ESTABLISHMENT OF CELL SUSPENSION CULTURE OF *Melastoma malabathricum* L. FOR THE PRODUCTION OF ANTHOCYANIN

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ESTABLISHMENT OF CELL SUSPENSION CULTURE OF *Melastoma malabathricum* L. FOR THE PRODUCTION OF ANTHOCYANIN

by

KOAY SUAN SEE

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DEDICATION

For my beloved...

Dad, Mum, Huat, Chuin, Yih & Feng

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KOAY SUAN SEE

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
B5	Gamborg
BA	6-benzylaminopurine
CHI	Chalcone isomerase
CHS	Chalcone synthase
CRD	Complete randamised design
CV/g-FCM	Colour value per gram fresh cell mass
CV/flask	Colour value per flask
Cy	Cyanidin
DCM	Dry cell mass
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
FCM	Fresh cell mass
HPLC	High performance liquid chromatography
JA	Jasmonic acid
LS	Linsmaier and Skoog
MeJA	Methyl jasmonate
MS	Murashige and Skoog
NAA	1-naphthylacetic acid
PC	Paper chromatography
RSA	Radical scavenging activity
UV	Ultraviolet
UVB	Ultraviolet B

v/v	Volume per volume
w/v	Weight per volume

PEMBANGUNAN KULTUR AMPAIAN SEL *Melastoma malabathricum* L. UNTUK PENGHASILAN ANTOSIANIN

ABSTRAK

Kultur kalus yang diinisiasi daripada eksplan daun aseptik dan dikultur dalam medium pejal MS + 1 mg/L BA + 6 mg/L NAA + 30 g/L sukrosa adalah stabil dan menghasilkan indeks pertumbuhan 8 selepas 26 kitar subkultur. Kalus yang diperolehi adalah rapuh. Pendedahan kepada cahaya berkeamatan 1000 – 1500 lux dan suhu 24 ± 2 ^{o}C di dalam bilik kultur menggalakkan pertumbuhan dan pigmentasi kalus di hujung kitar kultur. Kalus yang rapuh dan tumbuh cepat digunakan untuk memulakan dan membangunkan kultur sel di dalam medium cecair MS + 0.25 mg/L BA + 0.5 mg/L NAA + 30 g/L sukrosa. Sel titisan yang diperolehi adalah stabil dan dapat dikekalkan dengan menginokulasikan 1.25 g sel ke dalam 50 mL medium di dalam kelalang Erlenmeyer 250 mL dan disubkultur pada selang masa 14 hari. Selepas 10 hingga 12 subkultur, penghasilan pigmen mula diperhatikan dan sel-sel semakin berpigmen. Penghasilan pigmen adalah paling tinggi pada hari ke-9 pengkulturan di mana kultur berada di dalam fasa eksponen pertumbuhan. Satu sel titisan berpigmen yang stabil yang dibangunkan dengan menginokulasikan 0.8 g sel berpigmen ke dalam 50 mL medium di dalam kelalang Erlenmeyer 250 mL dan disubkultur setiap 9 hari dapat dikekalkan selama 24 bulan. Keputusan eksperimen menunjukkan makronutrien tidak memainkan peranan penting dalam peningkatan penghasilkan pigmen. Pendedahan yang berterusan kepada cahaya berkeamatan 301 – 600 lux, suhu 20 ± 2 ^{o}C dan kepekatan sukrosa 45 g/L dapat meningkatkan penghasilan pigmen. Sehubungan itu, kultur sel *M. malabathricum* L. dapat dikekalkan dalam medium cecair MS + 0.25 mg/L BA + 0.5 mg/L NAA + 30 g/L

sukrosa sebagai medium pertumbuhan bagi kitaran kultur selama 14 hari dan dipindahkan ke dalam medium cecair MS + 0.25 mg/L BA + 0.5 mg/L NAA + 45 g/L sukrosa, didedah kepada cahaya berkeamatan 301 – 600 lux dan 22 ± 2 ^{o}C serta dipungut hasilnya selepas 9 hari pengkulturan untuk tujuan penghasilan pigmen. Pigmen dapat diekstrak daripada sel dengan menggunakan metanol berasid, dipekatkan di bawah aliran gas nitrogen yang perlahan dan disimpan di dalam keadaan gelap pada 4 ^{o}C. Analisis kimia dengan menggunakan teknik kromatografi kertas, spectrofotometri dan kromatografi cecair berprestasi tinggi ke atas ekstrak pigmen yang diperolehi daripada daun, petiol dan buah pokok induk serta kalus dan sel yang segar dan kering yang telah melalui hidrolisis asid membolehkan pengenalan aglikon utama sebagai sianidin. Penentuan IC_{50} dan ujian DPPH yang dijalankan ke atas ekstrak menunjukkan sianidin yang diperolehi mempunyai aktiviti pemusnahan radikal yang tinggi, satu petunjuk bagi keupayaan antioksidan yang tinggi. Ciri ini menjadikan pigmen ini bukan sahaja dapat dijadikan pewarna asli, malah dapat digunakan sebagai supplemen nutraseutikal.

ESTABLISHMENT OF CELL SUSPENSION CULTURE OF *Melastoma malabathricum* L. FOR THE PRODUCTION OF ANTHOCYANIN

ABSTRACT

Callus culture initiated from the aseptic leaf explants and maintained on solidified MS medium + 1 mg/L BA + 6 mg/L NAA + 30 g/L sucrose was stable and produced an average growth index of 8 after 26 subculture cycles. The callus was friable. Continuous exposure to light intensities of 1000 – 1500 lux and temperature of 24 ± 2 ^{o}C in the culture room promoted callus growth and pigmentation towards the end of culture cycle. Using the friable and fast growing callus, the cell suspension culture was initiated and established in liquid MS medium + 0.25 mg/L BA + 0.5 mg/L NAA + 30 g/L sucrose. The cell line was stable and was maintained by inoculating 1.25 g of cells into 50 mL medium in 250 mL Erlenmeyer flasks and subcultured every 14 days. After 10 to 12 subcultures, pigment production was observed and the cells slowly became pigmented. Pigment production was the highest on day 9 of the culture period when the culture was at its exponential phase of growth. A stable pigmented cell line could be maintained for 24 months by inoculating 0.8 g of the pigmented cells into 50 mL of medium in 250 mL Erlenmeyer flasks and subcultured every 9 days. Results showed that macronutrients did not play an important role in enhancing pigment production. Light intensities of 301 – 600 lux, temperature of 22 ± 2 ^{o}C and sucrose concentration of 45 g/L were found to enhance pigment production. Thus, the cell suspension culture of *M. malabathricum* L. could be maintained in liquid MS medium + 0.25 mg/L BA + 0.5 mg/L NAA + 30 g/L sucrose as the proliferation medium for 14 days of culture cycle and transferred into liquid

MS medium + 0.25 mg/L BA + 0.5 mg/L NAA + 45 g/L sucrose, exposed to light intensities of 301 – 600 lux and 22 ± 2 ^{o}C, and harvested at the end of 9 days of culture period for pigment production. The pigment could be extracted from the cells using acidified methanol, concentrated under a gentle flow of nitrogen gas and kept in the dark at 4 ^{o}C. Chemical analyses via paper chromatography, spectrophotometry and high performance liquid chromatography on the acid-hydrolysed pigment extracts from the leaves, petioles and fruits of the mother plant as well as the extracts of fresh and dried callus and cells enabled the identification of the major aglycon from these sources as cyanidin. The determination of IC_{50} and DPPH test carried out on the extracts showed they possess high radical scavenging activity, an indicator for high antioxidant activity. This feature makes the pigment a potential nutraceutical supplement besides natural colourant.

CHAPTER 1. INTRODUCTION

From the earliest times colours have played an important role in the life of man. Tens of thousands of years ago bodypainting was already part of the ritual connected with waging war and funerals. The red dye from henna (*Lawsonia inermis* L.) was used by the ancient Greeks and Romans as a cosmetic, especially for giving human hair a redsheen.

Plants are the main natural resources for many pigments used by mankind. Colours that can be obtained from plant products include blue pigments from *Indigofera* spp. and *Haematoxylum campechianum* L., yellow from *Crocus sativus* L., red from *Rubia cordifolia* L., orange from *Bixa orellana*, brown from *Peltophorum pterocarpum* and black from *Macaranga tanarius* (L.) Muell. Arg. (Lemmens and Wulijarni-Soetjipto, 1991). Major classes of plant pigments are chlorophylls, carotenoids, flavonoids and quinones.

The rich flora of our country presents many sources of pigments. Today, some local plants that are being used widely as food colourants include the Anatto (*B. orellana*) for red, *Clitoria ternatea* for blue, the tubers of *Curcuma longa* L. for yellow and the leaves of *Pandanus amaryllifolius* Roxh. for green. Besides the colour, people also appreciate the typical flavour and taste given to the food by the plant product.

Natural colourants are often present in low concentrations in the plant tissues and in many cases, they are more expensive than the synthetic dyes. Since the introduction of synthetic dyes, the food industry use almost exclusively synthetic food colourings as they proved to be purer and cheaper to produce. Nevertheless, synthetic dyes are not always harmless for human beings. Some synthetic dyes have

proven to be carcinogenic. Others used in foods have been associated with behavioural disturbances such as hyperactivity and learning disorders in children. Moreover, their waste products cause environmental pollution. These facts have created awareness and many are looking for natural dyes, especially for use in foods and drinks.

Plant products can be divided into primary plant metabolites and secondary metabolites. Primary plant metabolites are those metabolites essential to the life of the plant. They include sugars, amino acids and nucleotides synthesised by plants to be used to produce essential polymers. Typically primary metabolites are found in all species within broad phylogenetic groupings, and are produced using the same or nearly the same metabolic pathway.

During the long evolutionary course, plants have evolved various strategies for survival and reproduction, one of which is the production of a myriad of small molecular weight compounds, also known as the secondary metabolites. These compounds are not directly involved in the normal developmental growth or reproduction of plants but they are believed to play vital roles in the physiology and ecology of the plants that produce them, particularly as defence elements against pests and pathogens besides colourful pigments to attract insects for pollination. They are often species-specific and thus have long been of prime importance in taxonomic research. The importance of secondary metabolites is apparent in some of the earliest annals of human history. For example, *Papaver somniferum*, the original source of morphine and codeine, was known to the Sumerians in 4000 B.C.E. as *hul gil* (joy-plant).

Today, secondary metabolites are used as food flavours, dyes, poisons, perfumes, scented oils in aromatherapy and in industrial products such as rubber and

oils. The benefits of some of these substances to human have only recently been discovered by scientists, which include boosting the immune system, protecting the body from free radicals and lethal to pathogenic microorganisms.

The anthocyanins, a subclass of flavonoids, constitute one of the major groups of natural pigments and are responsible for many of the colours of both fruits and vegetables as well as flowers. In recent years numerous studies have been carried out to characterise the profiles of the anthocyanins of different natural products and they are used as an alternative to the synthetic colourants in the food industry (Harborne and Grayer, 1988). One of the main advantages of anthocyanins is their hydro-solubility which facilitates their incorporation into different food-stuffs (Marz-Pop *et al.*, 2006).

Malaysia is one of the twelve megabiodiversity countries in the world. One of its local plant, *Melastoma malabathricum* L., has been found to be an important dye-producing plant. The roots are used in mixtures for red dye, the leaves in mixture for purple dye and the fruits used for dyeing cloth black (Lemmens and Wulijarni-Soetjipto, 1991).

The plant bears light to dark magenta-pink flowers. The fruits are like berries with small seeds embedded in a mess of purplish pulp. The fruits are sweet. When they are eaten the pulp will stain the mouth and tongue black. The pigments in the plant are due to the presence of anthocyanins. Thus, *M. malabathricum* L. has been identified as a potential local source for anthocyanin production.

Biotechnology offers new tools to improve production of plant products. Today, plant cell, tissue and organ cultures are used to obtain rapid asexual multiplication of plant cells or plants under sterile conditions. It is based on the fact that many plant cells have the ability to regenerate into a whole plant (totipotency).

Single cells, plant cells without cell wall (protoplasts), pieces of leaves, or roots can often be used to generate a new plant on artificial culture media, given the required nutrients and plant growth regulators. The whole new plantlets are genetically identical to their parent plants. Alternatively, portions of organs or tissues can be cultured to produce an unorganised mass of cells called callus (soft tissue that forms over a cut surface). Friable calli can further be used to establish cell suspension cultures. The establishment of cell suspension culture is important as it enables manipulations of various factors, either physical or chemical, to mass produce the desired secondary metabolite.

The advantages of plant tissue and cell cultures over living plants, in terms of secondary metabolite production, are clear. In the laboratory, growth conditions can be controlled, therefore, reproducible yields of end product can be achieved. Growth parameters such as pH, temperature, medium components and other environmental factors can be optimised to obtain metabolite production, preferably significantly higher than in the living plants. Separation of target compounds is much easier, too, due to lower complexity of the cultured materials. Lastly, large-scale growth of plant cells in liquid culture in bioreactors as a source of secondary products can be achieved and ultimately commercialised.

With the increasing awareness concerning the health risks caused by synthetic food colourants, the focus now is to look for pigments from natural resources to substitute the synthetic ones. Anthocyanins have great potential to be natural food colourant.

Besides having bright attractive colours and high water solubility, recent research have found that anthocyanins possess antioxidant, anti-inflammatory, anticarcinogenic, antiatherogenic, antibacterial and antiviral properties (Mazza,

2000). Thus, anthocyanins could be an ideal natural colourant which are less toxic, less polluting, less health hazardous, non-carcinogenic and non-poisonous and at the same time provide as nutritive enriched food with therapeutic effects.

The potential to produce anthocyanins through plant cell cultures is being considered as a valuable alternative to the use of synthetic dyes. Anthocyanins from plant tissue cultures have been successfully obtained in a number of plant species, such as carrot (Kinnersley and Dougal, 1980; Gläβgen *et al.*, 1992), *Catharanthus roseus* (Filippini *et al.*, 2003), strawberry (Mori *et al.*, 1993), cowberry (Andersen, 1985), bilberry (*Vaccinium myrtillus*) (Madhavi *et al.*, 1998), *Ajuga reptans* (Callebaut *et al.*, 1990), *Ajuga pyramidalis* (Madhavi *et al.*, 1996), *Oxalis linearis* (Meyer and Van Staden, 1995), *Tradescantia pallida* (Shi *et. al*. 1992) and purple sweet potato (Terahara *et al.*, 2004). To our knowledge, the application of tissue and cell culture technique for the production of anthocyanins from our local plants has not been developed. Thus the objectives of this study are:

1. To develop *in vitro* culture techniques for the production of plantlets, induction of callus and establishment of cell suspension cultures of *M. malabathricum* L.,
2. To optimise the physical and chemical factors for maximum production of the anthocyanins in the cell suspension cultures,
3. To identify and quantify the major anthocyanidin in the pigment of the established cell suspension cultures of *M. malabathricum* L.,
4. To determine the antioxidant activity of the cell extract of *M. malabathricum* L.,
5. To incorporate the pigment into various foodstuff, toileteries and cosmetic products.

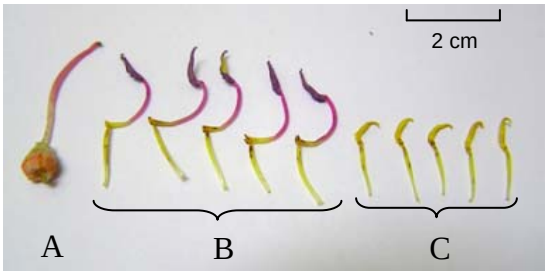
CHAPTER 2. LITERATURE REVIEW

2.1. *Melastoma malabathricum* L.

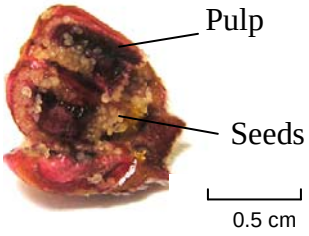
2.1.1. Plant morphology

M. malabathricum L. belongs to the Melastomaceae family. It is also called the Singapore Rhododendron, Straits Rhododendron or Sendudok (in Bahasa Malaysia). It is a shrub with an average height of one meter but it may grow up to three meters tall. The stems are square, reddish and covered with small bristly scales. The leaves are narrow, lance-shaped, tapered at both ends, slightly rough, especially on the adaxial surface, with three prominent longitudinal veins. The flowers are big, with five free petals that are from light to dark magenta-pink in colour (Plate 2.1a) and are borne at the ends of the twigs in short influorescences. The flowers are short-lived and they last only a day. The flower has ten stamens of two different kinds: five larger ones with yellow filaments and purple curved upper parts including the anther and five smaller ones with yellow and straight filaments and yellow anthers. The style is long and pink with green stigma (Plate 2.1b) (Henderson, 1974). The fruits are berry-like capsules with numerous seeds coated with red, sweet astringent pulp (Plate 2.1c). The seeds are dimorphic: with or without embryos. Fertile seeds are folded or spiral, triangular to D-shaped in outline, 0.45 – 0.8 mm long, 0.35 – 0.6 mm wide, 0.17 – 0.3 mm thick, with testa which is light yellow or pale to dark cream-coloured. Seeds without embryo are similar to the fertile seeds but smaller, 0.3 – 0.5 mm long, 0.2 – 0.3 mm wide, 0.2 mm thick, appear collapsed, dented, or wrinkled and with completely black or reddish-black testa (Scher, n.d.). The fruits are often eaten by children. The pulp will stain the mouth and tongue black. In fact, the word melastoma is Greek for "black mouth".

(a)



(b)



(c)

Plate 2.1. Morphology of *M. malabathricum* L.

(a) The flower
(b) The pistil and the stamens
A - The pistil
B - Five larger stamens
C - Five smaller stamens
(c) Longitudinally cut fruit showing the red pulp and seeds.

The colours in the plant are due to the presence of anthocyanins. Though the anthocyanins are easily recognised as flower pigments, their occurrence is not restricted to flowers but include all parts of the plant. The fruit yields a black dye while the roots a pink dye.

2.1.2. Habitat and distribution

Members of the Melastomaceae family are mostly tropical. There are about 160 genera (Hsuan Keng, 1986) with about 180 species in 25 genera common both in the mountains and the lowlands of Malaya (Henderson, 1974). Latiff *et al.* (1999) reported the presence of seven genera and 11 species of the Melastomaceae family in Pulau Tioman.

M. malabathricum L. is widely distributed, from Madagascar, India to Australia. It is very common in Southeast Asia. It is a common shrub in previously cleared land and waste places. Its pioneering characteristic is due to its ability to grow in areas of poor nutrient soil such as in areas with phosphorus deficient and acidic soil (Pengnoo *et al*., 2007). In Malaysia, it grows wild along almost every highway.

The plant plays a significant role in various ecological systems. The fruits are the favourites of birds like the flowerpeckers (such as *Dicaeum cruentatum*) and doves which also disperse the seeds, squirrels and monkeys. The plant is the host for caterpillars of butterflies such as the Common Sailor (*Neptis hylas*) and the Grey Count (*Tanaecia lepidea*). Carpenter bees can often be seen pollinating the flowers. Being among the first to colonise wasteland, the plant helps to prevent soil erosion and to allow regeneration of other vegetation in such places.

2.1.3. Traditional medicinal uses of *M. malabathricum* L.

In Malaysia, Indonesia and India, various parts of the plant are used in folk medicines. The leaves are pounded and applied as paste on cuts or wounds to stop bleeding (Latiff and Zakri, 2000; Indu and Razali, 1998).

The roots are used to prepare decoction to treat diarrhea (Lin, 2005) and the young leaves are eaten for the same purpose (Andersen *et al.*, 2003, Indu and Razali., 1998). A handful of young premature leaves are taken raw to cure dysentery (Sajem and Gosai, 2006).

The plant was also traditionally used to tone up the uterus after childbirth so as to strengthen the womb and accelerate healing. Its flowers, seeds and leaves are used to reduce white vaginal discharge and indigestion (Indu and Razali, 1998).

2.1.4. Phytochemical and bioactivity studies of *M. malabathricum* L.

The usage of the plant in traditional medicine relies on its chemical compounds. Yoshida *et al.* (1992a) reported the isolation of dimeric hydrolysable tannins, malabathrins B, C, D, and eleven known tannins (nobotanins B, D, G, H, J, pterocarinin C, casuarictin, strictinin, pedunculagin and two galloylglucoses) from it's leaves. Further investigation of the leaf extract led to the isolation of another seven C-glucosidic ellagitannins including three new complex tannins, named malabathrins A (6), E (11) and F (14) which were composed of C-glucosidic ellagitannin and a flavan-3-ol linked through a C-C bond (Yoshida *et al.*, 1992b). One of the major functions of tannins in plants is thought to act as a herbivory barrier, because of the astringent taste they impart to the herbivores. Industrially, tannins are used to transform raw animal skins into leather due to their ability to cross-link with the protein.

Din *et al.* (2002) reported the presence of steroid/triterpene and saponin in the leaves of a few species of *Melastoma* found in Malaysia. Nisya (2002) detected the presence of four flavonoid aglycones, chrysoeriol, 3,5-dimethyl myricetin, naringenin and auresidin from the plant extracts using thin layer chromatography. Two-dimension chromatography revealed the presence of chrysoeriol-7-glucoside, 3,5-dimethyl myrisetin, naringin and auresidin-4-rhamnoside. All the substances were detected in the *in vitro* seedlings but auresidin and auresidin-4-rhamnoside could not be detected in the mother plant. Tannin was not detected in both the *in vitro* seedlings and the mother plant.

When the aqueous methanolic extracts of the fresh leaves and flowers were separately partitioned with solvents and column chomatographed on silica gel and Sephadex LH-20, Ali (2004) isolated three urs-12-ene pentacyclic triterpenoids, namely ursolic acid, 2α-hydroxyursolic acid and asiatic acid, β-sitosterol 3-O-β-D-glucopyranoside, glycerol 1,2-dilinolenyl-3-O-β-D-galactopyranoside and glycerol 1,2-dilinolenyl-3-O-(4,6-O-isopropylidene)-β-D-galactopyranoside from the leaves. The ethyl acetate-soluble part of the flower yielded ellagic acid and six flavonoids which were identified as quercetin, kaempferol, kaempferol 3-O-α-L-rhamnopyranoside, kaempferol 3-O-β-D-glucopyranoside, kaempferol 3-O-β-D-galactopyranoside and kaempferol 3-O-(2",6"-di-O-E-p-coumaryl)-β-D-galactopyranoside. The last compound isolated was uncommon as it was only isolated once before from a plant.

Susanti (2006) reported that the n-hexane, ethyl acetate and methanol extracts of the leaves of *M. malabathricum* L. yielded three new compounds, 2,5,6-trihydroxynaphtoic carbonic acid, methyl-2,5,6-trihydroxynaphtalene carbonate and flavonol glycoside derivative. The n-hexane extract of the roots contained betulinic

acid, serrat-14-en-16-one and 2-(2'-hydroxyvinyl)-1-methyl-4-propoxyphthalate. The ethyl acetate extract of the flowers yielded three compounds, kaempferol-3-O-β-D-glucoside, kaempferol and naringenin while the methanol extract of the flowers was found to contain kaempferol-3-O-(2",6"-di-O-p-trans-coumaroyl)-glucoside and kaempferol-3-O-β-D-glucoside. The ethyl acetate extract of the fruits afforded betulinic acid, while the n-hexane extract of the stems produced α-amyrin.

A study on the antibacterial activity carried out by Ali (2004) showed that asiatic acid from the leaves, as well as ellagic acid, quercetin and kaempferol from the flowers were effective against *Bacillus cereus* and *Staphylococcus aureus*. When the pure compounds and crude extracts were subjected to antimicrobial, antioxidant, anti-inflammatory and cytotoxic assays, Susanti (2006) found the methanolic extract of the fruits of *M. malabathricum* L. exhibited the strongest inhibition against bacteria, *Bacillus subtilis*, *Streptococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* with minimum inhibitory concentration values of 62.5, 62.5, 125.0 and 62.5 µg/mL, respectively, in the antimicrobial assay. The antioxidant assay carried out using FTC (ferric thiocyanate) and DPPH (2,2-diphenyl-1-picrylhydrazyl) (UV and ESR spectroscopic) methods showed that kaempferol-3-O-(2",6"-di-O-p-trans-coumaroyl)glucoside, kaempferol-3-O-β-D-glucose, kaempferol, hyperin, quercetin and quercitrin showed strong antioxidative activities with inhibition of more than 90 % in the FTC method. Quercetin was found to be the most active as radical scavenger in DPPH-UV and ESR method with IC_{50} (the concentration needed for 50 % inhibition *in vitro*) of 0.69 and 0.65 µM, respectively. α-Amyrin and kaempferol-3-O-(2",6"-di-O-p-trans-coumaroyl)glucoside demonstrated the strongest activities in the antiinflammatory assay of TPA (12-O-tetradecanoylphorbol-13-acetate)-induced mouse ear oedema with IC_{50} of 0.11 and

0.34 mM/ear, respectively. The ethyl acetate extract of the leaves displayed high activity with an inhibition of 94.3 %. Kaempferol-3-O-(2”,6”-di-O-p-trans-coumaroyl)glucoside gave an IC_{50} of 5.6 µM in the PAF (platelet activating factor) anti-inflammatory assay. The cytotoxicity study carried out using MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay on MCF7 (a human breast cancer) cell line showed that kaempferol-3-O-(2”,6”-di-O-p-trans-coumaroyl)glucoside and naringenin were active in inhibiting cell proliferation of MCF7 with IC_{50} of 0.28 and 1.3 µM, respectively.

Kim *et al.* (2000) reported that the ethanolic extract, methanolic extract, ruthin, and quecertin of the plant might contain a substance that lowered the blood pressure and heart rate and increased the intestinal activity in the anaesthetised adult albino Wistar rats. The methanolic extracts indicated antiviral activities against the herpes simplex virus-1 and poliovirus as well as cytotoxic activities against murine and human cancer cell lines (3LL, L1210, K562, U251, DU145, MCF7) (Lohézic-Le Dévéhat *et al.*, 2002). Beside its positive effects on human health, Muhammad *et al.* (1997) found that the methanolic extracts of this plant was moderately effective against *Bursaphelenchus xylophilus*, the pine wood nematode.

2.2. Plant pigments

2.2.1. Anthocyanins

Anthocyanins are categorised in a subclass of flavonoids which in turn is a subclass of plant polyphenols. They are probably the most important group of visible plant pigments besides chlorophyll (Kong *et al.*, 2003). By far, more than 6400 different flavonoid compounds have been described in plants (Martens *et al.*, 2003).

Anthocyanins (in Greek, *anthos* means flower, and *kyanos* means blue) are water-soluble pigments widely present in various plant species, belonging to the phenolic class of flavonoids. They are largely responsible for the attractive colours of flowers, leaves, fruits, fruit juices and wines. They form the range of red and blue colourations seen in the higher plants. They commonly occur in flowers or fruits but may appear transiently or permanently in any other organ such as vegetables, roots, tubers, legumes and cereals. Chemically, an anthocyanin consists of an anthocyanidin or aglycon and sugar(s) and a commonly present third component, an acyl acid.

The anthocyanidins are substituted flavylium (2-phenylbenzopyrylium) salts differing in the number and position of free or methylated hydroxyl groups with the typical A-ring benzoyl and B-ring hydroxycinnamoyl systems. Figure 2.1 shows the structural formula of the flavylium cation and the ring-numbering convention (Michael Eskin, 1979).

Anthocyanidins are less stable and rarely found in plants. Glycosylation usually happens at C-3 or C-3 and C-5, forming the corresponding anthocyanin. This glycosylation does not only cause a reduction of maximum wavelength absorption (Table 2.1) but also confers higher stability and increased solubility of the compounds. In order of relative abundance, the sugars found with anthocyanidins are glucose, rhamnose, galactose, xylose, arabinose and glucuronic acid (Francis, 1999).

The glycosides, in turn, can be acylated. One or more molecules of the aromatic acids such as *p*-coumaric, ferulic and caffeic or the aliphatic acids such as malonic and acetic may be esterified to the sugar molecule (Francis, 1999). The acyl group is always attached to the sugar substituted in the 3-position (Harborne, 1964). Acylation of anthocyanins is found to be important for the stabilisation of

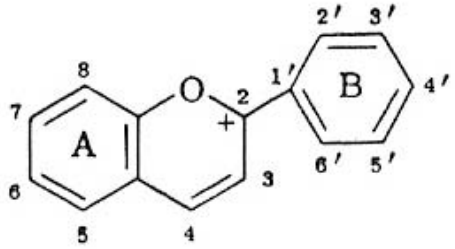


Figure 2.1. The flavylium cation

Table 2.1. Six most common anthocyanidins, their glycosides and maximum wavelength absorptions (Stintzing and Carle, 2004).

Anthocyanin	R_3	$R_{3'}$	$R_{5'}$	$\lambda_{vis\text{-}max}$ (nm)
Pelargonidin	H	H	H	520
Cyanidin	H	OH	H	535
Delphinidin	H	OH	OH	546
Peonidin	H	OCH_3	H	532
Petunidin	H	OCH_3	OH	543
Malvidin	H	OCH_3	OCH_3	542
Pelargonidin 3-glucoside	Glc	H	H	516
Cyanidin 3-glucoside	Glc	OH	H	530
Delphinidin 3-glucoside	Glc	OH	OH	543
Peonidin 3-glucoside	Glc	OCH_3	H	536
Petunidin 3-glucoside	Glc	OCH_3	OH	546
Malvidin 3-glucoside	Glc	OCH_3	OCH_3	546

Refer to Figure 2.1 for positions 3, 3’ and 5’.
Glc – glucose.

anthocyanin pigments (Brouillard, 1988).

It is estimated that more than 540 anthocyanin pigments have been found in nature (Anderson and Francis, 2004). New anthocyanins are constantly reported by researchers. Eighteen naturally occurring anthocyanidins have been identified. Among them, the six common anthocyanidins in higher plants include pelargonidin (3,5,7,4'-tetrahydoxyflavylium cation), cyanidin (3,5,7,3',4'-pentahydoxyflavylium cation), delphinidin (3,5,7,3',4'5'-hexahydoxyflavylium cation), peonidin (3,5,7,4'-tetrahydoxy-3'-methoxyflavylium cation), petunidin (3,5,7,3',4'-pentahydoxy-5'-methoxyflavylium cation) and malvidin (3,5,7,4'-tetrahydoxy-3',5'-dimethoxyflavylium cation) (Michael Eskin, 1979). These structures which differ in their hydroxylation and methoxylation patterns produce colour shades from orange-red (pelargonidin) to blue-violet (delphinidin) at a non-physiological pH around 1. In general, hydroxylation induces a bathochromic shift, while methylation of hydroxyl groups reverses this trend (Stintzing and Carle, 2004) (Table 2.1). In higher plants, cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin are distributed by 50, 12, 12, 12, 7 and 7 %, respectively (Zhang *et al.*, 2005).

In nature, the glucosides of the three non-methylated anthocyanidins (cyanidin, delphinidin and pelargonidin) are the most widespread, being present in 80 % of pigmented leaves, 69 % of fruits and 50 % of flowers (Kong *et al.*, 2003). Cyanidin is the most common anthocyanidin, and the 3-glucoside is the most active antioxidant anthocyanin and showed the highest ORAC (oxygen radical absorbance capacity) activity, which was 3.5 times stronger than Trolox (vitamin E analogue), while pelargonin had the lowest antioxidant activity but was still as potent as Trolox (Wang *et al.*, 1997a).

Anthocyanins undergo reversible structural transformations with changes in pH, which is accompanied by dramatic changes in colour. At pH 3 or below, the colour of anthocyanins ranges from orange to bluish-red, depending on the chemical structure, and exists predominantly as a flavylium cation. As the pH is raised, hydration and proton transfer reactions can occur with the generation of a number of different chemical structures: the first reaction produces a colourless carbinol pseudobase which can undergo ring opening to a chalcone pseudobase, the latter reactions give rise to quinonoidal bases, with formation of purplish quinonoid anions after further deprotonation (Brouillard, 1988). Due to these transformation with pH, anthocyanin application for food systems were typically sought in acidic food (pH < 3) to assure a predominance of the flavylium cation (Giusti and Wrolstad, 2003).

Current emphasis is on attempts to make colourants containing anthocyanins more stable. Considerable research has emphasised the possibilities of copigmentation such as association of anthocyanins with themselves or with a series of other compounds like flavonoids, polysaccaharides, proteins, tannins, and other polyphenolic compounds, glycosylation and chelation with metal such as aluminium, magnesium or iron. Bordignon-Luiz *et al*. (2007) reported that copigmentation of anthocyanins from the crude extracts of Isabel grapes (*Vitis labrusca* L.) with tannic acid (1 : 1, w/v) increased the half-life time by 187 hours compared to the control samples.

2.2.2. Anthocyanin biosynthesis pathway

Anthocyanin pigments are assembled from two different streams of chemical raw materials in the cell: both starting from the C2 unit acetate (or acetic acid) derived from photosynthesis. One stream involves the shikimic acid pathway to

produce the amino acid phenylalanine while the other stream, the acetic acid pathway, produces three molecules of malonyl-Coenzyme A, a C3 unit. These streams meet and are coupled together by CHS, which forms an intermediate chalcone via a polyketide folding mechanism that is commonly found in plants. The chalcone is subsequently isomerised by the enzyme chalcone isomerase (CHI) to the prototype pigment naringenin, which is subsequently oxidised by enzymes like flavonoid hydroxylase and coupled to sugar molecules by enzymes like UDP-O-glucosyl transferase to yield the final anthocyanins (Figure 2.2).

Anthocyanin biosynthetic enzymes are located at the cytoplasmic face of the endoplasmic reticulum while the site of anthocyanin accumulation could be the central vacuole. In the vacuole, anthocyanin pigments could be found dissolved uniformly in the vacuolar solution, or in discrete regions of the vacuoles as intensely pigmented globules called anthocyanoplasts or the anthocyanic vacuolar inclusions (AVIs) (Nozue *et al*. 2003).

2.2.3. Anthocyanins found in *M. malabathricum* L.

Lowry (1972) reported the anthocyanins in the petals of *M. malabathricum* L. as Mv-3,5-diglucoside and the fruit pulp as Cy-3-glucoside and Cy-3,5-diglucoside. Janna *et al.* (2006) reported that the highest anthocyanin concentration was obtained from flowers in S3 stage that is when the petals were fully-formed but the flower is not yet opened, as compared to other developmental stages. The extracted anthocyanins were quite stable, except for those exposed to the light where the degradation level reached more than 50 %. Increase in pH, even within the acidic range (pH 0.5 – 3.0) caused the colour to fade. The colour of the extracts changed from red at pH 0.5, fading and finally turned to light red at pH 3.0. High storage

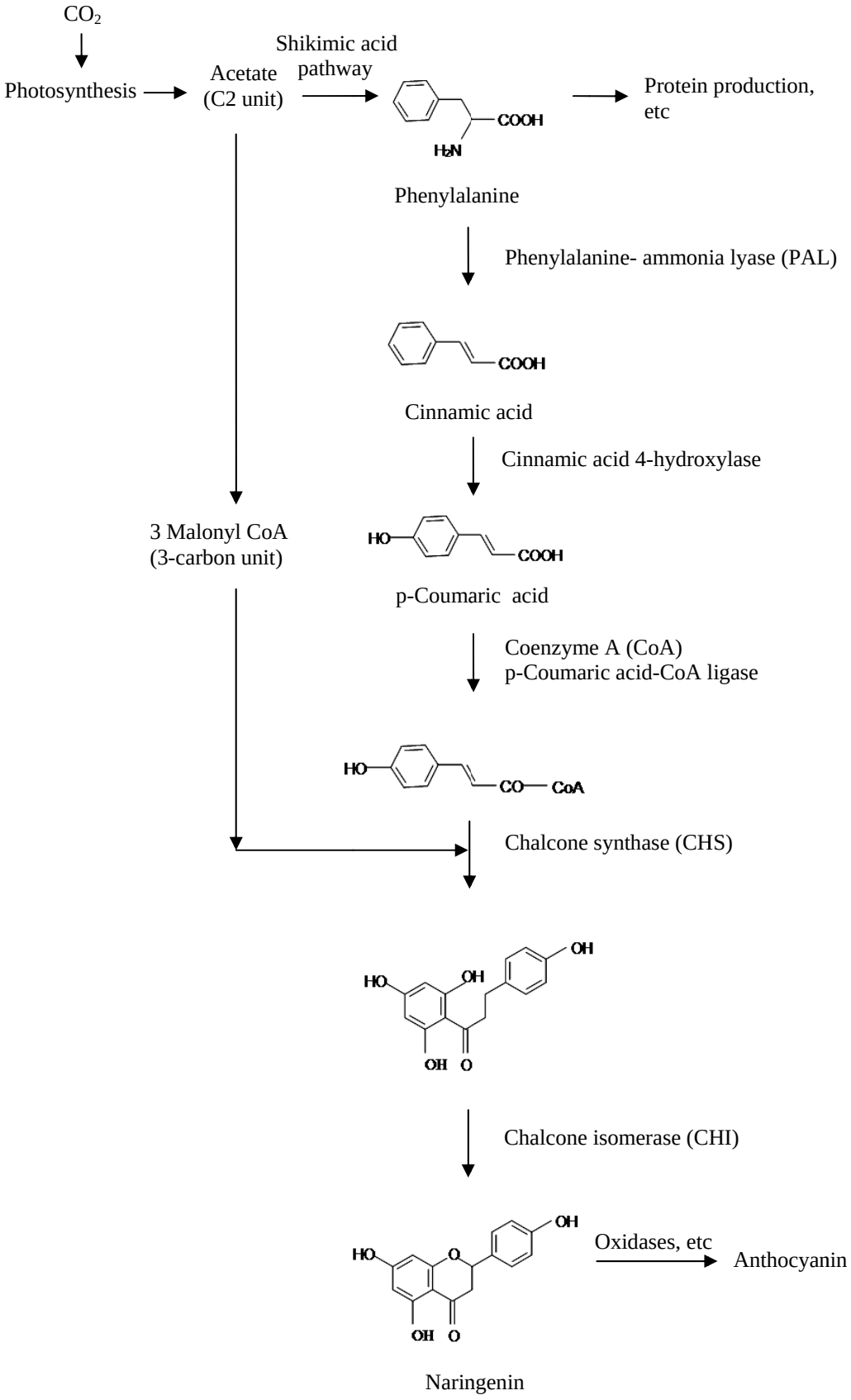


Figure 2.2. Anthocyanin biosynthetic pathway (Adopted from Sullivan, 1998).

temperature (31 °C) was also found to cause higher level of degradation compared to lower temperatures (25 °C). These researchers concluded that the suitable storage condition for coloured anthocyanin pigments from *M. malabathricum* L. as acidic solution (pH 0.5 and 1.0) kept in the dark and at low temperature (4 °C).

2.2.4. Importance of anthocyanins to plants

The most significant function of anthocyanins is their ability to impart colour to the plants to attract animals for pollination and seed dispersal. They are thus important in the co-evolution of plants and animals. Anthocyanins *in vivo* absorb the green and yellow wavebands of light, commonly between 500 and 600nm (Field *et al.*, 2001). Foliage appears red because of the subtraction of yellow-green light from the spectrum of light reflected from the leaf's surface.

UV radiation, part of the radiation received by the Earth from the Sun, can damage plant proteins and cause lower photosynthetic efficiency. It also damages the plant DNA and induces mutations. Anthocyanins have been found to protect plants from the damage caused by exposure to UV radiation. Stapleton and Walbot (1994) reported that there was significant increase in CPD (cyclobutane pyrimidine dimer), the result of dimerisation of adjacent pyrimidines on the same strand of DNA, in green (without anthocyanins) compared to purple (with anthocyanins) sheath tissue of maize plants after 5 and 10 seconds of irradiations with UV-C (wavelengths 100 – 280 nm) sources and after 500, 1000 and 2000 seconds of irradiations with UV-B (wavelengths 280 – 315 nm) sources. CPD damage is the best-studied type of UV-induced damage to DNA.

When leaves receive light energy above their saturation point, they show a reduction in their capacity for photosynthesis, termed photoinhibition. Under severe

conditions the chloroplasts generate reactive oxygen species, which have the potential to destroy thylakoid membranes, damage DNA, and denature proteins associated with photosynthetic electron transport (Gould, 2004). Anthocyanins have been shown in many plant species to reduce both the frequency and severity of photoinhibition, as well as to expedite photosynthetic recovery. In the red-osier dogwood (*Cornus stolonifera*), for example, a 30-minute exposure to strong white light reduced the quantum efficiency of photosynthesis by 60% in red leaves, but by almost 100% in acyanic leaves (Field *et al.*, 2001).

Besides this, by intercepting the high-energy quanta, anthocyanic cell vacuoles can prevent important photolabile molecules from degradation by green light (Gould, 2004). The silver beachweed (*Ambrosia chamissonis*) holds large amounts of thiarubrine A, a photolabile and potent defence compound that is toxic to insects, bacteria, and fungi. Page and Towers (2002) reported that the tissues in *A. chamissonis* that contain thiarubrine A were shielded from light by a sheath of cells containing a mix of two anthocyanins, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-(6'-*O*-malonylglucoside), which absorbed quanta that would otherwise lead to the destruction of thiarubrine A, and thereby contribute significantly to the defensive armoury.

Anthocyanins have also been reported to serve as warning signals against autumn-colonising animal pests (Hamilton and Brown, 2001) and anthocyanic tissues were found to be less often attacked by herbivores (Costa-Arbulu *et al.*, 2001). California maple aphids, for example, readily colonised yellow-orange leaves of Japanese maples, but they largely ignored the red ones (Furuta, 1986).

In general, anthocyanins are believed to increase the antioxidant response of plants in order to uphold the regular physiological status in tissues directly or

indirectly affected by biotic or abiotic stress factors (Stintzing and Carle, 2004). Gould (2004) has described the anthocyanins as the Swiss army knife of the plant kingdom.

2.2.5. Health benefits of anthocyanins to human

Oxidative damage in the human body plays an important causative role in disease initiation and progression (Jacob and Burri, 1996). Damage from free radicals and reactive oxygen species has been linked to some neurodegenerative disorders (Rao and Balachandran, 2002) and cancers (Goodwin and Brodwick, 1995), and oxidation of low-density lipoprotein is a major factor in the promotion of coronary heart disease and atherosclerosis (Frankel *et al.*, 1993).

Recent studies have shown that many flavonoids and related polyphenols which are present in fruits and vegetables are natural antioxidants. Many of them indeed are more effective antioxidants *in vitro* than vitamins E or C (Rice-Evans *et al.*, 1997). Einbond *et al.* (2004) identified cyanidin-3-O-b-glucopyranoside, an anthocyanin antioxidant, from the semi-purified aqueous fractions of the tropical fruit star apple (*Chrysophyllum cainito*), Surinam cherry (*Eugenia uniflora*), and jaboticaba (*Myrciaria cauliflora*) as well as delphinidin-3-O-b-glucopyranoside from *E. uniflora*. Antioxidation helps to prevent or delay the onset of major degenerative diseases of aging, including cancer, heart disease, cataracts and cognitive dysfunction (Mazza, 2000). Thus, the human health condition could be partly controlled through the dietary intake of plant polyphenols (Passamontia *et al*., 2003). These compounds act as antioxidants by donating hydrogen to highly reactive radicals, thereby preventing further radical formation.

The anthocyanin fractions from red wine have been reported to suppress the growth of human colon cancer cells (HCT-115) and human gastric cancer cells (AGS) (Kamei *et al.,* 1998). The anthocyanins in purple coloured sweet potato and red cabbage were found to suppress colon carcinogenesis induced by 1,2-dimethylhydrazine and 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine in rats (Hagiwara *et al.,* 2002). Anthocyanins from bilberry (*V. myrtillus*) have been reported to induce apoptosis of human leukemia and human colon carcinoma cells *in vitro* (Katsube *et al.,* 2003). The tart cherry anthocyanins and anthocyanidins were able to inhibit intestinal tumor development in Apc^{Min} mice (a model for human colon cancer) and growth of human colon cancer cell lines (HT-29 and HCT-115) *in vitro* (Kang *et al.,* 2003).

Structural variations in anthocyanins seem to influence their antioxidant activities. A few studies have found anthocyanidins more effective than their respective anthocyanins in the inhibition of certain human cancer cell lines. Meiers *et al.* (2001) found that cyanidin (the anthocyanidin) preferentially inhibited the growth of the human vulva carcinoma cell line A-431 when compared to cyanidin-3-β-D-galactoside (the anthocyanins) and over-expressed the epidermal growth factor (EGFR). By inhibiting the EGFR it shut off downstream signalling cascades and thus inhibited the growth of the cancer cells.

Zhang *et al.* (2005) compared the growth inhibitory effects of anthocyanidins and anthocyanins on five human cancer cell lines: AGS (stomach), HCT-116 (colon), MCF-7 (breast), NCI H460 (lung), and SF-268 (Central Nervous System, CNS). Four anthocyanins (cyanidin-3-glucoside, cyanidin-3-galactoside, delphinidin-3-galactoside and pelargonidin-3-galactoside) were found not effective in inhibiting cell proliferation of all the five human cancer cell lines when they were

applied at 200 µg/mL. However, at 200 µg/mL, the anthocyanidins tested showed different level of cell proliferation inhibitory activity. Malvidin inhibited AGS, HCT-116, NCI H460, MCF-7 and SF-268 cell growth by 69, 75.7, 67.7, 74.7 and 40.5 %, respectively. Pelargonidin was found to inhibit AGS, HCT-116, NCI H460, MCF-7 and SF-268 cell growth by 64, 63, 62, 63 and 34 %, respectively. On the other hand, cyanidin, delphinidin and petunidin inhibited the breast cancer cell growth by 47, 66 and 53 %, respectively.

These researchers explained that a free hydroxyl group at the 3-position in the flavylium moiety in anthocyanidins contributed to the inhibition of cell proliferation activity. In anthocyanins, the hydroxyl group at 3-position is substituted by various sugar moieties and hence prevent it from being inhibitory to cancer cell proliferation. Besides this, they also found that the number of hydroxyl and methoxyl groups in the B ring of anthocyanidins strongly influenced the growth inhibition of the studied cancer cell lines. The highest inhibitory activity was demonstrated by malvidin with hydroxyl groups at 3’ and 4’ positions and methoxy groups at 3’ and 5’ positions.

Hou *et al.* (2003) found that only those anthocyanidins with an ortho-dihydroxyphenyl moiety in the B-ring could induce apoptosis in human leukemia cells (HL-60). Besides their effects on cancer cells, Lazzé, *et al.* (2003) reported that the anthocyanidins were more effective to protect cells from lipid peroxidation compared to their anthocyanin counterparts. They showed that although pretreatment of rat smooth muscle cells (SMC) with the delphinidin, cyanidin, and their glycoside and rutinoside derivatives were able to protect the cells against tert-butyl-hydroperoxide-induced lipid peroxidation (an oxidative damage) and cell toxicity, only cyanidin and delphinidin were found to be able to protect the hepatoma cells

(MH1C1) against lipid peroxidation and cell toxicity. The glycosylated anthocyanins were ineffective. Cyanidin and delphinidin were also more effective in preventing DNA damage caused by single strand breaks with DNA damage reduced by 56 % in SMC cells and by 40 % in MH1C1 cells.

Yoshimoto *et al.* (2001) reported that the cyanidin-type anthocyanin (cyanidin-3-(6,6'-caffeylferulylsophoroside)-5-glucoside) extracted from the Ayamurasaki purple-fleshed sweetpotato was superior to the peonidin type (peonidin-3-(6,6'-caffeylferulylsophoroside)-5-glucoside) in its antimutagenic activity against the reverse mutation induced by Trp-P-1 on strain TA 98 of *Salmonella typhimurium*.

Chung *et al.* (2005) found that the antioxidative activities of the water extract, 50 % and 95 % ethanolic extracts of *Graptopetalum paraguayense* correlated with both the total phenol and anthocyanin contents. They reported that the 50 % ethanolic extract was effective in scavenging DPPH radical and showed a higher reducing power than the water or 95 % ethanolic extracts. However, there were no significant differences ($p > 0.05$) in the lipid-peroxidation-prevention effects among the extracts.

Besides their ability to inhibit proliferation of cancer cell and their antioxidant properties, other health benefits associated with anthocyanin extracts have been reported. Significant vasoprotective and anti-inflammatory activities of *V. myrtillus* anthocyanosides were found in rats and rabbits (Lietti *et al.*, 1976), while protection against the permeability-increasing action of hypertension was demonstrated in rats (Detre *et al.*, 1986). Besides this, anthocyanins isolated from tart cherries were found to be able to reduce inflammation-induced pain in rats (Tall *et al.*, 2004) and anthocyanins isolated from the fruit extract of *Ribes nigrum* L., was