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RESEARCH ARTICLE

In vitro antifungal effect of *Rosmarinus officinalis* essential oil on *Aspergillus niger*

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ABSTRACT

Background: The development of *Aspergillus niger* fungi at the surface and in the food products is indeed very often seen, especially in the tins. It is essential to find a solution for a better conservation using natural bioactive molecules such as essential oil (EO) of rosemary. **Aims and Objectives:** The aim of our study is to detect *in vitro* the antifungal effect of *Rosmarinus officinalis* (Rosemary) EO, against the fungal strain *A. niger* contaminating various food products and responsible for invasive fungal infection. **Materials and Methods:** The EO of *R. officinalis* is picked in the city of Tebessa-Algeria, and extracted by steam distillation using a Linkens Nickerson-type device. The chemical analysis is performed by ion-exchange gas chromatography coupled to mass spectrometry. The determination of the activity and the minimal inhibitory concentrations of the EO are performed by the method of incorporation in sabouraud agar medium. **Results:** We found that the EO contain 14 components, the major one is 1.8 cineol (63.65%), the study of its antifungal activity on selection of a major foods contaminated by *A. niger* shows an activity on all strains tested with a minimum inhibitory concentration of 0.5%. We also remark a fungistatic effect. **Conclusion:** Therefore, conclusions of this study can solve the problem of poor preservation of food products and the health risks associated with exposure to mold in particular *A. niger* food source.

KEY WORDS: *Rosmarinus Officinalis*; Essential Oil; *Aspergillus Niger*; Antifungal Activity

INTRODUCTION

Microorganisms can sometimes be part of food products composition; they are also in many cases essential to their maturation, but when their proportion becomes important, it can lead to considerable deterioration of food products with harmful consequences for both human health and economy. These microorganisms include bacteria, viruses, and fungi; they colonize several ecological niches;[1] and are transmitted to the food product through the water, air or during their preparation. The most often implicated specie is *Aspergillus niger*, this filamentous fungus is present in the air and dust, it is widely used in biotechnology thanks to these production capabilities of a wide range of organic acids, and it is a valuable source of extracellular enzymes. However, it is also known by the production of mycotoxins such as ochratoxin A (with known nephrotoxic effect) and fumonisin present especially in ordinary drinks,[2] both are classified as carcinogenic. In addition, a filamentous fungus is known to be the major causative agent of invasive aspergillosis. The inhibition of fungal growth by synthetic chemicals commonly used led to adverse effects on consumer

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health, due to different environmental problems and residual toxicity: Carcinogenicity, teratogenicity, hormonal imbalance, spermatic toxicity, etc.[1] Moreover, invasive fungal infections are difficult to treat with currently available antifungal.[3]

Some plants or their extracts traditionally used in food preservation showed high inhibitory capacity of toxigenic molds,[4] among these plants, *Rosmarinus officinalis* (rosemary) is an aromatic plant belonging to Lamiaceae family, it has been used during thousands of years for both culinary and medicinal proposes, due to its aromatic properties and health benefits.[5] This plant is endemic in the area of Tebessa-Algeria it has widely shown good results as antibacterial, otherwise, there are no works on the antifungal activity of its essential oil (EO).

In this work, we propose an evaluation of the antifungal effect of EO of the considered plant against the fungus "*A. niger*" issued from contaminated foods. The results will tell us about the relevance of its use for healthy and longtime food conservation with a better organoleptic quality.

MATERIALS AND METHODS

Vegetal Material

Location and characteristics of the harvest place

Our samples were collected in the region of Tebessa, located in north-eastern of Algeria and it belongs to the field of the Saharan Atlas at Algerian-Tunisian eastern border[6] with the following geographical coordinates: Latitude 35.42° North, longitude 8.12° West, and 863 m above sea level.[7] The total area of the city of Tebessa is 13.878 km^2[8] and its bioclimatic is semi-arid; in the period between 1972 and 2009, the annual rainfall averages were ranged from 307 to 625 mm and the temperature was around 15°C.[9]

Drug and extraction of EO

The leaves and flowering tops of *R. officinalis* were harvested early by dry weather; drying was carried out away from light and moisture for 10 days. The extraction of EO was performed by steam distillation using a Likens Nickerson-type device.[10] The distillations of vegetal material were made in batches of 100 g during 2 h, and the EO was collected by decantation, and then, it was stored in a refrigerator at 4°C in dark glass vials sealed.

Chemical analysis of the EO

The analysis is performed in gas phase by gas chromatography coupled with mass spectrometry (GC-MS) using a device type Varian GC 3400 Saturn MS ion trap, with commercial library National Institute of Standards and Technology). The device is equipped with a DB5-MS column (25 mm long, 0.32 mm internal diameter, and 1.0 μm film thickness). The initial temperature of 60°C was maintained for 1 min and then was increased at a rate of 3°C/min to 200°C and maintained at this value for 15 min. The temperatures of the injector and detector were, respectively, 250°C and 285°C. The pressure of the helium carrier gas at the column head was set at 138 KPa. The injected amount of EO was 1 μl in splitless mode. The assay was performed by internal standardization.[11]

Antifungal Activity

The antifungal activity was determined by method of incorporation of the oil tested in the agar sabouraud.

Culture of test-objects stem

Sixteen strains of fungi of the genus *A. niger*, isolated and collected on various foods (canned tomato, cheese, and harissa), are retained in our study.

The identification of the strains is based on macroscopic and microscopic characteristics.[12]

- Macroscopic (growth rate, aspect, topography, size, and color of colonies).
- Microscopic (hyphe conidiophore, phialides, and conidia) is performed by carrying out a spreading of the sample between slide and slip cover, then the preparation is stained by blue cotton. The objective 40 is used to highlight and identify the most important elements.

Determination of the minimum inhibitory concentration (MIC)

The MIC of EO are determined by incorporation method in agar;[13] the seeding is done as recommended by the Clinical and Laboratory Standards Institute document.[14,15] The EO is diluted in dimethyl sulfoxide and incorporated into the sabouraud medium previously melted and cooled at 45°C so as to obtain a series of dilution of 0.25%, 0.5%, 0.75%, and 1%. The strains were then inoculated at the medium surface by wide strips (no more than four strains per petri dish) (Figure 1).

- Incubation.

The inoculated Petri dishes were incubated at room temperature, and the observation was made every day for 5 days. The same strains tested in each Petri dish were seeded in the same order in a straight line on a witness sabouraud agar without EO and were incubated in the same conditions (control Petri dish).

Determining activity type

A transplant was performed from inhibition zones if existing on a Sabouraud medium free of EO, after incubation at room temperature:

- When there is no regrowth, the concentration is said fungicidal.
- If there the strain grows, the concentration activity is said fungistatic.[16]

RESULTS

EOs isolated from aromatic plants have wide applications in perfumery, flavor, cosmetic, and pharmaceutical industries. They have been used since ancient times and despite many of them being substituted by synthetic ones.[17] In what follows, we will study the chemical composition of our EO to determine its main components, while comparing their amounts with other EO from other countries. Then, we will study its effects against *A. niger* fungus that contaminate food products.

Chemical Composition of the EO of *R. officinalis*

The chromatography result of the extracted EO has identified 14 significant chemical compounds and three unidentified ones representing 3.54% (Table 1).

The studied EO contains only terpene components, where the majority was represented by monoterpenes (95.6%) and only one sesquiterpène bisabolol (0.86%). On the other hand, this EO is characterized by wealth of 1.8 cineole (63.65%) and camphor (14.23%); these rates are high compared to other profiles from other countries described in Table 1. The chemotype of the plant is then: *R. officinalis* 1.8 cineole.

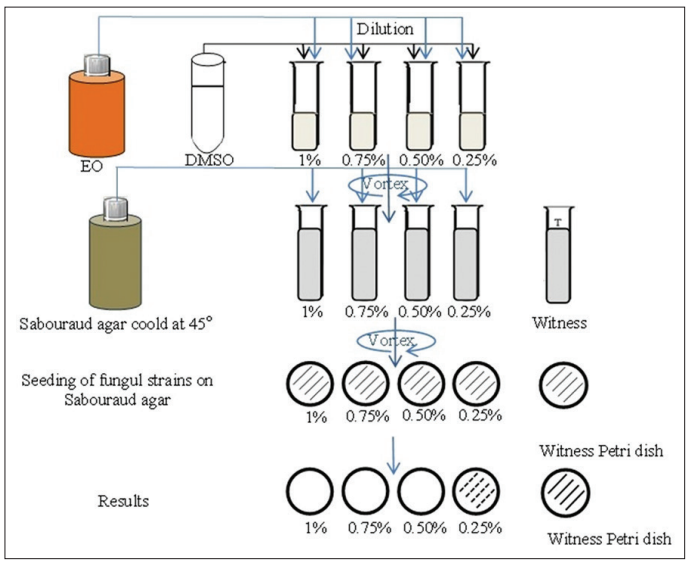

Figure 1: Experimental protocol

Identification of the Fungal Strains

The strain grown rapidly, it has a woolly and granular form with a pleated and soft terrain, its size is between 1.5 and 2 cm and has a white color first and then turns black.

The microscopic observation strain have identify septate and hyaline hyphe, hyaline conidiophores, and smooth wall, with a length between 400 and along 3000 µm; they are becoming darker at the top and ending in a globular vesicle with the size diameter 30-75 µm. The phialides cover the entire surface of the bladder with globular conidia, brown-black color, very rough. The diameter of the strain is 4-5 µm.

Antifungal Activity of EO

Compared to cultures in control dishes, the colonies of the 16 tested strains of *A. niger* showed a very weak growth at 0.25% concentration of EO. From a concentration of 0.50%, we noted a complete absence of growth for the 16 treated strains.

Therefore, the MIC value of the EO on the studied strains of *A. niger* was 0.50% (Figure 2).

We also noticed that *A. niger* have grown after being stopped by the presence of EO and its action against the strains tested is then fungistatic (Table 2).

DISCUSSION

The comparative study of chemical analysis of our EO of *R. officinalis* with those of Saudi Arabia,[18] Spain, Morocco, Tunisia, and Switzerland[19] showed that bisabolol and 4-terpineol appears only in rosemary of Tebessa-Algeria; we also note a lower content of α-pinene (4.93%). Furthermore, α terpineol, β-pinene, and p-cymene are only present in our sample and that of Saudi Arabia. The Camphor has a rate

Table 1: Chemical composition of the essential oil of rosemary extracted in comparison with others chromatographic profiles

Components sample region	α pinène	β pinène	p-cymène	Limonene	1,8 cinéol	Camphor	Borneol	4-terpineol	α-terpineol	Bornyl acetate	Bisabolol
(Tébessa-Algeria)	4.93	4.55	0.46	0.61	63.65	14.23	1.80	0.64	4.24	0.49	0.86
Saudi Arabia	19.48	1.20	1.28	/	23.16	4.10	4.51	/	2.43	/	/
Spain	18-26	/	/	2.5-5	16-25	13-21	2-4.5	/	/	0.5-2.5	/
Morroco-Tunisia	9-14	/	/	1.5-4	38-55	5-15	1.5-5	/	/	0.1-1.5	/
Switzerland	20.61	/	/	3.73	17.95	12.99	13.27	/	/	4.77	/

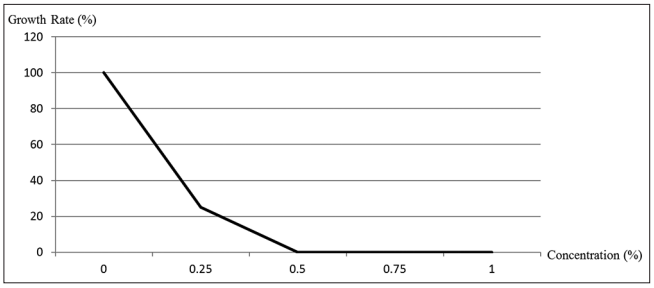

Figure 2: Minimum inhibitory concentration curve

Table 2: MIC variation range and type of EO activity of *Rosmarinus officinalis* on *Aspergillus niger*

Species	MIC (%)	Activity type
Aspergillus niger (*n*=16)	0.50	Fungistatic (*n*=16)

n: Number of strain, MIC: Minimum inhibitory concentration, EO: Essential oil

of 14.23%, comparable to that established by the European Pharmacopoeia samples type of Spain, Morocco, and Tunisia.

According to the literature, the results of the macroscopic and microscopic identification obtained, indicate that the examined strain corresponds to the species *A. niger*.[20-22]

In addition, we note an antifungal activity of the EO of *R. officinalis* which is probably related to the monoterpene oxide:1.8 cineole,[23] major compound with 65.63% of the overall composition of the oil. This component will cause an increase in the permeability of the cell membrane, thereby facilitating the entry of other active compounds especially: 4-terpineol.[24] Several authors have shown that in addition to the major compounds, minor ones such 4-terpineol (0.64%) significantly contribute to the activities of EO by synergistic action.[25,26] Antifungal activity can also be associated with camphor (14.23%); this compound is known as a fungicidal and also antibacterial.[27] One cannot also overlook the role of limonene (0.61%) which is known for its action on the permeability of the cytoplasmic membrane of fungal cells, causing a loss of inclusions.[26]

Several research studies reveal that the activity of EO components usually results in the morphological changes of the hyphe and direct disturbance of the fungal cell membrane,[28,29] for example, hydrophobic components constituting the EO increase the permeability of the cell membrane, causing leakage of the bacterial contents and fungal cells.[30]

Furthermore, the lipophilic nature of the hydrocarbon backbone of the EO components and the hydrophilic nature of their functional groups play a very important role in the antimicrobial activity,[31] which is also influenced by the cyclic structure of hydrocarbons.[23]

Despite the difficulty of developing an antifungal molecule,[32] the activity of the components of our EO of *R. officinalis* on the filamentous fungus *A. niger* and its MIC, reinforces the idea of using this type of molecules to preserve food products by slowing their deterioration, prevent, and inhibit the secretion of toxic substances and thus preserve human health. Finally, we highly encourage the researchers to propose others studies on the antifungal activity of *R. officinalis* collected from other regions against *A. niger,* to compare the results and find more advantages of using its EO to preserve food products.

CONCLUSION

Rosemary is a very abundant and endemic plant in Tebessa region situated in north-eastern of Algeria. The extraction of its EO and its analysis by GC-MS has identified 14 components with a predominance of monoterpenes. The chemotype is *R. officinalis* 1.8 cineol and this major component is often used by the pharmaceutical industry; it is also considered useful for the treatment of bronchitis, sinusitis, and rheumatism. The action of a low concentration of EO on 16 strains of *A. niger* collected from various food products has given an antifungal activity on all strains tested with a fungistatic effect. These results open the prospects for the use of this EO as a food preservative in substitution of chemical molecules known by their harmful effects.

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