CHAPTER 3

Results

3.1 Evaluation of major constituents in purple rice bran extracts

The dichloromethane extract was greasy green whereas the methanol extract was dark-purple powder (Figure 29). The percentage yield of dichloromethane and methanol extracts were presented as 13.285 ± 3.762 and 7.910 ± 0.351, respectively. The contents of some main components in each extract were determined using spectrophotometry and HPLC. The chromatograms of standard phenolic acids, flavonoids and anthocyanins were presented in Figure 30-A and 31-A, while the chromatograms of gamma-oryzanol and tocols were shown in Figure 32-A and 33-A. The methanol extract of purple rice bran contained higher amounts of total phenolic compounds and total flavonoids than the dichloromethane extract of purple rice bran (Table 1). The dichloromethane extract has higher contents of lipophilic compounds, gamma-oryzanol and tocols. Delta- and gamma-tocotrienols were found in the dichloromethane extract (Figure 33-B). Protocatechuic acid and vanilic acid were the phenolic compounds detected in the methanol extract of purple rice bran under our HPLC system (Figure 30-B). The major anthocyanins in the methanol extract of purple rice bran were cyanidin-3-glucoside and peonidin 3-*O*-glucoside (Figure 31-B).

(A) **(B)**

Figure 29. Purple rice bran extracts; dichloromethane extract **(A)** and methanol extract **(B)**

Table 1. Major compounds in purple rice bran extracts

Compounds	Contents (mg/g extract)	
	Dichloromethane	Methanol
Colorimetric methods		
- Total phenolic compounds	33.68 ± 1.15	91.31 ± 5.16
- Total flavonoids	25.90 ± 0.80	53.10 ± 2.66
HPLC analysis		
Hydrophilic compounds		
- Protocatechuic acid	ND	1.00 ± 0.66
- Vanilic acid	ND	0.73 ± 0.39
- Cyanidin-3-glucoside	ND	1.24 ± 0.27
- Peonidin 3-*O*-glucoside	ND	0.76 ± 0.12
Lipophilic compounds		
- Gamma-oryzanol	12.56 ± 0.00	5.87 ± 0.01
- Delta-tocotrienols	0.013 ± 0.001	ND
- Gamma-tocotrienols	0.107 ± 0.005	0.014 ± 0.001

Values are expressed as mean ± SD

ND: Not detected

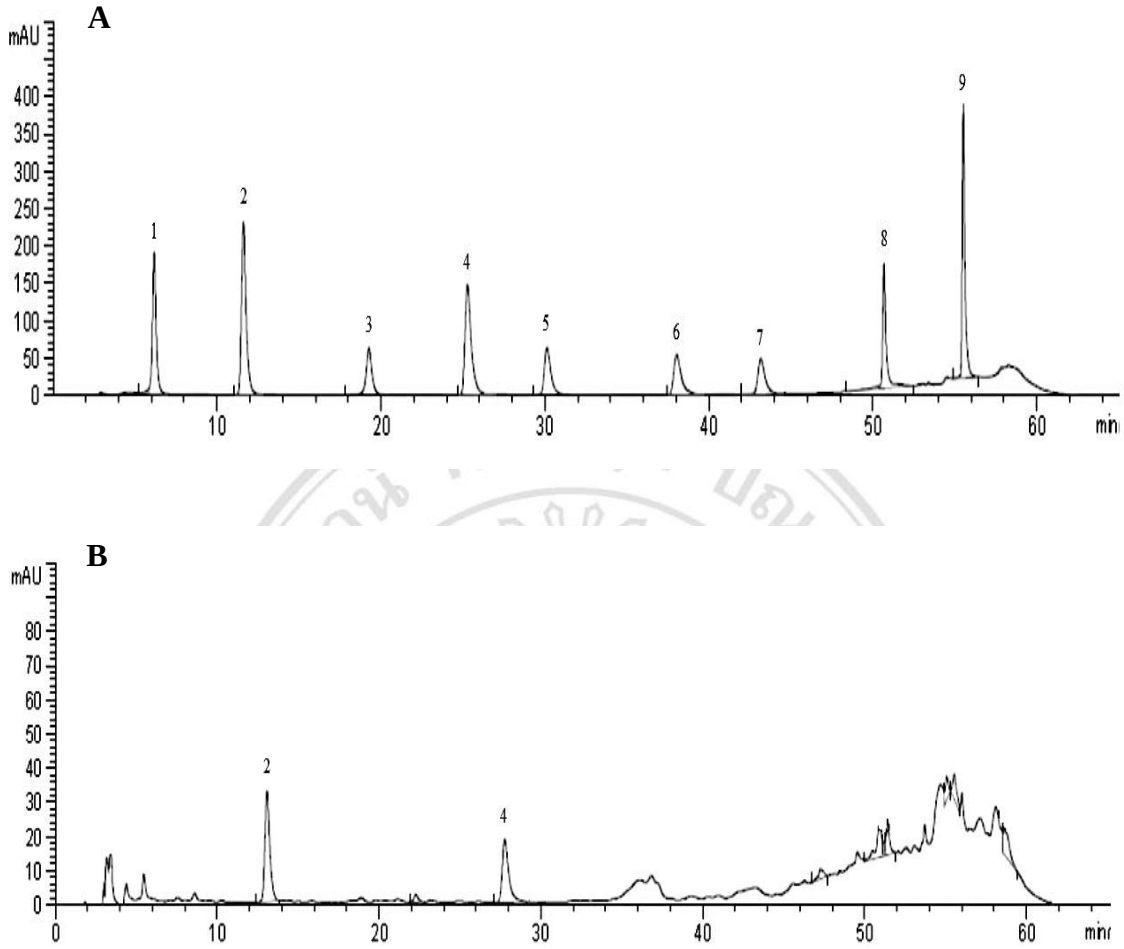

Figure 30. Chromatograms of standard phenolic acids and flavonoids (A) and some phenolic acids in methanol extract of purple rice bran (B). Peaks 1; gallic acid, 2; protocatechuic acid, 3; catechin, 4; vanilic acid, 5; epicatechin, 6; *p*-coumaric acid, 7; ferulic acid, 8; rutin and 9; quercitin.

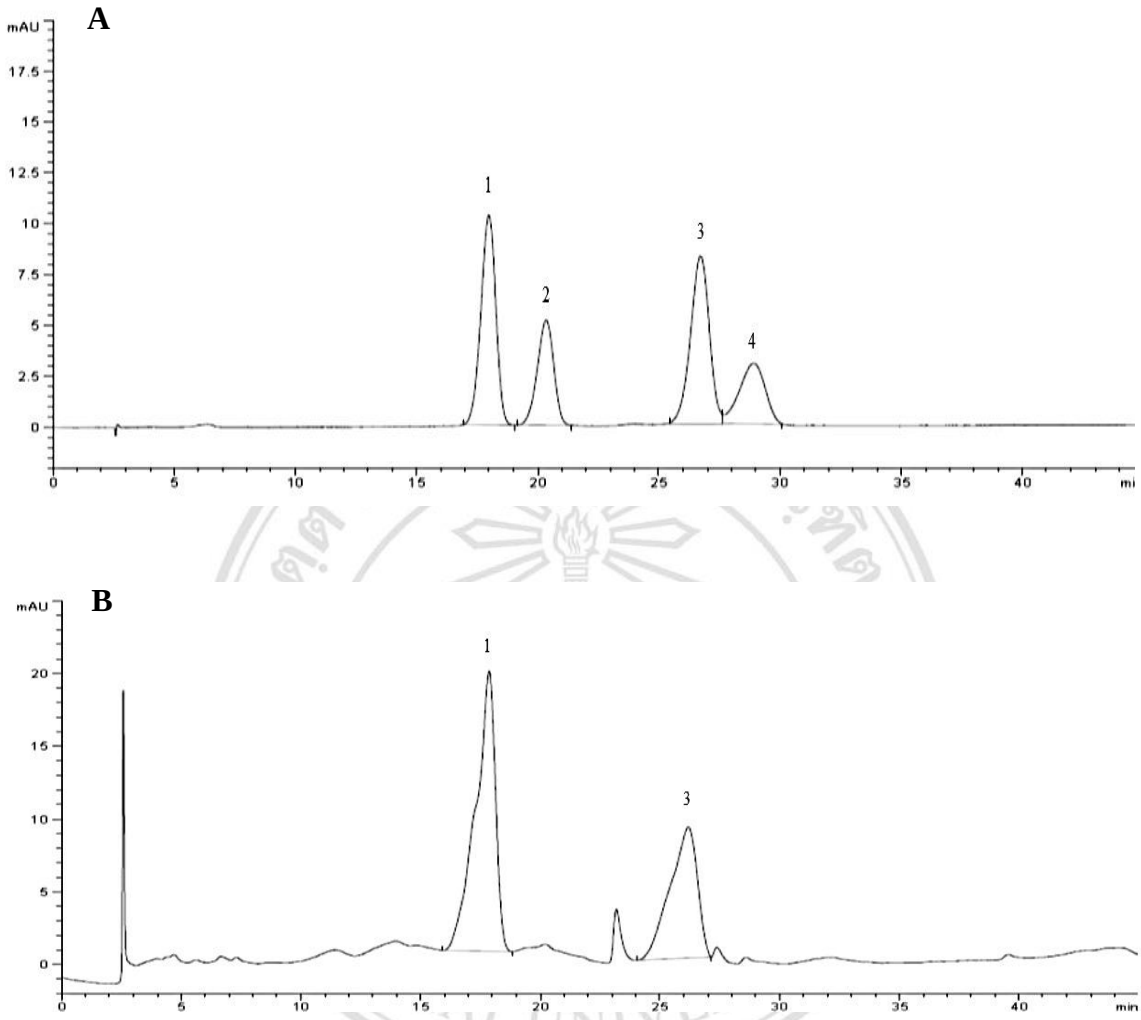

Figure 31. Chromatograms of standard anthocyanins (A) and some anthocyanins in methanol extract of purple rice bran (B). Peaks 1; cyanidin-3-O-glucoside, 2; cyanidin-3-rutinoside, 3; peonidin-3-O-glucoside and 4; malvidin-3-glucoside.

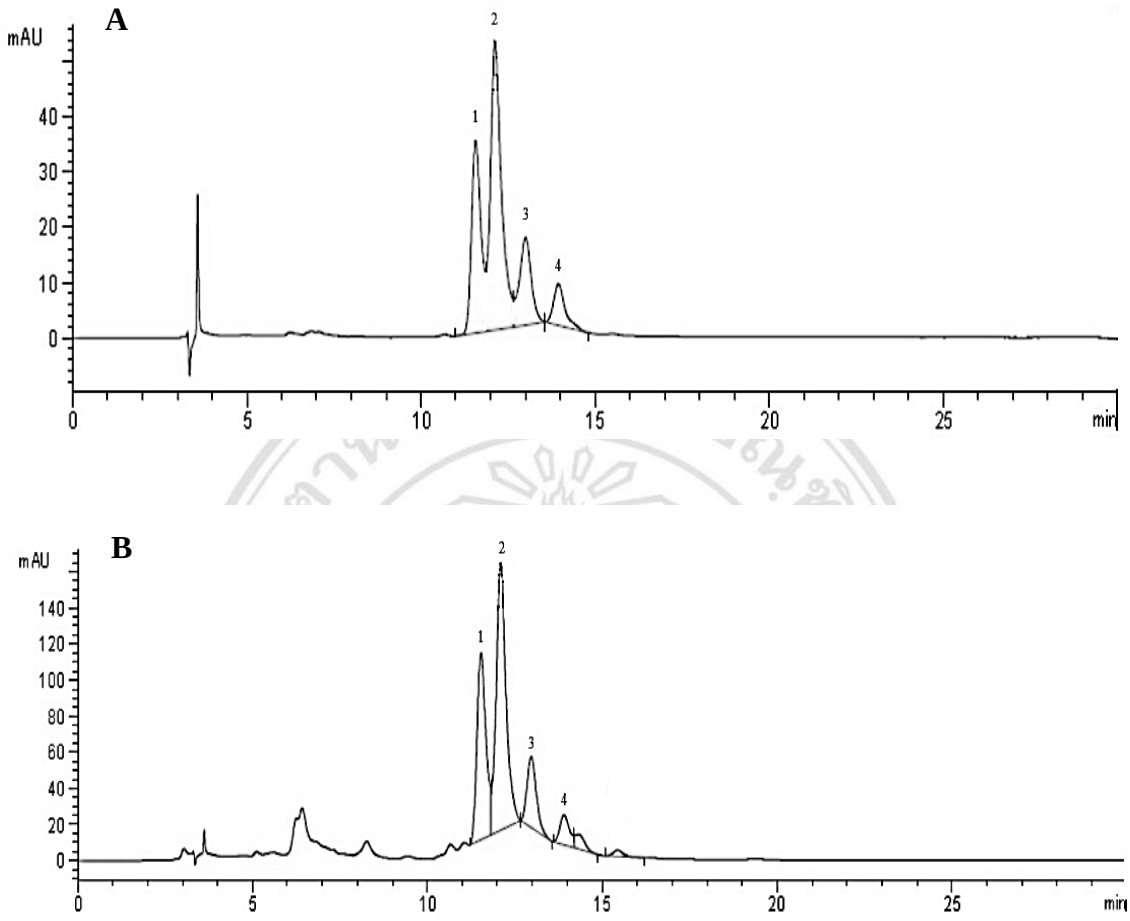

Figure 32. Chromatograms of standard gamma-oryzanol (A) and some gamma-oryzanol in dichloromethane extract of purple rice bran (B). Peaks 1; cycloartenyl ferulate, 2; 24-methylene cycloartanyl ferulate, 3; campesteryl ferulate and 4; β-sitosteryl ferulate.

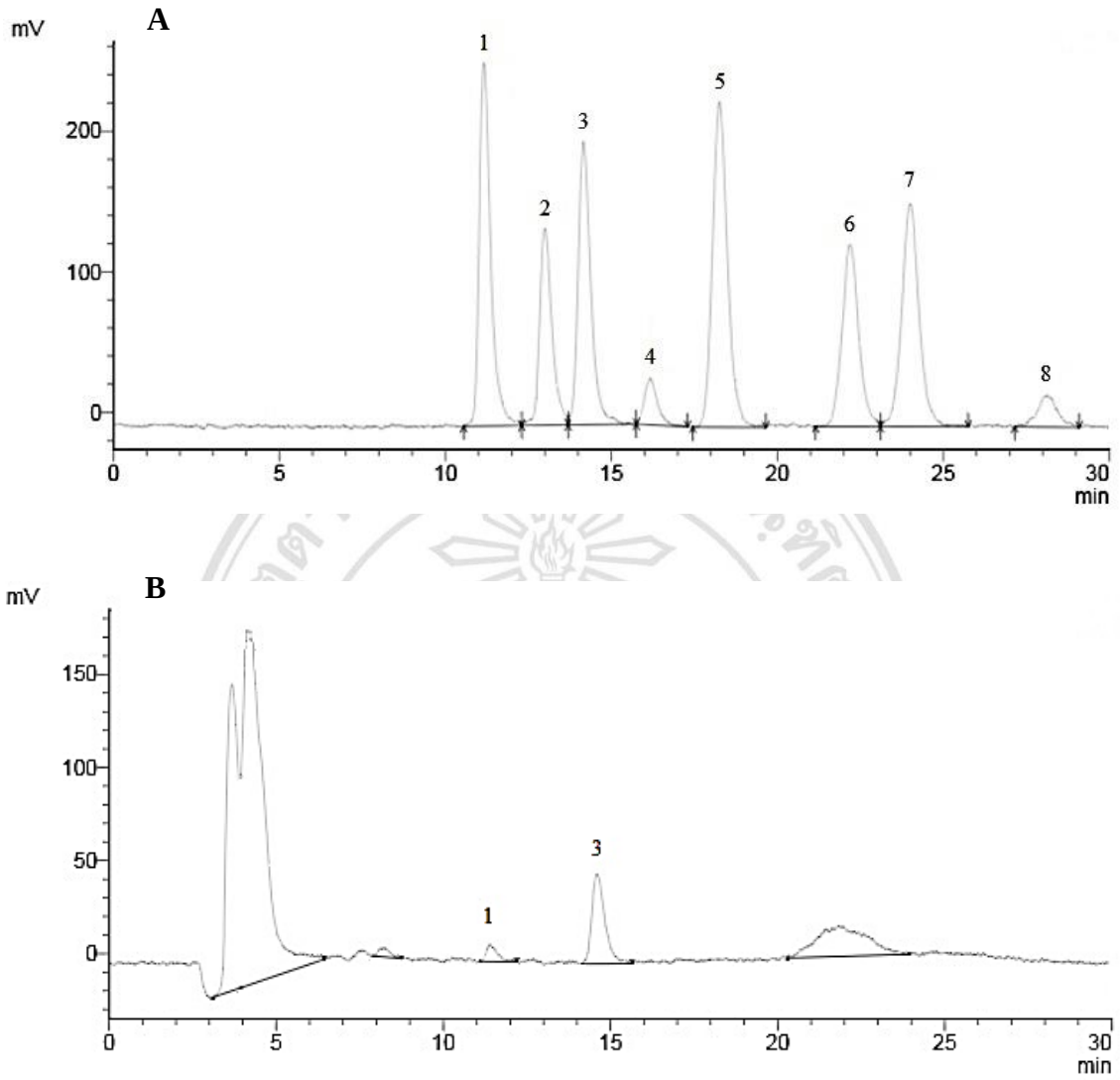

Figure 33. Chromatograms of standard tocols (A) and some tocols in dichloromethane extract of purple rice bran (B). Peaks 1; δ-tocotrienol, 2; β-tocotrienol, 3; γ-tocotrienol, 4; α-tocotrienol, 5; δ-tocopherol, 6; β -tocopherol, 7; γ-tocopherol and 8; α –tocopherol.

3.2 Effect of purple rice bran extracts on micronucleus formation in rat liver

The 28 day treatment of dichloromethane or methanol extracts of purple rice bran did not affect on food and water intake and body weight of rats. Moreover, these extracts did not induce the development of micronucleated, binucleated and mitotic hepatocytes (Figure 34) compared with vehicle control group. Thus, the consumption of dichloromethane and methanol extracts of purple rice bran was not toxic in rats (Table 2, Figure 35).

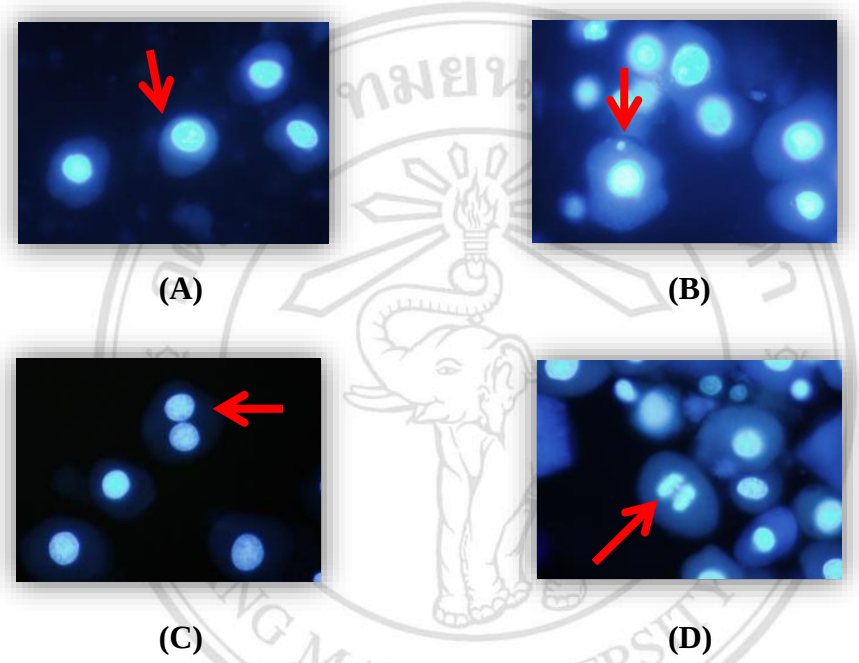

Figure 34. Microscopic morphology of hepatocytes; normal hepatocytes (A), micronucleus (B), binucleated hepatocytes (C) and mitotic hepatocytes (D)

3.3 Effect of purple rice bran extracts on xenobiotic metabolizing enzymes

The dichloromethane and methanol extracts did not alter the activities of both phase I and phase II enzymes. However, low dose of dichloromethane extract significantly induced the activities of CYP 1A1, 1A2, CPR and UGT (Table 3, Figure 36-37). Using Western blot analysis, it also increased the protein expression of CYP 1A1/2 but it had no effect on CPR and UGT protein expressions (Table 4, Figure 38).

Table 2. Effect of purple rice bran extracts on body weight and micronucleus formation in rat liver

Treatments	Body weight (g)		MN (per 1,000 Hep)	MN cells (per 1,000 Hep)	BNH (per 1,000 Hep)	MI (%)
	Initial	Final				
5% Tween-80	110.0 ± 19.8	279.2 ± 22.9	2.95 ± 0.76	2.83 ± 0.58	1.18 ± 0.30	1.19 ± 0.41
100 mg/ kg bw DME	100.0 ± 4.5	280.8 ± 14.6	3.04 ± 0.64	2.83 ± 0.66	1.35 ± 0.30	1.37 ± 0.28
500 mg/ kg bw DME	101.7 ± 9.3	288.3 ± 33.1	3.37 ± 0.59	3.08 ± 0.60	1.42 ± 0.44	1.45 ± 0.27
100 mg/ kg bw ME	100.0 ± 0.0	291.7 ± 20.2	3.46 ± 1.03	3.46 ± 1.03	1.19 ± 0.22	1.36 ± 0.23
500 mg/ kg bw ME	98.3 ± 4.1	283.3 ± 11.3	3.41 ± 0.81	3.32 ± 0.87	1.25 ± 0.24	1.34 ± 0.27

Values are expressed as mean ± SD

DME; Dichloromethane extract, ME; Methanol extract, MN; Micronucleus, BNH; Binucleated hepatocyte, MI; Mitotic index, Hep; Hepatocytes

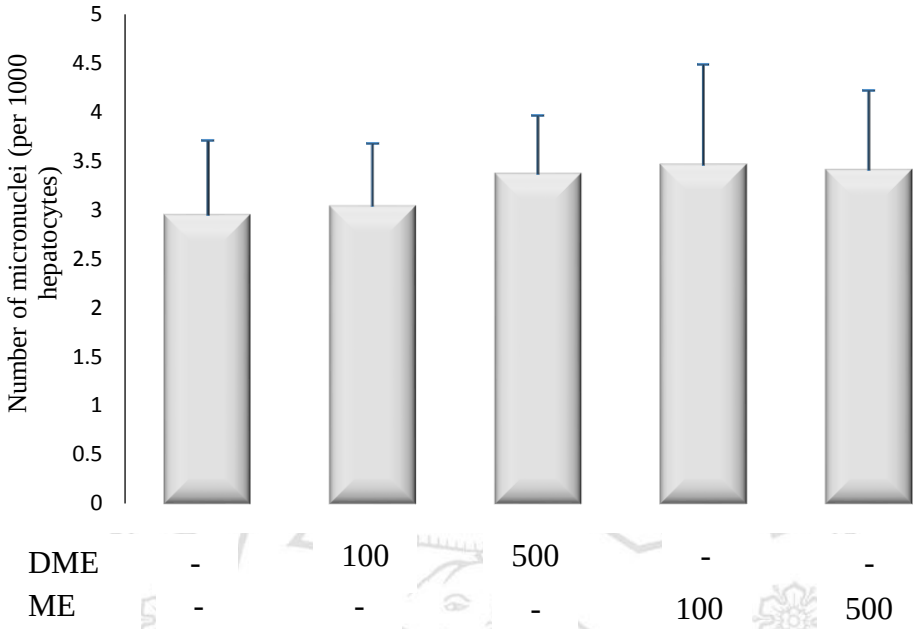

Figure 35. The number of hepatic micronuclei of purple rice bran extracts-treated rats

Table 3. Effect of purple rice bran extracts on the activities of xenobiotic metabolizing enzymes in rat liver

Treatments	CYP 1A1 ($x10^{-2}$ pmole/min/ mg protein)	CYP 1A2 ($x10^{-3}$ pmole/min/ mg protein)	CYP 3A2 (pmole/min/ mg protein)	CPR ($x10^{-3}$ U/ mg protein)	GST ($x10^{-2}$ U/ mg protein)	UGT ($x10^{-3}$ U/ mg protein)
5% Tween-80	18.058 ± 3.228	24.958 ± 2.596	12.541 ± 1.64	9.904 ± 0.957	70.439 ± 4.993	36.065 ± 4.256
100 mg/ kg bw DME	25.568 ± 3.058*	31.574 ± 5.414*	13.061 ± 0.657	12.047 ± 1.940*	82.042 ± 13.740	58.391 ± 22.370*
500 mg/ kg bw DME	21.332 ± 3.502	27.993 ± 4.255	12.249 ± 1.372	10.886 ± 1.290	79.815 ± 8.110	35.856 ± 13.490
100 mg/ kg bw ME	18.995 ± 2.546	25.322 ± 2.113	11.428 ± 0.801	9.899 ± 0.740	69.823 ± 8.240	33.924 ± 8.000
500 mg/ kg bw ME	18.202 ± 2.884	24.933 ± 3.790	11.146 ± 0.588	10.140 ± 1.470	73.414 ± 14.840	40.208 ± 9.790

Values are expressed as mean ± SD

DME; Dichloromethane extract, ME; Methanol extract, CYP; Cytochrome P450, CPR; NADPH-cytochrome P450 reductase, GST; glutathione *S*-transferase, UGT; UDP-glucuronyltransferase

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

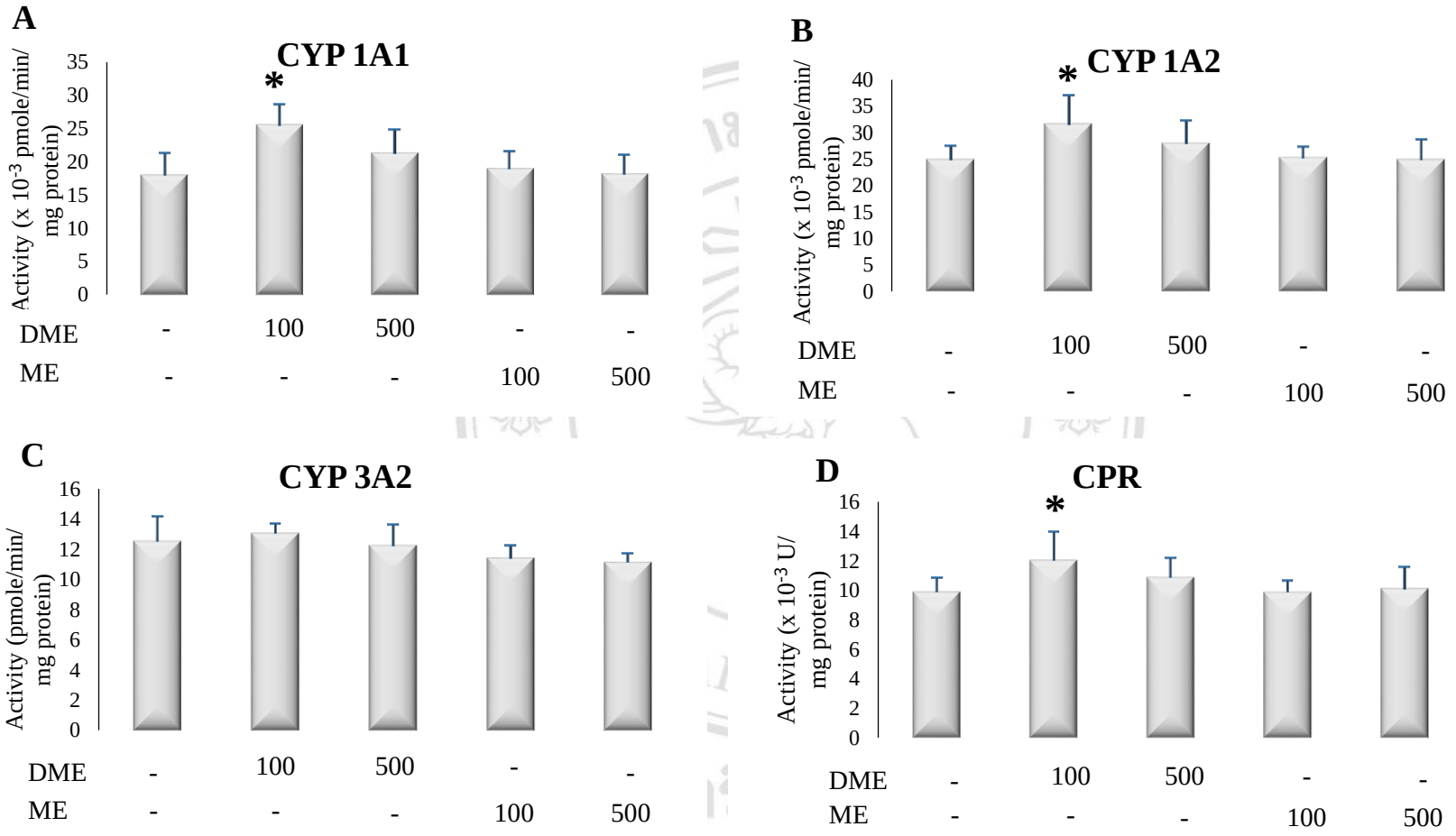

Figure 36. The activities of phase I xenobiotic metabolizing enzymes in rat livers treated by purple rice bran extracts

A; CYP; Cytochrome P450, CPR; NADPH-cytochrome P450 reductase

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

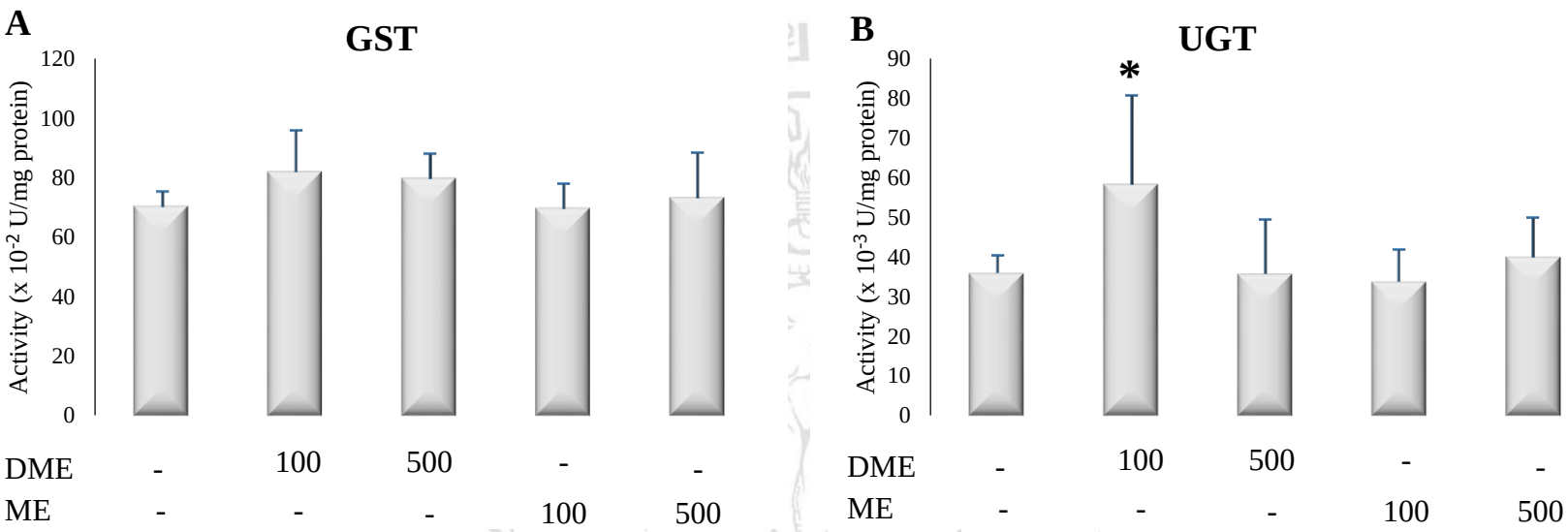

Figure 37. The activities of phase II xenobiotic metabolizing enzymes in rat livers treated by purple rice bran extracts

A; glutathione *S*-transferase and B; UDP-glucuronyltransferase

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

Table 4. Effect of purple rice bran extracts on the expression of xenobiotic metabolizing enzymes in rat livers using Western blot analysis

Treatments	Fold change				
	CYP 1A1/2	CYP 3A1/2	CPR	GST	UGT
5% Tween-80	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000
100 mg/ kg bw DME	1.200 ± 0.133*	1.188 ± 0.301	1.083 ± 0.061	0.945 ± 0.007	1.038 ± 0.034
500 mg/ kg bw DME	1.018 ± 0.138	0.753 ± 0.066	0.932 ± 0.126	0.958 ± 0.066	1.077 ± 0.052
100 mg/ kg bw ME	1.028 ± 0.125	1.026 ± 0.164	0.953 ± 0.107	0.906 ±0.113	1.018 ± 0.024
500 mg/ kg bw ME	1.013 ± 0.045	1.110 ± 0.127	0.979 ± 0.066	1.012 ± 0.147	1.067 ± 0.073

Values are expressed as mean ± SD

DME; Dichloromethane extract, ME; Methanol extract, CYP; Cytochrome P450, CPR; NADPH-cytochrome P450 reductase, GST; glutathione *S*-transferase, UGT; UDP-glucuronyltransferase

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

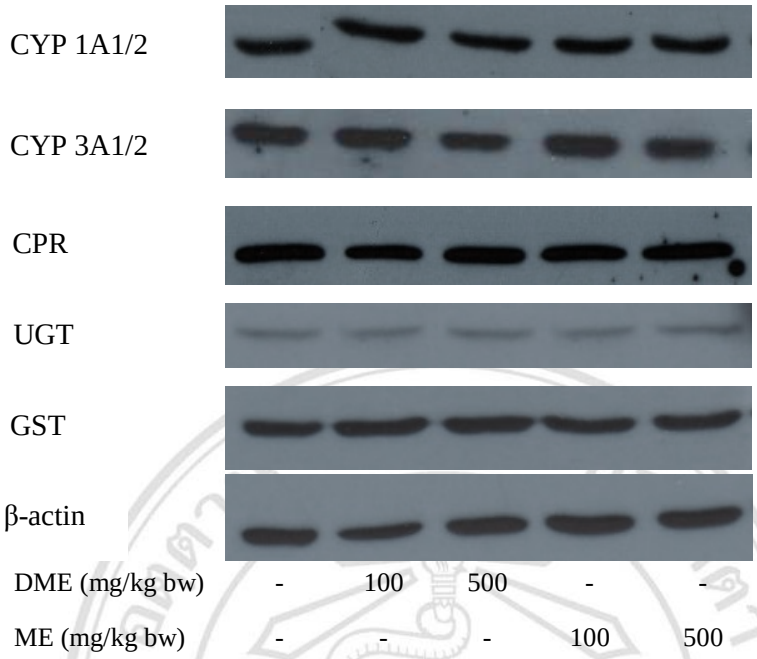

Figure 38. Protein expression of xenobiotic metabolizing enzymes in the livers of purple rice bran extracts treated rats

3.4 Optimization of AFB_1 on micronucleus formation in rat liver

The results showed that AFB_1 could increase number of micronuclei, binucleated hepatocytes and mitotic cells without body weight change when compared with control group. The administration of AFB_1 at dose 200 μg/kg bw could enhance more number of micronuclei and binucleated hepatocytes in the livers of rats than AFB_1 at dose 100 μg/kg bw. There was no significant difference in number of micronuclei and binucleated hepatocytes between single and double injections (Table 5, Figure 39). However, double injection of AFB_1 at 200 μg/kg bw significantly induced CYP 1A2 activity and reduced GST activity in rat liver (Table 6, Figure 40). From this reason, double injection of 200 μg/kg bw of AFB_1 was chosen for further investigation.

Table 5. Effect of AFB_1 administration on micronucleus formation in rat liver

Treatments		Body weight (g)		MNH (per 1,000 Hep)	MN cells (per 1,000 Hep)	BNH (per 1,000 Hep)	MI (%)
AFB_1 (μg/kg bw)	Day	Initial	Final				
0	-	100.0 ± 0.0	265.0 ± 13.2	3.00 ± 0.50	2.83 ± 0.29	1.43 ± 0.37	1.66 ± 0.75
100	4	102.8 ± 2.5	260.0 ± 8.2	8.74 ± 3.59*	7.74 ± 3.30*	2.23 ± 0.99*	3.58 ± 1.26*
100	0, 4	101.7 ± 2.6	260.0 ± 10.0	8.67 ± 2.02*	7.83 ± 2.08*	2.08 ± 0.45*	3.13 ± 0.21*
200	4	100.0 ± 5.0	261.7 ± 10.4	14.49 ± 3.91*,¥	13.66 ± 3.79*,¥	3.66 ± 0.55*,¥	5.26 ± 0.89*,¥
200	0, 4	101.2 ± 2.0	257.0 ± 9.1	14.39 ± 3.79*,¥	12.74 ± 3.47*,¥	3.03 ± 0.17*,¥	3.71 ± 0.35*,¥

Values are expressed as mean ± SD

MN; Micronucleus, BNH; Binucleated hepatocyte, MI; Mitotic index, Hep; Hepatocyte

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

¥ Significantly different at $p < 0.05$ compared to 100 μg/kg bw of AFB_1-treated group

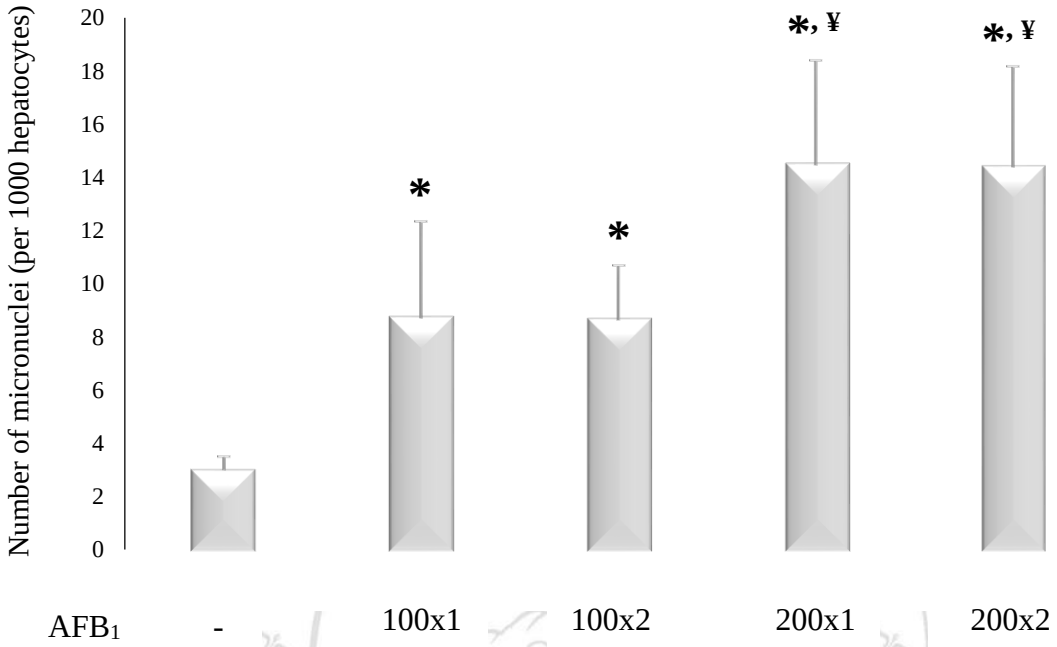

Figure 39. The frequencies of hepatic micronuclei induced by AFB_1

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

¥ Significantly different at $p < 0.05$ compared to 100 µg/kg bw of AFB_1-treated group

Table 6. Effect of AFB_1 treatments on the activities of some major xenobiotic metabolizing enzymes in AFB_1 metabolism in rat liver

Treatments		CYP 1A2 ($x10^{-3}$ pmole/min/mg protein)	GST ($x10^{-2}$ U/mg protein)
AFB_1 (μg/kg bw)	Day		
0	-	5.776 ± 2.270	42.313 ± 1.783
100	4	12.197 ± 1.643	42.324 ± 1.499
100	0, 4	13.102 ± 11.436	39.828 ± 3.383
200	4	17.749 ± 14.026	42.089 ± 2.685
200	0, 4	24.434 ± 9.706*	38.571 ± 3.312*

Values are expressed as mean ± SD

CYP; Cytochrome P450, GST; glutathione *S*-transferase

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

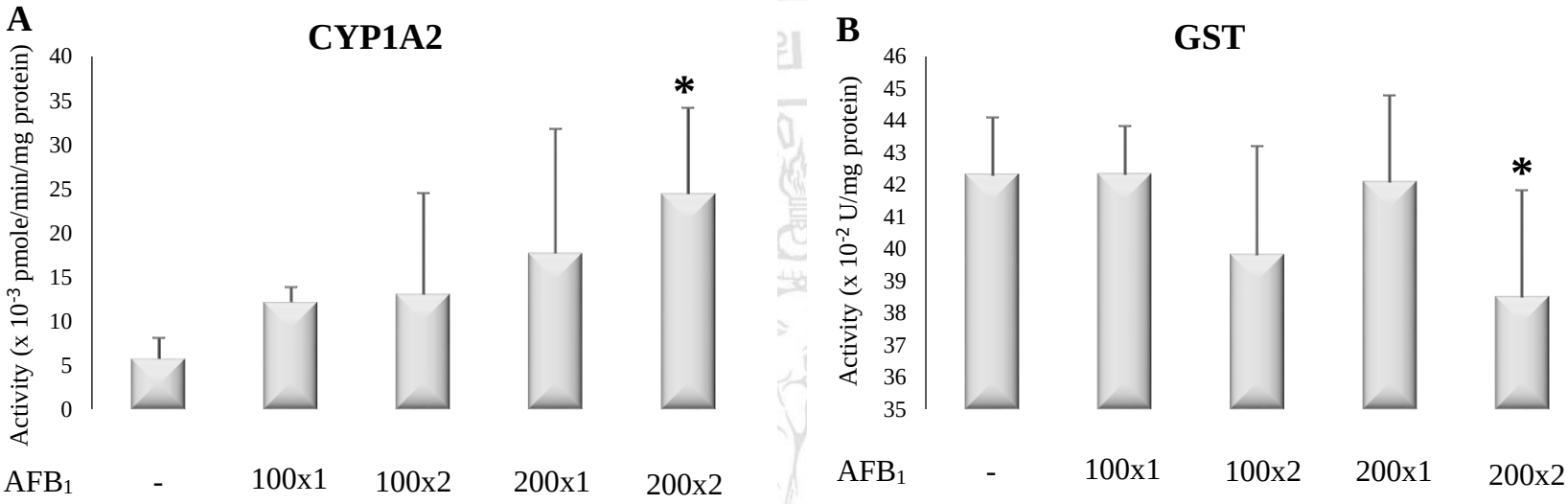

Figure 40. The activities of some major xenobiotic metabolizing enzymes induced by AFB_1

A; cytochrome P450 1A2 and B; glutathione *S*-transferase

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

3.5 Effect of purple rice bran extracts on micronucleus formation induced by AFB_1

The dichloromethane and methanol extracts of purple rice bran are able to reduce the formation of liver micronuclei by AFB_1 administration, concurrent with the other indicators of cell proliferation including binucleated and mitotic hepatocytes. Between the two doses of extracts, 100 and 500 mg/kg bw, they were not statistically different of these cell biomarkers (Table 7, Figure 41). These data predicted that purple rice bran extracts could be an inhibitor of liver cell damage-induced by strong carcinogen.

3.6 Inhibitory mechanism of purple rice bran extracts on xenobiotic metabolizing enzymes induced by AFB_1

During the modification of xenobiotic metabolism, AFB_1 induced phase I but reduced phase II enzyme activities. Outstandingly, the treatment of purple rice bran extracts can modulate the activities of phase I and stimulate the activities of phase II enzymes. Interestingly, the high dose of each extract has stronger effectiveness than the lower dose (Table 8, Figure 42-43). At the same time, rats received either dichloromethane or methanol extracts also significantly exhibited the reduction of phase I enzymes expression containing CYP 1A1/2, 3A2 and CPR (Table 9, Figure 44-45). Notably, the high concentration of methanol extract demonstrated higher potency for improvement of AFB_1-initiated hetapatic damage. These results revealed that the purple rice bran extracts might attenuate aflatoxicity through modulation of phase I and II xenobiotic metabolizing system.

Table 7. Effect of purple rice bran extracts on micronucleus formation induced by AFB_1

Treatments	Rat body weight (g)		MNH (per 1,000 Hep)	MN cells (per 1,000 Hep)	BNH (per 1,000 Hep)	MI (%)
	Initial	Final				
5% Tween-80	99.0 ± 6.5	284.0 ± 26.3	1.80 ± 0.67	1.70 ± 0.57	0.82 ± 0.21	0.85 ± 0.21
AFB_1 200 µg/kg bw	97.0 ± 5.7	280.0 ± 27.6	8.68 ± 1.67*	8.28 ± 1.64*	1.82 ± 0.19*	1.93 ± 0.38*
AFB_1 + 100 mg/ kg bw DME	97.9 ± 4.9	285.0 ± 15.0	3.78 ± 0.56**	3.71 ± 0.49**	1.31 ± 0.50**	1.23 ± 0.39**
AFB_1 + 500 mg/ kg bw DME	95.8 ± 3.8	283.3 ± 8.2	4.08 ± 0.38**	3.99 ± 0.32**	1.37 ± 0.21**	1.17 ± 0.13**
AFB_1 + 100 mg/ kg bw ME	98.6 ± 5.6	278.6 ± 18.9	3.99 ± 0.41**	3.92 ± 0.34**	1.36 ± 0.37**	1.31 ± 0.25**
AFB_1 + 500 mg/ kg bw ME	95.8 ± 3.8	274.2 ± 9.7	4.16 ± 0.60**	3.99 ± 0.83**	1.30 ± 0.16**	1.34 ± 0.26**

Values are expressed as mean ± SD

DME; Dichloromethane extract, ME; Methanol extract, MN; Micronucleus, BNH; Binucleated hepatocytes, MI; Mitotic index, Hep; Hepatocytes

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

** Significantly different at $p < 0.05$ compared to a positive group (AFB_1-treated group)

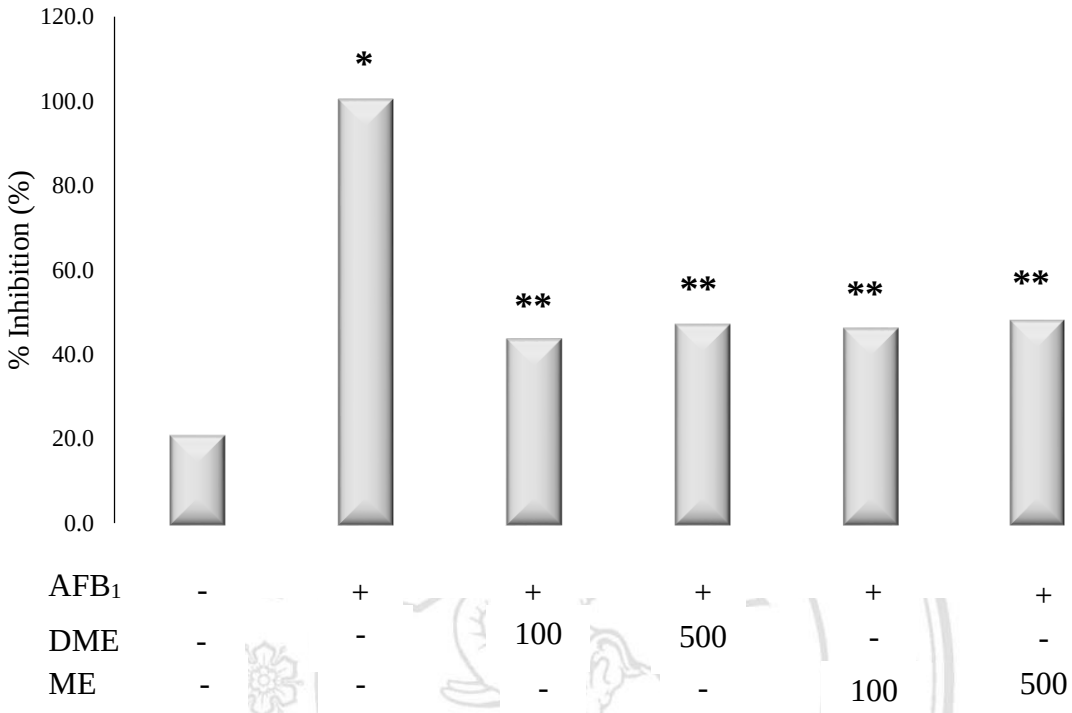

Figure 41. Inhibitory effect of purple rice bran extracts on micronucleus formation induced by AFB_1

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

** Significantly different at $p < 0.05$ compared to a positive group (AFB_1-treated group)

Table 8. Effect of purple rice bran extracts on the activities of phase I xenobiotic metabolizing enzymes activated by AFB_1 in rat liver

Treatments	CYP 1A1 ($x10^{-3}$ pmole/min/mg protein)	CYP 1A2 ($x10^{-3}$ pmole/min/mg protein)	CYP 3A2 ($x10^{-3}$ pmole/min/mg protein)	CPR ($x10^{-3}$ U/mg protein)
5% Tween-80	16.969 ± 1.867	11.818 ± 2.150	34.841 ± 7.193	12.471 ± 1.190
AFB_1 200 µg/ kg bw	19.895 ± 3.935*	17.151 ± 1.496*	38.111 ± 18.126	12.708 ± 2.180
AFB_1 + 100 mg/ kg bw DME	15.766 ± 2.112**	10.939 ± 1.507**	28.123 ± 4.756**	13.548 ± 2.940
AFB_1 + 500 mg/ kg bw DME	14.702 ± 3.785**	9.840 ± 1.301**	25.325 ± 3.683**	10.305 ± 1.590**
AFB_1 + 100 mg/ kg bw ME	15.904 ± 2.347**	11.738 ± 1.312**	25.259 ± 8.112**	11.037 ± 1.860
AFB_1 + 500 mg/ kg bw ME	14.721 ± 1.020**	9.687 ± 1.046**	17.368 ± 2.089**	9.576 ± 1.620**

Values are expressed as mean ± SD

DME; Dichloromethane extract, ME; Methanol extract, CYP; Cytochrome P450,

CPR; NADPH-cytochrome P450 reductase

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

** Significantly different at $p < 0.05$ compared to a positive group (AFB_1-treated group)

Table 9. Effect of purple rice bran extracts on the activities of phase II xenobiotic metabolizing enzymes activated by AFB_1 in rat liver

Treatments	GST ($x10^{-2}$ U/mg protein)	UGT ($x10^{-3}$ U/mg protein)
5% Tween-80	72.470 ± 8.851	39.861 ± 3.781
AFB_1 200 µg/ kg bw	56.946 ± 6.397*	41.797 ± 6.468
AFB_1 + 100 mg/ kg bw DME	63.066 ± 11.182	46.858 ± 4.689
AFB_1 + 500 mg/ kg bw DME	62.943 ± 3.803	42.940 ± 5.772
AFB_1 + 100 mg/ kg bw ME	71.386 ± 8.105**	37.579 ± 4.665
AFB_1 + 500 mg/ kg bw ME	78.282 ± 6.550**	48.403 ± 7.268**

Values are expressed as mean ± SD

DME; Dichloromethane extract, ME; Methanol extract, GST; Glutathione *S*-transferase,

UGT; UDP-glucuronyltransferase

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

** Significantly different at $p < 0.05$ compared to a positive group (AFB_1-treated group)

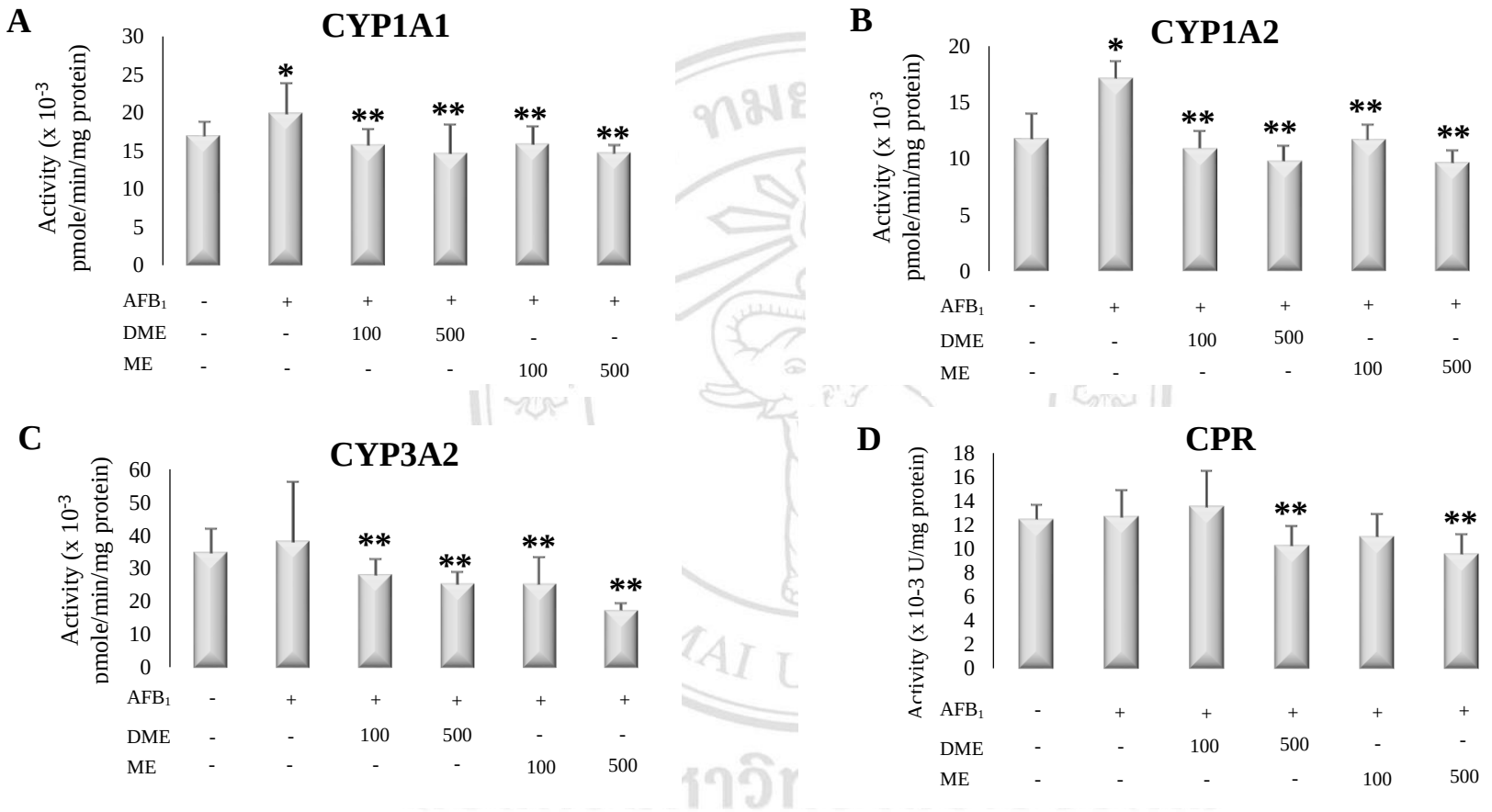

Figure 42. Effect of purple rice bran extracts on the activities of phase I xenobiotic metabolizing enzymes activated by AFB_1 in rat liver

A; cytochrome P450 1A1, B; cytochrome P450 1A2, C; cytochrome P450 3A2 and D; NADPH-cytochrome P450 reductase

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

** Significantly different at $p < 0.05$ compared to a positive group (AFB_1-treated group)

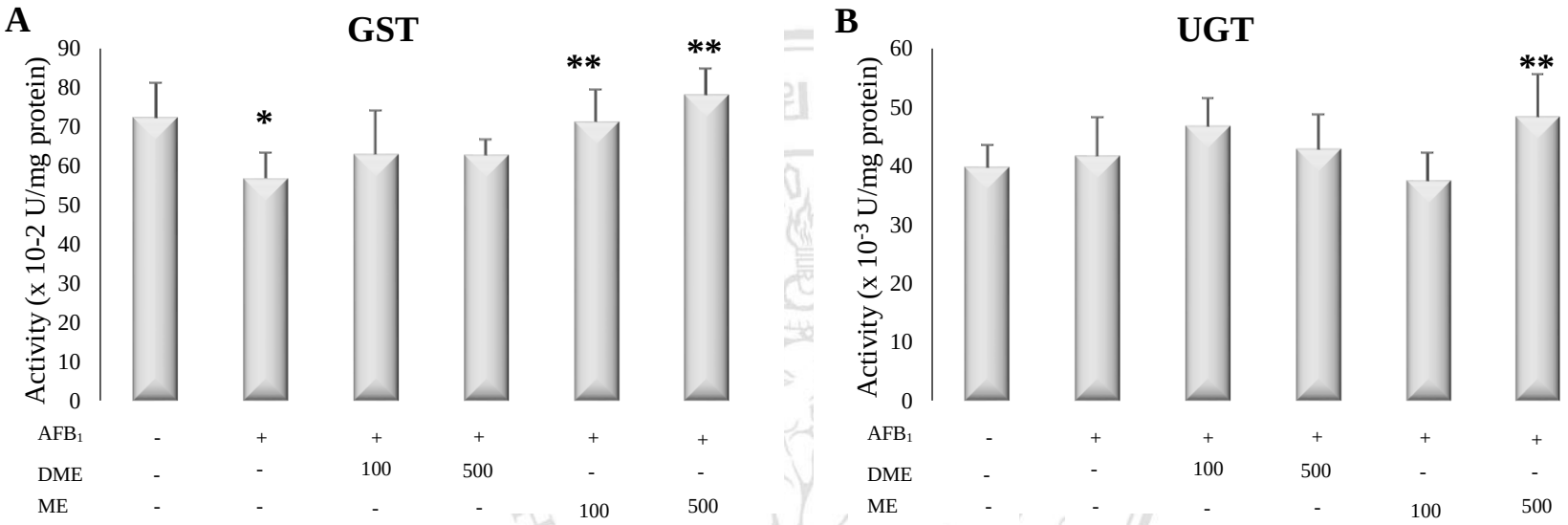

Figure 43. Effect of purple rice bran extracts on the activities of phase II xenobiotic metabolizing enzymes activated by AFB_1 in rat liver

A; glutathione *S*-transferase and B; UDP-glucuronyltransferase

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

** Significantly different at $p < 0.05$ compared to a positive group (AFB_1-treated group)

Table 10. Effect of purple rice bran extracts on the expression of xenobiotic metabolizing enzymes induced by AFB_1

Treatments	Fold change				
	CYP 1A1/2	CYP 3A1/2	CPR	GST	UGT
5% Tween-80	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000	1.000 ± 0.000
AFB_1 200 µg/ kg BW	1.359 ± 0.220*	1.147 ± 0.102	1.264 ± 0.155	1.030 ± 0.049	0.968 ± 0.046
AFB_1 + 100 mg/ kg BW DME	1.147 ± 0.126	1.107 ± 0.091	1.110 ± 0.289	0.945 ± 0.007	0.983 ± 0.061
AFB_1 + 500 mg/ kg BW DME	0.937 ± 0.275**	0.923 ± 0.132**	0.930 ± 0.164**	0.958 ± 0.066	1.037 ± 0.061
AFB_1 + 100 mg/ kg BW ME	1.000 ± 0.319**	1.011 ± 0.120	1.164 ± 0.262	0.906 ±0.113	1.020 ± 0.078
AFB_1 + 500 mg/ kg BW ME	0.831 ± 0.138**	0.763 ± 0.126**	0.819 ± 0.042**	1.012 ± 0.147	1.040 ± 0.066

Values expressed as mean ± SD

DME; Dichloromethane extract, ME; Methanol extract, CYP; Cytochrome P450, CPR; NADPH-cytochrome P450 reductase, GST; Glutathione *S*-transferase, UGT; UDP-glucuronyltransferase

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

** Significantly different at $p < 0.05$ compared to a positive group (AFB_1-treated group)

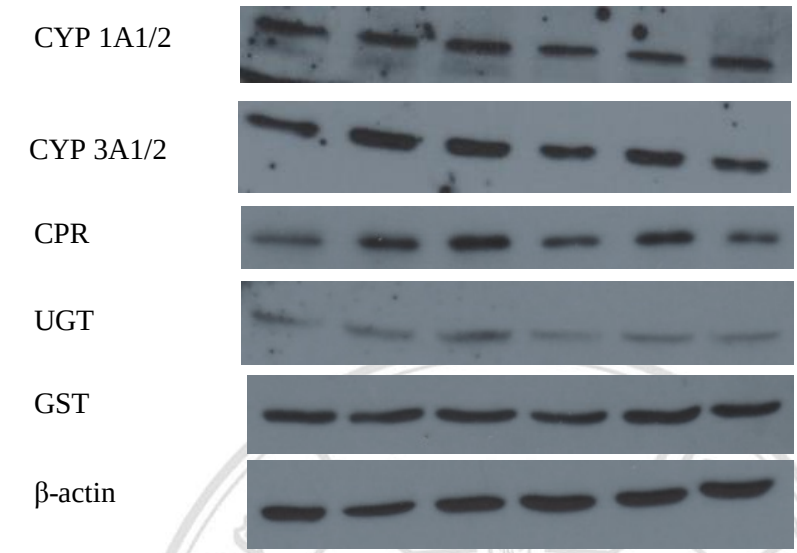

Figure 44. Protein expression of liver xenobiotic metabolizing enzymes activated by AFB_1 and treated with purple rice bran extracts

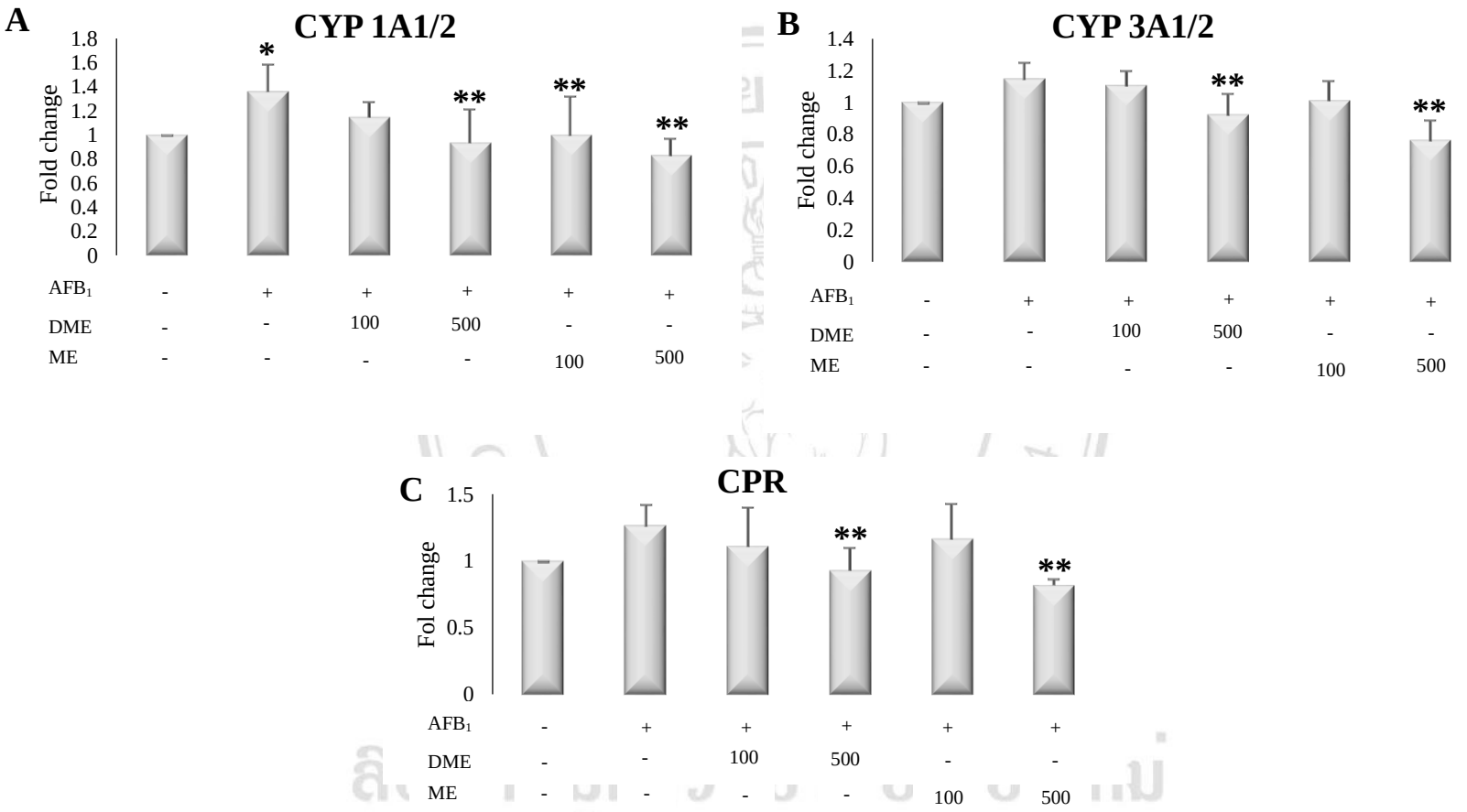

Figure 45. Effect of purple rice bran extract against AFB_1 treatment on the protein expression of xenobiotic metabolizing enzymes

A; cytochrome P450 1A1/2, B; cytochrome P450 3A and C; NADPH-cytochrome P450 reductase

* Significantly different at $p < 0.05$ compared to a control group (5% Tween-80 treated group)

** Significantly different at $p < 0.05$ compared to a positive group (AFB_1-treated group)