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Phytochemical Profile and Anticholinesterase and Antimicrobial Activities of Supercritical versus Conventional Extracts of *Satureja montana*

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Winter savory *Satureja montana* is a medicinal herb used in traditional gastronomy for seasoning meats and salads. This study reports a comparison between conventional (hydrodistillation, HD, and Soxhlet extraction, SE) and alternative (supercritical fluid extraction, SFE) extraction methods to assess the best option to obtain bioactive compounds. Two different types of extracts were tested, the volatile (SFE-90 bar, second separator vs HD) and the nonvolatile fractions (SFE-250 bar, first and second separator vs SE). The inhibitory activity over acetyl- and butyrylcholinesterase by *S. montana* extracts was assessed as a potential indicator for the control of Alzheimer's disease. The supercritical nonvolatile fractions, which showed the highest content of (+)-catechin, chlorogenic, vanillic, and protocatechuic acids, also inhibited selectively and significantly butyrylcholinesterase, whereas the nonvolatile conventional extract did not affect this enzyme. Microbial susceptibility tests revealed the great potential of *S. montana* volatile supercritical fluid extract for the growth control and inactivation of *Bacillus subtilis* and *Bacillus cereus*, showing some activity against *Botrytis* spp. and *Pyricularia oryzae*. Although some studies were carried out on *S. montana*, the phytochemical analysis together with the biological properties, namely, the anticholinesterase and antimicrobial activities of the plant nonvolatile and volatile supercritical fluid extracts, are described herein for the first time.

KEYWORDS: *Satureja montana*; supercritical fluid extraction; HPLC-DAD; anticholinesterase activity; Alzheimer's disease; antimicrobial activity

INTRODUCTION

Satureja montana, commonly called winter or mountain savory, is a perennial flowering plant, evergreen shrub belonging to the Lamiaceae family, which is native to warm temperate regions of the Mediterranean. This aromatic herb, found in nature and also cultivated, has been used for hundreds of years as a food condiment, as a tea, and also to garnish salads. In addition, various medicinal benefits have been reported for this plant, in particular, upon the whole digestive system. With respect to the biological properties and economic value, European and international patents were recently registered for *S. montana* extracts, claiming their anti-infertility properties (*1*). Other bioactivities described for *S. montana* include potent anti-HIV-1 activity of its aqueous extracts (*2*) and antiproliferative activity on human erythroleukemic K562 cells of the herb essential oil, which contained α-terpineol as the major constituent (*3*). The antimicrobial activity of *S. montana* essential oils is well documented (*4*−*7*). In addition, Cetkovic et al. reported that various *S. montana* L. subsp. *kitaibelii* extracts expressed inhibitory activity against both Gram-positive and Gram-negative bacteria (*8*).

The Local Food Nutraceuticals Consortium, which aimed to study traditional foods known to have potential against aging-related pathologies and to develop new products to be used as high-quality food supplements, studied a wide number of plant species including *S. montana*, which revealed a high activity on antioxidant, anti-inflammatory, and mood-disorder related assays as well as on xanthine oxidase (XO) and myeloperoxidase-catalyzed guaiacol oxidation (G−OH) enzyme inhibition assays (*9*). More recently, Cetkovic et al. reported on the antioxidant activity of various *S. montana* L. subsp. *kitaibelii* extracts (*8*), and *S. montana* phenolic constituents thymol and carvacrol, present in the essential oil (55% in total), were considered by Cavar et al.

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to be responsible for the overall reactivity of the savory oil toward DPPH (*4*). However, the antiradical activity of a savory essential oil from Slovenia, with respect to DPPH, was greater than that determined for its major components carvacrol (41.5%) and thymol (8.6%) (*10*).

Antioxidants may be applied as neuroprotective agents in the early stage of Alzheimer's disease (AD) (*11*). Although the origin of AD remains unknown, the depletion of neurotransmitters such as acetylcholine caused by the enzymes acetylcholinesterase (AChE) is clearly related to this disease (*12*). The mechanism of action of the available drugs for treating AD is based mainly on the inhibition of the enzyme AChE. Although a variety of natural AChE inhibitors are already known (*13*), the screening of plants used to treat memory dysfunction for AChE inhibitory activity continues to be pursued (*14*, *15*). Recently, butyrylcholinesterase (BChE) received particular attention because it is a coregulator of cholinergic neurotransmission and its activity is increased in AD and associated with all neuropathological lesions in this disease (*16*). Although selective BChE inhibitors of synthetic origin based on quinazolinimines and lipoic acid have been reported (*11*), the search for natural substances, more efficient and less expensive than those currently in use for patient's palliative care and treatment, is a mandatory research branch.

The promising bioactivities described for *Satureja* species encouraged us to further investigate *S. montana* and to compare various extraction methods focusing on the enrichment of active principles in the extract. Hydrodistillation (HD) and Soxhlet extraction (SE) are traditional techniques to recover compounds from aromatic plants. However, some drawbacks can be attributed to them, namely, hydrolysis and thermal degradation for HD and solvent contamination for SE. To overcome these limitations, supercritical fluid extraction (SFE) was used as an alternative method. SFE with CO_2 is a versatile technique, very suitable to obtain different plant extracts, because the manipulation of process parameters such as temperature and pressure of the supercritical fluid can change its solvent power (*17*, *18*). Hence, the main objectives of this work are as follows: (i) to produce *S. montana* extracts by SFE and by conventional methods and to characterize the chemical profile of each extract; (ii) to study the effect of the extraction method on the bioactivities of the extracts; (iii) to investigate both AChE and BChE inhibition and give new perspectives for the plant extracts as potential nutraceuticals against neurodegenerative disorders; (iv) to evaluate the influence of the extraction methodologies over the microbial susceptibility of different bacteria and fungi strains.

MATERIALS AND METHODS

Plant Material. Aerial parts (leaves and flowers) of *S. montana* (cultivated at Centro de Investigación y Tecnología Agroalimentaria, Ejea de los Caballeros, Zaragoza, Spain, 2005) were frozen with liquid N_2 and ground with a commercial mill. Different particle sizes were obtained and separated with a series of sieves (1.0, 0.8, 0.6, 0.5, 0.4, 0.3, 0.2, and 0.1 mm). After this process, the fractions were kept at −20 °C in dark bottles. The initial moisture content of the plant material was 11.8%.

SFE Apparatus. The SFE apparatus used in this work was described in detail by Reis-Vasco et al. (*19*). Briefly, it is composed by a diaphragm pump, an extraction vessel (1 L), and two separators (0.27 L), operating in series. A back-pressure regulator is used to control the pressure, which is measured with a Bourdon type manometer, and the total volume of CO_2 is determined with a dry test meter. A preset temperature in the extraction vessel is reached with the aid of a water jacket. The CO_2 (99.995% purity) used in the studies was supplied by Air Liquid (Lisbon, Portugal).

Extraction of Essential and Volatile Oils. The essential oil was isolated by HD in a Clevenger apparatus, for 4 h, using 40 g (dw) of plant material with a mean particle size of 0.6 mm (extract C). For volatile oil isolation by SFE, plant material (100 g dw) was placed inside the extractor between two layers of glass wool, and the extraction conditions were as follows: pressure of 90 bar, temperature of 40 °C, mean particle size of 0.6 mm, and flow rate of 1.1 kg/h. To obtain a pure volatile oil free of waxes in the second separator (extract A), a pressure of 80 bar and a temperature of −8 °C, in the first separator (to allow waxes precipitation), and a pressure of 20 bar and a temperature of −15 °C, in the second one (to allow the volatile oil precipitation), were selected. These were the experimental conditions that represented the best compromise between yield and volatile oil composition. Moreover, the amounts of waxes and volatile oil were determined gravimetrically (w/w).

Extraction of the Nonvolatile Fraction. After HD, the plant residue (5 g) was dried and subjected to a Soxhlet extraction with acetone (SE), for 5 h, to obtain the nonvolatile components (extract D). After SFE at 90 bar, 40 °C, 0.6 mm particle size, and 1.1 kg/h of CO_2 flow rate, the same plant matrix was submitted to an extraction, changing only the pressure to 250 bar, for 4 h (SFE extracts B1 and B2, obtained in the first and second separators, respectively, operated at 90 bar/40 °C and 20 bar/20 °C).

Gas Chromatography−Mass Spectrometry (GC-MS) Analysis of the Essential Oil. GC quantitative analyses were performed in a Hewlett-Packard 5890 gas chromatograph (HP, Waldbronn, Germany), equipped with a flame ionization detector (FID) and a fused-silica DB-5 capillary column (J&W; 30 m × 0.25 mm i.d., film thickness = 0.25 μm; Folsom, CA). Oven temperature was programmed isothermally to 40 °C, during 2 min, then was raised, at 3 °C/min to 230 °C, and finally increased at 5 °C/min to 310 °C, and held at this temperature for 15 min. The injector and detector were set at the same temperature, 310 °C. Helium was used as carrier gas at a flow rate of 24 cm/s, and the split ratio was 1:50. The percentage composition of the oils was computed by the normalization method from the GC peak areas without using response factors.

The GC-MS system consisted of a Perkin-Elmer Autosystem XL gas chromatograph (Perkin-Elmer, Shelton, CT) equipped with a DB-1 fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 μm; J&W Scientific Inc., Agilent Technologies, Santa Clara, CA), and interfaced with a Perkin-Elmer Turbomass mass spectrometer (software version 4.1; Perkin-Elmer, Shelton, CT). Oven temperature was programmed from 45 to 175 °C, at 3 °C/min, subsequently at 15 °C/min to 300 °C, and then held isothermal for 10 min; injector and detector temperatures, 280 and 290 °C, respectively; transfer line temperature, 280 °C; ion trap temperature, 220 °C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; ionization current, 60 μA; scan range, 40−300 u; scan time, 1 s.

The identity of the components was assigned by comparison of their retention indices, relative to C9−C18 *n*-alkanes indices (Supelco, Bellefonte, PA) and GC-MS spectra from a homemade library, constructed on the basis of the analyses of reference oils, laboratory-synthesized components, and commercial available standards.

HPLC-DAD Analysis of the Nonvolatile Fraction. For HPLC purposes, the following chemicals and standards were purchased: acetonitrile (ACN), (99.9%) and methanol (MeOH, 99.9%) (Panreac, Barcelone, Spain); acetic acid (99.8%) (Sigma-Aldrich, Buchs, Switzerland); ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid, 99%), caffeic acid (3,4-dihydroxycinnamic acid, 97% predominantly trans), syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid, 98%), gallic acid (3,4,5-trihydroxybenzoic acid, 97%), gentisic acid (2,5-dihydroxybenzoic acid, 98%), coumaric acid (*trans*-4-hydroxycinnamic acid, 98%), protocatechuic acid (3,4-dihydroxybenzoic acid, 97%), vanillic acid (4-hydroxy-3-methoxybenzoic acid, 97%), and (+)-catechin hydrate ((2*R*,3*S*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol, 98%) (Sigma-Aldrich, Buchs, Switzerland); chlorogenic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate), ≥95%) (Extrasynthèse, Genay, France) and (−)-epicatechin green tea ((2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol, ≥98%) (Sigma-Aldrich, Buchs, Switzerland). Ultrapure water was obtained from Milli-Q water purification systems (Millipore, Bedford, MA). The stock solutions of individual standards (1.00 mg/L) and samples were prepared in HPLC grade MeOH and used to prepare the working standard mixtures at the desired concentration. To prevent photodegradation, after experimental procedure, the stock solutions and working standards were wrapped in aluminum foil and stored at −4 °C.

HPLC-DAD analyses were carried out on an Agilent 1100 series LC system (Agilent Technologies, Waldbronn, Germany), composed by the

following modules: vacuum degasser (G1322A), quaternary pump (G1311A), autosampler (G1313A), thermostated column compartment (G1316A), and diode array detector (G1315B). Data acquisition and instrumental control were performed by the software LC3D ChemStation (version rev. A. 10.02[1757]; Agilent Technologies). Analyses were performed on a Tracer excel 120 ODS-A column, 150 mm × 4.0 mm, 5 μm particle size (Teknokroma, Barcelone, Spain). The mobile phase consists of a mixture of acetonitrile (solvent A) and acetic acid aqueous solution, pH 2.8 (solvent B). The applied gradient was 0−40 min, 1−32.2% A; 40−45 min, 32.2−40% A; 45−50 min, 40−1% A; and hold at 1% A for 10 min. The flow rate was 1.0 mL/min, the analyses were performed at 26 °C, and the injection volume was 10 μL with a draw speed of 200 μL/min. The detector was set at 280 nm. For identification purposes, a standard addition method was used by spiking the samples with the pure standards, as well as by comparing the retention parameters and UV−visible spectral reference data. The composition was determined through the normalized peak areas at 280 nm.

Anticholinesterase Assays. Ellman's assay (*20*) was used to screen the in vitro anticholinesterase activities of five *S. montana* extracts. Absorbency of the colored ion was detected at 410 nm on a double-beam spectrophotometer Shimadzu equipped with thermostatic cell holders operating on the kinetic mode. Appropriate disposable plastic Plastibrand cuvettes were used in the kinetic experiments, and absorbance data were acquired by means of UV Probe software. Enzyme activity (percent) and enzyme inhibition (percent) were calculated from the rate of absorbance change with time ($V = \Delta Abs/\Delta t$) data as follows:

$$\text{enzyme inhibition (\%)} = 100 - \text{enzyme activity (\%)}$$

$$\text{enzyme activity (\%)} = 100 \times V/V_{max}$$

Maximum rates (V_{max}) are obtained when no inhibitor is used, whereas V is the rate obtained in the presence of the inhibitor.

The following pa reagents were purchased from Sigma-Aldrich (Germany): AChE from bovine erythrocytes, BChE from human serum, acetylthiocholine iodide (ATchI), *S*-butyrylthiocholine iodide (BTchI), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), KH_2PO_4, KOH, and $NaHCO_3$. Deionized/sterilized water was used to prepare the buffer (pH 8.0), substrate, and DTNB solutions.

The extracts of *S. montana* and the controls (rivastigmine and donepezil) were initially dissolved in DMSO and diluted with distilled water until concentrations decrease to values between 0.044 and 4.4 mg/mL, to yield the final concentrations for the enzymatic test between 1 and 100 μg/mL. No inhibition was detected by residual DMSO (< 0.5%) at the reaction cuvette.

The absorbance data along the reaction time was taken for 4 min under a controlled temperature of 30 °C. A mixture of freshly prepared 0.1 M phosphate buffer (pH 8.0; 200 μL), enzyme solution in freshly prepared phosphate buffer [AChE (1.32 U/mL) or BChE (0.44 U/mL); 5 μL], DTNB 0.01 M in aqueous $NaHCO_3$ (0.02 M; 5 μL), and plant extract (5 μL) solution were kept for 15 min at 30 °C in a heated water bath, and then the substrate reagent ATchI or BTchI aqueous solution 0.022 M (5 μL) was added to start the enzymatic reaction. At least four replicates were made. Assays without the plant extract were carried out to determine the average V_{max}. Also, white assays without the enzyme and plant extract were performed to check for any nonenzymatic hydrolysis of the substrate. The final concentrations of chemicals in the test were as follows: [AChE] = 0.03 U/mL, [BChE] = 0.01 U/mL, [plant extract] = 1−100 μg/mL, [DTNB] = 0.0002273 M, [ATchI] = [BTchI] = 0.0005 M.

Statistical Data Analysis and Regression. Enzyme inhibition assays were conducted in quadruplicate, and all tabulated results were compared using Student's *t* test. A P value of < 0.05 was considered to be significant.

IC_{50} values were determined for the active extracts using nonlinear regression analysis (Statistical Software Origin Pro 8, OriginLab Corp.) toward a dose−response curve (enzyme inhibition vs extract concentration).

Microbial Susceptibility Tests. The antimicrobial activities of the five extracts of *S. montana* were initially screened using the paper disk diffusion method (*21*, *22*). When the extract was active, the minimum inhibitory and lethal concentrations (MIC, MLC) were assessed by the dilution method (*22*).

The microorganisms used in the tests belong to the American Type Culture Collection (ATCC) and to the Centraalbureau voor Schimmelcultures (CBS) collections, from the United States and The Netherlands, respectively. Additional fungus strains kept in our laboratory were also used. With regard to pathogenic bacteria, tests were carried out with *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 8739), *Listeria monocytogenes* (ATCC 7644), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enteritidis* (ATCC 13076), and *Staphylococcus aureus* (ATCC 25923). With respect to fungi, the pathogenic yeast *Candida albicans* (ATCC 10231) and the following phytopathogenic filamentous fungi were used: *Alternaria alternata* (CBS 108.41), *Biscogniauxia mediterranea* (CBS 101016), *Botrytis* spp., *Byssochlamys fulva* (CBS 146.48), *Colletotrichum coffeanum* (CBS 396.67), *Fusarium culmorum* (CBS 129.73), *Pyricularia oryzae* (CBS 433.70), *Rhizopus* spp., and *Stachybotrys chartarum* (CBS 414.95). For the disk diffusion method, fungal growth was performed on potato dextrose agar at 25 °C, whereas bacteria were incubated in nutrient agar at 37 °C. Paper disks of 6.4 mm were placed on the agar, and a solution of each extract (300 μg) in DMSO (15 μL) was applied on each disk. Chloramphenicol and actidione were used as positive controls for bacteria and fungi, respectively, and three replicates were made. After incubation, the nearest diameter of the inhibition zone was measured. For the dilution method, a series of dilutions starting at 500 μg/mL to 1.95 μg/mL were made for the extracts, whereas chloramphenicol dilutions ranged between 50 and 0.195 μg/mL. Bacteria were incubated at 35 °C for 16−20 h, and two replicates were made.

RESULTS AND DISCUSSION

GC-MS Analysis. The volatile extracts of *S. montana* obtained by HD and by SFE were submitted to a GC-MS analysis to determine the effect of the extraction method on the chemical composition and on the biological activities of each extract.

Thirty-five components were identified in the volatile HD and SFE extracts, and the oxygenated monoterpenes were the most abundant ones, followed by monoterpene hydrocarbons. Carvacrol, *p*-cymene, thymol, and γ-terpinene were the major compounds detected in HD and SFE volatile extracts (**Table 1**). Smaller amounts of bisabolene, *trans*-caryophyllene, α-terpinene, and myrcene were also detected in the HD and SFE extracts. With the exception of γ-terpinene (8.9% in HD; 4.3% in SFE) and thymoquinone (0.2% in HD; 2.9% in SFE), there are no significant differences between the chemical profiles of both extracts. Despite some variations that may occur in *S. montana* essential oil composition, due to development stages and/or geographical conditions, it can be characterized by a high percentage of monoterpene phenols such as carvacrol and thymol (*4*). However, the main components of essential oils vary according to the species studied. For example, the essential oil *Satureja horvatii* Silic, obtained by HD, has thymol as the major component (63.37%), whereas γ-terpinene (7.49%) and carvacrol (4.67%) are minor constituents (*23*).

HPLC-DAD Analysis. Identification and quantification of phenolic compounds can give vital information related to a plant's health benefits (*24*). Thus, the nonvolatile fractions composed by SFE extracts B1 and B2 and by SE extract D were analyzed by HPLC-DAD, in terms of their phenolic composition. For identification purposes, standard addition methodology was applied by spiking the samples with 11 pure standards, under standard instrumental conditions. All 11 phenolic acids and flavanols were present in the three extracts (B1, B2, and D), even though differences in composition of each individual compound were observed. The relative percentage of each compound detected in the analyzed extracts at 280 nm is summarized in **Table 2**. The highest levels of gallic, caffeic, syringic, and gentisic acids as well as (−)-epicatechin were detected in extract D, whereas (+)-catechin and protocatechuic, ferulic, chlorogenic, and vanillic acids were predominant in extract B2. Only coumaric

Table 1. Yield and Percentage Composition of *Satureja montana* Volatiles Obtained by Hydrodistillation (HD) and Supercritical Fluid Extraction (SFE)[a]

component	RI	HD	SFE
α-thujene	1010	0.6	0.3
α-pinene	1017	0.6	0.3
camphene	1032		
sabinene	1060	0.2	0.1
1-octen-3-ol	1062	0.1	0.1
β-pinene	1063	0.8	0.6
myrcene	1081	1.0	0.6
α-phellandrene	1093	0.2	0.2
δ-3-carene	1100	0.1	t
α-terpinene	1109	1.7	1.2
p-cymene	1117	12.8	10.1
1,8-cineole	1122	0.5	0.4
β-phellandrene	1122	0.5	0.4
limonene	1124	0.4	0.3
cis-β-ocimene	1145		0.1
γ-terpinene	1158	8.9	4.3
trans-sabinene hydrate	1166	0.5	0.7
terpinolene	1191	0.2	0.1
cis-sabinene hydrate	1201	0.1	0.2
linalool	1210	0.8	0.7
borneol	1279	0.7	0.7
terpinen-4-ol	1292	0.7	0.4
α-terpineol	1305	0.2	0.2
carvone	1366		0.2
thymoquinone	1371	0.2	2.9
carvacrol methyl ether	1381	0.1	
thymol	1419	11.0	10.9
carvacrol	1431	52.2	52.7
unknown	1507	0.1	0.1
β-bourbonene	1507	0.1	0.1
trans-β-caryophyllene	1551	1.3	1.5
trans-bergamotene	1579	0.2	0.2
γ-muurolene	1581	0.1	0.1
germacrene D	1606	0.2	0.3
β-bisabolene	1636	2.0	2.5
thymohydroquinone	1651	0.4	0.5
β-caryophyllene oxide	1707	0.2	0.2
α-bisabolol	1776		t
yield % (w/w)		1.5	1.4
identified components (%)		99.4	96.2
monoterpene hydrocarbons		27.8	20.3
oxygenated monoterpenes		66.8	70.4
sesquiterpene hydrocarbons		4.4	5.1
oxygenated sesquiterpenes		0.2	0.2
others		0.1	0.1

[a] SFE at 90 bar, 40 °C, mean particle size of 0.6 mm and flow rate of 1.1 kg/h; RI, retention indices relative to C9−C18 *n*-alkanes on the DB-5 column; t, trace (<0.05%).

acid content was more pronounced in extract B1. Studies on the composition of the nonvolatile fraction *S. montana* have been previously reported by Ćetković et al. (*24*). They analyzed different extracts obtained by maceration with a methanol/water mixture (70%), evaporation of methanol, and successive extraction with petroleum ether, chloroform, ethyl acetate, and *n*-butanol and concluded that the last two extracts showed the highest phenolic content. With the exception of gallic acid, their qualitative composition is similar to that obtained in the present work for the conventional acetone and supercritical extracts. However, the relative percentages of the components are quite different, catechin and epicatechin being the major phenolics detected in ethyl acetate and *n*-butanol extracts.

Table 2. Relative Percentage of Phenolic Compounds Detected in Three Extracts from the Nonvolatile Fraction of *Satureja montana* Determined by HPLC-DAD at 280 nm

	extracts[a] (%)		
phenolic compound	B1	B2	D
gallic acid	1.35	0.14	1.93
protocatechuic acid	0.18	0.38	0.03
gentisic acid	0.10	0.03	0.37
chlorogenic acid	0.60	0.75	0.29
(+)-catechin	0.25	0.67	0.25
vanillic acid	0.21	1.03	0.38
caffeic acid	0.46	0.21	0.92
syringic acid	0.32	0.19	0.91
(−)-epicatechin	0.20	0.75	0.80
coumaric acid	0.52	0.22	0.39
ferulic acid	0.40	0.42	0.29

[a] B1, supercritical fluid extract from first separator; B2, supercritical fluid extract from second separator; D, Soxhlet extract.

Enzyme Inhibition. The inhibition studies of acetylcholinesterase and butyrylcholinesterase revealed that essential oil and volatile extracts (HD and SFE) of *S. montana* inhibit both enzymes (**Table 3**). The best results were obtained with HD extract, which inhibits significantly ($P < 0.001$) AChE and BChE at 10 and 22 μg/mL, respectively. The bioactivity exhibited by HD and SFE extracts is expected to be due to carvacrol, their major component (**Table 1**), which is known to possess AChE inhibitory activity (*25*). The other major constituents, thymol, *p*-cymene, and γ-terpinene, present in lower amount, also possess antiacetylcholinesterase activity (*25*, *26*). Thymohydroquinone, which is more effective than carvacrol as acetylcholinesterase inhibitor (*25*), is also present as a minor constituent.

In contrast with the essential oil/volatile extracts, the nonvolatile fraction did not affect AChE activity but exerted a significant ($P < 0.001$) inhibition against BChE, even at 5 μg/mL (extract B2) and 22 μg/mL (extract B1). As shown in **Table 3**, extract D (Soxhlet acetone extraction) did not affect either enzyme's activity, at least at the concentrations tested. The composition of the studied fractions is quite complex (**Table 2**) and includes some anticholinesterase compounds, such as gallic acid, that promoted ca. 55% AChE inhibition at 1.36 g/L (*27*), as well as chlorogenic and caffeic acids (*28*). In addition, ferulic acid is known to inhibit the formation and extension of β-amyloid fibrils from amyloid β-peptide in a dose-dependent manner (*29*), an activity that gives an added-value to the bioactive extracts as potential nutraceuticals against Alzheimer's disease.

According to Li et al., phenolic acids are among the main antioxidative phytochemicals that have benefits to human health, reducing the risks of some age-related impairments including Alzheimer's disease (*30*). When extracts obtained from the nonvolatile fraction are compared (**Table 2**), extract B2 has the best relative percentages of (+)-catechin and vanillic, chlorogenic, and protocatechuic acids. The last two acids are the standard phenolic acids with the best antioxidant capacity, followed by caffeic, *p*-hydroxybenzoic, gentisic, ferulic, vanillic, syringic, and *p*-coumaric acids (*30*). In addition, Kanski et al. indicated that ferulic acid, present in higher amounts in B1 and B2 supercritical extracts, greatly reduces free radical damage in neuronal cell systems, suggesting the importance of naturally occurring antioxidants in the therapeutic intervention against neurodegenerative disorders, such as Alzheimer's disease, in which oxidative stress is implicated (*31*).

The inhibition levels obtained with the above extracts are lower than those obtained for the reference drugs rivastigmine (BChE) and donepezil (AChE) at the same concentrations and assay

Table 3. Inhibition (Percent) of Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) by *Satureja montana* Extracts

S. montana extract		final concentration (μg/mL)	AChE inhibition[a] (%)	BChE inhibition[a] (%)
volatile oil fraction	SFE at 90 bar	100	35 ***	53 ***
	extract A	22	24 *	33 ***
		10	5	11 *
		5	<5	11 *
		1	<5	8
essential oil fraction	HD	100	57 ***	62 ***
	extract C	22	53 ***	34 ***
		10	40 ***	21 *
		5	<5	9
		1	<5	<5
nonvolatile fraction	SFE at 250 bar	100	<5	49 ***
	extract B1	22	<5	27 ***
		10	<5	<5
nonvolatile fraction	SFE at 250 bar	100	<5	60 ***
	extract B2	22	<5	53 ***
		10	<5	33 ***
		5	<5	21 ***
		1	<5	<5
	SE extract D	100	<5	<5
controls[c]	rivastigmine	100	100 ***	100 ***
		10	88 ***	100 ***
		5	59 ***	100 ***
	donepezil	100	100 ***	100 ***
		10	100 ***	75 ***
		5	100 ***	70 ***

[a] Enzyme inhibition significantly different from that of a sample without extract: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. [b] SFE, supercritical fluid extraction. [c] Rivastigmine and donepezil are standard drugs used to treat patients with Alzheimer's disease and were tested as positive controls and for comparison purposes.

conditions (**Table 3**); nonetheless, it is important to note that the comparison is made between pure compounds and crude extracts, in which the concentration of each component is quite small.

The simple dose−response logarithm model was fitted successfully to the inhibition data points, allowing the prediction of IC_{50} values. Extract B2 showed the best value for BChE inhibition (IC_{50} = 34 μg/mL), whereas HD extract C inhibited both enzymes ($IC_{50,AChE}$ = 45 μg/mL; $IC_{50,BChE}$ = 52 μg/mL). All other extracts exhibited for both enzymes IC_{50} values > 96 μg/mL.

Microbial Susceptibility. The antimicrobial activity of the volatile oil fraction (HD and SFE extracts) was more pronounced than that revealed by the nonvolatile fraction, with the exception of SFE extract B2 (**Table 4**). The volatile oil and nonvolatile extracts A and B2, respectively, both obtained by SFE, have antibacterial activity over *B. cereus* and *B. subtilis*. In fact, the inhibition diameter caused by extract A over *B. subtilis* is of the same order of magnitude (47 mm) as that exhibited by chloramphenicol (46 mm), the positive control, at the same concentration (**Table 4**). These extracts are bacteriostatic and bactericidal, presenting both MIC = MLC = 62.5 μg/mL for *B. cereus*, whereas chloramphenicol gave MIC = 6.3 μg/mL and MLC = 12.5 μg/mL. With regard to *B. subtilis*, supercritical volatile oil extract A (MIC = MLC = 15.6 μg/mL) is more effective than extract B2 (MIC = MLC = 62.5 μg/mL), presenting an MLC lower than that of chloramphenicol (MLC > 50 μg/mL, MIC = 3.1 μg/mL). These effects are more pronounced than those

Table 4. Antibacterial Activity of *Satureja montana* Extracts: SFE versus Conventional Extraction[a]

	Satureja montana extract[b]						
	volatile fraction		nonvolatile fraction			control[c]	
	SFE (A)	HD (C)	SFE (B1)	SFE (B2)	SE (D)	I	II
bacteria							
Bacillus cereus	**20**	10	−	**19**	−	24	45
Bacillus subtilis	**47**	12	11	**34**	−	30	46
Enterococcus faecalis	11	10	−	−	−	29	43
Escherichia coli	11	11	−	−	8	29	42
Listeria monocytogenes	11	12	−	11	−	31	45
Pseudomonas aeruginosa	−	−	−	−	−	−	23
Salmonella enteritidis	11	−	−	−	−	32	44
Staphylococcus aureus	10	11	−	9	−	27	41
fungi							
Alternaria alternata	−	−	−	−	−	−	−
Biscogniauxia mediterranea	−	−	−	−	−	54	70
Botrytis spp.	14	14	−	−	−	−	20
Candida albicans	11	13	−	10	−	−	14
Colletotrichum coffeanum	9	−	−	−	−	16	24
Fusarium culmorum	−	−	−	−	−	12	18
Pyricularia oryzae	14	10	−	10	−	40	63
Rhizopus spp.	−	−	−	−	−	11	19
Stachybotrys chartarum	9	10	−	−	−	−	−

[a] Results were determined using the paper disk diffusion method and are expressed by the diameter of the inhibition zones (mm); (−) inhibition diameter <6.4 mm. [b] A solution of the compounds (300 μg) in DMSO (15 μL) was applied on the disk. [c] Chloramphenicol for bacteria and actidione for fungi were used as positive control. A solution of the control (I, 30 μg; II, 300 μg) in DMSO (15 μL) was used.

previously reported (*5*) for the hydrodistilled essential oil of *S. montana* L. and of *Satureja cuneifolia* Ten., collected in Croatia, against *B. subtilis* (250 μg/mL).

Mild activities (9−12 mm) against other bacteria such as *E. faecalis*, *E. coli*, *L. monocytogenes*, *S. enteritidis*, and *S. aureus* were also detected for HD and SFE extracts from the volatile fraction, whereas the nonvolatile SFE extract B2 was active only over *Bacillus* spp., *L. monocytogenes*, and *S. aureus* (**Table 4**). However, HD essential oils of the plant were reported to have a promising activity against *S. aureus*, *L. monocytogenes*, and *E. coli* (*7*, *32*), suggesting that location and stages of development, among other parameters, may influence essential oil composition and consequently its antimicrobial activity. With regard to the effect of the extracts over the fungi, it was observed that, once again, the volatile oil fractions SFE and HD revealed the most prominent antifungal activity (**Table 4**). The phytopathogen *Botrytis* spp. and the indoor allergic and toxic mold *S. chartarum* were both susceptible to volatile extracts A and C, exhibiting a promising activity when compared to the control used. However, *P. oryzae*, which causes rice blast disease and is considered to be a bioterrorism agent, was inhibited by SFE extract A, but was less affected by HD extract C. *C. albicans*, which is a causal agent of opportunistic oral and genital infections in humans, was revealed to be sensitive to the nonvolatile B2 and volatile SFE extracts, particularly to the volatile fraction extract HD (13 mm) when compared to the control, which led to a 14 mm inhibition diameter. The nonvolatile fraction obtained by conventional Soxhlet extraction with acetone (extract D) did not affect significantly any of the bacteria and fungi tested.

The bioactivity exhibited for the essential oils may result from their high content in carvacrol. This monoterpene phenol was described as the most active component responsible for the antimicrobial effects of *Satureja* essential oils (*4*, *6*, *7*). However, the presence of other major constituents such as thymol, *p*-cymene, and

γ-terpinene, as well as the great number of minor constituents, can also play a role in the plant bioactivity.

Conclusions. The extraction method proved to influence the bioactivity of the extracts. The supercritical nonvolatile fractions selectively and significantly inhibited butyrylcholinesterase, even at low concentrations, whereas the nonvolatile conventional extract did not affect this enzyme. Both supercritical fluid extracts exhibited a chlorogenic acid content higher than the Soxhlet extract. However, the one obtained in the second separator is characterized by the highest contents in vanillic acid, protocatechuic acid, and (+)-catechin, being also the most efficient extract. The volatile fractions were not selective and inhibited both acetyl- and butyrylcholinesterase, and the hydrodistilled essential oil was more active than the volatile supercritical extract regarding the inhibition of both enzymes. Concerning the antimicrobial activities, they were detected only for the volatile fractions, and the supercritical extract was considerably more active against both *Bacillus* species tested. The chemical profile of the supercritical volatile and nonvolatile fractions by GC-MS and HPLC-DAD analyses, respectively, also supports the bioactivity exhibited by the plant extracts.

This work highlights the importance of *S. montana* as a dietary adjuvant and shows the benefits of employing supercritical fluid extraction for the production of extracts with antimicrobial and anticholinesterase activities.

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