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Food Chemistry 83 (2003) 371–382

Food Chemistry

www.elsevier.com/locate/foodchem

Screening of antioxidant and antimicrobial activities of anise (*Pimpinella anisum* L.) seed extracts

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Received 26 April 2002; received in revised form 11 February 2003; accepted 21 February 2003

Abstract

In this study, antioxidant and antimicrobial activities of water and ethanol extracts of anise (*Pimpinella anisum* L.) seed (PAS) were investigated. The antioxidant properties of both extracts of PAS were evaluated using different antioxidant tests, including reducing power, free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, and metal chelating activities. Twenty μg/ml of water and ethanol extracts exhibited 99.1 and 77.5% inhibition of peroxidation in linoleic acid system, which was greater than the same concentration of α-tocopherol (36.1%). These various antioxidant activities were compared with synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and α-tocopherol. The water extract of PAS exhibited greater antioxidant capacity than that of ethanol. Antimicrobial activity tests were carried out using disc diffusion methods with 10 microbial species.

Keywords: *Pimpinella anisum* L; Antioxidant activity; Antimicrobial activity; Anise; Seed

1. Introduction

Reactive oxygen species (ROS), which include free radicals such as superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals (OH·) and non free-radical species such as H_2O_2 and singled oxygen ($^1O_2$), are various forms of activated oxygen (Gülçin, Oktay, Küfrevioğlu, & Aslan, 2002; Halliwel & Gutteridge, 1999; Yıldırım, Mavi, Oktay, Kara, Algur, & Bilaloğlu, 2000). The importance of free radicals and reactive oxygen species (ROS) has attracted increasing attention over the past decade (Gülçin, Büyükokuroğlu, Oktay, & Küfrevioğlu, 2002). These molecules are exacerbating factors in cellular injury and aging process (Lai, Chou, & Chao, 2001). ROS have aroused significant interest among scientists. Their broad range of effects on biological and medicinal systems has drawn the attention of many experimental works (Büyükokuroğlu, Gülçin, Oktay, & Küfrevioğlu, 2001). In living organisms, various ROS can form in different ways. Normal aerobic respiration stimulates polymorphonuclear leukocytes and macrophages, and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of ROS include tobacco smoke, certain pollutants, organic solvents, and pesticides (Davies, 1994; Halliwell & Gutteridge, 1989; Robinson, Maxwell, & Thorpe, 1997). ROS can cause lipid peroxidation in foods, which leads to the deterioration of the food (Miller, Diplock, & Rice-Evans, 1995; Sasaki, Ohta, & Decker, 1996). In addition, reactive oxygen species induce some oxidative damage to biomolecules like lipids, nucleic acids, proteins and carbohydrates. Their damage causes ageing, cancer, and many other diseases (Aruoma, 1994). As a result, ROS have been implicated in more than 100 diseases, including malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, and cancer (Alho & Leinonen, 1999; Duh, 1998; Hertog, Feskens, Hollman, Katan, & Kromhout, 1993; Tanizawa, Ohkawa, Takino, Ueno, Kageyama, & Hara, 1996; Yıldırım, Mavi, & Kara, 2001).

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doi:10.1016/S0308-8146(03)00098-0

$O_2^{\cdot-}$ is an oxygen-centred radical with selective reactivity. This species is produced by a number of enzyme systems in autooxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen. It can also reduce certain iron complex such as cytochrome *c*. H_2O_2 is not a radical and can be formed in vivo by many oxidize enzymes such as superoxide dismutase. It can cross membranes and may slowly oxidize a number of compounds. OH· is a highly reactive oxygen-centred radical, which attacks all proteins, deoxyribonucleic acid (DNA), and polyunsaturated fatty acid (Aruoma, 1998).

ROS are continuously produced during normal physiologic events and are removed by antioxidant defence mechanisms (Halliwell, Gutteridge, & Cross, 1992). There is a balance between the generation of ROS and inactivation of ROS by the antioxidant system in organisms. Under pathological conditions, ROS are overproduced and result in lipid peroxidation and oxidative stress. ROS are formed when endogenous antioxidant defences are inadequate. The imbalance between ROS and antioxidant defence mechanisms leads to oxidative modification in cellular membrane or intracellular molecules (El-Habit, Saada, & Azab, 2000).

Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers (Duh, 1998; Yen & Duh, 1994). Recently, interest has considerably increased in finding naturally occurring antioxidant for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects such as carcinogenecity (Ito, Fukushima, Hasegawa, Shibata, & Ogiso, 1983; Zheng & Wang, 2001). Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods (Kinsella, Frankel, German & Kanner, 1993; Lai et al., 2001; Pryor, 1991). Hence, the studies on natural antioxidant have gained increasingly greater importance.

Pimpinella anisum L. Umbelliferae is an annual herb and a grassy plant with white flowers and small green to yellow seeds, which grows in Turkey, Iran, India, Egypt, and many other warm regions of the world (Pourgholami, Majzoob, Javadi, Kamalinejad, Fanaee, & Sayyah, 1999; Zargari, 1989). Chemical studies have demonstrated that the PAS contain anethole (Chandler & Hawkes, 1984; Fujita & Nagasawa, 1960), estragole (Zargari, 1989), eugenol (Monod & Dortan, 1950), pseudoisoeugenol (Reichling, Kemmerer, & Sauer, 1995), methylchavicol and anisaldehyde (Wagner, Bladt, & Zgainski, 1984), coumarins, scopoletin, umbelliferon, estrols (Burkhardt, Reichling, Martin, & Becker, 1986), terpene hydrocarbons (Kartnig, Moeckel, & Mauns, 1975), polyenes and polyacetylenes (Schulte, Rucker, & Backe, 1970) as the major compounds.

It was reported that *P. anisum* had several therapeutic effects on several conditions such as digestive, gynaecologic, neurologic, and respiratory disorders (Aboabrahim, 1970). The aqueous extract of this plant has been reported to delay the onset of picrotoxin-induced seizures in mice (Abdul-Ghani, El-Lati, Sacaan, & Suleiman, 1987). It was demonstrated that the PAS had ovicidal activity against stored-product insects (Tunç Berger, Erler, & Dağlý, 2000). Eugenol and estragole have anaesthetic, hypothermic, muscle relaxant (Boskabady & Ramazani, 2001), and anticonvulsant activities (Dallmeier & Carlini, 1981). In addition, anethole possesses muscle relaxant effect.

P. anisum is primarily grown for its fruits, commercially called "seeds" that are currently used for flavouring. The essential oil from *P. anisum* fruits is also valuable in perfumery and in medicine (Ernts, 1989; Santos et al., 1998; Simon, Chadwick, & Craker, 1980). *Pimpinela* species are very common in Turkey. There are more than 20 *Pimpinela* species in Anatolia plant flora. *P. anisum* is one of the most common species, which grows in different regions of Turkey (Baytop, 1999). In Turkish folk medicine, this plant, especially its seeds, have been used as appetizer, tranquillizer and diuretic drug. Especially, this plant was extensively used in liquor production in Turkey (Asımgil, 1997; Baytop, 1999). However, there is no information about antioxidant and antimcrobial activities of the seeds of this plant. The purpose of the present study was to evaluate the antioxidant and antimicrobial activity of water and ethanol extracts of PAS and to determine their antioxidative and antimicrobial actions.

An important goal of this research was to examine a mixed extracts with effective antimicrobial activity. The spoilage and poisoning of foods by microorganisms is a large problem that has not yet been brought under adequate control despite the range of robust preservation techniques available. Consumers are increasably avoiding foods prepared with preservatives of chemical origin. Natural alternatives are therefore needed to achieve sufficiently long shelf-life of foods and a high degree of safety with respect to pathogenic microorganisms. Hence, the investigations on spoilage and poisoning of foods by microorganisms are very important.

2. Materials and methods

2.1. Chemicals

Ammonium thiocyanate was purchased from E. Merck. Ferrous chloride, polyoxyethylenesorbitan monolaurate (Tween-20), α-tocopherol, 1,1-diphenyl-2-picryl-hydrazyl (DPPH·), 3-(2-Pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), nicotinamide adenine dinucleotide (NADH), butylated

hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and trichloracetic acid (TCA) were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Muller Hinton agar was also obtained from Oxoid Ltd. (Basingstoke, Hampshire, England, CM337). All other chemicals used were of analytical grade and were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany).

2.2. Plant material and extraction

PAS was obtained from a local market at Erzurum, Turkey. For water extraction, 25 g sample was put into a fine powder in a mill and was mixed with 500 ml boiling water by magnetic stirrer for 15 min. Then, the extract was filtered over Whatman No. 1 paper. The filtrates were frozen and lyophilized in lyophilizator at 5 μmHg pressure at −50 °C (Labconco, Freezone 1L). For ethanol extraction 25 g sample was put into a fine powder in a mill and was mixed with 500 ml ethanol. The residue was re-extracted until extraction solvents became colourless. The obtained extracts were filtered over Whatman No. 1 paper and the filtrate was collected, and then ethanol was removed by a rotary evaporator (RE 100 Bibby, Stone, Staffordshire England, ST15 OSA) at 50 °C to obtain dry extract. Both extracts were placed in a plastic bottle, and then stored at −20 °C until used.

2.3. Total antioxidant activity determination

The antioxidant activity of PAS was determined according to the thiocyanate method (Mitsuda, Yuasumoto, & Iwami, 1966). For stock solutions, 10 mg of each PAS extracts was dissolved in 10 ml water. Then, the solution, which contains different concentration of stock PAS solution or standards samples (10, 20, and 50 μg/ml) in 2.5 ml of potassium phosphate buffer (0.04 M, pH 7.0), was added to 2.5 ml of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). Fifty millilitres linoleic acid emulsion contained 175 μg Tween-20, 155 μl linoleic acid, and 0.04 M potassium phosphate buffer (pH 7.0). On the other hand, 5.0 ml control was composed of 2.5 ml linoleic acid emulsion and 2.5 ml, 0.04 M potassium phosphate buffer (pH 7.0). The mixed solution (5 ml) was incubated at 37 °C in a glass flask. The peroxide level was determined by reading the absorbance at 500 nm in a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland) after reaction with $FeCl_2$ and thiocyanate at intervals during incubation. During the linoleic acid oxidation, peroxides are formed, which oxidize Fe^{+2} to Fe^{+3}. The latter ions form a complex with SCN^- and this complex has a maximum absorbance at 500 nm. Therefore, high absorbance indicates high linoleic acid oxidation. The solutions without added extracts were used as blank samples. All data on total antioxidant activity are the average of duplicate analyses. The % inhibition of lipid peroxidation was calculated by following equation:

$$\%\ \text{Inhibition} = 100 - [(A_1/A_o) \times 100]$$

where A_o is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample of PAS extracts (Duh, Tu, & Yen, 1999).

2.4. Reducing power

The reducing power of PAS extracts was determined by the method of Oyaizu (1986). Different concentrations of PAS extracts (2.7–13.4 μg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 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, which was then centrifugated for 10 min at 1036×*g* (MSE Mistral 2000, UK). 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. Increased absorbance of the reaction mixture indicates increased reducing power.

2.5. Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity of PAS extracts was based on the method described by Liu, Ooi, and Chang (1997) with slight modification. Superoxide radicals are generated in PMS–NADH systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). In this experiments, the superoxide radicals were generated in 3 ml of Tris–HCl buffer (16 mM, pH 8.0) containing 1 ml of NBT (50 μM) solution, 1 ml NADH (78 μM) solution and sample solution of PAS extracts (from 12.5 to 62.5 μg/ml) in water. The reaction started by adding 1 ml of phenazine methosulphate (PMS) solution (10 μM) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured against blank samples. L-Ascorbic acid was used as a control. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\%\ \text{Inhibition} = [(A_o - A_1)/A_o] \times 100$$

where A_o is the absorbance of the control, and A_1 is the absorbance of PAS extracts or standards (Ye, Wang, Liu, & Ng, 2000).

2.6. Free radical scavenging activity

The free radical scavenging activity of PAS extracts was measured by the 1,1-diphenyl-2-picryl-hydrazil (DPPH·) method proposed by Blois (1958). Briefly, 0.1 mM solution of DPPH· in ethanol was prepared and 1 ml of this solution was added 3 ml of PAS extracts solution in water at different concentrations (12.5–62.5 μg/ml). Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The DPPH· concentration in the reaction medium was calculated from the following calibration curve, determined by linear regression (R^2: 0.9545):

$$\text{Absorbance} = 0.0036 \times [\text{DPPH}]$$

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

$$\text{DPPH}\cdot \text{ Scavenging Effect}(\%) = [(A_o - A_1/A_o) \times 100]$$

where A_o is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample of PAS extracts.

2.7. Metal chelating activity

The chelating of ferrous ions by the PAS extracts and standards was estimated by the method of Dinis, Madeira, and Almeida (1994). Briefly, extracts (12.5–62.5 μg/ml) were added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. All test and analyses were run in triplicate and averaged. The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated using the formula given bellow:

$$\%\ \text{Inhibition} = [(A_o - A_1)/A_o] \times 100$$

where A_o is the absorbance of the control, and A_1 is the absorbance in the presence of the sample of PAS extracts or standards. The control does not contain $FeCl_2$ and ferrozine, complex formation molecules.

2.8. Scavenging of hydrogen peroxide

The ability of the PAS extracts to scavenge hydrogen peroxide was determined according to the method of Ruch, Cheng, and Klaunig (1989). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically measuring absorption with extinction coefficient for H_2O_2 of 81 $M^{-1}cm^{-1}$. Extracts (12.5–62.5 μg/ml) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). Absorbance of hydrogen peroxide at 230 nm was determined 10 min later against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging of both PAS extracts and standard compounds was calculated:

$$\%\ \text{Scavanged}\ [H_2O_2] = [(A_o - A_1)/A_o] \times 100$$

where A_o is the absorbance of the control, and A_1 is the absorbance in the presence of the sample of PAS extracts or standards.

2.9. Determination of total phenolic compounds

Total soluble phenolics in the PAS extracts were determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977) using gallic acid as a standard phenolic compound. Briefly, 1.0 ml of extract solution containing 1.0 g extracts in a volumetric flask was diluted with distilled water (46 ml). One millitre of Folin-Ciocalteau reagent was added and the content of the flask mixed thoroughly. Three minutes later, 3 ml of Na_2CO_3 (2%) was added, and the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm. The concentration of total phenolic compounds in the PAS extracts was determined as microgram of gallic acid equivalent using an equation obtained from the standard gallic acid graph:

$$\text{Absorbance} = 0.0008 \times \text{Gallic acid}(\mu g)$$

2.10. Preparation of test microorganisms

Pseudomonas aeruginosa (ATCC 9027, gram negative), *Escherichia coli* (ATCC 9837, gram negative), *Proteus mirabilis* (Clinical isolate, gram negative), *Citrobacter koseri* (Clinical isolate, gram negative), *Enterobacter aerogenes* (Clinical isolate, gram negative), *Staphylococcus aureus* (ATCC 6538, gram positive), *Streptococcus pneumoniae* (ATCC 49619, gram positive), *Micrococcus luteus* (Clinical isolate, gram positive), *Staphylococcus epidermidis* (Clinical isolate, gram positive), and *Candida albicans* (ATCC 10231) microorganism strains were employed for determination of antmicrobial activity.

Bacteria and fungi were obtained from the stock cultures of Microbiology Laboratory, Department of Microbiology, Medical Faculty, Atatürk Universty, Erzurum. The bacterial and fungal stock cultures were maintained on Muller Hinton Agar (Oxoid CM 337, Basingstoke, Hampshire, UK) slants, respectively,

which were stored at 4 °C. For the purpose of antimicrobial evaluation, ten microorganisms were used. These bacteria were maintained on Blood agar base (Oxoid CM55, Basingstoke, Hampshire, UK). The fungus was maintained on Sabouraud-dextrose agar (Oxoid CM41, Basingstoke, Hampshire, UK), which is often used with antibiotics for the isolation of the pathogenic fungi.

2.11. Antimicrobial cctivity determination

Agar cultures of the test microorganisms were prepared as described by Mackeen et al. (1997). Three to five similar colonies were selected and transferred with loop into 5 ml of Tryptone soya broth (Oxoid CM129, Basingstoke, Hampshire, UK). Tryptone soya broth is a highly nutritious versatile medium, which is 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 disc were impregnated with 250 μg of the water or ethanol extracts. The both PAS extract dissolved in sterile water for the assay by magnetic stirrer. Then the paper discs placed onto Mueller Hinton agar (Oxoid CM337, Basingstoke, Hampshire, UK). The inoculum for each organism was prepared from broth cultures. The concentration of cultures was to 10^8 colony forming units (1×10^8 cfu/ml). The results were recorded by measuring the zones of growth inhibition surrounding the disc. Clear inhibition zones around the discs indicate the presence of antimicrobial activity. All data on antimicrobial activity are the average of triplicate analyses. Netilmicin (30 μg/disc), amoxicillin-clavulanic acid (20–10 μg/disc), ofloxacin (5 μg/disc, BBL™ Sensi disc™), and antifungal micanozale nitrate (40 μg/disc, DRG International) were used as reference standards, which recommended by the National Committee for Clinical Laboratory Standards (NCCLS).

2.12. Statistical analysis

Experimental results were mentioned as mean ± S.D. of three parallel measurements. P values <0.05 were regarded as significant and P values <0.01 as very significant.

3. Results and discussion

3.1. Total antioxidant activity determination in linoleic acid emulsion

Total antioxidant activity of PAS extracts was determined by the thiocyanate method. Both PAS extracts exhibited effective antioxidant activity at all concentrations. The effects of various amounts of water and ethanol extracts of PAS (from 10 to 50 μg/ml) on peroxidation of linoleic acid emulsion are shown in Figs. 1 and 2. The antioxidant activity of both PAS extracts increased with increasing concentration. The different concentration of water and ethanol extracts (10, 20 and 50 μg/ml) of PAS showed higher antioxidant activities than that of 20 μg/ml concentration of α-tocopherol. The percentage of inhibition in linoleic acid

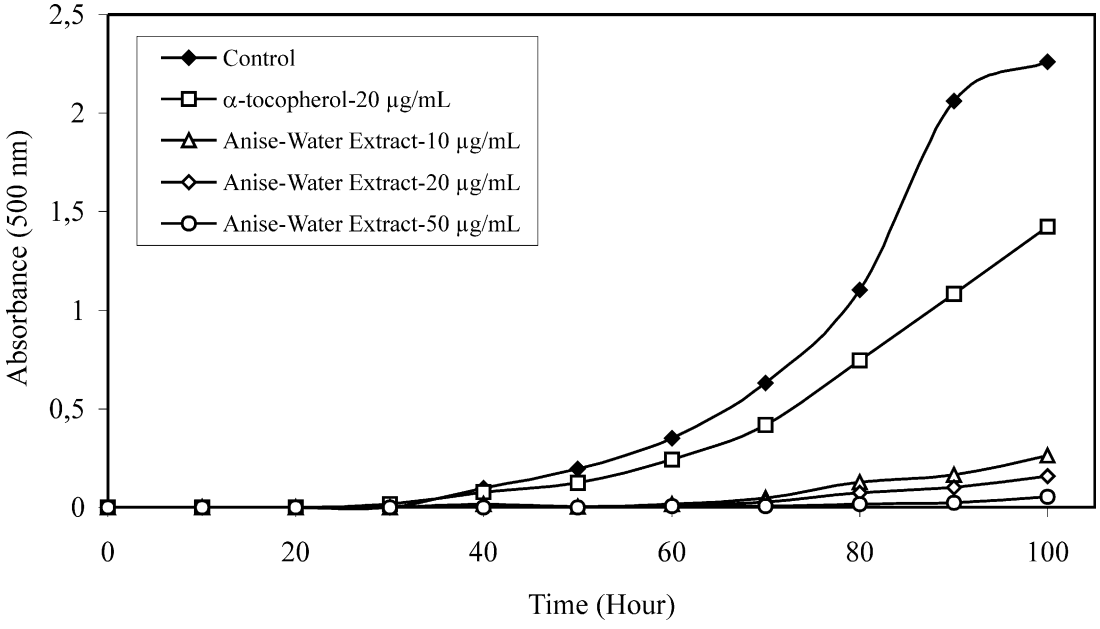

Fig. 1. Antioxidant activity of different concentrations of water extracts of PAS and α-tocopherol in the linoleic acid emulsion (PAS: *Pimpinella anisum* L. seed).

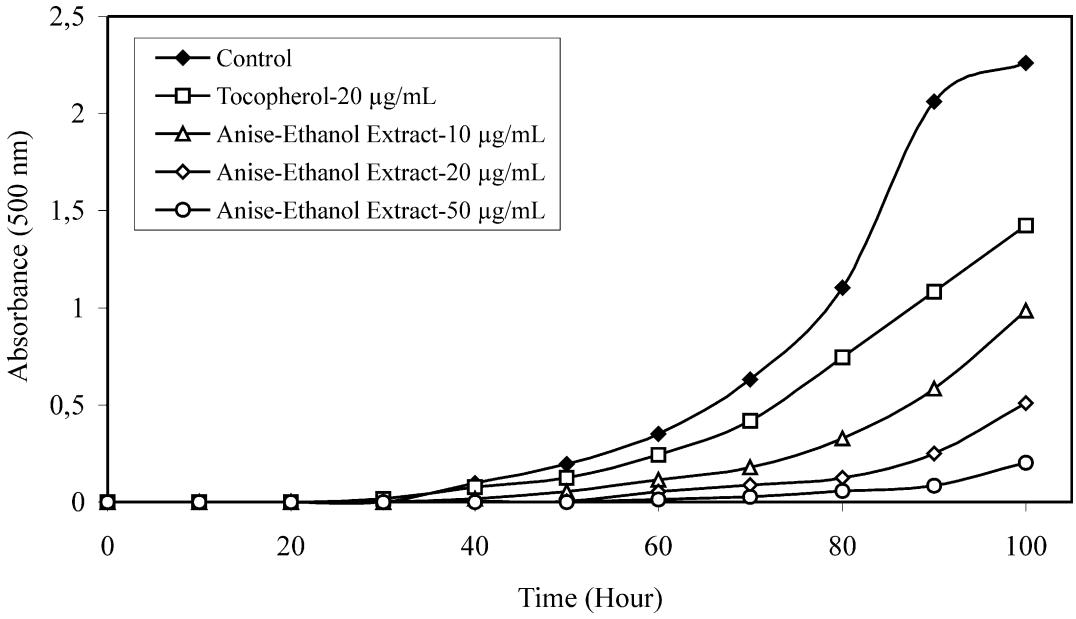

Fig. 2. Antioxidant activity of different concentrations of ethanol extracts of PAS and α-tocopherol in the linoleic acid emulsion (PAS: *Pimpinella anisum* L. seed).

system was 88.3, 94.1, 97.6, 56.5, 77.5, and 91.2%, respectively, and greater than that of 20 µg/ml of α-tocopherol (36.1%).

3.2. Reducing power

Fig. 3 shows the reductive capabilities of PAS extracts compared with BHA, BHT and α-tocopherol. For the measurements of the reductive ability, we investigated the Fe^{3+}–Fe^{2+} transformation in the presence of PAS extracts using the method of Oyaizu (1986). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir, Kanner, Akin, & Hadas, 1995). The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997; Yıldırım et al, 2001). Like

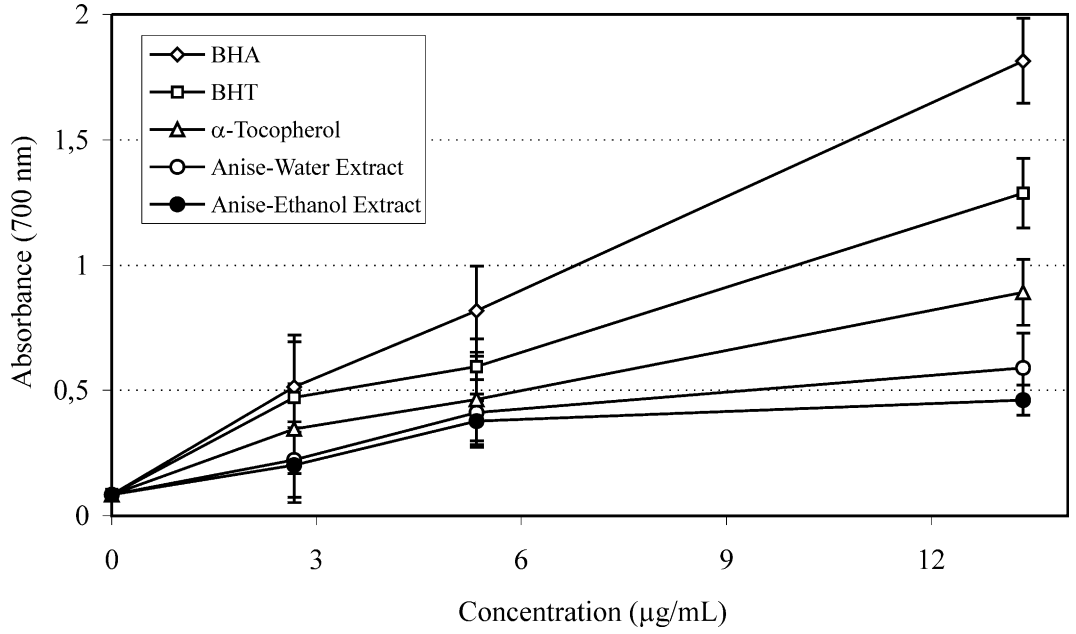

Fig. 3. Reducing power of water and ethanol extracts of PAS, BHA, BHT, and α-tocopherol. (Spectrophotometric detection of the Fe^{+3}–Fe^{+2} transformation, PAS: *Pimpinella anisum* L. seed, BHA: Buthylated hydroxyanisole, BHT: Buthylated hydroxytoluene).

the antioxidant activity, the reducing power of PAS extracts increased with increasing amount of sample. All of the amounts of both PAS extracts showed higher activities than control and these differences were statistically significant ($P<0.01$). Reducing power of water and ethanol extracts of PAS and standard compounds exhibited the following order: BHA > BHT > α-tocopherol > water extract of PAS > ethanol extract of PAS.

3.3. Superoxide anion scavenging activity

In the PMS–NADH–NBT system, superoxide anion derived from dissolved oxygen by PMS–NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Fig. 4 shows the % inhibition of superoxide radical generation by 12.5, 25, and 62.5 μg/ml of water and ethanol extracts of PAS and comparison with same concentrations of BHA, BHT, and α-tocopherol. Both extracts of PAS had strong superoxide radical scavenging activity and exhibited higher superoxide radical scavenging activity than and BHT and α-tocopherol. The results were found statistically significant ($P<0.05$). As seen in Fig. 4, the percentage inhibition of superoxide generation by 62.5 μg/ml concentration of BHA, water and ethanol extracts of PAS was found as 98.7, 97.8 and 95.6% and greater than that of same doses of BHT and α-tocopherol (88.3 and 80.5%), respectively. Superoxide radical scavenging activity of those samples showed the following order: BHA > water extract of PAS > ethanol extract of PAS > BHT > α-tocopherol.

3.4. Free radical scavenging activity

DPPH· is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares, Dins, Cunha, & Ameida, 1997). The reduction capability of DPPH· radicals was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. Hence, DPPH· is often used as a substrate to evaluate antioxidative activity of antioxidants (Duh et al., 1999). Fig. 5 illustrates a significant ($P<0.05$) decrease in the concentration of DPPH radical due to the scavenging ability of the extracts of PAS and standards. We used BHA, BHT and α-tocopherol as standards. The scavenging effect of water and ethanol extracts of PAS and standards on the DPPH radical decreased in that order: BHA > α-tocopherol > BHT > water extract > ethanol extract, which were 88.02, 86.38, 64.07, 53.22, and 34.49%, respectively, at the concentration of 12.5 μg/ml. These results indicates that both PAS extracts have a noticeable effect on scavenging free radical. Free radical scavenging activity also increased with increasing concentration.

3.5. Metal chelating activity

The chelating of ferrous ions by the extracts of PAS was estimated by the method of Dinis, Madeira, and Almeida (1994). 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 colour of the complex. Measurement

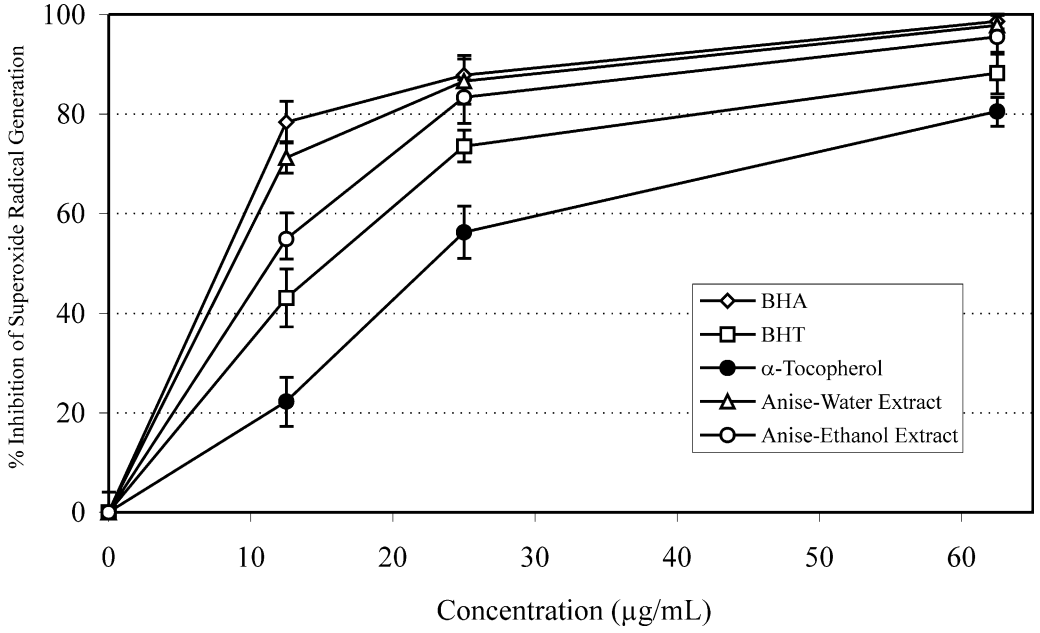

Fig. 4. Superoxide anion radical scavenging activity of water and ethanol extracts of PAS, BHA, BHT, and α-tocopherol by the PMS–NADH–NBT method. (PAS: *Pimpinella anisum* L. seed, BHA: buthylated hydroxyanisole, BHT: buthylated hydroxytoluene).

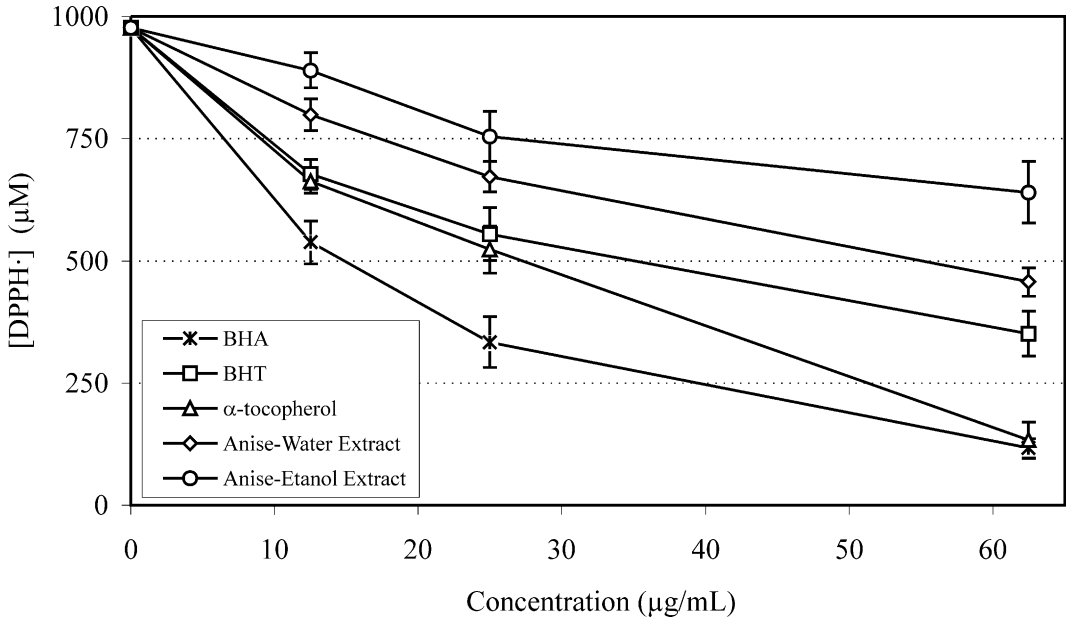

Fig. 5. Free radical scavenging activity of water and ethanol extracts of PAS, BHA, BHT, and α-tocopherol. (PAS: *Pimpinella anisum* L. seed, BHA: buthylated hydroxyanisole, BHT: Buthylated hydroxytoluene).

of colour reduction therefore allows estimating the metal chelating activity of the coexisting chelator (Yamaguchi, Ariga, Yoshimara, & Nakazawa, 2000). In this assay both extracts of PAS and standard compounds are interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and are able to capture ferrous ion before ferrozine.

As shown in Fig. 6, the formation of the Fe^{2+}–ferrozine complex is not complete in the presence of water and ethanol extracts of PAS, indicating that both extracts of PAS chelate with the iron. The absorbance of Fe^{2+}–ferrozine complex was linearly decreased dose dependently (from 12.5, 25, and 62.5 μg/ml). The difference between both extracts of PAS and the control was statistically significant ($P<0.05$). The percentages of metal scavenging capacity of 62.5 μg/ml concentration of water and ethanol extracts of PAS, α-tocopherol, BHA, and BHT were found as 33.1, 15, 43, 74.8, and 40.6%, respectively. The metal scavenging effect of both extracts of PAS and standards decreased in the order of BHA > α-tocopherol > BHT > water extract > ethanol extract of PAS.

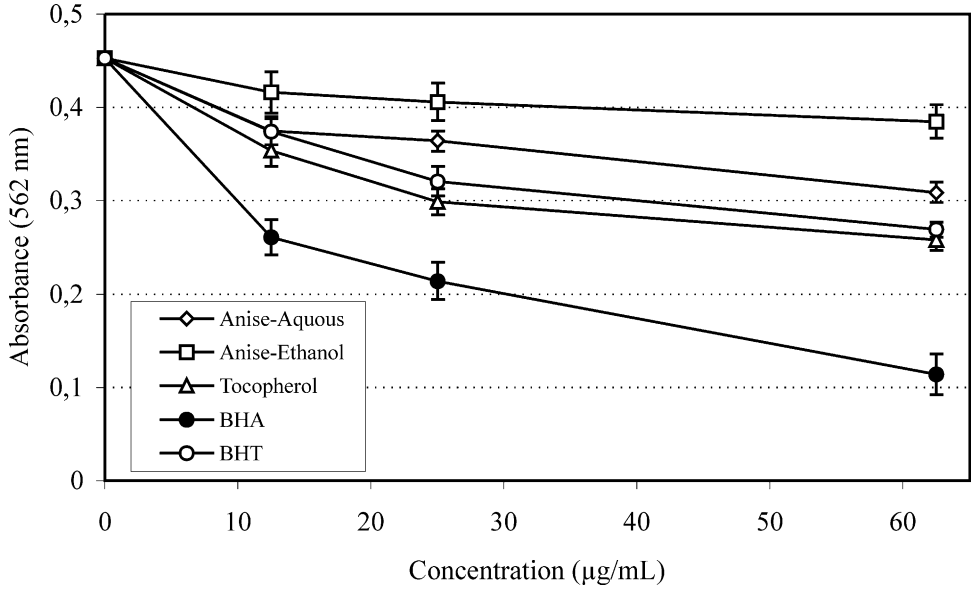

Fig. 6. Metal chelating effect of different amount of water and ethanol extracts of PAS, BHA, BHT, and α-tocopherol by 1,1-Diphenyl-2-picryl-hydrazyl radicals. (PAS: *Pimpinella anisum* L. seed, BHA: buthylated hydroxyanisole, BHT: buthylated hydroxytoluene).

Metal chelating capacity was significant, since it reduced the concentration of the catalysing transition metal in lipid peroxidation (Duh et al., 1999). It was reported that chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion (Gordon, 1990). The data obtained from Fig. 6 reveal that both extracts of PAS demonstrate a marked capacity for iron binding, suggesting that their action as peroxidation protector may be related to its iron binding capacity.

3.6. Scavenging of hydrogen peroxide

The ability of the both extracts of PAS to scavenge hydrogen peroxide was determined according to the method of Ruch et al. (1989). The scavenging ability of water and ethanol extracts of PAS on hydrogen peroxide is shown in Fig. 7 and compared with that of BHA, BHT and α-tocopherol as standards. PAS extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. Of water and ethanol extracts 62.5 μg/ml of PAS exhibited 44.3, and 31.5% scavenging activity on hydrogen peroxide, respectively. In the other hand, BHA, BHT, and α-tocopherol exhibited 37.5, 86, and 57% hydrogen peroxide scavenging activity at the same dose. These results showed that both PAS extracts had stronger hydrogen peroxide scavenging activity. Those values are close to that of BHA, but lower than that of BHT and α-tocopherol. There was statically significant correlation between those values and control ($P < 0.05$). The hydrogen peroxide scavenging effect of 62.5 μg/ml concentration of the both extracts of PAS and standards decreased in the order of BHT > α-tocopherol > water extract of PAS > BHA > ethanol extract of PAS. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells (Halliwell, 1991). Thus, removing H_2O_2 as well as $O_2^{\bullet -}$ is very important for protection of food systems.

3.7. Determination of total phenolic compounds

Phenols are very important plant constituents because of their radical scavenging ability due to their hydroxyl groups (Hatano, Edmatsu, Mori, Fujita, & Yasuhara, 1980). In water and ethanol extracts of PAS (1 mg), 30.0 and 77.5 μg gallic acid equivalent of phenols was detected. There was no relationship between total phenols and total antioxidant activity in PAS extracts. According to Velioglu, Mazza, Gao, and Oomah (1998), who examined 28 plant products, in many cases the high antioxidant activity was not correlated with the phenol content; probably other factors played major roles as antioxidants. The phenolic compounds may contribute directly to the antioxidative action (Duh et al., 1999). It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when up to 1.0 g daily ingested from a diet rich in fruits and vegetables (Tanaka, Kuei, Nagashima, & Taguchi, 1998).

3.8. Antimicrobial activity

Disc diffusion methods are extensively used to investigate the antibacterial activity of natural substances and plant extracts. These assays are based on the use of

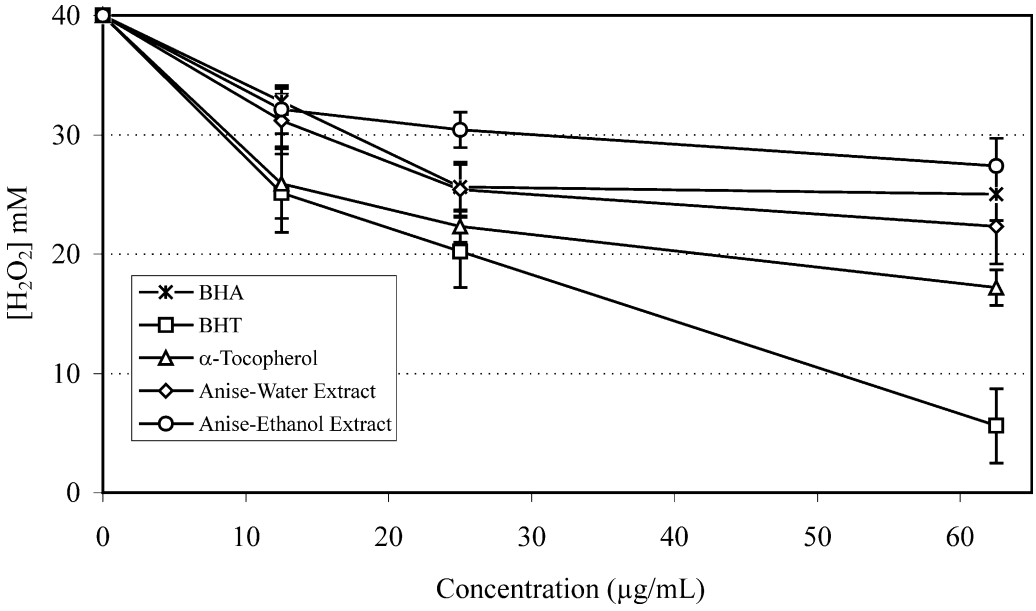

Fig. 7. Hydrogen peroxide scavenging activities of water and ethanol extracts of PAS, BHA, BHT, and α-tocopherol. (PAS: *Pimpinella anisum* L. seed, BHA: buthylated hydroxyanisole, BHT: buthylated hydroxytoluene).

Table 1
Antimicrobial activities of water and ethanol extracts of PAS, and miconazole nitrate, amoxicillin-clavulanic acid, ofloxacin, and netilmicin. (PAS: *Pimpinella anisum* L. seed)

Microorganisms	Diameter of extracts of PAS zone (mm)		Antimicrobial agent (mm)[a]			
	Water	Ethanol	MN	ACA	O	N
Pseudomonas aeruginosa	ND	7	–	ND	ND	10
Escherichia coli	ND	7	–	15	23	25
Proteus mirabilis	9	9	–	24	26	25
Citrobacter koseri	7	8	–	22	15	24
Staphylococcus aureus	9	9	–	15	12	27
Streptococcus pneumoniae	11	8	–	15	24	18
Enterobacter aerogenes	8	8	–	12	23	23
Micrococcus luteus	9	10	–	19	20	22
Staphylococcus epidermidis	8	9	–	24	21	25
Candida albicans	8	ND	20	–	–	–

[a] MN: Miconazole nitrate (40 μg/disc), ACA: Amoxicillin-clavulanic acid (20–10 μg/disc), O: Ofloxacin (5 μg/disc), N: Netilmicin (30 μg/disc), ND: Not detected activity at this concentration.

discs 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 discs means that holes or cylinders are preferably used (Bartner, Pfeiffer, & Bartner, 1994).

In this study, nine different microbial and one fungi species were used to screen the possible antimicrobial activities of both PAS extracts. Of the species used, *Staphylococcus aureus* is one of the most common of the gram-positive bacteria causing food poisoning. Its source is not the food itself but the humans who contaminate foods after they have been processed (Rauha et al., 2000). All of the extracts showed strong antibacterial activity against this bacterium (*Staphylococcus aureus*). Most of the bacterial species and the fungi species were inhibited antimicrobial activity as it is shown in Table 1. However, the antimicrobial activity of water extract of PAS was not detected against *Pseudomonas aeruginosa* and *Escherichia coli*. *E. coli*, which is a gram-negative bacterium, belonging to the normal flora of humans. However, an enterohemmoragic strain of *E. coli* has caused serious food poisoning, and preservatives to eliminate its growth are needed. *Candida albicans* is the microbe responsible for most clinical yeast infections, e.g. in mouth infections. Miconazole nitrate (40 μg/disc), amoxicillin-clavulanic acid (20–10 μg/disc), ofloxacin (5 μg/disc), and netilmicin (30 μg/disc) were used as positive controls for bacteria and fungi.

4. Conclusion

Both extracts of PAS showed strong antioxidant activity, reducing power, DPPH radical and superoxide anion scavenging, hydrogen peroxide scavenging, and metal chelating activities when compared with different standards such as BHA, BHT, and α-tocopherol. In addition, 250 μg of both PAS extracts possessed noticeable antimicrobial activity against gram positive and gram negative bacteria when compared with standard and strong antimicrobial compounds such as miconazole nitrate, amoxicillin-clavulanic acid, ofloxacin, and netilmicin.

The results of this study show that the extract of PAS can be used as easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry. However, the components responsible for the antioxidant and antimicrobial activities of both extracts of PAS are currently unclear. Therefore, it is suggested that further works should be performed on the isolation and identification of the antioxidant components in PAS.

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