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Supercritical CO_2 extract and essential oil of bay (*Laurus nobilis* L.) – chemical composition and antibacterial activity

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Abstract: The present study deals with the supercritical carbon dioxide (SC-CO_2) extraction and hydrodistillation (HD) of dried bay leaves (*Laurus nobilis* L.). The chemical composition and antibacterial activity of the SC-CO_2 extract and essential oil (EO) from dried leaves of bay were compared to each other and literature data. Qualitative and quantitative analyses of the SC-CO_2 extract and EO were performed using GC–FID and GC–MS analytical methods. A significant difference in the chemical composition of the SC-CO_2 extract and EO was observed. The EO comprised high contents of monoterpenes and their oxygenated derivates (98.4 %), principally 1,8-cineole (33.4 %), linalool (16.0 %) and α-terpinyl acetate (13.8 %), sabinene (6.91 %) and methyl eugenol (5.32 %). The SC-CO_2 extract comprised twice less monoterpenes and their oxygenated derivates (43.89 %), together with sesquiterpenes (12.43 %), diterpenes (1.33 %) and esters (31.13 %). The major components were methyl linoleate (16.18 %), α-terpinyl acetate (12.88 %), linalool (9.00 %), methyl eugenol (8.67 %), methyl arachidonate (6.28 %) and eugenol (6.14 %). An investigation of the antibacterial activity of bay SC-CO_2 extract and EO was completed on different *Staphylococcus* strains using the broth macrodilution method. *Staphylococcus intermedius* strains were the most susceptible to both the SC-CO_2 extract and EO (*MIC* = 640 μg/ml).

Keywords: *Laurus nobilis*; bay; supercritical extraction; essential oil; antibacterial activity; gas chromatography.

INTRODUCTION

Dried leaves and the essential oil (EO) of bay (*Laurus nobilis* L.) are used extensively in the food industry for seasoning of meat products, soups, and fishes.[1]

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Several studies have evaluated the potential role of bay EO as an antimicrobial and antifungal agent,[2–4] as well as the antioxidant properties of leaves extracts.[5–8] Recently, bay extracts obtained by solvent extraction were studied for their cytotoxic activity.[9,10]

The EOs and plant extracts are generally obtained by hydrodistillation (HD) and solvent extraction (SE), although these methods suffer certain disadvantages. During HD, extensive hydrolysis and thermal degradation phenomena can be induced, giving in any case a product with a characteristic off-odor. SE can give an oil but, due to a high content of waxes and/or other high molecular mass compounds, often gives rise to a concentrate with a scent very similar to that of the material from which it was derived. A further drawback of SE is that small amounts of organic solvents can pollute the extraction product. Supercritical fluid extraction (SFE) can be used for the production of flavors and fragrances from natural materials and can constitute a valid alternative to both of the above-mentioned processes.[11] Tuning of the process parameters (pressure, temperature) enables tuning of the selectivity of supercritical carbon dioxide (SC-CO_2) towards desirable fractions as well as complete separation of the phases so that a solvent-free extract can be obtained. Several research groups investigated SC-CO_2 extraction in order to isolate biologically active compounds from *Laurus nobilis* leaves,[4,8,12,13] berries[14] and seeds.[15] The chemical composition of the EO and extracts isolated from bay leaves were studied by different researchers.[4,12,13,16–22]

Previously investigated bay EO isolated by HD was reported for its inhibitory effects on the pathogens[21] in following order: *Escherichia coli* O157:H7 > > *Staphylococcus aureus* > *Staphylococcus typhimurium* > *Listeria monocytogenes*. Bouzouita *et al.*[2] reported that the high content of 1,8-cineole in the EO of *L. nobilis* L. contributed to its weak antimicrobial activity on two bacteria (*Lactobacillus plantarum* and *E. coli*) and a fungus (*Geotrichum candidum*). Santoyo *et al.*[4] reported that a SC-CO_2 extract had the strongest antimicrobial activity against *S. aureus* ATCC 25923, substantial activity against *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC, 10145, *E. coli* ATCC 11 775 and *Candida albicans* ATCC 60193 strains while the fungi *Aspergillus niger* ATCC 16404 was the least susceptible.

In this study, SC-CO_2 extraction and hydrodistillation of dried bay leaves were compared with respect to their efficiency and selectivity. Thus, the yield and chemical composition of the SC-CO_2 extract and EO obtained by HD of bay leaves were investigated and are discussed herein. The antibacterial activity of bay SC-CO_2 extract and EO was investigated against chosen *Staphylococcus* strains.

EXPERIMENTAL

Plant material

Dried leaves of bay (*Laurus nobilis* L.) originating from Montenegro (2007) were used for the SC-CO_2 extraction and HD. The plant material was milled in a blender and sieved to the fraction with average particle diameter of 0.8–0.9 mm.

Supercritical carbon dioxide extraction

Extraction with SC-CO_2 was preformed in a previously described[23] pilot-plant-scale supercritical fluid extractor (Autoclave Engineers SCE Screening System) with a 150 ml extraction cell. Commercial carbon dioxide (99 % purity, Messer Tehnogas, Belgrade, Serbia) was used for the extraction. The SC-CO_2 extraction was performed under a pressure of 10 MPa and at a temperature of 40 °C (density of SC-CO_2, 630 kg/m^3). The initially used mass of the plant material was 24 g and the solvent rate was 0.3 kg/h.

Hydrodistillation

Plant material (24 g) and water (500 ml) were placed in a Clevenger-type apparatus. The EO was isolated by HD for 4 h. The obtained EO was kept in a sealed vial at 4 °C until required.

GC/FID/MSD

The qualitative and quantitative analyses of the SC-CO_2 extract and EO were performed using Hewlett–Packard GC–FID and GC–MS analytical methods. In the first instance, a model HP-5890 Series II chromatogram, equipped with a split-splitless injector, HP-5 capillary column (25 m×0.32 mm, film thickness 0.52 μm) and a flame ionization detector (FID), was employed. Hydrogen was used as the carrier gas (1 ml/min). The injector was heated at 250 °C, the detector at 300 °C, while the column temperature was linearly programmed from 40 to 260 °C (4 °C/min). GC–MS analyze was realized under the same analytical conditions, using a model HP G1800C Series II GCD analytical system equipped with an HP-5MS column (30 m×0.25 mm×0.25 μm). Helium was used as the carrier gas. The transfer line (MSD) was heated at 260 °C. The EI mass spectra (70 eV) were acquired in the scan mode in the *m*/*z* range 40–400. In each case, the sample in a solution in hexane (1 μl) was injected in the split mode (1:30). Identification of constituents was performed by matching their mass spectra and Kovats indices (I_K) with those obtained from authentic samples and/or the NIST/Wiley spectra libraries, different types of search (PBM/NIST/AMDIS) and available literature data (Adams, 2007).[25] Area percents, obtained by the integration of corresponding chromatograms (FID), were used for quantification of the individual components.

Antibacterial activity

The investigation of the antibacterial activity of the SC-CO_2 extract and EO was performed on six *Staphylococcus* strains originating from dogs, cattle, humans and vistual so of animal origin. The investigated strains were isolated from ear and tonsils swabs and from cheese and raw milk samples. A reference strain *S. aureus* ATCC 25923 (Becton Dickinson) was also included in the investigation.

The antimicrobial effects of the plant extracts were investigated by the broth macrodilution method according to CLSI (Clinical and Laboratory Standards Institute, 2008) prescribed references [26,27] for antimicrobial susceptibility testing. A single modification of the method concerned the fact that the plant extracts were used instead of antibiotics, but the principle of the procedure as well as the means of preparation and culture media were not altered. The antimicrobial activity of the plant extracts was investigated in concentrations (ex-

pressed in μg/ml): 1280; 640; 320; 160; 80; 40; 20 and 10. Mueller Hinton II broth (cation adjusted, CAMHB, Becton Dickinson), was used in the investigation. Bromocresol Purple 1.6 % (Merck) in a final concentration of 0.2/200 v/v for the gram-positive bacteria and Phenol Red 1 % in a final concentration of 1/200 v/v for the gram-negative bacteria were added to the CAMHB to obtain bacterial growth visibility. The desired inoculum density of 5×10^5 CFU/ml was achieved by preparing a suspension of the bacteria of approximately 1×10^8–2×10^8 CFU/ml, which was the same density as the McFarland standard 0.5 (Becton Dickinson). The prepared suspension was diluted 10 times to obtain a final inoculum density of approximately 1×10^7–2×10^7 CFU/ml and 50 μl of this suspension was applied to the CAMHB, after which the number of bacteria in the media was approximately 5×10^5/ml. The active substance gentamicin sulfate purity 685 μg/mg (Sigma) was used for comparative antibiotic susceptibility testing. The media were incubated at 37 °C for 18 h. The *MIC* values were taken as the lowest extract concentration in the broth with no visible bacterial growth.

RESULTS AND DISCUSSION

The yield of the EO was 1.43 % after 4 h of HD, which has been in accordance with previously published data.[11,12,17,18] Ozek *et al.*[13] reported oil yields (on a dry weight basis) of 2.6 % for hydro- and 1.9 % for steam distillation after 3 h (coastal line of Turkey). Carreda *et al.*[12] isolated 0.90 % of EO from bay leaves (southern Sardinia, Italy) after 4 h. Recently, a novel microwave method was applied to the hydrothermal extraction of essential oil from bay leaves.[18] This study[18] revealed that the yield of EO obtained by HD in a Clevenger-type apparatus equipped with an electric mantle heater for 1 h (traditional method) was 0.784 %, while the yields of EO obtained by HD with a 200 and 300 W microwave system for 1 h were 0.813 and 1.132 %, respectively. Verdian-Rizi *et al.*[19] obtained 0.654–1.132 % of EO from the aerial parts of bay in different vegetative stages after 4 h.

In the present study, the yield of bay SC-CO_2 extract obtained by a single-stage SC-CO_2 extraction was 1.37 % after 1.4 h of extraction ($m_{CO_2}/m_{solid} = 16.67$). Ozek *et al.*[13] reported similar yields of bay SC-CO_2 extract, 1.34 % (8 MPa and 40 °C) and 1.13 % (8 MPa and 50 °C). Carreda *et al.*[12] isolated a SC-CO_2 extract by fractional separation at 9 MPa and 50 °C (waxes were entrapped in the first separator set at 9 MPa and –10 °C, the oil was recovered in the second separator at 1.5 MPa and 10 °C). In the mentioned study,[12] the authors reported a yield of essential oil fraction of 0.82 % after 4 h ($m_{CO_2}/m_{solid} = 21.44$).

The results of chemical analyses of the obtained SC-CO_2 extract and essential oil (EO) accomplished by GC–FID and GC–MSD are presented in Table I. Thirty-four components were detected and identified in the EO of bay obtained by HD. The EO comprised mostly oxygenated monoterpenes (78.77 %) and hydrocarbon monoterpenes (19.68 %). Sesquiterpenes (1.06 %) and their oxygenated (0.53 %) were also found in the EO of bay. The main components in the EO were 1,8-cineole (33.4 %), linalool (16.0 %), α-terpinyl acetate (13.8 %), sabinene (6.91 %), methyl eugenol (5.32 %), α-pinene (4.39 %) and β-pinene (3.52 %). A si mi-

lar chemical composition of the oil extracted from bay leaves was observed by several authors. [12,13,17–21] In these papers, 1,8-cineole was reported to be the main component in the bay EO isolated by HD, whereby its content was in the range of 23.51–60.72 %.

TABLE I. Percentage composition of the compounds identified in the SC-CO_2 extract and EO (mass %)

Component	I_K (Kovats index)	SC-CO_2 Extract	*EO*
p-Xylene 871.6		0.44	–
α-Thujene 919.2		–	0.55
α-Pinene 924.8		–	4.39
Camphene 938.9		–	0.30
Sabinene 965.0		–	6.91
β-Pinene 967.2		–	3.52
Dehydro-1,8-cineole 984.4		–	0.21
β-Myrcene 985.1		–	0.14
α-Phellandrene 997.1		–	0.17
δ^3-Carene 1002.7		–	0.24
α-Terpinene 1009.3		–	0.42
p-Cymene 1017.7		–	0.41
Limonene-*β*-phellandrene 1020.9		–	1.59
1,8-Cineole 1025.0		2.53	33.4
γ-Terpinene 1051.3		–	0.74
cis-Sabinene hydrate	1061.5	0.25	0.30
Terpinolene 1080.7		–	0.33
Linalool 1096.3		9.00	16.0
δ-Terpineol 1161.0		0.49	0.57
Terpinen-4-ol 1170.3		0.90	2.38
p-Cymen-8-ol 1175.5		0.23	–
α-Terpineol 1184.5		2.54	2.83
Nerol 1227.0		0.44	0.19
Linalyl acetate	1250.4	0.58	0.34
4-Thujen-*2a*-yl acetate	1296.1	0.20	0.28
Bornyl acetate	1278.7	0.27	0.47
δ-Terpinyl acetate	1310.1	0.55	0.68
exo-2-Hydroxycineole acetate	1335.8	0.31	0.20
α-Terpinyl acetate	1343.8	12.88	13.8
Eugenol 1352.8		6.14	1.77
β-Elemene 1383.8		0.69	–
Methyl eugenol	1400.4	8.67	5.32
β-Caryophyllene 1409.8		0.87	0.43
α-Guaiene 1429.7		0.18	–
α-Humulene 1444.1		0.71	–
allo-Aromadendrene 1451.2		0.16	–
Germacrene D	1472.0	0.55	–
β-Selinene 1476.8		0.33	–
Bicyclogermacrene 1487.3		0.72	0.36
Germacrene A	1493.0	0.39	–

TABLE I. Continued

Component	I_K (Kovats index)	SC-CO_2 extract	*EO*
γ-Cadinene 1504.7		0.29	–
δ-Cadinene 1514.4		0.32	0.27
trans-Cadina-1,4-diene 1522.5		0.41	–
α-Cadinene 1534.0		0.79	–
Dauca-5,8-diene 1565.9		0.56	–
Spathulenol 1567.9		0.79	0.27
Caryophyllene oxide	1572.7	0.46	0.26
Viridiflorol 1581.4		0.49	–
Ledol 1592.3		0.21	–
Dihydro-*cis*-*α*-copaene-8-ol 1608.7		0.20	–
Eremoligenol 1619.5		0.37	–
β-Eudesmol 1640.0		1.45	–
Shyobunol 1680.3		0.25	–
Sedanenolide 1712.4		1.21	–
Neocnidilide (sedanolide)	1717.7	0.36	–
Oplopanone 1729.1		0.17	–
Neophytadiene isomer I	1806.8	0.26	–
Dehydrosaussurea lactone	1823.8	0.35	–
Hexahydrofarnesyl acetone	1835.0	0.40	–
Methyl palmitate[a] 1915.4		1.49	–
Eremanthin (vanillosimin)	1981.0	0.20	–
Methyl linoleate	2087.2	16.18	–
Methyl petroselinate[b]	2092.2 5.95		–
Phytol 2102.4		1.33	–
Methyl stearate[c] 2117.5		1.23	–
Methyl arachidonate	2215.1	6.28	–

[a]Methyl hexadecanoate; [b]methyl *cis*-6-octadecenoate; [c]methyl octadecenoate

Sixty-three components were detected of which fifty two were identified (93.0 %) in the bay SC-CO_2 extract. The supercritical extract comprised mostly oxygenated monoterpenes (43.2 %) and fatty acid esters (31.13 %), followed by sesquiterpene hydrocarbons (7.26 %) and their oxygenated derivates (5.17 %), hydrocarbons (2.60 %), phthalides (1.57 %), diterpenes (1.33 %) and monoterpene hydrocarbons (0.69 %). The most abundant components in the SC-CO_2 extract were methyl linoleate (16.18 %), *α*-terpinyl acetate (12.88 %), linalool (9.00 %), methyl eugenol (8.67 %), methyl arachidonate (6.28 %) and eugenol (6.14 %). A comparison of the chemical composition of the SC-CO_2 extract and that of the EO revealed significant differences. The SC-CO_2 extract comprised more than two times less monoterpene hydrocarbons and oxygenated monoterpenes (43.89 %) in comparison to EO (98.4 %). Carreda *et al.*[12] studied the chemical composition of fractions of the SC-CO_2 extract during 4 h. According to this study,[12] the lighter compounds (hydrocarbon monoterpenes) were extracted almost completely during the first extraction hour, the content of oxygenated monoter-

penes decreased to a minor extent with time, content of hydrocarbon sesquiterpenes increased significantly with time, while the content of oxygenated sesquiterpenes did not change much after the 3rd hour.

Buttery *et al.*[28] stated that 1,8-cineole is the major aroma component of bay oil, followed by linalool. In addition, substances present in lower concentrations, such as eugenol and (*E*)-isoeugenol, and especially the non-identified compounds at trace levels, possessing a pepper-like odor, have to be considered as key aroma compounds with a marked influence on the overall odor and flavoring quality of the leaves.[27] In the present study, the contents of eugenol and methyl eugenol were two times higher then in the EO. A significant difference in the 1,8-cineole content in the EO and extract was also observed. The SC-CO_2 extract in this study had a very low content of 1,8-cineole (2.53 %) and high contents of eugenol (6.14 %) and methyl eugenol (8.67 %) compared to those previously reported for an SC-CO_2 extract.[12] This can be result of the shorter extraction time applied in the present study (1.4 h), since Carreda *et al.*[12] observed remarkable differences in the contents 1,8-cineole and methyl eugenol after the first and fourth hour of extraction (1,8-cineole, 30.98 *vs.* 2.05 % and methyleugenol, 6.85 *vs.* 16.42 %). Ozek *et al.*[13] identified high contents of 1,8-cineole (40.2–43.0 %) and low contents of eugenol and methyl eugenol (0.7–0.8 %) in SC-CO_2 extracts obtained at 8 MPa and at temperatures of 40 and 50 °C.

According to the *MIC* values given in Table II, bay EO and SC-CO_2 extract had the same antibacterial activity against the investigated *S. intermedius* and *S. aureus* strains. One of the *S. intermedius* strains was more susceptible to the presence of the SC-CO_2 extract and EO, with an *MIC* value of 640 μg/ml. However, the antibacterial activities against the other *Staphylococcus* strains were lower with an *MIC* value of 1280 μg/ml.

TABLE II. The minimum inhibitory concentrations (MIC) of the bay SC-CO_2 extract measured by the broth macrodilution (BMD) test

Bacterial strain	Origin of the examined strains	*MIC* / μg ml^{-1}		
		EO SC	-CO_2 extract	Gentamicin
S. aureus ATCC 25923	Reference strain	1280	1280	≤0.5
S. intermedius	Ear swab from dog	640 640		2
S. intermedius	Ear swab from dog	1280 1280		1
S. aureus	Feta cheese	1280	1280	1
S. aureus	Milk sample from cow with masititis	1280 1280		1
S. aureus	Tonsil swab from human	1280 1280		2

Antibacterial activity of the SC-CO_2 extract and EO isolated from bay leaves could be the result of high contents of linalool (SC-CO_2, 9.00 %; EO, 16.00 %), α-terpinyl acetate (SC-CO_2, 12.88 %; EO, 13.8 %), methyl eugenol (SC-CO_2,

8.67 %; EO, 5.32 %), eugenol (SC-CO_2, 6.14 %; EO, 1.77 %) and α-terpineol (SC-CO_2, 2.54 %; EO, 2.83 %), which were previously reported to have antibacterial activity.[29] High contents of methyl esters were identified in the SC-CO_2 extract (methyl linoleate, 16.18 %; methyl arachidonate, 6.28 %). The high antibacterial activity of eugenol was previously reported.[30] Fatty acids and fatty acid methyl esters were also reported to have significant antibacterial and antifugal activity.[31] In the present study, despite the much lower content of 1,8-cineole in the SC-CO_2 extract, the high contents of eugenol, methyl eugenol, and methyl esters[31] together with other active components (*e.g.*, linalool, α-terpinyl acetate) could contribute to its antibacterial activity.

CONCLUSIONS

In this study, similar yields of EO and SC-CO_2 extract were observed, although the supercritical extraction was a less time-consuming process. This study reported significant antimicrobial activity of bay EO and SC-CO_2 extract against the tested *Staphylococcus* strains. Despite having much lower contents of monoterpenes and their oxygenate derivates, which are generally considered to be responsible for antibacterial activity, the SC-CO_2 extract had the same antibacterial activity as the EO. The high contents of eugenol, methyl eugenol and fatty acid methyl esters together with other active components (*e.g.*, linalool, α-terpinyl acetate, 1,8-cineole) in the SC-CO_2 extract could contribute to its overall antibacterial activity. One of the *S. intermedius* strains was more susceptible to both bay EO and SC-CO_2 extract than the other strains. The presented results indicate that bay EO and SC-CO_2 extract could be considered for use not only as a spice and flavoring agent but also as preservative in the food industry.

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ИЗВОД

НАТКРИТИЧНИ ЕКСТРАКТ И ЕТАРСКО УЉЕ ЛОВОРА (*Laurus nobilis* L.) – ХЕМИЈСКИ САСТАВ И АНТИБАКТЕРИЈСКА АКТИВНОСТ

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У раду је испитана надкритична екстракција и хидродестилација осушених листова ловора (*Laurus nobilis* L.). Приказана је упоредна анализа хемијског састава и антибактеријске активности надкритичног екстракта и етарског уља као и поређење истих са литературним подацима. За квалитативну и квантитативну анализу хемијског састава надкритичног екстракта и етарског уља коришћене су GC–FID и GC–MS аналитичке методе. Хемијски састав надкритичног екстракта и уља ловора био је веома различит. Најзаступљеније компоненте у етарском уљу били су монотерпени и њихови кисеонични деривати (98,4 %), пре свега 1,8-

-цинеол (33,4 %), линалоол (16,0 %), α-терпинил-ацетат (13,8 %), сабинен (6,91 %) и метил--еугенол (5,32 %). Надкритични екстракт ловора садржао је два пута мању количину монотерпена и њихових кисеоничних деривата у односу на етарско уље (43,89 %) поред сесквитерпена (12,43 %), дитерпена (1,33 %) и естра (31,13 %). У надкритичном екстракту најзаступљеније компоненте били су метил-линолеат (16,18 %), α-терпинил-ацетат (12,88 %), линалоол (9,00 %), метил-еугенол (8,67 %), метил-арахидонат (6,28 %) и еугенол (6,14 %). Антибактеријско деловање надкритичног екстракта и етарског уља ловора испитивано је на сојевима *Staphylococcus* применом макродилуционе методе у бујону. Сојеви *Staphylococcus intermedius* били су најосетљивији на надкритични екстракт и етарско уље ловора при чему су вредности *MIC* биле 640 μg/ml.

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