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Antifungal activity of *Eugenia caryophyllata, Cinnamomum* sp., *Mentha piperita,* and *Thymus vulgaris* essential oils against *Aspergillus niger*

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Eugenia caryophyllata, Cinnamomum sp., *Mentha piperita* ve *Thymus vulgaris* esansiyel yağların *Aspergillus niger* suşuna karşı antifungal aktivitesi

Abstract: *Aspergillus* species are pollutants found in both food and air. The increase in the metabolic activity of *Aspergillus* leads to spoilage of foodstuffs and large economic losses. In addition, some *Aspergillus* species have the ability to produce aflatoxins and ochratoxins, secondary metabolites called, namely, mycotoxins. Especially mycotoxins are very important in terms of food safety and human health. Since the protection of human and animal health and the prevention of economic losses is a very important issue, our study aimed to determine the antifungal activity of *Eugenia caryophyllata* Thunb., *Cinnamomum* sp., *Mentha piperita* L. and *Thymus vulgaris* L. essential oils (EO) against *Aspergillus niger* Tiegh. NRRL 321 strain. In the second step, MIC and MFC values of EOs were determined. It was determined that the MIC value of *Cinnamomum sp, M. piperita* and *T. vulgaris* EOs was 0.01 µL/mL, and *E. caryophyllata* EO was 0.5 µL/mL. It was determined that *E. caryophyllata, Mentha piperita* and *Thymus vulgaris* EOs completely inhibited radial colony growth at MIC, 2xMIC and 4xMIC values. It was determined that the inhibition of radial growth of *Cinnamomum* sp. EO varies depending on the concentration, and the inhibition rate increases as the concentration increases. As a result, evaluations should be made considering the in vivo conditions that the tested EOs showed strong antifungal activity against the *A. niger* strain.

Key words: Antifungal activity, cinnamon oil, clove oil, peppermint oil, thyme oil

Özet: *Aspergillus* türleri hem gıdalarda hem de havada bulunan kirleticilerdir. *Aspergillus* cinsinin metabolik aktivitesindeki artış, gıda maddelerinin bozulmasına ve büyük ekonomik kayıpların oluşmasına yol açmaktadır. Ayrıca bazı *Aspergillus* türleri, aflatoksinler, okratoksinler olarak adlandırılan sekonder metabolitleri yani mikotoksinleri üretme yeteneğine sahiptir. Özellikle mikotoksinler, gıda güvenliği ve insan sağlığı açısından oldukça önemlidir. İnsan ve hayvan sağlığının korunması ve ekonomik kayıpların önlenmesi çok önemli bir konu olması sebebiyle, çalışma amacımız *Aspergillus niger* Tiegh. NRRL 321 suşuna karşı *Eugenia caryophyllata Thunb., Cinnamomum* sp., *Mentha piperita* L. ve *Thymus vulgaris* L. esansiyel yağlarının (EO) antifungal aktivitesi belirlenmiştir. İkinci adımda ise EO'ların MİK ve MFK değerleri belirlendi. *Cinnamomum* sp., *M. piperita* ve *T. vulgaris* EO'ların MİK değerinin 0,01 µL/mL, *E. caryophyllata* EO'nun ise 0,5 µL/mL olduğu tespit edilmiştir. *E. caryophyllata, M. piperita* ve *T. vulgaris* EO'ların MİK, 2xMİK ve 4xMİK değerlerinde radyal koloni gelişimini tamamen inhibe ettiği belirlendi. *Cinnamomum* sp. EO'nun ise radyal büyümenin inhibisyonu konsantrasyona bağlı olarak değiştiği, konsantrasyon arttıkça inhibisyon oranın arttığı belirlenmiştir. Sonuç olarak test edilen EO'ların *A. niger* suşuna karşı güçlü antifungal aktivite gösterdiği, in vivo koşullar göz önüne alınarak değerlendirmelerin yapılması gerekmektedir.

Anahtar Kelimeler: Antifungal aktivite, karanfil yağı, kekik yağı, nane yağı, tarçın yağı

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1. Introduction

Aspergillus species are very common both indoors and outdoors, producing numerous small, airborne spores and versatile metabolites. It has been determined that their presence in the air causes various ailments of the lungs, liver, skin and muscles (Klich et al., 2009; Kumar et al., 2017). Studies have shown that there is a relationship between upper respiratory tract, cough, wheezing and asthma symptoms in susceptible individuals, as well as severe respiratory tract infections in immunocompromised individuals and the presence of mould indoors. Studies examining indoor mould distributions have reported that the presence of *Aspergillus* species or teleomorphs are present in almost every environment and affects human health. In addition, mycotoxins (aflatoxins, fumonisins, ochratoxins, etc.) produced by the *Aspergillus* species have been found to be carcinogens, mutagens, teratogens and immunosuppressants (Klich et al, 2009; Prakash et al., 2015; Kumar et al., 2017). In addition to mycotoxin production, *Aspergillus* species also produce enzymes that can degrade many different organic substrates (Fogarty, 1994; Hua et al., 2014). Some species are xerophilic and can thrive at relatively low humidity. Indoors, they can thrive on wood, paper, paint, glue and even dirty metal doors when humidity is high. *Aspergillus* species in soil tend to occur in subtropical and temperate climates, with most of the pathogenic species in these environments (Klich, 2002; Hua et al., 2014). The most frequently reported species in soil are *A. fumigatus* Fresen, *A. niger* Tiegh., *A. flavus* Link, and *A. terreus* Thom. All four of these species are equally distributed in all biomes. It is probably not a coincidence that these are the most frequently reported species in human disease (Klich, 2002; Klich et al., 2009; Hua et al., 2014).

Since ancient times, medicinal and aromatic plants have found wide use in many different fields. They have been widely used in various industrial areas such as traditional medicine, agriculture, food (as preservatives and sweeteners), perfumery, cosmetics and the pharmaceutical industry. Regarding the biological properties of essential oils (EOs) and their plant extracts, they have recently gained significant scientific popularity and interest. The relationships between the composition and biological properties of EOs have been investigated. Phenolic compounds in EOs have been identified and accepted in the literature as bioactive components with antimicrobial activity. (Burt, 2004; Nedorostova et al., 2009).

Therefore, to fill these gaps, the antifungal activity of commercially available EOs from *Eugenia caryophyllata, Cinnamomum* sp., *Mentha piperita* and *Thymus vulgaris* against the *A. niger* strain was evaluated. First of all, the antifungal activity was determined by the agar well diffusion method. Then, minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC) of EOs were determined. After the MIC values were determined, the effect of EOs at different concentrations on the radial growth of *A. niger* was investigated.

2. Materials and Method

2.1. Materials

E. caryophyllata and *Cinnamomum* sp. EOs Kırıntı Baharat A.Ş. (Kocaeli, Türkiye), *M. piperita* and *T. vulgaris* EOs Toroslar Group A.Ş. (Adana, Türkiye) were provided.

A. niger Tiegh. NRRL 321 stock culture was first inoculated on Potato Dextrose Agar (PDA) medium, and the culture was revived to check its purity.

2.2. Method

2.2.1. Preparation of the spore suspension

Aspergillus strain was grown on slanted Malt Extract Agar (MEA) media at 25°C until well sporulated (7-10 days). Spores were collected by adding sterilized Tween 80 solution (0.1% v/v). Spore concentration was set at McFarland 0.5 density. The suspensions prepared to determine the MIC value were then diluted 1:10 with distilled water to obtain the final working inoculum, with a density of 2-5x10^5 cfu/mL.

2.2.2. Determination of antifungal activity by agar well diffusion method

The antifungal activity of *E. caryophyllata, Cinnamomum* sp., *M. piperita* and *T. vulgaris* EOs was used in the method specified in NCCLS M44-A, modified by drilling a 6 mm diameter well with a cork borer set instead of a disc. For the agar well diffusion method, after planting 0.1 mL of the prepared spore solutions on the PDA medium according to the smear plate method, 20 µL of EO was added to each opened well. Zone diameters were measured after 3-5 days of incubation at 25°C (Snoussi et al., 2018; Mseddi et al., 2020). The study was carried out in 3 parallels. Amphotericin B (20 IU, 21,19 mcg, Bioanalyse, Türkiye) and fluconazole (25 mcg, Himedia, India), antifungal discs were used as positive controls.

2.2.3. Determination of minimum inhibitory concentration (MIC)

MIC values were determined with minor modifications to the standard NCCLS M38-A2 method. RPMI 1640 medium (Himedia, India) containing 0.165 M MOPS with 2X essential oil and 3% DMSO was dispensed with a multichannel pipette into the wells of rows B and G microdilution plates in 100 µL volumes. Then 100 µL of spore solution was added to it. The final concentrations were 4, 2, 1, 0.5, 0.25 and 0.01 µL/mL. Row A of microplates contains RPMI 1640 broth + 3% DMSO medium containing 4 µL/mL EO and is determined as a negative control, and the last row (H) is only RPMI 1640 3% DMSO medium + culture and is defined as a positive control. Microplates were evaluated after 24-48 hours of incubation at 25°C. The first well without growth was determined as the MIC value. The MFC value was determined after the incubation at the appropriate temperature and time by planting with the drip planting method from the well defined as the MIC value and the subsequent two wells into the PDA medium (Snoussi et al. 2018; Mseddi et al., 2020). The study was carried out in three parallels.

2.2.4. Effect of molds on radial growth of mycelium

Soliman and Badeaa (2002) and Gámez-Espinosa et al. (2021) by modifying the methods specified. To analyze the effect of essential oils on the radial growth of mould mycelium, the *A. niger* was grown on a PDA medium at 25°C for 7-10 days. PDA media containing 3% DMSO at 1⁄4xMIC, 1⁄2xMIC, MIC, 2xMIC, and 4xMIC concentrations of EOs whose MIC values were determined were prepared and poured into 60 mm diameter petri dishes. Single-point planting was done in the middle of the petri dish with the help of a needle loop from cultures developed in PDA medium. Petri dishes were incubated at 25°C for 7-14 days, and colony diameters were measured after incubation. As a control, inoculation was made on a PDA medium containing 3% DMSO. Petri dishes were incubated at 25°C for 7-14 days. Zone diameters were measured at the end of incubation. The study was carried out in three parallels. Inhibition of radial growth was calculated with the following equation: %I=(C-T)/C*100 (C: growth diameter (mm) in the control plate, T: growth diameter (mm) in the essential oil-containing petri dish), I: inhibition (%)).

2.2.5. Statistical analysis

Results are given as mean (M) ± standard deviation (SD).

3. Results

The data obtained in this study were collected through the sequential application of different techniques to evaluate the antifungal activity of *E. caryophyllata, Cinnamomum* sp., *M. piperita* and *T. vulgaris* EO against *A. niger* NRRL 321. The first step was determining whether EOs have antifungal activity against the tested *A.niger* NRRL 321. For this purpose, the agar well diffusion method was used. Agar diffusion method results in mm are given in Table 1.

According to the results in Table 1, it was found that EOs of *E. caryophyllata, Cinnamomum* sp., *M. piperita*, and *T. vulgaris* against the tested *A. niger* NRRL 321 strain significantly inhibited the growth of amphotericin B antifungal agent. It was determined that *T.vulgaris* EO formed a very high inhibition zone compared to the positive

control amphotericin B antifungal. In the second step of the study, the MIC, MFC, and MFC/MIC values of the tested EOs were evaluated using the microdilution method; the results are given in Table 2.

Table 1. Agar disc diffusion method zone diameters in mm (M±SD)

EO	*Aspergillus niger* NRRL 321
Cinnamomum sp.	9.54 ± 0.56
E. caryophyllata	12.60 ± 1.33
M. piperita	13.08 ± 1.85
T. vulgaris	36.20 ± 2.61
AMP	13.29 ± 3.48
FLU	90.00 ± 0.01

Table 2. MIC, MFC value and MFC/MIC ratio of selected EOs in µL/mL against *Aspergillus niger* NRRL 321

EO	MIC	MFC	MFC/MIC
Cinnamomum sp.	0.01	0.01	1
E. caryophyllata	0.5	4	8
M. piperita	0.01	0.01	1
T. vulgaris	0.01	0.01	1

When evaluated considering the microplate concentrations with the microdilution method, the MIC value of the first well where no growth was observed was determined. Accordingly, according to the results in Table 2, the MIC value of *Cinnamomum* sp., *M. piperita* and *T. vulgaris* EOs was determined to be 0.01 µL/mL. The MIC value of *E. caryophyllata* EO was determined to be 0.5 µL/mL. After the MIC value was determined, the wells with the MIC value on the plates and the next two wells were inoculated into the PDA medium by drip planting method. Afterwards, the PDA petri dishes were incubated at the appropriate time and temperature. The first concentration at which growth was not observed after incubation was determined as MFC. The MFC value of *Cinnamomum* sp., *M. piperita*, and *T. vulgaris* EOs was determined to be 1 µL/mL. The MFC value of *E. caryophyllata* EO was determined to be 4 µL/mL. As mentioned in Gatsing et al., 2009, Snoussi et al., 2018 and Mseddi et al. (2020), when we evaluated whether the EOs were fungicidal (MFC/MIC ratio ≤ 4) or fungistatic (MFC/MIC ratio > 4), it was determined that *Cinnamomum* sp., *M. piperita* and *T. vulgaris* EOs were fungicidal agents. *E. caryophyllata* EO was determined to be a fungistatic agent.

In the third step of our study, the effect of EOs, whose MIC value was determined, on the radial growth of *A. niger* NRRL 321 strain was also evaluated. While determining the effect on radial growth, radial growth inhibition of EOs at 1⁄4xMIC, 1⁄2xMIC, MIC, 2xMIC, and 4xMIC concentrations was investigated in line with MIC values. The results regarding radial growth inhibition are given in Table 3. It was determined that *T. vulgaris* EO completely inhibited the radial growth of the tested *A. niger* NRRL 321 strain from 1⁄2xMIC concentration. *E. caryophyllata* and *M. piperita* EO were found to inhibit radial growth at the MIC value and all subsequent concentrations. *Cinnamomum* sp. EO was found to inhibit radial growth at the MIC value and the following concentrations, with a maximum inhibition of 46.41 ± 3.20%. As clearly seen in Table 3, the inhibition rate increases with the concentration.

4. Discussions

The results of this study highlight the antifungal activities of herbs and essential oils, offering a promising area of research for alternative therapies and food safety.

T. vulgaris EO inhibited radial growth by inhibiting *A. flavus, A. parasiticus* and *A. ochraceus* isolates (Soliman and Badeaa, 2002). Kumar et al. (2008) reported that *T. vulgaris* EO at a concentration of 0.7 µL/mL completely inhibited the radial growth of *A. flavus, A. terreus* and *A. fumigatus*, while it provided 89.56% ± 3.06% inhibition on *A niger*. Al-Shahrani et al. (2017) determined the MIC and MFC values of *T. vulgaris* EO, which they obtained from Saudi Arabia, as 10 mg/mL for *A. flavus* and *A. niger*.

Mahboubi and Kazempour (2014) determined that the MIC and MFC values of *M. piperita* EO against *A. niger* were 0.5 µL/mL and 1 µL/mL, respectively, while Hu et al. (2019) found 2 mg/ml for *A. niger*. Desam et al. (2019) 1.0 µl of *M. piperita* EO gave an inhibition zone of 30.08 ± 0.08 mm against *A. fumigatus* isolate and 17.23 ± 0.23 mm for *A. variecolor*, and the MIC value of *A. fumigatus* and *A. variecolor* determined 0.50 µg/mL and 10 µg/mL respectively for isolates. The study determined that the MIC and MIC values of *M. piperita* EO were 0.01 µL/mL and completely inhibited radial growth at MIC, 2xMIC, and 4xMIC concentrations.

Cinnamomum sp. EO was also effective on *Aspergillus* species (Thanaboripat et al., 2007; Pekmezovic et al., 2015; Lappa et al., 2017; Moghadam et al., 2019). Hu et al. (2019) found that the MIC value of *Cinnamomum* sp. EO was 0.0625 mg/mL for *A. niger*, and Yan et al. (2021) determined it as 62.50 µL/L.The study determined that a MIC value of 0.01 µL/mL and 4xMIC concentration inhibited *A. niger* strain by 46.41% ± 3.20%. Similarly, the activity of *E. caryophyllata* EO on *Aspergillus* species was determined (Soliman and Badeaa, 2002; Vijayalakshmi et al., 2014). Hu et al. (2019) reported that the MIC value of *Eugenia caryophyllata* EO against *A. niger* isolates was 0.25 mg/mL. This study determined that the MIC value of *E. caryophyllata* EO against the tested *Aspergillus* strain

Table 3. Radial growth inhibition rate (in %)

EO	1⁄4 x MIC	1⁄2 x MIC	MIC	2 x MIC	4 x MIC
E. caryophyllata	59.83 ± 7.86	78.18 ± 4.90	100.00 ± 0.01	100.00 ± 0.01	100.00 ± 0.01
Cinnamomum sp.	0.00 ± 0.01	0.00 ± 0.01	43.18 ± 6.15	43.07 ± 5.42	46.41 ± 3.20
M. piperita	61.55 ± 0.27	81.42 ± 5.43	100.00 ± 0.01	100.00 ± 0.01	100.00 ± 0.01
T.vulgaris	100.00 ± 0.01	100.00 ± 0.01	100.00 ± 0.01	100.00 ± 0.01	100.00 ± 0.01

was 0.5 µL/mL, and the lowest radial growth inhibition was 50% even at 1⁄4xMIC concentration.

As a result, the antimicrobial properties of herbal sources are important when considering the problem of drug resistance. However, safety and toxicity assessments are required before using these plant oils for medicinal or food purposes. It is concluded that this study will encourage further research in the future and may assist in developing alternative therapeutic agents.

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