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Mycelial Inhibitory Effect of Propolis Extract against Two Phytopathogenic *Alternaria* species

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Abstract: Propolis has been used by humans for years due to its antimicrobial and pharmaceutical properties. However, in recent years, its use as an agricultural fields antifungal agent has beeen discussed. In this study, inhibitory effect of ethanolic extract of Propolis (EEP) was investigated against two phytopathogenic *Alternaria* species. Inhibitory effect of EEP were done by contact phase against *Alternaria* species. Aiming to evaluate the mycelial growth of the pathogen, mycelial discs were placed in Petri dishes with 0, 250, 500, 1000, 2000, and 4000 µL L^{-1} of EEP. The *in vitro* experiments were carried out with three replicates depending on a completely randomized parcels design. Additionally, the chemical analyses of propolis components obtained from Muğla province were determined by high-performance liquid chromatography (HPLC) analyses. According to the HPLC results of the propolis used, phenological chemicals such as galangin, pinocembrin, quercetin, chrysin and naringenin were found in significant amounts. Based on the results of the study, EEP was found to inhibit the growth of *Alternaria* species in a dose dependent manner. The highest inhibitory effect against *A. alternata* and *A. solani* was detected in the high dose (4000 µL L^{-1}) application of EEP, 91.8 and 86.8%, rspectively. EEP has a high antifungal effect against *Alternaria* spp. because it contains flavonoids and aromatic compounds. Our results highlighted the need for further research to apply them as a safe alternative to chemical pesticides.

Keywords: Propolis, *Alternaria solani*, *Alternaria alternata*, Inhibitory effect, Alternative control

1. Introduction

The genus *Alternaria* was first introduced by Nees [1], kingdom Mycota, phylum Ascomycota, class Dothideomycetes, order Pleosporales belongs to the family Pleosporaceae [2]. *Alternaria* includes many saprophytic, endophytic and pathogenic species that are widespread throughout the world and cause significant economic losses everywhere [3]. Approximately 400 plant species, including various fruit, vegetable, ornamental and weed species, a wide range of economically important crops, are hosts to *Alternaria* species [4, 5]. *Alternaria* infections usually occur on the leaves and stems of the host plant [6]. Leaf spots are specific with black necrotic lesions surrounded by chlorotic halos. Leaf necrosis may result in reduced marketability of leaf-consumed vegetables. Additionally, it can cause yield loss in fruit trees by narrowing the photosynthesis area [7]. *Alternaria* spp. causes spots on fruits and also causes postharvest losses [8]. Among these species, *Alternaria solani* and *Alternaria alternata* cause early blight disease in many plants.

A. alternata is the most widespread species of this genus [9], has a wider host range than other species and is pathogenic on a wide range of plants such as potatoes, pomegranate, almond, kiwi, cactus, tomato, ginseng, citrus, banana, and pepper water hyacinth [10]. *A. alternata* shows good change especially on organic materials

in humid environments and causes the spoilage of many economically valuable products [11, 12]. *A. alternata* is an endophytic species and a destructive plant pathogen. Due to its necrotrophic nature, it causes severe damage to plants and harvested crops. Seedlings attacked by this disease rarely survive the infection [13, 14]. In the genus *Alternaria*, *A. alternata* is the most documented species and can produce more than 30 mycotoxins [15]. The pathogen is often transmitted in various ways, including water, insects, agricultural equipment, and infected seed. Spores of A.alternata can enter the leaves, stems and fruits of the plant [16].

Early blight caused by *A. solani* Sorauer is an economically important and widely distributed disease throughout the world on crops belonging to Solanaceae, Cucurbitaceae, Brassicaceae families [17, 18]. The pathogen continues its life on plant residues in the soil. The infection is mostly transmitted through soil but can also be transmitted through seeds. During the seedling period, it causes the seedlings to die by creating necrotic spots on the cotyledon leaves. However, spores on dead seedlings cause the disease to spread [19]. Spores of the pathogen are easily transported by air currents, wind-blown soil, splashing rain and irrigation water [20]. Although the disease occurs at all stages of plant development, it causes damage to the stem, leaves, flowers and fruits at temperatures between 24-29°C and in high humidity conditions caused by heavy rainfall [21, 22]. 32-57 % range of yield loss is caused by early blight every year in the different area of the tomato cultivation [23].

Alternaria species have a wide host range, a long and active vegetative phase, and are very difficult to control [24]. Many methods such as crop rotation, resistant varieties, soil fumigation and fungicide application are used to control the early blight disease [25, 26]. For controlling the early blight disease, synthetic fungicides are used as both seed and spray treatments, include Captan, Ridomil, Strobilurin, Iprodione, Mancozeb, Carbendazim, Chlorothalonil [27-31], but indiscriminate use of fungicides may lead to toxic residues, development of fungicide resistance environmental pollution and carcinogenic products [32].

In recent years, some natural substances have been used to combat diseases against pathogens. One of these natural products is propolis. Propolis is a resinous substance with bioactivity such as antibacterial, antiviral and antifungal, ranging in color from dirty yellow to dark brown and semi-solid at room temperature, formed by the biochemical changes of resinous substances and plant secretions collected by worker honeybees from different parts of plants such as shoots, flowers and buds with enzymes secreted by the glands in the head of the bees [33, 34]. Bees use propolis on the inside walls of their hives and other living spaces. In addition, bees use propolis to close holes and cracks in the hive, to repair combs, to stick them together, to facilitate defense or to narrow the hive entrance. The main propolis producing countries are China, Argentina, Uruguay, Chile, Brazil, Canada and some Eastern European countries. Japan imports large amounts of propolis from Brazil and China. Brazil is the most advanced country in propolis production. Tons of propolis produced in Brazil are exported to Japan and processed in this country. Raw propolis generally consists of 50% resin, 30% wax, 10% essential oils, 5% pollen and various organic and inorganic compounds. The biological activity of propolis is due to phenolic compounds, flavonoids, aromatic acids, phenolic acid esters, triterpenes, lignin, etc. [35]. In addition, elements such as Mg, Ca, I, K, Na, Cu, Zn, Mn and Fe, vitamins B1, B2, B6, C and E and many fatty acids have been identified in propolis. Propolis contains enzymes such as succinic dehydrogenase, glucose-6-phosphatase, adenosine triphosphatase and acid phosphatase [36]. Researchers have reported that PEE have antifungal effects against *Alternaria* species [37-40]. In this study, inhibitory effect of ethanolic extract of Propolis (EEP) was investigated to prevent the growth of two important species of *Alternaria*, including *Alternaria solani* and *Alternaria alternata* under *in vitro* conditions.

2. Materials And Methods

Pathogenic fungal isolates

The used highly pathogenic fungal species including *A. solani* (ET 66 isolate) and *A. alternata* (LAa 21 isolate) obtained from the Fungal Collection of Atatürk University of Agriculture Faculty, Department of Plant Protection and Fungal collection Mustafa Kemal University of Agriculture Faculty, Department of Plant Protection, respectively [41, 42]. Fungal isolates were aseptically subcultured and purified by serial transfers onto Petri dishes containing 25 mL of potato dextrose agar (PDA-Difco, 39 mL L^{-1}). Plates were incubated in the dark at 25±1°C for 7 days and culture was stored at 4°C for in the refrigerator.

Propolis collection and extraction

Raw propolis sample was collected from Muğla province of Türkiye in 2021 [43]. Propolis sample was stored in the freezer (-18°C) until further processing. After the sample stored in the freezer were ground, this ground propolis was mixed with 100 mL of 80% ethanol (C_2H_5OH). This mixture was kept in a dark room for five days, stirred 2 times a day during this period, and filtered with filter paper (Whatman No. 1) at the end of the period. EEP was prepared by isolating the propolis components from the wax. The alcohol in the combined filtrate was concentrated by evaporation with a rotary evaporator (IKA RV10- Germany). The finally mixture was stored in the dark glass at 4°C until further biological tests [44]. The component analysis of propolis extract was determined by using HPLC-DAD (high-performance liquid chromatograph-diode array detection) method.

Antifungal effect of EEP

Antifungal effect of EEP were done by contact phase against *Alternaria* species [45]. For determination of contact phase effect, various concentrations (0, 250, 500, 1000, 2000, and 4000 µL L^{-1}) of EEP were added to flasks containing molten PDA. Nearly 25 mL of enriched media was poured into each plastic Petri plate (90 mm). A fungal disc (5 mm in diameter) were cut from the edge of 7-days-old cultures of *Alternaria* species grown on PDA, and was placed at the center of each Petri plate. The plates without the EEP were used as control teratment. All Petri plates were sealed using parafim, incubated in the dark at 25±1°C for 10 days. The experiments were carried out with three replicates depending on a completely randomized parcels design. The diameter of developed colonies was measured when fungal mycelium covered one petri plate in control treatment to calculate the inhibition effect. The mycelial growth inhibition (MGI) was calculated by the following formula.

$$\textbf{MGI (\%) =[(dc-dt)/dc] x 100}$$

Where dc was the mycelium diameter in a control Petri plate, and dt was the mycelium diameter in the EEP-treated Petri plate [46].

Statistical analysis

Analysis of variance (ANOVA) was applied to the values regarding the obtained *in vitro* test results, and the differences between the averages were compared with the LSMeans Differences Student's test at the significance level of $P \leq 0.01$. The statistical analyses were accomplished with the JMP IN packet statistic program (SAS Institute, Carry, NC, 13.0 PC version).

3. Results And Discussion

Chemical composition of EEP

The HPLC-DAD analysis results of the main components of propolis extract showing significant antimicrobial activity are given in Table 1. A total of 17 active substances were determined in PE. It was determined that propolis samples contained high levels of phenolic chemicals. Depending on the presence and amount of flavonoids (Apigenin, Galangin, Kaempferol, Naringenin, Pinocembrin, Quercetin) and aromatic compounds (Caffeic acid, Caffeic acid phenethyl ester, Gallic acid, trans-Ferulic acid, trans-iso Ferulic acid, trans-Cinnamic acid, 3,4-Dimethoxycinnamic acid) in propolis extracts, It was found to be effective against *A. alternata* and *A. solani*.

Greenaway et al. [47] found that propolis contains different chemical compounds such as polyphenols (flavonoid aglycones, phenolic acids and their esters, phenolic aldehydes etc.), sesquiterpene quinones, coumarins, steroids, amino acids and inorganic compounds. In previous studies, flavonoids (Apigenin, Galangin, Kaempferol, Naringenin, Pinocembrin, Quercetin) and aromatic compounds (Caffeic acid, Caffeic acid phenethyl ester, Gallic acid, trans-Ferulic acid, trans-iso Ferulic acid, trans-Cinnamic acid, 3,4-Dimethoxycinnamic acid) in the composition of propolis extracts played an important role in the degradation of essential enzymes of pathogenic isolates and in membrane integrity, and cells died [48-64]. Our findings revealed that samples with high total phenolic content have high antioxidant effects. Similar to our findings, Aygun [65] reported that although the chemical composition of propolis is complex, its antimicrobial effect is due to phenolic acids, phenolic acid esters, terpenes and flavonoids.

Table 1: Components of propolis extract identified by HPLC-DAD

Major components[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 Mutlu Yılmaz et al. [43]. *Analysis results include µg g^{-1} amounts of liquid propolis in 1 mL.

The biocontrol effect of EEP on *Alternaria* species

The effects of six various concentrations (0, 250, 500, 1000, 2000, and 4000 µL L^{-1}) of EEP were evaluated for their inhibitory effects on *A. solani* (ET 66 isolate) and *A. alternata* (LAa 21 isolate) mycelial growth using the contact phase technique under *in vitro* conditions. The biocontrol effect of EEP are given in Table 2. The EEP concentrations were found to be significant according to the statistical analysis results ($p \leq 0.01$) of the *in vitro* experiment. The percentage of growth inhibition in the pathogenic fungi was increased by increasing the concentration of the EEP. The highest radial growth was found on control plates (42.1 mm and 42.0 mm). EEP showed the highest antimicrobial activity at a concentration of 4000 µL L^{-1} against *A. alternata* (LAa 21 isolate) and *A. solani* (ET 66 isolate) with an inhibition zone of 3.5 mm and 5.6 mm for contact phase technique, respectively. Again, EEP showed the lowest antimicrobial activity at a concentration of 250 µL L^{-1} against *A. alternata* (LAa 21 isolate) and *A. solani* (ET 66 isolate) with an inhibition zone of 19.2 mm and 22.3 mm, respectively. EEP doses showed percentage inhibition rates of *A. solani* (ET 66 isolate) ranging from 46.9 to 86.8%. The 4000 µL L^{-1} dose produced the most potential antifungal effect (86.8%), followed by the 2000 µL L^{-1} dose (74.9%). *A. alternata* (LAa 21 isolate) showed inhibition rates from EEP dosages between 54.4 and 91.8%. The highest antifungal effect was determined at 4000 µL L^{-1} dose (91.8%), followed by 2000 µL L^{-1}dose (82.8%). High dose application of EEP showed a high inhibitory effect against *A. alternata* than *A. solani*, and the inhibitory effect of EEP was found to be close to each other, depending on the *Alternaria* species and dose (Table 2).

In vitro studies conducted in petri plates the various doses of EEP inhibited both *Alternaria* spp. to varying degrees. The highest antifungal effect was obtained at 4000 µL L^{-1} dose between 91.8% and 86.8% in *A. alternata* (LAa 21 isolate) and *A. solani* (ET 66 isolate) pathotypes, respectively. In a similar study Özcan et al. [66] reported that methanol extracts of propolis from five different regions of Turkey against *Alternaria alternata* and Fusarium oxysporium f. sp. melonis. All the propolis extracts showed high inhibition at a concentration of 5 %. The antifungal effects of four different doses (0.5,1.0, 2.5 and 5.0 %) of EEP -3 on *Alternaria alternata*, Fusarium sp., Ulocladium sp., Botrytis cinerea, Penicillium expansum and Trichoderma reesei were evaluated *in vitro* using the agar dilution method. Although significant differences occurred among the extract doses, the results indicated that EEP-3 inhibited the mycelial growth of the six fungal pathogen evaluated [67]. Pobiega et al. [68] have tested the antifungal effect of ethanol extracts of propolis from Polish against many fungal pathogens such as *Alternaria solani*, Aspergillus niger, and Penicillium expansum. Results

showed that the MIC values ranked between 4 and 32 mg mL^{-1}. Similar to our findings against *Alternaria* spp., the inhibition values against *Alternaria* spp., Penicillium spp., Aspergillus spp. and Fusarium spp. at 50% propolis dose were found to be 79.26%, 78.15%, 74.81% and 71.62%, respectively, and and the difference was significant compared to the control application without propolis [69]. The antifungal activity of EEP was evaluated against different fungal pathogens such as P. digitatum, P. expansum, P. italicum, *A. alternata*, A. carbonarius, and Botrytis cinerea, being all of pathogen susceptible towards the EEP [70]. For *in vitro* assay, the alcohol-based propolis against *Alternaria* brassicicola was found to be 60 mg ml^{-1} with inhibition zones above 4.2 cm and 4.3 cm, respectively [71]. In this study, six propolis preparations at three different doses were tested against Fusarium oxysporum, *Alternaria alternata*, and Verticillium dahliae. In all tests, the smallest mean radial growth was determined in V. dahliae with 19.60 mm, followed by *A. solani* with 32.87 mm, and F. oxysporum with 48.20 mm [72]. The propolis microcapsules exhibited good slow release effect and good inhibitory effect on the development of *Alternaria alternata* growth which the colony diameter of the control was 41.38 % higher than the treatment at day six [73].

Table 2: Inhibitory effect of different concentration of EEP against *Alternaria* species

Concentration (µL L^{-1})	***A. solani* (ET 66 isolate)**		***A. alternata* (LAa 21 isolate)**	
	Mycelial growth (mm)[1]	**MGI (%)**	**Mycelial growth (mm)[1]**	**MGI (%)**
0 (Control)	42.0 a*	0.0	42.1 a	0.0
250	22.3 b	46.9	19.2 b	54.4
500	18.1 c	56.9	16.3 c	61.4
1000	14.5 d	65.5	11.9 d	71.8
2000	10.6 e	74.9	7.3 e	82.8
4000	5.6 f	86.8	3.5 f	91.8
$CV_{(0.01)}$	3.2		2.8	

[1]The mean mycelial growth of *Alternaria* species was determined at 10 days after inoculation. Based on five replicate plates, each observation. *Mean values followed by different letters within the column are significantly different according to LSD Test (P≤ 0.01). MGI: Mycelial growth inhibition rate (%). CV: Coefficient of variation

4. Conclusions

In the present study, the antifungal effect of EEP was determined against *A. solani* and *A. alternata* under *in vitro* conditions. The inhibitory effect against *Alternaria* species increased depending on the dose of EEP. The highest antifungal effect against *A. alternata* (LAa 21 isolate) was obtained from high dose (4000 µL L^{-1}) application of EEP and *A. alternata* was followed by *A. solani* (ET 66 isolate). It was determined that the propolis extract had a high capacity in terms of antimicrobial activity at increasing doses for *in vitro* assay. The high antimicrobial activity of EEP is due to flavonoids and aromatic compounds. Therefore, EEP can be used for controlling *A. solani*, and *A. alternata* and may be used as alternative control to synthetic chemicals. However, further studies are needed to explain the application time, dose, cost and mechanism of action of selected EEP.

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