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Protective effect of pomegranate (*Punica granatum* Linn.) juice against hepatotoxicity and testicular toxicity induced by ethanol in mice

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Abstract. Ethanol commonly causes hepatotoxicity and testicular toxicity after chronic consumption. Silymarin, a commercial drug for protective and curative treatment of liver disease, was used as a standard drug. This study aimed to evaluate the protective activities of pomegranate juice on liver damage and sperm quality impairment caused by ethanol consumption. The experiment was conducted on 5 groups of male mice. Group I as negative control received distilled water, group II as positive control received ethanol 400 mg/100 gBW for 14 days, group III received ethanol and silymarin at dose of 2.5 mg/100 gBW, group IV-V received ethanol and pomegranate juice at doses of 1000 and 2000 mg/100 gBW, respectively. Groups III-V was done by pretreatment with drug and pomegranate juice for 10 days and co-treatment with ethanol for 14 days. Pomegranate juice revealed the hepatoprotective activities by reduction of liver damage including lymphocytic infiltration, microvesicular steatosis and necrosis area. Moreover, it also significantly decreased the elevation of blood alanine amino transferase (ALT, a marker enzyme of liver injury)) and bilirubin. Furthermore, it also acted as a protectant of testicular toxicity by increase in blood testosterone and improvement of sperm quality. These results were a physiological evidence to support the use of pomegranate juice as a protectant of liver damage and testicular toxicity caused by ethanol consumption.
Key Words: Pomegranate (*Punica granatum* Linn.), ethanol, protective activities, hepatotoxicity, testicular toxicity.

Introduction. Liver is an important organ for metabolism or detoxification of foreign compounds entering our bodies (Athar et al 1997) and it is one of our best antioxidant supplied organ (Feher et al 1982). It is commonly damaged by drugs and alcoholic drinks. Sharma et al (2012) found that rats received 40 % ethanol (v/v) 2.0 ml/100 gBW for 21 days caused hepatotoxicity. Toxic substance such as reactive molecules (oxidant) generated during the metabolism of alcohol in the liver. These can cause oxidative stress and consequently caused liver damage and liver disease (Fernandez-Checa et al 1997). As well as, ethanol also affect spermatogenesis (Anderson et al 1983). However, many plants were used as hepatoprotectants by considering theirs antioxidant properties such as *Picrorhiza kurroa* (Anandan et al 1999), *Elephantopus scaber* (Rajesh & Latha 2001), *Hibiscus sabdariffa* (Liu et al 2006), *Silybum marianum* (Das & Vasudevan 2006) and *Sida veronicaefolia* (Sharma et al 2012).

Pomegranate (*Punica granatum* Linn.) is a potential medicinal plant of family Punicaceae (Heber 2011). The peel of fruit was used as a traditional remedy against malaria infection (Dell Agli et al 2010). The peel and seed had protective role against hepatotoxicity induced by carbon tetrachloride (CCl_4) (Abdou et al 2012). While, the juice was commonly used as a healthful beverage (Basu & Penugonda 2009), since it is a natural rich source of polyphenol, flavonoid and other antioxidants (punicalagin, ellagic acid, gallotannin, anthocyanin, etc.) (Yance & Valentine 1999). Pharmacological properties of the juice were anti-inflammatory (Adams et al 2006), anticancer (Adams et

al 2010), suppression of joint damage in rheumatoid (Shukla et al 2008), a cardioprotectant (Sumner et al 2005) and protection of hepatotoxicity induced by carbon tetrachloride (Pirinccioglu et al 2012). More over, it also increased the production of antioxidants in the sperm which improved its quality (Turk et al 2008). Thus, this study aims to evaluate the protective roles of pomegranate juice against the hepatotoxicity and testicular toxicity induced by ethanol oral administration in mice.

Material and Method

Chemicals and drug. Alanine aminotransferase assay kit (ALTL acc. to IFCC, Roche/Hitachi cobas c system, Roche Diagnostics GmbH, D-68296 Mannheim, USA.), bilirubin assay kit (BILTS, total protein special, Roche/Hitachi cobas c system) total protein assay kit (total protein gen 2 by colorimetric method, Roche/Hitachi cobas c system, Sanhofer Strasse 116, D-68305 Mannheim, USA.), testosterone radioimmunoassay kit (The DSL-400 ACTIVE$^{(R)}$ Testosterone Coated-Tube RIA kit, Diagnostic System Laboratories, Inc., USA) and standard drug, silymarin (Pharma supply Co., Ltd., Thailand).

Friut juice preparation. Fresh mature pomegranate fruits were purchased from local market at Khon Kaen province, Thailand. They were cleaned and cut, collected the aril part and minced by mortar. The fruit juice was obtained, then filtered through cotton mesh and evaporated in hot air oven at 45°C until it was concentrated at 1000 and 2000 mg/ml. The oral administration was done at dose of 1.0 ml/100 gBW.

Animals. Adult male mice, ICR strain, 8 week-old and 35-40 g was obtained from the National Laboratory Animal Center, Nakornpathom province, Thailand. They were housed under a 12:12 of light-dark cycle and at 25 ± 1°C. The standard pellet diet (Chareanpogapan Ltd., Thailand) and water *ad libitum* were available. The experiments were performed after the experimental protocol had been approved by the Institutional Animal Ethics Committee, Khon Kaen University, Thailand (Reference No. 0514.1.12.2196).

Experiment. The experiment was conducted on 5 groups of 6 animals each. Group I served as a negative control, received distilled water 0.5 ml/100 gBW for 24 days, group II served as a positive control, pretreated with distilled water 0.5 ml/100 gBW for 10 days and co-treated with ethanol 400 mg/100 gBW for 14 days, group III pretreated with silymarin 2.5 mg/100 gBW for 10 days and co-treated with ethanol for 14 days, groups IV-V pretreated with pomegranate juice 1000 and 2000 mg/100 gBW for 10 days and co-treated with ethanol for 14 days, respectively. At the end of treatment, assessment of hepatoprotective activity was done by determining blood alanine aminotransferase (ALT), total protein, bilirubin and percentage of liver damage. Meanwhile, the protective effect on testicular toxicity was assessed by evaluation of gonadal index, blood testosterone level and sperm quality.

Gonadal index evaluation. After 24 h of the last treatment, the body weight and testicular weight were recorded. The relative of testicular weigh/body weight were expressed as gonadal index.

Biochemical evaluation. At the end of treatment, blood samples were collected by cardiac puncture and centrifuged at 1,700 rpm for 5 min at room temperature. Plasma samples were obtained and used for testosterone assay, determination of blood bilirubin total protein and alanine aminotransferase (ALT), a biomarker enzyme of liver injury (Schomaker et al 2013).

Sperm quality analysis. After blood sampling, epididymis and vas deferens of all groups were excised and torn with needles (No. 25) in 2 ml of 0.9 % NaCl and then incubated at 35 ^{o}C for sperm quality investigation including total sperm counts and

percentage of viable sperms following the method of Yokoi et al (2003), motile sperms were evaluated by the method of Sonmez et al (2005) and abnormal sperms were investigated by method of Atessahin et al (2006).

Liver histological observation. Livers of all groups were collected and immediately fixed in Bouin's solution for 48 h, and then were processed by paraffin method. They were sectioned at 5 µm thickness and stained with haematoxylin & eosin (H & E). The sections were observed for histological changes of the architecture under light microscope. The percentage of the liver damage was investigated by using microscopic grid (Olympus Ltd., Japan).

Statistical analysis. All data were expressed as mean ± standard deviation ($\bar{x} \pm SD$), each parameter was separately analysed by one-way analysis of varience (ANOVA). Duncan's test was used to compare the different results among groups. A value of $P<0.05$ was considered as statistically significant (Zar 1991).

Results and Discussion. Protective effect of pomegranate juice against hepatotoxicity induced by ethanol oral administration for 14 days is presented in tables 1 & 2.

Table 1

Effect of *Punica granatum* juice (PJ) on blood alanine aminotransferase (ALT), bilirubin and total protein of mice received ethanol (EtOH)

Treatment (mg/100 gBW) N=6	$\bar{x} \pm SD$		
	ALT (U/L)	*Bilirubin (mg/dl)*	*Total protein (g/dl)*
0	35.00 ± 07.00^{a}	0.02 ± 0.01^{a}	4.76 ± 0.23^{a}
EtOH 400	58.14 ± 11.50^{b}	0.13 ± 0.05^{c}	4.37 ± 0.27^{b}
EtOH + Silymarin 2.5	30.83 ± 09.00^{a}	$0.04 \pm 0.02^{a,b}$	5.10 ± 0.28^{a}
EtOH + PJ 1000	32.17 ± 06.70^{a}	$0.08 \pm 0.04^{b,c}$	4.98 ± 0.31^{a}
EtOH + PJ 2000	31.29 ± 10.81^{a}	$0.08 \pm 0.04^{b,c}$	5.07 ± 0.35^{a}

N - number of experimental animals; different alphabet within column means significant difference ($P<0.05$); same alphabet within column means non-significant difference ($P>0.05$).

Table 2

Percentage of liver damage in mice received ethanol (EtOH), silymarin and pomegranate juice (PJ)

Treatment (mg/100 gBW) N=6	*% of liver damage ($\bar{x} \pm SD$)*			*Total*
	Necrosis area	*Microvesicular steatosis*	*Lymphocytic infiltration*	
0	0.12 ± 0.10^{a}	0.53 ± 0.21^{a}	0.53 ± 0.21^{a}	3.21^{a}
EtOH 400	0.74 ± 0.63^{a}	10.05 ± 0.76^{d}	1.76 ± 0.76^{b}	12.55^{b}
EtOH + Silymarin 2.5	0.70 ± 0.67^{a}	$3.9. \pm 1.43^{a,b}$	0.84 ± 0.41^{a}	5.47^{c}
EtOH + PJ 1000	0.16 ± 0.11^{a}	$7.45 \pm 1.86^{b,c}$	1.08 ± 0.21^{a}	8.69^{c}
EtOH + PJ 2000	0.38 ± 0.31^{a}	$5.63 \pm 1.86^{b,c}$	$c.61 \pm 0.28^{a}$	6.62^{c}

N - number of experimental animals; different alphabet within column means significant difference ($P<0.05$); same alphabet within column means non-significant difference ($P>0.05$).

There was a significant elevation in the level of blood ALT in group received ethanol (58.14 ± 11.50 U/L), which was higher level than that of the control group (35.00 ± 07.00 U/L). Meanwhile, it also significantly increased blood bilirubin and decreased total protein (Table 1). This evidence implied that liver was damaged. Hepatotoxicity was characterized by the increase in blood ALT, alkaline phosphatase (ALP) and bilirubin (Sharma et al 2012). An enormous amount of oxidants were generated during ethanol metabolism in liver and affected on mitochondria in cell to diminish energy production. This occurrence causes cell inflammation, cell damage and ended with cell necrosis

(Gracia-Rutz et al 1994). Whereas, the groups pretreated with silymarin 2.5 mg/100 gBW) and pomegranate juice at doses of 1000 and 2000 mg/100 gBW exhibited an ability to protect the hepatotoxicity by decreasing the level of ALT and blood bilirubin. As well as, the significant decrease in blood total protein was also found. These results were supported by the reducing of liver histopathological changes including lymphocytic infiltration (sign of liver inflammation), microvesicular steatosis (fatty liver) and necrosis area (death of hepatocytes) (Figure 1). They were found 5.47, 8.67 and 6.62 % of liver damage in groups pretreated with silymarin and pomegranate juice, respectively, which significantly decreased as compared to the control (12.55 %) ($P<0.05$). These results revealed the biological evidence of protective role of pomegranate juice on hepatotoxicity.

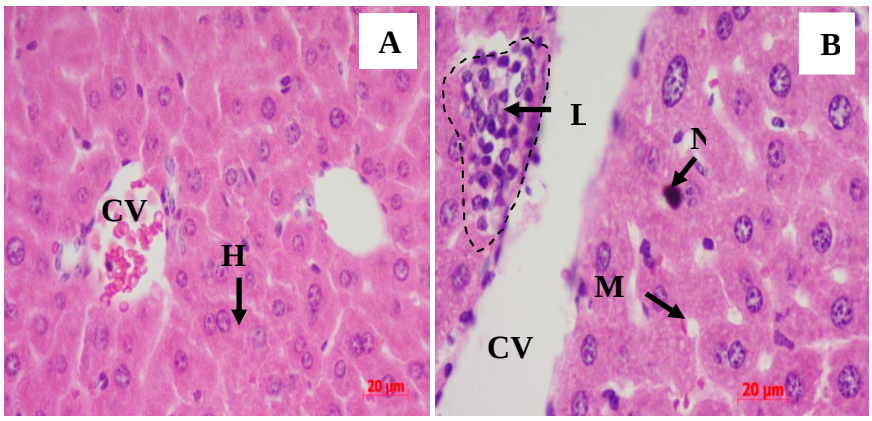

Figure 1. X-section of liver (H & E, x400), presentation of liver damage. A: normal structure, hepatocyte (H) arrangement surrounding the central vein (CV); B: abnormal structure, lymphocytic infiltration (L), microvesicular steatosis (M) and necrosis cell (N).

Feher et al (1982) claimed that liver disease caused by free radical reaction can be prevented by administration of antioxidants. Silymarin is an extract of *Silybum marianum* fruit and seed; it had a potent antioxidant property and was used as protective and curative drug of liver disease (Das & Vasudevan 2006). Previous finding had shown that nutrient and beverage containing high level of antioxidants decreased the risk of many diseases causing by oxidants (Sumner et al 2005). Futhermore, it also revealed the ability to protect against hepatotoxicity induced by chemicals (Liu et al 2006; Kirakosyan et al 2003; Pirinccioglu et al 2012; Sharma et al 2012).

The protective effect of pomegranate juice on testicular toxicity it is shown in tables 3 & 4.

Table 3

Gonadal index and blood testosterone level of mice received ethanol (EtOH), silymarin and pomegranate juice (PJ)

Treatment (mg/100 gBW) N=6	$\bar{x} \pm SD$	
	Gonadal index ($x10^{-2}$)	*Testosterone (ng/ml)*
0	$0.370 \pm 0.03^{a,b}$	11.77 ± 2.94^{a}
EtOH 400	0.355 ± 0.03^{a}	01.29 ± 0.36^{c}
EtOH + Silymarin 2.5	$0.358 \pm 0.02^{a,b}$	09.04 ± 1.38^{b}
EtOH + PJ 1000	0.396 ± 0.03^{b}	02.96 ± 0.94^{d}
EtOH + PJ 2000	$0.384 \pm 0.02^{a,b}$	06.84 ± 1.27^{c}

N - number of experimental animals; different alphabet within column means significant difference ($P<0.05$); same alphabet within column means non-significant difference ($P>0.05$).

Gonadal index of all groups were not changed. It is well known that the testicular weight did not determine the testicular function, while the blood testosterone is a marker in the diagnosis of male infertility (Chandra et al 2012). Group received ethanol revealed a significant decrease in blood testosterone (T) (1.29 ± 0.36 ng/ml) compared to the control group (11.22 ± 2.94 ng/ml). Meanwhile, the hormonal levels were 9.04 ± 1.38, 2.96 ± 0.94, 6.84 ± 1.27 ng/ml in the groups pretreated with silymarin and pomegranate juice, respectively, which were significantly increased when compared to the group received ethanol alone (Table 3). Vanthiel et al (1983) reported that ethanol and its metabolites were a testicular toxin, which caused a reduction in luteinizing hormone (LH) binding to Leydig cell and affected on its T synthesis. The decrease in blood T level was found as dose dependent manner in alcoholic patients (Mendelson et al 1977). Testosterone is an important hormone which roles on the spermatogenesis and sperm maturation (Jones 1977). Through, the incidence of the decrease in blood T may have adverse effect on sperm quality. Our results were found that all parameter of sperm quality including total sperm count, percentage of viable sperms and abnormal sperms were impaired in mice after ethanol oral administration for 14 days. While, the groups pretreated with silymarin and pomegranate juice for 10 days before co-treatment with ethanol for 14 days, they were found the protective activity on testicular toxicity by considering their sperm quality improvement and increasing of blood T level (Table 3 & 4). This finding was consistent to the work of Kulkarni et al (2009), they reported that alcohol decreased serum T level and correlated with the increase in testicular oxidative stress. Furthermore, this incidence caused adverse effect on sperm quality (Kefer et al 2009). Previous studies also found that natural antioxidants which can scavenge free radicals having great potential to attenuate the disease caused by oxidant such as liver disease, cardiovascular disease and neurogenerative disorder (Kirakosyan et al 2003) and also had an ability to improve male fertility (Sonmez et al 2005).

Table 4

Sperm quality of mice received ethanol (EtOH), silymarin and pomegranate juice (PJ)

Treatment (mg/100 gBW) N=6	$\bar{X}$ ± *SD*		
	Total sperms (x10^6 cells/each)	*% of viable sperms*	*% of abnormal sperms*
0	46.30 + 7.51^a	87.59 ± 2.18^a	19.22 ± 1.81^a
EtOH 400	33.98 ± 2.64^b	75.82 ± 7.13^b	56.77 ± 10.32^c
EtOH + Silymarin 2.5	45.41 ± 5.90^a	85.82 ± 3.42^a	31.85 ± 7.99^b
EtOH + PJ 1000	46.40 ± 6.85^a	90.07 ± 2.23^a	34.62 ± 4.20^b
EtOH + PJ 2000	45.44 ± 6.98^a	90.06 ± 3.30^a	32.50 ± 4.11^b

N - number of experimental animals; different alphabet within column means significant difference ($P<0.05$); same alphabet within column means non-significant difference ($P>0.05$).

Conclusion. The present study revealed the biological evidence that may support the use of pomegranate juice as a protectant of the liver damage and the testicular toxicity caused by ethanol consumption.

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