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Antioxidant, antibacterial, and anticandidal activities of an aquatic plant: duckweed (*Lemna minor* L. Lemnaceae)

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Abstract: Duckweed (*Lemna minor* L. Lemnaceae) is a widespread, free-floating aquatic macrophyte, a source of food for waterfowl and a shelter for small aquatic invertebrates. It grows quickly and reproduces faster than other vascular plants. The objective of this study was to determine the antioxidant, antiradical, antimicrobial, and anticandidal activities of duckweed using different in vitro methodologies. For evaluation of antioxidant and antiradical activities, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical scavenging, 1,1-diphenyl-2-picryl-hydrazyl (DPPH·) free radical scavenging, total antioxidant activity by ferric thiocyanate, total reducing power by potassium ferricyanide reduction method, superoxide anion radical scavenging, hydrogen peroxide scavenging, and ferrous ions chelating activities were calculated. In addition, α-tocopherol and trolox (a water-soluble analogue of tocopherol), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) were used as the reference antioxidant compounds. At the 45 μg mL^{-1} concentrations of lyophilized water extract (WELM) and ethanol extract (EELM), showed 100% and 94.2% inhibition, respectively, on lipid peroxidation of linoleic acid emulsion. On the other hand, BHA, BHT, α-tocopherol, and trolox demonstrated inhibition of 92.2%, 99.6%, 84.6%, and 95.6%, respectively, on peroxidation of linoleic acid emulsion at the same concentration. In addition, the total phenolics and flavonoids in WELM and EELM were determined as gallic acid and quercetin equivalents, respectively. Furthermore, an important goal of this study was to determine the inhibitory effects of WELM and EELM against 21 bacteria and 4 fungi yeast species by using the disk-diffusion method. In our results, it was observed that WELM and EELM had an antibacterial effect against *Staphylococcus epidermidis, Staphylococcus saprophyticus, Staphylococcus warneri, Citrobacter freundii, Citrobacter koseri, Neisseria lactamica, Neisseria sicca, Micrococcus luteus, Bacillus cereus, Bacillus subtilis, and Streptococcus pneumoniae*, and an anticandidal effect against *Candida parapsilosis and Candida glabrata*. Consequently, this plant is a promising source of natural food antioxidants.

Key words: Duckweed, *Lemna minor*, antioxidant activity, antimicrobial activity, radical scavenging

Bir su bitkisinin antioksidan, antibakteriyel ve antikandidal aktivitesi: su mercimeği (*Lemna minor* L. Lemnaceae)

Özet: Su mercimeği (*Lemna minor* L. Lemnaceae), yaygın, herhangi bir yere bağlı olmayan, yüzebilen, su kuşları için gıda ve küçük akuatik omurgasızlar için de barınak olan makrofit bir bitkidir. Hızlı bir şekilde büyür ve diğer vasküler bitkilerden daha hızlı çoğalır. Bu çalışmanın amacı, farklı in vitro metotlar kullanarak su mercimeğinin antioksidan, antiradikal, antimikrobiyal ve antikandidal aktivitelerini belirlemektir. Su mercimeğinin antioksidan ve antimikrobiyal aktivitelerini değerlendirmek için 2,2'-azino-bis(3-etilbenztiyoazolin-6-sülfonik asit) ($ABTS^{\bullet+}$) radikal giderme, 1,1-difenil-2-pikril-hidrazil serbest radikal (DPPH·) giderme, ferrik tiyosiyanat metoduna göre total antioksidan aktivite, potasyum ferriksiyanit indirgeme metoduna göre indirgeme kuvveti, süperoksit anyon radikal giderme, hidrojen peroksit giderme ve ferröz iyonları kelatlama metotları kullanıldı. Ayrıca, α-tokoferol ve α-tokoferolün suda çözünen bir analoğu olan troloks, bütillenmiş hidroksianisol (BHA) ve bütillenmiş hidroksitoluen (BHT) referans antioksidan maddeler olarak kullanıldı. Su mercimeğinin liyofilize edilmiş su (WELM) ile etanol ekstraktının (EELM), 45 μg mL^{-1} konsantrasyonunda linoleik asit emülsiyonunun peroksidasyonunu sırasıyla % 100 ve % 94,2 inhibe ettiği belirlendi. Diğer taraftan aynı konsantrasyonda BHA, BHT, α-tokoferol ve troloksun linoleik asit emülsiyonunun peroksidasyonunu sırasıyla % 92,2, % 99,6, % 84,6 and % 95,6 inhibe ettiği gözlendi. Bunun yanısıra, WELM ve EELM'de bulunan toplam fenolik ve flavonoit maddeler sırasıyla gallik asit ve kuersetin ekivalent olarak belirlendi. Ayrıca, WELM ve EELM'nin yirmibir bakteri ve dört mantar (maya) türüne karşı inhibitor etkileri disk-difüzyon metodu kullanılarak test edildi. Sonuçlarımıza göre WELM ve EELM *Staphylococcus epidermidis, Staphylococcus saprophyticus, Staphylococcus warneri, Citrobacter freundii, Citrobacter koseri, Neisseria lactamica, Neisseria sicca, Micrococcus luteus, Bacillus cereus, Bacillus subtilis* ve *Streptococcus pneumoniae* ye karşı antibakteriyel etkiye ve *Candida parapsilosis* ve *Candida glabrata* ye karşı da antikandidal etkiye sahip olduğu gözlendi. Sonuç olarak, bu bitki bir doğal gıda antioksidanı olarak umut vermektedir.

Anahtar sözcükler: Su mercimeği, *Lemna minor*, antioksidan aktivite, antimikrobiyal aktivite, radikal giderme

Introduction

Duckweed (*Lemna minor* L. Lemnaceae) is a widespread, free-floating aquatic macrophyte, a source of food for waterfowl, and a shelter for small aquatic invertebrates. It grows quickly and reproduces faster than other vascular plants. Duckweed is a suitable plant model for the toxicity evaluation of many substances due to its small size, rapid growth, and ease of culturing (1). The effects of the fungicide folpet on the enzymatic defenses against oxidative stress in duckweed fronds were examined (2). It has been shown to possess immunomodulatory properties, namely the ability to enhance phagocytosis (3). Moreover, the copper-induced changes in the activities of antioxidant enzymes in duckweed fronds were studied (4). Besides these effects, duckweed has been widely used as a raw material for the production of analgesic and antipyretic remedies (5).

Apiogalacturonans have been previously isolated from duckweed and preliminarily characterized (6). The apiogalacturonan fragment appeared to be part of a more complicated pectin isolated from fresh duckweed and identified as lemnan (7).

Reactive oxygen species (ROS), which include free radicals such as superoxide anion radicals ($O_2^{\bullet-}$), hydroxyl radicals (OH·), and non-free radical species such as H_2O_2 and singlet oxygen ($^1O_2$), are various forms of activated oxygen. These molecules are exacerbating factors in cellular injury and the aging process (8-10). ROS are continuously produced during normal physiologic events and they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. However, they are removed by antioxidant defense mechanisms. There is a balance between the generation of ROS and the inactivation of ROS by the antioxidant system in organisms. Oxidative stress occurs when the production of ROS is beyond the protective capability of the antioxidant defenses (11,12). Under pathological conditions, ROS are overproduced and this results in oxidative stress. ROS are formed when endogenous antioxidant defenses are inadequate. The imbalance between ROS and antioxidant defense mechanisms leads to oxidative modification in the cellular membrane or intracellular molecules (13,14).

Antioxidants can protect the human body from free radicals and ROS effects and can retard the progress of many chronic diseases as well as lipid peroxidation (15-17). The most commonly used antioxidants at the present time are BHA, BHT, and propylgallate and *tert*-butyl hydroquinone (18). However, their safety has recently been questioned due to toxicity, liver damage and possible carcinogenicity (19). Thus, the development of safer antioxidants from natural origins has been of interest.

As far as our literature survey could ascertain, no prior information was available on the in vitro total antioxidant activity, reducing power, DPPH· free radical scavenging, $ABTS^{\bullet+}$ radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, or metal chelating activities of duckweed extracts given here. Furthermore, we present here the antibacterial activity of WELM and EELM against 21 clinically isolated bacterial species and 4 clinically isolated fungal species.

Materials and methods

Chemicals

The following chemicals were obtained from Sigma-Aldrich GmbH in Sternheim, Germany: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$); riboflavin; methionine; BHA; BHT; nitroblue tetrazolium (NBT); the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH·); 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine); linoleic acid; α-tocopherol; polyoxyethylenesorbitan monolaurate (Tween-20); and trichloroacetic acid (TCA). Ammonium thiocyanate was purchased from Merck. Mueller Hinton agar was obtained from Oxoid Ltd. (Basingstoke, Hampshire, England, CM337). All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Plant material and identification

Duckweed (0.25 kg) was collected during September (2006) from the Hamamderesi area of Erzurum province, Turkey. It was separated from other plants and dried in the shade at room temperature until it achieved a constant weight. After drying, it was kept in a refrigerator at +4 °C until use. The plants were identified by senior taxonomist Dr. Yusuf Kaya, Sciences Faculty, Department of Biology, Atatürk University, Erzurum.

Extraction procedures

Extraction was carried out as described previously (20). Aquatic duckweed plants were shade-dried initially. For water extraction, 25 g of air-dried duckweed was ground into a fine powder in a mill and mixed with 400 mL of boiling water with a magnetic stirrer for 15 min. Then the extract was filtered through cheese-cloth and Whatman No. 1 paper, consecutively. The filtrates were frozen at -84 °C in an ultra-low temperature freezer (Sanyo, Japan) and lyophilized in a lyophilizator at 5 mmHg pressures at -50 °C (Labconco, Freezone, Japan) (21).

In order to determine the ethanol extraction, a 25 g sample of duckweed was ground into a fine powder in a mill and mixed with 500 mL of ethanol, and then evaporated. The residue was re-extracted under the same conditions until extraction solvents became colorless. The obtained extracts were filtered through Whatman No. 1 paper and the filtrate was collected; then the ethanol was removed using a rotary evaporator (RE 100 Bibby, Stone, Staffordshire, England) at 40 °C to obtain a dry extract. Both extracts were placed in a plastic bottle and then stored at -20 °C until used (22).

Determination of total phenolics content

The total phenolic contents were determined according to the procedure described by Slinkard and Singleton (23) with the slight modification of using a Folin-Ciocalteu phenolic reagent. Gallic acid was used as a standard phenolic compound. Briefly, 1 mg of WELM or EELM in a volumetric flask was diluted with 23 mL of distilled water. Then 0.5 mL of Folin-Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 3 min, 1.5 mL of Na_2CO_3 (2%) was added and then the mixture was allowed to stand for 2 h, with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer. The amounts of total phenolic compounds in the WELM and EELM were determined as micrograms of gallic acid equivalent, using an equation that was obtained from a standard gallic acid graph (R^2: 0.9944).

Absorbance (λ_{760}) = 0.0019 × Totol phenols (μg) + 0.0353

The content of total phenolics in each extract was calculated by employing the standard above curve prepared using gallic acid and expressed as micrograms of gallic acid equivalents (GAE).

Determination of total flavonoids

The amounts of total flavonoids in both extracts were determined as follows: the WELM and EELM solutions (1 mg) were diluted with 4.3 mL of ethanol containing 0.1 mL of 10% aluminum nitrate and 0.1 mL of 1 M aqueous potassium acetate. After 40 min of incubation at room temperature, the absorbance was determined spectrophotometrically at 415 nm. The total flavonoids concentration was calculated using quercetin as the standard (24):

Absorbance (λ_{415}) = 0.1158 × Totol flavonoids (μg) – 0.0114

The contents of total flavonoids in the WELM and EELM were calculated from the standard above curve prepared using quercetin and expressed as micrograms of quercetin equivalents (QE).

Total antioxidant activity determination by ferric thiocyanate method

The total antioxidant activity levels of WELM, EELM, and the standards were calculated according to the ferric thiocyanate method (25). For the stock solutions, WELM and EELM were diluted with the solvent, used for extraction to a suitable concentration (1 μg mL^{-1}) for analysis. Then the solutions, which contained the same concentration of stock WELM and EELM solutions, or standard samples (from 15 μg mL^{-1} to 45 μg mL^{-1}) in 2.5 mL of sodium phosphate buffer (0.04 M, pH 7.0), were added to 2.5 mL of linoleic acid emulsion in the above-mentioned buffer. Therefore, 10 mL of the linoleic acid emulsion was prepared by mixing and homogenizing 31 μL of linoleic acid, 35 μg of Tween-20 as an emulsifier, and 10 mL of phosphate buffer (pH 7.0). On the other hand, the same volume of control solution was composed of 5 mL of linoleic acid emulsion and 5 mL of sodium phosphate buffer (pH 7.0, 0.04 M). Five milliliters of this mixed solution was incubated at 37 °C in a polyethylene flask. The peroxide levels were determined by reading the absorbance at 500 nm in a spectrophotometer at intervals during incubation (Shimadzu, UV-1208 UV-VIS Spectrophotometer, Japan) after reaction with $FeCl_2$ and thiocyanate. During the linoleic acid peroxidation, peroxides are formed, which leads to the oxidation of Fe^{2+} to Fe^{3+}. The latter ions form a complex with ammonium thiocyanate and this complex has a maximum absorbance of 500 nm (26). This step was repeated every 5 h until the control reached its maximum absorbance value. The percentage inhibition values were calculated at this point (30 h). High absorbance indicates a high linoleic acid emulsion peroxidation. The solutions without WELM and EELM were used as blank samples. All data on total antioxidant activity are the averages of triplicate experiments. The inhibition percentage of lipid peroxidation in linoleic acid emulsion was calculated with the following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = 100 - \left(\frac{As}{Ac} \times 100\right)$$

where A_C is the absorbance of the control reaction which contains only linoleic acid emulsion and the sodium phosphate buffer, and A_S is the absorbance in the presence of the sample WELM and EELM or standard compounds (27).

Total reduction capability

The stock samples prepared for the ferric thiocyanate method were used for this and other assays. The reducing power of WELM and EELM was determined by Oyaizu's method (28). Different concentrations of WELM and EELM (15-45 μg mL^{-1}) in 1 mL of distilled water were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50 °C for 20 min. Aliquots (2.5 mL) of trichloroacetic acid (10%) were added to the mixture. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. The mean absorbance values were plotted against the concentration and a linear regression analysis was carried out. Increased absorbance of the reaction mixture indicates an increase in reduction capability.

Chelating activity of ferrous ions (Fe^{2+})

The chelating of ferrous ions by WELM, EELM, and the standards was performed according to the

method of Dinis et al. (29). The reaction was performed in an aqueous medium. Briefly, WELM and EELM (15 μg mL^{-1}) in 0.4 mL was added to a solution of 2 mM $FeCl_2$ (0.2 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.4 mL) and the total volume was adjusted to 4 mL of ethanol. Then the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage of the inhibition of ferrozine-Fe^{2+} complex formation was calculated by using the following formula:

$$\text{Ferrousion } (Fe^{2+}) \text{ chelating effect}(\%) = \left(1 - \frac{As}{Ac}\right) \times 100$$

where A_C is the absorbance of control and A_S is the absorbance in the presence of WELM, EELM, or the standards. The control contains $FeCl_2$ and ferrozine, complex formation molecules (30,31).

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging assay of WELM and EELM was carried out following the procedure of Ruch et al. (32) with slight modification (33). To this aim, a solution of H_2O_2 (43 mM) was prepared in a phosphate buffer (0.1 M, pH 7.4). WELM and EELM (at the 15 μg mL^{-1} concentration) in 3.4 mL of phosphate buffer were added to 0.6 mL of H_2O_2 solution (0.6 mL, 43 mM). The absorbance value of the reaction mixture was recorded at 230 nm. The blank solution contained the phosphate buffer without H_2O_2. The concentration of H_2O_2 in the assay medium was determined using a standard curve:

Absorbance (λ_{230}) = 0.038 × [H_2O_2] + 0.4397

The percentage of H_2O_2 scavenging of WELM, EELM, and the standard compounds was calculated using the following equation:

$$H_2O_2 \text{ scavenging effect}(\%) = \left(1 - \frac{As}{Ac}\right) \times 100$$

where A_C is the absorbance of the control and A_S is the absorbance in the presence of the sample WELM, EELM, or standards (34,35).

Radical scavenging activity

Free radical scavenging capacity of WELM and EELM was determined and compared to that of BHA, BHT, α-tocopherol, and trolox by using the DPPH·, $ABTS^{\bullet+}$, and superoxide anion radical scavenging methods.

*DPPH· **free radical scavenging activity.*** The DPPH· assay provides basic information on the antiradical activity of the extracts. The radical scavenging activity of the WELM and EELM was determined spectrophotometrically by monitoring the disappearance of DPPH· at 517 nm, according to the methodology of Blois (36), previously described by Gülçin et al. (37). The bleaching rate of a stable free radical, DPPH·, is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH· absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, a 0.1 mM solution of DPPH· (10^{-3} M) was prepared in ethanol and 0.5 mL of this solution was added to 1.5 mL of WELM and EELM solution in ethanol at different concentrations (15-45 μg mL^{-1}). These solutions were vortexed thoroughly and incubated in the dark. Thirty minutes later, the absorbance was measured at 517 nm against the blank samples. The lower absorbance of the reaction mixture indicates higher DPPH· free radical scavenging activity. A standard curve was prepared using different concentrations of DPPH·. The DPPH· scavenging capacity was expressed as mM in the reaction medium and calculated from the calibration curve determined by linear regression (R^2: 0.9845):

Absorbance (λ_{517}) = 9.692 × [DPPH·] + 0.215

The capability to scavenge the DPPH· radical was calculated using the following equation:

$$\text{DPPH} \cdot \text{ scavenging effect}(\%) = \left(1 - \frac{As}{Ac}\right) \times 100$$

where A_C is the absorbance of the control which contains 0.5 mL of DPPH· solution and A_S is the absorbance in the presence of WELM or EELM (20,34).

*$ABTS^{\bullet+}$ **radical cation decolorization assay.*** The spectrophotometric analysis of the $ABTS^{\bullet+}$ radical scavenging activity of WELM and EELM was

determined according to the method of Re et al. (38). This method is based on the ability of antioxidants to quench the long-lived $ABTS^{\bullet+}$ radical cation, a blue/green chromophore with characteristic absorption at 734 nm. The $ABTS^{\bullet+}$ cation radical was produced by the reaction between 2 mM $ABTS^{\bullet+}$ in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 4 h. Before usage, the $ABTS^{\bullet+}$ solution was diluted to achieve an absorbance of 0.750 ± 0.025 at 734 nm with a phosphate buffer (0.1 M, pH 7.4). Then 1 mL of $ABTS^{\bullet+}$ solution was added to 3 mL of WELM or EELM solution in ethanol at different concentrations (15-45 μg mL^{-1}). After 30 min, the percentage inhibition at 734 nm was calculated for each concentration relative to a blank absorbance. Solvent blanks were run in each assay. The extent of decolorization was calculated as the percentage of reduction of absorbance. For preparation of a standard curve, different concentrations of $ABTS^{\bullet+}$ were used. The $ABTS^{\bullet+}$ concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (R^2: 0.9841):

$$\text{Absorbance } (\lambda_{734}) = 4.6788 \times [ABTS^{\bullet+}] + 0.199$$

The scavenging capability of the $ABTS^{\bullet+}$ radical was calculated using the following equation:

$$ABTS^{\bullet+}\text{ scavenging effect}(\%) = \left(1 - \frac{As}{Ac}\right) \times 100$$

where A_C is the initial concentration of the $ABTS^{\bullet+}$ and A_S is the absorbance of the remaining concentration of $ABTS^{\bullet+}$ in the presence of WELM or EELM (31,39).

Superoxide anion radical scavenging activity. Superoxide radicals were generated by the method described by Zhishen et al. (40). Superoxide radicals were generated in riboflavin, methionine, and illuminate and assayed by the reduction of NBT to form blue formazan. All solutions were prepared in a 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed using fluorescent lamps (20 W). The concentration of WELM or EELM in the reaction mixture was 15 μg mL^{-1}. The total volume of the reactant mixture was 5 mL and the concentrations of the riboflavin, methionine, and nitro blue tetrazolium (NBT) were 1.33×10^{-5}, 4.46×10^{-5}, and 8.15×10^{-8} mol mL^{-1}, respectively. The reactant was illuminated at 25 °C for 40 min. The photochemically reduced riboflavin generated $O_2^{\bullet-}$, which reduced NBT to form blue formazan. The un-illuminated reaction mixture was used as a blank. The absorbance was measured at 560 nm. WELM or EELM was added to the reaction mixture, in which $O_2^{\bullet-}$ was scavenged, thereby inhibiting the NBT reduction. Decreased absorbance of the reaction mixture indicates an increased superoxide anion scavenging activity. The inhibition percentage of superoxide anion generation was calculated by using the following formula:

$$O_2^{\bullet-}\text{ scavenging effect}(\%) = \left(1 - \frac{As}{Ac}\right) \times 100$$

where A_C is the absorbance of the control and A_S is the absorbance of WELM, EELM or the standards (41,42).

Preparation of test microorganisms

Gram-positive bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Staphylococcus warneri, Staphylococcus xylosus, Micrococcus luteus, Bacillus cereus, Bacillus subtilis*, and *Streptococcus pneumonia*), gram-negative bacteria (*Escherichia coli, Citrobacter freundii, Citrobacter koseri* (formerly called *Citrobacter diversus*), *Enterobacter aerogenes, Neisseria lactamica, Neisseria sicca, Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa, Pseudomonas fluorescens, Klebsiella pneumonia*, and *Klebsiella oxyttoca*), and fungi yeast (*Candida albicans*-ATCC 90028, *Candida tropicalis*-clinical isolate, *Candida parapsilosis*-ATCC 22019, and *Candida glabrata*-clinical isolate) were employed for determination of antimicrobial and antifungal activity. Microorganisms that can be pathogenic for humans and animals were used in this study. The strains of bacteria and fungi were isolated and identified from patients (human and/or animal) and foods (milk and cheese).

Bacteria and fungi yeast were obtained from the stock cultures (clinical isolates and standard strains) of the Microbiology Laboratory, Department of Microbiology, Veterinary Faculty, Atatürk University, Erzurum. The bacterial and fungal stock cultures were

maintained on Mueller Hinton Agar (Oxoid CM 337, Basingstoke, Hampshire, UK) slants, respectively, which were stored at 4 °C. For the purpose of antibacterial evaluation, 21 microorganisms were used. These bacteria were maintained on a Blood Agar Base (Oxoid CM55, Basingstoke, Hampshire, UK). The yeast was maintained on Sabouraud-dextrose agar (Oxoid CM41, Basingstoke, Hampshire, UK), which is often used with antifungal for the isolation of pathogenic fungi. Clinical strains of bacteria and yeast isolates were identified by conventional biochemical tests (43) and confirmed by the API test system (BioMèrieux, Marcy l'Etoile, France) (44).

Determination of antimicrobial and antifungal activities

The antibacterial activity of the WELM and EELM was determined by a disk-diffusion test. Agar cultures of the test microorganisms were prepared as described by Gülçin et al. (45). For this purpose, 3 to 5 similar colonies were selected and transferred with loops into 5 mL of Tryptone soya broth (Oxoid CM129, Basingstoke, Hampshire, UK). Tryptone soya broth is a highly nutritious and versatile medium, recommended for general laboratory use and used for the cultivation of aerobes and facultative anaerobes, including some fungi. The broth cultures were incubated for 24 h at 37 °C. For screening, sterile, 6-mm diameter filter paper disks were impregnated with 45 µg (at the concentration of 1 µg mL^{-1}) of the water or ethanol extracts. In total, 45 µg of extract was loaded into each disk. Both the WELM and EELM were dissolved in sterile water for the assay with a magnetic stirrer. The extraction solvents without the plant extract were used as the control samples. Positive controls were prepared with the same solvents, which were used to dissolve the plant extracts. Then the paper disks were placed onto Mueller Hinton agar (Oxoid CM337, Basingstoke, Hampshire, UK). The inoculums for each organism were prepared from broth cultures. The concentration of the cultures was to 10^8 colony forming units (1×10^8 CFU mL^{-1}). The results were recorded by measuring the zones of growth inhibition surrounding the disks. Clear inhibition zones around the disks indicated the presence of antimicrobial activity (45). All data on antimicrobial activity are the average of triplicate analyses. Ampicillin (10 µg $disk^{-1}$), amoxicillin (25 µg $disk^{-1}$), cefuroxime (30 µg $disk^{-1}$), and antifungal miconazole nitrate (40 µg $disk^{-1}$, DRG International) were used as reference standards to determine the sensitivity of one strain or isolate in each tested microbial species, as recommended by the Clinical and Laboratory Standards Institute (46). Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.

Statistical Analysis

The experimental results are expressed as mean ± standard deviation (SD) of triplicate measurements and analyzed by SPSS (version 11.5 for Windows 2000, SPSS Inc.). One-way analysis of variance was performed according to ANOVA procedures. Significant differences between the means were determined by Duncan's multiple range tests. $P < 0.05$ was regarded as significant and $P < 0.01$ was very significant.

Results

The yield of crude extracts and total phenolics content of WELM or EELM are shown in Table 1. No significant differences in scavenging potential could be determined among the different amounts of WELM and EELM ($P < 0.05$). For determining the total phenolic contents, calibration curves were obtained using known quantities of standard gallic acid. The phenolic compounds of 1 mg of WELM and EELM ranged from 22.0 ± 0.8 to 16.7 ± 0.0 µg GAE, respectively. WELM possessed the highest phenolic compounds. The content of total flavonoids in WELM and EELM was determined spectrophotometrically and found to be 4.5 ± 0.12 and 17.4 ± 0.1 µg quercetin equivalents, respectively.

Table 1. The yield of crude extracts, total phenolics content and total flavonoid content of duckweed extracts (WELM: Water extract of duckweed; EELM: Ethanol extract of duckweed).

	WELM	EELM
Yield (%)	13.2	18.4
Total phenolics (µg mg^{-1} extract)	22.0 ± 0.8	16.7± 0.0
Total flavonoids (µg mg^{-1} extract)	4.5 ± 0.2	17.4 ± 0.1

The effects of different concentrations (15-45 μg mL^{-1}) of WELM and EELM on lipid peroxidation of linoleic acid emulsion are shown in Figures 1 and 2. The percentage inhibition of lipid peroxidation of 45 μg mL^{-1} of WELM and EELM was found to be 100% and 94.2%, respectively, and their activities are greater than that of α-tocopherol (84.6%) and similar to that of trolox (95.6%) at the same concentration.

Figure 3 shows the reducing power of the WELM, EELM, and standards (α-tocopherol and trolox) using the potassium ferricyanide reduction method. The reducing power of the tested samples increased with the increasing concentration of the samples (from 15 to 45 μg mL^{-1}). At these different concentrations, WELM and EELM showed an effective reducing power (Figure 3). When these results were compared

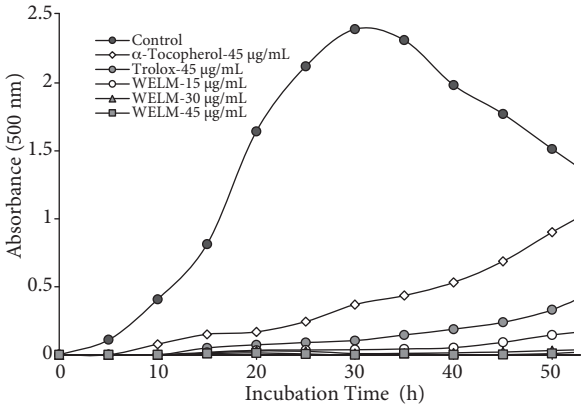

Figure 1. Total antioxidant activity of different concentrations (15-45 μg mL^{-1}) of WELM, α-tocopherol, and trolox (45 μg mL^{-1}) (WELM: Water extract of duckweed).

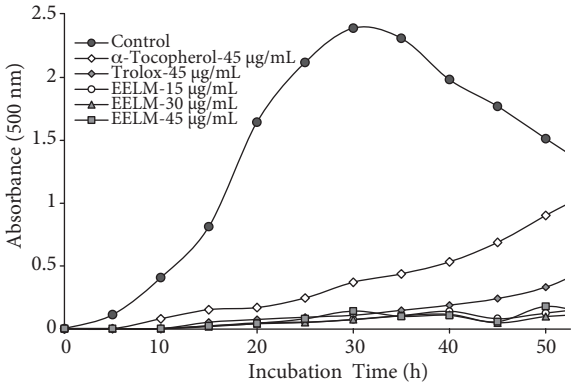

Figure 2. Total antioxidant activity of different concentrations (15-45 μg mL^{-1}) of EELM, α-tocopherol, and trolox (45 μg mL^{-1}) (EELM: Ethanol extract of duckweed).

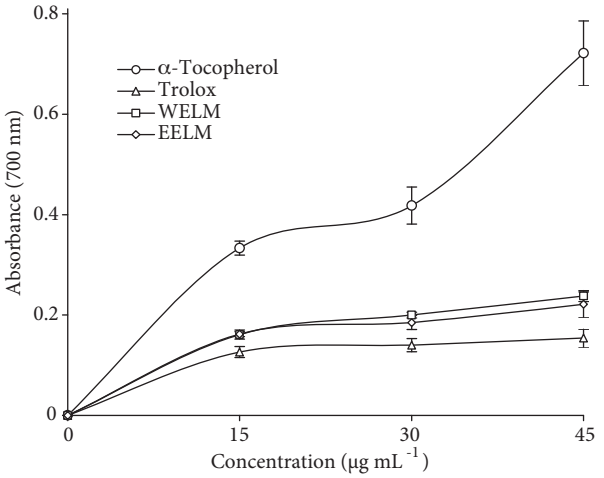

Figure 3. Reductive potential of different concentrations (15-45 μg mL^{-1}) of WELM, EELM, α-tocopherol, and trolox (WELM: Water extract of duckweed; EELM: Ethanol extract of duckweed).

to the control values, statistically significant differences were found ($P < 0.05$). The reducing power of WELM, EELM, and the standard compounds exhibited the following order: α-tocopherol > WELM ≈ EELM > trolox.

The ferrous ion chelating activities of WELM, EELM, α-tocopherol, and trolox are shown in Table 2. In regards to this Table, the absorbance of Fe^{2+}-ferrozine complex was decreased at a 15 μg mL^{-1} concentration of WELM or EELM. The difference between the 15 μg mL^{-1} concentration of WELM, EELM, and the control was found to be statistically significant ($P < 0.01$). At the above-mentioned concentration of 15 μg mL^{-1}, WELM and EELM exhibited marked ferrous ion chelating. The metal chelating effect of those samples decreased in the order of BHA > WELM > EELM > BHT > trolox > α-tocopherol. The data obtained from Table 2 reveal that WELM and EELM demonstrate a marked capacity for iron binding, suggesting that their main action as peroxidation protectors may be related to their iron binding capacity.

The ability of WELM and EELM to scavenge hydrogen peroxide was determined according to the method of Ruch et al. (32) as shown in Table 2, and was then compared with that of BHA, BHT, α-tocopherol, and trolox as standards. WELM and EELM were capable of scavenging H_2O_2. At the 15 μg mL^{-1} concentration, WELM and EELM exhibited 92.3

Table 2. Comparison of hydrogen peroxide (H_2O_2) scavenging activity, ferrous ion (Fe^{2+}) chelating activity, and superoxide anion radical ($O_2^{\bullet}$) scavenging activity of WELM, EELM, and standard antioxidant compounds such as BHA, BHT, α-tocopherol, and trolox at the concentration of 15 μg mL^{-1} (BHA: Butylated hydroxyanisole; BHT: Butylated hydroxytoluene; WELM: Water extract of duckweed; EELM: Ethanol extract of duckweed).

	H_2O_2 scavenging activity (%)	Ferrous ion chelating activity (%)	Superoxide scavenging activity (%)
BHA	36.4 ± 3.5	69.9 ± 7.5	75.3 ± 6.5
BHT	34.3 ± 4.1	60.0 ± 9.3	70.2 ± 7.1
α-Tocopherol	39.3 ± 2.9	31.3 ± 5.5	22.2 ± 3.3
Trolox	25.5 ± 3.3	45.2 ± 6.2	16.0 ± 1.9
WELM	92.3 ± 2.8	63.0 ± 6.9	38.8 ± 3.1
EELM	85.7 ± 1.1	61.0 ± 6.0	23.0 ± 2.4

± 2.8% and 85.7 ± 1.1% scavenging activity. These results showed that WELM and EELM had effective hydrogen peroxide scavenging activity. At the 15 μg mL^{-1} concentration, the hydrogen peroxide scavenging effect of WELM, EELM, and the 4 standard compounds decreased in the order of WELM > EELM > α-tocopherol > BHA > BHT > trolox.

Figure 4 illustrates a significant decrease ($P < 0.01$) in the concentration of the DPPH· radical due to the scavenging ability of WELM, EELM, and the standards. BHA, BHT, α-tocopherol, and trolox were used as references for radical scavengers. The scavenging effect of WELM, EELM, and the standards on the DPPH· radical decreased in the order of BHA > α-tocopherol > BHT > trolox > WELM ≈ EELM. The free radical scavenging activity of these samples also increased with increasing concentration.

WELM and EELM exhibited effectual radical cation scavenging activity. As seen in Figure 5, both extracts had effective $ABTS^{\bullet+}$ radical scavenging activity in a concentration-dependent manner (15-60

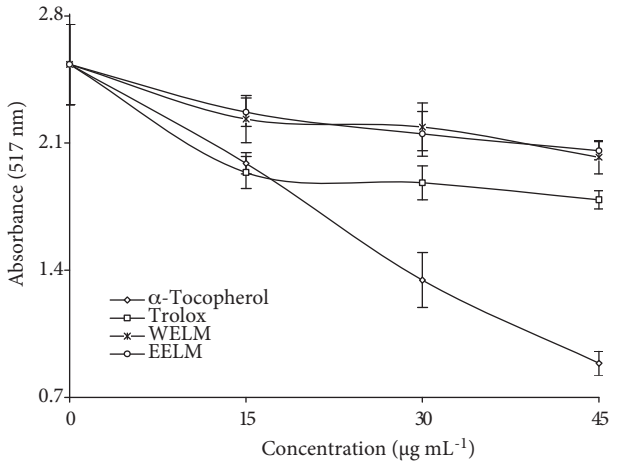

Figure 4. DPPH· free radical scavenging activities of different concentrations (15-45 μg mL^{-1}) of WELM, EELM, α-tocopherol, and trolox (DPPH·: 1,1-diphenyl-2-picryl-hydrazyl; WELM: Water extract of duckweed; EELM: Ethanol extract of duckweed).

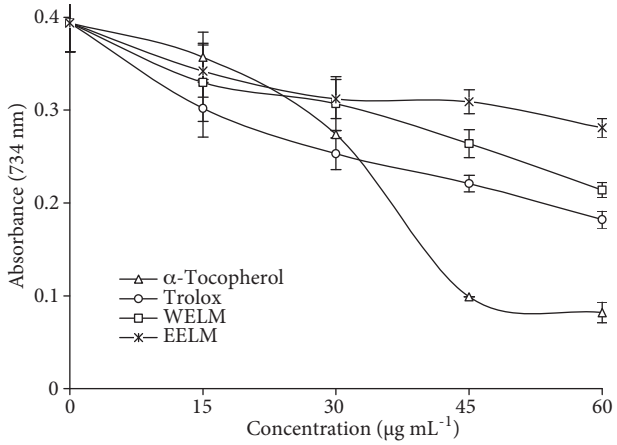

Figure 5. $ABTS^{\bullet+}$ radical scavenging activity of different concentrations (15-60 μg mL^{-1}) of WELM, EELM, α-tocopherol, and trolox ($ABTS^{\bullet+}$: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); WELM: Water extract of duckweed; EELM: Ethanol extract of duckweed).

μg mL^{-1}). There is a significant decrease ($P < 0.01$) in the concentration of $ABTS^{\bullet+}$ due to the scavenging capacity of all WELM and EELM concentrations. Moreover, the scavenging effect of WELM, EELM, and the standards on the $ABTS^{\bullet+}$ decreased in that order: BHA > BHT > α-tocopherol > trolox > WELM > EELM at the concentration of 60 μg mL^{-1}, respectively.

Table 2 shows the inhibition percentage of superoxide radical generation by a 15 μg mL^{-1} concentration of WELM, EELM, and the standards. As can be seen from Table 2, the percentage inhibition of superoxide anion radical generation by a 15 μg mL^{-1} concentration of WELM and EELM was found to be 38.8 ± 3.1% and 23.0 ± 2.4%, respectively.

In this study, 21 different bacterial and 4 different *Candida* species were used to screen the possible antibacterial and antifungal activities of both WELM and EELM (see Table 3). Most of the gram-positive and gram-negative bacterial species and the *Candida*

Table 3. Antibacterial and antifungal activity of WELM and EELM against bacteria strains based on disk-diffusion assay (WELM: Water extract of duckweed; EELM: Ethanol extract of duckweed). [a]Antimicrobial and antifungal results are the averages of triplicate measurements. [b] AMP: Ampicillin (45 mg $disk^{-1}$); AMC: Amoxicillin (45 mg $disk^{-1}$); CEF: cefuroxime (45 mg $disk^{-1}$); Miconazole nitrate (45 mg $disk^{-1}$); ND: Not detected activity at this concentration; S: Sensitivity; R: Resistant.

		Diameter of extract zone (mm)[a]		Antimicrobial agent (mm)[a,b]						Antifungal activity (mm)[a,b]		
		WELM	EELM	AMP		AMC		CEF		WELM	EELM	MN[a]
				S	R	S	R	S	R			
Staphylococcus aureus	ATCC 6538	ND	ND	-	11	-	10	-	16	-	-	-
Staphylococcus epidermidis	Clinical isolate	8	8	27	-	27	-	31	-	-	-	-
Staphylococcus saprophyticus	Clinical isolate	7	8	30	-	28	-	36	-	-	-	-
Staphylococcus warneri	Clinical isolate	9	9	27	-	26	-	39	-	-	-	-
Staphylococcus xylosus	Clinical isolate	ND	7	25	-	26	-	33	-	-	-	-
Escherichia coli	ATCC 9837	ND	ND	-	15	-	13	25	-	-	-	-
Citrobacter freundii	ATCC 8090	7	7	23	-	23	-	32	-	-	-	-
Citrobacter koseri	Clinical isolate	7	8	21	-	22	-	27		-	-	-
Enterobacter aerogenes	ATCC 13048	ND	ND	-	ND	-	ND	-	10	-	-	-
Neisseria lactamica	ATCC 23970	10	11	25	-	25	-	29	-	-	-	-
Neisseria sicca	Clinical isolate	10	10	24	-	26	-	30	-	-	-	-
Proteus vulgaris	ATCC 49990	ND	ND	-	ND	-	ND	18	-	-	-	-
Proteus mirabilis	ATCC 29906	ND	ND	-	ND	-	ND	18	-	-	-	-
Micrococcus luteus	Clinical isolate	8	9	22	-	21	-	37	-	-	-	-
Pseudomonas aeruginosa	*ATCC 9027*	ND	ND	-	ND		ND	-	11	-	-	-
Pseudomonas fluorescens	ATCC 13525	ND	ND	-	14	-	15	28	-	-	-	-
Bacillus cereus	ATCC 10987	7	7	24	-	22	-	38	-	-	-	-
Bacillus subtilis	ATCC 6633	8	8	20	-	21	-	35	-	-	-	-
Klebsiella pneumonia	ATCC13883	ND	ND	-	ND	-	ND	23	-	-	-	-
Klebsiella oxyttoca	ATCC43863	ND	ND	-	9	-	11	20	-	-	-	-
Streptococcus pneumoniae	ATCC 49619	8	8	24	-	27	-	37	-	-	-	-
Candida albicans	ATCC 90028	-	-	-	-	-	-	-	-	ND	ND	20
Candida tropicalis	Clinical isolate	-	-	-	-	-	-	-	-	ND	ND	22
Candida parapsilosis	ATCC 22019	-	-	-	-	-	-	-	-	7	8	19
Candida glabrata	Clinical isolate	-	-	-	-	-	-	-	-	7	7	23

species were inhibited by WELM and EELM. However, the antibacterial activity of WELM was not detected against *Staphylococcus aureus, Escherichia coli, Staphylococcus xylosus, Enterobacter aerogenes, Proteus vulgaris Proteus mirabilis, Pseudomonas fluorescens, Klebsiella pneumonia*, or *Klebsiella oxyttoca*. In the same manner, EELM had no effect against these microorganisms. Both extracts had inhibition effects on the generation of *Candida parapsilosis* and *Candida glabrata* (see Table 3). Ampicillin (10 μg $disk^{-1}$), amoxicillin (25 μg $disk^{-1}$) and cefuroxime (30 μg $disk^{-1}$) were used as positive controls for bacteria. Miconazole nitrate (40 mg $disk^{-1}$) was used as a positive control for antifungal activity.

Discussion

Recently, research in nutrition and food science has focused on plant products with potential antioxidant and antimicrobial activities. Such products are also rich in fiber, have no cholesterol and contain antioxidants such as carotenoids and flavonoids and other phenolic compounds. Flavonoids are an important group of natural compounds, which can prevent coronary heart disease and have antioxidant properties. It has been reported that flavonoids represent a class of naturally occurring compounds, mainly found in fruits, vegetables, and cereals (47). It has been further reported that phenolic compounds are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation. According to other reports, a highly positive relationship between total phenols and antioxidant activity was found in many plant species (22). As can be seen Figures 1 and 2 and Table 1, the current results indicate that there is a positive correlation between total antioxidant activity and the total phenolic content of WELM. Different results have been reported about this; some authors have found a correlation between phenolic content and antioxidant activity (48).

Total antioxidant capacity is widely used as a parameter for food, pharmaceutical, and medicinal extracts. In this study, the antioxidant activity of WELM and EELM was compared to that of BHA, BHT, and α-tocopherol and its water-soluble analogue, trolox. The antioxidant activity of the WELM, EELM, BHA, BHT, α-tocopherol, and trolox has been evaluated in a series of in vitro tests: DPPH· free radical scavenging, $ABTS^{\bullet+}$ radical scavenging, scavenging of superoxide anion radical-generated non-enzymatic systems, total antioxidant activity by ferric thiocyanate method, reducing power by Fe^{3+}-Fe^{2+} transformation, hydrogen peroxide scavenging, and metal chelating activities.

The ferric thiocyanate method measures the amount of peroxide, the primary product of lipid oxidation, produced during the initial stages of oxidation (49). The total antioxidant activity of WELM, EELM, α-tocopherol, and trolox was determined by the ferric thiocyanate method in the linoleic acid system. WELM, EELM, and the standard compounds exhibited effective antioxidant activity in this system.

Fe^{3+}-Fe^{2+} transformation was investigated to determine the measurements of the reductive ability of WELM and EELM by using the method of Oyaizu (28). Its results on the reducing power demonstrate the electron donor properties of WELM and EELM, thereby neutralizing free radicals by forming stable products. The outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging.

Chelation of ferrous ions (Fe^{2+}) may provide important antioxidative effects by retarding metal-catalyzed oxidation. Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The effective ferrous ion chelators may also afford protection against oxidative damage by removing iron (Fe^{2+}) that may otherwise participate in an HO• generating Fenton reaction.

Ferric (Fe^{3+}) ions also produce radicals from peroxides, although the rate is 10-fold less than that of ferrous (Fe^{2+}) ions (50). The Fe^{2+} ion is the most powerful pro-oxidant among the various species of metal ions. Fe^{2+} is able to generate free radicals from peroxides by Fenton reactions and may be involved in the progression of human cardiovascular disease. Thus, antioxidants capable of chelating with Fe^{2+} will minimize the ion's concentration and inhibit its capacity to catalyze free radical formation, which will result in protection against oxidative damage. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex

formation is disrupted, resulting in a decrease in the red color of the complex. Measurement of color reduction therefore allows for the estimating of the metal chelating activity of the coexisting chelator. In this assay, WELM and EELM are interfered with by the formation of ferrous and ferrozine complexes, suggesting that they possess chelating activities and are able to capture ferrous ion before ferrozine.

Hydrogen peroxide can be formed in vivo by many oxidizing enzymes, such as superoxide dismutase. Hydrogen peroxide is not very reactive; however, it can sometimes be toxic to cells because it may give rise to hydroxyl radicals within the cells. The addition of hydrogen peroxide to cells in a culture can lead to transition metal ion-dependent OH radicals' mediated oxidative DNA damage (42).

DPPH· is a long-lived nitrogen radical. Many antioxidants that react quickly with transient radicals, such as peroxyl radicals, may react slowly or may even be inert to DPPH· (51). DPPH· has also been widely used for the evaluation of free radical scavenging effectiveness of various antioxidant substances (49). In the DPPH· assay, the antioxidants were able to reduce the stable radical DPPH· to the yellow-colored diphenyl-picrylhydrazine. This method is based on the reduction of an alcoholic DPPH· solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction. DPPH· is usually used as a reagent to evaluate the free radical scavenging activity of antioxidants (28).

Generation of the $ABTS^{\bullet+}$ radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of solutions of pure substances, aqueous mixtures, and beverages (37,50). A more appropriate format for the assay is a decolorization technique in which the radical is generated directly in a stable form prior to reaction with putative antioxidants. The improved technique for the generation of $ABTS^{\bullet+}$ described here involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between $ABTS^{\bullet+}$ and potassium persulfate (52).

Superoxide anion radicals are produced by a number of enzyme systems in auto-oxidation reactions and by non-enzymatic electron transfers that univalently reduce molecular oxygen. They can also reduce certain iron complexes, such as cytochrome c (42). Superoxide anion is derived from dissolved oxygen by the riboflavin/methionine/illuminate system. In this method, the superoxide anion reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. Antioxidants are able to inhibit the blue NBT formation. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of the superoxide anion in the reaction mixture.

Herbs with antimicrobial properties have been widely used both traditionally and commercially to increase the shelf-life and safety of foods (53). Many naturally occurring extracts from edible and medicinal plants, herbs, and spices have been shown to possess antimicrobial functions and could serve as a source for antimicrobial agents against food spoilage and pathogens (54).

The antimicrobial and antifungal activities of WELM and EELM were calculated according to the disk-diffusion method. This method is extensively used for investigation of the antibacterial activity of natural substances and plant extracts. These assays are based on the use of disks as reservoirs containing solutions of substances to be examined. In the case of solutions with a low activity, however, a large concentration or volume is needed. The limited capacity of the disks means that holes or cylinders are preferably used (55).

Conclusion

According to data of the present study, when compared to standard antioxidant compounds such as BHA and BHT, the natural antioxidant a-tocopherol, trolox (a water-soluble analogue of tocopherol), WELM and EELM were found to be effective antioxidants and antiradicals in different in vitro assays, including reducing power, DPPH· radical, $ABTS^{\bullet+}$ radical, and superoxide anion radical scavenging; hydrogen peroxide scavenging; and metal chelating activities. Moreover, our studies showed that WELM and EELM are good sources of antioxidants for food, medicines, and pharmaceuticals. Based on the discussion above, these extracts can be use for

minimizing or preventing lipid oxidation in food products, retarding the formation of toxic oxidation products, maintaining nutritional quality and prolonging the shelf-life of foods and pharmaceuticals. Furthermore, our results exhibited that WELM and EELM had moderate antibacterial and anticandidal activities. Besides these properties, WELM and EELM could be used as preservatives in food products to prevent microbial spoilage.

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