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Antimicrobial Activity and Essential Oil Composition of a New *T. argyrophyllum* (C. Koch) Tvzel var. *argyrophyllum* Chemotype

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Abstract: Water-distilled essential oils from flowers and stems of *Tanacetum argyrophyllum* var. *argyrophyllum* from Turkey were analyzed by GC and GC/MS. The flower oil of *T. argyrophyllum* var. *argyrophyllum* was characterized with camphor 29.7%, borneol 12.0%, 1,8-cineole 8.4% and bornyl acetate 6.1%. Stem oil was characterized with camphor 26.6%, 1,8-cineole 17.5%, and borneol 15.0%. Our previous research and literature on the essential oil of this plant reported oils with high thujone content unlike the present study. Antibacterial activity of the oils were evaluated for five Gram-positive and five Gram-negative bacteria by using a broth microdilution assay. The highest inhibitory activity was observed against *Bacillus cereus* for stem oil (125 μ g/mL) when compared with positive control chloramphenicol it showed the same inhibition potency. However, the same oil showed lower inhibitory activity against *B. subtilis*. The flower oil did not show significant activity against the tested microorganisms. DPPH radical scavenging activity of the *T. argyrophyllum* var. *argyrophyllum* oil was investigated at 15 and 10 mg/mL concentrations. However, the oils did not show any significant activity when compared to positive control α-tocopherol. Both of the oils showed toxicity to *Vibrio fischeri* in the TLC-bioluminescence assay.

Key words: *Tanacetum argyrophyllum* var. *argyrophyllum*, Asteraceae, essential oils, camphor, borneol, 1,8-cineole, bornyl acetate, chemotypes, DPPH radical scavenging, antibacterial activity, *Vibrio fischeri* toxicity.

1 INTRODUCTION

Tanacetum argenteum is represented in Turkey with three varieties[1)]. Previous reports on *T. argyrophyllum* var. *argyrophyllum* revealed essential oil composition[2,3)] and chemistry[4)] of this plant. New sesquiterpene lactone tanargyrolide together with seven known compounds α-amyrin acetate, dihydro-1-carvone, 8α-hydroxyanhydrov erlotorin, tanachin, tabulin, isospiciformin and dentatin A were isolated from *T. argyrophyllum* var. *argyrophyllum*[4)]. In our previous investigation on the essential oils of this plant from Gumushane – Turkey we had reported the main composition of the flower oil as α-thujone 62.8%, and leaf oil as α-thujone 51.8% and 1,8-cineole 11.1%[2)]. Another investigation of this plant from Bingol-Turkey by a different research group reported the main essential oil composition as α-thujone 69.9% and β-thujone 5.6%[3)].

Comparison of main essential oil components of *T. argyrophyllum* var. *argyrophyllum* cited from previous reports together with the present data are given in Table 1.

As a part of our phytochemical and biological investigation of *Tanacetum* species, here we report on the essential oil composition and antibacterial, cytotoxic, radical scavenging properties of a new *T. argyrophyllum* var. *argyrophyllum* chemotype from Turkey.

2 EXPERIMENTAL

2.1 Plant materials

Plant material *T. argyrophyllum* var. *argyrophyllum* was collected during the flowering period in 20 July 2006 from Van – Edremit at 2001 m altitude. Voucher specimens have been deposited at the Herbarium of the Faculty of Science, Istanbul University (Voucher no. ISTE 83746), Turkey. Plant material was identified by Dr. Kerim Alpınar.

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Table 1 Comparison of % Main Essential Oil Components from Previous Reports of *T. argyrophyllum* var. *argyrophyllum* Together with Present Data

Literature → / Main Components ↓	[3]	[2] Fl.	[2] Lf.	A Fl.	B St.
1,8-Cineole	3.2	3.7	11.1	8.4	17.5
α-Thujone	69.9	62.8	51.8	–	tr
β-Thujone	5.6	4.3	4.7	–	–
Camphor	1.1	2.2	2.6	29.7	26.6
Bornyl acetate	–	–	–	6.1	3.3
Borneol	–	0.2	0,7	12.0	15.0

Fl. – Flower Oil
Lf. – Leaf Oil
St. – Stem Oil

2.2 Methods

2.2.1 Isolation of the essential oils

Flower and stems (100 g each) of the plant sample A and B from Van-Edremit location were separately subjected to hydrodistillation for 4 h using a Clevenger- type apparatus to produce the oils. Yellow colored oils were obtained in 0.95 % (A) and 0.6 % (B) (v/w) yields.

2.2.2 Essential oil analysis

The essential oil analyses were carried out simultaneously by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) systems.

2.2.2.1 Gas chromatography-mass spectrometry analysis

The GC-MS analysis was performed with an Agilent 5975 GC-MSD system with Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) and helium as carrier gas (0.8 mL/min). Oven temperature was programmed to 60℃ for 10 min. and raised to 220℃ at rate of 4℃/min. Temperature kept constant at 220℃ for 10 min. and then raised to 240℃ at a rate of 1℃/min. Mass spectra were recorded at 70 eV with the mass range *m/z* 35 to 450.

2.2.2.2 Gas chromatography analysis

The GC analyses were done with an Agilent 6890N GC system. FID detector temperature was set to 300℃ and same operational conditions applied to a duplicate of the same column used in GC-MS analyses. Simultaneous auto injection was done to obtain the same retention times. Relative percentage amounts of the separated compounds were calculated from integration of the peaks in FID chromatograms. The result of analysis is given in Table 2.

2.2.2.3 Identification of components

Identification of essential oil components were carried out by comparison of their retention times with authentic samples or by comparison of their relative retention indices (RRI) to series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS Library, Adams Library, MassFinder 2.1 Library)[26,27] and in-house "Başer Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data[28-30] was used for identification.

2.2.3 Antibacterial activity assay

Five gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermis* ATCC 12228, *Bacillus cereus* NRRL B-3711, *Bacillus subtilis* NRRL B-4378, Meticillin resistant *S. aureus* (Clinical isolate)) and five gram-negative bacteria (*Escherichia coli* NRRL B-3008, *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter aerogenes* NRRL 3567, *Proteus vulgaris* NRRL B-123, *Salmonella typhimurium* ATCC 13311) were used in this study. The minimum inhibitory concentration (MIC) values were determined for both of the oils, on each organism by using microplate dilution method[31]. Stock solutions of the oils (2 mg/mL) and standart antibacterial compound chloramphenicol were prepared in the liquid medium Mueller Hinton Broth (MHB, containing 25% DMSO - for solubility enhancement of the oil). Serial dilution of the initial concentrations was prepared on 96-well microlitre plates containing equal amounts of distilled water. Bacterial suspension concentrations were standardized to McFarland No:0.5 after incubation 24 h at 37℃ in MHB. Cultures were mixed with essential oils and were incubated 24 h at 37℃. Minimum inhibitory concentrations (MIC: µg/mL) were detected at the minimum concentration where bacterial growth was missing. 1% 2,3,5-Triphenyltetrazolium chloride (TTC, Aldrich St. Louis MO, USA) was used as an indicator of bacterial growth. Essential oil-free solutions were used as negative control and chloramphenicol was used as a positive control. All the experiments were performed in triplicate and means of results were given for the MIC values of the oils. The results of antibacterial activity test are given together with *Vibrio fischeri* toxicity in Table 3.

2.2.4 *Vibrio fischeri* cytotoxicity assay

5µL of 2mg/mL ethanol solutions of the essential oils were applied on HPTLC plates (Merck Darmstadt, GERMANY) by the help of Automatic TLC Sampler 4 (Camag

Table 2 Essential Oil Composition of A and B.

RRI	Name	A (%)	B (%)
1014	Tricyclene	0.3	0.3
1032	α-Pinene	2.9	2.4
1035	α-Thujene	tr	–
1076	Camphene	4.6	4.5
1093	Hexanal	tr	tr
1118	β-Pinene	1.3	1.3
1132	Sabinene	0.3	0.3
1135	Thuja-2,4(10)-diene	tr	tr
1176	α-Phellandrene	tr	–
1188	α-Terpinene	0.1	0.2
1195	Dehydro 1,8-cineole	0.1	0.1
1195	Iso amyl isobutyrate	0.1	–
1203	Limonene	0.4	0.3
1213	1,8-Cineole	8.4	17.5
1220	*cis*-Anhydrolinalool oxide	tr	–
1255	γ-Terpinene	0.2	0.5
1280	p-Cymene	0.7	1.2
1285	Iso amyl isovalerate	tr	–
1290	Terpinolene	0.1	0.2
1299	2-Methylbutyl-3-methyl butyrate	0.1	tr
1355	1,2,3-trimethyl benzene	0.1	0.1
1430	α-Thujone	–	tr
1439	γ-Campholene aldehyde	tr	tr
1445	Filifolone	–	0.1
1452	α,*p*-Dimethyl styrene	tr	tr
1450	*trans*-Linalool oxide (Furanoid)	0.4	–
1474	*trans*-Sabinene hydrate	0.1	0.3
1479	Linalool-7-oxide-3-one	0.3	0.1
1497	α-Copaene	–	tr
1499	α-Campholene aldehyde	0.1	tr
1522	Chrysanthenone	0.4	0.2
1532	Camphor	29.7	26.6
1538	*trans*-Chrysanthenyl acetate	–	1.0
1547	Dihydro achillene	0.1	0.1
1553	Linalool	0.6	tr
1556	*cis*-Sabinene hydrate	–	0.3
1568	1-methyl-4-acetylcyclohex-1-ene	0.1	0.2
1571	*trans*-*p*-Menth-2-ene-1-ol	0.1	0.2
1582	*cis*-Chrysanthenyl acetate	4.8	–
1586	Pinocarvone	–	1.0
1588	Bornyl formate	tr	tr
1591	Bornyl acetate	6.1	3.3
1600	Isophorone	tr	–
1611	Terpinene-4-ol	1.1	1.9
1612	β-Caryophllene	–	0.3
1617	Lavandulyl acetate	1.3	–
1638	*cis*-*p*-Menth-2-ene-1-ol	0.1	0.2
1648	Myrtenal	0.3	0.2
1642	*cis*-Linalooloxide acetate	0.4	–
1651	Sabina ketone	–	tr
1670	*trans*-Pinocarveol	–	0.8
1682	δ-Terpineol	tr	0.3
1683	trans-Verbenol	–	0.3
1686	Lavandulol	1.7	–
1706	α-Terpineol	0.7	1.1
1719	Borneol	12.0	15.0
1725	Verbenone	tr	–
1722	Cabreuva oxide II	0.2	0.1
1751	Carvone	tr	–
1758	*cis*-Piperitol	0.1	tr
1764	*cis*-Chrysanthenol	2.0	3.0
1768	Cabreuva oxide IV	0.2	tr
1793	α-Campholene alcohol	2.4	2.3
1804	Myrtenol	0.2	0.2
1811	*trans*-*p*-Mentha-1(7),8-diene-2-ol	0.1	0.1
1838	(E)-β-Damascenone	–	tr
1845	*trans*-Carveol	0.3	0.2
1857	Geraniol	0.1	–
1864	p-cymene-8ol	0.1	0.1
1896	*cis*-*p*-Mentha-1(7),8-diene-2-ol	0.1	0.1
1904	Ethyl-3-phenyl propionate	–	0.1
1945	1,5-Epoxy salvial-4(14)-ene	–	0.1
1958	(E)-β-Ionone	–	tr
1969	cis-Jasmone	–	0.1
1988	2-Phenylethyl-2-methylbutyrate	tr	–
2008	Caryophyllene oxide	0.2	0.4
2016	Isoamyl phenyl acetate	0.1	–
2037	Salvial-4(14)-ene-1-one	tr	0.1
2050	(E)-Nerolidol	0.1	1.4
2056	13-Tetradecanolide	–	0.1

RRI	Name	A (%)	B (%)
2071	Humulene epoxide II	–	tr
2073	*p*-Mentha-1,4-diene-7-ol	tr	0.1
2074	Caryophylla-2(12),6(13)-diene-5-one	tr	0.1
2113	Cumin alcohol	–	0.1
2144	Spathulenol	0.5	0.9
2183	(E)-Sesquilavandullol	0.5	–
2186	Eugenol	–	tr
2214	Phenyl ethyl tiglate	tr	tr
2247	*trans*-α-Bergamotol	–	0.1
2278	Torilenol	0.2	tr
2257	β-Eudesmol	0.5	0.6
2298	Decanoic acid	0.1	–
2316	Caryophylladienol I	0.3	0.1
2324	Caryophylladienol II	0.4	0.4
2300	Tricosane	tr	–
2389	Caryophyllenol I	0.1	0.1
2369	Eudesma-4(15),7-diene-1β-ol	0.1	tr
2384	1-Hexadecanone	0.1	0.1
2392	Caryophyllenol II	tr	0.1
2500	Pentacosane	0.1	–
2607	1-Octadecanol	–	0.1
2622	Phytol	–	0.1
2700	Heptacosane	0.1	–
2857	Palmito-γ-lactone	–	tr
2900	Nonacosane	tr	–
2931	Hexadecanoic acid	0.2	0.6
Monoterpenes		10.9	11.2
Oxygenated Monoterpenes		74.3	77.0
Sesquiterpenes		0.1	0.4
Oxygenated Sesquiterpenes		3.3	4.5
Others		0.9	1.2
Total		89.5	94.3

RRI: Relative Retention Indices
tr: Trace (<0.1%)
A: Tanacetum argyrophyllum var. argyrophyllum – Flower Oil
B: Tanacetum argyrophyllum var. argyrophyllum – Stem Oil

Muttenz,Switzerland). Freeze-dried, luminescent *Vibrio fischeri* microorganisms obtained from the kit were inoculated on the medium provided by the kit(ChromadexTM Irvine CA, USA). Culture of the microorganism was incubated for 24-30 hours at 28℃. Previously prepared HPTLC plates were dipped into the freshly grown luminescent culture with an automatic immersion device(Camag Muttenz,Switzerland)and excess of the culture removed from the plates with a squeegee. Plates were photographed at −30℃ with CCD camera of BioLuminizer(Camag Muttenz,Switzerland). Cytotoxicity of the oils were detected as black spots on the photographs[32]. The results of *Vibrio fischeri* toxicity activity test are given together with antibacterial activity results in Table 3.

2.2.5 DPPH radical scavenging activity assay

Antioxidant activities of the oils were determined with DPPH radical protocol[33]. A modified protocol for HPTLC-DPPH[34]was used. Stock solutions of the oils(10 and 15 mg/mL), positive control α-tocopherol(Aldrich, St. Louis MO, USA)(10 and 15 mg/mL)and DPPH(0.1 mM)(Aldrich, St. Louis MO, USA)were prepared with CH_3OH. 200 μL of the oil solutions were mixed with 1000 μL of DPPH solution as well as positive controls and essential oil free blank controls in 1.5 mL Eppendorf tubes and vortexed for 2 min. After incubating all the samples and controls for one hour in dark at room temperature, 2 μL of them were applied on an aluminium 60 F254 TLC Plate(Merck Darmstadt, GERMANY)with 5 mm band length by the help of Linomat 5 TLC applicator system(Camag Muttenz, Switzerland). After preparing samples and controls on the TLC; plates were scanned at 517 nm with a TLC Scanner 3(Camag Muttenz, Switzerland)and absorbance of the bands were detected. Percent of DPPH scavenging property was calculated according to % DPPH Scav. Prop. = [(AControl-ASample)/AControl] × 100 formula. The results of antioxidant property activity test is given in Table 4.

3 RESULTS

Essential oil compositions of *T. argyrophyllum* var. *argyrophyllum* flowers(A)and stems(B)are given in Table 2. Eighty three compounds were identified representing 89.5 % of flower(A)oil. Camphor 29.7%, borneol 12.0%, 1,8-cineole 8.4% and bornyl acetate 6.1% were found to be main components of the oil A. Eighty five compounds were identified representing 94.3% of stem(B)oil. Camphor 26.6%, 1,8-cineole 17.5% and borneol 15.0% are found to be main components of oil B. Previous reports indicate *Tanacetum* essential oils with high amounts of camphor, borneol and 1,8-cineole[5-11]. However previous reports on essential oil composition of *T. argyrophyllum* var. *argyrophyllum* are different than the present results. Previously reported *T. argyrophyllum* var. *argyrophyllum* oil had a high content of α-thujone but it did not contain camphor, borneol and bornyl acetate in high amounts[2-3]. 1,8-Cineole is present in all of the oils both in present study and previous reports. However thujone is completely missing in the present study except in the stem oil where it is present in trace amount.

Table 3 Antibacterial Activity (MIC, μg/mL) and Vibrio fischeri Toxicity of the Oils A and B.

Microorganism	A (μg/mL)	B (μg/mL)	+ C. (μg/mL)
Staphylococcus aureus	500	500	62.5
Methycillin resistant S. aureus	125	500	62.5
Staphylococcus epidermis	500	500	31.2
Bacillus cereus	500>	125	125
Bacillus subtilis	125	500	62.5
Escherichia coli	500	500	62.5
Pseudomonas aeruginosa	500>	500	31.2
Enterobacter aerogenes	250	500	62.5
Proteus vulgaris	500>	250	62.5
Salmonella typhimurium	250	500	125
Vibrio fischeri	Toxic	Toxic	N.A.

A: *Tanacetum argyrophyllum* var. *argyrophyllum* – Flower Oil
B: *Tanacetum argyrophyllum* var. *argyrophyllum* – Stem Oil
+ C. :Positive Control (chloramphenicol)
N.A. : Not Available

Table 4 DPPH Scavenging Evaluation of Oil A and B.

Concentration	A (%) *	B (%) *	α-tocopherol (%) *
15 mg/mL	12.7±4.10	13.7±2.30	94.6±0.96
10 mg/mL	3.8±2.26	5.3±3.36	94.5±0.79

A: Tanacetum argyrophyllum var. argyrophyllum – Flower Oil
B: Tanacetum argyrophyllum var. argyrophyllum – Stem Oil
* Results are given in means of three parallel experiments with S.D.

T. argyrophyllum var. *argyrophyllum* stem oil (B) showed the same level of activity against the food pathogenic bacteria *Bacillus cereus* (125 μg/mL) when compared to the positive control chloramphenicol (125 μg/mL). Similarly flower oil (A) showed relatively mild inhibitory activity against *B. subtilis* (125 μg/mL), and methycillin resistant *S. aureus* (125 μg/mL) when compared to the positive control chloramphenicol (62.5 μg/mL). However stem oil (B) oil did not show any significant activity to the other microorganisms tested in this series suggesting its selectivity. *B. cereus* is a food pathogen which is responsible for some of the food poisoning diseases by its ability to produce enterotoxin[12,13]. Flower and stem oils showed toxicity to *Vibrio fischeri* with HPTLC-*Vibrio fischeri* toxicity assay which is used to evaluate possible general toxicity of the oils as an initial indicator. The toxicity was observed at low concentrations when compared to vitamin C.

DPPH radical scavenging activity was observed on both flower (A) and stem (B) oils with 15 mg/mL and 10 mg/mL concentration. Both oils showed insignificant DPPH scavenging activity when compared to the positive control α-tocopherol in both concentrations.

4 DISCUSSION

There are many examples of chemovariation in the essential oils of *Tanacetum* species which is very well documented especially for *T. vulgare*[14-18]. In recent years chemovariation in other *Tanacetum* species are also reported such as chemovariation observed in *T. chiliophyllum* var. *chiliophyllum*, *T. nubigenum* and *T. cadmeum* ssp. *orientale* [6,7,10,19-23]. Previously essential oils of *T. argyrophyllum* var. *argyrophyllum* with high content of α-thujone were reported however to the best of our knowledge there is no previous report on the oil of this species with high content of camphor, borneol and 1,8-cineole. Borneol is produced in plants by carbocationic rearrangement of

α-terpinyl cation and quenching the resultant bornyl cation with water. Folding of the α-terpinyl cation in this rearrangement is achieved by special enzymes. Camphor is produced by enzymatic oxidation of borneol. However α-thujone is produced by rearrangement of terpinen-4-yl cation to thujyl cation continued with further oxidation, reduction steps [24-25]. Absence of thujone in the present study together with low content of borneol and camphor in previous studies suggests these differences are observed due to different chemovarieties of the plant. As explained before the formation of camphor, borneol and α-thujone have different biosynthetic pathways which employ special enzymes in the process. This suggests differences observed in this study could be related to variances in plants DNA or regulation of the enzymes which are responsible for formation of these compounds. Only significant antibacterial activity is observed on stem oil (B) against *B. cereus* when compared to positive control chloramphenicol. Both of the oils showed insignificant DPPH-scavenging activity. Both oils showed toxicity to *Vibrio fischeri* when compared to positive control Vitamin C.

5 CONCLUSIONS

Essential oil compositions of *T. argyrophyllum* var. *argyrophyllum* flower and stem oils from Van-Turkey were investigated. Camphor, borneol and 1,8-cineole rich oils were observed unlike the previous literature. The differences encountered in our previous research and literature on this plant suggested chemovariation in this plant. However comparison of DNA profiles of this plant from various locations could prove existence of chemotypes and determine further variation in *T. argyrophyllum* var. *argyrophyllum*.

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