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Studies on the Antimicrobial and Antioxidant Activity and Chemical Composition of the Essential Oils of *Kitaibelia vitifolia*

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The objective of this study was to evaluate the potential use of the essential oil of *Kitaibelia vitifolia* Willd. in the pharmaceutical and food industries. Antimicrobial and antioxidant activities of essential oil of *K. vitifolia* isolated by hydrodistillation using a Clevenger-type apparatus were assessed. GC/FID and GC/MS analyses were used to determine the major components of the essential oil as sclareoloxide (*cis* A/B) 17.9%, sclaral 10.9%, labda-7,13,14-triene 10.6% and sclareol 9.5%. The antimicrobial activity of the essential oil was evaluated against the bacterial strains: *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *P. mirabilis* ATCC 14153, and *Bacillus subtilis* ATCC 6633; and fungal strains: *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404. Antimicrobial activity was tested using a broth dilution procedure for determination of minimum inhibitory concentration (MIC). The essential oil of *K. vitifolia* showed strong antimicrobial activity. Antioxidant activities of the essential oils were evaluated using the DPPH and hydroxy radical scavenging, lipid peroxidation and metal chelating assays. The results for antioxidant activity were compared with control antioxidants, ascorbic acid, gallic acid, α-tocopherol and BHT. Results showed that the essential oil possesses antioxidant activity, with total antioxidant capacity of 95.4±0.7 μg AA/g and IC_{50} values of 5.45±1.45 μg/mL for DPPH free radical scavenging activity, 26.5±1.6 μg/mL for inhibitory activity against lipid peroxidation, 79.4±0.4 μg/mL for hydroxyl radical scavenging activity, and 39.9±0.7 μg/mL for metal chelating activity.

Keywords: *Kitaibelia vitifolia*, Essential oil, Antimicrobial activities, Antioxidant activities.

The use of traditional medicinal plants for primary health care and other purposes has progressively increased worldwide in recent years. Many plant secondary metabolites and essential oils have antimicrobial properties that make them successful in the treatment of bacterial, fungal and viral infections [1-3]. The different parts of plants are used to treat effectively a number of diseases. Their antioxidant and antimicrobial properties affect a range of physiological processes in the human body, thus providing protection against both free radicals and growth of undesirable microorganisms. Free radicals, as highly reactive intermediaries, lead to oxidative tissue damage and, therefore, potential damage. The accumulation of these radicals causes serious health problems, including cardiovascular diseases, premature aging, cancer, and inflammatory diseases [4-9]. However, synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), widely known for their ability to terminate the chain reaction of lipid peroxidation, have been proved to be carcinogenic and cause liver damage [10]. Both bacterial resistance to a large number of antibiotics and the capacity of plants to synthesize biologically active substances are reasons for the increasing importance given to the use of plant-derived products in bacterial control. The use of plants in the food industry to replace synthetic preservatives, antioxidants and other food additives has increased significantly over the last years [11] No previous studies on the biological activity, chemical constituents and traditional uses of *Kitaibelia vitifolia* Willd. (*Malvaceae*) have been reported.

Results on the qualitative and quantitative chemical composition of the essential oil of *K. vitifolia* are given in Table 1. A total of 41 components, accounting for 96.6% of the oil, were identified. Analysis showed that 82.3% of the oil characterized (96.6%) was made up of 15 components present in the sample at a concentration higher than 1%, and that more than half of the sample fraction characterized (48.9%) contained as few as four dominant components, sclareol-oxide (*cis*-A/B) (17.9%), sclaral (10.9%), labda-7,13,14-triene (10.6%) and sclareol (9.5%). The essential oil sample is extremely diterpenic in character, with the diterpenes present being predominantly of the labdane type.

The antimicrobial results obtained by the dilution method (MIC) are given in Table 2. Minimum inhibitory concentrations were determined for 8 selected indicator strains. The results presented in Table 2 reveal antimicrobial activity of the essential oil of *K. vitifolia* within the concentration range of 15.6 μg/mL to 62.5 μg/mL. The highest susceptibility to *K. vitifoila* essential oil among the bacteria tested was exhibited by *B. subtilis* ATCC 6633 (MIC = 15.6 μg/mL), followed by *S. aureus* ATCC 25923, *E. coli* ATCC 25922 (MIC = 31.2 μg/mL), *K. pneumoniae* ATCC 13883, *P. vulgaris* ATCC 13315 and *P. mirabilis* ATCC 14153 (MIC = 62.5 μg/mL). Among the fungi, *A. niger* ATCC 16404 (MIC = 31.2 μg/mL) showed the highest susceptibility, and *C. albicans* ATCC 10231 (MIC = 62.5 μg/mL) the lowest.

The results show that the essential oil of *K. vitifolia* possesses antioxidant activity, with total antioxidant capacity of 95.4±0.7 μg AA/g. IC_{50} values were determined for each measurement (Table 3).

Antioxidant and antimicrobial properties of essential oils and various extracts of many plants are of great interest in both fundamental science and food industry, since their potential use as

Table 1: Chemical composition of the essential oil tested.

Constituents	KIE	KIL	%
Linalool	1091.6	1095	0.3
Lilac aldehyde C	1134.0	n/a	0.8
Eugenol	1351.2	1356	0.3
1a,2,5,5-Tetramethyl-*cis*-1a,4a,5,6,7,8-hexahydro-γ-chromene	1366.7	1370	0.5
trans-β-Damascenone	1373.3	1383	0.8
Isopatchoula-3,5-diene	1378.5	1377	0.3
4-(2,6,6-Trimethylcyclohexa-1,3-dienyl) but-3-en-2-one	1405.9	1440	0.7
Geranyl acetone	1443.3	1453	0.5
trans-β-Ionone	1475.4	1487	0.4
γ-Patchoulene	1491.2	1502	0.5
11-*nor*-Drim-7-en-9-one	1563.4	n/a	0.4
Silphiperfol-5-en-3-one A	1568.4	1575	0.3
cis-Dihydro-mayurone	1583.2	1595	0.7
Selina-3,11-dien-6α-ol	1631.0	1642	0.8
Cyperotundone	1691.6	1695	0.7
3-Acetoxy-β-ionone	1753.4	1752	0.7
β-Acoradienol	1769.4	1762	0.3
Drimenone	1772.8	1792	0.6
8-α-Acetoxyelemol	1783.6	1792	2.6
Fukinanolide	1790.5	1798	0.4
Khusinol acetate	1824.6	1823	5.1
Sclaral (sclareolide lactol)	1833.8	n/a	10.5
Sclareoloxide	1862.6	n/a	3.7
1-(3-Hydroxypropyl)-5,5,8a-trimethyldecahydronaphthalen-2-ol	1874.1	n/a	0.3
Flourensadiol	1880.2	1869	1.1
8,13-Cedranediol	1909.3	1897	1.1
cis-γ-Curcumenyl isobutyrate	1917.0	1920	2.2
Sclareoloxide (cis-A/B)	1931.0	n/a	17.3
2-Furoic acid, 2-methyloct-5-yn-4yl ester**	1944.0	n/a	0.8
(3*E*,5*E*,7*E*)-6-Methyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3,5,7-octatrien-2-one	1959.2	n/a	0.7
Labda-7,13,14-triene	1971.0	1978	10.2
Manool oxide	1990.8	1987	2.8
n.i.*	2011.9	-	0.6
Labda-7,12(*E*),14-triene	2020.0	2036	3.0
Manool	2038.9	2056	0.4
n.i.*	n/a	-	0.3
Labda-7,14-diene-13-ol	2068.0	2096	1.7
Isoabienol**	2085.7	2124	0.5
trans-Phytol	2095.9	2112	2.7
n.i.*	2156.5	-	0.5
n.i.*	n/a	-	0.4
n.i.*	n/a	-	0.4
n.i.*	2213.5	-	0.6
n.i.*	2219.2	-	0.5
Sclareol (labd-14-ene-8,13-diol)	2228.1	2222	9.1
Labd-7,13-dien-15-ol	2262.6	2291	6.3
Tricosane	2279.1	2300	0.7
Pentacosane	2481.9	2500	0.3

KIE=Kovats (retention) index experimentally determined (AMDIS, uncorrected values)
KIL=Kovats (retention) index - literature data [14]
**=tentative identification; n.i*=not identified; n/a=not available

Table 2: Minimum inhibitory concentrations (MIC) of essential oil of *K. vitifolia*.

MIC µg/mL			
Microbial strains	**Essential oil**	**Amracin**	**Ketokonazol**
Staphylococcus aureus ATCC 25923	31.3	1.0	/
Klebsiella pneumonia ATCC 13883	62.5	0.5	/
Escherichia coli ATCC 25922	31.3	1.0	/
Proteus vulgaris ATCC 13315	62.5	0.5	/
Proteus mirabilis ATCC 14153	62.5	0.5	/
Bacillus subtilis ATCC 6633	15.6	0.3	/
Candida albicans ATCC 10231	62.5	/	2.0
Aspergillus niger ATCC 16404	31.3	/	1.0

natural additives has emerged from a growing tendency to replace synthetic antioxidants with natural ones. The obtained results show that the essential oil of Serbian *K. vitifolia* has good antimicrobial and antioxidant activity under the experimental conditions used.

Experimental

Chemicals: All chemicals and reagents were of analytical grade and purchased from Sigma Chemical Co. (St Louis, MQ, USA), Aldrich Chemical Co. (Steinheim, Germany) and Alfa Aesar (Karlsruhe, Germany).

Table 3: Antioxidant activity of the essential oil of *K. vitifolia*.

Sample	Total antioxidant capacity (µg AA/g)	[a]IC_{50} (µg/mL) DPPH scavenging activity	Inhibitory activity against lipid peroxidation	Metal chelating activity	Hydroxyl radical scavenging activity
Essential oil	95.5±0.7	5.5±1.5	26.5±1.6	39.9±0.7	79.4±0.4
GA		3.8±0.7	255.4±11.7	-	59.1±1.1
AA		6.1±0.3	> 1000	-	160.6±2.3
BHT		15.6±1.3	1.0±0.2	-	33.9±0.8
α-tocopherol		-	0.5±0.1	-	-

[a]IC_{50} values were determined by nonlinear regression analysis. Results are mean values ± SD from three experiments

Spectrophotometric measurements: Spectrophotometric measurements were performed using a UV-VIS spectrophotometer MA9523-SPEKOL 211 (ISKRA, Horjul, Slovenia).

Plant material: The test plant was collected at Ilijak Hill (Central Serbia) in May/June 2009. The species was identified and the voucher specimen deposited at the Department of Botany, Faculty of Biology, University of Belgrade (16350 BEOU, Lakušić Dmitar).

Isolation of essential oils: Thirty g of air-dried plant material was subjected to hydrodistillation for 3 h using a Clevenger-type apparatus, according to the standard procedure reported in the European Pharmacopoeia [12].

Analytical gas chromatography (GC/FID): GC/FID analysis of the oil was carried out using a HP-5890 Series II GC apparatus [Hewlett-Packard, Waldbronn (Germany)], equipped with a split-splitless injector and an automatic liquid sampler (ALS) attached to a HP-5 column (25 m x 0.32 mm, 0.52 µm film thickness) and fitted to a flame ionization detector (FID). Carrier gas flow rate (H_2) was 1 mL/min, split ratio 1:30, injector temperature 250°C, detector temperature 300°C, while column temperature was linearly programmed from 40-260°C (at rate of 4°/min). Solutions of plant extracts in methanol (~1%) were consecutively injected by ALS (1 µl, split mode). Area percent reports, obtained as a result of standard processing of chromatograms, were used as the basis for quantification purposes.

Gas chromatography/mass spectrometry (GC/MS): The same analytical conditions as those mentioned for GC/FID were employed for GC/MS analysis, along with a HP-5MS column (30 m 0.25 mm, 0.25 µm film thickness), using the HP G1800C Series II GCD system [Hewlett-Packard, Palo Alto, CA (USA)]. Instead of hydrogen, helium was used as the carrier gas. The transfer line was heated at 260°C. Mass spectra were acquired in EI mode (70 eV), in the m/z range 40-450. Solutions of plant extracts in methanol (~1%) were injected by ALS (200 nL, split mode). The constituents were identified by comparison of their mass spectra with those from Wiley 275 and NIST/NBS libraries, using different search engines. The experimental values for retention indices were determined using calibrated Automated Mass Spectral Deconvolution and Identification System software [13] compared with those from available literature [14] and used as an additional tool to approve MS findings.

Test microorganisms: The antimicrobial activity of the essential oil was tested *in vitro* against the following bacteria: *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Proteus mirabilis* ATCC 14153, and *Bacillus subtilis* ATCC 6633, and fungi: *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404. The fungi were cultured on potato-glucose agar for 7 days at 20ºC under alternating light and dark conditions. They were recultured on a new potato-glucose substrate for another 7 days. The culturing procedure was performed 4 times until pure culture was obtained. The identification of the test microorganisms was confirmed by the Laboratory of Mycology, Department of Microbiology, Institute Torlak, Belgrade, Serbia.

Minimum inhibitory concentration (MIC): The minimum inhibitory concentrations (MIC) of the extract and cirsimarin against the test bacteria were determined by the microdilution method in 96 multi-well microtiter plates [17]. All tests were performed in Muller–Hinton broth (MHB) with the exception of the yeast, in which case Sabouraud dextrose broth was used. A volume of 100 µL stock solutions of oil (in methanol, 200 µL/mL) and cirsimarin (in 10% DMSO, 2 mg/mL) was pipetted into the first row of the plate. Fifty µL of Mueller Hinton or Sabouraud dextrose broth (supplemented with Tween 80 at a final concentration of 0.5%, v/v, for analysis of oil) was added to the other wells. A volume of 50 µL from the first test well was pipetted into the second well of each microtiter line, and then 50 µL of scalar dilution was transferred from the second to the twelfth well. Ten µL of resazurin indicator solution (prepared by dissolving a 270 mg tablet in 40 mL of sterile distilled water) and 30 µL of nutrient broth were added to each well. Finally, 10 µL of bacterial suspension (10^6 CFU/mL) and yeast spore suspension (3×10^4 CFU /mL) was added to each well. For each strain, the growth conditions and the sterility of the medium were checked. The standard antibiotic amracin was used to control the sensitivity of the tested bacteria, whereas ketokonazol was used as a control against the tested yeast. Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37°C for 24 h for the bacteria and at 28°C for 48 h for the yeast. Color change was then assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of 3 values was calculated, and the obtained value was taken as the MIC for the tested compounds and standard drug.

Determination of total antioxidant capacity: The total antioxidant activity of the essential oil of *K. vitifolia* was evaluated by the phosphomolybdenum method [16]. The assay is based on the reduction of Mo (VI) – Mo (V) by antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acid pH. A total of 0.3 mL of sample extract was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then, the absorbance of the solution was measured at 695 nm using a spectrophotometer against the blank after cooling to room temperature. Methanol (0.3 mL) in place of extract was used as the blank. Ascorbic acid (AA) was used as the standard and total antioxidant capacity was expressed as mg of ascorbic acid per g of dry extract.

Determination of DPPH free radical scavenging activity: The method used by [17] was adopted with suitable modifications from [18]. DPPH (2,2-dephenyl-1-picrylhydrazyl) (8 mg) was dissolved in MeOH (100 mL) to obtain a concentration of 80 µg/mL. Serial dilutions were carried out with the stock solution (1 mg/mL) of the extract. Solutions (2 mL each) were then mixed with DPPH (2 mL) and allowed to stand for 30 min for any reaction to occur, and the absorbance was measured at 517 nm. Ascorbic acid (AA), gallic acid (GA) and butylated hydroxytoluene (BHT) were used as reference standards and dissolved in methanol to make the stock solution with the same concentration (1 mg/mL). Control sample was prepared containing the same volume without test compounds or reference antioxidants. Ninety-five % methanol was used as a blank. The DPPH free radical scavenging activity (%) was calculated using the following equation:

$$\% \text{ inhibition} = ((A_c - A_s)/A_c)*100$$

The IC_{50} value, defined as the concentration of the test material that leads to 50% reduction in the free radical concentration, was calculated in µg/mL through a sigmoidal dose-response curve.

Determination of inhibitory activity against lipid peroxidation: Antioxidant activity was determined by the thiocyanate method [19]. Serial dilutions were carried out with the stock solution (1 mg/mL) of the extracts, and 0.5 mL of each solution was added to linoleic acid emulsion (2.5 mL, 40 mM, pH 7.0). This was prepared by mixing 0.2804 g linoleic acid, 0.2804 g Tween-20 as emulsifier in 50 mL 40 mM phosphate buffer and the mixture was then homogenized. The final volume was adjusted to 5 mL with 40 mM phosphate buffer, pH 7.0. After incubation at 37ºC in the dark for 72 h, a 0.1 mL aliquot of the reaction solution was mixed with 4.7 mL of ethanol (75%), 0.1 mL $FeCl_2$ (20 mM) and 0.1 mL ammonium thiocyanate (30%). The absorbance of the mixture was measured at 500 nm and the mixture was stirred for 3 min. Ascorbic acid, gallic acid, α-tocopherol and BHT were used as reference compounds. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to the linoleic acid emulsion in the test sample and reference compound, was used. Inhibition percent of linoleic acid peroxidation was calculated using the equation above.

Measurement of ferrous ion chelating ability: The ferrous ion chelating ability was measured by the decrease in absorbance at 562 nm of the iron (II)-ferrozine complex [19, 20]. One mL of 0.125 mM $FeSO_4$ was added to 1.0 mL sample (with different dilutions), followed by 1.0 mL of 0.3125 mM ferrozine. The mixture was allowed to equilibrate for 10 min before the absorbance was measured. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and ferrozine only) using the equation above.

Determination of hydroxyl radical scavenging activity: The ability to inhibit non site-specific hydroxyl radical-mediated peroxidation was carried out according to the method described by [21]. The reaction mixture contained 100 µL of extract dissolved in water, 500 µL of 5.6 mM 2-deoxy-D-ribose in KH_2PO_4–NaOH buffer (50 mM, pH 7.4), 200 µL of premixed 100 µM $FeCl_3$ and 104 mM EDTA (1:1 v/v) solution, 100 µL of 1.0 mM H_2O_2 and 100 µL of 1.0 mM aqueous ascorbic acid. Tubes were vortexed and incubated at 50ºC for 30 min. Thereafter, 1 mL of 2.8% TCA and 1 mL of 1.0% TBA were added to each tube. The samples were vortexed and heated in a water bath at 50ºC for 30 min. The extent of oxidation of 2-deoxyribose was estimated from the absorbance of the solution at 532 nm. The percentage inhibition values were calculated from the absorbance of the control (Ac) and of the sample (As), where the controls contained all the reaction reagents except the extract or positive control substance. The values are presented as the means of triplicate analyses. The IC_{50} value, defined as the concentration of the test material that leads to 50% reduction in the free radical concentration, was calculated as µg/mL through a sigmoidal dose-response curve.

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