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The *In Vitro* Antifungal Evaluation of Propolis Extract against *Botrytis cinerea* Pers.

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Abstract*—Botrytis cinerea* is one of the most economically damaging pathogens of vegetables and fruits, both in the field and in storage. Synthetic fungicides used against pathogens threaten both human health and nature and cause resistance. One of the alternative methods against disease agents is the eco-friendly product propolis. The aim of this study was to investigate the inhibitory effect of ethanolic extract of Propolis (EEP) against Botrytis cinerea Pers. isolates (ET 33, ET 63, and Hatay) under laboratory conditions. Inhibitory activity of EEP was done by contact phase technique against the pathogen. For this purpose, at first, EEP collected from Muğla province was prepared using 80% ethanol. Then 0.25, 0.50, 1, 2.5, and 5µL mL^{-1} concentrations of this EEP were provided and added to potato dextrose agar (PDA) media. Petri plates containing different concentrations of EEP were inoculated with mycelial disks of fresh fungal cultures and incubated at 25 ± 1ºC until the colony in the control treatment filled the petri dishes. The experiments were carried out in a randomized plot design with three replicates. In addition, propolis was analyzed by high-performance liquid chromatography (HPLC). According to the HPLC analysis of the propolis, phenolic chemicals such as galangin, pinocembrin, quercetin, chrysin, and naringenin were found in significant amounts. EEP was found to inhibit the growth of B. cinerea isolates in a dose-dependent manner. The highest antifungal effect against B. cinerea (ET 63 isolate) was detected in the high-dose (5 µL mL^{-1}) application of EEP, 53.9%. Also, EEP has a low antifungal effect against isolates of B. cinerea ET 33 and Hatay. In conclusion, the study suggests propolis as a potent natural antifungal agent against B. cinerea.*

***Keywords*—** *Botrytis cinerea, Propolis, Antifungal activity, Phenolic compounds, Non-chemical control*

I. INTRODUCTION

Among *Botrytis* species, *Botrytis cinerea* Pers. (teleomorph: *Botryotinia fuckeliana*) has been documented to infect more than 200 host plant species, such as grape, strawberry, apple, kiwi, raspberry, blackberry, fig, citrus fruits, plum, tomato, cucumber, pepper, onion, bean, lettuce, artichoke, and ornamental plants (Elad and Stewart, 2004). *Botrytis* spp. are polyphagous fungi with diverse ecology, biology, morphology, and host spectrum (Elad et al., 2007). The pathogen is a necrotrophic fungus that can infect several parts of the host plant from the stage of seedling to the fruit ripening stage (Yahaya et al., 2015). Botrytis, also known as gray mold, is a genus of anamorphic fungi belonging to the hyphomycetes class with about 28 species of pathogenic importance (Dewey and Grant-Downton, 2016).

The pathogen enters the host plant through wounded tissues. It survives on plant residues and in the soil in various forms such as conidia, mycelium, and sclerotia. Fungus spores formed on diseased plant parts are easily spread by irrigation water, wind, agricultural equipment and insects. The conidia of the pathogen germinate in the presence of water and dew, forming germ tubes and entering the plant tissues through wounded tissues. Disease symptoms usually start on the leaves as brown spots or patches, which change into a grayish, furry mold (Williamson et al., 2007). Especially, young plants keel over and die very quickly once affected. Over-watering, over-crowding, and over-feeding are conditions that encourage molds growth. The pathogen can continue to cause disease on fruits after harvest as well as before harvest. Among *Botrytis* species, *B. cinerea* is the most studied and frequently observed species. This pathogen has attracted a lot of attention and has become a model pathogen, especially in the control against *Botrytis* species. Due to the increasing damage caused by *B. cinerea* and the high degree of extension to different botanical families of plants, the pathogen was given more importance in recent times (Drobota and Drobota, 2008). Currently, little information exists on the damage and cost of Botrytis diseases. However, the pathogen has been reported to cause annual losses of $10 billion to $100 billion (Boddy, 2016).

Cultural, chemical, and biological control methods can be used against the disease agent. Several synthetic fungicides, such as boscalid, cyprodinil, penthiopyrad, pyrimethanil, fenhexamid, fluopyram, imazalil, isofetamid, and adepidyn, are used in the chemical control of *B. cinerea* (Bardas et al., 2008; Avenot and Michailides, 2010; Grabke et al., 2013). Over a decade ago in Florida, approximately $1000 per season was spent on preharvest fungicides to control Botrytis fruit rot of strawberries (Haydu and Legard, 2003). Genescope (2002) reported that fungicides for *B. cinerea* control cost approximately €540 million in 2001 which represented about 10% of the global fungicide market.

The threat that synthetic fungicides pose to environmental and human health and the resistance of pathogens have increased the importance of alternative control methods to chemical control. Therefore, these difficulties suggest an urgent need for the exploration of other alternative measures, such as the use of biofungicides to control Botrytis disease. The need for alternative measures for *B. cinerea* control and the development of natural crop protective products to replace

synthetic fungicides are being sought by researchers and growers (Combrinck et al., 2011). Biocontrol agents and biofungicides can be good alternatives to chemical fungicides for *B. cinerea* control. These alternative practices can help meet consumers' desire for more natural and healthy foods (Martínez-Romero et al., 2008).

The number of substances in the chemical structure of propolis varies depending on various factors. As a result of studies carried out on different samples, over 300 compounds have been detected in propolis (Bankova et al., 2000). The average propolis production can vary from 10 g to 300 g per colony each year. Meanwhile, propolis production varies depending on the bees, climate, forest diversity, and trapping mechanisms. Propolis collected from the hive is raw and must be purified before use. The most practical solvent for raw propolis is 96% ethanol (Pietta et al., 2002). The composition of propolis generally consists of 5% organic compounds, 5% pollen, 10% aromatic and essential oils, 30% wax, and 50% resin (Russo et al., 2004). In addition, propolis contains some micro and macro minerals such as vitamins (A, C, D, E, and B1, B2, and B6), niacin, folic acid, iron, calcium, copper, nickel, zinc, magnesium, manganese, vanadium, strontium, and cobalt (Bankova, 2005). Aromatic acids, flavonoids, diterpenic acids, and phenolic compounds have been reported as the main components responsible for the biological activities of propolis (Silici and Kutluca, 2005).

During the last few years, significant efforts have been developed to identify natural products for controlling the diseases of crops, and the use of natural compounds, such as the ethanolic extracts of propolis, has been suggested as an approach to reduce certain phytopathogenic fungi (Giovanelli, 2008). Therefore, the use of natural products such as propolis to control fungal diseases in plants is considered a promising alternative to synthetic fungicides due to their lower negative impact on the environment (Ordóñez et al., 2011). Propolis is collected by honeybees (*Apis mellifera*) in the form of resin from plants, especially buds, stems, and leaves (Al-Ani et al., 2018). Propolis is a mixture of pollen, wax, and resin in trace amounts, ripened by the bees' salivary enzymes, and is a sticky, uniquely scented, mucilaginous substance containing significant levels of essential oils and used for many purposes within the hive (Doğan and Hayoğlu, 2012). Propolis has many beneficial biological activities as well as antibacterial, antifungal, and antiviral properties (Bankova et al., 2000). Some natural substances rich in flavonoids and phenolic acids have been reported to exhibit antifungal activity against plant pathogenic fungi such as *Botrytis cinerea*, *Aspergillus niger*, and *Alternaria alternata* (Mohammadzadeh et al., 2007; Castro et al., 2009), yet there is little work with propolis on plant pathogens. The antifungal activity of propolis extracts is usually evaluated by examining mycelium growth or spore germination. The evaluation of plant pathogen mycelium growth has been described by previous studies (Ghaly et al., 1998; Özcan et al., 2004; Erkmen and Ozcan, 2008; Soylu et al., 2008; Candir et al., 2009; Meneses et al., 2009; Yang et al., 2011; Temiz et al., 2013; Yanar et al., 2016; Embaby et al., 2019; Dudoit et al., 2020; Hosseini et al., 2020; Er, 2021; Yilmaz et al., 2023; Erdogan and Guzel, 2025). This study aims to evaluate the inhibitory activity of ethanolic extract of Propolis (EEP) *in vitro* against *Botrytis cinerea* Pers. isolates (ET 33, ET 63, and Hatay) under laboratory conditions.

II. Materials and Methods

A. Fungal Pathogen

The highly pathogenic fungal isolates used included *Botrytis cinerea* [ET 63 (strawberry), Hatay (tomato), and ET 33 (eggplant)] obtained from the Fungal Collection of Atatürk University of Agriculture Faculty, Department of Plant Protection and Fungal collection of Mustafa Kemal University of Agriculture Faculty, Department of Plant Protection, respectively (Tekiner et al., 2020; Soylu et al., 2020; Akça and Tozlu, 2022). Potato Dextrose Agar (PDA; Difco brand; 39 mL L^{-1}) was used as the medium in the study. Fungal isolates were aseptically subcultured and purified by serial transfers onto Petri dishes containing 25 mL of PDA medium. Plates were incubated in the dark at 25±1°C for 7 days, and the culture was stored at 4°C in the refrigerator.

B. Collection of Propolis Sample and Extraction

The crude propolis sample was collected from Muğla, Türkiye, in 2021 (Yilmaz et al., 2023). The propolis sample was stored in the freezer at -18°C until use. The frozen propolis sample was ground into fine powder. This ground propolis was taken and mixed with 100 mL of 80% ethanol. This mixture was kept in a dark room for a week, stirred 2 times a day during this period, and filtered with filter paper (Whatman No. 1) at the end of the period. The alcohol in the combined filtrate was concentrated by evaporation with a rotary evaporator (IKA RV10, Germany). The final mixture was kept in the dark glass at 4°C until further biological tests (Krell, 1996). An analysis of the HPLC-DAD (high-performance liquid chromatograph-diode array detection) profile of EEP was performed by using a Shimadzu HPLC-DAD system (Shimadzu Corporation, Kyoto, Japan).

C. Evaluation of Inhibition of Fungal Growth

Antifungal effects of EEP were done by the contact phase technique against *B. cinerea* isolates (Soliman and Badeaa, 2002). For determination of contact phase effect, different concentrations (0.25, 0.50, 1, 2.5, and 5 µL mL^{-1}) of EEP were added to flasks containing molten PDA (Curifuta et al., 2012). 25 mL of enriched media was poured into each plastic Petri plate (90 mm). Then, the 5 mm discs of the test fungi were cut with a mushroom drill from the periphery of 7 day-old-cultures of *B. cinerea* isolates and inoculated upside down separately onto each assay Petri plate. The plates without the EEP were used as a control treatment. The Petri dishes were sealed using parafim and incubated in the dark at 25 ± 1ºC for 7 days. Experiments were carried out with three replicates depending on a completely randomized parcel design. The diameter of developed colonies was measured when fungal mycelium covered one petri plate in the control treatment with a ruler. The inhibitory percentage (IP) was calculated by the following formula (Aminifard and Mohammadi, 2013).

$$IP\ (\%) = [(dc-dt)/dc] \times 100$$

Where dc was the mycelium diameter in a control Petri plate, and dt was the mycelium diameter in the EEP-treated Petri plate. The antifungal effect of the studied EEP was evaluated according to the following criteria: inhibition 30 to 40% means= low activity; 50 to 60% means = moderate activity; 60 to 70% means = good activity; >70% means = significant activity (Abd-Ellatif et al., 2011).

D. Statistical Analysis

The obtained data were subjected to Analysis of Variance (One-Way ANOVA), and when significant, the means were compared using the LSMeans Differences Student's test ($P \leq 0.01$). JMP IN software version 13.0 was used for the analysis.

III. Results and Discussion

A. HPLC-DAD Analysis

The HPLC-DAD analysis results of propolis extract are presented in Table I. A total of 17 major compounds were found in PE. It was determined that the propolis sample contained high levels of phenolic compounds. Depending on the presence and number of flavonoids (apigenin, galangin, kaempferol, naringenin, pinocembrin, quercetin) and aromatic compounds (caffeic acid, caffeic acid phenethyl ester, gallic acid, trans-Ferulic acid, trans-isoferulic acid, trans-cinnamic acid, 3,4-dimethoxycinnamic acid) in PE, it was found to be moderately effective against *B. cinerea* isolates (Table I).

Similar to our results, other studies have reported that propolis consists of different main components such as flavonoids, aromatic acids, and phenolic acid esters (Bankova et al., 2000; Pietta et al., 2002; Chen et al., 2008). Previous studies have also reported that compounds such as cinnamaldehyde, benzyl cinnamate, methyl cinnamate, caffeic acid, cinnamyl cinnamate, and cinnamoylglucine, among the components detected in propolis samples, are responsible for antifungal, antibacterial, antiviral, anti-inflammatory, and anti-carcinogenic activities against many microbial agents (Bufalo et al., 2009). Our results revealed that samples with high total phenolic content have high antioxidant effects. Aygun (2017) reported that the antimicrobial effect of propolis is due to phenolic acid, phenolic acid esters, terpenes, and flavonoids. Phenolic compounds are classified as natural antimicrobial metabolites because they inhibit the growth of many phytopathogens (Xu et al., 2018).

B. In Vitro Antifungal Activity

The antifungal effects of different concentrations (0.25, 0.50, 1, 2.5, and 5 µL mL^{-1}) of EEP were evaluated on B. cinerea isolates (ET 33, ET 63, and Hatay) radial growth used for the contact phase technique under *in vitro* conditions. The inhibitory effect of EEP is presented in Table II. The EEP concentrations were found to be significant according to the statistical analysis results ($p \leq 0.01$) in the experiment. The percentage of growth inhibition in the pathogenic fungi was partially increased by increasing the concentration of the EEP. The highest radial growth was found on control plates (43.8 mm, 43.4 mm, and 42.5 mm). EEP showed the highest antifungal effect at a concentration of 5 µL mL^{-1} (high-dose) against *B. cinerea* (ET 63 isolate) with an inhibition zone of 19.6 mm for contact phase technique. EEP showed the lowest inhibitory effect at a concentration of 0.25 µL mL^{-1} against isolates of *B. cinerea* ET 33, Hatay, and ET 63 with an inhibition zone of 40.9 mm, 40.1 mm, and 40.0 mm, respectively. EEP doses showed percentage inhibition rates of *B. cinerea* (ET 63 isolate) ranging from 5.9 to 53.9%. The highest antifungal effect was detected at a rate of 53.9% in high-dose application (5 µL mL^{-1}) application. *B. cinerea* (Hatay isolate) showed inhibition rates from EEP dosages between 7.7 and 43.8%. Similarly, *B. cinerea* (ET 33 isolate) showed inhibition rates from EEP dosages between 6.5 and 37.0%. High-dose application of EEP showed a higher inhibitory effect against the ET 63 isolate of *B. cinerea* than against the ET 33 and Hatay isolates of *B. cinerea*, and the antifungal effect of EEP varied depending on the isolates of *B. cinerea* and the dose (Table II).

As a result of petri dishes, all doses of EEP showed low antifungal activity against ET 33 and Hatay isolates of *B. cinerea*. Only high-dose EEP application showed moderate antifungal activity against the ET 63 isolate of *B. cinerea*. Other doses showed low antifungal activity. The antifungal effects of propolis extracts at different levels against different or similar pathogens are due to the differences in the chemical content of propolis. Similar results have been supported by many previous studies (Chee, 2002; Shehu et al., 2015). Similar to our results, in a study to determine the antifungal effects of different doses of propolis (0.5%, 1%, 2%, 3%, and 4%) against the pathogens *Penicillium digitatum*, *Aspergillus niger*, *Aspergillus parasiticus*, *Fusarium oxysporum* f. sp. *melonis*, *Alternaria alternata*, and *Botrytis cinerea*, the most effective dose was 4%, and *A. alternata* and *P. digitatum* were determined to be the most susceptible pathogens (Özcan, 1999). Curifuta et al. (2012) applied different doses of Chilean propolis (0.5%, 1.0%, 2.5%, and 5%) against *A. alternata*, *Fusarium* spp., *Ulocladium* spp., *B. cinerea*, *Penicillium expansum*, and *Trichoderma reesei* and reported that all doses showed antifungal effects at different rates. Quintero-Ceron et al. (2014) investigated the fungistatic activity of Colombian propolis against *Aspergillus niger*, *Penicillium* sp., *Rhizopus oryzae*, and *Botrytis cinerea in vitro* and reported that the most sensitive pathogen was *A. niger* (at 0.09% w/v concentration), followed by *Penicillium* sp. (0.42% w/v), *R. oryzae* (0.53% w/v), and *B. cinerea* (1.09% w/v). Davari et al. (2016) found *Sclerotinia sclerotiorum* more sensitive to propolis extract compared to *B. cinerea*. The inhibition of *B. cinerea* mycelium growth by different propolis extract concentrations was reported to range from 11% at low concentration to 59.55% at the highest concentration. Propolis extract at concentrations of 0.1% and 0.2% showed less effect on the mycelial growth of *B. cinerea* as the causal organism of grey mold in broccoli. All the concentrations showed inhibitory effect (approximately 31.63%) against *B. cinerea* until six days of incubation. This effect partly increased on seven days of incubation (Elwan et al., 2017). Both propolis extracts (EPE

and APE) exhibit very good antifungal effects against the studied phytopathogenic microorganisms (*Botrytis cinerea* and *Fusarium oxysporum*). However, they have different effects on phytopathogenic fungi (Ouahab et al., 2023). *In vitro* experiments determined that propolis ethanolic extract and chitosan applications significantly inhibited the mycelial growth rate and sporulation of *B. cinerea* but did not completely inhibit pathogen development. In particular, 20 mL PEE and 3% and 5% chitosan doses showed fungistatic effects (Stefanski et al., 2024). In contrast to our results, Sadallah (2025) reported that propolis extracts obtained using 90% ethanol and subjected to sonication had significant inhibitory effects on *B. cinerea* mycelium growth in a preliminary bioassay.

TABLE I. Major compounds of propolis extract identified by HPLC-DAD.

Major compounds[1]	**Amounts found (µg mL^{-1}) ***
Gallic acid	30.28
Epigallocatechin gallate	24.34
Caffeic acid	292.55
p-Coumaric acid	116.68
trans-Ferulic acid	86.00
trans-iso Ferulic acid	225.25
3-4-Dimethoxycinnamic acid	142.16
Quercetin	468.02
trans- Cinnamic acid	44.29
Naringenin	367.28
Apigenin	287.01
Kaempferol	172.73
Krisin	419.76
Pinocembrine	958.08
Galangin	959.83
Caffeic acid phenethyl ester	2102.26
trans- Chalcone	443.85

[1]HPLC-DAD analysis results are shared in the article of Yilmaz et al. (2023); *Analysis results include µg g^{-1} amounts of liquid propolis in 1 mL

TABLE II. Antifungal effect of EEP on the mycelial growth of *Botrytis cinerea* isolates.

Concentration (µL mL^{-1})	**ET 33 isolate**			**ET 63 isolate**			**Hatay isolate**		
	MG (mm)[1]	**Inhibition (%)**	**AE**	**MG (mm)**[1]	**Inhibition (%)**	**AE**	**MG (mm)**[1]	**Inhibition (%)**	**AE**
Zero (Control)	43.8 a*	0.0	-	42.5 a	0.0	-	43.4 a	0.0	-
0.25	40.9 b	6.5	low activity	40.0 ab	5.9	low activity	40.1 b	7.7	low activity
0.50	39.0 c	10.9	low activity	37.6 b	11.6	low activity	38.4 c	11.5	low activity
1	36.5 d	16.6	low activity	33.5 c	21.2	low activity	35.8 d	17.5	low activity
2.5	32.3 e	26.1	low activity	28.0 d	34.1	low activity	30.3 e	30.3	low activity
5	27.6 f	37.0	low activity	19.6 e	53.9	moderate activity	24.4 f	43.8	low activity
$CV_{(0.01)}$	3.2			7.7			2.5		

[1]The mean mycelial growth of *Botrytis cinerea* isolates was determined at 7 days after inoculation; based on three replicate plates, each observation; Prior to statistical analysis, arcsine transformation was done; *Mean values followed by different letters within the column are significantly different according to the LSD Test ($P \leq 0.01$); MG: Mycelial growth; AE: Antifungal effect; CV: Coefficient of variation

IV. Conclusion

In the present study, the inhibitory effect of EEP was determined against isolates of *B. cinerea* under *in vitro* conditions. The antifungal effect against *B. cinerea* partially increased depending on the dose of EEP. The highest inhibitory effect against *B. cinerea* (ET 63 isolate) was obtained from a high-dose (5 µL mL^{-1}) application of EEP. It was found that the propolis extract had a moderate capacity in terms of antifungal activity at increasing doses for *in vitro* assay. The moderate antifungal effect of EEP is due to flavonoids and aromatic compounds. Therefore, EEP can be used for controlling *B. cinerea* and may be used as an alternative control to chemicals. The inclusion of propolis extract in crop protection strategies will help to ensure the balance of agroecosystems and the safety of harvested products. The results obtained support the importance of further *in vivo* investigations into the antifungal capacity of propolis.

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