

THE EFFECT OF EXTRACTION METHODS ON ANTIOXIDANT AND ENZYME INHIBITORY ACTIVITIES AND PHYTOCHEMICAL COMPONENTS OF *Galium aparine* L.

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Abstract: *Galium aparine* L. is an annual herbaceous plant of Rubiaceae family. It has therapeutic effects as contains various bioactive components. The aim of this study was to investigate the effects of extraction methods on the amount of phytochemical components of *G. aparine*. The change in the amount of bioactive components directly affects antioxidant and antidiabetic activity. In this study, three different extraction methods, soxhlet, maceration and ultrasonic, using methanol as solvent, were used and the extractions were performed using of *G. aparine*. The phytochemical components of the extracts was analyzed both qualitatively and quantitatively. The soxhlet extraction showed that it contained the highest amount of flavonoids, tannins and phenolic compounds, compared to the other two extraction methods. Obtained soxhlet extraction contained 131.827 g quercetin acid equivalent (QE) flavonoids, 825.4 g as gallic acid equivalent (GAE) phenolic compounds, 366.998 mg as gallic acid (TAE) of tannins. Due to the high amount of phenolic compounds, flavonoids and tannins it contains, soxhlet extract has been shown to have α -amylase inhibition effect (46%) and much more radical scavenging activity than other extracts.

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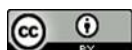
Özet: *Galium aparine* L. (yoğurt otu), Rubiaceae familyasına ait yıllık otsu bir bitkidir. Çeşitli biyoaktif bileşenler içerdiğinden terapötik etkilere sahiptir. Bu çalışmanın amacı ekstraksiyon yöntemlerinin bitkinin biyoaktif bileşen miktarına etkisini incelemektir. Biyoaktif bileşenlerin miktarındaki değişiklik doğrudan antioksidan ve antidiyabetik aktiviteyi etkiler. Bu çalışmada yoğurt otu kullanılarak çözücünün metanol olduğu soxhlet, maserasyon ve ultrasonik olmak üzere 3 farklı ekstraksiyon yöntemi kullanılmıştır. Ekstraktların fitokimyasal bileşeni hem kalitatif hem de kantitatif olarak analiz edilmiştir. Ekstraktlarda en fazla flavonoid, tanen ve fenolik bileşik içeren ekstraktın, soxhlet ekstraksiyonu sonucu elde edilen ekstrakt olduğu bulundu. Soxhlet ekstraktı, 131.827 kuersetin asit eşdeğeri (QE)/g, flavonoidler, 825.4 gallik asit eşdeğeri (GAE)/g, fenolik bileşikler 366.998 mg tallik asit eşdeğeri (TAE)/g tanenler içerir. İçerdiği fenolik bileşik, flavonoidler ve tanenlerle, soxhlet ekstraktının α -amilaz inhibisyon etkisine (%46) ve diğer ekstraktlardan çok daha fazla radikal temizleme etkinliğine sahip olduğu gösterilmiştir.

Introduction

Free radicals are constantly produced by biochemical processes such as respiration, which represent an important part of aerobic life and our metabolism. Human body has routine antioxidant mechanisms to passify many free radicals, including produced superoxide ion, hydrogen peroxide and hydroxyl radicals, and protect the body against their harmful effects. However, under severe oxidative stress conditions where antioxidant defense is insufficient, biochemical changes caused by reactive oxygen species (ROS) are known to cause various diseases such as cancer, atherosclerosis, arthritis, inflammation and neurodegeneration (Shirwaikar *et al.* 2006). Many researches have been performed to investigate how to reduce or prevent oxidative diseases. It

is focused on reducing the levels of oxidative stress in the body by increasing the amount of natural antioxidants with high consumption of vegetables and fruits. Natural antioxidants, especially polyphenolic compounds are safe and have antiradical activities. For this reason, extensive studies have been conducted recently to identify plants with antioxidant properties (Pohl & Lin, 2018, Kamble & Gacche, 2019).

There are approximately 350.000 plant species in the world and 80.000 of them are edible. However, it is estimated that about 150 species are bred for human consumption or as a feed source for animals (Füleky, 2009). It is known that foods rich in polyphenols increase the body's antioxidant capacity. More than 8,000 such



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compounds identified from different plant species are considered to be rich sources of secondary metabolites (Pandey & Rizvi, 2009). However, the risk of getting many diseases such as cancer and diabetes decreases when a diet rich in plant polyphenols is consumed (Pandey & Rizvi, 2009, Young & Woodside, 2001). The fact that polyphenols have single oxygen pacifiers, metal chelators, hydrogen donors and ferric hemoglobin act as reducers according to their redox properties. Due to its redox properties, polyphenols act as single reducers of oxygen, metal chelators, hydrogen donors and ferric hemoglobin and show an antiradical activity in the body (Rice-Evans *et al.* 1995, Rice-Evans *et al.* 1997, Prior *et al.* 2005, López *et al.* 2007, Cíz *et al.* 2008, Gebick & Banasiak 2009). Natural products of plant origin such as flavonoids, terpenoids, steroids also have different pharmacological properties, including antioxidant and antitumor activity (Aslantürk *et al.* 2017).

It is known that the α -amylase enzyme is effective in controlling blood sugar level in treatment of diabetes. There are many studies investigating the potential of plant extract to be used instead of enzyme inhibitors taken as medicines in treatment of diabetes (Soud *et al.* 2004, Tilia & Sarikurkcu, 2020).

Galium aparine L. is a herbaceous perennial plant belonging to the Rubiaceae family, widely grown in Europe and Asia (Senio *et al.* 2018). It is a natural antioxidant containing anthraquinones, iridoids, alkanes, flavonoids, tannins, polyphenolic acids and it is used in treatment of various diseases such as diabetes, cancer and hypertension (Saeed & Javed 2007).

Recently, researchers have been trying to isolate antioxidants and phytochemical constituents from plants as they act an alternative therapeutic (Airaodion *et al.* 2019). However, the effect of extraction method on α -amylase inhibition, antioxidant activity and phenolic compound amount in *G. aparine* has not been reported. The main aim of this study was to evaluate the effect of extraction method on enzyme inhibition, antioxidant activity and phytochemical compound amount in *G. aparine*.

Materials and Methods

Instrumentation

Extraction of *G. aparine* was done with an ultrasonic bath (Çalışkan, Ankara, Turkey) with a constant frequency of 40 kHz and a power setting of 60 W. The antioxidant activity, enzyme inhibitory activity and phytochemical components of *G. aparine* extracts were measured using a UV-Vis spectrophotometer (SpectraMax Plus 384 Microplate Reader, California, USA). A vortex mixer, (IKA MS3, SIEHE-IKA from Germany) was used to for mix samples.

Materials and Chemicals

Galium aparine was bought from a herbalist in İstanbul, Turkey. 1,1-diphenyl-2-picryl-hydrazyl (DPPH), [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS), potassium persulfate ($K_2S_2O_8$), iron

(III) chloride ($FeCl_3$), sodium carbonate (Na_2CO_3), citric acid monohydrate were purchased from Sigma (USA). N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD), ethanol, methanol, sulphuric acid, chloroform, acetic acid and sodium acetate were purchased from Merck. All solvents were of analytical grade.

Extraction

Soxhlet extraction, ultrasound assisted extraction and maceration were used as extraction methods. For each method, 5 grams of plant samples were weighed and used. The soxhlet extraction was performed with 150 ml of methanol, the ultrasound assisted extraction was performed in 150 ml of methanol for 30 minutes and for maceration method, 5 grams sample was kept in methanol for 1 day. The filtration for soxhlet extraction was carried out and the sample was preserved at 4°C.

Determination of antioxidant activity

DPPH free radical scavenging activity

Assay for DPPH free radical scavenging activity is often used to evaluate the antioxidant capacity of compounds. The Brand-Williams method was used to test whether sample will bleach the stable DPPH radical. Thus, the DPPH radical scavenging activity was measured (Brand-Williams *et al.* 1995). 0.75 mL of plant extract was added over 1.50 mL of DPPH solution prepared in ethanol (0.05 mM). The mixture was kept at room temperature for 30 minutes. Then, the absorbance at 517 nm was measured in the UV-Vis spectrophotometer. The scavenging activity of DPPH radical was calculated using the following equation (Cioancă *et al.* 2015):

$$DPPH\text{scavenging} (\%) = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right] \times 100 \text{ (Eq. 1)}$$

where A_{control} shows the absorbance of the control (DPPH solution without sample) and A_{sample} shows the absorbance of the test sample.

ABTS radical scavenging activity

This method, developed by Arnao *et al.* (2001) is based on the reduction of radical and color loss by adding antioxidants on the $ABTS^{+}$ radical cation formed by $K_2S_2O_8$ oxidation of ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)]. Blue/green colored $ABTS^{+}$ radical gives strong absorption in 600-750 nm (Arnao *et al.* 2001).

7.4 mM ABTS solution and 2.6 mM potassium persulfate solution were mixed equally and kept in the dark for 12 hours at room temperature. 1 mL of the ABTS radical solution was diluted with an absorbance of 1.1 ± 0.02 at 734 nm in the spectrophotometer by adding about 60 mL of methanol. 150 μ L of sample solutions and 2850 μ L of $ABTS^{+}$ radical solution were left in dark for 2 hours for incubation. The control solution was prepared using distilled water instead of the sample. The absorbance at 734 nm was measured in the spectrophotometer. In the calculations, ABTS% radical scavenging effect was found with the following equation (Cioancă *et al.* 2015).

$$ABTS \bullet + scavenging(\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}} \right] \times 100 \text{ (Eq. 2)}$$

where $A_{control}$ shows the absorbance of the control (ABTS solution without sample) and A_{sample} shows the absorbance of the test sample.

DMPD radical scavenging activity

The method developed by Fogliano *et al.* (1999) was applied for this antioxidant capacity test. DMPD turns into cation radical form (DMPD⁺) in acidic pH or in the presence of oxidant. After 100 mM DMPD solution was prepared, radical was formed by adding 100 mL of 0.1 M acetate buffer (pH 5.3) and 0.2 mL of 0.05 M FeCl₃ onto 1 mL of this solution. Next, the sample solution and the control solution prepared by replacing the sample with distilled water were added. After 10 minutes, the absorbance at 505 nm was measured on spectrophotometer. DMPD⁺ radical scavenging activity was calculated according to the equation below (Cioancă *et al.* 2015).

$$DMPD \bullet + scavenging (\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}} \right] \times 100 \text{ (Eq. 3)}$$

where $A_{control}$ shows the absorbance of the control (ABTS solution without sample) and A_{sample} shows the absorbance of the test sample.

Quantitative phytochemical analysis

Determination of total flavonoid content

Total flavonoid content was determined using the colorimetric method (Zhishen *et al.* 1999). 0.25 mL of standard quercetin (20-200 mg/mL) or extract solution (1 mg/mL) was taken and mixed with 1.25 mL distilled water. 75 µL of 5% (m/v) sodium nitrite and 150 µL of 10% (m/v) aluminum chloride solution were added. It was waited for 5 minutes before adding 0.5 mL of 1M sodium hydroxide solution. The mixture was completed to 2.5 mL with distilled water and the absorbance at 510 nm was measured in the spectrophotometer. The results were expressed as quercetin mg equivalent for 1 gram extract.

Determination of total phenolic compounds

The method developed by Slinkard-Singleton (1977) was used for total phenolic compound evaluation. After adding 45 mL distilled water, 1 mL Folin-Ciocalteu reagent to 1 mL sample solutions at a concentration of 1000 µg/mL, the mixture was thoroughly mixed with a shaking machine. After standing for 3 minutes in this mixture, 3 mL of 2% (w/v) Na₂CO₃ was added and incubated for 2 hours on the shaking machine. After incubation, the absorbances of the mixtures at 760 nm were measured. Distilled water was used instead of extract as blind and gallic acid was used as standard. Total phenolic substance content is expressed as the equivalent of the standard used per µg in 1 gram sample.

Determination of total tannin content

The total tannin content was determined by the method of Schanderl (1970). 1 mL of the plant extract mixed with 0.5 mL Folin's phenol reagent and 5 mL of

35% Na₂CO₃ was added. The mixture was allowed to stand for 5 min at room temperature. The blue color produced was read at 640 nm using UV/visible spectrophotometer. The tannin content was calculated by calibration curve of tannic acid and the results were expressed as tannic acid equivalent (mg/g).

Qualitative phytochemical analysis

Different methods have been used to qualitatively identify the phytochemical components of methanol extracts obtained by different extraction techniques (Harborne 1973, Trease & Evans 1989, Sofowora 1993, Odebiyi & Sofowora 1978, Roopashree 2008).

Test for tannins

0.5 ml extract and 20 ml of distilled water were boiled in a tube. 0.1% ferric chloride (FeCl₃) was added after filtering. Appearance of brownish green coloration showed the presence of tannins.

Test for terpenoids

0.5 ml extract and 2 ml of chloroform were mixed and sulphuric acid was added. Formation of red brown coloration showed the presence of terpenoids.

Test for phenols

1 ml extract and 2 ml of distilled water were mixed and few drops of 10% FeCl₃ was added. Blue colour showed the presence of phenols.

Test for quinones

1 ml extract and 1 ml of concentrated sulphuric acid were mixed. Formation of red colour showed the quinones.

Test for steroids

0.5 ml extract, 2 ml chloroform and 1 ml of sulphuric acid were mixed. Formation of reddish brown showed the presence of steroids.

Enzyme inhibitory activity

The extracts were analyzed for their α-amylase inhibitory activities using the method reported by Zengin *et al.* (2015). 25 µL of sample extract was mixed with 50 µL of alpha amylase in phosphate buffer (pH 6.9 containing 6 mM sodium chloride). After 10 min at 37°C, 50 µL of starch solution (0.05%) was added to start the reaction. After 10 min of incubation at 37°C, the reaction was stopped using 25 µL of HCl (1 M) and 100 µL of iodine- potassium iodide solution was added. Acarbose, a known α-amylase inhibitor was used as a standard drug. The absorbance was measured at 630 nm. The α-amylase inhibitory activity was calculated by using following equation (Cioancă *et al.* 2015):

$$\text{Percentage of inhibition}(\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}} \right] \times 100 \text{ (Eq. 4)}$$

where $A_{control}$ shows the absorbance of the control (ABTS solution without sample) and A_{sample} shows the absorbance of the test sample.

Results

Antioxidant activity of Galium aparine

Results of the in vitro antioxidant activities using three different assays (DPPH radical scavenging, ABTS radical scavenging, DMPD radical scavenging) were expressed as percent inhibition. Among the extracts obtained as a result of soxhlet, ultrasonic and maceration extraction methods, the soxhlet extract showed the highest radical scavenging activity and the extract obtained by maceration showed the lowest antioxidant activity. Results of DPPH, ABTS and DMPD scavenging activities are shown in Fig. 1.

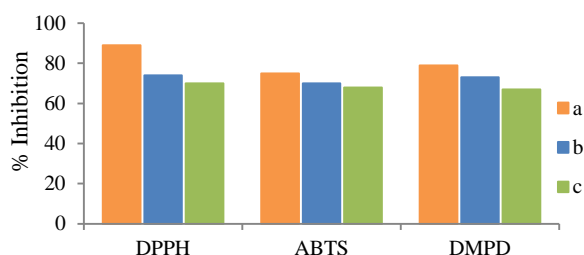


Fig. 1. DPPH, ABTS, DMPD radical scavenging activities of soxhlet (a), ultrasonic (b) and maceration (c) extracts.

In the DPPH test, the ability of *G. aparine* to act as a donor for hydrogen atoms or electrons to reduce DPPH to DPPH-H was spectrophotometrically measured, and the radical scavenging activity of the soxhlet extract was found to be greater than that of other extracts.

Neelam & Khan (2012) reported that methanol is the best solvent for cationic radical activity like DPPH. The methanol extract showed greater activity than the butylated hydroxy anisole used as standard. Studies in the literature support that when methanol is used, the radical scavenging activity increases.

In the ABTS test, the reaction between ABTS and potassium persulfate forms the ABTS radical cation (ABTS⁺) and a blue green color is observed. When the antioxidant is present, the radical reverts to a colorless state. In a study conducted by Bokhari *et al.* (2013), it was shown that *G. aparine* has ABTS radical scavenging activity.

DMPD radical cation (DMPD⁺) is generated through a reaction between DMPD and potassium persulfate and is subsequently reduced in the presence of hydrogen-donating antioxidants. Among the extracts, soxhlet was found to have the highest DMPD scavenging activity with 80%.

Qualitative phytochemical screening

The phytochemical analysis of the extracts obtained using different extraction techniques is shown in Table 1. Phytochemical analysis showed the presence of tannins, phenolic compounds, steroids, terpenoids and quinones in the plant extract. It is clear from the results that the tannins reached to high rates by soxhlet extraction, were at low rates by maceration and moderate concentration by ultrasonic method (Table 1). Soxhlet extract contains

more terpenoid and phenolic compounds compared to other extracts. It was observed that quinones could not be obtained by maceration extraction. Steroids were represented with higher amounts in soxhlet extraction method compared with the other two methods. General results showed that soxhlet is the best extraction method in this experiment of phytochemicals.

Table 1. Qualitative phytochemical screening.

Chemical Constituents	Soxhlet Extract	Ultrasonic Extract	Maceration Extract
Tannins	+++	++	+
Terpenoids	+++	+	++
Phenols	+++	+	+
Quinones	++	+	-
Steroids	++	+	+

+ = low concentration, ++ = moderate concentration, +++ = high concentration, - = absent.

Quantitative phytochemical screening

Table 2 summarizes the result of various quantitative phytochemical analysis of different extracts. The level of flavonoid content was found higher in soxhlet extract [131.827 mg quercetin acid equivalent (QE)/g] and lower in maceration extract (32.47 QE/g). When total phenolic compound amounts were measured, soxhlet extract was found to have the highest amount of phenolic compound with 825.4 µg gallic acid equivalent (GAE)/g. Maceration extract and ultrasonic extract have 186.7 µg GAE/g, 145.8 µg GAE/g phenolic compound, respectively. The level of tannin was found higher in soxhlet extract [366.998 mg gallic acid equivalent (TAE)/g] and lower in maceration extract (293.27 TAE/g).

Table 2. Quantitative phytochemical screening.

	Total Flavonoid Content (mg QE/g)	Total Phenolic Compound (µg GAE/g)	Total Tannin Content (mg TAE/g)
Soxhlet Extract	131.827± 0.008	825.4 ± 0.015	366.998 ± 0.004
Ultrasonic Extract	30.8 ± 0.009	145.8 ± 0.012	318.7 ± 0.0010
Maceration Extract	32.47 ± 0.010	186.7 ± 0.011	293.27 ± 0.013

Each value is the average of three analyses ± standard deviation.

In the study of Lakic *et al.* (2010), obtained total phenolic compound amounts of extracts by maceration method using methanol (2.44-4.65 mg and 4.57-5.16 mg)GAE / g dry extract) and flavonoid amount (6.38-13.40 µg and 15.56-17.96 µg QE / g dry extract). When compared with other similar studies, soxhlet extraction and the use of 100% methanol appeared to be the most effective extraction methods for the total amount of phenolic compounds and flavonoids.

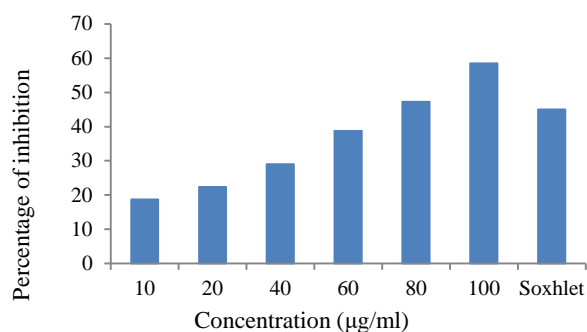


Fig. 2. α -amylase inhibitory activity of acarbose and soxhlet extract.

Inhibitory activity of α -amylase

Acarbose (at a concentrations 100 $\mu\text{g}/\text{mL}$) showed 58.45% inhibitory effects on the α -amylase activity. The inhibition effect of acarbose used as standards in different concentrations is given in Fig. 2. Soxhlet extract showed an effect close to the inhibition of 80 $\mu\text{g}/\text{ml}$ of acarbose with 45% inhibition. Soxhlet extract was the only extract showing α -amylase inhibition. The anti-diabetic potency was defined by the inhibition of α -amylase activity.

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Therefore, it is only soxhlet extract that has anti-diabetic properties among the extracts.

Conclusion

It has been shown that phytochemical compounds of *Galium aparine* has antioxidant and antidiabetic properties. Several in vitro studies have reported the antioxidant potential of *G. aparine* (Lindsey *et al.* 2002, Bokhari *et al.* 2013). Extracts from the seeds of the *G. aparine* also have therapeutic effects due to their phytochemical activity (Farcas *et al.* 2018). Since the extraction method used changes the phytochemical component of the extract, it also changes the antioxidant or antidiabetic activity. According to the results obtained, the most effective method for antioxidant and antidiabetic effect is the soxhlet extraction.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Conflict of Interest: The authors have no conflicts of interest to declare.

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