CHAPTER IV

RESULTS

Yield of Solvent Extracts, Total Polyphenol Contents and Phenolic Profiles

The results of the extract yields and total polyphenol contents obtained from the different extractions are summarized in Table 1. It can be seen that the amount of extractable components expressed as percent dry weight of leaves (DL) from 9.82% of 1-butanol extraction to 16.28% of 100 % methanol extraction. The extractions by 50 % aqueous methanol (15.95 %) and hot water (13.10 %) also gave relatively high yields of the extracts.

In terms of total polyphenol content, the values of the Folin-Ciocalteu determination were expressed as gram equivalents of either chlorogenic acid, rutin or gallic acid per 100 g dry leaves (DL) or dry extract (DE). The results showed that the four extracts had significantly different values of total polyphenol contents (Table 1). The highest value were obtained from the solvent extraction by 100% methanol followed by 50 % aqueous methanol, 1-butanol and hot water, with their relative extractable effectively of phenolics in the ratio of 3.4:1.9:1.0:1.1, respectively. There extractable values appeared to highest when they were expressed based on chlorogenic acid followed by rutin and gallic acid, with the ratio of 2.4:1.9:1, respectively. The ratio should reflect the presence of chlorogenic acid and rutin as major phenolic in the mulberry leaves as reported previously (Yan *et al.*, 2004; Chu *et al.*, 2006)

When the total phenol value was expressed based on the dried weight of its own extract, it appeared that the 100% methanolic extract contained phenolic

compounds (based on chlorogenic acid equivalent) up to 21.6% compared with only 12.1% in 50% aqueous methanol extract 10.5 % 1-butanol extract and 8.3 % hot water extract (Table 1).

A partial characterization of phenolic constituents in each solvent extract of mulberry leaves was conducted in order to compare the phenolic profiles of all the four extracts. This was performed by using a combination of diode array detection (DAD) and positive electrospray ionization mass spectrometry (ESI^+), couple to a HPLC. This HPLC-DAD-MS method allowed accurate structure elucidation of individual phenolics based on their MS and UV characteristics. By using a C_{18} reverse-phase column with the mobile phase consisting of A:water/formic acid (99.9/0/1) and B: acetonitile/formic acid (99.9/0.1) in a step gradient manner from 0% to 100% B in 50 min, a simultaneous separation of about 19 phenolics were obtained as shown by the chromatograms in Figure 9.

Identification of these individual phenolic compounds was achieved by comparing the UV absorption spectra and MS data obtained from this study (Table 2) with these reported in literature (Kim *et al.*, 1999; Nomura, 1999; Kim, Gao, and Kang, 2000; Doi, *et al.*, 2001; Lee, *et al.*, 2002; Chae *et al.*, 2003; Chu *et al.*, 2006).

It can be seen that most of the flavonoids detected were glycosides in which their mass spectra showed both the protonated molecule $[M+H]^+$ and the ion corresponding to the protonated aglycone $[A+H]^+$. The latter is formed by losing of the moieties of glucose, galactose and rhamnose moieties from the glycosides. Thus, based on the structure identification, we concluded that the phenolic constituents in mulberry leaves contained at least two phenolic groups of chlorogenic derivatives and flavonol derivatives (Table 2). Among these, rutin (peak no. 8), isoquercetin (peak no.

9), kaempferol glycosides (peak no. 10, 11) and chlorogenic acid (peak no. 2) appeared to be the major phenolic constituents.

Comparison of phenolic profiles in various solvent extracts, based on the HPLC Chromatograms (Figure 9) showed that both the 100% methanol extract and 50% aqueous methanol extract showed lesser extractable content and the hot water extract, as expected, showed quite different chromatogram.

Table 1 Extract Yields and Total Polyphenol Contents of Different Solvents Extracts of Mulberry Leaves[1].

Extract	Extract Yield[2]	Total phenol					
	% DL	Chlorogenic acid		Rutin		Gallic acid	
		% DL[3]	%DE[3]	% DL[3]	%DE[3]	% DL[3]	%DE[3]
100% Methanol extract	16.28 ± 1.62[a]	3.52 ± 0.44[a]	21.55 ± 0.54[a]	2.78 ± 0.24[a]	17.06 ± 0.23[a]	1.50 ± 0.11[a]	9.23 ± 0.25[a]
50% Methanol extract	15.95 ± 2.19[a]	1.92 ± 0.28[b]	12.05 ± 0.14[b]	1.52 ± 016[a]	9.56 ± 0.31[a]	0.81 ± 0.08[b]	5.12 ± 0.81[b]
1-Butanol extract	9.82 ± 1.29[b]	1.03 ± 0.08[b]	10.50 ± 0.55[b]	0.82 ± 0.14[b]	8.34 ± 0.30[b]	0.44 ± 0.05[c]	4.45 ± 0.40[c]
Hot water extract	13.10 ± 1.26[a]	1.08 ± 0.14[c]	8.27 ± 0.26[c]	0.86 ± 0.03[b]	6.53 ± 0.18[b]	0.46 ± 0.05[c]	3.54 ± 0.21[c]

[1] Values are the average of thee independent tea preparations. All CVs are less than 5.0% and values in the same column followed by different letters are significantly difference ($p \leq 0.05$).

[2]Grams of extract per 100g of (DL) dried leaves

[3]Express as grams of gallic acid, rutin and chlorogenic acid per 100g of (DL) dried leaves or per 100g of (DE) dried extract of extract respectively.

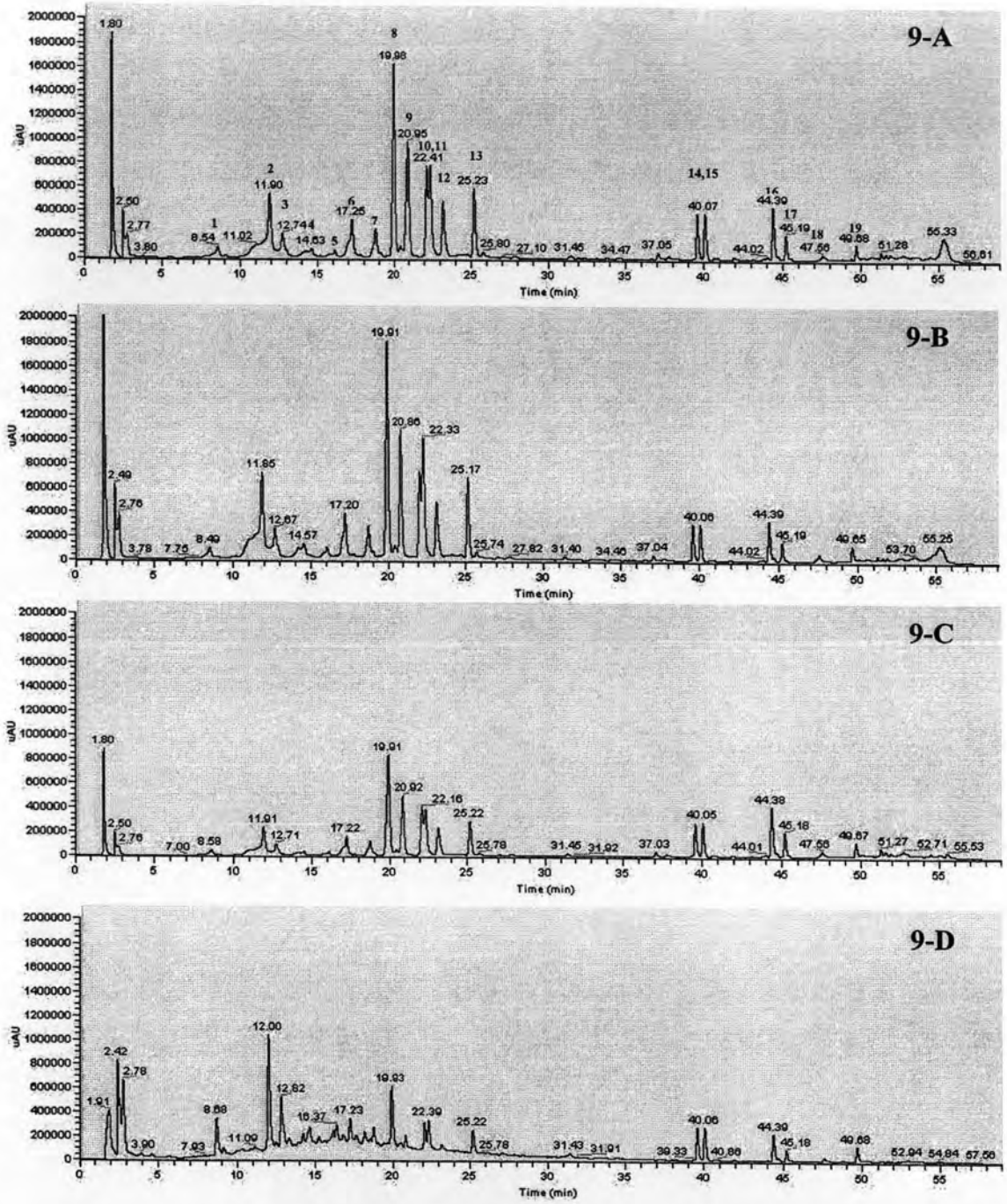

Figure 9 Chomatogram at 260 nm of various mulberry tea extracts as determined by HPLC (9-A) 100% methanol extract, (9-B) 50% aqueous methanol extract, (9-C) 1-butanol extract and (9-D) Hot water extract.

Table 2 LC-DAD-MS Characteristics of Phenols Identified in the 100% Methanol Extract of Mulberry Leaves

Compound	RT (min)	λ_{max}	$[M+H]^+$	Fragment ions	Proposed structure
1	8.4	296, 326	355	163	5-*O*-Caffeoylquinic acid
2	11.8	296, 326	355	163	3-*O*-Caffeoylquinic acid (Chlorogenic acid)
3	12.7	296, 326	355	163	Caffeoylquinic acid isomer
4	14.5	296, 326	538	163, 355	Caffeoylquinic acid derivatives
5	16.0	260, 280, 360	773	303	Quercetin –glucosyl-rhamnosyl-galactopyranoside
6	17.2	266, 314	757	303, 611	Quercetin dicoumaryl glycoside
7	18.6	266, 354	741	287, 611	Kaempferol dicoumaryl glycoside
8	19.9	254, 354	611	303	Quercetin-3-*O*-rhamnosylglucoside (Rutin)
9	20.9	254, 354	465	303	Quercetin-3-glucoside (Isoquercetin)
10	22.2	262, 350	595	287, 449	Kaempferol-3-*O*-rhamnosylglucoside
11	22.4	254	551	303	Quercetin 3-(6-malonylglucoside)
12	23.2	257	449	287	Kaempferol 3-glucoside (Astragalin)
13	25.2	262	535	287	Kaempferol 3-(6-malonylglucoside)
14	40.07	279		293, 391	Unknown
15	40.18	279		293, 391	Unknown
16	44.40	296, 326	677	355, 515	Tricaffeoylquinic acid
17	45.20	296, 326	677	355, 515	Tricaffeoylquinic acid
18	47.56	279	579	291, 411	Procyanidin dimmer B2 [epicatechin-(4β-8)-epicatechin]

Antioxidant Capacity

Free radical scavenging activity of tested samples was measured spectrophotometrically by DPPH assay, superoxide radical-scavenging assay, hydroxyl radical-scavenging assay and lipid peroxidation assay. IC_{50} values (concentration of extracts required to scavenge 50% of free radicals or to prevent lipid peroxidation by 50%) were calculated from the regression equations prepared from the concentrations of the extracts and percentage inhibition of free radical formation/ percentage inhibition of lipid peroxidation in different systems of assay, including DPPH assay, superoxide radical-scavenging assay, hydroxyl radical-scavenging assay and lipid peroxidation assay. IC_{50} values were compared with the IC_{50} value of standard chemical in each system to assess the antioxidant property of mulberry leave extracts (Table 3). A lower IC50 value indicates greater antioxidant activity.

All extracts exhibited the various free radical scavenging. The concentration of an antioxidant needed to decrease the initial free radical concentration by 50% (IC_{50} value) is a parameter widely used to measure antioxidant activity. Its IC_{50} value among mulberry leaves extracts obtained a range of DPPH scavenging activity of 356-743 μg/mL, superoxide radical scavenging of 302-375 μg/mL, hydroxyl radical scavenging of 396-543 μg/mL, and prevention of lipid peroxidation of 442-801 μg/mL

Among those, the 100% methanol extract showed the most scavenging by the lowest value of IC_{50} value followed 50% aqueous methanol, water and 1-butanol extracts, respectively and a dose dependent manner in DPPH assay (Table 3). The results provide a direct comparison of the antioxidant capacity with Trolox. In the TEAC assay, the extracts of mulberry leaves had values range of 2450-622 μM Trolox equivalent.

Table 3 Antiradical Capacity of Different Solvents Extracts of Mulberry Leaves[1].

Sample	Antioxidant Capacity DPPH method			Superoxide radical scavenging	Hydroxyl radical scavenging	Prevention of lipid peroxidation
	IC_{50} value [2] (μg/mL)	ARA[3] (%)	TEAC value[4] (μmol Trolox)	IC_{50} value [2] (μg/mL)	IC_{50} value [2] (μg/mL)	IC_{50} value [2] (μg/mL)
BHT	42 ± 2.0	100.0 ± 3.0	5621.0 ± 24.3	18 ± 0.5	39 ± 2.8	27 ± 1.2
Quercetin	56 ± 0.8	82.8 ± 1.8	3558.2 ± 9.7	9 ± 0.6	38 ± 6.9	46 ± 0.6
Rutin	67 ± 1.1	72.1 ± 1.2	1771.6 ± 14.1	22 ± 0.4	42 ± 1.1	59 ± 2.4
Chlorogenic acid	72 ± 0.6	66.4 ± 2.5	3643.2 ± 13.3	-	-	-
100% Methanol extract	356 ± 0.9[a]	70.0 ± 3.0[a]	2449.9 ± 13.8[a]	302 ± 1.6[a]	396 ± 1.7[a]	442 ± 1.3[a]
50% Methanol extract	544 ± 0.7[b]	45.8 ± 2.1[b]	2365.7 ± 57.7[a]	354 ± 2.4[b]	467 ± 0.6[b]	563 ± 7.5[b]
1-Butanol extract	606 ± 0.2[b]	41.0 ± 1.1[b]	1725.0 ± 17.3[b]	375 ± 4.9[b]	514 ± 0.4[c]	570 ± 3.1[b]
Hot water extract	743 ± 0.3[c]	33.6 ± 1.3[c]	622.4 ± 12.2[c]	333 ± 2.2[c]	543 ± 0.9[d]	801 ± 0.9[c]

[1] Values are the average of three independent sample preparations. All CVs are less than 5.0% and values in the same column followed by different letters are significantly difference ($p \leq 0.05$).

[2] IC_{50} (Inhibitory of concentration) is concentration (μg/mL) of antioxidant needed to decrease the initial free radicals concentration by 50%.

[3] Antiradical activity expressed as percent inhibition of DPPH radical and calculated based on concentration of 500 μg/mL of extract or100 μg/mL of standard chemical

[4] TEAC value expressed as μmol Trolox per g of dried leaves and standard chemical were measured at 100 μg/mL.

Inhibition of Cell Proliferation on HepG2 and Normal Human Liver Cells

In the present study, we focused on the effects of mulberry leaves extracts on the growth inhibition of HepG2 hepatoma cells. This was determined by Cell Counting Kit-8 (CCK-8). It was found that the IC_{50} of various crude extracts of the mulberry leaves ranged from 21.51 μg/mL of 1-butanol extract to 204.17 μg/mL of hot water extract. The growth inhibition of HepG2 cells appeared to be in a dose dependent manner as shown in Figure10. The percentage of inhibition of cell proliferation was found to be high with the organic extracts and relatively low with the hot water extract. The results were expressed as a percentage relative to the control cell number. The IC_{50} concentration of each mulberry leaves extracts after 12 h of treatment was chosen for the subsequent experiments.

The results on the effect of 100% methanol extract on the cell viability of the HepG2 compared to normal human liver cells are shown in Figure 11. The normal human liver cells were appeared to be more viable than HepG2 cells as shown by the higher IC_{50} (273.91 μg/mL) values than the HepG2 cells (33.11 μg/mL). At the dose ranging from 0 to 500 μg/mL, the viability of normal liver cells decreased with an increase of dosage. For HepG2 cells, a dose-dependent growth inhibitory effect was observed. At IC_{50} concentration of normal liver cell was higher 8.3 fold than IC_{50} concentration of HepG2 cells.

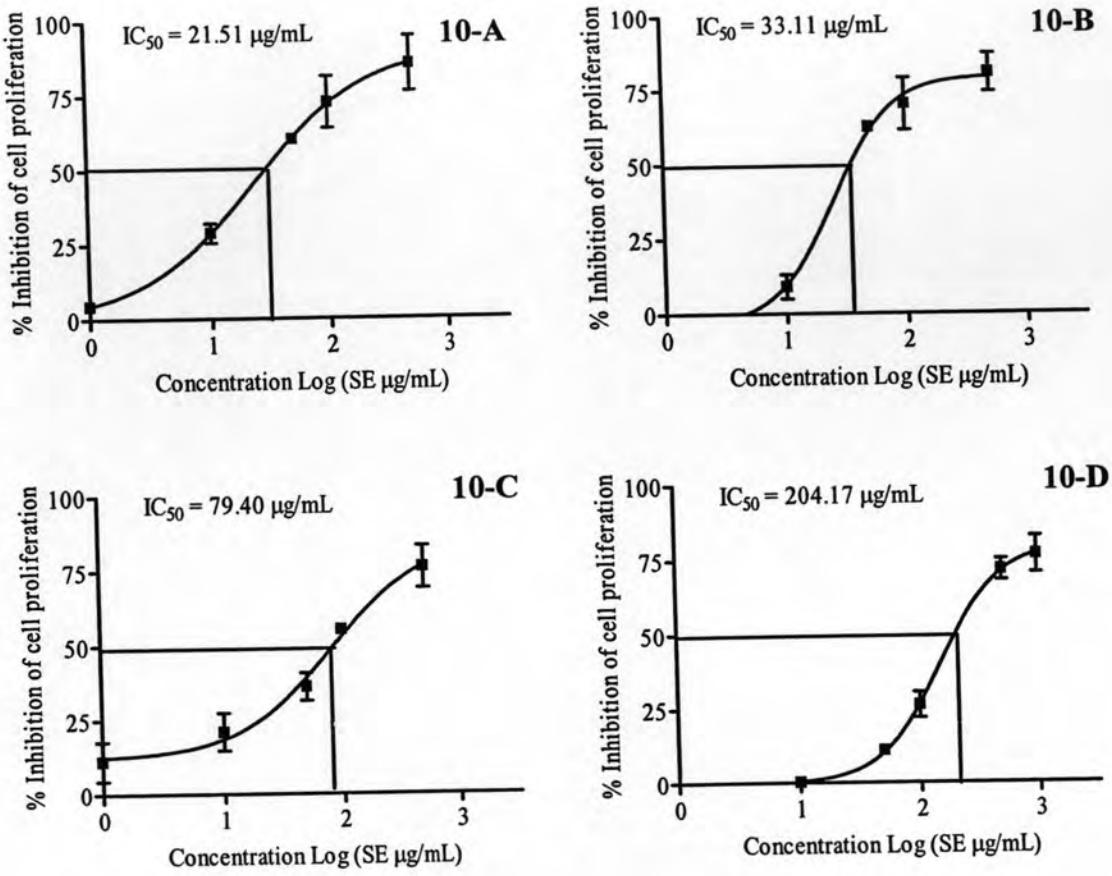

Figure 10 Inhibition of cell proliferation (%) of HepG2 cells. Cells were treated with various concentrations of the mulberry leaf for 12 h. Percentage of cell proliferation was determined by CCK-8 assay as described under Materials and Methods section. In descending order of potency: (10-A) 1-butanol (IC_{50} = 21.51 µg/mL), (10-B) 100% methanol (IC_{50} = 33.11 µg/mL), (10-C) 50% aqueous methanol (IC_{50} = 79.40 µg/mL), and (10-D) hot water (IC_{50} = 204.17µg/mL). Each value represents means ± SEM (n = 3) from two independent experiments. IC_{50} was represented in the antilog of the x-axis value at the inflection point of each sigmoid-curve fit.

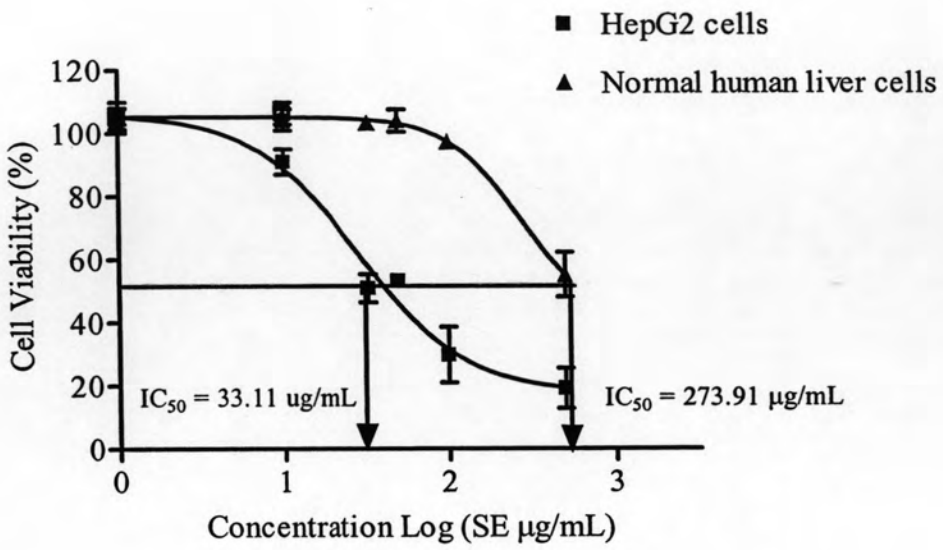

Figure 11 Cell viability of HepG2 (■) and normal human liver (▲) cells exposed with 100% methanol extract. Each value represents means ± SEM (n = 3) from two independent experiments. IC_{50} was represented in the antilog of the x-axis value at the inflection point of each sigmoid-curve fit.

Effect on Cell Cycle Progression

In order to examine the extract cellular stage at which mulberry tea extracts induced growth inhibition the frequency of cell cycle distribution of HepG2 cells after the leaf extract treatment was studied. In doing this, the treated HepG2 cells were fixed stained with propidium iodide (PI) and assayed for cell cycle distribution by flow cytometric analysis. The analysis was performed to identify G2/M arrest of cell cycle based on their light scattering properties and DNA content. As shown in Figure 12, it can be seen that HepG2 cells treated with 100 % methanol, 50 % aqueous methanol and 1-butanol extracts appeared to decrease in their G1 phase with slightly increased in the S phase and significantly increased in the G2/M phase. On the other hand, the result of hot water extract showed no significant change of the cell cycle distribution as compared with the control.

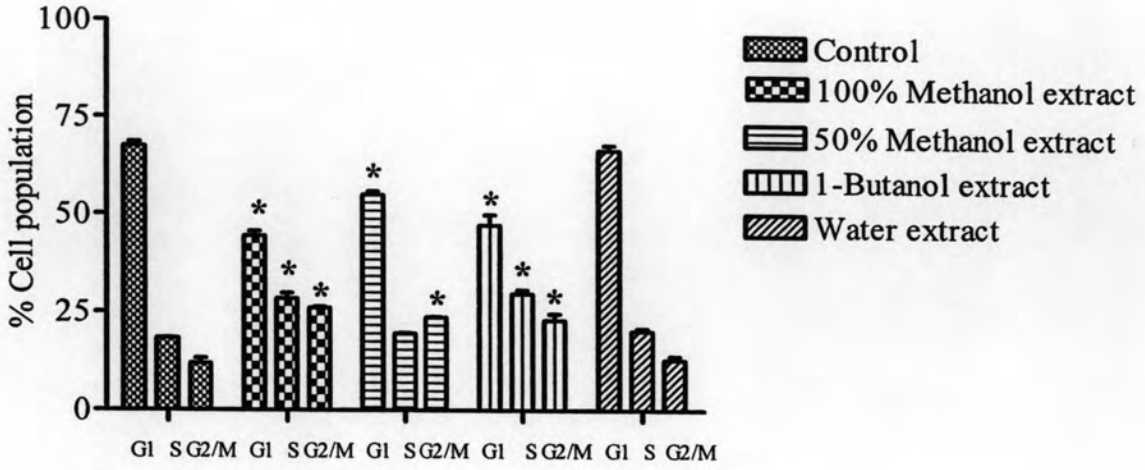

Figure 12 The effect of the various mulberry leaf extracts on cell cycle distribution of HepG2 cell line. The cells were incubated with IC_{50} concentration of each extracts for 12 h. The cells were collected and staining with PI solution and subjected to flow cytometric analysis. The results were reproducible in two additional independent experiments, * $p \leq 0.001$. The p value is determined by comparing with control group.

Apoptotic Induction

For the study of the effect on apoptotic induction of HepG2 cells were incubated for 12 h with the IC_{50} concentrations of mulberry extracts (33 μg/mL for 100% methanol, 80 μg/mL for 50% aqueous methanol, 22 μg/mL for 1-butanol and 205 μg/mL for hot water extract). Apoptotic cells were identified by the Vybrant® Poly Caspase Assay Kit employs to detect active caspases that is based on a fluororescent inhibitor of caspases (FLICA™) combined with PI exclusion assay and followed by flow cytometric analysis. It was found that the organic extracts of mulberry leaves effective induced cell apoptosis of HepG2 cells by activation of caspases significantly increased after incubated for 12 h (Figure 13). Again, the water extract had little effect on the apoptotic assay.

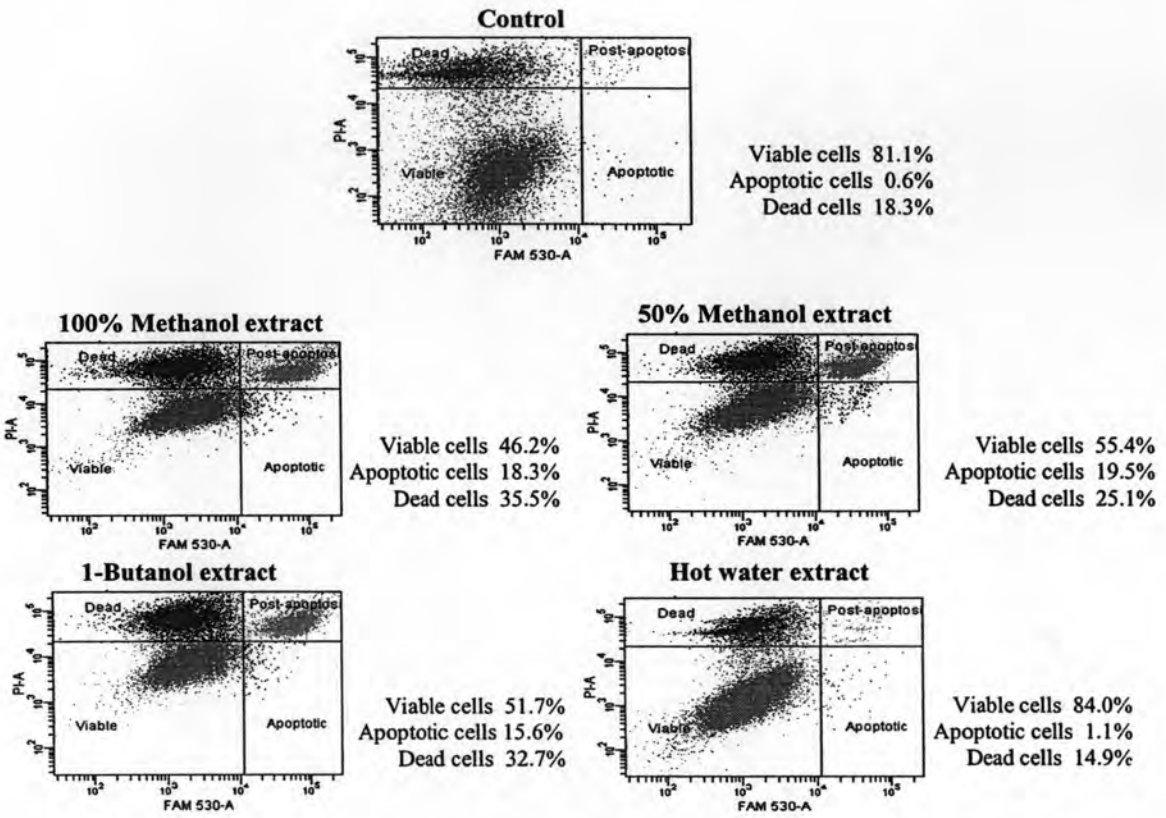

Figure 13 Quantitative analysis of apoptosis cells on HepG2 cells treated with the various of mulberry leaf extracts. HepG2 cells were incubated with IC50 concentration of each extracts for 12 h. and the percentage of apoptosis HepG2 cells were determined by FLICA™ coupled with PI staining and followed by flow cytometric analysis.

Inhibition of human topoisomerase IIα

The topoisomerase activities affected by the mulberry leaf extracts were tested in vitro using the human drug screening anti-topoisomease kit (Figure 14 and 15). Figure 14 shows the results of typical detection of anti-topoisomease IIα activity assayed under two different concentrations of cell proliferation of 100% methanol extracts (at IC_{20} concentration of 13 μg/mL and at IC_{50} concentration of 33 μg/mL in lanes 7-10), 50 % aqueous methanol extract (at IC_{20} concentration of 32 μg/mL and at IC_{50} concentration of 79 μg/mL in lanes 11-14), 1-butanol extract (at IC_{20} concentration of 9 μg/mL and at IC_{50} concentration of 21 μg/mL in lanes 15-18) and water extract (at IC_{20} concentration of 82 μg/mL and at IC_{50} concentration of 204 μg/mL in lanes 19-22). Supercoiled DNA (lane 1 and 2) was also included in the assay as markers. The inhibitory activity was measured based on the relative intensity of supercoiled DNA band in the reaction that contained the tested compounds, in comparison to the solvent negative control. The higher intensity of this band indicated a higher inhibition on topoisomease enzymes by the tested extracts. Lane 3 and 4 represented control reaction showing normal Topoisomerase IIα activity without the presence of the tested extracts. In these lanes, supercoiled converted into relaxed DNA, indicating only the activity of topoisomease IIα enzyme. The effect of solvent on topoisomerase activity was tested by adding DMSO to a final concentration of 1 % in the mixture (lanes 5 and 6). The same intensity of the band indicated that the presence of DMSO at a level 1 % did not interfere the enzyme activity. The mulberry leaf extracts of all the solvent at concentration of IC_{20} (μg SE/mL) significantly slightly inhibited topoisomease II although the hot water extract should only slightly.

In order to determine the IC_{50} value of each extract for the topoisomerase IIα activity, the concentration of each extracted was varied and the enzyme activity was recorded. The results are showed in Figure 15. It can be seen that the water extract had a lower IC_{50} (223.80 μg/mL) value than organic extract (concentration of IC_{50} 18.67 μg/mL for 100% methanol, 32.36 μg/mL for 1-butanol and 42.30 μg/mL for 50% aqueous methanol).

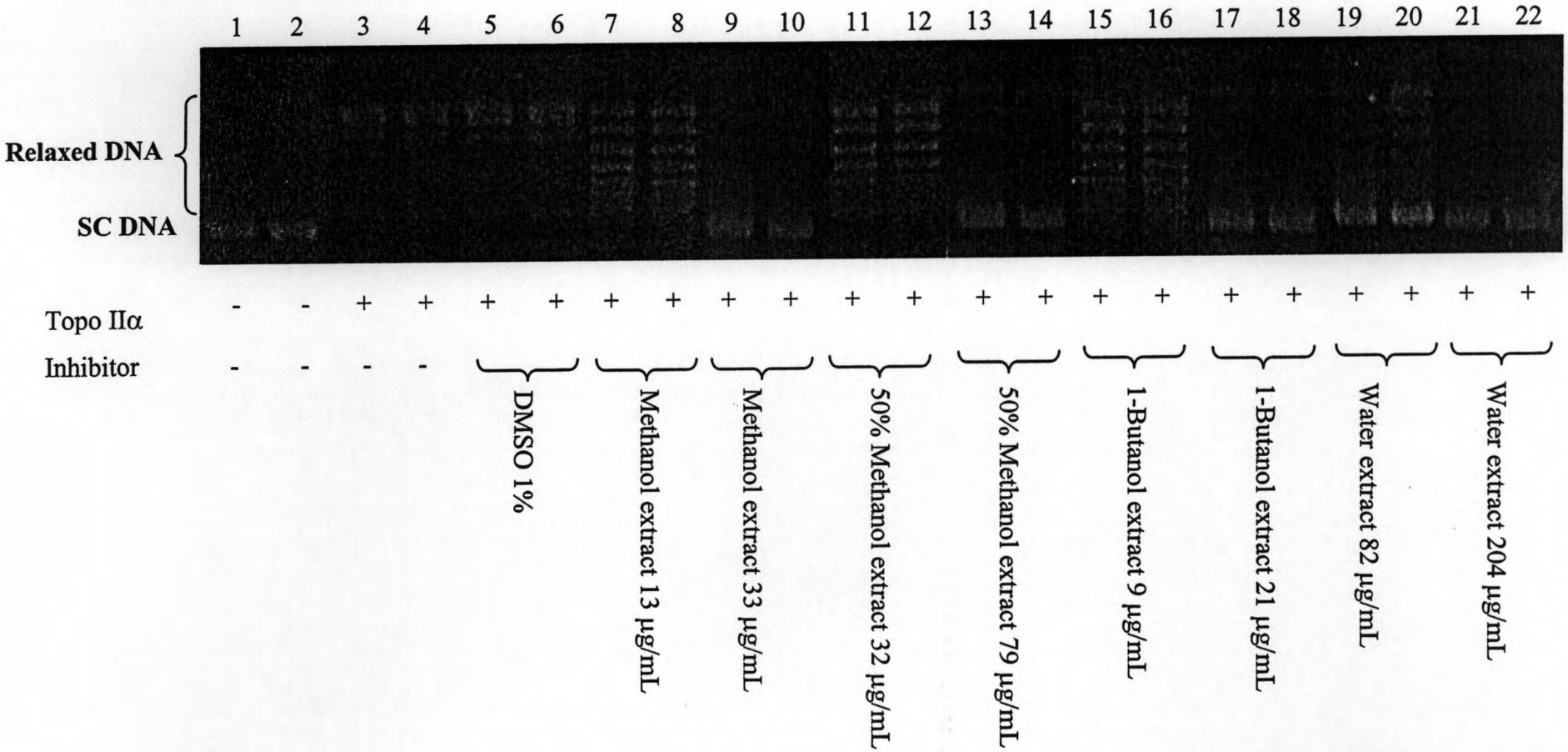

Figure 14 Representative relaxation assay for determining the inhibition of catalytic activity on human topoisomerase IIα by extracts of mulberry leaves. Substrate supercoiled (SC) pRYG DNA (1 µL or 0.2 µg) was incubated with 4 units (2 µL) of human DNA topoisomerase IIα. SC, supercoiled pRYG DNA marker.

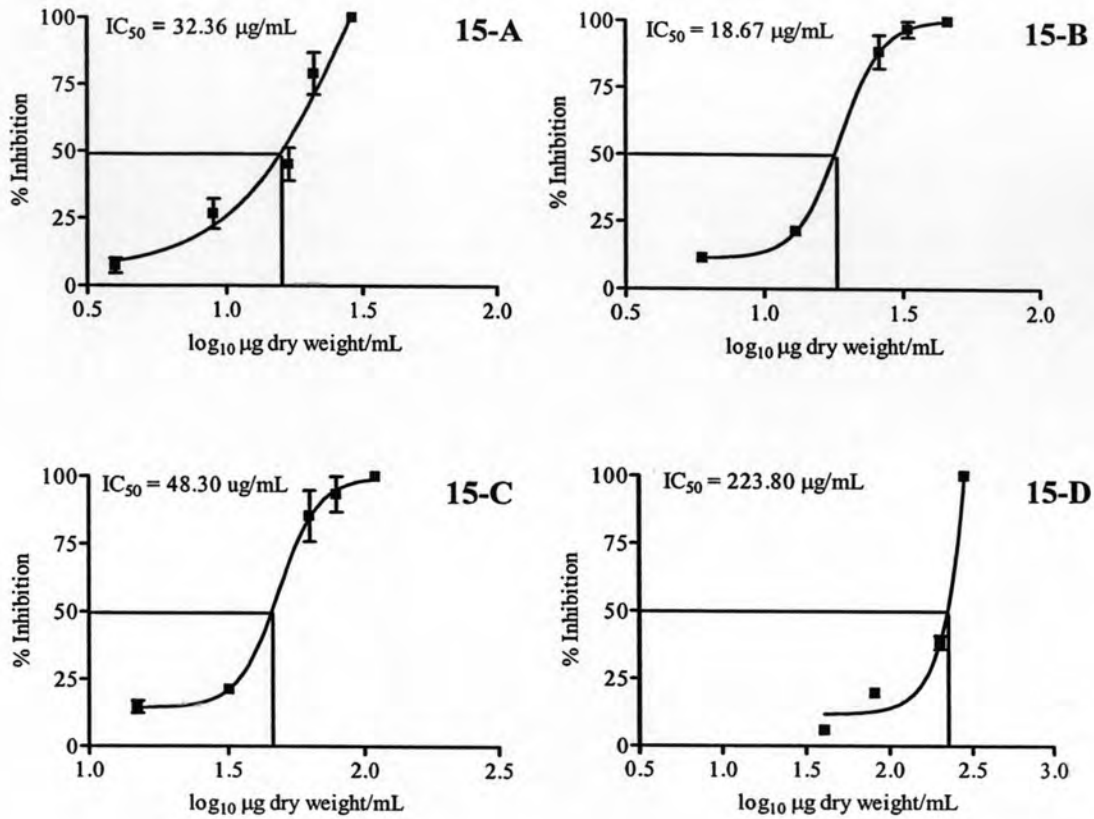

Figure 15 Anti-topoisomerase IIα activity effected by concentrations of various of mulberry leaf extracts. Values are expressed as relative activity of topoisomerase II enzyme of tested samples to that in solvent negative control. Assays were performed using a TopoGen® human drug screening kit as described in the methods section. (15-A) 1-butanol extract, (15-B) 100% methanol extract, (15-C) 50% aqueous methanol extract and (15-D) hot water extract. Each value is a average of at least duplicate measurements from independent two experiments.

The Expression of topoisomerase IIα , $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ Proteins in HepG2 Cells

Based on the previous results, the 100% methanol leaf extract with most potent effect was subsequently used for studying the expression of topoisomerase IIα, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ in HepG2 cells. In this study the effect of 100% methanol extract on the cell cycle regulatory proteins topoisomerase IIα , $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ was observed after treatment at 12 h by examining the treated HepG2 cells using immunoblot analysis. As shown in Figure 16, the treatment showed in an increase of topoisomerase IIα and decreased $p27^{Kip1}$ proteins, with absolutely no effect on the expression of $p21^{Cip1/Waf1}$. The results also showed that the concentration of 13 and 33 μg/mL of 100% methanol extract could induce a significant change of the expression of topoisomerase IIα (decreased by 17% and 55%) and $p27^{Kip1}$ (increased by 12% and 36%) proteins when compared with the untreated HepG2 cells. These results suggested that mulberry leaf 100% methanol extract affected the cell cycle and viability of HepG2 cells. For regards of this experimental treatment, the measurement of β-actin was used as an internal control for experimental error. The observed the expression of topoisomerase IIα , $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ proteins was not affect from protein loading as confirmed thought to be present at a constant level of β-actin.

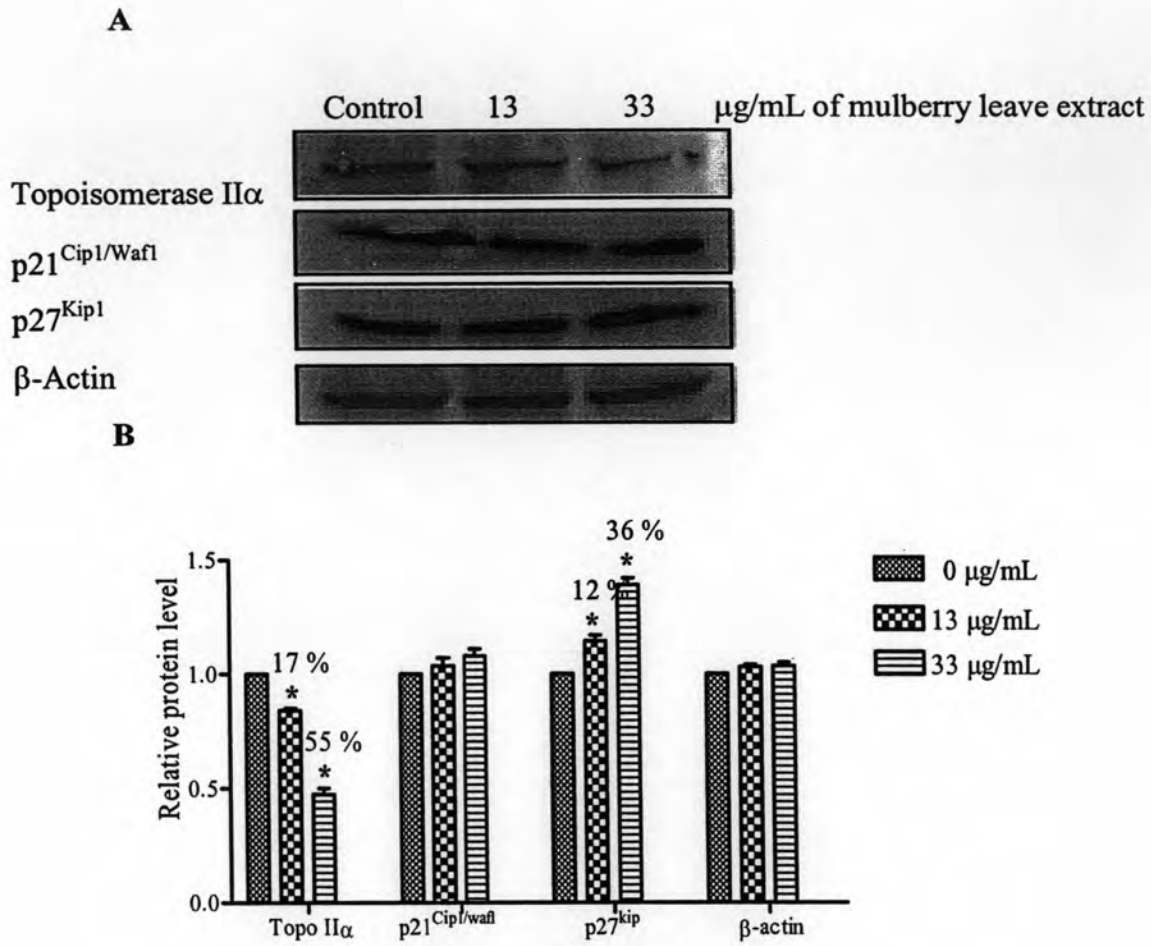

Figure 16 The expression of topoisomerase IIα, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ proteins in HepG2 cells treated with 100% methanol extract of mulberry leaves. Total cell lysates were prepared and subjected to SDS-PAGE followed by Western immunoblotting. Membranes with probed with antibodies to The topoisomerase IIα , $p21^{Cip1/Waf1}$, $p27^{Kip1}$ and β-actin, followed by peroxidase-conjugated appropriated secondary antibodies, and visualized by enhanced chemiluminescence detection system (A) and plotted as relative protein level (B). The densitometric data shown above the bar graph are presented as percentage changes as compared with their respective control.