

## Beneficial effects of commercial Assam green tea infusion on the microbial growth and oxidative stability of cooked beef

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### Abstract

Natural preservatives having the great antioxidant and antimicrobial activity have been utilized in the food industry for many years. In the present study, the effect of two brands of commercial Assam green tea infusion (represented by A and B) and 0.02% BHA/BHT on microbial growth, anti-lipid oxidation and color change were investigated in cooked beef. The green tea concentration has influenced to the results. It was found that A and B at the concentration of 250 mg/mL significantly reduced the population of *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium* and *E. coli* in the cooked beef to an undetectable level within 2 days of storage at 4°C. A and B also exhibited higher anti-lipid oxidation activity compared to 0.02% BHA/BHT, and control. Assam green tea infusions in cooked beef significantly increased  $\Delta L^*$  value and decreased  $\Delta a^*$  and  $\Delta b^*$  value ( $p \leq 0.05$ ). These indicate that Assam green tea infusion might be a potential candidate as a natural preservative for beef and other types of food.

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### Introduction

Ready-to-eat (RTE) foods are very popular in nowadays modern convenience society. Cooked beef is one of the popular RTE foods. However, it is prone to microbial spoilage and could be a potential foodborne microorganisms harbor as a result of their high nutritional value. A number of foodborne pathogens have been found in precooked beef under refrigerated storage conditions (Hubbert *et al.*, 1996; Ahn *et al.*, 2007). Those include *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes* and *Staphylococcus aureus*. Lipid oxidation and discoloration are also the major causes of their deterioration resulting from their high fat and high iron content. The iron content accelerates the oxidative process leading to deterioration in beef flavor, color and nutritional value (Ho *et al.*, 2009). These make beef products unacceptable and unsafe for the consumer, particularly in the new era of health concerned society.

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) mixture are an effective synthetic food additive capable of preventing both lipid oxidation and microbial contamination (Ahn, 2003; Mitsumoto *et al.*, 2005; Su *et al.*, 2008). However, there is a limitation allowance of the mixture of BHA and BHT for food application to not more than 0.01% each by weight (FAO, 2006). In

addition, the interest of substitution of the synthetic food additives by the natural ones is increasing, therefore, many investigations have been conducting in order to find natural antioxidant and antimicrobial agents to replace the BHA and BHT as activities *in vitro* (Ahn, 2003; Aksu and Maya, 2005).

Green tea used in this study, the Assamica tea, called *Camellia sinensis* var. *assamica* or *Camellia assamica*, one of tea species that has height 10 -15 m, with the leaves length more than 100 mm, thin, glossy with yellow-white flower, 2.5 - 4 cm in diameter with 5 petals and light green pistil (Jain, 1999). This species is the most cultivated tea because it can grow well in the tropical region. Compared with other tea, green tea is one of the undoubted potent natural preservatives applied to many foods as antioxidant and antimicrobial agents (Wang *et al.*, 1997; Tang *et al.*, 2001; An *et al.*, 2004; Mitsumoto *et al.*, 2005; Zhu *et al.*, 2005; Almajano *et al.*, 2008; Su *et al.*, 2008; Kristanti and Punbusayakul, 2009). Various studies have demonstrated antimicrobial activity of green tea extract *in vitro* against some potent pathogens, such as *Shigella dysenteriae*, *Salmonella* sp, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* (Chou *et al.*, 1999; Sakanaka *et al.*, 2000; Wang *et al.*, 2000). The antioxidant activity of green tea extract has also been verified in raw, frozen, cooked meat patties (Tang *et al.*, 2001; Jo *et al.*, 2003; Mitsumoto *et al.*, 2005) and sausage (Bozkurt, 2006). However,

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antimicrobial and antioxidant activities of the extract are influenced by many factors, including pH, microorganisms, food component, temperature and natural component properties (Ahn *et al.*, 2007), and up until now, there is no report of using tea infusion directly for those purposes in cooked beef. Therefore, in this study, the effectiveness of commercial Assam green tea infusion to preserve cooked beef from microbial growth and lipid oxidation were examined. These will provide more information of using Assam green tea infusion and the feasibility of using it as antimicrobial and anti-lipid oxidation in other complex foods.

## Materials and Methods

### Materials

Two commercial Assam green teas (represented by A and B) used in this experiment were purchased from some available supplier in Chiang Rai, Thailand. Assam green tea A and B were produced from the loose leaf Assamica green tea, harvested from Assamica tea native to Doi Mae Salong, Thailand. These two brands have been selected because of their high-level antioxidant and antimicrobial activities (Kristanti and Punbusayakul, 2009). BHA and BHT were purchased from Sigma-Aldrich Co, USA. Microbiological media, including Modified Oxford (MOX) and Xylose Lysine Deoxycholate (XLD) agar were purchased from Merck, Germany, while petrifilm for *E. coli*/coliform count and *S. aureus* express count plate were purchased from 3M Microbiology Thailand, Ltd. The fresh beef purchased from available supplier in Chiang Rai, Thailand.

### Bacteria strains and growth condition

Microorganisms purchased from Microbiological Resources Center, Thailand Institute of Scientific and Technological Research (TISTR) and DMST culture collection, Department of Medical Sciences Thailand, were used in this study. Four tested isolates were *Staphylococcus aureus* strain TISTR 1466, *Listeria monocytogenes*, *Salmonella typhimurium* strain TISTR 292, *Escherichia coli* strain TISTR 780. The four bacterial strain were cultured in 10 mL brain heart infusion (BHI) broth and incubated at 37°C for 24 h. The cultures were then transferred to 100 mL BHI broth and the microbes were allowed to grow for 24 h. The broth was centrifuged at 5,000 g for 15 min at 4°C. The culture was then washed and resuspended with 0.1% peptone water to get approximately 7 log CFU/mL (counted by haemocytometer). Specific agar including, Modified Oxford (MOX) and Xylose Lysine Deoxycholate (XLD) were prepared for *L.*

Table 1. Assam green tea infusion preparation for cooked beef antimicrobial and anti-lipid oxidation investigations

Volume of stock 250 mg/ml tea infusion (mL)	Distilled water (mL)	Tea final concentration (mg/mL)
0	100	0
12.5	87.5	31.3
25	75	62.5
50	50	125
100	0	250

*monocytogenes* and *S. typhimurium*, respectively, while express count plate and petrifilm were used for counting *S. aureus* and *E. coli*/coliform.

### Preparation of Assam green tea infusion

Different concentrations of Assam green tea infusion were prepared by mixing different volume of the stock Assam green tea infusion (250 mg/mL) and distilled water as shown in Table 1 in order to get 0, 31.3, 62.5, 125 and 250 mg/mL of Assam green tea infusion. Then, the extract was sterilized by passing through a membrane (pore size of 0.45 µm). For antimicrobial activity determination, three different Assam green tea infusion concentrations were used : 0, 125 and 250 mg/mL, while five different concentrations of Assam green tea infusion were used to determine anti-lipid oxidation activity in cooked beef. The 0.02% of BHA and BHT mixture was prepared by mixing 0.01% BHA and 0.01% BHT solution in ethanol.

### Preparation of cooked beef

The beef cut into 96 pieces of 6 X 6 X 1.5 cm (~25 g) and 160 pieces of 6 X 6 X 1.5 cm (~35 g) for antimicrobial and anti-lipid oxidation activity investigation, respectively. To obtain the sterile (bacteria-free) beef, beef was sterilized by autoclave at 121°C for 20 min. For antimicrobial activity investigation, the sterile beef were then dipped into 24 mL of 7 log CFU/mL of stock cultures prepared in above section to obtain the final inoculum size of 5 log CFU/g.

### Experimental design

To access the potential of Assam green tea infusion for the shelf life of cooked beef, antimicrobial and anti-lipid oxidation test system were performed. In antimicrobial test, petri dish containing inoculated cooked beef and two of commercial green tea (A and B) with three different concentration (0, 125 and 250 mg/mL) were constructed. The petri dishes were statically incubated at 4°C and the microbial analysis was determined everyday for 7 days. BHA/BHT and control experiments using sterilized water were also conducted at the same condition.

In the case for anti-lipid oxidation activity analysis, the cooked beef was separately dipped into 100 mL of 0, 31.3, 62.5, 125 and 250 mg/mL

Assam green tea infusions and 0.02% BHA/BHT solution for 30 sec. Then, the treated cooked beef was incubated in refrigerator at 4°C cold room for 7 days. The oxidative stability of cooked beef was monitored by using color (10 g), peroxide value (PV) (20 g) and thiobarbituric acid reactive substance (TBARS) (10 g) everyday throughout the storage time.

### Microbial analysis

Microbial determination was carried out according to the method of (Ahn, 2003). Briefly, the sample (25 g) was aseptically mixed with 225 mL of 0.1% sterile peptone water. The mixture was blended for 2 min in a stomacher. Serial dilution was carried out using 0.1% sterile peptone water and 0.1 mL of the appropriate dilution was spread in duplicate onto an MOX agar and XLD agar to determine the population of *L. monocytogenes* and *S. typhimurium*, respectively. Whereas, one milliliter of an appropriate dilution was dropped onto petrifilm of *E. coli* coliform and a Staph express count plate to determine the populations of *S. aureus* and *E. coli*, respectively. All of the samples were incubated at 37°C for 24 hr.

### Anti-lipid oxidation activity

The anti-lipid oxidation activity of Assam green tea infusions in cooked beef was investigated by means of PV and TBARS, during the refrigerated storage (at 4°C) for 7 days. PV refers to the total concentration of peroxides and hydroperoxides obtained as a result of an oxidative reaction. TBARS is the determination of secondary oxidation products, such as aldehydes, ketones, hydrocarbons and alcohols after the oxidative reaction. The higher peroxide value and TBARS indicate the increase of lipid peroxides products obtained as a result of rancidity in cooked beef.

### PV determination

PV determination was carried out according to the previously method (Prasetyo et al., 2008). The cooked beef (20 g) was ground by a blender at high speed for 2 min. The ground sample was put into a 250 mL Erlenmeyer flask. Iso-Propanol (32 mL) was added to the sample and was then homogenized with a blender at high speed for 30 sec. Then, hexane (64 mL) was added and the mixture was homogenized again at high speed for another 30 sec. After that, the mixture was centrifuged at 5,000 rpm for 15 min at 4°C, and the supernatant (n-hexane phase) was collected, evaporation was carried out by rotary evaporator under reduced pressure at 30°C to remove the solvent. The extracted beef lipid was mixed with 5 mL of acetic acid-chloroform (3:2 v/v) solution

and vortexed for 2 to 4 sec. Fifty microliters of 30% (w/v) ammonium thiocyanate solution was added to the sample and vortexed for 2 to 4 sec. Then, fifty microliters of 1% (v/v) ferrous iron solution was added and vortexed for 2 to 4 sec. The sample was incubated for 5 min at room temperature. The absorbance at 470 nm were read using UV/Visible spectrophotometer (Lamda 35, Perkin Elmer Life And Analytical Sciences, Inc, USA). This entire procedure was conducted in subdued light. The standard curve of Fe (III) were freshly prepared by diluting the working stock solution (10.4 µg/mL) with different volume of chloroform-acetic acid (3:2) in order to get the concentration of 2.5, 5, 7.5, and 10 µg/mL. The working stock solution was made by dissolving Fe (III) in 1% HCl. The peroxide values were expressed as (mEq) of peroxides per kilogram of the sample, which were calculated using the following equation.

$$\text{Peroxide value (mEq of peroxides/kg)} = (A_s - A_b) / (55.84 \times m_o \times m \times 2)$$

Where  $A_s$  was absorbance of sample;  $A_b$  was absorbance of the blank;  $m$  was slope of the Fe (III) calibration;  $m_o$  was mass in gram of the sample (20 g); 55.84 was atomic weight of iron.

### TBARS determination

TBARS used to determine lipid oxidation in the sample was assessed in triplicate by Tardladgis, Watts and Younathan (1960) with minor modification. The cooked beef (10 g) was ground by a blender at high speed for 2 min. The ground sample was mixed with 50 mL of 10% trichloroacetic acid (TCA) solution for 30 sec at high speed in a blender. The mixture was filtered using a Whatman filter paper No 1. Five milliliters of the filtrate were mixed with 5 mL of 0.02 M thiobarbituric acid (TBA) solution and heated in a 95°C bath for 20 min. Then, the absorbance was read at 532 nm by UV-Visible spectrofotometer (Lamda 35, Perkin Elmer Life And Analytical Sciences, Inc, USA). The standard curve of malonaldehyde (0-5 nM/mL) was freshly prepared by acidification of TEP (1,1,3,3-tetraethoxypropane). TBARS values were calculated as mg malonaldehyde (MAD)/kg sample according to the standard curve.

### Color of the cooked beef determination

The cooked beef (10 g) was subjected to color evaluation by the  $L^*$ ,  $a^*$  and  $b^*$  (lightness, redness, and yellowness) coordinates using the ColorQuest®XT (HunterLab, Associates Inc., Reston, Virginia, USA).  $\Delta L^*$ ,  $\Delta a^*$ , and  $\Delta b^*$  value were then calculated by the color differentiation of the first day and the evaluated day color throughout the storage time.

### Phenolic compounds determination

One milliliter of tea infusion was filtered through a polytetrafluoroethylene (PTFE) filter and the filtrate was subjected to High Performance Liquid Chromatography (HPLC) to measure the concentration of phenolic compounds including catechins (catechin (C); epicatechin (EC); gallic acid (GC); catechin gallate (CG); epigallocatechin (EGC); gallic acid gallate (GCG); epicatechin gallate (ECG); epigallocatechin gallate (EGCG), gallic acids and caffeine in the tea infusion. HPLC analysis was conducted in a Agilent Technology 1100 series system equipped with a quaternary pump, a degasser, a thermostatic autosampler with a reverse phase C18 column and a photodiode array detector (DAD). An acetonitrile and 0.05% trifluoroacetic acid (130:870, V/V) were used as the mobile phase at a flow rate of 2 mL/min, and detection was at wavelength at 280 nm. The concentration of investigated phenolics were determined, which based on the standard chromatographic data.

### Statistic analysis

All data were subjected to analysis of variance (ANOVA) by SPSS software. The statistical significance of the differences between green tea infusions were accepted statistically significant when  $p < 0.05$ .

## Results

### Antimicrobial effects of tea infusion in cooked beef

The antimicrobial effects of tea infusion in cooked beef were investigated against four tested bacteria. The results are shown in Figure 1. *S. aureus* population in cooked beef treated by 250 mg/mL of A and B tea infusion were decreased to an undetectable level within the first day of storage, while less inhibition effect at the lower concentrations were observed in cooked beef indicated by the longer time required (2 days) to reduce *S. aureus* to the undetectable level (Figure 1A). Whereas, the complete reduction of *S. aureus* was obtained in cooked beef treated by the mixture of 0.02% BHA/BHT within 3 days.

Figure 1B shows the survival of *L. monocytogenes* in cooked beef containing Assam green tea infusion stored at 4°C for 7 days. It was found that *L. monocytogenes* was completely inactivated by the 250 mg/mL of the green tea A and B by 3 days, whereas 4 days was required to inactivate *L. monocytogenes* by other lower concentrations of the green tea A and B and by BHA/BHT.

The survival of *S. typhimurium* in cooked beef applied with different concentrations of Assam green

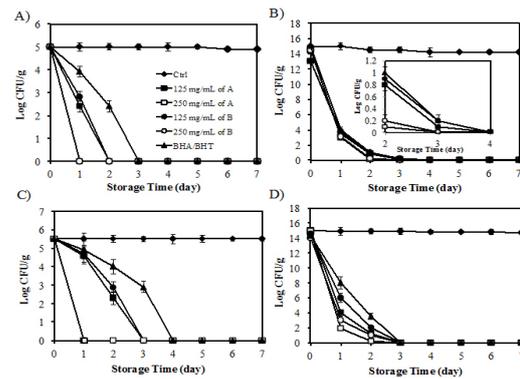


Figure 1. Population of *S. aureus* (A), *L. monocytogenes* (B), *S. typhimurium* (C) and *E. coli* (D) in the cooked beef incubated at 4°C for 7 days.

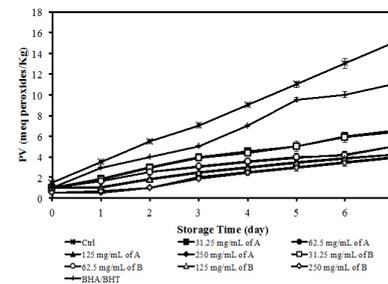


Figure 2. PV (meq peroxide/kg) in the cooked beef containing different concentrations of Assam green tea infusions and 0.02% BHA/BHT over 7 days of storage at 4°C.

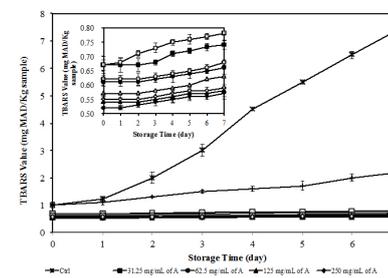


Figure 3. TBARS (mg MAD/kg) in the cooked beef containing different concentrations of Assam green tea infusions and 0.02% BHA/BHT over 7 days of storage at 4°C; (inset) enlarged TBARS of the cooked beef containing different tea concentrations.

tea infusion and stored at 4°C for 7 days is shown in Figure 1C. It was found that besides 250 mg/mL of A tea infusion, all concentrations of Assam green tea infusion and 0.02% BHA/BHT required 3 and 4 days, respectively, to inactivate the *S. typhimurium*; only 1 day was required by 250 mg/mL of A tea infusion to reduce *S. typhimurium* to an undetectable level. The less inhibitory effect was also observed in *E. coli*; the time required to completely inactivate this bacteria was 3 days (Figure 1D). Moreover, it was notable that all tested bacteria still survive throughout the storage time without additional tea infusion and 0.02% BHA/BHT, and the high concentration (mg/mL) of tea infusions were required to accelerate bacterial reduction of *S. aureus*, *L. monocytogenes*,

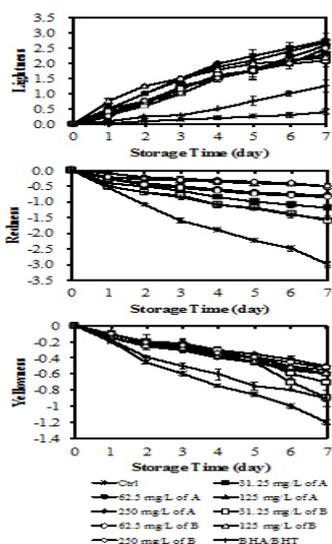


Figure 4. Lightness ( $\Delta L^*$ ), redness ( $\Delta a^*$ ) and yellowness ( $\Delta b^*$ ) value in the cooked beef containing different concentration of assam green tea infusions and 0.02% BHA/BHT over 7 days of storage at 4°C

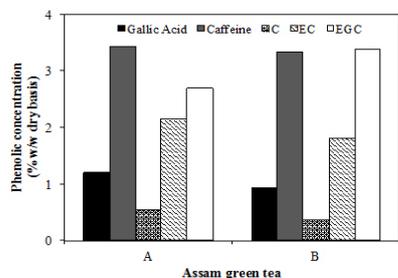


Figure 5. Phenolic compounds in Assam green tea infusion (% w/w dry basis)

*S. typhimurium* and *E. coli*.

*Anti-lipid oxidation of tea infusion in cooked beef*

The antioxidation effects of treatments, as measured by PV and TBARS values in cooked beef over 7 days of refrigerated storage are shown in Figure 2 and 3, respectively. PV and TBARS value in all samples of cooked beef increased throughout the storage time at 4°C. However, the PV and TBARS values increasing rate in cooked beef treated with the Assam green tea infusion were significantly lower than those of 0.02% BHA/BHT treated beef and the control, as indicated by the lower slope. These suggest that lipid oxidation was effectively retarded by the green tea A and B infusion than the BHA/BHT mixture. The green tea A and B at the highest concentration (250 mg/mL) exhibited the highest anti-lipid oxidation activity on the cooked beef throughout the storage time compared to the other concentrations as well as the BHA/BHT mixture. Moreover, the antioxidant activity of the Assam green tea infusion significantly increased with the tea infusion concentration ( $p \leq 0.05$ ).

*Effect of tea infusion on cooked beef color change*

The treatment effects on color changes of cooked beef during the refrigerated storage are shown in Figure 4. The addition of tea infusion significantly increased the lightness ( $\Delta L^*$ ) values of the cooked beef, whereas those of 0.02% BHA/BHT did not significantly change over the 7 days of storage. The addition of tea infusion also contributed to maintain the redness ( $\Delta a^*$ ) value of cooked beef. On day 7, the cooked beef containing assam green tea infusion in all concentrations were more red than the other treatments. Among all treatments, A at the concentration of 250 mg mL<sup>-1</sup> significantly provided the highest  $\Delta a^*$  values ( $p \leq 0.05$ ). Significant differences of yellowness ( $\Delta b^*$ ) values were observed in the control and the Assam green tea infusion treated cooked beef during the refrigerated storage. Assam green tea infusion prevented a decreased in  $\Delta b^*$  values in the cooked beef when compared to control and BHA/BHT.

*Main bioactive compounds in Assam green tea infusion*

Figure 5 shows the main bioactive compounds in Assam green tea infusion. In the figure showed that total concentration of catechins is higher than gallic acid and caffeine. Green tea A has 0.54% of C and 2.16% of EC content which higher than those of green tea B. While EGC was the only compound in green tea B observed higher than those of green tea A; about 2.69% and 3.37% of EGC were observed in green tea A and B, respectively.

**Discussion**

The shelf life of precooked beef is relatively short and generally depends on the degree of microbial cross-contamination, lipid oxidation and color changes. Some plant extracts have been recognized as antimicrobials as well as antioxidants in foods. In this study, the effect of Assam green tea on the inhibition of the growth of foodborne pathogens and the development of lipid oxidation associated with color changes were investigated in cooked beef systems. Based on the results, the addition of Assam green tea infusion and BHA/BHT inactivated *S. aureus*, *L. monocytogenes*, *S. typhimurium* and *E. coli* for 1 to 3 days. Some previous studies showed that polyphenols cause inhibition of a wide range of microorganisms. Indeed, the antimicrobial activity of polyphenols are well documented (An et al., 2004; Ho et al., 2009). Comparing the four bacteria studied, Assam green tea infusion showed relatively the best inhibitory activity against *S. aureus* followed

by *L. monocytogenes*, *S. typhimurium* and *E. coli*, maybe due to its cell wall structure and the presence of outer membrane (Zhao *et al.*, 2001). In general, gram positive bacteria are more susceptible to green tea infusion than Gram negative bacteria (An *et al.*, 2004). This can be attributed to the differences in the cell envelope composition between Gram-positive and Gram-negative bacteria, which affect permeability and susceptibility of these organisms to different compounds (Sikkema *et al.*, 1995).

The permeability of the cell wall of the Gram-negative bacteria is generally less efficient than Gram-positive probably because of the presence of the high level of phospholipids in the cell wall compared with Gram positive bacteria (Palumbo *et al.*, 1997). Gram-negative bacteria possess an outer membrane and unique periplasmic space not found in Gram-positive bacteria (Palumbo *et al.*, 1997). The resistance of Gram-negative bacteria towards antimicrobial substances is related to the hydrophilic surface of their outer membrane which is rich in lipopolysaccharide molecules, presenting a barrier to the penetration of numerous preservatives molecules and is also associated with the enzyme in periplasmic space, which are capable of breaking down the molecules introduced from outside (Gao *et al.*, 1999). These results were consistent with the results obtained by previous reports (Chou *et al.*, 1999; An *et al.*, 2004; Fernandez-Lopez *et al.*, 2005; Mitsumoto *et al.*, 2005; Tiwari *et al.*, 2005).

In addition, the susceptibility of four tested bacteria by Assam green tea infusion were higher than those of by 0.02% of BHA/BHT and control. Since the natural antimicrobial compounds more preferably applied into the foods instead of the synthetic preservatives (BHA/BHT) due to their health benefits and safety, Assam green tea infusion is some of great interest. We also found that large concentration or volume of tea infusion added could provide the greatest effect. Among all concentration, 250 mg/mL of A and B tea infusion had the highest inhibitory effect against four tested strain. Hence, the antimicrobial activities are influenced by microorganism, food component and the concentration of extract.

Assam green tea infusion contributed significantly to antioxidant activities, indicated by the reduction in the formation of PV/TBARS and color stability of cooked beef. This might be resulted from their antioxidant effects and their contribution of pigments. Besides as antimicrobial agents, polyphenolic compounds containing in green tea are primarily known as antioxidant agents that can retard the free radical chain reaction during oxidation process (Cuppet, 2001; Michalczyk and

Zawislak, 2008). Tea infusion in this study showed more concentration of catechins (C, EC and EGC) than gallic acid and caffeine (Figure 5). However, the antimicrobial and anti-lipid oxidation effect of green tea infusion were not resulted from individual catechins but a combination effect of all catechins. The ability of Assam green tea retained more redness during cooking might result in consumers avoiding undercooked beef.

Results of our analysis suggest that Assam green tea infusion might provide additional barrier to microbial growth, lipid oxidation and color degradation products. It has significant potential for use in food preservatives especially to overcome short shelf life of precooked meat that mentioned earlier. However, further studies of the adverse effects on the organoleptic properties by high concentration of green tea infusion are needed to ensure the quality of meat products.

## Conclusions

The addition of the Assam green tea infusion significantly reduced the number of *S. aureus*, *L. monocytogenes*, *S. typhimurium* and *E. coli*, retarded lipid oxidation and color degradation. These suggest that the Assam green tea infusion might be a potential natural preservative used to extend shelf life by improving the microbial safety, possibly showing the same effect to oxidative and color stability of cooked meat and other meat products.

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