CHAPTER IV

RESULTS AND DISCUSSION

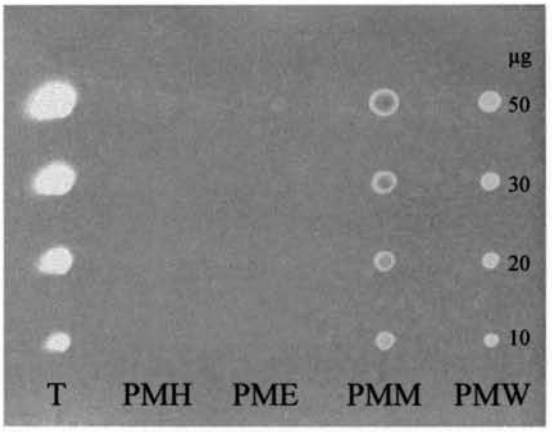
1. Preliminary screening for the antioxidative activities of various *P. mirifica* crude extracts

In order to select the crude extracts (PMH, PME, PMW and PMM) of *P. mirifica* with strong antioxidative activity for further study, the step of preliminary screen was first performed. This was carried out by a simple DPPH assay on a TLC plate. By spotting on a TLC plate with various *P. mirifica* extracts by using different amount of samples (10-50 μg) and Trolox (for control) followed by spraying with the DPPH solution, it was found that the extracts of PMM and PMW exhibited higher antioxidative activity than PME and PMH.

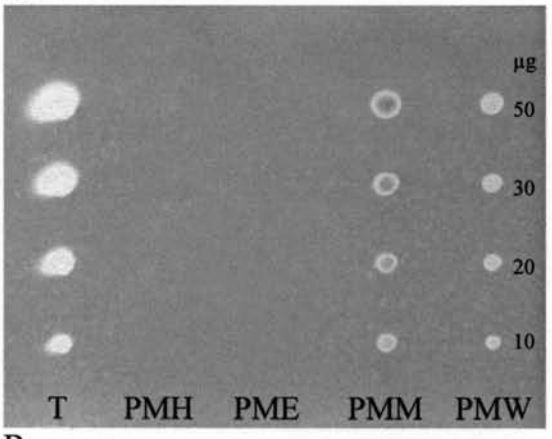
As shown in Figure 4, PMM and PMW showed positive DPPH assay only after 2 min (Figure 4A) after spraying with the DPPH solution whereas PME and PMH did not showed positive results after 35 min (Figure 4C).

The DPPH assay is based on the measurement of the reducing ability of antioxidants toward the DPPH radical. The stable DPPH radical is widely used to evaluate the antiradical/antioxidant properties of synthetic and natural phenols (Litwinienko & Ingold, 2003). It is easy to perform, highly reproducible and comparable with other methods (Gil et al., 2000).

The antioxidative activity can be quantitated by measuring the decrease of its absorbance (Prior, Wu and Schaich, 2005). EC_{50} is inversely related to the antioxidative capacity of a compound, as it expressed the amount of antioxidant



A.



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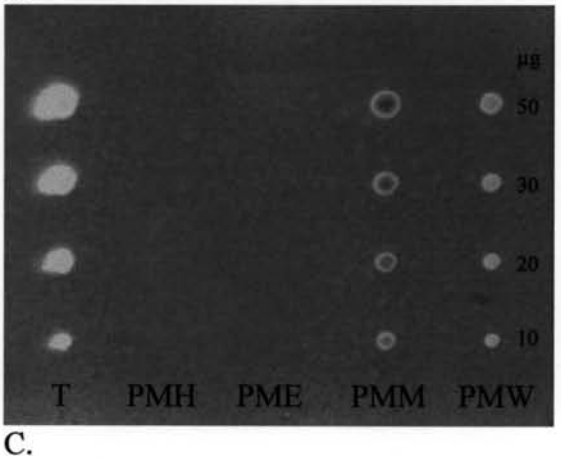

C.

Figure 4 Preliminary screening for the antioxidative activities of various *P. mirifica* extracts. Spots of Trolox (T), PMH, PME, PMM and PMW at 10, 20, 30 and 50 µg

needed to decrease the radical concentration by 50%. The lower EC_{50} means the higher the antioxidant capacity of from lower to upper row under visible light after spraying with DPPH for 2 min (A), 10 min (B) and 35 min (C)

compound. EC_{50} of Trolox, PMM, PMW, genistein and daidzein was 0.003, 0.194, 0.248, 13.79 and 14.26 mg/ml, respectively (Table 2). Additionally, there were no antioxidative activities of PME and PMH found (Table 4).

Therefore, PMM and PMW exhibited certain degree of antioxidative activities compared with the standards genistein, daidzein and Trolox. Both PMM and PMW were used for further studies.

Table 2 EC_{50} of Trolox, PMM, PMW, genistein and daidzein from DPPH assay

Sample	EC_{50}	slope	intercept	R^2	SEM
Trolox	0.003	-17.72	98.75	0.99	0.08
PMM	0.194	-0.25	97.99	0.99	2.39
PMW	0.248	-0.20	98.59	0.99	14.11
PME	0	0	0	0	0
PMH	0	0	0	0	0
GEN	13.79	0.00	96.90	0.97	89.35
DAI	14.26	0.00	97.05	0.87	52.25

2. Characterization of *P. mirifica* extracts

PMM and PMW were characterized for their chemical patterns, constituents and contents by HPLC. The HPLC condition was first validated using daidzein and genistein as standard markers. The system showed its percentage variable coefficient of both within-run and between-run precision under 2.5% and the percent recovery between 95-105%. Data were shown in appendix B. The linear regression (R^2) of the

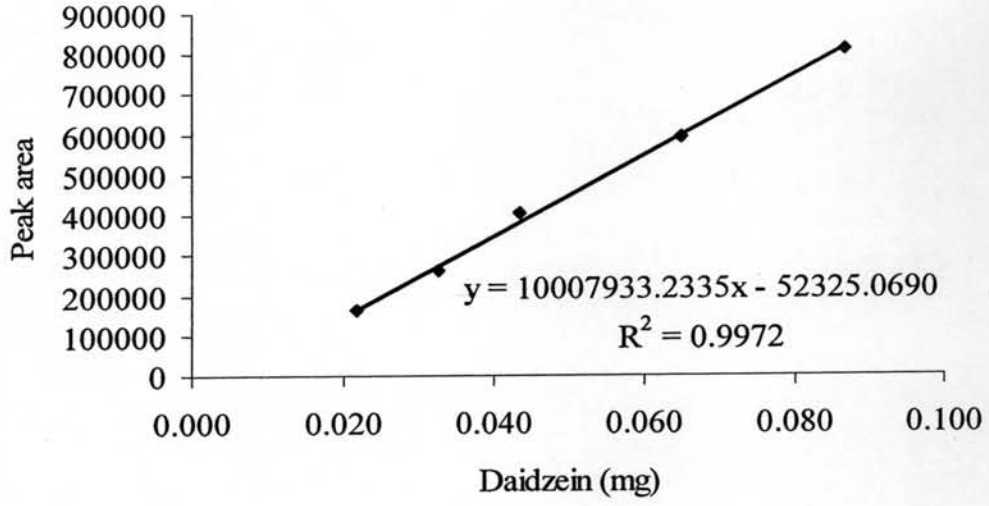

Figure 5 Standard curve of daidzein by HPLC analysis

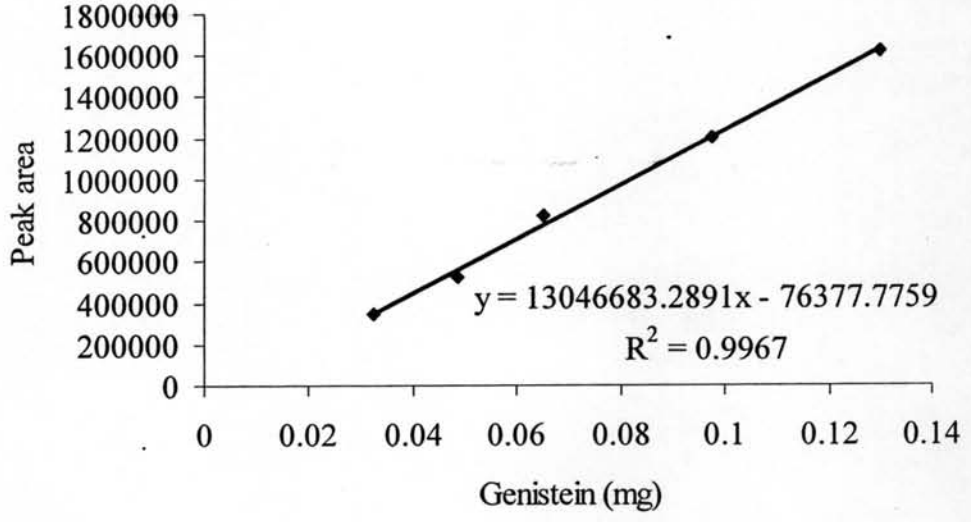

Figure 6 Standard curve of genistein by HPLC analysis

standard curves of daidzein and genistein were 0.9972 (Figure 5) and 0.9967 (Figure 6), respectively.

The HPLC chromatograms of PMM and PMW are shown in Figures 7A and 7B, respectively. It can be seen that PMM and PMW showed its own distinctive HPLC chromatogram. PMM showed two major peaks at the retention times of 39.1 and 43.3 min which were identified as daidzein and genistein by comparing with the retention times of the standards daidzein and genistein (Figure 7C).

For the water extract PMW, on the other hand, its HPLC chromatogram appeared to have several peaks eluted at the retention times between 3 to 40 min. The highest peak was the one with the retention time at 25.1 min. The peaks of daidzein and genistein were also detected but only daidzein could be quantitated.

In terms of quantity, daidzein and genistein in the extracts were determined by using daidzein and genistein standard curves. It was found that daidzein was present in PMM and PMW with the content of 0.556% and 0.076 % in the extract, respectively. For genistein, its content was found to be 0.551 % in PMM.

In this study, all constituents in the extract were not purified and identified. However, compounds that present in the *P. mirifica extracts* in this study might include the natural isoflavonoids such as miroestrol, deoxymiroestrol, isomiroestrol, daidzein, genistein, kwakhurin, coumestrol, tuberosin, daidzin, puerarin, and mirificin. Since many studies have been reported about these constituents in the *P. mirifica* root (Chansakow et al., 2000a, Chansakow et al., 2000b, วันชัย ดีเอกนามกูล และ ชาลี ทองเรือง, 2544).

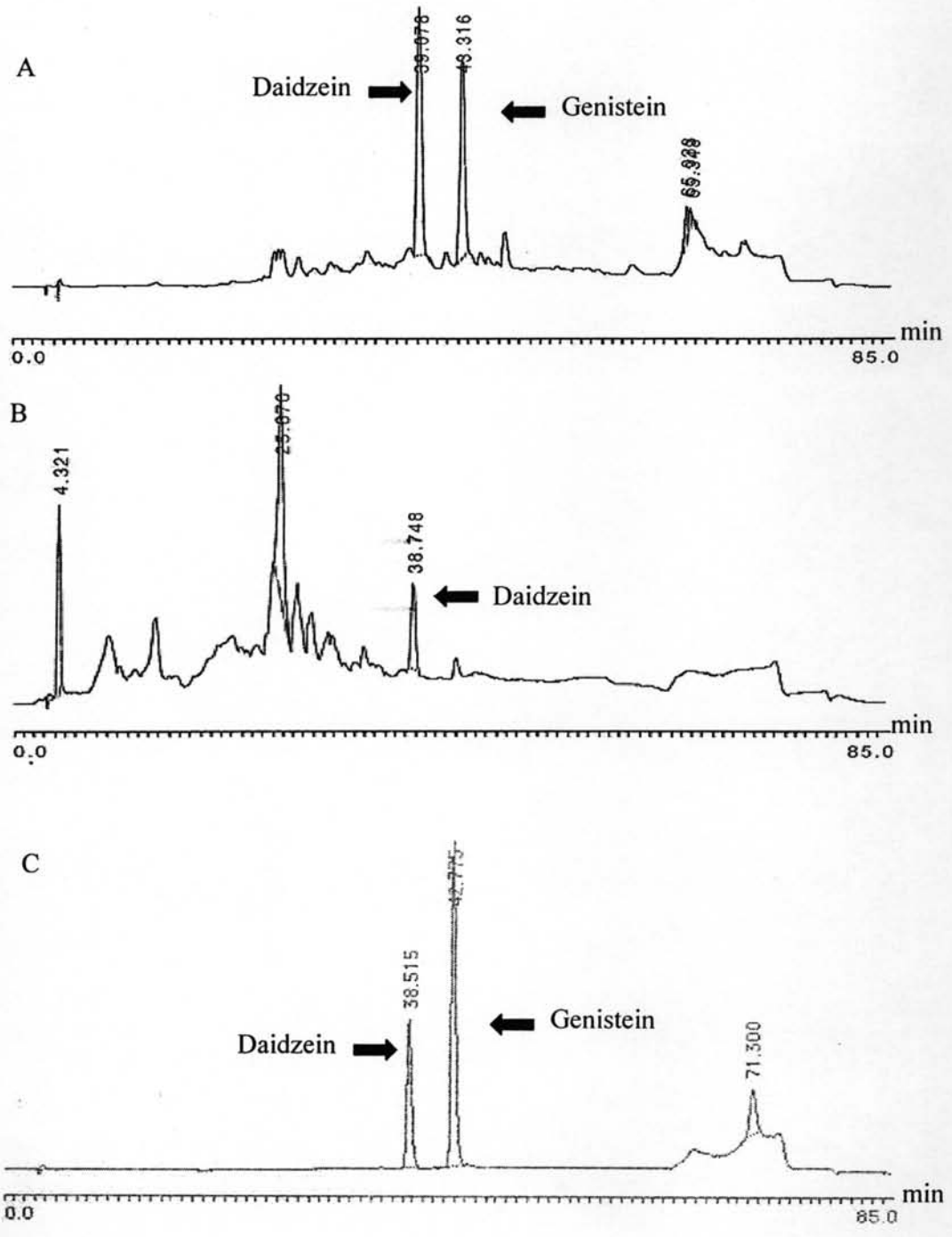

Figure 7 HPLC chromatograms of *P. mirifica* extracts of PMM (A), PMW (B) and the solution of mixed standards of daidzein and genistein (C)

Furthermore, the TLC technique was used to separate and investigate the antioxidative activities of each compound in the extracts. Figure 8A and 8B show the distinctive pattern of compounds contained in PMM and PMW at the wavelength of 254 and 366 nm, respectively. After spraying the plate with the DPPH solution (Figure 8C), several of PMM bands especially in the middle and upper part of the chromatogram exhibited antioxidative activities against the DPPH radical. The compounds showing the antioxidative activity in PMM included genistein and daidzein. This result showed that the synergistic effect of several substances in PMM, are important for the antioxidative activity of PMM.

The chromatogram of PMW shows that substances having the antioxidative activity mostly stay at the origin of the streak. Antioxidative activities of PMM and PMW, therefore, were the results from different pattern of compounds.

3. Cell proliferation experiments

To investigate the effect of the *P. mirifica* extracts on HT-22 neuronal cells, HT-22 cells were treated with various concentrations of PMM and PMW for 24 h. The cell number was then determined by the SRB assay. As shown in Figure 9 , it can be seen that the cell number of HT-22 cells treated with 10, 50 and 100 μg/ml of PMM were significantly lower than that of the control cells (p value ≤ 0.05). This clearly suggested that PMM could inhibit cell proliferation of HT-22.

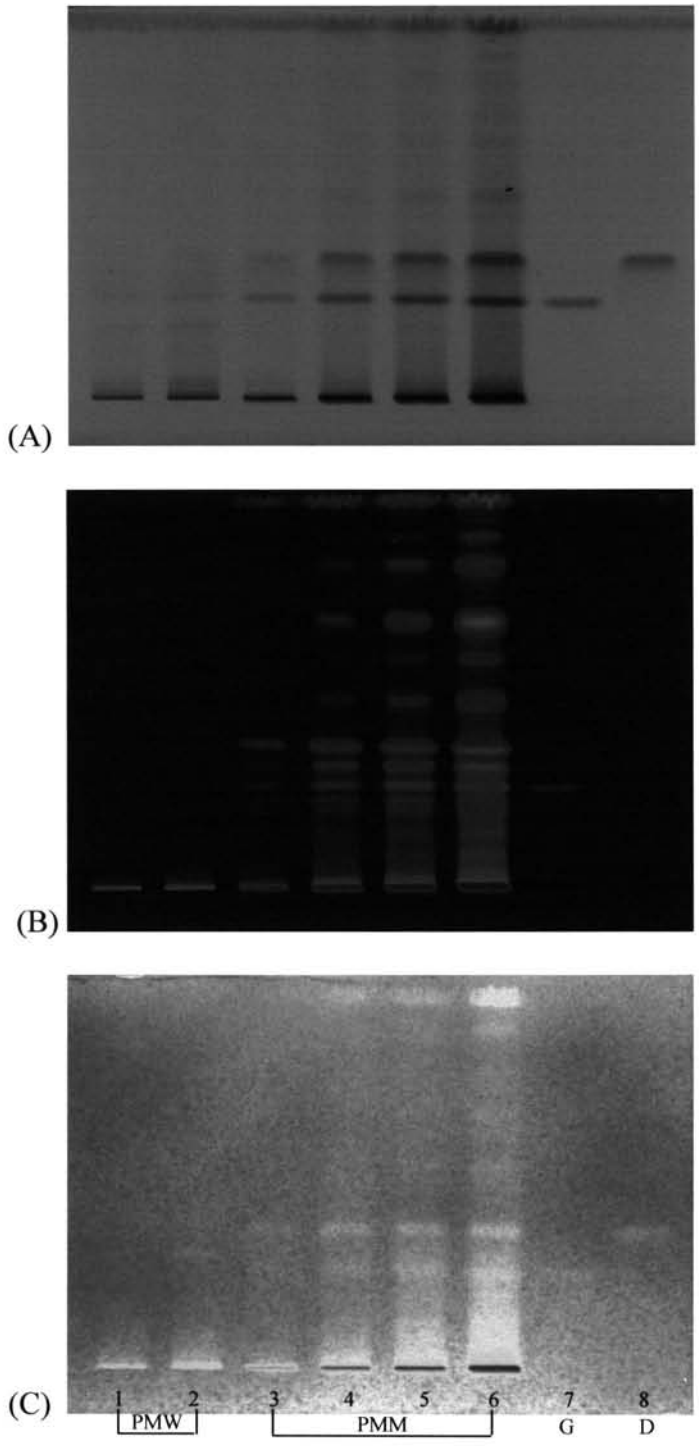

Figure 8 TLC chromatograms of PMW (1: 30 μg, 2: 50 μg), PMM (3: 10 μg, 4: 30 μg, 5: 50 μg, 6: 100 μg), daidzein (7: 0.1 μg) and genistein (8: 0.1μg) at 254 nm (A), 366 nm (B) and after the DPPH spray (C)

On the other hand, the HT-22 cells treated with PMW at the concentration of 0.01, 0.1, 1, 10 and 100 μg/ml showed cell number of 89, 90, 86, 91 and 87% of the control cells, respectively. This suggested that PMW had only slight effect on the HT-22 cell proliferation which was not related to the concentration of PMW. Therefore, the observed cell inhibition might not directly the results of the chemical constituents ofPMW.

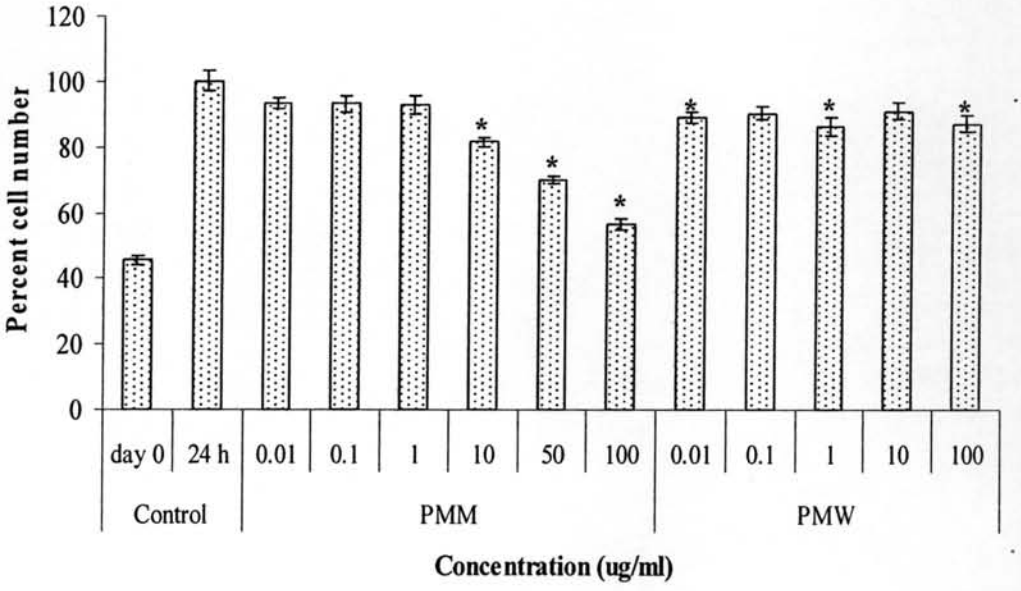

Figure 9 Cell number of HT-22 cells treated with various concentrations of PMM and PMW by SRB assay

4. Glutamate toxicity to HT-22 cells

The immortalized mouse hippocampal cell line HT-22 cells, which resemble neuronal precursor cells were used as a model for studying the mechanism of oxidative glutamate toxicity. To induce oxidative stress to HT-22 cells, glutamate

were added to cells. Glutamate induces oxidative stress by inhibiting the uptake of cystine into the cells via the cystine/glutamate transport system. Cystine is required for the synthesis of glutathione, the major intracellular antioxidant. Receptor-independent generation of oxidative stress by glutamate is termed oxidative glutamate toxicity (Tan, Schubert and Maher, 2001). The resulting loss of the cytosolic antioxidant, glutathione, effects the elevation of intracellular reactive oxygen species (ROS), which are neurotoxic (Li, Maher and Schubert, 1998, Murphy et al., 1989).

In this study, firstly HT-22 cells were treated with various concentrations of glutamate with and without ethanol to determine the effect of solvent on cell number when 1% ethanol was used to dissolve the extract. Figure 10 showed the absorbance of SRB between cells treated with various concentrations of glutamate in the presence and absence of 1% ethanol. The results showed no significant different between cells treated with 1% ethanol and without ethanol both in the presence or absence of glutamate. Therefore, 1% ethanol can be used in further experiments.

In glutamate toxicity investigation, HT-22 cells were treated with various concentrations of glutamate in 1% ethanol. The 50% killing dose of glutamate was determined. Figure 11 showed cell number in log scale when treated with different concentrations of glutamate for 24 h compared to the control cells. Glutamate exhibited its toxicity to the cells in a dose-dependent manner. HT-22 cells were killed by 50% of the control cells with glutamate at the concentration of 3.6 mM. Therefore, 3.6 mM of glutamate was the LC_{50} dose for HT-22 cells.

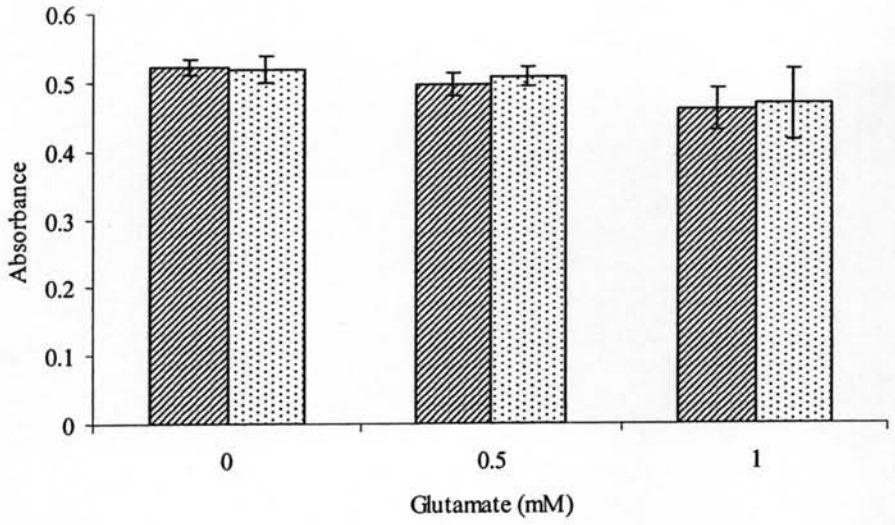

Figure 10 Absorbance of SRB between cells treated without (bar with stripes) and with (bar with dots) ethanol

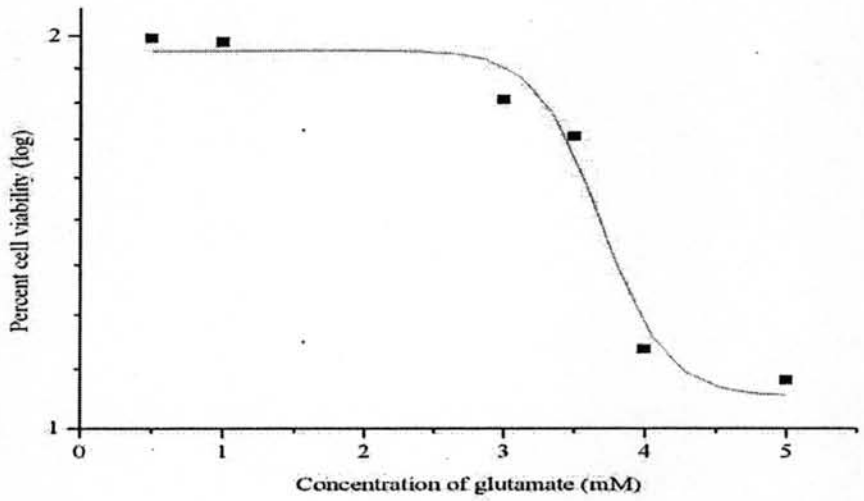

Figure 11 Percent cell number (in log scale) of HT-22 cells treated with different concentrations of glutamate for 24 h compared to the control cells

5. Neuroprotection of *P. mirifica* extracts against glutamate-induced toxicity

To investigate the protection activity of PMM and PMW against glutamate-induced toxicity, HT-22 cells were treated with different concentrations of PMM and PMW compared to Trolox, genistein and daidzein in the presence of 3.5 mM of glutamate.

The morphology of cells were examined by phase contrast microscopy whilst cells were treated with 3.5 mM of glutamate alone or with different concentrations of PMM and Trolox, PMW, genistein and daidzein for 24 h as shown in Figure 12. Control cells showed normal morphology with a high confluence (Figure 12A). There were changes in morphology and confluence of cells treated with 3.5 mM of glutamate for 24 h. More rounded up cells and a lower cell number were obvious in the glutamate-induced toxicity cells (Figure 12B). As shown in Figure 12 F and E, PMM at the concentration of 100 and 50 µg/ml exhibited clear neuroprotection against the glutamate-induced toxicity. Cells were still in normal morphology shape and more confluent than cells treated with only glutamate. Cells treated with lower levels of PMM (1 and 10 µg/ml) exhibited more rounding up and were less confluent (Figures 12C and D). Figure 12 (G-I) showed a dose-reponse protection of Trolox against glutamate toxicity. The higher concentration of Trolox, the more confluent of cells was found. Cells treated with PMW, daidzein and genistein against 3.5 mM of glutamate did had similar morphological pattern like cells treated with glutamate alone (Figures 12 J-L) and showed no indication of protection.

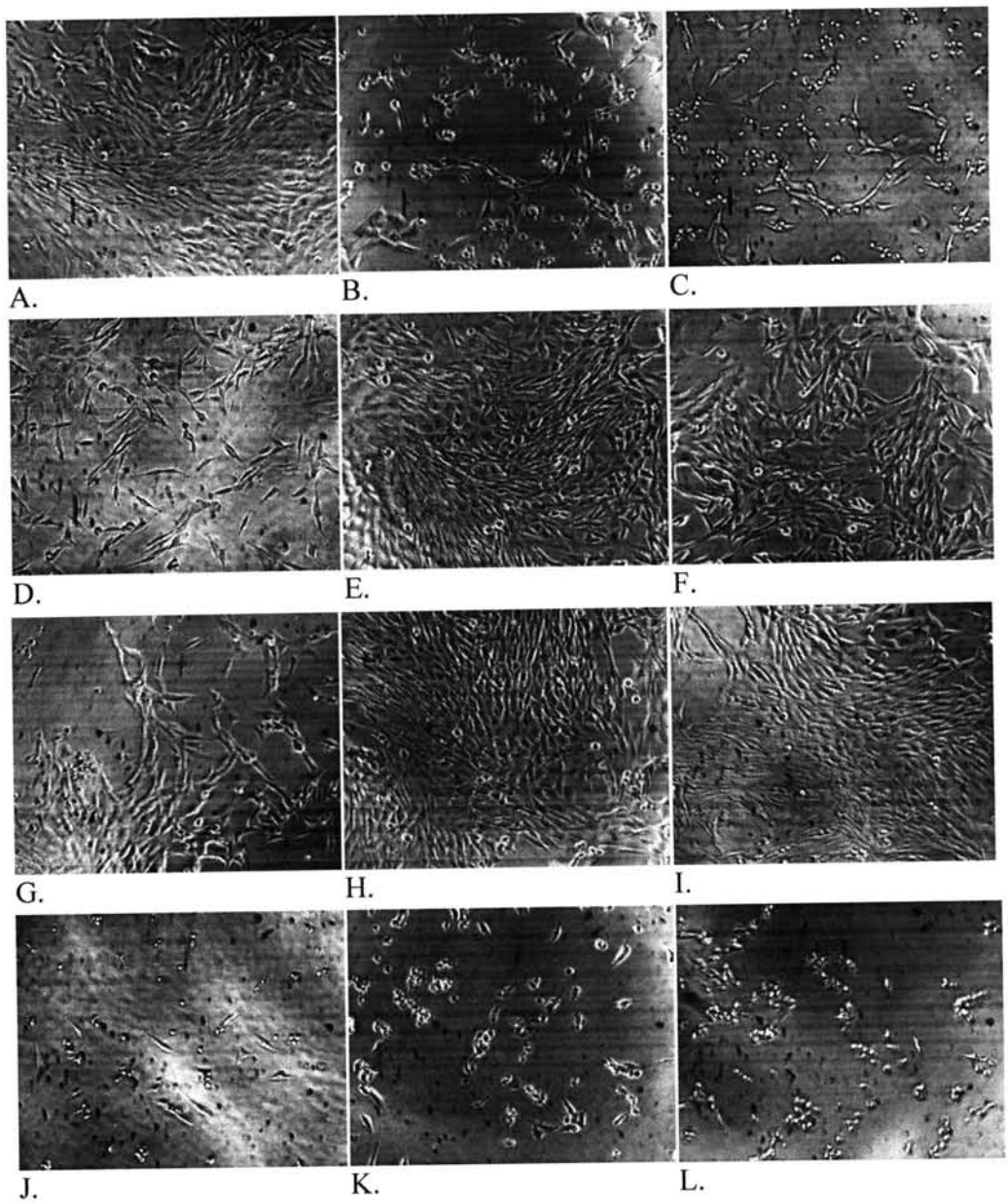

Figure 12 Morphology of HT-22 cells: control cells (A), cells treated with 3.5 mM of glutamate (B), cells treated with 3.5 mM of glutamate in the presence of PMM at 1 μg/ml (C), 10 μg/ml (D), 50 μg/ml (E) and 100 μg/ml (F), Trolox at 26.4 μg/ml (G), 132 μg/ml (H) and 264 μg/ml (I), 10 μg/ml of PMW (J), 2.54 ng/ml of daidzeine (K) and 2.7 ng/ml of genistein (L). Cells were examined by phase contrast microscopy (10x magnification)

To investigate cell apoptosis from glutamate toxicity and the neuroprotection of 100 and 50 μg/ml of PMM and 264 μg/ml of Trolox, the nuclei of cells were stained with DAPI. The nucleus of cell normally is generally round tout, as cells undergo apoptosis, the nucleus shrinks. Figure 13A showed the nucleus of the control cells most of which exhibited a large round morphology. In contrast, most of cells treated with glutamate alone showed a small elongated nucleus indicative of the apoptosis process (Figure 13B). The neuroprotection by 100 and 50 μg/ml of PMM and 264 μg/ml of Trolox against glutamate toxicity were confirmed by using the DAPI staining technique to observe the morphology of the nucleus of HT-22 cells (Figure 13C-E). Most of the nuclei still maintained round morphology and less cells contained apoptotic nuclei than cells treated with only glutamate.

The patterns of neutral red dye uptake of cells treated with different concentrations of PMM compared to Trolox against glutamate toxicity and to the control cells are shown in Figure 14. Neutral red is a vital dye which is endocytosed by viable cells and internalized inside lysosomes. For the control cells, (Figure 14A) the neutral red dye was taken up and retained in cells in the cytoplasm around the nucleus. The nucleus of cells can be readily distinguish as the clear rounded area at the middle of cells. Cells treated with glutamate alone were smaller than the control cells and the nucleus could not be seen clearly. There were no nucleus zone was found and neutral red dye was filled up the whole cell (Figure 14B). Cell treated with 50 μg/ml (Figure 14D) and 100 μg/ml of PMM (Figure 14C) and 264 μg/ml of Trolox (Figure 14F) against glutamate toxicity still exhibited normal morphology and confluency.

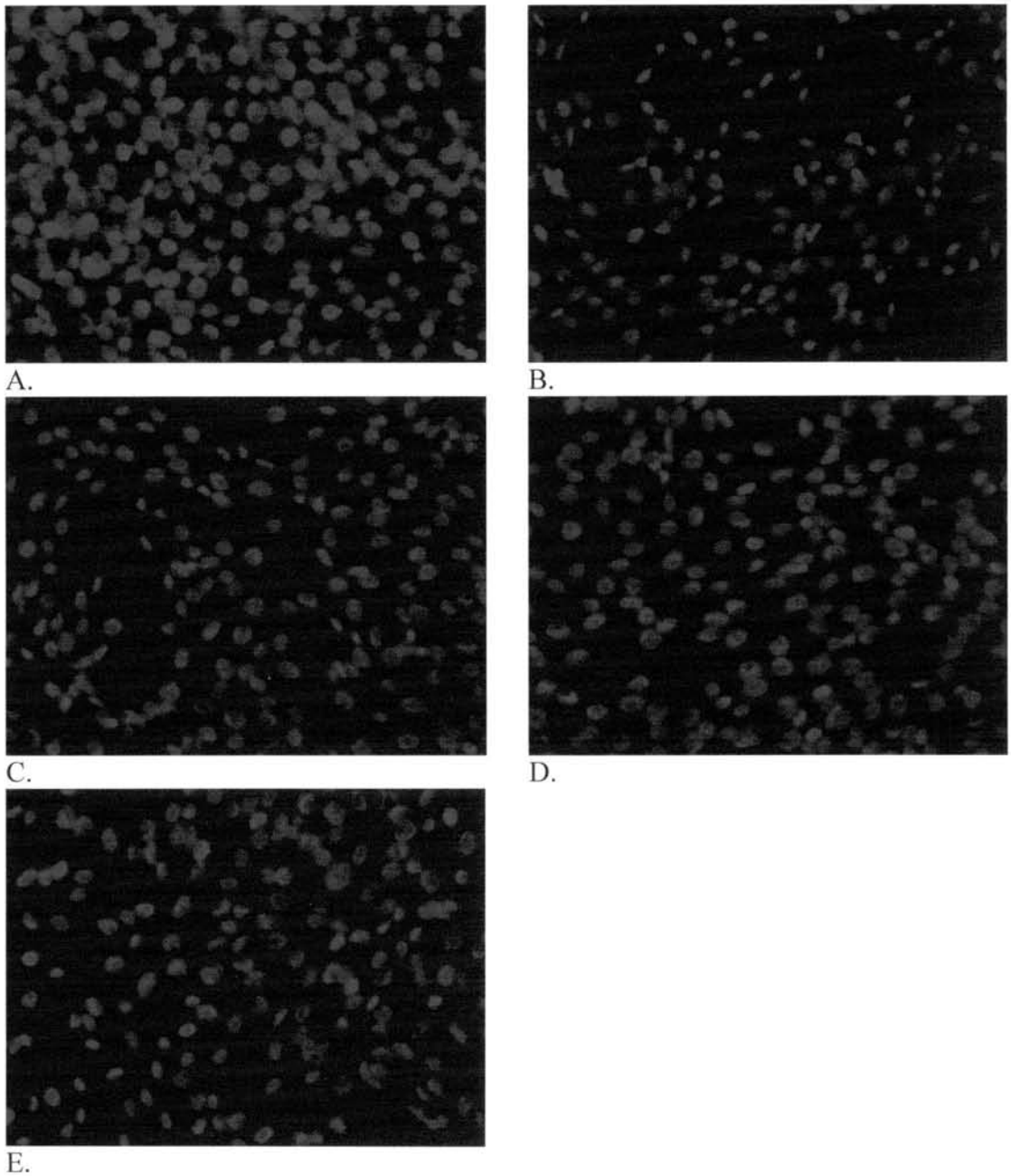

Figure 13 Nucleus of cells stained with DAPI: control cells (A), cells treated with 3.5 mM of glutamate (B), cell treated with 100 μg/ml (C), 50 μg/ml (D) of PMM and 264 μg/ml of Trolox (E) against 3.5 mM glutamate. Cell were examined by fluorescent microscopy (20X magnifications)

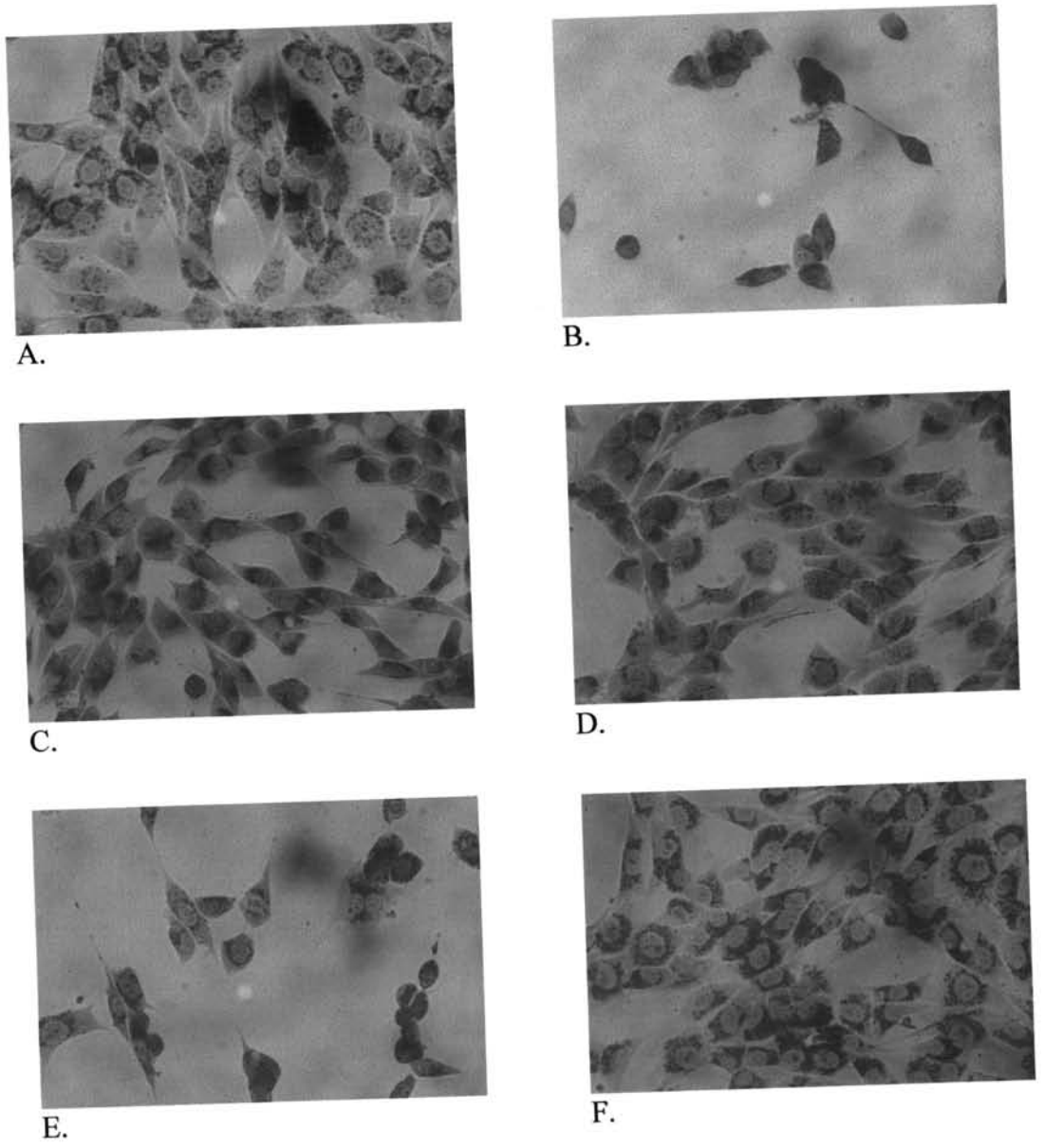

Figure 14 Neutral red dye uptake of cells: control cells (A), cells treated with 3.5 mM of glutamate (B), cells treated with 100 µg/ml (C), 50 µg/ml (D) and 10 µg/ml (E) of PMM against 3.5 mM of glutamate and cells treated with 264 µg/ml of Trolox (F) against 3.5 mM of glutamate. Cells were examined by microscope (40X magnification)

To qauntitate the neuroprotection against glutamate toxicity of PMM, PMW, Trolox, genistein and daidzein, the SRB assay was utilized. The results from the SRB assay (Figure 15) showed that cell number of HT-22 cells treated with 26.4, 132 and 264 μg/ml of Trolox against glutamate were 63, 78, and 83 % of the control cells, respectively.

They were significantly different from that of cells treated with only glutamate (46%). Therefore, Trolox at the concentration of 26.4, 132 and 264 μg/ml were found to have neuroprotective activity against 3.5 mM of glutamate. The result from this study showed that the glutamate-induced toxicity involving in the oxidative damage by ROS could be attenuate by Trolox. Therefore, the glutamate-induced toxicity in the HT-22 cells is the suitable model for studying the neuroprotection of antioxidants.

For PMM, cell number of HT-22 cells treated with different concentrations of PMM are shown in Figure 15. The number of cells treated with 50 μg /ml of PMM against glutamate was 72% of the control cells, which is significantly different from that of cells treated with only glutamate (46%). Therefore, PMM at the concentration of 50 μg/ml was found to have neuroprotective activity against 3.5 mM of glutamate.

Nonetheless, from previous proliferation experiments, the results indicated that PMM at high concentration could inhibit cell proliferation. This explained the non-dose response of PMM protection when treated with 50 μg/ml of PMM against 3.5 mM of glutamate (72%) and 100 μg/ml of PMM against glutamate (57%).

However, number of cells treated with 50 and 100 μg/ml of PMM alone was 70 and 56% of the control cells. In the presence of glutamate, the cell numbers of HT-22 cells treated with 50 and 100 μg/ml of PMM were not decreased by glutamate toxicity. Therefore, glutamate did not have an effect on decreasing number of cells

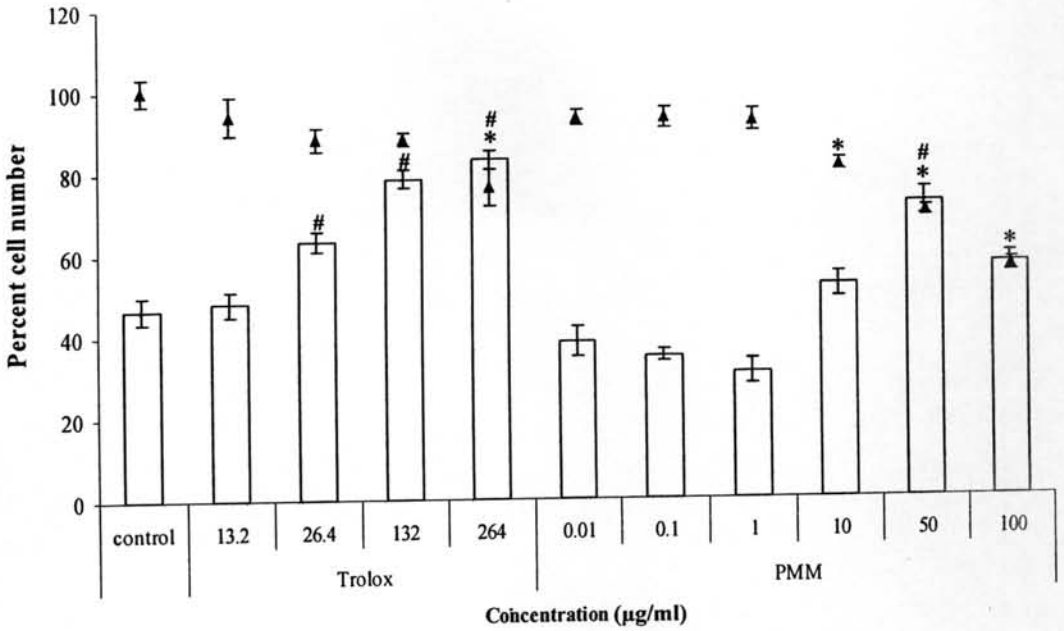

Figure 15 Cell number of HT-22 cells treated with different concentrations of Trolox and PMM with (bar) and without (triangle) 3.5 mM of glutamate, p value < 0.05 : compared to control cells with glutamate (#) and without glutamate (*)

treated with 50 and 100 µg/ml of PMM. In other way, PMM at the concentration of 50 and 100 µg/ml could protect HT-22 cells from glutamate toxicity.

Moreover, 50 and 100 µg/ml of PMM completely blocked the toxicity of glutamate similar to Trolox (264 µg/ml). However, daidzein and genistein at the concentrations of 0.25 to 2.5 and 0.27 to 2.7 µg/ml, respectively (Table 24), which cover the amount of daidzein and genistein found in 50 and 100 µg/ml of PMM extract did not exhibit any protection activity. Therefore, the activity of genistein or daidzein alone, has no effect on the protective activity of PMM. The neuroprotective activity of PMM against glutamate toxicity in HT-22 might be the result from many compounds in the extract that support each other.

Cell number of HT-22 cells treated with other concentrations of PMM and all concentrations of PMW (Tables 21 and 23) against glutamate toxicity was not significantly different from that of cells treated with glutamate alone.

PMW exhibits the lack of neuroprotective activity in this study. The poor penetration of the polar antioxidants through the cell membrane might be the explanation. A possible contributory mechanism to the antioxidative activity of isoflavonoids has been proposed is that they might able to stabilize membranes by decreasing membrane fluidity. Localization of isoflavonoids into membrane interiors and increased restrictions on the fluidity of membrane components could sterically hinder diffusion of free radicals and thereby decrease the kinetics of free radical reactions (Arora et al., 2000).

For optimal antioxidant capacity, a compound must orient within the membrane structure to facilitate donation of hydrogen atoms to phospholipid radicals (Bisby, 1990). Alpha-tocopherol is one of the most potent inhibitors of lipid peroxidation in microsomal preparations (Bors and Saran, 1987) as the hydrophobic phytyl chain results in effective partitioning into the lipid bilayer and optimal spatial distribution of the hydrogen-donating chromanol head group.

In conclusion, PMM but not PMW exhibited the neuroprotective activity against oxidative stress from glutamate toxicity in HT-22 cells. Therefore, the antioxidative activity of PMM against ROS were further investigated.

6. Antioxidative activity determined by ORAC assay and DCFH-dA assay

Since the formation of peroxyl radicals is a key step in lipid peroxidation and H_2O_2 is thought to convert into more reactive species, therefore this study would

focused on the antioxidative activity of PMM against the peroxyl radical and hydrogen peroxide by the ORAC and the DCFH-dA assays, respectively.

The ORAC assay measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects classical radical chain breaking antioxidative activity by H-atom transfer. The inhibition is observed as a preservation of the fluorescent signal. The protection was quantitated by calculating area under the curve (AUC) from the experimental sample. After subtracting the AUC from blank (Net AUC), the resultant difference is the protection conferred by the antioxidant compound being tested.

By applying the ORAC assay to PMM, its antioxidative activity against peroxyl radical was determined. The ORAC value was calculated based on Trolox activity. PMM has the ORAC value at 0.07 (Table 3). This result showed that PMM has much lower antioxidative activity against peroxyl radical than Trolox. Therefore, the direct scavenging property of PMM against peroxyl radical might not be the key protection mechanism of PMM against glutamate toxicity in HT-22 cells.

Table 3. Calculation for the ORAC value, linear equation, slope, intercept, and R^2 of PMM and PMW compared with Trolox from ORAC assay

Sample	Trolox	PMM
ORAC value	1.00 ± 0.078	0.07 ± 0.004
Slope	7.183	0.532
Intercept	3.576	11.018
R^2	0.998	0.998

To determine the antioxidative activities of PMM against hydrogen peroxide in HT-22 cells, the DCFH-dA assay was performed. DCFH-dA is freely permeable and enters the cell where cellular esterases hydrolyze the acetate moieties, making a polar structure that remains in the cell. ROS in the cells will oxidize the DCFH-dA, yielding the fluorescent product 2,7-dichlorofluorescin (DCF) (Rothe and Valet, 1990). Average fluorescent intensity of cells treated with PMM and Trolox as positive control were shown in Figure 16.

Cells treated with only H_2O_2 exhibited significantly high fluorescent intensity when compared to the control cells. The fluorescent intensity of cells treated with all concentrations of PMM (0.01 to 100 μg/ml), Trolox (13.2 -528 μg/ml) were significantly lower than the fluorescent intensity of cells treated with H_2O_2 alone. This result reveals that in HT-22 cells, PMM exhibits excellent antioxidative activity against hydrogen peroxide. As H_2O_2 is converted into more reactive species, the most important of which is the hydroxyl radical ($OH^{\bullet}$). The neuroprotection mechanism of PMM, therefore, should be involved with the antioxidative activity against hydrogen peroxide or other radicals generated from hydrogen peroxide.

To investigate the same antioxidative activity of daidzein and genistein in the extract, the concentration of daidzein and genistein in this experiment were set to be in the range that covers the amount of daidzein and genistein in the extracts. It was found that daidzein (0.0003-2.54 μg/ml) and genistein (0.0003-2.7 μg/ml) did not significantly lower the intensity of DCFH-dA (Tables 8 and 9). This result confirms that genistein or daidzein in the extract did not showed the antioxidative activity against hydrogen peroxide in HT-22 cells.

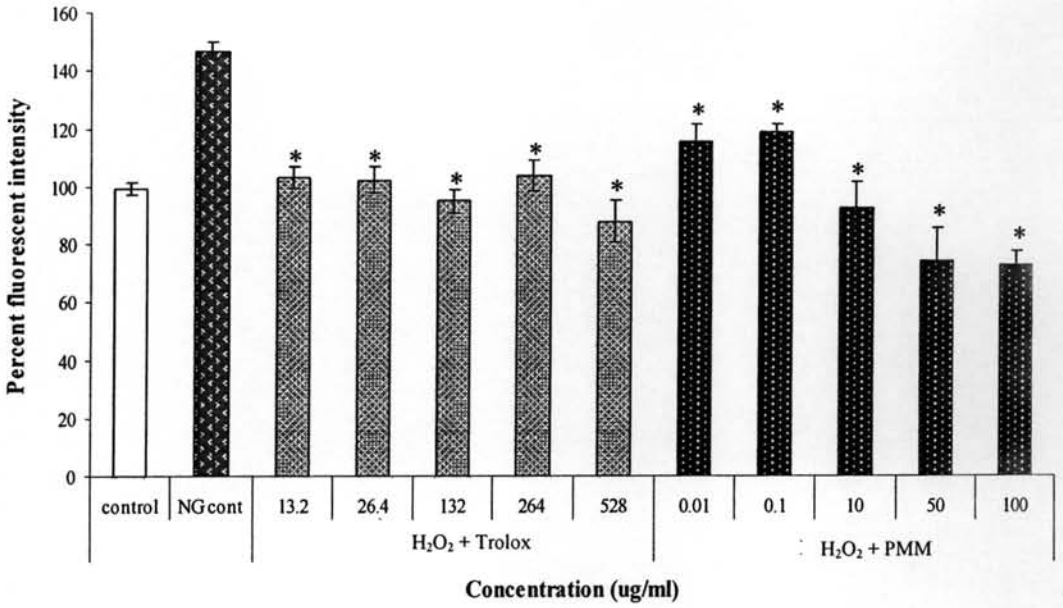

Figure 16 Average fluorescent intensity of cells treated with hydrogen peroxide in the presence of various concentrations of Trolox and PMM compared to negative control (NG cont) treated with hydrogen peroxide alone (* , p value < 0.05)

Another interesting point is the antioxidative activity of PMM in this assay was better than Trolox. This result indicated that PMM has a better activity to decompose hydrogen peroxide or other radicals generated from hydrogen peroxide such as hydroxyl radical than Trolox.

Since PMM is a mixture of polyphenolic compound such as genistein, daidzein, deoxymiroestrol, miroestrol and other substances (Chansakow et al., 2000a). Apart from directly scavenging hydrogen peroxide and other radicals to inhibit chain initiation and break chain propagation, the neuroprotection of PMM could be resulting from many compounds that mediate in different pathways to support each other both enzymatic or non-enzymatic pathway (i.e. synergistic protection) to maintain the free

radical defense system in HT-22 cells. PMM could be involve in suppressing the formation of free radicals by generating appropriate antioxidant enzymes such as catalase, glutathione peroxidase, peroxidase or increasing intracellular glutathione to reduce the generation of ROS.

It has been reported that chronic dietary administration of isoflavones significantly elevated the activities of catalase, SOD, glutathione peroxidase, and glutathione reductase in murine skin. In human endothelial cells, isoflavones slightly increased intracellular-reduced glutathione levels approximately by 10 to 30%. The ability of isoflavones to increase cellular GSH (reduced glutathione) might be important for their action in biological system (Guo et al., 2002).

The explanation that PMM involves an inhibition of the glutamate-induced toxicity cascade such as attenuation of calcium fluxes to protect the HT-22 cell is also possible. Since many flavonoids protect HT-22 cells from glutamate toxicity by increasing intracellular glutathione, directly lowering levels of ROS and preventing the influx of calcium (Ishige, Schubert and Sagara, 2001). Moreover, the effect of PMM to the process of repairing the damage and reconstitute cell membrane or the inhibition of particular apoptosis pathways should not be eliminated.

Another interesting point is the concentration of PMM showing the protection against glutamate toxicity was found to inhibit cell proliferation. Therefore, PMM should be further purified and investigated for the toxicity and protective activity of each pure substance in the extract.

In conclusion, the neuroprotection of PMM involving in the scavenging activity against hydrogen peroxide and the radicals formed by hydrogen peroxide but not the peroxyl radicals has been proved in this study.