Original article

In vitro antioxidant and antibabesial activities of the extracts of *Achillea millefolium*

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Abstract

Despite many phytochemical and pharmacological investigations, to date, there are no reports concerning the antibabesial activity of extracts of *A. millefolium* against *B. canis*. This study was aimed at investigating the biological activities of *A. millefolium* against the *Babesia canis* parasite and to identify its chemical ingredients. The water (WE), ethanol (EE) and hexane/acetone (H/AE) extracts of plant aerial parts were screened for total phenolic content (TPC), total flavonoid compound (TFC), DPPH free radical-scavenging activity and its antibabesial activity assay. In this study, imidocarb diproprionate was used as a positive control. The H/AE and EE extracts were analysed using gas chromatography–mass spectroscopy (GC–MS).

In the EE extract, the main compounds were 17.64% methyl octadec-9-ynoate, 16.68% stigmast-5-en-3-ol(3α,24S) and 15.17% hexadecanoic acid. In the H/AE extract, the main compounds were 34.55% 11-decyldocosane, 14.31% N-tetratetracontane, 8.22% β-caryophyllene, and 7.69% N-nonacosane. Extract of EE contained the highest content of phenolics followed by H/AE and WE. The concentration of flavonoids in EE, H/AE and WE extracts showed that TFC was higher in the EE samples followed by H/AE and WE. The antioxidant activities were highest for AA, followed by EE, WE and H/AE. The antibabesial assay showed that the WE, EE and H/AE extracts of *A. millefolium* were antagonistic to *B. canis*. At a 2 mg/mL concentration, it showed 58.7% (± 4.7%), 62.3% (± 5.5%) and 49.3% (± 5.1%) inhibitory rate in an antibabesial assay, respectively.

Considering these results, the present findings suggest that *A. millefolium* extracts may be a potential therapeutic agent and that additional studies including *in vivo* experiments are essential.

Key words: yarrow, plant extracts, babesiosis, tick borne diseases

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Introduction

Canine babesiosis is a common and clinically significant tick-borne disease, caused by the intracellular apicomplexan hematozoan parasites of the genus *Babesia* and is characterized by haemolytic anaemia, fever, splenomegaly, with occasional haemoglobinuria and death (Adaszek and Winiarczyk 2008, Adaszek et al. 2009, Łyp et al. 2016). Canine babesiosis is a growing veterinary problem in many regions throughout the world, including Africa, America, Asia, Australia, and Europe (Carcy et al. 2015, Solano--Gallego et al. 2016). The drug of choice used to treat babesiosis caused by the large *Babesia* species in Poland is imidocarb dipropionate, which has been associated with side effects and drug resistance in *Babesia canis* (Vial and Gorenflot 2006). The most frequently described side effects associated with this drug are pain at the site of injection and cholinergic signs (anorexia, hypersalivation, abdominal pain, vomiting and diarrhoea). The toxic effect of an overdose of imidocarb dipriopionate is nephrotoxicity (Solano--Gallego et al. 2016). The other treatments used with varying success for *Babesia* infections include combinations of atovaquone, azithromycin, diminazene, clindamycin, quinuronium sulfate, pentamidine, artesunate, tick peptides and plant extracts.

In the past, medicinal plants were studied as an important alternative source of new antibabesial compounds (Subeki et al. 2004, Kasahara et al. 2005, Murnigsih et al. 2005, Elkhateeb et al. 2005, 2007a, b, 2008, Nakao et al. 2009, AbouLaila et al. 2010a, b, c, 2012). *Achillea millefolium* L. (family Compositae) is a plant known as yarrow, it is native to Europe, and also grows wild all around Asia, North Africa and North America. *A. millefolium* is recognized as a powerful medicinal plant, which is widely distributed and has been used medicinally for thousands of years. *A. millefolium* showed a protective effect against H_2O_2 -induced oxidative damage in human erythrocytes and leucocytes (Konyalioglu and Karamenderes 2005), antibacterial (Pirbalouti et al. 2011) and antiprotozoal (Murnigsih et al. 2005, Naidoo et al. 2005, Vitalini et al. 2011) activity, and its flowers and leaves may be used to treat wounds, ulcers, diarrhoea, skin injuries, gastrointestinal disorders, flu, fever and urinary infections (Newall et al. 1996, Silva and Santana 1995, Luize et al. 2005). Water extract of *A. millefolium* showed strong activity against *Babesia gibsoni* (Murnigsih et al. 2005).

Despite many phytochemical and pharmacological investigations, there are no reports on the antibabesial activity of extracts of *A. millefolium* against *B. canis*. We therefore investigated the biological activities of *A. millefolium* against *B. canis* parasite and analysed its chemical ingredients, antioxidant potential, phenolic and flavonoid contents.

Materials and Methods

Plant material and sample preparation

Achillea millefolium L. (local name: krwawnik pospolity) herbs were obtained from a local market (Herb Confectioning Company FLOS, Elzbieta and Jan Glab, Morsko, Poland). The plant samples were ground to a fine powder. The samples (50 g) were subjected to enhanced solvent extraction (Dionex ASE150 Accelerated Solvent Extractor system, Sunnyvale CA). Water extract (WE) conditions were as follows: three extraction cycles, temperature 100°C, pressure 15 MPa, heat time 5 min, static time 5 min, 60% flush volume, purge 100 s. Ethanol (96%) and hexane/acetone (9/1, v/v) extracts (EE and H/AE, respectively) conditions were as follows: three extraction cycles, temperature 80°C, pressure 15 MPa, heat time 5 min, static time 5 min, 60% flush volume, purge 300 s. Extracts were condensed (Heidolph, Hei-VAP Precision), dried under nitrogen (Dionex SE400) at 25°C for 4 h, lyophilized (FeeZone 2.5 Labconco) at -45°C for 24 h and stored at -70°C until analysis.

GC-MS

Samples (20 mg/mL in DMSO for EE and H/AE) were separated by gas chromatography (Thermo TRACE GC Ultra) and identified with a mass spectrometer (Thermo ITQ 1100), operated in the electron ionization mode, scanning from 30 to 625 m/z. A DB-5MS capillary column (30 m length, 0.25 mm i.d.) was used for the GC system. The temperature program was set up from 50 °C to 200 °C with 7 °C/min, and He was used as a carrier gas. The injection volume was 1 μL. The compounds separated by GC were identified by matching the experimental mass spectra with those from the NIST/EPA/NIH Mass Spectral Library 2.0. The water extract was not studied using GC-MS.

Determination of total phenolic content

The total phenolic content (TPC) of the *A. millefolium* extracts was determined using the Folin-Ciocalteu reagent following the slightly modified method of Stanković (2011). TPC was calculated as gallic acid equivalents (GAE) in milligram per grams of dry material (dw) on the basis of a standard curve of gallic acid ($Y = 0.0015x + 0.0026$, $R^2 = 0.9956$). The results were expressed as means ($\pm$ SD) for three replications.

Determination of total flavonoids content

The total flavonoid content (TFC) was measured by a colorimetric assay following a slightly modified method described by Dewanto et al. (2002). TFC of the extract were expressed as mg catechin equivalents (CAE) per grams of dry material (dw) on the basis of a standard curve of (+)-catechin ($Y = 0.0011x + 0.0233$, $R^2 = 0.9944$). The results were expressed as means ($\pm$ SD) for three replications.

DPPH free radical-scavenging activity

The ability of the plant extracts to scavenge DPPH (1,1-diphenyl-2-picryl hydrazyl) free radicals was assessed by the method described by Shekhar and Anju (2014). The scavenging ability (I) was calculated as follows: I (%) = 100 x (Ac-As) / Ac. Where, Ac is the absorbance of the control, As is the absorbance of the sample. L-ascorbic acid (AA) was used as a positive control. The antioxidant activity is expressed in terms of the percentage of inhibitory activity (%) on the basis of a standard curve of AA ($Y = 3.307x + 9.6653$, $R^2 = 0.7999$), EE ($Y = 3.2287x - 0.3187$, $R^2 = 0.9818$), WE ($Y = 2.9411x - 10.003$, $R^2 = 0.9679$) and H/AE ($Y = 0.4638x + 6.0947$, $R^2 = 0.8922$). The results were expressed as means for three replications.

In vitro test for anti-babesial and haemolytic activity

The anti-babesial assay was performed against *B. canis in vitro* according to the reported method (Subeki et al. 2004). The percentage of parasitaemia of *B. canis* was determined by counting the number of parasitized cells in 1000 erythrocytes. The inhibitory activities of the plant extracts (20 mg/mL) against *B. canis* were classified as follows: at an inhibition rate of parasites less than 50% - the extract was considered to be inactive, from 50-80% - moderate activity, and over 80% - strong activity (Murnigsih et al. 2005). In this study, imidocarb diproprionate was used as a positive control (group I - 0.01 mg/mL and group I - 0.01 mg/mL) and PBS with DMSO 0.1% as a negative control. 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 parasitaemia in sample. The haemolytic activity of plant extracts was checked by the reported method of Powell et al. (2000) using heparinized dog blood.

Statistical analysis

All quantitative analyses were expressed as mean value $\pm$ SD for three replicates. Differences between treatments for each parameter studied were determined by using the one-way ANOVA test at 95% confidence interval. The statistical differences between the treatment groups were estimated using the Tukey test for multiple comparisons. The IC_{50} values were calculated by linear regression analysis. Data were analysed using Statistica software, version 8.0. *P* values less than 0.05 were considered significant.

Results

In the EE extract the main compounds were methyl octadec-9-ynoate (17.64%), stigmast-5-en-3-ol(3α,24S) (16.68%) and hexadecanoic acid (15.17%), followed by 10AH-2,12A-methano-H,4Hcyclopropa[5,6][1,3] dioxolo[2',3']cyclopenta[1',2':9,10]cyclodeca[1,2-D][1,3]dioxin15OL,1A,2,7A,13,14,14A-hexahydro-1,1,6,6,9,9,11,13-octamethyl-[1AR(1Aα,2α,7Aα,7BS*,10Aα,12Aα,13α,14Aα,15R*)] (4.77%), butyric acid (1aR)-1aα,2β,5,5a,6,9,10,10aα-octahydro-5β,5aβ-, 3.55%2H-pyran,2-(7heptadecynyloxy)tetrahydro-(4.00%), 9A-(acetyloxy)-2,4A,7B-trihydroxy-3-(hydroxylmethyl)-1,1,6,8-tetramethyl-5-oxo-1A,1B,2,4A,5,7A,7B,8,9,9A-decahydro1Hcyclopropa[3,4]benzo[1,2-E]azulen--9-yl acetate (3.75%), 2,4,10,11-tetrakis(acetyloxy)-7A--hydroxy1,1,3,6,9-pentamethyl-1A,2,3,4,4A,5,6,7,7 A, 10,11,11A-dodecahydro-1H-cyclopenta[A]cyclopropa[F]cycloundecen-7-yl-acetate (3.47%), methyl commate D (3.45%), (-)-α-santonin (2.91%), luteolin-6,8-C--α-diglucoside (2.75%), β-caryophyllene (2.42%), dotriacontane (2.37%), aldrin (1.94%), 1-acetyl-5-ethyl-2--[3-(2-hydroxyethyl)-1hindol-2-yl]-α-methyl-methyl ester (1.58%), arachidonic acid methylester (1.51%), luteolin-6,8-C-α-diglucoside (1.47%), 7-tetracyclo[6.2.1.0 (3.8)0(3.9)] undecanol,4,4,11,11-tetramethyl (1.44%), chamazulen (1.40%), methyl abiate (1.11%). Two components were identified on 3 and 2 peaks: luteolin--6,8-C-α-diglucoside, which is also known as lucenin-2 (2.75%, 1.47%, 0.60%, total: 4.82%), and 9A-(acetyloxy)-2,4A,7B-trihydroxy−3-(hydroxylmethyl)-1,1,6,8--tetramethyl-5-oxo-1A,1B,2,4A,5,7A,7B,8,9,9A--decahydro-1H-cyclopropa[3,4]benzo[1,2-E]azulen-9-yl acetate (2.29%, 1.46%, total: 3.75%), respectively (data not shown).

In the H/AE extract the main compounds were 11-decyldocosane (34.55%), N-tetratetracontane (14.31%), β-caryophyllene (8.22%) and N-nonacosane (7.69%), followed by β-phellandrene (4.64%), (E)-germacrene D (4.64%), nopol (3.33%), (-)-caryophyllene oxide (3.21%), 1-terpineol (2.14%), α-campholenaldehyde (1.40%), sabinene (1.34%), α-humulene (1.30%), luteolin-6,8-C--α-diglucoside (1.05%), camphene (1.01%), cyclolongifolene oxide (1.01%). Four components were identi-

Table 1. Total polyphenolic contents (mg GAE/g dw) of WE, EE and H/AE extracts of *A. millefolium*.

Group	Mean	Range	SD
WE	13.96[a]	13.10-14.80	0.85
EE	71.33[b]	70.10-72.40	1.15
H/AE	60.33[c]	58.20-62.10	1.97

Table 2. Total flavonoid contents (mg CAE/g dw) of WE, EE and H/AE extracts of *A. millefolium*.

Group	Mean	Range	SD
WE	20.86[a]	18.90-23.10	2.11
EE	47.33[b]	45.20-49.00	1.94
H/AE	33.93[c]	32.40-35.20	1.41

Table 3. DPPH radical scavenging ability at 30 µg/mL of the extracts of *A. millefolium*.

Group	Mean %	Range %	SD	IC_{50} µg/mL
WE	74.40[a]	71.22-76.80	2.87	20.40
EE	83.23[b]	79.90-87.48	3.87	18.06
H/AE	20.29[c]	18.11-22.53	2.21	94.67
AA	93.82[d]	90.00-97.57	3.78	12.19

fied on 2 peaks: sabinene (1.34%, 0.09%, total: 1.43%), 3-carene (0.19%, 0.19%, total: 0.38%), α-humulene (0.19%, 1.30%, total: 1.49%), and cis-ocimene (0.09%, 0.13%, total: 0.22%), and one compound was identified on 3 peaks: (E)-germacrene D (0.1%, 4.29%, 0.25%, total: 4.64%) (data not shown).

The amount of TPC in various yarrow extract of the species *A. millefolium* was reported as mg of gallic acid equivalent per gram of dry material (Table 1). Extract of EE contained the highest content of phenolics (71.33 mg GAE/g dw) followed by H/AE (60.33 mg GAE/g dw) and WE (13.96 mg GAE/g dw). Significant differences were found in TPC between all extract types.

The amount of TFC in samples was determined using the spectrophotometric method with aluminium chloride. The TFC of extracts was expressed as mg of catechin equivalent per grams of dry material (Table 2). The concentration of flavonoids in EE, H/AE and WE extracts showed that TFC was higher in EE samples (43.33 mg CAE/g dw) followed by H/AE (33.93 mg CAE/g dw) and WE (20.87 mg CAE/g dw). Significant differences were found in TFC between all extract types.

In the DPPH radical scavenging assay, antioxidants react with DPPH, and convert it to the yellow coloured 1,1-diphenyl-2-picryl hydrazine. The degree of discolouration indicates the radical-scavenging potential of the sample. In our study, all extracts were able to reduce the stable, purple coloured radical DPPH to the yellow-coloured 1,1-diphenyl-2-picryl hydrazine reaching 50%

of scavenging activity with an IC_{50} as follows: 18.06 µg/mL for EE, 20.40 µg/mL for WE and 94.67 µg/mL for H/AE. The antioxidant activities were highest for AA (positive control), followed by EE, WE and H/AE. Significant differences were found in antioxidant activity between all extract types (Table 3).

The crude WE, EE and H/AE extracts were tested for antibabesial activity. The results showed that the WE, EE and H/AE extracts (at a concentration of 2 mg/mL) of *A. millefolium* were antagonistic to *B. canis*. At a 2 mg/mL concentration, it showed 58.7% (± 4.7%), 62.3% (± 5.5%) and 49.3% (± 5.1%) inhibitory rate for an antibabesial assay, respectively. Significant differences were not found in antibabesial activity between three studied groups (WE, EE, H/AE) and positive control group I - 0.01 mg of imidocarb diproprionate/mL (Table 4).

Haemolytic activity for the cytotoxic study was analysed against dog red blood cells using Triton X-100 as a positive control. The control showed a strong haemolytic effect with 100% lysis after 60 min. All extracts in our study showed very low haemolytic effect < 0.5% (Table 5).

Discussion

The EE and H/AE extracts show the presence of major phytoconstituents like hydrocarbons, terpenes, fatty acids, steroids, flavonoids, phenols, alkaloids and glicosides. Five compounds, α-campholenaldehyde,

Table 4. Inhibitory rates of *A. millefolium* extracts against *B. canis*.

Group	Mean %	Range %	SD
2 mg/mL			
WE	58.6[b]	55.0-64.0	4.72
EE	62.3[bd]	57.0-68.0	5.50
H/AE	49.3[bc]	45.0-55.0	5.13
I* - 0.01 mg/mL	59.0[b]	56.0-63.0	3.60
I* - 0.001 mg/mL	29.6[a]	25.0-34.0	4.50
0.2 mg/mL			
WE	7.6[b]	6.0-9.0	1.52
EE	8.0[b]	7.0-9.0	1.00
H/AE	8.6[b]	7.0-10.0	2.33
I* - 0.01 mg/mL	59.0[a]	56.0-63.0	3.60
I* - 0.001 mg/mL	29.6[a]	25.0-34.0	4.50
0.02 mg/mL			
WE	4.6[b]	3.0-6.0	1.52
EE	4.3[b]	2.0-6.0	2.08
H/AE	5.3[b]	4.0-7.0	1.52
I* - 0.01 mg/mL	59.0[a]	56.0-63.0	3.60
I* - 0.001 mg/mL	29.6[a]	25.0-34.0	4.50

I* - imidocarb diproprionate

Table 5. Haemolytic activity, as a percentage of haemolysis caused by 0.1% Triton X-100, for extracts and control.

Group	Mean (%)	Range (%)	SD
WE	0.43[b]	0.33-0.55	0.11
EE	0.38[b]	0.36-0.40	0.02
H/AE	0.49[bd]	0.41-0.59	0.09
PBS buffer	0.29[bc]	0.26-0.36	0.05
0.1% Triton X-100	100[a]	-	-

nopol, β-caryophyllene, α-humulene and (E)-germac-rene D were present in both extracts. Comparing the present data with those previously reported in the literature for the methanol extracts from *A. millefolium*, a differences were observed. For example, glycosylated phenolic compounds e.g. luteolin 7-O-glucoside, apigenin 7-O-glucoside and caffeic acid glucoside (Yassa et al. 2007), chlorogenic acid, rutin, dicaffeo-ylquinic acid and apigenin 4-O-glucoside (Vitalini et al. 2011) which were found to be the major compounds in previous studies were not detected in our work. Moreover, one of the major compounds of our extracts has not previously been reported (e.g. luteolin-6,8-C--α-diglucoside).

Other authors reported different results regarding contents of polyphenolic compounds depending on the origin of the sample (wild or commercial) and the type of preparation of the extract (Dias et al. 2013). Some researchers have reported TPC content in herbal parts of *A. millefolium* from 2.74 to 9.55 mg/g dw (Wojdyło et al. 2007, Georgieva et al. 2015) and even more than 100 mg/g dw (Keser et al. 2013). In our study, the EE contained the highest content of phenolics followed by H/AE and AE (Table 1).

Flavonoids were previously detected in methanolic (Vitalini et al. 2011) and water (Eghdami and Sadeghi 2010) extracts of *A. millefolium*. The main constituents were kaempferol, apigenin and luteolin, mainly found as 7-O-glucosides and 7-malonylglucosides (Guédon et al. 1993, Ayoobi et al. 2017). Both the antioxidant and anti-inflammatory properties of this herb have been attributed to its flavonoid content (Ayoobi et al. 2017). In this respect, flavonoids produce various clinical characteristics such as antiproliferative (Huo et al. 2013), antibacterial (Candan et al. 2003, Stojanovic et al. 2005) and antiplasmodial (Vitalini et al. 2011)

effects. Using GC/MS in our study, for the EE extract, the one main flavonoid compound luteolin-6,8-C--α-diglucoside was determined, to the best of the authors knowledge for the first time. In contrast, 11 other flavonoids, namely vicenin-2, luteolin-3',7-O-diglucoside, luteolin-7-O-glucoside, rutin, apigenin-7-O-glucoside, luteolin, apigenin (Kyslychenko 2014) and chrysoeriol, diosmetin, genkwanin and acacetin (Kyslychenko 2014) – were identified by other authors in the methanol extracts of yarrow.

The antioxidant properties of *A. millefolium* have previously been reported in hydroalcoholic, methanolic and aqueous extracts (Candan et al. 2010, Trumbeckaite et al. 2011, Vitalini et al. 2011, Georgieva et al. 2015). In our study, the antioxidant activity of EE, H/AE, AE extracts are largely dependent on its chemical composition. From a general point of view, phenols were confirmed to possess strong antioxidant activity. In particular, oxygenated monoterpenes are mainly responsive to the antioxidant potential of the plant extracts. Monoterpene hydrocarbons should also be taken into account for the antioxidative activity observed. On the other hand, sesquiterpenes hydrocarbons have a very low antioxidant activity (Rubero and Baratta 2000).

Research involving plant extracts for parasite control on animals is a developing area in the world. Several *in vitro* studies have proven the potential use of medical herbs for the control of the *Babesia* spp. Of the plant extracts studied by Subeki et al. (2004, 2005a, b, c, 2007) many plants were found to have appreciable anti-*B. gibsoni* activity. Moreover, the extracts of *Berberis vulgaris* (Elkhateeb et al. 2007a), *Rosa damascena* (Elkhateeb et al. 2007b) showed activity against *B. gibsoni*. Murnigsih et al. (2005) screened 22 aqueous extracts of plants traditionally used to treat malaria in Java Island (Indonesia) for anti-*B. gibsoni* activity. Yarrow was one of thirteen species found to display strong inhibitory activity (over 80% inhibition at a 1 mg/mL concentration). To date, research into the efficiency of various plant extracts against the protozoa of the genus *Babesia* isolated from dogs was limited only to *B. gibsoni*. The available literature provides no data on the efficiency of plant extracts against *B. canis*. This paper is the first to evaluate the inhibition efficiency of *A. millefolium* extract on the growth of *B. canis* in *in vitro* conditions and to compare its inhibition efficiency with that of imidocarb, the drug commonly used in the therapy of canine babesiosis. The results indicate that the inhibition efficiency of the studied extract is similar to the inhibition efficiency of imidocarb at a concentration of 0.01 mg/mL. Both the results of our observations and the literature data show that *A. millefolium* extracts exhibit strong antibabesial (against *B. gibsoni* and

B. canis) activity, therefore, further research into their therapeutic efficiency under clinical conditions is necessary.

In the present study, extracts from *A. millefolium* exhibit important antibabesial activity against *B. canis*. Results revealed that yarrow extracts have a potent effect on the inhibition of the growth of *B. canis*. Clinical applications of these materials require further investigation.

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