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Antifungal activity of essential oils and crude extracts of *Santolina africana* from Tunisia and Morocco

M. Khammassi [1], M. Ben Mansour [2], A. Laabidi [1], I. Amri[1], M. Boudkhili [3], L. Hamrouni [1], H. Greeche [3], A. Khaldi [1], B. Jamoussi [4]

[1]*National Institute of Researches on Rural Engineering, Water and Forests, BP.10 Ariana 2080, Tunisia.*
[2]*Central polytechnic Private School of Tunis, 3 Street Mohamed V,Le Kram,1015 Tunis, Tunisia*
[3]*National Institute of Medicinal and Aromatic Plants, Sidi Mohamed Ben Abdellah University, Fez, Morocco.*
[4]*Chemistry Laboratory, Higher Institute of Education and Continuous Training, 43 Rue de la Liberté, 2019, Le Bardo, Tunisia.*

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khammassi_marwa@yahoo.fr

Abstract

Essential oils of *Santolina africana* from Tunisia and Morocco were extracted by hydro distillation and analyzed by GC-FID and GC-MS to determine their chemical composition. The main compounds of essential oil from Tunisian plant were artemisia ketone (42.96%), isoborneol (24.30%) and santoline alcohol (6.05). In the Moroccan essential oil, artemesia ketone (39.34%), santoline alcohol (14.84%) and isoborneol (7.86%) were the major components. Furthermore, antifungal activity of essential oils, methanolic and water extracts was tested against five phytopathogenic fungi (*Fusarium oxysporum*, *Fusarium solani*, *Fusarium graminearum*, *Botrytis cinerea* and *Bipolaris sorokiniana*) by using disc-diffusion method.Results showed that essential oils have an important antifungal effect against *B.sorokiniana* with a growth inhibition percentage of 100% at the concentration of 6µl /ml.

1. Introduction

For many centuries, treatment with medicinal plants was the only resource available for numerous ethnic groups. Today, plants are still used in traditional medicine to treat, alleviate or prevent many diseases. The treatment of diseases with medicinal herbs has become the first treatment of choice [1- 2]. It is more beneficial than synthetic products, ease of use, treatment efficacy, affordable cost and minimal side effects [3].

The genus *Santolina* is constituted by a taxonomically complex group of species whose botanical classification is periodically revised. This genus belonging to the *Compositae* family (*Asteraceae*) is represented by more than 10 species widely distributed in Mediterranean area [4].

Santolina has been used in traditional medicine for its healing effects [5-6].It is used for its antispasmodic, antiseptic and anti-inflammatory effects [7].

One species of *Santolina* known as *Santolina africana* (= *Ormenis africana*) is grown as an ornamental. It is a shrub with strong odour that grows wild among the rocks on arid regions [8].

This plant is endemic in North Africa (Tunisia, Algeria and Morocco). In Tunisia, it is traditionally used for its hypoglycemic effect. It is also beneficial in the treatment of stomacal pain. The inflorescences of this plant are mixed with honey and used for the treatment of the cardialgia ulcer [9]. The flowerheads and roots of *O. africana* essential oil demonstrated potent of antibacterial properties [10]. In Moroccan popular medicine, *Santolina africana* is mainly used as abortifacient, emmenagogue and vermifuge. It is also known for its antidiabetic properties [11].

Because of these properties, extracts and essential oils from aromatic and medicinal plants have been widely investigated in the search as alternative to chemical product .The aim of this study is to characterize the composition of essential oils of *Santolina africana* from Tunisia and Morocco. Furthermore, the antifungal effect of oils, methanolic and water extracts was investigated against five phytopathogenic fungi.

2. Materials and Methods

2.1. Plant Material

Five samples of *S. africana* were collected in January 2011 from the region of Lekrib, gouvernorate of Siliana in North of Tunisia and from Taounate, a province in the Fès region in Morocco. The plant was identified by Dr Ridha El Mokni, Department of Life Sciences, Faculty of Sciences of Bizerte.

2.2. Extraction of Essential oils

The essential oils have been extracted from (100 g) air-dried aerial parts (leaves and stems) by hydro distillation for 3h, using a Clevenger-type apparatus according to the European Pharmacopeia method [12]. Oils were dried over with anhydrous sodium sulfate and stored in sealed glass vials at 4°C until analyses. The amount of oils obtained was measured and the yields were calculated based on the dried weight.

2.3. Methanolic and water extract preparation

The air-dried aerial parts of *S.africana* (50 g) were powdered and then extracted with 500 ml of methanol or distilled water by using maceration process. The crude extracts were filtered through Whatman No. 4 filter paper and evaporated under reduced pressure at 45°C with a rotary evaporator. The dried extracts were stored at 4 °C for further use [13].

2.4. Essential oil identification

The essential oils composition was identified by GC–FID and GC–MS analyses. GC analysis oils were carried out on an HP5890-series II gas chromatograph (Agilent Technologies, CA, USA) equipped with Flame Ionization Detectors (FID) under the following conditions: the fused silica capillary column, apolar HP-5 and polar HP Innowax (30m–0.25mm ID, film thickness of 0.25 mm). The oven temperature was held at 50°C for 1 min then programmed at a rate of 5°C / min to 240°C and held isothermal for 4 min. The carrier gas was nitrogen at a flow rate of 1.2 ml /min; injector temperature: 250°C, detector: 280°C; the volume injected: 0.1 ml of 1% solution (diluted in hexane) in splitless mode. The percentages of the constituents were calculated by electronic integration of FID peak areas without the use of response factor correction.

GC/MS was performed in a Hewlett Packard 5972 MSD System. An HP-5 MS capillary column (30m–0.25mm ID, film thickness of 0.25 mm) was directly coupled to the mass spectrometry. The carrier gas was helium, with a flow rate of 1.2 ml / min. Oven temperature was programmed (50°C for 1 min, then 50–240°C at 5°C/ min) and subsequently held isothermal for 4 min. Injector and detector temperatures were 250°C and 280°C respectively. The injected volume was 0.1 ml of 1% solution (diluted in hexane) in splitless mode. The software adopted to handle mass spectra and chromatograms was ChemStation. The identification of the compounds was based on mass spectra (compared with Wiley 275.L, 6th edition mass spectral library). Further confirmation was done from Retention Index data generated from a series of alkane retention indices (relative to C9–C28 on the HP-5and HP-20M columns) [14].

2.5. Fungal species and in vitro antifungal activity

The Tunisian National Institute of Agronomic Research (INRAT) provided the 5 test fungal species: *Fusarium oxysporum*, *Fusarium solani*, *Fusarium graminearum*, *Botrytis cinerea* and *Bipolaris sorokiniana*. *Fusarium* species are soil fungi. Some are plant pathogens, causing root and stem rot, vascular wilt or fruit rot. Other species cause storage rot and are important mycotoxin producers.

Botrytis cinerea is a necrotrophic fungus that affects wine grapes whereas *Bipolaris sorokiniana* causes a wide variety of cereal diseases.

The fungal strains were maintained in Potato Dextrose Agar (PDA) medium and were stored at 4°C in 1ml of glycerol 25% at -8 °C.

Antifungal activity was studied by using an in vitro contact assay which produces hyphal growth inhibition [15]. Essential oils was dissolved in 1ml of Tween 20 (0.1% v/v) and methanolic extracts were evaporated and dissolved in Dimethyl Sulfoxide (DMSO). Extracts and oils, were then added into 20 ml PDA at 40°C to obtain different final concentrations. 5 mm diameter discs of the test fungal species were cut from one week-old cultures on PDA plates and inoculated at the centre of each PDA plate (90 mm diameter). The plates were then incubated at 24°C for 7 days. PDA plates treated with Tween 20 (0.1%) and DMSO without essential oil and crude extracts were used as negative control.

Tests were repeated in triplicate. Growth inhibition was calculated as the percentage of inhibition of radial growth relative to the control using the following formula [15]:

$\%$ Inhibition = $[(C - T/C)] \times 100$,

where C is the average of three replicates of hyphal extension (mm) of controls and T is the average of three replicates of hyphal extension (mm) of plates treated with essential oils or extracts.

2.6. Statistical analysis

The results were expressed as average values of three replicates and were subjected to analysis of variance (ANOVA) using the SPSS (Version 13.0) software. This type of analysis was used to test the effect of the concentration of essential oils and extracts on the mycelial growth of the tested strains. Differences between means were tested through the Student-Newman-Keuls and p values ≤ 0.05 were considered significantly different.

3. Results and discussion

3.1.Yield and composition of essential oils

Oils yields obtained from the air-dried aerial parts of Tunisian and Moroccan *S. africana* were 0.71% and 0.82% respectively. The yield of Moroccan essential oil (0, 82%) is almost similar to that reported by Lmachraa et al. (2014) [11].

Forty eight and thirty nine constituents which represented 99.74% and 97.58% of the total essential oils of *S. africana* aerial parts from Morocco and Tunisia respectively were identified. The main constituents of the essential oil extracted from Morrocan plant were artemisia ketone (39.3%), santolina alcohol (14.8%) and isoborneol (7.8%), whereas artemisia ketone (42.96%) and isoborneol (24.30%) were most prevalent in Tunisian oil (Table 1). In previous works, the most abundant components in *Santolina africana* oil collected from the region of Téjrouine in North West of Tunisia were terpinen- 4-ol (54.96%), α-terpineol (14%) and borneol (8.37%) [16].

Wherease, the prevalent constituents in *Santolina africana* essential oil from Djebel Kesra in the West Center of Tunisia were 1,8-cineole (12.94%), β-eudesmol (10.49%), terpinene-4-ol (6.97%) and γ-cadinene (6.55%) [10].

Results showed that essential oils from Morocco and Tunisia were characterised by a high percentage of oxygenated monoterpenes, followed by monoterpene hydrocarbons and sesquiterpene hydrocarbons. Similar composition was found in the research conducted on the fresh aerial parts of *Achillea fragrantissima* from Libanon. In fact, essential oil from this species was rich on oxygenated monoterpenes (56.66%) and the main compounds were artemisia ketone (29.97%) and α-Thujone (13.34%) [17]**.**

In other work , the essential oils extracted by hydrodistillation from the flowers of *Santolina africana* from Algeria were characterized by a large amount of monoterpene hydrocarbons (27.56%) and sesquiterpenes hydrocarbons (26.89%), whereas the oxygenated monoterpenes (6.42%) and oxygenated sesquiterpenes (0.86%) represent the low contents in the leaves oil from *S. africana* [18].

3.2. Antifungal activity of essential oils and extracts

The present study investigated the *in vitro* antifungal activity of the aerial part extracts and essential oils of *S. africana* against five fungi. The effects of tested samples on mycelia growth after an incubation period of 7 days at 24°C are summarized in tables 2 and 3.

Both oils showed an important antifungal activity against *B. sorokiniana* with a growth inhibition percentage of 100% at the concentrations of 6µl /mL (Fig.1). However*, F.solani, F.oxysporum, F.graminarium* and *B.cinerea* were found to be more resistant at this concentration. The inhibitory effect of essential oils on the growth of fungi is less important for concentrations ranged from 2 to 5µl/mL.

All the tested fungi were susceptible to essential oils and extracts of *S. africana* aerial part with different degrees. The susceptibility of fungi to plant extracts and oils, on the basis of growth inhibition percentage, varied according

to fungi strains and concentrations. Active substances of essential oils act to make the cell membranes of fungi permeable, causing leakage [19].
The antifungal activity of *S. africana* oils is mainly attributed to its richness on artemisia ketone, isoborneol and santolina alcohol.

Table1: Chemical composition of Tunisian and Moroccan *S. africana* essential oil

N°	Compounds	RI	*S. africana* essential oils %		Methods of identification
			Morocco	Tunisia	
1	Santolinatriene	907	0.8	0.6	MS, RI
2	α-tricyclene	927	0.4	0.7	MS, RI
3	α-Pinene	937	1.6	3.0	MS, RI, Co-GLC
4	Camphene	954	3.5	1.9	MS, RI
5	α-Sabinene	975	3.2	2.3	MS, RI
6	β-pinene	979	7.1	3.1	MS, RI, Co-GLC
7	β-myrcene	991	0.8	1.2	MS, RI , Co-GLC
8	α-Terpinene	1017	0.2	0.3	MS, RI , Co-GLC
9	*p*-Cymene	1025	0.2	0.3	MS, RI
10	Santolina alcohol	1031	14.8	6.1	MS, RI
11	Artemisia ketone	1062	39.3	42.9	MS, RI
12	(*Z*)-sabinene hydrate	1075	0.6	0.4	MS, RI
13	α-Terpinolene	1089	0.6	0.6	MS, RI, Co-GLC
14	Linalool	1098	2.4	0.6	MS, RI , Co-GLC
15	α-Thujone	1105	0.2	0.2	MS, RI
16	β-Thujone	1116	0.2	0.2	MS, RI, Co-GLC
17	*Trans*-Pinocarveol	1136	0.3	0.3	MS, RI
18	Menthone	1153	0.3	0.4	MS, RI, Co-GLC
19	Isoborneol	1162	7.8	24.3	MS, RI , Co-GLC
20	Terpinen-4-ol	1177	0.2	-	MS, RI, Co-GLC
21	*p*-cymen-8-ole	1182	0.6	0.8	MS, RI
22	α-terpineol	1189	0.7	0.6	MS, RI, Co-GLC
23	Myrtenal	1196	0.8	0.3	MS, RI
24	(*E*)- Piperitol	1201	0.2	0.4	MS, RI
25	(*E*)-Carveol	1212	0.3	0.6	MS, RI
26	Carvacrol methyl ether	1246	0.2	0.2	MS, RI
27	α-santalene	1417	0.2	-	MS, RI
28	(*Z*)-b-Farnesene	1445	0.2	-	MS, RI
29	α-humulene	1454	0. 6	0.2	MS, RI, Co-GLC
30	Allo-aromadendrene	1460	1.7	0.2	MS, RI
31	Germacrene D	1475	0.3	-	MS, RI, Co-GLC
32	Bicyclogermacrene	1492	0.2	-	MS, RI
33	α -Muurolene	1501	0.1	0.3	MS, RI
34	β-bisabolene	1504	0.3	0.2	MS, RI
35	γ-Cadinene	1513	0.5	0.2	MS, RI
36	δ-cadinene	1524	0.2	-	MS, RI
37	α -cadinene	1539	0.2	0.3	MS, RI, Co-GLC
38	β-Calacorene	1545	0.3	-	MS, RI
39	Germacrene B	1558	0.2	0.2	MS, RI
40	Germacrene D-4-ol	1572	0.2	0.4	MS, RI
41	Spathulenol	1578	0.4	0.3	MS, RI
42	Caryophyllene oxide	1583	0.1	0.2	MS, RI, Co-GLC

43	Guaiol	1590	0.3	0.3	MS, RI
44	β –oplopenone	1607	4.5	1.3	MS, RI
45	Epi-α - Cubenol	1615	0.3	0.3	MS, RI
46	(*Z*,*E*)-farnesol	1707	0.5	0.2	MS, RI
47	(*Z*,*Z*)-farnesol	1710	0.1	-	MS, RI
48	(*E*,*E*)-farnesol	1727	0.2	-	MS, RI
Yied			**0.82±0.045**	**0.71±0.025**	
Total identification :			99.749	97.584	
Monoterpene hydrocarbons% :			18.529	14.053	
Oxygenated monoterpenes %:			69.184	78.595	
Sesquiterpenes hydrocarbons% :			5.055	1.713	
Oxygenated sesquiterpenes %:			6.844	3.185	

RI. Retention Index; MS. mass spectrometry; Co-GLC: co-injection; -: not detected; [a] :apolar HP-5 MS column.

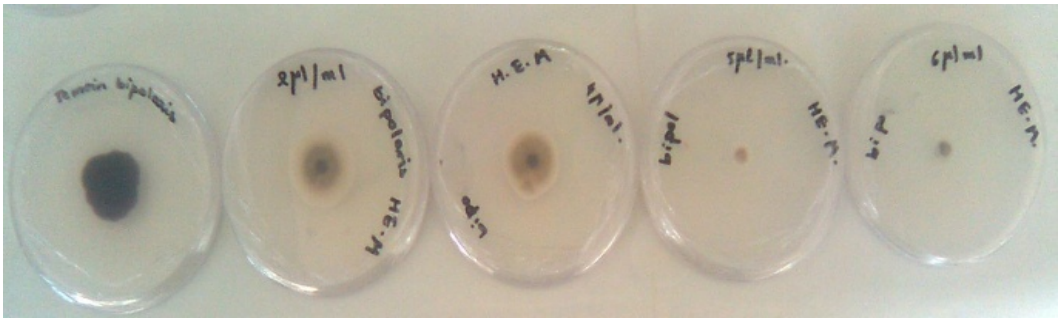

Figure1: *The effect of Moroccan essential oil on mycelia growth of B. sorokiniana* after an incubation of 7 days at 24°C

A wide variety of essential oils are known to possess antimicrobial properties and in many cases this activity is due to the presence of active constituents, mainly attributed to isoprenes such as monoterpenes, sesquiterpenes and related alcohols, hydrocarbons and phenols [18]. Monoterpenes diffuse into pathogens and damage cell membrane structures [20-21].

Table 2: Antifungal activity of essential oils of *S. africana* at different concentrations

Extracts	Origin	fungi	Growth reduction (%)			
			2µl ml^{-1}	4µl ml^{-1}	5µl ml^{-1}	6µl ml^{-1}
EO	Morocco	*F.solani*	18.34±10.94 bB	31.78±4.23ab AB	40.67±3.95 c AB	45.12±3.81c A
		F.oxysporum	29.45±5.48 b C	39.71±3.54 a BC	45.47±1.46 bc B	58.01±3.47 b A
		F.graminarium	45.79±1.66 a B	48.11±3.75 a B	51.92±2.71 b B	65.65±0.70 b A
		B.cinerea	12.42±0.94 b C	44.99±5.39 a B	53.27±3.79 b B	66.29±5.57 b A
		B.sorokiniana	9.88±5.66 b D	24.18±5.08 b C	64.29±0.57 a B	100±0 a A
EO	Tunisia	*F.solani*	4.43±2.04 a B	9.61±3.04 ab B	11.83±4.06 c B	29.60±3.88c A
		F.oxysporum	1.18 ±0.09 a C	4.14 ±0.87 b C	12.40±4.08 c B	31.36±1.09 c A
		F.graminarium	13.18±2.92 a D	25.43±7.16 a C	40.63±2.46 b B	58.36±1.35 b A
		B.cinerea	1.76 ±0.49 a B	2.35±0.32 b B	17.05±0.83 c A	22.35±1.66 d A
		B.sorokiniana	1.96±1.66 a C	15.59±7.54 ab C	50.99±0.33.aB	100 ±0 a A

Note: Means in the same column with the same lower case letter and means in the same rows with the same upper case letter are not significantly different according to the Student–Newman–Keuls test (p =0.05). Values are means ±SE.

It should be mentioned that there are no background antifungal studies of *Santolina africana* aerial part essential oil against phytopatologic fungi, while some studies have been reported the antifungal activity of flowers essential oil of *Santolina africana* against *Aspergillus flavus, Aspergillus niger* and *Candida albican* [18].
In the current study, both methanolic and aqueous extracts, from Tunisian and Moroccan plants were shown to exhibit, *in vitro*, a significant antifungal activity that depend on extracts concentrations and fungi strains.
As shown, in Table 3, the aqueous extract at the concentration of 40µl.ml^{-1}was more active against *B. cinerea* and *B. sorokiniana.* However, at this concentration, *F. graminarium* and *B. sorokiniana* were most sensitive to methanolic extracts.
Methanolic extracts were better or equally effective against the fungi strains with the exception that aqueous extract could not inhibit *F. solani.* Extract procedure may be relevant, of the use of plants in traditional medicine [22].
The antimicrobial action of the extracts can be attributed to astringent nature of the phenolic constituents including tannins and other polyphenols present in the extracts [23].

Table 3: Antifungal activity of methanolic and water extracts of *S.africana* at different concentrations

Extracts	Origin	fungi	Growth reduction (%)		
MeOH	Morocco		10µl.ml^{-1}	20µl.ml^{-1}	40µl.ml^{-1}
		F.solani	25.75±2.74 a C	39.07±0.86 b B	46.09 ±0.08 c A
		F.oxysporum	2.36±0.75 c B	11.81 ±0.94 d B	26.94±0.61 d A
		F.graminarium	30.78±0.83b C	48.13±4.02 b B	65.33±3.66 b A
		B.cinerea	17.64±1.66 b C	28.23±4.99 c B	41.17±1.66 c A
		B.sorokiniana	43.34±5.47 a B	62.06±5.27 aA	75.80±6.71 aA
	Tunisia		10µl.ml^{-1}	20µl.ml^{-1}	40µl.ml^{-1}
		F.solani	7.33±1.3 c B	20.15±3.9 c B	44.61±4.6 c A
		F.oxysporum	28.45±1.66 b C	37.31±1.34 b B	58.16±3.28 b A
		F.graminarium	68.33±2.35 a B	69.29 ±1.83 a B	84.13 ±3.02 a A
		B.cinerea	14.25±3.12 c C	37.46±3.5 b B	49.39±1.69 d A
		B.sorokiniana	34.06±8.39 b A	50.44±9.47 b AB	73.21 ±2.52 b A
Aqueous	Morocco		10µl.ml^{-1}	20µl.ml^{-1}	40µl.ml^{-1}
		F.solani	1.36±0.03 d A	2.05±0.90 c A	2.73±0.87 c A
		F.oxysporum	34.65±5.89 b A	43.04±5.68 b A	47.83 ±5.55 b A
		F.graminarium	21.61±4.30 c A	33.61 ±2.97 b A	39.06±6.75 b A
		B.cinerea	18.82±3.32 c C	69.41±1.66 a B	85.88±1.66 aA
		B.sorokiniana	49.24±3.35 a B	69.7±11.9 aA	84.25±13.08 a A
	Tunisia		10µl.ml^{-1}	20µl.ml^{-1}	40µl.ml^{-1}
		F.solani	0.7±0.99 c A	1.4±0.13 c A	2.13±1.02 d A
		F.oxysporum	19.18±2.18 b B	37.68±3.27 b A	44.91±0.29 b A
		F.graminarium	1.92±0.7 c B	30.04±4.64 b A	38.38±1.48 c A
		B.cinerea	54.65±4.49 a B	68.57±8.58 a B	91.8±9.4 a A
		B.sorokiniana	46.78±5.98 a B	77.02±3.62 a A	88.5±1.13 a A

Note: Means in the same column with the same lower case letter and means in the same rows with the same upper case letter are not significantly different according to the Student–Newman–Keuls test (p =0.05). Values are means ±SE.

Different modes of action of phenolic [25] and terpene compounds have been reported in several reviews [24-26]. Some researchers have suggested that the phenolic components of the plant extracts, when crossing the cell membrane, interact with the enzymes and proteins of the membrane and cause an opposite flow of protons and produce a flux of protons towards the cell exterior which induces change in the cell and ultimately its death [27-28]. Other researcher attributed the inhibitory effect of these plant extracts to the hydrophobicity characters of these extracts and their components. This enables them to partition in the lipids of the fungal cell wall membrane and

mitochondria, disturb their structure and render them more permeable. Cell death can be caused by leaking of ions and other cell contents [29].

Conclusion

Essential oils extracted from Tunisian and Moroccan *S.africana* were characterized by a high percentage of oxygenated monoterpenes. They exhibited highly significant antifungal activity against the tested antifungal strains. The present work showed that essential oil extracted from this plant was a potentially good source of antifungal agent and that further investigation is worthwhile to isolate and evaluate biologically active compounds from the essential oil.

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