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Evaluation of antifungal activities of the ethanolic extract of the macrofungus *Suillus collinitus* against dermatophytes

Ayşegül AKKOYUNLU[1*], Görkem DÜLGER[2], Başaran DÜLGER[3]
[1]*Duzce University, Graduate Education Institute, Department of Biology, Konuralp/Düzce, Türkiye.*
[2]*Duzce University, Medicine Faculty, Department of Medical Biology, 81620, Konuralp/Düzce, Türkiye*
[3]*Duzce University, Science and Arts Faculty, Department of Biology, 81620, Konuralp/Düzce, Türkiye*
[*]*aysegulgungor84@gmail.com,* [2]*gorkemdulger@duzce.edu.tr,* [3]*basarandulger@duzce.edu.tr*

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Suillus collinitus makrofungusunun etanolik ekstraktının dermatofitlere karşı antifungal aktivitelerinin değerlendirilmesi

Abstract: In this study, the ethanolic crude extracts obtained from the macrofungus *Suillus collinitus* (Fr.) O. Kuntze (*Suillaceae, Bolatales*) were investigated for their antifungal activities against dermatophytes, including the isolates *Microsporum gypseum* (E. Bodin) Guiart & Grigoraki, *Trichophyton rubrum* (Castell.) Sabour. and *Epidermophyton floccosum* (Harz) Langeron & Miloch., using both the agar well diffusion method and tube dilution methods. The ethanol extracts demonstrated strong antifungal activity, with mean inhibition zones observed at different concentrations. The widest zone of inhibition was recorded at 1500 mg/mL, measuring 20.8 mm for *M. gypseum*, 18.2 mm for *T. rubrum*, and 16.8 mm for *E. floccosum*. The MIC values observed were 50 µg/mL for *M. gypseum*, 60 µg/mL for *T. rubrum*, and 70 µg/mL for *E. floccosum*, respectively. Terbinafine as the comparison antifungal agent gave MIC values of 10 µg/mL for both *E. floccosum* and *T. rubrum*, and 20 µg/mL for *M. gypseum*. These findings against dermatophytes support our observations regarding the use of this macrofungus among the public. Besides, the findings of this screening study are a preliminary step to further pharmaceutical researches on the relevant macrofungus in the future.

Key words: *Epidermophyton floccosum*, *Microsporum gypseum*, *Trichophyton rubrum*, agar well diffusion, MIC.

Özet: Bu çalışmada, makrofungus *Suillus collinitus* (Fr.) O. Kuntze (*Suillaceae, Boletales*) türünden elde edilen etanollü ham ekstraktların, *Microsporum gypseum* (E. Bodin) Guiart & Grigoraki, *Trichophyton rubrum* (Castell.) Sabour. ve *Epidermophyton floccosum* (Harz) Langeron & Miloch. izolatları gibi dermatofitlere karşı antifungal aktiviteleri hem agar kuyucuk difüzyon yöntemi hem de tüp dilüsyon yöntemleri kullanılarak incelenmiştir. Etanol ekstraktları, farklı konsantrasyonlarda belirgin bir antifungal aktivite sergilemiştir. En geniş inhibisyon zonu, 1500 mg/mL'de gözlemlenmiş olup, *M. gypseum* için 20.8 mm, *T. rubrum* için 18.2 mm ve *E. floccosum* için 16.8 mm olarak kaydedilmiştir. Gözlemlenen MIC değerleri sırasıyla *M. gypseum* için 50 µg/mL, *T. rubrum* için 60 µg/mL ve *E. floccosum* için 70 µg/mL olmuştur. Karşılaştırma amaçlı kullanılan antifungal ajan terbinafin, *E. floccosum* ve *T. rubrum* için 10 µg/mL, *M. gypseum* için ise 20 µg/mL MIC değerleri vermiştir. Dermatofitlere karşı elde edilen bu bulgular, bu makromantarın halk arasında kullanımını desteklemektedir. Ayrıca, bu tarama çalışmasının bulguları, gelecekte ilgili makromantar üzerinde yapılacak daha ileri farmasötik araştırmalar için ön adım niteliğindedir.

Anahtar Kelimeler: *Epidermophyton floccosum*, *Microsporum gypseum*, *Trichophyton rubrum*, agar kuyu difüzyon, MIC

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1. Introduction

Fungal pathogens called dermatophytes are responsible for a group of disorders collectively referred to as dermatophytosis. Three genera of fungi–*Epidermophyton* Sabour., *Microsporum* Gruby, and *Trichophyton* Malmsten –cause the majority of dermatophytic infections. Human keratin is broken down by microorganisms including *M. gypseum* (E. Bodin) Guiart & Grigoraki, *T. rubrum* (Castell.) Sabour., eventually leading to tissue digestion. Additionally, these organisms can lead to superficial infections affecting the outer layers of the skin, hair, and nails. It is crucial to acknowledge that treating fungal infections can be quite difficult, as the fungi tend to become resistant to the antifungal drugs that are currently supplied (Shikwambana and Mamokone, 2020; Gupta et al., 2023).

Terbinafine is the primary treatment for superficial mycoses; however, the increasing incidence of terbinafine resistance throughout Europe and the occurrence of side effects such as epigastralgia, nausea and tachycardia necessitate the development of different antifungal agents against dermatophytes (Keller, 2012; Moreno-Sabater et al., 2022). Macrofungi contain active compounds that can treat fungal infections, making their screening for fungicidal agents crucial due to the rise in fungal pathogen resistance (Kumar et al., 2021). In Türkiye, many macrofungi are used to treat skin diseases, including those caused by fungal pathogens. However, information on these macrofungi is primarily limited to local communities within specific regions.

Suillus collinitus (Fr.) O. Kuntze (*Suillaceae, Boletales*) is found throughout Europe. It is an ectomycorrhizal fungus, forming mutually beneficial symbiotic relationships with several species of *Pinus*. Although not widely regarded as highly valuable, *S. collinitus* is reported to be edible when thoroughly cooked. To reduce the risk of an adverse

reaction to these mushrooms, some people have found it advisable to discard the cap skin of all species from the *Suillus* Gray genus (Watling and Hills, 2005).

During routine field excursions, it was established that *S. collinitus* is employed for the treatment of injuries. It was also noticed that this fungus's extracts, prepared as a pomade, were used to counteract foot peeling and nail thickening.

Consequently, the purpose of this work was to examine the antifungal properties of the ethanolic extract of *S. collinitus* against dermatophytes, which has traditionally been used by locals to treat fungal diseases.

2. Materials and Method

2.1. Microorganisms

The dermatophytes used in this study came from the culture at the Mycology Research Laboratory in the Biology Department, Düzce University, Düzce, Türkiye. *M. gypseum, T. rubrum* and *E. floccosum* (Harz) Langeron & Miloch. as dermatophytes were used as test fungal pathogens. All fungi were stored on Sabouraud Dextrose Agar (SDA Agar) (Oxoid, Hampshire, England) slants in the refrigerator at 4 °C, before use.

2.2. Macrofungal materials

The macrofungus *S. collinitus* was collected from Bolu-Abant Road, Bolu, Türkiye (40°39’31’’ N, 31°24’17’’ E, Alt. 920 m) in July, 2023. Voucher specimens (BD-505-1) of the macrofungus was deposited in the Department of Biology of Düzce University in the author’s collection.

2.3. Preparation of crude extracts

The macrofungal samples were pulverized after being dried at 40 °C in an oven. Using Soxhlet apparatus, each batch of dry powdered macrofungal material (50 g) was extracted for 24 hours using 150 mL of 95% ethanol from Merck, Darmstadt, Germany. First, Whatman filter paper No. 1 was used to filter the extracted mixture. A 32.8% ethanol yield was obtained by vacuum-evaporating the filtrate solvent in a rotary evaporator set at 55 °C. The dehydrated extract was kept at -20 °C in sterile screw-capped vials with labels. Dimethyl sulfoxide (DMSO) was used to dissolve the ethanol, which was found as sticky black solids, to a final concentration of 1 g/mL for first screening.

2.4. Preparation of inoculum

The fungal test microorganisms are revitalized at 27 °C on PDA (Potato Dextrose Agar) (Oxoid, Hampshire, England) for 14 days. Spores of revitalized fungus were collected from cultures on agar plates after 7 days of incubation as described by Broekaert et al. (1990). The sporangial suspension concentration was estimated with regards to the conidium and spores forming fungi, and micro-dilution standardized by the Clinical and Laboratory Standards Institute (CLSI, 2018) which involves an inoculum of spores adjusted spectrophotometrically to 2.5×10^5 cfu/mL at wavelength of 530 nm of 0.11 O.D. (Castilho et al., 2015). The fungal spore suspension was stored in 20% glycerol at -4 °C to avoid contamination and growth.

2.5. Determination and antifungal activity

The antifungal activity of the macrofungus *S.collinitus* extract was determined by the agar well diffusion method (Holder and Boyce, 1994; Jamuna et al., 2013). The PDA medium was prepared according to the manufacturer’s instructions (39 g/L). The fungal culture was evenly spread over the medium by sterile cotton swabs. A sterile cork borer made wells (6 mm) in the medium. A volume of 200 µL of extracts was transferred into each well, incubated at 30 °C for 48-72 h, and the plates were then observed for the formation of clear zones around the wells indicating the presence of antifungal activity (Ambikapathy et al., 2011). The zone of inhibition was measured using caliber (all inhibition minus 6 mm of the well) and recorded. Ethanol (96%) and Terbinafine obtained from pharmacy 200 µL each were used as the negative and positive control, respectively.

2.6. Minimum ınhibitory concentration (MIC)

MIC values of *S. collinitus* extract were determined using the tube dilution method (Koneman et al., 1997). The fungal spore inoculum of 100 µL of 2.5×10^5 dilution was inoculated into test tubes with (1800 µL) Malt Extract Broth (Oxoid, Hampshire, England) in eight different test tubes and the macrofungal extract was serially diluted, ranging from 100 mg/mL to 800 mg/mL. A volume of 100 µL of each extract dilution was mixed in each incubated test tube, incubated at 30 °C for 48-72 h, and then examined for visual turbidity. The results of the extracts were compared with a standard positive control (Terbinafine 20 µg/mL).

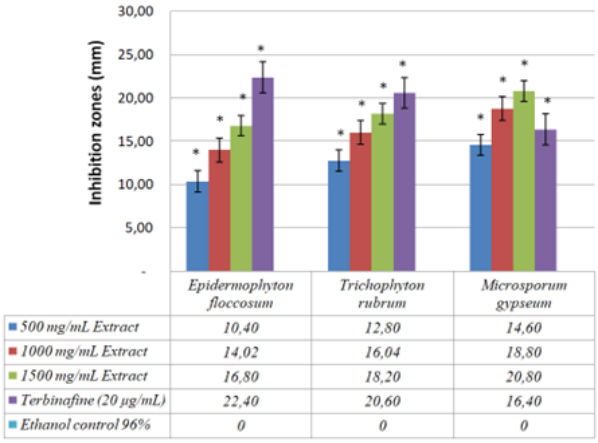
2.7. Data analysis

Statistical analyses were conducted using IBM SPSS Statistics Version 27. Experimental results obtained from the agar well diffusion assay were expressed as the mean of triplicate measurements (n=3). One-way analysis of variance (ANOVA) was used to evaluate significant differences among the groups. For data sets exhibiting normal distribution as evaluated by normality tests, Tukey's post hoc test was applied. Additionally, a Kruskal-Wallis test was conducted to compare the MIC values among *E. floccosum*, *T. rubrum*, and *M. gypseum*. Statistical significance was defined as $p<0.05$ with a 95% confidence interval.

3. Results

According to the data in Figure 1, the antifungal activity of the tested macrofungal extract on three dermatophyte fungi species increased with the applied concentration. The effects on *E. floccosum*, *T. rubrum* and *M. gypseum* tended to increase at 500, 1000 and 1500 mg/mL extract concentrations, respectively. However, this activity remained at lower levels compared to terbinafine used as positive control. For example, for *E. floccosum*, the effect of the macrofungal extract at 500 mg/mL concentration was 10.4 mm, while this effect reached 16.8 mm at 1500 mg/mL. In contrast, the effect of terbinafine at 20 µg/mL was higher at 22.4 mm. Similarly, the activity of *T. rubrum* extract increased with increasing concentration, but terbinafine showed higher antifungal activity. Notably, *M. gypseum* extract showed a stronger antifungal effect (20.8 mm) at the highest concentration than the standard Terbinafine (16.4 mm). The ethanol control group did not show any antifungal activity, confirming that the test conditions were appropriate. Inhibition zones (mm) were analyzed using one-way ANOVA followed by Tukey's post hoc test to determine the differences between the extract concentrations (500, 1000, and 1500 mg/mL) and the

standard antifungal agent Terbinafine (20 µg/mL) for each dermatophyte species. The results revealed statistically significant differences (*p<0.05) between the extract concentrations and Terbinafine, as well as among the extract concentrations themselves.



	Epidermophyton floccosum	*Trichophyton rubrum*	*Microsporum gypseum*
500 mg/mL Extract	10,40	12,80	14,60
1000 mg/mL Extract	14,02	16,04	18,80
1500 mg/mL Extract	16,80	18,20	20,80
Terbinafine (20 µg/mL)	22,40	20,60	16,40
Ethanol control 96%	0	0	0

Figure 1. Inhibition zones (mm) observed in agar well diffusion assay using different concentrations of test extracts and standard antifungal agent Terbinafine against selected dermatophytes. Statistical analysis using One-way ANOVA followed by Tukey's post hoc test revealed significant differences in the zones of inhibition between the extract concentrations and Terbinafine (* p<0.05). Error bars represent standard deviation (n=3).

In conclusion, the antifungal effect of the macrofungal extract on dermatophytes was observed, but it should be noted that it was more limited than terbinafine.

Regarding the MIC results (Table 1), the extract exhibited high to moderate (80-100% inhibition) antifungal activity against all three dermatophyte species, ranging from 50 µg/mL for *M. gypseum*, 60 µg/mL for *T. rubrum*, and 70 µg/mL for *E. floccosum*. Furthermore, Terbinafine, the comparative antifungal agent, resulted in MIC values between 10 µg/mL for *E. floccosum* and *T. rubrum*, and 20 µg/mL for *M. gypseum*. What's noteworthy is that the extract's effectiveness against all dermatophytes was not as strong as that of the standard Terbinafine. A Kruskal-Wallis test was conducted to compare the MIC values of the test extract in three dermatophyte species, *E. floccosum*, *T. rubrum* and *M. gypseum*. The analysis did not reveal any statistically significant difference between the groups (p>0.05).

Table 1. MIC values of the ethanolic extract of *S. collinitus* and standard Terbinafine*

Test Microorganisms	**MIC (µg/mL)**	**Standard (Terbinafine 20 µg/mL)**
Epidermophyton floccosum	70	10
Trichophyton rubrum	60	10
Microsporum gypseum	50	20

4. Discussions

The objective of this study was to evaluate the antifungal properties of an ethanolic extract of *S. collinitus* against dermatophytes. The findings indicate that the antifungal activity of the extract increased in a concentration-dependent manner in the three species tested (E. *floccosum*, *T. rubrum*, and *M. gypseum*). However, the activity generally remained lower than that of terbinafine. Interestingly, the zone of inhibition of M. gypseum at the highest concentration (20.8 mm) exceeded that of terbinafine (16.4 mm), indicating possible species-specific interactions. Such findings are critical for determining targeted applications for *S. collinitus* extracts in antifungal therapy. The increased zones of inhibition at higher extract concentrations support the hypothesis that the potency of the extract is concentration-related. However, the limited efficacy compared to terbinafine highlights the need for further improvements. MIC results further confirm this pattern, with the extract showing MIC values of 50 µg/mL for *M. gypseum*, 60 µg/mL for *T. rubrum*, and 70 µg/mL for *E. floccosum*. In contrast, terbinafine exhibited significantly lower MIC values, confirming its superior antifungal potential.

The results of this study are partially consistent with previous reports on the antimicrobial and antifungal properties of *S. collinitus*. Recent investigations have emphasized the antimicrobial capabilities of mushrooms, and various antibacterial compounds have been identified, primarily found in higher Basidiomycetes and certain Ascomycetes. These active compounds are secondary metabolites, including terpenoids, quinone or lactone derivatives, alkaloids, and other similar compounds. Additionally, high molecular weight molecules like polysaccharides or proteins contribute to mushrooms' anti-infectious properties. The bioactive chemicals also include certain derivatives that are sulfurated and chlorinated, which are frequently found in macromycetes (Hamers et al., 2020).

Remarkably, the literature review revealed that just one study had assessed the antibacterial activity of *S. collinitus*. According to Yamac and Bilgili (2006), disk diffusion and microdilution methods were used to investigate the antimicrobial activities of dichloromethane, ethyl acetate, and ethanol extract obtained from *S. collinitus* against test microorganisms such as *Salmonella typhimurium* NRRL-B-4440, *Escherichia coli* ATCC 25922, *Enterobacter aerogenes* NRRL-B-3567, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* NRLL-B-4337, *Bacillus subtilis* NRRL-B-558 as bacterial cultures, *Candida albicans* ATCC 10259, and *Saccharomyces cerevisiae* NRRL-Y-2034 as the yeast cultures. The *S. collinitus* dichloromethane extract was found to be the most effective against bacteria and yeast. The dichloromethane extract exhibited the greatest antifungal activity (>20 mm) against *S. cerevisiae* NRRL-Y-2034. For *S. aureus* ATCC 25923, the inhibitory zone varied between 15-20 mm. The inhibitory zone, which measured 10-15 mm, had a moderate antifungal efficacy against *Candida albicans* ATCC 10259. The MIC values of these extracts ranged from 31.25 to 250 µg/mL.

Compared to our study, this difference, which was partially seen in the same species, was due to genotype, chemotype, and geographical origin, it is thought that parameters cause environmental and soil conditions. One of the reasons for this difference can be explained by the difference in the strains and protocols of the test microorganisms used in the studies. The fungal compounds responsible for the resulting antifungal effect are thought to originate from terpenoids,

quinone or lactone derivatives, and alkaloids, which are macrofungal secondary compounds.

The limited number of studies conducted on *S. collinitus*, have begun to reveal the medicinal potential of this mushroom. According to one study, the methanolic extract of *S. collinitus* induced apoptosis in a human breast tumor cell line and had a p53-mediated influence on the normal cell cycle distribution (Vaz et al., 2012).

In another study, the reducing and radical-scavenging capacities of *S. collinitus* were assessed, along with its ability to prevent lipid peroxidation in liposome solutions. *S. collinitus* was also shown to have a high tocopherol content (Heleno et al., 2010). Furthermore, Akata et al. (2012) reported that *S. collinitus* has a 71.94% free radical scavenging rate of 1,1-diphenyl-2-picrylhydrazyl (DPPH). Moreover, Froufe et al. (2011) found the median effective concentration (EC50) values (14.05, 2.97, and 1.20 mg/mL) for radical scavenging, lipid peroxidation activity suppression, and *S. collinitus* reducing power, indicating the plant's strong antioxidant capacity. In another study, Emsen et al. (2019) used chromosomal aberration (CA), micronucleus (MN), nuclear division index (NBI), and mitotic index (MI) analyses to investigate the effects of acetone and water extracts of *S. collinitus* on genotoxicity and proliferation of human cells.

In conclusion, although *S. collinitus* has been shown to have strong antioxidant, antimicrobial and antifungal properties, it appears that this fungus exhibits a more limited effect compared to effective agents like terbinafine, particularly in treating dermatophyte fungal infections. However, these findings provide important insignts into the potential medicinal use of *S. collinitus* and highlight the need for further research to more comprehensively evaluate its biological activities.

Conflict of Interest

The authors declared no conflicts of interest.

Authors' Contribution

All authors participated in the conceptualization, design, analysis, and writing of the study. They have all reviewed and approved the final manuscript.

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