PRODUCTION OF BETALAIN COMPOUNDS FROM DRAGON FRUIT *Hylocereus costaricensis* *IN VITRO* PLANT CELL CULTURE SYSTEMS

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UNIVERSITI SAINS MALAYSIA

2020

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by

WINSON KOE WEI SHENG

Thesis submitted in fulfillment of the requirements for the degree of Master of Science

July 2020

ACKNOWLEDGEMENT

First and foremost, I would like to offer my thanks to God Almighty for bestowing upon me wisdom, understanding, perseverance, strength and the peace of mind to complete this research.

I express my appreciation and gratitude to my principal supervisor Professor Dr. Sreeramanan Subramaniam, co-supervisors Dr. Chew Bee Lynn and Associate Professor Dr. Kathiresan Sathasivam for their advices, guidance and support which were imperative throughout this research.

I am truly indebted to the Agricultural Crop Trust (ACT) for awarding me with scholarship to pursue my Master's degree. I would like to thank every generous sponsor at ACT. It is the goodwill and generosity of ACT which enabled me to continue and complete my postgraduate studies. The School of Biological Sciences, Universiti Sains Malaysia deserves no less credit for providing the necessary facilities required throughout my research.

I wish to express my gratitude to all my lab mates and friends who provided me a constant source of positivity and encouragement, making my challenging research journey much more enjoyable. As for all those who have directly or indirectly contributed to the birth of this thesis, thank you very much.

Last but not least, I am ever grateful to my parents for their love and unwavering support.

WINSON KOE

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

%	Percent
°C	Degree Celsius
µg/g	Microgram per gram
µM	Micromolar
$\mu mol\ m^{-2}\ s^{-1}$	Micromole per square metre per second
2,4-D	2,4-dichlorophynoxyacetic acid
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
cm	Centimetre
cm^2	Centimetre square
CO_2	Carbon dioxide
cv.	Cultivar
DAS	Days after sowing
DM	Dry mass
DNA	Deoxyribonucleic acid
$FADH_2$	Flavin adenine dinucleotide, reduced
FM	Fresh mass
g	Gram
g/mol	Grams per mole
GA_3	Gibberellic acid
h	Hour
IBA	Indole-3-butyric acid
$L\ mol^{-1}\ cm^{-1}$	Litre per mole per centimetre
LED	Light emitting diode

mg/g	Milligram per gram
mg/L	Milligram per litre
mg/mL	Milligram per millilitre
mL	Millilitre
mm	millimetre
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog
NAA	Napthaleneacetic acid
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NH_4^+	Ammonium ion
nm	Nanometre
NO_3^-	Nitrate ion
PAL	Phenylalanine ammonia lyase
PGR	Plant growth regulator
RLG	Relative light germination
rpm	Revolutions per minute
SE	Standard error
sp./ spp.	Species/ Species (plural)
TDZ	Thidiazuron
UAE	Ultrasonic-assisted extraction
v/v	Volume per volume
W	Watt
w/v	Weight per volume
$\bar{x}$	Mean
P_{fr}	Phytochrome active form that initiates biological responses

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PENGHASILAN SEBATIAN BETALAIN DARIPADA *Hylocereus costaricensis* DENGAN MENGGUNAKAN SISTEM *IN VITRO* KULTUR SEL TUMBUHAN

ABSTRAK

Hylocereus costaricensis lebih popular dikenali sebagai pitaya atau buah naga yang berwarna ungu-merah diklasifikasikan dalam famili Cactaceae dan merupakan salah satu sumber pewarna semula jadi yang amat menjanjikan disebabkan oleh buah naga kaya dengan sebatian betalain. Objektif utama kajian ini adalah untuk menginduksi kalus, menambah baik proliferasi kalus dan meningkatkan kandungan sebatian betalain melalui pendekatan *in vitro*. Anak benih aksenik *H. costaricensis* telah dicambah serta ditumbuhkan di atas medium MS separuh kepekatan yang digelkan dengan 5 g/L agarose dan dikekalkan di bawah pencahayaan putih sejuk pendarfluor. Di peringkat awal, potensi kalus induksi telah dikaji dengan menggunakan segmen epicotil dan kotilidon daripada anak benih aksenik yang berusia dua bulan dan dikulturkan di atas medium MS separuh kekuatan yang ditambahkan dengan pelbagai kepekatan NAA dan TDZ secara berasingan. Kesan pencahayaan (pencahayaan fotokala 16 jam dan kegelapan) terhadap induksi kalus juga dikaji. Secara umumnya, eksplan kotiledon yang dikulturkan di bawah pencahayaan fotokala 16 jam lebih optimum untuk menginduksikan kalus. Namun, kalus padat terbentuk bersama-sama dengan akar di bawah pengaruh NAA manakala kalus berbentuk butir terbentuk di media yang ditambah dengan TDZ. Kepekatan dan kombinasi pikloram dan 2,4-D juga diuji dalam percubaan mendapatkan kalus rapuh. Penggunaan 10 µM pikloram dapat menginduksikan kalus rapuh. Indeks pertumbuhan kalus dalam medium separuh kepekatan MS yang ditambah dengan 10 µM pikloram adalah 2.1 kali ganda dan 3 kali ganda lebih tinggi daripada 5 µM picloram + 10 µM 2,4-D dan 25 μM picloram masing-masing pada subkultur ketiga, oleh itu, optimum sebagai medium proliferasi. Kalus bertukar dari warna putih ke warna kuning muda dengan sedikit tompok merah selepas beberapa kali subkultur. Kalus yang berwarna merah telah diasingkan dan terus disubkulturkan manakala kalus berwarna kuning juga turut disubkulturkan secara berasingan. Dalam kalus merah, penambahan tyrosine sebagai pendahulu kepada laluan biosintetik betalain telah mengurangkan jisim kalus. Kandungan betalain telah ditingkatkan masing-masing dengan penambahan 50 μM tyrosine dan 200 μM leucine sebanyak 1.6 kali ganda dan 2.56 kali ganda. Namun demikian, proliferasi kalus terjejas. Elisitasi kalus kuning dengan 100 mg/L ekstrak yis meningkatkan kandungan betalain sebanyak 2 kali ganda tanpa menjejaskan jisim kalus. Kandungan betalain juga dapat ditingkatkan sebanyak 1.23 kali ganda dengan menggunakan 20 μM argentum nitrat manakala jisim kering kalus dan kandungan fenolik telah bertambah dengan penggunaan 40 μM argentum nitrat. Penemuan ini berguna untuk meningkatkan jisim kalus serentak dengan peningkatan kandungan betalain. Pencahayaan adalah satu strategi yang berpotensi untuk meningkatkan jisim kalus dan kandungan betalain. Kandungan betalain dalam kalus kuning telah meningkat sebanyak 3.8 kali ganda manakala dalam kalus merah telah meningkat sebanyak 4.8 kali ganda kandungan betalain ($p < 0.01$), masing-masing dengan pencahayaan yang dibekalkan oleh diod pemancar cahaya yang bewarna merah. Kajian ini melaporkan protokol yang mudah dan cekap untuk menghasilkan sebatian betalain melalui kaedah *in vitro* dari *H. costaricensis*. Justeru, kajian ini membolehkan penghasilan sebatian betalain secara berskala komersial di bawah persekitaran yang terkawal, bebas daripada ketidakstabilan pengaruh iklim dan tanah.

PRODUCTION OF BETALAIN COMPOUNDS FROM *Hylocereus costaricensis IN VITRO* PLANT CELL CULTURE SYSTEMS

ABSTRACT

Hylocereus costaricensis more popularly known as the purplish-red pitaya or dragon fruit belongs to the Cactaceae family and is one of the next promising biocolourant because of its abundance in betalain compounds. The primary objectives of this study are to induce callus, improve the callus proliferation and enhance betalain content through *in vitro* means. Axenic seedlings of *H. costaricensis* were raised in half-strength MS medium, supported on 5 g/L agarose and cultured under the cool white fluorescent lights. In the preliminary study on callus induction, epicotyl and cotyledonary segments of two-month-old axenic seedlings were examined for their callus induction potential when supplemented with various concentrations of NAA and TDZ. The effects of illumination (16-h photoperiod and darkness) on callus induction were also investigated. Generally, cotyledonary explants cultured under 16-h photoperiod was optimum for callus induction. Under the influence of NAA, compact callus with roots were formed while in TDZ supplemented medium formed granular callus. Different concentrations and combinations of picloram and 2,4-D were tested for the induction of friable callus. Picloram at 10 µM picloram induced friable callus with substantial callus biomass. The growth index of callus in half-strength MS medium supplemented with 10 µM picloram was 2.1-fold and 3-fold greater compared to 5 µM picloram + 10 µM 2,4-D and 25 µM picloram respectively in the third subculture cycle, therefore optimum for the proliferation of callus. Callus changed from white colour to pale yellow with some red variegated regions after few subcultures. The red regions of callus were separated and continue subcultured, meanwhile the pale yellow callus was also subcultured. In red callus cultures, addition of precursor tyrosine significantly reduced callus biomass. Total betalain content were enhanced by 1.6-fold with 50 µM tyrosine and 2.56-fold with 200 µM leucine, however, at the expense of callus proliferation. Elicitation of yellow callus cultures with 100 mg/L yeast extract enhanced betalain content by 2-fold without affecting callus biomass. Betalain content could also be improved by 1.23-fold with 20 µM silver nitrate while callus dry mass and total phenolic content were elevated at 40 µM silver nitrate. This is useful in simultaneously increasing callus biomass and improved betalain content. Utilization of light is a promising strategy for enhanced production of secondary metabolites in callus cultures of *H. costaricensis*. Both yellow and red callus cultures incubated under red LED lights enhanced total betalain content by 3.8-fold and 4.8-fold, significantly higher ($p < 0.01$) than cool white LED lights (control treatment). The present investigation reports a simple and efficient protocol for *in vitro* betalain production from *Hylocereus costaricensis*. This study may enable the future commercial production of betalain compounds under a controlled environment, independent of climate and soil conditions.

CHAPTER 1

INTRODUCTION

Hylocereus costaricensis (commonly known as red purple dragon fruit or pitaya) is a climbing vine species grouped under the Cactaceae family. It first gained recognition among ornamentalists for its large creamy white flowers and later on being widely grown as a fruit crop (Le Bellec *et al.*, 2006). *Hylocereus* spp. are endemic to Latin America and is now cultivated in Taiwan, Vietnam, Malaysia, Thailand, Indonesia, northern Australia and southern China (Alimi *et al.*, 2012).

The red pitaya has now gained popularity for its attractive deep red purple colouration contributed by betalain pigments. Betalain compounds comprised of red-violet betacyanins and yellow betaxanthins. It can only be found in thirteen families of the order Caryophyllales (Moreno *et al.*, 2008). In this study, the compounds of interest are betalains. Betalains have been extracted and identified from *H. polyrhizus* (Rebecca *et al.*, 2010). The betalain contents in both flesh and peel have been reported (Esquivel *et al.*, 2007b). Their stability in pH 3-7 range made this class of pigments a promising natural colour source over anthocyanins in colouring fruit juices, ice cream and dairy products (Jackman and Smith, 1996; Azeredo, 2009). These pigments also conferred additional nutritional values and their most vital role as an antioxidant (Kanner *et al.*, 2001; Wybraniec and Mizrahi, 2002; Nurliyana *et al.*, 2010; Tenore *et al.*, 2012). Recent studies have focused on the betalain pigments and antioxidant activity of *Hylocereus* spp. as they have preventive qualities against oxidative stress related disorders (Wu *et al.*, 2006).

In vitro culture systems are an attractive alternative over conventional field cultivation of whole plants in terms of its seasonal, geographical and environmental independence. Plant tissue culture also offers an aseptic, controlled production conditions, uniformity in yield and continuous supplies of easily extractable products (Rao and Ravishankar, 2002; Mulabagal *et al.*, 2004; Georgiev *et al.*, 2008). The shortcomings of unsustainable and inconsistent supply of secondary metabolites from intact plants can be overcome using plant tissue culture. Under optimized conditions, the efficacy of plant tissue culture to accumulate secondary metabolites at relatively higher levels compared to intact plants has been proven, for instance, berberin from *Coptis japonica* (Matsubara *et al.*, 1989), shikonin from *Lithospermum erythrorhizon* (Kim and Chang, 1990) and rosmarinic acid from *Coleus blumei* (Petersen and Simmonds, 2003). Biotechnological approaches such as cell line selection, optimization of medium conditions, metabolic engineering, precursor feeding and elicitation are ways to enhance secondary metabolites (Bourgaud *et al.*, 2001).

1.1 Rationale of research

In the recent decade, increasing health consciousness has caused resurgence towards natural colourants (Samantaa and Agarwal, 2009). Synthetic azo-dyes have been the underlying reason for hyperactivity in children and are known to be carcinogenic (Ward, 1997; Chung, 2016). A group of British researchers had established a link between the risk of hyperactivity and ingestion of foods containing dyes and synthetic preservatives in preschool children (Bateman *et al.*, 2004). Concerns on the use of synthetic colourants have prompted various industries to replace them with naturally derived edible biocolourants (Azeredo, 2009).

Betalains are commercially available as powders and concentrates from beetroot, *Beta vulgaris* (Cai *et al.*, 2005). Beetroot is currently the betalain-producing plant with economic value (Zrÿd and Christinet, 2004). However, betalains from beet root are associated with a distinctly unpleasant 'earthy' or 'musty' flavour caused by geosmin and pyrazine derivatives (Lu *et al.*, 2003). These attributes damper its application in dairy products. Furthermore, beet root has high nitrate which may be converted into carcinogenic nitrosamine (Stintzing and Carle, 2007). The oxidation of co-occuring phenols masking the original tint of betaxanthins deemed the yellow beet root unsuitable as food colourants (Stintzing *et al.*, 2002b). The drawbacks of beet root, toxic saponin and lectin in *Phytolacca*, dopamine in *Celosia* and saponin in *Amaranthus* have sparked interest to source betalains from other plants.

Cactus fruits such as *Hylocereus* genus have been highly regarded as a promising betalain source devoid of these drawbacks (Stintzing *et al.*, 2003). The conventional methods of propagation are not efficient for producing a large number of plants within short periods of time (Gengatharan *et al.*, 2015). Any large scale production of natural colourants would require a vast amount of land and the disposal of high volumes of crop waste and processed residues, reflecting an unsustainable practice (Rao and Ravishankar, 2002). Chemical synthesis of betalains is not feasible, are low yielding, and incurs a high cost as multiple steps are involved in the production an important intermediate compound known as betalamic acid (Gandía-Herrero *et al.*, 2006). Despite spray drying being a simple and direct method, it is an unsustainable practice (United States Patent No. US9028891B2, 2015).

Field grown pitaya is laborious and time consuming while the seasons of production varies between countries of northern and southern hemispheres (Mercado-Silva, 2018). Pitayas are susceptible to infections such as *Enterobacter cloacae* and *Fusarium solani* causing soft rot disease (Masyahit *et al.*, 2009; Rita *et al.*, 2013), *Gilbertella persicaria* interfering with postharvest storage (Guo *et al.*, 2012) while *Bipolaris cactivora*, *Cladosporium cucumerinum* and *Rhizopus stolonifera* mainly affecting the fruits (Oeurn *et al.*, 2015) have caused severe yield losses in plantations. Anthracnose and stem canker are also prevalent in pitaya plants (Jumjunidang *et al.*, 2016). Environmental changes caused fluctuations in Malaysian dragon fruit production through the outbreak of soft rot disease from year 2011 to 2013 due to high rainfall and humidity (Zainudin and Ahmad Hafiz, 2015). Moreover, betalain content is dependent on the maturity of cultivated dragon fruit (Phebe *et al.*, 2009).

Considering the fact that cultivated dragon fruits are intended for human consumption and the inconsistency in yield of secondary metabolites, tissue culture techniques such as callus and cell cultures can circumvent the impediments in the production of betalains (Georgiev *et al.*, 2008). Since there were only few reports for the production of betalains on this plant, therefore, this study sought to investigate the potential of *in vitro* seedlings to induce callus and initiate cell suspension cultures that can produce the desired betalain compounds. This study also compares and contrast the betalain content and antioxidant potential from callus, cell culture, seedlings, fruit pulp and fruit peel of *H. costaricensis*. These findings can serve as a sustainable method of producing betalain compounds that are 'natural' with value added antioxidant properties, potentially omitting the use of their synthetic counterparts.

1.2 Research objectives

This study was carried out with the following main objectives:

i. To optimize conditions for *H. costaricensis in vitro* seed germination and seedling growth to be used as a source of explant,

ii. To establish callus culture of *H. costaricensis*,

iii. To initiate cell suspension culture of *H. costaricensis*,

iv. To study the effect of elicitors and amino acids on callus culture,

v. To determine the betalain content and antioxidant profiles of *in vitro* cultures and *H. costaricensis*.

CHAPTER 2

LITERATURE REVIEW

2.1 The Cactaceae

The Cactaceae is the most represented succulent plant family which comprised of more than 30 botanical families of succulent plant species. They have the ability to temporarily store water in one or more organs and are termed as caudiciform plants (Oldfield, 1997). Cactus (plural: cacti, cactuses, cactus) are dicotyledonous plants which houses between 120 and 200 genera consisting of approximately 1500 to 2000 species. Although they are endemic to the New World, certain economically appreciated species have been naturalized in warmer regions of the Old World (Rojas-Aréchiga and Vázquez-Yanes, 2000).

Their wide distribution stretches from the Valley of Death which is above sea level to extreme altitudes in the Andes exceeding 4800 metres (Hussain *et al.*, 2018c) and from the almost rainless Atacama Desert to tropical rain forests receiving yearly rain fall with over 2000 mm (Oldfield, 1997). Their adaptations to intense growth conditions are possible through the modification of stems for water storage, waxy surfaces and reduction or absence of leaves (Luders and McMahon, 2006).

Most cacti have shallow roots enabling them to exploit light rainfall in competition with more deeply rooted plants in the surrounding. Cacti have distinctive stems which exhibit short shoot-long shoot architecture. Areoles (modified axillary buds) covered with spines (modified leaves) are the short shoot whereas the body of stem is the long shoot (Oldfield, 1997). Vegetative branch or flower may originate from areoles. The spines function as a protective measure against herbivores, reflecting light, shading the cactus to reduce water loss and to condense fog (Anderson and Brown, 2001).

The diverse forms, exquisite shoots and fascinating flowers of the Cactaceae family made them mainly appreciated for their ornamental values. Christmas cactus (*Schlumbergera truncata*) is the most commercially grown cacti for this purpose (Mizrahi *et al.*, 1997). Many members of the genera *Echinocereus*, *Stenocereus*, *Opuntia*, *Myrtillocactus* and *Hylocereus* are cultivated for their edible qualities. *Epithelantha bokei* is cultivated as forage for livestock. The Mexicans consume young cladodes (flattened leaf like stems) of *Opuntia* and *Nopalea* as a traditional vegetable dish known as 'nopalitos' which are highly regarded for their high fibre, phenylalanine, leucine and vitamins such as ascorbic acid and retinol (Nobel, 2002). Mucilage of cactus imbibes large amounts of water for usage during unfavourable conditions is used in industrial applications as natural thickener (Saag *et al.*, 1975); and increased shelf-life of strawberries (Del-Valle *et al.*, 2005).

In order to thrive in harsh environments, cactus spp. are conceived to developed effective defence system made of phytochemicals including terpenes, flavonoids and alkaloids that have remarkable bioactivities against human diseases (Harlev *et al.*, 2013). The high bioactive compounds such as betalains, phenolics and antioxidant activity of cactus fruits have gained attention as healthy food supplement and added value functional food (da Silva *et al.*, 2018). A detailed review on the therapeutic and curative properties of cactus was documented by Shetty *et al.* (2012). Stems of *Lophocereus schottii* can retard cancer cells and diabetes whereas stems of *Selenicereus grandiflorus* are administered as a homeopathic medicine to treat urinary tract infections and exert digitalis effect on the heart.

2.2 *Hylocereus* genus

2.2.1 History and distribution

The *Hylocereus* originated principally from the tropical and subtropical forest regions of Mexico, Central and South America. Their exact origin is not known but it is believed to be around southern Mexico, El Salvador and the Pacific side of Guatemala and Costa Rica. From these centers of origin, they spread to tropical and subtropical regions of America, Australia, Middle East and Asia (Mizrahi *et al.*, 1997; Lim, 2012b). The name *Hylocereus* was derived from the Greek terminology '*hyle*' which means 'forest'. It is believed to describe the habitat of *Hylocereus*. However, certain species can live in open and dry woodland areas that receive occasional rainfall (Anderson and Brown, 2001). Queen of the Night, Night Blooming Cereus, Strawberry Pear, Belle of the Night, Cinderella plant, Pitaya or Pitahaya are the common English names of the *Hylocereus*. They are often known by their vernacular names such as Caliz in the Philippines, Thanh Long in Vietnamese language, Huǒ long guǒ ("fire dragon fruit") or Lóng zhū guǒ ("dragon pearl fruit") in Chinese, PāPipi Pua in Hawaii and buah naga or dragon fruit in Malaysia (Alimi *et al.*, 2012).

The *Hylocereus* was introduced into Indochina (composed of modern day Vietnam, Laos and Cambodia) by French priests in the nineteenth century. It was first grown exclusively for the King and later popularize among affluent families. More recently, the pitaya has become an important economic fruit crop in South East Asia (Nerd *et al*., 2002). They naturalized and adapted very well to the new habitat in Indochina making the indigenous people to believe that dragon fruit was endemic to their region (Mizrahi *et al.*, 1997). In the year 1995, dragon fruit made its maiden large scale appearance in Vietnam by the name Dragon Fruit Pearl (Thang Loy in native Vietnamese language).

The dragon fruit has been mass cultivated and marketed in over 20 countries as a novel horticultural crop including Thailand, Taiwan, Malaysia, Cambodia, Indonesia, Laos and Japan (Nobel, 2002; Mizrahi, 2014). Today, they are cultivated for fruit production in the Philippines, Bahamas, Taiwan, Mexico, Japan (Okinawa), Myanmar, Malaysia, Sri Lanka, West Indies, Bermuda, Indonesia, southern China (Mercado-Silva, 2018); Vietnam, Israel, Nicaragua, Columbia, northern Australia and is gaining attention in the United States (Merten, 2003).

The Mexicans have been actively involved in cactus related research but the transfer of knowledge has been restricted due to many valuable publications not disseminated or translated to other languages (Mizrahi *et al.*, 1997). However, more reports on this fruit have been published in scientific databases between year 1994 and year 2012 and have since recorded a total of 198 publications (Mizrahi, 2014).

Dragon fruit was first introduced in Malaysia by Golden Hope Plantations situated in Sungai Wang Estate, Perak in the late 1990s. Its commercial cultivation then started in Sitiawan, Kluang and Kuala Pilah (Halimi and Satar, 2007). Pitaya acreage in Malaysia increased from 47.3 hectares in 2002 to 927.4 hectares in 2006 recording 2532.6 tons for local and export markets with an estimated production value worth RM 12.6 million (Cheah and Zulkarnain, 2008).

2.2.2 Botany and habitat

Pitaya belongs to the vine cacti of *Hylocereus* genus classified under the botanical family Cactaceae. *Hylocereus* genus comprised of around 16 species (Le Bellec *et al.*, 2006). Pitaya is a perennial, climbing cactus with fleshy, triangular and jointed green stems. Stem segments are crawling, creeping or clambering with many branches, joints, ribs may grow up to 20 feet long and visible aerial roots which facilitate anchorage up tree trunks (Zee *et al.*, 2004; Harlev *et al.*, 2013). These epiphytic cacti require a solid support such as trellis used in Vietnam and live trees used in Mexico. In Israel, two "wired walls" of five feet in height on both sides provide easy access to growers for pollination and harvesting fruits (Mizrahi, 2014).

An average of 2-4 short, brown spine is found on areoles, with few hairs or bristles. Flowers are nocturnal, hermaphroditic, fragrant, white within and sometimes reddish outside (Weiss *et al.*, 1994), bearing a large size around 40 cm and ranked among the largest of the angiosperm flowers (Barthlott and Hunt, 1993). This gave the *Hylocereus* spp. recognition as an important plant with ornamental values (Anderson and Brown, 2001). The fruits have medium-large berry appearance with bracts or scales on the fruit peel resembling the appearance of a dragon. The fruit pulp is embedded with many, small edible seeds and has a pleasant flesh texture almost similar to kiwi, juicy, delicate and taste like a cross between kiwi and pear with mild sweetness (Gunasena *et al.*, 2007).

Like other cacti, *Hylocereus* spp. adopt the crassulacean acid metabolism (CAM) with remarkably high water usage efficiency (Nobel and De La Barrera, 2004). This feature made them robust enough to cope in new environments and appear in a wide ecological and climatic spectrum (Gunasena *et al.*, 2007). Even though these vine cacti prefer to grow near regions surrounded by trees, certain species such as *H. costaricensis* are able to grow well under full sunlight. However, extreme dry conditions and intense sunlight may lead to burning of their stems (Le Bellec *et al.*, 2006). Interestingly, *Hylocereus* spp. are cacti plants that uptake considerably higher amount of water than normal desert plants as they originated from shady areas with high humidity and high precipitation (Merten, 2003). Excessive rainfall may lead to flower drop and fruit rot (Zee *et al.*, 2004).

2.2.3 Taxonomy

The related species of *Hylocereus* spp. belong to vine cacti of the subfamily Cactoideae and of the tribe Cacteae (Wybraniec and Mizrahi, 2002). Most *Hylocereus* spp. are diploid (2n = 22) except *H. megalanthus* which is allotetraploid (2n = 4× = 44) (Lichtenzveig *et al.*, 2000; Tel-Zur *et al.*, 2004).

Many contradictions and confusions on the botanical classification have arisen since the cultivation and hybridization of different pitaya species. The adoption of generic or vernacular names made their classification challenging (Mizrahi *et al.*, 1997) while their similar morphological characteristics and environmental conditions between species rendered their classification even more difficult. The common practice of defining species is solely based on the criterion of fruit morphology (Le Bellec and Vaillant, 2011).

The plant's description based on Britton and Rose (1963) classification is shown below:

Kingdom	:	Plantae (plants)
Sub kingdom	:	Trancheobiota (vascular plants)
Super division	:	Spermatophyta (seed plants)
Division	:	Magnoliophyta (flowering plants)
Class	:	Magnoliopsida (dicotyledons)
Order	:	Caryophyllales
Family	:	Cactaceae (cactus family)
Sub family	:	Cactoideae
Tribe	:	Hylocereae
Genus	:	*Hylocereus*
Species	:	*Hylocereus costaricensis* (F.A.C. Weber) Britton & Rose

2.2.4 Phytochemistry

The genus *Hylocereus* is a wealthy reservoir of various phytochemicals. Their common chemical constituents are betalains, phenolic acids, sterols, fatty acids, flavonoids and terpenes (Ibrahim *et al.*, 2018). Metabolite profiling of red and white pitayas and their peels using GC-TOF-MS analysis identified 29 primary metabolites out of total 37 metabolites detected, among which were 15 amino acids and amines, 8 sugars or alcohols, 4 organic acids and 2 fatty acids (Suh *et al.*, 2014).

A comprehensive review on the phytochemicals in genus *Hylocereus* was presented by Ibrahim *et al.* (2018). The authors compiled a total of 151 compounds mainly found in *H. polyrhizus, H. undatus, H. costaricensis* and a few other *Hylocereus* genotypes. These phytochemicals consisted of 69 betalain compounds, 10 flavonoids, 16 phenolic acids and phenylpropanoids, 15 sterols and triterpenes, 30 fatty acids and aliphatic compounds and 11 other compounds such as tocopherols, squalene, citramalic acids which accounted for the medicinal properties of pitayas.

Pitaya peel extracts contained predominantly triterpenoids and sterols. GC-MS analysis on red flesh pitaya peel revealed the presence of major compounds β-amyrin, α-amyrin, octacosane, γ-sitosterol, octadecane, 1-tetracosanol, stigmast-4-en-3-one and campesterol. The major compounds of white flesh pitaya peel were β-amyrin, γ-sitosterol, octadecane, heptacosane, campesterol, nonacosane and trichloroacetic acid, hexadecyl ester. The β-amyrin, β-sitosterol and stigmast-4-en-3-one were found to be responsible for the cytotoxic activities against human gastric cancer cell lines (MGC-803), human breast cancer cell line (Bcap-37) and human prostate cancer cell line (PC3) (Luo *et al.*, 2014).

Two new compounds taraxast-20-ene-3α-ol and taraxast-12,20(30)-dien-3α-ol were successfully isolated and identified from chloroform extracts of *H. undatus*.

These pentacyclic triterpenes produced a protective microvascular effect in intraperitoneally treated rabbits by reducing permeability and increased capillary resistance as demonstrated by the higher inhibition percentage of Evan's Blue leakage by taraxast-12,20(30)-dien-3α-ol (70.1 %) than a naturally occurring flavonoid Troxerutin (64.5 %) (Gutiérrez *et al.*, 2007).

Pitaya seed oils are rich in fatty acids linoleic, oleic and palmitic acids. Protocathechuic acid, p-coumaric, p-hydroxybenzoic, vanillic acid, gallic acid, caffeic acid and syringic acid were the seven antioxidative phenolic acids detected. Besides, phytosterols such as stigmasterol, campesterol, β-sitosterol and cholesterol were identified (Lim *et al.*, 2010). Additionally, GC-MS analysis on *H. polyrhizus* stems detected 4 main compounds. These compounds were 5-cedranone (73.05 %), β-selinene (7.25 %), eucalyptol (6.54 %) and terpinolene (3.69%), representing 91.25 % of the total oil composition. These oxygenated terpenes have been credited for the antimicrobial, anti-cytotoxic and antioxidant potential of pitaya (Ismail *et al.*, 2017). Six major flavonoids such as kaempferol 3-O-β-D-robinobioside, kaempferol 3-O-β-D-rutinoside, isorhamnetin 3-O-β-D-ribinobioside, isorhamnetin 3-O-beta-D-rutinoside, kaempferol 3-O-β-D-glucopyranoside and isorhamnetin 3-O-β-D-glucopyranoside have been identified from the flower of *H. undatus* (Yi et *al.*, 2012).

A total of 121 volatiles constituted of terpenes, aldehydes, alcohols, paraffins, esters, acids, ketones and miscellaneous compounds were isolated from the fruit pulp of yellow pitaya *H. megalanthus*. Aroma extraction dilution analysis identified nine odour-active compounds which could influence flavour (Célis *et al.*, 2012). In a separate study, nineteen aroma volatiles were identified from six varieties of *Hylocereus* and their compositions varied according to storage treatments or varieties. Alcohols, hydrocarbons, a ketone, an ester and a furan were the constituents of the volatiles. Aldehydes were the most abundant volatile, representing 90 % of the total amount (Obenland *et al.*, 2016).

Most betacyanins are 5-*O*-glucosides such as betanin is present in all plants containing betacyanins and is the major red-violet pigment in red beet root. Chromatographic and spectrophotometric methods revealed that the main betacyanin profile consisted typically of six pigments as observed in *H. polyrhizus*, *H. purpusii*, *H. costaricensis* and a few *Hylocereus* hybrids. The three major betacyanins identified were betanin, phyllocactin and hylocerenin. Betanin and phyllocactin were the predominant betacyanins in all *Hylocereus* fruits with variation in the betacyanin composition. Fruits of *H. polyrhizus* exhibited higher percentages of hylocerenin and phyllocactin. The high contents of hylocerenin and isohylocerenin were responsible for the fluorescent appearance of *H. polyrhizus* fruit pulp. The most prominent pigment in *H. costaricensis* was phyllocactin which contributed almost 4 times higher than betanin content. The highest pigment content recorded was in *H. costaricensis* 0.39 mg/g of fruit pulp (Wybraniec and Mizrahi, 2002). Similar HPLC profile of betacyanin pigments were reported, the major peaks of 1/1', 2/2' and 3/3' corresponded to the pigment betanin, phyllocactin and hylocerenin and their respective isoforms (Naderi *et al.*, 2012). Minor betacyanins in peel and flesh of fruits of different *Hylocereus* species were subsequently elucidated by GS-MS, LC-ESI-MS/MS, HPLC and ^{1}H and ^{13}C nuclear magnetic resonance (Wybraniec *et al.*, 2007). Unlike beet root which contains both betaxanthin and betacyanin, a previous study by Stintzing *et al.* (2002a) established the fact that pigments from *Hylocereus* is totally devoid of betaxanthins. However, betaxanthin was reported a few years later (Esquivel *et al.*, 2007b).

2.2.5 Ethnobotany

During early civilization, aerial parts of white fleshed dragon fruit *H. undatus* were used to prevent abortion because its spines were believed to ‘hold back the foetus’ (Ankli *et al.*, 1999). The utilization of *H. undatus* was found in ancient records of the Mayans where leaves and flowers have been used in traditional medicines for its diuretics, cicatrizing and hypoglycemic actions (Gutiérrez *et al.*, 2007). *Hylocereus* plants were also traditionally recognized for their wound disinfection properties. The people of Yucatec Maya in Northern Belize consumed dragon fruit as a remedy for digestive system disorder such as diarrhea until symptoms ceases (Blanco and Thiagarajan, 2017). In southern China, the flowers of *H. undatus* have been long used as folk medicine in the treatment of diabetes, bronchitis, tuberculosis, cough, mumps, cervical lymph node tuberculosis and hyperactivity (Wu *et al.*, 2011). Their flowers were also imported from the Mayans (Anderson, 2006) and traditionally cooked with pork ribs to make healthy soup (Xia *et al.*, 2004).

2.2.6 Beneficial properties and uses of *Hylocereus*

2.2.6(a) Nutritional

The rich nutritional profile of pitaya is a potential source for human alimentation. World Health Organization (WHO) has recommended the daily dragon fruit intake as 25 g (Zhuang *et al.*, 2012). Pitayas are abundant in water content and vitamins B_1, B_2 and B_3. Vitamin B_1 increases energy production and carbohydrate metabolism, vitamin B_2 functions as a multivitamin and increases appetite while vitamin B_3 lowers bad cholesterol, improves skin complexion, prevents hypertension and enhances eyesight. Vitamin C aids in enhancement of immune system, wound healing, alleviating asthma and cough (Le Bellec *et al.*, 2006; Jaafar *et al.*, 2009).

The high levels of calcium and phosphorus are responsible for promoting healthy teeth and reinforce bones. Glucose found in pitaya lowers blood glucose in type 2 diabetic patients (Jaafar *et al.*, 2009; Perween *et al.*, 2018). The high fiber content of pitaya increases bowel movement and protects from cancer (Jaafar *et al.*, 2009). White pitaya contained 0.26 mg/100g, 3.4 µg/100g and 1.4 µg/100g of vitamin E, lycopene and beta carotene respectively making them a distinguished healthy fruit (Charoensiri *et al.*, 2009). High phenolic content is usually correlated with high radical scavenging activity. Pitayas are abundant in bioactive compounds such as flavonoids, phenolics, polyphenols, phytoalbumin, iron, carotene and betalains (Choo and Yong, 2011). The high flavonoid content in dragon fruit act against heart related diseases (Hussain *et al.*, 2018c).

The proximate composition of red pitaya accounted up to 87.30 % moisture, 10.10 g crude fibre, 1.48 g carbohydrate, 0.70 g ash, 0.23 g fat and 0.16 g protein. Their mineral contents were potassium (56.96 mg), sodium (50.15 mg), magnesium (28.30 mg), phosphorus (23.0 mg), zinc (13.87 mg), calcium (5.70 mg), iron (3.40 mg) and copper (0.031 mg). The rich macroelement and microelement contents contribute to risk reduction of chronic diseases such as hypercholesterolemia, cardiovascular diseases, anaemia and diabetes mellitus (Khalili *et al.*, 2006).

Pitaya seed oils are abundant in natural antioxidants and contain high levels of polyunsaturated fatty acids, tocopherols, sterols and phenolics (Lim *et al.*, 2010). The grainy seeds of dragon fruit are a rich source of dietary essential fatty acids (EFAs) linoleic acid and linolenic acid. These polyunsaturated fatty acids are required for proper bodily functions such as having beneficial effects on the hair and skin because they are unable to be synthesized by our body. The seed oils of *H. polyrhizus* and *H. undatus* only contributed approximately 1-2 % of total fresh weight and the average seed oils were 29.5 % and 32.0 % respectively. Despite its contrastingly low seeds to fruit pulp ratio, pitaya seed oil presented higher linoleic acid content than canola, sesame seeds and flaxseeds (Ariffin *et al.*, 2009). Linoleic acid (466 g/kg) was reported to be the most abundant fatty acid in *Hylocereus* spp. (Villalobos-Gutiérrez *et al.*, 2012). Chemah *et al.* (2010) highlighted the high fatty acid contents in *H. megalanthus* (660 g/kg), *H. undatus* (540 g/kg) and *H. monocanthus* (480 g/kg). In other study, the efficacy of incorporating seed oils into lipstick formulation has been evaluated (Kamairudin *et al.*, 2014).

2.2.6(b) Industrial

The compatible physiocochemical properties of betalains are also used to establish dye-sensitized solar cells (Narayan, 2012) and in the removal of harmful dyes from water sources (Priyantha *et al.*, 2015). Dragon fruit foliage is a potential adsorbent for the removal of non-oxidisable and non-degradable heavy metals and synthetic dyes. The direct discharging of these harmful substances into water sources affect the amount of light penetrating into water and lowering photosynthetic rate of aquatic plants. Thus, the anaerobic degradation of dyes into hazardous substances suffocates aquatic organisms (Sivaraj *et al.*, 2001; Waranusantigul *et al.*, 2003). Untreated dye effluents degrade water quality and cause skin, cancer, liver, kidneys related problems and reproductive dysfunction in humans (Ibrahim and Sani, 2014). Dragon fruit peel was found to be particularly effective towards the removal of alcian blue and methylene blue (Mallampati *et al.*, 2015). The mechanism of absorbing harmful manganese ions was through the replacement of potassium ions on its functional groups (Priyantha *et al.*, 2013). These studies highlighted the functionality of dragon fruit as an environmental friendly, low cost and easily available bioadsorbent material for the removal of dyes and harmful metal ions from water source.

Reports have indicated the abundance of pectin, a complex polysaccharide from dragon fruit peel (Jamilah *et al.*, 2011). Pectin is a heterogeneous structural polysaccharide containing α-1,4-linked D-galacturonic acid residues (Ridley *et al.*, 2001). Pectin has been widely used as thickeners, emulsifiers and gelling agents in dairy products, soft drinks, desserts and jams. Furthermore, its applications are also widespread in the medical field (Voragen *et al.*, 1995; Jitpukdeebodintra and Jangwang, 2009). Pectin is in high demand as their consumption has exceeded 20,000 tons a year (Ptichkina *et al.*, 2008). Pectins from pitaya can be used to obtain a high dietary fiber powder, with high water-holding capacity, high swelling capacity, high oil holding capacity and high glucose retention. This powder can serve as a functional ingredient to improve human health (Zhuang *et al.*, 2012). Red pigments of dragon fruit are used prominently in the processing of colourants which have a huge market demand in the food colouring industry. These colourants are stable during processing and storage (Herbach *et al.*, 2007; Ortiz-Hernández and Carrillo-Salazar, 2012). The powder of pitaya could be used as natural coloring agent or as health supplement (Yusof *et al.*, 2012). More recently, researchers have also been tapping into its potential applications as biocolourants in cosmetics (Azwanida *et al.*, 2014; Thu and Mon, 2015; Sandriani *et al.*, 2017) and dairy products (Gengatharan *et al.*, 2016).

2.2.6(c) Pharmacological

In addition to their relevance as a colouring agent, the nutrients and minerals of dragon fruit can prevent risk factors such as diabetes mellitus, cardiovascular disease, hypercholesterolemia and improving eyesight (Khalili *et al.*, 2006). Bioactive metabolites of *Hylocereus* species have demonstrated therapeutic effects such as hepatoprotective properties (Galati *et al.*, 2005; Latif *et al.*, 2012), antimalarial activities (Hilou *et al.*, 2006), anti-spasmodic, anti-proliferative, radio protective, anti-inflammatory and anti-proliferative activities (Kaur *et al.*, 2018). Betalain compounds in dragon fruit extract have been associated to the improvement of haemoglobin and erythrocyte levels in pregnant women with anaemia (Widyaningsih *et al.*, 2017).

Betalains and other bioactive compounds found in *Hylocereus* spp. are responsible for their wide range of desirable pharmacological activities effective for treating various diseases. Topically applied aqueous flower and leaf extracts of *H. undatus* showed beneficial wound healing therapy in diabetic rats. The plant extracts significantly elevated tensile strength, hydroxyproline level, total proteins and DNA content at wounding site of diabetic rats. A shorter period of epithelization, total absence of congestion and oedema was also noted (Perez *et al.*, 2005). The treatment with dragon fruit plant extracts showed comparable outcome with commercially available Bioplacenton gel in the effective reduction of wound diameter of both diabetes induced rats and non-diabetic rats. Biopsy and histopathological outcomes showed that thinnest granulation tissues and highest score of re-epithelialization were found in pitaya extract treated rats (Tahir *et al.*, 2017). The wound healing ability was attributed to antioxidant and anti-inflammatory properties of dragon fruit. Polyphenols activate nitric oxide and drive the process of tissue granulation, endothelial function and accelerate the growth of macrophages and inhibit cell proliferation. Quercetin modulates cytokines and growth factors which plays a role in forming granulation tissue, inhibits reactive oxygen species and lessen excessive tissue inflammation.

Diabetes is often associated with the risk of cardiovascular complications. Excessive glucose can increase oxidative stress in diabetic patients. The efficacy of aqueous *H. undatus* pulp extract in controlling oxidative deterioration and reducing aortic stiffness in streptozotocin induced diabetic rats was reported by Swarup *et al.*

(2010). The same group of researchers discovered that lipid peroxidation activity was increased as indicated by the elevated levels of malondialdehyde (MDA) while the total antioxidant capacity (TAC) and superoxide dismutase (SOD) was reduced in diabetic rats as compared to control treatment and rats treated with pitaya extract. More recent evidence highlighted that rats with induced insulin resistance attenuated dyslipidemia, decreased insulin resistance, atherosclerotic changes and hypertriglyceridemia after being administered with whole fractions from *H. polyrhizus* juice. Antioxidant, soluble fibre and micronutrient contents of red pitaya played an integral role in synergistically contributing to the anti-insulin properties by reversing insulin resistance and its side effects (Omidizadeh *et al.*, 2014).

Treatment with ethanolic extract of *H. undatus* showed anti-proliferative effect towards human hepatocellular carcinoma cell line (HepG2) in a dose-dependent manner. Polyphenols were the main compounds in producing this effect (Li *et al.*, 2013). Pitaya extract exhibited high inhibitory activity against nitric oxide induced proliferating sodium MCF-7 cells. Nitric oxide scavenging activity was approximately 80 % when tested with Griess reagent (Jayakumar and Kanthimathi, 2011). Polyphenols as antioxidant in dragon fruit were accountable for scavenging free radicals and impeding cancer cell proliferation (Kraft *et al.*, 2005; Fresco *et al.*, 2010). Therapeutic potential against breast cancer was also manifested by red pitaya extract. Pitaya extract downregulated estrogen gene receptors by targeting pathways leading to cell cycle arrest and apoptosis (Guimarães *et al.*, 2017). Sub-fractionated pitaya extracts yielded higher betacyanin and antioxidant contents than whole extract (Tenore *et al.*, 2012). Pitaya seeds are also rich source of natural antioxidant (Adnan *et al.*, 2011). Anti-proliferative and antioxidant properties of ethanolic extracts of *H.*

costaricensis improved sperm quality and increased sperm count in mice through the action of scavenging ROS (Aziz and Noor, 2010).

Anti-inflammatory effect of ethanolic extracts from *H. polyrhizus* flesh was reported by Macias-Ceja *et al.* (2016). The distortion of mucosal architecture, epithelial barrier alteration, cellular infiltration, aberrant changes of pro-inflammatory molecules, and loss of body weight are characteristics of Crohn's disease. The presence of several compounds such as polyphenols, flavonoids and fatty acids were detected among which ellagic acid, p-HEPA-AC and luteolin were linked to the anti-inflammatory effects. These compounds ameliorated inflammation by decreasing myeloperoxidase activity responsible for mucosa dysfunction and inflammation in colonic tissues of 2,4,6-trinitrobenzesulphonic (TNBS) treated mice. Additionally, gene expression of pro-inflammatory cytokines was repressed.

There is a considerable amount of literature on the antimicrobial potential of dragon fruit. Chloroform extracts of red pitaya and white pitaya peels have been found to potentiate antimicrobial properties against a fairly broad spectrum of Gram-positive (*Bacillis cereus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Staphylococcus aureus*) and Gram-negative bacteria (*Eschericia coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Yersinia enterocolitica*) followed by hexane and ethanol extracts. Both species exhibited inhibition zones between 7 to 9 mm and inhibited bacterial growth with the MICs range of 1.25 – 10.00 mg/mL. Chloroform extracts of red pitaya exhibited better activity than white pitaya. An interesting finding was that *Campylobacter jejuni* was not inhibited by *H. polyrhizus* but was inhibited by *H. undatus* (Nurmahani *et al.*, 2012). The methanolic extract of red pitaya was significantly more effective against *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* than gentamicin. Red pitaya flesh possessed better antimicrobial properties than its white flesh counterpart as observed in the larger zones of inhibition for all four gram positive and six gram negative bacteria. Alkaloids, glycosides, volatile oils and tannins present in pitaya potentiated the antibacterial activity against pathogenic food microorganisms (Khalili *et al.*, 2012). Terpenoids and alkaloids detected in n-hexane fractions of red pitaya peel showed antibacterial activity against *Staphylococcus aureus* (Amalia *et al.*, 2015). Terpenoids could lead to the destruction of porin causing disarray in cell wall permeability and hampering the influx of nutrition, leading to eventual cell death. Alkaloids function by disrupting the arrangement of peptidoglycan on bacteria cell wall, leading to improper cell wall formation and causing bacterial cell wall to collapse (Amalia *et al.*, 2015). Polyphenolic fractions of *H. polyrhizus* have a broader antimicrobial activity in comparison to whole extracts and able to inhibit food borne pathogens including yeast and molds (Tenore *et al.*, 2012).

Prebiotics are food components that selectively stimulate the activity and growth of probiotics. Prebiotics should be able to enter the large intestine without being digested by the upper gastrointestinal tract and capable of enhancing the growth of beneficial bacteria while suppressing the growth of pathogenic bacteria (Thammarutwasik *et al.*, 2009). Glucose, fructose and oligosaccharides are major carbohydrates in dragon fruit. Mixed oligosaccharides extracts of dragon fruit demonstrated higher hydrolysis and enzymatic resistance against artificial human gastric juice and α-amylase hydrolysis respectively when compared to standard prebiotic inulin. The oligosaccharides also promoted the growth of good bacteria such as *Lactobacillus delbrueckii* and *Bifidobacterium bifidum* (Wichienchot *et al.*, 2010). Besides, a high amount of oligosaccharides (87.6 %) from dragon fruit flesh will reach the colon. Prebiotic properties of pitaya were evident through the increased amount of bifidobacteria, lactobacillus and decreased amount of clostridium and bacteroides in fecal fermentation. Oligosaccharides of dragon fruit metabolized by intestinal microbiota are broken down into acetic acid, lactic acid, propionic acid and butyric acid. These compounds have been credited for the inhibition of Caco-2 cells, thus, minimizing the risk of colon cancer (Dasaesamoh *et al.*, 2016).

2.2.7 Hylocereus costaricensis

The plant material used in this study was dragon fruit *H. costaricensis* (Plate 2.1). *H. costaricensis* is categorized with branches which are the stoutest in the genus. The stems are three-angled, green and turn bluish gray or whitish. *H. costaricensis* is widely distributed in Costa Rica, Panama, Guatemala and Nicaragua (Anderson and Brown, 2001; Nobel, 2002). The large globose, scarlet fruits have an average mass of 250-600 g with diameter of 10-15 cm, the peel is covered with large scales of different sizes with a deep red pulp on the interior (Vaillant *et al.*, 2005). *H. costaricensis* bears fruits with super red flesh and red skin. Their ovoid shape made them easily distinguishable from *H. polyrhizus* which bears oblong fruits.

To date, *in vitro* studies on the production of betalain from dragon fruit is limited. Callus induction from fruit pulp has been reported in *H. polyrhizus* (Wee *et al.* 2018) but yet to be induced from *in vitro* raised seedlings. Rumiyati *et al.* (2017) conducted a simple experiment by evaluating the concentration of sucrose and 2,4-D to induce callus from stem explants of *Hylocereus* species, but no phytochemical analysis was carried out to assess their betalain and antioxidant contents. In this study, investigation was carried out on the presence of betalain and the antioxidant profile of *H. costaricensis* pulp and peel, *in vitro* germinated seedlings and in cell and callus cultures.

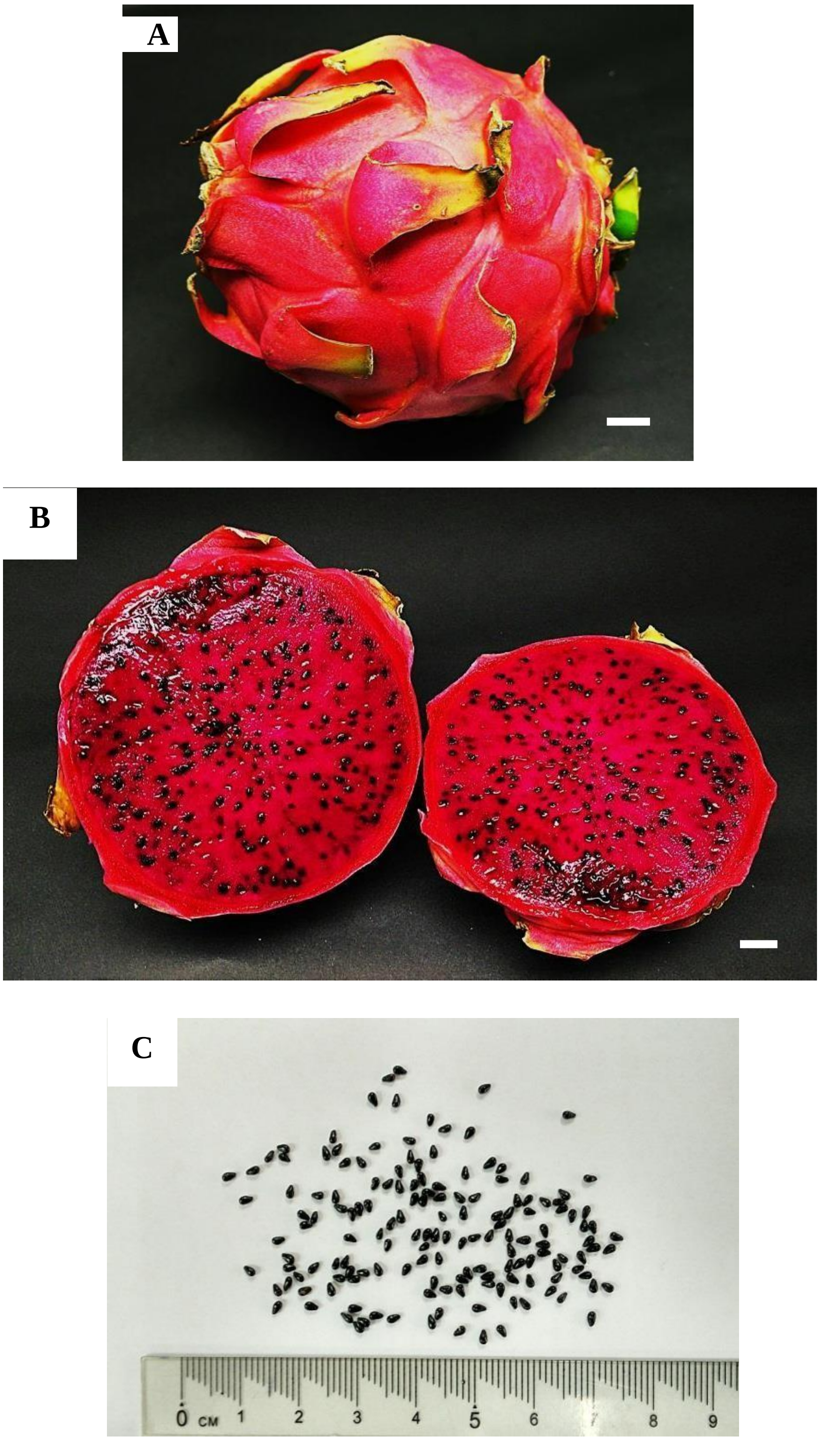

Plate 2.1: The dragon fruit *Hylocereus costaricensis*.

A) Whole fruit

B) Transverse section of dragon fruit revealing magenta coloured pulp studded with many small, black seeds

C) Seeds

* Scale bar = 1 cm for A and B.

2.3 Plant Secondary Metabolites

Compounds produced by living organisms can be divided into primary and secondary metabolites. Primary metabolites (e.g. carbohydrates, amino acids, lipids) are involved in fundamental life processes such as photosynthesis and respiration. Secondary metabolisms encompass pathways derived from primary metabolism but are not associated with growth, reproduction and development (Seigler, 1998). Secondary metabolites are low molecular weight compounds produced widely throughout the plant kingdom that play vital roles in interaction with their environment (Zhang *et al.*, 2004). These complex compounds are often synthesized in minute amounts (less than 1% dry weight) largely dependent on a plant's developmental and physiological stage (Oksman-Caldentey and Inzé, 2004). By the year 1985, scientists have discovered more than 20,000 secondary metabolites with this repository increasing by at least 1000 new secondary metabolites each year (Seigler, 1998). Unlike most animals which are mobile and have the ability to obtain food and defend against predators, sessile plants have developed alternative strategy through secondary compounds. These compounds have recognized ecological functions which affect plant survival. Secondary metabolites play roles in repelling herbivorous pest and other competing plant species, to attract symbionts and potential pollinators while some are the excretion or end products of a biosynthetic pathway (Bell, 1981; Bennett and Wallsgrove, 1994). Besides their important role in plants environmental adaptation, secondary metabolites such as pharmaceuticals, fragrances, pesticides, flavours and dyes have been of genuine interest to humans (Oksman-Caldentey and Inzé, 2004).