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Isolation of the Volatile Oil from *Satureja thymbra* by Supercritical Carbon Dioxide Extraction: Chemical Composition and Biological Activity

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Satureja thymbra L. is well known in Italy by the popular name of "Santoreggia sarda". It grows only in Sardinia and nowadays it is restricted to the slope of the Colle San Michele in Cagliari.
The composition of the aromatic extracts obtained by supercritical CO_2 and by hydrodistillation and their antifungal activity is reported. The collected extracts were analyzed by GC-FID and GC-MS methods. No significant differences were observed in the composition of the volatile extracts depending on the extraction method. The results showed the presence of thymol, γ-terpinene, β-caryophyllene, *p*-cymene, carvacrol and borneol as main components. 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*, *T. mentagrophytes* var. *interdigitale*, *Trichophyton rubrum*, *T. verrucosum*, *Microsporum canis*, *M. gypseum*, *Epidermophyton floccosum*, *Aspergillus niger*, *A. fumigatus* and *A. flavus*. The volatile extracts revealed a wide-spectrum antifungal activity. They were fungicidal and similarly potent against yeasts, dermatophyte and *Aspergillus* stains, with MICs ranging from 0.16 to 0.32 μL.mL^{-1}.

Keywords: *Satureja thymbra* L., essential oil, supercritical CO_2, antifungal activity.

The genus *Satureja* (*Lamiaceae*) is native to the Mediterranean region of Europe, western Asia, North Africa, the Canarian Islands and South America. This genus comprises about 15 species of herbaceous perennials and subshrubs [1a]. The most common *Satureja* specimen is *S. thymbra* L., which is sampled in countries of the Mediterranean region for use as a local spice as well as an herbal home remedy [1b]. Previous investigations on the chemical composition of the essential oil of *S. thymbra* indicated that it contains carvacrol and thymol as major components [1b-1d]. On the other hand, biological activity studies have established that the essential oil possesses significant antibacterial and antifungal activities [1e,1f]. It is evident that the phytochemical content of the essential oils for *Satureja* species varied greatly, depending on the period examined, and showed large prevalence of phenolic compounds and thymol as the major component [1f].

Many studies in several phenol-rich Lamiaceae species have shown that the predominance of carvacrol or thymol in their essential oils is associated to climatic conditions [1g,2a,2b]. Usually the sum of phenolic compounds or the four major constituents (carvacrol, thymol, *p*-cymene and γ-terpinene) was positively associated with higher air temperatures. In addition, chemotypes differentiation in a specific area is also related to intrinsic factors such as sexual polymorphism or genetic mechanism [2b].

There are several reports of the essential of *S. thymbra* from various sites of the Mediterranean area [1a-1g,2a,2b, 3-6]. In Italy, it grows only in Sardinia and nowadays it is restricted to the slope of the Colle San Michele in Cagliari. In 1989 Capone *et al.* [1b] reported the chemical composition and the antibacterial activity of essential oil steam distilled from *S. thymbra* gathered in 1984 and 1985 in the Colle San Michele in Cagliari. Our results represent the first detailed report of the composition of the volatile compounds of Sardinian *S. thymbra* extracted by mean of Supercritical CO_2, in comparison with hydrodistillation.

In the supercritical extraction the extractor pressure and temperature was set to 90 bar and 40°C, respectively. The

volatile oil was recovered in the separator that worked at 15 bar and 10°C. We performed a preliminary run drawing the extract, in separate vials, after each hour of extraction for four hours. The oil recovered after 60, 120, 180 and 240 minutes of extraction was: 1.3 g, 0.4 g, 0.3 g and 0.1 g, respectively with a total yield of 2.1%.

It is possible to group the components of *Satureja thymbr*a essential oil in four classes: hydrocarbon monoterpenes (HM), oxygenated monoterpenes (OM), hydrocarbon sesquiterpenes (HS) and oxygenated sesquiterpenes (OS) on the basis of the chemical structure. The quantitative results arranged as percentages of the various families, are reported in Figure 1.

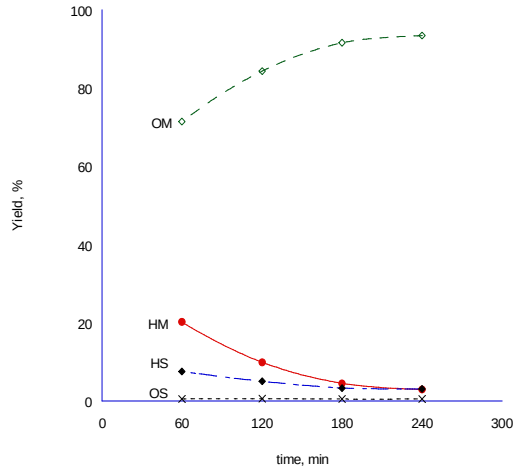

Figure 1: Evolution of *Satureja thymbr*a volatile extract composition with extraction time. Percentage of the various families at different extraction times: hydrocarbon monoterpenes (HM), oxygenated monoterpenes (OM), hydrocarbon sesquiterpenes (HS) and oxygenated sesquiterpenes (OS).

Percentage of OS was independent of the extraction time. Decreasing percentages of HM, HS were found as the extraction time increased; on the contrary, OM compounds showed a continuous percentage increase at increasing extraction times. The behaviour of single compounds as well as of compounds families can be explained on the basis of the structure of the vegetable material and the mass transfer mechanisms present during SFE. Since, as a rule, internal diffusion is the mass transfer controlling factor during SFE of essential oils [7a,7b] it is reasonable to hypothesize that inside the vegetable lighter compounds have shorter diffusion times than heavier ones.

In Table 1 are also reported, in the SFE column, the analytical results concerning the sample obtained, at the above-mentioned conditions, putting together all fractions in a single vial. In the HD column are shown the area percentages of the components of the hydrodistilled oil. The extract obtained by SFE, at a yield (w/w) of 2.1%, contained mainly: thymol (64.0%), γ-terpinene (7.2%), carvacrol (5.2%), β-caryophyllene (6.5%) and *p*-cymene (6.3%).

Table 1: Retention index (RI) and chromatographic area percentages of compounds found in *Satureja thymbr*a essential oil obtained by Supercritical CO_2 extraction (SFE) and by hydrodistillation (HD).

RI	Compound	SFE	HD
930	α-Thujene	0.2	0.5
937	α-Pinene	0.4	1.4
953	Camphene	0.3	0.7
980	ß-Pinene	0.5	0.8
992	Myrcene	0.4	0.8
1006	α-Phellandrene	tr.	0.2
1020	α-Terpinene	1.0	1.7
1027	*p*-Cymene	**6.3**	**9.8**
1032	Limonene	0.6	1.0
1040	(*Z*)-ß-Ocimene	0.2	0.3
1051	(*E*)-ß-Ocimene	0.2	0.3
1062	γ-Terpinene	**7.2**	**9.8**
1070	*cis*-Sabinene hydrate	0.4	tr.
1090	Terpinolene	tr.	0.2
1098	*trans*-Sabinene hydrate	0.2	-
1100	Linalool	0.3	0.3
1167	Borneol	**2.3**	**2.0**
1179	Terpinen-4-ol	0.3	0.5
1247	Carvacrol, methyl ether	1.7	1.7
1294	Thymol	**64.0**	**57.3**
1302	Carvacrol	**5.2**	**4.4**
1419	ß-Caryophyllene	**6.5**	**5.3**
1453	α-Humulene	0.3	0.2
1477	γ-Muurolene	0.2	tr.
1494	Valencene	0.2	tr.
1524	δ-Cadinene	0.3	0.2
1581	Caryophyllene oxide	0.7	0.6
1901	Not identified compound	0.2	tr.
	Total identified	99.9	98.8
	Per cent yield	2.1	3.3

tr: traces

The comparison with the hydrodistilled oil (yield: 3.3%) did not reveal important differences; hydrocarbons and oxygenated monoterpene were dominant in the oil (27.4% and 66.2%) and also contains above mention compounds in high percentage (57.3%, 9.8%, 4.4%, 5.3% and 9.8%, respectively).

The studies previously carried out by Capone *et al*. in 1989 [1b] on *Satureja thymbr*a collected in Cagliari, showed that γ-terpinene (32-40%), thymol (22-29%) and *p*-cymene (11-13%) were the main compounds. In most cases, thymol constitutes the major component of the oil: Greece [1f], Turkey [5] and Lebanon [6]. Carvacrol was the dominant component of essential oils from *S. thymbra* in island of Ikaria (Greece) [1g] and in Turkey [1a]. Karousou *et al*. [3] analyzed 13 plants collected from different localities of Crete (Greece), they found that the plants collected from the dry dwarf-shrub formations of the lowland have high carvacrol content whereas those collected from the more mesic timber or highland formations have a high thymol content. Also Skoula *et al*. [4], in plant material collected from Crete, Greece, in the summer of 2001, found carvacrol and thymol-type.

Antifungal activity of the volatile extracts of *S. thymbra* from Sardinia was signalised owing to data presented in Table 2. They were fungicidal and similarly potent against yeasts, dermatophyte and *Aspergillus* stains, with MIC values ranging from 0.16 to 0.32 $\mu L.mL^{-1}$. The antifungal activity of the extracts can the associated with high content of phenols, particularly thymol, which proved to be effective against these strains [7c]. To our knowledge this

Table 2 Antifungal activity (MIC and MLC) of *Satureja thymbr*a oil against for *yeasts*, dermatophyte and *Aspergillus* strains.

Strains	HD		SFE		Fluconazole		Amphotericin B	
	MIC[(a)]	MLC[(a)]	MIC	MLC	MIC[(b)]	MLC[(b)]	MIC	MLC
Candida albicans ATCC 10231	0.32	0.32-0.64	0.32	0.32-0.64	1	>128	N.T[(c)]	N.T
C. tropicalis ATCC 13803	0.32	0.64	0.32	0.64	4	>128	N.T	N.T
C. krusei H9	0.32	0.32-0.64	0.32	0.32	64	64-128	N.T	N.T
C. guillermondii MAT23	0.32	0.32	0.32	0.32	8	8	N.T	N.T
C. parapsilosis ATCC 90018	0.32	0.64	0.32	0.64	<1	<1	N.T	N.T
Cryptococcus neoformans CECT 1078	0.16	0.32	0.16	0.32	16	128	N.T	N.T
Trichophyton mentagrophytes FF7	0.16	0.32	0.16	0.32	16-32	32-64	N.T	N.T
T. mentagrophytes var. *interdigitale* CECT 2958	0.16	0.32	0.16	0.32	128	≥128	N.T	N.T
T. rubrum CECT 2794	0.16	0.16	0.16	0.32	16	64	N.T	N.T
T. verrucosum CECT 2992	0.16	0.32	0.16	0.32	>128	>128	N.T	N.T
Microsporum canis FF1	0.16	0.16	0.16	0.16	128	128	N.T	N.T
M. gypseum CECT 2905	0.16	0.32	0.16	0.16	128	>128	N.T	N.T
Epidermophyton floccosum FF9	0.16	0.16	0.16	0.16	16	16	N.T	N.T
Aspergillus niger ATCC16404	0.32	1.25	0.32	1.25	NT	NT	1-2	4
A. fumigatus ATCC 46645	0.32	0.64	0.32	0.64	NT	NT	2	4
A. flavus F44	0.32	0.64	0.32	0.64	NT	NT	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

is the first report on the antifungal activity of this species against dermatophyte strains. These results highlight the potential utilization of these extracts in dermatomycoses, aspergillosis and candidosis, which are common infections caused by filamentous fungi and by yeasts that can be severe in immunocompromised patients.

Experimental

Materials: Aerial parts of *Satureja thymbr*a were collected during the flowering stage from the locality of Colle S. Michele, Cagliari (Sardinia, Italy). Plants were identified at the Laboratorio di Botanica Farmaceutica Università degli Studi di Cagliari), where a voucher specimen is deposited (1079 Herbarium CAG). Vegetative 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.

Hydrodistillation: Hydrodistillation (HD) was performed for three hours in a circulatory Clevenger-type apparatus according to the procedure described in the European Pharmacopoeia [8].

SFE extraction: Supercritical CO_2 (purity 99%) extractions were performed in a laboratory apparatus equipped with a 400 cm^3 extraction vessel, E, operate in the single-pass mode by passing CO_2 through the fixed bed of charged vegetable particles. Waxes and essential oil were recovered in two separator vessels connected in series, S_1 and S_2, 300 and 200 cm^3, respectively. The cooling of the first separator was achieved by using a thermostated bath, accuracy of 0.1°C). The second separator allowed the discharge of the liquid product at desired time intervals. A high pressure diaphragm pump, P, with a maximum capacity of 6 kg/h, pumped liquid CO_2 at the desiderate flow rate. Carbon dioxide was then heated to the desired extraction temperature in a thermosted oven (accuracy of 0.02°C). The extraction was carried out in a semibatch mode by charging the vegetable matter in the vessel, followed by a continous flow solvent. Carbon dioxide flow was monitored by a calibrated rotameter located after the last separator. The CO_2 delivered during an extraction test was measured by a dry test meter. Temperatures and pressures along the extraction apparatus were measured by thermocouple and Bourdon-tube test gauges, respectively. Pressure was regulated by high pressure valves under manual control. Experiments were carried out at 90 bar and 40°C 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. Extractions were 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.

GC and GC/MS analysis: Analysis of the volatile extracts were carried out by gas chromatography (GC) and by gas chromatography-mass spectrometry (GC-MS) under the experimental conditions as reported earlier [9]. Mass Spectra Libraries were used as references [10,11a].

Antifungal strains: Antifungal activity of the essential oils 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 four type strains from the Colección Española de Cultivos Tipo (*T. mentagrophytes* var. *interdigitale* CECT 2958, *Trichophyton rubrum* CECT 2794, *T. verrucosum* CECT 2992, *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 –70ºC. Prior to antifungal susceptibility testing, each isolate was inoculated on Sabouraud agar to ensure optimal growth characteristics and purity.

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 [11b], M27-S3 [12a] and M38-A2 [12b] for yeasts and filamentous fungi, respectively. The serial doubling dilution of each extract was prepared in dimethyl sulfoxide (DMSO), with concentrations ranging from 0.04 to 5 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.

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