CHEPTER 1

INTRODUCTION

1.1 Statement of problem

In recent years, the depletion of the ozone layer causes the increasing of ultraviolet (UV) radiation at the Earth's surface. Based on sufficient evidence of carcinogenicity studies in human, ultraviolet (UV) radiation is known to be a human carcinogen. UV radiation also induces a number of biological responses, including development of erythema, edema, sunburn cell formation, hyperplasia, immune suppression, DNA damage, photoaging, melanogenesis, skin cancer and DNA damage at molecular levels (Matsumura and Ananthaswamy *et al.*, 2004).

The electromagnetic spectrums of UV light can be subdivided into three ranges, which are UVA, UVB and UVC. UVA radiation (320-360 nm) is absorbed into the epidermis and passes through the dermis. The radiation participates in formation of free radicals which cause of damage to cellular proteins, lipids, and saccharides, and it is responsible for aging and elasticity of the skin (Trautinger *et al.*, 2001). UVB radiation (280-320 nm) is absorbed mainly by epidermis of skin and is more harmful than UVA. Studies of the effect of UVA and UVB radiation on molecular levels in keratinocyte cells have shown that UVB was about 1000 times more genotoxic than UVA (de Gruijl *et al.*, 2002). Moreover, the UVB-induced keratinocytes have shown DNA mutations mainly cyclobutane-pyrimidine dimers which were dimers between two adjacent pyrimidines, such as thymine-thymine

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dimers and thymine-cytosine dimers. These dimers are blockade of transcription and lead to activation the p53 protein which can induce apoptosis of irradiated keratinocytes (de Gruijl *et al.,* 2002, and Sinha and Hader *et al.,* 2002). UVC radiation (200-280 nm), the short wavelengths, is the most dangerous radiation to all living organisms. UVC can generate highly reactive chemical intermediates, such as hydroxyl and oxygen radicals, which also in turn can damage DNA (Sinha and Hader *et al.,* 2002). The mutations that are caused by the UVB and UVC-directed DNA damage are commonly seen in skin cancers (Matsumura and Ananthaswamy *et al.,* 2004).

To protect and reduce the harmful effects of UV radiation, synthetic and natural cosmetic products are interested; especially botanical cosmetic products. It has been reported that some plant extracts with UV absorption properties can be used in photo-protective product as enhancer of physical or chemical sunscreens to minimize the damage can reduce the human body suffers from UV radiation exposure (Afaq *et al.,* 2002). The polyphenols, commonly present in edible plants, are known to have beneficial properties, including antioxidant, anti-inflammatory and photo-protective effect to prevent or reduce the UV irradiation effects (Afaq *et al.,* 2002 and Svobodova *et al.,* 2003). In this work, Thai plant extracts will be examined for their total phenolic contents (TPCs), cytotoxicity, mutagenicity, antimutagenicity, antityrosinase and also investigated for the effect on morphological changes-induced by UV radiation. Data from this study will serve as preliminary finding on the novel botanical cosmetic ingredients which may contain substances to protect or recover the effect of UV radiation such as capable of scavenging reactive oxygen species (ROS), reactive nitrogen species (RNS).

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1.2 Literature reviews

1.2.1 Background and related studies

In this work, the plant extracts of *Terminalia chebula* Retzius, *Terminalia bellerica*, *Etlingera elatior* (Jack) R.M. Smith, *Rosa damascena* and *Rafflesia kerrii* Meijer were used to investigate for novel properties in cosmetic applications.

1.2.1.1 *Terminalia chebula* Retzius

Terminalia chebula Retzius (*T. chebula* Retz.) belongs to *Terminalia* and family of Combretaceae. It is commonly known as smo-thai in Thailand. It is also found in the Southern Asia, Africa, India and Thailand.

Figure 1.1 The fruit of *Terminalia chebula* Retzius (http://eksotiskefrugter.emu.dk/billeder/myrobalan2/pict0001.jpg, 5 September 2010)

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The fruits of *T. chebula* Retz. were used as a traditional medicine such as chronic cough, asthma, sore throat, colic pain, diabetes (Barthakur and Arnold *et al.*,1991; Chattopadhyay and Bhattacharyya *et al.*, 2007) and anti-cancer (Saleem *et al.*, 2002). Besides, *T. chebula* Retz. extract was reported to have strong anti-

anaphylactic action, anti-inflammatory and analgesic properties (Shin *et al.*, 2001; Chattopadhyay and Bhattacharyya *et al.*, 2007).

In 2002, Saleem and co-workers (Saleem *et al.*, 2002) studied on the biological activities of the crude extract of *T. chebula* Retz. on several malignant cell lines. The dried fruits of *T. chebula* Retz. were extracted in 70% methanol. The crude extract was tested for cytotoxicity on several malignant cell lines including human breast cancer cell line (MCF-7), mouse breast cancer cell line (S115), human osteosarcoma cell line (HOS-1), human prostate cancer cell line (PC-3) and the human prostate cell line (PNT1A). The *T. chebula* Retz. extract showed the highest cell viability with 50% inhibition concentration (IC_{50}) value on PNT1A cell line at 44.0 ± 2.3 μg/ml and weakest on PC-3 cell line with IC_{50} value at 38.0 ± 1.2 μg/ml. In addition, the phenolic compounds such as chebulanic acid, tannic acid and ellagic acid which were isolate from the crude extract of *T. chebula* Retz. were tested for cell growth inhibition activity on HOS-1. Chebulinic acid showed the highest cell growth inhibition activity with IC_{50} value at 53.2 ± 0.16 μM following by tannic acid and ellagic acid with IC_{50} values at 59.0 ± 0.19 μM and 78.5 ± 0.24 μM, respectively.

In 2009, Reddy and co-workers (Reddy *et al.*, 2009) studied on the biological activity of chebulagic acid which was isolated from the dried fruit of *T. chebula* Retz. The compound was investigated for the enzymatic inhibition activities of cyclooxygenase (COX-1, COX-2) and 5-lipooxygenase (5-LOX) which are key enzymes involved in inflammation and carcinogenesis. The result revealed that chebulagic acid showed potential COX–LOX dual inhibition activity with IC_{50} values at 15 ± 0.288, 0.92 ± 0.011 and 2.1 ± 0.057 μM for COX-1, COX-2 and 5-LOX, respectively. Anti-proliferative activity of chebulagic acid was evaluated in five

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different cancer cell lines such as breast carcinoma (MDA-MB-231, HCT-15), colon cancer (COLO-205) DU-145, prostate cancer (DU-145) and chronicmyeloid leukemia (K-562). It was found that chebulagic acid showed the highest activity against on COLO-205 cell line. Chebulagic acid was also studied for antioxidant properties with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay and 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) decolorization assay. Chebulagic acid showed DPPH radical scavenging activity in the concentration dependent manner with IC_{50} value at 1.4 ± 0.0173 µM and strongly inhibition of ABTS radical was observed with an IC_{50} value at 1.7 ± 0.023 µM.

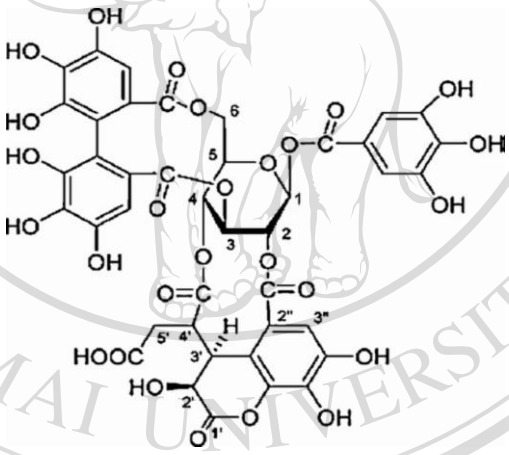

Figure 1.2 Chemical structure of chebulagic acid (Reddy *et al.*, 2009)

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Moreover, in 2010, Kim and co-worker (Kim *et al*., 2010) studied on the inhibitory effect of 1,2,3,4,6-penta-*O*-galloyl-β-D glucose (PGG) from *T. chebula* Retz. extract (Figure 1.3) on elastase and hyaluronidase. Elastase is the only enzyme that can break down elastin which is an important component of elastic fibers of connective tissue and tendons. Whereas, collagen is an important component in bone and joint composition, both elatin and collagen from a network under epidermis and

are decreased naturally with age. Hyaluronidase is an enzyme that degrades high molecular weight hyaluronic acid (hyaluronan, HA), which leads to inflammation, angiogenesis (involving the growth of new blood vessels), fibrosis, and collagen deposition in wound healing.

PGG was isolated from the fruit of *T. chebula* Retz. The result revealed that PGG has no cytotoxicity in rabbit articular chondrocytes up to concentration of 5 mg/mL and it showed elastase and hyaluronidase inhibitory activities with IC_{50} values of 57 μg/mL and 0.86 mg/mL, respectively. According to western blot analysis, treatment of PGG on rabbit articular chondrocytes significantly induced the type II collagen expression. The type II collagen expression was increased when the concentration of PGG was increased in dose dependent manner. This suggested that PGG might have an influence on skin conditions when used in cosmeceutical formulation as an anti-aging ingredient with no cytotoxicity.

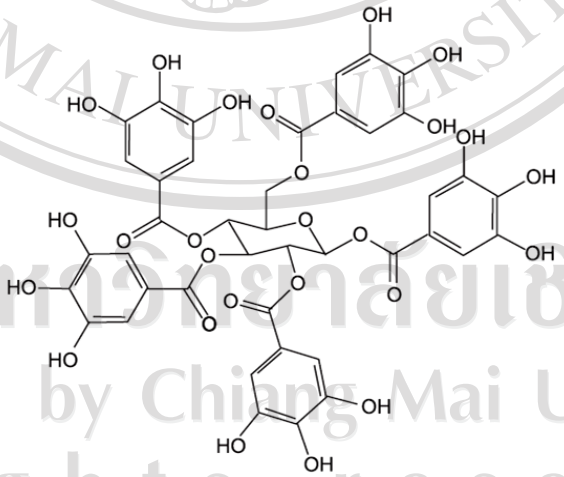

Figure 1.3 Chemical structure of 1,2,3,4,6-penta-*O*-galloyl-β-D glucose (PGG)

(Kim *et al*., 2010)

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1.2.1.2 *Terminalia bellerica*

Terminalia bellerica (*T. bellerica*) belongs to the family of Combretaceae. It is found in South-East Asia, including Thailand. The fruit of *T. bellerica* is used in traditional medicine to treat anemia, asthma, cancer, colic, cough, diarrhea, dyspepsia, dysuria, headache, hoarseness, hypertension, inflammations and rheumatism.

Figure 1.4 The fruit of *Terminalia bellerica*

(http://174.123.135.195/uploads05/57/0/Untitled-11530675090.jpg, 21 September 2010)

T. bellerica was reported to contain many chemical substances such as termilignan, thannilignan, 7-hydroxy-3,4 (methylenedioxy) flavone and anolignan B (Valsaraj *et al.*, 1997), gallic acid, ellagic acid, β-sitosterol (Anand *et al.*, 1997), arjungenin, belleric acid, bellericoside (Nandy *et al.*, 1989) and cannogenol 3-*O*-β-D-galactopyranosyl-(1→4)-*O*-α-L-rhamnopyranoside (Yadava and Rathore *et al.*, 2001).

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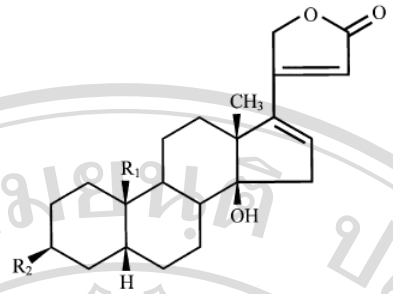

Figure 1.5 Chemical structure of cannogenol 3-*O*-β-D-galactopyranosyl-(1→4)-*O*-α-L-rhamnopyranoside from the seed of *T. bellerica*. (Yadava and Rathore *et al.*, 2001)

In 2005, Elizabeth (Elizabeth *et al*., 2005) studied on antimicrobial activity of the crude and methanol extracts from dried fruit of *T. bellerica* against 9 strains of human microbial pathogens which were *Staphylococcus aureus* (ATCC 9144), *Streptococcus pneumoniae* (UTI isolate), *Salmonella typhi* (NCTC 8393), *Salmonella typhimurium* (ATCC 23564), *Escherichia coli* (entero pathogen) *Eschericha coli* (uropathogen), *Pseudomonas aeruginosa* (ATCC 25619), *Yersinia enterocolitica* (ATCC 9610) and *Candida albicans* (ATCC 2091) using disc-diffusion method. Crude aqueous extract at 4 mg concentration showed clear zone of inhibition ranging from 15.5 to 28.0 mm. The crude aqueous extract showed highly antimicrobial activity on *S. aureus* at 28.0 mm and showed lower activity on *Y. enterocolitica* at 15.5 mm of zone inhibition, respectively. The methanol extract of *T. bellerica* was strongly inhibitory to *P. aeruginosa* and *S. aureu* with forming 14.0 to 30.0 mm zone of inhibition, respectively. Moreover, the minimal inhibitory concentrations (MICs) of the crude and methanol extracts were examined by broth dilution technique with concentration from 300 to >2400 µg/ml for crude extract and from 250 to >2000

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µg/ml for methanol extract. The result indicated that crude and methanol extracts of *T. bellerica* were highly effective against *S. aureus* with lower MIC values.

In 2009, Sabu and co-worker (Sabu *et al*., 2009) studied on the antidiabitic and antioxidant activities of *T. bellerica* extract in alloxan (2,4,5,6-tetraoxypyrimidine; 2,4,5,6-pyrimidinetetrone) induced hyperglycemia mices. *T. bellerica* aqueous extract prevented alloxan-induced hyperglycemia significantly after 6^{th} day of oral administration and there was 54% decreased on 12^{th} day. Oxidative stress usually occurs after alloxan is treated. After administration of *T. bellerica* extract, oxidative stress was observed to be significantly lowered level. The antioxidant activities of the extract were investigated by determination the level of lipid peroxidation (LPO) in mice liver and by examined the activity of enzyme superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) in blood and liver in mice model. The results indicated that the extract has reduced the serum glucose level after alloxan induced diabetic mice by 54% when compared with the control. In addition, it was found that the extract showed the reduction of the lipid peroxidation in mice liver. Moreover, the extract was inhibited the activity of the superoxide dismutase (SOD) and glutathione peroxidase (GPx) which caused the decreasing of the level of glutathione (GSH) and the activity of catalase (CAT).

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1.2.1.3 *Etlingera elatior* (Jack) R.M. Smith

Etlingera elatior (Jack) R.M. Smith (*E. elatior* (Jack) R.M. Smith) is in Zingiberaceae species. It is a native plant in Southeast Asia including Malaysia, Indonesia and Thailand. It is commonly known as Dalar which is used traditionally as medicine and as a flavor additive in Southeast Asia dishes.

Figure 1.6 *Etlingera elatior* (Jack) R.M. Smith

(http://xchange.teenee.com/up01/post-128309-1259462524.jpg, 4 June 2010)

In recent year, Jaafar and co-worker (Jaafae *et al.*, 2007) analyzed the essential oil from leaves, stems, flowers and rhizomes of *E. elatior* (Jack) R.M. Smith. The essential oils were extracted using the hydrodistillation method and analyzed by GC-MS. The result suggested that the essential oils from leaves, stems, flowers and rhizomes showed percentage yield of volatile constituents at 0.0735%, 0.0029%, 0.0334% and 0.0021%, respectively. The major compounds of the essential oil in the leaves contained caryophyllene, *ß*–pinene, and (*E*)-*ß*-farnesene at 15.36%, 19.7% and 27.90%, respectively. Whereas, the stem essential oil was found (*E*)-5-dodecane (26.99%) and 1,1-dodecanediol diacetate (34.26%). The essential oils of the flowers contained the major compounds such as 1,1-dodecanediol diacetate (24.38%) and cyclododecane (47.28%). The essential oils of the rhizomes contained the major compounds of 1,1- dodecanediol diacetate (40.37%) and cyclododecane (34.45%), respectively.

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Chan and co-worker (*Chan et al.*, 2007) investigated on the total phenolic content (TPC), antioxidant activity (AOA) and antibacterial activity of leaves of *Etlingera* species. Five species of *Etlingera* which consisted of *E. elatior, E. fulgens, E. maingayi, E. littoralis* and *E. rubrostriata* were studied. The extract of leaves *E. elatior* showed the highest total phenolic content and antioxidant activity following *E. rubrostriata*, *E. littoralis, E. fulgens* and *E. maingayi*, respectively. Moreover, the extract of leaves *E. elatior* showed the highest total phenolic content (3550 ± 304 mg GAE/ 100 g) following by the extract from inflorescences in flower (295 ± 24 mg GAE/ 100 g) and the extract from rhizomes (187 ± 46 mg GAE/ 100 g). The antibacterial activities of the extracts were screened by disc-diffusion method. The experiment was performed by using the gram-positive bacteria (*Bacillus cereus, Micrococcus luteus,* and *Staphylococcus aureus*) and gram-negative bacteria *(Escherichia coli, Pseudomonas aeruginosa*, and *Salmonella cholerasuis*). It was found that the leaves of *Etlingera* species exhibited antibacterial activity against only gram-positive bacteria, but not very effective against gram-negative bacteria. Leaves of *E. elatior, E. fulgens*, and *E. maingayi* exhibited antibacterial activity on three bacteria. The leaves of *E. rubrostriata* exhibited antibacterial activity on *B. cereus* and *S. aureus.* In contrast, the leaves of *E. littoralis* exhibited antibacterial activity on *S. aureus* only.

In 2008, Chan and co-worker (Chan *et al.*, 2008) studied on tyrosinase inhibition activity of leaves from 26 ginger species. The plant extracts were determined for tyrosinase inhibition activity by dopachrome method. The results suggested that the extract of leaves *E. elatior* showed strongest tyrosinase inhibition

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activity value at 55.2% following by *E. fulgens* (49.3%), *E. maingayi* (42.6%), *E. rubrostriata* (29.5%) and *E. littoralis* (22.0%), respectively.

In 2009, Chan and co-worker (Chan *et al.*, 2009) have isolated caffeoylquinic acids (CQA) and chlorogenic acid (CGA) from the leaves of *Etlingera* species. Five *Etlingera* species which consisted of *E. elatior*, *E. fulgens, E. maingayi, E. rubrostriata* and *E. littoralis* were used in this study. It was found that caffeoylquinic acids were obtained from *E. elatior E. fulgens* and *E. rubrostriata*. CGA was found only in leaves of *E. elatior* and *E. fulgens*. Caffeoylquinic acids and chlorogenic acid were revealed to show scavenging free radicals activity, inhibiting propagation of the lipid peroxidation (Savadova *et al.*, 2003) and chelating metal ions property (Kono *et al.*, 1998).

1.2.1.4 *Rosa damascena*

Rosa damascena (R. damascena) is originally introduced from the Middle East into Western Europe. It is one of the most important *Rosa* species for the flavor and fragrance industries that has been traditionally used for centuries. In addition, *R. damascena* has been traditionally used for treatment of constipation in human, antitussive, anti-nociceptive, hypoglycemic and bronchodilator (Zargari *et al.*, 1992, Shafei *et al.*, 2003, Rakhshandeh *et al.*, 2008, Gholamhoseinian, *et al.*, 2009 and Boskabady *et al.*, 2006). In recent years, antioxidant, antibacterial and antimicrobial activities of *R. damascena* extract have been demonstrated.

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Figure 1.7 *Rose damascena*

(http://www.uni-graz.at/~katzer/pictures/rosa_15.jpg, 21 September 2010)

In 2004, Ozkan and co-worker (Ozkan *et al*., 2004) have studied on antimicrobial and antioxidant properties of the fresh flower (FF) and spent flower (SF) extracts of *R. damascena*. The result suggested that the both extracts of *R. damascena* have shown antibacterial activity against fifteen spices of bacteria including *Aeromonas hydrophila, Bacillus cereus, Enterobacter aerogenes, Enterococcus feacalis, Escherichia coli, Klebsiella pneumoniae, Mycobacterium smegmatis, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens, Salmonella enteritidis, Salmonella typhimurium, Staphylococcus aureus* and *Yersinia enterocolitica*. The most sensitive bacterium was found to be *S. enteritidis* for FF extract and *M. smegmatis* for SF extract. Moreover, the antioxidant capacity was determined by the phosphomolybdenum method. The antioxidant capacity of the FF extract was higher than that of the SF extract. The antioxidant capacity of extracts

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revealed at values range from 372.26 ± 0.96 to 351.36 ± 0.84 mg/g extract, respectively.

In 2010, Sharafi and co-worker (Sharafi *et al*., 2010) studied on antibacterial, antioxidant, cytotoxicity of Hela cell after treated with methanolic and aqueous extracts of *R. damascena*. It was found that the methanolic extract was contained the total phenolic contents (TPCs) higher than aqueous extracts. TPCs of the methanolic and aqueous extracts were 132.67 ± 3.51 and 117.33 ± 6.81 μg GAE /mg of extract, respectively. The antibacterial activity of *R. damascena* extracts were tested on *Escherichia coli, Staphylococcus aureus, Streptococcus faecalis, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. It was found that both methanolic and aqueous extracts exhibited antibacterial activity of the five kinds of bacteria. *S. aureus* was equally sensitive to both methanolic and aqueous extracts. Moreover, the extracts were shown activity for decreasing the levels of cholesterol/HDL and LDL/HDL ratios, urea nitrogen, creatinine and uric acid in blood of mice animal model. Moreover, these results suggested the application of *R. damascena* extract as a natural antioxidant and health promoting agent.

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1.2.1.5 *Rafflesia kerrii* Meijer

Rafflesia kerrii Meijer is a member of the genus *Rafflesia* in the family *Rafflesiaceae*. It is found in Southeast Asia such as rainforest in Southern of Thailand, peninsular Malaysia, Indonesia and Philippines (Meijer *et al.*, 1984). The local Thai names are Bua Phut or Bua Toom. It is a kind of parasite flowering plant in wild plants such as genus *Tetrastigma* (*T. leucostaphylum, T. papillosum* and *T. quadrangulum*). The red flowers have a diameter of 50–90 cm with the odor of rotten

meat to attract flies for pollination (Barkman *et al*., 2008). In Thai traditional medicine, a decoction of flower buds is used to help restore the female uterus after giving birth, and also used for treatment of fever and backache (Kanchanapoom *et al*., 2007).

Figure 1.8 *Rafflesia kerrii Meijer*

(http://www.ubcbotanicalgarden.org/potd/rafflesia_kerrii1.jpg, 21 September 2010)

Recently, the structural determination of five compounds in *R. kerrii* Meijer have been reported (Kanchanapoom *et al*., 2007). There were four hydrolyzable tannins and one phenylpropanoid glucoside. These compounds were identified as 1,2,4,6-tetra-*O*-galloyl-β-D-glucopyranoside, 1,2,6-tri-*O*-galloyl-β-D-glucopyranoside,1,4,6-tri-*O*-galloyl-β-D-glucopyranoside and 1,2,4-tri-*O*-galloyl-β-D-glucopyranoside of hydrolyzable tannins and syringin of phenylpropanoid glucoside. Their chemical structures were shown in Figure 1.9.

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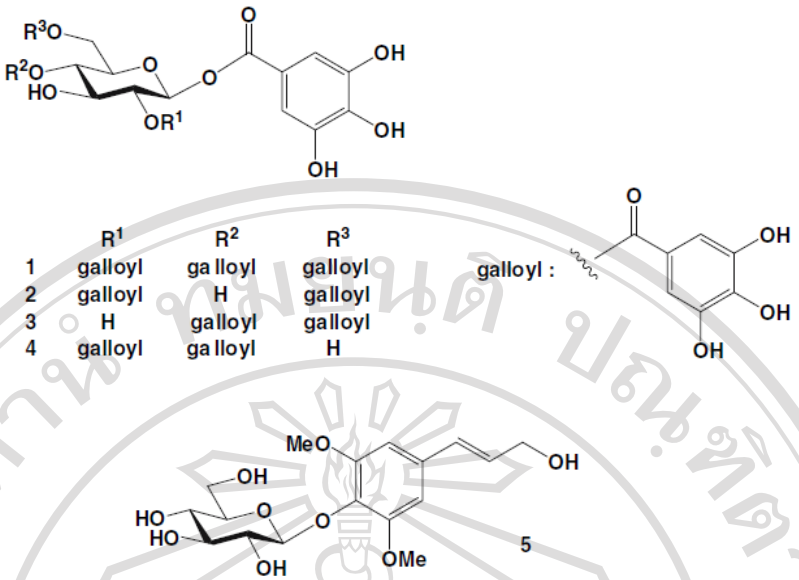

Figure 1.9 Chemical structures of four hydrolysable tannins (1-4) as 1,2,4,6-tetra-*O*-galloyl-β-D-glucopyranoside, 1,2,6-tri-*O*-galloyl-β-D-glucopyranoside, 1,4,6-tri-*O*-galloyl-β-D-glucopyrano side and 1,2,4-tri-*O*-galloyl-β-D-glucopyranoside and one phenylpropanoid glucoside (5)

In 2004, Wiart and co-worker (Wiart *et al*., 2004) studied on the antibacterial and antifungal activities of fifty plants in Malaysia. The disc-diffusion method was used to screen with six pathogens such as *Bacillus cereus, Bacillus subtilis, Candida albicans, Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus.* It was found that the extract of *Rafflesia hasseltii* showed the highest inhibition activity against *B. cereus, B. subtilis, P. aeruginosa* and *S. aureus*. In addition, the extract of *R. hasseltii* showed highly effective on accelerating the wound healing process in rat model (Mahmood *et al.*, 2009).

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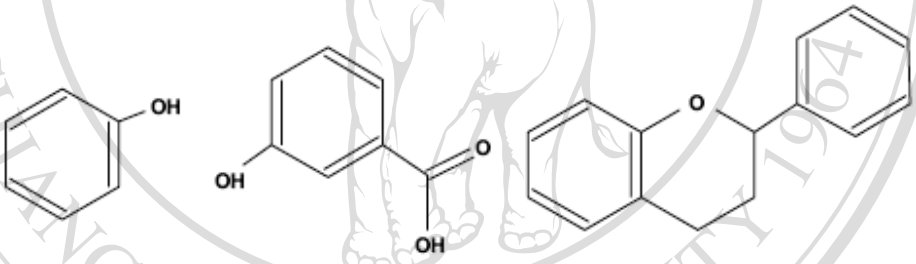
1.2.2 Phenolic compounds

Phenolics are plant secondary metabolites which are commonly found in herbs, vegetables and fruits. They are encompassed approximately 8000 biologically active compounds which one phenol molecules to polymeric structures. There are three important groups which are phenolic acids, flavonoinds and polyphenols. On the basis of the structural of phenolic acids, they are contained of phenol with carboxyl group thrust the structural of polyphenols are contained at two phenol ring as shown in Figure 1.10. Whereas, the flavonoids compose of two benzene rings joined by a linear three carbon chain (Harborne *et al.*, 1984 and Marinova *et al.*, 2005).



Phenol Phenolic acids Flavonoids

A B C

Figure 1.10 Structural of phenols (A), phenolic acids (B) and flavonoids (C) (http://www.foodnetworksolution.com/uploaded/phenolic%20compond.bmp, 24 November 2011)

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Phenolic compounds showed varieties of the biological activities such as antioxidant, antimutagenic, anticarcinogenic, antiviral, antibacterial, immune-stimulating, antiallergic, antihypertensive, hypholesterlemic and anti-inflammatory including helped to neutralize free radicals in living things. Free radicals, also known

simply as radicals, are organic molecules responsible for aging, tissue damage, and possibly some diseases. Therefore, the phenolic compounds play an important role in the inhibition of free radical reaction and capable of scavenging reactive oxygen species (ROS), reactive nitrogen species (RNS) and chelating transition metal ions (Michalak *et al.*, 2006 and Dejeridant *et al.*, 2007). In recent years, a great deal of works have been done on the determination of natural antioxidants in many sources for their use in cosmetic and food.

1.2.3 Cytotoxicity assay

Cytotoxicity is used to screen for toxicity of chemical compounds. The cytotoxicity is commonly tested on the cell line culture. Cell culture can be used to screen for toxicity both by estimation of the basal functions of the cell (i.e. those processes common to all types of cells) and by tests on specialized cell functions. Cellular damage will inevitably result in loss of the ability of the cell to maintain and provide energy for metabolic cell function and growth. Metabolic activity assays are based on these promises, usually mitochondrial activities are measured. Cytotoxicity is demanded calculation of 50% inhibitory concentration (IC_{50}) value, is a quantity of the chemical or drug required to cause death of 50% of the cell culture (Rubinstein *et al.*, 1990, Papazisiz *et al.*, 1997, Banasiak *et al.*, 1999 and Houghton *et al.*, 2007).

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1.2.3.1 MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay has been widely used for cell proliferation. Mosmann (Mosmann *et al.*, 1983) established the MTT colorimetric tatrazolium assay. The experiment was measured

the activity of mitochondrial reductase. This enzyme can reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole to purple formazan. The formazan is then solubilized with dimethyl sulfoxide (DMSO) and the abosorbance at 570 nm is measured by spectrophotometer.

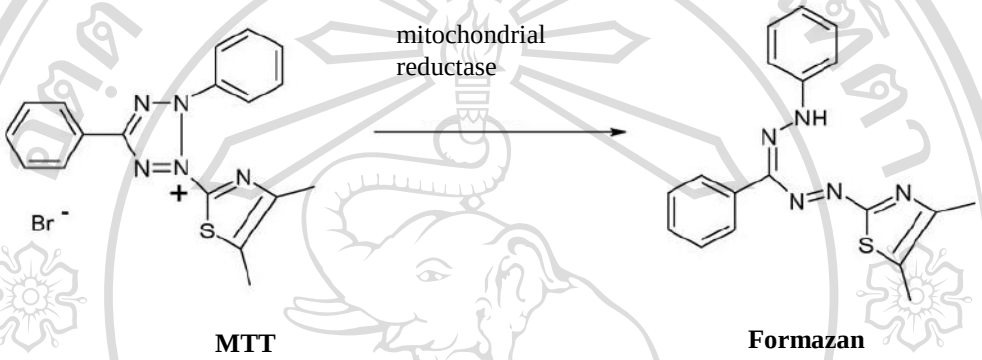

Figure 1.11 MTT reduction in live cells by mitochondrial reductase results in the formation of insoluble formazan (http://en.wikipedia.org/wiki/MTT_assay, 21 September 2010)

These reductions occur only when mitochondrial reductase was active. Therefore, the conversion is often used as a measure of viable (living) cells. It is important that other viability assays (such as dye exclusion method) sometimes has to be used to confirm the result.

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1.2.3.2 XTT assay (Scudiero *et al.*, 1988)

XTT assay (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2-tetrazolium-5-carboxanilide) was used for determination of cell proliferation in reaction which was similar to MTT assay. However, XTT assay showed highest efficient in assessing the viability of cells. The concentration determined by absorbance at 570 nm. XTT assay

were developed by introducing positive or negative charges and hydroxy groups to the phenyl ring of the tetrazolium salt that better with sulfonate groups (DMSO) added directly or indirectly to the phenyl ring. Moreover, the advantage of these compounds is that viable cells convert non-soluble formazan to water-soluble formazan which less step reduce of solubilization of product when compared with MTT assay.

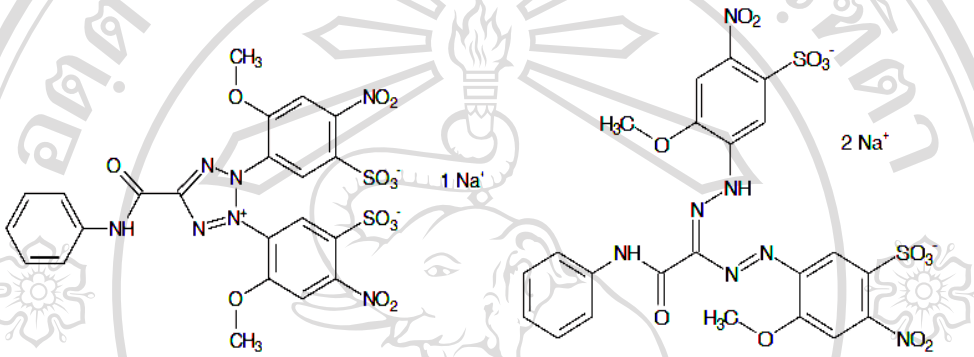

Figure 1.12 Structures of XTT and formazan

1.2.3.3 Sulforhodamine B (SRB) assay

Sulforhodamine B (2-(3-diethylamino-6-diethylazaniumylidene-xanthen-9-yl)-5-sulfo-benzene sulfonate) assay is used for cell density determination which is based on the measurement of cellular protein contents (Skehan *et al.*,1990). SRB dye binds to basic amino acids of cellular proteins and colorimetric evaluation provides an estimate of total protein mass which is related to cell number. SRB assay is performed at absorbance 585 nm. The advantages of this method are better linearity, high sensitivity and stable (Papazisiz *et al.*, 1997).

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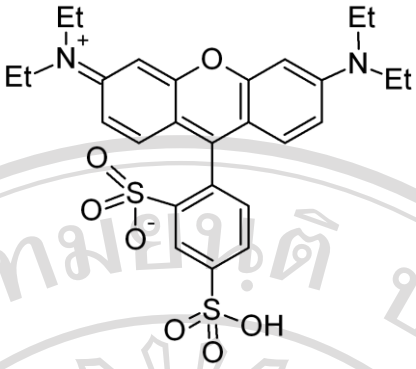

Figure 1.13 Chemical structure of sulforhodamine B

1.2.3.4 Dye exclusion assay

Dye exclusion assay is used for determination of the number of viable cells which present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cells do not. The viable cell will show a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm under light microscope. However, this method is still very labour-intensive and subject to observer error. These problems can be overcome with the developed assay based on the reduction of MTT to a formazan by living cells but not by dead cells (Freimoser *et al.,* 1999).

1.2.3.5 Dye inclusion assay

Dye inclusion assay is provided the quantitative estimation of the number of viable cells in a culture. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. The cells were measured by absorbance at 550 nm and calculated the % cell viability.

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1.2.4. Mutagenicity assay by Ames test

Mutations can be caused by copying errors in the genetic material during cell division and by exposure to radiation, chemicals and viruses which can occur randomly under cellular control during the processes such as meiosis or hypermutation. Mutagenicity test aims to the proposed drug or chemical of inducing DNA damage (mutation) such as inducing alterations in chromosomal structure or promoting changes in nucleotide base sequences. Mutagenicity test is carried out *in vitro* and *in vivo* such as Ames test.

Ames test is widely known as bacteria reverse mutation assay for identifying substances that can produce genetic damage and leads to gene mutation. *Samonella typhimurium* that different mutations in various genes in histidine operons were commonly used in the test. These bacteria had no synthesized the required amino acid, histidine and therefore unable to grow and form colonies. When, adding the mutagen, the bacteria can restore the gene's function to synthesize histidine, cell grow and form colonies. Moreover, the experiment are carried out in both presence and absence of S9 microsomal fraction (S9 fraction) and co-factor (NADH and NADPH) which are used for stimulation of the metabolic system in the bacterial cells. The genotypes of the *Samonella* strains are shown in Table 1.1. The bacterial strains were different in characteristic mutation in *his*G, *his*D and *his*C such as bacterial strain TA98 was mutated *hisD3052* that caused base-pair substitution mutations in a nitrogen base of triplet codon histidine gene. For example, a leucine (GAG/CTC) was substituted by proline (GGG/CCC) in histidine gene. The deletion or insertion of *hisG46* in bacterial strain TA100 has caused frameshifts mutations of nucleotide sequences. (Mortelmans *et al*., 2000).

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Table 1.1 The genotypes of the *Samonella* strains which were used in Ames test.

Allele	Strain	DNA target	Reversion event
hisG46	TA 100 TA 1535	-G-G-G-	Base-pair substitution
hisD3052	TA 98 TA 1538	-C-G-C-G-C-G-C-G-	Frameshifts
hisC3076	TA 1537	+ 1 frameshift (near –C-C-C- run)	Frameshifts
hisD6610	TA 97	-C-C-C-C-C-C- (+ 1 cytosine at run of C's)	Frameshifts
hisG428	TA 102 TA 104	TAA (ochre)	Transitions/transversions

1.2.5 Antimutagenicity assay

Antimutagen is an agent that can reduce the number of spontaneous or induced mutations. It can prevent the mutagen from interact with DNA such as chemical, enzymatic inactivation, reduce the adverse effects of mutagens by modulating cellular changes induced after DNA damage. Antimutagenicity assay was modified from the bacterial mutation assay or Ames test has been reported.

In 2006, Tate *et al.* have investigated on the effect of blackberry extracts on UVC-induced mutagenesis in *S. typhimurium* TA100. The eight ripe blackberries (Arapaho, Chester, Chickasaw, Choctaw, Hull, Kiowa, Navajo, and Triple Crown) extracts were prepared (Tate *et al.*, 2006). The Ames test was performed to measure the rate of reversion mutation of *S. typhimurium* TA100. The test was used as an initial screen to determine the mutagenic potential of new chemicals or compounds. After addition of blackberry extracts to UVC-induced *S. typhimurium* TA100, it was

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found that Arapaho and Choctaw blackberry extracts had no significant effect on mutagenesis with 2% and 7% of suppression, respectively. Whereas, Navajo and Chester extracts showed significant inhibitory of mutagenesis; equally 76 % suppression. The results implied that some varieties of blackberries contained natural substances that might inhibit error-prone DNA repair after UVC-induced mutagenesis.

Cariño-Cortés *et al.* (2007) have studied on antimutagenicity of *Stevia pilosa* and *Stevia eupatoria* by using the Ames test. *S. pilosa* and *S. eupatoria* are plants which grow from the southwestern of The United State to northern Argentina. These plants are used for various purposes in traditional medicine as herbal remedies for stomachache, and diuretic, antiinflammatory and antihypertensive agents (Cariño-Cortés *et al.*, 2007). Methanol extracts from leaves, root and flowers of the two species were used for the Ames test in with and without metabolic activation. It has been revealed that inhibitory effect of both species on the mutagenicity induced by 2-aminoanthrace (2-AA) in the stain *S. typhimurium* TA98. The best antimutagenic effect was found in leaves of both species and the flowers of *S. eupatoria* (99%). Moreover, the flower extract from *S. pilosa* and the root extract from *S. eupatoria* showed about 93% of inhibition when using *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine as a mutagen in *S. typhimurium* TA100 strain. In addition, the radical oxidation potential of the extracts with 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method was also examined. It was revealed that DPPH radicals were reduced by all extracts which acted as antioxidants with an efficiency of more than 90% reduction.

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1.2.6 Tyrosinase activity in melanogenesis mechanism

Melanogenesis takes place in the melanosome which is a distinct cytoplasmic organelle of melanocyte. This process is the production of melanin. Melanin is the pigment in our hair, skin, and eyes that protects us against sunlight damage. Two major types of melanins are produced by mammalian melanocytes such as eumelanins (black or brown) and pheomelanins (yellow-red). Eumelanins are produced in the course of oxidation of tyrosine (and/or phenylalanine) to *o*-dihydroxyphenylalanine (DOPA) and dopaquionone which further undergoes cyclization to 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (del Marmol & Beermann, 1996). Whereas, pheomelanins (yellow-red) are initially synthesized the same pathway as eumelanins, but DOPA undergoes cysteinylation, directly or by the mediation of glutathione. The end product of this reaction, cysteinyl DOPA, further polymerizes into various derivatives of benzothiazines as shown in Figure 1.14

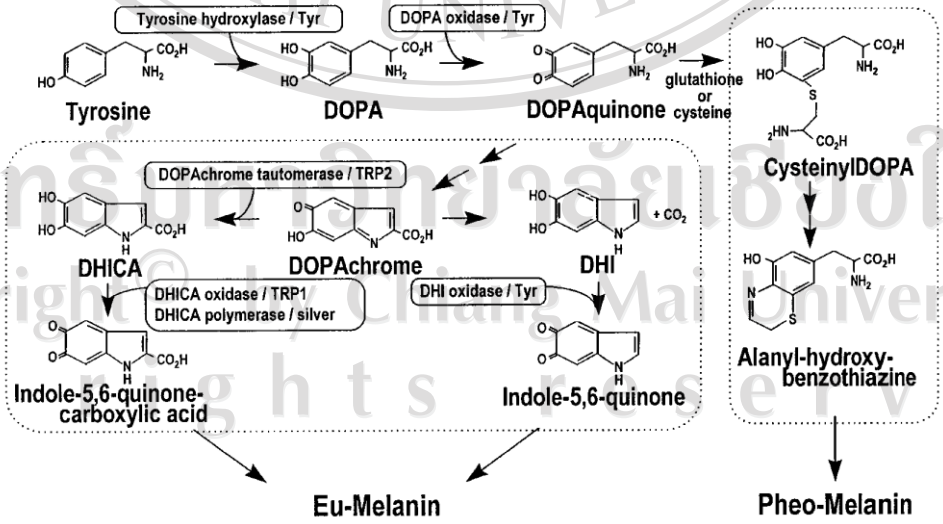

Figure 1.14 Eumelanins and pheomelanins biosynthesis pathway

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Tyrosinase (EC 1.14.18.1), a copper-containing monooxygenase, is the most important enzyme in mammalian melanin synthesis which catalyzes the rate-limiting step of melanin biosynthesis. Abnormal tyrosinase production is involved in the levels of melanin accumulation. Tyrosinase inhibitors are important constituent of cosmetic products and depigmenting agents for the treatment of hyperpigmentation. In recent years, the searching for inhibitors of melanin product from natural medicine to develop the constituent of cosmetic product was increasing.

In 2003, Miyasawa and co-worker (Miyasawa *et al.*, 2003) were investigated on the inhibitor of tyrosinase activity in black rice bran. The extract was prepared in methanol and re-extracted with hexane, chloroform, ethyl acetate and water. From the result, the ethyl acetate extract showed the % inhibition tyrosinase activity value at 80.5% with concentration of 0.4 mg/ml. The fraction which consisted of protocatechuic acid, methyl ester and procatechuic acid showed % inhibition tyrosinase activity value at 75.4% and 60.1%, respectively.

In 2011, Fujii and co-worker (Fujii *et al.*, 2011) have studied on inhibition tyrosinase acivity effect of rose hip (*Rosa canina* Linn.) on melanogenesis in mouse melanoma cells and on pigmentation in brown guinea pigs. It was found that the extract of rose hip was inhibited the intracellular tyrosinase activity in dose-dependent manner. The extract showed the % inhibition tyrosinase activity value at 71.7 % with concentration of 250 µg/ml. Moreover, the extract of rose hip also showed inhibitory effect on UVB-induces pigmentation in guinea pigs skin.

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1.2.7 Morphological changes of UV treated cell lines

The morphology changes can observe by electron microscopy (EM) such as the cells undergo in apoptosis in comparison to necrosis, or the morphological changes of dermis after induced by UV or chemical.

In 2009, Kitajima and Yamaguchi (Kitajima and Yamaguchi *et al.*, 2009) have studied on the effects of silybin treatment on keratinocytes and stimulated the expression of basement membrane component proteins. Silybin is a plant-derived flavonoid from *Silybum marianum*. The keratinocytes were treated with botanical extracts and their morphological changes were observed and compared with retinoic acid-treated. Retinoic acid (RA) has been effective for improving wrinkles on skin; however, it has also been reported that RA induced skin irritation. It was found that silybin inhibited confluent-induced keratinocyte differentiation and modulates the production of basement membrane components like RA. These results strongly suggest that silybin might be a potential agent for the prevention and safe treatment of skin aging and wrinkling.

Recently, Seo *et al.* (2010) have investigated on the anti-photoaging effect of fermented rice bran extract on UV-induced normal skin fibroblast. It was reported that co-fermentation of rice bran with *Lactobacillus rhamonsus* and *Saccharomyces cerevisiae* significantly reduced the melanin synthesis of the resulting extract to B16F1 melanoma cells. The effect of fermented rice bran extracts (FRBE) on fibroblast skin cell against UVB-induced damage *in vitro*, human skin fibroblast was studied. It was revealed that rate of cell growth was reduced upon exposure of fibroblasts to UVB radiation (30 mJ/cm^2) and the cells did not recover after FRBE

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treatment. The results implied that FRBE did not induce morphological changes and necrosis in a dose-dependent manner after UVB treated.

1.3 Research objectives

1.3.1 To investigate cytotoxicity of Thai plant extracts in normal and cancer mouse fibroblast skin cell lines

1.3.2 To evaluate mutagenic activity of Thai plant extracts and inhibition of mutagenicity by Thai plant extracts

1.3.3 To investigate antityrosinase activity of Thai plant extracts

1.3.4 To investigate recovery effect on morphological changes which were induced by UVA and UVB of Thai plant extracts in normal and cancer mouse fibroblast skin cell lines

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