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Screening of the antioxidative and antimicrobial properties of the essential oils of *Pimpinella anisetum* and *Pimpinella flabellifolia* from Turkey

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Abstract

The aerial parts of two endemic *Pimpinella* [*Pimpinella anisetum* Boiss. & Ball. and *Pimpinella flabellifolia* (Boiss.) Benth. ex Drude] were hydro-distilled to produce oils in the yields of 2.07% (v/w) and 2.61% (v/w), respectively. The oils were analysed by GC and GC/MS. Twenty-one and nineteen components were identified, representing 99.5% and 99.7% of the oils, respectively. The main compounds of *P. anisetum* were (E)-anethole (82.8%) and methyl chavicol (14.5%), whereas limonene (47.0%), (E)-anethole (37.9%) and α-pinene (6.0%) were the major constituents of *P. flabellifolia*. The oils were screened for their possible antioxidant activities by two complementary test systems, namely DPPH free radical-scavenging and β-carotene/linoleic acid systems. In the first case, *P. anisetum* oil exerted greater antioxidant activity than that of *P. flabellifolia* oil with an IC_{50} value of 5.62 ± 1.34 μg/ml. In the β-carotene/linoleic acid test system, the oil of *P. anisetum* was superior to *P. flabellifolia* with 70.5% $\pm$ 2.86 inhibition rate. Essential oils of the plants studied here were also screened for their antimicrobial activities against six bacteria and two fungi. The oils showed moderate antimicrobial activity against all microorganisms tested.
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Keywords: *Pimpinella anisetum*; *Pimpinella flabellifolia*; Essential oil; Antimicrobial activity; Antioxidant activity; GC–MS

1. Introduction

The genus *Pimpinella* is of around 150 species of mostly annuals and perennials with entire or pinnate leaves and dainty, star-shaped flowers borne in umbrella-like heads. These are followed by small, oval shaped fruits. Species occur naturally in Eurasia, Africa, and South America (Könemann, 1999).

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, umbelliferone, estrols (Burkhardt, Reichling, Martin, & Becker, 1986), terpene hydrocarbons (Kartnig, Moeckel, & Mauns, 1975), polyenes and polyacetylenes (Schulte, Rucker, & Backe, 1970) which are the major compounds of the plants in the genus *Pimpinella*.

Some of the members of this genus were cultivated by Egyptians, Greeks and Romans for their aromatic seeds

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used in medicine and as a condiment. They have also been used as popular aromatic herbs and spices since antiquity, and have been cultivated throughout Europe. Their fruits have been used for medicine and in cooking, and are listed in British, German and European pharmacopoeia. For medicinal purposes, they are used to treat dyspeptic complaints, and also as a mild expectorant (Fujimatu, Ishikawa, & Kitajima, 2003). The essential oils from the fruits of some *Pimpinella* species are also valuable in perfumery and in medicine (Ernst, 1989; Santos et al., 1998; Simon, Chadwick, & Cracker, 1980).

Lipid peroxidation is a complex process occurring in aerobic cells and reflects the interaction between molecular oxygen and polyunsaturated fatty acids. Radicals are known to take part in lipid peroxidation, which causes food deterioration, aging organisms and cancer promotion (Ashok & Ali, 1999; Cerruti, 1994). Reactive oxygen species are also reported to be involved in asthma, inflammation, arthritis, neurodegeneration, Parkinson disease, mongolism and perhaps dementia (Adams & Odunze, 1991; Perry et al., 2000). Antioxidants act as radical-scavengers, and inhibit lipid peroxidation and other free radical-mediated processes: therefore, they are able to protect the human body from several diseases attributed to the reactions of radicals (Nizamuddin, 1987; Takao, Kiatani, Watanabe, Yagi, & Sakata, 1994). Use of synthetic antioxidants to prevent free radical damage has been reported to involve toxic side effects (Cornwell et al., 1998; Faure, Lissi, Torres, & Videla, 1990; Feher & Pronai, 1993), making attractive the search for antioxidant and scavenger natural compounds.

To prolong the shelf-life of fruits and vegetables, the growth of microbial populations must be controlled and several post-harvest processes, such as washing and removal of damaged tissues, are employed to reduce initial high counts. It is well known that clean sanitation in essential in keeping the microbial population to a minimum, because storage life is shorter with high initial microbial loads (Bolin, Stafford, King, & Huxsoll, 1977). On the other hand, most of the evidence indicates that the native microorganisms should not be destroyed completely, because they control the growth of any contaminating pathogens (Watada, 1997).

The exploration of naturally occurring antimicrobials for food preservation receives increasing attention due to consumer awareness of natural food products and a growing concern of microbial resistance towards conventional preservatives (Gould, 1995). Many spices and herbs exert antimicrobial activity due to their essential oil fractions. Nychas (1995) reported antimicrobial activity of essential oils from oregano, thyme, sage, rosemary, clove, coriander, garlic and onion against both bacteria and fungi. Phenolic components, present in essential oils, have been known to possess antimicrobial activity and some are classified as Generally Recognized as Safe (GRAS) substances and therefore could be used to prevent post-harvest growth of native and contaminant bacteria (Kabara, 1991; Singh, Singh, Bhunia, & Simmon, 2001).

Members of the genus *Pimpinella* are very common in Turkey. There are more than 20 species in Anatolia. In Turkish folk medicine, these plants, and especially their seeds, have been used as appetizers, tranquillizers and diuretic drugs. Especially, some of these plants were extensively used in liquor production in Turkey (Asimgil, 1997; Baytop, 1997).

The aim of the present work is to study in vitro antioxidant and antimicrobial activities of the essential oils of *Pimpinella anisetum* and *Pimpinella flabellifolia* in addition to their chemical compositions. Numerous techniques are available to evaluate the antioxidant activities of compounds and complex mixtures such as plant extracts. Despite the various methods, just one procedure cannot identify all possible mechanisms characterising an antioxidant. Therefore, oils obtained by hydrodistillation were screened for their possible antioxidant activities by two complementary test systems, namely DPPH free radical-scavenging and β-carotene/linoleic acid systems.

2. Materials and methods

2.1. Collection of plant material

P. anisetum was collected from Sakaltutan Pass, Erzincan-Turkey on 04 July, 2003, while *P. flabellifolia* was collected from Gurun-Kangal turnoff, Sivas-Turkey on 17 June, 2003. Both plants were collected during their flowering season. The voucher specimens have been deposited at the Herbarium of the Department of Biology, Cumhuriyet University, Sivas, Turkey (CUFH Voucher No. AA 3334 and 3327, respectively).

2.2. Isolation of the essential oils

The air-dried and finely ground aerial parts of *P. anisetum* and *P. flabellifolia* were submitted to hydrodistillation for 3 h using a Clevenger-type apparatus (yields 2.07% and 2.61% v/w, respectively). The oils were dried over anhydrous sodium sulphate and, after filtration, stored at +4 °C until tested and analysed.

2.3. Gas chromatography analysis

2.3.1. GC alone

The essential oil was analyzed using a Hewlett–Packard 5890 II gas chromatography (GC) equipped with a FID detector and HP-5MS capillary column (30 m × 0.25 μm, film thickness 0.25 μm). Injector and

detector temperatures were set at 220 and 290 °C, respectively. Column temperature was gradually increased from 50 to 240 °C with a rate of 3 °C/min. Helium was the carrier gas at a flow rate of 1 ml/min. Diluted samples of oils (1/100 in acetone, v/v) of 1.0 µl were injected manually in the splitless mode. Quantitative data were obtained electronically from FID area percent data without the use of correction factors.

2.3.2. Gas chromatography/mass spectrometry analysis

Gas chromatography/mass spectrometry (GC/MS) analysis of diluted solutions of the essential oils was performed under the same conditions as GC alone (column, oven temperature, flow rate of the carrier gas), using a Hewlett–Packard 5890 II GC equipped with a Hewlett–Packard 5972 mass selective detector in the electron impact mode (70 eV). Injector and MS transfer line temperatures were set at 220 and 290 °C, respectively. *n*-Alkanes were used as reference points in the calculation of the Kovats Indices (K.I.). Tentative identification of the compounds, based on the comparison of their relative retention time and mass spectra with those of NBS75K library data of the GC/MS system and literature data (Adams, 2001).

2.4. Antioxidant activity

2.4.1. DPPH assay

Hydrogen atom- or electron-donation ability of the corresponding oils was measured from the bleaching of the purple-coloured methanol solution of DPPH. This spectrophotometric assay uses stable 2,2′-diphenylpicrylhydrazyl (DPPH) radical as a reagent (Burits & Bucar, 2000; Cuendet, Hostettmann, & Potterat, 1997). Fifty microliters of various concentrations of the oils in methanol were added to 5 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature the absorbance was read against a blank at 517 nm. Inhibition of DPPH free radical in percent (I%) was calculated in following way:

$$I\% = (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}})x100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated form the graph plotting inhibition percentage against extract concentration. Tests were carried out in triplicate.

2.4.2. β-Carotene–linoleic acid assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius, Venskutonis, Van Beek, & Linssen, 1998). A stock solution of β-carotene–linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 ml of chloroform (HPLC grade) and 25 µl linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then 100 ml of distilled water saturated with oxygen (30 min 100 ml/min) were added with vigorous shaking. 2.5 ml of this reaction mixture were dispersed to test tubes and 350 µl portions of the oils prepared at 2 g/l concentrations were added and the emulsion system was incubated for up to 48 h at room temperature. The same procedure was repeated with the synthetic antioxidant, butylated hydroxytoluene (BHT), curcumin and ascorbic acid as positive controls, and a blank. After this incubation period, absorbances of the mixtures were measured at 490 nm. Antioxidative capacities of the oils were compared with those BHT and blank.

2.5. Antimicrobial activity

2.5.1. Microbial strains

The essential oils were individually tested against a panel of microorganisms, including *Streptococcus pneumoniae* IK3, *Bacillus cereus* RK75, *Acinetobacter lwoffii* ATCC 19002, *Escherichia coli* Hak59,*Klebsiella pneumoniae* A137, *Clostridium perfringens* Kukens–Turkey, *Candida albicans* A117 and *Candida krusei* ATCC 6258. Bacterial strains were cultured overnight at 37 °C in Mueller Hinton agar (MHA), with the exception of *S. pneumoniae* (MHA containing 50 ml citrate blood/l) and *C. perfringens* (under anaerobic conditions). Yeasts were cultured overnight at 30 °C in Sabouraud dextrose agar.

2.5.2. Antimicrobial screening

The disc diffusion method was employed for the determination of antimicrobial activities of the essential oils (NCCLS, 1999). The MICs of the essential oils against the test microorganisms were determined by the broth microdilution method (NCCLS, 1997). All tests were performed in duplicate.

2.5.3. Disc diffusion method

The agar disc diffusion method was employed for the determination of antimicrobial activities of the essential oils in question (NCCLS, 1997). Briefly, a suspension of the tested microorganism (0.1 ml of 10^8 cells per ml) was spread on the solid media plates. Filter paper discs (6 mm in diameter) were impregnated with 15 µl of the oil and placed on the inoculated plates. These plates, after standing at 4 °C for 2 h, were incubated at 37 °C for 24 h for bacteria and at 30 °C for 48 h for the yeasts. The diameters of the inhibition zones were measured in millimeters. All the tests were performed in duplicate.

2.5.4. Determination of minimum inhibitory concentration

A broth microdilution broth susceptibility assay was used, as recommended by NCCLS, for the determination of the minimum inhibitory concentration (MIC) (NCCLS, 1999). All tests were performed in Mueller Hinton Broth (MHB; BBL) supplemented with Tween 80 detergent (final concentration of 0.5% (v/v)), with the exception of the yeasts (Sabouraud dextrose broth-SDB + Tween 80). Bacterial strains were cultured overnight at 37 °C in MHA and the yeasts were cultured overnight at 30 °C in SDB. Test strains were suspended in MHB to give a final density of 5×10^5 cfu/ml and these were confirmed by viable counts. Geometric dilutions ranging from 0.036 mg/ml to 72.0 mg/ml of the essential oils were prepared in a 96-well microtitre plate, including one growth control (MHB + Tween 80) and one sterility control (MHB + Tween 80 + test oil). Plates were incubated under normal atmospheric conditions at 37 °C for 24 h for bacteria and at 30 °C for 48 h for the yeasts. The bacterial growth was indicated by the presence of a white "pellet" on the well bottom.

3. Results and discussion

3.1. Chemical composition of the essential oils

The results obtained by GC and GC–MS analysis of the essential oils of *P. anisetum* and *P. flabellifolia* are presented in Table 1. In the first case, twenty-one compounds were identified, representing 99.5% of the total oil. On the other hand, nineteen compounds were determined for *P. flabellifolia*, representing 99.7% of the total oil. Oil yields of the plants were determined as 2.07% (v/w) and 2.61% (v/w), respectively. As a result of GC and GC–MS analyses, (E)-anethole was the major compound for the both oil samples studied (82.8% and 37.9%, respectively). Additionally, other major compounds were methyl chavicol (14.5%) for *P. anisetum* oil, limonene (47.0%) and α-pinene (6.0%) for *P. flabellifolia* oil.

As far as our literature survey could ascertain, there are few reports on chemical compositions of the essential oils obtained from the genus *Pimpinella*. There are some studies related to the various biological activities and chemical compositions of the oils isolated from *P. anisum* (Fujimatu et al., 2003; Kreydiyyeh, Usta, Knio, Markossian, & Dagher, 2003; Santos et al., 1998).

GC and GC–MS analyses revealed that benzene derivatives are represented in high amount (97.6%, approximately) in *P. anisetum* oil, followed by hydrocarbon monoterpenes (15.0%, approximately). On the other hand, monoterpene hydrocarbons (61.2%, approximately) and benzene derivatives (38.4%) were the main compound classes for *P. flabellifolia* oil. Based on the previous reports carried out on aniseed oils, *trans*-anethole (Kreydiyyeh et al., 2003), geijerene (Kubeczka & Ullmann, 1980), zingiberene (Velasco-Negueruela, Perez-Alonso, Perez de Paz, Pala-Paul, & Sanz, 2003) and compounds included in arylpropanoids class (*for*

Table 1
Chemical composition of *P. anisetum* and *P. flabellifollia* essential oils (diluted 1/100 in acetone v/v)[a]

K.I.[b]	Components	Composition (%)	
		P. anisetum	*P. flabellifolia*
898	α-Pinene	0.2	6.0
913	Camphene	–[c]	0.1
937	Sabinene	tr[d]	0.3
940	β-Pinene	tr	3.0
955	β-Myrcene	0.1	1.2
969	α-Phellandrene	–	0.1
992	*p*-Cymene	tr	–
995	Limonene	1.1	47.0
1030	γ-Terpinene	tr	3.5
1080	Linalool	–	tr
1105	Mentha-2,8-dien-1-ol, *trans*-para	–	tr
1122	Mentha-2,8-dien-1-ol, *cis*-para	–	tr
1126	Geijerene	0.1	–
1172	Terpinen-4-ol	–	tr
1190	α-Terpineol	–	tr
1196	Methyl chavicol	14.5	–
1262	(Z)-anethole	0.2	0.1
1277	Anisaldehyde (para)	0.1	0.1
1301	(E)-anethole	82.8	37.9
1406	α-Ylangene	tr	–
1451	β-Caryophyllene	tr	–
1465	γ -Elemene	–	tr
1485	Aromadendrene	tr	–
1515	γ-Himachalene	0.2	–
1519	Germacrene D	0.1	–
1532	α-Zingiberene	0.1	–
1533	Bicyclogermacrene	–	0.1
1547	β-Bisabolene	tr	–
1550	Methyl isoeugenol[e]	–	0.3
1562	δ-Cadinene	tr	–
1622	Spathulenol	tr	–
	Total	99.5	99.7

[a] Relative percentages of the compounds were obtained electronically from FID area percent data.
[b] Kovats index on non-polar HP-5 ms column in reference to *n*-alkanes.
[c] Not detected.
[d] Trace $\leqslant 0.06\%$.
[e] Correct isomer not determined.

Table 2
Antioxidative capacities of the essential oils of *P. anisetum* and *P. flabellifolia*[a]

Plant oils and controls	Test system	
	DPPH (μg/ml)	β-Carotene/linoleic acid (% inhibition rate)
P. anisetum	5.62 ± 1.34	70.5 ± 2.86
P. flabellifolia	8.49 ± 1.76	59.5 ± 2.64
BHT	18.0 ± 0.40	96.6 ± 1.29
Curcumine	7.8 ± 0.32	89.3 ± 2.14
Ascorbic acid	3.80 ± 0.17	94.5 ± 1.86

[a] Results are means of three different experiments.

Table 3
Antimicrobial capacities of the essential oils of *P. anisetum* and *P. flabellifolia*[a]

Microorganisms	*P. anisetum*		*P. flabellifolia*	
	Disc diffusion[b]	MIC[c]	Disc diffusion	MIC
Escerichia coli	10.00 ± 1.00	72.0	8.00 ± 1.05	–
Streptococcus pneumoniae	11.00 ± 1.75	18.0	12.00 ± 1.43	9.00
Bacillus cereus	9.00 ± 1.32	72.0	9.00 ± 1.45	72.0
Clostridium perfringens	11.00 ± 1.25	4.5	11.00 ± 1.89	2.25
Candida albicans	14.00 ± 1.43	36.0	11.00 ± 1.72	36.0
Candida krusei	16.00 ± 1.79	36.0	13.00 ± 1.44	36.0
Acinetobacter lwoffii	18.00 ± 1.42	18.0	15.00 ± 1.46	36.0
Klebsiella pneumoniae	9.00 ± 1.33	72.0	–	72.0

[a] Results are means of three different experiments.
[b] Diameter of inhibition zone including disc diameter of 6 (mm).
[c] MIC, minimum inhibitory concentration (as mg/ml).

instance, *trans*-epoxypseudoisoeugenyl 2-methylbutyrate) (Santos et al., 1998), are the characteristic compounds for *Pimpinella* oils.

3.2. Antioxidant activity

Essential oils were individually assessed for their possible antioxidative activities by employing two complementary tests; DPPH free radical-scavenging and β-carotene/linoleic acid assays. Free radical-scavenging capacities of the corresponding oils were measured by DPPH assay and the results are shown in Table 2. *P. anisetum* oil exerted greater antioxidant activity than that of *P. flabellifolia* oil with an IC_{50} value of 5.62 ± 1.34 μg/ml. In the case of the linoleic acid system, the oil of *P. anisetum* was superior to that of *P. flabellifolia*, with 70.5% ± 2.86 inhibition rate.

Based on a report by Avlessi et al. (2004), methyl chavicol and anethole are the main constituents responsible for the antioxidative activities of the oils which contain them. In our study, total percentage of these compounds present in *P. anisetum* oil is 97.3%, whereas it is 37.9% for *P. flabellifolia* oil. It is extremely important to point out that, antioxidant capacity of *P. anisetum* oil could be attributed to the presence and high percentages of these compounds.

3.3. Antimicrobial activity

As can be seen in Table 3, essential oils obtained from *P. anisetum* and *P. flabellifolia* were found to have moderate antimicrobial activity against all microorganisms tested. In general, *P. anisetum* oil exhibited stronger activity than did *P. flabellifolia* oil.

In the case of *P. anisetum*, results from the disc diffusion method, followed by measurements of minimal inhibition concentration (MIC), indicate that, *C. perfringens* is the most sensitive microorganism with the lowest MIC value (4.50 mg/ml). Other sensitive microorganisms are *S. peumoniae* and *A. lwoffii* with the same MIC values (18 mg/ml). According to the results obtained from *P. flabellifolia* oil, *C. perfringens* is again the most sensitive microorganism with the lowest MIC value (2.25 mg/ml), followed by *S. peumoniae* (9.0 mg/ml). In addition to these findings, *C. albicans* and *C. krusei* (yeasts) exhibited sensitivity to both oils with an MIC value of 36 mg/ml.

According to Park, Baek, Bai, Oh, and Lee (2004), anethole (and probably its isomers) is mainly responsible for the antimicrobial activity of the plant oils which contain them. As can be seen from Table 1, (E)-anethole is the main constituent in both plant oils studied. For that reason, antimicrobial activities presented here could be attributed to the presence of anethole isomers (especially the high percentage of (E)-anethole in *P. anisetum* oil).

The results presented here can be considered as the first information on the antioxidative and antimicrobial properties of *P. anisetum* and *P. flabellifolia*, two endemic species of Turkish flora. This may also contribute to knowledge of the antioxidative and antimicrobial potentials of *Pimpinella* species reported elsewhere.

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