CHAPTER 5

Discussion

The efficiency of anticancer and antioxidant activities of *M. oleifera* and *P. palatiferum* leaf extracts was examined both *in vitro* and *in vivo*. Discussions on the results of these two aspects are presented in this chapter.

5.1 Phytochemical components and safety evaluation of the extracts

5.1.1 Phytochemical Components

Phytochemical analysis of *M. oleifera* and *P. palatiferum* extracts was simple chemical reactions and the results were colors or precipitate which indicated the main groups of substances. *P. palatiferum* extract was found to contain more cardiac glycoside, terpenoid and saponin than the *M. oleifera* extract. These compounds are effective on the blood circulation system and have antioxidant activity (Tepe *et al*., 2005). *M. oleifera* extract contained high amount of flavonoid, phenolic, steroid, reducing sugar and anthraquinones. They are good for health and could inhibit free radicals, anti-inflammation, anti-bacteria and reduce blood pressure (Ferguson *et al*., 2004; Chumark *et al*., 2008 and Yammuenart *et al*., 2008).

In the old days, pharmaceutical utilization of medicinal plants was carried out by inherited traditional methods. At present, however, there have been attempts to look for scientific evidence to support the use of medicinal plants for utmost benefit. Research on the chemical constituents in the plants provides important and useful information since the therapeutic properties of medicinal plants depend on the activities of the chemical components in each plant. Sometimes the activities are due to synergetic property of many chemicals. Application of medicinal plants in the treatment of

diseases comparable with modern medicine should be under the quality control. Therefore, chemical components particularly the active ingredients should be elucidated. Research on the chemical composition must be carried out. Study on the phytochemical constituents and their bioactivities is important not only for the treatment of diseases but also for adding the value to those medicinal plants. Phytochemial compounds of current interest are phenolic compounds and flavonoids since they have a wide range of bioactivities especially the antioxidant activity (Ghasemzadeh *et al.*, 2010). It is well known that free radicals cause many serious diseases such as cancer and coronary heart disease (Pham-Huy *et al*., 2008).

5.1.2 Safety evaluation of the extracts

The bacterial mutation test is primarily used to confirm the gene safety of the substances before other tests, e.g. anticancer, were performed. (Ghazali *et al*., 2011). From our results, *M. oleifera* and *P. palatiferum* extracts had no mutagenic effect on *S. typhimurium* TA98 whether the activating enzyme was used or not. There had been a report that aqueous extract of *M. oleifera* at 0.2-5.0 mg/pl had no mutagenic effect on *S. typhimurium* TA98 and TA100 (Charoensin and Wongpoomchai, 2010). In this study, although higher concentrations of aqueous ethanol and hexane extracts (25-100 mg/ml) were used, mutation did not occur. Moreover, *P. palatiferum* extract at the same range of concentration was not able to induce bacterial cell mutation. It is, therefore, confirmed by our results that the aqueous ethanol and hexane extracts from *M. oleifera* and *P. palatiferum* leaves were not the mutagenic substances.

Our genes are prone to come in contact with toxic substances at all time either from the external environment or in the body. We are therefore at more risk for various diseases from the environment. However, our body has some genetic repair mechanisms which normalize the body. Inefficient repair will lead to permanent abnormality and result in many illnesses. Research on the mutagenic activities of different substances in the environment will give a clue to toxic substances against the genes which will reduce the risk of contact or intake of these substances. For example, to find out whether the medicinal plant in use is toxic to a gene, could be done by testing the mutagenic activity on the bacteria. It is mostly used in the analysis of genetic mutagens because it is simple

and convenient such as modified AMES test (Maron and Ames,1893; Matsushima *et al*, 1978). Its principle is to induce *Salmonella typhimurium* which could not synthesize an amino acid histidine and could not grow in the medium without histidine to mutate and synthesize histidine and is capable to grow in the non-histidine medium. The colonies which appear are mutant colonies, the number of which varies with the activity of the mutagen. The metabolism of toxic substance could be simulated in mutating the DNA as it may occur in the body. It is therefore a well known method and recommended as a preliminary test to obtain a basic information before a long term examination for anticancer activity.

5.2 Investigation of the antiproliferative effect on colon cancer cell lines

MTT assay was used for toxicity test on the cancer cells. MTT will be changed to purple color compound, formazan by mitochondrial dehydrogenase which is active in the living cells (Mosmann, 1983). This purple compound is dissolved in DMSO and the intensity of color is directly proportional to the amount of living cells. The concentration of the extract giving 50% cancer cell survival (IC_{50}) is the comparative value of the extract in inhibiting the cancer cells. In this study, each extract inhibited colon cancer cell proliferation differently. This might be due to the difference in cell properties and the phenotypic and genotypic characters of the three types of cells as well as the active constituents in the crude extract from each part of the plant or from different plants as suggested by Tima *et al* (2010). Moreover, the use of different solvents might give different proportions of bioactive substances which in turn, give different antiproliferative activity. Aqueous extract was used in the present study because the two plants are usually consumed with water e.g. hot soup or tea infusion. As for ethanol and hexane extracts, there are many reports on its effective inhibition of cancer cell proliferation (Padee *et al*., 2009: Tima *et al*., 2010). From the result of cell viability (Figure 6, 7 and 8), it was shown that the *M. oleifera* extracts had better antiproferative activity on all types of cells than the *P. palatiferum* extracts. Moreover, the aqueous ethanol and hexane extracts of *M. oleifera* had better antiproliferative effect on all types of tested colon cancer cell lines than other plant extracted with the same solvent e.g. *M. hortensis* which has higher IC_{50} than that of *M. oleifera* (Tansuwanwong

et al., 2007). Although the *P. palatiferum* extract did not show high activity on antiproliferation of cells, it had a tendency to inhibit colon cancer cell proliferation of all types. Therefore, it is possible to purify the extracts and analyze the active ingredients in order to develop into colon cancer drugs or dietary supplement for combined use with other chemotherapeutic agents in the future. Moreover, the ability of each extract to differently inhibit cell division and multiplication (Figures 7, 8 and 9) may depend on the main active substances in the plants and the solvents. For examples, ethanol extract of *M. oleifera* contains more flavonoids than that of *P. palatiferum* or anthaquinones are found only in the *M. oleifera* extract. There was a report on the cell toxicity test of ethanol extract which gave IC_{50} only 0.32 µg/ml for myeloma cell line and the activity depended on the concentration of the extract (Parvathy and Umamaheshwari, 2007). However, the solvent used was different from that in the present research and the tested cells were cell suspension which were entirely different from the colon cancer cells. Besides, it was reported that many compounds from the methanol extract of *M. oleifera* seeds i.e. methyl N-{4[(α-L-rhamnopyranosyl) benzyl]} carbamate, 1-*O*-phenyl-α-L-rhanopyranoside, 4-((β-D-glucopyranosyl) -(1→3) – (α -L-rhamnopyranosyl)] phenylacetonitrile, methyl N-{4[(α-L-rhamnopyranosyl) benzyl]} cabamate and methyl N-{4[(4'-*O* acetyl-α-L-rhamnopyranosyl) benzyl]} cabamate at the concentration of 100 ppm, did not show any toxicity on cells and inhibition of cell division against many cancer cells e.g. MCF-7 (mammary cancer cells) SF-268 (central nervous system cells) NCI-H460 (lung cancer cells) and HCT-116 (colon cancer cells) (Francis *et al.*, 2004). Extracts of *M. oleifera* bark with ethanol and water were also tested on the inhibition of lung cancer cell (COR L-23) and prostate gland cancer cell (PC-3) division. The IC_{50} was found to be more than 50 µg/ml indicating no inhibiting activity (Costa-Lotufo *et al*., 2005; Saetung *et al*., 2005; Ahmed *et al*., 2011). Nevertheless, there was a report on the toxicity test of aqueous extract of *M. oleifera* leaves on the cervical cancer cells (HeLa) (Nair and Varalakshmi, 2011) but it was not toxic against the leucocytes. Therefore, this extract exhibited therapeutic anti-cancer activity without damaging the leucocytes which are important for the immune system. As for *P. palatiferum*, although there are many reports on the lowering of blood sugar and its use in the alternative medicine for diabetes patients (Chayarop *et al*., 2010; Khonsund *et al*., 2011) but reports on anti-cancer are very few. Mai *et al* (2011) isolated

pure compounds i.e. lignans and palatiferins from *P. palatiferum* which exhibit moderate toxicity on cancer cells 2KB and HepG2 but botutin and lupeol show better anti-cancer activity against the two types of cells. There was a report that a plant in the same family i.e. *Pseuderanthemum carruthersii* contains anti-cancer substance against HeLa cervical cancer cells and mammary cancer cells MCF-7 (Vo *et al*., 2012). However, some substances from this research were able to inhibit cell division according to the level of concentration but were limited by the time which the activities exist. It was supported by Prasad *et al* (2008) who found that lupeol, a pure compound, was able to inhibit the multiplication of PC-3 cells on the concentration but not on the time basis. Thus the concentration-dependent activity, not the time –dependent, could be explained by the pharmaco-kinetic mechanism of the active substance which depends on the concentration (Ruth *et al*., 2005). In this study, the phytochemical substances extracted from *M. oleifera* and *P. palatiferum* with water, ethanol and hexane varied with the kind of substances and different solvents used (Table 3). These phytochemical substances had been reported to have inhibitory or preventive activity on cancer. Their mechanism, once enter the body, is to promote some enzyme actions. Some enzymes deactivate the carcinogens which enter the body (Bunpo *et al*., 2005). Moreover, all the extracts of *M. oleifera* and *P. palatiferum* leaves at 1000 µg/ml were able to inhibit cell division of colon cancer cells HCT15, SW48 and SW480 independent of the increasing time (Figures 7 (A, E, F); 8 (D, E, F) and 9 (A, B, D, F). This might be due to the different concentrations of the extracts at various time could totally inhibit the cell division. Therefore, the dose of the extract should be increased to see if the inhibition still exist. If similar result is obtained, indicating that the extract is still effective in inhibiting cell division and the concentration in the range 500-1000 µg/ml may be additionally investigated. This tendency to inhibit cancer cell division is the primary mechanism indicating that the plant extract may stimulate or send signal via any mechanism to interfere with the cell division.

The induction of cell cycle arrest, cell apoptosis and DNA fragmentation have been suggested for mechanism of action of plant extracts in cancer cell inhibition (Ma *et al*., 2006; Tian *et al*., 2007). Besides, the research has been explored in the molecular level in some plant extracts. Ze *et al*., (2005) reported the cytotoxicity of three cycloartane triterpenoids isolated from the *Cimicifuga dahurica* on solid tumor (HepG2), blood tumor (HL-60) and drug resistant tumor (R-HepG2) cell lines with the suggestion that those three compounds possessed potentially cytotoxic activitiy on cancer cell lines via apoptosis and G2/M arrest which was resulted from the inhibition of cdc2 protein expression. Some medicinal plants such as *Elaeagnus glabra* and *Pluchea indica,* showed the inhibitory effects on cancer cell invasion, primarily via suppression of activity of matrix metalloproteinase (MMP, type 2 and 9), the enzymes involved in cancer invasion and metastasis activity (Ohtsuki *et al*., 2008; Li *et al*., 2009). Although this research is only a preliminary study and only antiproliferative activity with MTT assay was studied, its results could support the folk use of *M. oleifera* and *P. palatiferum* as anticancer plants. Furthermore, this study is a promising first step towards further investigation concerning the anticancer efficiency of these 2 plant species. The combination of MTT with SRB assay to confirm their antiproliferative activity, the comparison of their ability to synthetic anticancer drug and the determination of mechanisms of action as conducted in other plant species (Prasad *et al*., 2005; Manosroia *et al*., 2006; Prasad *et al*., 2010; Sowemimo *et al*., 2011) should be investigated in our extensive researches.

5.3 Determination of antioxidant property

5.3.1 Test of antioxidant activities in the plant extracts

Since it was found that leaf extracts of *M. oleifera* and *P. palatiferum* have inhibitory potential on the colon cancer cell division, it would be interesting to know whether these two medicinal plants have antioxidant potential. There are some reports that many medicinal plants such as *Andrographis paniculata, Centella asiatica, Curcuma longa, Semecarpus anacardium* and *Aloe vera* have antioxidant property and inhibit various cancer cells (Tansuwanwong *et al*., 2007). Therefore, the extracts of *M. oleifera* and *P. palatiferum* were tested for the amount of antioxidant by ABTS and

DPPH radicals methods including total phenols and vitamin C. It was found that the activities of *M. oleifera* and *P. palatiferum* extracts were in the similar degree. *In vitro* analysis based on the mechanism of single electron transfer by ABTS and DPPH methods indicated that the total antioxidant ABTS was 0.003-0.018 TEAC µg/mg extract and DPPH was 0.66-1.51 GAE µg/mg extract (Table 7) which were less than ABTS activity of methanol extracts (5.18±0.19 TEAC µg/mg extract) and aqueous extract (4.26±0.43 TEAC µg/mg extract) from the roots of *Uvaria rufa* Blume and DPPH radical of methanol extract (8.60±0.04 GAE µg/mg extract) and ethyl acetate (9.61±0.25 GAE µg/mg extract) from *T. triandra*. This might be due to the lesser amount of phenol in *M. oleifera* and *P. palatiferum* extracts than that in the root extract of *Uvaria rufa* Blume. There is a report that phenol is more sensitive in the elimination of free radicals than other bioactive substances (Tiwari, 2005). In the present study, phenols from *M. oleifera* and *P. palatiferum* extracts were 1.66±0.33 – 2.45±0.84 µg/ml and 0.97±0.04 – 1.61±0.41 µg/ml respectively whereas the phenol from *Uvaria rufa* Blume ethanol and aqueous root extracts were 29.43±0.12 µg/ml and 24.17±0.69 µg/ml respectively. The disadvantage of ABTS and DPPH methods is that ABTS and DPPH radicals are not natural precursors for free radicals in the cells or body (Thaipong *et al*., 2006). Therefore Oxygen Radical Absorbance Capacity Assay (ORAC) and Total Radical-trapping Antioxidant Parameter (TRAP) methods are interesting since analyses of hydrogen atom transfer and use of peroxy radical are closer to body condition than using ABTS or DPPH radicals (Phan *et al*., 2005) methods. However, these two methods are easily done with common instruments. So they are well known methods for testing natural antioxidant activity. The total content of phenolic compounds from *M. oleifera* and *P. palatiferum* extracts were 0.97±0.04 – 2.45±0.84 µg/ml and vitamin C content was 1.18±0.22 – 11.87±1.30 µg/ml. *M. oleifera* extract contained highest amount of vitamin C, i.e. 11.87±1.30 µg/ml (Table 7) This is an advantage since most people prefer the form of infusion drink which is an easy and convenient mean of consumption to obtain the value of the extract. The ethanolic extract of *M. oleifera* contained highest total phenol i.e. 11.87±1.30 µg/ml (Table 7). Since its chemical components are flavonoid and polyphenol which have hydroxyl (-OH) group (highly polar functional group), these substances are then dissolved in the highly polar solution (Iris and Strain, 1996). Consequently, the ethanolic extract shows good antioxidant

activity. This is in accordance with the work of Dorman *et al*. (2003) on the medicinal plant extracts with high phenolic content that they are also very active in eliminating ABTS and DPPH radicals. Vitamin C is an antioxidant which plays an important role in getting rid of ROS in plants which have their own eliminating mechanism (Asada, 1999). Superoxide dismutase catalyzes reactive O_2 to H_2O_2 (Ushimaru *et al*., 2006) and H_2O_2 is eliminated by combining with vitamin C and converting to water, whereas vitamin C is converted to monodehydroascorbate by the action of ascorbate peroxidase (APX). In order to conserve the vitamin C level in the cells, monodehydroascorbate is reduced by monodehydroascorbate reductase to vitamin C but a part of it is changed to dehydroascorbate (DHA). In this step DHA reductase (DHAR) catalyzes DHA to vitamin C again (Bode *et al*., 1990). Although, the *in vitro* test shows antioxidant activity of *M. oleifera* and *P. palatiferum*, it does not mean that consumption of these two medicinal plants could eliminate free radicals in the body. Therefore, to prevent, treat and cure the disease effectively, determination of the level of over oxidative condition in the cells or body must be carried out by measuring the antioxidative ability or the amount of important biomolecules which are destroyed or damaged from the oxidized condition of the body or contain excess of free radicals. It is essential to conduct the *in vivo* test to confirm the antioxidative property of the plants.

5.3.2 Effect of antioxidant activities in laboratory rats

Although *M. oleifera* and *P. palatiferum* exhibited antioxidant activity in the *vitro* experiment, it does not mean that the consumption of these two plants will be able to eliminate the free radicals in the body. Therefore, for effective prevention, treatment and therapeutic measures, the level of over oxidative condition in the body has to be examined. This could be determined from the antioxidative ability or the amount of key biomolecules damaged from the oxidation or the over presence of free radicals. In the assessment of antioxidant activity *in vivo*, the aqueous extracts of *M. oleifera* and *P. palatiferum* leaves were used since the aqueous extracts exhibited good antioxidant capacity *in vitro*. Additionally, the most popular consumption ways are in the form of drink or boiled soup. In this research, the aqueous extracts of *M. oleifera* and *P. palatiferum* were found to reduce MDA which is the product of lipid peroxidation in the experimental rats given the extracts continuously for 60 days. It is indicated that

they could inhibit the free radicals either through the preventive antioxidant mechanism and inhibit scavenging antioxidants or the chain breaking antioxidant reaction is terminated (Dizdaroglu and Karakaya, 1999). There are enzymic systems and natural processes in the body to control free radicals and prevent them from damaging the cells or tissues (Baskin and Salem, 1997). Since the experiment was done in healthy animals without any limited condition such as inducing stress or injury. Thus, the healthy body has an equilibrated antioxidants system which is mostly enzymes e.g. SOD, CAT and GSH-Px (Artur *et al*., 1992). Moreover, Anila and Vijayalakshmi (2003) found that the flavonoids from *Mangifera indica* and *Emblica officinallis* extracts enhanced the efficiency of SOD and CAT activity in the experimental rats. In the present work, it was found that SOD level in the red blood cells increased and correlated with a decrease in MDA. SOD is an important enzyme for free radical inhibition (Dekker *et al*., 1996). The increase in SOD level with an increase in the dose of the extracts was probably due to the presence of certain substances which promote the synthetic efficiency of SOD in the erythrocytes. Due to the high antioxidative efficiency of SOD, its increase in the body resulted in a better removal of lipid peroxidation reaction as seen from the subsequent reduction of MDA in the experimental rats receiving the plant extracts. (Figure 9 and 10).

Good antioxidants should be able to be absorbed or transferred into the cells with sufficient concentration to exert their effect. They should be highly specific in combining with the free radicals and eliminate them completely and have no impact on gene expression (Baskin and Salem, 1997). The extracts should be concentrated enough to have the antioxidant activity. If the concentration is not high enough, the antioxidant might not exhibit or show less activity. However, good antioxidants must have low concentration but if the concentration is increased, they might turn to be synergistic to oxidation (Rajalakshmi and Narasimhan, 1996). In this study, the extracts were employed in 4 doses. *M. oleifera* extract of 180 mg/kg BW and *P. palatiferum* extract of 15 mg/kg BW were efficient as antioxidants.

Prevention of cancer includes the induction of cell cycle arrest, causing apoptosis and inhibiting intracellular signaling of cell multiplication. The main inhibitory activity at this stage is the inhibition of cyclooxygenase 2 (COX-2) (Chun

and Surh, 2004; Dannerberg *et al*., 2005) and inhibition of transcription factor activity: activator protein-1 (AP-1) or nuclear factor-kappaB (NF-kB) (Bharti and Aggarwal, 2002; Surh, 2003; Bode and Dong, 2004). There are reports that many plants could inhibit cancer via the mentioned mechanism e.g. roots of *Rhinacanthus nasutus* (L.) Kurz contain antioxidative substances such as flavonoids, anthraquinones, triterpenes, sterols and naphthoquinones as well as inhibitory activity on cervical cell division (HeLaS3 cells). They also cause apoptosis, nuclear fragmentation, DNA ladder, inhibit cell cycle at G2 and M stages and enhance caspase-3 action (Gotoh *et al*., 2004). *Michelia compressa* is able to induce more cells at G0/G1 stage or less at S stage because it induces hepatic cancer cells Hep G2 and SK-Hep-1 to enter G1 stage and inhibits DNA synthesis. Thus, cell multiplication is inhibited (Hsieh *et al*., 2005). Woo *et al*. (1997) had reported that *Michelia compressa* contain antioxidants such as 7-oxoaporphin and alkaloids. *Allium sativum* Linn. is able to inhibit the reduction of hydrogen peroxide production or reduce secretion of TNF-∞ as well as inhibit activator protein-1 (AP-1) and c-Jun N-terminal kinases (JNKs) which control protein synthesis. The production of NF-KB is then reduced (Erikssona *et al*., 2005). There was a report that *Allium sativum* Linn. contains active substances such as allicin and S-allylmercaptocysteine which are antioxidants (Kamanna and Chandrasekhara, 1982). Essack, (2006) studied the anti-cancer activity of extracts from plants in the Family Menispermaceae against 3 types of cancer cells i.e. Hela, CHO and MCF-7 and found that the main substances obtained destroyed the majority of cancer cells by induction of apoptosis. He also reported that these plants contained antioxidants such as alkaloid, triterpene and flavonoids. Antioxidants which inhibit cancer cells e.g. flavonoids are effective against prostaglandins, cyclin D1 and cyclooxygenase 2 (COX-2), reduce apoptosis. They also inhibit caspases, p21, p27, Bax; reduce angiogenesis by lowering VEGF, PDGF, FGF; reduce the distribution of cancer cells by affecting the molecules of ICAM-1, uPA, tPA, MMP and TIMP. Moreover, the flavonoids also inhibit kinase which affects the survival of cancer cells i.e. PI3K, Fyn, JAK1, Akt, MKK4, Raf1 and inhibit kinase which affects the distribution of cancer cells i.e. MEK1 and MEK4 (Banjerdpongchai, 2012). It is therefore possible that the efficiency in the inhibition of colon cancer cell division is due to the antioxidant activity of *M. oleifera* and *P. palatiferum*. The experimental results indicated that *M. oleifera* contains many antioxidants such as flavonoids, vitamin C,

total phenols and phenolic whereas *P. palatiferum* contains flavonoids, saponin, cardiac glycoside, terpenoid and reducing sugar. These substances have antioxidant activity, reduce cell degeneration and may have a role in preventing cancer. The extracts from these two plants are rich in flavonoids and other antioxidants. They should be promoted as health promoting products in the future. When these two plants are developed as medicine and gained popularity or are widely used, they will later become economic plants