



**Evaluation of anti-ageing properties of *Moringa oleifera* Lam., *Centella asiatica* (L.) Urban, *Clitoria ternatea* L. and *Cosmos caudatus* Kunth. for potential application as cosmeceuticals**

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## Declaration

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This thesis is submitted to the degree of Doctor of Philosophy at Newcastle University. The research in this thesis was performed in the Department of Agriculture at the School of Natural and Environmental Sciences, under the supervision of Dr. Edward Okello (primary supervisor) and in the Department of Dermatological Sciences at the Institute of Cellular Medicine, under the supervision of Prof. Mark Birch-Machin (secondary supervisor). I declare that the contents of this thesis are my own work unless stated otherwise and have not been presented or accepted in any previous application for a degree or any other qualification at this or any other university.



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## Abstract

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**Background.** In 2010, the launch of the Malaysian Economic Transformation Programme (ETP) changed the agriculture sector in Malaysia from “traditional” agriculture into an agribusiness sector. The National Key Research Area (NKRA) in the agriculture sector focuses on raising the status of local herbs for potential commercialisation. Application of local herbs in the cosmetics industry seems to be a promising investment with the current high demand for natural and organic cosmetics worldwide. Given this scenario, this study focused on investigating the anti-ageing properties of selected medicinal plants which are considered as underutilised in Malaysia, as scientific evidence to support their traditional and cosmetic usages is lacking.

**Aim.** This project aims to add agricultural and commercial value to the selected medicinal plants; *Moringa oleifera* Lam., *Centella asiatica* (L.) Urban, *Clitoria ternatea* L. and *Cosmos caudatus* Kunth. which were investigated for their anti-ageing properties (antioxidant, anti-collagenase, anti-elastase and anti-tyrosinase activities) and their protective effects against oxidative-stress induced by hydrogen-peroxide and UV radiation *in vitro*.

**Methods:** Two extracts per plant species were prepared using water and 70% ethanol to mimic either the traditional methods or industrial extract preparations. The total phenolic content (TPC) was determined by the Folic Ciacalteau method. High-performance liquid chromatography-mass spectrophotometry was performed to characterise the individual components of the extracts and to chemically authenticate the plant samples. Antioxidant activities were determined using diphenyl-picryl hydrazine (DPPH), 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS). The results are expressed as Trolox equivalent (mg/g dried extract) and IC<sub>50</sub> (μg/mL). The anti-collagenase and anti-elastase activities were measured by fluorescence assays and anti-tyrosinase activity using a colourimetric assay. The

results are expressed as percentage inhibition (%) compared to control (untreated enzymes).

HaCaT cells (human keratinocytes) were used to investigate the protective effect against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity by measuring the percentage cell viability of HaCaT treated with plant extracts compared with the control (untreated HaCaT). The protective effect against UV radiation was evaluated using polymerase chain reaction (PCR) targeting mitochondrial DNA (mtDNA) damage as a biomarker.

**Results:** In the antioxidant assays. *M. oleifera* ethanol extract (MOE) showed the highest antioxidant compared with other extracts, with 941 µM/mg, 135 µM/mg, 312 µM/mg of Trolox equivalent (TE)/mg dried extract in the ABTS, DPPH and FRAP assays respectively. Other plant extracts also demonstrated antioxidant activities, but at different levels between assays. The correlation analysis of TPC vs. antioxidant activities of all plant extracts showed a strong positive correlation (R=0.74), which suggests that phenolics might be responsible for the observed activities.

The collagenase, elastase and tyrosinase assays showed that *C. caudatus* and *C. ternatea* extracts were able to inhibit the enzymes in a dose-dependent manner. At the highest concentration tested (1 mg/mL), *C. caudatus* water extract (CCW) inhibited collagenase, elastase and tyrosinase activities by 48.7%, 64% and 72.6% respectively, while its ethanolic extract (CCE) inhibited collagenase and elastase by 46.7% and 26% respectively. *C. ternatea* extracts (CTW and CTE) inhibited collagenase, whereas CTE showed higher inhibition compared with CTW (70.1% vs. 82.1% respectively). Both extracts also inhibited elastase with 28% and 24 % inhibition for CTW and CTE respectively. *C. asiatica* extract showed collagenase inhibition, where its ethanol extract (CAE) inhibited collagenase at 77.4%. Meanwhile, *M. oleifera* extracts exhibited very low (6-22%) or no activity against the enzymes.

In the  $\text{H}_2\text{O}_2$ -induced cytotoxicity assay, only the water extracts of *M. oleifera* (MOW), *C. asiatica* (CAW) and *C. ternatea* (CTW) demonstrated significant protective effects ( $p<0.05$ ). Such activity was absent in the ethanol extracts of the plant species, mainly due to the lower concentrations that had to be used since the ethanol extracts were more toxic to HaCaT compared with the water extracts. Similar results were observed in the UV-induced mtDNA damage assay where the water extracts showed significant protective effects compared to the ethanol extracts. In the UV filter experiment, the water extracts of *C. asiatica*, *C. ternatea*, *C. caudatus* (CAW, CTW, CCW) and MOE showed significant protective effects against UV-induced mtDNA damage ( $p <0.05$ ). The UV spectra of each plant extract demonstrated UV absorbing properties, which may explain the protective effect observed. Other than that, the antioxidant properties of the extracts could also play a role by lowering UV-induced ROS level in the cells. This is further supported by the comparison of potency, which showed that MOE exerted protective effects in both UV filter and pre-incubation experiments, where MOE was shown to have the highest antioxidant activities in comparison with the other extracts in the DPPH, ABTS and FRAP assays.

**Conclusion.** The findings in this study demonstrate that the prepared extracts of the selected medicinal plants have high potential to be developed into antioxidant, anti-wrinkle, hypopigmentation agents and UV-filters. Significant protective effects against the targets of interest were observed that may provide a scientific explanation for the putative traditional and commercial use of the plants. These findings should therefore increase the value of these underutilised medicinal plants.



## List of abbreviations

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Abbreviation	Description
µL	microlitre
µM	micromolar
1,10-PNT	1,10-phenanthroline
ABTS	Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid
AL4	Forward primer
AS1	Reverse primer
ATP	Adenosine triphosphate
CAE	<i>Centella asiatica</i> ethanol extract
CAT	catalase
CAW	<i>Centella asiatica</i> water extract
CCE	<i>Cosmos caudatus</i> ethanol extract
CCW	<i>Cosmos caudatus</i> water extract
CPD	Cyclobutene pyrimidine dimers
Ct	Number of cycles (in PCR) that cross a threshold
CTE	<i>Clitoria ternatea</i> ethanol extract
CTW	<i>Clitoria ternatea</i> water extract
DMEM	Dulbecco's modified eagle media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	Diphenyl-1-1-picrylhydrazyl
ECM	Extracellular matrix
EGCG	Epigallocatechin gallate
EPPs	Entry point projects
ETC	Electron transport chain
ETP	Economic Transformation Programme
FCS	Foetal calf serum
FRAP	Ferric reducing antioxidant power
g	gram

GA (E)	Gallic acid (equivalent)
GRAS	Generally recognized as safe
GSH	Glutathione
GSH-Px	Glutathione peroxidase
HA	Hyaluronic acid
HaCaT	Human keratinocyte cell line
L-DOPA	L-3,4-dihydroxyphenylalanine
M	Molar
MAAPV	<i>N</i> -Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl
mg	milligram
ml	millilitre
mM	millimolar
MMP	Matrix metalloprotein
MMPs	Matrix metalloproteinases
MnSD	Manganese superoxide dismutase
MOE	<i>Moringa oleifera</i> ethanol extract
MOW	<i>Moringa oleifera</i> water extract
mtDNA	Mitochondrial DNA
MT-MPs	Membrane-type matrix metalloproteins
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHEK	Normal human epidermal keratinocyte
NKEA	National Key Economic Area
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RNS	Reactive nitrogen species
ROS	Reactive oxygen species

rpm	Revolution per minute
SANA	<i>N</i> -Succ-(Ala) 3-nitroanilide
SED	Standard erythemal dose
SOD	Superoxide dismutase
TAC	Total anthocyanin content
TE	Trolox equivalent
TIMPs	Tissue inhibitors metalloproteinases
TPC	Total phenolic content
TRP	Tyrosinase-related protein
TYR	Tyrosinase



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# **Chapter 1**



# Introduction

---

## 1.1 Introduction

The agricultural sector in Malaysia has been in transition to become an agribusiness sector since the launch of the Malaysian Economic Transformation Programme (ETP) in 2010. One of the main agenda in the agriculture National Key Economic Area (NKEA) policy is to uplift the status of local herbs for commercialisation (Ministry of Agriculture Malaysia, 2010). Applications of local herbs in the cosmetics industry may represent a promising investment, given the current worldwide trend for natural and organic cosmetics. The estimated market for natural and organic cosmetic is predicted to grow from USD 8 billion in 2013 to approximately USD 16 billion by 2020 (Grand View Research, 2016). At the national level, the emerging trend of "*halal*" cosmetics in compliance with Sharia law contributes to the further relevance of the use of herbs in the cosmetics industry. The "*halal*" cosmetic industry in the Southeast Asia region was valued at USD 945.8 million in 2015 and is expected to expand to a compound annual growth rate (CAGR) of 10.2% (Future Market Insight (FMI), 2015; Hassali *et al.*, 2015). Malaysia is at an advantage for the industry in terms of this industry because the Malaysian tropical rainforest has been predicted to harbour more than 2000 species of flora, representing a rich source of exotic and unique natural ingredients (Burkill, 1966).

Local traditional ethnobotanical knowledge suggests that many herbs possess remarkably high medicinal value, and some herbs have been utilised in traditional skin care or as cosmetics. For example, a specific plant or plant parts, commonly known as "*ulam*" by local people, is believed to promote a younger looking skin complexion and to prolong age. "*Ulam*" usually falls within the group of vegetables that contain high-value phytochemicals, such as the

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<sup>1</sup> The term "*halal*" originated from an Arabic word meaning lawful or permissible, and "*halal*" cosmetics must not contain porcine or non-Sharia law compliance animal by-products and are typically alcohol free.

phenolics that are known to have beneficial effects on health and the skin. “*Ulam*” may be referred to as “Malaysian herbs”, “vegetable salad” or “Malay salad” in the scientific literature, and it has been reported to have high antioxidant potential (Huda-Faujan *et al.*, 2007; Liliwirianis *et al.*, 2011; Reihani and Azhar, 2012). Meanwhile, antioxidants have been postulated to play a significant role in the management of oxidative stress-related skin agieng. In the present study, four medicinal plants were selected to be investigated for their anti-ageing properties with potential exploitation as cosmetic ingredients. These are considered to be underutilised in Malaysia and lacking in scientific evidence to support their traditional and cosmetic usages. The selected medicinal plants are *Moringa oleifera* Lam., *Centella asiatica* (L.) Urban, *Clitoria ternatea* L. and *Cosmos caudatus* Kunth.

# **Chapter 2**



## **Chapter 2: Literature Review**

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### **2.1 Malaysian agriculture: from agriculture to agribusiness**

The agricultural sector plays a significant role in Malaysia's economic development. It provides rural employment, increases rural incomes and ensures national food security. In 2009, the sector contributed merely 4 % of Malaysia's gross national income and was traditionally known as the poor man's sector. In 2010, the agriculture sector was identified as a Malaysian National Key Economic Area (NKEA) in the national Economic Transformation Programme (ETP). The launch of the Malaysian ETP initiated the transformation of the sector from "traditional" agriculture to an agribusiness sector. The sector continues to expand, and in 2015 provided a contribution of 8.9 % to the national income. The Agriculture NKEA, as identified in the ETP focuses on selected sub-sectors which have high-growth potential, and the herbal industry is one of the 16 entry point projects (EPPs) that have been identified as the sources of national economic growth (Ministry of Agriculture Malaysia, 2010).

### **2.2 The cosmetics industry provides a global market for local herbs**

In response to the transition of the Malaysian agriculture sector to agribusiness, the use of local herbs in the cosmetics industry seems to be very promising. Recent trends in global and national cosmetic markets indicate a huge market for local herbs, and the market for organic cosmetics is predicted to grow from approximately USD 8 billion in 2013 to USD 16 billion by 2020 (Grand View Research, 2016), and this figure doubles to USD 32 billion when natural cosmetics are included (Kline Group, 2015). Moreover, the Asian Pacific remains the fastest growing region for the cosmetics industry in the recent years, with a market value estimation of USD 60 billion (US Department of Commerce, 2015). In 2013, the total trade volume in cosmetics and toiletries, both import and export, in Malaysia was approximately USD 407

million (Department of Statistic Malaysia, 2013). The new emerging “*halal*” cosmetic market which was valued at USD 945.8 million in the Southeast Asia region in 2015 (Future Market Insight (FMI), 2015). This market segment is believed to contribute to the reductions in cosmetic imports and to promote local cosmetic manufacturing in Malaysia (Hassali *et al.*, 2015). Factors such as the large Muslim population, public awareness and rising concerns about the source of cosmetics ingredients among Muslim and other concerned consumers contribute to increasing demand for plant-based cosmetics, which further supports the relevance of the utilisation of local herbs in the cosmetics industry.

### **2.3 Phytocosmetics: a beauty secret from the past**

The term phytocosmetics is relatively new in the cosmetic industry. However, the use of plants in cosmetics has been known since time immemorial, as evidenced in historical documentation such as Eber’s and Smith’s papyri from the Ancient Egyptian civilization (Leake, 1952), and the “*De Materia Medica*”<sup>2</sup>, “*De compositione medicamentorum secundum locos*”<sup>3</sup> and “*De compositione medicamentorum per genera*”<sup>4</sup> from the Greek and Roman empires (Staub *et al.*, 2016; Grivas, 2017). Cosmetics recipes and ingredients are mentioned in detail in historical texts and are mostly derived from herbs. In Egypt, the traditional practice of using herbs or natural ingredients for cosmetic purposes has continued until the present day, as evidenced in a recent case study of 376 Alexandrian women (Elansary *et al.*, 2015).

Traditional Malaysian knowledge also includes valuable information on the usage of plants for cosmetics and medicine. The current Malaysian population consists of Malays (67.4%), Indians (24.6%), Chinese (7.3%) and other minorities (0.7%) (Department of Statistic Malaysia, 2010). Of the minorities, one group of indigenous people called the “*orang asli*” are believed to have

---

<sup>2</sup> *De Materia Medica* = “On the Medical Material”.

<sup>3</sup> *De compositione medicamentorum secundum locos* = “On the Composition of Drugs by Parts”.

<sup>4</sup> *De compositione medicamentorum per genera* = “On the Composition of Drugs by Type.”

great influence on the traditional practices in Malaysia (Alsarhan *et al.*, 2014). Their knowledge of plants for food and medicine is extensive, mainly because they live very close to nature and most “*orang asli*” villages are located near the edges of the national rainforests in Kelantan, Pahang, Negeri Sembilan and Kedah (Jeba *et al.*, 2010). Plant species with medicinal properties as used by the “*orang asli*” have been reported in several studies, such as: 146 species of plants used by local traditional practitioners in Kelantan (Ong and Nordiana, 1999); 62 plant species used by the “*orang asli*” in Perak (Jeba *et al.*, 2010); 39 species plants used by the *Kensiu* tribe of the “*orang asli*” in Kedah (Mohammad *et al.*, 2012); and 25 plant species used in postpartum practices in Perak and Negeri Sembilan (Othman *et al.*, 2014). Although there is no specific compilation of herbs for cosmetics recorded based on traditional Malaysian knowledge, the previously mentioned study by Ong and Nordiana (1999) has reported an “*ulam*” called *Curcuma longa* L. (synonym *Curcuma domestica* Valeton), which should be consumed fresh to improve skin complexion. The beneficial effects of “*ulam*” on the skin are well-known by locals, and this knowledge has been passed on orally across the generations.

## **2.4 “*Ulam*” as a source of cosmetics ingredients**

In Malaysia, “*ulam*” refers to certain parts of or the whole plant, which is consumed as a vegetable salad. Some “*ulam*” is cooked or partly cooked before eating, but most of the time it is eaten raw and fresh. Some popular “*ulam*” are being studied mainly for their antioxidant properties and are referred to as “Malaysian herbs”, “vegetable salad” or “Malay salad” in the literature (Huda-Faujan *et al.*, 2007; Liliwirianis *et al.*, 2011; Reihani and Azhar, 2012). The antioxidant properties of “*ulam*” have been linked to the presence of phenolic compounds which are known to have beneficial health effects and to reduce the risk of many chronic diseases such as diabetes, heart disease, cancer and ageing (Balasundram *et al.*, 2006). The use of “*ulam*” as cosmetics ingredients is highly relevant given the current trends demanding

organic, natural and “halal” cosmetics as supported by the market analysis described in the previous section. Moreover, natural ingredients from herbs, fruit and vegetables are expected to provide fuel for the cosmetics industry, as they are excellent sources of antioxidants (Grand View Research, 2016).

## **2.5 The medicinal plants selected for the study**

Given the Malaysian agriculture NKEA and the current trends in the cosmetics industry as previously discussed, four medicinal plants were selected for this study. The selected plants were *M. oleifera* Lam., *C. ternatea* L., *C. asiatica* (L.) Urb. and *C. caudatus* Kunth., as shown in Figure 2.1. These plants are underutilised in Malaysia and scientific evidence supporting their traditional and cosmetic usages is lacking. The criteria for selection were as follows.

- Potential mass cultivation**

All the selected plants are listed as agriculturally important plant species in the Malaysian NKEA with high potential for mass cultivation due to their high resistance to disease and drought. This is critically important to balance the demand and supply of raw materials for industrial applications. Therefore, further research on the potential commercial application of the plants is needed.

- Ethno-pharmacological benefits for the skin**

All the selected plants or plant parts used in the study are in the category of “ulam” based on their ethnopharmacologically defined benefits for the skin either by consumption or application on the skin as a paste. Additionally, the application of food plants in cosmetics products is usually perceived as safer by consumers.

- **Limitations in the scope of the study (anti-ageing properties)**

An extensive literature search was performed to further support the selection of plants in this project. The literature search was performed using the keywords of each plant's scientific name, and later was narrowed down by adding the keywords "ageing" or "aging" and "skin ageing" or "skin aging" to the search terms. As expected, very few research studies were found for these plant species in the anti-ageing studies, except for *C. asiatica* which has been relatively well-researched in this area.



A. *Moringa oleifera*



B. *Clitoria ternatea*



C. *Centella asiatica*



D. *Cosmos caudatus*

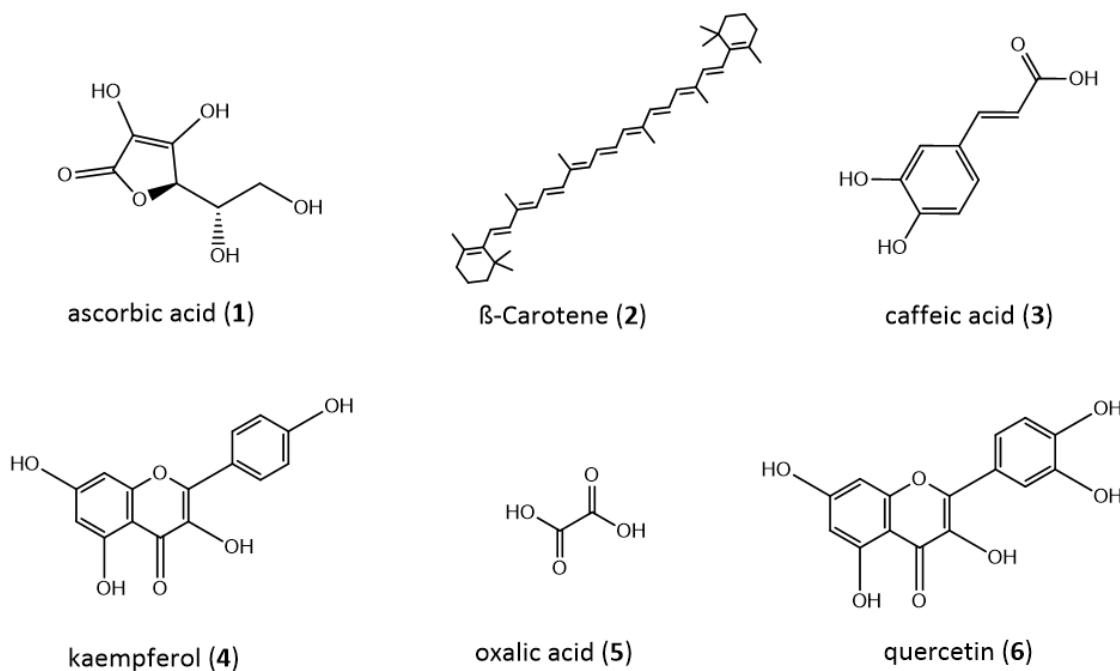
**Figure 2.1: Figure shows the selected plant species in the study.** The English and local names for each of the plants are: horseradish tree/kacang kelor (A); butterfly pea/bunga telang (B); gotu kola/pegaga (C); and wild cosmos/ulam raja (D). Images A,B,C and D retrieved from Crasto ( 2014), Aruna (2009), Anon (2014) and Morad (2011) respectively.

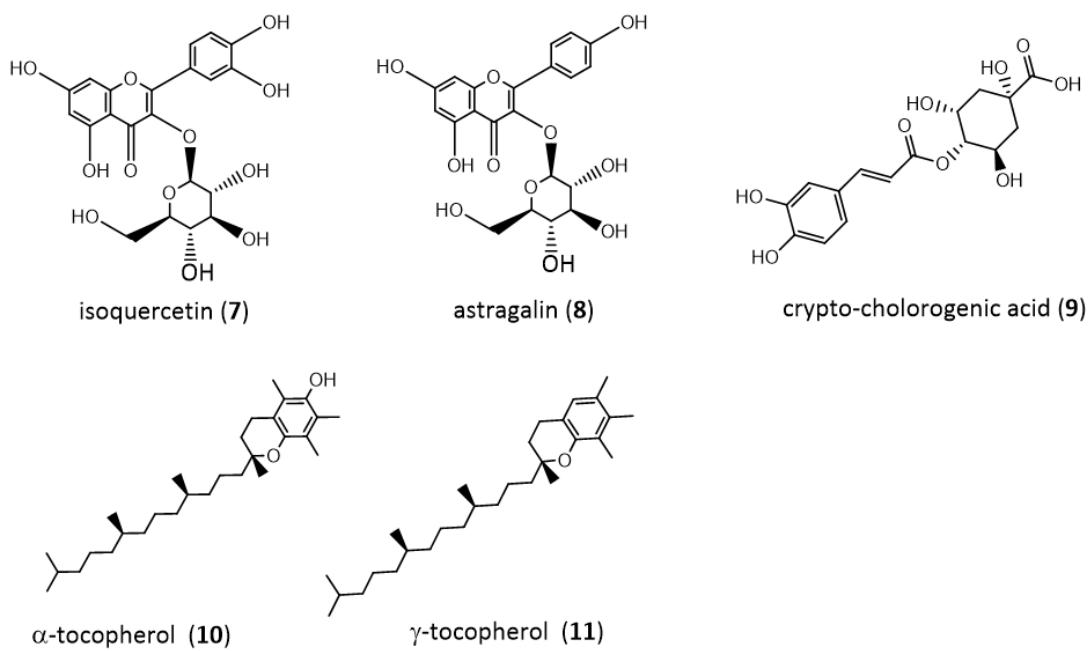
### 2.5.1 *Moringa oleifera* Lam.

*Moringa oleifera* Lam. (Moringaceae) is a perennial softwood tree growing to approximately 5 to 10 m in height (Palada, 1996). It originates from the south of Asia, where it grows in the Himalayan foothills and is believed to be native to India, Pakistan, Bangladesh and Afghanistan (Paliwall *et al.*, 2011). Nowadays, the plant has spread to South and Central America, Mexico, Hawaii and the Asian wider region, where it is commercially grown due to its economic and commercial value (Palada, 1996; Paliwall *et al.*, 2011). *M. oleifera* is referred to with various names, such as the horseradish tree, drumstick tree, benzolive tree, kelor, remunggal, marango, mlonge, moonga, mulangay, nébéday, saijhan, sajna or ben oil tree, depending on region of cultivation (Farooq *et al.*, 2012). The medicinal potential of *M. oleifera* has long been recognised in the Ayurvedic and Unani medicinal system. Almost all parts of the plant (root, bark, gum, leaf, fruit (pods), flowers, seed and seed oil) are used in the indigenous medicine to treat various ailments and diseases, including as an abortifacient, an antidote for centipede, scorpion or spider bites or stings, bactericide, diuretic, and a tonic (Palada, 1996; Mishara *et al.*, 2011). Although the cultivation of *M. oleifera* is increasing, the plant is still considered to be underutilised in Southeast Asian countries (Andarwulan *et al.*, 2012). In Malaysia, the traditional usage of *M. oleifera* focus on the leaves. A survey by Nordiana and Ong (1999) recorded the use of *M. oleifera* for constipation (cooked leaves) and swollen breasts due to childbirth (pounded leaves). However, the most common use of the leaves is as a salad or “ulam”, particularly to benefit the skin. The consumption of *M. oleifera* leaves is also typical in Indonesia and Africa due to its high nutritional content. It has been reported that the powdered leaves are used as a supplement for pregnant and breastfeeding women and as an alternative and inexpensive solution to treat malnutrition (Sambou Diatta, 2001; Fuglie, 2005). The high antioxidant potential of *M. oleifera* leaves is attributed to phenolics, flavonoids and vitamins, and can play a role in defence against skin ageing as believed by the elderly

(Sreelatha and Padma, 2009; Vongsak, Sithisarn, Mangmool, *et al.*, 2013; Vongsak *et al.*, 2014).

However, the scientific evidence of the protective effect of the leaves against skin ageing is still lacking based on the extensive literature search performed. Some of the phytoconstituents present in *M. oleifera* leaves are as shown in Figure 2.2. The leaves of *M. oleifera* consist of ascorbic acid (1), beta-carotene (2), caffeic acid (3), calcium, choline, copper, fiber, iodine, iron, kaempferol (4), oxalic acid (5) and phosphorus (Duke, 1999). Flavonols such as quercetin (6) and kaempferol are the most commonly detected flavonoids in the leaves extract, where their 3-O-glucosides were found; isoquercetin (7) and astragalin (8), together with crypto-chlorogenic acid (9) (Vongsak *et al.*, 2013; Vongsak, Sithisarn and Gritsanapan, 2013; Vongsak *et al.*, 2014). The leaves also contain  $\alpha$ - and  $\gamma$ -tocopherol (10 and 11) (Sanchez-Machado *et al.*, 2006).



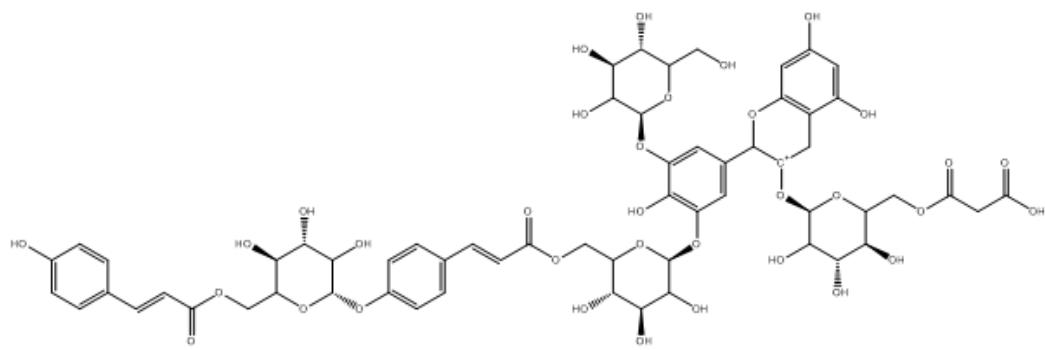


**Figure 2.2: Chemical structure of major phytoconstituents in *M. oleifera* leaves.**

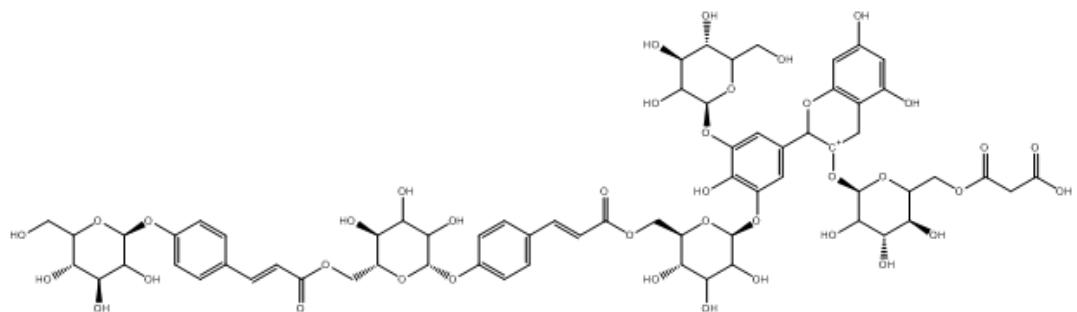
### 2.5.2 *Clitoria ternatea* L.

*Clitoria ternatae* L. (Leguminosae) or the butterfly pea originates from tropical Asia and was later distributed to South and Central America, the East and West Indies, China and India (Devi *et al.*, 2003). There are at least 60 species in the genus *Clitoria*, and *Clitoria ternatea* L. is the most frequently reported species (Devi *et al.*, 2003). The plant is perennial and easily identified by its conspicuous blue and white flower resembling a conch-shell (Mukherjee *et al.* 2008). The traditional usage of *C. ternatea* are extensive, from medicinal to food dyes for its naturally vivid blue colour. There have been many pharmacological activities reported for the plant, such as antimicrobial, antipyretic, anti-inflammatory, anticonvulsant, diuretic, anaesthetic, antidiabetic and insecticidal (Mukherjee *et al.*, 2008; Kosai *et al.*, 2015). Previous research has focused on the roots, seeds and leaves of the plants for its common uses as a laxative and a brain and nerve tonic in the Ayurvedic medicinal system. The root juice is mixed with honey and ghee to improve mental health and memory in children or it is used as a laxative when

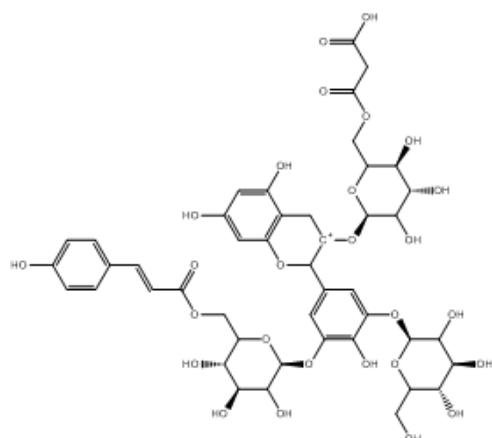
combined with ginger (Mukherjee et al. 2008). However, there is a lack of research on the biological activities of the flowers to explain their traditional and potential uses. Apart from ornamental purposes, the flowers have traditionally been consumed in a salad or prepared as an herbal tonic drink to promote younger looking skin complexion and a defence against skin ageing (Kaisoon et al. 2011; Lijon et al. 2017). Although a few authors have reported the use of *C. ternatea* flower extract as a cosmetics ingredient (Tantituvanont, 2008; Kamkaen and Wilkinson, 2009; Lijon et al., 2017), comprehensive studies of the *C. ternatea* flowers with respect to skin ageing are lacking. The flowers have been reported to have antioxidant activities, mainly due to anthocyanin content (Kamkaen and Wilkinson, 2009; Kaisoon et al., 2011; Iamsaard et al., 2014; Chayaratanasin et al., 2015). Anthocyanins are polyphenols with known antioxidant potential that play a significant role in the prevention of diseases such as cancer and cardiovascular diseases as well as defence against skin ageing (Miguel 2011). The plants are also used as forage for livestock, as they are highly palatable and nutritive (Gomez and Kalamani, 2003). The anthocyanin derivatives of ternatins, a group of (poly)acylated anthocyanins such as the delphinidins are the compounds that cause the bluish color of this species. Figure 2.3 shows the ternatins C1-C5 (1-5) and preternatins A3 (6) and C4 (7) that have been isolated from a young *C. ternatea* flowers (Terahara, 1998; Kazuma et al., 2003a).



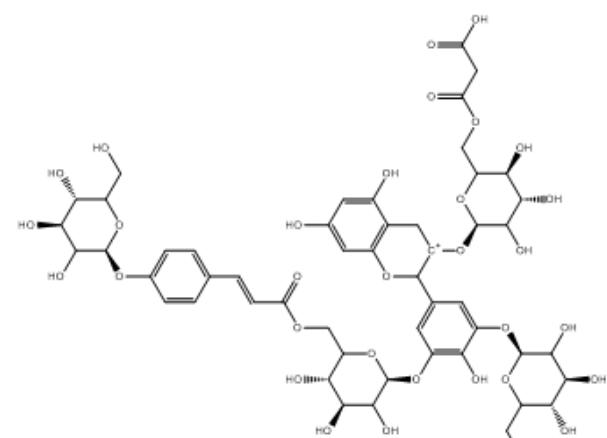
Ternatin C1(1)



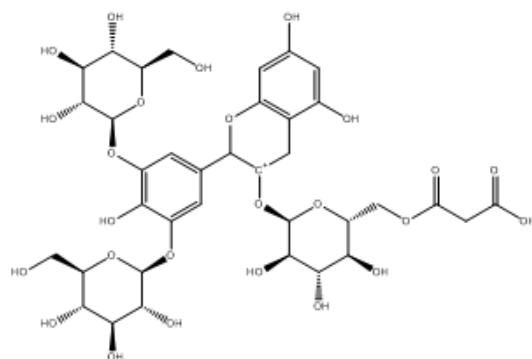
Ternatin C2 (2)



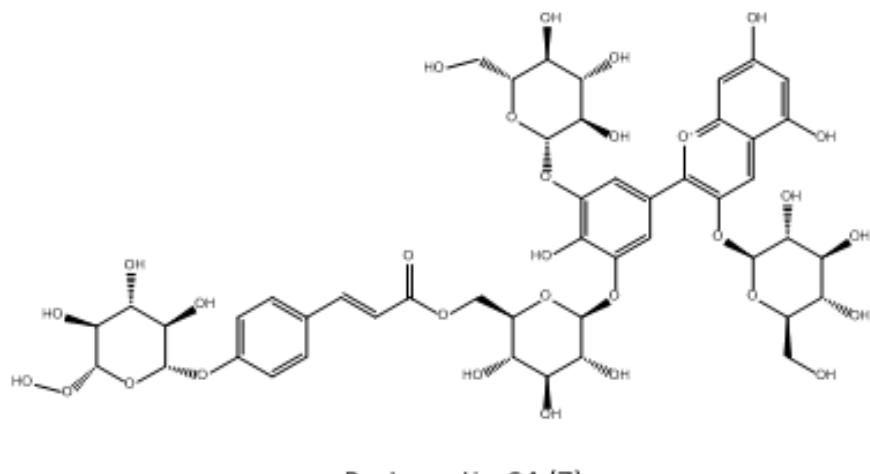
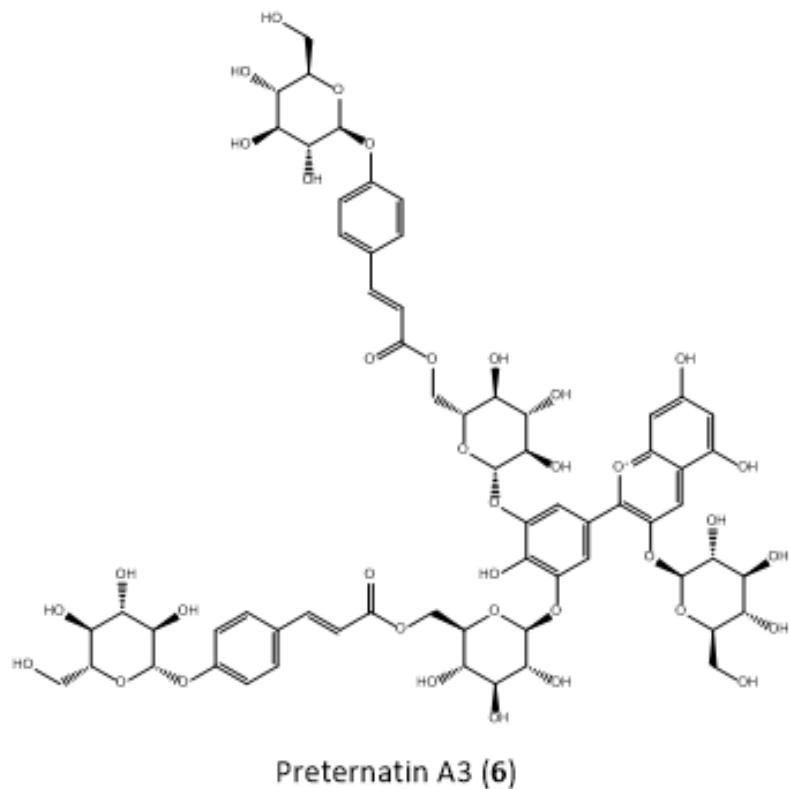
Ternatin C3 (3)



Ternatin C4 (4)

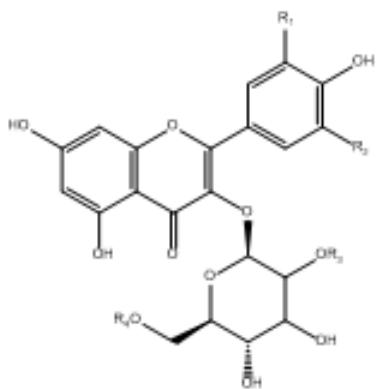


Ternatin C5 (5)



**Figure 2.3: Anthocyanins present in the flowers of *C. ternatea*.**

Other than that, flavonol glycosides of kaempferol, quercetin and myricetin, including three malonylated flavonol glycosides; kaempferol 3-*O*-(2"-*O*-α-rhamnosyl-6"-*O*-malonyl)-β-glucoside, quercetin 3-*O*-(2"-*O*-α-rhamnosyl-6"-*O*-malonyl)-β-glucoside, and myricetin 3-*O*-(2"-*O*-α-rhamnosyl-6"-*O*-malonyl)-β-glucoside also have been found in the flower petals, as shown in Figure 2.4 (Kazuma *et al.*, 2003b).



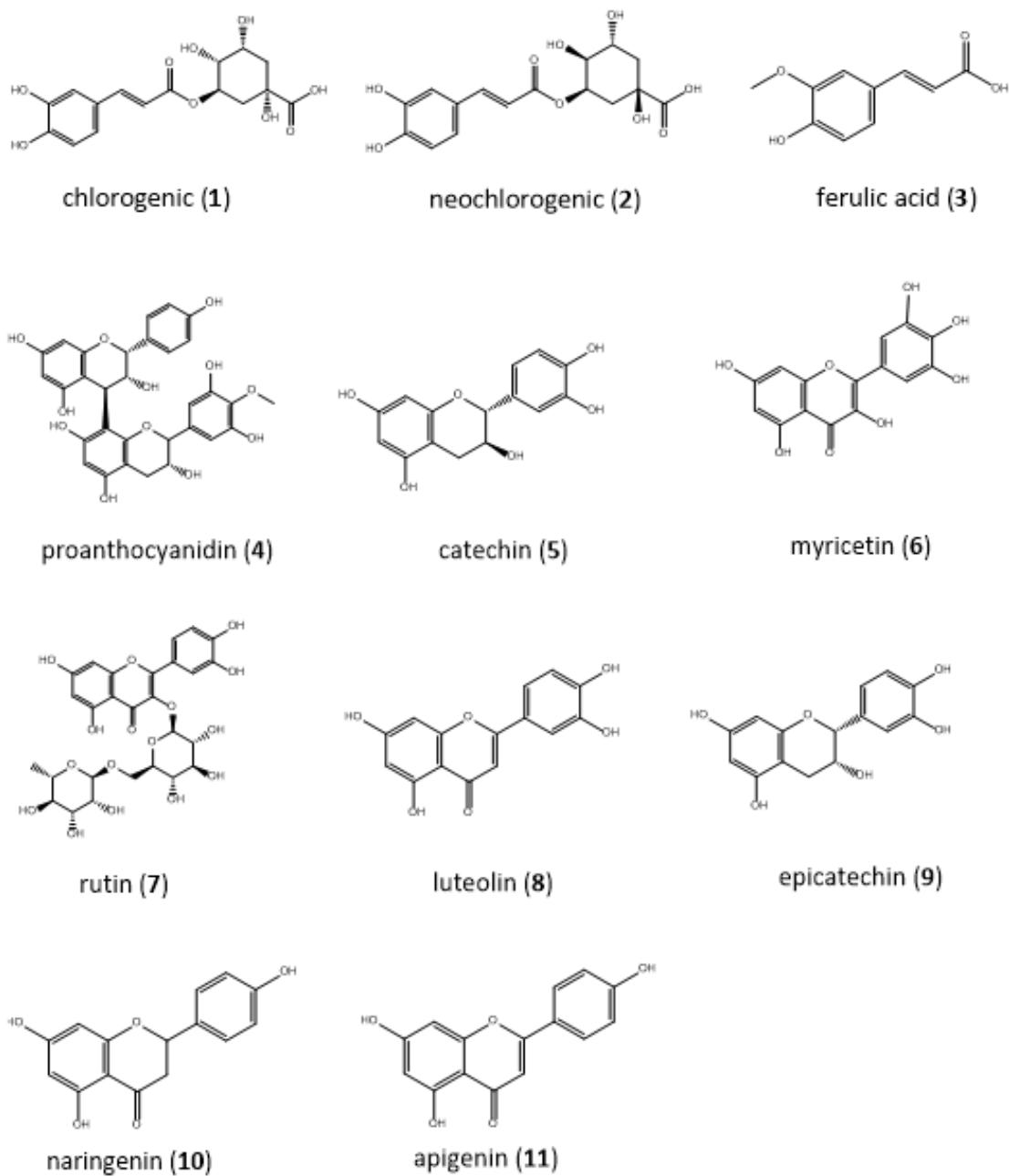
R<sub>1</sub>    R<sub>2</sub>    R<sub>3</sub>                    R<sub>4</sub>

1	H	H	rhamnosyl	malonyl
2	OH	H	rhamnosyl	malonyl
3	OH	OH	rhamnosyl	rhamnosyl
4	OH	H	rhamnosyl	rhamnosyl
5	H	H	rhamnosyl	rhamnosyl
6	H	H	rhamnosyl	H
7	OH	H	rhamnosyl	H
8	OH	OH	rhamnosyl	H
9	H	H	rhamnosyl	rhamnosyl
10	OH	H	H	rhamnosyl
11	OH	OH	H	rhamnosyl
12	H	H	H	H
13	OH	H	H	H
14	OH	OH	H	H

**Figure 2.4: Flavonols glycosides present in the flowers of *C. ternatea*.** **1**, kaempferol 3-*O*-(2"-*O*-α-rhamnosyl-6"-*O*-malonyl)-glucoside; **2**, quercetin 3-*O*-(2"-*O*-α-rhamnosyl-6"-*O*-malonyl)-β-glucoside; **3**, myricetin 3-2<sup>G</sup>-rhamnosylrutinoside; **4**, quercetin 3-2<sup>G</sup>-rhamnosylrutinoside; **5**, kaempferol 3-2<sup>G</sup>-rhamnosylrutinoside; **6**, kaempferol 3-neo- hesperidoside; **7**, quercetin 3-neohesperidoside; **8**, myricetin 3-neohesperidoside; **9**, kaempferol 3-rutinoside; **10**, quercetin 3-rutinoside; **11**, myricetin 3-rutinoside; **12**, kaempferol 3-glucoside; **13**, quercetin 3-glucoside; **14**, myricetin 3-glucoside. Structures adapted from Kazuma *et al.*(2003b).

### 2.5.3 *Cosmos caudatus* Kunth.

*Cosmos caudatus* Kunth. (Asteraceae) is an annual herb originating from Latin and Central America, which later spread to the Asian region via the Philippines (Moshawih *et al.*, 2017). The usages of the plant are quite extensive, from ornamental and culinary to medicinal. In Asian countries such as Malaysia and Indonesia, the plant is believed to have anti-ageing properties that can promote a younger looking skin complexion. *C. caudatus* is called “Ulam Raja” by locals in Malaysia, which is translated as the ‘King’s Salad’. The belief is followed with the traditional practice of eating the plant’s leaves and young shoots as a salad, either cooked or fresh, to defend against skin ageing. The pharmacological activities of the plant can be antioxidant, anti-microbial, anti-fungal, anti-diabetic, anti-hypertensive, and anti-inflammatory, as well having bone protective effects (Cheng *et al.*, 2015). Among these activities, the antioxidant property has often been reported (Shui *et al.*, 2005; Andarwulan *et al.*, 2010; Sumazian *et al.*, 2010; Ahmad; Hassan *et al.*, 2012; Mediani *et al.*, 2012; Mediani *et al.*, 2013; Mohamed *et al.*, 2013; Javadi *et al.*, 2015). The antioxidant capacity of *C. caudatus* has been reported to be the highest among 21 tropical plant extracts studied, and comparable to the synthetic antioxidants (Mustafa *et al.*, 2010). This is believed to be attributable to its phenolic, flavonoid and vitamin C content (Andarwulan *et al.*, 2010). Some of the individual bioactive compounds in the plant extracts are hydroxycinnamic acid derivatives (chlorogenic (1), neochlorogenic (2), crypto-chlorogenic, caffeic and ferulic acids (3)) and flavonoids (quercetins, pro-anthocyanidin (4), catechin (5), rutin (6), myricetin (7), luteolin (8), epicatechin (9), naringenin (10), kaempferol and apigenin (11)), as shown in Figure 2.5. The extensive literature search indicated that there is, nevertheless, a paucity of data on this species (Shui *et al.*, 2005; Ahmed; Mediani *et al.*, 2012; Andarwulan *et al.*, 2012; Javadi *et al.*, 2014).

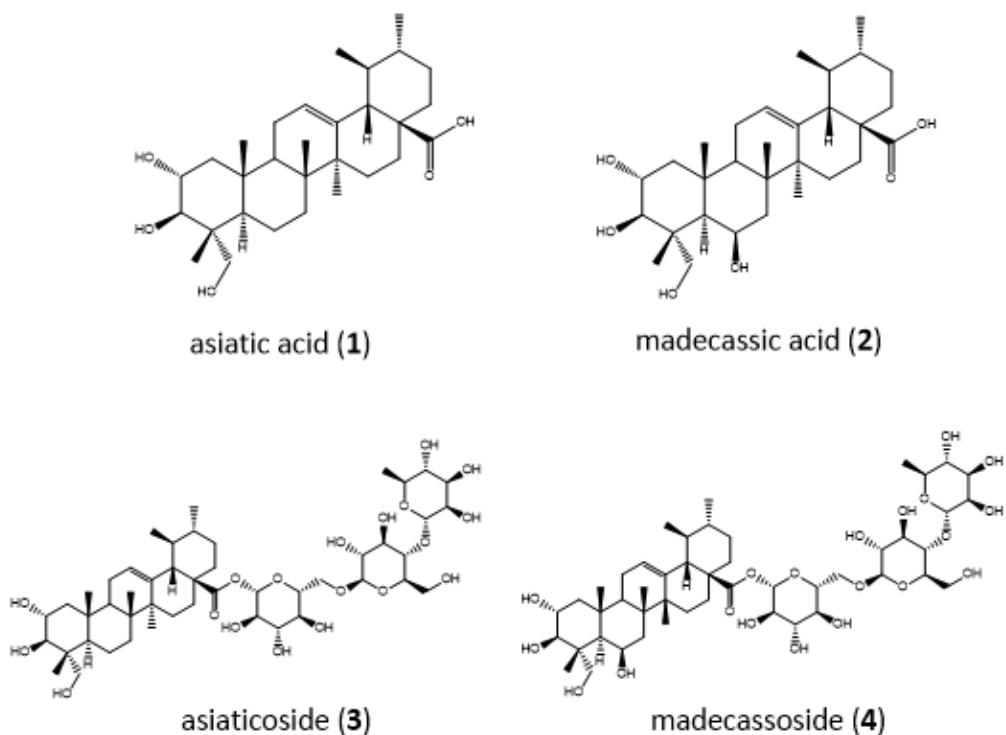


**Figure 2.5: Major phytoconstituent in *C. caudatus*.**

#### 2.5.4 *Centella asiatica* (L.) Urb.

*Centella asiatica* (L.) Urb. is a creeping, perennial plant from the Apiaceae family and a well-recognised medicinal herb in Asia. It is commonly known as Gotu-kola, Asian pennywort, Indian pennywort or tiger herb. In traditional Asian medicine, *C. asiatica* has been used for dermatological conditions such as to improve small wounds, burns, for wound healing and as

an anti-inflammatory agent for eczema (Bylka *et al.*, 2013). In Malaysia, *C. asiatica* is usually eaten as “ulam” and is believed by older folks to promote young looking healthy skin. The functional properties of this plant species were reviewed by Seevaratnam and Banumathi (2012), and antioxidant activity was most frequently reported. In comparison to the other plant species selected in this study, there is a relatively well-established literature on its benefits to the skin, mainly for wound healing (Somboonwong *et al.*, 2012; Wu *et al.*, 2012); but comprehensive studies of *C. asiatica* with respect to defense against skin ageing are still lacking. The main active compounds present in *Centella asiatica* are pentacyclic triterpenes such as asiatic acid (1), madecassic acid (2), and their respective glycosides derivatives; asiaticoside (3) and madecassoside (4), where a trisaccharide moiety are linked to its aglycone, as shown in Figure 2.6 (Inamdar *et al.*, 1996; James and Dubery, 2009; Rumalla *et al.*, 2010). These compounds are used as biomarkers to authenticate the plant species, and responsible for many of its bioactivities (Maquart *et al.*, 1990; Hashim, 2011; Bylka *et al.*, 2013).



**Figure 2.6: Major phytoconstituents present in *C. asiatica*.**

## 2.6 Overview: skin ageing

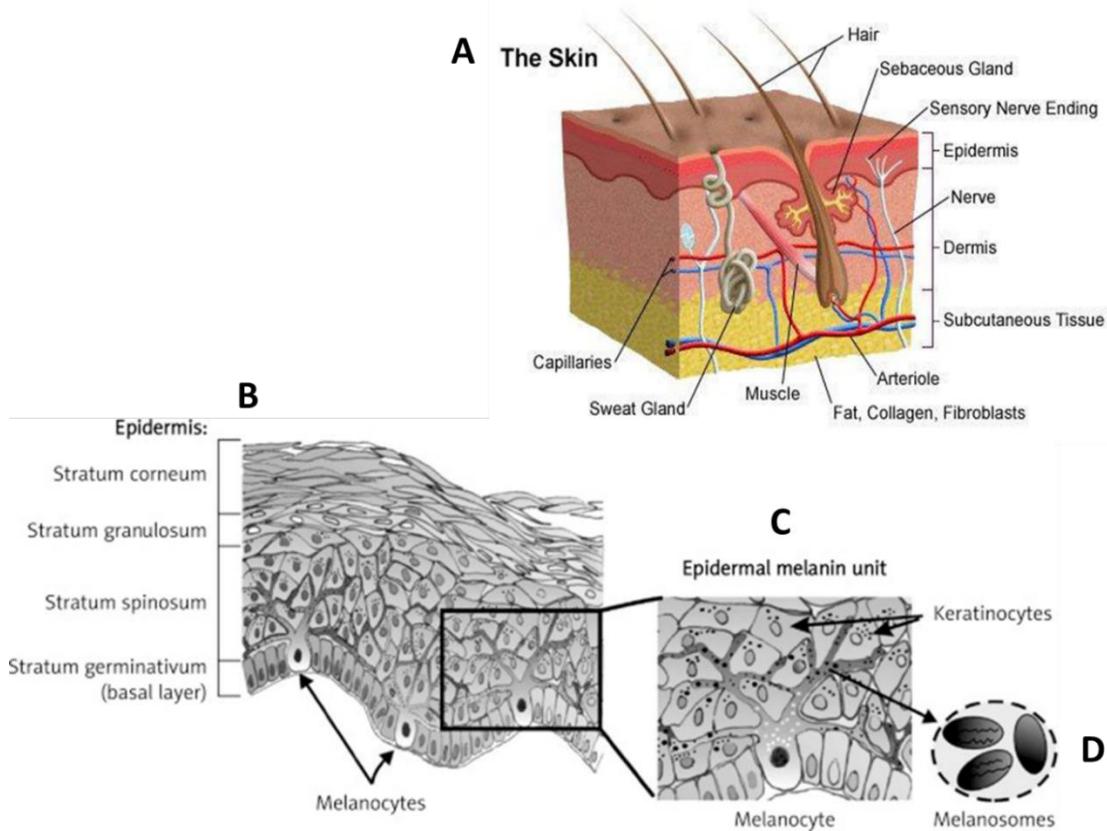
This thesis focuses on potential applications of plant extracts in cosmetic product development. “Anti-ageing” properties, with respect to skin ageing and cosmetic products, include skin lightening and the reduction of skin spots, skin renewal and stimulation, plumping to give smoother skin and moisturising (Center for the Promotion of Imports from Developing Countries (CBI), 2016). Skincare products with “anti-ageing” effects are the most sought after by consumers for their multiple activities.

## 2.7 Skin ageing (intrinsic and extrinsic)

There are two distinct types of skin ageing: intrinsic and extrinsic skin ageing. Intrinsic ageing is a consequence of chronological ageing and is determined by the individual genetic profile and the passage of time. Meanwhile, extrinsic skin ageing, also termed as “photo-ageing”, is caused by external factors such as ultraviolet light, smoking, the effects of chemicals, nutrition, and pollution (Fisher *et al.*, 2002; Farage *et al.*, 2008; Situm and Sjerobabski-Masnec, 2010). Both types of ageing are characterized by the loss of the structural integrity of the skin, and particularly the loss of skin elasticity, the reduction of local vascularization and the appearance of conspicuous lines and wrinkles on the skin surface. However, photo-ageing involves worse conditions of coarse wrinkles with the presence of hyperpigmentation and inflammation (Farage *et al.*, 2008).

## 2.8 Anatomy of the skin

The human skin consists of three layers, the epidermis, dermis and hypodermis, as illustrated in Figure 2.7A and further described below.



**Figure 2.7: Cross-section of the skin and its appendages (A), the layers of the epidermis (B), the epidermal melanin unit (C) and melanosome (D).** Images retrieved from Cichorek *et al.* (2013).

### 2.8.1 The epidermis

The epidermis is the outer layer of the skin that provides a barrier to the external environment.

It consists of two main cell types, which are keratinocytes (95%) and melanocytes (5%)

(Kolarsick *et al.*, 2011). The epidermis is arranged into four sub-layers as shown in Figure 2.7B.

The *stratum corneum* or horny layer is the outermost layer of the epidermis and is exposed to the environment. This layer of the epidermis protects the underlying tissue from infection, dehydration, chemical and mechanical stress. It contains nonviable cells (corneocytes) and keratin scale (5-10 layers) that are continuously shed and replaced by the underlying layers through the differentiation (cornification) of the living keratinocytes into corneocytes (Tagami, 2008; Calleja-Agius *et al.*, 2011). In the process, the cell membrane is replaced by a

layer of ceramides which is covalently bonded to structural proteins making the *stratum corneum* a compact barrier membrane (Tagami, 2008; Amen *et al.*, 2013).

The *stratum granulosum* or granular layer is a thin layer of 1-3 cells thickness of granulated squamous cells containing sulphurated proteins rich in the amino acid cysteine (Burlando *et al.*, 2010; Eckhart *et al.*, 2013). The keratinocytes in this layer also contain enzymes that have the potential to degrade vital cell organelles such as the nuclei, so that the cells can gradually differentiate into the corneocytes of the *stratum corneum* (Eckhart *et al.*, 2013).

The *stratum spinosum* or spinous layer contains multilayered cuboidal cells in 5-15 layers found between the *stratum granulosum* and *stratum germinativum* (basal layer). The keratinocytes in this layer synthesise several types of keratins that aggregate to form intermediate filaments, and later produce cell membrane connecting structures called desmosomes (Haftek *et al.*, 1997). The filamentous structures are anchored to desmosomes that strengthen the adhesion among cells and provide the resistance of the skin to frictional forces.

The *stratum germinativum/basale* or basal layer is the deepest layer of the epidermis that contains a single layer of cuboidal cells. The keratinocytes in this layer possess all typical cell organelles and the only layer that is capable of proliferating (Burlando *et al.*, 2010). These are the stem cells of the epidermis. Melanocytes reside in the basal layer of the epidermis forming the epidermal melanin unit, shown in Figure 2.7C surrounded by approximately 30-40 keratinocytes which favour the transport of melanosomes from the melanocytes to keratinocytes (Cichorek *et al.*, 2013), as shown in Figure 2.7D. Other specialised cells present in this layer are Langerhan cells, which form part of immune response, and Merkel cells, which are involved in the touch response (Kolarsick *et al.*, 2011).

### **2.8.2 The dermis**

The dermis is the second compartment beneath the epidermis and is 3-5 mm thick. It is characterized by an extensive extracellular matrix (ECM) containing the matrix proteins collagen and elastin that provide strength, firmness and elasticity to the tissue. It also contains hair follicles, sweat glands, capillaries and nerves that provide the senses of touch and heat (see Figure 2.2A). Fibroblast cells are the primary cell type in the dermis that can move across the tissue and produce precursors of the extracellular matrix that self-assemble to form collagen and elastin fibres. The integrity of the extracellular matrix depends on the equilibrium of the deposition of collagen, elastin and other matrix protein such as proteoglycans and glycosaminoglycan and the degradation processes caused by matrix-metalloprotein (MMP) released from the fibroblasts and keratinocytes.

### **2.8.3 The hypodermis**

The hypodermis or subcutaneous tissue is located below the dermis and is not part of the skin. Its purposes are to provide insulation, a reserve energy fuel for metabolism, and a cushion or padding for potentially damaging or traumatic events because the hypodermis contains mainly adipose tissue and fat. It also consists of blood and lymphatic vessels.

## **2.9 Five anti-ageing theories in personal care and the cosmetics industry**

Many anti-ageing theories have been proposed, but no single theory can explain all of the mechanisms involved in skin ageing. In the personal care and cosmetics industries, five well-known theories are accepted (Wilson, 2008).

### **2.9.1 The wear-and-tear theory**

This was first introduced by Dr. August Weismann, a German biologist, in 1882 (Winther, 2001). This theory proposes that accumulated damage to the vital parts of the cells, tissues and organs will wear them out, leading to death (Jin, 2010; Goldsmith, 2014).

### **2.9.2 The neuroendocrine theory**

The neuroendocrine theory was proposed by Dr. Vladimir Dilman in 1954 (Dilman and Dean, 1992). It suggests that the hypothalamus loses its ability to precisely regulate the release of hormones which regulate certain metabolic cascades and autonomic activities of the body (Tosato *et al.*, 2007; Mitteldorf, 2013). The dysregulation of stress mediator hormones such as cortisol could affect the expression of ECM enzymes through binding domains on gene promoter regions or direct interactions with transcription factors such as Activating Protein 1 (AP-1) and the nuclear factor-  $\kappa$ B (NF-  $\kappa$ B), resulting in the increased breakdown of collagen (Fisher *et al.*, 2002; Chen and Lyga, 2014).

### **2.9.3 The cross-linking theory**

This theory was first introduced by Dr. Johan Bjorksten in 1941 (Bjorksten, 1942, 1968). According to this theory, the progressive linking together of large vital molecules causes impairment of intracellular transport, slowing the biological process and therefore, reduces the safety margins of vital functions (Bjorksten and Tenhu, 1990). The cross-linking of skin protein collagen has been shown to be partly responsible for wrinkles and other age-related changes in the skin (Gkogkolou and Böhm, 2012; Snedeker and Gautieri, 2014).

### **2.9.4 The telomere theory**

This concept was developed by Alexei Olovnikov and John Watson in 1972 (Olovnikov, 1971, 1973; Watson, 1972). Telomeres are nucleic acid sequences located at the end of chromosomes, and they provide extra buffer protection to the DNA from enzymatic degradation or fusion (Kosmadaki and Gilchrest, 2004). With the continuation of cell division, telomeres eventually shorten, and the cells cease to divide themselves leading to cell death (Ning *et al.*, 2003; Dellambra and Dimbri, 2008; Jin, 2010). This theory is supported by the phenomenon of cellular senescence, a dormant state in which cells remain alive but can no

longer divide, which was discovered by Dr. Leonard Hayflick (Hayflick, 1965; Shay and Wright, 2000).

### **2.9.5 The free radical theory**

This was first introduced by Harman (1956), based on the chemical nature and ubiquitous presence of free radicals in living systems. Any molecule that possesses a free electron and is highly reactive is known as a “free radical”, and it can steal electrons from paired electrons in neighbouring molecules and thereby other reactive species are created, which may be either reactive oxygen species (ROS) or reactive nitrogen species (RNS) (Halliwell, 2006; El-Bahr, 2013). The accumulation of oxidative damage in cells, DNA, proteins and lipids is believed to be a major contributor to ageing (Harman, 1992).

## **2.10 Oxidative stress and skin ageing**

Of all of these theories, the free radical theory is the most deeply rooted and widely accepted (Harman, 1991; Alfredo *et al.*, 2014). Oxidative stress, a condition where the oxidant is surplus over the antioxidant, is another concept associated with this theory. In this condition, the innate antioxidant defense system is unable to maintain the redox balance within cells (Rinnerthaler *et al.*, 2015; Ahsanuddin *et al.*, 2016).

### **2.10.1 The role of UVR**

One of the major environmental factors causing extrinsic or photo-ageing is ultraviolet radiation (UVR). Both UVA (320 nm-400 nm) and UVB (290-320 nm) radiation are equally responsible for skin damage because UVA (long wavelength) penetrates deeper into the skin compartments of the epidermis and dermis and UVB (short wavelength) photons are more active than UVA photons, despite their penetration being limited to the epidermis (Krutmann and Humbert, 2011). Whenever UVR hits the skin, some of the photons are absorbed the natural chromophores such as porphyrins and flavins, vitamin K and B6 derivatives, bilirubin,

urocanic acid, advanced glycation end (AGE) products, and even DNA (Young, 1997) but, following an excitation phases the chromophores become electron donors that react with other electron acceptors to form free radicals. Free radicals or ROS generated from UVR such as the superoxide anion ( $O_2^{\bullet-}$ ), hydroxyl radical ( $\bullet OH$ ), or singlet oxygen ( $^1O_2$ ) (Fisher *et al.*, 2002; Krutmann and Humbert, 2011; El-Bahr, 2013) can cause oxidative damages to DNA, lipids and proteins, which may result in altered protein expression and reduced cellular function (Finkel and Holbrook, 2000; Sinha and Häder, 2002). It is also known that UV can cause direct damage to DNA, including DNA strand breaks and the formation of pyrimidine- and purine-dimers (Sinha and Häder, 2002; Rastogi *et al.*, 2010).

### **2.10.2 The role of mitochondria**

Mitochondria are organelles found within the cytoplasm, which function to generate energy for the cell in the form of adenosine triphosphate (ATP) (Fawcett, 1981; Krutmann and Schroeder, 2009). The mitochondria consist of an outer membrane, an inner membrane folded into cristae, and their own circular 16 kb double-stranded DNA, present in multiple copies attached to the inner membrane (Fawcett, 1981). Mitochondrial DNA (mtDNA) encodes the gene for 13 polypeptide subunits, which are part of the oxidative phosphorylation or electron transport chain (ETC) that produces energy and free radicals (Hüttemann *et al.*, 2007). It is estimated that up to 1% of mitochondrial ETC leads to the formation of  $O_2^{\bullet-}$  from the leakage of electrons (Turrens, 2003; El-Bahr, 2013). The electrons fed into complexes I and II are transferred to complex III, and finally to complex IV where intermediate molecules are retained until four electrons are deposited at the molecular oxygen to produce water (Turrens, 2003; Hüttemann *et al.*, 2007; El-Bahr, 2013). However, the electrons can leak prematurely from complexes I and III before reaching complex IV and produce  $O_2^{\bullet-}$ , the precursor of most ROS (Boveris and Cadenas, 2000; Liu *et al.*, 2002; Turrens, 2003). Under normal conditions,

$\text{O}_2^{\bullet-}$  dismutates into hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) spontaneously or is catalysed by manganese superoxide dismutase (MnSD), and later converted into water by catalase and glutathione peroxidase to lower  $\text{O}_2^{\bullet-}$  levels (Aguilar *et al.*, 2016). However, the conversion is not entirely efficient, and  $\text{H}_2\text{O}_2$  may be partially reduced into extremely reactive hydroxyl radical ( $\text{OH}^{\bullet}$ ) (Halliwell, 2006; Krutmann and Schroeder, 2009; El-Bahr, 2013). It is suggested that ROS production from the respiratory chain can cause damage to mtDNA, and as mtDNA encodes polypeptides subunits for the respiratory chain, this can lead to errors in gene expression resulting in dysfunctional subunits (Harman, 1956; Bandy and Davidson, 1990; Sanz and Stefanatos, 2008). The dysfunctional subunits later contribute to further leakage, and this increases oxidative damage (Bandy and Davidson, 1990). Furthermore, mtDNA has limited repair mechanisms, and has a higher mutation rate due to its close proximity to ETC, and mitochondria are lacking in protective histones (Birch-Machin *et al.*, 2013).

### **2.10.3 The role of ECM enzymes and tyrosinase**

Oxidative stress induced by UV, mitochondrial-produced-ROS or any other oxidants has also been shown to affect the levels of extracellular matrix (ECM) enzymes (Pittayapruet *et al.*, 2016), and melanin-producing-tyrosinase.

#### **2.10.3.1 Matrix metalloproteinases or MMPs**

Matrix metalloproteinases (MMPs) are zinc-dependent enzymes involved in the remodelling and degradation of extracellular matrix proteins (Philips *et al.*, 2011). There are 23 different forms of human MMPs and they are divided into six major groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type matrix metalloproteinases (MT-MMPs) and others (Konttinen *et al.*, 1999; Visse and Nagase, 2003). MMPs are categorised according to their peptide domain, substrate specificity and sequence similarity as shown in Table 2.1 (Snoek-van Beurden and Von Den Hoff, 2005). In human skin, the MMPs responsible for collagen

degradation are secreted by epidermal keratinocytes and epidermal fibroblasts, and their activity is regulated by the tissue inhibitors metalloproteinases (TIMPs) (Philips *et al.*, 2009, 2011; Bourboulia and Stetler-Stevenson, 2010). However, substantial evidence has suggested that UV radiation elevates at least three different MMPs in human skin *in vivo*, which are the interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), and 92kDa gelatinase (MMP-9) (Fisher *et al.*, 1996, 1998, 2002; Brenneisen *et al.*, 2002). The levels of these enzymes significantly increase after 24 hours of UV exposure (Fisher *et al.*, 1996), together with the upregulation of the transcription factor AP-1 that stimulate the expression of MMPs genes (Fisher *et al.*, 1996, 1998). UV-induced MMP-1 initiates the cleavages of fibrillary collagen type I and type II, which are later further degraded by elevated levels of MMP-3 and MMP-9 (Sternlicht and Werb, 2009). The combined actions of MMP-1, 3, and 9 have the capacity to degrade most of the proteins that comprise the dermal extracellular matrix. In the absence of perfect repair, the accumulation of MMP-mediated collagen damage contributes to the phenotype of photoaged skin.

**Table 2.1: Members of the MMP family and their substrates.** (Snoek-van Beurden and Von Den Hoff, 2005).

Subgroup	MMP	Name	Substrate
1. Collagenases	MMP-1	Collagenase-1	Col I, II, III, VII, VIII, X, gelatin
	MMP-8	Collagenase-2	Col I, II, III, VII, VIII, X, aggrecan, gelatin
	MMP-13	Collagenase-3	Col I, II, III, IV, IX, X, XIV, gelatin
2. Gelatinases	MMP-2	Gelatinase A	Gelatin, Col I, II, III, IV, VII, X
	MMP-9	Gelatinase B	Gelatin, Col IV, V
3. Stromelysins	MMP-3	Stromelysin-1	Col II, IV, IX, X, XI, gelatin
	MMP-10	Stromelysin-2	Col IV, laminin, fibronectin, elastin
	MMP-11	Stromelysin-3	Col IV, fibronectin, laminin, aggrecan
4. Matrilysins	MMP-7	Matrilysin-1	Fibronectin, laminin, Col IV, gelatin
	MMP-26	Matrilysin-2	Fibrinogen, fibronectin, gelatin
5. MT-MMP	MMP-14	MT1-MMP	Gelatin, fibronectin, laminin
	MMP-15	MT2-MMP	Gelatin, fibronectin, laminin
	MMP-16	MT3-MMP	Gelatin, fibronectin, laminin
	MMP-17	MT4-MMP	Fibrinogen, fibrin
	MMP-24	MT5-MMP	Gelatin, fibronectin, laminin
	MMP-25	MT6-MMP	Gelatin
6. Others	MMP-12	Macrophage metalloelastase	Elastin, fibronectin, Col IV
	MMP-19		Aggrecan, elastin, fibrillin, Col IV, gelatin
	MMP-20	Enamelysin	Aggrecan
	MMP-21	X MMP	Aggrecan
	MMP-23		Gelatin, casein, fibronectin
	MMP-27	C MMP	Unknown
	MMP-28	Epi lysin	Unknown

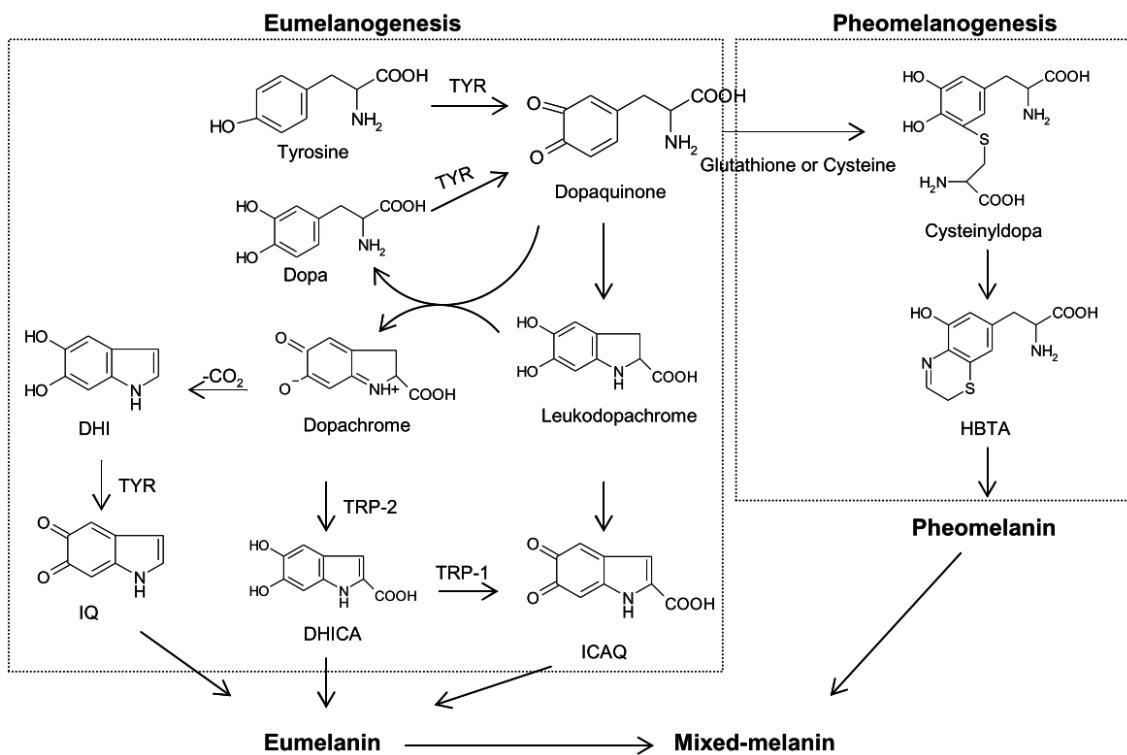
MMPs are categorized according to the organization of their peptide domains, their substrate specificity, and their sequence similarity. MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase.

### 2.10.3.2 Elastase

Elastase is an enzyme capable of breaking down elastin, which is an insoluble elastic fibrous protein that together with collagen determines the elasticity of the skin (Mecham *et al.*, 1997). There are at least two types of dermal elastases available in the skin, which are neutrophil elastase and skin fibroblast elastase (Szendroi *et al.*, 1984; Redini *et al.*, 1988). It has been evidenced that elastase activity is upregulated during the ageing process, where an age-dependent increase of dermal elastase activity is observed in mice of different age (Imokawa and Ishida, 2015). Similarly, repeated exposure to UV also stimulates the skin-fibroblast-derived elastase, where higher elastase activity has been observed *in vivo* in the UV-irradiated skin compared with the non-irradiated skin (Tsuji *et al.*, 2001; Imokawa and Ishida, 2015).

### 2.10.3.3 Tyrosinase

Photo-aged skin is characterized by the presence of age spots and hyperpigmentation. These characteristics are the result of an excess or overproduction of melanin (skin pigment) by the melanocytes, which is primarily caused by excessive exposure to the sun, ROS and inflammatory mediators produced in the skin when exposed to UV (Hearing, 1999; Costin and Hearing, 2007; Hakozaki *et al.*, 2010). Many targets have been identified to control the hyperpigmentation of the skin, and the most classic target is the inhibition of enzymes involved in melanin synthesis (Ebanks *et al.*, 2009; Hakozaki *et al.*, 2010). Melanin synthesis or melanogenesis is initiated with tyrosine oxidation to dopaquinone by the tyrosinase in the melanosome, and later proceeds spontaneously through a series of oxidation reactions to form eumelanin or pheomelanin, as illustrated in Figure 2.8 (Chang, 2009). While the three enzymes tyrosinase, TRP-1 and TRP-2 are involved in melanogenesis, tyrosinase is the key enzyme in catalyzing the synthesis, and it therefore becomes the target in the control of hyperpigmentation (Chang, 2012). Furthermore, the regulation of tyrosinase activity and melanin production has also been shown to be controlled by a post-translational regulation of pre-existing enzymes (Iozumi *et al.*, 1993). So, in view of applications in the cosmetics industry, tyrosinase inhibitors are the most popular and widely used hypopigmentation agents (Pillaiyar *et al.*, 2017).



**Figure 2.8: Biosynthetic pathway of melanogenesis.** TYR, tyrosinase; TRP; tyrosinase related protein; dopa, 3, 4-dihydroxyphenylalanine; DHICA, 5, 6-dihydroxyindole-2-carboxylic acid; DHI, 5, 6-dihydroxyindole; ICAQ, indole-2-carboxylic acid-5, 6-quinone; IQ, indole- 5, 6-quinone; HBTA, 5-hydroxy-1, 4-benzothiazinylalanine. Image obtained from Chang (2009).

## 2.11 Skin anti-ageing strategies

Therapies for skin ageing can be divided into invasive and noninvasive procedures. Invasive approaches include chemical peeling, rejuvenation using visible light devices and the injection of rejuvenating agents, fillers and implants that involve surgery (Ganceviciene *et al.*, 2012).

### 2.11.1 Chemical peeling

Chemical peeling is a method used to cause the chemical ablation of defined skin layers to induce an even and tight skin from the regeneration process. It can be classified into superficial, medium-depth and deep peeling, depending on the substances and concentrations used (Velasco *et al.*, 2004; Fischer *et al.*, 2010; Shehnaz Z, 2015). Although chemical peeling can give a significant improvement of the skin over a short period, the treatment can have adverse effects such as irritation, redness, inflammation and

hyperpigmentation (Handog *et al.*, 2012; Nikalji *et al.*, 2012). Complications arise from an improper use of phenol in deep peeling, which can result in even worst conditions including cardiac arrhythmia and kidney damage. These chemicals are also toxic to all cells (Velasco *et al.*, 2004).

### **2.11.2 Laser treatment**

Laser treatment using visible light devices can be classified into ablative and non-ablative resurfacing (Preissig *et al.*, 2012; Ibrahimi *et al.*, 2015). Ablative laser treatment results in significant outcomes promoting collagen formation and tightening the skin, as the treatment vapourises skin tissue by heating water molecules into gas and giving a skin peeling effect (Preissig *et al.*, 2012). Ablative resurfacing removes the epidermal layer, but non-ablative treatment keeps the epidermal intact and is less invasive, thus producing a gentler effect of dermal collagen stimulation. However, both ablative and non-ablative laser resurfacing can cause side effects such as scarring, discolouration or hyperpigmentation, postoperative erythema and infections (Tanzi and Alster, 2003; Hunzeker *et al.*, 2009). Fractionated laser resurfacing has been introduced for both ablative and non-ablative treatments to minimise complications while maintaining the efficacy of treatment (Manstein *et al.*, 2004), and initial reports of non-ablative fractionated devices emphasise an almost complete absence of prolonged side effects (Fisher and Geronemus, 2006; Gruber *et al.*, 2008). Nonetheless, fractionated ablative lasers have been reported to cause severe complications like those from traditional non-fractionated devices (Avram *et al.*, 2009; Hunzeker *et al.*, 2009; Ramsdell, 2012).

### **2.11.3 Injections of dermal fillers**

Fillers are products injected within or beneath the skin to rejuvenate it and to improve its physical features (Haneke, 2006). There are at least three types of fillers: short-term, long-

term and permanent fillers. These are categorized based on their durability in the tissue (Haneke, 2006; Ahn and Rao, 2014). Some fillers such as hyaluronic acid (HA) derived fillers are able to imitate the function of skin HA to hold water and provide improved structural properties in the skin, as well as being able to stimulate fibroblasts to express Col-1, MMP-1 and TIMP-1 (Tammi *et al.*, 2002; Wang *et al.*, 2007; Jäger *et al.*, 2012). Other types of fillers such as calcium hydroxylapatite (CaHA) create a long-term filling effect by stimulating endogenous collagen production, while polymethyl methacrylate (PMMA) provides a scaffolding for the dermis to recover its original thickness (Ahn and Rao, 2014). Although the substances used for fillers are usually non-immunogenic, complications such as pain, bruising and edema commonly occur following the procedures, and more serious complications can include visual impairment (Rzany *et al.*, 2009; Li *et al.*, 2015; Townshend, 2016).

#### **2.11.4 Topical cosmeceutical products**

A moderate and noninvasive approach such as the application of topical cosmeceutical products are generally preferred and are perceived as a safer option ( Ayob *et al.*, 2016). Antioxidants such as vitamins, polyphenols and flavonoids are the main anti-ageing agents usually employed in a cosmeceutical product that acts by reducing or neutralizing free radicals, later affecting skin ageing through various mechanisms as explained in section 2.10. Vitamin C (L-ascorbic acid) stimulates and regulates collagen synthesis, influences the expression of antioxidant enzymes and stimulates MMP-1 inhibitors (Nusgens *et al.*, 2001; Pullar *et al.*, 2017). Vitamin B<sub>3</sub> (niacinamide) also stimulates collagen synthesis, prevents oxidative damages and reduces hyperpigmentation in the skin (Matts *et al.*, 2002; Ortonne and Bissett, 2008), while vitamin E ( $\alpha$ -tocopherol) prevents oxidative damage and inflammation, downregulates MMP-1 through AP-1 binding, and suppresses melanogenesis (Nachbar and Korting, 1995; Wu *et al.*, 2008; Kamei *et al.*, 2009). Polyphenols, which are

abundant in fruit and vegetables, usually have multi-action effects on skin ageing. Epigallocatechin gallate (EGCG), for example, reduces UVB-induced MMP-1, MMP-8 and MMP-13. It also suppresses as well as modulates immune responses and assists in wound healing (Katiyar *et al.*, 1999; Hsu *et al.*, 2003; Bae *et al.*, 2008). Most polyphenols have the ability to absorb UV, and thus could prevent the molecular and cellular damage caused by UV, and are also able to inhibit MMP-1 and elastase that regulate skin matrix degradation (Thring *et al.*, 2009; Nichols and Katiyar, 2010; Zillich *et al.*, 2015).

## **2.12 Summary and conclusion**

From all of the above, the use of local herbs in the cosmetics industry seems to be relevant and able to add commercial value to under utilised medicinal plants and to provide novel cosmeceuticals application to exploit the plants. This potential is further supported by the high market demand for plant-based cosmetics worldwide and the preference for topical skin ageing management. Further exploration of the potential application of the plants selected as cosmetics ingredients is needed, including as antioxidants, anti-wrinkle, anti-hyperpigmentation and sunscreen agents.

## **2.13 Aim and Objectives**

### **2.13.1 Problem statement**

The herbal and cosmetics industries foresee a huge market for medicinal plants especially with increasing preference for organic and natural products. The application of the local herbs as cosmetic ingredients is very promising. However, the common challenge in the commercialization of the local herbs are the lack of supply of raw materials and the paucity of data giving scientific validation of the pharmacological effects of plants against skin ageing.

## **2.13.2 Aim and objectives of the project**

This project aims to investigate the anti-ageing properties of the selected medicinal plants for potential application as cosmetics ingredients. The objectives of this study are:

1. To evaluate the antioxidant activity and its correlation with TPC in each of the plant extracts (Chapters 3 and 4)
2. To investigate the inhibitory activities of the extracts against enzymes implicated in the reduction of skin elasticity and the declining aesthetic appearance of the skin, which includes anti-collagenase, anti-elastase and anti-tyrosinase activity (Chapter 5)
3. To investigate the protective effect of the plant extracts against hydrogen peroxide ( $H_2O_2$ )-induced cytotoxicity in HaCaT cells (Chapter 6)
4. To investigate the protective effect of the plant extracts against UV-induced mitochondrial damage (mtDNA) in HaCaT cells (Chapter 7)

# **Chapter 3**



## **Chapter 3: Phytochemical analysis of the plant extracts**

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### **3.1 Introduction**

Most of the preparation of the ethno-medical plants use water for the extraction of the plant material, either by boiling or infusion. It has been reported that 69.2 % of herbal preparations by locals in Malaysia was by decoction (Mohammad *et al.*, 2012). However, in laboratory settings and at a larger industrial scale, organic solvents are usually employed in extraction procedures, due to their efficiency. Ethanol is the most commonly employed solvent in plant extraction because it is easily available in high purity, low in cost, completely biodegradable and GRAS (generally recognized as safe) in the food industry (Rodriguez-Perez *et al.*, 2016). Sometimes, a co-solvent such as 70% ethanol is used in the extraction of plant materials to target compounds with mid-polarity. Several studies using co-solvent systems have shown that 70% ethanol extraction results in high yields, the maximum presence of phytochemicals and high activity levels such as antioxidant (Paulucci *et al.*, 2013; Vongsak, Sithisarn, Mangmool, *et al.*, 2013; Wei *et al.*, 2015).

Medicinal plants contain a mixture of different compounds, and there are no universal extraction methods that are suitable for all plants. An extensive review of the literature was performed to select suitable extraction methods, taking into consideration the following factors. Firstly, an efficient extraction time is needed for the appropriate experimental procedures without affecting the extracted compounds. Secondly, an efficient extraction temperature to avoid the oxidation and degradation of thermo-labile compounds; and finally, the choice of an efficient extraction solvent should take into consideration for safe application at the industrial level and optimum extraction yields. Therefore, two extracts (water and 70% aqueous ethanol) of each plant species were prepared for this study to mimic the traditional and potential industrial application.

### **3.1.1 Objectives**

The aim of this chapter is to investigate the major phytochemical components of the plant extracts. The objectives of this chapter are as below:

- 1) To prepare two extracts (water and 70% ethanol) to mimic traditional and industrial preparations
- 2) To determine and compare the TPC of the prepared extracts
- 3) To determine the TAC in the *C. ternatea* extracts
- 4) To determine potentially active compounds in the plant extracts

## 3.2 Materials and methods

### 3.2.1 Plant materials

Dried leaves of *M. oleifera* and *C. asiatica* were purchased from G. Baldwins & Co., UK. The dried leaves of *C. caudatus* and the dried flowers of *C. ternatea* were purchased from Selangor Herbs, Malaysia and Siam Herbarium, Thailand respectively. All sample vouchers were deposited in the Medicinal Plants Research Group's Herbarium at Newcastle University.

### 3.2.2 Extraction

#### 3.2.2.1 70% ethanol extraction

The previously used method of extraction was adapted with modifications (Vongsak, Sithisarn, Mangmool, *et al.*, 2013). The dried *M. oleifera*, *C. asiatica*, *C. ternatea* and *C. caudatus* were pulverized into fine powder using an electrical blender. Each powdered sample (100 g) was macerated with 500 mL of 70% ethanol for 72 h at room temperature with occasional shaking. The extract was vacuum-filtered using Whatman no.1 filter paper and the marc was rinsed with 70% ethanol (100 mL). The marc was discarded. The extract was further filtered at least three times followed by rotary evaporation (Rotavapor® R-210, Buchi) at 40 °C to remove excess ethanol. The remaining extract was freeze-dried and stored at -20 °C until further used. The final freeze-dried sample was weighed for yield calculation (see section 3.2.3). Dried samples were labelled as MOE, CAE, CTE and CCE for *M. oleifera*, *C. asiatica*, *C. ternatea* and *C. caudatus* extracts respectively.

#### 1.1.1.1 Water extraction

The previously used methods were followed with modifications (Thring *et al.*, 2009; Islam *et al.*, 2014). Powdered samples of each of *M. oleifera*, *C. asiatica*, *C. caudatus* and *C. ternatea* (50 g) were weighed and extracted in 500 mL of freshly boiled de-ionised water for 30 minutes.

The extract solutions were allowed to cool down with frequent shaking before sonication for

15 minutes. Each extract was vacuumed-filtered using a Whatman no.1 filter paper at least three times and the marc was discarded. Finally, the extract was freeze-dried and stored at -20 °C until further used. The final freeze-dried sample was weighed for yield calculation (see section 3.2.3). Dried samples were labelled as MOW, CAW, CTW and CCW for *M. oleifera*, *C. asiatica*, *C. ternatea* and *C. caudatus* extracts respectively.

### **3.2.3 Extraction yield formula**

Percentage yield was calculated using the formula below:

$$\text{Percentage yield (\%)} = \frac{\text{Final lyophilized weight in grams}}{\text{Initial weight of powdered samples in grams}} \times 100 \quad (3.1)$$

The yield was also expressed as (weight) grams per 100 g of dried plant materials.

### **3.2.4 Qualitative analysis of the phytochemicals**

The qualitative analysis of the phytochemicals was followed from the previous method as described in Table 3.1 (Khanam *et al.*, 2014). The presence and/or absence of alkaloids, saponins, terpenoids, phenolics and flavonoids was evaluated based on the presence of precipitation, bubble foam formation, presence of coloured rings/solutions and the disappearance of coloured reagents.

**Table 3.1: Protocols for qualitative phytochemicals analysis**

Tests	Compounds	Protocols
Dragendorff's reagent	Alkaloids	The extract (10 mg) was stirred with 2 mL of Dragendorff reagent (Sigma Aldrich, UK). The formation of opaque precipitate in the golden yellow solution showed the presence of alkaloid.
Froth test	Saponin	The extract (10 mg) was diluted with 2 mL of water and made up to 5 mL. The suspension was shaken in a test tube for 15 minutes. The development of foam indicated the presence of saponins. If the bubbles remain after 15 minutes to one hour, the test is positive for saponins. The height of bubbles that remain after 30 minutes was measured.
Salwoski test	Terpenoid	The extract (10 mg) was added with 2 mL of chloroform followed by 2 mL concentrated sulphuric acid (12 M) to form a layer. Reddish-brown colour at the interface indicated the presence of terpenoids.
Ferric Chloride test (FeCl <sub>3</sub> )	Phenolic	The extract (10 mg) was dissolved in 2 mL of distilled water and a few drops of 5% ferric chloride were added. Bluish black colour indicated the presence of phenolic compounds.
Alkaline reagent test	Flavonoid	A few drops of sodium hydroxide (0.5 M) were added to the extracts (1 mg/mL) to give an intense yellow colour. The disappearance of colour after the addition of dilute hydrochloride acid (6 M) showed the presence of flavonoid.

### 3.2.5 Total phenolic content

TPC was determined using the Folin-Ciocalteu (F-C) method in a 96-well plate (Chattuwatthana and Okello, 2015). In the assay, either gallic acid (GA) standards solution (20  $\mu$ L), plant samples (20  $\mu$ L) or solvents (20  $\mu$ L) for blanks were mixed with 10 % (v/v) F-C working solution (100  $\mu$ L) at room temperature and then left for 5 minutes. Later, 7.5% (w/v) sodium carbonate solution (80  $\mu$ L) was added to the assay wells, and the microplate was incubated in the dark for 60 minutes. The absorbance of the solution in the assay wells was read at 750 nm using a microplate reader (SpectraMax Plus<sup>384</sup>, Molecular Device Corporation). Background reading was corrected by subtracting the absorbance value of the blank. TPC was expressed as GA equivalent in 1 mg of plant extract.

#### 3.2.5.1 GA equivalent $\mu$ g/mg extract calculation

The GA standard absorbance values were plotted against the final concentration of GA in the well to obtain the GA standard equation ( $y = ax$ ) that crosses the origin. GA equivalent  $\mu$ g/mg extract was calculated as below:

$$GA \text{ equivalent } \frac{\mu\text{g}}{\text{mg}} \text{ extract} = \frac{y}{a} \times DF \quad (3.2)$$

where  $y$  is the absorbance value,  $a$  is the coefficient and  $DF$  is the dilution factor to achieve 1 mg.

The selected absorbance values should lie within the GA standard regression, as an over-concentrated sample may reach the limit of the instrument (OD 4.0). If necessary, the sample was diluted accordingly to obtain a suitable absorbance level for the calculation.

### 3.2.6 Total anthocyanin content

The TAC for *C. ternata* was determined using a pH-differential method using prepared reagents as described in Table 3.2 (Lee *et al.*, 2005). The method was modified accordingly to

suit a 96-well plate measured using a micro plate reader (SpectraMax Plus<sup>384</sup>, Molecular Device Corporation). Two sets of test sample (50  $\mu$ L) were pipetted into the 96-well plate in triplicates. One set was added with buffer with pH 1.0 (250  $\mu$ L) and the other set was added with buffer with pH 4.5 (250  $\mu$ L). The plate was shaken for 15 s before reading at 500 nm and 700 nm. The readings from blank (water) were subtracted before TAC calculation.

(Note: The OD readings of the samples were determined prior to assay to ensure that the values were within the limit of the instrument (OD = 4.0). Otherwise, appropriate dilution was made).

**Table 3.2: Protocol for TAC buffers**

Buffer	Protocol
a) pH 1.0 buffer (potassium chloride, MW:74.55 g/mol, 0.025 M)	1.86 g KCl was weighed into a beaker and 980 mL of distilled water was added. The solution was mixed, and pH was measured, and adjusted to pH 1.0 ( $\pm$ 0.05) with 6.3 mL of HCl. The volume was made up to 1L with distilled water to obtain 0.025 M potassium chloride pH 1.0.
b) pH 4.5 buffer (sodium acetate, MW:82.03 g/mol, 0.4 M):	54.43 g $\text{CH}_3\text{CO}_2\text{Na}\cdot 3\text{H}_2\text{O}$ was weighed in a beaker, and 960 mL of distilled water was added. The solution was mixed, and pH was measured, and adjusted to pH 4.5 ( $\pm$ 0.05) with 20 mL of HCl. The volume was made up to 1L with distilled water to obtain 0.4 M sodium acetate pH 4.5.

### 3.2.6.1 TAC calculation

TAC was calculated using the formula below:

$$\text{TAC} = (A \times \text{MW} \times \text{DF}_1 \times \text{DF}_2 \times 1000) / \epsilon, \quad (3.3)$$

where, A = (A<sub>520nm</sub> – A<sub>700nm</sub>) pH 1.0 – (A<sub>520nm</sub> – A<sub>700nm</sub>) pH 4.5; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF<sub>1</sub> = dilution factor to correct for the working concentration, DF<sub>2</sub> = dilution factor for any pre-dilution made to obtain the working

concentration,  $\epsilon = 19170$  molar extinction coefficients, in  $\text{L} \times \text{mol}^{-1} \times \text{cm}^{-1}$ , for cyd-3-glu; and 1000 = factor for conversion from g to mg.

The final concentration of the extract in the well was 200  $\mu\text{g}/\text{mL}$ , therefore  $DF_1 = 5$ . The ratio between the test samples and the buffer should be 1 to 4 parts, so that the buffer capacity is not affected. For that reason,  $DF_1$  should always be 5. If the sample was pre-diluted, TAC should be multiplied by  $DF_2$  (reciprocal of the dilution made to obtain the working concentration).

Note: The molar extinction coefficient was recalculated to correct the difference in path-length between a cuvette and a microplate's well (see Appendix A).

### 3.2.7 High resolution liquid chromatography-ultraviolet-mass spectrometry (LC-UV-MS)

The samples were sent for analysis at Kews Garden, London. The extracts were reconstituted in the extraction solvent at 10 mg/mL prior to chemical characterisation using LC-UV-MS/MS analysis. Analyses were performed on a Thermo Scientific system consisting of an 'Accela' U-HPLC unit with a photodiode array detector and an 'LTQ Orbitrap XL' mass spectrometer fitted with an electrospray source (Thermo Scientific, Waltham, MA, USA). Chromatography was performed on 5  $\mu\text{l}$  sample injections onto a 150-mm x 3-mm, 3  $\mu\text{m}$  Luna C-18 column (Phenomenex, Torrance, CA, USA). The mobile phase gradients for each plant species were as follows:

- *M. oleifera* extracts: 400  $\mu\text{L}/\text{min}$  mobile phase gradient of  $\text{H}_2\text{O}/\text{CH}_3\text{OH}/\text{CH}_3\text{CN} + 1\% \text{HCOOH}$ : 90:0:10 (0 min), 90:0:10 (5 min), 0:90:10 (60 min), 0:90:10 (65 min), 90:0:10 (67 min), 90:0:10 (70 min) followed by return to starting conditions and equilibration in starting conditions for 5 min before the next injection.
- *C. asiatica* extracts: 400  $\mu\text{L}/\text{min}$  mobile phase gradient of  $\text{CH}_3\text{CN}/\text{H}_2\text{O}/\text{CH}_3\text{CN} + 1\% \text{HCOOH}$ : 0:90:10 (0 min), 0:90:10 (5 min), 80:10:10 (60 min), 80:10:10 (65 min), 0:90:10

(67 min), 0:90:10 (70 min) followed by return to starting conditions and equilibration in starting conditions for 5 min before the next injection.

- *C. ternatea* extracts: 400  $\mu$ L/min mobile phase gradient of  $\text{H}_2\text{O}/\text{CH}_3\text{OH}/\text{CH}_3\text{CN} + 1\%$   $\text{HCOOH}$ : 90:0:10 (0 min), 90:0:10 (5 min), 0:90:10 (60 min), 0:90:10 (65 min), 90:0:10 (67 min), 90:0:10 (70 min) followed by return to starting conditions and equilibration in starting conditions for 5 min before the next injection.
- *C. caudatus* extracts: 400  $\mu$ L/min mobile phase gradient of  $\text{H}_2\text{O}/\text{CH}_3\text{OH}/\text{CH}_3\text{CN} + 1\%$   $\text{HCOOH}$ : 90:0:10 (0 min), 90:0:10 (5 min), 0:90:10 (60 min), 0:90:10 (65 min), 90:0:10 (67 min), 90:0:10 (70 min) followed by return to starting conditions and equilibration in starting conditions for 5 min before the next injection.

The ESI source was operated with polarity switching and the mass spectrometer was set to record high resolution (30 k resolution) MS1 spectra ( $\text{m/z}$  125–2000) in positive mode using the orbitrap and low resolution MS1 spectra ( $\text{m/z}$  125– 2000) in negative mode and data dependent MS2 and MS3 spectra in both modes using the linear ion trap. Detected compounds were assigned by comparison of accurate mass data (based on ppm), and by available MS/MS data with reference to the published compound assignment system (Schymanski *et al.*, 2014) and with supportive UV spectra.

### 3.2.8 Phytochemical databases search

Two accessible phytochemical databases were used to search for the potential active phytochemicals from the plant extracts.

**(a) Dr. Duke's Phytochemical and Ethnobotanical Databases:**

<https://phytochem.nal.usda.gov/phytochem/search>

**(b) MAPS/MPD3 Medicinal Plants Database for Drug Design:**

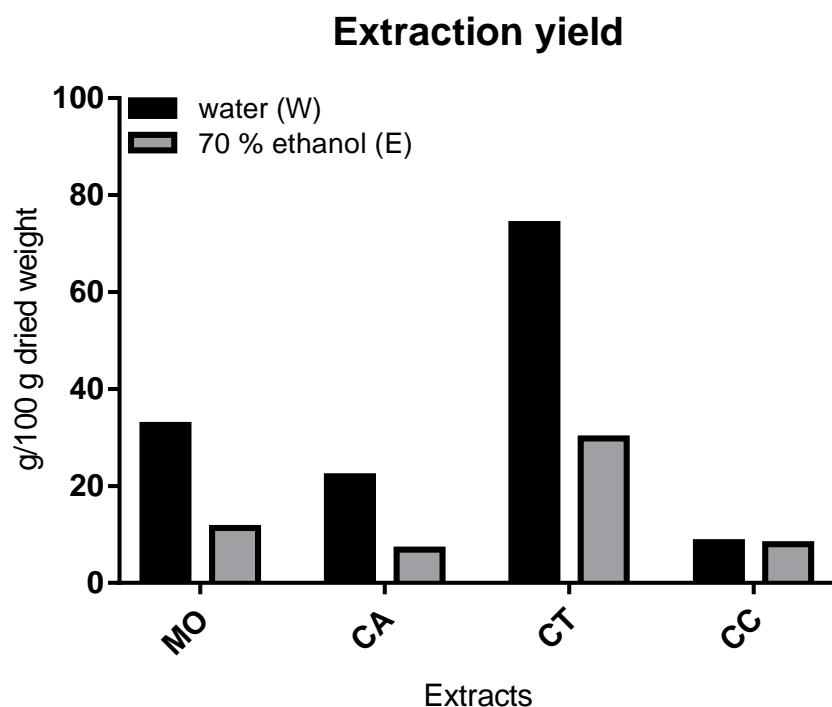
<http://bioinform.info/search.html>

The searches were cross-referenced with manual searches using other literature databases such as Scopus.com and ScienceDirect which were not covered by the databases. MAPS includes data from PubMed, PubPubmed Central, Pubchem, NPACT , Drug Bank and PhytoAmp (Ashfaq *et al.*, 2013). Meanwhile, Dr.Duke's database is from the U.S. Department of Agriculture from 1999-2016 based on the *Handbook of Phytochemical constituents of GRAS Herbs and Other Economic Plants* (U.S. Department of Agriculture, 2016).

### 3.3 Results

#### 3.3.1 Higher yield observed from extraction with water compared to 70 % ethanol

Higher yields were observed from the water extraction in comparison to the 70% ethanol, as shown in Figure 3.1, except for *C. caudatus* extracts where similar yields were obtained between CCW and CCE. The percentage yields for each plant extracts were: MOW 33%, CAW 22%, CTW 74%, CCW 8.4%, MOE 11%, CAE 7%, CTE 15% and CCE 4%.



**Figure 3.1: Extraction yield.** Figure shows the yield for water and 70% ethanol extraction for each of the plant species expressed as g/ 100 g dried weight. Abbreviations: MO= *M. oleifera*, CA: *C. asiatica*, CT: *C. ternatea* and CC: *C. caudatus*.

### 3.3.2 Qualitative analysis

The qualitative phytochemical analysis suggested the presence and absence of the following compounds in the water and 70 % ethanol extracts (Table 3.3). Phenolics and flavonoids were present in all extracts, saponins were absent in both *M. oleifera* extracts but present in the other plant extracts, and terpenoids were only found in the 70% ethanol extracts of *C. asiatica*, *C. ternatea* and *C. caudatus*. Meanwhile, alkaloids were absent or below the detection limits in all plant extracts. The qualitative analysis indicates that more of the groups of compounds are present in the 70% ethanol extracts as compared to the water extracts.

**Table 3.3: Qualitative analysis of phytochemicals presents in the plant extracts.**

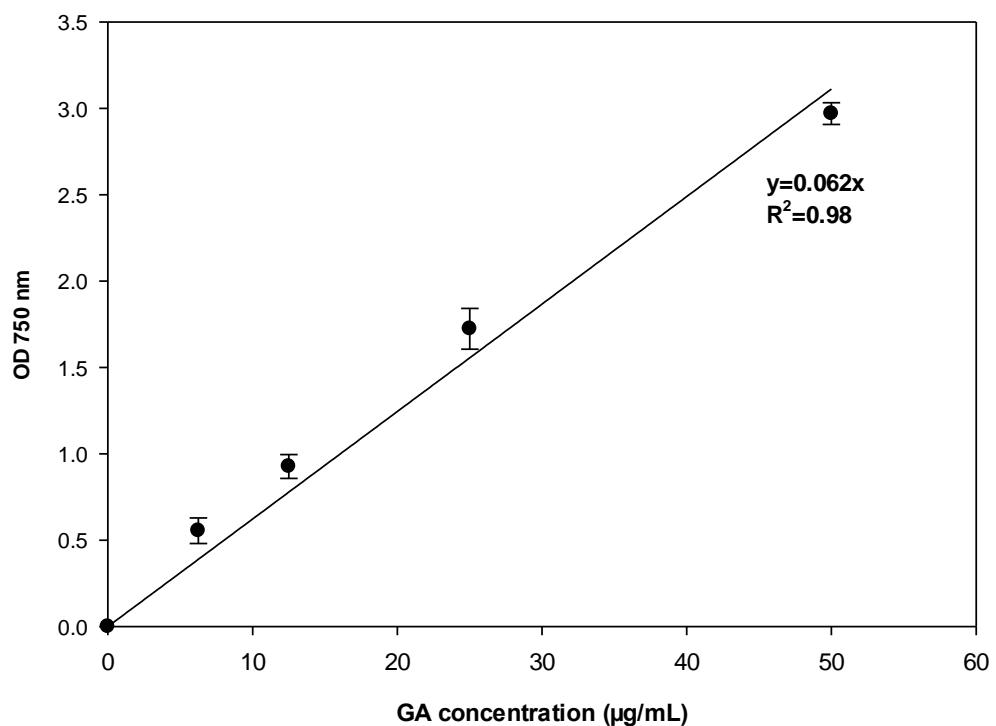
TEST	TEST COMPOUND	Water extracts				70% ethanol			
		MO	CA	CT	CC	MO	CA	CT	CC
Dragendorff	Alkaloids	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
Froth's	Saponins	(-)	(+)	(+)	(+)	(-)	(+)	(+)	(+)
Salwoski	Terpenoids	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)
FeCl <sub>3</sub>	Phenolics	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Alkaline reagent	Flavonoids	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)

For the froth test; bubbles that remain after 15 minutes are considered (+) for saponin.

Abbreviations: MO (*M. oleifera*), CA (*C. asiatica*), CT (*C. ternatea*) and CC (*C. caudatus*).

### 3.3.3 GA standard regression lines

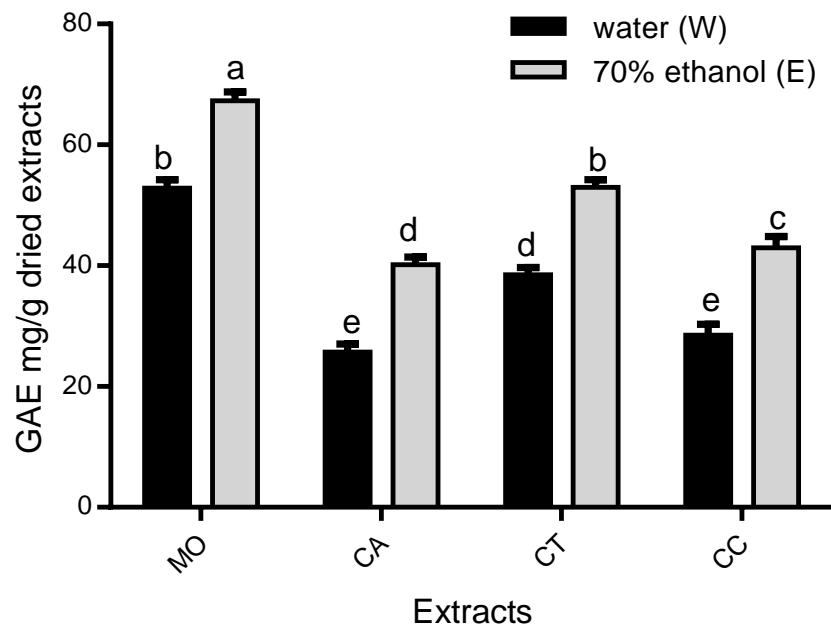
The regression lines for GA dissolved in water and in 70% ethanol were plotted individually to find the best fit line. Data for GA in water and in 70% ethanol were pooled together to get the final standard regression line used to determine the TPC. Data were pooled together as no significant difference was observed in the slopes of the two regression lines ( $y = 0.067x$  and  $y = 0.065x$ ) for GA in water and 70% ethanol. Hence, the equation from the pooled data ( $y = 0.062x$ ) was used to calculate the TPC of all samples (Figure 3.2).



**Figure 3.2: GA standard regression line.** The figure shows the regression line for GA standard (6.25-50 µg/mL) passing through the origin. Data represent the mean  $\pm$  SD of pooled data. (N=6 for each concentration).

### 3.3.4 Total phenolic content (TPC)

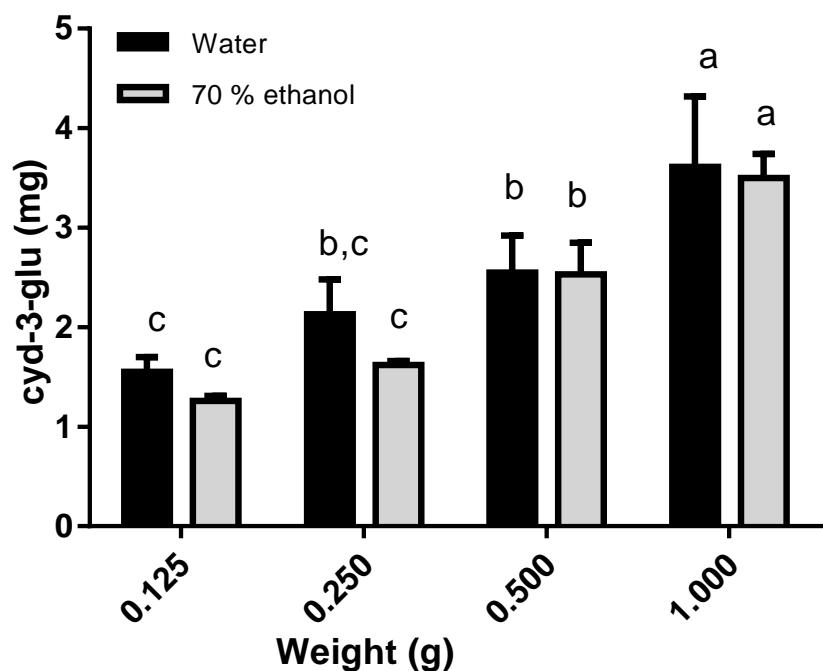
The values of TPC were higher in the ethanolic extracts compared with water extracts for all plant species (see Figure 3.3). MOE had the highest TPC of all extracts with the lowest observed in CAW and CCW. The TPC values from the highest to lowest were MOE > CTE, MOW > CCE > CTW, CAE > CCW, CAW.



**Figure 3.3: Comparison of total phenolic content (TPC).** The figure shows the TPC comparison between water and 70% ethanol extracts expressed as Gallic acid equivalent (GAE) mg/g extracts. Data represent the mean  $\pm$  SD of three replicates. Different letters indicate data with significant differences at  $p<0.05$ .

### 3.3.5 Similar values of TAC observed in *C. ternatea* extracts (CTW and CTE)

*C. ternatea* flower extracts were assessed for the TAC. There was no significant difference between the TAC in the two extracts. The TAC values for both water and ethanolic extracts were  $\sim 3.5$  mg cyd-3-glu equivalent/g extracts.



**Figure 3.4: Total anthocyanin content (TAC) for CTW and CTE.** Different letters indicate significant mean differences ( $p<0.05$  considered as significant). Data represent mean  $\pm$  SD of three replicates.

### 3.3.6 Compounds characterised by high resolution liquid chromatography-ultraviolet-mass spectrophotometry (LC-UV-MS)/MS

#### 3.3.6.1 *Moringa oleifera* Lam.

The compounds detected in *M. oleifera* extracts were chlorogenic acids derived from -O-coumaryl-, caffeoyl-, and feruloyl-ester, apigenin flavone glucosides (vicenin-2, vitexin and isovitexin) and flavonol glycosides of quercetins, kaempferols and isorhamnetins (see Table 3.4). Marumoside A, a nitrogen-containing phenolic glycoside, was observed at a retention time of 3.7 min with *m/z* value of 298.12, and this has been previously isolated from *M. oleifera* leaves as glycosides of 4'-hydroxyphenylethanamide (Sahaktipichan *et al.*, 2011). Several isomers of niazimins (thiocarbamates) were detected at retention times of 29.2, 32.0 and 36.9 min in MOE, but these were absent or below the detection limit in MOW. These compounds were the first natural carbamates known to be present in *M. oleifera* species, thus chemically-authenticating the plant samples (Faizi *et al.*, 1992, 1994).

**Table 3.4: Compounds assigned from LC-UV-MS/MS analysis of MOW and MOE.**

No.	Assigned compound# (or isomer)	Retention time (min)	Molecular formula	( <i>m/z</i> )	Ion	MOW ppm#	MOE ppm#
1.	Phenylalanine	2.9	C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>	166.086	[M + H] <sup>+</sup>	2.259	0.994
2.	Marumoside A	3.7	C <sub>14</sub> H <sub>19</sub> NO <sub>6</sub>	298.129	[M + H] <sup>+</sup>	0.893	3.576
3.	Tryptophan	4.3	C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	205.097	[M + H] <sup>+</sup>	1.776	0.459
4.	3- <i>O</i> -Coumaroylquinic acid	6.7	C <sub>16</sub> H <sub>18</sub> O <sub>8</sub>	339.109	[M + H] <sup>+</sup>	2.082	1.640
5.	5- <i>O</i> -Coumaroylquinic acid	6.9	C <sub>16</sub> H <sub>18</sub> O <sub>8</sub>	339.108	[M + H] <sup>+</sup>	0.667	1.640
6.	Caffeoylquinic acid	8.0	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	355.103	[M + H] <sup>+</sup>	0.680	1.975
7.	Feruloylquinic acid	8.7	C <sub>17</sub> H <sub>20</sub> O <sub>9</sub>	369.118	[M + H] <sup>+</sup>	0.484	2.063
8.	Benzyl hexosyl-pentoside	10.9	C <sub>18</sub> H <sub>26</sub> O <sub>10</sub>	420.187	[M + NH <sub>4</sub> ] <sup>+</sup>	0.018	2.779
9.	4- <i>O</i> -Coumaroylquinic acid	12.2	C <sub>16</sub> H <sub>18</sub> O <sub>8</sub>	339.108	[M + H] <sup>+</sup>	0.578	1.817
10.	Vicenin-2	12.5	C <sub>27</sub> H <sub>30</sub> O <sub>15</sub>	595.165	[M + H] <sup>+</sup>	0.263	1.585
11.	Hexenyl dihexoside	12.8	C <sub>18</sub> H <sub>32</sub> O <sub>11</sub>	442.230	[M + NH <sub>4</sub> ] <sup>+</sup>	2.041	1.544
12.	Butyl pentosyl-hexoside	14.0	C <sub>16</sub> H <sub>30</sub> O <sub>10</sub>	400.218	[M + NH <sub>4</sub> ] <sup>+</sup>	0.668	1.792
13.	Phenylethyl pentosyl-hexoside	16.0	C <sub>19</sub> H <sub>28</sub> O <sub>10</sub>	434.202	[M + NH <sub>4</sub> ] <sup>+</sup>	0.328	2.988
14.	Hexenyl pentosyl-hexoside	16.6	C <sub>17</sub> H <sub>30</sub> O <sub>10</sub>	412.219	[M + NH <sub>4</sub> ] <sup>+</sup>	1.013	2.056
15.	Vitexin	18.6	C <sub>21</sub> H <sub>20</sub> O <sub>10</sub>	433.114	[M + H] <sup>+</sup>	2.163	3.363
16.	Hexenyl pentosyl-hexoside	19.1	C <sub>17</sub> H <sub>30</sub> O <sub>10</sub>	412.218	[M + NH <sub>4</sub> ] <sup>+</sup>	0.503	1.983
17.	Hexenyl pentosyl-hexoside	19.2	C <sub>17</sub> H <sub>30</sub> O <sub>10</sub>	412.218	[M + NH <sub>4</sub> ] <sup>+</sup>	0.649	2.929
18.	Isovitexin	19.4	C <sub>21</sub> H <sub>20</sub> O <sub>10</sub>	433.114	[M + H] <sup>+</sup>	1.401	3.641
19.	Quercetin hexosyl-rhamnoside	19.7	C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	611.162	[M + H] <sup>+</sup>	1.078	2.665
20.	Quercetin hexoside	20.4	C <sub>21</sub> H <sub>20</sub> O <sub>12</sub>	465.103	[M + H] <sup>+</sup>	0.124	1.306

No.	Assigned compound# (or isomer)	Retention time (min)	Molecular formula	(m/z)	Ion	MOW ppm#	MOE ppm#
21.	Quercetin malonyl-hexoside	22.2	C <sub>24</sub> H <sub>22</sub> O <sub>15</sub>	551.103	[M + H] <sup>+</sup>	0.007	2.112
22.	Quercetin malonyl-hexoside	22.3	C <sub>24</sub> H <sub>22</sub> O <sub>15</sub>	551.103	[M + H] <sup>+</sup>	0.102	2.783
23.	Quercetin malonyl-hexoside	22.9	C <sub>24</sub> H <sub>22</sub> O <sub>15</sub>	551.103	[M + H] <sup>+</sup>	0.551	3.436
24.	Hexanol, pentosyl-hexoside	23.2	C <sub>17</sub> H <sub>32</sub> O <sub>10</sub>	414.234	[M + NH <sub>4</sub> ] <sup>+</sup>	0.790	3.518
25.	Kaempferol hexoside	23.7	C <sub>21</sub> H <sub>20</sub> O <sub>11</sub>	449.109	[M + H] <sup>+</sup>	2.343	0.240
26.	Isorhamnetin hexoside	24.2	C <sub>22</sub> H <sub>22</sub> O <sub>12</sub>	479.120	[M + H] <sup>+</sup>	2.186	3.126
27.	Kaempferol malonyl-hexoside	25.5	C <sub>24</sub> H <sub>22</sub> O <sub>14</sub>	535.109	[M + H] <sup>+</sup>	0.090	0.763
28.	Kaempferol malonyl-hexoside	25.6	C <sub>24</sub> H <sub>22</sub> O <sub>14</sub>	535.108	[M + H] <sup>+</sup>	0.651	1.293
29.	Isorhamnetin malonyl-hexoside	26.5	C <sub>25</sub> H <sub>24</sub> O <sub>15</sub>	565.119	[M + H] <sup>+</sup>	0.077	1.794
30.	Niaziminic A or B	29.2	C <sub>16</sub> H <sub>23</sub> NO <sub>6</sub> S	358.133	[M + H] <sup>+</sup>	Nd	3.114
31.	Niaziminin A or B	32.0	C <sub>18</sub> H <sub>25</sub> NO <sub>7</sub> S	400.144	[M + H] <sup>+</sup>	Nd	4.750
32.	Niaziminin A or B	36.9	C <sub>18</sub> H <sub>25</sub> NO <sub>7</sub> S	400.144	[M + H] <sup>+</sup>	Nd	3.226

All compounds assigned by comparison of accurate mass data (based on ppm#), and by interpretation of available MS/MS and/or UV spectra. nd = not detected or below detection limit.

### 3.3.6.2 *C. asiatica* (L.) Urb.

The constituents detected in *C. asiatica* extracts were phenolic compounds and triterpene saponins (see Table 3.5). The phenolics detected were assigned as chlorogenic acids derived from caffeoyl-, dicaffeoyl, caffeomalonyl, dicaffeomalonyl-, ferulyl- and syringoyl-esters, which were observed in both CAW and CAE. Flavonols such as quercetin and kaempferol eluting at 22.7 and 26.2 min were detected in CAE only. The triterpene saponins detected in the extracts consisted of various derivatives and isomers of asiaticosides, centellosides, centoic acids and madasiatic acids, which has been used as biomarker components for quality assessment and authentication for this species (Inamdar *et al.*, 1996; Chong and Aziz, 2011; Seevaratnam and Banumathi, 2012). While chlorogenic acids were detected in both CAW and CAE, the flavonols and the madasiatic acids were detected only in CAE.

**Table 3.5: Compounds assigned from LC-UV-MS/MS analysis of CAW and CAE.**

No.	Assigned compound <sup>#</sup> (or isomer)	Retention time (min)	Molecular formula	(m/z)	Ion	CAW ppm <sup>#</sup>	CAE ppm <sup>#</sup>
1.	Caffeoylquinic acid	3.8	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	353.087	[M - H] <sup>-</sup>	1.958	1.788
2.	3-O-Caffeoylquinic acid	4.3	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	353.088	[M - H] <sup>-</sup>	2.298	2.751
3.	Caffeoyl-O-malonylquinic acid	5.5	C <sub>19</sub> H <sub>20</sub> O <sub>12</sub>	439.088	[M - H] <sup>-</sup>	1.384	2.568
4.	5-O-Caffeoylquinic acid	7.6	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	353.088	[M - H] <sup>-</sup>	1.449	1.364
5.	4-O-Caffeoylquinic acid	8.5	C <sub>16</sub> H <sub>18</sub> O <sub>9</sub>	353.088	[M - H] <sup>-</sup>	2.383	1.619
6.	Caffeoyl-O-malonylquinic acid	11.1	C <sub>19</sub> H <sub>20</sub> O <sub>12</sub>	439.088	[M - H] <sup>-</sup>	2.135	1.520
7.	Feruloylquinic acid	13.0	C <sub>17</sub> H <sub>20</sub> O <sub>9</sub>	367.103	[M - H] <sup>-</sup>	2.646	2.074
8.	Syringoylquinic acid	13.2	C <sub>16</sub> H <sub>20</sub> O <sub>10</sub>	371.098	[M - H] <sup>-</sup>	1.123	2.074
9.	Trihydroxy-lupenedioic acid O-[hexosyl-rhamnosyl- hexoside] ester	15.4	C <sub>48</sub> H <sub>78</sub> O <sub>21</sub>	1035.502	[M + HCOO] <sup>-</sup>	1.125	1.367
10.	Quercetin hexuronide	15.8	C <sub>21</sub> H <sub>18</sub> O <sub>13</sub>	477.067	[M - H] <sup>-</sup>	1.243	0.937
11.	Trihydroxy-lupenedioic acid O-[hexosyl-rhamnosyl- hexoside] ester	16.5	C <sub>48</sub> H <sub>76</sub> O <sub>21</sub>	1033.487	[M + HCOO] <sup>-</sup>	1.389	1.863
12.	Di-O-caffeooylquinic acid	17.3	C <sub>25</sub> H <sub>24</sub> O <sub>12</sub>	515.119	[M - H] <sup>-</sup>	0.996	1.082
13.	Di-O-caffeooylquinic acid	17.5	C <sub>25</sub> H <sub>24</sub> O <sub>12</sub>	515.119	[M - H] <sup>-</sup>	1.082	0.597

No.	Assigned compound <sup>#</sup> (or isomer)	Retention time (min)	Molecular formula	(m/z)	Ion	CAW ppm <sup>#</sup>	CAE ppm <sup>#</sup>
14.	Dicaffeoyl-malonylquinic acid	18.0	C <sub>28</sub> H <sub>26</sub> O <sub>15</sub>	601.119	[M - H] <sup>-</sup>	0.073	0.643
15.	Di-O-caffeoylequinic acid	18.3	C <sub>25</sub> H <sub>24</sub> O <sub>12</sub>	515.119	[M - H] <sup>-</sup>	1.082	0.102
16.	Dicaffeoyl-malonylquinic acid	19.0	C <sub>28</sub> H <sub>26</sub> O <sub>15</sub>	601.120	[M - H] <sup>-</sup>	2.302	1.387
17.	Dicaffeoyl-malonylquinic acid	19.3	C <sub>28</sub> H <sub>26</sub> O <sub>15</sub>	601.119	[M - H] <sup>-</sup>	0.771	1.487
18.	Asiaticoside A (madecassoside), asiaticoside B, or asiaticoside G	19.8	C <sub>48</sub> H <sub>78</sub> O <sub>20</sub>	1019.506	[M + HCOO] <sup>-</sup>	0.255	0.255
19.	Asiaticoside, centellasaponin A, centellasaponin C, centellasaponin D, or isomer	20.1	C <sub>48</sub> H <sub>78</sub> O <sub>19</sub>	1003.512	[M + HCOO] <sup>-</sup>	0.702	0.703
20.	Centelloside D, or centellasaponin B	20.4	C <sub>42</sub> H <sub>68</sub> O <sub>16</sub>	873.448	[M + HCOO] <sup>-</sup>	0.456	0.525
21.	Asiaticoside, centellasaponin A, centellasaponin C, centellasaponin D, or isomer	21.0	C <sub>48</sub> H <sub>78</sub> O <sub>19</sub>	1003.512	[M + HCOO] <sup>-</sup>	0.762	0.095
22.	Asiaticoside, centellasaponin A centellasaponin C, centellasaponin D, or isomer	21.3	C <sub>48</sub> H <sub>78</sub> O <sub>19</sub>	1003.512	[M + HCOO] <sup>-</sup>	0.215	0.882
23.	Dicaffeoyl-succinoylquinic acid	21.7	C <sub>29</sub> H <sub>28</sub> O <sub>15</sub>	615.135	[M - H] <sup>-</sup>	0.721	0.819
24.	Asiaticoside E	21.9	C <sub>42</sub> H <sub>68</sub> O <sub>15</sub>	857.454	[M + HCOO] <sup>-</sup>	1.508	1.508
25.	Quercetin	22.7	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>	301.035	[M - H] <sup>-</sup>	Nd	1.598
26.	Asiaticoside, centellasaponin A, centellasaponin C, centellasaponin D, or isomer	23.5	C <sub>48</sub> H <sub>78</sub> O <sub>19</sub>	1003.512	[M + HCOO] <sup>-</sup>	0.822	1.061
27.	Asiaticoside C, Asiaticoside, centellasaponin A,	23.7	C <sub>50</sub> H <sub>80</sub> O <sub>20</sub>	1045.524	[M + HCOO] <sup>-</sup>	2.066	1.597
28.	Asiaticoside, centellasaponin A, centellasaponin C, centellasaponin D, or isomer	23.9	C <sub>48</sub> H <sub>78</sub> O <sub>19</sub>	1003.512	[M + HCOO] <sup>-</sup>	1.429	1.240
29.	Asiaticoside, centellasaponin A, centellasaponin C, centellasaponin D, or isomer	24.1	C <sub>48</sub> H <sub>78</sub> O <sub>19</sub>	957.506	[M - H] <sup>-</sup>	1.550	0.463
30.	Centelloside E	24.2	C <sub>48</sub> H <sub>76</sub> O <sub>19</sub>	1001.496	[M + HCOO] <sup>-</sup>	1.882	0.663
31.	Asiaticoside, centellasaponin A, centellasaponin C, centellasaponin D, or isomer	24.3	C <sub>48</sub> H <sub>78</sub> O <sub>19</sub>	1003.512	[M + HCOO] <sup>-</sup>	1.429	0.393

No.	Assigned compound <sup>#</sup> (or isomer)	Retention time (min)	Molecular formula	(m/z)	Ion	CAW ppm <sup>#</sup>	CAE ppm <sup>#</sup>
32.	Asiaticoside, centellasaponin A, centellasaponin C, centellasaponin D, or isomer	24.6	C <sub>48</sub> H <sub>78</sub> O <sub>19</sub>	1003.511	[M + HCOO] <sup>-</sup>	1.240	0.094
33.	Triferulic acid	25.1	C <sub>30</sub> H <sub>26</sub> O <sub>12</sub>	577.135	[M - H] <sup>-</sup>	0.914	1.018
34.	Asiaticoside D, or F	26.0	C <sub>48</sub> H <sub>78</sub> O <sub>18</sub>	987.518	[M + HCOO] <sup>-</sup>	2.045	2.045
35.	Kaempferol	26.2	C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>	285.040	[M - H] <sup>-</sup>	Nd	1.949
36.	Centoic acid, terminolic acid, madecassic acid, or isothankunic acid	30.0	C <sub>30</sub> H <sub>48</sub> O <sub>6</sub>	549.343	[M + HCOO] <sup>-</sup>	Nd	1.284
37.	Centoic acid, terminolic acid, madecassic acid, or isothankunic acid	30.5	C <sub>30</sub> H <sub>48</sub> O <sub>6</sub>	549.342	[M + HCOO] <sup>-</sup>	2.267	0.173
38.	Centoic acid, terminolic acid, madecassic acid, or isothankunic acid	30.7	C <sub>30</sub> H <sub>48</sub> O <sub>6</sub>	549.342	[M + HCOO] <sup>-</sup>	1.830	0.282
39.	Centoic acid, terminolic acid, madecassic acid, or isothankunic acid	31.7	C <sub>30</sub> H <sub>48</sub> O <sub>6</sub>	549.343	[M + HCOO] <sup>-</sup>	Nd	1.830
40.	Madasiatic acid, 2,3,23-trihydroxy-20-ursen-28-oic acid, centellasapogenol A, or ursen-28-oic acid, centellasapogenol A, or 2,3-dihydroxy-5-(hydroxymethyl)-24-norolean-12-en-28-oic	32.0	C <sub>30</sub> H <sub>48</sub> O <sub>5</sub>	487.343	[M - H] <sup>-</sup>	Nd	1.804
41.	Madasiatic acid, 2,3,23-trihydroxy-20-ursen-28-oic acid, centellasapogenol A, or ursen-28-oic acid, centellasapogenol A, or 2,3-dihydroxy-5-(hydroxymethyl)-24-norolean-12-en-28-oic	33.6	C <sub>30</sub> H <sub>48</sub> O <sub>5</sub>	533.348	[M + HCOO] <sup>-</sup>	Nd	1.162
42.	Madasiatic acid, 2,3,23-trihydroxy-20-ursen-28-oic acid, centellasapogenol A, or ursen-28-oic acid, centellasapogenol A, or 2,3-dihydroxy-5-(hydroxymethyl)-24-norolean-12-en-28-oic	34.2	C <sub>30</sub> H <sub>48</sub> O <sub>5</sub>	975.693	[2M - H] <sup>-</sup>	Nd	1.235

No.	Assigned compound <sup>#</sup> (or isomer)	Retention time (min)	Molecular formula	(m/z)	Ion	CAW ppm <sup>#</sup>	CAE ppm <sup>#</sup>
43.	Madasiatic acid, 2,3,23-trihydroxy-20-ursen-28-oic acid, centellasapogenol A, or ursen-28-oic acid, centellasapogenol A, or 2,3-dihydroxy-5-(hydroxymethyl)-24-norolean-12-en-28-oic	34.4	C <sub>30</sub> H <sub>48</sub> O <sub>5</sub>	533.348	[M + HCOO] <sup>-</sup>	Nd	2.081
44.	Madasiatic acid, 2,3,23-trihydroxy-20-ursen-28-oic acid, centellasapogenol A, or ursen-28-oic acid, centellasapogenol A, or 2,3-dihydroxy-5-(hydroxymethyl)-24-norolean-12-en-28-oic	38.0	C <sub>30</sub> H <sub>48</sub> O <sub>5</sub>	533.349	[M + HCOO] <sup>-</sup>	Nd	2.193

All compounds assigned by comparison of accurate mass data (based on ppm<sup>#</sup>), and by interpretation of available MS/MS and/or UV spectra. nd = not detected or below detection limit.

### 3.3.6.3 *Clitoria ternatea* Lam.

The constituents detected in *C. ternatea* extracts were flavonol glycosides consisting of the aglycones of quercetin, myricetin and kaempferol and which included three malonylglycosides of kaempferol eluting with retention times of 22.4 min, 22.8 min and at 25.6 min (see Table 3.6). The detection of glycosides and malonylglycosides of kaempferol, quercetin and myricetin in the extracts are in agreement with the known constituents of *C. ternatea* flowers (Kazuma *et al.*, 2003a, 2003b). The other main constituents detected were assigned as acylated delphinidin glycosides, which included a series of ternatins known to occur in the flowers of *C. ternatea* (Kazuma *et al.* 2003b), thus supports the chemical authentication of the plant material in this study. Other detected constituents included coumaroyl-sucrose and -glucose, observed from their  $[M + NH_4]^+$  ions.

**Table 3.6: Compounds assigned from LC-UV-MS/MS analysis of CTW and CTE.**

No.	Assigned compound <sup>#</sup> (or isomer)	Retention time (min)	Molecular formula	(m/z)	Ion	CTW ppm <sup>#</sup>	CTE ppm <sup>#</sup>
1.	Phenylalanine	2.9	C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>	166.086	[M + H] <sup>+</sup>	0.752	1.414
2.	Coumaroylsucrose	3.5	C <sub>21</sub> H <sub>28</sub> O <sub>13</sub>	506.188	[M + NH <sub>4</sub> ] <sup>+</sup>	2.259	2.141
3.	Tryptophan	4.4	C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	205.097	[M + H] <sup>+</sup>	0.418	1.832
4.	Coumaroylglucose	7.7	C <sub>15</sub> H <sub>18</sub> O <sub>8</sub>	344.134	[M + NH <sub>4</sub> ] <sup>+</sup>	0.921	1.357
5.	Ternatin A3 or C2*	9.6	C <sub>66</sub> H <sub>75</sub> O <sub>39</sub>	1491.391	M <sup>+</sup>	2.006	1.188
6.	Myricetin rhamnosyl- rhamnosyl-glucoside	13.5	C <sub>33</sub> H <sub>40</sub> O <sub>21</sub>	773.214	[M + H] <sup>+</sup>	1.132	2.387
7.	Ternatin B4 or C1*	14.3	C <sub>60</sub> H <sub>65</sub> O <sub>34</sub>	1329.337	M <sup>+</sup>	1.862	3.148
8.	Myricetin neohesperidoside	16.0	C <sub>27</sub> H <sub>30</sub> O <sub>17</sub>	627.157	[M + H] <sup>+</sup>	2.143	2.143
9.	Quercetin hexosyl- rhamnosyl-rhamnoside	16.4	C <sub>33</sub> H <sub>40</sub> O <sub>20</sub>	757.222	[M + H] <sup>+</sup>	4.028	2.575
10.	Quercetin hexosyl- rhamnoside	17.6	C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	611.163	[M + H] <sup>+</sup>	3.074	2.469
11.	Ternatin B2 or B3*	18.1	C <sub>75</sub> H <sub>81</sub> O <sub>41</sub>	1637.429	M <sup>+</sup>	2.328	2.628

No.	Assigned compound <sup>#</sup> (or isomer)	Retention time (min)	Molecular formula	(m/z)	Ion	CTW ppm <sup>#</sup>	CTE ppm <sup>#</sup>
12.	Kaempferol hexosyl-rhamnosyl-rhamnoside	18.8	C <sub>33</sub> H <sub>40</sub> O <sub>19</sub>	741.225	[M + H] <sup>+</sup>	2.435	3.015
13.	Delphinidin 3-O-[4-hydroxycinnamoyl-rhamnosyl-glucoside], 5-O-(6-O-malonyl-glucoside), bis-O-[3,4-dihydroxycinnamoyl-glucoside]	19.2	C <sub>75</sub> H <sub>81</sub> O <sub>42</sub>	1653.422	M <sup>+</sup>	1.692	1.692
14.	Quercetin hexosyl-rhamnoside	19.8	C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	611.162	[M + H] <sup>+</sup>	1.765	2.567
15.	Kaempferol hexosyl-rhamnoside	20.1	C <sub>27</sub> H <sub>30</sub> O <sub>15</sub>	595.167	[M + H] <sup>+</sup>	2.089	3.736
16.	Kaempferol hexosyl-rhamnoside	20.2	C <sub>27</sub> H <sub>30</sub> O <sub>15</sub>	595.167	[M + H] <sup>+</sup>	3.316	3.736
17.	Quercetin hexoside	20.4	C <sub>21</sub> H <sub>20</sub> O <sub>12</sub>	465.104	[M + H] <sup>+</sup>	2.747	2.618
18.	Ternatin B2 or B3*	21.3	C <sub>75</sub> H <sub>81</sub> O <sub>41</sub>	1637.429	M <sup>+</sup>	2.847	2.554
19.	Ternatin B4 or C1*	21.7	C <sub>60</sub> H <sub>65</sub> O <sub>34</sub>	1329.339	M <sup>+</sup>	2.689	2.96
20.	Ternatin B1*	22.2	C <sub>90</sub> H <sub>97</sub> O <sub>48</sub>	1945.520	M <sup>+</sup>	3.022	2.775
21.	Kaempferol rhamnosyl-malonyl-glucoside	22.4	C <sub>30</sub> H <sub>32</sub> O <sub>18</sub>	681.168	[M + H] <sup>+</sup>	2.686	2.774
22.	Kaempferol rhamnosyl-malonyl-glucoside	22.8	C <sub>30</sub> H <sub>32</sub> O <sub>18</sub>	681.168	[M + H] <sup>+</sup>	3.405	3.317
23.	Kaempferol hexosyl-rhamnoside	23.3	C <sub>27</sub> H <sub>30</sub> O <sub>15</sub>	595.167	[M + H] <sup>+</sup>	2.812	2.409
24.	Kaempferol hexoside	23.6	C <sub>21</sub> H <sub>20</sub> O <sub>11</sub>	449.109	[M + H] <sup>+</sup>	2.677	3.078
25.	Ternatin D1*	24.9	C <sub>84</sub> H <sub>87</sub> O <sub>43</sub>	1783.468	M <sup>+</sup>	3.826	3.074
26.	6''-Malonylastragalin	25.6	C <sub>24</sub> H <sub>22</sub> O <sub>14</sub>	535.109	[M + H] <sup>+</sup>	1.567	1.567
27.	Quercetin	28.7	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>	303.051	[M + H] <sup>+</sup>	Nd	2.808

All compounds assigned by comparison of accurate mass data (based on ppm<sup>#</sup>), and by interpretation of available MS/MS and/or UV spectra. nd = not detected or below detection limit.

### 3.3.6.4 *Cosmos caudatus* Kunth.

The compounds detected in the *C. caudatus* leaf extracts by high resolution LC-UV-MS/MS in the positive ionisation mode were assigned as amino acids, flavonol glycosides, including those derived from quercetins and kaempferols, and flavone C-glycosides derived from apigenins (vicienin-2 and vitexin) (see Table 3.7). Flavonoids and amino acids have been shown to occur in this plant species, especially in the leaves (Abas *et al.*, 2003; Andarwulan *et al.*, 2010, 2012; Mediani *et al.*, 2013). Other compounds detected in the extracts of this plant species were assigned as glycosides of hydrocarbon alcohols, including a diglycoside of 1-octen-3-ol.

**Table 3.7: Compounds assigned from LC-UV-MS/MS analysis of CCW and CCE.**

No.	Assigned compound <sup>#</sup> (or isomer)	Retention time (min)	Molecular formula	(m/z)	Ion	CCW ppm <sup>#</sup>	CCE ppm <sup>#</sup>
1.	Phenylalanine	2.9	C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>	166.087	[M + H] <sup>+</sup>	3.160	2.016
2.	Alanylleucine, leucylalanine, isoleucylalanine	3.0	C <sub>9</sub> H <sub>18</sub> N <sub>2</sub> O <sub>3</sub>	203.140	[M + H] <sup>+</sup>	3.451	2.368
3.	Phenylalanylalanine or alanylphenylalanine	4.0	C <sub>12</sub> H <sub>16</sub> N <sub>2</sub> O <sub>3</sub>	237.124	[M + H] <sup>+</sup>	4.180	2.366
4.	Tryptophan	4.3	C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	205.098	[M + H] <sup>+</sup>	2.759	2.954
5.	Leucylvaline or Valylleucine	4.4	C <sub>11</sub> H <sub>22</sub> N <sub>2</sub> O <sub>3</sub>	231.171	[M + H] <sup>+</sup>	3.119	3.638
6.	Glutamylleucine, glutamylsoleucine or leucylglutamic acid	5.5	C <sub>11</sub> H <sub>20</sub> N <sub>2</sub> O <sub>5</sub>	261.145	[M + H] <sup>+</sup>	2.802	1.768
7.	Glutamylphenylalanine or phenylalanylglutamic acid	6.9	C <sub>14</sub> H <sub>18</sub> N <sub>2</sub> O <sub>5</sub>	295.130	[M + H] <sup>+</sup>	3.191	2.276
8.	Leucylleucine or leucylsoleucine	7.4	C <sub>12</sub> H <sub>24</sub> N <sub>2</sub> O <sub>3</sub>	245.187	[M + H] <sup>+</sup>	3.266	1.839
9.	Leucylleucine or leucylsoleucine	9.3	C <sub>12</sub> H <sub>24</sub> N <sub>2</sub> O <sub>3</sub>	245.187	[M + H] <sup>+</sup>	2.818	1.88
10.	Vicienin-2	12.5	C <sub>27</sub> H <sub>30</sub> O <sub>15</sub>	595.167	[M + H] <sup>+</sup>	1.888	2.409
11.	Vitexin	18.6	C <sub>21</sub> H <sub>20</sub> O <sub>10</sub>	433.115	[M + H] <sup>+</sup>	4.772	4.079
12.	Quercetin hexoside	19.7	C <sub>21</sub> H <sub>20</sub> O <sub>12</sub>	465.104	[M + H] <sup>+</sup>	3.478	3.284
13.	Quercetin hexoside	20.3	C <sub>21</sub> H <sub>20</sub> O <sub>12</sub>	465.104	[M + H] <sup>+</sup>	4.338	2.747

No.	Assigned compound <sup>#</sup> (or isomer)	Retention time (min)	Molecular formula	(m/z)	Ion	CCW ppm <sup>#</sup>	CCE ppm <sup>#</sup>
14.	Quercetin pentoside	21.3	C <sub>20</sub> H <sub>18</sub> O <sub>11</sub>	435.094	[M + H] <sup>+</sup>	4.510	4.579
15.	Quercetin pentoside	21.9	C <sub>20</sub> H <sub>18</sub> O <sub>11</sub>	435.094	[M + H] <sup>+</sup>	4.441	3.522
16.	Quercetin pentoside	22.7	C <sub>20</sub> H <sub>18</sub> O <sub>11</sub>	435.094	[M + H] <sup>+</sup>	Nd	4.717
17.	Quercetin rhamnoside	23.6	C <sub>21</sub> H <sub>20</sub> O <sub>11</sub>	449.109	[M + H] <sup>+</sup>	2.944	1.653
18.	Di- <i>O</i> -Isopropylidene- <i>C</i> -methyl- <i>O</i> -methyl-hexose	25.2	C <sub>14</sub> H <sub>24</sub> O <sub>6</sub>	289.166	[M + H] <sup>+</sup>	5.551	5.343
19.	Kaempferol rhamnoside	27.0	C <sub>21</sub> H <sub>20</sub> O <sub>10</sub>	433.115	[M + H] <sup>+</sup>	4.079	3.571
20.	Heptanol pentosyl-hexoside	27.4	C <sub>18</sub> H <sub>34</sub> O <sub>10</sub>	428.251	[M + NH <sub>4</sub> ] <sup>+</sup>	5.785	2.632
21.	Di- <i>O</i> -Acetyl-di- <i>O</i> -isopropylidene- <i>glycero</i> -hexo-heptose	28.4	C <sub>17</sub> H <sub>26</sub> O <sub>9</sub>	392.193	[M + NH <sub>4</sub> ] <sup>+</sup>	4.263	3.932
22.	1-Octen-3-ol pentosyl-hexoside	29.8	C <sub>19</sub> H <sub>34</sub> O <sub>10</sub>	440.251	[M + NH <sub>4</sub> ] <sup>+</sup>	4.514	4.309

All compounds assigned by comparison of accurate mass data (based on ppm<sup>#</sup>), and by interpretation of available MS/MS and/or UV spectra. nd = not detected or below detection limit.

### 3.4 Discussion

The consumption of fruits and vegetables rich in polyphenols has been epidemiologically correlated with reduced risk of many chronic diseases, such as cardiovascular disease, diabetes, cancers, as well as ageing (Arts and Hollman, 2005; Pandey and Rizvi, 2009; Vauzour *et al.*, 2010; Lima *et al.*, 2014). Phenolic acids and flavonoids are the two main groups of polyphenols, which are abundant in fruits, cereals and vegetables (Lima *et al.*, 2014), and they are excellent antioxidants due to their unique structure that can accept electron, thus neutralizing and reducing the amount of damaging free radicals in tissue (Losada-barreiro and Bravo-díaz, 2017). The application of polyphenols together with other natural active compounds as active ingredients in the cosmetic industry has attracted attention for its beneficial effects on the skin (Mukherjee *et al.*, 2011; Zillich *et al.*, 2015).

In this study, the TPC of the medicinal plants investigated was quantified and compared. Additionally, individual compounds were analysed using high resolution LC-UV-MS/MS to determine other potentially active compounds in the plant extracts. MOE had the highest TPC with 67.3 mg GAE/g dried extracts, while MOW contained 52.8 mg GAE/g dried extract. Similarly, a previous study to maximize the TPC extraction from *M. oleifera* leaves has suggested that 70% ethanol extraction results in the highest TPC (Vongsak, Sithisarn, Mangmool, *et al.*, 2013). The HPLC-MS analysis detected the presence of chlorogenic acids of -*O*-coumaryl-, caffeoyl-, and feruloyl-ester, apigenin flavone glucosides and flavonol glycosides of quercetin and kaempferol derivatives, believed to be the active phenolics and flavonoids in *M. oleifera* leaves (Vongsak *et al.*, 2014), and this is supported by a database searched (U.S. Department of Agriculture, 2016). Other potentially active compounds in *M. oleifera* could be marumoside A and niazinamins, which are unique to the plant species (Faizi *et al.*, 1992, 1994; Sahakitpichan *et al.*, 2011).

CAW had the lowest TPC with 25.7 mg GAE/g of dried extract, while CAE had significantly higher TPC with 40.1 mg GAE/g dried extract. The analysis of individual compounds showed the presence of chlorogenic acid derivatives and the flavonols quercetin and kaempferol. However, the flavonols were detected in CAE only, which is consistent with the findings of higher TPC quantified in CAE compared with CAW (see Figure 3.3). A study comparing the TPC between various parts of the plant species has also shown that the leaves have the highest TPC compared with the roots or petiole (Zainol *et al.*, 2003). Its other active compounds are the pentacyclic triterpenes detected either as glycosides or saponins of their respective triterpenes, triterpenic acids and their glycosides derivatives. Apart from the phenolics, many of the activities exhibited by this plant species are due to the triterpenes (Maquart *et al.*, 1990; Tang *et al.*, 2011; Somboonwong *et al.*, 2012; Zahara *et al.*, 2014).

*C. ternatea* flower extracts (CTW and CTE) were investigated for their TPC and TAC. The TPC for CTE was significantly higher than CTW with 53.0 mg GAE/g vs. 38.5 mg GAE/g, while the TAC of the two extracts, expressed as cyd-3-glu equivalent/g extracts showed no significant difference ( $3.5 \pm 0.2$  vs.  $3.6 \pm 0.7$  mg/g). Rabeta and An Nabil (2013) showed similar higher TPC values of extract obtained with an organic solvent (methanol) compared to the aqueous extracts of the flowers, with 61.7 mg GAE/g and 20.7 mg GAE/g respectively, which is in accord with the current finding. Previously reported TAC values for the aqueous *C. ternatea* flower extract were lower than the findings in this study (Chayaratanasin *et al.*, 2015). The individual phenolic constituents of *C. ternatea* extracts consisted of flavonol glycosides and anthocyanins, and acylated delphinidin glycosides, including a series of ternatins. The anthocyanins are potentially the most active flavonoids in the flowers of *C. ternatea* as many of the reported activities are due to these compounds.

The TPC of *C. caudatus* extracts (CCW and CCE) were 28.5 mg GAE/g vs. 43.0 mg GAE/g dried respectively. *C. caudatus* is the least studied plant species of all the selected plants; however, its TPC has been reported in several studies. A methanolic extract of *C. caudatus* was measured to contain 70.4 mg GAE/g dried extract, which was higher than the measured TPC in this study (Mustafa *et al.*, 2010). Meanwhile, other reported TPC levels in 80% methanol and 80% ethanol extracts of *C. caudatus* were 22.3 and 19.3 g GAE/100 dried weight respectively (Mediani *et al.*, 2013). These findings were lower compared with the results in the present study, where the TPC of the water and ethanol extracts were equivalent to 239.4 and 172.0 g GAE/100 g dried weight respectively. The LC-MS analysis showed the presence of flavonoids such as flavonol glycosides of quercetins and kaempferol derivatives, and flavone glucosides, which are in agreement with findings in previous studies (Abas *et al.*, 2003; Andarwulan *et al.*, 2010; Ahmad; Mediani *et al.*, 2012; Mediani *et al.*, 2013).

The comparison of the two extracts showed that extraction yield and TPC are strongly dependent on the extraction methods used. However, it is also important to consider other factors associated with the plant samples, including environmental and genetic factors which may also influence the chemical composition of the extracts (Coelho *et al.*, 2016; Boneza and Niemeyer, 2018; Di Vittori *et al.*, 2018; Escriche and Juan-Borrás, 2018). Other than that, the presence of other potentially active compounds should also be considered to influence the investigated anti-ageing properties; hence the importance of LC-MS analysis in this study.

### **3.5 Conclusion**

The plant extracts prepared in this study showed the presence of phenolics such as flavonoids in the 70% ethanol extracts, which contained higher total phenolic content than the water extracts. However, water extraction is a better method to obtain higher yield per dried sample weight. The findings in this chapter are important as they will provide an understanding of the

potential active constituents that may cause the protective effects investigated in this study, and the data are significantly important for future pharmacological and clinical test.



# **Chapter 4**



## **Chapter 4: Antioxidant activities of the plant extracts and their correlation with total phenolics content**

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### **4.1 Introduction**

Antioxidants play a significant role in the prevention of skin ageing due to their ability to neutralise and reduce free radicals in the skin. Skin cells, like other cells in the body utilise oxygen for aerobic respiration. It has been shown that, under normal physiological conditions, aerobic respiration contributes to the production of reactive oxygen species (ROS) (Turrens, 2003; Hüttemann *et al.*, 2007; El-Bahr, 2013). The effects of other environmental factors then lead to further increases in ROS in the skin, resulting in a condition called oxidative stress when the innate defence system becomes deficient (Trenam *et al.*, 1992; Stojiljković *et al.*, 2014; Rinnerthaler *et al.*, 2015; Ahsanuddin *et al.*, 2016). Given an understanding of the role of ROS and other reactive species in causing damage to the skin, antioxidants have been included as functional active ingredients in skin care products as a part of the management of skin (Augustyniak *et al.*, 2010; Pouillot *et al.*, 2011; Pandel *et al.*, 2013). Polyphenols found in the roots, stems, flowers and leaves of plants are synthesised as metabolites which protect against infection and ultraviolet radiation (Harborne and Williams, 2000; Ferdinando *et al.*, 2014). The chemical structures of these compounds which can accept free radicals make them excellent antioxidants. Therefore, plant extracts have been evaluated for their antioxidant activities, and the correlation of such properties with total phenolic content analysed.

#### **4.1.1 Generation of ROS in the skin**

Free radicals can be divided into two types; reactive oxygen and reactive nitrogen species as shown in Table 4.1 (El-Bahr, 2013; Nimse and Pal, 2015). Under normal physiological conditions, ROS are generated from the uptake of oxygen and the leakage of electrons in the mitochondrial ETC (Turrens, 2003). The generation of ROS and RNS is elaborated below to

provide a more detailed understanding of the mechanism of action and of assays used for the evaluation of the antioxidants.

**Table 4.1: Classification of free radicals**

Reactive oxygen species (ROS)	Reactive nitrogen species (RNS)
<ol style="list-style-type: none"> <li>1. Singlet oxygen (<math>{}^1\text{O}_2</math>)</li> <li>2. Superoxide anion radical (<math>\text{O}_2^{\bullet-}</math>)</li> <li>3. Hydroxyl radical (<math>{}^{\bullet}\text{OH}</math>)</li> <li>4. Alkoxy radical (<math>\text{RO}^{\bullet}</math>)</li> <li>5. Peroxyl radical (<math>\text{ROO}^{\bullet}</math>)</li> <li>6. Hydrogen peroxide (<math>\text{H}_2\text{O}_2</math>); a precursor</li> <li>7. Lipid hydroperoxide (LOOH)</li> </ol>	<ol style="list-style-type: none"> <li>1. Nitric oxide (<math>\text{NO}^{\bullet}</math>)</li> <li>2. Nitric dioxide (<math>\text{NO}^{\bullet}_2</math>)</li> <li>3. Peroxynitrate (<math>\text{OONO}^{\bullet-}</math>)</li> </ol>

Apart from the mitochondrial ETC, ROS are produced when the skin is exposed to external stimuli such as ultraviolet light, responses to infection, and wound healing, as well as possible underlying pathological conditions (Cals-Grierson and Ormerod, 2004; Rinnerthaler *et al.*, 2015). These stimuli activate the immune system to produce appropriate responses. For example, the invasion of the skin by microorganisms such as bacteria, viruses and fungi stimulates phagocytic cells to undergo respiratory bursts (Trenam *et al.*, 1992). During this process, NADPH oxidase is activated which produces superoxide anions ( $\text{O}_2^{\bullet-}$ ) as a preventive measure (Fu *et al.*, 2014). The  $\text{O}_2^{\bullet-}$  is then converted into hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) by superoxide dismutase (SOD). These reactions are as shown in equations 4.1 and 4.2.



Then, the  $\text{H}_2\text{O}_2$  can react with reduced transition metals to produce a highly reactive hydroxyl radical ( ${}^{\bullet}\text{OH}$ ) via the Fenton reaction, as shown in equation 4.3 (Winterbourn, 1995; El-Bahr, 2013).



Radical species, and especially those capable of abstracting hydrogen, will attack the polyunsaturated fatty acid (PUFA) and initiate a lipid peroxidation chain reaction (Halliwell and Gutteridge, 1985). The oxidation of PUFA generates a fatty acid radical ( $\text{L}^\bullet$ ) that can react with  $\text{O}_2$  to form a fatty acid peroxy radical ( $\text{LOO}^\bullet$ ), as shown in equations 4.4 and 4.5, where PUFA is referred as LH. The peroxy radicals can further oxidize PUFA and initiate new chain reactions producing lipid hydroperoxide ( $\text{LOOH}$ ), which usually breaks down to aldehydes as shown in equations 4.6 and 4.7.



Meanwhile, RNS such as nitric oxide ( $\text{NO}^\bullet$ ) are produced by nitric oxide synthase (NOS) from arginine, and the  $\text{NO}^\bullet$  and  $\text{O}_2^\bullet-$  can react together to produce peroxynitrite ( $\text{ONOO}^-$ ), as shown in equation 4.8 and 4.9.



#### 4.1.2 Mechanism of antioxidants

Antioxidants are substances that can delay or prevent the oxidation damage caused by ROS or RNS. They can be classified as endogenous or exogenous antioxidants. Endogenous antioxidant systems in the skin are either enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT) and GSH-peroxidase (GSH-Px), or non-enzymatic antioxidants such as glutathione (GSH),  $\alpha$ -tocopherol, ascorbate,  $\beta$ -carotene and melanin (Sun, 1990;

Steenvoorden and Beijersbergen Van Henegouwen, 1997). The SOD catalyses the dismutation of  $O_2^-$  to  $O_2$  and  $H_2O_2$ , as previously described in equation 4.2, and the  $H_2O_2$  is converted to water and  $O_2$  to prevent the formation of toxic  $OH^\bullet$  by CAT, as shown in equation 4.10 (Sun, 1990). Meanwhile, GSH-Px reduces  $H_2O_2$  and organic hydroperoxides using glutathione (GSH) as a substrate to produce glutathione disulphide (GSSG) and water, as shown in equations 4.11 and 4.12.



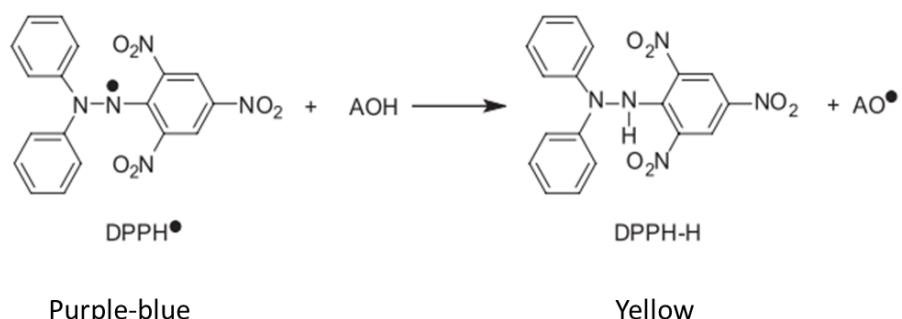
The non-enzyme endogenous antioxidants include low molecular weight antioxidants and metal binding proteins such as ferritin, myoglobin, metallothionein, coenzyme Q10, glutathione, melatonin, polyamines, transferrin, lactoferrin, albumin, ceruloplasmin, uric acid and bilirubin (Mironczuk-Chodakowska *et al.*, 2018). These proteins protect the skin from reactive species by either rapidly deactivating radicals and oxidants or inhibiting the formation of new reactive species by binding transition metal ions. Exogenous antioxidants such as ascorbic acid, vitamin E, carotenoids and polyphenols are supplied to the skin either by consumption or topical application, and also exert their activity against free radicals via similar mechanisms (Nimse and Pal, 2015).

#### 4.1.3 Antioxidant assays

Many antioxidant assays have been employed to measure the antioxidant capacities of plant and food components (Moon and Shibamoto, 2009). However, the most commonly used methods in the literature which are employed in this study are described below.

#### 1.1.1.1 2,2'-Diphenyl-1-picrylhydrazyl assay

The 2,2'-diphenyl-1-picrylhydrazyl (DPPH) assay is the most commonly used antioxidant assay due to its feasibility and ease of use. A synthetic, purple-blue and stable free radical of DPPH<sup>•</sup> is formed by dissolving the DPPH in organic solvents such as methanol or ethanol overnight. This free radical changes into yellow in the presence of antioxidants (see Figure 4.1) which are measurable at a wavelength of 571 nm. The antioxidant capacities are either expressed as IC<sub>50</sub>, which is the concentration capable of scavenging 50% of the free radicals present or compared to a standard equivalent such as Trolox or vitamin C (Floegel *et al.*, 2011).

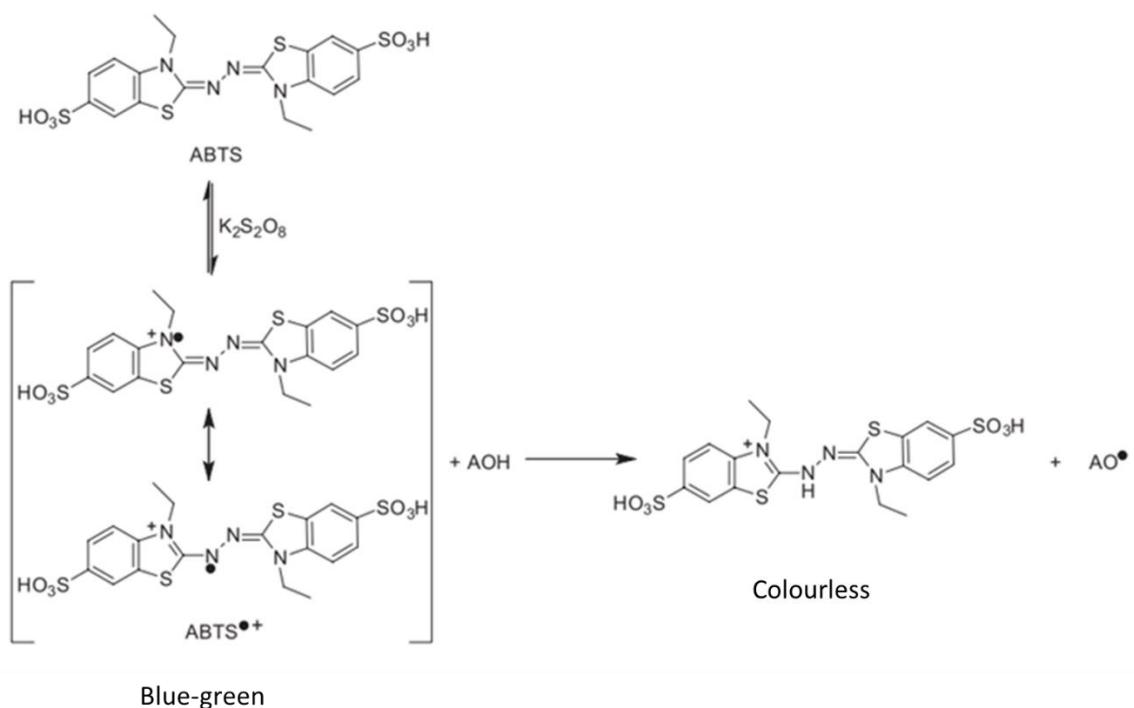


**Figure 4.1: Reaction of the stable free radical DPPH<sup>•</sup> with an antioxidant (AOH).** Image retrieved from Oliveira *et al.*, 2014.

#### 1.1.1.2 2,2'-Azino-bis-3-ethylbenzthiazoline-6-sulphonic acid assay

The 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay was originally based on the activation of metmyoglobin with hydrogen peroxide in the presence of ABTS to produce radical cations (Re *et al.*, 1999). An improved assay applicable to both lipo- and hydrophilic compounds was then developed in which the radical cation is formed without intermediates, and the preformed radical is more stable compared to that from the traditional method (Re *et al.*, 1999). The blue-green ABTS<sup>•+</sup> cation is preformed from the oxidation of ABTS salt with potassium persulfate (see Figure 4.2) and later is reduced or becomes colourless in the presence of antioxidants (Oliveira *et al.*, 2014). The maximum absorption is measured at 734

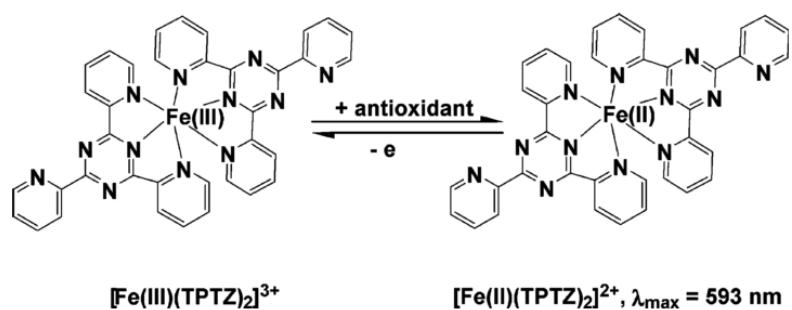
nm, and the antioxidant capacities are again either expressed as IC<sub>50</sub> or the standard equivalent such as Trolox or vitamin C.



**Figure 4.2: Oxidation of ABTS by potassium persulfate to generate ABTS<sup>•+</sup> and its reaction with an antioxidant.** Image retrieved from Oliveira *et al.*, 2014.

#### 1.1.1.3 Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) assay was developed by Benzie and Strain (1996). The method is based on the reduction of the ferric 2, 4, 6-tripyridyl-s-triazine complex (Fe<sup>3+</sup>-TPTZ) to the intensely blue ferrous form (Fe<sup>2+</sup>-TPTZ) and the intensity is measurable at 593 nm (see Figure 4.3). The method is inexpensive, simple and highly reproducible (Benzie and Strain, 1996). The redox potential of Fe (III) salt is comparable to that of ABTS, and the two assays are quite similar, except that the FRAP assay is performed under acidic conditions while the ABTS assay is at neutral (Huang *et al.*, 2005).



**Figure 4.3: FRAP reaction.** Image retrieved from Huang *et al.*, 2005.

#### 4.1.4 Objectives

The objective of this chapter is to investigate the antioxidant activities of the plant extracts and their correlation with the measured TPC in Chapter 3. Several methods of antioxidant measurements were employed to ensure the consistency of the activities exhibited, which include DPPH, ABTS and FRAP assays.

## 4.2 Materials and methods

### 4.2.1 Plant extracts and Trolox preparation

**Trolox standard (1000 µM):** 6.3 mg Trolox (MW: 250.29 g/mol) was weighed and dissolved in 25 mL of either water or 70% ethanol to obtain 1000 µM of Trolox stock. The stock was serially diluted at 1:1 dilution to obtain working concentrations of 500, 250, 125 and 62.5 µM.

**Plant extract stock (30mg/mL):** 300 mg of freeze-dried plant extracts was dissolved in 10 mL of either water or 70% ethanol to obtain 10 mL of 30 mg/mL stock. The stock was serially diluted to prepare working solutions of the plant extracts. Appropriate working concentrations were prepared for the DPPH, ABTS and FRAP assays to achieve final concentrations of 1000, 500, 250, 125 and 62.5 µg/mL in the wells.

### 4.2.2 DPPH assay

#### 4.2.2.1 DPPH reagent preparation

**a) DPPH stock (0.24 mg/ mL):** 24 mg of DPPH powder was weighed and dissolved in 100 mL of methanol to obtain 100 mL of 0.24 mg/mL DPPH stock. The stock was kept overnight in the dark at 4 °C to allow the complete dissolution of DPHH. The DPPH radical prepared was stable for at least 2-7 days but needed to be freshly diluted every day.

**b) DPPH working solution:** The prepared DPPH stock solution was diluted at a ratio of 1:5 with methanol to achieve a DPHH working solution with a final absorbance of approximately 1.0-1.2.

#### 4.2.2.2 DPPH assay protocol

The previous protocols were followed with some modifications using the prepared DPPH reagents (Pyrzynska and Pękal, 2013; Chattuwatthana and Okello, 2015). In a 96-well plate, Trolox (15 µL), plant samples (15 µL) and water or 70% ethanol (15 µL) as controls were added with DPPH working solution (285 µL) and properly mixed. The plate was incubated in the dark

at 30 °C for 30 minutes before a reading was taken at a wavelength of 517 nm using a microplate reader SpectraMax Plus<sup>384</sup> (Molecular Device Corporation). All experiments were performed in triplicates. Background absorbance was corrected by subtracting the absorbance value of a blank (water or 70% ethanol alone). The DPPH radical scavenging activity was expressed as Trolox equivalent (TE) in µM/ mg of extracts obtained from the Trolox standard curve. The final Trolox concentrations in the reaction mixture were 50, 25, 12.5, 6.25 and 3.33 µM (15 µL in 300 µL mixture: 1/20).

#### **4.2.3 ABTS assay**

##### **4.2.3.1 ABTS reagent preparation**

**a) 5 mM phosphate buffered saline (PBS), pH 7.4:** 9.0 g of sodium chloride (NaCl: 58.44 g/mol), 0.37 g of sodium dihydrogen phosphate (NaH<sub>2</sub>PO<sub>4</sub>: 119.98 g/mol) and 0.74 g of disodium hydrogen phosphate (Na<sub>2</sub>HPO<sub>4</sub>.12H<sub>2</sub>O: 141.96 g/mol) were weighed and dissolved in deionised water. The volume was made up to 1 L and the pH was adjusted to 7.4 using hydrochloric acid (HCl) or sodium hydroxide (NaOH).

**b) 15 mM ABTS:** 80 mg of the ABTS salt (MW: 514.62 g/mol) was weighed and dissolved in 10 mL of deionised water. A light turquoise green solution was obtained.

**c) 5 mM potassium persulfate (K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>):** 13.2 mg of potassium persulfate (MW: 270.32 g/mol) was weighed and dissolved in 10 mL of deionised water. A clear solution was obtained.

**d) ABTS stock:** The previously prepared 15 mM ABTS (5 mL) was added to 5 mM potassium persulfate (5 mL) to initiate a reaction, as ABTS radical is obtained from a 1:1 chemical reaction between 7 mM ABTS and 2.45 mM potassium persulfate. The solution was kept in the dark at 4 °C and remained stable for at least two days.

**e) ABTS working solution:** The preformed ABTS radical (1 mL) was diluted with 5-mM PBS (49 mL) to obtain a 50-mL working solution with an absorbance value of approximately 0.7-1.0 at 734 nm. The 50-mL working solution was sufficient for a full 96-well plate.

#### 4.2.3.2 ABTS assay protocol

The previous protocol was followed with some modifications using the prepared reagents (Chattuwatthana and Okello, 2015). In a 96-well plate, either Trolox standard solution (10 µL), plant sample (10 µL) or a solvent (10 µL) for control were thoroughly mixed with ABTS working solution (290 µL) in the assay wells. The microplate was subsequently incubated in the dark at 37°C for 6 minutes. All experiments were performed in triplicate. The absorbance of the solution in each assay well was determined using a microplate reader (SpectraMax Plus384, Molecular Device Corporation) at a wavelength of 734 nm. Background absorbance was corrected by subtracting the absorbance value of blank (water/70% ethanol). The ABTS radical scavenging activity was expressed as Trolox equivalent (TE) µM/mg extracts obtained from the Trolox standard curve. The final Trolox concentrations in the assay were 33.3, 16.7, 8.3, 4.2 and 2.1 µM (10 µL in 300 µL mixture:1/30)

#### 4.2.4 FRAP assay

##### 4.2.4.1 FRAP reagent preparation

**a) 0.2 M sodium acetate buffer:** 27.2 g of sodium acetate trihydrate,  $C_2H_3O_2Na \cdot 3H_2O$  (MW: 136.1 g/mol) was dissolved in deionised water and brought up to 1 L to obtain a 0.2 M sodium acetate buffer.

**b) 40-mM HCl:** 12.5 M concentrated HCl (320 µL) was made up to 100 mL using deionised water to obtain 40 mM HCl.

**c) 10 mM 2, 4,6-tri [2-pyridyl]-s-triazine (TPTZ):** TPTZ (31 mg) was weighed and dissolved in 10 mL of 40 mM HCl. The solution was heated up in a water bath (~50 °C) to facilitate the dissolution. This reagent was made fresh on the day of the experiment.

**d) 20 mM ferric chloride (FeCl<sub>3</sub>):** 54 mg of FeCl<sub>3</sub>.6H<sub>2</sub>O (MW: 270.29 g/mol) was weighed and dissolved in 10 mL of deionised water. The reagent was made fresh on the day of the experiment.

**e) FRAP working reagents:** FRAP reagent was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of FeCl<sub>3</sub> to make 75 mL of FRAP reagent. The reagent remained stable for one day.

#### **4.2.4.2 FRAP assay protocol**

The previously described method was followed with some modifications using the prepared reagents (Settharaksa *et al.*, 2014). In a 96-well plate, either Trolox (30 µL), sample (30 µL) or solvent (30 µL) was added to FRAP working reagent (270 µL) and mixed well. The plate was incubated for 30 minutes at room temperature before reading at 593 nm. Background absorbance was corrected by subtracting the absorbance value of blank (water or 70% ethanol). The FRAP activity was expressed as Trolox equivalent (TE) in µM/ mg of extract obtained from the Trolox standard curve. The final Trolox concentrations in the reaction mixture were 100, 50, 25, 12.5 and 6.25 µM (10 µL in 300 µL mixture: 1/10).

#### **4.2.5 Percentage inhibition (%)**

The percentage inhibition (%) of the free radicals in each assay was calculated using the formula below.

$$\text{Percentage inhibition (\%)} = \left(1 - \frac{(S)}{(C)}\right) \times 100 \quad (4.13)$$

where, S is the sample corrected absorbance value, and C is the corrected absorbance value of the control.

#### 4.2.6 Trolox standard for DPPH, ABTS and FRAP assays

**DPPH and ABTS assays:** The Trolox equivalent for DPPH and ABTS assays were either obtained from the regression lines constructed from percentage inhibition (%) vs. Trolox standard concentrations ( $\mu\text{M}$ ). As the concentration of Trolox increases, the percentage inhibition against DPPH<sup>•</sup> or ABTS<sup>•</sup> also increases. So, the regression line from this graph results in a positive slope ( $y = ax$ ).

**FRAP assay:** Trolox standard was obtained from the regression lines of OD 593 nm against concentration. With increasing Trolox concentration, the absorbance value at 593 nm also increases, resulting in a positive slope of the regression line ( $y = ax$ ).

#### 4.2.7 Trolox equivalent $\mu\text{M}/\text{mg}$ extract for each assay

The Trolox equivalent  $\mu\text{M}/\text{mg}$  extracts for each DPPH, ABTS and FRAP assays were calculated as below:

$$x = (y/a) \times DF_1 \times DF_2 \quad (4.14)$$

where  $y$  is the OD value of the sample,  $a$  is the slope of the Trolox regression line,  $DF_1$  is the dilution factor for either DPPH ( $DF=20$ ), ABTS ( $DF=30$ ) and FRAP ( $DF=10$ ) assay and  $DF_2$  is the dilution factor used to achieve the value for 1 mg extracts from the working concentration.

It is essential to make sure that the sample absorbance value is not outside the range of Trolox standard. lies within the Trolox standard regression line. Sample absorption values that lie outside the regression line needs to be diluted, and the Trolox equivalent should be corrected by multiplying with the  $DF_2$ .

## 4.3 Results

### 4.3.1 ABTS assay; Trolox equivalent (TE)

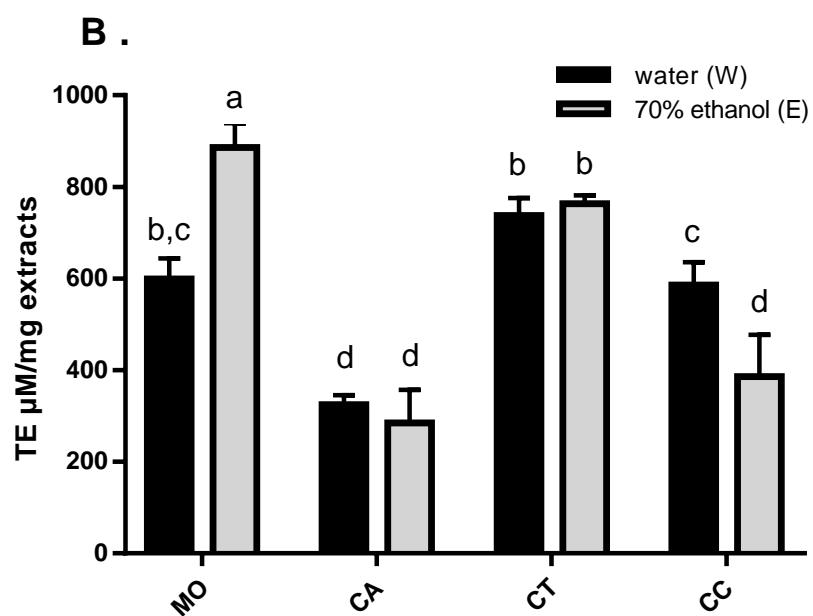
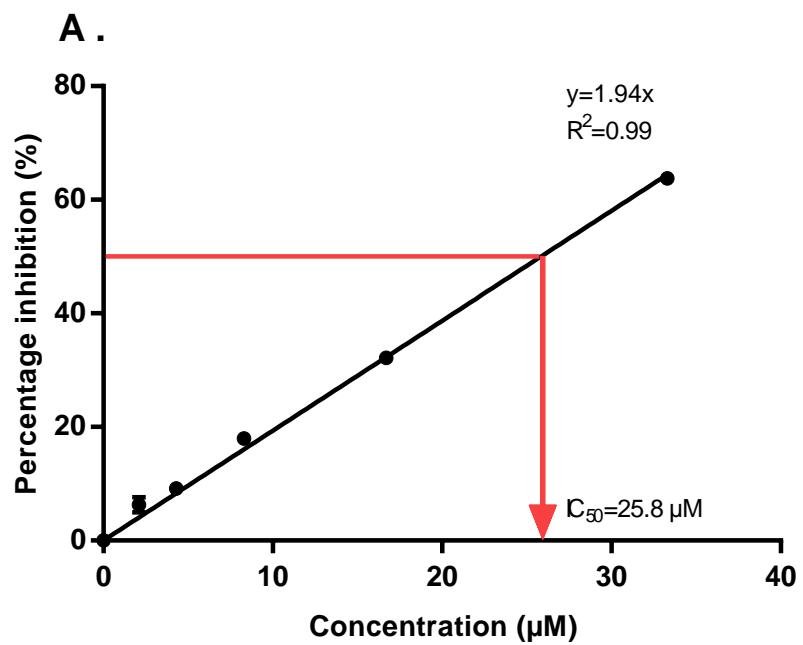
The  $IC_{50}$  of Trolox was 25.8  $\mu\text{M}$ , and the values are Trolox equivalent (TE) in  $\mu\text{M}/\text{mg}$  were as shown in Figure 4.4. MOE had the highest TE, while both *C. asiatica* extracts (CAW and CAE) had the lowest. TE values for all extracts from highest to lowest were MOE (941  $\mu\text{M}/\text{mg}$ ) > CTE (764  $\mu\text{M}/\text{mg}$ ) > CTW (738  $\mu\text{M}/\text{mg}$ ) > MOW (599  $\mu\text{M}/\text{mg}$ ) > CCW (586  $\mu\text{M}/\text{mg}$ ) > CCE (386  $\mu\text{M}/\text{mg}$ ) > CAW (325  $\mu\text{M}/\text{mg}$ ) > CAE (285  $\mu\text{M}/\text{mg}$ ).

### 4.3.2 DPPH assay; Trolox equivalent (TE)

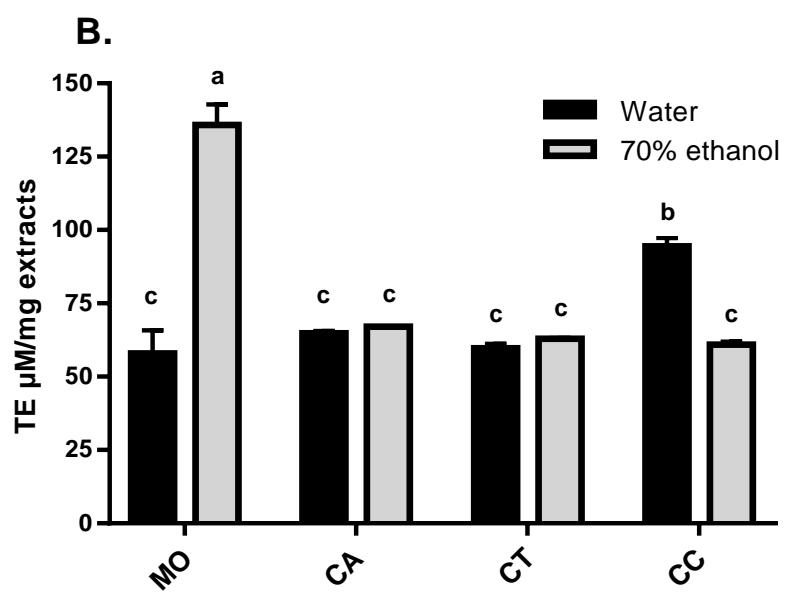
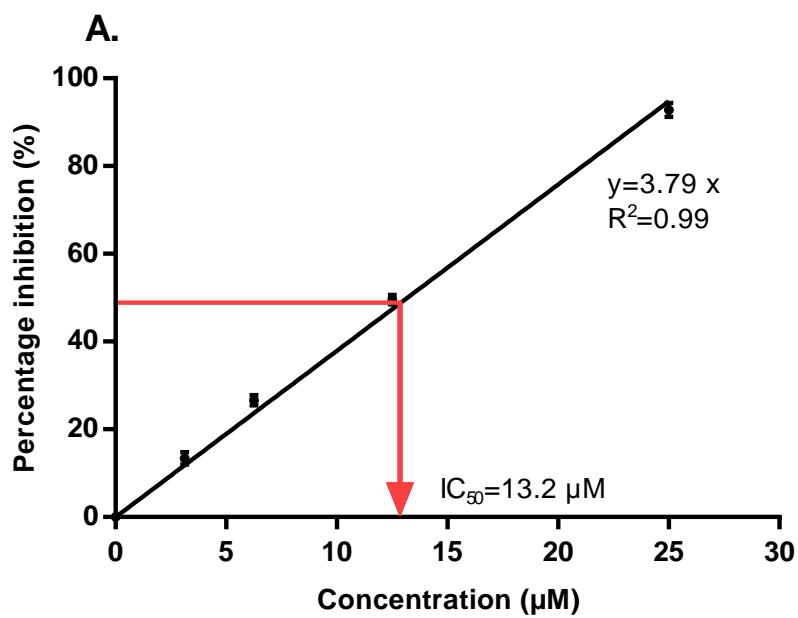
The  $IC_{50}$  for Trolox was 13.2  $\mu\text{M}$ , and the Trolox equivalent (TE) in  $\mu\text{M}/\text{mg}$  extracts for all plant extracts are shown in Figure 4.5. MOE had the highest TE followed by CCW, while other extracts showed no significant mean differences between each other. TE values of all extracts from highest to lowest were MOE (135  $\mu\text{M}/\text{mg}$ ) > CCW (95  $\mu\text{M}/\text{mg}$ ) > CAE (67  $\mu\text{M}/\text{mg}$ ) > CAW (65  $\mu\text{M}/\text{mg}$ ) > CTE (63  $\mu\text{M}/\text{mg}$ ) > CCE (61  $\mu\text{M}$ ) > CTW (60  $\mu\text{M}/\text{mg}$ ) > MOW (58  $\mu\text{M}/\text{mg}$ ).

### 4.3.3 FRAP assay; Trolox equivalent (TE)

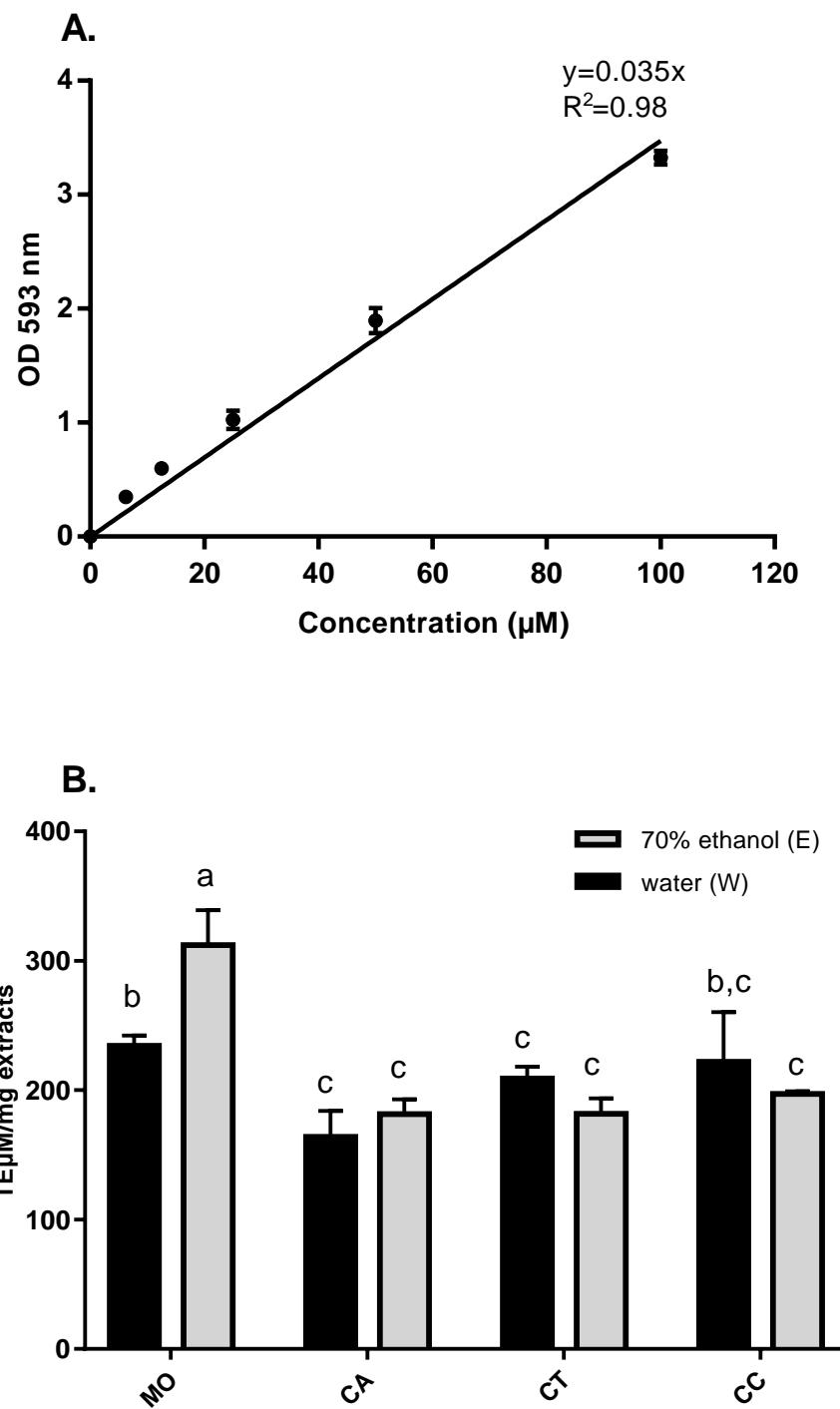
The Trolox standard regression line and the Trolox equivalent (TE) in  $\mu\text{M}/\text{mg}$  extract for all plant extracts are as shown in Figure 4.6. TE values of all extracts from highest to lowest were MOE (312  $\mu\text{M}/\text{mg}$ ) > MOW (234  $\mu\text{M}/\text{mg}$ ) > CCW (222  $\mu\text{M}/\text{mg}$ ) > CTW (209  $\mu\text{M}/\text{mg}$ ) > CCE (197  $\mu\text{M}/\text{mg}$ ) > CTE (182  $\mu\text{M}$ ) > CAE (182  $\mu\text{M}/\text{mg}$ ) > CAW (164  $\mu\text{M}/\text{mg}$ ).



**Figure 4.4: ABTS assay; Trolox regression line (A) and Trolox equivalent (TE) for all extracts (B): ABTS assay.** Different letters indicate significant mean differences with  $p<0.05$  considered as significant. Data are the mean  $\pm$  SD of triplicates.



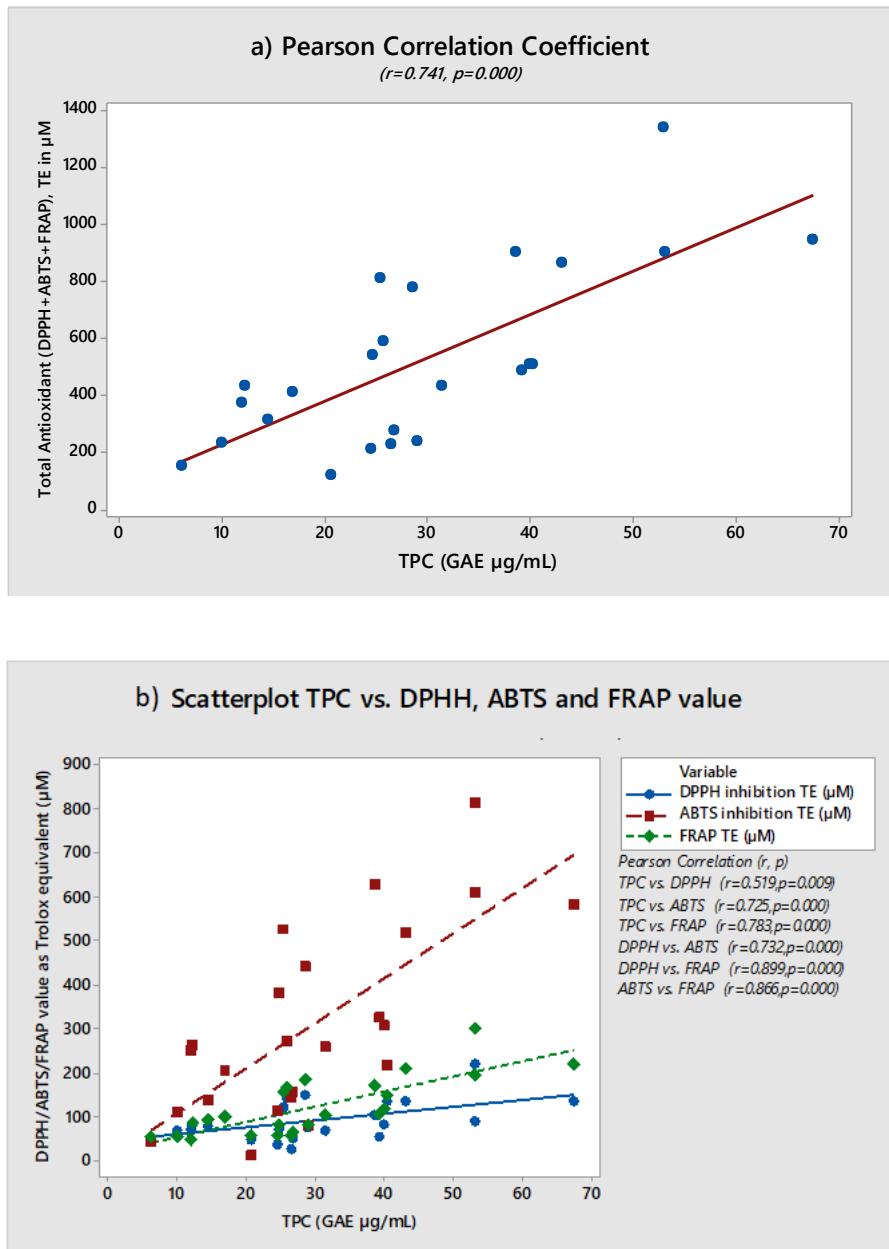
**Figure 4.5: DPPH assay; Trolox regression line (A) and Trolox equivalent for all extracts (B).**  
 Different letters indicate significant mean differences with  $p<0.05$  considered as significant.  
 Data are the mean  $\pm$  SD of triplicates.



**Figure 4.6: The Trolox regression line (A) and Trolox equivalent for all extracts (B).** Different letters indicate significant mean differences with  $p < 0.05$  considered as significant. Data are the mean  $\pm$  SD of triplicates.

#### 4.3.4 Correlation analysis

A strong positive correlation was observed between total antioxidant activity vs. TPC ( $r=0.742$ ), and between antioxidant activity measured using individual antioxidant assays vs. TPC, as shown in Figure 4.7.



**Figure 4.7: Positive correlation between TPC and antioxidant activities.** a) Correlation between TPC and total antioxidant, and b) Correlation between TPC and antioxidant activity measured by each assay. Each data point is the mean of triplicates.

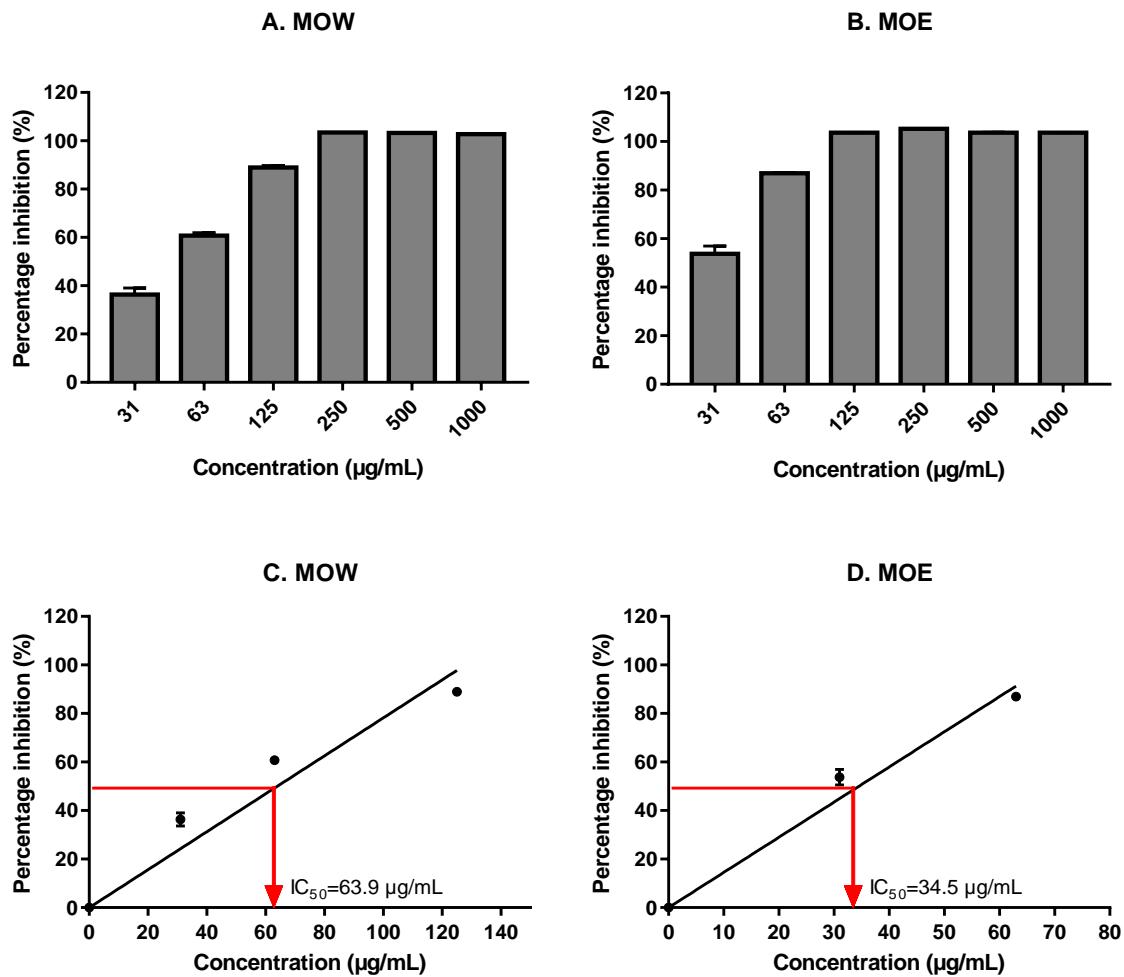
#### 4.3.5 Dose response and IC<sub>50</sub>: ABTS and DPPH assay

##### 4.3.5.1 *Moringa oleifera* Lam.

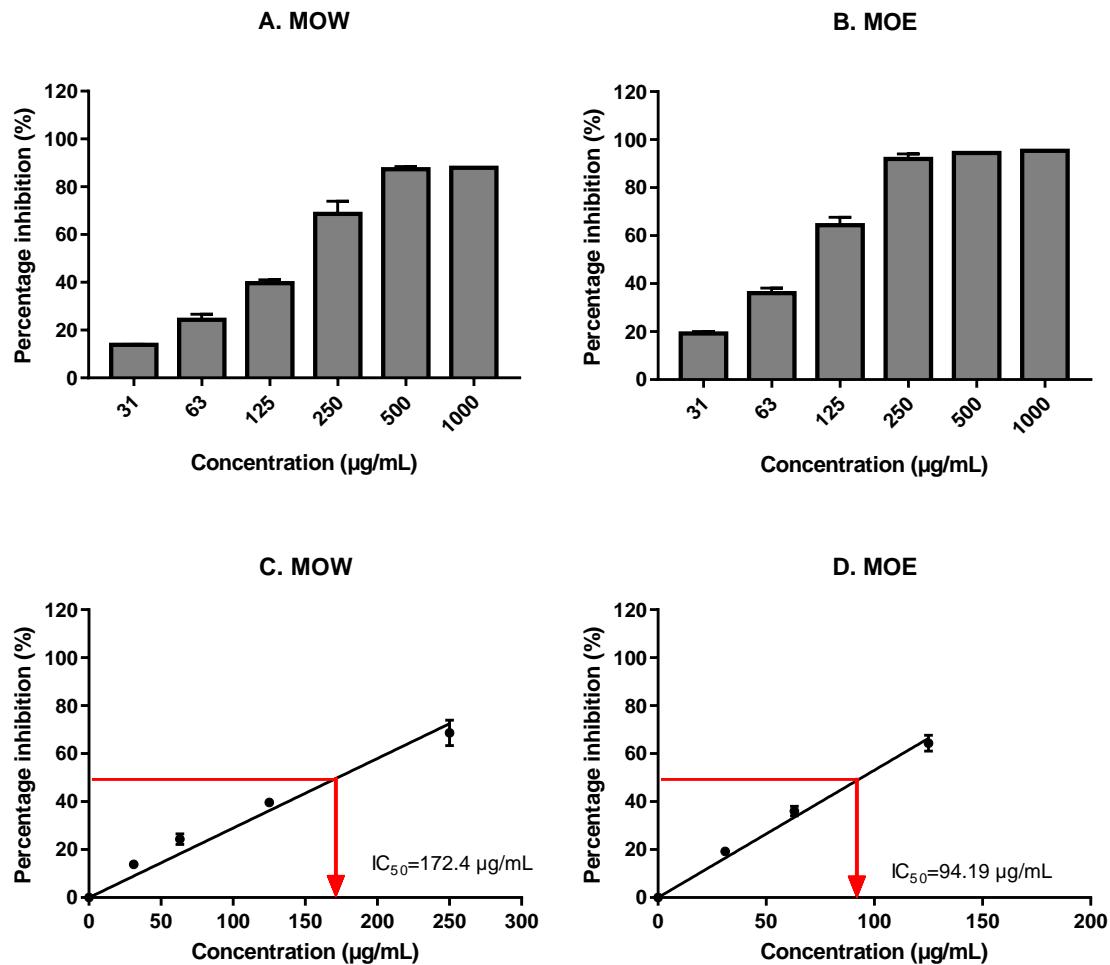
The IC<sub>50</sub> values for MOW and MOE in both assays were as shown in Table 4.2. MOE was more potent than MOW with lower IC<sub>50</sub> values observed in both assays. The free radical scavenging activities of both extracts were concentration-dependent, and the best-fitted regression lines were used to obtain IC<sub>50</sub> values in both assays (see Figures 4.8 and 4.9).

**Table 4.2: IC<sub>50</sub> values for *M. oleifera* extracts.**

Samples	ABTS IC <sub>50</sub> (µg/mL)	DPPH IC <sub>50</sub> (µg/mL)
MOW	63.9	172.4
MOE	34.5	94.2



**Figure 4.8: Dose-response and  $\text{IC}_{50}$  of MOW and MOE in ABTS assay.** A and B show the dose-response, and C and D the regression lines for MOW and MOE. Data represent the mean  $\pm$  SD of triplicates.



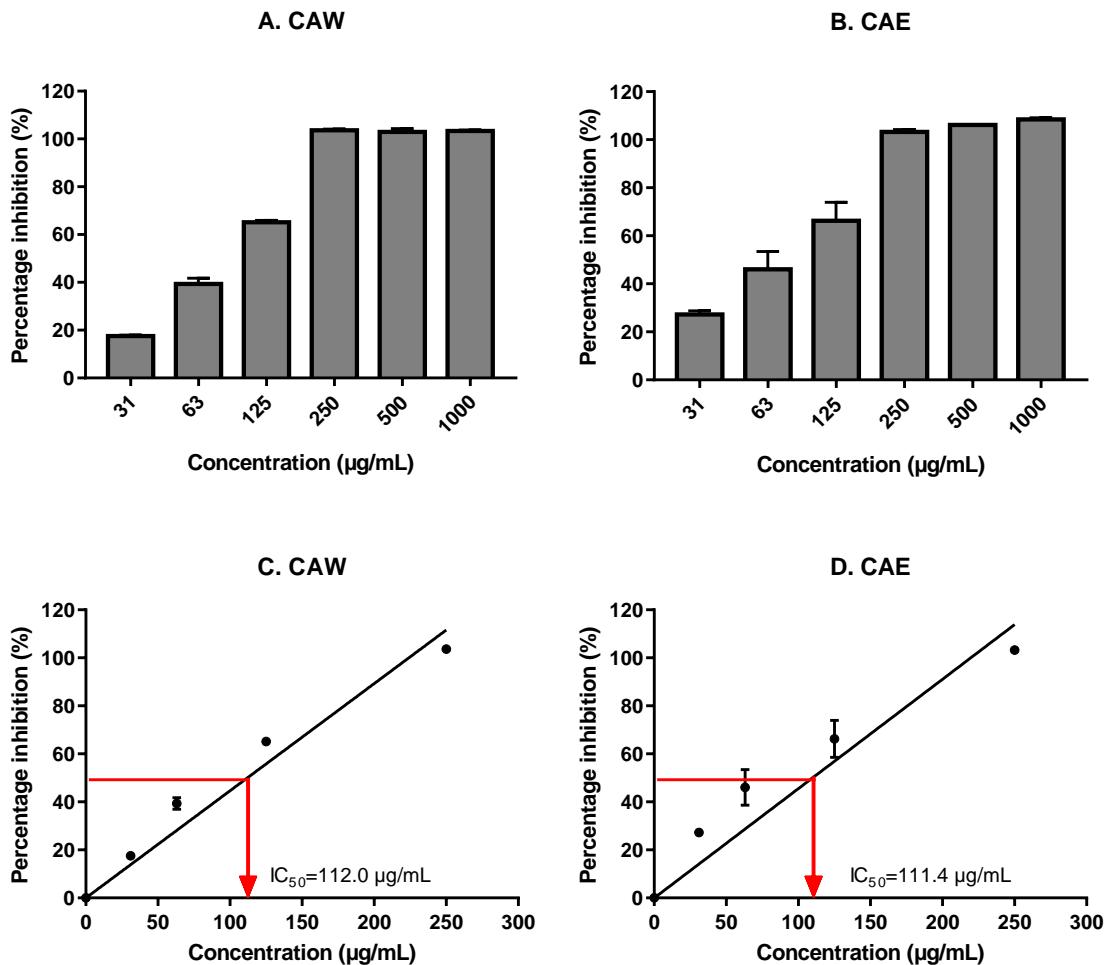
**Figure 4.9: Dose-response and  $\text{IC}_{50}$  of MOW and MOE in DPPH assay.** A and B show the dose-response and C and D the regression lines for MOW and MOE. Data represent mean  $\pm$  SD of triplicates.

#### 4.3.5.2 *Centella asiatica* (L.) Urb.

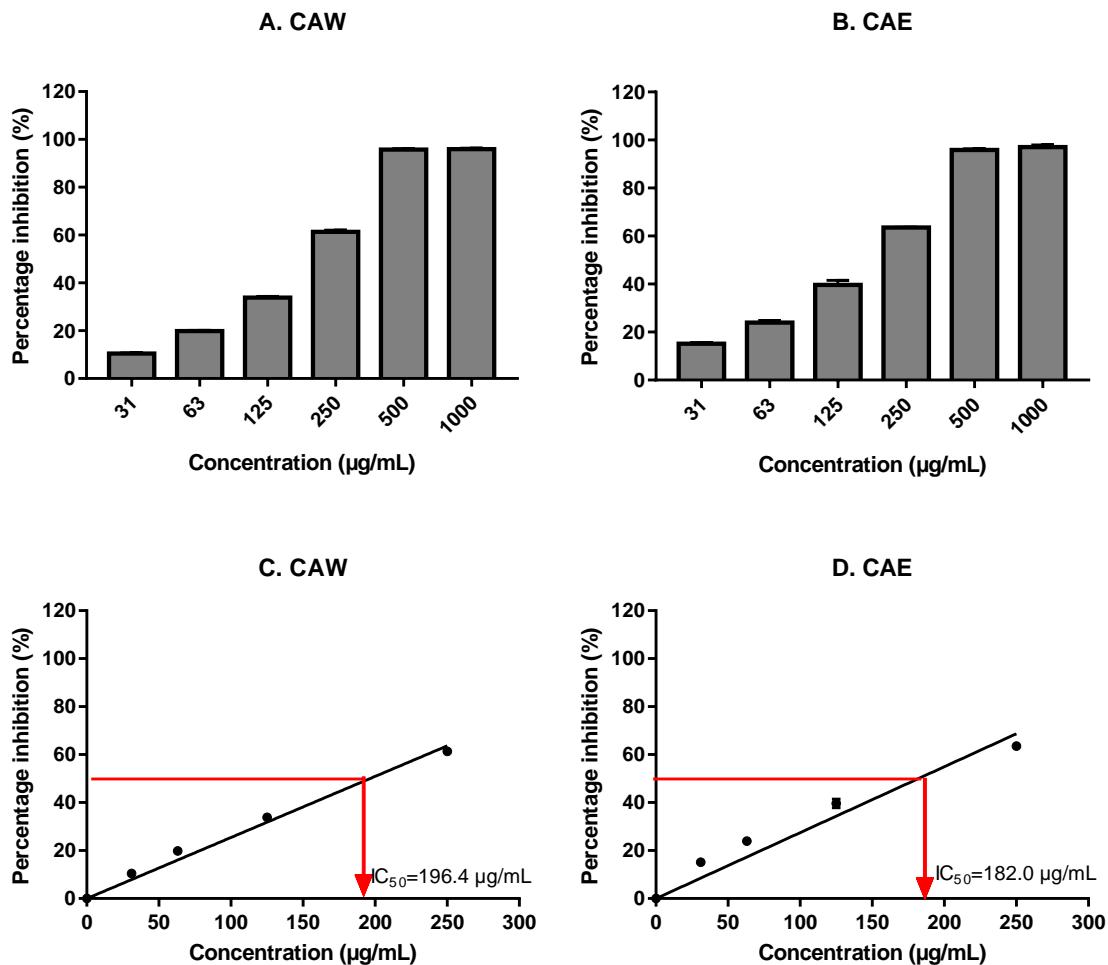
The IC<sub>50</sub> values for CAW and CAE in both assays were as shown in Table 4.3. CAE was more potent than CAW with lower IC<sub>50</sub> values observed in both assays. The free radical scavenging activities for both extracts were concentration-dependent, and the best-fitted regression lines were used to obtain IC<sub>50</sub> values in both assays (see Figures 4.10 and 4.11).

**Table 4.3: IC<sub>50</sub> values for *C. asiatica* (L.) Urb. extracts.**

Samples	ABTS IC <sub>50</sub> (µg/mL)	DPPH IC <sub>50</sub> (µg/mL)
CAW	112.0	196.4
CAE	111.4	182.0



**Figure 4.10: Dose-response and  $\text{IC}_{50}$  of CAW and CAE in ABTS assay.** A and B show the dose-response and C and D the regression lines for CAW and CAE. Data represent mean  $\pm$  SD of triplicates.



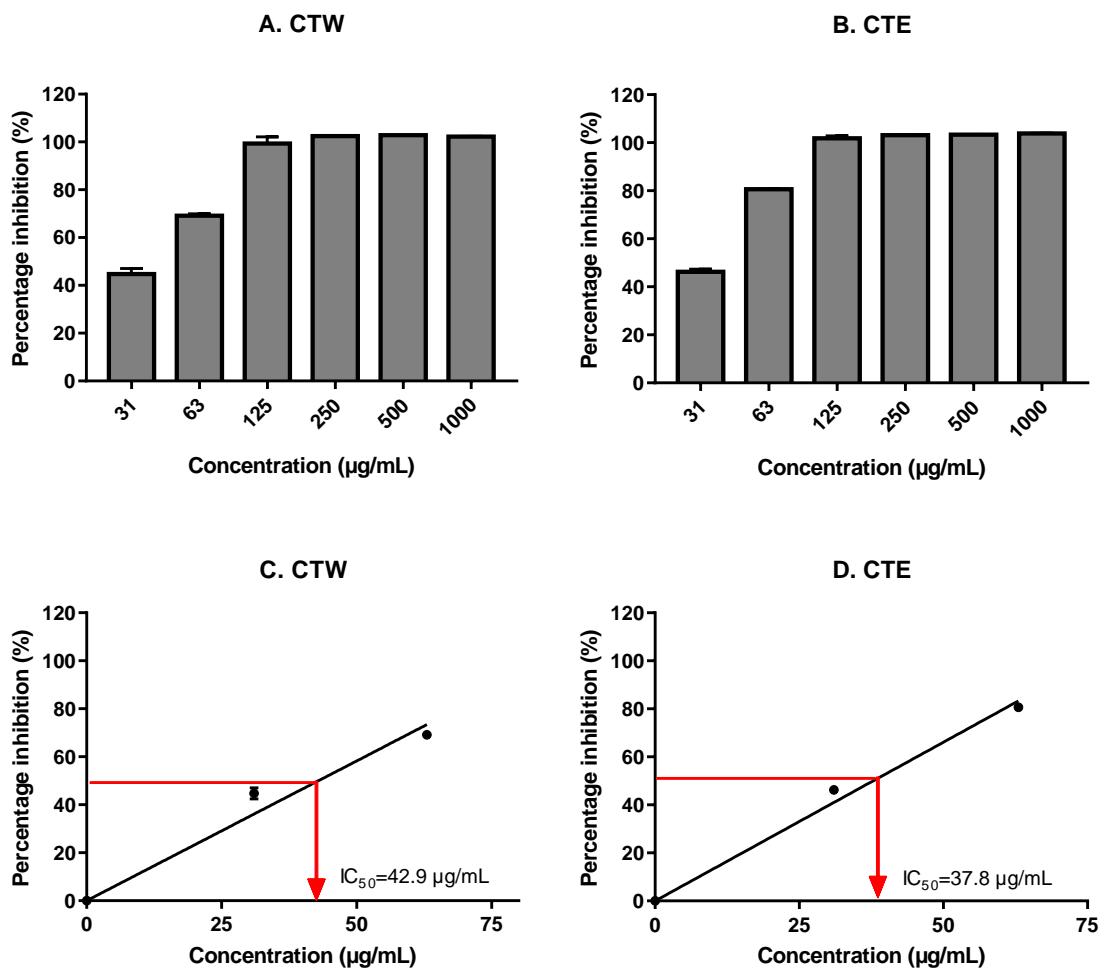
**Figure 4.11: Dose-response and  $\text{IC}_{50}$  of CAW and CAE in DPPH assay.** A and B show the dose-response and C and D the regression lines for CAW and CAE. Data represent mean  $\pm$  SD of triplicates.

#### 4.3.5.3 *Clitoria ternatea* L.

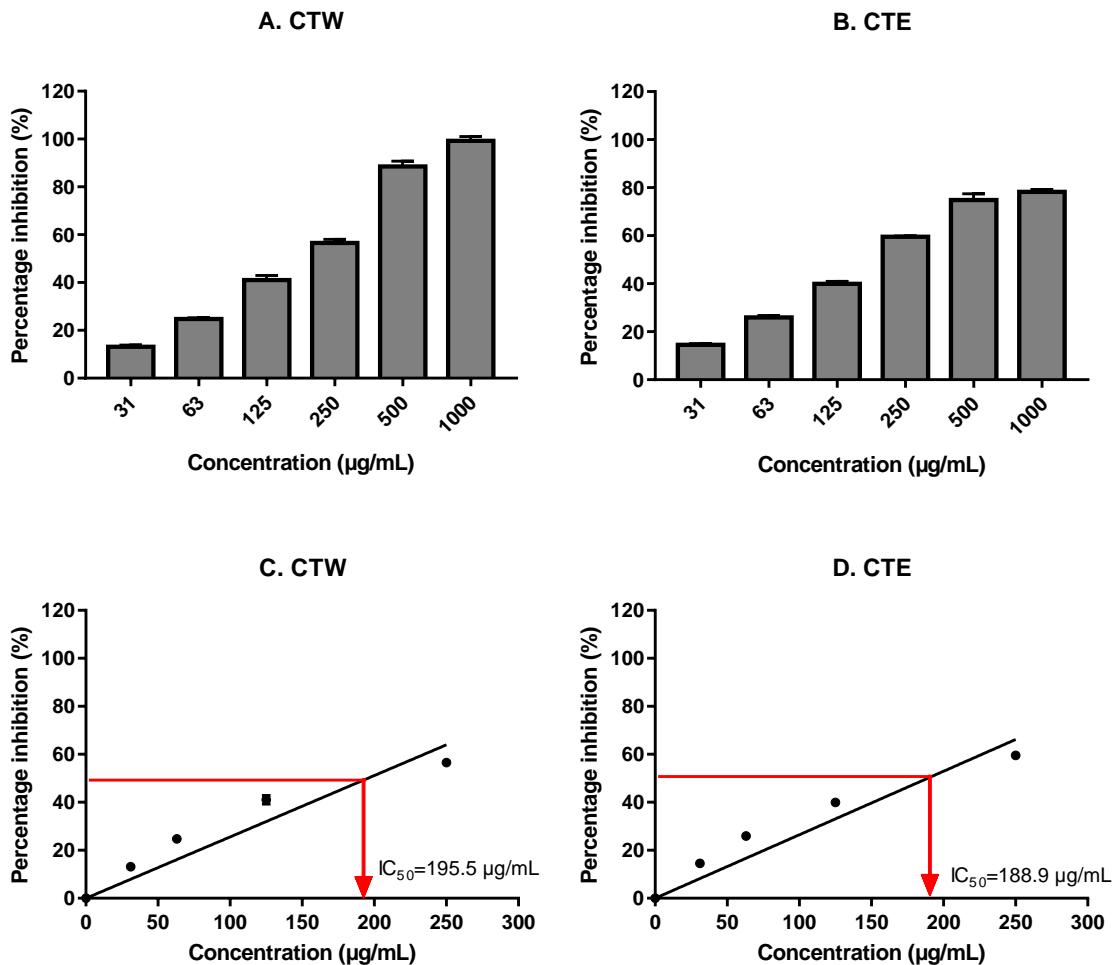
The IC<sub>50</sub> values for CTW and CTE in both assays were as shown in Table 4.4. CTE was more potent than CTW with lower IC<sub>50</sub> values observed in both assays. The free radical scavenging activities for both extracts were concentration-dependent, and the best-fitted regression lines were used to obtain IC<sub>50</sub> values in both assays (see Figures 4.12 and 4.13).

**Table 4.4: IC<sub>50</sub> values for *C. ternatea* L. extracts.**

Samples	ABTS IC <sub>50</sub> (µg/mL)	DPPH IC <sub>50</sub> (µg/mL)
CTW	42.9	195.5
CTE	37.8	188.9



**Figure 4.12: Dose-response and  $\text{IC}_{50}$  of CTW and CTE in ABTS assay.** A and B show the dose-response and C and D the regression lines for CTW and CTE. Data represent mean  $\pm$  SD of triplicates.



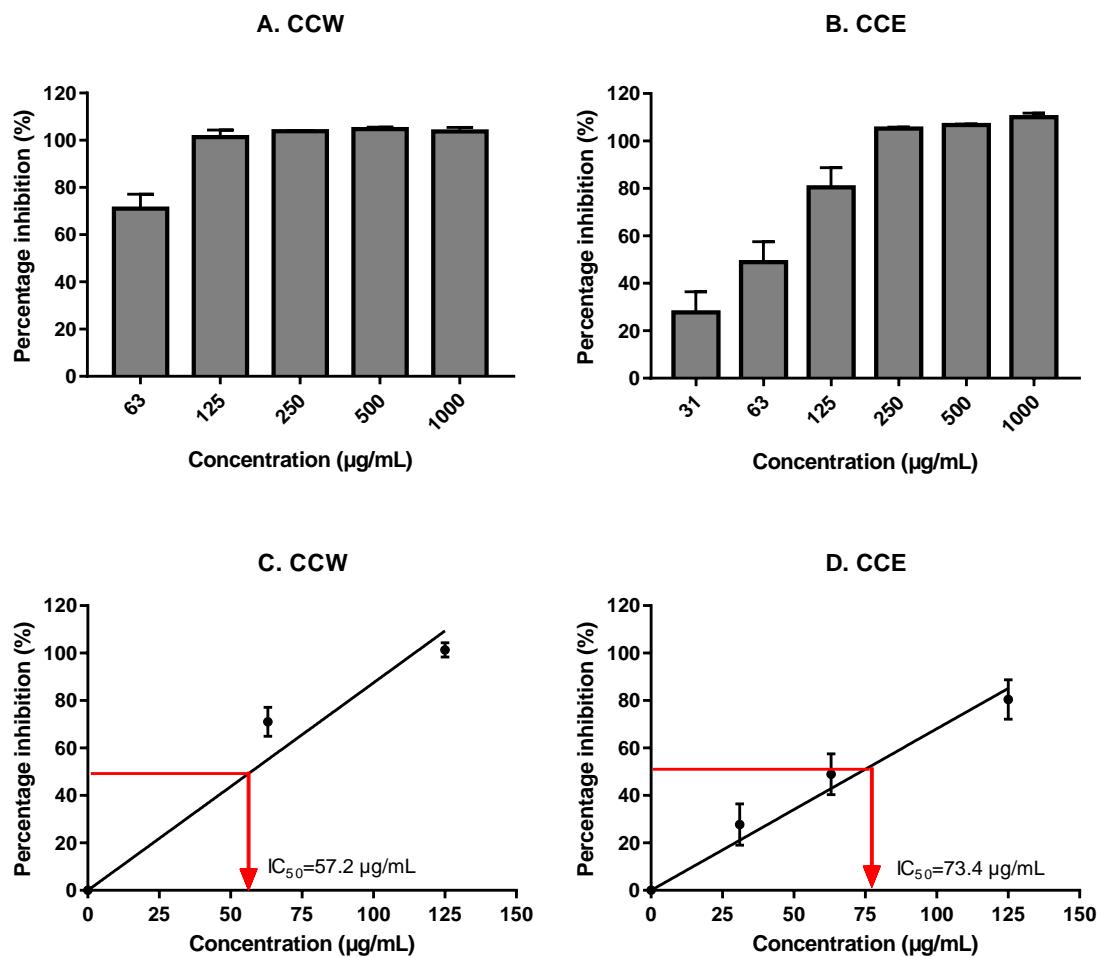
**Figure 4.13: Dose-response and  $\text{IC}_{50}$  of CTW and CTE in DPPH assay.** Figure A and B show the dose-response and C and D the regression lines for CTW and CTE. Data represent mean  $\pm$  SD of triplicates.

#### 4.3.5.4 *Cosmos caudatus* Kunth.

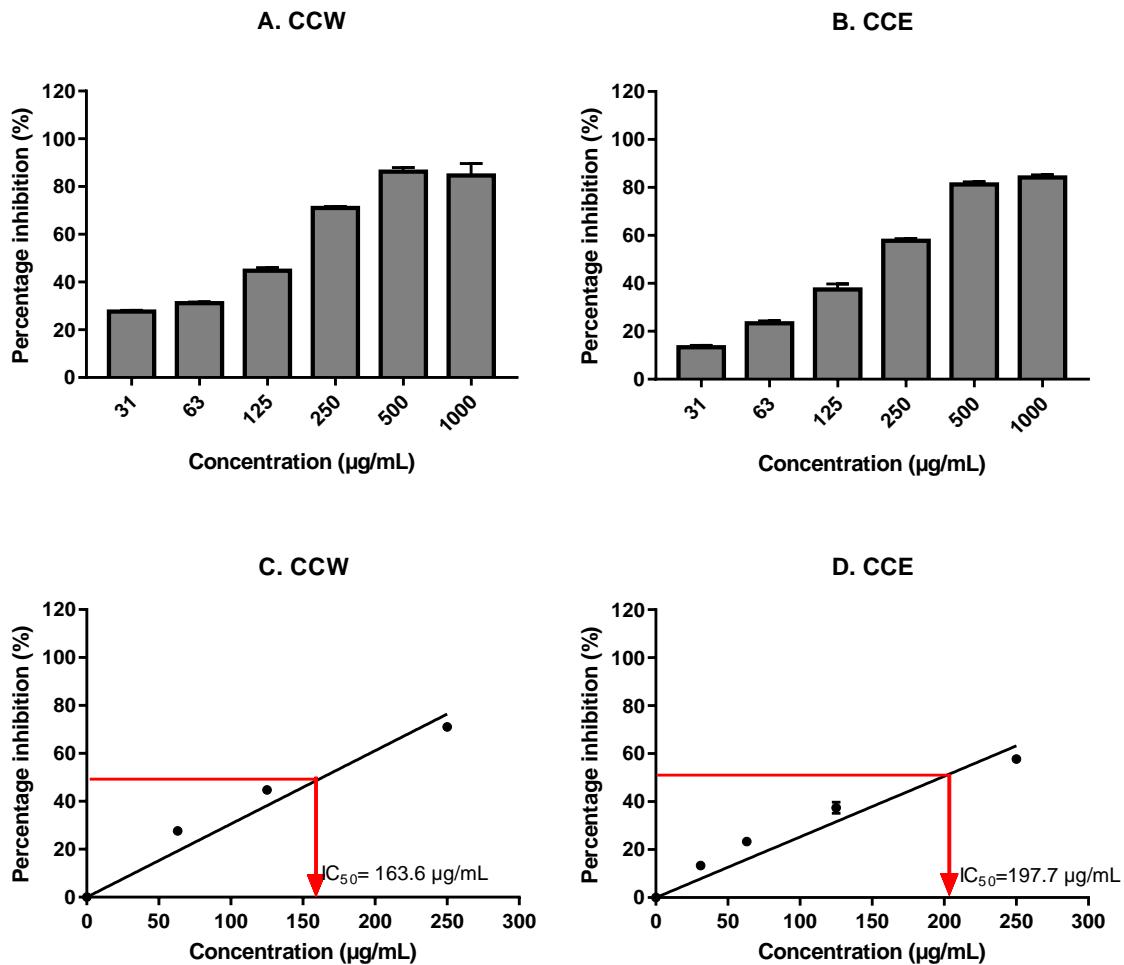
The IC<sub>50</sub> values for CCW and CCE in both assays were as shown in Table 4.5. CCW was more potent than CCE with lower IC<sub>50</sub> values observed in both assays. The free radical scavenging activities for both extracts were concentration-dependent, and the best-fitted regression lines were used to obtain IC<sub>50</sub> values in both assays (see Figures 4.14 and 4.15).

**Table 4.5: IC<sub>50</sub> values for *C. caudatus* Kunth. extracts.**

Samples	ABTS IC <sub>50</sub> (µg/mL)	DPPH IC <sub>50</sub> (µg/mL)
CCW	57.2	163.6
CCE	73.4	197.7



**Figure 4.14: Dose-response and  $\text{IC}_{50}$  of CCW and CCE in ABTS assay.** A and B show the dose-response and C and D the regression lines for CCW and CCE. Data represent mean  $\pm$  SD of triplicates.



**Figure 4.15: Dose-response and  $\text{IC}_{50}$  of CCW and CCE in DPPH assay.** A and B show the dose-response and C and D the regression lines for CCW and CCE. Data represent mean  $\pm$  SD of triplicates.

#### 4.3.6 Summary of antioxidant activity (IC<sub>50</sub> values)

The IC<sub>50</sub> values of all the plant extracts were summarised and compared as shown in Table 4.6.

In comparison to other extracts, MOE showed the highest antioxidant capacities in both ABTS and DPPH assays, as shown by the IC<sub>50</sub> values. However, the observed IC<sub>50</sub> values of all extracts were lower than of Trolox standard, which were as expected due to the difference in purity between Trolox and the extracts.

**Table 4.6: Comparison of IC<sub>50</sub> values of all plant extracts.**

Plant species/Standard	Samples	ABTS IC <sub>50</sub> (µg/mL)	DPPH IC <sub>50</sub> (µg/mL)
Positive control	Trolox	6.51	3.32
<i>M. oleifera</i>	MOW	63.9	172.4
	MOE	34.5	94.2
<i>C. asiatica</i>	CAW	112.0	196.4
	CAE	111.4	182.0
<i>C. ternatea</i>	CTW	42.9	195.5
	CTE	37.8	188.9
<i>C. caudatus</i>	CCW	57.2	163.6
	CCE	73.4	197.7

#### 4.4 Discussion

Epidermal and dermal antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as non-enzymatic antioxidants such as  $\alpha$ -tocopherol, ubiquinol-9, ubiquinone-9, ascorbic acid and dehydroascorbic acid, are significantly decreased after UV irradiation (Shindo *et al.*, 1993). Thus, one of the strategies use to combat skin ageing is the inclusion of active ingredients in cosmetics products to augment the depleted antioxidant defense system.

In this study, plant extracts were evaluated for their antioxidant potential using three different antioxidant assays. Plant extracts contain a mixture of compounds that exert antioxidant activity through different mechanisms, and thus it is necessary to make comparisons of the activities exhibited in each assay to ensure consistency (Moon and Shibamoto, 2009). A correlation analysis performed between TE vs. TPC (see Figure 4.7) showed a positive correlation. Similar correlations have also been shown in several other studies, suggesting that phenolics may have been responsible for the activity (Li *et al.*, 2009; Mustafa *et al.*, 2010; Piluzza and Bullitta, 2011; Mello and Quadros, 2014).

In all of the DPPH, ABTS and FRAP assays, MOE exhibited the highest levels of antioxidant activity, while the other plant extracts did showed variation between assays. Factors such as variability in the phytochemical components in the extracts may explain these results. Although the antioxidant activities of *M. oleifera* have previously been reported (Sreelatha and Padma, 2009; Vongsak, Sithisarn, Mangmool, *et al.*, 2013), a comparison between the plants selected in this study has not been reported, and there is variability in the results of other studies due to the assays and samples used. *M. oleifera* 70% ethanol was investigated by Vongsak *et al.*, (2013) and was also shown to exhibit the highest antioxidant activities in comparison to water extracts. The active antioxidant compounds in *M. oleifera* leaves such as

crypto-chlorogenic acid, isoquercetin and astragalin may have contributed to the demonstrated antioxidant activities (Vongsak *et al.*, 2014).

Both *C. asiatica* extracts (CAE and CAW) exhibited the lowest antioxidant capacities in comparison with all other extracts. In Chapter 3, it was shown that the TPC values of both extracts were also the lowest, which may have contributed to the lower antioxidant activity. Several studies comparing the effect of extraction solvents have demonstrated that *C. asiatica* extracted using a co-solvent system of water-ethanol exhibited higher antioxidant activity and TPC in comparison with 100% ethanol and water extracts (Hamid *et al.*, 2002; Rahman *et al.*, 2013), which is in agreement with the results in this study. A dose-dependent effect was observed in both assays for CAW and CAE, which suggests that, despite the lower antioxidant activities, there are compounds in the extracts that possess antioxidant potential (Singh *et al.*, 2014).

There are at least 60 species of the genus *Clitoria*, but the blue-flowered *C. ternatea* contains high levels of anthocyanins, which are excellent antioxidants (Sivaprabha *et al.*, 2008; Nair *et al.*, 2015). In Chapter 3, the values of TAC of *C. ternatea* extracts (VTW vs. CTE) were found to not significantly differ. However, measurement of their antioxidant activity showed that CTE had higher antioxidant levels compared with CTW. This result may be explained by the presence of quercetin in CTE, whilst in CTW this compound was not present or was below the detection limit (see Table 3.5). However, these results contradict those of Kamkaen and Wilkinson (2009) where the aqueous extract of *C. ternatea* had higher antioxidant capacity than that of the ethanol extract. In this study, a co-solvent system of 70% ethanol was used instead of pure ethanol, which has a higher polarity and is a better solvent for polar compounds, thus perhaps explaining the contradictory result (Vongsak, Sithisarn, Mangmool, *et al.*, 2013).

In comparisons with the other plant extracts, CCW had significantly higher antioxidant activities than CCE, despite the higher TPC observed in CCE (see Chapter 3). Studies of this plant species are very limited in number but several which have evaluated the antioxidant potential suggest that they are good source of antioxidants (Shui *et al.*, 2005; Mustafa *et al.*, 2010; Ahmad; Mediani *et al.*, 2012; Hassan *et al.*, 2012). A study by Mustafa *et al.*, (2010) comparing the antioxidant activity of 21 selected tropical plants showed that *C. caudatus* had the highest IC<sub>50</sub> value, at levels statistically comparable to those of a synthetic antioxidant. Several other studies comparing Malay vegetables or “ulam” also showed *C. caudatus* to rank among those with the highest antioxidant levels (Abas *et al.*, 2006; Reihani and Azhar, 2012; Chan *et al.*, 2014).

The results in this chapter suggest that all of the prepared plant extracts can scavenge free radicals and are able to reduce metal ions through reduction-oxidation (redox) mechanisms (López-Alarcón and Denicola, 2013). These properties are important in modulating the protective antioxidant effect in the skin by inhibiting the initiation of free radicals (a reaction chain breaker) or by interrupting the Fenton reaction (a metal ion chelator) (Gorinstein *et al.*, 2013).

## 4.5 Conclusion

The levels of antioxidant activity of the plant extracts are positively correlated with the total phenolic content, suggesting that phenolics are responsible for the antioxidant activities exhibited. In this study, all of the ethanolic extracts of the plant species were shown to have better antioxidant potential, except for *C. caudatus*. Although their potency differs, the antioxidant activities demonstrated suggest that all of the prepared plant extracts can scavenge free radicals, which may be useful in the management of skin ageing since they are potential sources of natural antioxidants.



# **Chapter 5**



## **Chapter 5: Protective effects against extracellular matrix enzymes: anti-collagenase, anti-elastase and anti-tyrosinase activities of the plant extracts**

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### **5.1 Introduction**

The enzymes involved in the degradation of extracellular matrix (ECM) and melanogenesis and are more specific targets compared with molecular approaches. Thus, the inclusion of inhibitors for these enzymes as active ingredients in cosmetics formulations is preferred in the cosmetics industry. Studies to explore novel natural inhibitors for these enzymes are increasing (Lee *et al.*, 2001; Thring *et al.*, 2009; Moon *et al.*, 2010; Kacem, 2013; Chattuwatthana and Okello, 2015), and commonly used assays for screening and evaluation are discussed below.

#### **5.1.1 Anti-collagenase assay**

One of the most effective types of therapy to improve the structure of type-1 collagen in the ECM is by inhibiting collagenase activity (Mukherjee *et al.*, 2012). Collagenase acts by cleaving the X-gly bond of collagen. It also has the ability to cleave similar bonds in synthetic peptide that contains the sequence of –Pro-X-Gly-Pro, where X could be almost any amino acid, provided that the amino terminus is blocked (Van Wart and Steinbrink, 1981). Collagenase from the bacteria *Clostridium histolyticum* is commonly used in the assessment of an anti-collagenase agent, since the bacterial collagenase degrades collagen in both physiological and *in vitro* conditions (Thring *et al.*, 2009; Roy *et al.*, 2013). The substrates used with the enzyme are either a fluorescence-labelled-collagen or a synthetic peptide such as N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala (FALPGA) (Van Wart and Steinbrink, 1981; MolecularProbes, 2001; Thring *et al.*, 2009). In the fluorescence assay, the labelled substrate is digested in the presence of collagenase, and the labelled fragments fluoresce at 485 ± 10 nm excitation and

530 ± 10 nm emission with Ex/Em<sup>5</sup> optimal at 495/515 nm. Lower fluorescent readings are observed in the presence of an inhibitor (MolecularProbes, 2001). Meanwhile, a collagenase assay using FALPGA shows maximum absorption at 345 nm (Van Wart and Steinbrink, 1981), with lower optical density suggesting higher collagenase activity on FALPGA (BioVision, 2016).

### 5.1.2 Anti-elastase assay

Elastin is one of the ECM proteins that provides elasticity to the connective tissues, and any damage to elastin leads to declining skin resilience (Robert *et al.*, 1984; Mecham *et al.*, 1997; Daamen *et al.*, 2007). Elastases, which are enzymes that are involved in the breakdown of elastin, have also been shown to increase with ageing and after UV irradiation and further cause reduction in skin resilience, thus also needing to be taken into account in skin aging management (see Chapter 2: 2.10.3.2). There are two commonly used assays to evaluate inhibitors for this enzyme, where a commercially available porcine elastase is used with either a fluorescent-labelled substrate or a synthetic peptide called *N*-Succ-(Ala)-3-nitroanilide (SANA). In the fluorescent assay, the digested substrates release fluorescent fragments that fluoresce at 485 ± 10 nm excitation and 530 ± 10 nm emission with Ex/Em optimal at 495/515 nm which correlates with elastase activity (MolecularProbe, 2001). Similarly, the release of *p*-nitroaniline from the digested SANA changes the reaction mixture from colourless to yellow, which is measurable at 410-495 nm using a spectrophotometer (Lee and Choi, 1999).

### 5.1.3 Anti-tyrosinase assay

An overproduction of skin melanin that causes facial hyperpigmentation is aesthetically undesirable. Another target enzyme in the formulation of cosmetics products to manage this problem is the tyrosinase, which catalyses the biosynthesis of melanin (see Chapter 2: 2.10.3.3). A commercially available mushroom tyrosinase is usually used in the anti-tyrosinase

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<sup>5</sup> Ex/Em = Excitation/Emission

assay in the evaluation of potential tyrosinase inhibitors (Chang *et al.*, 2007; Khan, 2007; Vardhan *et al.*, 2014; Zeitoun *et al.*, 2016). This assay uses either L-DOPA or L-tyrosine as a substrate, in which the L-DOPA is oxidized into L-DOPA-quinone and water by tyrosinase, whilst the L-tyrosine is oxidized into L-DOPA, and then the same reaction proceeds. The intensity of L-DOPA-quinone is measurable at 475-495 nm using a spectrophotometer.

#### **5.1.4 Chapter objectives**

This chapter aims to investigate potential applications of the plant extracts as anti-wrinkles and anti-hyperpigmentation agents. The objectives of this chapter are further elaborated below:

- ✓ To investigate the anti-collagenase activity of the prepared plant extracts
- ✓ To investigate the anti-elastase activity of the prepared plant extracts
- ✓ To investigate the anti-tyrosinase activity of the prepared plant extracts

## 5.2 Materials and methods

### 5.2.1 Anti-collagenase assay

#### 5.2.1.1 Materials and reagent preparation

An EnzChek Gelatinase/Collagenase Assay kit was purchased from ThermoFisher Scientific, UK (Code: E-12055; Lot#:1717422). The kit was supplied with 1 vial of lyophilized collagenase purified from *C. histolyticum* (500 units/vial), 1 vial of 1-10 phenantroline, monohydrate (MW: 198.22, 30 mg/vial), 5 vials of DQ gelatine from pig skin conjugated with fluorescein (1 mg/vial) and 50 mL of 10 X reaction buffer (0.5 M Tris-HCl, 1.5 M NaCl, 50-mM CaCl<sub>2</sub> and 2 mM sodium azide, pH 7.6). The kit was stored in -20 °C upon receipt. Reagents were prepared according to the protocols from the manufacturer with modifications as described in Table 5.1.

**Table 5.1: Reagents preparation for collagenase fluorescence assay**

No.	Reagent	Protocol
1.	1 mg/mL DQ gelatine stock solution	1 mL of deionised water was added directly to one of five vials. The mixture was agitated and sonicated in a water bath at 50 °C for 5 minutes to facilitate dissolution.  <b>Working concentrations (200 µg/mL):</b> The stock solution was added to 4.0 mL of the 1x reaction buffer to obtain 5 mL of 200 µg/mL working solution. The working solution was stored at 4 °C in the dark. Freezing and thawing were avoided as background fluorescence increases.
2.	1x reaction buffer	20 mL of the 10x reaction buffer was diluted in 180 mL of deionised water. The 200-mL 1X reaction buffer was enough for all assays.
3.	500 units/mL collagenase stock	The content of the collagenase vial was added to 1.0 mL deionised water. The reconstituted collagenase was stored in small vials at -20 °C until needed. The frozen enzyme was stable for up to 6 months without any loss of activity.  <b>Working concentration (0.8 units/mL):</b> 80 µL of collagenase stock was made up to a total volume of 50 mL using the 1X reaction buffer to obtain 0.8 units/mL of collagenase working solution.

No.	Reagent	Protocol
4.	1, 10-phenanthroline inhibitor (1,10-PNT)	1 mL of ethanol was added to the 1, 10-PNT vial. Later, the solution was made up to 15 mL using the 1x reaction buffer to obtain 10 mM 1, 10-phenanthroline inhibitor. <b>Working concentrations:</b> 400 µL of the 10 mM 1, 10-PNT inhibitor was added to 1.6 mL of 1X reaction buffer to obtain 2 mM 1, 10-PNT. Later, the 2 mM was serially diluted to obtain 0.5, 0.25, 0.125 and 0.0625 mM solutions. The final 1, 10-PNT concentrations were 0.5, 0.25, 0.125, 0.0625 and 0.0325 mM respectively in 200 µL reaction mixture.
5.	Plant extract preparation	Plants extracts were prepared in either water or 70% ethanol at 20 mg/mL as stock and stored at -20 °C. The working concentrations were prepared appropriately depending on the required final concentrations.

### 5.2.1.2 Collagenase assay protocol

The protocol was followed according to the manufacturer's suggestions with some modifications. In a 96 well-plate, 50 µL of collagenase (0.8 units/mL) was added to 50 µL samples or inhibitor and allowed to react for 15 minutes at 37 °C. After pre-incubation, 100 µL of substrate was added to the reaction well. The plate was further incubated for 120 minutes at 37 °C and fluorescence was measured every 15 minutes at Ex/Em of 485 ± 10/530 ± 15 nm emission, where interval measurements were obtained to ensure the enzyme was active. Control and treatments were compared at t=60 min, expressed as percentage inhibition compared to the control (see section 5.2.5).

**Table 5.2: Volume of reagent pipetted into each well in collagenase fluorescence assay.** The final concentrations of collagenase and DQ-gelatine in the wells were 0.2 units/mL and 100 µg/mL.

	Control (C)	Samples/standard inhibitor (µL)	Blank (µL)
Collagenase, 0.8 units/mL	50	50	0

Treatment(s)	50 (water)	50 (samples/inhibitor)	50 (solvent)
1 X Working buffer	0	0	50
Substrate, 400 µg/mL	100	100	100
<b>Total volume (µL)</b>	<b>200</b>	<b>200</b>	<b>200</b>

## 5.2.2 Anti-elastase assay

### 5.2.2.1 Materials and reagent preparation: colormetric assay

Elastase type-1 from porcine pancreas (E1250-25mg; Lot#: SLBN4280V, SucAla<sub>3</sub>-pNA (SANA) (Sigma: S4760-25 mg; Lot #: SLBM 7561V), Trizma base (T1503-250 mg; Lot #: SLBM7135V), and EGCG (E4268-100mg) were purchased from Sigma Aldrich, UK. Reagents were prepared as suggested by the manufacturer as described in Table 5.3.

**Table 5.3: Reagents preparation for anti-elastase colorimetric assay.**

Reagent	Protocol
Working buffer, 100 mM Tris-HCl buffer	12.1 g of Trizma-base (MW: 121.14 g/mol) powder was weighed and added to 1 L of water. The buffer was mixed until fully dissolved and the pH was adjusted to 8.0 at 25 °C with 1M of HCl. The buffer was stored at room temperature until required.
Substrate, 4.4 mM SANA	20 mg of SANA (MW: 451.43 g/mol) was weighed and dissolved in 10 mL of working buffer at with increasing temperature (~30 °C) and frequent stirred for 30 minutes to completely dissolve the SANA. The substrate solution was aliquoted in 5 small vials containing 2 mL each. The vials were stored in the fridge at 2-8 °C until required or at -20 °C for longer storage.
Elastase working solution, 0.34 unit/mL	100 µL of the elastase solution (*68 units/mL) was diluted in 19.9 mL of cold buffer (2-8 °C) to obtain 0.34 units/mL working elastase. The working solution was aliquoted into 10 small vials containing 2 mL each. The elastase vials were stored in the fridge at 2-8 °C until required.  *Data was obtained from Supplier's Certificate of Analysis (CAS) for the product.

EGCG working solution, 1.0 mg/mL	5.0 mg of EGCG (MW: 458.37) was weighed and dissolved in 5 mL of either water to obtain 1.0 mg/mL of EGCG. The solution was aliquoted into small vials and stored at -20 °C until required.
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#### **5.2.2.2 Pre-experiments investigating effect of enzyme concentrations and solvent**

**Enzyme concentration:** Elastase working solutions of varying concentrations were prepared appropriately from the enzyme stock to achieve final enzyme concentrations of 0.01, 0.02, 0.1 and 0.2 units/mL in assay wells. In a 96-well plate, a volume of 10 µL from each of the elastase working solutions was added to 4 mM SANA (20 µL), and the reaction volume was made up to final a reaction mixture of 300 µL with the working buffer. Enzyme activity was measured every 10 minutes for 60 minutes at 410 nm wavelength using a spectrophotometer. Enzyme activity was plotted against time.

**Solvent:** Water (10 µL) or 70% ethanol (10 µL) was allowed to react with 0.3 unit/mL elastase (selected based on previous assay) for 15 minutes. After the preincubation, 4 mM SANA (20 µL) was added and the reaction volume was made up to 300 µL with the reaction buffer. The absorbance reading was measured at 410 nm using a spectrophotometer and percentage activity of treated elastase at minute 60 was compared to control, untreated or buffer (see section 5.2.5).

#### **5.2.2.3 Anti-elastase colormetric protocol**

In a 96-well plate, elastase (10 µL) was preincubated with treatments (20 µL) for 15 minutes. Later, reaction buffer (250 µL) and 4 mM SANA were added to make a total volume of 300 µL reaction mixture. The plate was further incubated for 60 minutes and an absorbance reading was measured at 410 nm. Blank was subtracted from the readings and percentage inhibition compared to control was calculated (see section 5.2.5).

**Table 5.4: Volume for each reagent in elastase colorimetric assay.** The final concentration for elastase and SANA in the wells were 0.1 units/mL and 0.29 mM SANA.

Working reagent	Control ( $\mu$ L)	Samples/standard inhibitor ( $\mu$ L)	Blank ( $\mu$ L)
Elastase, 0.34 units/mL	10	10	0
Treatment(s)	20	20	20
100-mM Tris-HCl buffer	250	250	260
Substrate, 4.4 mM SANA	20	20	20
<b>Total volume (<math>\mu</math>L)</b>	<b>300</b>	<b>300</b>	<b>300</b>

#### 5.2.2.4 Materials and reagent preparation: fluorometric assay

An EnzChek® Elastase Assay Kit (E-12056) containing three vials of 1 mg DQ elastin from bovine neck ligament-conjugated with fluorophore, 30 mL of 10X reaction buffer (1 M Tris-HCl with 2 mM sodium azide, pH 8.0), 1 vial of elastase from pig pancreas and 1 vial of 500  $\mu$ g *N*-Methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl (MAAPV) ketone was purchased from ThermoFisher Scientific, UK. Reagents were prepared as suggested by the manufacturer as described in Table 5.5.

**Table 5.5: Reagent preparation for anti-elastase fluorescence assay.**

Reagent	Protocol
DQ elastin substrate (1mg/1mL)	1 mL of water was pipetted into the vial of 1 mg of DQ elastin substrate and mixed thoroughly to dissolve. Reconstituted DQ elastin (1mg/mL) was stored in aliquots at 4-8 °C until use or at -20° C for longer storage. Repeated freezing and thawing was avoided as it can increase background fluorescence readings.
1X reaction buffer	18 mL of the 10X reaction buffer was diluted with 162 mL of water to obtain 180 mL of 1X reaction buffer. The buffer was enough for the reactions of six 96-well plates with sufficient excess to prepare dilutions and working concentrations.
DQ elastin substrate working solution (100 µg/mL)	The stock substrate (1 mg/mL) was diluted by adding 9 mL of 1X reaction buffer to obtain 10 mL of 100 g/mL DQ elastin.
Elastase stock (100 units/mL)	500 µL was pipetted into the enzyme vial to obtain 100 units/mL enzymes. The enzyme was stored in aliquots at -20°C for longer storage up to 6 months.
Elastase working solution (0.5 units/mL)	100 µL of the elastase stock was diluted with 19.9 mL of 1X reaction buffer to obtain 20 mL of elastase working solution. This working solution was enough for 200 reactions.

#### **5.2.2.5 Anti-elastase fluorescence assay protocol**

This assay was performed according to the suggestions by the manufacturer with some modifications (MolecularProbe 2001). Porcine pancreatic elastase (100 µL) was allowed to react with each plant samples (50 µL) in a 96-well plate in the dark at 25 °C for 15 minutes. After the preincubation, the elastin working solution (50 µL) was added to each well to make a final reaction volume of 200 µL, and the microplate was further incubated in the dark at 25 °C for 120 minutes. Fluorescence were measured every 15 minutes at Ex/Em of 485 ± 10/530 ± 15 nm emission to ensure the enzyme was active. Controls and treatments were compared at t=60 min, expressed as percentage inhibition compared to control (see section 5.2.5).

**Table 5.6: Volume of reagents pipetted into each well for fluorescence assay.** The final concentration for elastase and substrate were 0.25 units/mL and 0.25 µg/mL respectively.

Working reagent	Control (µL)	Samples/standard inhibitor (µL)	Blank (µL)
Elastase, 0.5 units/mL	100	100	0
Treatment(s) or positive controls (MAAPV and EGCG)	50 (water)	50 (samples/inhibitor)	100 (1:1 of buffer: water)
Substrate, 100 µg/mL	50	50	50
<b>Total volume (µL)</b>	<b>200</b>	<b>200</b>	<b>200</b>

### 5.2.3 Anti-tyrosinase assay

#### 5.2.3.1 Materials and reagent preparation

Tyrosinase (T3824-25 KU; 5771 units/mg solid), L-tyrosine were purchased from Sigma Aldrich, UK. Reagents were prepared as described in Table 5.7.

**Table 5.7: Reagent preparation for tyrosinase assay pre-experiment.**

Reagent	Protocol
A buffer, 0.1 M sodium dihydrogen phosphate (NaH <sub>2</sub> PO <sub>4</sub> )	1.19 g of NaH <sub>2</sub> PO <sub>4</sub> .H <sub>2</sub> O (MW; 119.98 g/mol) was weighed and dissolved in 100 mL of water. The mixture was stirred for 10 minutes to completely dissolve the compound. The final concentration of A buffer was 0.1 M.
B buffer, 0.1 M disodium hydrogen phosphate dihydrate (Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O)	1.77 g of Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O (MW; 177.99 g/mol) was weighed and dissolved in 100 mL of water. The mixture solution was stirred for 10 minutes to completely dissolve the compound. The final concentration of stock B buffer was 0.1 M.
Working buffer, 50 mM phosphate buffer (pH 6.8)	51 mL of A buffer stock was added to 49 mL of B buffer stock to produce 0.1 mM of sodium phosphate buffer. Later, the solution was made up to 200 mL with water. The final concentration of the buffer solution was 50 mM, pH 6.8. The final pH was confirmed with a pH meter.
Tyrosinase stock (53 670 units/mL)	The tyrosinase powder (9.3 mg with 5771 units/mg solid) was dissolved with 1 mL of working buffer to obtain 53 670 units/ mL

	tyrosinase solution. The enzyme stock was stored at -20 °C and was stable for at least 6 months without any significant loss in activity. Required tyrosinase working solution was prepared from this stock.
Tyrosinase working solution (300, 600 and 1200 units/ mL)	Tyrosinase stock (44.8 µL) was diluted with the working buffer (19.95 mL) to obtain 20 mL of 2400 units/mL tyrosinase. The other tyrosinase working solution was prepared by serial dilution (1:1).
1 mM L-tyrosine working solution	18.1 mg of L-tyrosine (MW: 181.19 g/mol) was weighed* and dissolved in 100 mL of working buffer to obtain 1-mM L-tyrosine. The mixture was stirred for 30 minutes to completely dissolve the L-tyrosine. The final concentration of L-tyrosinase in the reaction mixture was 0.8 mM.
2 mM L-tyrosine working solution	36.2 mg of L-tyrosine (MW: 181.19 g/mol) was weighed* and dissolved in 100 mL of working buffer to obtain 2-mM L-tyrosine. The mixture was stirred for 30 minutes to completely dissolve the L-tyrosine. The final concentration of L-tyrosinase in the reaction mixture was 1.6 mM.

\*Note: An aluminum weighing boat instead of plastics was used because the plastic weighing boat tend to create electrostatic reaction upon contact with L-tyrosine.

#### **5.2.3.2 Pre-experiments investigating effect of enzyme concentration, substrate concentration, temperature and solvent**

**Enzyme concentration:** Tyrosinase working solutions of varying concentrations were prepared appropriately to achieve tyrosinase final concentrations of 30, 60 and 120 units/mL in the wells (see Table 5.7). In a 96-well plate, a volume of 20 µL from each of the tyrosinase working solutions was added to 1 mM L-tyrosine (160 µL) and 50-mM phosphate buffer (20 µL) to make a total volume of 200 µL reaction mixture. Blank contained L-tyrosine (160 µL) and reaction buffer (40 µL). Enzyme activity was measured every 10 minutes-interval for 60 minutes at 450 nm wavelength using a spectrophotometer. The corrected OD readings were plotted against time.

**Substrate concentration:** In a 96-well plate, a 20 µL of 300 units/mL tyrosinase (selected based on the assay above) was added to 2 mM L-tyrosinase (160 µL) and 50 mM phosphate buffer

(20  $\mu$ L) to make a total volume of 200  $\mu$ L reaction mixture. Blank contained L-tyrosine (160  $\mu$ L) and reaction buffer (40  $\mu$ L). The final concentrations of tyrosinase and L-tyrosine were 30 units/mL and 1.6 mM respectively. The absorbance reading was measured at 450 nm at 25 °C using a spectrophotometer and percentage activity of treated elastase at minute 60 was compared to control, untreated or buffer (see section 5.2.5).

**Temperature:** The protocol above for substrate concentration was repeated. One experiment proceeded with tyrosinase activity at 25 °C and the other one at 37 °C. After 60 minutes of reactions, the absorbance readings were measured at 450 nm and percentage activity (see 5.2.5) was calculated and compared. The final concentrations of tyrosinase and L-tyrosine were 30 units/mL and 1.6 mM respectively.

**Solvent:** Water (20  $\mu$ L) or 70% ethanol (20  $\mu$ L) was allowed to react with 300 units/mL tyrosinase for 15 minutes. After the preincubation, 2 mM L-tyrosine (160  $\mu$ L) and reaction buffer (20  $\mu$ L) were added to make a total of 200  $\mu$ L reaction volume. The reaction proceeded for 60 minutes at 25 °C before an absorbance reading was measured at 410 nm using a spectrophotometer. The final concentrations of tyrosinase and L-tyrosine were 30 units/mL and 1.6 mM respectively. Percentage activity of treated tyrosinase at minute-60 was compared to control, untreated or buffer (see 5.2.5).

#### 5.2.4 Anti-tyrosinase assay

The previous protocol was modified according to the optimal experimental conditions investigated in the pre-experiments (Moon *et al.*, 2010). In a 96-well plate, samples (20  $\mu$ L) and kojic acid at varying concentrations (20  $\mu$ L) were treated with 300 units/mL tyrosinase (20  $\mu$ L) for 15 minutes. After preincubation, 2 mM L-tyrosine (160  $\mu$ L) and reaction buffer (20  $\mu$ L) was added to make a total volume of 200  $\mu$ L reaction mixture, and further incubated for 60 minutes at 25 °C. The enzyme activity was measured every 10 minutes-interval at 450 nm to

ensure the enzyme was active throughout the incubation period. Percentage inhibition compared to control was calculated and compared (see section 5.2.5). Control and blank were prepared as in Table 5.8.

**Table 5.8: Volume of reagent pipetted in the tyrosinase assay.** The final concentration of tyrosinase and substrate were 30 units/mL and 1.6 mM.

Reagents	Control ( $\mu$ L)	Standard compound ( $\mu$ L)	Sample ( $\mu$ L)	Blank well ( $\mu$ L)
Tyrosinase (300 units/mL)	20	20	20	-
Treatments (water)	20 (water)	20 (kojic acid)	20 (samples)	20 (water)
Substrate, L-tyrosine (2 mM)	160	160	160	160
Working buffer (50 mM phosphate buffer)	0	0	0	20
<b>Total volume (<math>\mu</math>L)</b>	<b>200</b>	<b>200</b>	<b>200</b>	<b>200</b>

### 5.2.5 Percentage inhibition and activity

The percentage inhibition and activity of collagenase, elastase and tyrosinase were calculated using the formulae below:

$$\text{Enzyme percentage inhibition (\%)} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100 \quad (5.1)$$

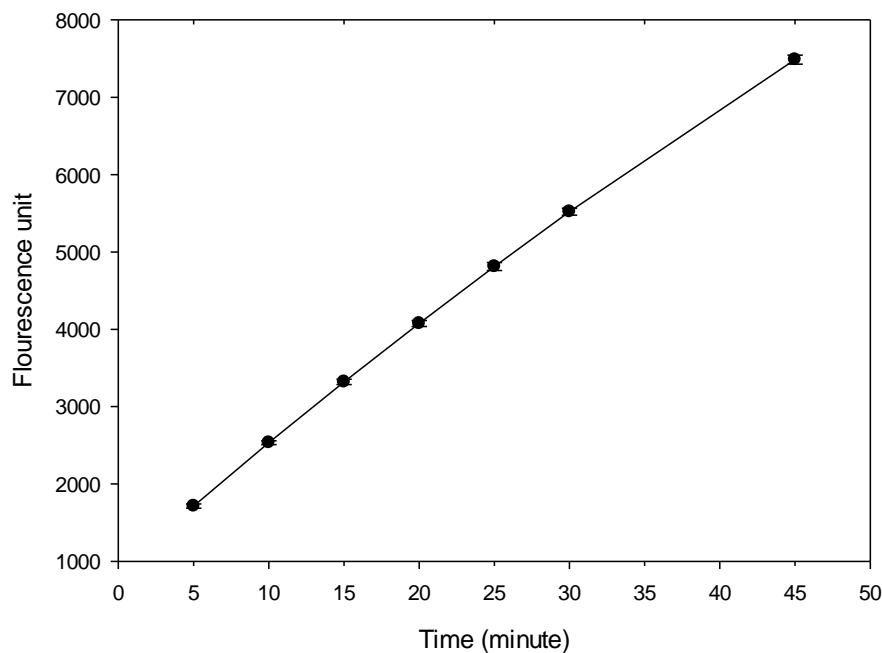
$$\text{Enzyme percentage activity (\%)} = \frac{\text{OD sample}}{\text{OD control}} \times 100 \quad (5.2)$$

## 5.3 Results

### 5.3.1 Anti-collagenase: pre-experiment assay

#### 5.3.1.1 Correlation of collagenase activity with digested gelatine

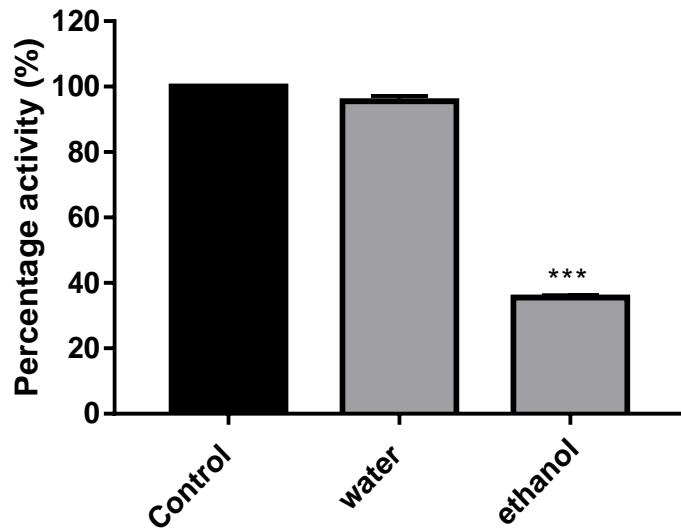
The activity of collagenase was measured by the amount of product produced from the digestion of gelatine attached with a fluorophore. The activity of collagenase over time was demonstrated to be correlated with the fluorescent unit (Figure 5.1).



**Figure 5.1: Collagenase activity over time.** Data are mean  $\pm$  SD of triplicates.

### 5.3.1.2 Effect of solvent on collagenase activity

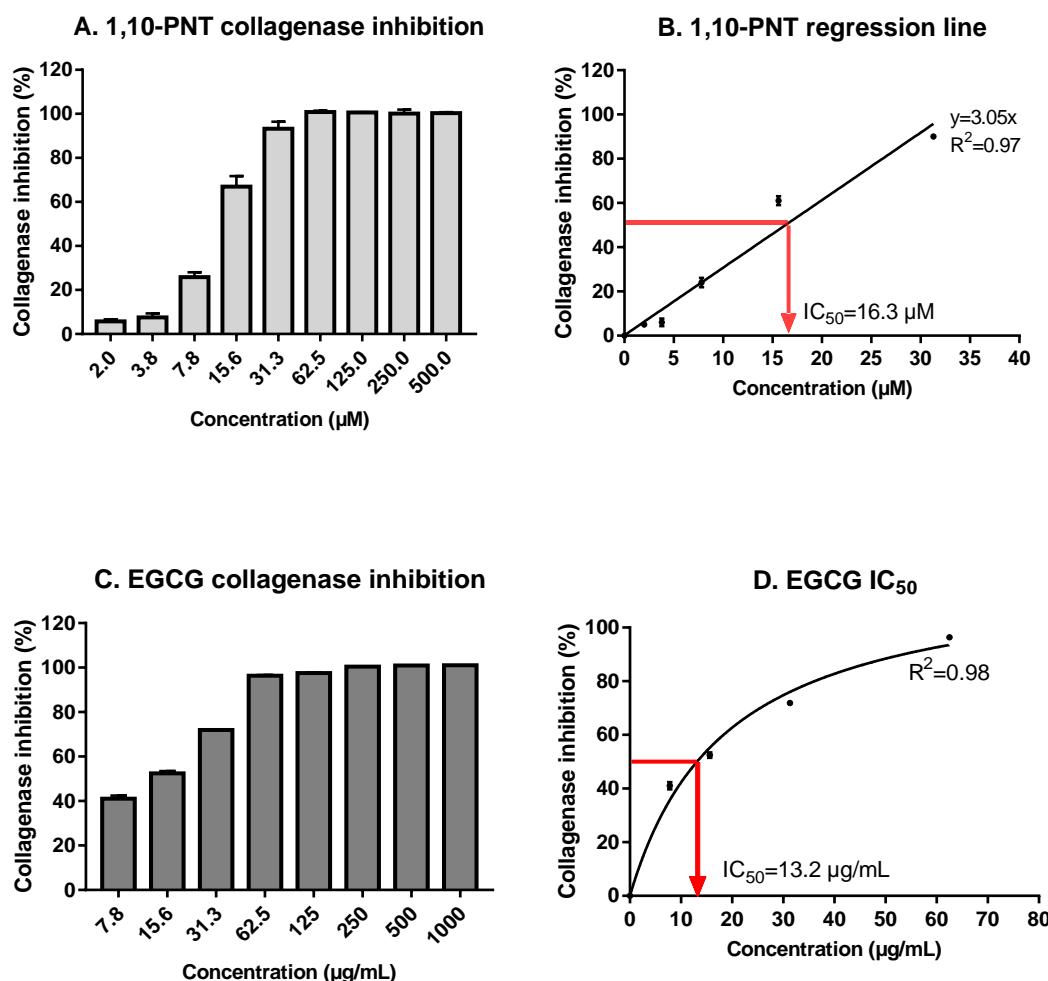
Water showed no effect on the collagenase activity, but 70% ethanol had a significant inhibitory effect (Figure 5.2). Thus, to reduce the effect of ethanol in the experiment, the ethanolic plant samples were prepared at a higher concentration and later diluted to ensure the final concentration of ethanol in the samples was the lowest (< 0.05%).



**Figure 5.2: Inhibitory effect of 70% ethanol on collagenase activity.** Data are mean  $\pm$  SD of triplicates.

### 5.3.1.3 Collagenase inhibition and $IC_{50}$ : 1,10-PNT and EGCG positive controls

1, 10-PNT exhibited a dose-dependent inhibition of collagenase activity, where 100% inhibition was observed at 63 to 500  $\mu$ M (see Figure 5.3 A). A standard regression line was constructed from the 2.0-31.3  $\mu$ M data,  $R^2=0.97$  with  $IC_{50}$  obtained at 16.3  $\mu$ M (see Figure 5.3.1 B). Similarly, EGCG also showed a dose-dependent collagenase inhibition with 100% inhibition observed at 62.3-500  $\mu$ g/mL (see Figure 5.3 C). An  $IC_{50}$  of 13.2  $\mu$ g/mL was obtained from a nonlinear best fitted line at 7.8-62.5  $\mu$ g/mL,  $R^2=0.98$  (see Figure 5.3 D).

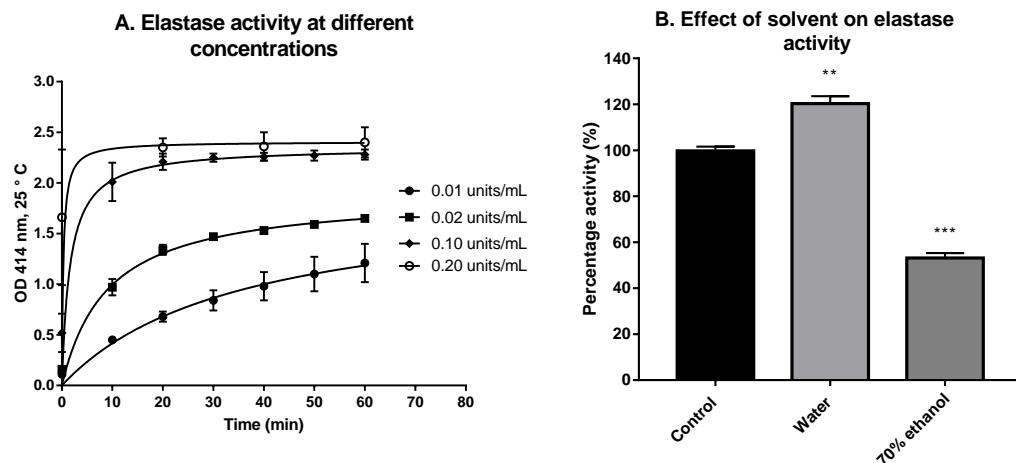


**Figure 5.3: 1,10-PNT and EGCG collagenase inhibition and  $IC_{50}$ .** Dose response and  $IC_{50}$  graphs for 1,10-PNT (A and B) and EGCG (C and D). Data represent mean and SD of triplicates.

### 5.3.2 Anti-elastase: pre-experimental assay

#### 5.3.2.1 Enzyme's concentrations and solvent effect: colormetric assay

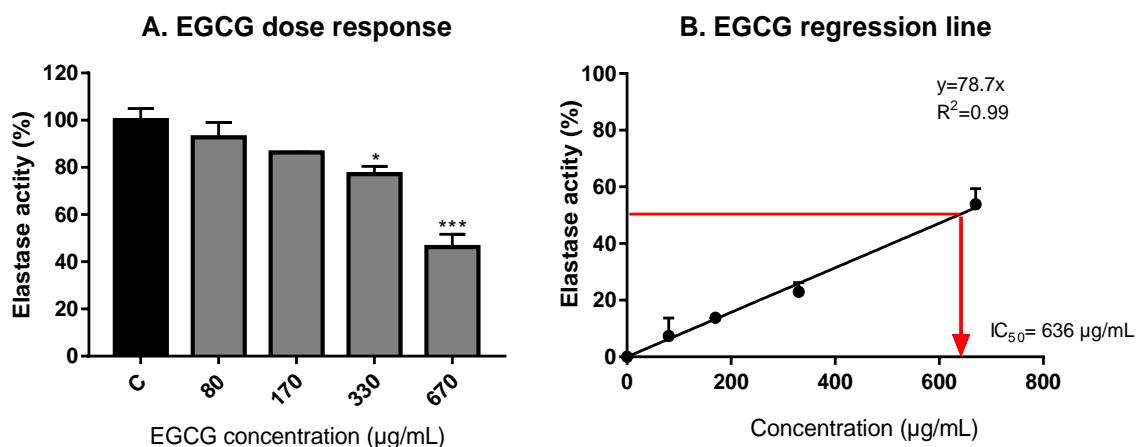
Studies on the colormetric anti-elastase assay use varying experimental conditions (Thring *et al.*, 2009; Moon *et al.*, 2010; Sahasrabudhe and Deodhar, 2010; Azmi *et al.*, 2014), and thus the optimal enzyme concentration was investigated in the pre-experimental assay. Final elastase concentrations of 0.1 and 0.2 units/mL were shown to have rapid reactions where all substrates were digested within 10 minutes, whilst 0.01 and 0.02 units/mL elastase seemed to have optimal OD readings of 1.0-1.5 after 60 minutes reaction (Figure 5.4 A). A 0.01 unit/mL elastase concentration was used in the inhibitory assay so as to allow the proper observation of the potency of elastase inhibition. Water was shown to increase elastase activity, but 70% ethanol showed an inhibitory effect as compared to control (buffer), as shown in Figure 5.4 B. Therefore, appropriate dilutions were made for the ethanolic extracts to minimize the effect of ethanol on enzyme activity.



**Figure 5.4: Colorimetric assay; effect of elastase concentration (A) and solvent on enzyme activity (B).** Data are the mean  $\pm$  SD of triplicates.

### 5.3.2.2 EGCG dose-response and $IC_{50}$ : colormetric assay

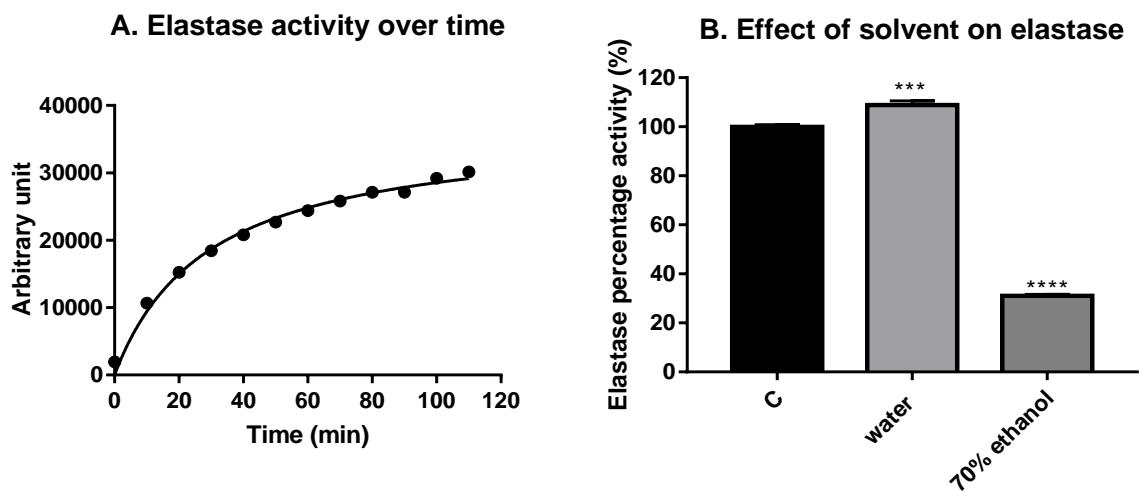
EGCG was used as the positive control in the colormetric assay and it was shown to exhibit significant elastase inhibition at 330 and 670  $\mu\text{g/mL}$  with 22.9 and 53.7% inhibition respectively (Figure 5.5 A). An  $IC_{50}$  of 636  $\mu\text{g/mL}$  was obtained from the best fit regression line,  $R^2=0.99$ , as shown in Figure 5.5 B. However, a preliminary assay showed that this method is not suitable with one of the plant species (*C. ternatea*) in the study as the plant's pigment masked elastase inhibitory activity. Therefore, a fluorescence assay was used to evaluate all of the plant samples for anti-elastase activity.



**Figure 5.5: EGCG dose response (A) and  $IC_{50}$  (B) in anti-elastase colorimetric assay.** Data are the mean  $\pm$  SD of triplicates.

### 5.3.2.3 Elastase activity over time and solvent effect: fluorescence assay

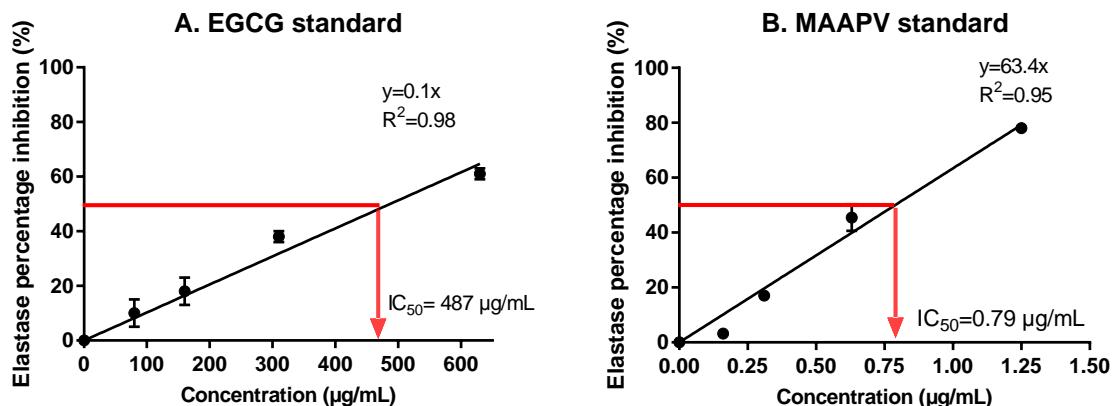
The fluorescence assay showed that elastase activity over time correlates with the fluorescence emitted (Figure 5.6 A). Similar effects were observed with water and 70% ethanol on elastase as those in the colormetric assay, where water increases but the 70% ethanol inhibits the enzyme activity (Figure 5.6 B). Therefore, appropriate dilutions were made for the ethanolic extracts to minimize the effect of ethanol on enzyme activity.



**Figure 5.6: Elastase activity over time (A) and the effect of solvent on enzyme activity (B) in fluorescence assay.** Data are the mean  $\pm$  SD of triplicates. \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$ .

#### 5.3.2.4 EGCG and MAAPV standards: fluorescence assay

EGCG and MAAPV showed concentration-dependent inhibition of elastase activity, with  $IC_{50}$  487  $\mu\text{g/mL}$  and 0.79  $\mu\text{g/mL}$  respectively as shown in Figure 5.7. MAAPV was demonstrated to be a more potent inhibitor than the EGCG standard of green tea origin.

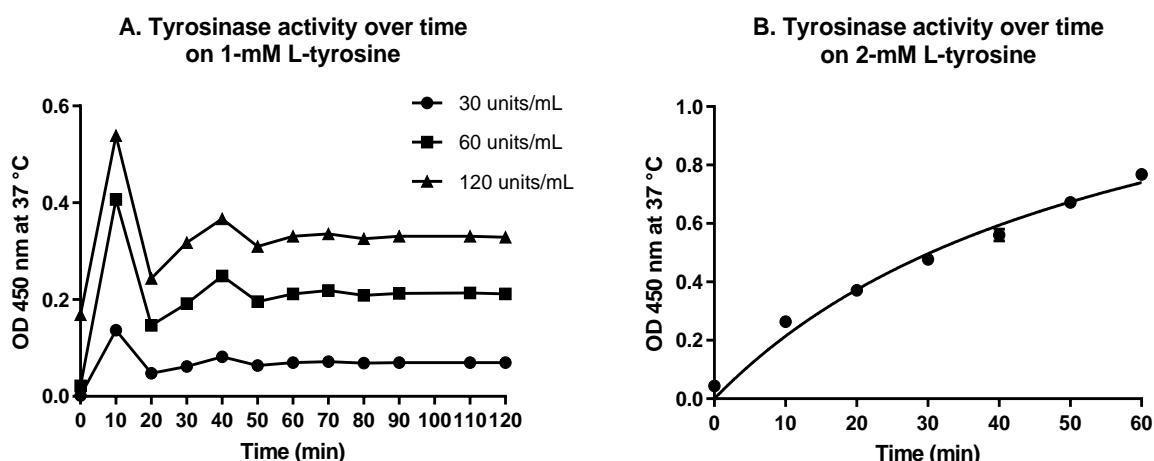


**Figure 5.7: Fluorescence assay; EGCG (A) and MAAPV (B) standards.** Data are the mean  $\pm$  SD of triplicates.

### 5.3.3 Anti-tyrosinase: pre-experiment assay

#### 5.3.3.1 Effect of enzyme's and substrate's concentration

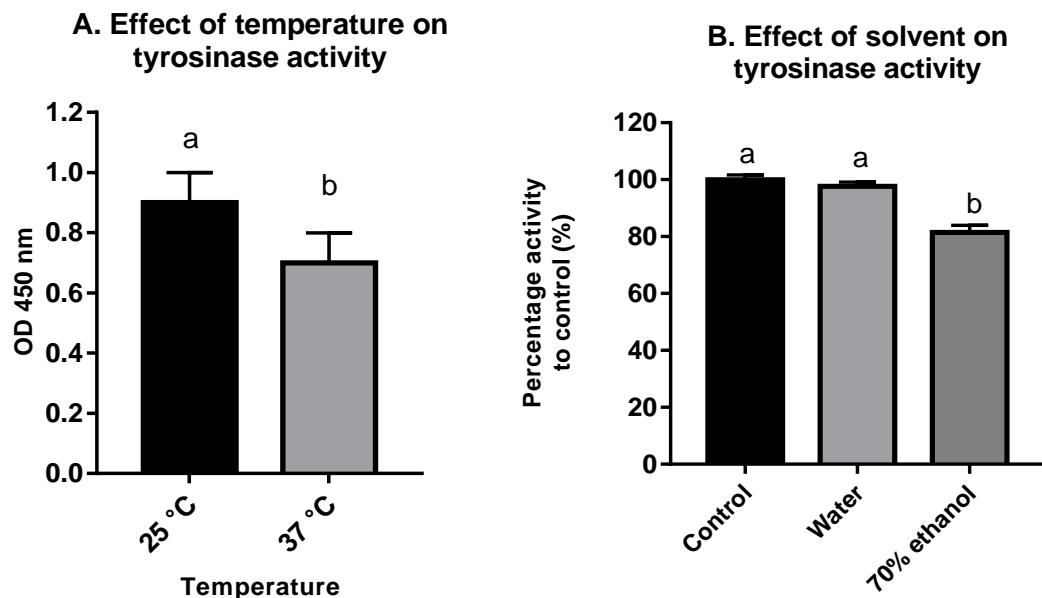
Tyrosinase activity was concentration-dependent, where at 120 units/mL the reaction was too rapid and lower reaction rates were observed at 60 and 30 units/mL (Figure 5.8 A). This result also suggests that a higher concentration of substrate is required to achieve optimal experimental conditions, as with 1 mM L-tyrosine all the substrate was utilised within 10 minutes. Subsequent experiment of 30 units/mL tyrosinase with 2 mM L-tyrosine were shown to be better conditions for the assay, as the final absorbance reading was at approximately 1.0 (Figure 5.8 B).



**Figure 5.8: Tyrosinase activity at different concentrations on 1 mM L-tyrosine (A) and, tyrosinase activity (30 units/mL) on 2 mM L-tyrosine (B). Data are the mean of triplicates.**

### 5.3.3.2 Effect of temperature and solvent on tyrosinase activity

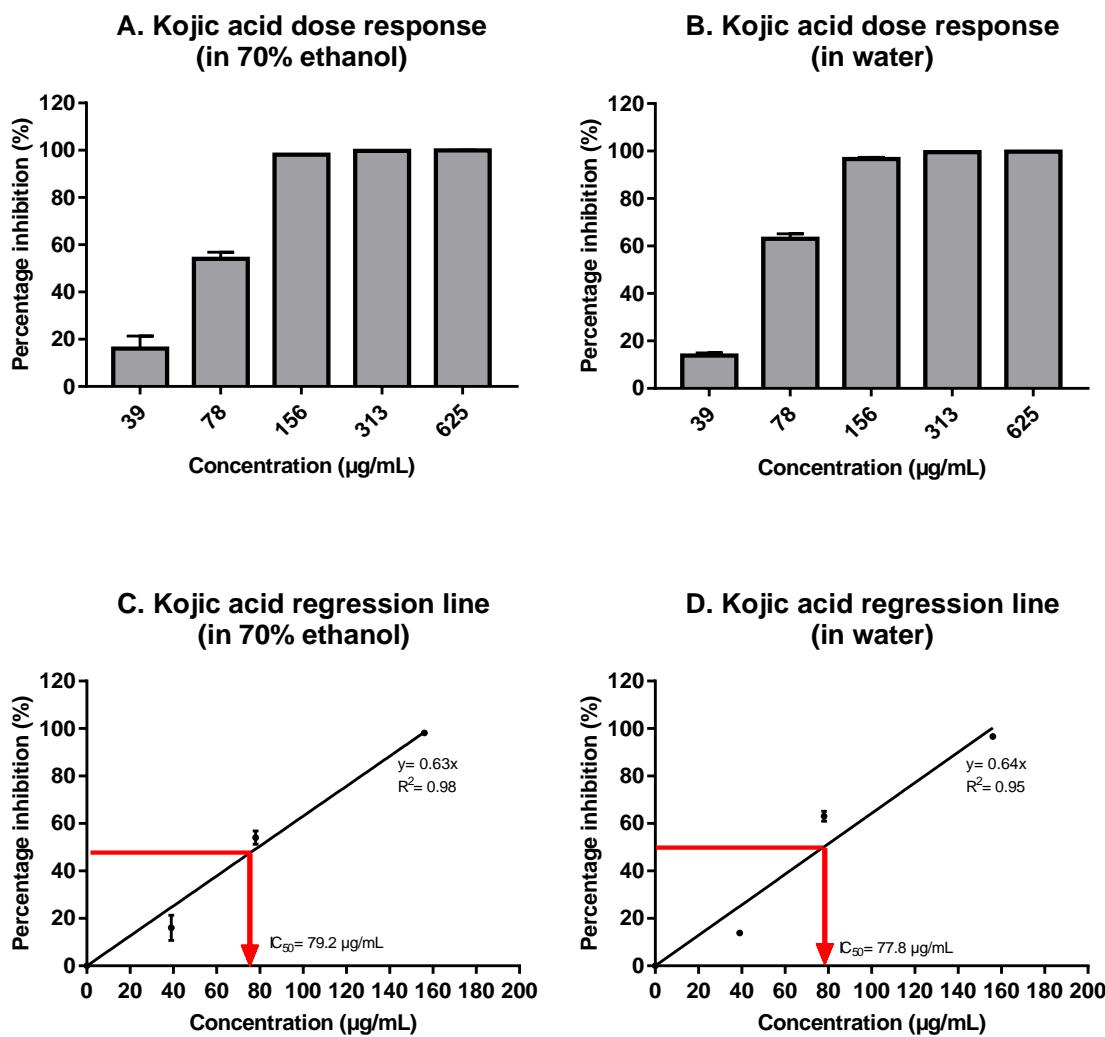
Temperatures of 25 and 37 °C have been reported in the anti-tyrosinase studies (Lee *et al.*, 2009; Moon *et al.*, 2010; Azmi *et al.*, 2014; Vardhan *et al.*, 2014), and thus the effect of temperature on tyrosinase activity was investigated. A reaction temperature of 25 °C was shown to provide better condition for tyrosinase as compared with 37 °C (Figure 5.9 A), and this temperature accords with the suggested optimal temperature for the enzyme's activity. Meanwhile, 70% ethanol was demonstrated to cause a significant 20% inhibitory effect on tyrosinase activity compared with the control (Figure 5.9 B), and thus appropriate sample preparation for the ethanolic extracts were made to ensure the final ethanol concentration in the well was the lowest. Water was shown to have no significant effect on the enzyme's activity.



**Figure 5.9: Effect of temperature (A) and solvent on tyrosinase activity (B).** Data are the mean and SD of triplicates. Different letters indicate significant mean difference at  $p<0.05$ .

### 5.3.3.3 Kojic acid standard

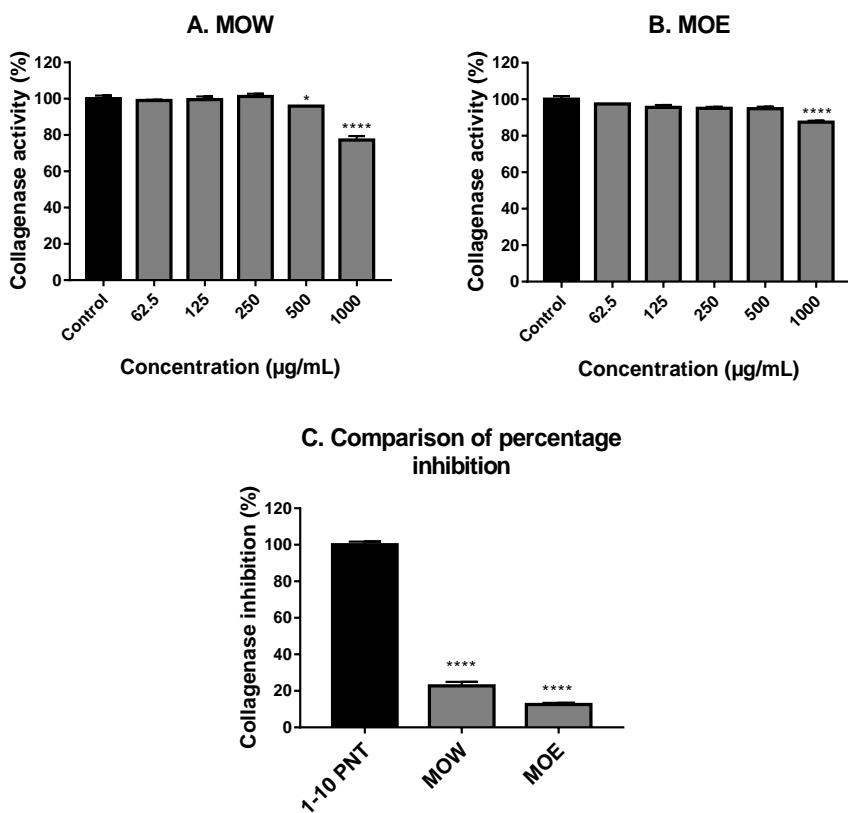
Kojic acid standards dissolved in 70% ethanol and water showed dose-dependent tyrosinase inhibition, where 100% inhibition was observed at 156-625  $\mu\text{g/mL}$ , as shown in Figure 5.10 A and B. Kojic acid in both 70% ethanol and water showed almost similar  $\text{IC}_{50}$  values, at 79.2 and 77.8  $\mu\text{g/mL}$  respectively. The regression lines used to interpolate the  $\text{IC}_{50}$  were as shown in Figure 5.10 C and D.



**Figure 5.10: Kojic acids tyrosinase inhibition (A and B) and  $\text{IC}_{50}$  (C and D).** Data are the mean and SD of triplicates.

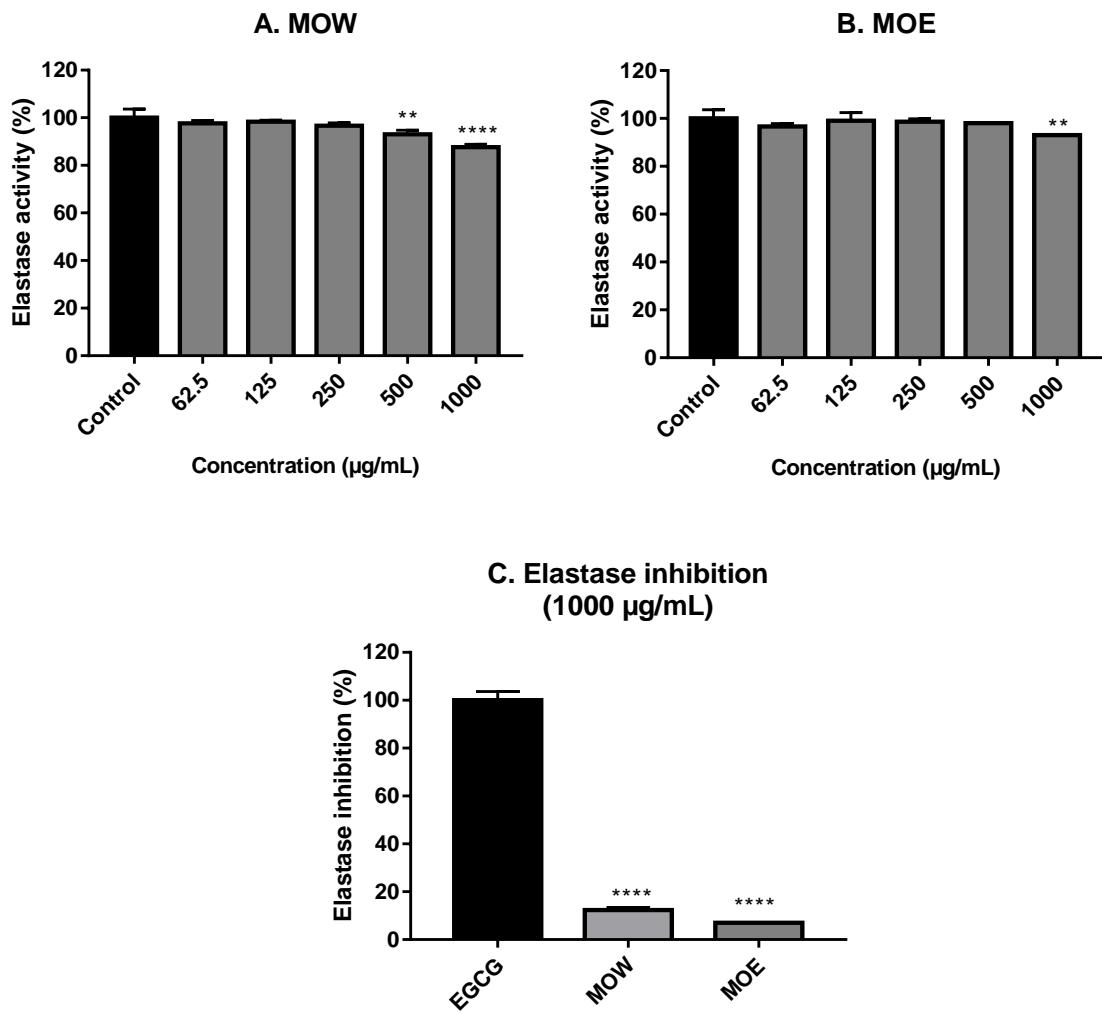
### 5.3.4 *Moringa oleifera* Lam.

**Anti-collagenase:** The anti-collagenase assay showed a lack of dose-dependent activity in both MOW and MOE extracts, where no inhibitory activity was observed from 62-250  $\mu$ g/mL by MOW and from 62-500  $\mu$ g/mL by MOE (see Figure 5.11 A and B). A significant reduction (5%) in activity compared to control was observed in MOW, and both extracts inhibited collagenase activity at 1000  $\mu$ g/mL. The values of percentage inhibition for both extracts (MOW and MOE) at 1000  $\mu$ g/mL were 22.7% and 12.6% respectively, which are very low compared with 1-10 PNT collagenase inhibitor (62.5  $\mu$ M), as shown in Figure 5.11 C.



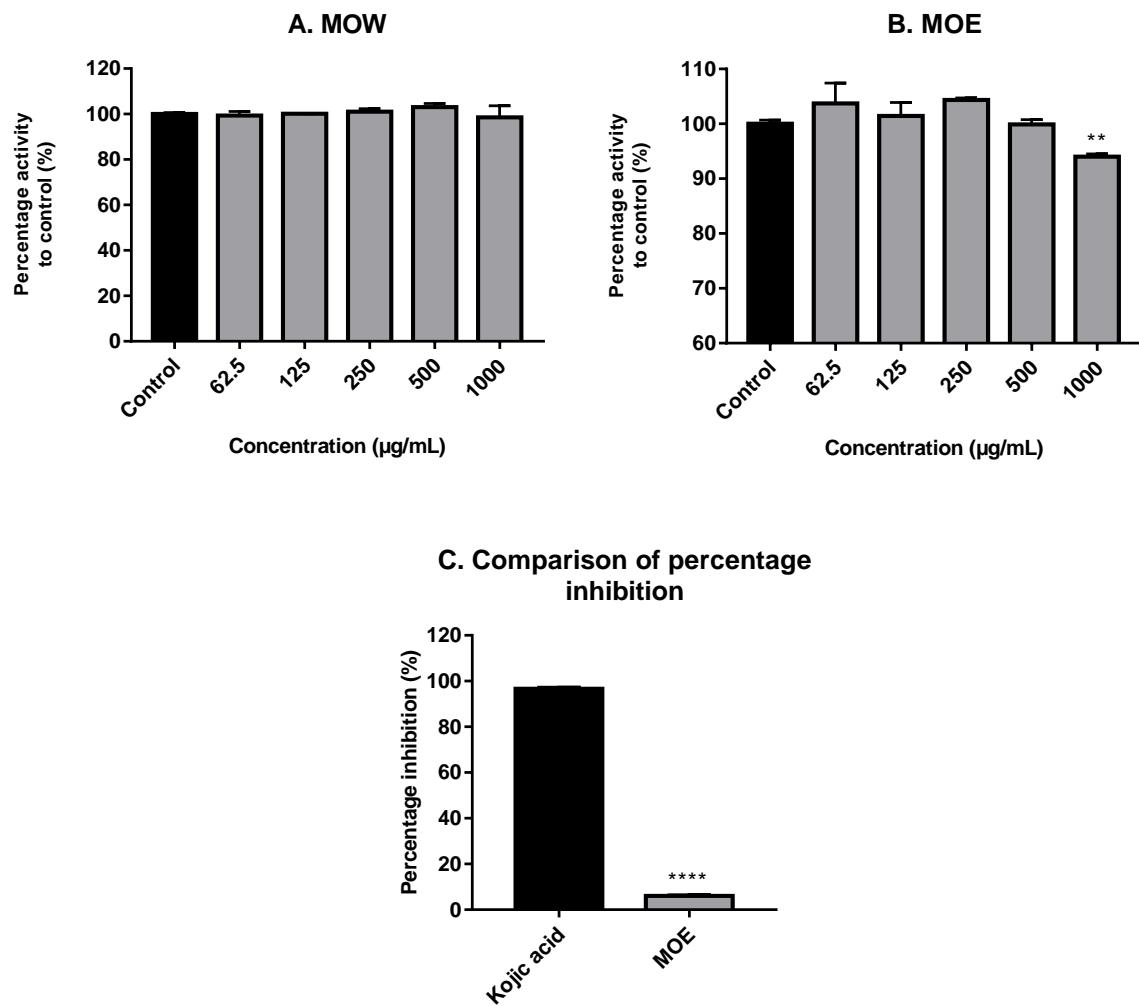
**Figure 5.11: Anti-collagenase activity and percentage inhibition of MOW and MOE.** Data are mean  $\pm$  SEM of triplicates. \* $p$ < 0.5 and \*\*\*\* $p$ < 0.0001 represent significant mean differences compared with control (untreated) in A and B or 1,10-PNT in C.

**Anti-elastase:** In both extracts, a lack of a dose-dependent effect was observed, where low inhibition was observed at 500 and 1000  $\mu\text{g/mL}$  in MOW and at 1000  $\mu\text{g/mL}$  in MOE (Figure 5.12 A and B). A comparison of percentage inhibition showed that MOW and MOE to inhibit elastase with 12 % and 7% inhibition respectively (Figure 5.12 C).



**Figure 5.12: Anti-elastase activity and percentage inhibition of MOW and MOE.** Data are mean  $\pm$  SEM of triplicates. \*\* $p < 0.01$  and \*\*\*\* $p < 0.0001$  represent significant mean differences compared with control (untreated) in A and B or EGCG in C.

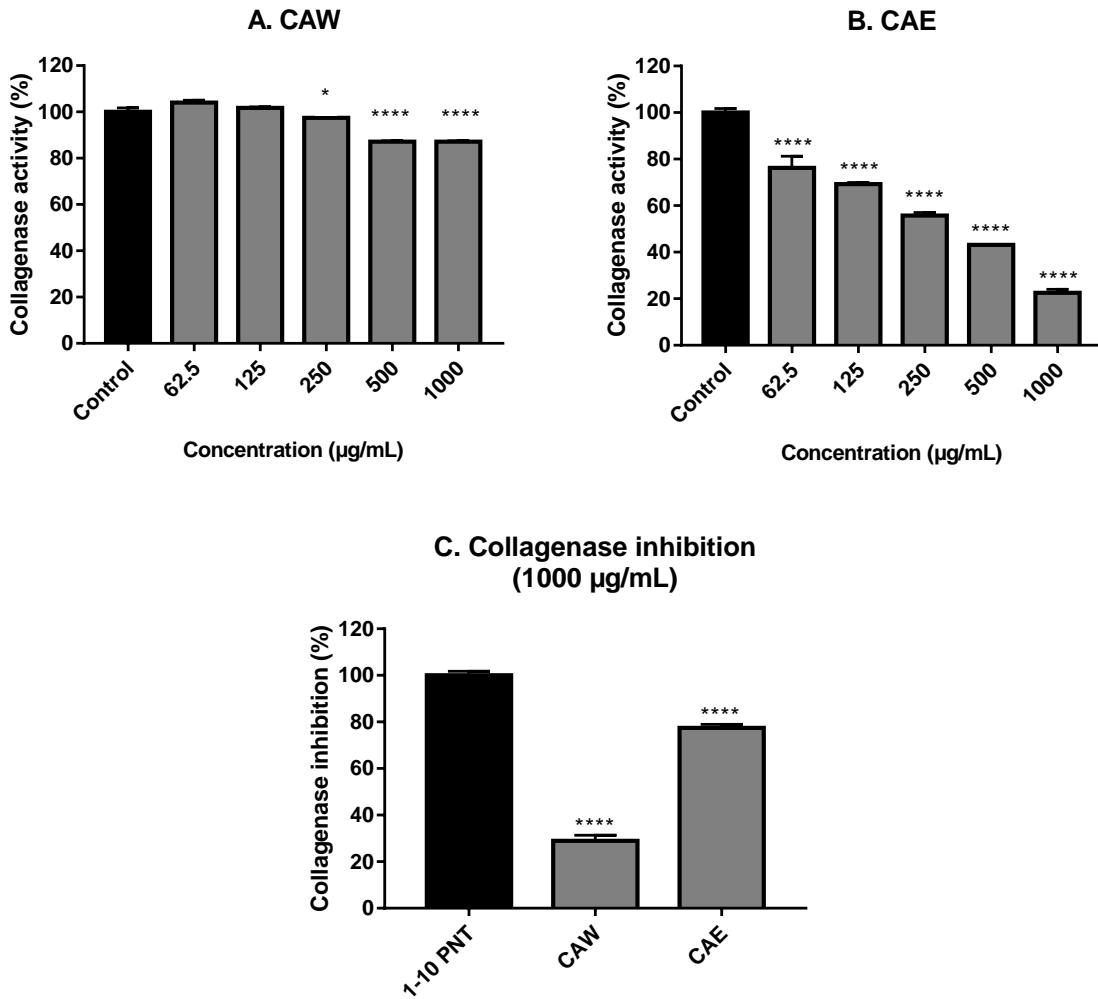
**Anti-tyrosinase:** MOW showed no inhibition of tyrosinase, while MOE inhibited tyrosinase at the highest concentration, 1000 µg/mL (Figure 5.13 A and B). The level of tyrosinase inhibition exhibited by MOE was 6.0%, which is very low compared with kojic acid, as shown in Figure 5.13 C.



**Figure 5.13: Anti-tyrosinase activity and percentage inhibition of MOW and MOE.** Data are mean  $\pm$  SEM of triplicates. \*\* $p < 0.01$  and \*\*\*\* $p < 0.0001$  represent significant mean differences compared with control (untreated) in A and B or kojic acid in C.

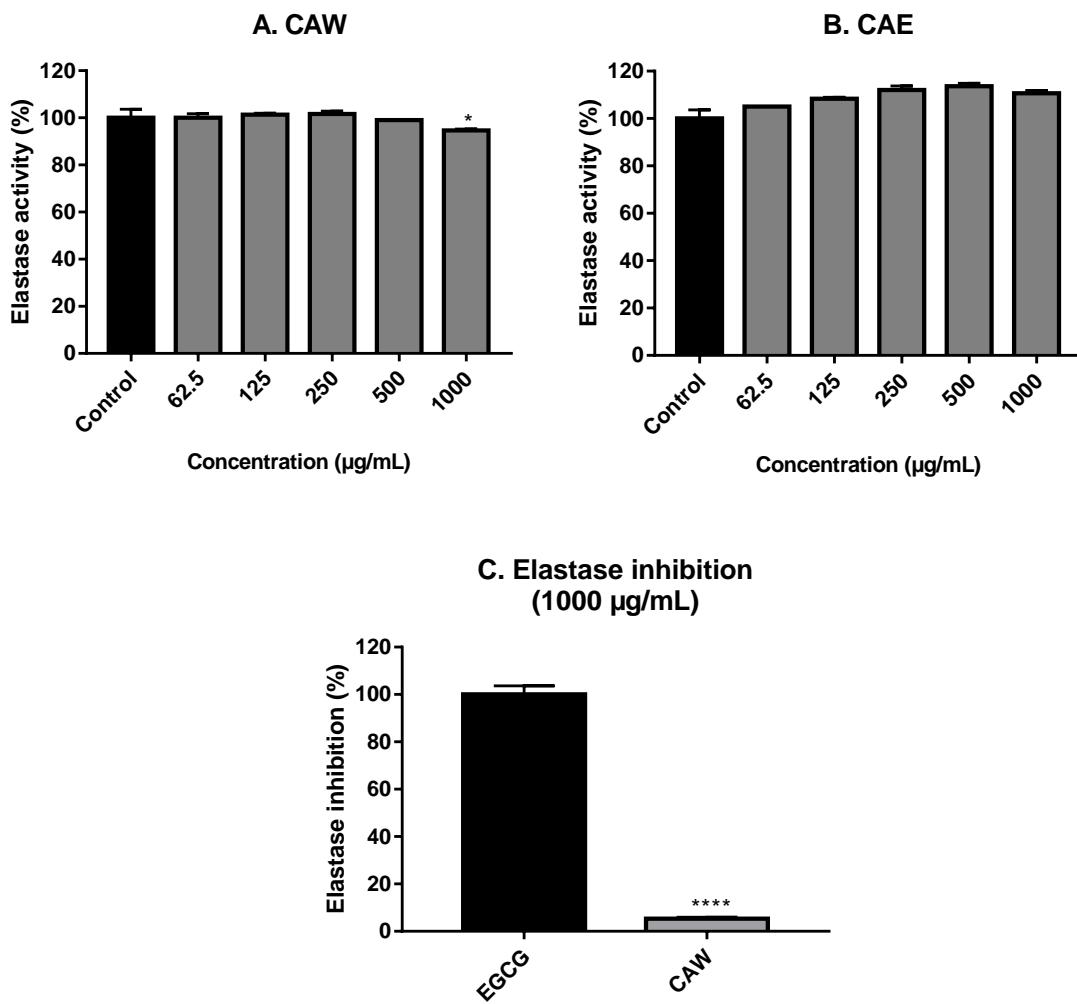
### 5.3.5 *Centella asiatica* (L.) Urb.

**Anti-collagenase:** CAW showed a lack of dose-dependent inhibition, while CAE showed strong dose-dependent inhibition activity against collagenase activity (Figure 5.14 A and B). Comparison of percentage inhibition at 1000 µg/mL showed that CAE has higher collagenase inhibition compared with CAW, with 77.4% and 29.0% respectively (Figure 5.14 C).



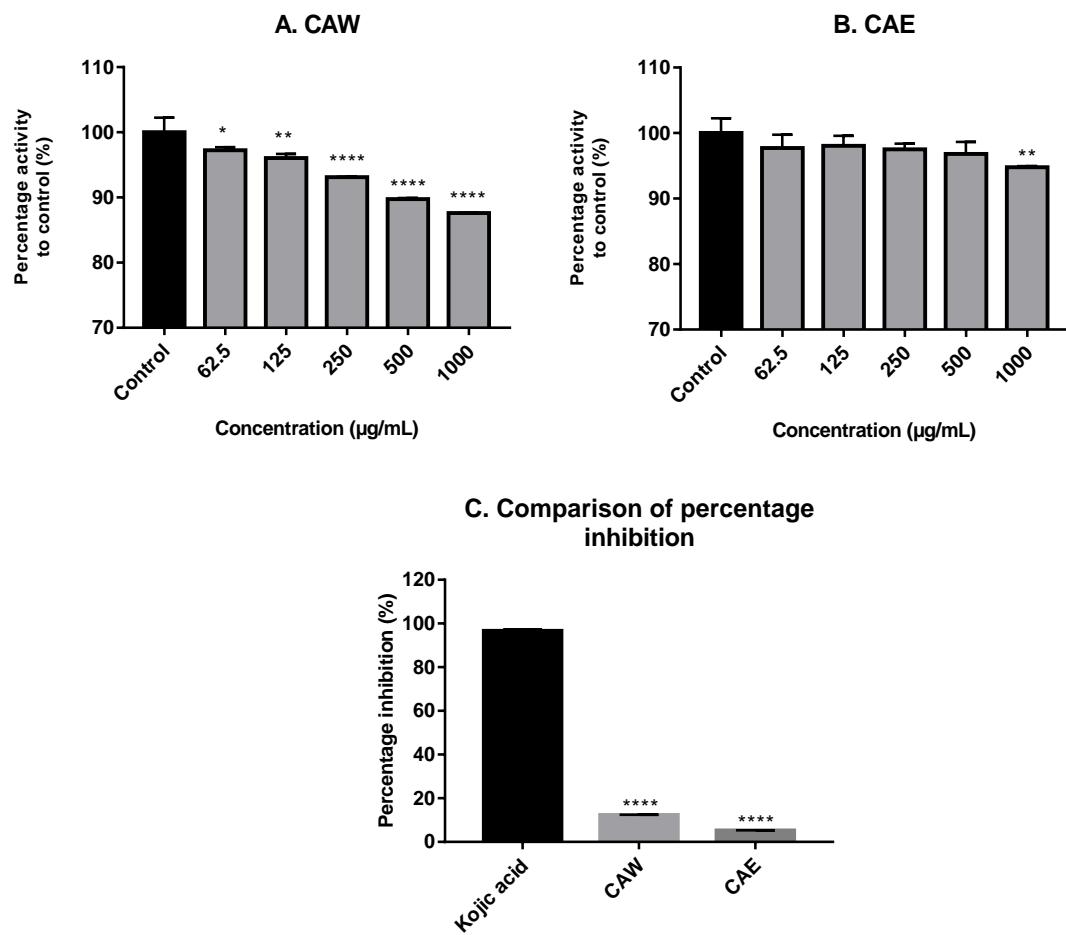
**Figure 5.14: Anti-collagenase activity and percentage inhibition of CAW and CAE.** Data are mean  $\pm$  SEM of triplicates. \* $p < 0.5$  and \*\*\* $p < 0.0001$  represent significant mean differences compared with control (untreated) in A and B or 1,10-PNT in C.

**Anti-elastase:** In both extracts, no dose-dependent effects were observed. Very low elastase inhibition was observed at 1000  $\mu\text{g}/\text{mL}$ , while no inhibitory activity was observed in CAE at any concentrations (Figure 5.15 A and B). CAW at 1000  $\mu\text{g}/\text{mL}$  showed 5% elastase inhibition, which is very low compared with EGCG (Figure 5.15 C).



**Figure 5.15: Anti-elastase activity and percentage inhibition of CAW and CAE.** Data are mean  $\pm$  SEM of triplicates. \* $p < 0.5$  and \*\*\*\* $p < 0.0001$  represent significant mean differences compared with control (untreated) in A and B or EGCG in C.

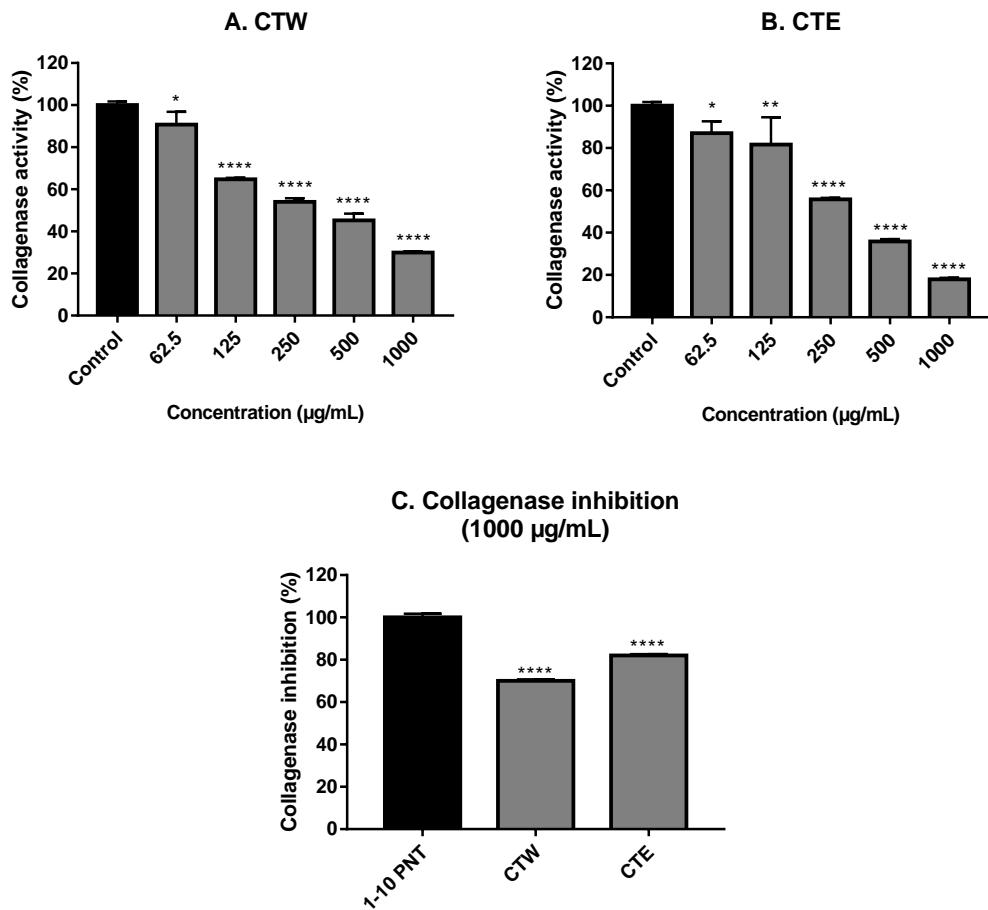
**Anti-tyrosinase:** CAW showed a concentration-dependent tyrosinase inhibition at 62.5-1000  $\mu\text{g/mL}$ , but CAE lacked dose-dependent effect inhibition where very low inhibition was observed at 1000  $\mu\text{g/mL}$  (Figure 5.16 A and B). The percentage inhibition exhibited at 1000  $\mu\text{g/mL}$  were 12.4% and 5.2% for CAW and CAE respectively (Figure 5.16 C).



**Figure 5.16: Anti-tyrosinase activity and percentage inhibition of CAW and CAE.** Data are mean  $\pm$  SEM of triplicates. \* $p < 0.5$  and \*\*\* $p < 0.0001$  represent significant mean differences compared with control (untreated) in A and B or kojic acid in C.

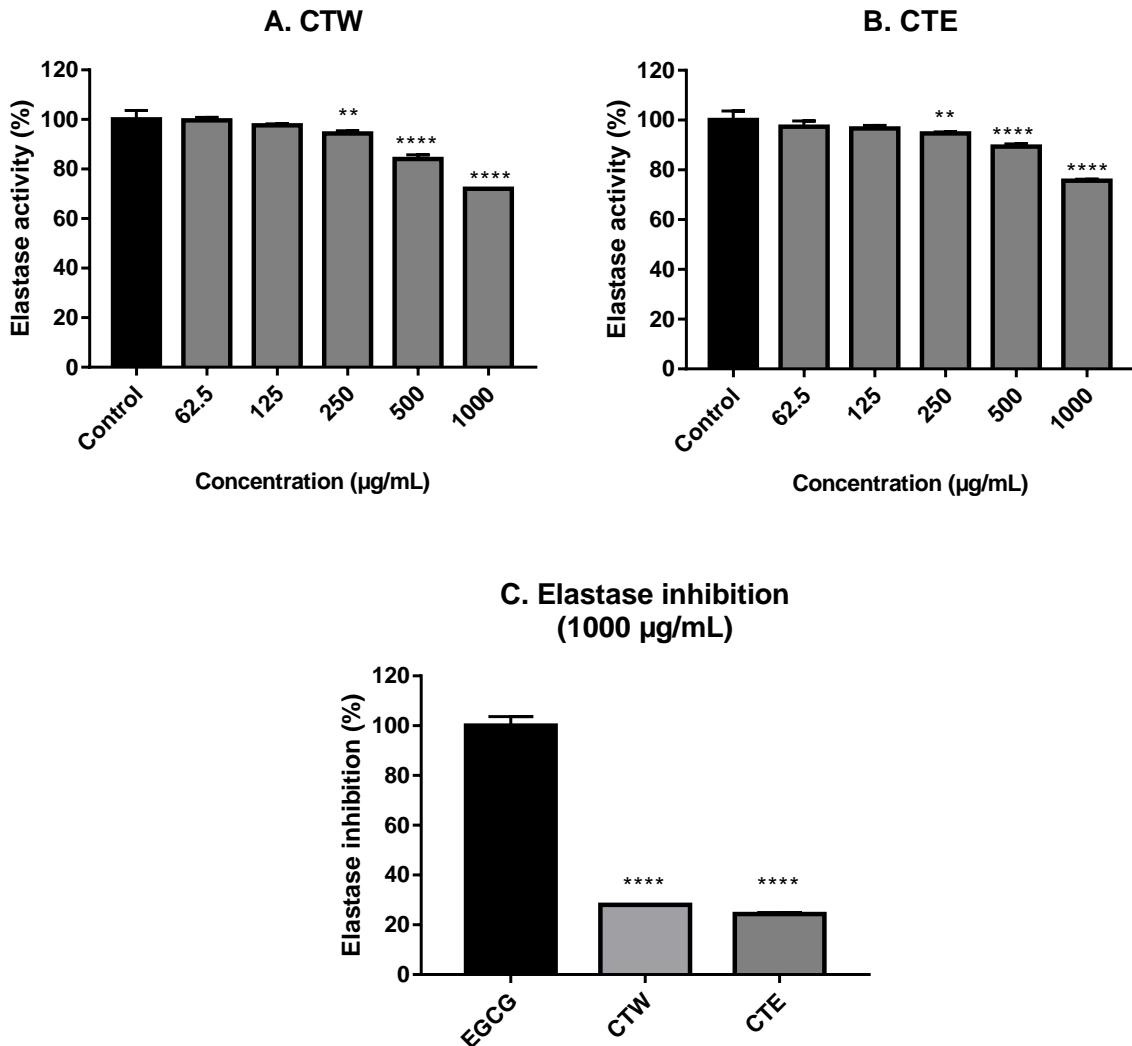
### 5.3.6 *Clitoria ternatea* L.

**Anti-collagenase:** Both *C. ternatea* extracts (CTW and CTE) extracts showed a dose-dependent inhibition against collagenase at 62.5-1000  $\mu\text{g/mL}$  (Figure 5.17 A and B). A comparison of percentage inhibitions between CTW and CTE showed CTE to be a better collagenase inhibitor (82.1%) than CTW (70.1 %), as shown in Figure 5.17 C.



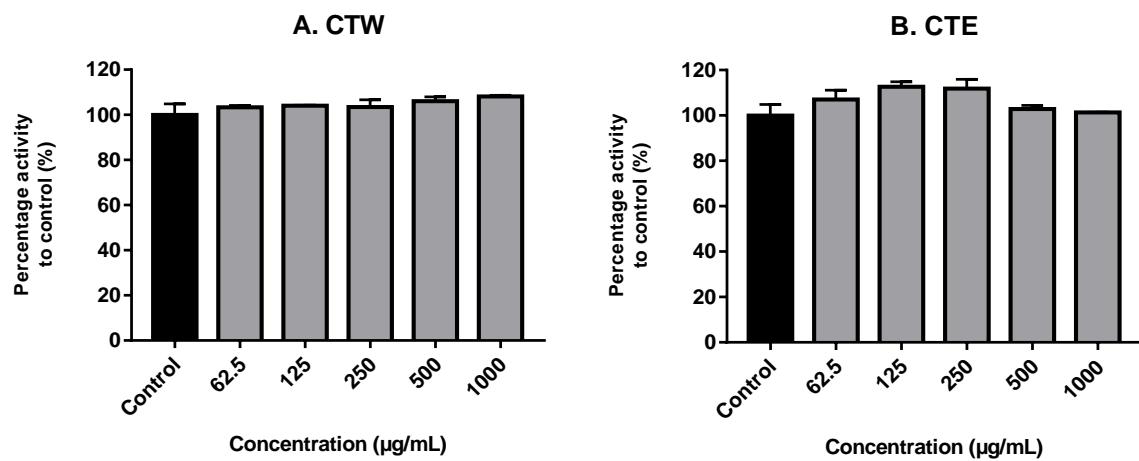
**Figure 5.17: Anti-collagenase activity and percentage inhibition of CTW and CTE.** Data are mean  $\pm$  SEM of triplicates. \* $p < 0.5$ , \*\* $p < 0.01$  and \*\*\* $p < 0.0001$  represent significant mean differences compared with control (untreated) in A and B or 1,10-PNT in C.

**Anti-elastase:** Both extracts of *C. ternatea* (CTW and CTE) showed a dose-dependent inhibition of elastase at 125-1000 µg/mL (Figure 5.18 A and B). Elastase activity was inhibited by 28 % and 24 % by CTW and CTE treatments respectively (Figure 5.18 C).



**Figure 5.18: Anti-elastase activity and percentage inhibition of CTW and CTE.** Data are mean  $\pm$  SEM of triplicates. \*\* $p < 0.01$  and \*\*\*\* $p < 0.0001$  represent significant mean differences compared with control (untreated) in A and B or EGCG in C.

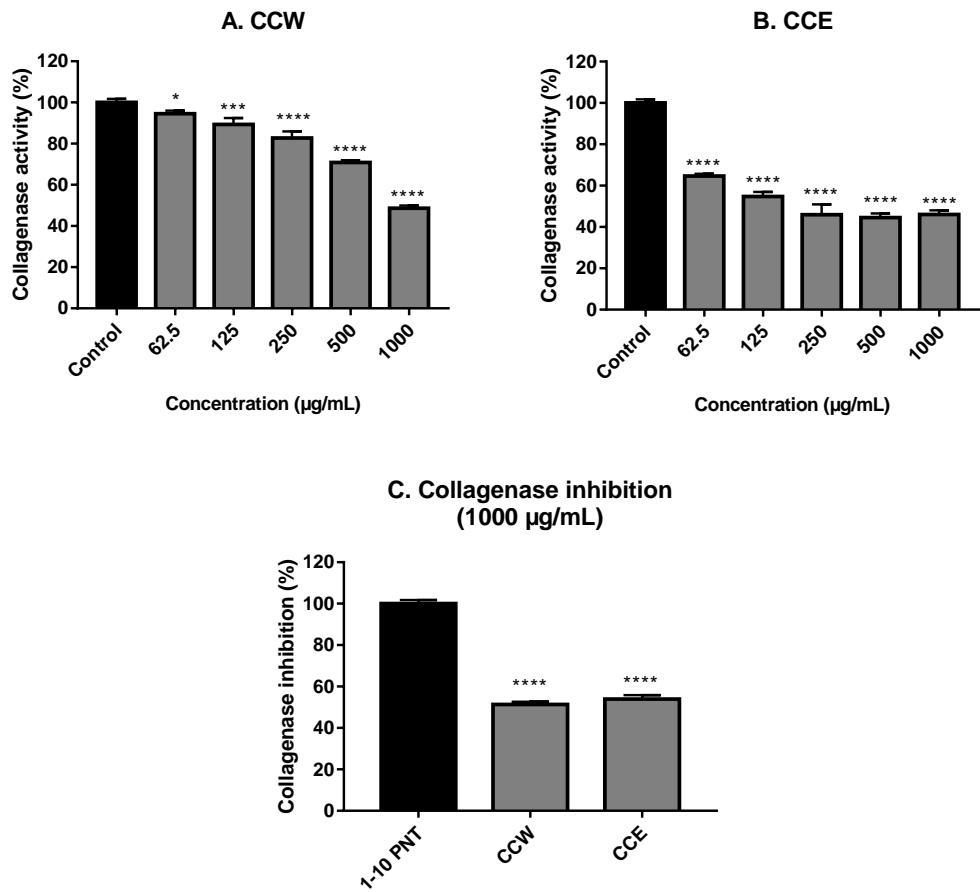
**Anti-tyrosinase:** Neither *C. ternatea* extract (CTW or CTE) exhibited any inhibitory activity against tyrosinase (Figure 5.19 A and B).



**Figure 5.19: Anti-tyrosinase activity of CTW and CTE.** Data are mean  $\pm$  SEM of triplicates.

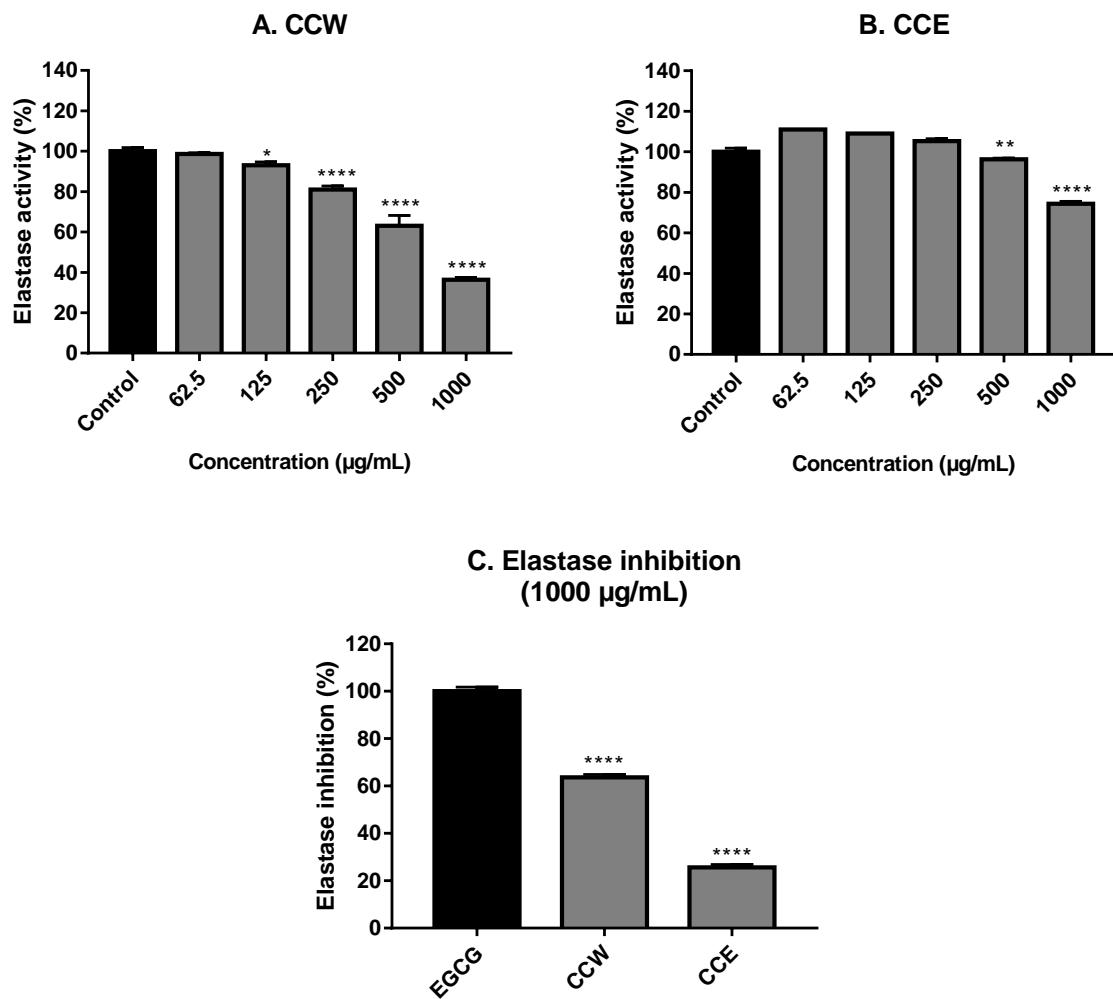
### 5.3.7 *Cosmos caudatus* Kunth.

**Anti-collagenase:** CCW showed dose-dependent inhibition of collagenase, but CCE showed no increase in inhibition at 250-1000 µg/mL (Figure 5.20 A and B). A comparison of collagenase inhibition at 1000 µg/mL between CCW and CCE showed collagenase inhibition of 48.7% and 46.7 % respectively (Figure 5.20 C).



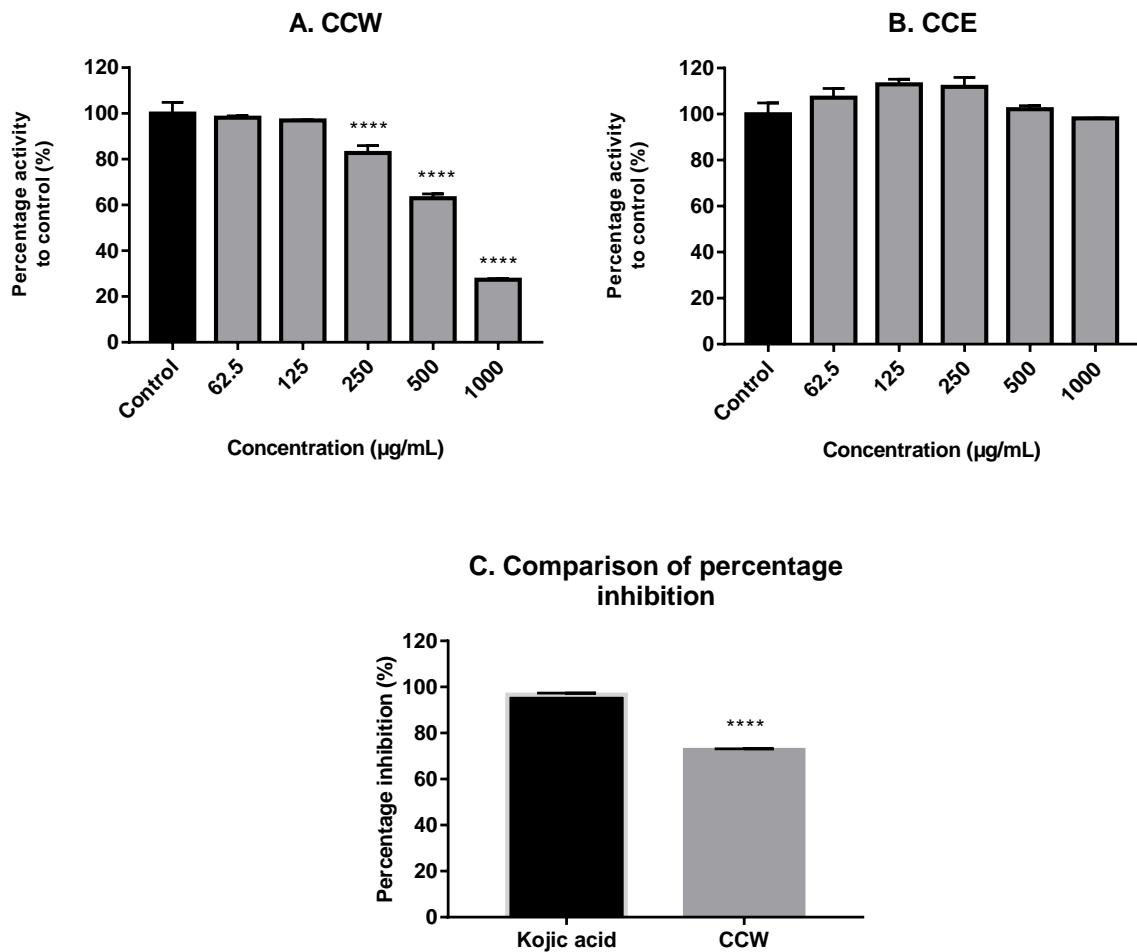
**Figure 5.20: Anti-collagenase activity and percentage inhibition of CCW and CCE.** Data are mean  $\pm$  SEM of triplicates. \* $p<0.5$ , \*\* $p<0.001$  and \*\*\* $p<0.0001$  represent significant mean differences compared with control (untreated) in A and B or 1,10-PNT in C.

**Anti-elastase:** CCW showed a strong dose-dependent inhibitory effect on elastase activity compared to CCE, where inhibition was observed at 500 and 1000 µg/mL (Figure 5.21 A and B). A comparison of percentage inhibition at 1000 µg/mL showed CCW to have higher elastase inhibition than CCE with 64 % and 26 % inhibition respectively (Figure 5.21 C).



**Figure 5.21: Anti-elastase activity and percentage inhibition of CCW and CCE.** Data are mean  $\pm$  SEM of triplicates. \* $p < 0.5$ , \*\* $p < 0.01$  and \*\*\*\* $p < 0.0001$  represent significant mean differences compared with control (untreated) in A and B or EGCG in C.

**Anti-tyrosinase:** A dose-dependent inhibitory effect was observed in CCW, but no inhibitory effect was observed of tyrosinase when treated with CCE (Figure 5.22 A and B). The calculated percentage inhibition of CCW at 1000 µg/mL showed 72.6% tyrosinase inhibition (Figure 5.22 C).



**Figure 5.22: Anti-tyrosinase activity and percentage inhibition of CCW and CCE.** Data are mean  $\pm$  SEM of triplicates. \* \*\*\*\*p< 0.0001 represents significant mean differences compared with control (untreated) in A and B or kojic acid in C.

## 5.4 Discussion

Skin ageing can be noticed according to several key signs such as fine lines and wrinkles, changes in skin tone and texture, dullness of the skin surface, visible pores, blotchiness, age spots and dryness (Farage *et al.*, 2013). Of these characteristics, the appearance of fine line and wrinkles is the most common and prominent sign of skin ageing due to the degradation of the extracellular matrix (ECM) proteins such as collagen and elastin (Sander *et al.*, 2006). The synthesis of these proteins is reduced with progressing age but their breakdown also occurs at higher rates, especially in photoaged skin with the induction of matrix metalloproteinases that make the skin condition even worse (Jin *et al.*, 2001). Other than that, hyperpigmentation due to the excessive synthesis of melanin on the face and neck is one of the skin problems that is aesthetically undesirable. Apart from its protective role, melanin has been shown to react with DNA and act as a photosensitizer that produces ROS after UV radiation (Kvam and Tyrrell, 1999). Skin ageing therapies such as dermal fillers, laser treatment and chemical peeling are some of the methods used to manage wrinkles and hyperpigmentation but, although these methods can give immediate results, they come with serious health complications (see Chapter 2:2.11). Therefore, a moderate approach such as the inclusion of anti-wrinkle and hypopigmentation agents in cosmetic products is usually preferred as the effects are usually limited to the site of application and are considered to be safer due to their specificity. In this chapter, plant extracts were evaluated for potential application as anti-wrinkle and hypopigmentation agents.

### 5.4.1 *M. oleifera*: low anti-collagenase, anti-elastase and anti-tyrosinase

In the anti-collagenase assay, it was shown that both MOW and MOE had very low anti-collagenase activity with a lack of dose-dependent effects. Similar trends were observed in anti-elastase and anti-tyrosinase assays where both extracts showed either very low or no

activity against the enzymes. Shin (2015) found that *M. oliefera* aqueous and ethanol extracts inhibited collagenase activity of 33.3% and 41.0% respectively at 1000 µg/mL. However, in this study the extracts were demonstrated to exhibit only 12.6% (MOE) and 22.7% (MOW) collagenase inhibition at the same concentration. Although higher activity was reported by Shin, (2015), the activity was measured using a colormetric assay, which may be affected by background readings from the extract's pigment, as compared to the fluorescence assay used in this study. An *in vitro* study using NHDF<sup>6</sup> cells showed no inhibitory activity of *M. oliefera* aqueous and ethanol extracts against MMP-2 and MMP-9 enzymes (Watthanaudomchai *et al.*, 2012). Meanwhile, Zeitoun *et al.* (2016) showed that *M. oliefera* ethanol extract inhibits tyrosinase activity of 65%, which is quite high compared to the 5% inhibition observed in this study. Their extract preparation was similar to that in this study, but the inclusion of the stem as well as the source of the plant may have affected level of activity. There are very limited studies which present data on direct enzyme inhibition using *M. oliefera* extract. Its active compounds such as quercetin, myricetin and kaempferol have been shown to exhibit tyrosinase inhibition but these are considered as weak inhibitors compared with the kojic acid standard (Chang, 2012). Therefore, the plant extracts may not be active as inhibitors of collagenase, elastase and tyrosinase.

#### **5.4.2 *C. asiatica*: a potential inhibitor of collagenase and tyrosinase**

*C. asiatica*, as an extract or isolated compound (asiaticoside, madecassoside, asiatic acid and madecassic acid), has been reported to stimulate fibroblast proliferation and collagen synthesis and to assist in wound healing processes (Maquart *et al.*, 1990; Somboonwong *et al.*, 2012; Wu *et al.*, 2012; Bylka *et al.*, 2013). In this study, *C. asiatica* extracts were shown to inhibit collagenase and tyrosinase, where significant dose dependent inhibitory effects were

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<sup>6</sup> NHDF = Normal Human Dermal Fibroblast

observed in CAE treated collagenase and CAW treated tyrosinase (Figure 5.17 and 5.18).

However, very low or no activity was observed in the anti-elastase assay for both extracts. A study using asiaticoside-enriched fractions of *C. asiatica* extracted using n-butanol and methanol also showed significant inhibitory activity against MMP-1 (collagenase) (Nema *et al.*, 2013). The same study also showed that enriched *C. asiatica* significantly inhibited elastase, suggesting that asiaticoside might be responsible for both activities. Aqueous extracts of *C. asiatica* have been shown to exhibit very low levels of anti-collagenase activity at 5% (Thring *et al.*, 2009), which is similar to the findings in this study. An *in vitro* study using human skin fibroblasts suggested that *C. asiatica* extract may exert an anti-wrinkle effect through the indirect regulation of collagenase and elastase by enhancing the expression of TIMP-1<sup>7</sup> mRNA (Wu *et al.*, 2012). The enhancement of this protein expression inhibits MMPs activity in degrading the skin's connective tissue (Bourboulia and Stetler-Stevenson, 2010). Meanwhile, the anti-tyrosinase activity of CAW is thought to be attributed to flavonols and hydrocinnamic acids, which have been detected in the extracts, rather than the active triterpenoids (see Chapter 3). A study by Puttarak *et al.* (2016) showed that pentacyclic triterpene-enriched *C. asiatica* extract inhibited tyrosinase with an IC<sub>50</sub> value of 104.8 µg/mL, while the individual active compounds (asiatic acid, madecassic acid, asiaticoside, and madecassoside) themselves showed no anti-tyrosinase activity. Flavonols are weak tyrosinase inhibitors (Chang, 2012), but hydroxycinnamic acid and its derivatives have been shown to inhibit melanin synthesis through various mechanisms including the inhibition of tyrosinase, suggesting potential synergy in the activity observed. Dicaffeoylquinic acids isolated from coffee beans, which are also detected in *C. asiatica* extracts (see Chapter 3), have been shown to have twofold anti-tyrosinase activity compared with arbutin and ascorbic acid standards (Iwai *et al.*, 2004). This

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<sup>7</sup> TIMP=Tissue Inhibitor Matrix Metalliproteinase

is further supported by Tabassum *et al.* (2016), who found that 4,5-O-dicaffeoylquinic acid significantly reduced melanin synthesis and tyrosinase activity in a dose-dependent manner in melanocytes with no cytotoxicity effect observed *in vivo*. Thus, it can be postulated that the anti-collagenase and anti-tyrosinase activity exhibited by CAW and CAE are due to the presence of pentacyclic triterpenes and hydrocinnamic acids respectively.

#### **5.4.3 *C. ternatea*: potential inhibitor of collagenase and elastase**

Both *C. ternatea* extracts showed dose-dependent effects in anti-collagenase and anti-elastase activity, but no activity was observed in the anti-tyrosinase assay. *C. ternatea* extract from the leaves has been shown to possess both anti-collagenase and anti-elastase activity (Mukherjee *et al.*, 2012). However, an extensive literature search suggests that this is the first report of anti-collagenase and anti-elastase activity of the flowers of *C. ternatea*. The presence of different classes of flavonoids such as flavonols and anthocyanins in both extracts, as shown in Chapter 3, may have caused the observed activity. Quercetin, a flavonol, has been shown to significantly inhibit collagenase as well as elastase (Melziq *et al.*, 2001; Sin and Kim, 2005). Similarly, delphinidin, kaempferol and myricetin have also been shown to exhibit both collagenase and elastase activity, but at lower levels than quercetin (Sartor *et al.*, 2002). However, *C. ternatea* extracts (CTW and CTE) did not show the expected tyrosinase inhibition. Anthocyanins have been shown to inhibit both mushroom and human tyrosinase (Jhan *et al.*, 2016). Therefore, the issue of the stability of the compounds in experimental conditions at different values of pH and temperature may have diminished the anticipated activity (Devi *et al.*, 2012; Hellström *et al.*, 2013; Oancea and Drăghici, 2013). This is further supported by a study by Hwang *et al.* (2013) which showed that liposome-encapsulated anthocyanin, which is more stable and has a longer half-life is able to increase the inhibition of melanin synthesis

in human A375 melanoma cells through the enhancement of tyrosinase inhibition. This may explain the absence of anti-tyrosinase activity found in this study.

#### **5.4.4 *C. caudatus*: potential inhibitor of anti-collagenase, anti-elastase and anti-tyrosinase**

Both *C. caudatus* extracts showed significant dose-dependent inhibition against collagenase, elastase and tyrosinase, except in the anti-tyrosinase assay where CAE did not show any activity. An extensive literature search shows that these activities have not yet been reported for *C. caudatus* extracts (Cheng *et al.*, 2015; Chan *et al.*, 2016; Moshawih *et al.*, 2017). Flavonols glycosides of quercetin and kaempferol derivatives, and two apigenins (vicenin-2 and vitexin) are the flavonoids detected in both extracts. These compounds, as previously discussed, have been shown to inhibit collagenase and elastase activity (Melziq *et al.*, 2001; Sartor *et al.*, 2002; Sin and Kim, 2005), while apigenins may contribute to anti-collagenase activity as the compounds were shown to inhibit collagenase in RAW 264.7 macrophage cells (Lee *et al.*, 2007). In the anti-tyrosinase assay, CCW exhibited the highest tyrosinase inhibition of 72.6% at 1000 µg/mL compared with all other extracts. The flavonoids detected, as previously mentioned, may be responsible for the observed activity. Quercetin has been shown to be a competitive tyrosinase inhibitor able to inhibit melanin production dose-dependently in B16 melanoma cells (Chen and Kubo, 2002; Fujii *et al.*, 2009). Although some conflicting results have been reported on the role of quercetin in melanogenesis (Choi and Shin, 2016), it is postulated that the inhibitory activity of quercetin is strictly dependent on the dosage introduced, where the inhibitory activity against tyrosinase would then be observed as shown by Yang *et al.* (2011).

### **5.5 Conclusion**

The anti-collagenase, anti-elastase and anti-tyrosinase activity of the plant extracts varies irrespective of the TPC. *M. oleifera* extracts were shown to have the highest TPC but, neither

extracts were active as inhibitors of the enzymes investigated. In general, flavonoids such as flavonols, flavanes and anthocyanins are the compounds responsible for the anti-collagenase, anti-elastase, and anti-tyrosinase activity exhibited by the extracts.



# **Chapter 6**



## Chapter 6: *In vitro* protective effect against hydrogen peroxide-induced cytotoxicity in HaCaT cells

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### 6.1 Introduction

In Chapter 4, plant extracts were demonstrated to have antioxidant activities that may play a role in providing a protective effect against induced-oxidative stress in the skin. These antioxidant properties are further investigated in cell cultures, where oxidative stress is induced by hydrogen peroxide. In the presence of transition metals, hydrogen peroxide can generate a hydroxyl radical, which is the most reactive free radical through the Fenton reaction (see equation 4.3). Hydrogen peroxide has been extensively used to induce oxidative stress in cell culture, and has been shown to be cytotoxic at  $\geq 50 \mu\text{M}$  depending on cell type and experimental conditions (Ines *et al.*, 2010; Kumar *et al.*, 2010; Liu *et al.*, 2012).

#### 6.1.1 HaCaT as an *in vitro* model to study skin ageing

HaCaT cells are a spontaneously immortalized aneuploid<sup>8</sup> human keratinocyte cell line derived from adult human skin (Boukamp *et al.*, 1988). The cell line is widely used in scientific research due to its ability to proliferate and maintain the capacity for epidermal differentiation. With a transformed phenotype, the cell line is immortal and clonogenic like cancer cells, but it is non-tumorigenic. In comparison to keratinocytes of rodent origin, which are prone to transformation and spontaneous neoplastic<sup>9</sup> conversion (Sanford and Evans, 1982), HaCaT is relatively resistant. Boukamp *et al.* (1988) demonstrated that the cell line's DNA fingerprint remains unaffected by continuous culturing, transformation or chromosomal alteration. Given these properties of HaCaT, this cell line is suitable for skin research, representing 80-95% of the human keratinocytes in the epidermal layer of the skin (Kolarsick *et al.*, 2011). It is the first

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<sup>8</sup> A condition with an abnormal chromosome number compared to the wild type

<sup>9</sup> Abnormal new growth of tissue; often tumorigenic

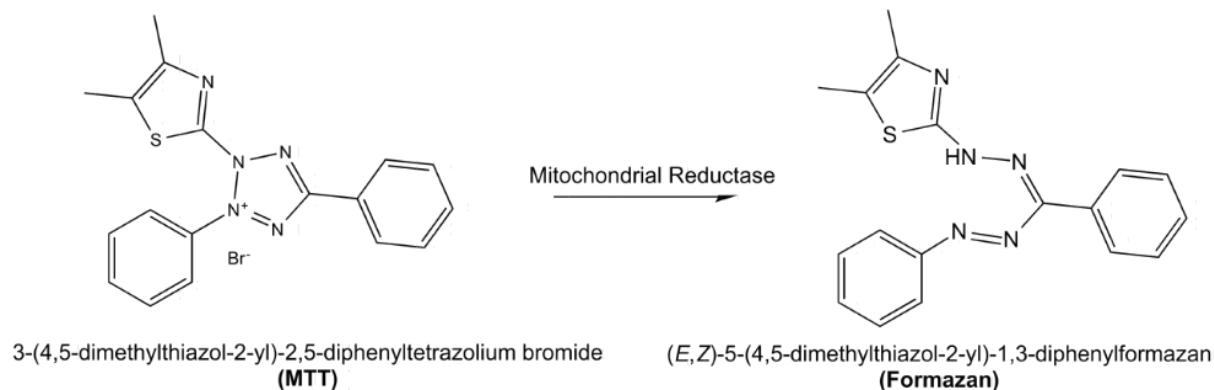
line of defense against various factors implicated in the ageing of the skin. Normal human epidermal keratinocytes (NHEK) are another commonly used cell model for keratinocyte research, but issues such as ethics, donors and ease of maintenance make HaCaT a better option for this study (Ines *et al.*, 2010; Liu *et al.*, 2012, 2016; Park *et al.*, 2012).

### 6.1.2 Principles of the assay

The principles of the methods used in this chapter are described below.

#### 6.1.2.1 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

MTT is the most commonly used method to measure cell viability, because it is simple, fast and economical. The mitochondrial oxidoreductase in the cells cleave the MTT dye and produce insoluble formazan (Figure 6.1). Formazan is soluble in organic solvents and will produce a deep purple colour measurable at 570 nm.



**Figure 6.1: Reduction of MTT (yellow) to formazan (purple) by mitochondrial reductase.**

#### 6.1.2.2 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

The MTS assay has the same principle as the MTT assay. MTS has advantages over MTT because the formazan produced is soluble in the cell culture medium and no organic solvent is needed. An additional step to dissolve the formazan, as with MTT, is not required in this

assay. In both assays, the optical density (OD) measured at 570 nm (MTT) and 490 nm (MTS) is directly proportional to the number of living cells.

### **6.1.3 Chapter aim and objectives**

This chapter aims to investigate the protective effect of the plant extracts against hydrogen peroxide-induced cytotoxicity in HaCaT cells. However, some interference was observed using the MTT assay for this experiment and the MTS assay was later considered. Therefore, the objectives of this chapter are outlined as below:

- ✓ To evaluate the suitability of both MTT and MTS assays for the experiment
- ✓ To determine the optimal hydrogen peroxide concentration for the experiment
- ✓ To investigate the protective effect of the plant extracts against hydrogen peroxide-induced cytotoxicity

## 6.2 Materials and Methods

### 6.2.1 Cell culture

**Preparation of reagents and materials:** Dulbecco's modified eagle media (DMEM), containing 4.5 g/L glucose, (+) L-glutamine and (-) pyruvate, trypsin and foetal calf serum (FCS), was purchased from Lonza, UK. Phosphate buffered saline (PBS) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich, UK. General reagents for cell culture maintenance were as described in Table 6.1.

**Table 6.1: Preparation of general cell culture reagents.**

Reagent	Protocol
Complete DMEM	Complete DMEM was prepared by adding 50 mL of FCS and 5 mL of pen-strep to obtain complete DMEM with 10% FCS and 1% pen-strep. The complete DMEM was used in culturing, maintenance of HaCaT and assays.
Freezing medium	The FCS used for freezing the HaCaT cells contained 10% DMSO to reduce the formation of ice crystals that can puncture the plasma membrane. 45 mL of FCS was added to 5 mL of sterile DMSO and mixed. The medium was added to the cells to be frozen.

#### 6.2.1.1 Culturing, splitting, maintenance and freezing

**Culturing and splitting:** A confluence HaCaT in a T-75 flask was received from the Newcastle University Medical School at passage 6 (P-6). Under a sterile fume hood, the medium used was aspirated, and cells were gently rinsed with PBS (10 mL). Later, trypsin (2 mL) was added to the flask and incubated for 5 minutes at 37 °C to facilitate the activity of trypsin on HaCaT. After incubation, the flask was gently tapped, and cells were observed under the microscope to ensure that they had detached from the flask. Under the hood, complete DMEM (5 mL) was added and the cell mixture was equally divided into three new T-175 flasks. The volume in each flask was made up to 15 mL with complete DMEM (see Table 6.2 for reference). The flask was further incubated at 37 °C with 5% CO<sub>2</sub> until confluent.

**Table 6.2: Flask size with volume of PBS, trypsin and DMEM for maintenance.**

Flask (size)	PBS (mL)	Trypsin (mL)	Complete DMEM (mL)
T25 (25 cm <sup>2</sup> )	5	1	7
T75(75 cm <sup>2</sup> )	10	2	15
T175 (150 cm <sup>2</sup> )	15	5	35

**Freezing:** Confluent T-175 flasks (P-7) were observed under the microscope to ensure the cells were free from contamination and infection. Under the hood, the media used was aspirated and cells were rinsed with PBS (10 mL). One flask was passaged as previously described (see culturing and splitting). For freezing cells, the media used was aspirated, rinsed with PBS (10 mL) and added with trypsin (5 mL). The flask was incubated for 5 minutes in the incubator. After incubation, cells were added with complete DMEM (5 mL) and transferred in equal amounts into two universal tubes. The tubes were centrifuged for 5 minutes at 15000 rpm/min. After centrifugation, the media was carefully removed without disturbing the cell pellets. The cell pellet in each tube was added with 3 mL of freezing medium (see Table 6.1) and combined. Cells in the freezing medium were equally divided into cryotubes (1mL/tube), labelled (with the cells' name, date, and passage number) and frozen at -80 °C overnight in a container containing propanol to facilitate freezing. The cryotubes were transferred into a nitrogen tank for storage.

**Thawing:** The cryotubes from the nitrogen tank were brought to room temperature. Cells were transferred into a universal tube, complete DMEM (5 ml) was added and centrifuged for 5 minutes at 1500 rpm/min. The medium was removed and the protocol for culturing cells was followed.

### **6.2.1.2 Cell counting**

Cell counting involved three steps: preparation of the cell suspension, mounting on the haemocytometer and counting.

**Cell suspension preparation:** Confluent cells were trypsinised and made up to 10 mL using complete DMEM. A sample of the cell solution (0.5 mL) was pipetted into an Eppendorf tube. Later, the cells (100  $\mu$ L) were gently mixed with 0.4% trypan blue (100  $\mu$ L) in a new Eppendorf tube. Trypan blue causes dead cells to appear blue while living cells appear clear.

**Cell mounting:** A coverslip was carefully placed on a sterile haemocytometer. The cells treated with trypan blue were pipetted (20  $\mu$ L) into a small groove on the haemocytometer and subsequently drawn by capillary action to fill the counting chamber under the coverslip (see Figure 6.2 A).

**Cell counting:** The counting grids have 9 large squares with 1  $\text{mm}^2$  area and 0.1 mm depth. Hence, the total counting grid had a total volume of 0.9  $\text{mm}^3$  (3 mm x 3 mm x 0.1 mm), as shown in Figure 6.2 B. Cells were counted in four large corner squares and the central square where the cells that fall on the top and left of the grid were counted “in” and those on the bottom and right were counted “out” (see Figure 6.2 C). The totals of cells counted were summed and averaged.

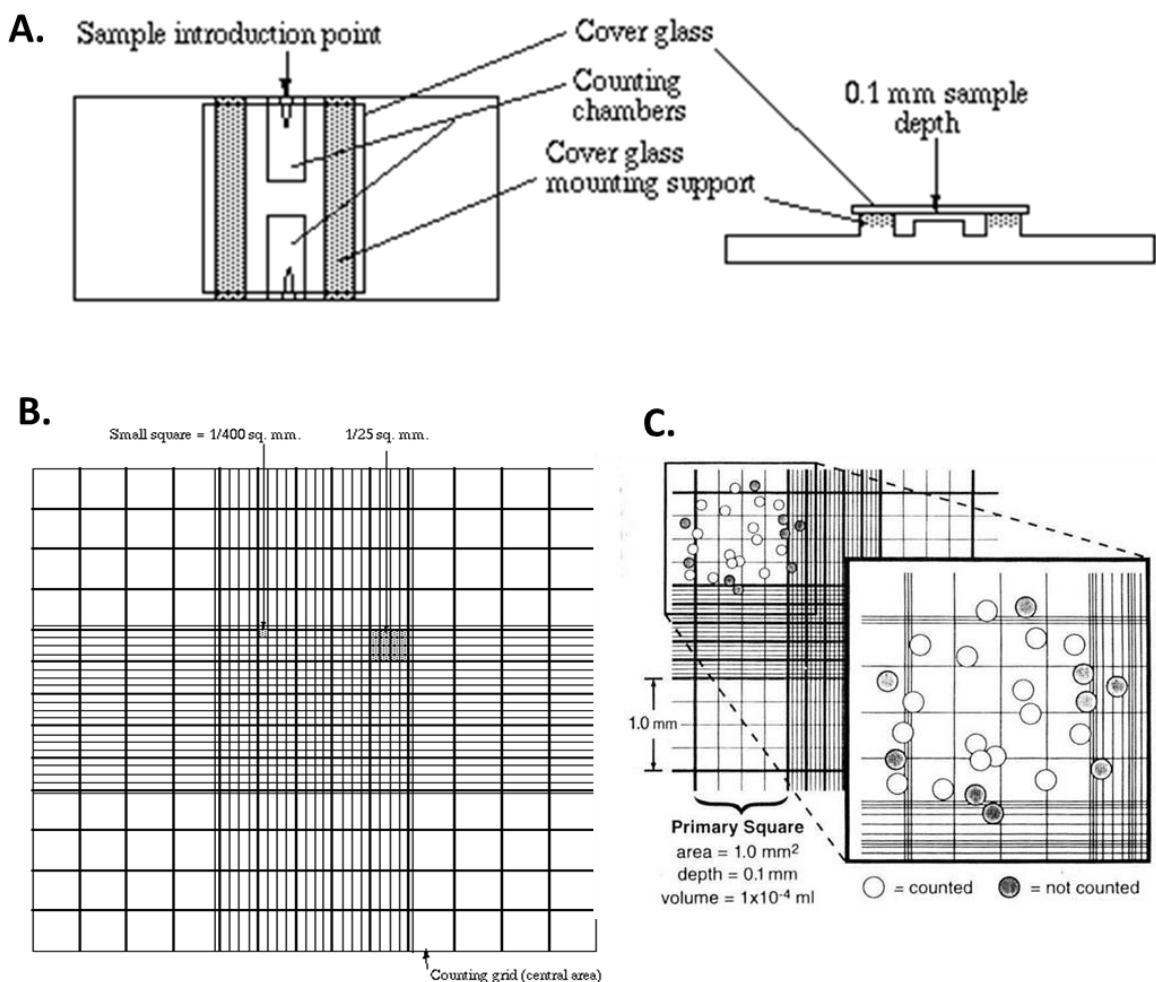
#### 6.2.1.3 Total cell calculation

The formula used to calculate cell total is:

$$\text{Total cells} = \left( \frac{\text{Total cells counted}}{\# \text{ of squares}} \right) \times \text{Dilution factor} \times 10^4 \text{ cells/mL} \times \text{volume (mL)} \quad (6.1)$$

For example, 325 cells were counted in 4 corner squares and the central big squares. Samples were diluted 1:1 with trypan blue. The total cell volume was 5 mL.

$$\text{Total cells} = (325 \text{ cells}/5) \times 2 \times 10,000 \text{ cells/mL} \times 5 \text{ mL} = 650 \times 10^4 \text{ cells}$$



**Figure 6.2: Hemocytometer (A), counting grid (B) and counting system (C).**

### 6.2.2 MTT assay

**Preparation of reagents and materials:** The MTT yellow powder (M5655-500MG) and hydrogen peroxide were purchased from Sigma Aldrich, UK. The MTS solution was purchased from Promega, UK. Reagents for the MTT assay were prepared as suggested by the manufacturer (see Table 6.3), while the MTS solution was used as supplied.

**Table 6.3: MTT reagents preparation.**

Reagent	Protocol
MTT stock (5 mg/mL)	The MTT yellow powder was weighed (50 mg) in a beaker and dissolved in 10 mL of PBS. The solution was sonicated for 5 minutes and filtered through a 0.2 $\mu$ M filter to obtain a

	homogenous cell culture grade reagent. The stock was aliquoted in small 1-2 mL vials and frozen at 20 °C until further use.
MTT-DMEM working reagent (0.5 mg/mL)	1 mL of the MTT stock was diluted in 9 mL of phenol-free complete DMEM.

#### 6.2.2.1 MTT assay protocol

After overnight incubation, the phenol-free DMEM was carefully aspirated using an automated aspirator from the 96-well plate. The cells were rinsed with PBS (100 µL) and fresh DMEM was added (100 µL). Later, MTT-DMEM working reagent (100 µL) was pipetted into each well using a multi-channel pipette. The plate was further incubated for 4 h at 37 °C. After incubation, the medium was aspirated and 100 µL of DMSO: ethanol (1:1) was added. The plate was read at 570 nm using a plate reader and the percentage cell viability was calculated (see 6.2.4).

#### 6.2.3 MTS assay protocol

After overnight incubation, the phenol-free DMEM was aspirated from the 96-well plate. Cells were rinsed with PBS (100 µL) and added with fresh complete DMEM (100 µL). Later, the MTS One Solution Cell Proliferation reagent (20 µL) was added to each well and the plate was incubated for 4 h at 37 °C. After incubation, the OD reading was measured at 490 nm and the percentage cell viability was calculated (see 6.2.4).

#### 6.2.4 Percentage cell viability

Cell viability was calculated using the following formula.

$$\text{Cell viability (\%)} = \frac{\text{Treatment (OD treated well)}}{\text{Control (OD nontreated well)}} \times 100 \quad (6.2)$$

OD treatment and control are the corrected values of absorbance from blank.

## **6.2.5 Pre-experiment to determine the experimental conditions**

### **6.2.5.1 Effect of phenol red on HaCaT growth**

A preliminary experiment showed that phenol red interfered with the MTT assay (data not shown). An experiment to investigate the effect of DMEM with and without phenol on HaCaT growth was performed. A confluent T-175 flask was passaged (1:3) into two new flasks. One flask was grown in complete DMEM (1% pen-strep and 10% FBS) with phenol red, and the other flask was grown in complete DMEM without phenol red. Any morphological changes when the cells reached confluence were observed.

### **6.2.5.2 Determination of optimal cell concentration and incubation time**

HaCaT (100 µL) at different cell concentrations (10 000, 25 000, 50 000 and 100 000 cells/ well) was seeded in a 96-well plate. Three sets of plates with these cell concentrations were prepared. The plates were left in an incubator overnight at 37 °C in a humidified environment, where the outer well of the 96-well plate was filled with PBS (100 µL) to avoid evaporation of DMEM. Then, the plate was placed in a loosely covered sterile plastic container, and a sterile-folded blue rolled wet with PBS was placed on the bottom of container. After 24 h, a plate was performed with the MTT assay, whilst the other were changed with fresh DMEM and further incubated. The same procedures were repeated until all plates were performed with the MTT assay.

### **6.2.5.3 Experiment to compare the efficiency of MTT vs. MTS assay**

HaCaT (100 µL) at different concentrations was seeded in the 96-well plate (5000, 10,000, 15,000, 20,000 and 25,000 cells/well) and allowed to settle overnight in a humidified environment. Two sets of cell concentrations were seeded in the 96-well plate, one set for the MTT assay and the other for MTS. Four plates with the same experimental set-up were

prepared. After each 24 h, MTT/MTS assays were performed. OD values at 570 nm/490 nm at each cell concentration were plotted against time (24, 36, 48 and 72 h).

#### **6.2.5.4 Effect of hydrogen peroxide at different concentrations on HaCaT**

The hydrogen peroxide reagents were prepared as shown in Table 6.4.

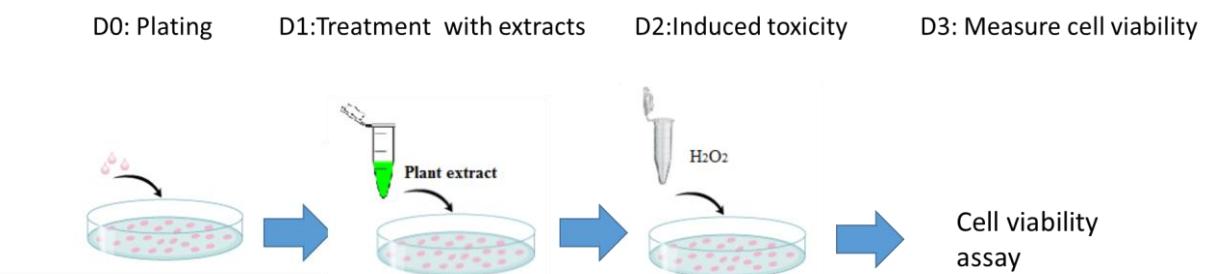
**Table 6.4: Hydrogen peroxide reagent preparation.**

Reagent	Protocol
1 M H <sub>2</sub> O <sub>2</sub>	5.67 mL of the 30 % H <sub>2</sub> O <sub>2</sub> (8.82 M) was diluted with 44.33 mL sterile water to obtain 50 mL of 1 M H <sub>2</sub> O <sub>2</sub> . The solution was filtered through a 0.2 µM filter to obtain a cell culture grade reagent.
2000 µM H <sub>2</sub> O <sub>2</sub>	20 µL of 1 M H <sub>2</sub> O <sub>2</sub> was diluted with 9.98 mL of sterile water to obtain 10 mL of 2000 µM H <sub>2</sub> O <sub>2</sub> solution. The solution was used as a stock to make the working H <sub>2</sub> O <sub>2</sub> reagents (30, 60, 150, 300, 600, 1200, 1500 and 1800 µM). All the reagents were kept at 4 °C.
1200 µM H <sub>2</sub> O <sub>2</sub>	24 mL of 1000 µM H <sub>2</sub> O <sub>2</sub> was diluted with 16 mL of water to obtain 40 mL of 1200 µM H <sub>2</sub> O <sub>2</sub> . 20 µL of this working reagent was added to 100 µL volume of medium in the 96 wells plate to reach 200 µM of H <sub>2</sub> O <sub>2</sub> as the final concentration (DF = 6).

**Experimental protocol:** HaCaT (100 µL) was seeded in a 96-well plate at 10,000 cells/well and allowed to settle overnight. The next day, the medium was aspirated and fresh complete phenol-free DMEM (100 µL) was pipetted into each well. Later, hydrogen peroxide working reagents (20 µL), as prepared in Table 6.4, were pipetted into the wells to obtain final concentrations of 0, 5, 10, 25, 50, 100, 200, 250 and 300 µM (DF = 6). After 24 h, the medium was aspirated and rinsed with PBS (100 µL). The MTS assay was performed as previously described. The experiment was repeated for 48 and 72 h of incubation.

#### 6.2.6 Protective effect of the plant extracts in H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity

HaCaT (100 µL) at 10 000 cells/well were plated and allowed to settle overnight at 37 °C. The medium was aspirated and fresh-complete DMEM was added (100 µL). Plant extracts at appropriate concentrations (20 µL) were added and the plate was incubated for 24 h. The medium was aspirated, cells were rinsed with PBS (100 µL) and added with fresh complete DMEM (100 µL). Later, 20 µL of 1200 µM H<sub>2</sub>O<sub>2</sub> was added to induce toxicity, except in the positive control wells where water (20 µL) was added. The plate was further incubated for 24 h before the MTS assay was performed. The final concentration of hydrogen peroxide in the well was 200 µM (DF = 6). The percentage cell viability compared to the control was calculated (see 6.2.4). The experimental sequence is illustrated in Figure 6.3.



**Figure 6.3: Experimental design.** On day 1, treatment with the plant extracts were introduced to HaCaT. On Day 2, cytotoxicity was induced with hydrogen peroxide and on day 3 cell viability was measured. This experimental design was modified from Kumar *et al.* (2010).

## 6.3 Results

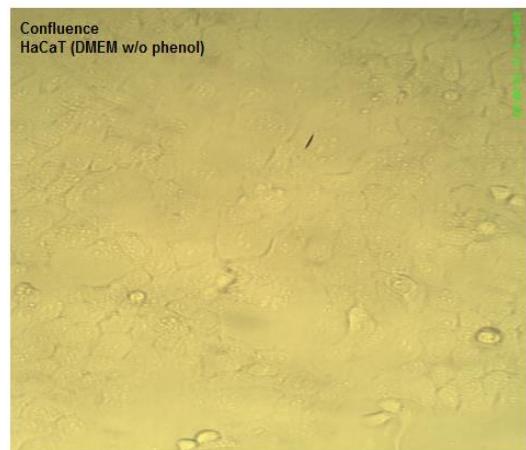
### 6.3.1 Phenol red has no effect on HaCaT growth

HaCaT cultured in regular DMEM vs. phenol-free DMEM reached confluence at the same time (2-3 days after splitting). There was no morphological difference between the two sets of HaCaT, suggesting that phenol red has no significant effect on its growth (see Figure 6.4). Therefore, all subsequent experiments were performed using phenol-free DMEM to reduce background interference.

**A. +phenol**



**B. -phenol**

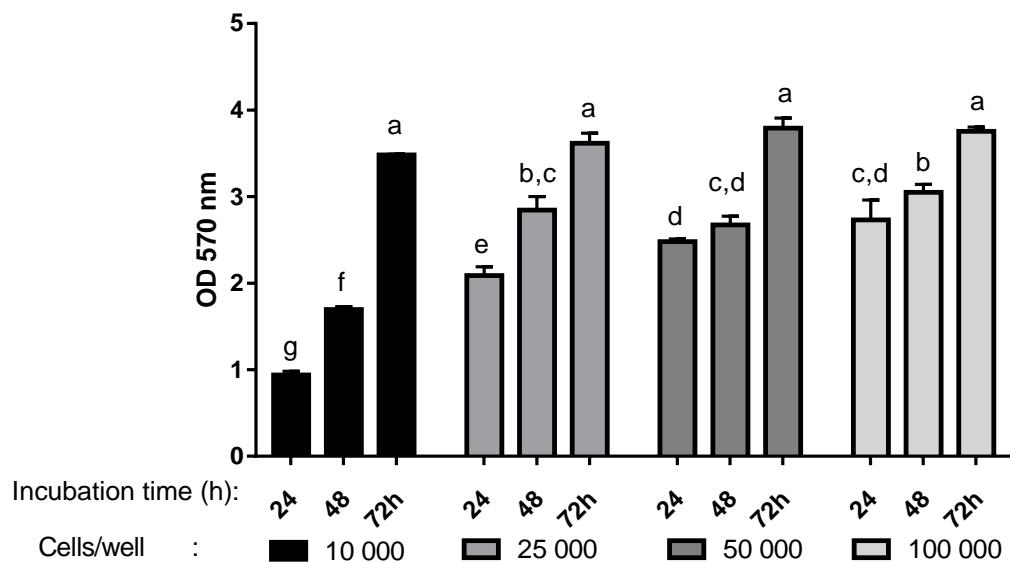


**Figure 6.4: Effect of DMEM with (A) and without phenol red (B) on HaCaT growth.**

### 6.3.2 Determination of optimal cell number and incubation time

**Cell number:** Cell concentration at 10,000 cells/well and 25,000 cells/well showed linear growth from 24-72 h as compared with cells at higher concentrations (50,000 and 100,000 cells/well) (see Figure 6.5). Optimal cell number was deduced to be in the range of 10,000-25,000 cells/well.

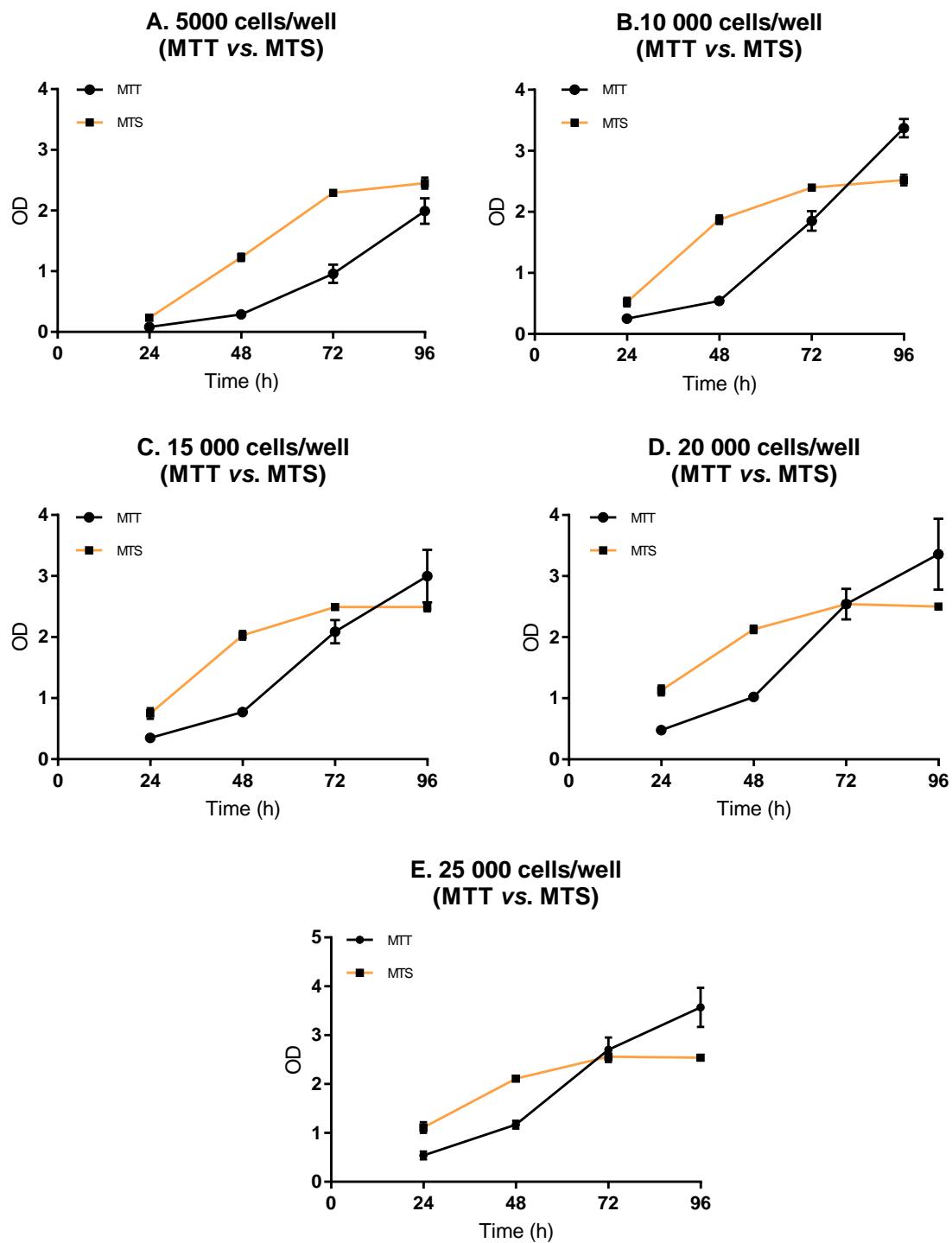
**Incubation time:** Incubation times (24 h and 48 h) seem to be the optimal incubation time for 10,000 and 25,000 cells/well because after 72h the OD readings reached the limit of the spectrophotometer (OD 4.00).



**Figure 6.5: OD readings at 570 nm after 24, 48 and 72 h at different cell concentrations.** Different letters indicate significant mean differences ( $p<0.05$ ). Data are the mean  $\pm$  SD of four replicates.

### 6.3.3 Comparison of MTT and MTS assays

The formazan produced in the MTT assay was sometimes aspirated with the medium used prior to the addition of MTT solvent, which affected the final readings of the experiment. In this experiment, the MTS assay showed consistent readings with small standard deviations (SDs) at all incubation times compared to the MTT assay (Figure 6.6). At higher cell concentrations, discrepancies between readings were observed (with large SDs) in the MTT assay, particularly after 72h and 96 h of incubation.



**Figure 6.6: Comparison of MTT vs. MTS optical density (OD) readings.** Figures show measurements at 5000 (A), 10 000 (B), 15 000 (C), 20 000 (D) and 25 000 (E) cells/well. Data are the mean  $\pm$  SD of six replicates.

#### 6.3.4 Cytotoxicity of hydrogen peroxide on HaCaT induced at 24, 48 and 72 h

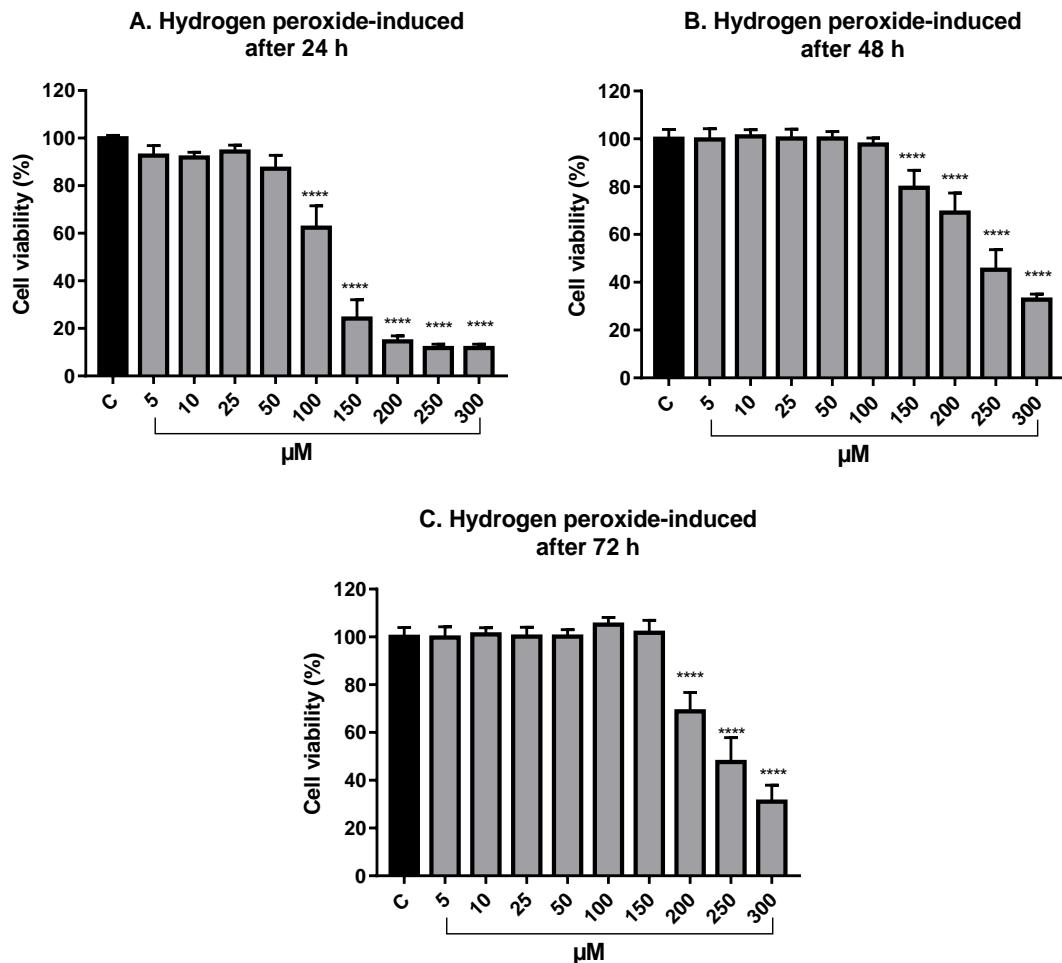
Figure 6.7 A, B and C shows the results for toxicity induced at days 1, 2 and 3 respectively.

Toxicity induced at day 1:  $\text{H}_2\text{O}_2$  concentrations of 5-50  $\mu\text{M}$  were non-toxic to HaCaT. At 100 and 150  $\mu\text{M}$ ,  $\text{H}_2\text{O}_2$  induced cell cytotoxicity causing  $33.7 \pm 3.46$  and  $75.8 \pm 7.86$  % cell death respectively. At higher concentrations of  $\text{H}_2\text{O}_2$  (200-300  $\mu\text{M}$ ), 85-90% cell death was observed.

Toxicity induced at day 2:  $\text{H}_2\text{O}_2$  concentrations of 5-100  $\mu\text{M}$  were non-toxic to HaCaT. Significant reductions in cell viability were observed in HaCaT treated with 150, 200, 250 and 300  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  compared with the control well. The percentages of cell death observed at the respective concentrations were  $20.4 \pm 7.23$  % (150  $\mu\text{M}$ ),  $30.8 \pm 8.20$  % (200  $\mu\text{M}$ ),  $54.8 \pm 8.43$  % (250  $\mu\text{M}$ ) and  $67.2 \pm 2.26$  % (300  $\mu\text{M}$ ).

Toxicity induced at day 3:  $\text{H}_2\text{O}_2$  concentrations of 5-150  $\mu\text{M}$  were non-toxic to HaCaT. Treatment with 200, 250 and 300  $\mu\text{M}$  caused  $31.2 \pm 7.98$  %,  $52.33 \pm 10.15$  % and  $68.9 \pm 6.87$  % cell death respectively. This experiment showed the concentration- and time-dependence of the effect of  $\text{H}_2\text{O}_2$  on HaCaT.

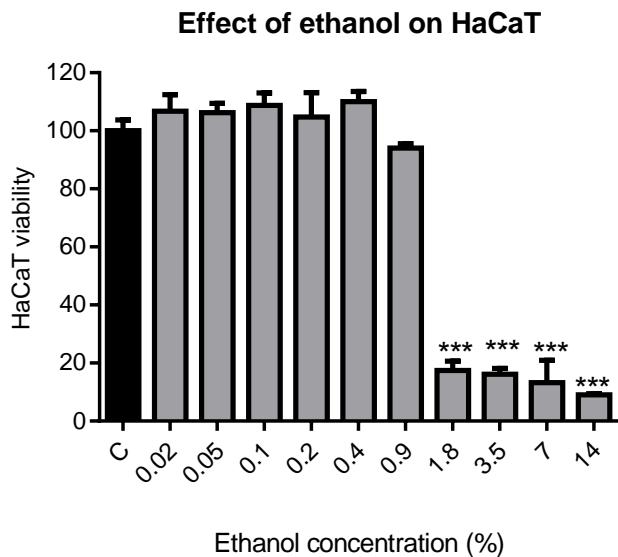
Therefore, an  $\text{H}_2\text{O}_2$  concentration of 200  $\mu\text{M}$  ( $\sim 30$  % cell death) induced at day 2 was selected as the most appropriate concentration for the experiment.



**Figure 6.7: Cytotoxicity effect of hydrogen peroxide on HaCaT after 24 h (A), 48 h (B) and 72 h (C).** Data are the mean  $\pm$  SEM of two experiments performed in six replicates for each treatment. \*\*\* $p<0.0001$ .

### 6.3.5 Effect of solvent on HaCaT

Figure 6.8 shows that ethanol concentrations of  $< 1\%$  did not cause significant cell death compared with the untreated control group. Therefore, ethanolic samples were prepared appropriately to ensure that the final concentration of ethanol was  $< 1\%$ .



**Figure 6.8: Cytotoxicity of ethanol on HaCaT.** Data are the mean  $\pm$  SD (n=8) and \*\*\* $p < 0.001$ .

### 6.3.6 Cytotoxicity effect of the plant extracts on HaCaT

Table 6.5 shows the cytotoxicity effect of the extract alone on HaCaT. All the plant extracts have different cytotoxicity effects on HaCaT. MOE is more toxic than MOW, CAE is more toxic than CAW, CTE is more toxic than CTW and CCE is more toxic than CCW. In general, all the ethanol extracts from each plant species were more toxic than the aqueous extract.

**Table 6.5: Cytotoxic and non-cytotoxic concentrations of the plant extracts on HaCaT.** The marked (\*) concentrations showed significant reductions in percentage viability compared with control (untreated HaCaT) and are considered cytotoxic to HaCaT at \* $p < 0.05$ .

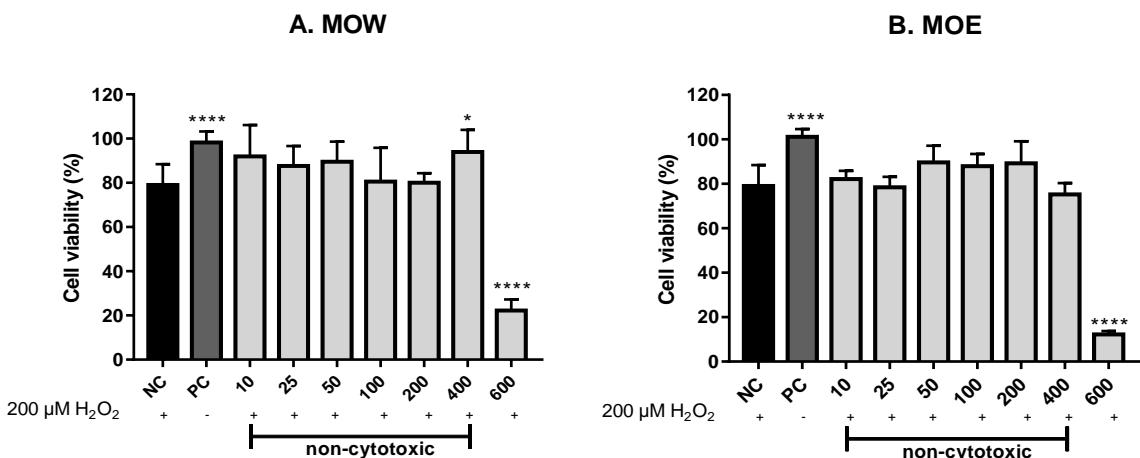
Plant species	Extracts	Concentration ( $\mu\text{g/mL}$ )	Highest nontoxic concentration ( $\mu\text{g/mL}$ )
<i>M. oleifera</i> Lam.	MOW	10, 25, 50, 100, 200, 400, 600, 800*, 1000*	600
	MOE	10, 25, 50, 100, 200, 400, 600*, 800*, 1000*	400
<i>C. asiatica</i> (L.) Urban.	CAW	250, 500, 1000, 1500*, 2000*, 2500*, 3000*	1000
	CAE	50, 100, 250, 500, 500*, 600*, 800*, 1000*, 2000*	250
<i>C. ternatea</i> L.	CTW	50, 100, 250, 500, 1000*, 1500*, 2000*, 2500*, 3000*	500

	CTE	50, 100, 200, 300, 400, 600*, 800*, 1000*, 1500*	400
<i>C. caudatus</i> Kunth	CCW	500, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000	8000
	CCE	20, 40, 60, 80, 100*, 125*, 250*, 500*	80

### 6.3.7 Protective effect against hydrogen peroxide-induced cytotoxicity

#### 6.3.7.1 *M. oleifera* Lam.

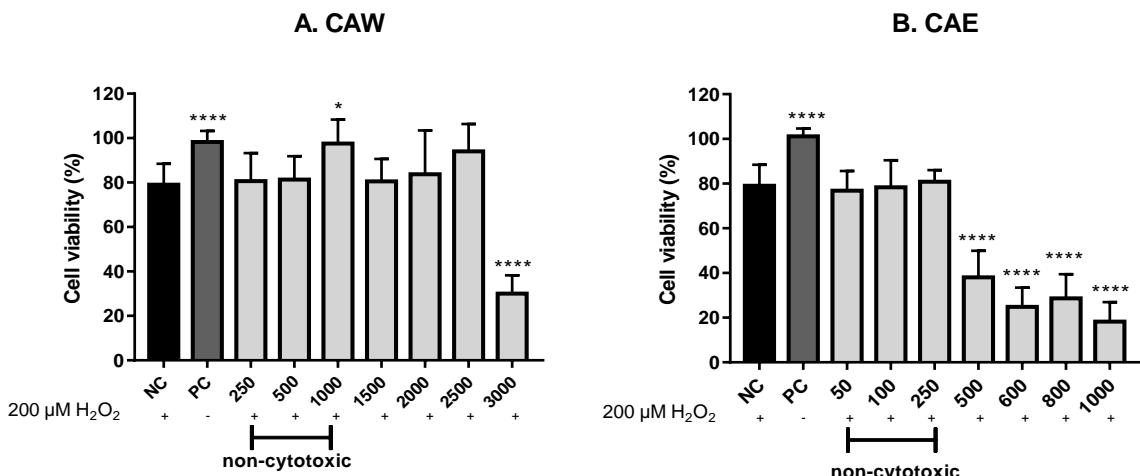
MOW and MOE (10-400 µg/mL) did not show any protective effect against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in HaCaT, except for MOW at 400 µg/mL where significantly high cell viability (94%) was observed compared with the negative control (NC), as shown in Figure 6.9. At 600 µg/mL, both MOW and MOE were toxic to HaCaT.



**Figure 6.9: Protective effect of MOW (A) and MOE (B) against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity (200 µM).** The mean differences between positive control (PC) and treatments were compared with negative control (NC). Data are the mean and SEM of three experiments with \*p<0.05 and \*\*\*\*p< 0.0001.

### 6.3.7.2 *C. asiatica* (L.) Urban

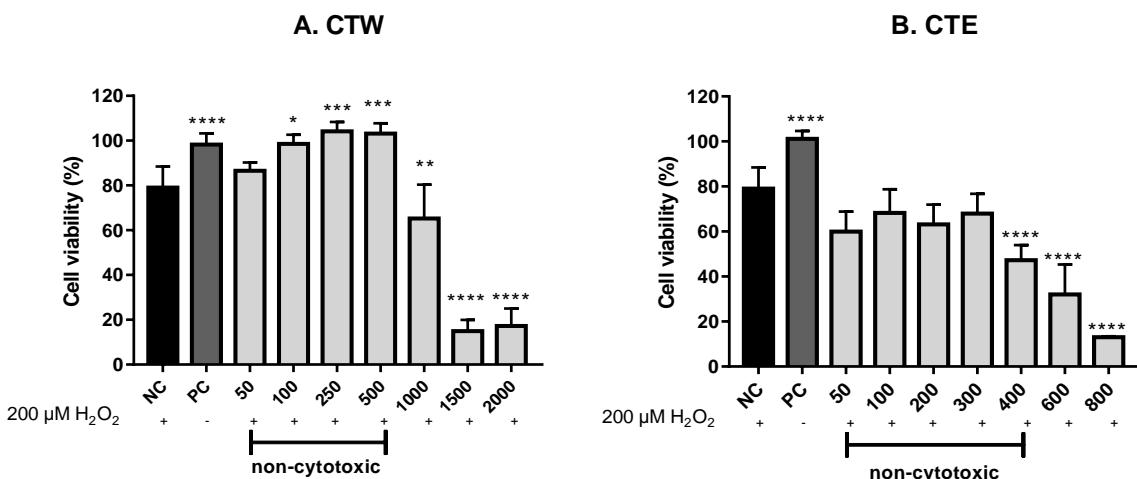
HaCaT treated with CAW at 1000  $\mu\text{g}/\text{mL}$  showed significantly higher percentage viability (98 %) than NC (79 %). Meanwhile CAE treatment did not show any protective effect (see Figure 6.10 A and B).



**Figure 6.10: Protective effect of CAW (A) and CAE (B) against  $\text{H}_2\text{O}_2$ -induced cytotoxicity (200  $\mu\text{M}$ ).** The mean differences of positive control (PC) and treatments were compared with negative control (NC). Data are the mean and SEM of three experiments with \* $p<0.05$  and \*\*\*\* $p<0.0001$ .

### 6.3.7.3 *C. ternatea* L.

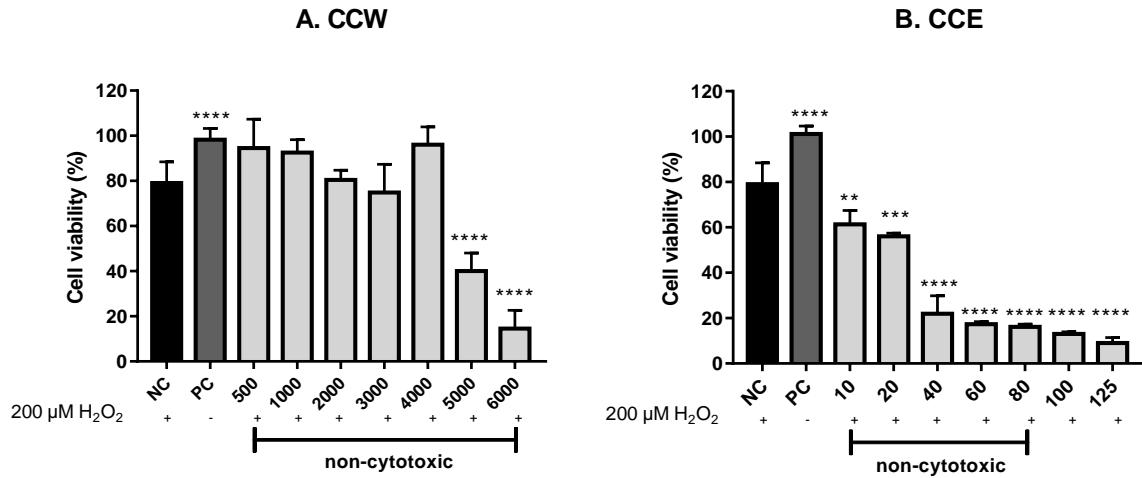
As shown in Figure 6.11 A, CTW concentrations of 100, 200 and 250 µg/mL protect HaCaT against hydrogen peroxide-induced cytotoxicity with significantly higher percentage viability observed (99 %, 104 % and 103 % respectively) compared with NC (79 %). CTW concentration of 50 µg/mL seemed to protect HaCaT, with 87 % cell viability but this figure was not significantly different from the NC. CTE treatment did not show any protective effect (see Figure 6.11 B).



**Figure 6.11: The effect of CTW (A) and CTE (B) against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity (200 µM).** The mean differences between positive control (PC) and treatments compared with negative control (NC) were determined using ANOVA. Data are the mean and SEM of three experiments with \*p<0.05 and \*\*\*\*p< 0.0001.

#### 6.3.7.4 *C. caudatus* Kunth.

HaCaT treated with 500, 1000 and 4000  $\mu$ g/mL seemed to protect HaCaT against hydrogen peroxide with higher cell viability observed (95 %, 93 % and 96 % respectively) but the differences were not significant when compared with NC (see Figure 6.12 A). CCE did not show any protective effect at any concentration (see Figure 6.12 B).



**Figure 6.12: The effect of CCW (A) and CCE (B) against  $\text{H}_2\text{O}_2$ -induced cytotoxicity (200  $\mu\text{M}$ ).** The mean differences between positive control (PC) and treatments compared with negative control (NC) were determined using ANOVA. Data are the mean and SEM of three experiments with \*\* $p<0.01$  and \*\*\*\* $p<0.0001$ .

## 6.4 Discussion

### 6.4.1 Determination of optimal experimental conditions

MTT vs. MTS: Initially, the MTT assay was used to determine cell viability. However, the replicates within the same sample showed a lack of precision in the absorbance readings, which affects the statistical significance of the experimental results. In the MTS assay the variation between replicate readings was reduced and this assay was used for the experiment. Other methods to determine cell viability were also considered, such as the trypan blue exclusion assay (Wang *et al.*, 2010), but MTS and MTT were faster and more time-efficient.

Solvent effect: Most organic solvents were cytotoxic, including ethanol. In the experiment, the use of ethanol in the well was limited to concentrations <1% as this level was determined to be non-cytotoxic to HaCaT. Although DMSO has been widely used in cell and tissue culture to dissolve samples with concentrations ranging from 0.5% to 10% (Da Violante *et al.*, 2002; Alerico *et al.*, 2015), ethanol was used in this study to obtain homogenously soluble extract solutions because the ethanol was used during the extraction of plant materials.

Hydrogen peroxide dose: The cytotoxic effect of H<sub>2</sub>O<sub>2</sub> has been shown to be dependent on cell type, cell number, length of exposure and the concentration used (Halliwell *et al.*, 2000; Gülden *et al.*, 2010; Ines *et al.*, 2010; Kumar *et al.*, 2010). An initial experiment using 100 µM of H<sub>2</sub>O<sub>2</sub> to induce oxidative stress in HaCaT showed that the rate of cell proliferation can diminish the cytotoxic effect under the present experimental design (data not shown). Therefore, a pre-experiment to investigate the effect of different H<sub>2</sub>O<sub>2</sub> concentrations on cell viability was performed (see Figure 6.7), and a stronger concentration of H<sub>2</sub>O<sub>2</sub> (200 µM) inducing ~30% cell death was used in the later experiments.

#### 6.4.2 Protective effect of the plant extracts against hydrogen peroxide-induced cytotoxicity

Hydrogen peroxide is a physiological constituent of living cells and is continuously produced via diverse cellular pathways. The effects of H<sub>2</sub>O<sub>2</sub> have been widely found to cause cellular oxidative damage such as apoptosis, loss of cell viability, morphological and nuclear damage, and lipid peroxidation, as well as changes in the levels of catalase, SOD and GPx antioxidant enzymes (Wijeratne *et al.*, 2005; Prasanna and Sreelatha, 2014). In this experiment, the protective effect of plant extracts against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity was investigated, and MOW, CAW and CTW showed significant protective effects.

The aqueous extract of *M. oleifera* (MOW) showed a significant protective effect against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in HaCaT, but the effect was only observed at the highest non-toxic concentration of MOW, which is at 400 µg/mL. Meanwhile, the ethanol did not show any protective effect at any concentration. Extracts of *M. oleifera* leaves have been described as ameliorating oxidative stress in several *in vitro* and *in vivo* studies (Jaiswal *et al.*, 2013; Prasanna and Sreelatha, 2014; Abdul Hisham *et al.*, 2018), but studies using human keratinocytes are very limited. In a study by Prasanna and Sreelatha (2014), *M. oleifera* ethanol extract was shown to provide a protective effect against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress, with significant improvements in cell proliferation, suppression of apoptotic events and lipid peroxidation, and significant enhancement of antioxidant enzymes. An *in vivo* study by Jaiswal *et al.* (2013) also showed improvements in antioxidant enzymes and significant reductions in lipid peroxidation with aqueous *M. oleifera* extract treatment in diabetic-induced oxidative stress. Another study by Abdul Hisham *et al.* (2018) suggests a potential synergy of hydro-ethanol extracts of *M. oleifera* and *C. asiatica* in modulating the protective effect against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in human fibroblast cells. These studies provide

evidence for the protective effect of *M. oleifera* extracts against oxidative stress, mainly due to its phenolic content, which supports the finding of a protective effect exhibited by MOW. However, the mechanism of action may be different for MOE, such as increase in antioxidant enzymes or suppression of apoptotic and lipid peroxidation events, thus explaining the result observed.

*C. asiatica* is a well-known medicinal plant in the Southeast Asian countries for its medicinal properties in treating various ailments, including dermatological conditions (Bylka *et al.*, 2013; Zahara *et al.*, 2014). In this experiment, HaCaT treated with CAW at the highest concentration (1000 µg/mL) exhibited a protective effect against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity with 98% cell viability compared with non-treated samples. Meanwhile, no protective effect was observed with CAE treatment. The protective effect of *C. asiatica* against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress has been shown *in vitro* and *in vivo* (Hussin *et al.*, 2009; Kim *et al.*, 2011; Mahanom *et al.*, 2011; Abdul Hisham *et al.*, 2018). An *in vitro* study by Kim *et al.* (2011) showed that pre-treatment of human fibroblast cells with hydro-ethanolic extracts of *C. asiatica* is able to provide a protective effect against H<sub>2</sub>O<sub>2</sub>-induced premature senescence, with decreased levels of p53 protein and the expression of genes involved in apoptosis, cell growth, transcription, senescence and DNA replication observed in treated cells. Meanwhile, *in vivo* studies have showed that *C. asiatica* reduces H<sub>2</sub>O<sub>2</sub>-induced oxidative stress by modulating lipid peroxidation observed with lower malonaldehyde (MDA) levels or low density lipids (Hussin *et al.*, 2009; Mahanom *et al.*, 2011; Ayala *et al.*, 2014). *C. asiatica* has also been shown to synergistically improve antioxidant enzymes in H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in human dermal fibroblast when combine with *M. oleifera* (Abdul Hisham *et al.*, 2018). These studies support the protective effect of CAW found in this study, whilst CAE may exert its activity

against oxidative stress through different mechanisms, such as modulating gene expression at molecular levels (Kim *et al.*, 2011).

In this experiment, CTW showed a dose-dependent protective effect against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity, with higher cell viability observed at 100, 250 and 500 µg/mL, while CTE did not show any such activity. Studies on the use of the flowers of *C. ternatea* against skin ageing are very limited, but their ability to provide protection against oxidative stress has been shown in several studies. The aqueous extract of *C. ternatea* flowers was shown to inhibit protein glycation and oxidation *in vitro* due to scavenging activity against different form of free radicals (Chayaratanasin *et al.*, 2015). The glycation of protein forming advanced glycation end products (AGEs), especially at the early stages, could generate free radicals and cause oxidative damage (Smith and Thornalley, 1992), and this reaction can occur in collagen to eventually produce wrinkles, as previously mentioned in the cross-linking theory of skin ageing (see Chapter 2, section 2.9.3). Moreover, the aqueous extract of *C. ternatea* flowers has also been shown to inhibit lipid peroxidation and suppress ROS (Phrueksanan *et al.*, 2014; Yimdee *et al.*, 2014; Nair *et al.*, 2015). The potential protective effect of *C. ternatea* against skin ageing is further supported by a study by Kolakul and Sripanidkulchai (2017) investigating the anti-ageing potential of the flowers of *Lagerstroemia speciosa* and *Lagerstroemia floribunda*, where similar anti-ageing properties were investigated as in this study. In their study, the ethanolic extracts of *L. speciosa* and *L. floribunda* flowers exhibited cytoprotective effects against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in human keratinocytes, where higher cell viability and lower numbers of apoptotic cells were observed in treated HaCaT. Like *C. ternatea*, these flowers also contain high levels of phenolics, such as phenolic acids flavonoids and anthocyanins.

The extracts from *C. caudatus* (CCW and CCE) did not show any significant protective effect in this experiment. Although higher cell viability was observed at several non-toxic concentrations of CCW, the differences were not statistically significant. Its scavenging activities against different forms of free radicals is well established (Andarwulan *et al.*, 2010; Mustafa *et al.*, 2010; Sumazian *et al.*, 2010), and has been shown in Chapter 4. Therefore, it should also be considered that the protective effect exhibited by extracts of this plant may be different than the investigated effect in this study. In comparison to its respective water extract, the ethanolic extracts of the plant species have higher toxic effects on HaCaT, thus limiting the range of concentrations that can be tested.

## 6.5 Conclusion

*M. oleifera*, *C. asiatica* and *C. ternatea* water extracts were able to provide protective effects against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in HaCaT. The absence of a protective effect with *C. caudatus* extracts and other ethanolic extracts needs further investigation. A more detailed mechanistic study is necessary to further support the observed results and to clarify the absence of activity in the respective plant extracts.

# **Chapter 7**



## **Chapter 7: Protective effect against UV-induced mitochondrial DNA (mtDNA) damage**

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### **7.1 Ultraviolet radiation, mitochondria and skin ageing**

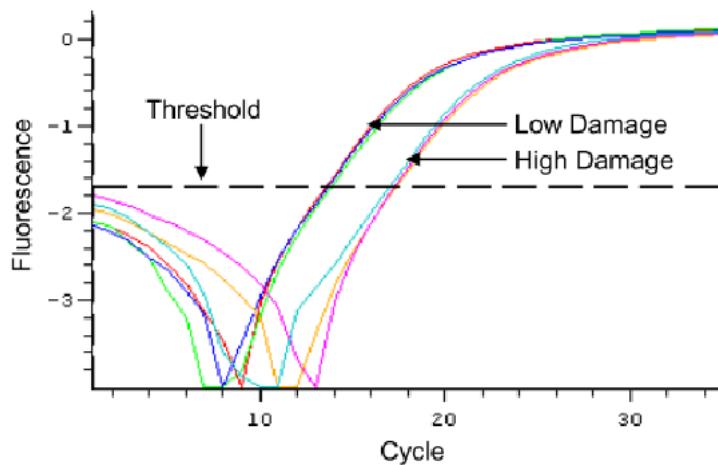
The role of ultraviolet (UV) radiation and mitochondria in skin ageing have been previously discussed (see Chapter 2, sections 2.10.1 and 2.10.2). UV radiation has been shown to cause molecular and cellular damage, oxidative stress, inflammation and suppression of the immune system in the skin (Finkel and Holbrook, 2000; Fisher *et al.*, 2002; Sinha and Häder, 2002; Farage *et al.*, 2008; Rastogi *et al.*, 2010). Meanwhile, the mitochondrial electron transport chain (ETC) can leak, producing superoxide radicals ( $O_2^{\cdot-}$ ) which are later converted into other oxidants such as hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radicals ( $\cdot OH$ ) (Halliwell, 2006; Krutmann and Schroeder, 2009). UV may interact with mitochondria either by generating ROS or directly causing damage to mtDNA which produces dysfunctional ETC subunits that later further increase oxidative stress in the skin (Bandy and Davidson, 1990). In this chapter, the plant extracts were investigated for their protective effects against UV-induced mtDNA damage.

#### **7.1.1 Mitochondrial DNA (mtDNA) as a biomarker for skin ageing**

In this chapter, UV-induced mtDNA damage was used as a biomarker for skin ageing. The mtDNA suffers more damage than nuclear DNA because it lacks protective histone, and is located very close to ETC at the inner membrane that continuously produces ROS (Birch-Machin *et al.*, 2013). Moreover, mtDNA has only limited repair mechanisms, and therefore accumulates damage and oxidative stress is further increased. The principle of the method used is described below.

### 7.1.1.1 Principle of mtDNA damage using polymerase chain reaction (PCR)

The principle of the qPCR assay of DNA damage is based on the fact that any kind of DNA lesion can slow down or impede the progression of DNA polymerase, resulting in lower amplification (Hunter *et al.*, 2010; Furda *et al.*, 2014). Therefore, the control and treated DNA samples will be amplified to different extents and those with less damage will be amplified to a greater extent compared to those with more damaged DNA. The principle of the method is further explained by the qPCR amplification plot shown in Figure 7.1. Ct value or the number of cycles when the amplified DNA crosses a threshold will differ between intact and damaged DNA. Intact DNA will have a low Ct value (or high copy number) but the damaged DNA will have a high Ct value (or low copy number) because the intact DNA will be amplified faster than the damaged DNA, thus giving a higher Ct value and vice versa.

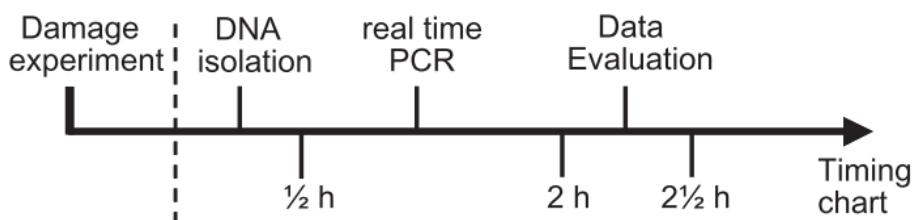


**Figure 7.1: The qPCR amplification plot.** The plot shows two samples run in triplicate, as shown by the coloured lines, one with low mtDNA damage and one with high mtDNA damage.

### 7.1.1.2 Benefits of qPCR

The qPCR method is beneficial because the mtDNA can be amplified from the total nuclear DNA without any need for isolation or purification (Hunter *et al.*, 2010; Furda *et al.*, 2014). Only a very small amount of sample is needed for the qPCR reaction (1-2 ng) (Hunter *et al.*,

2010), which is significantly important when the samples are of limited quantity. A rapid, gene-specific and simplified real time PCR protocol has been developed to accurately quantify mtDNA lesions induced by oxidative stress based on the interference of DNA polymerase activity (Rothfuss *et al.*, 2010). The method was modified accordingly to suit our experimental design. The efficiency of the method is illustrated in Figure 7.2, showing that it is a less time-consuming method that could be performed within 3 hours. Additionally, the method allows the amplification of a very small region of mtDNA lesions up to a size of 1000 bp amplicon.



**Figure 7.2: Experimental flow chart for qPCR methods damage assay.** The timing chart for the qPCR method consists of DNA isolation, qPCR and data evaluation that can be performed within 3 hours after the damage assay. Image from Rothfuss *et al.* (2010).

### 7.1.2 Chapter aim and objectives

The aim of this chapter is to investigate the protective effect of the plant extracts against UV-induced mtDNA damage. The specific objective of this chapter is to investigate the role of plant extracts (UV filters and antioxidants) in providing a protective effect against UV-induced mtDNA damage.

## 7.2 Materials and methods

### 7.2.1 Materials and instruments

A QIAamp genomic and RNA kit (50 DNA preps) for the purification of up to 50 kb genomic DNA was purchased from Qiagen, UK. The kit includes 50 QIAamp Mini Spin Columns, QIAGEN Proteinase K, buffers and 2 mL collection tubes. 2X SensiMix Hi-ROX containing hot-start DNA polymerase, SYBR Green I dye, dNTPs, stabilizers and 5-carboxy-X-rhodamine, succinimidyl ester (ROX) was purchased from Bioline, UK, and a MicroAmp Fast Optical 96-Well Reaction Plate was purchased from ThermoFisher Scientific, UK. The primers were designed and supplied by Eurofins, UK.

The instruments used in this study were seven 6-foot (ft) iSOLde Cleo performance 100 W-R lamps (Cleo) (iSOLde, Germany) fitted to a sun bath machine as a source of UV, a DMc150 Monochromator (Bentham Instrument Ltd., UK) to measure UV irradiance, a SpectroMax 250 microplate reader for absorbance reading and a Nanodrop machine for DNA quantification.

### 7.2.2 Samples

The freeze-dried samples were prepared in the same solvents used in the extraction process. For the 70% ethanol extract, the samples were prepared at higher concentration and diluted with water.

### 7.2.3 Determination of non-toxic concentration of the plant extracts on HaCaT

10,000 cells/well were seeded in a 96-well plate and allowed to acclimatize overnight. The media used was removed, rinsed with PBS (100 µL) and fresh new media (100 µL) was added to the control well, while plant extracts at varying concentrations were added to the treatment wells. The plate was further incubated for another 24 h at 37 °C. The MTS assay was performed the next day as described in Chapter 6, section 6.2.3.

#### 7.2.4 Cell culture and plating

All the cell culture work was performed according to the cell culture general protocols as described in Chapter 6 unless otherwise stated.

**Cell plating and treatments:** Confluent-cultured HaCaT was trypsinised and counted into 2.5  $\times 10^5$  cells/3 mL which were plated in the petri dish and allowed to settle overnight. The DMEM used was removed and the cells were rinsed with 1.5 mL PBS. Later, 2 mL of fresh phenol-free DMEM with 1% pen-strep was added. Phenol-free DMEM without FCS was used because phenol and FCS were shown to affect the assays.

#### 7.2.5 Calculation of UV irradiance and exposure time

The UV lamp (Cleo lamp) was turned on to warm up for 10-15 minutes. The appropriate lamp to be used was checked and the UV radiation was measured using a UV-detector. The UV irradiance from the Cleo lamp for each UVA, UVB and UVC was determined as in Table 7.1. The irradiance and exposure time of UV in minutes were calculated using the formula below:

$$\text{Irradiance} = \text{meter reading} \times \text{calibration factor} \quad (7.1)$$

$$\text{Time (min) of exposure for each SED} = \frac{\text{SED}}{(\text{SED}/\text{min of UV dose})^*} \quad (7.2)$$

$$\text{SED}/\text{min of UV dose}^* = \text{UV dose} \times \text{Cleo lamp calibration factor for SED/min} \quad (7.3)$$

For example, a meter reading of 8.84 resulted in the UVA irradiance calculation of:

$$\text{Irradiance} = 8.84 \times 1.19 \text{ mW/cm}^2 = 10.51 \text{ mW/cm}^2$$

And the total time of exposure to result in 2.0 standard erythema damage<sup>10</sup> (SED) is:

$$\text{Time of exposure (min)} = 2 \text{ SED} / (8.93^{-2} \text{ SED}/\text{min}) = 22.3 \text{ min}$$

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<sup>10</sup> Standard erythema damage (SED) is a weighted measure of radiant exposure equivalent to 100 Jm<sup>-2</sup> (Lucas *et al.*, 2006).

**Table 7.1: Calibration factors for Cleo lamp.** These factors are used to determine irradiance for each UVA, UVB and UVC from the lamp, with the calculated irradiance and SED per minute for a meter reading of 8.84.

Calibration factor	Irradiance (mW/cm <sup>2</sup> )
<b>Total UV</b>	$1.20^{+00}$
<b>UVC (250-280 nm)</b>	$0.00^{+00}$
<b>UVB (280-315 nm)</b>	$8.50^{-03}$
<b>UVA (315-400 nm)</b>	$1.19^{+00}$
<b>Weighted</b>	$1.70^{-03}$
<b>SED / min</b>	$1.01^{-02}$
	$1.06^{+01}$
	$0.00^{+00}$
	$7.51^{-02}$
	$1.05^{+01}$
	$1.50^{-02}$
	$8.93^{-02}$

## 7.2.6 MtDNA extraction, collection and quantification

### 7.2.6.1 Collection of cells

The media used was removed from the UV-exposed HaCaT and the cells were rinsed with PBS (1 mL). Trypsin (1.5 mL) was added to each petri dish which was then incubated at 37 °C for 5 minutes. After incubation, cells were observed under the microscope to make sure that all the cells were lifted and DMEM with 10 % FCS and 1% pen-strep (1.5 mL) was added to stop the activity of trypsin. A cell-scraper was used to remove all attached cells and to facilitate their collection. Cells were transferred to a 15 mL test tube and centrifuged for 5 min at 1200 rpm to collect the cell pellet. The media was removed, and the cell pellet was collected in a 1.5 mL Eppendorf tube. The cell pellets were stored at -20 °C until DNA extraction.

### 7.2.6.2 DNA extraction

The protocols for total nuclear and mitochondrial DNA extraction and collection were followed as described and suggested by the manufacturer (Qiagen, 2016). The collected cell pellet was resuspended with PBS (200 µL) and transferred to a centrifuge tube. Later, Proteinase K (20 µL) was added to the tubes and mixed to degrade any protein present. Lysis buffer of buffer AL from the kit (200 µL) was added to the samples to facilitate the lysis of the cell membrane. Samples were vortexed for 15 s and incubated at 56 °C for 10 minutes to lyse the samples.

Following incubation, ethanol (200 µL) was added to the samples to assist in DNA purification, and each sample was vortexed for 15 s before collection.

#### **7.2.6.3 DNA collection and purification**

The DNA solution in ethanol was later added to a QIAamp Spin Column and centrifuged at 8000 rpm for 1 minute to allow the DNA to adsorb to the spin column. The flow-through solution was discarded, and washed buffer or buffer AW1 (500 µL) was added to wash the column, and the column was centrifuged at 8000 rpm for 1 minute. The flow-through was discarded, and the column was washed with another wash buffer or buffer AW2 (500 µL) and centrifuged at 14 000 rpm for 3 minutes. The flow-through was again discarded and the column was spun at 14000 rpm for 1 minute to remove any remaining buffer AW2.

#### **7.2.6.4 DNA elution and quantification**

Later, elution buffer or buffer AE (60 µL) was added to the column, and the samples were incubated at 20 °C for 5 minutes and centrifuged for 1 minute at 8000 rpm to elute the DNA from the membrane into a 1.5 mL Eppendorf tube. This was repeated using the eluted flow-through to increase DNA yield. The DNA was stored at 4 °C until use or at -20 °C for long-term storage. DNA concentrations were determined using a ND-1000 NanoDrop Spectrophotometer (Thermo Scientific, UK) at a wavelength of 260 nm. The tip of the NanoDrop Spectrophotometer was cleaned with buffer AE (blank) without touching the tip. Later, the extracted DNA sample (1.5 µL) was added. The total DNA was calculated by multiplying the concentration obtained (ng/µL) by the total volume of extracted DNA (60 µL). The DNA quality was assessed using NanoDrop at 260/280 and 260/230 absorbance ratios, where a pure sample has values of absorbance ratios of approximately 1.8-2.0 and 2.0+ respectively.

## 7.2.7 Polymerase chain reaction (PCR): amplifying 1 kb fragment of mtDNA

The PCR hood was sterilized with 70% ethanol and RNase/DNase free water was treated with UV under the PCR hood for 20 minutes to sterilize the reagent and working environment. After sterilisation, the UV lamp was switched off prior to placing the samples under the hood. In the meantime, the PCR machine was switched on to warm up. DNA template and Mastermix preparation for PCR were prepared using the sterilized RNase/DNase free water.

### 7.2.7.1 Preparation of DNA template and PCR mastermix

DNA samples were thawed at room temperature and vortexed for 15 s to mix. In the PCR hood, the DNA solution was diluted in water to obtain sufficient volume, at approximately 1 mL in volume and at 6 ng/ $\mu$ L in concentration. Later, PCR mastermix was prepared on ice. The forward (AL4) and reverse primer (AS1) stocks were diluted with water at 1:10 to obtain 10  $\mu$ M of each forward and reverse primer working solutions. The sequences for the forward (AL4) and reverse (AS1) primers are CTGTTCTTCATGGGGAAGC and AAAGTGCATACCGCCAAAAG respectively (Rothfuss *et al.*, 2010). Sufficient volumes required for 2X SensiMix Hi-ROX reagent, forward (AL4) and reverse primers (AS1) were prepared for the number of reactions performed. The 2X SensiMix Hi-ROX reagent, forward primer (AL4), reverse primer (AS1), water and samples were assembled to a final volume of 20  $\mu$ L/well in a MicroAmp Fast Optical 96-well reaction plate. The volumes of each component were as shown in Table 7.2. The final concentrations for 2X SensiMix Hi-ROX Reagent, primers and DNA samples in the wells were 1x, 0.25  $\mu$ M and 1.2 ng/ $\mu$ L respectively.

**Table 7.2: Mastermix components for 1x PCR per 20 µL volume reaction.**

Component	1x (per 20µL reaction)
2x SensiMix Hi-ROX Reagent	10
Forward primer (10 µM)	0.5
Reverse primer (10 µM)	0.5
Water	7.0
Sample	2.0

### 7.2.7.2 PCR conditions

The following conditions were used for the qPCR run: pre-incubation phase of 10 min at 95 °C followed by 30 cycles each of 98 °C for 15 seconds, 60 °C for 15 seconds, and 72 °C for 55 seconds; and a final stage of 72 °C for 7 minutes. Melting curves for all DNA samples were added immediately after the reactions. All real-time qPCR runs for the analysis of mtDNA damage were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, UK) with the results viewed using StepOne Software V2.3 (Applied Biosystems, UK).

### 7.2.8 Pre-experiment assay: effect of UV on mtDNA damage

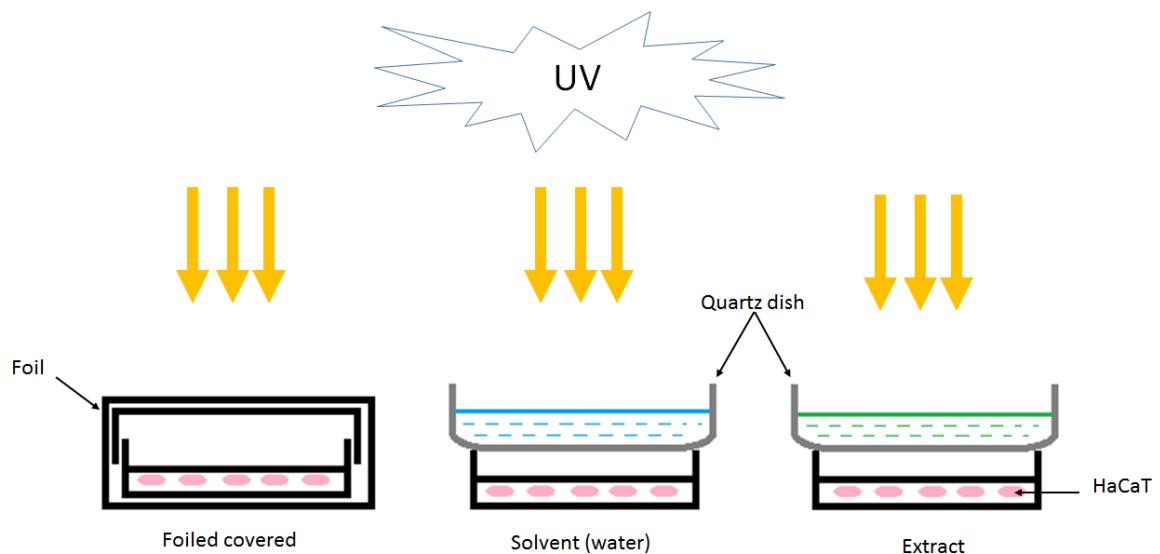
An assay was carried out prior to the actual experiments.  $1.5 \times 10^5$  cells in 3 mL of complete DMEM were added to a dish and allowed to settle overnight. The control and UV-treated dishes were prepared in duplicate. The next day, the media used was removed, cells were rinsed with PBS (1.5 mL) and fresh phenol free DMEM with 1% pen-strep (3 mL) was added. The dish covers were removed, and the controls were covered with aluminium foil, while the UV-treated dishes were left uncovered. The reading of the UV lamp was obtained using the DMc150 Monochromator and the time to cause 2.0 SED was determined as previously described (see section 7.2.5). Later, the dishes were covered with the lids and transported to the cell culture lab. The media was removed, and cells were rinsed with PBS (1.5 mL) and trypsin (1.5 mL) was added to each dish. After 5 minutes of incubation (37 °C), complete DMEM (1.5 mL) was added and the cells were scraped off using a circular motion and

transferred to a 15 mL test tube. Each dish was rinsed with complete DMEM (2 mL) to obtain a total of 5 mL cells solution. The 15 mL tubes were centrifuged to pellet the cells at 1200 rpm for 5 minutes. Later, the media was aspirated, and a small volume was left to make sure that the pellet was not disturbed. The samples were either stored at -20 °C until further use or extracted for mtDNA analysis. The same procedures were repeated for all dishes.

### **7.2.9 Investigating the role of plant extracts as UV absorbers in providing a protective effect against mtDNA damage**

#### **7.2.9.1 Protective effect of the plant extracts at 2 SED**

$2.5 \times 10^5$  cells/mL of HaCaT were plated in the 35 mm dish and settled overnight. A higher cell number was used to increase the total yield of DNA as compared to the pre-experiment assay, where  $1.5 \times 10^5$  cells/mL was plated per dish. The next day, the medium used was removed, cells were rinsed with PBS and phenol-free DMEM with 1% pen-strep (3 mL) was added for UV treatment. The cells were irradiated with the Cleo lamp for ~20 minutes or at 2 SED (refer to section 7.2.5). Later, the DNA was extracted, and qPCR was performed as described in sections 7.2.6 and 7.2.7. The experimental set-up during UV exposure to evaluate the physical protective effect of the extracts against UV-induced mtDNA damage is as illustrated in Figure 7.3, which was modified from the previous method used by Brugé *et al.* (2014). Plant extract (3 mL) was placed in a quartz dish placed on top of the dish containing the cells. Water was used as a negative control and cells were covered with foil for the positive control.



**Figure 7.3: Experimental set-up to evaluate physical protective effect of the extracts against UV-induced mtDNA damage.**

#### 7.2.9.2 UV absorption by the plant extracts

Plants extracts were prepared at the required concentrations, and 3 mL of each sample solution was pipetted into a glass cuvette. Absorbance readings from 190-800 nm wavelengths were taken using a Cary UV-Visible Spectrophotometer (Varian Inc., USA), with the output viewed using the Cary WinUV Kinetics Application (Varian, Inc.). The blank (water) was subtracted from the UV scan for each sample reading.

#### 7.2.9.3 Determination of HaCaT viability at different SEDs

10, 000 cells/well HaCaT was plated in a 96-well plate and allowed to settle overnight. The next day, the media used was removed, and the cells were rinsed with PBS (100  $\mu$ L) and phenol-free DMEM with 1% pen-strep (100  $\mu$ L) was added. An initial reading of UV irradiance from the sunbath machine was taken prior to the experiment and the time needed for each SED was calculated using the formula given in section 7.2.5. The exposure times for each SED are tabulated in Table 7.3. During the assay, the control wells were covered with foil and the

foil was moved to the next row after each exposure time (for example, after 10.5 min, the foil was moved to cover the rows adjacent to the control). These steps were repeated for all SEDs. After the UV treatment, the media was removed, cells were rinsed with PBS (100  $\mu$ L) and fresh complete phenol-free DMEM (100  $\mu$ L) was added. MTS solution (20  $\mu$ L) was added to each well and the plate was incubated for 4 h before reading at 490 nm using the Spectromax<sup>260</sup> plate reader.

**Table 7.3: Calculated exposure time for each SED with UV dose of 9.36.**

SED	Meter Reading	Time (min)	mJ/cm <sup>2</sup>				
			Total unweighted UV	Total weighted UV	UVC	UVB	UVA
0.99	9.36	10.5	7046.68	10.02	0.00	50.12	6993.60
2.01	9.36	21.3	14294.69	20.34	0.00	101.68	14187.03
4.00	9.36	42.3	28388.04	40.38	0.00	201.92	28174.24
5.99	9.36	63.4	42548.50	60.53	0.00	302.65	42228.05
7.99	9.36	84.5	56708.96	80.67	0.00	403.37	56281.87
9.99	9.36	105.7	70936.54	100.91	0.00	504.57	70402.29

#### **7.2.9.4 UV filtering experiment: protective effect of the plant extracts against UV-induced mtDNA damage at 4 SED**

$2.5 \times 10^5$  cells/dish were plated in the 35 mm dishes and left to settle overnight at 37 °C with 5% CO<sub>2</sub>. The next morning, the medium used was removed, cells were rinsed with PBS (1.5 mL) and phenol-free DMEM with 1% pen-strep (3 mL) was added for UV treatment. Plant extracts (3 mL) at the required concentrations were added to the quartz dish placed on top of the dish containing cells (see Figure 7.3). The neagative control (water) and positive control (foil covered dish) were exposed to UV concurrently with those cells physically covered with plant extracts. The cells were irradiated with Cleo lamps for 4 SED exposure time. Later, the cells were collected, the DNA was extracted, and qPCR was performed as described in sections 7.2.6 and 7.2.7.

#### **7.2.10 Investigating the role of plant extracts as antioxidants in providing protective effect against mtDNA damage**

##### **7.2.10.1 Pre-incubation experiment**

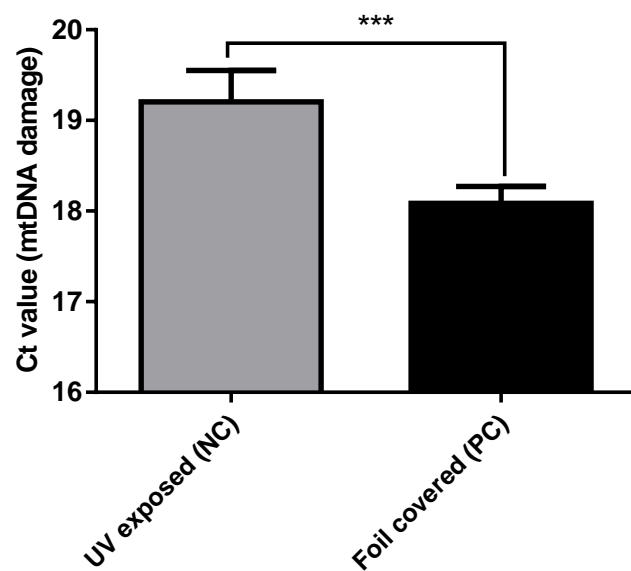
$2.5 \times 10^5$  cells were plated in the 35 mm dishes and left to settle overnight. The next day, the medium used was removed, and the cells were rinsed with PBS (1.5 mL) and plant extracts

made up in complete DMEM were added at the required concentrations (3 mL). The dishes were further incubated for another 24h at 37 °C. After 24h of pre-incubation, the cells were irradiated with Cleo lamps for 4 SED exposure time. Later, the cells were collected, the DNA was extracted, and qPCR was performed as described in sections 7.2.6 and 7.2.7.

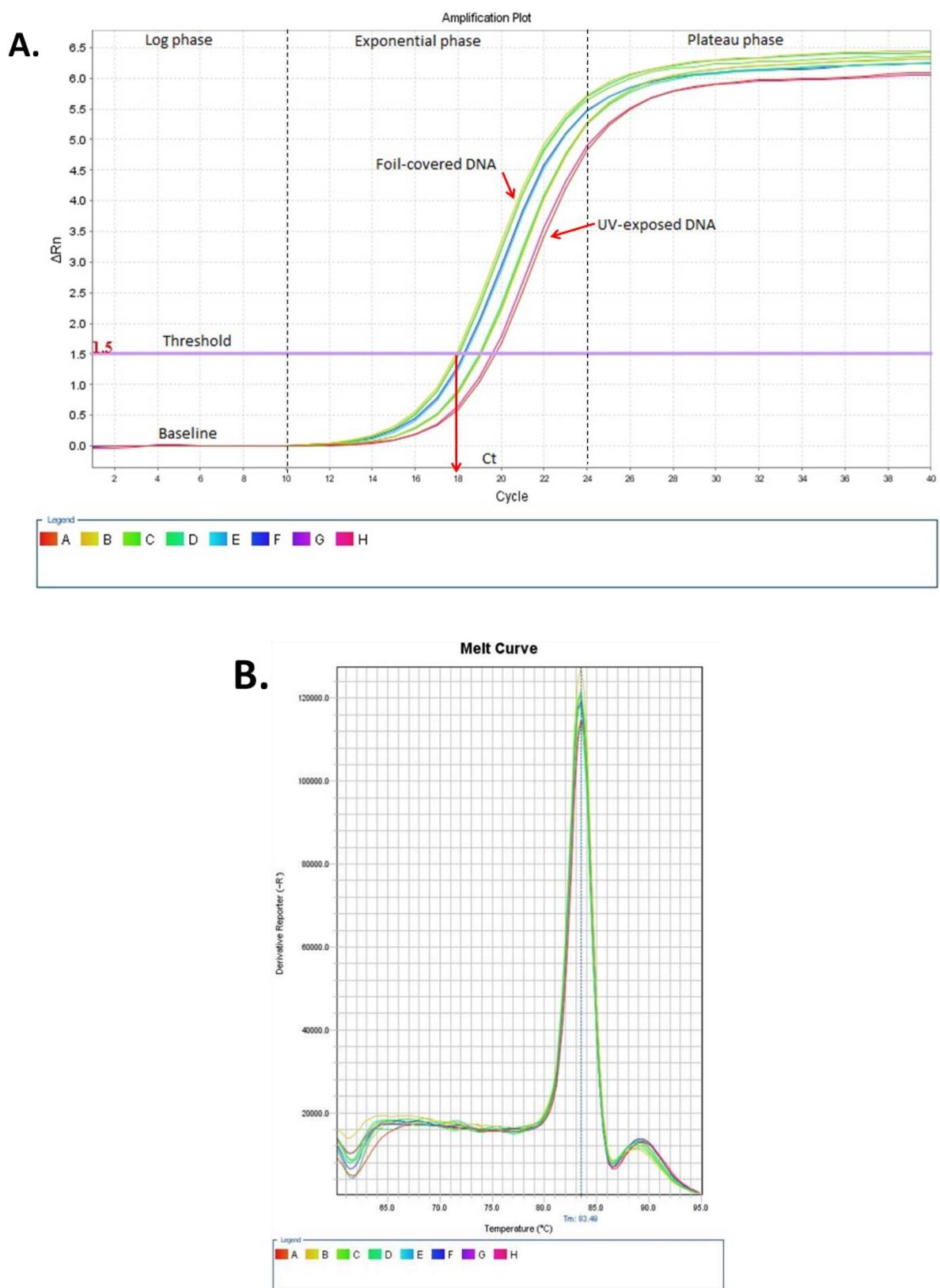
## 7.3 Results

### 7.3.1 Pre-experiments: UV causes significant mtDNA damage at 2 SED

A pre-experiment assay was performed to evaluate the efficiency of the method. In the experiment, a UV exposure of 2 SED or 20 minutes causes significant mtDNA damage to exposed-HaCaT as compared to the foil-covered HaCaT (see Figure 7.4). The DNA sample from the foil-covered sample was amplified more rapidly than the UV-exposed samples due to the lower level of damage, resulting in a higher Ct value (see Figure 7.5 A), and all of the DNA samples were shown to be melted at the same temperature (84 °C), suggesting the specificity of the reactions (see Figure 7.5 B). Therefore, the experiment proceeded with the same conditions.



**Figure 7.4: MtDNA damage caused by UV radiation in exposed HaCaT.** Significant mtDNA damage (\*\*p<0.001, t-test analysis) is shown in the exposed HaCaT. NC=Negative control and PC=positive control.



**Figure 7.5: PCR amplification plot of DNA samples from UV-exposed and foil-covered HaCaT (A) and PCR melt curves of the DNA samples (B).**

### **7.3.2 Toxicity effect of the plant extracts on HaCaT**

The effect of the plant extracts alone on HaCaT was determined prior to the assay. The plant extracts showed different toxic effects on HaCaT, where all the ethanolic extracts were more toxic than the water extracts. The cytotoxic and non-cytotoxic concentrations of each of the plant extracts are shown in Table 7.4, and the selected concentrations for the UV assay shown in Table 7.5, are the highest non-toxic concentrations of the extracts on HaCaT. An average of the highest two non-toxic concentrations were selected if the highest non-toxic concentration showed lower cell viability than the previous concentration, such as observed in MOW and CTE. For CCW, the highest non-toxic concentration tested was selected due to limited sample availability.

**Table 7.4: Cytotoxic and non-cytotoxic concentrations of the plant extracts on HaCaT.** The marked (\*) concentrations showed significant reductions in percentage viability compared with control (untreated HaCaT) and are considered cytotoxic to HaCaT at  $*p<0.05$ .

Plant species	Extracts	Concentration (µg/mL)	Highest nontoxic concentration (µg/mL)
<i>M. oleifera</i> Lam.	MOW	8, 16, 31, 63, 125, 250, 500, 1000*, 2000*, 4000*	500
	MOE	8, 16, 31, 63, 125, 250, 500*, 1000*, 2000*, 3000*, 4000*	250
<i>C. asiatica</i> (L.) Urban.	CAW	8, 16, 31, 63, 125, 250, 500, 500, 1000, 2000, 4000*	4000
	CAE	8, 16, 31, 63, 125, 250*	125
<i>C. ternatea</i> L.	CTW	8, 16, 31, 63, 125, 250, 500, 1000, 1500, 2000*, 2500*, 3000*, 4000*	1500
	CTE	8, 16, 31, 63, 125, 250, 500, 1000*, 2000*, 3000*, 4000*	500
<i>C. caudatus</i> Kunth	CCW	8, 16, 31, 63, 125, 250, 500, 1000, 2000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000	10,000
	CCE	8, 16, 31, 63, 125*, 250*	63

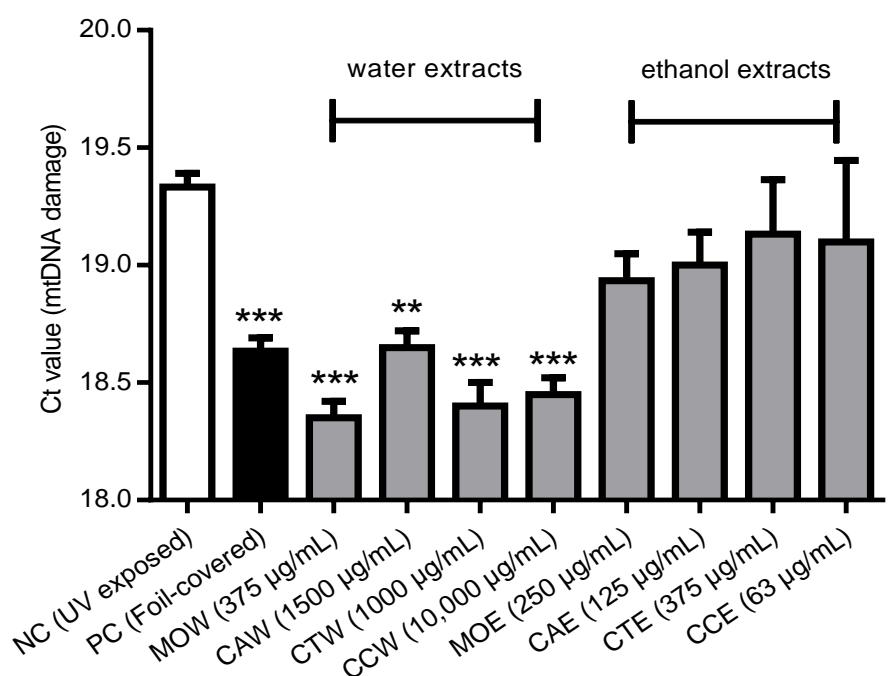
**Table 7.5: Selected non-toxic concentrations for UV treatment.**

Toxicity level on HaCaT	Samples extracts	Nontoxic concentration for UV assays (µg/mL)
Decreases	CCE	63
	CAE	125
	MOE	250
	CTE	375
	MOW	375
	CTW	1000
	CAW	1500
	CCW	10000

### 7.3.3 Investigation the role of plant extracts as UV filters in providing a protective effect against mtDNA damage

#### 7.3.3.1 Protective effect against UV-induced mtDNA damage at 2 SED

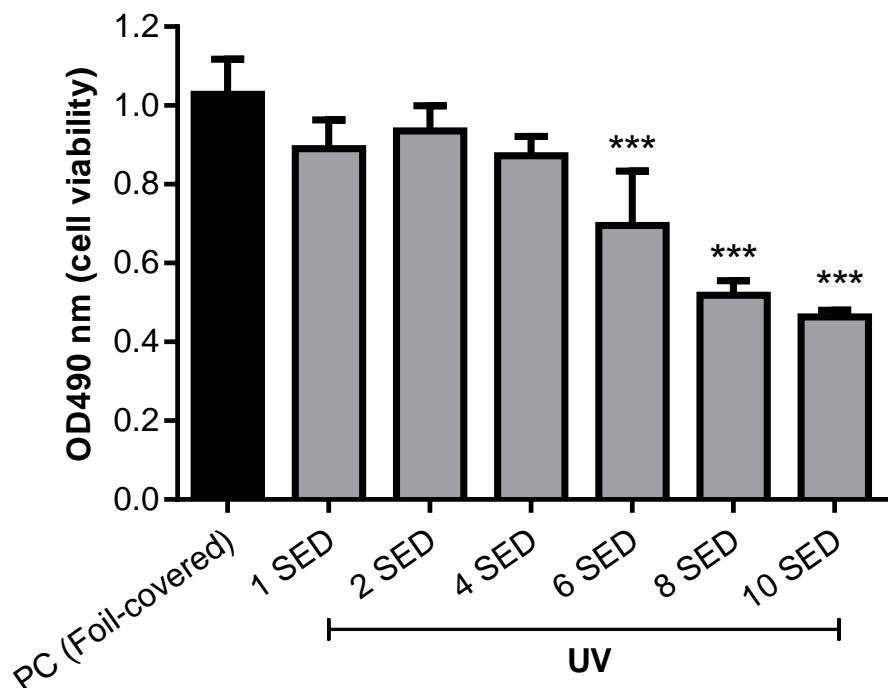
In this experiment, all the water extracts of each plant species (MOW, CAW, CTW and CCW) provide significant protective effects against UV-induced mtDNA damage when compared with UV-exposed HaCaT (see Figure 7.6). Meanwhile, none of the ethanolic extracts of the plant species (MOE, CAE, CTE and CCE) provided any protective effect. A further experiment at higher SED was performed to further confirm the protective effect.



**Figure 7.6: Protective effects of the plant extracts against UV-induced mtDNA damage at 2 SED (UV filter experiment).** NC= negative control and PC= positive control. Data are the mean and SD of triplicates (n=3).

### 7.3.3.2 HaCaT viability at different SED

In this experiment, UV exposure at 1, 2 and 4 SED (10.5, 21.3 and 42.3 minutes respectively) did not cause any significant cell death to HaCaT, as shown in Figure 7.7. Meanwhile at higher SED of 6, 8 and 10 or UV exposure for 63.4, 84.5 and 105.7 minutes respectively caused significant cell death compared with the control. This experiment suggests that 4 SED is the maximum UV exposure which can be used for the UV-induced mtDNA damage assay, and this level was investigated in the subsequent experiment.

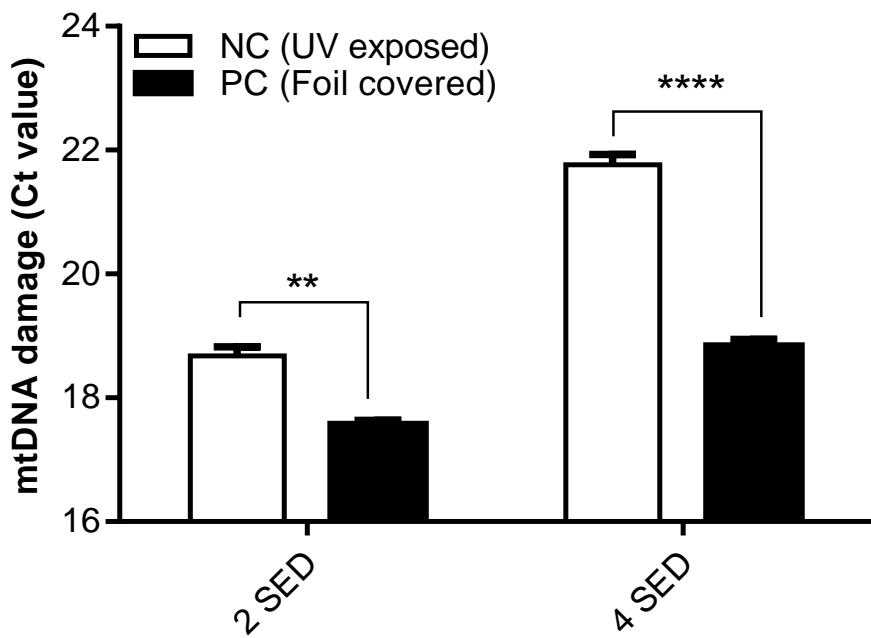


**Figure 7.7: HaCaT viability at different SED of UV exposure.** Data represent the mean and SD (n=6).

### 7.3.3.3 Comparison of UV-induced mtDNA damage at 2 SED vs. 4 SED

Longer UV exposure to HaCaT resulted in higher mtDNA damage, where a larger difference in Ct was observed between UV-exposed vs. foil-covered HaCaT at 4 SED (see Figure 7.8). At 2 SED, the observed Ct difference between the negative control (NC) and positive control (PC)

was approximately 1 Ct, whereas a difference of 3 Ct was observed at 4 SED. A larger Ct difference between the NC and PC can reduce a false negative result, and therefore the subsequent experiment was conducted at 4 SED to further confirm the protective effect of the plant extracts against UV-induced mtDNA damage.

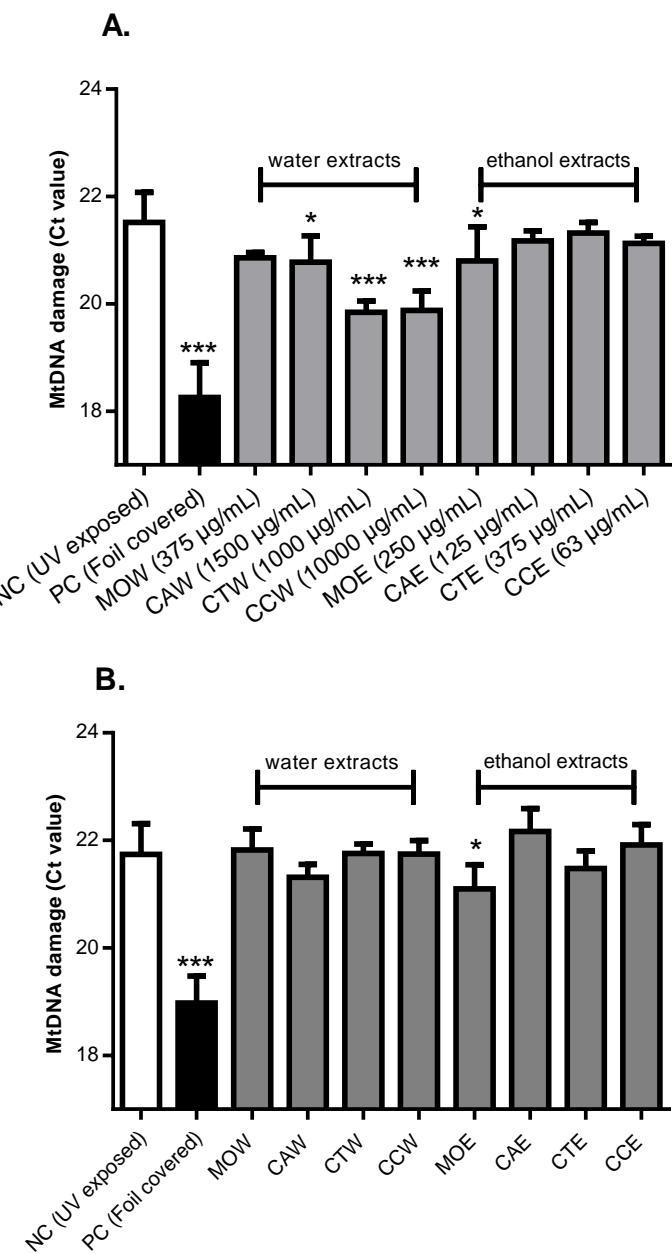


**Figure 7.8: Comparison of UV-induced mtDNA damage at 2 SED vs. 4 SED.** Data represent mean and SEM of two independent experimental runs with three technical PCR replicates for each DNA sample. NC= negative control and PC= positive control, where \*\* $p<0.01$  and \*\*\*\* $p<0.0001$ .

#### 7.3.3.4 Protective effect against UV-induced mtDNA damage at the highest non-toxic concentrations and at 63 µg/mL

The water extracts of *C. asiatica* (CAW), *C. ternatea* (CTW), *C. caudatus* (CCW) and the *M. oleifera* ethanol (MOE) extracts showed significant protective effects against UV-induced mtDNA damage (see Figure 7.9 A). None of the ethanol extracts showed any protective effect against UV-induced mtDNA damage. A comparison of the potency of the plant extracts was performed at 63 µg/mL, which is the lowest non-toxic concentration for all plant extracts, and

only MOE provided a significant protective effect against UV-induced mtDNA damage of  $p<0.05$  compared to UV-exposed HaCaT (see Figure 7.9 B). At this concentration, none of the other extracts showed any protective effect, suggesting a dose-dependent protective effect of the plant extracts.

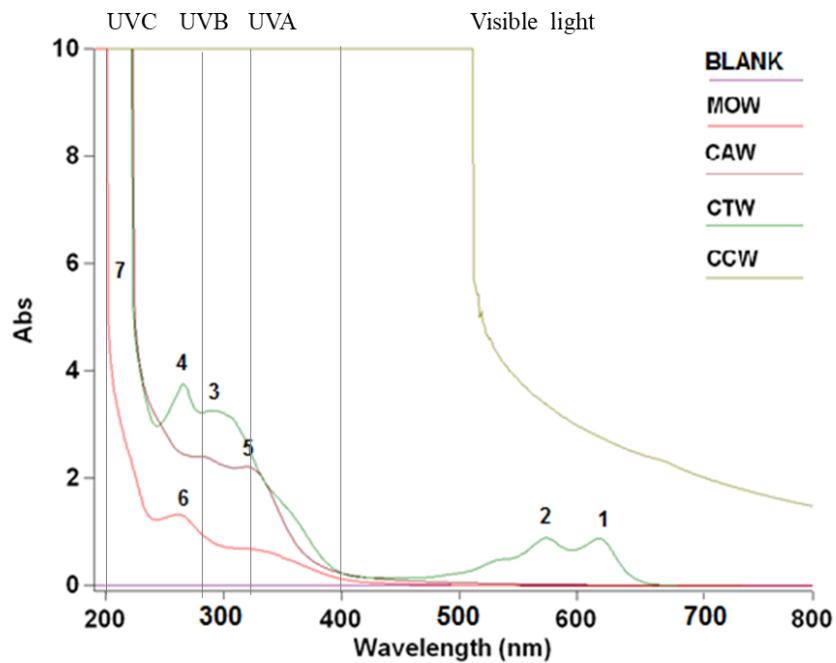


**Figure 7.9: UV filter experiment: protective effect against UV-induced mtDNA damage at the highest non-toxic concentration (A) and at 63 µg/mL (B).** Data are the mean and SEM of two experiments with three PCR technical replicates for each DNA samples with  $p<0.05$  considered as significant. NC= negative control and PC= positive control.

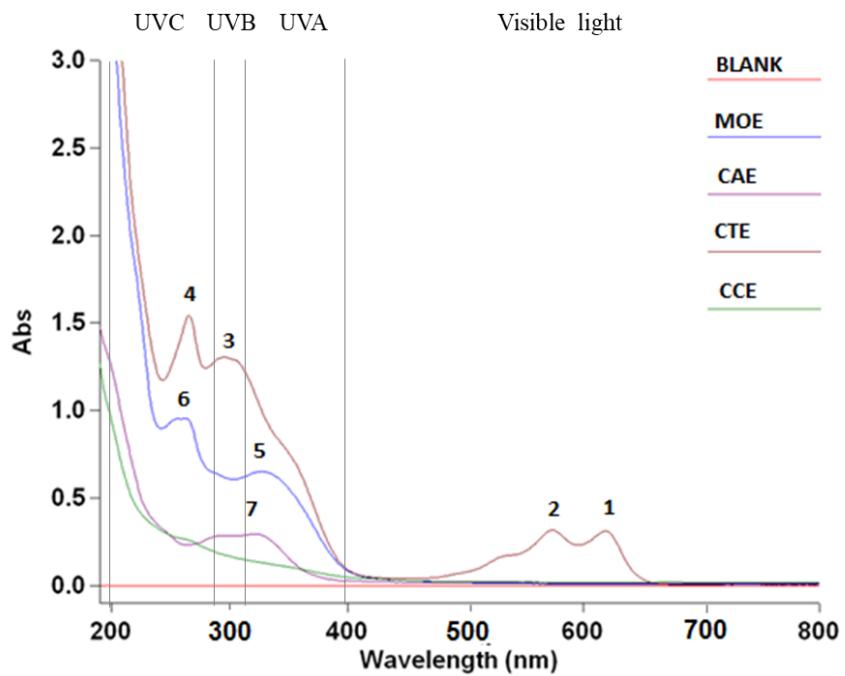
### 7.3.3.5 UVA, UVB and UVC filtering ability of the plant extracts

The plant extracts exhibited different values of UVA (320-400 nm), UVB (290-320 nm) and UVC (200-290 nm) absorptivity. The absorption spectra of each of the plant extracts at the highest non-toxic concentrations are shown in Figure 7.10 with the peak details tabulated in Table 7.6. At each of the concentrations, the MOW showed UVC absorption, while the MOE showed UVC and UVA absorption. Both CAW and CAE showed UVA absorption. *C. ternatea* extracts (CTW and CTE) showed absorption in the visible light, UVB and UVC ranges. No absorption peak was shown by CCE, but the CCW completely blocked UV and visible light. Comparison of the UV absorption profiles of all extracts at lower concentrations (63 µg/mL) showed similar absorption patterns, but with fewer peaks identified for both water and ethanol extracts (see Figure 7.11 and Table 7.7). All identified peaks were in the UVC range shown by MOW, CTW, MOE, CAE and CTE, and in the visible light range shown by CTW and CTE.

### A. Water extracts at highest non-toxic concentration



### B. Ethanol extracts at highest non-toxic concentration

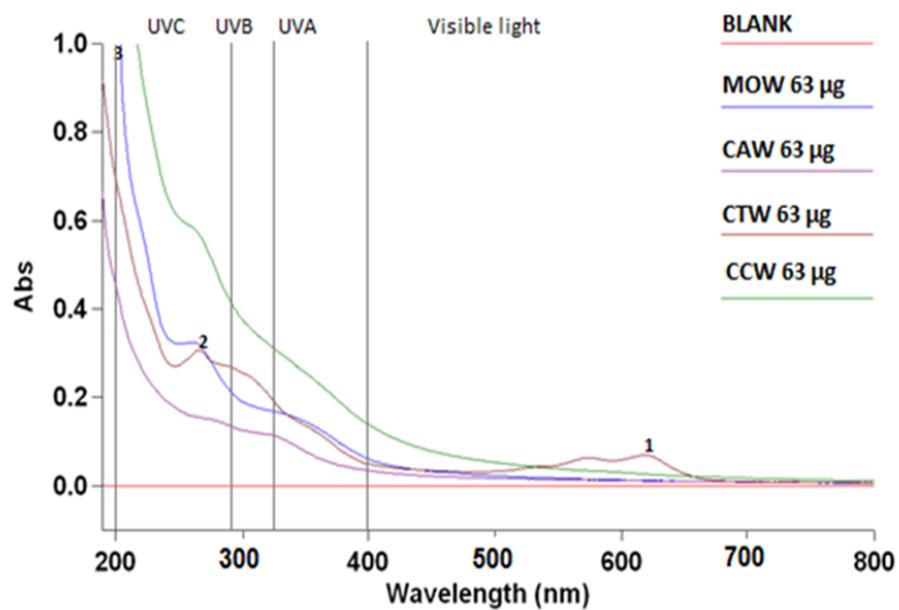


**Figure 7.10: UV absorption by the water (A) and ethanol (B) extracts at the highest non-toxic concentration.** Peak details (wavelength and absorption) for A and B are summarized in Table 7.6.

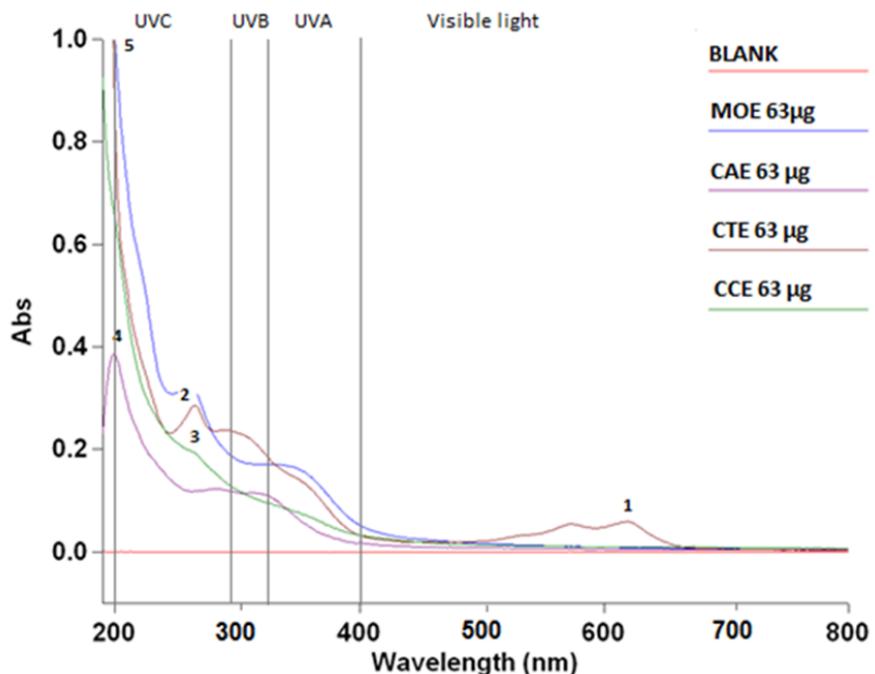
**Table 7.6: Peak details (wavelength in nm and absorption) of the extracts at the highest non-toxic concentrations as shown in Figure 7.10 A and B.**

Spectra (nm)	Water extracts				Ethanol extracts			
	MOW	CAW	CTW	CCW	MOE	CAF	CTE	CCE
UVC (200-290)	<b>6</b> (262, 1.324)	-	<b>4</b> (266, 3.779)	-	<b>6</b> (263, 0.955)	-	<b>4</b> (265, 1.544)	-
	<b>7</b> (202, 5.913)	-	<b>3</b> (292, 3.317)	-	-	-	<b>3</b> (295, 1.305)	-
UVB (290-300)	-	-	-	-	-	-	-	-
UVA (320-400)	-	<b>5</b> (321, 2.284)	-	-	<b>5</b> (327, 0.652)	<b>7</b> (321, 0.295)	-	-
Visible light (400-800)	-	-	<b>1</b> (618, 0.927), <b>2</b> (574, 0.933)	-	-	-	<b>1</b> (618, 0.311), <b>2</b> (573, 0.316)	-

### A. Water extracts at 63 µg/mL concentration



### B. Ethanol extracts at 63 µg/mL concentration



**Figure 7.11: UV absorption of the water (A) and ethanol (B) extracts at 63 µg/mL.** The patterns of absorption at 63 µg/mL were similar to those at higher concentrations for all the extracts, with fewer identified peaks observed. Peak details (wavelength and absorption) for A and B are summarized in Table 7.7.

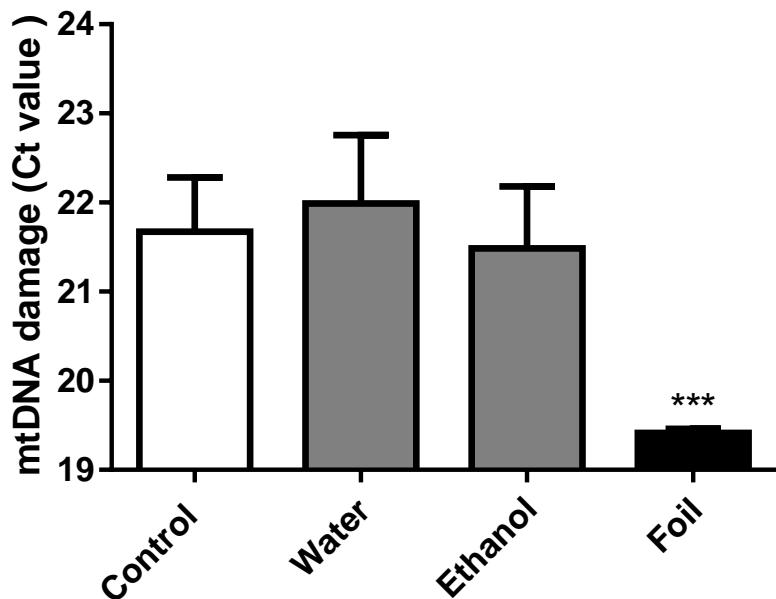
**Table 7.7: Peak details (wavelength in nm and absorption) of the extracts at 63 µg/mL as shown in Figure 7.11 A and B.**

Spectra (nm)	Water extracts				Ethanol extracts			
	MOW	CAW	CTW	CCW	MOE	CAE	CTE	CCE
UVC (200-290)	<b>3</b> (191, 3.888)	-	<b>2</b> (266, 0.307)	-	<b>5</b> (194, 1.257)	<b>4</b> (199, 0.385)	<b>3</b> (191, 2.972)	-
UVB (290-300)	-	-	-	-	-	-	-	-
UVA (320-400)	-	-	-	-	-	-	-	-
Visible light (400-800)	-	-	<b>1</b> (618, 0.07)	-	-	-	<b>1</b> (619, 0.059)	-

### 7.3.4 Investigation of the role of plant extracts as antioxidants in providing a protective effect against UV-induced mtDNA damage

#### 7.3.4.1 Vehicles (solvents) had no significant effect on UV-induced mtDNA damage

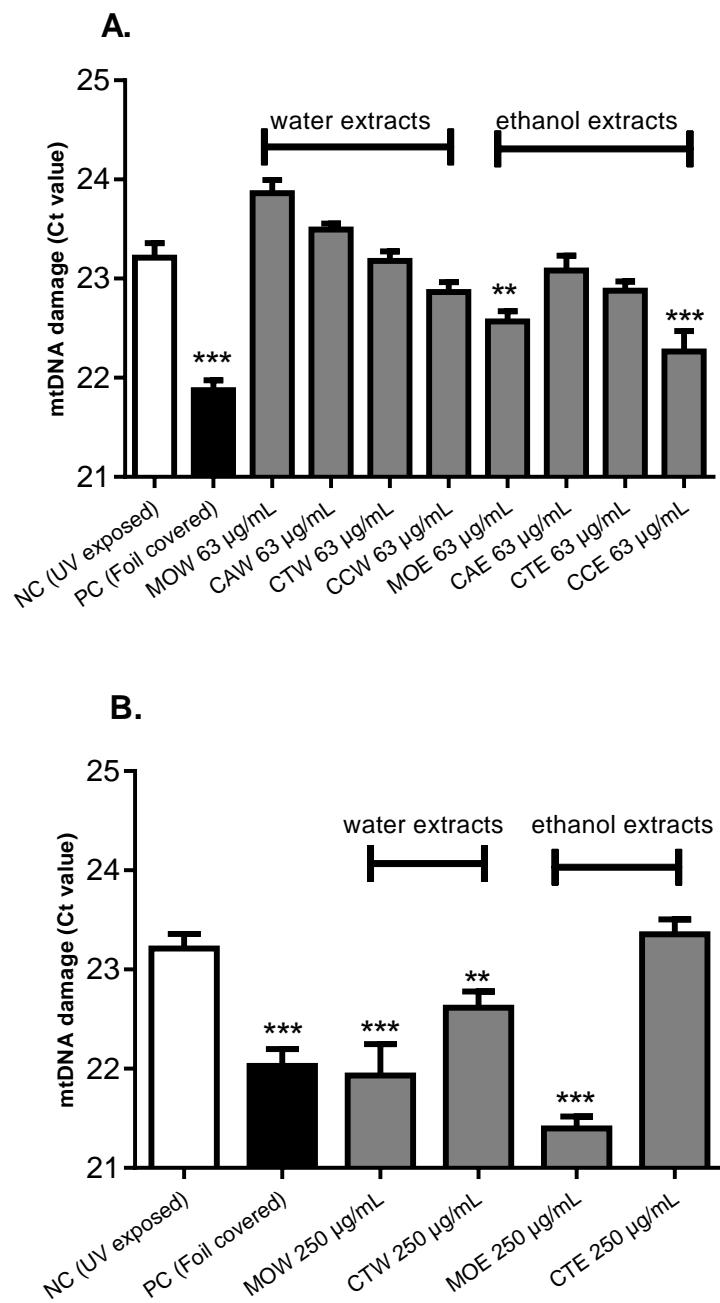
The effect of vehicles alone on mtDNA damage in UV-treated HaCaT was investigated and the results showed that neither water nor ethanol had any significant effect (see Figure 7.12). In this experiment, the pre-treatment of HaCaT with water and ethanol (1%) for 24 h did not show significant effects on UV-induced mtDNA damage. Therefore, the pre-treatment of HaCaT with the plant extracts indicate the effect of the extracts rather than the vehicles.



**Figure 7.12: Effect of vehicle (solvent) on mtDNA damage: preincubation experiment.** Data are the mean and SEM of two independent experimental runs in three technical replicates for each DNA sample with \*\*\* $p<0.001$  compared with control.

#### **7.3.4.2 Pre-incubation experiment: protective effect of the plant extracts against UV-induced mtDNA damage**

A comparison of potency at 63 µg/mL showed that only MOE and CCE protected HaCaT against UV-induced mtDNA damage (see Figure 7.13 A). None of the water extracts gave any significant protective effect to HaCaT. However, at the higher concentration (250 µg/mL), MOW and CTW showed significant protective effects, while MOE showed a higher protective effect at this concentration suggesting a dose-dependent effect (see Figure 7.13 B). Further experiments on other extracts were not performed either due to limitations in sample availability or cytotoxicity effects of the extracts on HaCaT.



**Figure 7.13: Pre-incubation experiment: protective effect of the plant extracts against UV-induced mtDNA damage at 63 µg/mL (A) and 250 µg/mL (B).** Data represent mean and SEM of two independent experiments with three technical PCR replicates. NC=negative control and PC=positive control with \*\* $p<0.01$  and \*\*\* $p<0.001$  compared with NC.

## 7.4 Discussion

Many sunscreen creams incorporate organic and inorganic compounds such as avobenzone, zinc oxide and titanium dioxide in their formulations to absorb, reflect or scatter UV, thus reducing its harmful effect on the skin (Beasley and Meyer, 2010). However, these compounds can also cause adverse effects such as contact and photocontact dermatitis, and some are cosmetically unacceptable because of their opaque quality that causes the skin to appear white (Gasparro *et al.*, 1998; Yap *et al.*, 2017). Antioxidants are sometime used in sunscreen formulations to complement or boost the protective effect of UV filters (Galanakis *et al.*, 2017). Therefore, the plant extracts considered in this research were investigated for their ability to provide protective effects against UV-induced mtDNA damage in HaCaT for potential application as either UV-filters or antioxidants. In this chapter, a novel method of assessment was utilised evaluating the protective effect of the plant extracts against photoageing.

### 7.4.1 Pre-experiment assay: proof of concept of the use of qPCR to assess mtDNA damage as a biomarker for skin ageing

The use of qPCR to assess DNA damage has been extensively used, as this method has been shown to have many benefits (Hunter *et al.*, 2010; Furda *et al.*, 2014). In the pre-experiment assays, UV was shown to induce mtDNA damage in HaCaT as compared with non-exposed cells, expressed as mean in qPCR Ct values (see results in section 7.3.1). The results were also graphically presented in amplification plots of the DNA samples which showed that the intact DNA sample was amplified faster compared with damaged DNA samples, with PCR melt curves showing that all the samples were melting at the same temperature. This assay provides proof of concept of the principle behind the method used, where any damage to the DNA will impede or slow down the progression of DNA polymerase (Hunter *et al.*, 2010; Furda *et al.*, 2014), and comparisons between intact and damaged DNA can be performed as the reaction

starts at the same time, as shown by the melt curves. However, the initial UV filter experiment showed that at 2 SED UV exposure (20 minutes), only the water extracts exhibited protective effects against UV-induced mtDNA damage (see Figure 7.6). These results may be explained by the fact that at the concentrations used, all the water extracts were non-toxic to HaCaT and higher concentrations were used in comparison to the ethanol extracts, where lower concentrations had to be used due to the cytotoxicity effect. However, to avoid any false negative results, UV exposure at 4 SED (40 minutes) with higher Ct difference between the negative and positive controls (exposed *vs.* covered HaCaT) were used in the subsequent experiments. Longer UV exposure to HaCaT induces higher level of damage as shown by a comparison of Ct differences between negative and positive controls at 2 SED and 4 SED, where differences of 1 Ct *vs.* 3 Ct respectively were observed (see Figure 7.8).

#### **7.4.2 UV filter experiment: potential application of the plant extracts as UV filters**

In the UV filter experiment, the water extracts of *C. asiatica*, *C. ternatea*, *C. caudatus* (CAW, CTW, CCW) and the ethanol extract of *M. oleifera* (MOE) showed significant protective effects against UV-induced mtDNA damage (see Figure 7.9 A). These extracts showed different levels of protective effect, mainly due to the concentrations used. However, a comparison of potency at similar concentration between all extracts showed that only MOE exhibited a significant protective effect (see Figure 7.9 B), suggesting a potential dose-dependent effect of the plant extracts.

A review of the photo-protection given by herbal extracts as an alternative to synthetic UV-filters by Radice *et al.* (2016) showed that the photo-protective activity of the plant extracts considered in this study has not yet been reported. Therefore, the results in this experiment may provide a potential novel application of extracts from these plant species as UV filters. In comparison to the extracts from leaves, studies on the photo-protection given by of *M.*

*oliefera* species have been limited to the seed, and particularly the lipid fraction (Gaikwad and Kale, 2011). However, it is possible that the protective effect exhibited by MOE is due to its ability to absorb UV radiation, and particularly UVA and UVC (see results section 7.3.3.5), thus reducing the amount of UV exposure to the cells which causes damage. Similarly, CAW and CTW also showed either UVA, UVB or UVC absorption. Meanwhile, CCW completely blocked UV radiation at the concentration used. This is further supported by the phytochemical analysis of the extracts, showing the presence of photoprotective compounds that can screen UV, such as phenolic acids and flavonoids (see Chapter 3). These compounds are known to be natural UV filters that work either by filtering, absorbing or blocking the UV (Pereira *et al.*, 2009; Agati *et al.*, 2013; Radice *et al.*, 2016; Galanakis *et al.*, 2017). For example, flavonoids such as quercetin and rutin have been shown to have strong absorption peaks in the UVA range (373 nm and 341 nm respectively), and their investigated sun protective factors (SPFs) have been shown to be similar to that of homosalate, which is a reference compound used to establish FDA standard sunscreen agents (Choquenet *et al.*, 2008). The same study also showed that the effectiveness of quercetin and rutin against UV is influenced by the concentrations used, with a dose-dependent effect observed. This supports the results of the present study. Phenolic acids such as caffeic, ferulic, *p*-coumaric, *p*-hydroxybenzoic and chlorogenic acids, which are present in almost all of the plant extracts studied, as well as anthocyanins (*C. ternatea*) and triterpenoids (*C. asiatica*), have been shown to have maximum absorption in the UVA, UVB and UVC range (Burdulis *et al.*, 2007; Spagnol *et al.*, 2015; Rojas *et al.*, 2016). Therefore, the protective effect against UV-induced mtDNA damage exhibited by the plant extracts in this experiment may have been contributed by their UV absorption properties.

#### 7.4.3 Pre-incubation experiment: protective effect against UV-induced mtDNA damage

In the previous chapter, some of the plant extracts were shown to have protective effects against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress (see Chapter 6), and the protective effect exhibited is suggested to be due to their antioxidant activity. In this chapter, UV was used as a source of oxidative stress and the protective effect against UV-induced mtDNA damage was investigated. A comparison of potency at 63 µg/mL showed that only MOE and CCE protected HaCaT from UV-induced mtDNA damage, whilst none of the water extracts showed any protective effect. At higher concentration (250 µg/mL), *C. ternatea* extracts (CTW and CTE) were selected to be compared with *M. oleifera* extracts (MOW and MOE) due to the positive activity exhibited in the previous chapter. At this concentration, MOW, MOE and CTW exhibited significant protective effects against UV-induced mtDNA damage, suggesting a potential dose-dependent protective effect.

Excessive UV exposure to the skin is known to cause oxidative damage to lipids, proteins and DNA, either directly or indirectly through ROS generation (Fisher *et al.*, 2002; Farage *et al.*, 2008; Ahsanuddin *et al.*, 2016). An extensive literature search showed that no studies have been published on the protective effect of *M. oleifera*, *C. caudatus* and *C. ternatea* extracts against UV-induced mtDNA damage. However, the plant extracts have been shown to have anti-radical and metal ion chelating properties mainly due to their phenolic content (see Chapter 4), which may play a role in modulating the protective effect displayed in this experiment. Phenolics have been shown to play a role in the prevention of UV-induced skin damage (Svobodová *et al.*, 2003), and their mechanisms of action include prevention of DNA damage and regulation of DNA repair (Afaq and Katiyar, 2011). It is possible that pre-treatment of HaCaT with the plant extracts reduces the effect of UV not only by absorbing UV radiation (Radice *et al.*, 2016), but also by interfering with the generation of ROS in the cells

(see Chapter 4), thus reducing mtDNA damage. The inhibition of ROS generation can occur either through the inhibition of lipid peroxidation, hydrogen peroxide, nitric oxide production or the depletion of antioxidant enzymes (Katiyar and Mukhtar, 2001; Sharma *et al.*, 2007). The pre-treatment of keratinocytes with an antioxidant has also been shown to reduce the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in mtDNA (Lee *et al.*, 2013), which is formed via oxidative damage to the DNA. Other than that, the topical application of polyphenols *in vivo* has been shown to reduce the formation of cyclobutene pyrimidine dimers (CPD), and to promote the repair of UV-induced CPDs in a dose-dependent manner through an IL-12-dependent DNA repair mechanism (Schwarz *et al.*, 2005; Meeran, Mantena and Katiyar, 2006; Meeran, Mantena, Elmets, *et al.*, 2006). In a different study, polyphenol supplementation has been shown to successfully restore D-loop mutations in mtDNA, with an increase in the levels of antioxidant enzymes in alcoholic rats (Reddyvari *et al.*, 2017). Although the damage was not induced by UV, the assessment of D-loops was similar to that in this study. All the studied extracts contain high TPC, and the fact that MOE has the highest TPC with the highest potency of the protective effect exhibited accords with these speculated mechanisms. Further potential mechanisms of action which may be exerted by various groups of polyphenols have been discussed by Svobodová *et al.* (2003) and Nichols and Katiyar (2010).

#### 7.4.4 Limitations

The qPCR mtDNA damage assay is a rapid and sensitive method with many strengths (Meyer, 2010). However, the exact mechanisms of action of the extracts in exerting their protective effect need further specific assays. Since the assay can detect all types of damage based on the impairment of DNA polymerase progression (Hunter *et al.*, 2010; Furda *et al.*, 2014), it cannot distinguish between different types of DNA damage, but this knowledge may be useful in understanding the potential mechanisms of action involved.

## 7.5 Conclusion

The plant extracts studied have high potential to be developed into UV-filters and antioxidant agents in commercial cosmetic products. MOE showed the highest potency of protective effect against UV-induced mtDNA damage in both UV-filter and antioxidant experiments, while some of the water extracts showed significant activity at higher concentrations. The protective activity may be due to the phenolic content, but more specific assays are needed to understand the potential mechanisms of action involved.

# **Chapter 8**



## Chapter 8: General discussion and conclusion

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This study was designed in response to the Malaysian Economic Transformation Programme (ETP) in the agricultural sector, where one of the main items in the agriculture National Key Economic Area (NKEA) agenda is to raise the status of local herbs for commercialisation (Ministry of Agriculture Malaysia, 2010). There are many herbal products available in the market now; however, their application in the cosmetics industry may represent a more promising investment according to the current trends and the demand for natural and organic cosmetics (Future Market Insight (FMI), 2015; Hassali *et al.*, 2015). This study aims to add agricultural and commercial value to the selected medicinal plants. Therefore, four medicinal plants (*M. oleifera* Lam., *C. asiatica* (L.) Urban, *C. ternatea* L. and *C. caudatus* Kunth.) were selected for an investigation of their anti-ageing properties with potential exploitation as cosmetics ingredients. These plants are considered to be underutilised in Malaysia and scientific evidence to support their traditional and cosmetic usages is lacking.

Skin ageing is a complex degenerative process of the skin caused by many factors such as genetics, nutrition, chemical agents and exposure to UV radiation (Farage *et al.*, 2008; Situm and Sjerobabski-Masnec, 2010; Farris and Krol, 2015; Lephart, 2016). It can be classified into two types, intrinsic and extrinsic skin ageing, which differ in causal factors and the characteristics of the aged skin (Raschke and Elsner, 2010; Situm and Sjerobabski-Masnec, 2010; Farage *et al.*, 2013). The exact mechanisms involved in the pathogenesis of skin ageing are yet to be fully understood, but substantial evidence suggests that they share similar fundamental potential mechanisms of action (Fisher *et al.*, 2002).

Many theories have been proposed to explain the pathogenesis (Jin, 2010; Mercado-Sáenz *et al.*, 2010; Alfredo *et al.*, 2014), and the accumulation of oxidative cellular damage to skin cells, proteins, lipids and DNA as proposed in the free radical or oxidative stress theory of skin ageing

is widely accepted (Rinnerthaler *et al.*, 2015; Ahsanuddin *et al.*, 2016). Additionally, most of the skin ageing targets relevant to cosmetics applications have also been shown to be associated with oxidative stress in the skin. “Anti-ageing” properties with respect to skin ageing and cosmetic products include skin lightening and the reduction of skin spots, skin renewal and stimulation, plumping for smoother skin and moisturising (Center for the Promotion of Imports from Developing Countries (CBI), 2016). Hence, this thesis examines the anti-ageing properties of medicinal plants relevant to cosmetics applications, which include their anti-oxidant, anti-collagenase, anti-elastase and anti-tyrosinase activities. Their protective effects against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity and UV-induced mtDNA damage in HaCaT cells (keratinocytes) are also investigated.

## **8.1 Potential application as antioxidant, anti-wrinkle, hypo-pigmentation and UV filter agents**

Antioxidants are any molecules that are capable of being oxidizing in preference to other molecules, and they can interact with free radicals and terminate the chain reaction (Oroian and Escriche, 2015). In plants, polyphenols are excellent antioxidants found in roots, stems, leaves, flowers and leaves which are capable of protecting themselves from oxidative stress induced by UV or other environmental factors (Pouillot *et al.*, 2011). Natural antioxidants such as vitamins, phenolic acids and flavonoids are used in the cosmetics industry as a preventive measure against skin ageing due to oxidative stress (Costa and Santos, 2017). Therefore, the two extraction methods used in this study also targeted polyphenols, where extraction in water was used to mimic the traditional preparation and 70% ethanol extraction was used due to its feasibility and because it is recognized as safe in industrial applications.

In Chapter 4, the plant extracts studied were investigated for their antioxidant potential, where their properties to scavenge diverse types of free radicals and their ability to chelate

metal ions were assessed. The correlation of the antioxidant activities of the extracts with their respective TPC was also assessed. The antioxidant assays showed that all the plant extracts can scavenge free radicals (DPPH and ABTS) and chelate metal ions, suggesting the potential application of the plant extracts as antioxidant agents in cosmetics products. The antioxidant activities vary depending on the extraction methods used to prepare the plant extracts, and aqueous ethanol extracts have higher antioxidant potential than water extracts. In the experiments, MOE showed the highest potential compared with all other extracts in all of the assays employed (DPPH, ABTS and FRAP). Although the scavenging activities of the plant species have been reported (Zainol *et al.*, 2003; Kamkaen and Wilkinson, 2009; Sreelatha and Padma, 2009; Mustafa *et al.*, 2010; Vongsak, Sithisarn, Mangmool, *et al.*, 2013), these activities may differ due to factors associated with the plant samples, such as processing and extraction methods, and genetics (Coelho *et al.*, 2016; Boneza and Niemeyer, 2018). Therefore, the comparison between the water and ethanol extracts of the plant species that showed different results than those previously reported further enhances the importance of the experiments conducted in this study.

In the assessments for potential collagenase, elastase and tyrosinase inhibition, no pattern of differences in activity was observed between the water and ethanol extracts. *M. oleifera* extracts (MOW and MOE) either showed very low or no inhibition against collagenase, elastase and tyrosinase. *C. asiatica* extracts (CAW and CAE) showed inhibition against collagenase and tyrosinase, while *C. ternatea* extracts (CTW and CTE) inhibited collagenase and elastase, but not tyrosinase. Meanwhile, *C. caudatus* extracts (CCW and CCE) showed potential application as inhibitors against all enzymes. Targeting these enzymes is significant in cosmetics applications for anti-wrinkle and hypo-pigmentation agents, which are preferable due to their localized effects (Tsuji *et al.*, 2001; Brenneisen *et al.*, 2002; Hakozaki *et al.*, 2010).

Of all the plant species, *C. caudatus* and *C. ternatea* showed the highest potential due to their activities against collagenase, elastase and tyrosinase. The observed activities of *C. caudatus* and *C. ternatea* extracts against these enzymes could be attributed to the presence of flavonoids such as quercetin and kaempferol that have been shown to inhibit collagenase, elastase and tyrosinase in similar assays or cell cultures (Melziq *et al.*, 2001; Chen and Kubo, 2002; Sin and Kim, 2005; Fujii *et al.*, 2009). Anthocyanins, which are present in *C. ternatea* extracts, have also been shown to inhibit collagenase, elastase and tyrosinase (Sartor *et al.*, 2002; Jhan *et al.*, 2016). More importantly, to the best of the present author's knowledge these are the first reports of the activity of these plant species against those enzymes; therefore, suggesting that these are novel findings relevant to their application in the cosmetics industry and indicating that the plants will have high agricultural value for mass cultivation.

In the cell culture experiments described in Chapter 6, the plant extracts were investigated for their protective effects against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in HaCaT. Previously, all the ethanol extracts of all plant species have been shown to have higher scavenging and metal chelating activities compared to their respective water extracts. However, in this experiment only the water extracts of *M. oleifera*, *C. asiatica* and *C. ternatea* showed significant protective effects (*p*< 0.05), whereas none of the ethanol extracts did. These results may be explained by the high toxicity of the ethanol extracts to HaCaT, and lower concentrations were used compared with the water extracts. In general, several studies have shown a protective effect of *M. oleifera*, *C. asiatica* and *C. ternatea* extracts against oxidative stress, which supports the observed results in this chapter. *M. oleifera* extracts show improvements in cell proliferation, the suppression of apoptotic events and lipid peroxidation, and a significant enhancement of antioxidant enzyme levels *in vitro* and *in vivo* (Jaiswal *et al.*, 2013; Prasanna and Sreelatha,

2014; Abdul Hisham *et al.*, 2018). *C. asiatica* has been shown to decrease the p53 protein and the expression of genes involved in apoptosis, cell growth, transcription, senescence and DNA replication in fibroblast cells (Kim *et al.*, 2011), and lower malonaldehyde (MDA) levels or low density lipids *in vivo* (Hussin *et al.*, 2009; Mahanom *et al.*, 2011; Ayala *et al.*, 2014). Meanwhile, *C. ternatea* was shown to inhibit lipid glycation and lipid peroxidation and to suppress ROS (Phrueksanan *et al.*, 2014; Yimdee *et al.*, 2014; Chayaratanasin *et al.*, 2015; Nair *et al.*, 2015). However, few studies have been conducted on their protective effect against oxidative stress in HaCaT cells; hence, pointing to the significance of this experiment.

The protective effect of the plant extracts against UV-induced mtDNA damage was also investigated, where the role of plant extracts as UV filters and antioxidants was assessed. In the UV filtering experiment, the observed protective effect shown by the plant extracts (CAW, CTW, CCW and MOE) was potentially dose-dependent, where only MOE showed protective effects at the lowest non-toxic concentration. It is suggested that the flavonoids, which are natural UV filters that are present in the extracts, contributed to the protective effect in this experiment by reducing the amount of UV reaching the HaCaT cells (Pereira *et al.*, 2009; Agati *et al.*, 2013; Radice *et al.*, 2016; Galanakis *et al.*, 2017). This conclusion is further supported by the UV spectra for each of the plant extracts showing the ability to absorb UV; thus, suggesting potential applications of the plant extracts as UV filters. Additionally, it is also possible that the plant extracts modulate the protective effect against UV-induce mtDNA damage through their anti-radical and metal-chelating properties (see Chapter 4), which were exhibited by MOE, CCE and CTW in the pre-incubation experiment. Phenolics have been shown to play a role in the prevention of UV-induced skin damage through various mechanisms (Svobodová *et al.*, 2003), and it is possible that the extracts interfere with UV-induced ROS generation in HaCaT (Katiyar and Mukhtar, 2001; Sharma *et al.*, 2007).

## 8.2 Strengths and limitations

This thesis has shown the potential application of the plant extracts as antioxidants, and anti-wrinkle, hypo-pigmentation and UV filter agents, where some of the activities found are the first reports to the best of the author's knowledge. For example, the anti-collagenase, anti-elastase and anti-tyrosinase activities of *C. caudatus* extract have not previously been reported, and neither have the anti-collagenase and anti-elastase activities of *C. ternatea* extracts. Although the protective effect of the plant extracts against oxidative stress has been shown, studies using HaCaT cells and assessment of UV-induced mtDNA damage are limited. This suggests that further research is required for cosmetics application of the plant species studied. Other than that, this thesis has also shown that the enzymatic colorimetric assays, which are commonly used, are not suitable for the assessment of plant extracts with coloured pigment. This will be particularly important in future research assessing other plant species of agricultural importance.

As for the limitations of this study, further specific assays are required to elucidate the potential mechanism of action of the plant extracts in more detail. In the cell culture assays, limited range of concentrations can be tested for the ethanol extracts due to their toxic effect to HaCaT; hence, no protective effect was observed (Chapter 6). The activity of individual pure active compounds has not been investigated in this thesis because the extracts contain more than one active compound that could work in synergy or antagonistically. However, the LC-MS data for each of the plant extracts provide important clues for the future standardization of the extracts.

Another point that needs to be considered is the concentrations used in the experiments were in the higher range (Chapters 4 and 5). In Chapters 4 and 5, the selected concentrations range was from 63-1000 µg/mL, so that the samples with weak activity level could be investigated.

However, in the case of extracts with potent antioxidant level; the concentration range may affect the  $IC_{50}$  values obtained. Although the  $IC_{50}$  values were shifted from the regression line in certain extract samples, the results did not affect the comparison between samples and standard (Trolox) because the level difference was apart. This limitation could be overcome by selecting a lower range of concentrations for the extracts with high antioxidant potential.

This issue is also observed in the experiments in Chapters 6 and 7, where high concentrations were selected for certain samples that have low cytotoxicity level on HaCaT. Therefore, these samples should be further investigated at lower concentrations for their observed their protective effects against  $H_2O_2$ -induced cytotoxicity and UV-induced mtDNA damage. Their application as active ingredients need further investigation to properly develop an effective and sustainable formulation. The most commonly used concentrations of an active in a formulation is from 1-5% and sometimes can be up to 10% depending on the potency and activity of the active ingredient (FDA, 2018). Further optimisation of the extracts for proper final product development such as extraction procedures for optimal active extracts or isolation of active compounds will resolve the issue. The intended use or function of the extract may also influence the concentration used in a formulation, in which a UV absorber agent (zinc oxide and titanium dioxide) may comprise up to 25% of the total formulation (FDA, 2018) suggesting that the high concentration used may be benefited for other usage.

It should also be noted that the antioxidant assays used (Chapter 4) were based on the chemical reaction between an antioxidant and a pre-formed radical. These assays are widely used to evaluate antioxidant activities of plant-based extracts (Moharram and Youssef, 2015; Djouonzo *et al.*, 2018; Lim *et al.*, 2019; Samsonowicz *et al.*, 2019; Sridhar and Linton, 2019). Despite the strength of the assays used (see Chapter 4, section 4.1.3), there are also limitation that need to be considered (Huang *et al.*, 2005; Pinchuk *et al.*, 2012; Schaich *et al.*, 2015; Apak

*et al.*, 2016). Since these radicals are pre-formed *in vitro*, the reported activity may not represent the actual activity *in vivo*; but the assays suggest their ability to react and participate in a redox reaction that could represent the mechanism involved in lowering oxidative stress in the skin. This is supported by the results in Chapters 6 and 7 in experiments using HaCaT cells, where significant protective effects were observed in H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity and UV-induced mtDNA damage of some of the prepared extracts that correlate with their antioxidant activities as measured in Chapter 4. Though the assays are very simple, fast and convenient, lack of standardization of the experimental procedures in the literature causes discrepancy in standard values for comparison (Apak *et al.*, 2016). It is very crucial to have a positive standard in each experiment runs for comparison to ensure the reproducibility and consistency of the standard values.

In Chapters 4 and 5, the positive controls used were Trolox (antioxidant activity), EGCG (anti-collagenase and anti-elastase) and kojic acid (anti-tyrosinase). These controls had shown potent antioxidant and inhibition against the studied enzymes compared to the extracts. The IC<sub>50</sub> values of Trolox in DPPH and ABTS assays were 3.32 and 6.51 µg/mL (see Table 4.6), whereas the extracts had less potent activities. Similarly, the IC<sub>50</sub> values for EGCG in the anti-collagenase and anti-elastase fluorescence assays were 13.2 and 487 µg/mL, while kojic acid in the tyrosinase assay was 79.2 µg/mL. Though the extracts had lower activity than the positive control, it should be considered that extracts preparation has not being optimised; thus the results. With proper optimisation of the extracts preparation, an optimal activities comparable with the controls may be achieved. As previously mentioned, lack of standardization in the *in vitro* assays results in variation in the gold standard values of the control for comparison (Moon *et al.*, 2010; Abdul Wahab, 2014; Azmi *et al.*, 2014; Himamura *et al.*, 2014; Mandrone *et al.*, 2015). Therefore, it is very important to run a positive control

for each of the assay replicates. Despite the limitation, the results in these *in vitro* assays are crucial in screening samples prior to cell culture and *in vivo* studies.

### 8.3 Conclusion

In conclusion, the results presented in this thesis suggest that the selected medicinal plants have potential to be developed as cosmetic ingredients. Their respective target of actions may differ depending on the extraction methods used to prepare the extracts and some extracts may have multi-action activities. Of all the extracts studied, the *M. oleifera* and *C. ternatea* extracts showed the highest potential to be developed as antioxidant ingredients, with significant protective effects observed in terms of H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity and UV-induced mtDNA damage in HaCaT cells. *C. ternatea* flower extracts also exhibited significant inhibition against collagenase and elastase activities, suggesting potential application as anti-wrinkle ingredients. Other extracts such as *C. asiatica* exhibited low antioxidant activities, but with potential application as collagenase and tyrosinase inhibitors. Meanwhile, the *C. caudatus* extracts have multi-action activities such as antioxidant, anti-collagenase, anti-elastase and anti-tyrosinase activities. Therefore, the results in this study represent potential commercial value and future agricultural potential in terms of mass cultivation of the selected medicinal plants. Their putative traditional usages are also supported with scientific evidence. However, it should be noted that further optimisation in extract preparations are required to obtain extract with high potency. Other than that, additional experiments are required to further elucidate the mechanism of the protective effects observed.



# **Chapter 9**



## **Chapter 9: Future direction and recommendation**

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This study focuses on the evaluation of the extracts from medicinal plants with agricultural potential for application as cosmetic ingredients. The results obtained provide significant data on the potential development of each of the plant extracts as antioxidant, anti-wrinkle, hypo-pigmentation and UV filter agents. Most of the plant extracts have exhibited multi-action anti-ageing activities, which are sought after by consumers of cosmetics. Future directions and recommendations for further research area are suggested as follows:

The comparison between the water and aqueous ethanol extracts of each of the plant species has showed that their activities depend on the extraction methods used to prepare the extracts. Therefore, an evaluation of different methods to prepare the plant extracts should be performed to obtain more potent and stable extracts with high extraction yields. This is particularly important in balancing the supply and demand chain of the raw materials and cosmetics applications. The phytochemical analysis data presented in Chapter 3 provides an important basis of solvent selection for future research. The identification, fractionation and isolation of individual phytoconstituents from each of the plant extracts are also required to gain fuller understanding of the role of individual compounds responsible for the activities measured.

The process of skin ageing in humans is complex (eg. differs between ethnic groups, environment and life-style) and may involve multiple pathways. This study focuses on various anti-ageing properties of the plant extracts; however, specific mechanistic assays are required to elucidate and further understand the mechanisms of action involved in each of the exhibited anti-ageing properties. For example, assessment of proteins involved in the hypothesized pathways will allow further elucidation of the mechanisms of action in  $H_2O_2$ -induced cytotoxicity and UV-induced mtDNA damage experiments.

Anti-collagenase, anti-elastase and anti-tyrosinase activities require further investigation using cell culture experiments, which will provide information on the effect of cellular interactions with the plant extracts on the anti-ageing properties exerted. Some of the suggested cells for the assays are fibroblast cells (HDFn) for anti-collagenase and anti-elastase activities, and B16 melanoma cells for anti-tyrosinase activity.

Another interesting aspect that needs further investigation is the stability of the plant extracts and their active components. In general, polyphenols at very high concentrations are also prone to auto-oxidation which could reduce the observed activity. Some of the active components in the plant species investigated, such as anthocyanins in *C. ternatea*, are sensitive to light and pH changes, which will affect the activity exerted. Although most cream formulations include stabilizers or preservatives to solve such problem, studies on this aspect may explain the absence of activity in some of the plants investigated.

With further research, the selected medicinal plants will be shown to have high potential for commercialisation as cosmetic ingredients. This will also lead towards a positive impact on the Malaysian agriculture sector.

# **Publication**



## In vitro protective effects of an aqueous extract of *Clitoria ternatea* L. flower against hydrogen peroxide-induced cytotoxicity and UV-induced mtDNA damage in human keratinocytes

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The traditional practice of eating the flowers of *Clitoria ternatea* L. or drinking their infusion as herbal tea in some of the Asian countries is believed to promote a younger skin complexion and defend against skin aging. This study was conducted to investigate the protective effect of *C. ternatea* flower water extract (CTW) against hydrogen peroxide-induced cytotoxicity and ultraviolet (UV)-induced mitochondrial DNA (mtDNA) damage in human keratinocytes. The protective effect against hydrogen peroxide-induced cytotoxicity was determined by 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, and mtDNA damage induced by UV was determined by polymerase chain reaction. Preincubation of HaCaT with 100, 250, and 500 µg/ml CTW reduced cytotoxicity effects of H<sub>2</sub>O<sub>2</sub> compared with control (H<sub>2</sub>O<sub>2</sub> alone). CTW also significantly reduced mtDNA damage in UV-exposed HaCaT ( $p < .05$ ). CTW was chemically-characterized using high resolution liquid chromatography-mass spectrometry. The main compounds detected were assigned as anthocyanins derived from delphinidin, including polyacylated ternatins, and flavonol glycosides derived from quercetin and kaempferol. These results demonstrated the protective effects of *C. ternatea* flower extracts that contain polyacylated anthocyanins and flavonol glycosides as major constituents, against H<sub>2</sub>O<sub>2</sub> and UV-induced oxidative stress on skin cells, and may provide some explanation for the putative traditional and cosmetic uses of *C. ternatea* flower against skin aging.

### KEYWORDS

antioxidant, *Clitoria ternatea*, cytotoxicity, HaCaT, hydrogen peroxide, mitochondrial DNA, oxidative stress, skin aging

### 1 | INTRODUCTION

*Clitoria ternatea* L. (Leguminosae) or the butterfly pea originates from tropical Asia and was later distributed to South and Central America, East and West Indies, China, and India (Zingare, Zingare, Dubey, & Ansari, 2013). There are at least 60 species in the genus *Clitoria*, and *C. ternatea* is the most frequently reported species (Zingare et al., 2013). The plant is a perennial and has conspicuous blue and white flowers resembling a conch-shell (Mukherjee, Kumar, Kumar, & Heinrich, 2008).

The traditional uses of *C. ternatea* are extensive, ranging from medicinal to food dyes for its naturally vivid blue colour. There have

been many pharmacological activities reported for the plant such as antimicrobial, antipyretic, anti-inflammatory, anticonvulsant, diuretic, anaesthetic, antidiabetic, and insecticidal (Kosai, Sirisidthi, Jiraungkoorskul, & Jiraungkoorskul, 2015; Mukherjee et al., 2008; Zingare et al., 2013). Previous research has focused on the roots, seeds, and leaves of the plant for their uses as a laxative, brain and nerve tonic in the Ayurvedic medicinal system. The root juice in particular was mixed with honey and ghee to improve mental health and memory in children or used as laxative when combined with ginger (Mukherjee et al., 2008). Root extracts improved memory in a number of *in vivo* studies, whereas an extract of leaves, stems, flowers, and fruits was sedative *in vivo* (Howes & Houghton, 2003). However, there

is a lack of research on the biological activities of the flowers to explain their traditional and potential uses. Apart from ornamental purposes, the flowers have been traditionally consumed in salads or drank as an herbal tonic to promote a younger skin complexion and defend against skin aging (Kaisoon, Siriamornpun, Weerapreeyakul, & Meeso, 2011; Lijon, Meghla, Jahedi, Rahman, & Hossain, 2017). Although there are some reports for the use of *C. ternata* flower extract as a cosmetic ingredient (Kamkaen & Wilkinson, 2009; Lijon et al., 2017; Tantituvanont, Werawatganone, Jiamchaisari, & Manopakdee, 2008), there is a paucity of data on the application of *C. ternata* flower extract in skin aging and scientific evidence to support this use is lacking.

*C. ternata* flower has been reported to have antioxidant potential, mainly due to its anthocyanin content (Chayaratana, Barbier, Suanpairintr, & Adisakwattana, 2015; Iamsaard et al., 2014; Kaisoon et al., 2011; Kamkaen & Wilkinson, 2009; Miguel, 2011). Many anthocyanins are known to have antioxidant effects, which may have an important role in the prevention of many diseases such as cancer, cardiovascular diseases, and skin aging; although there may be issues with their oral bioavailability (Howes & Simmonds, 2014; Miguel, 2011), thus topical use may be more appropriate for their relevance to dermatological conditions. Substantial evidence suggests oxidative damage from reactive oxygen species (ROS) that may originate from various factors such as genetic, lifestyle, nutrition, chemicals, and exposure to ultraviolet (UV) could lead to the deterioration of the skin surface, leading to wrinkles, hyperpigmentation, and sagging of the skin, which are aesthetically undesirable (Farage, Miller, & Maibach, 2010; Fisher et al., 2002; Gragnani et al., 2014; Sjerobabski-Masnec & Situm, 2010). Many therapies and strategies such as chemical peeling, injection of rejuvenating agents, fillers or implants, surgery, nutraceuticals, or application of cosmetic products have been developed to target skin aging via different mechanisms (Ganceviciene, Liakou, Theodoridis, Makrantonaki, & Zouboulis, 2012). Of these approaches, antiaging "skin care" is highly sought after, particularly those formulated with organic and naturally derived ingredients (Grand View Research Report, 2016; US Department of Commerce, 2015). Therefore, in this study, we investigated the potential antiaging properties of *C. ternata* flower water extract using a human keratinocytes cell line (HaCaT), assessing the protective effects against hydrogen peroxide-induced cytotoxicity and UV-induced mitochondrial DNA (mtDNA) damage, to scientifically support the putative traditional benefits of *C. ternata* flower extract for the skin, with potential development as an "antiaging" ingredient in cosmetic products.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals, reagents, and materials

The diphenyl-picryl hydrazine (DPPH) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid; ABTS), hydrogen peroxide (18.2 M) were purchased from Sigma Aldrich, UK. Dulbecco Modified Eagle Medium (DMEM), Fetal Calf Serum, and trypsin were purchased from Lonza, UK. The solution 3-(4, 5-dimethylthiazol-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) was purchased from Promega, UK. QIAamp DNA Mini Kit for total DNA extraction was purchased from Qiagen, UK. The primers were designed and supplied by Eurofins, UK. The 2× SensiMix Hi-ROX and MicroAmp Fast Optical 96-Well Reaction Plate were obtained from Bioline, UK and ThermoFisher Scientific, UK.

### 2.2 | Plant material

Plant material was purchased from Siam Herbarium, Thailand. The voucher sample was deposited in the Herbarium of Medicinal Plant Research Group, Newcastle University (CT/NNAZ/001).

### 2.3 | Extract preparation

Previous methods were followed with some modification (Islam, Parvin, Uddin, & Mazid, 2014; Thring, Hili, & Naughton, 2009). Briefly, powdered plant material was weighed (50 g) and extracted in 500 ml of freshly boiled deionized water for 30 min. The extract solution was allowed to cool down with frequent shaking before sonication for 15 min. The extract was vacuum-filtered for at least three times and the marc was discarded. Finally, the extract was freeze-dried and stored at -20 °C until used. Water was selected as solvent to reflect the traditional method for extract preparation.

### 2.4 | Antioxidant assays

The DPPH and ABTS assays were adapted from previous protocols (Chattuwatthana & Okello, 2015).

### 2.5 | Cell line and cell culture

Normal human keratinocytes (HaCaT) cell line was obtained from the Medical School of Newcastle University. The cells were maintained in complete DMEM (10% Fetal Calf Serum and 1% pen-strep) with 5% CO<sub>2</sub> at 37 °C.

### 2.6 | Cytotoxicity assay

Ten thousand cells/well were plated into a 96-well plate overnight at 37 °C. The next day, the medium was aspirated, cells were rinsed with 100 µl PBS and fresh DMEM was added (100 µl). HaCaT was treated with varying concentrations (50–2,000 µg/ml) of the plant extracts for 24 hr to determine the nontoxic concentrations. The number of viable cells after treatments was measured using MTS assay, expressed as the percentage of viable cell to untreated cells. The MTS assay was performed as described by the manufacturer (Promega, UK). Briefly, the medium was aspirated and cells were rinsed with PBS (100 µl) and fresh DMEM (100 µl) was added. The MTS solution (20 µl) was added into each well and the plate was incubated for 4 hr at 37 °C before reading at 490 nm with Spectromax Plus 384® and viewed with Softmax Pro V5 software.

### 2.7 | Hydrogen peroxide induced-cytotoxicity assay

Ten thousand cells/well were plated in a 96-well plate overnight at 37 °C. HaCaT cells were preincubated with the plant extracts at

different concentrations for 24 hr, prior to exposure to  $\text{H}_2\text{O}_2$  (200  $\mu\text{M}$ , negative control) from a freshly prepared 1,000  $\mu\text{M}$  stock solution. Positive control was the untreated HaCaT. Cell viability was determined by MTS assay as described above after 24 hr.

## 2.8 | UV-induced mtDNA damage assay

$2.5 \times 10^5$  cells/3 ml of HaCaT in complete DMEM were seeded in 35-mm culture dishes and allowed to settle overnight at 37 °C. The used medium was removed and cells were rinsed with 1 ml PBS and subsequently incubated with *C. ternatae* water extract (CTW) diluted in complete DMEM to a total volume of 3 ml for 24 hr at 37 °C. After incubation, the medium was removed, washed with 1 ml of PBS and 3 ml of phenol-free DMEM with 1% pen-strep was added to each dish. Later, the dish was placed under the UV lamps (seven 6 foot iSOLde Cleo performance 100 W-R lamps (Cleo); iSOLde, Germany) fitted into a custom built irradiation unit for ~40 min, equivalent to 4.0 standard erythema damage. The UV source delivered a flux of 40  $\text{mJ}/\text{cm}^2$  between 300 and 400 nm wavelength and the dose received by the samples was measured with a DMC150 Monochromator (Bentham Instruments Ltd., UK). After exposure, the cells were scrapped in a circular motion and observed under the microscope to ensure all cells were collected prior to DNA extraction.

## 2.9 | DNA extraction

The protocol for total DNA extraction was followed as recommended by the manufacturer using a QIAamp DNA Mini Kit (Qiagen, 2016). Briefly, the collected cell pellets were suspended in PBS (200  $\mu\text{l}$ ), added with Proteinase K (20  $\mu\text{l}$ ), and lysis buffer (200  $\mu\text{l}$ ). The mixture was vortexed (15 s) and incubated (56 °C) for 10 min, before adding absolute ethanol (200  $\mu\text{l}$ ). The mixture was further vortexed (15 s) and added to QIAamp Spin Column for centrifugation (8,000 rpm for 1 min). The flow-through solution was discarded and the column was washed with washing buffer 1 (500  $\mu\text{l}$ ) and centrifuged (8,000 rpm for 1 min). The flow-through was discarded, washed with washing buffer 2 (500  $\mu\text{l}$ ), and centrifuged (14,000 rpm for 3 min). The flow-through was discarded and the column was spun (14,000 rpm for 1 min). Finally, DNA was eluted with an elution buffer (60  $\mu\text{l}$ ) and stored at 4 °C for 1 day prior to use.

## 2.10 | qPCR procedures

DNA samples were thawed and vortexed (15 s) to mix. DNA samples were diluted with RNase/DNase free water to obtain the required concentration (6 ng/ $\mu\text{l}$ ). The protocol was performed on ice. The forward (AL4: CTGTTCTTTCATGGGGAGC) and reverse primers (AS1: AAAGTGCATACCGCCAAAG) were diluted with water to obtain a 10  $\mu\text{M}$  of forward and reverse primers working solutions. Later, the PCR mastermix, 2 $\times$  SensiMix Hi-ROX, and primers were assembled on ice. Then, 18  $\mu\text{l}$  of the mastermix working solution was added into each well of a MicroAmp Fast Optical 96-Well Reaction Plate and 2  $\mu\text{l}$  of DNA samples were added and thoroughly mixed. The final concentrations for 2 $\times$  SensiMix Hi-ROX, each primer and samples were 1 $\times$ , 0.25  $\mu\text{M}$  and 1.2 ng/ $\mu\text{l}$  respectively. The PCR was run using StepOnePlus Real Time PCR system (Applied Biosystem,

UK). The PCR conditions composed of preincubation phase of 10 min at 95 °C followed by 30 cycles of 98 °C for 15 s, 60 °C for 15 s, and 72 °C for 55 s; with a final stage of 72 °C for 7 min. A threshold in the linear range of the amplification plot was set. The number of cycles at which the fluorescence crossed the threshold (Ct) were used to compare the mtDNA damage between samples. DNA sample with a low Ct value indicates low damage (high copy number) and a high Ct value indicates high damage (low copy number).

## 2.11 | UV absorption

Three millilitre of the plant samples were pipetted into a cuvette and the absorption spectrum from 190 to 800 nm was determined using a Cary Spectrophotometer™. Blank (water) subtracted from the background reading.

## 2.12 | Phytochemical analysis

Previous methods were followed for qualitative analysis (Khanam, Wen, & Bhat, 2014), total phenolic content (Chattuwatthana & Okello, 2015), and total anthocyanin content (Lee, Durst, & Wrolstad, 2005).

## 2.13 | Liquid chromatography-serial mass spectrometry (LC-MS/MS)

The extract was reconstituted at 10 mg/ml prior to chemical characterisation using LC-UV-MS/MS analysis. Analyses were performed on a Thermo Scientific system consisting of an "Accela" U-HPLC unit with a photodiode array detector and an "LTQ Orbitrap XL" mass spectrometer fitted with an electrospray source (Thermo Scientific, Waltham, MA, USA). Chromatography was performed on 5  $\mu\text{l}$  sample injections onto a 150  $\times$  3 mm, 3  $\mu\text{m}$  Luna C-18 column (Phenomenex, Torrance, CA, USA) using the following 400  $\mu\text{l}/\text{min}$  mobile phase gradient of H<sub>2</sub>O/CH<sub>3</sub>OH/CH<sub>3</sub>CN +1% HCOOH: 90:0:10 (0 min), 90:0:10 (5 min), 0:90:10 (60 min), 0:90:10 (65 min), 90:0:10 (67 min), 90:0:10 (70 min), followed by return to start conditions and equilibration in start conditions for 5 min before the next injection. The ESI source was operated with polarity switching and the mass spectrometer was set to record high resolution (30 k resolution) MS1 spectra (*m/z* 125–2,000) in positive mode using the orbitrap and low resolution MS1 spectra (*m/z* 125–2,000) in negative mode and data dependent MS2 and MS3 spectra in both modes using the linear ion trap. Detected compounds were assigned by comparison of accurate mass data (based on ppm), and by available MS/MS data, with reference to the published compound assignment system (Schymanski et al., 2014) and with supportive UV spectra.

## 2.14 | Statistical analysis

ANOVA (Dunnet's test) was used to compare differences between test groups and the negative control ( $\text{H}_2\text{O}_2$  only) using Graphpad Prism Software.

### 3 | RESULTS

#### 3.1 | Antioxidant activities

The antioxidant activities for CTW against DPPH and ABTS free radicals showed concentration-dependent effects (Figure 1). Both assays showed that CTW extract can scavenge free radicals, although the potency is lower than the positive control (Trolox; Table 1). The DPPH and ABTS assays are based on the ability of the extract constituents to donate an electron to the preformed radical resulting in decreasing absorbance (Badarinath et al., 2010; Tirzitis & Bartosz, 2010). Two assays for evaluation were used to ensure consistency of the activity (Moon & Shibamoto, 2009).

#### 3.2 | Effect of $H_2O_2$ on HaCaT cell viability induced after 24, 48, and 72 hr proliferation

Incubation of HaCaT with  $H_2O_2$  showed a concentration-dependent and time-dependent cytotoxicity. As expected, the higher  $H_2O_2$  concentration introduced to HaCaT, the more potent the cytotoxic effect. However, the inverse correlation observed between  $H_2O_2$  concentrations and the time point of when  $H_2O_2$  was exposed to HaCaT suggests that the cytotoxic effect is dependent on cell concentration. Longer incubation of HaCaT allowed the cells to proliferate, and therefore, reducing the cytotoxic effect of  $H_2O_2$  (Ohguru, Fukuda, Sasabe, & Tano, 1999). Significant decrease in cell viability was observed at 100 (30%), 200 (70%), 250 (80%), and 300  $\mu M$  (90%), with  $H_2O_2$  induced after 24 hr of proliferation. After 48 hr (Figure 2b), significant reduction in cell viability was observed at 150 (20%), 200 (30%), 250 (55%), and 300 (70%). After 72 hr (Figure 2c), significant reduction in cell viability was observed at 200 (30%), 250 (50%), and 300 (70%). Considering the experimental design, where the cells were allowed to acclimatize overnight and treated with plant extracts for 24 hr before  $H_2O_2$  exposure, a concentration of 200  $\mu M$   $H_2O_2$  induced after 48 hr of proliferation was selected as an appropriate concentration to induce toxicity, to assess the protective effect of the plant extracts against  $H_2O_2$ .

#### 3.3 | In vitro protective effects against 200 $\mu M$ $H_2O_2$

The protective effect of CTW against  $H_2O_2$  induced toxicity on HaCaT was evaluated at concentrations ranging from 50 to 2,000  $\mu g/ml$ . CTW

TABLE 1  $IC_{50}$  values for CTW and Trolox standard in DPPH and ABTS assays

Assays	CTW ( $\mu g/ml$ ) $IC_{50}$	Trolox standard ( $\mu g/ml$ ) $IC_{50}$
DPPH	195.5	3.32
ABTS	42.9	6.51

Note.  $IC_{50}$  value for all assays were obtained from the best fit line constructed from the dose-response graphs at which 50% inhibition was observed. A lower  $IC_{50}$  value indicates higher antioxidant potency. CTW = *C. ternata* water extract; DPPH = diphenyl-picryl hydrazine; ABTS = 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid).

concentrations of 50, 100, 250, and 500 were nontoxic to HaCaT as indicated in Figure 3. However, CTW concentrations of 1,000, 1,500, and 2,000  $\mu g/ml$  were toxic to HaCaT. Preincubation of HaCaT with CTW with 100, 250, and 500  $\mu g/ml$  reduced the toxic effect of  $H_2O_2$  on HaCaT as indicated by the significantly higher percentage cell viability (10–20%) at the specified concentrations, compared with negative control,  $p > .05$ . A lower CTW concentration (50  $\mu g/ml$ ) did not show any significant protective effect on HaCaT.

#### 3.4 | In vitro protective effect against UV-induced mtDNA damage

CTW concentration of 250  $\mu g/ml$  was selected for UV assay based on the activity in the  $H_2O_2$  assay. The positive control (protected HaCaT from UV) resulted in significantly lower mtDNA damage compared with the negative control (exposed HaCaT). Similarly, pretreated HaCaT with CTW showed significantly lower mtDNA damage compared with the negative control,  $p < .05$  (Figure 4). Analysis of CTW spectra absorption showed high absorption in the UVB and UVC range (Table 2). Two peaks were also identified in the visible light wavelength range.

#### 3.5 | Phytochemical analysis

Qualitative and quantitative analysis of phytochemicals indicated the presence of saponins and phenolics, including flavonoids, with total phenolic content and TAC values of  $38.5 \pm 1.21$  and  $3.61 \pm 0.71$  mg/ml, respectively (Table 3).

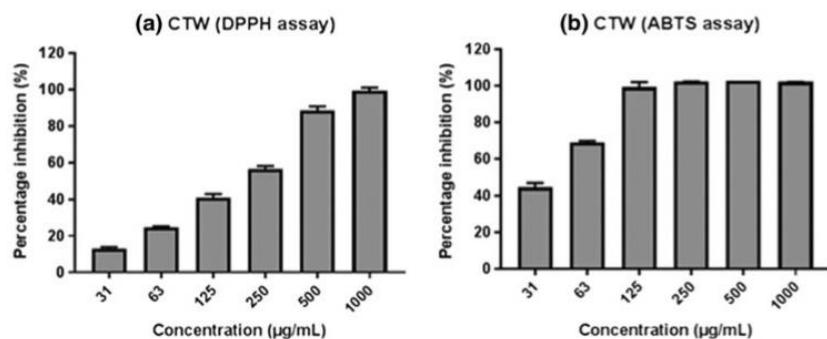
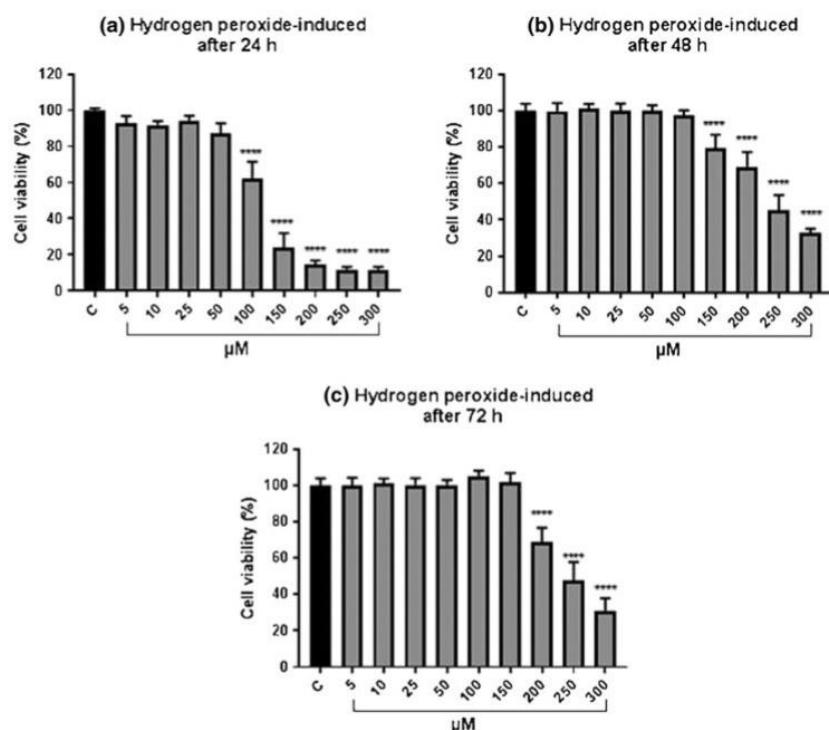
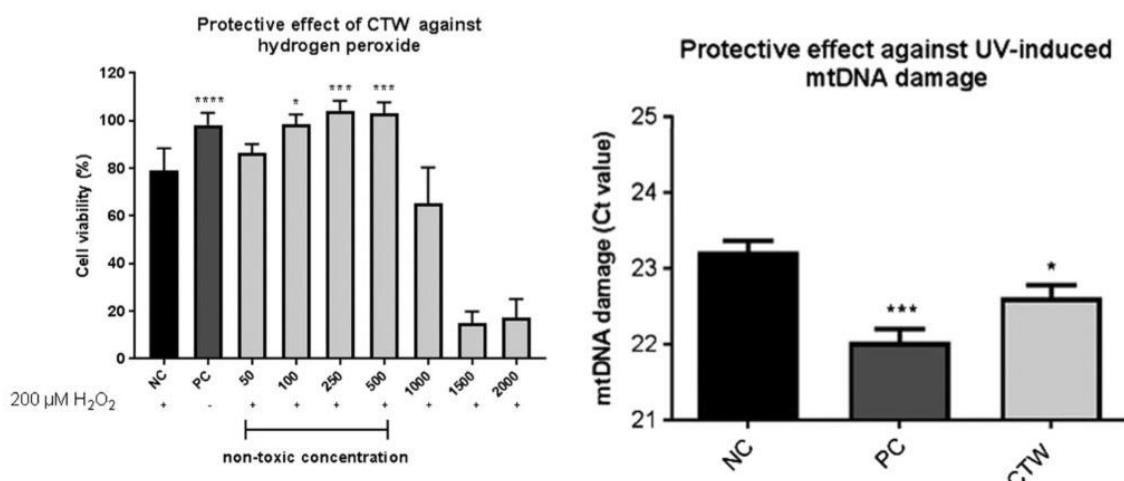


FIGURE 1 Diphenyl-picryl hydrazine and ABTS assays. Antioxidant activities of CTW against diphenyl-picryl hydrazine and ABTS radicals. Data are the mean  $\pm$  SD ( $n = 4$ ). ABTS = 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); CTW = *C. ternata* water extract



**FIGURE 2** Cytotoxicity of  $\text{H}_2\text{O}_2$  on HaCaT at different concentrations induced after 24, 48, and 72 hr proliferation with 24 hr incubation time. Percentage viability compared with untreated control (positive control) using Dunnett's test and  $p < .05$  is considered significant. Data are the mean  $\pm$  SEM of two independent experiments run with six replicates for each treatment



**FIGURE 3** Protective effect of *C. ternatea* water extract (CTW) against hydrogen peroxide-induced cytotoxicity. Figure shows the percentage cell viability of preincubated HaCaT with CTW in  $\text{H}_2\text{O}_2$ -induced oxidative stress. Data are the mean  $\pm$  SEM of three experiments with four replicates for each treatment. Differences between mean of treatments to negative control were determined by Dunnett's test with  $p < .05$  considered as significant. Negative control (NC) =  $\text{H}_2\text{O}_2$  alone and positive control (PC) = untreated HaCaT

**FIGURE 4** Protective effect of *C. ternatea* water extract (CTW) against ultraviolet (UV)-induced mitochondrial DNA (mtDNA) damage. Figure shows comparison of the mean differences  $\pm$  SEM of two experiments with three polymerase chain reaction replicates between the negative control (NC), positive control (PC), and treatment (CTW) as determined by Dunnett's test (\* $p < .05$  and \*\*\* $p < .001$  compared with NC)

**TABLE 2** *Clitoria ternatea* water extract absorption spectra

Spectra (nm)	Peak observed (nm) and OD
UVC (200–290)	266 (3.767), 289 (3.264),
UVB (290–320)	294 (3.261)
UVA (320–400)	—
Visible light (400–800)	573 (0.884), 619 (0.875)

Note. The absorption spectra for *C. ternatea* water extract. Peaks were observed in the visible light, UVB and UVC range. OD = optical density.

**TABLE 3** The qualitative analysis of phytochemical, TPC and TAC

Test compounds	CTW sample
1. Alkaloids (Dragendorff's)	(−)
2. Saponins (Froth)	(+)
3. Terpenoids (Salwoski)	(−)
4. Phenolics (Ferric chloride)	(+)
5. Flavonoids (Alkaline reagent)	(+)
6. Aldehyde (Schiff)	(−)
7. TPC as mg gallic acid equivalent /g dried extract	38.5 ± 1.21
8. TAC as mg cyanidin-3-glucoside equivalent /g dried extract	3.61 ± 0.71

Note. The presence or absence of compound denoted with (+) or (−). Data for TPC and TAC were mean ± SD ( $n = 4$ ). TPC = total phenolic content; TAC = total anthocyanin content; CTW = *Clitoria ternatea* water extract.

### 3.6 | Liquid chromatography-serial mass spectrometry (LC-MS/MS)

The assigned compounds detected in the CTW using high resolution LC-MS/MS are presented in Table 4. The main constituents detected included flavonol glycosides that were assigned from their observed  $[M + H]^+$  ions and supportive UV spectra and MS/MS interpretation. The detected flavonol glycosides consisted of the aglycones of quercetin, myricetin, and kaempferol, and included three malonylglycosides of kaempferol eluting with retention times of 22.4, 22.8, and at 25.6 min. The detection of glycosides and malonylglycosides of kaempferol, quercetin, and myricetin in the CTW investigated in this study is in agreement with the known constituents of *C. ternatea* flowers (Kazuma, Noda, & Suzuki, 2003a, 2003b). Other main constituents detected in CTW were assigned as acylated delphinidin glycosides from their observed  $M^+$  ions, with supportive UV spectra and MS/MS interpretation. These included a series of ternatins, which are known to occur in the flowers of *C. ternatea* (Kazuma et al., 2003b), thus supports the chemical authentication of the plant material investigated in this study. Other detected constituents in CTW included coumaroyl-sucrose and -glucose, observed from their  $[M + NH_4]^+$  ions; and the amino acids phenylalanine and tryptophan, observed from their  $[M + H]^+$  ions, as indicated in Table 4.

## 4 | DISCUSSION

The use of plants as cosmetics has been known since time immemorial, with evidence provided from cosmetic recipes recorded on the

Egyptian's Smith and Ebers papyri (Leake, 1952). Many plants, including herbs, fruits, and vegetables, contain polyphenols, which are widely documented as potent antioxidants that can scavenge free radicals.

*C. ternatea* flower extract has been reported to be used as a source of useful cosmetic ingredients, however, there is a paucity of data on the application of *C. ternatea* flower extract in skin aging (Kamkaen & Wilkinson, 2009; Lijon et al., 2017; Tantituvanont et al., 2008), and scientific evidence to support or understand its use in cosmetic products is also lacking.

This study demonstrated that pretreatments of human keratinocytes (HaCaT) with an aqueous *C. ternatea* flower extract significantly protected HaCaT cells against  $H_2O_2$ -induced cytotoxicity and UV-induced mtDNA damage.  $H_2O_2$  can generate the hydroxyl radical, a highly reactive free radical, in the presence of transition metals such as iron and copper through a reaction known as the Fenton reaction (El-Bahr, 2013; Winterbourn, 1995). Exposure to UV radiation has been shown to be one of the most damaging factors to the skin, as it can cause direct damage to cells, protein, and DNA, or may induce various signalling transduction pathways to mediate the damage (Fisher et al., 2002). It was suggested that UV initiates the molecular responses in human skin through the generation of ROS, where ROS generated from UV could oxidize and cause the activation of cell surface receptors triggering the downstream signal transduction pathways (Fisher et al., 2002). UV also activates NADPH oxidase, which generates  $H_2O_2$ , leading to further increase in oxidative stress (Fisher et al., 2002). It should also be considered that exposure to UV radiation is also associated with the development of melanomas (Shain & Bastian, 2016) in addition to the impact on skin aging.

In this study, the major constituents detected in the *C. ternatea* flower extract (Table 4) were assigned as kaempferol, quercetin, and myricetin glycosides and acylated anthocyanins derived from delphinidin. Flavonols and their glycosides, and anthocyanins, are widely documented to have potential benefits to human health, which may be mechanistically associated with antioxidant effects (Howes & Simmonds, 2014; Sivaprabha et al., 2008; Soto-Vaca, Gutierrez, Losso, Xu, & Finley, 2012). It may therefore be hypothesized that the ability of the *C. ternatea* flower extract to scavenge free radicals (Figure 1) is due to the presence of flavonol glycosides and anthocyanins, which reduced the oxidative stress arising from  $H_2O_2$  and UV exposure, thus explaining the reported protective effects against UV-induced skin damage. It is therefore emphasized that extracts of *C. ternatea* flower should be chemically characterized (as in this study) when subjected to further pharmacological tests relevant to cosmetic and therapeutic use, and when investigated in clinical studies, such that the constituents can be appropriately associated with the effects observed.

There is a paucity of data on the potential protective effects of *C. ternatea* flower extract against skin aging. However, other botanical extracts rich in anthocyanins or anthocyanidins have been reported to provide defence against UV-induced oxidative stress in human keratinocytes, including black soybean seed, orange extracts, and pomegranate, and the constituents delphinidin and cyanidin 3-O-glucoside (Cimino, Ambra, Canali, Saija, & Virgili, 2006; Cimino, Cristani, Saija, Bonina, & Virgili, 2007; Svobodová, Rambouskova, Walterova, & Vostalova, 2008; Tsotsi et al., 2008). For example, pretreatment and post-treatment with an anthocyanin rich extract (from bilberry) prevented UV

**TABLE 4** Compounds assigned from Liquid chromatography–serial mass spectrometry analysis of the *Clitoria ternatea* flower water extract

Assigned compound <sup>a</sup> (or isomer)	Retention time (min)	Molecular formula	(m/z)	Ion	ppm <sup>a</sup>
1. Phenylalanine	2.9	C9H11NO2	166.086	[M + H] <sup>+</sup>	0.752
2. Coumaroylsucrose	3.5	C21H28O13	506.188	[M + NH4] <sup>+</sup>	2.259
3. Tryptophan	4.4	C11H12N2O2	205.097	[M + H] <sup>+</sup>	0.418
4. Coumaroylglucose	7.7	C15H18O8	344.134	[M + NH4] <sup>+</sup>	0.921
5. Ternatin A3 or C2	9.6	C66H75O39	1,491.391	M <sup>+</sup>	2.006
6. Myricetin rhamnosyl-rhamnosyl-glucoside	13.5	C33H40O21	773.214	[M + H] <sup>+</sup>	1.132
7. Ternatin B4 or C1	14.3	C60H65O34	1,329.337	M <sup>+</sup>	1.862
8. Myricetin neohesperidoside	16.0	C27H30O17	627.157	[M + H] <sup>+</sup>	2.143
9. Quercetin hexosyl-rhamnosyl-rhamnoside	16.4	C33H40O20	757.222	[M + H] <sup>+</sup>	4.028
10. Quercetin hexosyl-rhamnoside	17.6	C27H30O16	611.163	[M + H] <sup>+</sup>	3.074
11. Ternatin B2 or B3	18.1	C75H81O41	1,637.429	M <sup>+</sup>	2.328
12. Kaempferol hexosyl-rhamnosyl-rhamnoside	18.8	C33H40O19	741.225	[M + H] <sup>+</sup>	2.435
13. Delphinidin 3-O-[4-hydroxycinnamoyl-rhamnosyl-glucoside], 5-O-[6-O-malonyl-glucoside], bis-O-[3,4-dihydroxycinnamoyl-glucoside]	19.2	C75H81O42	1,653.422	M <sup>+</sup>	1.692
14. Quercetin hexosyl-rhamnoside	19.8	C27H30O16	611.162	[M + H] <sup>+</sup>	1.765
15. Kaempferol hexosyl-rhamnoside	20.1	C27H30O15	595.167	[M + H] <sup>+</sup>	2.089
16. Kaempferol hexosyl-rhamnoside	20.2	C27H30O15	595.167	[M + H] <sup>+</sup>	3.316
17. Quercetin hexoside	20.4	C21H20O12	465.104	[M + H] <sup>+</sup>	2.747
18. Ternatin B2 or B3	21.3	C75H81O41	1,637.429	M <sup>+</sup>	2.847
19. Ternatin B4 or C1	21.7	C60H65O34	1,329.339	M <sup>+</sup>	2.689
20. Ternatin B1	22.2	C90H97O48	1,945.52	M <sup>+</sup>	3.022
21. Kaempferol rhamnosyl-malonyl-glucoside	22.4	C30H32O18	681.168	[M + H] <sup>+</sup>	2.686
22. Kaempferol rhamnosyl-malonyl-glucoside	22.8	C30H32O18	681.168	[M + H] <sup>+</sup>	3.405
23. Kaempferol hexosyl-rhamnoside	23.3	C27H30O15	595.167	[M + H] <sup>+</sup>	2.812
24. Kaempferol hexoside	23.6	C21H20O11	449.109	[M + H] <sup>+</sup>	2.677
25. Ternatin D1	24.9	C84H87O43	1,783.468	M <sup>+</sup>	3.826
26. 6"-Malonylastragalin	25.6	C24H22O14	535.109	[M + H] <sup>+</sup>	1.567

<sup>a</sup>All compounds assigned by comparison of accurate mass data (based on ppm), and by interpretation of available MS/MS and/or ultraviolet spectra.

damage by reducing ROS production, membrane lipid peroxidation, and depletion of intracellular glutathione in HaCaT (Svobodová et al., 2008). The same study also showed similar suppression of H<sub>2</sub>O<sub>2</sub>-induced damage as reported here. In a different study using the RAW 264.7 macrophage cell line, the total *C. ternatea* extract and its flavonol fraction were able to suppress ROS generated by NADPH oxidase and mitochondria, whereas the anthocyanin fraction inhibited NO production (Nair, Bang, Schreckinger, & Andarwulan, 2015). This further supports the protective effect of *C. ternatea* against skin aging.

We assessed the UV-induced mtDNA damage, as mtDNA is more susceptible to oxidative damage because of its lack of protective histones and close location to the ROS producing system (electron transport chain; Birch-Machin, Russell, & Latimer, 2013; Krutmann & Schroeder, 2009). UV-induced mtDNA damage has been measured in a previous study using qPCR, which is based on the observation that UV-induced damage can cause strand breaks in the mtDNA (Kalinowski, Illenye, & Houten, 1992). The principle of the qPCR mtDNA damage assay is that any kind of DNA lesion can slow or block the progression of DNA polymerase, in which DNA samples with fewer lesions will be amplified to a greater extent, compared with DNA sample with more extensive damage (Furda, Santos, Meyer, & Houten, 2014). A study by Rothfuss, Gasser, and Patenge (2010) showed that the D-loop region of the mtDNA is more

vulnerable to ROS-induced mtDNA damage, thus was the primer selected in this study to target the D-loop.

*C. ternatea* flower extract significantly reduced mtDNA damage caused by UV radiation, suggesting potential prevention of oxidative stress arising from mitochondrial dysfunction. It also has the ability to absorb UVB and UVC (Table 2) that may play a role in modulating the observed protective effects. The ability to act as a UV filter suggests further evaluation for its potential addition to sun protection topical products would be warranted. A cosmetic formulation containing an anthocyanin extract of purple sweet potato improved UV absorption, so reduced the amount of UV reaching the epidermis (Chan, Lien, Lai, Huang, & Liao, 2010), further indicating the relevance of anthocyanins for their protective role against UV radiation. The observed results from this study provide the first scientific evidence and rationale for the potential antiaging and skin-protective properties of a chemically characterized *C. ternatea* flower extract.

## 5 | CONCLUSION

This study demonstrated that an aqueous extract of *C. ternatea* flowers that contains flavonol glycosides and acylated anthocyanins as major

constituents was able to protect human keratinocytes from  $H_2O_2$ -induced cytotoxicity and UV-induced mtDNA damage, providing scientific evidence for its traditional and potential uses as a defence against skin aging. The results justify the further evaluation of *C. ternatea* flowers and constituents for their ability to protect skin against the effects of UV exposure, from both the cosmetic and therapeutic perspectives.

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#### CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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#### REFERENCES

Badarinath, A. V., Rao, K. M., Chetty, S. M. S., Ramkanth, S., Rajan, T. V. S., & Gnanaprakash, K. (2010). A review on in vitro antioxidant methods: Comparisons, correlations and considerations. *International Journal of PharmTech Research*, 2(2), 1276–1285.

Birch-Machin, M. A., Russell, E. V., & Latimer, J. A. (2013). Mitochondrial DNA damage as a biomarker for ultraviolet radiation exposure and oxidative stress. *The British Journal of Dermatology*, 169(Suppl), 9–14.

Chan, C. F., Lien, C. Y., Lai, Y. C., Huang, C. L., & Liao, W. C. (2010). Influence of purple sweet potato extracts on the UV absorption properties of a cosmetic cream. *Journal of the Society of Cosmetic Chemists*, 61(5), 333–341.

Chattuwalltha, T., & Okello, E. J. (2015). Anti-collagenase, anti-elastase and antioxidant activities of *Pueraria candolleana* var. *mirifica* root extract and *Coccinia grandis* fruit juice extract: An in vitro study. *European Journal of Medicinal Plants*, 5(4), 318–327.

Chayaratana, P., Barbieri, M. A., Suanpairin, N., & Adisakwattana, S. (2015). Inhibitory effect of *Clitoria ternatea* flower petal extract on fructose-induced protein glycation and oxidation-dependent damages to albumin in vitro. *BMC Complementary and Alternative Medicine*, 15(1), 1–9.

Cimino, F., Ambra, R., Canali, R., Sajja, A., & Virgili, F. (2006). Effect of cyanidin-3-O-glucoside on UVB-induced response in human keratinocytes. *Journal of Agricultural and Food Chemistry*, 54(11), 4041–4047.

Cimino, F., Cristani, M., Sajja, A., Bonina, F. P., & Virgili, F. (2007). Protective effects of a red orange extract on UVB-induced damage in human keratinocytes. *BioFactors*, 30(2), 129–138.

El-Bahr, S. M. (2013). Biochemistry of free radicals and oxidative stress. *Science International*, 1(5), 111–117.

Farage, M. A., Miller, K. W., & Maibach, H. I. (2010). *Textbook of aging skin*. Berlin Heidelberg: Springer-Verlag. ISBN: 9783540896555.

Fisher, G. J., Kang, S., Varani, J., Bata-Csorgo, Z., Wan, Y., Datta, S., & Voorhees, J. J. (2002). Mechanisms of photoaging and chronological skin aging. *Archives of Dermatology*, 138(11), 1462–1470.

Furda, A., Santos, J. H., Meyer, J. N., & Houten, B. V. (2014). Quantitative PCR-based measurement of nuclear and mitochondrial DNA damage and repair in mammalian cells. *Methods in Molecular Biology*, 1105(4), 419–437.

Ganceviciene, R., Liakou, A. I., Theodoridis, A., Makrantonaki, E., & Zouboulis, C. C. (2012). Skin anti-aging strategies. *Dermato-Endocrinology*, 4(3), 308–319.

Gragnani, A., Cornick, S. M., Chominski, V., Ribeiro de Noronha, S. M., Correa de Noronha, S. A. A., & Ferreira, L. M. (2014). Review of major theories of skin aging. *Advances in Aging Research*, 3(4), 265–284.

Grand View Research Report, 2016. Organic personal care market size and forecast by product (skin care, hair care, oral care, and cosmetics), by region (North America, Europe, Asia Pacific, Latin America, and Middle East & Africa) and trend analysis from 2014 to 2025. Grand View Research Report: 70. Available at: <http://www.grandviewresearch.com/industry-analysis/organic-personal-care-market> [Accessed December 22, 2016].

Howes, M.-J. R., & Houghton, P. J. (2003). Plants used in Chinese and Indian traditional medicine for improvement of memory and cognitive function. *Pharmacology Biochemistry and Behavior*, 75, 513–527.

Howes, M.-J. R., & Simmonds, M. S. J. (2014). The role of phytochemicals as micronutrients in health and disease. *Current Opinion in Clinical Nutrition and Metabolic Care*, 17, 558–566.

Iamsaard, S., Burawat, J., Kanla, P., Arun, S., Sukhorum, W., Sripanidkulchai, B., ... Kondo, H. (2014). Antioxidant activity and protective effect of *Clitoria ternatea* flower extract on testicular damage induced by ketocanozole in rats. *Journal of Zhejiang University Science. B (Biomed & Biotechnology)*, 15(6), 548–555.

Islam, M. S., Parvin, S., Uddin, M. N., & Mazid, M. A. (2014). Antidiabetic and antioxidant activities of decoctions of *Coccinia grandis* Linn. and *Centella asiatica* (L.) on Alloxan-induced diabetic rats. *Bangladesh Pharmaceutical Journal*, 17(1), 86–91.

Kaisoon, O., Siriamornpun, S., Weerapreeyakul, N., & Meeso, N. (2011). Phenolic compounds and antioxidant activities of edible flowers from Thailand. *Journal of Functional Foods*, 3(2), 88–99.

Kalinowski, D. P., Illenye, S., & Houten, B. V. (1992). Analysis of DNA damage and repair in murine leukemia L1210 cells using a quantitative polymerase chain reaction assay. *Nucleic Acids Research*, 20(13), 3485–3494.

Kamkaen, N., & Wilkinson, J. M. (2009). The antioxidant activity of *Clitoria ternatea* flower petal extracts and eye gel. *Phytotherapy Research*, 23(4), 1624–1625.

Kazuma, K., Noda, N., & Suzuki, M. (2003a). Malonylated flavonol glycosides from the petals of *Clitoria ternatea*. *Phytochemistry*, 62, 229–237.

Kazuma, K., Noda, N., & Suzuki, M. (2003b). Flavonoid composition related to petal colour in different lines of *Clitoria ternatea*. *Phytochemistry*, 64, 1133–1139.

Khanam, Z., Wen, C. S., & Bhat, I. U. H. (2014). Phytochemical screening and antimicrobial activity of root and stem extracts of wild *Eurycoma longifolia* Jack (Tongkat Ali). *Journal of King Saud University - Science*, 27(1), 23–30.

Kosai, P., Sirisidthi, K., Jiraungkoorskul, K., & Jiraungkoorskul, W. (2015). Review on ethnomedicinal uses of memory in boosting butterfly pea, *Clitoria ternatea*. *Journal of Natural Remedies*, 15(2), 1–6.

Krutmann, J., & Schroeder, P. (2009). Role of mitochondria in photoaging of human skin: The defective powerhouse model. *Journal of Investigative Dermatology*, 14(1), 44–49.

Leake, C. D. (1952). The chief Egyptian medical papyri. In *The old Egyptians medical papyri* (pp. 7–17). The University of Kansas Press.

Lee, J., Durst, R. W., & Wrolstad, R. E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative study. *Journal of AOAC International*, 88(5), 1269–1278.

Lijon, M. B., Meghla, N. S., Jahedi, E., Rahman, M. A., & Hossain, I. (2017). Phytochemistry and pharmacological activities of *Clitoria ternatea*. *International Journal of Natural and Social Sciences*, 4(1), 1–10.

Miguel, M. G. (2011). Anthocyanins: Antioxidant and/or anti-inflammatory activities. *Journal of Applied Pharmaceutical Science*, 1(6), 7–15.

Moon, J. K., & Shibamoto, T. (2009). Antioxidant assays for plants and food components. *Journal of Agriculture and Food Chemistry*, 57, 1655–1666.

Mukherjee, P. K., Kumar, V., Kumar, N. S., & Heinrich, M. (2008). The Ayurvedic medicine *Clitoria ternatea*—From traditional use to scientific assessment. *Journal of Ethnopharmacology*, 120(3), 291–301.

Nair, V., Bang, W., Schreckinger, E., & Andarwulan, N. (2015). Protective role of Ternatin Anthocyanins and Quercetin glycosides from butterfly pea (*Clitoria ternatea* Leguminosae) blue flower petals against Lipopolysaccharides (LPS)-induced inflammation in macrophage cells. *Journal of Agriculture and Food Chemistry*, 62(28), 6355–6365.

Ohguru, N., Fukuda, M., Sasabe, T., & Tano, Y. (1999). Concentration dependent effects of hydrogen peroxide on lens epithelial cells. *The British Journal of Ophthalmology*, 83(9), 1064–1068.

Qiagen. 2016. Protocol: DNA purification from blood or body fluids (Spin protocol). In QIAamp® DNA Mini and Blood Mini Handbook. Available at <https://www.qiagen.com/ch/resources/download.aspx%3Fid%3D%0A62a200d6-faf4-469b-b50f-2b59cf738962%26lang%3D%0Aen>. [Accessed October 4, 2017].

Rothfuss, O., Gasser, T., & Patenge, N. (2010). Analysis of differential DNA damage in the mitochondrial genome employing a semi-long run real-time PCR approach. *Nucleic Acids Research*, 38(4), 1–10.

Schymanski, E. M., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H. P., & Hollander, J. (2014). Identifying small molecules via high resolution mass spectrometry: Communicating confidence. *Environmental Science and Technology*, 48(4), 2097–2098.

Shain, A. H., & Bastian, B. C. (2016). From melanocytes to melanomas. *Nature Reviews Cancer*, 16(6), 345–358.

Sivaprabha, J., Supriya, S., Sumathi, S., Padma, P. R., Nirmaladevi, R., & Radha, P. (2008). A study on the levels of nonenzymic antioxidants in the leaves and flowers of *Clitoria ternatea*. *Ancient Science of Life*, 27(4), 28–32.

Sjerobabski-Masnec, I., & Situm, M. (2010). Skin aging. *Acta Clinica Croatica*, 48(3), 515–518.

Soto-Vaca, A., Gutierrez, A., Losso, J. N., Xu, Z., & Finley, J. W. (2012). Evolution of phenolic compounds from color and flavor problems to health benefits. *Journal of Agricultural and Food Chemistry*, 60(27), 6658–6677.

Svobodová, A., Rambouskova, J., Walterova, D., & Vostalova, J. (2008). Bilberry extract reduces UVA-induced oxidative stress in HaCaT keratinocytes: A pilot study. *BioFactors*, 33, 249–266.

Tantituvanont, A., Werawatganone, P., Jiamchaisari, P., & Manopakdee, K. (2008). Preparation and stability of butterfly pea color extract loaded in microparticles prepared by spray drying. *Thai J. Pharm.*, 32, 59–69.

Thring, T. S. A., Hili, P., & Naughton, D. P. (2009). Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants. *BMC Complementary and Alternative Medicine*, 9(27), 1–11.

Tirzitis, G., & Bartosz, G. (2010). Determination of antiradical and antioxidant activity: Basic principles and new insights. *Acta Biochimica Polonica*, 57(1), 139–142.

Tsoyi, K., Park, H. B., Kim, Y. M., Chung, J. I., Shin, S. C., Shim, H. J., ... Kim, H. J. (2008). Protective effect of anthocyanins from black soybean seed coats on UVB-induced apoptotic cell death *in vitro* and *in vivo*. *Journal of Agriculture and Food Chemistry*, 56(22), 10600–10605.

US Department of Commerce, 2015. Cosmetics & toiletries market overviews 2015. U.S. Commercial Service. Available at: [http://www.trade.gov/industry/materials/ITA.FSC.Cosmoprof.2015\\_final2.pdf](http://www.trade.gov/industry/materials/ITA.FSC.Cosmoprof.2015_final2.pdf) [Accessed June 18, 2017].

Winterbourn, C. C. (1995). Toxicity of iron and hydrogen peroxide: The Fenton reaction. *Toxicology Letters*, 82/83, 969–974.

Zingare, M. L., Zingare, P. L., Dubey, A. K., & Ansari, M. A. (2013). *Clitoria ternatea* (APARAJITA):A review of the antioxidant, antidiabetic and hepatoprotective potential. *International Journal of Pharmacy and Biological Science*, 3(1), 201–213.

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# **Appendices**



## Appendix A

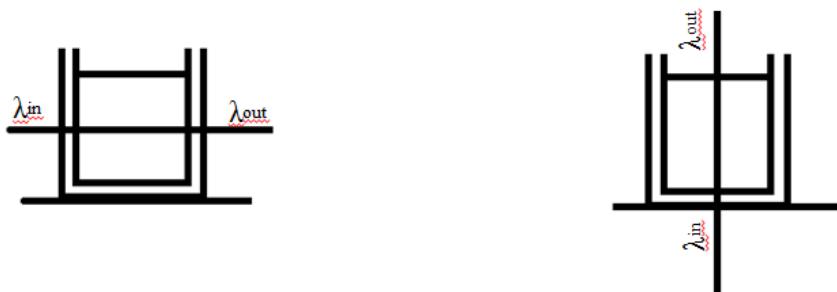
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The total anthocyanin was expressed as cyd-3-glu mg/g extract and calculated using the formula below (Lee *et al.*, 2005).

$$\text{TAC} = A \times \text{MW} \times 1000) / \varepsilon, \quad (\text{A.1})$$

where  $A = (A_{520\text{nm}} - A_{700\text{nm}}) \text{ pH} 1.0 - (A_{520\text{nm}} - A_{700\text{nm}}) \text{ pH} 4.5$ ; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu);  $\varepsilon = 2600$  molar extinction coefficient, in  $\text{L} \times \text{mol}^{-1} \times \text{cm}^{-1}$ , for cyd-3-glu; and 1000 = factor for conversion from g to mg.

The molar extinction for cyanidin-3 glucoside at 1 cm path length was  $26900 \text{ L} \times \text{mol}^{-1}$  (Lee *et al.*, 2005) was determined based on the path length travelled through regular 1 cm cuvette (polystyrene) from side to side (see Figure A.1 a). However, for the 96-well plate read by the Spectromax<sup>384</sup> microplate reader, the wavelength travelled vertically (Figure A.1 b). Hence, the path total path length travelled using the plate was equal to the volume contained in the well, with the assumption of no effect of plate height travelled on absorption.



a) Wavelength movement using cuvette: Side to side

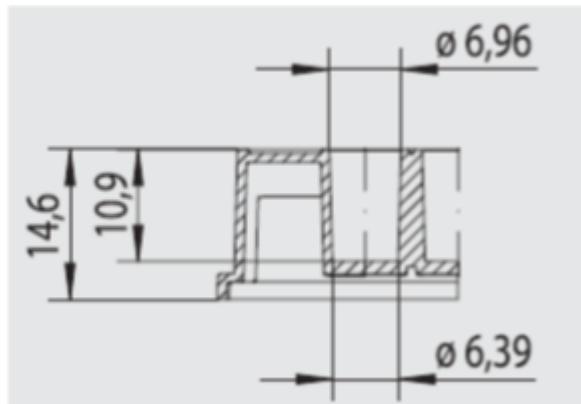
b) Wavelength movement using plate reader: Bottom to up

**Figure A.1: Comparison of wavelength movement using cuvette and plate reader.**

**Determination of path length travelled:** The path length was determined based on the microplate profile provided from the supplier (Figure A.2). Hence; the height of volume (path length travelled) was calculated as below:

If 384  $\mu\text{L}$  (total volume/well) = 10.9 mm, then for 250  $\mu\text{L}$ ,

The height travelled =  $(250 \mu\text{L} \times 10.9 \text{ mm})/384 \mu\text{L} = 7.13 \text{ mm}$



**Figure A.2: Well profile of flat bottom microplate.** Figure shows the well profile of flat bottom clear microplate in mm. The maximum volume for each well is 382  $\mu\text{L}$ . Image obtained from [www.gbo.com/bioscience](http://www.gbo.com/bioscience).

**Molar absorptivity for 7.13 mm path length:** From the Lambert-Beers law of absorption (Equation A.2), the absorption of light in a sample solution is dependent on the concentration  $c$  of the dissolved molecule, the specific molar extinction coefficient  $\epsilon$  at a defined wavelength  $\lambda$  and the path length  $d$ .

#### Equation A.2: Lambert-Beer's Law

$$A_\lambda = -\log_{10} = \alpha_\lambda \cdot c \cdot d$$

Hence, the molar extinction coefficient is:

#### Equation A.3: Molar extinction coefficient equation

$$\alpha_\lambda = A_\lambda / c \cdot d$$

From the equation for molar extinction coefficient (Equation A.3), the path length is inversely correlated with  $\alpha_\lambda$  or  $\epsilon$ , which is as the path length decreases, the  $\epsilon$  increases.

Hence, if the A and C are constants, the molar extinction coefficient at 7.13 mm could be determined as below:

If the  $\epsilon$  was  $26900 \text{ L. mol}^{-1}$  = 1 cm, hence at 7.13 mm, the  $\epsilon$  was;

The  $\epsilon$  at 7.13 mm =  $(7.13 \text{ mm} \times 26900 \text{ L. mol}^{-1})/10 \text{ mm}$

=  $19179 \text{ L. mol}^{-1}$



# **Reference**



## Reference

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Abas, F., Lajis, N. H., Israf, D. A., Khozirah, S. and Kalsom, Y. U. (2006) 'Antioxidant and nitric oxide inhibition activities of selected Malay traditional vegetables', *Food Chemistry*, 95(4), pp. 566–573.

Abas, F., Shaari, K., Lajis, N. H., Israf, D. A. and Yusoff, U. K. (2003) 'Antioxidative and radical scavenging properties of the constituents isolated from *Cosmos caudatus* Kunth.', *Natural Product Sciences*, 9(4), pp. 245–248.

Abdul Hisham, E. E., Rofiee, M. S., Khalid, A. M., Jalaluddin, A. F., Mohammad Yusof, M. I., Idris, M. H., Ramli, S., James, R. J., Wee, J. Y., Teh, L. T. and Salleh, M. Z. (2018) 'Combined extract of *Moringa oleifera* and *Centella asiatica* modulates oxidative stress and senescence in hydrogen peroxide-induced human dermal fibroblasts', *Turkish Journal of Biology*, 42, pp. 1–12.

Abdul Wahab, N. (2014) 'Assessment of antioxidant capacity, Anti-collagenase and Anti-elastase Assays of Malaysian Unfermented Cocoa Bean for Cosmetic Application', *Natural Products Chemistry & Research*, 2(3).

Afaq, F. and Katiyar, S. K. (2011) 'Polyphenols: skin photoprotection and inhibition of photocarcinogenesis', *Mini-Reviews in Medicinal Chemistry*, 11(14), pp. 1200–1215.

Agati, G., Brunetti, C., Di, M., Ferrini, F., Pollastri, S. and Tattini, M. (2013) 'Functional roles of flavonoids in photoprotection: new evidence, lessons from the past', *Plant Physiology and Biochemistry*, 72, pp. 35–45.

Aguilar, T. A. F., Navarro, B. C. H. and Pérez, J. A. M. (2016) *Endogenous antioxidants : a review of their role in endogenous oxidative stress, A Master Regulator of Oxidative Stress: The Transcription Factor NrF2*. Available at: <https://www.intechopen.com/books/a-master-regulator-of-oxidative-stress-the-transcription-factor-nrf2/endogenous-antioxidants-a-review-of-their-role-in-oxidative-stress> (Accessed: 12 January 2018).

Ahn, C. S. and Rao, B. K. (2014) 'The life cycles and biological end pathways of dermal fillers', *Journal of Cosmetic Dermatology*, 13(3), pp. 212–223.

Ahsanuddin, S., Lam, M. and Baron, E. D. (2016) 'Skin aging and oxidative stress', *AIMS Molecular Science*, 3(2), pp. 187–195.

Alerico, G. C., Beckenkamp, A., Vignoli-Silva, M., Buffon, A. and Von Poser, G. L. (2015) 'Proliferative effect of plants used for wound healing in Rio Grande do Sul state, Brazil', *Journal of Ethnopharmacology*, 176, pp. 305–310.

Alfredo, G., Mac;, C. S., Verônica, C., Ribeiro de Noronha, S. M., Alves Corrêa de Noronha, S. A. and Ferreira, L. M. (2014) 'Review of major theories of skin aging', *Advances in Aging Research*, 03(04), pp. 265–284.

Alsarhan, A., Sultana, N., Al-Khatib, A. and Abdul Kadir, M. R. (2014) 'Review on some Malaysian traditional medicinal plants with therapeutic properties', *Journal of Basic and Applied Sciences*, 10(4), pp. 149–159.

Amen, N., Mathow, D., Rabionet, M., Sandhoff, R., Langbein, L., Gretz, N., Jäckel, C., Gröne, H. J. and Jennemann, R. (2013) 'Differentiation of epidermal keratinocytes is dependent on glucosylceramide : ceramide processing', *Human Molecular Genetics*, 22(20), pp. 4164–4179.

Andarwulan, N., Batari, R., Sandrasari, D. A., Bolling, B. and Wijaya, H. (2010) 'Flavonoid content and antioxidant activity of vegetables from Indonesia', *Food Chemistry*, 121(4), pp. 1231–1235.

Andarwulan, N., Kurniasih, D., Apriady, R. A., Rahmat, H., Roto, A. V. and Bolling, B. W. (2012) 'Polyphenols, carotenoids, and ascorbic acid in underutilised medicinal vegetables', *Journal of Functional Foods*, 4(1), pp. 339–347.

Anon (2014) *What has made pegaga a miracle herb?*

Apak, R., Özyürek, M., Guclu, K. and Capanoglu, E. (2016) 'Antioxidant activity/capacity measurement: 1. Classification, physico-chemical principles, mechanisms and electron transfer ( ET )-based assays', *Journal of Agriculture, Food and Chemistry*, 64(5), pp. 997–1027.

Arts, I. C. W. and Hollman, P. C. H. (2005) 'Polyphenols and disease risk in epidemiologic studies', *American Journal of Clinical Nutrition*, 81, p. 317S–325S.

Aruna (2009) *Clitoria ternatea as a nootropic.*

Ashfaq, U. A., Mumtaz, A., Qamar, T. U. and Fatima, T. (2013) 'MAPS database: Medicinal plant Activities, Phytochemical and Structural database', *Bioinformation*, 9(19), pp. 993–995.

Augustyniak, A., Bartosz, G., Cipak, A., Duburs, G., Horáková, L., Luczaj, W., Majekova, M., Odysseos, A. D., Rackova, L., Skrzylewska, E., Stefek, M., Strosová, M., Tirzitis, G., Venskutonis, P. R., Viskupicova, J., Vraka, P. S. and Zarković, N. (2010) 'Natural and synthetic antioxidants: an updated overview.', *Free Radical Research*, 44(10), pp. 1216–1262.

Avram, M. M., Tope, W. D., Yu, T., Szachowicz, E. and Nelson, J. S. (2009) 'Hypertrophic scarring of the neck following ablative fractional carbon dioxide laser resurfacing', *Lasers in Surgery and Medicine*, 41(3), pp. 185–188.

Ayala, A., Muñoz, M. F. and Argüelles, S. (2014) 'Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal', *Oxidative Medicine and Cellular Longevity*, 5, pp. 1–32.

Ayob, A., Awadh, A., Hadi, H., Jaffri, J., Jamshed, S. and Ahmad, H. A. (2016) 'Malaysian consumers' awareness, perception, and attitude toward cosmetic products: Questionnaire development and pilot testing', *Journal of Pharmacy and Bioallied Sciences*, 8(3), pp. 203–209.

Azmi, N., Hashim, P., Hashim, D. M., Halimoon, N. and Majid, N. M. N. (2014) 'Anti-elastase, anti-tyrosinase and matrix metalloproteinase-1 inhibitory activity of earthworm extracts as potential new anti-aging agent', *Asian Pacific Journal of Tropical Biomedicine*, 4(Suppl 1), pp. S348-52.

Bae, J. Y., Choi, J. S., Choi, Y. J., Shin, S. Y., Kang, S. W., Han, S. J. and Kang, Y. H. (2008) '(-) Epigallocatechin gallate hampers collagen destruction and collagenase activation in ultraviolet-B-irradiated human dermal fibroblasts: Involvement of mitogen-activated protein

kinase', *Food and Chemical Toxicology*, 46(4), pp. 1298–1307.

Balasundram, N., Sundram, K. and Samman, S. (2006) 'Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses', *Food Chemistry*, 99(1), pp. 191–203.

Bandy, B. and Davidson, A. J. (1990) 'Mitochondrial mutations may increase oxidative stress: implications for carcinogenesis and aging?', *Free Radical Biology and Medicine*, 8(2), pp. 523–539.

Beasley, D. G. and Meyer, T. A. (2010) 'Characterization of the UVA protection provided by avobenzone, zinc oxide, and titanium dioxide in broad-spectrum sunscreen products', *American Journal of Clinical Dermatology*, 11(6), pp. 413–421.

Benzie, I. F. and Strain, J. J. (1996) 'The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay.', *Analytical Biochemistry*, 239(1), pp. 70–76.

BioVision (2016) *Collagenase Activity Colorimetric Assay Kit*. Available at: <http://www.biovision.com/documentation/datasheets/K792.pdf> (Accessed: 21 July 2017).

Birch-Machin, M. a, Russell, E. V and Latimer, J. a (2013) 'Mitochondrial DNA damage as a biomarker for ultraviolet radiation exposure and oxidative stress.', *British Journal of Dermatology*, 169(Suppl.2), pp. 9–14.

Bjorksten, J. (1942) 'Chemistry of duplication', *Chemical Industries*, 49, p. 2.

Bjorksten, J. (1968) 'The crosslinking theory of aging', *Journal of the American Geriatrics Society*, 16(4), pp. 408–427.

Bjorksten, J. and Tenhu, H. (1990) 'The crosslinking theory of aging-added evidence.', *Experimental Gerontology*, 25, pp. 91–95.

Boneza, M. M. and Niemeyer, E. D. (2018) 'Cultivar affects the phenolic composition and antioxidant properties of commercially available lemon balm (*Melissa officinalis* L.) varieties', *Industrial Crops and Products*, 112(1), pp. 783–789.

Boukamp, P., Petrussevska, R. T., Dirk, B., Hornung, J., Markham, A. and Fuseing, N. E. (1988) 'Normal keratinization in a spontaneously immortalized aneuploid human keratinocytes cell line', *Journal of Cell Biology*, 106(3), pp. 761–771.

Bourboulia, D. and Stetler-Stevenson, W. G. (2010) 'Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): positive and negative regulators in tumor cell adhesion', *Seminars in Cancer Biology*, 20(3), pp. 161–168.

Boveris, A. and Cadena, E. (2000) 'Mitochondrial of hydrogen peroxide regulation by nitric oxide and the role of ubisemiquinone', *IUBMB Life*, 50(11), pp. 245–250.

Brenneisen, P., Sies, H. and Scharffetter-Kochanek, K. (2002) 'Ultraviolet-B irradiation and matrix metalloproteinases; from induction via signalling to initial events', *New York Academy of Science*, 973, pp. 31–43.

Bruge, F., Tiano, L., Astolfi, P., Emanuelli, M., Damiani, E. and Bruge, F. (2014) 'Prevention of UVA-induced oxidative damage in human dermal fibroblasts by new UV filters, assessed using a novel in vitro experimental system', *PLoS ONE*, 9(1), pp. 1–11.

Burdulis, D., Ivanauskas, L., Dirse, V., Kazlauskas, S. and Razukas, A. (2007) 'Study of diversity of anthocyanin composition in bilberry (*Vaccinium myrtillus* L.) fruits', *Medicina*, 43(12), pp. 971–977.

Burkill, I. (1966) *A Dictionary of the Economic Products of the Malay Peninsula*. 2nd edn. Kuala Lumpur: Ministry of Agriculture and Cooperatives.

Burlando, B., Verotta, L., Laura, C. and Botiinini-Massa, E. (2010) 'The skin: morphophysiological traits and disease', in *Herbal Principles in Cosmetics*. Florida: Taylor and Francis Group, pp. 1–7.

Bylka, W., Znajdek-Awiżeń, P., Studzińska-Sroka, E. and Brzezińska, M. (2013) 'Centella asiatica in cosmetology', *Advances in Dermatology and Allergology*, 30(1), pp. 46–49.

Calleja-Agius, J., Brincat, M. and Borg, M. (2011) 'Skin connective tissue and ageing', *Best Practice and Research Clinical Obstetrics and Gynaecology*, 27, pp. 727–740.

Cals-Grierson, M. M. and Ormerod, A. D. (2004) 'Nitric oxide function in the skin', *Biology and Chemistry*, 10(4), pp. 179–193.

Center for the Promotion of Imports from Developing Countries (CBI) (2016) *Exporting anti-ageing extracts to Europe*. Available at: <https://www.cbi.eu/node/2532/pdf/> (Accessed: 19 June 2017).

Chan, E. W. C., Ping Tan, Y., Jia Chin, S., Yi Gan, L., Xian Kang, K., Hong Fong, C., Qi Chang, H. and Chern How, Y. (2014) 'Antioxidant properties of selected fresh and processed herbs and vegetables', *Free Radicals and Antioxidants*, 4(1), pp. 39–46.

Chan, E. W. C., Wong, S. K. and Chan, H. T. (2016) 'Ulam herbs of *Oenanthe javanica* and *Cosmos caudatus*: an overview on their medicinal properties', *Journal of Natural Remedies*, 16(4), p. 137.

Chang, T. S. (2009) 'An updated review of tyrosinase inhibitors', *International Journal of Molecular Sciences*, 10(6), pp. 2440–2475.

Chang, T. S. (2012) 'Natural melanogenesis inhibitors acting through the down-regulation of tyrosinase activity', *Materials*, 5(9), pp. 1661–1685.

Chang, T. S., Ding, H. Y., Tai, S. S. K. and Wu, C. Y. (2007) 'Mushroom tyrosinase inhibitory effects of isoflavones isolated from soygerm koji fermented with *Aspergillus oryzae* BCRC 32288', *Food Chemistry*, 105(4), pp. 1430–1438.

Chattuwatthana, T. and Okello, E. (2015) 'Anti-collagenase, anti-elastase and antioxidant activities of *Pueraria candolleana* var. *mirifica* root extract and *Coccinia grandis* fruit juice extract: an in vitro study', *European Journal of Medicinal Plants*, 5(4), pp. 318–327.

Chayaratana, P., Barbieri, M. A. Iejandro, Suanpairin, N. and Adisakwattana, S. (2015)

'Inhibitory effect of *Clitoria ternatea* flower petal extract on fructose-induced protein glycation and oxidation-dependent damages to albumin in vitro', *BMC Complementary and Alternative Medicine*, 15, p. 27.

Chen, Q. X. and Kubo, I. (2002) 'Kinetics of mushroom tyrosinase inhibition by quercetin', *Journal of Agricultural and Food Chemistry*, 50(14), pp. 4108–4112.

Chen, Y. and Lyga, J. (2014) 'Brain-skin connection: stress, inflammation and skin Aging', *Inflammation and Allergy-Drug Targets*, 13(3), pp. 177–190.

Cheng, S. H., Barakatun-Nisak, M. Y., Anthony, J. and Ismail, A. (2015) 'Potential medicinal benefits of *Cosmos caudatus* (Ulam Raja): a scoping review', *Journal of Research in Medical Sciences*, pp. 1000–1006.

Choi, M. H. and Shin, H. J. (2016) 'Anti-melanogenesis effect of quercetin', *Cosmetics*, 3(18), pp. 1–16.

Chong, N. J. and Aziz, Z. (2011) 'A systematic review on the chemical constituents of *Centella asiatica*', *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 2(3), pp. 445–459.

Choquenet, B., Couteau, C., Paparis, E. and Coiffard, L. J. . (2008) 'Quercetin and rutin as potential sunscreen agents: determination of efficacy by an *in vitro* method', *Journal of Natural Product*, 71, pp. 1117–1118.

Cichorek, M., Wachulska, M., Stasiewicz, A. and Tyminska, A. (2013) 'Skin melanocytes: Biology and development', *Postepy Dermatologii i Alergologii*, 30(1), pp. 30–41.

Coelho, M., Rocha, C., Cunha, L. M., Cardoso, L., Alves, L., Lima, R. C., Pereira, M. J., Campos, F. M. and Pintado, M. (2016) 'Influence of harvesting factors on sensory attributes and phenolic and aroma compounds composition of *Cymbopogon citratus* leaves infusions', *Food Research International*, 89, pp. 1029–1037.

Costa, R. and Santos, L. (2017) 'Delivery systems for cosmetics: from manufacturing to the skin of natural antioxidants', *Powder Technology*, 322, pp. 402–416.

Costin, G. E. and Hearing, V. J. (2007) 'Human skin pigmentation: melanocytes modulate skin color in response to stress.', *The Federation of American Societies for Experimental Biology (FASEB) Journal*, 21(4), pp. 976–994.

Crasto, A. M. (2014) *Moringa oleifera kills 97% of pancreatic cancer cells in vitro*.

Daamen, W. F., Veerkamp, J. H., Hest, J. C. M. and Kuppevelt, T. H. (2007) 'Elastin as a biomaterial for tissue engineering', *Biomaterials*, 28(30), pp. 4378–4398.

Dellambra, E. and Dimbri, G. P. (2008) 'Chapter 7: Cellular senescence and skin aging', in *Skin Aging Handbook: An Integrated Approach to Biochemistry and Product Development*. New York: William Andrew Inc., pp. 129–135.

Department of Statistic Malaysia (2010) *Population Distribution and Basic Demographic Characteristic Report 2010*. Available at: <https://www.dosm.gov.my> (Accessed: 27 June 2017).

Devi, B. P., Boominathan, R. and Mandal, S. C. (2003) 'Anti-inflammatory, analgesic and antipyretic properties of *Clitoria ternatea* root', *Fitoterapia*, 74, pp. 345–349.

Devi, P. S., Saravanakumar, M. and Mohandas, S. (2012) 'The effects of temperature and pH on stability of anthocyanins from red sorghum (*Sorghum bicolor*) bran', *African Journal of Food Science*, 6(24), pp. 567–573.

Dilman, V. and Dean, W. (1992) *The neuroendocrine theory of aging and degenerative disease*. Pensacola: The Center for Bio-Gerontology.

Djouonzo, P., Fotsing, M., Rachel, L. and Yamthe, T. (2018) 'Novel saponin and benzofuran isoflavanoid with *in vitro* anti-inflammatory and free radical scavenging activities from the stem bark of *Pterocarpus erinaceus* (Poir)', *Phytochemistry Letters*, 28, pp. 69–75.

Duke, J. A. (1999) *Moringa oleifera (Moringaceae)*.

Ebanks, J. P., Wickett, R. R. and Boissy, R. E. (2009) 'Mechanisms regulating skin pigmentation: The rise and fall of complexion coloration', *International Journal of Molecular Sciences*, 10(9), pp. 4066–4087.

Eckhart, L., Lippens, S., Tschachler, E. and Declercq, W. (2013) 'Cell death by cornification', *Biochimica et Biophysica Acta*, 1833, pp. 3471–3480.

El-Bahr, S. (2013) 'Biochemistry of free radicals and oxidative stress', *Science International*, 1(5), pp. 111–117.

Elansary, H. O., Mahmoud, E. A., Shokralla, S. and Yessoufou, K. (2015) 'Diversity of plants, traditional knowledge, and practices in local cosmetics: a case study from Alexandria, Egypt', *Economic Botany*, 69(2), pp. 114–126.

Escriche, I. and Juan-Borrás, M. (2018) 'Standardizing the analysis of phenolic profile in propolis', *Food Research International*, 106(1), pp. 834–841.

Faizi, S., Siddiqui, B. S., Saleem, R., Siddiqui, S., Aftab, K. and Gilani, A. (1994) 'Novel hypotensive agents, niazimin A, niazimin B, niazimin A and niazimin B from *Moringa oleifera*: isolation of first naturally occurring carbamates', *Journal of Chemical Society Perkin Transaction 1*, pp. 3025–3040.

Faizi, S., Siddiqui, B. S., Saleem, R., Siddiqui, S., Aftab, K. and Gilani, A. (1992) 'Isolation and structure elucidation of novel hypotensive agents, niazinin A, niazinin B, niazimicin and niaziminin A + B from *Moringa oleifera*: the first naturally occurring thiocarbamates', *Journal of Chemical Society Perkin Transaction 1*, (23), p. 3237.

Farage, M. A., Miller, K. W., Elsner, P. and Maibach, H. I. (2008) 'Intrinsic and extrinsic factors in skin ageing: a review', *International Journal of Cosmetic Science*, pp. 87–95.

Farage, M. A., Miller, K. W., Elsner, P. and Maibach, H. I. (2013) 'Characteristics of the aging skin', *Advances in Wound Care*, 2(1), pp. 5–10.

Farooq, F., Rai, M., Tiwari, A., Khan, A. A. and Farooq, S. (2012) 'Medicinal properties of *Moringa oleifera* : an overview of promising healer', *Journal of Medicinal Plant Research*,

6(27), pp. 4368–4374.

Farris, P. and Krol, Y. (2015) 'Under persistent assault: understanding the factors that deteriorate human skin and clinical efficacy of topical antioxidants in treating aging skin', *Cosmetics*, 2(4), pp. 355–367.

Fawcett, D. W. (1981) 'Mitochondria', in *The Cell*. 2nd edn. Philadelphia: W. B. Saunders Company, pp. 410–468.

FDA (2018) *CFR - Code of Federal Regulations Title 21*.

Ferdinando, M. D., Brunetti, C., Agati, G. and Tattini, M. (2014) 'Multiple functions of polyphenols in plants inhabiting unfavorable Mediterranean areas', *Environmental and Experimental Botany*, 103, pp. 107–116.

Finkel, T. and Holbrook, N. J. (2000) 'Oxidants, oxidative stress and the biology of ageing', *Nature*, 408(11), pp. 239–247.

Fischer, T. C., Perosino, E., Poli, F., Viera, M. S. and Dreno, B. (2010) 'Chemical peels in aesthetic dermatology: an update 2009', *Journal of the European Academy of Dermatology and Venereology*, 24(3), pp. 281–292.

Fisher, G. H. and Geronemus, R. G. (2006) 'Short-term side effects of fractional photothermolysis', *Dermatologic Surgery*, 31, pp. 1245–1249.

Fisher, G. J., Datta, S., Talwar, H. S., Wang, Z.-Q., Varani, J., Kang, S. and J., V. J. (1996) 'Molecular basis of sun-induced premature skin ageing and retinoid antagonism', *Nature*, 379, pp. 335–339.

Fisher, G. J., Kang, S., Varani, J., Bata-Csorgo, Z., Wan, Y., Datta, S. and Voorhees, J. J. (2002) 'Mechanisms of photoaging and chronological skin aging.', *Archives of Dermatology*, 138(11), pp. 1462–1470.

Fisher, G. J., Talwar, H. S., Lin, J., Lin, P., McPhillips, F., Wang, Z., Li, X., Wan, Y., Kang, S. and Voorhees, J. J. (1998) 'Retinoic acid inhibits induction of c-Jun protein by ultraviolet radiation that occurs subsequent to activation of mitogen-activated protein kinase pathways in human skin in vivo', *Journal of Clinical Investigation*, 101(6), pp. 1432–1440.

Floegel, A., Kim, D. O., Chung, S. J., Koo, S. I. and Chun, O. K. (2011) 'Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods', *Journal of Food Composition and Analysis*, 24(7), pp. 1043–1048.

Fu, X., Peng, Y., Hu, Y., Shi, Y., Yao, M. and Zhang, X. (2014) 'NADPH oxidase-1 and its derived reactive oxygen species mediated tissue injury and repair', *Oxidative Medicine and Cellular Longevity*, 1, pp. 1–10.

Fuglie, L. J. (2005) 'The Moringa Tree; a local solution to malnutrition ?', *Educational Concerns for Haiti Organization (ECHO) Technical Note*, pp. 1–34.

Fujii, T., Ikeda, K. and Saito, M. (2009) 'Inhibitory effect of rose hip (*Rosa canina* L.) on melanogenesis in mouse melanoma cells and on pigmentation in brown guinea pigs',

*Bioscience, Biotechnology and Biochemistry*, 73(9), pp. 1989–1993.

Furda, A., Santos, J. H., Meyer, J. N. and Van Houten, B. (2014) 'Quantitative PCR-based measurement of nuclear and mitochondrial DNA damage and repair in mammalian cells', *Methods Molecular Biology*, 1105, pp. 419–437.

Future Market Insight (FMI) (2015) *Halal cosmetics market : Indonesia and Malaysia most lucrative countries in Southeast Asia*. Available at: <http://www.futuremarketinsights.com/press?release/apac?halal?cosmetics?market> (Accessed: 19 June 2017).

Gaikwad, M. and Kale, S. (2011) 'Formulation and *in vitro* evaluation for sun protection factor of *Moringa oleifera* Lam. (Family-Moringaceae) oil sunscreen cream', *International Journal of Pharmacy and Pharmaceutical Sciences*, 3(4), pp. 371–375.

Galanakis, C. M., Tsatalas, P. and Galanakis, I. M. (2017) 'Phenols from olive mill wastewater and other natural antioxidants as UV filters in sunscreens', *Environmental Technology and Innovation*, 9, pp. 160–168.

Ganceviciene, R., Liakou, A. I., Theodoridis, A., Makrantonaki, E. and Zouboulis, C. C. (2012) 'Skin anti-aging strategies', *Dermato-Endocrinology*, pp. 308–319.

Gasparro, F. P., Mitchnick, M. and Nash, J. F. (1998) 'A review of sunscreen and efficacy', *Photochemistry and Photobiology*, 68(3), pp. 243–256.

Gkogkolou, P. and Böhm, M. (2012) 'Advanced glycation end products: key players in skin ageing?', *Dermato-Endocrinology*, 4(3), pp. 259–270.

Goldsmith, T. C. (2014) *An Introduction to Biological Aging Theory*. Azinet Press. Available at: [http://www.azinet.com/aging/aging\\_theory\\_introduction.pdf](http://www.azinet.com/aging/aging_theory_introduction.pdf) (Accessed: 12 January 2018).

Gomez, M. S. and Kalamani, A. (2003) 'Butterfly pea (*Clitoria ternatea*): a nutritive multipurpose forage legume for the Tropics: an overview', *Pakistan Journal of Nutrition*, 2(6), pp. 374–379.

Gorinstein, S., Böhm, V., Schaich, K. M., Özyürek, M. and Güçlü, K. (2013) 'Methods of measurement and evaluation of natural antioxidant capacity/activity', *Pure Application Chemistry*, 85(5), pp. 957–998.

Graber, E. M., Tanzi, E. L. and Alster, T. S. (2008) 'Side effects and complications of fractional laser photothermolysis: experience with 961 treatments', *Dermatologic Surgery*, 34(3), pp. 301–305.

Grand View Research (2016) *Organic Personal Care Market Size and Forecast By Product (Skin Care, Hair Care, Oral Care, and Cosmetics), By Region (North America, Europe, Asia Pacific, Latin America, and Middle East & Africa) And Trend Analysis From 2014 To 2025*, Grand View Research Report. Available at: <http://www.grandviewresearch.com/industry-analysis/organic-personal-care-market> (Accessed: 22 December 2016).

Grivas, C. (2017) '5th International Phytocosmetic and Phytotherapy Congress', in *Exploring Greek Texts on Cosmetics in Imperial Rome*. Patras: International Society for Phytocosmetic

Science, p. 36.

Gülden, M., Jess, A., Kammann, J., Maser, E. and Seibert, H. (2010) 'Cytotoxic potency of H<sub>2</sub>O<sub>2</sub> in cell cultures: impact of cell concentration and exposure time', *Free Radical Biology and Medicine*, 49(8), pp. 1298–1305.

Haftek, M., Simon, M., Kanitakis, J., Maréchal, S., Claudy, A., Serre, G. and Schmitt, D. (1997) 'Expression of corneodesmosin in the granular layer and stratum corneum of normal and diseased epidermis', *British Journal of Dermatology*, 137(6), pp. 864–873.

Hakozaki, T., Swanson, C. L. . and Bissett, D. L. (2010) 'Hyperpigmentation in aging skin', in *Textbook of Aging Skin*. Berlin Heidelberg: Springer-Verlag, pp. 495–501.

Halliwell, B. (2006) 'Reactive species and antioxidants: redox biology is a fundamental theme of aerobic life', *Plant Physiology*, 141(2), pp. 312–322.

Halliwell, B., Clement, M. V. and Lee, H. L. (2000) 'Hydrogen peroxide in the human body', *FEBS Letters*, 486, pp. 10–13.

Halliwell, B. and Gutteridge, J. M. C. (1985) 'Role of free radicals and catalytic metal ions in human disease: an overview', *Molecular Aspects Medicine*, 8, pp. 1–85.

Hamid, A. A., Shah, Z. M., Muse, R. and Mohamed, S. (2002) 'Characterisation of antioxidative activities of various extracts of *Centella asiatica* (L.) Urban', *Food Chemistry*, 77(4), pp. 465–469.

Handog, E., Singzon, I. and Datuin, M. S. (2012) 'Chemical peels for acne and acne scars in Asians: evidence based review', *Journal of Cutaneous and Aesthetic Surgery*, 5(4), pp. 239–246.

Haneke, E. (2006) 'Skin rejuvenation without a scalpel: fillers', *Journal of Cosmetic Dermatology*, 5(2), pp. 157–167.

Harborne, J. B. . and Williams, C. A. (2000) 'Advances in flavonoid research since 1992', *Phytochemistry*, 55(6), pp. 481–504.

Harman, D. (1956) 'Aging: a theory based on free radical and radiation chemistry', *Journal of Gerontology*, 11(3), pp. 298–300.

Harman, D. (1991) 'The aging process: major risk factor for disease and death.', *Proceedings of the National Academy of Sciences*, 88(6), pp. 5360–5363.

Harman, D. (1992) 'Free radical theory of aging', *Mutation Research*, 275, pp. 257–266.

Hashim, P. (2011) 'Centella asiatica in food and beverage applications and its potential antioxidant and neuroprotective effect', *International Food Research Journal*, 18(4), pp. 1215–1222.

Hassali, A., Dawood, O. T., Al-Tamimi, S. and Verma, A. K. (2015) 'Malaysian cosmetic market: current and future prospects', *Pharmaceutical Regulatory Affairs: Open Access*, 04(04), pp. 10–13.

Hassan, S. A., Mijin, S., Yusoff, U. K., Ding, P. and Wahab, P. E. M. (2012) 'Nitrate, ascorbic acid, mineral and antioxidant activities of *Cosmos caudatus* in response to organic and mineral-based fertilizer rates', *Molecules*, 17, pp. 7843–7853.

Hayflick, L. (1965) 'The limited *in vitro* lifetime of human diploid cell strains', *Experimental Cell Research*, 37(3), pp. 614–636.

Hearing, V. J. (1999) 'Biochemical control of melanogenesis and melanosomal organization', *Journal of Investigative Dermatology*, 4, pp. 24–28.

Hellström, J., Mattila, P. and Karjalainen, R. (2013) 'Stability of anthocyanins in berry juices stored at different temperatures', *Journal of Food Composition and Analysis*, 31(1), pp. 12–19.

Himamura, T. S., Umikura, Y. S., Amazaki, T. Y., Ada, A. T., Ashiwagi, T. K., Shikawa, H. I., Atsui, T. M., Ugimoto, N. S., Kiyama, H. A. and Keda, H. U. (2014) 'Applicability of the DPPH assay for evaluating the antioxidant capacity of food additives – Inter-laboratory evaluation Study –', *Analytical Sciences*, 30, pp. 717–721.

Hsu, S., Bollag, W. B., Lewis, J., Huang, Q. I. N., Singh, B., Sharawy, M., Yamamoto, T. and Schuster, G. (2003) 'Green tea polyphenols induce differentiation and proliferation in epidermal keratinocytes', *Journal of Pharmacology and Experimental Therapeutics*, 306(1), pp. 29–34.

Huang, D., Ou, B. and Prior, R. L. (2005) 'The chemistry behind antioxidant capacity assays', *Journal of Agricultural and Food Chemistry*, 53, pp. 1841–1856.

Huda-Faujan, N., Noriham, A., Norrakiah, A. S. and Babji, a. S. (2007) 'Antioxidative activities of water extracts of some Malaysian herbs', *International Food Research Journal*, 14(1), pp. 61–68.

Hunter, S., Jung, D., Giulio, R. Di and Meyer, J. (2010) 'The QPCR assay for analysis of mitochondrial DNA damage, repair, and relative copy number', *Methods*, 51(4), pp. 444–451.

Hunzeker, C. M., Weiss, E. T. and Geronemus, R. G. (2009) 'Fractionated CO<sub>2</sub> laser resurfacing: our experience with more than 2000 treatments', *Aesthetic Surgery Journal*, 29(4), pp. 317–322.

Hussin, M., Hamid, A. A., Mohamad, S., Saari, N., Bakar, F. and Dek, S. P. (2009) 'Modulation of lipid metabolism by *Centella asiatica* in oxidative stress rats', *Journal of Food Science*, 74(2), pp. H72–H78.

Hüttemann, M., Lee, I., Samavati, L., Yu, H. and Doan, J. W. (2007) 'Regulation of mitochondrial oxidative phosphorylation through cell signaling', *Biochimica et Biophysica Acta (BBA)*, 1773(12), pp. 1701–1720.

Hwang, J. M., Kuo, H. C., Lin, C. T. and Kao, E. S. (2013) 'Inhibitory effect of liposome-encapsulated anthocyanin on melanogenesis in human melanocytes', *Pharmaceutical Biology*, 51(1), pp. 941–947.

Iamsaard, S., Burawat, J., Kanla, P. and Arun, S. (2014) 'Antioxidant activity and protective effect of *Clitoria ternatea* flower extract on testicular damage induced by ketoconazole in

rats', 15(6), pp. 548–555.

Ibrahimi, O. A., Saedi, N. and Kilmer, S. L. (2015) 'Laser-based treatment of the aging face for skin resurfacing: ablative and non-ablative lasers', in *Surgery of the Skin*. Elsevier Inc., pp. 549–560.

Imokawa, G. and Ishida, K. (2015) 'Biological mechanisms underlying the ultraviolet radiation-induced formation of skin wrinkling and sagging I: reduced skin elasticity, highly associated with enhanced dermal elastase activity, triggers wrinkling and sagging', *International Journal of Molecular Sciences*, 16(4), pp. 7753–7775.

Inamdar, P. K., Yeole, R. D., Ghogare, A. B. and de Souza, N. J. (1996) 'Determination of biologically active constituents in *Centella asiatica*', *Journal of Chromatography A*, 742(1), pp. 127–130.

Ines, D., Sonia, B., Fatma, B. A., Souhail, B., Hamadi, A., Hamida, T. and Basma, H. (2010) 'Date seed oil inhibits hydrogen peroxide-induced oxidative stress in human epidermal keratinocytes', *International Journal of Dermatology*, 49, pp. 262–268.

Iozumi, K., Hoganson, G. E., Pennella, R., Everett, M. A. and Fuller, B. B. (1993) 'Role of tyrosinase as the determinant of pigmentation in cultured human melanocytes', *Journal of Investigative Dermatology*, 100(6), pp. 806–811.

Islam, S., Parvin, S., Uddin, N. and Mazid, A. (2014) 'Antidiabetic and antioxidant activities of decoctions of *Coccinia grandis* Linn. and *Centella asiatica* ( L.) on alloxan-induced diabetic rats', *Bangladesh Pharmaceutical Journal*, 17(1), pp. 86–91.

Iwai, K., Kishimoto, N., Kakino, Y., Mochida, K. and Fujita, T. (2004) 'In vitro antioxidative effects and tyrosinase inhibitory activities of seven hydroxycinnamoyl derivatives in green coffee beans', *Journal of Agricultural and Food Chemistry*, 52(15), pp. 4893–4898.

Jäger, C., Brenner, C., Habicht, J. and Wallich, R. (2012) 'Bioactive reagents used in mesotherapy for skin rejuvenation in vivo induce diverse physiological processes in human skin fibroblasts in vitro- a pilot study', *Experimental Dermatology*, 21(1), pp. 72–75.

Jaiswal, D., Rai, P. K., Mehta, S., Chatterji, S., Shukla, S., Rai, D. K., Sharma, G., Sharma, B., Khair, S. and Watal, G. (2013) 'Role of *Moringa oleifera* in regulation of diabetes-induced oxidative stress', *Asian Pacific Journal of Tropical Medicine*, 6(6), pp. 426–432.

James, J. T. and Dubery, I. A. (2009) 'Pentacyclic triterpenoids from the medicinal herb, *Centella asiatica* (L.) Urban', *Molecules*, 14(10), pp. 3922–3941.

Javadi, N., Abas, F., Hamid, A. A., Simoh, S., Shaari, K., Ismail, I. S., Median, A. and Khatib, A. (2014) 'GC-MS-based metabolite profiling of *Cosmos caudatus* leaves possessing alpha-glucosidase inhibitory activity', *Journal of Food Science*, 79, pp. C1130–C1136.

Javadi, N., Abas, F., Median, A., Abd, A., Khatib, A., Simoh, S. and Shaari, K. (2015) 'Effect of storage time on metabolite profile and alpha-glucosidase inhibitory activity of *Cosmos caudatus* leaves: GC-MS-based metabolomics approach', *Journal of Food and Drug Analysis*, 23(3), pp. 433–441.

Jeba, A., John, S., Kalusalingam, A., Chellappan, D. K., Gopinath, R., Radhamani, S., Husain, H. A., Muruganandham, V. and Promwichit, P. (2010) 'Ethnomedical survey of plants used by the Orang Asli in Kampung Bawong, Perak, West Malaysia', *Journal of Ethnobiology and Ethnomedicine*, 6(5), pp. 1–6.

Jhan, J. K., Chung, Y. C., Chen, G. H., Chang, C. H., Lu, Y. C. and Hsu, C. K. (2016) 'Anthocyanin contents in the seed coat of black soya bean and their anti-human tyrosinase activity and antioxidative activity', *International Journal of Cosmetic Science*, 38(3), pp. 319–324.

Jin, H. C., Jin, Y. S., Hai, R. C., Mi, K. L., Choon, S. Y., Rhie, G. E., Kwang, H. C., Kyu, H. K., Kyung, C. P. and Hee, C. E. (2001) 'Modulation of skin collagen metabolism in aged and photoaged human skin *in vivo*', *Journal of Investigative Dermatology*, 117(5), pp. 1218–1224.

Jin, K. (2010) 'Modern biological theories of aging', *Aging and Disease*, 1(2), pp. 72–74.

Kacem, R. (2013) 'Phenolic compounds from medicinal plants as natural anti-elastase products for the therapy of pulmonary emphysema', *Journal of Medicinal Plants Research*, 7(44), pp. 3499–3507.

Kaisoon, O., Siriamornpun, S., Weerapreeyakul, N. and Meeso, N. (2011) 'Phenolic compounds and antioxidant activities of edible flowers from Thailand', *Journal of Functional Foods*, 3(2), pp. 88–99.

Kamei, Y., Otsuka, Y. and Abe, K. (2009) 'Comparison of the inhibitory effects of vitamin E analogues on melanogenesis in mouse B16 melanoma cells', *Cytotechnology*, 59(3), pp. 183–190.

Kamkaen, N. and Wilkinson, J. M. (2009) 'The antioxidant activity of *Clitoria ternatea* flower petal extracts and eye gel', *Phytotherapy Research*, 23(April), pp. 1624–1625.

Katiyar, S. K., Challa, A., McCormick, T. S., Cooper, K. D. and Mukhtar, H. (1999) 'Prevention of UVB-induced immunosuppression in mice by the green tea polyphenol (-)-epigallocatechin-3-gallate may be associated with alterations in IL-10 and IL-12 production', *Carcinogenesis*, 20(11), pp. 2117–2124.

Katiyar, S. K. and Mukhtar, H. (2001) 'Green tea polyphenol (-)-epigallocatechin-3-gallate treatment to mouse skin prevents UVB-induced infiltration of leukocytes, depletion of antigen-presenting cells, and oxidative stress.', *Journal of Leukocyte Biology*, 69(5), pp. 719–726.

Kazuma, K., Noda, N. and Suzuki, M. (2003a) 'Flavonoid composition related to petal color in different lines of *Clitoria ternatea*', *Phytochemistry*, 64(6), pp. 1133–1139.

Kazuma, K., Noda, N. and Suzuki, M. (2003b) 'Malonylated flavonol glycosides from the petals of *Clitoria ternatea*', *Phytochemistry*, 62(2), pp. 229–237.

Khan, M. T. H. (2007) 'Molecular design of tyrosinase inhibitors: a critical review of promising novel inhibitors from synthetic origins', *Pure and Applied Chemistry*, 79(12), pp. 2277–2295.

Khanam, Z., Wen, C. S. and Bhat, I. U. H. (2014) 'Phytochemical screening and antimicrobial activity of root and stem extracts of wild *Eurycoma longifolia* Jack (Tongkat Ali)', *Journal of*

Kim, Y. J., Cha, H. J., Nam, K. H., Yoon, Y., Lee, H. and An, S. (2011) 'Centella asiatica extracts modulate hydrogen peroxide-induced senescence in human dermal fibroblasts', *Experimental Dermatology*, 20(12), pp. 998–1003.

Kline Group (2015) *The Move towards Truly Natural Products Aims to Strengthen the Natural Personal Care Market*. Available at: [http://www.klinegroup.com/news/natural\\_personal\\_care\\_market1-29-15.asp](http://www.klinegroup.com/news/natural_personal_care_market1-29-15.asp) (Accessed: 18 June 2017).

Kolarsick, P. A. J., Kolarsick, M. A. and Goodwin, C. (2011) 'Anatomy and physiology of the skin', *Journal of the Dermatology Nurses' Association*, 3(4), pp. 203–213.

Konttinen, Y. T., Ainola, M., Valleala, H., Ma, J., Ida, H., Mandelin, J., Kinne, R. W., Santavirta, S., Sorsa, T., López-Otín, C. and Takagi, M. (1999) 'Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis.', *Annals of the Rheumatic Diseases*, 58(11), pp. 691–7.

Kosai, P., Sirisidhi, K., Jiraungkoorskul, K. and Jiraungkoorskul, W. (2015) 'Review on ethnomedicinal uses of memory in boosting butterfly Pea, *Clitoria ternatea*', *Journal of Natural Remedies*, 15(2), pp. 71–76.

Kosmadaki, M. G. and Gilchrest, B. A. (2004) 'The role of telomeres in skin aging/photoaging', *Micron*, 35(3), pp. 155–159.

Krutmann, J. and Humbert, P. (2011) 'Skin Aging', in *Nutrition for Healthy Skin: Strategies for Clinical and Cosmetic Practice*. Berlin Heidelberg: Springer-Verlag, pp. 15–24.

Krutmann, J. and Schroeder, P. (2009) 'Role of mitochondria in photoaging of human skin: the defective powerhouse model', *Journal of Investigative Dermatology*, 14(1), pp. 44–49.

Kumar, S., Seal, C. J., Howes, M. J. R., Kite, G. C. and Okello, E. J. (2010) 'In vitro protective effects of *Withania somnifera* (L.) Dunal root extract against hydrogen peroxide and beta-amyloid<sub>(1-42)</sub>-induced cytotoxicity in differentiated PC12 cells', *Phytotherapy Research*, 24(10), pp. 1567–1574.

Kvam, E. and Tyrrell, R. M. (1999) 'The role of melanin in the induction of oxidative DNA base damage by ultraviolet A irradiation of DNA or melanoma cells', *Journal of Investigative Dermatology*, 113(2), pp. 209–213.

Leake, C. D. (1952) 'The chief Egyptian medical papyri', in *The Old Egyptian Medical Papyri*. Kansas: University of Kansas Press, pp. 7–17.

Lee, C. H., Wu, S. B., Hong, C. H., Chen, G. S., Wei, Y. H. and Yu, H. S. (2013) 'Involvement of mtDNA damage elicited by oxidative stress in the arsenical skin cancers', *Journal of Investigative Dermatology*, 133(7), pp. 1890–1900.

Lee, J., Durst, R. W. and Wrolstad, R. E. (2005) 'Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study', *Journal of AOAC International*, 88(5), pp. 1269–1278.

Lee, J. H., Zhou, H. Y., Cho, S. Y., Kim, Y. S., Lee, Y. S. and Jeong, C. S. (2007) 'Anti-inflammatory mechanisms of apigenin: inhibition of cyclooxygenase-2 expression, adhesion of monocytes to human umbilical vein endothelial cells, and expression of cellular adhesion molecules', *Archives of Pharmacal Research*, 30(10), pp. 1318–1327.

Lee, K. K., Cho, J. J., Park, E. J. and Choi, J. D. (2001) 'Anti-elastase and anti-hyaluronidase of phenolic substance from *Areca catechu* as a new anti-ageing agent', *International Journal of Cosmetic Science*, 23, pp. 341–346.

Lee, K. K. and Choi, J. D. (1999) 'The effects of *Areca catechu* L. extract on anti-aging', *International Journal of Cosmetic Science*, 21, pp. 285–295.

Lee, S. H., Sancheti, S., Sancheti, S. and Seo, S. Y. (2009) 'Potent antielastase and antityrosinase activities of *Astilbe chinensis*', *American Journal of Pharmacology and Toxicology*, 4(4), pp. 127–129.

Lephart, E. D. (2016) 'Skin aging and oxidative stress: Equol's anti-aging effects via biochemical and molecular mechanisms', *Ageing Research Reviews*, 31, pp. 36–54.

Li, X., Du, L. and Lu, J. J. (2015) 'A novel hypothesis of visual loss secondary to cosmetic facial filler injection', *Annals of Plastic Surgery*, 75(3), pp. 258–260.

Li, X., Wu, X. and Huang, L. (2009) 'Correlation between antioxidant activities and phenolic contents of radix *Angelicae sinensis* (Danggui)', *Molecules*, 14(12), pp. 5349–5361.

Lijon, B., Meghla, N. S., Jahedi, E., Rahman, A. and Hossain, I. (2017) 'Phytochemistry and pharmacological activities of *Clitoria ternatea*', *International Journal of Natural and Social Sciences*, 4(1), pp. 1–10.

Liliwirianis, N., Musa, N. L. W., Zain, W. Z. W. M., Kassim, J. and Karim, S. A. (2011) 'Premilinary studies on phytochemical screening of Ulam and fruit from Malaysia', *E-Journal of Chemistry*, 8(S1), pp. S285–S288.

Lim, S., Choi, A., Kwon, M., Joung, E., Shin, T., Lee, S., Kim, N. and Kim, H. (2019) 'Evaluation of antioxidant activities of various solvent extract from *Sargassum serratifolium* and its major antioxidant components', *Food Chemistry*, 278, pp. 178–184.

Lima, G. P. P., Vianello, F., Corrêa, C. R., Arnoux, R., Campos, D. S. and Borguini, M. G. (2014) 'Polyphenols in fruits and vegetables and its effect on human health', *Food and Nutrition Sciences*, 5(5), pp. 1065–1082.

Liu, L., Xie, H., Chen, X., Shi, W., Xiao, X., Lei, D. and Li, J. (2012) 'Differential response of normal human epidermal keratinocytes and HaCaT cells to hydrogen peroxide-induced oxidative stress', *Clinical and Experimental Dermatology*, pp. 772–780.

Liu, Y., Fiskum, G. and Schubert, D. (2002) 'Generation of reactive oxygen species by the mitochondrial electron transport chain', *Journal of Neurochemistry*, 80(5), pp. 780–787.

Liu, Y. H., Lin, Y. S., Huang, Y. W., Fang, S. U., Lin, S. Y. and Hou, W. C. (2016) 'Protective effects of minor components of curcuminoids on hydrogen peroxide-treated human HaCaT keratinocytes', *Journal of Agricultural and Food Chemistry*, 64(18), pp. 3598–3608.

López-Alarcón, C. and Denicola, A. (2013) 'Evaluating the antioxidant capacity of natural products: a review on chemical and cellular-based assays', *Analytica Chimica Acta*, 763, pp. 1–10.

Losada-barreiro, S. and Bravo-díaz, C. (2017) 'Free radicals and polyphenols: the redox chemistry of neurodegenerative diseases', *European Journal of Medicinal Chemistry*, 133, pp. 379–402.

Lucas, R., McMichael, T., Smith, W. and Armstrong, B. (2006) *Solar ultraviolet radiation: global burden of disease from solar ultraviolet radiation*, WHO. Available at: [http://www.who.int/uv/health/solaruvradfull\\_180706.pdf](http://www.who.int/uv/health/solaruvradfull_180706.pdf) (Accessed: 1 March 2018).

Mahanom, H., Azizah, A. H., Suhaila, M., Nazamid, S. and Maznah, I. (2011) 'Effect of *Centella asiatica* extract and powder on hydrogen peroxide-treated Sprague-Dawley rats', *Journal of Tropical Agriculture and Food Science*, 39(1), pp. 1–10.

Mandrone, M., Lorenzi, B., Venditti, A., Guarini, L., Bianco, A., Sanna, C., Ballero, M., Poli, F. and Antognoni, F. (2015) 'Antioxidant and anti-collagenase activity of *Hypericum hircinum* L.', *Industrial Crops and Products*. Elsevier B.V., 76, pp. 402–408.

Manstein, D., Herron, G. S., Sink, R. K., Tanner, H. and Anderson, R. R. (2004) 'Fractional photothermolysis: a new concept for cutaneous remodeling using microscopic patterns of thermal injury', *Lasers in Surgery and Medicine*, 34(5), pp. 426–438.

Maquart, F. X., Bellon, G., Gillery, P., Yanusz, W. and Borel, J. P. (1990) 'Stimulation of collagen synthesis in fibroblast cultures by a triterpene extracted from *Centella asiatica*', *Connective Tissue Research*, 24(2), pp. 107–120.

Matts, P. J., Oblong, J. E. and Bissett, D. L. (2002) 'A review of the range of effects of niacinamide in human skin', *International Federation Society of Cosmetic Chemistry*, 5(4), pp. 285–289.

Mecham, R. P., Broekelmann, T. J., Fliszar, C. J., Shapiro, S. D., Welgus, H. G. and Senior, R. M. (1997) 'Elastin degradation by matrix metalloproteinases', *Journal of Biological Chemistry*, 272(29), pp. 18071–18076.

Mediani, A., Abas, F., Khatib, A., Maulidiani, H. ., Shaari, K., Choi, Y. H. and Lajis, N. H. (2012) '1H-NMR-based metabolomics approach to understanding the drying effects on the phytochemicals in *Cosmos caudatus*', *Food Research International*, 49(2), pp. 763–770.

Mediani, A., Abas, F., Khatib, A. and Tan, C. P. (2013) '*Cosmos Caudatus* as a potential source of polyphenolic compounds: optimisation of oven drying conditions and characterisation of its functional properties', *Molecules*, 18, pp. 10452–10464.

Mediani, A., Abas, F., Tan, C. P., Khatib, A. and Lajis, N. H. (2012) 'Influence of growth stage and season on the antioxidant constituents of *Cosmos caudatus*', *Plant Foods for Human Nutrition*, 67(4), pp. 344–350.

Meeran, S. M., Mantena, S. K., Elmets, C. A. and Katiyar, S. K. (2006) '(-)-Epigallocatechin-3-gallate prevents photocarcinogenesis in mice through interleukin-12-dependent DNA repair', *Cancer Research*, 66(10), pp. 5512–5520.

Meeran, S. M., Mantena, S. K. and Katiyar, S. K. (2006) 'Prevention of ultraviolet radiation-induced immunosuppression by (-)-epigallocatechin-3-gallate in mice is mediated through interleukin-12-dependent DNA repair', *Clinical Cancer Research*, 12(7 I), pp. 2272–2280.

Mello, L. D. and Quadros, G. P. (2014) 'Correlation between antioxidant activity and total phenolic content with physicochemical parameters of blended extracts of *Camellia sinensis*', *Acta Scientiarum: Health Science*, 36(1), p. 97.

Melziq, M. F., Loser, B. and Ciesielski, S. (2001) 'Inhibition of neutrophil elastase activity by phenolic compounds from plants', *Pharmazie*, 56(12), pp. 967–970.

Mercado-Sáenz, S., Ruiz-Gómez, M. J., Morales-Moreno, F. and Martínez-Morillo, M. (2010) 'Cellular aging: theories and technological influence', *Brazilian Archives of Biology and Technology*, 53(6), pp. 1319–1332.

Meyer, J. N. (2010) 'QPCR: a tool for analysis of mitochondrial and nuclear DNA damage in ecotoxicology', *Ecotoxicology*, 19, pp. 804–811.

Ministry of Agriculture Malaysia (2010) *Agriculture National Key Economic Area (NKEA)*. Available at: <http://www.moa.gov.my/en/nkea> (Accessed: 14 August 2017).

Mironczuk-Chodakowska, I., Witkowska, A. M. and Zujko, M. E. (2018) 'Endogenous non-enzymatic antioxidants in the human body', *Advances in Medical Sciences*, 63, pp. 68–78.

Mishara, G., Singh, P., Verma, R., Kumar, S., Srivastav, S., Jha, K. K. and Khosa, R. L. (2011) '*Moringa olifera*- An important medicinal plant: a review of its traditional uses, phytochemistry and pharmacological properties', *Der Pharmacia Lettre*, 3(2), pp. 141–164.

Mitteldorf, J. J. (2013) 'How does the body know how old it is? Introducing the epigenetic clock hypothesis', *Biochemistry*, 78(9), pp. 1048–1053.

Mohamed, N., Sahhugi, Z., Ramli, E. S. and Muhammad, N. (2013) 'The effects of *Cosmos caudatus* (ulam raja) on dynamic and cellular bone histomorphometry in ovariectomized rats', *BMC Research Notes*, 6(239), pp. 1–6.

Mohammad, N. S., Milow, P. and Ong, H. C. (2012) 'Traditional medicinal plants used by the Kensiu tribe of Lubuk Ulu Legong, Kedah, Malaysia', *Ethno-Medicine*, 6(3), pp. 149–153.

Moharram, H. A. and Youssef, M. . (2015) 'Methods for determining the antioxidant activity: a review', *Journal of Food Science and Technology*, 11(4), pp. 31–42.

MolecularProbe (2001) *EnzChek® Elastase Assay Kit (E-12056)*. Available at: <https://tools.lifetechnologies.com/content/sfs/manuals/mp12056.pdf> (Accessed: 4 June 2015).

MolecularProbes (2001) *EnzChek® Gelatinase/Collagenase Assay Kit*. Available at: <https://tools.thermofisher.com/content/sfs/manuals/mp12052.pdf> (Accessed: 21 July 2017).

Moon, J. and Shibamoto, T. (2009) 'Antioxidant assays for plant and food components', *Journal of Agricultural and Food Chemistry*, 57(1), pp. 1655–1666.

Moon, J. Y., Yim, E. Y., Song, G., Lee, N. H. and Hyun, C. G. (2010) 'Screening of elastase and tyrosinase inhibitory activity from Jeju Island plants', *EurAsian Journal of Biosciences*, pp. 41–53.

Morad, A. F. (2011) *Cosmos caudatus Kunth*.

Moshawih, S., Manraj, S. C., Zuraini, A. and Zainul, A. (2017) 'A comprehensive review on *Cosmos caudatus* (Ulam Raja): pharmacology, ethnopharmacology, and phytochemistry', *International Research Journal of Education and Sciences*, 1(1), pp. 14–31.

Mukherjee, P. K., Kumar, V., Kumar, N. S. and Heinrich, M. (2008) 'The Ayurvedic medicine *Clitoria ternatea*: from traditional use to scientific assessment', *Journal of Ethnopharmacology*, 120(3), pp. 291–301.

Mukherjee, P. K., Maity, N., Nema, N. K. and Sarkar, B. K. (2011) 'Bioactive compounds from natural resources against skin aging', *Phytomedicine*, 19(1), pp. 64–73.

Mukherjee, P., Nema, N., Maity, N. and Sarkar, B. (2012) 'Standardized *Clitoria ternatea* leaf extract as hyaluronidase, elastase and matrix-metalloproteinase-1 inhibitor', *Indian Journal of Pharmacology*, 44(5), p. 584.

Mustafa, R. A., Hamid, A. A., Mohamed, S. and Bakar, F. A. (2010) 'Total phenolic compounds, flavonoids, and radical scavenging activity of 21 selected tropical plants', *Journal of Food Science*, 75(1), pp. C28–C35.

Nachbar, F. and Korting, H. C. (1995) 'The role of vitamin E in normal and damaged skin', *Journal of Molecular Medicine*, 73(1), pp. 7–17.

Nair, V., Bang, W. Y., Schreckinger, E., Andarwulan, N. and Cisneros-Zevallos, L. (2015) 'Protective role of ternatin anthocyanins and quercetin glycosides from butterfly pea (*Clitoria ternatea* Leguminosae) blue flower petals against lipopolysaccharide (LPS)-induced inflammation in macrophage cells', *Journal of Agricultural and Food Chemistry*, 63(28), pp. 6355–6365.

Nema, N. K., Maity, N., Sarkar, B. K. and Mukherjee, P. K. (2013) 'Matrix metalloproteinase, hyaluronidase and elastase inhibitory potential of standardized extract of *Centella asiatica*', *Pharmaceutical Biology*, 51(9), pp. 1182–1187.

Nichols, J. A. and Katiyar, S. K. (2010) 'Skin photoprotection by natural polyphenols: anti-inflammatory, antioxidant and DNA repair mechanisms', *Archives of Dermatological Research*, 302(2), pp. 71–83.

Nikalji, N., Patil, S., Sakhya, J., Godse, K. and Nadkarni, N. (2012) 'Complications of medium depth and deep chemical peels', *Journal of Cutaneous and Aesthetic Surgery*, 5(4), p. 254.

Nimse, S. B. and Pal, D. (2015) 'Free radicals, natural antioxidants, and their reaction mechanisms', *Royal Society of Chemistry Advances*, 5(35), pp. 27986–28006.

Ning, Y., Xu, J. F., Li, Y., Chavez, L., Riethman, H. C., Lansdorp, P. M. and Weng, N. P. (2003) 'Telomere length and the expression of natural telomeric genes in human fibroblasts', *Human Molecular Genetics*, 12(11), pp. 1329–1336.

Nusgens, B. V., Humbert, P., Rougier, A., Colige, A. C., Haftek, M., Lambert, C. A., Alain, R., Creidi, P. and Lapière, C. M. (2001) 'Topically applied vitamin C enhances the mRNA level of collagens I and III, their processing enzymes and tissue inhibitor of matrix metalloproteinase-1 in the human dermis<sup>1</sup>', *Journal of Investigative Dermatology*, 116(6), pp. 853–859.

Oancea, S. and Drăghici, O. (2013) 'PH and thermal stability of anthocyanin-based optimised extracts of romanian red onion cultivars', *Czech Journal of Food Sciences*, 31(3), pp. 283–291.

Oliveira, S. De, De Souza, G. A., Eckert, C. R., Silva, T. A., Sobral, E. S., Favero, O. A., Ferreira, M. J. P., Romoff, P. and Baader, W. J. (2014) 'Evaluation of antiradical assays used in determining the antioxidant capacity of pure compounds and plant extracts', *Química Nova*, 37(3), pp. 497–503.

Olovnikov, A. (1971) 'Principle of marginotomy in the synthesis of polynucleotides at a template', *Doklady Akademii nauk SSSR*, 201(16), pp. 394–397.

Olovnikov, A. M. (1973) 'A theory of marginotomy; the incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon', *Journal of Theoretical Biology*, 41(1), pp. 181–190.

Ong, H. C. and Nordiana, M. (1999) 'Malay ethno-medico botany in Machang, Kelantan, Malaysia', *Fitoterapia*, 70(5), pp. 502–513.

Oroian, M. and Escriche, I. (2015) 'Antioxidants: characterization, natural sources, extraction and analysis', *Food Research*, 74, pp. 10–36.

Ortonne, J. P. and Bissett, D. L. (2008) 'Latest insights into skin hyperpigmentation', *Journal of Investigative Dermatology, Symposium Proceedings*, 13(1), pp. 10–14.

Othman, R., Razak, N. I. and Ishak, N. (2014) 'International Conference on Multidisciplinary Trends in Academic Research (GTAR) 2015', in *Ethnobotanical study of traditional knowledge on Ppant used in traditional bath (mandi serom) among Malay midwives in Perak and Negeri Sembilan*. Bandung: Global Illuminators, pp. 291–296.

Palada, M. C. (1996) 'Moringa (*Moringa oleifera* Lam.): a versatile tree crop with horticultural potential in the subtropical United States', *Horticultural Science*, 31(5), pp. 794–797.

Paliwall, R., Sharma, V. and Pracheta (2011) 'A review on horse radish tree (*Moringa oleifera*): a multipurpose tree with high economic and commercial importance', *Asian Journal of Biotechnology*, 3, pp. 317–328.

Pandel, R., Poljšak, B., Godic, A. and Dahmane, R. (2013) 'Skin photoaging and the role of antioxidants in its prevention', *International Scholarly Research Notices: Dermatology*, pp. 1–11.

Pandey, K. B. and Rizvi, S. I. (2009) 'Plant polyphenols as dietary antioxidants in human health and disease', *Oxidative Medicine and Cellular Longevity*, 2(5), pp. 270–278.

Park, J. M., Cho, J. K., Mok, J. Y., Jeon, I. H., Kim, H. S., Kang, H. J. and Jang, S. Il (2012) 'Protective effect of astragalin and quercetin on ultraviolet (UV)-irradiated damage in HaCaT cells and Balb/c mice', *Journal of the Korean Society for Applied Biological Chemistry*, 55(3),

pp. 443–446.

Paulucci, V. P., Couto, R. O., Teixeira, C. C. C. and Freitas, L. A. P. (2013) 'Optimization of the extraction of curcumin from *Curcuma longa* rhizomes', *Brazilian Journal of Pharmacognosy*, 23(1), pp. 94–100.

Pereira, B. K., Rosa, R. M., Silva, J. da, Guecheva, T. N., Oliveira, I. M. de, Ianistcki, M., Benvegnú, V. C., Furtado, G. V., Ferraz, A., Richter, M. F., Schroder, N., Pereira, A. B. and Henriques, J. A. P. (2009) 'Protective effects of three extracts from Antarctic plants against ultraviolet radiation in several biological models', *Journal of Photochemistry and Photobiology B: Biology*, 96(2), pp. 117–129.

Philips, N., Auler, S., Hugo, R. and Gonzalez, S. (2011) 'Beneficial regulation of matrix metalloproteinases for skin health', *Enzyme Research*, (12), pp. 1–4.

Philips, N., Conte, J., Chen, Y. J., Natrajan, P., Taw, M., Keller, T., Givant, J., Tuason, M., Dulaj, L., Leonardi, D. and Gonzalez, S. (2009) 'Beneficial regulation of matrixmetalloproteinases and their inhibitors, fibrillar collagens and transforming growth factor- $\beta$  by *Polypodium leucotomos*, directly or in dermal fibroblasts, ultraviolet radiated fibroblasts, and melanoma cells', *Archives of Dermatological Research*, 301(7), pp. 487–495.

Phrueksanan, W., Yibchok-Anun, S. and Adisakwattana, S. (2014) 'Protection of *Clitoria ternatea* flower petal extract against free radical induced hemolysis and oxidative damage in canine erythrocytes', *Research in Veterinary Science*, 97(2), pp. 357–363.

Pillaiyar, T., Manickam, M. and Namasivayam, V. (2017) 'Skin whitening agents: medicinal chemistry perspective of tyrosinase inhibitors', *Journal of Enzyme Inhibition and Medicinal Chemistry*, 32(1), pp. 403–425.

Piluzza, G. and Bullitta, S. (2011) 'Correlations between phenolic content and antioxidant properties in twenty-four plant species of traditional ethnoveterinary use in the Mediterranean area', *Pharmaceutical Biology*, 49(3), pp. 240–247.

Pinchuk, I., Shoval, H., Dotan, Y. and Lichtenberg, D. (2012) 'Evaluation of antioxidants: Scope, limitations and relevance of assays', *Chemistry and Physics of Lipids*, 165(6), pp. 638–647.

Pittayapruet, P., Meephansan, J., Prapapan, O., Komine, M. and Ohtsuki, M. (2016) 'Role of matrix metalloproteinases in photoaging and photocarcinogenesis', *International Journal of Molecular Sciences*, 17(868), pp. 1–20.

Pouillot, A., Polla, L. L., Tacchini, P., Neequaye, A., Polla, A. and Polla, B. (2011) 'Natural antioxidants and their effects on the skin', in Dayan, N. and Kromidas, L. (eds) *Formulating, Packaging, and Marketing of Natural Cosmetic Products*. New Jersey: John Wiley & Sons, Inc., pp. 239–257.

Prasanna, V. and Sreelatha, S. (2014) 'Synergistic effect of *Moringa oleifera* attenuates oxidative stress induced apoptosis in *Saccharomyces cerevisiae* cells: evidence for anticancer potential', *International Journal of Pharmaceutical and Biological Sciences*, 5(2), pp. 167–177.

Preissig, J., Hamilton, K. and Markus, R. (2012) 'Current laser resurfacing technologies: a review that delves beneath the surface', *Seminars in Plastic Surgery*, 26(3), pp. 109–116.

Pullar, J. M., Carr, A. C. and Vissers, M. C. M. (2017) 'The roles of vitamin C in skin health', *Nutrients*, 9(8), pp. 1–27.

Puttarak, P., Brantner, A. and Panichayupakaranant, P. (2016) 'Biological activities and stability of a standardized pentacyclic triterpene enriched *Centella asiatica* extract', *Natural Product Sciences*, 22(1), pp. 20–24.

Pyrzynska, K. and Pękal, A. (2013) 'Application of free radical diphenylpicrylhydrazyl (DPPH) to estimate the antioxidant capacity of food samples', *Analytical Methods*, 5(17), pp. 4288–4295.

Qiagen (2016) *QIAamp DNA and Blood Mini Handbook*. Available at: <http://www.qiagen.com/knowledge-and-support/resource-center/resource-download.aspx?id=67893a91-946f-49b5-8033-394fa5d752ea&lang=en> (Accessed: 17 February 2017).

Rabeta, M. S. . and An Nabil, Z. (2013) 'Total phenolic compounds and scavenging activity in *Clitoria ternatea* and *Vitex negundo* Linn.', *International Food Research Journal*, 20(1), pp. 495–500.

Radice, M., Manfredini, S., Ziosi, P., Dissette, V., Buso, P., Fallacara, A. and Vertuani, S. (2016) 'Herbal extracts, lichens and biomolecules as natural photo-protection alternatives to synthetic UV filters. A systematic review.', *Fitoterapia*, 114, pp. 144–162.

Rahman, M., Hossain, S., Rahaman, A., Fatima, N., Nahar, T. and Uddin, B. (2013) 'Antioxidant activity of *Centella asiatica* (Linn.) Urban: impact of extraction solvent polarity', *Journal of Pharmacognosy and Phytochemistry*, 1(6), pp. 27–32.

Ramsdell, W. (2012) 'Fractional CO<sub>2</sub> laser resurfacing complications', *Seminars in Plastic Surgery*, 26(3), pp. 137–140.

Raschke, C. and Elsner, P. (2010) 'Skin aging: a brief summary of characteristic changes', in *Textbook of Aging Skin*. Springer-Verlag Berlin Heidelberg, pp. 37–43.

Rastogi, R. P., Richa, Kumar, A., Tyagi, M. B. and Sinha, R. P. (2010) 'Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair', *Journal of Nucleic Acids*, 2010, pp. 1–32.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. (1999) 'Antioxidant activity applying an improved ABTS radical cation decolorization assay', *Free Radical Biology and Medicine*, 26, pp. 1231–1237.

Reddyvari, H., Govatati, S., Matha, S. K., Korla, S. V., Malempati, S., Pasupuleti, S. R., Bhanoori, M. and Nallanchakravarthula, V. (2017) 'Therapeutic effect of green tea extract on alcohol induced hepatic mitochondrial DNA damage in albino wistar rats', *Journal of Advanced Research*, 8(3), pp. 289–295.

Redini, F., Tixier, J.-M., Petitou, M., Choay, J., Robert, L. and Hornebeck, W. (1988) 'Inhibition of leucocyte elastase by heparin and its derivatives', *Biochemistry Journal*, 252, pp. 515–519.

Reihani, S. F. S. and Azhar, M. E. (2012) 'Antioxidant activity and total phenolic content in aqueous extracts of selected traditional Malay salads (Ulam)', *International Food Research*

Journal, 19(4), pp. 1439–1444.

Rinnerthaler, M., Bischof, J., Streaubel, M. K., Trost, A. and Ritchter, K. (2015) 'Oxidative stress in aging human skin', *Biomolecules*, 5(2), pp. 545–589.

Robert, L., Jacob, M. P., FRances, C., Godeau, G. and Hornebeck, W. (1984) 'Interaction between elastin and elastase and its role in the aging of the arterial wall, skin and other connective tissues. A review\*', *Mechanisms of Aging and Development*, 28, pp. 155–166.

Rodriguez-Perez, C., Mendiola, J. A., Ibáñez, E. and Segura-Carretero, A. (2016) 'Green downstream processing using supercritical carbon dioxide, CO<sub>2</sub>-expanded ethanol and pressurized hot water extractions for recovering bioactive compounds from *Moringa oleifera* leaves', *Journal of Supercritical Fluids*, 116, pp. 90–100.

Rojas, J., Londoño, C. and Ciro, Y. (2016) 'The health benefits of natural skin UVA photoprotective compounds found in botanical sources', *International Journal of Pharmacy and Pharmaceutical Sciences*, 8(3), pp. 13–23.

Rothfuss, O., Gasser, T. and Patenge, N. (2010) 'Analysis of differential DNA damage in the mitochondrial genome employing a semi-long run real-time PCR approach', *Nucleic Acids Research*, 38(4), pp. 1–10.

Roy, A., Sahu, R., Matlam, M., Deshmukh, V., Dwivedi, J. and Jha, A. (2013) 'In vitro techniques to assess the proficiency of skin care cosmetic formulations', *Pharmacognosy Reviews*, 7(14), p. 97.

Rumalla, C. S., Ali, Z. and Aruna, D. (2010) 'Two new triterpene glycosides from *Centella asiatica*', *Planta Medica*, 76, pp. 1018–1021.

Rzany, B., Becker-Wegerich, P., Bachmann, F., Erdmann, R. and Wollina, U. (2009) 'Hyaluronidase in the correction of hyaluronic acid-based fillers: a review and a recommendation for use', *Journal of Cosmetic Dermatology*, 8(4), pp. 317–323.

Sahakitpichan, P., Mahidol, C., Disadee, W., Ruchirawat, S. and Kanchanapoom, T. (2011) 'Unusual glycosides of pyrrole alkaloid and 4'-hydroxyphenylethanamide from leaves of *Moringa oleifera*', *Phytochemistry*, 72(8), pp. 791–795.

Sahasrabudhe, A. and Deodhar, M. (2010) 'Anti-hyaluronidase, anti-elastase activity of *Garcinia indica*', *International Journal of Botany*, 6(3), pp. 299–303.

Sambou Diatta, B. (2001) *Supplementation for pregnant and breast feeding women with Moringa oleifera powder*. Available at: <http://shiningseasimports.com/wp-content/uploads/2014/04/Moringa-Powder-Sambou-Diatta-Researcher.pdf> (Accessed: 12 January 2018).

Samsonowicz, M., Regulska, E., Karpowicz, D. and Le, B. (2019) 'Antioxidant properties of coffee substitutes rich in polyphenols and minerals', *Food Chemistry*, 278, pp. 101–109.

Sanchez-Machado, D. I., Lopez-Cervantes, J. . and Rios Vazquez, N. . (2006) 'High-performance liquid chromatography method to measure a- and g-tocopherol in leaves, flowers and fresh beans from *Moringa oleifera*', *Journal of Chromatography A*, 1105, pp. 111–114.

Sander, C. S., Sercu, S., Ziemer, M., Hipler, U. C., Elsner, P., Thiele, J. and Merregaert, J. (2006) 'Expression of extracellular matrix protein 1 (ECM1) in human skin is decreased by age and increased upon ultraviolet exposure', *British Journal of Dermatology*, 154(2), pp. 218–224.

Sanford, K. K. and Evans, V. J. (1982) 'A quest for the mechanism of " spontaneous " malignant transformation in culture with associated advances in culture technology', *Journal of National Cancer Institute*, 68(6), pp. 895–913.

Sanz, A. and Stefanatos, R. K. a (2008) 'The mitochondrial free radical theory of aging: a critical view', *Current Aging Science*, 1, pp. 10–21.

Sartor, L., Pezzato, E., Dell'aica, I., Caniato, R., Biggin, S. and Garbisa, S. (2002) 'Inhibition of matrix-proteases by polyphenols: chemical insights for anti-inflammatory and anti-invasion drug design', *Biochemical Pharmacology*, 64(2), pp. 229–237.

Schaich, K. M., Tian, X. and Xie, J. (2015) 'Hurdles and pitfalls in measuring antioxidant efficacy: a critical evaluation of ABTS, DPPH, and ORAC assays', *Journal of Functional Foods*, 14, pp. 111–125.

Schwarz, A., Maeda, A., Kernebeck, K., van Steeg, H., Beissert, S. and Schwarz, T. (2005) 'Prevention of UV radiation-induced immunosuppression by IL-12 is dependent on DNA repair', *Journal of Experimental Medicine*, 201(2), pp. 173–179.

Schymanski, E. L., Jeon, J., Gulde, R., Fenner, K., Ru, M., Singer, H. P. and Hollender, J. (2014) 'Identifying small molecules via high resolution mass spectrometry: communicating confidence', *Environmental Science and Technology*, 48(4), pp. 2097–2098.

Seevaratnam, V. and Banumathi, P. (2012) 'Functional properties of *Centella asiatica* (L.): a review.', *International Journal of Pharmacy and Pharmaceutical Sciences*, 4(5), pp. 8–14.

Settharaksa, S., Madaka, F., Sueree, L., Kittiwut, S., Sakunpak, A., Moton, C. and Charoenchai, L. (2014) 'Effect of solvent types on phenolic, flavonoid contents and antioxidant activities of *Syzygium gratum* (Wight) S.N', *International Journal of Pharmacy and Pharmaceutical Sciences*, 6(2), pp. 114–116.

Sharma, S. D., Meeran, S. M. and Katiyar, S. K. (2007) 'Dietary grape seed proanthocyanidins inhibit UVB-induced oxidative stress and activation of mitogen-activated protein kinases and nuclear factor- B signaling in in vivo SKH-1 hairless mice', *Molecular Cancer Therapeutics*, 6(3), pp. 995–1005.

Shay, J. W. and Wright, W. E. (2000) 'Hayflick, his limit, and cellular ageing', *Nature Reviews: Molecular Cell Biology*, 1(10), pp. 72–76.

Shehnaz Z, A. (2015) 'Chemical peels for post acne hyperpigmentation in skin of color', *Journal of Pigmentary Disorders*, 02(02), pp. 1–5.

Shin, M. S. (2015) *The study of anti-aging cosmetics of Moringa oleifera*, Master's of Oriental Medicine Cosmetic Science. Daegu Hanny University: Korea.

Shindo, Y., Witt, E. and Packer, L. (1993) 'Antioxidant defense mechanisms in murine epidermis and dermis and their responses to ultraviolet light', *Journal of Investigative*

*Dermatology*, 100(3), pp. 260–265.

Shui, G., Leong, L. P. and Wong, S. P. (2005) 'Rapid screening and characterisation of antioxidants of *Cosmos caudatus* using liquid chromatography coupled with mass spectrometry.', *Journal of Chromatography B*, 827(1), pp. 127–138.

Sin, B. Y. and Kim, H. P. (2005) 'Inhibition of collagenase by naturally-occurring flavonoids', *Archives of Pharmacal Research*, 28(10), pp. 1152–1155.

Singh, S., Singh, D. R., Banu, V. S. and Avinash, N. (2014) 'Functional constituents (micronutrients and phytochemicals) and antioxidant activity of *Centella asiatica* (L.) Urban leaves', *Industrial Crops and Products*, 61, pp. 115–119.

Sinha, R. P. and Häder, D.-P. (2002) 'UV-induced DNA damage and repair: a review', *Photochemical and Photobiological Sciences*, 1(4), pp. 225–236.

Situm, M. and Sjerobabski-Masnec, I. (2010) 'Skin aging', *Acta Clinica Croatica*, 48(3), pp. 515–518.

Sivaprabha, J., Supriya, J., Sumathi, S., Padma, P. R., Nirmaladevi, R. and Radha, P. (2008) 'A study on the levels of nonenzymic antioxidants in the leaves and flowers of *Clitoria ternatea*', *Ancient Science of Life*, 27(4), pp. 28–32.

Smith, P. R. and Thornalley, P. J. (1992) 'Mechanism of the degradation of non-enzymatically glycated proteins under physiological conditions: studies with the model fructosamine, Nε-(1-deoxy-d-fructos-1-yl)hippuryl-lysine', *European Journal of Biochemistry*, 210(3), pp. 729–739.

Snedecker, J. G. and Gautieri, A. (2014) 'The role of collagen crosslinks in ageing and diabetes: the good, the bad, and the ugly', *Muscles, Ligaments and Tendons Journal*, 4(3), pp. 303–308.

Snoek-van Beurden, P. A. M. and Von Den Hoff, J. W. (2005) 'Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors', *BioTechniques*, 38(1), pp. 73–83.

Somboonwong, J., Kankaisre, M., Tantisira, B. and Tantisira, M. H. (2012) 'Wound healing activities of different extracts of *Centella asiatica* in incision and burn wound models: an experimental animal study', *BMC Complementary and Alternative Medicine*, 12(103), pp. 1–7.

Spagnol, C. M., Oliveira, T. S., Isaac, V. L. B., Corrêa, M. A. and Salgado, H. R. N. (2015) 'Validation of caffeic acid in emulsion by UV-spectrophotometric method', *Physical Chemistry*, 5(1), pp. 16–22.

Sreelatha, S. and Padma, P. R. (2009) 'Antioxidant activity and total phenolic content of *Moringa oleifera* leaves in two stages of maturity', *Plant Foods for Human Nutrition*, 64(4), pp. 303–311.

Sridhar, K. and Linton, A. (2019) 'In vitro antioxidant activity of Kyoho grape extracts in DPPH and ABTS assays: estimation methods for EC50 using advanced statistical programs', *Food Chemistry*, 275, pp. 41–49.

Staub, P. O., Casu, L. and Leonti, M. (2016) 'Back to the roots: a quantitative survey of herbal drugs in Dioscorides? *De Materia Medica* (ex Matthioli, 1568)', *Phytomedicine*, 23(10), pp.

Steenvoorden, D. P. T. and Beijersbergen Van Henegouwen, G. M. J. (1997) 'The use of endogenous antioxidants to improve photoprotection', *Journal of Photochemistry and Photobiology B: Biology*, 41(1–2), pp. 1–10.

Sternlicht, M. and Werb, Z. (2009) 'How matrix metalloproteinases regulate cell behavior', *Annual Review of Cell and Developmental Biology*, 17(12), pp. 463–516.

Stojiljković, D., Pavlović, D. and Arsić, I. (2014) 'Oxidative stress, skin aging and antioxidant therapy', *Scientific Journal of the Faculty of Medicine*, 31(4), pp. 207–217.

Sumazian, Y., Syahida, A., Hakiman, M. and Maziah, M. (2010) 'Antioxidant activities, flavonoids, ascorbic acid and phenolic contents of Malaysian vegetables', *Journal of Medicinal Plants Research*, 4, pp. 881–890.

Sun, Y. (1990) 'Free radicals, antioxidant enzymes and carcinogenesis', *Free Radical Biology and Medicine*, 8, pp. 583–599.

Svobodová, A., Psotova, J. and Walterova, D. (2003) 'Natural phenolics in the prevention of UV-induced skin damage. A review', *Biomedical Papers*, 147(2), pp. 137–145.

Szendroi, M., Meimon, G., Bakala, H., Frances, C., Robert, L., Godeau, G. and Hornebeck, W. (1984) 'On the presence of a metalloprotease in human skin fibroblasts that degrades the human skin elastic fiber system', *Journal of Investigative Dermatology*, 83(3), pp. 224–229.

Tabassum, N., Lee, J. H., Yim, S. H., Batkhuu, G. J., Jung, D. W. and Williams, D. R. (2016) 'Isolation of 4,5-O-dicaffeoylquinic acid as a pigmentation inhibitor occurring in *Artemisia capillaris* Thunberg and its validation in vivo', *Evidence-based Complementary and Alternative Medicine*, pp. 1–11.

Tagami, H. (2008) 'Functional characteristics of the *stratum corneum* in photoaged skin in comparison with those found in intrinsic aging', *Archives of Dermatological Research*, 300(suppl.1), pp. 1–6.

Tammi, M. I., Day, A. J. and Turley, E. A. (2002) 'Hyaluronan and homeostasis: a balancing act', *Journal of Biological Chemistry*, 277(7), pp. 4581–4584.

Tang, B., Zhu, B., Liang, Y., Bi, L., Hu, Z., Chen, B., Zhang, K. and Zhu, J. (2011) 'Asiaticoside suppresses collagen expression and TGF-β/Smad signaling through inducing Smad7 and inhibiting TGF-βRI and TGF-βRII in keloid fibroblasts', *Archives of Dermatological Research*, 303(8), pp. 563–572.

Tantituvanont, a (2008) 'Preparation and stability of butterfly pea color extract loaded in microparticles prepared by spray drying', *Thai J. Pharm.*, 32, pp. 59–69.

Tanzi, E. L. and Alster, T. S. (2003) 'Single-pass carbon dioxide versus multiple-pass Er:YAG laser skin resurfacing: a comparison of postoperative wound healing and side-effect rates', *Dermatologic Surgery*, 29(1), pp. 80–84.

Terahara, N. (1998) 'Eight new anthocyanins, ternatins C1–C5 and D3 and preternatins A3 and

C4 from young *Clitoria ternatea* flowers', *Journal of Natural Products*, 61(11), pp. 1361–1367.

Thring, T. S. A., Hili, P. and Naughton, D. P. (2009) 'Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants.', *BMC Complementary and Alternative Medicine*, 9(27), p. 11.

Tosato, M., Zamboni, V., Ferrini, A. and Cesari, M. (2007) 'The aging process and potential interventions to extent life expectancy', *Clinical Interventions in Aging*, 2(3), pp. 401–12.

Townshend, A. (2016) 'Blindness after facial injection', *Journal of Clinical and Aesthetic Dermatology*, 9(12), pp. E5–E7.

Trenam, C. W. ., Blake, D. R. . and Morris, C. J. (1992) 'Skin inflammation: reactive oxygen species and the role of iron', *Journal of Investigative Dermatology*, 99, pp. 675–682.

Tsuji, N., Moriwaki, S., Suzuki, Y., Takema, Y. and Imokawa, G. (2001) 'The role of elastases secreted by fibroblasts in wrinkle formation: implication through selective inhibition of elastase activity.', *Photochemistry and photobiology*, 74(2), pp. 283–90.

Turrens, J. F. (2003) 'Mitochondrial formation of reactive oxygen species', *Journal of Physiology*, 552(2), pp. 335–344.

U.S. Department of Agriculture (2016) *Dr . Duke's Phytochemical and ethnobotanical databases*. Available at: <https://phytochem.nal.usda.gov/phytochem/search> (Accessed: 12 January 2018).

US Deparment of Commerce (2015) *Cosmetics & Toiletries Market Overviews 2015*, U.S. Commercial Service. Available at: [http://www.trade.gov/industry/materials/ITA.FSC.Cosmoprof.2015\\_final2.pdf](http://www.trade.gov/industry/materials/ITA.FSC.Cosmoprof.2015_final2.pdf) (Accessed: 18 June 2017).

Vardhan, A., Khan, S. and Pandey, B. (2014) 'Screening of plant parts for anti-tyrosinase activity by tyrosinase assay using mushroom tyrosinase', *Indian Journal of Science Research*, 4(1), pp. 134–139.

Vauzour, D., Rodriguez-Mateos, A., Corona, G., Oruna-Concha, M. J. and Spencer, J. P. E. (2010) 'Polyphenols and human health: prevention of disease and mechanisms of action', *Nutrients*, 2(11), pp. 1106–1131.

Velasco, M. V. R., Okubo, F. R., Robeiro, maria E., Steiner, D. and Bedin, V. (2004) 'Facial skin rejuvenation by chemical peeling: focus on phenol peeling', *Brazilian Annals of Dermatology*, 79(1), pp. 91–99.

Da Violante, G. *et al.* (2002) 'Evaluation of the cytotoxicity effect of dimethyl sulfoxide (DMSO) on Caco2/TC7 colon tumor cell cultures', *Biological and Pharmaceutical Bulletin*, 25(12), pp. 1600–1603.

Visse, R. and Nagase, H. (2003) 'Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry', *Circulation Research*, 92(5), pp. 827–839.

Di Vittori, L., Mazzoni, L., Battino, M. and Mezzetti, B. (2018) 'Pre-harvest factors influencing the quality of berries', *Scientia Horticulturae*, 233(1), pp. 310–322.

Vongsak, B., Sithisarn, P. and Gritsanapan, W. (2013) 'Simultaneous determination of cryptochlorogenic acid, isoquercetin, and astragalin contents in *Moringa oleifera* leaf extracts by TLC-densitometric method', *Evidence-based Complementary and Alternative Medicine*, 2013(1), pp. 1–7.

Vongsak, B., Sithisarn, P. and Gritsanapan, W. (2014) 'Simultaneous HPLC quantitative analysis of active compounds in leaves of *Moringa oleifera* Lam.', *Journal of Chromatographic Science*, 52(7), pp. 641–645.

Vongsak, B., Sithisarn, P., Mangmool, S., Thongpraditchote, S., Wongkrajang, Y. and Gritsanapan, W. (2013) 'Maximizing total phenolics, total flavonoids contents and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method', *Industrial Crops and Products*, 44, pp. 566–571.

Wang, F., Garza, L. A., Kang, S., Varani, J., Orringer, J. S., Fisher, G. J. and Voorhees, J. J. (2007) 'In vivo stimulation of de novo collagen production caused by cross-linked hyaluronic acid dermal filler injections in photodamaged human skin', *Archives of Dermatology*, 143(2), pp. 155–163.

Wang, P., Henning, S. M. and Heber, D. (2010) 'Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols', *PLoS ONE*, 5(4), pp. 1–10.

Van Wart, H. E. . and Steinbrink, R. (1981) 'A continuous spectrophotometric assay for *Clostridium histolyticum* collagenase', *Analytical Biochemistry*, 113, pp. 356–365.

Watson, J. D. (1972) 'Origin of concatemeric T7DNA', *Nature New Biology*, 239(10), pp. 197–201.

Watthanaudomchai, A., Chayopas, P. and Sutthanut, K. (2012) 'The 4th Annual Northeast Pharmacy Research Conference of 2012 "Pharmacy Profession in Harmony"', in *Matrix metalloproteinase-2 and -9 inhibitory effects of 5 different Thai herbal extracts*, p. 279.

Wei, L., Zhang, W., Yin, L., Yan, F., Xu, Y. and Chen, F. (2015) 'Extraction optimization of total triterpenoids from *Jatropha curcas* leaves using response surface methodology and evaluations of their antimicrobial and antioxidant capacities', *Electronic Journal of Biotechnology*, 18(2), pp. 88–95.

Wijeratne, S. S. K., Cuppett, S. L. and Schlegel, V. (2005) 'Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in Caco-2 human colon cells', *Journal of Agricultural and Food Chemistry*, 53(22), pp. 8768–8774.

Wilson, N. (2008) 'Market evolution of topical anti-aging treatments', in *Skin aging Handbook; An Integrated Approach to Biochemistry and Product Development*. New York: William Andrew Inc., pp. 15–31.

Winterbourn, C. C. (1995) 'Toxicity of iron and hydrogen peroxide: the Fenton reaction', *Toxicology Letters*, 82–83(C), pp. 969–974.

Winther, R. G. (2001) 'August Weismann on germ-plasm variation', *Journal of the History of Biology*, 34, pp. 517–555.

Wu, F., Bian, D., Xia, Y., Gong, Z., Tan, Q., Chen, J. and Dai, Y. (2012) 'Identification of major active ingredients responsible for burn wound healing of *Centella asiatica* herbs', *Evidence-based Complementary and Alternative Medicine*, pp. 1–13.

Wu, S., Gao, J., Dinh, Q. T., Chen, C. and Fimmel, S. (2008) 'IL-8 production and AP-1 transactivation induced by UVA in human keratinocytes: roles of D- $\alpha$ -tocopherol', *Molecular Immunology*, 45(8), pp. 2288–2296.

Yang, Y. M., Son, Y. O., Lee, S. A., Jeon, Y. M. and Lee, J. C. (2011) 'Quercetin inhibits alpha-MSH-stimulated melanogenesis in B16F10 melanoma cells', *Phytotherapy Research*, 25(8), pp. 1166–1173.

Yap, F. H. X., Chua, H. C. and Tait, C. P. (2017) 'Active sunscreen ingredients in Australia', *Australasian Journal of Dermatology*, 58(4), pp. e160–e170.

Yimdee, J., Uabundit, N., Chaisiwamolkol, K., Iamsaard, S., Hipkaeo, W. and Wattanathorn, J. (2014) 'Evaluation of cognitive enhancing effects of *Clitoria ternatea* flowers water extract in normal male mice', *Asian-Pacific Journal of Science and Technology*, 14(3), pp. 59–66.

Young, A. R. (1997) 'Chromophores in human skin', *Physics in Medicine and Biology*, 42(5), pp. 789–802.

Zahara, K., Bibi, Y. and Tabassum, S. (2014) 'Clinical and therapeutic benefits of *Centella asiatica*', *Pure and Applied Biology*, 3(4), pp. 152–159.

Zainol, M. K. ., Abd-Hamid, A. ., Yusof, S. . and Muse, R. (2003) 'Antioxidative activity and total phenolic compounds of leaf, root and petiole of four accessions of *Centella asiatica* (L.) Urban', *Food Chemistry*, 81, pp. 575–581.

Zeitoun, H., Khoury, R. El, Beyrouthy, M. El, Salameh, D. and Lteif, R. (2016) 'Phytochemicals screening and anti-tyrosinase activity of Senegalese herbal extracts', *International Journal of Innovative Research in Science, Engineering and Technology*, 5(11), pp. 19781–19789.

Zillich, O. V., Schweiggert-Weisz, U., Eisner, P. and Kerscher, M. (2015) 'Polyphenols as active ingredients for cosmetic products', *International Journal of Cosmetic Science*, 37(5), pp. 455–464.

