CHAPTER 4

Results

According to studies on the anti-cancer and antioxidant properties of *M. oleifera* and *P. palatiferum* leaf extracts by feeding aqueous extracts from *M. oleifera* at doses of 60, 120, 180 and 240 mg/kg BW and from *P. palatiferum* at doses of 5, 10, 15 and 20 mg/kg BW to male rats for a consecutive period of sixty days, satisfactory research findings were obtained and the data obtained was presented in three parts. The first part consisted of phytochemical components and safety tests of the plants extracted by different solvents. After having known the components and safety of the extracts from both plant species, the next phase of the experiment was conducted. The second part comprised data from studying the anti-cancer properties consisting of efficiency in suppressing colorectal cancer cell proliferation and IC_{50}. The last part comprised data obtained from studying antioxidant properties *in vitro* and *in vivo*. Based on the results from *in vitro* study, aqueous extracts of *M. oleifera* and *P. palatiferum* were chosen for *in vivo* antioxidant studies because the aqueous extracts showed good antioxidant effects. Furthermore, both types of plants are usually consumed with water. The details of the research findings will be categorized as the next step.

4.1 Phytochemical components and safety evaluation of the extracts

4.1.1 Phytochemical Components

Preliminary phytochemical analysis of aqueous, ethanol and hexane leaf extracts of *M. oleifera* and *P. palatiferum* indicated that they contained cardiac glycoside, flavonoid, phenolic, reducing sugar, saponin, steroid, tannin and terpenoid. Glycone was found only in the *P. palatiferum* extract and anthraquinones was found only in the *M. oleifera*. However, alkaloid and coumarin were not found in any extracts. (Table 3)

Table 3 Phytochemical analysis of extracts of *Moringa oleifera* Lam. and *Pseuderanthemum palatiferum* (Nees) Radlk.

Chemical components	Aqueous extracts of *M. oleifera*	Ethanolic extracts of *M. oleifera*	Hexane extracts of *M. oleifera*	Aqueous extracts of *P. palatiferum*	Ethanolic extracts of *P. palatiferum*	Hexane extracts of *P. palatiferum*
alkaloid	-	-	-	-	-	-
anthraquinones	++	+	+	-	-	-
cardiac glycoside	+	+	+	++	++	++
coumarin	-	-	-	-	-	-
flavonoid	++	++	++	+	+	+
glycone	-	-	-	+	++	++
phenolic	++	++	++	+	+	+
phlobatannins	-	-	-	-	++	++
reducing sugar	++	++	++	+	++	++
saponin	+	+	+	++	++	+
steroid	++	+	+	+	+	+
tannin	+	+	++	+	+	+
terpenoid	+	+	+	++	++	++

Key - = Absence of constituent; + = Presence of constituent; ++ = Present in high concentrations.

4.1.2 Safety evaluation of the extracts (Ames test)

Aqueous, ethanol and hexane extracts from the leaves of *M. oleifera* and *P. palatiferum* had no mutagenic effect. The number of mutant colonies of the tested bacterium at all concentrations of the extracts was significantly lower than the positive control ($P<0.001$) but was not different from the negative control either in the presence or absence of the activating enzyme. (Table 4 and 5)

Table 4 Number of mutant colonies of *S. typhimurium* TA98 induced by *M. oleifera* extracts with or without activated enzyme**.**

Treatment	Mutant colony	
	+S9 mix	-S9 mix
0 (DMSO)	28.00 ± 6.63	18.00 ± 10.57
2-AA (0.5 μg/pL)	103.00 ± 7.21	-
AF-2 (0.025 μg/pL)	-	248.33 ± 31.68
aqueous extracts of *M. oleifera* (25 μg/pL)	27.50 ± 5.36	22.00 ± 2.45
aqueous extracts of *M. oleifera* (50 μg/pL)	30.17 ± 2.86	22.80 ± 5.36
aqueous extracts of *M. oleifera* (100 μg/pL)	33.83 ± 8.41	23.20 ± 4.09
ethanol extracts of *M. oleifera* (25 μg/pL)	28.50 ± 7.74	20.33 ± 1.51
ethanol extracts of *M. oleifera* (50 μg/pL)	33.50 ± 3.73	22.33 ± 2.16
ethanol extracts of *M. oleifera* (100 μg/pL)	36.67 ± 6.80	22.80 ± 5.36
hexane extracts of *M. oleifera* (25 μg/pL)	35.67 ± 3.15	25.77 ± 2.18
hexane extracts of *M. oleifera* (50 μg/pL)	40.97 ± 2.12	30.67 ± 2.42
hexane extracts of *M. oleifera* (100 μg/pL)	42.16 ± 2.86	35.15 ± 5.77

NB: The data shown are the average ± standard deviation from 3 independent tests which the natural occurring colonies were already subtracted. 2-AA and AF-2 are the standard substances giving positive result with and without metabolic activation respectively. 2-aminoanthracene (2-AA), 2 - aminofluorene (AF2)

Table 5 Number of mutant colonies of *S. typhimurium* TA98 induced by *P. palatiferum* extracts with or without activated enzyme**.**

Treatment	Mutant colony	
	+S9 mix	**-S9 mix**
0 (DMSO)	29.67 ± 7.00	23.17 ± 3.37
2-AA (0.5 μg/pL)	372.00 ± 22.44	-
AF-2 (0.025 μg/pL)	-	313.17 ± 19.00
aqueous extracts of *P. palatiferum* (25 μg/pL)	21.67 ± 2.88	23.17 ± 3.54
aqueous extracts of *P. palatiferum* (50 μg/pL)	27.83 ± 5.19	24.83 ± 9.39
aqueous extracts of *P. palatiferum* (100 μg/pL)	41.40 ± 5.03	25.40 ± 6.69
ethanol extracts of *P. palatiferum* (25 μg/pL)	27.76 ± 7.55	22.00 ± 3.41
ethanol extracts of *P. palatiferum* (50 μg/pL)	29.00 ± 5.29	22.50 ± 6.60
ethanol extracts of *P. palatiferum* (100 μg/pL)	35.83 ± 2.71	28.83 ± 9.26
hexane extracts of *P. palatiferum* (25 μg/pL)	40.02 ± 3.34	28.66 ± 9.37
hexane extracts of *P. palatiferum* (50 μg/pL)	42.46 ± 3.15	32.00 ± 1.00
hexane extracts of *P. palatiferum* (100 μg/pL)	46.26 ± 2.73	34.00 ± 2.65

NB: The data shown are the average ± standard deviation from 3 independent tests which the natural occurring colonies were already subtracted. 2-AA and AF-2 are the standard substances giving positive result with and without metabolic activation respectively. 2-aminoanthracene (2-AA), 2 -aminofluorene (AF2)

It has been shown from Tables 4 and 5 that extracts from *M. oleifera* and *P. palatiferum* leaves caused no mutation to *S. typhimurium* TA98 cells. Therefore, they are considered to be safe for using in the testing of efficiency of the extracts in suppressing colorectal cancer cell proliferation.

4.2 The antiproliferative effect of the extracts from *M. oleifera* and *P. palatiferum*

The aqueous, ethanol and hexane extracts of *M. oleifera* and *P. palatiferum* were found to be toxic against the three types of colon cancer cells as the concentration and time increased. The *M. oleifera* extracts were more effective in inhibiting cell proliferation than the *P. palatiferum* extracts (Figure 7, 8 and 9).

Aqueous extract of *M. oleifera* leaves at the concentrations of 100, 250 and 500 µg/ml was able to inhibit the cell division of colon cancer cell HCT15 according to the concentration of the extract and increasing time. However, at 1000 µg/ml, there was no significant difference on the inhibition of cell proliferation as the time increased. It was found that the extract at 250 and 500 µg/ml, 72 hrs was efficient in inhibiting cell proliferation. The suitable and recommended concentration is 250 µg/ml because of its lower concentration and efficiency in inhibiting cell proliferation of HCT15 cancer cells (Figure 7(A)). This extract was also able to inhibit colon cancer cells SW48 at varying concentrations and time. At the concentrations of 100, 250 and 500 µg/ml at 48 and 72 hrs, their ability to inhibit cell division was more or less similar. However, at 1000 µg/ml, 72 hrs, the inhibitory efficiency against SW48 was highest (Figure 7(B)). Moreover, the extract was also found to inhibit the proliferation of colon cancer cell SW480 at increasing concentrations and time. (Figure 7(C))

Aqueous extract of *P. palatiferum* leaves was able to inhibit cell proliferation of colon cancer cell HCT15 at various concentrations and increasing time (Figure 7(D)). The extract at the concentrations of 100, 250 and 500 µg/ml inhibited cell proliferation of colon cancer cell SW48 and SW480 according to the concentration of the extract and increasing time. However, at 1000 µg/ml, there was no significant difference on the inhibition of cell proliferation as the time increased (Figure 7 (E and F)).

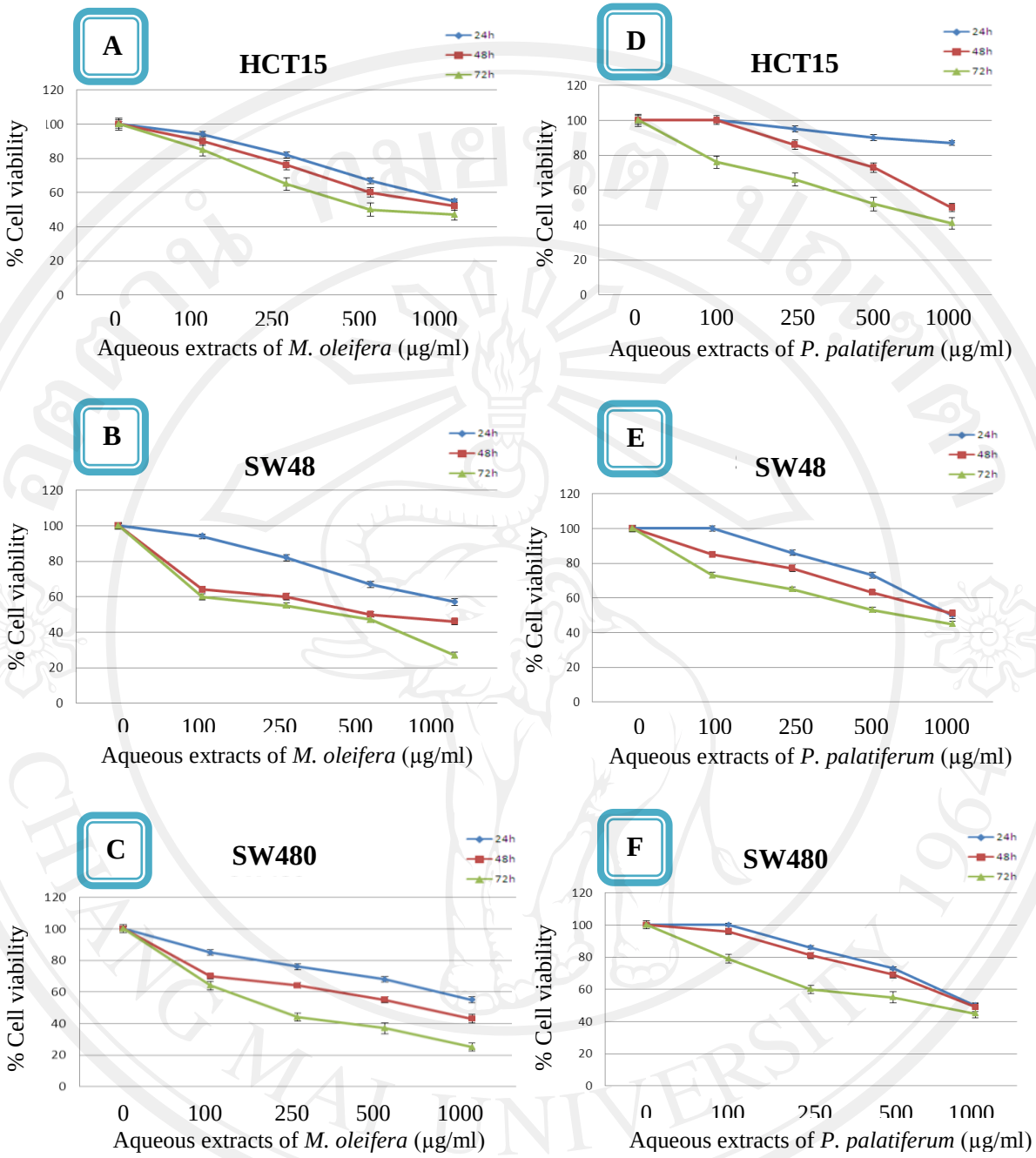

Figure 7 The antiproliferative effect of aqueous extracts from the leaves of *M. oleifera* and *P.palatiferum* on 3 types of human colon cancer cell line; HCT15, SW48 and SW480.

Ethanol extract of *M. oleifera* leaves was able to inhibit cell proliferation of colon cancer cell HCT15 and SW48 according to its concentration and increasing time (Figure 8(A)). At the concentrations of 100, 250 and 500 µg/ml; Hrs 24 and 48, inhibition of cell proliferation is rather low and indifferent. Whereas, at Hr 78, the inhibition was most prominent (Figure 8(B)). The extract also inhibited colon cancer cell SW480 with increasing concentration and time but at 1000 µg/ml, Hrs 24 and 48, there was no difference in the inhibition of cell proliferation. (Figure 8(C))

Ethanol extract of *P. palatiferum* leaves at the concentrations of 100, 250 and 500 µg/ml could inhibit HCT15 and SW48 colon cancer cells at different concentrations and increasing time. At 1000 µg/ml, the inhibition was not different as time increased (Figure 8(D, E)). The extract also inhibited SW480 with increasing concentrations and time. At 100, 250 and 500 µg/ml; Hrs 24 and 48, the inhibitory ability was rather close. At 1000 µg/ml, the inhibition was not different as the time increased. (Figure 8(F))

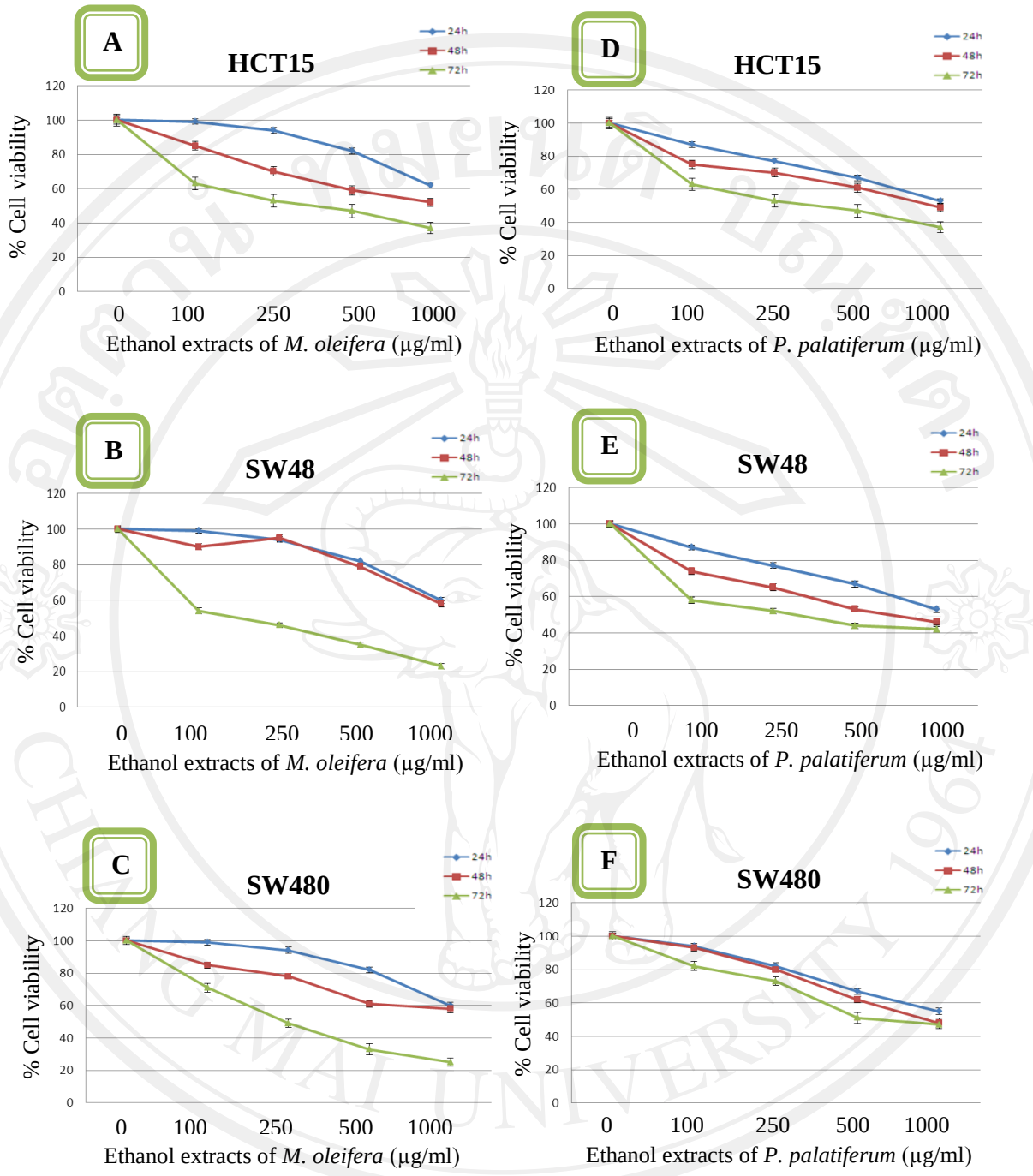

Figure 8 The antiproliferative effect of ethanol extracts from the leaves of *M. oleifera* and *P. palatiferum* on 3 types of human colon cancer cell line; HCT15, SW48 and SW480.

Hexane extract of *M. oleifera* leaves inhibited cell proliferation of colon cancer cell HCT15 at various concentrations but the time period had no effect on the inhibition of cell proliferation (Figure 9(A)). However, the extract inhibited SW 48 cell division at the concentrations of 100, 250, 500 and 1000 µg/ml; Hrs 48 and 72 and the inhibitory ability was not different (Figure 9(B)). It was also able to inhibit SW480 according to the concentrations of the extract and increasing time (Figure 9(C))

Hexane extract of *P. palatiferum* leaves inhibited cell proliferation of colon cancer cell HCT15 at various concentrations but the time period had no effect on the inhibition of cell proliferation (Figure 9(D)). It also inhibited SW48 at the concentrations of 100, 250 and 500 µg/ml; Hrs 48 and 72 and the inhibitory ability was not different. However, at 1000 µg/ml, the inhibitory efficiency was highest (Figure 9(E)). The extract was also inhibited SW480 at the increasing concentrations. AT 100, 250 and 500 µg/ml; Hrs 24 and 48, the inhibitory ability was very close but at 1000 µg/ml, the inhibition of cell proliferation did not vary with the increasing time. (Figure 9(F)).

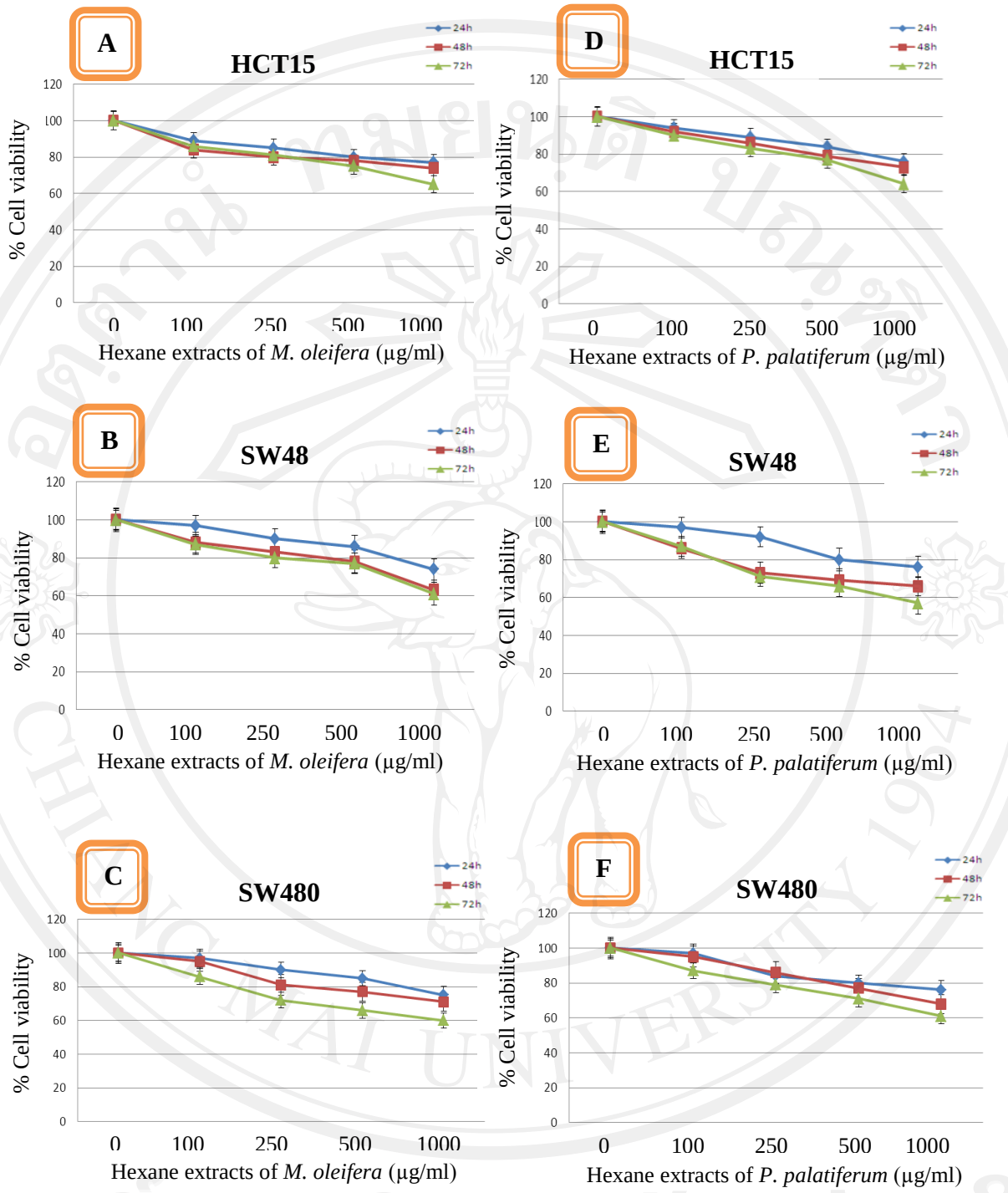

Figure 9 The antiproliferative effect of hexane extracts from the leaves of *M. oleifera* and *P.palatiferum* on 3 types of human colon cancer cell line; HCT15, SW48 and SW480.

The IC_{50} at 72 hours indicated that the ethanol extract of *M. oleifera* tended to inhibit cell proliferation of all the tested cell lines better than that of the aqueous and hexane extracts. SW48 was found to be the most sensitive cell lines to the *M. oleifera* extract. (Table 6)

Table 6 Toxicity (IC_{50} μg/ml) of *M. oleifera* and *P. palatiferum* extracts on 3 types of colon cancer cell lines. Data are representative of three independent experiments (mean ± SD).

Treatment	Colon Cell lines	IC_{50} (μg/ml)
Aqueous extracts of *M. oleifera*	HCT15	266.67 ± 18.53
	SW48	105.47 ± 23.50
	SW480	200.26 ± 27.64
Ethanol extracts of *M. oleifera*	HCT15	264.83 ± 23.33
	SW48	102.40 ± 16.08
	SW480	197.20 ± 32.52
Hexane extracts of *M. oleifera*	HCT15	325.65 ± 32.12
	SW48	283.90 ± 25.31
	SW480	317.22 ± 27.89
Aqueous extracts of *P. palatiferum*	HCT15	520.33 ± 24.74
	SW48	503.75 ± 30.21
	SW480	511.49 ± 21.22
Ethanol extracts of *P. palatiferum*	HCT15	508.98 ± 26.87
	SW48	500.39 ± 28.47
	SW480	511.74 ± 31.21
Hexane extracts of *P. palatiferum*	HCT15	647.39 ± 30.65
	SW48	568.19 ± 31.73
	SW480	613.76 ± 39.11

4.3 Antioxidant efficiency of the extracts from *M. oleifera* and *P. palatiferum*

The test of antioxidant properties was divided into two parts. The first part was an antioxidant test *in vitro* by ABTS, DDPH, ascorbic acid and total phenolic assays methods. According to the results from the first part that aqueous extracts of *M. oleifera* and *P. palatiferum* leaves exhibited good antioxidant capabilities *in vitro*, the aqueous extract was then an interesting candidate for using in the second part. In addition, most people prefer to consume the extracts in the form of tea or boiled soup. Therefore, the aqueous extracts were selected for the test of antioxidant activities *in vivo* by the TBARS and SOD methods.

4.3.1 Test of antioxidant activities in the plant extracts

It was found that all the extracts from *M. oleifera* and *P. palatiferum* exhibited the similar antioxidant capacities. According to the tests of antioxidant activities by the ABTS method, aqueous extracts of *M. oleifera*, ethanol extracts of *M. oleifera*, hexane extracts of *M. oleifera*, aqueous extracts of *P. palatiferum*, ethanol extracts of *P. palatiferum* and hexane extracts of *P. palatiferum* were found to have TEAC values of 0.018, 0.014, 0.010, 0.016, 0.013 and 0.006 µg trolox/mg extract, respectively. Tests of antioxidant activities by the DPPH method found aqueous extracts of *M. oleifera,* ethanol extracts of *M.oleifera*, hexane extracts of *M. oleifera*, aqueous extracts of *P. palatiferum*, ethanol extracts of *P. palatiferum* and hexane extracts of *P. palatiferum* to have GAE values of 1.37, 1.51, 1.36, 1.20, 1.36 and 0.66 µg gallic acid/mg extract, respectively. *M. oleifera* extracts from ethanol contain high total phenols at 2.45 ± 0.84 µg/ml, followed by aqueous extracts of *M. oleifera*, hexane extracts of *M. oleifera*, aqueous extracts of *P. palatiferum*, ethanol extracts of *P. palatiferum* and hexane 1.66 ± 0.33, 1.61 ± 0.41, 1.29 ± 0.08 and 0.97 ± 0.04 µg/ml, respectively. As for ascorbic acid, aqueous extracts of *M. oleifera* contains the highest amount at 11.87 ± 1.30 µg/ml, followed by hexane extracts of *M. oleifera*, aqueous extracts of *P. palatiferum*, hexane extracts of *P. palatiferum*, ethanol extracts of *M. oleifera* and ethanol extracts of *P. palatiferum* at 11.16 ± 0.44, 11.13 ± 0.48, 10.60 ± 0.58, 8.59 ± 0.27 and 8.18 ± 0.22 µg/ml, respectively (Table 7).

Table 7 Suppression or elimination of ABTS and DPPH oxidants by *M. oleifera* and *P. palatiferum* leaf extracts and the amount of total phenols and ascorbic acid in the extracts.

Treatment	ABTS (TEAC µg/mg extract)	DPPH (GAE µg/mg extract)	Total phenols (µg/ml)	Ascorbic acid (µg/ml)
aqueous extracts of *M. oleifera*	0.018	1.37	2.02 ± 0.33	11.87 ± 1.30
aqueous extracts of *P. palatiferum*	0.016	1.20	1.61 ± 0.41	11.13 ± 0.48
ethanol extracts of *M. oleifera*	0.014	1.51	2.45 ± 0.84	8.59 ± 0.27
ethanol extracts of *P. palatiferum*	0.013	1.36	1.29 ± 0.08	8.18 ± 0.22
hexane extracts of *M. oleifera*	0.006	1.36	1.66 ± 0.33	11.16 ± 0.44
hexane extracts of *P. palatiferum*	0.003	0.66	0.97 ± 0.04	10.60 ± 0.58

4.3.2 Test of antioxidant activities in laboratory rats

The study of anti-lipid peroxidation properties in serum and liver was conducted by orally administering male rats with aqueous extract from the leaves of *P. palatiferum* at doses of 5, 10, 15 and 20 mg/kg BW and from *M. oleifera* at doses of 60, 120, 180 and 240 mg/kg BW for a consecutive period of sixty days**.** Efficiency in reducing malondialdehyde levels was measured by the TBARS method.

The experimental groups which received the extracts from both plant species at high doses were found to have significantly lower serum MDA levels ($P < 0.05$) than the control group and they were in the dose dependent manner. The reductions of MDA levels in experimental groups which received *M. oleifera* extracts at doses of 120, 180 and 240 mg/kg BW were 24.02%, 32.86% and 38.15%, respectively. In addition, the reductions of MDA levels in rats received extracts from *P. palatiferum* at doses of 10, 15 and 20 mg/kg BW were 13.82%, 23.29% and 24.02%, respectively. The groups which received *M. oleifera* extracts at a dose of 60 mg/kg BW and *P. palatiferum* leaf extracts at a dose of 5 mg/kg BW were found to have lower trends of reduced MDA levels than the control group without differences with statistical significance as shown in Figure 9 and Table 8.

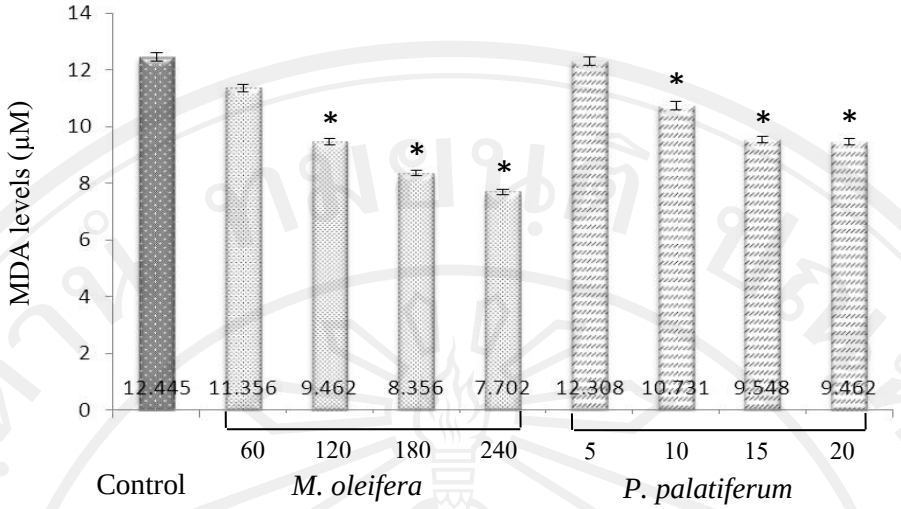

Figure 10 The anti-peroxidative activity of serum of male rats treated with *M. oleifera* at doses of 60, 120, 180 and 240 mg/kg BW and *P. palatiferum* at doses of 5, 10, 15 and 20 mg/kg BW for 60 days, the stars indicate significant difference at $P<0.05$.

Table 8 Reduced MDA percentages in the serum of male rats treated with extracts from *M. oleifera* at doses of 60, 120, 180 and 240 mg/kg BW/day and extracts from *P. palatiferum* leaves at doses of 5, I 0, 15 and 20 mg/kg BW/day for a period of 60 days in comparison with the control group.

Treatment		MDA level (µM) (mean±SD)	Percentage decrease in MDA level
	Control	12.45 ± 0.15 [a]	
	60 mg/kg. BW/day	11.36 ± 0.14 [a]	8.76
M.oleifera	120 mg/kg. BW/day	9.46 ± 0.12 [b]	24.02
	180 mg/kg. BW/day	8.36 ± 0.11 [c]	32.86
	240 mg/kg. BW/day	7.70 ± 0.09 [d]	38.15
	5 mg/kg. BW/day	12.31 ± 0.16 [a]	1.12
	10 mg/kg. BW/day	10.73 ± 0.14 [b]	13.82
P.palatiferum	15 mg/kg. BW/day	9.55 ± 0.12 [c]	23.29
	20 mg/kg. BW/day	9.46 ± 0.12 [d]	24.02

[a,b, c and d] are significant mean difference at $P<0.05$. The data are expressed as mean and standard deviation (SD.)

Concerning studies on MDA levels in the liver, the findings showed the experimental group which received *M. oleifera* extracts at doses of 180 and 240 mg/kg BW to have reduced MDA levels by as much as 57.81% and 66.45%, respectively. For *P. palatiferum*, however, only the extract at a dose of 20 mg/kg BW could significantly reduced MDA levels ($P<0.05$) when compared to the control group. The MDA levels were found to have reduced by as much as 35.22% while other experimental groups were only found to have downward trends in MDA levels from the control group as shown in Figure 10 and Table 9.

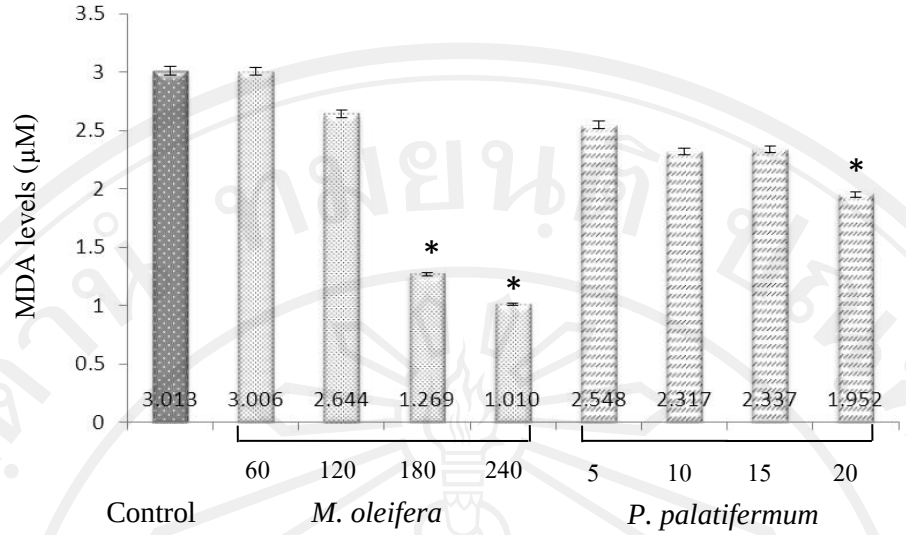

Figure 11 The anti-peroxidative activity of liver of male rats treated with *M. oleifera* at doses of 60, 120, 180 and 240 mg/kg BW and *P. palatiferum* at doses of 5, 10, 15 and 20 mg/kg BW for 60 days, the stars indicate significant difference at P<0.05.

Table 9 Reduced MDA percentages in the livers of male rats treated with extracts from *M. oleifera* at doses of 60, 120, 180 and 240 mg/kg BW/day and extracts from *P. palatiferum* leaves at doses of 5, 10, 15 and 20 mg/kg BW/day for a period of 60 days in comparison with the control group.

	Treatment	MDA level (µM) (mean±SD)	Percentage decrease in MDA level
	Control	3.01 ± 0.04 [a]	
	60 mg/kg. BW/day	3.00 ± 0.04 [a]	0.33
M.oleifera	120 mg/kg. BW/day	2.64 ± 0.03 [a]	12.29
	180 mg/kg. BW/day	1.27 ± 0.01 [b]	57.81
	240 mg/kg. BW/day	1.01 ± 0.01 [c]	66.45
	5 mg/kg. BW/day	2.55 ± 0.0 [a]	15.28
	10 mg/kg. BW/day	2.32 ± 0.14 [a]	22.92
P.palatiferum	15 mg/kg. BW/day	2.34 ± 0.12 [a]	22.26
	20 mg/kg. BW/day	1.95 ± 0.12 [b]	35.22

[a,b and c] are significant mean difference at $P<0.05$. The data are expressed as mean and standard deviation (SD.)

Investigation on SOD in the erythrocytes indicated that SOD in the rats administered with *M. oleifera* extract 180 and 240 mg/kg BW and *P. Palatiferum* extract 10, 15 and 20 mg/kg BW increased significantly ($P<0.05$) when compared with the control. The increase in SOD was also dose dependent. (Figure 11.)

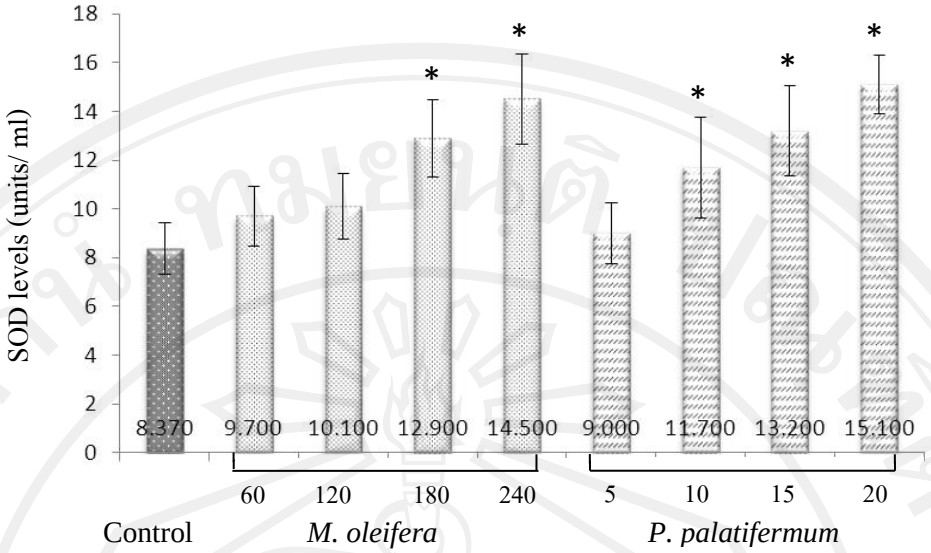

Figure 12 SOD of erythrocytes of male rats treated with *M. oleifera* at doses of 60, 120, 180 and 240 mg/kg BW and *P. palatiferum* at doses of 5, 10, 15 and 20 mg/kg BW for 60 days, the stars indicate significant difference at P<0.05.