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Chemical composition and antioxidant and antimicrobial activity of essential oil of *Artemisia annua* L. from Bosnia

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ABSTRACT

Hydrodistilled volatile oil obtained from the aerial parts of *Artemisia annua* L., cultivated near Sarajevo, Bosnia, was analyzed by GC–MS. More than one hundred compounds were identified, representing 95.5% of the total oil. The major constituents of essential oil were oxygenated monoterpenes, artemisia ketone (30.7%) and camphor (15.8%). Isolated essential oil was tested for radical-scavenging ability using the stable DPPH radical, the ABTS radical, for reducing power ability with a test based on the reduction of ferric cations, for reducing ability of hydroxy radical in ORAC assay, and for metal chelating ability using the ferrozine assay. In all tests oil did not show a prominent antioxidant activity, but still comparable with thymol, an already known antioxidant. The screening of antimicrobial activity of oil was individually evaluated against representatives of Gram-positive, Gram-negative bacteria and fungi, using the agar diffusion method. All tested microorganisms were inhibited by essential oil. To the best of our knowledge, this is the first report of antimicrobial activity of essential oil of *A. annua* against *Haemophilus influenzae*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Micrococcus luteus* and *Candida krusei* microbial strains. The antioxidant, antibacterial and antifungal activity of essential oil of *A. annua* from Bosnia is presented here for the first time and extends our knowledge in the range of valuable biological activities and possible roles in therapy associated with this medicinal herb.

1. Introduction

Artemisia annua L. (Asteraceae) is a fragrant annual herb widely distributed in Asia, Europe, and North America. The use of this plant (commonly named Sweet Wormwood, Sweet Annie, Sweet Sagewort, Annual Wormwood or Qinghaosu) in Chinese traditional medicine was recorded before 168 BC and the Chinese naturalist Li Shi-Zhen described the use of *A. annua* for treatment of malaria and other diseases in his 1596 book "Compendium of Materia Medica". In 1971 artemisinin, a sesquiterpene with antimalarial properties has been isolated (Klayman, 1985). Since then *A. annua* has become one of the most extensively investigated plants in recent years. The search for the other concerned active compounds has led to discovering and isolation of many phytochemicals, such as monoterpenoids, sesquiterpenoids, flavonoids and coumarins, and aliphatic and lipid compounds (Bhakuni et al., 2001, 2002). Besides its antimalarial activity (Bhakuni et al., 2002) *A. annua* also shows anti-inflammatory, antipyretic (Huang et al., 1993), anticancer (Zheng, 1994), antifungal (Liu et al., 2001), antiparasitic (Kim et al., 2002), antiulcerogenic (Foglio et al., 2001), and cytotoxic (Nibret and Wink, 2010) activities. The essential oil composition of this medicinal plant has been studied thoroughly and hundreds of components have been identified up to date (Brown, 2010). Camphor, artemisia ketone, germacrene D and 1,8-cineole, are usually found as the main components (Ahmad and Mishra, 1994; Tellez et al., 1999; Malik et al., 2009; Brown, 2010). Generally is accepted that variability of chemical composition of essential oil of *A. annua* depends of geographical origin and stage of plant development (Verma et al., 2011; Lenardis et al., 2011; Bhakuni et al., 2002; Brown, 2010; Holm et al., 1998).

Thus, there is still a considerable research interest in the assay of composition and/or biological properties of essential oil of *A. annua* from different geographical origin. In the present work, we investigated the essential oil composition of *A. annua* cultivated in Bosnia and Herzegovina. In addition, the aim of this study was to determine the antioxidant activity and antimicrobial activity of the isolated essential oil, using the microbial and fungal strains that have not been reported to date.

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2. Materials and methods

2.1. Plant material and reagents

A. annua was cultivated from the seeds of wild growing plant. The plant was sown at Kiseljak, near Sarajevo (43°57′0″N, 18°5′0″E), at altitude 525 m.s.m., in a March 2009.

This area climate exhibits influences of oceanic, humid continental and humid subtropical zones, with four seasons and uniformly spread precipitation. The proximity of the Adriatic Sea moderates this area climate somewhat, although the mountains to the south of the city of Sarajevo greatly reduce this maritime influence. The average yearly temperature is 13.5 °C, although in the year 2009 temperatures were very high, ranking from 15 to 17 °C in March 2009 to 25–27 °C in August 2009 (Federalni hidrometeorološki zavod Bosne i Hercegovine, 2009).

The crop of *A. annua* grew in June 2009, and the plant was harvested in August 2009, before the blooming, dried at room temperature, and grounded. Voucher specimen (No. AA-09-12) of plant is deposited at the Faculty of Science, University of Sarajevo, Bosnia and Herzegovina.

2.2. Chemicals

All applied reagents were of the highest purity available and purchased from the Sigma–Aldrich Chemical Company (Germany).

2.3. Sample preparation

Air-dried plant material was subjected to hydrodistillation for 2 h. The essential oil was extracted with dichloromethane and dried over anhydrous sodium sulphate. For the GC–MS analysis, sample of essential oils was dissolved in dichloromethane and, for antioxidant and antimicrobial assays, samples were dissolved in dimethylsulfoxide. Thymol was used as a positive probe for antioxidant, and ampicillin for antimicrobial assays, and it was prepared in the same way as tested samples. All determinations were carried out in triplicate.

2.4. Microbial strains

The antimicrobial activity of essential oils was evaluated using a panel, which included laboratory control strains obtained from the American Type Culture Collection: Gram-positive bacteria, *Staphylococcus aureus* ATCC 6538 and ATCC 25923, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Streptococcus pneumoniae* ATCC 49619, *Micrococcus luteus* ATCC 4698, and Gram-negative bacteria *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027 and ATCC 27853, *Haemophilus influenzae* ATCC 49766, and one fungus *Candida krusei* ATCC 6258.

2.5. Gas chromatography–mass spectrometry analysis of essential oil

Volatile compounds from the aerial parts of the plant were analyzed by GC–MS using Hewlett-Packard GC–MS system (GC 6890 series II; MSD 6890 series II). The GC conditions were: fused silica HP-5 column, carrier gas He (1.1 mL/min), temperature programme: 3 °C/min from 60 °C to 240 °C; the injection port temperature was 250 °C; detector temperature was 280 °C. Ionization of the sample components was performed in the EI mode (70 eV). The linear retention indices, RI, for all compounds were determined by injection of the sample with a solution containing the homologous series of C_8–C_{26} *n*-alkanes (Van Den Dool and Kratz, 1963).

The identification of essential oil constituents was accomplished by visual interpretation, comparing their retention indices and mass spectra with literature data (Adams, 2007), by computer library search (HP Chemstation computer library NBS75K.L, NIST/EPA/NIH, Mass Spectral Library 2.0 and Mass Finder 4 Computer Software and Terpenoids Library), and by the laboratory database.

2.6. 1,1-Diphenyl-2-picrylhydrazyl radical-scavenging activity (DPPH)

The ability of the essential oil constituents to donate hydrogen atom or electron and scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined by the slightly modified method of Brand-Williams et al. (1995). The concentrations of the tested samples were ranged from 10.00 to 0.10 mg/mL. A portion of sample solution (200 μL) was mixed with 3.0 mL of 5.25×10^{-5} mol/L DPPH· in absolute ethanol. Decreasing of absorbance of tested mixtures was monitored every 1 min for 30 min at 517 nm using Perkin-Elmer Lambda 25 UV/Vis spectrophotometer. Absolute ethanol was used to zero the spectrophotometer, DPPH· solution was used as blank sample, and thymol was used as a positive probe. The DPPH· solution was freshly prepared daily, stored in a flask covered with aluminium foil, and kept in the dark at 4 °C before measurements.

The radical-scavenging activity of the tested samples, expressed as percentage inhibition of DPPH, was calculated according to the formula IC (%) = $[(A_0 - A_t)/A_0] \times 100$, where A_t is the absorbance value of the tested sample and A_o is the absorbance value of blank sample, in particular time. Percent inhibition after 30 min was plotted against concentration, and the equation for the line was used to obtain the IC_{50} value. A lower IC_{50} value indicates greater antioxidant activity.

2.7. Evaluation for reducing power

The reducing power test is based on reduction of ferric to ferrous by the potent antioxidant. In presence of cyanide ions, and adding a new amount of Fe^{3+}, blue color of $Fe_4[Fe(CN)_6]_3$ develops. The reducing power of samples was determined by slightly modified method of Yen and Duh (1994), as describes below. Sample of 1.0 mL of various dilutions (from 10.00 to 0.01 mg/mL) was mixed with 2.50 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of 1% potassium-ferricyanide. The mixtures were incubated at 50 °C for 20 min. After incubation 2.5 mL of 10% trichloracetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Upper layer (0.5 mL) of solution is mixed with 2.5 mL of distilled water and 100 μL of 0.1% $FeCl_3$ and the absorbance was measured at 700 nm. Controlling sample contained 1.0 mL distilled water, 2.5 mL of phosphate buffer, 2.5 mL of 1% potassium-ferrocyanide and 2.5 mL of 10% trichloracetic acid. Blank sample contained 1.0 ml distilled water, 2.5 mL of phosphate buffer, 2.5 mL of 1% potassium-ferricyanide and 2.5 mL of 10% trichloracetic acid. Thymol was used as a positive control.

The reducing power of samples was calculated by the following formula: RP (%) = $(A_B - A_A) \times 100$; where: RP – reducing power; A_B – absorption of controlling sample (100%); A_A – absorption of tested sample. Percent inhibition was plotted against concentration, and the equation for the line was used to obtain the RP_{50} value. The lower RP_{50} value indicates greater reducing power ability.

2.8. 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical-scavenging activity (ABTS)

The ABTS method is based on the reduction of the green ABTS radical cation (7.00 mmol/L) that was obtained by its oxidation with equal volume of potassium persulphate (2.45 mmol/L), (Scalzo et al., 2005) for 12–16 h at 4 °C in the dark. On the day of analy-