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Anthemis wiedemanniana essential oil prevents LPS-induced production of NO in RAW 264.7 macrophages and exerts antiproliferative and antibacterial activities *in vitro*

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Anthemis wiedemanniana is known in folk medicine for the treatment of microbial infections, cancer and also urinary and pulmonary problems. In this study, the chemical composition of the essential oil from *A. wiedemanniana* was evaluated and its antibacterial activity was tested against 10 bacterial strains. The oil was also tested for its potentiality to inhibit nitric oxide production in RAW 264.7 macrophages and for its cytotoxicity against four human cancer cell lines. *A. wiedemanniana* oil, rich of oxygenated monoterpenes (25.4%), showed a good antibacterial activity against Gram-positive bacteria and a good activity against the two Gram-negative bacteria, *Escherichia coli* and *Proteus vulgaris*. Besides that, it exhibited a high inhibitory effect on the LPS-induced nitrite production and a strong cytotoxic activity, especially against amelanotic melanoma (C32) and large lung cell carcinoma (COR-L23) cell lines.

Keywords: *Anthemis wiedemanniana*; essential oil; antimicrobial activity; inhibition of NO production; antiproliferative activity

1. Introduction

The genus *Anthemis* L., tribe Anthemideae and subtribe Anthemidinae, is one of the most important genera of the Asteraceae family and comprises about 210 species (Fernandes, 1976). The genus is represented in the flora of Turkey by 81 taxa belonging to 51 species (Güner, Özhatay, Ekim, & Baser, 2000). Previous chemical studies seem to indicate that sesquiterpene lactone patterns are systematically important within the genus (Çelik et al., 2005).

Anthemis wiedemanniana Fisch. & C.A. Meyer (section *Cota*) is distributed in the Middle and the Eastern Anatolian regions of Turkey (Güner et al., 2000) where it is known as 'Papatya', a common name given to plants whose flowers resemble those of Roman and German chamomile (Strid & Tan, 1999). Infusions are used in Turkish Traditional medicine, especially for treating abdominal pain and as a folk remedy for cough and cold (Honda et al., 1996; Teixeira Da Silva, 2004), and the aerial parts are used in many commonly consumed beverages. Recently, the essential oil of *A. wiedemanniana* from

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Turkey and its antimicrobial activity has been studied (Kivcak et al., 2007). In this article, we investigate its anti-inflammatory, cytotoxic and antimicrobial potential. To our knowledge, no studies on cytotoxicity and anti-inflammatory activity of *A. wiedemanniana* have been reported so far.

2. Results and discussion

Hydrodistillation of the dry aerial parts of *A. wiedemanniana* yielded 0.13% w/v of a light yellowish oil. Fifty-six components were identified (Table 1), representing 92.7% of the total oil, which was characterised by a similar amount of oxygen containing monoterpenes (25.4%) and fatty acids and esters (24.2%) followed by oxygen-containing sesquiterpenes (10.1%). The presence of irregular monoterpenes (5.9%), such as *cis*-chrysanthenol (3.2%) and *cis*-chrysanthenyl acetate (1.9%) was noteworthy. The presence of hexahydrofarnesyl

Table 1. Essential oil composition of *A. wiedemanniana*.

K_i[a]	K_i[b]	Component	Identification[c]	(%)[d]
970	1243	2-Pentyl furan	R_i, MS	0.5
998		Yomogi alcohol	R_i, MS	0.1
1024	1280	*p*-Cymene	R_i, Co-GC, MS	0.4
1034	1213	1,8-Cineole	R_i, Co-GC, MS	6.2
1048	1663	Phenyl acetaldehyde	R_i, Co-GC, MS	0.7
1074	1450	*cis*-Linalool oxide (furanoid)	R_i, MS	0.8
1087	1455	*trans*-Linalool oxide (furanoid)	R_i, MS	1.0
1096	1474	*trans*-Sabinene hydrate	R_i, MS	1.1
1102	1553	Linalool	R_i, Co-GC, MS	3.8
1116	1449	β-Thujone	R_i, MS	2.1
1120	1638	*cis*-*p*-Menth-2-en-1-ol	R_i, MS	0.5
1125	1540	Chrysanthenone	R_i, MS	0.7
1138	1664	*trans*-Pinocarveol	R_i, MS	0.8
1154	1587	Pinocarvone	R_i, MS	0.5
1161	1455	*cis*-Chrysanthenol	R_i, MS	3.2
1175	1611	Terpineol 4	R_i, Co-GC, MS	2.3
1180	1864	*p*-Cymen-8-ol	RI, MS	2.2
1189	1706	α-Terpineol	R_i, Co-GC, MS	1.8
1201		Safranal	R_i, MS	0.2
1205	1723	Verbenone	R_i, MS	1.0
1219	1845	*trans*-Carveol	R_i, MS	0.5
1257	1582	*cis*-Chrysanthenyl acetate	R_i, MS	1.9
1291	2471	Indole	RI, Co-GC, MS	0.3
1301	2240	Carvacrol	R_i, Co-GC, MS	5.8
1329	1948	Piperitenone	R_i, MS	0.6
1343		β-Maaliene	R_i, MS	1.2
1355	2186	Eugenol	R_i, MS	2.5
1453	1673	(*E*)-β-farnesene	R_i, MS	0.6
1484	1957	β-Ionone	R_i, Co-GC, MS	0.2
1485	2354	Dihydroactinidiolide	R_i, MS	2.7
1542	2047	Elemol	R_i, MS	0.4
1575	2150	Spathulenol	R_i, MS	2.0
1580	2008	Caryophyllene oxide	R_i, Co-GC, MS	1.3
1608	2098	β-Oplopenone	R_i, MS	0.6
1635	2187	T-cadinol	R_i, MS	0.4
1642	2208	α-Muurolol	R_i, MS	0.9
1651	2255	α-Cadinol	R_i, MS	1.8
1653	2257	β-Eudesmol	R_i, MS	2.6
1711		Pentadecanal	R_i, MS	0.6
1758	2672	Tetradecanoic acid	R_i, Co-GC, MS	1.1
1843	2131	Hexahydrofarnesyl acetone	R_i, MS	8.3
1969	2931	Hexadecanoic acid	R_i, Co-GC, MS	10.5

(*continued*)

Table 1. Continued.

K_i[a]	K_i[b]	Component	Identification[c]	(%)[d]
2093		9,12-Octadecadienoic acid methyl ester	R_i, Co-GC, MS	0.4
2122	3157	9,12-Octadecadienoic acid	R_i, Co-GC, MS	12.2
		Total		92.7
		Irregular monoterpenes		5.9
		Monoterpene hydrocarbons		0.4
		Oxygen-containing monoterpenes		25.4
		Sesquiterpene hydrocarbons		1.8
		Oxygen-containing sesquiterpenes		10.1
		Hydrocarbons		3.2
		Carbonylic compounds		9.8
		Fatty acids and esters		24.2
		Phenols		8.3
		Others		3.6

Notes: [a]retention indices relative to C_8–C_{24} alkanes on the HP-5 MS column.
[b]Linear retention index on a HP Innowax column.
[c]R_i, retention index; Co-GC, co-injection with authentic compound; and MS, mass spectrum.
[d]*t*, trace, less than 0.05%.

Table 2. MIC values ($\mu g\,mL^{-1}$) of the essential oil from aerial parts of *A. wiedemanniana* and MIC of the reference antibiotic.

Bacterial strain	MIC	MBC	*G*
Gram-positive			
Bacillus cereus (ATCC 11778)	50	50	1.56
Bacillus subtilis (ATCC 6633)	25	50	1.56
Staphylococcus aureus (ATCC 25923)	12.5	12.5	3.12
Staphylococcus epidermidis (ATCC 12228)	6.25	12.5	6.25
Streptococcus faecalis (ATTC 29212)	50	100	>100
Gram-negative			
Escherichia coli (ATCC 25922)	50	50	3.12
Proteus mirabilis (ATCC 25933)	100	100	100
Proteus vulgaris (ATCC 13315)	50	100	100
Pseudomonas aeruginosa (ATCC 27853)	>100	>100	12.5
Salmonella typhi Ty2 (ATCC 19430)	100	>100	>100

Note: G, Gentamicyne (MIC).

acetone (8.3%) that contributes to the pleasant smell of the oil with its fresh jasmine floral celery odour was also interesting. The fatty acid fraction of the oil was essentially constituted by 9,12-octadecadienoic acid (12.2%) and hexadecanoic acid (10.5%). Our results present some similarities with data obtained by Kivcak et al. for the essential oil of *A. wiedemanniana* collected in Izmir-Bozdag (Turkey) on May 2003. Particularly, the main constituents are the same [(linalool (12.8%), 1,8 cineole (8.5%), hexadecanoic acid (6.1%)], even if their percentages are different. The essential oil from *A. wiedemanniana* was assayed for its *in vitro* antibacterial activity using the broth dilution method (Barry, 1976) towards 10 bacterial strains (Table 2). Our data show that the sample was more active against Gram-positive bacteria, particularly against *Staphylococcus epidermidis* (minimum inhibitory concentration, MIC = $6.25\,\mu g\,mL^{-1}$) and *Staphylococcus aureus*

(MIC = 12.5 μg mL^{-1}). *Escherichia coli* and *Proteus vulgaris* were the most affected Gram-negative bacteria (MIC = 50 μg mL^{-1}). When these data are considered together with the composition of the essential oil, it appears likely that the antibacterial activity is due to the presence of carvacrol and 1,8-cineole, but it may also be linked to other oxygen-containing compounds such as linalool and eugenol, present in appreciable amounts in the oil (Dorman & Deans, 2000).

Nitric oxide (NO) is recognised as a mediator and regulator in pathological reactions, especially in acute inflammatory responses (Surh et al., 2001). High levels of NO cause a variety of pathophysiological processes, including inflammation (MacMicking, Xie, & Nathan, 1997) and carcinogenesis (Ohshima & Bartsch, 1994). To evaluate the inhibition of NO production activity of *A. wiedemanniana* essential oil, the murine monocytic macrophage cell line RAW 264.7 was employed as a model for inhibition of inflammation mediators, such as NO. Indomethacin was used as a reference drug. It is well characterised that the treatment of RAW 264.7 macrophages with LPS (1 μg mL^{-1}) for 24 h induces NO production (26.4 μM from the basal level of 0.6 μM) (Y.H. Kim, J. Kim, Park, & H.P. Kim, 2007), as assessed by measuring the accumulation of nitrite, a stable metabolite of NO, in the media based on Griess reaction. As shown in Figure 1, incubation of RAW 264.7 cells with the essential oil of *A. wiedemanniana* induced a significant inhibitory effect on the LPS-induced nitrite production. At 200 μg mL^{-1}, the essential oil showed 93% inhibition of LPS-induced NO production in RAW 264.7 cells. This result is important if compared to the reference drug indomethacin which showed 95% inhibition at the same concentration. The essential oil showed an IC_{50} value more significant than reference drug indomethacin (41.2 and 52.8 μg mL^{-1}, respectively) and it did not show any cytotoxicity on RAW 264.7 cells at tested concentrations (data not shown). This activity may be due to the presence of monoterpenes: different previous studies demonstrated that essential oils and some constituents, such as 1,8-cineole, terpinen-4-ol, α-terpineol and linalool, exert anti-inflammatory activity (Loizzo et al., 2009).

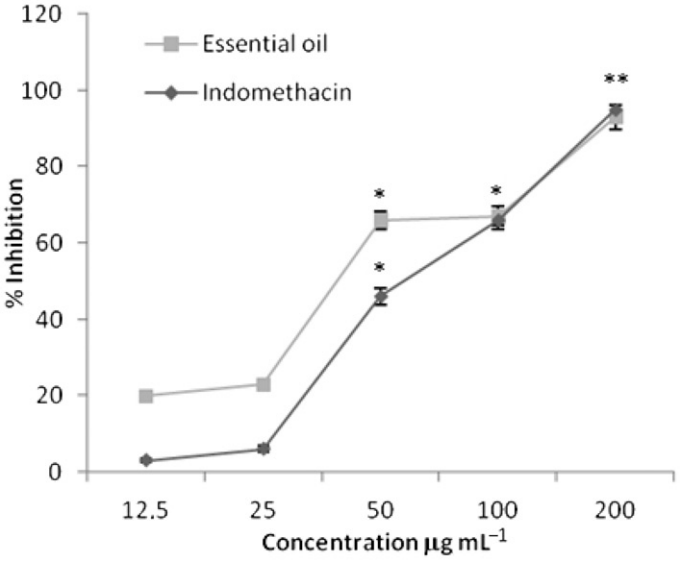

Figure 1. Inhibition of NO production in LPS-induced RAW 264.7 macrophages by *A. wiedemanniana* essential oil. Data are expressed as mean ± SD ($n = 3$), $p < 0.05$.

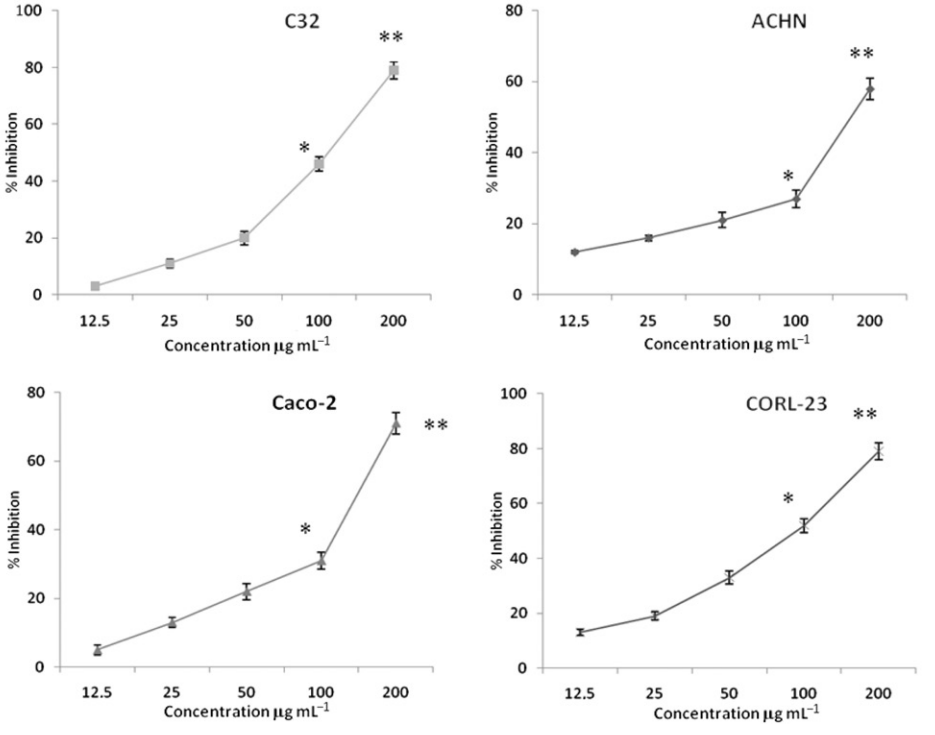

Figure 2. Cytotoxic activities against human tumour cell lines of the essential oil obtained from *A. wiedemanniana*. Exposure time 48 h. Data are expressed as mean ± SD ($n = 3$), $^{*}p < 0.05$, $^{**}p < 0.01$. ACHN: renal cell adenocarcinoma; C32: amelanotic melanoma cells; COR-L23: large lung cell carcinoma; Caco-2: colorectal adenocarcinoma. Vinblastine (IC_{50} of 2 µg mL^{-1}) was used as the positive control.

The essential oil of *A. wiedemanniana* was finally evaluated for its *in vitro* cytotoxic properties on four human cancer cell lines (amelanotic melanoma C32, large lung cell carcinoma COR-L23, colorectal adenocarcinoma Caco-2 and renal cell adenocarcinoma ACHN) by MTT test. After 48 h of treatment, the cytotoxicities of the essential oil were determined. The cytotoxic effects of the essential oil on the growth of human tumour cell lines are given in Figure 2, which shows the percentage of inhibition of cell growth *versus* the concentration of essential oil. The oil showed strong *in vitro* cytotoxic properties on COR-L23 and C32 cell lines (79% of inhibition of proliferation at 200 µg mL^{-1}). Concentrations of oil for which each cell line's growth was inhibited by 50% (GI_{50}) were calculated from the curve. GI_{50} values obtained were 93.20 ± 2.87 µg mL^{-1} for COR-L23, 108.07 ± 2.98 µg mL^{-1} for C32, 155.82 ± 3.35 µg mL^{-1} for Caco-2 and 179.02 ± 3.51 µg mL^{-1} for ACHN. Different compounds that proved to be active on tumour cells are present in our oils, such as carvacrol (Ait M'Barek et al., 2007), palmitic acid (Harada et al., 2002) and 1,8-cineole (Moteki et al., 2002).

3. Experimental

3.1. *Plant material*

Aerial parts of *A. wiedemanniana* Fisch. & C.A. Meyer were collected in June 2007 at the full flowering stage in Eskisehir, Sögüt near Oluklu Village (Turkey), 980 m a. s. l. Samples

are deposited at the Department of Biology, Çanakkale Onsekiz Mart University with voucher No. Çelik 2200-2205.

3.2. *Isolation of the essential oil*

The oil from air-dried and ground aerial parts of the plant was isolated by hydrodistillation for 3 h using a Clevenger-type apparatus, according to the method described earlier (Cardile et al., 2009). The oil was dried over anhydrous sodium sulphate and stored under N_2 at +4°C in the dark until tested and analysed. The sample yielded 0.13% of yellowish oil (on a dry mass) with a pleasant smell.

3.3. *GC and GC–MS analysis*

Analytical gas chromatography was carried out on a Perkin Elmer Sigma 115 gas chromatograph fitted with an HP-5 MS capillary column (30 m × 0.25 mm), 0.25 μm film thickness, as described previously (Tenore et al., 2011).

3.4. *Components identification*

Most of the constituents were identified by gas chromatography by comparing their retention indices (*I*) with those of the literature (Davies, 1990; Jennings & Shibamoto, 1980) or with those of authentic compounds available in our laboratories. The retention indices were determined following (Adams, 2007; Tenore et al., 2011). These results are shown in Table 1, where the compounds present in traces (<0.05%) or in concentrations of 0.1% have not been reported. Compounds present in trace amounts in the oil were: benzaldehyde, *cis*-verbenol, (E)-2-nonenal, carvotanacetone, γ-nonalactone and (*E*,*E*)-α-farnesene. Compounds whose concentration was determined to be 0.1% were hexanol and caryophylladienol I. Also, the alkanes tricosane (0.7%), pentacosane (1.4%), heptacosane (0.6%) and nonacosane (0.5%) are not reported in Table 1.

3.5. *Antimicrobial assay*

The antibacterial activity was evaluated by determining the MIC and the minimum bactericidal concentration (MBC) using the broth dilution method (Barry, 1976), as described previously (Tenore et al., 2011). The bacterial strains used, selected as representative of the class of Gram-positive and Gram-negative, are listed in Table 2.

3.6. *Cell culture*

The murine monocytic macrophage cell line RAW 264.7 (European Collection of Cell Cultures, London, UK) was grown in plastic culture flask in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (penicillin/streptomycin) under 5% CO_2 at 37°C. After 4–5 days, cells were removed from culture flask by scraping and treated as described previously (Conforti et al., 2010). A serial of concentrations of the samples, ranging from 12.5 to 200 μg mL^{-1}, were prepared from the stock solutions by serial dilution in DMEM and minimal amount of DMSO to give a volume of 100 μL in each well of a microtitre plate (96-well). Then, cells were cultured with vehicle, essential oil, in the presence of 1 μg mL^{-1} LPS for 24 h.

The amelanotic melanoma C32 (ATCC No.: CRL-1585), large lung cell carcinoma COR-L23 (ECACC No.: 92031919), colorectal adenocarcinoma Caco-2 (ATCC No.: HTB-37) and renal cell adenocarcinoma ACHN (ATCC No.: CRL-1611) human cell lines were used in this experiment. The COR-L23, ACHN and C32 cells were cultured in RPMI

1640 medium, while Caco-2 cells were cultured in DMEM medium. Both mediums were supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin. For the assay, a final concentration of 2×10^4 cells was prepared. 100 µL well^{-1} of cell suspension were seeded in 96-well microtitre plates and incubated to allow for cell adhesion. After 24 h, the cells were treated with the serial concentrations of the samples. In this study, 100 µL well^{-1} of each concentration was added to the plates in three replicates to obtain final concentrations of 12.5, 25, 50, 100 and 200 µg mL^{-1}. By these serial dilutions, the final mixture used for treating the cells contained not more than 0.5% of the solvent (DMSO), the same as in the solvent control wells. The culture plates were kept at 37°C with 5% (v/v) CO_2.

3.7. *Assay for cytotoxic activity*

Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay reported by Tubaro et al. (1996) with some modifications (Conforti et al., 2009). Cytotoxicity was expressed as LD_{50}, which is the concentration that inhibits the proliferation of treated cells by 50% with reference to the control (untreated cells). For each cell line, there was a linear relationship between cell number and absorbance, measured at 550 nm in both control and drug-treated wells. Vinblastine was used as positive control.

3.8. *Inhibition of NO production in LPS-stimulated RAW 264.7 cells*

The presence of nitrite, a stable oxidised product of NO, was determined in cell culture media by Griess reagent (1% sulphanamide and 0.1% *N*-(1-naphtyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4) (Green et al., 1982). Also, 100 µL of cell culture supernatant was removed and combined with 100 µL of Griess reagent in a 96-well plate, followed by spectrophotometric measurement at 550 nm using a microplate reader (GDV DV 990 B/V, Roma, Italy). Nitrite concentration in the supernatants was determined by comparing with a sodium nitrite standard curve.

3.9. *Statistical analysis*

Data were expressed as means ± SD. Statistical analysis was performed using Student's *t*-test. Differences were considered significant at $p \leq 0.05$. The inhibitory concentration 50% (LD_{50}) was calculated from the Prism dose–response curve (statistical program) obtained by plotting the percentage of inhibition *versus* the concentrations.

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