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Ameliorative effects of ethanolic constituents of Bangladeshi propolis against tetracycline-induced hepatic and renal toxicity in rats

Short running title: Propolis protects hepatonephrotoxicity

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Abstract

The study reports the phenolic composition of propolis from Bangladesh and its ameliorative effects against tetracycline-induced hepatonephrotoxicity in rats. Male Wistar Albino rats (n=18) were randomly divided into three following groups: (1) normal control (2) tetracycline-treatment (200 mg/kg/rat) and (3) tetracycline (200 mg/kg/rat) + propolis (100 mg/kg/rat) treatments. The ethanolic extract of propolis contained major phenolic acids as well as a flavonoid, rutin. Oral exposure to tetracycline caused severe hepatic and renal damage as indicated by significant alterations in liver marker enzymes in rat serum: bilirubin and protein concentrations, lipid profile and markers of kidney function when compared with controls. The observed biochemical perturbations were accompanied by histopathological changes. Co-administration with propolis extract, however, prevented the changes in

biochemical parameters, as revealed by maintenance of cell membrane integrity and regulation of lipid profile and the conservation of the histoarchitecture.

Practical application

Propolis is a resinous honeybee product which is becoming increasingly popular due to its potential contributions to human health. The phenolic compounds identified in propolis from Bangladesh were effective against tetracycline-induced hepatic and renal toxicity. Propolis may be a promising natural product in reducing the effects of chronic liver and kidney damage.

Keywords: Propolis, Bangladesh, phenolic compounds, tetracycline, hepatonephrotoxicity, HPLC

1. Introduction

Tetracyclines (TTC) are a well-recognised antibiotic family with a broad spectrum of antibacterial activities (Yin et al., 2006). TTC have been prescribed for the treatment of a wide range of bacterial infections since the 1950s (Eliopoulos et al., 2003). They are also used against intracellular chlamydial infections, mycoplasmas, rickettsiae, protozoan parasites and others non-infectious conditions. Bacterial resistance to TTC has, however, limited their effectiveness in human and veterinarian therapy (Eliopoulos et al., 2003). In human, TTC may induce severe microvesicular steatosis in liver, which may life-threatening (Yin et al., 2006). Overexposure to TTC at high blood concentrations can lead to life-threatening adverse effects, including liver and kidney injury (Shabana et al., 2012, Abdel-Daim and Ghazy, 2015, Yin et al., 2006). Microvesicular steatosis, oxidative tissue injury, and impaired mitochondrial functions are the possible contributing factors for TTC-induced hepatic and renal toxicity (Abdel-Daim and Ghazy, 2015, Yin et al., 2006, Asha et al., 2007).

Propolis is a generic name given for a resinous, gummy and balsamic product from honeybees (Toreti et al., 2013). Bees (*Apis mellifera*) collect resin from plant bark and buds and enrich the resin with salivary secretions, wax and pollen (Oliveira et al., 2010). Since

ancient times, propolis has been popular as a traditional medicine, especially in Asia, Eastern Europe and South America, used for its reported broad spectrum of biological activities (Burdock, 1998, Schmidt et al., 2014). The natural mastic and its extracts have numerous applications in the treatment of various diseases due to its described antimicrobial, antiseptic, antimycotic, antioxidative, antitumor, anti-inflammatory, cytotoxic, anti-ulcer, and immunomodulatory activities as well as its cardioprotective properties (Ahmed et al., 2017, Abubakar et al., 2014, Laskar et al., 2010, Silva et al., 2017). In recent years, the successful medical application of propolis has further led to its extensive use as a food additive in beverages and its application in cosmetics; it is now recognised as safe substance for humans (Daleprane and Abdalla, 2013, Lotfy, 2006, Tosi et al., 2007).

Recently, we investigated the anti-oxidant, anti-inflammatory and anti-nociceptive properties of propolis from Bangladesh and reported relatively high concentrations of total polyphenols (80.16 mg), flavonoids (10.95 mg), tannins (51.69 mg), ascorbic acid (19.51 mg) and reducing sugars (35.14 mg) per g of propolis (Tanvir et al., 2018). In addition, the propolis extract was highly reactive against free radicals with robust reducing potentials. Studies using a mouse model further revealed the anti-inflammatory properties of the ethanolic extract of propolis and its efficacy as analgesic medicine (Tanvir et al., 2018). The bioactive chemical profile of Bangladeshi propolis and its potential application in reducing the effects of drug-induced toxicity have, however, not been investigated. The present study aims to investigate the bioactive phenolic compounds in the ethanolic extract of propolis from Bangladesh using HPLC and the potential effects of propolis extract against TTC-induced hepatonephrotoxicity. These effects were confirmed by examining the changes in hepatic marker enzymes, bilirubin and protein metabolism, lipid metabolism and renal markers in Wistar Albino rats. Additionally, histopathological examination of liver and kidney tissues was undertaken to support the biochemical findings.

2. Materials and methods

2.1. Drugs and chemicals

Reference standards of gallic, tannic, vanillic, benzoic, L-ascorbic, trans-cinnamic and salicylic acids as well as rutin, catechin, quercetin, pyrogallol, and naringin were purchased from Sigma-Aldrich (USA). The tetracycline antibiotic, tetracycline hydrochloride, was purchased from ACME Laboratories Limited, Dhaka, Bangladesh and ethanol (100%) and

dimethyl sulfoxide (DMSO) were purchased from Merck Co. (Germany). All the chemicals used in this study were of analytical grade.

2.2. Propolis collection and extract preparation

Apis genus stingless bee propolis was collected from PROSHIKA Integrated Agricultural Farm Trust, Manikgonj, Dhaka division, Bangladesh in summer 2016. The collected propolis samples were cut into small pieces. The ethanolic extract of propolis (10%) was prepared by soaking propolis (10 g) in AR ethanol (100%) for 24 hours and then shaken (150 rpm) at 30°C for 48 hours. The collected extract solutions were passed through Whatman no. 1 filter paper. The collected filtrates were concentrated in a rotary evaporator (Buchi, Tokyo, Japan) at a controlled temperature (40°C) and under a reduced pressure (100 psi). The yield extracts were collected and were stored at -20°C for HPLC analysis and for use in the hepatonephrotoxicity study.

2.3. Analysis of phenolic compounds using HPLC

To identify and determine the phenolic acids and flavonoids in Bangladeshi propolis, HPLC with UV detection was used, based on previously established methods (Kumazawa et al., 2004, Afroz et al., 2016) with some slight modifications. Propolis extract (1 g) was dissolved in 10 mL methanol. The organic layer was collected by centrifugation at 6000 rpm for 10 min, followed by microfiltration using a 0.45 µm syringe filter (Sartorius AG, Germany). The filtrate (5 mL) was then passed through a 0.20 µm nylon membrane filter (Sigma, USA). Finally, an aliquot of 50 µL was diluted with methanol (10 mL) and was loaded on the HPLC system equipped with UV detector (SPD-20AV, Serial no.: L20144701414AE, Shimadzu Corporation, Japan). A Luna Phenomenex, HPLC column C18 100Å (150 x 4.6 mm, 5 µm) was used. The detection wavelength was between 200 and 450 nm, with a specific monitoring conducted at 265 nm. The chromatographic conditions used for the HPLC system were as described previously (Afroz et al., 2016).

The phenolic acids and flavonoid compounds in the propolis extract were identified by comparing the retention times (RT) between analytes and the reference standards to yield the area ratios for determination of the concentration of the analytes.

2.4. Experimental animals and treatment schedule

The study was conducted on adult male Wistar Albino rats weighing between 150 g and 200 g (aged 12-15 weeks). The rats were bred and maintained in an institutional animal house

facility under standard conditions of ventilation, room temperature ($25 \pm 2^\circ\text{C}$) and relative humidity (45-70%). The rats were housed in clean polypropylene cages and received a natural light/dark cycle (12/12h). The rats had access to standard laboratory food and water *ad libitum*. The study was carried out following the ethical guidelines approved by Bangladesh Association for Laboratory Animal Science. The experimental protocol of the study was approved by the Gono Bishwabidyalay Bioethics Advisory Committee [Approval number GBBAC 2016-06].

All animals were acclimatised for one week prior to the study. The animals were randomly divided into the normal control group (n=6) and treatment groups (n=12; 2 x 6) and were maintained for the study period of 28 days, as follows:

1. Normal control: Rats were administered 5% DMSO in normal saline (0.9% sodium chloride solution) per orally (p.o.) with standard laboratory diet for 28 days.
2. TTC: Rats were administered TTC at 200 mg/kg/rat, p.o. (dissolved in normal saline) for 28 days with standard laboratory diet. The rats in this group served as negative control.
3. TTC + Propolis: Rats were administered TTC (200 mg/kg/rat, p.o.) dissolved in normal saline and propolis extract (100 mg/kg/rat, p.o.) dissolved in 5% DMSO in normal saline for 28 days. The rats in this group served as the treatment group.

The propolis dose used in this study was based on previous studies (Ahmed et al., 2017, Ahmed et al., 2012) and the TTC dose was established based on our findings of the pilot study and reports by Shabana et al. (2012). During the investigation, the animals were observed for any abnormal clinical signs. Changes in body weight (BW) were monitored on a weekly basis.

2.5. Sacrifice of animals

At the end of the experimental period (28 days), the rats in each group were deeply anaesthetised with a ketamine hydrochloride injection (100 mg/kg) and sacrificed prior to dissection. Blood samples (3 mL) were collected using a heparinised syringe from the inferior vena cava. The blood samples were transferred into plain tubes and allowed to coagulate at an ambient temperature for 30 min. The serum was separated by centrifugation at 2500 rpm for 10 min and was stored at -20°C for subsequent biochemical analysis. In addition, the liver and kidney tissue samples were collected immediately after sacrifice and preserved in formalin solution (10%) for histopathological examinations as indicated below.

2.6. Biochemical assessment

The serum samples from each of the groups were used for the determination of the levels of serum glutamate-pyruvate transaminase (SGPT), glutamate-oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), total bilirubin (TB), total protein (TP), total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C) and creatinine, urea and uric acid levels using standard assay kits (HUMAN GmbH, Germany) on a Humalyzer 300 auto-analyzer, Germany. The serum very low-density lipoprotein cholesterol (VLDL-C) concentrations were calculated based on the Friedewald formula (Friedewald et al., 1972): where $VLDL-C = TG/5$

2.7. Histopathological examination

Following dissection, the liver and kidney tissues were fixed in 10% formalin. The fixed tissues were embedded in paraffin and then cut into serial sections (5 μ m thickness) using a rotary microtome (Leica RM 2145 Rotary Microtome, Germany). Each of the tissue sections was stained with hematoxylin and eosin (H & E) dye. Photomicrographs at 6100 x magnification were captured using a normal-spectrum fluorescence microscope (Olympus DP 72) with an attached digital camera (Olympus DP 72, Japan). The histopathologist was blinded to the treatment assignments of the different experimental groups during histological examination. The liver and kidney slides from each rats were examined, and the changes were scored as none (1), mild (2), moderate (3) and severe (4) pathological changes.

2.8. Statistical analysis

The results are expressed as the mean values $\pm$ standard deviations (SD). The data were analyzed using Microsoft Excel 2010 (Redmond, Washington) and SPSS (Statistical Packages for Social Science, version 20.0, IBM Corporation, New York). The comparisons of the biochemical data between different study groups were conducted using a one-way Analysis of Variance (ANOVA) and statistical analyses were conducted using Tukey's test. A value of $p < 0.05$ was considered as statistically significant.

3. Results

3.1. HPLC analysis of phenolic compounds

The results of HPLC analysis are presented in table 1 and figure 1 which includes four phenolic acids and one flavonoid based on their retention time.

3.2. Effects of Bangladeshi propolis on TTC-induced hepatonephrotoxicity

The effects of TTC and the propolis extract on rat body weight (BW) gain are shown in table 2. No deaths were observed in any of the study groups over the entire period of 28 days. At the end of the treatment period, there were no significant changes in BW gain observed between the treatment groups, although TTC alone treated rats showed slight decrease in BW gain when compared to normal controls. The co-administration of propolis, however, with TTC treatment resulted in BW gains to near that of the controls. In addition, several clinical signs were observed in the TTC alone treated rats, these included weakness with reduced physical activity, slightly liquid stools, loss of hair, decreased food consumption but increased water intake, excessive sweating with slightly reddish eye (during the last 7 days of treatment). The observed clinical signs were significantly less in propolis co-treated rats.

The exposure to TTC for consecutive 28 days significantly affected multiple biochemical parameters in rats (Figure 2). The animals treated with TTC alone had significantly ($p < 0.05$) elevated levels of hepatic marker enzymes, such as SGPT, SGOT and ALP activities compared to rats from the normal control group. Furthermore, TTC treatment increased serum TP and TB levels compared to controls (Figure 3). Conversely, a significant ($p < 0.05$) reduction in serum SGPT, SGOT and ALP activities as well as TP and TB levels were observed in the rats co-treated with propolis and TTC compared with TTC alone treated rats.

Sub-chronic treatment of TTC significantly altered the lipid profile in the animals (Figure 4). A significant rise in the levels of circulating TGs, TC and VLDL-C and a corresponding non-significant decrease in HDL-C levels were found in TTC alone treated rats when compared with normal controls. Again, co-treatment with propolis significantly decreased the serum TGs, VLDL-C levels and non-significantly TC level while increased the serum level of HDL-C when compared to the parameters for the TTC alone treated rats.

The detrimental effects of TTC on marker of renal function were confirmed by the significant ($p < 0.05$) elevation in serum levels of urea and uric acid when compared to normal control rats. This elevation was reduced in those rats co-treated with propolis and TTC. Serum creatinine levels were unchanged in any of the treated rats (Table 3).

Microscopic examination of the hepatic tissues of control rats revealed a normal histoarchitecture with clear and well-defined central vein (CV) in the centre of the lobule. The hexagonal hepatocytes were observed in a regular arrangement radiating from the CV towards the periphery of the lobule. Furthermore, the nuclei of the cells showed a regular vesicular structure with uniform and normal cytoplasm (Figure 5a & b). In contrast, the histology of TTC-treated rats indicated certain degenerative changes in the liver, including a severely disrupted arrangement of the hepatocytes originating from CV towards the periphery of the lobule, cell necrosis at the periphery of the CV and in the lobule, vascular congestion in the CV associated with inflammatory cell infiltration and oedema at different locations of the lobule (Figure 5c & d). However, the liver sections of TTC + propolis group exhibited improvement in all of the investigated histological parameters with a mild to moderate degree of disarrangement of hepatocytes, necrosis, inflammatory infiltrates and mild congestion in the CV and oedema in the lobule (Figure 5e & f and Figure 7).

Histopathology of kidneys of the control animals showed normal glomeruli and renal tubules (Figure 6a & b). In contrast, rats treated with TTC alone had significant degenerative changes in the renal glomeruli but non-significant changes in the tubules associated with tubular vacuolization (Figure 6c & d). However, the propolis treatment (TTC + propolis) group showed marked improvement with mild glomerular necrosis and tubular vacuolization (Figure 6e & f and Figure 7).

4. Discussion

To our knowledge, this study is the first to identify phenolic compounds in propolis from Bangladesh and to report the protective effects of propolis extract against hepatonephrotoxicity induced by TTC treatment in rats. The improvement in the biochemical markers of liver and kidney functions in this group were further confirmed histopathologically.

Reactive oxygen and nitrogen species (hydroxyl radicals, peroxy radicals, peroxide anions, superoxide anions, singlet oxygen, hydrogen peroxide and nitric oxide) formed by mitochondrial biochemical reactions and the metabolism of environmental and dietary xenobiotics may lead to oxidative damage of cellular lipids, proteins and nucleic acids. Overproduction of these reactive species may result in the oxidative stress, implicated in the

cellular mechanism of aging processes, hepatotoxicity, some phases of atherosclerosis and liver injury (Afroz et al., 2014b). Plant phenolics are aromatic compounds with one or more hydroxyl groups that potentially contribute to the functional quality and play significant role in the protection against oxidative stress and help to minimise molecular damage (Tanvir et al., 2015b).

The propolis from Bangladesh was observed to contain four phenolic acids (including gallic, benzoic and salicylic acid and pyrogallol) and a single flavonoid (rutin). Gallic acid has been previously reported in propolis originating from China and Poland (Bonvehí and Coll, 1994, Krol et al., 1996). Similarly, propolis samples collected from Brazil, Iran and Turkey were also reported to contain benzoic acid (Haghdoost et al., 2016, Alencar et al., 2007, Silici and Kutluca, 2005) while propolis from Poland was found to contain salicylic acid in propolis (Krol et al., 1996). Moreover, propolis samples from Tamil Nadu, India were reported to contain the flavonoid, quercetin, while propolis from northern India was found to contain pinocembrin and galangin (Thirugnanasampandan et al., 2012, Laskar et al., 2010).

Gallic acid (3,4,5-trihydroxybenzoic acid) is a derivative of benzoic acid containing three functional hydroxyl groups in its structure and acts as a strong radical scavenger in various oxidative stress disorders, including cardiovascular diseases, neurodegenerative diseases, cancer or during aging process. Due to its diverse pharmacological activities, gallic acid and its derivatives are now considered as promising lead compounds for new drug development (Nayeem et al., 2016). Salicylic acid (2-hydroxybenzoic acid) is another phytochemical which is naturally occurring in various plants, having potential anti-inflammatory effects, contributed by its inhibitory action on prostaglandin synthesis. A number of studies have confirmed the role of salicylates as antioxidants, an effect that may be influenced by its strong binding capacity with iron during lipid peroxidation (Randjelović et al., 2015). Benzoic acid (C_6H_5COOH) is a naturally occurring phenolic acid, commonly used as additive to foods and feeds. The major applications of benzoic acid in food, beverage and pharmaceutical industries are in the forms of its sodium and potassium salts (C_6H_5COONa and C_6H_5COOK , respectively) when sodium benzoate is commonly used as preservative and anti-corrosive agent (Maki and Takeda, 2000). Pyrogallol (1,2,3-trihydroxybenzene) is a phenolic allelochemical and is synthesized by plants. It is believed that the pyrogallol identified in propolis from Bangladesh may act as an important defensive agent against invading microbes. In addition to phenolic acids, rutin (3,3',4',5,7-pentahydroxyflavone-3-rhamnoglucoside) is an important flavonoid observed in this study in propolis from

Bangladesh. Previously, samples of propolis originating from Brazil, Uruguay and China were reported to contain rutin, determined from its powder form as well as in its alcohol extract (Bonvehí and Coll, 1994). The various pharmacological benefits of rutin include its activities as antioxidant, anti-inflammatory, anticancer, anti-diabetic effects, inhibition of platelet aggregation and reduction of blood cholesterol levels. It has therefore been exploited in human medicine and nutrition (Al-Dhabi et al., 2015, Afroz et al., 2016). Moreover, phenolic compounds are likely responsible for several pharmacological and biochemical pathways accompanied by the strong anti-radical, anti-inflammatory, antimicrobial activities as well as cytotoxic properties of propolis (Haghdoust et al., 2016).

In this study, an investigation of liver and kidneys of the experimental rats indicated that rats exposed to TTC alone experienced a marked decrease in BW gain compared to controls. Similar observations have been reported by Dietz et al. (1991), in which a dose-related reduction in the BW was noted in mice treated with tetracycline. The underlying mechanism might be attributed to the toxic effects of overexposure to antibiotics, interfering with the absorption of nutrients in the gut or acting indirectly through the central nervous system and/or endocrine system and resulted in the reduced food and water intake observed (Hossen et al., 2017). However, propolis co-administration with TTC improved the parameter compared to normal control and negative control rats.

In animals, the liver plays pivotal role in the detoxification of xenobiotics; the effects of TTC and the propolis extract on hepatic function were evaluated by measuring the serum activities of SGPT, SGOT and ALP enzymes. The serum TP and TB levels were also investigated to determine the extent of the liver toxicity and damage (Tanvir et al., 2015a). Oral treatment with TTC led to a profound increase in the serum SGPT, SGOT and ALP activities in animals that could be potentially attributed to liver damage and impaired biosynthesis of the enzymes. Altered permeability of cell membranes due to TTC toxicity likely allowed cellular enzymes to leak out from the cytosol of the hepatocyte into the systemic circulation, indicating the drug-induced necrosis and inflammatory reactions (Paul et al., 2016, Hossen et al., 2017). These findings are consistent with the results of Shabana et al. (2012) and (Asha et al., 2007). However, co-treatment with propolis extract significantly reduced the serum SGPT, SGOT and ALP levels in rats in comparison with the TTC-alone treatment group, demonstrating that the possible action of propolis in maintaining membrane integrity and thus restricting the leakage of these enzymes. The protective mechanism can be attributed to the phenolic compounds (including gallic acid, benzoic acid, salicylic acid, pyrogallol and rutin),

tannins, ascorbic acid, and vitamin E in this propolis extract all of which have inhibitory activities on membrane lipid peroxidation with robust antioxidant activities (Tanvir et al., 2018, Paulino et al., 2014, Hossen et al., 2017, Ahmed et al., 2017). Moreover, a significant rise in the TB level following TTC administration is one of the most important clinical indicators of the severity of necrosis and the TP levels in serum may also indicate the severity of hepatic injury (Afroz et al., 2014a). The results recorded in the present study are in accordance with a previous study (Shabana et al., 2012). In our study, supplementation with propolis extract restored the TB and TP levels, indicating the role of propolis in liver protection. It is plausible that the antioxidants present in the ethanolic extract of propolis (Tanvir et al., 2018) conferred protection of the liver by preserving the membrane integrity (Tanvir et al., 2015a).

Altered lipid metabolism is another important indication of liver toxicity (Hossen et al., 2017). The increase in TG and cholesterol levels (TC and VLDL-C) along with a decreased HDL-C level in serum in TTC administered rats, suggesting the possible effects of TTC on membrane permeability of the hepatocytes and/or impaired expression of the genes involved in lipid metabolism (Yin et al., 2006). Yin et al. (2006) reported that TTC administration at high dose resulted in altered gene expression (induction or suppression), of which, 141 genes were involved in lipid metabolism (including β -oxidation of fatty acids in mitochondria and peroxisomes, biosynthesis of triglycerides and cholesterol). High dose TTC administration may cause inhibition of β -oxidation of free fatty acids as well as secretion of lipoproteins from liver and lead to steatogenic hepatotoxicity (Yin et al., 2006, Fréneaux et al., 1988). In addition, necrotic damage of the hepatocytes causes an increased release of cholesterol from cells into the blood circulation (Santhosh et al., 2006). The blockage of the bile duct and subsequent decreased secretion of cholesterol into the duodenum may also result in higher cholesterol levels in the serum (Tanvir et al., 2015a). The data of the present study corroborated with findings reported by Shabana et al. (2012). However, the serum levels of TG, TC, VLDL-C and HDL-C in propolis co-treated animals revealed that propolis had significant capacity to restore these levels when compared to normal control and TTC alone treated rats. Propolis has been reported to modulate lipid and lipoprotein metabolism. Daleprane and Abdalla (2013) claimed that propolis administration reduced the hepatic TG synthesis in rats and diminished liver cholesterol and TG levels. In LDL receptor knockout mice (LDLr^{-/-}), the administration of Brazilian propolis, which is rich in phenolics (polyphenols and flavonoids), significantly decreased the triglyceride and cholesterol levels

and increased HDL-C level in plasma (Daleprane et al., 2012). Similarly, we speculate that the phenolic compounds present in Bangladeshi propolis have the potential to inhibit cholesterol absorption as well as its biosynthesis in liver and to increase the excretion of bile acids; this will require further investigation.

The present study also focused on the possible effects of TTC and propolis on kidney function. Serum urea and uric acids levels were significantly higher and the creatinine concentration was non-significantly higher in the rats administered with TTC alone, indicating TTC-induced nephrotoxicity. The findings on kidney markers are in accord with results of earlier studies (Shabana et al., 2012, Abdel-Daim and Ghazy, 2015). The exact mechanism of TTC-induced kidney injury is still unexplained. Oxidative stress as well as reduced antioxidant enzymes may be possible risk factors (Abdel-Daim and Ghazy, 2015). The findings of this study have indicated that propolis has potential protective effects against renal damage induced by toxic levels of TTC in animals. It is plausible that the presence of bioactive phenolic acids, flavonoids, tocopherol and ascorbic acids in Bangladeshi propolis (Tanvir et al., 2018), may confer nephroprotection in animals, as reported in another study involving Croatian propolis on diabetic nephropathy (Oršolić et al., 2012), where antioxidant potential of the propolis have also been highlighted.

The TTC-induced hepatic and renal toxicity was consistent with the observed necrosis of the hepatocytes and renal tubules, respectively. Degenerative necrosis, vascular congestion with inflammatory cells infiltration and edematous spaces were frequently observed at different locations of the lobule in the liver of the TTC-treated rat group. Furthermore, degenerative necrosis of the renal epithelium and localised vacuolisation were also observed in the kidney tissues of the same TTC treatment group. The histopathological findings of the study provided clear evidence of hepatic and renal toxicity in rats following high oral doses of TTC. Our findings are in agreement with a previous study on liver and kidney histopathology following similar TTC administration (Shabana et al., 2012). The hepatorenal protective effects of co-administered propolis extract were further confirmed by the significant improvements in liver and kidney histoarchitecture. The phenolics (including gallic acid, benzoic acid, salicylic acid, and pyrogallol), flavonoids (rutins), antioxidant vitamins (ascorbic acids and tocopherols) and others antioxidants/antiradicals in the ethanolic extract of Bangladeshi propolis (Tanvir et al., 2018) have synergistic effects which confer protection against TTC-induced histopathological alterations, and this may be *via* scavenging of free radicals and the regulation of cholesterol levels, which is consistent with the biochemical

findings. The study lacks to establish any potential toxic effects of propolis in rats. Further study is warranted to establish the toxicity of propolis and to elucidate the molecular mechanisms of hepato- and nephroprotection shown by propolis.

5. Conclusion

To the best of our knowledge, our study is the first to report the phenolic composition of propolis from Bangladesh and its hepato- and nephro-protective effects against TTC-induced toxicity in an animal model. The TTC-induced tissue injuries were confirmed biochemically and histopathologically. Our findings indicate that propolis from Bangladesh is a rich source of natural bioactive principles due to its phenolic acids and flavonoid content such as rutin. The phenolic constituents of propolis likely improve the structural integrity of cell membrane and ameliorate the histological changes and biochemical perturbations.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Tables:

Table 1: Phenolic acids and flavonoid compounds detected in propolis using HPLC

Serial No.	Standard compound	Retention time (min)	Area	Concentration (mg/g)
1	Tannic acid	3.178	-	ND
2	Gallic acid	3.696	1191972	0.109 ± 0.001 ^a
3	Catechin	3.809	-	ND
4	Pyrogallol	5.212	2868570	0.201 ± 0.002 ^a
5	Naringin	6.095	-	ND
6	Vanillic acid	7.531	-	ND
7	Rutin	8.264	2868570	132.112 ± 1.155 ^b
8	Benzoic acid	9.893	740207	0.530 ± 0.005 ^a
9	Trans-cinnamic acid	16.722	-	ND
10	Quercetin	19.263	-	ND
11	Salicylic acid	19.623	877049	0.033 ± 0.001 ^a

The data are expressed as mean ± SD (standard deviation), n=3. Different letters (a, b,) in the same column indicate significant difference at p<0.05. Limit of detection: LOD

ND: Not detected.

Table 2: The effects of TTC and ethanolic extract of propolis on body weight (BW) gain in different treated rats

Group	Initial BW (g)	14 days BW (g)	Final BW (g)	BW gain (%)
Normal Control	168.98 ± 15.51 ^a	191.27 ± 16.82 ^a	229.8 ± 10.10 ^a	26.32 ± 7.67 ^a
TTC	197.23 ± 8.28 ^b	214.73 ± 8.79 ^b	257.33 ± 10.27 ^b	23.33 ± 2.37 ^{a,b}
TTC + Propolis	169.30 ± 18.96 ^a	192.57 ± 13.89 ^a	244.77 ± 13.71 ^a	30.98 ± 4.45 ^{a,b}

The data are presented as the mean ± SD of six rats. Different superscript (a, b) in the same column denotes significant differences at $p < 0.05$; % of BW gain = [(final BW – initial BW)/final BW] X 100.

Table 3: The effects of TTC and ethanolic extract of propolis on serum kidney markers in different treated rats

Group	Creatinine (mg/dL)	Urea (mg/dL)	Uric acid (mg/dL)
Normal Control	0.87 ± 0.15 ^a	33.14 ± 9.71 ^a	1.42 ± 0.35 ^a
TTC	0.95 ± 0.23 ^a	65.59 ± 7.24 ^b	2.47 ± 0.93 ^b
TTC + Propolis	1.05 ± 0.22 ^a	42.58 ± 13.01 ^a	1.53 ± 0.47 ^{a,b}

The data are presented as the mean ± SD of six rats. Different superscript (a, b) in the same column denotes significant differences at $p < 0.05$.

Figure Legends

Figure 1: Chromatogram of phenolic acid standards: (1) tannic acid, (2) gallic acid, (3) pyrogallol, (4) vanillic acid, (5) benzoic acid, (6) trans-cinnamic acid, and (7) salicylic acid.

Figure 2: The effects of TTC and ethanolic extract of propolis on serum glutamate-pyruvate transaminase (SGPT), glutamate-oxaloacetate transaminase (SGOT) and alkaline phosphatase (ALP) activities in different treated rats.

The data are presented as the mean ± SD of six rats. Different letters (a, b) in the each column denotes significant differences at $p < 0.05$.

Figure 3: The effects of TTC and ethanolic extract of propolis on serum (a) total bilirubin and (b) total protein levels in different treated rats.

The data are presented as the mean $\pm$ SD of six rats. Different letters (a, b) in the each column denotes significant differences at $p < 0.05$.

Figure 4: The effects of TTC and ethanolic extract of propolis on serum lipid profile in different treated rats.

The data are presented as the mean $\pm$ SD of six rats. Different letters (a, b) in the each column denotes significant differences at $p < 0.05$.

Figure 5: Photomicrograph of H&E-stained liver sections. (a & b) Liver sections from control rats showing normal histoarchitecture with hepatic lobule and uniform arrangement of polyhedral hepatocytes radiating from the central vein (CV) towards the periphery of the lobule. The nuclei showing normal vesicular structure with uniform cytoplasm. (c & d) Liver sections from TTC alone treated rats showing a severe disruption in the arrangement of hepatocytes around the CV and in the lobule, cell necrosis around the CV and in the lobule, vascular congestion, inflammatory cells infiltration and edema (e & f) Liver sections from TTC plus propolis treated rats showing remarkable degree of preservation in cell arrangement and slight necrosis in the lobule with inflammatory infiltrates (Magnification: 40x, scale bar: 20 μ m).

Figure 6: Photomicrograph of H&E-stained kidney sections. (a & b) Kidney sections from normal control rats demonstrating normal morphology of the renal parenchyma with well-defined glomeruli (GM) and renal tubules. (c & d) Kidney sections from negative control rats demonstrating marked necrosis of the glomerulus, degenerative changes in the tubular epithelium and tubular vacuolization. (e & f) Kidney sections from TTC plus propolis treated rats showing remarkable degree of morphological preservation with mild to minimal necrosis of the glomeruli and tubular vacuolization (Magnification: 40x, scale bar: 20 μ m).

Figure 7: Semi-quantitative scoring of the histopathological changes as determined by histopathological examination of liver and kidney slides. Scoring was performed as follows: none (1), mild (2), moderate (3) and severe (4) pathological changes.

Each column presents means $\pm$ SD of six rats; column with different letters (a,b) denotes significant differences at $p < 0.05$.

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