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Research Article

Investigating the antifungal effects of Carvacrol and 1,8-cineole on *Candida albicans, Aspergillus flavus, Trichoph yton rubrum* and *Epidermophyton flucosum*

Behnoush Salami Naserian[1], Mohammad Hadi Fakoor[2], Azar Sabokbar[1*], Somayeh Talebi[3]

1. Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, Iran.
2. Department of Microbiology, Hidaj Branch, Islamic Azad University, Hidaj, Iran.
3. Department of Microbiology, Shahre-Qods Branch, Islamic Azad University, Tehran, Iran.

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ABSTRACT

There are many fungal agents that can infect and clean the skin. The aim of this study was to observe the antifungal effect of Carvacrol and 1.8 cineole on fungi. In this descriptive-cross-sectional study, Carvacrol and 1.8 cineol essential oils were supplied by the microbial collection of the Scientific and inisitue Research Organization of Iran. The fungal strains *Candida albicans*, *Aspergillus flavus*, *Trichophyton rubrum*, and *Epidermophyton flucosome* were used in this study. Agar diffusion and macrodilution broth were used. Statistical tests were used to investigate the antimicrobial effect of Carvacrol essential oil and 1.8 cineole. Combination effect of carvacrol and 1,8 cineol against fungal species by disc diffusion method showed that these compounds had the greatest effect on Candida and carvacrol was a more effective than 1,8 cineol. A synergistic study of carvacrol with fluconazole, ketoconazole, and terbinafine showed stronger effects against fungal species. The amount of MIC and MFC obtained from carvacrol against *Candida albicans*, *Aspergillus flavus, Trichophyton rubrum* and *Epidermophyton flucosome* was 250, 125 – 62.5 - 125 and 62.5 and 125,62.5 ppm and this amount for 1,8 cineole, respectively, is 15, 7.8 - 15, and 7.8 -31, 7.8 - 31 and 7.8 ppm. In order to the side effects and high toxicity of common antifungals, it is recommended to use herbal compounds in combination with these antifungals and by conducting clinical studies, toxic effects, pharmacological and its side effects should also be considered.

1. Introduction

Fungal infections have been increasing in recent years due to the increasing number of high-risk patients, especially immunocompromised patients (Pinto et al., 2009). Dermatophytosis are common infections caused by members of the genus Candida and filamentous fungi, especially dermatophytes. Superficial candidiasis and dermatophytosis can be very serious in immunocompromised patients (Yin et al., 2013; Krom et al., 2014). Also, the World Health Organization has estimated that 55 million people died in the world in 2011, and one third of this population was responsible for the death of infectious diseases, and also, this statistic can be due to the presence of

*Corresponding author: Azar Sabokbar
E-mail address: sabokbar@kiau.ac.ir

microorganisms that are resistant to the drugs were used (Varga et al., 2015).

Cutaneous fungal infections caused by the Trichophyton, Microsporium and Epidermophyton are among the most important not resolved public health problems (Sudbery et al., 2004). Trichophyton species such as T. mentagrophytes, T. itenteridigital and T.rubrum are known for causing many skin disorders in humans (Seebacher et al., 2008). Varies of clinical presentation observed from mild scaling to acute inflammation. Some species have acquired resistance to certain antifungal drugs. Therefore, explore to new treatment options are needed. Antifungal treatment based on azoles and terbinafine are actually very good drugs for acute dermatophytosis, but the results of treatment depended on the size and location of the complication, the causative factor and the patient's health status are very diverse (Mandras et al., 2016; Nenoff et al., 2007).

Despite the introduction of new antifungal drugs, they are still limited. Increasing fungal resistance to drugs, expensive treatment, and the fact that most available antifungal drugs have only static activity and are not lethal emphasize the research for new strategies (Escribano et al., 2011; Marchese et al., 2016). The common occurrence of skin infections by dermatophytes and the limited number of antifungal drugs that are effective against them prompt researchers to search for new antifungal agents (Fani et al., 2011). Recently, the frequency of skin fungal infections has increased. However, some modern antifungal treatments still cause significant side effects in some patients. In other cases, resistance to treatment with certain drugs, in addition to the need to prolong the treatment period, has been reported (Elaissi et al., 2012). It has also been proven in recent research that carvacrol and 1,8-cineole have good antimicrobial effects on various bacteria and also *C. albicans* (Vardar-Ünlü et al., 2010; Sujono et al., 2019). However, it seems that carvacrol was more effective (Chami et al., 2004).

In 2004, Takahashi et al. investigated the antimicrobial and antifungal properties of 26 Eucalyptus species. They showed that the extracts of Eucalyptus globus, E. maculata, and Eucalyptus vamilanis significantly inhibited the growth of T. mentagrophytes, but these extracts did not show strong antibacterial activity against Gram-negative bacteria such as Escherichia coli (Takahashi et al., 2004). In 2017, İşcan et al. investigated the antibacterial and anti-candidal activity of common compounds against 21 bacterial pathogens and Candida species using the standard dilution broth method (CLSI-M7-A7 and M27-A2). In total, the tested compounds showed a better inhibitory effect on Candida species than bacteria. The most effective microbial growth inhibiting components were determined as carvacrol, thymol, cumin alcohol, terpinen-4-ol, α-terpineol, levantolol, estragole and thymoquinone (İşcan, 2017).

The purpose of this study was to investigate the antifungal effect of two compounds Carocrol and 1, 8-cineole on Candida, Trichophyton, Epidermophyton and Aspergillus, which has not been researched so far.

2. Materials and Methods

2.1. Preparation of fungal samples used in experiments

Fungal strains T. rubrum PTCC 12, E. floccosum PTCC 6, A. flavus PTCC 29, C.albicans PTCC 18 were prepared from the microbial collection of the Scientific and Industrial Research Organization of Iran. Tests such as morphological examination (microscopic and macroscopic) were used to confirm the identity of dermatophytes, Candida and filamentous strains.

2.1.1. Macroscopic observation

Candida colonies appeared after 1-2 days, dermatophyte colonies after 14 days, and Aspergillus colonies after 1 week (for sporulation) (Fakoor et al., 2012). The growth period, color of the back and top of the colony, the surface of the colony, the microscopic shape of the aerial mycelium and other characteristics of the colonies were investigated.

2.2. Methods of preparing fungal suspension solution

Dermatophyte, Candida and Aspergillus fungi were cultured in Sabouraud dextrose agar medium with chloramphenicol and cyclohexamide inside the test tube in a slanted manner and incubated in a refrigerated incubator at 30 ˚C for 1 to 2 weeks. After Candida growth,

fungal suspension was prepared. Then, the concentration of the suspension was prepared using a spectrophotometer at a wavelength of 760 nm, the amount of light transmitted or transition was 75-77%, so that the concentration of the suspension was equal to 1-4× 10^4 CFU/ml. In order to prepare filamentous fungi, water and PST (Physiological Salt) solution were added to the tube of the medium and then vortexed to separate the spores completely. Then, the material containing spores was read with a spectrophotometer until the concentration of 1-4× 10^6 CFU/ml was obtained. The final suspension was prepared to perform a drug sensitivity test and MIC test based on CLSI (Mohammadpour et al., 2012). Then, a separate control from the desired substance for each fungus (positive control well: fungus without drug; RPMI medium + fungal suspension - negative control well: drug without fungus; RPMI medium + diluted stock drug - DMSO control well: fungal suspension + DMSO) It was cultured and according to the international recipe of the laboratory, the reading was performed after 48 hours (Rasooli et al., 2008).

2.3. Preparation of carvacrol compound, 1,8 cineol

In order to use the composition of carvacrol (98%, Merck) and 1,8 cineol (99%, Merck), their ready solution was prepared from the market.

2.4. Preparation of medicinal dilutions

To prepare medicinal stock based on the recommended CLSI standard, fluconazole powder (prepared with a concentration of 64 µg/ml) and ketoconazole was weighed using a sensitive scale to weigh 0.1 g of the powder with 1 ml of dimethyl sulfoxide or DMSO as a solvent added to eliminate possible contamination and was kept as a primary stock at -20 degrees Celsius until use. To use terbinafine drops, 1 microliter of the drop was dissolved in 100 microliters of dimethyl sulfoxide (Sarkhani Moghaddam et al., 2018; Zhang et al., 2011).

2.5. Examining the antifungal effects of the isolates

The antifungal activity of carvacrol and 1,8 cineole against dermatophyte, Candida and Aspergillus fungi was studied. Disk diffusion and macrodilution methods were used to investigate the antagonistic effects and antifungal production (Rasooli et al., 2008).

2.5.1. Investigating the antifungal effect of the combination of carvacrol and 1,8 cineol by disc diffusion method

In this research, Sabouraud dextrose agar medium containing cyclohexamide and sterile chloramphenicol was used. Fungal suspensions were prepared and 100 microliters of each of the fungal suspensions was cultured separately on the surface of the medium by a sterile swab. The amount of 5, 10, and 15 microliters of carvacrol and 1,8 cineol combined or combined extract was poured on the blank disks. After 7-14 day incubation time at 30 °C and 3 repetitions for each extract, the average of inhibition zone diameter was measured (Rasooli et al., 2008). In order to more accurately compare and evaluate the accuracy of the results of these two compounds on different fungal species, the prepared antifungal was used by disk diffusion method (Zargani Nejad et al., 2019). For this purpose, terbinafine drops were used to evaluate the anti-candidal effects of fluconazole powder with a concentration of 64 µg/ml and the amount of 0.012 grams, and to evaluate the anti-aspergillosis effects of ketoconazole powder, and to evaluate the antifungal effect of T. rubrum and E. flucosum species. Finally, the synergistic effect between carvacrol and species-specific antifungal was investigated in the same way.

2.5.2. Determining the minimum growth inhibitory concentration (MIC) by macrodilution method

In this research, RPMI1640 medium was used to determine MIC. The procedure is that 10 cc of medium was poured into each tube and different concentrations of carvacrol and cineol were prepared. 100 microliters of fungal suspension with a concentration of 1 × 10^4 CFU/ml for filamentous fungi and 1 × 10^3 CFU/ml added for Candida. For further investigation, positive control (without carvacrol and cineole), negative control (medium without

fungus) and DMSO control well were prepared for each sample and were incubated at 30 ˚C. The changes and growth of fungi were investigated during 7 to 14 days. The lowest concentration that prevented the growth of the fungus was considered as the MIC of the antifungal agent (Rasooli et al., 2008). This test was performed three times for each fungal isolate in separate tubes. To prepare the drug stock based on the recommended standard, CLSI (M100, 30th ed. January 2020) the powder of fluconazole prepared with a concentration of 64 µg/ml dimethyl sulfoxide (DMSO). Ketoconazole was weighed 0.1 g of the powder with 1 ml of dimethyl sulfoxide(DMSO) added. It was kept at -20 degrees Celsius as a primary stock until use. To use terbinafine drops, 1 microliter of the drop was dissolved in 100 microliters of dimethyl sulfoxide.

2.5.3. Determination of Minimum Fungicidal Concentration (MFC)

According to the definition, MFC is the lowest concentration of antifungal activity in which 99.9% of microorganisms are killed. To determine the MFC, using a sampler, remove from the MIC tube and the tube after the MIC that is growing and the tubes before the MIC and put it Sabouraud dextrose agar and after culture for 1-2 weeks, any dilution in the plate prevents complete growth or there were less than 3 colonies, it was considered as MFC (approximately equivalent to 99-99.5% of killing activity) (Mohammadpour et al., 2012).

3. Results

3. 1.The results of drug investigation on different species by disc diffusion method

3.1.1. The results of investigating the anti-candidacy effect of carvacrol and 1,8 cineol compound by disk diffusion method

In the disk diffusion method to determine the sensitivity of carvacrol, the results showed that the best amount to inhibit the growth of *C. albicans* is 10 and 15 microliters, and complete inhibition of growth was seen. Figure 1 also shows the trend of concentration changes over time. In the disk diffusion method to determine the sensitivity of 1.8 cineol, the results showed that the highest average of inhibition zone diameter observed after 4 days was 32mm. Therefore, no significant difference was observed between any of the groups with different volumes average, and there was no difference between different volumes in terms of inhibition of lack of growth. Figure 1 also shows the trend of growth changes over time.

3.1.2. The results of investigating the anti-candidal effect of fluconazole and the combination of carvacrol and fluconazole by disk diffusion method

The results of this study indicated that with the amount of 15 microliters of fluconazole as the only volume used (which was prepared with a concentration of 64 µg/ml), the inhibition zone diameter was 32 mm after the third day, only in the first 3 days of growth completely. The fungus was prevented. Since the effect of carvacrol was stronger than 1, 8 cineol, the synergy of carvacrol and fluconazole was performed, which has far more potential effects than the effect of fluconazole alone on C. albicans. Figure 2 shows the changes in the growth of C. albicans with fluconazole and with the combination of carvacrol and fluconazole over time.

3.1.3. Investigating and comparing the effect of different drugs (carvacrol, 1,8 cineole, fluconazole, synergy of carvacrol and fluconazole) on C. albicans

The results of Table 1 show that the probability value is less than 0.05, so there is at least a significant difference between the two drugs, and the highest average inhibition zone diameter is related to carvacrol, and this drug has the highest and 1.8 cineol the lowest effect on Candida species. To further investigate the differences, post hoc test with Bonferroni correction was used, the results of which are shown in Table 2. According to table 2, it can be seen that there is a significant difference between the drugs carvacrol and 1,8 cineol. But no significant difference was observed between other drugs.

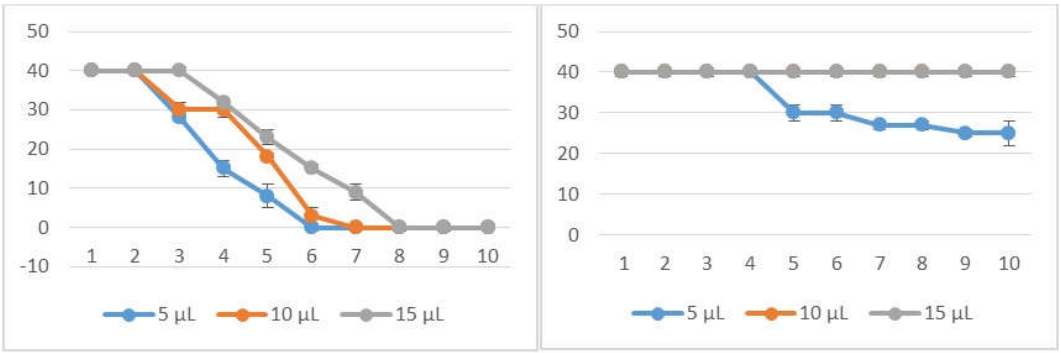

Figure 1. Changes in the growth of *C. albicans* with carvacrol (right side) and with the 1,8 cineol over time (left side)

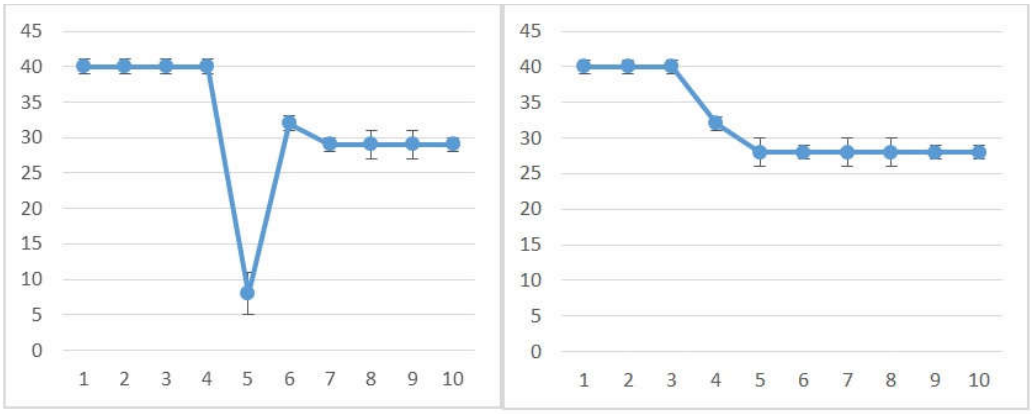

Figure 2. Changes in the growth of *C. albicans* with fluconazole (right side) and with the combination of carvacrol and fluconazole over time (left side)

Table 1. Comparison of the effects of different drugs, carvacrol, 1,8 cineole, fluconazole, synergy of carvacrol and fluconazole on C. albicans

drug	inhibition zone diameter (mm)	standard deviation	The result of the Kruskal-Wallis test
Carvacrol	37.47	5.24	p-*Value*<0.001
Cineol	16.36	16.84	
Fluconazole	32	5.65	
Carvacrol and fluconazole	31.60	9.81	

Table 2. Pair-by-pair comparison of the effects of drugs on Candida albicans

Drug comparison	The result of post hoc test with Bonferroni correction
Carvacrol-cineol	p-*Value*<0.001
Carvacrol-Fluconazole	p-*Value*=0.330
Carvacrol- Carvacrol and fluconazole	p-Value=0.980
Cineol-Fluconazole	p-Value=0.52
Cineol-carvacrol and fluconazole	p-Value=0.152
Fluconazole- Carvacrol and Fluconazole	p-Value=1.00

3.1.4. The results of investigating the anti-aspergillosis effect of the combination of carvacrol and 1, 8 cineole by disk diffusion method

Figure 4 shows the trend of growth changes over time. As can be seen, the results showed that the best amount to inhibit the growth of A. flavus was 15 microliters and the highest inhibition zone diameter mean 34.70 and 40 mm (non-growth) was observed. The highest inhibition zone diameter mean against 1.8 cineol was 12.6 mm. Figure 4 also shows the trend of changes in growth over time and the inhibition zone diameter in all volumes has decreased over time.

3.1.5. The results of investigating the anti-aspergillosis effect of ketoconazole and the combination of carvacrol and ketoconazole by disk diffusion method

The results of this study indicated that with the amount of 15 microliters of ketoconazole, the inhibition zone diameter was 35 mm after the second day. Inhibiting the complete growth of the fungus in the same amount was observed using carvacrol, which indicates that this compound is more effective than ketoconazole against A. flavus. Figure 5 shows the decreasing trend of the aura of lack of growth over time. In the next step, two compounds of carvacrol and ketoconazole were synergized, which had far more potential effects than each of the compounds individually on A. flavus, so that no growth was observed. The result of the Mann-Whitney test showed that there was no significant difference between the average of these two groups with different volumes at the level of 0.05 (p-Value>0.05). Figure 5 shows the growth trends of the two groups over time, which has remained constant over time.

3.1.7. Investigation and comparison of the effect of carvacrol, 1,8 cineol, ketoconazole and synergy of carvacrol and ketoconazole on A. flavus

Kruskal-Wallis test was used to compare the effect of drugs and then post hoc test with Bonferroni correction was used to compare two by two drugs. Table 3 shows that the probability value is significant at the 0.05 level. Therefore, there are at least 2 drugs whose effects are significantly different from each other. Based on this table, cineole had the least effect and carvacrol and the combination of carvacrol and ketoconazole had the most effect on A.flavus. A post hoc test was used to compare two by two drugs, the results of which are shown in Table 4. Table 4 shows that there is a significant difference between carvacrol and cineole, carvacrol with the combination of carvacrol and ketoconazole, cineole with ketoconazole, and cineole with the combination of carvacrol and ketoconazole.

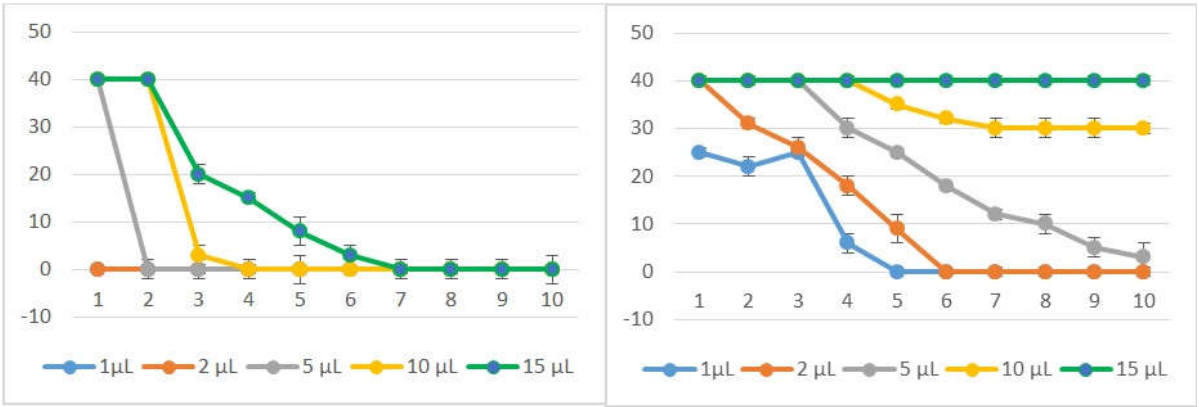

Figure 3. Changes in the growth of A. flavus with carvacrol (right) and cineole 1 and 8 over time (left)

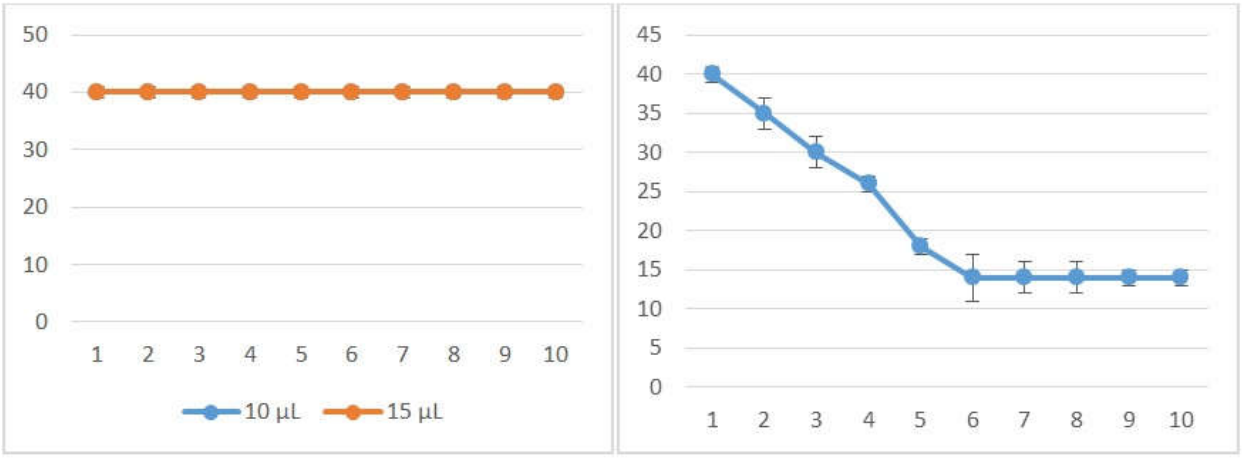

Figure 4. Changes in the growth of A. flavus with ketoconazole (right) and with the combination of carvacrol and ketoconazole over time (left)

Table 3. Comparison of the effect of different drugs (carvacrol, 1,8 cineol, ketoconazole and synergy of carvacrol and ketoconazole) on A. flavus

drug	inhibition zone diameter (mm)	standard deviation	The result of the Kruskal-Wallis test
Carvacrol	23.44	16.36	p-*Value*<0.001
Cineol	4.98	12.34	
Ketoconazole	21.90	10.05	
Carvacrol and ketoconazole	40	0.00	

Table 4. Pair by pair comparison of drug effects on A. flavus

Drug comparison	The result of post hoc test with Bonferroni correction
Carvacrol- Ketoconazole	p-*Value*=0.001
Carvacrol- Carvacrol and ketoconazole	p-*Value*=0.002
Cineol- Ketoconazole	p-Value=0.024
Cineol-carvacrol and ketoconazole	p-*Value*<0.00
Ketoconazole - Carvacrol and Ketoconazole	p-Value=0.065

3.1.8. The results of investigating the combination of carvacrol and 1, 8 cineole on T. rubrum by disk diffusion method

In this method to determine the sensitivity of carvacrol, using amounts of 2, 5, 10 and 15 microliters, the results showed that the best amount to inhibit the growth of T. rubrum is the amount of 10 and 15 microliters, and the largest inhibition zone diameter 35 mm was seen. According to the result of the Kruskal-Wallis test, it can be seen that the probability value is less than 0.05 (p-Value<0.05). Figure 6 shows the trend of growth changes over time. The results of zinc 1,8 cineole showed that this fungus was less sensitive to it and growth was seen in all amounts after the first day. According to the result of the Kruskal-Wallis test, it can be seen that the probability value is more than 0.05 (p-Value> 0.05). Therefore, no significant difference was observed between the groups with different volumes in terms of average, and the largest inhibition zone diameter mean was observed with a size of 12.30 mm and a volume of 15 µL. Figure 6 also shows the trend of growth changes over time, which has a decreasing trend.

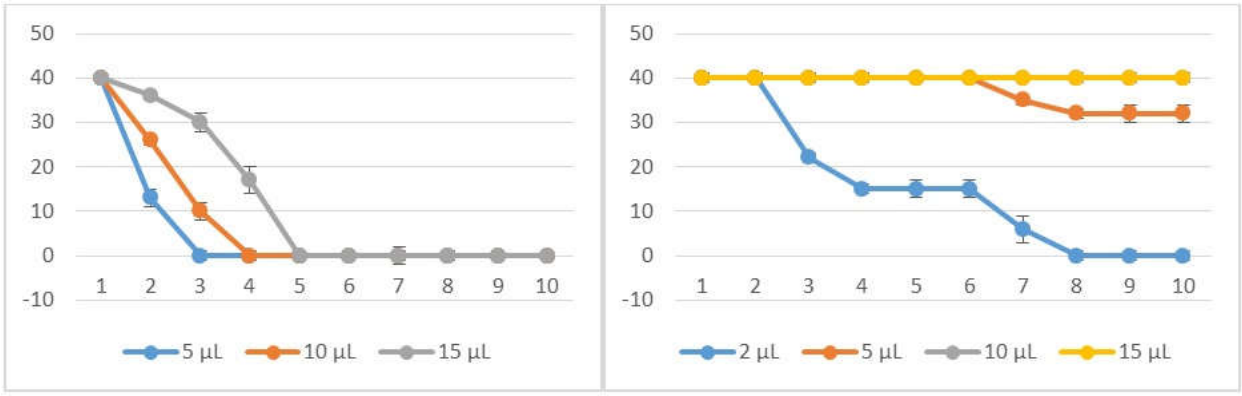

Figure 5. Growth trends of *T. rubrum* with carvacrol (right) and 1, 8 cineol (left) over time

3.1.9.The results of terbinafine and combination of carvacrol and terbinafine on T.rubrum by disk diffusion method

To evaluate the reliability of the results, the antifungal effect of terbinafine was investigated as a positive control. The results of this study indicate that complete growth inhibition was observed with 15 microliters of terbinafine. Then the synergy of two compounds of carvacrol and terbinafine, which also had much stronger effects on T. rubrum, so that no growth was observed. The result of the Mann-Whitney test showed that there was no significant difference between the average of these two groups at the level of 0.05 (p-Value>0.05). Figure 7 shows the growth trends of the two groups over time. An example of the growth and non-growth halo of this fungus against the combination of terbinafine and carvacrol is shown in Figure 7.

3-1-10. Investigating and comparing the effect of different drugs (carvacrol, 1,8 cineol, terbinafine and the combination of carvacrol and terbinafine) on T. rubrum

Kruskal-Wallis test was used to compare the effect of drugs and then post hoc test with Bonferroni correction was used to compare two by two drugs. The results of Table 5 show that at least there was a significant difference between the two drugs, and carvacrol was more effective than 1,8 cineol, and the results of combining it with terbinafine also prevented complete growth. A post hoc test was used to compare two by two drugs, the results of which are shown in Table 6. According to the results of Table 6, it can be seen that there is a significant difference between carvacrol and 1.8-cineole, 1.8-cineole and terbinafine, 1.8-cineole and the combination of carvacrol and terbinafine.

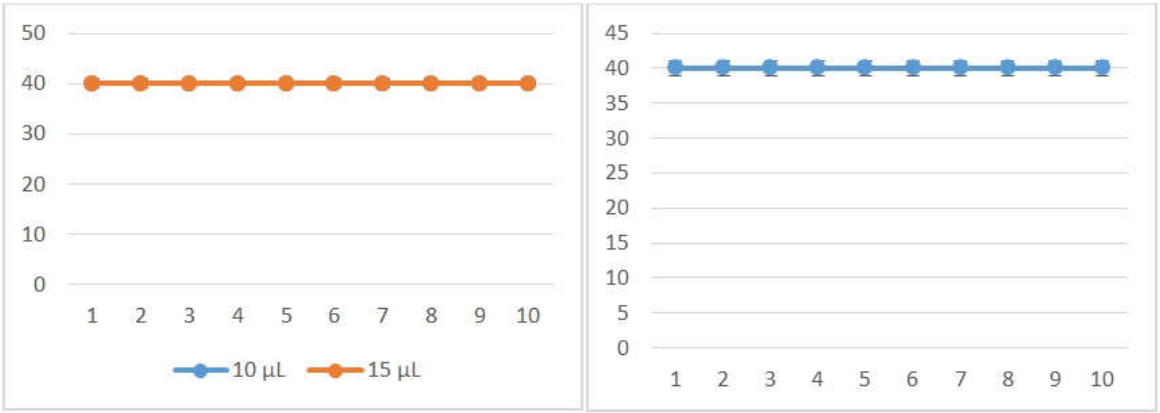

Figure 6. Changes in the growth of T. rubrum under terbinafine (right) with the combination of carvacrol and terbinafine in two groups (left) over time

Table 5. Comparison of the effect of different drugs (carvacrol, 1,8 cineol, terbinafine and the combination of carvacrol and terbinafine) on T. rubrum

drug	inhibition zone diameter (mm)	standard deviation	The result of the Kruskal-Wallis test
Carvacrol	33.10	12.88	p-*Value*<0.001
Cineol	8.40	14.52	
Terbinafine	40	0.00	
Carvacrol and terbinafine	40	0.00	

Table 6. Pair-by-pair comparison of drug effects on Trichophyton rubrum

Drug comparison	The result of post hoc test with Bonferroni correction
Carvacrol-1,8 cineol	p-*Value*<0.001
Carvacrol-terbinafine	p-*Value*=0.910
Carvacrol- Carvacrol and terbinafine	p-Value=0.385
1,8 cineol-terbinafine	p-*Value*<0.001
1,8cineol-carvacrol and terbinafine	p-*Value*<0.001
Terbinafine - Carvacrol and terbinafine	p-Value=1.00

3.1.11. The results of investigating the combination of carvacrol and 1, 8 cineol on E. flucosum by disk diffusion method

To determine the sensitivity of carvacrol using amounts of 2, 5, 10 and 15 microliters, the results showed that the best amount to prevent the growth of E. flucosum is the amount of 10 and 15 microliters, and the largest halo of non-growth was seen with a diameter of 30mm. According to the result of the Kruskal-Wallis test, it can be seen that the probability value is less than 0.05 (p-Value<0.05). Figure 8 shows the trend of growth changes over time. Also, the results of 1, 8 cineol investigation showed that this fungus was less sensitive to carvacrol and growth was seen in all amounts. The highest inhibition zone diameter was observed 25 mm after 2 days with the addition of 15 microliters of the compound to the disc. According to the result of the Kruskal-Wallis test, it can be seen that the probability value is less than 0.05 (p-Value<0.05). Figure 7 also shows the trend of growth concentration changes over time.

3-1-12. The results of examining terbinafine and the combination of carvacrol and terbinafine on E. flucosum by disk diffusion method

The results of this study indicate that complete growth inhibition was observed with 15 microliters of terbinafine. Figure 8 shows the trend of concentration changes over time. Then the synergy of two compounds of carvacrol and terbinafine took place, which had much stronger effects than each of the compounds individually on this fungus, and no growth was observed. The result of the Mann-Whitney test showed that there was no significant difference between the average of these two groups at the level of 0.05 (p-Value>0.05). Figure 8 shows the trend of growth changes over time.

3.1.13. Investigating and comparing the effect of different drugs (carvacrol, 1,8 cineol, terbinafine and the combination of carvacrol and terbinafine) on E. flucosum

Kruskal-Wallis test was used to compare the effect of drugs and then post hoc test with Bonferroni correction was used to compare two by two drugs. The results of Table 7 show that the test result is significant at the 0.05 level. It means that there is a significant difference between at least two drugs and carvacrol was the most effective drug against E.flucosum. In order to determine which pairs of drugs have significant differences with each other, a post hoc test with Bonferroni correction was used,

the results of which are shown in Table 8. Table 8 shows that there was no significant difference between carvacrol and terbinafine, carvacrol with carvacrol and terbinafine, terbinafine with carvacrol and terbinafine, but other drugs had significant differences with each other.

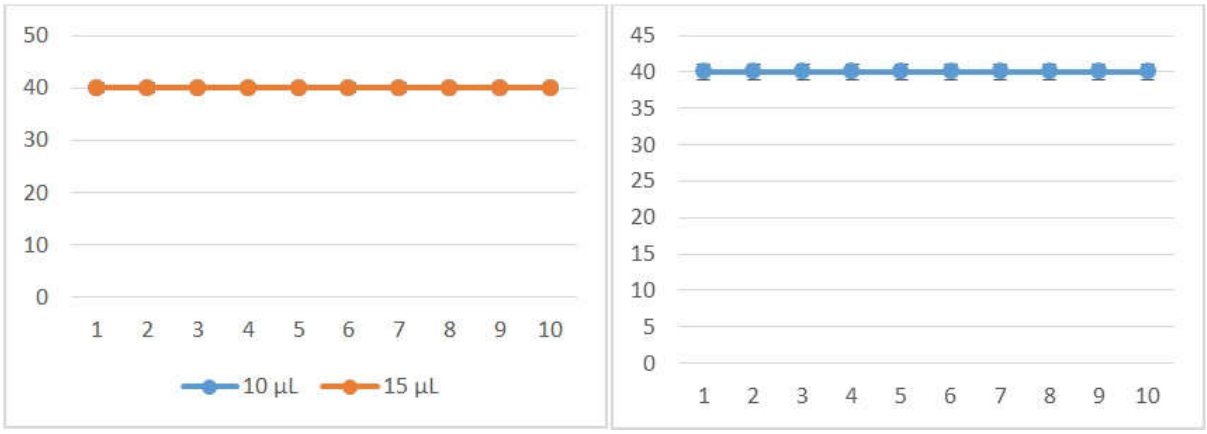

Figure 7. Changes in growth of *E. flucosum* with carvacrol (right) and 1,8-cineol (left) over time

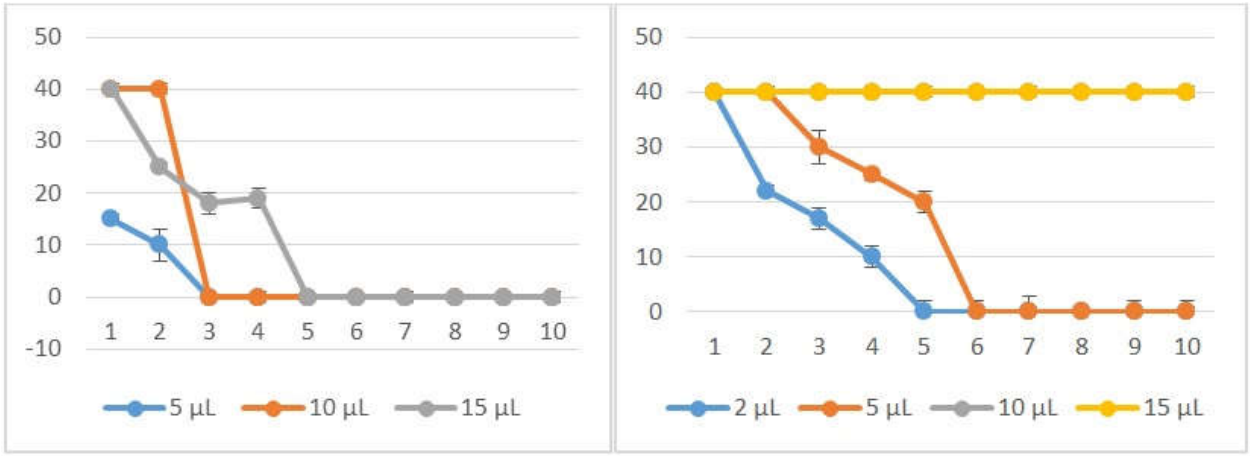

Figure 8. Changes in the growth of *E. flucosum* with terbinafine (right) and the combination of carvacrol and terbinafine (left) over time.

Table 7. Comparison of the effect of different drugs (carvacrol, 1,8 cineole, terbinafine and the combination of carvacrol and terbinafine) on E. flucosum

drug	inhibition zone diameter (mm)	standard deviation	The result of the Kruskal-Wallis test
Carvacrol	26.10	17.79	p-*Value*<0.001
Cineol	6.90	13.14	
Terbinafine	40	0.00	
Carvacrol and terbinafine	40	0.00	

Table 8. Pair-by-pair comparison of drug effects on Epidermophyton flucosum

Drug comparison	The result of post hoc test with Bonferroni correction
Carvacrol-terbinafine	p-*Value*=0.161
Carvacrol- Carvacrol and terbinafine	p-*Value*=0.026
1,8 cineol-terbinafine	p-*Value*<0.001
1,8cineol-carvacrol and terbinafine	p-*Value*<0.001
Terbinafine - Carvacrol and terbinafine	p-Value=1.00

3.2. The results of examining the composition of carvacrol, 1, 8 cineole by macrodilution method on the species used

In the macrobroth dilution method to determine the sensitivity of the combination of carvacrol and 1, 8 cineol against 4 isolates of C. albicans, A. flavus, T. rubrum and E.flucosum, it showed that the MIC and MFC obtained for C. albicans isolates for carvacrol were 6400 and 3200 ppm, and For 1, 8 cineol, it was 600 and 400 ppm, respectively. While the MIC and MFC levels for A. fumigatus isolate with carvacrol effect were 1600 and 800 ppm and for 1, 8 cineol were 600 and 400 ppm. The MIC and MFC of carvacrol for E.flucosum were 3200 and 1600 ppm, respectively, and for 1, 8 cineol, 800 and 400 ppm (Table 9).

Table 9. The results of investigating the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of different concentrations of carvacrol and 1, 8 cineole for the used fungal species.

			Dilutions used							
Species name	PPM compound concentration		200	400	600	800	1600	1250	3200	6400
Candida albicans	Carvacrol	Tube	-	-	-	-	-	-	-	+
		Plate	-	-	-	-	-	-	+	+
	1, 8 Cineol	Tube	-	-	-	+	+	+	+	+
		Plate	-	-	-	+	+	+	+	+
Aspergillus flavus	Carvacrol	Tube	-	-	-	-	-	+	+	+
		Plate	-	-	-	-	-	+	+	+
	1, 8 Cineol	Tube	-	-	-	+	+	+	+	+
		Plate	-	-	-	+	+	+	+	+
Trichophyton rubrum	Carvacrol	Tube	-	-	-	+	+	+	+	+
		Plate	-	-	-	-	-	+	+	+
	1, 8 Cineol	Tube	-	-	-	-	+	+	+	+
		Plate	-	-	-	+	+	+	+	+
Epidermophyton flucosum	Carvacrol	Tube	-	-	-	-	-	-	+	+
		Plate	-	-	-	-	-	+	+	+
	1, 8 Cineol	Tube	-	-	-	-	-	+	+	+
		Plate	-	-	-	+	+	+	+	+

4. Discussion

Today, the number of people suffering from fungal diseases is increasing. Superficial mycoses diseases are increasing in the world's population at the present time and more than 20-25% of the world's population are suffering from it. Social life, contact with animals, corticosteroids, anticancer drugs and some other factors contribute to the increase of infections (Gräser et al., 2008). Fungi are eukaryotic in nature, so it is not easy to obtain antifungal drugs with selective toxicity on the host. Most of the antifungal drugs used in medicine have side

effects, and a large number of antifungal substances are obtained from microorganisms that have toxic effects on humans. Also, antibiotic resistance is a serious threat to human health, especially with weak immunity, which requires action to find cheap and effective antimicrobial substances (Ewais et al., 2014).

The spread of opportunistic fungal diseases in susceptible people the increasing drug resistance and, on the other hand, the adverse effects of chemical antifungal drugs have caused interest in research on the antifungal effects of carvacrol and 1,8 cineole (Lima et al., 2013; Langeveld et al., 2014). Researchers have conducted many studies to investigate antifungal drugs of herbal origin. In recent years, the identification of plants bioactive components for different medicinal purposes, many efforts have been made on Trichophyton depending on the type of plant species, the phytochemical compounds in the composition and extracts are different and they show many antimicrobial effects (İşcan 2017).

In this regard, the present study has been determined the antifungal activity of carvacrol, 1,8 cineol and the combined effect of carvacrol with ketoconazole, fluconazole and terbinafine antifungals on four species of C. albicans, A. flavus, T. rubrum and E. flucosum by disc diffusion and macrodilution methods. The results of this study using Kruskal-Wallis, Mann-Whitney statistical tests and post hoc test with Bonferroni correction showed that carvacrol had the most effect against Candida alicense, T. rubrum, E. flucosum and A.s flavus, respectively. The average growth halo in them is 37.47, 33.10, 26.10 and 23.44 mm. MIC results by macrodilution method showed that A. flavus, T. rubrum and E. flucosum and finally C.albicans were more sensitive respectively (ppm 1600, 3200, 3200, 4800 respectively). As the results of this study show, A. flavus was inhibited by a lower concentration of carvacrol, which means it is more sensitive, while C. albicans had the highest level of resistance.

In the study of Saad et al. in 2010, they investigated the anti-candidal effect of Thymus maracanus and Thymus brusonti extracts, which have significant amounts of carocarol, and their synergy with amphotericin B and fluconazole. The MIC of these two compounds along with amphotericin B and fluconazole mg/ml were 0.49, 0.27, 0.37 and 0.3, respectively. In addition, the results showed that the synergistic effect of combinations with fluconazole is stronger than its combination with amphotericin B (Saad et al., 2010). In the present study, after investigating the effect of fluconazole and its combination with carvacrol against Candida, a far better antifungal effect than fluconazole alone was observed. Also, the effect of ketoconazole against Aspergillus was lower than that of carocarol. But the effect of the combination of ketoconazole and carvacrol against Aspergillus was slightly higher than the combination of fluconazole and carvacrol against Candida, which could be due to the difference in the species and method used. The use of these herbal compounds probably reduce the minimum effective dose of drugs. Therefore, toxicity, side effects and cost of treatment are reduced (Rabadia et al., 2011).

in 2019 Yılmaz et al . investigated the anticandidal effect of carvacrol. This substance was evaluated for its anti-candidacy activity against 5 human pathogenic strains according to the microdilution method. The MIC of carvacrol is 250-500 µg/ml against Candida species, which indicates relatively weak inhibitory activity compared to fluconazole as a standard (16-0.5 µg/ml) (Yılmaz et al., 2019). The MIC results by macrodilution method in our study were similar to this study, and Candida had a MIC of 250 ppm and showed less sensitivity than carvacrol in this method, but the disk diffusion results showed that carvacrol alone had a better effect than fluconazole alone showed on Candida species. Also, the calculated MIC for T.rubrum, E. flucosum and A. flavus are 3200, 3200 and 1600 ppm, respectively. According to these results, it can be seen that C. albicans has the most resistance and A. flavus is the most sensitive species to carvacrol. The results of these studies were completely in line with our study, as we showed using the macrodilution method that Candida albicans and A. flavus were the most sensitive species to 1,8 cineole, and T. rubrum and E. flucosum were the most resistant species to this substance. In the disk diffusion method, after Candida, which had the highest average non-growth, T. rubrum and E. flucovum were in the next ranks and showed more resistance.

Conclusion

Our findings showed that in the disk diffusion method, carvacrol and 1,8 cineole exerted the greatest effect on C. albicans and carvacrol was a more effective compound than 1,8 cineole. The data of this study showed that carvacrol was more effective than fluconazole and ketoconazole, but the effect of terbinafine alone is also noteworthy and it completely inhibited the growth of the fungus. The carvacrol in combination with routine antifungals, especially fluconazole and ketoconazole, acted much stronger in preventing the growth of fungi, and due to the side effects and high toxicity of these drugs, it is recommended to use them in combination with these oily compounds such as carvacrol. As a result, it provides a good opportunity for the pharmaceutical industry.

Refereces

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