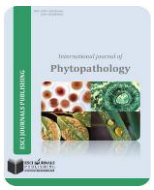

Available Online at ESci Journals
International Journal of Phytopathology
ISSN: 2305-106X (Online), 2306-1650 (Print)
http://www.escijournals.net/phytopathology

IMPACT OF PRE- AND POST–HARVEST APPLICATIONS OF NATURAL ANTIMICROBIAL PRODUCTS ON APPLE AND PEAR SOFT ROT DISEASE

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A B S T R A C T

Three natural antibacterial compounds including bacteriocin like substance (BLS) produced from lactic acid bacteria (LAB), ethanolic extract of propolis (EEP), and nine plant extracts were evaluated against soft rot *Bacillus* strains. Testing in vivo these compounds were evaluated to control pear and apple soft rot disease. Among eight BLS tested, BLS of LAB2, LAB105 and LAB 107 exhibited the highest antibacterial activity as indicated by the formation of clear inhibition zone. Propolis extracts exhibited significant antibacterial activity against all tested soft rot *Bacillus* strains and it was noticed that the antibacterial activity was concentration dependent. Among nine plant extracts tested, extracts of *Eucalyptus globulus* and *Psidium guajava* exhibited the highest antibacterial activity. All tested antibacterial products significantly decreased apple and pear soft rot severity caused by Bacillus altitudinis compared to untreated control. The highest reduction percentage of soft rot severity was recorded for EEP followed by BLS from LAB and plant extracts tested, respectively. Combined pre-and post-harvest treatments of apple and pear with antimicrobial compounds proved to be more effective in reducing the soft rot severity and improved the physical and chemical properties of fruits during storage in both years of the study. The natural antimicrobial agents used in this study were promising compounds, since it seems to be more safe, economical and great potential for extending the shelf life and improve the quality of fruits. Therefore, the application of these compounds in the control of apple and pear soft rot could be advantageous for consumers, producers, and the environment.

Keywords: Natural products, antimicrobial; bacteriocin, propolis, plant extracts, soft rot disease; pre and post–harvest, apple, pear.

INTRODUCTION

Bacterial soft rot was a destructive disease of fruits and vegetables worldwide and caused economic loss estimated between 15–30 percent of the harvested crop (Narayanasamy, 2006). This disease can be caused by more than six genera of pectolytic bacteria, including *Erwinia, Pseudomonas, Clostridium, Cytophaga, Xanthomonas* and *Bacillus* (Lund, 1983; Agrios, 2007). During the past years efforts have been increased to develop alternatives to pesticides such as biological control. The use of natural antimicrobial substances may be effective to protect and improve the quality of fruits by having an antimicrobial effect, inhibiting spoilage and avoiding oxidative processes. These substances can be defined as the compounds produced by living organisms with strong potential as sanitizing agents such as secondary metabolites from plants, bacteriocins, organic acids from bacteria, lysozyme from eggs and propolis from honey bee (Meyer *et al*., 2002; Kayser and Kolodziej, 1997).

Bacteriocins are antimicrobial peptides with a bactericidal mode of action. Bacteriocin producing species have now been identified among all the genera that comprise the LAB including *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc* and *Pedicoccus* as well as several *Enterococcus* (Jack *et al*., 1995; O'Sullivan *et al*., 2002; Motta and Brandelli, 2002; Settanni and Corsetti, 2008). In the study conducted by Cladera–Olivera *et al*. (2006), they used bacteriocin–like

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substance produced from *Bacillus licheniformis* P40 to control the potato soft rot caused by *Erwinia carotovora*. In recent years, bacteriocins from the generally recognized as safe LAB, have received significant attention as a novel approach to the control of pathogens in foods (Klaenhammer,1993; Settanni *et al*., 2005).

Propolis (bee glue) is a resinous hive product collected by honey bees (*Apis mellifera carnica*) from living plants and composed of resins (flavonoids and related phenolic acids), wax, essential oils, pollen and organic compounds (Burdock, 1998; Bankova *et al*., 2000). The antibacterial activity of propolis and its extracts against Gram-positive and Gram-negative strains was reported by Focht *et al*., (1993), Kayser and Kolodziej (1997) and Ivancajic *et al*., (2010). They found that propolis had antibacterial activity against a wide range of Gram–positive rods but had a limited activity against Gram–negative bacilli wherein the antimicrobial activity mainly related to flavonoids and aromatic compounds. Also, Lima *et al*., (1998) found that propolis exhibited antimicrobial activity against the post–harvest pathogens *B. cinerea* and *P. expansum*. Plant extracts for the control of plant diseases are emerging as alternatives to conventional synthetic chemicals as they are generally safe to humans and environmentally friendly. Researches focused on plant–derived natural bactericides and their possible applications in agriculture to control plant bacterial diseases are being intensified as these are having enormous potential to inspire and influence modern agrochemical research **(**Bergeron *et al.,* 1995**;** Zhang *et al*., 2005). Many researchers reported that plant extracts such camphor (*Eucalyptus globulus*), guava (*Psidium guajava*), white mulberry (*Morus alba*); fruit peel of pomegranate (*Punica granatum*) and orange (*Citrus sinensis*); seeds of fennel (*Foeniculum vulgare*), cumin (*Cuminum cyminum*); roots of liqurice (*Glycyrrhiza glabra*) and fruits pumpkin (*Cucurbita pepo*) showed significant antibacterial activity against common food borne causing diarrhea and food spoilage bacteria (Caili *et al*., 2006; Derakhshan *et al*., 2008; Gupta *et al*., 2008; Bendaoud *et al*., 2009; Quattrucci *et al*., 2013 ; Shruthi *et al*., 2013). However, the actual use of the natural products to control postharvest pathogens of fruits, particularly apple and pear pathogens is still limited. Therefore, the main objectives of this study were (i) to evaluate *in vitro* antimicrobial activity of the bacteriocin like substance (BLS) produced by lactic acid bacteria, ethanolic extract of propolis (EEP) and some plant extracts against tested soft rot *Bacillus* strains, (ii) to compare the efficacy of pre–, post– and combined pre– and post–harvest applications of these treatments for controlling soft rot disease of apple and pear fruits during storage and(iii) to determine changes in physical and chemical properties during storage of fruits treated with those natural products.

MATERIAL AND METHODS

Soft rot *Bacillus* strains and media: Six virulent soft rot *Bacillus* strains (AB4, AB5, AB6, PB1, PB5, and PB6) isolated from apple and pear collected from Egyptian markets and identified as *Bacillus altitudinis* (AB4, AB5, AB6) and *B. pumilus* (PB1, PB5, and PB6) (Elbanna *et al*., 2014) were used. For purity check, all the strains were streaked on nutrient agar, and then fresh single colonies were inoculated in nutrient broth and incubated at 28^{o}C on rotary shaker at 200 rpm for 24 h. The bacterial cells were adjusted to10^{8} CFU/ml.

Preparation of bacteriocin like substance (BLS): Eight LAB strains namely LAB 2, LAB 9, LAB 11, LAB 13, LAB 58, LAB 100, LAB 105 and LAB 107 isolated from fermented milk and dairy products were screened for their antibacterial activity against the soft rot *Bacillus* strains. Based on morphological, API 50 CHL kits and 16s rRNA, Strains (LAB 9 and LAB 11), (Lab 13 and LAB 58), (Lab 100 and LAB 105) and (LAB 2 and LAB 107), were characterized previously by Elbanna *et al*., (2010) as *Lactobacillus rhamnosus* (Accession No. HQ177094), *Lactobacillus casei* (Accession No.HQ177095), *Lactobacillus paracasei* (Accession No.HQ177096), *Lactobacillus* sp., respectively.

Bacteriocin like substances were produced from selected LAB according the method described by Cladera–Olivera *et al*., (2006) with slight modification. For this, each selected fresh strain was grown in Man Rogosa and Sharpe (MRS) medium and incubated at 37^{o}C for 72 h. After incubation period, cell free supernatants were obtained by centrifugation of the bacterial cultures at 5000×*g* for 30 min. The crude extracts were further purified by precipitation with 50% (w/v) ammonium sulfate. Partial purified bacteriocin–like substances were concentrated to 20 fold by resuspending the precipitated pellets in 50 mM phosphate buffer (pH 7.2). To eliminate the inhibitory effect organic acids and salts, concentrated BLS was dialyzed three times against the same buffer at 4°C for 24 h. To eliminate the inhibitory effect of hydrogen peroxide, BLS was treated with catalase (1 mg

/ml). The final treated supernatant containing (mg/ml) was filtrated through miliporefilter (0.45 µm), designated as BLS and stored at –20°C for further use.

Preparation of Propolis extract: Propolis extracts were prepared by the method described by Krell (1996) with slight modification. Propolis sample was collected from Fayoum governorate. Hand collected propolis was kept in a dry place and stored at 4°C until its processing. The sample was cut into small pieces, grounded and extracted with 70% ethanol (1:10, w/v) under shaking at 300 rpm at room temperature for 48 h. The ethanol extract was then filtered through a Whatman No.4 filter paper and the alcohol removed by evaporation at room temperature. The dried propolis extract was kept cool at 4°C until use. Propolis extract was diluted by water to give final concentration of 2.5, 5, 7.5 and 10 mg/ml.

Preparation of plant extracts: Leaves of camphor (*Eucalyptus globulus*), guava (*Psidium guajava*), white mulberry (*Morus alba*); fruit peel of pomegranate (*Punica granatum*) and orang (*Citrus sinensis*); seeds of fennel (*Foeniculum vulgare*) and cumin (*Cuminum cyminum*); roots of liqurice (*Glycyrrhiza glabra*) and fruits pumpkin (*Cucurbita pepo*) were collected from the different fields of Fayoum governorate, Egypt. The fresh plant parts were washed with tap water, dried in open air protected from direct exposure to sunlight and ground in mortar and pestle and then micronized to fine powder using the Kenwood electric blender. Powdered part (100 g) of each plant was extracted with 250 ml ethanol (80%) as described by Adwan *et al*. (2006). Plant extracts were filtered through sterilized Whatman No.2 filter paper under vacuum and evaporated to dryness at 37°C. Final stock solution of each plant extract was prepared by dissolving 1 g of each dried extract in 10 ml of sterile distilled water and stored at–20°C for further use.

***In vitro* bioassay of natural antimicrobial compounds against soft rot pathogen *Bacillus* strains:** Antimicrobial activity of BLS, propolis and the plant extracts against soft rot *Bacillus* strains was assayed by the agar well diffusion method described by Wolf and Gibbons (1996). For this, nutrient agar was seeded with soft rot bacterial strains and poured into sterile petri dishes and left to solidify, then well 5 mm diameter were cut and filled with 50, 100 and 200 µl of BLS, propolis and the plant extracts stock solutions, respectively. All assays were performed in triplicates and 100 µl of satirized distilled water was added for well as control. The plates were left at 4°C for 2 h to allow diffusion of the substances and then incubated aerobically at 28°C for 24 h. Inhibition zone were determined by measuring the diameter of clear zones around wells.

Effect of pre–, post–harvest and combined application of natural antimicrobial products on soft rot incidence of apple and pear fruits: To evaluate the efficacy of BLS, propolis and some plant extracts on soft rot incidence of apple and pear fruits, pre–, post–harvest and combined of these treatments were conducted. For this, uniform and twelve years old trees of apple (Anna 116) and pear (Le–Cont) growing in a commercial or char at Aboksah, Fayoum governorate (Egypt) during seasons 2011/2012 and 2012/2013 were used for the field experiment. Based on the pathogenicity test in our previous study (Elbanna *et al*. 2014), the most virulent soft rot strain *B. altitudinis* (AB6 strain) was chosen for pre– and post–harvest studies.

Pre–harvest treatments: For pre–harvest treatment, fruits of the whole apple and pear trees were spread with BLS (1 mg/ml) of LAB strains LAB 2, LAB 105, and LAB 107, ethanolic extract of propolis (7.5 and 10 mg/ml) and plant extracts of *Eucalyptus globulus* and *Psidium guajava* (10 mg/ml) using hand pump actuated spray bottles each spray volume of 15 L per tree for four times intervals. The last spray was on week before the harvest. Sprayed and unsprayed trees with sterile distilled water were served as positive and negative control, respectively. The experiment was conducted in completely randomized block design with three trees as replicate for each particular. The fruits were harvested after one week of spraying. Sterilized cork borer (0.5 cm, diameter) was used to make a hole (2 cm depth) in the middle of each surface sterilized fruits as the method described by Kremer and Unterstenhöfen (1967). One hundred µl of fresh prepared *B. altitudinis* strain AB6 (10^8 cfu/ml) were injected into each hole fruit. The holes were closed again with the same removed cylinders of fruits. All treated fruits were placed in sterilized plastic boxes at highly humidity (about 95%) and stored at 4°C for 60 days.

All treatments were performed as three replicates, and each replicate (box) contained five healthy fruits. Percentage of disease severity of each fruit was determined on scale from 0-4 where 0 represented healthy and 1, 2, 3, 4 represented disease with lesion area < 25%, 26–50%, 51–75% and >75%, respectively.

Disease severity (DS) for each treatment was calculated by the following formula (De Boer *et al*. 1978).

Disease severity=

$$\frac{\sum fruits\ in\ each\ category\ x\ Category\ No.}{Total\ No.\ of\ fruits\ \mathrm{X}\ highest\ category}\mathrm{X}100$$

Post–harvest treatments: For the post–harvest experiment, uniform, healthy and bruises free fruits harvested from unsprayed trees with above treatments were surface sterilized with 2% (v/v) sodium hypochlorite for 2 min, washed with tap water and air dried. The fruits were dipped into the solution of all treatments as mentioned before under pre-harvest treatment for 2 min. Dipped and undipped fruits with sterilized distilled water served as positive and negative control, respectively. Subsequently, the inoculation method for this experiment was the same as one described under the pre–harvest experiment. All treatments were performed as three replicates, and each replicate (box) contained five healthy fruits.

Combined pre– and post–harvest treatments: In this experiment, pre–and post–harvest fruits treated with BLS, propolis or plant extracts as described above were used as combined treatments to study the accumulation effects of different treatments in decreasing soft rot of apple and pear fruits. For this, sprayed fruits of pre-harvest treatments were harvested and retreated by dipping it in solution of each treatment as described above in the post-harvest experiment. All treatments were performed as three replicates, and each replicate (box) contained five healthy fruits. All apple and pear fruits of all treatments were artificially infected with *Bacillus altitudinis* strain AB6 (10^8 CFU/ml). Disease severity was determined after 60 days storage period at 4°C. Increase percentage in reduction of soft rot severity which resulted from combined of pre–and post–harvest was calculated using the following equation:

$$\text{Combined effect Increase (\%)} = \frac{\text{pre or post harvest} - \text{combined}}{\text{pre or post harvest}}\ X100$$

Effect of natural antimicrobial products on physical and chemical properties of apple and pear fruits: Physical and chemical properties of fruits were assessed at harvest time and after 60 days cold storage at 4°C. Firmness values of each fruit were measured with the help of penetrometer (EFFIGI, 11MM Prob) for five fruits per treatment as described by Pocharski *et al.*, (2000). Total soluble solids (TSS) and total titratable acidity (TTA) were assessed in juice obtained from five fruits per replicate. TSS content was determined with a hand refractometer (Kernco, Instruments Co. Texas), total titratable acidity (TTA) was estimated as percent malic acid by titrating 5 ml of fruit juce with 0.1 NaOH using phenolphthalein as an indicator. Total phenolic content (TPC) of health apple and pear fruits was colorimetrically determined according to the method described by Singleton and Rossi (1965).

Statistical analysis: The data were analysed by ANOVA using SPSS version 11.5 statistical software (SPSS Inc., Chicago, IL, USA) the mean of all treatment were compared by the least significant difference (LSD) at 0.05 level of probability (Steel *et al*., 1997).

RESULTS

Antibacterial activity of BLS against soft rot pathogen *Bacillus* strains: Antibacterial activity of partially purified BLS from eight LAB against soft rot *Bacillus* strains were evaluated using agar diffusion method. Table 1 and Fig. 1 showed that all soft rot *Bacillu*s strains were significantly inhibited by all BLS produced from LAB strains used in this study. The highest antibacterial activity was recorded for LAB2, LAB105 and LAB107, which exhibit clear zone diameter 29.61, 27.50 and 25.89 mm, respectively. While, BLS from strain LAB100, LAB11 and LAB13 recorded the lowest inhibitory effects which were 23.72, 20.89 and 19.56 mm, respectively. Therefore, BLS from strains of LAB2, LAB105 and LAB107 were chosen as alternative antibacterial products to control soft rot disease in pre–and post–harvest experiments.

Antibacterial activity of ethanolic extract of propolis (EEP) against soft rot *Bacillus* strains: As shown in Table 2 and Fig. 2, all concentrations of EEP inhibited the bacterial growth of the tested soft rot *Bacillus* strains. It was noticed that, the antibacterial activity was increased by increasing the propolis concentrations. Whereas, the means of antibacterial activity against soft rot *Bacillus* strains were 30.33 and 24.33, 22.56 and 17.28 (mm) for 10, 7.5, 5 and 2.5 mg/ml of EEP, respectively.

Antibacterial activity of some plant extracts against soft rot *Bacillus* strains: Table 3 and Fig. 3 showed that all tested plant extracts exhibited various degrees of antibacterial activity against soft rot *Bacillus* strains. It was noticed that extracts of *Eucalyptus globulus*, *Psidium guajava*, *Glycyrrhiza glabra*, *Punica granatum* and *Cucurbita pepo* exhibited the highest antibacterial activity which were 24.33, 21.17, 20.72, 19.67 and 16.95 mm, respectively, followed by *Foeniculum vulgare* (13.17 mm), *Citrus sinensis* (11.45 mm), *Morus alba* (10.39 mm) and *Cuminum cyminum* (5.17 mm).

Table 1. Antimicrobial activity of bacteriocin-like substance produced from lactic acid bacteria against soft rot *Bacillus* strains from apple and pear fruits *in vitro*.

Bacteriocin Like Substance	Inhibition zone diameter (mm) Bacillus strains						Mean
	PB1	PB5	AB5	AB6	AB4	PB6	
Control (S.D.W)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lab 2*	27.67	30.00	31.00	28.67	29.33	31.00	29.61
Lab 9	21.00	16.67	20.67	20.00	17.67	20.33	19.39
Lab 11	20.67	20.33	19.67	22.67	20.67	21.33	20.89
Lab 13	19.33	20.00	17.33	20.33	21.67	18.67	19.56
Lab 58	20.67	15.00	16.67	19.67	15.67	17.00	17.45
Lab 100	26.33	24.67	20.33	25.67	23.67	21.67	23.72
Lab 105	25.67	27.67	27.67	27.33	28.33	28.33	27.50
Lab 107	25.67	27.33	25.00	24.67	26.67	26.00	25.89
Mean	20.78	20.19	19.82	21.00	20.41	20.48	
L.S.D at 5% for:	Bacteriocin like substance (BLS)						0.51**
	Bacterial strains						0.44
	Bacteriocin x Bacterial strains						1.24

*Lab 2, Lab 9, Lab 11, Lab13, Lab 58, Lab 100, Lab 105 and Lab 107, bacteriocin like substance produced from lactic acid bacterial strains.

**Least significant difference (LSD) at 0.05 level confidences was calculated to compare variances between treatments (Steel *et al*., 1997).

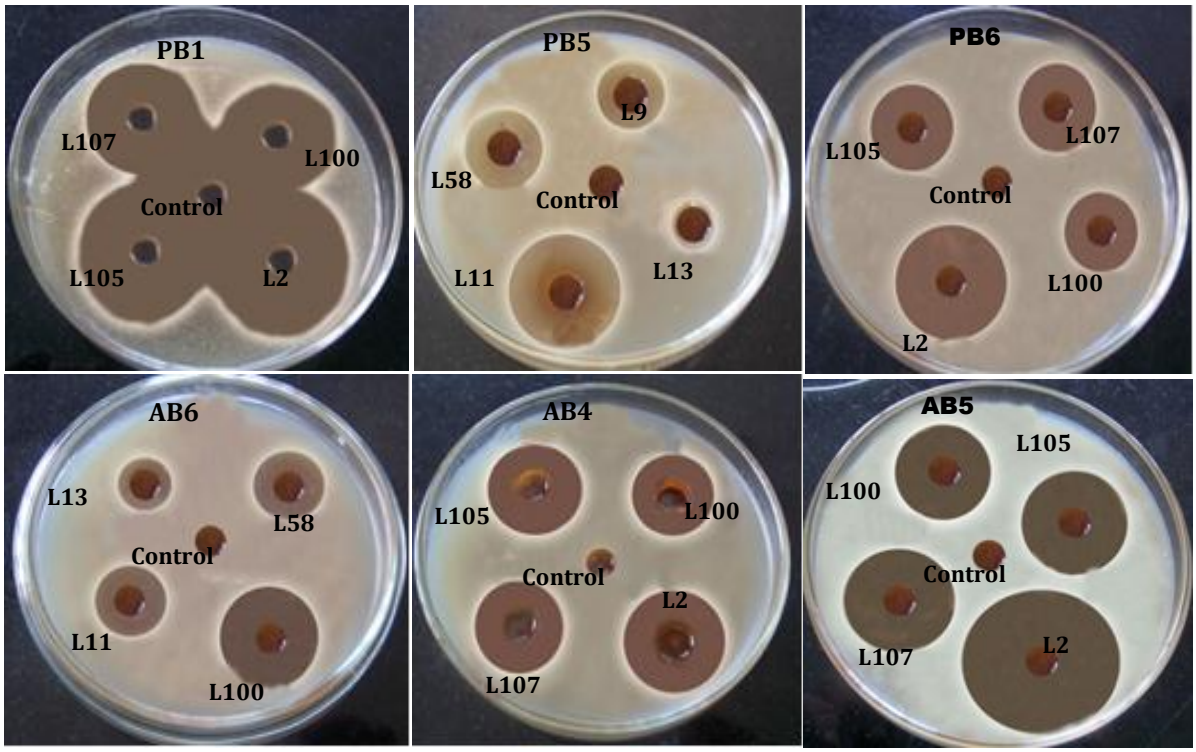

Figure 1. Antimicrobial activity of bacteriocin-like substances (BLS) produced from lactic acid bacteria against soft rot *Bacillus* strains isolated from apple and pear fruits. L 2, L 9, L 11, L 13, L 58, L 100, L 105 and L 107, bacteriocin like substance produced from lactic acid bacterial strains; PB1and BP6, soft rot *Bacillus* strains identified as *B. pumilus*; AB4 and AB5, soft rot *Bacillus* strains identified as *B. altitudinis*.

Table 2. Antimicrobial activity Ethanolic Extract (EEP) of Propolis against soft rot *Bacillus* strains *in vitro*.

Propolis concentrations (mg/ml)	Inhibition zone diameter (mm)						Mean
	Bacillus strains						
	PB1	AB5	AB4	AB6	PB5	PB6	
Control (S.D.W)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2.5	18.33	16.67	16.67	16.67	18.00	17.33	17.28
5	21.33	22.00	22.00	24.33	23.67	22.00	22.56
7.5	25.33	24.67	21.00	27.33	24.00	26.33	24.78
10	28.00	30.00	30.33	31.33	32.00	30.33	30.33
Mean	18.60	18.67	18.00	19.93	19.53	19.20	
L.S.D at 0.05	Ethanolic Extract of Propolis (EEP)						0.97*
	Bacillus strains						1.19
	Ethanolic Extract of Propolis x *Bacillus* strains						2.38

*Least significant difference (LSD) at 0.05 level confidences, calculated to compare variances between treatments (Steel *et al*., 1997).

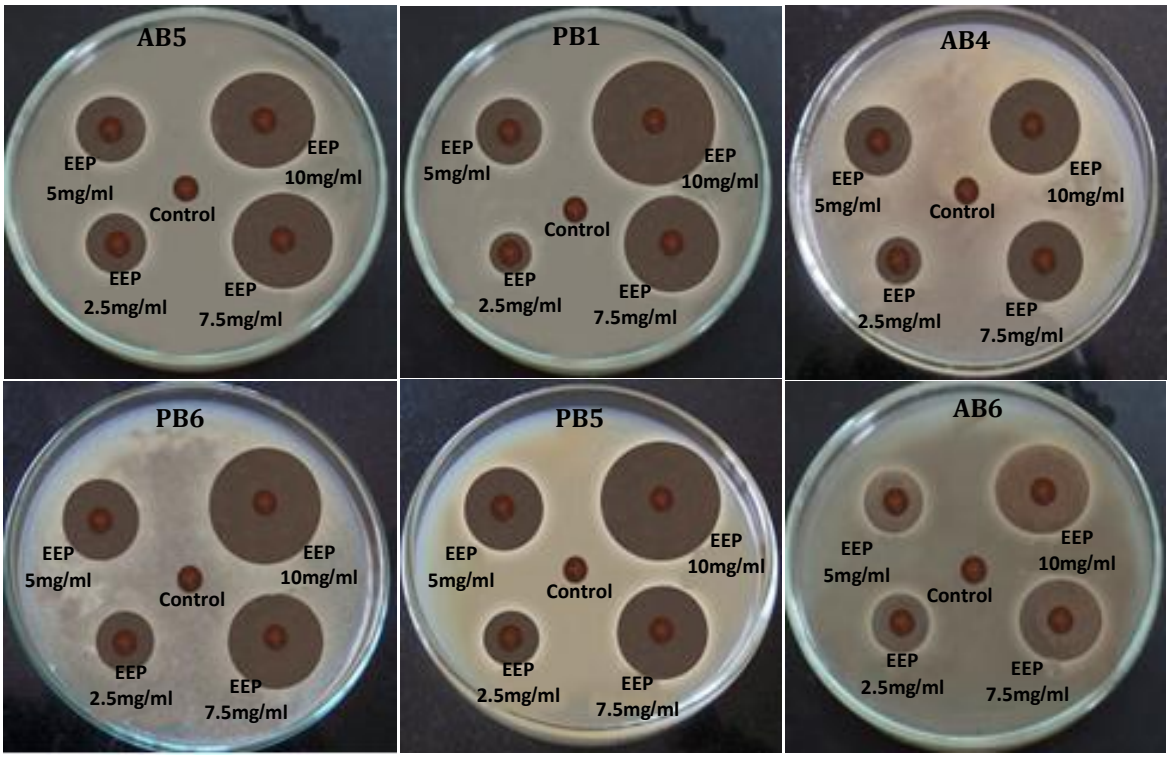

Figure 2. Antimicrobial activity ethanol extract of propolis (EEP) against soft rot *Bacillus* strains isolated from apple and pear fruits.EEP, ethanolic extract of propolis (2.5, 5, 7.5 and 10 mg/ml.); PB1, PB5and PB6, soft rot *Bacillus* strains identified as *B. pumilus*; AB4, AB5 and AB6, soft rot *Bacillus* strains identified as *B. altitudinis*.

Effect of pre–, post–harvest and combined application of BLS, EEP and some plant extracts on soft rot incidence of apple and pear fruits: Data presented in Tables 4 and 5 showed that all tested natural antibacterial products significantly decreased apple and pear soft rot severity that caused by *B. altitudinis* AB6 compared to untreated controls. In general, the highest average reduction percentage of soft rot severity was recorded for EEP, followed by BLS from LAB and plant extracts tested, respectively. However, the ability of these compounds to control soft rot disease was similar in both years. Regarding the effect of different concentrations of propolis extract tested, it was noticed that the antibacterial activity increased as propolis concentrations increased, whereas, EEP at 10 mg/ml exhibited the highest reduction percentage of soft rot disease severity of apple and pear which were 92.34, 88.93, 90.21 and 92.56 % during seasons 2011/2012 and 2012/2013, respectively.

Table 3. Antimicrobial activity of plant extract against soft rot *Bacillus* strains isolated from apple and pear fruits *in vitro*.

Plant extracts	Inhibition zone diameter (mm)						Mean
	Bacillus strains						
	AB4	AB5	AB6	PB1	PB5	PB6	
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Eucalyptus globulus	24.33	23.33	25.00	25.33	23.67	24.33	24.33
Cuminum cyminum	0.00	0.00	0.00	13.33	12.67	5.00	5.17
Foeniculum vulgare	15.67	15.67	15.00	0.00	15.67	17.00	13.17
Psidium guajava	20.33	20.00	21.00	22.33	21.33	22.00	21.17
Punica granatum	20.67	18.67	19.67	20.67	19.33	19.00	19.67
Citrus sinensis	14.67	5.00	5.00	14.67	15.00	14.33	11.45
Glycyrrhiza glabra	21.00	20.33	21.00	20.33	21.33	20.33	20.72
Cucurbita pepo	17.33	16.67	15.00	16.67	17.33	18.67	16.95
Morus alba	12.00	12.00	13.67	5.00	5.00	14.64	10.39
Mean	14.60	13.17	13.53	13.83	15.13	15.53	
L.S.D at 5% for	Plant extracts						1.84*
	Bacterial strains						1.50
	Plant extracts x bacterial strains						4.50

*Least significant difference (LSD) at 0.05 level confidences was calculated to compare variances between treatments (Steel *et al*., 1997).

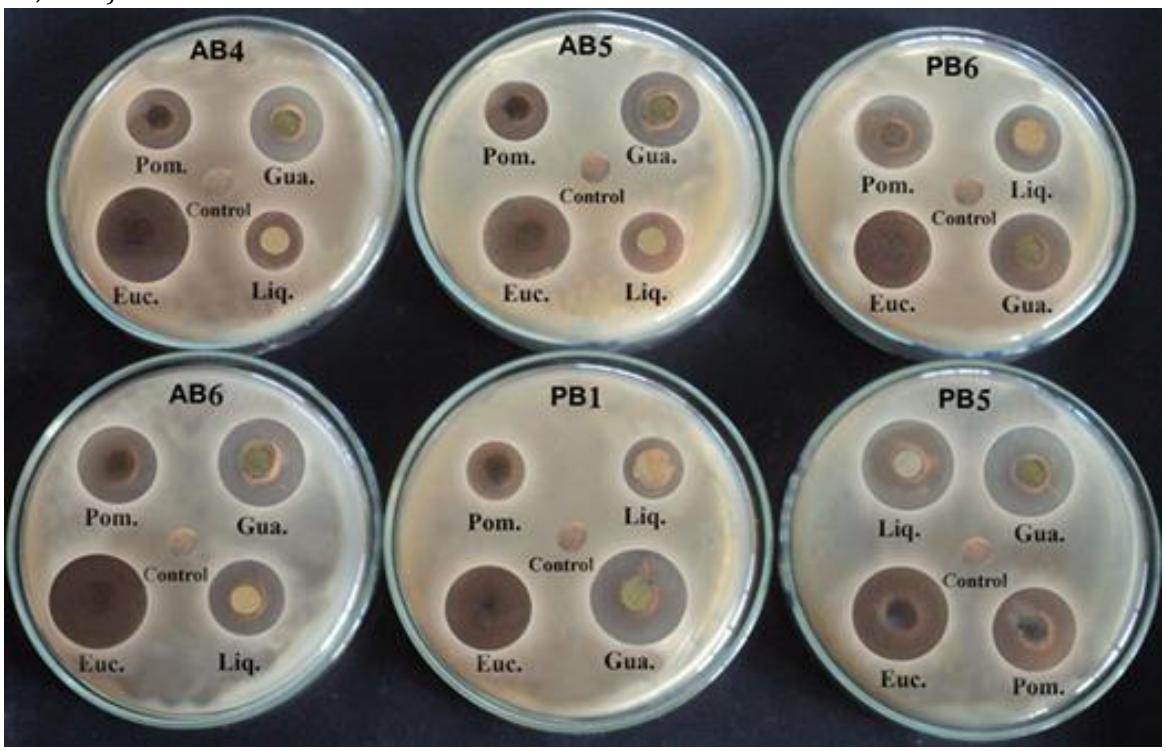

Figure 3. Antimicrobial activity of plant extracts against soft rot *Bacillus* strains isolated from apple and pear fruits.Pom, pomegranate fruit peel extract; Gua, guava leaves extract; Euc, camphor leaves extract; Liq, liqurice roots extract; AB4 , AB5 and AB6 , soft rot *Bacillus* strains identified as *B. altitudinis;* PB1, BB5 and PB6, soft rot *Bacillus* strains identified as *B. pumilus*

Among different BLS produced from tested LAB strains, BLS of strain LAB2 was the most effective in reducing the severity of soft rot disease which were 74.69, 78.49, 76.02 and 79.85 % during both seasons for apple and pear, respectively. Compared to control, plant extracts of *Psidium guajava* and *Eucalyptus globulus* significantly decreased the soft rot severity of apple and pear, whereas the reduction in disease severity of apple and pear fruits were 76.88, 74.28, 72.83 and 77.73% during both seasons, respectively.

Table 4. Effect of some natural antimicrobial compounds (bacteriocin like substance (BLS) produced lactic acid bacterial strains, Ethanolic Extract of propolis (EEP) and plant extracts on soft rot incidence of apple and pear fruits artificially inoculated with *Bacillus altitudinis* (AB6) in storage conditions at 4 ºC for 60 days during seasons 2011/2012.

Treatment[a]	Disease severity %						Mean		Reduction (%) of disease severity		Combined effect increase (%)			
	Time of application [b]													
	Pre- harvest		Post- harvest		Combined Pre and Post- harvest						relative to pre		relative to post	
	Apple	Pear	Apple	Pear	Apple	Pear	Apple	Pear	Apple	Pear	Apple	Pear	Apple	Pear
Control(-)	35.18	41.38	38.82	40.68	35.42	40.54	36.04	40.87	-	-	0.67	2.02	9.37	0.34
Control(+)	39.57	40.79	39.67	41.80	37.67	41.46	38.97	41.35	8.12	1.17	4.48	1.61	5.04	8.95
Lab 2 (mg/ml)	8.08	9.60	11.58	10.39	7.68	6.38	9.12	8.79	74.69	78.49	4.95	33.54	33.67	38.59
Lab105 (mg/ml)	10.45	10.20	10.98	10.60	8.15	8.13	9.86	9.98	72.64	75.58	22.00	20.29	25.77	23.30
Lab107 (mg/ml)	11.33	9.77	10.10	10.90	9.52	8.43	10.32	9.70	71.36	76.26	15.97	13.71	5.74	22.66
EEP (7.5 mg/ml)	3.75	6.62	5.15	7.24	2.79	3.65	3.84	5.84	89.34	85.71	25.60	44.86	45.82	49.58
EEP (10 mg/ml)	2.57	4.97	3.78	5.76	1.39	1.28	2.76	4.00	92.34	90.21	45.91	74.24	63.22	75.57
E. globulus(10 mg/ml)	7.90	13.82	9.78	11.69	7.31	7.15	8.33	10.88	72.83	73.37	7.46	48.26	25.25	38.83
P. guajava(10 mg/ml)	7.12	12.02	13.05	11.98	6.44	6.89	8.87	10.30	76.88	74.79	9.55	42.67	50.65	42.48
Mean	13.96	16.57	15.87	16.89	13..03	13.77	Apple	Pear						
L.S.D. at 0.05 for	Treatment =						0.86	0.87						
	Time of application =						0.50	0.50						
	Treatment x Time of application =						1.49	1.51						

[a]Treatments: (Lab 2 and Lab 107) and (Lab 105) bacteriocin like substance produced from *Lactobacillus paracasei* and *Lactobacillus* sp., respectively at rate 1mg/ml; EEP, Ethanolic Extract of Propolis at rate 7.5 and 10 mg/ml ; *E. globules* and *P. guajava*, plant extracts for Camphor (*Eucalyptus globules*) leaves and Guava(*Psidium guajava*) leaves at rate 10 mg/ml; Control (-) , trees and fruits were not treated or immersed with water and natural compounds; Control (+), trees and fruits were treated or immersed with sterilized distilled water. [b] Time of application: (i) Pre-harvest = treatments were applied as a spray to tree before harvesting; (ii) Post- harvest= treatments were applied as a fruits immerse after harvest; (iii) Pre and Post- harvest =combined pre and postharvest treatments.

Data in Tables 4 and 5 showed that combined of pre–and post–harvest treatments with natural antibacterial compounds resulted in highest reduction in soft rot disease severity of both apple and pear fruits stored at 4ºC during 60 day, followed by pre–and post–harvest treatments during both seasons, respectively. Relative to pre–harvest treatments, combined of pre- and post-harvest inducted increasing percentage in reduction of soft rot severity reached up to 45.91 and 58.0% in apple fruits, while it was 74.24 and 70.55% in pear fruits during 2011 and 2012, respectively. On the other hand, relative to post-harvest, combined of pre- and post-harvest treatments also showed increasing percentage in reduction of soft rot severity reached up to 63.22 and 67.01% in apple fruits, while was 75.57 and 79.92% in pear fruits during both seasons, respectively.

Table 5. Effect of some natural antimicrobial compounds (bacteriocin like substance (BLS) produced lactic acid bacterial strains, Ethanolic Extract of propolis (EEP) and plant extracts on soft rot incidence of apple and pear fruits artificially inoculated with *Bacillus altitudinis* (AB6) in storage conditions at 4 ºC for 60 days during 2012/2013.

Treatment[a]	Disease severity %						Mean		Reduction (%) of disease severity		Combined effect increase (%)			
	Time of application [b]													
	Pre- harvest		Post- harvest		Combined Pre and Post- harvest						relative to pre		relative to post	
	Apple	Pear	Apple	Pear	Apple	Pear	Apple	Pear	Apple	Pear	Apple	Pear	Apple	Pear
Control(-)	33.91	39.75	37.65	41.13	36.93	40.12	36.16	40.33	-	-	8.17	0.92	9.93	3.50
Control(+)	35.74	39.22	36.17	40.83	35.61	41.34	35.84	40.46	0.88	0.32	0.36	5.24	1.54	3.94
Lab 2 (mg/ml)	9.37	9.92	10.33	9.51	6.31	5.03	8.67	8.15	76.02	79.85	32.65	49.29	38.91	47.10
Lab105 (mg/ml)	9.99	10.00	11.13	11.14	7.08	7.35	9.40	9.50	74.00	76.52	29.12	26.50	36.38	34.02
Lab107 (mg/ml)	10.68	8.08	10.98	11.42	7.72	7.96	9.79	9.16	72.92	77.36	27.71	1.48	29.69	44.54
EEP (7.5 mg/ml)	6.62	5.07	6.41	6.42	2.78	3.56	5.27	5.01	85.42	87.61	58.00	29.78	56.63	79.92
EEP (10 mg/ml)	4.25	3.43	5.82	5.03	1.92	1.01	4.00	3.16	88.93	92.56	54.82	70.55	67.01	43.97
E. globulus(10 mg/ml)	9.10	12.22	9.35	11.28	5.69	6.32	8.05	9.94	77.73	75.43	37.47	48.28	39.14	49.55
P. guajava(10 mg/ml)	9.95	11.90	11.97	11.29	5.98	6.26	9.30	9.82	74.28	75.72	39.89	47.39	50.04	44.55
Mean	14.40	15.51	15.53	16.45	12.23	10.11	Apple	Pear						
L.S.D. at 0.05 for	Treatment =						0.57	0.59						
	Time of application =						0.99	0.34						
	Treatment x Time of application =						1.72	1.02						

[a]Treatments: (Lab 2 and Lab 107) and (Lab 105) bacteriocin like substance produced from *Lactobacillus paracasei* and *Lactobacillus* sp., respectively at rate 1mg/ml; EEP, Ethanolic Extract of Propolis at rate 7.5 and 10 mg/ml ; *E. globules* and *P. guajava*, plant extracts for Camphor (*Eucalyptus globules*) leaves and Guava(*Psidium guajava*) leaves at rate 10 mg/ml; Control (-) , trees and fruits were not treated or immersed with water and natural compounds; Control (+), trees and fruits were treated or immersed with sterilized distilled water. [b] Time of application: (i) Pre-harvest = treatments were applied as a spray to tree before harvesting; (ii) Post- harvest= treatments were applied as a fruits immerse after harvest; (iii) Pre and Post- harvest =combined pre and postharvest treatments.

Effect of natural products on physical and chemical properties of apple and pear fruits: The physical and chemical change in apple and pear fruits obtained from plants either sprayed with different natural compounds or unsprayed were evaluated at harvest time and after cold storage at 4ºC for 60 days.

Tables 6 and 7 showed that, firmness, TSS, TTA and TPC of apple and pear fruits, significantly increased in all treatments compared to control. In general, EEP and BLS from LAB2 were the most effective natural products to improve the quality of apple and pear fruits. Whereas, the highest firmness of apple and pear fruits was recorded for EEP at 10 mg/ml (5.58 and 5.80 kg/cm^2), followed by EEP at 7.5 mg/ml (5.17 and 5.77 kg/cm^2), BLS from LAB2 (5.05 and 5.78 kg/cm^2) and *Eucalyptus globulus* (5.10 and 5.47 kg/cm^2), respectively.

Regarding TSS content of apple, it was noticed that, the highest values were recorded for EEP at 10 mg/ml (13.83%), followed by EEP at 7.5 mg/ml (13.62%), *Eucalyptus globulus* (12.56%), *Psidium*

guajava (12.5%) and BLS of LAB2 (12.05%), respectively. While, TSS content of pear was 5.80, 5.78, 5.77, 5.47 and 5.45% for EEP (10 mg/ml), BLS of LAB2, EEP (7.5 mg/ml), *Eucalyptus globulus* and *Psidium guajava*, respectively. Also, Tables 6 and 7 showed that TTA significantly decreased by EEP, Also, Tables 6 and 7 showed that, the highest TPC contents in apple fruits were recorded for EEP (10 and 7.5 mg/ml) and BLS of LAB2 which were 1.33, 1.10 and 1.04 (mg/ml), respectively. While the highest TPC contents in pear fruits were 1.19 and 1.11 (mg/ml) for EEP (10 mg/ml) and BLS of LAB2, respectively.

Table 6. Effect of natural antimicrobial compounds treatments on some physical and chemical properties of apple (var. Anna116) fruits stored for 60 days at 4 ºC.

Treatments	Firmness (Kg/ Cm^2)			TSS (%)			TTA (%)			Total phenolic compounds (TPC) mg ml^{-1}		
	Period** (days)			Period (days)			Period (days)			Period (days)		
	0	60	Mean	0	60	Mean	0	60	Mean	0	60	Mean
Control*	3.87	3.39	3.63	11.10	11.10	11.05	0.54	0.41	0.48	0.43	0.28	0.36
Lab2 (mg/ml)	5.10	5.00	5.05	11.90	12.20	12.05	0.60	0.52	0.56	1.18	1.01	1.10
Lab105 (mg/ml)	4.87	4.27	4.57	11.68	12.08	11.88	0.61	0.60	0.61	1.00	0.99	1.00
Lab 107 (mg/ml)	4.30	3.20	3.75	11.17	11.60	11.39	0.60	0.55	0.58	0.81	0.76	0.79
EEP (7.5 mg/ml)	5.30	5.13	5.17	13.93	13.30	13.62	0.40	0.31	0.36	1.07	1.01	1.04
EEP (10 mg/ml)	6.03	5.13	5.58	13.42	14.23	13.83	0.38	0.25	0.32	1.36	1.30	1.33
E.golobulus (10mg/ml)	5.20	5.00	5.10	11.93	13.18	12.56	0.57	0.50	0.54	0.83	0.77	0.80
P. guajava (10 mg/ml)	5.03	4.37	4.70	11.90	12.59	12.25	0.88	0.72	0.80	0.75	0.45	0.60
Mean	4.96	4.42		13.87	12.54		0.57	0.48		1.01	0.82	
L.S.D. at 0.05 for:	Firmness			TSS			TAA			TPC		
Treatments (T) =	0.27***			0.43			0.03			0.11		
Period (P) =	0.18			0.29			0.02			0.06		
Teatment x Period =	0.39			0.61			0.04			0.15		

*Control= without natural compounds, **Period= (0) samples at harvest time, (60) samples after 60 day of storage at 4ºC., ***Least significant difference (LSD) at 0.05 level confidences was calculated to compare variances between treatments (Steel et al., 1997).

Table 7. Effect of natural antimicrobial compounds treatments on some physical and chemical properties of pear (var. Le-Conte) fruits stored for 60 days at 4ºC.

Treatments	Firmness (Kg/ Cm^2)			TSS (%)			TTA (%)			Total phenolic compounds (TPC) mg ml^{-1}		
	Period** (days)			Period (days)			Period (days)			Period (days)		
	0	60	Mean	0	60	Mean	0	60	Mean	0	60	Mean
Control*	5.30	5.00	5.15	12.83	13.50	13.17	0.33	0.30	0.31	0.54	0.46	0.50
Lab2 (mg/ml)	6.10	5.47	5.78	13.20	14.57	13.88	0.38	0.30	0.34	1.20	1.00	1.10
Lab105 (mg/ml)	5.80	5.00	5.40	12.52	13.20	12.86	0.38	0.31	0.34	1.11	1.00	1.11
Lab 107 (mg/ml)	5.60	5.00	5.30	12.57	14.10	13.33	0.35	0.31	0.33	0.82	0.80	0.81
EEP (7.5 mg/ml)	6.03	5.50	5.77	13.23	14.03	13.63	0.24	0.20	0.22	1.18	0.99	1.09
EEP (10 mg/ml)	6.20	5.40	5.80	14.00	14.60	14.30	0.21	0.19	0.20	1.37	1.02	1.19
E.golobulus (10mg/ml)	5.83	5.10	5.47	13.50	13.37	13.43	0.34	0.31	0.33	0.79	0.75	0.77
P. guajava (10 mg/ml)	5.73	5.17	5.45	12.73	14.03	13.38	0.41	0.30	0.36	0.94	1.01	0.98
Mean	5.83	5.20		13.07	13.90		0.33	0.28		0.99	0.88	
L.S.D. at 0.05 for:	Firmness			TSS			TAA			TPC		
Treatments (T) =	0.25***			0.37			0.27			0.10		
Period (P) =	0.16			0.25			0.01			0.07		
Teatment x Period =	0.36			0.52			0.04			0.15		

*Control= without natural compounds, **Period= (0) samples at harvest time, (60) samples after 60 day of storage at 4ºC., ***Least significant difference (LSD) at 0.05 level confidences was calculated to compare variances between treatments (Steel et al., 1997).

DISCUSSION

Bacterial soft rot is a major problem encountered in the fruits and vegetables during post-harvest storage in the Egyptian markets. Worth mentioning that, our previous study (Elbanna *et al*. 2014) showed that 64% of apple and pear soft rot bacterial strains were identified as members of the genus *Bacillus*. Among them *Bacillus altitudinis* was characterized as a new causative agent of bacterial soft rot. However, the use of the natural antibacterial compounds for the control of bacterial diseases in plant fruits is considered as alternative to chemical bactericides due to their low negative impact on environment. In the present study, three different natural compounds namely: BLS produced from LAB, different concentrations of EEP, and plant extracts were evaluated against tested soft rot *Bacillus* strains. Among eight LAB strains tested against soft rot *Bacillus* strains, bacteriocins of LAB2, LAB105 and LAB 107 recorded the highest antibacterial activity as indicated by the formation of clear zone diameters which were 29.61, 27.50 and 25.89 mm, respectively. The antibacterial activity of the neutralized and catalase treated BLS in this study indicated that the major antibacterial activity in all BLS was most likely due to their antimicrobial peptides not to organic acids or hydrogen peroxide produced by LAB strains. In this context, De Vuyst *et al*. (1994), Lavermicocca *et al*. (2000) and Schnürer *et al*. (2005) reported that LAB display a wide range of antimicrobial activities. Amongst these activities, the production of lactic acid and acetic acid were obviously the most important. However, certain strains of LAB are further known to produce bioactive molecules such as ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, reuterin, and reutericyclin. Many strains also produce bacteriocins and bacteriocin–like molecules that display antibacterial activity. Bacteriocins are generally considered to act at the cytoplasmic membrane and dissipate the proton motive force through the formation of pores in the phospholipid bilayer (Montville *et al*., 1995). Brötz *et al*. (1998) and Dalmau *et al*. (2002) mentioned that antimicrobial peptides may have diverse mechanisms of action, but the cytoplasmic membrane is the target for most bacteriocins. Likewise, the BLS appears to exert its activity by disrupting the functional barrier of membranes of soft rot *E. Carotovora* (Cladera–Olivera *et al*., 2006). However, even though bacteriocin has been widely used in food preservation and medical applications, they are still limited to control soft rot during post–harvest.

With regard to the effect of EEP against the tested soft rot *Bacillus* strains, it was noticed that the antibacterial activity increased as propolis concentrations increased. It was also found that EEP at 10 mg/mL exhibited the highest reduction percentage of soft rot disease severity of apple and pear which were 92.34, 88.93, 90.21 and 92.56 % during seasons 2011/2012 and 2012/2013, respectively. Recently, it was reported that the propolis extracts were successfully used to inhibit growth of some plant pathogenic bacteria such as *Agrobacterium, Clavibacter, Erwinia, Pseudomonas, Ralstonia* and *Xanthomonas* (Basim *et al*., 2006). In this context, it was stated that propolis inhibits the bacterial growth by preventing cell division, thus resulting in the formation of pseudo-multicellular streptococci. In addition, propolis disorganized the cytoplasm, the cytoplasmic membrane and the cell wall causing a partial bacteriolysis and inhibited protein synthesis. However, different researchers have reported that propolis antibacterial activity is attributed to a number of phenolic compounds, mainly flavonoids, phenolic acids and their esters (Bankova *et al*., 2000; Kosalec *et al*., 2004; Katircio *et al*., 2006; Trusheva *et al*., 2006).

The use of plant extracts as antibacterial agents has been known for a very long time, however, in the present study, all tested plant extracts *in vitro* experiment exhibited an obvious decrease in the bacterial growth of the six soft rot *Bacillus* strains. The antibacterial activity of both camphor (*E. globulus*) and guava (*P. guajava*) leaf extracts was found as the most effective compounds against tested soft rot *Bacillus* strains. Similar results were obtained by Cock (2008) and Egharevba *et al*. (2010) who found that the methanol extract of *E. baileyana* and guava exhibited significant antibacterial activity against *B. cereus* and *B. subtilus*, respectively. The antimicrobial mechanisms of plant extracts on microorganisms may involve one or more disturbances such as cytoplasm granulation, cytoplasmic membrane rupture, inactivation or synthesis inhibition of intracellular and extracellular enzymes (Cowan, 1999). As reported by Babayi *et al*. (2004), the antibacterial activity of *Eucalyptus camaldulensis* against soft rot *Bacillus* strains may be due to compounds of the essential oils particularly cineol, cuminal, phellandrene, aromadendral, valerylaldehyde, geralniol, cymene, catechol, tannins, terpenes and isoprenoids, phenolics,

cardiac glycosides, sterols, saponins and flavonoids. Begum *et al*. (2004) reported that the antimicrobial activity of guava leaves is due to tannins, pentacyclic triterpenoid guajanoic acid, ascorbic acid, volatile oils, triterpenoids, flavonoids, guaijavarin.

In vivo experiments also supported the hypothesis that pre–harvest, post–harvest and combined pre–and post–harvest exogenous treatments of both natural antimicrobial compounds substantially minimize the disease severity of apple and pear fruits in storage. The results of this study indicated that all tested natural antimicrobial compounds treatments could significantly reduce severity of soft rot disease caused with *Bacillus altitudinis* of apple and pear fruits stored for 60 days at 4ºC compared to control treatments. In general, combined pre-and post-harvest treatments of fruits with natural antimicrobial compounds proved to be more effective in reducing the soft rot severity of apple and pear followed by pre–then post–harvest treatments during 2011 and 2012 seasons. As presented in Tables 4 and 5 and Figs 4 and 5, relative to pre–harvest treatments, combined pre and post-harvest inducted increasing percentage ranging from 0.67–45.91 and 0.36–58.0% in apple fruits and 0.92–70 and 2.02–74.24% in pear fruits, during both seasons, respectively. Furthermore, relative to post harvest, combined of pre and post-harvest treatments showed increasing percentage ranging from 5.04–63.22 and 0.34–75.57% in pear fruits during both seasons, respectively. These results are in agreement with results obtained by Bartz and Kelman (1986) who reported that BLS effectively inhibits the soft rot development in potato tubers. Also Rosalia *et al*. (2008) reported that, LAB isolated from fresh fruits and vegetables were found to produce organic acid substances that affected some phytopathogenes, causal of postharvest. Propolis water solutions applied as sprays on tomato fruits, resulted in the reduction of the severity of *Xanthomonas* bacterial disease of tomato fruits (Ordónez *et al*., 2011). However, the use of BLS or propolis to control soft rot disease still rare.

The results in Tables 4 and 5 indicated that *E. golobous* and *P. guajava* caused a significant reduction in disease severity of apple and pear fruits during both seasons. These extracts were most effective against soft rot disease when they were applied as combined pre–postharvest treatments. Many researchers reported that plant extracts from 20 plant species caused a reduction of the soft rot disease and suppressed the growth of *B. pumilus* (Krebs *et al*., 2006; Latha *et al*., 2009). However, the mechanisms of disease suppression by plant products have suggested that the active principles present in plant extracts may either act on the pathogen directly (Amadioha, 2000) or induce systemic resistance in host plants resulting in a reduction of the disease development (Kagale *et al*., 2004). The results in Tables 6 and 7 demonstrated that certain natural antimicrobial compounds as plant spraying treatments could be maintained and improved the physical and chemical parameters (firmness, TSS, TTA and TPC) of apple and pear fruits without any deterioration at harvest time or during cold storage. The tested compounds i.e., BLS, EEP and leaves extracts of *E. globulus* and *P. guajava* significantly increased fruit TSS and TPC contents and fruit firmness, while TTA was significantly decreased with EEP treatment in apple and pear fruits. Similar results reported that these compounds improve the physical and chemical properties of fruits (Antani and Ibrahim, 1986; Benvenuti *et al*., 2004).

CONCLUSION

To our knowledge the results of this study demonstrate by first time that combined pre-and post-harvest application of natural antimicrobial compounds could significantly reduce severity of soft rot disease caused by *Bacillus altitudinis* of apple and pear fruits stored at cold temperatures. Furthermore, the combined pre-and post-harvest application of the tested compounds did not negatively affect the physical and physicochemical aspects of the apple and pear fruits, and their sensory characteristics improved during the storage period. These findings reveal the potential application of the natural antimicrobial compounds, especially combined pre–and post–harvest may be an alternative to synthetic antibacterial agents, a useful and promising measure for controlling post–harvest decays for the commercial scale.

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