Effect of Peanut (*Arachis hypogaea* L.) Anthocyanin Extract on Cell Surface Hydrophobicity and Hemagglutination of *Escherichia coli* in Acidified Buffer Solution

Jonathan Mazon Barcelo
Department of Chemistry, School of Natural Sciences
Saint Louis University, Baguio City

The effects of peanut anthocyanin extract and commercial cranberry juice on the hydrophobicity and hemagglutination of *Escherichia coli* (ATCC 25922) in acidified buffer solution was investigated. Pure cranberry juice and peanut anthocyanin extracts containing >108.55 mg/mL cyanidin-3-glucoside (cyd-3-glu) equivalents significantly reduced the hydrophobicity of 2.78 x 10^9 CFU/mL E. coli after 8, 20 and 30 hours ($\rho<0.01$) compared to lower concentrations while a concentration of 144.73 mg/L cyd-3-glu equivalents resulted in the best hemagglutination inhibition of the same bacterial density of *E. coli* in less than 8 hours. The results show that peanut anthocyanin extract reduces cell surface hydrophobicity and inhibits the hemagglutination of E. coli in a time-dependent and dose-dependent mechanism through nonspecific interaction with the bacterial cell membrane.

KEYWORDS: cell surface hydrophobicity, *Escherichia coli*, mean hydrophobicity index, minimum hemagglutinating concentration, peanut anthocyanin extract, pH differential method, solid phase extraction

INTRODUCTION

The membrane surface of eukaryotic and prokaryotic cells carries a net hydrophobic character which is usually associated with the fatty acid chains of the amphipathic phospholipid bilayer. In aqueous solutions, the hydrophobic and hydrophilic domains self-associate to become energetically stable. In *Escherichia coli*, cell surface hydrophobicity is a determinant of epithelial adhesion, infection (Doyle, 2000) colonization and biofilm formation which

causes pathogenic strains of *E. coli* to outlast the host's immune response (Suman et al., 2005) and potentiate antibiotic resistance. The hydrophobicity of the surface of E. coli is attributed to Type 1 fimbriae (Otto et al., 1999), P-fimbiae (Uberos et al., 2011), curli fimbriae (Patel et al., 2010) and (C – (C, H)) functional groups on the bacterial cell surface (Hamadi et al., 2008) such as palmitic acid and cis-vaccenic acid (Yuk & Marshall, 2004) of phosphatidylethanolamine and phosphatidylglycerol (Simon & Sampaio, 2011).

Prokaryotic and eukaryotic cell membranes have patches which yield a net negative charge. Raicu and Propescu (2008) reported that the eukaryotic cell membrane generates mainly negative charges due to carboxyl group (COO^-) of sialic acid, while both prokaryotic and eukaryotic cells generate dihydrogen phosphate ($H2PO4^-$), hydrogen phosphate or biphosphate ($HPO4^{-2}$), and phosphate ($PO4^{-3}$) from the polar head of the phospholipids. With these premises, compounds which are hydrophilic and positively charged can potentially bind to the negative patches on the cell membrane and reduce the hydrophobicity of *E. coli*.

Anthocyanins carry a net positive charge on the pyran ring (Giusti et al., 1999; Gallik, 2011) which is responsible for their pigmentation at acidic pH and a potent hydrogen-donating activity. Anthocyanins contain polyphenolic structure and a formal charge on the oxygen of the benzopyrilium ring. At very low pH, anthocyanins have positive charge but at pH 4.5, the positive charge is lost (Gallik, 2011).

Anthocyanin-rich cranberry (*Vaccinium macrocarpon*) products have been reported to decrease the surface hydrophobicity of E. coli (Uberos et al., 2011; Wojnicz et al., 2012) and limit biofilm formation. However, most studies focused on the effect of proanthocyanidins on cell adhesion and hemagglutination of the E. coli. Prior and Lee (2005) reported that proanthocyanidins found in cranberries are also found in plums, avocado, peanut, curry and cinnamon. Their study also revealed that the linkages of proanthocyanidins in cranberry closely matched the linkages in the proanthocyanidins in peanuts. Peanuts have been described to contain the proanthocyanidins catechin and epicatechin in the monomeric, dimeric, trimeric and tetrameric forms, most of which are located in the peanut skin. Aside from proanthocyanidins, peanuts are also rich with anthocyanins, causing variations in the colors of peanut seed coats ranging from light brown to deep red, purple and blue (Chukwumah et al., 2009).

Sobolev and Cole (2004) reported that peanut skins are usually consumed with some peanut based products including in-shell

peanuts and specialty types of peanut butters. However, most of the peanut skins are removed during processing. It is estimated that world production of peanut skins is on the magnitude of 750,000 tons annually, and the current market is confined to low value animal feed applications. The use of peanut seed coats as sources of pharmaceutical compounds could potentially raise the market value of peanut seed coats.

Despite previous studies on determining the type of anthocyanins in peanuts (Cheng et al., 2009; Rongfeng et al., 2011), similarities of proanthocyanidins in peanuts and cranberries (Prior & Lee, 2005) and measuring the antioxidant capacities (Nepote et al., 2005; Shem-tov et al., 2012), there have been no studies done to investigate the effect of peanut anthocyanins on the surface characteristics of bacteria in acidic pH. Hence, this study was conducted to compare the effects of peanut seed coat anthocyanins and cranberry juice on the cell surface hydrophobicity and hemagglutination of *E. coli* in acidic pH. Specifically, the study aimed to determine the phytochemical components of cranberry juice and peanut anthocyanin extract, determine the total monomeric anthocyanin content of peanut anthocyanin extract, and determine the time and concentration required to reduce the hydrophobicity and inhibit the hemagglutination of the minimum hemagglutinating concentration of *E. coli*.

MATERIALS AND METHODS

Plant Material

The peanut cultivar (*Moket* variety) was obtained from Rosario, La Union. Raw peanut pods were manually shelled and oven dried at 105°C. Seed coats were manually removed from the peanut kernel, coarsely pulverized, and extracted with 85% methanol solution (1:4 w/v) at room temperature for 72 hours, filtered thrice using Whatman No. 1 Filter paper (0.45µm pore size) in a Soxhlet apparatus attached to a vacuum and defatted thrice using n-hexane. The extract was stored at 4°C until used.

Determination of Total Anthocyanin Content

The defatted seed coat extract was placed in a clean boiling flask,

evaporated at 40oC using a rotatory evaporator and passed though C18 Sep-Pak SPE cartridges (500 mg sorbent) which was previously activated with methanol followed by 0.01% aqueous HCl according to a modified methodology of Giusti et al. (1999) and Mc Donald (2001). Anthocyanins and other phenolic compounds were adsorbed onto the mini-column while sugars, acids, and other water-soluble compounds were removed using two column volumes of 0.01% aqueous HCl solution. Polyphenolic compounds were separated from anthocyanins using two column volumes of ethyl acetate. Anthocyanins which were adsorbed onto the column were subsequently eluted with methanol containing 0.01% HCl (v/v), placed in a rotatory evaporator at 40 OC to remove methanol and stored -20°C until used.

Total anthocyanin content (TAC) was determined using pH differential method (AOAC Official Method, 2005). Absorbance (A) was measured at 520 and 700 nm using five replicates. The unit for TAC was expressed as mg/L cyanidin-3-glucoside equivalents (cyd-3-glu eq, molar extinction coefficient of 26,900 L x cm^{-1} x mol^{-1} and molecular weight of 449.2 g x mol^{-1}). Buffer solutions (pH 1.0 and 4.5) were prepared for determining the absorbance of anthocyanins. Spectrophotometic readings were measured at pH 1.0 and 4.5 according to the following equation:

$$A = (A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5}$$

$$TAC\ (mg/L) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times l}$$

Preparation of Bacterial Suspensions

American Type Culture Collection *E. coli* strain 25922 was obtained from the Department of Science and Technology in La Trinidad, Benguet. The bacterium was reported to be hydrophobic and exhibited positive hemagglutination of guinea pig and human red blood cells (Mythreyi et al., 2011). The stock culture was prepared by inoculating a static Luria Broth medium (10.0 g tryptone, 5.0 g yeast extract and 10.0g NaCl in 1000mL distilled water; pH = 7.2) at 37°C for seven days. The test organism was initially evaluated for its hydrophobicity and hemagglutination.

Bacterial pellets were prepared from the stock sample by obtaining

5 mL, centrifuged for 15 minutes at 5000 rpm, washed three times with Phosphate Buffered Solution (PBS; 16.0 g NaCl, 0.4 g KCl, 0.4 g KH_2PO_4, and 2.3 g NaH_2PO_4 .2H2O in 1000 mL distilled water; pH = 7.1) until the supernatant was clear. The final suspension was prepared by adding 5mL of PBS to the bacterial pellets. Bacterial density was estimated using the absorbance of the suspension at 510 nm using a Vis spectrophotometer (PD 303) using PBS as the blank solution. The absorbance of the minimum hemagglutinating concentration (MHC) was 0.6438 AU corresponding to a bacterial density of 2.78×10^9 CFU/ mL. Using sterilized glass test tubes, three replicates were prepared for each control (cranberry juice) and experimental groups (25%, 50%, 75% and 100% anthocyanin extracts).

Phytochemical Analysis of Cranberry Juice and Peanut Anthocyanins

Phytochemical analysis was performed to determine the compounds in cranberry juice and peanut anthocyanin extract according to the methodology published by Aguinaldo, Espeso, Guevara and Nonato (2005). The cranberry juice and peanut sample used in the control group and experimental groups were chosen from five cranberry juice and six peanut cultivars based on their ability to inhibit hemagglutination of 2.78×10^9 CFU/ mL *E. coli* after 30 minute incubation at 37°C. The commercially available cranberry juice cocktail (Ocean Spray Cranberries, Inc., Lakeville-Middleboro, MA) was used. According to Liu et al, (2006) Ocean Spray cranberry cocktail (pH=2.3) contains 27% (w/w) cranberry juice.

Preparation of Treatment and Control Groups

Bacterial suspensions from Luria Broth were centrifuged for 15 minutes at 5,000 rpm to obtain bacterial pellets. Bacterial pellets were washed three times using PBS and adjusted to a density of 2.78×10^9 CFU/ mL using the identified spectrophotometric absorbance. After the final wash, 0.5 mL of commercial cranberry juice cocktail was used to suspend the bacterial pellets in the control group. For the experimental groups, 25%, 50%, 75% and 100% concentrations (v/v) of peanut anthocyanin extracts were added to bacterial suspensions in PBS and incubated for thirty hours at 37°C. The pH values in all treatment groups were maintained adjusted to 2.3 using 6N Hydrochloric acid.

Hydrophobicity Assay

The aqueous-aqueous partition method described by Perez et al. (1998) was modified in the hydrophobicity assay. Bacterial pellets were suspended in 3 mL of PBS and adjusted to a density of $2.78x10^9$ CFU/ mL using a Vis spectrophotometer (PD 303). An equal amount of n-hexane was added to the test tube, vortexed for one minute then kept still for thirty minutes to allow the two aqueous phases to separate. After separation of the two phases, n-hexane was removed using a micropipette and transferred to a clean quartz cuvette. The absorbance of n-hexane was determined at 410 nm and recorded as OD_a (Optical Density After). The absorbance was compared to the optical density of pure hexane at 410 nm, which was referred to as OD_b (Optical Density Before). A lesser absorbance value of hexane implies a decreased hydrophobicity of *E. coli*. Hydrophobicity difference in all samples was expressed as hydrophobicity index (HI) using the following formula:

$$HI\ (\%) = \frac{ODb - ODa}{ODb} \times 100$$

Hemagglutination Assay

The protocol described by Adebayo (2004) was modified to prepare 10% and 0.5% red blood cell suspensions. Blood sample was collected from healthy guinea pigs via cardiac puncture, mixed with EDTA and placed in sterile glass test tubes. The blood samples were centrifuged for 15 minutes at 3,000 rpm to separate red blood cells and plasma. Blood plasma was discarded through the use of micropipette. The red blood cells were washed three times using PBS (pH = 7.1).

The weight of the packed red blood cells was obtained and suspended in PBS nine times greater than the packed cell mass to yield a 10% mass to mass blood suspension. In a separate test tube, 0.5 mL of the 10% suspension was mixed with 9.5 mL of PBS to prepare a 10 mL 0.5% working suspension. A small sample of the 0.5% suspension was viewed under light microscope to evaluate cell integrity. Five replicates were prepared per control and treatment groups. The suspensions were stored in the refrigerator (4^oC) until

used.

The protocol of Adebayo (2004) was used to determine the hemagglutination of *E. coli*. In a sterilized test tube, 400μL of bacterial cell suspension was added with an equal volume of working erythrocyte suspension, mixed in a vortex for one minute, and incubated at 37°C for 30 minutes. A negative control (red blood cell suspension) and a positive control (untreated *E. coli* in red blood cell suspension) were prepared to serve as basis for positive hemagglutination. The results were visually evaluated by comparing the results with the positive and negative control groups. A drop of the suspension was viewed under oil immersion (1000x magnification) to characterize red blood cell and bacterial morphology after positive hemagglutination result. Hemagglutination was expressed using the symbols (+++) for strong reaction, (++) for moderate reaction, (+) for weak reaction and (–) for a negative reaction.

Statistical Analysis

Data were analyzed using the statistical software SPSS 18.0. Two-way Analysis of Variance was used to compare the mean hydrophobicity indices of between and within groups in hydrophobicity assay and interaction effects of peanut anthocyanin concentration and time of exposure in reducing hydrophobicity of *E. coli*. Post hoc Tukey Honestly Significant Difference (HSD) was used to determine where the significance lies among the mean hydrophobicity indices among different treatments and time of exposures. Means were considered to be significantly different when the ϱ value is less than 0.01 ($\varrho<0.01$).

RESULTS AND DISCUSSION

Description of the Crude Peanut Extract

Out of 200 g of dried seed coats, 35 g of peanut crude extract was obtained using methanolic extraction (17.5% yield). The peanut extract was slightly viscous and appeared dark reddish brown. After Solid Phase Extraction, the resulting total anthocyanin extract was 10 g (5.0% yield). The anthocyanin extract was stored in a clean Erlenmeyer flask sealed with paraffin film at -20°C until used. The extraction process was repeated based on the amount needed in the study.

Total Monomeric Anthocyanin Content of Peanut Anthocyanin Extract

Using pH differential method, the total monomeric anthocyanin content of peanut seed coats (*Moket* variety) was determined to be 144.73 ± 0.42 mg/L cyd-3-glu equivalents or roughly 0.72 mg /g peanut seed coat (n=5). The result was lower compared to the study of Rongfeng et al. (2011) and Cheng et al. (2009) which are 1.16 mg /g and 11.0 mg /g, respectively. The lower monomeric anthocyanin content of peanuts used in the study could be attributed to the different variety of peanut used and the method of extraction. Cheng et al. (2009) used Black Kingkong peanut varieties, used deionized water for extraction and stirred the peanut seed coats during extraction while the present study extracted anthocyanins from dark pink *Moket* variety using methanol without stirring or shaking.

Phytochemical Analysis of Cranberry Juice and Peanut Anthocyanin Extract

Table 1 shows that the commercial cranberry juice used in the study was positive for gums and mucilages, reducing sugars, phenolic compounds, 2-deoxysugars, leucoanthocyanins, cyanidins, and tannins. The peanut anthocyanin extract was positive for leucoanthocyanins and cyanidins only. The results imply that solid phase extraction efficiently isolated the anthocyanin pigments from peanut seed coats.

Table 1.

Summary of Phytochemical Analysis of Commercial Cranberry Juice and Peanut Anthocyanins*

Name of Test	Test for	Cranberry Juice	Peanut Anthocyanin Extract
Lead Acetate Test	Gums and mucilages	(+)	(-)
Fehling's Test	Reducing sugars	(+)	(-)

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Table 1. (Continued...)

Summary of Phytochemical Analysis of Commercial Cranberry Juice and Peanut Anthocyanins*

Name of Test	Test for	Cranberry Juice	Peanut Anthocyanin Extract
Ferric chloride Test	Tannins	(+)	(-)
Millon's Test	Phenolic compounds	(+)	(-)
Dragendorff Test	Alkaloids	(-)	(-)
Mayer's Test	Alkaloids	(-)	(-)
Keller-Killiani Test	2-deoxysugars	(+)	(-)
Leibermann-Burchard Test	Unsaturated Steroids, triterpenes	(-)	(-)
Borntrager's Test	Anthraquinones	(-)	(-)
Modified Borntrager's Test	Anthraquinones	(-)	(-)
Bate-Smith and Metcalf Test	Leucoanthcyanins	(+)	(+)
Wilstatter Cyanidin Test	γ-benzopyrone nucleus	(+)	(+)
Froth Test	Saponins	(-)	(-)
Gelatin Test	Tannins	(-)	(-)
Guignard Test	Cyanogenic glycosides	(-)	(-)

*Ocean Spray Cranberry Juice Cocktail

Effect of Time of Exposure and Concentration of Peanut Anthocyanin on Hydrophobicity of *E. coli*

At pH 2.3, the minimum hemagglutinating concentration (2.78×10^9 CFU/mL) of *E. coli* (ATCC 25922) showed a mean hydrophobicity index of 33.73 ± 3.59 (n=5). The net hydrophobicity of E. coli could be a result of the self-association of polar and non-polar components of the bacterial cell membrane. The cell membrane of E. coli was reported to carry a weak negative charge (Raicu & Propescu, 2008) and net hydrophobicity. This explains why *E. coli* displayed affinity towards n-hexane.

Cranberry juice and peanut anthocyanin extract decreased the hydrophobicity of *E. coli* in a time dependent and concentration dependent fashion (Figure 1). The results showed that 100% anthocyanin extract (144.73 mg/L cyd-3-glu eq) required the shortest time (20 hours) to decrease the hydrophobicity of *E. coli* compared

to lower concentrations of peanut anthocyanins and pure cranberry juice cocktail. The lowest possible mean hydrophobicity index in anthocyanin extract and cranberry was 1.43 even after 30 hours of exposure.

In the study, *E. coli* and peanut anthocyanins were placed in acidic buffer solutions (pH = 2.3). An increased acidity of the buffer solution may have increased the hydrophobicity of *E. coli*. Moorman et al. (2008) reported that a significant increase in the hydrophobicity of *Listeria innocua* and decrease in membrane fluidity are adaptation mechanisms to an acidic environment. When the cell membrane is exposed to an acidic environment, there is an increase in the synthesis of saturated fatty acids and decrease in the unsaturated fatty acid synthesis to prevent the flow of protons into the cell.

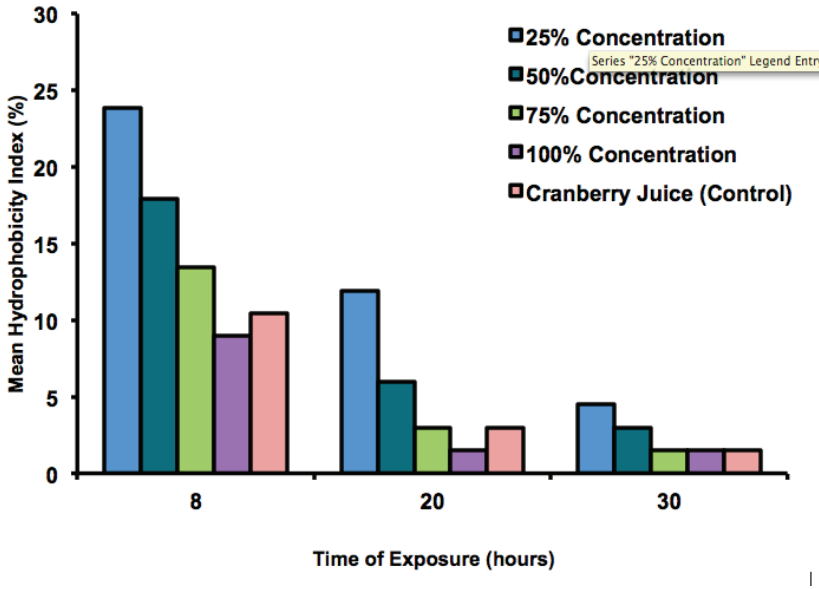

Figure 1. **Effect of Peanut Anthocyanin Extracts and Cranberry Juice on Mean Hydrophobicity Index of *E. coli* after 8, 20, and 30 Hours**

Yuk and Marshall (2004) reported that acid adaptation increases palmitic acid and decreases *cis*-vaccenic acid in the membrane lipids of *E. coli* ATCC 43895 and ATCC 25922. Higher amounts of long branched saturated fatty acids increase hydrophobicity of the cell membrane but decrease in membrane fluidity. In early studies, cranberry juice was thought to decrease epithelial adhesion of *E. coli*

due to its ability to acidify the urine. This study, however, suggests that acidification of urine may promote increased hydrophobicity and consequently, higher bacterial adhesion of *E. coli*.

On the other hand, peanut anthocyanin molecules could have existed as flavylium cations at the same acidic buffer solution (pH = 2.3) where the bacteria have been immersed (Figure 2) (Brouillard et al., 1999). This cation is characterized to bear a positive charge on the benzopyrilium moiety. The positively charged moiety may have interacted with the negatively charged patches on the bacterial cell membrane, causing a shift in the net charge of the cell surface of the bacteria. In addition, anthocyanin molecules at pH 2.3 may have caused self-association of anthocyanin molecules especially on the hydroxyl-bearing catechol group of the molecule, resulting in a stack-like formation of anthocyanin molecules (Rein, 2005). The net hydrophobicity of the bacterial cell membrane may have decreased due to the formation of anthocyanin complexes with each other, causing polar moieties to attach to the bacterial cell membrane while exposing other polar moieties on the cell surface.

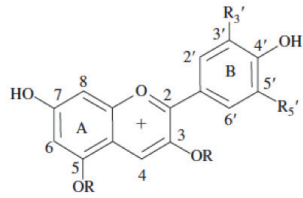

Figure 2. **Cation Form of Anthocyanin Molecule**

Retrieved from Brouillard, R., Figueiredo, P., & George, F. (1999). Malvin Z-chalcone: An unexpected new open cavity for the ferric ion. *Phytochemistry*, *50*, 1391-1394. Retrieved from http://repositorio-cientifico.uatlantica.pt/bitstream/10884/503/1/phyto2.pdf

Brouillard et al. (1999) also discussed that in strongly acidic aqueous solutions, the flavylium cation is highly stable at acidic pH. The positive charge is delocalized through all the pyrilium moieties but carbons 2 and 4 are more positively charged. At pH 4, one of the OH group loses a proton producing a quinoid base. Since the pH of the medium was acidic, the anthocyanin molecule is expected to be protonated and stable. This explains why the results were already observed after 8 hours of exposure.

Cranberry juice cocktail may have reduced the hydrophobicity of *E. coli* in a different mechanism because it contains hydrophilic

proanthocyanidins (Liu et al., 2008). Aside from its anthocyanins, other compounds such as proanthocyanidins and reducing sugars may also have reduced the hydrophobic character of other structures of *E. coli*. Based on the results, the inability of cranberry juice and peanut anthocyanins to completely reduce the hydrophobicity of *E. coli* implies that the bacterial cell membrane has other hydrophobic structures that promote its affinity towards hydrophobic compounds; however, this was not further investigated in the study.

Interaction Effects of Time of Exposure and Concentration of Peanut Anthocyanin Extract on Mean Hydrophobicity Index of *E. coli*

Based on the results of Two Way Analysis of Variance (Table 2), simple main effect analysis revealed that time of exposure caused significant effects on the mean hydrophobicity indices, ($F(2, 74) = 514.913$, $\varrho < 0.01$). The concentration of peanut anthocyanin also caused significant differences on the mean hydrophobicity indices ($F(4, 74) = 109.550$, $\varrho < 0.01$). There was also a significant interaction effect between time of exposure and concentration of peanut anthocyanin on the mean hydrophobicity indices ($F(8, 74) = 14.829$, $\varrho < 0.01$).

Table 2.

Two Way Analysis of Variance of Effects of Time of Exposure and Concentration of Peanut Anthocyanin on Mean Hydrophobicity Index

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	0.002[a]	14	0.000	113.333	0.000
Intercept	0.386	1	0.386	388804.35	0.000
Time of Exposure	0.001	2	0.001	514.913	0.000
Concentration	0.000	4	0.000	109.550	0.000
Time of Exposure *Concentration	0.000	8	1.47E-005	14.829	0.000
Error	5.96E-005	60	9.93E-007		
Total	0.388	75			
Corrected Total	0.002	74			

* R squared = 0.964 (Adjusted R squared)
* $\alpha = 0.01$

A post hoc Tukey Honestly Significant Difference test was employed to determine where the significant differences lie among the different concentrations and time of exposure. Among the different concentrations of peanut anthocyanins (Table 3), the greatest reduction in mean hydrophobicity index was caused by 100% peanut anthocyanin extract. The result showed no significant difference with 75% anthocyanin extract and cranberry juice. Among the time of exposure, a 30 hour exposure caused the greatest reduction in the mean hydrophobicity indices of all treatments. The result shows significant difference to a 20-hour exposure and 8-hour exposure.

Table 3.

Comparison of Mean Hydrophobicity Indices in Different Concentrations of Peanut Anthocyanins and Time of Exposure

Treatment	**Time of Exposure**			
	8 hours	**20 hours**	**0 hours**	**Mean**
25% Anthocyanin Concentration	23.88	11.94	4.48	13.43[a]
50% Anthocyanin Concentration	17.91	5.97	2.99	8.96[b]
75% Anthocyanin Concentration	13.43	2.99	1.49	5.97[c]
100% Anthocyanin Concentration	8.95	1.49	1.49	3.98[c]
Cranberry Juice	10.45	2.99	1.49	4.98[c]
Mean	14.93[a]	5.07[b]	2.39[c]	

*Means indicated by different letters indicate significant difference at α=0.01

Commercial cranberry cocktails contain 13.6 mg/L cyd-3-glu equivalents based on the study of Barnes et al. (2005), which is higher compared to the results in the study. Other studies reported that cranberry juice cocktails contained 1.08 mg/100 mL (Lee et al., 2008) to 2.5 mg/100 mL anthocyanins (Cunningham et al., 2004) which is estimated to be within the range of 12 to 25 mg/L anthocyanin concentration. In the study, peanut anthocyanin extract contains lesser monomeric anthocyanin content compared to cranberry. This explains why a higher concentration of peanut anthocyanins is required to reduce the hydrophobicity of *E. coli*.

Effect of Time of Exposure and Concentration of Peanut Anthocyanin Extract on Hemagglutination of *E. coli*

The hemagglutinating ability of *E. coli* (ATCC 25922) was evaluated upon the addition of 200μL of bacterial suspension in PBS (pH=7.1) to an equal volume of 0.5% guinea pig RBC suspension after 30 minute incubation at 37°C. Upon microscopic observation in 1000x magnification, red blood cells appeared clumped together. Clumping of bacterial cells was also observed near the red blood cells. Morphologically, the surface of red blood cells appeared uneven (Figure 3).

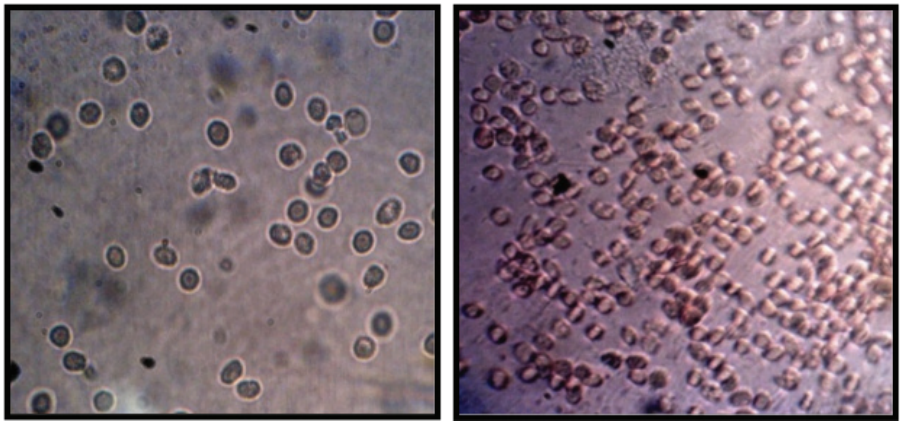

Figure 3. **Morphology of Guinea Pig Erythrocytes in Negative Control and Positive Control with E. coli (1000x magnification)**

Higher concentrations of anthocyanin extract inhibited hemagglutination of *E. coli* (2.78×10^9 CFU/mL) after 8, 20 and 30 hours (Table 4). Lower concentrations of anthocyanin extract did not inhibit hemagglutination of *E. coli* even after 30 hours of exposure.

Hemagglutination occurs when bacterial cells attach to the receptor sites in red blood cells, forming cross-links and visible clumps (Mithreyi et al., 2011). According to Turner et al. (2005), *E. coli* strain ATCC 25922 possesses Class II P-fimbriae which are also found in uropathogenic *E. coli* strains. This indicates that *E. coli* (ATCC 25922) could adhere to uroepithelial cells. Positive hemagglutination therefore indicates the presence of P-fimbriae in *E. coli*. Bacterial pellets were suspended in

peanut anthocyanins and cranberry juice prior to hemagglutination assay, suggesting that the inhibition of peanut anthocyanins and cranberry juice may have acted on hemagglutinating structures in *E. coli*, preventing its ability to react with the receptor sites in guinea pig red blood cells during the hemagglutination assay.

Prior and Lee (2005) reported that cranberry juice cocktails contain proanthocyanidin aside from other compounds reported in this study. Apparently, proanthocyanin seems to be absent in the peanut anthocyanin extract since the use of solid phase extraction was able to isolate anthocyanin pigments only. This claim is supported by the results from the phytochemical analysis (Table 1), since cranberry juice is positive for reducing sugars, tannins, phenolic compounds, gums and mucilages, cyanidin-based compounds and deoxysugars. These compounds could have influenced the ability of cranberry juice to reduce hydrophobicity and inhibit hemagglutination of *E. coli*. Flavonoids and reducing sugars were previously reported to alter the cell surface and fimbriae of *E. coli* (Mulvey 2002; Beecher 2004; Howell et al., 2005) although the mechanism was not clearly understood. In this study, the mechanism of action of peanut anthocyanins on P-fimbriae was not further investigated.

Table 4.

Hemagglutination by E. coli as Affected by Duration of Exposure and Concentration of Anthocyanins

Treatment	**Length of Exposure**		
	8 hours	**20 hours**	**30 hours**
25% Anthocyanin Concentration	++	++	++
50% Anthocyanin Concentration	++	++	++
75% Anthocyanin Concentration	+	+	-
100% Anthocyanin Concentration	-	-	-
Cranberry Juice	-	-	-

According to Uberos et al. (2011), the incubation of *E. coli* with cranberry syrup in the same concentration significantly reduced hydrophobicity but not hemagglutination but the study revealed a different result. Cranberry juice reduced hemagglutination first before reducing the hydrophobicity of *E. coli*, suggesting that compounds in

the cranberry such as proanthocyanidins may have caused changes in P-fimbriae conformation of *E. coli* while cranberry anthocyanins may have reduced cell membrane surface hydrophobicity at a later time. The combination of different compounds in cranberry juice seems to exert different mechanisms in altering the surface properties of *E. coli*.

In the study, a 75% anthocyanin extract (108.55 mg/L cyd-3-glu eq) reduced hydrophobicity first before inhibiting hemagglutination after 20 hours while a 100% anthocyanin extract (144.73 mg/mL cyd-3-glu eq) had the same effect with cranberry juice. Liu et al. (2006) suggested that proanthocyanidins change the conformation of fimbriae. This could explain why cranberry juice inhibited hemagglutination better than pure anthocyanin extract. The result (Table 3) suggests that anthocyanins could also inhibit hemagglutination more effectively in concentrations greater than 108.55 mg/L cyanidin-3-equivalents. However, inhibition of hemagglutination by anthocyanins might be a result of electrostatic alteration of P-fimbriae, not because of conformational change. It is highly likely that the effects of peanut anthocyanins on the cell surface properties of *E. coli* are based on non-specific interactions with the molecules of the bacterial cell membrane.

CONCLUSION

Peanut seed coats contain lower anthocyanin content than commercial cranberry juice but exhibit the same activity in reducing hydrophobicity and inhibiting hemagglutination *E. coli*. Apparently, cranberry juice inhibits hemagglutination better than peanut anthocyanins but both can reduce the hydrophobicity of *E. coli* despite the lower anthocyanin content of peanut seed coats. At acidic pH, peanut anthocyanin extract reduces the cell surface hydrophobicity of *E. coli* in a time-dependent and dose-dependent manner through non-specific binding to the cell membrane surface. Further studies are recommended to optimize the extraction of anthocyanin from peanut seed coats, and determine the effects of peanut anthocyanins on other microorganisms at various pH levels, temperature and concentrations.

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