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Fate of *Escherichia coli* and *E. coli* O157:H7 in apple juice treated with propolis extract

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Abstract- Fruit juices are targets of spoilage moulds, yeasts and acid tolerant bacteria. They might be contaminated with bacteria from raw materials, environment, packaging and during the handling of the product. These contaminations have frequently resulted in the spoilage of fruit juice and consequently commercial losses. The objective of this study was to determine the influence of propolis in apple juice against *Escherichia coli* and *E. coli* O157:H7 strains of the spoilage and pathogenic bacteria. For this purpose, apple juice was obtained from fresh apples and then was pasteurised. The pH value, titrable acidity (as % malic acid) and Brix degree of this apple juice were 3.72 ± 0.10, 0.67 ± 0.05% and 12.1 ± 0.01, respectively. Propolis extract at 1, 2 and 5% concentrations were tested to determine of *E. coli* and *E. coli* O157:H7 inhibition using paper disc diffusion method. The control treatment had no propolis extract. The apple juices were contaminated with these bacteria, and the activity of propolis was observed at first, 18th, 24th, 48th and 72nd hours at 4 and 25 ^{o}C. The number of cells in the tubes was counted using serial dilution method. Results indicated that propolis extract at 2 and 5% concentrations had significant antimicrobial activity against *E. coli* and *E. coli* O157:H7, therefore we can conclude that propolis extract is worthy of further study as a natural preservative for the foods prone to microbial spoilage.

Key words: propolis extract, antibacterial activity, apple juice, *Escherichia coli* and *E. coli* O157:H7.

INTRODUCTION

Unpasteurised fruit juices have desirable flavour characteristics but they have short shelf life due to microbial and enzymatic spoilage. Contamination could arise from raw materials, environment, handling, process and packaging. Faecal contamination has been implicated to the use of soiled and unwashed apples as the source of *Escherichia coli* in some apple cider outbreaks (Besser *et al*., 1993; CDC, 1997). In 1980s, before recognition of *E. coli* O157:H7 as a human pathogen, an outbreak of haemolytic uremic syndrome likely from *E. coli*, was reported in apple cider (Parish, 1997).

Escherichia coli is a Gram negative, rod shaped, facultative anaerobe bacteria (Phillips, 1999; McClure, 2000) that known as a part of the intestinal flora of humans and warm-blooded animals and is considered a good indicator for faecal contamination. Only a few *E. coli* strains are pathogenic to humans. Pathogenic strains can cause infections of the urinary tract, lungs (pneumonia), blood (bacteria), and intestines. For example, *E. coli* O157:H7 is the most widespread verotoxigenic type of this species. The survival of *E. coli* O157:H7 in acidic fruits and fruit juices, most notably the apple products, has been well documented (Zhao *et al*., 1993; Fisher and Golden, 1998). In many researches, it has been reported that the involvement of *E. coli* O157:H7 in foodborne illness associated with the consumption of acidic foods and/or drinks such as apple cider and yogurt which has drawn attention to the acid resistance properties of this pathogen, and many subsequent studies have demonstrated that this bacterium can survive in a variety of acidic foods and drinks that has low pH (Morgan *et al*., 1993; Miller and Kasper, 1994; Leyer *et al*., 1995; Semanchek and Golden, 1996; Tsai and Ingham, 1997; Adhikari, 2005). In addition to their survival in low pH-foods, the development of acid resistance by *E. coli* O157:H7 may provide additional protective effect against heat, salt and irradiation of the foods (Buchanan *et al*., 1998; Cheng and Kaspar, 1998; Leenanon and Drake, 2001).

Propolis is a resinous substance collected by *Apis mellifera* L. from various tree buds, and the bee use propolis for coating hive parts and also to seal the cracks and crevices in the hive. It is a natural honeybee product and has different biological activities. The antibacterial (Keskin *et al*., 2001; Kartal *et al*., 2003) antifungal (Silici *et al*., 2005; Koc *et al*., 2007), antioxidant (Orhan *et al*., 1999; Kolankaya *et al*., 2002) and anticarcinogenic (Ozkul *et al*., 2005) activities of the Turkish propolis extracts were reported. In last two decades, several studies on the antibacterial activity of propolis have been conducted, and their results showed considerable similarity (Moreno *et al*., 1999; Sforcin *et al*., 2000).

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As stated above much of the interest in the properties of propolis has so far focused on its possible role in human health (Cafarchia *et al*., 1999). Some chemical studies of propolis have shown the presence of flavonoids and related compounds like phenolic acids, which may be responsible for their antibacterial activity (Marcucci, 1995). Many studies have shown that fatty acid esters, phenolic compounds and cinnamic acid were the main constituents of the propolis, and some of them were shown antibacterial activity. For example, chemical analysis of the propolis extracts (poplar type) from Kayseri region indicated the presence of poplar compounds: mainly phenols, phenolic acids, esters and flavonoids (Popova *et al*., 2005). The researchers stated that propolis samples originating from Central Anatolia (Kayseri) showed high antibacterial activity and displayed very similar phenolic and flavonoid contents. But some researchers stated that different substance combinations are essential for the biological activity of the various propolis samples (Kujumgiev *et al*., 1999).

However, very few attempts have been made to assess the antimicrobial properties of propolis in foods. Han *et al*. (2001) demonstrated that propolis extracts can serve as good chemical preservatives for pork products and may contribute to promote human health as a natural product.

Silici *et al*. (2005) and Koc *et al*. (2007) have stated that propolis has significant antifungal activity against yeasts and moulds isolated from the some spoiled fruit juices. Silici *et al*. (2005) indicated that propolis had significant antifungal activity against some yeast isolates (*Candida famata*, *Candida glabrata*, *Candida kefyr*, *Candida parapsilosis*, *Candida pelliculosa and Pichia ohmeri*) isolated from spoiled fruit juices, and it was concluded that the propolis was worthy of further study as a natural preservative for the foods tended to fungal spoilage.

Therefore, the aim of this study was to determine the effectiveness of propolis in apple juice against the *E. coli* and *E. coli* O157:H7 known as spoilage and/or pathogenic bacteria.

MATERIAL AND METHODS

In this study, raw apple was squeezed for the juice in our laboratory directly from Turkish Golden apples (*Malus sylvestris* Miller cv. Golden) and kept in refrigerator (at 4 ^{o}C) for about 1 h before the use.

Physicochemical analyses of the apple juice. pH value of the fresh apple juice was measured with a HANNA pH meter (HANNA Instruments, Italy) and the acidity by titration with N/10 NaOH in the presence of phenolphthalein and expressed as percent malic acid. Brix degree of the juice was measured using a refractometer (Reichert AR 700 Automatic Refractometer, USA).

Preparation of test bacteria. *Escherichia coli* ATCC 25922 and *E. coli* O157:H7 ATCC 33150 strains were used to determine the antibacterial activities of propolis in the apple juice. The bacteria obtained from stock culture were grown in Nutrient broth (Merck, Darmstadt-Germany) and was incubated at 37 ^{o}C for 18 h. Final cell counts of the bacteria were about 10^8 CFU/mL.

Preparation of propolis extracts. Propolis sample was collected from Kayseri region (Central Anatolia) in Turkey. Hand collected propolis was kept in desiccated and dark conditions until the processing. Voucher specimen was deposited in Department of Microbiology, Medical Faculty of Erciyes University, Kayseri, Turkey. An aliquot of crude propolis (7 g) was dissolved in 100 mL of the 80% ethanol by shaking at 50 ^{o}C for three days protected from light. The resulting aqueous-ethanol extract was filtered three times through paper filter (Whatman No. 42, England) and evaporated at 50 ^{o}C to remove all the solvent. Percent yield was 48.95%. Then, the resin obtained (3.43 g) was dissolved in absolute ethanol (8 mL) for the determination of inhibitive effect as main stock at 30% concentration.

Paper disc diffusion method. The propolis was tested for antibacterial activity against *E. coli* and *E. coli* O157:H7 using the paper disc diffusion method (Sagdic, 2003; Sagdic and Ozcan, 2003). Nutrient agar (Merck, 25 mL) was inoculated with 1% fresh broth culture (Stock cultures contained 8.09 ± 0.13 log CFU/mL *E. coli* and 8.23 ± 0.35 log CFU/mL *E. coli* O157:H7) and poured in sterile 9 cm Petri dishes. Propolis extract at 1, 2 and 5% concentrations were tested to determine of *Escherichia coli* and *E. coli* O157:H7 inhibition. Fifty microlitres of concentrations of the propolis extract or absolute ethanol (control) were applied to a sterile paper disc (4 mm in diameter). After evaporation of solvent in a biological safety cabinet with laminar flow (Nuaire Laminar Flow Products, USA), the discs were placed on the agar surface inoculated with the test bacteria. The plate contained four paper discs soaked with each concentration of the propolis extract (50 µL) and a disc with absolute ethanol (50 µL) as control. The plates were incubated at 37 ^{o}C for 18 h. Then, inhibition zones were recorded in millimetre (mm) (Sagdic, 2003; Sagdic and Ozcan, 2003). All experiments were conducted in duplicate and the results are expressed as average values of inhibition.

Effect of the propolis extract on the growth of *E. coli* and *E. coli* O157:H7 in apple juice. The inhibitory effect of the propolis extract (at 2 and 5% concentrations) was measured using serial dilution method. The propolis extract (2 and 5%) was added to tubes containing 10 mL of sterilised (100 ^{o}C for 30 min) apple juice. The tubes were inoculated with activated bacteria cultures (0.1 mL, approximately 10^6 CFU/mL) and then incubated at 25 and 4 ^{o}C for 4 days. At first, 18th, 24th, 48th and 72nd hours, the number of cells in the tubes was counted using serial dilution method in Sorbitol-MacConkey (SMAC, Merck) and Eosin Methylene Blue (EMB, Merck) agars.

The control tube was prepared as described above except the propolis extract. Microbial counts (as log CFU/mL) were carried out in a biological safety cabinet with laminar flow (Nuaire Laminar Flow Products).

RESULTS AND DISCUSSION

The results showed that pH value, titrable acidity (as % malic acid) and Brix degree of the fresh apple juice were 3.72 ± 0.10, 0.67 ± 0.05% and 12.1 ± 0.01, respectively.

Paper disc diffusion method. *In vitro* antibacterial activity of the propolis extract against *E. coli* and *E. coli*

O157:H7 are shown in Table 1. The propolis extract at 2 and 5% concentrations showed inhibitory effect (inhibition ≥ 9 mm) against both *E. coli* and *E. coli* O157:H7. However, 1% concentration of the propolis extract was inactive against the tested bacteria. Again, as might be expected, the control (absolute ethanol) had no inhibitory effect on these organisms. The effects of propolis extract against the two test bacteria were similar, and these bacteria were affected by ≥ 2% concentration of the propolis.

TABLE 1 - Antibacterial activity of the propolis extract concentrations against the test bacteria

Bacteria	Inhibition zone diameter (mm)		
	1%	2%	5%
Escherichia coli	–*	9	15
E. coli O157:H7	–	10	14

* -: inactivity.

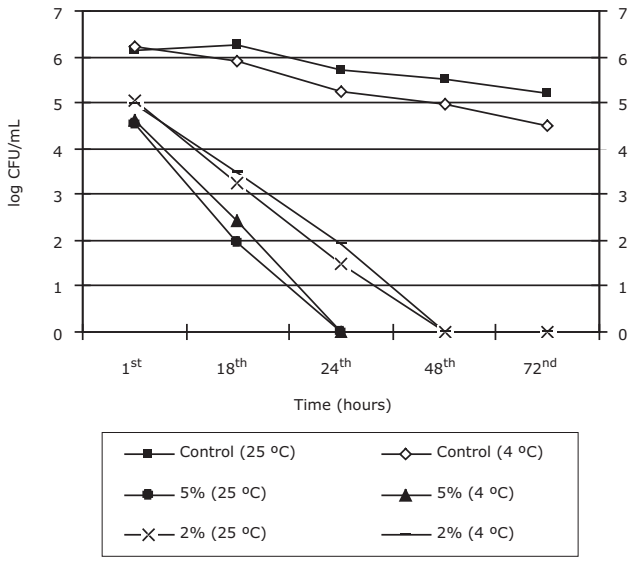

FIG. 1 - Fate of *Escherichia coli* in apple juice with propolis

Effect of the propolis extract on the growth of *E. coli* and *E. coli* O157:H7 in apple juice. Antibacterial activity of the propolis extract against *E. coli* and *E. coli* O157:H7 was shown in Fig. 1 and 2. Propolis extract at 2 and 5% concentrations had bactericidal effects against *E. coli* and *E. coli* O157:H7 at the end of 24th h of the storage at 25 ºC. However, these concentrations had same effect at the end of 48th h of the storage at 4 ºC. This result indicates that propolis extract seemed to have higher bactericidal effect at 25 ºC than that of at 4 ºC.

In contrast to the propolis extract groups, in control groups at 25 and 4 ºC, the counts of the *E. coli* and *E. coli* O157:H7 were approximately 5-6 and 4-5 log CFU/mL at the end of the 24th h of the storage, respectively. It has been well known that *E. coli* and *E. coli* O157:H7 can survive in acidic foods e.g. apple cider (Morgan *et al*., 1993; Miller and Kasper, 1994; Leyer *et al*., 1995; Semanchek and Golden, 1996; Tsai and Ingham, 1997; Adhikari, 2005).

Reinders *et al*. (2001) reported that *E. coli* O15:H7 was inhibited by praline and caffeic acid in apple juice. Again, Roller and Seedhar (2002) found that treatment with 1 mM of carvacrol or cinnamic acid delayed spoilage of fresh-cut kiwifruit and honeydew melon at chill temperatures without adverse sensory consequences. Kisko and Roller (2005) determined that when inoculated at a level of 4 log CFU/mL into unpasteurised apple juice (pH 3.20 ± 0.06), *E. coli* O157:H7 survived for up to 3 and 19 days at 25 and 4 °C, respectively. Consequently, treatment of the juice with 1.25 mM carvacrol or *p*-cymene reduced the numbers of *E. coli* O157:H7 to undetectable levels within 1-2 days at both storage temperatures. Same researchers reported that the use of chitosan in the treatment of fruit juices however may potentially lead to an increased risk of food poisoning from *E. coli* O157:H7 (Kisko *et al*., 2005).

In this study, *E. coli* and *E. coli* O157:H7 were strongly inhibited in apple juice by both 2% and 5% concentrations of the propolis extract, and these findings were in line with the previous studies stated above.

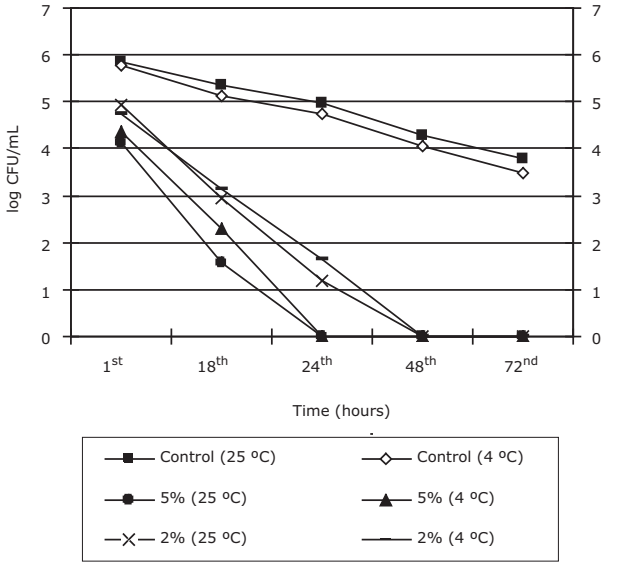

FIG. 2 - Fate of *E. coli* O157:H7 in apple juice with propolis

CONCLUSIONS

When inoculated into apple juice, *E. coli* and *E. coli* O157:H7 survived no more than 24 h in presence of the propolis extract at 25 °C storage temperature. At the 2% and 5% concentrations, propolis extract reduced the numbers of *E. coli* and *E. coli* O157:H7 to undetectable levels within 24th h. The propolis extract might be used as a bactericidal agent against *E. coli* and *E. coli* O157:H7 in apple juices increasing shelf-life and improving the safety of the samples, particularly when stored at room temperatures. The results of this research indicate that there might be a great potential for the propolis extract as an alternative natural food preservative agents if it is organoleptically acceptable that entails further study.

REFERENCES

Adhikari S.D. (2005). *The* impact of organic acids and pH on the virulence factor expression of *E. coli* O157:H7. MSc Thesis in Food Science, North Carolina State University, USA.

Besser R.E., Lett S.M., Weber J.T., Doyle M.P., Barrett T.J., Wells J.G., Griffin P.M. (1993). An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. JAMA, 269: 2217–2220.

Buchanan R.L., Edelson S.G., Snipes K., Boyd G. (1998). Inactivation of *Escherichia coli* O157:H7 in Apple Juice by Irradiation. Applied Environ. Microbiol., 64: 4533-4535.

Cafarchia C., De Lauranthis N., Milillo M.A., Losacco V., Puccini, V. (1999). Antifungal activity of Apulia region propolis. Parassitologia, 41: 587-590.

Centers for Disease Control and Prevention (CDC). (1997). Outbreaks of *Escherichia coli* O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider-Connecticut and New York, October 1996. Morb. Mortal. Wkly. Rep., 46: 4–8.

Cheng C.M., Kaspar C.W. (1998). Growth and processing conditions affecting acid tolerance in *Escherichia coli* O157:H7. Food Microbiol., 15: 157-166.

Fisher T.L., Golden D.A. (1998). Fate of *Escherichia coli* O157:H7 in ground apples used in cider production. J. Food Prot., 62: 1372–1374.

Han S.K., Yamauchi K., Park H.K. (2001). Effect of nitrite and propolis preservative on volatile basic nitrogen changes in meat products. Microbios, 105: 71-75.

Kartal M., Yildiz S., Kaya S., Kurucu S., Topcu G. (2003). Antimicrobial activity of propolis samples from different regions of Anatolia. J. Ethnopharmacol., 86: 69-73.

Keskin N., Hazir S., Baser K.H.C., Kurkcuoglu M. (2001). Antibacterial activity and chemical composition of Turkish propolis. Z. Naturforsch., 56c: 1112-1115.

Kisko G., Roller S. (2005). Carvacrol and *p*-cymene inactivate *Escherichia coli* O157:H7 in apple juice. BMC Microbiology, 5: 36-44.

Kisko G., Sharp R., Roller S. (2005). Chitosan inactivates spoilage yeasts but enhances survival of *Escherichia coli* O157:H7 in apple juice. J. Applied Microbiol., 98: 872–880.

Koc N., Silici S., Mutlu-Sariguzel F., Sagdic O. (2007). Antifungal activity of propolis in four different fruit juices. Food Technology and Biotechnology, 45: 57–61.

Kolankaya D., Selmanoglu G., Sorkun K., Salih B. (2002). Protective effects of Turkish propolis on alcohol-induced serum lipid changes and liver injury in male rats. Food Chem., 78: 213-217.

Kujumgiev A., Tsvetkova I., Serkedjieva Y.U., Bankova V., Christov R., Popov S. (1999). Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. J. Ethnopharmacol., 64: 23-240.

Leenanon B., Drake M.A. (2001). Acid stress, starvation and cold stress affect poststress behavior of *Escherichia coli* O157:H7 and non-pathogenic *Escherichia coli*. J. Food Prot., 64: 970-974.

Leyer G.J., Wang L.L., Johnson E.A. (1995). Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. Applied Environ. Microbiol., 61: 3752-3755.

Marcucci M.C. (1995). Propolis: chemical composition, biological properties and therapeutic activity. Apidology, 26: 83–99.

McClure P. (2000). The impact of *E. coli* O157 on the food industry. World J. Microbiol. & Biotech., 16: 749-755.

Miller L.G., Kaspar C.W. (1994). *Escherichia coli* O157:H7 acid tolerance and survival in apple cider. J. Food Prot., 57: 460-464.

Moreno M.I.N., Isla M.I., Cudmani N.G., Vattuone M.A., Sampietro A.R. (1999). Screening of antibacterial activity of Amaicha del Vale (Tucuma´n, Argentina) propolis. J. Ethnopharmacol., 68: 97–102

Morgan D., Newman C.P., Hutchinson D.N., Walker A.M., Rowe B., Majid F. (1993). Verotoxin producing *Escherichia coli* O157 infections associated with consumption of yogurt. Epidemiol. Infect., 111: 181-187.

Orhan H., Marol S., Hepsen I.F., Sahin G. (1999). Effects of some probable antioxidants on selenite-induced cataract formation and oxidative stress-related parameters in rats. Toxicology, 139: 219-232.

Ozkul Y., Silici S., Eroglu E. (2005). The anticarcinogenic effect of propolis in human lymphocytes culture. Phytomedicine, 12: 742-747.

Parish M.E. (1997). Public health and nonpasteurized fruit juices. Crit. Rev. Microbiol., 23:109-119.

Phillips C.A. (1999). The epidemiology, detection and control of *Escherichia coli* O157. J. Sci. Food Agricul., 79: 1367-1381.

Popova M., Silici S., Kaftanoglu O., Bankova V. (2005). Antibacterial activity of Turkish propolis and its qualitative and quantative chemical composition. Phytomedicine, 12: 221-228.

Reinders R.D., Biesterveld S., Bijker P.G.H. (2001). Survival of *Escherichia coli* O157:H7 ATCC 43895 in a model apple juice medium with different concentrations of proline and caffeic acid. Applied Environ. Microbiol., 67: 2863–2866.

Roller S., Seedhar P. (2002). Carvacrol and cinnamic acid inhibit microbial growth in fresh-cut melon and kiwifruit at 4^{o} and 8 ^{o}C. Let. Applied Microbiol., 35: 390–394.

Sagdic O. (2003). Sensitivity of four pathogenic bacteria to Turkish thyme and oregano hydrosols. Food Sci Tech. – LWT, 36: 467-473.

Sagdic O., Ozcan M. (2003). Antibacterial activity of Turkish spice hydrosols. Food Control, 14: 141-143.

Semanchek J.J., Golden D.A. (1996). Survival of *Escherichia coli* O157:H7 during fermentation of apple cider. J. Food Prot., 59: 1256-1259.

Sforcin J.M., Fernandes J.R.A., Lopes C.A.M., Bankova V., Funari S.R.C. (2000). Seasonal effect on Brazilian propolis antibacterial activity. J. Ethnopharmacol., 73: 243–249.

Silici S., Koc N., Mutlu-Sariguzel F., Sagdic O. (2005). Mould inhibition in different fruit juices by propolis. Arc. Lebensmittelhyg., 56: 87-90.

Tsai Y.W., Ingham S.C. (1997). Survival of *Escherichia coli* O157:H7 and *Salmonella* spp. in acidic condiments. J. Food Prot., 60: 751-755.

Zhao T., Doyle M.P., Besser R.E. (1993). Fate of enterohemorrhagic *Escherichia coli* O157:H7 in apple cider with and without preservatives. Applied Environ. Microbiol., 59: 2526–2530.