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RESEARCH ARTICLE

Protective Effects of Cinnamon, Cinnamaldehyde and Kaempferol against Acetaminophen-Induced Acute Liver Injury and Apoptosis in Mouse Model

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A B S T R A C T

Liver diseases are among the major health problems in Pakistan. The present study investigated the mechanism of hepatoprotection by cinnamon, cinnamaldehyde and kaempferol in Acetaminophen (APAP)-induced liver injury. Qualitative phytochemical analysis was performed for standardization of cinnamon ethanolic extract. For *in-vivo* evaluation, Balb/c mice were administered with cinnamon extract (200 mg/kg i.g.), cinnamaldehyde (10 mg/kg i.g.) and kaempferol (10 mg/kg i.g.) for 14 days followed by administration of APAP (200 mg/kg i.p.). At the end of trial, mice were dissected, and blood, liver and spleen samples were collected for biochemical, histopathological and apoptotic genes expression analysis. Statistical analysis was performed for significance of results. The results showed that the hepatic damage due to APAP administration for 8 hours in mice was apparent with increased severity. Cinnamon extract, cinnamaldehyde and kaempferol pretreatment suggested ameliorative effects on organ injury induced by APAP by decreasing the elevated serum levels of total proteins and bilirubin. In addition, APAP exerted severe alterations on liver histology without affecting spleen histology alongwith upregulation of Bad, Bax and Caspase-3 and downregulation of Bcl-2. However, cinnamon, cinnamaldehyde and kaempferol pretreatment ameliorated APAP-induced liver alterations and apoptosis, possibly through their antioxidant activity. In addition, cinnamaldehyde and kaempferol possessed comparable protective potential at 20-times less dose as compared to cinnamon extract alone, suggesting therapeutic potential at lower dose in APAP-induced liver injury and apoptosis.

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INTRODUCTION

Hepatic disorders account for increased morbidity and mortality, alleviation of which is challenge to the society. Exposure of xenobiotics including environmental pollutants, alcohol, toxicants (Khan *et al.*, 2009), food additives, viral load and many drugs like aminoglycosides, ibuprofen and acetaminophen (APAP) (Mazer and Perrone, 2008) to the body results in metabolic alkalosis (Hussain and Khan, 2017), brain dysfunction, cardiac arrhythmias and hepatotoxicity (Sirisha *et al.*, 2013). Drug-induced liver injury is responsible for about 50% cases of total liver failure characterized by fibrosis and cirrhosis (Somova *et al.*, 2013). APAP, the widely used antipyretic drug, although is safe at prescribed dosage but an overdose can cause liver injury due to centrilobular apoptosis resulting in acute liver failure. Apoptosis is initiated by the activation of caspases and lysosomal enzymes (Moles *et al.*, 2018). APAP-induced acute liver injury in mice is extensively studied to investigate stress-induced inflammation and acute hepatitis (Yan *et al.*, 2018).

Cinnamon is a common spice generally used in South East Asia. It is accessible in two main forms, i.e., *Cinnamomum cassia* and *C. zeylanicum*, the latter is also named as *C. verum* or 'true cinnamon' (Rao and Gan, 2014). The major constituents of cinnamon include essential oils, cinnamaldehyde, kaempferol, cinnamic acid and little coumarin and based on these phytoconstituents, cinnamon is reported to have antidiabetic (Sahib, 2016),

anti-oxidative (Shahid *et al.*, 2018), antimicrobial, anti-inflammatory (Gunawardena *et al.*, 2015) and anticancer properties (Sadeghi *et al.*, 2019). Cinnamaldehyde, isolated from cinnamon bark (60-70% present in cinnamon bark essential oil) is an aromatic aldehyde described to have therapeutic activities (Xue *et al.*, 2011). Chao *et al.* (2008) reported that low concentrations of cinnamaldehyde could reduce cellular burden of reactive oxygen species by inhibiting TNF-α and interleukin-1β expressions. Another compound present in cinnamon is kaempferol described to have anticancer, anti-inflammatory, antioxidant activities (Wang *et al.*, 2018).

Presently, there are limited experimental studies for evaluating cinnamon phytoconstituents comparatively. The present study was designed to investigate the ameliorative effects of cinnamon, cinnamaldehyde and kaempferol on liver injury and apoptosis due to APAP in experimental mice.

MATERIALS AND METHODS

Cinnamon phytoconstituents and Acetaminophen: Cinnamaldehyde and Kaempferol were purchased from Fluka Research Chemicals and Solarbio Life Sciences respectively. Powdered Acetaminophen (99% purity) was purchased from Sigma Aldrich and reconstituted in water.

Preparation of plant extract: The *C. zeylanicum* bark was identified and deposited in the Herbarium (voucher specimen number 244-1-18), Department of Botany, University of Agriculture, Faisalabad, Pakistan. Plant extract was prepared by macerating 100g powdered sample in 1000 ml of solvent (Ethanol, 80%). Finally, the filtered and dried extract (1:10 w/v) was stored at 4ºC until analysis.

Measurement of reducing power, diphenyl picrylhydrazyl (DPPH)-radical scavenging activity and phytochemical screening of plant extract: The reducing potential and DPPH-radical scavenging activity of the plant extract were determined by following previously described procedures (Wiczkowski *et al*., 2013; Yadav *et al*., 2014). The results were expressed as µg/mL and a percentage of absorbance of a control DPPH solution respectively. The qualitative phytochemical analysis of the extract was performed according to the previously described method (Kala *et al*., 2012).

Animal grouping and drug administration protocol: Healthy adult Balb/c mice (20-23g) were maintained in the animal housing facility at the temperature 25±4°C and relative humidity 55±2%. The experimental mice were provided with local feed, mouse chow diet # 14 (Table 1) *ad libitum* till the completion of trial. After 1 week of acclimatization, mice were divided into 4 groups (n=12); Group I was control group, whereas Group II, III and IV were administered orally with cinnamon bark extract (200 mg/kg), cinnamaldehyde (10 mg/kg) and kaempferol (10 mg/kg) respectively for 14 days. At 15th day of the experiment, each group was further divided into two subgroups (n=6) and exposed to vehicle (normal saline) or APAP (200 mg/kg) intraperitoneally for 8 hours. Then, mice were decapitated and blood and organ samples were collected for analysis.

Blood and tissue collection: Blood samples were collected in clot activator tubes (Gel & Clot Activator, Xinle, China) and centrifuged (80–2 Centrifugal Machine, China) at 1010 x g for 15 min. The serum was collected and stored at -30°C until analysis. The collected liver and spleen samples were placed in freshly prepared buffered formalin solution (10%) for 7 days.

Biochemical parameters: The serum samples were used for evaluation of biochemical markers determined by using microplate spectrophotometer (Thermo Scientific Multiskan GO™ equipped with SkanIt software version 4.1). Serum total protein and albumin were determined by using commercial biochemical kits (Total Protein and Albumin BioRays Lab, Pakistan, Cat#1405TP and Cat#1405AB respectively). Total bilirubin and direct bilirubin concentrations were detected by using commercially available kit; Bilirubin (QCA J-G, Spain, Ref#99 20 93). Lactate dehydrogenase (LDH) was detected by using commercial biochemical kit (LDH Innoline®, Merck (pvt) Ltd, France, Ref#5.17653.0001).

Histopathological analysis: Histopathological analysis of collected tissue samples was performed in accordance with previous method (Arsad *et al*., 2014) using a light microscope (IM-910 IRMECO Gmbh & Co; Germany). The extent of organ injury was evaluated based on pathologic lesions in each section (Mann *et al.*, 2012).

Gene expression analysis of liver tissues: From liver tissue samples, total RNA was extracted by TRIZOL (ThermoFisher Scientific, Massachusetts, USA) method as described previously (Chomczynski and Sacchi, 1987). RevertAid kit (#K1622, ThermoFisher Scientific) and qRT-PCR was performed by using Maxima SYBR Green/ROX Master Mix (#K0221, ThermoFisher Scientific) on C1000 Touch Thermal™ Cycler. Primer sequence is presented in Table 2, provided by Invitrogen, Karlsruhe, Germany.

Table 1: Mice diet composition

Sr. #	Feed constituents	Composition (%)
1	Crude protein	20
2	Crude fiber	4.5
3	Crude Fat	6
4	Total Ash	6
5	*NFE	63.5
6	**Total carbohydrates	63.5
7	***Energy value (kJ/100 g)	1641.5

*NFE: Nitrogen free extracts; $\text{NFE}\ (\%) = 100 - (\text{moisture}\ \% + \text{crude protein}\ \% + \text{crude fat}\ \% + \text{crude fiber}\ \% + \text{ash}\ \%)$

**Total carbohydrates; $\text{Total carbohydrates}\ (\%) = 100 - (\text{crude protein}\ \% + \text{crude fat}\ \% + \text{crude fiber}\ \% + \text{ash}\ \%)$

***Energy value; $\text{Energy value}\ \left(\frac{\text{kJ}}{100\text{g}}\right) = (17 \times \text{protein}) + (37 \times \text{fat}) + (17 \times \text{carbohydrate})$

Statistical analysis: Data were assessed statistically by applying one-way ANOVA and DMR post hoc test and expressed as Mean±SE. SPSS software (version 21.0) was used with level of significance 5%.

RESULTS

Qualitative and quantitative phytochemical analysis of cinnamon: Cinnamon extract was exposed to phytochemical investigation for identification and recognition of phytoconstituents of cinnamon. For assessing the fundamental role of phytoconstituents in quality characteristics, cinnamon ethanolic extract was analyzed for antioxidant assays by using reducing power and DPPH assays as shown in Table 3. Antioxidant ability of the extract was expressed as IC_{50} (µg/mL) and the results showed IC_{50} values for cinnamon ethanolic extract was 89.03 as compared to that of ascorbic acid standard 53.94. Overall, cinnamon has potent free radical scavenging potential. Cinnamon ethanolic extract was exposed to phytochemical investigation qualitatively to identify phytoconstituents. The findings of chemical tests for identification and recognition of phytoconstituents in cinnamon ethanolic extract showed the presence of flavonoids, phenols, saponins, fats and fixed oils, carbohydrates and terpenoids, while alkaloids, tannins and glycosides were absent.

Cinnamon, cinnamaldehyde and kaempferol protect mice from APAP-induced elevated protein profile: Results of protein profile showed significant increase ($P<0.05$) in total protein, albumin, globulin levels and albumin to globulin ratio after 8 hours of APAP exposure. Cinnamon, cinnamaldehyde and kaempferol pretreatment restored APAP-dependent elevations in proteins levels (Fig. 1).

Cinnamon, cinnamaldehyde and kaempferol effects on total bilirubin levels: Results of serum bilirubin showed significant increase in bilirubin levels after 8 hours of APAP exposure. Pretreatment with cinnamon, cinnamaldehyde or kaempferol restored APAP-dependent elevations in bilirubin levels (Fig. 2A, 2B, 2C). Results of serum LDH levels showed significant increase ($P<0.05$) after 8 hours of APAP exposure. The pretreatment of mice with cinnamon, cinnamaldehyde or kaempferol restored APAP-dependent elevations in LDH level (Fig. 2D).

Effects of cinnamon, cinnamaldehyde and kaempferol on liver and spleen histopathology: To determine the severity of hepatic damage by APAP, histopathological examination of the liver was performed which indicated liver injury and marked degenerative changes at 8 hours APAP-post exposure. These results reveal that APAP administration induces severe liver injury. Histopathological examination showed that cinnamon pretreatment reduced the severity of liver injury. The degenerative changes at 8 hours APAP exposure were also attenuated by pretreatment of cinnamaldehyde or kaempferol (Fig. 3). Histopathological examination of mouse spleen exhibited little histological disorganization following 8 hours APAP exposure. Furthermore, the histopathological investigation of spleen sections did not show significant vascular alterations or necrotic lymphocytes in APAP exposure mice group. Cinnamon or its phytoconstituents pretreated mice group showed no significant inflammatory changes. Pretreated groups showed normal splenic architecture with minimized lymphoid follicles and normal sinuses (Fig. 4).

Effects of pretreatment on intrinsic and extrinsic apoptotic factors in mice with APAP-induced liver injury: The contribution of pro-apoptotic and apoptotic markers during APAP-induced cell injury was studied to focus the role of pretreatment on apoptotic signaling pathway. Marker proteins, such as Bad, Bax and Bcl-2, of intrinsic apoptosis and caspase-3, of extrinsic apoptosis were detected. Pretreatment with cinnamon, cinnamaldehyde and kaempferol suppressed the increased tissue Bax expressions, while increasing the expressions of Bcl-2. The mice group treated with APAP only did not show much increase in Bad levels in liver tissues. On the other hand, CE+APAP group mice expressed higher levels of Bad comparatively. However, pretreatment with cinnamaldehyde or kaempferol resulted in remarkable decrease in this level as shown in Fig. 5A, 5B and 5C. The increase in pro-apoptotic protein (Bax) and decrease in anti-apoptotic protein (Bcl-2) by APAP activated the caspase cleavage to form cas-3. The levels of cas-3 was significantly increased in mice exposed with APAP, whereas pretreatment with cinnamon, cinnamaldehyde and kaempferol suppressed these increased levels as shown in Fig. 5D. Overall, these results confirmed that pretreatment with cinnamon or its phytoconstituents inhibits the protein expression of cas-3 in mice which may be due to downregulation of Bax and upregulation of Bcl-2 signaling pathway.

Table 2: Sequence of primers (oligonucleotides)

Gene	Forward primer (5' - 3')	Reverse primer (5' - 3')
Bad	TTGATCTAGGGCATGTCGGG	TGGACACTGAGAGAGGACGG
Bax	CATCGCACTCACCCCTGTAG	ATCCATCCCCCTGACTCTCC
Bcl-2	GCTACCGTCGTGACTTCGC	CCCCACCGAACTCAAAGAAGG
Cas-3	CTCGCTCTGGTACGGATGTG	TCCCATAAATGACCCCTTCATCA
β-actin*	GGCTATAGTCACCTCGGGGC	GTAATAATGCGGCCGGTCTG

* Housekeeping/reference gene for qRT-PCR data analysis.

Table 3: Cinnamon ethanolic extract reducing power and % inhibition of DPPH *in-vitro* analysis

Concentration (µg/mL)	Reducing power		% inhibition of DPPH	
	Ascorbic acid std. (Mean±SE)*	Cinnamon ethanolic extract (Mean±SE)*	Ascorbic acid std. (Mean±SE)*	Cinnamon ethanolic extract (Mean±SE)*
10	0.278±0.008	0.155±0.001	28.87±2.18	27.88±2.56
50	0.347±0.003	0.275±0.055	56.75±1.86	46.90±5.42
100	0.457±0.002	0.378±0.012	69.56±2.56	60.46±4.42
200	0.877±0.051	0.519±0.041	79.67±2.22	65.48±2.05

* Average of 3 replicates, expressed as Mean ± SE.

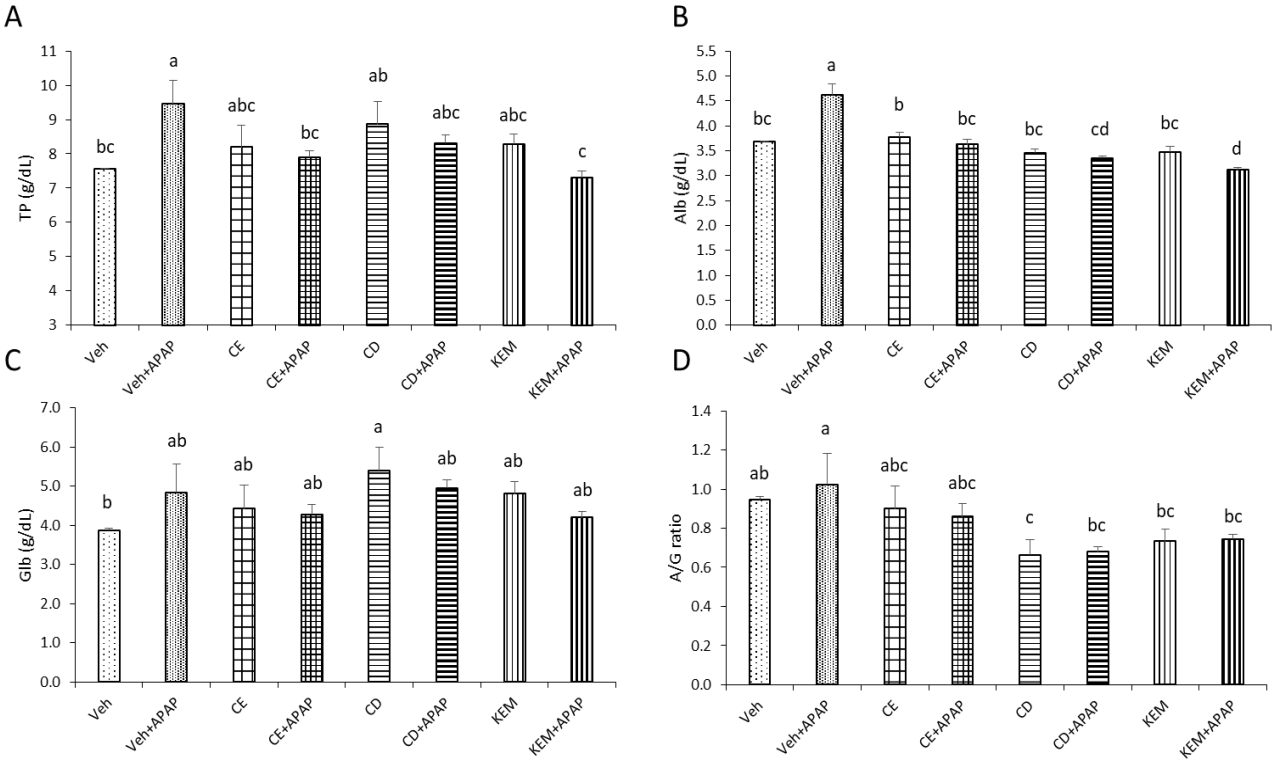

Fig. 1: Effects of cinnamon, cinnamaldehyde and kaempferol on APAP-induced elevated serum protein profile. Pretreatment with cinnamon ethanolic extract, cinnamaldehyde and kaempferol for 14 days protected mice from elevated protein levels due to APAP exposure at 0 and 8 hours as assessed by measuring serum (A) Total protein (B) Albumin (C) Globulin and (D) Albumin to Globulin ratio. The groups with different alphabets are different significantly. APAP, acetaminophen; CD, cinnamaldehyde; CE, cinnamon ethanolic extract; KEM, kaempferol; Veh, vehicle.

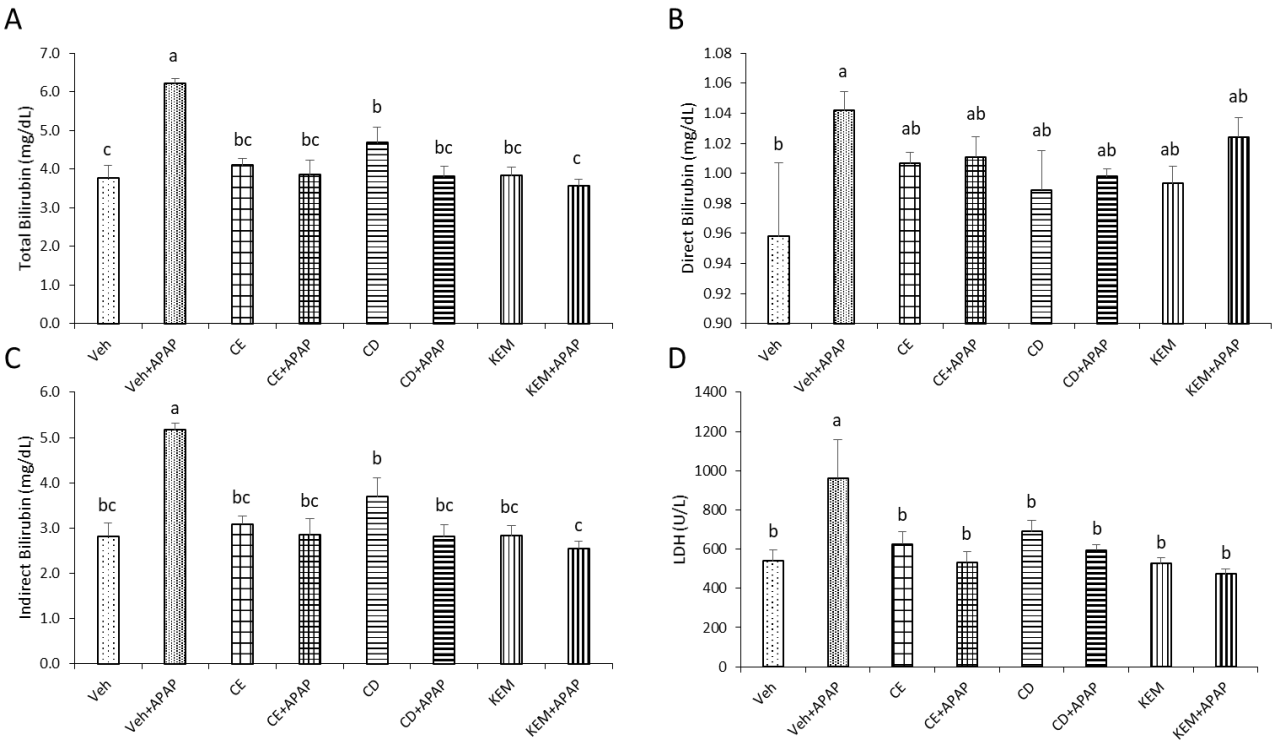

Fig. 2: Effects of cinnamon, cinnamaldehyde and kaempferol on APAP-induced elevated serum bilirubin and LDH levels. Pretreatment with cinnamon ethanolic extract, cinnamaldehyde and kaempferol for 14 days protected mice from elevated bilirubin and enzymatic levels due to APAP exposure at 0 and 8 hours as assessed by measuring serum (A) Total bilirubin (B) Direct bilirubin (C) Indirect bilirubin and (D) LDH levels. The groups with different alphabets are different significantly. APAP, acetaminophen; CD, cinnamaldehyde; CE, cinnamon ethanolic extract; KEM, kaempferol; Veh, vehicle.

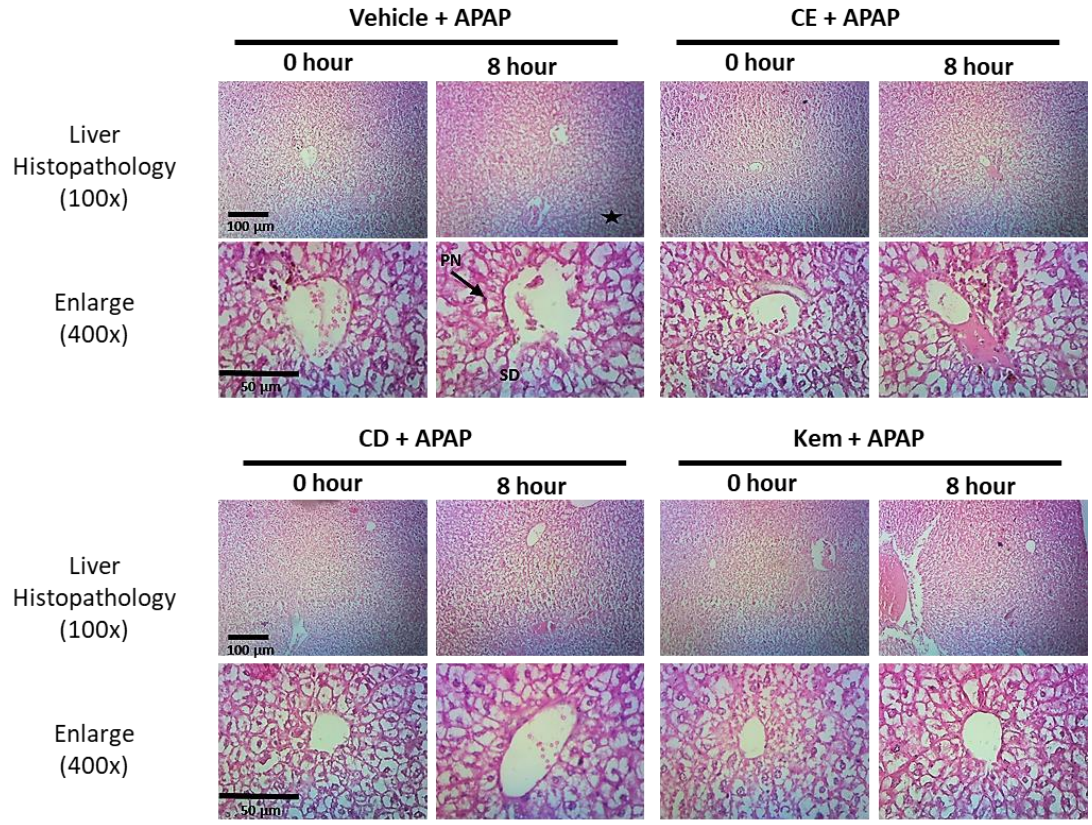

Fig. 3: Effects of cinnamon, cinnamaldehyde and kaempferol on APAP-induced altered liver histopathology. Pretreatment with cinnamon ethanolic extract, cinnamaldehyde and kaempferol for 14 days followed by 0 and 8 hours APAP exposure affected the liver histopathology (H and E stain, 100x and 400x). APAP, acetaminophen; CD, cinnamaldehyde; CE, cinnamon ethanolic extract; KEM, kaempferol; PN, pyknotic nuclei; SD, sinusoidal dilatation; Star★, cellular infiltration.

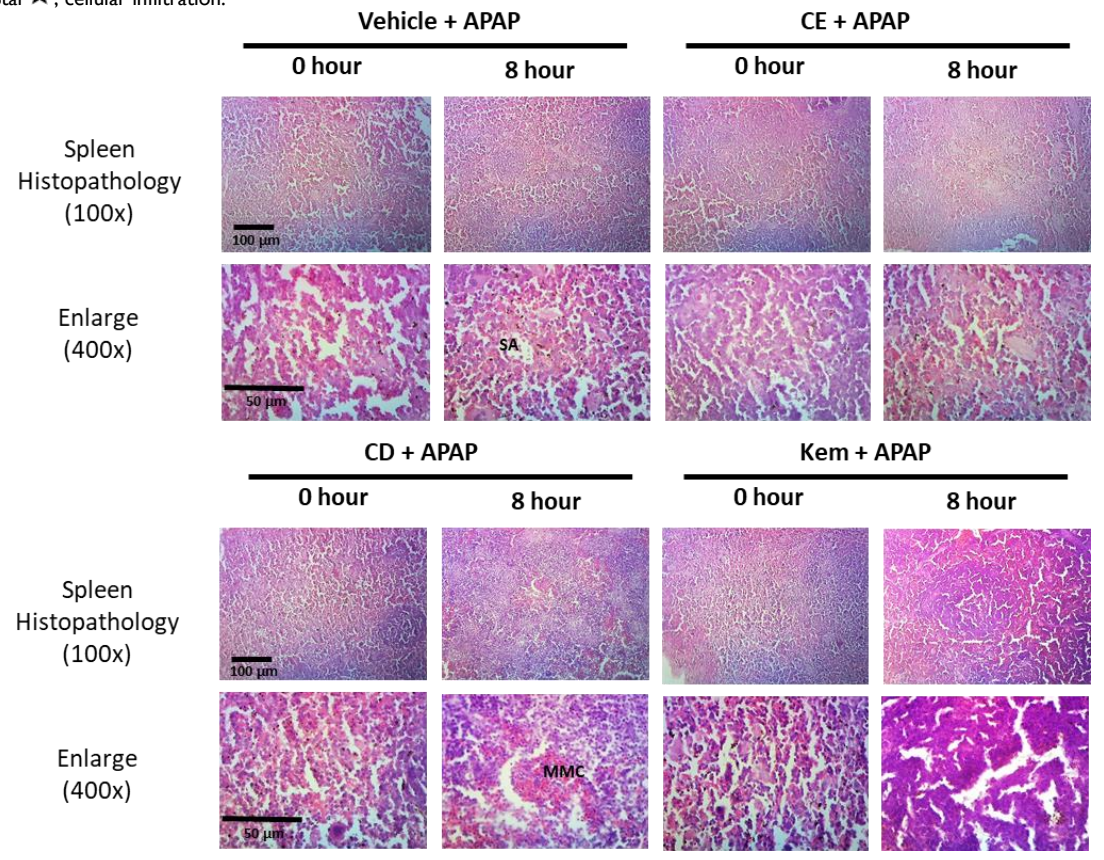

Fig. 4: Effects of cinnamon, cinnamaldehyde and kaempferol on APAP-induced spleen histopathology. Pretreatment with cinnamon ethanolic extract, cinnamaldehyde and kaempferol for 14 days followed by 0 and 8 hours APAP exposure did not affect photomicrographs of spleen tissue sections (H and E stain, 100x and 400x). APAP, acetaminophen; CD, cinnamaldehyde; CE, cinnamon ethanolic extract; KEM, kaempferol; MMC, melanomacrophage centre; SA, splenic arteriole.

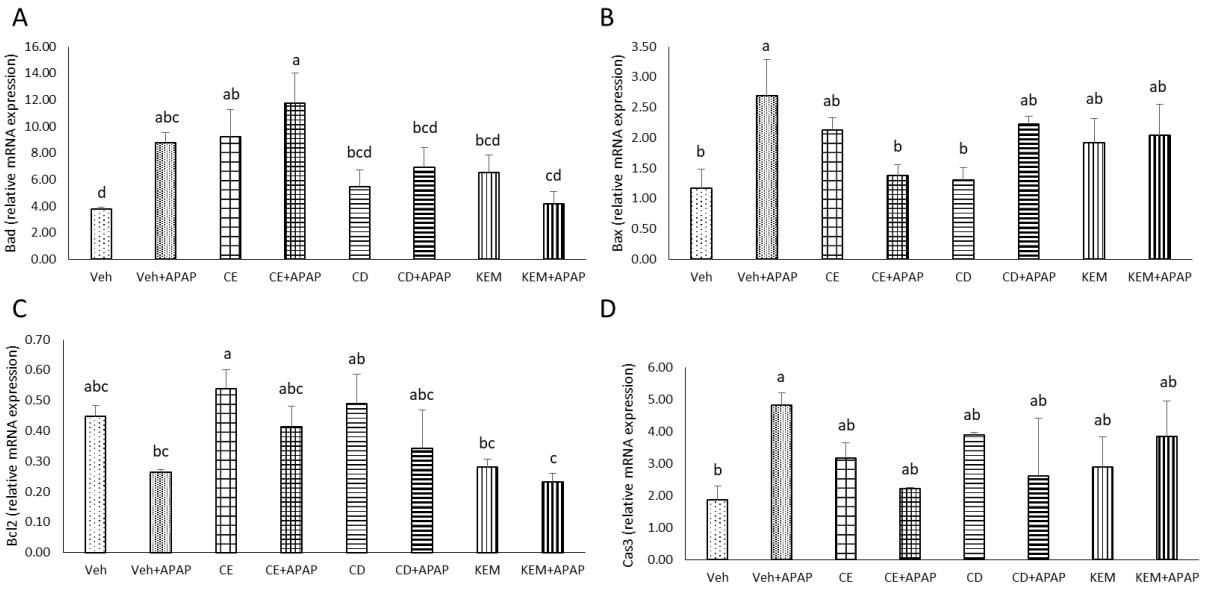

Fig. 5: Suppression of apoptotic factors with pretreatment of cinnamon, cinnamaldehyde and kaempferol. Pretreatment with cinnamon ethanolic extract, cinnamaldehyde and kaempferol for 14 days followed by 0 and 8 hours APAP exposure affected the apoptotic factors measured in liver tissues including (A) Bad (B) Bax (C) Bcl-2 and (D) Cas-3. The groups with different alphabets are different significantly. APAP, acetaminophen; CD, cinnamaldehyde; CE, cinnamon ethanolic extract; KEM, kaempferol; Veh, vehicle.

DISCUSSION

Cinnamon plant has got significant attention for the phytochemical investigations, though there is little evidence about the isolation and pharmacological characterization of cinnamon phytoconstituents. In this study, we demonstrated the phytochemical and pharmacological properties of cinnamon and its phytoconstituents; cinnamaldehyde and kaempferol. We found that the protective effects of cinnamon bark extract, cinnamaldehyde and kaempferol against APAP-induced liver injury and apoptosis involve antioxidant and anti-apoptotic mechanism.

Phytoconstituents obtained from plants differ in structure, but with similar antioxidant and curative potential act synergistically (Kroes, 2014). In the present study, qualitative phytochemical tests of cinnamon extract indicated the presence of major phytochemicals except that of alkaloids, tannins and glycosides. From results of reducing power and DPPH data, we established the antioxidant potential of cinnamon ethanolic extract. Further, DPPH results were in consistent with Abeysekera *et al*. (2017), who described that ethanolic extract of cinnamon bark possess more (107.69±2.01%) free radical scavenging activity (mg Trolox equivalents/g of cinnamon) as compared to that of methanolic extract (60.49±0.48%) free radical scavenging activity (mg Trolox equivalents/g of cinnamon). We found that cinnamon ethanolic extract possess antioxidant potential comparable with ascorbic acid standard.

From results of *in-vivo* evaluation of cinnamon, cinnamaldehyde and kaempferol, we found the hepatic metabolic function markers (proteins, bilirubin and LDH synthesis) synchronize with the histopathological alterations in the liver without significant affecting the spleen histology in the APAP and phytoconstituents treated mice groups. Our study revealed that hepatic metabolic function was significantly affected by APAP overdose as indicated from elevated total proteins, albumin and bilirubin as well as LDH enzymatic activity in serum. The significant rise in total protein levels after IP administration of 200 mg/kg APAP dose was similar to that of previous study (Hussain *et al.*, 2019). The increased biochemical levels in the serum due to APAP exposure was ameliorated with the pretreatment of cinnamon, cinnamaldehyde and kaempferol. Bilirubin is a metabolic hemoglobin product which is transported to the liver and conjugated with glucuronic acid and secreted through bile. All these mediators are the biomarkers of liver injury in experimental animal models. The elevation of these mediators specifies increased hepatocellular damage followed by increased hepatocytic membrane permeability. Pretreatment of cinnamon, cinnamaldehyde and kaempferol followed by APAP exposure significantly lowered the levels of these biomarkers equivalent to that of vehicle control group. This effect is similar to that of previous report that serum biochemical levels become normalize after healing of hepatic parenchyma and regeneration of hepatocytes (Khalid *et al.*, 2018).

Histopathological analysis of liver showed significant vascular and inflammatory changes along with sinusoidal dilatation which were similar to that of previous studies (Hussain *et al.*, 2018; Weng *et al*., 2019). After pretreatment of cinnamon, cinnamaldehyde or kaempferol, the severity of liver injury was prevented. To analyze the splenic status, we conducted histopathological analysis of spleen from each mice group. Analysis of spleen showed no significant histopathological change or any vascular injury in APAP-treated mice group compared with the pretreated mice. A search of literature revealed no previous reports of APAP-induced splenic damage but the lesion in this tissue was detected at 600 mg/kg dosage of APAP in 4-8 hours after dosing (Placke *et al*., 1987). In the current study, no significant APAP-associated inflammatory changes in splenic architecture was observed which could have suggested toxicity.

In order to focus on the mechanistic pathways altered by APAP exposure and the signaling events that play an important role in liver injury, the expression of apoptotic markers was analyzed by qRT-PCR. The level of apoptotic mediators was increased in APAP-treated mice group. The protective effect of cinnamon, cinnamaldehyde and kaempferol may be connected with the reduction in levels of apoptotic mediators (Bax and Cas-3). In this way, the modulatory function of cinnamon, cinnamaldehyde and kaempferol in apoptosis was reflected in the downregulation of apoptotic mediators raised in APAP exposure. It is hard to elucidate the obvious mechanism of APAP-induced acute liver injury owing to its diversity and complexity. Overall, these results indicate that cinnamon and its phytoconstituents; cinnamaldehyde and kaempferol presented protective potential against APAP-induced liver injury as observed from measuring serum markers as well as histopathological and gene expression analysis of the liver. The assessment of phytochemicals-related side-effects requires further study that should progress for the exploration of molecular pathways involved in the pathogenesis of complications arising from drug induced liver injury.

Conclusions: The results revealed that cinnamon ethanolic extract has potent antioxidant activity attributable to the presence of phytoconstituents. APAP administration to mice induced acute liver injury as documented by hepatic biochemical (total proteins, bilirubin and LDH) and histopathological data. Pretreatment with cinnamon, cinnamaldehyde and kaempferol ameliorated the APAP-induced acute liver injury. The mechanism of this protection could be associated with the anti-apoptotic actions of cinnamon, cinnamaldehyde and kaempferol. It is noteworthy that both cinnamaldehyde and kaempferol possessed comparable antioxidant and anti-apoptotic potential at 20-times less dose than that of cinnamon ethanolic extract.

Declarations

Ethics approval and consent to participate: This work did not involve any human data. The study was approved by Institutional Biosafety and Bioethics Committee (IBC) by Permission No. 504/ORIC and Synopsis Scrutiny Committee by Permission No: DGS/533-36, University of Agriculture, Faisalabad, Pakistan. The animals in the present study were cared for and treated in accordance with the Guidelines of National Biosafety Committee 2005, Punjab Biosafety Rules 2014 and Punjab Animal Health Act 2019.

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Authors contribution: Conceptualization, ZH and JAK; Formal analysis, JAK, FM, and RZA; Investigation, ZH and JAK; Methodology, ZH, JAK, MIA and RZA; Project administration, ZH, JAK MIA; Resources, JAK, MIA, FM and RZA; Software, ZH, and RZA; Supervision, JAK; Validation, FM and RZA; Visualization, JAK and FM; Writing – original draft, ZH and JAK; Writing – review & editing, ZH, JAK, MIA, FM, and RZA.

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