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Chemical Composition and Biological Activity of Volatile Extracts from Leaves and Fruits of *Schinus terebinthifolius* Raddi from Tunisia

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Abstract: Volatile oils composition from leaves and ripe fruits of pink pepper (*Schinus terebinthifolius* Raddi) growing in Tunisia were investigated using GC-FID and GC-MS techniques. Volatile oil extraction was achieved by hydrodistillation (HD) using a Clevenger apparatus and by supercritical fluid extraction (SFE) using carbon dioxide. All plant organs, gave SFE extracts chiefly composed by α-pinene, α-phellandrene, β-phellandrene, germacrene D and bicyclogermacrene. In the case of the fruits, both extraction techniques gave volatile oils of similar composition; whereas the comparison between the HD and SFE leave oils revealed important differences in the content of α-pinene (6.1 % *vs* traces), α-phellandrene (22.7 % *vs* 0.8 %) and β-phellandrene (14.6 % *vs* 1.2 %). All volatile samples were evaluated against yeasts and dermatophyte strains, being more active against *Cryptococcus neoformans*, particularly the volatile oil from the fruits, with MIC values of (0.32-0.64) mg/mL. Moreover, this oil revealed an inhibitory effect on germ tube formation in *C. albicans* at sub-inhibitory concentration. At the concentration of MIC/8 the inhibition of filamentation was more than 70 %.

Keywords: *Schinus terebinthifolius*; supercritical carbon dioxide extracts; essential oil; antifungal activity.
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1. Introduction

Schinus terebinthifolius Raddi (Anacardiaceae) is a perennial tree indigenous to South and Central America and can also be found in semitropical and tropical regions of the United States and Africa. It is known by a variety of common names including "aroeira-vermelha", "aroeira-pimenteira", Brazilian pepper, Christmas-berry, pink pepper, poivre rose [1]. In Brazil, *S. terebinthifolius* dried fruits are also marketed as a substitute for black pepper and are occasionally found as a pink seeded adulterant in *Piper nigrum* (Black Pepper) in other countries. Many medicinal properties have been attributed to this plant, such as antioxidant, wound healing, antitumor and antimicrobial activities. Biological activities in vitro and in vivo are reported for *Schinus terebinthifolius* extracts, like antibacterial, antifungal, antileishmanial and cicatrizing [2-5]. Plants found in different geographic regions might have different medicinal properties based on the difference in essential oil chemical composition [1,6].

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Although several studies have determined the biological activities of this plant, all of them have been conducted on South American vegetable matter, where it is native, and a few has been conducted on samples from African continent. Bendaoud et al., reported the composition, the antioxidant and the anticancer activities of the volatile oils of the fruits collected in southern of Tunisia [7]. The major constituents identified were: α-phellandrene (34.4 %), γ-cadinene (18.0 %), β-phellandrene (10.6 %), *p*-cymene (7.3 %), α-pinene (6.5 %) and α-terpineol (5.6 %). The essential oil was extracted from fresh leaves, collected in Zimbabwe and its antibacterial, anti-fungal as well as antioxidant activities were determined by Gundidza et al. [8]. The most abundant component found in this study were sabinene (40.7 %), α-pinene (30.3 %), α-phellandrene (9.9 %) , β-pinene (7.9 %) and myrcene (6.6 %).

The objective of the present study was to investigate the chemical composition of the volatile oils extracted from leaves and ripe fruits of *S. terebinthifolius* collected in Tunisia and evaluate their inhibitory effect on the growth of yeasts and filamentous fungi.

2. Materials and Methods

2.1. Plant Materials

Leaves and fruits of *Schinus terebinthifolius* were collected in November 2013 from Zaghoun (North of Tunisia) and authenticated according to the flora of Tunisia by the botanist Dr. Fethia Harzallah-Skhiri (Institut Supérieur de Biotechnologie, Monastir, Tunisia). Voucher specimens were deposited at the Institut Supérieur de Biotechnologie, Monastir, Tunisia. Before utilization, the vegetable matter was ground with a Malavasi mill (Bologna, Italy) taking care to avoid overheating, to obtain particles size in the range (250÷425) μm.

2.2. Hydrodistillation

Hydrodistillation (HD) was performed for 3 h in a circulatory Clevenger-type apparatus according to the procedure described in the European Pharmacopoeia [9].

2.3. SFE extraction

Supercritical CO_2 (SFE) extractions were performed 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. Extraction was carried out in a semi batch mode: batch charging of vegetable matter and continuous flow solvent. The *Schinus terebinthifolius* extracts were obtained working at 90 bar and 40 °C (CO_2 density, ρ_{CO2} = 0.287 g cm^{-3}) in the extraction vessel, at 90 bar and –10 °C in the first separator and at 20 bar and 15°C in the second one.

2.4. GC and GC/MS analysis

Qualitative analysis was carried out by means of gas chromatography-mass spectrometry (GC/MS) and the quantitative composition was accomplished by gas chromatography (GC/FID). GC-MS analyses were carried out in a gas chromatograph (Agilent, Model 6890N, Palo Alto, CA) equipped with a split-splitless injector, an autosampler Agilent model 7683 and two different Agilent fused silica capillary columns (30 m × 0.25 mm i.d., film thickness 0.25 μm) of different polarities (HP-5, 5 % phenyl-methylpolysiloxane; DB-WAXetr, polyethylene glycol). GC conditions used were: programmed heating from (60 to 250) °C at 3 °C/min followed by 20 min under isothermal conditions. The injector was maintained at 250 °C. Helium was the carrier gas at 1.0 mL/min; the sample (1 μL) was injected in the split mode (1:10). The GC was fitted with a quadrupole mass spectrometer, MS, Agilent model 5973 detector. MS conditions were as follows: ionization energy 70 eV, electronic impact ion source temperature 200 °C, quadrupole temperature 150 °C, scan rate 3.2 scan/sec, mass range (30÷480) u. Software adopted to handle mass spectra and chromatograms was ChemStation. Samples were run in chloroform with a dilution ratio of 1:100. The volatile compounds were identified by both their retention indices and their mass spectra. Retention indices, calculated by linear

interpolation relative to retention times of a series of *n*-alkanes, were compared with those of authenticated samples from our database [10]. Mass spectra were compared with reference spectra from a home-made library or from literature data [11,12]. Analytical GC/FID was carried out in a gas chromatograph (Agilent, Model 7890A, Palo Alto, CA), equipped with a flame ionization detector (FID), an autosampler (Agilent, Model 7683B), Agilent HP5 fused silica column (5 % phenyl-methylpolysiloxane), 30 m × 0.25 mm i.d., film thickness 0.25 μm, and a Agilent ChemStation software system. Oven temperature was settled at 60 ºC, raising at 3 ºC/min to 250 ºC and then held 20 min at 250 ºC; injector temperature: 250 ºC; carrier gas: helium at 1.0 mL/min; splitting ratio 1:10; detectors temperature: 300 ºC. Percentage of individual components was calculated based on GC/FID peak areas without FID response factor correction. Three replicates were performed for each sample. The average of these three values and the standard deviation were determined for each compound identified.

2.5. Antifungal activity

2.5.1 Fungal strains

The antifungal activity of the essential oils from fruits and leaves (HD_F and HD_L) and the SFE extract from the fruits (SFE_F) was evaluated against yeasts and filamentous fungi: one clinical *Candida* strain isolated from recurrent cases of vulvovaginal (*C. krusei* H9, *C. guilliermondii* MAT23); three *Candida* type strains from the American Type Culture Collection (*C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803 and *C. parapsilopsis* ATCC 90018); one *Cryptococcus neoformans* type strain from the Colección Espanõla de Cultivos Tipo (*C. neoformans* CECT 1078); three dermatophyte clinical strains isolated from nails and skin (*Epidermophyton floccosum* FF9, *Trichophyton mentagrophytes* FF7 and *Microsporum canis* FF1), and four dermatophyte type strains from the Colección Espanõla de Cultivos Tipo (*T. mentagrophytes* var. *interdigitale* CECT 2958, *T. rubrum* CECT 2794, *T. verrucosum* CECT 2992, and *M. gypseum* CECT 2908). All strains were stored in Sabouraud dextrose broth with 20 % glycerol at -80 ºC and subcultured in Sabouraud dextrose agar (SDA) or Potato dextrose agar (PDA) before each test, to ensure optimal growth conditions and purity.

2.5.2. Antifungal activity

A macrodilution broth method was used to determine the minimal inhibitory concentrations of the oil (MICs) according to the Clinical and Laboratory Standards Institute (CLSI) reference protocols M27-A3 (CLSI 2008), M27-S3 (CLSI, 2008) and M38-A2 (CLSI 2008) for yeasts and filamentous fungi, respectively [13-15]. Briefly, inoculum suspensions were prepared at appropriate densities in RPMI 1640 broth (with L-glutamine, without bicarbonate, and the pH indicator phenol red) from SDA or PDA cultures and distributed into 12×75 mm glass test tubes. Inoculum densities were confirmed by viability counts on SDA. The serial doubling dilution of the volatile extracts was prepared in dimethyl sulfoxide (DMSO), with concentrations ranging from (0.16 to 10) mg/mL. Final concentration of DMSO never exceeded 1 %. Oil-free growth controls and DMSO control tubes, were also included. The test tubes were incubated aerobically at 35 ºC for 48 h/72 h (*Candida* spp. and *Cryptococcus neoformans*) or at 30 ºC for 7 days (dermatophytes). MIC values were determined as the lowest concentration of the oil causing full growth inhibition. Quality control was performed by testing fluconazole with the reference strains *C. parapsilopsis* ATCC 22019 and *C. krusei* ATCC 6258 and the results were within the predetermined limits. To measure minimal lethal concentrations (MLCs), 20 μL samples were taken from each negative tube, plus the first tube showing growth (to serve as a growth control) after MIC reading to SDA plates and incubated at 35 ºC for 48 h/72 h (*Candida* spp. and *Cryptococcus neoformans*) or at 30 ºC for 7 days (dermatophytes). MLC values were determined as the lowest concentration of the oil causing fungal death. All experiments were performed in triplicate and repeated whenever the results of each triplicate did not agree. A range of values is presented when different results were obtained.

2.5.3. Germ tube inhibition assay

In order to determine the effect of the volatile oil on the yeast-mycelium transition, the suspensions of *C. albicans* strains ATCC 10231, from overnight cultures in SDA were prepared in NYP medium (*N*-acetylglucosamine [Sigma; 10^{-3} mol/L], Yeast Nitrogen Base [Difco; 3.35 g/L], proline [Fluka; 10^{-3} mol/L], NaCl [4.5 g/L], and pH 6.7±0.1 [16]. The suspensions were adjusted to obtain a density of yeast cell suspensions at $(1.0\pm0.2) \times 10^6$ CFU/mL and distributed into glass test tubes in a volume of 990 µL. Each dilution of essential oil was added into the cell suspension tubes, in 10 µL volumes, to obtain appropriate sub-inhibitory concentration. After incubation at 37 ºC without agitation for 3 h, the treated and untreated yeast cells were counted for germ tube formation under light microscope and the percentage of germinating cells was calculated. A formation of germ tube was considered positive when the germinating tube was at least as long as the diameter of the blastospore. The results are presented as averages ± standard deviations (SD) of three separate experiments.

3. Results and Discussion

The amounts of the volatile oil obtained by HD and SFE from fruits of *S. terebinthifolius* were 2.6 and 2.1 % (w/w, dry matter), respectively. The leaf oil was yielded at 0.4 and 0.3 %, for HD and SFE respectively. The oils were analyzed by GC-FID and GC-MS and the qualitative and quantitative compositions are presented in Table 1. Both the volatile oils obtained by HD and SFE from ripe fruits, were characterized by high content of α-phellandrene (39.6 *vs* 36.2 %), β-phellandrene (22.9 *vs* 21.3 %), α-pinene (18.4 *vs* 13.2 %) and germacrene D (10.0 *vs* 19.6 %) respectively. The volatile oils composition obtained from leaves has shown significant quantitative variations according to the extraction methods (HD to SFE): α-pinene decreased from 6.1 % to traces, α-phellandrene from 22.7 % to 0.8 % and β-phellandrene from 14.6 % to 1.2 %. However germacrene D increased from 23.6 % to 39.8 %. The other sesquiterpene components did not show large variations but they increased also in the CO_2 supercritical extract. By deduction the essential oil obtained by hydrodistillation from leaves of *S. teribinthifolius* was dominated by hydrocarbons monoterpenes (51.0 %). The CO_2 supercritical extract was dominated by sesquiterpenes hydrocarbons (80.3 %). The main difference between SFE and HD oils was the content of sesquiterpenes which are higher in the SFE products (extracted from leaves and fruits): 92.2 % *vs* 46.9 % and 24.5 % *vs* 12.1 %. The differences observed may be due to the fact that the hydrodistillation induces migration of volatile compounds from the inside of the secretory structures up to the leaf surface, followed by their subsequent evaporation. Therefore, low molecular weight compounds are easily taken from the vegetable matrix, while supercritical CO_2 emulates an organic solvent, improving the extraction of high molecular weight compounds.

By referring to the literature, our results reinforce previous data reported by Bendaoud et al., on volatile oil extracted from fruits collected in Tunisia (Sfax, Southern East) [7]. They identified α-phellandrene, γ-cadinene, β-phellandrene as major constituents and found an high content of monoterpene hydrocarbons (62.8 %), a medium content of oxygenated monoterpenes (6.3 %) a markedly higher quantity of sesquiterpene hydrocarbons (26.8 %).

Concerning the leaf oil composition none has been conducted in Tunisia, so our data are the first. This profile is not similar to others previously described for this plant isolated from other regions of the Africa and of the world [8].

Leaf and fruit essential oils for Brazilian *S. terebinthifolius* were characterized by a high percentage of sesquiterpene and monoterpene hydrocarbons. Limonene (14.2 and 17.0 %) and germacrene D (11.4 and 10.9 %) were the main components of the oils obtained from the leaves and berries of *S. terebinthifolius*, respectively [17]. Silva et al., reported that the main components of the essential oil of fresh leaves submitted to water distillation were *p*-cymen-7-ol (22.5 %); 9-*epi*-(*E*)-caryophyllene (10.1 %), carvone (7.5 %) and verbenone (7.4 %) [18]. It is well known that these sorts of variations are due to the part of the plant used as well as geographical origin, harvesting time, growing conditions and of extraction methods [19].

Table 1. Identified Compounds in leaves and fruits of *Schinus terebinthifolius* volatile oil extracted by Hydrodistillation (HD) and by Supercritical CO_2 at 90 bar 40 °C (SFE).

Compound	RI (HP-5)	RI LITERATURE	HD_L	SFE_L	HD_F	SFE_F
α-thujene	929	930	0.5	-	-	-
α-pinene	937	939	6.1	t	18.4	13.2
sabinene	976	975	-	-	0.1	0.1
β-pinene	979	979	0.3	-	0.4	0.3
myrcene	992	991	0.6	t	1.0	0.9
α-phellandrene	1006	1003	22.7	0.8	39.6	36.2
α-terpinene	1018	1017	1.2	t	0.2	t
o-cymene	1027	1026	4.2	1.0	4.1	2.9
β-phellandrene	1032	1030	14.6	1.2	22.9	21.3
γ-terpinene	1061	1060	0.8	t	-	-
terpinolene	1089	1089	-	-	0.2	0.2
δ-elemene	1338	1338	0.6	t	0.2	t
α-copaene	1376	1377	0.4	0.8	t	0.2
β-elemene	1391	1391	2.4	1.9	-	-
(*E*)-caryophyllene	1418	1419	0.9	1.7	t	0.2
β-copaene	1428	1432	t	0.9	-	-
α-himachalene	1448	1451	-	-	t	0.1
γ-muurolene	1477	1480	0.8	2.4	-	-
germacrene D	1481	1485	23.6	39.8	10.0	19.6
β-selinene	1485	1490	0.8	3.1	-	-
δ-selinene	1490	1493	0.6	1.1	-	-
bicyclogermacrene	1495	1500	4.8	7.5	t	0.2
α-muurolene	1499	1500	0.5	1.7	-	-
trans-β-guaiene	1502	1503	-	-	t	0.2
germacrene A	1503	1509	4.4	6.7	-	-
γ-cadinene	1513	1514	t	1.3	-	-
n.i.	1520	-	0.9	1.4	0.4	0.1
δ-cadinene	1523	1523	2.2	4.8	0.4	0.2
germacrene B	1555	1561	2.0	3.6	0.7	1.3
spathulenol	1575	1578	0.7	3.0	-	-
viridiflorol	1589	1593	tr	0.4	-	-
cis-isolongifolanone	1609	1613	-	-	0.2	0.3
n.i.	1610	-	0.2	0.9	-	-
trans-isolongifolanone	1627	1627	1.0	2.5	0.5	1.0
epi-α-muurolol	1641	1642	0.9	2.9	-	-
α-muurolol	1645	1646	t	1.0	-	-
β-eudesmol	1648	1651	-	-	t	0.2
n.i.	1651	-	-	-	0.7	0.3
α-cadinol	1653	1654	1.3	7.2	-	-
eudesma-4(15),7-dien-1-β-ol	1683	1688	t	0.8	-	-
Total identified			97.9	95.2	98.9	99.6
Hydrocarbon monoterpenes			51.0	3.0	86.8	75.1
Hydrocarbon sesquiterpenes			44.7	80.3	11.3	23.0
Oxygenated sesquiterpenes			2.2	11.9	0.8	1.5

Compounds listed in order to their elution on the HP-5 column. Mean and standard deviation of 3 samples. Standard deviation were recorded, values were insignificant and omitted from the Table to avoid congestion; t: trace, i.e., percentage lower than 0.1 %; n.i.: not identified compound.

The highest antifungal activity of investigated samples was observed against *Cryptococcus neoformans*, being the volatile oil from the fruits the more active, with MIC values of (0.32-0.64) mg/mL (Table 2).

Table 2. Antifungal activity (MIC and MLC) of *Schinus terebinthifolius* leaf and fruit oil obtained by HD and SFE for *yeasts* and dermatophyte strains.

Strains	SFE_F		HD_F		HD_L		Fluconazole	
	MIC [a]	MLC [a]	MIC	MLC	MIC	MLC	MIC	MLC [b]
Candida albicans ATCC 10231	1.25	1.25-2.5	2.5	2.5	2.5	2.5	1	>128
Candida tropicalis ATCC 13803	2.5	2.5	5	5	5.0	5.0	4	>128
Candida krusei H9	1.25	1.25-2.5	5	5-10	>5.0	>5.0	64	64-128
Candida guillermondii MAT23	1.25	2.5	2.5	5	2.5	>5.0	8	8
Candida parapsilosis ATCC 90018	5.0	5.0	5	5	>5.0	>5.0	<1	<1
Cryptococcus neoformans CECT 1078	0.64-1.25	1.25-2.5	0.32-0.64	0.64-1.25	1.25	5.0	16	128
Trichophyton mentagrophytes FF7	2.5-1.25	2.5-1.25	1.25	2.5-1.25	1.25	1.25	16-32	32-64
Microsporum canis FF1	1.25	2.5	1.25-2.5	2.5	1.25	1.25	128	128
T. rubrum CECT 2794	1.25	1.25	0.64-1.25	1.25	0.64	0.64	16	64
M. gypseum CECT 2905	2.5	5	2.5	2.5	1.25	2.5	128	>128
Epidermophyton floccosum FF9	1.25	1.25-2.5	1.25	1.25	0.64	1.25	16	16
T. mentagrophytes var. *interdigitale* CECT 2958	2.5	2.5	1.25-2.5	2.5	1.25	2.5	128	≥128
T. verrucosum CECT 2992	2.5	2.5	2.5	2.5	1.25	2.5	>128	>128

[a] MIC and MLC were determined by a macrodilution method and expressed in mg/mL (W /V).
[b] MIC and MLC were determined by a macrodilution method and expressed in µg/mL (W/V).

For dermatophytes the oil from leaves was more active with MIC values of (0.64-1.25) mg/mL, showing fungicidal activity for *Tricophytum mentagrophytes*, *Microsporon canis* and *T. rubrum.*

Crytococcosis is an invasive fungal infection, caused by *C. neoformans* or *C. gattii*. The clinical situation of crytococcosis varies from asymptomatic infections to severe pneumonia and respiratory failure. In immunocompromised patients, the disease may extend to the brain and trigger neurological troubles such as meningoencephalitis, a disease responsible for more than 600,000 death/year worldwide [20,21]. Dermatophytes are frequently resistant to the standard treatment, and the rate of relapse is unacceptably high, especially in case of onychomycosis [22]. For this reason, it is important to find alternative or complementary treatments for superficial mycosis by dermathophytes. In this study dermatophytes showed the highest susceptibility to essential oil from the fruits (HD_F).
The oils were less effective against *Candida* spp. Nevertheless, the sample more active against *Candida albicans* (SFE_F) was also found to inhibit more than 70 % of filamentation at concentration of MIC/8 (0.16 mg/mL) (Table 3).

Table 3. Influence of sub-inhibitory concentrations of *Schinus terebinthifolius* extract SFE_F on germ tube formation of *Candida albicans* ATCC 10231.

Control[(a)]	MIC/16	MIC/8	MIC/4	MIC/2	*MIC*
84 ± 3	*37±3*	*14±3*	*5±1*	*4±1*	*0±0*

[a] Untreated samples
[b] Absolute concentration in mg/mL

The yeast-mycelium transition in *C. albicans* is described as a significant virulence factor in this species [23]. These findings add significant information to the antifungal activity of *Schinus terebinthifolius*.

In conclusions, the plant has a very long history of use, this is the first time Tunisian *Schinus terebinthifolius* volatile oil has been studied for the chemical composition and biological activity. The oils, evaluated against yeasts and dermatophyte strains, are more active against *Cryptococcus neoformans*, particularly the volatile oil from the fruits, with MIC values of (0.32-0.64) mg/mL.

This study indicated that *Schinus terebinthifolius* oils may possess antifungal activity and can be exploited as an ideal treatment for for topical formulations in the management of superficial mycoses.

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