DOI 10.24425/pjvs.2021.136795

Original article

Anti-babesial potential and chemical composition of essential oil from yarrow *Achillea millefolium*

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Abstract

Essential oils from plants used in traditional medicine are known as a rich source of chemically diverse compounds with specific biological activities. *Achillea millefolium* essential oil (AEO) was screened for *in vitro* activity against *Babesia canis*. The AEO was obtained by hydrodistillation and analysed by gas chromatography coupled to mass spectrometry (GC-MS). GC-MS revealed the presence of 47 compounds in the essential oil. Those present in the highest concentrations were chamazulene (34.45%), β-caryophyllene (8.93%), (E)-germacrene D (7.55%), patchoulene (7.27%), β-guaiene (4.62%), α-humulene (4.59%), santolina epoxide (4.41%), ethyl iso-allocholate (2.97%), aromadendrene (2.62%), and neoclovenoxid-alkohol (2.46%). AEO was found to be active *in vitro* against *B. canis*, with 50% inhibitory concentration (IC50) values of 0.06 mg/mL, as compared to imidocarb, with IC50 = 0.007 mg/mL. The study confirms that essential oil from *A. millefolium* has anti-babesial properties *in vitro*.

Key words: *Achillea millefolium*, anitbabesial activity, essential oil

Introduction

Babesiosis is a tick-transmitted disease caused by piroplasms of the genus *Babesia* and characterized by haemolytic anaemia and fever, with occasional haemoglobinuria and death (Zygner et al. 2013). Ticks may be able to transmit a number of piroplasms species to cattle (*B. bigemina*, *B. bovis*), horses (*B. caballi*, *Thieleria equi*), cats (*B. cati*, *B. felis*), dogs (*B. canis*, *B. gibsoni*, *B. rossi*, *B. vogeli*, and *B. vulpes*), and humans (*B. divergens*, *B. durcani*, *B. microti*, and *B. venatorum*) (Adaszek and Winiarczyk 2008, Adaszek et al. 2009, Łyp et al. 2016). In Europe two large canine *Babesia* species (*B. canis* and *B. vogeli*) are the most

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frequently detected piroplasms in domestic dogs. Babesiosis can be controlled through tick management or with anti-babesial drugs, or by a combination of these approaches. Babesiosis is commonly treated with drugs such as imidocarb: 3,3'-bis(2-imidazolin-2-yl)-carbanalidae, diminazene aceturate: 4,4′(azoamino)dibenzamidine, triclosan: 2',4',4'-tricloro-2'-hydroxyphenil ether, and azithromycin with atovaquone (Köster et al. 2015). In Poland, the drug of choice to treat babesiosis caused by large *Babesia* species (e.g. *B. canis*) is imidocarb dipropionate. However, due to the observance of side effects of this drug or drug resistance (Vial and Gorenflot 2006), the search for new agents to treat babesiosis has been increased.

Plants are recognized as important sources of antiprotozoal compounds for the development of drugs against numerous tropical diseases, including babesiosis. Examples of natural anti-babesial products include *Achillea millefolium*, *Arcangelisia flava*, *Azadirachta indica*, *Baeckea frutescens*, *Berberis vulgaris*, *Blumea balsamifera*, *Brucea javanica*, *Catharanthus roseus*, *Curcuma zedoaria*, *Garcinia benthamiana*, *Lansium domesticum*, *Morinda citrifolia*, *Peronema canescens*, *Rauvolfia serpentina*, *Rosa damascene*, *Strychnos lucida*, *Swietenia macrophylla* and *Uncaria gambir* (Subeki et al. 2004, Mutnigsih et al. 2005, Elkhateeb et al. 2007a,b,c, Nakao et al. 2009, Guz et al. 2019). Some of the compounds identified in these plants have been reported to have anti-babesial activity, including nerolidol (a sesquiterpene present in essential oils of several plants), artesunate (an artemisinin derivative present in *Artemisia annua*), (−)-epigallocatechin-3-gallate (present in green tea *Camellia sinensis*), and gossypol (1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'dimethyl[2,2'-binaphthalene]8,8'-dicar-boxaldehyde, present in *Gossypium* spp.) (Klayman et al. 1984, Arruda et al. 2005, AbouLaila et al. 2010, Mosqueda et al. 2012). Bringmann et al. (2020) findings show that naphthylisoquino-line alkaloids and their analogs possess strong activity against *B. canis* and might provide a potential alternative to therapeutic agents currently used for the treatment of *Babesia*-caused protozoan diseases.

Achillea millefolium (yarrow or milfoil) belongs to the family Asteraceae and is traditionally and widely believed to be a beneficial medicine for respiratory infections, digestive ailments, and inflammatory conditions. It also has antimicrobial (Candan et al. 2003) and antifungal (Aydin and Sevindik 2018) properties. Yarrow essential oil at high concentrations may exhibit some activity against *Trypanosoma cruzi* (Santoro et al. 2007). An *in vitro* study investigated the anti-*Babesia canis* activity of nine essential oils (Guz et al. 2020), among which essential oils from *A. millefolium*, *Eugenia caryophyllus* and *Citrus grandis* proved to be the most active (IC50 values of 51.0, 60.3 and 61.3 µg/mL, respectively).

In view of the high global prevalence of canine babesiosis and the need for new therapeutic options, an ethnopharmacological survey was conducted to test for anti-babesial activity. The *in vitro* anti-*Babesia canis* activity of *A. millefolium* essential oil (AEO) was investigated.

Materials and Methods

Plant material

Achillea millefolium L. (local name: 'krwawnik pospolity') was obtained from a local market (Herb Confectioning Company FLOS, Elżbieta and Jan Głąb, Morsko, Poland). A voucher specimen (No. DFD-AM-22) has been deposited in the herbarium of the Department of Fish Diseases and Biology, Faculty of Veterinary Medicine, University of Life Sciences, Lublin, Poland.

Essential oil distillation

The vegetable material (50 g) together with 400 mL of distilled water was subjected to hydrodistillation for 3 h in a Deryng apparatus to produce oil according to the method recommended by the Polish Pharmacopoeia VI (2002). The oil was dried over anhydrous sodium sulphate and stored in a dark glass bottle at -20°C until analysis.

GC-MS analysis

Essential oil was diluted 100 times using *n*-hexane to achieve a 1 mL volume, and then 10 µL C12 and C19 was added to the diluted oil as an internal standard mixture solution (1 mg/mL in toluene). Samples thus prepared were subjected to quantitative GC–MS determination, as described by Kowalski and Wawrzykowski (2009). The GC-MS analysis was performed on a TRACE GC Ultra Thermo chromatograph linked to a Thermo ITQ 1100 mass spectrometer using a DB-5 capillary column. The parameters set for the DB-5 column were 30 m × 0.32 mm i.d., 0.25 µm film thickness, column, oven temperature 50°C for 1 min, then 3°C/min to 200°C for 10 min. Helium was used as the carrier gas at a flow rate of 1 mL/min. The injector and detector temperatures were 200°C. A mass selective detector was operated in electron impact mode with ionization energy of 70 eV, a scan time of 0.5 s, and a mass range of 40-870 AMU. The qualitative analysis was based on MS spectra, which were compared with spectra from the NIST library (National Institute of Standards and Technologies, Mass Spectra Libraries).

Molecular examination

Babesia canis parasites were obtained from the blood of dogs with clinical babesiosis. One ml of the blood was used for *in vitro* tests, and 1 ml for molecular study to establish the strain of the parasite. DNA for PCR was extracted from EDTA-anticoagulated whole blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. The amplification of *B. canis* DNA was performed using the forward primer BAB GF2 (5'-GTC TTG TAA TTG GAA TGA TGG-3'), and the reverse primer BAB GR2 (5'-CCA AAG ACT TTG ATT TCT CTC-3'), which amplify a 559-bp region of the 18S rRNA gene of *B. canis* according to the method described by Adaszek and Winiarczyk (2008). After the completion of amplification, all the PCR products were purified using QIAquick spin columns (Qiagen), eluted in 50 *μl* of Tris 10 mM, pH 7.6, and sequenced at the Polish Academy of Sciences, Warsaw, Poland. DNA sequences were assembled and edited using SeqMan (DNAstar, Lasergene, Madison, USA), and ClustalV alignments to the published *B. canis* 18S rRNA gene (GenBank accession numbers: EU622792).

In vitro test for anti-babesial activity

The anti-babesial assay was performed *in vitro* against *B. canis* according to the method described by Subeki et al. (2004) with our own modification. Heparinized blood from a normal dog was washed three times with Vega y Martinez (VYM) solution ($CaCl_2$ x $2H_2O$ – 16.0 mg, KCl – 400.0 mg, KH_2PO_4 – 1415.4 mg, $MgSO_4$ x $7H_2O$ – 154.0 mg, Na_2HPO_4 x $7H_2O$ – 1450.0 mg, NaCl – 7077.0 mg and dextrose – 20.5 mg in 1L H_2O containing 0.25 mM of adenine and 0.5 mM of guanosine) supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL), and then the blood was washed twice with RPMI 1640. After washing, erythrocytes were re-suspended to a final cell volume of 6% in a culture medium consisting 60% RPMI 1640 (with 0.02% Tween 20) and 40% normal dog serum. The erythrocyte suspension was mixed with parasitized erythrocytes to obtain 0.7% parasitaemia at the start of incubation. The test was performed in a 96-well culture plate, with each well containing 25 μL of parasitized erythrocyte suspension and 25 μL of AEO. The final concentrations of the AEO were 0.1, 0.01, and 0.001 mg/mL. The plate was incubated at 37°C under a humidified atmosphere of 5% CO_2. After leaving the plate in an incubator for 72 h, a Giemsa-stained thin smeared specimen was prepared. The percentage of *B. canis* parasitaemia was determined by counting the number of parasitized cells in 1000 erythrocytes. The inhibitory activity of the plant extracts against *B. canis* were classified as follows: at a parasite inhibition rate less than 50% the extract was considered to be inactive, 50% to 80% indicated moderate activity, and over 80% was considered strong activity (Murnigsih et al. 2005). A positive control containing the reference antimalarial drug imidocarb dipropionate (Sigma Aldrich Ltd, Poznań, Poland) and a negative control with medium and Tween-20 (Sigma Aldrich Ltd, Poznań, Poland) solution were used in the experiment. The inhibitory rate (IR) was calculated according to the following formula: IR = [(A-B)/A] x 100 (%), where: A – percentage of parasitaemia in control, B – percentage of parasitaemia in sample. The 50% inhibitory concentrations (IC50) relative to the drug-free control responses were estimated by linear interpolation using Excel software.

Statistical analysis

All quantitative analyses were expressed as mean ± SD for three replicates. Differences between treatments for each parameter were determined by one-way ANOVA at a 95% confidence interval. The statistical differences between the treatment groups were estimated using the Tukey test for multiple comparisons. Data were analysed using Statistica software and Excel 2010. P values less than 0.05 were considered significant.

Results

GC-MS analysis

The essential oil contained 47 compounds (100% of the total oil), including 19 sesquiterpenes (77.38%) and 16 monoterpenes (7.93%), as well as diterpenes (4.81%), fatty acids (1.88%), and others (8.00%). The GC-MS analysis revealed the highest concentration of chamazulene (34,45%), followed by β-caryophyllene (8.93%), (E)-germacrene D (7.55%), and patchoulene (7.27%) (Fig. 1). Compounds found in small amounts were β-guaiene (4.62%), α-humulene (4.59%), santolina epoxide (4.41%), ethyl iso-allocholate (2.97%), aromadendrene (2.62%), 'neoclovenoxid-alkohol' (2.46%), ledenoxid-(II) (1.93%), δ-cadinene (1.62%), δ-3-carene (1.44%), 4-thujanol (1.30%), γ-gurjunene (1.28%), α-campholenaldehyde (1.19%), α-pinene (1.14%), 8A-(Acetyloxy)-2A-[(acetyloxy) methyl]-3,3A,6B-trihydroxy-1,1,5,7-tetramethyl-4-oxo-1,1A,1B,1C,3,3A,4,6A,6B,7,8,8A-dodecahydro-2AHcyclopropa[5',6']benzo[1',2':7,8]azuleno[5,6-B] oxiren-8-yl acetate (0.99%), dotriaconate (98%), 4-thujanol, cis- (0.94%), α-muurolene (0.86%), β-caryophyllene oxide (0.77%), isochiapin B (0.66%), 3',4',7-trimethylquercetin (0.60%), 10,13-octadecadiy-

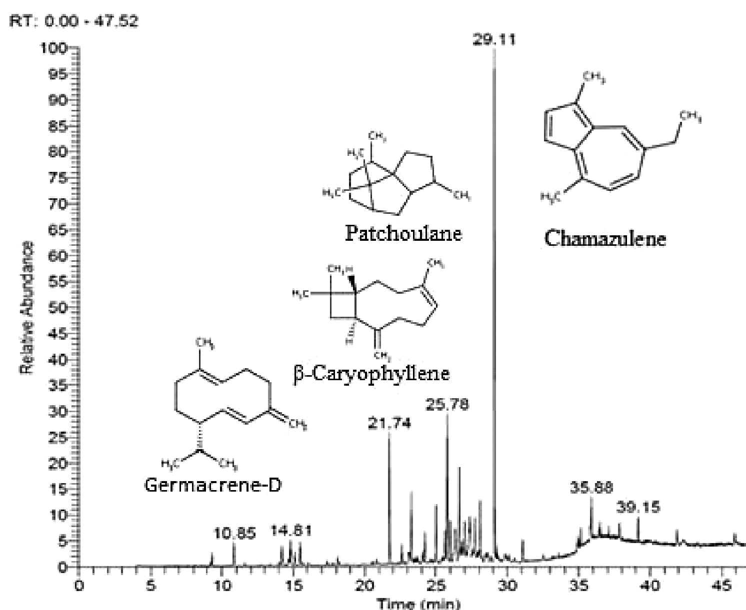

Fig. 1. GC-MS total ion chromatogram of AEO showing four major chemical constituents of the essential oil. Retention times (RT) of major compounds: β-caryophyllene, pathoulene and chamazulene were 21.74, 25.78 and 29.11 min, respectively. Germacrene-D was identified on 3 peaks (RT: 20.82, 23.27 and 24.04 min, respectively).

Table 1. Inhibitory rates of *Achillea millefolium* essential oil (AEO) against *B. canis*. The averages marked by the same letter did not significantly differ at a $p<0.05$. Data are presented as mean, range and standard deviation (SD), n=3.

Group	Mean %	Range %	SD
AEO - 0.1 mg/mL	73.6 [a]	60.0 – 86.0	13.0
AEO - 0.01 mg/mL	29.6 [b]	21.0 – 38.0	8.5
AEO - 0.001 mg/mL	5.0 [c]	2.0 – 8.0	3.0
I* 0.01 µg/mL	61.0 [a]	52.0 – 71.0	9.5
I* 0.001 µg/mL	30.3 [be]	23.0 – 38.0	7.5

I*, imidocarb diproprionate.

Table 2. IC50 values of AEO against *B. canis*.

Samples	Equation	r^2	IC50 (mg/mL)
AEO	y = 610.21x + 13.489	0.9239	0.06
Imidocarb	y = 3411.1x + 26.889	1	0.007

noic acid, methyl ester (0.50%), retinol, acetate (0.40%), myrtenal (0.33%), sabinene (0.33%), 6,9,12,15-docosatetraenoic acid, methyl ester (0.28%), sabinyl acetate (0.28%), sabinol (0.26%), α-copaene (0.23%), γ-terpinene (0.23%), cis-verbenol (0.20%), 1,3-cyclohexadiene-1-methanol,4-(1-methylethyl) (0.13%), cis-ocimene (0.12), methyl linoleate (0.11%), ç-ionone, methyl- (0.09%), cymene (0.08%) and 6,9,12-octadecatrienoic acid, methyl ester (0.05%). Three components were identified on three peaks: germacrene D (6,39%, 0.80% and 0.36%, respectively), α-humulene (2.43%, 1.70% and 0,46%, respectively) and sabinene (0.25%, 0.04% and 0.04%, respectively), whereas δ-3-carene was identified on two peaks (1.41% and 0.03%, respectively).

Molecular examination

Based on the similarities between sequences of 18S RNA gene fragment, isolated *Babesia* protozoa were classified as *Babesia canis* EU622792 (99,8-100% similarity).

In vitro test for anti-babesial activity

The AEO was tested for anti-babesial activity and was shown to be antagonistic to *B. canis*. At concentrations of 0.1 mg/mL, 0.01 mg/mL and 0.001 mg/mL, it showed 73.6% (± 13.0%), 29.6% (± 8.5%) and 5.0% (± 3.0%) inhibitory rates, respectively, in the anti-babesial assay (Table 1). AEO (at 0.1 mg/mL) and imidocarb dipropionate (at 0.01 μg/mL) were found to display moderate inhibitory activity (parasite inhibition rates from 50% to 80%). AEO was found to be active *in vitro* against *B. canis*, with 50% inhibitory concentration values of 0.06 mg/mL, as compared to imidocarb with IC50 = 0.007 mg/mL (Table 2).

Discussion

A number of studies have attempted to demonstrate the efficacy of plant extracts against *Babesia* spp. (Subeki et al. 2004, Murnigsih et al. 2005, Elkhateeb et al. 2007a,b,c, AbouLaila et al. 2010, Guz et al 2019, 2020). *A. millefolium* essential oil has been shown to contain sesquiterpenes (chamazulene, β-caryophyllene, germacrene-D, and nerolidol) and monoterpenes (β-pinene, sabinene, camphor, 1,8-cineole, terpinen-4-ol, and borneol), some with antimicrobial and anti-parasitic activity (Mockute et al. 2003, Bączek et al. 2015, Sevindik et al. 2016). In the present study, GC-MS analysis of the composition of the essential oil revealed a very interesting profile of chemical constituents. The main compounds identified in the AEO were sesquiterpenes: chamazulene: 7-ethyl-1,4-dimethylazulene, β-caryophyllene: (1R,4E,9S)-4,11,11-trimethyl-8--methylidenebicyclo[7.2.0]undec-4-ene, germacrene D: (1*E*,6*E*,8*S*)-1-methyl-5-methylidene-8-propan-2--ylcyclodeca-1,6-diene and patchoulene: 4,10,11,11--tetramethyltricyclo[5.3.1.01,5]undecane. Sesquiterpenes have been found in a large number of species of the genus *Achillea* (Nemeth 2005). Chamazulene is probably responsible for the physiological activity of yarrow and has anti-inflammatory, antioxidant, and radical scavenging activity, as well as strong antifungal properties against dermatophytes (Jamalian et al. 2012). However, there are no reports on anti-parasitic effects of chamazulene. Martinez-Diaz et al. (2015) found that dihydrochamazulene and *trans*-caryophyllene extracted from *Artemisia absinthium* essential oil were effective against *T. cruzi* and *T. vaginalis*. Essential oils rich in caryophyllene from various plant species have been tested on *Trypanosoma cruzi*, *T. brucei*, *Trichomonas vaginalis*, *Plasmodium* spp. and *Leishmania* spp (Cheikh-Ali et al. 2011, Polanco-Hernández et al. 2012). Stokes et al. (2007) concluded that the cruzain inhibitory activity exhibited by *Eugenia* sp. nov. 'San Bosco' leaf essential oil is due to the presence of high concentrations of two sesquiterpene hydrocarbons, zingiberene and germacrene D. There are no reports on anti-babesial effects of chamazulene, caryophyllene, patchoulene or germacrene D, the most abundant compounds of AEO identified in this study.

Our results demonstrated that the essential oil from *A. millefolium* had a considerable effect on *B. canis* survival. While imidocarb inhibited 61.0% of parasites at a concentration of 0.01 μg/mL, 29.6% of parasites were killed in the presence of the AEO at a concentration of 0.01 mg/mL, and 73.6 % at 0.1 mg/mL. This evidence verifies the anti-parasitic effect of the AEO and suggests that its anti-babesial property is most likely attributable to sesquiterpene compounds.

Conclusions

The data obtained from GC-MS analysis led to the investigation of 47 essential oil components in this ecotype, of which most were monoterpenes and sesquiterpenes. Chamazulene, β-caryophyllene, (E)-germacrene D and patchoulene were among the major sesquiterpenes found in *A. millefolium*. In this study, essential oil from *A. millefolium* exhibited important anti-babesial activity against *B. canis*. The results revealed that yarrow essential oil has a potent inhibitory effect on the growth of *B. canis*.

Acknowledgements

This research was supported by the University of Life Sciences in Lublin, Poland (Grant No. DS-1).

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