Antifungal Properties of Eucalyptus Camaldulensis Essential Oil Against Aspergillus Spp

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ABSTRACT

Eucalyptus Essential oils has a variety of bioactivities as fungicidal, bactericidal, insecticidal and other therapeutic properties . This investigation was conducted to assess the antifungal properties of Eucalyptus camaldulensis essential oil alone and in combination with clotrimazole against Aspergillus spp that isolated from patients. The in laboratory results appeared that the development of Aspergillus spp was discouraged by the essential oil at all tested concentration (100 , 50 and 25%) and the effect was increased with the increasing of concentration. The antifungal drugs clotrimazole , itraconzole and griseofulvin also affect the development of Aspergillus spp and the fungus A. fumigatus was less sensitive to essential oil and drugs than the other species of Aspergillus. The outcome shown that the combination of clotrimazol with essential oil was more effective than each one alone and the combination enhanced the antifungal activity with an additive interaction.

1. Introduction

Aspergillus species are much of the time recognized as the causative of serious contagious infections, especially in patients with immunocompromised systems. Most infections caused by Aspergillus spp. are caused by Aspergillus fumigatus, although infections can also be caused by Aspergillus flavus, Aspergillus niger, and Aspergillus terreus, which are not fumigatus species. (Paulussen et al., 2017 ; Rozaliyani et al., 2022). Rich sources of bioactive chemicals viewed in plants have been shown as useful against various illnesses and diseases (Tan et al., 2022). Leaves of E. camaldulensis are known to possess several biological and pharmacological activities, including antifungal activity against several pathogenic contagious fungi (Mehani et al., 2022 ; Al-abedi et al., 2023). When four normal form mold fungi (Aspergillus flavus, A. niger, A. terreus, and Fusarium culmorum) were developed, the essential oil from E. camaldulensis was tested for its antifungal activity. The results showed that the oil had great antifungal activity, with A. niger showing the highest percent of inhibition (91.66%) (Elgat et al., 2020). Antifungal medications of various kinds are being used to treat aspergillosis. In any case, resistance to these antifungal drugs has been increasing over the long run in various Aspergillus species. Numerous azole resistance mechanisms have been distinguished in A. fumigatus, with the most very much described mechanism being distinguished. Moreover, the usage of these customary antifungal medicines has been restricted because of their adverse consequences (Verweij et al., 2020). Nazzaro et al., (2017) stated that mixing essential oils with antifungal medications can help overcome the issue of fungal drug resistance. The authors observed a combination of some essential oils and azol- dependent medications to have synergistic antifungal action. (Rosato, et al., 2010).

Based on above information we postulate that the combination of azole with the essential oil of Eucalyptus essential oil may be affected the growth of Aspergillus spp that cause aspergillosis .Research has shown that combining medications with natural compounds is a different approach to treating multi drug resistance (MDR) in fungi (Sharma, et al., 2022). Therefore, this studyś was to assess the impact of Eucalyptus camaldulensis essential oil alone and in combination with azole drug against Aspergillus spp. that cause aspergillosis.

2. Material and Methods

Source of essential oil

The Directorate of Agrarian Research, Ministry of Science and Technology, provided the essential oil of *Eucalyptus* spp.

Source of antifungal drug

The antifungal medications (itraconazole, griseofulvin, and chlorrimazole) utilized in this investigation were purchased as standard solutions from a pharmacy in the province of Wasit.

Effect of essential oil on growth of *Aspergillus* spp

to assess the essential oil's effect on *Aspergillus* spp. development using the disc diffusion method. Different essential oil concentrations (100, 50, and 25%) were created by consolidating the essential oil with dimethyl sulphoxide. The examined growths' 1 milliliter spore solution was combined with 100 milliliters of PDA, then added to a sterile petri plate and allowed to solidify. Five-millimeter paper disks that had been autoclave-sterilized were soaked in essential oil at concentrations of 100, 50, and 25% before being placed in each plate. As a control, paper discs were soaked in dimethyl sulfoxide. To permit the solution to diffuse, the agar plate was left in the room temperature for two hours. Following that, each plate was incubated for a predetermined measure of time at 25℃. The inhibitory zones were then measured in millimeters after that. (Muthomi *et al*., 2008).

Effect of antifungal drugs on growth of *Aspergillus* spp

Using the discs diffusion method, the effects of antifungal medications (clotrimazole, griseofulvin, and itraconazole) on *Aspergillus* spp. growth were estimated using three different medication concentrations (10 µg/ml, 5 µg/ml, and 2.5 µg/ml). Paper discs (5 mm) that have been autoclaved and sterilized are placed in a petri dish containing PDA that has already been inoculated with the target fungi. The paper discs are then soaked in each concentration of the antifungal medication. For two hours, the agar plates were maintained at room temperature, permitting the solution to diffuse. After that, each plate was incubated for a predetermined measure of time at 25 °C. After that, the zone of hindrance was estimated in millimeters (mm). (Mukherjee *et al*., 2003).

Effect of the combination of Clotrimazole with essential oil on growth of *Aspergillus fumigatus*

To assess the antifungal activity of *Eucalyptus* essential oil in combination with Clotrimazole on growth of *Aspergillus fumigatus* by using the approach of the disc diffusion. 5ml of Clotrimazole in a concentration 5µg /ml wer mixed with 5ml of each concentration (100, 50, and 25%) of *Eucalyptus* essential separately. Paper discs (5mm) have been sterilized with the use of the autoclave then soaked afterwards in each concentrations and put it in a petri dish contain PDA inoculate previously with tested fungus. Paper discs (5 mm) were soaked in distilled water as the negative control and just Clotrimazole as the positive control. Agar plates have been kept at the temperature of the room for a 2h period, which allows for the solution diffusion. After that, each plate has been incubating at 25°C for the specified period of time. The zone of inhibition has been estimated afterward in (mm) (Mukherjee *et al*., 2003).

The analysis of the synergy between clotrimazole and essential oil was done by looking at the table value for 1 df (3.84) and the computed $\chi 2$ value. The calculated $\chi 2$ value can be determined using the accompanying formula: if the calculated $\chi 2$ value is greater than the tabulated value, it shows a synergistic effect; on the off chance that the tabulated value is greater than the calculated $\chi 2$ value, it represents an additive effect. (Goyal *et al*., 2022).

$$ZE = ZC + ZO\ (1 – ZC /100)$$

$$\chi 2 = (ZCO – ZE)^2 / ZE$$

ZE = expected inhibition zone

ZC = inhibition zone by Clotrimazole alone

ZO = inhibition zone by *Eucalyptus* essential oil alone

ZCO = inhibition zone by combination

$\chi 2$ = Calculated Chi squared

Statistical Analysis

Using the Statistical Package for the Social Sciences (SPSS) software, the information were statistically evaluated and subjected to analysis of variance (ANOVA). The Least Significant Difference (LSD) test was utilized to analyze differences between treatments at the likelihood threshold of less than 0.05.

3. Results and discussion

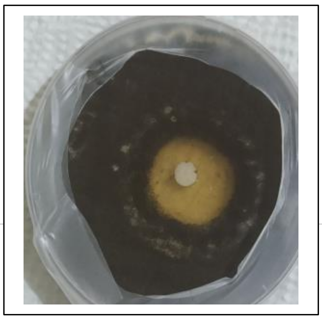
Effect of essential oil on growth of *Aspergillus* spp by disc diffusion method

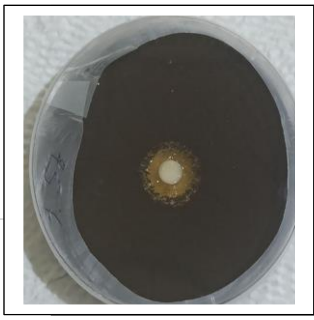
The findings of this study, which are shown in Table (3-4) and Figure (3-9), demonstrated that *Eucalyptus* essential oil inhibited the development of *Aspergillus* spp. and that the effect increased with increasing oil concentration. The inhibition zones of *A. niger*, *A. flavus*, and *A. fumigatus* were, respectively, (29.67, 27.67, 26.33mm) at the concentration 100 percent and (8.33, 7.67, 7.00mm) at the concentration 25%. The highest mean of the inhibition zone was recorded on account of *A. niger* (17.56mm), and the lowest one was recorded on account of *A. fumigatus* (15.22mm). The results also demonstrated that *A. niger* was the most sensitive, while *A. fumigatus* was more resistant.

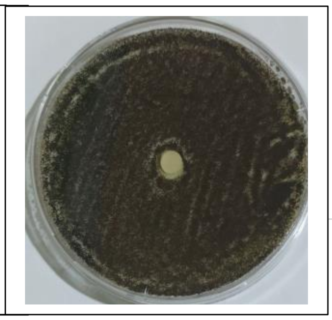
Table (1) Effect of *essential oil* of Eucalyptus on *Aspergillus* spp by disc diffusion method

Treatment		Inhibition zones (mm)			Means
Extract	Concentrations (%)	*A. niger*	*A. flavus*	*A. fumigatus*	
Essential oil	100	29.67	27.67	26.33	27.89
	50	14.67	13.67	12.33	13.56
	25	8.33	7.67	7.00	7.67
Control		0			
LSD (0.05)		0.934 N.S			0.539
Means		17.56	16.33	15.22	
LSD (0.05)		0.539			

Each value is a mean of three replicates







a **d**

a **b** **c** **d**

Figure (1) Effect of essential oil on *Aspergillus niger* by disc diffusion method (a- 100 , b- 50 , c- 25% , d- control)

According to the study's findings, *Eucalyptus* essential oil has strong antifungal activity against the fungus it was tested on. This could be because the oil contains antifungal compounds. Flavonoids and tannins are among the several phytochemical substances tracked down in *Eucalyptus*. Yet, what was in them differed depending on the species (Nasr *et al*., 2019).

Previous studies (Kim and Park, 2012; Siramon, Ohtani, and Ichiura, 2013) have assessed the efficacy of oil derived from *Eucalyptus* spp. as an antifungal against a variety of pathogenic growths.

Strong antimicrobial properties of *Eucalyptus* essential oils enable them to combat a variety of pathogenic microbes (Rocha *et al*., 2015; Yangui *et al*., 2017). The phenolic compounds found in *Eucalyptus* extracts are among the most potent antifungal agents found in plants, which may explain their effectiveness against fungus. Actually, phenolic chemicals' hydrophobic nature makes it easier for them to enter the lipid membrane of fungus (Zabka and Pavela, 2013).

Using the disc-diffusion method, Dogan *et al*,. (2017) investigated the essential oil activity of *E. globulus* against *Candida* and found that it has a decent antifungal potential with inhibition zone of up to 25 mm. In comparison to the studied antifungals, fluoroscytosine and amphotericin B, which had hindrance zones of 18 and 20 mm, respectively, the oil inhibition zone resulted in a greater value.

In vitro analysis is used by Jaradat *et al*., (2023) to determine the antimicrobial activities of the essential oil extracted from *E. camaldulensis* leaves. The essential oil of *E. camaldulensis* was found to contain 52 components. Of them, p-cymene (38.64%) and aromadendrene are the primary chemical elements.*Protease vulgaris* and methicillin-resistant *Staphyococcus aureus* were both significantly inhibited by the essential oil. It has a potent anti-*Candida albicans* as well.

A related study revealed that the essential oils of *Eucalyptus* and other medicinal plants demonstrated efficacy against other fungal species, including *Penicillium* species, *Cladosporium*, and *Alternaria*, as well as antifungal activity against *A.Niger, A.Flavus, A.Parasiticus, A.Fumigatus*, and *A.Ochraceus* (Abdi-Moghadam *et al*.,2023).

Arun *et al*., (2024) carried out a study to examine the biosynthesis of fluconazole, itaconazole, and ketoconazole containing oil/water (O/W) nanoemulsions (NE) using *Eucalyptus* essential oil and its impact on mucor-mycosis and aspergillosis. The results showed that Flucaconazole showed the highest antifungal activity among all formulations against *Aspergillus* strains. MTCC 277. mixture of fluconazole and essential oils.

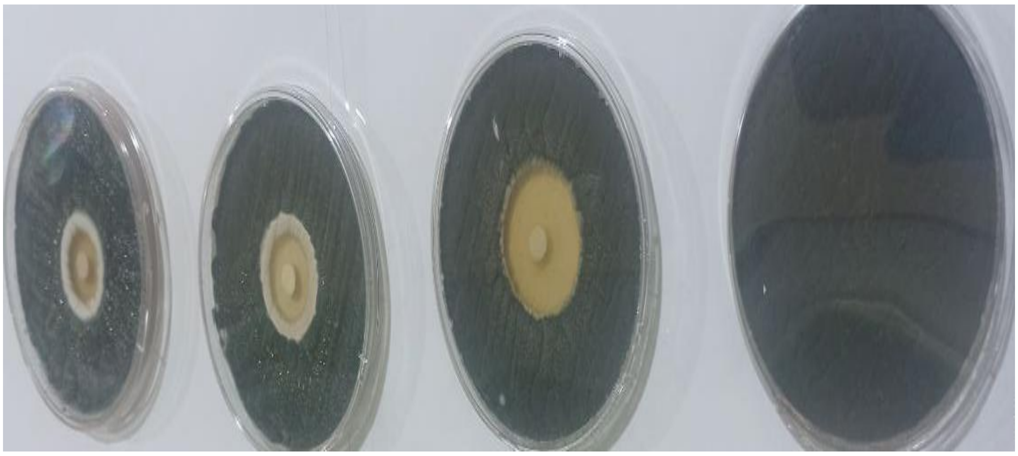
Effect of antifungal drugs on growth of *Aspergillus* spp by disc diffusion method

The data that obtained from this study were presented in Table (2) and Figure (2). The result revealed that the antifungal drugs clotrimazole had exhibited the best *in vitro* activity against *Aspergillus* spp and the activity was proportional with the concentration. The maximum hindrance zone was determinded in the case of *A. niger* and clotrimazole at the concentration 10µg/ml which was (34.33mm) and the lowest hindrance zone was measured in the case of *A. fumigatus* and itraconazol at the concentration 2,5µg/ml which was (6.00mm). Additionally, the results indicated that *A. niger* was the more sensitive fungus, with a hindrance zone mean of (15.852 mm), compared to *A. flavus* and *A. fumigatus*, which had inhibition zone means of (14.519 mm) and (13.185 mm), respectively.

Table (2) Effect of antifungal drugs against *Aspergillus* spp by disc diffusion method

Treatment		Inhibition zones (mm)			Mean
Antifungal	Concentrations	*A.niger*	*A. flavus*	*A. fumigatus*	
Clotrimazole	10µg/ml	34.33	31.50	28.50	31.44
	5µg/ml	18.50	15.67	13.33	15.83
	2.5 µg/ml	8.33	7.33	6.67	7.44
Griseofulvin	10µg/ml	23.50	22.50	20.67	22.22
	5µg/ml	12.83	11.83	10.33	11.67
	2.5µg/ml	7.50	6.67	6.33	6.83
Itraconazole	10µg/ml	19.50	18.50	17.50	18.50
	5µg/ml	11.50	10.33	9.33	10.39
	2.5µg/ml	6.67	6.33	6.00	6.33
LSD (0.05)		0.8817			0.5091
Means		15.852	14.519	13.185	
LSD (0.05)		0.2939			

Each value is a mean of three replicates



A b c d

Figure (2) Effect of clotrimazole on *A. fumigatus* by disc diffusion method (a- 25 , b- 50 , c-

100 % , d- control)

Our study's findings showed that *A. fumigatus* was less sensitive to clotrimazole than *A. niger*, which was consistent with those of Amareshwar *et al*., (2010) who discovered that *A. niger* was more sensitive to the medication and that *A. fumigatus'* inhibition zone was 26 mm as opposed to 32 mm for *A. niger*. In terms of minimum inhibitory concentration (MIC) against *A. fumigatus*, *A. flavus*, and *A. niger*, clotrimazole has the lowest value. According to a study by Raksha *et al*., (2018) clotrimazole shown 100 percent sensitivity to all *Aspergillus* species, while itraconazole demonstrated 66.67% sensitivity. The study evaluated the susceptibility of *Aspergillus* species: *A. fumigatus*, *A. niger*, *A. flavus*, and *A. terrus* to clotrimazole.

A similar investigation was carried out by AL-Jobori *et al*., (2015) to examine the in vitro activities of musk and clotrimazole against *Aspergillus fumigates*, *Aspergillus niger*, and other pathogenic fungi. The results showed that the diameters of the hindrance zones were 32 mm for *A. fumigatus* and 36 mm for *Aspergillus niger*. Tokarzewski *et al*., (2012) observed that *A. niger* was more susceptible to antifungal drugs such clotrimazole and itraconazole, which is in agreement with the findings of our investigation. Azole drugs, such as itraconazole and clotrimazole, neutralize fungus by preventing the formation of ergosterol. When ergosterol synthesis is inhibited, the fungal cytoplasmic membrane is impaired both structurally and functionally, which results in cell content leakage, lysis, and ultimately death (Shalini *et al*., 2011; Noël de Tilly and Tharmalingam, 2022).

Effect of clotrimazole with essential oil on growth of *Aspergillus fumigatus* by disc diffusion method

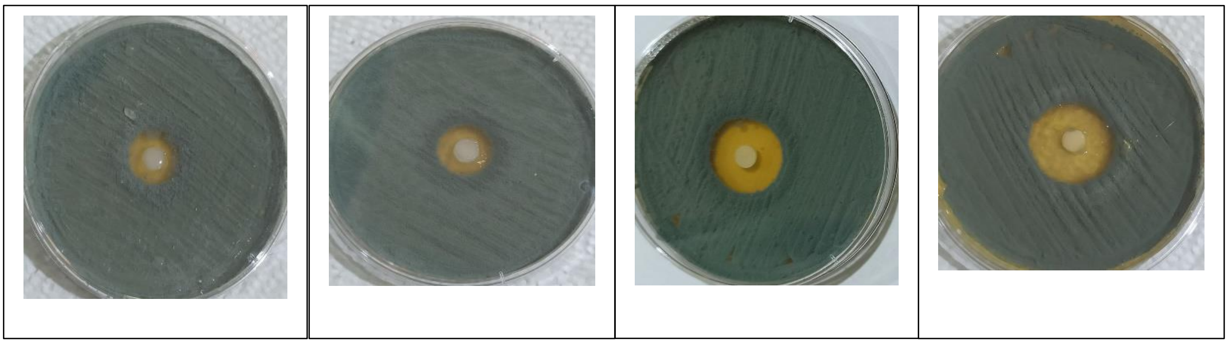
The study's findings, which are shown in Tables (3) and Figure (3), showed that the development of *A. fumigatus* was considerably impacted by the combination of essential oil and clotrimazole as opposed to the combination of essential oil and clotrimazole alone. The effect was also concentration-dependent. Zones of inhibition against the tested fungus were seen when clotrimazole and essential oil were combined; these values varied from (23.50 mm to 32.33 mm). Comparing the essential oil instance at 100 % concentration with clotrimazole (5µg/ml) to that of clotrimazole alone (13.33mm), the highest restraint zone was obtained at (32.33mm). This was followed by (29.50mm) and (23.50mm) at 50% and 25% concentrations, respectively. Based on statistical research, clotrimazole alone and all combo treatments differ significantly from one another.

Table 3: Effect of clotrimazole with essential oil on growth of *Aspergillus fumigatus* by disc diffusion method

Treatments	Concentrations	Inhibition zon(mm)
Essential oil	100% oil + clotrimazole -5 µg/ml	32.33
	50 % oil + clotrimazole -5 µg/ml	29.50
	25 % oil + clotrimazole -5 µg/ml	23.50
Clotrimazole	Clotrimazole - 5 µg/ml alone	13.33
Essential oil	Oil 100% alone	26.33
Essential oil	Oil 50% alone	12.33

Essential oil	Oil 25% alone	7.00
Control		0
LSD 0.05		1.011

Each value is a mean of three replicates



a b c d

Figure (3): Effect of essential oil with clotrimazole on *A.fumigatus*

a- Clotrimazole alone (5mg/ml) , b- Cotrimazole with oil 25%, c- Cotrimazole with oil 50% , d- Clotrimazole with oil 100%

The results also showed that the combination between antifungal drug clotrimazole and the essential oil exhibited an additive effect because the value of chi squared ($\chi 2$) was lower than the tabulated value (3.84) Table 4.

Table (4) Chi squared ($\chi 2$) test to determine the type of interaction between clotrimazole and *essential oil* against A. *fumigatus*

Treatment and Concentrations		**Inhibition zone (mm)**				**Calculated $\chi 2$**	**Type of Interaction**
Essential oil 100%	**clotrimazole 5µg/ml**	**Essential oil (alone)**	**clotrimazole (alone)**	**Expected**	**Observed (Combin.)**		
		26.33	**13.33**	**36.15**	**32.33**	**0.40**	**Additive**

The outcome shown that the combination of clotrimazole and essential oil produced superior inhibitory zones against fungus. This may be because the oil contains physiologically active phytochemicals that enhance clotrimazole's antifungal properties. Around the world, the primary treatment for many medical conditions is the combination and synergistic interaction of antimicrobial medicines with numerous herbal formulation of distinct natural bioactive chemicals (Zhou *et al*., 2016). Triazoles were tested by Sabatelli *et al*., (2006) against a variety of clinically significant pathogenic growths, including yeast and mould. They discovered that *Aspergillus* and *Candida* species showed resistance to azole antifungals, so consolidating commercial antifungal medications with naturally happening bioactive compounds from plants will be a complementary and alternative strategy to combat resistance and recurrent incidences of fungal infection.

Research indicates that the oils from *Eucalyptus citriodora* have synergistic effects with the antifungal medication nistatin, and this combination may boost nistatin's antimicrobial efficacy against skin conditions (Kirui, 2014). Pereiraa *et al*., (2014) investigated the impact of consolidating *Eucalyptus* essential oil with an antibiotic against *Pseudomonas aeruginosa*. Their findings indicated that in 55% of the cases, the combination of the antibiotic and essential oil showed an additive effect, indicating an increase in antibacterial activity. The use of *Eucalyptus globulus* components (oils and extracts) may be a valuable source of bioactive chemicals and a viable therapy choice for *P. aeruginosa*-related respiratory infectious illnesses. The antibacterial activity of two *Eucalyptus camaldulensis* essential oils was assessed by Knezevic P *et al.*, (2016) against a number of clinical isolates that were resistant to medications for *Acinetobacter baumanii.* Using all three tested antibiotics - ciprofloxacin, gentamicin, and polymicin B- essential oils demonstrated both synergistic effects and antibacterial action.

A study by Bohmova *et al*., (2019) assessed the synergistic interactions between essential oils of *Syzygium aromaticum* and the conventional antifungal medication clotrimazole against isolates of *Malassezia pachydermatis*. The results suggested that clotrimazole could be more effective when used in combination with essential oils. The synergistic effects of *Eucalyptus* oil and antibiotics were investigated by Cui *et al*., (2021) and Iseppi *et al*., (2021). All studies concluded that the combined action of the two agents was more antibacterial than either agent working alone against resistant bacteria. A related study discovered that antifungal cream and turmeric essential oil, *Aloe vera* gel, and *Candida* spp. have a synergistic effect against *Aspergillus* spp. and *Candida* spp. Additionally, the combination of antibiotic creams and natural plant products is a promising approach for developing new antifungal agents (Ogidi *et al.,* 2021). Ahmad *et al*., (2023) discovered that the antifungal activity of ketoconazole against *Candida albicans* is enhanced by clove and *Eucalyptus* oils, and that the combined action is greater than that of ketoconazole alone. According to Arun *et al*., (2024), *Eucalyptus globulus* essential oil increases the azole compound's antifungal efficacy against *Aspergillus* and *Mucor* spp.

4. Conclusion

In this study, we investigate the antifungal activity of essential oil of *Eucalyptus camendulensis* plant and antifungal drugs (clotrimazole, itraconzole and griseofulvin) against aspergillus spp. that isolated from patients. We found that the essential oils of *E. camendulensis* plant showed an interesting biological activity on *Aspergillus* spp . Antifungal studies have proven the effectiveness of essential oils alone and in combination with antifungal clotrimazole against A. *fumigatus*. Therefore, it can be used the essential oils of *E. camendulensis* to enhance the activity of antifungal drugs against *Aspergillus* spp ,

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