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Chemical Composition, Antioxidant and Antitumor Activity of *Thymus vulgaris L.* Essential Oil

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Abstract: The genus Thymus are important medicinal plants, highly recommended due to a variety of therapeutic properties of their essential oils, normally known as thyme oil. Scientific validation of traditional uses and phytochemical and antitumour activity evaluation of essential oils from *Thymus vulgaris L.* was performed. The hydrodistillated oils obtained from wild thyme species were analyzed by gas chromatography-mass spectrometry (GC-MS). Forty two components in total were identified representing more than 97.6% of the oil composition, with thymol (54.26%), γ-terpinene (9.50%), *p*-cymene (7.61%), carvacrol (4.42%), terpinolene (3.27%), α-terpinene (2.36%), α-terpineol (1.63%) and α-tujene (1.52%) in *T. vulgaris L.* as major constitutes. The antioxidant activity of thyme essential oil was assessed by DPPH assay, the concentrations that led to IC_{50} is 210, 150, 96 and 53 µg/mL at 10, 30, 60 and 90 min. incubation, respectively. The *T. vulgaris L.* essential oil revealed the most effective one against A-549, MCF-7 and HepG2 cell lines, presenting IC_{50} values of 75, 60 and 41µg/mL, respectively.

Key words: *T. vulgaris* · Essential Oils · Chemical Composition · Cytotoxic · Antioxidant Activity

INTRODUCTION

Essential oils are chemical products formed by odoriferous essences from a great diversity of plants. They are volatile liquid insoluble in water, highly soluble in alcohol and ether. Essential oils are complex mixtures of different chemical compounds. The aerial parts of *Thymus* species have been highly recommended; they were generally used as herbal teas, condiments and spices, so as for various medicinal purposes [1]. Many ethno medicinal properties are attributed to infusions, decoctions and essential oils of the aerial parts of *Thymus* species, due to their tonic, carminative, antispasmodic, antimicrobial, antioxidant, antiviral and expectorant activities [2, 3]. The genus *Thymus* comprising of around 300 species of perennial, aromatic herbs and subshrubs is predominantly found in Mediterranean region, Asia, Southern Europe and North Africa [4]. *Thymus* species are considered as medicinal plants due to their pharmacological and biological properties [4-6]. Its properties are due to its main components, thymol and carvacrol [7]. The genus *Thymus* has numerous species and varieties and their essential oils have been already studied [8]. However, there are considerable research interests to continue with studying due to many other biological properties *Thymus* essential oils may possess [9]. *Thymus vulgaris* L. a significant aromatic plant with around 100 species in the world is widely used for medicinal purposes as well as in culinary dishes. Most of these species grow in the wild and are harvested from the countryside, with only five percent cultivated commercially. *Thymus vulgaris* L. is a perennial herb indigenous in Asia, Europe, America and Africa. It is widely used in folk medicine in the treatments of variety of diseases such as gastroenteric and bronchopulmonary disorders [10]. Garden thyme has natural antibiotic properties as a consequence of the presence of thymol which constitutes around 50 percent of the total essential oils. Carvacrol is also of importance in this respect [11]. Thyme oil is among the world's top ten essential oils regarding its use as a food additive [1]. Essential oil content of thyme has been reported from 0.32 to 4.9% [12]. Thymol and carvacrol constituted the main phenolic compound of Thyme oil. The major non-phenolic compounds were linalool and p-cymene [13, 14]. It has been reported that its essential oils possesses numerous

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biological activities including anti-worm, antiseptic, antispasmodic, antimicrobial and antioxidant [15, 16]. Therefore, our primary objective was to characterize the essential oils of *T. vulgaris L.* and to evaluate their antioxidant and antitumor activity.

MATERIAL AND METHODS

Plant Material: Leaves of *Thymus vulgaris* were collected in March 2014 from local markets in Jazan, KSA. The leaves were randomly collected from plant parts and shade dried. The plant material was dried naturally on laboratory benches at room temperature (28-30°C) for 5 days until crisp.

Essential Oil Extraction: A hundred grams of dried aerial parts of *T. vulgaris L.* were placed in a flask and 400 ml of distilled water was added. Sycamore leaves were extracted by hydrodistillation using a Clevenger-type apparatus for 4 h. Water was heated to produce steam that carried the most volatile fractions of the aromatic material with it. The watery phase was extracted with dichloromethane (3×50 mL) and dried with anhydrous sodium sulfate. The dichloromethane solution of the volatiles was concentrated to 5 ml by evaporation under vacuum in a rotary evaporator at 30°C under reduced pressure. The volatile phase from *T. vulgaris L.* was stored at 4°C prior to further analyses. The volatile phase in dichloromethane was performed for gas chromatography and mass spectrometry analysis. The volatiles yield calculated about the dry matter.

Physical Properties: The physical properties, such as appearance, refractive index, specific gravity and color, of the essential oil extracted were determined using the Food Chemical Codex method [17]. The refractive index and specific gravity were measured at 20°C.

Gas Chromatographic (GC) Analysis: GC analysis was performed by using Hewlett-Packard model 5890 equipped with flame ionization detector (FID). A silica capillary Rtx-5ms column (30 m × 0.25 mm i.d.) was used. For fatty acid analysis, the oven temperature was programmed from 50 to 240°C at a rate of 3°C/min. Helium was used as the carrier gas, at flow rate 1.1 mL/min. The injector and detector temperatures were 220 and 250°C, respectively. For analysis of the extracted coconut aroma, the oven temperature was maintained initially at 50°C for 6 min and then programmed from 50 to 240°C at a rate of 3°C/min. The injector and detector temperatures were 220 and 240°C, respectively. The retention indices (Kovats index) of the separated volatile compounds were calculated with hydrocarbons (C8–C22, Aldrich Chemical Co.) as references. The relative concentration of each individual compound was determined by comparing the peak area of the compound in each chromatogram with that of γ-decalactone (IS), assuming all response factors were 1.

Gas Chromatographic-mass Spectrometric (GC-MS) Analysis: The analysis was carried out using a coupled gas chromatography Hewlett-Packard (5890)/ mass spectrometry Hewlett- Packard-MS (5970). The ionization voltage was 70 eV, mass range m/z 39-400 amu. The GC condition carried out as mentioned above. The components of oils were identified by comparison of their mass spectra and retention indices with those published in the literature [18], comparison of their mass spectra of peaks with those obtained from GC-MS library and by comparison of their relative retention times with those of authentic samples on a capillary column [19].

Biological Activity

DPPH Radical-Scavenging Activity: The free-radical scavenging activity of *T. vulgaris L.* Essential oil was measured by 2,2-diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich) using the method described by Shimada *et al*. [20]. One milliliter of the essential oil at known concentration was added to 0.25 ml of a DPPH methanolic solution. The mixture was shaken vigorously and left standing at room temperature for 30 min in the dark. The absorbance of the resulting solution was then measured at 515 nm and corresponded to the ability of the essential oil to reduce the stable radical DPPH to the yellow colored diphenylpicrylhydrazine. The antiradical activity was expressed as IC_{50} (µg/mL), the extract dose required to cause a 50% inhibition. Absorption of a blank sample containing the same amount of methanol and DPPH solution acted as negative control. All determinations were performed in triplicate. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Scavenging ability (\%)} = [(\Delta A_{515\ \text{of control}} - \Delta A_{515\ \text{of sample}}) / \Delta A_{515\ \text{of control}}] \times 100.$$

Cytotoxicity in Human Tumor Cell Lines and Normal Primary Culture

Cell Propagation, Maintenance and Treatments: Three human tumor cell lines were used: MCF-7 (breast adeno carcinoma), A-549 (Human alveolar basal epithelial) and HepG2 (hepatocellular carcinoma).

Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine (MCF-7 and A-549) or in DMEM supplemented with 10% FBS, 2 mM glutamine,100 U/mL penicillin and 100 mg/mL streptomycin (HepG2 cells), at 37°C, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7 and A-549or 1.0×10^4 cells/well for HepG2) in 96-well plates. Experiments were conducted when the cells were in the logarithmic growth phase. We used the following concentrations 25, 50, 75 and 100 µg/mL of the study essential oil and the cells were incubated with these compounds for 48 h at a cell density of 10,000 cells per well of 96 well plate A positive control, doxorubicin (*M*r = 579.9), was used as a known cytotoxic natural agent giving 100% inhibition ((IC_{50}, 2.0 µg/mL). Dimethyl sulfoxide (DMSO) was the vehicle used for dissolution of the tested compound and its final concentration on the cells was less than 0.5%.

Cytotoxicity Test: Cell viability was measured by neutral red uptake assay according to Reptto *et al*. [21]. Briefly, the neutral red uptake assay provides a quantitative estimation of the number of viable cells in a culture. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. Briefly, Neutral red working solution 0.4 µg/mL was incubated overnight at 37°C the same as treated cells. In each well of the incubated cells, culture media was removed and 100 µL of neutral red medium were added then incubated for 2 hrs to allow for vital dye incorporation into living cells. The neutral red media was removed and rapid rinsed with 150 µL DPBS buffer. Dye was extracted from the cells by adding 150 µL extraction buffer (1% acetic acid: 50% ethanol (96%): 49% deionized H_2O) followed by rapid agitation for at least 10 min on micrometer plate shaker. The red color intensity was measured at 530 and 645 nm as excitation and emission wavelengths in a micro titer plate reader spectrophotometer. Using a 4 parameter logistic curve, the relation between used concentrations and neutral red intensity value, IC_{50} of tested compounds was calculated.

RESULTS AND DISCUSSION

Physical Properties: The essential oil isolated by hydro distillation of the aerial parts of *T. vulgaris* was purple yellow oil, with a yield of 1.77% (w/w), based on dry weight respectively. The appearance, solubility, refractive index, specific gravity, acid value and ester value of the essential oil extracted with hydro distillation are provided in Table 1. The refractive index, specific gravity, acid value and ester value of the essential oil were 1.47; 0.95; 3.52 and 3.45, respectively, which indicates high quality and purity of the volatile oil.

Table 1: Physical Characteristics of essential oils of *T. vulgaris L*

Physical Properties	Values
Color	purple yellow
Yield	1.77%
Solubility	Soluble in 80% alcohol
Refractive index	1.47
Specific gravity	0.95
Acid value	3.52
Ester value	3.45

Chemical Composition of Thyme Essential Oil: Essential oils are very complex natural mixtures which can contain about 30 –60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20 – 70%) compared to others components present in trace amounts. Generally, these major components determine the biological properties of the essential oils. The components include two groups of distinct biosynthetical origin [5, 22]. The main group is composed of terpenes and terpenoids and the other of aromatic and aliphatic constituents. Chemical composition of the essential oil of *T. vulgaris* was shown in Table 2. Forty two components were identified in the *T. vulgaris* essential oil that represented 97.6% of the oils. The major components were thymol (54.26%), γ-terpinene (9.50%), *p*-cymene (7.61%), carvacrol (4.42%), terpinolene (3.27%), α-terpinene (2.36%), α-terpineol (1.63%) and α-tujene (1.52%). The results also showed that essential oils extracted from *T. vulgaris* contained oxygenated monoterpenes and hydrocarbons monoterpenes. Our results confirm earlier reports that major volatile constituents obtained from the aerial parts of *T. vulgaris* were thymol, carvacrol, p-cymene, γ-terpinene and β-caryophyllene [23-27]. Many studies on the chemical composition of the oils from the plants belonging to the genus Thymus were conducted, including *T. serpyllum, T. algeriensis* and *T. vulgaris* [28, 29]. Our results on chemical profiling of the *T. serpyllum* essential oil are in agreement with some other studies [30], where α-pinene and carvacrol were reported to be the major oil components [31].

Table 2: Chemical composition of the essential oils of *T. vulgaris L*

Peak	Component	Rt	Formula	Relative concentration (%)
1	3-Hxenol	5.211	$C_6H_{12}O$	0.10
2	α-Tujene	6.636	$C_{10}H_{16}$	1.52
3	α-Pinene	6.801	$C_{10}H_{16}$	1.31
4	Camphene	7.131	$C_{10}H_{16}$	0.75
5	Sabinene	7.640	$C_{10}H_{16}$	0.84
6	3-Otenol	7.705	$C_8H_{16}O$	0.36
7	3-Otanone	7.871	$C_8H_{16}O$	0.20
8	β-Myrcene	7.966	$C_{10}H_{16}$	0.67
9	3-Otanol	8.043	$C_8H_{18}O$	0.21
10	α-Pellandrene	8.303	$C_{10}H_{16}$	0.10
11	δ-3-Carene	8.440	$C_{10}H_{16}$	0.11
12	α-Terpinene	8.571	$C_{10}H_{16}$	2.36
13	ρ-Cymene	8.750	$C_{10}H_{14}$	7.61
14	Sylvestrene	8.839	$C_{10}H_{16}$	0.34
15	1,8-Cineol	8.911	$C_{10}H_{18}O$	0.57
16	*cis*-Oimene	8.974	$C_{10}H_{16}$	0.22
17	β-Oimene	9.211	$C_{10}H_{16}$	0.20
18	γ-Terpinene	9.504	$C_{10}H_{16}$	9.50
19	*cis*-Sabinene	9.683	$C_{10}H_8O$	0.10
20	3-Nonenol	9.876	$C_9H_{20}O$	0.12
21	3-Nonene	10.041	C_9H_{18}	0.22
22	α-Terpinolene	10.144	$C_{10}H_{16}$	3.27
23	Linalool	10.328	$C_{10}H1_8O$	0.93
24	Terpineol	10.874	$C_{10}H_{18}O$	1.37
25	Carene	11.267	$C_{10}H_{16}$	0.35
26	*E*-Citral	11.307	$C_{10}H_{16}O$	0.54
27	3,4-Octadienal	11.486	$C_8H_{14}O$	0.23
28	Verbenol	11.717	$C_{10}H_{16}O$	0.12
29	*endo*-Borneol	11.870	$C_{10}H_{18}O$	0.10
30	4-Terpineol	12.091	$C_{10}H_{18}O$	1.44
31	α-Terpineol	12.353	$C_{10}H_{18}O$	1.63
32	Dihydrocarvone	12.501	$C_{10}H_{16}O$	0.10
33	Decanal	12.565	$C_{10}H_{20}O$	0.12
34	9-ρ-Menthenol	12.680	$C_{10}H_{16}O$	0.23
35	2,6-Octadienal	13.364	$C_8H_{12}O$	0.14
36	Anisole	13.411	$C_7H_{18}O$	0.23
37	Geraniol	13.681	$C_{10}H_{18}O$	0.10
38	Citral	13.964	$C_{10}H_{16}O$	0.24
39	Thymol	14.403	$C_{10}H_{14}O$	54.26
40	Carvacrol	14.560	$C_{10}H_{14}O$	4.42
41	Octadienoic acid	14.948	$C_{18}H_{12}O$	0.10
42	Geranic acid	15.435	$C_{10}H_{16}O_2$	0.30

In addition the thymol (30%) and carvacrol (20%) was reported to be the second main component of the wild thyme oils [32], while thymol being the third major component 18% in the wild thyme oil, after the content of γ-terpinene (22%) and *p*-cymene (20%) [33]. This characteristic seems to be common for all *Thymus* spp. essential oils [34] and is frequently attributed to the origin, environmental conditions and developmental stage of the sourcing plant material [35]. Although *T. algeriensis* is one of the rarest *Thymus* species, various authors already testified the occurrence of different oil chemo types, such as thymol, linalool, carvacrol and geranyl acetate and terpinyl acetate the first two being the most common ones [36].

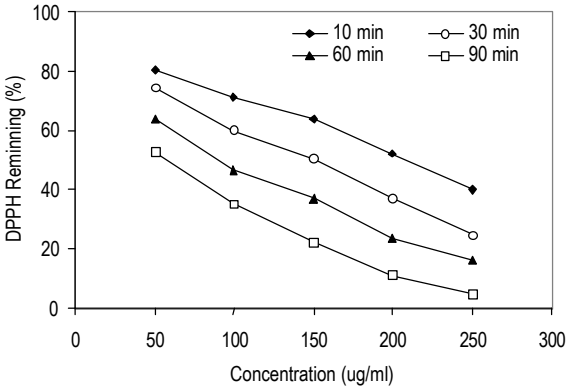

Fig. 1: Antioxidant activity of *T. vulgaris* essential oil at 10, 30, 60 and 90 minute incubation

Antioxidant Activity: The radical scavenging capacity of thyme essential oil increased in a concentration dependent manner. The values for 50% scavenging activity (IC_{50}) are presented in Figure 1. The antioxidant activity of thyme essential oil was assessed by DPPH assay, evaluating the H-donating or radical scavenging ability of the oil using the stable radical DPPH as a reagent. The concentrations that led to 50% inhibition (IC_{50}) for thyme essential oil is 210, 150, 96 and 53 µg/mL at 10, 30, 60 and 90 minute incubation, respectively. It seems to be a general trend that the essential oils which contain oxygenated monoterpenes and/or sesquiterpenes have greater antioxidative properties [37, 38]. Hence, many aromatic plants are today considered as the most important sources for the extraction of compounds with strong antioxidant activity. Thyme is two spices widely used in folk medicine, cosmetics and the flavoring of food products [39, 40]. Antioxidant activity exhibited by essential oil justifies traditional uses of thyme herbs. The observed antioxidant potential should be addressed to the phenolic oil constituents [36, 41], while the oil chemo-protective efficacy against oxidative stress-mediated disorders is mainly due to its free radical scavenging.

Cytotoxic Activity for Human Tumor Cell Lines and Normal Liver Primary Culture: The effects of *T. vulgaris* essential oil on growth of breast adeno carcinoma (MCF-7), human alveolar basal epithelial (A-549) and hepatocellular carcinoma (HepG2) are presented in Figure 2 as concentrations that caused 50% of the cell growth inhibition (IC_{50}). *T. vulgaris* oil was the most potent in all tested human cell lines, presenting IC_{50} values ranging from 75, 60 and 41µg/mL for A-549, MCF-7 and HepG2, respectively. The *T. vulgaris* essential oil

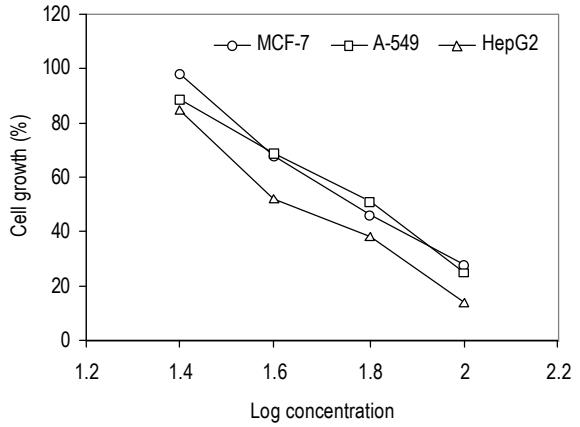

Fig. 2: Effects of *T. vulgaris* essential oil on growth of A-549, MCF-7 and HepG2 cell lines

showed no efficacy against non tumor cell line PLP2 in concentrations higher than 450 µg/mL. Up till now, various authors have reported antitumor activities of essential oil and their constituents. For example, the thyme oil appears to be the most effective against HepG2 cell line [42]. The thyme oil which contains carvacrol as its major constituent has an important *in-vitro* cytotoxic activity against tumor cells. Our thyme essential oil inhibited the viability of several tumor cell lines in a concentration dependent manner [38]. Tsukamoto *et al.* [43] reported that the thymol, which is the major constituents in our essential oil, might be involved in the stimulation of active proliferation of pulp fibroblasts. Whether the thymol alone, or in combination with other oil constituents is responsible for the observed cytotoxicity against tumor cells, still remains to be revealed, presenting an important limitation of the study. At non toxic concentrations, thyme extract was also identified as a natural antimutagen with the possibility of enhancement of error free DNA repair [44].

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