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Production of Functional Sausage Using Pomegranate Peel and Pistachio Green Hull Extracts as Natural Preservatives

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ABSTRACT

This study investigated partial replacement of nitrite by Pomegranate Peel (PPE) and Pistachio Green Hull Extracts (PGHE) in cooked sausages and their effects on oxidative, microbial, and physicochemical properties of the samples. To this end, 250, 500, 750, 1,000, and 1,250 ppm of the two extracts and 100, 80, 60, 40, and 0 ppm of nitrite were added to the sausages and the peroxide and TBARS values, microbial tests, sensory evaluation, and color factors were measured during 30 days storage at 4°C. Antioxidant and antimicrobial properties of both treatments were as well as the control, or sometimes better than it. PGHE treatments had better color factors compared to PPE treatments. Sensory scores of PPE_3 and $PGHE_3$ (containing 60 ppm nitrite and 750 ppm of extracts) were not significantly different compared to the control. Thus, reduction of nitrite up to 50% and replacement of it by PPE or PGHE do not cause great changes in quality parameters of sausage and improve its functional properties.

Keywords: Functional food, Nitrite, *Pistacia vera* L., *Punica granatum* L.

INTRODUCTION

Several synthetic antioxidants are often added to prevent oxidative and microbial spoilage of meat products (Shah *et al.*, 2014). Sodium or potassium nitrite can be used as effective antioxidants in cured meat products. These additives have four major roles: color improvement, antimicrobial activity, antioxidative activity, and taste improvement. Despite advantages of these additives, formation of nitrosamines is one of the potentially carcinogenic and mutagenic effects (Zarringhalami *et al.*, 2009). Hence, many researchers have evaluated reduction of nitrite by natural counterparts (Pegg and Shahidi, 2000). Also, consumers have a tendency toward natural meat products that contain lower amounts of nitrite and higher amounts of natural functional ingredients. Plant extracts are rich sources of phenolic compounds and can be substituted for synthetic preservatives such as nitrite (Shah *et al.*, 2014). In previous studies, partial replacement of nitrite by natural materials such as peppermint essential oil, annatto pigments, and red grape extract has been investigated in different meat products (Moarefian *et al.*, 2012; Zarringhalami *et al.*, 2009; Riazi *et al.*, 2015). It should be noted that agricultural waste products are rich in polyphenolic compounds, cheap and economical sources, and can be used instated of essential oils and medicinal plants, which are more expensive.

Pomegranate (*Punica granatum* L.) is one of the oldest fruits that contain the highest amount of total phenolic compounds in comparison with other fruits. Pomegranate peels and seeds are the main waste fractions of industrial processing of pomegranate fruit, which are produced in a large scale (Goula and Lazarides, 2015). Pomegranate peel has been widely studied by many researchers because it contains natural antioxidants such as phenolic acids and flavonoids that possess anti-mutagenic, anti-cancer, anti-inflammatory and anti-cardiovascular diseases (Li *et al.*, 2006; Negi *et al.*, 2003). Regarding the antimicrobial activity of PPE, Al-Zoreky (2009) noted that

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80% methanolic extract of pomegranate peel inhibited the growth of *L. monocytogenes*, *E. coli, S. aureus*, and *Y. enterocolitica*. Kanatt *et al.* (2010) indicated that PPE had stronger antioxidant activity than BHT in chicken meat products. Pistachio (*Pistacia vera* L.) is another native fruit of Iran with high annual production. After harvesting and gathering the pistachio, the soft outer hull of pistachio is immediately separated from the hard woody shell; the hull is the largest portion of waste product of pistachio that makes up about 35-45% of the pistachio nut (Barreca *et al.*, 2016). Because of high levels of phenolic compounds, the green hull of pistachio can potentially be used as an antioxidant and natural preservative. Rajaei *et al.* (2010) investigated the antimicrobial effect of pistachio green hull extract on the gram positive and negative bacteria and concluded that it was effective against gram-positive ones such as *B. cereus* and *S. aureus*. Also, its antioxidant effect was studied by Goli *et al.* (2005), showing good antioxidative effect in soybean oil up to 0.06%.

Although there are a few studies on reduction of nitrite in meat products formulation, no information is available about replacement of PGHE and PPE with reduction of nitrite in sausage formulation. Therefore, we aimed to study the effects of pomegranate peel and pistachio green hull extracts as partial replacers of nitrite on oxidative, microbial, and physicochemical characteristics of cooked beef sausage.

MATERIALS AND METHODS

Chemicals

TriChloroacetic Acid (TCA), sodium chloride, sodium thiosulphate, gallic acid, Folin-Ciocalteu reagent, sodium carbonate, thiobarbituric acid, starch, methanol, and culture media were purchased from Merck Co. (Darmstadt, Germany). DPPH (1, 1-DiPhenyl-2-PicrylHydrazyl) was purchased from Sigma-Aldrich Co. (St. Louis. Missouri, USA). Chloroform and acetic acid were purchased from Mojallali Laboratory (Tehran, Iran). All other reagents and chemicals used in the study were of analytical grade.

Preparation of Pomegranate Peel and Pistachio Green Hull Extracts

The pomegranate peels (*Malase torshe saveh* variety) and pistachio green hulls (*Ahmad aghaei* variety) were dried, powdered in a grinder (Moulinex, France) to reach particles sizes of 0.5 to 2 mm for pistachio green hull and 0.2 mm for pomegranate peel and then packed and stored at -20^{o}C until extraction. Dried powders were extracted according to the methods reported by Yasoubi *et al.* (2010) and Goli *et al.* (2005). The concentrated extracts were freeze- dried. The dried extracts were stored in black bags in the freezer (-20^{o}C) until the day of experiments.

Preparation of Low-Nitrite Sausages

Sausage samples were manufactured according to the traditional formulation. The formulations of both treatments had constant ingredients including beef meat (55%), ice (22.4%), soybean oil (10%), starch (4%), gluten (3%), soy protein isolate (3%), salt (1.5%), $Na_5P_3O_{10}$ (0.3%), red and black pepper (0.5%) and nutmeg (0.3%). The variant ingredients of sausage formulations are shown in Table 1. The components of each formulation were mixed separately in a cutter (Seydelmann, Aalen, Germany) and were filled in polyamide casings and labelled, then cooked at 75^{o}C for 1 hour. After cooking, the sausages were cooled with cold water and chilled at 2°C for 8 hours. Finally, the sausages were stored in a refrigerator (at 4±1°C) for 29 days.

Total Phenolic Contents and Radical Scavenging Activity of Extracts

The amounts of Total Phenolic Contents (TPC) of the extracts were measured according to the Folin-Ciocalteu method (Yasoubi *et al.*, 2010), and the results were expressed as Gallic Acid Equivalents per gram dry weight of samples (mg GAE g^{-1} dw).

Radical scavenging activity was determined according to Rajaei *et al.* (2010) method with some modifications. A sample of 0.3 mL of

Table 1. Abbreviations used for different treatments in this study.[a]

Treatment	Variant		
	Nitrite (ppm)	PPE (ppm)	PGHE (ppm)
C	120	-	-
PPE_1	100	250	-
PPE_2	80	500	-
PPE_3	60	750	-
PPE_4	40	1000	-
PPE_5	-	1250	-
$PGHE_1$	100	-	250
$PGHE_2$	80	-	500
$PGHE_3$	60	-	750
$PGHE_4$	40	-	1000
$PGHE_5$	-	-	1250

[a] C: Control; PPE: Pomegranate Peel Extract, PGHE: Pistachio Green Hull Extract.

different concentrations of extracts was mixed with 2.7 mL of methanolic solution of DPPH radicals (0.1 mM). After shaking, the reaction mixture was incubated for 30 mins in dark place at room temperature and absorbance was read at 517 nm. Radical scavenging activity was calculated as follows:

%Radical Scavenging Activity (%RSA)= $[(A_{control}-A_{sample})/A_{control}]\times100$

Where, A stands for Absorbance. Thirty microliter methanol plus 2.7 mL of DPPH solution was applied as a control. IC_{50} value, which is the inhibitory concentration of extract at which 50% of DPPH radicals of the solution are scavenged by the extract, was also measured.

Effect of Thermal Processing on Radical Scavenging Activities of Extracts

Both extracts were heated (at 75 and 100°C for 15, 30, and 45 minutes) and then, radical scavenging activities of the extracts were measured by DPPH method.

Peroxide Value

Peroxide Values (PV) of the sausages were measured on days 1, 8, 15, 22, and 29 of storage. For PV measurement, sausage's oil was extracted according to Fölch *et al.* (1957) method with some modifications. Then, peroxide value was measured according to the AOAC (1999) method.

Thiobarbituric Acid Reactive Substances (TBARS) Value

On days 1, 8, 15, 22, and 29 of storage, TBARS values were measured according to Pfalzgraf *et al.* (1995) method. TBARS content was calculated from a calibration curve using MalonDiAldehyde (MDA) as a standard (0.05-0.5 µM).

Microbiological Evaluation

Serial dilutions of the samples were prepared in the tubes containing peptone water (0.1%) and the selective medium of each bacterium was used for its identification and counting (Viuda-Martos *et al.*, 2010). Microbial tests including total viable count, coliforms (Viuda-Martos *et al.*, 2010), molds and yeasts (Anonymous, 2008) and *C. perfringens* determination (Riazi *et al.*, 2015) were performed after 1, 8, 15, 22, and 29 days of storage. The results were reported as $\log_{10}$ cfu g^{-1} sample.

Color Evaluation

Color of samples were measured on the first and last days of storage at 4˚C using the

Hunter Lab Colourflex Colorimeter (Hunter Associated Lab, Inc., Reston, VA, USA) against a white standard tile. The CIE L* (Lightness), a* (redness), and b* (yellowness) values were measured. Also, the Hue angle and Chroma were calculated with the following formula:

Chroma= $\sqrt{a*^2 + b*^2}$ Hue angle= $\tan^{-1}\frac{b*}{a*}$

pH Measurement and Chemical Analyses

For pH measurement, 10 g of minced sample was homogenized with 100 mL water in a homogenizer (IKA-T18, Staufen, Germany). Then, the pH value of the resulting slurry was measured with the pH meter (Metrohm, pH Lab 827, Herisau, Switzerland) (Viuda-Martos *et al.*, 2010). Chemical analyses including moisture, protein, fat and ash content were carried out according to AOAC (1999) methods.

Sensory Evaluation

On day 8 of storage, sensory analysis of the samples was performed to evaluate the color, odor and taste of each sausage sample by 30 semi-trained panelists (12 males and 18 females) with an age range of 22–45 years, who were selected for sensory evaluation of the samples. The assessors gave scores from 0 to 100 (100: Excellent, 75: Good, 50: Fair, 25: Poor and 0: Terrible) for each parameter of sausages of different formulations (Moarefian *et al.*, 2012).

Statistical Analysis

All experiments were carried out in triplicate and mean values with standard deviation were reported. Analysis Of Variance (ANOVA) was conducted and differences between variables were tested for significance by one-way ANOVA with Least Significant Difference (LSD) test. All statistical analyses were performed using SAS software (9.4). A statistical difference at $P< 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Total Phenolic Contents of Extracts

Phenolic compounds, which are produced by many plants, cause antioxidative properties of many vegetables and fruits (Kanatt *et al.*, 2010). TPC of pomegranate peel and pistachio green hull dried extracts were 451.7± 5.3 and 180.1±9.3 mg GAE g^{-1} dw, respectively. Basiri *et al.* (2015) found the TPC of the Pomegranate Peel Extract (PPE) to be 420.58 ± 9.1 mg tannic acid equivalent (TAE) g^{-1}. The most dominant compounds responsible for the antioxidative potential of the pomegranate peel are gallic acid, ellagic tannins and ellagic acid (Negi *et al.*, 2003). Similar to PGHE's result, Grace *et al.* (2016) declared that the TPC of the PGHE was 140.11 mg GAE g^{-1} dw. The most numerous phenolic compounds of PGHE are gallic acid, 4-hydroxybenzoic acid, protocatechuic acid, eriodictyol-7-O-glucoside, isorhamnetin-7-O-glucoside, isorhamnetin-3-O-glucoside, 3-O-rutinoside, quercetin, naringin, and catechin (Barreca *et al.*, 2016).

Radical Scavenging Activity of Extracts

The Radical Scavenging Activity (RSA) of PPE and PGHE are shown in Figure 1. PPE showed an excellent RSA with an IC_{50} of 0.12 mg dry extract mL^{-1}. RSA of PPE had a significant gradual to sharp increasing trend between 20 and 200 mg kg^{-1} ($P< 0.05$). However, no additional increase in RSA was observed after 200 mg kg^{-1} concentration of extract ($P<0.05$). Also, Basiri *et al.* (2015) observed a gradual to sharp increase in RSA of PPE between 50 and 500 ppm and there was no additional increase in RSA between 500 and 1,000 ppm. Rajan *et al.* (2011) reported the IC_{50} value of aqueous extract of pomegranate rind to be 0.13 mg mL^{-1} that is inconsistent with our result. PGHE also showed a good RSA with an IC_{50} of 0.35 mg dry extract mL^{-1}. Similar to PPE, RSA of PGHE increased up to 500 ppm, after which it was constant. As expected, PPE with higher

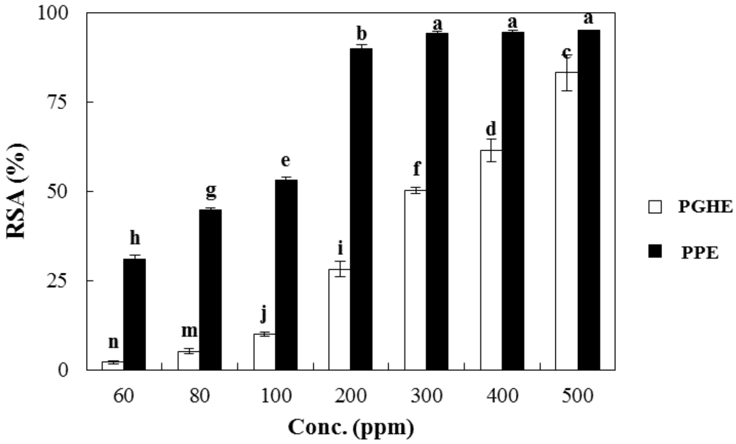

Figure 1. Radical Scavenging Activity (RSA) of Pomegranate Peel (PPE) and Pistachio Green Hull Extracts (PGHE) at different concentrations. The different letters on the bars indicate significant differences (P< 0.05).

TPC had stronger RSA and lower IC_{50} value compared to PGHE.

For industrial application of extracts in sausages formulation, the effect of thermal processing on the RSA of extracts were performed (Figure 2). As can be seen in Figure 2, thermal processing increased RSA of both extracts and antioxidant activities of the extracts were increased by increasing heating temperature and time. Similar to our finding, Kang *et al.* (2007) observed that free radical scavenging activity and total phenolic contents of American ginseng were significantly increased by thermal processing. These increases of RSA and TPC can be related to the release of the phenolic acids bound with glucosides or amine functionalities. It should be noted that the rate of the increase in RSA of PGHE was higher than PPE.

Peroxide Value

Table 2 shows changes in PV of the control (containing 120 ppm nitrite), PPE and PGHE-formulated sausages. The amounts of hydroperoxides as rudimentary oxidation products that are produced by attack of oxygen on the double bond of fatty acids at the initial stage of oxidation are measured by PV test (Qin *et al.*, 2013). Peroxide values ranged over 0.50 to 1.83 meq O_2 kg^{-1} oil for all treatments. The PV values increased in the treatments up to the middle of the storage time. Then, PV values decreased to the end of storage period. The increase of PV may be due to the

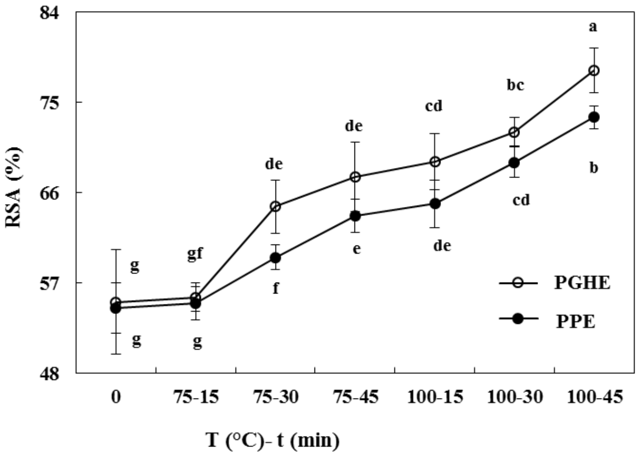

Figure 2. Effect of thermal processing on DPPH Radical Scavenging Activity (RSA) of Pomegranate Peel (PPE) and Pistachio Green Hull Extracts (PGHE). Different letters on the lines indicate significant differences (P< 0.05).

production of new hydroperoxides that takes place faster than their decomposition (Qin *et al.*, 2013). The decrease of PV may support this hypothesis that, as hydroperoxides are so reactive, they decompose and give rise to secondary oxidation products that are responsible for the increase of TBARS value (Berasategi *et al.*, 2011). Also, after induction period, a period at which oxidative changes are minimal, hydroperoxides' decomposition rate is faster than their formation rate that yield to a wide variety of decomposition products, including aldehydes, ketone, acid, etc. (Chaijan *et al.*, 2006). Georgantelis *et al.* (2007) reported the same decrease in PV of beef burger. As can be seen in Table 2, PV values of all treatments (except $PPE_{(1, 2, 3)}$ on the 8th day and $PGHE_2$ on the 29th day, which have significantly (P< 0.05) more PV values compared to the control) are significantly lower than the control or do not have significant difference compared to the control throughout storage period. This indicates that PPE and PGHE could reduce the formation of hydroperoxides as well as, or even better than, nitrite. Qin *et al.* (2013) also reported that utilization of pomegranate rind powder extract could reduce PV value of raw ground pork meat the same as BHT and as well as, or better than, pomegranate juice and pomegranate seed powder extract. Moreover, samples with higher PPE and PGHE levels showed lower PV and more inhibitory effect throughout storage period, which is due to the higher amounts of phenolic compounds. This suggests that PPE and PGHE retard lipid oxidation in a dose dependent manner. Hence, $PPE_{(4, 5)}$ and $PGHE_{(4, 5)}$ were the best samples from the viewpoint of this parameter. This is agreement with the research done by Juntachote *et al.* (2006), who noticed that higher concentrations of dried galangal powder and its ethanolic extract reduced PV value in cooked ground pork meat greater than lower concentrations, and it followed concentration dependant manner. In contrast to our result, Moarefian *et al.* (2012) stated that higher concentrations of peppermint essential oil added to sausage caused prooxidative effect and increased PV value. PV values of PGHE treatments were lower than PPE treatments up to 8th day of storage. But, on the 15th day of storage, there were no significant differences among treatments, except $PGHE_5$ which had significantly lower PV (0.65 meq O_2 kg^{-1} oil) compared to others, and on the 22nd and 29th days of storage, there were no significant differences among treatments, except PPE_5, which had the lowest PV (0.53 meq O_2 kg^{-1} oil) among others.

Thiobarbituric Acid Reactive Substances Values

Table 3 shows changes of TBARS values of

Table 2. Peroxide values of sausages (meq O_2 kg^{-1} oil) produced by different amounts of nitrite, PGHE and PPE during 29 days of storage at 4˚C. [a]

	Day				
Treatment	1	8	15	22	29
C	1.23±0.15^{aB}	1.53 ± 0.05^{bcA}	1.66 ± 0.11^{aA}	1.60 ± 0.17^{aA}	1.13 ± 0.05^{bcdB}
PPE_1	0.96±0.05^{bC}	1.66 ± 0.11^{abA}	1.56 ± 0.11^{abcA}	1.56 ± 0.05^{abA}	1.20 ± 0.17^{bcB}
PPE_2	1.13±0.11^{abC}	1.83 ± 0.15^{aA}	1.46 ± 0.05^{bcB}	1.40 ± 0.17^{bB}	0.93 ± 0.05^{deC}
PPE_3	1.13±0.15^{abC}	1.76 ± 0.20^{aA}	1.43 ± 0.05^{cB}	1.03 ± 0.05^{cC}	0.9 ± 0.10^{eC}
PPE_4	1.10±0.10^{abAB}	1.13 ± 0.11^{deAB}	1.23 ± 0.15^{dA}	1.03 ± 0.05^{cBC}	0.86 ± 0.05^{eC}
PPE_5	0.53±0.05^{cdC}	1.10 ± 0.10^{efB}	1.53 ± 0.05^{abcA}	0.53 ± 0.05^{dC}	0.53 ± 0.05^{fC}
$PGHE_1$	0.70±0.10^{cC}	1.33 ± 0.15^{cdB}	1.66 ± 0.15^{aA}	1.73 ± 0.11^{aA}	1.23 ± 0.25^{bcB}
$PGHE_2$	0.61±0.12^{cdB}	0.85 ± 0.15^{gB}	1.53 ± 0.05^{abcA}	1.70 ± 0.17^{aA}	1.56 ± 0.11^{aA}
$PGHE_3$	0.56±0.05^{cdD}	0.86 ± 0.15^{gC}	1.65 ± 0.13^{abA}	0.96 ± 0.05^{cC}	1.26 ± 0.11^{bB}
$PGHE_4$	0.56±0.05^{cdC}	0.88 ± 0.12^{fgB}	1.10 ± 0.10^{dA}	0.93 ± 0.11^{cAB}	1.06 ± 0.11^{bcdeAB}
$PGHE_5$	0.50±0.10^{dB}	0.56 ± 0.05^{hB}	0.65 ± 0.15^{eB}	0.93 ± 0.11^{cA}	1.03 ± 0.05^{cdeA}

[a] Values are mean±Standard Deviation (SD) of three replicates. Different lowercase letters within the same column are different by LSD test (P< 0.05). Different uppercase letters within the same row are different by LSD test (P< 0.05). All abbreviations are defined under Table 1.

Table 3. TBARS values of sausages (µmole MDA kg^{-1} sample) produced by different amounts of nitrite, PGHE and PPE during 29 days of storage at 4˚C.[a]

	Days				
Treatment	1	8	15	22	29
C	$3.01±0.08^{cdefB}$	$3.33 ± 0.11^{aA}$	$3.40 ± 0.23^{abA}$	$2.88 ± 0.06^{cB}$	$2.79 ± 0.05^{fgB}$
PPE_1	$2.74±0.03^{gC}$	$2.92 ± 0.11^{bcdeB}$	$2.96 ± 0.01^{cdB}$	$3.61 ± 0.04^{aA}$	$2.96 ± 0.03^{defB}$
PPE_2	$2.90±0.05^{efgC}$	$2.66 ± 0.10^{eD}$	$2.93 ± 0.17^{cdC}$	$3.63 ± 0.10^{aA}$	$3.17 ± 0.06^{bcdB}$
PPE_3	$2.92±0.04^{defC}$	$2.94 ± 0.09^{bcdeC}$	$2.83 ± 0.16^{dC}$	$3.68 ± 0.09^{aA}$	$3.24 ± 0.12^{abcB}$
PPE_4	$2.89±0.05^{efgC}$	$2.84 ± 0.38^{cdeC}$	$2.94 ± 0.12^{cdBC}$	$3.51 ± 0.10^{aA}$	$3.28 ± 0.09^{abAB}$
PPE_5	$2.88±0.05^{fgC}$	$3.00 ± 0.05^{bcdBC}$	$3.11 ± 0.19^{bcdB}$	$3.56 ± 0.15^{aA}$	$3.44 ± 0.08^{aA}$
$PGHE_1$	$3.07±0.05^{bcdAB}$	$2.78 ± 0.29^{deBC}$	$3.31 ± 0.26^{abA}$	$2.62 ± 0.16^{dC}$	$2.69 ± 0.12^{gC}$
$PGHE_2$	$3.05±0.10^{bcdeB}$	$3.41 ± 0.19^{aA}$	$3.24 ± 0.19^{abcAB}$	$3.12 ± 0.14^{bAB}$	$2.95 ± 0.28^{efB}$
$PGHE_3$	$3.21±0.11^{bAB}$	$2.86 ± 0.24^{cdeB}$	$3.30 ± 0.34^{abA}$	$3.01 ± 0.017^{bcAB}$	$3.03 ± 0.18^{cdeAB}$
$PGHE_4$	$3.13±0.07^{bcB}$	$3.13 ± 0.15^{abcB}$	$3.42 ± 0.17^{abA}$	$3.19 ± 0.12^{bB}$	$3.14 ± 0.02^{bcdeB}$
$PGHE_5$	$3.38±0.24^{aAB}$	$3.22 ± 0.08^{abAB}$	$3.52 ± 0.10^{aA}$	$3.14 ± 0.27^{bB}$	$3.06 ± 0.12^{cdeB}$

[a] Values are mean±Standard Deviation (SD) of three replicates. Different lowercase letters within the same column are different by LSD test ($P< 0.05$). Different uppercase letters within the same row are different by LSD test ($P< 0.05$). All abbreviations are defined under Table 1.

the control (containing 120 ppm nitrite), PPE and PGHE-formulated sausages. TBARS value measures the amount of secondary oxidation products, especially MalonDiAldehyde (MDA), which may cause off-flavor in fatty foods (Yasoubi *et al*., 2010). TBARS values ranged over 2.62 to 3.61 µmol MDA kg^{-1} sample for all treatments. Since, PV values of PPE samples decreased on 22^{nd} day of storage, TBARS values increased rapidly in that time. This increase shows that the hydroperoxides have partially decomposed, giving rise to secondary oxidation products, that is in agreement with the results reported by Berasategi *et al.* (2011), about the use of aqueous extract of *Melissa officinali* in bologna sausages. TBARS values of PGHE samples increased up to day 15 of storage. Thereafter, TBARS values of samples decreased at the last day of storage for PPE and from day 15 up to the end of storage for PGHE treatments, which agrees with previously published results (da Silveira *et al.*, 2014). Rubio *et al.* (2008) pointed out that decrease in TBARS value during storage could be related to MDA reaction with amino acids, sugars and nitrite in the sample. Also, another reason for this decrease may be due to the fact that MDA and other carbonylic compounds derived from lipid oxidation are not permanently stable and, after some time, decomposes to organic acids and alcohols that are not determined by this method, yet. Another possibility may be the utilization of MDA by *Enterobacteria* and *Pseudomonas*, which have the ability to metabolize carbonylic compounds (da Silveira *et al.*, 2014). At the beginning of storage time, no significant differences ($P< 0.05$) were observed among most treatments compared to the control. All PPE samples had lower TBARS values than the control on the 8^{th} and 15^{th} days of storage and there were no significant differences among treatments.
Reduction in the TBARS values of PPE treatments can be related to the presence of PPE, which might have neutralized oxidation products. On the 22^{nd} day of storage, the extent of secondary oxidation of all PPE treatments was higher than the control, and on the 29^{th} day of storage, secondary oxidation in the control and PPE_1 were less than other samples. In the case of PGHE samples, TBARS values fluctuated among treatments. No significant differences were observed among most treatments and the control up to day 15 of storage, after which, TBARS values of treatments were significantly higher than the control, compared to which some treatments did not have significant differences. This fact indicates that the antioxidative activity of both extracts decreased in the last week of storage due to their decomposition. Wang *et al.* (2014) utilized Green Tea Polyphenols (GTP) and

Grape Seed Extract (GSE) as antioxidants in dry-cured bacon and observed that only GTP treated bacons had lower TBARS values compared to the control, compared to which the TBARS values of GSE treated samples did not have significant difference. According to Greene and Cumuze (1982), the acceptability limit of TBARS value for meat products is about 12 µmole MDA kg^{-1} sample. In our study, all samples had lower TBARS values than this limit throughout storage period and, therefore, they were acceptable. Besides, PPE treatments had significantly lower TBARS values in comparison to PGHE treatments up to 15^{th} day of storage.

Microbial Evaluation

In general, the standard Total Viable Count (TVC) increased throughout storage in both formulations and it ranged from 2.15 to 3.54 logcfu/g (Table 4). Other researchers (Qin *et al.*, 2013; Kanatt *et al.*, 2010) observed similar increase in the total viable count of raw ground pork meat and chicken products, respectively. There were no significant differences (P< 0.05) in TVC among the control, PPE, and PGHE treatments, but there were exceptions for PPE treatments, which, on the 1^{st} and 15^{th} days of storage, had significantly (P< 0.05) lower TVC compared to the control. This is in agreement with other studies about pomegranate peel extract incorporating in meat products (Kanatt *et al.*, 2010; Qin *et al.*, 2013). Results of microbial analysis show that PPE and PGHE have been able to control microbial growth in the product or sometimes better than nitrite, because nitrite dosage gradually decreases in the treatments and plant extract dosage increases, showing the potential of extract to act as well as nitrite. No growth of *C. perfringens*, yeasts and molds and coliforms were observed in all the samples during storage. This might be due to destruction of these bacteria during cooking of sausage at 75ºC that is above their death point (57ºC). Other factors could be hygienic factors such as high-quality raw materials, good handling during the processing and packaging, enough heat treatment of the product and good storage conditions. Additionally, different ingredients used in the formulation of the product such as nitrite, phosphates, condiments and natural extracts that are proved to possess antibacterial and/or bacteriostatic effects, might have inhibited their growth (Gadekar *et al.*, 2014). The antimicrobial activity of PPE has been studied by many researchers. Al-Zoreky (2009) found the inhibitory effect of pomegranate peel extract against the growth of *L. monocytogenes*, *E. coli*, *S. aureus*, and *Y. enterocolitica*. Hayrapetyan *et al.* (2012)

Table 4. Total viable count of sausages (log cfu g^{-1}) produced by different amounts of nitrite, PGHE and PPE during 30 days of storage at 4˚C. [a]

Treatment	Days				
	1	8	15	22	29
C	2.47±0.07^{aD}	2.84 ± 0.05^{abC}	3.08 ± 0.03^{aB}	3.15 ± 0.14^{aB}	3.35 ± 0.10^{bcA}
PPE$_1$	2.31±0.07^{bcD}	2.74 ± 0.05^{abcC}	2.79 ± 0.04^{cC}	2.95 ± 0.10^{bB}	3.32 ± 0.08^{bcA}
PPE$_2$	2.29±0.09^{cD}	2.70 ± 0.14^{cC}	2.80 ± 0.04^{cBC}	2.96 ± 0.06^{bB}	3.32 ± 0.13^{bcA}
PPE$_3$	2.30±0.07^{cD}	2.80 ± 0.05^{abcC}	2.86 ± 0.23^{bcBC}	3.08 ± 0.17^{abB}	3.35 ± 0.12^{bcA}
PPE$_4$	2.31±0.12^{bcD}	2.79 ± 0.09^{abcC}	2.87 ± 0.17^{bcBC}	3.02 ± 0.12^{abAB}	3.24 ± 0.05^{cA}
PPE$_5$	2.15±0.09^{dC}	2.83 ± 0.10^{abB}	2.90 ± 0.09^{bcB}	2.99 ± 0.14^{abB}	3.40 ± 0.15^{abA}
PGHE$_1$	2.52±0.02^{aE}	2.74 ± 0.05^{abcD}	2.93 ± 0.05^{abcC}	3.09 ± 0.09^{abB}	3.36 ± 0.10^{bcA}
PGHE$_2$	2.48±0.11^{aD}	2.73 ± 0.05^{abcC}	2.95 ± 0.06^{abcB}	3.04 ± 0.07^{abB}	3.45 ± 0.03^{abA}
PGHE$_3$	2.43±0.04^{abE}	2.71 ± 0.07^{bcD}	2.93 ± 0.04^{abcC}	3.10 ± 0.05^{abB}	3.42 ± 0.05^{abA}
PGHE$_4$	2.45±0.06^{aE}	2.73 ± 0.07^{abcD}	2.97 ± 0.03^{abC}	3.14 ± 0.05^{aB}	3.46 ± 0.07^{abA}
PGHE$_5$	2.43±0.04^{abE}	2.85 ± 0.08^{aD}	2.97 ± 0.06^{abC}	3.16 ± 0.03^{aB}	3.54 ± 0.02^{aA}

[a] Values are mean±Standard Deviation (SD) of three replicates. Different lowercase letters within the same column are different by LSD test (P< 0.05). Different uppercase letters within the same row are different by LSD test (P< 0.05). All abbreviations are defined under Table 2.

found that pomegranate peel extract could effectively inhibit *L. monocytogenes*'s growth in meat pâté. Rajaei *et al.* (2010) concluded that mostly gram-positive bacteria (*B. cereus* and *S. aureus*) were inhibited by both crude and purified PGHE and they were more sensitive in comparison to gram- negative bacteria. Phenolic compounds have the ability to denature enzymes. They can also inhibit the growth of microorganisms by binding to substrates such as minerals, vitamins and carbohydrates, and making them inaccessible for microorganisms. However, the most probable mechanism is that phenolics can be adsorbed to the cell wall of microorganisms and cause disruption of the cell membrane (Basiri *et al.*, 2015).

Color Evaluation

The L*, a*, b*, Hue angle and chroma values of the control (containing 120 ppm nitrite), PPE and PGHE-formulated sausages during storage period are presented in Table 5. As there were no significant differences ($P< 0.05$) among color parameters of samples throughout storage, the color parameters of the 29th day of storage are reported. The L* value significantly decreased ($P< 0.05$) with the addition of PPE in all the PPE samples compared to the control and the product became slightly darker. This was in agreement with other researches in this field such as Qin *et al.*(2013) and EI-Gharably and Ashoush (2011) who utilized pomegranate peel powder and powder extract in pork meat and beef sausage, respectively. There were no significant differences between treatments, except PPE_5 that had no nitrite and lower L* value compared to other treatments. In PGHE samples, the L* value of $PGHE_1$ was significantly ($P< 0.05$) lower than the control. $PGHE_{(2, 3, 4)}$ did not have significant differences with the control and $PGHE_5$ had the highest L* among all treatments. The a* value also, significantly decreased in all the PPE and PGHE samples compared to the control except $PGHE_1$, which did not have significant difference compared to the control. No-nitrite samples (PPE_5 and $PGHE_5$) had the same and the lowest a* value amongst the treatments. This is in agreement with findings of EI-Gharably and Ashoush (2011). Nitrite causes production of nitrosomyoglobin and after cooking it changes to nitrosohemochrome as a denatured protein (Parthasarathy and Bryan, 2012). Thus, in low-nitrite products, a* decreases. The b* value of PPE samples were significantly lower than the control, compared to which the PGHE samples did not have significant differences, except PPE_5 and $PGHE_5$ that had the highest b* value among the treatments and control. In a general view, it was noticeable that PGHE treatments had higher L*, a* and b* values than PPE

Table 5. Color factors of sausages produced by different amounts of nitrite, PGHE and PPE on the 29th day of storage at 4˚C.[a]

Treatment	Color factors				
	Lightness (L*)	Redness (a*)	Yellowness (b*)	Hue angle	Chroma
C	59.54±0.17[b]	12.37 ± 0.14[a]	17.13 ± 0.10[c]	54.13 ± 0.30[d]	21.13 ± 0.14[a]
PPE_1	56.22±0.71[d]	10.05 ± 0.64[de]	16.23 ± 0.11[de]	58.21 ± 1.61[bc]	19.09 ± 0.35[d]
PPE_2	55.81±0.41[de]	10.25 ± 0.58[d]	15.44 ± 0.33[f]	56.35 ± 0.98[bcd]	18.53 ± 0.59[de]
PPE_3	55.07±0.24[e]	9.45 ± 0.73[e]	15.61 ± 1.06[ef]	58.65 ± 3.71[b]	18.27 ± 0.54[e]
PPE_4	55.12±0.70[e]	9.96 ± 0.53[de]	16.18 ± 0.52[def]	58.26 ± 1.61[bc]	19.01 ± 0. 52[d]
PPE_5	54.05±0.44[f]	6.61 ± 0.26[f]	17.90 ± 0.14[b]	69.70 ± 0.59[a]	19.08 ± 0.21[d]
$PGHE_1$	58.64±0.53[c]	12.26 ± 0.32[ab]	16.85 ± 0.08[cd]	53.86 ± 0.60[d]	20.84 ± 0.24[ab]
$PGHE_2$	59.08±0.42[bc]	11.56 ± 0.17[bc]	16.75 ± 0.30[cd]	55.27 ± 0.21[d]	20.35 ± 0.34[bc]
$PGHE_3$	59.16±0.44[bc]	11.31 ± 0.38[c]	17.06 ± 0.45[c]	56.32 ± 1.59[bcd]	20.47 ± 0.17[bc]
$PGHE_4$	58.97±0.23[bc]	11.23 ± 0.27[c]	16.74 ± 0.32[cd]	56.05 ± 0.92[cd]	20.16 ± 0.26[c]
$PGHE_5$	60.85±0.69[a]	7.23 ± 0.27[f]	19.30 ± 0.43[a]	69.40 ± 1.05[a]	20.61 ± 0.35[abc]

[a] Values are mean±Standard Deviation (SD) of three replicates. Different lowercase letters within the same column are different by LSD test ($P< 0.05$). All abbreviations are defined under Table 2.

treatments. Addition of PPE made all color parameters of sausages lower than the control. However, addition of PGHE did not change color parameters, except a* value. Van Ba *et al.* (2016) observed that sausages containing 0.01 % nitrite had higher a* values compared to sausages containing *shiitake* by-product extract; also, addition of that extract resulted in higher b* values that is agreement with this study's results. Salejda *et al.* (2016) utilized walnut green husk in cooked sausage as a preservative and observed that a* and L* values decreased and b* increased compared to the control, in agreement with our findings. PPE_4 and $PGHE_4$ samples had lower nitrite content in comparison with other treatments and did not have significant differences with each other's and could be chosen as the best samples in terms of color attribute. It can be concluded that reduction of nitrite to 40 ppm to gain the appropriate color was possible in this study mainly in PGHE treatments. Hue angle of sausage samples with reduced nitrite or without any nitrite increases during storage period (Moarefian *et al.*, 2012). In our study, with the increase of PPE and PGHE concentrations and reduction of nitrite and a* value, hue angle increased and the highest degrees were observed in PPE_5 and $PGHE_5$. This is agreement with the results obtained by EI-Gharably and Ashoush (2011) and Moarefian *et al.* (2012). Chroma results indicated that PPE and PGHE treatments had lower values than the control, except $PGHE_{(1, 5)}$, which did not have significant differences with the control. Also, chroma values of PGHE treatments were significantly higher than PPE treatments. Red beet powder incorporated in beef sausage also caused reduction of chroma value compared to the control (EI-Gharably and Ashoush, 2011).

pH Changes and Chemical Analysis of Sausages

The pH changes ranged over 6.00-6.41 among treatments and there was not significant effect during storage time (data not shown). The moisture, fat, protein and ash contents of the control and all treatments ranged from 61.83-62.93, 15.16-15.70, 14.99-15.15, and 2.40-2.94%, respectively. Addition of the different concentrations of nitrite, PPE and PGHE did not have any significant effect ($P< 0.05$) on these contents (data not shown). Salejda *et al.* (2016) also reported that addition of walnut green husk to cooked sausages did not affect the chemical composition of those products.

Sensory Evaluation

The results of sensory evaluation of the control (containing 120 ppm nitrite), PPE and PGHE-formulated sausages are shown in Table 6. $PPE_{(1, 2, 3)}$ and $PGHE_{(1, 2, 3)}$ samples had the highest scores for taste and odor, which did not have significant differences with the control ($P< 0.05$). Odor and taste of treatments 4 and 5 of both PPE and PGHE had significantly lower scores compared to the control ($P< 0.05$), probably because the darker color of these treatments affected the panelists' mentality to give lower scores, while different concentrations of extracts did not seem to have any considerable effect on taste and odor of the products. Color of $PGHE_1$, $PGHE_2$ and $PGHE_3$ treatments did not have significant differences compared to control, but all PPE treatments had significantly lower color scores compared to the control ($P< 0.05$). PPE_5 and $PGHE_5$ gained the lowest scores of color among the treatments and the control because of lack of nitrite in them. Qin *et al*. (2013) found that although pomegranate rind powder extract caused changes in the color of raw ground pork meat, the overall acceptability of the product did not have significant differences compared to the meat including synthetic antioxidant. As a result, nitrite dosage can be reduced up to 60 ppm, which is a harmless concentration from the view of nitrosamine formation and can be replaced by 750 ppm of PPE or PGHE without any great change in the acceptability of the sample, and resulting in healthier product.

CONCLUSIONS

PPE and PGHE are inexpensive, abundant, and economic sources of phenolics in comparison with essential oils or synthetic antioxidants.

Table 6. Sensory scores of sausages produced by different amounts of nitrite, PGHE and PPE on the 8th day of storage at 4˚C.[a]

	Sensory Parameters		
Treatment	Color	Odor	Taste
C	69.16±20.43[a]	68.33 ± 14.58[a]	68.33 ± 14.58[a]
PPE_1	56.66±22.67[b]	61.66 ± 19.40[abcd]	65.83 ± 19.12[ab]
PPE_2	57.50±14.89[b]	63.33 ± 14.28[abc]	67.50 ± 14.89[a]
PPE_3	55.83±18.19[b]	61.66 ± 14.28[abcd]	63.33 ± 17.03[abc]
PPE_4	44.16±14.20[c]	59.16 ± 13.90[bcd]	56.66 ± 18.49[cd]
PPE_5	20.83±17.47[e]	57.50 ± 21.92[bcd]	57.50 ± 16.28[bcd]
$PGHE_1$	74.16±16.71[a]	65.83 ± 19.12[ab]	68.33 ± 19.62[a]
$PGHE_2$	75.00±17.37[a]	62.50 ± 20.50[abcd]	70.00 ± 21.17[a]
$PGHE_3$	68.33±21.70[a]	64.16 ± 16.97[abc]	65.83 ± 21.25[ab]
$PGHE_4$	56.66±18.49[b]	56.66 ± 17.28[cd]	55.83 ± 15.65[cd]
$PGHE_5$	31.66±19.62[d]	54.16 ± 20.84[d]	54.16 ± 19.78[d]

[a] Values are mean±Standard Deviation (SD) of three replicates. Different lowercase letters within the same column are different by LSD test (P< 0.05). All abbreviations are defined under Table 2.

They showed high TPC and great antioxidant activities. PV and TBARS values of treatments including reduced amount of nitrite and increased amount of plant extracts were the same or sometimes better than the control sample and lower than reported standard for sausages. Microbial evaluation showed the efficacy of nitrite and both extracts having complementary effects to hold total viable count and growth of pathogenic bacteria below the threshold limit. Color of the PGHE samples were better than PPE samples and hunter-lab results showed that color factors of PGHE samples were nearer to the control, which contained 120 ppm nitrite. Reduction of nitrite by 50% was not detectable by sensory evaluators. Finally, these extracts can be applied with reduced amounts of nitrite up to 50% in cooked sausages to enhance the functional properties of the product and lower the formation of carcinogenic nitrosamines in sausage.

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تولید سوسیس فراسودمند با استفاده از عصاره های پوست انار و پوست سبز پسته به عنوان دو نگهدارنده طبیعی

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چکیده

در این تحقیق تاثیر جایگزینی بخشی از نیتریت در سوسیس پخته شده با عصاره های آبی پوست انار (PPE) و پوست سبز پسته (PGHE) بررسی گردید و تاثیر آن ها بر خواص اکسایشی، میکروبی و فیزیکوشیمیائی نمونه ها مطالعه شد. به ترتیب ۲۵۰، ۵۰۰، ۷۵۰، ۱۰۰۰ و ۱۲۵۰ ppm از دو عصاره و ۱۰۰، ۸۰، ۶۰، ۴۰ و ۰ ppm از نیتریت به سوسیس ها اضافه شد ومقادیر پراکسید و TBARS، آزمون های

میکروبی، ارزیابی حسی و فاکتورهای رنگ در طی ۳۰ روز نگهداری در دمای $4\ ^{o}C$ اندازه گیری شدند. خواص ضداکسایشی و ضد میکروبی هر دو تیمار عصاره همانند کنترل و در برخی موارد بهتر از آن بود. تیمارهای PGHE از نظر عامل رنگ بهتر از تیمارهای PPE بودند. نتایج آزمون حسی PPE_3 و $PGHE_3$ (حاوی ۶۰ ppm نیتریت و ۷۵۰ ppm از عصاره ها) در مقایسه با کنترل اختلاف معنی داری نداشتند. بنابراین، کاهش نیتریت تا ۵۰ درصد و جایگزینی آن با PPE یا PGHE باعث تغییرات زیادی در پارامترهای کیفی سوسیس نشده و خواص فراسودمند آن را بهبود می بخشد.