Study on the antimicrobial activity of Ethanol Extract of Propolisagainst enterotoxigenic Methicillin-Resistant *Staphylococcus aureus* in lab prepared Ice-cream

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Abstract

The objective of this study was to investigate the antimicrobial activity of ethanol extract of propolis against enterotoxigenic strain of MRSA which inoculated into lab prepared ice cream. EEP was added to ice cream in 3 concentrations (150, 300 and 600 mg/L). The prepared ice cream was divided into 2 groups, one stored at freezer temp. at (-5℃), while the other was kept in deep freezer temp. at (-20℃). MRSA could not be counted from the 4th, 2nd and 1st week of storage at freezer temp, while at deep freezer temp. MRSA could not be enumerated from the 3rd, 1st week and 3rd day of storage in portions contained 150, 300 and 600mg/L EEP, respectively.
Keywords: Propolis, *MRSA*, Ice cream.

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Introduction

In the last few decades staphylococcal food poisoning has been reported as the third cause of foodborne illness in the world (Zhang *et al*., 1998). *Staphylococcus* can usually be treated with antibiotics, but some strains of *Staphylococci* have become resistant to antibiotics that once destroyed it (WebMD, *2010*). Recently, the increasing prevalence of *MRSA* has become a worldwide public health problem. It is the causative pathogen of the majority of nosocomial infections that lead to long hospitalization stays, and high morbidity and mortality rate (Raghukumar *et al.,* 2010). In 1995 the first foodborne outbreak of *MRSA* was described and caused the death of five out of twenty-one diseased patients (Kluytmans *et al.*, 1995).

In the context of the lack acceptability of synthetic preservatives, there is a growing interest of introducing natural additives to food. Propolis is an interesting alternative to be considered in new applications of food technology as it is extensively used, as phytochemical ingredient, in functional foods at levels that may confer health benefits (International Food Information Service, 2005). Propolis chemical composition is complex and varies according to its botanical and phyto-geographical origin, but in general, propolis in nature is composed of 30% wax, 50% resin and vegetable balsam, 10% essential and aromatic oils, 5% pollens and 5% various other substances, including organic debris (Greenaway *et al.,* 1991*;* Bonvehi *et al.*, 1994; Burdock, 1998; Bankova and Marcucci, 2000; Kalogeropoulos *et al.,* 2009 and Petrova *et al.,* 2010).

Bees use propolis as a protective barrier against intruders by sealing holes in their honeycombs (Burdock, 1998; Salatino *et al.*, 2005 and Sforcin, 2007). Moreover, propolis is responsible for the low incidence of bacteria and moulds within the hive as it has antibacterial and antifungal properties. (Bankova *et al.,* 2000). The antimicrobial effect of propolis is due to its components that are mostly of phenolic nature,

mainly flavonoids, as the simple phenols, phenolic acids and polyphenols are active antimicrobial agents (Cowan, 1999 and Ishida *et al., 2011*). Propolis has been used as a popular remedy in folk medicine, in apitherapy, as a constituent of biocosmetics, health foods and in numerous other purposes (Bankova *et al.,* 2000 and Banskota *et al.*, 2001).

The antibacterial, antifungal and antioxidant properties of propolis are combined with the fact that several of its constituents present in food and/or food additives, and generally recognized as safe (GRAS) (Burdock, 1998), make it an attractive candidate as a natural preservative in new food applications. This meets the demand for natural antioxidants and antimicrobials, fueled by the increasing consumer awareness for natural, minimally processed foods with traditional preservatives absent or at very low concentrations (Han and Park, 1995 and Tosi *et al., 2007*). Kilic *et al.,* (2005) and Raghukumar *et al.,* (2010) documented the antimicrobial activity of propolis extract against *MRSA*, so this study was performed to investigate the effect of ethanol extract of propolis on *MRSA* inoculated into lab prepared ice cream, as *Staphylococci* might contaminate ice-cream from non-hygienic serving tools and handlers.

Materials and Methods

Collection and extraction of propolis: Propolis samples were collected from different regions of Egypt. The obtained crude propolis was stored at -18°C in a domestic freezer till its use. The frozen propolis was grounded using a blender as described by Haddadin *et al.,* (2008). Propolis samples were extracted according to the method described by Biscaia and Ferreira (2009). 5 g of propolis were placed inside a paper timber and submitted to 6 h Soxhlet extraction at a maximum temperature of 60°C, using 150 ml of solvent (ethanol). Waxes from extract were removed by 3 consecutive steps of maintaining at -18°C overnight and filtration at 0°C. The resulting extracts were evaporated at reduced pressure at a low temperature (<40°C) in a rotary evaporator to afford a concentrated ethanolic extract of propolis (EEP). The extract was transferred to small glass vials with a small amount of methanol which evaporated. Different concentrations were prepared (50, 100, 150, 200, 400, 600, 800, 1000 mg/L) by dissolving the final Ethanol Extract Propolis (EEP) in distillated water to determine the minimum inhibitory concentration (MIC).

Estimation of MIC of EEP: Estimation of MIC was performed according to method (disk diffusion method) prescribed by Silva, *et al.,* 2008.

Preparation of MRSA cellular suspension: Enterotoxigenic *MRSA* strain was obtained from Food Hygiene Department, Faculty of Veterinary Medicine, Assiut University. The inocula was prepared from MRSA pure culture, incubated in Brain Heart Infusion (BHI) agar for 24 h at 35°C. One milliliter of the culture was serially diluted to obtain the count of the organism/ml. One tenth (0.1) ml of the obtained cellular suspension (7×10^8 CFU/ml) was added to 100 ml of brain heart infusion agar, tempered at 45°C, to obtain a final count of 7×10^5 CFU/ml, then mixed and poured in Petri dishes and left for 1 h to solidify.

Detection of MIC: MIC was determined using agar disk diffusion method (150 mg/L).

Manufacturing of ice cream in lab: Ice cream powder was added to 2 litre of cool sterilized milk then manufactured according to the label of instructions of the produced company. Two ml of the previously prepared *MRSA* cellular suspension (7×10^8 CFU/ml) were added to ice cream and mixed thoroughly, to obtain initial count of 7×10^5 CFU/ml. Ice cream was divided into 4 portions and 3 concentrations (150, 300 & 600mg/L) of EEP were added to 3 portions and the 4th one was kept as a control (EEP free). Each portion was divided into 2 portions, one was kept at freezer (0±2°C) and the other was stored at deep freezer (-20°C). Samples were taken to detect the initial count before addition of EEP and after hardening of ice cream portions at the first 3 days and then weekly for 4 weeks for screening the antimethicillin-resistan *Staphylococcus aureus* (*MRSA*) activity of EEP. The *MRSA* count/g was determined using pour plate technique on BHI agar.

Table-1: Effect of different concentrations of ethanol extract propolis (EEP) on enterotoxigenic Methicillin-Resistant Staphylococcus aureus (MRSA) in lab prepared ice cream stored at freezing temperature (-5°C).

Storage period	Control	Ethanol Extract of propolis (EEP)					
		150 mg/L	Red.%*	300mg/L	Red.%*	600mg/L	Red. %*
Initial count	7×10^5	7×10^5	-	7×10^5	-	7×105	-
Zero time	2×10^4	8.7×10^3	56.5	2×10^3	90	8.6×102	95.7
1st day	5.9×10^4	1.1×10^4	81.35	4×10^3	93.2	1×103	98.3
2nd day	1.1×10^5	9×10^3	91.8	1×10^3	99	5.7×102	99.48
3rd day	9.5×10^4	6.3×10^3	93.36	8×10^2	99.15	2×102	99.79
1st week	7×10^4	7×10^2	99	1.2×10^2	99.8	N.D**	100
2nd week	5.2×10^4	4.8×10^2	99.07	N.D**	100	-	-
3rd week	9×10^3	70	99.2	-	-	-	-
4th week	3.8×10^3	N.D**	100	-	-	-	-

* Reduction % **Not Determined

Table-2: Effect of different concentrations of ethanol extract propolis (EEP) on enterotoxigenic Methicillin-Resistant Staphylococcus aureus (MRSA) in lab prepared ice cream stored at deep freezing temperature (-20°C)

Storage period	Control	Ethanol Extract of propolis (EEP)					
		150 mg/L	Red.%*	300mg/L	Red.%*	600mg/L	Red. %*
Initial count	7×10^5	7×10^5	-	7×10^5	-	7×10^5	-
Zero time	8×10^3	3×10^3	62.5	7×10^2	91.25	2.5×10^2	96.87
1st day	1.1×10^4	1.2×10^3	89	1×10^3	90.9	1×10^2	99
2nd day	5×10^4	9×10^2	98.2	5×10^2	99	1.2×10^2	99.76
3rd day	1.7×10^4	1×10^2	99.4	1×10^2	99.4	N.D**	100
1st week	7.5×10^3	80	98.9	N.D**	100	-	-
2nd week	1.3×10^3	80	93.8	-	-	-	-
3rd week	1×10^3	N.D**	100	-	-	-	-
4th week -	-	-	-	-	-	-	

* Reduction % **Not Determined

Results and Discussion

Results are shown in Table-1 and Table-2. Recently, consumers think that the natural food preservatives are better and safer than synthetic ones as they are considered the reason of many carcinogenic and teratogenic attributes as well as residual toxicity. The investigation of natural sources, which offer an unique pool of chemically diverse substances, is a valid approach to the search for new antimicrobials with chemical scaffolds that differ from known antibiotics would have less tendency to generate microbial resistance and health problems. In the last decades, propolis has gained wide acceptance by people from many western and eastern countries. The effect of propolis samples that were collected by Raghukumar *et al.*, (2010) against MRSA was so much higher than that obtained by our propolis extract (MIC= 64mg/L), while the anti*MRSA* effect of 2 of 3 proplis samples groups collected by Kilic *et al*., (2005) were closely related to ours (MIC= 161.9 and 101 µg/ml).

The data presented in Table-1 showed that, The count of *MRSA* in control samples was decreased gradually from (2×10^4 CFU/g) to (3.8×10^3 CFU/g) at the 4th week. The count of *Methicillin-resistant S.aureus*, in ice-cream sample portion contained 150mg/L ethanol extract of propolis (EEP) and stored at freezing temp. at zero time was (8.7×10^3 CFU/g) with (65.5%) reduction percent then reduced gradually to reach (70 CFU/g) at 3rd week with reduction percent (99.2 %), while through the 4th week *MRSA* was showed no visible colonies to be enumerated but could be isolated. In the ice cream portion with concentration of 300mg/L EEP *MRSA* count was reduced gradually from (2×10^3 CFU/g) at Zero time to (1.2×10^2CFU/g) with reduction percent of (99.8%). At 1st week count of *MRSA* was diminished gradually from (8.6×10^2 CFU/g) at

Zero time to (2×10^2 CFU/g) at the 3rd day in the portion with 600mg/L EEP. On the other hand, *MRSA* could be isolated till 2nd and 1st week in samples contained 300 and 600 mg/L EEP, respectively, representing a public health hazard. In relation to control portion *MRSA* count was reduced to 100% at 4th, 2nd and 1st week in ice cream samples with 150mg, 300mg, 600mg EEP/L and stored at freezing temperature (-5°C), respectively.

From the data illustrated in Table 2 it is evident that, the anti*MARSA* effect of propolis in ice cream contained 150mg/L EEP and kept at deep freezing temperature reduced the count of MRSA from (3×10^3 CFU/g) to (80 CFU/g) during 2 weeks. *MRSA* count, in ice cream portion with added 300 mg/L EEP, diminished to (1×10^2 CFU/g) at the 3rd day from 7×10^2 CFU/g at 0 time. A very marked reduction in *MRSA* count occurred at Zero time (2.5×10^2 CFU/g) which subsequently decreased gradually till became 1.2×10^2 CFU/g at the 2nd day. Though *MRSA* could not be enumerated from 3rd, 1st week and 3rd day in concentrations of 150, 300 and 600mg/L EEP at deep freezing temp., yet it assimilated a threat to consumer health as it could be isolated from the samples, respectively. The count of *MRSA* in control samples was decreased gradually from 8×10^3 to 1×10^3 C.F.U/g at the 3rd week.

In general we can say that, the anti-*MRSA* effect of propolis increase by time. The decrease in reduction percent in ice cream samples with 150mg/L EEP and kept at deep freezing temperature is not due to the weakness of EEP effect by time, but it is due to the decrease in *MRSA* count in control samples, which contributed to deep freezing effect, and fixation in the EEP effect in the 1st and 2nd week of storage resulting in diminution of the gap between the *MRSA* count in control sample and that one with 150mg/L EEP.

From this study we are not recommending the use of propolis as a food preserver only but also as a treatment for the resistant strain-infections such as *MRSA*. According to our knowledge, so far the represented study is the first one that search on the antimethicilline resistant *S.aureus* activity of Egyptian propolis.

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Conflict of interest

Authors declare that they have no conflict of interest.

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