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Scientific Research

Assessment of the Antioxidant Effects of *Urtica dioica* Aqueous Extract on Free Radical Scavenging, Determination of Total Phenol and Flavonoid Content, and the Bioactive Compound Groups

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ABSTRACT

Urtica dioica L., commonly known as nettle is a perennial herbaceous plant that is widely distributed throughout the world. This plant is rich in antioxidant compounds and possesses anti-inflammatory, anticancer, and antiallergic properties. Therefore, the aim of this study was to determine the bioactive compound groups, total phenolic content, total flavonoid content, and antioxidant activity of aqueous leaf extract of nettle plant. Fourier-transform infrared spectroscopy (FTIR) was used for identifying the bioactive compound groups. Additionally, the total phenolic content was measured using the Folin-Ciocalteu method, the total flavonoid content was determined by the aluminum chloride colorimetric method, and the antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. The results showed that the aqueous leaf extract of nettle contained 59.83 mg GAE/g of total phenols, 29.44 mg QE/g of total flavonoids, and exhibited antioxidant activity with 66.47% and 81.32% inhibition of DPPH and ABTS radicals, respectively. This study demonstrates that the aqueous leaf extract of nettle is a significant source of natural antioxidants and can be considered as a suitable alternative to synthetic antioxidants in food and pharmaceutical industries.

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1- Introduction

In recent years, an increasing amount of scientific literature has confirmed a strong correlation between various illnesses, such as cardiovascular diseases, inflammatory conditions, cancer, osteoporosis, etc. and elements like reactive oxygen species, reactive nitrogen species, and oxidative stress. Reactive oxygen species, in the form of free radicals, often manifest their adverse impacts by targeting unsaturated fatty acids in cellular membranes, initiating a series of destructive processes including lipid peroxidation, reduced membrane flexibility, impairment of antioxidant enzymes and damage of membrane proteins. These detrimental mechanisms ultimately result in cellular inactivation or death. Therefore, antioxidants have gained considerable attention as promising agents to counteract the detrimental effects of free radicals [2-1].

Antioxidants are found in both natural and synthetic forms. Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), ascorbyl palmitate (AP), butyl gallate (BG), octyl gallate (OG), and dodecyl gallate (DG), are added to food products as an effective way to inhibit free radicals. However, the use of synthetic antioxidants might lead to undesired side effects. So, natural antioxidants, especially those derived from plants, are considered as safer alternatives. [3].

Plant extracts are rich sources of antioxidants and bioactive compounds, such as polyphenols, flavonoids etc. which are widely used in the food and packaging industries. These extracts are derived from various parts of plants, including leaves, fruits, seeds, and roots, are generally distinguished by their high phenolic compound content, contributing to their potent antioxidant characteristics [4-5].

Nettle (*Urtica dioica L.*) is a perennial herbaceous plant belonging to the Urticaceae family, and distributed across various regions, including Europe, Asia, North Africa, and North America. This plant typically grows up to one meter in height and features upright, sturdy stems that support its rough, serrated leaves, which are positioned in opposite configuration [6].

Recent researches have demonstrated that nettle can be beneficial in both regular diet and medical treatments, particularly for conditions such as rheumatic diseases, urinary tract infections, and allergies [7-8].

Nettle is rich in various chemical compounds, particularly in its leaves, where a substantial amount of bioactive compounds such as terpenoids, carotenoids (for example beta-carotene, neoxanthin, violaxanthin), fatty acids (specifically palmitic, cis-9,12-linoleic, and alpha-linolenic acids), polyphenolic compounds, essential amino acids, chlorophyll, vitamins (A, B, C, E, and K), tannins, carbohydrates, sterols, polysaccharides, lectins, and minerals can be found [9]. Moreover, the aqueous extract of nettle leaves contains phenolic acids (like chlorogenic and caffeic) as well as the flavonoid isorhamnetin. In addition, the flavonoid glycosides in nettle leaves have immune-enhancing, anti-cancer, anti-inflammatory, antioxidant, and anti-allergic properties [8].

A variety of solvents, with specific properties, are used for plant-based active compounds extraction. These solvents include water, ethanol, methanol, ethyl acetate, methyl acetate, dichloromethane, hexane, and etc. The selection of an

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appropriate solvent for active compounds extraction depends on the physicochemical properties of the active compound, its chemical structure, the extraction environment, cost, and other factors. Extracted plant-based bioactive compounds are affected by various factors such as plant type, growing conditions (soil type, light, moisture and temperature), solvent type, extraction method (mechanical, organic solvents, modern techniques like microwave, ultra sound etc.) plant growth stage, and so on. In the present study the maceration method with water as the solvent applied to extract active compounds from nettle leaves with the aim of investigating total phenolic and flavonoid content, antioxidant activity, and identification of bioactive functional groups in the Nettle leaves aqueous extract.

2- Materials and method

2-1- Chemicals

Folin-Ciocalteu reagent, quercetin, ABTS radical, and DPPH radical were provided from Sigma Co. (USA), Sodium carbonate was obtained from Samchun Co. (Korea), Sodium persulfate, methanol, sodium hydroxide and aluminum tri-chloride were of Merck chemical Co. (Germany).

2-2- Preparation of Aqueous Extract of Nettle Plant

The maceration method was used to obtain the aqueous extract of nettle leaves. For this purpose, the collected plant samples were dried in the shade away from direct sunlight for 5 days and crushed using a grinder before extraction. Then, 50 g of the powdered leaves was added to 500 ml of distilled water and stirred in a shaking incubator for 24 h. The resulting solution was filtered and centrifuged at 5000 rpm for 10 minutes. Thereafter, vacuum rotary evaporator was utilized to remove the solvent and finally, the dried extract was collected in dark glass containers and stored in the refrigerator until testing [11].

2-3- Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) of the prepared extract was determined using the Folin-Ciocalteu reagent, based on the procedure described by Alizadeh et al. (2022). Briefly, 0.2 ml of the extract was mixed with 1 ml of Folin-Ciocalteu reagent. Then, 2 ml of 7% sodium carbonate solution was added to the previous mixture. Obtained mixture was incubated at room temperature for 30 min and the absorbance was read at 765 nm. The TPC was expressed as mg gallic acid equivalent (mgGAE/g) of the extracted samples on dry basis using a gallic acid standard curve [12].

2-4- Determination of Total Flavonoid Content (TFC)

To determine the total flavonoid content of the prepared extract, 500 µL of the extract was mixed with 300 µL of sodium nitrate (5%) and stirred and incubated at room temperature for 6 minutes. Then, 300 µL of aluminum chloride (10%) was added, and the mixture was again incubated at room temperature for 6 minutes with stirring. Then, 2 ml of 1 M NaOH was added, and the absorbance was immediately read at 510 nm. The TFC was reported as mg of quercetin equivalent per g of extract (mgQE/g) [13].

2-5- Determination of Antioxidant Activity

2-5-1- Measurement of DPPH Radical Scavenging Activity

DPPH radical scavenging activity was measured according to the method of Hojjati et al. (2021). First, 0.1 ml of the extract was mixed with 3.9 ml of 0.2 mM DPPH methanolic solution and incubated

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for 30 min at room temperature in the dark. Then, the absorbance of the sample was read at 517 nm. The DPPH radical scavenging activity of the extract was calculated using the following equation [14]:

DPPH scavenging activity (%) = ($A_{control} - A_{sample}$) / $A_{control} \times 100$

Where $A_{control}$ and A_{sample} are the absorbances of the control and sample solutions, respectively.

2-5-2- Measurement of ABTS Radical Scavenging Activity

The antioxidant activity of the extract was investigated by using the ABTS radical scavenging assay. First, an ABTS cationic solution was prepared by mixing equal volumes of 45.2 mM potassium persulfate and 7 mM ABTS solution and allowing the mixture to stand at room temperature for 16 h. Then, 0.1 ml of the extract or methanol was mixed with 3.9 ml of the ABTS solution. The mixture was incubated at room temperature for 6 min, and the absorbance was read at 734 nm. The ABTS radical scavenging activity of the extract was calculated using the following formula [15]:

ABTS scavenging activity (%) = ($A_{control} - A_{sample}$) / $A_{control} \times 100$

Where $A_{control}$ and A_{sample} are the absorbances of the control and sample solutions, respectively.

2-6- Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was employed to identify the functional groups present in the nettle leaves aqueous extract. The analysis was carried out in the spectral range of 500-4000 cm^{-1} using a Bruker Tensor II FTIR spectrometer (Germany) [16].

2-7- Statistical analysis

The data were analyzed using a one-way ANOVA with SPSS version 18. Graphs were created using Excel, and means were compared using Duncan's multiple range test at a 95% confidence level.

3- Results and Discussion

3-1- Total Phenol and Flavonoid Content

Polyphenols are a class of compounds found in many plants. The most common forms of polyphenols are flavonoids, phenolic acids, catechins, anthocyanins, and isoflavones. They play an important role in the prevention of degenerative diseases, especially diseases such as cardiovascular disease and cancer. It is supposed that polyphenolic compounds may limit oxidative stress due to their antioxidant effect and inhibit neurodegenerative diseases by preventing DNA damage. On the other hand, flavonoids are indeed plant pigments, which classified as phenolic compounds. They are responsible for vegetables and fruits bright and vivid colors and considered as the most effective protective agents against cancer and heart disease [17-18]. Figure 1 shows the results of total phenol and flavonoid content determination. The results revealed that nettle leaves aqueous extract contained 59.83 ± 0.46 mg GAE/g of total phenols and 29.44 ± 0.22 mg QE/g of total flavonoids. This indicates that water is a suitable solvent for extracting phenolic acids and glycosides that may be present in the plant, which increases the extraction yield of these compounds compared to other organic solvents such as ethanol or methanol. Flores et al. (2022) reported that the aqueous extract of nettle had a total phenolic content of 21.9 mg GAE/g [4]. In the study carried out by Zeković et al.

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(2023), the total phenol content of the ethanol and methanol extracts of nettle was found to be 21.60 mg GAE/g and 11.71 mg GAE/g, respectively. The total flavonoid content of the ethanol and methanol extracts in their study was 22.50 mg QE/g and 38.79 mg QE/g, respectively [19]. Noshad et al. (2022) indicated that the ethanol extract of nettle leaves contained 65.42 mg GAE/g of total phenols and 22.19 mg QE/g of total flavonoids [20]. According to their obtained results, the most effective solvent for phenolic and flavonoid compounds extraction was methanol but methanol has been known as a hazardous solvent for the environment. Therefore, it is preferable to use solvents such as water for extraction purposes. However, no significant degradation of phenolic compounds in nettle extracts has been reported regardless of the type of solvent (organic or hydroalcoholic solvents). Horozić et al. (2022) investigated the phenolic compounds of nettle (fresh and dry samples). Their findings showed that the aqueous extracts of fresh and dry samples had total phenol contents of 14.34 mg GAE/g and 63.59 mg GAE/g and total flavonoid contents of 9.52 mg QE/g and 25.77 mg QE/g, respectively [21]. In another study, Ebrahimi et al. (2015) inspected the effect of three different solvents (water, methanol 80%, and chloroform) on the extraction of phenolic and flavonoid compounds from nettle leaves. Their results demonstrated extraction with water led to the highest extraction yield [22]. Some researchers [23-24] have reported that habitat has a significant impact on the accumulation of phenolic compounds in nettle leaves. Therefore, the results of this study are consistent with those reported by other researchers [12]. However, the difference between the levels of phenolic compounds in various studies may be due to factors such as plant variety, habitat, extraction conditions, and solvent type.

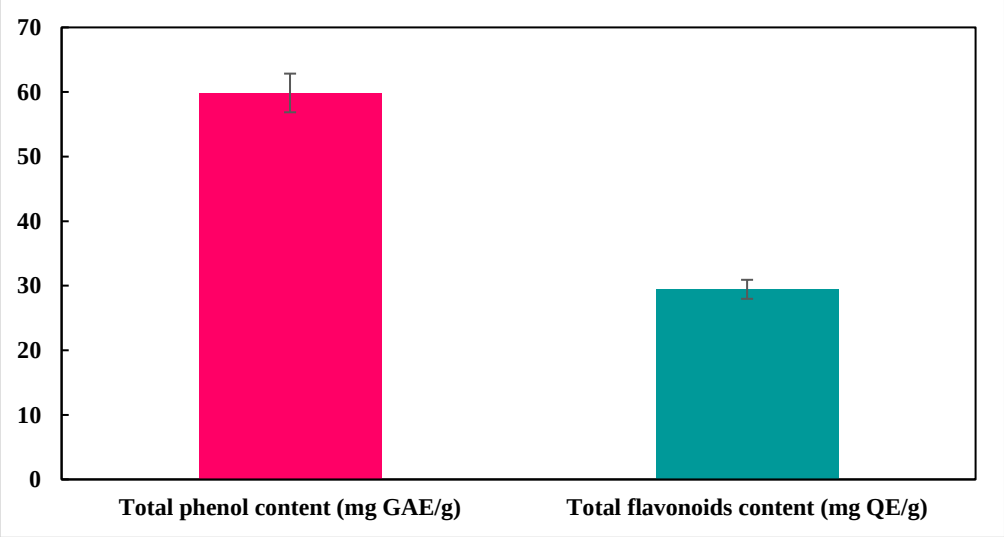

Fig. 1. Total phenolic content (TPC) and total flavonoid content (TFC) of nettle (*Urtica dioica* L.) leaf aqueous extract.

3-2- Antioxidant Activity

Antioxidant activity can be occur through various mechanisms, including chelating metal ion catalysts, peroxides decomposition, preventing further hydrogen abstraction and free radical scavenging [19]. Antioxidants generally eliminate free radicals and detoxify the

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physiological system. Free radicals are normally produced either during the body's natural metabolic processes or be obtained from the environment. The main function of antioxidants is to prevent chain reactions and other oxidation reactions by eliminating free radicals. Therefore, antioxidants are very important for health [18-1]. Antioxidant activity of the extract against DPPH and ABTS free radicals is shown in Figure 2. The results indicated that the aqueous extract of nettle leaves effectively inhibited DPPH and ABTS free radicals by 66.47±1.04% and 81.23±0.92%, respectively. Based on Flores et al. (2022) research DPPH and ABTS free radical scavenging activity of aqueous, ethanol, and methanol extracts of nettle leaves were of 91.1, 90.2, and 87.7% (for DPPH) and 90.8, 48.2, and 58.4% (for ABTS), respectively [4]. These findings were in accordance with the results of the present study. Gülçin et al. (2004) found that nettle has strong antioxidant activity, therefore can be used as a natural antioxidant in the food supplements pharmaceutical industries [19]. Koczka et al. (2019) evaluated the antioxidant activity of ethanol, chloroform, and hexane extracts of nettle. The results indicated that the ethanol and hexane extracts had the highest (21.75%) and lowest (25.55%) DPPH free radical scavenging activity, respectively [1]. In addition, Stanojević et al. (2016) showed that the ethanol-water extract of nettle has high antioxidant activity. According to some studies, the predominant antioxidant activity of phenolic compounds is due to the presence of the hydroxyl functional groups in their structure. The antioxidant effect of these extracts is not only owing to the phenolic compounds presence, but also is because of the synergistic effects of these compounds with other isolated biological molecules from plant materials [24].

Almasi (2016) investigated the antioxidant activity of ethanol extract of nettle leaves for stabilizing soybean oil in two ways: direct addition of the extract to the oil and use of an active starch film containing the extract. The results showed that the DPPH scavenging power and oil stability index in the sample containing nettle extract at a concentration of 800 ppm on the last day of the storage period was not significantly different from the obtained values in the sample containing 100 ppm of the extraction. The use of a film containing nettle extract resulted in maintenance of the soybean oil oxidative stability at a satisfactory level during storage [25]. However, rapeseed oil containing 100 ppm TBHQ was more stable than the ones with 100 ppm and 800 ppm of nettle extract as a natural antioxidant [26]. In another conducted study, the effect of nettle leaf extract on neural stem cells in an oxidative environment was investigated. The results of this study demonstrated that the nettle leaf extract has neuroprotective effects and can modulate the conditions of applied oxidative stress in the laboratory. So, improves the nervous system disfunction caused by the free radicals [27]. Regarding the results of the researches on the antioxidant activity of nettle leaf extract and the results of this study, it can be concluded that the nettle leaf extract used in this work had higher antioxidant effect.

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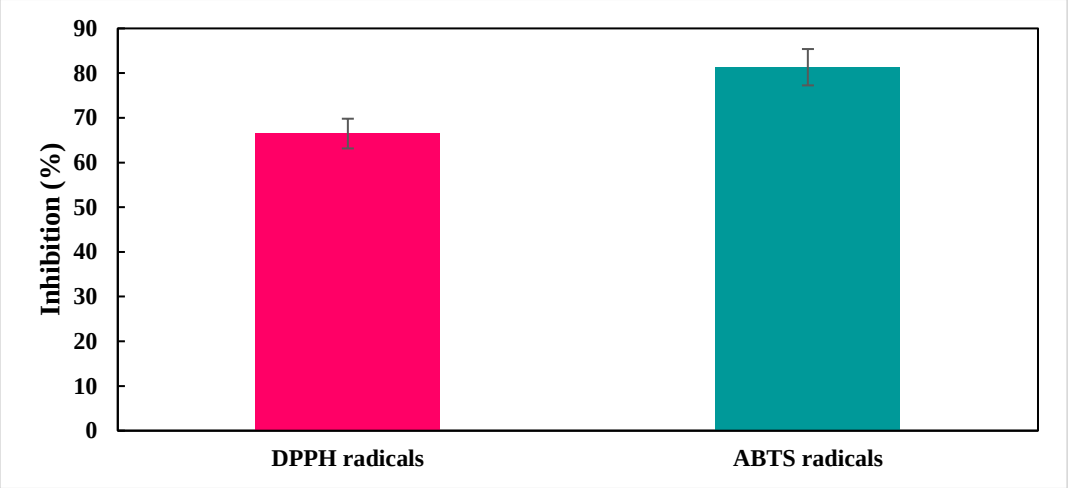

Fig. 2. Antioxidant activity of nettle (*Urtica dioica* L.) leaf aqueous extract.

3-3- Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy is one of the most important methods for identifying functional groups and the structure of organic compounds. FTIR spectrum of the nettle leaf extract is shown in Figure 3. The sharpest peaks were recorded at 3295.15 cm^{-1}, 1607.57 cm^{-1} and 1395.53 cm^{-1}. These peaks are mainly characterizing the C-H, O-H and C-O stretching vibrations in ethers, esters, carboxylic acids, tannins, flavonoids, and other metabolites. The presence of a peak in the range of 1245.73 cm^{-1} showed the presence of aliphatic and carbonyl stretching vibrations. The peaks in the region of 1500-1300 cm^{-1} related to aromatic rings and the presence of C=C bonds, which indicated the presence of polyphenols, relating to phenolic groups with bending vibrations at 1392.53 cm^{-1}. The stretching and deformation vibrations of methyl and methylene groups were seen at 1607.57 cm^{-1}, while the asymmetric and symmetric vibrations of these groups were recorded as a peak in the range of 2970-2800 cm^{-1}. The presence of a peak in the range of 3600-3000 cm^{-1} was associated with the C-H stretching vibrations of phenols and alcohols. In addition, the peaks in the range of 3000-2850 cm^{-1} were related to CH bonds [17 and 28].

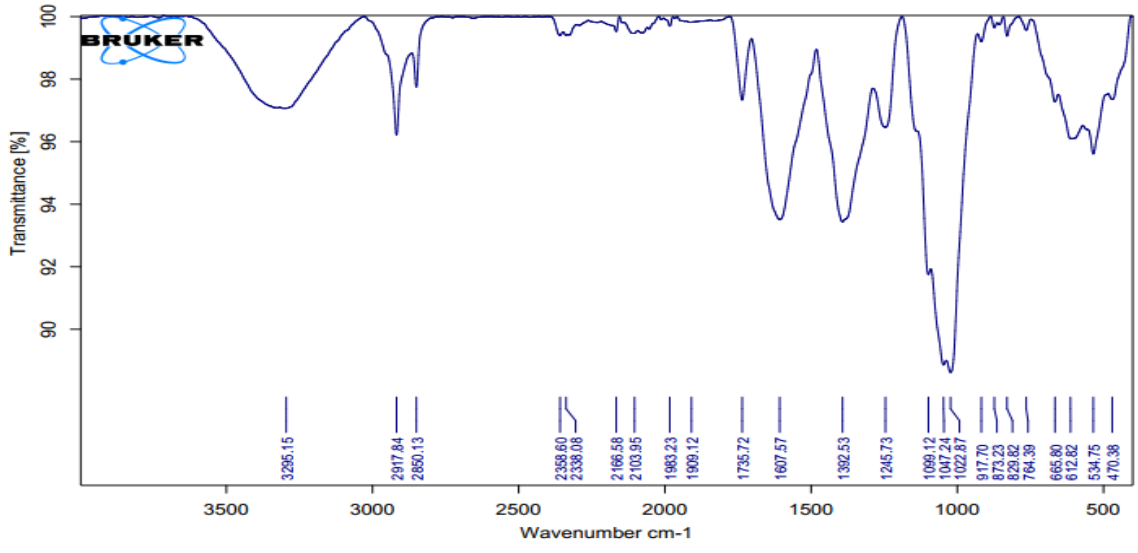

Fig. 3. FTIR spectrum of nettle (*Urtica dioica* L.) leaf aqueous extract.

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4- Conclusion

The results of this study confirmed that the aqueous extract of nettle plant is a rich source of phenolic and flavonoid compounds and possess a high free radical scavenging activity against DPPH and ABTS radicals. Therefore, nettle leaves aqueous extract can be considered as a promising source of natural antioxidants and consequently, a suitable substitute for synthetic ones in the food products.

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ارزیابی اثرات آنتی‌اکسیدانی عصاره آبی گیاه گزنه (*Urtica dioica*) بر مهار رادیکال‌های آزاد، سنجش محتوای فنول و فلاونوئید کل و تعیین گروه‌های عاملی زیست فعال

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چکیده

گیاه گزنه با نام علمی *Urtica dioica* L.، گیاهی علفی و چند ساله است که در سرتاسر جهان گسترش یافته است. این گیاه سرشار از ترکیب‌های آنتی‌اکسیدانی و دارای خاصیت ضدالتهاب، ضد سرطان و ضدحساسیت است. از این‌رو، هدف از این مطالعه، تعیین گروه‌های عاملی زیست فعال، محتوای فنول کل، فلاونوئید کل و فعالیت آنتی‌اکسیدان عصاره آبی برگ گیاه گزنه بود. جهت شناسایی گروه‌های عاملی زیست فعال از روش طیف‌سنجی تبدیل فوریه فروسرخ استفاده شد. همچنین برای اندازه‌گیری محتوای فنول کل از روش فولین–سیوکالتو، محتوای فلاونوئید کل از روش رنگ سنجی آلومنیوم کلراید، فعالیت آنتی‌اکسیدانی به روش مهار رادیکال‌های ۲و۲–دی فنیل ۱– پیکریل هیدرازیل (DPPH) و ۲،۲– آزینو بیس–۳– اتیل بنزو تیازولین – ٦– سولفونیک اسید (ABTS) استفاده گردید. نتایج نشان داد که عصاره آبی برگ گزنه دارای ۵۹/۸۳ mg GAE/g فنول کل، ۲۹/٤٤ mgQE/g کل بود و فعالیت آنتی‌اکسیدانی آن بر اساس مهار رادیکال آزاد DPPH و ABTS به ترتیب ٦٦/٤۷ و ۸۱/۳۲ درصد بدست آمد. این مطالعه نشان می‌دهد که عصاره آبی برگ گزنه منبع مهمی از آنتی‌اکسیدان‌های طبیعی است و می‌تواند جایگزین مناسبی برای آنتی‌اکسیدان‌های مصنوعی در نظر گرفته شود.

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