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

**OW PHUI SAN REBECCA**

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

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

**INSTITUTE OF BIOLOGICAL SCIENCES**
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# 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 100ºC; and stored under -20ºC, 4ºC 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 100ºC and from water extraction. Pigments stored at -20ºC under both extraction methods showed minimal change compared to those stored at RT and 4ºC.

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 ($EC_{50}$) 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 µm glass microfiber filters, and 0.45µm and 0.22µm 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, *mat*K and 5-GT gene were successfully isolated and characterized. Bioinformatic analysis on the *mat*K 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 mat*K 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 *mat*K 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 100ºC; disimpan di bawah suhu -20ºC, suhu bilik dan 4ºC. Dalam kaedah pengektrakan 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 100ºC dan kaedah pengekstrakan air. Pigmen yang disimpan pada -20ºC dalam kedua-dua kaedah pengekstrakan menunjukkan perubahan yang minimum berbanding dengan sampel lain yang disimpan di suhu bilik dan 4ºC.

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 µm, serta penapis membran nilon 0.45µm dan 0.22µm. 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 *mat*K dan 5-GT telah diasingkan dan dicirikan. Analisis bioinfomatik gen *mat*K 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 *mat*K 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 *mat*K dan 5-GT diklon dari *Hylocereus polyrhizus*.

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*- Great is Thy faithfulness, morning by morning,*
*new mercies I see, countless blessings, Thank You –*
*Lamentations 3:22-23*

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# LIST OF FIGURES

# LIST OF TABLES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 3D | 3-Dimension |
| 5-GT | 5-glucosyltransferase |
| λ | Lambda |
| µg | Microgram |
| µl | 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 aicd |
| 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-dihydrophenylalanine |
| LC-MS/MS | Liquid Chromatography-Tandem Mass Spectrometry |
| *mat*K | *Maturase*K |
| 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

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