CHAPTER I

INTRODUCTION

Free radicals and related species have attracted a great deal of attention in recent years. They are mainly derived from oxygen (reactive oxygen species/ROS) and nitrogen (reactive nitrogen species/RNS), and are generated in our body by various endogenous systems, exposure to different physicochemical conditions or pathological states called pro-oxidant effect (Devasagayam et al., 2004, Papas, 1999). Normally, their rate of production does not exceed the capacity of the body to dispose of them. However, under certain circumstances when the body's natural defenses are compromised (e.g., following exposure to sunlight, smoking or in individuals with a genetic predisposition), a condition known as oxidative stress may result. Consequently, overproduction of ROS and RNS can lead to premature aging and a variety of diseases including cancer, ischemic damage following stroke, cardiovascular disease, arthritis, atherosclerosis, and a host of neurodegenerative disorders, such as Alzheimer's disease (Baskin and Salem, 1997; Chanwitheesuk, Teerawutgulrag and Rakariyatham, 2005).

The skin is always in contact with oxygen and is increasingly exposed to ultraviolet (UV) irradiation. Therefore, the risk of photo-oxidative damage of the skin induced by reactive oxygen species (ROS) has increased substantially. The term reactive oxygen species not only collectively includes oxygen-centered radicals such as the super-oxide anion ($O_2^{\cdot-}$) and the hydroxyl radical ($OH^{\cdot}$), but also some non-radical species, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), among others, all being produced in the skin upon UV irradiation (Scharffetter-Kochanek, 1997). The ROS also play a substantial role in collagen metabolism. They not only directly destroy interstitial collagen, but also inactivate TIMPs (tissue inhibitors of matrix-metalloproteinases) and induce the synthesis and activation of matrix-degrading metalloproteases which are responsible for the connective tissue degradation in photoaging, tumor invasion, and metastasis (Brenneisen et al., 1998; Scharffetter-Kochanek et al., 2000). Less severe photoaging changes result in wrinkling, scaling, dryness, and uneven pigmentation consisting of hyper- and hypo-pigmentation (Pinnell, 2003). Moreover, exposure to UV irradiation has also shown direct and indirect melanogenic effect on melanocyte to produce pigment melanin (Busca and Ballotti, 2000). Although this pigment in human skin is a major defense mechanism against ultraviolet light from the sun, including UVA and UVB that reach the earth's surface, the production of abnormal pigmentation such as melasma, freckles, senile lentigines, and other forms of melanin hyperpigmentation could be a serious aesthetic problem (No et al., 1999). To counteract the harmful effects of ROS and protect the overproduction of melanin, antioxidants and whitening agents have thus been the subject of many studies.

The antioxidants are now known to play an important role in protection against disorders caused by oxidative damage. The term "antioxidants" refers to compounds that can delay or inhibit the oxidation of lipid or other molecules by inhibiting the initiation or propagation of oxidative chain reaction, and which can thus prevent or repair damage done to the body's cells by oxygen. They act in one or more of the following ways: reducing agents, chelating agents and reactive oxygen/nitrogen species scavengers. One method to achieve control of biological oxidation is via antioxidant enzymes (e.g., superoxide dismutase (SOD), catalase, and glutathione peroxidase) while another is via small, nonproteinaceous antioxidant molecules such as vitamin C, E, ubiquinone, β-carotene and polyphenols (Baskin and Salem, 1997). Among the synthetic types, the most frequently used to preserve food are butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), both of which, however, could be toxic (Moure et al., 2001). Vitamin C has been proposed, for a long time, as a biological antioxidant. It can donate a hydrogen atom to the vitamin E-derived phenolate radical, thus regenerating its activity. However, in the presence of ferric ions, vitamin C is a potent potentiator of lipid peroxidation (Devasagayam, et al., 2004). Vitamin E is one of the best quenchers of singlet oxygen, and can have antioxidant, neutral or pro-oxidant effect. Beta-carotene also can behave as a pro-oxidant in the lungs of smokers. In the case of phenolic compounds, the ability of the phenolics to act as antioxidants depends on the redox properties of their phenolic hydroxyl groups, which allow them to act as reducing agents, hydrogen-donating antioxidants, and oxygen quenchers (Devasagayam et al., 2004; Chanwitheesuk et al., 2005). However, phenolic compounds have sometimes been found to exhibit pro-oxidant properties (Halliwell, Gutteridge, and Cross, 1992).

For acquired skin hyperpigmentation, many modalities of treatment are available including chemical agents or physical therapies, but none are completely satisfactory. The most popular depigmenting agent is hydroquinone (dihydroxybenzene, HQ) introduced for clinical use since 1961. However, because of the hazard of its long-term treatments, the use of hydroquinone in cosmetics has been banned by the European Committee (24 th Dir. 2000/6/EC) and formulations are available only by prescription of physicians and dermatologists. The majority of naturally occurring tyrosinase inhibitors has been described as consisting of a phenolic structure or of metal-chelating agents (Nerya et al., 2004). Kojic acid (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one) is an antibiotic produced by many species of *Aspergillus* and *Penicillium.* The depigmenting action of kojic acid is attributed to the chelating ability of copper in tyrosinase enzyme, a rate-limiting enzyme in melanogenesis (Briganti, Camera and Picardo, 2003). Nonetheless, kojic acid exhibited mild cytotoxicities in the MTT assay and it is too unstable to be stored for a long time (Kim et al., 2004).

The higher manufacturing cost and toxicity of synthetic antioxidants and skin whitening agents have created a need for identifying alternative natural, and probably safer, sources of antioxidants for use in the food and cosmetic industry. Natural substances are presumed to be safe since they occur in plant foods, and are seen as more desirable than their synthetic counterparts. *Phyllanthus emblica*, for example, is a plant that has been reported as being a multifunctional natural product (Chaudhuri, 2004).

Phyllanthus emblica Linn (also called *Emblica officinalis*) is commonly known as amla. It is a member of the family Euphorbiaceae. *P. emblica* is distributed in subtropical and tropical areas of Southeast Asia, particularly in China, India, Indonesia, the Malay Peninsula, Bangladesh, Nepal, Pakistan, Uzbekistan, Sri Lanka and Thailand (Scartezzini and Speroni, 2000; Zhang et al., 2001; Zhang et al., 2004). In Thailand, it is known as "Ma Kham Pom". The plant is a tree of small or moderate size with a greenish-gray bark and greenish-yellow flowers, formed in axilla clusters. The feathery leaves are linear-oblong, with a rounded base and obtuse or acute apex. The tender fruits are green, fleshy, globose and shining, and changes to light yellow or brick-red when mature (Summanen, 1999). The fruit, similar in appearance to the common gooseberry, is usually called "Indian gooseberry". Commonly edible and abundantly available fresh fruits of *P. emblica* are collected during the months of November through January (Ghosal, 1996).

Chemical investigation of this plant led to the isolation of several novel sesquiterpenoids from the roots, new organic acid gallates and polyphenols from the fruit juice, and new ellagitannins and flavonoids from the branches and leaves. Some of these substances were estimated for their anti-proliferative activities and the antitumor activities of phenols might be linked to their anti-inflammatory properties (Jeena, Kuttan and Kuttan, 2001; Zhang et al., 2004). Due to the presence of the conjugated ring structures and hydroxyl groups, most phenolic compounds have the potential to function as antioxidants by scavenging the superoxide anion, singlet oxygen and lipid peroxyl radicals and stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species (Anila and Vijayalakshmi, 2003).

This herb has been used for thousands of years in India for its medicinal value and is commonly used within the traditional healing system of Ayurveda. The fruit is the most commonly used plant part, with the fresh fruit being preferred. The fresh (or dry) fruit is used in traditional medicines for the treatment of diarrhea, jaundice and inflammations and its extracts possessed potent anti-pyretic and analgesic activity (Perianayagam et al., 2004). The air-dried fruit is also used for the treatment of cancer in Tibetan and Egyptian medicines (Zhang et al., 2004). Recently, its fruits have been tested for their antiviral activity particularly for inhibiting reverse transcriptase in the replication of retroviruses like HIV-1 (Scartezzini and Speroni, 2000). Other studies have shown that the fruit exhibited the immunomodulatory effects (Suresh and Vasudevan, 1994; Ganju et al., 2003) and has a hypolipidemic effect (Mathur et al., 1996; Anila and Vijayalakshmi, 2002). *P. emblica* fruit also possesses hepatoprotective effect (Jeena, Joy and Kuttan, 1999; Jeena and Kuttan, 2000). The root, leaves and bark have been used for treating eczema, wart, diarrhea and headache after a fever in the Southwest of China, and the root is also used as an astringent and hematostatic agent in Nepal (Zhang et al., 2001). The active principles or extracts of *P. emblica* have been shown to possess several pharmacologic actions such as ability to reduce cytotoxic effects of known clastogen like caesium chloride (Ghosh, Sharma and Talukder, 1992). Moreover, it has been used for treatment of several disorders including common cold, scurvy, cancer and heart diseases (Khopde et al., 2001).

P. emblica L. has been reported to have antioxidant (Khopde et al., 2001; Anila and Vijayalakshmi, 2003; Chaudhuri et al., 2004; Chaudhuri, 2004); anti-diabetic (Sabu and Kuttan, 2002), antiulcerogenic (Sairam et al., 2002; Bafna and Balaraman, 2005), and antitussive activities (Nosal, Mokry and Hassan, 2003). It is also regarded in these world cultures as a traditional immunomodulatory and a natural adaptogen and as an important traditional medicine with "broad prospects" (Xia et al., 1997).

As a skin care ingredient, *P. emblica* fruit extract has been shown to have multi-functional benefits. Low molecular weight tannins (<1000), namely emblicanin A and emblicanin B, along with pedunculagin and punigluconin are the key ingredients of *P. emblica* fruit extracts (Chaudhuri et al., 2004). The tannin-based *P. emblica* extract has been found to have broad-spectrum antioxidant activity (Chaudhuri, 2002), excellent iron- and copper-chelating ability and matrix-metalloproteinases (MMPs) inhibitory activity (Chaudhuri et al., 2004). The effect of *P. emblica* on reactive oxygen species includes the scavenging of singlet oxygen, superoxide anion radical and hydroxyl radical. Among many iron- and copper-chelates tested, only the complex of 5 mM *P. emblica* extract and iron (or copper) showed the absence of any water coordination (that is, the complex is fully and firmly saturated and there is no room for any pro-oxidant activity via the formation of oxo-ferryl or oxo-cupryl radical). Therefore, *P. emblica* extract has been found to prevent oxidative stress-induced damage caused by ROS and is also pro-oxidant free (Chaudhuri and Puccetti, 2002). Furthermore, *P. emblica* also exhibits inhibitory effect on the activity of collagenase and stromelysin-1 (collagen-degrading enzymes), which is increased by UV light. Reduction in matrix-metalloproteinase (MMPs) activity helps reduce wrinkling and loss of elasticity of the skin. *P. emblica* thus helps maintain a younger looking skin and acts as anti-aging agent. Though its primary applications within the skin care industry are as a photo-protective agent and skin lightener, it offers a host of other benefits for the skin (Chaudhuri et al., 2004; Chaudhuri, 2004). However, the effect of *P. emblica* locally grown in Thailand has not been fully evaluated in comparison with other established sources. Consequently, *P. emblica* locally produced in Thailand should be investigated for its antioxidant, anti-collagenase and anti-tyrosinase activities in order to establish more relevant information which can support and promote the use of Thai natural plant products for cosmetic applications.

In the present study, the fruits of *Phyllanthus emblica* locally grown in Kanjanaburi province, Thailand were extracted and investigated for:

1. Their antioxidant activity including hydrogen-donating activity; ability to scavenge hydroxyl radical, which is the highly toxic ROS (Halliwell, Gutteridge and Aruoma, 1987; Halliwell et al., 1995; Brenneisen et al., 1998) as well as its possible pro-oxidant activity.

2. Their anti-collagenase activity was evaluated using collagenase type IV (MMP-2) as a model enzyme because this enzyme is able to degrade basement membrane compounds including type IV and type VII collagen (Seltzer et al., 1989; Malina et al., 2004), as well as native type I collagen (Tournier et al., 1994; Aimes and Quigley, 1995).

3. Their anti-tyrosinase activity was also determined for their potential use in the treatment of hyperpigmentation disorders (Nerya et al., 2004).

In addition, extracts obtained from different extracting solvents were compared to identify the most appropriate solvent or extraction method which provided the best antioxidant/whitening activities.