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Full Length Research Paper

Antifungal effect of the extract of propolis on the growth of three species of *Epidermophyton flucosum*, *Trichophyton violaseum* and *Trichophyton tonsorans* in laboratory environment

Shahin Gavanji[1], Mohammad Javad Asgari[2]*, Ramin Vaezi[3] and Behrouz Larki[2]

[1]Young Researchers Club, Khorasgan Branch, Islamic Azad University, Khorasgan, Iran.
[2]Department of Soil Science, Islamic Azad University, Khorasgan Branch, Isfahan.
[3]Science and Research Campus, Islamic Azad University, Tehran, Iran.

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Cutaneous infections in human include a wide variety of diseases. Most of these infections are caused by dermatophytes and the resulting disease is called dermatophytosis. Dermatophytosis brings many problems for human hence, studies and new researches about this disease can be useful in controlling it with fewer side effects. So, in this study the antidermatophytal features of propolis of bee were tested. In this test, first of all, the alcoholic extract of propolis was prepared and the minimum inhibitory concentration (MIC) of it on three dermatophyte species; *Trichophyton violaseum*, *Triehophyton tonsorans* and *Epidermophyton flucosum* were examined in laboratory environment. The results indicated that the extract of propolis has a good antifungal activity against these three species. The MIC of the extract of propolis per mm in the culture medium was determined equal to 2\5 against these three species of fungi. In this study, we saw that the propolis of bee has a useful antifungal activity against dermatophytes.

Key words: Propolis, *Trichophyton tonsorans*, *Trichophyton violaseum*, *Epidermophyton flucosum*, dermatophytosis.

INTRODUCTION

Lexically, propolis consists of two words; the first word is pro which means forward and defense while the second word is polis which it means to defend the city (Tosi et al., 1996).

Propolis is a substance similar to wax and it is a production of bee and due to the interference of many factors, its appearance may vary widely (Fernandes et al., 2007). Normally it is a solid, pasty and sticky substance. Its color is greenish brown to brown depending on the origin of resins, duration and condition of storage (Krol, 1996; Ghisalberti, 1979). Bees collect this substance from buds of flower, leaf, bark and other parts of plants and sometimes from the gums which are used for grafting plants (Greenaway et al., 1990). Bees use propolis for filling the gaps, narrowing the ventilation holes, tightening the valve of flight, repairing fracture, polishing the interior wall of hive and removing dead objects which could not be removed by bees themselves (Ghisalberti, 1979; Burdock, 1998; Ebadi and Ahmadi, 1990). Furthermore, mixing small amount of propolis with wax can close the cells from laying. Propolis can destroy the bacilli of larvae and reduce the possibility of infection in the growth of larvae and pupa. It also diminishes the growth of those bacteria that decompose the tissue of dead animal (Meresta and Meresta, 1988; Miagan and Sulimanovic, 1982).

The use of this substance dates back to about 3000 BC and in many parts of the world it has been used as a healing factor in local and traditional medicine. Egyptians,

*Corresponding author. E-mail: asgari627@yahoo.com, shahin.gavanji@khuisf.ac.ir.

Greeks and Romans used propolis for healing and treating some skin lesions. The ancient Egyptians also used it to mummify the bodies (De Vecchi and Drago, 2007).

Naturally, propolis is composed of 30% wax, 50% gum, 10% essential fats, 5% pollen and aromatic substances of plants (Ebadi and Ahmadi, 1990; Burdock, 1998; Kettel, 1995). Chemical compositions of propolis are very complicated and more than 300 compositions have been identified in its samples. In fact the composition of propolis depends on the plant source and local flora (Tosi et al., 1996). Propolis contains aliphatic and aromatic acids, esters, flavonoids, sugars, glycerol, phosphoric acid, vanillin, Myristin, thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, vitamins C, E, A, iron, manganese, copper, calcium, vanadium, aluminum, strontium, silicon, zinc, sodium, iodine and magnesium in different amounts and also amino acids in small amounts which are mostly arginine and proline. Propolis can be mixed with saliva and as a result, succinic dehydrogenase, glucose, phosphatase, adenosine triphosphatase, acid phosphatase and beta-amylase are produced in its contents (De Vecchi and Drago, 2007; Krell, 1999; Scifo, 2004).

The biological activity of propolis is mainly because of the numerous chemical constituents it contained such as flavonoid, caffeic acid, ferulic acid, coumaric acid and esters. Propolis has been used widely for its antimicrobial activities against a wide range of microorganisms and also because of its anti-inflammatory, anesthesia, antioxidant, anti-tumor, anti-ulcer and liver protection activities.

Several studies have shown that propolis has deterrent effect on at least 21 bacterial species, 9 species of fungi, 3 species of protozoa and a wide range of viruses (Sforcin, 2007). These studies have identified the effect of propolis on pathogenic fungi including different species of dermatophytes and *Candida* (De.Azevedo et al., 1986; Murad et al., 2002; Salatino, 2005).

Studies which have been carried out in Iran are very limited, but in the study of Avijegan et al. (2006), the fungal effect of the extract of *Echinophora platyloba* on a number of dermatophytes was examined and its effect on *Trichophyton schoenlinii* and *Trichophyton verroocusom* was desirable.

In fact, dermatophytosis or ringworm is the most important and prevalent fungal skin disease. The distribution of the dermatophytosis is worldwide so that 10% of the population of world is infected by dermatophyte infections. Dermatophytes are transmitted by direct contact by infected human, animal or soil. Based on microscopic features, dermatophytes are divided into three genus: *Microsporum*, *Epidermophyton* and *Trichophyton*. They are keratinophilic fungi which cause infection in keratinous tissues like skin, nail and hair. According to the existing reports, more than 100 million dollars have been spent only in U.S annually for the drug treatment by griseofulvin. Developing countries also spend a lot of money for the treatment. Therefore, by doing such a research to achieve nonsynthetic and natural drugs we can prevent many complications of chemical drugs, and also brings about economic savings (Lui, 2002).

According to the studies, propolis is non-toxic and it has no side effects, hence people can use it in concentration of 1/4 mg kg or equivalent to 70 mg per day. After treatment with different concentrations of propolis and its extracts, it does not cause any changes neither in the total concentration of fat, triglycerides, cholesterol and high density lipoprotein (HDL) cholesterol nor in the specific activity of low density lipoprotein (LDH) and aspartate aminotransferase (AST) (Fernandes et al., 2007).

In this study, we examined the effect of alcoholic extract of propolis on the growth of three species of *Epidermophyton flucosum*, *Trichophyton tonsorans* and *Trichophyton violaseum*. These species were taken from the patients with dermatophytosis and examined in the culture medium sabouraud chloramphenical cycloheximide agar (SCC) which was made in the QUELAB Company of UK.

MATERIALS AND METHODS

Sampling of patients

At first, patients with dermatophytoses were sampled and the samples were inoculated in the culture medium of SCC. After the growth of colony, macroscopic, microscopic and physiological characteristics of the colony were used to determine their genus and species, then three species of *E. flucosum*, *T. violaseum* and *T. tonsorans* were isolated.

Preparing extract

It has been found that the biological activities of a sample depend on a methodology which is used for preparing the extract of the sample. The most common materials used for the preparation of the extract in biological method are ethanol, methanol and water (De Vecchi and Drago, 2007). In this study, the samples of propolis were grinded and for preparation of extract, 10 g of them were weighed carefully. Then they were poured in 250 ml balloon and by adding ethanol 96%, the volume of sample was reached to 100 mm, then the mixture was stirred very well. For 3 days, this practice was repeated once or twice every day. The mixture was kept in a warm and dark place for 1 to 2 weeks. After this period of time, the mixture was filtered and was placed in a refrigerator at the temperature of 1 to 4°C for 1 day so that the solution was filtered again and the obtained extract was kept in a dark and impervious container. The remaining alcohol in suspension was completely separated by Soxhlet apparatus and this pure alcoholic extract was obtained. The procedure was in the way that alcoholic solution was put in a cylindrical tube under which there was a filter case. Soxhlet apparatus consists of two output tubes; one of them is attached to faucet and the other to vacuum pump. Owing to the fact that, this solvent of alcohol evaporates in a temperature below the temperature for water evaporation, within a few hours, it was completely isolated from the solution and alcoholic extract of propolis was collected in the case.

Preparing dilutions from the crude alcoholic extracts

First of all, 800 mm of the culture medium of SCC was selected and then about 100 mm of it was divided into 8 separate Erlenmeyer flasks. In the next stage, the alcoholic extract of propolis was added to the first to seventh Erlenmeyer flasks in amounts of 20, 10, 5, 2/5, 1/25, 625 and 312% mm, respectively. Therefore, dilutions of 0/2, 0/1, 0/05, 0/025, 0/0125, 0/00625 and 0/00312 per mm of the culture medium were obtained. The alcoholic extract of propolis was not added to the eighth Erlenmeyer flask and it was used as control. All of the Erlenmeyer flask**s** were sterilized 15 min in the autoclav**e** with temperature of 121℃ and pressure of 15 pound p er square inch (lb/in^2). Then the contents of each Erlenmeyer flask were equally divided in 10 Petri dishes thus in every experiment 80 Petri dishes of culture medium of SCC which had different dilution of the extract of propolis were obtained.

The culture of fungal colony of the species of *T. tonsorans*, *E. Flucosum* and *T. violaseum* in the culture medium of SCC obtained from the samples of clinical patients was inoculated in the center of Petri dishes containing different dilutions of alcoholic extract of propolis. In this way, each species was inoculated in seven dilutions of the existing extracts in culture medium and then they were kept in incubator with the temperature of 25℃.

Daily inspection and examination of the growth`s rate of fungal colony in the culture medium contains different dilutions of propolis's extract.

All inoculated Petri dishes were inspected every day and after observing the first evidences of growth, the diameter of fungal colony for each fungal strain and dilution was separately measured and recorded by digital calipers (Mitutoyo, made in Japan).

RESULTS

All Petri dishes containing different dilutions of alcoholic extract of propolis which was inoculated with fungi were kept in incubator for 1 week in the temperature of 25℃. After a week, the rate of colony`s growth were examined and the diameter of their colony was measured. The rate of changes were recorded in the Table 1 daily.

As shown in Table 1, *T. tonsorans* in dilutions of 20, 10, 5, 2/5 and 1/25 ml of alcoholic extract of propolis in 100 ml of culture medium of SCC, was observed with no growth but in dilutions of 0/625 and 0/312 after a week of incubation in the temperature 25℃, colonies with t he diameter of 2/8 and 4/36 were formed, respectively. It means that by reducing the concentration of alcoholic extract in culture medium, the rate of colony`s diameter increased. Therefore, the minimum inhibitory concen-tration (MIC) of growth for *T. violaseum* was determined.

About *E. flucosum* in dilutions of 20, 10, 5, 2/5, 1/25 during all days of examination, no colony`s growth was observed. But in dilution of 0/625 and 0/312 after a week of incubation, colonies with diameter of 2/17 and 3/22 cm were formed. MIC of growth for *E. flucosum* was determined equal to 1/25.

DISCUSSION

Several groups of researchers have focused on the biological activities of the constituent components of propolis. Propolis is composed of compounds such as gum, wax, volatile oil and pollen. One of the most extensive subjects to research about propolis is its antimicrobial properties that has been found by many researchers which proved the effect of propolis on different bacterial species and other microorganisms. One of the most important goals in this study was examining the effect of alcoholic extract of propolis on pathogenic dermatophytes to benefit from herbal medicines with fewer side effects to treat diseases resulting from dermatophytes. So, to fulfill this goal further researches are necessary.

Various studies have shown that after prescribing propolis to mice or human, it does not cause any side effects and according to Brudock (1998), the scope of DL 50 of propolis is from 2 to 7/3 g/kg in mice, but he suggests that safe concentration for human is about 1/4 mg/kg/day or about 70 mg/day. After treatment with different concentrations of propolis (1,3,6 mg/kg/day), different extracts (water or ethanol) and prescribed time (30,90 and 180 day), no particular change was observed neither in total concentrations of lipids, triglycerides, cholesterol and HDL cholesterol nor in specific activities of LDH and AST.

Although after using propolis, contact dermatitis and allergy have been reported in very few cases. The ethanol and water extract of propolis have an anti-allergy activity that prevent it from releasing histamine in mast cells. But concentrations higher than 300 mg of propolis activate mast cell indirectly and increase the release of inflammatory mediators which can lead to allergy in people who are sensitive to propolis. It has been determined that antimicrobial activity of propolis might be due to direct action on microorganisms (Sforcin, 2007).

The required propolis was prepared from Chadegan (a city located in Esfahan Province). The most common extracts which are used in biological methods are the extracts of ethanol, methanol and water with different concentrations (Tosi et al., 1996). In this study, we used ethanol extract of propolis with the concentration of 10%.

Studies have shown that propolis has anti-bacterial, anti-protozoan, anti-viral and antifungal properties in large amounts (Koc, 2005; Sforcin, 2007; Fernandes et al., 2007). It has been clear that despite the important effect of propolis on inhibiting the growth of gram-positive bacteria, it has little effect on gram-negative bacteria (Koc, 2005).

Studies on pathogenic fungi indicate that propolis has a large effect on fungal diseases particularly candidiasis (De.Azevedo et al., 1986; Murad et al., 2002).

In this study, we used methanol extract of propolis and we observed proper effects of propolis on inhibiting the growth of *E. flucosum*, *T. violaseum* and *T. tonsorans*. In another study, antifungal activity of propolis on different species of *Candida* was examined and the sensitivity of *Candida albicans* with regard to other species of *Candida* such as *Candida tropicalis* was reported more (Salatino, 2005).

Table 1. The rate of colony`s growth of *E. Flucosum*, *T. violaseum* and *Trichophyton tonsorans* in the culture media containing different delusions of alcoholic extract of propolis during 10 consecutive days in the terms of cm.

Dilution		0.312	0.625	1.25	2.5	5	10	20
Diameter of fungi	**Day**							
T. tonsorans	1	4.35	2.89	-	-	-	-	-
	2	4.89	3.05	-	-	-	-	-
	3	5.09	3.26	-	-	-	-	-
	4	5.30	3.59	-	-	-	-	-
	5	5.59	3.89	-	-	-	-	-
	6	5.89	4.25	-	-	-	-	-
	7	5.89	4.16	-	-	-	-	-
	8	5.89	4.16	-	-	-	-	-
	9	5.89	4.16	-	-	-	-	-
	10	5.89	4.16	-	-	-	-	-
T. violaseum	1	2.12	1.14	-	-	-	-	-
	2	2.50	1.39	-	-	-	-	-
	3	2.98	1.71	-	-	-	-	-
	4	3.60	2.26	-	-	-	-	-
	5	4.10	2.79	-	-	-	-	-
	6	4.97	3.74	-	-	-	-	-
	7	4.97	3.74	-	-	-	-	-
	8	4.97	3.74	-	-	-	-	-
	9	4.97	3.74	-	-	-	-	-
	10	4.97	3.74	-	-	-	-	-
E. flucosum	1	3.21	2.39	-	-	-	-	-
	2	3.59	2.52	-	-	-	-	-
	3	3.69	2.70	-	-	-	-	-
	4	3.81	2.78	-	-	-	-	-
	5	3.89	3.1	-	-	-	-	-
	6	3.93	3.1	-	-	-	-	-
	7	3.93	3.1	-	-	-	-	-
	8	3.93	3.1	-	-	-	-	-
	9	3.93	3.1	-	-	-	-	-
	10	3.93	3.1	-	-	-	-	-

Koc (2005) has pointed out the antifungal effect of propolis in his research. He examined the antifungal effect of ethanol extract of propolis on *Trichophyton* mentagrophytis and *Trichophyton rubrum* in comparison with other antifungal drugs and then introduced propolis as a substance with effective antifungal activity (De Vecchi and Drago, 2007).

In our study, three species of *E. flucosum*, *T. violaseum* and *T. tonsorans* were also examined and the results indicated that propolis of bee has an excellent antifungal property against all these three species. In this study, MIC for these three species of *E. flucosum*, *T. violaseum* and *T. tonsorans* was determined equal to 0/0125 mm per each mm of culture medium.

Conclusion

Considering the obtained results, it is clear that the ethanol extract of propolis can be used against the examining dermatophytes in different concentrations. And also, with regard to the fact that the MIC of the three species has equal value, it is necessary to carry out studies on laboratory creatures in *in vivo* conditions, so that the profits and the side effects of this substance can be examined carefully.

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