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ARTICLE

Effects of Annatto (*Bixa orellana* L.) Seeds Powder on Physicochemical Properties, Antioxidant and Antimicrobial Activities of Pork Patties during Refrigerated Storage

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Abstract

This study investigated the effect of the powder produced by ball-milling the outer layer of annatto (*Bixa orellana* L.) seeds on the physicochemical properties as well as the antioxidant and antimicrobial activities of pork patties over 14 d of refrigerated storage (4± 1°C). Five pork patty treatments were produced containing three different concentrations of annatto seeds, 0.1, 0.25 and 0.5% (ANT0.1, ANT0.25, ANT0.5), 0.1% ascorbic acid (AA0.1), and a control (CTL). Based on the results, annatto seed powder appeared to show antioxidant activity. The Hunter color values of pork patties were affected by the addition of annatto seed powder, which increased the redness and yellowness values, but decreased the lightness of the patties (p<0.05). To evaluate the antioxidative effects of annatto on pork patties, thiobarbituric acid reactive substances (TBARS) and peroxide values (POV) were analyzed over 14 d of refrigerated storage. Treatments containing annatto seed showed lower TBARS and POV than control (CTL) samples (p<0.05). The volatile basic nitrogen (VBN) of the pork patties containing annatto seeds were lower than that of CTL at the end of storage (p<0.05). Although no differences in total bacterial counts were observed between control and treated patties, those containing annatto seeds had lower microbial counts for *Enterobacteriacease* than CTL or AA 0.1%. Therefore, annatto seed powder might be a good source of natural antioxidants for the production of meat products.

Keywords: Annatto seeds, antioxidant activity, lipid oxidation, pork patties

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Introduction

Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and propyl gallate (PG) are widely used to prevent oxidative degradation in the food industry. The oxidation of lipids and proteins is considered as a major cause of food spoilage, especially in meat and meat products. Thus, the use of synthetic antioxidants is the most effective method to control oxidation, which reduces the formation of undesirable oxidation products, enhances nutritional value and sensory quality and extends the shelf-life of muscle foods during storage (Jia *et al*., 2012; Shah *et al*., 2014). Since synthetic antioxidants may produce toxic compounds, consumers and meat technologists have become more interested in antioxidants from natural resources (Ibrahim *et al*., 2010; Jia *et al*., 2012). Recently, the utilization of natural antioxidants and antimicrobial agents extracted from various plants and fruits as food additives and preservatives has increased (Hinneburg *et al.,* 2006).

Annatto (*Bixa orellana* L) seed has been used as a natural colorant in many traditional foods found in Asia. Annatto ranks second place in economic importance worldwide among all natural colorants and its extract shows antimicrobial and antioxidant properties (Yolmeh *et al*., 2014a). The color of the pigment from the outer layer of annatto seeds ranges from yellow to red and is affected by the concentration of the color compounds (Taham *et al*., 2015). The main color pigments of annatto seeds are bixin and nor-bixin, extracted from the outer coating of the seeds (Taham *et al*., 2015). Several previous studies have measured the antioxidant activities and color properties of different annatto extracts (Cardarelli *et al*., 2008). On the

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other hand, numerous studies were performed based on the extraction of bixin compounds from annatto seeds using different techniques (Rodrigues *et al*., 2014; Taham *et al*., 2015; Yolmeh *et al*., 2014a). Although most of the studies focused on the determination or optimization of the extraction methods for the compounds and their antioxidant activities, studies on the antioxidative and antimicrobial effects of annatto seed extracts on meat and meat products are limited. Therefore, the objectives of the present study were to evaluate the efficacy of different level of annatto seed powder in preventing lipid and protein oxidation, and to determine the physicochemical and antioxidative activities of pork patties during 14 d of refrigerated storage.

Materials and Methods

Preparation of annatto seed powder

Annatto seeds were purchased from the local market in Central Highland, Vietnam. The fresh materials were oven-dried at 50°C for about 72 h until the weight stopped changing. Then, they were extracted using double distilled (dd)-water in combination with ultrasonic assistance at a frequency of 40 kHz with 300 W of generation power (Ultrasonic JAC 2010, 330W, Korea) at 30°C for 1 h. The resulting solution was filtered twice using a 200 μm mesh filter screen (Chung GyeSang Gong Sa, Korea), then was kept in the deep freezer at -70°C prior to lyophilization at -55°C (Ilshin freeze dryer, Korea) for about 5 d until it was completely dried. After drying, the product was ground with a ball-milling grinder (Planetary Mono Ball-Milling, Pulverisette, Fritsch, Germany) for 12 h at 400 rpm (Fig. 1). Finally, the annatto seed powder was used for further analysis to determine its phytochemical components and physicochemical characteristics, and was also used for the manufacture of pork patties to evaluate the antioxidative and antimicrobial activity during storage.

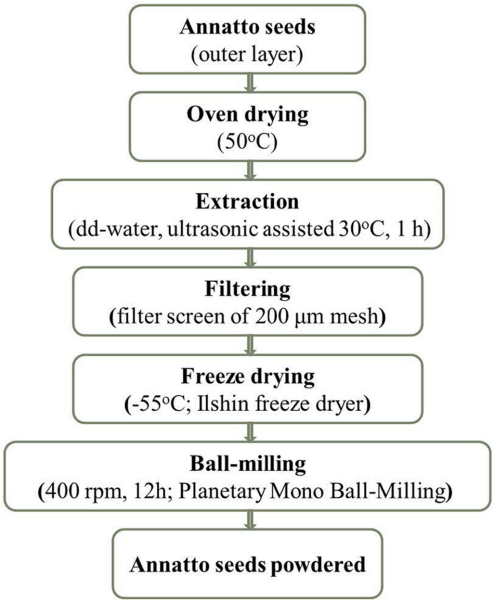

Fig. 1. Diagram of the procedure to prepare annato seeds powder.

Analytical methods for annatto seeds

Total carotenoids content

The amount of carotenoids in the powder was determined according to the methodology described by Scott (2001) with slight modification. The amount of carotenoids in the powder was determined as bixin (oil soluble) and nor-bixin (water soluble) equivalents. The absorbance was measured in a UV spectrophotometer, and the bixin concentration was calculated based on the absorption coefficient ($E^{1\%}_{1cm}$) of 3090 at 487 nm while the nor-bixin concentration was calculated using the absorption coefficient ($E^{1\%}_{1cm}$) of 2870 at 482 nm (Smith, 2006).

Total phenolic compounds, ascorbic acid content and Total flavonoid content

The total phenolic compounds in each extract were measured as gallic acid equivalents using the Folin-Ciocalteau's phenol reagent (FC reagent) according to the method described by Lin and Tang (2007). The ascorbic content was determined by using the method described by Park *et al.* (2008). Total flavonoids from the powder were determined and quantified according the aluminum chloride colorimetric method as described by Lin and Tang (2007) with slight modification.

Antioxidant assays of annatto seed powder

1,1-diphenyl-2-pycrylhydrazyl (DPPH) radical-scavenging activity was determined by the method described by Huang *et al.* (2006) with slight modification.

$$\text{DPPH radical} - \text{scavenging activity (\%)} = [(ABS_{ctl} - ABS_{spl}) / ABS_{ctl}] \times 100$$

Where: ABS_{ctl} is the absorbance value of the control group, and ABS_{spl} is the absorbance of the samples.

Ferrous ion-chelating activities of the leaf were evaluated by measuring the inhibition of the formation of a Fe^{2+} ferrozine complex using the method described by Le

et al. (2007) with some slight modification. The absorbance was then measured at λ = 562 nm with a UV − vis spectrophotometer (UV 1601).

The chelating activity was calculated as a percentage via the following equation:

Chelating effect capacity (%) =
$[(1 - ABS_{spl}/ABS_{ctl}] \times 100$

Where: ABS_{ctl} is the absorbance value of the control group, and ABS_{spl} is the absorbance of the samples.

The reducing power was measured via the method described by Huang *et al.* (2006). The absorbance at λ = 700 nm was measured and high levels of absorbance were regarded as reflective of high reducing power. Ascorbic acid (0-10 mg/mL) was used as the positive reference with the annatto powder.

The ABTS (2,2'-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) activity of the annatto seed powder was measured with the ABTS cation decolorization assay using the modified method described by Re *et al*. (1999). The absorbance was measured at λ = 734 nm after 20 min of reaction against the phosphate buffer saline blank. The results were expressed as the percentage of decolorization and calculated as mean values ± standard deviation (with *n*=3). The percentage of inhibition was calculated with the equation:

Inhibition percentage (% IP) =
$[(ABS_{ctl} - ABS_{spl})/ABS_{ctl}] \times 100$

Where: ABS_{ctl} is the absorbance value of the control group, and ABS_{spl} is the absorbance of the samples.

The total antioxidant capacity of the annatto seed powder was evaluated by the method described by Prieto *et al*. (1999). The total antioxidant activity of the samples was expressed as milligrams of ascorbic acid equivalents per gram of dried matter.

Physicochemical analyses

The pH of annatto powder was measured in a suspension resulting from blending one g sample with 10 mL of deionized water for 2 min, using a pH meter (Mettler-Toledo AG, 8603, Switzerland). The Hunter color values were measured at 5 different locations using a color reader (CR-10, Minolta Co. Ltd., Japan) and expressed as lightness (*L*), redness (*a*) and yellowness (*b*).

The water-holding capacity (WHC) and oil holding capacity (OHC) were determined according to Beuchat (1977) and the solubility (%) of the powder was analyzed according to Kha *et al*. (2010) with slight modifications.

Processing of pork patties

Five types of pork patties were prepared and the formulations are listed in Table 1. They consisted of a control (CTL) group of samples (without addition), a reference group (AA0.1), and three different concentrations (0.1, 0.25 and 0.5%) of annatto seed powder (ANT0.1, ANT 0.25, ANT0.5, respectively). Pork patties (approximately 65 g/patty) were formed using a conventional patty-maker to give average dimensions of 8.5 cm diameter and 1 cm thickness and then were stored for 14 d at 4±1 °C in a refrigerator.

The physicochemical properties and antioxidant activities of pork patties containing annatto seed powder during refrigerated storage

pH and color measurement

The pH values were measured using a pH-meter (MP-120, Mettler-Toledo, Switzerland), while the color of the patties was assessed using a Hunter color reader (CR-10, Minolta Co. Ltd., Japan). Ten measurements were made at different positions on the surfaces of the patty samples, and the mean values were calculated. Hunter color values were reported as lightness (*L*), redness (*a*) and yellowness (*b*). The total color changes (ΔE) between patties at day 1 and day 14 of storage were calculated with the equation

Table 1. Formulation of pork patties containing annatto seed powder as affected by different amounts of annatto seed (0.1-0.5%)

Ingredients (%)	Treatment*				
	CTL	AA0.1	ANT0.1	ANT0.25	ANT0.5
Raw meat	78.5	78.5	78.5	78.5	78.5
Fat	20	20	20	20	20
Salt	1.5	1.5	1.5	1.5	1.5
Ascorbic acid	-	0. 1	-	-	-
Annatto seed	-	-	0.1	0.25	0.5

*Treatments: CTL, patties without annatto seeds; AA=0.1% of ascorbic acid; ANT0.1, 0.25, 0.5=patties containing 0.1%, 0.25% and 0.5% of annatto seed powder, respectively.

below:

$$\Delta E_{1-14} = [(L_{14} - L_1)^2 + (a_{14} - a_1)^2 + (b_{14} - b_1)^2)]^{1/2}$$

Thiobarbituric acid reactive substances (TBARS)

The secondary products of lipid oxidation during the storage period were determined with the TBARS assay according to Shinnhuber and Yu (1977). The reactive substances were measured at 532 nm using a spectrophotometer (UV-1601, Shimadzu, Australia). TBARS values were calculated with the following equation:

TBARS value (mg malondialdehyde/kg) = optical density (O.D.) × 9.48/sample weight (g)

Peroxide values (POV)

POV were determined using the spectrophotometric method as described by Shantha and Decker (1994) with some modification. The absorbance of the sample was measured at 500 nm against a blank that contained all the reagents except the samples using a spectrophotometer (UV-1601, Shimadzu, Australia). The results of POV were expressed in milliequivalents of oxygen per kilogram of patty (meq/kg).

Total volatile basic nitrogen (VBN) measurements

Total VBN content was determined according to the micro-diffusion method introduced by Conway (1962) with slight modification. Total VBN values were expressed in mg/100g.

Microbial counts

Total plate counts (TPC) and violet red bile (VRB) agar were prepared for the determination of total viable counts and *Enterobacteriaceae*, respectively (Park and Chin, 2010). Finally, relevant colonies on the petri dish were counted and the results for TPC and VRB were expressed as Log CFU/g.

Statistical analysis

Data were analyzed and statistical comparisons were performed by using one-way or two-way analysis of variance (ANOVA). The significant differences were assessed with the Duncan post hoc test at p value < 0.05 using the Statistical Package for the Social Sciences (IBM SPSS version 20.0) software for Windows. Results were presented as the mean±standard deviation (n=3).

Results and Discussion

Phytochemical components and antioxidant activities of annatto seed powder

Phytochemical (Bixin, Nor-bixin, TPC, TFC and AA) compounds of annatto (*Bixa orellana* L.) seed powder are presented in Table 2. The antioxidant abilities are summarized in Figs. 2(A)-(D). The DPPH radical scavenging activities of ANT seed powder at different concentrations (1.0-10 mg/mL) ranged from 85.8 to 94.7%, and the concentration at 5.0 mg/mL showed the highest ability (Fig. 2A). It was observed that the DPPH radical scavenging activities of ANT seed powder were similar to those of AA. Ferrous ion chelating capability increased from 30.7%

Table 2. Phytochemical components and physicochemical properties of annatto (*Bixa orellana* L.) seed powder

Parameters		Mean ± SD
Phytochemical components	Carotenoids (mg bixin/g)	40.33 ± 0.32
	Carotenoids (mg nor-bixin/g)	31.61 ± 3.88
	TPC (mg GAE/g)[A)]	62.08 ± 2.21
	TFC (mg QE/g)[B)]	5.19 ± 1.52
	Ascorbic acid content (mg AA/g)[C)]	13.75 ± 4.0
	Total antioxidant capacity (mg AAE/g)[D)]	17.42 ± 0.45
Physicochemical properties	pH	5.73 ± 0.01
	Lightness (*L*)	40.97 ± 2.41
	Redness (*a*)	15.39 ± 1.33
	Yellowness (*b*)	5.73 ± 0.86
	Water holding capacity (%)	39.72 ± 2.21
	Oil holding capacity (%)	50.68 ± 3.81
	Solubility (%)	45.77 ± 2.41

[A]Total phenolic compounds was expressed as mg gallic acid equivalent /g d.m. of seed powder.
[B]Total flavonoid contents are expressed as mg quercetin equivalent /g d.m. of seed powder.
[C]Ascorbic acid content as mg /g d.m. of seed powder.
[D]Total antioxidant capacity is expressed as mg ascorbic acid equivalent /g d.m. of seed powder.

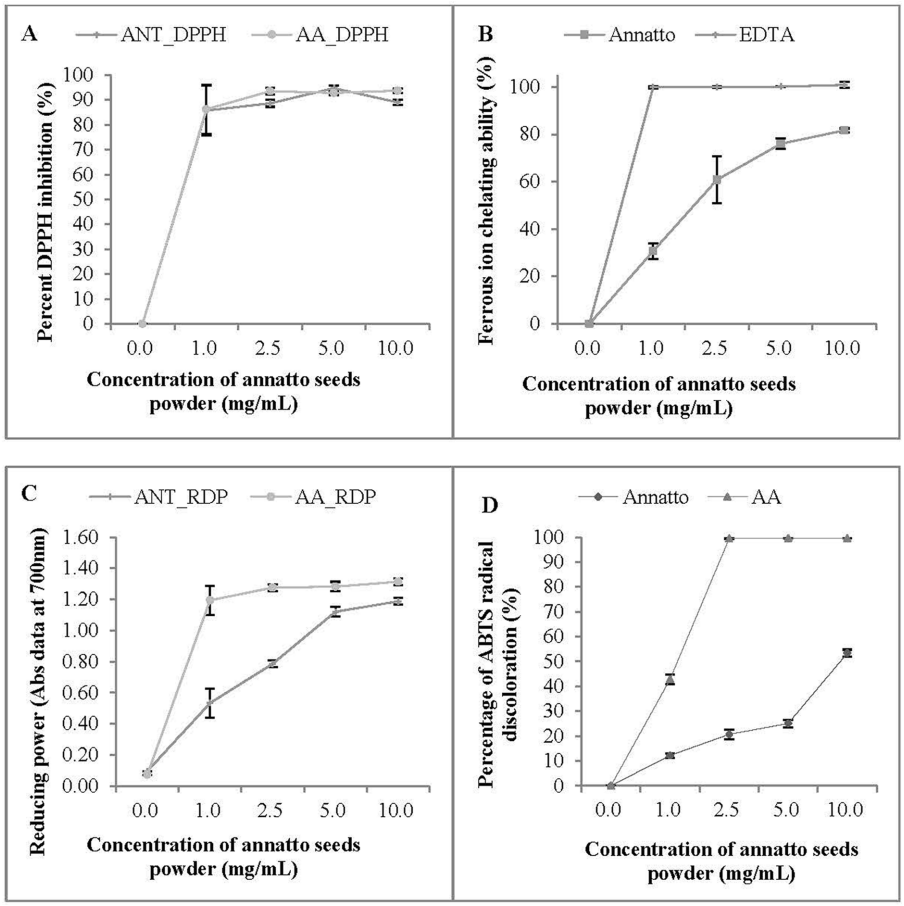

Fig. 2. In vitro antioxidant assays of annatto seeds powder: DPPH RSA (A), ferrous ion chelating ability (B), reducing powder (C) and ABTS radical discoloration (D).

to 81.65%, with increasing annatto seed concentration from 1.0 to 10.0 mg/mL (Fig. 2B). Compared to EDTA, ANT seed powder had lower ferrous ion chelation. Reducing power (Abs at 700 nm) ranged from 0.53 to 1.19, showing an increase with increasing concentration. The reducing power of ANT seeds was lower compared to AA (1.0-10 mg/mL) (Fig. 2C). The ABTS ability also had a similar trend to the other *in vitro* antioxidant assays, and lower concentrations tended to have lower ABTS ability. The ABTS ability increased linearly with increasing concentration and the highest value was recorded at 10 mg/mL (Fig. 2D). Based upon these results, annatto seed powder was confirmed to show antioxidant activity. Many studies have stated that the presence of phenolic compounds in food, especially in fruit, could be important for consumers to prevent some diseases and cancers because of their beneficial health properties, which may be associated with their antioxidant capacities. Thus, annatto seed powder could be used as a natural antioxidant in various foods, especially meat products.

Physicochemical properties of annatto seed powder

The physicochemical properties of annatto seed powder are presented in Table 2. The mean pH value of annatto seed powder was 5.73. Viuda-Martos *et al*. (2012, 2015) reported that pomegranate juice arils bagasse had a pH of 4.4, pomegranate juice whole fruit bagasse had a pH of 4.5 and the pH of fig (*Ficus carica* L.) powder ranged from 5.10 to 5.49. In Hunter color values, the lightness value for annatto powder was 40.97, which is relatively dark. The lightness value was compared to those of other powders such as pomegranate, ranging from 62.2 to 62.8, and fig (*Ficus carica* L.) powder, ranging from 49.72 to 68.62 (Viuda-Martos *et al*., 2012; Viuda-Martos *et al*., 2015). The redness (*a*) value for annatto seed powder is 15.39, while the yellowness (*b*) value is 5.73.

The WHC is an important characteristic in the interaction between water and proteins that occurs in various food systems (Traynham *et al*., 2007). The WHC and OHC of annatto seed powder is 39.72% and 50.68%, respectively (Table 2). Moreover, WHC is an important processing

parameter, which can enhance the cooking yield, especially in meat. The OHC is a techno-functional property that is influenced by the chemical structure of polysaccharides, which are present in plant resources and are highly related to their chemical and physical structures (Viuda-Martos *et al.*, 2015). Based on the results for OHC and WHC, annatto seed powder may have stronger affinity for oil than water. Therefore, annatto seed powder, which has relatively higher OHC than WHC, may be favorable in food items. In addition, the solubility of annatto seed powder was 45.77%. Since solubility is directly associated with the dispersion and appearance of final products, the high solubility of annatto seed powder might be more favorable in emulsifying meat systems, such as sausages. Based on these results, annatto seed powder might be suitable for the improvement of cooking yield and retarding lipid oxidation in meat products during storage.

The physicochemical properties of pork patties containing annatto seed powder during storage

pH values

Changes in the pH values of pork patties during storage are shown in Table 3. The pH ranged from 5.61 to 5.86. No significant differences between ANT0.1 and ANT0.25 were observed, but ANT0.5 and AA0.1 were higher than the CTL. The present study showed that the pH values increased during refrigerated storage (p<0.05). Usually, the pH values of meat or muscle foods show significant increases with increasing storage length and this observation has been reported by several authors (Kim and Chin, 2015; Park and Chin, 2010). Moreover, changes in the pH values of meat during storage have an important effect on the quality, especially in sensorial characteristics such as odor, color, and texture (Li *et al*., 2014). Several factors could contribute to the changes of pH during storage in muscle foods such as the generation of lactic acid, the production of volatile basic components and alkaline substances by the decomposition of meat by microbial organisms and endogenous enzymes such as ammonia groups and amine groups (Cai *et al*., 2015; Li *et al*., 2014). In addition, Miao *et al*. (2015) reported that the increase of pH values in fresh pork during refrigerated storage was mainly caused by bacterial decomposition of meat components.

Hunter color values

The Hunter color values of pork patties during storage are presented in Tables 3-5. The lightness (L) increased and the redness (a) decreased (p<0.05), while no changes in yellowness were observed during refrigerated storage (p>0.05). As shown in Table 3, the lightness decreased while redness increased with the addition of annatto seed powder, as compared to the control (CTL) or reference (AA0.1). This clearly indicated that the Hunter color values of pork patties were affected by the addition of annatto seed powder, which has a strong color and changed the color to a slightly dark red as a result of the bixin and nor-bixin pigments. Therefore, a high increase of redness and a decrease of lightness were observed with increased

Table 3. Physicochemical properties of pork patties containing annatto seed powder according to amount (No interaction)

		Parameters[1]								
		pH	L	a	b	TBARS	POV	VBN	TPC	VRB
Treatment (TRT) * day		NS	NS	*	NS	**	**	**	NS	NS
TRT [2]		**	**	**	**	**	**	*	NS	*
Storage (d)		**	**	**	NS	**	**	**	**	**
TRT	CTL	5.80^{b}	60.10^{a}	8.31^{e}	9.07^{b}	1.53^{a}	7.27^{a}	9.3^{ab}	4.30	4.03^{ab}
	AA0.1	5.86^{a}	57.33^{b}	11.03^{d}	9.34^{b}	0.22^{c}	1.69^{c}	9.6^{a}	4.42	4.09^{a}
	ANT0.1	5.64^{c}	56.02^{c}	13.44^{c}	16.11^{a}	0.95^{b}	3.44^{b}	8.4^{bc}	4.16	3.83^{abc}
	ANT0.25	5.61^{c}	51.31^{e}	16.15^{b}	16.09^{a}	0.30^{c}	1.51^{c}	8.3^{bc}	4.37	3.57^{c}
	ANT0.5	5.86^{a}	44.83^{f}	21.75^{a}	15.62^{a}	0.20^{c}	0.31^{d}	9.3^{ab}	4.16	3.66^{bc}
Storage days	1	5.65^{c}	52.59^{d}	16.29^{a}	13.54	0.29^{d}	2.07^{b}	6.9^{c}	2.64^{e}	2.23^{d}
	3	5.73^{b}	53.75^{c}	15.67^{a}	13.49	0.47^{c}	2.18^{b}	7.0^{c}	3.03^{d}	2.42^{d}
	7	5.71^{b}	53.96^{bc}	14.49^{b}	13.95	0.57^{bc}	2.46^{b}	7.3^{c}	4.10^{c}	3.60^{c}
	10	5.74^{b}	54.81^{ab}	13.08^{c}	13.67	0.69^{ab}	3.08^{a}	9.5^{b}	4.82^{b}	4.24^{b}
	14	5.86^{a}	55.20^{a}	12.38^{c}	13.60	0.78^{a}	3.07^{a}	13.1^{a}	6.77^{a}	6.50^{a}

Means with different superscript letters ($^{a-f}$) in the same column indicate significant differences at p<0.05. NS=not significant
*indicated significant at p<0.05, **: indicated significant at p<0.001.
[1]Parameters: L=Lightness, a=Redness, b=Yellowness, TBARS=thiobarbituric acid reactive substances, POV=Peroxides values, VBN=Total volatile basic nitrogen. TPC: Total plate count, VRB: Violet red bile. [2] Treatment: CTL=patties without addition, AA0.1=0.1% Ascorbic acid; ANT0.1, 0.25, 0.5 = 0.1%, 0.25% and 0.5% of annatto seed powder, respectively.

levels of annatto seed powder, which might not be acceptable for the consumer. The present results are consistent with other findings of a positive correlation between plant pigments and the surface colors of meat products (Rodriguez-Corpena *et al*., 2011). Since the strong color pigments from plant extracts were transferred to the pork patties during processing, the final color of the meat products were changed. Although the redness decreased in all treatments throughout storage, the rate of decrease was quite different from those without treatment ($p<0.05$, Table 4). Since the interaction between treatment and storage time had a significant effect on the redness (Table 3, $p<0.05$), data were separated by treatment according to storage time or storage time according to the treatment. As shown in Table 4, the rapid decreases in the redness of the CTL and ANT0.1 samples might be partially due to rapid lipid oxidation, in which oxymyoglobin was changed to metmyoglobin in the control and at lower levels of annatto seed powder. Moreover, the results showed that the decrease in redness within the first 3 d was slow, but rapidly increased thereafter. When the changes in displayed color between day 1 and day 14 (ΔE_{1-14}) are compared among treatments, CTL patties or those with 0.1% annatto seeds (ANT0.1) had more intense color deterioration than patties with higher levels (0.25 and 0.5%) of annatto seeds, or ascorbic acid (Table 5). This means that patties without annatto seeds and those with lower levels of annatto seeds (< 0.1%) had more rapid changes and less color stability during storage, whereas treatments with higher levels (> 0.25%) of annatto or ascorbic acid showed more resistance against discoloration than the control as shown in Table 6. The protective effect of annatto seed powder in preventing discoloration could be partially due to the polyphenol compounds or antioxidant agents in annatto seed powder. Several previous studies reported color changes in numerous raw meats as affected by various fruits during refrigerated storage. Rodriguez-Corpena *et al.* (2011) reported that avocado extracts preserved the color of raw pork during cold storage (4°C/15 d). Keokamnerd *et al.* (2008) concluded that rosemary extracts had an overall positive effect in maintaining the color stability and sensorial quality of chicken meat during storage. Ganhão *et al*. (2010) reported several types of natural resources extracts including strawberry, common hawthorn, dog rose, and blackberry that showed high capability to maintain the color of meat during storage, as compared to the control. On the other hand, several authors have reviewed the disadvantages of fruits or vegetables on the displayed color of meats,

Table 4. Physicochemical properties of pork patties with added annatto seed powder according to amount (Interaction)

Parameters[1]	Treatments[2]	Storage (d)				
		1	3	7	10	14
Redness (*a*)	CTL	10.20^{aE}	10.88^{aD}	8.93^{bD}	5.88^{cE}	5.68^{cD}
	AA0.1	11.53^{abD}	12.48^{aCD}	12.43^{aC}	9.70^{bcD}	9.03^{cC}
	ANT0.1	17.23^{aC}	15.35^{bBC}	12.75^{cC}	11.82^{cC}	10.07^{dC}
	ANT0.25	18.33^{aB}	16.58^{bB}	15.73^{cB}	15.38^{cdB}	14.73^{dB}
	ANT0.5	23.63^{aA}	22.19^{aA}	21.97^{aA}	20.66^{aA}	20.30^{aA}
TBARS (mg MDA/kg)	CTL	0.82^{cA}	1.18^{bcA}	1.55^{abcA}	1.88^{bA}	2.23^{aA}
	AA0.1	0.13^{aDE}	0.20^{aC}	0.23^{aC}	0.26^{aC}	0.26^{aC}
	ANT0.1	0.28^{dB}	0.76^{cB}	1.02^{bB}	1.32^{aB}	1.36^{aB}
	ANT0.25	0.21^{cC}	0.31^{bC}	0.26^{bcC}	0.30^{bC}	0.43^{aC}
	ANT0.5	0.19^{aCD}	0.20^{aC}	0.21^{aC}	0.22^{aC}	0.20^{aC}
POV (meq/kg)	CTL	5.98^{bA}	6.11^{bA}	6.47^{bA}	9.63^{aA}	8.18^{abA}
	AA0.1	1.58^{aB}	1.68^{aC}	1.70^{aC}	1.92^{aC}	1.59^{aCD}
	ANT0.1	1.88^{dB}	2.55^{cB}	3.57^{bB}	3.99^{bB}	5.19^{aB}
	ANT0.25	1.22^{bB}	1.28^{bC}	1.46^{bC}	1.35^{bC}	2.22^{aC}
	ANT0.5	0.32^{aB}	0.33^{aD}	0.30^{aD}	0.41^{aD}	0.17^{aD}
VBN (mg/100 g)	CTL	6.8^{bBC}	7.1^{b}	6.8^{b}	9.0^{bAB}	16.8^{aA}
	AA0.1	6.5^{bBC}	6.5^{b}	7.9^{b}	12.6^{abA}	14.5^{aA}
	ANT0.1	7.1^{bAB}	7.2^{b}	7.0^{b}	10.0^{aAB}	10.6^{aB}
	ANT0.25	7.5^{bA}	7.3^{b}	7.9^{b}	8.4^{bAB}	10.3^{aB}
	ANT0.5	6.4^{cC}	6.8^{bc}	7.6^{bc}	9.1^{bAB}	16.4^{aA}

Means with different lowercase superscript letters ($^{a-f}$) in the same row indicate significant differences at $p<0.05$. Means with different uppercase superscript letters ($^{A-E}$) in the same column indicate significant differences $p<0.05$.

[1)]Parameters: a=Redness, TBARS=thiobarbituric acid reactive substances, POV=Peroxides value, VBN=Total volatile basic nitrogen.

[2)]Treatments: see Table 1.

Table 5. Total color changes (ΔE_{1-14}) during refrigerated storage (between days 1 and 14) measured on the surface of raw pork patties

Treatment*	Mean ΔE_{1-14} ± Standard deviation
CTL	7.32 ± 0.25^{a}
AA0.1	3.44 ± 0.79^{b}
ANT0.1	8.15 ± 0.68^{a}
ANT0.25	4.31 ± 0.83^{b}
ANT0.5	4.76 ± 1.48^{b}

Means with different superscript letters (^{a,b}) in the same column indicate significant differences at $p<0.05$.
*Treatment: see on the Table 1.

Table 6. Slope values for TBARS, Lightness (L), redness (a) and VBN during refrigerated storage (from day 1 to 14) of raw pork patties

Treatment*	Parameters			
	TBARS slope	L slope	a slope	VBN slope
CTL	0.105^{a}	0.437^{a}	0.432^{a}	0.068^{a}
AA0.1	0.009^{b}	0.152^{bc}	0.241^{b}	0.068^{a}
ANT0.1	0.080^{a}	0.258^{b}	0.535^{a}	0.030^{b}
ANT0.25	0.013^{b}	0.066^{c}	0.245^{b}	0.021^{b}
ANT0.5	0.001^{b}	0.149^{bc}	0.242^{b}	0.069^{a}

Means with different superscript letters ($^{a-c}$) in the same column indicate significant differences at $p<0.05$.
*Treatment: see in Table 1.

which can be unacceptable to consumers. Since the addition of annatto seeds led to marked changes in the displayed color of the pork patties as compared with the control, the final product might not be accepted by consumers. Thus, the differences in color values between the annatto treated patties and control should be minimized with the addition of other ingredients that can control the color.

TBARS

Since there was an interaction between treatments and storage time with regard to TBARS values ($p<0.001$), the data were organized by treatment during storage or storage time according to the treatment (Table 4). The addition of annatto seed powder or ascorbic acid reduced the TBARS values. In fact, the TBARS values of CTL patties were highest at the beginning of storage, followed by patties containing 0.1% annatto. No differences in TBARS among patties treated with different amounts of annatto seed powder or with ascorbic acid 0.1% were observed during refrigerated storage. These results indicated that the TBARS values were affected by annatto seed powder during refrigerated storage, which might be partially due to the high level of total phenolic compounds in annatto seed powder, and could contribute to the retardation of lipid peroxidation. Numerous studies have reported that phenolic compounds from diverse plants play a major role in antioxidant activity (Kim *et al*., 2013; Maqsood *et al*., 2015). The donation of electrons and protons from phenolic and other bioactive compounds could prevent the initiation and propagation of lipid oxidation. Moreover, the inhibition of free radical reactions could be caused by the chelation of transition metal ions in addition to preventing oxygen attacks on radical chains (Jia *et al*., 2012; Shan *et al*., 2009). The spoilage value for chemical deterioration of meat and meat products is considered 1.0 mg MDA/kg meat and the TBARS values of the control patties indicated they began to spoil on storage day 3. However, the ANT0.1 samples reached the spoilage limit for TBARS value after 7 d of storage, while other treatments with higher amounts of ANT seeds did not even reach 1.0 during the entire storage period. These results were in agreement with the findings of Jia *et al*. (2012), who reported that the TBARS value in CTL pork patties was much higher than those of other samples containing black currant extract and exceeded 2.0 mg MDA/kg at storage day 6. The ANT0.25 and ANT 0.5 pork patties maintained low TBARS values and showed similar results as that of ascorbic acid (AA 0.1%) during storage up to 14 d. Lipids in meat and meat products are easily oxidized and form hydroperoxides, which decompose to secondary lipid oxidation products such as aldehydes and ketones, resulting in the production of rancid off-flavor, which might not be acceptable to consumers. Many types of fruit extracts from wild resources such as dog rose, blackberry, and hawthorn have shown very strong antioxidant activity, which can prevent the oxidation of lipids in raw pork patties during cold storage (Ganhão *et al*., 2010). Furthermore, Shan *et al*. (2009) reported that several natural extracts including cinnamon stick, oregano, clove or their by-products such as pomegranate peel and grape seeds added to raw sliced pork meat could act as inhibitors of lipid oxidation even up to 9 d of storage at room temperature (20°C). Therefore, the addition of ANT seed powder (from 0.25 to 0.5%) might be a good way to prevent lipid oxidation in meat products as shown in the lower TBARS slope of the ANT treatment rather than the control (Table 6).

Peroxide value (POV)

POV is widely used as a chemical test for primary lipid oxidation in muscle food during storage. The POV of pork patties containing ANT seed powder are shown in Table 4. CTL samples were the highest ($p<0.05$), followed by pork patties with 0.1% ANT. No significant differences between

AA0.1% and ANT0.25% were observed ($p>0.05$), whereas the pork patties containing ANT0.5% showed the lowest POV ($p<0.05$). Among the samples treated with annatto seed powder, pork patties with higher amounts of annatto had lower POV during storage. Furthermore, pork patties with annatto seeds combined with other products were more effective in lowering POV than those with annatto seeds alone. As shown in Table 4, POV tended to plateau at day 7 in all treatments ($p>0.05$), increasing rapidly thereafter up to day 14. The increases of POV after day 7 of storage could be partially due to the end point for the induction of lipid oxidation in pork patties. Peroxide value could not be qualified for the valuation of lipid oxidation due to the low stability of peroxides and the easy breakdown to non-peroxide compounds. However, a linear relationship has been observed between peroxide values and sensorial quality, especially in flavor scores during the initial stages of lipid oxidation (O'Brien, 2008). Therefore, high peroxide values usually mean poor flavor ratings in meat products, due to the presence of oxidized metabolites of fats.

Total volatile basic nitrogen (VBN)

VBN is one of the most important indicators of the freshness of meat, dairy or seafood products (Cai *et al*., 2015; Miao *et al*., 2015). It might be associated with the activity of several spoilage microorganisms and endogenous enzymes (Cai *et al*., 2015). The VBN values in all the samples showed a slight or no increase during initial storage (Table 4), but marked increase was observed after 7 d of storage. This might be due to the activity of spoilage bacterial and endogenous enzymes system in meat products. In the first 7 d of storage, the activity of microorganisms was still low, leading to a slight increase in the VBN value from 6.9 to 7.3 mg/100 g ($p>0.05$, Table 3). However, the VBN of all patties gradually increased ($p<0.05$) after 7 d and reached 9.5 on day 10 and 13.1 mg/100 g at by the end of storage (Table 3). At the end of storage, the VBN values of CTL samples AA0.1 and ANT0.5% did not show any difference and AA0.1 had higher VBN than samples treated with ANT01 and ANT0.25 (Table 4). Based on these results, it appears that treatment with very high amounts of ANT (0.5%) might causes protein degradation. Moreover, Miao *et al*. (2015) reported that the limit according to the national standard of China for hygienic standards for fresh meat from livestock was less than 15 mg/100 g of VBN in meat. Other countries have been considered VBN values less than 10 mg/100 g of meat. In this study, the control exceeded 10 mg/100 g of VBN after about 8 d of storage, however the other treatments took approximately 10 d or more to reach the same value, depending on the amount of annatto seed. Miao *et al*. (2015) showed that the VBN value of fresh pork had lower rates of increase and did not exceed the safety standard within the first 8 d of storage as compared to the control. Thus, pork patties containing ANT within the range of 0.1 to 0.25% could have reduced the VBN, resulting in the extension of shelf-life.

Microbial counts

The results of microbial count are shown in Table 3. Although no differences in total microbial counts were observed among the treated patties, the addition of ANT seed powder to pork patties reduced the microbial count of *Enterobacteriaceae*, as compared to the control or reference (AA, 0.1%) ($p<0.05$) when the amount of ANT was higher than 0.25%. There have been several reports on the antimicrobial activity of annatto seeds in *in vitro* cultures. The present results might be supported by Venugopalan and Giridhar (2012), who stated that the ethanolic extracts of annatto seeds are capable against *E. coli* and *B. cereus.* Moreover, Yolmeh *et al*. (2014b) reported that the annatto dye was able to retard the microbial growth of *E. coli* and exhibited bactericidal effects on mayonnaise. The pooled mean of microbial counts (TPC and VRB) in all of the patties increased with increasing storage time ($p<0.05$). At 3 d of storage, the microbial counts for *Enterobacteriaceae* did not increase ($p>0.05$); however, they started to increase after 7 d of storage in all patties, indicating that the samples underwent spoilage. Therefore, ANT seed powder could have not only antioxidant activity, but also the potential for antimicrobial activity in pork patties.

Conclusions

Annatto seed powder had antioxidant abilities as well as water and fat holding capacity which might be suitable for incorporation with meat products. The redness values of pork patties containing annatto seed powder increased due to the presence of bixin and nor-bixin pigments ($p<0.05$). Moreover, pork meat patties containing annatto seed powder were found to have reduced lipid deterioration, which resulted in lower TBARS and POV than the control sample. The VBN values of the pork patties containing annatto seeds were lower than those of CTL at the end of storage ($p<0.05$). The addition of annatto seeds to meat products at a level of 0.25% appeared to be optimal

for the manufacture of pork patties. Therefore, annatto seed powder could be a promising source for natural antioxidants and antimicrobial agents that could be used in the production of meat products to extend the shelf-life.

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