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Hepatoprotective Effects of *GongJin-dan* on Ethanol-mediated Experimental Liver Damage in Rats

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ABSTRACT

Background : A traditional Oriental medicine, *GongJin-dan* (GJD), is one of the most well-known tonic agents in Korea. Among 6 types of GJD components, antler, red ginseng, and Cornus fructus have shown antioxidant effects, while EtOH-induced tissue damage may be a consequence of oxidative stress.

Objectives & Methods : The hepatoprotective effects of GJD were evaluated on EtOH-mediated experimental liver damaged rats at 50, 100, 250 and 500mg/kg comparing with 100mg/kg of silymarin as a reference drug in the present study. Test substances were dosed once a day for 60 days with oral administration of 20% ethanol 2.5ml/100g body weight twice a day (equivalent to 7.9g ethanol/kg/day). Each of 8 rats per group was selected using body weight at 10 days after acclimatization. Experimental animals were sacrificed after 60 days of continuous oral treatment of test substances with 20% ethanol treatment, and changes on the body weight, liver weight, and serum AST and ALT were observed.

Results : There were dramatic decreases of body weight and increases of liver weight and serum AST and ALT. Similar inhibition effects on the EtOH-induced hepatic damages were detected between equal dosages of GJD and silymarin.

Conclusion : Based on these results, it is concluded that GJD showed clear hepatoprotective effects on EtOH-induced hepatic damage.

Key words : Hepatoprotective, *GongJin-dan*, Ethanol-mediated, Liver damage

Ⅰ. INTRODUCTION

Liver an important organ actively involved in metabolic functions is a frequent target of number of toxicants[1]. It is well known that a substantial increase in steatosis and fibrosis usually leads to potentially lethal cirrhosis of the liver in human[2]. The high global prevalence of these hepatopathies place them among the most serious disease. Although the pathogenesis of liver fibrosis is not quite clear, there is no doubt that reactive oxygen species play an important role in pathological changes in the liver, particularly in cases of alcoholic and toxic liver diseases[3].

Alcoholic liver disease remains one of the most

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common causes of chronic liver disease in the world[4]. Oxidative stress is known to play an important role in the pathogenesis of ethanol-induced liver injury[5-6].

As increase of the concern in the functional food and well being in life, the demands and consumption of functional food originated form natural sources are increased[7]. A traditional Oriental medicine, *GongJin-dan* (GJD) is one of the most famous tonic agents, in Korea and consisted of 4 herbs - Angelicae gigantis radix, Red ginseng, Corni fructus and Rehmanniae radix preparata, and 2 animal resources - antler and musk. These 4 types of herbal preparation and 2 types of animal resources were plastered using honey and coated by gold plates. Although, hypolipemic[8] and immune stimulated[9] effects of GJD have been evaluated using animal models, there are no evidences that GJD has antioxidant and related hepatoprotective effects, yet. Anyway, among 6 types of GJD components, antler[10] - one of animal resource component and Red ginseng[11] - one of herbal preparation component have been showed antioxidant effects. In addition, ursolic acid, main component of Corni fructus has been showed favorable antioxidant and it can ameliorated related EtOH-induced experimental hepatic damages in rats[12]. We, thus, hypothesized that GJD will show favorable hepatoprotective effects against ethanol mediated experimental liver damages.

II. Materials and Methods

1. Animals and Husbandry

One hundred-twelve female Wistar rats (6-wk old upon receipt, SLC, JAPAN) were used in this study. Animals were allocated four per polycarbonate cage in a temperature (20~25℃) and humidity (40~45%) controlled room. Light : dark cycle was 12hr : 12hr and feed (Samyang, Korea) and water were supplied free to access. About half of healthy rats (fifty-six rats) showed similar body weights (170 ~ 193g) were selected after 10 days of acclimatization, and forty-eight rats were induced the ethanol-mediated experimental hepatic damages, and eight rats were used as intact control. In the present study, all test articles are orally dosed once a day for 60 days with oral administration of 20% ethanol, same dosing scheme with test articles.

2. Test article and Formulation

GJD used in this study was purchased from Daegu Oriental Hospital of Daegu Hanny University (Daegu, KOREA) as listed in Table 1.

Table 1. Composition of *GongJin-dan* Used in This Study

Herbs	Scientific Names	Korean	Amounts (g/1 pill)
Antler(Cornus cervi parvum)	*Cervus elaphus* Linne	녹 용	0.683
Angelicae gigantis radix	*Angelica gigas* Nakai	당 귀	0.683
Red ginseng	*Panax ginseng* CA Mey.	홍 삼	0.683
Corni fructus	*Cornus officinalis* Sieb. Et Zucc	산수유	0.683
Rehmanniae radix preparata	*Rehmannia glutinosa* (Gaertner) Liboschitz	숙지황	0.683
Musk	*Moschus moschiferus* Linne	사 향	0.122
Honey	*Apis indica* Radoszkowski	꿀	2.506
Gold plate		금 박	0.006
Total	8 types		6.050

Deep brown gold-coated plasters, GJD was stored in a refrigerator at -20°C to protect from light and degeneration. 500, 250, 100 and 50mg/kg of GJD were homogenized in 5ml of distilled water. The appearances of homogenized GJD in distilled water are deep brown homogenous suspension, and it is well suspended upto 100mg/ml concentration levels in the present study. Silymarin (Sigma, USA) was used as Reference drug in this study. Four different dosages of GJD or 100mg/kg of silymarin were dosed by oral gavage using a sonde attached to 3 ml syringes containing test articles in a volume of 5ml/kg, once a day for 60 days. In addition, only distilled water was orally dosed in ethanol (EtOH) and intact controls, respectively

3. Induction of EtOH-mediated Hepatic damage

To induce EtOH-mediated hepatic damages, 20% EtOH (Merck, Germany) 2.5ml/100g body weight were orally dosed, twice a day (equivalent to 7.9g ethanol/kg/day) used distilled water as vehicle (*v/v*). In intact control rats, only distilled was orally dosed once a day for 60 days.

4. Measurement of Body Weights

Changes of body weight and its gains were calculated at one day before dosing, at dosing and every 10 days after test article and EtOH dosing until termination. At start of test article and EtOH treatment, all experimental animals were overnight fasted (water was not; about 18hr) to reduce the differences from feeding with at a termination day. In addition, body weight gains during experimental period were calculated as follow:

EQUATION 1. Body Weight Gains (g)

= Body weight gains throughout the whole experimental periods (60 days), body weight at a termination (Fasted) - at start of test article and EtOH treatment (Fasted)

5. Measurement of Liver Weights

At a termination, the wet-weights of individual livers was measured at g levels, and to reduce the differences from individual body weights, the relative weight (%) was calculated using body weight at a termination and absolute wet-weights as follow:

EQUATION 2. Relative Liver Weight (%)

= [(Absolute liver weight / Body weight at a termination) × 100]

6. Measurement of Serum AST and ALT levels

For detecting the serum AST and ALT levels, blood were collected at 1 day before initial dosing of test article and EtOH (base line), and at a termination (60 days after test article and EtOH treatment) from orbital plexus, and serum was separated with general methods from collected blood. Serum AST and ALT levels were detected using automated blood analyzer (Toshiba 200 FR, Japan) by the method of Reitman and Frankel[13]. In addition, the changes after test article and EtOH-dosing were also calculated to reduce the individual differences as follow:

EQUATION 3. Changes of Serum AST and ALT Levels (IU/L)

= Serum levels at a termination - Serum levels at 1 day before dosing

7. Histopathology and histomorphometry

After weighing of the livers, they were fixed in 10% neutral buffered formalin. After paraffin embedding, 3 ~ 4 μm sections were prepared. Representative sections were stained with hematoxylin and eosin (H & E) for light microscopic examination. After that, the

histological profiles of individual livers were observed. In addition, for detecting more detail histopathological changes, the percentages regions occupied by fatty changes in hepatic lobules were calculated as percentages between 1 field of liver (%/hepatic lobules), and the mean diameters (μm) of hepatocytes (at least 10 hepatocytes/livers were calculated) were also calculated by using automated CCD image analyzer (DMI, Korea) as histomorphometry.

Ⅲ. RESULTS

1. Changes on the Body Weights and Gains

Changes on the body weights and gains after EtOH and test article administration were summarized in Table 2 and Figure 1. Significant ($p<0.01$) decreases of body weight was detected from 10 days of EtOH-administration, and the body weight gains after EtOH-injection were also significantly ($p<0.01$) decreased in EtOH control compared to that of intact control. However, significantly ($p<0.01$ or $p<0.05$) increases on the body weights were detected in silymarin and all four different dosages of GJD treated groups compared to that of EtOH control from 20 ~ 40 days after administration, respectively.

Table 2. Changes on the Body Weight and Gains in Intact and EtOH-mediated Hepatic Damaged Rats

Groups	At start of administration[1]	At a termination[1]	Body weight gains after 60 days of administration
Controls			
Intact	164.63±5.78	258.38±8.63	93.75±9.44
EtOH	164.13±4.79	170.38±7.87*	6.25±6.39*
Silymarin	164.38±8.07	190.38±5.18*,#	26.00±10.49*,#
GJD treated as			
500mg/kg	164.25±6.88	203.75±10.39*,#	39.50±8.72*,#
250mg/kg	163.38±5.37	194.13±6.69*,#	30.75±8.86*,#
100mg/kg	163.38±6.52	191.38±8.50*,#	28.00±12.22*,#
50mg/kg	163.25±3.92	186.13±12.49*,##	22.88±14.51*,#

n=8; (Mean ± S.D., g); EtOH, 20% ethanol; GJD, *GongJin-dan*; [1] All animals were fasted overnight; * $p<0.01$ compared with intact control; # $p<0.01$ and ## $p<0.05$ compared with EtOH control.

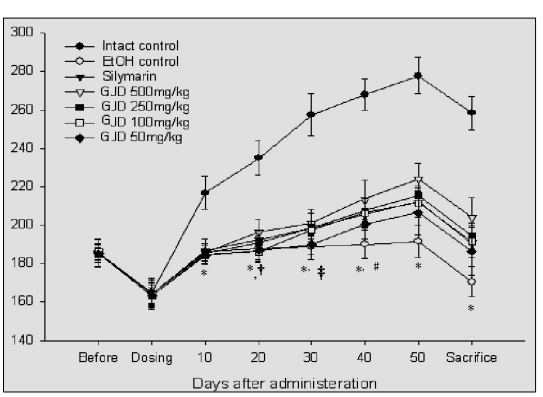

Fig. 1. Body Weight Changes in Intact and EtOH mediated Hepatic Damaged Rats

Note that significant ($p<0.01$) decreases of body weights were detected in all EtOH-treated rats as compared with intact control from 10 days after EtOH treatment (*), respectively. However, significant ($p<0.01$ or $p<0.05$) increases of body weight were detected in 500mg/kg of GJD administered groups from 20 days after start of test article administration (†), and from 30 days in silymarin, 250 and 100mg/kg of GJD treated groups, respectively as compared with EtOH control (‡). In 50mg/kg of GJD treated group, body weights were significantly ($p<0.01$ or $p<0.05$) increased from 40 days after administration (#). Mean ± SD of 8 rats. All animals at dosing and sacrifice were overnight fasted.

2. Changes on the Liver Weights

Changes on the liver weights after EtOH and test article administration were summarized in Table 3. Significantly ($p<0.01$) increases of absolute and relative liver weights were detected in EtOH control compared to that of intact control. However, significantly ($p<0.01$) decreases on the absolute and relative liver weights were detected in silymarin and all four different dosages of GJD-treated groups compared to that of EtOH control, respectively.

Table 3. Changes on the Liver Weights in Intact and EtOH-mediated Hepatic Damaged Rats

Groups	Absolute weights (g)	Relative weights (%)
Controls		
Intact	6.530±0.434	2.528±0.169
EtOH	11.162±0.952*	6.570±0.713*
Silymarin	9.457±0.885*,#	4.974±0.524*,#
GJD treated as		
500mg/kg	8.822±0.620*,#	4.348±0.488*,#
250mg/kg	9.234±0.846*,#	4.761±0.465*,#
100mg/kg	9.540±0.613*,#	4.997±0.436*,#
50mg/kg	9.742±0.846*,#	5.248±0.488*,#

n=8; (Mean ± S.D.); EtOH, 20% ethanol; GJD, *GongJin-dan*; Relative weights = (absolute weight / body weight at sacrifice) ×100; * $p<0.01$ compared with intact control; # $p<0.01$ compared with EtOH control.

3. Changes of Serum AST Levels

Changes on the serum AST levels after EtOH and test article administration were summarized in Table 4. The serum AST levels at a termination of all test substances-treated groups tested in this study were significantly ($p<0.01$) decreased compared to that of EtOH control, and the changes of serum AST levels after 60 days of dosing were also significantly ($p<0.01$) decreased in all treated groups, respectively.

Table 4. Changes on the Serum AST Levels in Intact and EtOH-mediated Hepatic Damaged Rats

Groups	Base lines (1 day before treatment)	At 60 days after test article and EtOH treatment (At sacrifice)	Change between before treatment and at sacrifice
Controls			
Intact	67.50±4.04	68.31±3.14	0.81±3.08
EtOH	67.38±4.75	169.25±10.74*	101.88±8.56*
Silymarin	67.88±3.87	117.88±14.54*,#	50.00±16.38*,#
GJD treated as			
500mg/kg	67.63±4.50	90.50±11.84*,#	22.88±11.46*,#
250mg/kg	67.88±2.95	106.25±15.57*,#	38.38±16.64*,#
100mg/kg	67.50±3.78	118.25±8.80*,#	50.75±9.69*,#
50mg/kg	67.75±2.92	120.50±8.86*,#	52.75±10.78*,#

n=8; (Mean ± S.D., IU/l); Serum AST levels were detected using automated blood analyzer (Toshiba 200 FR, Japan); EtOH, 20% ethanol; GJD, *GongJin-dan*; Change = [Serum levels at sacrifice – Serum levels at 1 day before initiation of test article or EtOH treatment]; * $p<0.01$ compared with intact control; # $p<0.01$ compared with EtOH control.

4. Changes of Serum ALT Levels

Changes on the serum ALT levels after EtOH and test article administration were summarized in Table 5. The serum ALT levels at a termination of all test article-treated groups tested in this study were significantly ($p<0.01$) decreased compared to that of EtOH control, and the changes of serum ALT levels after 60 days of treatment were also significantly ($p<0.01$) decreased in all administrated groups, respectively.

Table 5. Changes on the Serum ALT Levels in Intact and EtOH-mediated Hepatic Damaged Rats

Groups	Base lines (1 day before treatment)	At 60 days after test article and EtOH treatment (At sacrifice)	Change between before treatment and at sacrifice
Controls			
Intact	24.75±4.20	26.13±3.83	1.38±5.13
EtOH	24.88±3.80	66.50±3.46*	41.63±5.04*
Silymarin	25.00±3.70	47.13±6.36*, #	22.13±9.43*, #
GJD treated as			
500mg/kg	24.75±2.82	33.75±7.01*, #	9.00±7.17**, #
250mg/kg	25.00±2.73	42.25±3.28*, #	17.25±3.33*, #
100mg/kg	24.75±3.11	46.38±5.53*, #	21.63±6.07*, #
50mg/kg	24.88±3.27	52.38±7.56*, #	27.50±9.41*, #

n=8; (Mean ± S.D., IU/l); Serum ALT levels were detected using automated blood analyzer (Toshiba 200 FR, Japan); EtOH, 20% ethanol; GJD, *GongJin-dan*; Change = [Serum levels at sacrifice - Serum levels at 1 day before initiation of test article or EtOH treatment]; * $p<0.01$ and ** $p<0.05$ compared with intact control; # $p<0.01$ compared with EtOH control.

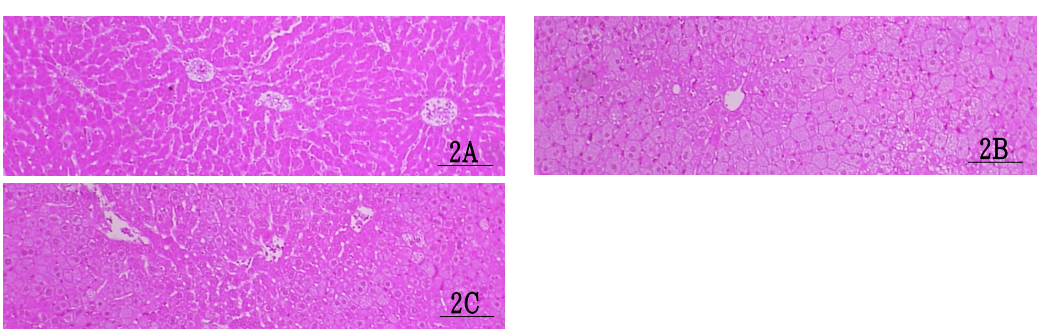

Fig. 2. Hepatic Histopathological Profiles Detected in Intact (A), EtOH (B) Controls and Silymarin (C) Treated Rats.

Note that no meaningful abnormal changes were detected in intact control, but severe fatty changes were detected in all EtOH-treated rats with hypertrophy of hepatocytes around centrolobular regions. However, these EtOH induced histopathological hepatic damages were dramatically inhibited by treatment of silymarin. All H&E stain; Scale bars = 80μm.

5. Changes on the Histopathology-Histomorphometry of Liver

Changes on the histopathology of the liver were shown Fig 2 ~ 3 and the changes on the histomorphometry of liver after EtOH and test article administration were summarized in Table 6. In intact control, no abnormal histopathological changes were observed (Fig 2A), but severe

hypertrophy due to fatty changes were detected in all EtOH-treated rats, mainly centrolobular regions (Fig 2B). However, these hepatic damages were dramatically inhibited by treatment of silymarin and all four different dosages of GJD in the present study (Fig 2C, 3). Consequently, the percentage of fatty changed regions in hepatic parenchyma were significantly ($p<0.01$) increased in EtOH control compared to that of intact control, and the mean diameters of hepatocytes in EtOH control were significantly ($p<0.01$) increased as results from fatty changes. However, both two fatty changed scores were significantly ($p<0.01$) decreased in all 5 treated groups tested in the present study, respectively (Table 6).

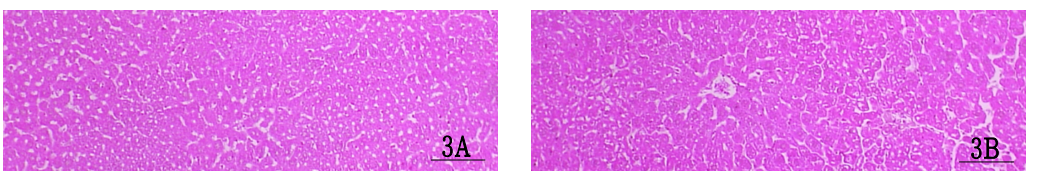

Fig. 3. Hepatic Histopathological Profiles Detected in GJD 500 (A) and 250 (B)mg/kg Treated Rats.

Although, classic EtOH-induced hepatic histopathological changes such as fatty changes were also detected in GJD 500 and 250mg/kg treated rats, they were dramatically ameliorated as compared with EtOH control, respectively. All H&E stain; Scale bars = 80μm.

Table 6. Changes on the Histomorphometry, Fatty Change Scores of Liver in Intact and EtOH-mediated Hepatic Damaged Rats

Groups	Percentages of fatty changed regions among hepatic lobules (%/hepatic lobules)	Mean diameters of hepatocytes (μm/hepatocytes)
Controls		
Intact	7.80±3.12	18.94±2.46
EtOH	91.86±4.10*	44.06±9.01*
Silymarin	73.11±6.10*, #	28.29±5.10*, #
GJD treated as		
500mg/kg	28.55±5.29*, #	22.98±4.01#
250mg/kg	65.07±4.89*, #	26.11±3.95*, #
100mg/kg	72.30±5.17*, #	28.56±2.25*, #
50mg/kg	80.97±4.62*, #	31.85±4.06*, #

n=8; (Mean ± S.D.); EtOH, 20% ethanol; GJD, *GongJin-dan*; Histomorphometrical analysis was conducted using automated image analyzer at prepared histological samples; * $p<0.01$ compared with intact control; # $p<0.01$ compared with EtOH control.

Ⅳ. DISCUSSION

Among 6 types of GJD components, antler[10], Ginseng steamed red[11], Cornus fructus[12] have been showed antioxidant effects and EtOH-induced tissue damage may be a consequence of oxidative stress[14], therefore, the hepatoprotective effects of GJD were evaluated on the EtOH-mediated experimental liver damaged rats at 50, 100, 250 and 500mg/kg comparing with 100mg/kg of

silymarin as reference drug in the present study.

Wei Yilin(危亦林) who was oriental clinician in the last era of Yuan(元代) mentioned the GJD in his famous book "Effective Prescriptions for Generation(世醫得效方)[15]" for the first time. Since the book, lots of clinicians have prescribed in clinical field usefully. The prescription, GJD, is consisted of four important medicinal stuffs to strengthen the feeble. *Cervus* for raising up vital energy(Yang energy, 陽氣), *Angelica* for promoting blood circulation, *Cornus* for raising up vital energy(Yin energy, 陰氣) and storing the goods in body and *Moschus* is for perforating every organ's stuck so that the general circulation can be promoted. Especially in this experiment 2 medicinal stuffs, *Ginseng Radix Rubra* to improve general condition and *Rehmanniae Radix Preparata* which can moist the body, are added in the GJD in order to duplicate the efficiency[15-8].

Test substances were orally dosed once a day for 60 days with orally administration of 20% ethanol 2.5ml/100g body weight twice a day (equivalent to 7.9g ethanol/kg/day). Each of 8 rats per group was selected using body weight at 10 days after acclimatization. Experimental animals were sacrificed after 60 days of continuous oral treatment of test substances with 20% ethanol treatment, and changes on the body weight, liver weight, serum AST, ALT were observed with histopathological changes of hepatic parenchyma. For detecting more detail histopathological changes, percentages of fatty changed regions in hepatic parenchyma and mean diameters of hepatocytes were also calculated as histomorphometry.

As results of 60 days of serial EtOH treatments caused hepatic damages, featuring dramatical decrease of body weight and increases of the liver weight, serum AST, ALT with fatty change-related histopathology of liver including dramatical increase of fatty changed regions and hypertrophy of hepatocytes. However, these changes from EtOH-treatment related hepatotoxicity were clearly reduced by treatment of silymarin and all four different dosages of GJD. Similar inhibition effects on the EtOH-induced hepatic damages were detected between equal dosages of GJD and silymarin. In addition, GJD showed a clear dose-dependent inhibition effects on the EtOH-induced hepatic damages in the present study.

The body weight decrease after EtOH treatments was considered as the results of the direct toxicity of EtOH and or indirect toxicity related to the liver damages, and the changes on the body weight after EtOH administration have been used as a valuable index in the efficacy tests[18-20]. In the present study, the body weight and gains during 60 days of administration periods were dramatically decreased in EtOH control compared to that of intact control as previously[18-20]. However, these decreases of body weight and gains were significantly ($p<0.01$ or $p<0.05$) inhibited by treatment of silymarin and all four different dosages of GJD, in which a clear dose-dependent inhibition on body weight and gain decreases induced by treatment of EtOH. The inhibition of body weight losses by treatment of silymarin and all four different dosages of GJD were considered as an indirect or direct evidence of their efficacy on the EtOH-induced hepatic damages since body weight was considered as a putative indicator of health. Similar inhibition of the body weight decreases were detected between equal dosage of GJD and silymarin.

As results of EtOH treatments, hepatic damages were induced, and the consequent results in severe fatty changes with increases of liver weights and liver-body weight ratio[20-1]. The inhibition of liver weigh increases by silymarin and dose-dependent inhibitions in all four different dosages of GJD were considered as direct evidence of their efficacy on the EtOH-induced hepatic damages, and similar inhibition of the liver weight increases were detected between equal dosage of GJD and silymarin.

AST, formerly known as SGOT, is a mitochondria-bound enzyme. It is found in several body tissues but is especially high in liver and striated muscle. Serum AST activity is elevated with skeletal muscle necrosis and hepatocellular necrosis. Elevated serum AST activity with no ALT elevation indicates muscle necrosis but AST activity rise more slowly than ALT in liver damages and indicates more complete cellular disruption because it leaks from the cell only with necrosis, not membrane instability[22]. ALT, formerly known as SGPT, is present in large quantities in the cytoplasm of hepatocytes. This enzyme enters the blood when liver cells are damaged or destroyed, and circulates for a few days. This enzyme is a sensitive indicator of active live damage but dose not indicates the cause or reversibility of the damage[22]. Damage to the liver after ethanol ingestion is a well-known phenomenon, and the obvious sign of hepatic injury is the leakage of cellular enzymes into plasma[23]. The increased levels of serum enzymes such as AST and ALT have been observed in alcohol administered rats, which indicate the increased permeability, damage and/or necrosis of hepatocytes[24]. The inhibition of serum AST and ALT elevations by treatment of silymarin and all four different dosages of GJD were considered as indirect evidences of their efficacy on the EtOH-induced hepatic damages, and similar inhibition of the serum AST and ALT level increases were detected between equal dosage of GJD and silymarin. GJD gave dose-dependent high hepatoprotective effect by reversing the changes produced by ethanol. The observed decreases in the activities of these enzymes shows that GJD, to some extent, preserves the structural integrity of the liver from the toxic effect of ethanol.

Moreover, alcohol administration produces a spectrum of histological abnormalities in the liver, as described earlier[25]. Liver histology of ethanol administered animal showed pathomorphologic alterations. These changes were predominant in the centrilobular region having reduced oxygen perfusion. Hepatic damage may be partially attributed to cytochrome P450 dependent enzyme activities in liver that tends to be present in greatest concentration near the central vein and lower near the peripheral sites[26]. Treatment with GJD reduced the morphological changes produced by ethanol and greatly reverted the microanatomy of the liver to normal.

V. CONCLUSION

Base on the results, it is, thus, concluded that GJD shows clear hepatoprotective effects on the EtOH-induced hepatic damages. The potency of GJD compares well with equal dose of silymarin with respect to all hepatic markers tested. Hence it merits further development for exploiting it as a therapeutic agent. Multiple mechanisms may interplay in its hepatoprotective effect and further

research on the mechanism of action of GJD is underway. In addition, further investigation is needed to identify which herbs and chemical components of GJD are responsible for these actions.

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