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Canarium ovatum Engl. (*Pili*) exocarp crude extract as functional food colorant incorporated in yogurt developed product

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Abstract

Canarium ovatum Engl. (*Pili*), a locally grown plant in the Philippines, bears highly pigmented fruits. In this research study, the deeply pigmented fruit exocarp was evaluated for phytochemical contents, functional properties and possible application to yogurt as a colorant. Spectrophotometric analysis of the extract revealed high phenolic and flavonoid content, particularly anthocyanins (17.5 mg CE/g DW of the sample). The pigment extract also exhibited potential antioxidant activities as determined by DPPH and FRAP assays and did not show any inhibitory activity against gut normal flora, *Escherichia coli*, but also failed to express cytotoxic activity against HCT116 colon cancer cell line. Stability tests showed decreased redness with increasing temperature or pH. The pigment exhibited excellent color retention in yogurt during the two-week storage at 4°C. Sensory evaluation showed a slight difference in over-all acceptability between natural and synthetic-colored yogurt. Thus, *Pili* exocarp extract can be used as a functional food colorant in yogurt.

1. Introduction

Food colorants are added to food to make it more appealing and appetizing. Majority of these colorants are synthetically prepared to pose increased concern on safety. Consequently, consumers are turning to naturally-derived colorants as alternatives. Interestingly, a vast source of colorants may also be derived from natural sources such as plants, animals, and even microorganisms. These sources are considered safe since they are part of the normal human diet. Among the popularly used plant sources are the brightly colored fruits and vegetables, e.g. grapes, blueberries, purple cabbage, red beet, and purple corn (Lakshmi, 2014). Anthocyanins, the chemical responsible for these pigments, have also been tapped as natural colorants for beverages, jellies, candies, and dairy products (Giusti and Wrolstad, 2003). In addition to the color they impart, anthocyanins were also reported to possess antioxidant activity (Wang *et al.*,1997; Kähkönen and Heinonen, 2003; Zheng and Wang, 2003), anti-inflammatory (*Li et al.*, 2014), anti-tumor and chemoprotective effects (Kamei *et al.*, 1995; Koide *et al.,* 1997; Wang and Stoner, 2008).

Several fruit-bearing trees grown in the Philippines produce highly pigmented fruits likely to be rich in phytonutrients, particularly anthocyanin, and thus, can also be potential sources of natural food dyes. *Canarium ovatum* Engl., locally known as *Pili,* is an indigenous tropical nut-producing tree belonging to the family Burseraceae. The trees are erect to spreading, reaching a height of 30 meters or more. The fruit is drupe with an ovoid to ellipsoid shape, 5.0-8.0 centimeters long, and weighing 15.7 to 45.7 grams. It is covered with a thin, smooth and shiny peel or skin called exocarp which turns from green to dark purple color when the fruit is fully ripe. Underneath is a fleshy and fibrous pulp or mesocarp layer. The hard endocarp or shell is elongated and trigonous with the pointed basal end and blunt apical end. It is nearly triangular in transverse section. Inside the shell is the milky kernel with two white cotelydons surrounded by a brown papery seed coat called testa (Coronel, 1996). Almost every part of the *Pili* tree is utilized either as food, feed, fuel, or handicrafts (Philippines, Department of Agriculture (Phil-DA), 2011), thus locally dubbed as "tree of hope". The tree itself provides good shade for other important crops such as abaca and cacao. From the trunk comes a valued aromatic exudate knew as "*Manila elemi"* which is becoming popular now in the perfumery and pharmaceutical industry. The young leaves are made into salads. It can also be used in the paper and dye making.

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The fruit pulp when blanched is commonly eaten raw by locals. It is also processed into flour, oil, paste, and other bakery products. The shell is made into handicrafts or used as materials for souvenir items. The nut, the most economically important part of the fruit, is popularly made into confectioneries, candies, and oil (Phil-DA, 2011). However, the brightly pigmented exocarp is usually discarded as waste after the nut and pulp were utilized. Owing to its deep pigmentation, the fruit exocarp is hypothesized to possess phytonutrients with potential functional properties. Thus, this research study aimed to assess the pigment extracts of *C. ovatum* exocarps as a functional food colorant. The results of this study will contribute another application opportunity of the rather regarded waste to the food industry. Also, this may lead to further utilization of the *Pili* pigment in the pharmaceutical and cosmetic industries as a natural colorant with functional properties.

2. Materials and methods

2.1 Chemicals and reagents

Ethanol used for extraction was bought from ACI Labscan (Bangkok, Thailand). Folin-Ciocalteau's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,4,6 -tri(2pyridyl)-S-triazine (TPTZ) reagents were purchased from Sigma-Aldrich (St. Loius, MO, USA). Standards catechin, ascorbic acid, and vanillin were purchased from Ajax Finechem (New Zealand) and Sigma-Aldrich (St. Loius, MO, USA). Commercially available impregnated discs of ampicillin and tetracycline used as control antibiotics for the antibacterial test were purchased from Oxoid (Thermo Scientific, United Kingdom). Pronadisa chromogenic agars were purchased from Asiagel Corporation, Philippines. All other chemicals and reagents used in the experiments were of analytical grade.

2.2 Sample preparation and extraction

Five kg of matured *Pili* fruits were purchased from a local market in Goa, Camarines Sur, Philippines. The maturity of fruits was determined based on the color of the fruit skin or exocarp which is dark purple to black when fully ripe. *Pili* fruits without physical damage were carefully selected, washed under running water, and allowed to dry at room temperature. Using a kitchen peeler, exocarps were collected and allowed to air dry for 2-3 days. Sample specimen was submitted to the Botany Section of the National Museum in Manila and was identified as *C. ovatum* Engl. Approximately 187 grams of dried exocarp were ground using a food blender. Ground samples were placed in a stoppered flask and submerged completely in 500 mL absolute ethanol for 24 hours. The mixture was then filtered through a Whatman filter paper. Adhering samples on the flask surfaces were collected using fresh portions of ethanol, filtered and combined with the previous filtrate. The mixture was concentrated under vacuum (Eyela evaporator, Japan) at 50^{o}C until the ethanol was completely evaporated. Pigment concentration was computed following the protocol of Aguinaldo *et al*. (2004) and samples were kept in sterile amber vials at refrigerated temperature until further analysis.

2.3 Determination of the total flavonoid content

Total flavonoid content was determined using the aluminum chloride colorimetric assay described by Marathakam *et al.* (2012). Triplicate samples were analyzed and absorbance reading was done at 510 nm using a spectrophotometer. The total flavonoid content was expressed as mg catechin equivalents (CE)/ g dry weight.

2.4 Determination of the total phenolic content

Total phenolic content of the pigment extract was quantified adopting the modified Folin-Ciocalteau colorimetric method described by Chew *et al.* (2011). Two mL of diluted pigment extract (0.5 mg/ml) was mixed with 1 mL of 1 N Folin-Ciocalteau reagent and allowed to stand for 5 minutes. The mixture was then added to 4 mL saturated sodium carbonate solution (60 g/L). The volume of the mixture was adjusted to 10 mL by adding distilled water. After 2 hours, the absorbance of the mixture was measured at 760 nm against a blank. Analysis was done in triplicates. Ascorbic acid solution prepared with different concentrations was used in constructing the standard curve. Results were expressed as mg ascorbic acid equivalent (AAE)/g dry weight of the sample.

2.5 Determination of the total anthocyanin content

Total anthocyanin content was determined by a spectrophotometric method using the protocol developed by Fuleki and Francis (1968) with slight modifications. One ml aliquot of the pigment extract was mixed with 2.5 mL of 1% (w/v) vanillin in ethanol and 2.5 mL of 9.0 N hydrochloric acid in ethanol. After 20 mins incubation, samples were read at 520 nm in a UV-VIS Spectrophotometer (APEL-100, Japan) and the result was expressed as mg catechin equivalent (CE)/gram dry weight sample. Standard calibration was made using catechin solution at different concentrations.

2.6 Determination of antioxidant activity by DPPH and

FRAP assay

The DPPH• (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity and the ferric-reducing antioxidant power of the pigment extract were used to determine its antioxidant activity following the method described by Yang and Zhai (2010). DPPH assay measures the reduction of DPPH solution in the presence of a hydrogen donating antioxidant. Reduction of the purple color of DPPH to yellow-colored diphenyl-picryl hydrazine signified positive results. Whereas, FRAP assay determines the ability of a sample to reduce ferric to ferrous ion by donating electrons. The percentage reduction of the DPPH free radicals was calculated using the following formula:

DPPH · Free radical scavenging activity (%) = (Ac - As) x 100/Ac (1)

Where:

Ac = absorbance of the control (no sample added)

As = absorbance of the sample

Ascorbic acid was used in constructing the standard calibration curve. Effective Concentration 50 (EC_{50}) value was calculated to further quantify the concentration of sample required to scavenge 50% DPPH free radical. On the other hand, FRAP was expressed as mmol $FeSO_4$ equivalents per gram dry weight of the sample. A standard curve was constructed using $FeSO_4$.

2.7 Test for antibacterial activity

Antibacterial activity of the pigment extract was evaluated using the paper disc diffusion method described by Ortez (2005). Inocula of approximately 10^8 CFU/mL of the standard test bacteria, i.e., *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853), were inoculated onto the sterile surface of Mueller-Hinton agar. Thirty microlilters of the different concentrations, i.e., 1.0, 5.0 and 10.0 mg/ml, of the pigment extract were impregnated into sterile Grade AA Whatman™ paper discs (6 mm in diameter) and allowed to dry. Commercially available impregnated antibiotic discs of ampicillin and tetracycline at 10 µg/mg were used as positive controls while the solvent methanol:acetone was used as negative control. After incubation at 35°C for 24 hours, the zone of inhibition (ZOI) or the diameter of the clearing zones around each disc was measured in millimeter.

2.8 Test for cytotoxic activity

The potential cytotoxicity activity of the pigment extract was tested on human colorectal cancer cell line (HCT 116 cells) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay adapted from the procedure of Mosmann (1983). The Inhibition Concentration 50 (IC_{50}) was computed using the software "*icpin"* which uses a linear interpolation of the graph of absorbance against concentration. Doxorubicin served as positive control while DMSO served as negative control.

2.9 Stability testing of the pigment extract at different pH and temperature

2.9.1 Temperature

Four test samples were prepared by dissolving the pigment extract (0.04 g) in distilled water (40 ml). The test samples were then incubated for 30 minutes at different temperatures mimicking processing and storage conditions: at 4°C (refrigerated), 25°C (ambient), 75°C (pasteurization), and 100°C (boiling/cooking). Spectral characteristics were determined. Each treatment was shot three times using a chromameter (Konica Minolta Chromameter CR400, Japan) calibrated against a white plate. The values obtained were used to assess the color differences between the treatments relative to 4°C using the formula (adapted from Cai and Corke, 1999):

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (2)$$

Hue angle was also calculated following the formula (adapted from McLellan *et al.,* 1994):

$$\text{Hue angle } (H^\circ) = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad (3)$$

Hue angle quantitatively indicates color shift such that 0° or 360° denoted red hues, 90° for yellow, 180° for green, and 270° for blue.

2.9.2 pH

Approximately 0.04 g of the extract was dissolved in buffer solution (40 ml) with different pH values, i.e., pH 4, pH 7 and pH10, and allowed to stand for 30 minutes. Munsell color and the spectral characteristics of the pigment extract was measured using the same chromameter. Results were expressed as L* (lightness), a* (+red/-green), and b* (+yellow/-blue). Each treatment was shot three times. Color differences relative to pH 4 and hue angle were also calculated.

2.10 Application of pigment extract as natural colorant to yogurt

2.10.1 Preparation of yogurt with Pili exocarp colorant

Natural colorant for the yogurt was prepared by mixing 2 g of the pigment extract with 20 ml honey. Honey was used as a carrier for the colorant and as a sweetener for the yogurt. The mixture was stirred gently until completely mixed. The *Pili* exocarp colorant was then added to 1,000 ml of full cream milk prior to pasteurization at 70-75^{o}C for 30 minutes. Probiotic starter (0.06% w/w) containing mix cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* was added when the mixture had cooled down to 45^{o}C. This was further incubated at 40-45^{o}C for about 7-10 hours until the pH drops to 4.5-4.6. Two other yogurts with and without artificial pigment served as controls.

2.10.2 Determination of color characteristics and retention

The color characteristics of the three prepared yogurt samples were measured using a chromameter (Konica Minolta Chromameter CR400, Japan) calibrated against a white plate. Results were expressed as L*(lightness), a*(+red/-green), and b*(+yellow/-blue). From these values, chroma (C) or color intensity/saturation was computed using this formula (adapted from Cai and Corke, 1999):

$$\text{Chroma (C)} = (a^{*2} + b^{*2})^{1/2} \quad (4)$$

Where in high C value denoted more intense color.

To determine color retention of the *Pili* exocarp colorant in yogurt, small vials containing 50 g of the yogurt were stored over a two-week period at refrigerated (4^{o}C) temperature. Color characteristics and pH were measured at day 0, 7 and 14 using the same chromameter and a pH meter, respectively. Duplicate samples were used for this purpose. Munsell color was also determined using the same chromameter. Percent color retention (%CR) was also calculated using the formula:

$$\text{Color retention (\%)} = \text{C value at Z storage time X } \frac{10^2}{C \text{ value at } O \text{ storage time}} \quad (5)$$

Color differences of the treatments relative to the initial reading at week 0 were computed using equation 2. Color shifts were also determined by computing the hue angle following equation 3.

2.11 Microbiological analysis

Microbiological analysis of the yogurt sample with the developed natural colorant was performed with slight modifications of the methods stipulated in the Bacteriological Analytical Manual (United States Food and Drug Administration (US FDA), 2001). Freshly prepared yogurt with *Pili* exocarp colorant was tested for Coliform, *Escherichia coli, Salmonella,* and Coagulase (+) *Staphylococcus aureus* as specified for yogurt and other fermented milk in the "Revised guidelines for the assessment of microbiological quality of processed food" (Department of Health- Food and Drug Administration (DOH-FDA), 2013). Pronadisa® Salmonella chromogenic agar, *E. coli*-Coliform chromogenic agar, and Baird-Parker Agar with Egg yolk suspension (*S. aureus)* were used for the analyses. Lactic acid bacteria were also quantified using de Man, Rogosa and Sharpe (MRS) agar. Twenty-five (25) grams of yogurt sample was mixed with 225 ml 0.1% peptone water producing a 10^{-1} dilution. Serial dilution was performed until 10^{-4}. After which, 0.1 ml aliquot was obtained from each dilution as well as from the undiluted yogurt sample and inoculated onto solidified sterile agar plates mentioned above. The inoculum was spread using a sterile, bent glass rod and allowed to dry for about 5 minutes. All culture plates (in duplicates) were then incubated at 37^{o}C for 24 hours. Following incubation, colonies of coliform (salmon to red colonies on *E. coli*-Coliform chromogenic agar), *E. coli* (dark blue to violet colonies on *E. coli*-Coliform chromogenic agar), *Salmonella* (magenta colonies on *Salmonella* chromogenic agar), and *S. aureus* (black colonies on Baird-Parker Agar with Egg yolk suspension) were counted. If positive for the target bacteria, colony forming unit per gram sample was computed using the following equation:

$$\frac{CFU}{g} = \frac{average\ of\ colonies\ counted}{volume\ of\ inoculum\ used}\ X\ dilution\ factor \quad (6)$$

where dilution factor is the reciprocal of the dilution used.

However, if results are negative, only no growth was reported in the results indicating the absence of the target bacteria from the prepared yogurt.

The lactic acid bacteria in the prepared yogurt were also quantified on de Man, Rogosa and Sharpe (MRS) agar using pour plate method. Sterile, molten paraffin was poured over the solidified agar surface forming an overlay in order to create an anaerobic condition that will facilitate the growth of the facultative anaerobic lactic acid bacteria. The culture plates were then incubated at 37^{o}C for 48 hours. After incubation, colonies were counted manually and CFU/g was calculated using equation 6.

2.12 Sensory acceptability of the developed yogurt

Consumer acceptability test of the yogurt samples was conducted through sensory evaluation. Fifty (50) untrained panelists were asked to assess the sensory attributes of the prepared yogurts (i.e., yogurt with *Pili* exocarp colorant, yogurt with artificial colorant, and yogurt without colorant). The appearance, color, aroma, taste, mouth-feel, and overall acceptability of the samples were evaluated using a 7-point hedonic consumer preference test.

2.13 Statistical analysis

Data analysis and statistical computation for the analysis of variance (ANOVA) and Duncan's Multiple Range Test were performed using SigmaStat version 3.1. Significant difference was defined at $\rho < 0.05$.

3. Results and discussion

3.1 Phytochemical content and antioxidant properties of the Pili pigment extract

A thick semi-solid, dark purple paste was extracted from the dried *Pili* exocarp. Total flavonoid, phenolic, anthocyanin content and antioxidant activities are presented in Table 1. The results support the hypothesis that the *Pili* exocarp being highly pigmented is rich in anthocyanin. Phenolic compounds such as anthocyanins are known natural antioxidant (Wang and Mazza, 2002; Kahkonen and Heinonen, 2003; Gnanavinthan, 2013). As revealed by the Effective Concentration (EC_{50}) value, a very small amount (0.0071 mg/ml) of the extract is required to reduce the amount of the radical DPPH in the solution to 50%. These phytochemicals may, therefore, be responsible for the significant radical scavenging activity and ferric reducing ability of the extract, indicating a promising antioxidant potential. Consistent with these results, Chew *et al.* (2011) reported comparable radical scavenging activity of the whole edible portion (pulp and skin) of *Canarium odontophyllum,* a botanical relative of *C. ovatum*.

3.2 Antibacterial and cytotoxic activities of the Pili pigment extract

In this study, the pigment crude extract from the *Pili* exocarp failed to inhibit the test bacteria *Staphylococcus aureus* ATCC 25923*, Escherichia coli* ATCC 25922*,* and *Pseudomonas aeruginosa* ATCC 27853 as shown by the absence of zone of inhibition in the disc diffusion assay. The results can be interpreted from two perspectives. First, this simply showed that the *Pili* pigment did not exhibit any inhibitory activity against the test bacteria, and hence, its phytochemical constituents do not have any antibacterial property. Secondly, on the other hand, the absence of inhibitory activity could also suggest a lesser toxicity against human gut flora as exemplified by the absence of inhibition against *Escherichia coli*, a known normal bacterial gut flora. Since it is the goal of the research to incorporate this pigment in yogurt which is consumed orally, the developed product should not have any antagonistic effect against beneficial microorganisms found in our colon. In addition, this also showed that the pigment extract may not exhibit inhibitory activity against probiotic microorganisms, the gram-positive bacteria *Lactobacillus* and *Streptococcus*, present in yogurt since it also failed to inhibit the gram-positive test bacteria, *Staphylococcus aureus*. This is further supported by the growth of lactobacilli in the developed yogurt presented below.

Similarly, MTT assay showed little or no cell death against the human colorectal cancer cell line HCT 116 indicating that the *Pili* pigment did not cause any cytotoxic activity even at the highest concentration tested, i.e., 50 µg/ml. The National Cancer Institute in the US limits the activity of crude extract at 50% inhibition (IC_{50}) after 72 hours of exposure to less than 30 µg/ml or below to be considered cytotoxic (Vijayarathna and Sasidharan, 2012). This inactivity against the test microbes and cancer cell lines could perhaps be attributed to the use of crude *Pili* extract where flavonoids exist in glycosidic form. Although this was not tested in this study, the glycosidic form of the flavonoids contained sugar molecules that decreased the effectiveness of the compound against some bacteria as mentioned by Rhee *et al*. (1994), Kapoor *et al*. (2007) and Parvathy *et al*. (2009). Likewise, in previous reports on cytotoxic activities of anthocyanin (Malik *et al.,* 2003; Zhang *et al.,* 2005), purified anthocyanidins, the aglycone of anthocyanin were used. The free hydroxyl group in 3 position of anthocyanidin may have contributed to their anti-proliferative activity of the compound. In the case of anthocyanin, activity was

Table 1. Total flavonoid, phenolic, anthocyanin contents and antioxidant activities of the *Pili* crude pigment extract.

Total flavonoid (mg CE/g DW)[1]	Total phenolic (mg AAE/g DW)[2]	Total anthocyanin (mg CE/g DW)	DPPH (% RSA at 1 mg/ml)[3]	EC_{50}/DPPH (mg/ml)	FRAP (mmolFeSO4/ g DW)
2.2	8.8	17.5	82.1	0.0071	32.0

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prevented by the substitution of the hydroxyl group by different sugar moieties.

3.3 Pigment stability of the Pili pigment extract

Colorimetric analysis using Konica Minolta Chromameter CR400 characterized the *Pili* pigment as red. When subjected to different temperatures, the redness was more pronounced at a lower temperature (4°C) and a significant reduction in redness (a*) was noted when the temperature was elevated (Figure 1). A similar effect was reported by Hou *et al.* (2013) on the anthocyanins isolated from black rice. Furthermore, color shifted from red to yellow-red when exposed to higher temperatures (70-100°C) as elucidated by the increase in hue angle values (Table 2). Computed value for color difference (ΔE*) relative to 4°C decreased as the temperature is increased. These results suggest that high temperature accelerated the degradation of anthocyanin resulting in color change. Hence, these indicate better color stability of the pigment at refrigerated temperature. In the same manner, pH also affected the color characteristic and stability (Table 2). Redness was again more pronounced at lower pH, i.e., pH 4 (Figure 1). At neutral (7) to higher (10) pH, this redness substantially decreased and resulted in the shift of the color from red to yellow-red. Similarly, in the study of Assous *et al.* (2014), the stability of the anthocyanin extracted from purple carrots was also observed at acidic pH (1-5) while rapid degradation occurred at pH 7.

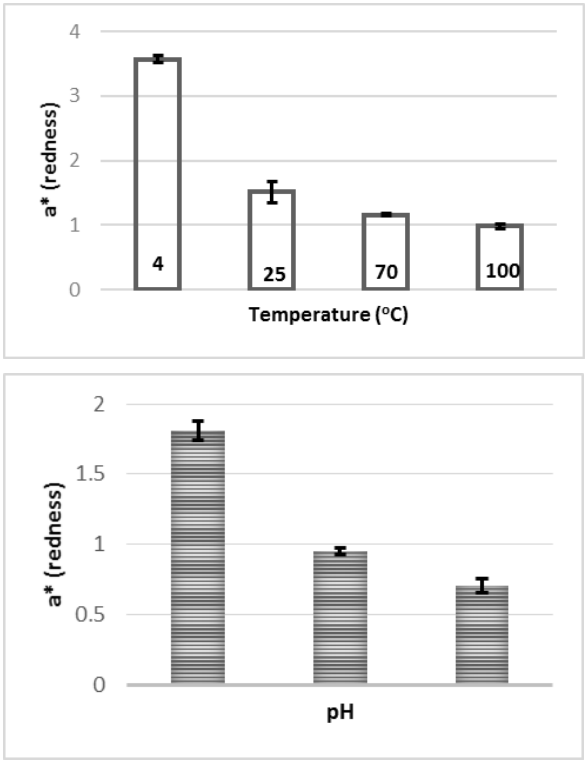

Figure 1. Effect of temperature (A) and pH (B) on redness of the *Pili* pigment extract.

3.4 Color characteristics and retention of Pili pigment extract

The *Pili* pigment extract which was found to be rich in anthocyanin imparted a uniform purplish color to the developed yogurt similar to a commercial blueberry yogurt. However, previous studies showed that naturally-derived colorants normally fade over time (Cai and Corke, 1999; Krammerer *et al.,* 2006; Wallace and Giusti, 2008). It is therefore vital to maintain the stability of the developed product amidst varying processing and storage conditions. In this study, the redness was more pronounced in yogurt with artificial colorant than yogurt with *Pili* exocarp colorant as shown in the a* values (Table 3). This is further supported by the higher or more intense/saturated color given by the C value. The developed yogurt with the natural colorant was also monitored in terms of pH and color characteristics for two weeks at refrigerated temperature (4°C). As shown in Table 4, the yogurt sample retained its redness based on the Munsell color over the two-week storage. Looking at the a* values, the redness was even enhanced with prolonged storage up to two weeks. Perhaps, this can be attributed to the lowering of pH. At a lower pH, anthocyanins were primarily present in the form of red flavylium cations (Wang *et al.*, 2013). Interestingly, there was an excellent color retention of the *Pili* pigment in the developed yogurt (Table 4). This was substantiated by the increase in color saturation as shown in the C value and % Color Retention obtained within the two weeks of storage. Similar results were obtained in the study on the retention of *Berberis boliviana* anthocyanin applied in yogurt (Wallace and Giusti, 2008) and yogurt supplemented with natural betacyanin extract from *Opuntia soehrensii* seeds stored at 4°C for four weeks (Caldas-Cueva *et al.*, 2016). This could be again due to the lowering of the pH of yogurt. During the fermentation process in yogurt, the probiotic bacteria, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus,* produced lactic acid resulting in the decrease in pH of yogurt. This was also evident on the yogurt prepared by Bashiti (2010) using native starters isolated from locally made yogurts. Interestingly, in the study of Arslan and Bayrakci (2016), phenolic contents and antioxidant activity of persimmon-supplemented yogurt were enhanced after two weeks of storage. Phenolic stability was also observed in yogurt containing *Berberis boliviana* anthocyanin over an eight-week refrigerated storage (Wallace and Giusti, 2008). These prove that the functional colorant from fruit and vegetable pigments would certainly add value to healthy products such as yogurt.

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Table 2. Effect of temperature and pH on the color stability of *Pili* pigment extract[1].

Temperature (°C)	Exposure time (mins)	L*[2]	a*[2]	b*[2]	ΔE*[3]	Hue Angle (H°)	Hue (Munsell)
4	30	22.42+0.24[a]	3.57+0.05[a]	2.50+0.08[a]	-	35.00	Red
25	30	19.45+2.04[b]	1.51+0.17[b]	1.19+0.04[b]	3.84	38.24	Red
70	30	21.27+0.17[ab]	1.16+0.01[c]	0.96+0.04[c]	3.08	39.61	Yellow-Red
100	30	21.48+0.10[ab]	0.98+0.04[d]	1.18+0.05[b]	3.05	50.29	Yellow-Red
pH	Exposure time (mins)	L*	a*	b*	ΔE*	H°	Hue (Munsell)
4	30	22.07+0.24[a]	1.81+0.07[a]	0.83+0.04[a]	-	24.63	Red
7	30	20.56+0.12[a]	0.95+0.02[b]	1.04+0.03[b]	1.75	47.59	Yellow-Red
10	30	21.18+0.01[a]	0.70+0.05[c]	1.16+0.00[c]	1.46	58.89	Yellow-Red

[1]Statistical analysis: a-d means different letters within the same column differs significantly ($p<0.05$)
[2]Color dimensions: L*=0 (black); L*=100 (diffused white); a*= red; -a*= green; +b*= yellow; -b*=blue
[3]ΔE* calculated relative to 4°C and pH 4

Table 3. Color comparison of the prepared yogurt samples[1].

Yogurt sample	L*[2]	a*[2]	b*[2]	Chroma
Yogurt with artificial red colorant	73.55+0.01[a]	17.01+0.01[a]	10.40+0.04[a]	19.94
Yogurt with *Pili* exocarp colorant	57.04+0.06[b]	9.20+0.05[b]	6.20+0.13[b]	11.09
Yogurt without colorant	83.27+0.22[c]	-0.88+0.01[c]	12.41+0.09[c]	12.44

[1]Statistical analysis: a-c means different letters within the same column differs significantly ($p<0.05$)
[2]Color dimensions: L*=0 (black); L*=100 (diffused white); a*= red; -a*= green; +b*= yellow; -b*=blue

Table 4. Color retention of yogurt with Pili exocarp colorant in a two-week storage period[1].

Week	pH	L*[2]	a*[2]	b*[2]	Chroma	ΔE*[3]	Hue Angle (H°)	Color retention (%)	Hue (Munsell)
0	4.6	57.04+0.06[a]	9.20+0.05[a]	6.20+0.13[a]	11.09	-	33.98	-	Red
1	4.4	62.66+0.60[b]	10.09+0.06[b]	4.35+0.17[b]	10.98	5.98	23.32	99.04	Red
2	4.3	64.93+0.00[c]	11.05+0.00[c]	4.17+0.00[bc]	11.81	8.35	20.68	107.49	Red

[1]Statistical analysis: a-c means different letters within the same column differs significantly ($\rho < 0.05$)
[2]Color dimensions: L*=0 (black); L*=100 (diffused white); a*= red; -a*= green; +b*= yellow; -b*=blue
[3]ΔE* calculated relative to 4°C and pH 4

3.5 Microbiological and sensory acceptability of the prepared yogurt with natural colorant

To ensure that the developed yogurt with natural colorant is safe for consumption, microbiological analyses were conducted. Coliforms including *E.coli* and other pathogenic organisms (*Salmonella, S. aureus*) were not detected as shown by the absence of growth on the chromogenic culture agar. On the other hand, lactic acid bacteria were quantified to be 2.5 x 10^6 CFU/g. This count passed the required number of viable lactic acid bacteria in yogurt (DOH-FDA, 2013). The value of yogurt as a probiotic product is due to the presence of these live microorganisms. These probiotic microorganisms such as lactic acid bacteria provide health benefits which includes but not limited to the control of intestinal infections, reduction of lactose intolerance, reduction of serum cholesterol levels, and anticarcinogenic activities as mentioned by Lourens-Hattingh and Viljoen (2001), Adolfsson *et al.* (2004) and Kechagia *et al.* (2013). Hence, any additives to yogurt, e.g. colorants, flavorings, or fruits, should not interfere with or inhibit the growth of probiotic bacteria.

The successful application as the natural colorant of the extracted pigment from *Pili* exocarp produced a uniform purplish-pink color in yogurt. Analysis of variance of the consumer acceptability test showed no significant difference between the yogurt incorporated with *Pili* exocarp pigment and with a commercial red food colorant when it comes to appearance, aroma, and taste. However, there is a significant difference between color and mouth-feel. For color, yogurt with artificial colorant was preferred over the other two yogurt samples due to the bright color appearance made by the artificial colorant, making the product more attractive. However, for the mouth-feel (i.e., consistency), the respondents preferred yogurt without any colorant. Comparing the

different criteria, taste obtained the lowest scores among the sensory parameters. Based also on written comments and feedbacks, most evaluators preferred yogurt to be sweeter. Highest values were given to color and appearance for both yogurt with artificial and natural colorant. This simply showed that consumers often rely on visual characteristics of food products for preference with inclination to colored foods. Consistent with these results, Garber *et al*. (2000) and Griffiths (2005) noted that color of the food or beverages also affects flavor perception. In addition, appetites were also stimulated by color (Lakshmi, 2014). Looking at the overall acceptability, respondents still preferred yogurt with artificial colorant followed by yogurt without colorant and the least was yogurt with *Pili* exocarp pigment. Interestingly, in spite of their preference for yogurt with artificial colorant, all the yogurt samples received grades between 5 (like slightly) and 6 (like moderately). This showed that generally the respondents slightly or moderately like the prepared yogurt. Although not performed in this study, prolonged storage may result in rheological and physical changes. These may lead to a reduction in sensory characteristics and consequently, decreased probability of consumer acceptability. Previous studies showed that titratable acidity, computed as % lactic acid, significantly increased due to the continued metabolic activity of the lactic acid bacteria (Serra *et al.*, 2009; Izadi *et al*., 2015; Arslan and Bayrakci, 2016). In the study of Izadi *et al.* (2015), the initial increase in firmness, apparent viscosity, and syneresis or the leaking of liquid from the gel due to the separation of whey were observed but this eventually decreased over time.

4. Conclusion

This research studied the potential application of pigment obtained from the exocarp of *Pili (Canarium ovatum* Engl) as a functional colorant to yogurt. The thick, semi-solid, dark purple paste extracted from the exocarp was found to possess antioxidant activity that could be attributed to the phytonutrients present particularly anthocyanin. The pigment extract was successfully incorporated in yogurt as a functional ingredient. The pigment extract was comparable with commercial colorant when applied to yogurt in terms of color and appearance. Furthermore, it presented excellent color retention over the two-week storage at refrigerated temperature (4^{o}C), hence, a potential alternative for the synthetic colorant. This study is the first to investigate the property and use of the *Pili* exocarp which is normally discarded as agricultural waste, thus results generated from this research endeavor may open opportunities for application for this valued crop.

Further isolation and quantification of the pigment extracted from the fruit exocarp using High Performance Liquid Chromatography are recommended to better elucidate its phytochemical profiles. It is also suggested that further purification and fractionation of the crude extract be done to isolate the bioactive compounds particularly anthocyanin and test these compounds against other cancer cell lines. Further studies may also be done on varying concentrations of the pigment extract to obtain the most appealing color without compromising the taste and quality of the product.

Conflict of Interest

The authors declare that there was no conflict of interest in the conduct of this study.

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