CHAPTER 4

DISCUSSIONS & CONCLUSIONS

4.1 Discussions

The five plant extracts consisted of *T. chebula* Retz., *T. bellerica, E. elatior* (Jack) R.M. Smith, *R. damascena* and *R. kerrii* Meijer were prepared and supplied by S&J International Enterprise Pubic Company Limited. These plants were found in Thailand and some have been reported to possess some striking biological activities. In this work, the plant extracts in hydroglycol which are commonly used in cosmetic ingraedint were investigated for their biological activities. The advantages of hydroglycol are colourless and odourless with boiling point higher than 180°C and freezing point lower than 30°C which is better than that of water and ethyl alcohol. In addition, hydroglycol has humectants and antimicrobial properties, and its safety has been reported for use in food, medicines and cosmetic applications. (Chulasiri *et al.*, 2011).

In this work, 70% hydroglycol was used as solvent for *T. chebula* Retz. and *T. bellerica* extraction, whereas 50% hydroglycol was used as solvent for *E. elatior* (Jack) R.M. Smith, *R. damascena* and *R. kerrii* Meijer extractions. The extracts were investigated for their physical characteristics such as color and the precipitation of plant extracts. Five kinds of plant extracts were suspended liquid and contained small

amount of precipitation in hydroglycol ranging from light brown to dark brown (Table 3.1). The plant crude extracts were studied for their biological activities without purification. Next, the five plant extracts were examined for the constituents by using Ultraviolet-visible spectrophotometer (UV-Visible), Infrared spectrometer (IR) and Nuclear magnetic resonance (NMR).

The UV spectrum of five extracts showed maximum absorbance (λ_{max}) at between 260-279 nm (Figure 3.2). The results showed that the extracts of *T. chebula* Retz., *T. bellerica*, *E. elatior* (Jack) R.M. Smith, *R. damascena* and *R. kerrii* Meijer showed maximum absorbance (λ_{max}) at 260, 275, 279, 263 and 271 nm, respectively. The results suggest that the extracts might contain phenolic and glycoside compounds as similar to previous reports of the five plant extracts. All plant extracts were examined by Infrared spectroscopy (IR). It was found that the extracts showed the highly absorption in the ranging from O-H stretching of solvent and aromatic compounds (Figure 3.3 A-E). The properties of the plant extracts were examined by Nuclear magnetic resonance (NMR). The results were showed in Figure 3.4 A-F. All the extracts contained unsaturated protons and glycosides. The result suggested that the five plant extracts contained the aromatic compounds which are phenolic compounds and glycosides that commonly occurred in natural products (Ersöz *et. al.*, 2002; Dai *et al.*, 2010).

Phenolics compounds have been considered as powerful antioxidants such as capable of scavenging reactive oxygen species (ROS), reactive nitrogen species (RNS) and chelating transition metal ions, leading to oxidation of a varieties of biomacromolecules, such as enzymes, proteins, DNA and lipids (Svobodova *et al.*, 2003; Michalak *et al.*, 2006; Dejeridant *et al.*, 2007 and Dai *et al.*, 2010). There are

many methods to study, the antioxidant properties of phenolic compounds such as Trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), ferric ion reducing antioxidant power (FRAP), cupric ion reducing antioxidant capacity (CUPRAC) assays and Folin-Ciocalteu method (Huang *et al*., 2005). In this study the Folin-Ciocalteu method was choosen for determination of total phenolic content due to the advantages of Folin-Ciocalteu method are commonly accepted assay and long-wavelength absorption of the chromophore minimizes interference from the sample colors (Huang *et al*., 2005 and Waterhouse *et al.,* 2002). The extract of *R. kerrii* Meijer showed the highest total phenolic content followed by *T. chebula* Retz., *T. bellerica* and *R. damascena*, which were quantified as 112.40 ± 0.08, 101.18 ± 0.07, 94.37 ± 0.03 and 34.23 ± 0.01 mg GAE/g of wet weight, respectively. The extract of *E. elatior* (Jack) R.M. Smith showed the lowest the total phenolic content as 14.90 ± 0.02 mg GAE/g of wet weight.

To evaluate the safety of all five plant extracts, MTT assay and dye exclusion method were performed to investigate the cytotoxic effect of the extracts by using normal mouse fibroblast L929 and mouse melanoma B16F10 cell lines. Both methods were calculated the cell viability. MTT assay was used in this study to determine the mitochondrial reductase activity in active cell lines (Mosmann *et al.,* 1983). Whereas, the dye exclusion method was used for determination of number of viable cells present in a cell suspension after adding trypan blue. According to results from MTT assay, all extracts showed low cytotoxicity on both fibroblast cell lines with 50% cytotoxicity (CD_{50}) values at ranges between 5.43 ± 0.18 and 39.39 ± 0.21 mg/ml for normal fibroblast cell lines but more cytotoxicity in mouse melanoma fibroblast cell

line. All of the extracts showed cytotoxicity with CD_{50} values at ranges between 2.00 ± 0.18 and 58.23 ± 0.18 mg/ml except the extract of *E. elatior* (Jack) R.M. Smith which showed more cytotoxicity against normal fibroblast cell lines rather than mouse melanoma fibroblast cell line. When comparison of these results, it was found that the extract of *T. bellerica* showed the highest cytotoxicity with CD_{50} value at 2.00 ± 0.18 mg/ml as shown in Table 4.1. From the result, the extract has affected on morphology of mouse melanoma B16F10 cell line which could be due to the active compounds that present in the extract such as gallic acid that exhibited anti-proliferative and inducred apoptotic death in various cancer cell lines in previously report (Yoshioka *et al.,* 2000, Kaur *et al*., 2005 and Pinmai *et al.,* 2008)

From dye exclusion assay, the extracts have shown similar cytotoxic effects as that of MTT assay. The extracts showed cytotoxicity with CD_{50} values in the range of 8.73 ± 1.40 – 22.93 ± 0.50 mg/ml for normal mouse fibroblast cell line and showed cytotoxicity with CD_{50} values in the range of 1.75 ± 0.63 – 43.17 ± 0.14 mg/ml in mouse melanoma fibroblast cell line as shown in Table 4.1. The extracts of *T. bellerica* showed the highest cytotoxicity followed by *E. elatior* (Jack) R.M. Smith and *R. damascena* in normal mouse fibroblast cell lines, respectively. Whereas, the extracts of *T. chebula* Retz. showed the highest cytotoxicity in mouse melanoma fibroblast cell line followed by *T. bellerica, E. elatior* (Jack) R.M. Smith and *R. damascena*, respectively. However, it has been noticed that the dark brown color and suspension of *T. chebula* Retz. and *R. kerrii* Meijer extracts may interfere on cell number determination in some cases especially in high concentrations of the extracts.

Table 4.1 Cytotoxicity of plant extracts on normal mouse fibroblast L929 and melanoma fibroblast B16F10 cell lines expressed as 50% cytotoxic dose (CD_{50}) values which were obtained in MTT assay and dye exclusion method.

Plant extracts	50% cytotoxic dose (mg/ml)			
	MTT assay		Dye exclusion	
	L929	B16F10	L929	B16F10
T. chebula Retz.	11.96 ± 0.80	4.35 ± 0.33	ND	1.75 ± 0.63
T. bellerica	5.43 ± 0.18	2.00 ± 0.18	8.73 ± 1.40	8.73 ± 1.56
E. elatior (Jack) R.M. Smith	19.85 ± 0.65	58.23 ± 0.18	10.92 ± 3.15	10.92 ± 4.20
R. damascena	17.13 ± 0.39	10.04 ± 0.24	22.93 ± 0.50	43.17 ± 0.14
R. kerrii Meijer	39.39 ± 0.21	26.47 ± 0.47	ND	ND

ND: not determine

When compared the result of cytotoxicity in both methods, the extract of *T. bellerica* showed the highest cytotoxicity amount all of the extracts for both cell lines in the MTT assay and normal mouse fibroblast L929 in the dye exclusion assay. *T. chebula* Retz. extract showed the highest cytotoxicity for melanoma fibroblast cell line in the dye exclusion assay. The cytotoxic dose of *E. elatior* (Jack) R.M. Smith extract in both cell lines was similar in the dye exclusion assay. On the other hand, the cytotoxic dose of this extract in the MTT assay showed higher cytotoxicity against normal fibroblast cell lines rather than mouse melanoma fibroblast cell line. The extract of *R. damascena* showed more cytotoxicity against normal fibroblast cell lines

than mouse melanoma fibroblast cell line in the dye exclusion assay which was contrast with the result of MTT assay.

In this study, the cytotoxic effects of the extracts on cell viability were investigated by two different methods: Trypan blue dye exclusion method and mitochondrial function (MTT assay). Cell viability assay using trypan blue dye exclusion method is more suitable to cells grown in suspension than to monolayers because dead cells can detach from monolayers and are therefore lost from the assay. This reason could justify the low CD_{50} values obtained by trypan blue dye exclusion method when compared to the other cytotoxicity evaluation methods. In some cases, the higher concentration of the dye or the concentration of the extracts may affect on morphological alterations and counting error due to the color of the extracts (Wilson *et al*., 2000). On the other hand, MTT assay is probably the most commonly used colorimetric indicators of cell viability. The advantage of MTT assay is easy to perform, and more reliable system. The MTT assay for cytotoxicity evaluations are objective and it can be automated using a personal computer with a microplate reader (Takeuchi *et al.*, 1991). In this study, the trypan blue dye exclusion methods were inappropriate for cytotoxicity evaluation of *T. chebula* Retz. and *R. kerrii* Meijer according to non detectable cell viability after treated with both extracts. Whereas, MTT assay was more reliable than dye exclusion methods when *T. chebula* Retz. and *R. kerrii* Meijer were tested. Moreover, the differences were found may be the function of time in which the extracts were incubated and contacted to the cell lines. (Wilson *et al.,* 2000; Samee *et al.,* 2002 and Andrighetti-Fröhner *et al.,* 2003).

The *T. bellerica* extract showed the highest cytotoxicity on both melanoma and normal fibroblast cell lines when compared with the five plant extracts by MTT

assay. According to Khosit and co-workers investigation, the *T. bellerica* extracts showed cytotoxicity on hepatocellular carcinoma and lung cancer cell lines rather than normal cells (Khosit *et al.,* 2008). Moreover, it has been reported the extract exhibited anti-proliferative effects in several cancer cell lines including Shiongi 115, breast cancer MCF-7, prostate cancer PC-3 and DU-145cells (Kaur *et al.*, 2005).

The *T.chebula* Retz. extract showed the second highest in cytotoxicity value on both melanoma and normal fibroblast cell lines when compared with the five plant extracts in MTT assay. According to Saleem and co-workers investigation, 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 and 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 (Saleem *et al*., 2002). Moreover, in the previous report, the *T.chebula* Retz. extract exhibited several pharmacological effects including antifungal, antiviral, anti-tyrosinase, anti-inflammatory and anti-mutagenic effect (Kaur *et al.,* 1998; Cheng *et al.,* 2003; Lee *et al.,* 2005; Reddy *et al.,* 2009 and Manosroi *et al.,* 2011). Furthermore, the *T.chebula* Retz. and *T. bellerica* extracts inhibited Eph tyrosine kinase receptors and their ephrin ligands which played a central role in several human cancer cells (Mahamed *et al.,* 2011).

The *R. damascena* extract showed the third highest cytotoxicity value on both melanoma and normal fibroblast cell lines when compared with the five plant extracts

by MTT assay. The *R. damascena* extract was used in perfumery and cosmetic industry. In addition, the extract was reported for treatment of in human health and biochemical activities such as antitussive, anti-nociceptive, hypoglycemic, bronchodilator, anti-inflammatory and antibacterial (Zargari *et al.*, 1992, Shafei *et al.*, 2003, Rakhshandeh *et al.*, 2008, Gholamhoseinian, *et al*., 2009 and Boskabady *et al.*, 2006). In recent study, it was found that the extract showed no cytotoxicity on human monocytic leukemia cell line (U937) when compared doxorubicin which was used as an anti-cancer chemotherapy drug (Kalim *et al.*, 2010).

The *R. kerrii* Meijer extract showed the less cytotoxicity on both melanoma and normal fibroblast cell lines when compared in the five plant extracts by MTT assay. The *R. kerrii* Meijer has no reported in biological activity due to the plant is a preserve. Whereas, *E. elatior* (Jack) R.M. Smith showed higher cytotoxicity on normal fibroblast cell lines rather than mouse melanoma fibroblast cell line. In order to confirm the cytotoxicity of the five plant extracts, the cytotoxicity was determined in sulphorhodamine B (SRB) or the higher eukaryotic cells system. The cytotoxicity data of the five extracts themselves have intrinsic value in defining toxic effects and are also important for designing more in biological activity.

Next, five extracts was also investigated for its mutagenic properties using Ames test (Mortelmans and Zeiger *et al.*, 2000). The Ames test was performed in histidine-dependent strains of *Salmonella typhimurium* TA98 for detection of frameshift mutations of *hisG46* and *S. typhimurium* TA100 for detection of base-pair substitution mutations of *hisD3052* in the presence and absence of metabolic activation (S9 microsomal fraction). The evaluation of mutagenicity was important as initiation test for complex mixtures because of the possibility that one or more

components (such as phenolic compounds) in the extracts that provided protection against chemically induced mutagenesis due to of phenolic compounds has affected on cell function in different mechanisms (Carino *et al.*, 2007). Initiation stage, phenolics may inhibit activation of procarcinogens by inhibition on phase I metabolizing enzyme, such as cytochorme P450, and also facilitated detoxifying and elimination of the carcinogens by induction of phase II metabolizing enzymes such as glutathione S-transferase (GST), NAD(P)H quinine oxidoreductase (NQO), and UDP-glucuronyltransferase (UGT) that may also limit the formation of the initiated cells by stimulating DNA repair (Kaur *et al.*, 1998; Hodek *et al.*, 2002 and Webster *et al.*, 2003). In previous report the flavonoids in plant extracts may also directly protected DNA form the eletrophilic methabolie of the mutagens (Marnewick *et al.*, 2000). In this study, mutagenicity of *T. chebula* Retz., *T. bellerica*, *E. elatior* (Jack) R.M. Smith, *R. damascena* and *R. kerrii* Meijer extracts had no mutagenic effects on *S. typhimurium* strain TA98 and TA100 in the presence or absence of metabolic activation.

For, antimutagenic activity of the five extracts against 2-AA and AF-2 mutagens in *S. typhimurium* strain TA 98 and TA100 were shown in Table 3.10-3.19. The extracts were also examined for the 50% inhibitory concentration values of the plant extracts (IC_{50}) which were represented for inhibition of mutagenic properties as shown in Table 3.20-3.21. The extracts of *T. chebula* Retz., *T. bellerica* and *R. kerrii* Meijer showed the strongly antimutagenic activity. The antimutagenicity of *T. chebula* Retz. was similar results to the previous report from Kaur and co-workers which studied hydrolyzable tannin from the fruit of *T. chebula* Retz. (Kaur *et al.*, 1998). Moreover, *T. bellerica* and *R. kerrii* Meijer extracts may contain active

compounds such as hydrolyzable tannin (Pfundstein *et al.*, 2010 and Kanchanapoom *et al*., 2007) which exhibit anti-mutagenicity in *S. typhimurium* strains TA98 and TA 100 induced with 2AA and AF-2. Whereas, the extracts of *R. damascena* and *E. elatior* (Jack) R.M. Smith did not showed the antimutagenic activity on both bacterial *S. typhimurium* strains in the presence and absence of metabolic activation. These results indicated that the *R. damascena* and *E. elatior* (Jack) R.M. Smith extracts might contain the active compounds which acted as co-mutagen with 2-AA and AF-2 mutagens in both bacterial strains or antimicrobial activity of *R. damascena* extract (Özkan *et al.*, 2004). The antimutagenicity of the *R. damascena* and *E. elatior* (Jack) R.M. Smith extracts have not been observed. In order to confirm the co-mutagen activity of the five plant extracts, the Ames test in higher eukaryotic cells such as micronucleus test (MNT) in mammalian cells should be further investigated (Cherdshewasart *et al.*, 2008 and Speit *et al.*, 2009).

The plant extracts were investigated for tyrosinase inhibitory activity by using the Dopachrome method. In this experiment, 3,4-Dihydroxy-L-phenylalanine (L-Dopa) was used as a substrate for tyrosinase assay. Tyrosinase is one of the key enzymes in mammalian melanin synthesis which catalyses the rate-limiting step of melanin biosynthesis (Chang *et al.*, 2009). In this study, all five plant extracts showed tyrosinase inhibitory activity. The results tyrosinase inhibitory activity of *T. chebula* Retz. and *E. elatior* (Jack) R.M. Smith extracts were similar to Manosroi (2011) and Chan (2008) reports, respectively (Manosroi *et al.*, 2011 and Chan *et al.*, 2008). The extract of *R. kerrii* Meijer showed the highest tyrosinase inhibitory activity which could be due to the active compounds content that presented in the extract. These phenolic compounds might contribute to the highest inhibitory activity of tyrosinase

in *R. kerrii* Meijer extract. According to Sakuma and co-worker study on the relationship between chemical structures and tyrosinase inhibitory action, the result revealed that the phenol derivatives containing of 2 or 3 hydroxyl groups have been reported to show oxidation-reduction potential in melanin biosynthesis (Sakuma *et al.,* 1999). In irreversible inhibition, covalent binding with the enzyme may cause its inactivation by altering the active site of the enzyme and/or by conformational changes to the protein molecule. Further studies are required to measure enzyme inhibition kinetics using Lineweaver-Burk plots with varying concentrations of L-DOPA as the substrate to classify the tyrosinase inhibitory activity as competitive, uncompetitive, mixed type and non-competitive inhibitors (Chang *et al.,* 2009 and Smit *et al.,* 2009).

Ultraviolet (UV) radiation in solar light has affected on growth and metabolic activity in organism such as induce production of reaction oxgen specices (ROS) that interact with proteins, lipids, and DNA thus altering cellular function (Heo *et al.,* 2010). In present, the skincare products contained plant derived as antioxidant ingredients have proven to be useful for the prevention of UV damage (Seo *et al.,*2010). In the previous report, it has been reported of plant extracts to protect UV damage such as rice band extract (Seo *et al.,* 2010), sulforaphane from broccoli extract (Shirasug *et al.,* 2010) and diphlorethohydroxycarmanol from algae (Heo *et al.,* 2010). In the present study, five plant extracts were investigated for their effect on morphological changes after treated with UVA and UVB radiation using normal and melanoma mouse fibroblast cell lines. The cell lines were exposured to UVA radiation with 0.30 J/cm^2 for 22 min 44 sec and UVB radiation with 30 mJ/cm^2 for 6 min 45 sec.

The results revealed that all the extracts have no effect on morphological recovery in both fibroblast cell lines after exposed to UVA and UVB irradiation. Moreover, the cell morphology of UV treated controls in both cell lines did not change upon treatment with UV when compared with untreated UV control group. The possible explanation of this is that the limitation of the UV light sources and the condition in UV irradiation equipment was not suitable for cell cultivation system such as the level of carbon dioxide (CO_2). For further study, the effect of the extract on morphological changes in both fibroblast cell line before treated with UV radiation should be performed for further study.

4.2 Conclusions

The biological activities of Thai plant extracts of *T. chebula* Retz., *T. bellerica*, *E. elatior* (Jack) R.M. Smith, *R. damascena*, and *R. kerrii* Meijer were investigated for cytotoxicity, mutagenicity, antimutagenicity, antityrosinase and morphological changes on cell lines after induced by UV radiation to screen for novel botanical cosmetic ingredients. This study suggested that the extracts of *T. chebula* Retz., *T. bellerica* and *R. kerrii* Meijer showed high potential applications for cosmetics according to their low cytotoxic effect, antimutagenicity and antityrosinase activities The extract of *R. damascena* showed low cytotoxic effect, antityrosinase activities. Whereas, *E. elatior* (Jack) R.M. Smith extract showed low cytotoxic effect and antityrosinase activities as similar to the result of *T. chebula* Retz., *T. bellerica*, *R. damascena*, and *R. kerrii* Meijer extracts. In contrast, *E. elatior* (Jack) R.M. Smith extract showed co-mutagen activity with 2-AA and AF-2 mutagens in both bacterial strains. In order to confirm the co-mutagen activity of the extracts, the

antimutagenicity test in higher eukaryotic cells such as mammalian cells and will be further investigated. The results suggested that the phenolic compounds in the extracts showed inhibitory effect of mutagens except the extracts of *E. elatior* (Jack) R.M. Smith and *R. damascena*. On the other hand, the phenolic contents of five plants extract were showed tyrosinase inhibitory activity. Moreover, the extracts have no effect on morphological recovery after treated with UVA and UVB radiation that the limitation of the UV light sources and the condition in UV irradiation equipment. All the biological activities of five plant extracts including cytotoxicity, mutagenicity, antimutagenicity, antityrosinase and morphological changes on cell lines after induced by UV radiation were shown in Table 4.2.

Table 4.2 Biological activity of five plant extracts

Plant extracts	Cytotoxicity (CD_{50} mg/ml)				Total Phenolic contents (mg /g)	Mutagenicity (87.34 mg/plate)				Antimutagenicity (IC_{50} mg/plate)				Anti-tyrosinase (IC_{50} mg/ml)	Morphological			
	MTT		Dye exclusion			TA98		TA100		TA98		TA100			UVA		UVB	
	L292	B16 F10	L292	B16 F10		+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9		L292	B16 F10	L292	B16 F10
***T. chebula* Retz.**	11.96	4.35	ND	1.75	101.18	-	-	-	-	+	+	+	+	39.96	-	-	-	-
T. bellerica	5.43	2.00	8.73	8.73	94.37	-	-	-	-	+	+	+	+	20.07	-	-	-	-
***E. elatior* (Jack) R.M. Smith**	19.85	58.23	10.92	10.92	14.90	-	-	-	-	-	-	-	-	20.00	-	-	-	-
R.damascena	17.13	10.04	22.93	43.17	34.23	-	-	-	-	-	-	-	-	13.00	-	-	-	-
***R. kerrii* Meijer**	39.39	26.47	ND	ND	112.40	-	-	-	-	+	+	+	+	1.27	-	-	-	-
	ND = not determined					(-) = extract showed no mutagenicity (+) = extract showed mutagenicity				(-) = the extract showed no antimutagenic activity (+) = the extract showed antimutagenic activity					(-) = the extract has no effect on morphological changes of cell line (+) = the extract has slightly effect onmorphological changes of cell line			