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Antioxidant mediated protective effect of *Bridelia tomentosa* leaf extract against carbofuran induced oxidative hepatic toxicity

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ABSTRACT

Bridelia tomentosa (B. tomentosa) is a traditional medicinal plant for treating diverse ailments. Hence, we designed our study to scrutinize the protective effect of the methanol extract of *B. tomentosa* leaf (BTL) against carbofuran-induced oxidative stress-mediated hepato-toxicity in Sprague-Dawley rats for the first time, along with the identification and quantification of phenolic acids and flavonoids by high-performance liquid chromatography (HPLC) and evaluation of antioxidant and antiradical activities of this extract. HPLC analysis confirmed the existence of tannic acid, gallic acid, salicylic acid, and naringin in *B. tomentosa* leaf extract which showed in-vitro antioxidant potentialities with DPPH, nitric oxide, hydrogen peroxide, and hydroxyl radical scavenging properties. Co-administration of *B. tomentosa* leaf extract with carbofuran showed dose-dependent significant protective effects of hepatic toxicity on serum markers such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyl-transferase, lactate dehydrogenase, total bilirubin, total protein, albumin, globulin, lipid profile, urea, uric acid, and creatinine. Carbofuran intoxication also revealed an upsurge in malondialdehyde (MDA) and a decline in cellular endogenous antioxidant enzyme levels in rats compared with the control group. However, *B. tomentosa* leaf extract co-treatment increased the levels of hepatic antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, and amended the MDA level. Similarly, histopathological evaluation further assured that BTL could keep the hepatocyte from carbofuran-induced damage. Therefore, all of our findings may conclude that the phenolic acids and flavonoids of *B. tomentosa* leaf extract are responsible to neutralize the toxic free radical-mediated oxidative hepatic damages.

1. Introduction

Nature always serves as a vast library of medicinal plants that stands as a golden mark to exemplify the numerous drug discovery with the greatest concern in the direction of the therapeutic development focused on innumerable diseases around the world. Treatment and/or prevention with widely available medicinal plants have a large impact on healthcare worldwide. Findings reported that about 80 % of people in most of the countries believe in medicinal plant-based traditional medicines for the necessities of human disorders [1]. Among the human disorders, the hepatic ailment is continuously providing health afflictions throughout the world owing to viral infections, environmental contamination, biochemical, chemotherapeutic, toxic agents, and most commonly unhealthy dietary habits [2]. As the liver plays a noticeable role in the metabolism and clearance of xenobiotic, the pathogenesis of chemical-induced hepatic damages is commenced by the metabolic alteration of this xenobiotic into reactive intermediate species, such as electrophilic compounds or free radicals, which significantly hamper the structure and functions of cellular macromolecules [3]. Pesticides are frequently used chemicals to look after agricultural crops from pests with the aim of improving yield. However, indiscriminate usage has caused their excessive accumulation in diverse

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environmental components from which humans and animals may exposure to these through the ingestion of pesticide-contaminated food and [4].

Carbofuran (2, 3- dihydro-2, 2-dimethyl-7-benzofuranol methyl carbamate) is a broad-spectrum pesticide that has higher mammalian toxicity [5]. Some Environmental Protection Agencies declared that carbofuran has been banned in developed countries, despite that, carbofuran continues to be widely used in developing countries like Bangladesh [6]. Due to its lipophilic nature, carbofuran accumulates in the fat depots and elicits its toxic effects on diverse organs such as the brain, liver, skeletal muscles, and heart [7]. Usually, carbofuran inhibits acetylcholinesterase (AChE) and increased the acetylcholine level at the synaptic junctions. Increased acetylcholine level causes hyper-excitation state that upsurges the flow of oxygen and converts them to reactive oxygen species (ROS) [8]. These reactive oxygen species are accountable for the membrane phospholipids' peroxidation, interrupting the usual lipid bilayer function and developing numerous pathological conditions. Therefore, Milanovic and his peers revealed that carbofuran produced myopathy by excessive formation of ROS leading to lipid peroxidation (LPO) through mitochondrial dysfunction [9]. Some earlier studies demonstrated that carbofuran administration produces oxidative stress by increasing the level of malondialdehyde (MDA) and altering the activities of cellular antioxidant enzymes in the liver, brain, kidney, heart, and erythrocytes [5,7,8]. Generally, cellular antioxidant enzymes control the generation of free radicals and neutralize them to maintain cellular homeostasis in normal physiological conditions. However, toxicants instigate the overproduction of reactive radicals that imbalance the redox state and produce oxidative stress [10]. At that time, nutritional antioxidant supplementation may enhance the defensive efficiency to combat toxic free radical-induced oxidative impairment [11]. Unfortunately, synthetic antioxidants are unsatisfactory because they exert serious long-term side effects such as skin allergies, gastrointestinal tract problems, and cancer risk. Therefore, people of diverse locations rely on phytochemicals as a source of natural antioxidants to treat chronic diseases [12]. Natural antioxidants are mainly categorized into three classes as phenolic compounds, vitamins, and carotenoids. Among them, polyphenolic compounds possess diverse biological activities such as antioxidant, antiradical, anticancer, anti-inflammatory, cardioprotective, and hepatoprotective effects [13].

Bridelia (Family: Phyllanthaceae) containing a total of 60 species are used as traditional medicines in Asia and Africa regions for the treatment of numerous diseases such as anti-diarrheal, anti-bacterial, anti-amebic, anti-anemic, anthelmintic, anti-inflammatory, anticonvulsant, antidiabetic, antiviral, antimalarial, hypoglycemic, and antinociceptive [14]. *Bridelia tomentosa* Blume (Local name: Khy, serai), is a small evergreen tree or large shrub and which is comprehensively found in Bangladesh in different forest areas for instance Chittagong, Dinajpur, Srimangal, and therefore Sylhet district [15]. It is used as a traditional medicinal plant in Thailand. Leaves and stem bark decoction are used against colitis. Leaves are also applied to treat traumatic injuries in herbal medicine [16]. Nonetheless, there are no substantial scientific reports concerning the phenolic and flavonoid compounds identification and hepatoprotective activity of methanolic extract of *B. tomentosa* leaf. Therefore, we aimed to determine phenolic acids and flavonoids contents of leaf extract of *B. tomentosa* and correlate their *in-vitro* antioxidant and antiradical potentialities with their protective activity against carbofuran-induced oxidative stress-mediated hepatocellular toxicity and to our knowledge, this is for the first time for the methanol extract of *B. tomentosa* leaf.

2. Materials and methods

2.1. Chemicals and reagents

Merck is a German multinational pharmaceutical, chemical and life sciences company from which folin – ciocalteu, methanol, sodium phosphate, and ammonium molybdate were purchased, whereas phosphoric acid, hydrogen peroxide, gallic acid, ascorbic acid, quercetin, tannic acid, vanillic acid, benzoic acid, salicylic acid, pyrogallol, catechin, naringin, rutin, and 1,1 diphenyl-2-picrylhydrazyl (DPPH) were procured from Sigma-Aldrich Co, U.S.A. We also acquired griess reagent, potassium ferricyanide, trichloroacetic acid, and ferric chloride from Ranbaxy lab, India. Carbofuran (CF) (Purity 98 %) was purchased from Shetu Corporation Limited, Dhaka, Bangladesh. In our study, all of these chemicals were of analytical grade and were used as received.

2.2. Plant material

Fresh leaves of *B. tomentosa* were collected from the Botanical garden of Jahangirnar University, Savar, Dhaka, Bangladesh in January 2017 and authenticated by National Herbarium, Bangladesh. A voucher specimen (Acc. No. DACB: 43751) was deposited in the National Herbarium, Bangladesh for future reference.

2.3. Extract preparation

The collected *B. tomentosa* leaves were first cleaned with tap water, and sun-dried and then dried further at a moderate temperature of 40 °C to make them suitable for grinding. A blender (CM/L7360065, Jaipan, and Mumbai, India) was used to grind into a fine powder from the dried leaves. According to Hossain et al. 1000 g of leaf powder was used for extraction by Soxhlet apparatus (Z556203, Aldrich® Soxhlet extraction apparatus, Darmstadt, Germany) at 65 °C temperature with methanol (5000 mL) as a solvent to form 20 % methanolic extract [17]. Thereafter, the extract was filtered through Whatman 1 filter paper and dried under reduced pressure (100 psi) by means of rotary evaporation at a temperature of 40 ± 2 °C. The gummy concentrate was collected and preserved at −20 °C for further analysis and was screened with respect to pharmacological properties; the yield was 235.20 g.

2.4. Phenolic acids and flavonoid contents detection by HPLC from B. tomentosa leaf

According to Wahed et al. HPLC coupled with a UV detector was employed to determine phenolic acids and flavonoids in the methanol extract of *B. tomentosa* leaf [18]. Briefly, 1 g of the *B. tomentosa* leaf extract was dissolved in 10 mL of methanol (HPLC grade), followed by centrifugation at 6000 rpm for 10 min, and then filtration through a 0.45 μm syringe filter (Sartorius AG, Germany). A 0.20 μm nylon membrane filter (Sigma, U.S.A.) was used to pass the filtrate (5 mL). An aliquot of 50 μL was diluted with 10 mL of methanol and loaded on the HPLC system (SPD-20AV, Serial no.: L20144701414AE, Shimadzu Corporation, Kyoto, Japan) equipped with a UV detector (SPD-20AV, Serial no.: L20144701414AE, Shimadzu Corporation, Kyoto, Japan). A Luna Phenomenex, C18 100A (150 × 4.60 mm, 5 μm) HPLC column, a linear gradient at a flow rate of 0.5 mL/min was used in this HPLC system and the total analytical time was approximately 35 min. A binary mobile phase consisting of solvent A (ultrapure water with 0.1 % phosphoric acid) and solvent B (pure methanol with 0.1 % phosphoric acid) was applied. Elution from the column was achieved with the following gradient: 0–10 min of solvent B, increased from 35 % to 55 %; 10–25 min of solvent B, increased to 62 %; 25–30 min of solvent B increased to 85 %, and the final composition was kept constant up to 35 min. 265 nm wavelength of the UV detector was set for detection. By comparing the retention times and UV spectra of reference standards, phenolic acids and flavonoids identification were accomplished. The concentrations of identified compounds in leaf extract were measured according to standard curves, and the outcomes were articulated in milligrams per gram of dry weight.

2.5. Determination of total phenolic and flavonoid content, and total antioxidant capacity

According to the method described by Hossain et al. total phenolic content of the extract was determined and expressed as mg of gallic acid equivalent (GAE)/ g of the extract [19]. In a comparable fashion, determination of the total flavonoid content, given as mg of quercetin equivalent (QE)/ g of extract, was accomplished by using the aluminum chloride colorimetric method described by Mondal et al. [20]. On the other hand, the total antioxidant capacity of the extract was evaluated following the phosphomolybdenum method described by Hossain et al. [19]. Then, the absorbance of solutions was measured with the aid of a Shimadzu UV PC-1600 spectrophotometer against a blank. Finally, the result of the antioxidant capacity is expressed as mg of ascorbic acid equivalent (AAE)/ g of extract

2.6. DPPH-free radical scavenging assay

DPPH scavenging activity of the extract was measured according to the standard procedure [21]. Briefly, in different test tubes, 1 mL of each extract or standard ascorbic acid of different concentrations (800, 400, 200, 100, 50, 25, 12.5, or 6.25 μg/mL) of the solutions were taken and 2 mL of 0.004 % DPPH freshly prepared solution in the solvent was added to each test tube to make the final volume of 3 mL and incubated the mixture in room temperature for 30 min in a dark place. After that, the absorbance was measured at 517 nm. The percentage (%) inhibition activity was calculated as: $\{(A_0 - A_1)/A_0\} \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard. The half-maximal inhibitory concentration (IC_{50}) was calculated by using regression analysis.

2.7. Nitric oxide radical scavenging assay

The nitric oxide scavenging capacity of extract was determined as described by the earlier procedure [21]. Various concentrations (160, 80, 40, 20, or 10 μg/mL) of extract and standard were taken in different test tubes. Then sodium nitroprusside (5 mM) in phosphate buffer was added to each test tube to make volume up to 1.5 mL. Solutions were incubated at 25 °C for 30 min and then 1.5 mL of Griess reagent was added to each test tube. The absorbance was measured at 546 nm. The percentage (%) inhibition was calculated as: $\{(A_0 - A_1)/A_0\} \times 100$; where A_0 is the absorbance of the control and A_1 is the absorbance of the extract or standard. The half-maximal inhibitory concentration (IC_{50}) was calculated by the linear regression method.

2.8. Hydrogen peroxide radical scavenging assay

The ability of the extracts to scavenge hydrogen peroxide was determined as described by Mondal et al. [22]. Different concentrations (12.5, 25, 50, 100, 200, 500 μg/mL) of extracts and standard (ascorbic acid) were prepared in distilled water. Then, these were added to a hydrogen peroxide solution (6 mL, 40 mM). 1 mL of each mixture was taken into a test tube and 3 mL of phosphate buffer solution was added to each mixture. A blank solution containing phosphate buffer without hydrogen peroxide was prepared. The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes. The percentage of hydrogen peroxide scavenging by the extracts and standard compound was calculated as follows: % Scavenged of $[H_2O_2] = [(A_0 - A_1)/A_0] \times 100$, Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract and standard. IC_{50} was calculated by the linear regression method.

2.9. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of the extract was measured by the published method [22]. 0.5 mL 2- deoxy-2-ribose solution (2.8 mM)

was mixed with 12.5 μL of different concentrations (12.5, 25, 50, 100, 200, 500 μg/mL) of sample extracts or standard (ascorbic acid). Then 1 mL of 200 μM FeCl$_3$, 1 mL of 1.04 mM EDTA, 0.5 mL of 1 Mm H2O2 and 0.5 mL of 1 mM of ascorbic acid were added to prepare the reaction mixture. After an incubation period of 1 h at 37 °C, 3.75 mL of 2.8 % TCA was added to the reaction mixture. 3.75 mL of 1 % TBA was added and kept at 100 °C for 20 min.. The absorbance was measured at 530 nm. The hydroxyl radical scavenging activity was calculated by the following equation: % of OH radical scavenging activity = $[(A_0 - A_1)/A_0] \times 100$, Where, A_0 was the absorbance of the blank and A_1 was the absorbance of sample and standard. IC_{50} was calculated by linear regression method.

2.10. Animals

Sprague-Dawley male rats (6–8 weeks) of 140–180 g and Swiss albino male mice (6–7 weeks) of 25–30 g were collected from Pharmacology Laboratory, Department of Pharmacy, Jahangirnagar University, and were acclimatized to normal laboratory conditions for one week prior to the study and were assessed to a pellet diet and water *ad libitum*. These animals were kept under normal laboratory conditions (temperature: 25 ± 2 °C, humidity: $55 \pm 5\%$, and 12 h light/dark cycles) mentioned in the Animals By-Laws approved by the Bangladesh Association for Laboratory Animal Science, whereas the experimental protocol was approved by the Biosafety, Biosecurity, and Ethical Committee of Jahangirnagar University [approval number: BBEC, JU/M 2018 (1) 3]. This analysis was carried out at the Department of Pharmacy, Jahangirnagar University, Savar, Dhaka, Bangladesh. Finally, after adaptation to normal laboratory conditions for 1 week, rats were used for carbofuran-induced hepatic toxicity study.

2.11. Acute toxicity study

Swiss albino male mice (n = 50) were randomly divided into five groups conveying ten animals of each group according to the organization for economic cooperation and development (OECD)-No.423 (2001) guideline to evaluate for acute toxicity study [23,24]. With the help of a stainless-steel needle attached to a plastic syringe, different doses of methanol extract (250, 500, 1000, 2000, and 4000 mg/kg body weight) were administered orally and inserted into the stomach through the esophagus. Then, all the animals were observed for mortality and clinical signs of toxicity (i.e. weight loss, general behavior, respiratory pattern, cardiovascular signs, motor activities, reflexes and changes in skin and fur texture) within 24 h with special care given during the first 4 h, and daily thereafter, for a total of 14 days.

2.12. Experimental design for hematological and hepatoprotective study

A total of 35 Sprague-Dawley male rats were randomly divided into five groups conveying seven animals of each group and were kept in the experimental period of 28 days, as follows:

Normal Control: Animals received 0.5 mL olive oil/rat with normal diet.

Negative Control (CF): Animals received CF alone at 1.5 mg/kg BW dissolved in olive oil (0.5 mL/rat).

Treatment group (CF + BTL 250): Animals received *B. tomentosa* leaf extract at a dose of 250 mg/kg BW/rat and CF (1.5 mg/kg BW) dissolved in olive oil (0.5 mL/rat).

Treatment group (CF + BTL 500): Animals received extract of *B. tomentosa* leaf at a dose of 500 mg/kg BW/rat and CF (1.5 mg/kg BW) dissolved in olive oil (0.5 mL/rat).

Standard group (CF + Silymarin 100): Animals received silymarin at a dose of 100 mg/kg BW/rat and CF (1.5 mg/kg BW) dissolved in olive oil (0.5 mL/rat).

Based on the findings of the acute toxicity study, LD_{50} of *B. tomentosa* leaf extract may be greater than 5000 mg/kg, so, to get a working dose,

divide 5000 by 10 to get 500 mg/kg BW. To get a low dose, divide 5000 by 20 to get 250 mg/kg BW. Whereas the carbofuran and *standard silymarin* dose were designated according to the findings of Wahed and co-workers [18]. All of the oral administrations were given in the morning between 09:30 am and 10:30 am and continued for 28 consecutive days. During experimentation, the rats were observed daily for any unusual behavior and death while body weight (BW) changes were observed on a weekly basis. We found that the CF-treated rats suffered from severe trembling after the administration of CF; however, conditions of all groups, except for the CF alone treated group, improved in the third and fourth weeks of treatment. After 28 days of treatment, rats in each group were injected with a deeply anesthetized drug (ketamine hydrochloride at 100 mg/kg) and sacrificed. Afterward, blood samples and liver tissues were collected for biochemical and histopathological examinations as indicated below.

2.13. Serum and liver tissue homogenate preparations

By using a heparinized syringe, blood samples (approximately 4 mL) were drained out from the inferior vena cava of rats in each group. Then, the blood samples were separated into 2 portions: one portion conveying 1 mL of blood which was placed into EDTA tubes for hematological analysis, while the remaining portion was placed into plain tubes at room temperature for 30 min before centrifugation at 3000 rpm for 10 min to yield the serum needed for subsequent biochemical analysis. At the same time, liver tissues were flensed immediately from the surrounding tissues and washed with ice-cold phosphate buffer saline, followed by weighing. Subsequently, Liver samples were homogenized with phosphate buffer saline (25 mM, pH 7.4) to produce an approximately 10 % (w/v) homogenate. Centrifugation was then done at 1700 rpm for 10 min, and the supernatant was collected prior to storage at −20 °C until biochemical analysis. For histopathological examination, a portion of the liver tissues was stored in 10 % formalin and the relative organ weight gain of the liver was calculated by dividing the liver weight by the final body weight of each rat according to the following formula: relative organ weight (%) = (wet organ weight/body weight) × 100 [25].

2.14. Evaluation of biochemical parameters in serum

We determined the biochemical parameters for liver function including, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP)), γ-glutamyl-transferase (GGT) and lactate dehydrogenase (LDH), total bilirubin (TB), total protein (TP), albumin (ALB), globulin (GLB), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C), urea, uric acid and creatinine levels by following the standard protocols of human commercial kits and by using Humalyzer 3500 (Human, Wiesbaden, Germany) [3].

2.15. Lipid peroxidation (LPO) assay

MDA is a renowned indicator of lipid peroxidation which was determined by the standard method [26]. Briefly, homogenate tissue (0.2 mL) was mixed with 8.1 % sodium dodecyl sulfate (0.2 mL), 20 % acetic acid (1.5 mL), and 8% thiobarbituric acid (1.5 mL). Subsequently, distilled water (4 mL) was added, and the mixture was heated at 95 °C in a water bath for 60 min. After finishing the heat, the mixture was then allowed to cool to room temperature, and the final volume was increased to 5 mL. The mixture of butanol/pyridine (15/1) was added, and the contents were allowed to vortex for 2 min. The mixture was then centrifuged at 3000 rpm for 10 min, the upper organic layer was extracted, and its absorbance was determined at 532 nm against a blank. Levels of MDA were expressed as nmol of thiobarbituric acid reactive substances (TBARS) per mg of protein.

The total protein in liver tissue homogenates was appraised by the method of Lowry et al. [27]. Concisely, 0.2 mL of the sample which was digested with 0.1 N sodium hydroxide was mixed with 2 mL of working reagent. The reagent consisted of 2% sodium carbonate, 0.1 N NaOH, 1.56 % copper sulfate, and 2.37 % sodium-potassium tartrate. The reaction mixture was incubated for 10 min at room temperature. The adding of 1 N Folin–Ciocalteu's phenol reagent (0.2 mL) was followed by 30 min of incubation at room temperature. Finally, the absorbance was measured at 660 nm using a spectrophotometer (Shimadzu UV PC-1600, Japan). Bovine serum albumin was used as the reference standard.

2.16. Evaluation of superoxide dismutase levels

According to Nandi and Chatterjee method, Superoxide Dismutase (SOD) level was assessed [28]. SOD assay was carried out by the pyrogallol autoxidation method. The assay mixture contained 1 mM Diethylenetriaminepentaacetic acid (DTPA), 40 μg catalase, 50 mM air-equilibrated Tris– cacodylate buffer (pH 8·5) and tissue homogenate in a final volume of 2 mL. The reaction was commenced by the adding of 100 μL of freshly prepared 2.6 mM pyrogallol solution in 10 mM HCl to attain a final concentration of pyrogallol of 0·13 mM in the assay mixture. The assay mixture was shifted to a 1.5 mL cuvette and the absorbance was measured at 420 nm using a spectrophotometer (Shimadzu UV PC-1600, Japan). Generally, one unit of SOD is described as the amount of enzyme required to cause 50 % inhibition of pyrogallol autoxidation per 3 mL of assay mixture. Therefore, Results have been expressed in units per mg protein for tissue homogenate. All the experiments were carried out in an air-conditioned room at 25 °C. Total Protein was estimated according to the method of Lowry et al. [27].

2.17. Evaluation of catalase levels

The activity of the catalase (CAT) enzyme was accomplished using hydrogen peroxide as a substrate following the method of Aebi [29]. Catalase catalyzes the decomposition of H_2O_2. In the ultraviolet range, H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in extinction at 240 nm. 0.1 mL of the tissue homogenate (approximately 0.1 mg protein) was mixed with 1.9 mL of the phosphate buffer (0.5 M, pH 7). The decrease in extinction was measured at 240 nm, 1 min interval for 3 min immediately after adding 1 mL of 11 mM H_2O_2 solution in buffer. A sample control was placed in the reference cuvette containing 0.1 mL of tissue homogenate and 2.9 mL of the buffer. The activity of catalase was calculated using the mmoles extinction coefficient 40 cm^{-1}. Units per mg protein can be calculated by following equation [(ΔA/min x 1000 × 3)/ 40 x mg protein in sample].

2.18. Evaluation of glutathione peroxidase levels

According to the method of Wahed et al., levels of endogenous glutathione peroxidase (GPx) in the hepatic tissues were assessed by using standard ELISA assay kits (CUSABIO, Baltimore, USA) [18]. To this end, the liver tissue homogenates were re-centrifuged at 12,000 rpm for 10 min at 4 °C using an Eppendorf 5415D centrifuges (Hamburg, Germany). Clean liver tissue supernatants were used for analysis and a standard assay procedure was carried out. The amounts of GPx were expressed as mIU/mL.

2.19. Histopathological evaluation

For histopathological studies, liver tissues were fixed in 10 % neutral buffered formalin. Liver tissue was trimmed (5 μm thickness) with the aid of a rotary microtome (HM 325, Thermo Scientific, U.K.) and embedded in paraffin wax. Afterward, Tissue sections were stained with hematoxylin and eosin and they were photographed with the help of an

Olympus DP 72 microscope (Tokyo, Japan) [18].

2.20. Statistical analysis

All the data are presented as mean ± standard error mean (SEM). The statistical analysis of the results was carried out with an SPSS (Statistical Packages for Social Science, version 20.0, IBM Corporation, New York, U.S.A.), Graphpad Prism (version 6.02), GraphPad Software Inc., San Diego, CA, U.S.A.), and Microsoft Excel 2013 (Redmond, Washington, U.S.A.). Data were subjected to one-way analysis of variance (ANOVA) with the aid of Tukey's multiple comparisons to analyze data sets. Differences were considered significant at 0.1 % and 5% levels of significance at $p < 0.001$ and $p < 0.05$.

3. Results

3.1. HPLC Analysis of B. tomentosa leaf extract

HPLC analysis revealed the presence of phenolic acid (Tannic, Gallic, and Salicylic acid) as well as flavonoids (Naringin) in the *B. tomentosa* leaf methanol extract (Fig. 1). Quantified phenolic acids and flavonoid compounds were presented in Table 1, where gallic acid was the most plentiful polyphenols in the leaf extract.

3.2. In-vitro antioxidant evaluation

3.2.1. Antioxidant potentials of B. tomentosa leaf extract

Quantity of total phenol in the methanol leaf extract of *B. tomentosa* was found to be 245.67 ± 0.38 mg/g, gallic acid equivalent, while the total flavonoid content was 143.51 ± 0.50 mg/g, quercetin equivalent. Similarly, total antioxidant capacity of *B. tomentosa* leaf extract was 175.63 ± 0.17 mg/g ascorbic acid equivalent.

3.2.2. In-vitro free radical scavenging tests

The outcomes of free radical scavenging assays are shown in Table 2. Among those assays, *B. tomentosa* leaf extract displayed a superior outcome in hydrogen peroxide radical scavenging (IC$_{50}$ value 61.54 ± 0.68 μg/mL) than other radical scavenging assays, yet, ascorbic acid showed the best consequence(IC$_{50}$ value 11.29 ± 0.45 μg/mL). Likewise, leaf methanol extract presented potential scavenging abilities in DPPH radical (IC$_{50}$ value 83.76 ± 0.44 μg/mL) whereas the ascorbic acid demonstrated an IC$_{50}$ value of 14.11 ± 0.24 μg/mL. *B. tomentosa* leaf extract also exhibited considerable nitric oxide and hydroxyl radical scavenging activity with IC$_{50}$ values of 198.74 ± 0.51 and

Table 1
Amount of phenolic and flavonoid compounds detected in *B. Tomentosa* leaf extract using HPLC analysis.

Standard compounds	Retention time of standard (min)	Retention time of *B. Tomentosa* (min)	Area	Concentration (mg/g)
Benzoic acid	7.812	–	–	–
Catechin	4.169	–	–	–
Cinnamic acid	4.920	–	–	–
Gallic acid	3.443	3.483	4,317,876	0.3464 ± 0.0011[d]
Naringin	5.095	5.150	116,367	0.0033 ± 0.0001[b]
Pyrogallol	5.212	–	–	–
Quercetin	19.003	–	–	–
Rutin	3.714	–	–	–
Salicylic acid	8.669	9.893	51,115	0.0095 ± 0.0001[c]
Tannic acid	2.470	2.280	50,631	0.0015 ± 0.0003[a]
Vanillic acid	3.924	–	–	–

Data are presented as means ± SEM, (n=3), Values in the same column with different superscripts are significantly different at $p < 0.05$. One way ANOVA followed by Tukey's multiple comparison was performed to analyze data set.

111.54 ± 1.18 μg/mL respectively.

3.3. Acute toxicity study

No death was observed at the highest dose (4000 mg/kg) of *B. tomentosa* leaf methanol extract in the Swiss albino male mice, therefore, the LD$_{50}$ dose for this animal should be greater than 4000 mg/kg and considered safe up to the dose of 4000 mg/kg. Moreover, there were also no signs of restlessness, respiratory distress, general irritation, coma or convulsion, hair loss, and significant bodyweight loss or gain.

3.4. In vivo hepatic-protective studies

3.4.1. Effect of B. tomentosa leaf extract on body weights and relative liver weight

The effects of *B. tomentosa* leaf extract on the body weights and relative liver weight are presented in Table 3. Compared with the control animals, the rats in the carbofuran (CF) treated group had a noteworthy decline in body weight and relative liver weight. On the other hand, the treatment groups which were treated with *B. tomentosa* leaf extract (BTL) displayed a significant amelioration in the body mass and
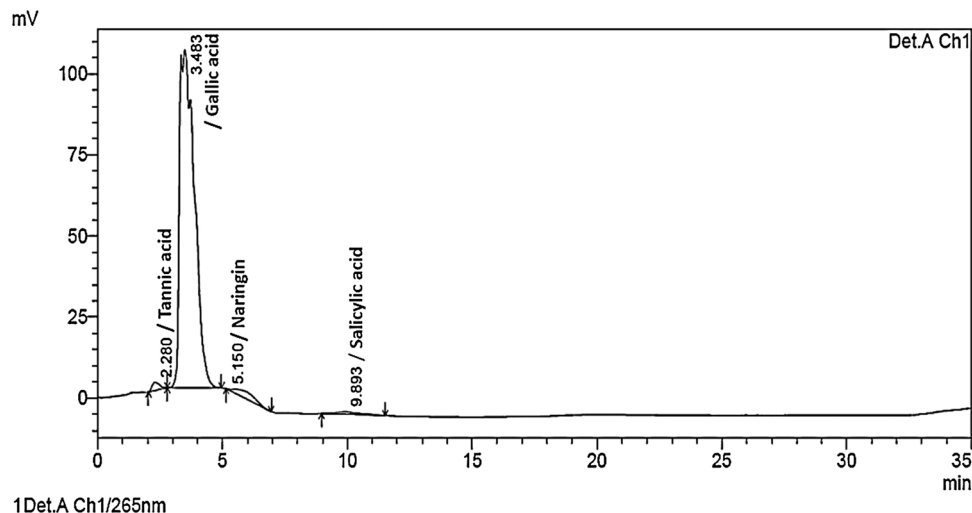
Fig. 1. HPLC chromatograph of *B. Tomentosa* leaf extract.

Table 2

Results of free radical scavenging tests of the leaf extracts of *B. Tomentosa* and standards.

Standard /Extracts	DPPH (IC$_{50}$ (μg/mL))	Nitric oxide (NO) (IC$_{50}$ (μg/mL))	Hydrogen peroxide (H$_2$O$_2$) (IC$_{50}$ (μg/mL))	Hydroxyl radical (OH)$^{•}$ (IC$_{50}$ (μg/mL))
Ascorbic acid	14.11 ± 0.24 [a]	49.04 ± 0.97 [a]	11.29 ± 0.45 [a]	25.89 ± 0.28 [a]
B. Tomentosa leaf extract	83.76 ± 0.44 [b]	198.74 ± 0.51 [b]	61.54 ± 0.68 [b]	111.54 ± 1.18 [b]

Values are the mean of triplicate experiments and represented as mean ±SEM. Values in same column with different superscripts are significantly different ($p < 0.05$). One way ANOVA followed by Tukey's multiple comparisons was performed to analyze data set.

Table 3

Effects of *B. Tomentosa* leaf extract on body weights and relative liver weight.

Parameters	Groups				
	Normal Control	Negative Control (CF)	Treatment Group (CF + BTL 250)	Treatment Group (CF + BTL 500)	Standard Group (CF + Silymarin)
Initial Body Weight (g)	152.08 ± 7.09	155.06 ± 5.13	158.05 ± 6.87	165.60 ± 9.29	155.39 ± 7.68
Final Body Weight (g)	211.55 ± 7.32	174.10 ± 6.86 [a]	185.20 ± 8.78	198.00 ± 4.77	192.91 ± 7.51
Body Weight Gain (g)	59.47 ± 5.29	19.04 ± 2.78 [b]	27.15 ± 4.45 [b]	32.40 ± 6.07 [a, x]	37.52 ± 2.24 [a, x]
Relative Liver Weight (%)	4.63 ± 0.15	3.56 ± 0.16 [b]	4.17 ± 0.09 [x]	4.40 ± 0.12 [y]	4.46 ± 0.07 [y]

Values are mean ± SEM (n = 7); Significant at [a] $p < 0.05$, [b] $p < 0.01$ when compared to normal control. Significant at [x]$p < 0.05$, [y]$p < 0.01$ when compared to negative control (CF) group.

relative liver weight to normal levels than the CF treated rats.

3.4.2. Effect of B. tomentosa leaf extract on biochemical parameters

Table 4 displays the effects of carbofuran, silymarin, and *B. tomentosa* leaf methanol extract on serum diagnostic markers in carbofuran-induced hepatic damage. Carbofuran alone treatment elevated levels of serum hepatic enzymes such ALT, AST, ALP, and LDH than the normal control group, co-treatment of both doses of BTL and silymarin, however, pointedly lowered the levels of these serum enzymes to normal levels. Carbofuran treatment caused a significant increment in GGT level for all groups compared to normal control, while, both doses of BTL and silymarin reduced its level substantially.

Like the serum hepatic enzymes, serum TB, TP, ALB, and GLB are vital markers for the assessment of hepatic function. Carbofuran treatment meaningfully raised levels of serum total bilirubin, whereas, *B. tomentosa* leaf extract significantly decreased this level in a dose-dependent manner to normal level. Carbofuran also increased globulin level significantly, while, 500 mg/kg BTL dose and Silymarin reduced the level as compared to CF treated group. On the other hand, a substantial reduction in serum total protein was witnessed in the negative control (CF) and BTL 250 treatment group, yet, this parameter was restored nearer to control levels in *B. tomentosa* leaf extract 500 mg/kg and standard silymarin. Similarly, the negative control (CF) and BTL 250 treatment group's ALB level was decreased pointedly and *B. tomentosa* leaf extract showed a dose-dependent noteworthy raise this level compared to intoxicated rats (Table 5).

Our experimental study also revealed that animals treated with carbofuran at a dose of 1.5 mg/kg evidently increased the TC, TG, LDL-C, and VLDL-C levels with respect to the control group. However, co-treatment with *B. tomentosa* leaf methanol extract pointedly abridged

these lipid profile levels in a dose-dependent manner. Unlikely, HDL-C level diminished in a remarkable manner when experimental rats were exposed to carbofuran compared with the control group nonetheless leaf extract 500 mg/kg dose co-treatment improved HDL-C level significantly. The standard group showed the best effect in these lipid profile levels (Table 6).

Carbofuran treated group demonstrated a considerable rise in serum urea, uric acid, and creatinine level, yet, co-treatment with leaf extract of *B. tomentosa* caused a dose-dependent amelioration of these markers levels with the standard silymarin (Table 7).

3.4.3. Effect of B. tomentosa leaf extract on lipid peroxidation

In Fig. 2, the outcomes with the treatment of carbofuran, *B. tomentosa* leaf methanol (BTL) extract, and silymarin are illustrated on the levels of malondialdehyde in the liver tissue of experimental rats. When compared with the control group, the malondialdehyde (MDA) levels were suggestively ($p < 0.01$) higher in carbofuran-intoxicated rats. However, the results showed that MDA level was substantially ameliorated by BTL in a dose-dependent manner as compare to carbofuran group and 500 mg/kg dose showed better outcome with the standard group, all these findings recommended the hepatocellular protection of BTL against CF-induced elevation of lipid peroxidation as evidenced by a significant decrease in MDA levels.

3.4.4. Effect of B. tomentosa leaf extract on liver antioxidant enzymes

Table 8 revealed the consequences of the cellular antioxidant enzymes such as SOD, CAT, and GPx levels in the hepatic tissues of experimental rats. There was a substantial ($p < 0.01$) decline in these antioxidant enzyme levels in rats only treated with carbofuran than the normal control group. Nevertheless, following the CF administration,

Table 4

Effects of *B. Tomentosa* leaf extract on serum hepatic marker enzymes.

Serum Hepatic Marker Enzymes	Groups				
	Normal Control	Negative Control (CF)	Treatment Group (CF + BTL 250)	Treatment Group (CF + BTL 500)	Standard Group (CF + Silymarin)
ALT (U/L)	68.60 ± 4.03	97.00 ± 2.43 [b]	67.40 ± 1.99 [y]	60.40 ± 2.71 [y]	54.44 ± 4.27 [y]
AST (U/L)	125.20 ± 7.82	180.60 ± 4.31 [b]	145.20 ± 3.70 [y]	140.00 ± 5.36 [y]	123.20 ± 5.41 [y]
ALP (U/L)	229.80 ± 8.77	320.40 ± 7.72 [b]	251.20 ± 5.65 [y]	233.40 ± 5.27 [y]	229.80 ± 6.57 [y]
GGT (U/L)	2.25 ± 0.15	5.82 ± 0.26 [b]	4.59 ± 0.25 [b, y]	4.11 ± 0.14 [b, y]	3.05 ± 0.23 [b, y]
LDH (U/L)	62.60 ± 3.96	112.80 ± 5.49 [b]	79.20 ± 3.44 [y]	67.80 ± 3.77 [y]	60.40 ± 5.80 [y]

Values are mean ± SEM (n = 7); Significant at [a] $p < 0.05$, [b] $p < 0.01$ when compared to normal control. Significant at [x]$p < 0.05$, [y]$p < 0.01$ when compared to negative control (CF) group.

Table 5
Effects of *B. Tomentosa* leaf extract on TB, TP, ALB, and GLB.

Biochemical Parameters	Groups				
	Normal Control	Negative Control (CF)	Treatment Group (CF + BTL 250)	Treatment Group (CF + BTL 500)	Standard Group (CF + Silymarin)
TB (mg/dL)	0.168 ± 0.007	0.219 ± 0.010 [b]	0.178 ± 0.004 [x]	0.162 ± 0.004 [y]	0.164 ± 0.004 [y]
TP(g/dL)	6.84 ± 0.06	5.81 ± 0.03 [b]	6.48 ± 0.03 [b, y]	6.70 ± 0.04 [y]	6.68 ± 0.03 [y]
ALB (U/L)	3.28 ± 0.04	2.46 ± 0.09 [b]	2.79 ± 0.09 [a, x]	3.06 ± 0.07 [y]	3.27 ± 0.06 [y]
GLB (g/dL)	1.82 ± 0.11	3.17 ± 0.12 [b]	3.00 ± 0.09 [b]	2.31 ± 0.12 [x]	2.10 ± 0.21 [y]

Values are mean ± SEM (n = 7); Significant at [a] $p < 0.05$, [b] $p < 0.01$ when compared to normal control. Significant at [x]$p < 0.05$, [y]$p < 0.01$ when compared to negative control (CF) group.

Table 6
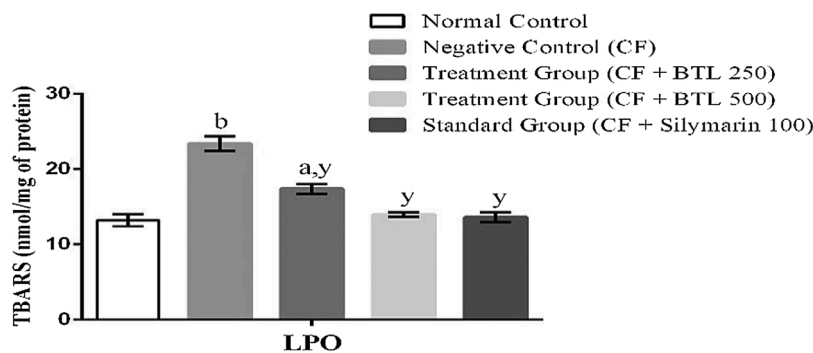Effects of *B. Tomentosa* leaf extract on serum lipid profiles.

Serum Lipid Profiles	Groups				
	Normal Control	Negative Control (CF)	Treatment Group (CF + BTL 250)	Treatment Group (CF + BTL 500)	Standard Group (CF + Silymarin)
TC (mg/dL)	76.80 ± 2.88	153.65 ± 8.33 [b]	126.17 ± 6.15 [b, x]	99.48 ± 5.53 [y]	83.00 ± 6.19 [y]
TG (mg/dL)	66.05 ± 4.03	127.74 ± 5.21 [b]	109.30 ± 3.52 [b, x]	98.87 ± 3.59 [a, y]	70.55 ± 2.65 [y]
HDL-C (mg/dL)	49.18 ± 3.45	28.52 ± 1.79 [b]	32.54 ± 1.12 [b]	40.12 ± 1.61 [x]	43.03 ± 1.95 [x]
LDL-C (mg/dL)	14.42 ± 5.33	99.58 ± 4.87 [b]	71.77 ± 6.07 [b, x]	39.59 ± 5.12 [a, y]	25.86 ± 6.95 [y]
VLDL-C(mg/dL)	13.21 ± 0.81	25.55 ± 1.04 [b]	21.86 ± 0.70 [b, x]	19.77 ± 0.72 [a, y]	14.11 ± 0.53 [y]

Values are mean ± SEM (n = 7); Significant at [a] $p < 0.05$, [b] $p < 0.01$ when compared to normal control. Significant at [x]$p < 0.05$, [y]$p < 0.01$ when compared to negative control (CF) group.

Table 7
Effects of *B. Tomentosa* leaf extract on serum renal markers.

Serum Renal Markers	Groups				
	Normal Control	Negative Control (CF)	Treatment Group (CF + BTL 250)	Treatment Group (CF + BTL 500)	Standard Group (CF + Silymarin)
Urea (mmol/L)	19.35 ± 1.03	32.80 ± 1.62 [b]	26.66 ± 0.75 [a, x]	21.76 ± 1.79 [y]	19.95 ± 0.71 [y]
Uric Acid (mmol/L)	1.74 ± 0.05	3.05 ± 0.08 [b]	2.63 ± 0.07 [a, x]	1.83 ± 0.07 [y]	1.83 ± 0.06 [y]
Creatinine (mmol/L)	0.64 ± 0.02	0.82 ± 0.01 [b]	0.72 ± 0.02 [x]	0.67 ± 0.02 [y]	0.65 ± 0.01 [y]

Values are mean ± SEM (n = 7); Significant at [a] $p < 0.05$, [b] $p < 0.01$ when compared to normal control. Significant at [x]$p < 0.05$, [y]$p < 0.01$ when compared to negative control (CF) group.

Fig. 2. Effects of *B. Tomentosa* leaf extract on LPO assay. Values are mean ± SEM (n = 7); Significant at [a] $p < 0.05$, [b] $p < 0.01$ when compared to normal control. Significant at [x]$p < 0.05$, [y]$p < 0.01$ when compared to negative control (CF) group.

Table 8
Effects of *B. Tomentosa* leaf extract on liver antioxidant enzymes.

Liver Antioxidant Enzymes	Groups				
	Normal Control	Negative Control (CF)	Treatment Group (CF + BTL 250)	Treatment Group (CF + BTL 500)	Standard Group (CF + Silymarin)
SOD (U/mg protein)	4.48 ± 0.12	1.78 ± 0.07 [b]	2.80 ± 0.09 [a, x]	3.93 ± 0.06 [y]	4.19 ± 0.09 [y]
CAT (U/mg protein)	27.72 ± 0.58	16.62 ± 0.75 [b]	20.17 ± 0.59 [b, x]	23.76 ± 0.35 [a, y]	27.84 ± 0.83 [y]
GPx (mIU/mL)	0.64 ± 0.02	0.82 ± 0.01 [b]	0.70 ± 0.02 [a, x]	0.68 ± 0.02 [y]	0.65 ± 0.01 [y]

Values are mean ± SEM (n = 7); Significant at [a] $p < 0.05$, [b] $p < 0.01$ when compared to normal control. Significant at [x]$p < 0.05$, [y]$p < 0.01$ when compared to negative control (CF) group.

oral co-administration of *B. tomentosa* leaf methanol (BTL) extract for 28 days pointedly refurbished the levels of these enzymes in a dose-dependent manner as compared to the carbofuran treated group. Standard Silymarin group revealed significant results for these antioxidant enzymes.

3.4.5. Effect of B. tomentosa leaf extract on histological examination of liver tissue

Histopathological examination of hepatic tissues of the normal control group displayed usual cellular architecture with the distinct arrangement, sinusoidal spaces, and central vein (Fig. 3A). However, degeneration of hepatocytes with centrilobular necrosis, inflammatory cell infiltrations, and vascular edematous congestion was observed in the only carbofuran-treated group (Fig. 3B). Co-administration of leaf extract at a dose of 250 mg/kg with carbofuran treatment showed some improvements with a moderate degree of hepatocytes degeneration with vascular edematous congestion and inflammatory cell infiltrations (Fig. 3C). Likewise, co-administration of *B. tomentosa* leaf extract at a dose of 500 mg/kg with carbofuran treatment reduced the hepatic damages with little necrosis and less vascular congestion and showed noticeable protection. (Fig. 3D). In Fig. 3E, treatment of CF-intoxicated rats with the standard drug, silymarin showed a substantial reduction in the degeneration of the cellular arrangement.

4. Discussion

The current experimental work was designed to identify and quantify the phenolic and flavonoid compounds and to evaluate *in-vitro* free radicals scavenging activities of *B. tomentosa* leaf extract and correlate these potentialities with protective activity against carbofuran induced hepatotoxicity.

Generally, pesticides exert their toxicity by inducing oxidative stress [30]. Carbofuran treatment induces the generation of reactive oxygen and nitrogen species (ROS and RNS) which are accountable for the peroxidation of membrane phospholipids that alter the normal function of the lipid bilayer and cause numerous pathological ailments [6]. Antioxidants with ROS and RNS scavenging capability may have great significance in the inhibition of oxidative stress. According to our findings, methanolic extracts of *B. tomentosa* leaf showed a worthy amount of antioxidant potentialities in total polyphenol, total flavonoid, and total antioxidant capacity assay. Apart from that, HPLC analysis verified the presence of tannic acid, gallic acid, salicylic acid and naringin in the leaf extract. These bioactive constituents exert diverse biological activities such as antioxidant, antiradical, anticancer, anti-inflammatory, cardio and hepatoprotective effects [31]. Similarly, *B. tomentosa* leaf methanol extract exhibited *in-vitro* antiradical potentialities. Stable radical scavenging activity is reflected in the antiradical properties of natural bioactive compounds. DPPH is a comparatively established free radical and it was ascertained that DPPH is reduced to the corresponding hydrazine when it reacts with the hydrogen donor moiety of the antioxidants [20]. Nitric oxide produced a highly reactive peroxy-nitrite anion which is associated with various carcinomas and inflammatory ailments [17]. Hydrogen peroxide and hydroxyl radicals cause severe injury to membranes, proteins, and DNA. Therefore, the removal of these radicals is important for diminishing oxidative stress [32] and that work was done by phenolic acids and flavonoids of our experimental plant. At first, Gallic acid is recognized as a potent scavenger of reactive oxygen and nitrogen species [33]. It owns anticancer, anti-bacterial, anti-depressant, antioxidant and anti-obesity effects [34]. The outcome of the earlier study evidently proved that gallic acid possesses promising protective effects in paracetamol-induced hepatic damage in mice [35]. Furthermore, Goudarzi and his peer revealed that gallic acid replenishes the actions of hepatic antioxidant enzymes and biomarkers in mercuric chloride-intoxicated rats, undoubtedly by scavenging of toxic ROS and improving the antioxidant defensive mechanisms [36]. Similarly, tannic acid has been found to scavenge DPPH radicals, ABTS radicals, superoxide anion, and hydrogen peroxide scavenging activities [37]. El-Khawaga demonstrated that tannic acid has the potentiality to mitigate the adverse effects induced by methomyl toxicity [38]. Due to antioxidant activity, tannic acid exerted its hepatoprotective action by reducing lipid peroxidation and increasing enzymatic and non-enzymatic antioxidants levels in carbon tetrachloride-induced toxicity [39]. After that, Salicylic acid and its derivatives are also capable to scavenge DPPH, nitric oxide, hydroxyl radicals [40] and
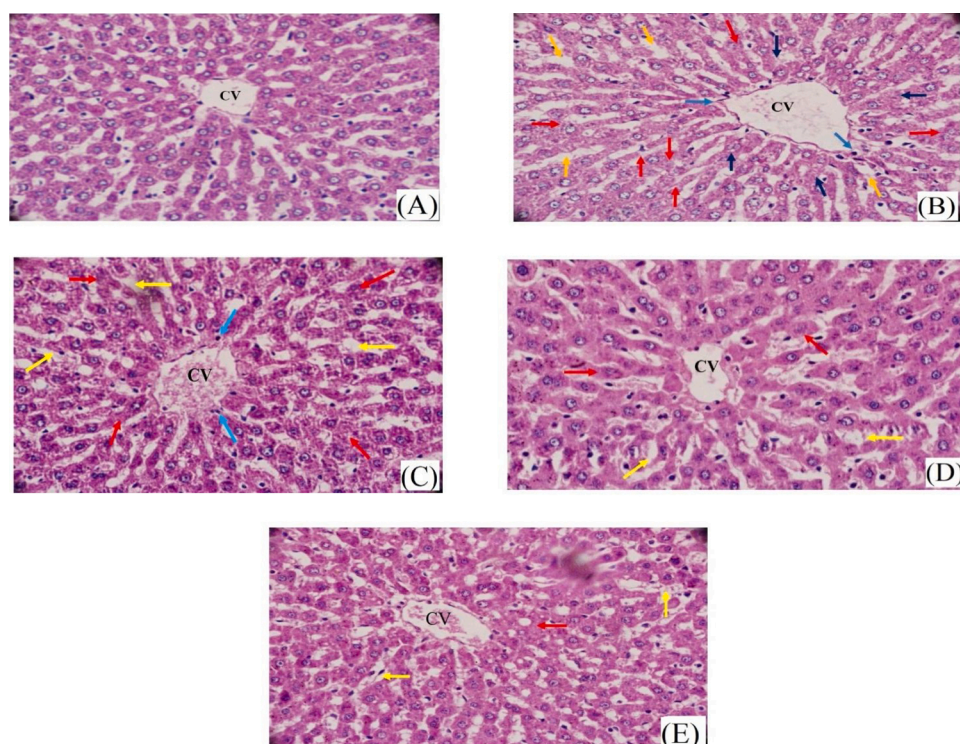
Fig. 3. Effects of *B. Tomentosa* leaf extract on histological examination of liver tissue against carbofuran induced histological changes in the investigational animals. **(A):** normal control, **(B):** Negative Control, (CF treated), **(C):** Treatment Group (CF + BTL 250), **(D):** Treatment Group (CF + BTL 500), **(E):** Standard Group (CF + Silymarin) [Magnification: 40X; scale bar: 20 μm]. (Black and red arrows indicate necrosis at the peripheral area of the central vein and the lobule respectively, while blue arrow delineates congestion in the central vein linked with profound inflammatory infiltrates and yellow arrow shows edema at different positions).

reduce the risk of cancer [41]. Salicylic acid is an inhibitor of oxidative stress which lessened superoxide anion radicals by acting on NADPH activity that reduced ROS in human endothelial cells [42]. Zhao and associates presented the protective effect of salicylic acid against sulfur dioxide-induced lipid peroxidation in mice [43]. Some scientific studies also concluded that salicylic acid reduced oxidative damage and exhibited protective effects against paclitaxel and cisplatin-induced neurotoxicity [44]. On the other hand, Naringin is a natural flavanone glycoside that possesses different biological and pharmacological properties [45] and showed potent hydroxyl radicals, superoxide, hydrogen peroxide, nitric oxide radical, and DPPH radical scavenging activity [46]. Shirani and his peers demonstrated the hepatoprotective effects of naringin against different chemical toxicants such as acetaminophen, doxorubicin, cyclophosphamide, cisplatin, and carbon tetrachloride [47]. In the same way, chronic naringin treatment mitigated oxidative damage, as supported by dropping of malondialdehyde level and refurbishment of superoxide dismutase, catalase and glutathione S-transferase levels against colchicine-induced oxidative damage [48]. Therefore, affluence with these phenolic compounds and flavonoids, *B. tomentosa* leaf methanol may show protective activity by lessening oxidative stress induced by carbofuran.

In toxicity studies, body weight and relative organ weights are vital criteria for the assessment of related organ damage. Carbofuran exposure over four weeks produced a substantial decrease in rat's body weight which might either be due to the direct pesticide-induced cholinergic or oxidative trauma of somatic cells and/or indirectly affect the nervous system which regulates feed and water intake as well as hampers absorption process of nutrients from the gut [25]. Toxic biomolecules of lipid peroxidation also caused cellular damage that reduced the body mass. Increased lipid peroxidation in the carbofuran-intoxicated rats of our study strengthened these outcomes as connected to the earlier study [3]. Moreover, the reduction in the relative liver weights was observed in our study on carbofuran treatment that could be endorsed by the rapid destruction of hepatocytes as a result of lipid peroxidation [49]. Nonetheless, co-treatment of *B. tomentosa* leaf extract with carbofuran amended the body and liver weights anomalies, which is illuminating of its protective effect by its antioxidant capabilities.

ALT, AST, and ALP are crucial indicators of hepatocellular injury. Usually, ALT catalyzes transamination reaction and an increment in ALT serum levels delineates liver damage. AST, an important enzyme in amino acid metabolism, is found in the liver, heart, skeletal muscle, kidneys, brain, and red blood cells. AST discharge into blood signifies liver damage. Likely, ALP belongs to the hydrolases family and the raise of this enzyme indicates hepato-biliary ailments [50]. Outcomes of the current experiment revealed that oral carbofuran administration led to a noteworthy increase in these enzyme activities in the serum. Due to the alteration of the membrane permeability of the hepatocytes, these cytosolic enzymes were liberated into the systemic circulation, demonstrating necrosis and inflammatory reactions [51]. However, rats treated with BTL suggestively refurbished these enzyme levels by its protective activity. Lactate dehydrogenase (LDH) found in the cytoplasm catalyzes the inter-conversion of lactate and pyruvate. The liberation of this enzyme indicated the opening of the cellular membrane or cellular death [52]. Jaiswal and his co-researchers validated that carbofuran dropped the hepatic LDH level with a rise of serum LDH which was similar to our study [53]. LDH level in serum was mitigated by leaf extract of *B. tomentosa* which showed its protective activity by phenolic acids and flavonoids of this plant. Gamma-glutamyltransferase is another microsomal enzyme found in hepatocytes and biliary epithelial cells. Augmented serum GGT levels are observed in chronic liver disease which is associated with bile duct damage and fibrosis [54]. Carbofuran treatment increased serum GGT level where co-administration of *B. tomentosa* leaf extract reduced the level near to the normal control group.

The serum total bilirubin, total protein, albumin, and globulin levels are also important markers for hepatocellular noxiousness. Bilirubin is a byproduct of haem which derived from blood cells breakdown and carried to the liver by serum albumin, where it is excreted into the bile after glucuronide conjugation. The upsurge in the bilirubin level indicates the severity of necrosis hepatic cells [55]. A significant elevation was perceived in the bilirubin level of carbofuran treated rats where *B. tomentosa* leaf methanol extract restored this level to typical. Serum total protein levels are responsible for the severity of the necrosis and the protein synthesis process [51]. Likely, Serum albumin levels are one of the standard tests of liver function because low albumin can point out severe liver disease [56]. Serum TP and ALB levels were evidently abridged in the carbofuran-treated group, while co-administration of leaf methanol extract of *B. tomentosa* with carbofuran amended their levels. Furthermore, Serum globulin has been specified as prognostic markers for certain hepatocellular malignancies [57]. In our study, serum globulin amount purposely augmented in carbofuran treatment group whereas co-administration of leaf extract of *B. tomentosa* reduced the level and ameliorated carbofuran induced toxicity. Therefore, observed outcomes were plausible by the phenolic acid and flavonoid of the leaf extract which confers protective properties to preserve the membrane integrity.

The Lipid profile is one of the most indicative biomarkers of hepatic tissue damage. Oxidative stress affected lipid metabolism by damaging cellular membrane integrity and causing some membrane lipids to be liberated into the systemic circulation [58]. The present study has demonstrated the significant increase of TC, TG, LDL-C, and VLDL-C as well as a substantial decrease in the serum HDL-C level in carbofuran treated rats. Increased TC level suggests that carbofuran has the ability to affect the cell membrane permeability and amplify cholesterol synthesis in the liver. Additionally, due to blockage of liver bile ducts initiating reduction or cessation of cholesterol secretion to the intestine may result in cholestasis, which illuminates the liver damage [59]. Raised triglycerides delivered a strong indication of the augmented hepatic glyceride synthesis that is proportionate to the concentration of fatty acid and glycerophosphate and may contribute to the increased VLDL-C, which is the precursor of bad cholesterol LDL-C [25]. Nevertheless, parallel administration of *B. tomentosa* leaf extract corrected the alterations of the serum lipid levels in the experimental rats. On the other hand, HDL-C is regarded as good cholesterol and a low level of HDL-C concentration has been shown to be associated with an increased risk of developing cardiovascular disease. The earlier report recommended that low levels of HDL-C were induced with carbofuran treatment in experimental animals [60]. Co-treatment with leaf extract of *B. tomentosa* pointedly resolved this level which reveals ameliorative effects against this pesticide-induced toxicity. Raised serum urea, uric acid, and creatinine levels intensely reflect oxidative stress-induced metabolic syndromes that are critical factors for the development of liver disease [61]. Uric acid and creatinine levels were detected to be elevated pointedly after carbofuran exposure, which was similar to previous study [62]. Outcomes of the present study signified that *B. tomentosa* leaf extract restored these levels to normal which may be plausible for the presence of phytochemicals such as tannic acid, gallic acid, and naringin.

The escalation in the Malonaldehyde (MDA) level is reflected at the beginning of oxidative stress in experimental animals that are exposed to detrimental toxicants. Toxicants exert their toxicity via the generation of free radicals (hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, nitric oxide radical, and peroxynitrite radical) and production of oxidative stress [6]. The findings of the current study revealed the substantial elevation in the levels of MDA with carbofuran treatment, proposing, thereby, the generation of this pesticide-induced oxidative damage in the hepatic tissues. Like the other carbamates, carbofuran inhibits Acetylcholinesterase (AChE) activity which increases acetylcholine concentration at the synaptic junctions. Augmented acetylcholine causes a hyper-excitation state that increases the flow of oxygen [7]. This exciting situation causes the generation of

reactive oxygen species that triggered oxidative cellular damage [30]. Some researchers demonstrated that another mechanism linked with the overproduction of reactive oxygen species involved the inhibition of cytochrome c oxidase and creatine kinase. [63,64]. Furthermore, carbofuran also tempted nitric oxide synthase action, which has been connected with the overproduction of superoxide anions [65]. These toxic ROS cause lipid peroxidation of polyunsaturated fatty acids of bio-membranes and generate several compounds such as alkanes, malanoaldehyde, and isoprostanes [66]. Jaiswal and co-researchers informed similar outcomes that carbofuran treatment increased MDA levels in the rat's liver and brain [67]. However, co-administration with *B. tomentosa* leaf extract pointedly abridged the levels of MDA in carbofuran-treated rats, signifying the protective effects of *B. tomentosa* against oxidative tissue damage. The diminution in MDA level may be due to the direct scavenging of ROS by phenolic acids and flavonoids of *B. tomentosa* which exhibited potent *in-vitro* radical scavenging activity.

To combat these reactive free radicals, our human body has some endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). These antioxidant enzymes inhibit oxidative damage principally through their free radical scavenging property [68]. SOD transforms the super-oxide radical to hydrogen-peroxide and molecular oxygen whereas catalase converts the hydrogen-peroxide to molecular oxygen and water, therefore, abolishing the pro-oxidative influence of the harmful radicals [69]. Similarly, Glutathione peroxidase, glutathione-s-transferase, and peroxidase are responsible to decompose toxic lipid hydro-peroxides to corresponding non-toxic lipid alcohols [68]. In addition, the presence of ample super-oxide radicals hinders the activity of catalase. Overproduction of singlet oxygen and peroxyl radicals also has an impact on SOD and catalase activities [70]. Therefore, the overproduction of ROS decreases these enzyme levels in the liver tissue. These consequences were quite similar to earlier studies where carbofuran exposure produces MDA levels and decreases the antioxidant levels of liver tissues in rats [3,17]. To combat this unbearable situation, dietary antioxidants would improve the damage caused by oxidative stress by inhibiting the initiation or propagation of chain reaction, performing as free radical scavengers, quenchers of singlet oxygen and reducing agents [71]. For this reason, *B. tomentosa* leaf extract supplementation improved these anti-oxidative enzyme activities in a significant manner that had been abridged by carbofuran. Phenolic acids and flavonoids of *B. tomentosa* leaf methanol extract may combat highly toxic free radical-induced oxidative damage. Moreover, significant *in-vitro* total polyphenol, total flavonoid, and total antioxidant capacity assay showed a good amount of antioxidant potentialities and free radicals scavenging activities of leaf extract validated the higher anti-oxidative properties of *B. tomentosa*. Hence, the identified phenolic compounds and flavonoids in *B. tomentosa* leaf extract might be accountable for its pragmatic protective activity. The significant feature of the histopathological evaluation of hepatic disease is the identification of the degree of histo-architectural alterations in hepatocytes [72]. Hepatic biochemical function tests have confirmed the findings of histopathological lesions detected in our present study. Toxic carbofuran produced reactive oxygen species-mediated molecular damage of membrane components of the hepatocytes. Degeneration and necrosis of hepatocytes, inflammatory cell infiltrations, and vascular congestion with edematous spaces were common features in carbofuran-intoxicated rat liver that were similar to our observations [18]. On the contrary, the liver section of the rats treated with *B. tomentosa* leaf extract and silymarin, displayed a notable degree of protection in the cellular arrangement. Therefore, our biochemical and histopathological findings collaboratively may propose that identified phenolic acids and flavonoids scavenged the toxic free radicals and inhibited oxidative injuries and the subsequent degeneration as well as necrosis of hepatic tissues.

5. Conclusion

Altogether, the results of the present study revealed that carbofuran induced perturbations in rat liver via oxidative stress, as depicted by the elevated levels of MDA and the reduced enzymatic activities of SOD, catalase, and GPx. It also caused significant changes in the levels of serum hepatic identifying enzymes, lipid profiles, total bilirubin, total protein, albumin, globulin, and histopathological architecture. On the other hand, the experimental rats that were co-treated with *B. tomentosa* leaf methanol extract followed by carbofuran administration suggested the ameliorative potentiality to reduce the oxidative stress and refurbishment of the aforementioned parameters to near normal values. On top of that, identified phenolic acids and flavonoids in *B. tomentosa* leaf extract exerted in vitro antiradical activity which may be accountable for diminishing oxidative stress induced by carbofuran. The authors further emphasize that more comprehensive studies are prerequisites for establishing the safety and efficacy of this plant extract as a therapeutic intervention.

Ethical approval

The study was conducted following the approval by the Biosafety, Biosecurity and Ethical Committee [Approval Number: BBEC, JU/M 2018 (1)3] of Jahangirnagar University, Savar, Dhaka, Bangladesh.

Author statement

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Sukalyan Kumar Kundu: Supervision

Declaration of Competing Interest

The authors report no declarations of interest.

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