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Antibacterial Activity of Pomegranate, Orange and Lemon Peel Extracts Against Food-Borne Pathogens and Spoilage Bacteria *In vitro* and on Poultry Skin

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Abstract: This study was conducted to determine the antimicrobial activity of methanol and ethanol extracts of peels of pomegranate (*Punica grana*), orange (*Citrus siensis*) and lemon (*Limona taris*) against four foodborne pathogens (*Listeria monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* (MRSA) and a food spoilage bacterium (*Pseudomonas fluorescens*. Inhibition tests were conducted *in vitro* using the disc diffusion and minimum inhibition concentration (MIC) assays with the Bioscreen Microbiology analyzer. The study also evaluated the antimicrobial activity of the extracts in situ by determining CFU/ml of bacteria recovered from rinsates of chicken skin treated with the peel extracts and by examining the microflora of treated skin samples using scanning electron microscopy (SEM). The antimicrobial activity of all extracts, except the pomegranate ethanol extract, were dependent on the concentration of extract that the bacteria were exposed to during the trials. Treating the inoculated chicken skin with 5 mg/ml of either the five extracts produced significant ($p<0.01$) reductions in CFU/ml of MRSA, *L. monocytogenes* and *P. fluorescens* recovered and the MRSA findings were supported by SEM observations. The antimicrobial activity of peel extracts of pomegranate, orange and lemon indicates that these extracts may be used as sanitizers to reduce microbial contamination of some foods and processing.

Key words: Plant extracts, antimicrobial activity, foodborne pathogens, spoilage bacterium, chicken skin

INTRODUCTION

Illnesses caused by zoonotic foodborne pathogens and drug resistance due to widespread antibiotic use remains a global health problem. A number of bacterial pathogens have been reported to be responsible for foodborne infections and diseases, thereby posing a health risks to humans worldwide (Karch *et al*., 2012). Foodborne gastroenteritis implicating pathogens such as *S.* Typhimurium (Barco *et al*., 2014), *Escherichia coli* (Bloch *et al*., 2012), *Staphylococcus aureus* (Lima *et al*., 2013) and *Listeria monocytogenes* (Choi *et al*., 2014) are well documented in the literature. Factors responsible for these episodes of illness in the consumers of contaminated food frequently include gross pre-harvest contamination, inadequate storage or cooking temperatures and post-process contamination (Gaulin *et al*., 2014).

To ensure that foods are safe for human consumption, efforts are focused on preventing contamination and reducing or eliminating microbial contaminants by physical (e.g. temperature), chemical (e.g. sanitizers) and other treatments (Rooney *et al*., 2004). However, foodborne illnesses continue to pose considerable threats to public health despite improved hygiene management systems and increased regulations. Additionally, several food spoilage organisms, including *Pseudomonas fluorescens*, although non-pathogenic, have been documented to cause great economic losses, therefore measures for their control are also required (Mundo *et al*., 2004).

The prevalence of foodborne, bacterial illnesses has led to recommendations for the use of natural antimicrobial substances (e.g. bacteriocins) in combination with novel technologies for controlling microbial contamination of foods, thereby improving both food quality and food safety (Galvez *et al*., 2010).

The use of natural antimicrobial compounds in food has gained much attention by consumers and the food

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industry (Benelli *et al*., 2010). Extracts of parts of plants and fruits have been documented to possess antibacterial, anti-fungal and anti-viral activities (Chen *et al*., 2014; Sah *et al*., 2011; Settanni *et al*., 2012). Although there are reports on the antibacterial activity of peels of pomegranate, orange and lemon using the disc diffusion method (Abdollahzadeh *et al*., 2011; Settanni *et al*., 2012), there is little information of the growth kinetics of bacteria exposed to these plant extracts. Furthermore, little electron microscopy has been conducted to examine the effects of plant extracts against bacteria on treated food surfaces. Moreover, the incorporation of these plant extracts into food packaging films is continually a major challenge for food industry. Much of the concern related to the use of fruit extracts as sanitizers during food production is not due to the efficacy of these compounds as sanitizers, but due to undesirable organoleptic changes that the sanitizers may produce in foods (Pohlman *et al*., 2002).

Therefore, the present study was conducted to determine the antimicrobial activity of extracts from pomegranates (*Punica grana*), oranges (*Citrus siensis*) and lemons (*Limona taris*) against two Gram-positive food-borne pathogens (MRSA and *L.. monocytogenes*), two Gram-negative pathogens (*E. coli* and *S.* Typhimurium) and one Gram-negative food spoilage bacterium (*P. fluorescens*). Antibacterial activity was measured using the disc diffusion and minimal inhibition concentration (MIC) assays. Additionally, antimicrobial activity of the extracts on inoculated chicken skin was measured by determining the number of bacteria (CFU/ml) recovered from rinsates of treated skin and by observing treated skin using scanning electron microscopy.

MATERIALS AND METHODS

Preparation of plant extracts: Pomegranate (*Punica grana*), orange (*Citrus sinensis*) and lemon (*Limona taris*) purchased from local grocery stores in Auburn, Alabama, USA.

Fruit surfaces were thoroughly washed with distilled water and then dried with disposable paper towels. Fruit skin was peeled from the three fruits using a peeler and peels were collected in separate containers. Peels were freeze-dried for 48 h and a food grinder was used to pulverize the dried skins. Twenty grams each of powdered sample were soaked for 24 h in 100 ml of organic solutions. Extract P1 was prepared by soaking pomegranate peel in 99.9% methanol, Extract P2 was prepared by soaking pomegranate peel in 99.5% ethanol, Extract O1 was prepared by soaking orange peel in 99.9% methanol, Extract O2 was prepared by soaking orange peel in 99.5% ethanol, Extract L1 was prepared by soaking lemon peel in 99.9% methanol and Extract L2 was prepared by soaking lemon peel in 99.5% ethanol. After soaking, extracts were filtered through No. 1 Watman filter paper and each extract was concentrated by heating at 50°C in a rotary evaporator to evaporate almost 90% of solvent. Extracts were stored in a desiccator at room temperature to ensure maximum evaporation of the solvent. Three concentrations (1, 10 and 100 mg/ml) of each extract was made by diluting the extracts in phosphate saline buffer (PBS).

Selection and source of microorganisms: Bacterial strains were obtained from Poultry Microbiological Safety and Processing Research Unit, Russell Research Center, U. S. Poultry National Research Center, Athens, GA, USA 30605. Stock cultures of isolates of methicillin-resistant *S. aureus* (MRSA), *L. monocytogenes*, *S.* Typhimurium, *E. coli* and *Pseudomonas fluorescens* were maintained on tryptic soy agar (TSA) slants (Difco, Becton Dickinson, Cockeysville, MD, USA) at 4°C.

Determination of antimicrobial activity using the agar diffusion method: The 5 bacterial strains were each inoculated into 50 ml of tryptic soy broth (TSB, Difco) then MRSA, *L. monocytogenes, S.* Typhimurium, *E. coli* were incubated for 18-24 h at 37°C, while *P. fluorescens* was incubated for the same time at 28°C. Cultures were then centrifuged at 3,400 g for 10 min, cell pellets were re-suspended in 0.1% peptone water and the optical density (O.D.) of each bacterial inoculum was adjusted to an O.D. equivalent to approximately 10^8 colony forming unit (CFU)/ml using a spectrophotometer at 600 nm. An aliquot of 0.1 ml of the bacterial cultures were spread on the surface of TSA plates and the surface of the plates were dried under a biological safety cabinet. Twenty-five µl aliquots of each plant extract in concentrations of 1, 10 and 100 mg/ml were pipetted onto sterile paper discs (6 mm diameter, Oxoid) and the discs were placed onto the surface of inoculated agar plates. Sterile discs pipetted with PBS were included in this study as control negative. All inoculated plates were incubated at 37°C for 24 h, with the exception of *P. fluorescens* which was incubated at 28°C. After incubation, the diameter of the zone of inhibition (mm) of bacterial growth around the discs was measured using Traceable Carbon Fiber Digital Calipers (Fisher Scientific, Inc., Pittsburg, PA). The experiment was performed in triplicate and the zones of inhibition were expressed as mean±SD.

Minimum inhibition concentration (MIC) assays of plant extracts: Solutions of 10 mg/ml concentrations of each plant extract was serially diluted (2-fold) in sterile TSB to produce extract concentrations of 0.62, 1.25, 2.5 and 5 mg/ml. Suspensions (0.1 ml) of 18-24 h cultures of each of the five bacterial isolates were added to

separate tubes containing 0.9 ml of TSB-plant extract (TSB-PE) mixtures (1:10 dilution) to produce a final concentration of 10^7 CFU/ml of each. Then, 0.3 ml volumes of each inoculated TSB-PE mixture were dispensed into wells of a Honeycomb multi-well plate (Lab Systems, Inc, Franklin, MA) (Hinton, 2013). Wells containing TSB plus 0.1 bacterial culture inoculum without plant extract served as the bacterial positive control. The inoculated multi-well plates were then placed in the incubator tray of Bioscreen C Microbiology Reader (Thermo Electron Corp., West Palm Beach, FL) operated by a computer with Growth Curves Software, v. 2.28 (Transgalactic Ltd., Helsinki, Finland). The microbiology reader recorded the O.D. of cultures at 600 nm during incubation at 37°C for 24 h but at 28°C for 24 h for *P. fluorescens* and this O.D. used to plot the growth curves of each of the five bacteria used in the study.

Antibacterial activity of extracts on chicken skin: Three chicken skin pieces (1 x 1 cm) in size were dipped into 10 ml of approximately 1 x 10^8 CFU separate suspension of each of the five test microorganisms and vortexed for 2 min at room temperature (25°C) to ensure uniform inoculum. The skins were stored in bacterial cultures at 4°C for 6 h in sterile bags to facilitate bacterial attachment to the skin surface. All inoculated skin samples were then air dried in a sterile petri dish in a laminar flow hood for 1 h at room temperature as earlier described (Gyawali *et al*., 2011). Thereafter, 50 µl of 2.5 or 5 mg/ml concentrations of each plant extract were added to separate skin pieces. In addition, inoculated chicken skin samples, which were not treated with any of the extracts (0 mg/ml concentration), were included in the study as controls. After treatement of the skin samples with plant extracts, each sample was added to 10 ml of 0.1% peptone solution and vortexed for 2 min (Lang *et al*., 2004). Serial 10-fold dilutions of the rinsates were performed in 0.1% peptone water and 100 µl of each dilution of the rinsates were plated in duplicate on TSA plates. The inoculated plates were incubated at 37°C for 24 h, except for *P. fluorescens* plates which were incubated at 28°C and bacterial colonies were counted and reported as average log CFU/ml.

Scanning Electron Microscopy (SEM) of skin samples: Chicken skin samples inoculated with MRSA and treated with the plant extracts were examined by SEM to observe possible morphological changes of bacterial cells attached to the skin (Gyawali *et al*., 2011). Samples were fixed by immersion in 1 ml of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 12 h and then post-fixed in 1% OsO_4 for 2 h. The fixed samples were dehydrated in an ethanol gradient (60, 70, 80, 90 and 100%) with 10 min of exposure per concentration and then will be critical point dried in CO_2. Finally, the slides were taped onto stubs, coated with gold and observed using SEM (Jeol USA, Inc., Peabody, MA 01960). Samples were examined at a magnification of 15000X and at a zoom level of 10 kv and 1 micron.

Statistical analyses: One-way analysis of Variance (ANOVA) of group means of inhibition zones and log CFU/ml was performed to determine significant differences alpha = 0.001 (SPSS 12.0.0.2).

RESULTS AND DISCUSSION

The antibacterial activities of the plant extracts tested against MRSA, *L. monocytogenes*, *S.* Typhimurium, *E. coli* and *P. fluorescens* by the agar disc diffusion method are displayed in Table 1. All the tested plant extracts showed antibacterial activity against the tested bacterial strains with the exception of P2. The antimicrobial activities of P1, O1, O2, L1 and L2 were significantly ($p<0.001$) higher against the Gram-positive (MRSA and *Listeria monocytogens*) compared with Gram-negative (*S.* Typhimurium and *E. coli*) pathogenic bacteria. For all four pathogenic microorganisms the degree of bacterial inhibition by the extracts was concentration related, i.e., lowest at 1 mg/ml and highest at 100 mg/ml. However, *P. fluorescens* was detected to be significantly ($p<0.001$) more susceptible to all the five extracts that were inhibitory compared with the other four bacteria tested. *P. fluorescens* was inhibited by all extracts even at the lowest concentration (1 mg/ml) tested.

There are published reports on the potential use of fruit extracts and essential oils as alternatives to control growth of microorganisms and to control diseases caused by these microorganisms (Hamdan *et al*., 2010; Nannapaneni *et al*., 2008). Antimicrobial, anti-oxidant, anti-inflammatory, anti-trypanosomal and cytotoxic properties of plant extracts are well documented in the literature (Hamdan *et al*., 2013; Hamdan *et al*., 2010; Nannapaneni *et al*., 2008). The findings in this study are in agreement with published reports that found similar antibacterial activity of peel extracts of the three fruits (Al-Zoreky, 2009; Hayrapetyan *et al*., 2012; Nannapaneni *et al*., 2008; Rakholiya *et al*., 2014; Tayel *et al*., 2012) on selected bacteria. It has also been demonstrated that the extracts of peels of pomegranate have synergistic activity when used in conjunction with other antimicrobial agents, therefore offering therapeutic potential (Dey *et al*., 2012; Jurenka, 2008). However, reports also exist on the failure of some of these extracts to inhibit certain bacteria and that different fractions of the extracts vary in their antimicrobial activities (Nannapaneni *et al*., 2008; Sah *et al*., 2011).

Table 1: Antimicrobial activity of different plant extracts against food borne and spoilage bacteria

Strain		MRSA	*L. monocytogenes*	*S.* Typhimurium	*E. coli*	*P. fluorescens*
Compound concentrations		Zones of inhibition				
P1	100	12.27±0.21^{a}	12.5±0.2^{a}	9.16±0.31^{b}	7.03±0.21^{c}	13.3±0.21^{d}
	10	9.13±0.15^{e}	10.16±0.21^{f}	7.2±0.26^{g}	0	10.57±0.31^{f}
	1	0	6.67±0.25^{h}	0	0	6.97±0.15^{h}
P2	100	0	0	0	0	0
	10	0	0	0	0	0
	1	0	0	0	0	0
O1	100	13.8±0.26^{a}	25.56±0.15^{b}	12.16±0.38^{c}	7.8±0.46^{d}	24.3±0.2^{e}
	10	10±0.26^{f}	20.96±0.21^{g}	8.66±0.25^{h}	0	20.3±0.2^{i}
	1	0	6.73±0.38^{j}	0	0	10.33±0.25^{k}
O2	100	14.2±0.26^{a}	23.06±0.15^{b}	10.3±0.36^{c}	0	22.67±0.25^{b}
	10	10.4±0.26^{d}	19.56±0.25^{e}	6.5±0.26^{f}	0	19.27±0.21^{e}
	1	0	6.73±0.38^{g}	0	0	10.37±0.25^{h}
L1	100	13.96±0.15^{a}	23.03±0.21^{b}	11.63±0.42^{c}	7.3±0.44^{d}	23.37±0.25^{b}
	10	9.16±0.21^{e}	18.36±0.32^{f}	8.07±0.31^{g}	0	19.43±0.25^{h}
	1	0	6.96±0.15^{i}	0	0	10.37±0.15^{j}
L2	100	13.43±0.25^{a}	23.4±0.26^{b}	10.2±0.26^{c}	6.6±0.26^{d}	22.03±0.25^{e}
	10	9.5±0.2^{f}	18.33±0.21^{g}	6.6±0.36^{h}	0	18.77±0.25^{g}
	1	0	6.83±0.15^{i}	0	0	9.3±0.26^{j}

Values are averages±SD deviation, n = 3. Within each row of every plant extract, different superscripts indicate significant differences ($p<0.001$) by one-way analysis of Variance (ANOVA). P1, Pomegranate peel (Methanol extraction); P2, Pomegranate peel (Ethanol extraction); O1, Orange peel (Methanol extraction); O2, Orange peel (Ethanol extraction); L1, Lemon peel (Methanol extraction); L2, Lemon peel (Ethanol extraction)

Although the solvents used to prepare the plant extract may have bactericidal activity at high concentrations, the concentration of solvents contained within the plant extract of this study displayed no antibacterial activity against the five micro-organisms tested (data not shown). We have observed antibacterial activity of pomegranate extracted using methanol and no antibacterial activity of pomegranate extracted using ethanol against the five micro-organisms tested. Other studies have also reported the antibacterial activity of methanol extracts of pomegranate (Braga *et al*., 2005; Dey *et al*., 2012). Although ethanol extracts of pomegranate peel failed to exhibit antibacterial effect in the present study, others have demonstrated that this extract may possess antibacterial activity (Guneidy *et al*., 2014). Solvents used for extraction may interfere with the biological effects of plants active ingredients (Rababah *et al*., 2010; Rakholiya *et al*., 2014). Additionally, peels of different strains of pomegranate may contain different amounts of antibacterial active components which may be affected by the extraction solvents, concentration and temperature used (Rababah *et al*., 2010; Rakholiya *et al*., 2014).

The findings in the current study, which indicated that Gram-positive bacteria tested were more susceptible to the plant extracts than the Gram-negative bacteria may be due to the ability of the extracts to penetrate the cell walls of the Gram-positive bacteria. Alvarez-Ordonez *et al.* (2013) reported that the plant extracts affected the molecular structure of the bacteria cell wall and caused leakage of intracellular contents. Sanchez *et al.* (2010) also reported that plant extracts were able to disrupt the cell membranes of *V. cholerae* cells, causing increased membrane permeability, a decrease in cytoplasmic pH, cell membrane hyperpolarization and a decrease in cellular ATP concentration. The outer membrane surrounding the cell wall of Gram-negative bacteria restricts the diffusion of hydrophobic compounds (Mahfuzul Hoque *et al*., 2007; Nascimento *et al*., 2000; Rakholiya *et al*., 2014; Ratledge and Wilkinson, 1988). Other studies have also found that Gram-negative are more susceptible to the antibacterial activity of plant extracts (Burt, 2004), while some studies have found that there was no significant differences in the susceptibility of the two groups of bacteria to plant extracts (Hamdan *et al*., 2013).

Minimal inhibition concentration of plant extracts against five bacteria: The growth curves of the five bacterial in the presence of different concentrations of plant extracts and their minimum inhibition concentration (MIC) are shown in Fig. 1. In Fig. 1 (a), the control MRSA strain in TSB grew from initial turbidity (~0.3, O.D. 600 nm) to achieve a maximum absorbance of 1-1.1 within 24 h of incubation at 37°C. The MIC values determined against MRSA were 2.5, 5, 5, 2.5 and 2.5 mg/ml for P1, O1, O2, L1 and L2, respectively. In Fig. 1(b), the MIC against *L.. monocytogenes* was determined to be 1.25 mg/ml for P1, O1, L1 and L2 and 2.5 mg/ml by O2. On the other hand, Figures 1 (c) and (d) show that the Gram-negative pathogenic bacteria (*S.* Typhimurium and *E. coli*) in the presence of the plant extracts still achieved their maximum growths and therefore had high MIC values which is above the tested concentrations by MIC assay. However, the MIC values against *P. fluorescens* were 1.25 mg/ml by P1, O1, O2, L1 and L2 extracts (Fig. 1e). Low MIC values have been reported for plant extracts for Gram-positive bacteria (Al-Zoreky, 2009;

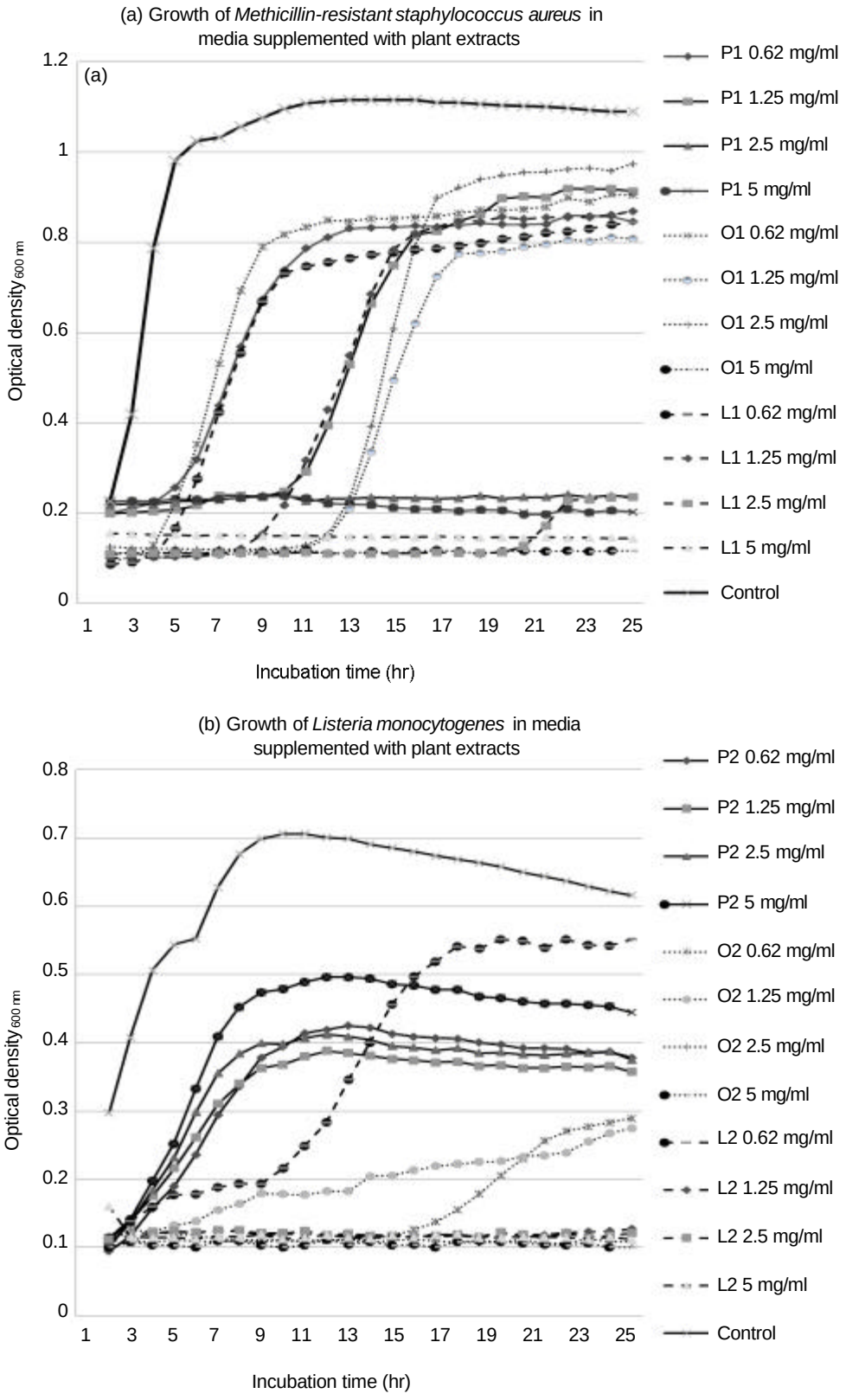
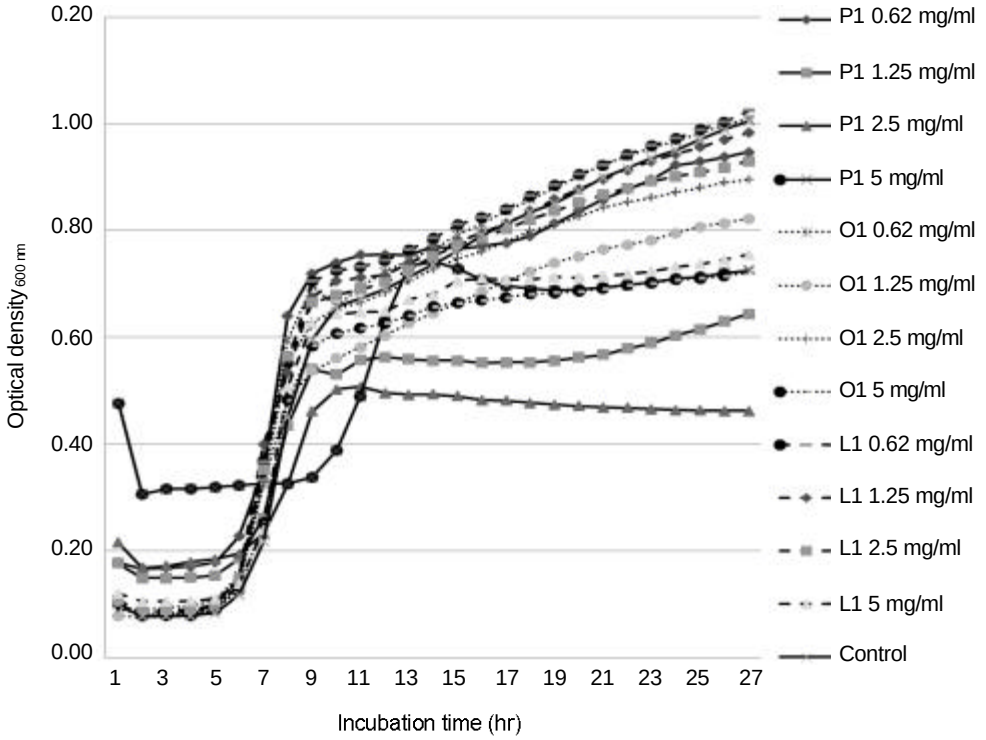
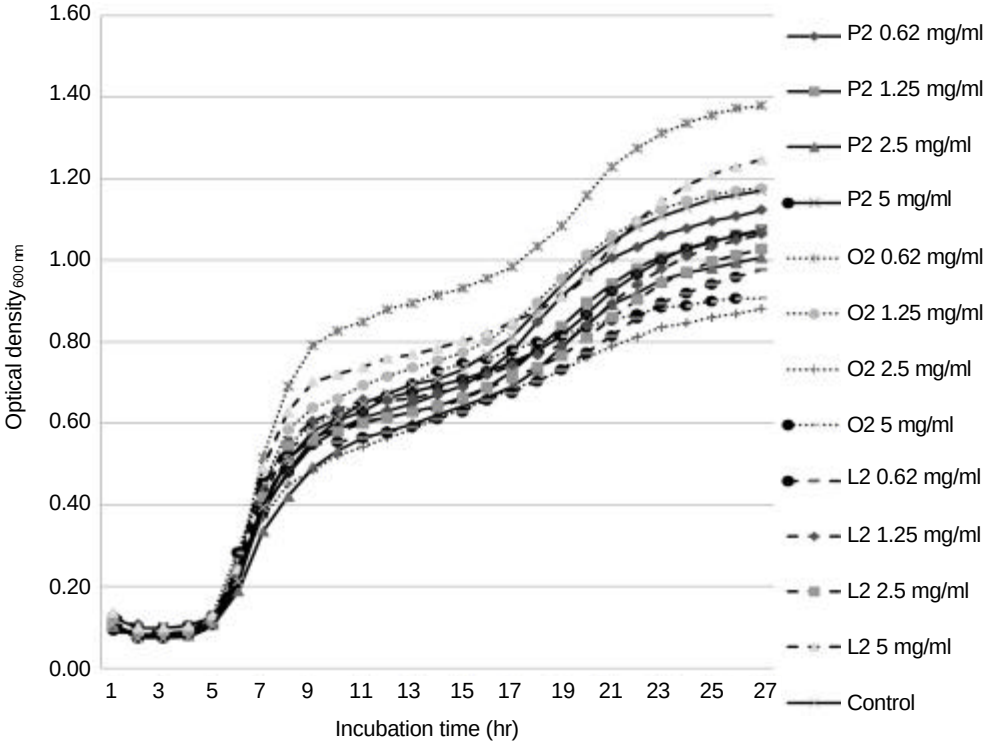


Fig. 1: Continue

(c) Growth of *Salmonella* Typhimurium in media supplemented with plant extracts



(d) Growth of *E. coli* in media supplemented with plant extracts



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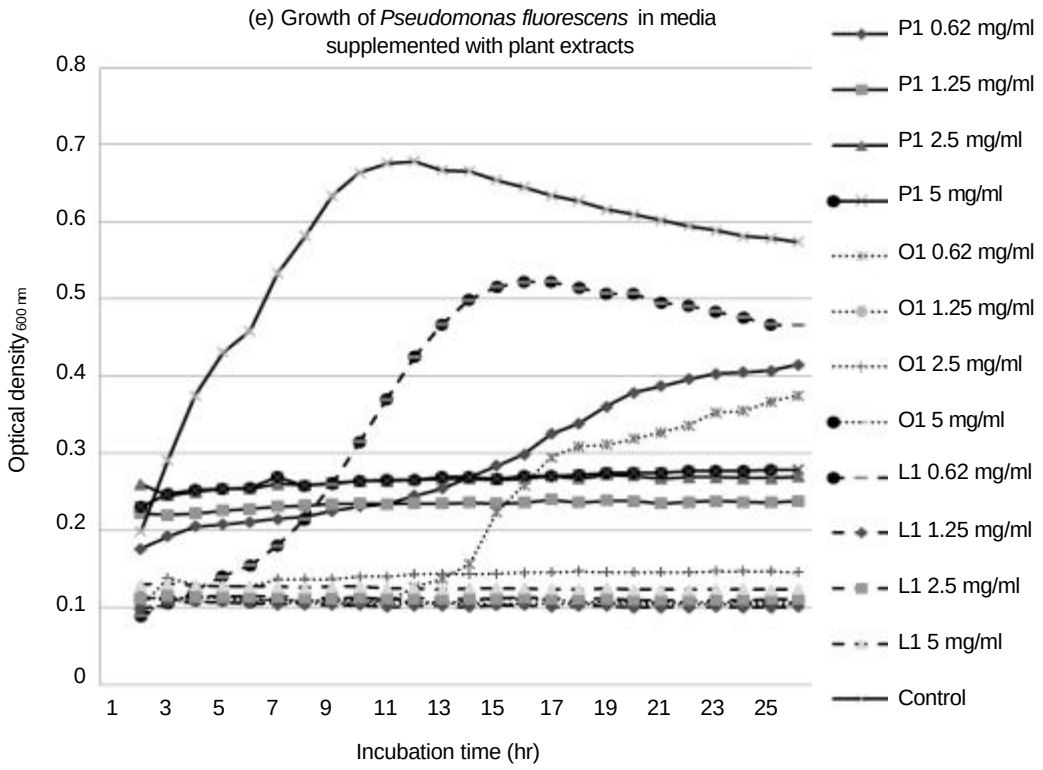

Fig. 1(a-e): Optical Density$_{600\ nm}$ of Methicillin-resistant *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli* and *Pseudomonas fluorescens* cultures incubated at 37°C for 48 h in media supplemented with plant peel extracts from pomegranate (*Punica grana*), (P1, P2), orange (*Citrus siensis*) (O1, O2) and lemon (*Limona taris*) (L1, L2), at concentrations of 0.62, 1.25, 2.5 and 5 mg/ml

Hayrapetyan *et al*., 2012; Tayel *et al*., 2012) and comparatively high values for Gram-negative bacteria (Al-Zoreky, 2009; Burt, 2004). It was also of interest that *P. fluorescens*, a Gram-negative bacterium was even more susceptible to the extracts with MIC value as low as 1.25 mg/ml. This finding is supported by the report of Farag *et al*. (1989) who found that *E. coli* was less resistant than *P. fluorescens* when tested with essential oils from some plant extracts. It is therefore evident that there is not only a variation of susceptibility to plant extract between Gram-negative and Gram-positive organisms but also there is a variation in susceptibility amongst the genera of Gram-negative bacteria. The action of these extracts therefore does not entirely depend on the cell wall structure alone but on other mechanisms as earlier suggested (Alvarez-Ordonez *et al*., 2013; Sanchez *et al*., 2010).

Antimicrobial activity of plant extracts on inoculated chicken skin: Figure 2(a-e) shows that the bacterial populations in the rinsate of chicken skin, were significantly reduced ($p<0.01$) from approximately 8 log CFU/ml in control samples of MRSA, *L. monocytogenes* and *P. fluorescens* to approximately 4.5~4.8 log CFU/ml at a concentration of 5 mg/ml for all tested plant extract except P2. However, reduction for *S.* Typhimurium and *E. coli* were not statistically significant ($p>0.01$). Valtierra-Rodriguez *et al*. (2010) evaluated *in vitro* and *in situ* skin model to test antibacterial activity of peel extracts from 28 edible plants, against *Campylobacter jejuni* and *Campylobacter coli*. These results indicated no *Campylobacter* were recovered from chicken skin inoculated with 10^5 CFU of *Campylobacte*r after incubation (48 h at 4°C) with any of these extracts mixtures.

Scanning electron microscopy of bacteria attached to chicken skin: SEM photographs of skin inoculated with MRSA are shown in Fig. 3(a-c). Figure 3(a) shows the control skin that were no treatment with plant extracts while in Fig. 3(b, c), shows a visible reduction of bacterial population of inoculated skin samples treated for 8 h with extracts P1 and and L1. SEM examination of chicken skin inoculated with MRSA and treated with plant extracts did not demonstrate any detectable changes in the cellular morphology of MRSA on control or treated

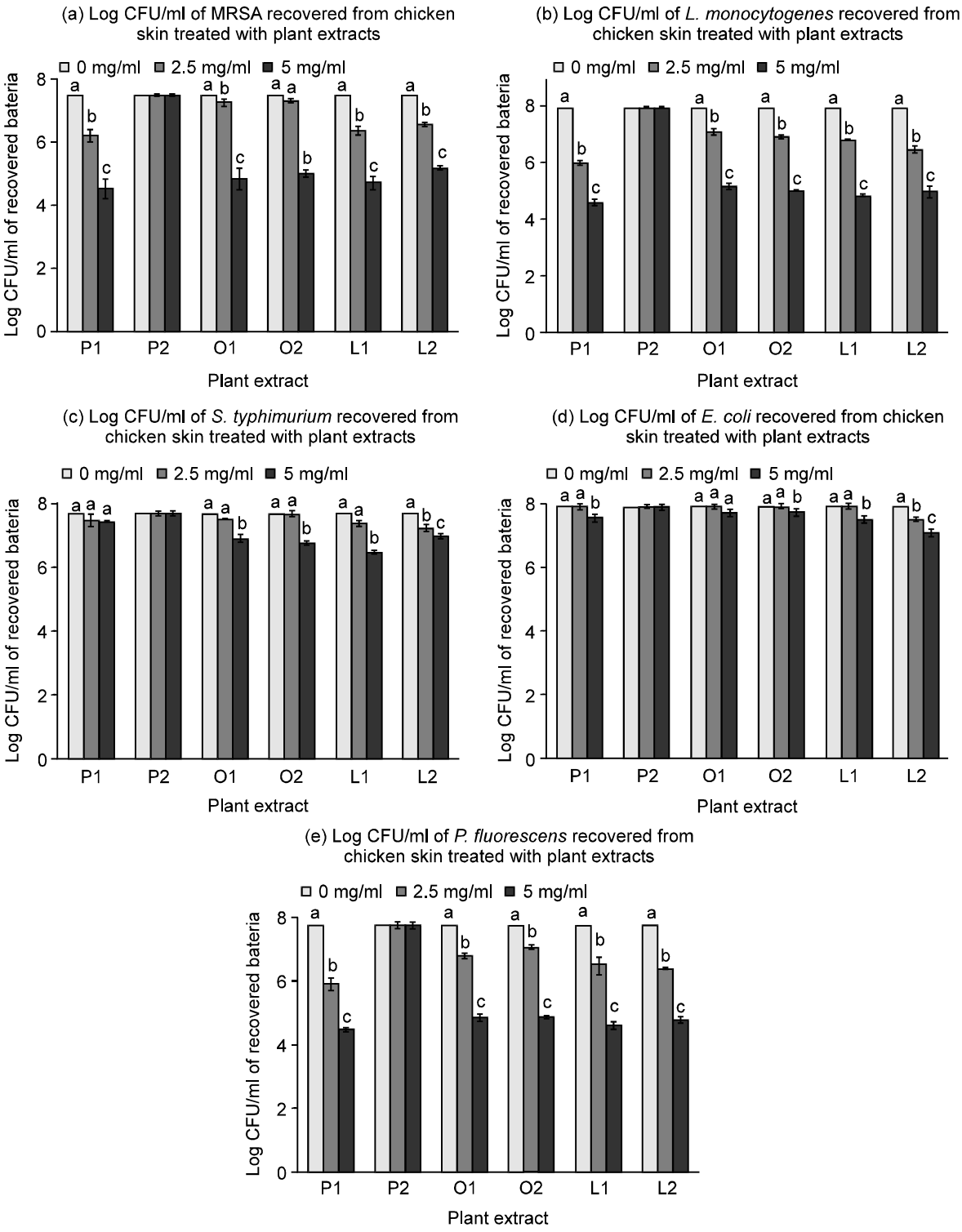

Fig. 2 (a-e): Log_{10} CFU/ml of bacteria recovered from rinsates of chicken skin inoculated with Methicillin-resistant *Staphylococcus aureus*, *Listeria mono-cytogenes*, *Salmonella* Typhimurium, *Escherichia coli* and *Pseudomonas fluorescens*, after treatment with plant peel extracts from pomegranate (*Punica grana*), (P1, P2), orange (*Citrus siensis*) (O1, O2) and lemon (*Limona taris*) (L1, L2) at concentrations of 0, 2.5 and 5 mg/ml. Different superscripts for each plant extract bars for the tested bacteria indicate significant differences ($p<0.001$)

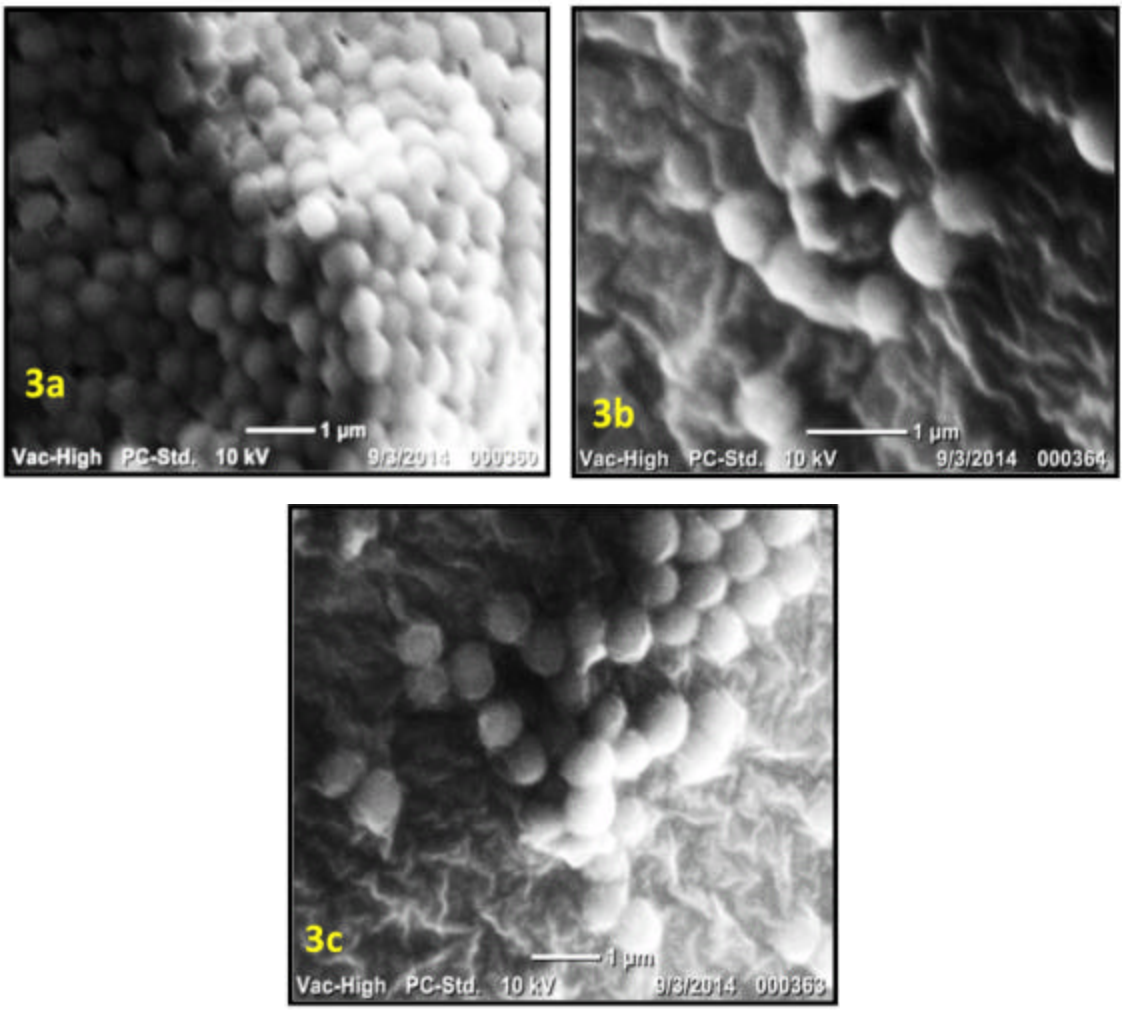

Fig. 3: Scanning electron microscope photographs of chicken skin inoculated with Methicillin-resistant *Staphylococcus aureus* (MRSA). 3a-Untreated control; 3b-skin treated with 5 mg/ml of pomegranate extract for 6 h; 3c-skin treated with 5 mg/ml of extract lemon peel extract for 6 h. Magnification of 15000X and at a zoom level of 10 kv and 1 micron

chicken skin samples. Similar morphology of MRSA on treated and non-treated skin may be because the bactericidal activity of the plant extracts was at cellular and molecular level (Alvarez-Ordonez *et al*., 2013; Sanchez *et al*., 2010).

Findings from this study indicate that these extracts possess antibacterial activity and may potentially be used as food preservatives or sanitizers. The source of these extracts may increase their acceptance by ever-increasing health conscious consumers.

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