EVALUATION OF THE ANTI-OXIDANT AND ANTI-AGING PROPERTIES OF EXTRACTS FROM FOUR TYPES OF PLANTS

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EVALUATION OF THE ANTI-OXIDANT AND ANTI-AGING PROPERTIES OF EXTRACTS FROM FOUR TYPES OF PLANTS

by

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LIST OF SYMBOLS, ABBREVIATION OR NOMENCLATURE

Abbreviations	Full description
$ABTS^{+}$	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate) radical cation
AP-1	Activator protein 1
AHA	Alpha hydroxyl acid
ANOVA	Analysis of variance
BROD	Benzyloxy-resorufin-*O*-dealkylase
BHT	Butylated hydroxytoluene
CAT	Catalase
CUPRAC	Cupric reducing antioxidant capacity
CYP1A	Subfamily of Cytochrome liver CYP450
CYP2B9/10	Subfamily of Cytochrome liver CYP450
CO_2	Carbon dioxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
DNA	Deoxyribonucleic acid
EC_{50}	Concentration of a drug that gives half-maximal response
ECM	Extracellular matrix
EDTA	Ethylenediamenetetraacetate
EGC	(-)-epicatechin-3-gallate
EGCG	(-)-epigallocatechin
EIA	Enzyme immunossay
ELISA	Enzyme-linked immunosorbent assay
EROD	Ethoxy-resorufin-*O*-dealkylase
EUP	Eupatorin

FBS	Fetal bovine serum
FTC	Ferric thiocyanate
FRAP	Ferric reducing antioxidant power
GAG's	Glycosaminoglycans
GPx	Glutathione peroxidase
GR	Slutathione reductase
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
HDFa	Adult human dermal fibroblasts
HPLC	High performance liquid chromatography
HSD	Tukey's honestly significant difference
HUVEC	Human umbilical vein endothelial cells
IC_{50}	Concentration giving 50% inhibition
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
L1	Larval stage 1
L3/4	Larval stage 3/4
L4	Larval stage 4
LB	Luria broth
MDI 301	picolinic acid-substituted ester of 9-cis retinoic acid.
MBC	Minimum bactericidal concentration
MDA	Malondialdehyde
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase

MMP-1	Matrix metalloproteinase-1 / collagenase
MMP-2	Matrix metalloproteinase-2 / 72-kd gelantinase
MMP-3	Matrix metalloproteinase-3 / stromelysin 1
MMP-9	Matrix metalloproteinase-9/ 92-kd gelatinase
mRNA	Messenger ribonucleic acid
MnSOD	Manganese-containing superoxide dismutase
MNPEs	Micronucleated polychromatic erythrocytes
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Na_2HPO_4	Disodium phosphate
N2	*C. elegans* strain, wild-type Bristol
NCE	Norchromatic erythrocytes
NGM	Nematode growth medium
NF-κB	Nuclear factor-κB
OS	*Orthosiphon stamineus*
OP50	*E. coli* strain
ORAC	Oxygen radical absorbance capacity
PAC	Proanthocyanins
PBS	Phosphate buffer
PCE	Polychromatic
PD	Population doubling
PDT	Population doubling time
PICP	Procollagen I C-peptide
POD	Peroxidase
RA	Rosmarinic acid

RARs	Retinoic acid receptors
RXRs	Retinoid X receptors
ROS	Reactive oxygen species
SEN	Sinensetin
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TEAC	Trolox equivalent antioxidant capacity
TLC	Thin layer chromatography
TIMPs	Tissue inhibitors of metalloproteinase's
TMF	3'-hydroxy-5,6,7,4'-tetramethoxyflavone
TNF	Tumor necrosis factor
TRAP	Total radical trapping antioxidant
TNF-α	Tumor necrosis factor
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
Z_{Ave}	Z average diameter value

PENILAIAN CIRI-CIRI ANTI-OKSIDA DAN ANTI-PENUAAN EKSTRAK DARIPADA EMPAT JENIS TUMBUH-TUMBUHAN

ABSTRAK

Ciri-ciri anti-penuaan ekstrak daripada empat jenis tumbuh-tumbuhan yang berbeza, iaitu *O. stamineus*, *C. hirta*, *P. sarmentosum* dan *C. caudatus* telah disiasat. Kaedah pengekstrakan yang mudah telah dijalankan sebelum analisis yang seterusnya. Asai DPPH menunjukkan bahawa ekstrak etanol *C. hirta* menunjukkan aktiviti memerangkap radikal yang tertinggi, dengan IC_{50} 33.3±1.8µg/ml manakala ekstrak etanol *P. sarmentosum* menunjukkan aktiviti memerangkap radikal yang terendah, dengan IC_{50} 660.4±14.4µg/ml. Toksisiti ekstrak tumbuh-tumbuhan telah diuji dengan menggunakan sel-sel fibroblast kulit manusia dewasa. *O. stamineus* dan *C. caudatus* adalah toksik kepada sel-sel pada kepekatan 100µg/ml. Kesan-kesan daripada ekstrak tumbuh-tumbuhan terhadap prokolagen I telah diuji dan didapati bahawa *O. stamineus*, *C. hirta* dan *C. caudatus* meningkatkan ekspresi PICP apabila diuji pada kepekatan 50, 100, 250 dan 500ng/ml. Untuk kesan perlindungan ekstrak tumbuh-tumbuhan pada kepekatan 50, 100, 250 dan 500ng/ml terhadap dos sublethal hidrogen peroksida pada 750µg/ml, hanya 50ng/ml *O. stamineus* menunjukkan perlindungan terhadap tekanan oksidatif. Ekstrak tumbuh-tumbuhan telah disiasat selanjutnya dengan menggunakan *C. elegans* sebagai model organisma in vivo. Nematod telah diradiasi dengan ultraungu B selama 3 minit dan dirawat dengan ekstrak tumbuh-tumbuhan pada kepekatan 1, 10 dan 100µg/ml, *O. stamineus* dan *P. sarmentosum* pada kepekatan 10µg/ml meningkatkan jangka hayat purata kepada 7.0±0.3 dan 6.9± 0.2 hari berbanding dengan 6.2±0.1 hari untuk nematod yang tidak dirawati. Liposome digunakan sebagai pembawa untuk menyampaikan *O. stamineus* kepada *C. elegans* untuk mengkaji keberkesanannya dalam menyampaikan jumlah

ekstrak yang konsisten kepada nematod. Walaupun kecekapan pengkapsulan yang tinggi telah diperolehi, tetapi formulasi liposom tidak memanjangkan jangka hayat *C. elegans*. Oleh itu adalah penting untuk menguji keberkesanan ekstrak tumbuh-tumbuhan secara in vivo dan in vitro untuk kajian anti-penuaan kerana penuaan kulit merupakan satu proses biologi yang kompleks.

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ABSTRACT

The anti-aging properties of extracts from four different plants, namely *O. stamineus*, *C. hirta*, *P. sarmentosum* and *C. caudatus* were investigated. A simple extraction method was carried out before further analysis. DPPH assay indicated that ethanol extract of *C. hirta* showed the highest radical scavenging activities, with IC_{50} of 33.3 ± 1.8 µg/ml whilst ethanol extract of *P. sarmentosum* showed the lowest radical scavenging activities, with IC_{50} of 660.4 ± 14.4 µg/ml. Toxicity of plant extracts was tested on adult Human Dermal Fibroblast (HDFa). *O. stamineus* and *C. caudatus* were toxic to cells at the concentration of 100µg/ml. The effects of the plant extracts on Type I pro-collagen production were tested and it was found that *O. stamineus*, *C. hirta* and *C. caudatus* accelerated PICP expressions when tested at the concentrations of 50, 100, 250 and 500ng/ml. For the cytoprotection effects of plant extracts at the concentrations of 50, 100, 250 and 500ng/ml towards the sub-lethal dose of hydrogen peroxide at 750µg/ml, only 50ng/ml of *O. stamineus* showed protection against the oxidative stress. Plant extracts were further investigated by using *C. elegans* as the model in vivo organism. The nematodes were irradiated with ultraviolet B for 3 minutes and treated with plant extracts at the concentrations of 1, 10 and 100µg/ml. *O. stamineus* and *P. sarmentosum* at the concentration of 10µg/ml increased the mean lifespan to 7.0 ± 0.3 and 6.9 ± 0.2 days respectively compared to 6.2 ± 0.1 days for untreated nematodes. Liposome was used as a carrier to deliver *O. stamineus* to *C. elegans* to study its efficacy in delivering consistent amounts of the extract to the nematodes. Even though high encapsulation efficiency was obtained, the liposomal formulation did not prolong the lifespan of the *C. elegans*. Therefore it is important

to test the efficacy of plant extracts both in vivo and in vitro for anti-aging studies as skin aging is a complex biological process.

CHAPTER 1

INTRODUCTION

1.1 Chronological aging and photoaging

Aging is a complex process involving progressive physiological changes that eventually lead to senescence in an organism, resulting in the decline of biological functions and the organism's ability to adapt to metabolic stress (Rabe *et al*., 2006).

Human skin undergoes two different types of aging; intrinsic and extrinsic aging. Intrinsic aging or chronological aging is a genetically programmed aging which depends on the passage of time while extrinsic aging is caused by environmental factors such as ultraviolet rays, harsh weather, pollution and cigarette smoke (McCullough and Kelly, 2006; Tigges *et al*., 2014). Chronological skin aging is characterized by fine wrinkles, reduced elasticity and paleness. The main factor contributing to human skin aging is continuous exposure to ultraviolet irradiation from the sun, resulting in photoaging. Photoaging is a cumulative process superimposed on intrinsically aging skin. It causes the skin to age prematurely and affects more severely individuals with lighter skin colour. Photoaged skin has uneven tone, leathery appearance, is coarsely wrinkled and affected with telangiectasia. Photoaged skin is also associated with increased development of benign and malignant neoplasms (Rabe *et al*, 2006). Solar ultraviolet (UV) rays, consisting of UVA (wavelength 320 – 400nm) and UVB (wavelength 290 – 320nm) radiation cause photoaging. Both UVA and UVB exert significant impairment to the skin, with UVA penetrating more deeply into the dermis layer while UVB is completely absorbed in the epidermis layer (Sjerobabski-Masnec and Šitum, 2010). In addition

to that, the UVA is 10-fold more abundant in sunlight compared to UVB (Zussman *et al*., 2010).

Ultraviolet radiation induces the formation of reactive oxygen species (ROS) in the photoaged skin. Reactive oxygen species are toxic to cells and damage macromolecules such as lipids, protein and cellular DNA (Wu and Cederbaum, 2003; Rabe *et al*., 2006).

1.2 Ultraviolet radiation and reactive oxygen species

Reactive oxygen species are generated in small amounts during mitochondrial metabolism. The hypothesis that reactive oxygen species lead to aging process was popularised by Harman (1956; Harman, 2006). Oxygen with reduction of one or two electrons produces reactive oxygen species such as superoxide radicals and peroxide radicals (Clancy and Birdsall, 2013; Lushchak, 2014). The imbalance between reactive oxygen species production and removal results in a state known as oxidative stress (Wu and Cederbaum, 2003). Reactive oxygen species cause DNA damage, such as mutations, degradation, deletions, single-strand breakage and rearrangements (Meng *et al*., 2009). Reactive oxygen species increase signal transduction pathways, leading to inhibition of protein-tyrosine phosphatase and finally up-regulation of nuclear transcription factor activator protein 1 (AP-1) and nuclear factor-κB (NF-κB). AP-1 is composed of c-Jun and c-Fos proteins, which regulates the transcription of genes for matrix metalloproteinase (MMP). The MMPs are a large group of proteolytic enzymes responsible for the degradation of collagen, elastin and other proteins in the extracellular matrix (Naylor *et al*., 2011). MMPs which are involved in matrix degradation include MMP-1 (collagenase), MMP-2 (72-kd gelantinase),

MMP-3 (stromelysin 1), and MMP-9 (92-kd gelatinase). For example, MMP-1 cleaves collagen type I, II and III whereas MMP-9 cleaves collagen type IV, V and gelatins (Krutman, 2009). AP-1 which is activated by the presence of reactive oxygen species will increase the transcription of MMP and consequently suppress type I collagen synthesis. Activation of NF-κB stimulate transcription of pro-inflammatory cytokines including interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor (TNF-α). Both AP-1 and NF-κB are triggered at low dose of ultraviolet radiation through an iron dependent mechanism (Rabe *et al*, 2006). The increase in expression of MMPs and decrease in the collagen synthesis lead to the reduction in the extracellular matrix.

Elastin decreases with age; however it is induced upon ultraviolet radiation (Lewis *et al*., 2004; Rabe *et al*., 2006). Elastin makes up about 2 – 4% of skin (Bernstein *et al*., 1994; Daamen *et al*., 2007) and is found in the upper and middle dermis layers. The function of elastin is to provide elasticity and resiliency to the skin. Normal elastic fibres network is disrupted in photodamaged skin causing accumulation of amorphous, abnormal elastin containing material (Knott *et al*., 2009). Solar elastosis is used to describe the elastin containing material. Solar elastosis is the histologic feature of photoaging and stains strongly with elastin-specific Verhoeff van Gieson stain. In addition to that, fibrillin microfibrills is the major component of elastic fibres (Kielty *et al*., 2002). Fibrillin made up of a group of three proteins, fibrillin-1, fibrillin-2 and fibrillin-3. Mutation in fibrillin-1 causes Marfan syndrome, a connective tissue disorder, which is associated with ocular, skeletal and cardiovascular defects (Kielty *et al*., 2005). Melanocytes are present in the epidermis

and function to produce melanin. Melanin is responsible for the skin colour and provides the protection against ultraviolet radiation.

1.3 Enzymatic and non-enzymatic antioxidants

The disturbance in the redox system can be counteracted by skin via a network of antioxidant systems. There are two important antioxidant activities in human body, namely enzymatic and non-enzymatic systems. The enzymatic antioxidants include glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT). Non-enzymatic antioxidants can be divided into lipophilic antioxidants (α-tocopherol, ubiquinol and ubiquinone) and hydrophilic antioxidants (ascorbic acid, dehydroascorbic acid, and glutathione). Generally, both enzymatic and non-enzymatic antioxidants are higher in epidermis than dermis (Pandel *et al*., 2013).

The function of superoxide dismutase is to convert superoxide radicals rapidly to form hydrogen peroxide while catalase converts hydrogen peroxide into water (Sander *et al*., 2002). There are various types of superoxide dismutase in human body, e.g. copper-zinc superoxide dismutase can be found in cytosol while manganese-containing superoxide dismutase (MnSOD) is presented in mitochondria. In a study carried out by Okada *et al* (1994) (Pandel *et al*., 2013), the responses of enzymatic and non-enzymatic antioxidants in epidermis and dermis of hairless mice towards solar light were compared. After irradiation, losses of catalase and superoxide dismutase activities were observed. α-tocopherol, ubiquinol 9, ubiquinone 9, ascorbic acid, dehydroxyascorbic acid and glutathione reductase activities were reduced by 26 – 93% in both epidermal and dermal layers. However

oxidised glutathione did not increase significantly. Based on the result obtained, they concluded that ultraviolet light is less damaging to the antioxidants in dermal layer (Pandel *et al*., 2013). The study of Poswig *et al* (1999) (Godic *et al*., 2014) performed repetitive exposure of UVA onto the skin. UVA irradiation increased the induction of MnSOD. The adaptive response of MnSOD enzyme contributed to the protective response of the skin during light hardening in phototherapy of various photodermatoses. In another study done by Sander *et al* (2002), it was shown that chronic and acute ultraviolet light exposure decrease the antioxidant enzyme expression and increase oxidative protein damage in the epidermis layer. They also concluded that the antioxidant enzymes are low in dermal layer. Tissues were taken from photodamaged skin with histologically confirmed solar elastosis, non UV-exposure skin as control and young skin without disease. All of the antioxidant enzymes within the stratum corneum were reduced in highly ultraviolet-exposed human skin if compared to young controls and intrinsically aged skin.

1.4 Antioxidants

1.4.1 Vitamin A

The treatment of photoaged skin can be categorised into primary, secondary and tertiary treatments. The two main forms of vitamin A found in nature are retinols and carotenoids. Retinols are found in animal food source and are the most biologically active form of vitamin A (Zussman *et al*., 2010). Carotenoids are found in fruits and vegetables and also shown to possess strong antioxidant capabilities. The common forms of carotenoids include β-carotene, α-carotene and β-cryptoxanthin (Zussman *et al*., 2010). Retinoic acid, a metabolite of vitamin A, exerts its effects on the nuclear receptor families by binding to the receptor, i.e. retinoic acid receptors (RARs) and

retinoid X receptors (RXRs). Ultraviolet radiation reduces the expression of nuclear retinoid receptors RAR-γ and RXR-α in vivo. As a result, ultraviolet irradiation causes vitamin A deficiency in the skin. However, pre-treatment with retinoic acid reduces the loss of RAR/RXR receptors (Wang *et al*.,1999; Karlsson *et al*., 2004).

Retinoic acid pre-treatment in advance was demonstrated in another study by using human fibroblast cells in vitro (Fisher *et al*., 2000). The exposure of ultraviolet irradiation was found to activate c-Jun protein which inhibited type I and III procollagen expression. However, human skin pretreated with 0.1% of all-trans-retinoic acid for 24 hours protected skin cells against loss of type I and III procollagen mRNA and protein by inhibition of ultraviolet-induced c-Jun protein (Fisher *et al*., 2000).

In another in vitro study performed by other group (Varani *et al*., 1998; Varani *et al*., 2001), retinoic acid stimulated the keratinocyte and fibroblast cells growth in monolayer culture, irrespective of the origin of cells isolated from sun-protected or sun-exposed sites. This implied that retinoic acid may counteract the effect of photodamaged cells as well as intrinsic aging. Although treatment with retinoic acid seems promising, use of this compound is known to be associated with several side effects. Treatment with retinoic acid may cause irritation, redness, peeling and burning of the skin (Zussmann *et al*., 2010).

On the other hand, MDI 301, a picolinic-acid-substituted 9-cis, a non-irritating retinoic acid ester was used to treat diabetic patients with foot ulcer. MDI 301 is reported to be less irritating than retinoic acid (Varani *et al*., 2003; Appleyard *et al*.,

2004) and pre-treatment with MDI 301 on hairless mice enhanced the wound closure time (Varani *et al*, 2002; Warner *et al*., 2008). Effects of MDI 301 and retinoic acid were evaluated on skin structure, MMP and procollagen expression (Zeng *et al*., 2011). Initially the percentage of MMP's activity was higher and tissue inhibitors of metalloproteinase's (TIMP's) activity was lower in diabetic subjects, type I procollagen synthesis was decreased and skin structure was deficit at the early stage of the study. However, 3 μM of MDI 301 was found to significantly reduce the activity of MMP-1 and MMP-9 by 29% and 40% respectively, while at the same time increase TIMP activity by 45%. In addition, MDI 301 also increased type I procollagen synthesis and was able to repair skin structure. 2 μM of retinoic acid did not affect skin structure significantly but was found to reduce MMP-1 activity (Zeng *et al*., 2011).

1.4.2 Vitamin E

Vitamin E provides protection against adverse conditions such as photoaging and is the major lipid-soluble antioxidants agent in human skin. The term vitamin E comprises both tocopherols and tocotrienols derivatives. Tocopherols and tocotrienols are further categorised into α, β, δ and γ, according to the position and number of methyl substitution on the chromanol ring. The antioxidant capability of the vitamin E depends on their ability to donate phenolic hydrogen to lipid free radicals (Kamal-Eldin and Appelqvist, 1996; Blokhina *et al*., 2003). Vitamin E can be found in food sources such as vegetable oils, nuts and green leafy vegetables.

Vitamin E has been recommended for the treatment of various skin disorders, such as yellow nail syndrome, prevention of the scar formation, melasma and atopic

dermatitis, although the supportive data is insufficient (Thiele *et al*., 2005). Nevertheless, oral supplementation of vitamin E may aid in chronic wound healing (Thiele *et al*., 2005). There are several studies indicating that dietary deficiency in vitamin E leads to an increase in reactive oxygen species and further accelerates skin aging (Nachbar and Korting, 1995; Stojiljković *et al*., 2014).

Although tocopherols and tocotrienols are closely related, it has been reported that these different forms of vitamin E showed varying biological activities. Tocotrienols have shown to possess unique biological activities when compared to tocopherols. The beneficial effects of tocotrienols and tocopherols on cell damage caused by oxidative stress were evaluated. Tocotrienols proved to be a better inhibitor compared to tocopherols against selenium deficiency-induced cell death (Saito *et al*., 2003; Saito *et al*., 2010).

In a study by Adachi and Ishii (2000), tocotrienols was found to increase the mean lifespan, but not maximum lifespan of the nematode *C. elegans*. In addition to that, pre-administration and post-administation of tocotrienols protected *C. elegans* from UVB irradiation. This protective effect was not observed with α-tocopherol acetate (Adachi and Ishii, 2000).

1.5 Plants of interest

Malaysia is blessed with vast green tropical vegetation and forest. The biodiversity of plants in Malaysia warrants investigation in view of the vast varieties and many of them are known in folklore to possess medicinal values. It is a normal practice for Malaysians to seek herbal and traditional remedies as an alternative treatment for disease or as tonic to maintain one's health.

In addition to that, polyphenols being the secondary metabolite in the plant are generally involved in defence against ultraviolet radiation. They possess several beneficial biological properties, such as antioxidant, anti-aging, anti-inflammation, anti-apoptotic, anti-artherosclerosis, anti-apoptotic, cardiovascular protection and improvement of the endothelial function (Chanudom *et al*., 2014). Thus, plants with high polyphenols content were chosen in this study.

1.5.1 *Orthosiphon stamineus*

Orthosiphon stamineus or locally known as 'misai kucing' has been studied extensively. *O. stamineus*, Benth, belongs to the family of Lamiaceae and is used for the treatment of epilepsy, gallstone, hepatitis, rheumatism, syphilis, eruptive fever and renal calculus (Akowuah *et al*., 2004). In Malaysia, *O. stamineus* is consumed as tea to improve health and treatment of diabetes, kidney, gall inflammation and gout. Bioactive compounds such as sterols, phenolic compounds and terpenoids can be found in *O. stamineus* (Tezuka *et al*., 2000; Chan and Loo, 2006). Among all these bioactive components, phenolic compounds are one of the most important compounds and closely correlated with the antioxidant property in *O. stamineus* crude extract (Khamsiah *et al*., 2006).

Leaves of *O. stamineus* are reported to possess the highest antioxidant properties compared to other parts of the plant attributed to its polyphenol (Akowuah *et al*., 2005; Farhan *et al*., 2012). The main polyphenols in *O. stamineus* leaves are sinensetin, eupatorin and 3'-hydroxy-5 6 7 4'-tetramethoxyflavone and caffeic acid derivatives such as rosmarinic acid (Alshawsh *et al*., 2012).

Antioxidant and antibacterial properties of methanol extract of *O. stamineus* were investigated by Ho *et al*., 2010. Whole parts of the *O. stamineus* were extracted at different concentrations 0%, 25%, 50%, 75% and 100% of methanol. Antimicrobial activity of the methanol extracts of *O. stamineus* against 9 species of bacteria was determined by using disc diffusion test. *O. stamineus* extracted with 50% and 75% methanol showed strong inhibition against *Vibrio parahaemolyticus*, a type of bacteria that causes mild gastroenteritis in human upon the consumption of infected seafood. The high antibacterial property was due to the high rosmarinic acid content in both extracts as determined by HPLC.

Acute toxicity of standardised extract of *O. stamineus* was evaluated in Sprague-Dawley rats. Male rats of 8 weeks old were chosen and administered as a single dose of 5000 mg/kg body weight orally on day 0. There were no deaths recorded and no signs of toxicity observed during the 14 days of experimental period. There were no behavioural changes such as body weight, food and water intake. The results of relative organ weight did not differ significantly from the control group. Hence, the oral acute toxicity of *O. stamineus* was expected to be higher than 5000 mg/kg of body weight (Abdullah *et al*., 2009).

Other than oral acute toxicity, genotoxicity of *O. stamineus* was evaluated as well by Muhammad *et al*. (2011). Aqueous extract of *O. stamineus* up to concentration of 5000 µg/plate did not show toxicity towards *Salmonella* tester strains and the number of revertant colonies over the background incidence was not increased. In the mouse bone marrow assay, the aqueous extract of *O. stamineus* did not change the polychromatic:normochromatic erythrocytes (PCE:NCE) ratio nor increase the incidence of micronucleated polychromatic erythrocytes (MNPEs). Moreover, no toxicity, myelotoxicity nor changes in liver cytochrome CYP1A (EROD) and 2B9/10 (BROD) activity were observed.

Besides, the diuretic and hypouricemic effects of the methanol extract of *O. stamineus* on Sprague-Dawley rats were studied (Arafat *et al*., 2008). Methanol and methanol water (1:1) leaves extracts of *O. stamineus* at 0.5g/kg were administered consecutively for a week. In addition to that, hypouricemic effect of methanol:water extract at various concentrations (0.25, 0.5, 1 and 2g/kg) were tested and allopurinol was used as the positive control (Arafat *et al*., 2008). A single dose of the extract (2g/kg) increased the sodium and potassium excretion significantly in the first 8 hours of the treatment. On the other hand, 0.5, 1 and 2g/kg of methanol:water extracts and allopurinol decreased the serum urate level in hyperuricemic rats 6 hours later after the extract was administered. The results suggested that methanol:water extract was potent as a diuretic and a hypouricemic agent in rats (Arafat *et al*., 2008).

1.5.2 *Clidemia hirta*

Clidemia hirta, known as 'senduduk bulu' locally, comes from the family of Melastomataceae. It is also known as Koster's curse (a Hawaiian name), which is a kind of weed that is commonly found in disturbed areas, such as landslides, old fields, plantations, roadsides, river banks, tree tips mounds, burned areas, fence rows and pastures. *C. hirta* is native to southern Mexico, South and North America, Northern Argentina and Bolivia (Stevens *et al*., 2001) and subtropics to Peninsular Malaysia, Hawaii and Fiji (Wester and Wood, 1977; Peters, 2005).

Methanol extract of *C. hirta* showed antibacterial property against 10 bacterial isolates (Musa *et al*., 2011). The crude extract displayed the best MIC (minimum inhibitory concentration) and minimum bactericidal concentration (MBC) against *Vibrio anginolyticus*, with the values of 0.39mg/ml and 6.25mg/ml respectively. Scanning Electron Microscope analysis showed that methanol extract of *C. hirta* at the concentration of 0.195mg/ml disrupted the cell surface and was able to inhibit the growth of *V. anginolyticus*. When thin layer chromatography plates were sprayed with 2,2-diphenyl-1-picryhydrazyl (DPPH), *C. hirta* extract showed potent radical scavenging activities indicated by colour changes from purple to yellow. The phytochemical test of methanol extract of *C. hirta* showed positive results for flavonoids, tannins, terpenoids and saponins while negative result for the presence of steroid. Tannins and flavonoids were antioxidants which act as free radical scavenger (Ayoola *et al*., 2008). Besides antioxidant, tannins in *C. hirta* also displayed antibacterial property (Chopra *et al*., 2007).

C. hirta is used to treat *Leishmania braziliensis* skin infections in Brazil (Franca *et al*., 1996; Braga *et al*., 2007). This species appeared as a serious weed in topical plantations such as rubber. *C. hirta* rebuilds disturbed areas and provides food for wildlife. The chemical compound hydrolysable tannins are toxic to goats (Murdiati *et al*., 1990; Hervás *et al*., 2003). Sheep was found to control most of the weed in plantations but will not consume *C. hirta* (Chee and Faiz, 2002).

1.5.3 *Piper sarmentosum*

P. sarmentosum, known as 'Kaduk' locally, is herbaceous plant belonging to the *Piperaceae* family, which can be found easily in tropical and subtropical region. The leaves are cordate, simple and alternate, 7 – 15cm wide and 5 – 10cm long. The phytochemical compounds in the plant are flavonoids, alkaloids, phenols, vitamin C, vitamin E, tannins and xantophylls (Chanwitheesuk *et al*., 2005, Hussain *et al*., 2009). Antioxidant activity of *P. sarmentosum* was attributed to the presence of these chemical compounds, especially xantophylls and vitamin E.

P. sarmentosum was shown to have pharmacological activities by several research groups. Various kinds of different extraction had been done, such as chloroform, ethanol, methanol and aqueous extraction on the plant. Myricetin, quercetin and apigenin were found in the aqueous-methanol extract of *P. sarmentosum* (Miean and Mohamed, 2001). Methanol extract of *P. sarmentosum* was found to contain naringenin (Subramaniam *et al*., 2003).

In Malaysia, *P. sarmentosum* has been used to treat diabetes mellitus and hypertension. In Thailand, *P. sarmentosum* is known as "Chaplu" locally and and water extract of the plant has been traditionally used to treat diabetic patients.

Three different extracts of *P. sarmentosum*, aqueous, methanol and hexane were used to test on hydrogen peroxide induced human umbilical vein endothelial cells (HUVEC). All the concentrations of *P. sarmentosum* were potent in decreasing the levels of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in hydrogen peroxide induced HUVECs (Hafizah *et al*., 2010). They proposed that extract of *P. sarmentosum* exhibited the protective effect on membrane damage by penetrating the lipid bilayers. However, the main component for its antioxidant activity was unknown.

A paper reported by Ugusman and colleagues (2012) revealed that aqueous extract of *P. sarmentosum* contained high total flavonoid content (48.57 ± 0.03 mg GAE/g DM) and total phenolic content (91.02 ± 0.2 mg QE/g DM). By using HPLC, they showed the presence of rutin and vitexin as the main flavonoids in aqueous extract of *P .sarmentosum*. Both of the compounds at the concentration of 150 – 400µM enhanced the viability of HUVEC induced by hydrogen peroxide. Therefore, rutin and vitexin were proposed to be involved in the protective effects against oxidative stress.

1.5.4 *Cosmos caudatus*

Cosmos caudatus Kunth, also known as 'Ulam Raja' (King's salad), belongs to the family Asteraceae. It is an edible plant and can be found in tropical areas, such as Mexico, Central America, South America, United States, Malaysia and Thailand (Shui *et al*., 2005). In Malaysia, *C. caudatus* has been used traditionally to reduce body heat, promote fresh breath, as an anti-aging agent, strengthening bone marrow, and to treat infections related to pathogenic microorganisms (Rasdi *et al*., 2010). Methanol extract of *C. caudatus* was reported to demonstrate a moderate antioxidant activity when tested using xanthine-xanthine oxidase enzymatic assay (Norhanom *et al*., 1999; Abas *et al*., 2003). Phytochemical screening of the leaves extract of *C. caudatus* displayed the existence of fatty acids, flavonoids, tannins, alkaloids, saponins and terpenoids (Harbone, 1998; Rasdi *et al*., 2010).

In a study performed by Faridah *et al*. (2006), antioxidant activities and the nitric oxide inhibition activity of *C. caudatus* were investigated. *C. caudatus* showed a better antioxidant activity than α-tocopherol but not butylated hydroxytoluene (BHT). *C. caudatus* displayed strong antioxidant activity in both thiobarbituric acid (TBA) method and ferric thiocyanate (FTC) method. Therefore it was suggested that consumption of *C. caudatus* provides dietary benefits to health as *C. caudatus* is able to scavenge free radical and gives protection against lipid peroxidation. They concluded that *C. caudatus* has strong antioxidant properties. This was supported by another study performed by Sukri (2012). *C. caudatus* extracts exhibited the highest DPPH (2,2-dipheyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid), ferric cyanide antioxidant activities and inhibition of linoleic acid oxidation. *C. caudatus* was reported to have high flavonoid and phenolic contents.

Other than antioxidant properties, antimicrobial property of different extracts of *C. caudatus* was studied as well (Rasdi *et al*., 2010). Antimicrobial potential of crude ethanol, n-hexane, diethyl ether and phosphate buffer saline (PBS) extracts of *C. caudatus* leaves were investigated. The tested concentrations were 1, 20 and 50mg/ml. 5 pathogenic microbial strains were tested, 2 Gram-positive strains (*Staphylococcus aureus* and *Bacillus subtilis*), 2 Gram-negative strains (*Pseudomonas aeruginosa* and *Escherichia coli*) and a fungal strain (*Candida albicans*). Higher concentration of extracts showed bigger zones of inhibition. All of the various extracts tested exhibited different levels of antimicrobial actions towards all of the strains tested, and all of the strains were liable to the extracts especially when treated with higher concentrations. The lowest MIC was exhibited by phosphate buffer saline extract (6.25 mg/ml on *E. coli* and *B. subtilis*) and the highest MIC was displayed by n-hexane extract (25 mg/ml on all the strains). As a conclusion, there were higher chances to find potential antimicrobial agents from ethanol, diethyl ether and n-hexane extract rather than phosphate buffer saline. Furthermore, the plants can be studied further as a new source of antibiotic agent if the compounds that responsible for the antimicrobial property can be isolated (Rasdi *et al*., 2010).

1.6 Scope of study

The present study aimed to investigate the antioxidant and anti-aging properties of plant extracts for topical use. Leaves of *O. stamineus*, *C. caudatus* and *P. sarmentosum* are edible plants and consumed by local people for health purpose. *C. hirta* is a weed that has been traditionally reported to possess wound healing properties. All of them have high antioxidant properties as discussed earlier. There is increasing demand towards natural products.

In addition to that, the efficacy of the plant extracts was tested in vitro by using cell culture. Other than that, the antioxidant and anti-aging properties of the plant extracts were studied in whole organisms, by using *C. elegans*. The nematode, a multicellular organism, shares 40% homologous similarity to human being, making it a good model in general biological process. In addition to that, *C. elegans* is very suitable to be used in the anti-aging study due to its short generation time, ease of culturing in the laboratory and production of lots of progeny. For conventional method, chemical compounds are delivered orally to the nematodes, either by mixing them with bacteria food source or by incorporating them into the nematode growth medium.

Besides conventional method, efficiency of liposome-mediated delivery was done as well. Plant extracts-loaded liposomes were fed to *C. elegans* and lifespan was determined. The whole study was conducted in several stages in order to achieve the objectives of the present study:

1. To carry out plant extraction for *C. hirta*, *O. stamineus*, *P. sarmentosum* and *C. caudatus*, and to test the radical scavenging activities of the extracts by DPPH assay.

2. To investigate the cytotoxicity of the plant extracts on fibroblast cells, effects on the Procollagen Type I C-Peptide (PICP) expression and cytoprotection against hydrogen peroxide (H_2O_2).
3. To evaluate the protective effects of plant extracts on the lifespan of *C. elegans* after stressed with UVB.
4. To prepare and examine the protective effects of plant extract-loaded liposomes.
5. To determine whether *C. elegans* is a good surrogate for predicting anti-aging properties of the plant extracts.

CHAPTER 2

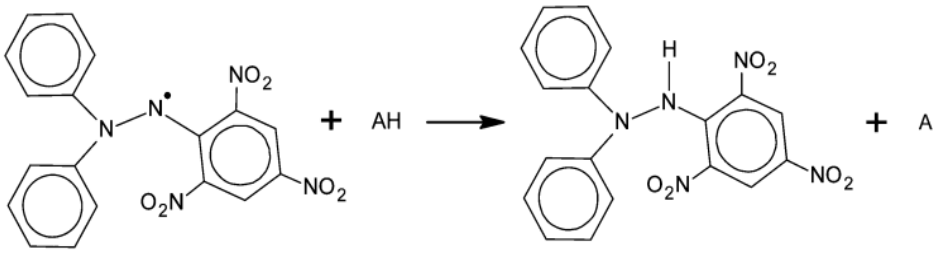
EXTRACTION METHODS AND RADICAL SCAVENGING ACTIVITY OF PLANT EXTRACTS

2. 1 Introduction

Antioxidant phytochemicals are valued as free radical scavengers which inhibit the propagation of free radical reactions, as well as protect human body from chronic and degenerative ailments (Terao and Piskula, 1997; Pham-Huy *et al*.,2008). Plant-derived antioxidants, such as phenolic compounds, are the largest category of phytochemicals (Ang, 2007). Phenolic compounds comprise one or more aromatic rings with one or more hydroxyl groups and are generally categorised as phenolic acid, flavonoids, stilbenes, coumarins and tannins (D'Archivio *et al*., 2007). Phenolic compounds are the secondary metabolites of plants and were reported to possess various biological functions. They may act as phytoalexins (Popa *et al*, 2008), antifeedants, attractant for pollinators, contributors to plant pigmentation, antioxidants and protective agents against UV light (Ignat *et al*., 2011). A category of such compounds, flavonoids, is found commonly in fruits, vegetables, wine, tea and coffee, and is responsible for two-thirds of the phenolics in our diet (Ang, 2007). Flavonoids have strong antioxidant activity, metal chelating potential (Tsao and Yang, 2003) and give protection against diseases such as cancer and heart disease (Cook and Samman, 1996; Beecher, 2003; Liu *et al*., 2008).

The antioxidant activities of phenolic compounds can be evaluated by several antioxidant assays. One of the simplest antioxidant tests is the DPPH assay. DPPH or 1,1-diphenyl-2-picrylhydrazyl is a stable free radical which has been used

extensively in many research to assess the radical scavenging activity of various plant materials. Upon accepting an electron or a hydrogen atom from a donor, it will become stable diamagnetic molecule of DPPH, which is non-radical. The reduced form of DPPH can be indicated by the color change, from purple to yellow. DPPH assay was first developed by Blois in 1958 and later several modifications were done. One of the parameters to interpret the results of DPPH is to determine the EC_{50} 'efficient concentration 50' EC_{50} values or 'Inhibition concentration 50' IC_{50} values. The meaning of IC_{50} is the concentration of the samples that causes 50% loss of DPPH (Tirzitis and Bartosz, 2010). DPPH assay can be run by using spectrophotometer in the wavelength of 515 – 528 nm (Pyrzynska and Pekal, 2013).



2,2`-diphenyl-1-picrylhydrazyl** **2,2`-diphenyl-1-picrylhydrazine

2,2'-diphenyl-1-picrylhydrazyl, the DPPH free radical, after combining with a hydrogen atom will be reduced to 2, 2'-diphenyl-1-picrylhydrazine (Pyrzynska and Pekal, 2013).

The purpose for this part of study is to do plant extraction and test the radical scavenging activities of the plant extracts by using DPPH assay.

2.2 Materials

Dried leaves of *C. hirta* were collected from Air Hitam Dam, Penang. Plant samples for the dried leaves of *O. stamineus*, *P. sarmentosum* and *C. caudatus* were

purchased from Herbagus Sdn. Bhd. All of the plants were identified by Dr. Rahmad Zakaria, a senior lecturer from School of Biological Science, Universiti Sains Malaysia (USM). Voucher specimens for *O. stamineus* (Voucher number:11545), *P. sarmentosum* (Voucher number: 11546) and *C. caudatus* (Voucher number: 11547) and *C. hirta* (Voucher number: 11035) were deposited at the Herbarium Department of School of Biological Science, USM. The leaves of *C. hirta* were air dried in the air-conditioned room for about 14 days until a constant weight was obtained. Ascorbic acid and mixed tocotrienols (containing α-, γ- and δ-tocotrienols of 12.3%, 20.6% and 5.3% respectively) were a token from Carotech Bhd. (Ipoh, Perak). 1,1-diphenyl-2-picrylhydrazyl (DPPH, molecular weight 394.32 g/mol) in powder form was purchased from Sigma-Aldrich (St. Louis, USA). 99.7% ethanol was bought from QRec™, Selangor, Malaysia. Gregar extractor was bought from Kontes Glass Company (Vineland, New Jersey, USA).

2.3 Methods

2.3.1 Ethanol extraction

All the leaves were extracted with 99.7% of ethanol (QReC™, Selangor, Malaysia). Before that, the leaves were blended with a domestic blender (Panasonic, Japan). The ratio of leaves to ethanol solvent was 1:20. The extraction process was run by using Gregar extractor for 24 hours at 80 °C. The solvent with dissolved plant substances was collected and subjected to centrifugation at 3000 rpm, for 15mins. Later on, rotary evaporation was carried out to remove the solvent and crude plant extract was collected. Crude plant extract was stored in freezer (-20°C) until further analysis.

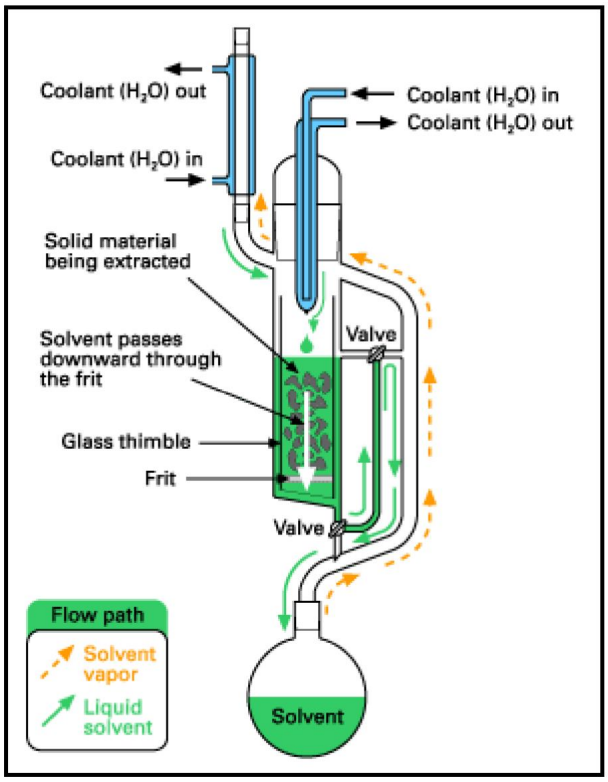


Figure 2.1 Gregar Extractor (Product Technical Notes, Chemglass Life Sciences, 2011)

2.3.2 Aqueous extraction

Dried leaves of *Cosmos caudatus* were extracted by using double-boiled method. The ratio of leaves to distilled water was 1:10. A fixed volume of boiling water was poured into Schott bottle with leaves. The Schott bottle was placed in a beaker with boiling water. One hour later, the hot water with extract was filtered out and leaves were discarded. Centrifugation at 3000rpm, for 15 minutes was done in order to remove the plant residue. The supernatant was kept and the pellet was thrown away. The dried form of plant extract was collected by using freeze-dried method (Labconco, USA).

2.3.3 DPPH radical scavenging method

DPPH radical scavenging activities were carried out according to the method performed by Lim (2012). Ascorbic acid and mixed tocotrienols were used as positive control. Stock solution of plant extracts was prepared at 10 mg/ml while ascorbic acid and mixed tocotrienols was prepared at 1 mg/ml. The plant samples were serially diluted down to concentrations of 2000µg/ml, 1000µg/ml, 500µg/ml, 250µg/ml and 125µg/ml respectively. For *C. hirta*, a series of concentrations in the range of 15.63 – 2000µg/ml were prepared as well. Ascorbic acid and mixed tocotrienols with concentrations ranging from 6.25µg/ml to 200µg/ml were prepared. 0.008g of DPPH powder was carefully weighed and dissolved in 100ml of 99.7% ethanol. A stock solution of 200µM was obtained and diluted to 50µM for the experiment use. Later on 50µl of sample was added to 950µl of DPPH in ethanol solution, vortexed for 10 seconds and incubated in the oven at 37 °C for 30 minutes. The samples were measured at wavelength 517nm with Multiskan Spectrum (Thermo Scientific, Finland). The experiment was carried out in the dark because DPPH is light sensitive. All the analysis was run in triplicates. The radical scavenging activities of the sample can be determined as below:

$$\% \text{ RSA} = \left[\frac{A_{blank} - (A_{sample} - A_{sample\ blank})}{A_{blank}} \right] \times 100\% \qquad \text{Eq. 2.1}$$

2.4 Statistical analysis

The experiments were run in triplicates. Data were presented as mean ± standard deviation. Data were analyzed by using one way analysis of variance (ANOVA) with Tukey's test to determine the significant difference between the variance. $P < 0.05$ was considered as statistically significant difference.

2.5 Results

2.5.1 Ethanol extraction

The leaves were blended into small pieces to increase the surface area of extraction. Gregar extractor allowed the extraction to be carried out in a closed loop system and therefore decrease the evaporation rate of solvent. Extraction of the leaves was carried out for 24 hours. After that, the discoloration of leaves can be observed and dark green coloured ethanol with leaves residues in powdered form was collected. The leaves residues were removed by using centrifugation. Rotary evaporation was done to remove the ethanol solvent and the whole process took about 2 – 3 hours. When the process completed, a sticky paste was left inside the round bottom flask. The crude ethanol extract was then removed from the flask by using spatula. Ethanol collected from rotary evaporation was reused for the next extraction. The yields of the plant extracts were about 6.4%, 16.0%, 2.4% and 14.4% from ethanol extracts of *O. stamineus*, *C. hirta*, *P. sarmentosum* and *C. caudatus* respectively. The crude ethanol extract was stored in bottle and kept in freezer (-20°C) until further analysis.

2.5.2 Aqueous extraction

Hot plate was used to boil water and then the boiling water was poured into Schott bottle containing the *C. caudatus* leaves. The Schott bottle was then placed in a beaker with boiling water in order to avoid the harmful effects of direct heating on the leaves. Besides, double boil is also a method practiced by the old folks when they are using the leaves as medication. A strong herbal smell will be noticed during the one hour of extraction. One hour later, the leaves were filtered immediately to avoid the continuous decoction. After the aqueous extract was cooled down, centrifugation