ANTIOXIDANT ACTIVITIES OF *Metroxylon sagu* EXTRACT AND ITS THERAPEUTIC EFFECTS ON CIGARETTE SMOKE EXPOSED MICE

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DECLARATION

The material in this thesis is original except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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ABSTRAK

Aktiviti Antioksidan ekstrak *Metroxylon sagu* dan kesan terapeutiknya pada pendedahan asap rokok pada tikus

Secara amnya terdapat kepelbagaian komponen semulajadi pada tumbuh-tumbuhan yang bersifat antioksidan melalui tindakannya dalam menentang radikal bebas dan menghalang pelbagai penyakit. *Metroxylon sagu* diekstrak menggunakan air melalui kaedah pengestrakan Pepejal-Cecair. Sebanyak 2.87% ekstrak akueus sagu (SAE) diperolehi. Penentuan aktiviti antioksidan SAE dilakukan menerusi kaedah Ferric Tiosianat (FTC), esei 2,2'-Azinobis-(3-etilbenzotiazolin-6-sulfonat) (ABTS) dan aktiviti pengikatan logam. SAE berpotensi menjadi sumber antioksidan semulajadi berdasarkan tindakannya dalam menghalang proses pengoksidaan asid linoleik, memerangkap radikal 2,2'-Azinobis-(3-etilbenzotiazolin-6-sulfonat) dan memiliki keupayaan mengikat logam. Tiga puncak dengan masa retensi pada 2.61 min, 4.30 min dan 4.70 min dikesan semasa pemisahan SAE oleh HPLC menggunakan kolum C_{18} fasa berbalik. Puncak pertama, SAE (Puncak 1) dipilih untuk kajian terperinci menggunakan FT-IR dan NMR memandangkan puncak tersebut adalah paling stabil. Berdasarkan pada spekrum FT-IR, kumpulan asas yang dikenalpasti pada SAE (Puncak 1) adalah kumpulan alkana, alkena, alifatik tak tepu, karbohidrat dan alkohol. Pengenalpastian SAE (Puncak 1) menggunakan ^{1}H NMR mendapati terdapat sepuluh keseimbangan proton yang berbeza, berkemungkinan berasal dari kumpulan alifatik dan karbohidrat (kawasan bagi komponen berkenaan terletak lebih kurang δ 0.8 sehingga δ 4.2). Spektra ^{13}C NMR bagi SAE (Puncak 1) memaparkan kehadiran dua belas karbon dengan sepuluh karbon terletak pada julat δ 60.3 – δ 81.5, dan selebihnya pada δ 92.3 dan δ 103.8. Komponen yang memiliki struktur tersebut berkemungkinan adalah karbohidrat, dengan sepuluh karbon adalah karbon bukan anomerik dan selebihnya berkemungkinan C-1 pada terminal penurunan dan C-1 yang terlibat dalam ikatan glikosidik. Ujian toksisiti *Artemia salina* menunjukkan SAE adalah tidak toksik apabila dibandingkan dengan potasium dikromat dengan toksisiti relatifnya iaitu 0.004. Bagi penentuan aktiviti antioksidan enzim (superoxid dismutase, glutation peroxidase dan katalase), kumpulan kawalan negatif menunjukkan aktiviti glutation peroxidase yang lebih tinggi pada tisu paru-paru berbanding kumpulan kajian. Bagi tisu hepar dan buah pinggang, kumpulan kajian menunjukkan aktiviti katalase lebih rendah berbanding kumpulan kawalan negatif manakala pada tisu otak, kumpulan kajian memiliki aktiviti katalase yang lebih tinggi berbanding kumpulan kawalan negatif. Penambahan SAE mempunyai kesan pelindung dalam mengurangkan radikal bebas yang dibebaskan melalui asap rokok sebelum membahayakan tisu berkenaan. Oleh itu, jumlah aktiviti glutation peroxidase dan katalase yang diperlukan adalah minimum untuk menghalang kerosakan yang lebih teruk pada tisu berkenaan yang berpunca daripada asap rokok. Berdasarkan pada keputusan kajian, pengambilan SAE dalam diet mampu melindungi perokok dan bukan perokok yang terdedah pada asap rokok.

ABSTRACT

Antioxidant Activities of *Metroxylon sagu* extract and its therapeutic effects on Cigarette Smoke Exposed Mice

It is known that a vast number of natural compounds in the plant kingdom possess antioxidant properties that can combat the deleterious effects of free radicals and thus prevent a number of diseases. Metroxylon sagu was extracted with water using the Liquid-Solid extraction method. 2.87% yield of sago aqueous extract (SAE) was obtained. Determination of the antioxidant activities of the SAE was carried out by the Ferric Thiocyanate (FTC) method, 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) assay and Metal Chelating Activity. SAE has been shown to be a potential natural antioxidant source, based on its action in inhibiting the peroxidation of linoleic acid, scavenging the 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) radicals and its metal chelating properties. Three peaks with retention times of 2.61 min, 4.30 min and 4.70 min, respectively, were detected on separation of SAE by HPLC using C_{18} reverse phase column. The first peak, SAE (Peak 1) was further investigated using FT-IR and NMR as it was the most stable peak. Based on the FT-IR spectrum, the principal bands identified in SAE (Peak 1) were those belonging to alkanes, alkenes, unsaturated aliphatics, carbohydrates and alcohols. Identification of SAE (Peak 1) using ^{1}H NMR found ten different equivalent protons, which could belong to aliphatic and carbohydrate regions (as the region indicative of these compounds is approximately δ 0.8 to δ 4.2). ^{13}C NMR spectrum of SAE (Peak 1) shows the presence of twelve carbons of which ten carbons were located within the range of δ 60.3 – δ 81.5, and the remaining two at δ 92.3 and δ 103.8, respectively. The possible compound with these structures could be carbohydrates, with ten non-anomeric carbons, and the remaining two probably are signals from C-1 at a reducing terminus and C-1 involved in a glycosidic lingkage. The brine shrimp lethality test shows that SAE is non-toxic when compared to potassium dichromate with relative toxicity of 0.004. For the determination of free radical scavenging enzyme activities (superoxide dismutase, glutathione peroxidase and catalase), the negative control group showed significantly higher glutathione peroxidase activities in the lung tissue compared to the experimental group. In liver and kidney tissues, the experimental group showed a significantly lower catalase activity compared to the negative control group and in brain tissue, higher catalase activities were observed in the experimental group compared to the negative controls. Thus, SAE supplementation has protective effects in reducing those free radicals that are released from sidestream cigarette smoke before causing extensive damage to the tissues. Thus, a lesser amount of glutathione peroxidase and catalase activity is needed to combat the oxidative stress induced from the cigarette smoke in those tissues. Based on these results, SAE supplementation might have a beneficial role in protecting smokers and non-smokers exposed to sidestream cigarette smoke.

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LIST OF ABBREVIATIONS

SAE	Sago Aqueous Extract
SSCS	Side-stream Cigarette Smoke
FTC	Ferric Thiocyanate
ABTS	2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate)
UV/Vis	Ultraviolet/Visible
UV	Ultraviolet
HPLC	High Performance Liquid Chromatography
FT-IR	Fourier Transform-Infrared
NMR	Nuclear Magnetic Resonance
IR	Infrared
SOD	Superoxide dismutase
GSH-Px	Glutathione Peroxidase
GSH	Glutathione
CAT	Catalase
Eq.	Equation
NADPH	The reduced form of NADP
$NADP^+$	Oxidised Nicotinamide Adenine Dinucleotide Phosphate
PUFA	Polyunsaturated Fatty Acids
LDL	Low Density Lipoprotein
HOCl	Hypochlorous acid
Cu, Zn-SOD	Copper, Zinc - Superoxide Dismutase
Mn-SOD	Manganese - Superoxide Dismutase
Fe-SOD	Iron - Superoxide Dismutase

BHT	Butylated Hydroxy Toluene
KBr	Potassium bromide
EDTA	Ethylene diamine tetra acetic acid
CHD	Coronary Heart Disease
TMS	Tetramethyl silane
D_2O	Deuterium oxide
LC_{50}	Lethal Concentration which causes the death of 50% of experimental animals
B	Mice fed on basal diet only
BSm	Mice fed on basal diet only + exposure to SSCS
BEx	Mice fed on basal diet + SAE
BExSm	Mice fed on basal diet + SAE + exposure to SSCS
BC	Mice fed on basal diet +Ascorbic acid
BCSm	Mice fed on basal diet + Ascorbic acid + exposure to SSCS

LIST OF SYMBOLS

g	gravity
cm	centimeter
m	meter
ft	feet
nm	nanometer
ppm	parts per million
mL	mililitre
mg	milligram
M	molar
mM	milimolar
min	minute(s)
mmol	milimoles
nmol	nanomoles
µL	microlitre
µm	micrometer
Log	logarithm
^{o}C	degree Celsius
^{1}H	hydrogen isotope (tritium)
^{13}C	carbon isotope (carbon thirteen)
$O_2^{\bullet -}$	superoxide radical
$NO^{\bullet}$	nitric oxide
$ONOO^{-}$	peroxynitrite
OH^{-} / $HO^{\bullet}$	hydroxyl radical

H_2O_2	hydrogen peroxide
$COO^{\bullet}$ / $ROO^{\bullet}$	peroxyl radical
O_2	oxygen
Fe^{3+}	ferric ion
Cu^{2+}	cuprous ion
$HO_2\bullet$	hydroperoxyl or perhydroxyl
H_2O	water
H^+	hydrogen ions
Fe^{2+}	ferrous ion
CH_2	methylene group
$R^{\bullet}$	alkyl radical
$RO^{\bullet}$	alkoxyl radical
ROOH	organic hydroperoxides
ROH	organic hydroxyl
RH	organic substrate
%	percentage

CHAPTER ONE

INTRODUCTION

1.1 Introduction

The sago tree, also known as "Rumbia", has been thoroughly studied for the production of sago starch, but the properties of other components, for example, the antioxidant capacities are less known. The washing from the sago starch extraction process is rich in antioxidant compounds that give it a golden brownish colour. On exposure to air, the liquid changes to a deep purple colour rapidly. Hence, this byproduct of the sago industry could be a rich source of antioxidant compounds which are known to protect the body from free radicals that can damage various systems in the body. The purpose of this research is to investigate the antioxidant properties of the aqueous extract of sago pith in the search for a natural, abundant and cheap source of antioxidant compounds.

To date, many studies have identified various health conditions and cardiovascular diseases to be prevented or treated with antioxidant supplementation (Harada *et al.* 2003; Nicolesu *et al.* 2001) and the list grows continuously. One of the factors contributing to the increasing incidence and severity of various cancers, degenerative pulmonary and cardiovascular diseases is cigarette smoke inhalation, known as side-stream cigarette smoke (SSCS) inhalation (Zhang *et al.* 2001). The free radicals that are generated in biological systems can cause oxidative damage, resulting in lipid peroxidation in the various organs.

The present research has also been designed to investigate the changes in the activities of the free-radical scavenging enzymes between mice fed on normal basal diet (negative controls) and those fed on basal diet supplemented with SAE (experimental group) or ascorbic acid (positive controls) when exposed to stress such as side-stream cigarette smoke (SSCS). The rationale for this approach is that elevated free radical levels will lead to increase activities of the free-radical scavenging enzymes and reduce levels of free radical will result in lowered activities of these enzymes. Supplementation with antioxidants might have a beneficial role in protecting the smokers and non-smokers exposed to SSCS by reducing risk from elevated free radical levels via the combined action of antioxidants as well as free radical scavenging enzymes.

Hence, investigations were carried out to find out if this is indeed true. The freeze-dried aqueous extract of *Metroxylon sagu* (SAE) was investigated for its antioxidant properties, using various chemical as well as enzymatic assays. The antioxidant activities were evaluated using the ferric thiocyanate (FTC) method (Kikuzaki & Nakatani, 1993; Rahmat *et al.* 2003), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS assay) (Cano *et al.* 1998, Yu *et al.* 2004), and metal chelating activity (Decker & Welch, 1990; Yen & Wu, 1999) by reading the absorbance values at different wavelengths using a UV/Vis Spectrophotometer. Assays were done to measure the antioxidant capacity of SAE via different approaches; by the inhibition of peroxidation, decolorization of ABTS radicals and based on chelating activity.

Further studies were carried out on the SAE using HPLC, FTIR and NMR to establish the chemical nature of the chemical compound in the extract. Those techniques are important as they provide information on the presence or absence of particular

functional groups and symmetry, electronic environment of the proton or carbon atoms, the quantity of protons present and nature of linkages between nearby nuclei.

The Brine Shrimp Lethality Test (Sam, 1993) was used to measure the toxicity of SAE relative to the control (potassium dichromate) by determining the lethal concentration for 50% mortality after six hours of exposure to SAE, known as the acute LC_{50} and the chronic LC_{50} (after 24 hours of exposure).

As for the experimental trials, forty-nine male mice (*Mus musculus* sp.) were divided into six groups, based on the different diets fed to them, comprising pellet and SAE or ascorbic acid and exposed to side-stream cigarette smoke (SSCS) for eight weeks. At the end of the experimental period, the mice were killed by cervical dislocation and the tissues (mainly lung, liver, kidney and brain) were isolated and used for assessment of the activities of free radical-scavenging enzymes, superoxide dismutase (SOD) (Marklund & Marklund, 1974), catalase (CAT) (Aebi, 1974) and glutathione peroxidase (GSH-Px) (Paglia & Valentine, 1967).

1.2 Objectives of the Research

The main objectives of this research are:-

(i) To extract the *Metroxylon sagu* components and determine the antioxidant activities of SAE using three different chemical assays.

(ii) To assess the activities of free radical-scavenging enzymes (SOD, GSH-Px and CAT) in the mice fed on normal basal diet and mice fed on diet supplemented with SAE when exposed to side-stream cigarette smoke.

CHAPTER TWO

LITERATURE REVIEW

2.1 Free Radicals

Free radicals are unstable and highly reactive molecules that occur ubiquitously in living things. These reactive compounds are generated in the human body during normal physiological functions but they can also be introduced from the environment (Ahmad, 1995). They have unpaired electrons which makes them highly reactive intermediates (Bagchi & Puri, 1998) compared to those without unpaired electrons. Borg (1993) stated that, *"A free radical is a molecule or molecular fragment with an unpaired valence electron"*, whereas Halliwell *et al.* (1995) define free radicals as "any species that contains one or more unpaired electrons and is incapable of independent existence".

Because of their instability, they react quickly with other compounds to capture the needed electron (Jakus, 2000) thus creating a free radical chain reactions which can be destructive to cells and tissues. However, in normal biological processes, free radicals play an important role in the removal of destructive bacteria and damaged cells and they also act as regulatory molecules in biochemical processes (Rice-Evans & Burdon, 1994). Therefore, free radicals can become highly reactive and cause "oxidative stress" if the production is not tightly controlled. Oxidative stress is a situation describing the steady state level of oxidative damage caused by the imbalance between the prooxidants and antioxidants in our body system (Rice-Evans & Burdon, 1994).

2.1.1 Types of Free Radicals

Free radicals are often generated by oxygen in eukaryotic cells. They are also known as Reactive Oxygen Species (ROS). The notation ($^{\bullet}$) in the following paragraph refers to the single remaining odd electron while ($^{-}$) means a radical anion. Major ROS are the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), nitric oxide radical ($NO^{\bullet}$) and hypochlorous acid (HOCl).

2.1.1.1 Superoxide Radical ($O_2^{\bullet -}$)

$O_2^{\bullet -}$ is a small and non-polarizable anion (Rice-Evans & Burdon, 1994) that is produced in the inner membrane of mitochondria from various pathways. One of them is the oxidation of xanthine or hypoxanthine to uric acid, by the dehydrogenase enzyme called xanthine oxidase (Eq. 1 & 2). By reducing oxygen to superoxide anion, these radicals are important sources of the initiation and propagation of several pathological processes *in vivo* (Sánchez-Moreno, 2002). Autoxidation of metal complexes may also produce the superoxide radical that becomes a precursor for other reactive species. Superoxide reacts rapidly with nitric oxide ($NO^{\bullet}$) to produce peroxynitrite $ONOO^{-}$, a potent agent of LDL oxidation *in vitro* (Leeuwenburgh *et al.* 1997). At the same time, $O_2^{\bullet -}$ triggers $HO^{\bullet}$ production by reducing the transition metal ions (either Fe^{3+} or Cu^{2+}) which then react with hydrogen peroxide in Fenton reactions (Rice-Evans & Burdon, 1994).

Xanthine oxidase

$$\text{Xanthine/Hypoxanthine} + O_2 \longrightarrow \text{Uric acid} + O_2^{\bullet -} \quad \text{(Eq. 1)}$$

$$O_2 + \text{NADPH} + H^{+} \rightarrow O_2^{\bullet -} + NADP^{+} \quad \text{(Eq. 2)}$$

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