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Original Article:

Evaluation of Antifungal and Antitumor Effects of Propolis

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Abstract

Introduction: One of the major concerns of the health system of countries is resistance to common fungicides by pathogenic strains. The World Health Organization places special emphasis on finding natural compounds with antifungal properties. In the present study, therefore, the antifungal and antitumor effects of ethanol extract of propolis were studied.

Materials and Methods: For this purpose, at first, M. furfur MF7 strain was prepared and cultivated. Then, propolis ethanol extract was prepared. The microbioassay method was used to study the effects of different concentrations of propolis extract on the growth of M. furfur, and the broth microdilution method was used to determine the MIC and MFC. Also, the effect of this extract on ergosterol biosynthesis was studied.

Results: The results of the study showed that the MIC of propolis ethanol extract on this pathogenic fungus is 10 mg/ml and its MFC is 20 mg/ml. A decrease in fungus growth was seen with an increase in the concentration of propolis ethanol extract, so that there was no growth in the concentration of 20 mg/ml ethanol extract of propolis. Also, with the increase in the concentration of the extract, the biosynthesis of ergosterol decreased, and at the concentrations of 15 and 20 mg/ml, a severe decrease in the biosynthesis of this compound was seen. Propolis reduced HT-29 cell line viability at 2000µg/mL.

Conclusion: In general, it was concluded that propolis ethanol extract is a suitable option for treating diseases caused by M. furfur. Studies in clinical conditions are needed.

Keywords: Extract, *Malassezia*, MIC, Propolis

1. Introduction

Pityriasis versicolor, commonly known as Tinea versicolor, is a frequent, benign, superficial fungal infection of the skin. It is caused by a type of yeast that naturally lives on skin. It can be attributed as a fungal skin infection caused by the dermatophyte *Malassezia furfur* (*Pityrosporum ovale*). Tinea versicolor is characterized by small, scaly, hypopigmented macules that are predominantly seen on the trunk, neck, and proximal limbs. The stain may disappear in cool weather and worsen in hot, humid weather. These are likely to be dry and scaly and may itch or hurt. This disease is detected by ultraviolet radiation, which appears fluorescent yellow-green when exposed to it [1,2]. These fungi are most likely to be found in the surface layer of the skin (stratum corneum) as well as hair follicles. Since fat is necessary for its growth and development, areas of the skin that have a lot of sebaceous glands provide a place for the fungus to live. This yeast is found in more than 90% of adults without causing an infection. However, Tinea versicolor is caused by its abnormal growth in the skin [3].

Topical treatment alone is used for most patients.

Systemic therapy is indicated with widespread involvement, recurrent infections, or when local therapy fails [4]. Because it is relatively easy to treat and recurrence is common, it is imperative that treatment be as safe, inexpensive, and convenient as possible. However, the chemical drugs used such as ketoconazole and imidazole have undesirable side effects and increase the possibility of pathogen resistance to these fungicides [5]. Therefore, it is necessary to study new treatment solutions based on natural compounds to deal with this disease.

Propolis is a gum collected by honeybees from the buds and leaves of trees and plants and mixed with pollen and also enzymes secreted by the bees. Bees use it as glue, general sealant and extruder for beehives [6]. Propolis, which has been known in traditional medicine since ancient times, has been considered as a useful substance in medicine and food products in recent years, because it has various biological properties, including antimicrobial, antioxidant, and anti-ulcer properties [7].

The antimicrobial properties of propolis have been widely reported. Park et al. (1998) reported that the growth of *Streptococcus*, an oral pathogen, was inhibited by ethanol extracts of propolis from different regions of Brazil [8]. Fernandes et al. (1995) demonstrated the antimicrobial activity of propolis against bacterial and yeast pathogens isolated from human infection [9]. In addition, Grange et al., (1990) reported that this compound was active against Gram-positive bacteria, but showed limited activity against Gram-negative bacteria [10]. The antimicrobial activity of propolis is reflected in its components, which may vary from region to region and season to season depending on its chemical composition. Flavonoids and esters of phenolic acids are generally responsible for the antimicrobial activity of propolis [11].

Colon cancer has been one of the most common malignancies in recent years, with a high mortality rate in patients [12]. Despite recent advances in the treatment of this disease, colon cancer has not yet been completely cured and more research is needed in this field. The treatment of this cancer includes chemotherapy, radiation therapy, and immunotherapy, which causes many side effects in patients leading to a decrease in the quality of life [13]. It seems that finding natural compounds with antitumor properties is of great importance [14]. The anticancer effects of propolis have been reported in many malignancies [15]. However, there is a need to study more about the effect of this compound on colon cancer.

Therefore, considering the wide spectrum of antimicrobial and antitumor activities of propolis, in this research, we decided to study the effect of this compound on Tina versicolor fungus and HT-29 colon cancer cell line for the first time. It is hoped that by conducting this research, a new treatment solution will be invented to deal with these diseases.

2. Materials and Methods

Preparation of propolis

Propolis was obtained from Sigma (CAT# P8904). To prepare the ethanolic extract, propolis was placed in 95% v/v ethyl alcohol in a closed glass container for 4 days at 37°C, with occasional shaking. Then, the ethanolic extract was filtered through Whatman grade 4 filter paper and evaporated on a rotary evaporator under reduced pressure at 60°C.

Preparation and cultivation of fungus

M. furfur (MF1) was obtained from the collection of pathogenic fungi of the mycology department of Pasteur Institute of Iran. After preparation, the fungus was cultured in Dixon agar medium and spore suspension was prepared from them. For this purpose, Dixon agar culture medium with chloramphenicol was used to prepare *M. furfur* spore suspension. Then, the culture medium was placed in an incubator for 10 days at 32°C to allow the fungus to sporulate.

Minimum inhibition concentration (MIC)

The MIC was determined by microbroth dilution method according to CLSI reference guidelines. For this purpose, 96-well plates were used. To conduct the experiment, fungus stock was first prepared. Stock preparation was done according to the following formula:

$$M_1V_1=M_2V_2$$

where M_1: number of spores counted, V_1: volume of spore suspension that should be diluted with medium, M_2: reference number of the standard amount of fungal spores 5 $x10^4$ and V_2: number of wells x 100 µL of medium.

To perform the microbroth dilution test, two rows of 96-well plates (127.71mm*85.43mm) were considered for each material. The first column is the environment control; the second column is the solvent control; the third column is the positive control; the fourth column is the negative control (100 microliters of the drug stock); and the highest concentration of the sample stock is poured into the fifth column. From the sixth column onwards, dilution of the compounds was poured into the plate; in this way, 100 µL of RPMI medium was poured in the sixth to twelfth columns,

and 100 µL was removed from the wells of the fifth column with the help of an 8-channel sampler and poured into the wells of the sixth column. After stirring, this process was continued until the twelfth column and 100 µl was discarded from the last column. In this way, the concentration of drugs was halved from the fifth column onwards. Finally, 100 µl of fungal stock was added to all the wells except the wells of the first column. After this step, the plates were placed in an incubator for 2 days at 35°C.

After two days of incubation, first the control results were read to confirm the accuracy of the test. After confirming the accuracy of the test through visual observation, the last clear well from the side of higher concentration to lower concentration was considered as MIC.

Minimal fungicidal concentration (MFC)

To determine the MFC of propolis, 10 microliters of the contents of the MIC well were removed and poured onto subrodextrose agar medium with chloramphenicol. These plates were incubated for 48 hours at 35°C. Then, the fungus growth was checked and the lowest concentration in which the fungus had no growth was considered as the MFC.

M. furfur growth and ergosterol biosynthesis

4 days after incubation, all the plates were placed in an oven at 60°C for one hour to inactivate the fungus, and then the fungal mycelia were separated and placed on aluminum foils that were weighed beforehand. The wet weight was recorded and were then placed in the oven to dry for 3 hours at 80°C. After three hours, the foils were removed and weighed again to obtain the dry weight of the fungus. In this way, the percentage of inhibiting the growth of fungi was calculated according to different concentrations of propolis. Then, IC_{50} (minimum concentration that leads to 50% inhibition of fungus growth) was also calculated.

Also, the ergosterol content of *M. furfur* was calculated after incubation. For this purpose, after the completion of the four-day incubation period, the fungi were separated and were placed at 60 °C for 3 hours. After drying, they were ground and then 25% potassium hydroxide alcoholic solution was added to them and vortexed. Then, they were placed in the oven at 80°C for 60 minutes. After reaching room temperature, a mixture of 3 mL of n-hexane and 1 mL of distilled water was added to each sample tube and vigorously vortexed for 3 minutes. After 10 minutes at room temperature, the n-hexane layer was separated and placed on a higher surface, and this phase was separated and transferred to large glass tubes and incubated at -20°C for 18-24 hours. After this period of time, 5 times the volume of absolute ethanol was added to the large glass tubes containing the transparent layer of the hexane phase. For the resulting solution, OD was read at wavelengths of 281.5 and 230 nm with a UV/VIS spectrophotometer.

Anti-tumor activity of Propolis

To investigate the antitumor activity of propolis, first, HT-29 cells were prepared and cultured in RPMI medium. Then, MTT test was used. Briefly, 5 x 10^4 HT-29 cells were cultured in 96-well plates and treated with 1, 10, 100, 1000 and 2000 µg/ml Propolis and 20 µl of MTT solution was added. Finally, after 3 hours of incubation, the intensity of light absorption at 570 nm was measured.

Statistical analysis

GraphPad Prism version 8 software was used for data analysis and drawing graphs. All data were presented as mean ± standard deviation and Tukey's post hoc test was used at the probability level of $P<0.05$ to check for significant differences between the means. One-way analysis of variance (ANOVA) was used to analyze the data

3. Results

MIC and MFC

The effect of propolis at concentrations of 0, 2, 4, 6, 8, 10, 15, and 20 mg/ml was studied on the inhibition of *M. furfur*, and the results are shown in Figure 1. As can be seen, increasing the concentration of propolis increases the inhibition percentage of this pathogenic fungus. The MIC of this compound was calculated 10 mg/ml against *M. furfur*, and since no bacterial growth was observed at a concentration of 20 mg/ml, this concentration was considered as the minimum fungicidal concentration (MFC). Therefore, it can be said that propolis has anti-*Malassezia* effects.

** and **** indicate significant differences at the probability level of $P<0.0001$ and $P<0.01$ compared to the control, respectively.

Effects of propolis on M. furfur growth

As can be seen in Table 1, with an increase in the concentration of propolis ethanol extract, the weight of the mycelium of *M. furfur* decreased, indicating the inhibition of *M. furfur*. According to the findings of this test, it was found that the ethanolic extract of this compound has strong anti-*malassezia* effects. Thus, no growth was observed in the concentration of 20 mg/ml of this extract. Therefore, it can be concluded that propolis ethanol extract at a concentration of 20 mg/ml can have fungicidal effects against *M. furfur*.

Effects of propolis on M. furfur ergosterol biosynthesis

The effect of propolis ethanol extract on ergosterol biosynthesis was concentration-dependent in a way that the inhibition percentage of ergosterol biosynthesis augmented by increasing the extract concentration (Figure 2). The highest percentage of inhibition of ergosterol biosynthesis of this extract was seen in the concentration of 20 mg/ml propolis ethanol extract

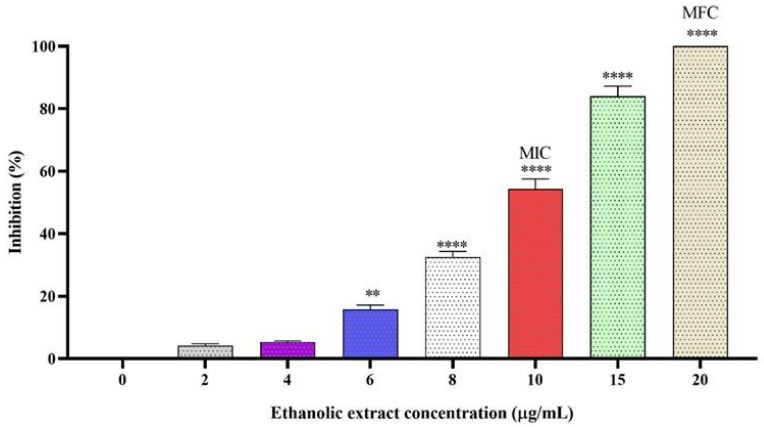

Figure 1. The effect of different concentrations of propolis ethanol extract on *M. furfur* and determination of MIC and MFC

Table 1. The effects of different concentrations of propolis on *M. furfur* growth

Propolis concentration (mg/mL)	Dry weight of *M. furfur* mycelium	Inhibition percentage
2	2.38±0.13	4.23
4	2.31±0.14	5.36
6	2.08±0.11	15.85
8	1.67±0.15	32.58
10	1.13±0.08	54.32
15	0.40±0.06	83.98
20	-	100

(100%). At a concentration of 20 mg/ml of propolis ethanol extract, complete inhibition of ergosterol biosynthesis was seen (100%). Therefore, one of the fungicidal mechanisms of propolis ethanol extract is the inhibition of ergosterol biosynthesis, which is confirmed in the current study.

Antitumor activity of Propolis

The concentration-time response of HT-29 cell line to the propolis is shown in Figure 3. As can be seen, the viability of cells decreased with increasing propolis concentration and time. The lowest viability

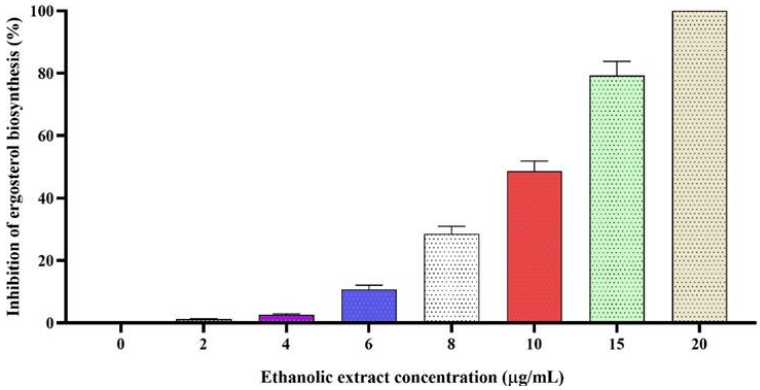

Figure 2. The effect of different concentrations of propolis ethanol extract on *M. furfur* on ergosterol biosynthesis

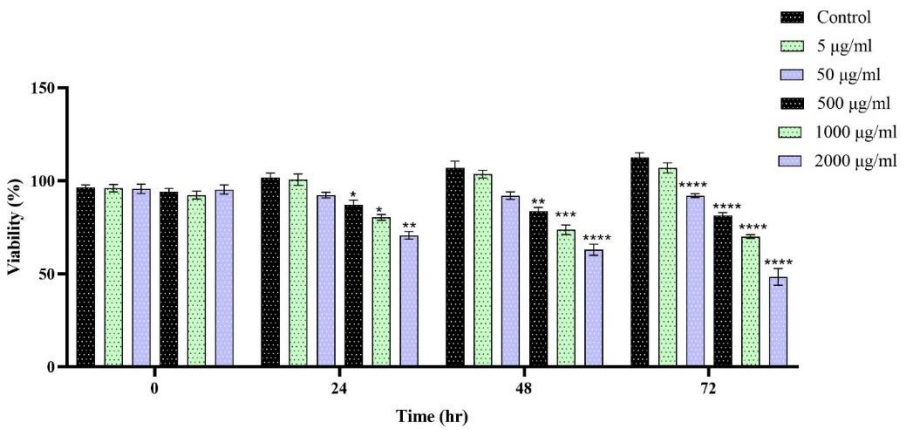

Figure 3. The viability percentage of HT-29 cell line treated with the different concentrations (µg/mL) of Propolis. *, **, *** and **** showed significant differences

of HT-29 cell line was seen in 2000 µg/mL of propolis at 72-hour treatment (Figure 3). Therefore, our results showed that propolis had anti-tumor effects on colon cancer cell line.

4. Discussion

The results of the present study showed that propolis ethanol extract was able to inhibit the growth of *M. furfur*, so that its MIC was calculated as 10 mg/ml and MFC as 20 mg/ml. These inhibitory and fungicidal effects were attributed to the reduction of ergosterol biosynthesis, an essential compound in the fungal wall.

Microbial resistance to antibiotics as well as to antifungal agents has increased in recent years and is considered a serious public health problem by the World Health Organization. Monitoring data from the Central Asia and Europe Antimicrobial Resistance Annual Report 2020 (CAESAR) shows that resistance to fungicides is widespread across Europe, as can be seen by the high percentage of resistance (10–50%) and resistance profiles. This strongly supports the global call for action against antimicrobial resistance [16]. Since new active molecules and alternative therapies are urgently needed, the use of old and traditional therapeutic resources needs attention and revision. *Malassezia* species are associated with several skin diseases such as ringworm, seborrheic dermatitis and dandruff, atopic dermatitis and psoriasis. Tinea versicolor, a chronic, superficial fungal disease that causes pigmented lesions, generally occurs on the upper trunk, neck, and arms and is associated with *M. furfur* [17]. Therefore, in this present study, the effects of propolis ethanol extract on Malassezia furfur were studied.

Typical raw propolis consists of 45-55% plant resin, 25-35% wax, 5-10% essential oil and aromatic compounds, 5% pollen and 5% other natural products [18]. In addition, propolis contains various types of other secondary plant metabolites, whose concentrations vary depending on the season, the geographical origin of the collection, and the proximity of the hive to certain plant sources. The main constituents of propolis collected from Europe, Asia, North America, etc. are characterized by many phenolic compounds including flavonoids, aromatic acids and their esters, which are often collected by honey bees from spruce buds. These compounds are the dominant components in poplar buds and show numerous biological and medicinal properties. Polyprenyl benzophenones and various diterpenes were the main compounds found in tropical propolis collected from tropical regions such as Brazil [19]. Several groups of researchers have proven that all types of propolis have antibacterial properties. Veiga et al. [20] reported that propolis has an antibacterial effect on gram-positive and gram-negative microorganisms, including multidrug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), while Yıldırım et al. [21] examined Turkish propolis against tuberculosis and found that its aqueous extract has anti-tuberculosis activity against different types of mycobacteria. In addition, many studies have documented the remarkable effect of propolis against many types of microorganisms, including yeasts, viruses, bacteria, and parasites [22]. In addition to the biological activities and medicinal properties of propolis, a number of studies showed that it has no toxicity and side effects in animal models or humans [23]. Despite many reports about the antimicrobial effects of propolis, the review of sources showed that the effects of this natural compound on the *M.furfur* have not been studied so far. Therefore, in the present study, the anti-*Malassezia* effects of

propolis were studied *in vitro*.

The results of the present study showed that ethanol extract of propolis has inhibitory effects on *M. furfur*. Different solvents are often used to extract different bioactive compounds from propolis, such as ethanol, water, glycerol, propylene glycol, methanol, DMSO (dimethyl sulfoxide) and trichloromethane, but ethanol is globally the most efficient and commonly used solvent for extract preparation for propolis [24]. Therefore, in the present study, the ethanolic extract of this compound was prepared.

Parameters commonly used to express antimicrobial activity are the diameter inhibition zones, MIC and MFC, which is the lowest concentration for which there is no growth, or death. Diameter inhibition zone is expressed in millimeters, whereas MIC and MFC are usually expressed in µg/ml, or sometimes as a percentage [25]. The percentage of inhibition of mycelium growth in the presence of propolis is also used as a parameter of antimicrobial activity. Therefore, in the present study, MIC and MFC were considered as parameters related to the anti-*Malassezia* activity of propolis ethanol extract, and the results showed that MIC and MFC were 10 and 20 mg/ml, respectively. Antifungal activity of propolis has been reported in many studies. For example, in the study of Manani et al., the ethanolic extract of propolis showed antifungal activity. The MIC of the ethanolic extract of propolis was 0.2 µL/mL for *M. gypseum*, 0.05 µL/mL for *M. nanum*, and 0.025 µL/mL for *M. canis* [26]. In the case of yeast, the most sensitive species to prolactin appear to be *C. albicans* (MIC90 = 0.05 µg/ml) as well as *C. glabrata* (MIC90 = 0.05 µg/ml), followed by *C. krusei* and *Trichosporon* spp, each with similar MIC90 (values of 0.05 µg/ml). The most effective anti-yeast propolis extract seems to be the Turkish extract, which shows lower MIC values (MIC90 = 0.05 µg/ml).

Several basic mechanisms have been proposed by different research groups in relation to the antimicrobial activity of propolis, including inhibition of cell division, nucleic acid synthesis, protein synthesis, inhibition of cytoplasmic membrane function, alteration of membrane permeability, reduction of biofilm formation ability, inhibition of bacteriolysis and reduction of bacterial resistance to some common antibiotics [6]. The fungal cell wall is the first barrier responsible for the growth, adaptation and regulation of permeability of fungal pathogens during infection. Corrêa and co-workers found that Brazilian propolis damages the integrity of the cell wall and cell membrane of *C. albicans*, causing leakage of intracellular organelles. This study hypothesizes that the antifungal effect of propolis is due to the capacity of polyphenols to form complexes with soluble proteins by disrupting chitin synthesis, leading to cell wall disruption [27]. After measuring the growth of *C. albicans* in the presence and absence of an osmotic protective agent (sorbitol), the results showed that the ethanolic extract of Polish propolis does not affect the cell wall. However, ergosterol and membrane depolarization assays suggest that the cell membrane may be a potential target for propolis [28]. The presence of phenolic compounds in propolis was considered responsible for the fungicidal activity. Propolis exhibits specific activity against *Penicillium italicum*, reducing the level of phosphorylated adenosine nucleotides in hyphae and disrupting the cell membrane, resulting in ion leakage [29]. Therefore, the anti-*Malassezia* effects of propolis ethanol extract, which was seen in the present study, can be attributed to its components, especially phenol and flavonoids.

We also studied the antitumor effects of propolis on HT-29 cells and the results indicated the antitumor effects of this compound. Antitumor effects of propolis have been reported in some studies [30]. The mechanism of antitumor effects of this compound was attributed to the effect on reactive oxygen species (ROS) production and induction of apoptosis [31]. Therefore, propolis with antitumor effects in a variety of malignancies can be considered as an adjuvant cancer treatment option. However, there is a need for clinical studies in this field.

5. Conclusion

In general, it can be concluded that propolis ethanolic extract has a strong potential for antifungal effects against *M. furfur*. These effects can be attributed to its components such as phenols. The mechanism of action of the antifungal effects of propolis in the present study was attributed to blocking the ergosterol biosynthesis. Also, this compound showed anti-tumor effects on HT-29 colon cancer cell line. The current study confirms that the ethanolic extract of propolis has antifungal and antitumor effects. Nevertheless, clinical studies are warranted in this field.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Author's contributions

Ebrahim Khademi wrote the draft and conducted the data analysis. Behdokht Jamali was responsible for managing the project and revising the manuscript.

Conflict of Interest

There were no conflicts of interest among authors.

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