

## Antioxidative Effects of Extracts of Cocoa Shell, Roselle Seeds and a Combination of Both Extracts on the Susceptibility of Cooked Beef to Lipid Oxidation

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**Abstract:** This aim of this study was to investigate the effects of Cocoa Shell (CS), Roselle Seeds (RS) and a combination of both extracts (CR), compared to synthetic antioxidants (BHT and  $\beta$ -tocopherol) on lipid oxidation in cooked beef stored at 4°C for up to 14 days. Extracts and synthetic antioxidants were added to ground beef at a concentration of 7.5 g lyophilised extract/kg ground beef. The extent of lipid oxidation was measured using the 2-Thiobarbituric Acid Assay (TBA) on days 1, 3, 7 and 14 of storage at 4°C. The phenolic content of ethanolic lyophilised extract of CS was significantly higher ( $p < 0.05$ ) than the phenolic content of RS. The antioxidant and free radical scavenging activity of CS a concentration of 7.5g L<sup>-1</sup> were significantly greatest among other extracts, except for CR in free radical scavenging activity. Lipid oxidation was significantly lower in cooked beef containing the extracts compared to synthetic antioxidants. Based on TBA numbers, the extracts tested were more effective than BHT and  $\alpha$ -tocopherol in inhibiting lipid oxidation in cooked-refrigerated beef. The study showed that the antioxidant capacity of phenolic compounds present in the ethanolic extract of CS have potential to reduce lipid oxidation of cooked-refrigerated beef.

**Key words:** Cocoa shell, roselle seeds, antioxidant activity, scavenging activity, lipid oxidation, cooked beef, chilling storage

### INTRODUCTION

Lipid oxidation is a major cause of quality deterioration in cooked meats. Synthetic antioxidants such as BHA and BHT are commonly used to prevent oxidative deterioration in a variety of foods. However, in recent years, synthetic antioxidants have come under scrutiny due to possible health risks and toxicity<sup>[1]</sup>. The development and utilization of natural effective antioxidants from inexpensive, renewable and abundant sources from agricultural industries is desirable due to the increased awareness among consumers in diet related health problems.

Many studies have shown that most of the by-products of food processing industries are potential source of natural antioxidants and could be exploited for the development of functional ingredients or nutraceuticals<sup>[2-5]</sup>. Antioxidant properties have been reported in blackcurrant seeds<sup>[6]</sup>, grape seeds<sup>[7]</sup>, red grape marc<sup>[8]</sup>, apple pomace<sup>[9]</sup>, citrus peels and seeds<sup>[10]</sup>. In addition, other by-products such as cottonseed meals<sup>[11]</sup> and rice hull<sup>[12]</sup> have been shown to be highly effective as antioxidants in cooked meats. However, few studies have been carried out to evaluate the potential extracts

prepared from residue sources in preventing lipid oxidation in meat products<sup>[13]</sup>.

In cocoa liquor manufacture, roasted cocoa beans are crushed and cotyledons/nibs are separated from the shells through a winnowing process. Approximately 10% of the whole dry bean weight is produced as cocoa by-product. Cocoa shell is the product of the testa and remnants of mucilage after the fermentation and drying process of fresh beans. MARDI<sup>[14]</sup> reported that an estimated amount of 6,500 ton of cocoa shell is produced annually in Malaysia. Fresh (unfermented) cocoa beans contain high levels of polyphenols. It is well-documented that there is a drop in the soluble polyphenol content of cocoa beans during the fermentation process. Kim and Keeney<sup>[15]</sup> suggested that a migration of epicatechin into the cocoa testa from the cotyledon during cocoa fermentation could be responsible for the drop in polyphenol content. The researchers implied that these compounds are largely concentrated in the shell of the beans. Azizah *et al.*,<sup>[4]</sup> reported that the bioactive components found in the extracts of cocoa shell could be flavonoids, which showed high antioxidant activity *in-vitro*.

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*Hibiscus sabdariffa*, Linn also known as Roselle, karkade and even mesta, is a herbal shrub plant popular in Middle east countries. It can be grown in warm countries like Malaysia, Indonesia, Thailand and Philippines. Roselle is a new commercial crop of Malaysia. The origin of roselle is uncertain, others believed that its home country is India. The calyces or petals of the flower are processed mainly to make herbal drink, beverages, jams and jellies. Roselle calyces have been reported to contain higher antioxidant properties compared to BHA and  $\alpha$ -tocopherol<sup>[16]</sup>. The high antioxidant activity was due to the polyphenol components<sup>[17]</sup>. In the manufacturing of roselle products, approximately 50% by weight of the raw materials are underutilised roselle capsules which are discarded and contain seeds. Roselle seeds have been reported to contain a high amount of protein, lipids and carbohydrate<sup>[18]</sup>, dietary fiber and certain minerals<sup>[19]</sup>. However, to date no study has been published on the antioxidative properties of these seeds.

Cocoa shells and roselle seeds are both residual from waste products of agricultural industries which may be a potential source of antioxidant components for food uses. To evaluate their antioxidative efficacy, in comparison to synthetic antioxidants, crude extracts from Cocoa Shell (CS), Roselle Seeds (RS) and a combination of both extracts (CR) were prepared and incorporated into ground beef and the extent of lipid oxidation based on TBA number was monitored up to 14 days at 4°C. The study also evaluated a synergistic effect of compounds present in CS and RS extracts on lipid oxidation. In addition, the antioxidant activity and scavenging effect of CS, RS and CR extracts were examined *in-vitro*.

## MATERIALS AND METHODS

**Materials:** Cocoa shell was obtained from KL-Kepong Cocoa Products Sdn Bhd., Port Klang, Selangor, Malaysia. Roselle seeds were collected from Jabatan Pertanian Rhu Tapai, Kuala Terengganu, Terengganu, Malaysia. The Australian beef (type: eyeround) was purchased from a local market. The fat content of the beef was 10%.

**Chemicals:** Ethanol,  $\beta$ -tocopherol, Butylated Hydroxytoluene (BHT), 2-Thiobarbituric Acid (TBA), 1,1-Diphenyl-2-Picrylhydrazyl (DPPH), polyoxyethylene-sorbitanmonolaurate (Tween 20) and linoleic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hexane and glacial acetic acid were purchased from Merck KGaA, (Darmstadt, Germany). Petroleum ether was purchased from Ajax Laboratory Chemicals, Auburn, Australia). All chemicals used were of analytical reagent grade.

**Preparation of samples:** Cocoa shells and roselle seeds (dried) were defatted according to the method of Sanbongi, Osakabe, Natsume, Takizawa, Gomi and Osawa<sup>[20]</sup> with modifications of defatting times. Samples were ground using a blender (MK-T3GN; Kuala Lumpur, Malaysia) and sieved through 300  $\mu$ m mesh in order to achieve uniform particles. Samples were soaked with n-hexane in a ratio of 1: 5. The mixture was stirred using a magnetic stirrer for 45 min at ambient temperature. Subsequently, the mixture was left overnight at room temperature and filtered through a Whatman No. 4 filter paper. Following filtration, the residue remaining was defatted again as described above. Cocoa shell was defatted twice and roselle seed was defatted three times. Following defatting, the residue remaining after filtration was left to dry overnight in a fume hood at room temperature. The samples were subsequently dried in an air-oven at 40°C for 1 h to remove n-hexane residue.

**Preparation of crude extracts:** Crude polyphenols of defatted CS and RS were extracted according to the method of Sanbongi *et al.*,<sup>[21]</sup> with modifications in which 70% (v/v) ethanol was used instead of 80% (v/v). CS and RS were diluted 7 fold of with 70% (v/v) ethanol. The mixture was stirred with a magnetic stirrer for 2 h at ambient temperature. The resultant mixture was left overnight and filtered through a Whatman No. 4 filter paper. Following filtration, the remaining residue was re-extracted as described above. The filtrate was concentrated using a rotary evaporator (Laborata 4000; Heidolph Instruments GmbH and Co. KG, Germany) at 45°C under reduced pressure to remove ethanol residues. The ethanolic extracts were lyophilized using a freeze-dryer (Virtis Co., Inc., New York, USA) at -50°C, 35 mmHg. Then, the resulting extracts were transferred into an air-tight container and stored at -80°C until further uses.

**Determination of total phenolic content:** Total phenolic content was estimated according to Velioglu, Mazza, CS, RS and CR lyophilised extracts were prepared at a concentration of 1 mg mL<sup>-1</sup>, whereby 4 mg of the extracts were weighed and dissolved in 4 mL of 70% ethanol. The ethanolic extracts (100  $\mu$ l) were transferred into test tubes and 0.75 mL of Folin-Ciocalteu's reagent (1: 10 with distilled water) were added, mixed and allowed to stand at room temperature for 5 min. 0.75 mL of 6% sodium carbonate was added and then gently mixed. Following standing at 28°C for 90 min, the absorbance was read at 725 nm using a UV-Visible spectrophotometer. A standard calibration (0.01 – 0.05 mg mL<sup>-1</sup>) curve was generated using gallic acid as a standard. The total phenolic content was expressed as Gallic Acid Equivalents (GAE) in milligrams per gram sample.

**Preparation of ground beef:** Beef meat was prepared according to the method of Tang, Kerry, Sheehan, Buckley and Morrissey<sup>[22]</sup> with modifications. Beef was cut into small pieces and homogenized in a blender<sup>[12]</sup>. Ground meat (40 g) was thoroughly mixed by hand with either 7.5 g kg<sup>-1</sup>[22] of the ethanolic lyophilised extracts of CS, RS and CR.  $\beta$ -Tocopherol and BHT (7.5 g/kg) was used for comparative purposes. Minced beef containing no additives was run as control. Beef samples were pressed into a mould (4 cm diameter, 1 cm height) and cooked for 30 min in a water bath at  $75 \pm 1^\circ\text{C}$  until an internal temperature of  $\sim 75^\circ\text{C}$  was reached. Following cooking, the beef was cooled, covered with oxygen-permeable polyvinyl chloride film and stored at  $4^\circ\text{C}$  until required further analysis.

**Determination of Thiobarbituric Acid (TBA) numbers:** The extent of lipid oxidation in cooked beef was monitored using the TBA assay on days 1, 3, 7 and 14, according to the method of Kirk and Sawyer<sup>[23]</sup>. Cooked beef (10 g) was chopped into small pieces and homogenised in a distillation flask with 100 mL distilled water for 2 min. A few drops of 4 M HCl were slowly added to bring to the pH to 1.5. The mixture was heated using an electric mantle and the distillate (5 mL) collected into a conical flask. A blank was prepared similarly using 100 mL distilled water without added sample. The distillate (5 mL) was transferred into a glass-stoppered tube and 5 mL of TBA reagent (0.02 M) was added. The mixture was incubated in boiling water for 35 min. After 10 min cooling, the absorbance was read against the blank at 538 nm using a UV-Visible spectrophotometer (UV-1601; Shidmadzu Corp., Kyoto, Japan). Lipid oxidation was expressed as 2-Thiobarbituric Acid Reactive Substances (TBAR) in mg Malondialdehyde (MDA)/kg beef.

**Determination of antioxidant activity:** The antioxidant activity of CS, RS and CR extracts was evaluated using the  $\beta$ -carotene bleaching method<sup>[24]</sup>.  $\beta$ -carotene solution (1 mL) (0.2 mg  $\beta$ -carotene/mL of chloroform) was transferred into a round-bottom flask containing 0.02 mL of linoleic acid and 0.2 mL of Tween 20. The chloroform was removed at  $40^\circ\text{C}$  using a rotary evaporator. The resultant mixture was immediately diluted with 100 mL of distilled water and mixed for 1-2 min to form an emulsion. A mixture prepared similarly without  $\beta$ -carotene was used as a blank. A control, containing 0.2 mL of 70% ethanol instead of extract, was also prepared. The emulsion (5 mL) was transferred into a test tube containing 0.2 mL of extract. The tubes were placed at  $50^\circ\text{C}$  in a water bath for 2 h. Absorbance was read at 470 nm using a UV-Visible spectrophotometer (UV-1601; Shidmadzu Corp., Kyoto, Japan). The percentage antioxidant activity was calculated using the following equation.

$$\text{Antioxidant activity (\%)} = \left( 1 - \frac{(A_0 - A_t)}{(A_0 - A_c)} \right) \times 100$$

Where,

$A_0$  and  $A_{c0}$  are the absorbance values measured at zero time of incubation for extracts and control, respectively.

$A_t$  and  $A_{ct}$  are the absorbance values for extracts and control respectively at  $t = 120$  min.

**Determination of free radical scavenging activity:** Free radical scavenging was evaluated using the DPPH free radical according to the method of Tang, Kerry, Sheehan and Buckley<sup>[25]</sup>. One mL of 0.2 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH) in 100% ethanol was added to test tubes containing 4 mL of extract. A control was prepared by adding 1 mL of DPPH solution to 4 mL of 70% ethanol. Following storage in the dark for 30 min, the absorbance was read at 517 nm using a UV-Visible spectrophotometer. The percentage free radical scavenging activity was calculated based on the following equation.

$$\text{Free radical scavenging activity (\%)} = \left( 1 - \frac{\text{Absorbance of sample at 517 nm}}{\text{Absorbance of control at 517 nm}} \right) \times 100$$

**Statistical analysis:** The Statistical Package for Social Sciences (SPSS ver.10.0) was used for statistical analysis. One-way analysis of variance (ANOVA) test was carried out to compare the means of TBA numbers in cooked meat and means of antioxidant activity and free radical scavenging of extracts. A t-test was used to analyse the results of total phenolic content. Differences between treatment means were determined at the 5% level.

## RESULTS AND DISCUSSION

Addition of extracts, BHT and  $\beta$ -tocopherol into beef at a concentration of  $7.5 \text{ g kg}^{-1}$  resulted in a significant reduction ( $p < 0.05$ ) of TBA numbers compared to the control. After 1 day of storage, RS exhibited the strongest inhibition on lipid oxidation compared to CS, CR and the synthetic antioxidants used (BHT and  $\beta$ -tocopherol), while  $\beta$ -tocopherol showed the least effect (Table 1). The antioxidant potency of the extracts was in the order of  $\text{RS} > \text{CR} > \text{CS} > \text{BHT} > \beta\text{-tocopherol}$ . This indicates cooked beef treated with BHT and  $\beta$ -tocopherol had lower oxidative stability.

On day 3, all the cooked beef irrespective of treatments, were more susceptible to lipid oxidation compared to the effect on day 1, based on the higher values of TBA with RS still having the strongest inhibition on lipid oxidation among the extracts and the synthetic antioxidants. The inhibitory effect on lipid oxidation of the three extracts observed after 3 days of storage was similar to the trend as day 1.

Table 1: The effects of Cocoa Shell (CS), Roselle Seed (RS) and a combination of cocoa shell and roselle seed (CR) extracts on oxidative deterioration of cooked beef, compared with BHT and  $\beta$ -tocopherol.

Treatment	TBA numbers (mg MDA kg <sup>-1</sup> cooked beef)			
	Time (days)			
	1	3	7	14
Control	9.32 ± 0.04a	10.90 ± 0.18a	12.95 ± 0.02a	16.55 ± 0.07a
CS	2.96 ± 0.02b	4.71 ± 0.02b	6.89 ± 0.06b	4.56 ± 0.04b
RS	2.43 ± 0.02c	3.16 ± 0.02c	6.15 ± 0.01c	6.45 ± 0.07c
CR	2.62 ± 0.02d	3.70 ± 0.03d	4.25 ± 0.04d	4.75 ± 0.03b
BHT	3.82 ± 0.03e	6.58 ± 0.02e	11.90 ± 0.04e	13.44 ± 0.09d
$\alpha$ -tocopherol	4.22 ± 0.02f	7.64 ± 0.02f	10.02 ± 0.03f	11.83 ± 0.04e

Values with different letters within the same column were significantly difference at the level of  $p < 0.05$ . TBA numbers were means of three determinations. Values indicate that the coefficient of variation was less than 5%. TBA numbers of cooked beef treated with cocoa shell (CS), roselle seed (RS) and a combination of both extracts (CR) compared to BHT and  $\alpha$ -tocopherol on days 1, 3, 7 and 14 at a concentration of 7.5 g ethanolic extract/kg beef.

Table 2: Mean phenolic content, antioxidant and free radical scavenging activities of Cocoa Shell (CS), Roselle Seed (RS) and a combination of both extracts at a concentration of 7.5 g L<sup>-1</sup>.

Ethanolic extract	Phenolic content (mg g <sup>-1</sup> extract)	Antioxidant activity (%)	Free radical scavenging activity (%)
CS	112.9 ± 0.6a	98.4 ± 2.2a	95.9 ± 2.0a
RS	018.3 ± 1.4b	60.3 ± 2.2b	87.9 ± 2.0b
CR	-	96.8 ± 0.9a	93.6 ± 2.0c

Data are means ± standard deviation of three determinations. Total phenolic content was expressed as gallic acid equivalents in milligrammes per gram extract. Antioxidant activity was measured using a  $\beta$ -carotene bleaching assay, while scavenging activity were estimated based on the capability of the extracts to scavenge the DDP radical. Values indicate that the coefficient of variation was less than 5%. Values with different letters within the same column were significantly difference at the level of  $p < 0.05$ . The phenolic content of CR was not carried out.

CR had the strongest inhibitory effect on lipid oxidation, followed by RS, CS,  $\beta$ -tocopherol and BHT on day 7. ANOVA indicated that the means of TBA numbers of cooked beef treated with extracts was significantly lower ( $p < 0.05$ ) compared to the studied synthetic antioxidants.

On day 14, CS had the strongest inhibition on lipid oxidation compared to other extracts. All cooked beef treated with RS, CR, BHT and  $\beta$ -tocopherol showed the significant high values ( $p < 0.05$ ) in TBA numbers. No significant difference was observed between CS and RS. TBA number for CS was lower compared to the effect on days 3 and 7. The inhibitory effect of the three extracts on lipid oxidation was in the order of CS  $\approx$  CR > RS >  $\beta$ -tocopherol > BHT.

To further evaluate the potential antioxidant compounds as contributed to an inhibitory effect on lipid oxidation, we estimated the total phenolic content of CS and RS. CS exhibited a significant higher ( $p < 0.05$ ) phenolic content compared to RS, with a value of six times higher than the latter (Table 2). High phenolic content in CS could be due to the migration of epicatechin from cocoa cotyledons to its shell during cocoa fermentation as suggested by Kim and Keeney<sup>[15]</sup>. A study done by Hammerstone, Lazarus, Mitchell, Rucker and Schmitz<sup>[26]</sup>, found that cocoa cotyledons are rich in phenolic compounds, such as epicatechin, catechin and

procyanidins. However, no study has been reported on the content of phenolics in roselle seeds.

To evaluate the antioxidant potencies of each extract and its combination at 7.5 g L<sup>-1</sup> two different model systems of antioxidant assay were used, namely  $\beta$ -carotene-linoleate bleaching and free radical scavenging activity. The antioxidant activity of the extracts was in order of CS  $\approx$  CR > RS. There was no significant difference in the antioxidant activity between CS and CR (Table 2). Results demonstrated that a combination of CS and RS extracts resulted in higher antioxidant activity compared to RS alone. At 7.5 g L<sup>-1</sup> free radical scavenging activity of CS was the highest among the extracts, with the value of 96% while RS showed the least activity (Table 2). However, the free radical scavenging activity of CS was not significantly different when compared to CR.

The antioxidative effect may have contributed to the high oxidative stability of cooked beef with the addition of CS on day 14 (Table 1). This effect could be due to the significant amount of phenolics present in the ethanolic extract of the CS (Table 2). In addition, a similar finding was also observed in cooked beef when added with CR. It could be postulated to be due to the synergistic effect between the compounds present in CS and RS. Catechins in tea, have been reported to inhibit lipid oxidation in cooked meats through radical scavenging abilities<sup>[27]</sup>. Nanjo, Goto, Seto, Suzuki, Sakai and Hara<sup>[28]</sup> showed tea phenolics to have an excellent scavenging effect on 1,1-diphenyl-2-picrylhydrazyl. In contrast, an addition of CS exhibited the highest TBA numbers in cooked beef compared to RS and CR on days 1, 3 and 7. This could be due to the slow permeate rate of antioxidant components into the lipid bilayer of the membrane structure of refrigerated-cooked beef.

Phenolic compounds present in cocoa beans have been shown to possess antioxidant activity against lipid oxidation<sup>[29]</sup>. Azizah *et al.*,<sup>[4]</sup> showed that phenolic compounds of cocoa shells exhibited a strong

antioxidant activity compared to synthetic antioxidants. The study showed that CS had a strong inhibitory effect towards lipid oxidation on day 14. This could be due to the scavenging effect of phenolic compounds which possess an important mechanism in inhibiting the lipid oxidation of cooked-refrigerated beef. Several studies have reported that phenolic-rich extracts showed an excellent oxidative stability when compared to synthetic antioxidants<sup>[26,30-32]</sup>.

Further research is necessary to clearly understand the relationship among antioxidant components present in these extracts and their mechanisms in inhibiting lipid oxidation in food products.

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