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Research Article

Inhibition Effect of Different Propolis Extracts against *Fusarium solani in vitro*

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Abstract
Propolis is a natural by-product created by honey bees and has been widely tested against various fungal pathogens causing damage to agricultural products. The diluent used in the extraction of propolis causes differences in the total phenolic compounds and antimicrobial properties of propolis. *Fusarium solani* is infectious to many cultivated plants, resulting in significant crop losses. In most plants, it leads to rot, wilt and necrotic spots and eventual plant death.
In this study, the antifungal effect against *F. solani* of propolis collected from Bingöl province of Turkey was evaluated using a total of 9 groups of applications. In order to determine the differences between preparations and the critical dose levels, 3 different preparations (Ethanol, (Dimethyl sulfoxide (DMSO), and pure propolis purified by supercritical fluid extraction method) were applied in 3 different concentrations (100µl, 200µl, and 600µl) in PDA medium.
The statistical results of the study showed that pure extracts of propolis are more effective at increasing doses (around 30 mm for 600 µl) against the mycelial growth of pathogen. Furthermore, ethanol extracts of propolis showed a moderate antifungal effect (around 33 mm for 600 µl), while the DMSO extracts were less effective (around 58 mm for 600 µl). This study is the first report showing the fungicidal activity of pure propolis purified by the SFE method against *F. solani*. The results of this study may shed light on the development of drug-based strategies against various fungal-borne phytopathogens in the future studies.

Keywords: *F. solani*, DMSO, ethanol, pure propolis, Turkey, antifungal activity

Farklı Propolis Ekstraktlarının *Fusarium solani*'ye Karşı Antifungal Etkisi

Öz
Propolis, bal arıları tarafından oluşturulan doğal bir yan üründür ve tarımsal ürünlerde zarara neden olan çeşitli fungal patojenlere karşı yaygın olarak test edilmiştir. Propolisin ekstraksiyonunda kullanılan seyreltici, propolisin toplam fenolik bileşikleri ve antimikrobiyal özelliklerinde farklılıklara neden olur. *Fusarium solani* birçok bitki türü için infeksiyözdür ve önemli ürün kayıplarına neden olur. Çoğu bitkide çürüklük, solgunluk, nekrotik lekeler ve sonunda ölüme yol açar.
Bu çalışmada, Türkiye'nin Bingöl ilinden toplanan propolisin *F. solani*'ye karşı antifungal etkisi toplam 9 uygulama grubu kullanılarak değerlendirilmiştir. Preparatlar ve kritik doz seviyeleri arasındaki farkları belirlemek için, 3 farklı preparasyon (Etanol, (Dimetil sülfoksit (DMSO) ve süperkritik sıvı ekstraksiyon yöntemi ile saflaştırılmış saf propolis) PDA ortamında 3 farklı konsantrasyonda (100µl, 200µl ve 600µl) uygulanmıştır.
Çalışmanın istatistiksel sonuçları, saf propolis ekstraktlarının artan dozlarda (600 µl için yaklaşık 30mm) patojenin misel büyümesine karşı daha etkili olduğunu gösterdi. Ayrıca, propolisin etanol ekstraktları orta düzeyde bir antifungal etki gösterirken (600 µl için yaklaşık 33mm), DMSO ekstraktları ise daha az etkiliydi (600 µl için yaklaşık 58mm). Bu çalışma, *F. solani*'ye karşı SFE yöntemiyle saflaştırılan saf propolisin fungisidal aktivitesini gösteren ilk rapordur. Bu çalışmanın sonuçları, ileride yürütülen çalışmalarda çeşitli fungal kaynaklı fitopatojenlere karşı ilaç temelli stratejilerin geliştirilmesine ışık tutabilir.

Anahtar Kelimeler: *F. solani*, DMSO, etanol, saf propolis, Türkiye, antifungal aktivite

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1. Introduction

Propolis (bee glue) is a natural sticky substance produced by honey bees (Apis mellifera) using resinous material collected from the buds and cracks of mainly poplar, horse chestnut and coniferous trees, beeswax and its salivary and enzymatic secretions (Gardana et al., 2007; Velikova et al., 2000). Propolis is primarily used by honey bees to cover cracks in the hive, to regulate humidity and temperature, to mummify large invading pests, as well as to protect the colony against pathogens such as bacteria, fungi, and viruses. In particular, the microbial defensive property of propolis and its success in preventing the decay and rotting of pests inside the hive has attracted the attention of many researchers in various scientific fields (Rufatto et al., 2017; Drescher et al., 2017). It has been reported that the chemical composition of propolis consists of more than 300 bioactive compounds depending mainly on environmental factors, the geographical areas, the season, and harvesting periods (Reference). The chemical composition of propolis consists of more than 300 bioactive compounds, including phenolic compounds (flavonoids and phenolic acids) and esters, alcohols, aldehydes, ketones, terpenes, coumarins, steroids, amino acids, inorganic substances, vitamins, many fatty acids and enzymes (Dias et al., 2012; Huang et al., 2014; Kustiawan et al., 2017).

The activities of antimicrobial, pharmacological, antioxidant, anti-inflammatory, healing, cytotoxic, and anti-tumoral of propolis were supported by numerous laboratory and clinical analyzes. Most of these bioactive properties are due to flavonoids and phenolic acids, which are phenolic compounds in propolis (Velazquez et al., 2007; Anjum et al., 2019). The ethanol preperation of propolis are widely used for antimicrobial purposes to date, different solvents such as methanol, DMSO (Dimethyl sulfoxide), water, acetone, ethyl acetate, olive oil, chloroform can also be rarely used for extraction purposes. The antifungal activity of propolis have mostly been demonstrated against various fungi types such as yeast and phytopathogen fungus using broth microdilution, agar well diffusion, agar dilution, and disc diffusion methods (Ota et al., 2002; Shehu et al., 2016; Gür et al., 2020). Ethanol extracts of propolis (PEE) were tested against yeast isolated from oncomyosis disease by Oliveira et al.,(2006). Propolis extracts showed the inhibition effect at dose-dependent manner by causing cell death against all yeasts (*Candida parapsilosis*, *C. tropicalis*, *C. albicans*, and *Trichosporon* spp), indicating that antifungal effect of propolis as a byproduct with can be considered another option in yeast treatment. Similarly, the inhibition ability of four honey bee products (honey, propolis, royal jelly, pollen) was evaluated against *Candida* spp. and *Trichosporon* spp. This study revealed that propolis and pollen are particularly successful in the management of fungal strains resistant to fluconazole derived fungicides (Koç et al., 2011). Until now, numerous documents have been reported regarding the anifungal effect of propolis against phytopathogenic fungi causing crop loss. Er (2021) reported that ethanol extracts of propolis (PEE) and water-based propolis (WBP) have high antimicrobial capacity for fungi F. graminearum, Alternaria brassicicola, Verticillium dahliae, and Pythium ultimum, with an inhibition rate of about 97%. Furthermore, it has been reported under in vivo conditions that propolis preparations of different concentrations of propolis significantly suppress the severity, rate and development of disease caused by fungi (*Podosphaera fuliginea*, *Botrytis cinerea*, *Rhizoctonia solani*, *Penicillium digitatum*, and *Sclerotium rolfsii*) in agriculturally important crops such as bean, grapevine, cucumber, and strawberry (La Torre et al., 1990; Özdemir et al., 2010; Guginski-Piva et al., 2015; Abd-El-Kareem et al., 2018). Its antifungal effect has also been confirmed against various fungi under in vitro conditions that reported by Curifuta et al., (2012), Araujo et al., (2016), Meneses et al., (2009), Pazin et al., (2019).

Fusarium spp, a member of the Ascomycota phylum with wide host range, is capable of causing disease in many crops of agricultural importance as well as human tissues. The genomes of most fungi of this genus encode host-specific virulence factors, which interferes with the physiological balance of the host resulting in necrosis and disruption of cell integrity (Porto et al., 2019). *Fusarium solani*, which has the potential to form colonies in soil and plants, is a species complex of more than 26 filamentous fungi in the family Nectriaceae (Summerell et al., 2010). Many methods such as biological control, fungicides, use of aromatic oils and other methods have been tried to minimize the product loss caused by the *Fusarium* genus and other soil-borne pathogens in cultivated crops (Erdoğan et al., 2014; Erdoğan et al., 2016; Koç et al., 2018).

In this present study, we aimed to determine and compare the effects of solutions extracted by Ethanol (70%) and DMSO (1%) of propolis and pure propolis obtained from Bingöl province of Turkey against the destructive fungus *F. solani* in a dose-dependent manner..

2. Material and Method

2.1. Isolation of fungal pathogen and preparation of the inoculum

F. solani, isolated from the infected parts of the common bean plants (*Phaseolus vulgaris* L.) and confirmed by morphological characterization, was selected as the pathogen isolate collected from Bingöl province in 2019 and used for experimental purposes in this study. *F. solani* isolate was kept for incubation at 25 °C for 7 days to cover the PDA surface fully after being planted into PDA medium.

2.2. Preparing Propolis Solutions

Ethanol and DMSO solvents were used to prepare the extracts of propolis collected from Bingöl province beekeepers. For ethanol extracts of propolis (PEE), 100 g of propolis ground by using a blender device was desolved in 400 ml of 70% ethanol for 15 days, filtered, and stored until use at -20 °C. Pure extracts of propolis (PEP) was obtained by Supercritical fluid extraction (SFE) method using CO2 solvent (Yamani et al., 2007; Porta, 1999; Pourmortazavi, 2004). Accordingly, 5.8 grams of pure propolis was recovered from 150 grams of raw propolis. Homogenization was achieved by vortexing with 1:1 ratio of ethanol and pure propolis. To obtain the DMSO extracts of propolis (PEDMSO), 1/4 ratio was used for propolis and DMSO (1%), incubated for 15 days at room temperature with occasional inversion, then the mixture was filtered to obtain extract.

2.3. Preparation of solid growth medium for antifungal tests and treatment groups

Potato Dextrose Agar (PDA) and dH2O, which are common media for fungi, were used for the isolation and growth of fungus isolates. The semi-liquid PDA medium containing 2.39 g of PDA and 60 ml of dH2O was autoclaved at 121 °C for 15 min and then poured to sterile 100 mm glass petri dishes after cooling to 45 °C. In order to establish the control and treatment groups of propolis, 600 µl, 200 µl, and 100 µl doses of 70%

ethanol only, 1% DMSO only, PEE, PEDMSO, and PEP solutions were added to sterilized PDA media and allowed to solidify (Çakar et al., 2021).

2.4. Determination of the inhibition efficiency of propolis and Statistical analysis

Eight mm diameter mycelial discs taken from PDA medium were cutting out and immediately placed in the center of petri dishes containing PDA media prepared before for all groups. To test the antifungal activity and prevent contamination by other pathogens, petri dishes were tightly covered with parafilm. After the petri dishes were incubated for 7 days at 24 ± 1ºC, the colony diameters were calculated by measuring the mycelium lengths in vertical and horizontal directions using ruler. Experimental outputs were recorded (Benjilali et al., 1984). The percentage inhibition rates of propolis preparations were evaluated based on the formula reported by Deans & Svoboda (1990). All antifungal experiments were conducted with 3 replications. Statistical significance of the treated groups mean with that of non-treated groups were evaluated by SPSS 17 package program. The ANOVA analysis was used to idetify the differences between the groups in the study followed by Duncan's multiple range tests to separate means. Differences were considered statistically significant if $P<0.05$. Percent inhibition (PI) of propolis solutions was calculated based on Equation 1.

$$\text{Inhibition (\%)} = \frac{gc - gt}{gc} \times 100$$

Where; gc, Mycelial colony diameter measured in the control set after the incubation period, ignoring the inoculum disc diameter; gt, Mycelial colony diameter measured after the incubation period, ignoring the inoculum disc diameter.

3. Results

Here, three different solutions of propolis were tested to determine their antifungal activity against the pathogen *F. solani.* The percentage inhibition rates and inhibition zone measurement of propolis extracts were evaluated statistically using SPSS program (Table 1).

Table 1. Statistical analysis data showing the antifungal activity of different propolis extracts against *F. solani*

No	Treatments		Average fungus diameter (mm)
1	NT (Only fungus)	Control Groups	77±1,155^{h}
2	Only DMSO (100µl)		74,67±2,333^{gh}
3	Only DMSO (200µl)		73,67±2,331^{gh}
4	Only DMSO (600µl)		72,67±1,453^{g}
5	Only Ethanol (100 µl)		71,67±1,667^{g}
6	Only Ethanol (200 µl)		67±0,577^{f}
7	Only Ethanol (600 µl)		64,67±0,882^{f}
8	PEDMSO (100µl)		59±0,577^{e}
9	PEDMSO (200µl)		59±0,577^{e}
10	PEDMSO (600µl)		58,33±0,882^{de}
11	PEE (100µl)		54,67±2,603^{d}
12	PEE % (200µl)		45±1,155^{c}
13	PEE % (600µl)		33,67±0,333^{ab}
14	PEP (100µl)		45,67±1,202^{c}
15	PEP (200µl)		37,33±1,453^{b}
16	PEP (600µl)		30,33±1,453^{a}

*a, b, c, d, e, f, g, h; means the difference between the averages with same letters in the same column is no significant, but different letters are significant ($p<0.05$); PEE: ethanol extracts of propolis; PEDMSO: DMSO extracts of propolis; PEP: Pure extracts of propolis; DMSO, dimethylsulphoxide; NT, no treatment.

As given in Table 1, we found that all solutions of propolis negatively affected fungal growth to some extent, which was supported by statistical analysis. Based on dose increase, most effective antifungal effect was statistically determined in PEP extracts within all treatment groups, followed by PEE and, PEDMSO, compared to control treatments. Statistically, no inhibition activity was observed at any dose of solvent treatments. While PEP applications showed an strong inhibition effect (approx. 30 mm for 600 µl), PEDMSO demonsrated minimal inhibition effect against fungal pathogen *F. solani*, up to approx. 58 mm for 600 µl.

Analyzes of percent inhibition zone rates were consistent with statistical results (Table 2). According to the results of the analysis, inhibition percentages were determined as 60.61% for PEP, 33.27% for PEE, and 25.25% for PEDMSO in 600 µl dose. Generally, it was determined that propolis solvents exhibited dose-dependent increased antifungal activity. Although the solvents alone showed slight activity on the fungal diameter, this was considered statistically insignificant.

Table 2. Chart showing the inhibition incidence of different propolis extracts against *F. solani*

Treatment	Inhibition Incidence (%)
Control (Only Fungus)	0
Only DMSO (100µl)	3,02
Only DMSO (200µl)	4,32
Only DMSO (600µl)	6,49
Only Ethanol (100 µl)	6,92
Only Ethanol (200 µl)	12,99
Only Ethanol (600 µl)	16,88
PEDMSO (100µl)	23,38
PEDMSO (200µl)	23,38
PEDMSO (600µl)	25,25
PEE (100µl)	29
PEE % (200µl)	41,56
PEE % (600µl)	33,27
PEP (100µl)	40,69
PEP (200µl)	51,52
PEP (600µl)	60,61

4. Discussion

F. solani is an important plant pathogen and soil saprophyte causing vascular wilt and root rot in many agroeconomic crops as well as animal organisms (Kriaa et al., 2015; Pérez-Hernández et al., 2020). To date, many chemical and biological treatment strategies such as fungicide-based applications, soil solarization, and biosolarization have been tried to eradicate this pathogen (Pérez-Hernández et al., 2014). Various preparations were evaluated by Ganesh & Dwivedi (2018) who reported that different doses of Carbendazim (a widely used systemic fungicide), *Trichoderma viride* (biological control agent), and *Thuja occidentalis* extract (a tree from the Pinales order) inhibited the mycelial growth of *F. solani*.

Recently, the antimicrobial properties of propolis have become a popular topic emprically tested against numerous bacterial, fungal and protozoan agents of animal and plant origin. Plus, extensive studies on studies related to pharmaceutical and human health effects have also gained value, mainly due to phenolic compounds such as flavonoids,
phenolic acids, derivates of caffeic acids, and other compounds such as terpenoids contained in propolis (Marini et al., 2012; Mavri et al., 2012; Falcão et al., 2014; Pereira et al., 2017; Alenezi et al., 2018).

The antimicrobial effect of PEE has been widely used against a variety of pathogens when compared to other solvents. Ertürk et al., (2011) different solvent extracts of propolis including acetone, ethyl acetate, chloroform, ethanol, methanol, DMSO, and water tested against fifteen microorganisms including fungus *C. albicans* and other bacteria using disk diffusion and Minimal Inhibition Concentration (MIC) method. According to this study, the weak activity of DMSO extracts was determined against some tested organisms, while other extracts showed high antimicrobial effect against all organisms except water extracts. This outputs shows that DMSO is not a effective solvent against some microorganisms, just like its moderate effect against *F. solani* in our study. Ugur & Aslan (2004) reported that PEE exhibited an antimicrobial effect on the growth of *C. albicans* at increasing doses compared to acetone extracts of propolis. Similarly, Ghasemi et al., (2017) recorded that PEE have a broad spectrum antibacterial activity against Gram-positive and Gram-negative bacteria compared to DMSO solutions. In another study using DMSO as diluent, it was determined that the various fungal pathogens (*Aspergillus fumigatus*, *Microsporum gypseum*, *M. canis*, and *C. albicans*) tested displayed a significant sensitivity in concentration-dependent response to DMSO-derived propolis suspension (Netíkóva et al., 2013), harmony with our results indicating the antifungal effect of propolis. The effectiveness of human pathogenic fungi (eight strains) against PEE and pure propolis was evaluated by Buchta et al., (2011). Thanks to the high flavonoid content of propolis, especially PEE had a negative effect especially on *T. mentagrophytes* and *C. albicans*.

Propolis derived preperations were also tried against some phytopathogenic fungi worldwide and nationally *in vitro* and *in vivo*, confirming its antifungal effect. Özyiğit (2020) reported that spore germination and mycelial growth of mold fungi (*A. flavus*, *A. niger*, *A. oryzae*, and *P. digitatum*) are susceptible to PEE at different concentration. *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *R. solani*, and *Macrophomina phaseolina* causing wilting, root, and root rot disease in tomatoes are assessed to determine the antifungal activity of PEE using agar dilution method *in vitro* conditions. Mycelial growth of all agents was significantly inhibited compared to control plates (Gül, 2019). This antifungal effect, which is higher in our study, may be due to the fact that the content of propolis varies significantly according to geographical origins or the fungal pathogen used reacts differently to propolis.

Studies revealing an inhibition relationship between propolis and *Fusarium* spp. are poorly studied that was reported for the first time by Kim et al., (2019) using the paper disc approach and resulted in the strong inhibition effect of propolis on a dose-based basis against the fungi studied (*F. solani*, *Rhizoctonia solani*, and *P. ultimum*), which is consistent with the results obtained from that of *F. solani* in our study. In Brazil, the

myclial growth of *F. proliferatum*, known as a plant and human pathogen, was inhibited by more than 70% by PEE, and this antifungal activity was associated with total flavonoid and antioxidant compounds found in propolis (Gregolin et al., 2019). Likewise, same extract absolutely inhibited the radial growth process of the fungus *F. oxysporum* (Ahmed et al., 2008; Petruzzi et al., 2020; Türk, 2017). These antifungal effects, which is higher than our study, may be due to the fact that the content of propolis may vary significantly depending on the geographical origin or that propolis reacts differently to the fungal pathogen used (*F. solani*).

According to Al-Ani et al., (2018), propolis has a moderate antifungal effect, while as reported by Özcan (1999), a concentration of 4% propolis can reduce the growth of *F. oxysporum* f. sp. *melonis* by up to 50%. These literatures associated with *Fusarium* spp are particularly consistent with the results of the PEE used in our study. The antifungal effect exhibited in certain proportions is due to effective compounds such as flavonoids and phenolics in propolis, which are responsible for disrupting cell membrane permeability, resulting in the loss of inorganic anions and cations such as nucleic acids, proteins and phosphate and potassium in their intracellular content, resulting in cell death (Shehu et al., 2016; Farnesi et al., 2009).

The mycelial growth of certain phytopathogenic fungi (*A. flavus*, *B. cinerea*, *A. tubingensis*, *Cladosporium cladosporioides*, *V. dahliae*, *Fulvia fulva* and *P. digitatum*) were suppressed by PEE *in vitro* PDA medium at increasing concentrations, even at low concentrations (Kurt & Şahinler 2003; Ezazi & Davari, 2018). Quiroga et al., (2006) showed that the two compounds in propolis (pinocembrin and galangin) are destructive and cytotoxic as synthetic drugs for some crucial fungi species (Xylophagous fungi, and yeast strains) and phytopathogenic fungi (*A. niger*, *Fusarium* spp., *Macrophomina spp*., *P. notatum*, *Phomopsis* sp., and *Thichoderma* spp.) that cause damage to many crops in the agroecosystem.

The antifungal effect of PEE against *Stemphylium vesicarium*, the causal agent of brown spot disease of pear, was demonstrated by controlling the mycelial growth of the fungus (Loebler et al., 2020). The antifungal effects of Colombian propolis were evaluated *in vitro* by Meneses et al., (2009), the results indicated that two major post-harvest diseases in papaya, avocado and mango, anthracnose and stem-end rot, are susceptible to EPEM (n-hexane/methanol extracts) and dichloromethane solutions of propolis in PDA culture media.
Four different preparations (0.5, 1.0, 2.5 and 5.0%) of the PEE showed an inhibition effect using the agar dilution method against agriculturally important fungi (*A. alternata*, *Fusarium* spp., *Ulocladium* spp., *B. cinerea*, *P. expansum*, and *T. reesei*) (Curifuta et al., 2012). Dumping off, late blight, root and crown rots disease (caused by *Phytophthora infestans*, *P. capsici*, and *P. parasitica*), which has agronomic importance, are extremely destructive for solanaceous and cucurbitous crops. In a study using methanol extracts of Turkish Propolis, it was reported that four different preparations (10, 7, 5, and 3 μg mL^{-1}) completely inhibited or even killed the mycelial growth of all fungi (Yanar et al., 2005).

5. Conclusions and Recommendations

Propolis is a highly popular honey bee product that has been tested against various microorganisms. In this study, we obtained extraction with three different methods and aimed to determine the antifungal effect against *F. Solani*. Based on the experimental and statistical results of three different concentrations, we determined that pure extracts of propolis (PEE) obtained by SFO method using CO_2 were more effective at dose-depended manner, followed by the ethanol and the DMSO extract. Our results indicate that solutions of propolis can be used as a plant protection product against the detrimental wilt agent *F. solani* for agricultural crops.

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