EFFECTS OF ANTHOCYANINS EXTRACTED FROM *Hibiscus rosasinensis* (RED LAYERED *Hibiscus*) FLOWERS AS A ANATURAL COLOURING AGENT ON PRE-COOKED CHICKEN SAUSAGES

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Abstract

Hibiscus rosasinensis flowers contain anthocyanins such as cyanidin and delphinidin that gives it characteristic deep red color. This study was conducted to evaluate the effectiveness of Hibiscus rosasinensis flower extract as a natural colorant and its effects on pre-cooked chicken sausages. Anthocyanins from H. rosasinensis flowers were extracted using ethanol and freeze-dried. Pre-cooked sausages were prepared by incorporating different levels of anthocyanins (0.05%, 0.10%, 0.15%, 0.20% and 0.25% w/w) and, 60 ppm $NaNO_2$ incorporated sausages were considered as the control. Sensory evaluation was carried out to identify the most appealing sausage compared to the control. The color, pH value, water holding capacity (WHC), 2-thiobarbituric acid-reactive substances (TBARS) values and total viable plate counts of sausages were evaluated during three weeks of storage at -18℃. The highest consumer overall acceptability was shown by the sausages incorporated with the 0.05% anthocyanin + 0.10% citric acid. Sensory evaluation results revealed that, the median scores given by panelists for odor, taste and texture were the same in all the treatments. Instrumental "a" and "b" color were significantly ($P<0.05$) lowered with the addition of anthocyanins. Whereas, increasing levels of anthocyanins in sausages have shown significantly high effects on antimicrobial properties. The optimum level of anthocyanin to be incorporated to the pre-cooked chicken sausages is 0.05% with 0.10% citric acid. Incorporation of Hibiscus rosasinensis anthocyanins has a considerable effect on antimicrobial properties of precooked chicken sausages.

***Keywords**: Anthocyanin, Hibiscus rosasinensis, Chicken sausages, $NaNO_2$*

INTRODUCTION

Colour is one of the most important quality attributes affecting the consumer's acceptance of food since it gives the first impression of food quality (Azza *et al*., 2011). People associate certain colors with certain flavors and the color of food can influence its perceived flavor (Delwiche, 2004). In fact, the colour of a food influences not only the perception of flavor, but also attraction, quality and subsequently, consumption. Therefore, the colourings are used in the food industry to imitate or improve a colour that is accepted by the consumer as natural, to concealment of natural variations in food colours, to balance colour loss due to light, inappropriate temperatures and dry storage conditions. In addition, food colourants provide identity to foods (Henry, 1996; Abd-El-Galeel, 2002). Food colourants are either natural or synthetic depending on the source. Natural colourants are extracted from renewable sources such as plant materials, insects, algae, etc. while the synthetic colorants are manufactured chemically and are the most commonly used dyes in the food, pharmaceutical and cosmetic industries (Aberoumand, 2011). Sausages are manufactured of two basic components; meat and nonmeat sources. The nonmeat ingredients are colors, binders, spices, preservatives, flavor enhancers etc., (Romans *et al*., 1994). Nitrates and nitrites play important roles in meat products by fixing the red color of meat products, developing unique flavor, and acting as antimicrobial and antioxidant substances

(Potter and Hotchkiss, 1995). The presence of nitrates and nitrites in food is associated with potential health risks (Pearson and Tauber, 1984; Romans *et al.*, 1994). The nitrates are not poisonous whereas nitrites are poisonous in large doses. Illness in adults due to nitrite has occasionally been reported, due to mistaken ingestion of large quantities of sodium nitrite (Lijinsky, 1976). Further the potential carcinogenic effect of nitrosamines formed has demanded to reduce nitrite for curing of meat in order to reduce the risk of nitrosamine formation in processed meat product and thereby the potential health risks (Sindelar and Milkowski, 2011).

As per the recommendation of Codex Committee on Food Additives and Contaminants (CCFAC), and international research findings, ingestion of dye is regulated under the acceptable daily intake (ADI) (Bessonov, 2011). In addition, "Sunset yellow" (110) and "Canthaxanthin" are used in sausage production as food colourants. "Sunset Yellow" is a synthetic colourant and derived from petroleum and has been linked to food allergies (aspirin allergies), and cancers (Rovina *et al*., 2016). Use of Sunset yellow is banned in several European countries and but commonly use in the United Kingdom (http://www.ukfoodguide.net/e110.htm). Due to above mentioned limitations and worldwide tendency towards the consumption of natural products, the interests on natural colourants has increased significantly (Huck and Wilkes**,** 1996). Furthermore, limited availability of red pigments has resulted special interest to the food industry (Lauro and Francis, 2000), therefore research into natural sources of red pigments have increased recently.

Anthocyanins are a group of natural colourant, which is widely present in the plant sources. *Hibiscus rosasinensis* flowers contain anthocyanins such as cyanidin and delphinidin that gives it characteristic deep red colour. Anthocyanins have antioxidant activities and play a vital role in prevention of neuronal and cardiovascular diseases, cancers and diabetes (Yazhen *et al.*, 2019). Furthermore, it has been shown that extracts of *Hibiscus rosasinensis* flowers also lower cholesterol content in blood serum and helps to prevent oxidation of low-density lipoprotein (LDL) and lowers blood pressure (Vankan and Shukla, 2011). This tendency may be due to phenolic structures of anthocyanins which show antioxidant activity towards a variety of easily oxidizable substances and might be part of anthocyanin defense mechanisms against free radical mediated damage (Onyesom *et al*., 2008). Anthocyanins are water-soluble glycoside of anthocyanidins. Anthocyanins are part of the C15 phenolics known collectively as flavonoids with the typical A-ring benzoyl and B-ring hydroxycinnamoyl system (Onyesom *et al*., 2008). Therefore, use of anthocyanins extracted from *Hibiscus rosasinensis* flowers in chicken sausages as a natural colourant would be as safe solution to improve sensory attributes and physico-chemical and other functional properties of sausages. The objectives of the present study were to evaluate the effects of anthocyanin extracted from *Hibiscus rosasinensis* (Red layered hibiscus) flowers on sensory, physico-chemical and microbiological properties of pre-cooked chicken sausages.

MATERIALS AND METHOD

Materials and chemicals

H. rosasinensis flowers were collected and kept in cold (4 °C) in polyethylene bags until the experiment started. Ingredients listed in the table 1 and artificial cellulose casings were used in the sausage production. Standard plate count agar and peptone water were purchased from OXOID Ltd., Basingstoke, Hampshire, England. The experiments were carried out at Cargills Quality Foods (Pvt) Ltd., Ja-ela, Meat Science Laboratory, Department of Animal Science, Faculty of Agriculture and Research Laboratory, Faculty of Science, University of Peradeniya.

Preparation of freeze-dried powdered Hibiscus extracts

Extraction of anthocyanins from *H. rosasinensis* flowers was carried out using the method as described by Vankan and Shukla, (2011) with slight modifications. One hundred grams (100 g) of fresh *H. rosasinensis* flower petals were weighed and cut into small pieces and thoroughly mixed with 200 mL of 95% (w/v) ethanol and kept for 3 hours at room temperature with stirring. The mixture was filtered using Whatman No 1 filter papers. The residue on the filter paper was re-extracted for three times with 100 mL of 95% (w/v) ethanol each time till the filtrate became almost colourless. Above-mentioned procedure was carried out six times to extract anthocyanin from fresh *Hibiscus* flowers. The combined filtrates were then concentrated using Rotovapor at 55 °C. Filtrates were kept in -18 °C with an angle to increase the surface area to freeze. Frozen samples were freeze-dried under 133 MBAR pressure and (-42) °C for 2 days and powdered *Hibiscus* extract was obtained.

Determination of moisture content and Hibiscus extract yield

Moisture content of fresh flowers was analyzed according to the AOAC, 1995. Weight of the powdered extract was taken after freeze-drying the filtrate and, extract yield was determined.

Preparation of the control chicken sausage sample

Meat chunks were ground through a 6.35 mm plate using to form uniform and small size particles. Mincing was done by using the meat mincer. The required amount of meat and all other ingredients (Table 1) were weighed according to the industry's formulation and the fat emulsion was prepared with the ingredients given in the table 1, and thoroughly mixed for 10 minutes until a fine fat emulsion formed. Then, all the other ingredients and the fat emulsion were mixed with meat in the bowl chopper. Ice flakes were added gradually to reduce heat generation and maintain the meat temperature around 0 °C. Chopping and mixing of ingredients were carried out for 10 minutes to have a uniform distribution of all ingredients in minced meat and to form a fine emulsion.

Then, the meat batter was transferred to the hand stuffer machine and stuffed manually into artificial cellulose casings (17 mm diameter). This was followed by linking, in which the batter filled casings were tied manually by twin threads, separating 9 cm long, discrete units of sausages. Cooking and smoking were carried out by using smoking chamber for 1.5 hours under 72 °C. After dipping the products in running water (25 °C) for 15 minutes the internal temperature was made to be lowered up to 30 °C. Then, the casings were removed manually and the sausages were vacuum-packaged in polyethylene+Nylon bags using multivac machine. Finally, the packaged sausages were stored in the freezer at a temperature of -18 °C until they were analyzed.

Preliminary trials to select the range of anthocyanins and citric acid for sausage formulation

A series of preliminary trials were conducted to select the most suitable range of extracted anthocyanins amounts and citric acid (INS 330) that could be incorporated to the chicken sausage. The range was selected as (0.05 to 0.25% for anthocyanin and citric acid was 0.1%).

Preparation of extracted anthocyanin incorporated chicken sausages and sodium nitrite incorporated sausage

This experiment was carried out to select the best consumer preferable amount of anthocyanins and citric acid to be incorporated. Five levels of anthocyanins (0.05, 0.1, 0.15, 0.2 and 0.25%) incorporated sausages were produced using the same sausage production procedure and conditions as given above, and 60 ppm of $NaNO_2$ incorporated sausage was produced as the control (Table 2). Extracted

anthocyanin amount used for the each treatment and citric acid (INS 330) were dissolved in 5 mL of chilled water prior adding to the sausage batter. During manufacturing of sausages, ice flakes quantity was replaced by extracted anthocyanin amount and sodium nitrite. So that ice flakes and anthocyanin extract+citric acid/nitrite accounted for 17.06% of the total mixture. The cooked sausages were vacuum-packed in poly-ethylene+Nylon bags and stored in freezer at -18° C.

Evaluation of consumer acceptance

Sausages were fried for two minutes, cooled down to room temperature and served with tap water and unsalted cracker between samples to cleanse the palate. Three digit treatment level specific codes were applied to each testing sample and the sample arrangement order was randomized for each panelist. Thirty trained panelists evaluated colour, juiciness, texture, taste and overall acceptability of the products using five-point hedonic scale (1= dislike extremely, 2= dislike moderately, 3= neither like nor dislike, 4= like moderately, 5= like extremely).

Table 1: Ingredients used in the production of the control batch of emulsified chicken sausages.

Ingredients used for sausage	Ingredients used for the fat emulsion
Boneless chicken meat	Soy protein isolates
Fat emulsion	Vegetable oil
Ice flakes	Sodium chloride
Isolated soy protein	Chilled water
Wheat starch	
Table Salt	
Sugar	
Milk powder	
Spice mixture	
Phosphate	
Citric acid	
Sodium nitrite	

Table 2: Anthocyanins from *H. rosasinensis* flowers incorporated sausages (ingredients used as a % of the total formulation)

Treatment	Extracted anthocyanin	Citric acid	Sodium nitrite	Chilled water	Ice-flakes
Control	0.00	0.00	0.06	5.00	12.00
Treatment 1	0.05	0.10	0.00	5.00	11.91
Treatment 2	0.10	0.10	0.00	5.00	11.86
Treatment 3	0.15	0.10	0.00	5.00	11.81
Treatment 4	0.20	0.10	0.00	5.00	11.76
Treatment 5	0.25	0.10	0.00	5.00	11.71

Evaluation of keeping quality parameters

Determination of pH, water holding capacity (WHC), and the colour

The pH, WHC of the samples were measured according to the AOAC protocol (AOAC, 1995). Colour of the samples was measured by using the Chroma Colour Meter.

Determination of 2-thiobarbituric acid-reactive substances (TBARS)

Two grams of sample was placed in a centrifuge tube to which 5 mL of a 10% (w/v) solution of trichloroacetic acid (TCA) were added and vortexed at high speed for two minutes. Five milliliters of 0.02 M aqueous solution of 2-thiobarbituric acid was then added to each centrifuge tube which was further vortexed for 30 seconds. Then, the samples were centrifuged at 3000 *g* for 10 minutes and the supernatants filtered through a Whatman No.3 filter paper. Filtrates were heated in a boiling water bath for 45 minutes, cooled to room temperature in ice, and the absorbance of the resulting pigment read at 532 nm using a spectrophotometer. TBARS values were calculated by multiplying the absorbance reading by a factor of 7.0, which was obtained from a standard curve prepared 1,1,3,3-tetramethoxy methane as a pre-cursor of malonaldehyde. TBA values were expressed as mg malonaldehyde kg^{-1} (7.0 × Absorbance). Inhibition of TBARS formation was determined using the following equation (Siu and Drapper, 1978).

$$\% \text{ Inhibition} = \frac{100 - 100 \text{ (TBARS value for the treated sample) x \%}}{\text{TBARS value for control sample}}$$

Determination of total viable plate count

Total viable plate count was carried out to determine the number of viable cells according to the SLSI 516 Part 1 Section 2. Number of colony forming units per gram of the product was calculated using the following formulae.

$$N = \frac{\Sigma C}{(N_1 + 0.1\, N_2)\, d}$$

N= Number of colony forming units, C = Sum of colonies counted in all the dishes retained, N_1 = Number of dishes retained in the 1st dilution, N_2 = Number of dishes retained in the 2nd dilution, d = Dilution factor corresponding to the 1st dilution.

Statistical analysis

The objective measurements were taken with three replicates. The experimental design was Complete Randomized Design (CRD). The sensory data were analyzed using the non-parametric procedure, according to the Friedman test. Data were analyzed SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) software programme. Means were compared using the Duncan's Multiple Range ($P< 0.05$) Test (DMRT).

RESULTS AND DISCUSSION

Preparation of freeze-dried powdered Hibiscus extracts

Anthocyanins are one of the most abundant natural pigments available in plants. Anthocyanins are the vacuolar dye found in almost every part of higher plants and water-soluble strong colours and have been used to colour food since historical times (Vankar and Shukla, 2011). The dry matter percentage of the *Hibiscus rosasinensis* flowers which were used as the anthocyanins source was 15.6%. In preparing anthocyanin rich phenolic extracts from plant materials, an acidified organic

solvent, most commonly methanol or ethanol is used. Ethanol and methanol denature the cell membranes, simultaneously dissolves the anthocyanins, and stabilizes them (Gonzalo, 2003). In this experiment ethanol was used for anthocyanins extraction as ethanol is safe for human consumption. The extracted pigment yield was 1.623 g/100 g of fresh petals. Candrasekhar *et al*., (2012) found that 50% (v/v) ethanol and acidified water resulted maximum anthocyanin extraction from red cabbage (390.6 mg/L).

Effect of incorporation of anthocyanin on sensory evaluation

Sensory evaluation was carried out with the intention of identifying the most appealing sausage sample among anthocyanin incorporated sausages and control sausages (Table 3). The highest median score for colour was observed with the Treatment 1. Therefore, 0.05% anthocyanin extract could be the most suitable anthocyanin amount responsible for the consumer's acceptance for the colour.

Consumer preference for colour has been decreased with the increasing amount of anthocyanin incorporation to the sausages. It might be due to increasing the dark colour of surface of the sausages when increasing the level of anthocyanin extract in sausages. Meanwhile, the median scores given by panelists for juiciness, taste and texture were same in all the treatments. The highest consumer overall acceptability was shown for the treatment 1 and it was not significantly different from the control sample.

Table 3: Effect of different anthocyanin incorporation levels in sausages on different sensory attributes

Treatment	Colour	Odor	Taste	Texture	Overall acceptability
T1	4.20^{a} (±0.68)	3.73^{a} (±0.77)	3.46^{a} (±0.87)	3.56^{a} (±0.98)	4.16^{a} (±0.74)
T2	3.50^{b} (±1.06)	3.43^{a} (±0.88)	3.50^{a} (±0.98)	3.70^{a} (±0.88)	3.33^{b} (±0.74)
T3	2.63^{c} (±0.74)	3.36^{a} (±0.64)	3.83^{a} (±0.74)	3.67^{a} (±0.74)	3.16^{b} (±0.65)
T4	2.76^{c} (±0.78)	3.43^{a} (±0.58)	3.43^{a} (±0.86)	3.67^{a} (±0.64)	2.63^{c} (±0.74)
T5	1.76^{d} (±0.88)	3.50^{a} (±0.64)	3.83^{a} (±0.54)	3.56^{a} (±0.88)	1.93^{d} (±0.67)
Control	4.06^{a} (±0.76)	3.30^{a} (±0.88)	3.36^{a} (±0.64)	3.46^{a} (±0.67)	4.06^{a} (±0.88)

Different letters within each column indicate significant differences at $P<0.05$. Parenthesis indicate standard deviation, Values indicate mean value of 3 replicates.

T1= 0.05% anthocyanin + 0.1% citric acid incorporated sausages
T2= 0.10% anthocyanin + 0.1% citric acid incorporated sausages
T3= 0.15% anthocyanin + 0.1% citric acid incorporated sausages
T4= 0.20% anthocyanin + 0.1% citric acid incorporated sausages
T5= 0.25% anthocyanin + 0.1% citric acid incorporated sausages
Control= $NaNO_2$ (60 ppm) incorporated sausages.

Analysis of keeping quality characteristics

Effect of storage period on colour values ("L", "a" and "b" value) of the sausages

Results showed that with the increasing level of anthocyanin incorporation to the sausages internal and external "L" values of the sausages have been increased (Table 4) and internal and external "a" and "b" values have been decreased. Hence, "L" values and "a" values could have a negative relationship. Carballo *et al*. (1994) also reported with decreasing the lightness value ("L" value) while increasing the redness value ("a" value). Irrespective of the treatment effect, the "L" values of all sausages have been increased in all the treatments during the storage period at -18 °C. Initially, the surface "L" value of T-5 sausages was significantly higher than all the other anthocyanin incorporated sausages. Surface "L" values of the sausages were higher than the internal "L" values of all the treatments.

Table 4: Mean L, a, b values of sausages incorporated with different level of anthocyanin extracts from *H. rosasinensis* and $NaNO_2$ incorporated sausages.

	Mean value for external colour of sausages			Mean value for internal colour of sausages		
Treatment	**Week 1**	**Week 2**	**Week 3**	**Week 1**	**Week 2**	**Week 3**
"L" value						
T1	26.7^{a} (±0.07)	27.9^{b} (±0.34)	30.5^{a} (±1.51)	23.4^{b} (±0.04)	24.2^{b} (±0.08)	24.9^{a} (±0.46)
T2	31.6^{b} (±0.1)	32.5^{c} (±0.47)	34.1^{c} (±0.91)	25.4^{b} (±0.37)	26.1^{c} (±0.33)	26.6^{b} (±1.23)
T3	35.1^{c} (±0.42)	35.5^{d} (±0.20)	36.1^{d} (±1.07)	27.7^{d} (±0.04)	28.2^{d} (±0.30)	29.4^{c} (±1.18)
T4	38.1^{d} (±1.94)	38.3^{e} (±0.23)	39.3^{e} (±0.11)	28.4^{e} (±0.14)	29.0^{e} (±0.12)	30.4^{c} (±0.04)
T5	39.1^{d} (±0.78)	39.1^{f} (±0.26)	39.5^{e} (±0.53)	31.2^{f} (±0.43)	31.2^{f} (±0.21)	32.7^{d} (±0.52)
Control	27.1^{a} (±0.07)	27.1^{a} (±0.33)	27.8^{a} (±1.48)	19.8^{a} (±0.04)	22.0^{a} (±0.15)	23.6^{a} (±0.65)
"a" value						
T1	6.0^{b} (±0.00)	5.9^{b} (±0.02)	5.7^{b} (±0.9)	7.4^{b} (±0.04)	7.6^{b} (±0.21)	7.8^{ab} (±0.03)
T2	6.1^{b} (±0.05)	5.6^{b} (±0.61)	5.4^{b} (±0.07)	6.8^{c} (±0.05)	7.1^{c} (±0.13)	7.2^{c} (±0.44)
T3	6.1^{b} (±0.07)	4.0^{c} (±0.09)	3.3^{c} (±0.19)	6.6^{c} (±0.12)	6.8^{d} (±0.12)	7.1^{c} (±0.15)
T4	6.0^{b} (±0.14)	3.3^{d} (±0.07)	3.2^{c} (±0.26)	6.3^{d} (±0.34)	6.7^{de} (±0.12)	7.6^{bc} (±0.19)
T5	5.3^{c} (±0.27)	3.8^{cd} (±0.33)	3.1^{c} (±0.12)	6.0^{e} (±0.06)	6.5^{e} (±0.14)	6.6^{d} (±0.32)
Control	9.8^{a} (±0.19)	7.6^{a} (±0.23)	7.1^{a} (±0.25)	7.8^{a} (±0.10)	8.0^{a} (±0.12)	8.2^{a} (±0.44)
"b" value						
T1	8.3^{b} (±0.05)	8.2^{b} (±0.02)	8.1^{b} (±0.83)	8.3^{b} (±0.16)	8.1^{b} (±0.02)	7.9^{b} (±0.41)
T2	7.2^{c} (±0.06)	6.5^{c} (±0.09)	6.1^{b} (±0.98)	6.4^{c} (±0.30)	5.8^{c} (±0.07)	5.4^{c} (±0.64)
T3	4.6^{d} (±0.04)	4.4^{d} (±0.07)	4.1^{c} (±0.62)	4.5^{d} (±0.00)	4.3^{d} (±0.04)	4.2^{d} (±0.54)
T4	2.8^{e} (±0.24)	2.7^{e} (±0.02)	2.5^{cd} (±0.6)	3.2^{e} (±0.05)	3.1^{e} (±0.01)	2.9^{e} (±0.76)
T5	1.2^{f} (±0.02)	0.5^{f} (±0.08)	0.3^{d} (±0.00)	0.4^{f} (±0.02)	0.3^{f} (±0.04)	0.2^{f} (±0.10)
Control	15.6^{a} (±0.03)	13.4^{a} (±0.25)	11.9^{a} (±2.14)	10.6^{a} (±0.06)	10.4^{a} (±0.66)	10.3^{a} (±0.19)

Different letters within each column indicate significant differences at $P<0.05$. Values indicate mean value of 3 replicates with standard deviation in parenthesis.

T1= 0.05% anthocyanin + 0.1% citric acid incorporated sausages
T2= 0.10% anthocyanin + 0.1% citric acid incorporated sausages
T3= 0.15% anthocyanin + 0.1% citric acid incorporated sausages
T4= 0.20% anthocyanin + 0.1% citric acid incorporated sausages
T5= 0.25% anthocyanin + 0.1% citric acid incorporated sausages
Control= $NaNO_2$ (60 ppm) incorporated sausages.

Perhaps, this may be due to accumulation of colour compounds and phenolic compounds of the extract on the surface of the product. The temperature of thermal processing of food has an effect on stability of anthocynins (Patras *et al*., 2010). However, knowledge on effect of food smoking on anthocynins is limited.

In the current study, citric acid was used with anthocyanin extract to give a desirable colour to the sausages. Vankar and Shukla (2011) reported that *Hibiscus* anthocyanin is redder and more intense in colour at low (acid) pH and bluer and less intense in colour at a higher pH. Pearson and Gillet (1996) reported that the use of citric acid spray gives a better surface appearance and helps to develop surface colour. The citric acid reduces surface pH of the sausages either coagulates the proteins at the surface (Pearson and Gillet, 1996).

As shown in the table 4, "a" values of the control sausage samples were higher ($P<0.05$) than that of all the anthocyanins incorporated sausage samples. Therefore, redness was significantly high in $NaNO_2$ incorporated sausages. In cured meat products, the nitrate compounds in sausage mixture reduce to nitrite in colour development process to form nitrosohemocrome, which gives the characteristic redness to cured sausages (Pearson and Tauber, 1984; Girard, 1992).
In terms of redness, surface "a" value of sausages for all the treatments decreased significantly during the storage time. This could be due to the photo-oxidation of the pigments, the redness of the sausage must have decreased. MacDougall (1982) suggested that the frozen meats are subjected to major colour problem during the storage due to photo-oxidation of the pigment. The rate of fading the pigment is influenced by the illumination level, storage temperature, packing method and muscle type. However, in the current study, internal "a" values of all the treatments were increased ($P<0.05$) during the storage period at -18 °C. The type of anthocyanin pigment, co-pigments, light, temperature, pH, metal ions, enzymes, oxygen, and antioxidants affect its stability (Khoo *et al*., 2017).

Perhaps, due to pH reduction, colour of the anthocyanin was shifted to red colour range from the colour purple range during the storage. Pork sausages containing 2% of anthocyanin rich coloured potato flakes did not affect the L^* and a^* value (CIE) of cooked sausages (Jayawardena *et al*., 2012).

According to the results $NaNO_2$ incorporated sausage samples were more yellowish than the anthocyanin incorporated sausages. Moreover, with the storage period, "b" value was decreased in all the samples. Though, value has been changed with the storage time the variation was not significant.

pH values of sausages incorporated with different level of anthocyanin extracts from H. rosasinensis and $NaNO_2$ incorporated sausages.

The addition of citric acid has contributed to reduce the pH values of anthocyanin incorporated sausages. Results shown that (Table 5), pH values of sausages in all the treatments were reduced during the storage period at -18 °C. During the storage period, pH values of anthocyanins incorporated sausages were similar and showed higher acidity than control sausages. The pH of control sample was significantly higher than the treatments. The pH could be altered by certain technological processes and by incorporation of non-meat ingredients (Claus *et al*., 1990; Claus and Hunt, 1991). Below pH 2, anthocynins become strongly red or orange due to the positive charges of the eight conjugate double bonds. When pH is high it becomes colourless and in alkaline conditions their colour is blue (Horbowicz *et al*., 2008).

Table 5: pH values of sausages incorporated with different level of anthocyanin extracts from *H. rosasinensis* and 60 ppm $NaNO_2$ incorporated sausages.

	pH values		
Treatment	Week 1	Week 2	Week 3
T1	6.31^a (±0.00)	6.28^a (±0.00)	6.24^a (±0.01)
T2	6.30^a (±0.01)	6.27^a (±0.01)	6.23^a (±0.00)
T3	6.31^a (±0.00)	6.28^a (±0.00)	6.24^a (±0.00)
T4	6.30^a (±0.00)	6.27^a (±0.00)	6.23^a (±0.00)
T5	6.30^a (±0.01)	6.27^a (±0.00)	6.23^a (±0.00)
Control	6.60^b (±0.01)	6.56^b (±0.00)	6.50^b (±0.01)

Different letters within each column indicate significant differences at P<0.05. Values indicate mean value of 3 replicates with standard deviation in parenthesis.

T1= 0.05% anthocyanin + 0.1% citric acid incorporated sausages
T2= 0.10% anthocyanin + 0.1% citric acid incorporated sausages
T3= 0.15% anthocyanin incorporated sausages + 0.1% citric acid
T4= 0.20% anthocyanin + 0.1% citric acid incorporated sausages
T5= 0.25% anthocyanin + 0.1% citric acid incorporated sausages
Control= $NaNO_2$ (60 ppm) incorporated sausages.

Thiobarbituric acid-reactive substances (TBARS) values of sausages incorporated with different level of anthocyanin extracts from H. rosasinensis and $NaNO_2$ incorporated sausages.

One of the oldest and most frequently used tests for assessing lipid oxidation in foods and other biological systems is the 2-thiobarbituric acid (TBA) test (Shahidi and Wanasundara, 1998).

The TBARS values increased (P<0.05) in all the treatments with storage period (Figure 1). Formation of malonaldehyde is occurred with oxidation of lipids during the storage of the sausages.

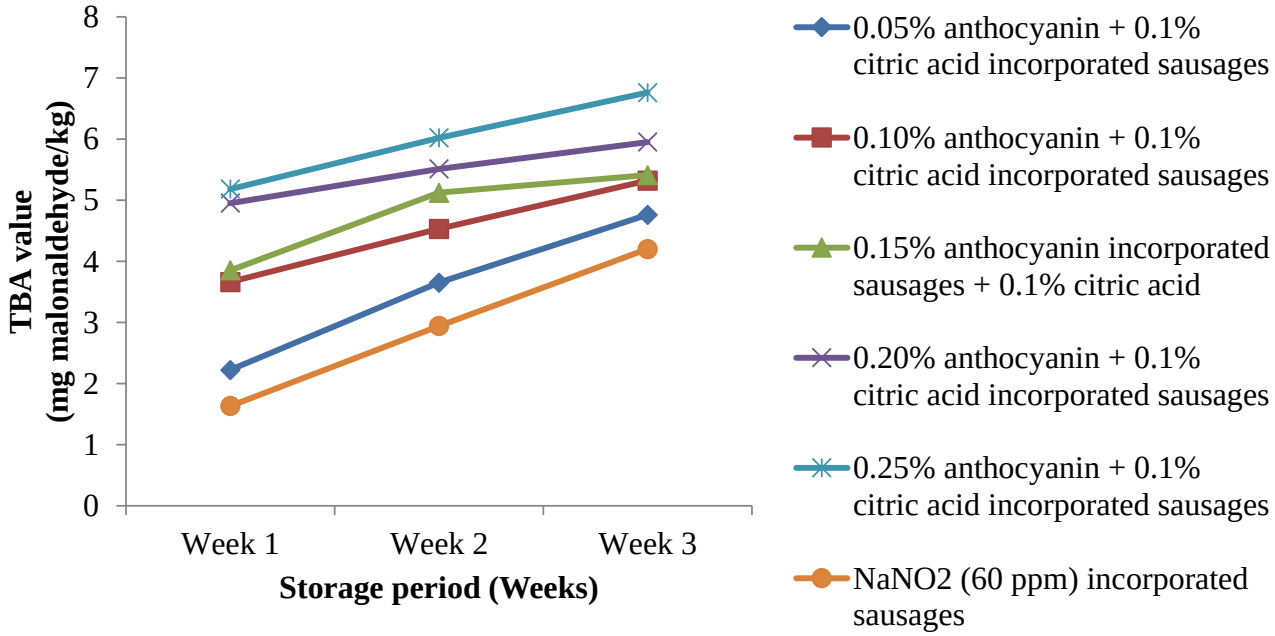

Figure 1: TBARS values of sausages during the storage period at -18 °C

Meanwhile, with addition of anthocyanin, TBARS vales have been increased and the highest TBARS values were observed in 0.25% anthocyanin incorporated sausage (T5) samples. Hence, increasing the amount of anthocyanin incorporation has increased the TBARS values. Use of the TBARS test in food and biological samples has shown some limitations on the evaluating their oxidative state, due to their chemical complexity (Shahidi and Wanasundara, 1998). Muchuweti *et al*., (2008) reported that anthocyanins extraction was carried out within the range of 525 to 527 nm wave length. Whereas, the TBARS values are also measured at the same wavelength range (532 nm). Hence it is clear that the presence of anthocyanins has interfered with the TBARS values and TBARS is not a suitable method to evaluate the antioxidant capacity of anthocyanins incorporated products. Further, Dugan (1995) has reported that sucrose and some other compounds can react with the TBA reagent to give a red colour that interferes with the TBA test. In acidic media anthocyanin also gives red colour and it might be the reason for above observations in this study.

Water holding capacity (WHC)

The WHC of meat and meat products is one of the most important factors affecting economic values and meat quality. WHC affects the weight changes during transport, texture and juiciness of the meat and meat products (Lawrie *et al*., 1991).

Figure 2 shows that, WHC for T1, T2 and T3 samples were significantly lower ($P<0.05$) than control sample. In addition, WHC has been decreased in all the treatments during the storage period at -18 °C (Figure 2). For successful sausage production it is important proteins to hold fat and water effectively (Puolanne, 2010). Zhang *et al*., (2014) has observed reduction of WHC with that with increasing contents of acetate cassava starch in pork sausages. The reduction of pH results the proteins to reduce their WHC, resulting in a drying effect (Huff-Lonergan, and Lonergan, 2005). However, with increasing amounts of anthocyanins and fixed amount of citric acid, WHC has shown an increasing trend.

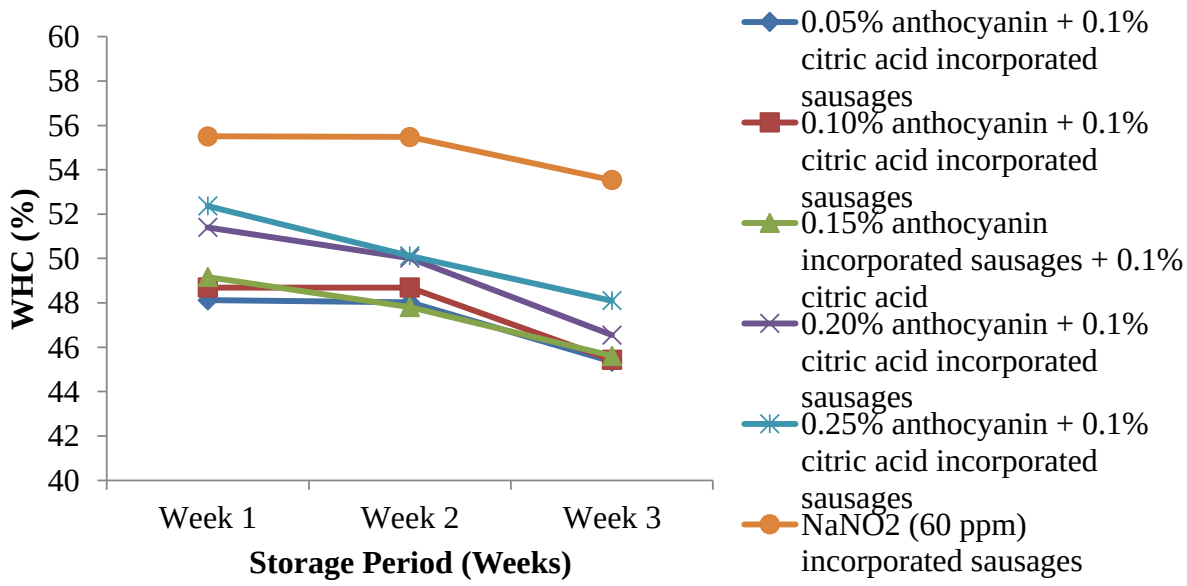

Figure 2: Water holding capacity of sausages during the storage period at -18 °C

Total viable plate counts

Total Viable Plate Count (TVPC) provides an indication of total population of microbes in products (Forrest *et al*., 1975). Results of the TVPC detection for six treatments after 1st week, 2nd week and 3rd week storage at –18 °C are shown in Figure 3.

A continuous increase of TVPC values was observed in all the sausage samples with storage period. The lowest microbial count was observed in 0.25% + 0.1% citric acid incorporated sausages. This could be due to the effect of on antimicrobial properties from citric acid and *Hibiscus* anthocyanins. It was clearly observed that, with the increasing level of anthocyanin extract incorporated to the sausages microbial count was decreased significantly ($P<0.05$). Cell culture studies, animal models, and human clinical trials have shown that anthocyanidins and anthocyanins possess antioxidative and antimicrobial activities (Khoo *et al.*, 2017). Anthocyanin-containing extracts exert these antimicrobial activities by several mechanisms and synergistic effects of many compounds such as phytochemicals including anthocyanins, weak organic acids, phenolic acids, and their mixtures etc. (Cisowska *et al*., 2011). All the anthocyanin incorporated sausage samples showed lower microbial count than 60 ppm $NaNO_2$ incorporated sausages. $NaNO_2$ is used in meat products as antimicrobial substances (Potter and Hotchkiss, 1995). However, is this experiment comparatively low level of (60 ppm) NaNO2 has been used.

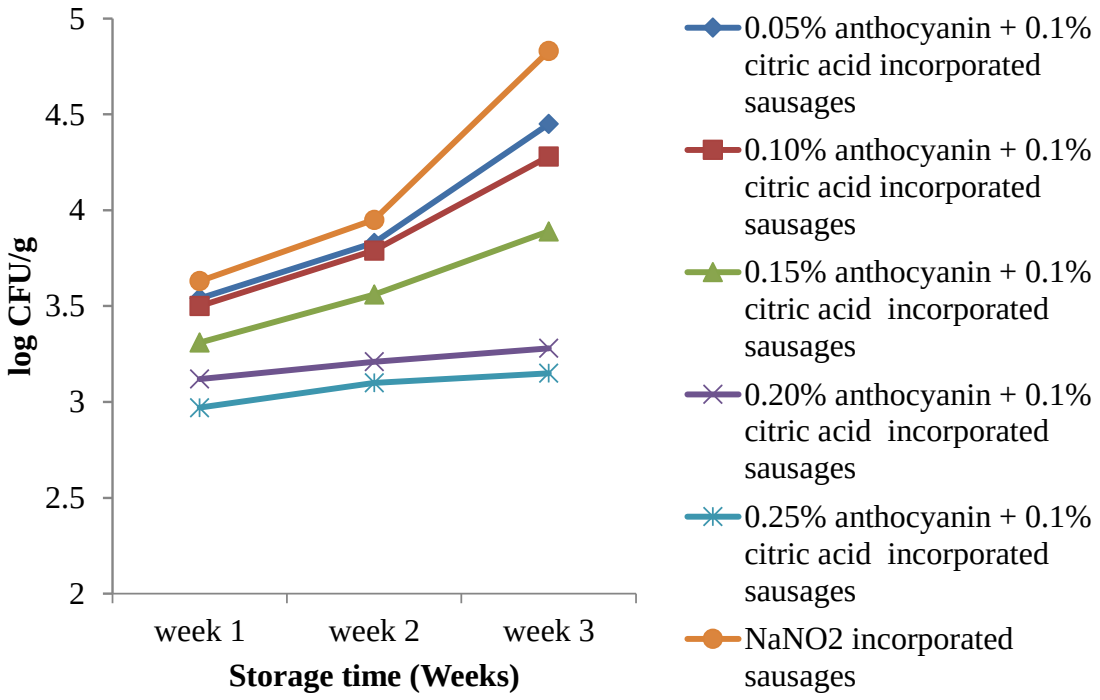

Figure 3: Effect of different treatments on Total Viable Plate Count (TVPC) change during 3 weeks of storage at -18 °C

CONCLUSION

The optimum level of anthocyanin extract to be incorporated into the sausage mixture without affecting its sensory attributes, physiochemical and keeping quality properties is 0.05%. With the incorporation of anthocyanins the instrumental "a" and "b" values of pre-cooked sausages are reduced while increasing the L value. Further incorporation of *Hibiscus rosasinensis* extract has a significant effect on antimicrobial properties of pre-cooked chicken sausages. However, long term storage evaluation of the anthocyanin incorporated pre-cooked sausages is recommended to carry out as the shelf life of precooked sausages is comparatively long.

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