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Antimycotic Activity of Essential Oil of *Rosemarinus officinalis* on Asexual Reproductive Stages of Foodstuff Fungi

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ABSTRACT

Essential oil of Rosemarinus officinalis was obtained by hydro distillation. The essential oil inhibited all three asexual reproductive stages of A. niger, A. flavus and A. awamori. Antifungal drug myconazole was used for comparison with the essential oil. The efficacy of essential oil varies with the Aspergillus sp. and asexual reproductive stage. At spore germination and sporulation A. awamori and mycelium growth A. flavus was found to be most sensitive against essential oil. Spore lysis was detected for all the species. Irreversible spore germination and sporulation inhibition was also observed. The effectiveness of higher concentration of essential oil was comparable with myconazole. Rosemary oil for its observed antifungal activities can be used as antimycotic additive to food stuff.

Key words: *Essential oil, Rosemarinus officinalis, Antimycotic, Aspergillus sp., Asexual reproduction, Spore lysis.*

INTRODUCTION

Various spices are commonly used to enhance the food flavor since ancient civilizations. It has been observed that some of these spices also increase the shelf life of foods, signifying their ability to inhibit spoilage and pathogenic microorganisms[1,2,3]. Currently, due to the 'clean label' phenomenon, the food industry faces the need to meet the customers increasingly concerns with food security and the use of synthetic additives, particularly preservatives. Similarly, there are also an increasing number of studies on the development of natural antibiotics and herbicides to discourage the use of pesticides demonstrably toxic to humans and the environment[4,5,6]. Moreover, the increased antimicrobial resistance among pathogenic microorganisms due to indiscriminate use of antimicrobial drugs, the focus has been directed toward medicinal plants for the treatment and prevention of various infectious diseases[7]. In a World health organization (WHO) survey, it was reported that traditional medicine using plant extracts are used by the majority of world's populations as a primary treatment of many diseases[8].

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The main reason behind the use and increased acceptance of natural biologically active compounds as antimicrobial agent is due to relatively low side effects[9]. Essential oils are highly complex, volatile, lipophilic liquid mixtures of intense and characteristic odor, originating from the secondary metabolism of plants, which can be obtained by various physicochemical methods[4,10,11,12]. They have variety of uses in daily life especially in pharmacology, medicine, agriculture, and food production. Chemically, each essential oil is a mixture of nearly 20-60 different constituents, two or three are major components found in high concentrations in the extracted oil e.g., terpenoids, alkaloids etc. and have great impact on living cells[13,14]. They seem to have no specific targets[15]. Due to their lipophilic action, essential oils can easily pass through membranes causing leakage of cell materials and finally death[16,17]. Rosemary (*Rosemarinus officinalis*) is one of medicinal, aromatic plant rich in important bioactive compounds known for their antibacterial, antifungal, antiviral, antioxidant and anti-inflammatory activities[18]. The constituent of essential oil varies from place to place depending on the soil, climate and the methods of extraction[19]. The aim of this study was to investigate the antifungal activity of essential oil of locally grown Rosemary (*Rosemarinus officinalis*) against two strains of *Aspergillus sp.* to contribute to the use of this oil for inhibiting growth on different reproductive stages, responsible for their spread.

MATERIALS AND METHODS

Essential oil isolation: The oil of *R. officinalis* was obtained by hydrodistillation. Dried, crushed leaves were water distilled using a Dean Starke apparatus[20]. The purity of the oil was checked and estimated to be >99%. Dimethyl sulfooxide (DMSO) was added to the extracted essential oil in concentration range of 5% (v/v) to enhance its solubility and diffusion.

Cultures: Three strains of *Aspergillus,* A *,flauus,* A niger and *A. awamori* were isolated[21] from decaying fruit were used in the present studies. These cultures were maintained on Sabouraud dextrose Agar (SDA)[22] and subcultured after every 20 days.

Identification of the strains: For fungal identification, fungal slide culture analysis was performed according to Ribeiro & Soares[23], with modifications proposed by the identification key of Taniwaki & Silva[24].

Antifungal studies: The basal medium used for the studies was SDA. Three dilutions of oil were prepared, i.e. 0.01, 0.05 and 0.10%, and one treatment of standard antifungal drug Myconazole 0.10% w/v. Oil dilutions were prepared by dispersing the calculated amount of oil in 0.3% agar solution and then adding to the rest of the medium. The standard of the antifungal drug was obtained by mixing a calculated amount of Myconazole powder in the medium. The control received no oil or antifungal drug.

Effects of EO on Spore Germination:

The cultures were cultivated in slants for five days to obtain a substantial number of spores. The spores were collected by adding 10mL of 0.9% saline solution to the slants. The spores were suspended in saline solution by gentle shaking. The spore suspension (500 µL) was evenly spread on the surface of the medium. The Petri dishes were incubated at 30°C for 7 h for *A. niger* and 10 h for *A. flavus.* These times were previously determined for maximum germination. The germinal tube equal to the spore size was considered as the spore germination index. The percentage of germinated spores was calculated by counting at least 300 spores in various microscope fields at random using a binocular microscope with video attachment Model Olympus BH-2. The germination percentage was calculated by the following formula:

$$\text{Germination \%} = \frac{\text{Number of germinated spores in particular treatment}}{\text{Total number of spores counted}} \times 100$$

During the counting of spores, the non-germinated spores were also observed for lysis and recorded.

Recovery test was performed for spores whose germination was inhibited by E.O. Such spores were inoculated on oil free SDA and incubated for observing germination daily till 10th day.

Effect of EO on Mycelium Growth:

The angled tubes (slants)[25] were used for studying the effects of oil on mycelium growth[26]. The cultures were grown on SDA for 24 h at 30°C. After 24 h, a piece of mycelium was taken from the growth front and transferred to the entry of the angled tube containing SDA having different concentrations of EO/antifungal drug. The tubes were then incubated at 30°C for 10 days. The increase in mycelium length was recorded after every two days. The growth inhibition percentage (GIP) was calculated after 10 days of mycelium growth by the following formula of Diniz et al.[27]:

$$GIP = \frac{\text{Growth in control – growth in presence of inhibitor}}{\text{Growth in control}} \times 100$$

Effect of EO on Sporulation:

The strains were grown on SDA for 24 h. Disks (5 mm diameter) covered with mycelium were cut and transferred into the same size wells in Petri dishes containing SDA with different concentrations of EO/drug. After an incubation period of Ten days at 30°C, the spores were harvested in 50 mL 0.9% saline containing 0.01% Tween 80. The spores in the suspension were counted by use of a hemo-cytometer. The results were expressed as a number of spores/mL of suspension.

RESULTS AND DISCUSSION

Anti-spore germination activity of E.O.:

The results of inhibition of spore germination by EO of *R. officinalis* in comparison with fluconazole are summarized in Fig. 1. Fluconazole was found to be most effective for inhibition of spore germination of *A. niger*, followed by *A. flavus* and *A. awamori*. The spore germination varies with the concentration of EO and *Aspergillus sp*. The spore germination was decreased with the increase in EO concentration. Similar observation was also reported by Rahman and Gul[28] for *Psamogeton canescens* oil against *Aspergillus sp.* and Thomson[29] for cinmon, thyme and clove oil against *Aspergillus*, *Mucor* and *Rhysopus sp*. Maximum inhibitory effect of EO was observed against *A. awamori* at 0.10% concentration where the germination of spores was reduced to 13.65%. The spores of *A. flavus* were most resistant among the *Aspergillus sp.* treated in the present studies. The inhibition effect of fluconazole 0.01% was comparable with essential oil 0.10% against *A. awamori* spores. It was observed that higher concentrations of essential oil of *R. officinalis* required for complete inhibition of germination of *A. niger* and *A. flavus* spores as compared to antifungal drug fluconazole. Adulaziz *et al.*[30] reported that rosemary oil had potent antifungal activities against fluconazole resistant fungi. Dragoni and Valloni[31] also reported different efficacy against moulds and attributed it the presence of boneol, α-pinene, euclyptol, camphor and limonene. The inhibition order of E.O. in all concentrations against *Aspergillus sp*. remained as follows:

A. awamori > *A. niger* > *A. flavus*

Lysis was also observed in some spores of all the three *Aspergillus* strains under study. Rahman and Gul[32] reported spore lysis of A. niger when exposed to *Thymus cerpyllum* essential oil. Tantaoui-Elaraki et al.[33] also observed the spore lysis of *Zygorrhynchus* sp when exposed to different concentrations of oregano and eucalyptus oils. Whereas, Rahman and Gul[28] and Tantaoui-Elraki et al.[34] observed no spore lysis, when *A. niger* spores were exposed to different concentrations of various oils.

In recovery test of our experiments, no germination was observed even after 10 days of incubation on oil free medium which led to the conclusion that the oil caused irreversible

inhibition of spore germination. The same phenomenon was observed by Rahman and Gul[28] for *Aspergillus sp.*, whereas, Tantaoui-Elaraki *et al*[25] have reported reversible inhibition of spore germination of *A. niger* and *Penicillium italicum* when treated with 1% eucalyptus oil.

Anti-mycelium growth activity of E.O.:

Growth inhibition percentage was calaculated on the most important stage of fungus responsible for its spread (Table 1). EO of *R. officinalis* was most active against *A. flavus.* The activity of EO was comparable with myconazole 0.1% at 1.0 concentration, against the all the *Aspergillus sp.* under study.

The effects of mycelium growth of different foodstuff fungi (*Aspergillus sp.*) in response to various concentrations of the *R. officinalis* oil and drug are presented in Fig. 2. The results presented here are the averages of three determinations. The *Aspergillus sp.* showed significant growth in control medium. Like spore germination, the mycelium growth inhibition was also varied with the concentration of oil and the species of the *Aspergillus. A. awamori* was the least tolerant against EO. The EO not only inhibited but also delayed the mycelium elongation. The inhibitory effects of the antifungal drug were comparable at different concentrations of the oil against various *Aspergillus* species. The inhibition of mycelial growth was directly related to the concentration of oil. The effects of the oil and antifungal drug on individual *Aspergillus* sp. were follows:

A. niger:

Myconazole 0.01% delayed the spread of mycelium till 4th day and remained suppressed i.e., the growth was restricted to only 21.74% as compared to control after 10 days. The growth of mycelium varied with concentration of Rosemary oil. The growth of mycelium at 0.01% and 0.05% oil level commenced at the same time but the growth rate was slow at 0.05 than at 0.01%. Rosemary oil 0.1% delayed mycelium elongation till 2nd day. 6 mm mycelium elongation in response to 0.10% oil was recorded after the 10th day. It was only 4.26 % greater than mycelium growth observed for myconazole 0.01%, over the same period of time. (Figure 2a). The ranking of drug and the oil with respect to their mycelium inhibition was as follows:

Myconazole 0.01% > EO 0.10% > EO 0.05% > EO 0.01%

A. fIavus:

Fig. 2b show that the effectiveness of EO was dependent on its concentration. The pattern of growth retardation of EO 0.05 & 0.01% was quite the same as for *A. niger*. The data presented here revealed that both myconazole 0.01% and EO 0.10% delayed the spread of mycelium till 2nd day. Further, inhibition of growth was also more or less the same after period of 10 days i.e., remained only 25.93 and 22.23 %, as compared to control, repectively.

The order of effectiveness of oil and the drug against the *Aspergillus sp*. after 10 days of growth period remained as:

EO 0.10% > Myconazole 0.01% > EO 0.05% > EO 0.01%

A. awamori:

Fig 2c revealed that *A. awamori* was found to be the most sensitive among *Aspergillus sp.* under study to both Myconazole and rosemary oil. The mycelium growth inhibition increased with the increase in concentration of oil. The results of antifungal drug myconazole 0.01% were comparable to oil 0.10 %. In both cases the mycelium growth was delayed till 4th day whereas the mycelium spread was 4 and 3 mm at 10th day, respectively. However, the rate of growth was initially higher in the presence of oil. The oil concentration 0.01 and 0.05 also delayed the initiation of growth up to 2nd day but the growth rate was less at 0.05 % oil concentration. The growth of mycelium at 10th day was in the range of 12 to 28 % in presence of EO 0.01 to 0.10 % as that of control.

The ranking of drug and the oil with respect to their mycelium inhibition was as follows:

Drug 0.05% > EO 0.10% > EO 0.05% > EO 0.01

Anti-sporulation activity of E.O.:

The spore formation is one of the important stages for continuing its existence. The present studies unveil that the sporulation was the most sensitive asexual reproductive stage of the isolated *Aspergillus sp.* Sporulation inhibition by myconazole and different concentrations of *R. officinalis* oil presented in Fig. 3. The sporulation inhibition vary with the concentration of E.O. and species of *Aspergillus*. The effect on sporulation by Myconazole 0.1% was analogous to E.O. 0.01 % against *A. flavus* where 100% inhibition was observed. The E.O. was more effective against *A. flavus* than *A. niger* and *A.awamori*. The sporulation inhibition of essential oil 0.10 % against *A. niger* was quite the same as by myconazole 0.01% i.e., 98.25 & 97.56 %, respectively. More or less the same effect was observed for *A. awamori.* In this case the sporulation inhibition % was 96.95 by myconazole 0.01% and 97.63 by E.O. 0.10%. Hmamouchi reported that the inhibition of sporulation was due to the destruction of mycelium or inhibition of fungal growth. The partial inhibition of sporulation may be due to the destruction of mycelium or inhibition of fungal growth[28]. Rahman and Gul[32] observed that the sporulation stage of different *Aspergillus sp.* was least affected as compared to other reproduction stages against *Thymus serpyllum* essential oil.

Table 1: GIP of *Aspergillus sp.* in different concentrations of EO of *R. officinalis* and Myconazole at mycelium producing stage

Treatments	Fungal Strains					
	A. niger		*A. flavus*		*A. awamori*	
	Average growth (mm)	GIP	Average growth (mm)	GIP	Average growth (mm)	GIP
Control	23.0	0.00	27.0	0.00	25.0	0.00
Myconazole, 0.01%	5.0	78.26	7.0	74.07	5.0	80.00
EO, 0.01%	20.0	13.04	12.0	55.55	9.0	64.00
EO, 0.05%	13.0	43.47	9.0	66.66	6.0	80.00
EO, 0.10%	6.0	73.91	6.0	77.77	3.0	88.00

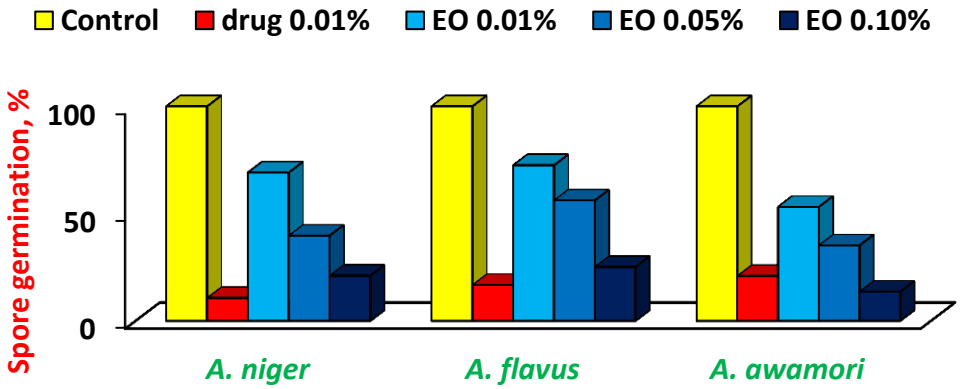

Fig. 1: Effects of Myconazole and EO on germination of *Aspergillus sp.*

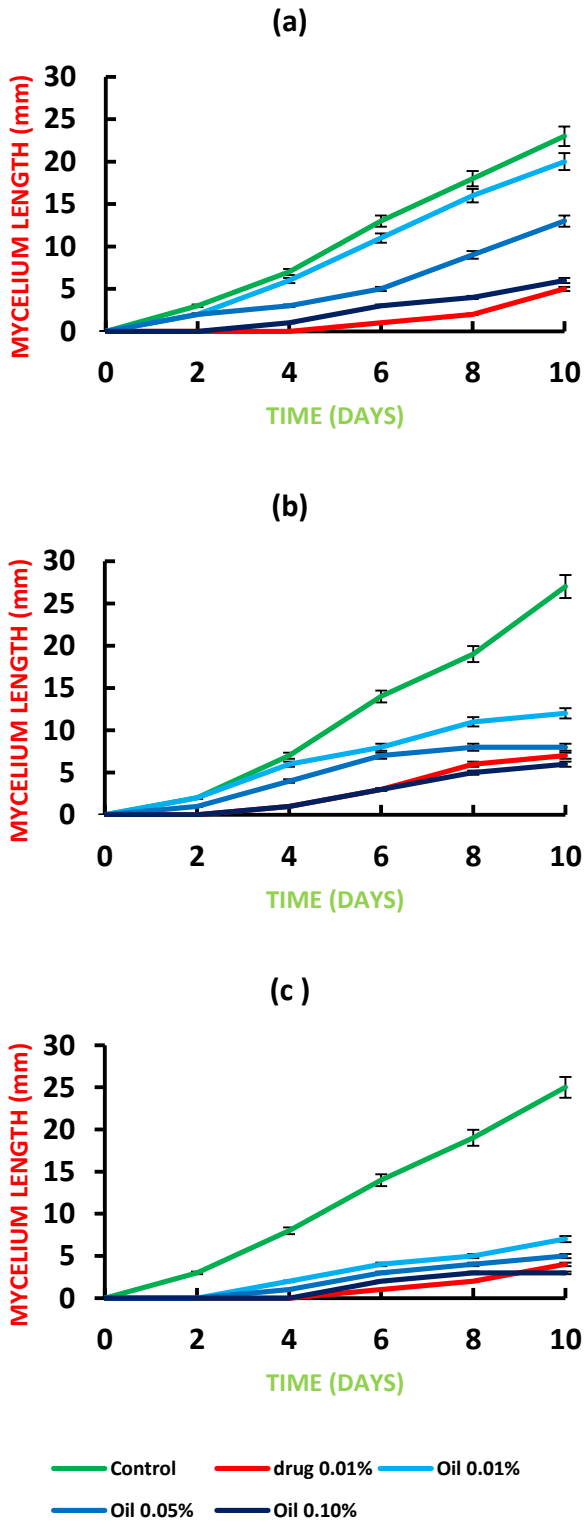

Fig. 2: Effects of Myconazole and EO on Mycelium growth of *Aspergillus sp.*
(a) *A. niger* (b) *A. flavus* (c) *A. awamori*

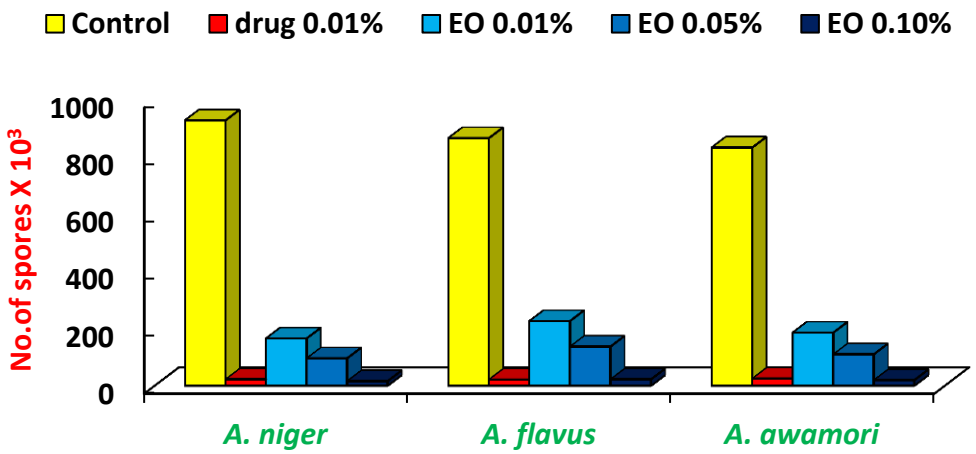

Fig. 3: Effects of Myconazole and EO on sporulation of *Aspergillus sp.*

CONCLUSIONS

From the current studies, it can be safely concluded that the essential oil of *Rosemarinus officinalis* possess potent antifungal activity and validate the ethno pharmacological claim. From the results it appears that essential oil of Rosemary have potential as food preservative.

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