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ORIGINAL RESEARCH PAPER

EFFICACY OF WALNUT LEAVES AND SWEET CHERRY STEMS AS NATURAL ANTIOXIDANTS IN RAW PORK PATTIES DURING FROZEN STORAGE

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Abstract: The effect of walnut leaf and cherry stem extracts on lipid oxidation, instrumental color, *p*H and antioxidant activity of raw pork patties stored at -18 °C for nine months was evaluated. Incorporation of the phenolic-rich extracts into pork patties reduced the extent of lipid oxidation significantly ($p<0.05$) and showed better results than butylated hydroxytoluene (BHT). Walnut leaf extract demonstrated a stronger lipid oxidation inhibitory effect than cherry stem extract. Addition of plant extracts significantly ($p<0.05$) increased the total phenolic content and the antioxidant activity of raw pork patties. *p*H values increased while total phenolic content and radical scavenging activity significantly decreased throughout the nine months storage period. The extracts determined color alterations but they were effective for inhibiting the browning and discoloration of frozen pork patties by reducing the loss of redness and the increase of yellowness. The present results indicated that walnut leaf and cherry stem extracts are effective in retarding lipid oxidation of raw pork patties during frozen storage.

Keywords: *antioxidant activity, color, lipid oxidation, meat products, pH, plant extracts*

INTRODUCTION

During the processing and storage of meat, lipid oxidation causes changes in color, flavor and nutritional value and affects meat safety [1, 2]. Deterioration of the meat color can be determined by the oxidation of meat lipids that causes myoglobin oxidation. The color of meat and its stability are important because any change in color is a source of difficulty in its fresh marketing. In addition, lipid oxidation results in the degradation of liposoluble vitamins and essential fatty acids, as well as the formation of free radicals and potentially toxic compounds such as hydroperoxides, aldehydes, ketones, isofurans, which give rancid odors and significantly affect human health [3, 4].
In fresh meat, lipid oxidation is influenced by factors such as fatty acid content, presence of endogenous or exogenous prooxidant and antioxidant substances [5]. For example, at the concentrations frequently used in meat (0.5-2.5 %), salt exerts a prooxidizing effect on lipids. In addition, salt accelerates the formation of metmyoglobin and the color change of fresh meat [6]. Also, meat mincing leads to increased exposure to air, resulting in an increase of the microbiological and oxidative degradation rate of the meat [5, 7].
A series of studies have shown the effects of oxidation on meat and meat products during storage in the frozen state [8 – 10].
In order to minimize or prevent lipid oxidation in meat and extend shelf life, the meat industry uses synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tertiary butyl hydroquinone (TBHQ) [11, 12]. However, due to their toxic effects, changing food habits and increasing consumer preference for the use of natural products, the research into the use of natural antioxidants in meat and meat products has intensified [5].
In previous studies, a series of extracts from natural sources have been added and have been shown to be effective for delaying lipid oxidation during frozen of meat and meat products [2, 7, 8, 13 – 17]. They showed a high antioxidant capacity determined mainly by the high content of phenolic compounds.
Walnut (*Juglans regia* L.) leaves have been often used to prepare teas and decoctions for the treatment of venous insufficiency, hemorrhoids, diarrhea, and microbial infections, skin inflammations, and ulcers, and also for their antidiarrheic, antihelmintic, depurative and astringent properties [18 – 20]. Keratolytic, hypoglycaemic, hypotensive, hepatoprotective, anti-scrofulous and sedative activities have also been described [21, 22]. In addition, previous studies reported that walnut leaves have demonstrated considerable anti-proliferative activities against various cancer cell lines [23, 24]. The medicinal properties of walnut leaves have been related to their high content of natural antioxidants such as ellagitannins, tocopherols and phenolic compounds, namely flavonoids and phenolic acids [25, 26].
In the traditional medicine, the sweet cherry (*Prunus avium* L.) stems are administered as sedatives and diuretics and to treat kidney stones. Hooman *et al*. [27] evaluated and validated in a clinical trial the claimed diuretic effect of the herb. The anti-inflammatory and diuretic properties of sweet cherry stems are related to their high content of secondary metabolites, particularly the phenolic acids and flavonoids [28]. Bastos *et al*. [29] observed that cherry stems have a greater antioxidant capacity than cherry fruits and, particularly, the hydrometanolic extracts, followed by decoctions and, finally, the infusions.

The objective of this work was to investigate the potential of using infusions from walnut leaves and sweet cherry stems to enhance the frozen storage oxidative stability of raw pork patties subjected to chilled storage. The TBARS value was used to monitor lipid oxidation in the meat samples. The color, *p*H, total phenolic content and antioxidant activity of frozen raw pork patties were also evaluated during nine months of storage at -18 ^{o}C. Butylated hydroxytoluene (BHT) was used as a positive control in this study.

MATERIALS AND METHODS

Preparation of plant extracts

Fresh walnut (*Juglans regia* L.) leaves and sweet cherry (*Prunus avium* L.) stems were collected from trees growning in the experimental orchard of the University of Craiova located at Râmnicu Vâlcea (Romania) research station (45°07'N/24°22'E). They were washed thoroughly with tap water, air dried in the shade and then ground into a powder in a coffee grinder (Bosch MKM6000, Germany). The extracts were prepared by adding 10 g of the dried powder to 100 mL of boiling distilled water and extracted for 60 min with occasional shaking. The extracts were filtered through filter paper (Whatman, No. 541, Wycombe, UK).

Preparation of raw pork patties

Fresh lean pork meat and back fat were purchased from a local market. They were clean, cut into cubes, ground through a 3 mm plate and mixed to contain 21 % back fat. The mixture was divided into four treatments as follows: I) C0 - Control (meat batter + 1.5 % salt + 5.5 % water); II) C1 - Control with BHT (meat batter + 1.5 % w/w salt + 5.5 % water + 0.1 % BHT); III) WLE (meat batter + 1.5 % w/w salt + 5.5 % walnut leaf extract); IV) CSE (meat batter + 1.5 % w/w salt + 5.5 % cherry stem extract). Salt and BHT were dissolved in water or plant extract prior to mixing with the meat batter. Immediately after adding all ingredients, meat samples were thoroughly mixed and made into patties manually (50 g each). The patties were aerobically packed in polyethylene bags and analyzed within 24 h, and after 3, 6, and 9 months of storage at -18 °C. For the analysis, the samples were thawed for 12 hours at 6 ± 2 °C.

*p*H

The pork patties (10 g) were homogenized with 50 mL of distilled water at high speed for about 1 min. The *p*H of the homogenate was measured using a multiparameter instrument Hanna HI255 (Italy).

Thiobarbituric acid reactive substances (TBARS) value

Lipid oxidation was monitored by measuring TBARS value during frozen storage. TBARS values of pork patties were determined using the extraction method described by Witte *et al*. [30] with minor changes. Briefly, the sample (5 g) was extracted in 12.5

mL of 20 % trichloroacetic acid with vigorous stirring then transferred to a 25 mL volumetric flask and diluted up to the volume with cold distilled water. Five mL extract was mixed with 5 mL of 0.02 M 2-thiobarbituric acid and heated at 100 °C for 35 minutes. After cooling, the absorbance was recorded at 532 nm with a Varian Cary 50 UV spectrophotometer (Varian Co., USA). A calibration curve of 1,1,3,3-tetraethoxypropane (Sigma-Aldrich) standard solutions was used to determine the concentrations of TBA reactive substances in samples. TBARS values were expressed as mg of malondialdehyde (MDA) per kg of meat sample.

Extraction of meat samples

Samples were weighed (10 g) in centrifuge tubes and extracted sequentially with 40 mL of methanol/water (50:50, v/v) at room temperature for 1 h. The tubes were centrifuged at 6000 rpm for 15 min, and the supernatant was recovered. Next, 40 mL of acetone/water (70:30, v/v) were added to the residue at room temperature, extracted for 60 min, and centrifuged. Methanol and acetone extracts were combined to make up 100 mL with distilled water and used to determine the ABTS antioxidant activity and the total phenolic content.

Total phenolic content

The total phenolic content of patties was evaluated by the Folin-Ciocalteu method as described by Singleton *et al*. [31]. The extracts (0.1 mL) made as described above were mixed with 6 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent was added to this mixture. After 30 sec to 8 min, 1.5 mL of sodium carbonate (20 % w/v) was added. The reaction mixture was diluted with distilled water to a final volume of 10 mL. The same procedure was also applied to the standard solutions of gallic acid. The absorbance at 765 nm of each mixture was measured on a Varian Cary 50 UV spectrophotometer (Varian Co., USA) after incubation for 30 min at 40 °C. Results were expressed as mg of gallic acid equivalents (GAE) per 100 g of meat sample.

ABTS antioxidant activity

Antioxidant activity of the samples was measured using an ABTS (2,29-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) procedure described by Re *et al*. [32]. The ABTS cation radical solution (ABTS+) was prepared by mixing 5 mL of a 7.0 mM ABTS solution and 88 µL of a 145 mM potassium persulfate solution and incubating in the dark at room temperature for 16 h. The ABTS+ solution was then diluted with 80 % ethanol to obtain an absorbance of 0.700 ± 0.005 at 734 nm. Twelve milliliters of ABTS+ solution (0.700 ± 0.005 absorbance) were added to 120 µL of sample extract and vigorously mixed in a vortex. After 6 minutes, the absorbance at 734 nm was read using ethyl alcohol as blank. The calibration curve was constructed using ethanol solutions with known concentrations of Trolox (100-2000 µM Trolox/L) and the results were expressed in µM Trolox per 100 g of meat sample.
All the reagents were of analytical grade (from Sigma Aldrich, Germany) and solutions were prepared using double-distilled water.

Statistical analysis

Three replications of the study were performed, and measurements of all parameters were taken in duplicate. The results were reported as mean ± standard deviation. Mean values for various parameters were compared by analysis of variance using the Statgraphics Centurion XVI software (StatPoint Technologies, VA, USA). Fisher LSD (least significant difference) test was applied for determining group differences at 95 % significance level.

RESULTS AND DISCUSSION

Initially, significant differences in *p*H ($p<0.05$) were found among treatments. The raw patties with BHT and those with walnut leaf extract had lower pH value compared with the control patties. This is in line with the findings of Das *et al*. [33] who reported that raw goat patties with BHT had lower *p*H value compared with the control patties and the patties with *Moringa* leaves extract. Naveena *et al*. [34] reported also that chicken patties with BHT had lower *p*H value compared with chicken patties with pomegranate juice and rind powder.

Over nine months of frozen storage, the *p*H values of the raw pork patties significantly increased ($p<0.05$) in all samples, probably as a result of the exposure of basic groups by protein denaturation. There were no significant differences ($p<0.05$) in the *p*H values between treatments in raw pork patties after nine months of storage in the frozen state.

The lipid oxidation products were measured by means of TBARS values, expressed as mg malonaldehyde per kilogram of meat sample. Changes in the TBARS values of raw pork patties during frozen storage are shown in Table 1.

Immediately after preparation, the extent of lipid oxidation in raw patties was relatively low in all samples, however TBARS values of raw pork patties treated with walnut leaf and cherry stem extracts were significantly ($p<0.05$) lower than those of the control without antioxidant and BHT treated samples.

A considerable increase in the TBARS values was observed in all groups during the nine months frozen storage (-18 ^{o}C) period. However, during the whole chilled storage, the amount of TBARS was significantly ($p<0.05$) lower in patties with extracts than in the control counterparts, which suggests that walnut leaf and cherry stem extracts were effective in protecting the raw pork patties from lipid oxidation. After nine months of frozen storage, the MDA level in the control patties increased to 2.17 mg MDA/kg, while in samples treated with walnut leaf and cherry stem extracts they were only 0.72 and 1.01 mg MDA/kg respectively (Table 1).

The ability to protect raw pork patties from lipid oxidation may be due to the fact that walnut leaves and cherry stems have an extremely high antioxidant capacity, attributed to the high content of antioxidant compounds, mainly phenolic acids and flavonoids.

At the end of the storage period, the TBARS values in the samples with added extracts were significantly lower than those in the BHT added samples, suggesting that plant extracts were more effective than BHT in retarding lipid oxidation. Amongst treated patties, the samples with walnut leaf extract had the lowest TBARS values by the end of the storage.

Table 1. *TBARS values, pH, total phenolic content and ABTS antioxidant activity of the raw pork patties during frozen storage for 9 months*[#]

	Storage period [months]			
Treatment	**0**	**3**	**6**	**9**
TBARS values [mg MDA/kg]				
C0	0.74 ± 0.03^{cA}	0.94 ± 0.03^{dB}	1.24 ± 0.05^{dC}	2.17 ± 0.12^{dD}
C1	0.28 ± 0.02^{bA}	0.45 ± 0.03^{cB}	0.75 ± 0.04^{cC}	1.22 ± 0.07^{cD}
WLE	0.12 ± 0.02^{aA}	0.25 ± 0.02^{aB}	0.43 ± 0.02^{aC}	0.72 ± 0.04^{aD}
CSE	0.16 ± 0.02^{aA}	0.32 ± 0.02^{bB}	0.53 ± 0.03^{bC}	1.01 ± 0.04^{bD}
***p*H**				
C0	5.83 ± 0.04^{cA}	6.04 ± 0.05^{bB}	6.16 ± 0.04^{aC}	6.28 ± 0.05^{aD}
C1	5.70 ± 0.03^{abA}	6.02 ± 0.02^{bB}	6.10 ± 0.05^{aC}	6.24 ± 0.05^{aD}
WLE	5.68 ± 0.05^{aA}	6.01 ± 0.03^{bB}	6.11 ± 0.03^{aC}	6.24 ± 0.04^{aD}
CSE	5.77 ± 0.04^{bcA}	5.92 ± 0.04^{aB}	6.11 ± 0.04^{aC}	6.20 ± 0.04^{aD}
Total phenolic content [mg GAE/100 g]				
C0	8.80 ± 0.30^{aA}	8.48 ± 0.40^{aA}	8.34 ± 0.30^{aA}	8.40 ± 0.50^{aA}
C1	8.65 ± 0.40^{aA}	8.56 ± 0.30^{aA}	8.49 ± 0.40^{aA}	8.36 ± 0.40^{aA}
WLE	17.03 ± 0.60^{cD}	15.92 ± 0.60^{cC}	14.02 ± 0.50^{cB}	12.55 ± 0.50^{cA}
CSE	12.97 ± 0.50^{bD}	12.08 ± 0.50^{bC}	10.58 ± 0.40^{bB}	8.98 ± 0.30^{bA}
ABTS antioxidant activity [mmol Trolox/100 g]				
C0	0.47 ± 0.02^{aD}	0.41 ± 0.01^{aC}	0.33 ± 0.02^{aB}	0.26 ± 0.01^{aA}
C1	0.60 ± 0.02^{bD}	0.53 ± 0.02^{bC}	0.47 ± 0.02^{bB}	0.38 ± 0.02^{bA}
WLE	0.76 ± 0.03^{dD}	0.70 ± 0.03^{dC}	0.60 ± 0.02^{cB}	0.52 ± 0.02^{dA}
CSE	0.71 ± 0.02^{cD}	0.64 ± 0.02^{cC}	0.56 ± 0.03^{cB}	0.45 ± 0.02^{cA}

[#] Different lowercase letters indicate significant difference at $p<0.05$ level between different treatments, while different uppercase letters are indicative of the same within each treatment during the storage period.

Similar studies have been conducted to assess the effect of plant extracts with high antioxidant activity on lipid oxidation in meat products during frozen storage. Sebranek *et al*. [8] showed that the rosemary extract was more effective than BHA/BHT for preventing lipid oxidation in precooked frozen pork sausage while Trindade *et al*. [35] reported that rosemary and oregano added individually or in combination, improved the lipid stability of beef burgers during storage at -20 °C for 3 months. Reihani *et al*. [2] have also reported that addition of extracts from ulam raja leaves (*Cosmos caudatus*) into beef patties reduced the extent of lipid oxidation for up to 10 weeks at -18 °C. Mint extract was also found effective on retarding MDA formation in irradiated lamb meat during chilled storage for 4 weeks [13].

The total phenolic content of pork patties is presented in Table 1. Walnut leaves extract significantly ($p<0.05$) increased the phenolics content of raw pork patties followed by cherry stems extract. Das *et al*. [33] observed an increase in phenolic content of goat meat patties prepared with *Moringa oleifera* leaves extract. Devatkal *et al*. [36] have also reported that extracts of kinnow and pomegranate byproducts significantly increased the total phenolic content of cooked chicken patties. The results indicated a significant ($p<0.05$) decrease in phenolics up to 9th month of frozen storage in pork patties treated with rich-phenolic extracts. At the end of the storage period, the patties with walnut leaf extract presented the highest phenolic content (12.55 mg GAE/100 g).

According to the ABTS assay, the WLE and CSE treatments both produced significantly ($p<0.05$) antioxidant activity compared to control and BHT samples (Table 1). Radical scavenging activity significantly decreased throughout the nine months storage period, in agreement with the previous studies [37]. The antioxidant activity was significantly ($p<0.05$) higher for the WLE than the CSE treatment both at the beginning and at the end of the storage period.

The evolution of color parameters is shown in Table 2. Lightness (L* value) was significantly ($p<0.05$) higher in control and BHT as compared to WLE and CSE added samples. Devatkal *et al*. [36] reported also that inclusion of extracts of pomegranate and kinnow fruit by-products resulted in lower L values. L* values decreased in all samples during the storage period. Lower L* values can affect the overall acceptability of meat products [38].

The a* values of WLE treated patties were higher than those of the control at day 0 but no significant difference was found at the end of the storage period.

All types of patties suffered a significant decrease in redness (a* values) during chilled storage, which is in agreement with previous studies [1, 36, 39, 40]. Decrement of the a* value indicates the change in color from red to brown which could be due to the formation of metmyoglobin as a result of pigment oxidation [41]. Several authors have linked the loss of redness in raw meats subjected to chilled storage to the occurrence of oxidative reactions [42, 43].

Walnut leaf and cherry stem extracts significantly ($p<0.05$) reduced the loss of redness during frozen storage of pork patties. Other authors have reported the effectiveness of phenolic-rich extracts at minimizing the color changes occurred during chilled storage of porcine meat [39, 42, 44]. CSE added patties had significantly higher ($p<0.05$) b* values than control patties, while no significant differences were found between the other treatments. In a previous study, b* values of pork patties increased with addition of extracted green tea leaf powder [44]. The b* value also decreased in all groups during storage.

Mean Hue values were significantly ($P<0.05$) higher in CSE followed by C0 and lowest in BHT and WLE treated samples which showed no significant difference. Hue values further increased with storage intervals in all the treatments. Overall means of chroma value (color intensity) was significantly higher in CSE than other treatments. Chroma value did not differ significantly between BHT, WLE and control treatments. Chroma showed a decreasing trend with storage interval in all the treatments.

Table 2. *Color parameters of the raw pork patties during frozen storage for 9 months*[#]

Color parameters	Treatment	Storage period [months]			
		0	3	6	9
L*	C0	74.16 ± 0.31^{cD}	70.65 ± 0.32^{cC}	67.14 ± 0.71^{bB}	65.38 ± 0.93^{bA}
	C1	72.60 ± 0.91^{bC}	70.43 ± 0.45^{cB}	68.27 ± 1.07^{bA}	67.19 ± 1.49^{bA}
	WLE	68.03 ± 0.86^{aC}	65.92 ± 0.83^{aBC}	63.81 ± 1.89^{aAB}	62.76 ± 2.48^{aA}
	CSE	68.47 ± 0.85^{aA}	68.17 ± 0.58^{bA}	67.88 ± 0.55^{bA}	67.73 ± 0.64^{bA}
a*	C0	10.72 ± 0.57^{aD}	8.51 ± 0.47^{abC}	6.30 ± 0.42^{abB}	5.19 ± 0.41^{aA}
	C1	11.65 ± 0.47^{bD}	9.55 ± 0.49^{cC}	7.45 ± 0.63^{bB}	6.40 ± 0.72^{bA}
	WLE	11.86 ± 0.48^{bC}	9.34 ± 0.90^{bcB}	6.83 ± 1.42^{abA}	5.58 ± 1.70^{aA}
	CSE	10.08 ± 0.79^{aD}	7.87 ± 0.36^{aC}	5.65 ± 0.33^{aB}	5.54 ± 0.53^{aA}
b*	C0	15.19 ± 0.89^{aC}	13.59 ± 0.72^{aB}	11.99 ± 1.39^{aA}	11.19 ± 0.69^{aA}
	C1	14.51 ± 0.75^{aA}	13.95 ± 0.82^{aA}	13.40 ± 1.09^{aA}	13.12 ± 1.26^{aA}
	WLE	15.09 ± 0.62^{aB}	13.89 ± 1.34^{aAB}	12.69 ± 2.09^{aAB}	12.09 ± 2.46^{aA}
	CSE	17.94 ± 1.22^{bC}	16.93 ± 0.53^{bBC}	15.91 ± 0.22^{bAB}	15.41 ± 0.54^{bA}
C	C0	18.59 ± 1.04^{aC}	16.03 ± 0.81^{aB}	13.55 ± 1.30^{aA}	12.34 ± 0.71^{aA}
	C1	18.61 ± 0.86^{aC}	16.91 ± 0.95^{aBC}	15.33 ± 1.24^{abAB}	14.60 ± 1.43^{abA}
	WLE	19.19 ± 0.76^{abC}	16.74 ± 1.61^{aBC}	14.42 ± 2.51^{aAB}	13.33 ± 2.93^{aA}
	CSE	20.58 ± 1.35^{bC}	18.67 ± 0.58^{bB}	16.89 ± 0.32^{bA}	16.06 ± 0.66^{bA}
h	C0	54.78 ± 0.64^{bA}	57.95 ± 1.02^{bB}	62.13 ± 1.52^{aC}	65.09 ± 1.72^{aD}
	C1	51.24 ± 0.68^{aA}	55.60 ± 0.28^{aB}	60.92 ± 0.62^{aC}	64.01 ± 1.01^{aD}
	WLE	51.84 ± 0.58^{aA}	56.07 ± 0.43^{aB}	61.87 ± 1.32^{aC}	65.71 ± 2.85^{aD}
	CSE	60.68 ± 1.49^{cA}	65.08 ± 0.89^{cB}	70.47 ± 0.80^{bC}	73.61 ± 1.31^{bD}

[#] Different lowercase letters indicate significant difference at $p<0.05$ level between different treatments, while different uppercase letters are indicative of the same within each treatment during the storage period.

CONCLUSIONS

The present results indicate the potential usage of walnut leaf and cherry stem extracts as efficient inhibitors of lipid oxidation and color deterioration during chilled storage of raw pork patties. Addition of walnut leaf and cherry stem extracts into the raw pork patties limited the oxidative reactions more than BHT, a synthetic antioxidant. These effects could be attributed to the presence of antioxidant phenolic compounds in the extracts, which act as efficient radical scavengers and metal chelators in vitro and retard methmyoglobin formation in patties.

According to the present results, the walnut leaf extract was more effective than cherry stem extract in preventing lipid oxidation and inhibiting the browning and discoloration of frozen pork patties by reducing the loss of redness and the increase of yellowness. Using these extracts as natural antioxidants could be an effective strategy to prolong the shelf life of frozen meat products.

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