Original Paper

Chemical Composition and Antifungal Activity of *Rosmarinus officinalis* Essential Oil against Four Pathogenic Fungi (*Fusarium oxysporum*, *Fusarium culmorum*, *Fusarium poae* and *Helminthosporium sativum*) of Wheat in Morocco

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This study aimed to characterize the chemical composition and antifungal activity of Moroccan medicinal and aromatic plant *Rosmarinus officinalis*. The essential oil (EO) were isolated by hydrodistillation using a Clevenger type apparatus and their chemical composition was identified by using gas chromatography-mass spectrometry. Monoterpenes were the major class of compounds with an abundance of 1.8-cineole (46.3%), camphene (13.4%) and α-pinene (9.5%), borneol (6,5%), camphor (4.8%) and terpinene-4- (3.6%). The antifungal activity of *Rosmarinus officinalis* against *Fusarium oxysporum*, *F. culmorum*, *F. poae* and *Helmintosporium sativum* pathogenic fungi revealed a significant inhibitory effect. The minimum inhibitory concentration as well as the fungicidal/fungistatic profile indicated that the essential oil of *R. officinalis* is very active allowing inhibition at the highest concentration and the fungal strains were therefore very sensitive. Obtained results suggested that *R. officinalis* essential oil might be a potential treatment for biocontrol of plant diseases.

Keywords: antifungal activity, biocontrol, *Rosmarinus officinalis*, phytopathognic fungi, essential oil

1 Introduction

The massive use of synthetic pesticides, including fungicides, to protect cereal crops in Morocco is not always effective due to the emergence of multi-resistant strains and their negative effects on the environment and human health. Studies have focused on medicinal and aromatic plants (MAP), which have a long tradition of use in both therapy and food preservation (Sartoratto et al., 2004).

Plants are a valuable source of a wide range of secondary metabolites that are used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, biopesticides and food additives (Al-Snafi 2018). Thus, pathogenic fungi are the main infectious agents in plants, causing changes at all stages of plant growth and development. In some cases, fungi are indirectly responsible for allergic and/or toxic disorders in consumers through the production of allergens and/or mycotoxins (Dellavalle et al., 2011).

Many types of essential oils obtained from different plants and fungi showed intense antifungal properties (Al-Fatimi et al., 2018). *Rosmarinus officinalis* belongs to the Lamiaceae family, which grows in semi-arid and sub-humid bio-climates. It is widely distributed in the eastern Rif, the eastern Middle Atlas, the eastern High Atlas and the highlands of the Oriental of Morocco. This plant has attracted more attention due to its various biological activities, including anti-hyperglycaemic (Naimi et al., 2017), antibacterial (Bozin et al., 2007), anticancer (Cheung and Tai, 2007), anti-inflammatory (DeMelo et al., 2011) and antioxidant activities (Pérez-Fons et al., 2010). Thus, the chemical profile of *Rosmarinus officinalis* is rich in essential oils, terpenoids, flavonoids and

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phenolic acids (Bozin et al., 2007; Bai et al., 2010), which opens up a wide range of applications, particularly as an antioxidant, antimicrobial and environmentally friendly green pesticide.

The antifungal activity of essential oils of *Rosmarinus officinalis* against *Fusarium oxysporum*, *F. culmorum*, *F. poae* and *Helminthosporium sativum* was studied under our laboratory conditions to verify possible inhibitory activity. The minimum concentration capable of inhibiting or preventing fungal growth was also determined among the fungal strains that showed inhibitory properties.

2 Material and Methods

2.1 Plant Sample Preparation

Samples of *Rosmarinus officinalis* were collected in May in the Eastern region of Morocco. A Botanist identified the plant. Aerial parts of the collected plant have been cleaned, dried at room temperature and in the shade for 2–3 weeks. The leaves were separated and then stored away from light until use.

2.2 Extraction of Essential Oil

The essential oil was extracted by hydrodistillation using a Clevenger-type apparatus. The distillations were carried out by boiling 200 g aerial parts of the fresh plant material in one litre of water for 1 h 30 min. The essential oils collected were stored in opaque glass at 4 °C in the dark with anhydrous sodium sulphate.

2.3 Gas Chromatography-Mass Spectrometry Analysis

Identification of EO composition were performed on gas chromatography coupled to mass spectrometry (GC/MS) and based on their Kovats index (KI). Samples were analyzed on a thermo Fischer capillary gas chromatograph coupled to the mass spectrometry system (model GC ULTRA S/N 210729).

The column used was a non-polar HP-5MS fused silica capillary column (60 m × 0.32 mm, film thickness 0.25 µm) under the following conditions: oven temperature program from 40 °C (2 min) to 260 °C at 2 °C·min^{-1} and the final temperature maintained for 10 min; injection temperature at 250 °C; carrier gas, debit rate 1 ml·min^{-1}. Injected sample volume was 1µl of essential oil diluted in hexane, splitless injection technique, ionization energy 70 eV, in electron ionization mode; ion source temperature 200 °C; mass range scan of m/z 40–650 and interface line temperature 300 °C.

2.4 Fungal Strains

The four fungi studied in this work, *Fusarium oxysporum*, *Fusarium culmorum*, *Fusarium poae* and *Helminthosporium sativum*, were isolated from infected soft wheat roots showing signs of severity during the months of April-May in North Western Morocco (Elyacoubi et al., 2012). The fungal strains were regularly maintained by transplanting on the nutritive medium PDA (Potato Dextrose Agar) and incubation at 25 °C in the dark.

2.5 In Vitro Antifungal Tests

The minimum inhibitory concentrations (MICs) of essential oil of *Rosmarinus officinalis* were determined according to the method reported by Remmal et al. (1993). Due to the immiscibility of essential oil to water and thus to the culture medium, emulsification was carried out using a 0.2% agar solution to promote contact between the germ and the compound. Dilutions are prepared at 1/10th, 1/25th, 1/50th, 1/100th, 1/200th, 1/300th and 1/500th.

In test tubes each containing 13, 5 ml of PDA medium autoclaved for 20 min at 121 °C and cooled to 45 °C, 1.5 ml of each of the dilutions was added to give the final concentrations of 1/100, 1/250, 1/500, 1/1,000, 1/2,000, 1/3,000 and 1/5,000 (v/v). After stirring, the contents of the tubes were placed in Petri dishes 5 cm in diameter. Controls, containing the culture medium and the 0.2% agar solution alone, were also prepared. Three replicates were made for each concentration and for each fungus.

After solidification of the medium, mycelia discs of 3 mm of diameter, taken from the periphery of a 7-day old young crop, were inoculated. Incubation was carried out in the dark at 25 °C. Mycelia growth was estimated by measuring the average perpendicular diameter of each fungus in mm.

Percentage inhibition I (%) of mycelia growth relative to the control was calculated according to the following formula:

$$I(\%) = \frac{(gh0 - ghC)}{gh0}$$

where: *gh0* – growth of the fungus (mm) on the culture medium without essential oil (control); *ghc* – growth of the fungus (mm) on the culture medium at a concentration "c" of the essential oil

The extract is considered:

- very active when it exhibits an inhibition of between 75 and 100%, in which case the fungal strain is said to be very sensitive;

- active when it has an inhibition between 50 and 75%, the fungal strain is said to be sensitive;
- moderately active when it has an inhibition between 25 and 50%, the fungal strain is said to be limited;
- slightly or not active when it has an inhibition between 0 and 25%, the strain is said to be little sensitive or resistant (Rotimi et al., 1988)

2.6 Fungicide-Fungistatic Profile of the Essential Oil

Based on antifungal tests that have showed total inhibition at the minimum inhibitory concentration (MIC), the mycelium discs were transferred to an untreated PDA medium, restoring favourable conditions to fungal growth. This transfer allowed us to evaluate the fungicidal or fungistatic profile of the essential oil.

3 Results and Discussion

The *R. officinalis* essential oil yield obtained was 2.1% (expressed in milliliter concerning 100 g of dry plant matter). The chemical profile showed that several compounds were principle in essential oil of *Rosmarinus officinalis*: namely 1.8 Cineol (46.32%), Camphene (134%) α-pinene (9.52%), Borneol (6.48%), Camphor (4.76%) and Terpinene-4- (3.56%) (Table 1).

Table 1 Chemical composition of *Rosmarinus officinalis*

KI	Compounds	*R. officinalis* (%)
924	α-thujene	1.6
936	α-pinene	9.52
940	camphene	13.4
967	sabinene	3.18
1013	p-Cym	1.87
1021	1.8 cineol	46.32
1088	α-Terpinolene	0.8
1144	camphor	4.76
1169	borneol	6.48
1176	Terpinene-4-ol	3.56
1188	1-α-Terpineol	0.66
1287	Bornyl acetate	0.27
1398	caryophyllene	0.69
1437	humulene	0.17
1465	γ-Murolene	0.15
1514	β-Himachalene	0.09
Total		93.52

KI – kovats retention index

Compared to previous reports, the yield of our plant was found to be lower than the yield obtained from samples collected in Algeria (Boukhoubza et al., 2021) and in Poland (Małgorzata Dzięcioł, 2021) and higher than the values obtained in Tunisia (Aouadi et al., 2021) and in Sardinia (Melito et al., 2019). The qualitative and quantitative composition and yield of the EO is influenced by the origin (Jamshidi et al., 2009), environmental conditions (Moghtader and Afzal, 2009), plant development stage (Ruberto and Baratta, 2000) and harvest time of plants (Melito et al., 2019).

Previously reported studies about the chemical composition of rosemary essential oil showed also a diversity in chemical profile and composition. Ramdani et al. (2021) have showed that eucalyptol (45.16%) was the main compound of *R. officinalis* oil, followed by 2-bornanone, with 24.74%. The main components were: 1.8-cineole (about 31%), α-pinene (17–20%) and camphor (17–19%) was presented by (Dzięcioł, 2021).The results founded by (Isman et al., 2008) showed that 1.8-cineole was the dominant compound, constituting on average 52.1% of the oil, other major constituents included α-pinene (avg. 9.8%), Camphor (avg.9.0%), and β-pinene (avg. 8.2%). The major constituents in *Rosmarinus officinalis* collected in Tunisia were 1.8-cineole (52.06%), α-pinene (15.35%), camphor (7.69%), β-pinene (5.74%) camphene (5.34%), borneol (2.28%) and β-caryophyllene (2.21%) (Aouadi 2021).

The antifungal activity against the fungi responsible for wheat diseases was evaluated by measuring the growth inhibition of fungal species mycelia tested in contact with *R. officinalis* essential oil at different concentrations (Figure 1).

The analysis of the *in vitro* effect of the essential oils revealed their antifungal activity against the studied pathogenic strains. Differences in efficacy were noted according to the dilutions of the essential oil and the fungi species.

Compared to the control, a clear reduction was noted in the diameter of the mycelia growth of fungi subjected to the action of essential oil at different concentrations. It can also be advanced that no fungal species could grow at the highest concentration (1/100 v/v) of *Rosmarinus officinalis* essential oil.

In the presence of *Rosmarinus officinalis* essential oil at the concentration 1/250, the development of the four fungal species was less important compared to the control. Indeed, the recorded values are of the order of 23.33, 35.33, 21.66 and 24 mm for the strains *F. oxysporum*, *F. culmorum*, *F. poae* and *H. sativum* respectively, the values of the controls being 45, 51.66, 44 and 49.66 mm respectively (Figure 2).

Measurements of the mean diameters of mycelia growth revealed inhibition rates of *Rosmarinus officinalis* essential oil expressed as percentages of inhibition (Table 2).

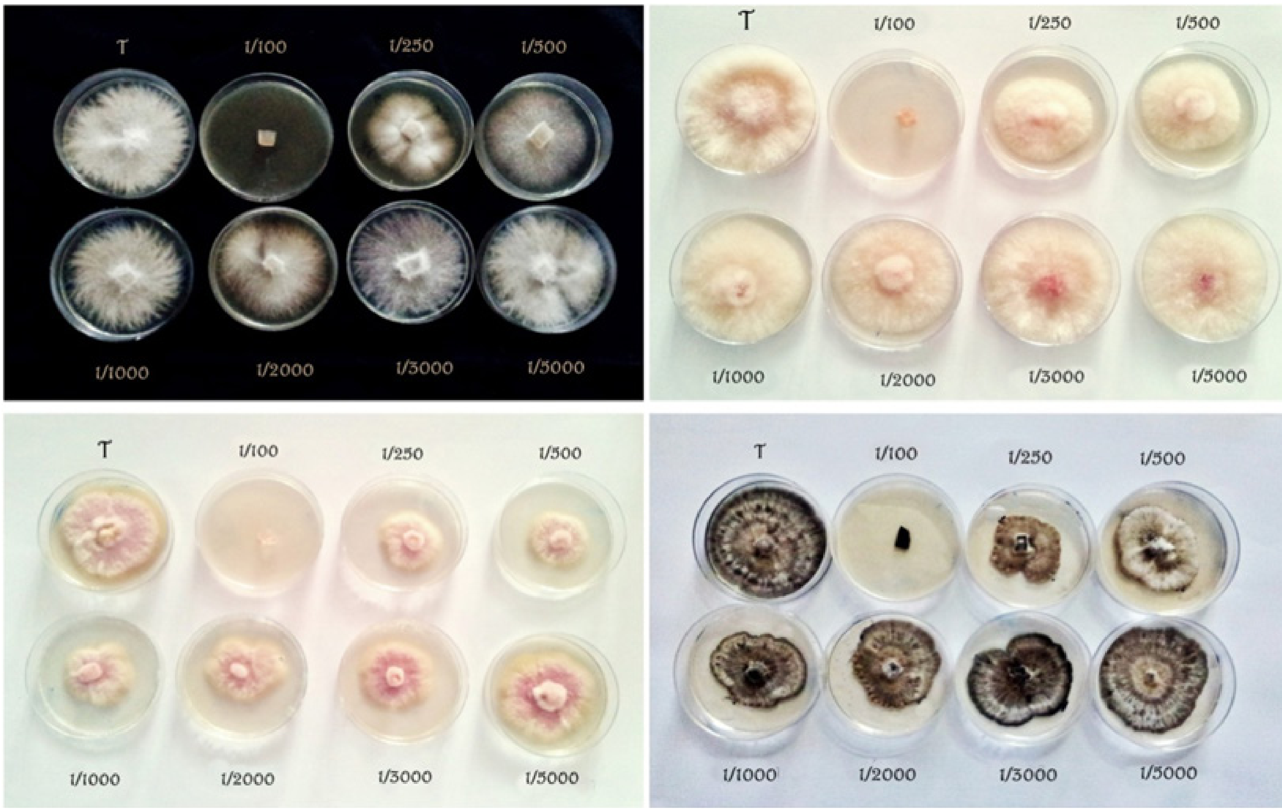

Figure 1 Effect of different concentrations of *Rosmarinus officinalis* essential oils on the studied fungal strains

The essential oil of *Rosmarinus officinalis* seemed to inhibit fungal strains growth at the highest concentration. At a concentration of 1/250 v/v, only the *H. sativum* species have showed a percentage of inhibition higher than 50% (between 50 and 75%), it is therefore sensitive and the essential oil is active. At the same concentration, the three other studied strains were limited and the essential oil is said to be moderately active.

Unreasonable use of pesticides in crop protection constitutes a serious threat to the environment and the health of the consumer. The search for substitutes to pesticides is becoming a necessity for the development of a sustainable agriculture that respects the different environmental components including humans.

According to several researches, the use of extracts of MAP or their essential oils seemed to be a very promising

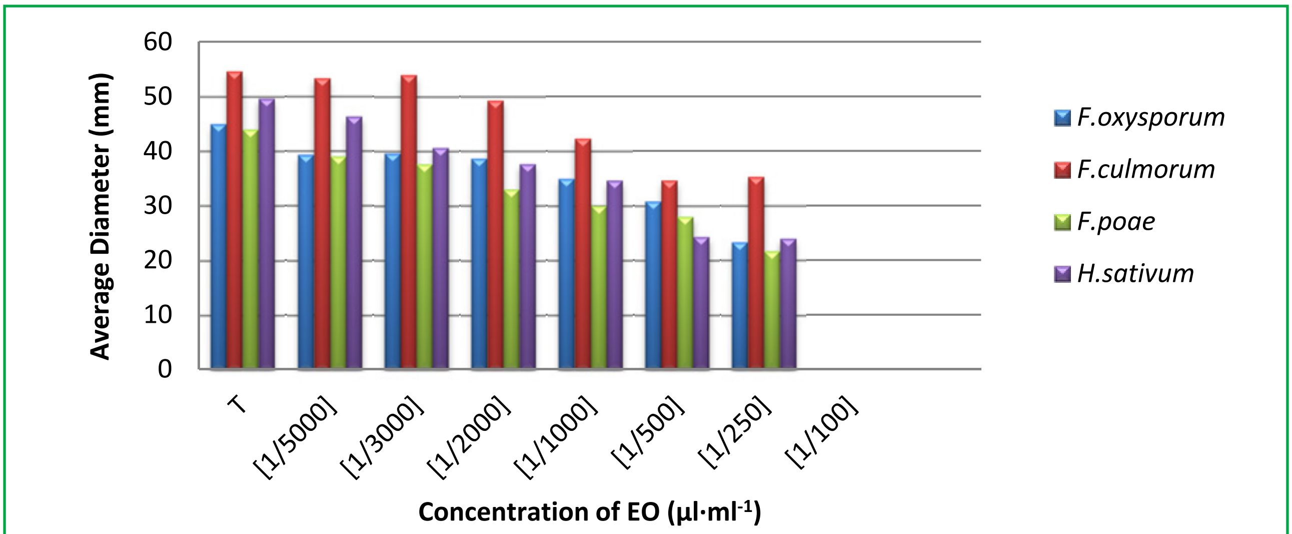

Figure 2 Effect of *Rosmarinus officinalis* essential oil on the mycelial growth of the four fungi studied

Table 2 Inhibition percentage of fungal species mycelia growth

Rosmarinus officinalis								
	T	[1/5000]	[1/3000]	[1/2000]	[1/1000]	[1/500]	[1/250]	[1/100]
F. oxysporum	0	12.6	11.86	14.08	22.22	31.48	48.15	100
F. culmorum	0	2.43	4.46	9.75	22.55	36.58	35.36	100
F. poae	0	11.3	14.4	25	31.81	36.36	28.04	100
H. sativum	0	6.7	18.12	24.16	30.2	51	51.67	100

way. Several compounds obtained from MAP showing nematicidal and fungicidal activities have been isolated mostly from genera belonging to the family Lamiaceae (Gommers & Bakker, 1988j).

Evaluation of the antifungal activity of the essential oil tested revealed a significant inhibitory effect of *Rosmarinus officinalis* on the four pathogenic fungi responsible for numerous diseases in wheat in the several regions of Morocco. This efficacy varies according to the studied species, the nature and the concentration of essential oil used, as confirmed by Hmiri et al. (2011). According to Chouhan et al. (2017), the use of volatile extracts of herbal and medicinal products has many advantages. Indeed, Essential oils showed low toxicity to the environment and can have an interesting biocidal activity.

The activity of these essential oils can be attributed to the existence of a wide variability of secondary metabolites. Observations published in Some studies have shown that the use of the whole essential oil provides an effect which is greater than that of the major components used together (Burt, 2004), suggesting that the minor components may have a synergistic effect or a potentiating influence (Pezzani et al., 2019).

The study of the fungicidal and/or fungistatic profile carried out on fungal strains showed a total growth inhibtion at MIC of 1/100 of the essential oil of *Rosmarinus officinalis*. Thus, the effect recorded was fungistatic (Figure 3).

The essential oil of *R. officinalis* showed a good performance as total growth inhibitor but only at the highest concentration. However, Fadel et al. (2000) also demonstrated the fungistatic effect of this oil. In the other hand, Ben Kaab et al. (2019) showed in their investigation a very interesting inhibitory effect of the essential oil of *Rosmarinus officinalis* on *Fusarimum culmorum*. As well, Ferdes et al. (2017) have confirmed a very significant effect of rosemary essential oil on the pathogenic fungus *Fusarium oxysporum*. These differences in the results can be due to the geographical origin of the medicinal plants used, their genotype and therefore a difference in chemical composition.

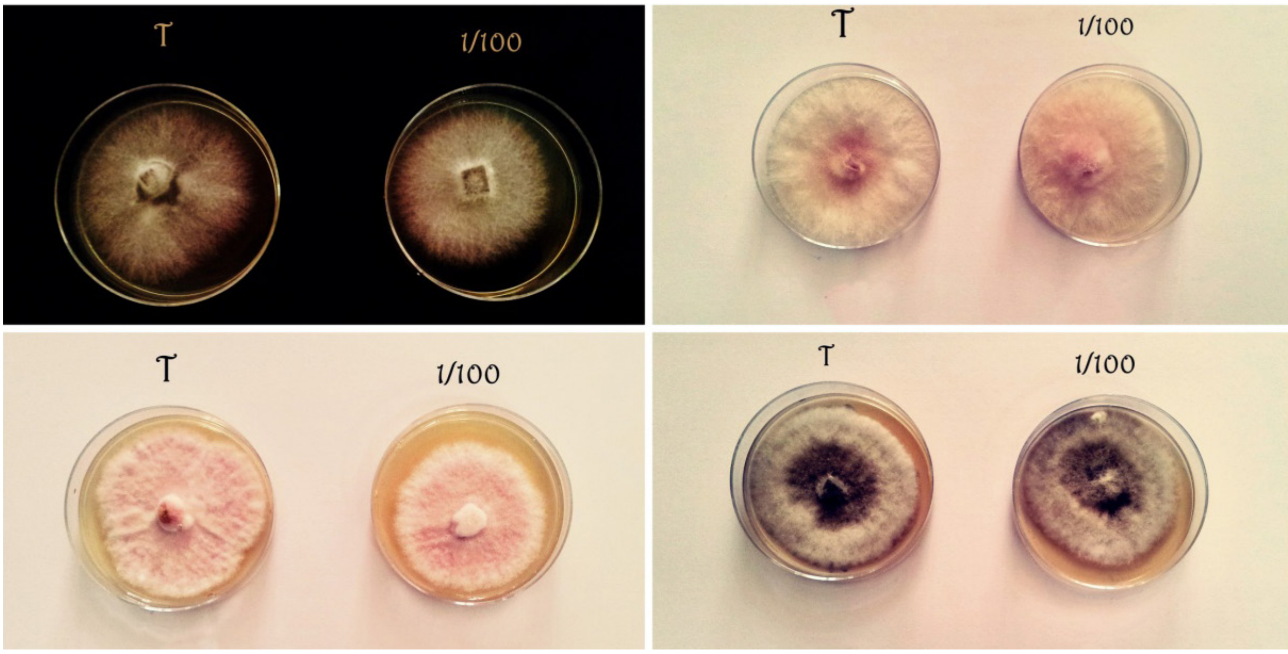

Figure 3 Fungicide-fungistatic effect of *Rosmarinus officinalis* on the four fungal species

4 Conclusion

This work highlights the chemical composition and the inhibitory effect of *Rosmarinus officinalis* against phytopathogenic strains, and thus the possibility of using secondary metabolites from medicinal and aromatic plants to naturally fight against crop enemies and encourages the enlargement of knowledge about these biomolecules.

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