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Antifungal Activity of Propolis in Four Different Fruit Juices

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Summary

Fruit juices and soft drinks are targets for spoilage yeasts, moulds and bacteria. The aim of this study is to examine the antifungal effect of ethanolic extract of Turkish propolis (EETP) treatments in four nonpasteurized fruit juices including apple, orange, white grape and mandarin against 6 different yeasts isolated from the corresponding spoiled juices. These isolated yeasts include: *Candida famata, C. glabrata, C. kefyr, C. pelliculosa, C. parapsilosis* and *Pichia ohmeri*. Minimum Inhibitory Concentration (MIC) ranges were determined responding to the National Committee for Clinical Laboratory Standards (NCCLS) M27-A that were slightly modified with broth microdilution method. In this study, the presence of propolis in apple (pH=3.9), orange (pH=3.7), white grape (pH=3.8) and mandarin (pH=3.4) juices ranging from 0.01 to 0.375 mg/mL inhibited the growth of all spoilage yeasts at 25 °C. MIC ranges of propolis were 0.02–0.375, 0.04–0.375, 0.01–0.185, 0.02–0.185 and 0.04–0.375 mg/mL in mandarin, apple, orange, white grape juices and RPMI medium, respectively. MIC ranges of Na benzoate, which was used as positive control, were 80–320, 80–320, 40–640, 40–80 and 320–1280 μg/mL in mandarin, apple, orange, white grape and RPMI medium as blank control, respectively. In terms of MIC ranges, propolis showed greater antifungal activity than Na benzoate. As a result, propolis had significant antimicrobial activity against the yeast isolates from spoiled fruit juices. It was concluded that propolis is worthy to study further as a natural preservative for foods prone to fungal spoilage.

Key words: propolis, antifungal activity, fruit juice, yeast isolation

Introduction

Fruit juices contain various concentrations of sucrose, which constitutes a very important component of the medium for the growth of fungi (*1*). Microbial spoilage is a serious problem for the food industry as fungal contamination can occur during processing as well as handling of the end products. Since yeasts can generally resist extreme conditions better than bacteria, they are often found in products with low pH and in those containing preservatives (*2*). Especially yeast spoilage has increased in recent years as a result of lower doses of preservatives and milder preservation processes, required for higher standards of food quality (*3*).

Chemical food preservatives have been used for centuries to prevent bacterial and fungal spoilage of foods. Sodium benzoate, potassium sorbate and their mixtures are commonly used as preservatives with a broad-spectrum activity against yeasts and moulds and are generally considered safe and well accepted world-wide (*4*).

The application of natural compounds with antimicrobial properties to food products might provide an alternative to the »chemical« preservatives currently em-

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ployed (*4*). Spices, herbs and plant essential oils added to foods primarily as flavouring agents have been shown to possess a broad range of antimicrobial activities (*5*).

In recent years attention has been focused on the use of propolis as a health supplement suited to consumers in developed countries. Propolis, a natural honey bee product, has different biological activities. It is a resinous substance collected by *Apis mellifera* L. from various tree buds, and used for coating hive parts and also sealing cracks and crevices in the hive. Ethanolic extract of Turkish propolis samples collected in various areas exhibited antibacterial (*6–11*), antifungal (*12*), antioxidant (*13,14*), and anticarcinogenic (*15*) properties. Very few attempts have been made to assess the antimicrobial properties of propolis in foods. Han *et al*. (*16*) reported that propolis extracts can serve as good chemical preservatives of pork meat products and can contribute to promoting human health. The aim of this study was to determine the effectiveness of propolis *in vitro* in various fruit juices and in agar media against 10 yeasts isolated from spoiled fruit juices.

Materials and Methods

Origin and chemical analysis of propolis

Propolis was hand collected in Kayseri, Central Anatolia, Turkey, and kept desiccated in dark until processing. Voucher specimen is deposited at the Department of Microbiology, Medical Faculty, Erciyes University, Kayseri, Turkey. An aliquot of crude propolis (7 g) was dissolved in 80 % ethanol by shaking at 50 °C for 3 days protected from light. The resulting aqueous ethanol extract was filtered three times through paper filter and concentrated at 50 °C. The resin obtained was dissolved in absolute ethanol to a final concentration of 3 mg/mL (EETP). This final solution was used for the antifungal assays.

Propolis content had previously been identified by GC-MS and the main compounds are shown in Table 1 (*17*). The main compounds were flavonoids (mainly chrysin) and their esters, aromatic and fatty acids, as well as alcohol and ketones (Table 1).

Isolation and identification of the test yeasts

Apple, mandarin, white grape and orange juices were stored at room temperature for 2 days. The yeast strains previously isolated from spoiled fruit juices were inoculated into Sabouraud Dextrose Agar (SDA) and incubated at 25 °C for 24–48 h. Ten strains belonging to 2 genera were isolated and identified using standard microbiological procedures which included identification based on the macroscopic and microscopic characteristics of the culture strains (*18,19*). For the yeast identification, API 20 C AUX (BioMerieux, Marcy I'Etoil, France) test kits were used. Isolates were again incubated at 25 °C for 24–48 h. After incubation, the cultures were streaked onto SDA, and working cultures from which inoculates were prepared for MIC were freshly prepared.

Quality control was performed by testing *Candida albicans* ATCC 90028 according to the recommendations of NCCLS document M27-A (*20*).

Microbial medium and fruit juices

Apple, mandarin, white grape and orange fruits were purchased from retail market in November 2004 in Kayseri, Turkey. After washing the whole fruit, the corresponding juices were prepared undiluted by direct squeezing and filtered through a disposable sterilized filter (Schleicher and Schuell, Germany) with a pore size of 0.45 μm.

RPMI 1640 broth medium (Sigma Chemical Company, Madrid, Spain) with L-glutamine, but without sodium bicarbonate, buffered at pH=7.0 with 0.165 M morpholinepropanesulphonic acid (MOPS) (Sigma, Madrid) was used for broth microdilution susceptibility testing.

For agar dilution test RPMI 1640 broth supplemented with 1.5 % Bacto Agar (Difco Laboratories, Detroit, MI, USA) and 2 % glucose, and buffered with MOPS was used.

Physicochemical analyses of the fruit juices

The pH was measured with a pH meter (HANNA Instruments, Italy) and the acidity was determined by titration with 0.1 M NaOH in the presence of phenolphthalein and expressed as percentage of citric, malic or

Table 1. Chemical composition of ethanol extract of Kayseri propolis sample

Compounds	TIC/%	RT/min	Compounds	TIC/%	RT/min
Fatty and aromatic acids			*Flavonoids*		
9-Octadecenoic acid	1.46	25.22	Chrysin	10.62	34.38
2-Propenoic acid	4.88	27.53	*Esters*		
Caffeic acid	3.45	29.19	Cinnamyl cinnamate	3.21	30.00
Alcohol, ketone and terpens			*Others*		
2-Naphtalene methanol	0.9	15.24	1-Phenanthrene carboxaldehyde	0.92	25.97
2-Propen-1-one	8.81	29.85	Benzenamine	1.67	26.60
4H-1-Benzopyran-4-one	6.73	31.29	Eicosane	3.88	37.15
Coumaran-5,6-diol-3-one	0.87	33.53	Heptacosane	7.97	34.32
Benzofuran-3-one	2.94	35.19	Cyclotrisiloxane	0.80	35.96

TIC/%: percentage of total ion current, GC-MS (*17*). The ion current generated depends on the characteristics of the compound concerned and it is not a true quantitative; RT: retention time

tartaric acid. Brix degree of the fruit juices was measured using a refractometer.

Antifungal agents

Propolis extract was diluted with fruit juices and added in concentrations ranging from 0.01–3 mg/mL. Na benzoate was dissolved with distilled water and added in concentrations ranging from 5–5120 μg/mL.

Susceptibility testing

Fruit juices were inoculated with pure cultures of yeasts originally obtained from the spoiled fruit juices. Broth microdilution testing of inoculum preparation in 0.5×10^3 to 2.5×10^3 CFU/mL concentrations was performed according to NCCLS guidelines by using the spectrophotometric method (*20*). The trays were incubated at 25 °C and were observed for the presence or absence of growth at 24–48 h. Aliquot of 100 μL of *Candida kefyr, C. parapsilosis*, *C. famata*, *C. glabrata*, *C. pelliculosa* or *Pichia ohmeri* was added to each juice dilution. The final bacteria cell concentration was 10^4–10^5 cells/mL**.** Microtiter plates were incubated at 25 °C overnight for 24 and 48 h. The minimum inhibitory concentration (MIC) value was considered the lowest concentration of EETP or Na benzoate that yields negative subcultures.

Results

The main compounds of EETP were previously identified and are listed in Table 1. The physicochemical characteristics of the fresh fruit juices are presented in Table 2. The fresh fruit juice samples had suitable technological properties. In the Microbiology and Clinical Microbiology Department of Gevher Nesibe Hospital (Erciyes University) the following yeasts were isolated from the four spoiled fruit juices: *C. kefyr*, *C. parapsilosis*, *C. famata*, *C. glabrata*, *C. pelliculosa* and *P. ohmeri* (Table 2).

The activity of EETP was investigated against the six yeast isolates. Table 3 shows that the addition of propolis to the four fruit juices at levels ranging from 0.01 to 0.375 mg/mL inhibited the growth of all the spoilage yeasts examined at 25 °C.

The most resistant strain to ETTP was *C. kefyr* with MICs in the range of 0.185–0.375 mg/mL in mandarin and apple juices, 0.02–0.185 mg/mL in the orange juice and 0.09–0.185 mg/mL in the white grape juice. In mandarin juice, *C. kefyr* was the most resistant yeast to Na benzoate with MIC range of 160–320 μg/mL after 48 h. In apple juice, the most sensitive yeast to ETTP was *C. pelliculosa* with MIC value of 0.04 mg/mL; the other yeasts were inhibited by the propolis extract in the range of 0.09–0.375 mg/mL after 48 h of treatment. In orange juice, the most resistant strain to both ETTP and Na benzoate was *C. kefyr*, with MIC ranges of 0.02–0.185 mg/L and 160–640 μg/mL, respectively.

C. parapsilosis was the most sensitive strain to propolis extract in grape juice, while the most resistant strains were *C. kefyr* and *C. glabrata*. MIC range of Na benzoate was from 40 to 80 μg/mL against the yeasts tested in grape juices. *C. glabrata* could not be grown in this juice.

C. kefyr and *C. parapsilosis* were the most resistant strains to Na benzoate. MIC ranges in *C. kefyr* and *C. parapsilosis* were 320–1280 and 640–1280 μg/mL, respectively.

Of the six strains of the yeasts isolated from spoiled fruit juices in this study, the yeasts of *Candida* genus, *C. kefyr* and *C. famata* were resistant to propolis in mandarin and apple juices at the maximum MIC concentration ranges of 0.185–0.375 and 0.09–0.375 mg/mL, respectively.

Table 2. Physicochemical characteristics of the fruit juices and test yeast strains isolated from spoiled fruit juices

Fruit juices	pH value	Titratable acidity/%	Brix degree	Isolated yeasts
Mandarin	3.30	0.90	12.0	*Candida parapsilosis, C. kefyr, C. famata*
Orange	3.57	0.93	13.2	*C. parapsilosis, C. glabrata, Pichia ohmeri*
Apple	4.00	0.34	14.9	*C. kefyr, C. famata*
White grape	3.80	0.78	12.1	*C. kefyr, C. pelliculosa*

Table 3. Minimum Inhibitory Concentration (MIC) ranges of propolis extract and Na benzoate in the four different fruit juices and RPMI medium

Yeasts	Fruit juices	EETP/(mg/mL)		Na benzoate/(μg/mL)	
		24 h	48 h	24 h	48 h
		MIC range	MIC range	MIC range	MIC range
Candida kefyr	Mandarin	0.09	0.185–0.375	80–320	160–320
(*N*=3)	Orange	0.02–0.09	0.02–0.185	80–640	160–640
	Apple	0.04–0.185	0.185–0.375	160–320	80–320
	White grape	0.04–0.09	0.09–0.185	40–80	80
	RPMI	0.04–0.185	0.09–0.375	320–640	320–1280
C. parapsilosis	Mandarin	0.04	0.09	80	160
(*N*=2)	Orange	0.01–0.04	0.01–0.09	80–160	80–320
	Apple	0.09	0.09	80–160	160
	White grape	0.02–0.04	0.02–0.04	40	40
	RPMI	0.09–0.185	0.185–0.375	640	640–1280

Table 3. continued

Yeasts	Fruit juices	EETP/(mg/mL)		Na benzoate/(μg/mL)	
		24 h	48 h	24 h	48 h
		MIC range	MIC range	MIC range	MIC range
C. famata	Mandarin	0.02–0.04	0.09–0.185	80–160	160
(*N*=2)	Orange	0.01–0.04	0.02–0.04	40–160	40–160
	Apple	0.09	0.09–0.375	80–160	160
	White grape	0.02–0.04	0.04–0.09	40–80	40–80
	RPMI	0.09–0.185	0.185	320–640	640
C. glabrata	Mandarin	0.09	0.185	80	160
	Orange	0.04	0.04	40	40
	Apple	0.09	0.185	160	160
	White grape	0.09	0.185	160	160
	RPMI	0.09	0.185	–	640
C. pelliculosa	Mandarin	0.04	0.09	80	160
	Orange	0.09	0.09	80	80
	Apple	–	0.04	80	160
	White grape	0.04	0.04	80	80
	RPMI	0.09	0.185	320	640
Pichia ohmeri	Mandarin	0.04	0.09	80	160
	Orange	0.04	0.04	40	80
	Apple	–	0.09	160	160
	White grape	0.02	0.04	80	80
	RPMI	0.185	0.185	640	640

– ineffective

Discussion

The results of the study showed that propolis extract was an effective antifungal agent at very low levels against yeasts associated with spoiled fruit juices. All of the six yeast strains were inactivated by 0.375 mg/mL of EETP. Much of the interest in the antifungal properties of propolis is focused on its possible role in human health (*21–26*). MICs as low as 0.01 μg/mL for Turkish propolis against some pathogenic fungi have been reported (*9*). However, other authors have found that 2–16 mg/mL of Brazilian propolis were necessary to inhibit the growth of some fungal strains (*25*). EETP concentrations needed to inhibit the growth of the strains used in this study were lower than those previously reported for a Brazilian propolis in assays with other fungi (*23*). It is possible that such differences are due to differences in the chemical composition among propolis samples collected in the regions of temperate and tropical climate. Salomao *et al*. (*25*) indicated that Bulgarian propolis was more effective than Brazilian propolis against bacteria. The activity of European propolis against a broad range of bacteria and some species of fungi has been associated with the presence of flavonoids and derivatives of caffeic acid in it (*23,26*). The antifungal agents in ethanolic extract of Turkish propolis are not known. Popova *et al.* (*8*) stated that propolis from Central Anatolia (Kayseri) showed high antibacterial activity and displayed very similar phenolic and flavonoid content. The composition of Kayseri propolis seems to be directly related to the bud exudates of *Populus* species.

Many studies have been reported on the antimicrobial activities and use of benzoates. In many countries, Na benzoate and potassium sorbate can be used in various food products, including pickles and soft drinks, up to 0.1 % (*27,28*). In the present study, it has been shown that EETP was more active against yeast isolated from spoiled fruit juices than Na benzoate, which presented MIC values ranging from 40 to 1280 μg/mL.

Propolis is one of the major hive products of bees and is rich in flavonoids, which are known for their antioxidant activities. Very few attempts have been made to date to assess the antimicrobial properties of propolis in foods. Han and Park (*29*) reported that propolis extracts can serve as chemical preservatives of pork meat products. Until now, propolis and propolis-based products have been consumed for health reasons but have not been used in fruit juice processing and preservation. In recent years attention has been focused on the use of propolis as a health supplement suited to consumers in developed countries. This is due to the fact that it is recognized around the world as a natural, healthy and beneficial product.

Conclusions

The results of this investigation further indicate the potential use of propolis as an alternative to chemical food preservative agents. However, given the strong aromatic flavour of this resin, it should be added in small amounts, so as not to affect the organoleptic qualities of the food.

Encouraging results of the use of natural products to control fungal contamination indicate that we should be able to develop natural fungicides that would be as effective as chemicals, and presumably safer for man and the environment. Several plants and plant extracts have thousands of years of history and their nontoxicity, at least at oral level, has been proven. This safety feature is very important in formulations of such products for commercial purposes because it has an impact on the cost of development and registration of a new product. The research and development costs of botanical fungicides from discovery to marketing are much lower compared to those of chemical preservatives.

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