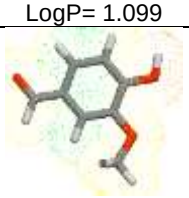
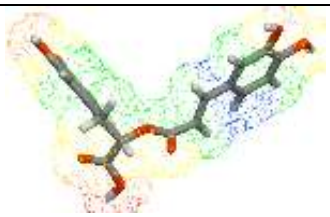
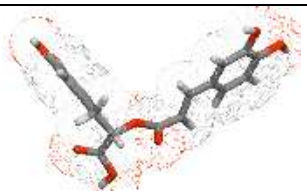
Table 6. The ADMET parameters of the following compounds from plant via pkCSM software

Property	Predicted Value				
	 Pyrocatechol	 Vanillic acid	 Vanillin	 Rutin	 Rosmarinic acid
Absorption					
Water solubility (log mol/L)	-0.762	-1.838	-1.308	-2.892	-3.059
Caco2 permeability (log Papp in 10^{-6} cm/s)	1.682	0.33	1.219	-0.949	-0.937
Intestinal absorption (human)(% Absorbed)	86.856	78.152	84.976	23.446	32.516
Skin Permeability (log Kp)	-2.618	-2.726	-2.832	-2.735	-2.735
P-glycoprotein substrate	No	No	No	Yes	Yes
P-glycoprotein I inhibitor	No	No	No	No	No
P-glycoprotein II inhibitor	No	No	No	No	No
Distribution					
VDss (human) (log L/kg)	-0.022	-1.739	-0.152	1.663	0.393
Fraction unbound (human) (Fu)	0.62	0.518	0.43	0.187	0.348
BBB permeability[a] (log BB)	-0.318	-0.38	-0.243	-1.899	-1.378
CNS permeability[b] (log PS)	-2.076	-2.628	-2.236	-5.178	-3.347
Metabolism					
CYP2D6 substrate	No	No	No	No	No
CYP3A4 substrate	No	No	No	No	No
CYP1A2 inhibitor	No	No	No	No	No
CYP2C19 inhibitor	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	No	No
CYP2D6 inhibitor	No	No	No	No	No
CYP3A4 inhibitor	No	No	No	No	No
Excretion					
Total Clearance (log ml/min/kg)	0.147	0.628	0.601	-0.369	0.25
Renal OCT2 substrate	No	No	No	No	No
Toxicity					
AMES toxicity	No	No	No	No	No
Max. tolerated dose (human) (log mg/kg/day)	-0.017	0.719	1.285	0.452	0.152
hERG I inhibitor	No	No	No	No	No
hERG II inhibitor	No	No	No	Yes	No
Oral Rat Acute Toxicity (LD50) (mol/kg)	2.14	2.454	1.937	2.491	2.811
Oral Rat Chronic Toxicity (LOAEL) (log mg/kg_bw/day)	2.313	2.032	2.007	3.673	2.907
Hepatotoxicity	No	No	No	No	No
Skin Sensitization	Yes	No	No	No	No
T.Pyriformis toxicity (log µg/L)	0.105	0.265	-0.014	0.285	0.302
Minnow toxicity (log mM)	2.194	1.926	1.899	7.677	2.698

[a]BBB (Blood-brain Barrier)

[b]CNS (Central Nervous System)

Table 7. Molecular lipophilicity potential (MLP) and topological polar surface area (TPSA) 3D structures of compounds from plant based on Molinspiration Cheminformatics.

Compounds	MLP	TPSA
Pyrocatechol		
	LogP= 1.0978	TPSA= 40.46
Vanillic acid		
	LogP= 1.099	TPSA= 66.76
Vanillin		
	LogP= 1.2133	TPSA= 46.53
Rosmarinic acid		
	LogP= 1.63	TPSA= 144.52
Rutin		
	LogP= -1.06	TPSA= 269.43

In the surface representation of the molecular lipophilicity potential, the hydrophobic surface is coded with purple and blue colors and the hydrophilic surface is coded with orange and red color

DISCUSSION

In previous studies of the plant, the antioxidant activities of ethyl acetate, chloroform, and butanol extracts from the aerial parts of the plant were examined by the DPPH method and it was found that ethyl acetate extract (33.928 mg ascorbic acid equivalent /g sample) showed the strongest radical scavenging activity. It was also found that 4,5-di-p-trans-coumaroylquinic acid compound (661.98 mg ascorbic acid equivalent/g sample) has higher scavenging activity than the stereoisomer of the di-p-coumaroylquinic acid compound, both of which were isolated from the plant [5]. In our current study by contrast, different extracts were obtained with various extraction methods and it was determined that the methanol extract had the strongest DPPH radical scavenging activity. The antioxidant activities of the ethanol extract obtained from the fruit of the plant were found to have moderate DPPH (IC_{50}: 11.52 µg /mL), ABTS (IC_{50}: 6.74 µg /mL) radical scavenging and

iron (III) ion reducing power activity (0.11 mmol Fe^{2+}/100 µg/mL) compared to the standard [32]. In our present study, the DPPH radical scavenging activities of the aerial parts of the plant were examined and the IC_{50} values of the methanol extracts obtained by all three methods were found to be 0.277-0.372 mg/mL. When we compared the results, fruit ethanol extract had stronger radical scavenging activity than methanol extracts. In the FRAP experiment, unlike above study, the chloroform extract from the aerial parts had strong FRAP values among the others. When radical cation scavenging activity of both studies was compared, polar extracts (ethanol and methanol) were found to have a higher ABTS radical scavenging effect compared to other extracts. In the study of Dakshayini and coauthors, The DPPH radical scavenging and iron (III) ion reducing power activities of the ethanol extract obtained from the fruit of the plant using the soxhlet method were found to be IC_{50}:142.2 µg/mL and 133.12µmol Fe^{2+}/g extract, respectively [33]. In our study, all methanol extracts by all three extraction methods showed lower DPPH radical scavenging activity than the ethanol extract from the fruit of plant. When the FRAP activity results were compared, it was found that the chloroform extracts (169-263 M Fe^{2+}/mg extract) obtained from the extraction methods showed higher iron (III) ion reducing power activity than ethanol extracts of fruit. In another study, the antioxidant activities of hexane, ethyl acetate, ethanol and methanol extracts obtained from the fruit of the plant using the soxhlet method were examined and it was determined that the methanol extract exhibited the strongest DPPH (IC_{50}: 219.7 ug/mL) radical scavenging activity [34]. When we compare the results of the present study with the above, the fruit methanol extracts had stronger DPPH free radical scavenging activity than methanol extract from (IC_{50}: 277-372 ug/mL) the plant's aerial parts. According to all the data in the literature, the antioxidant activity of the fruit of the plant has been generally examined, but there are a limited number of antioxidant studies of the plant's aerial parts. In this study, the effects of different solvents and extraction methods on the antioxidant capacity of the plant's aerial parts was examined in detail for the first time. In the study of Orhan and coauthors, butyrylcholinesterase and acetylcholinesterase inhibitory activity of 1 mg/mL concentration methanol:chloroform(1:1 v/v) extract prepared from plants was investigated and it was found to inhibit acetylcholinesterase and butyrylcholinesterase enzymes by 37.89% and 78.32% respectively[35]. In our present study, three extraction methods of methanol extracts had higher acetylcholinesterase enzyme inhibition (50.941-66.763%) activity than those found in the above studies. In another study, it was found that ethanol extract prepared using the soxhlet method from roots exhibited moderate inhibitory (IC_{50}: 492.3 µg/mL) activity on acetylcholinesterase enzymes [36]. To our knowledge, no studies have been conducted on the plant's anti-urease activity. In this study, urease inhibitory potential of different extracts of the plant were examined comparatively by us firstly.

In previous study, it was determined that 25R-spirost-4-en-3,12-dione tribulusterine and terrestribisamide were isolated from the methanol extract prepared from the fruit of the plant [38]. Protodioscin, neoprotodioscin and neoprototribestin compounds were isolated from a 40%ethanol extract prepared from the aerial parts of the plant by Combarieu and coauthors [38]. In another study, quercetin, isoquercitrin, rutin, isorhamnetin, kaempferol, isorhamnetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside compounds were isolated from the plant [39-42]. In another study, terrestrosin A-E, desgalactotigonin, F-gitonin, gitonin, desglucolanatigonin and (25R,S)- 5α - spirostan- 3β- ol - 3 - O-β3- D- galactopyranosyl(1-2)-β- D-glucopyranosyl (1-4)-β- D-galactopyranoside were isolated from an 80% ethanol extract prepared from the fruit of the plant [37]. It was determined by HPLC–DAD–ESI–MS that the ethanol extract obtained from the fruit of the plant contained quercetin 3,7 diglucoside+xyl, isomer of quercetin 3-*O*-arabinosyl galactoside, kaempferol-3-gentiobioside, isoquercitrin, astragaloside, sumaflavone, apigenin-6,8 glucoside+xyl, quercetin-3-gentiobioside [32]. According to our present knowledge, no studies have been reported regarding the effects of different extraction solvents/ methods on the chemical content of the plant. In this study, for the first time, the effects of different extraction methods on the chemical composition of the plant were analyzed in detail both qualitatively and quantitatively. According to the data obtained, pyrocatechol, vanillic acid, rosmarinic acid and vanillin compounds except the rutin compound were analyzed in this plant for the first time.

CONCLUSION

In this study, it was revealed that different extraction/solvents methods are significantly effective in determining the biological activity and chemical content of the plant. In antioxidant activity studies, it was determined that methanol solvent with maceration and soxhlet methods would be the best applications for radical scavenging activities. Maceration and soxhlet methods with chloroform were observed to be better in reducing iron and copper ions. It was determined that ultrasonic bath and soxhlet methods with methanol gave better results compared to other methods in anti-urease, calcium oxalate anti-crystallization and anticholinesterase activities, respectively. The ADMET predictions of phenolic compounds show that these

compounds are well absorbed and do not have toxic effects, suggesting that this species can be used as a natural medicinal and nutritional source in the future after detailed analysis tests.

Funding: This research received no external funding.
Acknowledgments: The authors thank Dr. Ahmet Dogan, Faculty of Pharmacy, Marmara University, Turkey for the plant identification.
Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

1. Ștefănescu R, Tero-Vescan A, Negroiu A, Aurică E, Vari CE. A Comprehensive Review of the Phytochemical, Pharmacological, and Toxicological Properties of *Tribulus terrestris* L. Biomolecules. 2020; 10 (5): 752-5.
2. Gupta D, Dubey PK. Effect of ethanolic extracts of *Tribulus terrestris*, *Phyllanthus niruri* and combination on calcium oxalate urolithiasis in rats. IJPBS. 2020; *11*(2): 6491-94.
3. Kostova I, Dinchev D. Saponins in Tribulus terrestris chemistry and bioactivity. Phytochem. Rev. 2005; 4(2-3): 111-37.
4. Basaiyye SS, Bafana A, Naoghare PK, Krishnamurthi K, Saravanadevi S. Reactive species metabolism and cytotoxicity of Tribulus terrestris L. alkaloid extracts in leukemic cell line. Indian J. Pharm. Sci. 2018;80 (4): 719-26.
5. Hammoda HM, Ghazy NM, Harraz FM, Radwan MM, ElSohly MA, Abdallah II. Chemical constituents from Tribulus terrestris and screening of their antioxidant activity. Phytochem.2013; 92: 153-9.
6. Gupta R. Ethnobotanical studies on medicinal plant: Gokhru (*Tribulus terrestris*). Int. J. Herb. Med. 2017;5 (6): 73-4.
7. Lopez-Jaen AB, Codoñer-Franch P, Valls-Bellés. V. Free radicals: A review. Journal of Pediatric Biochemistry. 2013;3(3):115-21.
8. Fang YZ, Yang S, Wu G. Free radicals,antioxidants, and nutrition. Nutrition.2002;18 (10):872-79.
9. Ramos-Tovar E, Muriel P. Free radicals, antioxidants, nuclear factor-E2-related factor-2 and liver damage. J. Appl. Toxicol.2020;40(1):151-68.
10. Chey WD, Leontiadis GI, Howden CW, Moss SF.ACG clinical guideline: treatment of *Helicobacter pylori* infection. Am. J. Gastroenterol. 2017;112 (2): 212–39.
11. Pan W, Zhang H, Wang L, Zhu T, Chen B, Fan J. Association between *Helicobacter pylori* infection and kidney damage in patients with peptic ulcer. Renal failure. 2019;41 (1): 1028-34.
12. Lau LF, Brodney MA. Therapeutic approaches for the treatment of Alzheimer's disease: an overview. Alzheimer's Disease. 2007;1-24.
13. Nixon RA. Autophagy, amyloidogenesis and Alzheimer disease. J. Cell Sci. 2007;120 (23): 4081-91.
14. Chien YC, Mansouri A, Jiang W, Khan SR, Gray JJ, McKee MD. Modulation of calcium oxalate dihydrate growth by phosphorylated osteopontin peptides. J. Struct. Biol. 2018;204 (2): 131-44.
15. Wesson JA, Johnson RJ, Mazzali M, Beshensky AM, Stietz S, Giachelli C, Liaw L, Alpers CE, Couser WG, Kleinman JG, Hughes J. Osteopontin is a critical inhibitor of calcium oxalate crystal formation and retention in renal tubules. J. Am. Soc. Nephrol. 2003;14 (1):139-47.
16. Chow K, Dixon J, Gilpin S, Kavanagh JP, Rao, PN. Citrate inhibits growth of residual fragments in an in vitro model of calcium oxalate renal stones. Kidney Int. 2004; 65 (5):1724-30.
17. Khoddami A, Wilkes MA, Roberts TH. Techniques for analysis of plant phenolic compounds. Molecules. 2013; 18: 2328-75. doi:10.3390/molecules18022328.
18. Biesaga M. Influence of extraction methods on stability of flavonoids. J. Chromatogr. A 2011; 1218: 2505–12.
19. Castro-Vargas HI, Rodríguez-Varela LI, Ferreira SRS, Parada-Alfonso F. Extraction of phenolic fraction from guava seeds (*Psidium guajava* L.) using supercritical carbon dioxide and co-solvents. J. Supercrit. Fluids 2010;51:319–24.
20. Vinatoru M. An overview of the ultrasonically assisted extraction of bioactive principles from herbs. Ultrason. Sonochem. 2001; 8: 303–13.
21. Domeño C, Blasco M, Sánchez C, Nerín C. A fast extraction technique for extracting polycyclic aromatic hydrocarbons (PAHs) from lichens samples used as biomonitors of air pollution: Dynamic sonication versus other methods. Anal. Chim. Acta. 2006; 569: 103–12.
22. Pires DE, Blundell TL, Ascher DB. pkCSM: predicting small-molecule pharmacokinetic and toxicity properties using graph-based signatures. J. Med. Chem. 2015;58 (9):4066-72.
23. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 1996; 239: 70–6.

24. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 1999; 26 (9-10):1231-37.
25. Wei F, Jinglou C, Yaling C, Yongfang L, Liming C, Lei P, Zhou D, Liang X, Ruan J. Antioxidant, free radical scavenging, antiinflammatory and hepatoprotective potential of the extract from *Parathelypteris nipponica* (Franch.et Sav.) Ching. J Ethnopharmacol. 2010; 130: 521-28.
26. Apak R, Güclü K, Ozyurek M, Karademir SE. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC Method. J. Agric. Food Chem. 2004; 52: 7970–81.
27. Ellman GL, Courtney KD, Andress V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 1961; 7 (2): 88-95.
28. Taskin T, Dogan M, Yilmaz BN, Senkardes I. Phytochemical screening and evaluation of antioxidant, enzyme inhibition, anti-proliferative and calcium oxalate anti-crystallization activities *of Micromeria fruticosa* spp. *brachycalyx* and *Rhus coriaria*. Biocatal. Agric. Biotechnol. 2020; 27: 101670.
29. Patlewicz G, Jeliazkova N, Safford RJ, Worth AP, Aleksiev B. An evaluation of the implementation of the Cramer classification scheme in the Toxtree software. SAR QSAR Environ Res. 2008;19 (5-6): 495-524.
30. Han Y, Zhang J, Hu CQ, Zhang X, Ma B, Zhang P. In silico ADME and toxicity prediction of ceftazidime and its impurities. Front. Pharmacol. 2019; 10:1-12.
31. Ahmed S, Hasan MM, Khan H, Mahmood ZA, Patel S. The mechanistic insight of polyphenols in calcium oxalate ürolithiasis mitigation. Biomed. Pharmacother. 2018; 106: 1292-99.
32. Tian C, Zhang Z, Wang H, Guo Y, Zhao J, Liu M. Extraction technology, component analysis, and in vitro antioxidant and antibacterial activities of total flavonoids and fatty acids from *Tribulus terrestris* L. fruits. Biomed. Chromatogr. 2019; 33 (4): e4474.
33. Dakshayini PN, Mahaboob BP. Phytochemical screening and in vitro antıoxidant potential of *Tribulus terrestris* fruit and Mesua ferrea flower extracts: a comparative study. Int. J. Pharm. Pharm. Sci. 2018;10 (3): 70-5.
34. Nessa F, Khan SA. Evaluation of free radical scavenging activity and toxic heavy metal contents of commercially available fruits of *Tribulus terrestris* Linn. European J. Med Plants. 2015; 9 (3): 1-14.
35. Orhan I, Sener B, Choudhary MI, Khalid A. Acetylcholinesterase and butyrylcholinesterase inhibitory activity of some Turkish medicinal plants. J. Ethnopharmacol. 2004; 91: 57–60.
36. Ghareeb DA, ElAhwany AM, El-Mallawany SM, Saif AA. In vitro screening for anti-acetylcholiesterase, anti-oxidant, anti-glucosidase, anti-inflammatory and anti-bacterial effect of three traditional medicinal plants. Biotechnol. Biotechnol. Equip. 2014;28 (6):1155-64.
37. Wu TS, Shi LS, Kuo SC. Alkaloids and other constituents from *Tribulus terrestris*. Phytochem. 1999; 50 (8):1411-15.
38. Combarieu ED, Lovati M, Mercalli E. Furostanol saponins from Tribulus terrestris. Fitoterapia. 2003;74: 583-91.
39. Qu NN, Yang SS. Extraction and determination of chemical constituents of flavonides in *Tribulus terrestris* L. J Tradit. Chin. Med. 2007; 9 (3):182-83.
40. Saleh NA, Ahmed AA, Abdalla MF.Flavonoid glycosides of *Tribulus pentandrus* and *T. terrestris*. Phytochem. 1982;21(8):1995-2000.
41. Alavia SHR, Yekta MM, Hadjiaghaee R, Ajani Y. Flavonoid Glycosides from *Tribulus terrestris* L. orientalis. Iran. J. Pharm. Sci. 2008; 4 (3):231-36.
42. Wang Y, Ohtani K, Kasai R, Yamasaki K. Steroidal saponins from fruits of *Tribulus terrestris*. Phytochem. 1997;45 (4): 811-17.