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RESEARCH ARTICLE

Screening for antifungal activity polyphenol content of *Origanum majorana* L. essential oil treated and non treated with salt

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Abstract

Origanum majorana L. essential oil has been known as an interesting source of antimicrobial and antioxidant compounds to be applied in food conservation.
This study aims to evaluate the antioxidant activity and to assess the efficient of *O. majorana* L. essential oil, treated (75 mM NaCl) and non treated (0 mM NaCl), in inhibiting the growth and survival of potentially pathogenic fungal strains such as *Engyodontium album*, *Acremonium strictum*, *Trichophyton verrucosum*. The antifungal potential was determined performing the disc diffusion assay.
These results suggest that essential oils from marjoram were more effective than those of three standard samples, in absence and presence of salt; and it could be regarded as a potential antifungal compound for controlling the growth of pathogen fungi and the occurrence of mycoses.

INTRODUCTION

Several plant families, especially the Lamiaceae, present prominent amounts of essential oil (yield >2 %) (Li et al., 2008) Given their broad variety of chemical characteristics and aroma, different species and biotypes of *Origanum* are widely used by the pharmaceutitical and cosmetic industries, as a food flavor, for fragrance in perfumes and in alcoholic beverages (Meda et al., 2005).
Oregano essential oils have been shown to possess antioxidant, antifungal, diaphoretic, carminative, antispasmodic and analgesic activities (Baser., 1994). In recent years, a large number of researches have reported the efficacy of essential oils from several *Origanum* species against bacterial strains (Novak at al., 2000). Essential oils also include antioxidants such as terpenoid and phenolic components. *O. majorana* L. have been used in folk medicine to treat many illnesses as a spasmodic, antimicrobial, digestive and aromatic (Meda et al., 2005). Some studies have found interesting antimicrobial activity in *Origanum* species, (Tommasi at al., 2009). The essential oil of aerial parts of *O.majorana* has a broad spectrum of antimicrobial activity. The efficacy of essential oil (EO) on bacteria (Baatour et al., current data) and fungi tested with essential oil compounds depend on the quality and quantity of the active substance contained in the raw material (Bouhdid at al., 2008). Recently, several natural plants have received much attention as sources of biological active substances including antioxidants. Natural antioxidants properties are mainly attributed to their phenolic contents, thus their antioxidants action is similar to synthetic phenolic antioxidants (Baydar at al., 2008). Phenolic compounds are well known as radical scavengers, reducing agents, hydrogen donors, and singlet oxygen (Carvalhinho at al., 2012). Therefore, natural antioxidants can protect the

human body from free radicals and could retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods (Fecka and Turek 2008).
The aim of the present research was to investigate; the antifungal and antioxidant activity of the essential oil in relation to the chemical composition.

1. Materials and methods

1.1 Fungal strains

The experiments were carried out on the following species: *Engyodontium album*, *Trichophyton verrucosum Acremonium strictum* isolated from Tunisian patients. They were subcultured on Sabouroud medium (Sab) and incubated at 30° C for 48h.

1.2 Screening for antifungal activity

Disc-diffusion assay.

Seeds of marjoram were collected in 2010 from a nursery located in Soliman in northeastern Tunisia (latitude 36° 41' 47 N; longitude 10° 29' 30 E; altitude 1500 m).
Essential oil was extracted, by hydrodistillation, from fresh shoots (50 g) during 90 min GC-FID and GC-MS according to Baâtour et al., (2012c).
The screening of selected essential oils for antimicrobial activity was done by the disc diffusion method, which is normally used as a preliminary check and to select between efficient control and treated essential oils (Lopez et al., 2005).
Antimicrobial tests were then carried out by the disc diffusion method (Murray et al., 1995) using 100 µl of suspension containing 10^4 spore/ml of fungi spread on sabouraud suiTab medium.
The essential oils extracted from the aerial part of Tunisian *O. majorana* as referred to (Baatour et al., 2015; Current data).
The sterile paper discs (8 mm in diameter) were impregnated with 80 µl of essential oil in 18 µl tween 80% (with sterile cones).
The first were impregnated with control oil (0 mM NaCl), the second with treated one (75mM NaCl). Negative controls were prepared using (100µl tween 80%). The inoculated plates were incubated at 37°C for 48 h with yeast. The activity of tested substances was estimated as the mean diameter of clear zones above each disc and by the growth of the strains inside these zones. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. The zones of inhibition with 0.1 mm diameter and over are considered. Each substance was tested in triplicate during 9 days. Results presented are the mean of three assays. The inhibition percentage of the growth was calculated relative to control without essential oil from the following equation:
% Inhibition = (dt-de) / dt * 100.
Dt (mm) = diameter of fungal growth in positive control disc.
De (mm) = diameter of fungal growth in essential oil disc.
Diameters of microbial inhibition zones were measured in mm.
The essential oil was:
-very active, when it has an inhibition between 75 and 100%: fungal strain was very sensitive;
-active, when inhibition was between 50 and 75%: fungal strain was sensitive;
-active, when it had a moderate inhibition between 25 and 50%; the strain is limited;
-little or no active when inhibition range between 0 and 25%; the strain is insensitive or resistant.
Growth inhibition of fungal strains against essential oil was expressed by the difference between the rayon R2 (area without EO) and the rayon R1 (area in front of EO) obtained by the average of three replicates.

1.3. Antioxidant Activity:

Determination of total phenolic content (TPC):

The level of total phenols in the marjoram essential oil was determined by using Folin–Ciocalteu reagent and external calibration with gallic acid. Marjoram essential oil, 180µl and 4 ml of Folin–Ciocalteu reagent were added and the contents mixed thoroughly. After 4 min, 1 ml of 18% Na_2CO_3 was added, and then the mixture was allowed to stand for 1 h at normal temperature. The absorbance was measured at 760 nm using a spectrophotometer. Total phenolic concentration was calculated as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve (Li et al., 2008).

2.3.5. Radical scavenging activity and antioxidant content

The scavenging activity of samples for the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was measured as described by Meda et al. (2005). Ethanolic extracts (3 ml) at different concentrations (0.04 to 1.00 g/ml) were added to 0.1 ml of DPPH (Sigma-Aldrich, Germany). Ethanolic solution (0.1 mM), served as the blank sample. The mixtures were maintained in the dark for 30 min at room temperature. The antioxidant content was determined using

standard curves for carvacrol, thymol and marjoram essential oil. The radical scavenging activity was calculated as follows:

% Inhibition = [(blank absorbance - sample absorbance)/blank absorbance] x 100.

The mean of three IC50 (concentration causing 50% inhibition) values from each sample was determined. Concentrations were expressed in µg/ml.

1.4 . Statistical analysis

Results are the means of 3 replicates. Data were subjected to one-way analysis of variance (ANOVA) as variability factor and mean comparison with Duncan post hoc test (Statistica). Means followed by different letters are significantly different at $P \leq 0.005$.

Result and Discussion

Antifungal activity of *Origanum* essential oils was screened against three human pathogenic fungi (T16: *Trichophyton verrucosum*; T3: *Acremonium strictum* and R18: *Engyodontium album*) (Fig 1-3).

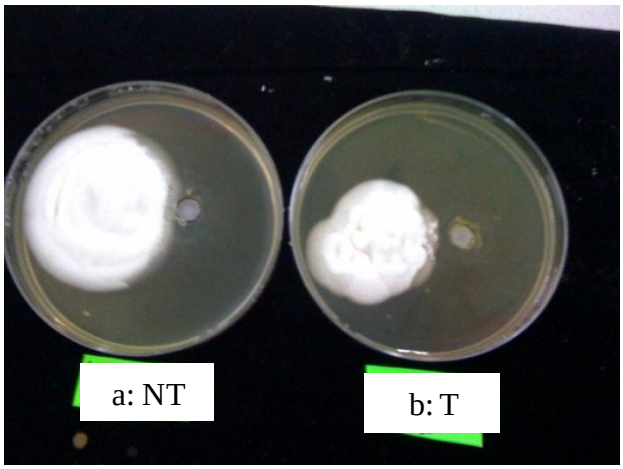

Figure 1: Inhibition test of *Origanum majorana* essential oil against *Engyodontium album*: (a): not traited oil (NT), (b): Traited oil (T).

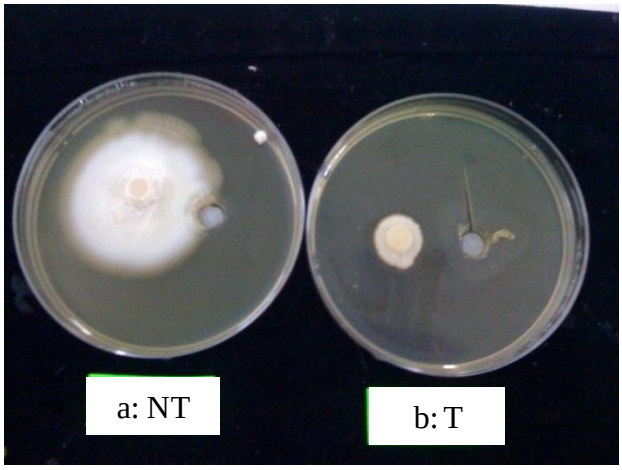

Figure 2: Inhibition test of *Origanum majorana* essential oil against *Acremonium strictum* : (a): not traited oil (NT), (b): Traited oil (T).

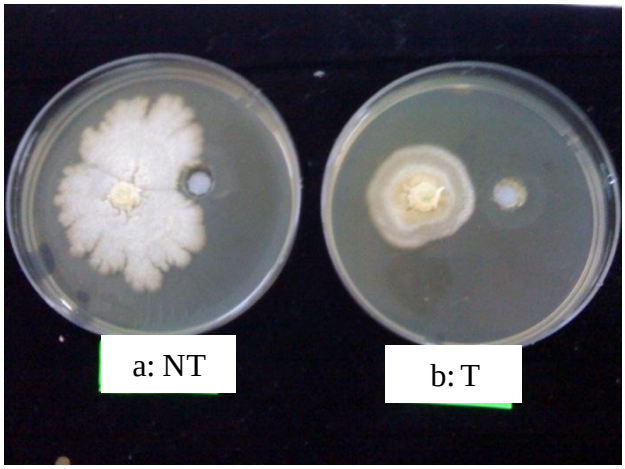

Figure 3: Inhibition test of *Origanum majorana* essential oil against *Trichophyton verrucosum* (a): not traited oil (NT), (b): Traited oil (T).

The antimicrobial activity against microorganisms, examined in the present study, and their potency were quantitatively assessed; by the presence or absence of inhibition zones and zone diameter. The results are given in Tab 1.

Table 1: inhibition diameter (en mm) of *Origanum majorana* L.

	Traited oil	**Untraited oil**	**Growth**
Champignons	**Diameter (mm)**		
Engyodontium album (T18)	(+) 5±0,2mm	(++) 10±0,18mm	There is a small growth of ***E. album*** containing the treated EO compared to untreated NT
Acremonium strictum (T3)	(-) 0	(-) 0	Total growth inhibition of ***A. strictum***
Trichophyton verrucosum (T16)	(+) 5±0,03mm	(+) 7,5±0,02mm	Small growth inhibition of ***T. verrucosum*** against traited EO

Disc diameter included in agar (5mm), - : absence of growth,
+: small growth, ++ : Average growth

Marjoram treated essential oil with 75 mM NaCl, showed no antifungal activity against the three fungal strains tested, as referred to inhibitions zones observed. The disc diameter zones of inhibition ranged from 5 mm to 10 mm (Tab 1). The control essential oil had an inhibitory activity only, against *Engyodontium album* (Tab 2).

Table 2: Inhibition Percentage of champignions

	Inhibition Pourcentage (%)	
	NT	T
Engyodontium album	16,73±1,71	40,16±0,4
Acremonium strictum	0	0
Trichophyton verrucosum	20±0,00	37,5±1,24

Fungal susceptibility to these essential oils, as determined by the direct contact method, showed that marjoram untreated (0 mM NaCl) oil produced a 10 mm and 7 mm in diameter inhibition zone against *Engyodontium album* and *Trichophyton verrucosum* thus presenting the highest inhibitory effects as compared to treated one (Tab 1).
According to our results obtained in Tab 1, *Acremonium strictum* was more sensitive than *Trichophyton verrucosum* and finally to *Engyodontium album*.
Marjoram oil showed a fungistatic activity against three fungal strains. Such an activity could be strictly related to their chemical composition. The results of chemical analysis of *O. majorana* were represented in a current paper of (Baatour et al., 2015; Current Data).
According to Tab 2, the inhibition percentage of tested oils depends on salt treatment against fungi. Indeed, inhibition percentage of EO tested varied between 16 and 100%. A total growth inhibition percentage of *Acremonium strictum* had been observed. But, this percentage varied between 16 to 40% for *Engyodontium album* and 20 to 37% for *Trichophyton verrucosum* respectively for treated and non treated oil. We can suggest that essential oil of marjoram treated or non treated with salt was active against different microorganisms tested. We can conclude that the essential oil of marjoram treated or not treated was active against different microorganisms tested. Therefore, it may be an antifungal alternative.
Cavanagh (2007), mentioned that the mechanism of action of essential oil on fungi is unclear but the majority of reports agree that volatiles oil result in morphological changes to the hyphae. The inhibition of the growth of fungal strains is related to volatile compounds contained in essential oils and the sensitivity of microorganisms Holley and Patel (2005). In addition fungi grow mainly on the surface of the agar medium and might be more susceptible to direct vapor contact.
Marjoram Tunisian essential oils was rich in terpinen-4-ol compound (Baatour et al., 2015; Data in press) who exhibit antifungal potential, which has already been described in the literature by Settanni et al. (2012) and Agastian et al.(2000).

Essential oil antioxidant activity

Salinity, characterizing arid regions may increase phenolic compound biosynthesis, as a response to the oxidative stress generated by the formation of reactive oxygen species in these hostile environments (Hammer et al., 2009). In contrast to our result, Agastian et al. (2000) reported an increase of polyphenol amount under increasing salinity. Navarro et al., (2006) showed increased total phenolics amount at moderate saline levels in red peppers (*Capsicum annuum* L.).
Phenolic compounds are well known as radical scavengers (Elena et al., 2009). Antioxidant capacity of plants is correlated with the content of phenolic compounds. In control, total phenolic contents (TPC) were 6.23 (mg gallic acid equivalent/g dry weight) mg GAE/g of marjoram essential oil (Fig.4).
Our results were in agreement with Sellamia et al. (2009) who reported that, TPC varied from 2.706 to 6.834 mg/g of dry weight of marjoram essential oils. Recently, Roby et al (2013), found that, the total phenolic contents were 8.10, 5.95, and 5.18 (mg gallic acid equivalent/g dry weight) for thyme, sage, and marjoram, respectively. However, a significant decrease in polyphenol accumulation occurred of about 25.82 %, respectively at 75 mM, with respect to the control (Fig.1). This decrease suggested the salt sensitivity of plant. Previous studies suggested that abiotic stresses (salinity) characterizing arid regions may increase phenolic compound biosynthesis, as a response to the

oxidative stress generated by the formation of reactive oxygen species in these hostile environments (Sellamia et al., 2009). In contrast to our result, Roby et al. (2013) showed an increase of polyphenol amount under increasing salinity. An increase in total phenolics amount at moderate saline levels was observed in red peppers (*Capsicum annuum* L.) (Sellamia et al., 2009).

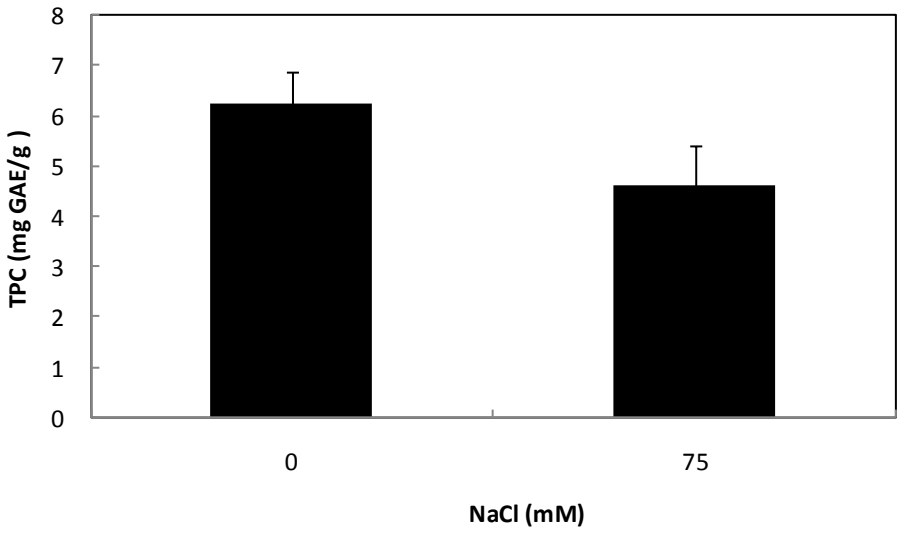

Figure 4: Effect of salinity in total phenolic contents (TPC) (mg gallic acid equivalent/g dry weight) mg GAE/g of areal part of Tunisian *Origanum majorana* essential oil

Antioxidant Activity

The antioxidant activities of marjoram oil was compared with the standard commercial synthetic antioxidants BHT, Ascorbic acid (Baatour et al., 2015; Current Data) terpinene 4-ol and marjoram essential oil (Tab 3). In absence and presence of salt, terpinene 4-ol (0.54 ± 0.01 µg/ml and IC50 = 0.80 ± 0.00 µg/ml respectively) were more effective than synthetic antioxidants BHT and ascorbic acid (IC50 = 2.82 ± 0.18 µg/ml and 4.79 ± 0.19 µg/ml, respectively) (Baatour et al., 2015; Current Data) and the essential oil obtained were more effective than these three standard samples in absence and presence of salt (Tab 3).

Table 3: Antioxidant activity (DPPH free radical-scavenging assay) of terpinene 4 ol and marjoram essential oil as compared to ascorbic acid, BHT (Baatour et al., 2015; in press).

IC50 (µg/ml)		
Compounds	**0 mM**	**75 mM**
Terpinene 4-ol	0.54 ± 0.01^{a}	0.80 ± 0.00 µg/ml^{b}
Essential oil (Data in press, 2015)	1.85 ± 0.02^{b}	2.99 ± 0.07^{a}

The main components in control medium, was characterized by high content of trans-sabinene hydrate and terpinene-4-ol. Under 75 mM NaCl, the chemotype (major 2, components) became cis sabinene hydrate/ terpinene-4-ol (Baâtour et al., 2012c). Thus these components had antimicrobial (Baatour et al., 2015, Current Data) and had stronger antifungal activity than antimicrobial activity (Erika et al., 2005).

Essential oil component of marjoram belongs to phenolics and flavonoids classes. So, we found a strong relationship among total phenol content, antioxidant and antimicrobial activity, as phenols are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups (Parsaeimehr et al., 2010). Therefore, the phenolic content of plants may be contributed directly to their antioxidant action (Wojdylo et al., 2007). Polyphenolic compounds have antimicrobial, antifungal (Breton, 2008) and antioxidant properties (Eyles et al., 2004) that protect plant tissues against environmental conditions. So, as it has been demonstrated by (Chan et al., 2012), the use of natural marjoram essential oils as green antioxidant and antifungal agents, could have industrial applications and incite scientific research.

Conclusion

The importance of these preliminary results is that fungi development can be controlled using plant essential oils. The use of marjoram essential oils against *Engyodontium album*, *Acremonium strictum*, *Trichophyton verrucosum* seems to be an interesting solution because of their safety. In conclusion, the essential oils of *O. majorana* showed interesting antifungal activity. Further investigations include quantitative tests in order to determine the concentration of essential oils (minimum inhibitory concentration) needed to exhibit antimicrobial activity against food related microorganism in order to use them as natural antimicrobial agents to extend the shelf life and to increase the safety of the processed food.

Our results indicate that essential oils from *Origanum majorana* L. could be used as green antioxidant and an alternative antifungal compound to control the growth of pathogen fungi.

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