CHAPTER IV

RESULTS AND DISCUSSIONS

This chapter presents the findings obtained from the study, which is divided into two parts; *In-vivo* and *In-vitro* study.

Effect of curcuminoids on liver function enzymes in ethanol-induced toxicity rats

Rats were received ethanol orally for sixty days (8 weeks) continuously to induce hepatotoxicity. As shown in Figure 12 and 13, serum AST and ALT levels were increased significantly from all rats received ethanol, comparing to the control group ($p \leq 0.05$) at week eighth. In the other hand, rats received glucose with caloric equivalence to the ethanol showed minimal changes on the AST and ALT levels. After 60 days the ethanol-induced toxicity rats were continuously received ethanol with a vehicle (carboxymethyl cellulose, CMC), silymarin (hepatoprotective agent) or various doses of curcuminoids for another 6 weeks. The results showed that the hepatoxicity rats continuously received ethanol with curcuminoids concentration at 500 and 750 mg/kg/day, the serum ALT and AST levels are decreased significantly ($p \leq 0.05$). These attenuation of the liver function enzymes effect of curcuminoids seemed to be similar to the effect on ethanol induced toxicity rats with silymarin. The rats received ethanol with the vehicle showed no changes of the serum levels of ALT and AST. (Figure 12 and 13)

From study of the serum ALP and LDH, we saw the slightly increase in both enzymes which indicated some hepatic cells damaged from the ethanol-induced toxicity rats. Our results also showed slightly decrease of both ALP and LDH in the groups of ethanol induced toxicity rats given silymarin or curcuminoids at 500 and 750 mg/kg/day but there was no statistic differences (Figure 14 and 15). The result from ethanol-induced toxicity rats suggests that curcuminoids can decrease AST and ALT liver function enzymes.

The most sensitive and widely used liver enzyme markers are the aminotrasferase, including AST and ALT [138,139]. AST and ALT are the reliable makers for liver cell function. It is established that AST can be found in liver, cardiac

muscle, skeletal muscle, kidney, brain, pancreas, lung, leukocyte and erythrocytes whereas ALT is mostly presented in liver. The increased levels of serum enzymes AST and ALT indicate the increased permeability and damage and/or necrosis of hepatocytes [140]. In a patient, the laboratory abnormalities have been reported in ALD. An AST level is elevated to a level of 2-6 times of the normal upper limits in severe alcoholic hepatitis. Almost all patients will show the elevation of AST more than ALT with both below 300 IU/ml. In 70% of patients, the AST/ALT ratio is higher than 2 [141]. In animal model, there is no exactly report which refers to serum AST and ALT levels in toxic substance-induced hepatocytes toxicity in rats. According to several toxic substance-induced hepatocytes toxicity experiments, the AST and ALT levels reported will compare with the normal rats. Normally, AST levels in substance-induced hepatocytes toxicity rats are increased more than normal rats to 1.2-3.3 fold and the ALT levels are more than 1.5-2.8 fold, comparing to the base line [142, 145]. ALP is a hydrolase enzyme, which is present in all tissues throughout the entire body, but particularly concentrated in the liver, bile duct, kidney, bone, and placenta [146]. Alcoholic liver disease patients who have steatosis and cholestatic normally show elevation of serum alkaline phosphatase. McGill, D.B., *et al* published that patients with fatty liver without hepatitis have normal serum bilirubin and alkaline phosphatase. Moreover, LDH is less specific than AST and ALT as a marker of hepatocyte injury. However, LDH can be generally used as marker for cell or tissue damages [138]. Our study of the liver function enzyme markers from the ethanol-induced hepatotoxicity rats had confirmed that curcuminoids at 500 mg/kg/day and higher decrease all the abnormal enzyme levels.

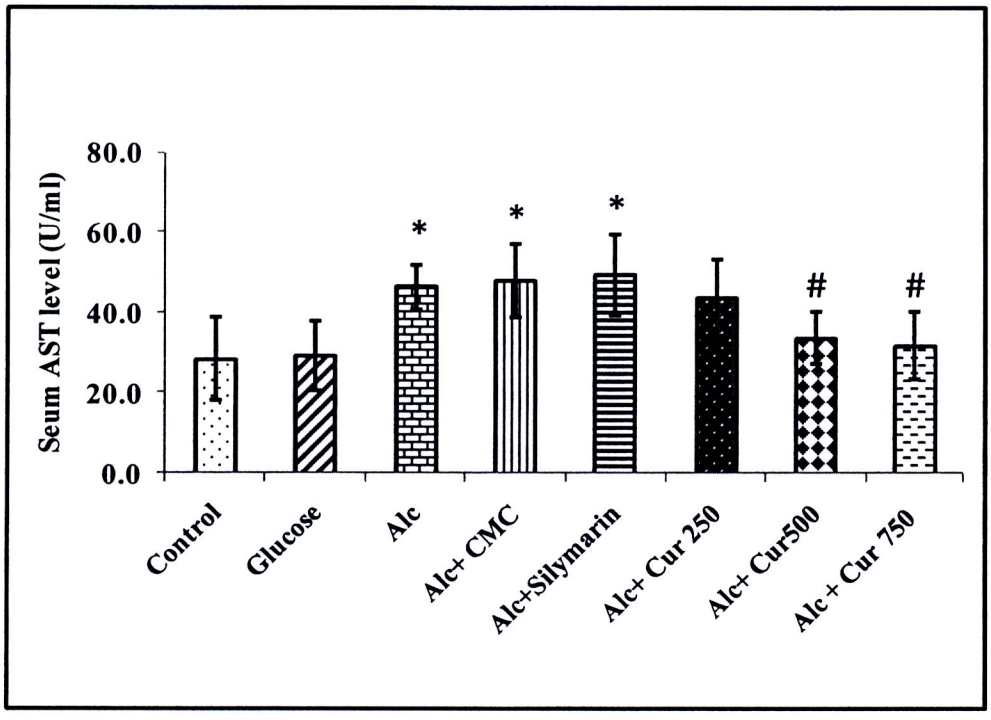


Figure 12 Effect of curcuminoids on AST level in ethanol stimulated rats.

Note: Rats were received ethanol (6 g/kg/day) for 8 weeks and then were given CMC, silymarin or curcuminoids (250, 500, 750 mg/ml) for another 6 weeks. Serum AST levels were measured with the liver function enzyme kits. Data were from 6 animals (n=6) and shown as mean ± SD. Data were analyzed statistic significantly by ANOVA ($p \leq 0.05$), comparing to the control group (*) or comparing to the ethanol induced hepatic toxicity (#). Alc: alcohol, CMC: carboxymethyl cellulose, Cur: curcuminoids, AST: aspartate aminotrasferase

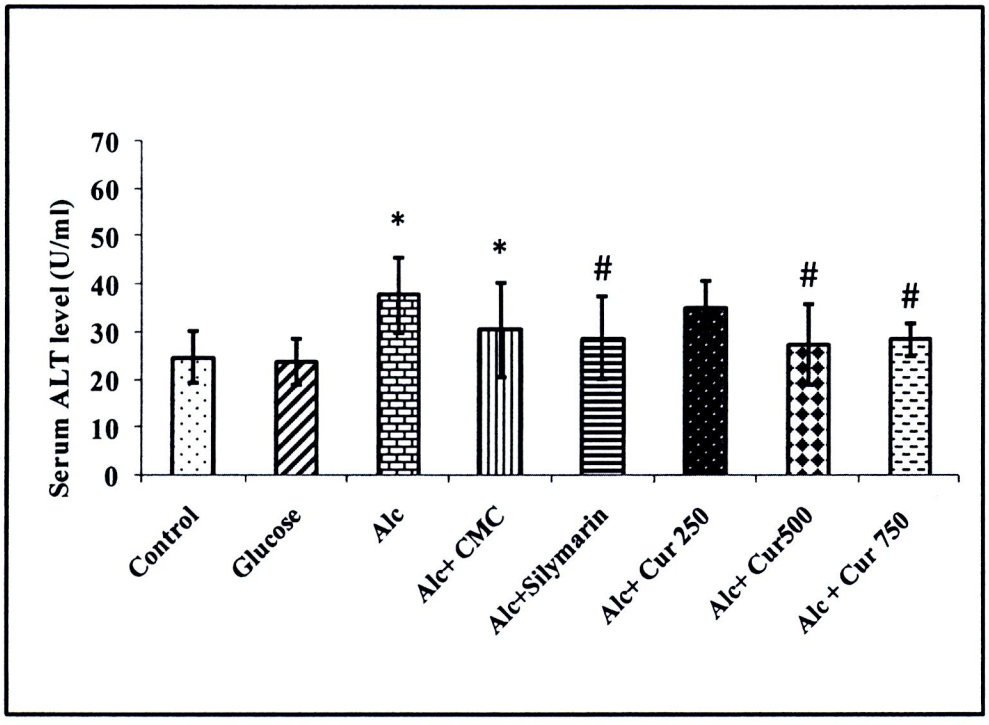


Figure 13 Effect of curcuminoids on ALT levels in ethanol-stimulated rat.

Note: Rats were received ethanol (6 g/kg/day) for 8 weeks and then were given CMC, silymarin or curcuminoids (250, 500, 750 mg/ml) for another 6 weeks. Serum ALT levels were measured with the liver function enzyme kits. Data were from 6 animals (n=6) and shown as mean ± SD. Data were analyzed statistic significantly by ANOVA ($p \leq 0.05$), comparing to the control group (*) or comparing to the ethanol induced hepatic toxicity (#). Alc: alcohol, CMC: carboxymethyl cellulose, Cur: curcuminoids, ALT: alanine aminotrasferase

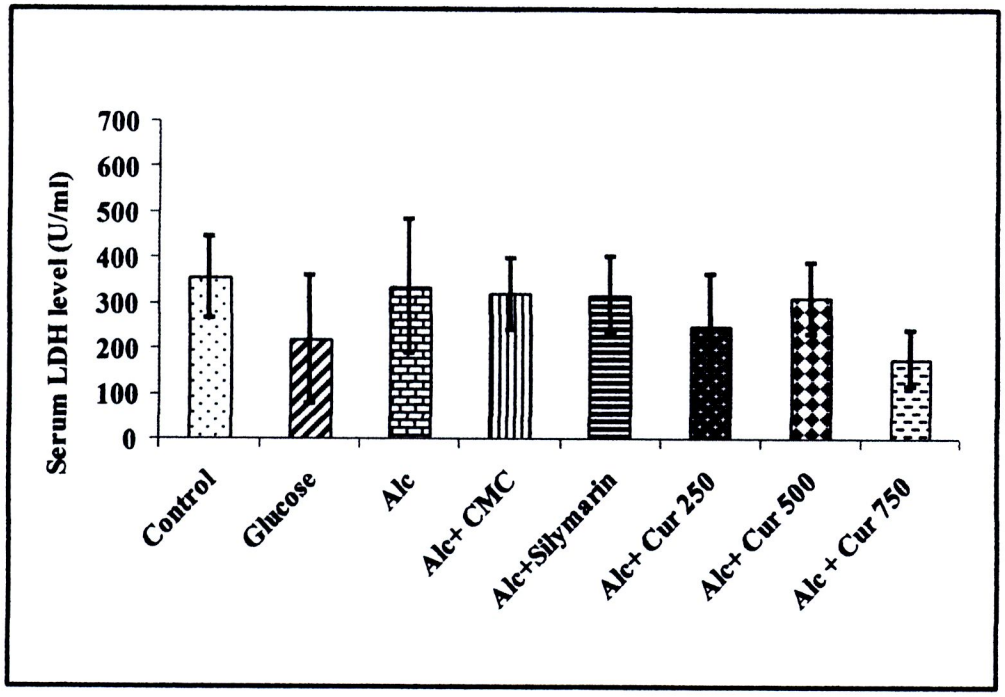


Figure 14 Effect of curcuminoids on LDH level in ethanol-stimulated rats.

Note: Rats were received ethanol (6 g/kg/day) for 8 weeks and then were given CMC, silymarin or curcuminoids (250, 500, 750 mg/ml) for another 6 weeks. Serum LDH levels were measured with the liver function enzyme kits. Data were from 6 animals (n=6) and shown as mean ± SD. Data were analyzed statistic significantly by ANOVA ($p \leq 0.05$), comparing to the control group (*) or comparing to the ethanol induced hepatic toxicity (#). Alc: alcohol, CMC: carboxymethyl cellulose, Cur: curcuminoids, LDH: lactate dehydrogenase

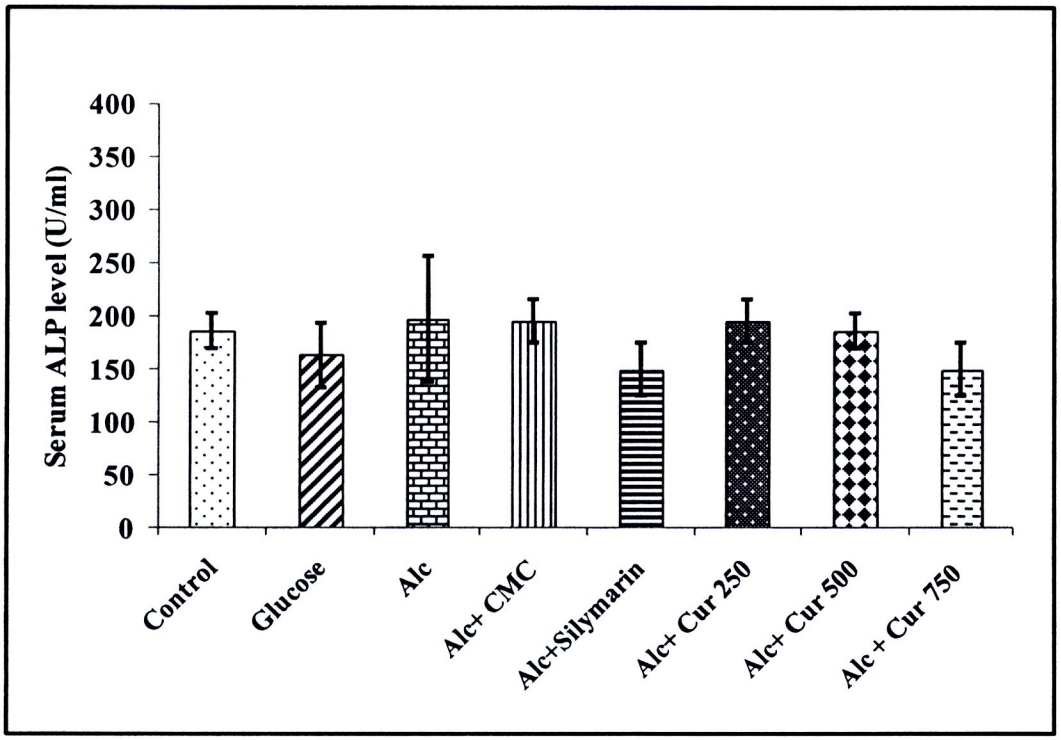


Figure 15 Effect of curcuminoids on ALP level in ethanol stimulated rats.

Note: Rats were received ethanol (6 g/kg/day) for 8 weeks and then were given CMC, silymarin or curcuminoids (250, 500, 750 mg/ml) for another 6 weeks. Serum ALP levels was measured with the liver function enzyme kits. Data were from 6 animals (n=6) and shown as mean ± SD. Data were analyzed statistic significantly by ANOVA ($p \leq 0.05$), comparing to the control group (*) or comparing to the ethanol induced hepatic toxicity (#). Alc: alcohol, CMC: carboxymethyl cellulose, Cur: curcuminoids, ALP: alkaline phosphatase.

Histological examination in ethanol induced hepatotoxicity.

The histological appearances of hepatocyte in a control rat showed normal liver histopathology which has no lipid deposition (Figure 16A) and inflammation lesion. The examination of the livers of rats treated with 6 g/kg ethanol for 8 weeks and then combined with CMC as a vehicle revealed degenerative changes such as microvesicular steatosis, macrovesicular steatosis, portal inflammation and lobular inflammation shown in Figure 16B, and 17A and 17B respectively. However, the histological changes of rats fed isocaloric equivalent glucose showed the development of mild steatosis (Figure 16C). It is possible that high calorie intake of glucose lead to imbalance of lipid metabolism resulting in accumulation of fat droplet and may be of inflammation. The rats stimulated with ethanol to induce hepatotoxicity and combined curcuminoids 250, 500 and 750 mg/kg confirmed the improvement of steatosis significantly including microvesicular steatosis, macrovesicular steatosis and mix micro- macrovesicular steatosis when compare to the ethanol-induced toxicity with CMC rat livers. In additions, 250, 500 and 750 mg/kg curcuminoids also attenuated portal and lobular inflammations (Table 7, 8).

Oxidative stress is an important factor in the pathogenesis of liver injury that partly come from alcohol metabolism. The increasing productions of free radicals attenuate the levels of antioxidants [6]. Lipid peroxidation caused by reactive oxygen species (ROS) result in the disarrangement and ultimately disruption of cell membranes, which lead to necrotic death [147]. Chronic ethanol administration to rodent has been demonstrated a variety of microscopic hepatic changes, including steatosis, hepatocellular oncotic necrosis, apoptosis, inflammatory cell infiltration, terminal hepatic venular sclerosis, proliferation of the smooth endoplasmic reticulum, and mitochondrial aberration [58]. The significant variation in animal model used in the study of ALD reflects the difficult task of developing a suitable model that exactly replicates the human prototypes [58]. Animal models have been administered ethanol chronically by various methods in attempt to develop liver lesion resembling those seen in human ALD. Simple inclusion of ethanol in the drinking fluid seldom causes high and sustained elevation of blood ethanol levels and only a moderate rise in liver triglyceride is observed [148]. Moreover, several experiments in animal model of ALD showed that, the type of dietary fat ingested by animal is a major factor in

alcohol-induced liver damages [148]. For example, polyunsaturated fatty acid such as corn oil and vegetable oil, promote destructive CYP2E1 activity by generating free radical , which attack polyunsaturated lipid (a process known as lipid peroxidation) [149]. Moreover, the environmental and genetic influences involve in modulating the pathology of tissue damage to mild pathology in some cases.

The histological damages by ethanol-induced toxicity in our study demonstrated some histological damages by ethanol induced toxicity, however there was less severe as judged by the changes comparing to the biopsy liver from ALD patient [61].

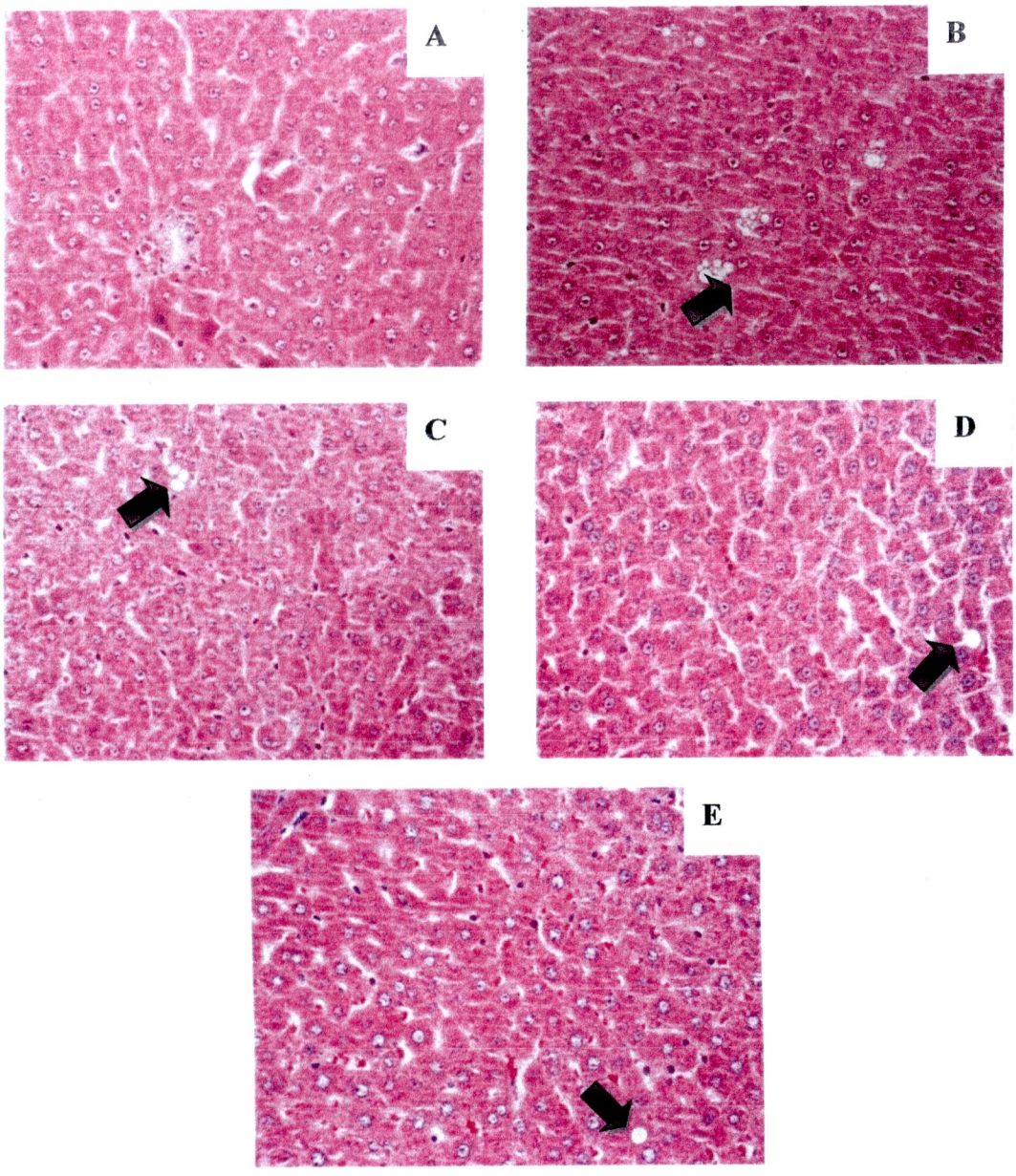


Figure 16 Histological appearances of steatosis in hepatocytes (H&E staining, magnification x400)

Note: 16A, hepatocytes a control rats, 16B, hepatocytes of ethanol-induced toxicity rats with CMC, demonstrating steatosis (black arrow), 16C, hepatocytes of isocaloric equivalence treated rat liver, 16D, hepatocytes of ethanol-induced toxicity rats with silymarin, 16E, hepatocytes of ethanol-induced toxicity rats with 500 mg/kg/day curcuminoids

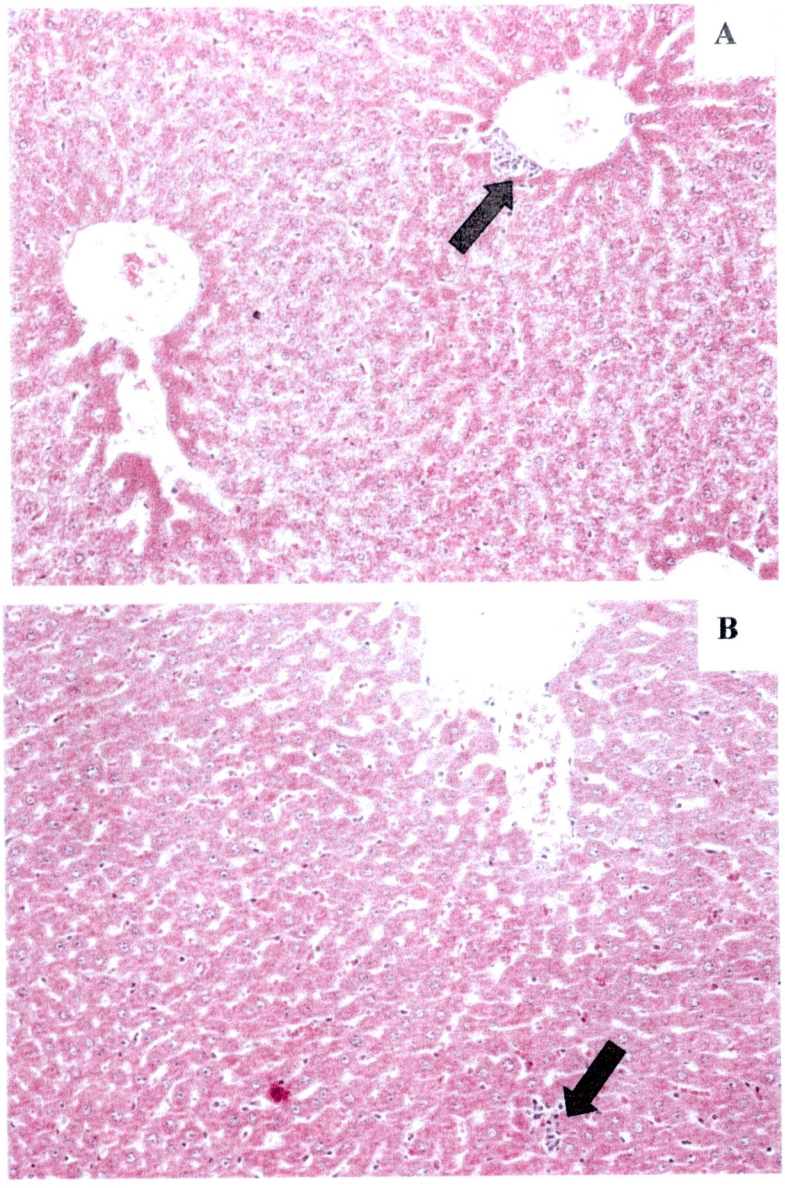


Figure 17 Histological changes of inflammation in ethanol –induced hepatocytes (H&E magnification, x200)

Note: 17A: Portal inflammation, 17B: lobular inflammation.

Table 7 Pathologic changes of fatty liver (steatosis) in alcohol-induced toxicity rats

Experimental groups	Fatty liver (% of parenchymal cells containing fat droplets)		
	Microvesicular	Macrovesicular	Mix-micro and macro vesicular
Control	1.09±0.55	0.93±0.72	0.99±0.69
Alc+CMC	1.91±0.49[a]	1.36±0.67	2.07±0.87[a]
Glucose (Isocaloric equivalence)	1.90±0.60[a]	1.28±1.24	2.09±1.46[a]
Alc+Silymarin	0.97±0.49[b]	0.62±0.54[b]	0.98±0.91[b]
Alc+250 mg Cur	1.25±0.45[b]	0.98±0.71	1.10±0.87[b]
Alc+ 500 mg Cur	0.52± 0.62[ab]	0.92±0.49[b]	0.60±0.69[b]
Alc+750 mg Cur	0.95±0.59[b]	0.62±0.65[b]	0.80±0.80[b]

Note: Data were from 6 rats (n=6) and were analyzed statistic significantly by *t*-test; All values expressed as mean ±SD

a: statistic significant comparing to control group, b: statistic significant comparing to Alc+CMC group ($p < 0.05$)

Alc: alcohol, CMC: carboxymethyl cellulose, Cur: curcuminoids

Table 8 Pathological changes of inflammation in alcohol-induced toxicity rats

Experimental groups	Inflammatory characteristics	
	Lobular inflammation (foci per x 200 field)	Portal inflammation (% of total portal triad)
Control	0.06±0.32	1.50±2.65
Alc+CMC	0.28±0.57	2.72±4.80
Glucose (Isocaloric equivalence)	0.33±0.59	2.28±5.35
Alc+Silymarin	0.22±0.55	0.98±2.18
Alc+250 mg Cur	0.11±0.47	0.00±0.00[ab]
Alc+ 500 mg Cur	0.67±0.90[a]	1.01±2.09
Alc+750 mg Cur	0.39±0.70	0.81±1.63

Note: Data were from 6 rats (n=6) and were analyzed statistic significantly by *t*-test; All values expressed as mean ±SD

a: statistic significant comparing to control group, **b**: statistic significant comparing to Alc+CMC group ($p < 0.05$)

Alc: alcohol, CMC: carboxymethyl cellulose, Cur: curcuminoids

Effect of curcuminoids on ethanol-induced lipid peroxidation in microsomal extraction.

Lipid peroxidation in microsomal extraction was measured by estimating the formation of thiobarituric acid reactive substances (TBARs). The TBARs calculated as malondialdehyde (MDA) content in microsomal extraction were assessed. Our result showed the moderate increases of MDA levels in ethanol stimulated with CMC rats. However, curcuminoids at concentration 250,500 and 750 mg/kg/day diminished ethanol-induced lipid peroxidation when compared with the ethanol supplement with CMC significantly. In additions, curcuminoids at higher dose trended to reduce MDA levels even lower than the control group. Lipid peroxidation is an autocatalytic mechanism leading to oxidative destruction of cellular membranes. Their destruction can lead to cell death and also the production of toxic and reactive aldehyde metabolites such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) [150]. Lipid peroxidation has been implicated in the pathogenesis of hepatic injury by free radical derivatives of ethanol metabolism and responsible for cell membrane damage and consequent release of the marker enzymes of hepatotoxicity [147]. The degree of lipid peroxidation may be moderated by antioxidant defenses. Our study demonstrated that curcuminoids diminished ethanol-induced lipid peroxidation. The hepatoprotective effect of curcuminoids against ethanol –induced liver injury may involve mechanisms related to free radical scavenging effect and inhibition of the propagating chain of lipid peroxidation [125]. On the other hand, silymarin had only minimal effect on lipid peroxidation in this study.

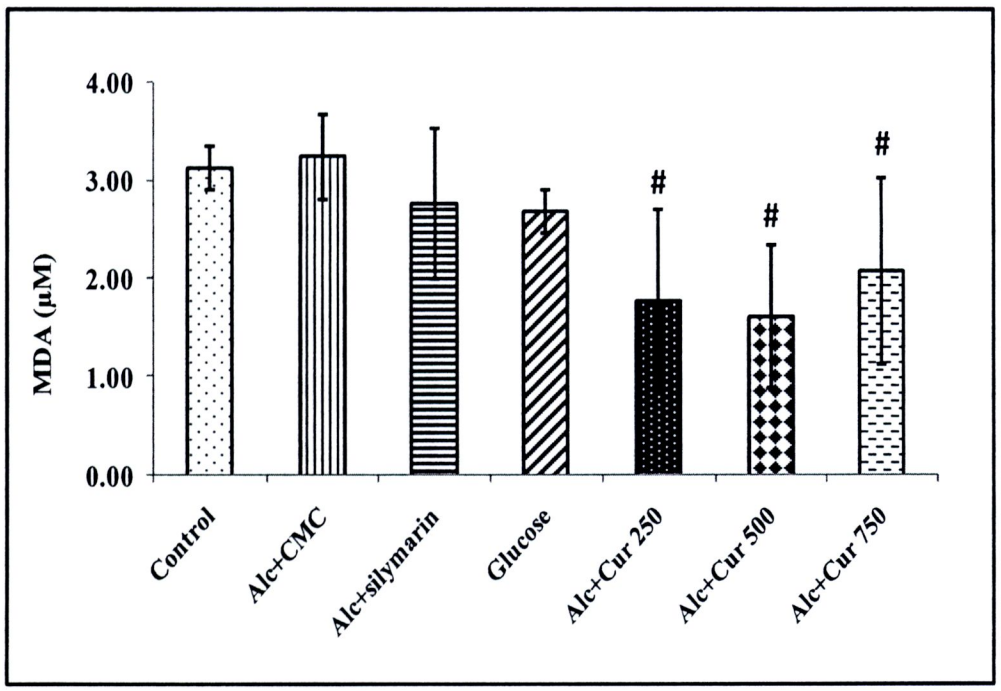


Figure 18 The effects of curcuminoids on lipid peroxidation in liver microsomal extraction from ethanol-induced toxicity rats.

Note: Data were from 6 separated experiments (n=6) and shown as mean ± SD of MDA level, Data were analyzed statistic significantly by ANOVA, comparing to the control ($p \leq 0.05$).

Alc: alcohol, CMC: carboxymethyl cellulose, Cur: curcuminoids

Effect of curcuminoids on superoxide dismutase activity in liver microsomal extraction.

Mitochondrias efficiently reduce oxidant under normal conditions via antioxidants mechanism including glutathione and superoxide dismutase (SOD) [151]. SOD converts O^{-}_{2} into a less toxic product. This enzyme is the first line in cell defense against oxidative stress [152]. The SOD activity was represented as % inhibition of the formation of formazan product (WST-1). Our results showed that the levels of hepatic SOD activity were not different among the groups. The level of hepatic SOD activity of the control group was 80.78% inhibition indicating that there was endogenous SOD in the rat microsome extract. The SOD activity of the ethanol treated with CMC group was slightly decrease to 76.05% inhibition as well as of the rat group with curcuminoids supplement (250,500,750 mg/kg) showed similar hepatic SOD activity (80.98, 76.73 and 79.30 % inhibition respectively) The effects of chronic ethanol exposure on SOD activity are controversial. Yang *et al* (2004) and Samuhasaneeto *et al* (2009) showed that there was no changes of SOD in the hepatocytes from chronically ethanol fed rats [125, 142]. In literature reviews suggest that cells contain a variety of antioxidant enzymes including SOD, catalase (CAT) and glutathione peroxidase (GPx). Both GPx and CAT can react with H_2O_2. Our study showed mild histological changes in both steatosis and inflammation in ethanol induced toxicity rats. Hence, the SOD activity in the liver might not alter. Moreover, the differences in SOD levels in the livers of rats chronically fed ethanol might be different upon rat stains, the dose, duration and route of ethanol administration.

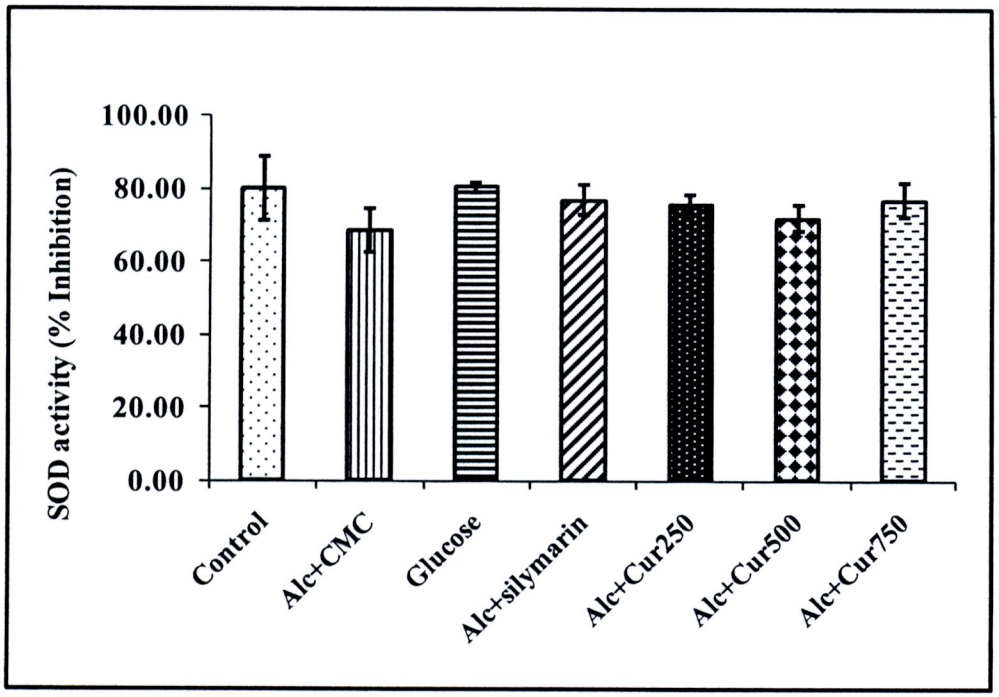


Figure 19 The effects of curcuminoids on superoxide dismutase in liver microsomal extraction from ethanol-induced toxicity rats.

Note: Data were from 6 separated experiments (n=6) and shown as mean ± SD of SOD activity (% inhibition), Data were analyzed statistic significantly by ANOVA, comparing to the control ($p \leq 0.05$). Alc: alcohol, CMC: carboxymethyl cellulose, Cur: curcuminoids

Acute oral toxicity test in normal rat

The purpose of this study was to investigate the potential toxicity of curcuminoids by acute oral toxicity in Sprague-Dawley rats. Acute toxicity test was performed by OECD guideline for testing of chemicals. These studies are used the single fixed doses 5000 mg/kg of curcuminoids.

The result showed that curcuminoids did not cause any mortality at a single dose of 5000 mg/kg. We observed that the rats fed with the high dose of curcuminoids were healthy. No unusual changes in behavior, ataxia, and signs of intoxication were observed. Serum AST liver function enzymes were not significantly different when compared to the baseline before curcuminoids treatment. However, ALP was decreased, and ALT and LDH enzymes activities were increased significantly compared to the baseline (Table 9). In these studies, the body weights of each rat were measured before treatment and once a week at day 7^{th} and day 14^{th} after treatment. The rat body weights and food consumption were increased every week. Therefore, curcuminoids did not appear to retard growth or affect food consumption.

The results of acute toxicity study in normal rats revealed that curcuminoids was not toxic, which is similar to many references of the safety of curcuminoids and turmeric in rats, guinea pigs and monkeys [153].

Table 9 Serum liver function enzymes activity of a single dose 5000 mg/kg curcuminoids in normal rats.

Treatment	AST (IU/L)	ALT (IU/L)	ALP (UI/L)	LDH (UI/L)
Baseline	42.51 ± 11.1	23.21 ± 1.4	529.34 ± 39.7	337.35 ± 189.2
Curcuminoids (5000 mg/kg)	44.26 ± 5.9	27.73± 2.3[a]	312.35 ± 17.9[a]	554.72 ± 295.2[a]

Note: Data were from 6 rats (n=6) and were analyzed statistic significantly by *t*-test; All values expressed as mean ±SD

a: Statistic significant comparing to baseline ($p < 0.05$)

Table 10 Body weights and food consumptions during the study period of a single dose 5000 mg/kg curcuminoids in normal rats

Time	Body weight (g)	Food intake (g/rat/day)
Day 0	295.83 ± 6.5	15.0± 0.81
Day 7	326.83 ± 9.1[a]	14.2 ± 0.66
Day 14	346.50 ± 8.7[a]	15.6 ± 0.43

Note: Data were from 6 rats (n=6) and were analyzed statistic significantly by *t*-test. a: Statistic significant, comparing to the baseline (Day0)($p < 0.05$). All values expressed as mean ± SD

Acute oral toxicity test in ethanol-induced hepatotoxicity rats.

Several studies investigate toxicity of the compound in normal rats. Small number of toxicological data is available regarding to the safety of curcuminoids in to ALD patients. As a part of a safety evaluation of curcuminoids on ethanol –induced toxicity rats, acute oral dose toxicity study was carried out after single oral dosing of curcuminoids in ethanol-induced toxicity rats.

Rats were induced hepatotoxicity by ethanol for 8 weeks as mentioned in the method. All the liver function enzymes were increased indicating the liver cells damages. Then, the rats were received curcuminoids at dose 5000 mg/kg. Our result showed that all hepatotoxicity rats were not dead. Moreover, curcuminoids at high dose could reduce all liver function enzymes especially ALT, ALP and LDH significantly (Table 11). Body weights of each rats were measured at the initiation before ethanol treatment and once a week throughout the study. The body weight gains were observed in all rats. This information strongly suggests that curcuminoids are safe in ethanol-induced toxicity rats. It has been shown in the several studies about curcuminoids safety in animals and humans [153,154]. For example, a phase one human trial with 25 subjects using up to 8000 mg of curcumin per day for 3 months found no toxicity of curcumin. Five other human trials using 1125-2500 mg of curcumin per day have revealed the safety [154]. Our study first demonstrates the safety of curcuminoids in the ethanol-induced toxicity. These imply that curcuminoids treatment should be safe in ALD patients.

Table 11 Serum liver function enzymes activity of a single dose 5000 mg/kg curcuminoids in ethanol-induced toxicity rats

Conditions	AST (IU/L)	ALT (IU/L)	ALP (UI/L)	LDH (UI/L)
Control (before ethanol treatment)	46.73 ± 8.6	23.51 ± 4.3	481.33 ± 62.0	516.78 ± 11.1
Ethanol induced toxicity	51.07 ± 11.1	62.77 ± 16.3[a]	573.2 ± 136.6	927.96 ± 286.9[a]
Curcuminoids treatment (5000 mg/kg/day)	47.21 ± 4.8	36.72 ± 7.6[ab]	151.98 ± 21.7[b]	251.73 ± 101.7[ab]

Note: Data were from 6 rats (n=6) and were analyzed statistic significantly by *t*-test. a: Statistic significance compared with the control (before ethanol-induced toxicity) ($p < 0.05$), b: Statistic significance compared with ethanol-induced toxicity rats ($p < 0.05$). All values expressed as mean ±SD

Table 12 Body weights and food consumptions during the study period of a single dose 5000 mg/kg curcuminoids in the ethanol-induced toxicity rats

Time	Body weight (g)	Food intake (g/rat/day)
Day 0	306.50 ± 4.5	13.0 ± 0.50
Day 15	350.83 ± 8.5[a]	12.6 ± 0.59
Day 30	376.50 ± 10.4[a]	15.3 ± 0.59[b]
Day 45	416.33 ± 18.5[a]	12.7 ± 0.83
Day 60	409.17 ± 12.7[a]	13.9 ± 0.04[b]
Day 75	433.17 ± 4.4[a]	12.5 ± 0.32

Note: Data were from 6 rats (n=6) and were analyzed statistic significantly by t-test. a: Statistic significance compared with the baseline (day 0) ($p < 0.05$), b: statistic significance compared with food intake day 15 ($p < 0.05$). All values expressed as mean ±SD.

HepG2 Cell culture

The human hepatocellular carcinoma (HepG2) cells characterized cell in an experiment, is human liver cancer cell lines that allow hepatocyte to be studied *in-vitro*. HepG2 cells have an epithelial-like morphology and contain one nucleus which different from primary hepatocytes display the typical cubic cell shape and often containing two nuclei [155]. HepG2 cells line have biosynthetic capabilities similar to those of normal hepatocytes. It provides a reproducible in vitro model for studying not only by drug but also by ethanol [158]. HepG2 cells have inducible cytochrome P450 2E1 and ADH which are associated with ethanol induced liver damage [159].

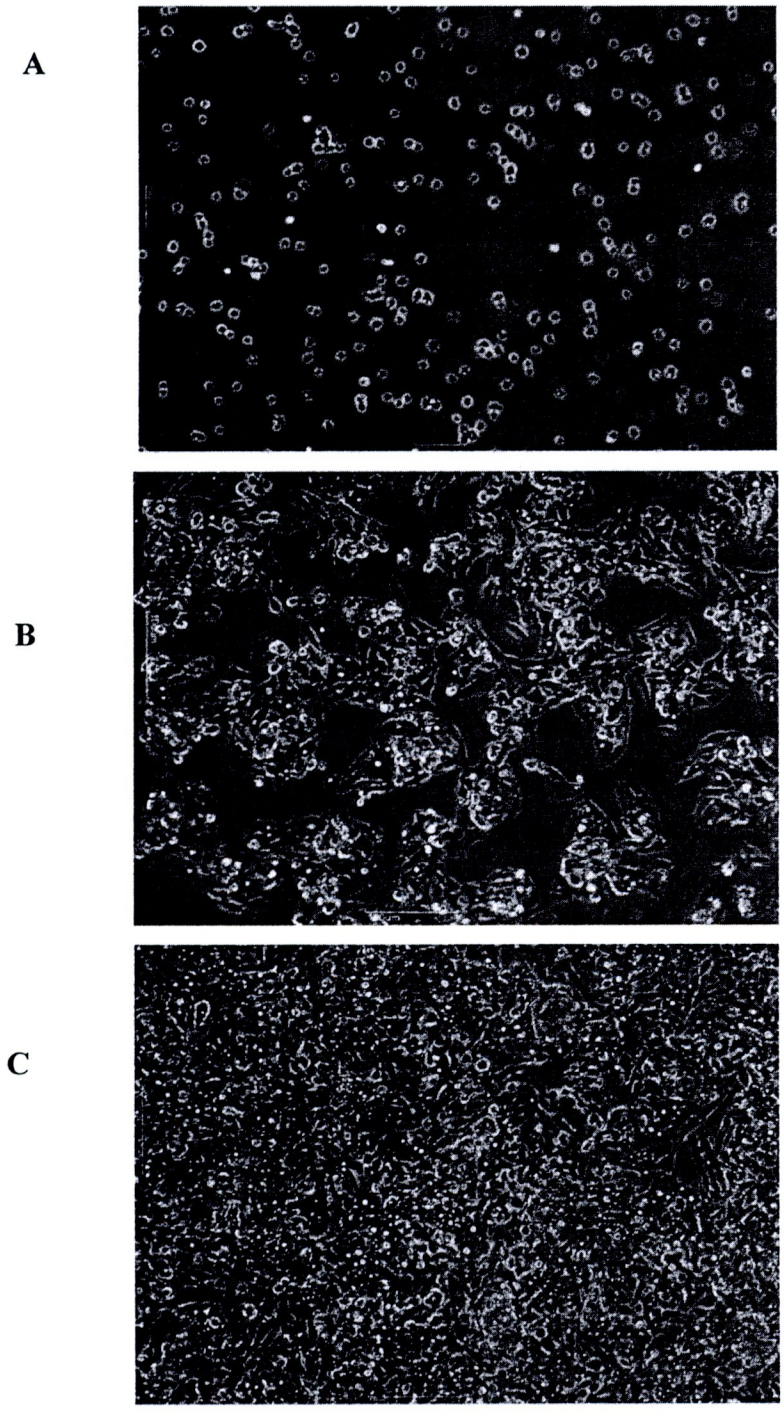


Figure 20 Morphological characters of monolayer HepG2 cells in cell culture flask.

Note: A, B, C: morphology of HepG2 cells at day 0, day 1 and day 3 (original magnification x100, scale bar = 100 μm)

Direct effect of ethanol on cytotoxicity in HepG2 cells

The direct effects of ethanol on cytotoxicity were investigated by MTT assay and measuring the release of cytosolic lactate dehydrogenase (LDH). HepG2 cells were cultured in various ethanol concentrations 1.25, 2.5, 5, 7.5 and 10 % (v/v) for 4, 8, 24, 48 hours. The results showed that various concentrations of ethanol and the different incubation times were associated with cell viability in doses and time dependent manners (Figure 21A).

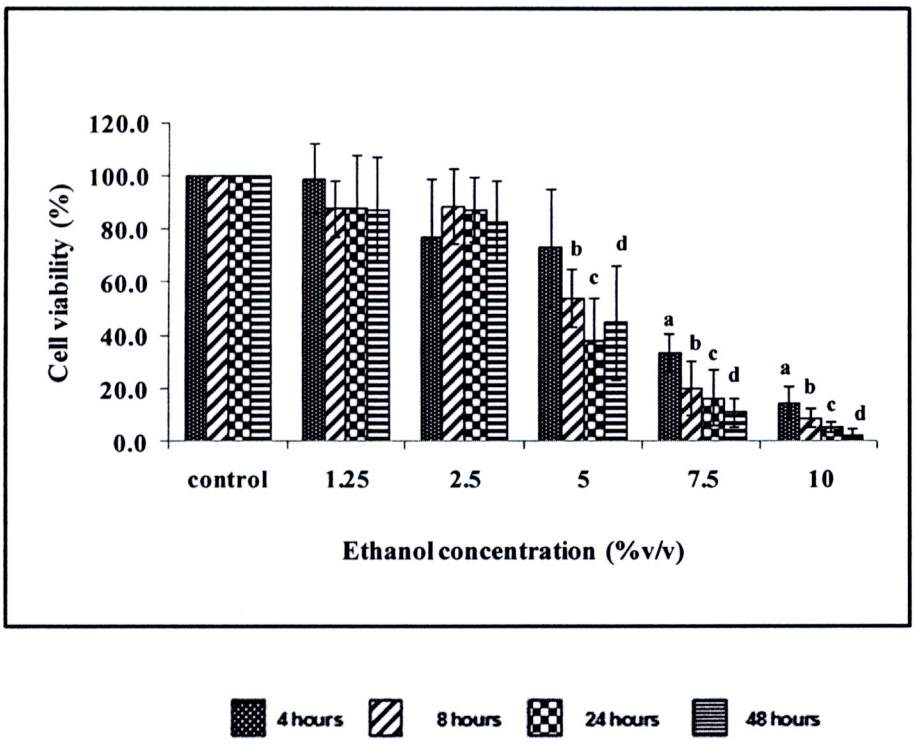
Then, the direct effect of various ethanol concentrations at 24 hours on cytotoxicity in HepG2 cells were examined by MTT assay and the release of LDH enzymes. The results demonstrated that at 24 hours ethanol decrease % cell viability in the HepG2 cells significantly at the concentration of 2.5, 5, 5.5, 6, 6.5, 7 and 7.5% (v/v) and the percentages of cell viability were 89, 70.0, 52.0, 18.9, 11.3, 10.2 and 10.8% respectively.

In this study, the direct effects of ethanol in HepG2 cells were also observed by the release of LDH into the medium. LDH is an enzyme that exists in many tissues including liver. When tissues or organs are damaged, LDH is released into the blood [156]. Therefore, LDH leakage can be used to indicate cell viability. The higher LDH leakage is explained as lower viability of hepatocytes. The data showed significantly LDH leakage at the concentrations 6, 6.5,7 and 7.5% (v/v) of ethanol.

The HepG2 cells, which retain differentiated parenchymal functions, may provide a reproducible in vitro for studying cytotoxicity induced by drugs and ethanol [157,158]. It has been reported to be a model for the study of alcoholic liver damage [160,161,162]. Alcohol-induced liver tissue damage results from toxic by produce of ethanol metabolites acetaldehyde and free radical from oxidative stress. The ethanol exposure may have a direct effect on cell structure and cell metabolism. Mechanisms of tissue damages include effects of ethanol on mitochondrial functions, membrane fluidity, immune system and oxidative stress [163,164]. These processes are vulnerable to disrupt liver cell and resulting in hepatocytes necrosis. The direct toxic effect of ethanol metabolisms are associated cytochrome P450 2E1 and alcohol dehydrogenase enzyme. ADH is the majority of ingested alcohol to acetaldehyde which is toxic metabolites in hepatocytes [155]. After alcohol exposure showed acetaldehyde-induced hepatocytes apoptosis and depletion of mitochondrial

glutathiones. Many studies showed that ethanol could induce hepatotoxicity to HepG2 cells [17]. Our study has confirmed that HepG2 cells incubated with 60-80 mmol which is equal to 3.7-5% (v/v) ethanol for 24 hours, showed an approximate 30-40% loss of cell viability as assessed by MTT assay and LDH release.

21A



21B

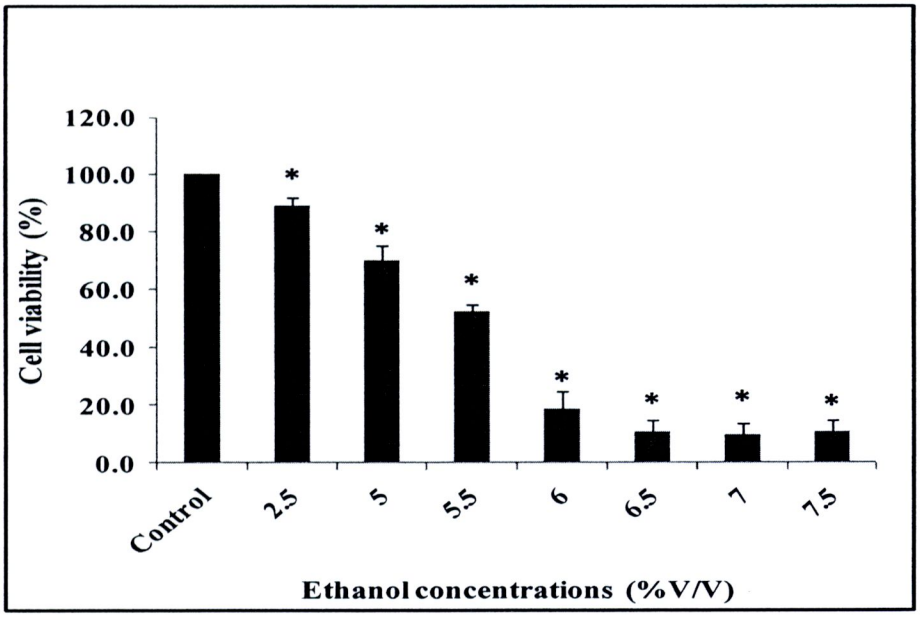


Figure 21 Direct effects of ethanol on cytotoxicity in HepG2 cells.

21C

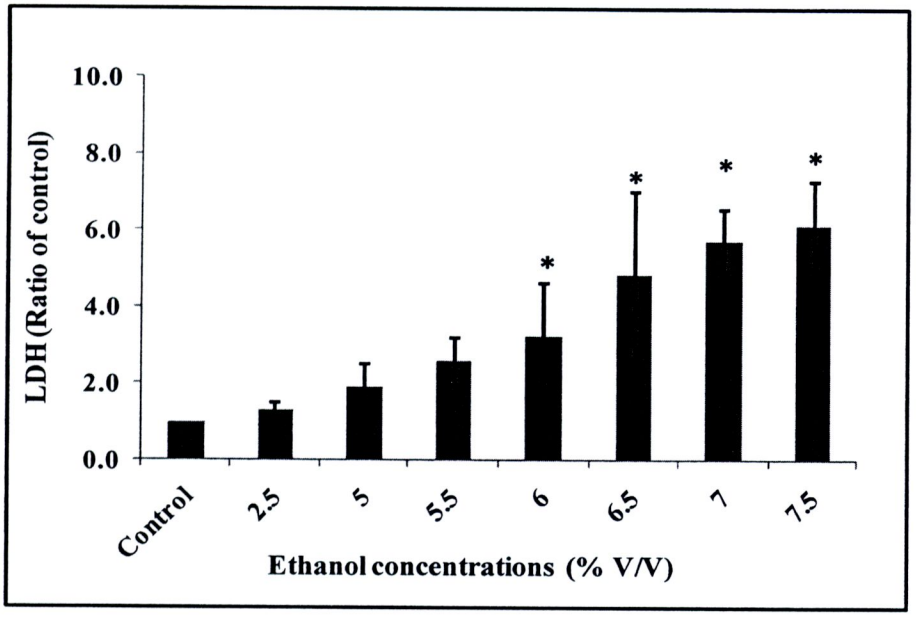


Figure 21 (cont.)

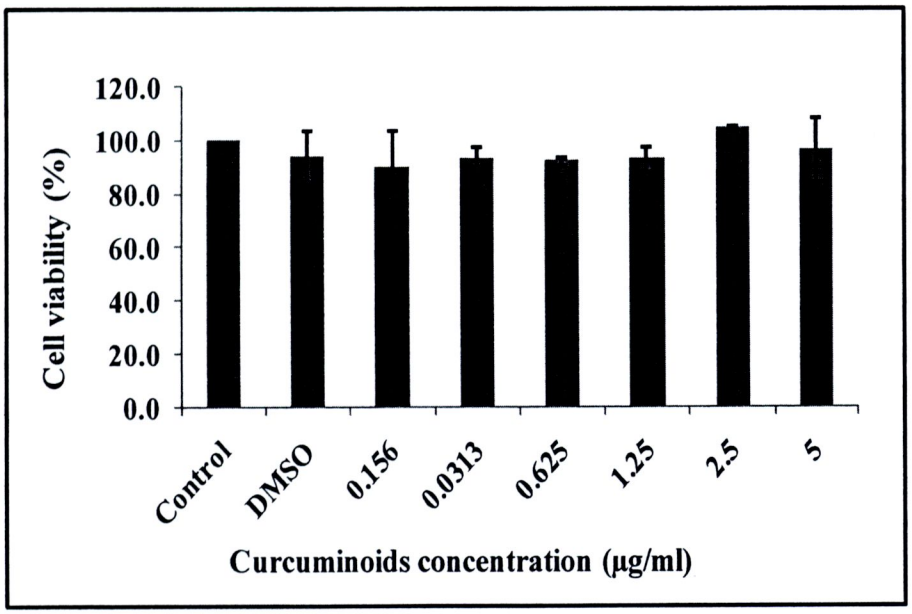
Note: 21A: MTT assay of various concentrations and time exposure of ethanol, 21B: MTT assay of various concentration of ethanol at 24 hours, 21C LDH activity measurement of various concentration of ethanol at 24 hours. Data were from 3-5 separated experiments (n=3-5) and shown as mean ± SD of % cell viability. Data were analyzed statistic significantly by ANOVA, comparing to control ($P \leq 0.05$), $^{a,b,c,d}P \leq 0.05$ comparing to control 4,8,24,48 hours respectively.

Direct effect of curcuminoids on cytotoxicity in HepG2 cells.

The direct effect of curcuminoids was also investigated by MTT and LDH assay. HepG2 cells were treated with a series of curcuminoids concentration, 0.156, 0.313, 0.625, 1.25, 2.5, 5 μg/ml for 24 hours. The MTT results as compared with the control, curcuminoids slightly decrease % cell viability in HepG2 cells. In order to examine the direct effect of curcuminoids, we also measured LDH releasing into the medium. There was no significance of LDH release at concentrations from 0.156 - 5 μg/ml. From this experiment, curcuminoids was found to be non toxic to HepG2 cell culture over a range of 0.156-5 μg/ml. Therefore, these concentrations are not toxic to cells which were used to examine in other experiments.

Previous report have shown the ability of curcumin to induced apoptosis in tumor cell by producing ROS, loss of mitochondrial membrane potential, and deoxyribonucleic acid (DNA) fragmentation [16, 17, 166]. Ghoneim, A.I., *et al* were evaluated the lowest used concentration of curcumin that showed an anti-lipid peroxidation, reduced cytochrome *c* release. However, higher concentration induced cytochrome *c* release, Caspase-3 activation, GSH depletion, necrosis and apoptosis in isolated rat hepatocytes [167].

22A



22B

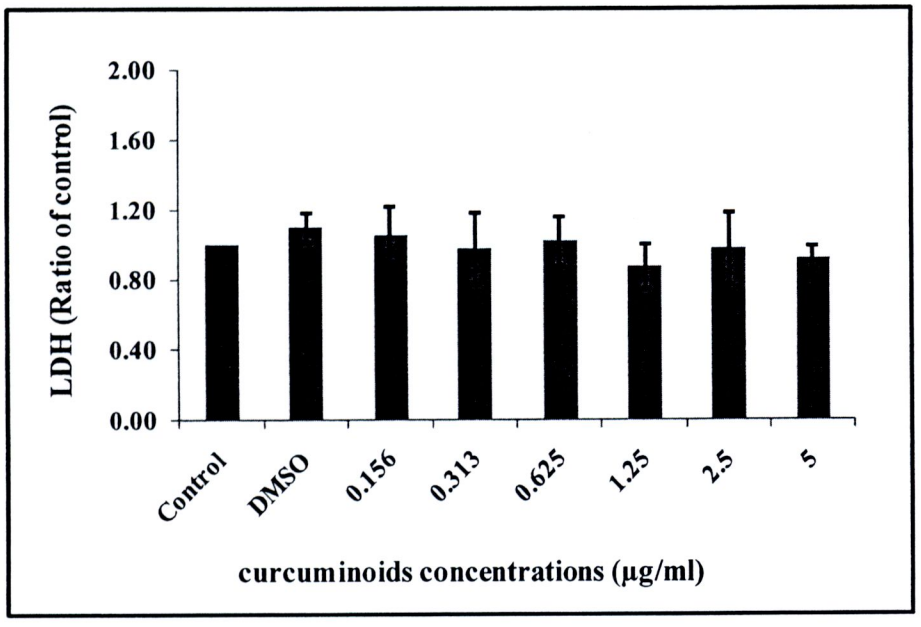


Figure 22 Direct effects of curcuminoids on cell viability in HepG2 cells.

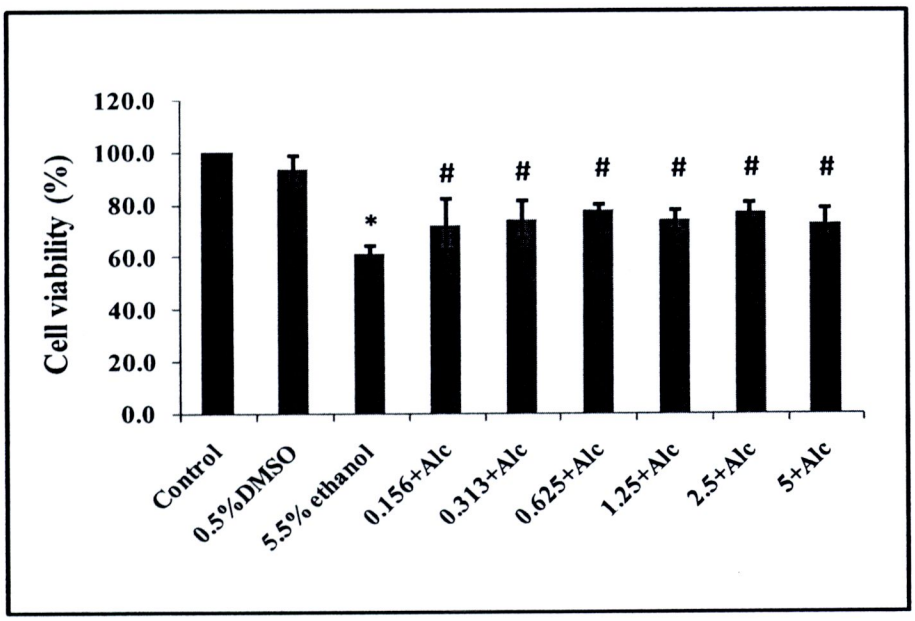
Note: 22A, MTT assay of various concentrations of curcuminoids at 24 hours 22B: LDH activity measurement of various concentrations of curcuminoids at 24 hours. Data were from 3 separated experiments (n=3) and shown as mean ± SD of % cell viability. Data were analyzed statistic significantly by ANOVA, comparing to control ($p \leq 0.05$)

Effect of curcuminoids on cell viability in ethanol stimulated HepG2 cells.

We examined effects of curcuminoids on ethanol induced toxicity in HepG2 cells. The effects of pretreatment with various concentrations of curcuminoids 0.156-5 μg/ml for 2 hours before co-incubating with 5.5% (v/v) of ethanol showed that curcuminoids at dose 0.156, 0.313, 0.625, 1.25, 2.5 and 5 μg/ml increase cell viability significantly in HepG2 cell by MTT assay. In addition, the effect of curcuminoids on ethanol-induced toxicity evaluated by LDH assay, trended to decrease 5.5% ethanol-induced LDH release. The previous studies were showed ethanol at 80 mmol which caused toxicity in HepG2 cell [161,162] and it is in the concentration range expected to reached in the liver of produce a subject drinking 60 g of alcohol per day [162] . This experiment, 5.5% (v/v) was used to induce hepatotoxicity as equal to 80.5 mmol ethanol. The toxicity of ethanol to the liver derives from a variety of mechanism such as the metabolism of ethanol by alcohol dehydrogenase, cytochrome P450 and oxidative stress [160,163]. A many studies suggested that oxidative stress mechanism in hepatocytes to play important role of ethanol induced toxicity [15, 16]. Over production of oxygen radicals leads to altered antioxidant enzyme activity, glutathione depletion, decreased DNA repair and lipid peroxidation [158]. These alterations have been recognized to be characteristic of alcohol-induced liver damage.

Curcumin, a polyphenol in curcuminoids derive from turmeric compound extract is well known for its antioxidant activity [15, 17]. It is able to reduce ROS formation [169] and decrease lipid peroxidation formation [170]

23A



23B

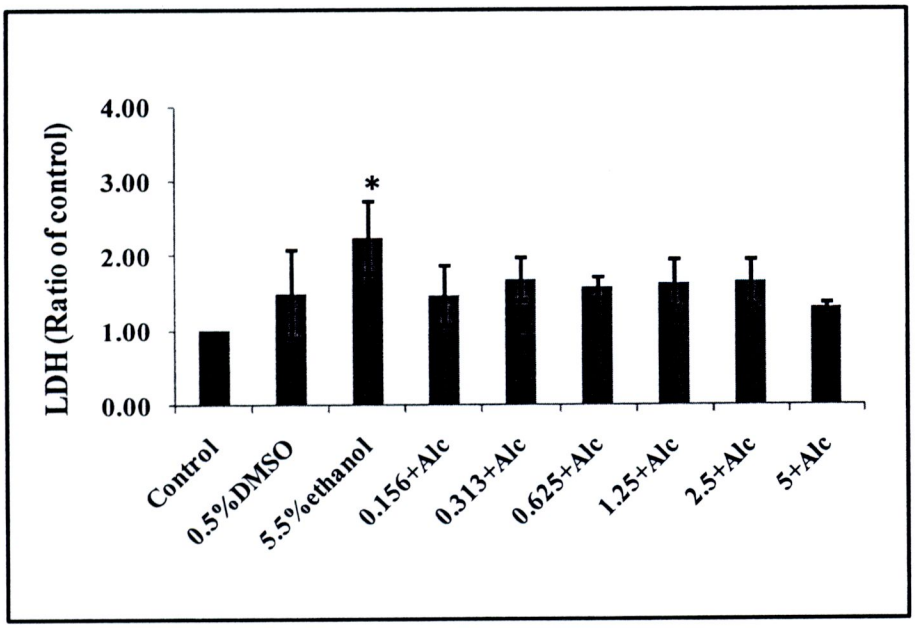


Figure 23 Effect of curcuminoids on cytotoxicity in ethanol stimulated HepG2 cells.

Note: 23A: various concentration of curcuminoids on cytotoxicity in ethanol stimulated HepG2 cells by MTT assays, 23B: various concentration of curcuminoids on cytotoxicity in ethanol stimulated HepG2 cells by LDH measurement. Data were from 3-4 separated experiments (n=3-4) and shown as mean ± SD. Data were analyzed statistic significantly by ANOVA, comparing to 5.5% v/v ethanol stimulated cells ($p \leq 0.05$)

Effect of curcuminoids on lipid peroxidation in ethanol stimulated HepG2 cells

The lipid peroxidation productions from the cell measured by TBARs assay as an representing molondialdehyde content. HepG2 cells with various concentrations of ethanol for 24 hours, increased MDA levels as a dose-dependent manger. We investigated the effect of curcuminoids on lipid peroxidation in ethanol-induced toxicity HepG2 cells. Data revealed that incubation with 5.5% ethanol increase MDA levels significantly comparing to control cells. When 5.5% ethanol-stimulated HepG2 cells were pre-incubated with various concentration of curcuminoids for 2 hours, we found that curcuminoids at concentrations 1.25, 2.5 and 5 μg/ml diminish ethanol-induced lipid peroxidation significantly, comparing to 5.5% ethanol-stimulated HepG2 toxicity at 24 hours (Figure 24 and 25).

Ethanol metabolism is directly involved in the production of reactive oxygen species (ROS) [171]. Lipid peroxidation is one of the indicators of oxidative stress. Lipid peroxidation results from interaction of unsaturated lipid components with oxygen free radicals, generated by ethanol metabolism [172]. A destruction of cell membrane causing by lipid peroxidation can lead to death. Lipid peroxidation was increased in ethanol-induced toxicity HepG2 cells which correlated to membrane peroxidation and membrane breakdown. Curcumin has been reported to prevent liver lipid peroxidation in ethanol induced hepatotoxicity [173]. The inhibition of peroxidation by curcumin is mainly attributed to scavenging of the free radical and inhibiting of propagating chain of lipid peroxidation. The antioxidant property of curcumin mainly involve in two sources such as its innate polyphenolic structure and its ability to affect activity of antioxidant enzyme [23, 174]. The antioxidant property of curcumin protects biomembrane against peroxidative damage. Our study also demonstrated curcuminoids could prevent lipid peroxidation production in ethanol induced toxicity and obtain an antioxidant property.

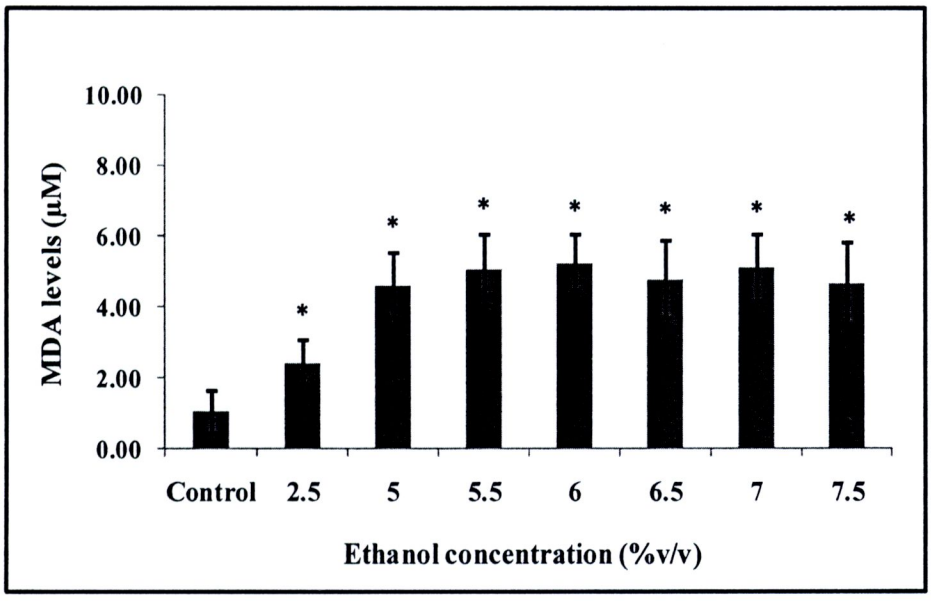


Figure 24 Effects of ethanol on lipid peroxidation in HepG2 cells.

Note: Data were from 4 separated experiments (n=4) and shown as mean ± SD of MDA levels. Data were analyzed statistic significantly by ANOVA, comparing to the control ($p \leq 0.05$).

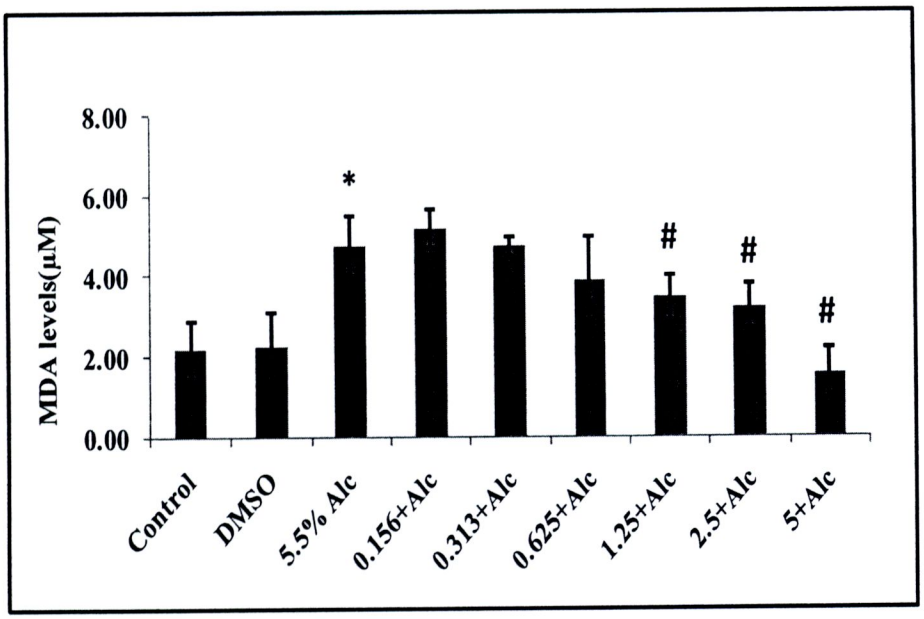


Figure 25 Effects of curcuminoids on lipid peroxidation in ethanol stimulated HepG2 cells.

Note: The ethanol-treated HepG2 cells were combined with various concentrations of curcuminoids. Data were from 4 separated experiments (n=4) and shown as mean ± SD of MDA level. Data were analyzed statistic significantly by ANOVA, comparing to the 5.5% ethanol stimulation ($p \leq 0.05$).

Effect of curcuminoids on NO production in ethanol stimulated HepG2 cells

HepG2 cells, stimulated with various concentrations of ethanol for 24 hours, increased NO production as a dose-dependent manger. This experiment, we used ethanol at concentration 7.5% (v/v) to induce NO generation. When 7.5% ethanol-stimulated HepG2 cells, the data showed that the cells were generated amounts of NO significantly, comparing with the control cells. HepG2 cells were pre-incubated with curcuminoids 2 hours and added 7.5% ethanol through 24 hours, the result showed that curcuminoids at 0.156 and 0.313 μg/ml trend to decrease NO productions (Figure 26 and 27). However, curcuminoids at higher concentration (0.625, 1.25, 2.5, 5 μg/ml) could not lowering NO product, in contrast curcuminoids seem to increase the NO production in ethanol-stimulated HepG2 cells

NO acts as an important mediator of vascular tone and neuronal transduction in low concentration, but also has cytotoxic effects at higher (μmolar) concentration. Nitrites and nitrates are increased in NO production in ALD has been linked to an increased tolerance to alcohol [41]. It has been demonstrated that enzymes iNOSs are unregulated in cirrhotic livers and they involve in pathological process [152,175] . They modulate different inflammatory cells and prolong cytokines secretions such as TNF-α, resulting in hepatitis [176]. Curcumin is reported to inhibit the NO production and expression of inducible nitric oxide syntase (iNOS) protein and mRNA in RAW 264.7 cells stimulated with lipopolysaccharides (LPSs) or interferon-γ [177]. Chan, M.M., *et al* have reported that curcumin can inhibit the iNOS gene expression in isolated BALB/c mouse peritoneal macrophages and in the liver of LPS-inject mince [178]. There is no report particularly demonstrate the effect of curcuminoids or other substance; desmethoxycurcumin and bisdesmethoxycurcumin on NO production in liver cells. In this study, we found the degree of NO production at the lower concentration (0, 156, 0.625) of curcuminoids and there effect is form the curcumin in curcuminoids acted as active substance to inhibit the NO in ethanol stimulated hepatotoxicity. We thought that the other substances in curcuminoids might have different effect on NO productions in this ethanol induced toxicity. It is necessary to further study the effect of demethoxycurcumin and bisdemethoxycurcumin on NO production

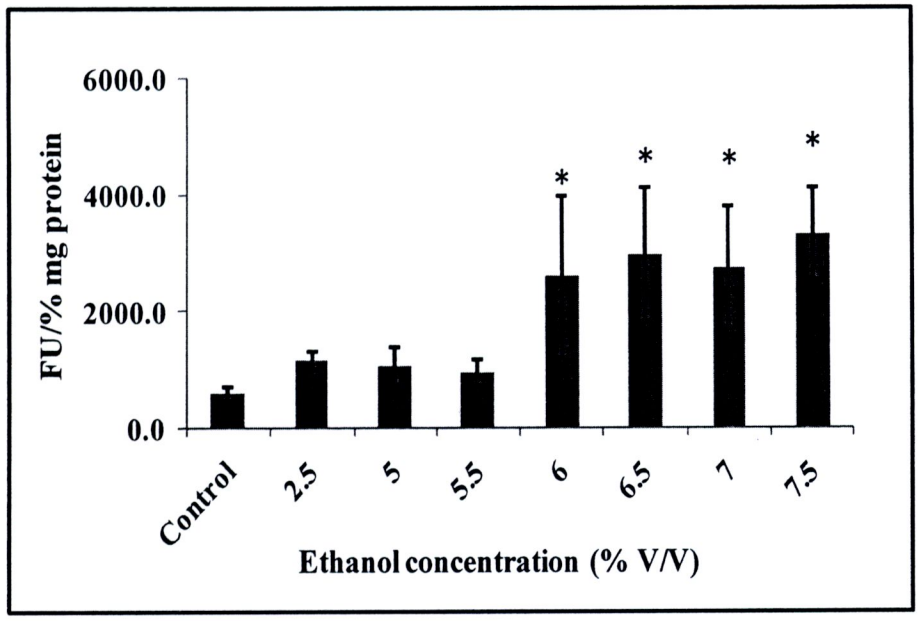


Figure 26 Nitric oxide (NO) production in ethanol stimulated HepG2 cells.

Note: Cells were stimulated with ethanol with various concentrations for 24 hours. Data were from 3-4 separated experiments (n=3-4) and shown as mean ± SD of fluorescent (unit) unit/%mg protein. Data were analyzed statistic significant by ANOVA, comparing to the control ($p \leq 0.05$)

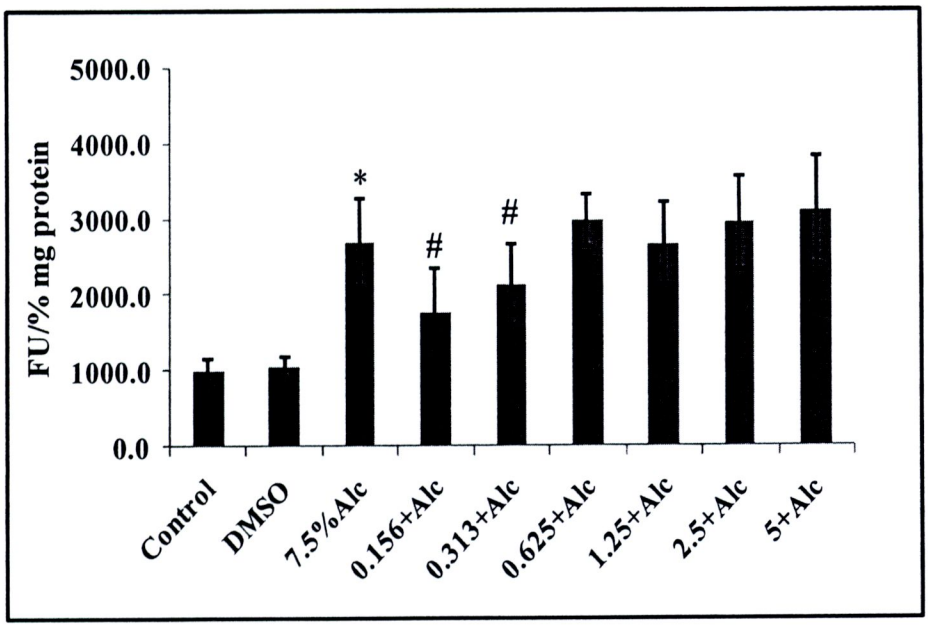


Figure 27 Effect of curcuminoids on NO production in ethanol stimulated HepG2 cells.

Note: Ethanol stimulated cells pre-incubated with various concentrations of curcuminoids. Data were from 3-4 separated experiments and shown as mean ± SD of fluorescent unit (FU)/%mg. protein: Data were analyzed statistic significantly by ANOVA, comparing to 7.5% ethanol induced toxicity cells ($p \leq 0.05$).