BIOACTIVE COMPOUNDS DETERMINATION IN FERMENTED LIQUID DRAGON FRUIT (*HYLOCEREUS POLYRHIZUS*)

Foong, J. H., Hon, W. M., & Ho, C. W.

Faculty of Applied Sciences, UCSI University,
No. 1, Jalan Menara Gading, UCSI Heights, Cheras 56000, Kuala Lumpur, Malaysia.

ABSTRACT. *Bioactive compounds have been intensively studied due to their beneficial effects on high cholesterol, cardiovascular disease and cancer. This study focused on the bioactive compounds (fatty acids, phytosterols, betacyanins and acetic acid) and other aspects (physical, microbiological, chemical and nutritional properties) of fermented liquid dragon fruit (Hylocereus polyrhizus) without pasteurization sample A (SA) and with pasteurization sample B (SB). Overall, SB exhibited significantly higher concentrations ($p<0.05$) of phytosterols, betacyanins, acetic acids, total phenolic content (TPC), total flavonoid content (TFC) and total flavanol assay (TFA) as compared to SA, except fatty acids; this suggested that heat treatment (75^{o}C for 15 s) may have exerted a favourable effect on the concentration of these bioactive compounds. Also, SB showed greater ($p<0.05$) radical scavenging capacity in 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay than SA. No microorganisms were detected in SA or SB which was possibly due to restricting internal factor such as low pH (3.94-4.00), high ethanol concentration (7.31-8.92%) and the presence of organic acids. In conclusion, the overall results suggested both SA and SB were microbiologically safe for consumption however heat pasteurization may exert a beneficial effect on concentrations of bioactive compounds.*

KEYWORDS. Bioactive compounds, dragon fruit, cholesterol, pasteurization, fermentation

INTRODUCTION

Over past decades, transitions in dietary habits and food consumption have been observed in developed and developing countries due to urbanization and accelerated economic status, and that has also led to the rise of diet-associated on-communicable diseases such as diabetes and hypertension at an alarming rate. The increased rates of these diseases have led to the rise of demand for functional foods, which are demonstrated to offer health benefits and reduce risk of chronic diseases beyond the widely accepted nutritional effects (Smith & Edward, 2010).

Fermented products are one of the popular functional food choices due to their good functional and nutritional properties (Stanton *et al.*, 2005). Development of new functional products can be expensive and challenging. One of the products introduced in recent years is the fermented dragon fruit drink. Red dragon fruit (*Hylocereus polyrhizus*) is well known in the fruit market as an excellent source of antioxidants and for its high content of nutrients, particularly potassium, as well as dietary fibre. Frequent consumption of red dragon fruit has been reported to improve overall digestive system, reduce cholesterol and prevent constipation. However, fresh dragon fruit can only last a maximum of 14 days at 10^{o}C and 5 days at room temperature. Consequently, it is desirable to process fresh fruit into fermented liquid dragon fruit in order to avoid waste and increase its marketability (Bellec *et al.*, 2009).

In recent years, different brands of fermented dragon fruit drink have been introduced to the local market. These brands include Pitacacti Delight, Forliko, Klau, Sunberry. The consumption of these fermented dragon fruit drinks is claimed improve overall body functions (Chew, 2009). The protective health effects was strongly believed to be due to its bioactive compounds since these compounds are commonly found in plant based diets.

Extensive studies have been done on cereals, legumes, nuts, oil, vegetables, fruits, tea and red wine to identify their bioactive compounds as well as their associated health benefits such as antioxidant, anti-carcinogen, cholesterol reducing as well as remitting other chronic diseases (Kris-Etherton *et al.*, 2002). However, there are only limited published studies on fermented dragon fruit drink. Therefore, the objective of this study is to determine bioactive compounds that are present in fermented liquid red dragon fruit, with and without pasteurization treatment.

MATERIALS AND METHODS

Raw materials

Fresh red dragon fruits (RDF) were purchased from Great Sun Pitaya Farm Sdn. Bhd., Selangor, Malaysia while organic raw cane sugar was purchased from a local hypermarket.

Chemicals

Chemical reagents used in this study were 4-(Dimethylamino) cinnamaldehyde (Sigma Aldrich, USA), 5α-cholestane (Sigma Aldrich, Israel), absolute ethanol (John Kollin Corporation, UK), ABTS chromophore (Merck, Germany), aluminium chloride hexahydrate (Fisher Chemical, UK), betanin (TCI, Japan), bis (trimethylsilyl) trifluroacetamide (Sigma Aldrich, USA), chloroform (Friendemann Schidmt, Australia), denatured ethanol (Bumi-Pharma, Malaysia), Folin Ciocalteu phenol reagent (Merck, Germany), glacial acetic acid (Merck, Germany), hydrochloric acid fuming 37% (Merck, Germany), methanol analytical grade (Fisher Scientific, UK), methanol HPLC grade (Merck, Germany), n-heptane (Merck, Germany), n-hexane (Merck, Germany), orthophosphoric acid (R&M Chemical, UK), peptone water (Merck, Germany), petroleum ether (Friendemann Schimdt, UK), plate count agar (BD, France), potassium hydroxide (Merck, Germany), potato dextrose agar (BD, France), purified nitrogen 99.999% (MOX, Malaysia), pyridine (Merck, Germany), sodium carbonate anhydrous (Merck, Germany), sodium chloride (Merck, Germany), sodium methylate (Merck, Germany) and sodium nitrite (Friendamann Schidmt, Australia). Milli-Q system (Millipore, USA) deionised water and ultra pure water was used throughout this study.

Sample pre-treatment

Red dragon fruits (RDF) with an average weight of 0.7-0.8 kg were rinsed with tap water and dried to remove dirt and residues. Subsequently, the skin of RDF was peeled and discarded while the flesh was cut into pieces, each with average thickness of 5 mm.

Fermentation

Stainless steel fermentation tanks with 2 L volume were autoclaved while utensils were cleaned and rinsed with hot water prior to fermentation. First, 1.4 kg of RDF and 280 g of raw cane sugar were weighed. Then, raw cane sugar (RCS) and RDF were arranged layer by layer in fermentation tanks as shown in FIGURE 1 below. Lastly, the fermentation tanks were closed tightly and stored in a clean cabinet for 8 weeks at 25^{o}C.

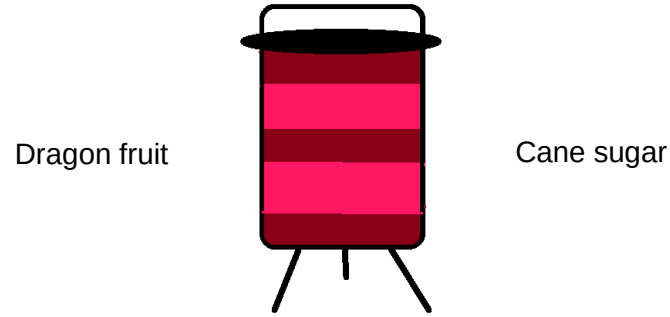

Figure 1. Arrangement of RDF and RCS in fermentation tank.

After 8 weeks storage, fermented samples were strained into 2 Schott Duhran bottles (1L), sample A (SA) and sample B (SB). SA was not subjected to any treatment while SB was pasteurized at 75^{o}C for 15 sec. SA and SB were then divided into sterilized universal bottles for further analyses. Samples were stored at -20^{o}C prior to analysis to ensure their freshness.

Analyses

a) Instrumental Analyses

i) Ethanol

The determination of ethanol content was done through automated injection method with slight modification from various studies (Wang *et al.* 2003; Jacobson, 2006). Sample was first diluted 40 times with ultra pure water and filtered into each vial using syringe filter of pore size 0.45 µm (nylon) prior to injection into gas chromatography (GC). All of the samples were assayed in triplicate. Peak area of sample was recorded and calculated based on equation y = 712.38, R^2 = 0.9971 developed from ethanol standard curve [0.1% -0.9% (v/v)].

The alcohol content of sample was determined using Agilent A7890 gas chromatography (Agilent Ins, US), equipped with a computer containing integrator software (ChemStation), a 30 m DB-Wax capillary column (250 µm id, film thickness: 0.25 µm) and a flame ionization detector (FID) (H_2: 30 ml/min and air: 400 ml/min). The flow rate of nitrogen gas was set at 25 ml/min while the temperature at injector port and detector was set at 250^{o}C and 300^{o}C, respectively, and split injection (97.5:1 split ratios) was used (0.1 µL for each injection). Oven temperature was controlled with a temperature elevation program during analysis, which was initially set at 40^{o}C for 5 min, increased to 250^{o}C at a rate of 50^{o}C/min and maintained for 0.8 min, with a total determination time of 10 min.

ii) Fatty acids (FA)

Lipid was extracted from sample according to Aldo *et al.* (2007) and Amanda *et al.* (2010) with slight modification. Sample (10 mL) was added with 15 mL of Folch reactive solution [chloroform/methanol; 2:1(v/v)]. The mixture solution was vortexed for 5 min, followed by the addition of 3.74 mL of 0.73% (w/v) sodium chloride solution. Mixture was centrifuged at 2000 rpm for 2 min to speed up the separation of organic and alcoholic-water layer. After centrifugation, the lower organic layer was transferred to another centrifuge tube by filtering through a glass funnel laid with 2 g of anhydrous sodium sulphate to remove remaining water while the upper layer was discharged. The solid residue remaining was re-extracted by repeating the extraction process. The combined lower layer organic solution was transferred to a round-bottom flask (RBF) and evaporated under vacuum at 40^{o}C until dry. Extracted lipid was stored at -20^{o}C until further analysis.

Fatty acids in sample were converted to fatty acid methyl esters (FAME) through transesterification process before injecting into GC. Extracted lipid (50 mg) was dissolved in 4.75 mL of hexane in a screw-capped bottle, followed by the addition of 250 µL of sodium methoxide. The mixture was vortexed vigorously for 10 sec and allowed to settle for 5 min. Subsequently, 3 mL of upper hexane layer was transferred to a new screw capped bottle and 1 mL of methanol was added. The mixture was again vortexed for 10 second settled for 5 min. Upper hexane layer was transferred into new screw capped bottle laid with 1 mm layer of anhydrous sodium sulphate and left to stand at room temperature for 30 min. Lastly, upper hexane layer was filtered into vial using nylon syringe filter (0.45 µm) and injected into GC for analysis.

Sample (1 µL) was injected into Perkin Elmer Instrument Autosystem XL gas chromatography equipped with a split-splitless injector and a flame ionization detector (FID). A polar fused silica capillary column SPTM-2380 of 30 m length, 0.25 mm internal diameter and 0.25 mm film thickness was used in FAME analysis. The flow rate of hydrogen gas and nitrogen gas was fixed at 38 mL/min and 18 psi respectively. The temperature of injector was set at 225^{o}C while FID was maintained at 230^{o}C. Initially, the column oven temperature was programmed to maintain at 140^{o}C and then increased to 200^{o}C at a rate of 4^{o}C/min, followed by increment to 205^{o}C at 0.5^{o}C/min and 225^{o}C at the rate of 1^{o}C/min. Column temperature was held at 225^{o}C for 2 min. The FAME peaks were identified by comparing the retention times of samples with standard 37 Component FAME mix (Supelco, USA). Analysis was done in triplicate and each peak area was computed and expressed as µg of fatty acid per mL of sample. The fatty acids concentration was quantified based on Equation 1 and Equation 2.

Equation 1:

Response factor = FA in standard peak area / FA standard concentration

Equation 2:

FA concentration in sample = FA in sample peak area/ Response factor

iii) Phytosterols

Determination of phytosterols involves a series of processes like saponification, extraction of unsaponifiables and silylation in order to convert phytosterols into volatile derivatives that can be detected by GC (Jill, 2011). The extracted lipid (250 mg) was weighed and added to 5 mL of 6% ethanolic KOH and 100 µL of 5α-cholestane in round-bottom flask (RBF). Three anti-bumping chips were added to ensure smooth boiling. Mixture was refluxed for 1 hr at low heat to avoid overheating in the saponification process. The saponified mixture was transferred to a centrifuge tube, followed by the addition of 10 mL of ultra pure water and 10 mL of petroleum ether (PE). The mixture was centrifuged at 3000 rpm for 15 min. After centrifugation, approximately 10 mL of upper PE layer was transferred to a clean centrifuge tube. The remaining mixture was re-extracted by repeating the addition of water, PE and centrifugation. Once 30 mL of PE layer were collected, 10 mL of ethanolic-water solution (1:1, v/v) was added into the PE solution. The mixture was vortexed for 10 sec and centrifuged at 4500 rpm for 5 min. The upper PE layer was again transferred to a new centrifuge tube. For further purification, the new PE layer was purified by repeating the addition of ethanolic-water solution and centrifugation process twice. After the final centrifugation, the PE layer was transferred into a new boiling tube and 0.5 g of anhydrous sodium sulphate was added. The entire tube was wrapped with aluminium foil and Parafilm before storing at room temperature in a cabinet overnight. The PE layer was evaporated under vacuum at 25^{o}C until almost dry. The residual solvent was purged to dryness at 55^{o}C with 99.9% nitrogen gas. Subsequently, 60 µL of Bis (trimethylsilyl) trifluoroacetamide (BSTFA) and 240 µL of pyridine were added into RBF and vortexed for 10 sec. The mixture was transferred into a screw capped bottle and heated at 70^{o}C for 45 min. It was allowed to

cool down before the addition of 1.7 mL of heptane. Lastly, the mixture was vortexed for 10 sec and filtered into a GC vial using a nylon syringe filter (0.45 µm).

Phytosterols was determined using Agilent A7890 gas chromatography (Agilent Ins, US) equipped with 30 m DB-Wax capillary column (320 µm id, film thickness: 0.25 µm) and a flame ionization detector (FID) (H_2: 30 ml/min and N_2: 30 ml/min). The temperature at injector port and detector was set at 250^{o}C and 325^{o}C, respectively, and splitless injection was used, in which injection volume was set at 1 µL. The initial column oven temperature was programmed at 250^{o}C and held for 1 min, and then it was increased to 300^{o}C at a rate of 2^{o}C/min. Phytosterol peaks were identified by comparison with the retention time of 3 main phytosterols, which are campesterol, stigmasterol and β-sitosterol. The concentration of campesterol ($y = 110824x – 363.04$; $R^2 = 0.99$), stigmasterol ($y = 39922x – 6.83$; $R^2 = 0.99$) and β-sitosterol ($y = 51150x – 98.03$; $R^2 = 0.99$) were quantified based on equation developed from standard curve. Its concentration was expressed as µg/mL of sample.

iv) Organic acids and betacyanins

Acetic acid standard solution with concentration ranging 50 ppm, 100 ppm, 200 ppm, 400 ppm and 800 ppm was prepared from glacial acetic acid (Merck, Germany) while betanin standard which consists of betanin and isobetanin (500 ppm, 1000 ppm, 2000 ppm, 4000 ppm and 8000 ppm) was prepared from betanin (TCI, Japan). The sample was first diluted 10 times with ultra pure water before filtering through nylon syringe filter (0.45 µm) into sample vial.

An Agilent 1200 series HPLC system fitted with G1322A degasser, C1328B injector, G1329A column oven and G1315D diode array detector (DAD) was employed. The column used throughout this study was a reversed phase Zorbax Eclipse Plus C18 column 3.5 Micron (2.1 x 150 mm). Sample injection volume was set at 10 µL while the elution was performed at a solvent flow rate of 0.2 mL/min. Temperature of column was maintained at 40^{o}C and detection under UV spectra was carried out at 210 nm (organic acids), 280 nm (catechins) as well as 534 nm (betacyanins) and indicated by peaks plotted in chromatograms.

The mobile phase was composed of 0.1% of ortho-phosphoric acid in ultra pure water (v/v) (Eluent A) and 0.1% of ortho-phophoric acid in methanol (v/v) (Eluent B). The ortho-phosphoric acid was added to the solution before filtering through 0.45µm nylon membrane filter disc (Millipore, England) and degassed. Table 1 shows the mobile phase composition for a binary gradient condition using dual pumping system.

Table 1. Mobile phase composition for binary gradient condition.

Elution time (min)	Eluent A (%)	Eluent B (%)
10	98	2
58	50	50
63	50	50
73	0	100
83	0	100
93	98	2

Peaks were identified by comparing the retention times of SA and SB with retention times foracetic acid and betanin standards. The amount of acetic acid and betacyanins was determined from the peak areas at 210 nm and 534 nm respectively based on the equation developed from acetic acids (50 – 800 ppm) $y = 1.5966x$, R^2=0.9996, betanin (500 – 8000 ppm) $y = 0.154x$, $R^2 = 0.9895$ and isobetanin standard curve (500 – 8000 ppm) $y = 0.1721x$, $R^2 = 0.9878$. Each sample was analysed in triplicate.

b) Chemical analyses

i) Total phenolic content (TPC)

Determination of total phenolic content was done according to Thoo *et al.* (2010) with slight modifications. Sample (1 mL) of 10-fold dilution was mixed with 1 mL of Folin-Ciocalteu reagent (10x dilution). After 3 min, 800 µL of 7.5% (w/v) anhydrous sodium carbonate was added to the mixture and vortexed well. Subsequently, the mixture was incubated in the dark at room temperature for 2 hr followed by absorbance measurement at 765 nm against blank using UV-vis spectrophotometer (UviLine9400, France). Results were expressed as mg of gallic acid equivalent (GAE) per 100 mL of sample based on the equation $y = 0.0396x$, $R^2 = 0.9975$ using gallic acid as standard.

ii) Total flavonoid content (TFC)

Determination of total flavonoid content (TFC) was performed according to procedures described by Ozsoy *et al.* (2007) and Thoo *et al.* (2010) with slight modifications. A sample (0.25 mL) was added to 1.25 mL deionized water. At time zero, 75 µL of 5% sodium nitrite was added into the mixture, followed by 150 µL of 10% aluminium chloride hexahydrate 5 min later. In the next 5 min, 0.5 mL of 1 M sodium hydroxide and 275 µL deionized water were added and mixed well. Immediately, absorbance of reaction mixture was measured at 510 nm against blank and total flavanoid content was expressed as catechin equivalent (CE) per 100 mL of sample based on the equation $y = 0.0033x$, $R^2 = 0.9991$ using catechin (50-800 µg/mL) as standard.

iii) Total flavanol assay (TFA)

Total flavanol assay was determined using p-dimethylaminocinnamaldehyde (DMACA) method as proposed in various studies (Ferreira *et al.*,2006 ; Silva *et al.,* 2007; Taylor & Giusti, 2009). The sample (160 µL) was mixed with 2.4 mL of DMACA solution [2% in 2N HCL in methanol (1:1, v/v)]. The mixture was vortexed and allowed to react at room temperature for 12 – 15 min. Absorbance of reaction mixture at 640 nm was measured against a blank (DMACA solution). The concentration of TFA was calculated based on the equation $y = 0.0022x + 0.039$, $R^2>0.99$ using catechin as standard. Results were expressed as mg catechin equivalent (CE) per 100 mL of sample.

iv) ABTS radical scavenging capacity

ABTS radical solution was prepared gently by mixing 10 mL of 7mM ABTS solution with 10 mL of 2.45 mM potassium per sulphate solution. The mixture was stored in a cabinet at room temperature for 12 – 16 hr before use. The ABTS radical solution was adjusted with ethanol to an absorbance 0.7±0.02 at 734 nm before use. The sample (25 µL) was added to 975 µL of ABTS radical solution and allowed to react for 6 min. The absorbance of the sample was measured at 734 nm against blank. The percentage of ABTS radical scavenging capacity was calculated as $[1- (A_e / A_c)] \times 100\%$ ($A_e = A_{734}$ in the presence of sample; $A_c = A_{734}$ of negative control solution). The ABTS was expressed as The ABTS was then expressed as micromoles of trolox equivalents antioxidant capacity (TEAC) using an equation obtained from standard curve of trolox (0.1–0.8 mM) $y = 120.1142x$, $R2 = 0.9984$ (Thoo *et al.*, 2010).

c) Physical analyses

i) pH

The pH of SA and SB were measured using pH meter (Mettler Toledo, Switzerland). Calibration was done using buffer solution of pH 7 and pH 4 prior to measurement. pH of sample was measured by immersing the probe into sample. Readings were recorded once there was no fluctuation shown.

ii) Total soluble solids (TSS)

Hand-held refractometer with detection Brix^{o} range of 0 – 32 Brix^{o} (Fisher Scientific, UK) was used to determine TSS of samples. Samples were equilibrated to room temperature before use. Refractometer was first calibrated with distilled water and a reading of 0 Brix^{o}obtained. A few drops of sample were placed on the prism surface with daylight plate closed gently. Refractometer was held in direction of natural light and reading was recorded (Vasquez & Mueller, 2009).

d) Microbiological analyses

Microbiological analyses were aimed at evaluating the safety of food based on the presence of micro-organisms. The microbiological analyses performed were total plate counts (TPCs) and yeast and moulds (YM). According to Centre for Food Safety (2007) and NSW Food Authority (2009), the safety limit of TPC for ready-to-eat foods is 10^4 to 10^5 CFU/g generally. Triplicates were done for each microbiological analysis for SA and SB.

i) Total plate counts (TPCs)

Plate count agar was prepared from Difco^{TM} Plate Count Agar powder (BD, France). A serial dilution of 10^{-1}, 10^{-2} and 10^{-3} were prepared. TPCs assay was carried out using spread plate method and incubated at 37^{o}C in incubator for 48±2 hours. Any light amber, slightly opalescent colonies formed were recorded (Christison *et al.,* 2007; Dolci *et al.,* 2008).

ii) Yeast and moulds(YM)

Potato dextrose agar (PDA) was prepared from Difco^{TM} Potato Dextrose Agar powder (BD, France) for detection of YM in SA and SB. 1 mL of 10% tartaric acid was added to into every 100 mL of media solution before media solidified. Dilution factor of 0, 10^{-1} and 10^{-2} were prepared. 0.1 mL of sample from each dilution was aseptically transferred to Petri dish and spread evenly. Petri dish was incubated at 25^{o}C in cabinet for 5 days. Any light amber, clear and slightly opalescent colonies were recorded (Ukuku & Sapers, 2006).

Statistical analysis

All experiment results were analysed using Minitab software (Minitab version 15.1.1.0). All numerical data were expressed as mean ± standard deviations. The repeatability of these experiment results was indicated with relative standard deviation expressed in percentage (%RSD) as shown in Equation 3. Significant differences (p<0.05) between the means were determined using one-way analysis of variance (ANOVA) with Tukey's test.

Equation 3:

%RSD = (Standard deviation/Mean) x 100

RESULTS AND DISCUSSION

Repeatability

Table 2. RSD (%) of fermented dragon fruit liquid sample A (SA) and sample B (SB).

Analyses	SA	SB
Physical		
pH	0.15	0.14
Total soluble solids	0.50	0.51
Microbiological		
Total plate count	N.A	N.A
Yeast and moulds	N.A	N.A
Gas chromatography (GC)		
Ethanol	1.09	0.47
Fatty acids		
Stearic acid (C16:0)	6.36	1.66
Oleic acid (C18:1n9c)	0.28	7.25
Phytosterols		
Campesterol	0.17	0.04
Stigmasterol	N.A	6.89
B-sitosterol	N.A	0.46
High performance liquid chromatography (HPLC)		
Acetic acid	4.38	6.00
Betanin	2.11	4.27
Isobetanin	1.44	4.25
Chemical analyses		
Total phenolic content (TPC)	0.04	0.89
Total flavonoid content (TFC)	2.25	1.80
Total flavanol assay (TFA)	0.86	1.35
ABTS radical scavenging activity	0.84	0.50

N.A Not applicable
RSD Relative standard deviation

The precision of physical, microbiological, GC, HPLC and chemical analyses were evaluated in terms of relative standard deviation (RSD) as shown in TABLE 2. Overall, the RSD ranged from 0.00 to 7.25. These low values of RSD below 10% indicated the small variability and high precision of all physical, microbiological, instrumental and chemical analyses (Bievre & Gunzler, 2005; California Department of Toxic Substance, 2006).

As compared to other analyses, GC analyses have a slightly higher range of RSD values from 0.04 to 7.25. One of the possible factors was manual injection of sample into GC in fatty acid analyses because injection accuracy, speed of injection and syringe handling technique are crucial in accurate quantisation of fatty acid methyl esters (FAME). It is possible that some loss of the high boiling components may have occurred during initial injection entry and also after the removal of needle due to vaporization and fractionation of sample from needle (Barwick, 1999). Apart from that, pre-treatment of sample prior to injection into GC may contribute to larger RSD as these are subjective to personal handling techniques and consistency.

Determination of Fatty Acids and Bioactive Compounds

Table 3. Fatty acids and bioactive compounds in sample A (SA) and sample B (SB).

Analyses	SA[x]	SB[x]
GC analysis		
Fatty acids		
Stearic acid (C18:0) (mg/L)	2.00±0.13[b]	1.52±0.03[a]
Oleic acid (C18:1n9c) (mg/L)	5.10±0.01[a]	3.52±0.26[b]
Phytosterols		
Campesterol (mg/L)	3.38±0.01[b]	3.64±0.00[a]
Stigmasterol (mg/L)	N.D	0.82±0.06[a]
B-sitosterol (mg/L)	N.D	2.83±0.01[a]
HPLC analysis		
Acetic acid (g/L)	5.15±0.23[b]	6.52±0.39[a]
Betanin (g/L)	13.24±0.28[a]	14.23±0.61[a]
Isobetanin (g/L)	7.31±0.11[b]	12.73±0.54[a]
Chemical analysis		
Total phenolic content (TPC) (mg/100mL GAE)	371.63±0.15[b]	396.12±3.61[a]
Total flavonoid content (TFC) (mg/100mL CE)	119.70±2.69[b]	140.51±2.52[a]
Total flavanol assay (TFA) (mg/100mL CE)	81.21±0.69[b]	100.91±1.36[a]

[x] Each value in table represents the mean value ± standard deviation
[a-b] Mean value with different superscript in each column differs significantly ($p<0.05$)
N.D Not detected
GAE Gallic acid equivalent
CE Catechin equivalent

Bioactive compounds refer to essential and non-essential compounds that occur in nature, are part of the food chain, and can be shown to have an effect on human health (Bielsalski *et al.,* 2009). They typically occur in small quantities and present as natural constituents in food that provide health benefits beyond the basic nutritional value of the product. Bioactive compounds have been intensively studied lately due to their beneficial effect on cholesterol, cardiovascular diseases and cancer shown in many studies (Kris-Etherton *et al.,* 2002). These bioactive compounds are usually divided into various groups based on their chemical structure and functions like phenolic compounds and its subcategory, flavonoids, antioxidants pigments like betalains and its subgroup betacyanins (Henriette, 2009) as well as phytosterols (Saura & Goni, 2009).

Apart from bioactive compounds, two main fatty acids, namely stearic acid and oleic acid, were emphasized in SA and SB due to their beneficial effect on cholesterol level. Stearic acid was reported to have "neutral" effect on LDL and cholesterol level due to its rapid transformation into oleic acid (Valentin *et al.,* 2005) while Craig *et al.* (2000) suggested that stearic acid reduced plasma cholesterol level by reducing cholesterol absorption and increased excretion of endogenous cholesterol in hamster study. Oleic acid is well-studied for its effectiveness in reducing cholesterol level and decreases the risk of cardiovascular disease as reported by Stoutjesdijk *et al.* (2000), Kalyana *et al.* (2003) and Ribeiro *et al.* (2009). The results obtained were in accordance with Khor (2011) who also found that stearic acid and oleic acid are the main fatty acids in red dragon fruits (RDF) seeds. It was suggested that fatty acids and phytosterols found in RDF seed penetrated out from seeds into fermented liquid during fermentation process. This is supported by Fadahunsi & Sunni (2010) as well as

Afolabi *et al.* (2011) who mentioned that fermentation improved the fatty acids composition and oil yield from seeds. SA has significantly higher concentration ($p<0.05$) of stearic acid and oleic acid than SB, which were 2.00 mg/L and 5.10 mg/L respectively. This suggested that heat pasteurization on SB caused the reduction in stearic acid and oleic acid concentration, which is in accordance with Agata *et al.* (2005) and Somboonsilp *et al.* (2011) who also reported the decrease of stearic acids in their studies of heat pasteurization on milk and juice respectively.

Phytosterols are natural components that are largely derived from vegetable oils, plants and cereals. Among various plant sterols structures that have been discovered, the phytosterols found in the highest abundance in most plants are sitosterol, campesterol and stigmasterol (Pascal & Segal, 2006). According to Isabelle *et al.* (2009), recommended daily intake of 2g of phytosterols reduced LDL-cholesterol concentration by 10% by inhibiting cholesterol absorption by 30-40%. Therefore, phytosterols fortified food products were given much interest. From Table 3, SB showed a significantly higher concentration ($p<0.05$) of phytosterols (campesterol, stigmasterol and β-sitosterol) in contrast to fatty acids as compared to SA. Stigmasterol and β-sitosterol were not detected in SA probably due to its low concentration that was beyond the detection limit. One of the possible reasons of lower phytosterol concentration in SA was oxidation. According to Soupas (2006), phytosterols are prone to oxidation due to the double bond at the 5-position and the percentage of oxidation was dependent on the lipid matrix, fatty acids composition and sterol content. For instance, oxidation of sitosterol was noted to increase with increasing unsaturation of lipid matrix. This probably explained the lower concentration of phytosterols in SA due to its higher content of unsaturated fatty acids. The absence of stigmasterol and β-sitosterol was also supported by Carreno *et al.* (2008) who pointed out that stigmasterol, followed by cholesterol and sitosterol has the greatest susceptibility to oxidation in oil-in-water emulsions due to their differences in their surface activity. Also, it was suggested that the pasteurization temperature and time were too short to cause the significant loss of phytosterols in SB. This was supported by Carren *et al.* (2008) who reported that milk subjected to Schaal oven condition at 65°C for 24 hours only slightly reduced phytosterol content by 4%. In addition, Linda (2002) also stated that chemical stability of heat pasteurized phytosterol-esters containing milk and yogurt were found to be similar to standard product.

Organic acid is one of the common byproducts formed during fermentation. The content of organic acid not only influences the flavours but also the stability, acceptability, nutrition and keeping quality of a product (Shui & Leong, 2002). Acetic acid was emphasized in this study due to its beneficial effect on lowering cholesterol level as reported by Fushimi *et al.* (2006) and Dash (2009) on cholesterol-fed rats. As analysed by HPLC, SB showed a significantly higher acetic acid concentration compared to SA. The possible explanation was the exposure of SB to heat pasteurization increased the rate of acetate esters hydrolysis. This was supported by Pascal *et al.* (2006) who stated that ethyl acetate is one of the most prevalent esters found in fermented wine. The breakdown of esters thus increased the concentration of acetic acid in liquid (Jackson, 2008). The result was further supported by the higher ethanol concentration of SB as shown in Table 5.

Besides that, SB was found to exhibit significant higher concentration ($p<0.05$) of isobetanin content as compared to SA. This showed that the effect of pasteurization (75°C for 15s) did not decrease the concentration of betanin and isobetanin in SB. Instead, it was likely that the temperature was not too high and the time was too short to cause significant degradation of betacyanins. This is supported by (Henriette, 2009) who reported that loss of betacyanin in dragon fruit juice acidified to pH 4 is less than 10% during pasteurization at 80°C for 5 min. Also, the low pH of SB at pH 4.0 and the presence of vitamin C naturally present in dragon fruit were reported by Henriette (2009) to improve the stability of betacyanin pigments. Therefore, the difference in betanin and isobetanin concentration of SA

and SB was more likely to be caused by some other extrinsic factors like exposure to oxygen and light during sample treatment. Light and oxygen have been identified as the major factor of colour deterioration instead of heat; each caused betalain degradation by 15.6% and 14.6% as reported by Woo *et al.* (2011). In addition, different structures of betalains also contributed to its stability. For instance, glycosylated structures are more stable than a glycons, probably because of the higher oxidation–reduction potentials of the former (Henriette, 2009). Since betacyanins are most often glycosylated, thus it is likely to be stable when subjected to heat pasteurization for a short time (Mortensen, 2006). Betacyanin was given much interest lately due to its stability as a natural food colorant as well as its excellent antioxidant properties (Rebecca *et al.*,2010; Woo *et al.*,2011). In addition, Tsai *et al.* (2011) also reported its potent beneficial effect of lowering cholesterol. There are at least seven identified betacyanins in *Hylocereus polyrhizus*, in which betanin, isobetanin, betanidin, isobetanidin being the major red-violet pigment and others such as plhyllocactin, isophyllocactin, and bougainvillein-R-I, where all have identical absorption spectra that contribute to the colour of pulp (Henriette, 2009; Rebecca *et al.*, 2010). Among these, only betanin and isobetanin were quantified in this study due to the unavailability of commercial standard. Plant source food bioactive compounds such as phenolic compounds have been recognized as having potential to reduce disease risk such as coronary heart disease, cancer and reduce cholesterol level, mainly arisingfrom its antioxidant properties (Yang *et al*., 2007; Kuraswamy & Satish, 2008; Zainoldin & Baba, 2012). Therefore, various spectrophotometric methods were used for the quantification of phenolic compounds in fermented RDF liquid, such as total phenolic content (TPC), total flavonoid content (TFC) and total flavanol assay (TFA). All of these assays are used to determine various structural groups phenolic compounds based on different principles (Chang *et al*., 2002). From Table 3, SB was found to exhibit significant higher ($p<0.05$) value of TPC, TFC and TFA as compared to SA.

Total phenolic content (TPC) assay measures both free and bound phenolic compounds. The significant higher value of TPC in SB could be explained by the higher concentration of betacyanins that present. This is because betacyanins could also contributed to the total phenolic due to the presence of phenol structure in the molecule (Zainoldin & Baba, 2012). Apart from that, Anup *et al.* (2011) also found that pasteurized aonla juice at 75^{o}C had a higher amount of gallic acid during storage. It was likely that the effect of pasteurization also contributed to the increased concentration of total phenolics compounds in SB. This was supported by Yang *et al.* (2007) who reported that pasteurization at 65^{o}C and 75^{o}C will not significantly decrease noni juice quality as most of the phenolics from plants present in a more stable conjugated form with glycosides or esters. In addition, the difference in TPC value between SA and SB may also be affected by the presence of other compounds like oleic acid and reducing sugars like fructose (Yu, 2008).

Red dragon fruits are rich in naturally-occurring flavonoids (Kunnika & Pranee, 2011). These flavonoids were reported to have a wide range of biological activities such as antioxidant, enzyme-inhibiting, and antibacterial effects (Lee, 2009). Total flavonoid content (TFC) was examined by aluminium chloride colorimetric method. This method does not detect all kinds of flavonoids, instead only flavones and flavonols were found to complex stably with aluminium chloride. Even though it is not suitable to obtain values of total flavonoids, it is sufficient for comparative analysis (Chang *et al.*, 2002). In contrast to Laura *et al.* (2010) and Igual *et al.* (2011) who reported that heat pasteurization generally reduced flavonoid content in juices, SB showed a significantly higher ($p<0.05$) TFC value than SA. Hence, it is suggested that the difference in TFC value was due to the variation in ripeness of red dragon fruit in SA and SB.

Total flavanol assay (TFA) is mainly used to determine flavanols such as epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC) and catechin (C), which are well-known for their strong antioxidant

properties (Cabrera *et al.* 2006). Among these, affinity of DMACA to (-)-epicatechin was reported to be a few folds higher than other individual flavanol compounds (Treutter, 1989). As shown in TABLE 3, SB showed a significantly higher (p<0.05) TFA value than SA. This was in accordance with results reported by Fuleki and Ricardo (2003) who mentioned that pasteurization increased the concentration of catechins in cold-pressed juice. Also, Zimmerman & Gleichenhagen (2011) reported that higher steeping temperature of green tea infusions resulted in higher amount of catechins. Thus, it was suggested that the higher concentration of TFA in SB was due to the higher rate of catechins diffused into liquid as the pasteurization temperature at 75^{o}C was not too high to cause significant degradation of catechins. This was supported by Ziaedini *et al.* (2010) who observed that catechin degradation occurred in the temperature range of 80-90^{o}C.

Antioxidant Scavenging Capacity

Table 4. Antioxidant scavenging capacity of sample A (SA) and sample B (SB).

Chemical analyses	Unit	SA[x]	SB[x]
ABTS radical scavenging capacity	mM TEAC	0.54±0.02[b]	0.59±0.01[a]

[x] Each value in table represents the mean value ± standard deviation
[a-b] Mean value with different superscript in each column differs significantly (p<0.05)
TEAC Trolox equivalent antioxidant capacity

ABTS radical-scavenging capacity assay was chosen instead of the commonly used DPPH assay due to the absorbance interruption at 517 nm by the similar purple colour of fermented RDF liquid. In ABTS radical scavenging capacity assay, ABTS was converted to its radical cation by the addition of potassium per sulphate. The radical cation is reactive towards most antioxidants which include phenolics, thiols, and vitamin C (Bhagat *et al.*, 2011). As shown in Table 4, SB exhibited a significantly higher (p<0.05) radical scavenging capacity than SA. This was probably due to the significantly higher concentration of betanin, isobetanin, TPC, TFC as well as TFA of SB than in SA. Even though SA showed a higher vitamin C concentration than SB, it was suggested that betacyanins exhibited stronger oxidative power than vitamin C. These were supported by Cai *et al.* (2003) who reported that betalains showed stronger antioxidative power than ascorbic acid and catechins in the plants of *Amaranthaceae* and Tenore *et al.* (2011) who found that betacyanins fractions exhibited the highest radical-scavenging capacity among flavonoids and phenolics in red pitaya fruits. Also, phenolic content was reported by Lee *et al.* (2003) to be highly correlated with antioxidant capacity from ABTS assay and Lee (2009) mentioned that phenolic and polyphenolic compounds constituted the main class of natural antioxidants present in plants that accounted for their strong antioxidant properties. Besides that, heat pasteurization may also be one of the factors that contributed to higher radical scavenging capacity as heat treatment may release bound antioxidants as free antioxidants as stated by Yang *et al.* (2007). The cause of variations in the ABTS assay may be that the ABTS working solution was not always the same age, hence the reaction of the solution with fermented RDF liquid might have been different during the determination time (Thaipong *et al.*, 2006) and the ABTS radical solution concentration is difficult to control (Cai *et al.*, 2003).

Physico-Chemical Properties

Table 5. Physico-chemical profile of sample A (SA) and sample B (SB).

Analyses	Unit	SA[x]	SB[x]
pH	-	3.94±0.01[b]	4.00±0.01[a]
Total soluble solids (TSS)	Brix^{o}	23.1±0.12[a]	22.7±0.12[b]
Ethanol	%	7.31±0.08[b]	8.92±0.04[a]

[x] Each value in table represents the mean value ± standard deviation
[a-b] Mean value with different superscript in each column differs significantly (p<0.05)

Physico-chemical analyses are commonly used in determining food stability because physical properties of a food affect the environment for chemical reactions and microbial growth. As shown in Table 5, it was found that SA and SB were significantly different (p<0.05) from the aspect of pH, TSS and ethanol content. This suggested that the pasteurization treatment of SB may be one factor that caused the significant difference (p<0.05) in the physical properties of fermented RDF liquid as compared to non-pasteurized SA. However the results obtained were in contrast with some of the previous findings which stated that no remarkable increase in pH was observed after different thermal treatment by Rivas *et al.* (2006) and Sampedro *et al.* (2009). Therefore, it was more likely that the difference in pH was due to the different amount of organic acids produced from two individual fermentation tanks. Even so, the pH range of SA and SB were found to be acceptable and similar to some other fermented fruit wine like palm sap wine (pH 4.0), coconut palm sap (pH 3.7), Jambal fruit wine (pH 3.5), mango wine (pH 3.7) and banana wine (pH 3.85) as reported by George (2008).

Total soluble solids (TSS) is a measure of overall sugar content of mixture. Besides being a parameter in assessing food physical stability, it is also often associated with food sweetness and consumer acceptability (Harker *et al.*, 2002). Sampedro *et al.* (2009) reported no significant variation in Brix^{o} for thermally treated orange juice. It is more likely that the significant difference in TSS of SA and SB was due to the difference in ripeness of fruit as higher Brix^{o} is found in riper fruit as suggested by Rivas *et al.* (2006).

Ethanol is one of the by-products formed from fermentation particularly by heterogenic lactic acid bacteria and yeasts as stated by Asli (2010). It is believed to be the main contributor for the alcoholic flavour in SA and SB due to its presence in large quantities as well as its high reactivity of primary alcohols (Chung *et al.*, 2008; Sumby *et al.*, 2010). Overall, SB has significant higher ethanol content than SA. The possible reason was the exposure of SB to high heat during pasteurization. According to Jackson (2008), temperature directly affects the rate of reactions particularly ester hydrolysis in wine during storage. This may explain the significantly (p<0.05)higher ethanol (Table 5) and acetic acid content (Table 3) in SB as accelerated ester hydrolysis resulted in more ethanol and organic acids being released into the sample (Sumby *et al.*, 2010). These results were also supported by Patricia *et al.* (2006) who reported that acetate esters hydrolysed more quickly than other ethyl esters and these significant decreases were found in wine subjected to higher temperature at 45^{o}C.

Microbiological Properties

Table 6. Microbiological profile of sample A (SA) and sample B (SB).

Microbiological analyses	Unit	SA	SB
Total plate count	CFU/mL	N.D	N.D
Yeast and mould	CFU/mL	N.D	N.D

N.D Not detected ; CFU Colonies forming unit

Microbiological analysis plays an important role in judging the quality and safety of a food product. The growth of microorganisms and presence of pathogenic microorganisms need to be considered as these will lead to food spoilage and food safety issues (Yousef & Calstrom, 2003). As shown in TABLE 6, no growth of aerobic mesophilic bacteria, yeast or moulds was observed in either SA or SB. This indicated that SA and SB fall under the satisfactory level of microbiological standards for ready-to-eat food, which is less than 10^4 CFU/mL of sample (Centre for Food Safety, 2007; NSW Food Authority, 2009). Therefore both SA and SB can be confirmed microbiologically safe for consumption.

The absence of microorganisms regardless of pasteurization could be due to the restricting internal factor of SA and SB such as low pH, presence of ethanol, carbon dioxide and organic acids that were formed during fermentation as suggested by Erbas *et al.* (2005). Similar results were reported in other fermented products like plant beverage and maize by Kantachote *et al.* (2008), Alobo & Offonry (2009), Namugumya & Muyanja (2009), Chelule *et al.* (2010) who also reported that the combined effect of low pH (3.5-4.0) and presence of organic acids and ethanol were found inhibitive to bacteria.

CONCLUSION

In summary, these results confirmed that both SA and SB were microbiologically safe for consumption due to the absence of micro-organisms. The effect of pasteurization suggested possible beneficial effects on bioactive compound concentration as SB showed significantly (p<0.05) higher amounts of phytosterols (campesterol, stigmasterol, β-sitosterol), betacyanins (betanin, isobetanin), acetic acids, total phenolic content (TPC), total flavonoid content (TFC) and total flavanol assay (TFA) as well as greater (p<0.05) radical scavenging capacity in ABTS assay as compared to SA. However, the pasteurization also reduced the concentration of fatty acids, vitamins and minerals in SB. Further study can be done to maximize the concentration of bioactive compounds while minimizing the loss of vulnerable vitamins and minerals.

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