ANTIOXIDATIVE AND ANTIMICROBIAL ACTIVITIES OF

*M. denticula AND M. tanarius*

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DECLARATION

I hereby declare that this dissertation entitled Antioxidative and Antimicrobial Activities of Macaranga Denticula and Macaranga tanarius and the work presented in it are on my own. Sources of finding reviewed herein have been duly acknowledged.

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ANTIOXIDATIVE AND ANTIMICROBIAL ACTIVITIES OF

*M. denticula* AND *M. tanarius*

The leaves of *M. denticula* and *M. tanarius* were extracted via reflux and the respective crude extracts were then fractionated using solvent-solvent extraction with 4 solvent with different polarity which yielded hexane (HEX), dichloromethane (DCM), n-butanol (BUT), and methanol aqueous (AQ) extracts. Total phenolic content, antioxidative and antimicrobial activities of the extracts were evaluated. The total phenolic content, determined according to the Folin-Ciocalteu method was varied between 115.58 mg GAE/g and 204.94 mg GAE/g. Antioxidative activities of the extracts were measured using the DPPH method. The antioxidative activities were then compared with that of butylated hydroxytoluene (BHT). The results showed that BUT extract of *M. tanarius* had the highest antioxidative activities among the fractions tested with the 64.23 % inhibition at the highest concentration (0.04 mg ml⁻¹) tested. These results showed strong association with the total phenolic content ($r^2 = 0.9684$), suggested that phenolic compounds are probably responsible for the antioxidative activity of the *Macaranga* sp. The antimicrobial activity of the extracts, which evaluated by the disc diffusion assays showed weak inhibition against the bacteria (*S. aureus*, *B. cereus*, *P. aeruginosa*, *S. typhimurium* and *E. coli*). Conclusively, *M. denticula* and *M. tanarius* extracts tested possesses antioxidative activities which rendered them suitable as potential therapeutics, thus made them excellent candidates for more detailed investigation.
ANTIOXIDATIVE AND ANTIMICROBIAL ACTIVITIES OF

*M. denticula* AND *M. tanarius*

Sebatian dalam daun-daun dari tumbuhan *M. denticula* dan *M. tanarius* diekstrak melalui refluks. Ekstrak-ekstrak mentah yang terhasil kemudian diekstrak berlanjutan melalui ekstraksi ceceair-ceceair dengan menggunakan 4 pelarut yang berlainan kepolaran. Ini meghasilkan 4 ektrak iaitu ekstrak n-butanol (BUT), diklorometana (DCM), heksana (HEX) dan akues metanol (AQ). Sifat-sifat antioksida, antimikrobal dan kandungan fenolik setiap sampel dinilai. Sifat antioksida telah diuji dengan kaedah perencatan radikal bebas DPPH secara relatif kepada butylated hydroxytoluene (BHT). Kajian ini menunjukkan keempat-empat ektrak dari setiap sampel muncul sebagai antioksida yang agak memuaskan di mana aktiviti antioksidanya lebih cekap berbanding dengan BHT.

Nilai kereletifan kesemua ektrak dalam setiap sampel adalah hampir sama antara satu sama lain menunjukkan mereka memiliki kecekapan yang hampir sama dalam kebolehan merencatkan radikal bebas DPPH. Kandungan fenolik yang didapati daripada setiap ektrak adalah berkadar langsung dengan kecekapan antioksida ektrak mencadangkan sebatian fenolik adalah penyumbang kepada sifat antioksida ektrak-ektrak yang dikaji.

Walau bagaimana pun, ektrak-ektrak menunjukkan keputusan yang lemah terhadap perencatan pertumbuhan bacteria-bakteria berikut: *S. aureus*, *B. cereus*, *P. aeruginosa*, *S. typhimurium* and *E. coli*, dalam ujian antimikrobal.
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CHAPTER 1

INTRODUCTION

1.1 Background of the study

Organic compound has a very strong tendency to react with atmospheric oxygen and oxidized. The reaction of organic compounds with oxygen, known as autoxidation, is the most common of all organic reactions. The reaction is a free radicals chain process which includes initiation, propagation, and termination step involving different types of free radicals (Barclay & Vinqvist, 2003). Oxidation reaction is intimately linked to free radicals, the highly reactive oxygen species (ROS) with one or more unpaired valence electrons (Vimala et al., 2003). It is commonly accepted that under the situation of oxidative stress, ROS such as superoxide (O$_2^-$), hydroxyl (OH$^-$) and peroxyl (‘OOH, ROO$^-$) radicals are generated (Huang et al., 2004). These ROS are produced in the mitochondrial cells in normal aerobic respiration and in others common biochemical reaction in our body (Halliwell & Gutteridge, 1990; Halliwell et al., 1992; Halliwell, 1994). However, free radicals production is enhanced by exogeneous sources e.g. atmospheric pollutants, diets high in fat, saturated oil, processed food, cigarette smoking,
drugs, xenobiotics and a stressful life (Atawodi, 2005). All these contributors are very much a typical part of today’s globalization lifestyle. Excess free radical can react with cell components, causing mutations in DNA and destroying cell proteins and lipids, which are closely associated with carcinogenesis (Floyd, 1999; Goodwin & Brodwick, 1995).

Lipid peroxidation is a free radicals chain reaction that involve the oxidative deterioration process of lipids containing any number of carbon-carbon double bonds. Oxidative modification or damage of plasma low-density-lipoprotein (LDL) in the human body plays an important causative role in the initiation and progression of coronary disease (Yun, et al., 2002; Cosgrove et al., 1987). Thus, the human body has an antioxidant defence mechanism that consists of natural antioxidant enzymes and vitamins. Antioxidant is substrates that neutralize the harmful effect of free radicals. They simultaneously degrading the excess free radicals in our body to non-reactive form (Mathew & Emilia, 2006). When age increases the antioxidant enzymes in our body are insufficient to scavenge and eliminate excess free radicals efficiently (Vimala et al., 2003). Perhaps this explains why antioxidant is always perceived as anti-aging.

Antioxidant is indeed a free radical scavenger. It has the electrophilic properties which allowed them to donate the hydrogen or electron to the free radical and stabilize it (Williams et al., 1995). It is a protective mechanism that may act independently or in a combination as anti-cancer or cardioprotective agents. Recently many reports have
suggested the importance of protective defense system in the living cell against damage caused by free radicals and active oxygen. There is an increasing interest in the protective biochemical function of dietary antioxidant, which can be a candidate for prevention of mutagenesis and carcinogenesis and, in some cases, extend the life span of animals (Osawa, 1992).

Accumulated evidence suggest that ROS can be scavenged through natural antioxidant compounds present in plants (Atawodi, 2005). The most widely used synthetic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are suspected of causing some safety concerns (Amarowicz et al., 2004; Howell, 1986; Ito et al., 1986; Namiki, 1990). As such, identification and development of safer, natural antioxidants is beneficial and promise more security to the consumer (Yun et al., 2002).

Sources of natural antioxidants are primarily plant phenolics, which may occur in all parts of the plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks (Pratt & Hudson, 1990). Phenolic compounds are products of secondary metabolism widely spread in the plant kingdom which are multifunctional and can act as free radicals terminators (Mathew & Emilia, 2006; Lee et al., 1999). Nowadays it is well known that phenolic compounds are highly responsible of the health effects derived from consumption of plant origin food. They play a key role as antioxidants due to the presence of hydroxyl substituents and their aromatic structure, which enables them to scavenge free radicals and as such, is a good source of natural antioxidants in human
diets. Among them flavonoids are particular attractive classes of polyphenols, as they occur often in significant concentrations.

The flavonoids are typical phenolic compounds and, therefore, act as potent metal chelators and free radical scavengers (Krishnakumar & Gordon, 1996; Pratt, 1992). They are powerful chain-breaking antioxidants and display a remarkable array of biochemical and pharmacological actions, some of which suggest that certain members of this group of compounds may significantly affect the function of various mammalian cellular systems.

In recent years, phytochemical constituents of plants with varied pharmacological, physiological and biochemical activities have received attention (Natarajan, et al., 2006). The genus Macaranga (Euphorbiaceae) comprises more than 300 species found mainly in the southern part of Asia (Webster, 1994). In South East Asia, the genus comprises about 51 species, 47 species are known from Borneo (Shaw, 1975). Local healers apply fresh or dried leaves of certain Macaranga sp to treat cuts, swellings, sores, bruises and boils (Singh et al., 1984; Fasihuddin, 1993; Nick et al., 1995). A previous phytochemical investigation on this plant has resulted in the isolation of a flavonoid and its derivative compounds (Hui et al., 1975; Tseng et al., 2001; Vinh et al., 2002; Sutthivaiyakit et al., 2002; Jang, 2004).
1.2 Objectives:

The main objectives of this study were

a) To determine the total phenolic contents in the *Macaranga sp.*

b) To investigate the antioxidative and antimicrobial activities of the extracts of *Macaranga sp.*

1.3 Scope of study:

In this study, two different species of *Macaranga sp.* were extracted and tested for their antioxidative activities. Both of the species: *Macaranga denticula* and *Macaranga tanarius* were collected in the main campus area of University Malaysia Sabah.

Scopes of works which were included in the investigation include total phenolics analysis and biological activity test on *Macaranga sp.* extracts. For determination of total phenolics content, *Macaranga sp.* extracts were tested by using Folin-Ciocalteu method (Folin & Ciocalteo, 1927). The *Macaranga sp.* extracts were also screened for its flavonoids, and flavonol contents. The antioxidative activity was carried out using Ferric thiocyanate method (Cuvrelier *et al.*, 1992), DPPH (Halliwell, 1997) and The antimicrobial activity was carried out using disc diffusion methods against *Staphylococcus aureus* (S 277), *Escherichia coli* (E91/026), *Bacillus cereus* (B 43/04B), *Pseudomonas aeruginosa* (A TCC) and *Salmonella typhimurium* (S100).
2.1 Free radical and antioxidant

2.1.1 Free Radicals

All atoms contain a nucleus, made up of protons and neutrons. Electrons move around the nucleus, usually associated in pairs. A free radicals is simply defined as any species that contains one or more unpaired electrons (Ames, 1989). The presence of unpaired electrons alter the chemical reactivity of an atom or molecule, usually making it more reactive than the corresponding nonradicals (Vimala et al., 2003). They are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electrons.

In chemistry, free radicals take part in radical addition and radical substitution as reactive intermediates. Reactions involving free radicals can usually be divided into three distinct processes: initiation, propagation, and termination (Rhodes, 2000).
Initiation reactions are those which result in a net increase in the number of free radicals. They may involve the formation of free radicals from stable species or they may involve reactions of free radicals with stable species to form more free radicals. Propagation reactions are those reactions involving free radicals in which the total number of free radicals remains the same. Termination reactions are those reactions resulting in a net decrease in the number of free radicals. Typically two free radicals combine to form a more stable species.

2.1.2 Classification of Free Radicals

Long lived radicals can be classified into two categories:

a. Stable Radicals

Purely organic radicals can be long lived if they occur in a conjugated $\pi$ system, such as the radical derived from $\alpha$-tocopherol (vitamin E). There are hundreds of known examples of heterocyclic thiazy radical s which show remarkable kinetic and thermodynamic stability, with only a very limited extent of $\pi$ resonance stabilization (Oakley, 1998).
b. Persistent Radicals

Persistent radical compounds are those whose longevity is due to steric crowding around the radical center and makes it physically difficult for the radical to react with another molecule (Banister, 1995). Examples of these include Gomberg's radical (triphenylmethyl), Fremy's salt (Potassium nitrosodisulfonate, (KSO₃)₂NO⁻), nitroxides, (general formula R₂NO⁻) such as verdazyls, nitronyl nitroxides, and azephenylenyls. The longest-lived free radical is melanin, which may persist for millions of years.

c. Biradicals are molecules containing two radical centers. Multiple radical centers can exist in a molecule.

In the upper atmosphere free radicals are produced through dissociation of the source molecules, particularly the normally unreactive chlorofluorocarbons by solar ultraviolet radiation or by reactions with other stratospheric constituents (Michael & Edward, 1983). These free radicals then react with ozone in a catalytic chain reaction which destroys the ozone, but regenerates the free radical, allowing it to participate in additional reactions. Such reactions are believed to be the primary cause of depletion of the ozone layer and this is why the use of chlorofluorocarbons as refrigerants has been restricted.
2.1.3 Formation and Sources of Free Radicals

The formation of radicals requires covalent bonds to be broken homolytically, a process that requires significant amounts of energy. The homolytic bond dissociation energy between two covalently bonded atoms is affected by the structure of the molecule as a whole, not just the identity of the two atoms, and radicals requiring more energy to form are less stable than those requiring less energy (Halliwell and Gutteridge, 1985). Homolytic bond cleavage most often happens between two atoms of similar electronegativity. In organic chemistry this is often the O-O bond in peroxide species or O-N bonds.

Free radicals and other reactive oxygen species, both inorganic and organic, collectively known as ROS are generated continuously via normal physiological processes, more so in pathological conditions (Afanas et al., 1983). ROS are partially reduced forms of atmospheric oxygen (O₂). They typically result from the excitation of O₂ to form singlet oxygen or from the transfer of one, two or three electrons to form a superoxide radical (O₂⁻), hydrogen peroxide (H₂O₂) or a hydroxyl radical (HO'), respectively.

There are two sources of free radicals, the endogenous sources where the free radicals are produced as toxic by-product of normal functions in our body and exogenous source where the free radical production is enhanced by external stimuli (Vimala et al., 2003).
Free radicals are often produced during aerobic process, such as metabolism, biochemical reactions in cells, detoxification in the liver and energy generation by the mitochondria. Superoxide Free Radicals (SFR) are common biological free radicals found inside the human body (Ames, 1989). They are produced in the mitochondria during aerobic metabolism when oxygen is used for oxidization of the food that we eat to produce energy. SFR and hydrogen peroxides are also generated as part of the immune system to attack and kill invading bacteria. Thus, the body does need and use some free radicals. However, excess free radicals are undesirable for they can attack biological molecules such as lipids, proteins, enzymes, DNA, and RNA, leading to cell or tissue injury associated with degenerative disease (Amarowicz et al., 2000).

On the other hand, free radical production is enhanced by exogenous sources e.g. diets high in fats, saturated oils, barbecued meat and processed food products. A stressful lifestyle, cigarette smoking and drugs also enhanced the free radical production (Floyd & Malgorzata, 1983). During bacterial or viral infections, phagocytes, i.e. neutrophils and monocytes, which defend against infections, are known to generate high levels of free radical. Excess free radical cause joint pains and body-aches, symptoms usually experienced during a viral fever. Free radicals also enter the body from chemicals in food coloring, preservatives and taste enhancers, as well as environmental pollution and pesticides.

As a natural by-product of the normal metabolism of oxygen in our body, ROS has important roles in cell signaling. However, during times of environmental stress ROS
levels can increase dramatically which can result in significant damage to cell structures. This cumulates into a situation known as oxidative stress. Cells are normally able to defend themselves against ROS damage through the use of enzymes such as superoxide dismutases and catalases (Cosgrove, 1987). Small molecule antioxidants such as Ascorbic acid (vitamin-C), uric acid, and glutathione also play important roles as cellular antioxidants.

2.1.4 Free Radicals in Biology

Free radicals play an important role in a number of biological processes, some of which are necessary for life, such as the intracellular killing of bacteria by neutrophil granulocytes. Free radicals have also been implicated in certain cell signalling processes. The two most important oxygen-centered free radicals are superoxide and hydroxyl radical (Johnson et al., 1983). They are derived from molecular oxygen under reducing conditions. However, because of their reactivity, these same free radicals can participate in unwanted side reactions resulting in cell damage. Many forms of cancer are thought to be the result of reactions between free radicals and DNA, resulting in mutations that can adversely affect the cell cycle and potentially lead to malignancy. Some of the symptoms of aging such as atherosclerosis are also attributed to free-radical induced oxidation of many of the chemicals making up the body. In addition free radicals contribute to alcohol-induced liver damage, perhaps more than alcohol itself. Radicals in cigarette smoke have been implicated in inactivation of alpha 1-antitrypsin in the lung. This process promotes the development of emphysema (Omenn et al., 1994).
Free radicals may also be involved in Parkinson's disease, senile and drug-induced deafness, schizophrenia, and Alzheimer's (Ito et al., 1986; Owen et al., 2000; Junqueira et al., 2004). The classic free-radical syndrome, the iron-storage disease hemochromatosis, is typically-associated with a constellation of free-radical-related symptoms including movement disorder, psychosis, skin pigmentary melanin abnormalities, deafness, arthritis, and diabetes. The free radical theory of aging proposes that free radicals underlie the aging process itself (Ames, 1989).

Free radical attacks on DNA, which is the genetic material of the cells, cause cells to die or mutate and possibly become cancerous. Free radicals may be involved in cancers of the lungs, cervix, skin, stomach, prostate, colon and esophagus (Pitt, 1994). Free radicals also attack blood fats which may lead to heart and blood vessel disease. Free radicals can also damage cellular enzymes. The processes which depend on these enzymes stop, leading to cell damage and death. Dormant enzymes can also be activated and this can result in tissue damage. Cells contain components called mitochondria which are responsible for respiration and energy production. Free radicals can damage the mitochondria, affecting the ability of the cell to produce the energy it needs to function. Substances which are toxic to nerves can also be released by free radicals, leading to nerve and brain damage, such as that seen in Parkinson's disease (Halliwell, 1997; Goodwin & Brodwick, 1995).

Free radicals may be involved in the loss of transparency of the lenses of the eye, leading to cataracts and macular degeneration. Free radicals may be involved in the inflammatory response seen in rheumatoid arthritis and asthma. Free radicals may also
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