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Original article

ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF AQUEOUS EXTRACT FROM *RAPHANUS RAPHANISTRUM* L.

Nurgün KÜÇÜKBOYACI[1]*, Ayşegül GÜVENÇ[2], Nilüfer N. TURAN[3], Ahmet AYDIN[4]

[1]Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, 06330 Ankara, TURKEY
[2]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Botany, 06100 Ankara, TURKEY
[3]Gazi University, Faculty of Pharmacy, Department of Pharmacology, 06330 Ankara, TURKEY
[4]Yeditepe University, Faculty of Pharmacy, Kayışdağı, Ataşehir, 34755 İstanbul, TURKEY

Abstract

The present study was carried out to investigate the antioxidant effects of aqueous extract of Raphanus raphanistrum L. (Brassicaceae) aerial parts by using various in vitro systems. The antioxidant activity of the plant was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging test, flow injection analysis-luminol chemiluminescence (FIA-CL) and thiobarbituric acid (TBA) assays. To estimate the total phenolic content, the assay using Folin-Ciocalteu reagent was used. In addition, mineral composition of the raw plant was examined by atomic absorption spectroscopy. Raphanus raphanistrum aqueous extract was found to possess DPPH free radical scavenging activity (IC_{50}=8.78 mg/mL), inhibitory effect on H_2O_2- and HOCl-luminol chemiluminescence (-logIC_{50}=3.73 and 3.1x10^{-2} mg/mL), and inhibitory activity toward lipid peroxidation (IC_{50}=63.33 µg/mL) compared to the references. The total phenolic content was 43.32 mg gallic acid/g extract. According to our results of mineral composition, the raw plant has lower amount of toxic elements (Pb, Cd and Al) than permissible level recommended by WHO. In addition, Ca level was found to be higher (16 mg/g) than daily intake of man. These observations suggest that Raphanus raphanistrum have a potential source of antioxidant and minerals of natural origin.

Key words: *Brassicaceae, Raphanus raphanistrum L., Antioxidant activity, Radical scavenging activity, Flow injection analysis, Luminol chemiluminescence, Mineral.*

Raphanus raphanistrum L. Sulu Ekstresinin Antioksidan Aktivitesi ve Total Fenolik İçeriği

Bu çalışmada Raphanus raphanistrum L. (Brassicaceae)'un toprak üstü kısımlarının sulu ekstresinin antioksidan etkileri çeşitli in vitro sistemler kullanılarak incelenmiştir. Bitkinin antioksidan aktivitesi 2,2-difenil-1-pikrilhidrazil (DPPH) serbest radikal süpürme testi, akışa injeksiyon analiz-luminol kemiluminesens (FIA-CL) ve tiobarbitürik asit (TBA)test sistemleri ile tespit edilmiştir. Total fenolik bileşenleri tespit etmek için Folin-Ciocalteu reaktifi kullanılmıştır. Ayrıca taze bitkinin mineral bileşimi atomik absorpsiyon spekroskopisi ile belirlenmiştir. Raphanus raphanistrum'un sulu ekstresi referanslarla karşılaştırıldığında DPPH serbest radikal süpürme aktivitesine (IC_{50}=8.78 mg/mL), H_2O_2- ve HOCl-luminol kemiluminesens üzerine inhibitor etkiye (-logIC_{50}=3.73 and 3.1x10^{-2} mg/mL) ve lipid peroksidasyona karşı inhibitör (IC_{50}=63.33 µg/mL)aktiviteye sahip bulunmuştur. Total fenolik bileşenler 43.32 mg gallik asit/g ekstredir. Mineral bileşimi sonuçlarımıza göre, taze bitki WHO tarafından belirtilen izin verilen düzeylerden daha düşük miktarda toksik elementler (Pb, Cd ve Al) içermektedir. Ayrıca, Ca seviyesi (16 mg/g) günlük alım miktarından daha yüksek bulunmuştur. Bu sonuçlar Raphanus raphanistrum bitkisinin doğal kaynaklı potansiyel bir antioksidan ve mineral kaynağı olduğunu göstermektedir.

Anahtar kelimeler: *Brassicaceae, Raphanus raphanistrum L., Antioksidan aktivite, Radikal süpürücü aktivite, Akışa injeksiyon analiz, Luminol kemiluminesens, Mineral.*

***Correspondence:** E-mail: nurgun@gazi.edu.tr; Tel: +90 312 202 31 77

INTRODUCTION

Oxidative stress play a crucial role in the development of aging process and some chronic diseases such as cancer, neurodegenerative and cardiovacsular diseases and diabetes in living organisms (1). Dietary antioxidants protect the body against free radicals. There is an increasing interest in the antioxidant effects of compounds derived from plants, which could be relevant in relation to their nutritional incidence and their role in health and disease. Bioactive natural substances having the additive and synergistic effects in plant food are responsible for their potent antioxidant activities (2-5).

A number of greens have been regarded as an essential part of the diet for many years in the Mediterranean region. Therefore, various greens have been picked from nature or purchased from the bazaar. Among them, *Raphanus raphanistrum* L. (Brassicaceae) is named "Turp otu" in the Aegean region of Turkey. The fresh aerial parts of the plant are picked from nature in winter before the flowering time and these parts are mostly consumed as a salad with olive and lemon juice after boiled (6). *R. raphanistrum* (wild radish), only one species in Turkey, is annual herbaceous plant usually branched from near base with erect branches 15-50 cm (7).

R. raphanistrum is also a dietary plant in Mediterranean region such as Italy and the leaves of the plant are used as anti-rheumatic in traditional medicine (8). The anti-inflammatory (8) and antioxidant (8-11) activities on hydroalcholic extracts of *R. raphanistrum* have been reported, however, no experimental study has been conducted so far evaluating the antioxidant effects of the aqueous extract.

To aim of this study was to determine total phenolic content and antioxidant activity of *R. raphanistrum*. The antioxidant activity of aqueous extract from the aerial parts of the plant were investigated by three different in vitro antioxidant test systems: 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging test, flow injection analysis-luminol chemiluminescence (FIA-CL) and thiobarbituric acid (TBA) assays. The results were compared with those obtained with references. The total phenolic content was determined by the Folin-Ciocalteu method. Furthermore, mineral composition of the raw plant was also examined by atomic absorption spectroscopy.

EXPERIMENTAL

Plant material

Raphanus raphanistrum L. (Brassicaceae) was collected from the vicinity of Bagarası, Aydın, Turkey in February 2007. The plant was identified by Professor Nezaket Adıgüzel, from the Department of Biology, Faculty of Science and Art, of Gazi University. Authenticated voucher specimen (GUE 2571) was deposited in the Herbarium of Faculty of Pharmacy, Gazi University.

Chemicals

All chemicals were analytical-reagent grade and obtained from the following sources: Luminol (5-amino-2,3-dihydro-phthalazinedione), ascorbic acid, sodium hydroxide, hexadecyltrimethyl ammonium bromide (HTAB), cobalt (II) chloride hexahydrate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, Na_2CO_3, *tert*-butyl-1-hydroxytoluene (BHT), sodium hypochlorite and quercetin (Sigma-Aldrich S.p.A., Milan, Italy); hydrogen peroxide, sodium chloride, potassium dihydrogenphosphate, gallic acid and methanol (Merck, Darmstadt, Germany).

10^{-3} M luminol stock solution was prepared by dissolving 0.0177 g of luminol in NaOH and, phosphate-buffered saline (PBS: 10 mM KH_2PO_4 and 150 mM NaCl, pH 7.4) was added up to 100.0 mL. PBS was used to control the acidity of the interacting system. HTAB (as a surfactant, final concentration was 10^{-5} M) was added into the working solution of 10^{-4} M luminol before adding PBS for maintaining luminol in basic environment (and 10^{-5} M in Co^{2+}

when the oxidant in use was H_2O_2). It was stored at 4°C and luminol solution was protected from light by a foil wrapper.

Hydrogen peroxide solutions were prepared daily by serial dilution of 100-volume hydrogen peroxide and protected from light by a foil wrapper. HOCl was prepared as described previously by Vissers et al. (12). NaOCl was diluted with PBS and the pH of the solution readjusted to 7.4. At this pH, the solution contains approximately 1:1 HOCl and NaOCl.

Extract preparation

The aerial part of plant material was dried at room temperature and under shade, and then powdered to a fine grade by using a laboratory scale mill. 10 g plant material was extracted with distilled H_2O at room temperature for two times (x 50 mL). The combined aqueous extract was lyophilized to give the crude dry extract (% 3.3).

Determination of total phenolic content

Total phenol content of *R. raphanistrum* aqueous extract was determined using the Folin-Ciocalteu technique (13). To 50 µL sample were added 250 µL of undiluted Folin-Ciocalteu reagent. After 1 min, 750 µL of 20 % (w/v) aqueous Na_2CO_3 were added, and the volume was made up to 5.0 mL with H_2O. After 2 h incubation at 25 °C, the absorbance was measured at 760 nm and compared to a gallic acid calibration curve. Total phenols were determined as gallic acid equivalents (mg gallic acid/g extract), and the values are presented as means of triplicate analyses.

Antioxidant activity

DPPH radical-scavenging activity

The ability of the extracts to scavenge DPPH free radicals were estimated to the method of Brand-Williams et al. (14). A 0.75 mL of the aqueous extract at different concentrations was mixed with 1.5 mL of a DPPH methanolic solution (20 mg/L). The controls contained all the reaction reagents except the extract or positive control substance. After 20 min incubation in darkness and at ambient temperature, the absorbance was recorded at 517 nm. The percent of DPPH decolorization of the sample was calculated according to the equation % decolorization = $[1 - (ABS_{sample} / ABS_{control})] \times 100$. The decolorization was plotted against the sample extract concentration, and a linear regression curve was established in order to calculate the IC_{50} (mg/mL) which is the amount of sample necessary to decrease by 50 % the absorbance of DPPH. BHT, quercetin and ascorbic acid were used as positive controls. All the analyses were carried out in triplicate and results were expressed means±SEM.

HOCl or H_2O_2-luminol chemiluminescence assay

The HOCl or H_2O_2-induced luminol chemiluminescence (CL) assay were performed as described by Sariahmetoglu et al. (15). The peristaltic pump was a Gilson Minipuls 3 and the injection valve was a Rheodyne RH-5020, obtained from Anachem (Luton, Bedfordshire, UK). The pump tubing has a suitable internal diameter, to deliver the required flow-rate. The remainder of the flow-injection manifold was constructed from PTFE tubing joined with low-pressure fittings from Anachem (UK). CL detection was carried out using a luminometer (Model:Lumi-Flo, Chrono-log,USA). Results were recorded on a chart recorder, (Model 706-707, Chrono-log, USA).

The oxidant stream was merged with a luminol/buffer reagent immediately before the luminometer. The total flow rate was 1 mL/min, shared equally between the luminol and the oxidant channel; the oxidant channel includes an injection valve in the middle, which allows making successive nominally 20 µL injections of the extracts. Mixed flow of ooxidant/antioxidant matches with luminol/buffer before the entrance to the flow cell.

The CL is measured as the photomultiplier output in mV; the effects of antioxidants were measured by the depression of the signal from its uninhibited level and were expressed as a percentage attenuation of the maximum CL due to the antioxidant. The sensitivity of extract is expressed as the inhibitor concentrations that elicited 50 % of the maximal responses (IC_{50}, mg/mL). IC_{50} values (mg/mL) of inhibitor effects of extracts and ascorbic acid were expressed as negative log M, using the Prism 3 Graph Pad program.

Thiobarbituric acid (TBA) test for antioxidant activity using liposomes

The lipid peroxidation activity was evaluated using the TBA test reported by Conforti et al. (16). The TBA reaction is because peroxidation of most membrane systems leads to formation of small amounts of free malondialdehyde (MDA) so, high levels of antioxidation lead to the most intense color. One molecule of MDA reacts with two molecules of TBA to yield a colored product, which in an acid environment absorbs light at 532 nm and it is readily extractable into organic solvents. It can, thus, be measured and quantified sprectrophotometrically. The intensity of color is a measure of MDA concentration. Absorbance at 532 nm was determined on a Perkin Elmer Lambda 40 UV/VIS spectrophotometer. The incorporation of any antioxidant compound in the mixture will lead to a reduction of the extent of peroxidation.

The lyophilised extract of *R. raphanistrum* was tested in phosphate buffered saline (5 mg/ml). Peroxidation was started by adding 0.1 mL $FeCl_3$ (1mM) and 0.1 mL ascorbic acid (1mM) followed by incubation at 37 °C for 20 min. Ascorbic acid is a well known anti-oxidant but also has pro-oxidant properties in the presence of certain transition metal ions, such as Fe or Cu. BHT in ethanol was added to prevent lipid peroxidation during the TBA test itself. Propyl gallate was used as a positive control in seven different consentrations ranging from 1 mg/mL to $6.4x10^{-5}$ mg/mL. Four replicate experiments were performed for each extract. Results are given as mean±SEM.

Percentage inhibition of lipid peroxidation was assessed by comparing the absorbance of the reaction mixture containing no inhibitor with that of the extract test reaction mixtures where the substance to be assessed was included. The absorbance readings of the extract alone and the liposomes alone were also taken in account as follows:

$$\% \text{ inhibition} = 100x \frac{(FRM-B)-(ET-B-EA)}{(FRM-B)}$$

Where FRM is the absorbance of the full reaction mixture (liposomes and iron source plus solvent water without the test substance), B is the absorbance of the blank mixture (liposomes only), ET is absorbance of the extract test mixture (full reaction mixture plus test substance), EA is the absorbance due to the extract alone. The half-maximal inhibitory concentration (IC_{50}) of the extract was calculated by linear regression analysis using PRISM software.

Mineral analysis

Plant material was digested in Microwave Acid Digestion system (17). 0.5 g raw plants materials were subjected to acid digestion with HNO_3 65 % and H_2O_2 30 % mixture in microwave digestion unit and analyzed using a Varian 30/40 model atomic absorption spectrophotometer. Pb, Cd, and Mn levels were determined by electrothermal atomisation techniques (Graphite Furnace System) (Varian, GTA-96). Cu, Fe, Al, Ca, Mg, and Zn levels were determined by Flame Atomic Absorption System (Varian, PSC-56). In this study, atomic absorption parameters were given in Tables 1 and 2.

Table 1. Graphite Furnace Atomic Absorption System.

Elements	Wavelength (nm)	Ash temperature (°C)	Atomisation temperature (°C)
Pb	217.0	400	2000
Cd	228.8	300	1800
Mn	279.5	800	2400

Table 2. Flame Atomic Absorption System.

Elements	Wavelength (nm)	Gas mixture
Cu	327.4	Air- acetylene
Fe	372.0	Air- acetylene
Ca	422.7	Nitrous oxide- acetylene
Mg	285.2	Air- acetylene
Zn	213.9	Air- acetylene

RESULTS AND DISCUSSION

In the present study, three different assays used to evaluate the antioxidant activity of the aqueous extracts of *R. raphanistrum*, were those of DPPH free radical scavenging activity, HOCl or H_2O_2-luminol chemiluminescence by flow injection analysis and thiobarbituric acid assays. Flow injection analysis is a rapid, and quantitative method, which can be used, coupled with chemiluminescence. Reactive oxygen species (H_2O_2, HOCl-, etc.) mediated oxidation of luminol produces a chemiluminescence peak and the inhibitory effect on the peak chemiluminescence is a useful tool for the detection and characterization of radical scavengers (15). DPPH free radical scavenging, TBA and chemiluminescence assays were often used to evaluate the radical scavenging activity (antioxidant capacity) of various compounds and medicinal plants (14,15,18,19).

The antioxidant activity results were given in Table 3. The antioxidant properties on DPPH radical scavenging was thought to be due to their hydrogen-donating ability. DPPH scavenger capacity of the extract was compared with the known antioxidant substances such as BHT, quercetin and ascorbic acid. Aqueous extract of the plant (IC_{50}= 8.78±0.75 mg/mL) was shown to possess DPPH radical scavenging activity compared to reference substances.

This systematic study identifies the direct antioxidant potential of *R. raphanistrum* growing in Turkey against a spectrum of oxidants (H_2O_2 or HOCl) by using FIA coupled to luminol chemiluminescence. A continuous CL signal from H_2O_2 (10^{-3} M) (in the presence of 10^{-4} M luminol and 10^{-5} M Co^{2+} in PBS at pH 7.4) was obtained. The H_2O_2-dependent CL signal was inhibited by the aqueous extract of *R. raphanistrum*. Ascorbic acid (chain-breaking reference antioxidant) (10^{-8}-10^{-3} M) (n=6) also inhibited the CL signal in a concentration-dependent manner. The –log IC_{50} values (mg/ml) were 3.73±0.95 and $1.4x10^{-4}$±$2.9x10^{-5}$ for the aqueous extract and ascorbic acid, respectively (Table 3). The continuous CL signal obtained from NaOCl (10^{-4}M), (in the presence of 10^{-4} M luminol in PBS at pH 7.4) was also inhibited by the aqueous extract of the plant (n=6). Ascorbic acid (10^{-4}-10^{-1} M) (n=6) also inhibited the CL signal in a concentration-dependent manner. The –log IC_{50} values were $3.1x10^{-2}$±$4.1x10^{-4}$ and $1.8x10^{-5}$±$2.0x10^{-6}$ mg/ml for the aqueous extract and ascorbic acid, respectively (Table 3).

The *in vitro* antioxidant activity tests were carried out using lipid peroxidation of liposomes where TBA is used to assess the efficacy of the compounds to protect liposomes from lipid peroxidation. Using liposomes prepared from bovine brain, the inhibition of lipid peroxidation was calculated as IC_{50}=63.33±2.62 µg/mL for the extract of *R. raphanistrum*. As reference, the IC_{50} of propyl gallate was 0.21±0.01 µg/mL (Table 3).

Table 3. Antioxidant activities of aqueous extract of *Raphanus raphanistrum*.

Samples	Total phenolic content [a]	DPPH [b]	HOCl - luminol CL [c]	H_2O_2 - luminol CL [c]	TBA [d]
Aqueous extract of *R. raphanistrum*	43.32±0.87	8.78±0.75	$3.1x10^{-2}$±$4.1x10^{-4}$	3.73±0.95	63.33±2.62
BHT	-	0.51±0.01	-	-	-
Quercetin	-	0.059±0.01	-	-	-
Ascorbic acid	-	0.09±0.01	$1.8x10^{-5}$±$2.0x10^{-6}$	$1.4x10^{-4}$±$2.9x10^{-5}$	-
Propyl gallate	-	-	-	-	0.21±0.01

Data are presented as mean values ± standard error of mean (±SEM).
BHT, quercetin, propyl gallate and ascorbic acid were used as positive control.
[a] Values expressed as mg gallic acid/g extract
[b] Values expressed as IC_{50} (mg/mL)
[c] Values expressed as –log IC_{50} (mg/mL)
[d] Values expressed as IC_{50} (µg/mL)

During the literature survey, several reports have been reported on antioxidant activities of *R. raphanistrum* alcholic extracts. For instance, the antioxidant activity of the 50 % methanol and ethanol extract of the *R. raphanistrum* growing in Greece and Turkey were assessed against DPPH free radical scavenging effect, inhibition of hydrogen peroxide, and Fe^{2+}-chelating activity as well as hypochlorous acid-induced oxyhaemoglobin bleaching and lipid peroxidation in mouse brain tissue, and the extracts were found to have antioxidant activity on investigated methods (10,11). In previous studies, hydroalcoholic extracts of the plant were tested for their in vitro antioxidant and antiradical properties (inhibition of linoleic acid oxidation, bovine brain liposomes peroxidation, and DPPH radical scavenging). The extracts exerted also radical scavenging and/or antioxidant properties, which contained the amount of phenolics (28 mg/g) (8,9).

Phenolic compounds have been shown to be responsible for the antioxidant activity of plant extracts (20). Therefore, the content of total phenols in the extract was investigated by the Folin-Ciocalteu method. The content of total phenols is expressed as gallic acid equivalents (mg gallic acid/g extract). The total phenolic content of the *R. raphanistrum* aqueous extract was 43.32 mg/g (Table 3).

Recently, numerous researchers have focused on trace elements in the environmental conditions due to the factors indispensable for the proper functions of living organisms. These elements are contained in enzymes and activate them, thus in an essential way influencing biochemical process in cells. Plant foods and medicinal plants can be toxic effects due to including toxic elements such as lead (Pb), cadmium (Cd) and aluminum (Al). However, these plants can be containing macro- and microelements, which have beneficial health effects (17,21). The results of the elements of *R. raphanistrum* in raw material were shown in Table 4. According to our results of mineral composition, the raw plant has lower amount of toxic elements (Pb, Cd and Al) than permissible level recommended by WHO. The material has low amounts of microelements (Cu, Fe, Mn and Zn) and Mg as a macroelement. In the raw material, Ca level was found to be higher (16 mg/g) than daily intake of man (22,23). This study reveals that aqueous extracts of *Raphanus raphanistrum* is a good source due to Ca level.

Table 4. Content of Elements in Raw Materials of *R. raphanistrum.*

Element	Content of elements (μg/g)	Recommended/permissible quantity (21,22)
Cu	27	1.5-3 (mg/day)
Fe	203	10-15 (mg/day)
Mn	42	2.0-5.0 (mg/day)
Ca	16.000	1.0-1.2 (mg/day)
Mg	2000	310-320 (mg/day)
Zn	14	12-15 (mg/day)
Pb	0.005	10 (mg/kg)
Cd	0.558	0.3 (mg/kg)
Al	278	-

It has been reported that reactive oxygen species contribute to various pathophysiological conditions and endogenous defense mechanisms have evolved to offer protection in these conditions (24). Determination of the naturally occurring antioxidant compounds will help to develop new drug candidates for antioxidant therapy. Therefore, in this study, the capacity to scavenge reactive oxygen radicals and inhibition of lipid peroxidation of *R. raphanasitrum* aqueous extract has been evaluated by using different techniques such as spectrophotometry and chemiluminescence. This study reveals that *R. raphanistrum* is a good candidate for a rich source of natural antioxidants and minerals. Phytochemical studies of the active principles based on the present results may help to discover the new drug candidates for the development of new antioxidants.

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