

**ISOLATION, PURIFICATION AND  
CHARACTERIZATION OF NATURAL  
RED PIGMENT FROM DRAGON FRUIT  
(*HYLOCEREUS POLYRHIZUS*)**

**OW PHUI SAN REBECCA**

**FACULTY OF SCIENCE  
UNIVERSITY OF MALAYA  
KUALA LUMPUR  
2012**

**ISOLATION, PURIFICATION AND CHARACTERIZATION OF  
NATURAL  
RED PIGMENT FROM DRAGON FRUIT  
(*HYLOCEREUS POLYRHIZUS*)**

**OW PHUI SAN REBECCA**

**THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT  
FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY**

**INSTITUTE OF BIOLOGICAL SCIENCES  
FACULTY OF SCIENCE  
UNIVERSITY OF MALAYA  
KUALA LUMPUR**

**2012**

## ABSTRACT

Stability, antioxidant properties, toxicology profile of betacyanins and selected target genes from *Hylocereus polyrhizus* were studied.

In the stability study, the pigments were obtained using water extraction and juice concentration; extracted at room temperature (RT) and 100°C; and stored under -20°C, 4°C and RT. In the water extraction method, the best weight: volume ratio was obtained using the ratio of 1:1. Pigments extracted at RT and from juice concentrate showed lower total betalain concentration changes as compared to samples extracted at 100°C and from water extraction. Pigments stored at -20°C under both extraction methods showed minimal change compared to those stored at RT and 4°C.

Analysis using the High Performance Liquid Chromatography (HPLC) confirmed the presence of betanin. Total polyphenol assay showed that there were 86.10mg of total polyphenolic compound in 0.50g of dried extract and this was further confirmed by the reducing power assay which showed an increase in the reducing capability from 0.18 to 2.37. The Vanillin-HCl assay which measures the amount of condensed tannin showed that the dried dragon fruit sample had an equivalent of 2.30mg catechin/g while the DPPH• radical scavenging activity determination showed that the effective concentration (EC<sub>50</sub>) for dragon fruit was 2.90 vitamin C equivalents/g dried extract.

X-ray crystallography, High Performance Liquid Chromatography (HPLC), Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) and Nuclear Magnetic Resonance (NMR) yielded a novel discovery of *myo*-inositol crystals. The purity level of crystals was verified using HPLC where a clean single peak was obtained, while LC-MS/MS was employed to provide a comparison with *myo*-inositol standard. NMR established the molecular structure and conformation of the crystals. This is the first time *myo*-inositol crystals were isolated and reported for *Hylocereus polyrhizus*.

Pigments for the toxicology screening were extracted with a customized six step filtration method: centrifugation, six layers of mira cloth, Whatman (No.6) filter papers, 1.6  $\mu\text{m}$  glass microfiber filters, and 0.45 $\mu\text{m}$  and 0.22 $\mu\text{m}$  nylon membrane filters. Toxicology analysis showed that the extract contained 750 cfu/g of total bacterial count (TBC) where the figure was well below the usual levels of < 1000 cfu/g that were reported in many other commercial fruit juice. Microorganism analysis on common foodborne pathogens, for example, yeast and mold, coliforms, *Escherichia coli* and *Salmonella* sp all resulted in negative. All targeted heavy metals (lead, mercury, arsenic, cadmium, tin and antimony), 52 organochlorine and 136 organophosphorus insecticides were not detected in this toxicology study.

In the molecular study, *matK* and 5-GT gene were successfully isolated and characterized. Bioinformatic analysis on the *matK* gene showed that it was unique and highly conserved within the cactus family.

The 5-GT gene, a pigment-producing gene similar to anthocyanin, has a signature domain for the plant secondary production gene (PSPG). The 3D structures of the predicted proteins were also generated and phylogenetic trees were drawn to show the relationship of *Hylocereus polyrhizus matK* and 5-GT genes to the corresponding genes in the database. Both sequences were characterized and successfully deposited in the GeneBank (NCBI) and were assigned the following accession numbers: JQ770196 and JQ770197.

This is the first time *matK* and 5-GT genes were cloned from *Hylocereus polyrhizus*.

## ABSTRAK

Kestabilan, kandungan antioksidan, profil toksikologi pigmen betasianin dan gen terpilih dari *Hylocereus polyrhizus* telah dikaji.

Dalam ujian kestabilan, pigmen telah diperolehi dengan menggunakan pengekstrakan air dan jus pekat; diekstrak pada suhu bilik dan 100°C; disimpan di bawah suhu -20°C, suhu bilik dan 4°C. Dalam kaedah pengekstrakan air, nisbah terbaik berat: isipadu adalah dengan menggunakan nisbah 1:1. Pigmen yang diekstrak pada suhu bilik dan dari jus pekat telah menunjukkan perubahan minimum dalam jumlah kepekatan betalain berbanding dengan sampel yang diekstrak pada 100°C dan kaedah pengekstrakan air. Pigmen yang disimpan pada -20°C dalam kedua-dua kaedah pengekstrakan menunjukkan perubahan yang minimum berbanding dengan sampel lain yang disimpan di suhu bilik dan 4°C.

Analisis dengan menggunakan Kromatografi Cecair Prestasi Tinggi (HPLC) mengesahkan kehadiran betanin. Assay jumlah polifenol menunjukkan bahawa terdapat 86.10mg jumlah kompaun polifenolik dalam 0.50g ekstrak kering dan ini disahkan dengan assay “reducing power” yang menunjukkan peningkatan keupayaan “reducing power” dari 0.18 ke 2.37.

Assay vanillin-HCl yang mengukur jumlah tannin pekat menunjukkan bahawa sampel kering buah naga bersamaan dengan 2.30mg catechin/g dan penentuan “DPPH• radical scavenging activity” menunjukkan bahawa kepekatan yang berkesan ( $EC_{50}$ ) bagi buah naga adalah setara dengan 2.90 vitamin C/g ekstrak kering.

X-ray kristalografi, Kromatografi Cecair Prestasi Tinggi (HPLC), Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) dan Getaran Magnetik Nuklear (NMR) menghasilkan penemuan novel kristal *myo*-inositol. Tahap ketulenan kristal telah disahkan dengan menggunakan HPLC di mana satu puncak tunggal bersih telah diperolehi, manakala LC-MS/MS digunakan untuk membuat perbandingan dengan standard *myo*-inositol. NMR telah menetapkan struktur molekul dan konformasi kristal. Ini merupakan kali pertama kristal *myo*-inositol telah diasingkan dan dilaporkan untuk *Hylocereus polyrhizus*.

Pigmen untuk pemeriksaan toksikologi telah diekstrak dengan kaedah penapisan enam langkah yang disesuaikan dengan menggunakan: “centrifugation”, enam lapisan kain mira; kertas Penapis Whatmann(No.6), penapis microfiber kaca 1.6  $\mu\text{m}$ , serta penapis membran nilon 0.45 $\mu\text{m}$  dan 0.22 $\mu\text{m}$ . Analisis toksikologi menunjukkan bahawa ekstrak mengandungi 750 cfu/g jumlah kiraan bakteria (TBC) di mana jumlah ini adalah jauh di bawah tahap biasa <1000 cfu/g yang dilaporkan di dalam jus buah-buahan komersil yang lain. Analisis mikroorganisma untuk mengesan patogen bawaan makanan yang biasa seperti yis dan kulapuk, koliform, *Escherichia coli* dan *Salmonella* sp kesemua keputusan kembali negatif.

Kesemua logam berat yang disasarkan (plumbum, merkuri, arsenik, cadmium, timah, dan antimoni), 52 racun serangga perosak organoklorin dan 136 racun serangga perosak organophosphorus tidak dikesan dalam kajian toksikologi ini.

Di dalam kajian molekular, gen *matK* dan 5-GT telah diasingkan dan dicirikan. Analisis bioinformatik gen *matK* menunjukkan bahawa ia adalah unik dan sangat terpelihara dalam keluarga kaktus sahaja. Gen 5-GT, gen yang terlibat dalam penghasilan pigmen seperti antosianin, mempunyai domain signature untuk gen penghasilan sekunder tumbuhan (PSPG).

Struktur 3D untuk protein yang diramalkan telah dijanakan dan pokok filogenetik telah dilukis untuk menunjukkan hubungan *matK* dan 5-GT *Hylocereus polyrhizus* kepada gen-gen sama di dalam pangkalan data. Kedua-dua turutan gen telah dicirikan dan didepositkan di dalam GenBank (NCBI) dan diberikan nombor kesertaan berikut: JQ770196 and JQ770197.

Ini merupakan kali pertama gen *matK* dan 5-GT diklon dari *Hylocereus polyrhizus*.



## ACKNOWLEDGEMENT

- Great is Thy faithfulness, morning by morning,  
new mercies I see, countless blessings, Thank You –

*Lamentations 3:22-23*

*First of all, I would like to express my utmost gratitude from the bottom of my heart to my supervisor, Dr. Chandran Somasundram for his constant guidance, immense contribution in time, constructive feedbacks and continuous advice in making this thesis possible. Secondly, my gratitude to Datuk Prof Dr Amru Nasrullah Boyce, my co-supervisor, for your valuable advice and opinions while supervising this thesis.*

*A big thank you also goes to Zuliana, Wei Lim, Kit, Daniel, Arina, Punitha, Wijen, Loo, Nadiah, Jasmine and Mr. Dorai for your continuous support and encouragement whilst working in the lab.*

*I couldn't have done this without my beloved family – dad, mom, Jonathan and Charis. My deepest appreciation for your unwavering prayers, unconditional love, words of wisdom, constant encouragement and care all along.*

*A note of thanks to Lee Huei, Bob for their friendship; my darling babies Zara Iman and Adhvik whose mere existence makes me believe that there are still miracles in the world. And those names I've failed to mention here, especially my friends, thank you for everything.*

*Last but not least, to my ever supportive boyfriend, Joshua, thank you for being my steady, thank you for understanding and your unlimited affection. I can't thank you enough.*

## LIST OF FIGURES

- Figure 2.1 *Hylocereus polyrhizus* plant with complex vine branching system
- Figure 2.2 Scaly and thorny appearance on every branch of the entire *Hylocereus polyrhizus* plant
- Figure 2.3 A *Hylocereus polyrhizus* flower
- Figure 2.4 A *Hylocereus polyrhizus* fruit
- Figure 2.5 Shikimate pathway in plants
- Figure 2.6 (a) Chemical structure of betalamic acid  
(b) Betalain and the resonating double bonds in the 1,2,4,7,7-pentasubstituted-1,7-diazaheptamethin system which attributes to the vast betalain colours.
- Figure 2.7 (a) An example of a betaxanthin: Indicaxanthin from *Opuntia ficus-indica*.  
(b) An example of a betacyanin: Betanin from *Beta vulgaris*.
- Figure 2.8 The initial biosynthetic pathway of betalains
- Figure 3.1 Pigment colour change extracted at RT and 100°C.
- Figure 4.1 Reducing power in *Hylocereus polyrhizus* pulp extract
- Figure 5.1 Example of crystals obtained after storage at 4 – 6°C for 7 days.
- Figure 5.2 View of crystal under a microscope at 10X magnification
- Figure 5.3 Orthographic projection of the asymmetric unit of *myo*-inositol along the *c* axis
- Figure 5.4 A spacefilled orthographic projection of the asymmetric unit of *myo*-inositol along the *c* axis
- Figure 5.5 A complete labelled ellipsoid plot of the two units of the asymmetric unit *myo*-inositol (C<sub>12</sub>H<sub>24</sub>O<sub>12</sub>)
- Figure 5.6 Crystal purity check using HPLC where the peak and retention time of the *myo*-inositol sample was observed at 4.8 minute
- Figure 5.7 Retention time of crystal sample observed using LC-MS/MS. One clean peak was obtained at 1.24 minute
- Figure 5.8 Retention time of *myo*-inositol standard observed using LC-MS/MS. One clean peak was obtained at 1.24 minute
- Figure 5.9 The Multichannel Analyser (MCA) scan from *myo*-inositol standard showing the precursor ion at 179.0 *m/z* and the product ion at 87.0 *m/z*

- Figure 5.10 One-dimensional  $^1\text{H}$  spectra of crystal sample
- Figure 5.11 One-dimensional  $^{13}\text{C}$  spectra of crystal sample
- Figure 5.12 Two-dimensional  $^1\text{H}/^1\text{H}$  spectra using COSY analysis on crystal sample
- Figure 6.1 A customized filtration method to obtain a pure and clarified *H. polyrhizus* extract
- Figure 7.1 PCR results with *matK* primers
- Figure 7.2 Nucleotide sequence of purified *matK* PCR product from *Hylocereus polyrhizus*
- Figure 7.3 The complete nucleotide sequence of *matK* gene and the amino acid sequence of its predicted product
- Figure 7.4 Comparison of the deduced amino acid sequence of *matK* gene from *Hylocereus polyrhizus* and sequences encoding the maturase K coding region from *Selenicereus boeckmannii* (AY015311), *Hylocereus peruvians* (AY015310), *Selenicereus vagans* (FN997113), and *Neoraimondia arequipensis* (AY015299). Amino acids were aligned using CLUSTALW
- Figure 7.5 Phylogenetic tree generated from the CLUSTALW multiple alignment programme to show the relationship between *matK* gene and the maturase K gene from *Selenicereus boeckmannii* (AY015311), *Hylocereus peruvians* (AY015310), *Selenicereus vagans* (FN997113), and *Neoraimondia arequipensis* (AY015299).
- Figure 7.6 Sequence analysis of *matK* gene
- Figure 7.7 The predicted coding region of *matK* domain within the nucleotide sequence with an ORF from the ATG position at 1173 and a in frame TGA termination codon for the coding region at position 1841
- Figure 7.8 The two conserved *matK* domain in the amino acid sequence where the domains are *matK/trnK* amino terminal region and Type II intron maturase
- Figure 7.9 3D structure of *Hylocereus polyrhizus* *matK* protein generated by a 3 state prediction (Helix/Strand/Coil)
- Figure 7.10 PCR results with GT5GENE primers
- Figure 7.11 Nucleotide partial sequence of 659bp from the purified PCR product of *Hylocereus polyrhizus* GT5GENE protein
- Figure 7.12 The complete nucleotide sequence of GT5GENE gene and the amino acid sequence of its predicted product

- Figure 7.13 Comparison of the deduced amino acid sequence of GT5 gene from *Hylocereus polyrhizus* and sequences from *Dianthus caryophyllus* (AB294380), *Volvox carteri* f. *nagariensis* (XM002952324) and *Forsythia x intermedia* (BAI65913)
- Figure 7.14 Phylogenetic tree generated from the CLUSTALW multiple alignment programme to show the relationship between GT5 gene from *Hylocereus polyrhizus* with *Dianthus caryophyllus* (AB294380), *Volvox carteri* f. *nagariensis* (XM002952324) and *Forsythia x intermedia* (BAI65913).
- Figure 7.15 Sequence analysis of GT5 gene
- Figure 7.16 The predicted open reading frame within the nucleotide sequence with an ORF from the ATG position at 87 and a TGA termination codon at position 329
- Figure 7.17 Alignment of the putative 5-O-glucosyltransferase (GT5) gene from *Hylocereus polyrhizus* with CaUGT2 (Curcumin glucosyltransferase), NtGT1b (a phenolic glucosyltransferase from tobacco) and B5GT (betanidin 5-O-glucosyltransferase).
- Figure 7.18 3D structure of *Hylocereus polyrhizus* GT5 protein generated by a 3 state prediction (Helix/Strand/Coil)

## LIST OF TABLES

Table 2.1	The six main groups of pigments
Table 2.2	Examples of betalains
Table 2.3	Approved colours for food industry in the European Union (Central and Eastern Europe, CEE) and in the Food and Drug Administration (FDA) of USA
Table 2.4	List of permitted and the most common naturally derived pigments from plants utilized in the current food industry
Table 2.5	Application of beet root powder as natural colour in food products
Table 3.1	Total betalain concentration of samples in different volumes of SDW
Table 3.2	pH measurement of samples in different volumes of SDW
Table 3.3	Total betalain concentration of samples at different temperatures
Table 3.4	pH measurement of samples at different temperatures
Table 3.5	Total betalain concentration of 5g of <i>Hylocereus polyrhizus</i> pulp extracted in 5ml of SDW at room temperature stored under various conditions
Table 3.6	pH measurement of samples stored under different conditions
Table 3.7	Total betalain concentration of 5g of <i>Hylocereus polyrhizus</i> pulp extracted in 5ml of SDW at 100 °C stored under various conditions
Table 3.8	pH measurement of samples stored under different conditions
Table 3.9	Total betalain concentration of 5 ml of <i>Hylocereus polyrhizus</i> juice concentrate extracted at RT stored under various conditions
Table 3.10	pH measurement of samples stored under different conditions
Table 3.11	Total betalain concentration of 5 ml of dragon <i>Hylocereus polyrhizus</i> concentrate extracted at 100 °C stored under various conditions
Table 3.12	pH measurement of samples stored under different conditions
Table 4.1	Retention time and peak area of <i>Hylocereus polyrhizus</i> sample and betanin standard
Table 4.2	Total polyphenolic content, flavonoid content and DPPH• radical scavenging activity
Table 5.1	LC-MS/MS analysis on <i>myo</i> -inositol standard and crystal sample

Table 6.1	Microorganisms detection from the toxicology analysis carried out on <i>Hylocereus polyrhizus</i> sample
Table 6.2	Heavy metal analysis from the toxicology analysis carried out on <i>Hylocereus polyrhizus</i> sample
Table 7.1	Consensus pattern of <i>matK</i> isolated from <i>Hylocereus polyrhizus</i> consistent with eight other deposited sequences
Table 7.2	The sequences of the super-family domains of <i>matK/trnK</i> amino terminal region and Type II intron maturase region
Table 7.3	Seven distinct patterns of <i>matK</i> isolated from <i>Hylocereus polyrhizus</i>
Table 7.4	Five distinct patterns of GT5 gene isolated from <i>Hylocereus polyrhizus</i>

## LIST OF ABBREVIATIONS

3D	3-Dimension
5-GT	5-glucosyltransferase
$\lambda$	Lambda
$\mu\text{g}$	Microgram
$\mu\text{l}$	Microliters
ADI	Acceptable Daily Intake
ATP	Adenosine triphosphate
cfu	Colony forming units
cyclo-DOPA	cyclo-dihydroxyphenylalanine
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanin
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	Effective concentration
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
kb	Kilobase
L-DOPA	L-5,6-dihydroxyphenylalanine
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
<i>matK</i>	<i>MaturaseK</i>
ml	Mililiters
NaCl	Sodium chloride
nm	Nanometers
NMR	Nuclear Magnetic Resonance
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
ppm	Parts per million
PSPG	Plant secondary production gene

PVP	Polyvinylpyrrolidones
RT	Room temperature
SDW	Sterile distilled water
TE	Tris-ethylenediaaminetetraacetic acid
USFDA	Food and Drug Administration (FDA) of USA
UV	Ultraviolet
WHO	World Health Organization



## LIST OF APPENDICES

- Appendix 1** Rebecca OPS, Zuliana R, Boyce AN and Chandran S (2008). Determining pigment extraction efficiency and pigment stability of dragon fruit (*Hylocereus polyrhizus*). Journal of Biological Sciences 8 (7): 1174 – 1180
- Appendix 2** Silver Medal Award in the University of Malaya Research Expo 2009
- Appendix 3** Rebecca OPS, Boyce AN and Chandran S (2010). Pigment identification and antioxidant properties of red dragon fruit (*Hylocereus polyrhizus*) African Journal of Biotechnology: 9 (10): 1450 – 1454
- Appendix 4** Rebecca OPS, Boyce AN and Chandran S (2012). Isolation and identification of myo-inositol crystals from dragon fruit (*Hylocereus polyrhizus*). Molecules 17: 4583 - 4594
- Appendix 5** The official result for all the analysis carried out on the *Hylocereus polyrhizus* extract from Consolidated Laboratory (M) Sdn Bhd
- Appendix 6** The official result for all the analysis carried out on the *Hylocereus polyrhizus* extract from Consolidated Laboratory (M) Sdn Bhd
- Appendix 7** List of the tested organochlorine insecticides
- Appendix 8** List of the tested organophosphorus insecticides
- Appendix 9** Wizard<sup>R</sup> SV gel and PCR purification Clean-up system (Promega) quick protocol
- Appendix 10** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW
- Appendix 11** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 10ml of SDW
- Appendix 12** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 15ml of SDW
- Appendix 13** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 25ml of SDW
- Appendix 14** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at RT for 10 minutes
- Appendix 15** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at 40°C for 10 minutes
- Appendix 16** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at 60°C for 10 minutes
- Appendix 17** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at 80°C for 10 minutes
- Appendix 18** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at 100°C for 10 minutes
- Appendix 19** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at RT for 10 minutes before keeping for one week at RT, exposed to light.
- Appendix 20** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at RT for 10 minutes after keeping for one week at RT, exposed to light.
- Appendix 21** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at RT for 10 minutes before keeping for one week at RT in the dark.

- Appendix 22** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at RT for 10 minutes after keeping for one week at RT in the dark.
- Appendix 23** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at RT for 10 minutes before keeping for one week at 4°C
- Appendix 24** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at RT for 10 minutes after keeping for one week at 4°C
- Appendix 25** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at RT for 10 minutes before keeping for one week at -20°C
- Appendix 26** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at RT for 10 minutes after keeping for one week at -20°C
- Appendix 27** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at 100°C for 10 minutes before keeping for one week at RT, exposed to light
- Appendix 28** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at 100°C for 10 minutes after keeping for one week at RT, exposed to light
- Appendix 29** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at 100°C for 10 minutes before keeping for one week at RT in the dark
- Appendix 30** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at 100°C for 10 minutes after keeping for one week at RT in the dark
- Appendix 31** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at 100°C for 10 minutes before keeping for one week at 4°C
- Appendix 32** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at 100°C for 10 minutes after keeping for one week at 4°C
- Appendix 33** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at 100°C for 10 minutes before keeping for one week at -20°C
- Appendix 34** pH measurement, absorbance reading and total betalain concentration of 5g of dragon fruit pulp in 5ml of SDW at 100°C for 10 minutes after keeping for one week at -20°C
- Appendix 35** pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at RT for 10 minutes before keeping for one week at RT, exposed to light
- Appendix 36** pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at RT for 10 minutes after keeping for one week at RT, exposed to light
- Appendix 37** pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at RT for 10 minutes before keeping for one week at RT in the dark

<b>Appendix 38</b>	pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at RT for 10 minutes after keeping for one week at RT in the dark
<b>Appendix 39</b>	pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at RT for 10 minutes before keeping for one week at 4°C
<b>Appendix 40</b>	pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at RT for 10 minutes after keeping for one week at 4°C
<b>Appendix 41</b>	pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at RT for 10 minutes before keeping for one week at -20°C
<b>Appendix 42</b>	pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at RT for 10 minutes after keeping for one week at -20°C
<b>Appendix 43</b>	pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at 100°C for 10 minutes before keeping for one week at RT, exposed to light
<b>Appendix 44</b>	pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at 100°C for 10 minutes after keeping for one week at RT, exposed to light
<b>Appendix 45</b>	pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at 100°C for 10 minutes before keeping for one week at RT in the dark
<b>Appendix 46</b>	pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at 100°C for 10 minutes after keeping for one week at RT in the dark
<b>Appendix 47</b>	pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at 100°C for 10 minutes before keeping for one week at 4°C
<b>Appendix 48</b>	pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at 100°C for 10 minutes after keeping for one week at 4°C
<b>Appendix 49</b>	pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at 100°C for 10 minutes before keeping for one week at -20°C
<b>Appendix 50</b>	pH measurement, absorbance reading and total betalain concentration of 5ml of dragon fruit juice concentrate extracted at 100°C for 10 minutes after keeping for one week at -20°C
<b>Appendix 51</b>	Dragon fruit sample HPLC analysis 1
<b>Appendix 52</b>	Dragon fruit sample HPLC analysis 2
<b>Appendix 53</b>	Dragon fruit sample HPLC analysis 3
<b>Appendix 54</b>	Retention time and area of dragon fruit sample obtained from HPLC
<b>Appendix 55</b>	Betanin standard from beet root in HPLC analysis 1
<b>Appendix 56</b>	Betanin standard from beet root in HPLC analysis 2
<b>Appendix 57</b>	Betanin standard from beet root in HPLC analysis 3
<b>Appendix 58</b>	Retention time and area of dragon fruit sample obtained from HPLC
<b>Appendix 59</b>	Gallic acid standard curve for total polyphenolic content determination
<b>Appendix 60</b>	Total polyphenolic content determination in dragon fruit sample
<b>Appendix 61</b>	Reducing power assay in dragon fruit sample

<b>Appendix 62</b>	Catechin standard for Vanillin-HCl assay in dragon fruit sample
<b>Appendix 63</b>	Vanillin-HCl assay in dragon fruit sample
<b>Appendix 64</b>	Vitamin C standard curve for DPPH <sup>•</sup> radical scavenging activity in dragon fruit sample
<b>Appendix 65</b>	% of inhibition in DPPH <sup>•</sup> radical scavenging activity in dragon fruit sample

# ISOLATION, PURIFICATION AND CHARACTERIZATION OF NATURAL RED PIGMENT FROM DRAGON FRUIT (*Hylocereus polyrhizus*)

<b>Abstract</b>		<b>ii</b>
<b>Abstrak</b>		<b>v</b>
<b>Acknowledgement</b>		<b>viii</b>
<b>List of Figures</b>		<b>ix</b>
<b>List of Tables</b>		<b>xii</b>
<b>List of Abbreviations</b>		<b>xiv</b>
<b>List of Appendices</b>		<b>xvi</b>
<b>Chapter 1</b>	Introduction	<b>1</b>
<b>Chapter 2</b>	Literature Review	<b>10</b>
2.1	Introduction to Dragon Fruit	
2.2	Botanical Description of <i>Hylocereus polyrhizus</i>	
2.2.1	Origin	
2.2.2	Taxonomy	
2.2.3	The plant	
2.2.4	The flower	
2.2.5	The fruit	
2.3	Economic Importance	
2.4	The Malaysian Scenario	
2.5	Secondary Metabolites	
2.5.1	General secondary metabolite pathway	
2.5.2	Role of secondary metabolite in plants	
2.5.3	Phytochemicals	
2.5.4	Phenolics and polyphenolics	
2.5.5	Significance to human health	
2.6	Plant Pigments	
2.7	Betalains	
2.7.1	Betalain structures	
2.7.2	Betalain biosynthesis	
2.7.3	Betalains in <i>Hylocereus polyrhizus</i>	
2.8	The Artificial Colouring Industry	
2.9	Dangers of Synthetic Dyes	
2.10	Food Colouring	
2.10.1	Available natural food dyes from plants	
2.11	Potential dye from <i>Hylocereus polyrhizus</i>	

<b>Chapter 3</b>	<b>Extraction Efficiency and Stability of Dragon Fruit (<i>Hylocereus polyrhizus</i>) Pigments</b>	<b>51</b>
3.1	Introduction	
3.2	Materials and Methods	
3.2.1	Plant material	
3.2.2	Pigment extraction	
3.2.3	Sample measurements	
	3.2.3.1 Determination of total betalain concentration in samples	
	3.2.3.2 pH measurement	
3.2.4	Efficiency of water volume to extract pigment	
3.2.5	Efficiency of temperature to extract pigment	
3.2.6	Stability of pigments extracted with water	
	3.2.6.1 Sample preparation	
	3.2.6.2 Stability test samples	
3.2.7	Stability of pigments from juice concentrate	
	3.2.7.1 Sample preparation	
	3.2.7.2 Stability test samples	
3.3	Results	
3.3.1	Efficiency of water volume to extract pigment	
3.3.2	Efficiency of temperature to extract pigment	
3.3.3	Stability of pigments extracted with water	
	3.3.3.1 Samples extracted at room temperature	
	3.3.3.2 Samples extracted at 100 °C	
3.3.4	Stability of pigments from juice concentrate	
	3.3.4.1 Juice concentrate extracted at room temperature	
	3.3.4.2 Juice concentrate extracted at 100°C	
3.4	Discussion	
<b>Chapter 4</b>	<b>Identification and Antioxidant Properties of Dragon Fruit (<i>Hylocereus polyrhizus</i>) Pigment</b>	<b>68</b>
4.1	Introduction	
4.2	Materials and Methods	
4.2.1	Plant Material	
4.2.2	Qualification of betacyanins using HPLC method	
	4.2.2.1 Reagent preparation	
	4.2.2.2 Sample preparation	
	4.2.2.3 HPLC	
4.2.3	Determination of Antioxidant Properties	
	4.2.3.1 Reagent Preparation	
	4.2.3.1.1 Reagents for total polyphenol determination	
	4.2.3.1.2 Reagents for reducing power assay	
	4.2.3.1.3 Reagents for Vanillin-HCl assay	
	4.2.3.1.4 Reagents for 1,1-diphenyl-2-picrylhydrazyl (DPPH') radical scavenging activity	
	4.2.3.2 Sample preparation	
	4.2.3.3 Determination of total phenolic contents and reducing power assay	
	4.2.3.4 Determination of flavonoid content (Vanillin-HCl assay)	

	4.2.3.5 1,1-diphenyl-2-picrylhydrazyl (DPPH <sup>*</sup> ) radical scavenging activity	
4.3	Results	
	4.3.1 Qualification of betacyanins using HPLC method	
	4.3.2 Antioxidant properties	
4.4	Discussion	
	4.4.1 Qualification of betacyanin using HPLC method	
	4.4.2 Antioxidant properties	
<b>Chapter 5</b>	<b>Isolation and Identification of <i>Myo</i>-Inositol Crystals from Dragon Fruit (<i>Hylocereus Polyrrhizus</i>)</b>	<b>86</b>
5.1	Introduction	
5.2	Materials and Methods	
	5.2.1 Sample Preparation	
	5.2.2 Purification	
	5.2.3 Crystallization	
	5.2.4 Crystal structure determination using X-Ray Crystallography	
	5.2.5 Qualification of crystal purity Using High Performance Liquid Chromatography	
	5.2.6 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) analysis	
	5.2.7 Nuclear Magnetic Resonance (NMR) analysis	
5.3	Results	
	5.3.1 Purification and crystallization	
	5.3.2 X-Ray crystallography analysis	
	5.3.3 Qualification of crystal purity using High Performance Liquid Chromatography	
	5.3.4 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) analysis	
	5.3.5 NMR analysis	
5.4	Discussion	
<b>Chapter 6</b>	<b>Toxicology Analysis of Betacyanin Extracted from Dragon Fruit (<i>Hylocereus polyrrhizus</i>)</b>	<b>116</b>
6.1	Introduction	
6.2	Materials and Methods	
	6.2.1 Plant Material	
	6.2.2 Filtration	
	6.2.3 Toxicology Analysis	
	6.2.3.1 Total bacterial count	
	6.2.3.2 Yeast and mould colony count	
	6.2.3.3 General coliform group detection	
	6.2.3.4 <i>Escherichia Coli</i> detection	
	6.2.3.5 <i>Salmonella</i> Sp. detection	
	6.2.3.6 Heavy metal analysis	
	6.2.3.7 Pesticide screening	
6.3	Results	
	6.3.1 Microorganisms Analysis	
	6.3.2 Heavy Metal Analysis	

6.3.3	Pesticide Screening	
6.4	Discussion	
<b>Chapter 7</b>	<b>Cloning and characterization of <i>matk</i> and 5-Glucosyltransferase Genes from Dragon Fruit (<i>Hylocereus polyrhizus</i>)</b>	<b>138</b>
7.1	Introduction	
7.2	Materials and Methods	
7.2.1	Plant Material	
7.2.2	Extraction of DNA from <i>Hylocereus polyrhizus</i> Pulp Tissue	
7.2.2.1	Preparation of reagents	
7.2.2.2	DNA extraction	
7.2.3	Preparation Of 1% Agarose Gel	
7.2.4	Quantification of DNA Template	
7.2.5	PCR of <i>Hylocereus polyrhizus</i> DNA	
7.2.5.1	<i>matk</i> gene primers	
7.2.5.2	5-GT gene primers	
7.2.5.3	Template preparation	
7.2.6	Purification of PCR Product	
7.2.7	Sequencing of PCR Product	
7.2.8	Bioinformatics	
7.3	Results	
7.3.1	PCR Results with <i>matk</i> Primers	
7.3.1.1	Nucleotide sequence of the <i>matK</i> protein	
7.3.1.2	BLAST analysis on nucleotide sequence of the <i>matK</i> protein	
7.3.1.3	ClustalW (2.1) multiple sequence alignment for <i>matK</i> gene	
7.3.1.4	Sequence analysis of <i>matK</i> gene	
7.3.1.5	Features of the <i>matK</i> gene	
7.3.2	PCR Results with GT5GENE Primers	
7.3.2.1	Nucleotide sequence of the GT5GENE protein	
7.3.2.2	BLAST analysis on nucleotide sequence of the GT5GENE protein	
7.3.2.3	ClustalW (2.1) multiple sequence alignment for GT5GENE gene	
7.3.2.4	Sequence analysis of GT5 gene	
7.3.2.5	Features of the GT5 gene	
7.4	Discussion	
<b>Chapter 8</b>	<b>General Discussion</b>	<b>186</b>
	<b>Literature Cited</b>	<b>196</b>
	<b>Appendices</b>	<b>227</b>