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Biological Assays and Chemical Composition of Volatile Oils of *Bupleurum fruticosum* L. (Apiaceae)

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Abstract: The composition of supercritical CO_2 extracts and essential oils obtained by hydrodistillation of *Bupleurum fruticosum* L., growing spontaneously in Italy and Portugal, and its antifungal activity is reported. The collected extracts were analyzed by GC-FID and GC-MS methods. The minimal inhibitory concentration (MIC) and the minimal lethal concentration (MLC) were used to evaluate the antifungal activity of the oils against *Candida albicans*, *C. tropicalis*, *C. krusei*, *C. guillermondii*, *C. parapsilosis*, *Cryptococcus neoformans*, *Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum canis*, *M. gypseum*, *Epidermophyton floccosum*, *Aspergillus niger*, *A. fumigatus* and *A. flavus*.

Keywords: *Bupleurum fruticosum* L.; essential oil; supercritical carbon dioxide; antifungal activity.

1. Introduction

Bupleurum fruticosum L. (Apiaceae) is a shrubby, perennial, evergreen plant up to 1-2 m high, the branches are glabrous, striated and reddish. The coriaceous leaves are blue-green, 3-7 cm long, with obovate or elliptical shape. Inflorescences are umbels organized with 8-12 yellow flowers. The

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fruit is a diachene. It is found throughout in the northern hemisphere, in Asia, in North America and in Europe, particularly in the Mediterranean regions [1]. The bloom period is from the end of May to August [2]. This plant has a quite unpleasant aroma as does the aerial part contains the aromatic compounds in great abundance. On the contrary, the fragrant and tasty fruits, which reminds one fennel, has been used as a spice [3]. Animals are repelled by this shrub, probably due to the presence of essential oils in branches and leaves [3]. In the south-western of Sardinia (Italy), the decoction of roots and stems is used as antirheumatic [4].

Essential oils obtained from plants collected in several origins were investigated. Manunta et al. [5] identified 22 constituents in the oil obtained from both the branch and leaf oils of *B. fruticosum*, grown in the Botanical Garden of the University of Urbino. They characterized the presence of γ-terpinene (48.6%), α-phellandrene (12.2%) and sabinene (12.0%) in the stems oil, while sabinene (39.7%) and β-phellandrene (38.7%) are the main compounds in the leaves oil. Giamperi et al.[6] analysed the essential oil from the epigean parts of *B. fruticosum*, harvested in Cirenaica (Libya); the oil was found to be rich in β-phellandrene (49.3%) and α-pinene (15.3%). Recently, Dugo et al.[7] analyzed the essential oil of Sicilian *B. fruticosum* leaves, which was rich in α-pinene (21.7%), β-phellandrene (21.3%) and β-pinene (13.2%). Otherwise, β-phellandrene (67.7%) and sabinene (9.3%) were the major components of *B. fruticosum* from Corsica [8]. The antispasmodic and anti-inflammatory activities were also reported for *B. fruticosum* oil [9].

The aim of this work was to investigate, for the first time, the volatile extracts of *B. fruticosum* obtained by supercritical CO_2 extraction. To our knowledge, despite some works published with the essential oils of *Bupleurum* sp.pl., this is the first report on the volatile oils of *B. fruticosum* growing spontaneously in Portugal and in Sardinia as well as their antifungal activity.

2. Materials and Methods

2.1. Plant Material

Aerial parts of *Bupleurum fruticosum* L. was collected during three different vegetative phases of the plants, before flowering (sample 1), during flowering (samples 2, 4) and during fruiting (samples 3, 5). The collection was made from two different sites: Cerca de Santa Comba (Central Portugal) (samples 4, 5) and Baunei (Central-eastern of Sardinia Island, Italy) (samples 1, 2, 3). Voucher specimens (numbers: LS355; 590) were deposited at the Herbarium of the Laboratory of Pharmacognosy (Faculty of Pharmacy, University of Coimbra, 300-548 Coimbra) and at the Herbarium CAG-Università di Cagliari (Viale S. Ignazio 13, 09123 Cagliari) respectively.

Vegetal material was air-dried in a hot air-drier at 40 °C with forced ventilation for two days. Before utilization, matter was ground with a Malavasi mill (Bologna, Italy) taking care to avoid overheating.

2.2. SFE apparatus

Supercritical CO_2 extractions were performed according Marongiu et al. [10] in a laboratory apparatus, equipped with a 320 cm^3 extraction vessel and two separator vessels of 300 and 200 cm^3 respectively, connected in series. Experiments were carried out at different conditions in the extraction section. In the first separator the temperature was set at -10 °C and the pressure at the same value as the extraction section. The second separator was set at 15 bar and 10 °C. Extraction was carried out in a semi batch mode: batch charging of vegetable matter and continuous flow solvent. About 180 g of material were charged in each run.

2.3. Hydrodistillation

Hydrodistillation (HD) was performed for three hours in a circulatory Clevenger-type apparatus up to exhaustion of the oil contained in the matrix, according to the procedure described in the European Pharmacopoeia [11].

2.4. GC and GC/MS analysis

Analysis of volatile oil was carried out by gas chromatography (GC) and by gas chromatography-mass spectrometry (GC-MS).

Analytical GC was carried out in a Hewlett Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph with HP GC ChemStation Rev. A.05.04 data handling system, equipped with a single injector and two flame ionization detectors (FID). A graphpak divider (Agilent Technologies, Part Number 5021-7148) was used for simultaneous sampling to two Supelco (Supelco Inc., Bellefont, PA, USA) fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane 30 m × 0.20 mm i.d., film thickness 0.20 μm), and SupelcoWax 10 (polyethyleneglycol 30 m × 0.20 mm i.d., film thickness 0.20 μm). Oven temperature was settled at 70 ºC, raising at 3 ºC.min^{-1} to 220 ºC and then held 15 min at 220 ºC; injector temperature: 250 ºC; carrier gas: helium, adjusted to a linear velocity of 30 cm/s; splitting ratio 1:40; detectors temperature: 250 ºC.

GC-MS analyses were carried out in a Hewlett Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.25 μm), interfaced with an Hewlett Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters as above; interface temperature: 250 ºC; MS source temperature: 230 ºC; MS quadrupole temperature: 150 ºC; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35-350 u; scans/sec: 4.51.

The identity of the components was assigned by comparison of mass spectra and retention indices for two different chromatographic stationary phases calculated by linear interpolation to the retention of a series of *n*-alkanes. Experimental data were compared with corresponding data of reference oils and commercial available standards banked at a home-made library or from literature data [12, 13]. Percentages of individual components were calculated based on GC peak areas without FID response factor correction.

2.5. Antifungal strains

Antifungal activity of the essential oils obtained during the fruiting phase of the plants collected in Portugal (sample A) and Italy (sample B) were evaluated against yeasts, dermatophyte and *Aspergillus* strains: two clinical *Candida* strains isolated from recurrent cases of vulvovaginal candidosis (*C. krusei* H9, *C. guillermondii* MAT23), three type strains from the American Type Culture Collection (*Candida albicans* ATCC 10231, *C. tropicalis* ATCC 13803, *C. parapsilosis* ATCC 90018) and one type strain from the Colección Española de Cultivos Tipo (*Cryptococcus neoformans* CECT 1078); three dermatophyte clinical strains isolated from nails and skin (*Epidermophyton floccosum* FF9, *Trichophyton mentagrophytes* FF7, *Microsporum canis* FF1) and two type strains from the Colección Española de Cultivos Tipo (*Trichophyton rubrum* CECT 2794, *M. gypseum* CECT 2908); and one *Aspergillus* clinical strain isolated from bronchial secretions (*A. flavus* F44) and two type strains from the American Type Culture Collection (*Aspergillus niger* ATCC 16404, *A. fumigatus* ATCC 46645).

The fungal isolates were identified by standard microbiology methods and stored on Sabouraud broth with glycerol at –70 ºC. Prior to antifungal susceptibility testing, each isolate was inoculated on Sabouraud agar to ensure optimal growth characteristics and purity.

2.6. Antifungal activity

A macrodilution broth method was used to determine the Minimal Inhibitory Concentrations (MIC) and Minimal Lethal Concentrations (MLC), according to Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) references documents M27-A3 [14], M27-S3 [15] and M38-A2 [16] for yeasts and filamentous fungi, respectively.

The serial doubling dilution of each oil was prepared in dimethyl sulfoxide (DMSO), with concentrations ranging from 0.16 to 20 µL/mL. Final concentration of DMSO never exceeded 2%. Recent cultures of each strain were used to prepare the cell suspension adjusted to 1-2x10^3 cells per mL for yeasts and 1-2x10^4 cells per mL for filamentous fungi. The concentration of cells was confirmed by viable count on Sabouraud agar. The test tubes were incubated aerobically at 35ºC for 48h/72h (*Candida* spp. and *Aspergillus* spp./*Cryptococcus neoformans*) and at 30ºC for 7 days (dermatophytes) and MICs, were determined. To evaluate MLC, aliquots (20 µL) of broth were taken from each negative tube after MIC reading, and cultured in Sabouraud dextrose agar plates. Plates were then incubated for 48 h at 35ºC (*Candida* spp. and *Aspergillus* spp.), 72h for *Cryptococcus neoformans* and 7 days at 30ºC (dermatophytes). In addition, two reference antifungal compounds, amphotericin B (Fluka) and fluconazole (Pfizer) were used to control the sensitivity of tested microorganisms. All tests were performed in RPMI medium. For each strain tested, the grow conditions and the sterility of the medium were checked in two control tubes. The innocuity of the DMSO was also checked at the highest tested concentration. All experiments were performed in triplicate and repeated if the results differed.

3. Results and Discussion

The oil were analyzed by GC and GC-MS and the qualitative and quantitative compositions are presented in Tables 1 and 2, where compounds are listed in order of their elution on a polydimethylsiloxane column.

All the essential oils obtained by hydrodistillation from the Italian *B. fruticosum* were characterized by high content of β-phellandrene, 71.4%-57.8%, and sabinene, 13.9%-12.1%.

The essential oils composition obtained from Sardinian species in different vegetative phases has shown significant quantitative variations: β-phellandrene increased from 57.8% to 71.4% and sabinene from 12.1% to 13.9%. The other components did not show large variations before, during and after the flowering period.

The chemical composition of Portuguese *B. fruticosum* is quite different from those of the Italian one. α-Pinene and β-pinene represent more than 70% of the total oil. Their contents were almost constant through the life-cycle stages, although the amount of α-pinene ranged from 37.3% in flowering phase to 42.7% in fruiting phase. β-Phellandrene and (E)-β-ocimene varied in the opposite direction, their content was higher in flowering period than in fruiting one.

The results showed that the Sardinian and Portuguese *B. fruticosum* belong to different chemotypes. The chemical composition of the Sardinian oil is very similar to the oils obtained from other *B. fruticosum* growing in the Mediterranean basin. Data reported earlier [5, 6, 8] reveals that β-phellandrene and sabinene were the main constituent of the essential oil from *B. fruticosum* gathered in Marches, Libya and Corsica. Instead, Portuguese sample could be included in the chemotype α-pinene/β-pinene previously referred by Lorente et al. [9] for plants collected in Spain.

Concerning the volatile extracts obtained by supercritical CO_2, no data are available in literature. *Bupleurum fruticosum* L. SFE extracts yields 0.1%-2.7%. The highest yield was found during the fruiting period: 2.7% Sardinian sample and 1.1% Portuguese sample.

Table 1. Retention index (RI) and chromatographic area percentages of compounds found in *Bupleurum fruticosum* essential oil obtained by hydrodistillation: **1** leaves Italy; **2** leaves and flowering umbels Italy; **3** leaves and umbels containing ripe seeds Italy; **4** leaves and flowering umbels Portugal; **5** leaves and umbels containing ripe seeds Portugal*.

RI (SPB-1)	RI (SuperW-10)	Compound	1	2	3	4	5
924	1029	α-Thujene	-	-	-	1.3	-
932	1028	α-Pinene	1.4	1.5	1.4	37.3	42.7
945	1074	Camphene	0.1	-	0.1	1.1	1.0
967	1125	Sabinene	12.1	12.2	13.9	1.0	2.5
973	1117	β-Pinene	0.1	-	-	33.0	33.5
982	1159	Myrcene	1.8	2.2	2.4	2.0	1.6
1000	1169	α-Phellandrene	1.0	1.2	1.7	0.1	0.1
1014	1271	p-Cymene	1.7	0.9	0.7	2.7	2.7
1014	1183	α-Terpinene	-	0.1	0.2	t	0.1
1024	1231	(Z)-β-Ocimene	-	0.9	-	1.0	-
1024	1215	β-Phellandrene	57.8	68.5	71.4	7.8	6.2
1037	1246	(E)-β-Ocimene	0.3	-	0.5	4.7	3.5
1049	1246	γ-Terpinene	0.4	-	0.5	1.7	1.4
1053	1455	trans-Sabinene hydrate	0.4	-	-	-	-
1080	1284	Terpinolene	0.1	-	-	0.2	0.1
1084	1553	cis-Sabinene hydrate	0.4	-	-	-	-
1084	1539	Linalool	t	-	0.4	-	-
1123	1644	cis-Verbenol	-	-	-	0.2	0.1
1123	1643	trans-Pinocarveol	-	-	-	-	0.1
1154	1660	Cryptone	4.3	2.0	-	t	-
1160	1592	Terpinen-4-ol	0.7	-	0.5	-	0.8
1160	1622	Myrtenal	-	-	-	0.2	0.1
1174	1667	Estragol	0.2	-	-	t	-
1174	1672	cis-Piperitol	0.2	-	-	-	-
1193	1823	trans-Carveol	0.6	-	-	-	-
1211	1770	Cuminal	0.6	-	-	-	-
1226	1423	Hexyl-2-methyl butirate	-	-	-	0.6	-
1226	1600	Carvacrol methyl ether	-	-	-	0.7	-
1236	1836	Geraniol	0.6	-	-	-	-
1247	1709	Phellandral	1.4	-	0.1	-	-
1258	1752	Decanol	2.4	-	-	-	-
1281	2200	Carvacrol	1.5	-	-	-	-
1360	1741	Geranyl acetate	3.0	-	-	-	-
1370	2002	Methyl eugenol	2.6	2.8	-	-	-
1371	1490	α-Copaene	t	-	1.5	0.1	t
1416	1593	(E)-Caryophyllene	-	-	-	0.5	-
1416	1596	β-Cedrene	-	-	-	0.5	-
1446	1661	α-Humulene	-	-	-	0.2	-
1470	1698	Germacrene D	-	-	0.2	1.1	0.4
1485	1723	(Z)-α-Bisabolene	-	-	0.2	-	-
1498	1719	β-Bisabolene	-	-	-	0.8	-
1510	1749	δ-Cadinene	-	-	0.1	-	-
1518	2215	Elemicin	0.3	-	-	0.1	-
1557	1966	Caryophyllene oxide	0.3	-	0.3	0.1	-
		Total identified	96.3	92.3	96.1	99.0	96.9
		Per cent yield	1.0	1.8	2.6	0.8	1.1

*Compounds listed in order to their elution on the SPB-1 column.

The main difference between SFE and SD oils was the content of sesquiterpenes which are higher in the SFE products (see Tables 1 and 2): i.e. for the Germacrene D (24.6% vs 1.1%). This result is rationalized as follows. Essential oil compounds are only slightly soluble in water, therefore. only low molecular weight compounds are taken from the vegetable matrix. Supercritical CO_2 emulates an organic solvent characteristics with changes in extraction conditions (temperature and pressure). Also, by using this process, high molecular weight compounds can be extracted from the *Bupleurum* spp. particles. The extend of extraction of these compounds must be optimize for SFE.

Table 2. Retention index (RI) and chromatographic area percentages of compounds found in *Bupleurum fruticosum* supercritical extracts: **1** leaves Italy; **2** leaves and flowering umbels Italy; **3** leaves and umbels containing ripe seeds Italy; **4** leaves and flowering umbels Portugal; **5** leaves and umbels containing ripe seeds Portugal.

RI (SPB-1)	RI (SuperW-10)	Compound	1	2	3	4	5
924	1029	α-Thujene	-	-	0.1	-	-
932	1028	α-Pinene	1.3	1.4	1.3	30.3	33.3
945	1074	Camphene	-	0.1	0.1	-	-
967	1125	Sabinene	12.0	12.1	13.9	1.0	2.5
973	1117	β-Pinene	0.4	0.3	0.6	19.1	21.9
982	1159	Myrcene	1.1	2.2	2.2	0.5	0.8
1000	1169	α-Phellandrene	1.0	1.3	1.8	-	-
1006	1152	3-Carene	-	0.1	0.4	-	0.1
1014	1271	p-Cymene	2.3	1.7	0.5	2.5	2.9
1019	1205	Limonene	0.5	0.9	5.2	-	0.1
1024	1231	(Z)-β-Ocimene	-	1.0	0.7	-	-
1024	1215	β-Phellandrene	53.0	64.9	65.1	5.7	6.9
1037	1246	(E)-β-Ocimene	0.1	0.2	0.2	2.3	2.1
1049	1246	γ-Terpinene	0.1	0.1	0.2	0.1	0.5
1080	1284	Terpinolene	-	0.2	0.1	0.1	-
1160	1592	Terpinen-4-ol	0.2	0.3	0.1	0.4	0.1
1370	2002	Methyl eugenol	9.8	4.0	1.2	0.2	0.1
1380	1535	β-Cubebene	0.1	0.4	0.2	0.4	0.2
1416	1593	(E)-Caryophyllene	0.2	0.1	0.1	0.1	0.3
1470	1698	Germacrene D	0.4	0.4	0.3	24.6	18.2
1481	1725	Bicyclogermacrene	0.1	0.1	0.2	0.9	0.7
1554	2110	Spathulenol	-	-	0.3	0.2	0.1
	Total identified		82.6	91.8	94.8	88.4	90.8
	Per cent yield		0.6	0.4	2.7	0.1	1.1

* Compounds listed in order to their elution on the SPB-1 column.

The essential oil is generally obtained by hydro- or steam distillation. This technique, even when do not induce extensive phenomena of hydrolysis and thermal degradation, give in any case a product with a characteristic off odour. Solvent extraction can give an oil, but on account of a high content of waxes and/or other high molecular mass compounds, often give rise to a concrete with a scent very similar to the material from which it was derived. A further drawback of this technique is that small amounts of organic solvents can pollute the extraction product. Supercritical fluid extraction, SFE, is a good technique for the production of flavours and fragrances from natural materials and can constitute a valid alternative to both the above-mentioned processes. In fact compressed carbon dioxide, CO_2, is able to solubilize hydrocarbon and oxygenated mono- and sesquiterpenes, the main essential oils constituents. The separation of the extractant is easy and

possible residues do not cause a risk for the human health. Indeed, CO_2 besides being safe, non-combustible and inexpensive, is non-toxic.

Evaluation of MIC and MLC of the oils showed a variability of inhibition among all the fungi tested (Table 3). Italian oil proved to be active against *Cryptococcus neoformans*, with MIC and MLC values ranging from 0.32-0.64 µL/mL. This oil might be useful for the control of cryptococcal infections. On the other hand, both oils showed low activity for *Candida* and *Aspergillus* strains, with MIC values ranging from 10 to 20µL/mL

Table 3. Antifungal activity (MIC and MLC) of *Bupleurum fruticosum* oil against for *yeasts*, dermatophyte and *Aspergillus* strains.

Strains	Portugal		Italy		Fluconazole		Amphotericin B	
	MIC[(a)]	MLC[(a)]	MIC[(a)]	MLC[(a)]	MIC	MLC	MIC[(b)]	MLC[(b)]
C. albicans ATCC 10231	5.0	5.0	1.25	2.5	1	>128	N.T[(c)]	N.T
C. tropicalis ATCC 13803	5.0	5.0	5.0	5.0	4	>128	N.T	N.T
C. krusei H9	5.0	5.0-10.0	2.5	2.5	64	64-128	N.T	N.T
Candida guillermondii MAT23	2.5	2.5-5.0	2.5	2.5	8	8	N.T	N.T
Candida parapsilosis ATCC 90018	5.0	2.5-5.0	2.5	5	<1	<1	N.T	N.T
Cryptococcus neoformans CECT 1078	1.25-0.64	1.25	0.32	0.64	16	128	N.T	N.T
T. mentagrophytes FF7	2.5	2.5	2.5	5	16-32	32-64	N.T	N.T
Microsporum canis FF1	1.25	1.25	1.25-2.5	1.25-2.5	128	128	N.T	N.T
Trichophyton rubrum CECT 2794	1.25	1.25	1.25	1.25	16	64	N.T	N.T
M. gypseum CECT 2905	2.5	2.5	2.5	5	128	>128	N.T	N.T
Epidermophyton floccosum FF9	1.25	2.5	1.25	1.25	16	16	N.T	N.T
Aspergillus niger ATCC16404	10.0	>20	5-10	20	N.T	N.T	1-2	4
A. fumigatus ATCC 46645	10-20	20	5-10	>20	N.T	N.T	2	4
A. flavus F44	>20	>20	10	>20	N.T	N.T	2	8

[a] MIC and MLC were determined by a macrodilution method and expressed in µL/mL(v/v).
[b] MIC and MLC were determined by a macrodilution method and expressed in µg/mL (w/v).
[c] Not tested. Results were obtained from 3 independent experiments performed in duplicate.

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