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Raisins: A kitchen cabinet item can restores the liver function and structure

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Abstract: The aqueous methanol extract of raisins (*Vitis vinifera*) was investigated in carbon tetrachloride (CCl_4)-induced hepatotoxic rats model. Where it was found to revert the alteration induced by CCl_4 in liver structure and function by improving the body weights, liver index, liver and bile duct specific enzymes, liver conjugative and synthetic markers, reduced glutathione and the total bilirubin/ albumin ratio while increasing the percent inhibition of lipid peroxidation in test groups treated with extract in doses of 400 and 800 mg/kg body weight as compared to negative control group only treated with CCl_4 3mL/kg that showed entirely opposite picture of all these parameters. Silymarin 100 mg/kg was used as reference hepatoprotective medicine in present study. In addition, histopathological studies of liver tissues of test groups displayed the restoration of liver anatomy. Therefore, raisins' extract proved to have liver protective, regenerative and antioxidant properties. These might reside in total phenolic content particularly in gallic acid and rutin in extract estimated and detected by spectrophotometric and high performance liquid chromatographic methods.

Keywords: Raisins, alanine aminotransferase, bilirubin, gallic acid, rutin.

INTRODUCTION

Liver is the second vital organ (after heart) of the body, plays critical role in excretion of wastes and xenobiotic metabolism (Hassan *et al.*, 2019). In this process, liver exposed to high concentration of toxicants such as food additives, drugs (acetaminophen, antibiotic, chemotherapeutic agents, etc), industrial chemicals and alcohol making it prone to damage (Pan *et al.*, 2019, Gulati *et al.*, 2018). Among chemicals used in industries like vinyl chloride, carbon tetrachloride and different fumigants, all contributing in accelerating acute to chronic liver problems in the world (Jeyadevi *et al.*, 2019, Lin *et al*., 2019), if these are not used with predefined safety precautions. Pakistan ranked at top among the most sufferers of the world in this regard (Majid *et al.*, 2019). Carbon tetrachloride (CCl_4) used as cleaning and defatting agent in industries, continuous inhalation of its fumes will induce the production of reactive metabolites (trichloromethyl; $OCCl_3$ and peroxytrichloromethyl; $OOCCl_3$) after passing through live metabolism that persuade lipid peroxidation and alter the functions of organelles like mitochondria, endoplasmic reticulum, etc, thereby involve in generating more and more reactive oxygen radicals and creating oxidative stress, which can damage each and every tissue of the body including liver (Hashem *et al*., 2019, El-Aarag *et al*., 2019). The main objective to treat such problems is to neutralize oxidative stress by upgrading the efficiency of antioxidant system of the body. In this esteem, functional foods including fruits, dry fruits, vegetables, etc, rich in polyphenol contents are beneficial in scavenging free radicals and minimizing oxidative stress.

Raisin (Urdu: Kismish) is one of the powerful dry fruits belongs to family *Vitaceae*, and Species *Vitis vinifera*. Interestingly, its color and variety depend on the type of grapes dried. These are cheaply available throughout the year, globally attracted and commonly used in cooking salty and sweet dishes (Khiari *et al.*, 2019). Raisins are rich in sugars (glucose, fructose and sucrose), vitamins (ascorbic acid, pyridoxine, riboflavin and thiamin), minerals (potassium, sodium, high magnesium and iron) and trace element boron (Çağındı and Ötleş, 2009, Fulgoni *et al.*, 2018). Beside these, soluble and insoluble fibers, tartaric acid, and polyphenols are the major constituents of raisins (Schuster *et a*l., 2017, Spiller *et al.*, 2003, Karadeniz *et al.*, 2000). Number of pharmacological activities of this dry fruits have been reported so far including antibacterial, antioxidant,

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antifungal, anticarcinogenic, antiinflammatory, antioxidant, antiobesity, cholesterol reducing, tooth and bone protective activities (Abdel-Hamid *et al.,* 2015, Abouzeed *et al.,* 2018, Di Lorenzo *et al.,* 2016, Olmo-Cunillera *et al.,* 2020, Wu, 2009). The present study was designed to investigate the effect of aqueous methanol fruits extract of raisins in reversing the alteration induced by CCl_4 in liver structure and function.

MATERIALS AND METHODS

Experimental dry fruit and preparation of its* aqueous methanol *fruit extract (AqMFEt)

Long golden yellow dry raisins were purchased from local market at Saddar and testified by taxonomist of Department of Botany, University of Karachi. The sample was kept (voucher No.: KU/BCH/SAQ/10) in Department of Biochemistry of same university. 10 g of cut and mashed raisins was repeatedly extracted for quadrate times (40 min per each) with 100 mL of distilled water: methanol (20:80) mixture at 37◦C, filtered through Whatman's filter paper No. 42, shifted into crucible and placed on boiling water bath to evaporate and obtained golden brown residue (18.9±1.34 g/ 100g of starting material) with constant weight and referred as AqMFEt (Azmi *et al.,* 2018).

Quantitative analysis of total phenols in AqMFEt through Spectrophotometric and High performance liquid chromatographic (HPLC) methods

The total phenolic content (g) of AqMFEt was estimated by Folin–Ciocatleu method using standard curve of gallic acid (Lateef and Qureshi, 2014). Secondly, HPLC system (Shimadzu) equipped with a pump of LC -10AC, column of Eclipse plus C18 (Agilent) and UV-Vis spectrophotometric (SPD-6AV) detector. Where 20 µL of extract was injected in developing solvent containing 50 % of each of water and methanol and run with a speed of 0.7 ml/minute at 260 nm. Gallic acid and rutin were purchased from Merck (Germany) and used as reference compounds to detect their presence in AqMFEt. The retention time, peak areas and relative concentrations were noted.

Hepatotoxin, reference drug and dissolving medium for AqMFEt

Carbon tetrachloride (CCl_4; BDH Chemicals, USA) was used as hepatotoxin to induce alteration in liver structure and function of rats. Silymarin (Silliver; Abbot Laboratories Pvt. Ltd., Pakistan) was used as reference hepatoprotective medicine**.** 0.05% DMSO (Dimethyl Sulphoxide; Fisher Scientific, UK) was used as dissolving medium for preparing and administering the doses of AqMFEt in rats of test group.

Experimental rats and protocol

Wistar female albino rats weighing from 180-190 g were bought from breading house of Dow University of Health Sciences (DUHS), Karachi. Experimental rats were kept by giving standard diet and drinking water *ad libitum* in completely hygienic and air conditioned animal house of the Department of Biochemistry, University of Karachi according to the internationally accepted guidelines for animal handling approved by ASRB (Advanced Study and Research Board) of same university. For trial, rats were divided into five groups (6 rats/ group) as normal and negative controls (group I and II), each of them treated with distilled water (1 mL/kg), reference group (III) treated with silymarin (100 mg/kg) and two test groups (group IV and V) treated with AqMFEt in doses of 400 and 800 mg/kg respectively. Each treatment was done once in a day in early morning orally for five consecutive days whereas CCl_4 3mL/kg diluted with olive oil in 1:1 ratio was given through intra-peritoneal injection on 3^{th} and 5^{th} day of trial in groups II, III, IV and V except group I (normal control). After 24 hrs of receiving last dose of CCl_4, rats were decapitated in order to collect blood from neck then serum was separated and used for analyzing biochemical parameters. In addition, livers were dissected out for recording their wet weights, estimating antioxidants parameters and histo-pathological studies (Qureshi *et al.,* 2019).

Physical, biochemical, antioxidant and histo-pathological studies

Physical parameters including percent body weight (PBW) of each group was calculated by formula (given below) after measuring initial and final body weight (IBW and FBW) of each rat with the help of weighing balance on 1^{st} and last day of trial. In addition, livers were also weighed to calculate liver index using following formula (Qureshi *et al.,* 2019).

PBW = [(FBW-IBW)/IBW] x100

Liver index (%) = [Liver weight / Final body weight] x 100

Biochemical markers including alanine transaminase (ALT), aspartate transaminase (AST), gamma glutamyltranspeptidase (GGT), alkaline phosphatase (ALP), total, conjugated and unconjugated bilirubin (TB, CB and UCB), total protein (TP), albumin (ALB), and globulin (G) levels were estimated by using commercially available reagent kits (Randox, UK) on automatic Beckman Coulter AU 480 analyzer. Whereas ratios of ALB/G, TB/ALB and percent increase / decrease in each of biochemical parameter were calculated by authentic formulae (Ardakani *et al.,* 2011, Qureshi *et al.,* 2019).

ALB / G Ratio = ALB /G

TB / ALB Ratio = TB /ALB

Percent increase / decrease in parameter = [At –Ax / Ax] x 100

Where At and Ax are the mean reading of treated group (III/IV/V) and group II respectively.

Liver homogenate was used to estimate the percent inhibition of reduced glutathione (GSH) and lipid peroxidation (LPO) by conducting manual methods (Mudassir *et al.,* 2018). Surgically dissected out liver tissues were soaked in 10% formaldehyde solution separately and used for histo-pathological studies with the assistance of Dr. Essa's diagnostic laboratory, Abul-Hasan Isphahani Road, Karachi. Photographs of the liver tissue slides were captured by using system microscope (Olympus BX51) and digital camera (Olympus DP72).

STATISTICAL ANALYSIS

Data of the study were expressed as mean ± SD (standard deviation) and analyzed through statistical package for social science (SSPS version 17) by using one-way analysis of variance (one-way ANOVA) and LSD (least significant difference) test. Differences between test and control groups found significant at $p<0.0001$, $p<0.001$, $p<0.01$ and $p<0.0.5$.

RESULTS

Spectrophotometric and HPLC analysis of AqMFEt

AqMFEt was found to contain as 0.35g of total phenolic content in term of gallic acid /g of extract. Whereas HPL-chromatogram of AqMFEt displayed the peaks of gallic acid and rutin with retention time (relative concentration) 3.379 (54.912µg) and 9.637 (3.195 µg) min respectively which were identified by comparing peaks of their standards with almost similar retention times (table 1 and fig. 1).

Physical, biochemical, antioxidant and histo-pathological studies

Test groups (IV and V) treated with AqMFEt in doses of 400 and 800 mg/kg produced significant gained ($p<0.000$) in PBW up to +5.49 and +6.97% respectively as compared to group II and III that showed remarkable reduction in weights up to -11.5 and -9.21% (table 2). On the other hand, LI in both test (IV and V) and reference (III) groups decreased than group II (table 2).

Silymarin (100 mg/kg) and AqMFEt (400 and 800mg/kg) were found effective ($p<0.0001$) in reference and test groups by dropping the values of ALT (196±16, 169± 55.99 and 94±27 U/L respectively) up to -94 to -97% as compared to group II administered with CCl_4 showed drastic elevation in the level of same enzyme (table 2). Similarly, AST (646±163, 697±147 and 397±92.5 U/L) levels were also found decreased ($p<0.001$) from -55 to -74% in groups III, IV and V respectively (table 2). In addition, same groups III, IV and V were showed suppressed levels of GGT (1.34±0.61, 1.67±0.50 and 1.45 ±0.99 U/L) and ALT (93±37, 102 ±9.1 and 100 ± 2.1U/L) up to -82 to -86% and -30 to -36% respectively (table 2). Group II showed significant increase in TB, CB and UCB. However, AqMFEt (400 and 800 mg/kg) treated test groups effectively demonstrated the decrease in the values of three of these bilirubins by showing -52 (0.21± 0.01 mg/dL)) to -68 (0.14±0.06 mg/dL) % reduction in TB, -62% in CB in each test group and -35 (0.11 ± 0.0 mg/dL) to -76 (0.04±0.06 mg/dL) % in UCB (table 2). Almost same significant reduction was observed in bilirubin profile in group III (table 2). On the other hand, the levels of TP was found increased more than 30% in groups III (5.81±0.23 mg/dL), IV (6.20±0.05 mg/dL) and V (6.19±0.07 mg/dL) and ALB from 18 to 50% in same three treated groups as 2.5±0.18, 3.12±0.18 and 3.17±0.14 mg/dL respectively in contrast with group II (table 2).

ALB/G and TB/ ALB ratios were vice versa to each other in groups III, IV and V (fig. 2). In the same way, the percent inhibition of GSH and LPO in test groups IV and V were entirely opposite to their inhibition found in group II (fig. 3).

Histo-pathological study of liver tissue of group II clearly displayed severe necrosis with inflammation and fatty deposition (ballooning) around dilated central vein. Less degree of these effects were also observed in liver tissue of group III treated with silymarin (100mg/kg). Whereas these harmful effects were completely vanished in liver tissues of test groups (IV and V) and their structures appeared close to normal group I (fig.4).

DISCUSSION

The detoxification property of liver makes it prone to damage as it is always in direct subjection to xenobiotic metabolism (Bencheikh *et al.*,2019). Liver damaging agents ranging from viruses, xenobiotics, number of medications, environmental toxins, work place chemicals and alcohol ingestion (Ezzat *et al.*,2020).These substances reported to produce free radicals /or reactive oxygen species (ROS) which injured liver structure and altered its function (Yang *et al.*,2019). Emulsification and digestion of food is another function of liver that severely affected in liver dysfunction (Mahmud *et al.,* 2012). Therefore, loss of appetite is the prime indication of acute or chronic liver damage, if it is not treated on time; it will create negative impact on muscle mass and body weight. The hepatotoxin CCl_4 of present study also displayed a drastic decreased in percent body weight of negative control and reference groups. Whereas test groups treated with AqMFEt (400 and 800mg/kg) showed a prominent gained in their body weights.

Another physical parameter liver index (LI) was calculated. High LI is actually the reflection of non-alcoholic fatty liver, metabolic syndrome, steatosis, fibrosis and cirrhosis (Dehnavi *et al.,* 2018). Study illustrate that damage in liver induced structural change in this tissue and increased the expression of transforming growth factor (TGF)-β which enhances the collagen

Table 1: Gallic Acid and Rutin in HPL-Chromatogram of AqMFEt at 260 nm

Gallic Acid	Standard	AqMFEt
Retention time (min)	3.456	3.379
Concentration (µg)	99.921	54.912
Rutin		
Retention time(min)	9.212	9.637
Concentration(µg)	99.608	3.195

Table 2: Effect of AqMFEt on physical and biochemical parameters

	Groups				
	I: Normal control	II: Negative control	III: Reference	IV: AqMFEt 400mg/kg	V: AqMFEt 800mg/kg
Physical parameters					
PBW	2.19 ± 1.41	-11.5 ± 6.87	-9.21 ± 1.10	5.49 ± 2.23[a]	6.97 ± 1.81[a]
LI	4.60 ± 0.89	9.30 ± 1.05	6.87 ± 0.75[a]	5.7 ± 0.16[a]	3.9 ± 0.25[a]
Biochemical parameters					
ALT U/L	47 ± 0.88	3288 ±1899	196 ± 16 [a](-94.03)	169 ± 55.99 [a](-94.8)	94 ± 27 [a](-97.1)
AST U/L	174 ±101.9	1579 ± 794	646 ± 163[a](-59.03)	697 ± 147[a](-55.8)	397 ± 92.5[a](-74.8)
GGT U/L	10 ± 0.00	9.67 ± 6.77	1.34 ± 0.61 [a](-86.1)	1.67 ± 0.50[a] (-82.7)	1.45 ± 0.99 [a](-85.0)
ALP U/L	99 ± 13	147 ± 20.9	93 ± 37 [a](-36.7)	102 ± 9.1 [b](-30.6)	100 ± 2.1 [b](-31.9)
TB mg/dL	0.12 ± 0.00	0.44 ± 0.24	0.22 ± 0.02[b](-50)	0.21 ± 0.01[b](-52.2)	0.14 ± 0.06[a](-68.1)
CB mg/dL	0.1 ± 0.01	0.27 ± 0.16	0.1 ± 0.00[b](-62.9)	0.1 ± 0.01[b](-62.9)	0.1 ± 0.01[b](-62.9)
UCB mg/dL	0.0 ± 0.00	0.17 ± 0.09	0.12 ± 0.05[b](-29.4)	0.11 ± 0.00[b](-35.2)	0.04 ± 0.06[a](-76.4)
TP mg/dL	5.25 ± 0.20	4.45 ± 0.19	5.81 ± 0.23b(+30.5)	6.20 ± 0.05[b](+39.3)	6.19 ± 0.07[b](+39.1)
ALB mg/dL	3.0 ± 0.16	2.11 ± 0.14	2.5 ± 0.18[a](+18.4)	3.12 ± 0.18[a](+47.8)	3.17 ± 0.14[a](+50.2)

Results expressed as mean ± SD (n=6). a & b=$p<0.0001$&$p<0.001$ respectively, when compared with group II. Values within bracket indicated the increase (+) / decrease (-) in parameter.

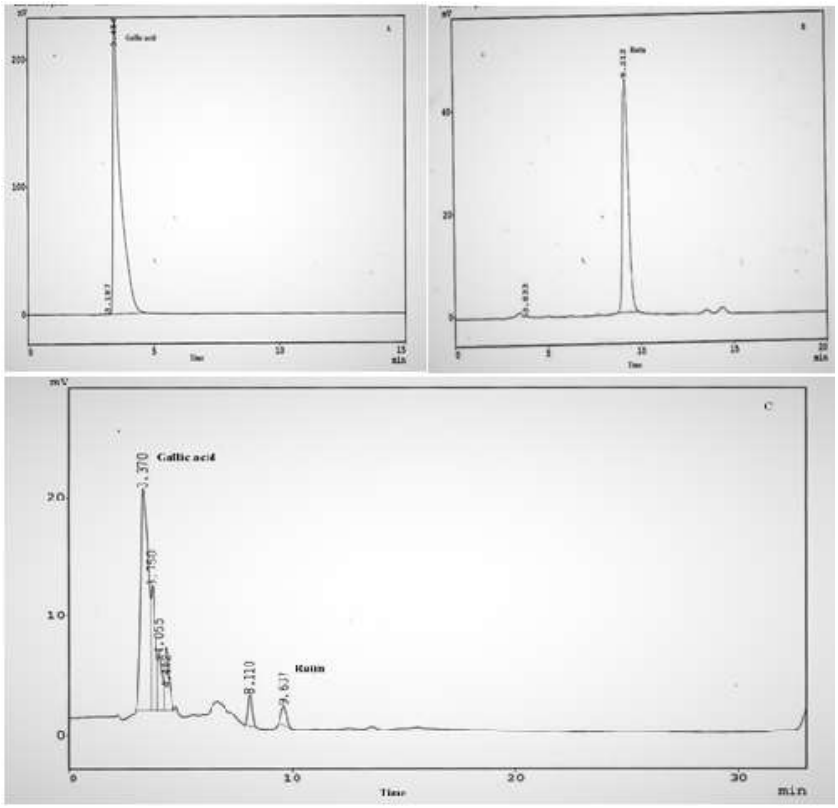

Fig. 1: HPL-chromatograms of standards (gallicacid; A and rutin; B) and AqMFEt (C)

synthesis by activating the hepatic stellate cells *via* Smad pathway that results in the accumulation of collagen fibers in hepatocytes (Lee *et al.*, 2019, Hashem *et al.*, 2019). In addition, steatosis (fatty deposition) in liver was reported in metabolic syndrome (Tanaka *et al.*, 2019). Therefore, accumulation of collagen fibers and fats in liver increase the weight of liver and ofcourse the LI. The high LI was also found in CCl_4 treated group in the present study while it decreased sharply in both test groups even the large dose of AqMFEt 800mg/kg brought its value very close to the value of LI calculated in normal group. Means AqMFEt somehow beneficial in decreasing the content of collagen fibers and fatty deposition in liver, thereby well suited for decreasing liver weight and LI.

Liver specific transaminases ALT and AST found in cytosol and mitochondria of hepatocytes, membrane injury or necrosis releases these enzymes in circulation in high amount (Shibabaw *et al.*, 2019, Jiang *et al.*, 2019). Similarly, elevated level of GGT and ALP in serum reflects the biliary damage (Anand *et al.*, 2019).The same was appeared by observing the increase levels of ALT, AST, ALP and GGT in negative control group while silymarin and both doses of AqMFEt of raisins significantly decreased the levels of these four enzymes in reference and test groups reflecting the hepato-biliary protective effects of this extract by reestablishing the integrity of cell membrane.

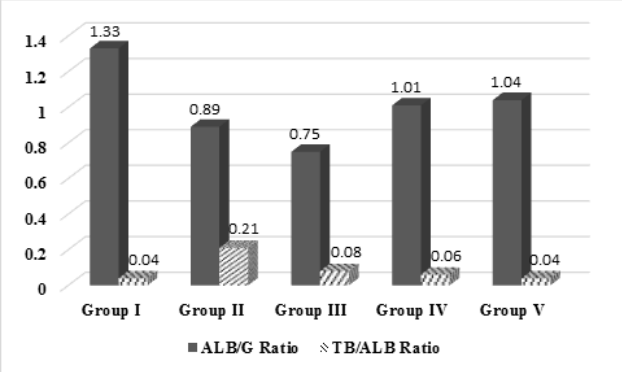

Fig. 2: Effect of AqMFEt on ALB/G and TB/ALB ratio
Each bar represented the mean (n=6). $p< 0.05$, when group IV and V compared with group II

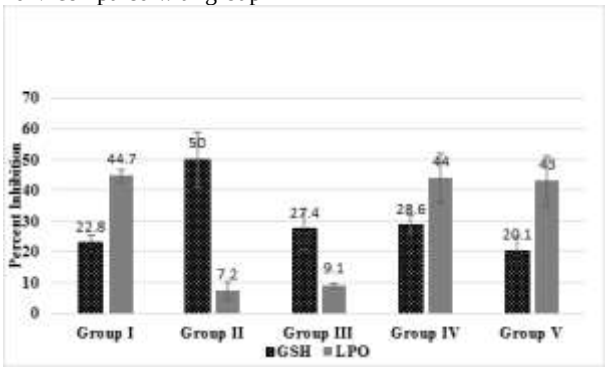

Fig. 3: Effect of AqMFEt on GSH and LPO
Each bar expressed as mean ± SD (n=6). $p< 0.0001$, when group IV and V compared with group II

Liver protective effect of AqMFEt of raisins was more pronounced by observing the betterment in liver conjugative and synthetic abilities in term of bilirubin and protein profiles. Hyperbilirubinemia occurs when there is increased total bilirubin (TB) concentration particularly unconjugated (UCB) one. This situation noticed in severe hemolysis, hepatitis and cirrhosis where conjugation reaction cannot be occurred in liver (Cachón *et al.*, 2017). Liver has unique property to conjugate UCB enzymatically with glucuronide and convert it into conjugated bilirubin (CB) which can easily excreted through bile in duodenum (Hamoud *et al.*, 2018). It only happens when there is ample amount of glucuronide available and liver function properly. Interestingly, raisins contained 60% sugar including glucose and fructose (Olmo-Cunillera *et al.*, 2020) which are very beneficial for producing glucuronide in body required for conjugation reaction. On the other hand, as raisins' extract found to normalize the levels of liver specific enzymes in serum by maintaining the integrity of hepatocytes' membrane, it also helps to accelerate the conjugation reaction smoothly. The same was observed in test groups treated with AqMFEt of raisins which showed 50% reduction in TB and UCB levels as compared to negative control group treated with only CCl_4 demonstrated high amount of these bilirubins.

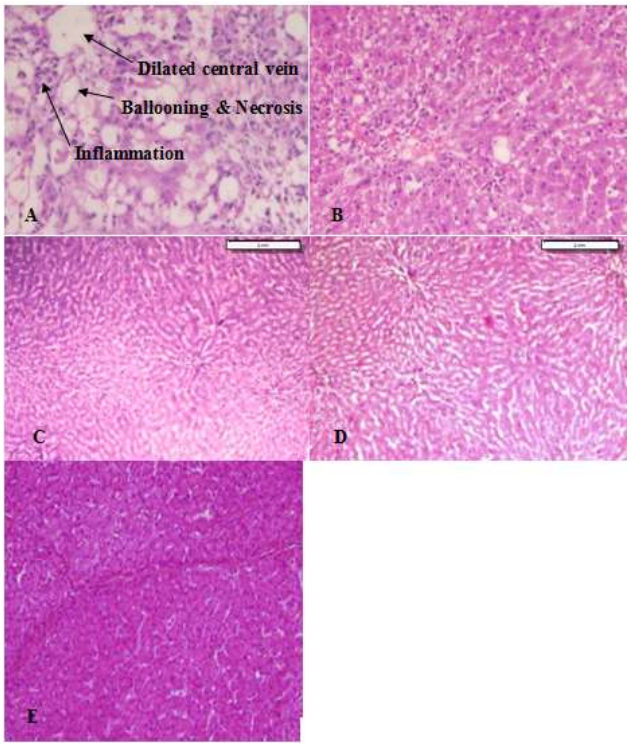

Fig. 4: Effect of AqMFEt on liver tissue. A= Group II (CCl_4 treated) that displayed fatty deposition (ballooning), necrosis and inflammation around central vein. B= Group III (silymarin 100 mg/kg) showed inflammation and ballooning. These features are completely subside in group IV and V (C and D) treated with AqMFEt (400 and 800mg/kg). E = Normal group I (distilled water 1mL).

Similarly, liver synthetic ability was found improved by observing more than 30% increase in TP and ALB concentrations in both test and reference groups especially AqMFEt in a dose of 800mg/kg was found more effective in increasing ALB concentration up to 50% in its respective test group as compared to negative control group that exhibited decrease in levels of TP and ALB. Almost all proteins except immunoglobulin and cent percent albumin synthesized by liver. Therefore depletion of TP and ALB in blood is the finest indicator of loss of liver synthetic ability (Qureshi *et al.,* 2019). However, their decreased levels may also be found in kidney problems where their (TP and ALB) excretion become increased in urine (Zhang *et al.,* 2019). In this esteem, low ALB/G ratio is a red signal of liver dysfunction, inflammation and cirrhosis that tells the amount of globulin is greater than ALB (Aysin *et al.*, 2019). The same was found in CCl_4 treated negative control group whereas this ratio becomes increased in both test groups.

On contrary, TB/ ALB ratio found increased in negative control group. UCB is lipophilic in nature and it can cross lipid bilayers including blood brain barrier, thereby its increase level not only increase TB level and produce jaundice but also create other complications like affecting brain (Mosallam *et al.*, 2019). Whereas, in both test groups, TB/ALB ratio become decreased and proved that AqMFEt of raisins is effective in minimizing liver damaging effect of CCl_4 and its associated complications.

CCl_4 induced oxidative stress suppress antioxidant enzymes and proteins like reduced glutathione (GSH) while elevate lipid peroxidation (LPO) that destabilize the cell membrane (Byun *et al.,* 2018, Qureshi *et al.,* 2019). The same was observed in negative control group while AqMFEt treated test groups showed the decreased in percent inhibition of GSH and increased in percent inhibition of LPO, thereby protecting the body tissues including liver from oxidative stress. This gives the strong evidence of antioxidant potential of AqMFEt of raisins which was also verified by histopathological studies of liver tissues like inflammation, necrosis, ballooning (fatty infiltration) and dilated central vein were observed in CCl_4 treated negative control group (Zamzami *et al.*, 2019). Unfortunately, few of these toxic effects were also observed in liver of reference group treated with silymarin (100mg/kg) while all these effects were completely disappeared in liver tissues of both test groups treated with AqMFEt (400 and 800 mg/kg) and restored the structure of liver compatible to the liver of normal group. In order to evaluate the secret constituent possibly involved in hepatoprotective effect of AqMFEt, the phytochemical analysis of extract was done and found that it contains nice amount of total phenols which was further supported by detecting the presence of gallic acid (non-flavonoid) and rutin (flavonoid) in the same extract through HPLC. Gallic acid and rutin are well reported as antioxidants, antiinflammatory and hepatoprotective in nature (Alam *et al.,* 2017, Farcas *et al.,* 2019, Zakariaa *et al.,* 2019).

CONCLUSION

The AqMFEt of raisins proved to be liver protective and regenerative by normalizing the levels of liver- and bile duct specific enzymes and improving the liver structure, synthetic, conjugative and antioxidant abilities that might resides in its total phenolic content especially gallic acid and rutin.

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