ESTABLISHMENT OF ANTHOCYANIN-PRODUCING CALLUS CULTURE OF *Taraxacum officinale* F. H. WIGG (ASTERACEAE)

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2020

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by

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

November 2020

ACKNOWLEDGEMENT

First of all, I would like to express my gratitude to my supervisor, Prof. Dr. K. Sudesh Kumar A/L C Kanapathi Pillai for his guidance throughout the completion of the research and thesis writing. I would like to thank him for the opportunity given to conduct research in his lab.

Secondly, I would like to express my appreciation to my co-supervisors, Prof. Dr. Shaida binti Sulaiman and Dr. Chew Bee Lynn for guiding me throughout the research. Their effort and patience in spending out precious time to discuss the project with me are greatly appreciated. Also, I would like to thank all the lab members from Lab 269 and Lab 409 for their help in accomplishing my postgraduate study.

Next, I would like to thank the administration of School of Biological Sciences, Universiti Sains Malaysia for providing facilities and equipment needed for my experiments. I would like to thank research assistants and research officers of School of Biological Sciences for their assistance.

Besides that, I am also grateful to my family members especially my parents who are always giving support and encouragement to me. Their supportive words and advices give me strength and determination to complete my work.

I would like to express my gratitude to Dr. Christine Stanly. Thank you for your advices, suggestions, supports, encouragement and motivation. I am deeply grateful for your guidance on the plant tissue culture techniques. Thank you for spending your time to share your expertise with me. Also, thanks to my fellow labmates, Marisa, Justin, Kavi and Arul, for their help whenever I encountered difficulties. Thanks to Bryan, Mei Ling and Dr. Ooi Kheng Leong for their help in my research project. Last but not least, thank you everyone for all the help.

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LIST OF ABBREVIATIONS

AC	Acetyl-CoA carboxylase
AHW	Acetic acid: hydrochloric acid: water
ANOVA	One-way analysis of variance
ANS	Anthocyanidin synthase
ATP	Adenosine triphosphate
BAW	*n*-butanol: acetic acid: water
BEH	Ethylene bridged hybrid
BEW	*n*-butanol-ethanol-water
BN	*n*-butanol-2 M ammonium hydroxide
BuH	*n*-butanol: HCl
CE	Capillary electrophoresis
CHI	Chalcone isomerase
CHS	Chalcone synthase
C4H	Cinnamate 4-hydroxylase
DCW	Dry cell weight
DF	Dilution factor
DFR	Dihydroflavonol 4-reductase
DHK	Dihydrokaempferol
DHM	Dihydromyricetin
DHQ	Dihydroquercetin
ESI	Electrospray ionization
FAB-MS	Fast atom bombardment mass spectrometry
FCW	Fresh cell weight
FDA	Food and drug administration
F3H	Flavanone 3-hydroxylase
F3’H	Flavonoid 3’-hydroxylase
F3’5’H	Flavonoid 3’,5’-hydroxylase
GI	Growth index
HPLC	High performance liquid chromatography
LC	Liquid chromatography
LED	Light emitting diode
LS	Linsmaier and Skoog

MS	Mass spectrometry
MS	Murashige and Skoog
M_W	Molecular weight
NAA	1-napthalene acetic acid
NAHNES	National health and nutrition examination survey
NMR	Nuclear magnetic resonance
PAL	Phenylalanine ammonia lyase
PC	Paper chromatography
PDA	Photodiode array
QTOF	Quadrupole-time-of-flight
RT	Retention time
TEM	Transmission electron microscopy
TLC	Thin layer chromatography
UFGT	UDP-glucose: flavonoid 3-O-glucosyltransferase
UPLC	Ultra performance liquid chromatography
WHO	World Health Organization
4CL	4-coumaryl-CoA ligase

PENUBUHAN KULTUR KALUS PENGHASIL ANTOSIANIN DARIPADA *Taraxacum officinale* F. H. WIGG (ASTERACEAE)

ABSTRAK

Taraxacum officinale (*T. officinale*) merupakan tumbuhan ubatan yang terdapat di kawasan yang mempunyai empat musim. Sebatian antosianin yang terdapat pada tangkai tumbuhan ini adalah sumber yang berpotensi untuk menjadi pewarna makanan. Kajian ini menumpukan pada faktor-faktor yang berlainan terhadap pengumpulan antosianin. Kesan jenis eksplan pada induksi kalus telah diuji di medium Murashige dan Skoog (MS) yang diperkaya dengan 0.5 mg/L 1-naphthaleneacetic acid (NAA). Kesan parameter yang berlainan (kepekatan NAA, saiz inokulum, jenis medium asas, kekuatan medium, kepekatan nitrogen keseluruhan, sumber karbon dan tahap gula) juga telah dikaji secara susunan. Mikroskopik cahaya dan mikroskopik elektron transmisi (TEM) kalus segar serta kromatografi kertas (KK) dan kromatografi cecair prestasi ultra (KCPU) antosianin juga dijalankan. Keputusan menunjukkan bahawa eksplan akar mempunyai nilai yang tertinggi untuk induski kalus (100%) dengan pembentukan kalus yang remah dan berwarna ungu. Kultur kalus pigmen dan tanpa pigmen telah ditubuhkan dengan subkultur secara pilihan dan ulangan pada selang masa empat minggu. Pertumbuhan kalus menunjukkan satu lengkung sigmoidal biasa dan memuncak pada hari ke-35 berdasarkan berat kering kalus. Kultur kalus pigmen dan tanpa pigmen berbeza dalam kemampuan pengumpulan antosianin. Pengumpulan pigmen adalah bergantung pada cahaya. Kalus yang dikultur dalam kegelapan menghasilkan semula pigmen selepas terdedah kepada cahaya dan induksi yang paling awal berlaku pada hari ke-3 inkubasi. Selain itu, kepekatan fruktosa dan galaktosa pada 3% (w/v) mempunyai kesan negatif terhadap kultur kalus. Kepekatan

sukrosa yang rendah daripada 0.8% tidak menyokong pertumbuhan kalus. Saiz inokulum yang optimum adalah 1.5 g. Medium penuh MS yang diubahsuai (50 mM nitrogen keseluruhan) dengan tambahan 1.0 mg/L NAA dan 2% sukrosa menghasilkan indeks pertumbuhan kalus yang lebih tinggi (705.9%) serta kandungan antosianin (2.03 mg/ g berat kering) 2.9 kali lebih tinggi apabila dibandingkan dengan medium induksi kalus (medium penuh MS + 3% sukrosa + 0.5 mg/L NAA), yang mempunyai indeks pertumbuhan sebanyak 620.2% dan kandungan pigmen sebanyak 0.69 mg/g berat kering. Analisis mikroskopik cahaya kalus *T. officinale* menunjukkan campuran sel berwarna ungu dan tanpa warna yang mempunyai bentuk yang berlainan. TEM kalus segar mendedahkan pigmen antosianin sebagai bahan osmofilik elektron-padat yang terkumpul di dalam vakuola tengah serta sepanjang tonoplas dalaman. Kehadiran sebatian antosianin dikenalpasti melalui pembentukan lapisan magenta di bawah sinaran cahaya lampu mengunakan KK dengan sistem pelarut yang berbeza. Keputusan KCPU menunjukkan bahawa kandungan antosianin lebih tinggi dalam kalus (34.5 μg/100 mg berat segar) daripada tangkai anak benih *T. officinale in vitro* (27.5 μg/100 mg berat segar). Sebatian yang lain (luteolin dan asid kafein) juga dikesan. Kajian ini menyediakan satu protokol yang efisien untuk penghasilan antosianin yang tinggi dalam kultur kalus *T. officinale*.

ESTABLISHMENT OF ANTHOCYANIN-PRODUCING CALLUS CULTURE OF *Taraxacum officinale* F. H. WIGG (ASTERACEAE)

ABSTRACT

Taraxacum officinale (*T. officinale*) is a medicinal plant distributed in the areas with temperate climate. Anthocyanin compound present in the petiole of this plant is a potential source of food colouring. Current study focuses on the effect of different factors on anthocyanin accumulation of *T. officinale* callus. Effect of explant type on callus induction was evaluated on Murashige and Skoog (MS) medium enriched with 0.5 mg/L 1-naphthaleneacetic acid (NAA). Effects of different parameters (NAA concentration, inoculum size, type of basal medium, medium strength, total nitrogen, carbon source and sugar level) were also investigated accordingly. Light microscopy and transmission electron microscopy (TEM) of fresh callus as well as paper chromatography (PC) and ultra performance liquid chromatography (UPLC) of anthocyanin were also carried out. Results demonstrated that root explants had the highest callus induction value (100%) with the formation of friable and purple calli. Pigmented and non-pigmented callus lines were established by selective and repeated subcultures at four-week intervals. Callus growth showed a typical sigmoidal curve and peaked at 35th day on dry cell weight basis. Pigmented and non-pigmented callus lines differed in the capabilities of anthocyanin accumulation. Pigment accumulation was light-dependent. Dark-grown callus restored pigmentation after exposure to light and the earliest induction was at day-3 of incubation. Besides, fructose and galactose at a concentration of 3% (w/v) were detrimental to the callus culture. Sucrose concentrations lower than 0.8% were not supporting callus growth. The optimum inoculum size was 1.5 g. Full-strength modified MS medium (50 mM total nitrogen)

fortified with 1.0 mg/L NAA and 2% sucrose resulted in higher callus growth index (705.9%) as well as 2.9-fold higher anthocyanin content (2.03 mg/g DCW) when compared with the callus induction medium (full-strength MS medium + 3% sucrose + 0.5 mg/L NAA), which had growth index of 620.2% and pigment content of 0.69 mg/g DCW. Light microscopic analysis of *T. officinale* callus revealed mixtures of purple pigmented and colourless non-pigmented cells with different shapes. TEM of fresh callus revealed anthocyanin pigments as electron-dense osmophilic materials that accumulated in the central vacuole and along the inner tonoplast. Formation of magenta bands under visible light in PC with different solvent systems indicated the presence of anthocyanin compound. Results of UPLC demonstrated higher anthocyanin content in the callus (34.5 μg/100 mg FCW) than in the petiole of *T. officinale* plantlet (27.5 μg/100 mg FCW). Other compounds (luteolin and caffeic acid) were also detected. The present study provides an efficient protocol for high anthocyanin accumulation of *T. officinale* callus culture.

CHAPTER 1

INTRODUCTION

Taraxacum officinale (*T. officinale*), commonly known as dandelion, is a perennial plant which is native to the warmer temperate zones of Northern Hemisphere. Besides being an alternative food source and a potential bio-indicator for metal pollution, it is well known as an important medicinal herb and has long been used as herbal medicine to cure various illnesses such as dyspepsia, arthritic diseases, as well as gall and liver malfunctions (Sweeney *et al.*, 2005; Schütz *et al.*, 2006; Grauso *et al.*, 2019). The bioactive constituents such as phenylpropanoids, flavonoids and terpenoids present in *T. officinale* exhibit a variety of health-beneficial effects like anti-carcinogenic, anti-inflammatory and anti-oxidative activities (Jeon *et al*., 2008; Choi *et al*., 2010; Saratale *et al.*, 2018).

As reported by Akashi *et al*. (1997), the purplish-red pigment on the petiole of *T. officinale* has been identified as cyanidin 3-(6”-malonyl) glucoside, which is one of the common anthocyanins found in the plant kingdom. Anthocyanins are plant-derived flavonoids that contribute to various attractive colours, ranging from scarlet to deep blue, in different parts of the plant such as petals, leaves, fruits and storage organs (Gould *et al.*, 2008). Anthocyanins are important to plants as pollinator attractors for the purpose of plant propagation as well as seed dispersal (Koes *et al.*, 1994; Gould *et al*., 2008; Miller *et al.*, 2011). Other than that, anthocyanins also provide light-filtering function and shield the plant tissues underneath from photodamage caused by excessive light exposure (Gould *et al.*, 2008; Zhang *et al.*, 2010).

In recent years, demands and preferences for natural colourants over artificial dyes has increased gradually among the public. The natural colourant industry has an

estimated global market volume of $291.7 million in 2014 and the value is projected to reach $387.4 million by 2021 (Appelhagen *et al.*, 2018). With the "clean label" trend, many food and beverages companies have made commitments to remove any artificial substances (e.g. synthetic colourants) from their products in order to meet changing market demands and legislative restrictions (Cortez *et al.*, 2017). The preference of consumers towards natural food colourant is mainly due to health and food safety issues regarding synthetic dyes as studies have shown that the chemicals used in the synthesis of artificial food colourants may exert some adverse effects on human health. For instances, it has been reported that the consumption of artificial food colourant had caused hyperactivity in children and allergenicity in sensitive individuals (McCann *et al*., 2007; Carocho *et al.*, 2014; Oplatowska-Stachowiak & Elliott, 2017).

Anthocyanins have been authorised as food additives by both the European Union (E-163) and the Food and Drug Administration (FDA) in United States (Andersen & Jordheim, 2013). They are promising alternatives to replace synthetic colourants used in food and beverages due to their low to no toxicity (World Health Organisation [WHO], 1982). Health-giving properties such as anti-oxidant, anti-inflammatory, anti-cancer and wound-healing properties (He & Giusti, 2010; Khoo *et al.*, 2017) offer an added benefit to anthocyanins as a substitute for their artificial counterpart. Moreover, the high stability of acylated anthocyanins in the aspect of pH, temperature and light as compared to the other pigments makes them suitable to be utilised in food products with longer shelf life (Francis, 1992; Dangles *et al.*, 1993; Giusti & Wrolstad, 2003).

The conventional method for obtaining anthocyanin pigments always involve whole plant extraction. Nevertheless, the current supply of natural anthocyanins from fresh plant materials faces several limitations. One of the restrictions is the inconsistency of the composition of anthocyanins that vary qualitatively and quantitatively with the growing conditions and seasons of the plant source, which indirectly affects the overall products' qualities (Scalzo *et al.*, 2013; Timmers *et al.*, 2017; Appelhagen *et al*., 2018). Other problems such as low extraction yield, loss of fresh plant materials due to pest/disease attack and pigment degradation during extraction process and storage also hinder their mass production (Zhang & Furusaki, 1999; Santos-Buelga & Williamson, 2003). Hence, there is a need to search for alternatives to overcome the bottlenecks.

Plant cell culture technique is a potential approach to traditional methods for mass production of high-value plant secondary metabolites, including anthocyanins. As compared to field cultivation, the production of anthocyanins by means of plant cell biotechnology is not subjected to the seasonal and geographical variations as well as other environmental conditions (Rao & Ravishankar, 2002; Hussain *et al.*, 2012). Thus, a consistent supply of anthocyanin compounds with uniform quality and yield can be accomplished (Rao & Ravishankar, 2002). In addition, the use of automated control systems could reduce labour cost and at the same time, improve the overall productivity (Hussain *et al.*, 2012). Other than that, this technology also enables researchers to select and control the types of pigments to be produced (Gould *et al.*, 2008). Also, the extraction and isolation of the desired compounds produced from plant cell cultures are more efficient and rapid as compared to the extraction from whole plant (Hussain *et al.*, 2012). Production of anthocyanins in plant cell cultures has been reported in *Vitis vinifera* (Pépin *et al.*, 1995), *Euphorbia* spp. (Yamamoto *et*

al., 1982), *Daucus carota* (Rajendran *et al.*, 1994) and *Perilla frutescens* (Zhong *et al.*, 1995).

Callus induction from selected parent plant with desirable characteristics is the first step to initiate an *in vitro* culture, followed by optimisation of the culture medium for maximum cell biomass and anthocyanin production. Growth and accumulation of the anthocyanins pigments depend greatly on abiotic as well as biotic factors such as sucrose, hormone effect, pH, temperature, nitrogen and phosphate concentration as well as light irradiation (Zhang & Furusaki, 1999; Gould *et al.*, 2008; Smetanska, 2008). Medium optimization for anthocyanin production from different plants has been demonstrated in *V. vinifera* (Do & Cormier, 1991), *Fragaria ananassa* (Nakamura *et al.*, 1999), *D. carota* (Narayan & Venkataraman, 2002), *Melastoma malabathricum* (Koay *et al.*, 2011) and *Cleome rosea* (Simões *et al.*, 2009).

Studies on the effect of plant growth regulators on the *in vitro* response of *T. officinale* have been reported (Bowes, 1970; Booth & Satchuthananthavale, 1974; Slabnik *et al.*, 1986; Ermayanti & Martin, 2011; Chong, 2016). The relationship between light-emitting diode (LED) light illumination and anthocyanin content in wild *T. officinale* has also been studied (Ryu *et al.*, 2012). However, only a few reports on the anthocyanin production of *T. officinale* callus culture are available (Akashi *et al.*, 1997; Chong, 2016; Martínez *et al.*, 2018). Akashi *et al.* (1997) established purplish-red callus line on cytokinin-rich medium and they have characterised the pigment as cyanidin 3-(6”-malonylglucoside). Chong (2016) reported the induction of purplish-red callus from *T. officinale* root explant cultured on NAA-supplemented MS medium. On the other hand, Martínez *et al.* (2018) studied the anthocyanin accumulation under the influence of glucose and sucrose as well as the effects of combination of BA and

NAA at different concentrations. Nevertheless, information on the influence of other factors such as basal medium, sugar types and total nitrogen on growth and anthocyanin accumulation of *T. officinale* callus culture are still lacking. Hence, the current study focused on the effects of different factors on the pigment accumulation of *T. officinale* callus and aimed to develop a modified medium for higher yield of anthocyanin via plant tissue culture approach.

1.1 Objectives

i. To establish anthocyanin-producing callus lines of *T. officinale* using root explant

ii. To investigate the effect of various factors (concentrations of 1-naphthaleneacetic acid, inoculum sizes, types of basal medium, different medium strengths, total nitrogen and sucrose concentration) for growth improvement and anthocyanin accumulation of *T. officinale* callus culture

iii. To determine the microscopic structure of *T. officinale* callus using light microscopy and transmission electron microscopy

iv. To quantify and compare the anthocyanin pigment presents in callus and petiole of *in vitro T. officinale* using ultra performance liquid chromatography

CHAPTER 2

LITERATURE REVIEW

2.1 *Taraxacum officinale*

2.1.1 Scientific classification

Taraxacum officinale (L.) Weber ex F. H. Wiggers, also known as dandelion, is a herbaceous, perennial plant which is classified under the family Asteraceae (formerly Compositae). Asteraceae is the largest family of the flowering plants. This cosmopolitan family consisted of 13 tribes, 84 genera and more than 240 species (Adedeji & Jewoola, 2008). Members of this family are highly advanced and easily recognised with worldwide distribution. Plants under family Asteraceae are mostly woody herbs and shrubs, while some are trees and climbing herbs (Adedeji & Jewoola, 2008).

Kingdom :Plantae

Class :Magnoliopsida

Order :Asterales

Family :Asteraceae (Compositae)

Tribe :Cichorieae

Genus :*Taraxacum*

Species : *T. officinale*

Members of the Asteraceae family are economically important as medicinal plants (e.g. *Hypochaeris radicata*), ornamental plants (e.g. *Chrysanthemum morifolium)*, alternative sources of natural rubber (e.g. *Taraxacum kok-saghyz*), natural sweeteners (e.g. *Stevia rebaudiana*), sources of cooking oil (e.g. *Helianthus annuus*) and others.

2.1.2 Common names

Dandelion, the common name of *T. officinale*, is derived from the French phrase 'dent de leon' and has the meaning of lion's tooth because of the toothed margin of the leaves (Grauso *et al.*, 2019). *Taraxacum*, a word which came from Greek, means disease remedy while *officinale* carries the meaning of medicinal or 'of the shop', as it was sold as a remedy for treatment of various sickness (Stewart-Wade *et al.*, 2002). Apart from that, the word *Taraxacum* had originated from Arab as 'tarachakum' (wild cherry), 'tarakshaqun' (wild chicory) and 'tarashqun' (bitter herb) (Stewart-Wade *et al.*, 2002). On the other side, its name was also believed to be altered from the Greek words: 'taraxis' (an eye disease), 'tarasos' (disorder), 'trogimon' (edible) and 'akeomai' (to cure or remedy) (Stewart-Wade *et al.*, 2002). There are many other common names of *T. officinale* which include blowball, canker-wort, Irish daisy, piss-in-bed, fairy clock, one-o-clocks, Lowenzahnwurzel (Germany), Pu Gong Ying (Chinese) and Kukraundha or Kanphool (Indian).

2.1.3 Botanical description

T. officinale is well-recognised by its yellow flowers and white puffballs (Figure 2.1). The plant has long, narrow, irregularly lobed leaves that form a basal rosette above the ground. The leaves have an average length of 5-40 cm and width of

0.7-15 cm. The width of the leaves decreases along the length from the base to the tip. The shape of the leaves usually varied from lobeless to toothed edges and from shallowly lobed to deeply lobed (Grauso *et al.*, 2019; Stewart-Wade *et al.*, 2002). The midrib of the leaves has a pale yellow-green to reddish-brown colour and is usually hollow (Collins, 2000). Generally, a whole plant of *T. officinale* develops around 5 to 10 flowering stems at the same time. The flower head (capitulum) is supported by a long and hollow leafless peduncle and is composed of up to 250 small, yellowish ligulate florets that made up a compositae (Stewart-Wade *et al.*, 2002). Soon after flowering, the inflorescences will turn into hairy, white puffballs (pappus) that bear numerous olive or brown conical seeds (3-4 mm in length, 1 mm width) (Stewart-Wade *et al.*, 2002).

In terms of the rooting system of *T. officinale*, the plant has a deep, branched tap root with an average length of 1-2 m and 2-3 cm in diameter (Stewart-Wade *et al.*, 2002). The root of the mature plant can proliferate below the level of other grass roots and thus help the plant to compete for survival (Stewart-Wade *et al.*, 2002). Apart from that, all parts of the plant contain lactiferous tissues (Evert, 2006) and secrete a latex rich in polyphenols when cut (Schütz *et al.*, 2005).

During flowering season, yellow flowers of *T. officinale* will open in daylight and close in the dark as they are light-sensitive (Kemper, 1999). Generally, it takes 2.8 days for blooming followed by 9.6 days of seed ripening in closed capitula (Martinková & Honěk, 2008; Martinkova *et al.*, 2011). Once maturation stage is reached, the bracts will open and parachute-like seeds will be dispersed by the wind for reproduction. The seeds can germinate immediately as they are viable before dispersal (Collins, 2000).

Figure 2.1 Common dandelion, *Taraxacum officinale* Web. Image retrieved from https://wemakedirtlookgood.com/2016/08/weed-opedia-dandelion/.

2.1.4 Habitats and distribution

T. officinale is native to temperate zones of Northern Hemisphere with warmer climate. As it has high tolerance to various climates, it is widely distributed in temperate and subtropical regions of the world (Stewart-Wade *et al.*, 2002). *T. officinale* can be found in many areas such as disturbed sites, waste ground, pastures, lawns, orchards, roadsides and urban habitats. It grows well in a wide range of soils but preferably chalky soils and nutrient-rich loamy soils with moderate humus content (Bond *et al.*, 2007). It is able to survive in soils with pH ranging from 4.8 to more than 7.6 (Stewart-Wade *et al.*, 2002). Moreover, *T. officinale* exhibits high light adaptivity which enables it to grow well either in full sunlight or diffused light in the shades of trees and buildings (Stewart-Wade *et al.*, 2002).

2.1.5 Uses of *T. officinale*

2.1.5(a) Medicinal values

T. officinale is an important and valuable medicinal herb which is well known for its diverse health-beneficial properties such as diuretic, digestive, choleretic, anti-oxidative and anti-rheumatic effects. It has long been used as a folk medicine for treatment of various ailments including dyspepsia, digestive complaints, gall and liver malfunctions as well as rheumatic and arthritic diseases (Sweeney *et al.*, 2005; Schütz *et al.*, 2006; Grauso *et al.*, 2019). Extracts from different parts of *T. officinale* possess different pharmacological effects. For instances, dandelion leaf extracts have been shown to exhibit anti-bacterial (Oseni & Yussif, 2012; Ionescu *et al.*, 2013), anti-viral (Rodríguez-Ortega *et al.*, 2013) and anti-oxidative activities (Choi *et al.*, 2010; Ghaima *et al.*, 2013) while root extracts enhance hepatic regenerative capabilities due

to its antifibrotic properties (Domitrović *et al.*, 2010). Leaf, flower or crude extracts of the plant were also found to exert hepatoprotective effect against liver cirrhosis (Park *et al.*, 2010; Colle *et al*., 2012). Moreover, dandelion extracts also exhibit anti-carcinogenic activities that inhibit the invasion of breast, prostate and liver cancer cells efficiently (Sigstedt *et al.*, 2008; Saratale *et al.*, 2018). Recent studies have also demonstrated the potential of dandelion extracts to be utilised as anti-obesity agent (Zhang *et al.*, 2008; Davaatseren *et al.*, 2013; González-Castejón *et al.*, 2014).

2.1.5(b) Food source

T. officinale is suitable to be utilised as a food source as it contains high levels of minerals, fibres, vitamins, essential fatty acids as well as some trace elements (de Padua *et al.*, 1999; Kemper, 1999; Escudero *et al.*, 2003). It had been demonstrated in previous studies that leaves of *T. officinale* had higher level of beta-carotene than carrot and higher contents of calcium and iron than lettuce and spinach (Bruneton, 1995; Shi *et al.*, 2008). In general, the entire plant of *T. officinale* is edible and different parts of the plant have been made into a variety of food products rich in various nutrients. For instances, dandelion leaves can be eaten cooked or raw as salad; dried leaves and roots have been made into digestive or dietary drinks like herbal teas; roasted and grinded dandelion roots are processed into caffeine-free beverage as coffee-substitute due to its bitter taste; and its inflorescences have been fermented into dandelion wine and beer. In addition, dandelion extracts have been utilised as flavour components in dairy desserts, candies and puddings (Leung & Foster, 1996; Grauso *et al.*, 2019). *T. officinale* have also been made into capsules, tinctures, tablets and juices as pharmaceutical products (Leung & Foster, 1996; Williams *et al.*, 1996).

2.1.5(c) Bio-indicator for metal pollution

Plants have always been a good candidate as potential biological monitors. By studying the amount and composition of microelements in the plants, one gets to know the chemical and geochemical characteristics of soil and its surroundings (Kuleff & Djingova, 1984). *T. officinale* is a potential bio-indicator for metal pollution as its leaves, shoots, and roots are capable of accumulating heavy metals such as arsenic, antimony, manganese, mercury and zinc (Kuleff & Djingova, 1984; Djingova *et al.*, 1986; Simon *et al.*, 1996; Bini *et al.*, 2012; Radulescu *et al.*, 2013). In addition, traits of *T. officinale* change in response to the pollution level as an adaptation to survive in heavily polluted areas. For examples, reduction in length and weight of the seeds followed by increase in the number of seeds were observed in *T. officinale* that grow in heavily contaminated areas (Savinov, 1998). Although other species such as mosses, lichens (Szczepaniak & Biziuk, 2003) and some trees (Sawidis *et al.*, 2011) also accumulate chemical elements, they have restricted distribution which limits their application and usage (Kuleff & Djingova, 1984). As such, *T. officinale* is a potential plant to be used as bio-indictor for heavy metal pollution as it is ubiquitous and distributed in various latitudes and altitudes (Kuleff & Djingova, 1984).

2.1.6 Phytochemical studies

Taraxacum officinale has been used as herbal medicine for the past decades to treat different kinds of diseases. Its health-promoting effects are attributed to the presence of various bioactive constituents inside the plant itself. These compounds consist of different types of plant secondary metabolites such as phenylpropanoids, flavonoids and terpenoids. Hydroxycinnamic acid derivatives, such as chicoric acid, monocaffeyltartaric acid and chlorogenic acid were reported to be the major phenolic

constituents present inside *T. officinale* while coumarins like chichoriin and aesculin were reported in dandelion's leaf extracts (Williams *et al.*, 1996; Budzianowski, 1997). In addition, a number of flavones glycosides namely luteolin 7-O-glucoside, luteolin 7-O-rutinoside, isorhamnetin 3-O-glucoside and apigenin 7-O-glucoside were characterised in the various extracts of dandelion (Wolbis *et al.*, 1993; Kristó *et al.*, 2002; Hu & Kitts, 2003). Furthermore, the presence of quercetin glycosides, has also been identified (Schütz *et al.*, 2005).

Apart from that, triterpenoids and sesquiterpenoids, which are classified under terpenoid essential oils, have also been characterised in the dandelion extracts (Kikuchi *et al.*, 2016). The presence of triterpene acids, in particular oleanolic and ursolic acids, were found predominantly in callus cells while α-amyrin and β-amyrin were detected both in the callus and wild plant. (Furuno *et al.*, 1993). On the other hand, Kisiel and Barszcz (2000) identified several sesquiterpenoids such as taraxinic acid derivatives, ixerin D, ainslioside and 11β, 13-dihydrolactucin in the dandelion root extracts. The presence of other triterpenoids and steroids namely stigmasterol, campesterol, lanosterol and taraxerol in various dandelion extracts has also been reported (Westerman & Roddick, 1981; Jung *et al.*, 2011; Rodríguez-Ortega *et al.*, 2013; Grauso *et al.*, 2019).

2.2 Plant pigments

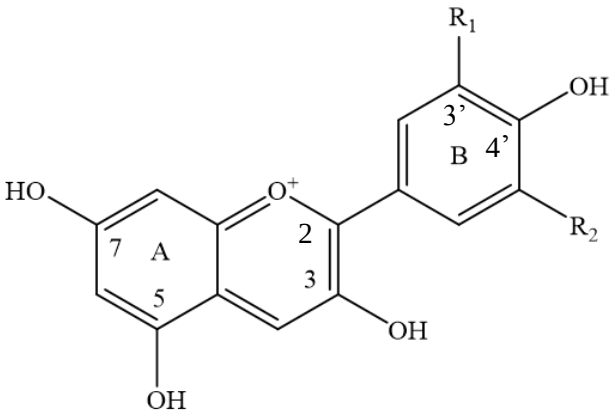
2.2.1 Anthocyanins

2.2.1(a) General biology and chemistry

Anthocyanins are important, water-soluble flavonoid compounds in nature that are responsible for a wide range of colourations (pink, scarlet, red, mauve, violet and blue) in flowers, leaves, fruits and storage organs of higher plants (Harborne, 1998).

Anthocyanins are less reported in liverworts, algae and other lower plants but some of them are detected in a few mosses and ferns (Bate-Smith & Swain, 1962). Besides being found in gymnosperms, they are present in most angiosperms except in the Caryophyllales (beets, cacti, bougainvillea, Amaranthus) where betalain pigment is predominant (Glover & Martin, 2012). The term "anthocyanins" was first introduced by Marquart in 1835 and the name was derived from the Greek words "anthos" and "kyanos" which mean "flower" and "dark blue", respectively (Delgado-Vargas *et al.*, 2000). Anthocyanins are also known as flavylium (2-phenylchromenylium) ions as they are derived from flavonol compounds. The chemical structure of anthocyanidins (Figure 2.2) consisted of a C15 skeleton with a chromane ring (ring-A) bearing a second aromatic ring (ring-B) in position 2 (C6-C3-C6) and with the attachment of one or more sugar units at different hydroxyl groups (Bate-Smith & Swain, 1962; Counsell *et al.*, 1979; Delgado-Vargas *et al.*, 2000). The empirical formula for flavylium ion of anthocyanins is $C_{15}H_{11}O^{+}$, with molecular weight of 207.25 g/mol (Khoo *et al.*, 2017).

Anthocyanins are anthocyanidins (phenyl-2-benzopyrilium) with attachment of sugar molecules while anthocyanidins are the aglycone form of anthocyanins



Compound	**R_1**	**R_2**	**Colour**
Pelargonidin	H	H	orange-red
Cyanidin	OH	H	magenta
Delphinidin	OH	OH	blue
Peonidin	OCH_3	H	magenta
Petunidin	OCH_3	OH	purple
Malvidin	OCH_3	OCH_3	purple

Figure 2.2 Structures of common anthocyanindins isolated from plants. Adapted from Zhang and Furusaki (1999); Delgado-Vargas et al. (2000).

(Bate-Smith & Swain, 1962; Counsell *et al.*, 1979). To date, more than 700 types of naturally occurring anthocyanins with dissimilar structures and 30 anthocyanindins have been characterised (Andersen & Jordheim, 2013; Zhang *et al.*, 2014; Appelhagen *et al.*, 2018). According to Castaneda-Ovando *et al.* (2009), the six most common anthocyanidins and their distribution in the plant kingdom are cyanidin (50%), delphinidin (12%), pelargonidin (12%), peonidin (12%), malvidin (7%) and petunidin (7%). These anthocyanidins were named after flower sources from which the pigments were first isolated by Willstätter and Everest (Zhang & Furusaki, 1999). The chemical structures of common anthocyanidins are listed in Figure 2.2.

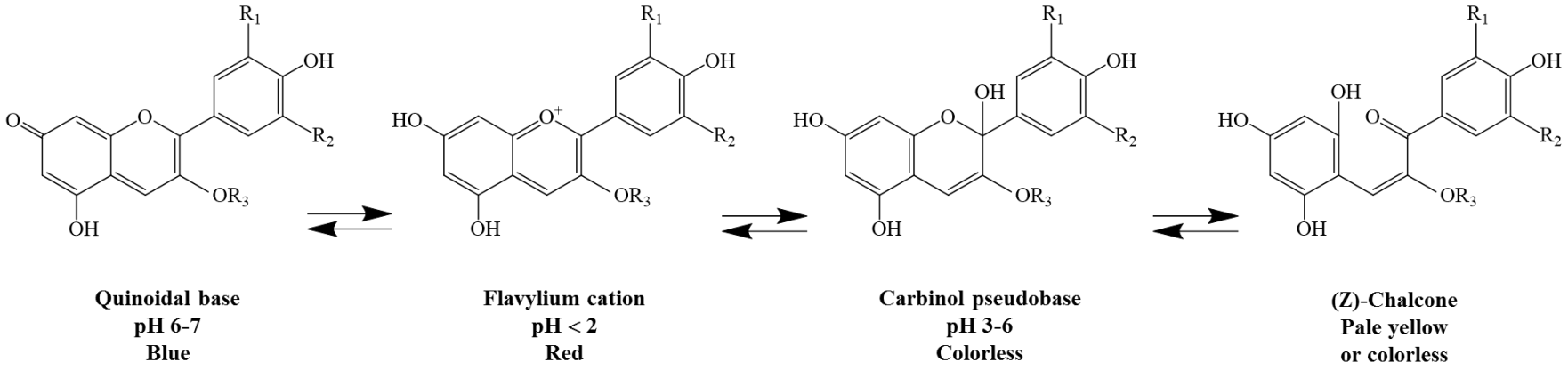
Generally, the variation of anthocyanins comes in several ways: (1) number and position of hydroxyl groups; (2) methylation on the hydroxyl groups; (3) type and number of the sugar units and the positions at which they are attached (4) acylation on sugar units and the type of acylating agent (Kong *et al.*, 2003; Castaneda-Ovando *et al.*, 2009; Rodriguez-Amaya, 2019). In addition, the colouration of anthocyanins is influenced by the substitution of hydroxyl and methoxyl groups on ring-B. An increase in the number of hydroxyl group increases the bluish shade of the compound while intensified redness would be observed with the increment in the number of methoxyl group (Delgado-Vargas *et al.*, 2000).

Cyanidin is the most common anthocyanidin and all the other types are derived from the cyanidin molecule by hydroxylation, methylation or glycosylation (Harborne, 1998). Hydroxylation and methylation normally occur at the aromatic ring-B while glycosylation takes place at the position 3, 5, and/or 7 of the hydroxyl group on the phenolic compound (ring-A). The sugar moieties attached to it can be any sugars such as glucose, rhamnose, arabinose and galactose in the form of mono-, di-, or

trisaccharide (Harborne, 1998). In some instances, the sugar unit at position 3 of ring-A is acylated by either aliphatic (e.g. malonic acid, acetic acid) or aromatic acids (e.g. ρ-coumaric acid, caffeic acid), forming acylated anthocyanins (Harborne, 1998). Other than that, anthocyanins also react with metals such as aluminium, iron or magnesium for stabilisation of the pigment complex and the reaction forms the intensely blue colouration as seen in mophead hydrangeas, cornflowers and *Commelina communis* (Brouillard & Dangles, 1994; Harborne, 2001; Glover & Martin, 2012).

2.2.1(b) Stability of anthocyanin colour based on pH

Anthocyanins are very sensitive to pH due to the ionic nature of its molecular structure (Turturică *et al.*, 2015). Anthocyanins undergo reversible structural transformation as well as colour change according to the pH of the aqueous solution (He & Giusti, 2010; Wrolstad & Culver, 2012). The structural transformation of anthocyanins is presented in Figure 2.3. In acidic aqueous solution with pH below 2, anthocyanins are predominantly in the form of flavylium cation and appear red. While in pH 3-6, the flavylium cation undergoes rapid hydration at C-2 and transforms into colourless carbinol pseudobase. Carbinol then forms (Z)-Chalcone by ring-opening tautomerization, where the latter can isomerize into (E)-Chalcone. At pH 6-7, deprotonation occurs on the flavylium cation and gives rise to blue quinoidal base (Khoo et al., 2017; Rodriguez-Amaya, 2019). Other than pH effect, glycosylation, hydroxylation and methylation as well as other factors such as temperature, light exposure, oxygen and presence of enzymes and metallic ions have also been showed to influence the stability of anthocyanin molecules (Francis & Markakis, 1989; Bridle & Timberlake, 1997; Castaneda-Ovando *et al*., 2009; Rodriguez-Amaya, 2019).



R_1 and R_2 = H, OH or OCH3, R_3 = sugar

Figure 2.3 Schematic representation of structural transformations of anthocyanins at different pH values. Adapated from Rodriguez-Amaya (2019).

2.2.1(c) Anthocyanin biosynthesis pathway

Anthocyanin biosynthetic pathway is well established in many plant species (Mol *et al*., 1989; Holton & Cornish, 1995; Liu *et al*., 2018). A generalised anthocyanin biosynthetic pathway is presented in Figure 2.4. Malonyl-CoA and ρ-coumaroyl-CoA are the active precursors for the formation of aromatic ring-A and ring-B of the flavan skeleton, respectively. Malonyl-CoA is formed via carboxylation of acetyl-CoA by acetyl-CoA carboxylase (AC), in the presence of adenosine triphosphate (ATP) while the biosynthesis of ρ-coumaroyl- CoA involves the phenylpropanoid pathway which starts with the deamination of the substrate L-phenylalanine to cinnamic acid by the action of phenylalanine ammonia lyase (PAL). Next, cinnamic acid is converted into ρ-coumaric acid by the action of cinnamate 4-hydroxylase (C4H) and transformed into its active form, ρ-coumaroyl-CoA by 4-coumaryl-CoA ligase (4CL). Subsequently, both aromatic rings derived from malonyl-CoA and ρ-coumaroyl-CoA are joined via condensation reaction mediated by chalcone synthase (CHS) to produce yellow coloured naringenin chalcone. It is then converted to the colourless naringenin via isomerisation mediated by chalcone isomerase (CHI). Next, naringenin is hydroxylated to form colourless dihydrokaempferol (DHK) by flavanone 3-hydroxylase (F3H). DHK can be further hydroxylated by flavonoid 3'-hydroxylase (F3'H) to yield dihydroquercetin (DHQ) or by flavonoid 3',5'-hydroxylase (F3'5'H) to form dihydromyricetin (DHM) (Delgado-Vargas *et al*., 2000; Liu *et al*., 2018).

Additionally, DHQ can also be converted to DHM by F3'5'H. The dihydroflavonols are then reduced to colourless leucoanthocyanidins (flavan-3,4-cis-diols) by dihydroflavonol 4-reductase (DFR). Subsequently, anthocyanidin synthase

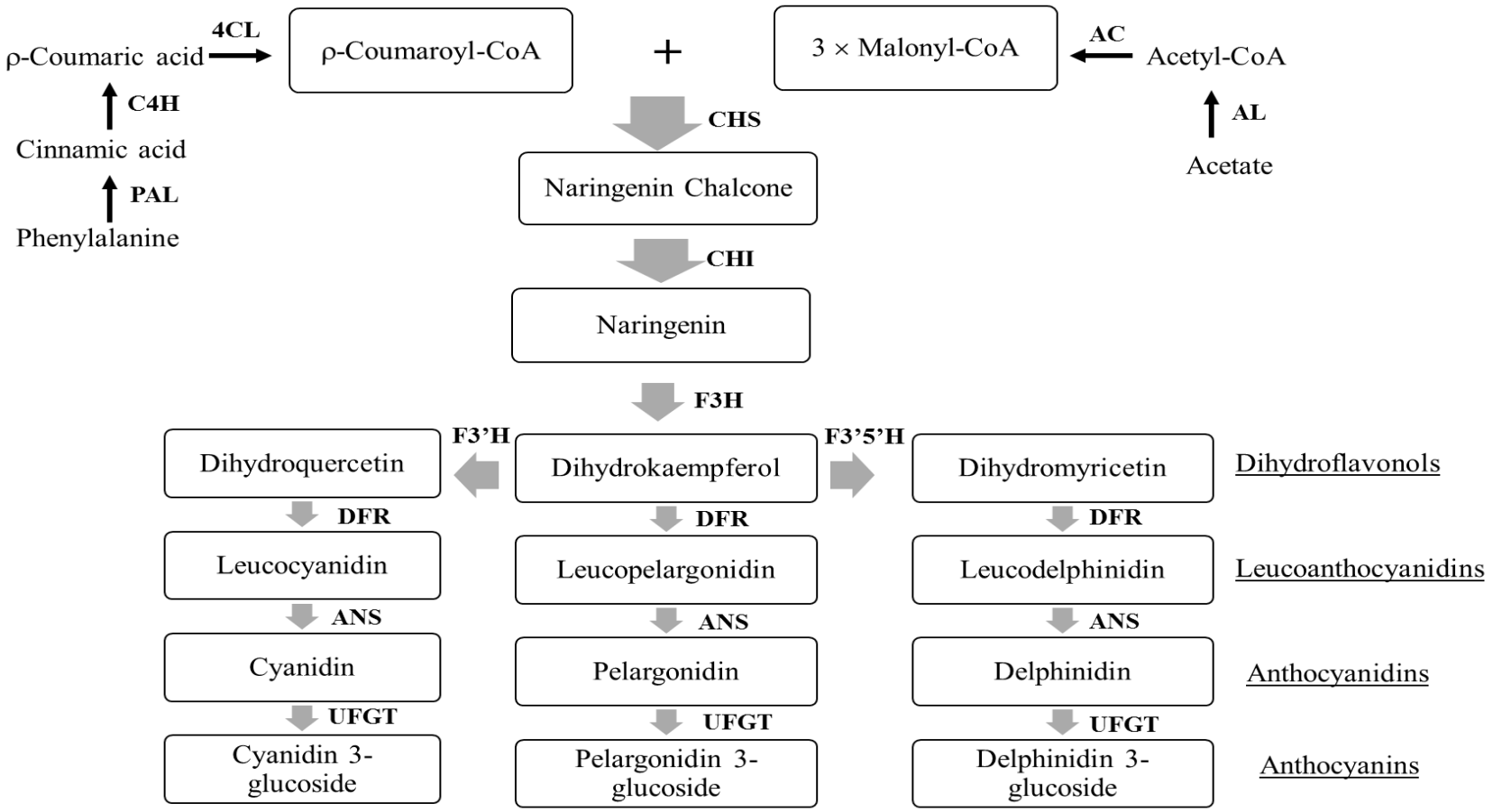


Figure 2.4 Schematic diagram of anthocyanin biosynthesis pathway. Enzymes involved: AL, acetate-CoA lyase; AC, acetate-CoA carboxylase; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaryl-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, F3'H, F3'5'H, flavonol hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanin synthase; UFGT, UDP-glucose: flavonoid 3-O-glucosyltransferase. Adapated from Delgado-Vargas *et al*. (2000) and Liu *et al*. (2018).

(ANS) catalyses the formation of colourless leucoanthocyanidins to coloured anthocyanidins. Glycosylation and acylation are the final steps in anthocyanin biosynthesis. Glycosyltransferase such as UDP-glucose: flavonoid 3-O-glycosyltransferase (UFGT) mediates the attachment of sugar unit to the anthocyanidin molecules. The C-3 position of the chromane ring is glycosylated first in order to stabilise the flavylium cation and subsequently the other positions. In some cases, the anthocyanins are further acylated by acyltransferase to form acylated anthocyanins (Delgado-Vargas *et al*., 2000; Liu *et al*., 2018).

CHS is the key enzyme for flavonoid biosynthesis as chalcone is the first common intermediate for all flavonoids. It had been demonstrated that accumulation of anthocyanins is closely related to the CHS activity (Ozeki, 1996; Akashi *et al*., 1997; Meng *et al*., 2004; Zhou *et al*., 2013). PAL is also an important enzyme as it is the entry point for the phenylpropanoid pathway where its end product, ρ-coumaroyl-CoA, is one of the active precursors for anthocyanin biosynthesis (Zhang & Furusaki, 1999). On the other hand, F'3H and F3'5'H contribute to the diversification of anthocyanins by determining the ring-B hydroxylation position and subsequently the colouration (Tanaka & Brugliera, 2013; Liu *et al*., 2018).

2.2.1(d) Importance of anthocyanins to plants

Bright and attractive colouration ranging from vivid red to purple violet are the common characteristics of anthocyanin-rich plant species. By imposing a strong contrast with the uniform green background of plant vegetation, bright colouration of fruits or flowers assists in plant propagation as well as seed dispersal by attracting various pollinators and fruit-eating animals (Koes *et al.*, 1994; Gould *et al.*, 2008; Miller *et al.*, 2011). Apart from that, anthocyanins, particularly those present in

vegetative organs, have also been implicated in plant defence mechanism by acting as warning signal (aposematic colouration) to repel potential insect herbivores (Hamilton & Brown, 2001; Gould *et al.*, 2008; Archetti, 2009). The red colouration signals elevated defensive compounds which could impair insect fitness and thus indirectly reduces herbivory of insects such as aphids (Archetti, 2009; Cooney *et al.*, 2012). In addition, anti-bacterial and fungicidal properties of anthocyanins also protect the plants against various infections caused by pathogenic microorganisms (Treutter, 2006; Schaefer *et al.*, 2008; Tellez *et al.*, 2016).

In addition, anthocyanins also serve as light attenuator to shield photosynthetic plant tissues from adverse effects of high irradiance (Gould *et al.*, 2008; Zhang *et al.*, 2010). Anthocyanins are generally distributed in the vacuoles of epidermal, palisade and spongy mesophyll cells (Chalker-Scott, 1999; Pietrini *et al.*, 2002; Steyn *et al.*, 2002; Merzlyak *et al.*, 2008). They function as light-filtering materials and protect the plant tissue from photoinhibition and photodamage by absorbing excess irradiance which would otherwise be absorbed by the chlorophyll pigments in the subjacent mesophyll cells (Gould *et al.*, 1995; Chalker-Scott, 1999; Hoch *et al.*, 2001; Hughes *et al.*, 2005; Merzlyak *et al.*, 2008). Other than that, studies have shown that anthocyanins, specifically those esterified with cinnamic acids, are able to absorb ultraviolet-B radiation and shield the surrounding plant tissues from destructive effect of harmful radiation (Tevini *et al.*, 1991; Woodall & Stewart, 1998; Ferreira da Silva *et al.*, 2012; Costa *et al.*, 2015). Furthermore, accumulation of anthocyanins in plant tissues is an indicator of stress in response to mechanical wounding (Gould *et al.*, 2002), osmotic stress (Shoeva *et al.*, 2017) and nutrient deficiency (Chalker-Scott, 1999; Steyn *et al.*, 2002). The red-pigmented compound is also able to protect the

plants from metal toxicity by its metal-chelating properties (Hale *et al.*, 2001; Hale *et al.*, 2002; Landi *et al.*, 2015).

2.2.1(e) Health benefits of anthocyanins to human

The beneficial effects of anthocyanins on human health have gained much attentions in recent years with the increased awareness on health issues. Examples of dietary anthocyanins include coloured fruits and vegetables (e.g. berries, grapes and purple cabbages) as well as processed beverages like red wines. According to the report from National Health and Nutrition Examination Survey (NHANES), daily intake of anthocyanins has been estimated to be 11.6 ± 1.1 mg/d for individuals aged ≥ 20 years (Sebastian *et al.*, 2015; Wallace & Giusti, 2015). On the other hand, Chinese Nutrition Society (2013) recommended a minimum daily intake of 50 mg anthocyanins in the diets for health purpose. The biological activities of anthocyanins such as anti-oxidant, anti-cancer, anti-diabetes, anti-obesity and neuroprotective activity have been investigated and reported in many cell cultures and animal studies (de Pascual-Teresa *et al.*, 2010; Tsuda, 2012; Smeriglio *et al.*, 2016; Khoo *et al.*, 2017; Li *et al.*, 2017; Rodriguez-Amaya, 2019). However, reports on human clinical trials are still lacking.

Among all the health-promoting effects, anthocyanins are well-known to be good anti-oxidant agents. As shown in earlier report, hydroxyl groups on 3' and 4' positions of the ring-B were important in determining the radical scavenging potential of flavonoids with a saturated 2,3- double bond while the anti-oxidant properties are closely associated with the patterns of hydroxylation and glycosylation (Wang *et al.*, 1997; Delgado-Vargas *et al.*, 2000). As reported by Tsuda *et al.* (1996), the higher the number of hydroxyl substituents, the higher the anti-oxidant activities of glycosylated anthocyanins. In an animal study, glycosides and aglycone forms of cyanidin,

pelargonidin and delphinidin extracted from *Phaseolus vulgaris* (seed coat) have been shown to inhibit lipid peroxidation efficiently attributed to its promising anti-oxidant activities (Tsuda *et al.*, 1996).

In addition to anti-oxidative effect, it was reported that anthocyanin-rich extracts inhibited the initiation and proliferation of several cancers, such as cervical cancer (Barrios *et al.*, 2010; Rugină *et al.*, 2012), blood cancer (Tsai *et al.*, 2014), fibrosarcoma (Filipiak *et al.*, 2014) and breast cancer (Faria *et al.*, 2010; Hui *et al.*, 2010). As for anti-obesity effect, Tsuda *et al.* (2003) reported that reduction of body weight gain and lipid accumulation were observed on obese mice which were fed with cyanidin 3-glucoside extracted from *Zea mays* for 12 weeks. Moreover, anthocyanins were also proven to reduce the deposition of cholesterols in the plasma and prevent atherosclerosis as well as myocardial infarction (Mink *et al.*, 2007; Mauray *et al.*, 2010; Cassidy *et al.*, 2013).

2.2.1(f) Potential use of anthocyanins: food colourant

Food colourant is important in the food industries as it helps to enhance the appearance of food products, ensuring the uniformity of the colours, providing colour identities to otherwise colourless food and also to restore colour loss during processing and storage (Cortez *et al.*, 2017; Sigurdson *et al.*, 2017). Recently, there is a growing trend in using natural pigments as a replacement for synthetic dyes in processed food and beverages. This is mainly due to increase public concerns on the food safety and the potential side-effects of artificial dyes. It has been reported that chemicals used to produce artificial colourants caused hyperactivity in children and allergenicity in sensitive individuals (McCann *et al.*, 2007; Carocho *et al.*, 2014; Oplatowska-