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Original article

# Antifungal Activity of Mentha Rotundifolia Essential Oil Against Fusarium Oxysporum

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**Abstract**

The antifungal activity of *Mentha rotundifolia* essential oil, harvested in Setif (Algeria) was evaluated *in vitro* against a phytopathogenic fungi *Fusarium oxysporum,* causing damage on tomato. The molecular identification of the strain was based on a comparison (BLAST) of the sequences obtained against a database and was often supplemented by microscopic observations. After "SANGER" sequencing of the PCR products, the sequences were received in FASTA format. Analysis of *M. rotundifolia* essential oil by Gas Chromatography/Mass Spectrometry method (GC-MS) identified 14 compounds. The 3-Cyclopenten-1-one, 2-hydroxy-3-(3-methyl-2-butenyl) - was the major constituent of this oil with a rate of about 89.09%. For this activity, we adopted the technique of direct contact on agar. *F. oxysporum* continued to grow on oil-free media at 1% and 0.1% (fungistatic effect); also on media with an oil concentration of 0.01%. While the explants taken from Petri dish with essential oil concentration of 2; 4 and 10% did not grow (fungicidal effect). The very interesting antifungal effect of *M. rotundifolia* essential oil indicates the potential of this plant species as a source of natural fungicidal material. The present study revealed that this mint exhibited antifungal effect against *F. oxysporum* which provided a scientific basis for the use of this species as a good source of antifungal compounds. This preliminary work could provide a basis for the determination of sufficient and effective concentrations for *in planta* studies for the biological control of natural active substances of *M. rotundifolia* against fungal diseases.

**Keywords:** Antifungal activity, *Mentha rotundifolia*, essential oil, Fusarium oxysporum, molecular identification, GC/MS.

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## INTRODUCTION

Over the past 20 years, there has been a sharp increase in diseases caused by fungi, which affect a very wide range of hosts. These diseases are caused by a surprisingly high number of fungal species (Bitar et al., 2013). Tomato fusariosis only attacks certain cultivars. Plants infected with this soil fungus has yellowing of the leaves and wilting spreading from the base of the stem. Initially, symptoms are only visible on one half of the surface of leaves, branches or plants, before spreading to the entire (Messiaen et al., 1993). The application of synthetic fungicides has led to a number of environmental and health problems, as they themselves are carcinogenic, teratogenic and highly toxic with a high duration of degradation (Lingk, 1991; Tian et al., 2011). New awareness aimed at reducing the use of chemical pesticides by developing alternative strategies or technologies to improve plant disease resistance and pathogen control is encouraged (Labiod et al., 2015). As a result, there has been growing interest in research into alternative pesticides and active antimicrobial compounds, including plant extracts and aromatic plant essential oils (Pradhanang et al., 2003, Kotan et al., 2013). The antifungal activity of *Mentha* species has been studied on different fungi (McKay and Blumberg, 2006; Mimica-Dukić and Bozin, 2008; Peixoto et al., 2009). The antifungal activity of *Mentha* extracts has been studied to a lesser extent. Nevertheless, extracts have shown interesting antifungal activity (Gulluce et al., 2007; Hussain et al., 2010). For this reason, the objective of this part is to assess the antifungal activity of the essential oil of *M. rotundifolia* against *Fusarium oxysporum* strain of fungus causing damage to tomato.

### Material and Methods

#### *Plant material and isolation of essential oil*

Aerial parts of *M. rotundifolia* were collected on June 2014 from Chouf Lakdad region (Setif, Algeria). Plant materials were identified by Pr. Boulaacheb Nacira. The samples were dried in the shade away from light at room temperature. After drying, a sample of 100 g was subjected to hydrodistillation for 3h, using a Clevenger (1928) apparatus type to produce essential oil. This was preserved in an amber flask at low temperature (4°C) until further analysis.

#### *Identification of components*

Identification of the components was based on: comparison of their GC retention indices (RI) with those of authentic compounds or literature data; computer matching with a mass spectral library and commercial libraries (WILLEY and NIST database/ChemStation data system), comparison of the signals in the $^{13}C$ NMR spectra: were recorded on a Bruker Avance-III 400 Fourier Transform spectrometer operating at 100 MHz for $^{13}C$ NMR equipped with a 5 mm probe, in deuterochloroform, with all shifts referring to internal tetramethylsilane (TMS), Ozen et al. (2011).

***Chromatographic analysis***

***Gas chromatography (GC) analysis***

Gas chromatography analyses of essential oil were analyzed by GC-MS on a Agilent Technologies GC 7890A model and injected with HP-5ms capillary column (30 m x 250 mm x 0.25 m), at an ionization voltage of 70 eV. The carrier gas (1 ml $min^{-1}$) was helium. The column temperature was increased 60 °C to 240 °C at a 4 °C/min rate, total run time 45 min. Diluted sample of 1.0 µl was injected in the split mode (20:1).

***Gas chromatography/mass spectrometry (GC/MS) analysis***

The essential oils were performed on Agilent Technologies GC 7890A with a built-in 5975 Triple Axis Detector MS system, equipped ionization system, HP5-ms (30 m x 250 mm x 0.25 mm) column and ionization energy (70 eV) for GC-MS detection. The helium was used a carrier gas at a flow rate (1 ml/min). The same column temperate was achieved with GC analysis given above in our laboratory, by comparison of their mass spectral fragmentation patterns (WILLEY and NIST database/ChemStation data system) and their retention indices (determined with reference to homologue series of normal alkanes).

***Isolation and purification of the fungal strain***

The microorganism tested is a pathogen isolated from an infected tomato. Isolation of the pathogen was followed by the protocol described by Zhu et al. (2001). Fragments from young tomato lesions (bay, leaves, stem) were disinfected with 5% diluted bleach for 5 minutes and deposited in petri dishes containing freshly prepared PDA supplemented with antibiotics "Gentamicine 80mg/2ml" (Zhu et al., 2001; Djeugap et al., 2009). After 4 to 6 days of incubation at 28 $^{o}$C, successive transplants on antibiotic-free environment strains purified the strain and the pure culture obtained was preserved at 4$^{o}$C.

***Macroscopic and microscopic identification of the fungal strain***

The identification of the pathogen was based on the macroscopic description (the growth rate, the face and reverse colour colony, the shape and appearance of the colony) and microscopic (the appearance of the partitioned or unbounded thalle, the shape and the disposition of spores, the structure of mycelium) referring to fungal systematic documents (Alexopoulos, 1979; Viennot-Bourgin, 1980; Botton et al., 1990; Pitt - Hocking, 1997).

***Molecular identification of the fungal strain***

Molecular identification of the fungal strain was carried out at the Conidia Expert Mould Laboratory in Lyon. The identification of the strain is based on a comparison (BLAST) of the sequences

obtained against a database. Identification is often supplemented by microscopic observations. After sequencing "SANGER" of PCR products, we receive the sequences in FASTA format.

***Antifungal activity of M. rotundifolia essential oil***

The incorporation of essential oil into the growing medium was done using the method of (Remmal et al., 1993). Due to the non-miscibility of essential oils to water and therefore to growing media, an emulsion of the oil was achieved thanks to a solution of agar agar at 0.2%. It allows to obtain in the middle a homogeneous distribution of compounds in the dispersed state, it gives the middle a viscosity capable of preventing the constituents of essential oils from reassociating after agitation and increasing as much as possible the germ/compound contact. The essential oil is diluted first to $1/10^{th}$ in the agar-agar solution. From the different tubes containing different concentrations of HEs, amounts of this dilution (2 mL) were added to the sterilized test tubes, which were cooled to 45°C, containing 18 mL of PDA. These tubes are then stirred at the Vortex and poured into petri dishes. Final concentrations (V/V) in essential oil are 10% (1/10), 4% (1/25), 2% (1/50), 1% (1/100), 0.1% (1/200) and 0.01% (1/300). Control boxes, containing the growing medium plus the agar-agar solution 0.2% alone, are also prepared. After solidifying the Potato Dextrose Agar, seeding is done by depositing a 6 mm diameter explant, taken from the periphery of a mycelian mat and from a seven-day culture. The petri dishes were sealed with Parafilm and incubated at room temperature for a week. Each test is repeated three times to minimize the experimental error. The antifungal activity of *M. rotundifolia* essential oil was evaluated *in vitro* by observing the inhibition of the growth of the fungus to be tested in contact with this oil at different concentrations. The results of this study are represented by the sensitivity of the strain to this oil (inhibition or development).

**Results**

***Chemical composition of M. rotundifolia essential oil***

The GC/MS chromatogram of the essential oil was given in Figure 1. The essential oil composition of *M. rotundifolia* was given in Table 1. Fourteen compounds were identified. The 3-Cyclopenten-1-one, 2-hydroxy-3-(3-methyl-2-butenyl) was found as the major constituent of this oil with a rate of about 89.09%. The remaining parts were monoterpenes and sesquiterpenes. Table 1 showed that the presence in *M. rotundifolia* essential oil of certain identical compounds (β-Pinene). Two minor compounds with levels not exceeding 0.5% of the total essential oil were identified as 1-Octen-3-ol, acetate (0.29%) and Borneol (0.38%).

**Table 1.** Chemical composition of *M. rotundifolia* essential oil.

| Compound Number | Retention Time (mn) | Retention Index | % | Name |
|---|---|---|---|---|
| 1 | 11.751 | 907 | 1.18 | α-Pinene |
| 2 | 12.951 | 947 | 0.54 | β-Pinene |
| 3 | 13.135 | 953 | 1.17 | β-Pinene |
| 4 | 13.337 | 959 | 0.68 | β-Pinene |
| 5 | 14.749 | 1001 | 1.74 | β-Terpinyl acetate |
| 6 | 17.304 | 1077 | 0.29 | 1-Octen-3-ol,acetate |
| 7 | 19.619 | 1144 | 0.38 | Borneol |
| 8 | 22.873 | 1237 | 0.52 | Carvone oxide |
| 9 | 26.509 | 1346 | 89.09 | 3-Cyclopenten-1-one, 2-hydroxy-3-(3-methyl-2-butenyl)- |
| 10 | 27.352 | 1372 | 0.77 | 3-Methyl-2-pent-2-enyl-cyclopent-2-enone |
| 11 | 27.548 | 1378 | 1.25 | Cinerolone |
| 12 | 28.212 | 1397 | 0.91 | Caryophyllene |
| 13 | 28.867 | 1419 | 0.70 | β-Farnesene |
| 14 | 30.078 | 1458 | 079 | β-Cubebene |

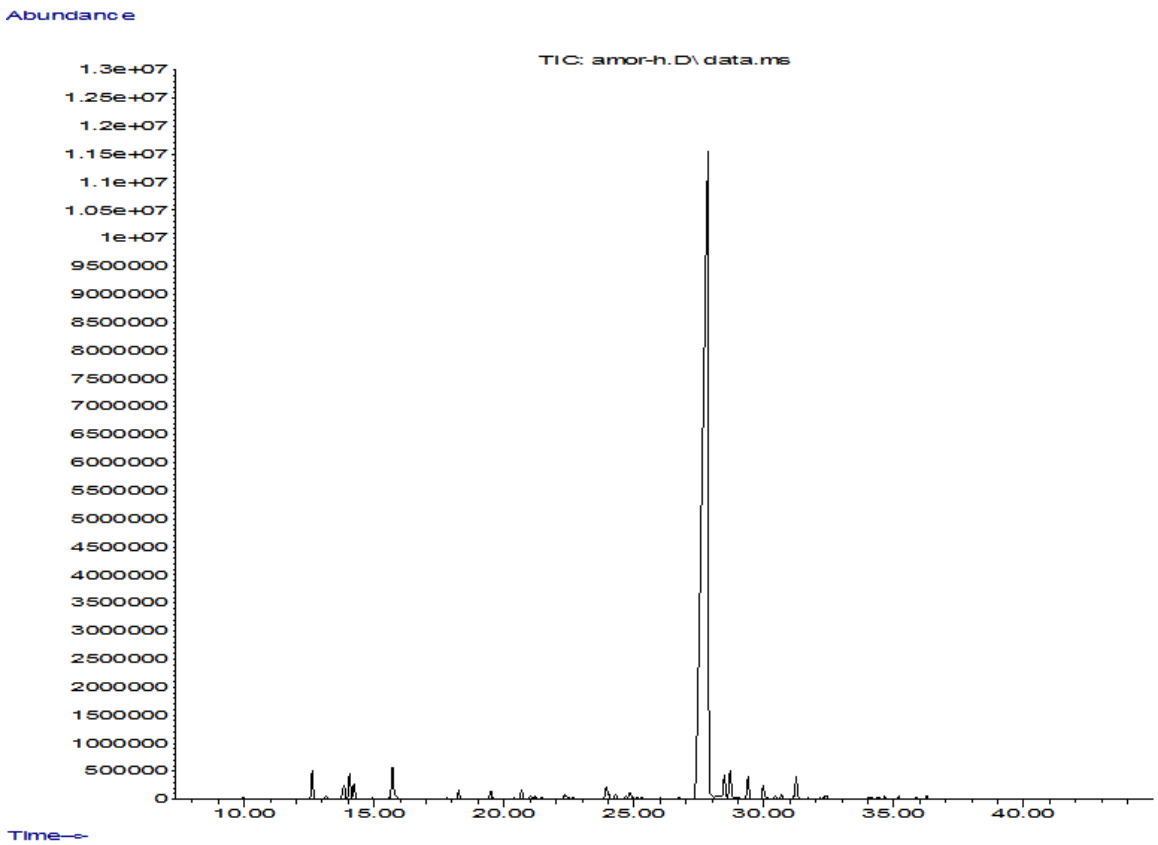


**Figure 1**. GC/MS Chromatogram of *M. rotundifolia* essential oil

***Macroscopic and microscopic identification of the strain***

After the isolation and incubation of the strain found on tomato, the identification of the fungal genus was made by referring to champion's identification keys (1997); Botton et al. (1990); Watanabe (2002); Navi et al. (1999) based on the macroscopic characteristics of the colonies (appearance, color, smell, shape, contour, etc.) and on microscopic traits (mycelium partition, spores and conidia shape, fruiting organ shape, etc.). Based on the microscopic traits retained after optical microscope observation with x4 and x10 magnifications, we were able to identify *Fusarium sp*. Macroscopic and microscopic observation of the strain (Figure 2) revealed the existence of the following traits: the color of its colony is whitish to pale pink on the face of the petri dish and pink on its lapel, the texture of the colony is fluffy, under microscope strain presented siloed myceliums with different types of conidia (microconidia, macroconidia and monophialidias) and intercalary or terminal chlamydospores.

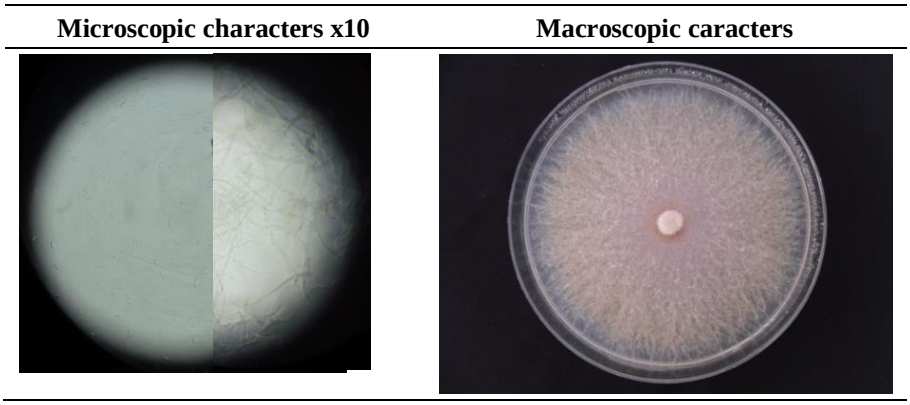


**Figure 2.** Macroscopic and microscopic observation of the fungal strain

***Molecular identification***

After "SANGER" sequencing of PCR products, FASTA sequences are represented by two sequences for the strain studied. The sequences are analyzed by BLAST on: internal data base, NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and unit (https://unite.ut.ee/analysis.php). Molecular identification of our strain gave *Fusarium oxysporum* (Figure 3).

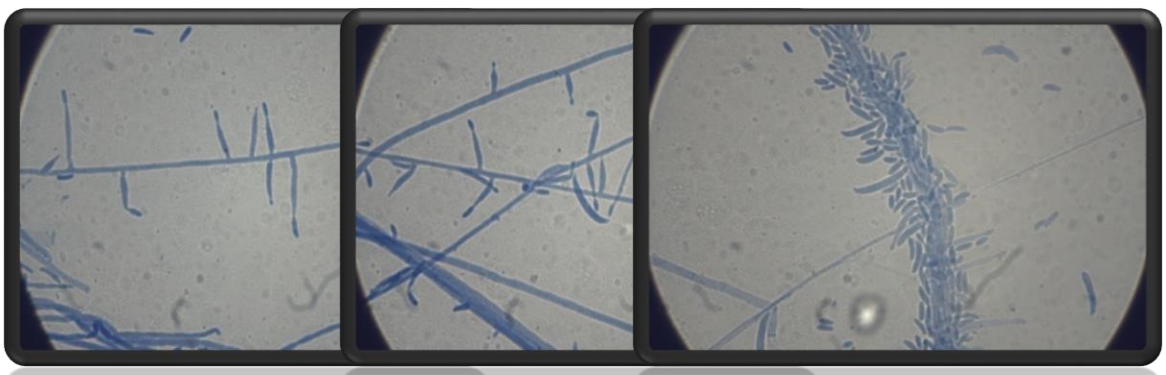

**Figure 3.** Microscopic profile of the *Fusarium oxysporum* strain (hyphae, microconidia, macroconidia).

***Antifungal activity of essential oil***

Essential oils have many biological activities according to their majority constituents. In the areas of plant health and agri-food, essential oils or their active compounds could also be used as protective agents against microorganisms, fungi and the process of food oxidation (Farhat, 2010). To assess the antifungal activity of *M. rotundifolia*'s essential oil, we adopted the technique of direct contact on agar. The results showed that essential oil has significant activity against the fungus tested (Figure 4). *F. oxysporum* grew on PDA with an oil concentration of 0.01% and did not develop on oil-free media at 0.1%; 1% ; 2% ; 4% ; 10%. The essential oil of *M. rotundifolia* completely inhibits the growth of this fungus at concentrations equal to 0.1%. Table 2 summarizes the results of inhibition of fungi growth by essential oil.

**Table 2.** Inhibition activity of *M. rotundifolia* essential oil on the growth of *F. oxysporum*

| | **Concentration of EO %** | | | | | | |
|---|---|---|---|---|---|---|---|
| Fungus tested | 0.01 | 0.1 | 1 | 2 | 4 | 10 | Control |
| *F. oxysporum* | + | - | - | - | - | - | + |

+ development, - inhibition

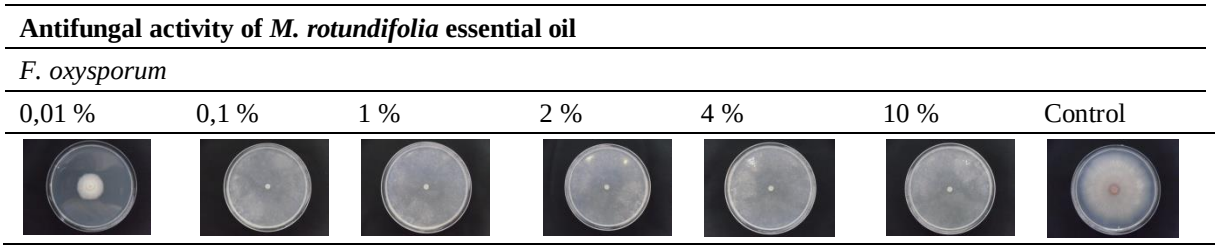


**Figure 4.** Activity to inhibit *M. rotundifolia* essential oil on fungus growth after 7 days of incubation

The explants of the fungus, which did not grow, were reseeded in new petri dishes devoid of oil. The fungus taken from the petri dishes with a concentration of 1 essential oil; 0.1 and 0.01% continued to grow (fungistatic effect). While the explants of the fungus taken from the petri dishes with a concentration of essential oil is 2; 4 and 10% did not grow (fungicide effect).

**Discussion**

With the 3-Cyclopenten-1-one, 2-hydroxy-3-(3-methyl-2-butenyl) as major component, the essential oil of our study does not exhibit the same chemical characteristics of the essential oils of *M. rotundifolia* of Algerian origin: Arab et al., (2016) found as the major compound piperitone oxide (72.87%), Brahmi et *al*., (2016) were able to identify pulegone (70.4%), Lehbab (2013) found the piperitoneone (33.06%), Brada et al., (2007) found piperitone oxide (Miliana: 31.4%, Rouina: 19.7%) and piperitenone oxide (Miliana: 27.8%; Rouina: 29.4%) representing chemotype 1. For the sample from Chlef, the major components are piperitenone (54.9%) and piperitenone oxide (17.6%) representing chemotype 2. While Brada et *al*., (2006) found piperitenone oxide with 38.6% in Rouina

and 23.5% in Miliana. Previous studies on the chemical composition of the essential oil of *M. rotundifolia* in other countries have revealed the existence of chemotypes with different major components, such as β-Caryophyllene (26.67%) in Beja and pulegone (32.09%) in Bizerte (Riahi et al., 2013), 2.4 (8), 6-p-menthatrien-2.3-diol in Cuba (Pino et al., 1999)**,** linolool (35.32%) in Egypt (Aziz and Abbass 2010), piperitol (57.6%) in Spain (Raya et al., 1990)**,** Pulgone (85%) (El Arch et *al*., 2003), Menthol (40.50%) (Derwich et al., 2010) in Morocco. Piperitenone oxide has been reported as a characteristic constituent of volatile oils of *M. rotundifolia* chemotypes with 80.8% in Uruguay (Lorenzo et al., 2002) and 81.5% in Germany (Nagell, Hefendehl, 1974). Differences in chemical composition observed for essential oils is likely related to abiotic factors such as climate specific regions of provenance samples, geographical factors such as altitude and soil type (Brada et al., 2007). According to Riahi et al. (2013), genetic factors should not be excluded in explaining the chemo-variation of essential oils. Moreover, chemical differences in the oil composition of plant species in relationships with harvesting season were reported (Giray et al., 2008).

Several studies have been carried out on the antifungal activity of *Mentha sp* essential oil (Oumzil et al., 2002; Norsati et al., 2011; Moghaddam et al., 2013; Moghtader, 2013; Mehani et al., 2015; Dehghanpour-Farashah and Taheril, 2016; Park et al., 2016; Reddy et al., 2017). However, few have been devoted to the antifungal activity of *M. rotundifolia* essential oil against this strain found on tomato. The essential oil of *M. rotundifolia* exerted a very strong antifungal activity against the fungus. Essential oils are more effective anfungals than polar extracts (Hajlaoui et al., 2009; Mkaddem et al., 2009; Teixeira et al., 2012). It inhibited their mycelian growth from a very low concentration of about 1/200 v/v. The fungal strain tested showed a high sensitivity to the phenol-rich essential oil of *M. rotundifolia*. Phenols have been shown to act by inactivating fungal enzymes that contain SH grouping in their active site (Farag et al., 1989; Celimene et al., 1999). Phenolic terpenes also act by attaching to the amine and hydroxylamine groups of microbial membrane proteins causing alteration of permeability and flight of intracellular constituents (Knowles et al., 2005; Lopez-Malo et al., 2005). The antifungal activity of the essential oil analyzed can be attributed mainly to its majority constituent, 3-Cyclopenten-1-one, 2-hydroxy-3-(3-methyl- 2-butenyl)- with a percentage of about 89.09%. According to El Ajjouri et al. (2010), the chemical composition of essential oils must be taken in its entirety. Indeed, the synergistic effect between different constituents, including minority constituents, should not be overlooked. The antifungal action of essential oils is due to an increase in the permeability of the plasma membrane followed by a rupture of the plasma membrane resulting in a leakage of cytoplasmic content and the death of yeast (Mann et al., 2000). The researchers explain that the maximum antibacterial and antifungal activity of plant essential oils is caused by chemical constituents containing heteroatomas such as oxygen (Dorman and Deans, 2000; Proestos et al., 2006 ; Ben-Bnina et al., 2010).

## Conclusion

The inhibitory power of mycelian growth of *M. rotundifolia* essential oil was demonstrated by the reduction or inhibition of mycelian growth with increased oil concentration. The very interesting antifungal effect of *M. rotundifolia* essential oil indicates the potential of this plant species as a source of natural fungicide material. This study revealed that this mint has an antifungal effect against *F. oxysporum*, providing a scientific basis for the use of this species as a good source of antifungal compounds. This preliminary work could provide a basis for determining sufficient and effective concentrations for *in planta* studies for biological control by natural active substances of *M. rotundifolia* against the agent *F. oxysporum* plant pathogen.

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