CHAPTER III

MATERIALS AND METHODS

Chemicals and Materials

Trolox standard (6-hydroxy-2,5,7,8-tetramethylchoman-2-carboxylic acid), 99% caffeine anhydrous, 98% gallic acid monohydrate, 90% (−)-epicatechin, were purchased from Aldrich (Milwaukee, WI). Biorad protein reagent was obtained from Bio-Rad (Hercules, CA). Tween 20, dimethyl sulfoxide, phosphate buffer saline (PBS) and methanol (HPLC grade) were obtained from Fisher Scientific (Hanover Park, IL). Quercetin-3-β-glucoside (>90%), 97% myricetin, were purchased from Fluka (Milwaukee, WI). Formic acid (>96%, ACS grade), gallic acid, 95% rutin hydrate, (−)-caffeic acid, quercetin dihydrate, rutin hydrate (>95%), 90% kaempferol, folin-ciocalteu's phenol reagent, 2,2-diphenyl-1-picryl hydrazyl approx. 90% (DPPH), penicillin-streptomycin, RNase A, trilox X-100, Eagle's modified minimum essential medium (EMEM), trypsin-EDTA, were obtained from Sigma (St. Louis, MO). 0.4% Typan blue stain, bovine serum, Eagle's Minimum Essential Medium (EMEM) were obtained from Gibco BRL (Grand Island , NY). Certified molecular biology agarose was obtained from Bio-Rad (Hercules, CA). The cell counting kit-8 (CCK-8) was from Dojindo Molecular Technologies (Gaithersburg, MD). Vybrant® FAM Poly Caspases Assay Kit was obtained from Invitrogen (Invitrogen Corporation, OR). The primary and secondary antibody of topoisomerase IIα, $p21^{Cip1/Waf1}$, $p27^{Kip1}$, β-actin, western blot's reagent and enhance chemiluminescence kit was from Amersham Life Sciences. A topoisomerase drug screening kit and human DNA

topoisomerase IIα were purchased from TopoGEN, Inc. (Columbus, OH).All the other materials used were obtained from Sigma (St. Louis, MO).

Methods

Plant Material

Fresh mulberry (*M. alba* L.) leaves were collected from the plantation field of the Silk Innovation Center of Mahasarakham University, Mahasarakham Province, Thailand. The leaves were cleaned, dried in a hot air oven at 60 °C and ground to fine powder in a mill. The resulting powder was then passed through an 80 mesh sieve and kept without expose to sunlight in a sealed aluminium bag at 4 °C until further use.

HepG2 Culture and Incubation Conditions

Human hepatoma carcinoma cells (HepG2, ATCC HB-8065) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Normal human liver cells (fresh primary human hepatocytes, Lot number H1013) were purchased from ADMET Technologies, (Durham, NC). HepG2 and normal human liver cells were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum and 100 IU penicillin/100 μg streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Cells were cultured in T75 flasks and were seeds once a week. Culture medium was changed three times a week.

Preparation of Aqueous and Organic Extracts of Mulberry leaves

The aqueous extract of mulberry leaves was prepared from 2.0 g leaf powder soaked in 200 mL boiling water for 10 min. The mixture was cooled down to room temperature before filtration using Whatman paper No 1 and then lyophilized. The freeze-dried solid extract was kept at -20 °C in plastic tubes protected from light. The solid extract was re-dissolved in double distilled water, filtered with a 0.22 µL syringe top filer prior to use in all assays. For preparation of organic extracts, their solvent extraction was carried out according to the method describe previously by Katsube *et al.*, 2006. Briefly, two grams of dried powdered mulberry leaves were extracted separately with 20 mL of various organic solvents (50% methanol, 100% methanol and 1-butanol), 3 h in the dark at room temperature, on a multi-magnestir platform. Each extract was separated by centrifugation (13,000 g, 10 min), the supernatant was removed, and the residue was re-suspended with 20 mL of the same solvent and the mixture was again separated by centrifugation. The two resulting supernatant were then combined and concentrated under vacuum to dryness and the residue was stored in the dark at -20 °C until analysis of chemical constituents. For the bioassays, the residue of each extract was redissolved in 1 mL of DMSO before use.

Total Polyphenol Content

The total polyphenol contents of various solvent extracts were measured as described by the modified Folin-Ciocalteu method (Nurmi *et al.*,1996). Briefly, 1 mL 1 N Folin-Ciocalteu reagent was added to a 1 mL sample and this mixture was allowed to stand for 2–5 min before the addition of 2 mL 20% Na_2CO_3. The solution was then allowed to stand for 10 more min before measuring the absorbance at 730 nm in a Beckman DU® 640 spectrophotometer (Fullerton, CA). The total

polyphenol content was expressed as milligram equivalents to the three standards used per milliliter aqueous extract or gram dried leaves. Equations obtained for standard curves were: $y = 0.0248x + 0.0036$, $r^2 = 0.98$ for gallic acid ; $y = 0.0107x - 0.0004$, $r^2 = 0.99$ for chlorogenic acid and $y = 0.0136x - 0.0026$, $r^2 = 0.99$ for rutin (where, y; absorbance at 730 nm; x: polyphenol concentration; r^2: correlation coefficient)

Characterization of Phenolic Compounds by HPLC-MS Analysis

HPLC-MS analysis of composition of phenolic compounds was determined according to method described previously by Yousef *et al.*, 2004. Five mg of each extracts sample (crude 100% methanol, 50% methanol, 1-butanol and hot water extracts) was dissolved in 1 mL aqueous methanol (1:1, v/v) and used for HPLC-MS analysis. Commercial standards were prepared at a concentration of 0.5 mg/mL in 50% methanol. The HPLC-MS analyses were performed with an LCQ Deca XP mass spectrometer (Thermo Finnigan Corp., San Jose, CA), MS version 1.3 SRI, electrospray ionization (ESI) in the positive ion mode (m/z 150-2000), with a photodiode array (PDA) detector (3-channels, 260, 280 and 360 nm), version 1.2 and autosampler version 1.2 and Xcalibur software for data processing. The HPLC separations were carried out on a 150 × 2.1 mm i.d., 5-μm Agilent Eclipse XDB C-18 column (Vydac, Western Analytical, Murrieta, CA). The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). A step gradient of 0%, 5%, 50%, 80%, 100% and 0% of solvent B at 3, 4, 30, 42, 50 and 58 min, respectively, a flow rate of 200 μL/min, and injection volume of 10 μL were employed. The injected volume contained approximately 50 μg of each sample or 5 μg each standard. The identification of compounds present in the tea sample was

based on the molecular weight, retention times, and UV-VIS spectra, comparisons with know standards and referenced form literature.

DPPH Radical Scavenging Activity

The ability of each extract to scavenge DPPH radical was determined by method as described previously (Cardador-Mart'inez, Loarca-Pína, and Oomah, 2002). Briefly, each samples or standard was added to a well in a 96-well flat-bottom visible light plate containing 200 μL of 150 μM DPPH solution (in 80% v/v aqueous methanol). 22 μL of samples were prepared in triplicate for each of four concentrations used. The plate was then covered and left in the dark at room temperature. After 10, 20, 30, 40, 50 and 60 min, absorbance at 520 nm was measured in the plate was read with an automatic reader Elx80810 ultra microplate reader (Biotek Instruments, Winooksi,VA) using the K.C. Junior computer program, exported, and analyzed in Microsoft Excel. A plot of $A_{520\ nm}$ and concentration was made for each time interval. The sample concentration with initial absorbance closest to that of the blank (DPPH + solvent) was chosen for final calculation of antiradical (ARA) activity defined by the following equation of Burda and Oleszek, 2001.

ARA = 100 x (1 − Absorbance of sample/Absorbance of control)

Antioxidant Capacity Assay

Total antioxidant capacity was determined by using the DPPH modified trolox equivalent antioxidant capacity (TEAC) as reported (Cardador-Mart'inez, Loarca-Pína, and Oomah, 2002). This assay which is based on the reaction of DPPH with trolox was used to compare between the radical scavenging activity of a compound and that of trolox, a water-soluble vitamin E analogue (Pietta, Simonetti, and Mauri,

1998; van den Berg, Haenen and Bast, 1999). Each sample or standard (chlorogenic acid, rutin, quercetin or kaempferol) (22 μL) was added to a well in a 96-well flat-bottom visible light plate containing 200 μL150 μM DPPH solution (in 80% v/v aqueous methanol). The plate was covered and left in the dark at room temperature. Trolox solutions in the 50-400 μM concentration ranges were employed for calibration. The decrease in absorbance at 520nm 30 min after addition of a compound was used for calculating the TEAC. The TEAC value which is an indicator of the antioxidant capacity of the sample relative to Trolox on a molar basis was calculated as follows (van den Berg, Haenen and Bast, 1999):

$$TEAC_{sample} = \Delta A_{sample}/(slope * [sample])$$

where ΔA_{sample} is the decrease in absorbance of the sample over 30 min and [sample] is the concentration of the sample in μM. The TEAC values were converted to mM TEAC/ g of sample.

Superoxide Radical Scavenging Activity

Superoxide radical scavenging activity was determined by using the modified riboflavin–light–NBT system as reported (Beauchamp and Fridovich, 1971). The assay was based on the capacity of the extracts to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in the presence of the riboflavin–light–NBT system. Briefly, each 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, NBT (75 μM) and 1 ml of each extract. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min illumination from a fluorescent lamp. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes

with reaction mixture were kept in the dark and served as blanks. The percentage inhibition of superoxide generation was measured by comparing the absorbance values of the control and those of the reaction mixture containing sample solution.

Hydroxyl Radical Scavenging Activity

The scavenger activity of each extract to scavenge hydroxyl radical was determined by method as described previously (Racchi *et al.*, 2002). This assay is based on the inhibition of the degradation of deoxyribose caused by the attack of hydroxyl radicals. Briefly, in the final volume 1.2 mL, the reaction mixture contained the following reagents at the final concentrations: $FeCl_3$ (25 μM) premixed with EDTA (100 μM) in KH_2PO_4/KOH buffer (pH 7.4) , 2-deoxy-D-ribose (2.8 mM), H_2O_2 (2.8 mM), ascorbic acid (100μM), and 12 μL of extract, or KH_2PO_4/KOH buffer (control sample). Samples were placed in a water bath at 37 °C for 1 h, and then 1 mL of 1% thiobarbituric acid and 1 mL of 2.8% trichloroacetic acid were added. The reaction mixture were heated in water bath at 80 °C for 20 min, kept in ice for 5 min, and then centrifuged for 5 min at 3000 rpm to separate the particles. The absorbance of the supernatant wad read in a spectrophotometer at 532 nm against a solution prepared as described but without ascorbic acid (blank) to correct for interference due to the sample color and thiobarbituric acid-reactive substances that might occur in the extracts.

The scavenger activity was expressed as the percentage inhibitory activity (IA%) of degradation of deoxyribose in the presence of the extract, relative to that the control sample (without the extract) using the following equation:

$$\text{IA\%} = 100 - \frac{\text{absorbance extract x 100}}{\text{absorbance control sample}}$$

Lipid Peroxidation Assay

The lipid peroxidation assay of the extracts was measured as described by the modified method from Choi *et al.*, 2002. Briefly, the reaction mixture contained 500 µL linoleic acid (20 mM), 500 µL Tris-HCl (100 mM, pH 7.5), 100 µL $FeSO_4.7H_2O$ (4 mM) and 100 µL of each extract. Linoleic acid peroxidation was initiated by addition of 100 µL of ascorbic acid (2mM), incubated for 30 min at 37 °C and terminated by the addition of 1 mL of 2.8% trichloroacetic acid. 1mL of the mixture was added with 250 µL of thiobarbituric acid in 50 mM NaOH, followed by heating for 10 min and then centrifuged for 5 min at 3000 rpm to separate the particles. The absorbance of the supernatant wad read in a spectrophotometer at 532 nm against a solution prepared as described but without ascorbic acid (blank) to correct for interference due to the sample color and thiobarbituric acid-reactive substances that might occur in the extracts.

The scavenger activity was expressed as the percentage inhibitory activity (IA%) of degradation of linoleic acid in the presence of the extract, relative to that the control sample (without the extract) using the following equation:

$$\text{IA\%} = 100 - \frac{\text{absorbance extract x 100}}{\text{absorbance control sample}}$$

Cell Viability

Cells were counted prior to all assays to ensure cell viability. This procedure used trypan blue, which stains only dead cells to differentiate between dead and alive cells. A cell suspension consisting of 50 μL cells suspended in media, 450 μL trypan blue was prepared. Ten μL of this cell suspension was added to each side of a hematocytometer and 5 squares per side were counted using a light microscope (Nikon Diaphot, Frank E. Fryer Co. Scientific Instruments, Canpentersville, IL) and the numbers of cells were averaged. To calculate the number of cells per ml the following equation was used:

Average number of cells per square x dilution factor x 10^4

Where dilution factor = 10 and 10^4 represents the volume counted

A dilution was made with media to get the appropriate number of cells for each assay.

Cell Cytotoxicity Assay

Cytotoxicity was determined according to method described previously by Ramirez-Mares, Chandra, and de Mejia, 2004. HepG2 or normal human liver cells undergoing exponential growth were trypsinized, suspended in fresh medium at density of 5 x 10^5 cells/ mL and inoculated in a 96-well plate flat-bottomed plate in volume of 100 μL per well. After 24 h at 37 °C incubation in a humidify atomosphere containing 5 % CO_2. The growth medium was then removed and 100 μL. PBS buffer containing various concentrations of each mulberry extract were added directly into the cells, each one in four replications. Control cultures received the same amount of PBS and blank wells contained serum-free media with no cells. After the sample are incubated under the same conditions for 12 h. At the end of the incubation, the

viability of the cells was determined by a cell counting kit-8 (CCK-8) according to the manufacture's protocol. Briefly, after the 12 h incubation with the treatment, media was removed and the wells were washed with PMS. Fresh media and CCK were added to the wells (100 μL media/10 μL CCK-8) and incubated for 2 h. The plates were read at 450 nm. In EL x 808 IU Ultra Microplate Reader equipped with KC-Junior software from Bio-Tek Instrument Inc. (Winooski, VT). The cytotoxicity of the tested crude extracts was determined by comparing the response of the cells with that of the PBS control. The following parameters were used to determine cytotoxicity as described previously by Monks *et al.*, 1991: IC_{50} (inhibitory concentration, 50%) = concentration resulting in 50%inhibition of net cell growth; IC_{50} = *%T/C*: [OD of treated cells/OD of control cells] x 100 = 50, where OD = optical density, *T* = test optical density, and *C* = control optical density.

Mulberry extracts was dissolved in DMSO at concentration of 250 mg/mL, and diluted in tissue culture medium before use. The concentration of DMSO (1%) did not affect the cell viability.

Cell Cycle Analysis

Flow cytometric analysis was used to evaluate cell cycle distribution of HepG2 using the method described previously (Jia, Han and Chen, 2005). Exponentially growing cells were incubated at density 5 x 10^5 cells/mL in 24 well plates. After cell attachment, the cells were incubated in the presence or absence of various mulberry extracts for 12 h and harvested by trypsinization. The cells were centrifuged at 400 g for 10 min. The medium was removed and then, the cells were rinsed with cold PBS, and processed for cell cycle analysis. Briefly, the cells were resuspended in 50 μL of cold PBS, to which 70% cold ethanol (450 μL) was added

and the cells were then incubated and fixed for overnight at –20 °C. After centrifugation, the pellet was washed twice with cold PBS, suspended in 500 μL PBS. The cells were kept on ice for 10 min and stained with propidium iodide (0.02 mg/mL containing 0.2 mg/mL RNase) at 37°C for 30 min. The cell cycle distribution was determined using a BD Biosciences LSR II analytical flow cytometer (BD Biosciences, San Jose, CA) equipped with BD FACSDiva software.

Assessment of Apoptosis

Flow cytometric analysis was used to evaluate apoptosis analysis of HepG2 using Vybrant® FAM Poly Caspases Assay Kit (Invitrogen Corporation, OR) with FAM-VAD-FMK FLICA reagent, a generic probe for the detection of most caspases. Briefly, HepG2 cells were plated in 6-wells plates at a density 5 x 10^5 cells/mL for 24 h. After cell attachment, the cells were incubated in the presence or absence of various mulberry extracts for 12 h and harvested by trypsinization. After treatments, apoptosis assay was performed according to the manufacture's instruction. The HepG2 cells were trypsinized and centrifuged at 400 g for 10 min. The supernatant was discarded and the cell pellet was washed with cold PBS and re-centrifuged. The cell re-suspension (300 μL) was added 10 μL of 30X FLICA working solution and mixed by flicking tubes. The cells were incubated for 1 h at 37°C and 5% CO_2 in the dark followed by centrifugation at 400 g for 10 min, two times. The supernatant was discarded and the pellet was re-suspended in 1X wash buffer (supplied by manufacture) and centrifuged again. The resulting pellet was finally re-suspended in 400 μL of 1X wash buffer and was stained with propiodium iodide (0.02 mg/mL containing 0.2 mg/mL RNase) for flow cytometric analysis using a BD Biosciences

LSR II analytical flow cytometer (BD Biosciences, San Jose, CA) equipped with BD FACSDiva software.

Human Topoisomerase II Catalytic Activity

Topoisomerase II catalytic activity was monitored via electrophoresis using a topoisomerase II drug screening kit (TopoGEN, Inc., Columbus, OH) as previously reported (Jo, de Mejia, and Lila, 2005). Briefly, 20 μL of reaction mixtures contained 50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, and 0.5 mM dithiothreitol. Supercoiled DNA (pRYG) provided in the kit was determined to be ideal for this assay since it contains a single, high affinity topoisomerase II cleavage and recognition site. After 1 μL (0.25 μg) of pRYG DNA was added, followed by the addition of 2 μL of test sample in solvent (1% DMSO unless specified otherwise), the reaction was initiated by adding 4 units (2 μL) of human DNA topoisomerase II and carried out at 37 °C for 75 min. The reaction was terminated by adding 2 μL of 10% sodium dodecyl sulfate (SDS) followed by digestion with 0.6 μL of proteinase K (1.25 units/μL, 25 μg/μL) at 52 °C for 15 min to degrade enzyme. After 2 μL of loading buffer (0.25% bromophenol blue and 50% glycerol) was added to the mixture, DNA was extracted from the mixture by adding 20 μL of chloroform:isoamyl alcohol (24:1), followed by centrifugation at 10000*g* for 20 s. A portion (15 μL) of the upper blue layer containing extracted DNA was loaded onto 1% agarose gel. Electrophoresis was conducted at 66 V (2 V/cm) for 5 h in TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) using Classic CSSU 2025 Electrophoretic Gel System, E-C Apparatus Corporation (Florida). Supercoiled DNA (pRYG) and relaxed DNA were included in the electrophoresis run as markers for DNA topology. Gels were then stained in 0.5

μg/mL ethidium bromide in distilled water for 30 min and destained for 15 min in distilled water prior to digital image acquisition using Kodak Image Station 440 CF (Eastman Kodak Co.). The optimized lane and band identification was analyzed using KODAK 1D Image Analysis Software version 3.5. The inhibitory activity was expressed as relative activity of topoisomerase enzyme in the presence of the test compound in comparison to that in the negative control solution. Solvent used in dissolving test compounds was added to the negative control solution to get an unbiased measurement of the effect of solvent on enzyme activity.

SDS-PAGE and Western Blot Analysis of the Levels of Topoisomerase IIα, $p21^{Cip1}$/Waf and $p27^{Kip1}$

Control and treated HepG2 cells were plated in T75 flasks at a density 5 x 10^5 cells/mL for 24 h. After cell attachment, the cells were incubated in the presence or absence of 100% methanol mulberry extracts for 12 h. The cells were then harvested by scraping the cells from the cultured flasks using a cell scraper and collected by centrifuge at 400g for 10 min. The cells were washed with ice-cold PBS and the whole cell lysate were then prepared by lysing the cell using sonication. Electrophoresis was carried out in a Phastsystem Kit (Amersham-Pharmacia, Inc., NJ) with an 8–25% polyacrylamide-gradient gel (Pharmacia). Extracted samples were centrifuged at 20,000 g for 15 min to eliminate any precipitate that might have formed during thawing. The supernatant (20 μL, 3 mg/mL) was added to 20 μL of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) with 5% β-mercaptoethanol (Sigma, MO). Samples were boiled for 5 min using the Laemmli buffer system. After the samples and molecular-weight standard had cooled down to room temperature, 30 μL of sample protein extracts were loaded into PhastSystem ready gels. A broad-

range protein standard (Bio-Rad Kaledoiscope prestained) containing pure recombinant proteins of the following molecular weights: 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa was used. The gels were run using the Phastsystem electrophoresis kit (Amersham-Pharmacia, Inc., NJ) at a 55 mA constant, and gels were run for 20 min (60–125 V). For electrophoresis, the gels were fixed for 20 min in methanol/acetic acid/ water (5:5:2) and stained with Coomassie Brilliant G-250 for 1 h. Then, gels were destained with two washes of 5 min with acetic acid/water (40%) and finally, washed once again with deonized water. Stained gels were soaked in 20 mL of blotting buffer at pH 8.3, which consisted of 10% (v/v) methanol, 25 mM Tris base, and 192 mM glycine, for 3 min. The membrane used was the Immune-Blot PVDF membrane. A Western blot sandwich was assembled by placing a sponge, a filter, and the gel, the PVDF membrane, another filter and sponge, avoiding the formation of bubbles. After the transfer was completed, the membrane was then saturated by incubation in 5% nonfat dry milk (NFDM) in 0.01% Tween in TBS (TTBS) buffer for 1 h, followed by probed with primary antibodies specific to topoisomerase II, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ and β-actin overnight at 4 °C in 3% BSA and TTBS buffer for 1 h. The membrane was washed 3 times in TTBS and then incubated for 1 h at room temperature at 1:15000 dilution HRP-conjugated anti-rabbit/mouse IgG (Amersham-Pharmacia, Inc., NJ) prepared in 3% NFDM in TTBS buffer. The membrane was washed 3 times for 5 min with 0.01% TTBS and prepared for detection using an ECL Advance Western Blotting Detection Kit substrate solution (Amersham-Pharmacia, Inc., NJ) following the recommendations of the manufacturer. After the substrate to the blot was added, the intensity of the bands was read in a Kodak Image Station 440 CF (Kodak, Rochester, NY) with a total exposure

time of 2 min and 8 captures. The relative protein level was calculated as the ratio of the optical density of topoisomerase II, $p21^{Cip1/Waf1}$, $p27^{Kip1}$ and that of β-actin.

Statistical Analysis

All values shown represent means ± standard deviation (SD). Statistical analyses for detection of significant differences between the control and treated groups were carried out by a one-way ANOVA followed by Dunnett's multiple comparison tests. A probability (p) value of < 0.05 indicated a significant difference. All other calculations were performed on the Prism 3.0 statistical program.