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Research Article
Downregulation of TLR4-NF-κB-p38 MAPK Signalling in Cholestatic Rats Treated with Cranberry Extract

[1]Aysam M. Fayed, [2]Esraa A. Abdalla, [3]Soha A. Hassan, [4]Mohammed A. Hussein and [1]Tamer M. Roshdy

[1]Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Menoufia, Egypt
[2]Department of Medical Laboratories, Faculty of Applied Medical Sciences, October 6 University, Sixth of October City, Egypt
[3]Department of Basic Science, Faculty of Dentistry, October 6 University, Sixth of October City, Egypt
[4]Department of Biochemistry, Faculty of Applied Medical Sciences, October 6 University, Sixth of October City, Egypt

Abstract
Background and Objective: Cholestasis is a liver disease that occurs when bile flow is restricted or blocked. Estrogen-induced cholestasis is marked by a reduction in bile flow and the accumulation of bile acids in the liver as well as liver damage. The aim was to evaluate the hepatoprotective effect on EE-induced cholestasis in rats of Cranberry Water Extract (CWE). **Materials and Methods:** Adult albino rats weighing approximately 150±10 g were divided into six groups of six animals each. As control groups, three groups (I, II and IV) and three experimental groups were used (III, V, VI). **Results:** Oral administration for 15 days of CWE (150 mg kg^{-1} b.wt.) in EE-treated rats (100 µg kg^{-1} 5 days b.wt.) improved serum cholesterol, bile acid and TBIL as well as hepatic SOD and GPx significantly. Also, CWE inhibited ALP, ALT, γ-GT activity as well as levels of TNF-α, NO, MMP-2 and MMP-9 and MDA in comparison with the EE treatment rats. On the other hand, the liver TLR4, NF-κB and p38MAPK gene expression was down regulated group of rats administrated with cranberry extract when compared with the EE-treated rats. CWE's prophylactic action II is more pronounced than prophylactic one. The hepatoprotective effects of cranberry in restoring normal liver functional ability were also supported by histopathological examination of liver tissues. **Conclusion:** The results show clearly that cranberry extract has a strong prophylactic effect in EE-induced cholestasis by normalizing the levels of TLR4, NF-κB and p38MAPK gene expression.

Key words: Cranberry extract, ethinylestradiol, hepatic cholestasis, oxidative stress biomarkers, matrix metalloproteinases, mulberries, kaempferol

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Corresponding Author: Mohammed A. Hussein, Department of Biochemistry, Faculty of Applied Medical Sciences, October 6 University, Sixth of October City, Egypt Tel: 0020124832580

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Depletion of bile flow due to obstruction of bile flow through intra-or extrahepatic bile ducts is defined as cholestasis[1]. The flow of bile is impaired at some point between the liver cells (which produce bile) and the duodenum (the first segment of the small intestine)[2]. Cholestatic reactions tend to be prolonged after the discontinuation of the causative agent, presumably because cholangiocyte repair and regeneration is slower than that of the hepatocyte and because bile secretory function may be slower to recover than other hepatocyte functions[3].

During pregnancy, estrogens administrated woman or woman treated with hormone replacement therapy during postmenopausal are susceptible to intrahepatic cholestasis[4].

A synthetic estrogen: 17α-ethinylestradiol (EE) is widely used to cause experimental cholestasis in rodents to examine molecular mechanisms involved in this disease[5]. EE decreased sinusoidal uptake of bile acids by down-regulating the expression of sodium taurocholate co-transporting protein[6]. At the canalicular level, EE treatment decreased the ATP-dependent taurocholate transport, which was thought to be due to impaired expression of bile salt export pump[7]. In addition, indirect mechanisms also play a crucial role due to estrogens influencing the secretion of pituitary GH that in turn can exert significant effects on liver gene expression concerning xenobiotic, bile acid and lipid metabolisms[8]. There exists a gender-differentiated secretion pattern in all mammals that leads to gender-dependent differences in xenobiotic clearance[9], lipid metabolism[10] and bile acid synthesis and transport[11]. Many herbal products have shown important antioxidant activity (5), which may be important in the treatment of several ill diseases, including cholestasis, in the treatment of medical plants[12]. Polyphenols derived from plants minimise liver injury induced cholestasis[13]. Phenolic compounds have recently received more attention and are one of those found in grapes and mulberries in particular[5,14]. Cranberry is the best source of flavonols[15,16]. Quercetin is the most abundant flavonols in cranberry and it varies from 11 to 25 mg/100 g, primarily as the 3-o-galactoside[17]. Cranberry is also the best source of myricetin, kaempferol[14,15] and 20 different flavonol glycosides[18,19]. No reports about the application of cranberry extract in the treatment of liver cholestasis in rats. As a continuation of our interesting research in pharmaceutical and medical importance of natural products[20-24]. A facile route to assay the preventive and therapeutic potential of cranberry water extract on rat hepatic cholestasis induced by EE was reported in this study.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Biochemistry, Faculty of Applied Medical Sciences, October 6 University, Egypt from January, 2019 to March, 2020).

Materials: Cranberry Water Extract (CWE) was purchased from Virgin Extracts (TM), China. EE and Tween 80 was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the other chemical products used in this study were analytical grade, maintained under standard conditions and supplied by standard commercial suppliers.

Animals: Approximately 150±10 g of female albino rats were purchased from the National Cancer Institute's animal home, University of Cairo, Giza, Egypt. They have been housed in plastic cages with stainless steel coverings. The animals were kept in a light-controlled room at 22±1°C and humidity of 55-60%. The animals were kept for 7 days to acclimatize and provided with a standard diet and water *ad libitum*.

Experimental setup: This experiment studied the prophylactic effect of CWE on liver cholestasis induced by EE. This experiment was carried out in accordance with the National Cancer Institute Animal Care guidelines. Adult female rats of 150±10 g were divided into six groups of six animals each as described in Table 1, according to the method adopted by Hussein[5].

Biochemical assays: Reflotron Plus Analyzer and Roche kits (Forrenstrasse2, 6343 Rotkreuz, Switzerland) were used to determine serum levels of cholesterol, bile acids, alanine aminotransferase (ALT), alkaline phosphatase (ALP), MMP-2, MMP-9 and gamma-glutamyl transpeptidase (µ-GT). The enzyme-linked immunosorbent assays (ELISA) have been used for the quantitative estimation of serum-necrosis factor-alpha tumour (TNF-α). The Griess reaction after quantitative conversion of nitrate to nitrate by reductase nitrate method was used to estimate a stable end product of NO serum nitrate level. In addition, Abnova enzyme-linked immunosorbent (ELISA) kits (Taipei City, Taiwan) was used to estimate MMP-2 and -9, liver superoxide dismutase (SOD), catalase (CAT), GSH, thiobarbituric acid reactive substance-using (ZeptoMetrix) kit.

Reverse-transcription-polymerase chain reaction (RT-PCR). For the RT-PCR procedure, the rats were deeply anaesthetized and transcranial perfused with ice-cold PBS[25]. The livers were quickly removed, the cortical tissues were dissected and the samples were stored at -80°C until analysis. Total RNA was extracted using TRIzol reagents (Invitrogen, Thermo Fisher

Table 1: Description of treatment groups

Groups	Group name	Treatment description
I	Normal control A	Days 1-15: 3 mL of distilled water orally
II	Normal control B	Days 1-15: 3 mL of tween 80, 1% orally
III	CWE (150 mg kg^{-1} b.wt.)	Days 1-15: oral suspension of CWE (150 mg kg^{-1} b.wt.) in tween 80, 1%[5]
IV	EE (100 µg kg^{-1} b.wt.)	Days 1-10: 3 mL of tween 80, 1% orally[23]
		Days 11-15: Subcutaneous injection of 100 µg EE kg^{-1} b.wt., in tween 80, 1% in a single daily dose[23]
V	CWE+EE (prophylactic I)	Days 1-10: Administration of CWE (150 mg kg^{-1} b.wt.) in tween 80, 1% orally
		Days 11-15: Administration of CWE (150 mg kg^{-1} b.wt.) in tween 80, 1% orally and subcutaneous injection of 100 µg EE kg^{-1} b.wt. in tween 80, 1%, in a single daily dose [24]
VI	EE+CWE (prophylactic II)	Days 1-5: Administration of CWE (150 mg kg^{-1} b.wt.) in tween 80, 1% orally and subcutaneous injection of 100 µg EE kg^{-1} b.wt., in tween 80, 1%, in a single daily dose
		Days 6-15: Administration of CWE (150 mg kg^{-1} b.wt.) in tween 80, 1% orally[24]

Scientific, Inc.) and then reverse-transcribed at 42°C for 60 min and at 95°C for 5 min to obtain single-strand cDNA with a Reverse Transcription System (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Single-strand cDNA was amplified using PCR with a 100 µl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, 200 µM dNTPs, 0.5 µM of sense and antisense primers and 2.5 units of Taq DNA polymerase (Promega Corporation). The primer sequences were as follows: Toll like receptor 4 (TLR4): Forward: 5-ATCATCCAGGAAGGCTTCCA-3, Reverse: 5-GCTGCCTCAG CAAGGACTTCT-3. Nuclear factor (NF)-κB: Forward: 5 CATGAAGAGAAGACAC-TGACCATGGAAA3, Reverse: 5 TGGATAGAGGCTAAGTGT AGACACG 3 , p38 mitogen-activated protein kinase (p38MAPK): Forward 5-CGAAATGACCG-GCTACGTGG-3, Reverse:5-CACTTCATCGTAGGTCAGGC-3, Beta-actin: Forward 5 GAGACCTTCAA CACCCCAGC 3, Reverse 5 ATGTCACGCACGATTTCCC 3.

Histological assessment: For the histological study, the liver was sliced and pieces fixed in a 10% formaldehyde solution. The tissues were embedded in paraffin wax. Sections of 5 mL thick were prepared and then stained with hematoxylin and eosin according to the methods of Bancroft and Steven[26].

Statistical analysis: For six different determinations, the results were expressed as Mean±SD. The data were evaluated statistically using With SPSS/18 Software. The statistical significance p-values<0.05 was considered[27].

RESULTS

Table 2 and 3 show the specific cholestatic and liver function biomarkers. EE (100 µg kg^{-1} b.wt.) administration for 5 days led to a significant increase of biochemical marker levels for ALT, ALP, total bilirubin, TBA, γ-GT as well as a level of MMP-2 and -9 while significantly decreased of plasma total cholesterol as compared with the normal control group (p<0.01), indicating acute hepatocyte damage. Also, significant depletion in ALT, ALP, total bilirubin, TBA, γ-GT, MMP-2 and MMP-9 levels as well as elevation cholesterol levels (p<0.01) in pre-and post-treatment of animals with CWE (150 mg kg^{-1} b.wt.) when compared to EE groups (p<0.01).

Table 4 show a significantly (p<0.01) decreased activities of liver antioxidant enzymes, while significantly increased liver malondialdehyde (MDA) as well as serum Nitric Oxide (NO) and tumour necrosis factor-alpha (TNF-α), were observed in the EE-treated rats as compared with the normal control group (p<0.01). Cranberry extract (150 mg kg^{-1} b.wt.) pre-and post-treatment significantly (p<0.01) decreased the liver MDA, TNF-α and NO level as compared to the EE-treated group.

Also in Table 5, liver reduced glutathione (GSH) superoxide dismutase (SOD) and catalase (CAT) are both implicated in the EE-treated rats as compared with the normal control group (p<0.01). Cranberry extract (150 mg kg^{-1} b.wt.) pre-and post-treatment significantly (p<0.01) enhanced liver GSH, SOD and CAT level as compared to the EE-treated group.

Figures 1-3 displayed the elevation of liver Toll like receptor 4 (TLR4), Nuclear factor (NF)-κB and p38 mitogen-activated protein kinase (p38MAPK) gene expression in EE (100 µg kg^{-1} b.wt.)-treated the group of rats compared with the control group. Administration of cranberry extract (150 mg kg^{-1} b.wt.), led to a statistically significant down-regulated of TLR4, NF-κB and p38MAPK gene expression relative to EE-treated group of rats after 5 days (p<0.05). Agarose gel electrophoresis images of TLR4, NF-κB, p38MAPK and β-actin by RT-PCR support the present results Fig. 4.

Table 2: Effect of cranberry extract on serum cholestatic indices in rats treated with ethinylestradiol (EE)

Groups	Treatment description	Cholesterol (U L^{-1})	Total bilirubin (µmol L^{-1})	Bile acids (µmol L^{-1})	ALP (U L^{-1})	ALT (U L^{-1})
I	Normal control A (distilled water-treated) 3 mL kg^{-1}	212.23±19.35^{a}	1.51±0.22^{a}	100.48±10.87^{a}	192.58±8.02^{a}	33.73±3.99^{a}
II	Normal control B (1% tween 80-treated) 3 mL kg^{-1}	207.47±15.09^{a}	1.56±0.24^{a}	105.63±5.82^{a}	191.73±11.78^{a}	35.80±5.68^{a}
III	Cranberry extract (150 mg kg^{-1} b.wt.)	198.40±5.22^{c}	1.53±0.20^{a}	93.07±8.55^{a}	198.78±10.38^{a}	36.95±4.21^{a}
IV	EE (100 µg kg^{-1} b.wt.)	148.67±7.06^{e}	2.16±0.19^{b}	433.74±14.54^{d}	336.48±20.07^{a}	79.05±7.51^{c}
V	Cranberry extract+EE (prophylactic I)	189.37±11.54^{b}	1.65±0.13^{a}	164.19±14.85^{c}	200.41±15.01^{a}	46.53±3.75^{b}
VI	EE+cranberry extract (prophylactic II)	161.31±7.38^{d}	1.83±0.22^{a}	190.44±11.67^{b}	222.04±13.40^{a}	50.17±3.35^{b}

Values are given as mean±SD for groups of six animals each. Data followed by the same letter are not significantly different, Values are statistically significant at *p<0.05. cranberry extract and ethinylestradiol (EE) treated rats were compared with normal control B rats. Experimental groups (5 and 6) were compared with ethinylestradiol (EE) treated rats

Table 3: Effect of cranberry extract on the activity of gamma-glutamyl transpeptidase (γ-GT) as well as a level of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) in serum rats treated with ethinylestradiol (EE)

Groups	Treatment description	γ-GT (U L^{-1})	MMP-2 (ng mL^{-1})	MMP-9 (ng mL^{-1})
I	Normal control A (distilled water-treated) 3 mL kg^{-1}	1.34±0.13^{a}	32.27±4.57^{a}	5.79±0.65^{a}
II	Normal control B (1% tween 80-treated) 3 mL kg^{-1}	1.33±0.10^{a}	29.81±3.81^{a}	5.53±0.55^{a}
III	Cranberry extract (150 mg kg^{-1} b.wt.)	1.30±0.12^{a}	32.18±3.30^{a}	5.83±0.68^{a}
IV	EE (100 µg kg^{-1} b.wt.)	2.61±0.25^{c}	68.55±4.90^{d}	22.16±2.60^{c}
V	Cranberry extract+EE (prophylactic I)	1.53±0.24^{a}	39.88±2.51^{b}	7.42±0.78^{ab}
VI	EE+cranberry extract (prophylactic II)	2.15±0.34^{b}	47.88±4.50^{c}	9.82±0.82^{b}

Values are given as mean±SD for groups of six animals each. Data followed by the same letter are not significantly different, values are statistically significant at *p<0.05

Table 4: Effect of cranberry extract on levels of liver malondialdehyde (MDA) as well as serum nitric oxide (NO) and tumour necrosis factor-alpha (TNF-α) in rats treated with ethinylestradiol (EE)

Groups	Treatment description	Liver MDA (nmol mg^{-1} protein)	Serum NO (µmol L^{-1})	Serum TNF-α (pg mL^{-1})
I	Normal control A (distilled water-treated) 3 mL kg^{-1}	1.78±0.20^{a}	12.60±2.73^{a}	32.18±4.30^{a}
II	Normal control B (1% tween 80-treated) 3 mL kg^{-1}	1.88±0.32^{a}	12.35±3.12^{a}	30.81±3.59^{a}
III	Cranberry extract (150 mg kg^{-1} b.wt.)	1.80±0.19^{a}	14.50±3.97^{a}	31.99±5.14^{a}
IV	EE (100 µg kg^{-1} b.wt.)	2.29±0.43^{a}	45.97±2.65^{d}	59.22±4.71^{d}
V	Cranberry extract+EE (prophylactic I)	1.90±0.21^{a}	19.89±2.45^{b}	38.88±4.12^{b}
VI	EE+cranberry extract (prophylactic II)	1.86±0.34^{a}	23.65±3.09^{c}	41.02±4.45^{c}

Values are given as mean±SD for groups of six animals each. Data followed by the same letter are not significantly different, values are statistically significant at *p<0.05. SOD: One unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1 min mg^{-1} protein and GPx: GSH consumed/min mg protein (µg)

Table 5: Effect of cranberry extract on liver reduced glutathione (GSH) superoxide dismutase (SOD) and catalase (CAT) levels in rats treated with ethinylestradiol (EE)

Groups	Treatment description	GSH (mg g^{-1} tissue)	SOD	CAT	Total protein (mg g^{-1} tissue)
I	Normal control A (distilled water-treated) 3 mL kg^{-1}	5.81±0.60^{a}	12.25±0.81^{a}	55.81±5.22^{a}	99.76±7.56^{a}
II	Normal control B (1% tween 80-treated) 3 mL kg^{-1}	5.64±0.58^{a}	11.78±0.54^{a}	55.26±3.46^{a}	102.08±6.73^{a}
III	Cranberry extract (150 mg kg^{-1} b.wt.)	5.78±0.66^{a}	11.64±1.14^{a}	56.46±4.38^{a}	107.92±7.40^{a}
IV	EE (100 µg kg^{-1} b.wt.)	3.65±0.48^{a}	5.51±042^{c}	30.11±4.00^{d}	62.51±3.11^{d}
V	Cranberry extract+EE (prophylactic I)	4.67±0.52^{a}	9.71±0.53^{a}	50.28±4.45^{b}	96.56±6.87^{b}
VI	EE+cranberry extract (prophylactic II)	4.47±0.34^{a}	8.53±0.87^{b}	44.10±5.41^{c}	81.25±5.25^{c}

Values are given as mean±SD for groups of six animals each. Data followed by the same letter are not significantly different, values are statistically significant at *p<0.05. Liver samples were collected 24 hrs after the last dose administration. Values are given as mean±SD for groups of eight animals each. SOD: One unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1 min mg^{-1} protein and CAT: H_2O_2 utilized/min mg protein (µmol)

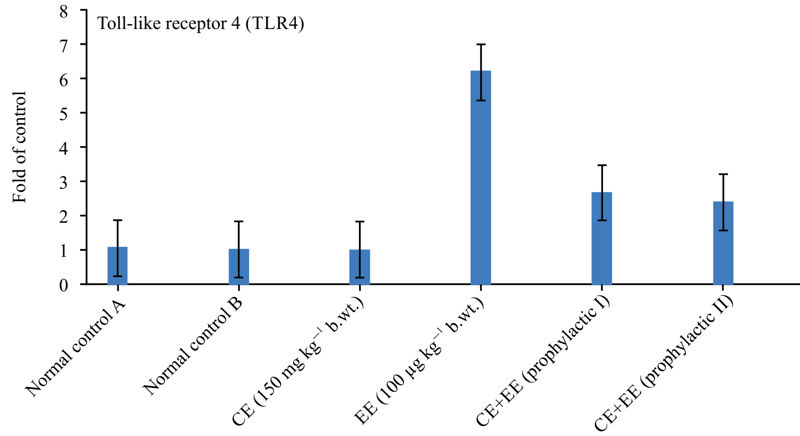

Fig. 1: Effect of cranberry extract (CE) (150 mg kg^{-1} b.wt.) on liver toll-like receptor 4 (TLR4) gene expression in ethinylestradiol (EE) induced liver cholestasis in rats
Representative bar diagram of three independent experiments is presented

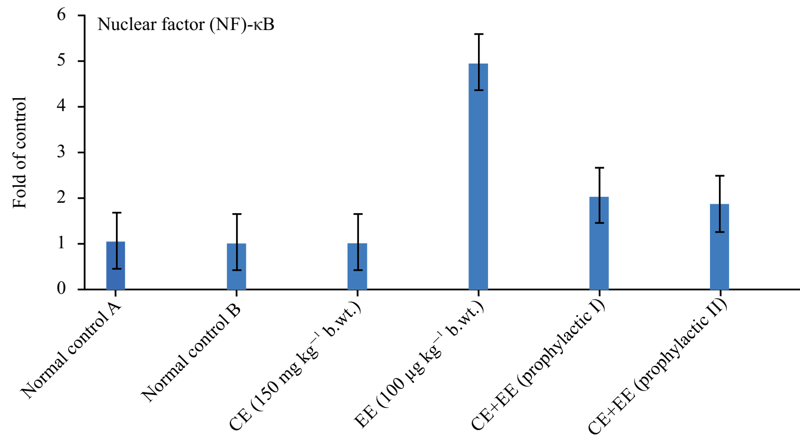

Fig. 2: Effect of cranberry extract (CE) (150 mg kg^{-1} b.wt.) on liver Nuclear factor (NF)-κB gene expression in ethinylestradiol (EE) induced liver cholestasis in rats
Representative bar diagram of three independent experiments is presented

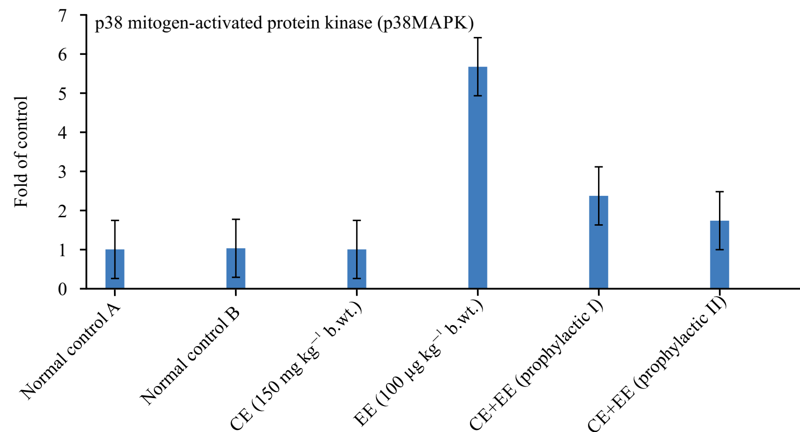

Fig. 3: Effect of cranberry extract (CE) (150 mg kg^{-1} b.wt.) on liver p38 mitogen-activated protein kinase (p38MAPK) gene expression in ethinylestradiol (EE) induced liver cholestasis in rats
Representative bar diagram of three independent experiments is presented

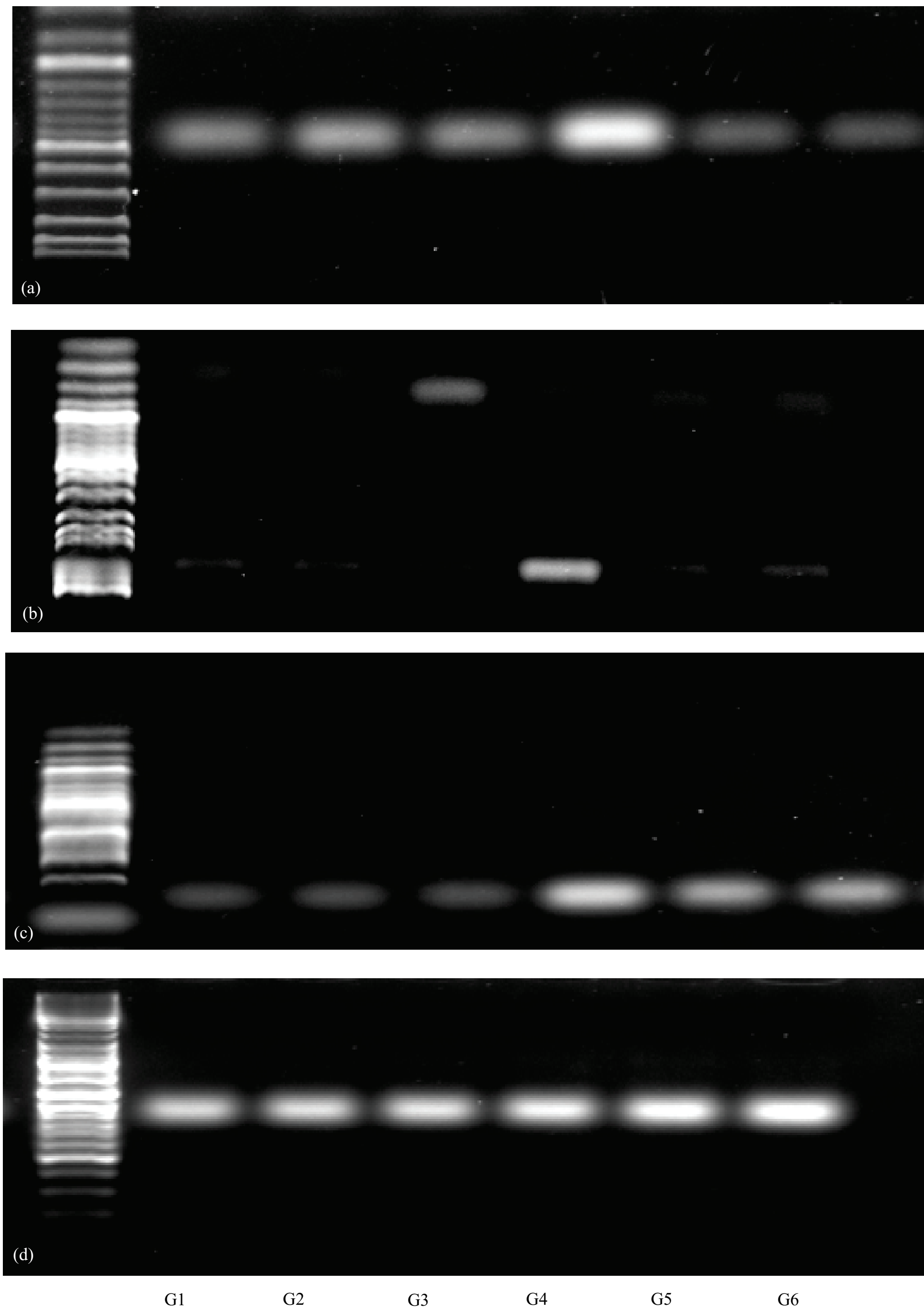

Fig. 4(a-d): An agarose gel electrophoresis shows PCR products of liver in different studied groups
(a) TLR4, (b) NF-κB, (c) p38MAPK, (d) Beta actin and M: DNA marker with 100 bp

Histopathology examination: Gallbladders sections from control as well as and CWE groups had normal histological appearance was consistent with the normal levels of ALT, ALP, bilirubin, bile acid Fig. 5(a-c). Generally, there were no observable changes in the architecture of livers of treated animals compared to the control.

In the group of rats treated with EE, bile canaliculi, necrosis and higher inflammatory cells infiltrate (Fig. 5d).

Also, bile canaliculus and hepatic cell necrosis were significantly reduced in comparison with the EE-treated group pre-and post-treatment of CWE Fig. 5(e-f).

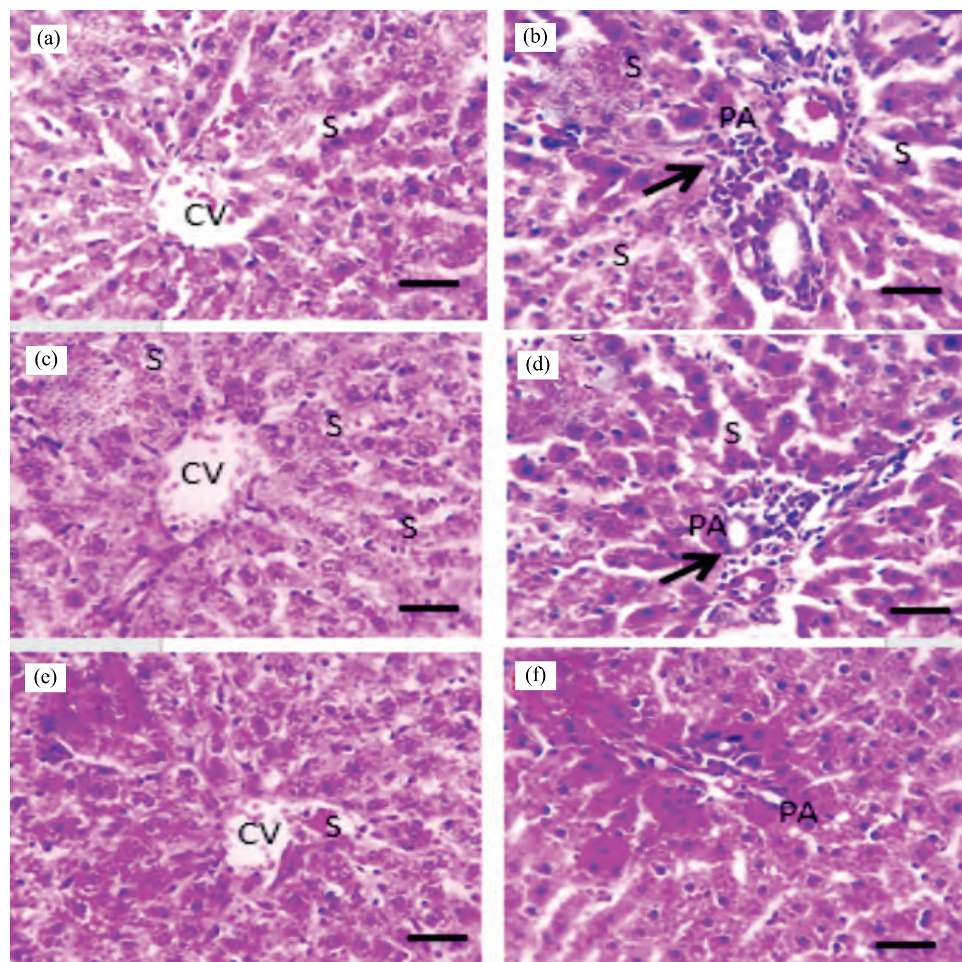

Fig. 5(a-f): Sections stained with hematoxylin and eosin (H and E, 400 X) histological examination of rats liver of different groups compared to normal control groups A and B, (a) Group I: Normal control A, (b) Group II: Normal control B, (c) Group III: Was administrate Cranberry extract (150 mg kg^{-1} b.wt.), (d) Group IV: Was administrate EE (100 µg kg^{-1} b.wt.), (e) Group V: Was administrate Cranberry extract+EE (Prophylactic I) and (f) Group VI: Was administrate EE+cranberry extract (prophylactic II)

CV: Central veins, PA: Portal area and S: Sinusoids in the control group

DISCUSSION

This study showed significant reductions in the levels of serum cholesterol and bile acids and biochemical high markers ALT, ALP, γ-GT, total bilirubin and MDA in EE-treated rats. Estrogens in susceptible women are well known to lead to intrahepatic cholestasis during pregnancy[25]. Liver cholestasis is associated with cell necrosis[24], increases in tissue lipid peroxidation are associated with increased oxidative stress due to MDA, ALT, ALP, γ-GT and bilirubin. The cholestatic activity of EE seems to affect the reduction of canal membrane bile excretory activity[24] and the reduction of bilirubin conjugated to plasma, a diagnostic symptom of obstructive jaundice[28]. EE has been shown to reduce serum cholesterol in combination with a rise in hepatic cholesterol[24,29]. Stimulates the activities of lipoprotein receptors at small densities and increases the lipoprotein linkage to the liver membrane[30] and increase hepatic catabolism of low-density lipoproteins. EE Hypocholesterolemic effect appears to be acting[29]. Also, after EE administration the decline in bile flow due to precipitation may cause the reduction in serum bile acid levels[13,24,29].

The present study showed significant protection against induced decreased serum cholesterol and bile acids through inhibition of squalene monooxygenase, a cholesterol biosynthesis rate-limiting enzyme when orally administered CWE (150 mg kg^{-1} b.wt.) The best source of anthocyanin is also cranberry[30]. Anthocyanin also stimulates the activity of

cholesterol-7α-hydroxylase, which results in the synthesis of bile acids and reduces levels of liver cholesterol[31].

CWE can inhibit TNF-α and NO in the treated EE group, according to the results presented here. Cell proliferation and death as well as gene expressions, such as TNF-α, NO and MDA, are regulated by free radicals[13]. Free radicals, oxidative stress and lipid peroxidation are present in cholestatic damage[24,29]. EE causes oxidative stress as well as increases the production of hepatic MDA and decreases the liver's enzymes antioxidant activity. It has been shown that in chronic cholestasis, the increased intrahepatic concentration of bile acids induces mitochondrial toxicity and free-radical generation[13].

The most widely studied mitogenic and fibrogenic factors are TNF-α, TGF-β1 and interleukin-6. CWE also inhibits the expression of proinflammatory cytokine[15]. Taken together, these results show that the cranberry extract antifibrotic effect is associated with mitogenic or fibrotic signalling blocks. TNF-α induced NO formation has been reported[16]. As a result of these inflammatory and destructive processes, increased NO production is recognized as a significant mediator in physiological and pathology processes[29].

In this study, after EE administration, a significant decrease in hepatic SOD and CAT activity was detected. CWE is also a potent reactive Oxygen Scavenger (ROS) species[24] and has normalised oxidative stress biomarkers (NO, MDA) which result in reduced oxidative stress, which contributes to the suppression of the inflammation of the hepatocytes by EE.

Cranberry containing anthocyanins seems to have a major health-improving property due to antioxidant and anti-inflammatory activity. What makes anthocyanins unique from other polyphenols is that they decrease ROS without inducing mitochondrial biogenesis or dismutase expression of manganese superoxide[30]. The wide effects on the liver and cardiovascular parameters of anthocyanins have to date been reported[31,32]. The intake of anthocyanidins was associated with a statistically significant reduction in cardiovascular risk[33,34].

Anthocyanin has also been confirmed to be able to reduce insulin resistance, improve glycemic control, reduce liver lipid build-up[35,36], reduce inflammatory markers such as (TNF-α)[37] decrease in liver oxidative stress (e.g. stress markers, MDA) as well as lower liver enzyme levels (e.g. ALT and AST)[38]. CWE is also a potent reactive oxygen (ROS) scavenger[39] and normalised by NO and MDA, resulting in lowered oxidation stress, thus helping to suppress hepatocyte inflammation with EE.

In the present study, a significant decrease in hepatic SOD and CAT activity was detected after EE administration. The activities of SOD and catalase (CAT) were changed in EE treatment groups during the experiment by cranberry administration. The GSH concentration was increased in the cranberry group compared to the control. The lipid peroxide, TG and TC levels were ameliorated by cranberry treatment. The results showed that cranberry extract protects the rats from EE-induced oxidative stress[39].

Our results show that the MMP-2 and -9 were involved in the serum and liver in rats exposed to EE (100 μg kg^{-1} b.wt.). Increased MMP-2 levels in the serum are associated with fetal inflammation and preterm labour[29], peripartum cardiomyopathy[35] and pre-eclampsia[40,41]. It was also reported that the secretions of MMP-2 and -9 were significantly suppressed during defective placental development, which leads to intrauterine growth restrictions[42]. In this study, MMP-2 and -9 are up-regulated. Both MMPs do indeed involved in EE-treated rats, which requires further study on disease functions[42,43]. Cholestasis is reportedly the main syndrome of hepatotoxicity[44].

On a related note, CWE containing quercetin has been shown to inhibit MMP-9 activity by several independent reports. Quercetin was reported to inhibit MMP-9 expression in both breast cancer cells and rheumatoid fibroblast-like synoviocytes to suppress their migration and invasion[45].

It is worth noting that cranberry extract therapy may reduce the MMP-2 and MMP-9 levels at both mRNA and protein levels, suggesting that cranberry extract suppression of MMPs might occur as soon as transcription.

Future investigations are needed to reveal the mechanisms underlying the inhibitory effects of cranberry extract on MMP-2 and MMP-9 as well as to identify potential targets of cranberry extract other than these two MMPs.

TLRs serve critical roles in the induction of innate and adaptive immunity[46]. Additionally, these receptors possess leucine-rich repeats in their extracellular region, which are responsible for the recognition of pathogen-associated molecular patterns and endogenous ‘danger’-associated molecular patterns and a Toll IL-1 receptor domain in the intracellular region that is required for the initiation of intracellular signalling[47]. Among the TLR family, TLR4 has been of particular interest because it is the primary receptor recognizing bacterial infections and endogenous ligands released following tissue injury. Endogenous ‘danger signals’, including high mobility group box protein 1 and heat shock proteins activate TLR4 signalling. The activation of TLR4 stimulates κB-α phosphorylation and degradation, which results in the nuclear translocation of NF-κB. Subsequently, NF-κB activation regulates the expression levels of inflammatory genes that are involved in innate immune responses and lead to the initiation of inflammation[46]. The

TLR4 and NF-κB signalling pathways are widely considered to mediate ischemic brain injury processes and to be a promising therapeutic target for ischemic stroke[46,47]. The present study demonstrated that cranberry extract downregulated the expression of TLR4 and inhibited the activation of NF-κB. The low expression of TLR4 following cranberry extract administration could weaken activation of the TLR4/NF-κB signalling pathway and attenuate inflammation, which in turn would reduce the liver cholestasis and inflammation-induced in EE-treated rats.

p38MAPK is an intracellular signalling enzyme[48] and involved in cellular inflammatory response and apoptosis under the condition of stress[49]. Liver inflammation by chastises leads to the activation of p38MAPK and TLR4/NF-κB gene transcription and translation to induce apoptosis, which constitutes a positive feedback path, resulting in impaired liver chastises, amplifying the inflammatory cascade and thus causing the death of liver cells, to achieve cell death. In liver cells, TLR4/NF-κB can induce P38MAPK activation and increase its activity, thereby inducing liver endothelial cell death and stimulating neutrophil function, leading to the increase of TLR4/NF-κB and the accumulation of neutrophils in lung tissue and damage its cells[50]. In our study, we found that cranberry extract significantly down-regulated P38MAPK protein expression in rat with EE induced liver cholestasis group. Rong *et al*. concluded that treatment with quercetin suppresses migration, invasion and metastasis through the p38MAPK signalling pathway in liver tissue[51].

Histopathological studies support the results of biochemistry. Fatty changes, vacuoles, forming space and losing cell boundaries in the liver were shown in EE-treated rats. Hussein[13] reported that marked changes in the liver such as vacuolated hepatocytes and congested sinusoids in EE treated rats. The oral administration of cranberry extract (150 mg kg^{-1} b.wt.) suppressed EE-induced histopathological changes in rats.

Administration of cranberry extract into EE treated rats liver showed normal histoarchitecture[52] reported. In the preliminary phytochemical screening of the cranberry extract, flavonoids like anthocyanins and quercetin have been identified. They can modulate the enzyme activity (SOD and CAT) and the heat reduction TLR4, NF-κB and p38MAPK and have important antihepatotoxic, antiallergic, anti-inflammatory, antiosteoporotic and even anti-tumour and antioxidant activity They can affect the behaviour of many cell systems [20,22]. The present study indicated the post and pre-treatment of Cranberry extract have an effect against liver cholestasis in rats. Downregulation of TLR4-NF-κB-p38 MAPK signalling in EE-treated rats could be explained the anti-cholestatic activity of Cranberry extract to optimize its pharmaceutical importance. Finally, it would appear that the no limitations for using cranberry extract as a protecting agent against EE-induced cholestasis because it is commonly used for the prevention and treatment of other diseases; urinary tract infections (UTIs), renal toxicity, kidney stones and enlarged prostate.

CONCLUSION

The results of this study concluded that Cranberry extract possesses a potent hepatoprotective action upon EE-induced hepatic cholestasis in rats. This may be due to its antioxidative activity with its ability to scavenge free radicals and inhibit lipid peroxidation, all of which are capable of hepatocellular injury.

SIGNIFICANCE STATEMENTS

This study discovers the anti-cholestatic activity of Cranberry extract that can be beneficial for the treatment of liver cholestasis. This study will help the researcher to uncover the critical areas that focus on evaluate of cranberry extract as a promising new agent in the treatment of liver cholestasis that many researchers were not able to explore. Thus, a new theory to explain the correlation between liver cholestasis and TLR4-NF-κB-p38 MAPK signalling may be arrived at.

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