CHAPTER 3

RESULTS

3.1 Chemical constituents of glutinous purple rice extracts

The contents of total phenolic compounds and flavonoids in methanol extract of glutinous purple rice seed were 95.25±4.40 mg gallic acid equivalents per gram extract and 63.11±3.91 mg of catechin equivalents per gram extract. One gram of the extract contained anthocyanins 11.72±2.17 mg cyanidin-3-glucoside equivalents per gram extract (Table 3-1).

The results of chemical constituents of acidified methanol extract of glutinous purple rice hull was shown in Table 3-1. The total phenolic and flavonoid contents were 95.55±4.20 mg of gallic acid equivalents per gram extract and 51.68±4.28 mg of catechin equivalents per gram extract, respectively. The amount of anthocyanins in the extract was 6.38±1.07 mg cyanidin-3-glucoside equivalents per gram extract.

The total phenolic compounds, flavonoids and γ–oryzanol contents detected in the dichloromethane extract of glutinous purple rice hull extract were 174.0±5.50 mg of gallic acid equivalents per gram extract, 220±7.48 mg of catechin equivalents per gram extract and 23.62±0.07 mg per gram extract, respectively. The anthocyanins were not detected in the glutinous purple rice hull extract (Table 3-1). The HPLC chromatograms of γ-oryzanol, the standard mixture of various forms of standard tocols, and the tocols in the dichloromethane extract of glutinous purple rice hull are shown in Figures S-8 and S-9 of Appendix E. One gram of the glutinous purple rice hull extract contained 65.32±0.87, 14.15±0.30, 87.45±0.15 and 44.08±0.34 micrograms of α–, β–, δ– and γ–tocopherols per gram extract, respectively and 10.93±0.16, 67.38±0.73 and 8.64±0.05 micrograms of β–, δ– and γ–tocotrienols per gram extract, respectively. The α–tocotrienol was not detectale in this glutinous purple rice hull extract.

Table 3-1 Chemical constituents of glutinous purple rice extracts

Parameters	% yield	Total phenolic contents (mg GAE/g extract)	Total flavonoid contents (mg CE /g extract)	Anthocyanins contents (mg C3G/g extract)
Purple rice seed				
Methanol extract	0.78	95.25±4.40	63.11±3.91	11.72±2.17
Purple rice hull				
Acidified methanol extract	3.18	95.55±4.20	51.68±4.28	6.38±1.07
Dichloromethane extract	0.16	174.0±5.50	220.0±7.48	N.A.

Values are expressed as mean±standard deviation.

GAE; gallic equivalents

CE; cathechin equivalents

C3G; cyanidin-3-glucoside equivalents

N.A.; not analysis

3.2 Acute oral toxicity of glutinous purple rice extracts

The acute oral toxicity of a single dose of acidified methanol and dichloromethane extracts of glutinous purple rice hull and methanol extract of glutinous purple rice seed was evaluated. Two thousands mg/kg body weight of each extracts did not produce any signs of toxicity or mortality in the rats during the 14 days of observation. Neither body weight nor internal organ weights of treated rats was significantly changed when compared to a control group (Tables 3-2 to 3-4). According to OECD guideline TG 425, oral administration of glutinous purple rice extracts lack acute toxicity.

Table 3-2 Effect of acidified methanol extract of glutinous purple rice hull in oral acute toxicity test

Parameters	control	2000 mg/kg bw of ACEH
Initial body weight (g)	199.0 ± 7.4	201.0 ± 9.6
Final body weight (g)	231.0 ± 8.9	240.0 ± 7.9
Body weight change (percent)	16.0 ± 4.5	19.0 ± 4.7
Food intake (g/rat/day)	11.5 ± 2.1	13.5 ± 4.4
Water intake (ml/rat/day)	14.0 ± 6.7	22.0 ± 9.6
Heart (g/100 g bw)	0.30 ± 0.01	0.32 ± 0.01
Lung (g/100 g bw)	0.41 ± 0.02	0.40 ± 0.02
Thymus (g/100 g bw)	0.15 ± 0.02	0.17 ± 0.03
Liver (g/100 g bw)	3.45 ± 0.05	3.56 ± 0.11
Pancreas (g/100 g bw)	0.34 ± 0.04	0.41 ± 0.12
Spleen (g/100 g bw)	0.24 ± 0.02	0.23 ± 0.01
Kidneys (g/100 g bw)	0.60 ± 0.06	0.67 ± 0.02
Stomach (g/100 g bw)	0.42 ± 0.04	0.47 ± 0.04
Fallopian tube (g/100 g bw)	0.17 ± 0.02	0.24 ± 0.10
Ovary (mg/100 g bw)	70.72 ± 1.05	62.12 ± 3.17
Adrenal gland (mg/100 g bw)	37.03 ± 5.69	41.94 ± 5.08

Values are expressed as mean ± standard deviation.

ACEH: acidified methanol extract of glutinous purple rice hull

Table 3-3 Effect of dichloromethane extract of glutinous purple rice hull in oral acute toxicity test

Parameters	control	2000 mg/kg bw of DCEH
Initial body weight (g)	196.0 ± 9.6	194.0 ± 5.5
Final body weight (g)	229.0 ± 8.7	230.0 ± 6.1
Body weight change (percent)	16.0 ± 4.3	18.0 ± 5.4
Food intake (g/rat/day)	16.5 ± 2.9	15.0 ± 2.6
Water intake (ml/rat/day)	17.0 ± 3.5	15.5 ± 5.4
Heart (g/100 g bw)	0.32 ± 0.03	0.30 ± 0.04
Lung (g/100 g bw)	0.47 ± 0.04	0.45 ± 0.02
Thymus (g/100 g bw)	0.17 ± 0.04	0.15 ± 0.02
Liver (g/100 g bw)	3.51 ± 0.23	3.20 ± 0.47
Pancreas (g/100 g bw)	0.40± 0.08	0.46 ± 0.08
Spleen (g/100 g bw)	0.23 ± 0.03	0.22 ± 0.03
Kidneys (g/100 g bw)	0.68 ± 0.05	0.64 ± 0.11
Stomach (g/100 g bw)	0.46 ± 0.06	0.48 ± 0.07
Fallopian tube (g/100 g bw)	0.19 ± 0.06	0.23 ± 0.04
Ovary (mg/100 g bw)	70.04 ± 22.49	68.20 ± 15.10
Adrenal gland (mg/100 g bw)	30.60 ± 6.52	39.50 ± 10.00

Values are expressed as mean ± standard deviation.

DCEH: dichloromethane extract of glutinous purple rice hull

Table 3-4 Effect of methanol extract of glutinous purple rice seed in oral acute toxicity test

Parameters	control	2000 mg/kg bw of MES
Initial body weight (g)	199.0 ± 7.4	192.0 ± 8.3
Final body weight(g)	231.0 ± 8.9	234.0 ± 8.2
Body weight change (percent)	16.0 ± 4.5	22.0 ± 8.2
Food intake(g/rat/day)	11.5 ± 2.1	15.5 ± 3.3
Water intake (ml/rat/day)	14.0 ± 6.7	21.0 ± 5.9
Heart (g/100 g bw)	0.30 ± 0.01	0.27 ± 0.09
Lung (g/100 g bw)	0.41 ± 0.02	0.40 ± 0.10
Thymus (g/100 g bw)	0.15 ± 0.02	0.14 ± 0.06
Liver (g/100 g bw)	3.45 ± 0.05	2.99 ± 0.54
Pancreas (g/100 g bw)	0.34 ± 0.04	0.33 ± 0.07
Spleen (g/100 g bw)	0.24 ± 0.02	0.21 ± 0.04
Kidneys (g/100 g bw)	0.60 ± 0.06	0.60 ± 0.06
Stomach (g/100 g bw)	0.42 ± 0.04	0.36 ± 0.14
Fallopian tube (g/100 g bw)	0.17 ± 0.02	0.23± 0.05
Ovary (mg/100 g bw)	70.72 ± 1.05	70.15 ± 1.50
Adrenal gland(mg/100 g bw)	37.03 ± 5.69	36.95 ± 5.10

Values are expressed as mean ± standard deviation.

MES: methanol extract of glutinous purple rice seed

3.3 Clastogenicity of glutinous purple rice extracts

Tables 3-5 to 3-7 show the clastogenicity of the acidified methanol and dichloromethane extracts of glutinous purple rice hull and methanol extract of glutinous purple rice seed using a micronucleus assay in regenerating liver tissue of male rats.

The initial and final body weights, water intake and food consumption of treated rats were not significantly different from the vehicle control group. All of glutinous purple rice extracts did not increase the number of mironuclei, micronucleated hepatocytes, mitotic hepatocytes and binucleated cells when compared to their control groups. It was indicated that the glutinous purple rice extracts did not exhibit clastogenicity in rat liver.

3.4 Anticlastogenicity of glutinous purple rice extracts

The anticlastogenicity of glutinous purple rice extracts was performed in rats initiated by diethylnitrosamine, a hepatomutagen and a hepatocarcinogen. The injection of diethylnitrosamine significantly increased the number of micronuclei, micronucleated hepatocytes and binucleated cells. The oral administration of the extracts for 28 days statistically reduced the number of micronuclei, micronucleated hepatocytes and binucleated cells in rat livers (Tables 3-8 to 3-10). Mitotic index was determined as cell division control.

The highest dose of acidified methanol extract of rice hull presented the strongest anticlastogenicity (54.02%) in rat liver (Table 3-11). The liver of rat-treated by acidified methanol extract of glutinous purple rice hull was further evaluated on inhibitory mechanism involving xenobiotic metabolizing enzymes.

Table 3-5 Clastogenicity of acidified methanol extract of glutinous purple rice hull in rat liver

Treatment	Body weight (g)		MNHEPs cell / 1,000 Hep	MNHEPs / 1,000 Hep	Mitotic index (%)	Binucleated cells / 1,000 hep
	Initial	Final				
Distilled water	117.0 ± 5.7	307.0 ± 8.4	2.82 ± 1.50	3.01 ± 2.82	0.20 ± 0.07	8.78 ± 6.29
100 mg/kg bw ACEH	115.0 ± 10.2	300.0 ± 16.2	2.55 ± 1.74	2.31 ± 1.29	0.15 ± 0.04	9.24 ± 9.29
300 mg/kg bw ACEH	116.0 ± 6.1	294.0 ± 23.0	1.99 ± 1.62	2.39 ± 2.16	0.16 ± 0.05	9.71 ± 1.86
1000 mg/kg bw ACEH	121.0 ± 9.7	292.0 ± 13.0	2.10 ± 1.26	2.20 ± 1.23	0.14 ± 0.01	9.77 ± 5.63

Value are expressed as mean ± standard deviation.

MNHEPs cell /1,000 Hep: number of micronucleated cells per 1,000 hepatocytes

MNHEPs/1,000 Hep: number of micronuclei per 1,000 hepatocyte

ACEH: acidified methanol extract of purple rice hull

Table 3-6 Clastogenicity of methanol extract of glutinous purple rice seed in rat liver

Parameters	Control	1000 mg/kg bw of MES
Initial body weight (g)	117.0 ± 5.7	113.0 ± 3.2
Final body weight (g)	307.0 ± 8.4	300.0 ± 6.6
MNHEPs cell / 1,000 Hep	2.82 ± 1.50	4.68 ± 1.35
MNHEPs / 1,000 Hep	3.01 ± 2.82	5.01 ± 1.33
Mitotic index (%)	0.20 ± 0.07	0.14 ± 0.06
Binucleated cells /1,000 Hep	8.78 ± 6.29	6.09 ± 3.96

Values are expressed as mean ± standard deviation.

MNHEPs cell /1,000 Hep: number of micronucleated cells per 1,000 hepatocytes

MNHEPs/1,000 Hep: number of micronucleus per 1,000 hepatocytes

MES: methanol extract of purple rice seed

Table 3-7 Clastogenicity of dichloromethane extract of glutinous purple rice hull in rat liver

Parameters	Control	500 mg/kg bw of DCEH
Initial body weight (g)	110.0 ± 4.1	114.0 ± 4.2
Final body weight (g)	287.0 ± 15.0	286.0 ± 11.5
MNHEPs cell / 1,000 Hep	3.37 ± 2.49	2.04 ± 0.79
MNHEPs / 1,000 Hep	4.62 ± 3.96	2.62 ± 1.01
Mitotic index (%)	0.14 ± 0.04	0.14 ± 0.13
Binucleated cells /1,000 Hep	2.37 ± 1.25	3.69 ± 2.44

Values are expressed as mean ± standard deviation.

MNHEPs cell /1,000 Hep: number of micronucleated cells per 1,000 hepatocytes

MNHEPs/1,000 Hep: number of micronucleus per 1,000 hepatocytes

DCEH: dichloromethane extract of purple rice hull

Table 3-8 Anticlastogenicity of acidified methanol extract of glutinous purple rice hull in the liver of diethylnitrosamine treated-rats

Treatment	Body weight (g)		MNHEPs cell / 1,000 Hep	MNHEPs / 1,000 Hep	Mitotic index (%)	Binucleated cells per 1,000 hepatocytes
	Initial	Final				
Distilled water+NSS	117.0 ± 5.7	307.0 ± 8.4	2.82 ± 1.50	3.01 ± 2.82	0.20 ± 0.07	8.78 ± 6.29
Distilled water+DEN	113.0 ± 4.8	303.0 ± 16.0	29.35 ± 2.85	35.30 ± 5.84	0.20 ± 0.11	11.62 ± 4.96
300 mg/kg bw ACEH+DEN	112.8 ± 7.6	310.0 ± 11.2	17.17± 3.46*	19.92 ± 1.30*	0.16 ± 0.16	9.80 ± 0.99
1000 mg/kg bw ACEH +DEN	116.0 ± 8.3	305.0 ± 4.9	14.02 ± 2.91*	16.32 ± 3.76*	0. 22 ± 0.11	6.47 ± 4.07*

Values are expressed as mean ± standard deviation.

*Significantly different from DEN control group, $p < 0.05$

MNHEPs cell /1,000 Hep: number of micronucleated cells per 1,000 hepatocytes

MNHEPs/1,000 Hep: number of micronuclei per 1,000 hepatocytes

NSS: normal saline

DEN: 30 mg/kg bw diethylnitrosamine

ACEH: acidified methanol extract of purple rice hull

Table 3-9 Anticlastogenicity of methanol extract of glutinous purple rice seed in the livers of diethylnitrosamine-treated rats

Treatment	Body weight (g)		MNHEPs cell / 1,000 Hep	MNHEPs / 1,000 Hep	Mitotic index (%)	Binucleated cells per 1,000 hepatocytes
	Initial	Final				
Distilled water+NSS	117.0 ± 5.7	307.0 ± 8.4	2.82 ± 1.50	3.01 ± 2.82	0.20 ± 0.07	8.78 ± 6.29
Distilled water+DEN	113.0 ± 4.8	303.0 ± 16.0	29.35 ± 2.85	35.30 ± 5.84	0.20 ± 0.11	11.62 ± 4.96
300 mg/kg bw MES+DEN	113.0 ± 7.6	310.0 ± 8.3	18.00 ± 6.03*	20.31 ± 7.65*	0.14 ± 0.05	3.28 ± 2.13*
1000 mg/kg bw MES +DEN	117.0 ± 4.4	283.0 ± 11.5	14.99 ± 3.08*	19.12 ± 4.28*	0.13 ± 0.05	3.55 ± 3.79*

Values are expressed as mean ± standard deviation.

*Significantly different from DEN control group, $p < 0.05$

MNHEPs cell /1,000 Hep: number of micronucleated cells per 1,000 hepatocytes

MNHEPs/1,000 Hep: number of micronuclei per 1,000 hepatocytes

NSS: normal saline

DEN: 30 mg/kg bw diethylnitrosamine

MES: methanol extract of purple rice seed

Table 3-10 Anticlastogenicity of dichloromethane extract of glutinous purple rice hull in the livers of diethylnitrosamine-treated rats

Treatment	Body weight (g)		MNHEPs cell / 1,000 Hep	MNHEPs / 1,000 Hep	Mitotic index (%)	Binucleated cells per 1,000 hepatocytes
	Initial	Final				
5% Tween + NSS	110.0 ± 4.1	287.0 ± 15.0	3.37 ± 2.49	4.62 ± 3.96	0.14 ± 0.04	2.37 ± 1.25
5% tween-80 + DEN	111.7 ± 5.2	280.0 ± 10.9	27.73 ± 5.12	32.92± 5.20	0.16 ± 0.07	8.75 ± 9.28
500 mg/kg bw DCEH + DEN	113.3 ± 4.0	284.0 ± 24.1	16.53 ± 4.04*	20.65 ± 6.37*	0.15 ± 0.04	4.65 ± 2.61*

Value are expressed as mean ± standard deviation.

*Significantly different from DEN control group, $p < 0.05$

MNHEPs cell /1,000 Hep: number of micronucleated cells per 1,000 hepatocytes

MNHEPs/1,000 Hep: number of micronuclei per 1,000 hepatocytes

NSS: normal saline

DEN: 30 mg/kg bw diethylnitrosamine

DCEH: dichloromethane extract of glutinous purple rice hull

Table 3-11 The inhibitory effect of glutinous purple rice extracts on micronucleus formation in the liver of diethylnitrosamine initiated rats

Parameters	Dose (mg/kg bw)	Percent inhibition
Glutinous purple rice hull		
Acidified methanol extract	300	43.88 ± 6.78
	1000	54.02 ± 7.64
Dichloromethane extract	500	37.27 ± 4.87
Glutinous purple rice seed		
Methanol extract	300	42.78 ± 7.98
	1000	46.14 ± 5.43

Value are expressed as mean ± standard deviation.

3.5 Effect of acidified methanol extract of glutinous purple rice hull extracts on activities and protein expression of xenobiotic metabolizing enzymes in rats

The administration 100 mg/kg bw of acidified methanol extract of glutinous purple rice hull for 28 days significantly induced glutathione *S*-transferase (GST), cytochrome P450 reductase (CPR), NADPH quinone oxidoreductase (NQO) and heme oxygenase (HO) activities while 300 and 1000 mg/kg bw did not induce those of activities (Table 3-2). Furthermore, the extract did not affect on the activity of UDP-glucuronyltransferase (UDP-GT) and it did not alter CPR and GST-alpha protein expressions when compared to a control group (Figure 3-1 and Table 3-13).

According to Table 3-11, the acidified methanol extract of glutinous purple rice hull presented the strongest anticlastogenicity, the possible chemopreventive mechanism of the extract on hepatocarcinogenesis involving xenobiotic metabolizing enzymes was evaluated. In Table 3-14, diethylnitrosamine did not significantly induce the activities of some xenobiotic metabolizing enzymes. The administration acidified methanol extract of glutinous purple rice hull for 28 days significantly enhanced the activities of GST, UDP-GT and NQO (Table 3-14). However, it did not affect on CPR and HO-1 activities. Table 3-15 and Figure 3-2 show DEN significantly induced CYP2E1 expression in rat liver. The acidified methanol extract of glutinous purple rice hull significantly suppressed CYP2E1 expression, but induced glutathione *S*-transferase alpha expression in a dose dependent manner.

Table 3-12 Effect of acidified methanol extract of glutinous purple rice hull on the activities of some hepatic xenobiotic metabolizing enzymes in rats

ACEH (mg/kg bw)	**CPR** **(x10^{-3} U/mg protein)**	**NQO** **(nmol/mg protein)**	**UGT** **(pmol/mg protein)**	**GST** **(x10^{-2}U/mg protein)**	**HO** **(nmol/min/mg protein)**
0	4.89 ± 2.92	2205.63 ± 414.03	3097.41 ± 551.87	35.22 ± 13.21	5.27 ± 0.98
100	11.03 ± 1.56*	4111.27 ± 916.48*	2891.72 ± 541.72	51.69 ± 14.04*	10.66 ± 1.09*
300	6.85 ± 1.10	2377.82 ± 291.38	2892.67 ± 858.67	32.78 ± 5.49	5.51 ± 1.97
1000	6.51 ± 2.15	2317.12 ± 430.85	3149.56 ± 777.25	36.33 ± 4.54	5.12 ± 2.39

Values are expressed as mean ± standard deviation.

*Significantly different from control group, $p<0.05$

ACEH; acidified methanol extract of purple rice hull

CPR ; cytochrome P450 reductase, HO; heme oxygenase, NQO; NADPH-quinone reductase,

UGT; UDP-glucuronyltransferase, GST; glutathione *S*-transferase

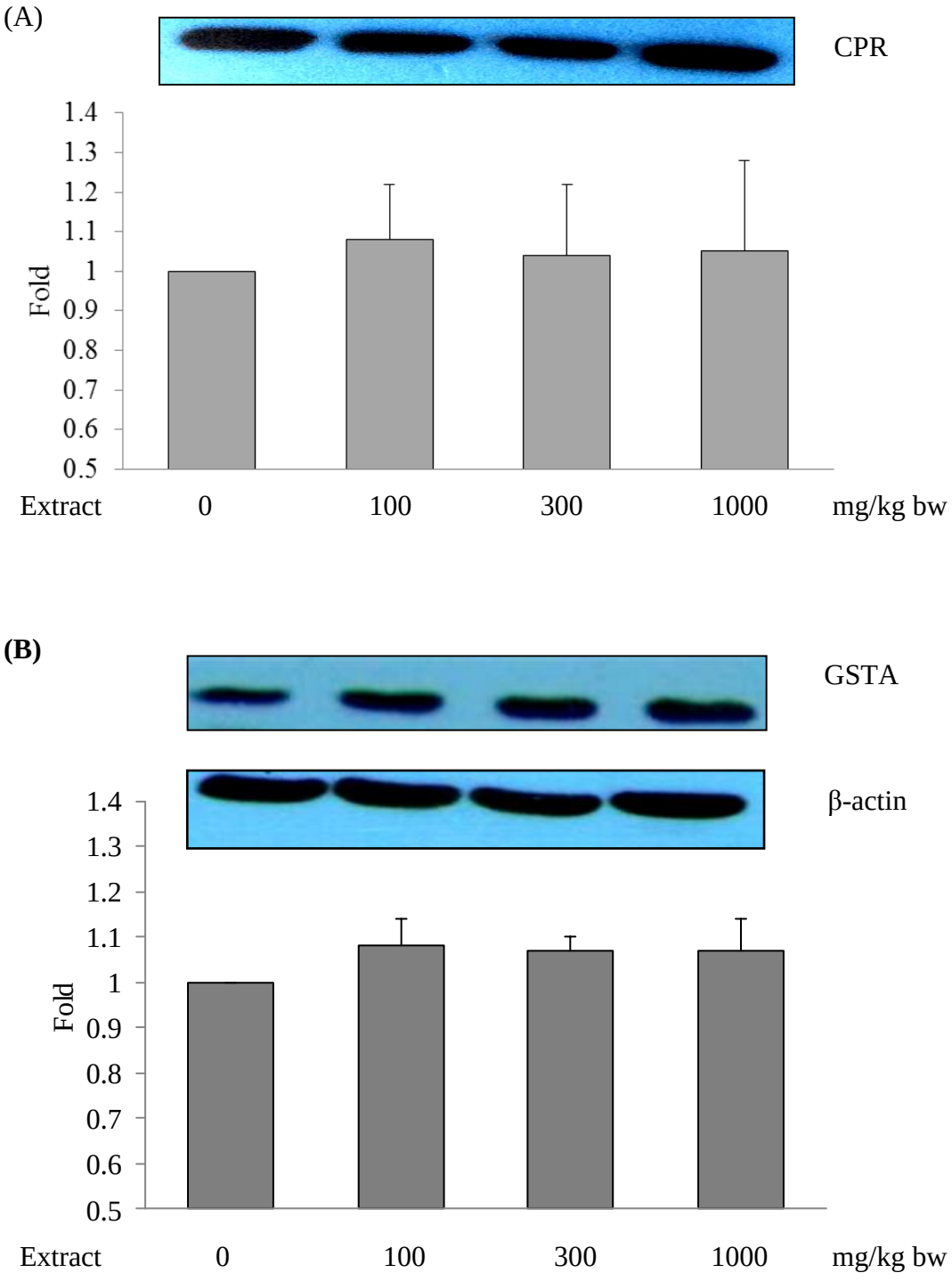

Figure 3-1 Western blot analysis of (A) cytochrome P450 reductase (B) glutathione *S*-transferase alpha in rat liver

Table 3-13 The fold change of some xenobiotic metabolizing enzymes expression in acidified methanol extract of glutinous purple rice hull treated rats

ACEH	**Fold change**	
(mg/kg bw)	**CPR**	**GST**
0	1.00±0.00	1.00±0.00
100	1.08±0.14	1.08±0.06
300	1.04±0.18	1.07±0.03
1000	1.05±0.23	1.07±0.07

Values are expressed as mean ± standard deviation.

ACEH; acidified methanol extract of purple rice hull,

CPR ; cytochrome P450 reductase , GST; glutathione *S*-transferase

Table 3-14 Effect of acidified methanol extract of glutinous purple rice hull extract on the activities of some hepatic xenobiotic metabolizing enzymes in diethynitrosamine-initiated rats

ACEH (mg/kg bw)	**CPR** ($x10^{-3}$ U/mg protein)	**NQO** (nmol/mg protein)	**UGT** (pmol/mg protein)	**GST** ($x10^{-2}$U/mg protein)	**HO** (nmol/min/mg protein)
Water+NSS	7.43 ± 3.00	2899.76 ± 845.45	655.75 ± 54.54	42.62 ± 4.40	12.24±7.16
Water+DEN	6.67 ± 1.84	3719.92 ± 677.35	832.75 296.67	47.85 ± 8.13	10.64±4.44
300 mg/kg bw+DEN	6.68 ± 2.07	4746.65 ± 7 71.96*	1312.64 ± 646.24*	50.14± 5.73	11.52 ±2.64
1000 mg/kg bw+DEN	6.20 ± 2.37	4952.29 ± 668.07*	1036.20 ± 255.89*	59.74 ± 13.75*	10.58± 7.00

Values are expressed as mean ± standard deviation

*Significantly different from control group, $p<0.05$

ACEH; acidified methanol extract of purple rice hull

CPR ; cytochrome P450 reductase, HO; heme oxygenase, NQO; NADPH- quinone reductase,

UGT; UDP-glucuronyltransferase, GST; glutathione *S*-transferase

NSS; normal saline

DEN; 30 mg/kg bw diethylnitrosamine

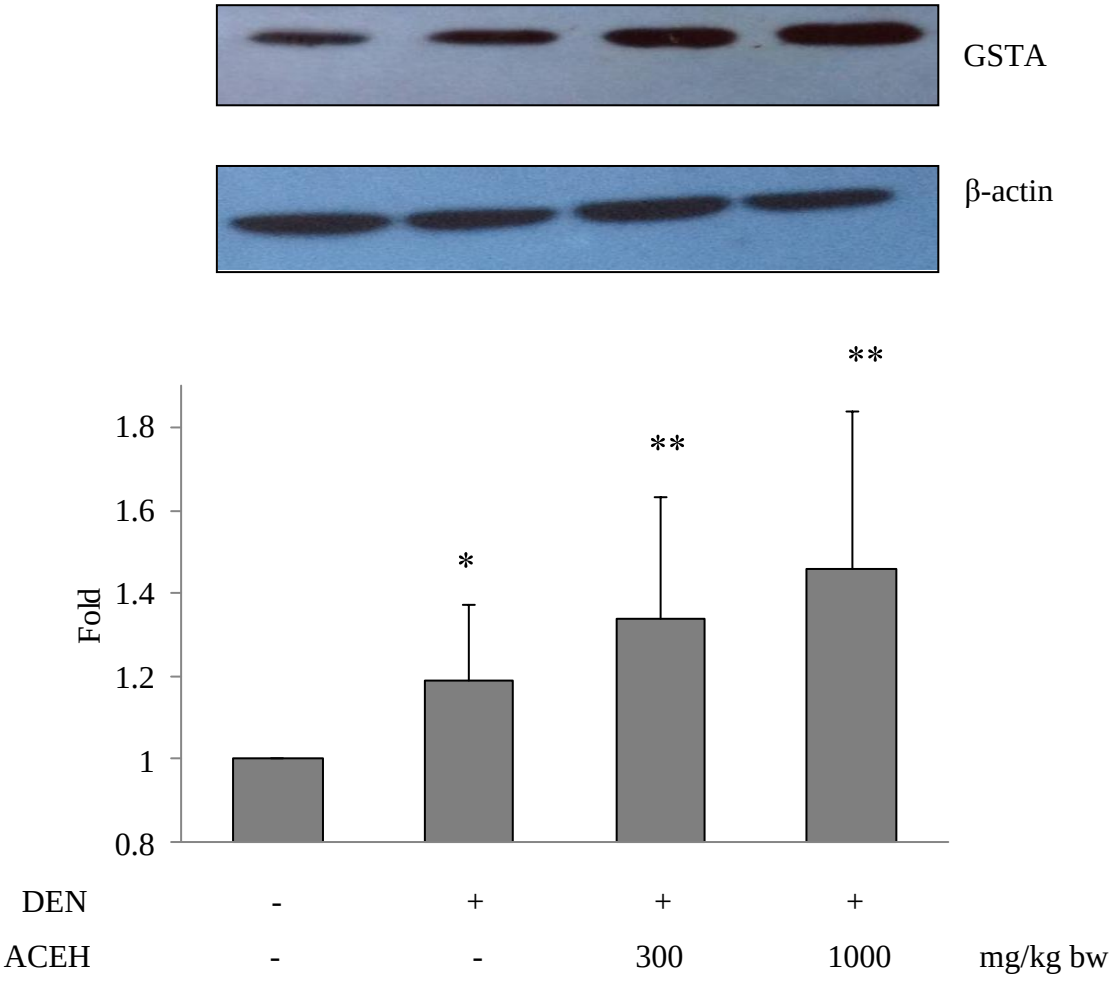

Figure 3-2 Western blot analysis of glutathione *S*-transferase alpha expression in rat livers

*significantly different from control group, $p<0.05$

**significantly different from DEN control group, $p<0.05$

ACEH; acidified methanol extract of purple rice hull

DEN; 30 mg/kg bw diethylnitrosamine

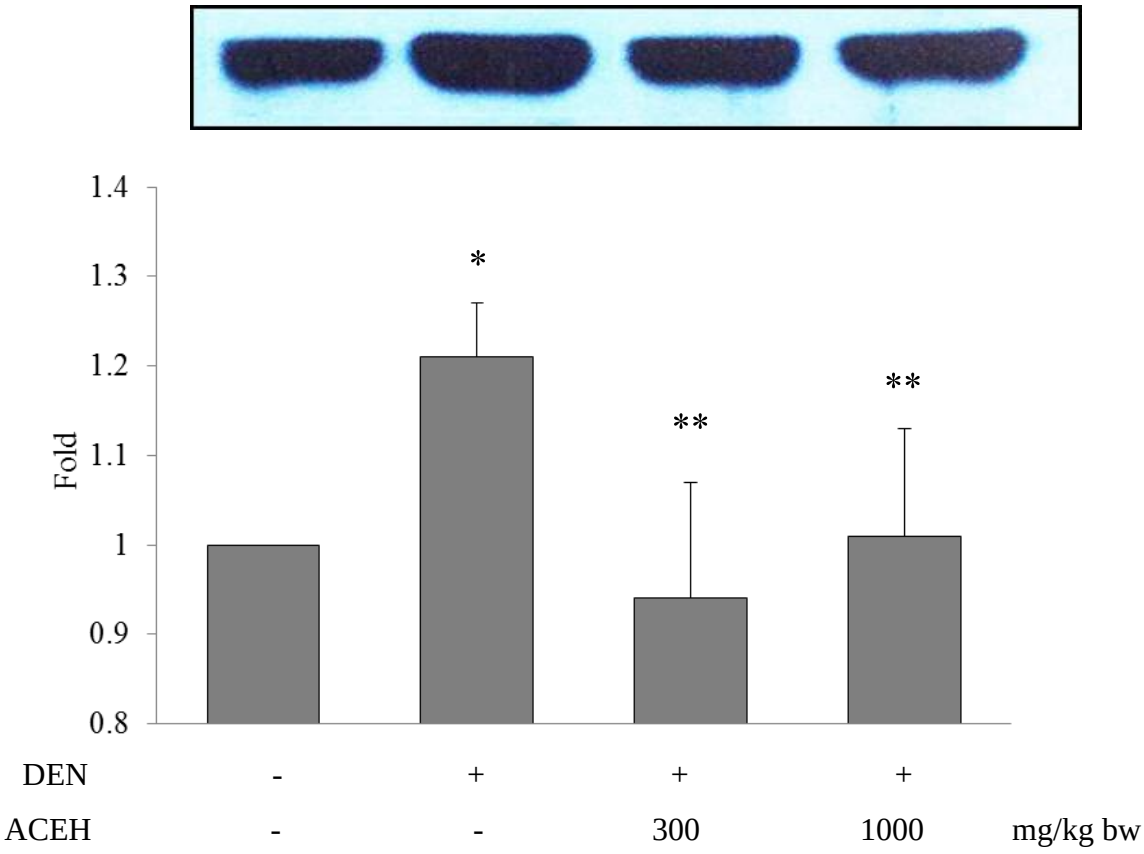

Figure 3-3 Western blot analysis of cytochrome P450 isoform 2E1 in rat livers

*significantly different from control group, $p<0.05$

**significantly different from DEN control group, $p<0.05$

ACEH; acidified methanol extract of purple rice hull

DEN; 30 mg/kg bw diethylnitrosamine

Table 3-15 The fold change of some xenobotic metabolizing enzymes expression in acidified methanol extract of glutinous purple rice hull against diethylnitrosamine initiated rats

ACEH (mg/kg bw)	**Fold change**		
	β -actin	**GST**	**CYP2E1**
Water+NSS	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Water+DEN	0.98 ± 0.04	1.19 ± 0.18*	1.21± 0.06*
300 mg/kg bw+DEN	1.00 ± 0.02	1.34 ± 0.29**	0.94 ± 0.13**
1000 mg/kg bw+DEN	0.99 ± 0.03	1.46 ± 0.38**	1.01 ± 0.12**

Values are expressed as mean ± standard deviation

*significantly different from control group, $p<0.05$

**significantly different from DEN control group, $p<0.05$

GST; glutathione *S*-transferase

NSS; normal saline

DEN; 30 mg/kg bw diethylnitrosamine