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Composition and potential of *Tanacetum haussknechtii* Bornm. Grierson as antioxidant and inhibitor of acetylcholinesterase, tyrosinase, and α-amylase enzymes

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ABSTRACT

The phytochemical composition of essential oils and extracts of *Tanacetum haussknechtii* were investigated with GC–FID–MS and LC–MS/MS techniques and evaluated against oxidation, acetylcholinesterase, tyrosinase, and α-amylase enzymes. The major volatile constituents of *T. haussknectii* were found to be α- and β-pinene, and borneol. Caffeoylquinic acid derivatives and flavonoids were detected in the aqueous, alcohol, and ethyl acetate extracts. In DPPH assay, the methanol extracts exhibited the highest activity. TEAC assay resulted with superiority of all methanol and the capitula ethyl acetate extract. In β-carotene bleaching assay, linoleic acid was the best protected by the ethyl acetate extract of flower. The flower oil inhibited higher acetylcholinesterase activity than the remaining extracts. The flower ethyl acetate extract was found as the most effective inhibitor of α-amylase. The herb and the leaf+stem water extracts possess highest inhibitory effect on tyrosinase.

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Abbreviations: AChE, acetylcholinesterase; BHT, butylated hydroxytoluene; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DOPA, 3,4-dihydroxyphenylalanine; EO, essential oil; GA, gallic acid; GAE, gallic acid equivalent; GC–FID, gas chromatography—flame ionization detector; LC–MS/MS, liquid chromatography–mass spectrometry; MS, mass spectrometry; QE, quercetin equivalent; TCI, thiocholine iodide; TE, Trolox equivalent.

Introduction

Using natural products instead of synthetics has become an important issue in the world. Vascular plants comprising approximately 300 thousand species are the main source for production of natural pharmaceuticals, organic agriculture, cosmetics. The pathogenesis of Alzheimer's disease is not fully discovered yet. There are several hypotheses on the pathogenesis of this disease. According to one of these hypotheses, healthy people have more acetylcholine neurotransmitter in their brain when compared with Alzheimer's patients. Inhibiting acetylcholinesterase that is responsible for breakdown of acetylcholine increases the availability of acetylcholine.[1] Therefore, searching for compounds that have inhibitory effect on AChE is considered to be an alternative to current drugs used for treatment of Alzheimer's disease. Until now, galanthamine from *Galanthus nivalis* L.[2], eserin

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from *Physostigma venenosum* L.[3], and huperzin A from *Huperzia serrata* (Thumb.) Trev.[4] were found to be pharmacologically active on inhibition of AChE. Galanthamine is prescribed in the United States and the European Union for Alzheimer's patients. In contrast, owing to its adverse side effects, eserine cannot be used in the treatments. Moreover, huperzin A is still under phase-II clinical trial in China.

It is estimated that 415 million people in the world diabetes is the most common endocrine disease. In Turkey, more than 7 million people have diabetes mellitus. Patients with diabetes experience significant morbidity and mortality from micro- and macrovascular complications. The cost of diabetes to society is very high, and it is escalating every year. Turkey has the highest age-adjusted comparative prevalence (12.8% comparative prevalence) and the third-highest number of people with diabetes in the Europe. [5] There are several approaches in therapy of diabetes. One of them is the inhibition of α-amylase enzyme to decrease digestion of carbohydrates. Nowadays, there is a serious demand for effective and safe inhibitors of α-amylase. In this context, natural compounds from plants and other sources may have a potential. Produced by *Actinoplanes* sp. fermentation, acarbose is the most common natural drug used for the treatment of diabetes clinically. The literature search revealed reports about the potential of *Tanacetum* species as antidiabetic agents: *T. vulgare* L.

Tyrosinase is an enzyme that occurs in mammalian melanocytes and is responsible for hydroxylation of monophenols to *o*-diphenols and catalization of *o*-diphenols to *o*-quinones. In the melanogenesis process, tyrosinase plays a key role in the hydroxylation of l-tyrosine and oxidation of L-DOPA to *o*-dopaquinone. After several non-enzymatic reactions, melanin is produced from the *o*-quinones. Inhibition of tyrosinase in the melanogenesis process may slow down the production of abnormal amounts of melanin and offers a solution for esthetic problems on human skin. Another important item, tyrosinase gene is expressed in the human substantia nigra. There is growing evidence that tyrosinase could play a central role in dopamine neurotoxicity as well as contribute to the neurodegeneration associated with Parkinson's disease.[6] In addition, tyrosinase is responsible for the color change such as browning of fruits and vegetables. In the literature, there are a number of papers about anti-tyrosinase effects of different types of phytochemicals. [7,8]

Reactive oxygen species are known to play a major role in the development of oxidative stress. Oxidative stress may lead to cardiac diseases, diabetes, inflammation, neurodegenerative disorders, cancer, anemia, and atherosclerosis in human body.[9] Many synthetic drugs are produced to control oxidative stress. However, the financial obligations, accessibility issues, and particularly side effects of these synthetic drugs are the main obstacles in the prevention of oxidative stress. Acquiring natural antioxidants from many plant sources with minimal side effects and low cost gained importance against synthetic drugs.

Asteraceae family contains approximately 23,000 species in the world. *Tanacetum* L. is the third largest genus within Asteraceae and has 160 species worldwide.[10] *Tanacetum* species spread to Europe, Asia, North Africa, and North America. Forty-six of these 160 species are grown naturally in Turkey.[11] *Tanacetum* species have been used to cure or lower the pain of several diseases traditionally as well as for food purposes.[12–14] Uses of *Tanacetum vulgare* (L.) (tansy) as anthelmintic and *Tanacetum parthenium* (L.) Schultz Bip. (feverfew) as antipyretic are among the well-known traditional uses.[15] Traditional uses of *Tanacetum* species are summarized in Table 1. *Tanacetum haussknechtii* (Bornm.) Grierson is an endemic of Turkey. Almost no published information about the chemistry and uses of *T. haussknechtii* is available, despite the local importance of the genus *Tanacetum* in the traditional health care system of Turkey. Phytochemical investigations on *Tanacetum* species resulted with high diversity of volatile and non-volatile constituents. A summary of previous phytochemical reports on *Tanacetum* species is presented in Tables 2 and 3.

In brief, α-pinene, β-pinene, camphene, sabinene, limonene, γ-terpinene, α-thujone, α-phellandrene, α-terpinene, *p*-cymene, bornyl acetate, borneol, chrysanthenyl acetate, camphor, α-terpineol, pinocarvone, β-caryophyllene, and caryophyllene oxide were reported as common volatiles[10], while sesquiterpene lactones and flavonoids were detected as non-volatile constituents in the extracts. [35,36]

To the best of our knowledge, there is no any report on biological activity of *T. haussknechtii*. In this study, we aimed to reveal phytochemistry and biological properties of *T. haussknechtii* for the

Table 1. Traditional uses of *Tanacetum* taxa in Turkey.

Plant Name	Local Name	Plant part	Use	Utilization method	Ref.
T. argyrophyllum (C. Koch) Tvzel. var. *argyrophyllum*	Yavşan	Aerial	Pulmonic disorders, cold, antipyretic, anti-inflammatory	Ext.-Decoc Int.-Infus.	[16]
T. balsamita L.	Kilicotu, Baga yapragi, Marsuvan	Aerial	Tonic, stimulant, antipyretic, headache, diuretic, gall bladder disorders, wound healing	Int.-Infus.	
T. punctatum (Desr.) Grierson	Sendel	Aerial	Emmenagogue, anti-inflammatory	Int.-Infus.	
T. balsamita subsp. *balsamitoides* (Sch. Bip.) Grierson	Giyakeçk	Flower stems	Diuretic, gall stones and stomachic	Int.-Infus.	
T. chiliophyllum (Fisch. & Meyer) Schultz var. *chiliophyllum*	Bevujan	Capitulum	Pulmonic disorders, colds, kidney stones, antipyretic	Int.-Decoc.	
T. coccineum (Willd.) Grierson subsp. *chamaemelifolium* (Sommier & Levier) Grierson	Pire otu	Flower	Insecticidal, anthelmintic	Powdered	[15]
T. cinerariifolium Schultz Bip.	Dalmacya pire otu	Flower	Insecticidal	Powdered	
T. vulgare L.	Solucan otu	Aerial	Stomachic, anthelmintic	Int.-Infus.	
T. parthenium (L.) Schultz Bip.	Gümüşdügme	Flower	Tonic, stimulant, antipyretic, headache, diuretic, stomachic, gall stone	Int.-Decoc.	
T. cadmeum Boiss. Heywood subsp. *cadmeum*	Ayvadanasi	Herb Fruit, Capitulum	Ulcer Stomach ache	Int.-Decoc. Int.-Infus.	[17]

*Ext.: external; Int.: internal; Decoc: decoction; Infus: infusion.

first time. The main aim of this paper was to give a first detailed characterization of the essential oil and extracts compositions and antioxidant, anti-acetylcholinesterase, anti-tyrosinase, and anti-amylase activities of an important but hitherto poorly studied traditional medicine of Turkey.

Materials and methods

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), gallic acid, butylated hydroxytoluene (BHT), quercetin, β-carotene, linoleic acid, Tween 20, Folin & Ciocalteu's phenol reagent (FCR), Tris (hydroxymethyl) aminomethane (ACS reagent), acetylcholinesterase (AChE) from *Electrophorus electricus* (electric eel, Type VI-S, 200–1000 units/mg protein), bovine serum albumin (BSA), acetylthiocholine iodide (ATCI), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), galanthamine, tyrosinase from mushroom (EC 1.14.18.1), 3,4-dihydroxy-L-phenylalanine (L-DOPA), 1-naphthyl acetate, Fast Blue B Salt (dye content 95%), kojic acid, α-amylase from porcine pancreas (Type VI-B, ≥10 units/mg solid), acarbose, and galanthamine hydrobromide from *Lycoris* sp. were purchased from Sigma-Aldrich (St. Louis, MO, USA). Starch-soluble extra pure iodine and potassium iodide were purchased from Merck (Darmstadt, Germany). Sodium phosphate, disodium phosphate, aluminum chloride, ultrapure water, methanol (MeOH), dimethyl sulfoxide (DMSO), and ethyl acetate (EtAc) were extra pure analytical grade. A C_9–C_{40} *n*-alkane standard solution was purchased from Fluka (Buchs, Switzerland).

General

Agilent 5975 GC-MSD system (Agilent, USA; SEM Ltd., Istanbul, Turkey) was equipped with the HP-Innowax FSC column (60 m × 0.25 mm id with 0.25 μm film thickness, Agilent, USA). The GC–FID analysis was performed with capillary GC using an Agilent 6890N GC system (SEM Ltd., Istanbul, Turkey). Microtiter plate assay was performed with Biotek Powerwave XS microplate reader. Ultrapure water (0.05 μS/cm) was obtained from a Direct-Q® Water Purification System (Germany). TLC sprayer Biostep

Table 2. Literature survey on volatile compounds of *Tanacetum* species.

Species	Major Compound	Ref.
T. argenteum (Lam.) Willd. ssp. *argenteum* (L.) All., *T. argenteum* (Lam.) Willd. *subsp. canum* (C. Koch) Grierson var. *canum* (C. Koch) Grierson	α-Pinene (67.9%, 53.6%), β-pinene (4.8%, 1.0%), 1,8-cineole (2.2%, 14.8%)	[89]
T. argenteum ssp. *Argenteum*	α-Pinene (36.7%), β-pinene (27.5%), 1,8-cineole (9.8%)	[18]
	α-Pinene (27.9%), 1,8-cineole (6.8%)	[19]
T. mucroniferum Hub. –Mor. et Grierson	1,8-cineole (21.9%), camphor (6.4%)	[20]
T. chiliophyllum (Fisch. et Mey.) Schultz Bip. var. *monocephalum* Grierson	Flower and stem: 1,8-cineole (8.3%, 2.5%), camphor (17.3%, 10.4%), root: hexadecanoic acid (37.5%)	[21]
T. densum (Lab.) Schultz Bip. ssp. *laxum* Grierson	α-Pinene (5.0%), epi-bicyclosesquiphellandrene (31.4%), α-cadinol (7.0%)	[90]
T. kotschyi (Boiss.) Grierson	Flower and stem: artemisia ketone (54.6%, 26.5%), longiverbenone (9.2%, 8.9%), artemisia alcohol (4.6%, 5.2%)	[22]
T. zahlbruckneri (Nab.) GRIERSON	Germacrene D (29.7%) and spathulenol (12%)	[23]
Tanacetum argyrophyllum (C. Koch) Tvzel. var. *argyrophyllum* (C. Koch) Tvzel.	*cis*-Thujone (69.9%)	[24]
T. parthenium (L.) Schultz Bip.varieties	Camphor (28.0%), chrysanthenyl acetate (30.0%)	[25,91]
T. nitens (Boiss. et NOE) Grierson	α-Pinene (4.6%), 1,8-cineole (27.6%)	[19]
T. macrophyllum (Waldst. et Kit.) Schultz Bip.	β-Eudesmol (89.5%)	[26]
T. alyssifolium (Bornm.) Grierson	Borneol (35.2%), α-thujone (24.6%)	[27]
T. aucheranum (DC.) Schultz Bip.	1,8-Cineole (23.8%), camphor (11.6%)	
T. sorbifolium (Boiss.) Grierson	Camphor (54.3%)	[28]
T. argyrophyllum var. *argyrophyllum, T. argenteum* ssp. *canum* var. *canum; T. praeteritum* (Horwood) Heywood ssp. *praeteritum* (Horwood) Heywood, *T. praeteritum* (Horwood) Heywood ssp. *massicyticum* Heywood	α-Thujone, 1,8-cineole	[29]
T. armenum (DC.) Schultz Bip.	Leaf and herba: 1,8-Cineole (31.0%, 11.0%), camphor (9.0%, 27.0%)	[30]
T. haradjanii (Rech. Fil.) Grierson	Camphor (16.0%)	
T. balsamita L.	Carvone (52.0%)	
T. balsamita L. ssp. *balsamita* L.	Carvone (51.0%) and β-thujone (20.8%)	[31]
	trans-Chrysantenol (22.3%) linalool oxide (1.5%), 1,8-Cineole (2.7%), camphor (7.5%), chrysantenyl acetate (19.7%)	[32]
T. punctatum (Desr.) Grierson	*trans*-β-Farnesene (30.6%), caryophyllene oxide (26.1%), β-caryophyllene (21.1%)	[33]
T. audibertii (Req.) DC	Artemisia ketone (39.8%), *trans*-linalyl oxide acetate (32.0%), 1,8-cineole (15.9%)	[34]

Desaga (SG e1 BS130.605) was used for spraying of enzyme and reagent solutions. Spectrophotometric measurements were performed with spectrophotometer UV-PharmaSpec 1700 (Shimadzu). TLC Silica gel 60 F_{254} aluminum sheet (Merck) was used for thin-layer chromatographic experiments.

Plant material

T. haussknechtii was collected in the flowering stage in Sivas: Kangal-Gürün road, 16 km, 1545 m, 39° 07′ 52.0″ N, 37° 14′ 33.2″ E (August, 2015) and identified by Dr. M. Tekin (Trakya University). Voucher specimens (ESSE No. 15011) are maintained in Herbarium of the Faculty of Pharmacy (Anadolu University, Eskişehir, Turkey).

Essential oil isolation

Air-dried plant material (leaf+stem and capitula) was separately subjected to hydrodistillation (3 h) using Clevenger-type apparatus according to European Pharmacopoeia. Essential oil yields were calculated on moisture-free basis. The oils were diluted with *n*-hexane (10%, v/v) for GC–FID and GC/MS analysis.

Table 3. Literature survey on non-volatile compounds of *Tanacetum* species.

Compound	Species	Ref
Dentatin A	*T. argenteum* ssp. *flabellifolium*	[37]
l-*epi*-Ludovicine C	*T. vulgare*	[38]
Deacetyl-β-cyclopyretrosine	*T. ferulaceum*	[39]
Reynosin	*T. parthenium*	[40]
Sivasinolide	*T. densum* ssp. *sivasicum*	[41]
1-α-Hydroperoxy-1-desoxo chrysanolide	*T. chiliophyllum* var. *heimerlei*	[42]
Costunolide	*T. parthenium*	[43]
Santamarin	*T. santolina*	[44]
Deacetyllaurenobiolide	*T. densum* ssp. *sivasicum*	[41]
Partenolide	*T. parthenium*	[40]
Spiciformine	*T. densum* ssp. *sivasicum*	[41]
Tamirin	*T. vulgare*	[38]
Tanacin		
Tatridin A	*T. ptarmicaeflorum*	[45]
Deacetyltulipinolide-1-β,10-α-epoxide	*T. densum* ssp. *amani*	[46]
Artecanin	*T. parthenifolium*	[47]
Canin	*T. macrophillum*	[48]
Apigenin	*T. albipannosum*	[49]
Luteolin	*T. cinerariifolium*	[50]
Hispidulin	*T. sibiricum*	[51]
Quercetin	*T. vulgare*	[52]
Axillarin	*T. vulgare*	
Naringenin	*T. sibiricum*	[53]

Preparation of extracts

The leaf+stem and capitula of *T. haussknechtii* were powdered and separately subjected to maceration in ethyl acetate, methanol, and water at room temperature (drug/solvent ratio 1:10) for 24 h with continuous shaking. The filtrated organic phases of each plant part were evaporated until dryness *in vacuo* (MeOH, EtAc) or lyophilized (water) and preserved in the freeze dryer until the assays were performed. The dried extracts were dissolved in methanol (10 mg/mL) and used as the stock solutions prior subjection to biological activity screening, total phenols content and total flavonoids content evaluations.

Gas-chromatographic analysis

The GC/MS and GC–FID analyses were performed with an Agilent 5975 GC-MSD and Agilent 6890N GC systems (Agilent, USA; SEM Ltd., Istanbul, Turkey) equipped with the HP-Innowax FSC column (60 m × 0.25 mm id with 0.25 μm film thickness, Agilent, USA) in conditions reported earlier. [54]

Identification and quantification of compounds

Identification of the volatile constituents was achieved as reported previously.[54] Briefly, identification of the individual compounds was based on the following: (i) comparison of the GC/MS Relative Retention Indices (RRI) of the compounds on polar column determined relative to the retention times of a series of n-alkanes (C_8–C_{40}), with those of authentic compounds or literature data; (ii) computer matching with commercial mass spectral libraries: MassFinder software 4.0, Adams Library, Wiley GC/MS Library (Wiley, New York, NY, USA), and Nist Library, and comparison of the recorded spectra with literature data. Confirmation was also achieved using the in-house "Başer Library of Essential Oil Constituents" database, obtained from chromatographic runs of pure compounds performed with the same equipment and conditions (Table 4).

The non-volatile secondary metabolites were identified based on MS fragmentation pattern obtained with MS/MS detector and were matched with the data given in the references listed in

Table 4. Chemical composition of *T. haussknechtii* essential oils.

No.	RRI[§]	RRI[#]	Compound	A, %	B, %	ID method
1.	1032	1032[56]	α-Pinene	14.5	12.3	a,b,c
2.	1043	1030 [57]	Santolinatriene	1.7	7.4	a,b,c
3.	1076	1076 [56]	Camphene	1.3	2.8	a,b,c
4.	1118	1118 [56]	β-Pinene	7.2	6.4	a,b,c
5.	1132	1132 [56]	Sabinene	1.0	1.1	a,b,c
6.	1213	1213[58]	1,8-Cineole	3.6	3.1	a,b,c
7.	1497	1497[56]	α-Copaene	0.7	1.0	a,b,c
8.	1668	1666 [59]	(*Z*)-β-Farnesene	1.1	1.1	a,b,c
9.	1704	1704[56]	γ-Muurolene	–	4.5	a,b,c
10.	1706	1706[60]	α-Terpineol	2.4	1.4	a,b,c
11.	1709	1685[61]	α-Terpinyl acetate	2.4	1.4	a,b,c
12.	1719	1719[60]	Borneol	17.2	20.5	a,b,c
13.	1726	1726 [60]	Germacrene D	7.4	–	a,b,c
14.	1755	1756 [60]	Bicyclogermacrene	1.3	0.7	a,b,c
15.	1805		α-Campholene alcohol	1.8	0.8	b,c
16.	1900	1899 [62]	*epi*-Cubebol	1.4	0.6	a,b,c
17.	1957	1929 [62]	Cubebol	3.9	1.4	a,b,c
18.	2131		1-αH-Himachal-4-en-1β-ol	3.3	2.5	b,c
19.	2234		2-Himachalen-7-ol	2.3	1.5	b,c
20.	2257	2258[56]	β-Eudesmol	2.7	0.8	a,b,c
21.	2300	2300[56]	Tricosane	–	2.0	a,b,c

* ≥1.0% Compounds, A: Leaf+stem EO, B: Capitula EO; [§] RRI calculated against *n*-alkanes (C_9–C_{40}) on polar HP-Innowax column; [#] RRI on polar column reported in the literature [a)] Identification based on retention index of genuine compounds on the HP-Innowax column; [b)] Identification on the basis of computer matching of the mass spectra from Başer, Adams, MassFinder, Wiley, and NIST libraries; [c)] Tentative identified on the basis of computer matching of the mass spectra from Wiley Library.

Table 5 and according to previously published procedure.[55] Detailed description of phenolic compounds identification together with spectral data is given in the supplementary material.

Antioxidant activity

Free radical scavenging assay (DPPH test)

The scavenging effect of the samples on DPPH free radical was determined using a modified method of Brand-Williams.[80] The solutions of the EOs (30 mg/mL), the extracts (10 mg/mL), and standard (0.1 mg/mL) were prepared in methanol (MeOH). Moreover, 100 µL of the sample (EO/extract/standard) solution was mixed with 100 µL DPPH solution (0.08 mg/mL in MeOH) in 96-flat bottom well plate cells. The mixtures were allowed to stand in the dark for 30 min. Decrease in the absorbance was recorded at 517 nm. Gallic acid was used as a positive control. The experiments were performed in triplicate. The free radical scavenging activity of the samples was expressed as percentage of inhibition calculated according to Eq. (1):

$$\%\ Inh = \left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right) \times 100, \qquad \text{(Eq 1)}$$

where $Abs_{control}$ is the absorbance of the control (containing all reagents except the test compound), Abs_{sample} is the absorbance of the sample with added DPPH. The IC_{50} values were obtained by plotting the DPPH scavenging percentage of each sample against the sample concentration. Data were analyzed using the *SigmaPlot* software (Version 12.0).

ABTS radical cation scavenging activity (TEAC assay)

Trolox equivalent antioxidant capacity of the samples was estimated toward $ABTS^{\bullet+}$ according to the procedure described by Re et al. [81] with slight modification. Moreover, 7 mM ABTS and 2.5 mM $K_2S_2O_8$ dissolved in 10 mL ultrapure water were incubated in the dark for 16 h at room temperature to create $ABTS^{\bullet+}$ free radical cation. Prior to the assay, the $ABTS^{\bullet+}$ solution was diluted with

Table 5. LC–MS/MS analysis results for *T. haussknechtii* extracts.

Rt	$[M-H]^-$	Fragments	Compound	Extract	Ref.
3.8	191	173, 127	Quinic acid	LM, CE, CW	[63]
3.8	499	353, 191, 173	*p*-Coumaroyl-Caffeoylquinic acid	CW	[64]
6.1	315	153, 108	Protocatechuic acid hexoside/Genistic acid hexoside	LM	[65]
6.3	341	179, 135	Caffeoyl glucose	HM	[66]
6.9	353	353, 191	3-Caffeoylquinic acid	HM, LM, CM, CE, CW	[67]
6.9	401	269, 161	Apigenin pentoside	HW, LW	[65]
7.4	387	369, 207, 163	Icariside b5/mediaresinol	HE, HW, LM, LW, CE, CW	[68]
8.4	479	317, 299, 271	Myricetin glucoside/quercetagetin glucoside	HM, CM	[69]
8.9	179	135	Caffeic acid	HW, LW, CM	[70]
9.1	609	301	Quercetin rutinoside	HM, CM	[71]
9.1	677	515, 497, 353, 179	Tricaffeoylquinic acid	HM, LM	[70]
9.2	351	333, 249	Unknown	HE, HW	
9.6	473	427, 221, 161	Unknown	HE, HW, LW, CW	
9.9	449	269, 207, 225	Apigenin derivative	LM	
10.0	349	183, 139	Methylgallate derivative	LW	
10.1	463	301 271	Quercetin glucoside	HM, LM, CM, CE	[69]
10.3	447	285, 151, 133	Luteolin glucoside	HM, HE, LM, CM, CE	[72]
10.4	493	331, 373, 316	Patuletin glucoside or methylmyricetin glucoside	HM, HE, CM, CE	[69,71]
10.7	593	285 255, 227	Luteolin rutinoside	HM, LM	[72]
11.0	577	269	Apigenin rutinoside	HM	[70]
11.5	477	462 315 299 271	Methylquercetin glucoside	HM, HE, LM, CE	[69]
11.3	515	353, 173, 335, 173	3,4-*O*-dicafeoylquinic acid	HM, LM, CM	[73]
11.8	515	191, 335 353, 135	1,3-*O*-dicafeoylquinic acid	HM, HE, LM, LE, CM, CE	[73]
11.8	288	270, 244 200	Unknown	CW	
12.4	515	353, 173, 173	4,5-*O*-Dicafeoylquinic acid	HM, LM, CM, CE	[73]
13.2	519	315, 357	Methylquercetin O-acetylglucoside	HM, CM, CE	[74]
13.5	639	315, 271, 161	Methylquercetin-O-hexoside-O-hexoside	HM	[75]
13.6	561	369, 351, 191, 173	Caffeoylquinic acid derivative	LW	[73]
14.1	515	353,191, 179, 173,	3,5-*O*-Dicafeoylquinic acid	HM,	[73]
14.4	367	193, 173, 134	3-Feruloylquinic acid	HM	[67]
16.1	426	333, 382, 163, 127	Coumaric acid derivative	HM, HE, LM, LE, CM	
16.4	447	285	Luteolin glucoside	HM	[72]
17.5	301	273, 179, 151	Quercetin	CM, CE, CW	[72]
17.5	285	133, 175, 151	Luteolin	HE, LM, LE, CM	[72]
17.8	315	300, 271, 228	Methylquercetin	HM, HE, LM, LE, CM, CE	[72]
18.3	460	378, 114	Unknown	HM	
19.5	785	665, 545, 421	Unknown C glucoside	HM, CM, CE	
19.5	327	309, 291, 229,211,	Unknown	HM, HE, HW, LM, LW, CE	
20.3	305	225, 207, 181	Unknown	HM, LW	
20.5	269	117	Apigenin	HM	[72]
20.9	299	284, 255, 137	Chrysoeriol	HE, LE	[76]
21.4	329	314, 299, 285, 271,199, 133	Unknown	HM, HE, LM, LE, CE	
22.6	359	344, 329, 314, 301, 285	Centaureidin/Jaceidin	HM, HE, LE	[77]
23.4	329	314, 299, 285, 271	Cirsiliol	HM, CM	[78]
23.6	299	271	Unknown	HM, CM	
24.8	313	298, 283, 255	Chrysoeriol methyl ether/Ermanin	HM, HE, LE, CM, CE	[79]

HM: Herb Methanol, **HE**: Herb Ethylacetate, **HW**: Herb Water, **LM**: Leaf and stem Methanol, **LE**: Leaf and stem Ethylacetate, **LW**: Leaf and stem Water, **CM**: Capitula Methanol, **CE**: Capitula Ethylacetate, **CW**: Capitula Water.

absolute ethanol to an absorbance 0.7–0.8 at 734 nm. The solutions of the EOs (30 mg/mL), the extracts (10 mg/mL), and Trolox (3.0; 2.0; 1.0; 0.5; 0.25; 0.125 mM) were prepared in MeOH. Moreover, 10 µL of the sample (EO/extract/standard) solution was mixed with 990 µL of $ABTS^{\bullet+}$ solution, and 10 µL of MeOH mixed with $ABTS^{\bullet+}$ solution was used as control. The mixtures were allowed to stand in the dark for 30 min at room temperature. Gallic acid (0.1 mg/mL) was used as positive control. Decrease in the absorbance was recorded at 734 nm to obtain the linear Trolox equation. Experiments were performed in triplicate. $ABTS^{\bullet+}$ scavenging activity of the samples was expressed as Trolox equivalent antioxidant capacity and calculated using linear equation obtained for Trolox ($y = 33.644x + 2.6523$, $r^2 = 0.9942$).

Lipid peroxidation inhibition (β-carotene bleaching test)

The lipid peroxidation inhibition of the samples was performed as described previously[82] with slight modification. Briefly, 1.2 mL of β-carotene solution (1 mg/mL) prepared in chloroform was mixed with Tween 20 (1200 mg) and linoleic acid (120 mg). Chloroform was evaporated fully under vacuum (at 40°C) and N_2. Then, 300 mL of oxygenated ultrapure water was added to the mixture and vigorously shaken. The emulsion obtained was freshly prepared before each experiment and stored in the dark. The solutions of the EOs (30 mg/mL), extracts (10 mg/mL), and BHT (standard, 1mg/mL) were prepared in MeOH. Moreover, 10 µL of the sample (EO/extract/standard) solution was mixed with 2 mL of β-carotene emulsion in 96-deep-well plate. In addition, 300 µL of this mixture was transferred to 96-well flat bottom plates, and the absorbance values were recorded (at 50°C) every 15 min of 120 min at 470 nm. Results were calculated according to Eq. (2):

$$\%AA = \left[1 - \frac{\left(Abs0_{sample} - Abs120_{sample}\right)}{Abs0_{control} - Abs120_{control}}\right] \times 100, \quad \text{(Eq 2)}$$

where AA is the antioxidant activity, $Abs0_{sample}$ and $Abs120_{sample}$ are the absorbance of the sample at 0 min and 120 min, $Abs0_{control}$ and $Abs120_{control}$ are the absorbance of the control at 0 min and 120 min.

Total phenol content

The total phenolic contents of the extracts were determined as gallic acid equivalent (GAE) using FC reagent using the method of Singleton.[83] Briefly, 50 µL of the sample, 3.9 mL ultrapure water, and 250 µL FCR were mixed in a test tube and vortexed. After 1–8 min incubation, 750 µL Na_2CO_3 (20%) was added to the mixture and vortexed again. The absorbance after 2 h at 25°C at 760 nm was compared with gallic acid calibration curve (y = 2.3503x+0.0347, r^2 = 0.9922). The experiment was performed in triplicate.

Total flavonoid content

The total flavonoid contents of the extracts were determined as quercetin equivalents (QE) using $AlCl_3$ reagent. [84] Briefly, 200 µL of the sample, 200 µL of $AlCl_3$ (2%), and 4.6 mL of absolute ethanol were mixed in tube. A blank consisting of 1 drop acetic acid instead of $AlCl_3$ was prepared for each sample as well. After 40 min at 25°C incubation, the absorbance was recorded at 415 nm. The total flavonoid content values were calculated by comparison with quercetin calibration curve (y = 2.6386x – 0.1381, R^2 = 0.9939).

Enzyme inhibitory activity

TLC assay for screening of ache inhibition

The anti-AChE activity assay on TLC plates described by Marston et al. [85] has been used for rapid screening of enzyme inhibition by plant EOs and extracts. The enzyme activity was detected by the conversion of naphthyl acetate into naphthol and the formation of the purple-colored diazonium dye with Fast Blue B salt. Inhibitors of AChE produced white spots on the dye-colored background of the TLC plate. Briefly, the plate was previously washed with acetone and dried to eliminate possible contaminants. Each sample was applied on the plate in three different amounts (EOs in 0.4, 0.1, 0.04 mg, the extracts in 0.1, 0.02, 0.005 mg) as spot, and the solvent was removed under nitrogen gas. After drying of the spots, the plate was sprayed with 2 mL of AChE solution (3.33 U/mL in Tris buffer, pH 8.0 with addition of BSA) using a TLC-sprayer. The plate with loaded samples was incubated for 20 min at 37°C in humid atmosphere. For this purpose, the Petri dish with water was also incubated in the oven. The inhibition of AChE activity was detected using the reagent consisting of freshly prepared mixture (1:4) of naphthyl acetate (2.5 mg/mL in abs. ethanol) and Fast Blue Salt (2.5 mg/mL in water). This reagent (4 mL) was sprayed onto the plate to give a purple coloration. The active samples were determined from the colorless areas occurred on purple ground (within 30 min).

Microtiter assay for determination of ache inhibition

Acetylcholinesterase (AChE) inhibition of the samples was evaluated using Ellman's method as previously reported [86] with slight modification. Three buffers were used: (**A**) 50 mM Tris-HCl (pH = 8.0, in ultrapure water); (**B**) 0.1% BSA in buffer A; (**C**) 0.1 M NaCl and 0.02 M $MgCl_2 \bullet 6H_2O$ in buffer A. In the 96-well flat bottom plates, 25 µL of the sample (EO/extract/standard), 50 µL of buffer B, and 25 µL of AChE (0.22 U/mL in buffer A) solution were pipetted with 8-channel automatic pipette (Eppendorf Research® plus, Germany) and incubated for 15 min at 25°C. Then, 125 µL of Ellman's reagent DTNB (3.0 mM in buffer C) and 25 µL of substrate ATCI (15 mM, in ultrapure water) were added. Hydrolysis of ATCI was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader (Biotek Powerwave XS, USA). The mixture was allowed to stand 15 min at 25°C, and the absorbance was recorded at 412 nm. Similarly, a blank (for eliminating the colors of the samples) was prepared by adding the sample solution to all reaction reagents and 25 µL of buffer instead of enzyme. The control wells contained all the reagents without the sample (the solvents of the samples instead were added). Galanthamine hydrobromide from *Lycoris* sp. (0.1 mg/mL) was used as positive control. The percentage inhibition was calculated according to Eq. (3):

$$\%Inh = \left[\frac{(Abs_{control} - Abs_{control\ blank}) - (Abs_{sample} - Abs_{sample\ blank})}{Abs_{control} - Abs_{control\ blank}}\right] \times 100 \qquad (\%\text{Eq } 3)$$

where $Abs_{control}$ and $Abs_{control\ blank}$ are the absorbance of the control and its blank, and Abs_{sample} and $Abs_{sample\ blank}$ are the absorbance of the sample and its blank. Data obtained from *in vitro* enzyme inhibition assays were expressed as the mean standard error (±SEM).

Tyrosinase inhibition

The tyrosinase inhibitory activity of the samples was measured using the modified dopachrome method with L-DOPA as substrate, as previously reported[87] with slight modifications. In 96-well flat bottom plates, 80 µL of sodium phosphate buffer (0.1 M, pH = 6.8), 40 µL of the sample (EO/extract/standard) solution, and 40 µL of tyrosinase (33 U/mL in buffer, pH = 6.8) were pipetted with 8-channel automatic pipette and incubated for 10 min at 23°C. The reaction was initiated with the addition of 40 µL of the substrate L-DOPA (2.5 mM in buffer) to the mixture. The absorbance was recorded at 475 nm after 15 min incubation at 23°C. The sample blanks contained all reaction reagents and 40 µL of buffer instead of enzyme. The tyrosinase inhibition activity of the samples was expressed as the kojic acid equivalent (KAE) and calculated using the linear equation obtained for kojic acid ($y = 1014.1X+11.798$, $r^2 = 0.9958$). Quercetin (1 mg/mL in methanol) was used as positive control.

α-Amylase inhibition

The effect of the samples on α-amylase was evaluated using the iodine/potassium iodide (I/KI) method. [88] Briefly, 25 µL sample solution and 50 µL α-amylase (0.8 U/mL in 20 mM of sodium phosphate buffer pH = 6.9) pipetted in 96-well flat bottom plates and incubated for 10 min at 37°C. The reaction was initiated with addition of the substrate solution (50 µL 0.05% soluble starch solution in ultrapure water). Then, the mixture was subjected to a second incubation for 10 min at 37°C. After incubation, the reaction was stopped with 25 µL of HCl (1 M). Finally, 100 µL of I/KI was pipetted to the wells. The sample blanks contained all reaction reagents and 50 µL of buffer instead of enzyme. The control wells contained all the reagents without the sample (the solvents of the samples instead were added). The percentage inhibition was calculated according to Eq. (4):

$$\%Inh = \left[\frac{(Abs_{control} - Abs_{control\ blank}) - (Abs_{sample} - Abs_{sample\ blank})}{Abs_{control} - Abs_{control\ blank}}\right] \times 100 \qquad (\text{Eq } 4)$$

where $Abs_{control}$ and $Abs_{control\ blank}$ are the absorbance of the control and its blank, and Abs_{sample} and $Abs_{sample\ blank}$ are the absorbance of the sample and its blank. The α-amylase inhibition activity of the samples was expressed as the acarbose equivalent (AE) and calculated using the linear equation obtained for acarbose ($y = 71.925X+41.653$, $r^2 = 0.993$).

Results and discussion

Our study aimed to elucidate further knowledge on the secondary metabolites of *T. haussknechtii* species and screened its volatile and non-volatile fraction for antioxidant, anti-AChE, anti-tyrosinase, and anti-α-amylase properties. The hydrodistillation of *T. haussknechtii* resulted with yellowish colored EOs with nice odor. The oil yields obtained from the leaf+stem and the capitula were 0.09% and 0.08%, respectively. Different parts of *T. haussknechtii* (herb, leaf+stem, capitula) were extracted with methanol, ethyl acetate, and water. The yields of the extracts are presented in Table 6. The GC–FID and GC/MS techniques allow us to determine qualitative and quantitative profiles of *T. haussknechtii* EOs. The list of detected volatile compounds with their relative percentages, retention indices, and method of identification is given in Table 4 in order of their elution on the HP-Innowax FSC column. The GC analysis of the leaf+stem and the capitula oils resulted with 60 and 56 constituents, representing 92.0% and 91.4% of the total oils, respectively. The EOs were characterized with high diversity of volatile constituents that were classified as mono- and sesquiterpene hydrocarbons and their oxygenated forms. The EOs from the leaf+stem and the capitula were characterized with high abundance of monoterpenes with α-pinene (14.5% and 12.3%, resp.), β-pinene (7.2% and 6.4%, resp.), santolinatriene (1.7% and 7.4%, resp.), and borneol (17.2% and 20.5%, resp.) as major constituents. In the literature, there are several reports about *Tanacetum* species with pinene and borneol as major volatiles: *T. argenteum* (Lam.) Willd. ssp. *argenteum* (L.) All., *T. argenteum* (Lam.) Willd. subsp. *canum* (C. Koch) Grierson var. *canum* (C. Koch) Grierson, *T. densum* (Lab.) Schultz Bip. ssp. *amani* Heywood, *T. densum* (Lab.) Schultz Bip. ssp. *amani* Heywood. [89–91]

A spectrophotometric evaluation of the extracts with FCR revealed that all the methanol extracts were characterized with high abundance of the phenols. The total phenols contents in the extracts ranged from 36.0 ± 0.46 to 5.94 ± 0.07 mg GAE/g extract. The total flavonoids contents in the extracts ranged from 30.44 ± 0.56 to 5.5 ± 0.14 mg QE/g extract. The extracts obtained with ethyl acetate from the capitula were found as rich with flavonoids. All results are given in Table 6.

LC–MS/MS analysis of *T. haussknechtii* extracts obtained with different polarity solvents resulted with identification of 47 phenolic compounds. Their mass spectral data are given in Table 5. According to LC–MS/MS results, the main compounds of the methanol extracts were 5-caffeoylquinic and dicaffeoylquinic acids (3,5/4,5) (Figure 1). An unknown C hexoside at m/z 785 $[M-H]^-$ was also determined in the herb and capitula methanol extracts. However, they were not detected in the leaf and stem extracts. That fact lets us to consider that the main source of this unknown C hexoside is the capitula. Beside 3,5-dicaffeoylquinic acid, quercetin and derivatives were also determined as dominant in ethyl acetate extracts. The water extracts were distinguished with considerably low amount of phenolic compounds. Representative LC–MS/MS chromatogram obtained for the methanol extract from *T. haussknechtii* herb is presented in Figure 2.

Antioxidant activity

Different antioxidant activity assays have been developed and applied for evaluation of antioxidative potential of natural products. Depending upon the reactions involved, the antioxidant activity assays can roughly be classified into two types: assays based on electron transfer (ET) and assays based on hydrogen atom transfer (HAT) reactions. For evaluation of *T. haussknechtii* antioxidant effects the both type assays were applied; ET: total phenols assay by Folin-Ciocalteu reagent, DPPH test, and Trolox equivalent antioxidant capacity assay; HAT: β-Carotene bleaching test. The results of *T. haussknechtii* EOs and extracts effects in different antioxidant activity assays are summarized in Table 7.

Table 6. Yield of extracts and essential oil, total phenols content, and total flavonoids content in *T. haussknechtii* extracts.

	Yield*				TPC**			TFC***		
	MeOH	EtAc	Water	EO	MeOH	EtAc	Water	MeOH	EtAc	Water
Herb	8.92	3.3	10.4	–	34.14 ± 0.7	16.59 ± 0.01	5.94 ± 0.07	12.82 ± 0.02	12.47 ± 0.08	5.5 ± 0.14
Leaf-stem	6.2	2.3	5.7	0.09	36.0 ± 0.46	14.78 ± 0.23	6.9 ± 0.19	10.0 ± 0.54	10.65 ± 0.06	5.99 ± 0.29
Capitula	8.4	2.6	5.44	0.08	34.82 ± 0.26	33.4 ± 0.35	16.61 ± 0.3	18.39 ± 0.27	30.44 ± 0.56	6.51 ± 0.39

* g extract/100g dried plant.
**Expressed as mg gallic acid equivalents in 1 g dried extract (GAE/g extr).
***Expressed as mg quercetin equivalents in 1 g dried extract (QE/g extr).

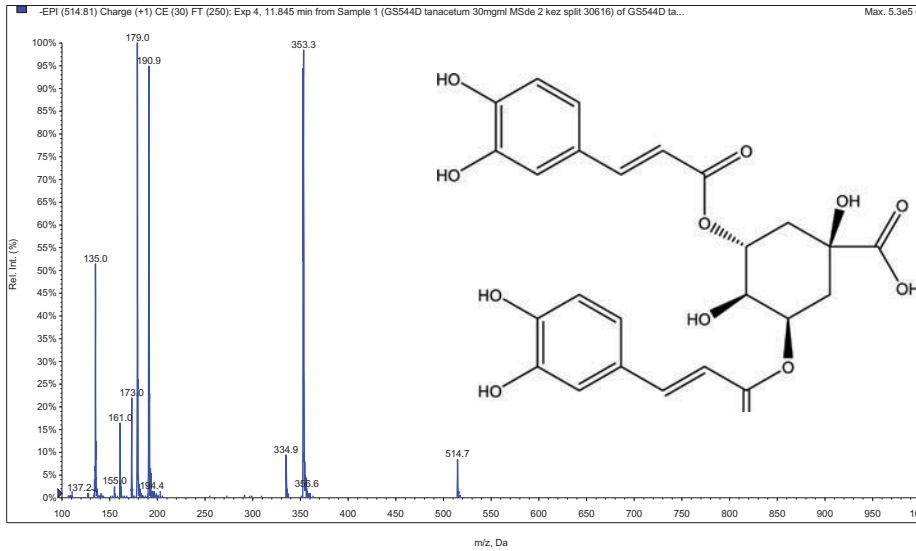

Figure 1. 3,5-Dicaffeoylquinic acid determined in the methanol and ethyl acetate extracts of *T. haussknechtii*.

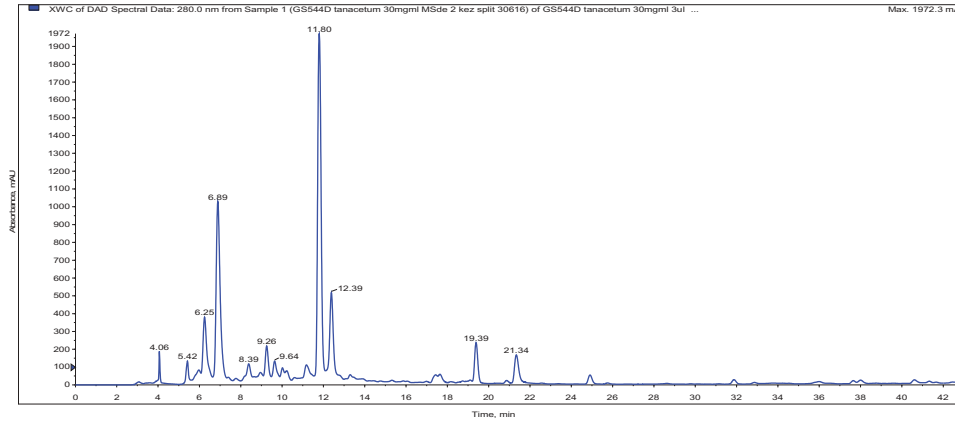

Figure 2. LC–MS/MS chromatogram of the methanol extract from the herb of *T. haussknechtii*. Description of the peaks is given in Table 5.

Free radical scavenging activity

The EOs and the extracts of *T. haussknechtii* were evaluated for free radical scavenging ability using DPPH reagent. The decrease in the absorbance at 517 nm induced by antioxidants in the samples was recorded and used for calculation of IC_{50} values. In DPPH assay, all the methanol extracts that are found as the richest ones in phenolics (34.14; 36.0; and 34.82 mg GAE/g extr.) exhibited the highest free radical scavenging activity (IC_{50} 0.12; 0.09; and 0.15 mg/mL). The extract obtained from the leaf+stem revealed the maximum activity with IC_{50} value 0.09 ± 0.01 mg/mL. The respective extracts obtained with ethyl acetate and water showed lower activity, and EOs were not active (Table 7).

Trolox equivalent antioxidant capacity

Antioxidant capacities of the EOs and the extracts were determined by their $ABTS^{\bullet+}$ free radical scavenging activity. The results were compared with Trolox and expressed as Trolox equivalents

Table 7. Antioxidant activity of *T. haussknechtii*[§] essential oils and extracts.

	DPPH, IC_{50}				TEAC, mM				β-Carotene Bleaching Test, %Inh			
	MeOH	EtAc	Water	EO	MeOH	EtAc	Water	EO	MeOH	EtAc	Water	EO
Herb	0.12 ± 0.01	0.48 ± 0.01	0.71 ± 0.02	NT	2.68 ± 0.16	1.78 ± 0.021	0.68 ± 0.03	NT	44.36 ± 4.68	24.45 ± 2.89	9.54 ± 0.8	NT
Leaf+Stem	0.09 ± 0.01	0.38 ± 0.02	0.74 ± 0.02	NA	2.64 ± 0.05	1.63 ± 0.03	0.73 ± 0.06	0.02 ± 0.005	46.89 ± 4.65	42.22 ± 2.27	14.65 ± 3.35	NA
Capitula	0.15 ± 0.002	0.2 ± 0.005	0.43 ± 0.02	NA	2.58 ± 0.14	2.64 ± 0.11	1.58 ± 0.22	0.1 ± 0.007	49.95 ± 4.35	53.68 ± 5.19	43.9 ± 4.83	NA
GA		0.001 ± 0.0001				2.89 ± 0.004				–		
BHT		–				–				58.45 ± 6.26		

[§]Values are the mean ± SD of triplicate samples from one experiment that is representative of three independent experiments;
NA not active; NT not tested.

(TE). ABTS$^{\bullet+}$ radical scavenging test resulted with superiority of all methanol extracts (2.68; 2.64; and 2.58 TE mM) and the capitula ethyl acetate extract (2.64 ± 0.11 TE mM). However, EOs did not demonstrate protection effect against lipid peroxidation in this test (Table 7).

Inhibition of lipid peroxidation

The effects of *T. haussknechtii* EOs and the extracts on lipid peroxidation were evaluated with the β-carotene/linoleic acid system. The β-carotene bleaching test uses a model lipid substrate (linoleic acid) in an emulsified form, and the method lies in between methods employing only model substrates (e.g., DPPH) and those using real lipids. Antioxidant activities of the samples were expressed as percent inhibition of lipid oxidation. EOs were not able to inhibit linoleic acid peroxidation at concentration 1.0%, but all the extracts obtained with MeOH and all the capitula extracts demonstrated significant inhibitory effect on the lipid peroxidation. In β-carotene bleaching assay, linoleic acid was the best protected by the capitula ethyl acetate (53.68 ± 5.19%) and methanol (49.95 ± 4.35%) extracts against the oxidative decomposition. The leaf+stem methanol extract also demonstrated noticeable protection effect (46.89 ± 4.65%) (Table 7).

In general, the alcohol extracts of *T. haussknechtii* demonstrated the highest antioxidant activity making the biggest contribution into antioxidant profile of the plant. However, the essential oil was found to be weak on scavenging of free radicals and inhibition of lipid peroxidation. Among the phenolic compounds detected in the extracts of *T. haussknechtii*, the caffeoylquinic acid derivatives and flavonoids are the major components. All these constituents have earlier been mentioned to have approved antioxidant activity. Actually, luteolin[92], quercetin[93], apigenin[94], caffeoylquinic acid derivatives[95], and caffeic acid[96] are important contributors into antioxidant status of the related extracts. In the literature, there are several reports about antioxidant properties of the above-mentioned phenols. [97,98]

Enzyme inhibition activity

Anti-acetylcholinesterase activity

The drugs approved and licensed for the treatment of neurodegenerative diseases inhibit AChE enzyme. Although, they belong to the same pharmacologically active group, they have different chemical structures: some of them possess some side effects. Besides, there is still no approved cure for the severe type of Alzheimer's disease. Therefore, there is still demand for discovery of new anti-Alzheimer drug candidates. In our study, screening of the samples for anti-acetylcholinesterase activity was performed using both the TLC rapid test and the spectrophotometric evaluation. In our experiments, the "dot-blot" type examination was performed for preliminary TLC-test of anti-AChE activity of the extracts and EOs. The TLC application was chosen, because it gives quick access to information concerning the activity of the samples examined. The test relies on the cleavage by acetylcholinesterase of 1-naphthyl acetate to form 1-naphthol, which in turn reacts with Fast Blue B salt to give a purple-colored diazonium dye. The preliminary test revealed that the water extracts of *T. haussknechtii* did not demonstrate inhibitory effect on AChE. Therefore, they were eliminated and was not included in the second TLC-test. The second TLC-test revealed that the methanol and ethyl acetate extracts and essential oils were found to have inhibitory effect on enzyme (Figure 3).

Microtiter plate assay was performed using Ellmans's reagent. Electric eel AChE was used as the enzyme source, while ATCI was employed as substrate of the reaction. The hydrolysis of ATCI was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at a wavelength of 412 nm utilizing a 96-well microplate reader. AChE enzyme inhibition effects were expressed as percent inhibition and compared with galanthamine hydrobromide. The capitula EO inhibited acetylcholinesterase activity (51.2 ± 2.5%) (Table 8). The EOs of *T. haussknechtii* were found to contain noticeable amounts of monoterpenoids with pinene and camphene skeletons, namely α-pinene (14.5–12.3%), β-pinene (7.2–6.4%), 1,8-cineole (3.6–3.1%) and borneol (17.2–20.5%). In a previous research, these constituents were found to be uncompetitive reversible inhibitors of AChE.[99] Therefore, anti-AChE

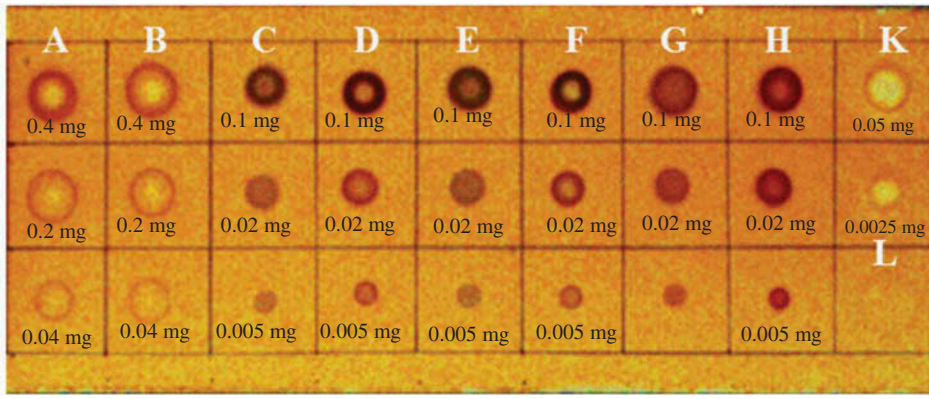

Figure 3. TLC-autography for screening of *T. haussknechtii* essential oils and extracts for anti-AChE activity. A: Stem+Leaf EO; B: Flower EO; C: Methanol extract of the herb; D: Ethyl acetate extract of the herb; E: Methanol extract of the stem+leaf; F: Ethyl acetate extract of the stem+leaf; G: Methanol extract of the flower; H: Ethyl acetate extract of the flower; K: Galanthamine; L: Galanthamine; M: Methanol (5 uL).

activity of the tested *T. haussknechtii* EOs may be related to the presence of these compounds. The extracts of *T. haussknechtii* demonstrated anti-AChE activity ranged between 19.2% and 37.1%. As resulted from LC-MS/MS analysis, the extracts contained caffeoylquinic acid and its derivatives as well as flavonoid constituents. These compounds have been earlier demonstrated to be cholinesterase inhibitors.[98]

Anti-amylase activity

Antidiabetic effects of the EOs and the extracts were estimated by their α-amylase enzyme inhibitory properties. Only extracts obtained with methanol and ethyl acetate showed noticeable inhibition of the enzyme. The capitula ethyl acetate extract was found to be the most inhibited (356.9 ± 0.06 mg AEs/g Extr) of α-amylase activity. However, the water extracts have no effect on α-amylase enzyme (Table 8). It should be considered that ethyl acetate extract of *T. haussknechtii* contained derivatives of caffeoylquinic acid and flavonoids. In fact, the above-mentioned compounds have earlier been reported to have antidiabetic effect. [100]

Anti-tyrosinase activity

Tyrosinase is a key enzyme in melanogenesis process as well as in pathogenesis of Parkinson's disease. Melanin is responsible for the protection against harmful ultraviolet irradiation, which can cause significant pathological conditions, such as skin cancers. Natural inhibitors of tyrosinase are in demand. Today, tyrosinase inhibitors from natural sources attract more attention owing to their safety, cost, and availability compared to chemically synthesized compounds. Different types of phenolics, flavonoids, stilbenes, chalkones, and hydroquinones have been reported as effective inhibitors of tyrosinase. Also, the literature search revealed reports about the effect of *Tanacetum parthenium* extracts on tyrosinase activity.[101] Considering the above mentioned, we investigated tyrosinase inhibitory activities (on L-DOPA) of *T. haussknechtii* EO and the extracts. The method described by Masuda with slight modifications was applied, and the activity was expressed as a well-known tyrosinase inhibitor kojic acid equivalents. The herb, capitula ethyl acetate extract, and the leaf+stem water extract possess the highest inhibitory effect (20.09–18.99 mg KAE/g extr) on tyrosinase. The extracts obtained with MeOH from the leaf+stem and capitula were not active (Table 8).

Table 8. Enzyme inhibitory activity of *T. haussknechtii*[§] essential oils and extracts.

	AChE, %Inh				α-Amylase*				Tyrosinase**			
	MeOH	EtAc	Water	EO	MeOH	EtAc	Water	EO	MeOH	EtAc	Water	EO
Herb	19.2 ± 2	23.1 ± 1.2	NA	NT	263.3 ± 0.05	305.6 ± 0.01	NA	NT	7.03 ± 0.002	20.09 ± 0.004	6.18 ± 0.004	NT
Leaf+Stem	20.6 ± 3.3	34.9 ± 3.7	NA	28.7 ± 2.5	320.7 ± 0.05	258.8 ± 0.03	NA	123.35 ± 0.02	NA	2.11 ± 0.001	24.27 ± 0.003	4.72 ± 0.001
Capitula	19.4 ± 1.2	37.1 ± 3.1	NA	51.2 ± 2.5	265.7 ± 0.04	356.9 ± 0.06	NA	157.38 ± 0,02	NA	18.99 ± 0.004	3.91 ± 0.002	9.27 ± 0.003
Galanthamine	100.0 ± 4.4				–				–			

[§] Values are the mean ± SD of triplicate samples from one experiment that is representative of three independent experiments; *Expressed as acarbose equivalents, (mg AEs/g); **Expressed as kojic acid equivalents (mg KAEs/g); NA: not active; NT: not tested.

Conclusion

The present work is the first investigation of *Tanacetum haussknechtii* essential oils and extracts obtained with different polarity solvents from different plant parts. We performed phytochemical investigations using GC–FID, GC/MS, LC–MS/MS, TLC, and spectrophotometric techniques. Based on biological activity assays, antioxidant properties and inhibitory effects of *T. haussknechtii* on acetylcholinesterase, α-amylase, and tyrosinase enzymes were investigated. The essential oils and extracts of the plant were found to contain valuable phytochemicals with significant biological activities. Our study is the first report about the antioxidant and inhibitory effects of this species against enzymes involved in important neurobiological, carbohydrate digestive, and skin pigmentation processes. The results provide strong scientific evidence for traditional uses of *Tanacetum* species. Finally, *T. haussknechtii* could be considered as the valuable source for isolation of active natural constituents for food supplements and therapeutic applications.

Declaration of interest

All authors of the manuscript declare that they do not have financial/commercial conflicts of interest.

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