Asian Journal of Chemistry Vol. 20, No. 6 (2008), 4823-4830

Antifungal Activity of Propolis Against Postharvest Disease Agent *Penicillium digitatum*

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The *in vitro* and *in vivo* antifungal activity of the propolis was evaluated against fungal pathogen *Penicillium digitatum*, causal agent of green mold of citrus fruits. The germination of conidia completely inhibited by 10, 50 and 100 µg mL^{-1} concentrations of propolis extracted in 70 % ethanol. The same concentrations of propolis extracted in 35 % ethanol also inhibited conidial germination by 31, 68 and 93 % respectively. The *in vivo* effect of propolis on the spoilage of Star Ruby grapefruits by *Penicillium* was also evaluated at room temperature. None of the concentrations of propolis extracted in 70 % ethanol prevented the fungal growth on artificially inoculated fruits. The 100 µg mL^{-1} propolis extracted in 70 % ethanol, however, provided complete inhibition of naturally occurring green mold disease on wounded but uninoculated control fruits.

Key Words: Antifungal activity, Post harvest disease, Propolis, *Penicillium*.

INTRODUCTION

About 25 % of the economical losses are caused by fungal and bacterial plant pathogens during harvesting, packing and transportation of harvested fruit and vegetables[1]. Green mold, caused by fungal pathogen *Penicillium digitatum* is an important post-harvest disease of citrus in many countries[2] including Turkey[3,4]. This disease is primarily controlled by the extensive use of fungicides, such as *ortho*-phenyl phenate, imazalil and thiabendazole as pre- or post-harvest treatments[2]. However consumer demands for pesticide-free organic food and the development of pathogenic strains that are resistant to currently used fungicides and ineffectiveness of such pesticides necessitates the development of environmentally friendly alternative methods for post

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harvest diseases[5]. Development of fungicide resistant strains of *P. digitatum* has occurred and no new fungicide is currently being used[5,6]. During last few years, considerable research efforts have been developed to identify effective alternative methods for controlling diseases of fruit, vegetable and crop plants. Alternative to synthetic chemicals that are of potential use in green mold disease control on citrus include antagonistic microorganism, natural plant- and animal-derived products with fungicidal properties and induced natural resistance of plants[7-10].

Propolis is a a natural brownish-green resinous product that honeybees collect from different plant exudates. Propolis is used to make the protective shield at the entrance of Beehive to fill the cracks in the hive, to attach the corners of frames to the grooves in the hive and also to polish the cells of the honeycomb[11]. The bodies of dead lizards, snakes and mice that have entered hives are sealed into the walls with bee glue, thereby protecting the colonies against the unpleasant odour and bacterial flora of the putrefying corpses. It possesses many biological activities such as antibacterial, antiviral, fungicidal, antitumour agents, *etc*.[12-18]. At least 200 compounds were identified in different propolis samples, with more than 100 in each one, including: fatty and phenolic acids and esters, substituted phenolic esters, flavonoids (flavones, flavanones, flavonols, dihydroflavonols, chalcones), terpenes, steroids, aromatic aldehydes and alcohols, sesquiterpenes, naphtalene and stilbene derivatives[19].

Although the antimicrobial activity of propolis has been demonstrated against human pathogenic fungi, bacteria and virus, very few *in vitro* studies have been conducted against plant pathogenic microorganism[20-23]. Almost no study has been conducted *in vivo* effects of propolis against plant pathogen. The aim of this study was to evaluate *in vitro* and *in vivo* antifungal activity of propolis against the most important post-harvest disease agent *P. digitatum*.

EXPERIMENTAL

Origin of propolis: Crude propolis from Hatay province (Eastern Mediterranean Region of Turkey) were hand gathered. The propolis exudates collected by bees (*Apis mellifera anatoliaca, Apis mellifera caucasica, Apis mellifera syriaca* and their hybrids) in Hatay were mainly from a mixture of wild and medicinal aromatic plant species such as *Medicago* spp., *Trifolium* spp., *Lathyrus sativus, Coronilla varia, Lotus* spp., *Pisum arvense, Origanum syriacum, Lavandula stoechas, Thymbra spicata, Adonis* spp., *Anagalis arvensis, Hordeum bulbesum, Aegilops ovata, Convovulus* sp., *Anthemis* sp., *Salvia multicaulis, Ferula communis* and *Petroselinum sativum* (parsley). The hand collected propolis was kept desiccated and in the dark until further processing.

Preparation of propolis extracts: Ethanolic extracts of propolis were prepared as described by Salomao *et al.*[16] and used throughout this work. Briefly, propolis were frozen to -20 °C, cut in small pieces and ground in a chilled mortar. It was extracted with 70 or 35 % ethanol (5 mL of ethanol:g of propolis) with agitation for a week at room temperature and were filtered. The filtered suspension was decanted by centrifugation at 10000 g for 20 min. The extract was kept in fridge at 4 ºC in the dark until use. The amount of dissolved principles was assessed by weight difference.

Fruit material: Grape fruit (*Citrus paradisi* cv. Star Ruby) were obtained from a local orchard and used shortly after harvest. Prior to use, fruits were thoroughly washed with tap water and surface sterilized by wiping with technical grade (75 %) ethanol.

Fungal culture and preparation of spore suspension: The fungal isolate used in the study was isolated from infected citrus fruit and maintained on potato dextrose agar (PDA, Merck). The culture was stored at 4 °C and sub-cultured once a month. Spore suspension was prepared from 2 week old PDA culture. The spores were removed from the surface of the culture, suspended in 5 mL of sterile distilled water containing 0.05 % (v/v) Tween 20 and filtered through sterile steel filter with 50 µm mesh. Spore concentration was determined using a haemocytometer and adjusted to 10^4 spores mL^{-1}.

***In vitro* studies:** The 20 % stock solution was prepared in 70 or 35 % ethanol and used for assessing its contact effects towards spore germination as described by Soylu *et al.*[10]. PDA containing 0.05 % (v/v) Tween 20 was autoclaved and cooled in a water bath to 40 ºC. Stock solution of propolis was filter sterilized through a 45 µm Millipore filter and subsequently mixed with sterile molten PDA to obtain final concentrations of 10, 50 and 100 µg mL^{-1}. The PDA was poured into Petri dishes (≈ 20 mL/plate), which were then seeded with a drops of 200 µL of *P. digitatum* conidial suspension at the concentration of 10^4 spores mL^{-1}. All inoculated Petri dishes were incubated for 24 h, at 25 ºC. Approximately 200 spores of *P. digitatum* were evaluated for germination rate and germ tube length per treatment within each replicate using microscope eyepiece graticule. Each treatment was replicated three times and the experiment was repeated twice. The growth values were obtained and then converted in to the inhibition percentage of spore germination in relation to the control treatment.

***In vivo* studies:** Since the highest antifungal activities were recorded with samples extracted in 70 % ethanol, this treatment was taken into consideration in order to determine the antifungal activities of propolis during *in vivo* experiments. Citrus fruits were assigned in equal numbers to three different groups. Fruits in the first and second groups were wounded (2 mm depth, 5 mm width) with cork borer at the equator and treated with propolis extracts by dipping into solution and left to dry. Fruits in the first group

were inoculated with a spore suspension. Propolis treated wounded fruits in the second group were sprayed with sterile water containing Tween 20 (control 1). Fruits in the third groups were not wounded but treated with propolis extracts by dipping into solution and left to dry. Fruits in this group were also sprayed with sterile water containing Tween 20 as in the second group (control 2). All treatments were incubated at 20 °C. Lesion diameter on treated fruits in each group was evaluated 7 d after treatments.

SPSS statistic program was performed for all calculations and the significance was determined by means of Duncan's Multiple Range Test ($p < 0.01$).

RESULTS AND DISCUSSION

In this study, antimicrobial activity of propolis, collected from Hatay province located at the Eastern Mediterranean Region of Turkey, was investigated against post-harvest citrus pathogen *P. digitatum*.

The chemical compositions of the alcohol extracts of propolis from the Hatay province used in this study were previously determined by GC-MS analysis[24]. The major compounds found in alcohol extracts of propolis were benzyl cinnamate (3.37 %), methyl cinnamate (2.23 %), caffeic acid (2.98 %), cinnamyl cinnamate (7.99 %) and cinnamoylgylcine (0.83 %). The total rates of the sesquiterpenes were 34.36 %. Beside, total of 13 fatty acids, 5 hydrocarbons, one alcohol and two ketones were determined. The amount of the compounds present in the propolis sample was higher than those found in propolis collected from Albania, Mongolia, Egypt and Bulgaria[19,25].

Different concentrations of propolis (10, 50 and 100 μg mL^{-1}) extracted in 35 or 70 % ethanol were prepared (Table-1) and investigated *in vitro* for inhibition of conidial germination (Table-2). The germination of conidia completely inhibited by all concentrations of propolis extracted in 70 % ethanol. The propolis extracted in 35 % ethanol also inhibited conidial germination by 31, 68 and 93 % at the concentrations of 0, 50 and 100 μg mL^{-1}, respectively (Table-2).

The chemical compositions of these sample used in present study supported earlier claims for antimicrobial activity of propolis[11,13]. This activity is reported to be due to flavonoids and aromatic acids and esters present in the resin[11], but the relationship between the structure and antibacterial activity of propolis constituents is unknown. The activity of European propolis against a broad range of bacteria and some species of fungi has been associated to the presence of flavonoids and derivatives of caffeic acid[14,19,25-28]. The mechanism of antimicrobial activity is complex and could be attributed to a synergism between phenolic and other compounds in the resin as suggested earlier[29]. Indeed, esters of phenolic acids and especially caffeates and ferulates have been identified as antibacterial, antifungal and antiviral principles of propolis[17,18].

TABLE-1
TREATMENTS USED DURING *in vitro* AND *in vivo* EXPERIMENTS

in vitro studies	*in vivo* studies
Water + Tween 20 (control)	Water + Tween 20 (control)
70 % Ethanol (control)	70 % Ethanol (control)
35 % Ethanol (control)	10 µg mL^{-1} Propolis in 70 % ethanol
10 µg mL^{-1} Propolis in 70 % ethanol	50 µg mL^{-1} Propolis in 70 % ethanol
50 µg mL^{-1} Propolis in 70 % ethanol	100 µg mL^{-1} Propolis in 70 % ethanol
100 µg mL^{-1} Propolis in 70 % ethanol	
10 µg mL^{-1} Propolis in 35 % ethanol	
50 µg mL^{-1} Propolis in 35 % ethanol	
100 µg mL^{-1} Propolis in 35 % ethanol	

Table-2
in vitro ANTIFUNGAL ACTIVITY OF PROPOLIS, USED AT VARIOUS CONCENTRATIONS, ON CONIDIAL GERMINATION OF *Penicillium digitatum*

Treatments	Conidial germination (%)	Inhibition (%)
Water + Tween 20 (control)	97.0	3.0
70 % Ethanol (control)	0.0	100.0
35 % Ethanol (control)	72.0	28.0
10 µg mL^{-1} Propolis in 70 % ethanol	0.0	100.0
50 µg mL^{-1} Propolis in 70 % ethanol	0.0	100.0
100 µg mL^{-1} Propolis in 70 % ethanol	0.0	100.0
10 µg mL^{-1} Propolis in 35 % ethanol	69.0	31.0
50 µg mL^{-1} Propolis in 35 % ethanol	32.0	68.0
100 µg mL^{-1} Propolis in 35 % ethanol	7.0	93.0

Antimicrobial effects of propolis against various fungal plant pathogens were previously reported[20,21,23] *in vitro* conditions. In addition to antifungal activities of the ethanolic extract of propolis used in present study, water extracts of 4 % propolis was also reported to be effective against to various fungal pathogens including *P. digitatum*[22].

Because of strong *in vitro* inhibitory effect of propolis extracted in 70 % ethanol, this treatment was used for *in vivo* studies. The results were presented in Table-3. Amongst the all concentrations, none of the concentrations has prevented the fungal growth on artificially wounded and inoculated fruits (Table-3, Fig. 1A). Similar results were also reported by Torre *et al.*[23] who found no significant antifungal effect of propolis against grey mold disease growth on strawberry fruits.

TABLE-3
in vivo ANTIFUNGAL EFFECT OF PROPOLIS ON THE LESION DIAMETER (mm) OF GREEN MOLD ON GRAPE FRUITS CV STAR RUBY 7 DAYS AFTER INOCULATION

Treatments	Unwounded uninoculated	Wounded uninoculated	Wounded + Inoculated
Water + Tween 20 (control 1)	0.00c*	26.91b	88.31a
70 % Ethanol control (control 2)	0.00c	16.22b	86.71a
10 µg mL^{-1} Propolis in 70% ethanol	0.00c	13.21b	86.27a
50 µg mL^{-1} Propolis in 70% ethanol	0.00c	9.01bc	85.02a
100 µg mL^{-1} Propolis in 70% ethanol	0.00c	0.00c	83.69a

*Means values followed by different small letters are significantly different according to Duncan multiple range test ($p < 0.01$).

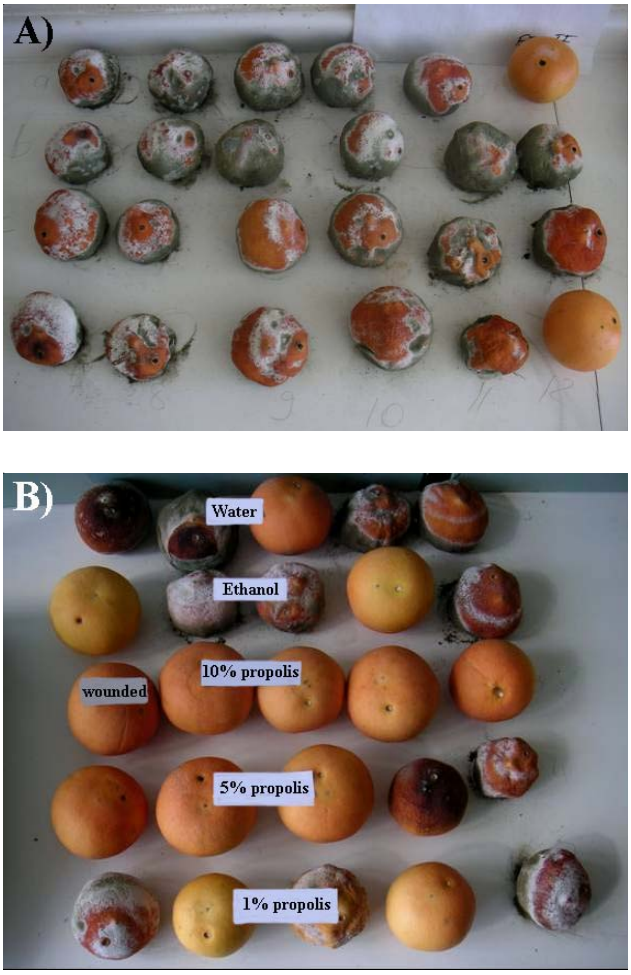

Fig. 1. Antifungal effects of propolis treatments on fungal growths on wounded and inoculated (A), wounded but uninoculated (B), unwounded and uninoculated (C) fruits

Although propolis did not effectively reduce pathogen growth on the artificially inoculated fruits (Fig. 1A), it may possess significant antifungal activity on control treatments which were not inoculated. On wounded but not inoculated fruits, only the 100 µg mL^{-1} propolis provided complete inhibition of pathogen growth (Table-3, Fig. 1B). The propolis concentration below 100 µg mL^{-1} had slight antifungal effect on the pathogen growth on wounded but uninoculated fruits. No disease development was observed on unwounded and uninoculated fruits (Table-3, Fig. 1C).

In conclusion, citrus fruits damaged during postharvest handling might be protected by high concentrations of propolis successfully. However, propolis could not control disease development once fungal infections were initiated on damaged fruits.

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(*Received*: 26 October 2007; *Accepted*: 13 March 2008) AJC-6460