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Full Length Research Paper

Chemical composition and antioxidant, antimicrobial, antispasmodic activities of the essential oil of *Thymus Fallax* Fisch. Mey

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The aim of this study was to investigate radical scavenging, antimicrobial and antiospasmodic activities *in vitro* and the composition of *Thymus fallax* Fisch. Mey. (labiatte) essential oil. The oil of *T. fallax* was analyzed by the GS-MS method. The sample was subjected to screening for possible antioxidant activity by using 2, 2-diphenyl-1- picrylhydrazyl (DPPH) and β-carotene-linoleic acid assays. Antimicrobial activities of the essential oil were determined on 9 micro-organisms using the agar-disc diffusion method. In rat ileum, direct effect of *T. fallax* essential oil was investigated on spontaneous contractions. GS/MS analysis of the oil resulted in the identification of 18 compounds, representing 92.41% of the oil; carvacrol (46.15%) was the main component. The essential oil exhibited strong antioxidant activity. It has antimicrobial activity on 8 of 9 microorganisms using the agar-disc diffusion method. *T. fallax* oil exhibited spontaneous contractions in rat ileum at 100% level at 0.1 mg/mL level. The essential oil of *T. fallax* has a strong antimicrobial, antioxidant, and antispazmodic activities as studied in our laboratory settings. *T. fallax* may therefore be regarded as a resourceful plant that can be used in the pharmaceutical and food industries.

Key words: *Thymus fallax*, antioxidant, antimicrobial, anti-spazmodic activity.

INTRODUCTION

Fresh and dried aromatic plants as well as their processed products have been widely used as flavourings since ancient times, however, during last few decades they also have become a subject for a search of natural antioxidants and antibacterial agents (Reynolds et al., 1996; Jones et al., 1999; Sokmen et al., 2004a; Sokmen et al., 2004b; Vardar-Unlu et al., 2003; Fecka et al., 2007; Li et al., 2008). Thyme (*T. fallax* Fisch Mey), belonging to the Lamiaceae family, is a pleasant smelling perennial shrub, which grows in several regions of the world such as Western Mediterranean, Southern Italy, Iran, and Turkey (Davis, 1982; Baytop, 1997). Such plants are represented in Turkish flora by 39 species. Thyme is used for seasoning, poultry, soups, and vegetables in herbal teas prepared for colds and flues as well. Thyme and its oil have been used as fumigants, antiseptics, antioxidants, and mouth washes (Davis, 1982; Davis, 2001; Baytop, 1997; Gulluce et al., 2007). There are several studies related to Thymus species but a limited number of studies are available on *T. fallax* (Tumen et al., 1999, Barazandeh et al., 2004; Ozturk et al., 2005). The chemical composition of *T. fallax* has previously been reported (Tumen et al., 1999; Barazandeh, 2004).

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Although antimicrobial and antioxidant activities of essential oil extract of *T. fallax* were investigated in previous studies (Ozturk et al., 2005; Ozgen et al., 2006), there was no research assessing the antispasmodic, antimicrobial, and antioxidant activities of essential oil of *T. fallax*.

The aim of the present study was to investigate the effect of antioxidant, antimicrobial, and antispasmodic activities of the essential oil of *T. fallax* from the Turkish flora. The antioxidant activity was determined with 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene-linoleic acid assays. The antimicrobial activity was measured with disk diffusion method. Its antispasmodic activity was determined *in vitro* in rat ileum in organ bath model.

MATERIAL AND METHODS

Plant material

T. fallax plants were collected from Imranli-Yoncabayiri, Taslikyamac (1950 m), Sivas, Turkey. The taxonomic identification was made during flowering season late February 2006. The voucher specimen was identified and deposited at the Herbarium of the Department of Biology, Cumhuriyet University in Sivas in Turkey (CUFH-Voucher No: ED 11011).

Isolation of the essential oil

The air-dried and finely ground aerial parts of *T. fallax* were subjected for 3 h to water distillation using a clevenger- type apparatus (Yield 2.9% v/w). The oil was dried over anhydrous sodium sulphate and after filtration stored at +4°C.

Gas chromatography mass spectrometry (GS/MS) analysis

The chemical composition of *T. fallax* essential oil was analyzed using a Shimadzu OP5000 GC-MS, equipped with a GL SCIENCE capillary column TC-5 (30 mx0.25 mm i.d., 0.25 mm) and a 70 eV EI Quadrapol detector. For GC–MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was the carrier gas, at a flow rate of 1.9 ml/min. Injector and MS transfer line temperatures were set at 250 and 280°C, respectively. Column temperature was initially set at 40°C and held for 2 min; then gradually increased to 125°C at the rate of 2°C/min, held for 2 min, and finally increased to 250°C at 5°C/min held for 2 min. Diluted samples (1:100 v/v, in acetone) of 1.0 µl amounts were injected manually and in split less manner. The components were identified by comparison with their relative retention time and MS (NBS75K library data of the GC–MS system) (Adams, 2001, 2008).

DPPH assay for detection of antioxidant activity

The method is based on the reduction of alcoholic DPPH solutions in the presence of a hydrogen donating antioxidant. DPPH solutions show a strong absorption band at 517 nm appearing as a deep violet colour. The absorption vanishes and the resulting decolorization is stoichiometric with respect to degree of reduction. The remaining DPPH, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant (Burits et al., 2000). Fifty microliter of various concentrations of the extracts in methanol was added to 5 ml of a 0.004% methanol solution of DPPH. After 30 min of incubation at room temperature, the absorbance was read against a blank at 517 nm. Inhibition free radical DPPH in percent (I %) was calculated in the following manner:

$$\text{Inhibition \%} = \frac{(A_{blank} - A_{sample})}{A_{blank}} x100$$

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 from the graph plotting inhibition percentage against extract concentration. Synthetic antioxidant reagent butylated hyroxytoluene (BHT) was used as the positive control and all tests were carried out in triplicate.

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 (Burits et al., 2000; Cuendet et al., 1997). This method is based on the loss of the yellow colour of β-carotene due to its reaction with radicals that are formed by linoleic acid oxidation in an emulsion. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants. This fact is used in the antioxidant activity evaluation of the essential oil in comparison with known, synthetic and natural antioxidants, namely BHT. A stock solution of β-carotene–linoleic acid mixture was prepared as following: 0.5 mg β-carotene was dissolved in 1.0 mL of chloroform (HPLC grade), to which 25 µL of linoleic acid and 200 mg of Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then 100 mL distilled water saturated with oxygen (30 min 100 mL/min) was added with a vigorous shaking: 2500 µL of this reaction mixture was dispersed into test tubes and 350 µL portion of the extracts prepared in ethanol at 2 g/L concentrations was added and the emulsion system was incubated up to 48 h at room temperature. Same procedure was repeated with positive control BHT and a blank. After this incubation period absorbance of the mixtures were measured at 490 nm. Antioxidative capacities of the extracts were compared with those BHT at the same concentration and blank containing only 350 µL ethanol.

Microbial strains

Antimicrobial and antifungal activities of the essential oil were evaluated against three gram-positive and five gram-negative bacteria and fungus by disk diffusion method. The microorganisms used were *Staphylococcus aureus* ATCC-25923, *Escherichia coli* ATCC-35218, *Pseudomonas aeruginosa* ATCC-27853, *Salmonella thyphi* NCTC-9394, *Klebsiella pneunomoniae* NCTC-5046, *Proteus vulgaris* RSHM-96022, *Bacillus subtilis* ATCC-6633, *Corynebacterium diphteriae* RSHM-633 and *Candida albicans* ATCC-10231. All these cultures were obtained from the culture collections of the Department of Health of Refik Saydam Hygiene Center Contagious Diseases Research Department (Ankara Turkey). Bacterial strains were cultured overnight at 37°C in Mueller Hinton agar (MHA-Oxoid-CM 337). The yeast was cultured overnight at 30°C in Sabouradud dextrose agar (Oxoid-CM41). All the experiments were carried out in triplicate and average and standard deviation (SD) were calculated for the inhibition zone diameters.

Table 1. Chemical composition of *T. fallax* essential oil.

No	Retention time (min)	Compound	Composition (%)
1	2.789	Etil asetate	9.11
2	6.354	α-buthyl benzyl alchol	4.48
3	12.770	α-pinene	3.30
4	13.555	Camphene	0.80
5	15.183	β-pinene	0.68
6	16.418	β-myrcene	2.74
7	17.100	β-Phellandrene	0.35
8	17.500	Delta-4-carene	0.23
9	18.000	α-terpinene	1.26
10	18.531	1.8cineol	8.20
11	20.667	γ-terpinene	8.97
12	21.908	α-terpinolene	0.14
13	39.215	Myrcenalasetat	0.63
14	41.987	Dihidrocarveal asetat	0.44
15	38.625	Carvacrol	46.0
16	45.983	Trans caryophylene	3.47
17	51.187	α-patchoulene	0.78
18	51.242	β-bisabolene	0.68
Total			92.41

Antimicrobial assay (disk diffusion assay)

Agar disc diffusion method was employed for the determination on antimicrobial activities of the essential oil in question (NCCLS, 1997; NCCLS, 999). A suspension of a test microorganism (0.1 mL from 108 cells per mL) was spread on the solid media plates. Filter paper discs (6 mm in diameter) were impregnated with 10 µL of the oil and placed on the inoculated plates. These plates, after staying 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 yeast. The diameters of the inhibition zones were measured in millimeters.

Antispasmodic activity

Animals

Male Wistar rats weighing 250-400 g were used. They were housed in a standard environmental condition and fed with relevant diet and water *ad libitum*. The study was approved by the Animal Ethics Committee of Cumhuriyet University Medical School.

Effect on rat ileum muscle strips preparation

The rats were killed by stunning and cervical dislocation. Small segments of ileum (2 cm long) were removed and mounted vertically in organ baths containing Tyrode solution of the following composition (mM): NaCl 136.0, KCl 5.0, MgCl2 0.98, CaCl2 2.0, NaH2PO4 0.36, NaHCO3 11.9, glucose 5.5, bubbled with air (37°C, pH 7.4). Spontaneous contractions of the ileum were observed without adding any drug. Following an equilibration period of 1 h, the study solution of essential oil of *T. fallax* added to the organ bath with cumulative doses (0.1, 0.5, and 1 mg/ml) and changes in amplitude and frequency of contractions was noted. In experiments examining the relaxation of the basal tonus of the ileum, paired segments of ileum were set up; one piece exposed to the study solution and the other receiving no treatment that served as the control. Data were expressed as mean ± SD and percentage. ANOVA was used analyses of frequency and amplitude of smooth muscle contractions. $P<0.05$ was considered as statistically significant.

RESULT AND DISCUSSION

Although several studies are available on the chemical composition, and antioxidant and antibacterial activities of different Thymus species (Sokmen et al., 2004a., Sokmen et al., 2004b, Vardar-Unlu et al., 2003); however, only a few studies have been carried out with *T. Fallax*. The results of the present study investigating the effect of *T. fallax* essential oil are being reported as the first study related to antioxidant, antispasmodic and antimicrobial effects in combination.

According to GC/MS results of the essential oil of *T. fallax* collected at Konak Bey Mountain in Malatya in Turkey by Tumen et al. (1999), carvacrol (68.1%), p-cymene (4.8%), β-caryophylene (3.8%), and γ-terpinene (3.6%) were the main constituents of the oil. Their results are similar to those of our study. We determined 18 compounds representing (92.4%) of the oil, with carvacrol (46.1%), ethyl acetate (9.1%), γ- terpinene (9.0%), and 1-8 cineole (8.2%) as the principal components comprising (72.4%) the essential oil (Table 1). In another study in Iran, thymol and γ -terpinene is reported as 65.1 and 10.8%, respectively in *T. fallax* (Barazandeh et al., 2004). This finding is different from the finding of Tumen et al. (1999) and our results. In the studies done in Turkey, carvacrol was found to be major component; however in

Table 2. Antimicrobial activity of the essential oil of *T. fallax* using agar disc diffusion method.

Microorganisms	Essential of T. fallax	Gentamycin	Nystatin
Staphylococcus aureus	90 ±1.2	23± 0.8	-
Escherichia coli	90±0.76	16±1.0	-
Pseudomonas aeruginosa	11±0	20±1.1	-
Salmonella thyphi	38±1.3	10±0.5	-
Klebsiella pneumonia	67±1.2	20±0.7	-
Proteus vulgaris	37±1.1	22±1.4	-
Bacillus subtilis	70±1.0	29±1.1	-
Corynebacterium diphteriae	62±0.8	23±1.1	-
Candida albicans	53±1.8	-	25±0.9

Results are means of three different measurements.

Table 3. Antioxidant activity of essential oil of *T. fallax* and positive control (BHT) with the free radical DPPH scavenging and β-carotene-linoleic acid methods.

	Inhibition IC_{50} (µg/ml) by DPPH	Inhibition % (µg/ml) by β-carotene-linoleic acid
T. fallax	215	71
Buthylated hydroxytoluen	10.5	100

the studies conducted in Iran, thymol (65.9%) was reported to be the major component. The season of the difference of chemical components may be due to temperature, height, geographical and geological differences. Apart from these differences, there are no reports on the chemical composition of the essential oil of *T. fallax* in the literature.

We found no study about the antioxidant and antimicrobial activities of *T. fallax* essential oil, but there was a study about antibacterial effect of *T. fallax* extract (Ozturk et al., 2005). In that study, strong antimicrobial properties of it was reported. In the present study, essential oil inhibited the growth of eight of nine microorganisms (except for *Pseudomonas aeruginosa*) (Table 2).

Free radical scavenging capacities of the essential oil measured in DPPH and β-carotene-linoleic acid assays were given (Table 3). IC_{50} value of *T. fallax* essential oil was found to be 215 µg/ml, while IC_{50} value for BHT was found to be 10.5 µg/mL. β-carotene-linoleic acid assay also identified the 71% inhibition. In the study of Ozgen et al. (2006), extract of *T. fallax* provided strong antioxidant activity.

In rat ileum, direct *T. fallax,* essential oil effect was investigated on spontaneous contractions. *T. fallax* has been inhibited both amplitude and frequency of spontaneous contractions with 100% at 0.1 mg/mL (Figure 1). The effect of essential oil was potent and concentration dependent, and fully reversible on washout. Thus, these actions do not appear to be the result of nonspecific properties of oils to modify the lipid environment of the cell membrane, but instead point to more precise changes. Thus, a variety of plant essential oils exert potent effects

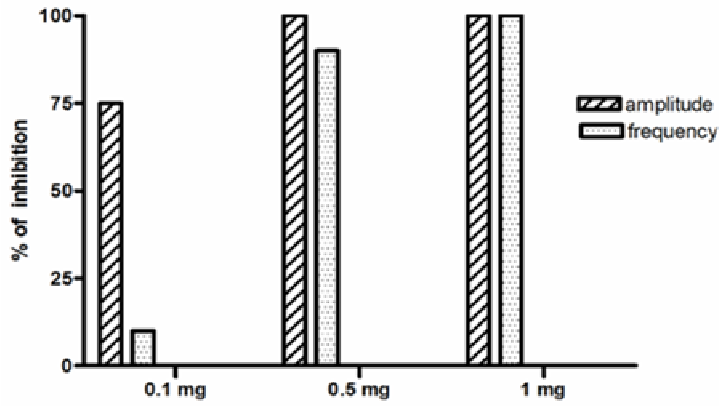

Figure 1. Effect of *T.fallax* essential oil (0.1, 0.5 and 1 mg) on rat ileum spontaneus contractions. From 0.1 to 1 mg of essential oil significantly reduced amplitude and frequency of rat ileum *in vitro* ($p<0.05$).

on isolated intestinal smooth muscle ostensibly via different mechanisms. Further studies are necessary to elucidate the underlying mechanism of action of *T. fallax* and to evaluate its likely participation as the active principle of infusates used in folk medicine.

This is the first study investigation antimicrobial, antispasmodic and antioxidant properties of the essential oil of *T. fallax* in combination in our laboratory setting. Owing to the strong antimicrobial, antioxidant, and anti-spasmodic activity tests of the essential oil of *T. fallax*, this plant may be regarded as a natural source that can after further studies.

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