RMIT UNIVERSITY

Genetic diversity, nutritional and biological activity of *Momordica cochinchinensis* (Cucurbitaceae)

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(Applied Biology and Biotechnology)

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Dilani Chathurika Wimalasiri

31 August 2015

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DEDICATION

This thesis is dedicated to my mother J. P Wimalasiri, father I. D

Wimalasiri, my brother Delan Wimalasiri and my husband Kalana

Katuwawala

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

7-AAD	7-aminoactinomycin D
ABTS	2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
AFLP	Amplified fragment length polymorphism
AMP	Ampicillin
ANOVA	Analysis of variance
APAF1	Apoptotic protease activating factor 1
BAK	BCL-2 antagonist or killer
BAX	BCL-2-associated X protein
BCL-2	B-Cell lymphoma gene 2
BID	BH3-Interacting domain
bp	Base pairs
°C	Degrees centigrade
CAMs	Cell-cell adhesion molecules
CCK-8C	Cell counting kit-8
CIE	International commission on illumination
CO_2	Carbon dioxide
CRTISO	Carotenoid isomerase
DIABLO	Direct inhibitor of apoptosis-binding protein with low pI
DNA	Deoxyribonucleic acid
DMAPP	Dimethylallyl diphosphate
DMEM	Dulbecco's modified eagle's medium
DOXP	2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathways
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate

DW	Dry weight
EC_{50}	Effective concentration 50%
EDTA	Ethylenediamine tetra acetate
FADD	FAS-associated domain
FasL	Fas ligand
FBS	Foetal bovine serum
FDA	Food and drug administration
FRAP	Ferric ion reducing antioxidant power
FW	Fresh weight
g	Gram
GGPP	Geranylgeranyl diphosphate
h	Hour
HPLC	High performance liquid chromatography
HYDB	β-carotene hydroxylase
IAP	Inhibitor of apoptosis
IPP	Isopentenyl diphosphate
IPPI	Isopentenyl diphosphate isomerase
ISSR	Inter simple sequence repeat
kDa	Kilodaltons
L	Litre
LCYE	Lycopene ε-cyclase
LSD	Least significant difference
MAPK	Mitogen-activated protein kinase
MCF-7	Michigan cancer foundation-7
MSE	Mean square error
min	Minute

mM	Millimolar
MOMP	Mitochondrial outer membrane permeabilisation
MPT	Mitochondrial permeability transition
MS	Mass spectrometry
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
nm	Nanometre
NMR	Nuclear magnetic resonance
NOXA	NADPH oxidase activator
PCA	Principal component analysis
PI3K	Phosphoinositide 3-kinase
PI	Propidium iodide
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDA	Photodiode array
PDS	Phytoene desaturase
PS	Phosphatidylserine
PSY	Phytoene synthase
PTEN	Phosphatase and tensin homolog
PTFE	Polytetrafluoroethylene
PUMA	p53 Up-regulated modulator of apoptosis
RAF	v-Raf-1 murine leukemia viral oncogene homolog
RAPD	Random amplified polymorphic DNA
RAS	Retrovirus-associated DNA sequences
Rb	Retinoblastoma protein
RFLP	Fragment length polymorphism

rpm	Revolutions per minute
RPMI	Roswell park memorial institute
SMAC	Second mitochondria-derived activator of caspase
TBE	Tris-borate-EDTA
THF	Tetrahydrofuran
TEM	Transmission electron microscope
TNF	Tumour necrosis factor
TP53	Tumour protein 53
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SSR	Simple sequence repeat
UPGMA	Unweighted pair group method analysis
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
V	Voltage
VDE	Violaxanthin de-epoxidase
VEGF	Vascular endothelial growth factor
ZDS	ζ-carotene desaturase
ZEP	Zeaxanthin epoxidase
v/v	Volume per volume
w/v	Weight per volume
µg	Microgram
µL	Microlitre
µm	Micrometre

THESIS PUBLICATIONS

Journal Publication

Wimalasiri D., Piva, T., Urban, S. & Huynh, T. (2015). Morphological and genetic diversity of *Momordica cochinchinensis* (Cucurbitaceae) in Vietnam and Thailand. Genetic Resources and Crop Evolution, 1-15. (Presented as Chapter 2). (http://link.springer.com/article/10.1007/s10722-015-0232-8)

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Wimalasiri, D., Piva, T. & Huynh, T. Diversity in nutrition and bioactivity of *Momordica cochinchinensis*. 3rd International Conference Sustainable Agriculture, Food and Energy), Ho Chi Minh City, Vietnam (November, 2015).

Manuscripts in preparation

Wimalasiri D., Piva, T., Urban, S. & Huynh, T. Anticancer activity of *Momordica cochinchinensis* (Cucurbitaceae) on breast cancer and melanoma cells.

Wimalasiri, D., Brkljaca, R., Piva T., Urban, S. & Huynh, T. Influence of genetic diversity and eco-geographical distribution on carotenoid content of *Momordica cochinchinensis* (Cucurbitaceae)

THESIS ABSTRACT

Momordica cochinchinensis (Lour.) Spreng belongs in the Cucurbitaceae family and is geographically restricted to South East Asia. The fruit possesses the highest amount of nutritionally important carotenoids (lycopene and β-carotene) of all known fruits and vegetables including tomatoes and carrots by more than 200 and 54 times, respectively. Little information is available on the influence of genetic diversity and eco-geographical differences on the nutritional and medicinal potential of the fruit. This information is essential to assist future agricultural practices for the commercialisation of *M. cochinchinensis* as a new fruit in the medicine, food and nutrition industries. This thesis elucidated the variation in morphology, genetics, nutritional content (carotenoids) and biological activity (anticancer) of *M. cochinchinensis* collected from Vietnam, Thailand and Australia.

M. cochinchinensis was morphologically and genetically diverse and the highest diversity was in Vietnam, indicating the presence of a diverse gene pool which contributed to superior nutritional and medicinal varieties. Molecular profiling of *M. cochinchinensis* samples based on morphology and molecular analyses (ISSR and RAPD) were in agreement and clustered the samples based on the country of origin. This study also indicated that the previously unknown origin of the Australian samples was Southern Vietnam, an important finding for tracking the parentage of the plants grown in Australia and analysing the gene flow of *M. cochinchinensis* to Australia.

The lycopene content was greater in samples from the Lam Dong and Lam Ha provinces of Central Vietnam as detected by the HPLC method and corresponded to

larger and heavier seeds of *M. cochinchinensis*. Northern Vietnam samples possessed the highest levels of combined lycopene and β-carotene. It is recommended that the varieties from Central and Northern Vietnam should be selected in future plant development for high carotenoids. Carotenoid accumulation was influenced by climatic factors, where lycopene accumulation was greatest at lower temperatures (<14°C) and higher elevations whilst β-carotene accumulation was greatest at temperatures between 27 to 33°C. These growth conditions should be replicated in future agriculture to enhance carotenoid accumulation in the fruit.

Four different analytical methods were investigated to determine the accuracy and potential of analysis of carotenoid content in the *M. cochinchinensis* aril samples. Colorimetry, which was linked with redness of the aril correlated with higher lycopene concentrations as quantified by chromatography (HPLC, UPLC) and UV-visible spectrophotometry but this was not the case for β-carotene. This simple method could be used to screen lycopene rich *M. cochinchinensis* in the field and will be useful for resource-poor farmers.

Water extracts of the aril of *M. cochinchinensis* had a higher cytotoxicity on breast cancer and melanoma cells than the hexane based extracts. This suggested that the anticancer bioactive compounds extracted from the arils are not the carotenoids. The cytotoxicity of the water-based extract was selective towards breast cancer (MCF7) and melanoma (MM418C1 and D24) cells and not against normal human dermal fibroblast cells (NHDF). Furthermore, treatment of cells with the water extract caused both apoptotic and necrotic cell death. The cytotoxicity of the water extract was greater for

samples obtained from Northern and Central Vietnam (>70% of cell death), especially those from the Ha Noi and Lam Dong provinces, respectively. High anticancer activity of the aril extract against melanoma cells were correlated with cool climates with low temperatures (<14°C) and a high precipitation during the driest month, indicating that these conditions facilitated the production of compounds responsible for anticancer activity.

The findings of this study are novel and will assist current and future agricultural practices for the commercialisation of *M. cochinchinensis* as a new fruit in the medicine, food and nutrition industries.

CHAPTER 1

Background and review of literature

1.1   Cucurbitaceae family

The Cucurbitaceae family consists of 800 species in 118 genera comprising monoecious (separate male and female flowers on the same plant) and dioecious (male and female flowers on different plants) species (Whitaker and Davis, 1962, Jeffrey, 1980). They are mostly annual, herbaceous, tendril bearing and frost sensitive vines (Jeffrey, 1980). The flowers are commonly greenish-white to yellow and they are typically small (Jeffrey, 1980). The staminate (male) flowers are borne on an inflorescence that can be either racemose or cymose (Gerrath et al., 2008). The pistillate (female) flowers are generally solitary with an inferior ovary. The fruit is large, fleshy, with a hard outer covering and is known as a pepo fruit type (Gerrath et al., 2008). Members of the Cucurbitaceae family are normally grown in tropical and subtropical regions with few representatives in cooler temperate climates (Bharathi and John, 2013). A common feature of cucurbits is the presence of cucurbitacins, toxic substances responsible for bitterness, that have been eliminated from fruits through selective breeding but which are present in leaves and stems (Chambliss and Jones, 1966).

1.1.1 Economic importance

Cucurbitaceae are economically important as the third most widely cultivated vegetable crop in the world (Yiqun and Zhanyong, 2011). They are commonly cultivated as edible fruits including cucumber, melon, pumpkin, watermelon and bitter gourd. According to the United Nations' Food and Agriculture Organization (FAO, 2014) the total production of cucumbers and gherkins was 2.1 million hectares in 2013. The average world yield was 71 million tonnes and about 82% of this world production was in Asia, which was led by China (50%) with a production of 29 million tonnes (FAO, 2014).

1.1.2 Taxonomical relationship within cucurbit crops

Cucurbitaceae can be divided into two subfamilies based on pollen characters as Zanonioideae and Cucurbitoideae (Jeffrey, 1964a). Zanonioideae is the smaller family with 19 genera and about 60 species, characterised by a complete uniformity of striate pollen grains which are prolate in shape and small (<40 µm) (van der Ham et al., 2010). In contrast, Cucurbitoideae consists of 111 genera and 740 species and has comparatively large (>40 µm) non striate and oblate-prolate shaped pollen grains (Jeffrey, 1964a, Jeffrey, 1964b, van der Ham et al., 2010). Cucurbitoideae is further sub-divided into 10 tribes mainly based on seed coat morphology, chloroplast DNA and nuclear DNA. These tribes include: Jollifieae (Momodiceae), Trichosantheae, Herpetospermeae, Bryonieae, Benincaseae, Schizopeponeae, Sicyeae, Coniandreae, Luffeae and Cucurbiteae (Jeffrey, 1980, Behera et al., 2011b, Esteras et al., 2011, Yiqun

and Zhanyong, 2011). Out of these tribes, Benincaseae, Cucurbiteae, Sicyeae and Momodiceae possess economic importance as functional foods (Figure 1.1).
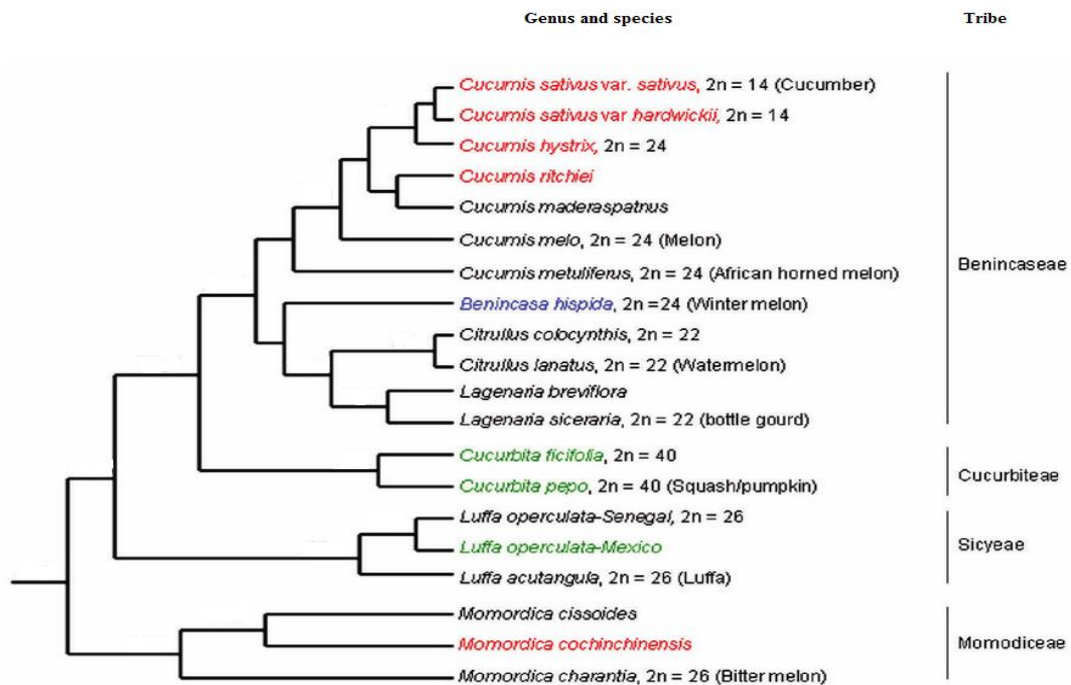
Figure 1.1: Phylogenetic relationship between economically important Momordiceae. Chromosome numbers and common names follow each species name (when available). The tribe to which the species belongs is shown to the right of vertical bars. Geographical origin of species: Green—America; Black—mainland Africa; Red—Asia; Blue—Australia. Figure obtained from Yiqun and Zhanyong (2011).

1.2   Momordica genus and representatives

The genus Momordica belongs in the tribe Momordiceae. Momordica is comprised of 59 species, with 47 species from African origin and 12 from South East Asia (Schaefer and Renner, 2010). Most species are perennial climbers and the flowers are 1−10 cm in diameter. About a third of the species are monoecious, the remaining are dioecious (Schaefer and Renner, 2010). Representatives of Momordica lack cucurbitacins which are responsible for bitterness in the fruit; the bitterness in *M. charantia* is due to the alkaloid momordecines (Jeffrey, 1980). Of the 12 species originated from South East Asia (Figure 1.2), nine are dioecious and three are monoecious (Behera et al., 2011b) but only six are economically important. These species include *M. charantia* L. and *M. balsamina* L. which belong in the monoecious group and *M. dioica* Roxb., *M. sahyadrica* Joseph & Antony, *M. cochinchinensis* (Lour.) Spreng. and *M. subangulata Blume* (ssp. Renigera (G. Don) W.J.J. deWilde) represent the dioecious group (Behera et al., 2011b). These species are morphologically different based on vegetative and reproductive characteristics with importance ranging from nutritional to ethno-botanical properties (Table 1.1 and Figure 1.3).
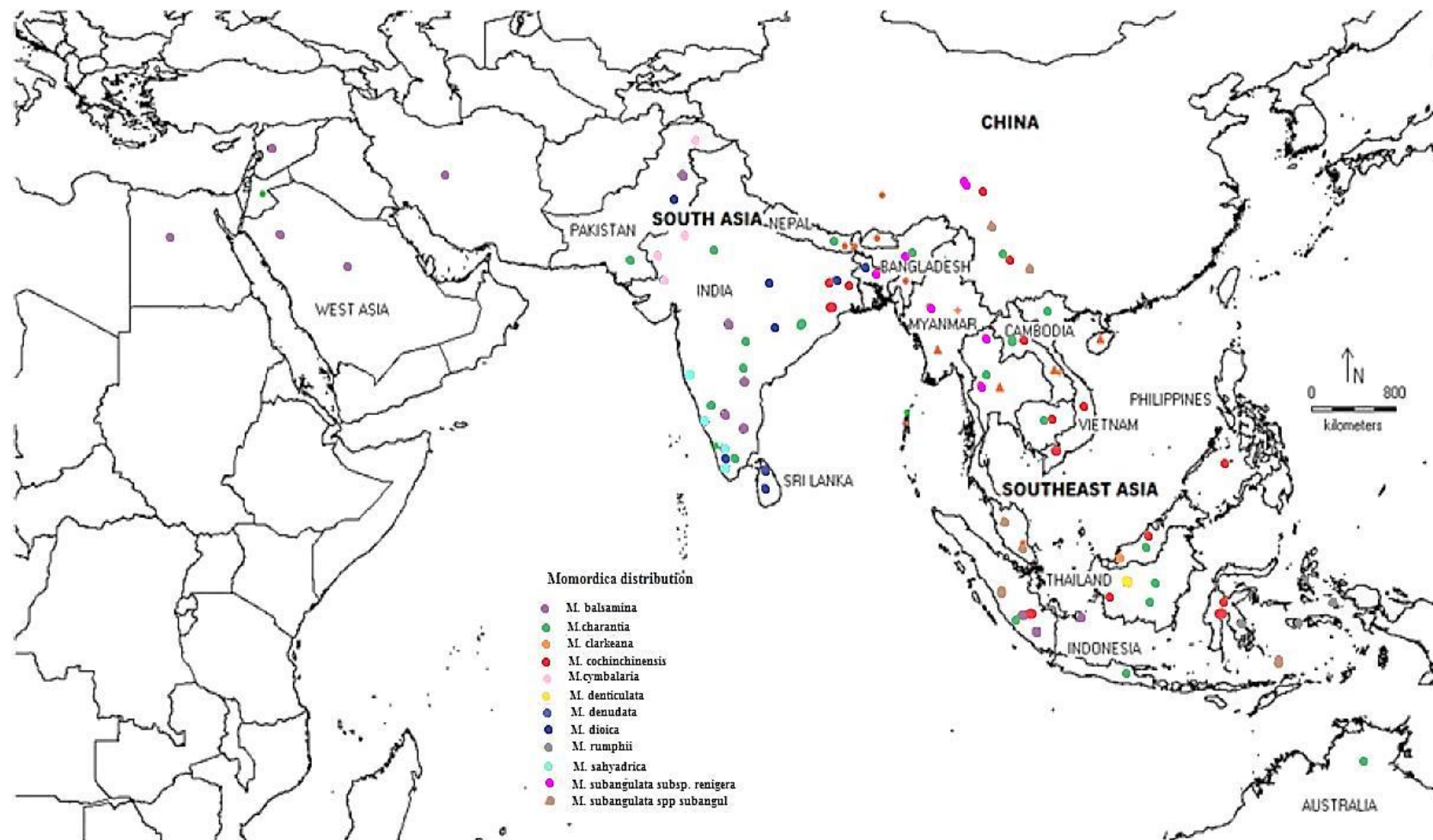
Figure 1.2: Distribution of Momordica in Asia and West Africa. Figure obtained from Behera et al. (2011a).

Table 1.1: Morphological, reproductive characters and growth habitats of economically important Momordica species in Asia.

Scientific Name	Sex type and habitat	Vegetative and reproductive morphology			Importance
		Leaf	Flower	Fruit shape	
M. balsamina L	Monoecious Annual	Leaves 3–5 lobed	Pale yellow to creamish yellow	Ovoid ellipsoid	Vegetable used as food (Lim, 2012) Ornamental (Lim, 2012) Antimalarial (Ramalhete et al., 2010) Antimicrobial and anti-inflammatory (Thakur et al., 2009)
M. charantia	Monoecious Annual	Leaf blade deeply palmately lobed (5-9)	Yellow flowers	Ovoid–ellipsoid to elongate fruits	Vegetable used as food (Lim, 2012) Anti-diabetics (Chen et al., 2003, Chen et al., 2005, Tan et al., 2008, Joseph and Jini, 2013) Antibacterial (Roopashree et al., 2008) Antiviral (Grover and Yadav, 2004, Pongthanapisith et al., 2013) Anti-inflammation and cholesterol lowering activities (Chen et al., 2003) Anticancer (Li et al., 2012a)
*M. cochinchinensis**	Dioecious Perennial	Leaf blade entire or palmately lobed (3-5)	White to ivory yellow in colour	Round to oval	Vegetable used as food (Lim, 2012), Antioxidant (Kubola and Siriamornpun, 2011) Anticancer (Tien et al., 2005) Vitamin A activity (Vuong et al., 2002)

(Continued)

Table 1.1 (Continued): Morphological, reproductive characters and growth habitats of economically important Momordica species in Asia.

Scientific Name	Sex type and habitat	Vegetative and reproductive morphology			Importance
		Leaf	Flower	Fruit shape	
M. dioica	Dioecious Perennial	Leaf blade broadly ovate lobed (3-5) or unlobed leaves	Lemon-yellow coloured	Broadly ovoid–oblong	Vegetable used as food (Lim, 2012) Anti-diabetic (Ilango et al., 2009) Antioxidant and hepato-protective (Shreedhara and Vaidya, 2006, Jain et al., 2008) Antimicrobial (Shrinivas et al., 2009) Anti-inflammatory (Shreedhara and Vaidya, 2006) Anti-lipid peroxidative (Ilango et al., 2009)
M. sahyadrica	Dioecious Perennial	Leaves are triangular cordate	Yellow flowers	Broadly ellipsoid or ovoid to fusiform	Vegetable used as food (Lim, 2012)
M. subangulata subsp., renigera	Dioecious Perennial	Leaf blade ovate, cordate, unlobed	Creamy flowers with a purple bull's-eye blotch on three inner petals	Fruits broadly ovoid or ellipsoid	Vegetable used as food (Lim, 2012)

*: Vegetative, reproductive morphology and importance of *M. cochinchinensis* will be discussed further in sections 1.4 and 1.5
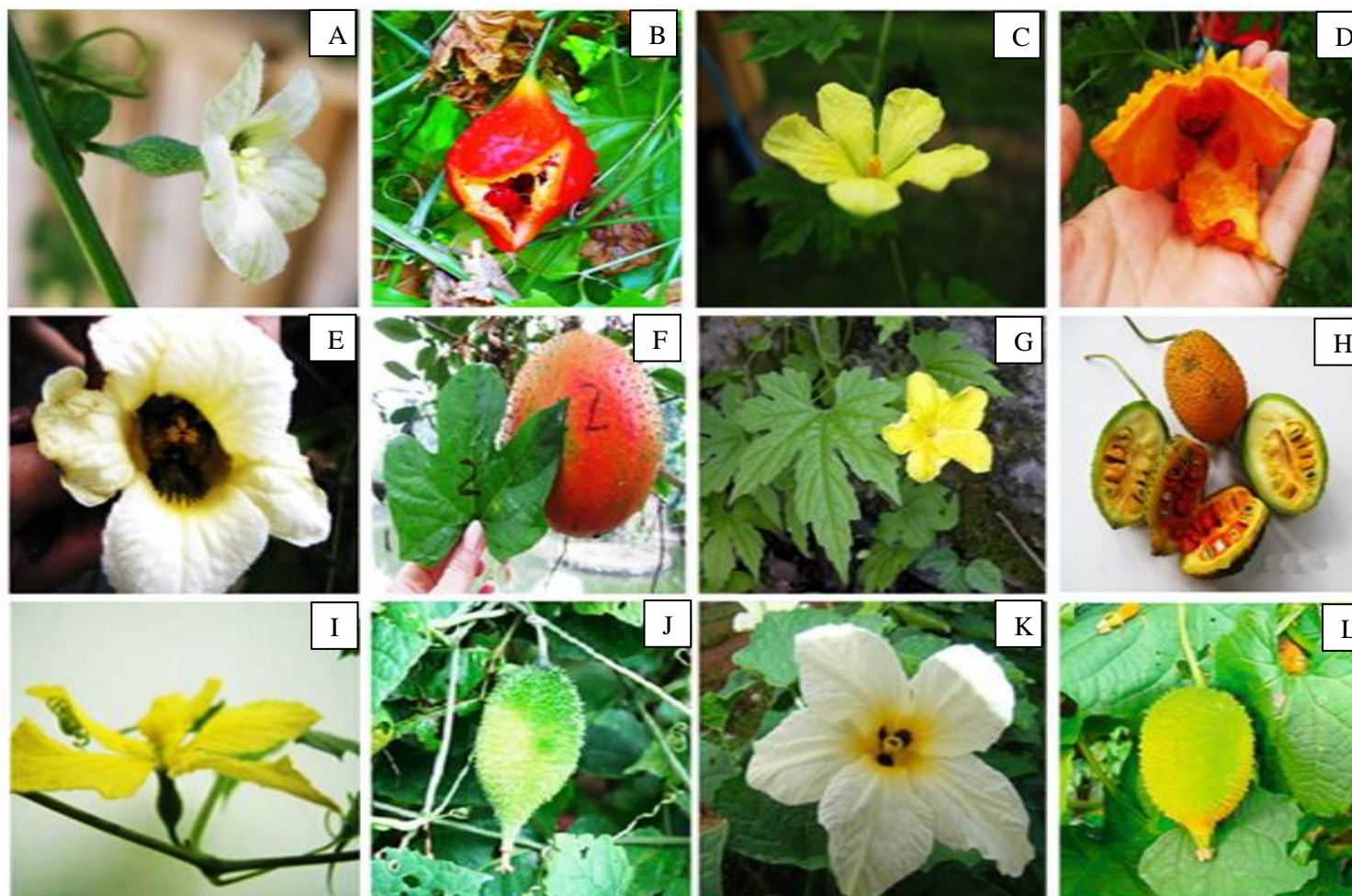
Figure 1.3: Fruit and floral morphology of economically important members of genus Momordica. A,B: *M. balsamina L* (Ken Fern, 2014), C,D: *M. charantia*, E,F: *M. cochinchinensis*, G,H: *M. dioica* (Rathnayake, 2013) I,J: *M. sahyadrica* (Bharathi and John, 2013) K,L: *M. subangulata subsp., renigera* (Behera et al., 2011a, Bharathi and John, 2013).

1.2.1　Origin and distribution

The genus Momordica originated in tropical Africa and the Asian species were likely the result of a long-distance dispersal event that occurred about 19 million years ago (Schaefer and Renner 2010). The monoecious species have evolved from dioecious species (Bharathi and John, 2013). The two monoecious species *M. charantia* and *M. balsamina L* were previously thought to originate in Asia (Walters and Decker-Walters, 1988, Marr et al., 2004, Joseph and Antony, 2008) but a study using mitochondrial and plastid DNA suggested they are African in origin (Schaefer and Renner, 2010).

Representatives of the genus Momordica are distributed throughout Africa and Asia but *M. charantia* is the only widely cultivated crop (Bharathi and John, 2013). There are two botanical varieties of *M. charantia*; *M. charantia* var. *muricata* (syn. var. *abbreviata*) is the wild variety which can be found in tropical Asia and Africa. In contrast, *M. charantia* var. *charantia* is the cultivated variety (Walters and Decker-Walters, 1988). The dioecious species, *M. dioica*, *M. cochinchinensis* and *M. subangulata* subsp. *renigera* are under domestication interference (Bharathi and John, 2013). *M. cochinchinensis* was hypothesised to have evolved in South Asia, probably in the Cochinchina region of Vietnam and the evolution was independent of *M. dioica* (Mondal et al., 2006). In contrast, morphological similarity and interspecific crossability suggested that *M. subangulata* ssp. Renigera (n = 56) originated from *M. dioica* (n = 28) and *M. cochinchinensis* (n = 28) as a result of a natural cross (Mondal et al., 2006, Bharathi et al., 2011a). *M. balsamina L* and *M. sahyadrica* are found in the wild (Bharathi and John, 2013); *M. balsamina L.* is distributed in western India, West Asia

(Figure 1.2) and Africa (Behera et al., 2011a) but *M. sahyadrica* is restricted to India (Bharathi and John, 2013) and genetically diverse from other members.

1.3 *Momordica cochinchinensis*

1.3.1 Distribution and vernacular names

Momordica cochinchinensis is indigenous to South East Asia including Vietnam, Thailand, China and India and has different vernacular names based on the countries in which it is found (Table 1.2). Multiple vernacular names which are reported in the same country might be due to the usage of a unique dialect in a tribe within a province, or a region of that country.

Table 1.2: Vernacular names of *M. cochinchinensis* based on the different Asian countries which it is grown. Adopted from Lim (2012).

Country	Vernacular name
Bangladesh	Kakrol
China	Da Ye Mu Bie Zi, Mu Bie, Mù-Biē-Guǒ, Mu Bie Zi, Teng Tong, Tu Mu Bie
India	Bhat Kerala, Golkara, Kakrol, Gangerua, Gulkakra, Kakur, Kakrol, Kantola, Kathaamla
Indonesia	Pupia, Torobuk, Toropu
Laos	Khaawz
Malaysia	Teruah
Philippines	Tabog-Ok, Tabog-Uak
Thailand	Bai-Khai-Du, Fakkhao, Phak-Khao, Khika-Khrua Yawd-Fak-Kao
Vietnam	Red Gac, Moc Miet Tu

1.3.2 Cultivation and propagation

Plants exists in the wild (Bharathi and John, 2013) or domestically as vines on home lattices in Thailand, Vietnam and India (Figure 1.2) (Kha, 2010) and cultivated under green-house conditions (*ex situ*) in Australia (Parks et al., 2013). *M. cochinchinensis* is cultivated through traditional methods where farmers randomly collect mature seeds (Kha et al., 2013), swollen root tubers (Sanwal et al., 2011) and grafting (Joseph et al., 2011) (Table 1.3). However, germination by seeds *in situ* is agriculturally not-suitable since it is not possible to identify male and female plants before flowering (Sanwal et al., 2011) and currently it is known that 40% of seeds produce male plants (Joseph and Bharathi, 2008). Seeds are also reported to have trypsin inhibitors which cause dormancy (Huang et al., 1999) and might be affected by environmental conditions. In contrast, seed dormancy was not affected in *ex situ* conditions but 80% of seeds produced male plants (Parks et al., 2013). Cultivation through root tubers and grafting have been found to be useful for getting fruits in a shorter time and guaranteeing fruit production, indicating that these methods are more suitable for the cultivation of *M. cochinchinensis* (Table 1.3).

Table 1.3: Cultivation method and propagation time (weeks) of *M. cochinchinensis*.

Cultivation method	Time (weeks)					Reference
	Germination	Leafing	Flowering	Fruiting	Dormancy	
Seed						
Traditional	3	na	12	32	Yes	(Kha, 2010)
Green House	2	7	24	44	No	(Parks et al., 2013)
Tubers	na	na	8	na	-	(Joseph and Bharathi, 2008)
Grafting	-	-	-	12	-	(Joseph et al., 2011)

na: No information available.

1.3.3 Botany

Botanical description is useful in plant identification and the analysing of the phylogenetic relationships between species. The botanical description of *M. cochinchinensis* in India is reported in the literature (Bharathi and John, 2013) (Table 1.4) but variations in the vegetative and reproductive characters exist based on the country and region the plant is grown in (Wimalasiri et al., 2015). These variations are reviewed in section 1.3.4.

Table 1.4: Vegetative and reproductive morphological characters of *M. cochinchinensis*. Adapted from Bharathi and John (2013).

Morphology	Parameter	Characteristics
Vegetative		
	Stem	Angular robust, glabrous
	Leaf shape and outline	Entire, broadly ovate or sub orbicular outline
	Leaf lobes	3–5 palmately lobed
	Leaf size	10-16 cm
	Root	Tuberous
Reproductive	Male flowers	
	Bract	Sub apical, cucullate, sub orbicular or reniform, 20–40 mm wide, scabrous, rounded at base, acute at apex, margins undulate, veins sub parallel, very prominent outside (Figure 1.4 B)
	Sepals	Coriaceous, 10–12, 4–8 mm, ovate-oblong or triangular, acute at apex, blackish, finely scabrid.
	Petals	Sub elliptic, conspicuously sub parallel veined, 3 scales, at the base of the blotched petals, protecting the nectary; inner 3 petals with purple bull's eye mark at base, filaments short, fleshy, 5–6 mm long, inserted at the base of the receptacle tube, anthers variable in size, 'S' shaped, connective swollen.
	Female flowers	
	Bract	Small or just as in male.
	Sepals	Linear oblong, 4–10 mm long or just as in male.
	Petals	Same as male flower.
	Ovary	Ellipsoid oblong, 12–15 mm long, densely soft muricate.
	Style	8–9 mm long.
	Fruit	
	Shape	Round or oval (Figure 1.4 D) and shortly rostrate at base. 10–15 cm in length and 6–10 cm width,
	Size	350 and 500 g or more.
	Weight	Unripe fruits are green turns orange or red on ripening
	Colour	(Figure 1.4 D,E).
	Pericarp	Densely tuberculate with uniformly short round conical structures and yellow mesocarp (Figure 1.4 F).
	Seeds	
	Size	Vary, usually 1.5–2, 0.8–1.2 cm.
	Shape	Broadly ovate penta-hexangular with flat sculptured surfaces and dentate margins (Figure 1.4 G)
	Testa	Black (Figure 1.4 E)
		Red aril (covering of the seeds) (Figure 1.4 F)
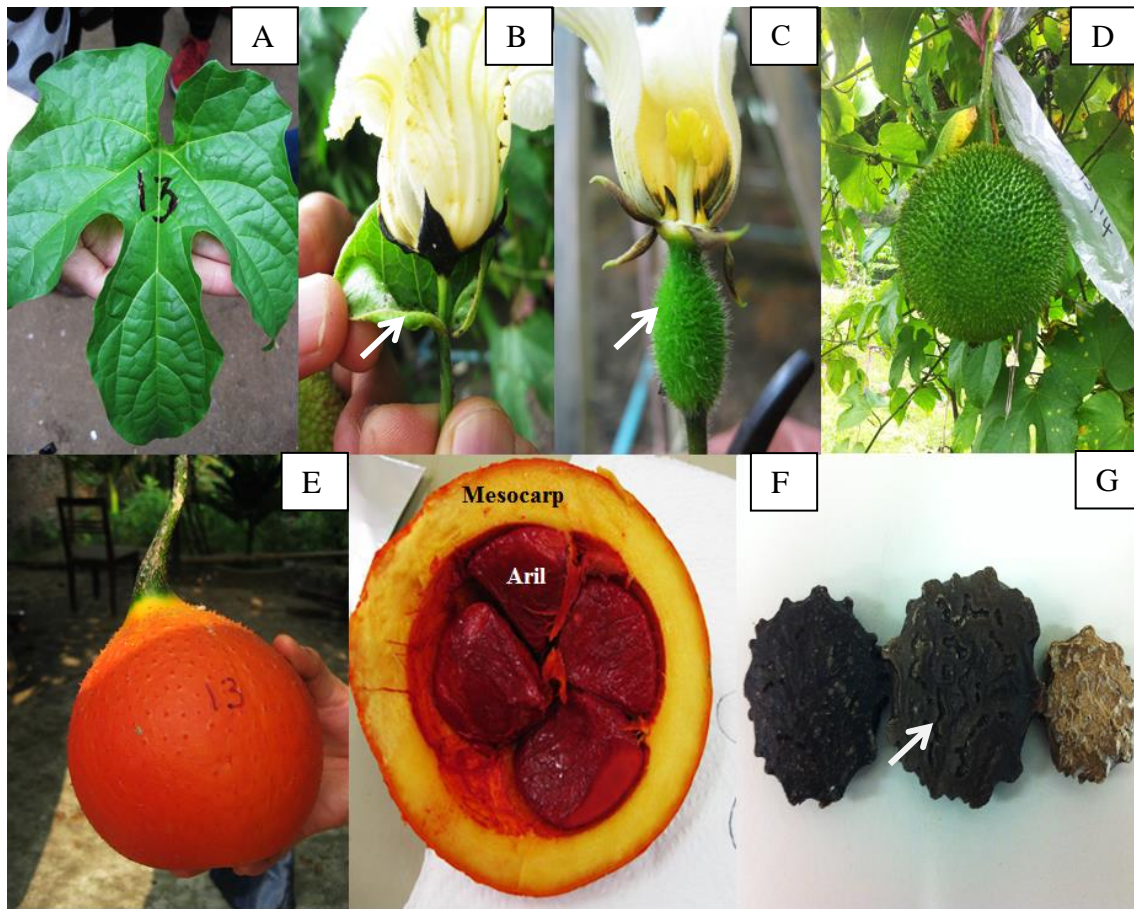
Figure 1.4: Morphology of leaves, flowers (male, female), fruits (immature, mature) and seeds of *M. cochinchinensi*s. A: Leaves, B: Male flower with broad reinform bracts (arrow), C: Female flower with scabrous ovary (arrow). D: Immature fruit, E: Mature fruit, F: Cross section of the fruit, G: Seeds with black testa (arrow).

1.3.4   Diversity

1.3.4.1   Morphological diversity

M. cochinchinensis is morphologically diverse based on the country and region the plant is grown in (Wimalasiri et al., 2015) and variations exist in vegetative (leaves) and reproductive (fruits and seeds) components.

Diversity in vegetative components:

There is morphological diversity in vegetative components of *M. cochinchinensis* with Southern Vietnam having individuals with three and five lobed leaves (Wimalasiri et al., 2015). However, it is not known whether the variations are related to genetic or environmental factors. Additionally, it is not known whether the diversity of vegetative components of *M. cochinchinensis* are related to economically important traits such as higher fruit yield (number per plant and size) or nutritional and phytochemical content.

Diversity in reproductive components:

The reproductive components, the fruit and seeds of *M. cochinchinensis*, have wide variations (Figure 1.4). The spine density of the fruits varies ranging from dense spines, hard and widely spaced (Vuong, 2000) to no spines (Wimalasiri et al., 2015). The fruit shape can be round, oblong (Vuong, 2000) or tapered (Wimalasiri et al., 2015). Whether these differences are genetically or environmentally influenced is

unknown but they might be influenced by abiotic factors including collection sites or soil nutrients. The seed weight varies from 0.88 to 4.64 mg and length varies from 18.03 to 34.84 mm (Wimalasiri et al., 2015). Likewise, the number of seeds per fruit varies from 7 to 54 (Wimalasiri et al., 2015). These morphological variations in seeds are influenced by country and region of collection (Wimalasiri et al., 2015). The fruits from Northern and Central Vietnam possess a greater seed weight and length (Wimalasiri et al., 2015) and might have potential for commercial seed oil production. Furthermore, large seeds may be favoured because they produce larger and more vigorous seedlings with better chances of survival (Rao et al., 2008). In contrast, the small seeds may have a selection advantage due to wider and more effective dispersal (Ruxton and Schaefer, 2012).

Selection based on morphological characteristics has been used to develop plant varieties with desirable qualities such as increased fungal resistance (Amaro et al., 2007) and increased nutritive value (Xu et al., 2013). For example, in capsicum, the fruit colour (orange fruits) is associated with high β-carotene levels, and in tomato lycopene content is associated with redder fruits (Guzman et al., 2010). Such improvements based on morphological selection have not been conducted in *M. cochinchinensis*. However, plant selection based on morphological characters might be subjective, vary based on plant growth environment and not all markers will be associated with important economic traits (e.g. fruit yield, quality and nutrients) (Jiang, 2013). This indicates the necessity of unbiased markers for the selection of genotypes in breeding programs for commercial propagation.

1.3.4.2 Molecular markers

DNA based molecular markers are superior to morphological markers since they provide an unbiased estimation of genetic diversity and do not depend on environment variability hence, establish a genetic relationship more precisely (Soller and Beckmann, 1983). Rapidly evolving regions of DNA are informative in detecting polymorphisms (base deletions, insertions and substitutions) beyond the species level (e.g. plant populations in different regions or countries). These polymorphisms can be detected by polymerase chain reaction (PCR) based methods which include the restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), microsatellites or simple sequence repeat (SSR), and single nucleotide polymorphism (SNP) methods. These methods have been extensively used in plant breeding to correlate genetic regions which contribute to agriculturally desirable traits (Jiang, 2013).

There are relatively few studies done on the genus Momordica on DNA molecular markers with only two studies performed on *M. cochinchinensis* (Table 1.5). However, there are no genetic markers identified for plants producing commercially desirable traits which include better seeds, fruit yield and nutrients. These markers will be important in breeding programs of *M. cochinchinensis* to select plants with desirable traits in its early stages of growth. In a study by Bootprom et al. (2012), found that *M. cochinchinensis* accessions could be grouped into two main clusters based on the gender of the plants. This suggested that the RAPD markers used in the study were useful in determining the gender of the *M. cochinchinensis* plants. Development of such markers will be important for the effective selection of female plants at early stages of growth as

well as for the selection of plants with higher fruit qualities such as nutrients (higher carotenoids).

Table 1.5: Molecular markers used in genetic diversity studies of Momordica species.

Taxon	Number of accessions	Marker type	Country of collection	Reference
M. charantia	45	RAPD	China	(Zhang et al., 2004)
	38	RAPD	India	(Dey et al., 2006)
	38	ISSR, RAPD	India	(Behera et al., 2008a)
	33	RAPD	India	(Resmi and Sreelathakumary, 2012)
	38	AFLP	India	(Gaikwad et al., 2008)
	38	RAPD, ISSR, AFLP	India	(Behera et al., 2008b)
	29	AFLP, SSR	China	(Kole et al., 2012)
	36	SSR	China	(Wang et al., 2010)
	50	RAPD, ISSR	India	(Behera et al., 2012)
M. diocia	29	RAPD	India	(Rasul et al., 2007)
M. cochinchinensis	25	RAPD	Vietnam, Thailand	(Bootprom et al., 2012)
	42	RAPD, ISSR	Vietnam, Thailand, Australia	(Wimalasiri et al., 2015)

1.3.4.3   Genetic diversity

M. cochinchinensis samples from Vietnam and Thailand have been shown to be genetically diverse based on RAPD and ISSR molecular markers (Bootprom et al., 2012, Wimalasiri et al., 2015). Both studies indicated a high degree of polymorphism (>90%) based on molecular markers. This suggested that the gene pool of *M. cochinchinensis* is diverse and might contribute to its potential nutritional and medicinal properties but the relationship is unknown.

The reproductive mechanisms of dioecious plants typically result in a high heterogenetic population as a result of exchange of the genetic material due to out-crossing breeding systems (Loveless and Hamrick, 1984, Hamrick et al., 1992).

Consistent with this, the dioecious members of the Momordica genus have shown a higher percentage of genetic diversity than the monoecious species (Bharathi et al., 2011b), possibly due to their pollination strategy, which involves wind and *Ctenoplectra* bees (Schaefer and Renner, 2008, Bharathi et al., 2011b). However, the reproductive success of this strategy can be hindered by self-incompatibility (Torres et al., 2002), geographical restrictions, the short flight distance of pollinators and environmental disturbances (e.g. fire).

Environmental variations can intensify genetic differences and result in the expression of phenotypes, which help to adapt to stress inducing conditions (abnormal temperature fluctuations and drought conditions) (Hoffmann and Hercus, 2000). Since *M. cochinchinensis* is found in both temperate and tropical climates (Wimalasiri et al., 2015), the climatic and eco-geographical differences may reflect the adaptive ability of this species, which will be important for its long term survival. Similarly, the dioecious *M. diocia* species have high genetic diversity (Rasul et al., 2007, Behera et al., 2008a) and suitable adaptation to their eco-geography (Pham et al., 2009, Hamasha et al., 2013).

As described in section 1.3.2, the seeds of *M. cochinchinensis* fruits are collected by farmers from the wild and exchanged with relatives or friends. This random exchange of seeds contributes to increase genetic diversity and gene flow due to human and environmental interactions (Vargas-Ponce et al., 2009, Du et al., 2011). Vegetative propagation is more popular to ensure fruiting and an effective strategy for preserving the genetic resources of *M. cochinchinensis*. Domestication via vegetative propagation reduces reproductive biology and the genetic diversity of the cultivated species (Miller

and Gross, 2011) relative to their wild population due to artificial selection and genetic drift through bottleneck effects (the change of alleles of a species due to random sampling) (Bartsch et al., 1999, Bourguiba et al., 2012). However, although vegetative propagation can be an effective means to artificially improve horticultural traits (e.g. high fruit yielding or ethno-pharmacological standards), the lack of gene flow in mono-culture populations will affect their long term survivability (Doebley et al., 2006) due to the loss of valuable genes, which enable the plants to adapt to environmental stress or pathogen resistance.

1.4   Phytochemicals

Phytochemicals are secondary metabolites produced as plant defence mechanisms against environmental stress conditions such as drought and extreme temperatures and play a major role in the adaptation of plants (Bourgaud et al., 2001). There are over 10,000 dietary phytochemicals derived from fruits and vegetables (Liu, 2004, Watson and Preedy, 2009). In this review, the main focus will be on carotenoids, phenolics and fatty acids as they are main components in *M. cochinchinensis*.

1.4.1   Carotenoids

Carotenoids consist of a 40-carbon conjugated polyene chain and account for around 600 pigments (Rodriguez-Amaya, 2001). This polyene chain is a chromophore, which absorbs light in the visible range of 400 to 550 nm, responsible for the characteristic colours that range from yellow to red (Rodriguez-Amaya, 2001). Carotenoids can be categorised into four main groups based on their chemical structure

(Table 1.6) and possess important health benefits (Hirayama et al., 1994, Viljanen et al., 2002).

Carotenoids are found in plants, photosynthesising bacteria and fungi (Rao and Rao, 2007). Animals are not capable of synthesising carotenoids and therefore, obtain these via dietary intake (Rodriguez-Amaya, 2001). In plants, carotenoids are found in chloroplasts (chlorophyll) and chromoplast membranes (carotenoid-associated proteins) (Vishnevetsky et al., 1999). They are synthesised *de novo* in photosynthetic plants and bacteria through the mevalonic acid and pyruvate 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (DOXP) pathways (Goodwin, 1980, Bramley, 2002). These biosynthetic pathways are regulated through a series of enzymes encoded by specific genes (Cunningham and Gantt, 1998, Hirschberg, 2001) leading to the final products lutein and violaxanthin (Figure 1.5).

Synthesis of carotenoids through the mevalonic acid pathway results in the formation of two key phytochemicals of interest, lycopene and β-carotene (Figure 1.5). Accumulation of lycopene has been correlated with the up-regulation of the phytoene synthase (PSY), phytoene desaturase (PDS), ζ-carotene desaturase (ZDS) and carotenoid isomerase (CRTISO) genes and the down-regulation of the lycopene ε-cyclase (LCYE) gene has been found to correlate with β-carotene and lutein synthesis in *M. cochinchinensis* (Hyun et al., 2012). This is similar to the enzymatic regulation, which leads to lycopene (Giuliano et al., 1993) and β-carotene (Ronen et al., 1999) accumulation in tomato. This enzymatic regulation will be important in future studies on molecular breeding for higher carotenoid producing varieties of *M. cochinchinensis*.

Table 1.6: Classification of carotenoids based on their chemical structure, colour, concentration and biological importance from major plant sources.

Structure	Name	Colour	Plant Source		Content (mg/g) FW	Importance
			Scientific name	Common name		
Acyclic	Lycopene	Red	*S. lycopersicum*	Tomato	0.03-0.20	Anticancer: prostrate lung digestive tract
			M. cochinchinensis	Red gac	3.05	and breast (Rao & Agarwal, 1999;
			C. lanatus	Watermelon	0.02-0.07	Weisburger, 2002)
	ζ-carotene	Yellow	*P. edulis*	Passion fruit	NA	Antioxidant (Rao & Agarwal, 1999)
	Phytoene	Colourless	*S. lycopersicum*	Tomato	NA	Anti- inflammation and anti-aging (Fuller et al., 2006)
	Neurosporene	Orange	*S. lycopersicum*	Tomato	NA	Antioxidant and UV-B radiation protector (Ramaprasad et al., 2013)
Cyclic	α-carotene	Yellow-orange	*D. carota*	Carrot	0.02-0.21	Anticancer: lung (Ziegler et al., 1996)
	β-carotene	Orange	*D. carota*	Carrot	0.05-0.26	Anticancers: prostrate, breast, lung,
			M. cochinchinensis	Red gac	0.10-0.77	gastrointestinal, cervical, ovarian and pancreatic (Mayne, 1996, Giovannucci, 1999, Stahl and Sies, 2003, Rao and Rao, 2007)
	γ-carotene	Yellow-orange	*S. lycopersicum*	Tomato	NA	Antioxidant (Rao & Agarwal, 1999)
	δ-carotene	Orange-red	*S. lycopersicum*	Tomato	NA	Antioxidant (Rao & Agarwal, 1999)

(Continued)

Table 1.6 (Continued): Classification of carotenoids based on their chemical structure, colour, concentration and biological importance from major plant sources.

Structure	Name	Colour	Plant Source		Content (mg/g) FW	Importance
			Scientific name	Common name		
Hydroxycarotenoids	Rubixanthin	Red-orange	*R. canina*	Rosehip	NA	Food additive
	α-cryptoxanthin	Yellow	NA	NA	NA	NA
	β-Cryptoxanthin	Orange	*C. annuum*	Red pepper	0.02	Pro-vitamin A, anti-cancer (Lian et al., 2006)
	Lutein	Yellow	*B. oleracea*	Kale	0.06-0.15	Make up the yellow pigment in the macula of the human retina, important in maintaining eye health (Bone et al., 1988, Rao and Rao, 2007)
	Zeaxanthin	Yellow-orange	*C. annuum*	Orange Pepper	0.02-0.09	Produces the yellow pigment in the macula of human retina (Mozaffarieh et al., 2003)
Epoxycarotenoids	Violaxanthin	Orange		Mango	0.02	Food additive

NA: Information not available
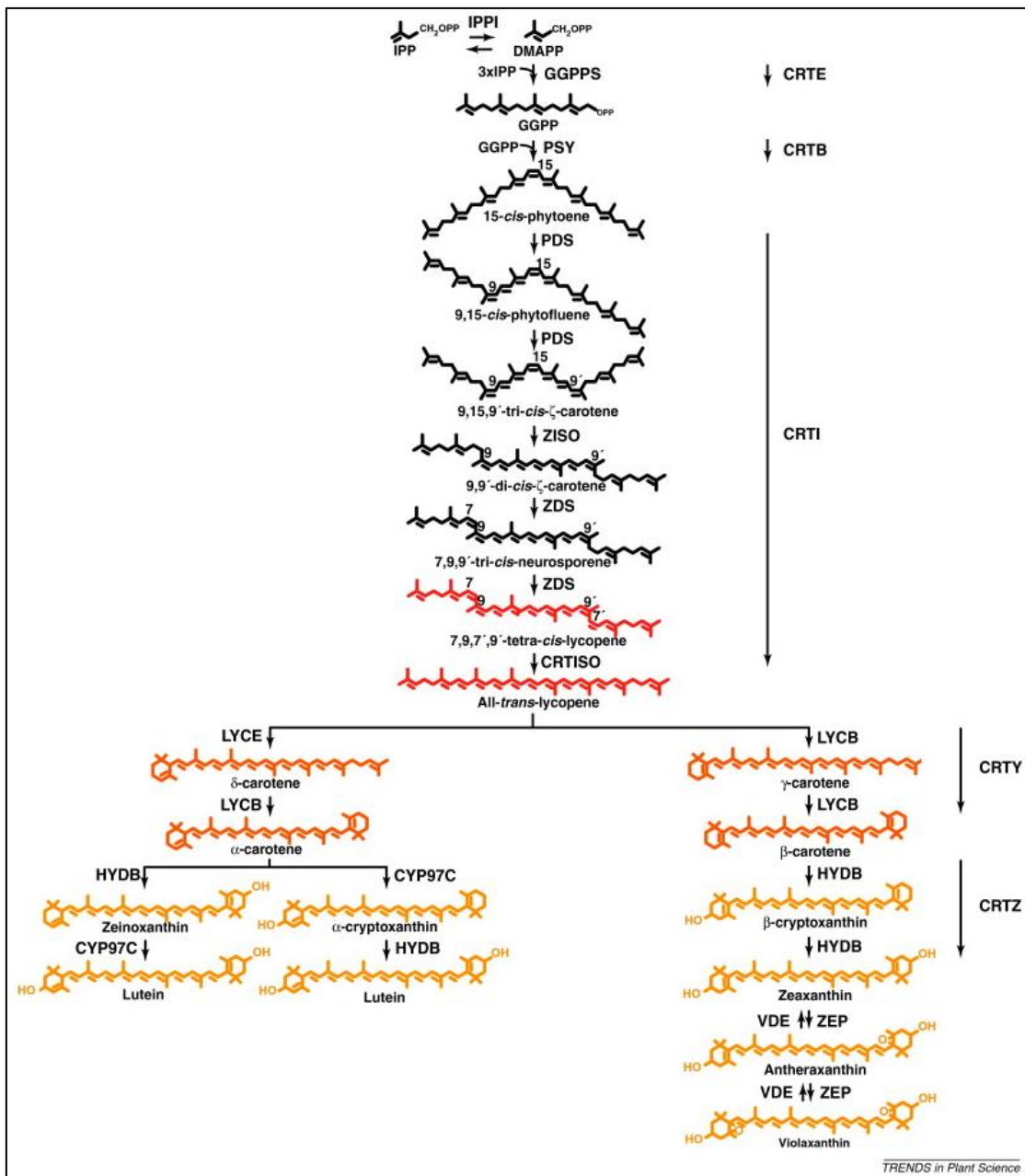
Figure 1.5: Carotenoid biosynthesis in plants through the mevalonic acid pathway. Abbreviations: CRTISO, carotenoid isomerase; CYP97C, carotene ε-ring hydroxylase; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; HYDB, β-carotene hydroxylase [non-heme di-iron hydroxylases, β-carotene hydroxylase (BCH) and heme-containing cytochrome P450 β-ring hydroxylases, CYP97A and CYP97B]; IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase; Z-ISO, ζ-carotene isomerase. Figure adapted from Farré et al. (2011).

1.4.1.1   Carotenoids in *M. cochinchinensis*

M. cochinchinensis is the richest source of carotenoids in all known fruits and vegetables. The two predominant carotenoids in the fruit of *M. cochinchinensis* are lycopene and β-carotene, with the aril containing a more than 7 times higher concentration of lycopene compared to β-carotene (Table 1.7) (Nhung et al., 2010). The lycopene concentration in the aril reported by Ishida et al. (2004) was more than 20 times higher than those for field grown cherry tomatoes (Kuti and Konuru, 2005). Likewise, the β-carotene concentration in *M. cochinchinensis* aril (Ishida et al., 2004) was more than 15 times higher than that of carrots (Gul et al., 2015).

Variations exist in the reported concentrations of lycopene and β-carotene in the fruit of *M. cochinchinensis* and the carotenoid content on a dry weight (DW) basis is higher than on a fresh weight (FW) basis (Table 1.7). However, the DW-based concentration of lycopene and β-carotene reported by Kubola and Siriamornpun, (2011) was 5 and 3.8 times higher, respectively than reported byAoki et al. (2002). Likewise, a discrepancy of up to 16 times for lycopene and 10 times for β-carotene was reported on a fresh weight basis (Table 1.7). These discrepancies might be due to differences in extraction solvents, fruit collection sites or variety.

The aril, mesocarp and peel of *M. cochinchinensis* are also rich in other minor carotenoids (Table 1.8).

Table 1.7: Lycopene and β-carotene concentration in different parts of the fruit (aril, mesocarp and peel) of *M. cochinchinensis* from Thailand and Vietnam.

Fruit part	Extraction method	Lycopene mg/g	β-carotene mg/g	Collection site	Reference
Aril	Homogenized fruit	0.45 (FW)	0.01 (FW)	Sakon Nakhon and Nakon Phanom province, Thailand	(Kubola et al., 2013)
	Chloroform: methanol (2:1)	0.49 (FW)	1.18 (FW)		
	Petroleum ether	0.30 (FW)	0.14 (FW)		
	Hexane	0.21 (FW)	0.12 (FW)		
	Hexane/acetone/ethanol (2:1:1)	7.02 (DW)	1.60 (DW)	Nakhon Phanom province	(Kubola and Siriamornpun, 2011)
	Ice cold methanol	2.23 (FW)	0.72 (FW)	Vietnam	(Ishida et al., 2004)
	Acetone	0.38 (DW)	0.10 (DW)	HCM city, Vietnam	(Aoki et al., 2002)
	THF	0.41 (FW)	0.08 (FW)	Northern Vietnam	(Vuong et al., 2006)
	THF and methanol (4:1)	3.28 (FW)	0.36 (FW)	Hai Duong province, Vietnam	(Nhung et al., 2010)
Mesocarp	Hexane/acetone/ethanol (2:1:1)	1.6–5.9 (DW)	3.0–5.4 (DW)	Nakhon Phanom province	(Kubola and Siriamornpun, 2011)
	Acetone	0.0009 (DW)	0.02 (DW)	HCM city, Vietnam	(Aoki et al., 2002)
Peel	Hexane/acetone/ethanol (2:1:1)	1.6–3.4 (DW)	1.6–5.9 (DW)	Nakhon Phanom province, Thailand	(Kubola and Siriamornpun, 2011)

FW: Fresh Weight, DW: Dry Weight

Table 1.8: Summary of minor carotenoids in different parts of *M. cochinchinensis* fruit.

Fruit part	Phytochemical	Concentration (mg/g)	Reference
Aril	α-carotene	0.11 FW	(Ishida et al., 2004)
	α-tocopherol	0.76 FW	(Vuong et al., 2006)
	Zeaxanthi	0.01 DW	(Kubola & Siriamornpun, 2011)
	β-cryptoxanthin	<0.01 DW	(Kubola & Siriamornpun, 2011)
Mesocarp	α-tocopherol	31.00 FW	(Vuong et al.,2006)
	Leutin	18.10 DW	(Kubola & Siriamornpun, 2011)
	Zeaxanthin	0.02 FW	(Aoki et al.,2002)
	β-cryptoxanthin	0.03 FW	(Aoki et al., 2002)
Peel	Leutin	52.02 DW	(Kubola & Siriamornpun, 2011)

FW: Fresh Weight, DW: Dry Weight

1.4.1.1.1 Pro-vitamin A activity

β-carotene can be converted into Vitamin A in the body and is called a pro-vitamin A precursor. The aril of *M. cochinchinensis* contains the highest concentration of β-carotene, over 15 times higher than in carrots (Gul et al., 2015). A clinical trial on Vietnamese children found that a 30 day supplement of fresh *M. cochinchinensis* aril increased plasma β-carotene and vitamin A concentration compared to the control group (steamed rice without aril) and a group given synthetic β-carotene, indicating that β-carotene in *M. cochinchinensis* aril was bio-available (Vuong et al., 2002). This bio-availability of β-carotene in *M. cochinchinensis* aril might be due to the presence of a higher amount of oil and fatty acids in the aril (reviewed in section 1.4.2) compared to synthetic β-carotene supplement, which has been reported to promote the intestinal absorption of β-carotene in humans (Castenmiller and West, 1998, Yeum and Russell, 2002, Maiani et al., 2009). This study suggested that dietary intake of *M. cochinchinensis*

aril has the potential to treat vitamin A deficiency, which is especially seen in third world countries.

1.4.1.1.2 Antioxidant activity

An antioxidant is a compound present in the diet which can lower the formation of reactive oxygen species (pro-oxidants and free radicals) produced during cell aerobic respiration (Pulido et al., 2000). Fruit extracts of *M. cochinchinensis* have shown anti-oxidative activities mainly due to their carotenoid and phenolic compounds (reviewed in section 1.4.3) (Kubola and Siriamornpun, 2011). In a study of the different parts of the fruit, the aril from the mature fruits had the highest antioxidant activity while the seed extract had the lowest activity (Kubola and Siriamornpun, 2011). Apart from ripe fruit, the homogenised extract of immature green fruit also showed antioxidant activity of 0.058 mg/g and 0.45 mg/g equivalent to ascorbic acid determined using the ferric ion reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assays, respectively (Bharathi et al., 2014).

The antioxidant activity of *M. cochinchinensis* is affected by moisture content, the drying and/or processing temperature. An aril powder, prepared with 10% maltodextrin by spray drying at 120°C with a moisture content of 4.9% was shown to have antioxidant activity equivalent to 1.4 mmol Trolox per gram in the 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay (Kha, 2010) but when the moisture content was increased (6%), the antioxidant activity was reduced (Kha et al., 2011). The processing temperature of 80°C caused isomerisation of 16% of the lycopene from the all-*trans* to the *cis* isoform which resulted in a 1.5 times higher antioxidant activity than

at 50°C (Phan-Thi and Wache, 2014). However, the difference in antioxidant properties between the lycopene isomers has not yet been clearly explained in the literature.

1.4.1.2   Factors affecting carotenoid content

Carotenoid biosynthesis is influenced by genetics, environmental factors, stage of maturity and post-harvest processing (Cazzonelli and Pogson, 2010). However, improvements in the carotenoid content through traditional agriculture by selection of the varieties with the highest carotenoid content and manipulation of the growth environments are still unexplored for *M. cochinchinensis*.

1.4.1.2.1   Genetic influences

Genotype affects the carotenoid content in different varieties and cultivars of fruits and vegetables but this is unknown for *M. cochinchinensis*. Identifying the genotype or cultivar that produces a higher amount of carotenoids is unknown and is reflected by the vast variation in the literature with up to 18 times and 16 times discrepancies for lycopene and β-carotene, respectively (Table 1.7). Selecting the best genotype is necessary in breeding to get consistent carotenoid yields.

Example from the literature include the content of lycopene and β-carotene varying significantly among cherry tomato varieties grown in Southern Italy and greenhouse conditions and being significantly influenced by the genotype with the best genotypes identified in commercial breeding programs (Kuti and Konuru, 2005, Toor and Savage, 2005, Lenucci et al., 2006). Additionally, the influence of genetic diversity on

the carotenoid content has been reported for carrot (Nicolle et al., 2004) watermelon (Perkins-Veazie et al., 2006), lettuce (Mou, 2005), sweet potatoe (Almeida Muradian and Penteado, 1992, Rodriguez-Amaya and Kimura, 2004, Vimala et al., 2011), papaya and banana (Wall, 2006) and cultivated citrus (Fanciullino et al., 2006) but not for *M. cochinchinensis*.

1.4.1.2.2   Eco-geographical influences

The country and region of origin of fruits reflects climatic and geographical differences including temperature, rainfall, sun exposure and elevation. These differences have been studied relative to the variation in carotenoid content of fruits such as tomatoes (Aherne et al., 2009), mangoes (Mercadante and Rodriguez-Amaya, 1998), cashew apples (Assunção and Mercadante, 2003) and apricots (Munzuroglu et al., 2003, Dragovic-Uzelac et al., 2007). Variation in the carotenoid content (Table 1.7) might be attributed to differences in collection sites of *M. cochinchinensis* but no systematic analysis has been conducted.

The carotenoid content is known to be increased by the temperature during fruit development and the optimum temperature for this has been identified for tomato. The optimum temperature for lycopene synthesis ranges between 12 to 32°C for tomatoes (Dumas et al., 2003) but this is unknown for *M. cochinchinensis*. Exposure to such a wide range of temperatures influences the production of plant secondary metabolites including carotenoids (Rice-Evans et al., 1996). Direct sunlight and excessive solar radiation is also known to reduce the bioaccumulation of carotenoids in tomato (Helyes et al., 2007, Inbaraj and Chen, 2008, Pék et al., 2011). *M. cochinchinensis* is found in tropical (>18°C

of minimum temperature) and temperate (<15°C minimum temperature) climates but the effect of temperatures have not been studied for carotenoid accumulation and this will be important when considering growth sites for future agricultural production.

Fruits grown under field conditions produce higher lycopene concentrations compared to hydroponic farms and this might be related to sun exposure, cultivation practices, water use and fertiliser use (Kuti and Konuru, 2005). Field-grown cherry tomatoes had a 1.6 times higher lycopene content compared to those grown under hydroponic conditions (Kimura and Rodriguez-Amaya, 2003, Kuti and Konuru, 2005). However, these conditions have not been studied for their effects on the carotenoid accumulation in *M. cochinchinensis*.

1.4.1.2.3   Stage of maturity

Ripening increases the total carotenoid levels in fruits (Speirs and Brady, 1991) with an increase of the acyclic (lycopene) and cyclic (β-carotene) carotenoids (Fraser et al., 1994, Bramley, 2002,) but a decrease of the hydroxycarotenoids (leutin) and epoxycarotenoids (violaxanthin) (Su et al., 2015). In *M. cochinchinensis*, while the lycopene and β-carotene levels were higher in mature (red aril, orange mesocarp) fruits (Nhung et al., 2010, Kubola and Siriamornpun, 2011), lutein was highest in medium ripe mesocarp (yellow) fruits (Kubola and Siriamornpun, 2011).

1.4.1.2.4   Post-harvest processing

Carotenoids are susceptible to oxidation during dehydration processes due to their unsaturated double bond system. Traditionally, the aril of *M. cochinchinensis* is air dried under the sun which causes lycopene and β-carotene to degrade (Chuyen et al., 2015), but methodologies have been developed to reduce this degradation, which is important for the commercial production in *M. cochinchinensis*-based products.

Several methods have been studied to determine the best way to use the *M. cochinchinensis* aril to produce a powder and an oil with reduced carotenoid degradation (Chuyen et al., 2015). Aril powder was produced by five different drying methods (vacuum, air, oven, spray and freeze drying) (Kha, 2010, Kha et al., 2011, Kha et al., 2013b). Drying reduced the carotenoid content in *M. cochinchinensis* aril due to their oxidation. However, the best method to produce aril powder was identified to be freeze-drying, which had carotenoid levels 14.5 times higher than that seen in spray dried arils (Kha et al., 2013b). High temperature and low moisture levels reduced the carotenoid content in the aril powder. A temperature of 60°C and a 15-18% moisture content retained carotenoids in the aril powder and these conditions were suitable for aril powder production (Mai et al., 2013a, Mai et al., 2013b). Furthermore, pre-treatments and addition of reducing agents decreased the oxidation of the carotenoids caused by drying. These treatments included pre-soaking the aril with ascorbic acid and sodium bisulphite (Kha et al., 2011), blanching in citric acid and streaming, use of a maltodextrin-gelatin mixture (1:1), addition of 0.2% vitamin C and vitamin E (Minh and Dao, 2013), which resulted in retention of the carotenoids during the drying process.

Aril oil is currently produced through pressing and extraction using organic solvents and water (Mai et al., 2013a). Aril oil is traditionally produced by manual pressing (Vuong and King, 2003) and the oil yield is improved by pre-treatment with microwaves (630 W, 65 min) and steam (20 min) (Kha et al., 2013b, Kha et al., 2014). Of the organic solvents used, chloroform:methanol (2:1) yielded >2 times more lycopene and >9 times more β-carotene than oil extracted by hexane (Kubola et al., 2013). Furthermore, oil extracted with water in the presence of enzymes (protease, cellulase, pectinase and α-amylase) increased the total carotenoid content by 10% compared to the non-enzymatic treated control (Mai et al., 2013a). This was attributed to the breaking of the cell wall, which facilitated the release of carotenoid pigments, indicating that these methods were more suitable for retaining carotenoids than simple drying techniques and could be used for oil production on the industrial scale.

1.4.1.2.5 Extraction

Carotenoids are not soluble in polar solvents due to their long linear aliphatic hydrocarbon chain and therefore, are extracted using non-polar organic solvents (Rodriguez-Amaya and Kimura, 2004). Solvents such as ethanol, acetone, petroleum ether, hexane, benzene and chloroform are routinely used for carotenoid extraction from fruits and vegetables (Rodriguez-Amaya and Kimura, 2004). However, solvent combinations such as methanol/tetrahydrofuran (THF) (50:50 v/v) ethanol/hexane (50:50 v/v), hexane/acetone/ethanol (50:25:25 v/v/v) are more efficient and give better extraction of carotenoids (Lin and Chen, 2003, Periago et al., 2004). Several extraction methods using various organic solvents and solvent combinations have been used for the

analysis of carotenoids in *M. cochinchinensis* (Table 1.7). Of these, hexane/acetone/ethanol (50:25:25 v/v/v) yielded the highest lycopene and β-carotene concentrations (Kubola & Siriamornpun, 2011), suggesting that it is the most suitable extraction method.

Other methodologies have been developed to reduce the degradation of the carotenoids and increase their concentration in foods during processing (Sadler et al., 1990, Gross, 1991). Saponification using 1-2 mL of 40-60% potassium hydroxide (KOH) removes unwanted lipids, chlorophylls and other water soluble impurities (Ausich and Sanders, 1999, Gross, 1991). Extraction methods such as microwave solvent extraction, pressurised accelerated solvent extraction and supercritical CO_2 extraction are alternative extraction methods. These methods reduce the extraction time and the organic solvent consumption (Sadler et al., 1990, Benthin et al., 1999, Baysal et al., 2000, Gómez-Prieto et al., 2003) and have been used with *M. cochinchinensis*.

1.4.1.2.6 Analysis

1.4.1.2.6.1 Chromatography

High Performance Liquid Chromatography (HPLC) is widely used to analyse the carotenoids in *M. cochinchinensis* (Ishida et al., 2004, Vuong et al., 2006, Kubola and Siriamornpun, 2011). HPLC is very effective at detecting, quantifying and separating carotenoids (Hart and Scott, 1995, Azevedo-Meleiro and Rodriguez-Amaya, 2004, de Rosso and Mercadante, 2007). The carotenoids are analysed by both normal phase and reversed phase HPLC. Normal phase HPLC uses the conventional stationary phases

(alumina and silica) and a mobile phase of low polarity (e.g. hexane) and thus, elutes the carotenoids fast with low selectivity and poor resolution. In contrast, reversed-phase chromatography employs a ODS (octadecylsilanyl) stationary phase (e.g. a silica support carrying bonded C18 alkyl chains) and polar (e.g. methanol) mobile phases (Amorim-Carrilho et al., 2014). Thus, hydrophobic carotenoids in the polar mobile phase adsorb to the hydrophobic stationary phase, allowing for longer retention times and a more efficient separation than normal phase HPLC (Amorim-Carrilho et al., 2014). Two types of reverse phase columns are used in the separation of carotenoids, namely, C30 and C18 (Rodriguez-Amaya, 2001). C30 columns can be used to discriminate several *cis*-isomers of the same carotenoid whilst C18 columns are used in less detailed analysis and to separate mixtures of carotenoids (Emenhiser et al., 1995, Schoefs, 2002).

Supplementing HPLC with a diode-array detector and automation has increased the analytical power of HPLC for fast throughput processing (Rodriguez-Amaya, 2001). Using such detectors, it is possible to analyse different carotenoids which elute on the full UV–Visible range (190–800 nm) and the identity of the eluted carotenoids can be determined based on their absorption spectra (Müller, 1997, Craft, 2001, Tzouganaki et al., 2002, Burns et al., 2003, Lin and Chen, 2003, Cortes et al., 2004, Barba et al., 2006). However, HPLC requires considerable technical expertise, expensive instrumentation and hazardous solvents and therefore, it is not accessible for resource-poor farmers. Therefore, alternative simple methods are necessary for use in the routine analysis and selection of carotenoid-rich fruits.

1.4.1.2.6.2 UV-Visible spectrophotometry

Absorbance spectrophotometry can be used to identify the major carotenoid pigments present in a mixture because it reflects the fingerprint of the pigments due to differences in the organisation of the IR conjugated double bond system (Schoefs, 2002). Once the pigments are identified, it is possible to use a set of equations to estimate their respective concentration because the absorbance is proportional to the concentration according to Beer Lambert's Law. However, these absorption spectra are solvent dependent and the absorbance maxima changes with the solvent used (Schoefs, 2002). Carotenoids absorb maximally at three wavelengths, resulting in a three-peak spectrum (Figure 1.6) but the λ-max shifts to longer wavelengths as the number of conjugated double bonds increases (Rodriguez-Amaya, 2001). Lycopene is linear, has 11 conjugated double bonds (Figure 1.5), is red and absorbs at the longest wavelengths (λ-max at 447, 470, 502 nm in petroleum ether) (Figure 1.6). Although β-carotene has the same number of conjugated double bonds as lycopene, it is yellow-orange and exhibits two absorption peaks at 450 and 477 nm in petroleum ether (Rodriguez-Amaya, 2001, Machmudah and Goto, 2013).

Carotenoids can be identified and quantified using UV-Visible spectrophotometry. However, crude carotenoid extracts have overlapping absorbance spectra because they contain several carotenoid compounds or one dominant carotenoid at a high concentration (Fish, 2012). This problem can be overcome by using fluorescence spectrophotometry for chlorophyll-related pigments but it cannot be used for carotenoid analysis, since their fluorescence is very weak (Schoefs, 2002). Therefore,

separation and purification of crude extracts are essential to precisely analyse the concentration of each carotenoid in a sample (Feltl et al., 2005).
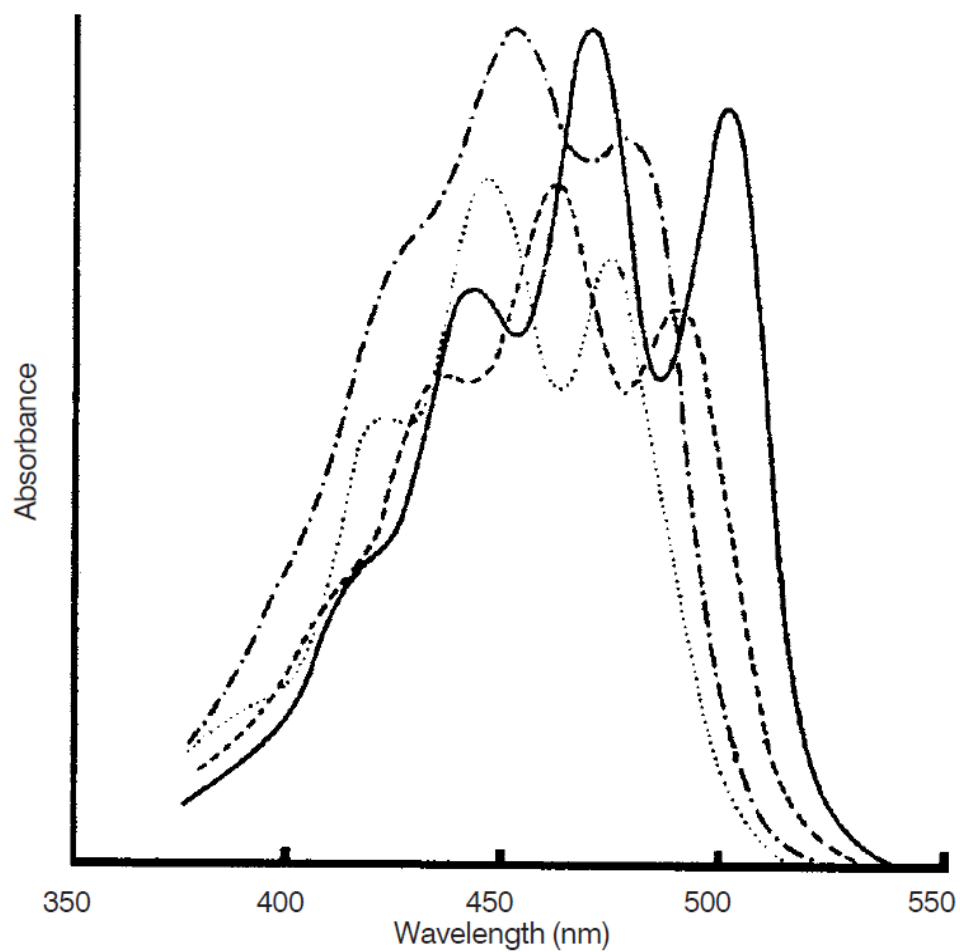
Figure 1.6: Visible absorption spectra of lycopene (−), γ-carotene (---), β-carotene (-.-.) and α-carotene (…) in petroleum ether. Figure was obtained from Rodriguez-Amaya and Kimura (2004).

1.4.1.2.6.3 Colorimetry

Visual colour is a reflection of the pigments present in a sample (Schoefs, 2002). The human eye detects the light by one of the three colour sensors of the retina: red, green, or blue. This information is not sent as individual colours but as a red/green signal, a yellow/blue signal, or a black/white signal (Schoefs, 2002). Therefore, visual evaluation of colour can be described by parameters such as hue, the dominant shade (saturation/chroma) and how much colour is present (lightness).

The amount of light reflected by an object can be converted into the above colour values by using a standard illuminant and a standard observer. To do this, in 1976 the CIE (International Commission on Illumination) adopted a standard method of calculating colour characteristics, known as the CIE Lab Colour Space. The lightness parameter L* ranges from black (L*=0) to white (L*=100) while the coordinates a* and b* indicate the colour on a rectangular-coordinate grid perpendicular to the L* axis. The colour at the grid origin is achromatic (e.g. grey; a*=0, b*=0). On the horizontal axis, positive and negative a* values indicate the hue of redness (positive values) and greenness (negative values), whereas on the vertical axis, b* indicates yellowness (positive values) and blueness (negative values).

These untransformed and mathematically transformed colour values and mathematically modified colour values have been used in studies of the carotenoids in fruits and vegetables including mangoes (Rungpichayapichet et al., 2015) and tomatoes (Akhtar et al., 1999, Arias et al., 2000, Murkovic et al., 2002, Hyman et al., 2004). Good linear correlations of the a* colour parameter with the total carotenoid content have been

reported for the dried aril of *M. cochinchinensis* (Kha, 2010, Mai et al., 2013a,) but this method has not been used for the analysis of carotenoids in fresh or frozen aril. However, this method could be useful for the selection of carotenoid-rich fruits in the field and small scale industrial settings.

1.4.2   Fatty acids

M. cochinchinensis aril and seeds are rich sources of fatty acids essentially linoleic and oleic acid (Table 1.9). Fresh and dried aril is composed of fatty acids such as oleic, palmitic and linoleic acids. Seeds are rich in stearic, linoleic, palmitic and oleic fatty acids (Vuong et al., 2002, Ishida et al., 2004, Mai et al., 2013b). The high amounts of linoleic and oleic acids found in the aril may potentially contribute to human health by reducing LDL-cholesterol levels as well as having other anti-antherogenic effects (Pariza, 2004). The presence of high amounts of fatty acids and oil in the aril can also facilitate the absorption and transportation of carotenoids into the body (Vuong et al., 2002, Vuong and King, 2003).

Notably, the CO_2 supercritical point extraction method has been found to be more suitable on the extraction of fatty acids; the yield was 37% to 42% (w/w) oil from dried aril (Kha et al., 2014) compared to extraction with petroleum ether in a soxhlet which yielded 18% to 34% (w/w) total fatty acids (Kha et al., 2013b).

Table 1.9: Fatty acid composition of *M. cochinchinensis* aril and seeds (% of total fatty acids).

Fruit part	Type of Fatty acid				Reference
	Oleic	Palmitic	Stearic	Linoleic	
Aril	32.0	29.0	7.7	28.0	(Ishida et al., 2004)
	34.1	22.0	7.1	31.4	(Vuong and King, 2003)
	59.5	17.3	7.5	13.9	(Mai et al., 2013a)
Seed	9.0	5-6	60.5	20.0	(Ishida et al., 2004)

1.4.3 Phenolic compounds

Phenolic compounds are the largest category of phytochemicals that are widely distributed in the plant kingdom and can be categorised into three major groups: flavonoids, phenolic acids and polyphenols (King and Young, 1999). These compounds are derived from the phenylpropanoid and acetate pathways (Hahlbrock and Scheel, 1989) and play an important role in plant growth, reproduction and defence mechanisms (Rice-Evans et al., 1996). In general, phenolic compounds possess diverse biological activities such as antiulcer (Carlo et al., 1993), anti-inflammatory (Hämäläinen et al., 2007, García-Lafuente et al., 2009), antioxidant (Rice-Evans et al., 1997, Zheng and Wang, 2001), cytotoxic and antitumour properties (Galati and O'Brien, 2004, Ghasemzadeh and Ghasemzadeh, 2011).

1.4.3.1 Flavonoids

The different parts of the fruit of *M. cochinchinensis* (e.g. aril vs mesocarp) have different concentrations of flavonoids and this distribution may contribute to attract predators for seed dispersal (Schaefer et al., 2004). The predominant flavonoids found

in the aril are rutin and luteolin, (Kubola and Siriamornpun, 2011). Myricetin is found in all parts of the fruit (aril, mesocarp and peel) but the levels in the aril are twice than found in the mesocarp (Kubola and Siriamornpun, 2011). The red aril contains a higher amount of rutin equivalent (RE) total flavonoid content (376 REmg/g DW) than found in mature (302 REmg/g DW) and immature mesocarp (285 REmg/g DW) (Kubola and Siriamornpun, 2011). Furthermore, the catechin equivalent (CE) total flavonoid content of homogenised fruit has been reported to be 1.32 CEmg/g FW (Bharathi et al., 2014).

1.4.3.2 Phenolic acids

The phenolic acid content of *M. cochinchinensis* varies depending on the region of the fruit and its maturity (Kubola and Siriamornpun, 2011). In this study, the green mesocarp contained the highest levels of ferulic acid and p-hydroxybenzoic acid, which was 8 times higher than that detected in the green peel (Kubola and Siriamornpun, 2011). The total phenolic content of the immature fruit aril was twice greater than that of the peel (Kubola and Siriamornpun, 2011). However, ripening reduced the total phenolic content in all parts of the fruit by more than 50% (Kubola and Siriamornpun, 2011).

1.5   Medicinal importance

1.5.1   Traditional medicinal uses of *M. cochinchinensis*

Seeds of *M. cochinchinensis* have been used in traditional Chinese medicine for over 1200 years as a preparation known as "Mubeizi". They have traditionally been

used to treat internal and external disorders such as inflammation, tinea, scrofula and skin infections such as sores, carbuncles and furuncles (Lim, 2012). The fresh aril and the oil is traditionally used by natives in Vietnam for promoting longevity and vitality (Burke et al., 2005). Aril is also used to make a tonic for children and lactating or pregnant women and to treat "dry eyes" (xerophthalmia) and night blindness (Burke et al., 2005).

All parts of the plant are medicinally important with their use ranging from topical to internal preparations against malnutrition to anticancer effects (Table 1.10) but this review will focus on the efficacy of *M. cochinchinensis* as an anti-cancer and chemo-preventive agent.

Table 1.10: Medicinally important phytochemicals in different components of the *M. cochinchinensis* plant.

Plant component	Phytochemical	Importance	Reference
Aril, mesocarp and peel*	Lycopene	Antioxidant	(Kubola and Siriamornpun, 2011)
	β-carotene	Vitamin A activity	(Vuong et al., 2002)
Seed	Trypsin inhibitory proteins (MCCTI-1, MCoTI-1, MCoTI-II, MCoTI-III)	NA	(Wong et al., 2004)
	Momordica saponins	Gastroprotective activity, improves endocrine function	(Chan et al., 2009, Wang et al., 2007)
	Momordica peptides	Toxic against a human melanoma cell line MM96L	(Hernandez et al., 2000)
	MCoCI	Anti-inflammatory	(Tsoi et al., 2004)
	Momorcochin-s	Ribosome-inactivating protein activity	(Bolognesi et al., 1989)
Leaves	Pentacyclic steroid	Antioxidant	(Wong et al., 2004)
	Tri-terpenoids	Antimicrobial activity	(Nantachit and Tuchinda, 2009)
Roots	Chondrillasterol	NA	(Hernandez et al., 2000)
	Trypsin inhibitors	Increases protein absorption	(Hernandez et al., 2000)
	Momorcochin glycoprotein	Abortifacient, antitumor, ribosome inactivating and immunomodulatory	(Yeung et al., 1987)
	Oleanolic acid glycosides	Antipruritic effect	(Matsuda et al., 1998)
	MG-1 and MG-2 glycosides	Hypoglycaemic activity	(Jalil et al., 1986)
	Steryl glycosides	NA	(Ng et al., 1986)

MCoCI: Chymotrypsin-specific potato type I inhibitor

*: The phytochemicals and their importance have been reviewed in detail in section 1.4.

NA: Not available

1.5.2 Cancer

Cancer is a disease characterised by uncontrolled cell growth and proliferation (Weinberg, 1996). It is initiated by inappropriate cell division as a result of genetic changes that transform normal cells into malignant cells through specific biological processes (Labi and Erlacher, 2015). In normal cells, proto-oncogenes and tumour suppressor genes encode many classes of proteins that help cells to grow, divide and proliferate in an orderly manner. In particular, proto-oncogenes stimulate cell growth, proliferation, differentiation but tumour suppressor genes slow down cell division, repair DNA mistakes or damage or induce apoptosis (Labi and Erlacher, 2015).

Proto-oncogenes can be activated into oncogenes by three mechanisms: chromosomal rearrangement, mutation and gene amplification (Croce, 2008). As an example, mutations in the RAS or RAF oncogenes can affect their product's active configuration, result in the activation of the protein kinase cascade (MAPK/ERK) and lead to abnormal cell growth (Figure 1.7) (De Luca et al., 2012). On the other hand, tumour suppressor genes are targeted in the opposite way where mutations will reduce the activity of the gene product (e.g. BRCA1 gene) (Vogelstein and Kinzler, 2004). These accumulated multiple mutations in proto-oncogenes and tumour suppressor genes in cancer cells avoid the G phase of the cell cycle, which regulates cell division and death (Croce, 2008) and thus, givees rise to cancer (Figure 1.7).

Cancer is categorised as the second leading cause of death, with 14.1 million estimated cases worldwide in 2015 and it is a major health problem in both developed

and developing countries (Torre et al., 2015). This review will focus on breast and skin cancer and the hallmarks that enhance their proliferative signalling.

1.5.2.1   Breast cancer

Breast cancer is a malignant tumour that originates in the cells of the breast. Breast cancer occurs predominantly in females, although men can also develop the disease, accounting for approximately 1% of cases. Globally, breast cancer is the most frequently diagnosed cancer in women, with an estimated 1.38 million new cases per year (Eccles et al., 2013). There are 458,000 deaths per year from breast cancer worldwide making it the most common cause of female cancer deaths in both the developed and developing world (Eccles et al., 2013).

1.5.2.2   Skin cancer

Skin cancer can be classified as melanoma and non-melanoma skin cancer (NMSC) (basal cell carcinoma, squamous cell carcinoma) (Narayanan et al., 2010). Melanoma is a process where normal melanocytes (melanin producing cells) undergo a malignant transformation (Lee et al., 2012). This cancer is less common than non-melanoma skin cancers but it has a high mortality rate (75%) if not detected at early stages (Jerant et al., 2000, Narayanan et al., 2010). Australia and New Zealand have the highest incidence and mortality rates in the world from melanoma due to the exposure to intense UV radiation (Sneyd and Cox, 2013). A large proportion of melanomas (40-60%) exhibit the BRAF ^{V600E} mutation, which results in the activation of the RAS or RAF oncogene and leads to abnormal cell growth through the MAPK/ERK cell

signalling pathway (Robert et al., 2015). Although drugs such as vemurafenib (Bollag et al., 2012) are commercially available to treat BRAF ^{V600E} mutated melanoma incidents, there still remains a need for treatments that extend survival and provide a better quality of life (Robert et al., 2015).
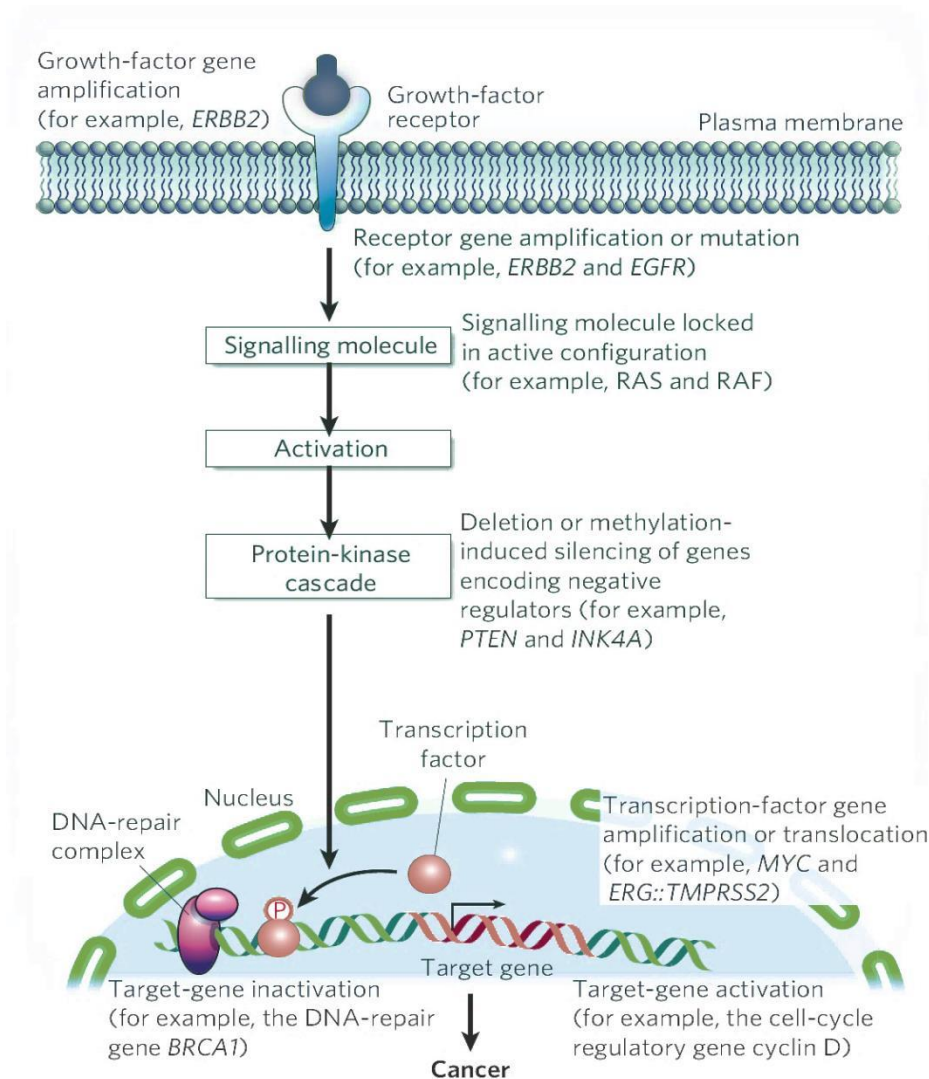
Figure 1.7: Development of cancer through regulation of oncogenes and tumour suppressor genes. Extracellular and intracellular signalling pathways can be disrupted at different points leading to multiple genetic and epigenetic alterations and ultimately leading to cancer. Figure adapted from Chial (2008).

1.5.2.3 Hallmarks of cancer

The difficulties associated with finding an effective mode to treat cancer are due to the characteristics in cancer cells which are called 'hallmarks' of cancer. Detailed below are six main biological capabilities acquired during the multistep development of human cancer, which result in cancer cell growth and survival by evading routine safeguard mechanisms and therapeutic agents (Hanahan and Weinberg, 2011). All these hallmarks have been shown to be involved in the development and progression of almost all cancers (Hanahan and Weinberg, 2011).

i. **Sustaining proliferative signalling**: this is recognised as the ability to sustain growth signals through mutations in oncogenes (e.g. mutations in oncogenes such as RAS signalling pathways) or by the disruption of negative feed-back mechanisms (e.g. mutations in PTEN amplify PI3K signalling). These mutations remove dependence on normal cell signalling and hence, give rise to excessive cell proliferation (Figure 1.7) (Chial, 2008).

ii. **Evading growth suppressing signals**: this mechanism allows cancer cells to promote cell proliferation and become insensitive to anti-growth stimuli (e.g. mutations in TP53 and Rb affect tumour suppressor pathways).

iii. **Activating invasion and metastasis:** this allows tumour cells to escape the primary site to a secondary site, where they can take up residence. This is usually due to altered functions of proteins like the cell-cell adhesion molecules (CAMs), which are involved in securing cells to their surroundings.

iv. **Enabling replicative immortality:** the ability of cancer cells to maintain replicative signalling, usually as a result of up-regulated expression of the telomerase enzyme, so as to inhibit telomere shortening and maintain telomere length (Hanahan and Weinberg, 2011).

v. **Inducing angiogenesis:** the ability to develop new blood vessels (angiogenesis), which allows for the continuous supply of oxygen and nutrients, required for the survival of tumour cells. Angiogenesis signals include the up-regulation of proteins like the vascular endothelial growth factor (VEGF) (Ferrara, 2009).

vi. **Resisting cell death:** the ability of cancer cells to bypass programmed cell death pathways such as apoptosis and autophagy. This is usually observed in collaboration with the overexpression of pro-survival proteins such as Bcl-2 (Dutta et al., 2012) and the loss of anti-apoptotic proteins (TP53) (Junttila and Evan, 2009).

1.5.2.4 Cell death

Cell death is a vital physiological process and investigation of cell death and its mechanisms is essential for the development of novel therapeutic strategies (Galluzzi et al., 2009). Cell death represents a heterogeneous process that can follow the activation of biochemical cascades and can be noticeable with different morphological features. There are three main kinds of cell death; apoptosis, necrosis and autophagy; these occur as physiological processes triggered by any mild cell injury or when cell function is disturbed (Ouyang et al., 2012). All these processes are involved in maintaining cellular

homeostasis because they impact on functions such as nutrient recycling, energy generation and the clearance of damaged proteins and organelles (He et al., 2013).

1.5.2.4.1   Morphological aspects of cell death

Necrosis: This is characterised morphologically by cellular swelling and disruption of the plasma membrane (Figure 1.8), resulting in a rapid release of cytoplasmic content and massive inflammation in the physiological environment surrounding the dying cell (Han et al., 2008).

Apoptosis: This does not involve inflammation and it has distinct cellular morphological changes (Figure 1.8) (Kerr et al., 1972, Han et al., 2008). The early stage of apoptosis is identified by cell shrinkage and apoptotic cells characterised by their small size, dense cytoplasm and thin organelles (Elmore, 2007). During the intermediate stage of apoptosis, cell blebbing takes place, which occurs when a cell is starting to fragment into apoptotic bodies. In the final stage of apoptosis, cells break up and form apoptotic bodies (Elmore, 2007). Apoptosis also induces nuclear alterations such as nuclear fragmentation and chromatin condensation (Kerr et al., 1972, Galluzzi et al., 2015).

Autophagy: This is a lysosomal degradative process, known as "self-eating", characterised by the presence of autophagosomes in cells. It also plays a significant role as a tumour suppressive mechanism in cancer development and progression (Hippert et al., 2006). Autophagy can also lead to a caspase-independent form of programmed cell

death (Lin and Baehrecke, 2015). Induction of autophagic cell death by anticancer agents is also an important modality of cancer treatment (Mehrpour et al., 2013).
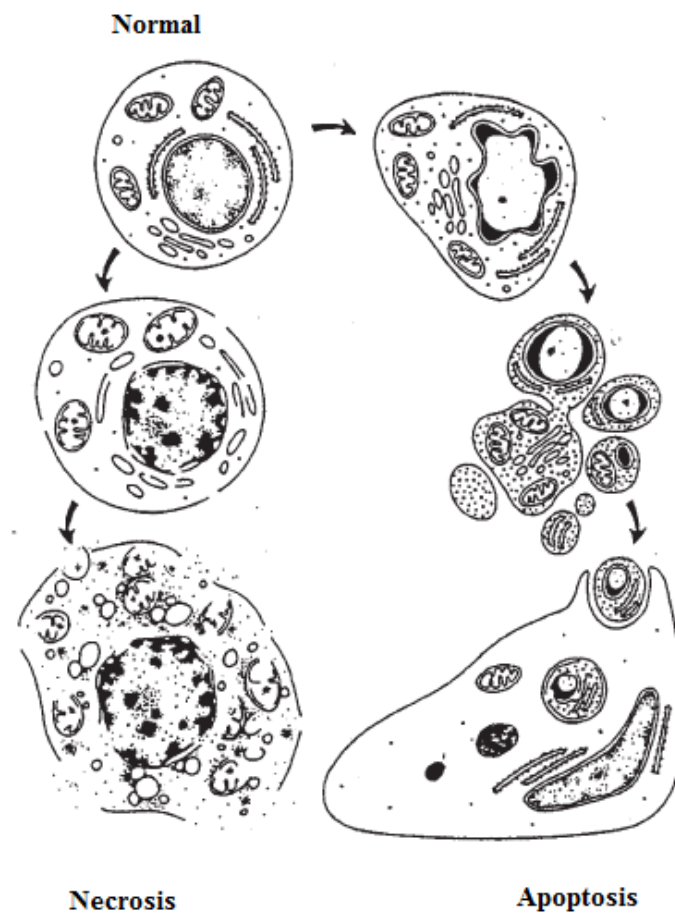
Figure 1.8: Morphologic features of apoptotic and necrotic cell death according to Kerr et al. (1995).

1.5.2.4.2   Biochemical aspects of apoptosis

Understanding the biochemical mechanisms of apoptosis is critical since it helps in the development of anticancer drugs that can target certain apoptotic genes or

pathways (Wong, 2011). Stimuli such as DNA damage, ER stress, nutrient starvation, reactive oxygen species (ROS) and activation of death receptors are able to activate molecular pathways leading to apoptosis (Galluzzi et al., 2009). For example, DNA damage can cause mitochondrial membrane permeabilisation (MMP) which can trigger apoptosis, necrosis and autophagic cell death (Galluzzi et al., 2009). Apoptosis cell death occurs through two pathways: the extrinsic cell receptor pathway and the intrinsic-mitochondrial pathway (Figure 1.9).

Extrinsic pathway: The cell-extrinsic pathway triggers apoptosis in response to engagement of cell death receptors; which are members of the tumour necrosis factor (TNF) receptor gene family (FAS, TNFR1, TRAILR), which bind to their respective ligands (e.g. FasL, TNF-alpha) (Ashkenazi, 2002). This results in recruitment of several proteins such as FAS-associated domain (FADD) protein (Mariño et al., 2014). Ligand-induced activation of cell-surface death receptors leads to rapid assembly of a death-inducing signalling complex (DISC) and activation of the apoptosis-initiating proteases caspase-8 and caspase-10. Then, these caspases activate the group of caspases responsible for causing cell death (caspase 3, 6 and 7); the latter can also be activated by the cell intrinsic pathway through caspase-9 (Mariño et al., 2014).

Intrinsic pathway: Most chemotherapy agents and irradiation treatments trigger tumour-cell apoptosis, through the cell-intrinsic pathway, as an indirect consequence of causing cellular damage. Stimuli for the intrinsic pathway include DNA damage, defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of survival factors or other types of severe cell distress (Ashkenazi, 2002). Initiation of this pathway requires p53 function but in cancer cells p53 is often inactivated and

dysfunctional, either directly through mutation of the TP53 suppressor gene or indirectly through p53 modulators (e.g. MDM2 protein). Induction of the p53 protein results in up-regulation of the oligomerisation of the BCL-2-associated X protein (BAX) and/or the BCL-2 antagonist or killer (BAK) proteins in the outer mitochondrial membrane (Adams and Cory, 2007). DNA damage also can stimulate the transactivation of genes encoding pro-apoptotic proteins (PUMA and NOXA) by p53 (Vousden and Lane, 2007), which can stimulate mitochondria to release apoptogenic factors such as cytochrome c and SMAC/DIABLO to the cytosol (Adams and Cory, 1998, Hunt and Evan, 2001). In the cytosol, cytochrome c binds and activates the adaptor protein APAF-1, forming an 'apoptosome' which activates the apoptosis-initiating protease caspase-9. In turn, caspase-9 activates the 'executioner' proteases caspase-3, 6 and 7. The mitochondrial SMAC/DIABLO proteins also promotes apoptosis by binding to the inhibitor of apoptosis proteins (IAP) preventing these factors from attenuating caspase activation (Mariño et al., 2014).
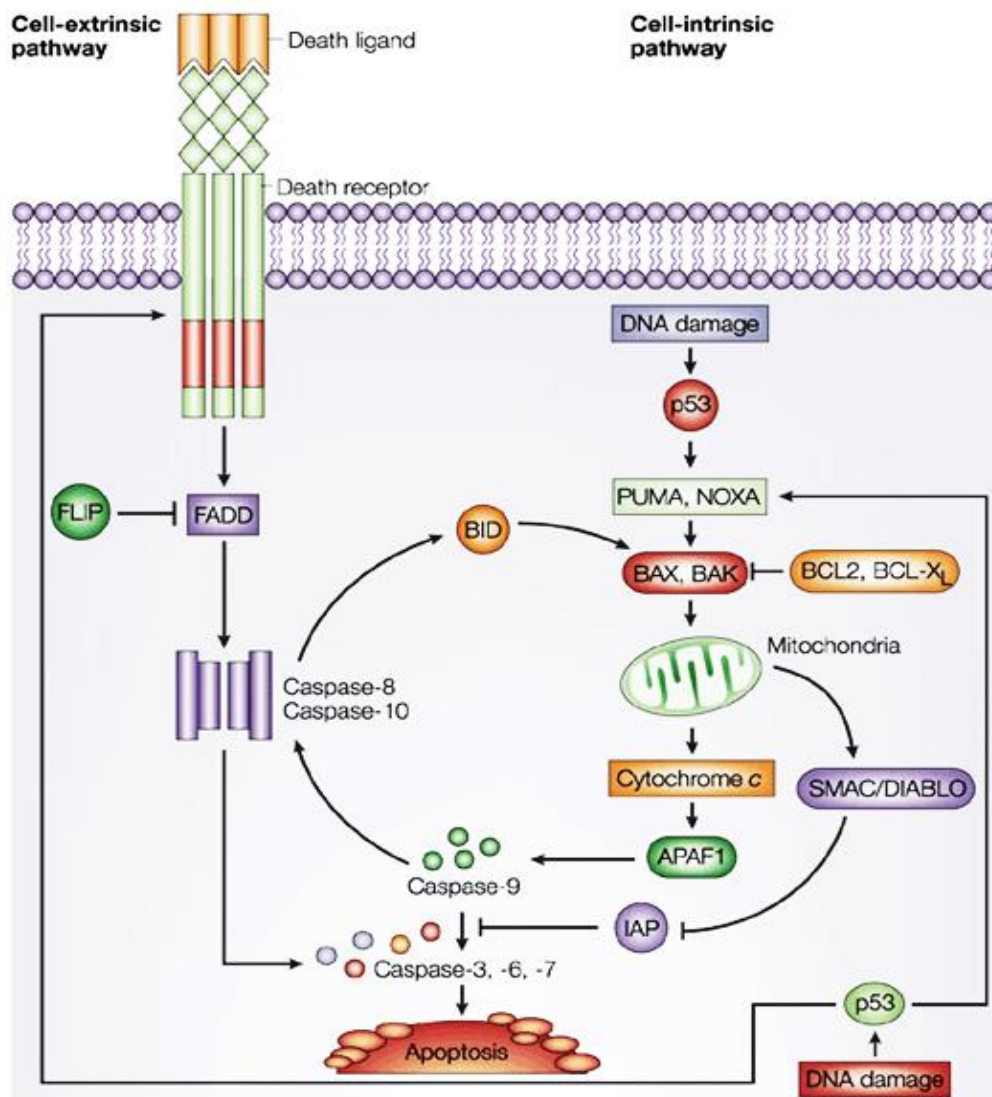
Nature Reviews | Cancer

Figure 1.9: Representation of the extrinsic and intrinsic pathways of apoptosis (Ashkenazi, 2002). FADD: FAS-associated domain, FLIP: Fas-associated death domain (FADD)-like interleukin 1b-converting enzyme (FLICE)-inhibitory protein, BAX: BCL-2-associated X protein, BAK: BCL-2 antagonist or killer, BID: BH3-interacting domain, PUMA: p53 up-regulated modulator of apoptosis, IAP: Inhibitor of apoptosis, APAF1: Apoptotic protease activating factor 1, NOXA: SMAC/DIABLO: Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI.

1.5.2.4.3 Analysis methods for cell death

There are different methods available for the detection of cell death *in vitro* (in cell cultures) and *in vivo* (in model organisms) relative to its morphological and biochemical characteristics (Figure 1.10) (Kroemer et al., 2009). Commonly used cell death analysis techniques include; microscopy (light, electron and fluorescence), immune-blotting, cytofluorometry and luminometry (Kroemer et al., 2009) (Figure 1.10). These experimental procedures depend on distinct technologies, which can be distinguished by specificity (techniques selective to apoptosis versus autophagy), sensitivity (lower detection limit), detection range (upper detection limit), precision (qualitative or quantitative) and throughput (e.g. low for electron microscopy-based methods versus high for automated procedures) (Philpott et al., 1996, Kroemer et al., 2009, Wong, 2011, Galluzzi et al., 2015). All these methods are prone to false-negative and/or false-positive results as well as misinterpretations (Kroemer et al., 2009) and hence, one method only cannot be used to confirm the cell death mode. This can be avoided by using at least two distinct methods that assess 1) the end-stage cell death, and 2) the mechanism of the cell death (Galluzzi et al., 2015).
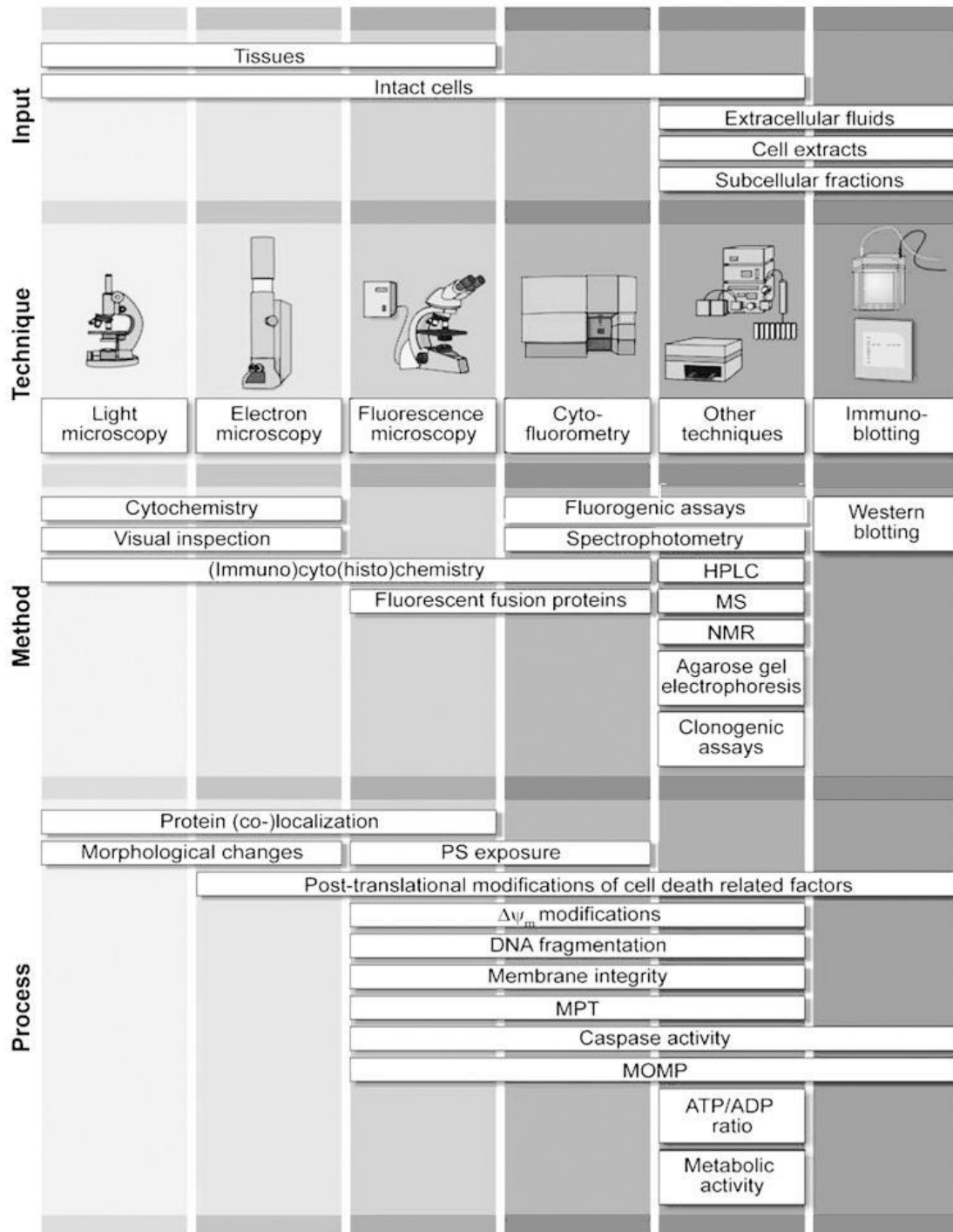
Figure 1.10: Methods used for the analysis of cell death. $\Delta\psi$m: mitochondrial transmembrane potential, HPLC: high-pressure liquid chromatography, MOMP: mitochondrial outer membrane permeabilisation; MPT, mitochondrial permeability transition, MS: mass spectrometry, NMR: nuclear magnetic resonance, PS: phosphatidylserine, SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Figure adapted from Kroemer et al. (2009).

1.5.2.5   Current cancer therapy

Successful cancer therapy requires selective killing of cancer cells with low toxicity to normal cells. Current cancer therapies, including surgery, radiation therapy, chemotherapy and immunotherapy, have developed rapidly over the past decade (Urruticoechea et al., 2010). However, side effects are often associated with existing therapies due to random cytotoxic effects on both cancer and normal cells. Although treatments have emerged (Urruticoechea et al., 2010, Zhao et al., 2013) that have reduced mortality by 20% between 1991 and 2010 period (Siegel et al., 2014), globally there is still a growing incident of cancers, indicating a lapse in current strategies. Therefore, there is still a need for less toxic and more potent anticancer agents for the treatment of cancer.

1.5.2.6   Natural health products in cancer

Natural products serve as both chemotherapeutic and chemo-preventive agents (Newman and Cragg, 2012). They are secondary metabolites and can be divided into four main groups based on the source from which they are derived: 1) microbial, 2) marine 3) animal and 4) plant (Harvey, 2008). More than 50% of the U S Food and Drug Administration (FDA) approved anticancer drugs are either natural products or their derivatives (Newman and Cragg, 2012). For example, a total of 25 natural product-derived drugs were approved for marketing from January 2008 to December 2013 (Butler et al., 2014).

Plant-derived products have been used to prevent and to treat various diseases for thousands of years and local and traditional knowledge has been the starting point for the development of many successful anticancer drugs (Sumner et al., 2015). More than 80% of the world population consider traditional plant-derived medicine as their source of primary health care (Bhanot et al., 2011, Figueroa et al., 2012). Several plant-derived compounds are currently employed in cancer treatment or are undergoing clinical trials (Table 1.11). Identification of potential candidates for plant-derived chemotherapy agents then allows for refinement through molecular structure and function research to optimise the efficacy of these phytochemicals (Gullett et al., 2010).

Plant derived health products are useful in chemoprevention because they possess bioactive phytochemicals (non-nutritive compounds) with health benefits and can be obtained through functional foods (Surh, 2003, Gullett et al., 2010). Functional foods with chemo-preventive phytochemicals and their mechanism of action have been extensively reviewed in the literature (Table 1.12). One such chemo-preventive agent is lycopene (Lin et al., 2011). Since *M. cochinchinensis* possesses the highest amount of lycopene of all known fruits and vegetables (Vuong et al., 2006), it should also be useful as a functional food but systematic research is necessary to test this hypothesis.

Table 1.11: Chemical classes of plant-derived compounds that are currently used in cancer treatment.

Class	Plant name	Drug	Trade name	Cancer used	Clinical trial	Reference
Vinca alkaloid	*Catharanthus roseus*	Vinblastine	Alkaban-AQ®, Velban®	Breast, lymphoma, germ-cell and renal cancer	Phase III/IV	(Gigant et al., 2005)
		Vincristine	Oncovin®	Leukaemia, lymphoma, breast, lung, paediatric solid cancers and others	Phase III/IV	(Mohammadgholi et al., 2013, Lu et al., 2003)
Taxane	*Taxus brevifolia*	Paclitaxel	Taxol®	Ovary, breast, lung, bladder, and head and neck cancer	Phase III/IV	(Rowinsky and Donehower, 1995, Malik et al., 2011)
		Docetaxel	Taxotere®	Breast cancer, lung cancer	Phase III/IV	(Montero et al., 2005)
Quinoline alkaloid	*Camptotheca acuminata*	Topotecan	Hycamtin ®	Ovarian, lung and paediatric cancer	Phase II/III	(Riemsma et al., 2010)
		Irinotecan	Campto ®	Colorectal and lung cancer	Phase II/III	(Xu and Villalona-Calero, 2002)
Acridone alkaloid	*Acronychia baueri*	Acronyciline	Experimental	Experimental	Phase II/III	(Nguyen et al., 2009)

Table 1.12: Dietary phytochemicals that possess chemo-preventive potential and their dietary sources.

Functional phytochemicals	Scientific name	Common name	Reference
6-Gingerol	*Zingiber officinale*	Ginger	(Shukla and Singh, 2007)
Capsaicin	*Capsicum annuum*	Chilli peppers	(Bley et al., 2012)
Curcumin	*Curcuma longa*	Turmeric	(Bar-Sela et al., 2010)
Diallyl sulfide	*Allium sativum*	Garlic	(Nicastro et al., 2015)
Epigallocatechin-3-gallate	*Camellia sinensis*	Green tea	(Boehm et al., 2009)
Genistein	*Glycine max*	Soy beans	(Li et al., 2012c)
Indole-3-carbinol	*Brassica oleracea* var. capitata	Cabbage	(Weng et al., 2008)
Lycopene	*Solanum lycopersicum*	Tomato	(Bhuvaneswari and Nagini, 2005)
Resveratrol	*Vitis vinifera*	Grapes	(Kaur et al., 2009)
Sulphoraphane	*Brassica oleracea* var. italica	Broccoli	(Gupta et al., 2014)

1.5.3   Anticancer activity of *M. cochinchinensis*

M. cochinchinensis aril has been shown to be effective as an anticancer agent against colon cancer (Tien et al., 2005). A water extract of the aril caused necrotic cell death in the colon 26-20 cell line through down-regulation of proteins involved in the G1-S phase of the cell cycle (cyclinA, Cdk2, p27waf1/Kip1) (Pucci et al., 2000). The compound responsible for anticancer activity was an unidentified 35 kDa protein (Tien et al., 2005). However, bio-activity of *M. cochinchinensis* aril on other cancer types, including melanoma and breast cancer, is unknown.

M. cochinchinensis seed water extracts have been reported to be effective agents against melanoma, lung, breast and gastric cancer cells (Table 1.13). Ethanol extracts of the seeds also showed anticancer activity on lung, breast, oesophageal carcinoma and melanoma cell lines (Zhao et al., 2010, Liu et al., 2012). Ethyl-ester extracts of the seeds suppressed cell growth and differentiation of mouse melanoma cells (B16F1) at a relatively low concentration of the extract (5 µg/mL) (Zhao et al., 2012). Furthermore, the water extract of the seeds had anticancer activity on the MDA-MB-231 breast carcinoma cell line (Meng et al., 2012). The bioactivity might be due to the compounds reported in the literature (Table 1.10) such as momordin (Tang and Eisenbrand, 1992), oleanolic acid (Murakami et al., 1966), trypsin inhibitors (Huang et al., 1999, Heitz et al., 2001, Tsoi et al., 2004, Wong et al., 2004) or tri-terpenoid esters (De Shan et al., 2001) but this has not yet been determined. Furthermore, the mechanism and mode of action of these compounds are not known.

Table 1.13: Anticancer activity of aril and seed extracts of *M. cochinchinensis*.

Fruit part	Extract	Effective concentration mg/mL	Cell lines tested	References
Aril	Water	1.25	Colon 26-20 cell line *in vivo* and *in vitro*	(Tien et al., 2005)
Seed	Water	10-100*	Lung (a549), breast (MDA-MB-321), oesophageal (TE-13) carcinoma cell lines and melanoma cell line (B16)	(Zhao et al., 2010)
	Ethyl ester	0.05	Melanoma cell line B16F1 (mice)	(Zhao et al., 2012)
	Ethanol	0.4	Breast cancer cell line MDA-MB-321	(Meng et al., 2012)
	Ethanol	0.8	Human gastric cell lines (SGC7901, MKN-28)	(Liu et al., 2012)
	Water	0.05	Breast cancer cell line ZR-75-30	(Zheng et al., 2014)

*: Varied between cell lines.

1.6   Justification and aims of the study

Momordica cochinchinensis (Cucurbitaceae) is native to South East Asia and known for its nutritional and medicinal properties. The plant was previously widespread throughout Asia and is now restricted to specific regions in Vietnam, Thailand and India (Behera et al. 2011a). *M. cochinchinensis* is genetically and morphologically diverse but there is little information available on the extent of this diversity. This lack of information available on the morphological and genetic variation of this species limits selective germplasm and cultivar collection for high aril yield, nutrition (increased amount of lycopene and β-carotene) or anticancer activity.

The aril of *M. cochinchinensis* is the richest source of lycopene and β-carotene with 70 times greater lycopene content than tomato and five times greater β-carotene content than carrots (Aoki et al., 2002b, Vuong et al., 2006). The genotype and environmental influences on lycopene and β-carotene concentration were evident in other carotenoid-rich fruits including tomatoes (Kuti and Konuru, 2005, Aherne et al., 2009), mangoes (Mercadante and Rodriguez-Amaya, 1998) and carrots (Nicolle et al., 2004) but the effects are unknown on *M. cochinchinensis*. This information is essential for the selection of the best cultivars for breeding and to determine the most suitable eco-geographical factors leading to high lycopene and β-carotene synthesis and accumulation.

The aril of *M. cochinchinensis* possessed anticancer activity against colon cancer with the active compound identified as a 35 kDa protein causing necrotic cell death (Tien et al., 2005) but the bioactivity on other cancer types, including breast cancer and melanoma is unknown. The variation in genotypes and environmental profiles likely influence secondary metabolite production and anticancer activity. However, the influences of these parameters are unknown for *M. cochinchinensis*. Filling these knowledge gaps will improve the applications of *M. cochinchinensis* in the agricultural, nutraceutical and medicinal industries. Therefore, this study aimed to investigate the genetic diversity, nutritional and biological activity of *M. cochinchinensis* and its effect on the end produce as a nutritionally and bio-active functional food.

The specific aims of this thesis were to:

1. Determine the morphological and genetic diversity of *M. cochinchinensis* collected from Thailand, Vietnam and Australia **(Chapter 2)**,

2. Compare the carotenoid concentration (lycopene and β-carotene) of *M. cochinchinensis* with other common lycopene and β-carotene containing fruits and vegetables **(Chapter 3)**,

3. Compare the carotenoid concentration of the aril of *M. cochinchinensis* using UPLC, UV-visible spectrophotometry and colorimetry methods with the standard HPLC method **(Chapter 3)**,

4.   Determine the most nutritious (higher lycopene and β-carotene) aril sample of *M. cochinchinensis* collected from Thailand, Vietnam and Australia **(Chapter 4)**,

5.   Determine the cytotoxic effect of water and hexane based aril extracts of *M. cochinchinensis* on breast cancer (MCF7 and BT474) and melanoma (MM418C1 and D24) cells **(Chapter 5)**,

6.   Determine the mechanism of cell death on the treated cancer cells using morphological and biochemical assays **(Chapter 5)**, and

7.   Determine the most bio-active aril sample of *M. cochinchinensis* collected from Thailand, Vietnam and Australia against breast cancer and melanoma cells and provide recommendations for the future direction of this industry **(Chapter 5)**.

CHAPTER 2

Morphological and genetic diversity of *Momordica cochinchinensis* in Thailand, Vietnam and Australia

2.1 Introduction

The genus *Momordica* (Cucurbitaceae) comprises 59 species, of which 47 are found in Africa and 12 in Southeast Asia (De Wilde and Duyfjer, 2010, Schaefer and Renner, 2010). *Momordica cochinchinensis* (Lour.) Spreng. has the common name of Teruah throughout Asia (De Wilde and Duyfjer, 2010) and is locally named as Red Gac (Vietnam), Fak Kao (Thailand), Mu Bei Zi (Beijing, China), Mak Kao (Laos) and Bhat Kerala (India) (Kubola and Siriamornpun 2011). *M. cochinchinensis* was previously widespread throughout Asia and it is now restricted to specific regions (Behera et al., 2011a). As described in chapter 1, the plant is a perennial dioecious vine with 3-5 palmately lobed leaves and white to ivory yellow flowers (Bharathi and John, 2013). Fruits are large (12-17 cm in diameter) and vary in shape (globose and oval) (Bharathi and John, 2013). Young fruits are green in colour and turn orange-red to dark red with maturity (Bharathi and John, 2013). Seeds are large, ovoid compressed with a wavy outline, black-brown with irregularly sculptured seed coat covered with red aril (Bharathi and John, 2013).

All parts of the *M. cochinchinensis* fruit are used, including the seeds and aril as oils (Vuong and King, 2003, Kubola and Siriamornpun, 2011), food additives (Vuong, 2004) and beverages (Kubola and Siriamornpun, 2011, Bootprom et al., 2012). Seeds and aril have anti-inflammatory (Kha et al., 2013a) as well as antioxidant and anticancer activities (Tien et al., 2005). The aril contains the highest amount of β-carotene and lycopene of any known lycopene/carotene rich fruits and vegetables (Aoki et al., 2002, Vuong, 2004).

M. cochinchinensis is underutilised despite its known nutritional and medicinal value (Sanwal et al., 2011). There are only a few studies conducted on crop development *in situ* and *ex situ* (Sanwal et al., 2011, Parks et al., 2013) and conservation by germplasm collection (Behera et al., 2011a). There has been only one study on the genetic diversity of collected germplasm from Thailand and Vietnam (23 and 2 representatives, respectively) (Bootprom et al., 2012). High genetic diversity was observed within the Thailand samples; however, the extent of the morphological and genetic diversity of *M. cochinchinensis* between Thailand and Vietnam was not analysed. The lack of information available on the morphological and genetic variation of *M. cochinchinensis* in its wide natural habitats limits selective germplasm collection and cultivar selection for either high aril yield or high nutrition (increased amount of lycopene and β-carotene).

Variation of fruit morphology is common in the Momordica genus (Bharathi and John, 2013); however, the extent of the variation observed in *M. cochinchinensis* based on where it is grown is unknown. Previously, in India, 60 accessions each of *M. dioica* roxb., *M. subangulata Blume subsp. Renigera* (G. Don) de Wilde and eight accessions of *M.*

cochinchinensis were studied for morphological variability (Bharathi and John, 2013). This led to the identification of two high fruit yielding varieties of the first two species for commercial propagation but not for *M. cochinchinensis* (Bharathi and John, 2013). Therefore, improved understanding of the morphological and genetic diversity within *M. cochinchinensis* in its natural habitats will be important for plant selection in agricultural applications, strategic germplasm collection and for the conservation of the species.

The aims of this chapter were to investigate the morphological diversity and the genetic diversity of *M. cochinchinensis* collected from natural habitats in Thailand and Vietnam as well as a recently cultivated variety from Australia.

2.2   Materials and Methods

2.2.1   Sample collection and eco-geographical data

Fruit, seeds and leaves of 53 samples of *M. cochinchinensis* were collected from Thailand, Southern Vietnam, Central Vietnam and Northern Vietnam (Tables 2.1 and 2.2). All the samples were collected by Dr Tien Huynh from their natural habitats, as reported by locals, during harvesting season (December-February) (Figure 2.1, Tables 2.1 and 2.2). All the fruits collected were at the same maturity stages of ripeness with orange or red coloured skin with soft texture. A recently cultivated variety from Australia (A1-A12) was provided by the Department of Primary Industries; NSW and used for comparison (Tables 2.1 and 2.2). After collection, all the fruits were cleaned; the aril was separated from the seeds,

transported to RMIT University in an insulated bag, separated into aliquots, covered with aluminium foil and stored at -20°C in darkness until required. Leaves of *M. cochinchinensis* were air dried and stored at 4°C until used for DNA extraction. Leaves of *M. charantia* (MC) were purchased from the Springvale market, Melbourne, Australia, air dried and stored at 4°C and used as an out-group for molecular comparison.

The geographical (altitude, latitude and longitude) and ecological (rainfall of wettest and driest month, observed minimum and maximum temperature, annual temperature range) data were obtained for each province using the DIVA-GIS spacial analysis software (Hijmans et al., 2004) (Table 2.1).
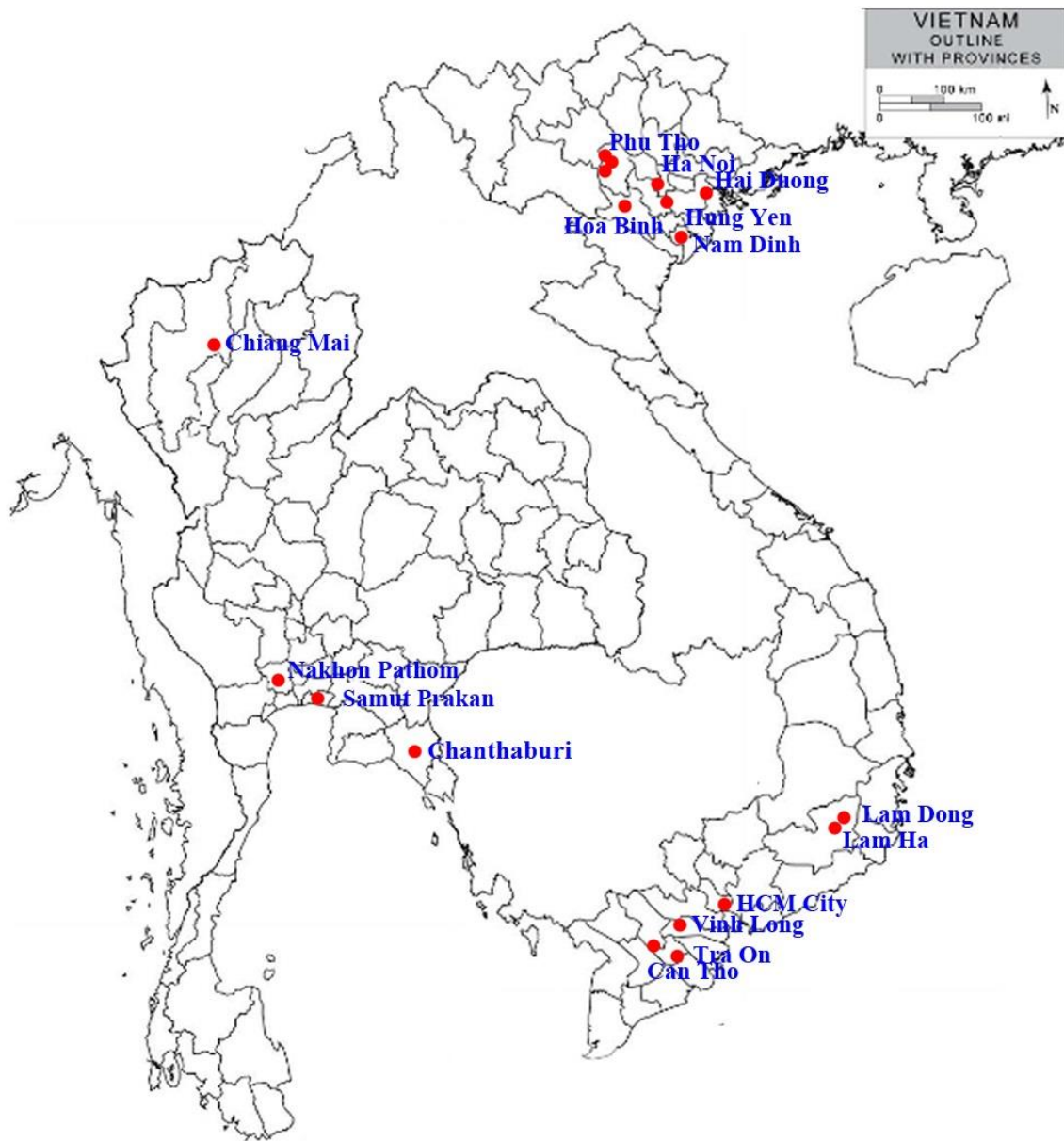
Figure 2.1: Geographical locations of *M. cochinchinensis* collected from Thailand and Vietnam. Figure obtained from DIVA-GIS spacial analysis software (Hijmans et al., 2004).

Table 2.1: *M. cochinchinensis* samples analysed based on country, province, latitude, longitude and bio-climate. Bioclimatic data of temperature and precipitation and altitude was obtained from DIVA-GIS spacial analysis software.

Country	Province	Sample number	Latitude	Longitude	Elevation (m)	Temperature °C			Precipitation (mm)			Climate[*]
						Maximum	Minimum	Annual range	Annual	Wettest month	Driest month	
S Vietnam	Can Tho	VS1	10.034320	105.775130	1	32.6	22.2	10.4	1524	254	2	Tropical
	Can Tho	VS2	9.996850	105.665870	3	32.4	22.0	10.4	1623	269	3	Tropical
	Vinh Long	VS3a,3b	10.248758	105.969566	2	33.2	21.9	11.3	1413	244	2	Tropical
	Tra On	VS4a,4b	9.965880	105.925140	3	32.5	22.1	10.4	1520	259	2	Tropical
	HCM City	VS7	10.757410	106.673439	6	34.7	21.1	13.6	1851	310	2	Tropical
N Vietnam	Hung Yen	VN8	20.814159	106.125992	4	33.1	14.4	19	1679	316	15	Temperate
	Hung Yen	VN9a,9b	21.648399	105.028999	71	32.2	11.6	20.6	1774	325	18	Temperate
	Hai Duong	VN10	20.897711	106.423561	3	32.8	14.2	18.6	1648	311	17	Temperate
	Hai Duong	VN11	20.919382	106.419062	3	33	13.5	19.5	1692	312	14	Temperate
	PhuTho	VN12	21.331640	104.953453	296	30.8	10.5	20.3	1574	300	10	Temperate
	PhuTho	VN13-15	21.445990	105.094719	38	32.3	11.3	20.9	1734	315	19	Temperate
	Ha Noi	VN16-20	20.866699	105.750000	9	33	13.5	19.5	1700	314	14	Temperate
	Ha Noi	VN21	20.985260	105.858501	9	33.1	13.5	19.6	1673	324	15	Temperate
	HoaBinh	VN22,23	20.829601	105.332893	195	31.6	11.7	19.9	1801	352	8	Temperate
	Nam Dinh	VN24-27	20.422930	106.173630	4	33.4	14.7	18.7	1663	336	27	Temperate
C Vietnam	Lam Dong	VC28-30	11.835772	108.336275	1112	27.9	13.3	14.6	1651	248	7	Temperate
	Lam Ha	VC31-32	11.793576	108.268610	925	28.1	13.8	14.3	1658	252	8	Temperate
Thailand	Nakhon Pathom	T1-T4	13.828790	100.039620	8	35.9	18.7	17.2	1237	257	6	Tropical
	Samutprakan	T5	13.576110	100.665420	2	34.5	20.4	14.1	1439	325	10	Tropical
	Chiang Mai	T6,T8	18.767000	99.026161	316	36.2	12.9	23.3	1163	244	7	Tropical
	Canthaburi	T7	12.878721	102.129135	79	32	17.4	14.6	1751	316	10	Tropical
Australia[a]	Newcastle	A1-A12	33.212281	151.225069	NA	25	18.0	NA	NA	NA	NA	Tropical

[*]: Regions with a minimum temperature < 15°C in coldest month was selected as temperate climate.

NA: Information not available, [a]: Grown in greenhouse conditions

2.2.2 Morphological characteristics

Only 38 *M. cochinchinensis* samples were used for morphology analysis since they had complete information of the fruit, seed and leaf data (Table 2.2 and Figure 2.2 A-G). Fruits were categorised as globose (round at both ends), globose-oval (round at the bottom and pointed at the top), oval (pointed at top and bottom) (Behera et al., 2011a) as well as tapered (pointed at top and bottom but elongated) (Figure 2.2 D). Fruit surface was categorised based on the spike density as being dense, medium or sparse (Figure 2.2 A-C). All seeds from each fruit were cleaned of aril and their weight and length recorded. Seed colour was categorised as blackish brown, brown or light brown (Figure 2.2 E). Leaves were categorised based on the number of lobes as three and five lobed (Figure 2.2 F-G). These morphological characters were assigned a code (Appendix 1) and used in UPGMA cluster analysis as described in section 2.3.1.

Table 2.2: List of *M. cochinchinensis* samples used in this study: Sample number, region of collection, fruit, seed and leaf morphology. Seed measurements are averages ± SD.

Sample	Collection date	Region Province	Fruit[b] Shape	Spike density	Seed[b] Weight (g)	Length (mm)	Number	Colour	Leaf[a] Lobes
VS1	10.12.11	Can Tho	Globose-oval	Dense	4.64±0.63	32.89±2.39	8	Blackish-brown	3
VS2	11.12.11	Can Tho	Globose-oval	Dense	2.47±0.17	27.08±1.01	7	Blackish-brown	3
VS3a	12.12.11	Vinh Long	Globose	Dense	1.89±0.74	25.98±1.11	7	Blackish-brown	3
VS3b*	12.12.11	Vinh Long			Not available				
VS4a	14.12.11	Tra On	Globose-oval	Dense	2.00±0.22	25.76±1.28	11	Blackish-brown	3
VS4b	14.12.11	Tra On			No fruit				5
VS7	16.12.11	HCM City	Globose-oval	Dense	2.61±0.28	28.80±1.95	15	Blackish-brown	3
VN8	21.12.11	Hung Yen	Tapered	Medium	4.19±0.46	32.96±2.53	13	Brown	3
VN9a	21.12.11	Hung Yen	Globose	Medium	3.27±0.19	28.35±1.69	21	Blackish-brown	3
VN9b	21.12.11	Hung Yen	Globose-oval	Medium	2.24±0.25	23.81±1.76	36	Blackish-brown	3
VN10	21.12.11	Hai Duong	Tapered	Medium	2.88±0.17	28.70±2.33	33	Blackish-brown	3
VN11	21.12.11	Hai Duong	Tapered	Medium	2.05±0.40	26.63±1.34	25	Brown	3
VN12	22.12.11	Phu Tho	Oval	Medium	4.37±0.80	34.84±1.45	17	Light brown	3
VN13	22.12.11	Phu Tho	Globose-oval	Medium	2.96±0.16	25.62±1.53	33	Brown	3
VN14	22.12.11	Phu Tho	Globose-oval	Sparse	2.77±0.29	25.61±1.54	28	Brown	3
VN15	22.12.11	Phu Tho	Globose-oval	Medium	2.15±0.20	23.65±1.09	23	Blackish-brown	3
VN16	20.12.13	Ha Noi	Oval	Dense	2.53±0.23	28.79±2.16	54	Blackish-brown	3
VN17	20.12.13	Ha Noi	Globose-oval	Medium	2.89±0.53	28.00±3.47	25	Blackish-brown	3
VN18	20.12.13	Ha Noi	Globose-oval	Dense	2.85±0.26	27.94±2.37	34	Blackish-brown	3
VN19	20.12.13	Ha Noi	Oval	Dense	2.59±0.54	26.50±2.70	22	Blackish-brown	3
VN20	20.12.13	Ha Noi	Globose-oval	Dense	2.58±0.20	26.21±1.71	33	Blackish-brown	3
VN21	20.12.13	Ha Noi	Globose-oval	Medium	3.13±0.18	28.71±1.45	21	Blackish-brown	3
VN22	21.12.13	Hoa Binh	Oval	Medium	2.05±0.29	25.90±1.91	42	Blackish-brown	3
VN23	21.12.13	Hoa Binh			No fruit				3

Table 2.2 (Continued): List of *M. cochinchinensis* samples used in this study: Sample number, region of collection, fruit, seed and leaf morphology. Seed measurements are averages ± SD.

Sample	Collection date	Region Province	Fruit[b] Shape	Spike density	Seed[b] Weight (g)	Length (mm)	Number	Colour	Leaf[a] Lobes
VN24	24.12.13	Nam Dinh	Tapered	Medium	1.75±0.20	25.70±1.66	34	Blackish-brown	3
VN25	24.12.13	Nam Dinh	Tapered	Medium	1.82±0.45	26.28±1.60	38	Blackish-brown	3
VN26	24.12.13	Nam Dinh	Tapered	Sparse	3.64±0.24	31.89±1.84	37	Blackish-brown	3
VN27	24.12.13	Nam Dinh	Tapered	Sparse	1.97±0.44	25.55±2.54	30	Blackish-brown	3
VC28	26.12.13	Lam Dong	Globose-oval	Medium	3.95±0.26	23.06±1.59	34	Blackish-brown	3
VC29	26.12.13	Lam Dong	Oval	Medium	3.94±0.30	31.90±1.59	34	Blackish-brown	3
VC30	26.12.13	Lam Ha	Globose-oval	Medium	3.63±0.48	30.05±3.08	34	Blackish-brown	3
VC31	26.12.13	Lam Ha			No fruit				3
VC32	26.12.13	Lam Ha	Globose-oval	Medium	3.69±0.31	28.66±1.76	30	Blackish-brown	3
T1	27.01.13	Nakhom Pathom	Oval	Dense	1.05±0.14	20.10±1.33	29	Brown	3
T2	27.01.13	Nakhom Pathom			No fruit				3
T3	27.01.13	Nakhom Pathom	Oval	Dense	1.59±0.09	23.73±2.01	22	Brown	3
T4	27.01.13	NakhomPathom	Oval	Dense	1.64±0.07	21.41±0.87	12	Brown	3
T5	27.01.13	Samut Prakan	Globose	Dense	0.88±0.89	18.03±1.14	54	Brown	3
T6	01.02.13	Chang Mai	Oval	Dense	1.68±0.15	23.18±1.16	43	Light brown	-
T7	05.02.13	Canthaburi	Oval	Dense	1.86±0.19	24.60±1.82	49	Brown	-
T8	22.01.14	Chang Mai	Oval	Dense	1.44±0.30	21.47±1.75	21	Light brown	3
A1	15.07.13	Newcastle			No fruit				3
A2	15.07.13	Newcastle			No fruit				3
A3[c]	15.07.13	Newcastle	Globose-oval	Dense	3.44±0.46	29.57±1.94	25	Blackish-brown	3
A4[c]	15.07.13	Newcastle	Globose-oval	Dense	2.58±0.37	29.42±1.90	35	Blackish-brown	3
A5-12*	27.05.13	Newcastle			Not available				

*: Aril was available but no fruit, seed and leaf data was available.

[a]: Leaves were used to extract DNA therefore only 42 samples were used for DNA extraction.

[b]: 38 samples used for morphology analysis

[c]: Aril was not available (seed data was provided by Department of Primary Industries, NSW, Australia)
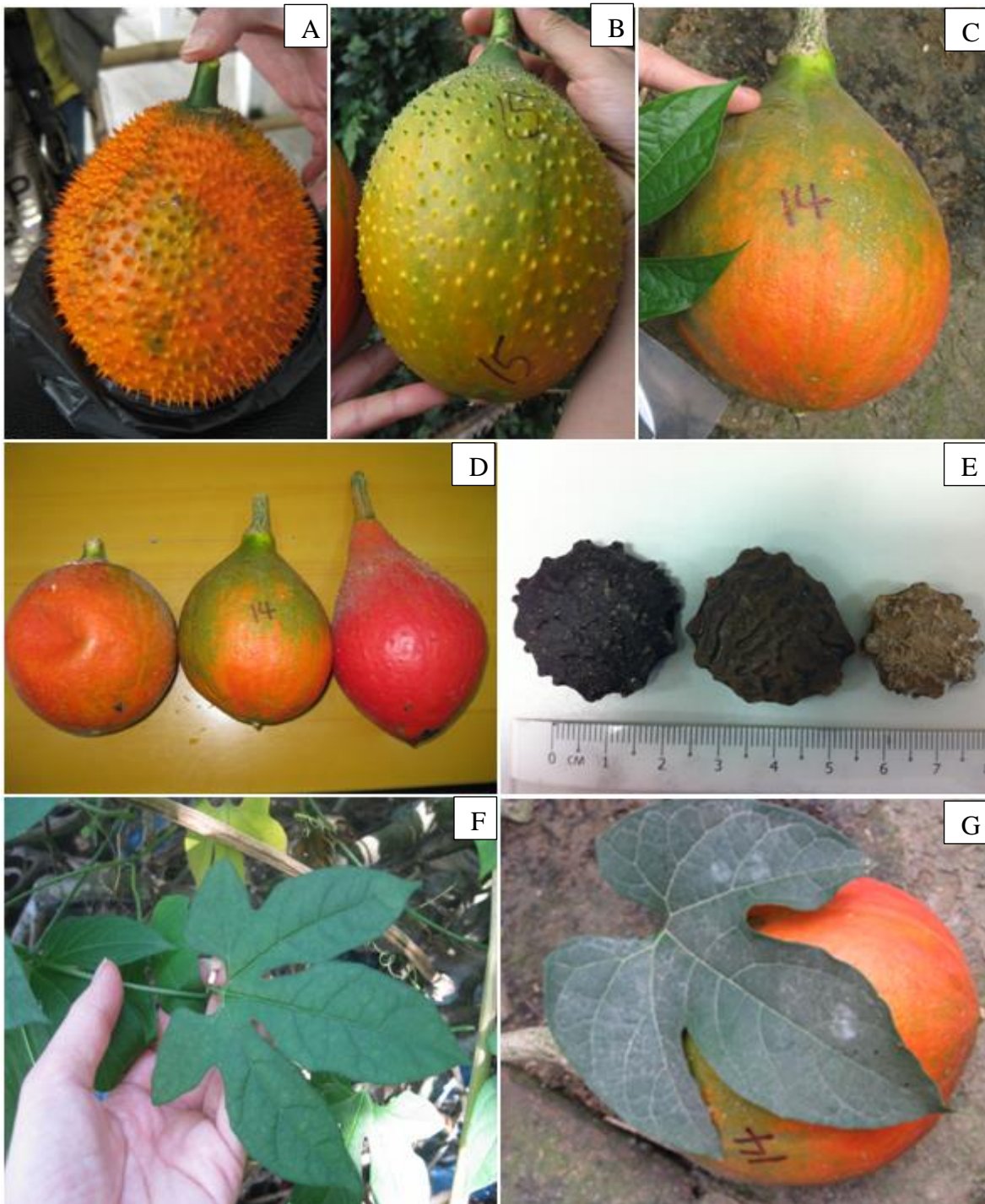
Figure 2.2: Fruit, seed and leaf morphology of *M. cochinchinensis*. A-C: Spike density of fruits categorised as dense (A), medium (B) and sparse (C). D: Fruit shapes categorised as round (left), globose-oval (centre) or tapered (right). E: Colour and size variations of mature seeds categorised as black- large (left), dark brown- large (centre), light brown-small (right). F-G: Leaf differences between five (F) and three lobes (G).

2.2.3 DNA isolation of *Momordica* samples

Out of the 53 samples collected, only 42 samples had leaves therefore those samples were used in DNA extraction (Table 2.2). DNA was extracted from dried leaves using the Favor Prep^{TM} Plant Genomic DNA Extraction Mini Kit (Favorgen, Taiwan) according to the manufacturer's instructions (Appendix 2). This extracted DNA was quantified against a GeneRuler^{TM} DNA Ladder Mix (Appendix 3) (Fermentas, Germany) by electrophoresis of 2 µL of the DNA extract with 2 µL gel loading dye (Fermentas, Germany) on a 1.4% (w/v) agarose (Bioline, Australia) gel in Tris-Borate- EDTA (TBE) buffer (1X) at 100 V for 1 hour and the gel was post-stained with 50 ng/L ethidium bromide. Bands were visualised over ultraviolet light in a Gel Doc^{TM}XR+ System with Image Lab^{TM}Software (Bio Rad, Australia). Extracted DNA was also stored at -20°C until required for RAPD and ISSR analysis.

2.2.4 Randomly Amplified Polymorphic DNA (RAPD)

Screening was performed on five randomly selected *M. cochinchinensis* samples (VS1, VS2, VS4, VN8, VN9a, A1) with 18 OPA primers along with another primer (OPW3), which was known to react specifically with *M. cochinchinensis* (Bootprom et al., 2012). Of the 19 primers tested, eight primers (OPA2, OPA7, OPA11, OPA13, OPA9, OPA18, OPA15 and OPW3) reacted and were further amplified with all *M. cochinchinensis* samples. The OPA9, OPA11, OPA13, OPA18 and OPW3 amplifications (Table 2.3) were

found to be more informative because they produced clear, distinct, non-smearing, polymorphic banding patterns and as a result they were used in cluster analysis.

For the above primer selection, amplification was performed as previously described (Sikdar et al., 2010) in a G-Storm (Gene Technologies, England) PCR system using a total reaction volume of 25 µL containing 12.5 µL Green Taq (Promega, USA), 1 µL primer (5 µM), 1 µL DNA (6 ng/µL) and 10.5 µL nuclease-free H_2O. Amplification conditions were as follows: initial denaturation at 94°C for 4 min followed by 45 cycles of 94°C for 1 min, 32-37°C for 1 min (Table 2.3), 72°C for 1 min and final extension at 72°C for 5 min. Amplified products were run on agarose gel and imaged as described.

Table 2.3: Details of sequence, annealing temperature, total bands and polymorphic bands of RAPD and ISSR primers used in analysis.

Primer	Sequence 5' to 3'	Annealing temperature ^{o}C	Total bands	No of polymorphic bands	Percentage of polymorphism
RAPD					
OPA09	GGGTAACGCC	37	19	19	100
OPA11	CAATCGCCGT	37	19	18	94.7
OPA13	CAGCACCCAC	37	18	18	100
OPA18	AGGTGACCGT	35	4	4	100
OPW03	GTCCGGAGTG	32	20	20	100
Total			80	79	98.7
ISSR					
$(GACA)_4$		55	20	20	100
$(GTG)_5$		55	25	25	100
Total			45	45	100

2.2.5   ISSR amplification

Screening was performed on six randomly selected samples (VS1, VS2, VN9a, VN10, VN11 and A1) of *M. cochinchinensis* with 4 ISSR primers. Only two of the four ISSR primers, $(GACA)_4$ and $(GTG)_5$, produced clear, distinct, reproducible banding patterns so these were selected for use on all the samples (Table 2.3). Amplification was as before with primers substituted using the cycle parameters as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 7 min and the amplified products were separated as described in section 2.2.4.

2.2.6   Data analysis

2.2.6.1 Morphology data analysis

38 samples which had all the information on fruit, seed and leaf morphology was used in analysis (Table 2.2). Seed weight and length were analysed using the Minitab version 16 statistical software. For normally distributed data, a one-way analysis of variance (ANOVA) was conducted and the least significant differences between the means of $p < 0.05$ were determined using Fisher's post-hoc test. Non-normally distributed data were analysed using the Kruskal Wallis test or $\log_{10}$ transformed and the significance difference between the means were determined using Fisher's post-hoc test. Statistical values of $p \leq 0.05$ were considered as being significant. A similarity matrix using

unweighted pair group method with arithmetic mean (UPGMA) for cluster analysis of morphological data (seed weight, length, colour, number of seeds, fruit shape, spike density and number of lobes per leaf) was constructed using NTSYSpc version 2.1 (Rohlf, 1992).

2.2.6.2 Molecular data analysis

Each fragment amplified with the two microsatellite primers, $(GACA)_4$ and $(GTG)_5$ and the five RAPD primers (OPA9, OPA11, OPA13, OPA18 and OPW3) was treated as a unit character and scored as a binary code (1-presence, 0-absence). Only clear distinct bands that were 1 mm apart were considered for scoring. The fragment data from the two primers were pooled to generate a binary matrix which was used for phylogenetic analysis. Phylogenetic analysis was performed using the software NTSYSpc version 2.1 (Rohlf, 1992) and the distance matrix was computed using Nei's genetic distance method (Nei, 1972) (Appendix 4 and 5). The distance matrix output was subjected to UPGMA analysis and was used to generate the dendrogram. A genetic distance of 0 represented identical samples and the highest genetic distance of 1.65 and 1.79 (RAPD and ISSR, respectively) suggested different species. Mantels' test (Mantel, 1967) was performed to analyse the correlation between the genetic matrices of ISSR analysis and RAPD using NTSYSpc version 2.1 (Rohlf, 1992).

2.3   Results

2.3.1   Morphology analysis

The morphological characters (fruit, seed and leaf) of *M. cochinchinensis* samples were different based on the country and region of collection. Seeds from Central Vietnam were the heaviest and largest, while those from Thailand were lighter and smaller in length (Figure 2.3 A-B). The highest number of seeds per fruit was observed in Thailand samples with >40 seeds per fruit (T5, T6 and T7) while those from South Vietnam (VS1, VS2 and VS3) had <10. Seed colour ranged from light brown to blackish-brown; however, the seeds from Thailand samples were brown and light brown compared to those from Vietnam, which were mostly blackish-brown. Fruit shape varied within Southern Vietnam (globose to globose oval), Northern Vietnam (globose, globose oval, oval and tapered), Central Vietnam (globose oval, oval) and Thailand (oval). Globose and oval shaped fruits were observed from both Vietnam and Thailand samples but tapered fruits were only observed from Northern Vietnam (Table 2.2). The surface of the fruits ranged from densely to sparsely spiny. Sparsely spiny surfaces were only observed in one Northern Vietnam accession (VN15, VN26, and VN27). Most leaves were three lobed (36 out of 38 samples) except for the VS4b and A3 samples which were five lobed (Figure 2.2 E-F).

2.3.2 Cluster analysis based on morphological data

Morphological characteristics of the fruit, seed and leaves were combined in cluster analysis. Based on the dendrogram generated, at the similarity level of 0.31, all 38 samples clustered into two main clusters (cluster A and B) according to the country of origin (Figure 2.4). Cluster A, which was separated early at a similarity level of 0.31, consisted of all the Thailand samples and was morphologically diverse from the Vietnam samples with less morphological diversity within the samples. At a similarity coefficient of 0.40, cluster B was divided into four sub clusters (sub cluster 2-5). These four clusters consisted of Northern Vietnam samples (sub cluster 2 and 4), a combination of both Northern and Central Vietnam samples (sub cluster 3) and Southern Vietnam samples (sub cluster 5), indicating a high morphological diversity within the Vietnam samples. The two samples from Australia clustered together with the Southern Vietnam samples, indicating morphological similarities (Figure 2.4).
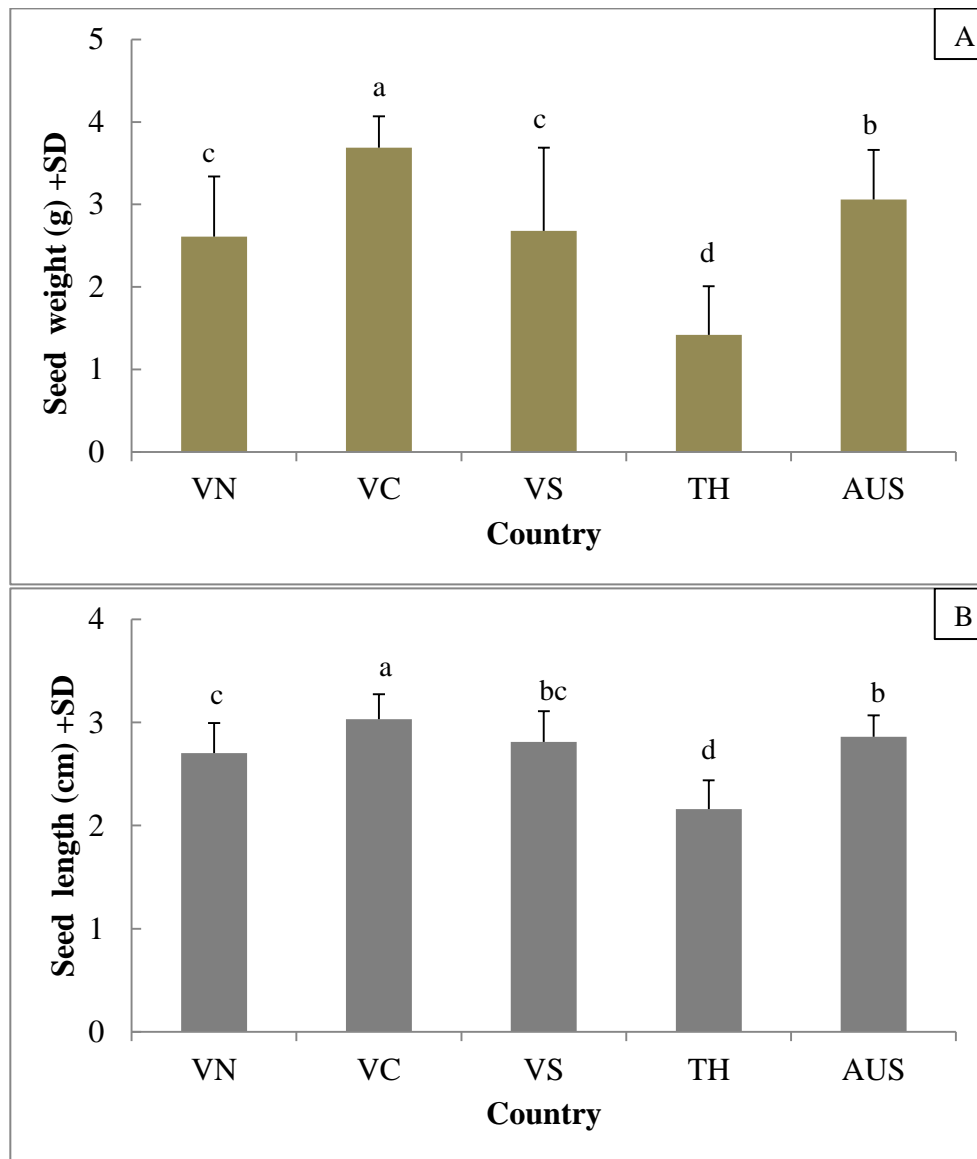
Figure 2.3: Seed weight (A) and length (B) of *M. cochinchinensis* based on the country of collection. Bars with different letters denote significant differences using Fishers's LSD (least significance differences) generated from one-way ANOVA for weight (df=4, F=258.72, p=0.000) and length (df=4, F=213.87, p=0.000). Regions and countries are presented as AUS (Australia), TH (Thailand), VN (Northern Vietnam), VC (Central Vietnam) and VS (Southern Vietnam).
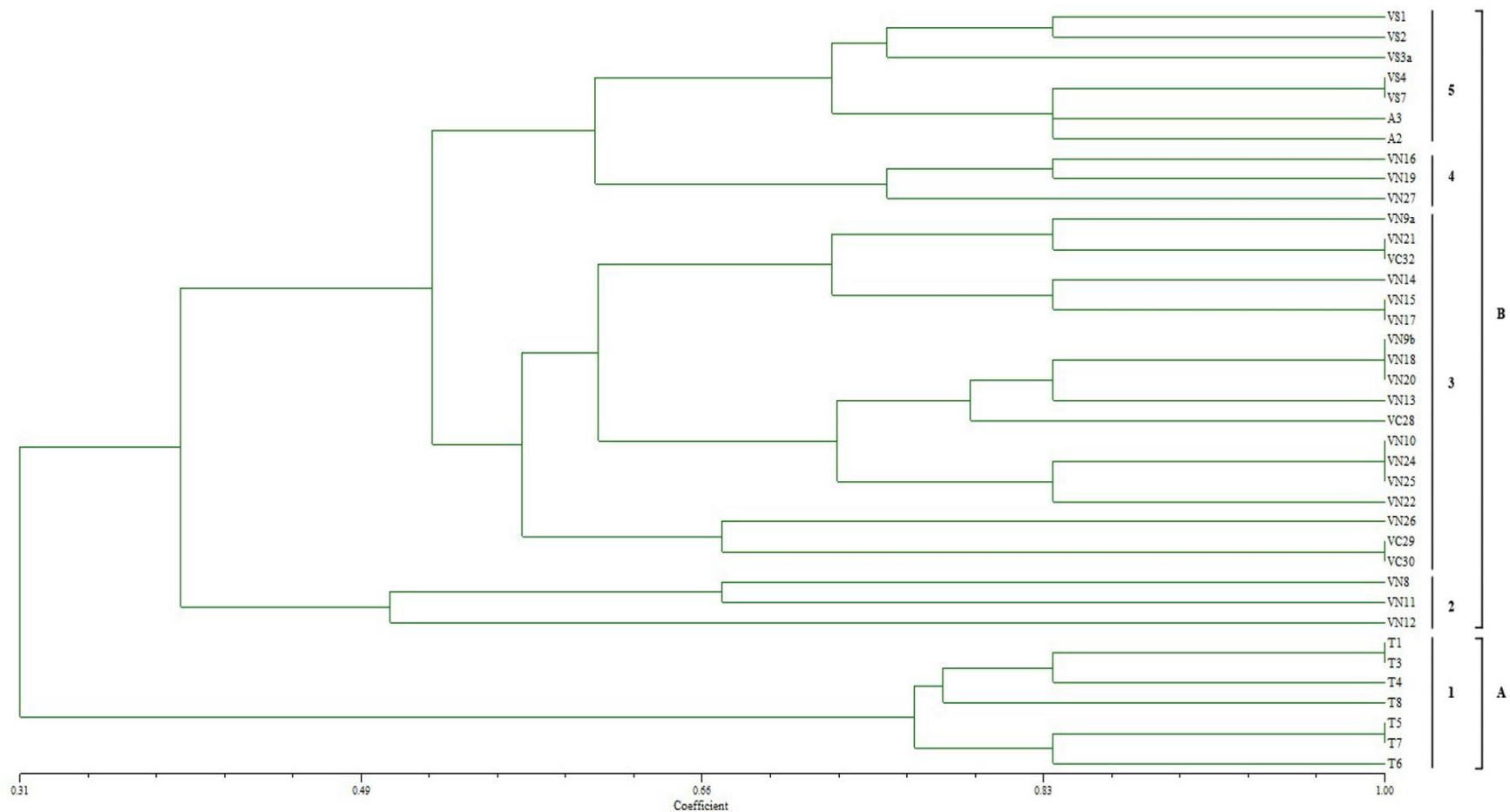
Figure 2.4: Dendrogram of 38 *M. cochinchinensis* samples based on overall morphological data including fruit, seed and leaf characteristics; constructed using the UPGMA cluster analysis method based on similarity value by NT-SYS version 2.1. Note: 0 distance indicated morphologically identical characteristics. Higher values indicated morphologically different characteristics.

2.3.3   RAPD amplification

The RAPD primers used (OPA9, OPA11, OPA13, OPA18 and OPW03) amplified all 42 *M. cochinchinensis* samples as well as the out-group (*M. charantia*). A total of 52 loci were amplified and they were all polymorphic in the range of 300-2500 bp (Figure 2.5 A). The banding profile was used to construct a dendrogram using Nei's distance matrix coefficient. The genetic distance ranged from 0 (between T1 and T5, indicating they are 100% similar) to 1.65 (between T1 and the out-group, *M. charantia*).

The final output was represented in a dendrogram containing all *Momordica* samples (Figure 2.6) and the 42 samples of *M. cochinchinensis* showed similarities according to the country of origin and the three clusters were assigned as cluster A-C. Vietnam samples were clustered together (cluster A and B). Cluster A contained all the samples from Southern Vietnam as well as from Australia at a genetic distance of 0.52. However, cluster A was further divided into subcluster 1 and 2 at a genetic distance of 0.36 which separated the samples from Southern Vietnam and Australia, respectively. Cluster B contained all the samples from Northern Vietnam (sub cluster 1) as well as those from Central Vietnam (sub cluster 2) at a genetic distance of 0.38. At a genetic distance of 0.76, group C contained all the samples from Thailand. The out-group (*M. charantia*) separated outside the main clusters as cluster D as expected since it is a different species.
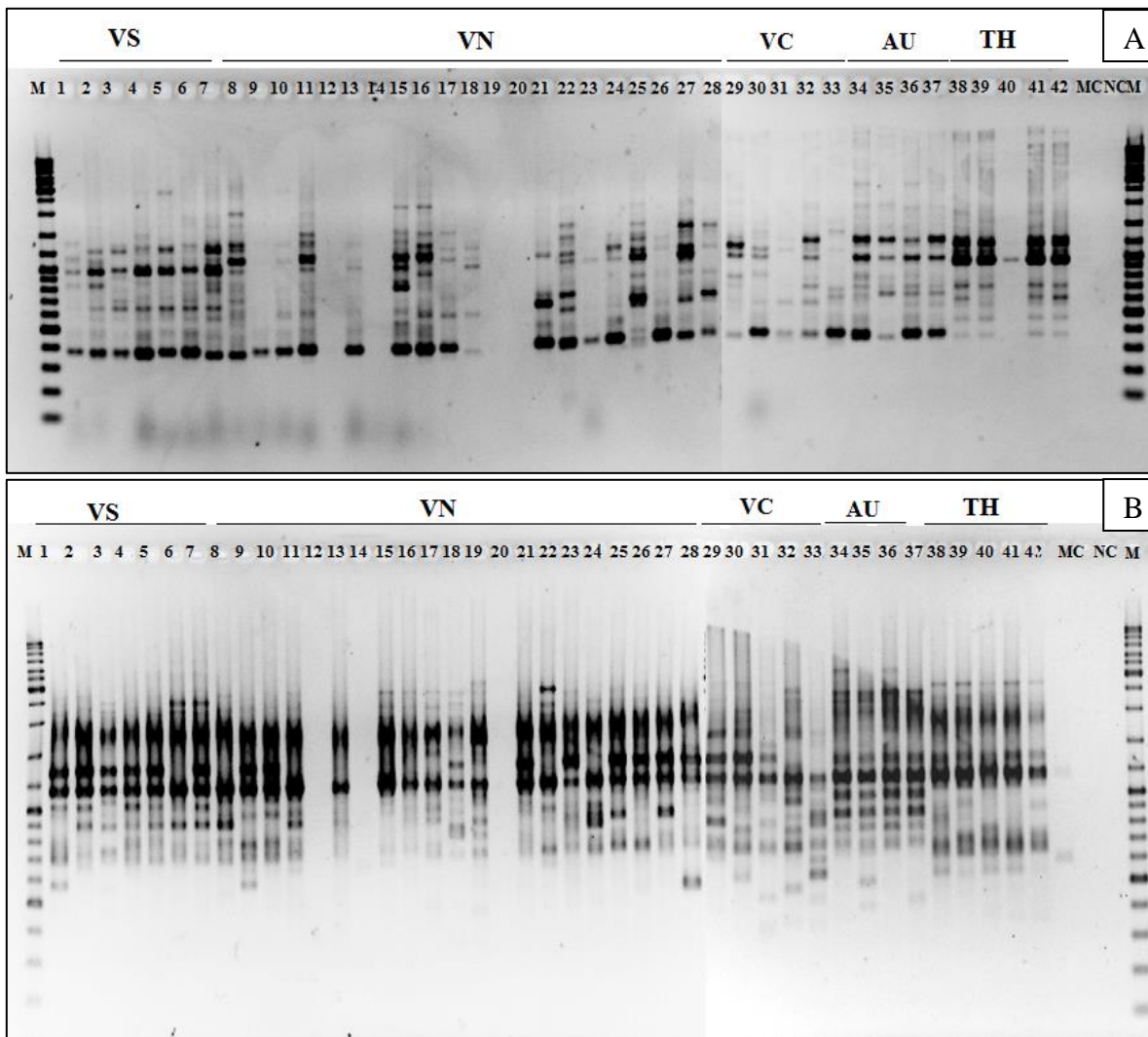
Figure 2.5: Gel electrophoresis of RAPD and ISSR amplified products. A: (OPA 09) amplification. Lane 1-7: VS1-7, Lane 8-28: VN8-27, Lane 29-33: VC28-31, Lane 34-37: A1-4, Lane 38-41: T1-4. Lane 42: T8, MC: *M. charantia*. B: (GACA)$_4$ amplification. Lane 1-7: VS1-7, Lane 8-28: VN8-27, Lane 29-33: VC28-31, Lane 34-37: A1-4, Lane 38-41: T1-4. Lane 42: T8, MC: *M. charantia*. 100 bp Gene Ruler^{TM} DNA ladder indicated by lane M and negative control is indicated by NC.
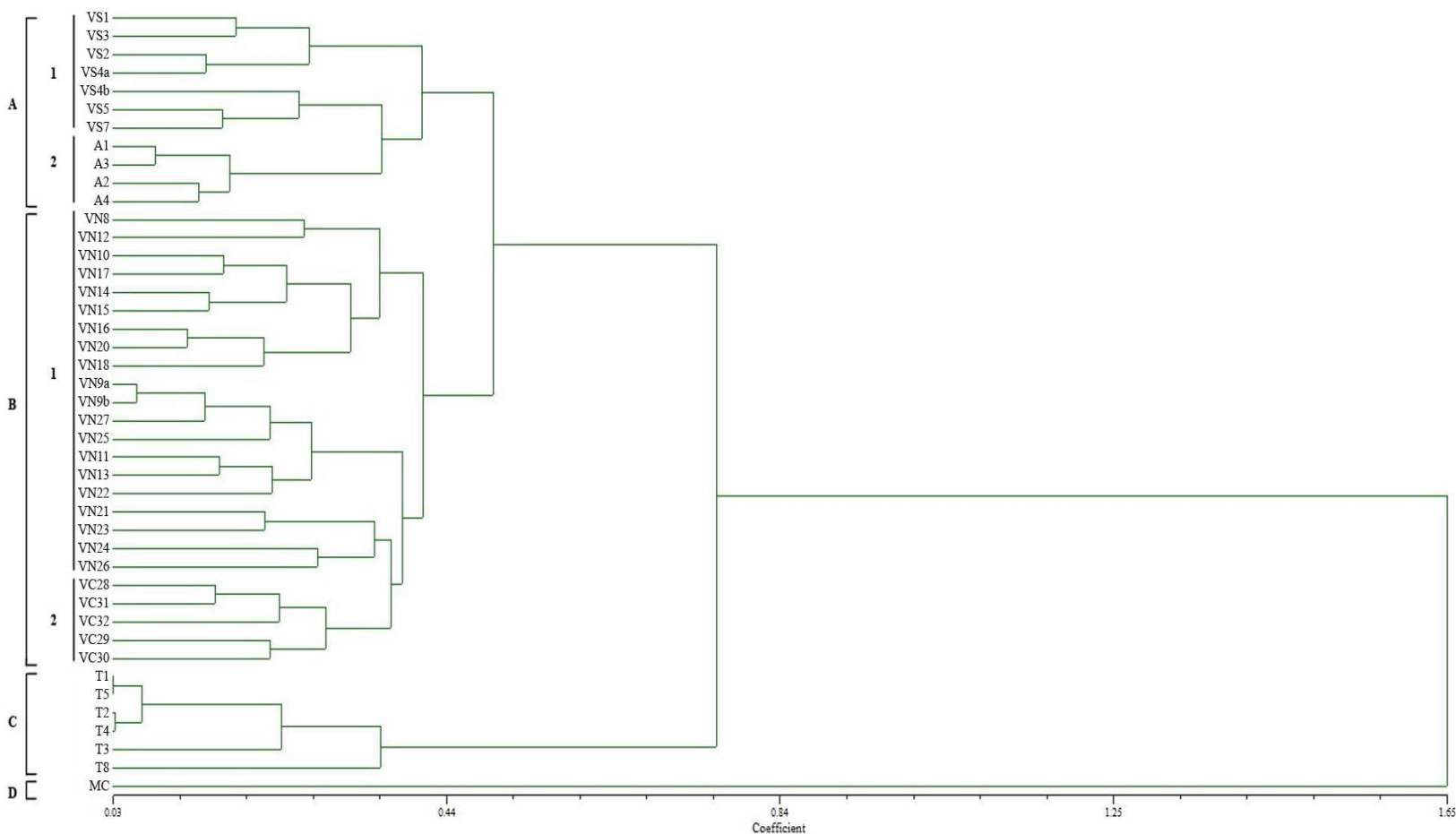
Figure 2.6: Dendrogram based on RAPD analysis of *Momordica* samples collected from Vietnam, Thailand and Australia and constructed using the UPGMA cluster analysis method, based on Nei's (1972) genetic distance value, with NT-SYS version 2.1. Coefficient values of 0 indicated genetically identical samples while a coefficient value of 1.63 indicated genetically different species.

2.3.4   ISSR amplification

The (GACA)$_4$ primer amplified all 42 *M. cochinchinensis* samples but not the out-group (*M. charantia*) despite three independent amplifications. The (GACA)$_4$ primer amplified 20 loci, which were all polymorphic in the range of 550-2500 bp (Figure 2.5 B). The (GTG)$_5$ primer amplified all 42 *M. cochinchinensis* samples as well as the out-group *M. charantia*. The amplification yielded 25 loci, which were all polymorphic in the range of 350-3000 bp.

A dendrogram was created from the combined results of (GACA)$_4$ and (GTG)$_5$ amplification, which produced a total of 38 loci in the range of 550–3000 bp with 100% polymorphism. The banding profile for the pooled ISSR primers was used to construct a dendrogram using Nei's distance matrix coefficient. The genetic distance ranged from 0 (indicating that T1-T3 samples were identical and 100% similar with each other) to 1.79 between T1 and *M. charantia*.

The dendrogram for the pooled ISSR primers produced four clusters (A-D) (Figure 2.7), *M. cochinchinensis* showed similarities according to the country of origin and the three clusters were assigned as cluster A-C (Figure 2.7). The samples from Vietnam clustered together as cluster A and B. Cluster A contained all the samples from Southern Vietnam as well as from Australia at a genetic distance of 0.54. However, the samples from Southern Vietnam (sub cluster 1) and Australia (sub cluster 2) were separated into two sub

clusters at a genetic distance of 0.08. Cluster B contained all the samples from Northern Vietnam (sub cluster 1) as well as from Central Vietnam (sub cluster 2) at a genetic distance of 0.50. At a genetic distance of 0.55, group C contained all the samples from Thailand. The out-group (*M. charantia*) separated outside the main clusters as cluster D, which was as expected because it is a different species.

The dendrograms constructed using RAPD and ISSR markers were highly correlated using Mantel's test at a significance level of 1, giving a correlation coefficient of 0.76. This showed a good correlation between the two types of molecular markers analysed in this study.
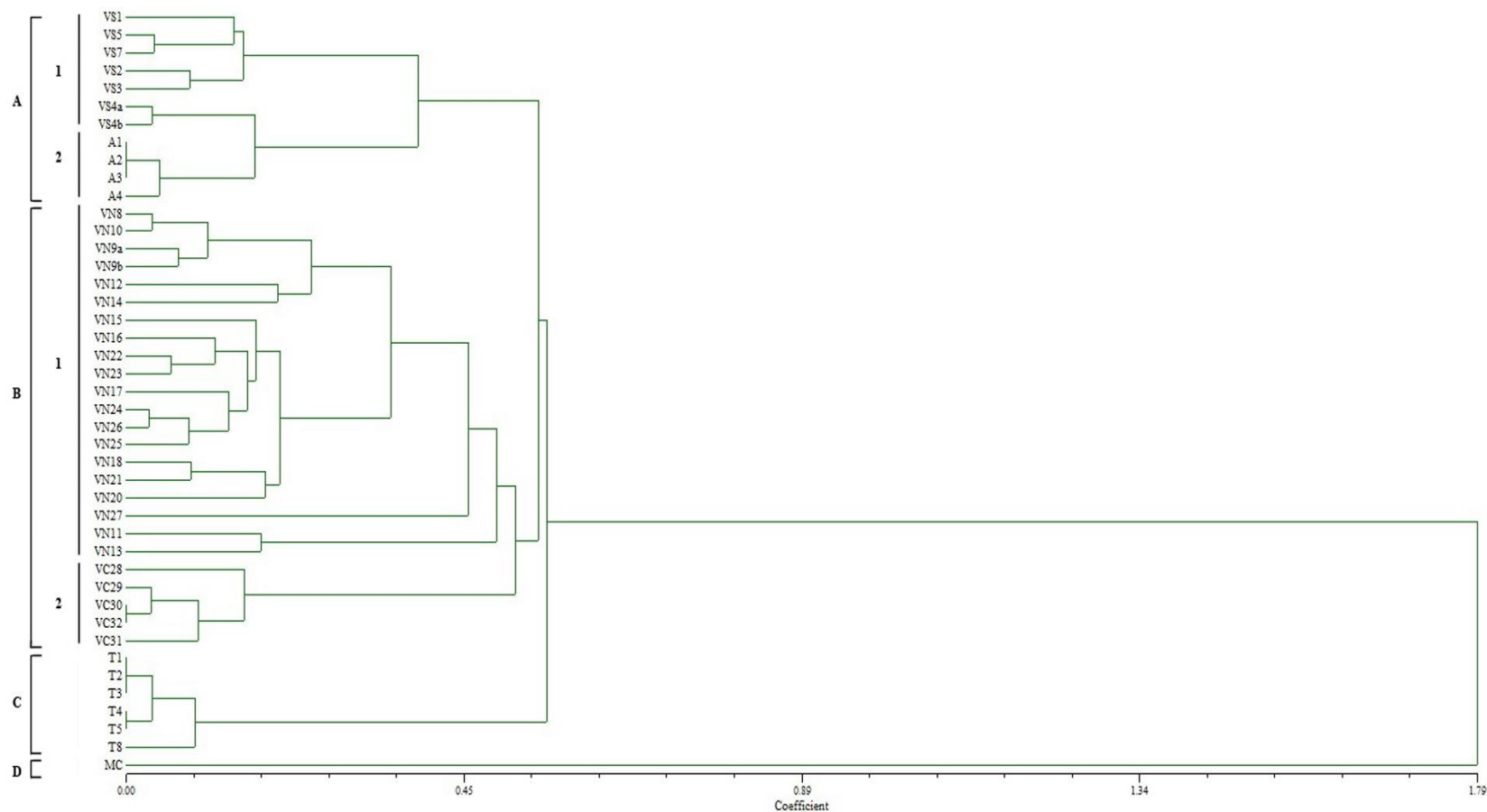
Figure 2.7: Dendrogram based on ISSR analysis of *Momordica* samples collected from Vietnam, Thailand and Australia using (GACA)$_4$ and (GTG)$_5$ primers, constructed using the UPGMA cluster analysis method based on Nei's (1972) genetic distance value with NT-SYS version 2.1 software. Genetic distance of 0 indicated genetically identical samples and 1.52 indicated genetically different species.

2.4   Discussion

2.4.1   Morphological diversity of *M. cochinchinensis*

The *M. cochinchinensis* samples showed high morphological variation with respect to fruit (shape and surface texture), seed (weight, length, number per fruit and colour) and leaves (number of lobes). The seeds from Vietnam had greater weight and length than seeds from Thailand. The highest diversity of fruit shape, surface texture and seeds were observed in the Vietnam samples, where the fruits had a high variation in seed weight and length and globose to tapered shapes with closely to sparsely spiny surfaces.

The high morphological variations within *M. cochinchinensis* were expected particularly considering the diverse environments and eco-geographic profiles from which the plants were collected. However, samples from the same environmental conditions (Thailand and Southern Vietnam) did not have the same morphological characters, indicating that the environment might not be responsible for the differences in phenotypes. A similar study which analysed fruit shape, fruit colour, fruit surface structure, number of fruits per plant and flower colour on *Cucumis sp.* also failed to differentiate the phenotype depending on their adaptability to various agro-climatic zones (Parvathaneni et al., 2011). However, the results from this study differs to that seen in the study of *Cucurbita maxima* D. and *Cucurbita pepo* L., which demonstrated that the phenotypic differences were based on eco-geographical factors such as temperature and geographical distribution (Liu et al.,

2013). Therefore, the high morphological variability of *M. cochinchinensis* samples in this study indicates the presence of different genotypes in Thailand and Vietnam. Furthermore, Southern Vietnam sample VS4b and Australian sample A3 possessed 5 lobed leaves indicating they might be closely related. However, a wider collection of morphological data from Southern Vietnam and Australia is necessary to strengthen the phenotypic relationship between these samples.

2.4.2   Genetic diversity of *M. cochinchinensis*

2.4.2.1   Polymorphism among the samples of *M. cochinchinensis*

The *M. cochinchinensis* samples analysed in this study showed a high level of polymorphism. The samples analysed covered three main regions of Vietnam (North, Central and South) which have different environmental conditions, Thailand (North and South) and recently cultivated samples in Australia. This study assessed samples collected from a wider variation of regions than an earlier study, which included 23 samples from Thailand and 2 samples from Vietnam (Bootprom et al., 2012). The RAPD and ISSR primers used in this study showed a high degree of polymorphism (>90%) and this is in agreement with the previous studies on *M. cochinchinensis* (Bootprom et al. 2012), *M. charantia* (Dey et al., 2006, Behera et al., 2008a) and *M. diocia* (Rasul et al., 2007).

Cluster analysis based on Nei's genetic distance was high, indicating the presence of different genotypes and a genetically diverse population of *M. cochinchinensis*. The

range of genetic distances among the 42 *M. cochinchinensis* samples varied from 0.00-0.55 in the ISSR analysis and 0.03-0.76 in the RAPD analysis. The high genetic diversity was expected considering the breeding system (out-crossing) of the genus Momordica (Trivedi and Roy, 1972). The range of genetic diversity is similar to that observed in 29 samples of *M. diocia* using 44 RAPD primers (Rasul et al., 2007), 38 genotypes of *M. charantia* using 116 RAPD primers (Dey et al., 2006) and 31 genotypes of pumpkin (*Cucurbita moschata* Duchesne ex Poiret) using 31 RAPD primers (Gwanama et al., 2000).

2.4.2.2   Genetic diversity of *M. cochinchinensis* between and within Thailand and Vietnam

The *M. cochinchinensis* samples collected from Thailand were different from Vietnam samples. Genetic diversity of a plant species between different countries reflects the long term evolutionary and ecological history such as habitat fragmentation shifts in geographical distribution, political boundaries, population isolation and most importantly breeding systems, seeds and dispersal mechanisms (Hamrick and Godt, 1996, Schaal et al., 1998, Rao and Hodgkin, 2002, Khan et al., 2009, Li et al., 2011). Between geographically separated species, gene flow is limited by pollen and seed dispersal. *M. cochinchinensis* in natural habitats is known to be pollinated by *Ctenoplectra* bees (Schaefer and Renner, 2010, Bharathi and John, 2013) and the pollen dispersal is limited by the short flight range of these bees. Seed dispersal of *M. cochinchinensis* is not efficient because the average seed weight is approximately 4 g and the seeds lack dispersal structures; therefore, natural gene flow will be limited between geographically distant samples. This suggests that the current

genetic diversity of *M. cochinchinensis* between Thailand and Vietnam may be due to limited gene flow among the samples, independent evolution and lack of human influence e.g. absence of plant and seed exchange between these countries. However, future research focusing on wider collection of samples from Thailand as well as other neighbouring countries (Cambodia and Laos) is needed to assess the gene flow of *M. cochinchinensis*.

The samples from Vietnam showed a higher level of genetic diversity than the samples from Thailand indicating the presence of a diverse gene pool and this will be useful for crop improvement. Both the RAPD and ISSR analyses clustered the Southern Vietnam samples apart from the Northern and Central Vietnam indicating the presence of different genotypes of *M. cochinchinensis* in Vietnam. High genetic diversity of a species within a country is expected due to its breeding system (out-crossing), selection by farmers and eco-geographical differences (Rao and Hodgkin, 2002, Yao et al., 2007, Li et al., 2011). This high diversity of *M. cochinchinensis* in Vietnam is possible due to the out crossing nature of this genus and its adaptation to the eco-geography of Southern (tropical), Central (temperate) and Northern Vietnam (temperate). Similar high genetic diversity, based on adaptation to eco-geography, has been reported in other species such as olive (*Olea europaea* L.), wild barley (*Hordeum spontaneum* C. Koch), rye (*Secale cereal* L.) and chilli pepper (*Capsicum baccatum* L.) (Dawson et al., 1993, Solouki et al., 2008, Akhavan et al., 2010, Mousavi et al., 2014).

The Australian samples were clustered together with the Southern Vietnam samples, which suggest that they share a common ancestor. This study will be important in tracking

the origin of cultivated *M. cochinchinensis*. A similar study on soya bean (*Glycine max* L. Merrill) (Li and Nelson, 2001) has shown the value in using molecular markers to track the origin of these plants. The intentional and unintentional seed, fruit and plant exchange from one region/country to another by human intervention increases the distribution of species and, as a long-term effect, the genetic structure of the species may change (Pairon et al., 2010, Zybartaite et al., 2011). However, from a horticultural perspective, identifying the origin and genotype of plants will be important in the selection of parents for breeding the best nutritional varieties (e.g. fruits that produce superior aril and seed for oil) of *M. cochinchinensis*.

2.4.3 Use of morphological and molecular markers in the analysis of genetic diversity of *M. cochinchinensis*

The morphological diversity based on seven quantitative traits of the plant samples (fruit, seed and leaf) and the molecular diversity based on five RAPD primers and 2 ISSR primers corresponded to each other. The groupings obtained from morphological analysis were matched with the groupings obtained from molecular analysis and indicated differences between the Thailand and Vietnam samples. However, the clustering based on molecular analysis was more refined in separating samples within Vietnam compared with morphology analysis. The lack of discrimination between varieties within Vietnam using morphological clustering may be due to the low number of quantitative traits that were analysed as well as the effect of genotype and environmental interaction. A similar low discrimination between morphological and molecular clusters has been reported for *M.*

diocia using 14 quantitative traits (Dey et al., 2006). However, plant selections based on morphological characters are economical and useful in the field (Rasul et al., 2007, Khan et al., 2009, Serçe et al., 2011). Therefore, other morphological (fruit weight, length, number of fruits per plant, spike density), physiological (salt and water and temperature tolerance), chemical (nutrient differences, e.g. amount of lycopene and β-carotene in the fruit) and biochemical (protein differences) markers could be studied for their effectiveness in predicting the genetic diversity of *M. cochinchinensis* and helping in marker-assisted plant selection.

Highly effective molecular markers (RAPD and ISSR) used in genetic analysis need to be informative and concord with each other (Behera et al. 2008a). The RAPD and ISSR markers used in this study were equally important in detecting polymorphism among the samples; they had a high correlation with each other ($r = 0.76$) and they were able to group samples based on country of origin. A similar high correlation between ISSR and RAPD markers has been reported in *M. charantia* (Behera et al., 2008a) using 29 RAPD and 15 ISSR primers.

2.5   Conclusion

The analysis of genetic diversity of plants in their natural habitats is necessary for selective germplasm collection and the selection of individuals for crop development. *M. cochinchinensis* possesses high nutritional and medicinal potential and research is necessary for the selection of the best plants for crop development and cultivation. This

study indicates that the *M. cochinchinensis* gene-pool in Vietnam is highly diverse and that the plant possesses a good adaptation to its eco-geography. The next chapters assess the influence of genetic diversity on the plant's nutritional quality (carotenoid) and on aspects of its biological activity (anticancer), which will facilitate the current and future breeding practices used with *M. cochinchinensis*.

CHAPTER 3

Comparison of methods used in the analysis of carotenoids in *Momordica cochinchinensis*

3.1   Introduction

Carotenoids are lipid-soluble antioxidants produced by most photosynthetic organisms and play an important role in human health (Rodriguez-Amaya, 2001, Rao and Rao, 2007). As described earlier in section 1.4.1, lycopene and β-carotene are highly nutritionally valuable carotenoids (Rodriguez-Amaya, 2001). Epidemiological and experimental studies have suggested a strong correlation between the intake of carotenoid-rich foods and a reduced risk of chronic degenerative diseases, cardiovascular diseases and age-related macular degeneration (Rao and Rao, 2007). The fruit of *M. cochinchinensis* is the richest known source of lycopene and β-carotene, containing 70 times more lycopene than tomatoes and 5 times more β-carotene than carrots (Aoki et al., 2002, Vuong et al., 2006). Since these carotenoids are valuable phytonutrients, many plant breeders and growers are interested in increasing the lycopene and β-carotene content in their crops. Thus, simple and inexpensive assays to quantify these carotenoids are desirable in order to test if plants have large levels of these phytonutrients.

The concentration of carotenoids in fruits and vegetables are affected by the analysis method. Chromatography, spectrophotometry and colorimetry methods have widely been used to quantify carotenoids (Schoefs, 2002). High-performance liquid chromatography (HPLC) is currently the most effective method to detect and quantify carotenoids in food and it is highly versatile, sensitive and selective (Hart and Scott, 1995, Azevedo-Meleiro and Rodriguez-Amaya, 2004, de Rosso and Mercadante, 2007). HPLC analysis of carotenoids usually uses C18 or C30 RP-columns, operated with isocratic or gradient elution with a wide variety of mixtures of different organic solvents as mobile phases, using UV–visible (450 nm), photodiode array or MS detection (Müller, 1997, Craft, 2001, Tzouganaki et al., 2002, Burns et al., 2003, Lin and Chen, 2003, Cortes et al., 2004, Barba et al., 2006). HPLC is the method of choice for quantifying carotenoids and is widely used to analyse carotenoids in *M. cochinchinensis* (Ishida et al., 2004, Kubola and Siriamornpun, 2011). However, it involves long analysis times, large quantities of solvents and is too slow and expensive for routine use for large numbers of samples.

Ultra-high performance liquid chromatography (UPLC or UHPLC) has emerged as a liquid chromatographic technique offering very rapid and efficient separation with increased signal-to-noise (S/N) ratio, enhanced peak resolution, a significantly reduced analysis time and less mobile phase solvent consumption (Welch et al., 2010). It is widely used in the pharmaceutical industry (Granado-Lorencio et al., 2010, Stinco et al., 2014, Klimczak and Gliszczyńska-Świgło, 2015). It has been used to analyse carotenoids in tomato (Li et al., 2012b) but not in *M. cochinchinensis*. However, all chromatographic methods require considerable technical expertise, expensive instrumentation and the use of

hazardous solvents, and so, they are not accessible to resource-poor farmers. Thus, a simple and inexpensive method to quantify carotenoids is essential for use in the field.

Conventional solvent extraction coupled with spectrophotometry has been used as a reliable and relatively fast method to quantify carotenoids (Fish et al., 2002, Davis et al., 2003, Scott, 2005). In this method, the concentration of carotenoids can be estimated by measuring the absorbance of the extract because the absorbance is proportional to its concentration as determined by Beer Lambert's Law. However, these absorption spectra are solvent dependent and the absorbance maxima changes with the solvent used. For lycopene, the typical UV-spectra consists of three absorbance maxima values in hexane (445, 471 and 502 nm) while for β-carotene there are two absorbance maxima (450 and 478 nm). These overlapping spectra can interfere with the measurement of carotenoid concentrations in a sample. While spectroscopic methods can be used to identify and quantify carotenoids present in a sample, the correct concentration might be compromised. Furthermore, this method requires the use of flammable, biologically hazardous solvents that pose personnel safety and environmental waste issues.

Visual colour evaluation is a convenient method that can be used to screen lycopene and β-carotene rich fruits (Arias et al., 2000). As described earlier in section 1.4.1.2.6.3, the most preferred methods for objective measurement of colour are the Tristimulus Hunter and CIE L*a*b* systems (Seroczyńska et al., 2006). Transformed colour values such as $(a*/b*)^2$ have been used to determine the lycopene content in field applications in tomato (D'Souza et al., 1992, Arias et al., 2000, López Camelo and Gómez, 2004), pumpkin and

squash (Itle and Kabelka, 2009) and sweet potato (Michael and Wilson, 1997) but not for *M. cochinchinensis*.

Though chromatography is thought to be the most accurate method of quantifying carotenoids, spectrophotometry and visual colour evaluation are much faster and require less technical expertise and cheaper instruments. Visual colour evaluation is much cheaper than spectrophotometry and has potential for routine use with *M. cochinchinensis* provided that its accuracy is acceptable.

The aims of this chapter were to, a) compare concentrations of carotenoids (lycopene and β-carotene) in *M. cochinchinensis* with *M. charantia*, *Solanum lycopersicum* and *Daucus carota* using the standard HPLC method, and b) compare carotenoid concentrations (lycopene and β-carotene) of *M. cochinchinensis* detected by UPLC, UV-visible spectrophotometry and colorimetry methods with the standard HPLC method to develop a method that can be used for routine analysis.

3.2   Materials and methods

3.2.1   Sample collection

Fifteen aril samples of *M. cochinchinensis* collected in 2011, from Southern Vietnam (VS1, 2 3a, 3b, 4a, 7), Northern Vietnam (VN 8,9a,9b) and Thailand (T1, T3-7) were processed as described in chapter 2 and the lycopene and β-carotene concentrations

were compared based on different analytical methods. Tomatoes and carrots are known to have high amounts of lycopene and β-carotene, respectively, and are important sources of these carotenoids in western diets. Therefore, fresh vine-ripened truss tomatoes (*Solanum lycopersicum*), tomato paste and fresh carrots (*Daucus carota*) were purchased from a local supermarket (Coles, Australia). The carotenoids were extracted on the same day and the extracts were used for comparison with *M. cochinchinensis*. *M. charantia* was used as another member representing the genus Momordica and its carotenoid content was compared with *M. cochinchinensis*. The fruit was purchased from Springvale Market, Victoria, Australia, and the aril was separated from the seeds and stored at -20°C until analysis.

3.2.2   Extraction of carotenoids

The aril of the *M. cochinchinensis* samples, *M. charantia* fruit, the flesh of tomatoes, the taproot of *D. carota* and tomato paste were used to extract carotenoids. The carotenoids in all the samples were extracted using a method described previously in literature (Barba et al., 2006). Two grams of the sample was placed in a vessel, protected from sunlight, and mixed with 100 mL of extraction solvent (hexane/acetone/ethanol: 50:25:25 v/v/v). The mixture was sonicated for 30 min and then 15 mL of distilled water was added to enhance phase separation. The upper hexane layer was separated and 1 mL was used for UV-Visible spectroscopic analysis. From the remaining volume, an aliquot of 10 mL of the extract was evaporated to dryness. The residue was dissolved to a final volume of 4 mL in HPLC and UPLC injection solvent (THF/acetonitrile/methanol:

50:25:25 v/v/v). The final solvent was filtered through 0.45 µm membrane filters and 20 µL was used for HPLC analysis and 1.4 µL was used for UPLC analysis. Since there were more than three samples from Southern Vietnam, Northern Vietnam and Thailand, carotenoid extraction was carried out only once per sample.

3.2.3   Carotenoid analysis

3.2.3.1   High Performance Liquid Chromatography (HPLC)

Carotenoid extracts were analysed via reverse phase analytical HPLC using solvents and conditions described previously (Barba et al., 2006). Samples were analysed using an isocratic method (90% CH_3CN/H_2O) on an Alltech Alltima HP C18 (250 x 4.6) 5 µm column at a flow rate of 1.0 mL/min. Analytical HPLC analyses were performed on a Dionex P680 solvent delivery system equipped with a PDA100 UV detector (operated using "Chromeleon" software). The column temperature was 30°C and UV detection was monitored at 475 nm. The concentrations of lycopene and β-carotene were quantified with reference to samples of commercial standards of lycopene and β-carotene (Sigma Chemical, St. Louis, USA) of known concentrations ranging from 10-400 µg/mL which were used to obtain a calibration plot. The regression equation was used to determine the lycopene and β-carotene content of the samples where, y = 1.16x - 4.369, R^2 = 0.971 for lycopene and y = 0.540x + 0.458, R^2 = 0.984 for β-carotene (Figure 3.1 A). The analyses were performed in triplicate for all the samples.

3.2.3.2 Ultra Performance Liquid Chromatography (UPLC)

M. cochinchinensis aril carotenoid extracts was analysed using an isocratic method (60% CH_3CN/H_2O) on a Waters Aquity UPLC^{TM}BEH fitted with a C18 column (2.1 × 50 mm, 1.7 µm packing) at a flow rate of 0.12 mL/min. The column temperature was 30°C and absorbance was read at 473 nm. The concentrations of lycopene and β-carotene of the samples were quantified by reference to commercial standards (Sigma Chemical, St. Louis, USA) as for HPLC. The regression equations for these were used to determine the lycopene and β-carotene content of the samples where, y = 0.7493x-9.1813, R^2 = 0.9968 for lycopene and y = 0.3035x-0.3097, R^2 = 0.9998 for β-carotene (Figure 3.1 B). The analyses were performed in duplicate for all *M. cochinchinensis* samples.
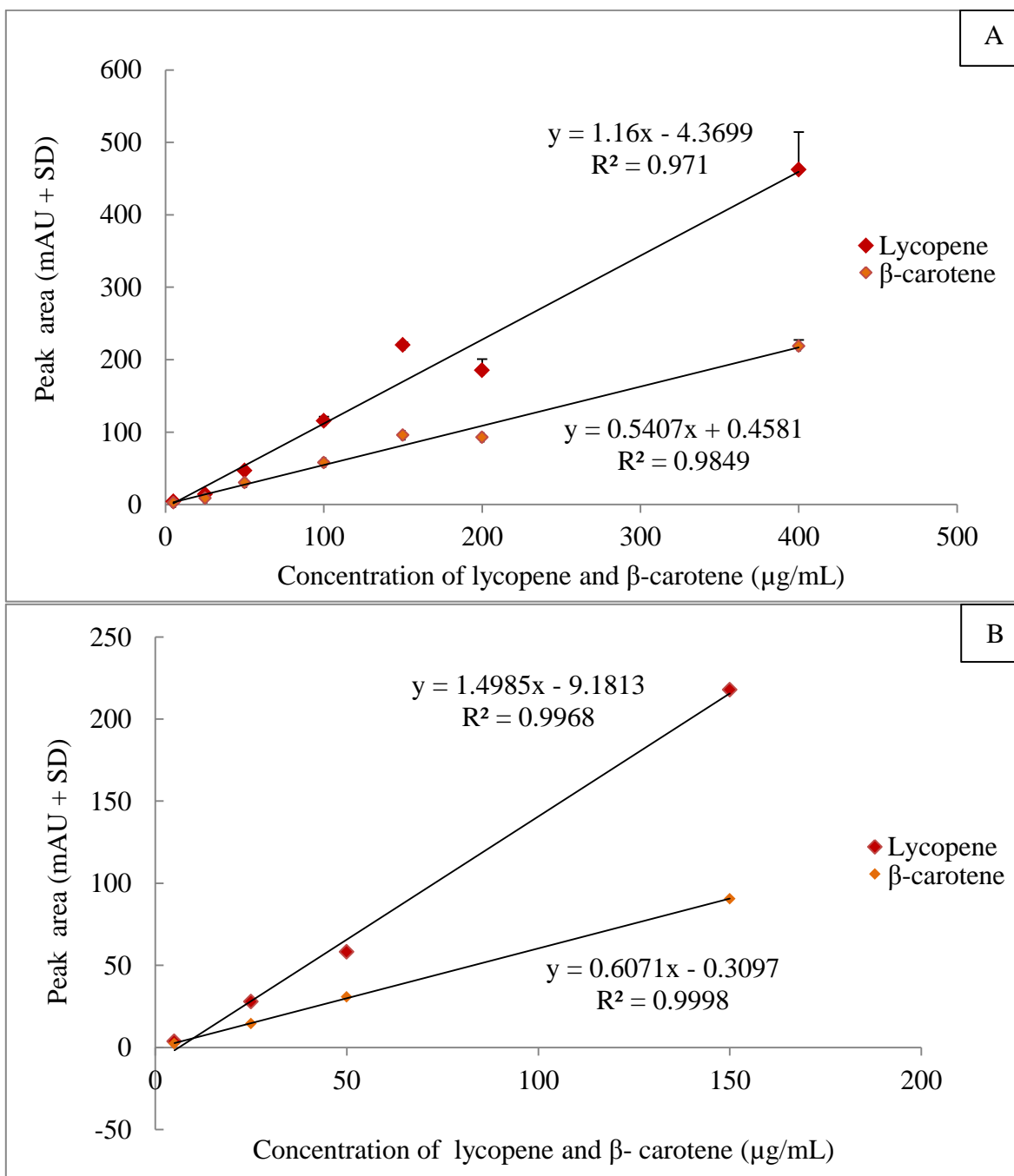
Figure 3.1: Calibration curves for lycopene and β-carotene standards analysed by HPLC (A) and UPLC (B).

3.2.3.3 UV- Visible spectrophotometry

Carotenoid extracts of *M. cochinchinensis* were placed in a 1 mL quartz cuvette and analysed using UV-visible spectrophotometry in a Cary® 50 UV-Vis spectrophotometer (Varian Inc, USA). UV visible spectra were recorded between 200-800 nm in hexane using a 1 nm interval.

The carotenoid content of the samples was calculated at the absorption maxima of lycopene (502 nm) and β-carotene (450 nm) using the equation: $C = (A_\lambda \times Fd)/\varepsilon$ (Biehler et al., 2010), where C was the unknown content of carotenoids in mol/L; A_λ was the absorbance maximum of lycopene and β-carotene; *F* was a dilution factor adjusting for extraction, drying and reconstitution processes; ε was the molar extinction coefficient in hexane (ε (L/mol) = 169000 for lycopene and 139000 for β-carotene) (Zechmeister et al., 1943); and *d* was the width of the cuvette (1 cm). Using the molar mass of lycopene (537 g/mol) and β-carotene (537 g/mol), the results were converted to mg/g fresh weight. The spectroscopic analyses were performed in triplicate for all *M. cochinchinensis* samples.

3.2.3.4 Colorimetry

The colour of the frozen aril samples was measured in triplicate using a tristimulus colorimeter (Konico Minolta, Chroma meter – CR 400, Japan) with a 8 mm measuring area and diffuse illumination/0° viewing angle. The data were recorded as $L*$, $a*$, $b*$, corresponding to indices of lightness, redness and yellowness, respectively, according to the CIE L*a*b* scale (Hunter, 1987). The $L*$ coordinate indicates darkness or lightness of colour and ranged from black (0) to white (1) (Figure 3.2). The a* and b* coordinates indicate the colour directions where a*>1 is the red direction and a*<0 is the green direction; b*>1 is the yellow direction and b*<0 is the blue direction (Itle and Kabelka, 2009) (Figure 3.2). Chroma indicates the saturation of colour, whereas as the value of chroma increases, the colour becomes more intense and as it decreases, the colour becomes duller. The hue was used to interpret the actual colour, for example, 0° = pure red, 45° = orange, 90° = yellow, 180° = pure green (Arias et al., 2000, Itle and Kabelka, 2009). Both chroma and hue were derived from a* and b* values (Arias et al., 2000) where, chroma = $(a*^2 + b*^2)^{1/2}$ and hue = $\tan^{-1}(b^*/a^*)$. The colour space values were transformed (e.g. a^*/b^*, $(a^*/b^*)^2$, a^{*4}) to improve the correlations with the lycopene and β-carotene content was quantified by HPLC.
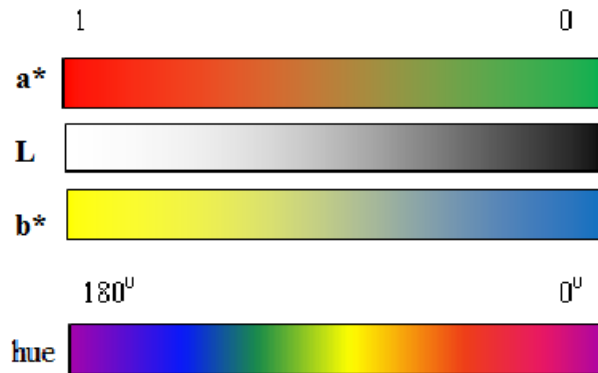
Figure 3.2: Representation of colour coordinates generated by adobe photoshop CS5 version 12.0 (Adobe, USA). The *L** coordinate indicates darkness (black=0) or lightness (white=1) of colour. The a* b*coordinates indicate the colour directions where a*>1 is the red direction and a*<0 is the green direction; b*>1 is the yellow direction and b*<0 is the blue direction. Hue was the actual colour.

3.2.4   Statistical analysis

The content of lycopene and β-carotene, as quantified by all methods, was analysed using Minitab statistical software (version 16). For normally distributed data, means were compared using one-way analysis of variance (ANOVA) with Fisher's post-hoc test. Least significant differences were calculated using the equation:

$$LSDx = t_{x \text{ (df for MSE)}} \sqrt{2MSE\left(\frac{1}{Ny}\right)}$$

where t is the critical value of the t distribution table; MSE is the mean square error obtained from the results of the ANOVA test; x is the the desired level of possibility and y

is the number of replicates. Non-normally distributed data were transformed or analysed using the non-parametric Kruskal Wallis test and the significance difference between the mean were analysed using Fisher's post-hoc test. Statistical values of P≤0.05 were considered as significantly different. Pearson's correlation was used to correlate carotenoid concentrations (lycopene and β-carotene) of *M. cochinchinensis* detected by the standard HPLC method with those from UPLC, UV-visible spectrophotometry and colorimetry. The *r* value represented the correlation coefficient between two variables (1= total positive correlation, 0 = no correlation, -1 = total negative correction) at a statistically significant level of P≤0.05.

3.3   Results

3.3.1   HPLC

The elution times for lycopene and β-carotene in HPLC were 28.2 and 35.1 min, respectively (Figures 3.3 and 3.4). The HPLC method indicated that the lycopene concentration of the aril of *M. cochinchinensis* ranged between 1.44-3.92 mg/g with an average of 2.37 mg/g. The highest lycopene concentration was seen in samples from Northern Vietnam, which had a value of 3.92 mg/g (FW). This was more than 30 times higher than that seen in *S. lycopersicum* (fresh), ~2 times greater than that of tomato paste and 5 times higher than that of *M. charantia* (Table 3.1). Likewise, the β-carotene concentration of the aril of *M. cochinchinensis* was greater in Northern Vietnam samples (2.19 mg/g) which was more than 200 times higher than seen in fresh carrots.
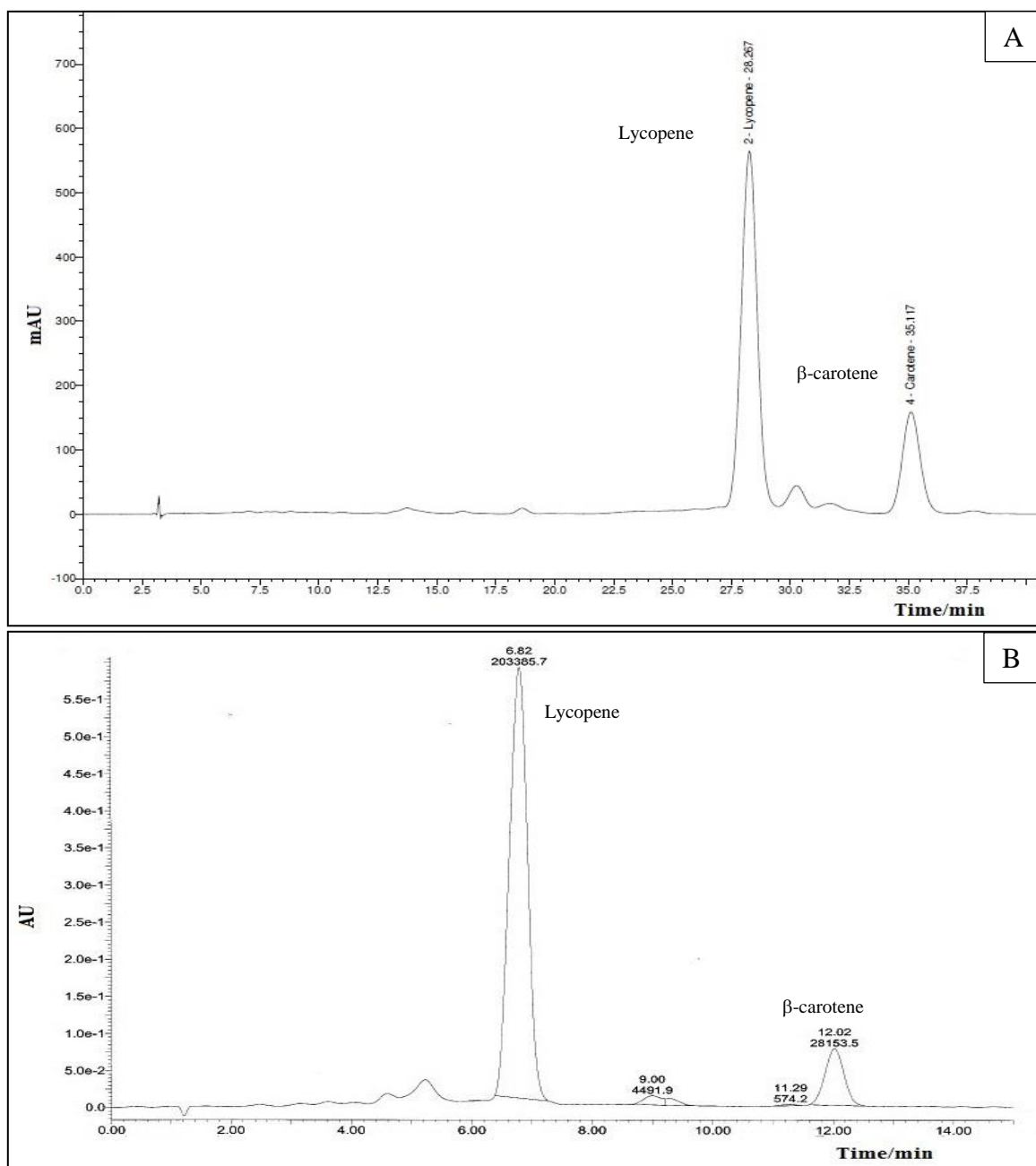
Figure 3.3: Chromatographs of lycopene and β-carotene standards detected by High Performance Liquid Chromatography (HPLC) (A) and Ultra Performance Liquid Chromatography (UPLC) (B).
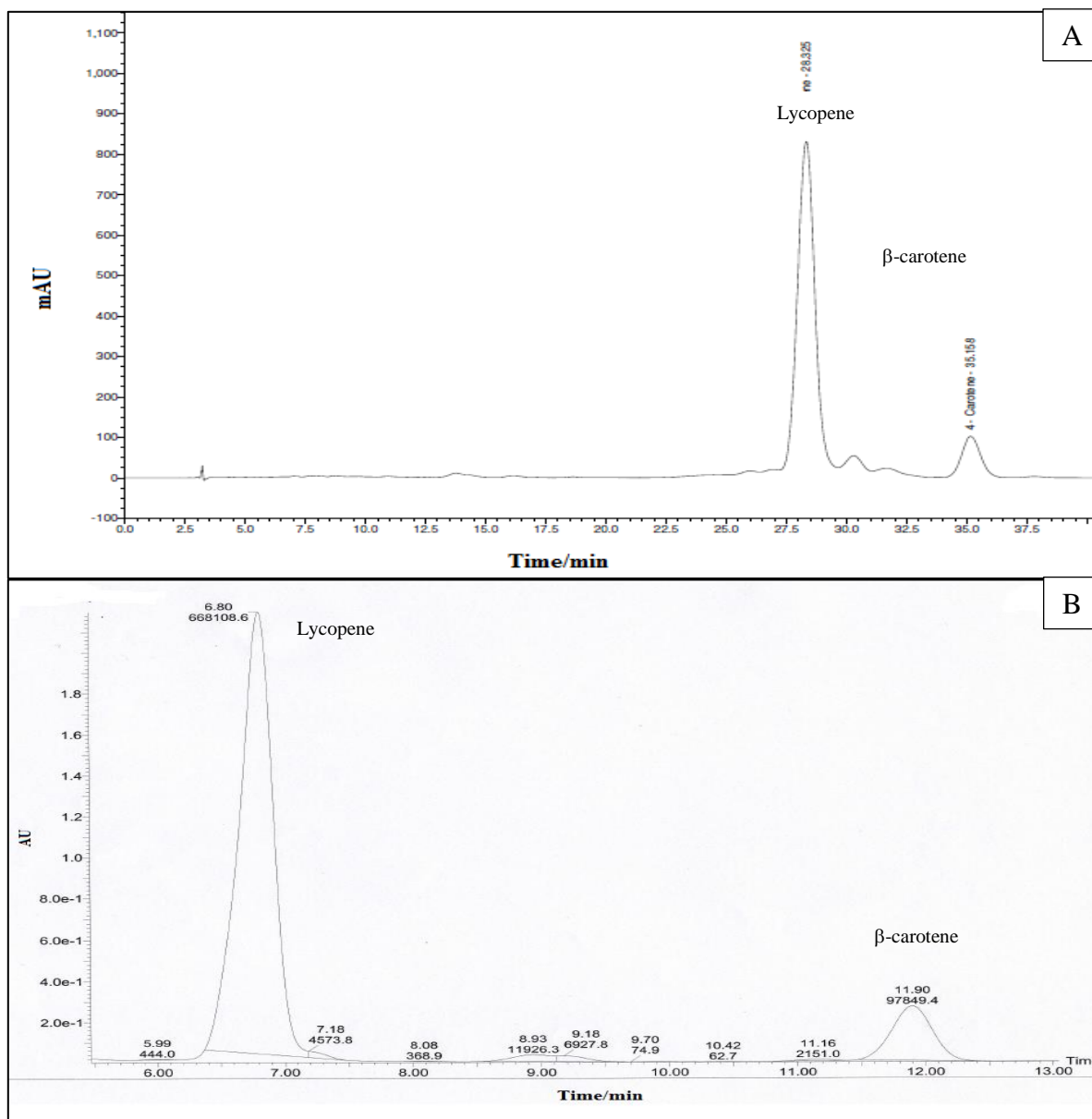
Figure 3.4: Chromatographs of lycopene and β-carotene of VN9a sample as detected by High Performance Liquid Chromatography (HPLC) (A) and Ultra Performance Liquid Chromatography (UPLC) (B).

Table 3.1: Lycopene and β-carotene concentrations of *M. cochinchinensis* and other carotenoid-rich fruits analysed by chromatographic, spectrophotometry and colorimetric methods.

Plant (fruit state)	Lycopene (mg/g)				β-carotene (mg/g)			
	HPLC	UPLC	Spectro-photometry	Colorimetry $(a*/b*)^2$	HPLC	UPLC	Spectro-photometry	Colorimetry $(a*/b*)^2$
M. cochinchinensis (frozen)								
TH (n=6)	2.52 ± 0.77^{ab}	3.67 ± 0.80^{a}	1.27 ± 0.55^{b}	2.01 ± 1.20^{ab}	1.55 ± 0.56^{a}	2.27 ± 0.76^{a}	1.57 ± 0.33^{a}	2.01 ± 1.20^{a}
VN (n=3)	3.92 ± 1.03^{ab}	5.71 ± 1.33^{a}	1.83 ± 0.77^{b}	3.50 ± 0.91^{ab}	2.19 ± 0.70^{a}	3.20 ± 1.02^{a}	2.13 ± 0.33^{a}	3.50 ± 0.91^{a}
VS (n=6)	1.44 ± 0.15^{ab}	2.06 ± 0.37^{ab}	0.72 ± 0.28^{b}	0.59 ± 0.19^{b}	1.34 ± 0.11^{b}	1.40 ± 0.16^{a}	0.90 ± 0.12^{b}	0.59 ± 0.19^{b}
Average	2.37 ± 0.35^{a}	3.44 ± 0.57^{a}	1.16 ± 0.16^{b}	2.53 ± 0.63^{a}	1.41 ± 0.28^{b}	2.10 ± 0.38^{ab}	1.41 ± 0.19^{b}	2.53 ± 0.63^{a}
M. charantia (fresh)	0.90 ± 0.00	-	-	-	ND	-	-	-
S. lycopersicum (fresh)	0.10 ± 0.00	-	-	-	ND	-	-	-
S. lycopersicum (paste)	2.53 ± 0.06	-	-	-	ND	-	-	-
D. carota (fresh)	ND	-	-	-	0.10 ± 0.00	-	-	-
LSD (*M. cochinchinensis* only)	0.71	1.07	0.33	0.63	1.22	1.19	0.39	0.63
LSD All[*]	0.59				1.20			

ND: Not detected

-: Not measured

[*]: Comparison of *M. cochinchinensis* (average) with other fruits based on HPLC analysis.

LSD: Least significant difference.

The average *M. cochinchinensis* values for lycopene and β-carotene not sharing a letter superscript were statistically significant (p<0.05)

3.3.2 UPLC

The retention times of lycopene and β-carotene in UPLC were 6.82 and 12.02 min, respectively, which were >20% less than for HPLC (Figures 3.3 and 3.4). Therefore, the total analysis time using the UPLC system was 3 times shorter than for HPLC. Furthermore, the UPLC utilised 8 fold less solvent than the HPLC, thus decreasing solvent costs. The carotenoids (lycopene and β-carotene) were just as well separated using UPLC than they were using HPLC (Figure 3.3). Furthermore, for the UPLC method the injection volume was 1.4 μL while HPLC required 20 μL, which reduced the volume of sample required for analyses by 93%.

The highest concentration of lycopene and β-carotene in the *M. cochinchinensis* aril was detected by UPLC and ranged from 2.06-5.71 mg/g between collection sites with an average of 3.44 mg/g and this was 1.5 times greater than that of HPLC (Table 3.1) but was not statistically different. However, the lycopene (r=0.99, P≤0.001) and β-carotene (r=0.97, P≤0.001) concentrations detected by UPLC showed good correlations to those of HPLC, indicating that both methods are suitable for the analysis of the relative amounts of carotenoids in *M. cochinchinensis* (Table 3.2, Figures A1 and A2).

Table 3.2: Pearson correlation coefficients (r) between values from chromatography, spectrophotometry and colorimetry methods for carotenoids from the aril of *M. cochinchinensis*.

Method	Lycopene				β−carotene			
	HPLC	UPLC	Spectrophotometry	Colorimetry	HPLC	UPLC	Spectrophotometry	Colorimetry
HPLC	-	-	-	-	-	-	-	-
UPLC	0.99***	-	-	-	0.97***	-	-	-
Spectrophotometry	0.95***	0.94***	-	-	0.79***	0.78***	-	-
Colorimetry								
L*	-0.62*	-0.66**	-0.58*	-	-0.13	-0.13	-0.57*	-
a*	-0.14	-0.12	-0.24	-	-0.06	-0.07	-0.21	-
b*	-0.59*	-0.62*	-0.59*	-	-0.12	-0.07	-0.54*	-
a*/b*	-0.59	0.64**	0.52*	-	0.05	0.03	0.45	-
$(a^*/b^*)^2$	-0.62*	0.68**	0.53*	-	0.03	0.03	0.45	-
$(a)^4$	-0.14	-0.12	-0.24	-	-0.05	-0.06	-0.20	-
$(a)^2$	-0.14	-0.12	-0.24	-	-0.06	-0.06	-0.20	-
$(a)^{0.5}$	-0.14	-0.12	-0.24	-	-0.06	-0.07	-0.20	-
L*(a*/b*)	0.45	0.50	0.39	-	-0.01	-0.03	0.32	-
Tan(a*/b*)	-0.35	-0.37	-0.31	-	-0.10	-0.17	-0.28	-
Hue	0.46	0.50	0.45	-	0.10	-0.05	0.46	-
$(b^*)^2$	-0.54*	-0.57*	-0.53*	-	-0.13	-0.07	-0.50	-
Chroma	-0.60*	-0.62*	-0.66**	-	-0.16	-0.11	-0.61*	-
a*b*	-0.65*	-0.68**	-0.67**	-	-0.12	-0.07	-0.60*	-
L* a*b*	-0.25	-0.3	-0.23	-	-0.06	-0.11	-0.18	-

*: p≤0.05, **: p≤0.01, ***: p≤0.001

3.3.3 UV-visible spectrophotometry

The lycopene concentrations estimated by UV-Visible spectrophotometry were significantly lower (P<0.05), at less than half the values for HPLC and UPLC (Table 3.1). However, the β-carotene concentrations detected by UV-Visible spectrophotometry were not significantly different from (P>0.05) HPLC and UPLC. Furthermore, the estimated concentration of lycopene and β-carotene by UV-Visible spectrophotometry was positively correlated with those by UPLC (r=0.938, P≤0.001 for lycopene, r=0.784, P≤0.001 for β-carotene) and HPLC (r=0.948, P≤0.001 for lycopene, r=0.793, P≤0.001 for β-carotene), indicating that all these methods were reliable in the analysis of the relative amounts of carotenoids in *M. cochinchinensis* (Table 3.2 and Figures A1 and A2).

3.3.4 Colorimetry

The lycopene concentrations estimated by the $(a^*/b^*)^2$ colour value were not significantly different than the lycopene quantified by HPLC and UPLC (Table 3.1). The raw colour values (L* a* and b*) and the calculated colour values a*/b*, $(a^*/b^*)^2$, chroma and a*b*) also showed moderate correlations with lycopene concentrations detected by both chromatographic methods (HPLC and UPLC), with the greatest being a*b* and $(a^*/b^*)^2$ (Table 3.2). However, the β-carotene concentrations detected by the $(a^*/b^*)^2$ colour value were higher than those by HPLC and UPLC (Table 3.1) and none of the raw or

calculated colour values showed a significant correlation with the β-carotene concentrations detected by UPLC and HPLC.

3.4   Discussion

3.4.1   Comparison between materials tested

The *M. cochinchinensis* aril possessed the highest lycopene concentration of the samples tested, which was >30 times that of *S. lycopersicum* (fresh), and twice that of tomato paste, which is the principal source of lycopene in Western diets (Clinton, 1998). The lycopene concentration of *M. cochinchinensis* was five times higher than that of the aril of *M. charantia,* which is a fruit well-known in Asia and belonged to the same genus as *M. cochinchinensis* (Joseph and Jini, 2013). This study was in agreement with previous studies which reported that *M. cochinchinensis* aril had 10-70 times greater amounts of lycopene than fresh tomatoes (Bauernfeind, 1972, Aoki et al., 2002, Vuong et al., 2006). However, the β-carotene concentrations reported in this study were more than twice higher than previously reported by Ishida et al. (2004). This may be due to differences in the extraction and analytical methods or differences in the samples (collection site and genotype) analysed.

3.4.2   Comparison between methods used

UPLC was the method of recommendation to quantify the lycopene and β-carotene content in *M. cochinchinensis*. It had a shorter analysis time per sample (<15 min) and required a smaller sample volume than conventional HPLC (Hung and Hatcher, 2011). Therefore, UPLC highlighted its ability to rapidly resolve the carotenoids of interest in *M. cochinchinensis* than HPLC (Table 3.3). However, the variability might be attributed to the differences in the columns used in HPLC and UPLC. This variability could be tested by using an internal standard such as β-apo-8'-carotenal (Hart and Scott., 1995). UPLC also improved peak efficiency (smaller peak width) and decreased the use of solvents due to the use of short columns packed with smaller particles (< 2 µm) (de Villiers et al., 2006, Guillarme et al., 2007, Guillarme et al., 2008). The minimum concentration of lycopene and β-carotene used in the standard curve was used as the limit of detection (LOD) for both UPLC and HPLC. However, in general, UPLC enables the detection of compounds of the interest at very low concentrations with low injection volumes because of the improved signal-to-noise ratio (Guillarme et al., 2007). Therefore, it could be useful in clinical studies to measure the carotenoid levels in plasma (Guillarme et al., 2008).

UPLC uses shorter columns with a smaller diameter, and thus, theoretically enabling the carotenoids to elute faster with less degradation in the column (Guillarme et al., 2007). The lycopene and β-carotene standards used in the calibration curve had similar peak areas (Figure 3.1) in both methods but the peak areas were slightly different when the same samples were analysed by both HPLC and UPLC (Figure 3.4). This could be due to

the difference in percentage recovery of the compounds, degradation in the column or sensitivity of the instrument. This can be tested by analysing the percentage recovery by spiking a known amount of sample with a known concentration of standard. However, there were no statistically significant differences in the concentration of lycopene and β-carotene measured using the two methods.

Both HPLC and UPLC methods were highly accurate, precise, high in reproducibility provided better separation, identification and quantification of individual carotenoids but they required complex sample extraction/preparation and pure carotenoid standards (Kimura and Rodriguez-Amaya, 1999, Barba et al. (2006). Furthermore, these chromatographic methods are too expensive, require expertise and are too slow for the routine screening of fruit from breeding programs designed to develop new cultivars with a greater carotenoid content.

Conventional spectrophotometric assays are reliable and simple methods for carotenoid quantification (Sadler et al., 1990, Fish et al., 2002). The estimated concentration of lycopene and β-carotene by UV-visible spectrophotometry was positively correlated with those by UPLC and HPLC. However, generally spectroscopic methods has low accuracy, specificity, linearity compared to chromatographic methods and also require the use of volatile organic solvents (hexane) to prepare each sample. For routine use this quickly multiplies into large volumes of hazardous waste that are costly to dispose of under strict environmental guidelines. Therefore, it is desirable to develop a method that could be

used in the field for routine analysis of carotenoid-rich *M. cochinchinensis* arils for future breeding programs.

The simple and rapid colorimetric method could be used to estimate or screen lycopene rich *M. cochinchinensis* in the field because the $(a*/b*)^2$ and a*b* colour space values were significantly correlated with the lycopene concentrations as determined by the chromatographic (HPLC and UPLC) and spectroscopic methods (Table 3.2). The $(a*/b*)^2$ colour space value has been used to compare lycopene concentrations among different genotypes (Hyman et al., 2004) and can be used to select lycopene rich *M. cochinchinensis* varieties. Although, the accuracy and specificity of this method was low compared to chromatographic method, this simple method is valuable to select lycopene rich fruits in the field as well as in commercial products and suitable in technologically challenged situations. Furthermore, colorimetric method has added advantages over chromatographic and spectroscopic methods in that it requires less time, is cost efficiency and did not require technical expertise and hence, can be used for field applications (Table 3.3). Although previous studies have reported that the b*, chroma and hue values were significant parameters for estimating the β-carotene concentrations in mangoes (Rungpichayapichet et al., 2015), apricots (Ruiz et al., 2008) and tomatoes, those values were not suitable for *M. cochinchinensis*. This might be due to the high lycopene content which provides the prominent red colour and shadows the orange colour provided by β-carotene.

Table 3.3: Advantages and disadvantages of methods used for the analyses of carotenoids (lycopene and β- carotene) in *M. cochinchinensis*.

Parameter	HPLC	UPLC	Spectrophotometry	Colorimetry
Time				
Extraction	+++	+++	++	+
Analysis	+++	++	++	+
Cost				
Solvent	+++	++	+++	-
Instrument	+++	+++	+++	+
Standards	++	++	-	-
Sample volume	+++	+	+++	-
Technical expertise	+++	+++	++	-
Quantification	+++	+++	+	-
Accuracy	+++	+++	++	-
Specificity	+++	+++	++	-
Linearity	++	+++	++	-
Precision	+++	++	++	-

+++: High, ++: Moderate, +: Less, -: Not required

3.5   Conclusion

The carotenoids in *M. cochinchinensis*, lycopene and β-carotene, were measured by chromatography (HPLC and UPLC), spectrophotometry and colorimetry methods. Both HPLC and UPLC methods accurately determined both lycopene and β-carotene concentration in *M. cochinchinensis* but UPLC had advantages of a lower analysis time and a lower solvent cost per sample. Spectrophotometry also can be used to estimate lycopene concentrations in *M. cochinchinensis* aril but it over estimated the β-carotene concentration and required large amounts of solvents. However, colorimetry could only be used to predict the lycopene concentration in the *M. cochinchinensis* fruits since the correlation factor was <68% but has the advantages of cost effectiveness, solvent free, did not require technical expertise and can be used for fast processing of mass quantities.

CHAPTER 4

Influence of eco-geographical distribution and climatic factors on carotenoid content of *Momordica cochinchinensis*

4.1 Introduction

Carotenoids are fat soluble pigments synthesized only in plants, photosynthetic bacteria and fungi (Giovannucci et al., 1995, Stahl and Sies, 1996, Baysal et al., 2000) through the mevalonic acid pathway using an isopentenyl pyrophosphate (IPP) precursor (Rosati et al., 2000). The main carotenoids are lycopene and β-carotene, which are the compounds responsible for the red and yellow-orange coloration, respectively. Both lycopene and β-carotene have numerous bio-medicinal and nutraceutical benefits (Rao and Rao, 2007). Both pigments are highly effective antioxidants due to their conjugated double bond system and free radical scavenging activity. Amongst carotenoids, lycopene has the highest singlet oxygen-quenching rate tested from biological systems (Perkins-Veazie et al., 2001) and is responsible for the reduction of cancers, cardiovascular diseases and macular dysfunction (Rao and Agarwal, 1999, Britton and Khachik, 2009, Kubola et al., 2013). Lycopene is the precursor of most cyclic carotenoids including β-carotene, which converts to produce vitamin A, an important requirement for the development of cell membranes, immunity and skin health (Rodriguez-Amaya and Kimura, 2004, Britton and Khachik, 2009, Hammond and Renzi, 2013).

M. cochinchinensis had the highest content of dietary β-carotene and lycopene, with levels 10-70 fold more than all known fruits and vegetables (Aoki et al., 2002, Vuong, 2004) including tomatoes (8.8-42.0 µg/g FW lycopene) (Rao and Rao, 2007) and carrots (47-11,210 µg/g FW β-carotene) (Gul et al., 2015). Despite its superior carotenoid content and phytochemical potential (Tables 1.7 and 1.8), *M. cochinchinensis* is genetically diverse and geographically restricted (Bootprom et al., 2012, Wimalasiri et al., 2015); it is not widely distributed outside of within South East Asia (Aoki et al., 2002). There is limited information on varietal differences and downstream effects on carotenoid accumulation, hindering the selection of breeding stock.

Carotenogenesis in other economically important fruits, including apricots (Dragovic-Uzelac et al., 2007), carrots (Leja et al., 2013) and tomatoes, has been influenced by geographical and environmental factors, including elevation (Rodriguez-Amaya and Kimura, 2004), rainfall (Brandt et al., 2003) and temperature (Leoni, 1991, Dumas et al., 2003), with carotenoid bio-accessibility impacted more by geographical location than varietal differences (Aherne et al., 2009) or vice versa (Leja et al., 2013). *M. cochinchinensis* has a diverse distribution in South East Asia from tropical to temperate regions with varying eco-geographical profiles (Wimalasiri et al., 2015), which could account for the nutritional variations previously reported (Reviewed in chapter 1.4). Cultivar selection and optimal growth conditions are essential for producing consistently higher quantities of carotenoids but in the case of *M. cochinchinensis* these parameters are unknown.

The carotenoids in *M. cochinchinensis* are commonly quantified using High Performance Liquid Chromatography (HPLC) (Ishida et al., 2004, Vuong et al., 2006, Kubola and Siriamornpun, 2011) but this standardised methodology is not available to primary producers in developing nations where *M. cochinchinensis* is endemic. Other analytical methods such as spectrophotometry or colour evaluation assays (Barba et al., 2006) could be used for rapid screening since they are simple, cost effective and routinely used in the quantification of lycopene and β-carotene in other common fruits and vegetables (Biehler et al., 2010, Fish, 2012, Soroka et al., 2012)..

The aims of this chapter were firstly, to elucidate the effect of geographical distribution of *M. cochinchinensis* on carotenoid content to assist in the breeding selection of superior cultivars that are morphologically and genetically diverse (Wimalasiri et al., 2015). Secondly, to investigate the eco-geographical factors that lead to high lycopene and β-carotene content so that these could be replicated in existing farms or locations selected to ensure consistent carotenoid content. Lastly, to determine the effect of detection methods on the variation of the carotenoid (lycopene and β-carotene) content of *M. cochinchinensis* to assist selection of carotenoid-rich fruits in resource-poor or technologically challenged situations.

4.2 Materials and methods

4.2.1 Sample collection

Fruits, seeds and leaves of 53 samples were collected from Thailand, Vietnam and Australia (Table 2.1 and 2.2). However, only 44 samples, belonging to 17 provinces of Vietnam, Thailand and Australia had comparable mature fruits and were suitable for the carotenoid analysis (Table 1.4). These fruits were processed as described in chapter 2. The morphological and genetic signatures of all the samples were described in chapter 2.

Table 4.1: Geographical and environmental climate of *M. cochinchinensis* from Vietnam (southern, northern and central), Thailand and Australia. Bioclimatic data was obtained from DIVA-GIS spacial analysis software (Hijmans et al. 2004).

Country	Province	Sample	Latitude	Longitude	Elevation (m)	Annual temperature ^{o}C			Precipitation (mm)			Climate*
						Max	Min	Difference	Annual	Wettest month	Driest month	
S Vietnam	Can Tho	VS1	10.034320	105.775130	1	32.6	22.2	10.4	1524	254	2	Tropical
	Can Tho	VS2	9.996850	105.665870	3	32.4	22.0	10.4	1623	269	3	Tropical
	Vinh Long	VS3a,3b	10.248758	105.969566	2	33.2	21.9	11.3	1413	244	2	Tropical
	Tra On	VS4	9.965880	105.925140	3	32.5	22.1	10.4	1520	259	2	Tropical
	HCM City	VS7	10.757410	106.673439	6	34.7	21.1	13.6	1851	310	2	Tropical
N Vietnam	Hung Yen	VN8	20.814159	106.125992	4	33.1	14.4	19.0	1679	316	15	Temperate
	Hung Yen	VN9a,9b	21.648399	105.028999	71	32.2	11.6	20.6	1774	325	18	Temperate
	Hai Duong	VN10	20.897711	106.423561	3	32.8	14.2	18.6	1648	311	17	Temperate
	Hai Duong	VN11	20.919382	106.419062	3	33.0	13.5	19.5	1692	312	14	Temperate
	PhuTho	VN12	21.331640	104.953453	296	30.8	10.5	20.3	1574	300	10	Temperate
	PhuTho	VN13-15	21.445990	105.094419	296	32.3	11.3	20.9	1734	315	19	Temperate
	Ha Noi	VN16-20	20.866699	105.750000	9	33.0	13.5	19.5	1700	314	14	Temperate
	Ha Noi	VN21	20.985260	105.858501	9	33.1	13.5	19.6	1673	324	15	Temperate
	Hoa Binh	VN22	20.829601	105.332893	195	31.6	11.7	19.9	1801	352	8	Temperate
	Nam Dinh	VN24-27	20.422930	106.173630	4	33.4	14.7	18.7	1663	336	27	Temperate
C Vietnam	Lam Dong	VC28,29	11.835772	108.336275	1112	27.9	13.3	14.6	1651	248	7	Temperate
	Lam Ha	VC32	11.793576	108.268610	925	28.1	13.8	14.3	1658	252	8	Temperate
Thailand	Nakhon pathom	T1,T3,T4	13.828790	100.039620	8	35.9	18.7	17.2	1237	257	6	Tropical
	Samut Prakan	T5	13.576110	100.665420	2	34.5	20.4	14.1	1439	325	10	Tropical
	Chiang Mai	T6,T8	18.767000	99.026161	316	36.2	12.9	23.3	1163	244	7	Temperate
	Chanthaburi	T7	12.878721	102.129135	79	32.0	17.4	14.6	1751	316	10	Tropical
Australia[a]	Newcastle	A5-A12	33.212281	151.225069	NA	25.0	18.0	NA	NA	NA	NA	Tropical

[*]: Regions with a minimum temperature < 15°C in coldest month was selected as temperate climate.

[a]: Plants grown in greenhouse condition with average temperature of 25°C

4.2.2   Extraction of carotenoids

Carotenoids of the *M. cochinchinensis* aril were extracted as previously described (Barba et al., 2006). The aril (2 g) was placed in a vessel protected from sunlight and mixed with 100 mL of extraction solvent (hexane/acetone/ethanol: 50:25:25 v/v/v) (Barba et al., 2006). The mixture was ultra-sonicated (Unisonics, Australia) for 30 min and then 15 mL of distilled water was added to enhance phase separation, whereby the upper hexane layer contained the carotenoids and the bottom water layer contained hydrophilic compounds and cell debris. 1 mL of the upper organic layer was used for UV-Visible spectroscopic analysis; 10 mL of the remaining hexane extract was evaporated to dryness using a rotary evaporator (Büchi Labortechnik AG, Australia). The residue was dissolved in THF/acetonitrile/methanol: 50:25:25 v/v/v to a final volume of 4 mL for HPLC analysis. The final extract was filtered through a 0.45 µm PTFE membrane filter (Labquip, Australia) and a 20 µL injection was employed for HPLC analysis.

4.2.3   Analyses of carotenoids

4.2.3.1   High Performance Liquid Chromatography (HPLC)

The HPLC analysis was performed as described in section 3.3.1

4.2.3.2 UV- Visible spectrophotometry

The UV-Visible spectrophotometry analysis was performed as described in section 3.3.3.

4.2.3.3 Colorimetry

The colorimetry analysis was performed as described in section 3.3.4

4.2.4 Statistical analysis

All analytical experiments were performed in triplicate and the values for the contents of lycopene and β-carotene were analysed using the Minitab statistical software (version 16). For normally distributed data, the means were compared using the one-way analysis of variance (ANOVA) with Fisher's test post-hoc. Least significant differences were calculated using the equation:

$$LSDx = t_{x \, (df \, for \, MSE)} \sqrt{2MSE(\frac{1}{Ny})}$$

where t is the critical value of the t distribution table; MSE is the mean square error, obtained from the results of the ANOVA test; x is the desired level of possibility and y is the number of replicates. Non-normally distributed data were transformed or analysed using the non-parametric Kruskal Wallis test and the significance difference between the means

were analysed using Fisher's post-hoc test. Statistical values of P≤0.05 were considered as significantly different. 36 *M.cochinchinensis* samples grown in its natural habitat in Vietnam (southern, central and northern) and Thailand was used to correlate carotenoid contents with eco-geographical factors. The Pearson's correlation was used to determine whether the carotenoids correlated with any of the eco-geographical factors (latitude, longitude, maximum temperature, minimum temperature, annual temperature range, annual precipitation, rainfall of wettest and driest month and elevation) or with the different analysis methods used (HPLC, UV-Vis spectrophotometry and colorimeter). The *r* value represented the correlation coefficient between two variables (1= total positive correlation, 0 = no correlation, -1 = total negative correction) and the statistically significant level was set at p≤0.05. Principal component analysis (PCA) for lycopene and β-carotene content of 44 *M. cochinchinensis* samples was carried out using Minitab statistical software (version 17).

4.3   Results

4.3.1   The effect of geographical distribution on carotenoid content in *M. cochinchinensis*

The carotenoid content of all tested samples was highly variable and ranged from 0.88 to 6.30 mg/g for lycopene and 0.25 to 5.70 mg/g for β-carotene (Figures 4.1 and 4.2). The highest lycopene content was observed in samples collected from the Lam Ha (6.30 mg/g) and Lam Dong (6.20 mg/g) provinces of Central Vietnam as detected by both HPLC

and UV-visible spectrophotometry. These samples were found to be more than seven times higher in lycopene content than those from the Chanthaburi province (0.88 mg/g) in Thailand (Figure 4.1). These higher lycopene levels had the maximum $(a^*/b^*)^2$ values, which corresponded to dark red aril, and were common in samples from Central and Northern Vietnam (Figures 4.1 and 4.3). The highest β-carotene content was observed in samples from a different province, specifically from Hoa Binh (5.70 mg/g) in Northern Vietnam, as detected by both HPLC and UV-visible spectrophotometry, and was more than 22 times higher than that of samples from the Samut Prakan province (0.25 mg/g) of Thailand (Figure 4.2). High levels of β-carotene did not correspond with the $(a^*)^4 \times 10^6$ colour values and the colouration of freshly extracted and frozen aril was visually difficult to compare (Figure 4.3).

The proportions of lycopene and β-carotene were highly variable and not accumulative or synergistic with the total carotenoid content. Some fruits possessed high lycopene and low β-carotene (Lam Ha 6.30 mg/g lycopene and 1.80 mg/g β-carotene) whilst others had equally high (Hoa Binh 5.10 mg/g lycopene and 5.70 mg/g β-carotene) or equally low (Chanthaburi 0.90 mg/g lycopene and 0.80 mg/g β-carotene) carotenoids. The fruits from most regions sampled contained more lycopene than β-carotene (15 of 17 regions), with the highest difference being a 10-fold higher lycopene than β-carotene content in samples from Samut Prakan region.

The PCA analysis grouped the three Central Vietnam samples together (VC28, VC29 and VC32) and collectively contained the highest lycopene concentrations, in

agreement with the UPGMA cluster analysis (Figures 4.4, 2.6 and 2.7). Samples from Southern Vietnam, Thailand and Australia was grouped together (Figure 4.4) but the T1 and T3 samples were separated from the other Thailand samples due to the high β-carotene levels. The highest variation in lycopene and β-carotene concentration was observed in Northern Vietnam samples and was in agreement with genetic and morphological clusters (Figures 4.4, 2.5, 2.6 and 2.7).
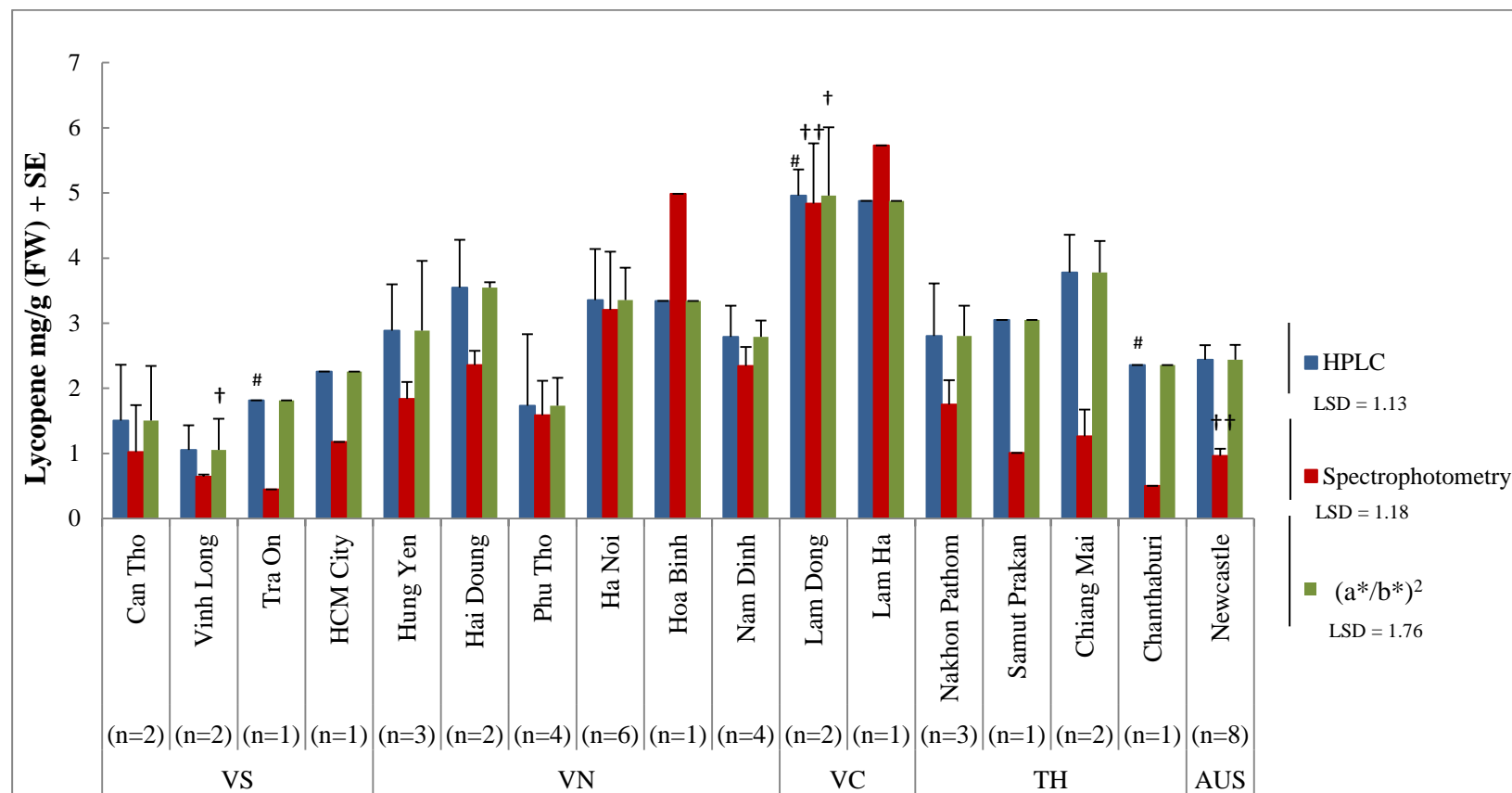
Figure 4.1: Lycopene content of *M. cochinchinensis* samples collected from different provinces and districts of Australia (AUS), Thailand (TH) and Vietnam (northern=VN, central=VC and southern=VS) as measured by HPLC, UV-visible spectrophotometry and $(a^*/b^*)^2$ colour value. Fisher's LSD (least significance differences) values were generated from $\log_{10}$ transformed data of lycopene detected by HPLC (df= 16, F= 2.72, p=0.01), UV- Visible spectrophotometry (df= 16, F= 2.1, p=0.042) and untransformed data of $(a^*/b^*)^2$ colour value (df= 16, F= 4.18, p=0.024). #, †† and † indicated statistical significance at p≤0.05 for lycopene compared with HPLC, spectrophotometry and $(a^*/b^*)^2$, respectively based on fruit collection sites.
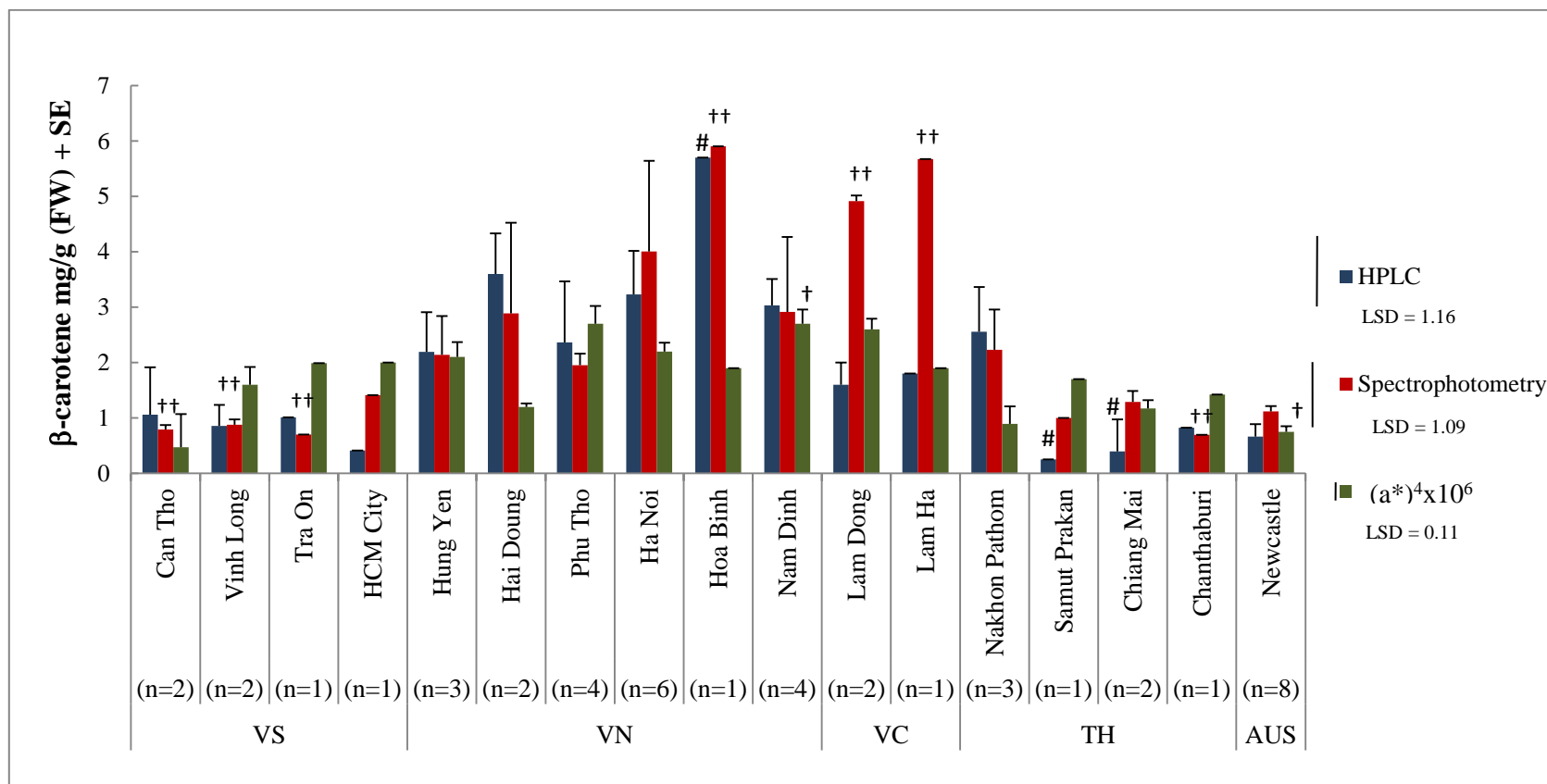
Figure 4.2: β-carotene content of *M. cochinchinensis* samples collected from different provinces and districts of Australia, Thailand and Vietnam (northern, central and southern) as measured by HPLC, UV-visible spectrophotometry and $(a^*)^4 x10^6$ colorimetry value. Fisher's LSD (least significance differences) values were generated from log_{10} transformed data of β-carotene detected by HPLC (df=16, F=4.18, p≤0.001) and UV-visible spectrophotometry (df=16, F=7.37, p≤0.001) and untransformed data of $(a^*)^4$ colour value (df= 16, F= 6.73, p≤0.001). #, †† and † indicated statistical significance at p≤0.05 for β-carotene compared with HPLC, spectrophotometry and $(a^*)^4 x10^6$, respectively based on fruit collection sites.
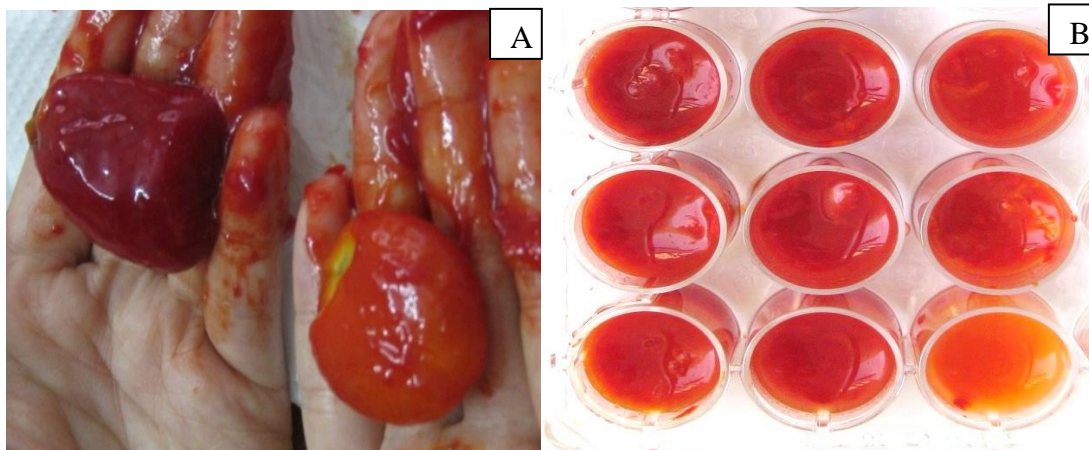
Figure 4.3: Colour differences of the aril of *M. cochinchinensis* fruits. (A) Fresh aril showing red (left) and orange (right) colouration from Tra On province. (B) Water homogenate of frozen samples showing red (top left corner) and orange (bottom right corner) colouration.
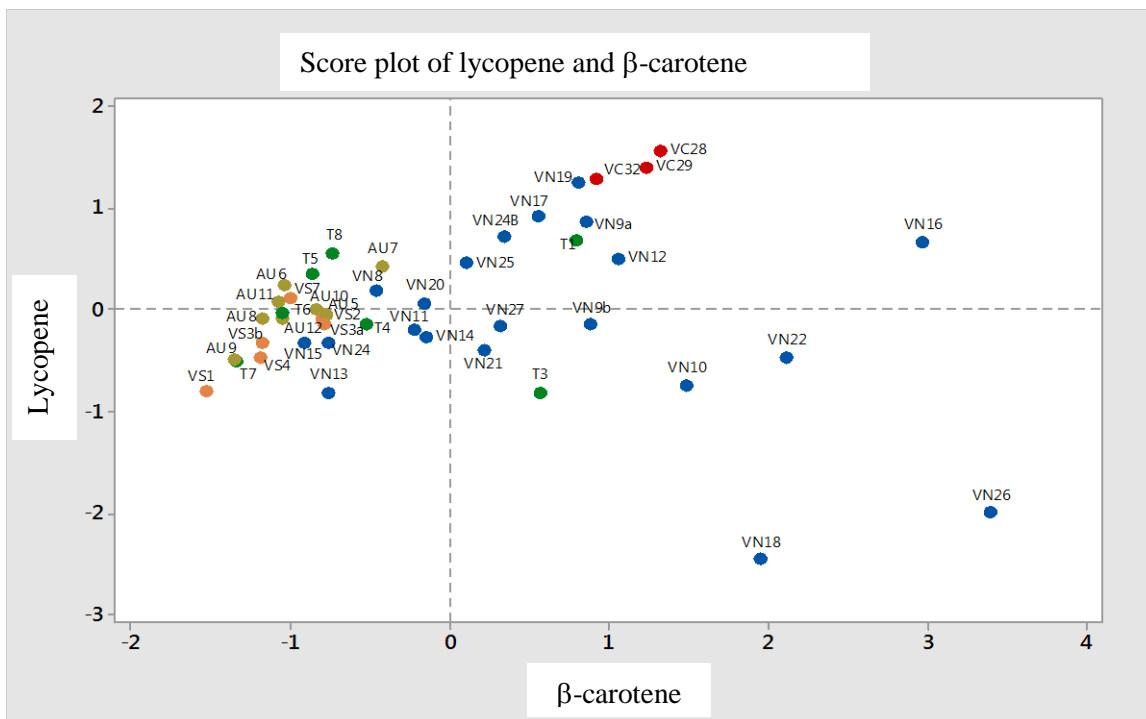
Figure 4.4: Score plot of lycopene and β-carotene of 44 *M. cochinchinensis* samples collected from Vietnam, Thailand and Australia constructed using the Principal Component Analysis method (PCA) with Minitab statistical software (Version 17). Country of collection was indicated as different colours; orange=Southern Vietnam, blue=Northern Vietnam, red=Central Vietnam, green= Thailand and olive green=Australia.

4.3.2   The effect of environmental factors

The samples from Australia was excluded from analysis to determine the correlation between carotenoid content and eco-geographical factors since they were grown in controlled environments compared to the samples from Vietnam and Thailand which were grown in their natural habitats. Of the seven environmental factors investigated in this study, lycopene accumulation was influenced by temperature and elevation whilst β-carotene was influenced by temperature and precipitation, irrespective of geographical distribution and provenance eco-profiles (Table 4.2).

Temperate cooler climates with low temperatures, high temperature fluctuations and high elevations were correlated with a high lycopene content (Table 4.2 and Figure A3). The highest lycopene content was observed in samples from Northern and Central Vietnam, which are cooler regions with low minimum temperatures (<14°C) and high temperature ranges (>18°C) (Table 4.1 and Figure 4.1). The correlation between minimum temperature and lycopene concentration was moderate, with an r^2 value of 0.28, which can interpret that 28% of the variability of lycopene concentration in *M. cochinchinensis* was due to the minimum temperature (Table 4.2). Samples from high elevations correlated with a high lycopene content, such as those from Lam Dong and Lam Ha, which are situated at 925-1112 m above sea level (Table 4.1 and Figure 4.2). In comparison, a low content of lycopene was seen in samples from Southern Vietnam, which has high minimum temperatures (>20°C) reminiscent of tropical climates and consequently low annual temperature ranges (<11°C) and low elevations (1-6 m).

The β-carotene content was significantly reduced in warmer tropical climates where the maximum temperatures exceeded 34°C (P<0.05, *r*=-0.416) (Table 4.2 and Figure A5) (e.g. Samut Prakan and HCM city). In contrast, a high β-carotene content was seen in samples from provinces that have cooler temperate climates (e.g. Lam Dong and Lam Ha). However, the β-carotene content was not correlated with elevation (Tables 2.1 and 4.2 and Figure 4.2). A high β-carotene content was also inversely correlated with moist conditions as measured by the annual precipitation (P<0.05, *r*=-0.400) and the precipitation during the wettest (P<0.05, *r*=-0.402) and driest month (P<0.05, *r*=-0.371) (Figure A4).

Table 4.2: Pearson correlation coefficients (*r*) and coefficient of determination (r^2) of carotenoids between eco-geographical factors (maximum and minimum temperature, annual temperature range, elevation and precipitation) and the carotenoid content of 44 *M. cochinchinensis* samples as measured by HPLC.

Factor	Details	Correlation coefficient (*r*)		Coefficient of determination (r^2)	
		Lycopene	β-carotene	Lycopene	β-carotene
Temperature	Minimum	-0.53[*]	-0.27[NS]	0.28	0.07
	Maximum	0.32[NS]	-0.42[*]	0.10	0.17
	Range	0.40[*]	0.28[NS]	0.16	0.08
Elevation	Elevation	0.38*	-0.06[NS]	0.14	0.00
Precipitation	Annual	0.25[NS]	0.40[*]	0.06	0.16
	Driest month	0.29[NS]	0.40[*]	0.09	0.16
	Wettest month	0.21[NS]	0.37[*]	0.04	0.14

[*]: Significant at P<0.05
[NS]: Not significant P>0.05

4.3.3   Comparisons between quantification assays

The lycopene concentration estimated by UV-Visible spectrophotometry and colorimetry methods was not significantly different (p<0.05) from HPLC. The estimated concentration of lycopene by UV-Visible spectrophotometry (r=0.802, p≤0.001) and $(a*/b*)^2$ value of colorimetry, (r=0.734, P≤0.001) was significantly correlated with HPLC (Table 4.3 and Figures 4.1 and A5). High lycopene content was confirmed by all three analyses and the samples with the highest lycopene content were consistently from the Central Vietnam regions, especially from Lam Dong and Lam Ha provinces. In contrast, low content of lycopene was inconsistent between the analyses with colour values of aril from Tra On and Chanthaburi being more than double the values obtained from chromatography and spectrophotometry quantification (Figure 4.2).

With respect to β-carotene quantification, there was a low correlation between the spectrophotometry and chromatography (r=0.49, p≤0.001) (Table 4.3 Figure A5) results. Both methods agreed in that the sample with the highest β-carotene content was from Hoa Binh province (Figure 4.2). Provinces in central Vietnam also produced fruit with a high β-carotene content (Lam Dong and Lam Ha provinces). However, this was only detected by spectrophotometry and not by HPLC, with discrepancies in the magnitude of 3-4 mg (Figure 4.2). The raw and transformed colour values detected by colorimetry had a low correlation with HPLC (Table 4.3 and Figure A5) resulting in trend lines that underestimated the β-carotene content by more than half in the sample from Hoa Binh province (Figure 4.2).

Table 4.3: Pearson correlation coefficients (*r*) between colorimetry, spectrophotometry and chromatography (HPLC) of 44 *M. cochinchinensis* samples.

Analytical test	Details	Correlation (r) with carotenoids (HPLC)	
		Lycopene	β-carotene
Colorimetry	L*	-0.512[**]	-0.031[NS]
	a*	0.311[**]	0.408[**]
	b*	-0.412[**]	0.127[NS]
	a*/b*	0.686[**]	0.069[NS]
	$(a*/b*)^2$	0.734[**]	0.063[NS]
	Hue	-0.161[NS]	-0.017[NS]
	Chroma	-0.216[NS]	0.188[NS]
	$(a*)^2$	0.302[**]	0.418[**]
	$(a*)^4$	0.288[NS]	0.427[**]
	$(a*)^{0.5}$	0.315[**]	0.402[**]
	a*b*	-0.216[NS]	0.283[NS]
Spectrophotometry	502 nm	0.802	NA
	450 nm	NA	0.490

[**] : Significant at P<0.05
[NS] : Not significant at P>0.05
NA: Not available

4.4   Discussion

4.4.1   The effect of geographical distribution on carotenoid content in *M. cochinchinensis*

The geographical distribution of the collected *M. cochinchinensis* fruit was related to the carotenoid content, with the best levels observed in samples from specific provinces; the diversity of carotenoid level was vast, ranging from 0.88-6.30 mg/g for lycopene and 0.25-5.70 mg/g for β-carotene. The carotenoid content was significantly different between provinces with the highest lycopene levels found in the samples from Central Vietnam in Lam Ha province and the highest β-carotene levels measured in the

samples from Northern Vietnam in Hoa Binh province. This carotenoid content was related to that of the morphology from the same provinces, with a notable character being that Central Vietnam samples had the heaviest and largest seeds (Wimalasiri et al., 2015). The same samples were genetically and morphologically clustered (Wimalasiri et al., 2015), and was reflected in PCA analysis with high lycopene and β-carotene contents. The highest diversity in lycopene and β-carotene concentrations were seen in the samples from Northern Vietnam and was in agreement with genetic and morphology clustering (Figures 2.5, 2.6 and 2.7). However, this diversity might be attributed by the larger number of Northern Vietnamese samples analysed in this study. Interestingly, the samples collected from Australia had similar levels of lycopene and β-carotene as did the Southern Vietnam samples and was grouped together indicating that the variety might have an influence on the nutrition content. However, the Thailand samples were not separated from the samples from Australia and Southern Vietnam as did for the genetic and morphology clustering indicating that there might be other factors, such as soil nutrition, micro-climatic differences and limited sampling per each location that are responsible for variations in carotenoids.

All samples analysed were genetically diverse based on molecular markers (Wimalasiri et al., 2015). However, there is no apparent genetic marker identified for lycopene content in *M. cochinchinensis*, which could make the fruit produce consistent higher yields of lycopene as observed for samples from Lam Ha province compared with Chanthaburi province. Studies on other carotenoid rich foods, such as sweet potato, pumpkin squash and tomato, have also found that the genotype and variety determined the lycopene, phenolic compound and antioxidant superiority of these fruits (Abushita

et al., 2000, Martínez-Valverde et al., 2002, Rodriguez-Amaya and Kimura, 2004, Lenucci et al., 2006, Guil-Guerrero and Rebolloso-Fuentes, 2009, Chandra et al., 2012). However, this is contradictory to other studies, which have suggested that the same varieties grown in separate countries with different climates had a diverse carotenoid content, indicating that geographical location had a greater impact than the variety (Rodriguez-Amaya and Kimura, 2004, Nishiyama et al., 2005, Sass-Kiss et al., 2005, Bergquist et al., 2006, Raffo et al., 2006, Maiani et al., 2009). This information on varietal differences and downstream influences on carotenoid accumulation was previously unknown for *M. cochinchinensis*. Further experiments are pivotal to elucidate if genetically identical varieties could be encouraged to produce higher lycopene levels for this potentially valuable crop. This information will be imperative for primary producers to facilitate the conservation, sharing and breeding of better varieties of *M. cochinchinensis* with consistently higher carotenoids.

4.4.2 The effect of environmental conditions

Of the seven environmental factors analysed, the temperature, elevation and precipitation were related to the carotenoid content of the *M. cochinchinensis* aril and therefore, they may have influenced the specific metabolic pathways for the production of lycopene, β-carotene or both. The optimum temperatures for lycopene synthesis are known to be between 16°C and 21°C for tomatoes (Martínez-Valverde et al., 2002) and, at temperatures above 30°C, lycopene is known to be converted to β-carotene (Tomes, 1963, Dumas et al., 2003, Nishiyama et al., 2005, Brandt et al., 2006). Therefore, high temperatures may have caused the low lycopene content observed in the samples from tropical Southern Vietnam and Thailand provinces,

where the maximum temperatures exceed 30°C compared to the samples from the temperate regions of Central Vietnam where the temperature does not exceed 28°C. Low temperatures at high altitudes with high UV radiation, particularly in Central Vietnam where the varieties are grown in mountainous provinves, may explain the high levels of carotenoids. This was consistent with other studies, which indicated that low temperatures, high altitudes and high UV radiation increased secondary metabolite synthesis in other fruits such as tomato, grape berry and passionfruit (Rodriguez-Amaya and Kimura, 2004, Pereira et al., 2006, Jaakola and Hohtola, 2010, Cechin et al., 2012). The influence of geography and climate has been found to override genetic control in tomatoes (Aherne et al., 2009) and this finding has been implemented using seasonal and environmental manipulations to improve lycopene synthesis and bio-accumulation (Rodriguez-Amaya and Kimura, 2004, Nishiyama et al., 2005, Sass-Kiss et al., 2005, Bergquist et al., 2006, Raffo et al., 2006, Maiani et al., 2009).

Interestingly, a *M. cochinchinensis* variety originated from Southern Vietnam (Wimalasiri et al., 2015) but grown in temperature-controlled glasshouses at 25°C in Australia did not produce higher levels of lycopene than the samples collected from Southern Vietnam. This suggests that variables other than temperature may be involved in carotenoid synthesis and bioaccumulation in *M. cochinchinensis*. Since there is a lack of genetic markers to predict higher carotenoid content, eco-geographical contributions could assist primary producers to maximise the carotenoid production. More experiments are currently underway with the same genotypes

growing in different provinces to determine the dominance of eco-geographic versus genetic influences on lycopene accumulation.

4.4.3 Comparisons between quantification assays

The estimation of lycopene in the aril of *M. cochinchinensis* was consistent across the chromatographic, colorimetric and spectrophotometric methods. All the methods were in agreement and indicated that the lycopene content was the highest in the samples collected from Central Vietnam. This indicates the potential applicability of plants from Central Vietnam as breeding stock to improve the lycopene content in the field.

High performance liquid chromatography (HPLC) is the standard method to quantify carotenoids in fruits and vegetables but it is too costly and inefficient for resource poor farmers and even for use in commercial scale quality control. The use of colorimetry and spectrophotometry to estimate lycopene is more feasible and this has been implemented in field applications for tomatoes (D'Souza et al., 1992, Arias et al., 2000, López Camelo and Gómez, 2004) and cucurbits such as pumpkins and squash (Itle and Kabelka, 2009). These methods have been used to detect differences among genotypes (Hyman et al., 2004) and could be used for *M. cochinchinensis* in future field assays for lycopene.

In comparison, the estimation of β-carotene was consistent with spectrophotometry but not colorimetry, compared with HPLC. The inconsistency of the colorimetry results may be due to interference from a high lycopene content. This has

been confirmed in other carotenoid studies when lycopene, xanthophyll and chlorophyll were co-localised in homogenates (D'Souza et al., 1992, Arias et al., 2000, Hyman et al., 2004, Barba et al., 2006, Khoo et al., 2008, Fish, 2012). Therfore, the colorimetry method may require additional separation, fractionation and purification for the accurate quantification of β-carotene in *M. cochinchinensis*.

4.5   Conclusion

This study confirms the nutritional quality of *M. cochinchinensis* from wild and cultivated samples obtained from Vietnam and Thailand. The fruit contained up to 200 times more lycopene than hydroponic tomatoes (Javanmardi and Kubota, 2006) and 54 times more β-carotene than carrots (Gul et al., 2015). The carotenoid levels in this study were more than expected; they were 3 times higher in lycopene and 8 times higher in β-carotene than the highest content previously reported for *M. cochinchinensis* (Aoki et al., 2002, Ishida et al., 2004, Kubola and Siriamornpun, 2011). Varieties with high carotenoids (lycopene and β-carotene) and grown under optimal eco-geographical parameters (optimum temperature, elevation and precipitation) were identified. These findings will be pivotal for the agricultural development of this fruit for the food and nutriceutical industries.

CHAPTER 5

Anticancer activity of *Momordica cochinchinensis* aril extracts on breast cancer and melanoma cells

5.1 Introduction

Cancer is a disease characterised by uncontrolled cell growth and proliferation initiated by inappropriate cell division. It is categorised as the second leading cause of death, with 14.1 million estimated cases in 2015 and causing a major health problem in both developed and developing countries in the world (Torre et al., 2015). Breast cancer is the second largest cancer type in the world and the most common malignancy among women (Ayob et al., 2014). However, Australia and New Zealand have the highest incidence and mortality rates from cutaneous melanoma in the world mainly due to exposure of the skin to high UV radiation (Sneyd and Cox, 2013). A rise in the incidence and mortality rates of these cancers has led to an increased emphasis on drug development, public health policies and awareness programs for reducing cancer (Stracci et al., 2005, Ayob et al., 2014).

More than 80% of the world's population consider traditional plant derived medicine as their source of primary health care (Bhanot et al., 2011, Figueroa et al., 2012). Thus, there is a continuing need for the development of new anticancer drugs and/or drug combinations, through methodical and scientific exploration of the enormous pool of plant based products. Currently, the most common approaches for

treating cancer include chemotherapy, surgery and radiotherapy (Jones et al., 2004). However, non-surgical therapies are associated with toxicity due to non-selective targets (Mukherjee et al., 2001). Plant-derived medicines have a long history of use in the treatment of cancer and over 60% of currently used anti-cancer agents are from natural sources (Lin, 2002, Gordaliza, 2007, Bhanot et al., 2011).

Fruits and vegetables are rich sources of phytochemicals with antioxidant, immune-modulatory and anti-cancerous properties (Wang et al., 1996, Cohen et al., 2000, Richman et al., 2012). Understanding the mechanism of cell death induced by phytochemicals is a key step in the development of a chemo-preventive or chemo-therapeutic drug. Phytochemicals can induce cell death by two well-known mechanisms: necrosis or apoptosis. These two forms of cell death differ from each other and can be distinguished from each other using morphology (time-lapse microscopy, flow fluorocytometry, and transmission electron microscopy), cell surface markers (phosphatidylserine exposure versus cell permeability by flow fluorocytometry), intracellular markers (oligonucleosomal DNA fragmentation by flow fluorocytometry, caspase activation, and cytochrome c release by western blotting) and intra-cellular markers (caspases, HMGB-1 and cytokeratin 18) (Vermes et al., 1995, Renvoizé et al., 1998, Hengartner, 2000, Saraste and Pulkki, 2000, Krysko et al., 2008).

The fruit mesocarp and aril of *Momordica cochinchinensis* is rich in phytochemicals such as carotenoids, flavonoids and phenolics with potential pro-vitamin A, antimicrobial and anticancer activities (Chuyen et al., 2015). The aril contains high levels of carotenoids such as lycopene and β-carotene (Kubola and Siriamornpun, 2011). These carotenoids are the most bio-active carotenoids present in

nature and possess antioxidant, anti-inflammatory, cardio protective and anticancer effects (Rao and Agarwal, 1999, Kris-Etherton et al., 2002, Weisburger, 2002, Mordente et al., 2011). A water extract of aril is known to have anticancer activity against colon cancer *in vivo* and *in vitro* by inducing necrosis (Tien et al., 2005). An unknown 35 kDa protein was shown to be the active compound of the extract (Tien et al., 2005). However, so far, it is unknown whether the water extract has the same activity on other cancer cells including breast cancer and melanoma.

M. cochinchinensis is genetically different and grown in diverse eco-geographical conditions (Wimalasiri et al., 2015). This might result in variation of the phytochemical composition in the fruits, which may have an effect on anticancer activity. Phytochemicals are produced as a plant's defence mechanism, helping it adapt to both micro and macro environments, such as water stress, temperature stress, UV light and disease (Li et al., 2013). These metabolites can be significantly influenced by many intrinsic and external factors, such as genetic differences within species, stage of growth and development, soil fertility, availability of water and light, competition with neighbouring plants and interactions with pathogens and parasites, such as bacteria, fungi, viruses and nematodes (Szakiel et al., 2011). Understanding the variability in anticancer activity of *M. cochinchinensis*, based on collection sites and their climatic factors, will be important for plant selection, conservation and for the production of fruits with consistent anti-cancer activity.

The aims of this chapter were firstly, to investigate the cytotoxicity effect of water and hexane/acetone/ethanol (2:1:1) aril extracts of *M. cochinchinensis* on melanoma (MM418C1 and D24) and breast cancer (MCF7 and BT474) cell lines.

Secondly, to determine the mechanism of cell deaths caused by the most active extract. Thirdly, to compare the anticancer activity of aril samples of *M. cochinchinensis* collected from Thailand, Vietnam and Australia.

5.2 Materials and methods

5.2.1 Sample collection

The aril of *M. cochinchinensis* samples were processed as described previously in chapter 2. The aril of the VC29 sample (Lam Dong, Central Vietnam) was used in cytotoxicity and apoptosis assays as well as morphological analysis (Table 5.1). Selected arils of 15 mature fruits from Thailand (n=3), Southern Vietnam (n=3), Central Vietnam (n=3), Northern Vietnam (n=3) and Australia (n=3) were used to compare their cytotoxic effects (Table 5.1).

Table 5.1: Eco-geographical factors and carotenoid content of *M. cochinchinensis* from Vietnam (southern, northern and central), Thailand and Australia. Bioclimatic data was obtained from DIVA-GIS spacial analysis software.

Country	Province	Sample code	Altitude (m)	Temperature °C Maximum	Minimum	Annual range	Precipitation (mm) Annual range	Annual	Wettest month	Driest month	Carotenoids mg/g Lycopene	β-carotene
S Vietnam	Can Tho	VS1	1	32.6	22.2	10.4	10.4	1524	254	2	0.3	1.0
	Vinh Long	VS3b	2	33.2	21.9	11.3	11.3	1413	244	2	1.3	0.8
	HCM city	VS7	6	34.7	21.1	13.6	13.6	1851	310	2	1.9	0.4
N Vietnam	Hung Yen	VN9a	71	32.2	11.6	20.6	20.6	1774	325	18	5.2	2.0
	Ha Noi	VN16	9	33.0	13.5	19.5	19.5	1700	314	14	7.5	5.3
	HoaBinh	VN22	195	31.6	11.7	19.9	19.9	1801	352	8	5.1	5.7
C Vietnam	Lam Dong	VC28	1112	27.9	13.3	14.6	14.6	1651	248	7	6.6	1.7
	Lam Dong	VC29*	1112	27.9	13.3	14.6	14.6	1651	248	7	5.8	1.5
	Lam Ha	VC32	925	28.1	13.8	14.3	14.3	1658	252	8	6.3	1.8
Thailand	Nakhon Pathom	TH4	8	35.9	18.7	17.2	17.2	1237	257	6	2.3	1.5
	Samut Prakan	TH5	2	34.5	20.4	14.1	14.1	1439	325	10	2.5	0.3
	Chiang Mai	TH8	316	36.2	12.9	23.3	23.3	1163	244	7	2.9	0.2
Australia	Newcastle	A8	NA	25.0	18.0	NA	NA	NA	NA	NA	1.6	0.5
	Newcastle	A10	NA	25.0	18.0	NA	NA	NA	NA	NA	2.1	0.8
	Newcastle	A12	NA	25.0	18.0	NA	NA	NA	NA	NA	1.7	0.7

* : Sample was identified as the most promising and tested in all the experiments.

NA: Information not available

5.2.2   Preparation of extracts

5.2.2.1   Hexane/acetone/ethanol (2:1:1) extract

The frozen aril (2 g) was placed in a vessel, protected from sunlight (covered in aluminium foil) and mixed with 100 mL of extraction solvent (hexane/acetone/ethanol (2:1:1). The mixture was ultra-sonicated (Unisonics, Australia) for 30 min and then 15 mL of distilled water was added to enhance phase separation, whereby the upper hexane layer contained the carotenoids and the bottom water layer, contained hydrophilic compounds and cell debris. The carotenoid extract was weighed, diluted to a concentration of 11 mg/g using delivery vehicles. Since carotenoids are water insoluble 0.1% DMSO, 1% Ethanol, Tween 40 and DMEM (Dulbecco's Modified Eagle's Medium) media with 10% FBS (Foetal Bovine Serum) were used as the delivery vehicles. For Tween 40 method, carotenoids in hexane extract was mixed with 20 µL of Tween 40 at 20 g/100 mL in acetone and the solvents were evaporated. The dried residue was then solubilized in 2 mL of serum-free medium and vigorously mixed. The extract was filter sterilised using 0.45 µm filters before introducing it to the cells, 10 µL of the extract was added to 96 well plates containing 100 µL of cells and media to give a final concentration of 1 mg/mL.

5.2.2.2   Water extract

The frozen sample (2 g) was placed in a vessel, protected from sunlight (covered in aluminium foil) and mixed with 100 mL of distilled water. The mixture was ultra-

sonicated for 30 min, filtered through Whatman® qualitative filter paper (Grade 1) and evaporated to dryness using a rotary evaporator (Büchi Labortechnik AG, Australia). The crude extract was weighed, diluted to appropriate working concentrations in the range of 2.75-22 mg/mL using milliQ water and mixed using a water-bath sonicator (Unisonics, Australia) for 10 min. The extract was filter sterilised using 0.45 μm filters and 10 μL of the extract was added to 96 well plates with 100 μL of cells in media to give a final concentration of 0.25-2 mg/mL.

5.2.3 Cell lines and culture

Human breast cancer MCF7 (wild type p53 tumour suppressor gene and caspase-3 deficient) and BT474 (mutated p53^{E285K} tumour suppressor gene), melanoma MM418C1 (mutated BRAF oncogene) and D24 (wild type BRAF oncogene) cell lines were provided by the School of Medical Sciences, RMIT University, Australia. The breast cancer and melanoma cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium) and RPMI-1640 media (Gibco, Life Technologies, USA), respectively, supplemented with 10% FBS (Serana, Melbourne, Australia), streptomycin and penicillin 1% v/v (Gibco, Life Technologies, USA) at 37°C in 5% CO_2 (Appendix 11). Human fibroblast cells (NHDF) were used as normal untransformed cells and were grown in DMEM media supplemented with 10% FBS, streptomycin and penicillin 1% v/v at 37°C in 5% CO_2.

5.2.4 *In vitro* cytotoxicity assay

The effect of the extracts on inhibition of cancer cell growth was determined using the CCK-8 (Cell Counting Kit-8) assay (Sigma-Aldrich, St Louis, USA). This assay measures cytotoxicity based on the conversion of a water-soluble tetrazolium salt, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), to a water-soluble formazan dye upon reduction by dehydrogenases in the presence of an electron carrier (Ishiyama et al., 1996). The melanoma and breast cancer cells were seeded in 96 well plates (Greiner Bio-One, Australia), 5000 cells/well and 3000 cells/well, respectively, along with 100 µL of fresh media. The cells were allowed to attach for 24 h before being treated with 10 µL of 11 mg/mL of the water extract or the hexane/acetone/ethanol (2:1:1) extract to give a final concentration of 1 mg/mL. After treatment, the plates were incubated for either 24 or 72 h.

In order to calculate the EC_{50} concentration for the water extract, the cells were treated with 10 µL of the extract at different concentrations (2.75-22 mg/mL). After 22 h and 70 h of treatment (total time including after the CCK-8 solution was added was 24 and 72 h, respectively), 10 µL of CCK-8 solution was added to each well of the 96 well plate containing treated and control samples. The plates were incubated in 37°C for 2 h and the absorbance was measured spectrophotometrically at 450 nm using a CLARIOstar® High Performance Monochromator Multimode Microplate Reader (BMG LABTECH) and the results were analysed using the BMG La Tech, MARS data analysis software (version 3.00R₂). The data were presented as proportional viability

(%) by comparing the treated cells with the untreated cells (control), using the following equation:

$$\text{Cell viability \%} = \frac{At - Ab}{Ac - Ab} \times 100$$

where *At* is the absorbance value of the treated cells, *Ab* is the absorbance of CCK-8 only, *Ac* is the negative control which included cells and CCK-8 only. Two types of controls were used; the media control consisted of cultured cells in 10% FBS-containing medium alone and the vehicle control (for the water extract) consisted of cells in 10% FBS-containing medium and 10 µL of sterile water. However, as both controls did not cause cytotoxicity, the media control was used to calculate cell viability.

5.2.5 Quantification of apoptosis by Annexin-V labelling

The percentage of cells dying by apoptosis was quantified using the Annexin V & Dead Cell kit (Millipore, Hayward, USA). One feature of the early stages of apoptosis is externalisation of phosphatidylserine (PS) to the outer surface of the cell membrane. This assay utilise phycoerythrin-labelled Annexin V to selectively bind to PS and helps to identify cells undergoing apoptosis. Furthermore, 7-AAD (7-aminoactinomycin D) DNA dye is used to differentiate viable and dead cells because 7-AAD is not permeable to viable and early apoptotic cells. This fluorescence dye intercalates double-stranded DNA with a high affinity for GC-rich regions of late apoptotic/necrotic or dead cells. Four populations of cells can be distinguished in this assay: non-apoptotic cells (Annexin V negative and 7-AAD negative), early apoptotic

cells (Annexin V positive and 7-AAD negative), late-stage apoptotic and dead cells (Annexin V positive and 7-AAD positive), and necrotic nuclear debris (Annexin V negative and 7-AAD positive).

The assay was performed according to the manufacturer's instructions. Briefly, MCF-7, MM418C1 and D24 cells were treated with 1 mg/mL of crude water extract for 24 and 72 h. Detached and adherent cells were collected by trypsinisation, collected abd transferred into 1.5 mL microcentrifuge tubes and centrifuged at 400 g for 5 min. The cell pellets were resuspended in 100 μL of fresh medium, to which 100 μL of Muse Annexin V & Dead Cell assay kit reagent was added. The content were mixed and incubated for 20 min at room temperature (RT) in the dark. The events for live, early apoptotic and late apoptotic/necrotic cells of 100 events were counted with the Muse Cell Analyser (Millipore, Hayward, CA, USA). All tests and analyses were conducted in triplicate experiments.

5.2.6 Detection of Apoptosis and necrosis by confocal microscopy

Annexin V-FITC and propidium iodide (PI) stains (Beckman Coulter) were used to detect apoptotic and necrotic cells under confocal microscopy. Briefly, the melanoma (5000 cells/well) and breast cancer cells (3000 cells/well) were seeded in 96 well plates and permitted to adhere for 24 h at 37°C in 5% CO_2. Then, the cells were treated with 1 mg/mL of crude water extract for 72 h. Annexin V-FITC and PI was diluted with binding buffer (Beckman Coulter) for a final concentration of 0.2 mg/mL and 0.1 mg/mL, respectively. The cells were incubated with Annexin V-FITC for 15 min and with PI for 5 min. The fluorescence images of treated and control cells of all three cell

lines were captured using a Nikon Eclipse Ti-E (inverted) confocal microscope (Nikon, Japan).

5.2.7 Cell morphology analysis by phase contrast microscopy

Morphological changes to water extract (1 mg/mL) treated and control cells at the 24 and 72 h time points were analysed using an inverted light microscope (Nikon Eclipse TS100, Japan). The images were captured using a Nikon digital camera (DS-Fi1).

5.2.8 Ultra-structural analysis of melanoma and breast cancer cells using transmission electron micrography (TEM)

Cells were treated with *M. cochinchinensis* water extract (2 mg/mL) for 72 h. Subsequently, the treated cells were washed with 0.1 M PBS and stained with 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3) for 30 min. The stained cells were centrifuged (400 g for 5 min at RT), rinsed with 0.1 M sodium cacodylate buffer (pH 7.3) twice and left overnight in the same buffer. The cells were fixed with 1% (v/v) osmium tetroxide and 1.5% potassium ferrocyanide for 1.5 h at RT and washed twice with distilled water for 10 min. Dehydration was conducted as follows; 50% (v/v) ethanol for 15 min followed by 70% (v/v) ethanol for 15 min, 90% (v/v) ethanol for 15 min, 95% (v/v) ethanol for 15 min, followed by 100% (v/v) ethanol for 30 min and repeated, finally twice with 100% (v/v) acetone for 30 min. Infiltration was carried out using a mixture of acetone and Spurr's resin mix (1:1) on a shaker overnight at RT. The next day, new acetone: Spurr's resin mix (1:1) was added

to the cells and left for 2 h. Then, 100% Spurr's resin was added to the cells and they were placed under vacuum for 2 h. This was followed by exchanging to fresh resin and continuing the infiltration for 2 h. Finally, the cells were embedded and cured at 70°C for 24 h. Sectioning was done using an ultra-microtome (Leica Ultracut UCT) to produce thin sections (<1 μm). The thin sections were washed with distilled water and dried on blotting paper. The sections were examined under a JEOL1010 transmission electron microscope equipped with a Gatan Orius SC600 CCD Camera.

5.2.9 Cytotoxicity among *M. cochinchinensis* samples from Thailand, Vietnam and Australia

The crude water extract from 15 samples, consisting of three samples each from Thailand, Vietnam (Northern, Central and Southern) and Australia were tested for their cytotoxicity using the CCK-8 assay. Breast cancer (MCF7) and melanoma (MM418C1 and D24) cells were plated in 96 well plates at a cell density of 3000 cells/well and 5000 cells/well, respectively, and were treated with crude water extract (1 mg/mL). Control cells were cultured in 10% FBS- containing tissue culture medium only. The plates were incubated for 72 h after adding the extract and the readings were taken as described previously (section 5.2.4). All tests and analyses were done for three individual experiments performed in duplicate.

5.2.10 Statistical analysis

The results were analysed with the one-way analysis of variance (ANOVA) using the Minitab statistical software (version 16). For normally distributed data, the

means were compared using the one-way analysis of variance (ANOVA) and Fisher's post-hoc test. The least significant differences were calculated using the equation:

$$LSDx = t_{x\ (df\ for\ MSE)} \sqrt{2MSE\left(\frac{1}{Ny}\right)}$$

where t is the critical value of the t distribution table; MSE is the mean square error, obtained from results from the ANOVA test; x is the desired level of possibility and y is the number of replicates. Pearson's correlation was used to test the correlation between cytotoxicity and eco-geographical parameters. Statistical values of $P<0.05$ were considered as significantly different. The effective concentration (EC_{50}) for cytotoxicity was derived from a nonlinear regression model (curve fit) based on a sigmoidal dose response curve (variable) and computed using GraphPad Prism version 6 (Graphpad). Three component principal component analysis (PCA) analysis for anticancer activity of the aril extract of *M. cochinchinensis* was carried out using Minitab statistical software (version 17).

5.3   Results

5.3.1   Effect of hexane/acetone/ethanol (2:1:1) and water extracts of *M. cochinchinensis* on breast cancer and melanoma cell viability

The water insoluble carotenoids were introduced to the cells using 0.1% DMSO, 1% Ethanol, Tween 40 and DMEM (Dulbecco's Modified Eagle's Medium) media with 10% FBS (Foetal Bovine Serum) as the vehicles. However, 0.1% DMSO, 1% Ethanol

and Tween 40 did not successfully dissolve the carotenoids in the aril extract but DMEM media supplemented with 10% FBS was effective in dissolving the carotenoids in aril extract. However, the carotenoids present in the hexane/acetone/ethanol (2:1:1) extract of the *M. cochinchinensis* sample had significantly lower activity than did the water extract (Figures 5.1-5.4). After 24 h, hexane/acetone/ethanol (2:1:1) extract reduced by 15% the cell viability of the MCF7 breast cancer (Figure 5.1) and D24 melanoma (Figure 5.4) cell lines but had no cytotoxic effect on BT474 breast cancer (Figure 5.2) and MM418C1 melanoma (Figure 5.3) cell lines at 24 h. When the cells were exposed to the hexane/acetone/ethanol (2:1:1) extract for 72 h the level of cytotoxicity was similar to that seen at 0 and 24 h and suggested that carotenoids in *M. cochinchinensis* had low activity.

The water extract induced a time-dependant cytotoxicity in the breast cancer and melanoma cell lines with the highest level of activity seen at 72 h (Figures 5.1- 5.4) and cell cells underwent morphological changes as seen in figures 5.1 to 5.4. This extract caused a 15-40% loss in cell viability in both breast cancer and melanoma cells at 24 h but no visible morphological changes were observed (Figures 5.1 and 5.2). At 72 h, the water extracts reduced cell viability by up to 70% in the MM418C1 cells. Treatment of the cells resulted in distinct morphological changes (detachment from the substrate, rounding of cells) in both MM418C1 and MCF7 cells. This reduction of cell viability was more than twice that seen at 24 h which suggest that the extract elicited a time-dependant activity in both melanoma cell lines (MM418C1 and D24) and in the MCF7 breast cancer cell lines. However, the breast cancer cell line BT474, was less sensitive to treatment with only 30% cell death observed at 72 h (Figure 5.2), which was lower

than seen for the other cell lines. Therefore, as a result we used both melanoma cell lines (MM418C1 and D24) but only one breast cancer cell line (MCF7) was used for further tests. Human dermal fibroblasts were used to represent a non-neoplastic cell line.

The water extract elicited a dose-dependent cytotoxicity on melanoma (MM418C1 and D24) and breast cancer (MCF7) cells. The MM418C1 cells were the most sensitive with a 80% loss in cell viability seen at 72 h for cells treated with 2 mg/mL water extract (Figure 5.5). At 24 h, low concentrations (0.25 and 0.5 mg/mL) of the water extract displayed a hormetic effect on the melanoma and breast cancer cells as well as on the primary dermal fibroblasts. Higher concentrations (1-2 mg/mL) of the water extracts caused cytotoxicity with up to a 30% loss in cell viability (Figure 5.5) observed at 24 h, which was less than that seen at of 72 h. Therefore 72 h time point was selected to determine the EC_{50} value because it showed the highest reduction in cell viability for all the examined cell lines (Figure 5.5). The most sensitive cell line was the MM148C1 melanoma cells which had an EC_{50} for 0.49 mg/mL. The EC_{50} for the MCF7 breast cancer cells was 0.59 mg/mL while for the D24 (melanoma) cells it was 0.73 mg/mL. The EC_{50} value for the human dermal fibroblast cells was >2 mg/mL (Figure 5.5), which suggests that the *M. cochinchinensis* water extract has selective cytotoxicity towards neoplastic cells.
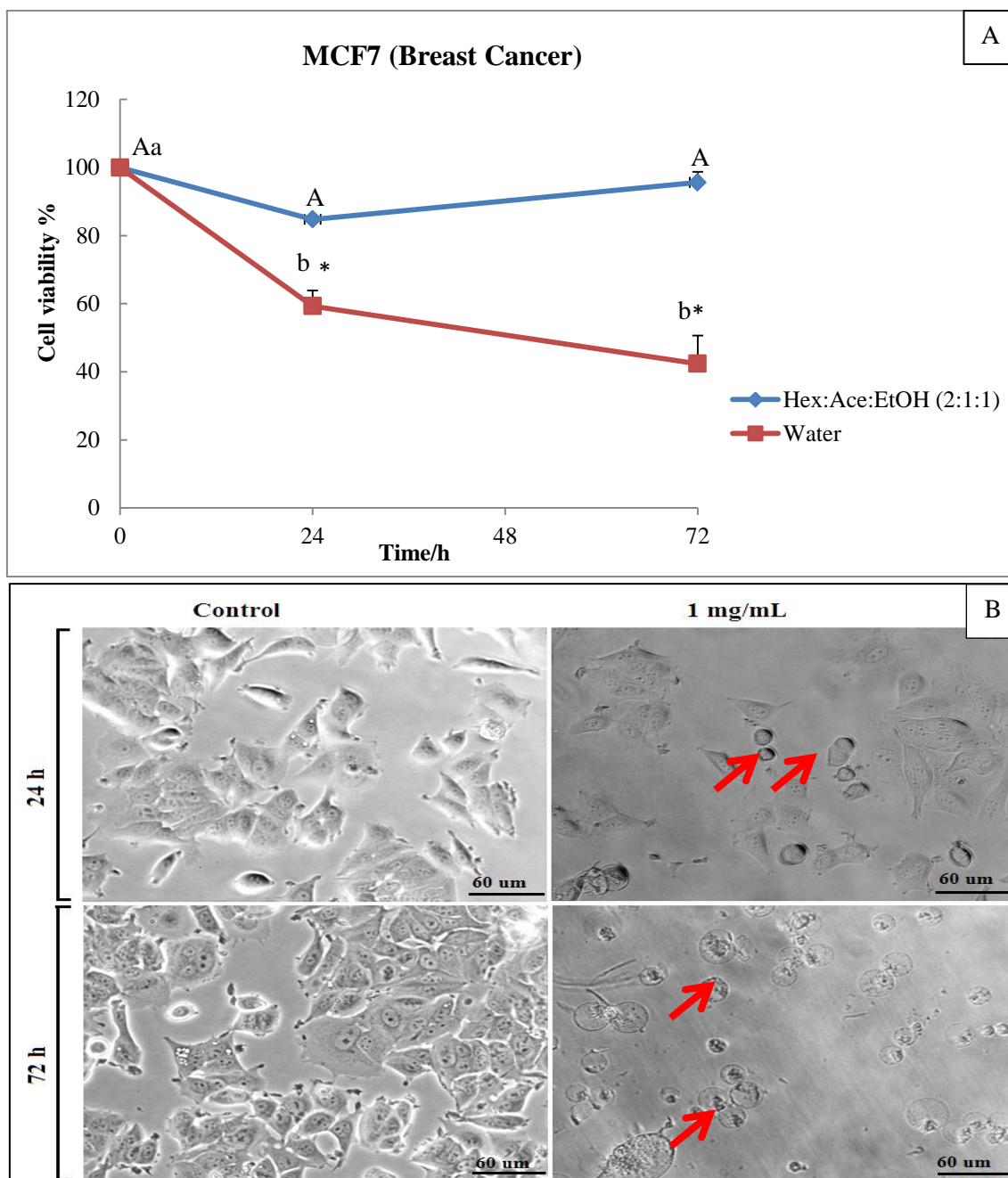
Figure 5.1: Effect of *M. cochinchinensis* water and hexane/acetone/ethanol (2:1:1) extracts of aril on cell viability of MCF7 breast cancer cell line compared with control. **A:** changes in cell viability of MCF7 cell line after treatment with water and hexane/acetone/ethanol (2:1:1) extracts for 24 and 72 h. 0 h indicated the time the extracts were added. **B:** Morphological changes (shown by solid arrow) of MCF7 cells when treated with 1 mg/mL aril water extract. Results are representative of three independent experiments. The values shown are average + SD compared to control. * indicates significance at p≤0.05 between water and hexane/acetone/ethanol (2:1:1) extracts at a corresponding time point. Error bars with different letters denote significant differences using Fishers's LSD (least significance differences) generated from one-way ANOVA compared to control at a corresponding time point.
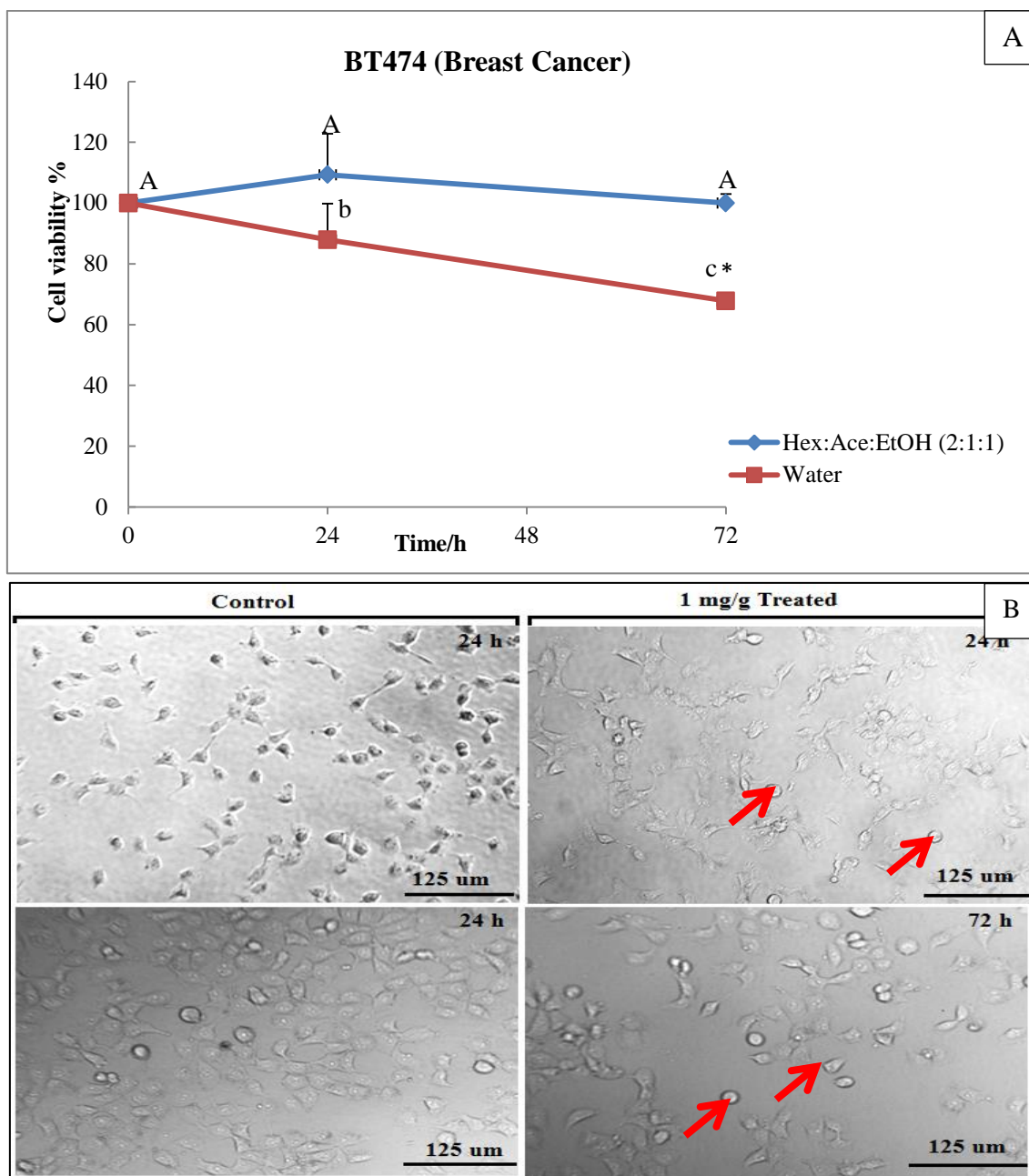
Figure 5.2: Effect of *M. cochinchinensis* water and hexane/acetone/ethanol (2:1:1) extracts of aril on cell viability of BT427 breast cancer cell line compared with control. **A:** changes in cell viability of BT474 cell line after treatment with water and hexane/acetone/ethanol (2:1:1) extracts for 24 and 72 h. 0 h indicated the time the extracts were added. **B:** Morphological changes (shown by solid arrow) of BT474 cells when treated with 1 mg/mL aril water extract. Results are representative of three independent experiments. The values shown are average + SD compared to control. * indicates significance at $p \leq 0.05$ between water and hexane/acetone/ethanol (2:1:1) extracts at a corresponding time point. Error bars with different letters denote significant differences using Fishers's LSD (least significance differences) generated from one-way ANOVA compared to control at a corresponding time point.
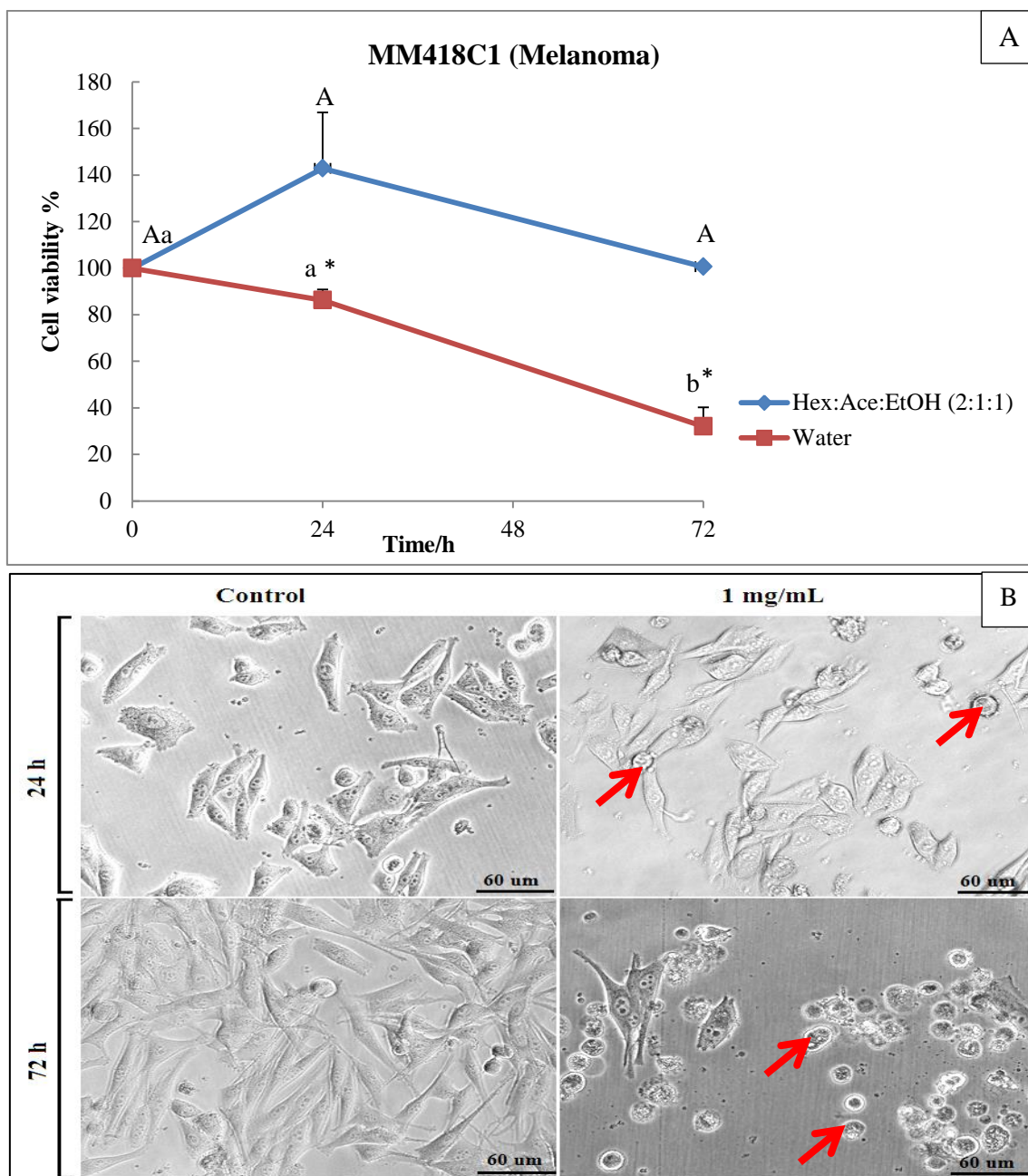
Figure 5.3: Effect of *M. cochinchinensis* water and hexane/acetone/ethanol (2:1:1) extracts of aril on cell viability of MM418C1 melanoma cell line compared with control. **A:** changes in cell viability of MM418C1 cell line after treatment with water and hexane/acetone/ethanol (2:1:1) extracts for 24 and 72 h. 0 h indicated the time the extracts were added. **B:** Morphological changes (shown by solid arrow) of MM418C1 cells when treated with 1 mg/mL aril water extract. Results are representative of three independent experiments. The values shown are average + SD compared to control. * indicates significance at $p \leq 0.05$ between water and hexane/acetone/ethanol (2:1:1) extracts at a corresponding time point. Error bars with different letters denote significant differences using Fishers's LSD (least significance differences) generated from one-way ANOVA compared to control at a corresponding time point.
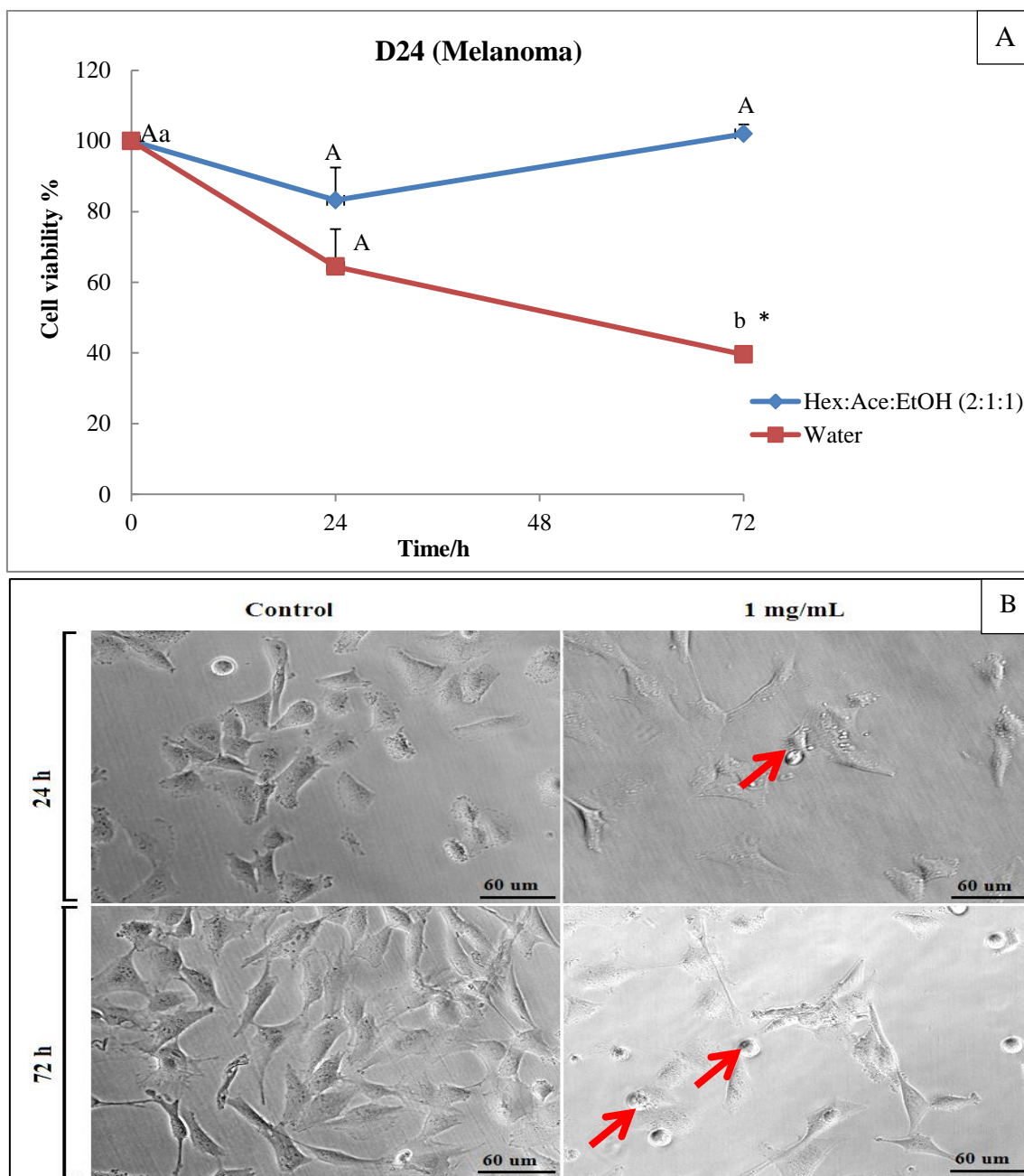
Figure 5.4: Effect of *M. cochinchinensis* water and hexane/acetone/ethanol (2:1:1) extracts of aril on cell viability of D24 melanoma cell line compared with control. **A:** changes in cell viability of D24 cell line after treatment with water and hexane/acetone/ethanol (2:1:1) extracts for 24 and 72 h. 0 h indicated the time the extracts were added. **B:** Morphological changes (shown by solid arrow) of D24 cells when treated with 1 mg/mL aril water extract. Morphological changes are shown by the solid arrow. The values shown are average + SD compared to control. * indicates significance at p≤0.05 between water and hexane/acetone/ethanol (2:1:1) extracts at a corresponding time point. Error bars with different letters denote significant differences using Fishers's LSD (least significance differences) generated from one-way ANOVA compared to control at a corresponding time point.
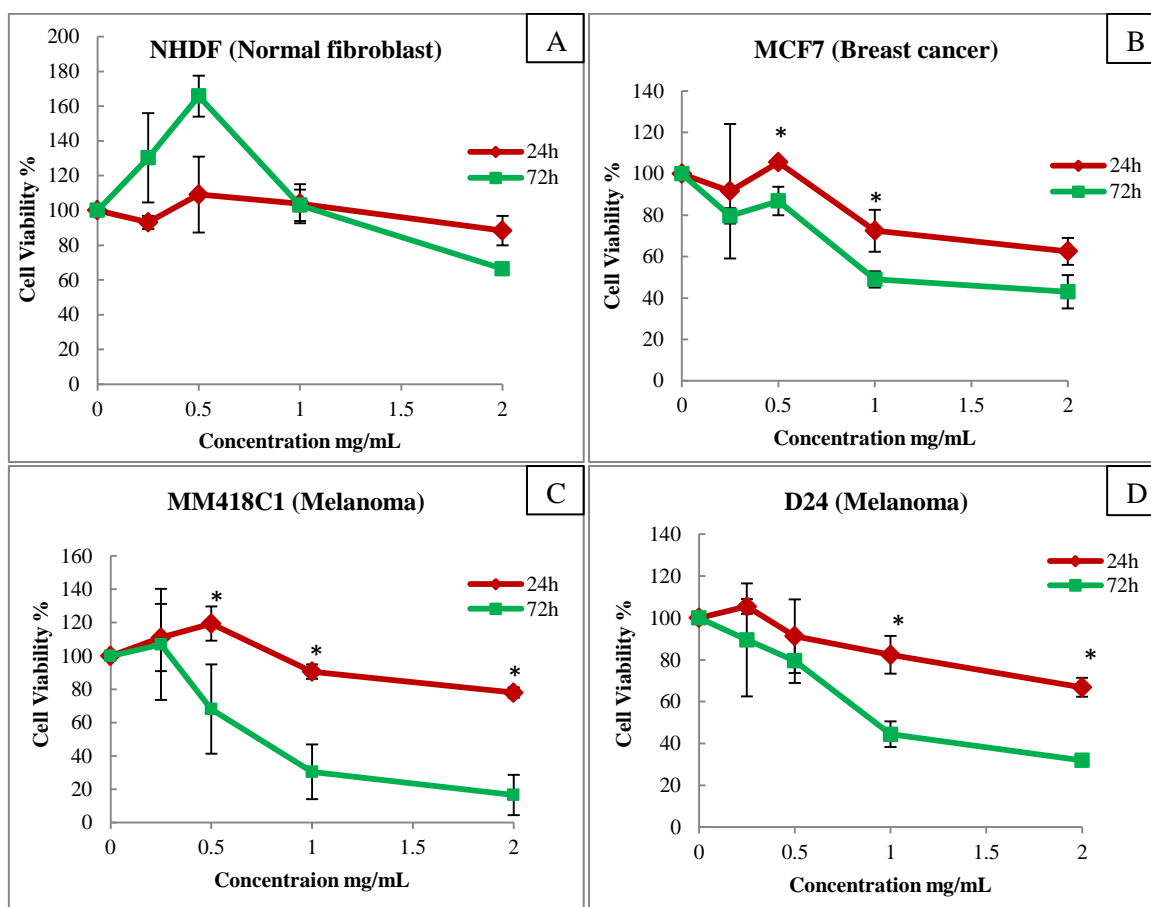
Figure 5.5: Effect of exposure time and concentration of *M. cochinchinensis* aril water extract on cell viability. The cells were treated with an aril water extract (0-2 mg/mL) for either 24 or 72 h. The cells were normal human dermal fibroblast (NHDF) (A), breast cancer cells (MCF7) (B), MM418C1 melanoma cells (C) and D24 melanoma cells (D). Results are representative of three independent experiments done in triplicate (n=3). The values shown are average ± SD.* indicates significance at p≤0.05 between the two time points at a corresponding concentration.

5.3.2   Assessment of apoptosis by flow cytometer

The *M. cochinchinensis* water extract induced apoptosis in the melanoma (MM418C1, D24) and breast cancer (MCF7) cells in a time-dependant manner as detected by muse flow cytometry. The melanoma and breast cancer cells were treated with 1 mg/mL water extract for 72 h. Annexin V was shown to bind the externalised plasma membrane of the cells, as shown by the right shift of the scatter plot (Figures 5.6, 5.7 and 5.8) compared with that of non-treated cells (control cells) indicating early apoptosis. Furthermore, this was confirmed by the AnnexinV-FITC/ PI double staining method using confocal microscopy, which indicated that a greater number of cells were stained green (Figure 5.9). The percentage of live cells decreased significantly in a time dependent manner in all three cell lines (Figures 5.6, 5.7 and 5.8) with the highest cell death (apoptosis and necrosis) observed for the MM418C1 cells after 72 h (47.9%) and this was in agreement with that seen in the cytotoxicity assay (Figure 5.3).

The percentages of late apoptotic/necrotic (Annexin V positive and 7-AAD positive) cells in the melanoma and the breast cancer cell lines were significantly different to that of their untreated controls (Figures 5.6 B and 5.7 B). This was also observed by confocal microscope analysis where the cells were stained with green (Annexin V) and red (PI) stains (Figure 5.9). However, early apoptotic events (Annexin V positive and 7-AAD negative) were not significantly different for the MM418C1 and MCF7 cell lines than for the corresponding controls (Figures 5.6 B and 5.7 B). The water extract-treated D24 melanoma cell line showed significantly more early apoptosis events at the 72 h time point, than the corresponding control (Figure 5.8). This was also

confirmed with the Annexin/PI staining method where the number of green stained cells (Annexin V) was higher than for those stained red (PI) (Figure 5.9).
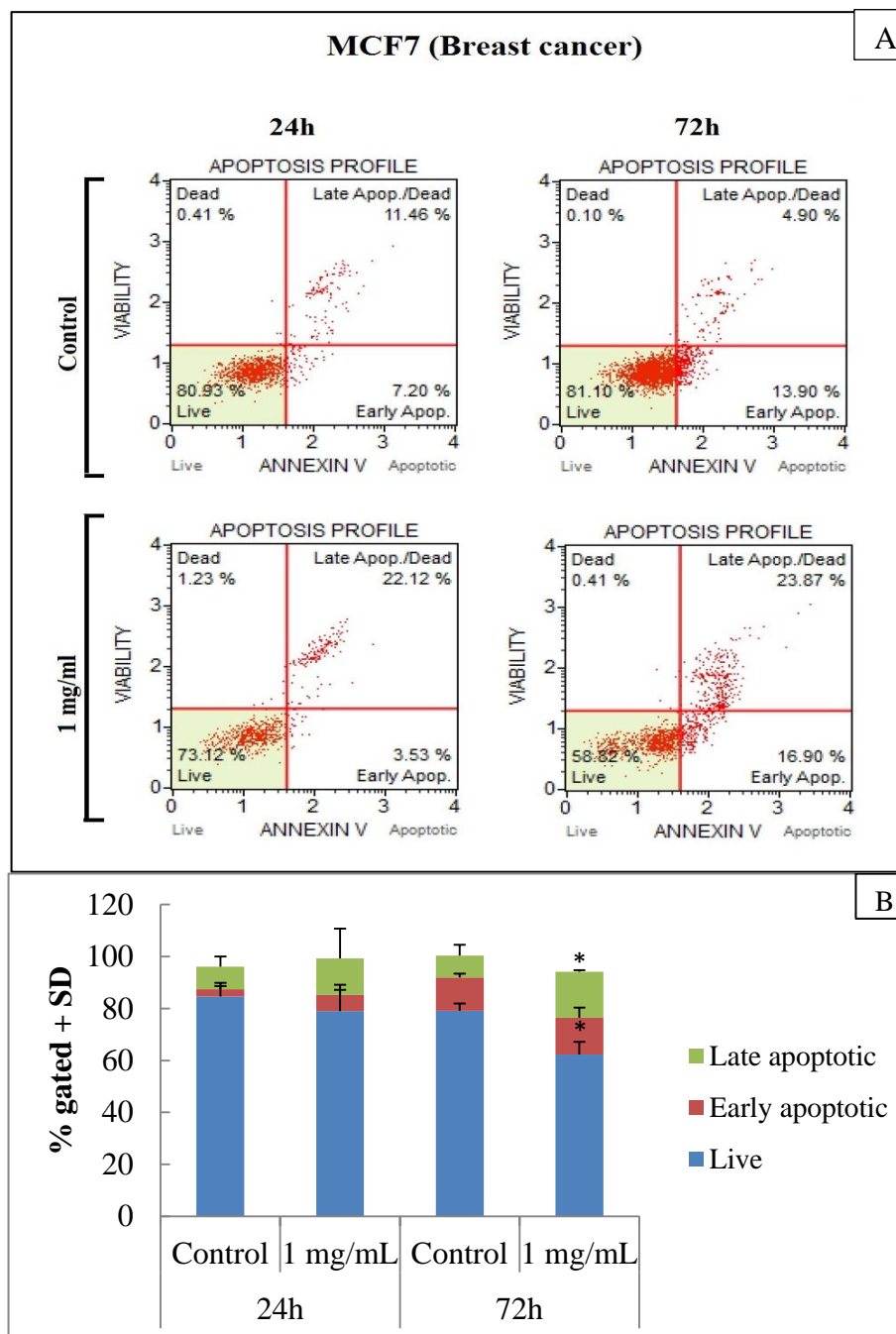
Figure 5.6: Time-dependant apoptotic effect of *M. cochinchinensis* aril water extract on MCF7 breast cancer cells when treated with 1 mg/mL water extract for 24 and 72 h. Following exposure, the cells were treated with AnnexinV/ 7AAD stain and analysed by muse flow cytometry. Percentages of live and apoptotic cells (early and late) are shown in histograms (B) which are representative of three independent experiments (n=3). * indicates significance at $p \leq 0.05$ compared with the control group.
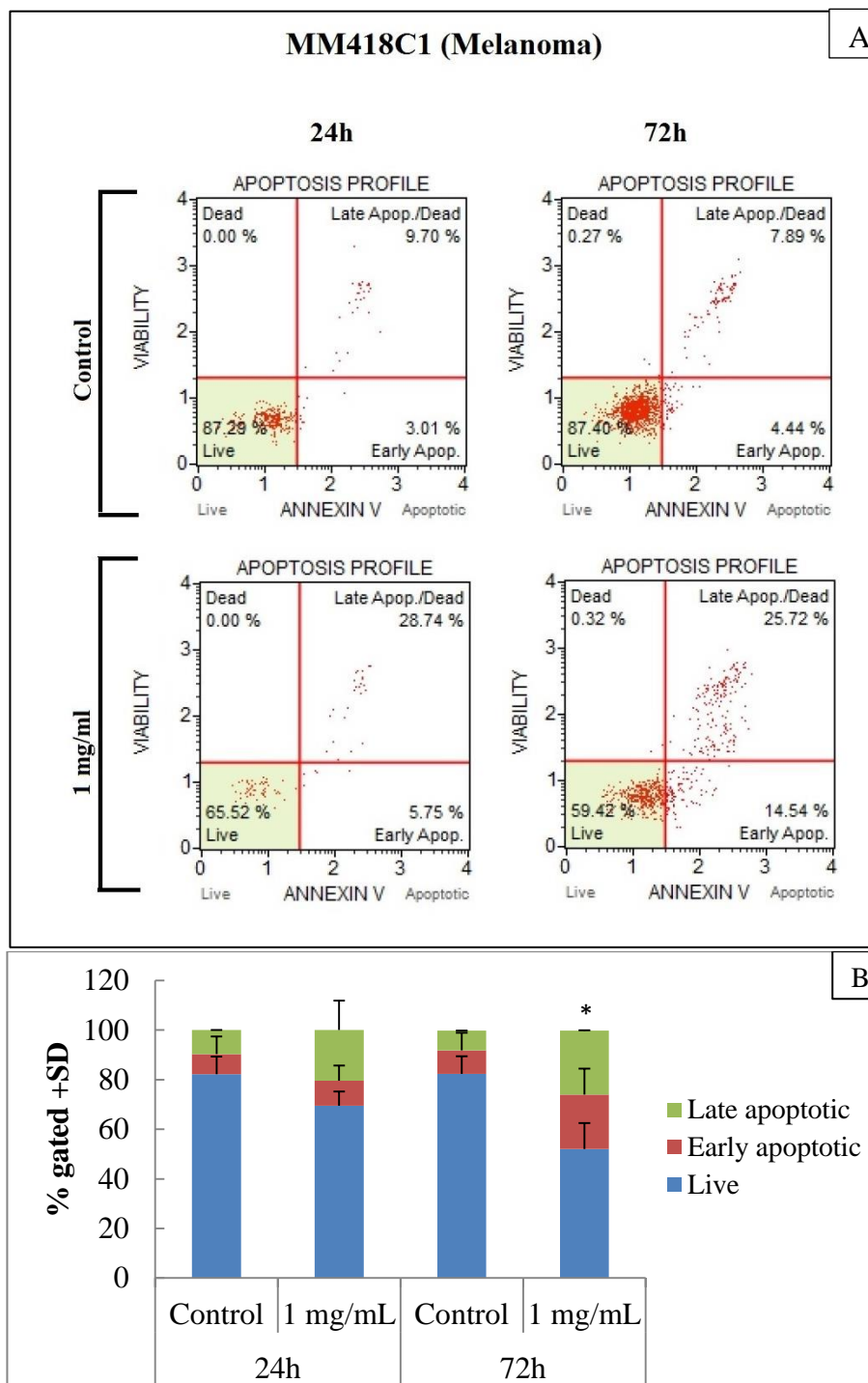
Figure 5.7: Time-dependant apoptotic effect of *M. cochinchinensis* aril water extract on MM418C1 melanoma cells when treated with 1 mg/mL water extract for 24 and 72 h. Following exposure, the cells were treated with AnnexinV/ 7AAD stain and analysed by muse flow cytometry (A). Percentages of live and apoptotic cells (early and late) are shown in histograms (B) which are representative of three independent experiments (n=3). * indicates significance at $p \leq 0.05$ compared with the control group.
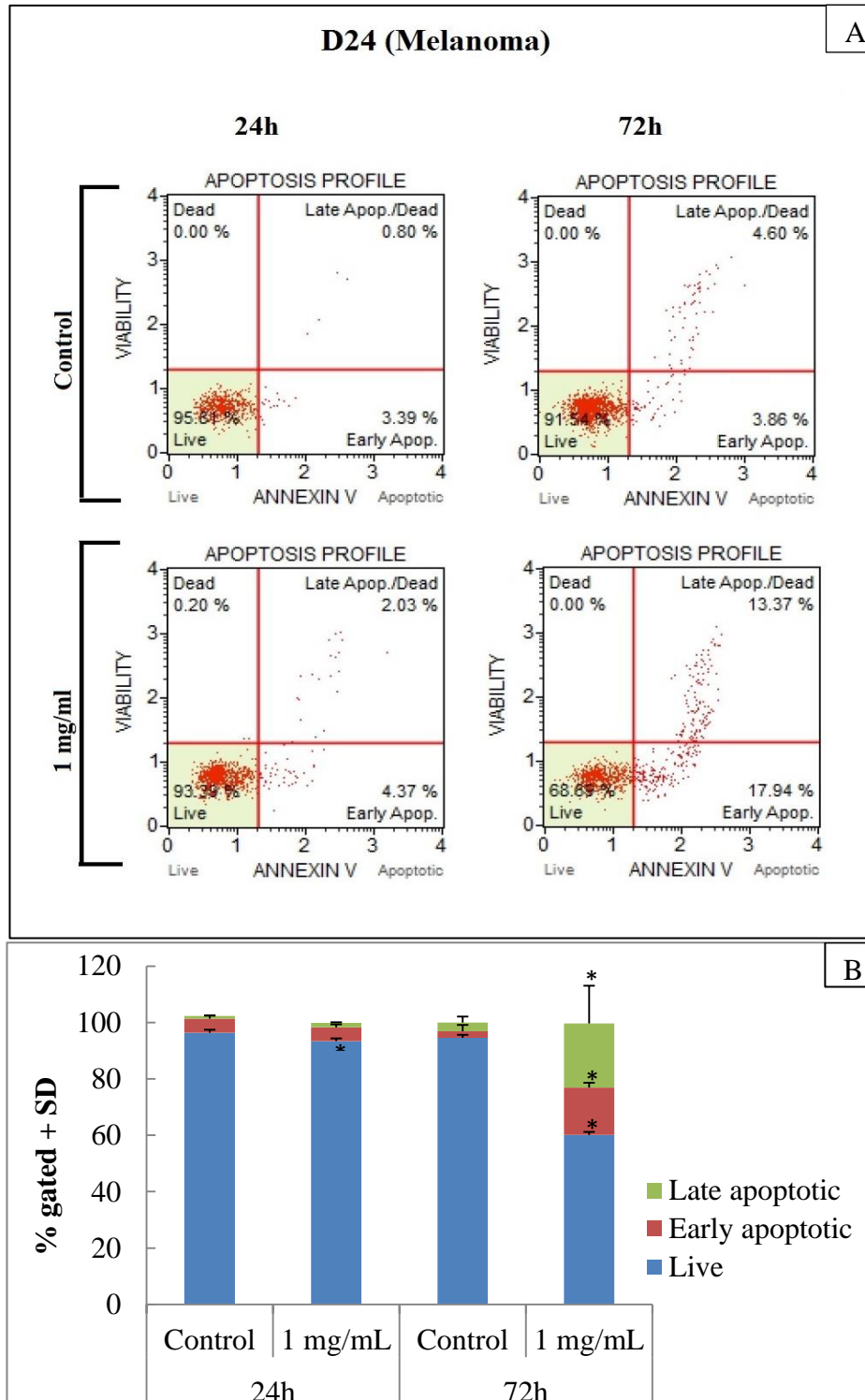
Figure 5.8: Time-dependant apoptotic effect of *M. cochinchinensis* aril water extract on D24 melanoma cells when treated with 1 mg/mL water extract for 24 and 72 h. Following exposure, the cells were treated with AnnexinV/ 7AAD stain and analysed by muse flow cytometry (A). Percentages of live and apoptotic cells (early and late) are shown in histograms (B) which are representative of three independent experiments (n=3). * indicates significance at p≤0.05 compared with the control group.
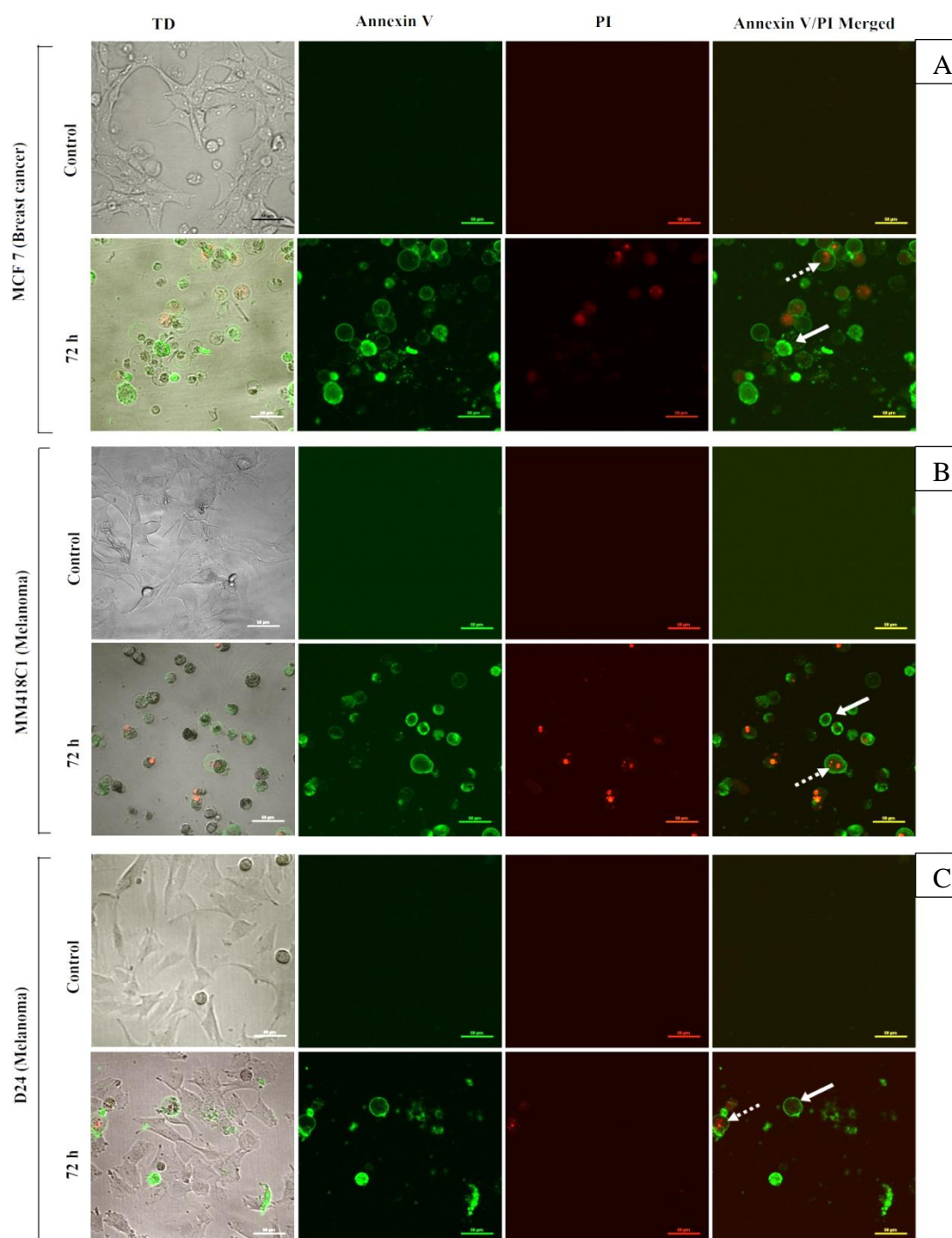
Figure 5.9: Water extract (1 mg/mL) of *M. cochinchinensis* induced apoptosis of breast cancer (A) MCF7) and melanoma (B) (MM418C1, C: D24) cells at 72 h, as detected by the Annexin V-FITC (green) /PI (red) double staining assay and observed under confocal microscopy. TD: the images were obtained by phase contrast microscopy. The solid-line arrows indicate early apoptotic cells. The dotted-line arrows indicate late apoptotic/necrotic cells.

5.3.3 Ultrastructure analysis of *M. cochinchinensis* water extract treated breast cancer (MCF7) and melanoma (MM418C1 and D24) cells

The effects of 2 mg/mL *M. cochinchinensis* aril water extract on the morphology of the cells treated for 72 h were observed under electron microscopy (Figure 5.10). The *M. cochinchinensis* water extract induced clear morphological changes in both breast cancer and melanoma cell lines. The untreated MCF7 breast cancer cells possessed a large nucleus with distinct nucleoli and did not have any peripheral heterochromatin (Figure 5.10 A). After treatment with the water extract for 72 h, these cells showed loss of microvilli, reduction of cytoplasmic volume and formation of cytoplasmic vacuoles as well as blebbing of plasma membranes (Figure 5.10 B-C). Furthermore, the chromatin was pushed towards the nuclear membrane (Figure 5.10 B-C), indicating characteristics of early apoptotic cells, but there were also vacuolisation of the cytoplasm (Figure 5.10 B) as well as a swollen endoplasmic reticulum, which was indicative of necrosis. This suggests that the water extract of *M. cochinchinensis* aril contained compounds that can induce both types of cell death in MCF7 breast cancer cells.

The untreated MM418C1 cells possessed large nucleoli within the nucleus and had no peripheral heterochromatin (Figure 5.10 D). After 72 h treatment with the *M. cochinchinensis* water extract chromatin condensation, shrinkage of the cytoplasmic volume and blebbing of the cytoplasm, indicative of apoptosis, were all observed (Figure 5.10 E). Furthermore, the late stage of the apoptotic or secondary necrotic types of cell death was also observed, as seen by the presence of highly condensed nuclei and ruptured cellular organelles in some cells (Figure 5.10 F). However, in the D24

melanoma cell line, clear chromatin condensation in the nucleus was not observed but the treated cells were different from the untreated control, as seen by the loss of microvilli on the plasma membrane, blebbing and shrinkage of the cell (Figure 5.10 G-I) (Chen et al., 2001). The nuclear chromatin was also marginated and less electron dense with highly convoluted nuclei, which was indicative of early apoptosis (Chen et al., 2001). Furthermore, these morphological changes agreed with the results with the Annexin V/7-AAD assay, which showed a high percentages of early apoptosis in this cell line (Figure 5.9 C), when it was treated with the water extract.
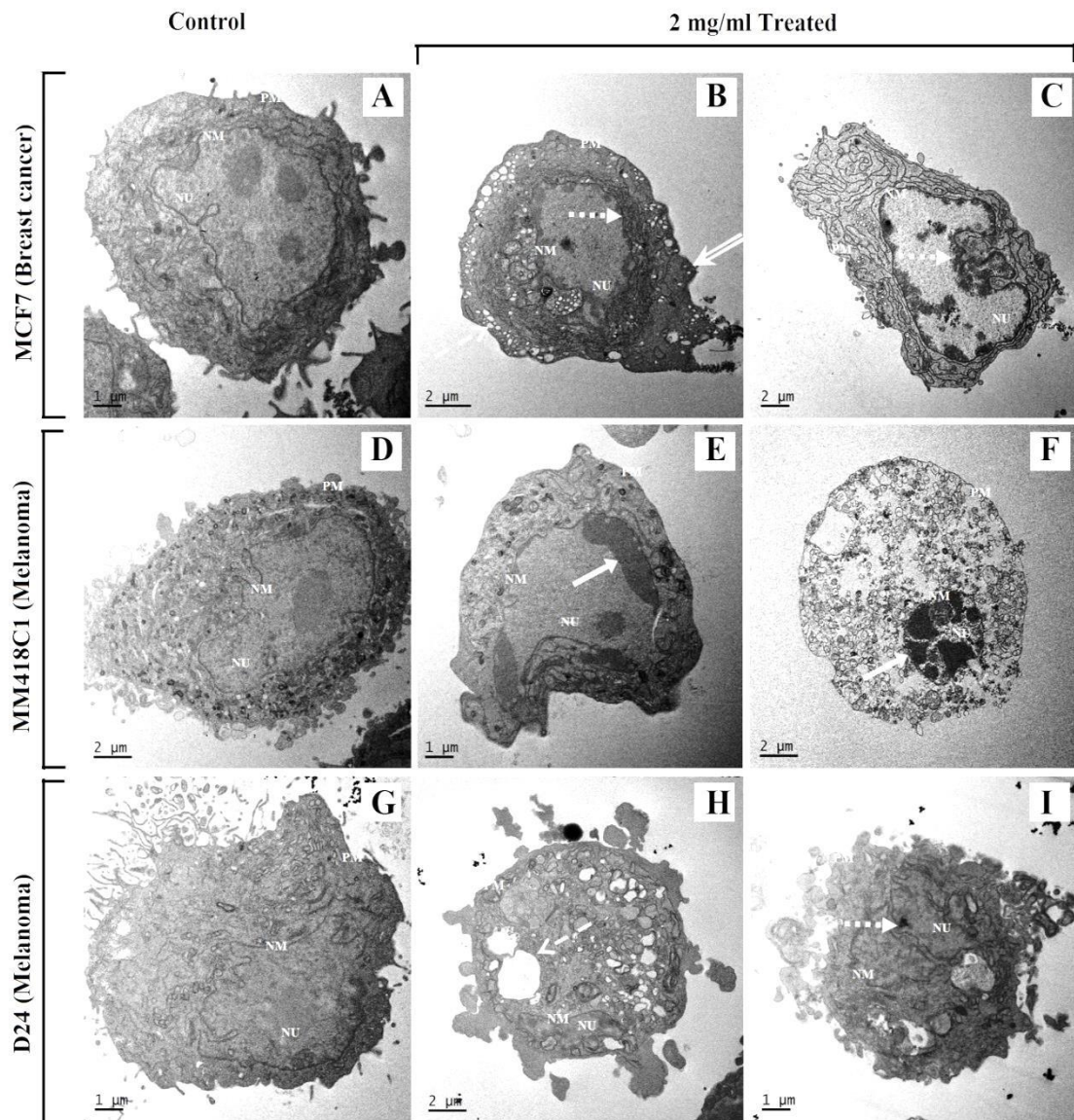
Figure 5.10: Ultrastructure analysis of breast cancer and melanoma cell lines treated with the water extract of the *M. cochinchinensis* aril. A-C: Control (A) and 2 mg/mL treated MCF7 cells with morphological alterations at 72 h. D-I: Control (D, G) and 2 mg/mL treated MM418C1 (E-F) and D24 (H-I) melanoma cells with apoptotic and necrotic cell death. Solid-line arrow: Chromatin condensation. Dotted-line arrow: Marginating of chromatin. Hatched-line arrow: Vacuolisation. Double-line arrow: Cell membrane blebbing. NU: Nucleus, NM: Nuclear membrane, PM: Plasma membrane.

5.3.4 Comparison of samples from Vietnam, Thailand and Australia

The effect of water extracts (1 mg/mL) from the arils of *M. cochinchinensis* samples collected from different regions were tested on the cancer cell lines (MM418C1, D24 and MCF7). The cytotoxicity of these extracts was variable and significantly affected by varietal differences ranging from 0-71% loss in cell viability (Figure 5.11). The extracts from Northern and Central Vietnam samples significantly reduced cancer cell viability and subsequently cell growth by up to 70% relative to untreated controls. However, not all samples from Central and Northern Vietnam showed similar high levels of activity and this might be due to the genetic differences between those samples. The least active samples were from Southern Vietnam on with the exception of VC32 on MCF7 and MM418C1 cells and TH4 on D24 cells (Figure 5.11). The anticancer activity of these water extracts on these cell lines was not correlated ($P>0.05$) to their lycopene and β-carotene concentrations, which suggests that these are not the active compounds in the extracts.

Anticancer activity of the 15 *M. cochinchinensis* aril extracts on melanoma (MM418C1 and D24) and breast cancer (MCF7) was analysed using three component PCA analysis and presented on a 2-D graph (anticancer activity of MM418C1 versus D24) (Figure 5.12). The samples with the highest anticancer activity against all three cell lines were from Northern and Central Vietnam (VN16, VC28 and VC29) and they were clustered in the bottom left quadrat of the PCA grouping equivalent to low cell viability on all cell lines, whilst the Southern Vietnam samples clustered on the bottom right quadrat equivalent to D24 cell-sensitivity but MM418C1 cell-resistance (Figure 5.12). The samples from Australia and Thailand clustered in the top left quadrat

equivalent to D24 cell-resistance and MM418C1 cell-susceptibility (Figure 5.12). This clustering based on anticancer activity of *M. cochinchinensis* aril water extract was random and did not group the samples based on geographical origin or varietal differences as did for morphology and genetic analyses (Figures 5.12, 2.5, 2.6 and 2.7).

The samples from Australia were excluded from the analysis of correlation between carotenoid content and eco-geographical factors since they were grown in controlled environments compared to the samples from Vietnam and Thailand which were exposed to environmental variations in their natural habitats (Table 5.2). The aril water extracts from the plants grown in cooler temperatures had higher cytotoxicity on MM418C1 (r=0.67, P=0.017) and D24 cell lines (r=-0.69, P=0.013) than for the plants grown in warmer climates, as seen by the good correlations and correlation of determination between the minimum temperature and cell viability (Table 5.2 and Figure A6). Furthermore, the samples collected from regions with high precipitation in the driest month correlated with high cytotoxicity of MM418C1 (r=-0.73, P=0.007) and D24 (r=-0.72, P=0.009) cell lines (Table 5.2 and Figure A6). Thus, can interpret that, >48% of variability in cytotoxicity of MM418C1 and D24 is due to minimum temperature and high precipitation in the driest month (Table 5.2 and Figure A6). However, arils collected from different eco-geographical conditions did not show any significant correlation with their cytotoxicity of MCF7 cells (Table 5.2).
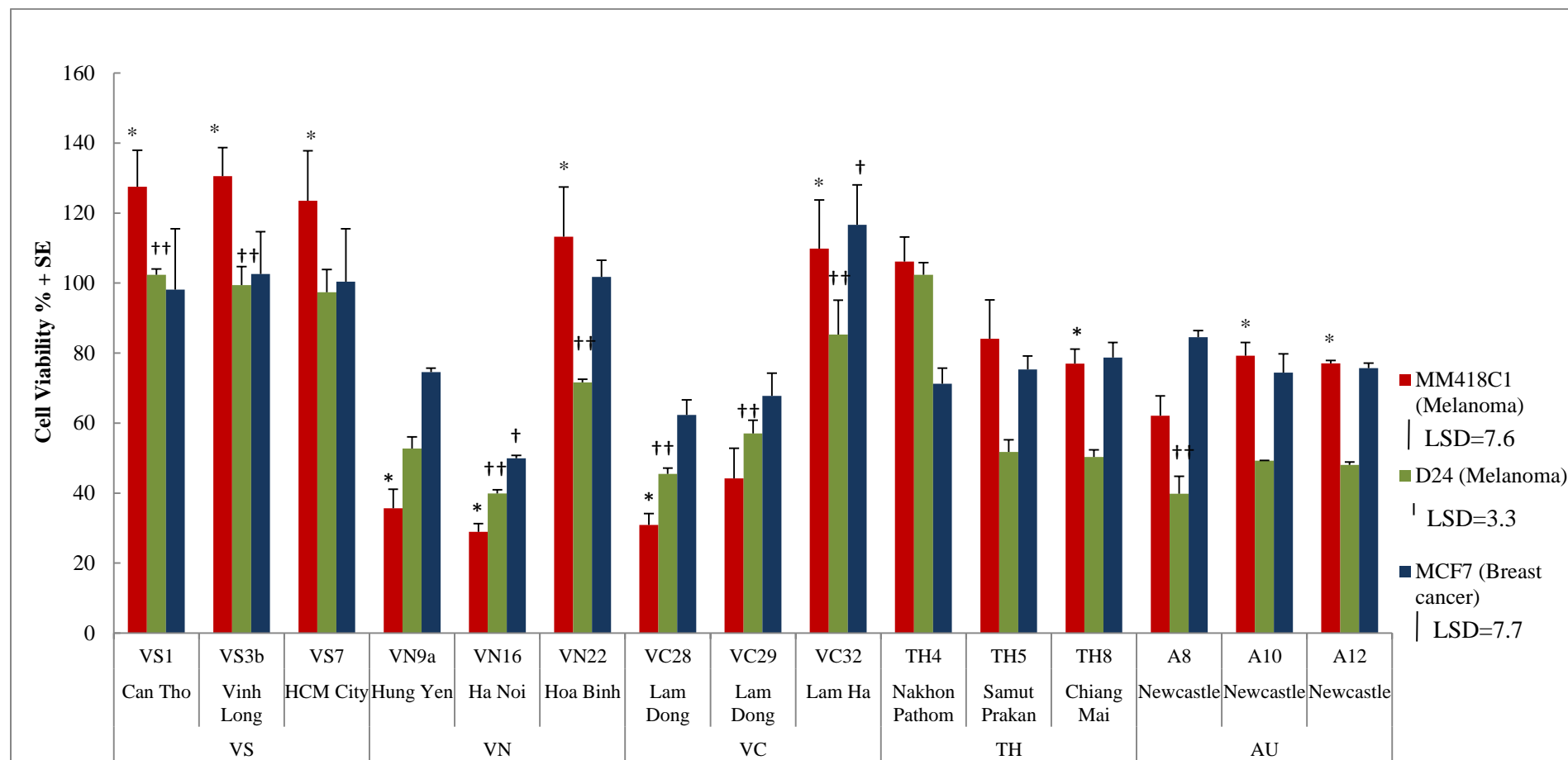
Figure 5.11: Comparison of cell viability of 1 mg/mL water extract of aril from Vietnam (southern, central and northern), Thailand and Australia using the CCK-8 assay. Fisher's LSD (least significance differences) values were generated from cell viability of MM418C1 (df= 14, F= 10.42, p=0.000), D24 (df= 14, F= 15.58, p=0.000) and MCF7 (df= 14, F= 2.22, p=0.069) cell lines. The results are means of three individual experiments which were performed in duplicate. *, †† and †† indicated statistical significance at p ≤ 0.05 on cell viability of MM418C, D24 and MCF7, respectively based on fruit collection sites.
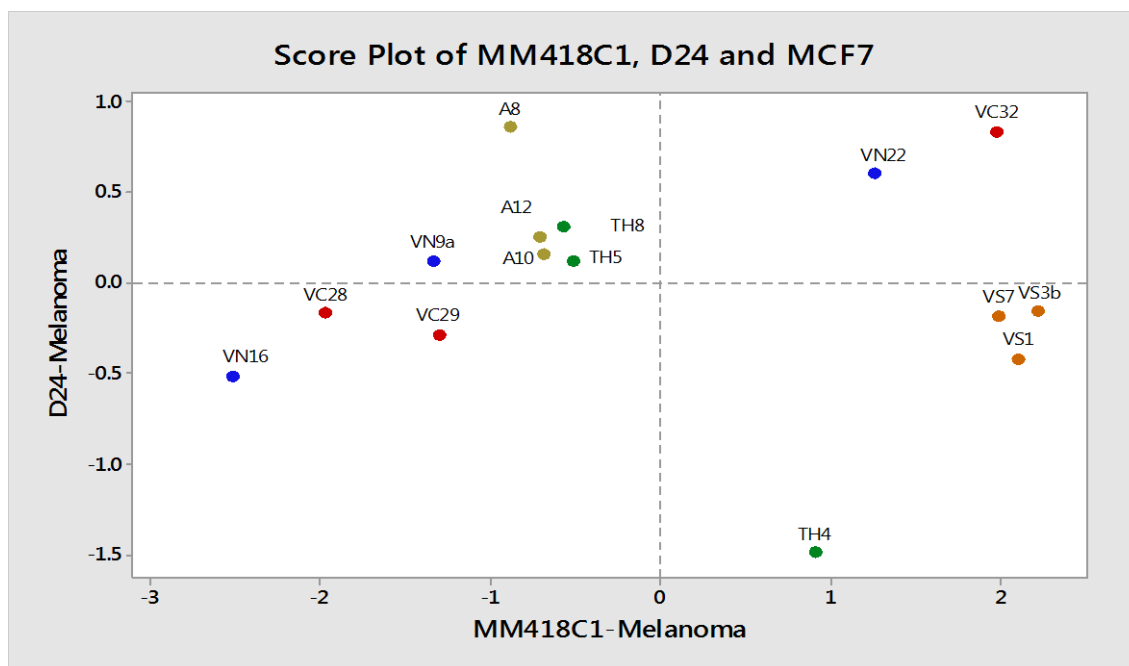
Figure 5. 12: Score plot of the anticancer activity of 15 *M. cochinchinensis* aril water extract against D24 and MM418C1 cells constructed using the Principal component analysis method (PCA) with Minitab statistical software (Version 17). Country of collection is shown by different colours; orange=Southern Vietnam, blue=Northern Vietnam, red=Central Vietnam, green=Thailand, olive green=Australia.

Table 5.2: Pearson correlation coefficients (r) and coefficient of determination (r^2) between eco-geographical factors (maximum and minimum temperature, annual temperature range, elevation and precipitation) and cell viability of water extract of *M. cochinchinensis* aril on breast cancer (MCF7) and melanoma (MM418C1 and D24) cells.

Factor	Details	Correlation (r) with cell viability %			Coefficient of determination (r^2)		
		MCF7	MM418C1	D24	MCF7	MM418C1	D24
Temperature	Minimum	0.33	0.67[*]	0.69[*]	-	0.45	0.48
	Maximum	-0.04	-0.34	-0.23	-	-	-
	Range	-0.39	0.46	-0.57	-	-	-
Elevation	Elevation	0.08	-0.40	-0.32	-	-	-
Precipitation	Annual	0.13	-0.19	-0.13	-	-	-
	Driest month	0.51	-0.73[*]	-0.72[*]	-	0.53	0.51
	Wettest month	0.05	-0.09	-0.26	-	-	-

[*]: Significant at $P<0.05$

173

5.4   Discussion

5.4.1   Cytotoxicity effect of water and hexane/acetone/ethanol (2:1:1) extracts

The water extract from the aril of *M. cochinchinensis* fruit elicited significantly higher cytotoxicity towards breast cancer (MCF7) and melanoma (MM418C1 and D24) cell lines than did the corresponding hexane/acetone/ethanol: 2:1:1 extract. The activity of the water extract of *M. cochinchinensis* aril samples might be due to the presence of active proteins as mentioned in a previous study (Tien et al., 2005). The activity on these cell lines was high and the EC_{50} concentration ranged from 0.49- 0.73 mg/mL. This was higher than seen in a previous study where an EC_{50} value of 1.25 mg/mL for a colon 26-20 cancer cell line was determined (Tien et al., 2005). The discrepancy between these values may be due to several reasons, such as the differences in the plant material (collection site, variety), the post-harvest methods (storage, extraction, processing, and detection) or variation in cell lines and should be the focus of future research to determine which factor specifically enhance anticancer activity.

The water extract displayed a time- and dose-dependent cytotoxicity, where the MM418C1 melanoma cell line was the most sensitive, having a EC_{50} value of 0.49 mg/mL at 72 h. This cell line possess a mutation in the BRAF gene ($BRAF^{V600E}$), which codes for the protein B-Raf (a protein kinase of the mitogen-activated protein kinase (MAPK) pathway) that normally regulates cell growth, proliferation and differentiation (Chapman et al., 2011). This mutation is common in a large proportion (40-60%) of melanomas (Chapman et al., 2011). Furthermore, the BRAF mutated MM418C1 cell

line was more sensitive to the water extract than the D24 cells, which possesses the wild type BRAF gene. Thus, this implies that the water extract might contain compounds that block the activity of the mutated BRAF protein (BRAF^{V600E}) or act on the RAS-mediated signalling pathway (MAPK/ERK). Similarly, the water extract was cytotoxic to the MCF7 cell line with an EC$_{50}$ value of 0.59 mg/mL. This cell line is ER-positive (oestrogen receptor- positive) and ~ 70% of human breast cancers, which are hormone-dependent and ER-positive (Lumachi et al., 2013). Thus, this study indicates the necessity to identify the active ingredients in the extract of the *M. cochinchinensis* aril, which could be developed as promising therapeutical agents for melanoma and breast cancer treatments.

Carotenoids are strong antioxidants and they are known to have anticancer activity as crude extracts or in purified form on prostrate, colon and breast cancer (Rao and Rao, 2007, Tanaka et al., 2012). The aril of *M. cochinchinensis* has the highest content of lycopene and β-carotene among known fruits and vegetables (Vuong et al., 2006). Carotenoids are fat soluble and commonly extracted using organic solvents. The extraction solvent, hexane/acetone/ethanol (2:1:1) used in this study contained these carotenoids but had less effect on both breast cancer and melanoma cell lines. This was in agreement with the preliminary results of a previous study (Tien et al., 2005) but the low activity might be due to poor solubility of carotenoids in aqueous medium, especially because there is a high amount of oil in the aril of *M. cochinchinensis* (Ishida et al., 2004, Vuong et al., 2006).

5.4.2 Water extract caused apoptosis and necrotic cell death in breast cancer and melanoma cells

The water extract of the aril from *M. cochnchinensis* induced cell death in melanoma and breast cancer cell lines at 72 h, as detected by the CCK8 assay, flow cytometry and confocal microscopy. The two well-known cell death mechanisms are apoptosis and necrosis, which possess distinct morphological and biochemical differences. During the initiation of cell death, cells lose contact with neighbouring cells and adherent cells detach from the surface and became round. Further in the process, the structural integrity of the plasma membrane is lost, the final endpoint at which a cell can no longer maintain its discrete identity from the environment (Galluzzi et al., 2015). These cells can be measured biochemically as the release of cytosolic enzymes, including lactate dehydrogenase, and the uptake of membrane-impermeant dyes, such as propidium iodide and 7-aminoactinomycin D (7-AAD) (Berghe et al., 2013). In this study, cell death was measured using the CCK-8 assay and the Annexin V/7-AAD flow cytometry methods but these methods did not confirm the mechanism by which these cells died.

Morphological hallmarks of apoptosis in the nucleus are chromatin condensation and nuclear fragmentation, which can be observed under transmission electron microscopy (Kerr et al., 1972). Several stages of nuclear apoptosis can be distinguished: in the first stage, there are rippled nuclear contours and partial chromatin condensation; the second stage is distinct in having marked peripheral chromatin condensation; and in the third stage, formation of nuclear small bodies takes place (Chen et al., 2001).

The crude water extract induced complex responses with different nuclear morphological features, which were dependant on the cell lines analysed. The melanoma cell line, MM148C1, displayed clear apoptotic morphological features with peripheral chromatin condensation, while the D24 (melanoma) and MCF7 (breast cancer) cell lines only showed partial chromatin condensation. The lack of apoptotic nuclei in the MCF7 cell line was expected since this cell line is caspase-3 deficient (Kagawa et al., 2001). Apoptosis is typically accompanied by the activation of a class of death proteases called caspases. Among these caspases, caspase-3 is essential for the nuclear morphological changes (e.g. chromatin condensation) that occurr during apoptosis (Jänicke et al., 1998, Porter and Jänicke, 1999).

Crude extracts can induce complex responses in cancer cells due to the presence of more than one bio-active molecule, each of which can trigger different cell death mechanisms. It is also possible for a certain dose of death-inducing agents, to induce both apoptosis and necrosis simultaneously in cells (Ankarcrona et al., 1995, Zong and Thompson, 2006). In this study, although early apoptotic nuclei were observed in cells, cytoplasmic vacuolisation (Figure 5.8 B and H), rupture of cellular organelles (Figure 5.8 F) and swelling of the endoplasmic reticulum (Figure 5.8 C) was indicative of necrotic cell death. This suggests a combination of death modes was elicited by this extract. This also suggests the presence of different classes of compounds in the extract and further analysis of the extract is needed to identify the active compounds.

5.4.3 **Anticancer activity was influenced by eco-geographical differences of *M. cochinchinensis***

Geographical distribution appeared to determine the anticancer activity of *M. cochinchinensis*. Cytotoxicity was significantly different based on the provenance of the fruit with the most active sample being from Ha Noi province in Northern Vietnam. The anticancer activity determined by cytotoxicity varied widely, causing up to 70% cell death. All the samples analysed were genetically different based on molecular markers and therefore, the observed variation in cytotoxicity might be due to genetic diversity. Genetic variation within a species can give rise to different compositions of biologically active secondary metabolites as reported in 25 commercially available Kava (*Piper methysticum*) medicinal plants (Martin et al., 2014).

Fruits originating from the same plant with an identical phytochemical composition will most likely possess the same level of cytotoxicity. The three samples from Australia originated from a single fruit and therefore, they had a low genetic variation (Wimalasiri et al., 2015), which could explain why they had similar levels of cytotoxicity. This suggests that genetics might be a predominant factor and that maintaining the same genetics could assure the consistency of bioactivity of the best genotypes. Interestingly however, although the Australian *M. cochinchinensis* variety originated from Southern Vietnam (Wimalasiri et al., 2015), it was grown in temperature-controlled glasshouses at 25ºC, which may explain why the Australian samples showed a higher cytotoxicity against the melanoma cell lines than samples from where the variety originated suggesting that variables other than genetics are responsible. The clustering based on anticancer activity of *M. cochinchinensis* aril water

extract did not group the samples based on geographical origin or varietal differences as did for morphology and genetic analyses. This might be due to other factors which are responsible for variations in anticancer compounds such as soil nutrition, micro-climatic differences and environmental differences. Furthermore, the lack of differentiation of clustering based on geographical origin based on PCA analysis might be due to the low number of samples from each location analysed in this study.

The samples collected from cool climates with a high precipitation rate during the driest month correlated with a high cytotoxicity of the aril extract towards the melanoma cells but not the breast cancer cells. This was expected since environmental stress conditions such as temperature and drought is known to cause higher amounts of secondary metabolites to be produced in other plant species (Gershenzon, 1984, Dixon and Paiva, 1995). However, more experiments are necessary (e.g. the same genotypes growing in different climates/environments) to confirm the relative dominance of eco-geographic and genetic influences on the anti-cancer activity of *M. cochinchinensis*. Furthermore, future research focusing on the micro-climate of the plant growth sites is necessary to confirm the environmental influence on the anticancer activity of *M. cochinchinensis*.

5.5   Conclusion

The water extract of the aril of *M. cochinchinensis* was shown to be cytotoxic to breast cancer and melanoma cells. The water extract had significantly higher (>70% cell death) cytotoxicity than the hexane/ethanol/acetone (2:1:1) extract, indicating that the active compounds are water soluble and are not carotenoids, which are water insoluble;

and this was in agreement with a previous study (Tien et al., 2005). The water extract elicited both apoptotic and necrotic cell death mechanisms indicating that the extract contained more than one type of bio-active molecule and was expected since it was a crude extract.

The samples with the best anticancer activity were from Ha Noi in Northern Vietnam and Lam Dong in Central Vietnam, indicating that plants from these regions should be considered for future agricultural developments. Furthermore, the anticancer activity was influenced by temperature and precipitation, where samples collected from locations with <14°C minimum temperatures and high precipitation rates during the driest month had high anticancer activity. This indicates that these environmental conditions could be manipulated to obtain higher bioactive compounds, which might contribute to anticancer activity.

CHAPTER 6

Synopsis and future directions

This thesis elucidated the influence of genetic diversity and growth environment on the nutritional and medicinal potential of *M. cochinchinensis*. The study fulfilled the aims by identifying the best genotypes and growth environments to facilitate optimal synthesis and accumulation of carotenoids and anticancer compounds.

6.1   Morphological and genetic diversity

The *M. cochinchinensis* plants in their natural habitats were morphologically and genetically diverse and this was related to their country of origin. High morphological and genetic diversity existed within Vietnam, contributing to genotypes with commercially viable traits, such as varieties producing bigger seeds, a higher carotenoid contents and a higher anticancer activity. The highest seed weight and length were from fruits collected from Central and Northern Vietnam. Selection of these varieties will be beneficial for seed oil production, which can be used in the treatment of disease conditions including inflammation, tinea, scrofula and skin infections such as sores, carbuncles and furuncles (Lim, 2012).

Large and heavy seeds were correlated with an increased carotenoid content and could be used to direct the selection of carotenoid-rich fruits prior to processing as well as for breeding carotenoid-rich *M. cochinchinensis* fruits. Seed weight and length have been linked with fruit yield (number per plant and larger fruits) in other tropical plants

181

such as camu-camu (Pinedo, 2013) and custard apple (Mariguele and Silva, 2010) but it is not known whether the same is true for *M. cochinchinensis*. Therefore, future research can study the effect of seed size and weight on the fruit yield of *M. cochinchinensis*, which will facilitate the commercial production of this fruit.

Traditionally, morphological characters are used to improve crops, such as that seen for tomato (Casals et al., 2011, Barrios-Masias and Jackson, 2014) and bitter gourd (Dey et al., 2006). Other morphological features that could contribute to improved agricultural production in terms of higher fruit yield, include fruit size, fruit weight, number of fruits per plant, aril weight, mesocarp weight and spike density (e.g. <3 spikes/10 cm^2, 4-10 spikes/ 10 cm ^{2}). Analysis of these parameters in future studies will facilitate the commercial production of *M. cochinchinensis*. No genetic markers have been identified for factors that are responsible for bigger seeds, fruit prediction (number of fruits per plant) and fruit size. Therefore, such markers will be important for the future breeding of *M. cochinchinensis*.

Morphological characteristics are influenced by both genotype and environment. The morphological traits measured in this study might be due to the differences in environment (e.g. soil nutrition, irrigation, and temperature differences), genetic variation of the plants or both. Hence future research is necessary to confirm the genotype and environment influence by analysing morphology traits generated by plants collected from difference locations but grown in a controlled environment. This will eliminate the influence of environment and will confirm the effect of genetics. Furthermore, growth of a specific variety (e.g. seeds of the fruits from Northern or Central Vietnam) in different natural environment (e.g. farms in tropical Thailand) or

greenhouse conditions (e.g. Australia) will confirm the influence of the growth environment on the morphological traits.

M. cochinchinensis in Vietnam, Thailand and Australia was genetically diverse and indicated the presence of different genotypes. The samples from Vietnam showed higher levels of genetic diversity than the samples from Thailand but future research focusing on a wider collection of samples from Thailand as well as other neighbouring countries (Cambodia and Laos) is needed to confirm this diversity. However, *M. cochinchinensis* samples collected from Thailand were genetically and morphologically different from Vietnam and Australian samples indicating that they might be a different variety or a sub-species of *M. cochinchinensis*.

M. cochinchinensis is not well known or widely distributed hence, a limitation of this study was the availability of single replicates of fruits in certain locations (e.g. HCM City, Samutprakan and Canthaburi), which limits the accurate comparison between collection sites, because it does not represent an entire population. Therefore, in future research, collection of adequate numbers of representatives from each province is necessary to confirm the presence of genetically different varieties within Vietnam and Thailand. Furthermore, future genetic studies using DNA sequencing methods will be important to draw more robust conclusions about the genetic variability of *M. cochinchinensis* between Vietnam and Thailand. This study found that the previously unknown origin of Australian samples were likely to be from Tra On province of Southern Vietnam and is vital in the tracking the origin of cultivated *M. cochinchinensis*.

6.2   Nutritional diversity

M. cochinchinensis based products have potential in the nutraceutical and functional food industries due the fruit's high carotenoid concentration. Currently, the commonly recognised source of lycopene is tomatoes and and for β-carotene it is carrots. The aril of *M. cochinchinensis* has been shown to contain up to 200 times more lycopene than hydroponic tomatoes (Javanmardi and Kubota, 2006) and 54 times more β-carotene than carrots (Gul et al., 2015). In this study, the carotenoid levels in the *M. cochinchinensis* samples were higher than expected and were 3 times higher for lycopene and 8 times higher for β-carotene compared to that previously reported for *M. cochinchinensis* (Aoki et al., 2002, Ishida et al., 2004, Kubola and Siriamornpun, 2011). The global carotenoid market value was estimated to be \$1.5 billion in 2014 and it is expected to reach nearly \$1.8 billion in 2019, with an annual growth rate of 3.9% (BCC, 2105). *M. cochinchinensis* can contribute to this expanding carotenoid market as an alternate source of lycopene and β-carotene.

The Lam Dong province of Central Vietnam and the Hoa Binh province of Northern Vietnam possessed genotypes that contained high levels of lycopene and β-carotene, respectively. Therefore, these varieties should be considered for future commercial production. *M. cochinchinensis* aril is currently being used in traditional Asian cuisine, predominantly to prepare a rice dish locally known as xôi gấc. The fruit has a short harvest period (November-December) (Kha, 2010) and therefore, the fresh fruit is only seasonally available in the market. Frozen and freeze dried aril can retain its nutrient content for almost a year making it available throughout the year in Asian

countries (Kha et al., 2013a). Products containing processed *M. cochinchinensis* aril include cooking powder, drinks, oil and cosmetics (Chuyen et al., 2015); freeze dried aril is also marketed in the name of Carogac™ in the USA.

The lycopene and β-carotene concentration of *M. cochinchinensis* from Central and Northern Vietnam was 8 to 20 times higher than that seen for samples from Thailand, respectively. In this study, no apparent genetic marker was identified for the superior lycopene and β-carotene levels in *M. cochinchinensis* from Central and Northern Vietnam. The levels of lycopene and β-carotene have been linked with the genes involved in their biosynthesis pathway (Smita et al., 2013a) in tomato, which has facilitated the identification of genotypes producing high carotenoid levels for commercial breeding programs (Isaacson et al., 2002, Smita et al., 2013b). Future studies focused on the expression of key genes involved in lycopene (CRTISO PSY) and β-carotene (LCYB) accumulation will facilitate the selection of breeding stock of *M. cochinchinensis* for the development of consistently high carotenoid synthesising varieties.

The clustering based on genetics and carotenoid content of *M. cochinchinensis* samples was in agreement. This suggests the possibility of the presence of genotypes responsible for the production of high levels of lycopene (e.g. VC28), β-carotene (e.g. VN26) or both (e.g. VN16). Future studies focusing on those genotypes in the development of breeding lines for higher carotenoid producing varieties is essential for the agricultural sustainability of this fruit.

The samples analysed were from its natural habitats and the carotenoid production might be attributed by the growth environment, as seen by the correlations between temperature, precipitation and elevation. Therefore, the influence of genotype and environment factors should be tested independently. As an example, seeds from different regions of Vietnam (southern, central and northern) should be grown in controlled environments (green house conditions) or the seeds and stem cuttings from the plants from Central (e.g. VC28) or Northern Vietnam (e.g. VN26 and VN16) should be grown in different environments (*in situ*). This will help to determine the relative effect of the genotype versus the environmental conditions, which will facilitate the selection of the best genotype and the most favourable environmental condition for growing the fruit in future agricultural production. Furthermore, the seeds and or stem cuttings of the plants from Central and Northern Vietnam with high lycopene and β-carotene should be grown in greenhouse conditions in a temperature range of 14-20°C to confirm the effect of low temperature in carotene synthesis of *M. cochinchinensis*. The environmental data used in this study were averages from a local weather station and not recorded where the fruits were actually grown. In future studies, environment data of the actual location of the plant should be compared to confirm the effect of environmental factors on carotenoid content of *M. cochinchinensis* fruit.

The colour intensity (redness) of the frozen aril was correlated with the lycopene content, indicating that fruits can be selected without chemical processing for commercial production. In future studies, similar colour matching could be applied to other parts of the fruit, such as the mesocarp and peel, which could help farmers to pre-select carotenoid-rich fruits for commercial benefit. Furthermore, colour comparisons of

frozen and fresh aril should be conducted to confirm the correlation between colour values and carotenoid content of *M. cochinchinensis* which will facilitate the use of colorimeter in the field. This method can also be developed to get correlation between the peel colour at the different fruit ripeness stages and the carotenoid content, which would facilitate the harvesting of the fruits when their carotenoid content is optimum.

6.3   Anticancer activity

The highest anticancer activity was found for the water based extract of the *M. cochinchinensis* aril and this was in agreement with a previous study (Tien et al., 2005). This previous study indicated that the compound responsible for anticancer activity on colon 26-20 cell line was a protein with a molecular weight of 35 kDa (Tien et al., 2005). However, it is not known whether the same protein was responsible for the effects elicited by the water extract on the breast cancer and melanoma cells analysed in this study. Therefore, future studies can be focused on the activity of purified 35 kDa protein and its correlation with bioactivity.

The water extract of the aril possessed selective cytotoxicity against both breast cancer and melanoma cells through triggering apoptosis and necrosis, indicating that the extract contains more than one bioactive compound. Isolated compounds have been shown to be more effective than a mixture of compounds in other studies (Harvey, 2008) and future research is necessary to fractionate (HPLC), purify (HPLC) and characterise (NMR) these compounds to identify the bioactive compounds in the water extract. Other studies on apoptosis (p53, Bcl-2, caspase-3) and necrosis (cyclin A, cdk-2) related genes and proteins will provide more information on the cell death pathways

involved in the cytotoxicity of the water extract, which will improve the understanding of the mechanism of action of the anticancer compounds (Kepp et al., 2011). Furthermore, *in vitro* and *in vivo* studies are necessary to determine the efficacy (absorbability, bioavailability and toxicity) of the aril extract.

The water extract of the aril was more effective towards the MM418C1 cell line, which possessed a mutation in the BRAF gene (BRAF^{V600E}) common in a large proportion (40-60%) of melanoma incidents (Chapman et al., 2011). *M. cochinchinensis* might have compounds that inhibit the activity of the mutated BRAF gene, like the drug Vemurafenib (Bollag et al., 2012) or compounds that act through the Raf/MEK/ERK cellular signalling pathway, but future research is necessary to identify and analyse the responsible compound(s) and their mode of action prior to genomic and proteomic elucidation.

Consumption of fruits and vegetables rich in phytochemicals is essential in reducing chronic diseases such as cancer (Willett, 2002, Liu, 2004). The fruit of *M. cochinchinensis* has potential to prevent cancer due to its high carotenoid content as well as to other potential anticancer compounds that have not yet been analysed. The results of this project suggest that the Lam Dong province of Central Vietnam and the Ha Noi province of Northern Vietnam possess genotypes with high anticancer activity. This should be considered for the possibility of introducing *M. cochinchinensis* to the market as a chemo-preventive fruit in the future. For this, plants from these regions should be selected for future breeding programs.

6.4   Overall conclusion

M. cochinchinensis is nutritionally and medicinally important and has potential to be developed as a functional food for the nutraceutical industry. The cluster analysis of carotenoids was in agreement with the genetic clustering indicating the presence of varieties responsible for the production of high lycopene and β-carotene contents. The varieties from Northern and Central Vietnam possessed the highest carotenoid (lycopene and β-carotene) content and anticancer bioactivity and should be selected as breeding stock for future commercial breeding and production programs. Specifically, plants from Lam Dong and Ha Noi provinces produced fruits with high lycopene content and high anticancer activity, which was influenced by low temperatures (<14°C). Therefore, varieties from these provinces should be selected for future crop development and commercialisation and to possibly introduce *M. cochinchinensis* as a functional fruit to the market. Furthermore, existing growth sites with low temperatures can be selected as farming sites for future increased crop production in order to attain the maximum levels of nutritional and medicinal potential of this fruit.

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APPENDICES

Appendix 1
Table A1: Codes assigned for fruit and seed morphology data of *M. cochinchinensis* samples.

Sample	Fruit		Seed		
	Shape	Spike density	Number	Weight and length	Colour
VS1	2	3	1	1	3
VS2	2	3	1	2	3
VS3a	1	3	1	2	3
VS4	2	3	2	2	3
VS7	2	3	2	2	3
VN8	3	2	2	1	2
VN9a	1	2	3	1	3
VN9b	2	2	4	2	3
VN10	3	2	4	2	3
VN11	3	2	3	2	2
VN12	4	2	2	1	1
VN13	2	2	4	2	2
VN14	2	1	3	2	2
VN15	2	2	3	2	3
T1	4	3	3	3	2
T3	4	3	3	3	2
T4	4	3	2	3	2
T5	4	3	5	3	2
T6	4	3	5	3	1
T7	4	3	5	3	2
T8	4	3	3	3	1
VN16	3	3	5	2	3
VN17	2	2	3	2	3
VN18	2	2	4	2	3
VN19	4	3	3	2	3
VN20	2	2	4	2	3
VN21	2	2	3	1	3
VN22	3	2	5	2	3
VN24	3	2	4	2	3
VN25	3	2	4	2	3
VN26	3	1	4	1	3
VN27	3	1	3	2	3
VC28	2	2	4	3	3
VC29	4	2	4	1	3
VC30	4	2	4	1	3
VC32	2	2	3	1	3
A2	1	3	1	2	3
A3	1	3	1	2	3

Fruit Shape: Globose=1, globose oval=2, tapered=3, oval=4
Spike density: Sparse=1, medium=2, dense=3
Seed number:<10=1, 11-20=2, 21-30=3, 31-40=4, >40=5
Seed weight and length: large-heavy=1, medium-heavy=2, small-light=3,
Seed colour: Light brown=1, brown=2, blackish brown=3

Appendix 2: Protocol for DNA extraction using Favor PrepTM Plant Genomic DNA

Extraction Mini Kit (Favorgen, Taiwan).

Step 1 – Tissue Dissociation

- Cut off 500 mg of fresh or frozen plant tissue or 5 mg (up to 100 mg) of dried sample.

- Grind the sample under liquid nitrogen to a fine powder. Transfer it to a microcentrifuge tube.

Step 2 – Lysis

- Add 400 μl FAPG1 Buffer and 8 μl RNase A (50 mg/l) into the sample tube and mix by vortexing.

- Incubate at 65 °C for 10 min. During incubation, invert the tube every 5 minutes. At the same time preheat the required volume of elusion buffer (200 μl per sample) at 65 °C.

- Add 130 μl of FAG2 buffer and mix by vortexing.

- Incubate in ice for 5 min.

- Place a filter column in a 2 ml collection tube.

- Apply the mixture from previous step to the filter column. Centrifuge for 3 min at 13,000 rpm.

- Discard the filter column and carefully transfer the clarified supernatant in the collection tube to a new microcentrifuge tube.

Step 3 – DNA binding

- Add 1.5 volumes of FAPG3 buffer (ethanol added) to the cleared lysate and mix immediately by vortexing for 5 sec. For example, add 750 μl FAPG3 buffer to 500 μL lysate.

- Place a FAPG column in a 2ml collection tube.

- Apply 750 μL of the mixture from the previous step to the FAPG column.

- Centrifuge at full speed (13,000 rpm) for 2 minutes.

- Discard the flow through in the Collection tube.

Step 4 – Wash

- Add 500 μL of W1 Buffer (ethanol added) to the column.

- Add 750 μL of Wash Buffer (ethanol added) to the column.

- Centrifuge at full speed (13,000 rpm) for 3 minutes to dry the column matrix.

Step 5 – DNA elusion

- Transfer dried FAPG column into a clean 1.5 mL microcentrifuge tube.

- Add 50-200 μL of preheated Elusion Buffer into the centre of the column matrix. Stand for 3 minutes until rhe Elusion Buffer is absorbed by the matrix.

- Centrifuge at full speed (13,000 rpm) for 2 minutes to elute purified DNA.

Appendix 3: Quantification of DNA (Invitrogen™)

DNA was separated on an agarose gel and compared to a known standard λ-PstI DNA ladder.

GeneRuler DNA Ladder Mix, ready-to-use

Appendix 4

Table A4: Distance matrix of RAPD analysis generated by the NT-SYS software.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
1	0.0																																										
2	0.2	0.0																																									
3	0.2	0.3	0.0																																								
4	0.3	0.1	0.3	0.0																																							
5	0.4	0.4	0.3	0.2	0.0																																						
6	0.5	0.5	0.4	0.3	0.3	0.0																																					
7	0.6	0.5	0.5	0.4	0.2	0.2	0.0																																				
8	0.4	0.6	0.5	0.6	0.7	0.4	0.5	0.0																																			
9	0.2	0.4	0.3	0.3	0.4	0.5	0.6	0.4	0.0																																		
10	0.3	0.5	0.4	0.4	0.5	0.5	0.7	0.4	0.1	0.0																																	
11	0.3	0.4	0.4	0.4	0.5	0.5	0.5	0.3	0.3	0.3	0.0																																
12	0.4	0.6	0.5	0.5	0.6	0.6	0.8	0.5	0.3	0.2	0.4	0.0																															
13	0.4	0.6	0.4	0.4	0.5	0.5	0.5	0.3	0.2	0.3	0.2	0.4	0.0																														
14	0.2	0.4	0.3	0.3	0.4	0.5	0.6	0.4	0.1	0.2	0.3	0.2	0.2	0.0																													
15	0.4	0.6	0.5	0.5	0.6	0.6	0.6	0.4	0.5	0.4	0.2	0.5	0.3	0.5	0.0																												
16	0.5	0.7	0.7	0.6	0.7	0.6	0.6	0.5	0.5	0.4	0.2	0.7	0.3	0.6	0.1	0.0																											
17	0.3	0.5	0.4	0.5	0.6	0.6	0.6	0.4	0.3	0.3	0.1	0.6	0.3	0.4	0.3	0.2	0.0																										
18	0.3	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.2	0.5	0.3	0.4	0.3	0.3	0.2	0.0																									
19	0.5	0.6	0.4	0.4	0.5	0.6	0.6	0.5	0.2	0.3	0.3	0.4	0.4	0.2	0.5	0.5	0.3	0.3	0.0																								
20	0.4	0.6	0.5	0.5	0.6	0.6	0.6	0.4	0.2	0.2	0.3	0.5	0.3	0.3	0.4	0.3	0.1	0.3	0.2	0.0																							
21	0.3	0.5	0.4	0.5	0.5	0.5	0.6	0.4	0.4	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.6	0.5	0.0																						
22	0.3	0.5	0.4	0.4	0.5	0.4	0.5	0.4	0.2	0.1	0.3	0.2	0.3	0.2	0.5	0.6	0.5	0.4	0.4	0.4	0.4	0.0																					
23	0.3	0.4	0.4	0.4	0.5	0.6	0.7	0.4	0.2	0.3	0.4	0.4	0.4	0.2	0.3	0.5	0.4	0.4	0.5	0.4	0.2	0.2	0.0																				
24	0.3	0.5	0.4	0.5	0.6	0.5	0.5	0.4	0.4	0.5	0.3	0.4	0.4	0.4	0.4	0.4	0.3	0.5	0.3	0.4	0.4	0.3	0.4	0.0																			
25	0.4	0.7	0.5	0.4	0.5	0.5	0.7	0.6	0.2	0.3	0.4	0.5	0.4	0.3	0.4	0.4	0.4	0.5	0.3	0.2	0.6	0.4	0.3	0.4	0.0																		
26	0.5	0.6	0.5	0.5	0.6	0.7	0.8	0.7	0.4	0.4	0.4	0.4	0.4	0.4	0.3	0.2	0.4	0.5	0.7	0.5	0.3	0.4	0.4	0.3	0.4	0.0																	
27	0.4	0.6	0.4	0.4	0.5	0.6	0.7	0.5	0.1	0.2	0.5	0.3	0.4	0.2	0.6	0.5	0.4	0.5	0.4	0.3	0.4	0.3	0.4	0.4	0.2	0.3	0.0																
28	0.4	0.6	0.6	0.6	0.7	0.5	0.6	0.4	0.4	0.5	0.4	0.4	0.3	0.4	0.4	0.5	0.5	0.4	0.7	0.6	0.4	0.3	0.3	0.5	0.4	0.3		0.0															
29	0.4	0.5	0.5	0.5	0.6	0.7	0.8	0.5	0.3	0.3	0.2	0.3	0.3	0.3	0.3	0.4	0.3	0.3	0.5	0.4	0.3	0.3	0.4	0.3	0.4	0.3	0.3		0.0														
30	0.7	0.9	0.7	0.7	0.8	0.9	0.9	0.7	0.4	0.3	0.3	0.4	0.3	0.6	0.4	0.3	0.3	0.4	0.4	0.3	0.7	0.5	0.7	0.5	0.4	0.3	0.3	0.4	0.2	0.0													
31	0.3	0.5	0.4	0.5	0.5	0.6	0.6	0.4	0.4	0.4	0.2	0.4	0.2	0.4	0.3	0.3	0.3	0.3	0.5	0.4	0.3	0.4	0.3	0.5	0.2	0.3	0.2	0.2	0.3		0.0												
32	0.4	0.6	0.6	0.6	0.7	0.8	0.9	0.5	0.3	0.4	0.4	0.5	0.4	0.4	0.5	0.5	0.3	0.4	0.6	0.5	0.3	0.4	0.3	0.4	0.5	0.5	0.3	0.2	0.3	0.3	0.2	0.0											
33	0.4	0.5	0.5	0.4	0.3	0.4	0.3	0.6	0.5	0.5	0.4	0.6	0.5	0.5	0.5	0.6	0.4	0.3	0.7	0.6	0.5	0.4	0.5	0.4	0.5	0.6	0.6	0.5	0.5	0.7	0.4	0.5	0.0										
34	0.3	0.5	0.4	0.5	0.3	0.5	0.4	0.5	0.4	0.4	0.3	0.5	0.4	0.4	0.5	0.5	0.3	0.3	0.6	0.4	0.4	0.4	0.4	0.3	0.6	0.4	0.5	0.4	0.4	0.7	0.3	0.4	0.1	0.0									
35	0.4	0.6	0.4	0.4	0.3	0.4	0.3	0.6	0.4	0.4	0.4	0.5	0.4	0.4	0.5	0.6	0.4	0.4	0.6	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.5	0.7	0.4	0.5	0.1	0.2			0.0								
36	0.3	0.4	0.3	0.3	0.2	0.5	0.4	0.5	0.2	0.3	0.3	0.4	0.3	0.2	0.4	0.5	0.3	0.3	0.5	0.4	0.4	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.5	0.3	0.4	0.2	0.1	0.1		0.0							
37	0.6	0.7	0.5	0.7	0.8	0.8	0.9	0.9	0.6	0.7	0.6	0.8	0.6	0.6	0.7	0.9	0.6	0.6	0.9	0.8	0.7	0.7	0.7	0.8	0.8	0.7	0.7	0.8	0.7	0.9	0.6	0.8	0.7	0.6	0.6	0.5	0.0						
38	0.8	0.9	0.8	0.9	0.9	1.1	1.1	1.3	1.0	1.1	0.8	0.9	0.7	0.8	0.8	1.0	0.9	0.8	1.1	1.2	1.0	1.0	1.0	0.9	1.2	0.7	1.1	0.9	0.8	1.1	0.7	1.2	0.9	0.8	0.8	0.7	0.1	0.0					
39	0.7	0.8	0.6	0.8	0.9	0.9	1.1	0.9	0.6	0.7	0.6	0.6	0.5	0.4	0.8	1.0	0.7	0.7	0.7	0.8	0.8	0.6	0.7	0.9	0.8	0.7	0.7	0.7	0.6	0.9	0.7	0.8	0.8	0.7	0.7	0.5	0.2	0.3	0.0				
40	0.7	0.8	0.6	0.8	0.8	0.9	0.9	1.1	0.8	0.9	0.7	1.0	0.6	0.8	0.7	0.9	0.8	0.7	1.2	1.0	0.9	0.8	0.9	0.8	1.0	0.7	0.9	0.8	0.7	0.9	0.6	1.0	0.8	0.7	0.7	0.6	0.1	0.0	0.3	0.0			
41	0.6	0.7	0.5	0.7	0.8	0.8	1.0	0.9	0.7	0.7	0.6	0.8	0.5	0.7	0.7	0.8	0.7	0.6	1.0	0.8	0.8	0.7	0.8	0.7	0.9	0.6	0.8	0.7	0.6	0.8	0.5	0.8	0.7	0.6	0.6	0.5	0.0	0.1	0.2	0.0	0.0		
42	0.7	0.8	0.6	0.8	0.9	0.9	1.1	0.9	0.4	0.5	0.6	0.8	0.5	0.6	0.7	0.7	0.6	0.7	0.7	0.6	0.7	0.6	0.5	0.9	0.6	0.6	0.6	0.7	0.6	0.7	0.7	0.6	0.8	0.7	0.7	0.5	0.3	0.5	0.3	0.4	0.3	0.0	
43	2	1	1	1	1	1	1	2	2	3	1.4	1.9	2	2	1	2	2	1	2	3	1	2	2	1.2	2	2	3	2	2	2.7	2	2.6	1	1	1	1	1	1.6	2	2	1	2	0

Appendix 5

Table A5: Distance matrix of ISSRanalysis generated by the NT-SYS software.

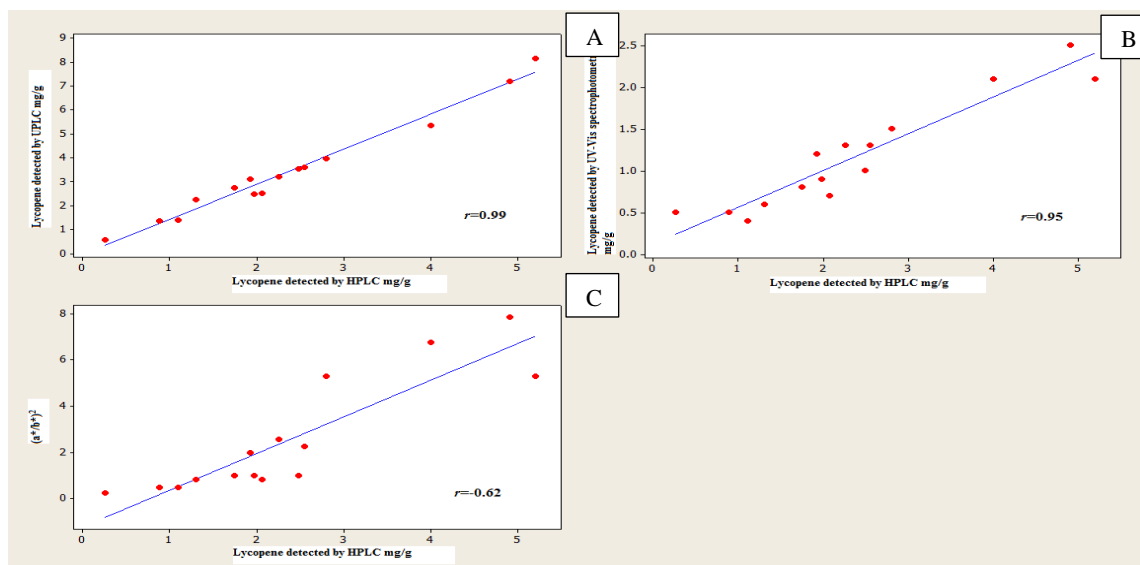|	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
1	0.0																																										
2	0.2	0.0																																									
3	0.2	0.1	0.0																																								
4	0.4	0.3	0.2	0.0																																							
5	0.3	0.4	0.2	0.0	0.0																																						
6	0.2	0.2	0.2	0.3	0.2	0.0																																					
7	0.1	0.1	0.1	0.2	0.2	0.0	0.0																																				
8	0.5	0.6	0.5	0.4	0.4	0.4	0.4	0.0																																			
9	0.3	0.6	0.6	0.5	0.4	0.6	0.5	0.1	0.0																																		
10	0.3	0.5	0.4	0.5	0.4	0.4	0.4	0.1	0.1	0.0																																	
11	0.4	0.5	0.4	0.5	0.4	0.4	0.4	0.0	0.1	0.1	0.0																																
12	0.8	1.0	0.8	0.7	0.6	0.6	0.7	0.5	0.7	0.7	0.6	0.0																															
13	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.2	0.3	0.3	0.2	0.4	0.0																														
14	0.6	0.8	0.6	0.7	0.5	0.5	0.5	0.5	0.7	0.7	0.5	0.2	0.3	0.0																													
15	0.5	0.6	0.5	0.5	0.4	0.4	0.5	0.2	0.3	0.3	0.2	0.3	0.2	0.5	0.0																												
16	0.4	0.4	0.4	0.5	0.4	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.4	0.3	0.2	0.0																											
17	0.5	0.5	0.5	0.6	0.5	0.4	0.4	0.3	0.4	0.3	0.3	0.3	0.3	0.5	0.3	0.2	0.0																										
18	0.3	0.3	0.3	0.4	0.3	0.3	0.2	0.4	0.4	0.3	0.3	0.5	0.4	0.6	0.3	0.2	0.2	0.0																									
19	0.4	0.4	0.4	0.5	0.4	0.3	0.3	0.3	0.4	0.3	0.3	0.5	0.2	0.6	0.3	0.3	0.2	0.1	0.0																								
20	0.3	0.3	0.3	0.4	0.4	0.3	0.2	0.3	0.4	0.3	0.3	0.5	0.3	0.5	0.4	0.2	0.3	0.1	0.1	0.0																							
21	0.5	0.5	0.5	0.6	0.5	0.4	0.4	0.3	0.4	0.3	0.3	0.4	0.2	0.5	0.1	0.2	0.2	0.1	0.2	0.0	0.0																						
22	0.4	0.4	0.4	0.5	0.4	0.4	0.3	0.4	0.4	0.3	0.3	0.3	0.3	0.4	0.3	0.2	0.2	0.1	0.1	0.1	0.2	0.0																					
23	0.4	0.4	0.4	0.5	0.4	0.4	0.4	0.3	0.3	0.2	0.2	0.3	0.3	0.5	0.3	0.2	0.1	0.1	0.2	0.2	0.1	0.1	0.0																				
24	0.4	0.4	0.4	0.5	0.5	0.4	0.3	0.4	0.5	0.4	0.4	0.4	0.4	0.4	0.3	0.1	0.2	0.1	0.2	0.2	0.2	0.1	0.1	0.0																			
25	0.6	0.5	0.4	0.6	0.6	0.6	0.5	0.6	0.6	0.5	0.5	0.4	0.6	0.5	0.4	0.2	0.3	0.2	0.3	0.3	0.3	0.2	0.2	0.1	0.0																		
26	0.4	0.4	0.4	0.5	0.4	0.4	0.3	0.4	0.4	0.3	0.3	0.5	0.4	0.4	0.3	0.1	0.2	0.1	0.3	0.1	0.2	0.1	0.1	0.0	0.1	0.0																	
27	0.5	0.5	0.5	0.7	0.8	0.7	0.6	0.7	0.6	0.5	0.6	0.7	0.7	0.8	0.6	0.3	0.5	0.4	0.6	0.4	0.5	0.4	0.3	0.2	0.2	0.2	0.0																
28	0.4	0.5	0.5	0.6	0.5	0.5	0.4	0.6	0.5	0.5	0.7	0.5	0.4	0.6	0.4	0.5	0.4	0.4	0.4	0.5	0.4	0.3	0.3	0.4	0.5	0.5	0.6	0.0															
29	0.3	0.5	0.5	0.5	0.4	0.5	0.4	0.6	0.4	0.5	0.7	0.5	0.4	0.6	0.4	0.5	0.5	0.5	0.6	0.5	0.6	0.5	0.4	0.5	0.7	0.5	0.6	0.1	0.0														
30	0.3	0.6	0.6	0.6	0.5	0.6	0.5	0.6	0.4	0.5	0.6	0.4	0.5	0.5	0.5	0.4	0.4	0.5	0.7	0.5	0.6	0.4	0.4	0.5	0.6	0.4	0.6	0.2	0.0	0.0													
31	0.4	0.4	0.5	0.6	0.5	0.5	0.4	0.7	0.6	0.6	0.7	0.5	0.4	0.7	0.5	0.5	0.5	0.5	0.6	0.6	0.6	0.5	0.5	0.5	0.7	0.5	0.6	0.1	0.1	0.1	0.0												
32	0.3	0.6	0.6	0.6	0.5	0.6	0.5	0.6	0.4	0.5	0.6	0.4	0.5	0.5	0.5	0.4	0.4	0.5	0.7	0.5	0.6	0.4	0.4	0.5	0.6	0.4	0.6	0.2	0.0	0.0	0.1	0.0											
33	0.6	0.4	0.3	0.2	0.2	0.4	0.4	0.6	0.7	0.7	0.7	0.8	0.6	0.8	0.7	0.7	0.6	0.7	0.8	0.6	0.9	0.7	0.6	0.7	0.7	0.7	0.7	0.8	0.7	0.7	0.6	0.7	0.0										
34	0.6	0.4	0.3	0.2	0.2	0.4	0.4	0.6	0.7	0.7	0.7	0.8	0.6	0.8	0.7	0.7	0.6	0.7	0.8	0.6	0.9	0.7	0.6	0.7	0.7	0.7	0.6	0.8	0.7	0.7	0.6	0.7	0.0	0.0									
35	0.6	0.4	0.3	0.2	0.2	0.4	0.4	0.6	0.7	0.7	0.7	0.8	0.6	0.8	0.7	0.7	0.6	0.7	0.8	0.6	0.9	0.7	0.6	0.7	0.7	0.7	0.6	0.8	0.7	0.7	0.6	0.7	0.0	0.0	0.0								
36	0.7	0.5	0.4	0.1	0.2	0.5	0.4	0.6	0.6	0.6	0.6	0.7	0.6	0.8	0.7	0.7	0.7	0.6	0.8	0.6	0.9	0.6	0.6	0.7	0.6	0.6	0.6	1.0	0.8	0.8	0.7	0.8	0.0	0.0	0.0	0.0							
37	0.5	0.6	0.5	0.6	0.6	0.7	0.6	0.6	0.5	0.4	0.5	0.7	0.4	0.7	0.6	0.5	0.6	0.6	0.6	0.6	0.4	0.6	0.5	0.5	0.5	0.5	0.4	0.8	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.0						
38	0.5	0.6	0.5	0.6	0.6	0.7	0.6	0.6	0.5	0.4	0.5	0.7	0.4	0.7	0.6	0.5	0.6	0.6	0.6	0.6	0.4	0.6	0.5	0.5	0.5	0.5	0.4	0.8	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.0	0.0					
39	0.5	0.6	0.5	0.6	0.6	0.7	0.6	0.6	0.5	0.4	0.5	0.7	0.4	0.7	0.6	0.5	0.6	0.6	0.6	0.6	0.4	0.6	0.5	0.5	0.5	0.5	0.4	0.8	0.6	0.6	0.7	0.6	0.6	0.6	0.6	0.6	0.0	0.0	0.0				
40	0.4	0.5	0.4	0.6	0.6	0.6	0.5	0.6	0.5	0.4	0.5	0.7	0.5	0.7	0.6	0.6	0.5	0.7	0.7	0.5	0.6	0.5	0.5	0.6	0.5	0.5	0.5	0.7	0.5	0.5	0.6	0.5	0.5	0.5	0.5	0.6	0.0	0.0	0.0	0.0			
41	0.4	0.5	0.4	0.6	0.6	0.6	0.5	0.6	0.5	0.4	0.5	0.7	0.5	0.7	0.6	0.6	0.5	0.7	0.7	0.5	0.6	0.5	0.5	0.6	0.5	0.5	0.5	0.7	0.5	0.5	0.6	0.5	0.5	0.5	0.5	0.6	0.0	0.0	0.0	0.0	0.0		
42	0.4	0.5	0.4	0.5	0.5	0.6	0.5	0.5	0.4	0.3	0.4	0.8	0.5	0.8	0.6	0.6	0.6	0.5	0.5	0.4	0.6	0.5	0.5	0.6	0.5	0.5	0.5	0.8	0.7	0.7	0.8	0.7	0.5	0.5	0.5	0.5	0.1	0.1	0.1	0.1	0.1	0.0	
43	1.7	1.6	1.7	1.7	1.4	1.3	1.3	1.7	2.5	1.8	1.4	1.6	1.7	1.4	1.4	1.3	1.5	1.4	1.4	1.3	1.2	1.4	1.4	1.4	1.8	1.4	2.3	2.5	2.5	2.5	2.4	2.5	1.6	1.6	1.6	1.6	2.4	2.4	2.4	2.5	2.5	2.3	0.0

Appendix 6

Figure A1: Scatterplots showing correlations between lycopene of 15 *M. cochinchinensis* aril extracts and analytical methods. A: Scatterplot showing correlations between lycopene and UPLC. B: Scatterplot showing correlations between lycopene and spectrophotometry. C: Scatterplot showing correlations between lycopene and $(a*/b*)^2$.
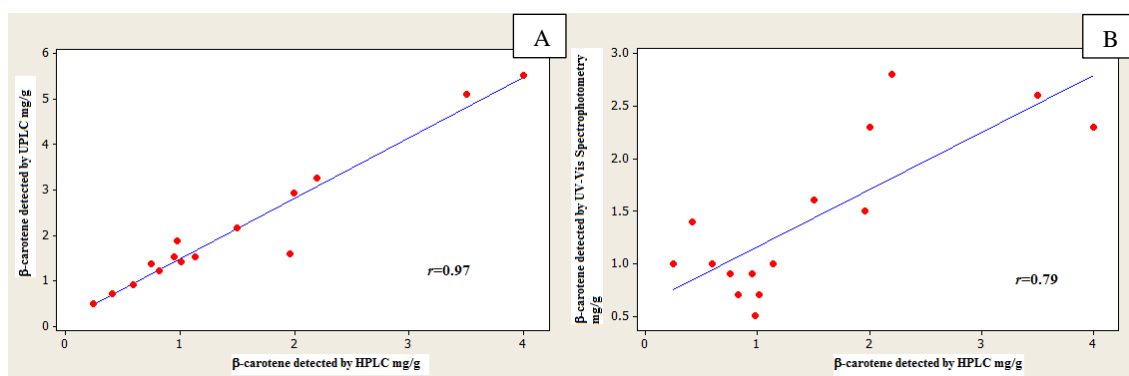

Figure A2: Scatterplots showing correlations between β-carotene of 15 *M. cochinchinensis* aril extracts and analytical methods. A: Scatterplot showing correlations between β-carotene and UPLC. B: Scatterplot showing correlations between β-carotene and spectrophotometry.
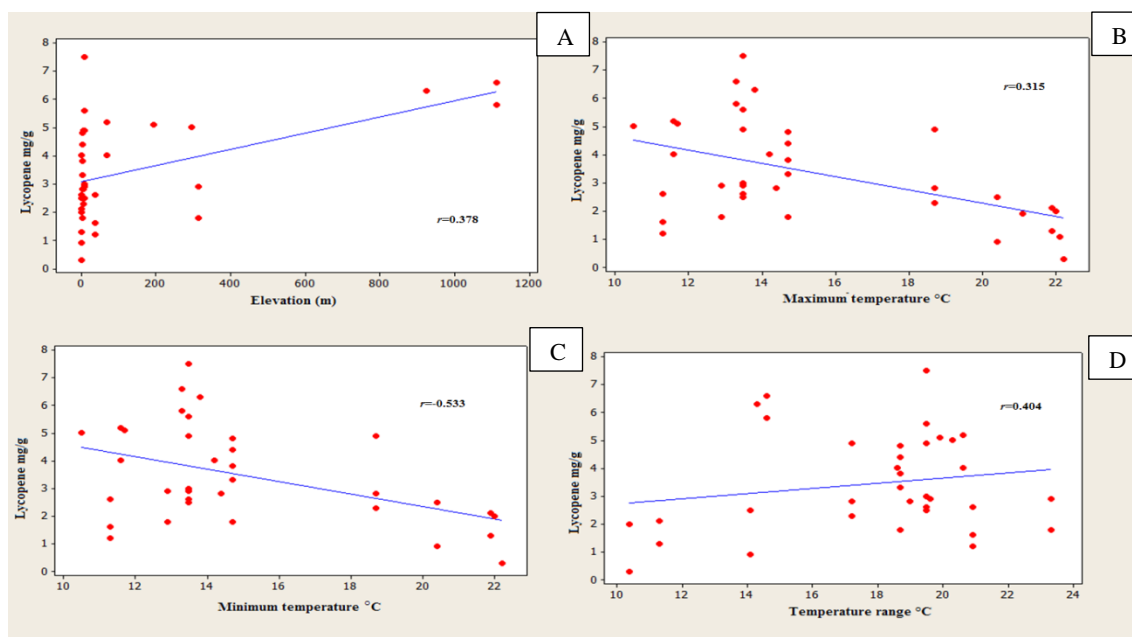
Appendix 8

Figure A3: Scatterplots showing correlations between lycopene concentration of 36 *M. cochinchinensis* aril extracts and different eco-geographical parameters, including elevation (A), minimum temperature (B), maximum temperature (C) and temperature range (D)
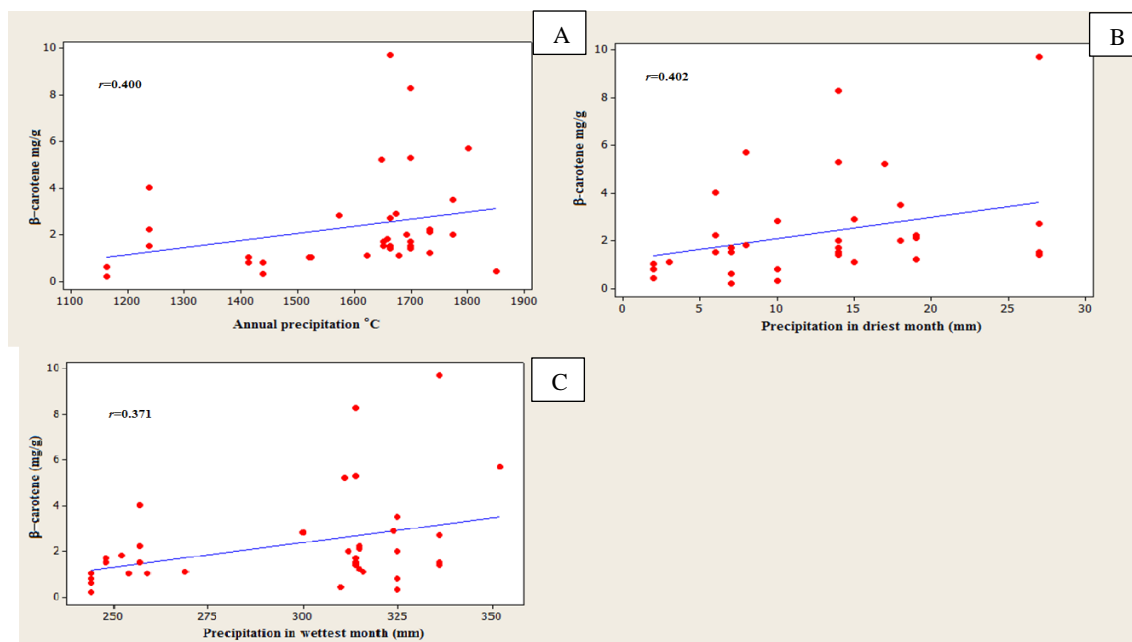

Figure A4: Scatterplots showing correlations between β-carotene concentration of 36 *M. cochinchinensis* aril extracts and different eco-geographical parameters, including annual precipitation (A), precipitation in driest month (B), precipitation in wettest month (C).
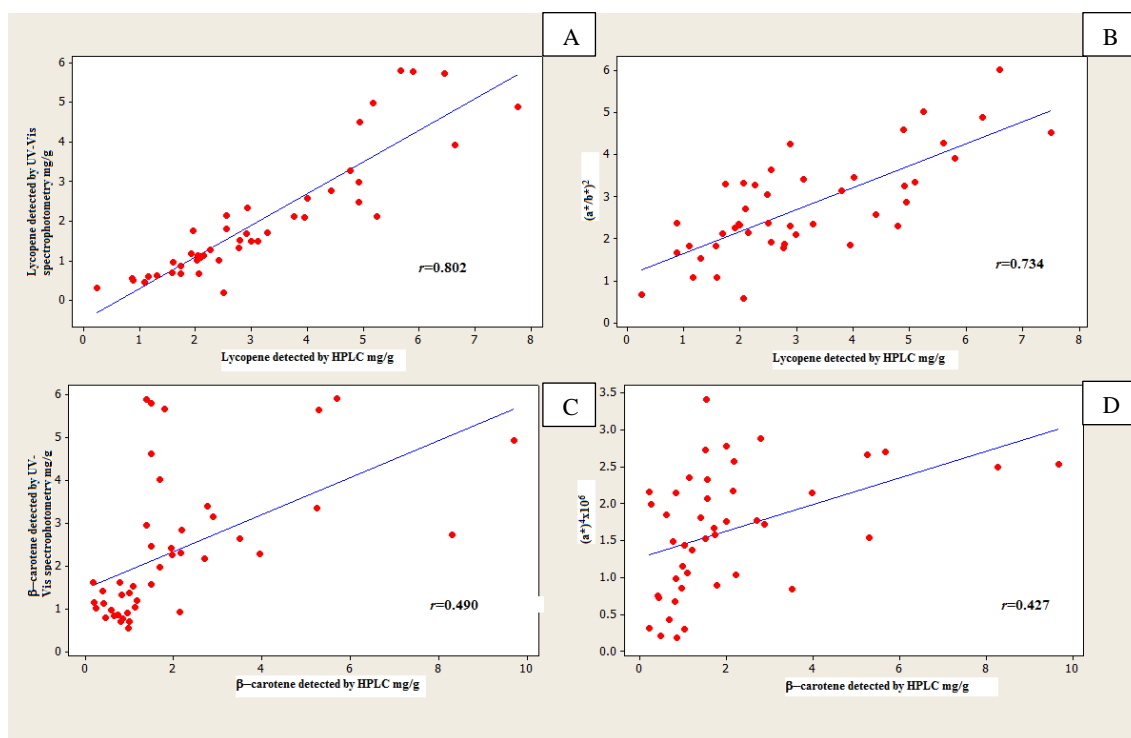
Appendix 9

Figure A5: Scatterplots showing correlations between carotenoids (lycopene and β-carotene) of 44 *M. cochinchinensis* aril extracts and analytical methods. A: Scatterplot showing correlations between lycopene and UV-Vis spectrophotometry. B: Scatterplot showing correlations between lycopene and $(a^*/b^*)^2$. C: Scatterplot showing correlations between β-carotene and UV-Vis spectrophotometry. D: Scatterplot showing correlations between β-carotene and $(a^*)^4 \times 10^6$.
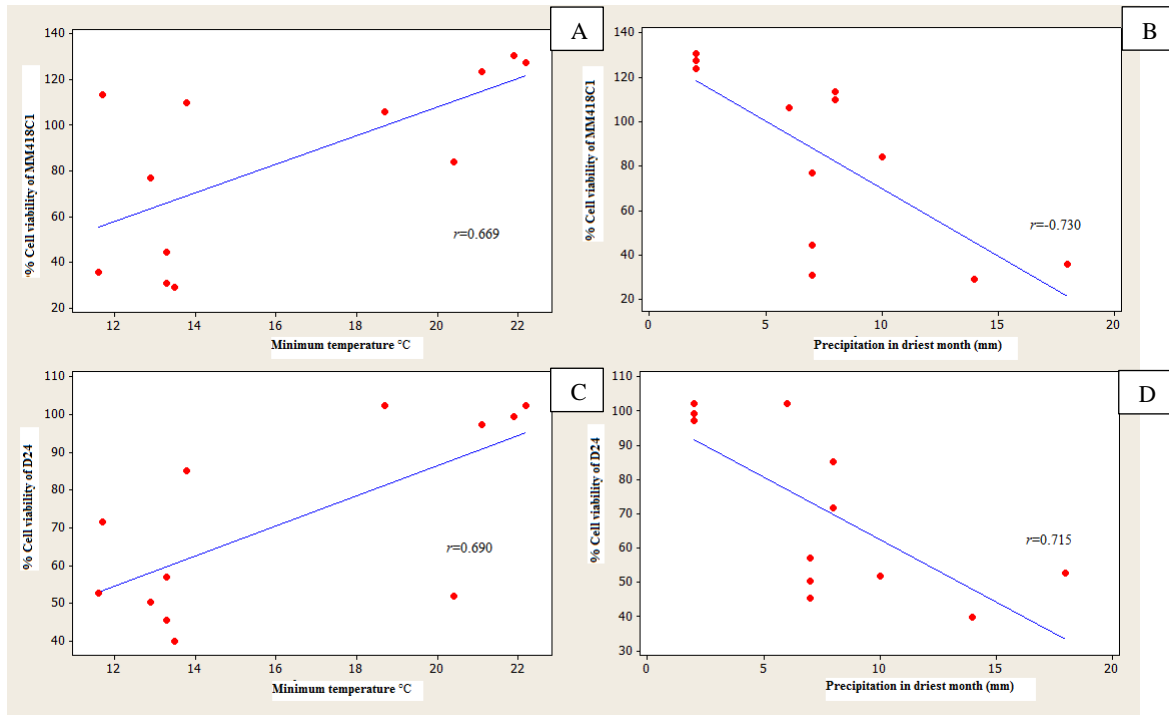
Appendix 10

Figure A6 : Scatter plots showing correlations between cytotoxicity of MM418C1 and D24 cell lines when treated with the aril water extract of 12 *M. cochinchinensis* samples and different parameters, including minimum temperature (A,C), precipitation in the driest month (B,D).

Appendix 11: Maintenance of cell culture and counting

Cells were grown in 75 cm^2 tissue culture flasks. Cells were supplemented with new media containing 10% FBS every second day. Once the cells were 80% confluent (4-5 days), the cells were subcultured using the following steps.

- Used tissue culture media was discarded

- The cells were washed twice with 5 mL of pre warmed (37°C) 1X PBS (Phosphate Buffered Saline).

- Next, 2.5 mL of TrypLE™ Express (without phenol red) was added to the cells and incubated for 2 min at 37°C.

- The cells and media were transferred into a 10 mL falcon tube

- 2.5 mL of fresh media (37°C) was added

- The cells were spun down the cells at 1000 rpm for 5 min

- The media was removed media and 1 mL of fresh tissue culture media (used for counting as described in the next section) was added

- The cells were divided into 75 cm^2 tissue culture flasks containing 20 mL of media supplemented with 10% FBS and antibiotics (streptomycin, penicillin 1% v/v).

Cell counting

10 µL of the cell suspension and 10 µL of trypan blue was mixed in a 100 µL microfuge tube. Then, 10 µL of the mixture was added to the Countess® Cell Counting Chamber Slides and The Countess™ automated cell counter (Invitrogen). All samples were processed and read within 2 min. The actual volume counted was 0.4 µL, the same as counting four (1 mm x 1 mm) squares in a standard haemocytometer.[6]