Commelina benghalensis attenuates cyclophosphamide induced hepatotoxicity by preserving hepatic mitochondrial activity through upregulating pro-mitochondrial proteins

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Abstract

Background

Anticancer therapy is largely impeded by its non-specificity and toxicity. They induce cell death by triggering oxidative stress. Cyclophosphamide (CP) is used as antineoplastic agent against solid tumours, lymphomas and leukaemias. Metabolites of CP will potentially damage the glutathione reserves and thereby induces cell death. Strategies that can attenuate this off-target effect of CP could be helpful in successful treatment of cancers with fewer side effects. *Commelina benghalensis (Linn)* belongs to apocynaceae family and is widely used in oriental traditional medicine. Despite of its medicinal value, its potential against drug induced hepatotoxicity is unknown. The present study evaluates the hepatoprotective effect of hydroethanolic extract of *Commelina benghalensis* (HECB) in rat model of CP induced hepatotoxicity.

Methods

Chemical characterization of HECB was carried out followed by DPPH assay. Liver weight, serum hepatic enzyme activity and hepatic antioxidant reserves were estimated after treatments at 50 and 100 mg/kg for 8 weeks. Inflammatory markers such as IL-6 and TNFα were analysed in the tissue lysates. Mitochondrial integrity was performed by analysing Complex I activity followed by estimating the NRF2 and Mitochondrial Transcriptional Factor A (TFAM) levels. Histopathological analysis of liver was carried out and phenobarbitone induced sleeping time was performed to confirm the hepatoprotective effect.

Results

Flavonoids and phenolic compounds were found at higher concentrations of 50 and 100 µg/ml with a significant free radical scavenging activity as displayed by DPPH assay. Administration of CP has resulted in increased liver weight, elevated serum hepatic enzyme activity along with Inflammatory markers, decreased hepatic antioxidant reserves, profound oxidative stress and impaired mitochondrial activity. Correspondingly, daily oral administration of HECB reduced actual and relative liver weights, normalized circulating hepatic enzyme activity as well as inflammatory markers, hepatic oxidative stress and restored antioxidant reserves. Further hepatic mitochondrial activity, NRF2 and TFAM levels were also improved. Hepatoprotective effect pronounced by HECB was further confirmed by histopathological analysis and phenobarbitone induced sleeping time. Nonetheless, the hepatoprotective effect was more prominently observed at 100 mg/kg dose.

Conclusion

Conclusively, the study provides preliminary evidence regarding hepatoprotective activity of HECB and the contribution of its antioxidant potential towards this pharmacological effect.

1. Background

Antineoplastic agents lack cellular specificity making them cytotoxic to normal cells even at the therapeutic doses [1]. The most vulnerable sites of toxicity are the rapidly dividing cells such as hair follicular cells, intestine epithelium, hepatocytes, gonadal cells and bone marrow cells [2]. Hence requisite for search of compounds which can lessen the off-target side effects of anticancer drugs in normal tissues are emphasised. Cyclophosphamide (CP), a nitrogen mustard and an alkylating agent is a widely used as antineoplastic and immunosuppressant. It is noted to be an effective therapeutic agent in managing auto immune diseases, lymphomas, leukemias and solid tumours [3]. Being a pro drug, CP when taken orally, rapidly gets converted by mixed-function oxidase enzymes (cytochrome P450 system) in the liver to its active metabolites. Major active metabolite of CP is 4-hydroxycyclophosphamide, a tautomer of aldophosphamide which is highly protein bound. Due to its high tissue distribution coefficient it is known to cross the placenta and known to be secreted through breast milk [4]. Detoxification of CP is mainly by oxidising 4-hydroxycyclophosphamide into its in-active metabolite called carboxyphosphamide by aldehyde dehydrogenase (ALDH). Even though the carboxyphosphamide remains to be the major detoxification product, a small proportion of aldophosphamide undergoes spontaneous beta elimination resulting in toxic metabolites like phosphoramide mustard and acrolein. It is known that majority of adverse toxic effects of CP are mediated by acrolein, which gets metabolised by glutathione depended alkylation resulting in drastic decrease in glutathione reserves and subsequent disturbance of redox balance in the cell [5]. This turns out to be the major reason for the declining usage of CP not withstanding of its therapeutic potency.

Oxidative stress induced by CP has immediate detrimental effects on all rapidly dividing as well as metabolically active tissues causing impairment in their physiology. Organs like liver and kidney are also known to be affected. Even though Liver injury from therapeutic doses of CP is rare, in cases such as myeloablative therapy, where higher doses of CP is given as in preparation for hematopoietic cell transplantation can result in sinusoidal obstruction syndrome leading to acute liver failure in 25–50% of patients [6],[7],[8]. Even though CP metabolite acrolein and redox imbalance are focussed to be the major perpetrators, the exact mechanism of the toxicity remains idiosyncratic. Currently no specific therapy is available for the idiosyncratic hepatic failure due to CP other than supportive management to prevent further damage. On the other hand, studies have corroborated the occupational exposure of CP amongst health care professionals, and reported several toxicity including reproductive, dermal and carcinogenic effects [9].

Commelina benghalensis (CB) L belonging to the apocynacae family is a native to the tropical and subtropical regions in Africa, Asia and The Pacific. The whole plant of CB was reported to contain alkaloids, volatile oils, wax, vitamin-C and higher levels of both lutein and β-carotene. In the northern Thailand, the plant is used as a post-partum medication, diuretic, febrifuge and anti-inflammatory [10],[11],[12]. Ethanol extracts of the plant is found to be active against *Candida albicans* infections and aqueous extract is active against *Escherichia coli* and *Staphylococcus aureus* infections justifying the traditional claims [13]. Despite of these cytoprotective studies, no profound evidence exists regarding the effect of this plant against drug induced toxicity. In this scenario the present study is aimed to evaluate the hepatoprotective effects of Hydro Ethanolic extract of CB (HECB) on CP induced hepato toxicity in SD rats.

2. Methods and Materials

2.1. Animals and Study Design:

Twenty-four male albino Sprague Dawley rats of 8 weeks age weighing approximately 130–150 g were used for the experimentation. Animals were acclimatized for a period of one week prior to experimentation. All animal care and experimental procedures for this study were approved by the Institutional Animal Ethics Committee (IAEC No: 09/NIPER/CPCSEA/351), according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and ARRIVE Guidelines. The animals were housed at 22 ± 2° C, maintained on a 12 h light-dark cycle, fed with regular Rat Chow and had free access to drinking water. Rats were randomly divided into four groups.

- Control group (n = 6) received daily oral dose of CMC for 8 weeks.
- CP group (n = 6) received a weekly once intraperitoneal CP for 8 weeks at a dose of 15 mg/kg Body weight [14].
- HECB50 (n = 6) received a weekly once intraperitoneal CP for 8 weeks at a dose of 15 mg/kg Body weight in addition to daily oral dose of 50mg/kg Body weight HECB suspended in CMC.
- HECB100 (n = 6) received a weekly once intraperitoneal CP for 8 weeks at a dose of 15 mg/kg Body weight in addition to daily oral dose of 100mg/kg Body weight HECB suspended in CMC.

2.2. Preparation of the extract:

The plant was collected from the State of Madhya Pradesh, India and was authenticated by Guwahati University, Assam, India. A voucher specimen no. 4653–54 was maintained. The selected parts of the plant were then shade dried for 15 to 20 days, chopped and pulverised to dry powder. Powdered crude drug of all parts of plant (800 g) was subjected for extraction process by maceration using solvents (70% ethanol and 30% water) at room temperature for 7 days. The extract was filtered and concentrated by vacuum dried to avoid the decomposition of natural metabolites. The yield was found to be approximately 15% w/w.

2.3. LC-MS analysis of HECB:

The ethanolic extract was further processed for LCMS (Water Bio Accord LC-MS System) analysis for Identification of Phytochemicals present in the extract. LC-MS (liquid chromatography-mass spectroscopy) analysis was carried out using Accucore C18 column (150*4.6 mm, 2.6µm) The detection was performed through direct injection mode with electron spray ionization (ESI) probe at Positive and negative mode. The sample flow rate was kept at 1mL/min and the capillary temperature was maintained at 280□C. The acquisition mode for mass was set from min range 50 (m/z) and Max of 1000 (m/z). Solvent composition was 0.5mm acetic acid in water (95%) for pump A and 5% Acetonitrile for pump B. The resulting total ion chromatogram is a time vs area plot which shows each phytoconstituents overall response based on the abundance of its molecular ions.

2.4. In Vitro Antioxidant Assays:

Total phenolic contents of HECB was determined by the modified Folin-Ciocalteu method [15]. Briefly, 500 µl of HECB at a concentration of 1,12.5,25,50,100 µg/ml are mixed with 1000 µl of 1:10 Folin-Ciocalteu's reagent and incubated at room temperature for 5 min. After this 900 µl of saturated (7.5%) sodium carbonate solution

was added and the reaction mixtures were further incubated at room temperature for 1 hour. The absorbance of the reaction mixtures was recorded at 765 nm and the total phenolic contents have been expressed as gallic acid equivalents mg/100 g of the extracts.

Total Flavanoid content of the HECB was estimated as follows. Breifly, 1,12.5,25,50,100 µg HECB is mixed with 1 ml of 95% methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water and vortexed for 5min. The resultant solution is incubated in dark for 30 min at room temperature. The absorbance of the reaction mixture was recorded at 415 nm. The flavonoid content of HECB was expressed as milligram quercetin equivalent/100 g of the extract [16].

The DPPH free-radical scavenging activity of HECB was estimated as described earlier [17]. A series of concentrations of HECB namely 1,12.5,25,50,100 µg/ml were mixed with 1000µL of methanolic solution of 0.1 mM DPPH. The mixtures were allowed to incubate for 30 min in the dark. The absorbance was measured at 523. An equal amount of DPPH and methanol were used as standard and blank, respectively. The scavenging activity was calculated using the following formula:

Scavenging (%) = $(A_{control} - A_{sample})/A_{control} \times 100$.

2.5. Blood collection, Serum preparation, Tissue sampling and Tissue Homogenate preparation:

The Day after the last dose of HECB, blood was collected from the orbital sinus and serum was prepared from it. Consequently, the animals were sacrificed by CO_2 asphyxiation and necropsied for the gross evaluation of the various organs. Liver was carefully dissected and washed with ice-cold physiological saline, dried over lint free tissue paper for few seconds and weighed. Organ weight to body weight ratio (Relative Organ Weight) was calculated. Liver was then homogenized (1:10 ratio) in RIPA buffer using handheld tissue homogenizer. The homogenate was centrifuged at 12000 RPM for 5 min at 4^0C using DuPont Sorvall ultracentrifuge to separate the nuclear debris. The resultant supernatant was used to estimate various biochemical parameters. Protein content in serum as well as tissue lysates was estimated by Bradford's method.

2.6. Hepatic function test:

Hepatic function test was performed by estimating the Alanine Aminotransferase (ALT), Aspartate Amino Transferase (AST) and Alkaline Phosphatase (ALP) activities in serum using commercially available kits (ALT kit from sigma Aldrich MAK052, AST kit from sigma Aldrich MAK055 and ALP kit from Abcam ab83369) as per manufacturer's protocol.

2.7. Hepatic protein carbonyls:

Protein carbonyl levels in Hepatic tissue lysate was measured by reacting carbonyl groups with 2,4-dinitrophenylhydrazine to form a 2,4-dinitrophenylhydrazone as described previously [18]. Quantified protein carbonyls were later normalised with protein content of tissue lysate.

2.8. Hepatic levels of Enzymatic and nonenzymatic antioxidants activity:

Copper-zinc SOD (Cu-Zn SOD) was assayed by the method of Marklund and Marklund [19]. The degree of inhibition of the auto-oxidation of pyrogallol at an alkaline pH by Cu-Zn SOD was used as a measure of the enzyme activity. The unit of enzyme activity is expressed as units per milligram of protein. The activity of Catalase (CAT) was measured as described previously [20]. Test is based on the principle that dichromate in acetic acid is reduced to chromic acid when heated in the presence of H_2O_2, with the formation of perchloric acid as an unstable intermediate; the chromic acetate thus produced is measured at 570 nm. The unit of enzyme activity is expressed as micromoles of H_2O_2 consumed per minute per milligram of protein.

Glutathione-S-transferase (GST) activity was analyzed by the method of Habig et al [21]. The conjugation of glutathione to 1-chloro-2, 4-dinitro benzene (CDNB) was measured as a nonspecific substrate for GST activity. The unit of enzyme activity is expressed as micromoles of CDNB used per minute per milligram of protein.

Glutathione (GSH) and glutathione disulphide (GSSG) levels were measured by the method of Rahman et al with minor modifications. 5,5'-Dithio (2-nitrobenzoic acid) is a disulfide compound that is readily reduced by sulfhydryl compounds, forming a brightly coloured yellow anion; the optical density of this yellow substance is measured at 412 nm [22], [23]. Glutathione and glutathione disulphide concentrations were expressed as micrograms per milligram of protein and data was expressed as GSH / GSSG ratio.

2.9. Mitochondrial isolation and Complex I Activity Assay:

Liver tissue is minced and placed in mitochondrial extraction buffer consisting of 50 mM Tris-HCl pH7.4, 100 mM KCl, 1.5 mM MgCl2, 1 mM EGTA, 50 mM HEPES and 100 mM sucrose and homogenised using a handheld homogenizer. The homogenates were then spun at 4°C at 850g for 10 minutes and the supernatant is collected. In the second spin, the supernatant was spun at 1000g for 10 minutes to separate nuclear pellet and finally spun at 10000g for 30 minutes to obtain the mitochondrial pellet. To obtain pure mitochondrial fraction, the mitochondrial pellet was resuspended in fresh ice cold mitochondrial extraction buffer and re-spun at 10000g for 10min [24]. Resultant mitochondria were stored at -80°C until subjected to analysis.

Mitochondria were later subjected to five freeze-thaw cycles to disrupt the mitochondrial membranes. The protein content of the mitochondrial membranes was estimated using bradford assay. For estimating the mitochondrial complex I assay the method described by Pollard AK et al., was employed [24]. Briefly, 30 µg of mitochondrial sample was added to a reaction mixture containing 920 µl of complex 1 buffer (25 mmol/L monobasic potassium phosphate, 120 µl of 50 mmol/L dichlorophenolindophenol (DCIP), 100 µl of 1mmol/L Antimycin A, 400 µl of 17.5 mmol/L Decylubiquinone, made up to 100 ml with ddH_2O) and 50 µl of 70 g/L fatty acid free BSA. The cuvette was allowed to equilibrate at room temp for 1 minute and the reaction was initiated by the adding 30 µl of 10 mmol/L NADH. Change in absorbance was recorded every 30s for 2.5 minutes. The mitochondrial complex I activity was measured by using the formula:

Enzyme activity (nmol NADH/min/mg) = (ΔAbsorbance/min×1,000)/[(extinction coefficient of DCIP×volume of sample used in 1 ml) × (sample protein concentration in mg/ml)]

The enzyme activity of the samples was represented as nmol/min/mg of protein.

2.10. Enzyme Linked Immunosorbant Assays:

Levels of inflammatory markers such as IL-6 and TNFα along with the pro-mitochondrial proteins such as NRF2 and TFAM were estimated in the hepatic tissue lysates using commercially available kits. The levels of each individual proteins were normalized to the protein content and expressed as nMoles/ mg protein.

2.11. Phenobarbitone induced sleeping time:

Phenobarbitone induced sleeping time was performed as described earlier [25]. Briefly, phenobarbitone at a dose of 40 mg/ kg body weight was administered intraperitoneally to the animals in each experimental group at the end of the study and the total sleeping time was recorded in minutes from onset to natural arousal.

2.12. Histopathology:

Liver tissue biopsies were washed with ice cold PBS and immediately fixed in 10% Neutral Buffered Formalin. After histo-kinetic processing, tissues are embedded in paraffin and sectioned into thin sections. Hepatic sections were then stained with haematoxylin and Eosin and mounted with DPX medium. Histopathological evaluations were performed at both 10X and 40X magnification for a better understanding.

2.13. Statistical Analysis:

Variance in the data was analysed by one-way ANOVA followed by Tukey HSD post hoc test. Data is represented as Mean ± SD and $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. HECB contains Flavonoids, Phenolic compounds and possess antioxidant potency:

HECB was subjected to biochemical analysis to quantify flavonoids and phenolic components. Although series of concentrations of HECB were tested positive to both phenolic and flavonoid content, 50 and 100 µg/ml of concentration have shown an appreciable concentration (Fig. 1A and 1B). In support to this phytochemical analysis, the in vitro antioxidant potential was analysed by DPPH radical scavenging assay. On the similar lines with the flavonoid and phenolic content of HECB an significant radical scavenging effect of pronounced by 50 and 100µg/ml concentrations (Fig. 1C).

Additionally, LC-MS based detection of HECB revealed the presence of various phytoconstituents that are enlisted in table no 1. Notably, phytosterols, flavanols, flavanol glycosides and carotenoids were identified in the HECB making it bioactive rich fraction. The representative total ion chromatogram is shown in Fig. 2.

3.2. HECB preserves anthomorphometric indices of liver:

A significant increase in the organ weight was observed in CP alone treated group of animals when compared to control group ($p < 0.05$) indicating hepatomegaly. Interestingly, HECB treatment could able to prevent the pathological increase in the liver mass at 100mg/kg dose but not at 50mg/kg dose ($p < 0.05$ HECB100 Vs CP). In agreement with this finding, the relative liver weight has also increased with the CP treatment and was

attenuated by 100mg/kg HECB but not with 50mg/kg dose ($p < 0.05$ HECB100 Vs CP). Data is shown in Fig. 3A and 3B.

3.3. Improvement of Hepatic function upon treatment with HECB:

Hepatic function test was performed by estimating the levels of ALT, AST and ALP in serum. It was observed that CP has significantly elevated the levels of hepatic enzymes indicating hepato toxicity ($p < 0.05$ CP Vs Con for ALT and $p < 0.01$ CP Vs Con for AST and ALP). Treatment with HECB at both doses could able to successfully reduce the hepatic enzymatic activities (except for ALT, where only 100mg/kg could show effect). Additionally, phenobarbitone induced sleeping time was performed in order to study the integrity of hepatic microsomal enzymatic arcade. Chronic exposure to CP has caused increase in the phenobarbitone induced sleeping time while HECB could able to normalize it only at 100 mg/kg dose but not at 50mg/kg ($p < 0.01$ HECB100 Vs CP). Data is shown in Fig. 4A and 4B.

3.4. HECB preserves hepatic antioxidant status and reduces Inflammatory stress:

Hepatic antioxidant status was estimated at both enzymatic and non-enzymatic levels in the liver tissue lysates. Administration of CP has strongly affected the enzymatic levels and reduced their activities. Activities of both cytosolic and mitochondrial isoforms of SOD were reduced with CP ($p < 0.01$ for both CP Vs Con). While, HECB at both doses could able to successfully increase the hepatic SOD activities (except for Cu-Zn SOD, where only 100mg/kg could show effect). In accordance to this, CP has also decreased the activities of Catalase and GST indicating extreme oxidative stress ($p < 0.01$ for both CP Vs Con). An appreciable degree of improvement in these enzymatic activities was observed with HECB at both doses. Data is shown in Fig. 5A-C.

Non enzymatic antioxidant status was evaluated by analysing GSH and GSSG levels. The ratio of these two crucial non enzymatic antioxidant indices was significantly decreased with CP administration indicating oxidative stress ($p < 0.01$ CP Vs Con). Although HECB at a dose of 50mg/kg couldn't restore this redox balance, there was a significant increase with 100mg/kg dose ($p < 0.01$ HECB100 Vs CP). Data is shown in Fig. 5D.

Although antioxidant enzymatic activities were evaluated, direct impact of free radical stress could be better emphasized with protein carbonylation quantification. In agreement with the previous findings, levels of protein carbonyls were higher in CP treated animals indicating oxidative damage ($p < 0.01$ CP Vs Con). Treatment with HECB was able to reduce the protein carbonyl levels only at a dose of 100mg/kg indicating an antioxidant effect. Data is shown in Fig. 5E.

Upregulation of inflammatory cytokines by CP in response to the oxidative stress was assessed by estimating the levels of IL-6 and TNFα using ELISA kits. CP administration has significantly induced hepatic IL-6 and TNFα levels indicating upsurge in inflammation ($p < 0.01$ CP Vs Con for both). Although HECB at a dose of

50mg/kg couldn’t reduce the hepatic inflammation, there was a significant decrease with 100mg/kg dose ($p < 0.05$ HECB100 Vs CP). Data is shown in Fig. 5F.

3.4. HECB preserves hepatic mitochondrial activity and upregulated Pro-Mitochondrial Proteins:

Hepatic Mitochondrial were subjected to complex I activity assay to understand the effect of HECB on mitochondrial function. Administration of CP has strongly reduced the complex I activity ($p < 0.01$ CP Vs Con). Interestingly, HECB at higher dose alone could able to successfully increase the complex I activity ($p < 0.01$ HECB100 Vs Con). With this evidence, the levels of pro-mitochondrial proteins such as NRF2 and TFAM were assessed. CP exposure to the experimental animals has strongly impacted the pro-mitochondrial proteins by reducing their levels ($p < 0.01$ CP Vs Con for both). Similar to the previous results, HECB at higher dose alone could able to successfully increase the NRF2 and TFAM levels ($p < 0.05$ HECB100 Vs Con). Data is shown in Fig. 6A-C.

3.5. HECB preserves hepatic histological architecture:

As mentioned in the previous sections, CP has altered the sensitive redox balance in the liver there by producing toxicity. Histopathological evaluation of CP alone received livers has revealed that an evident hepatocyte swelling with vacuolization, enlargement of central vein, dilated and congested blood sinusoids were observed. Atrophic changes of hepatic cells that surrounding the blood vessels was also observed in these livers. Although, treatment with HECB at a dose of 100mg/kg has restored the tissue architecture, there was an evident mononuclear inflammatory cell infiltration with central vein dilation was observed at 50mg dose. Histopathological reference images were shown in Fig. 7.

4. Discussion

Plants contain a wide variety of phytochemicals that can potentially improve human health. The might of plant products in healing different ailments can be dated back to pre-historic era. Phytochemicals possess immense antioxidant activity imparting cytoprotection. Nonetheless, several plants species need to be investigated for their medicinal properties. Drug induced toxicity is often fatal due to its cascading pathological events produced in the body over a period[26]. Plant extracts used in traditional medicine and the phytomolecules are known to successfully attenuate the drug induced toxicity[27]. In this scenario, the present study aims to investigate the pharmacological effect of HECB at two doses namely 50 and 100 mg/Kg body weight against CP induced hepatic damage. Study has employed SD rats as an experimental model and drug treatments were continued for 8 months.

Before the animal experiments were initiated HECB was subjected to flavonoid and total phenolic content analysis. Concentrations ranging from 1-100µg/ml were tested positive to the flavonoid and phenolic content (Fig. 1A and B). Nonetheless, 50 and 100µg/ml of HECB was found to have a significantly higher concentration of flavonoids and phenolic compounds. LC-MS analysis revealed the presence of phytosterols, flavanols, flavanol glycosides and carotenoids. Multiple studies have identified cytoprotective role of these

phytomolecules in both in-vitro and in-vivo studies [28–30]. To be more specific, some of these molecules have a hepatoprotective effect against various obnoxious stresses [31–33]. Findings of the free radical scavenging activity assay was also in agreement with these finding (Fig. 1C). As mentioned earlier, CP disturbs the sensitive glutathione redox cycle and induces cytotoxic effects[5]. The impact of oxidative stress can be better understood by estimating the levels of protein carbonylation in the organs. Free radicals derived from molecular oxygen are highly charged and they react with the proteins resulting in formation of stable carbonyls [34]. Correspondingly, administration of CP caused an upsurge in the levels of hepatic protein carbonyls, which was brought down by the HECB co-administration. When the antioxidant mediators are investigated, there was a dramatic decrease in the levels of SOD, Catalase, GST as well as non-enzymatic antioxidants with CP administration. Increase in oxidative stress with concurrent drop in the antioxidant reserves of the liver could initiate a cascading damage to the hepatic function. Administration of HECB could able to restore this delicate redox balance by increasing the levels of antioxidant enzymes and decreasing the free radical induced cellular injury (Fig. 5).

Inflammation and oxidative stress are interrelated and can be inter-activated in many pathological conditions. Studies have shown that CP causes surge in inflammatory cytokines in the tissue in response to the oxidative stress[35]. Phytomolecules are known to have a promising attenuating effect on the inflammatory cytokines in response to chemotherapy there by preventing off target effects. In agreement with these reports, HECB has normalized the hepatic IL-6 and TNFα levels there by preventing further deterioration of hepatic structure and function. Serum biomarkers such as ALT, AST and ALP serves as a direct indicator of the hepatic function[36]. Hepatocellular damage could release these crucial enzymes into the blood stress spiking their concentrations in the serum. Hence, these markers are often employed to evaluate hepatic dysfunction and are indicative of cellular damage, cell leakage, and the loss of cell membrane integrity in the liver. Elevated levels of ALT, AST and ALP levels upon administration of CP agrees with the hepatic oxidative stress (Fig. 4). Restoration of these biomarker levels with administration of HECB indicates a potent hepatoprotective activity. Histopathological examination also revealed an improvement in the tissue architecture with lesser hepatocyte vacuolization, enlargement of central vein, dilated and congested blood sinusoids (Fig. 6). When the gross pathology was evaluated by anthomorphometric analysis, the organ weight and the relative organ weights in the CP alone treated groups were higher than controls indicting the hepatomegaly and sinusoidal congestion [37].

Mechanistically HECB appears to impart cytoprotection by conserving the preserving the mitochondrial function as evident from the mitochondrial complex I activity assay and the levels of NRF2 and TFAM. Studies have shown that CP induces mitochondrial dysfunction and their by causes cellular death[38]. Notably, NRF2, a nuclear factor upon activated by multiple upstream molecules, transcriptionally upregulates TFAM and there by causes mitochondrial biogenesis and improved oxidative phosphorylation[39]. Reports have indicated that CB is rich in lutein and β-carotene which might have produced the antioxidant effect [10]. The levels of β-carotene can be as high as 31.33mg/100g of dry plant while the levels of lutein are 183mg/100g of dry plant as estimated by HPLC DAD method [40, 41]. Additionally, the presence of phenolic and flavonoid components in HECB with a potent free radical scavenging activity also supports the mito-stimulant effect exerted by HECB in the liver. Reports have shown that lutein and β-carotene have positive impact on the mitochondrial function and can prevent disease progression[42, 43]. Additionally multiple other antioxidants in HECB can be

attributed to its hepatoprotective activity [25]. This falls in line with the existing evidence regarding the ability of plant extracts to impart cytoprotection by conserving the antioxidant status of the cell [44].

5. Conclusion

Conclusively, HECB could able to successfully attenuate CP induced hepatotoxicity by preserving mitochondrial function and thereby reducing oxidative stress. The findings of the study have clearly indicated that HECB at 100mg/kg dose was found to be more effective than 50mg/kg.

Although, the studies lack deeper molecular studies to understand the upstream pathways which lead to the activation of the NRF2–TFAM pathway, the results of the study has provided primordial evidences behind mitoprotective and hepatoprotective effect of HECB in rodents. Further studies focussing on finding the phytomolecules in HECB could be helpful in developing potent drug candidates for managing drug induced hepatotoxicity.

Abbreviations

ALP - Alkaline Phosphatase; ALT - Alanine Aminotransferase; AST - Aspartate Amino Transferase; CNDB - 1-chloro-2,4-dinitro-benzene; DPPH - 2,2-diphenyl-1-picrylhydrazyl; GSH – Glutathione (Reduced); GSSG – Glutathione (Oxidized); NRF2: Nuclear Factor Erythroid 2 Related Factor 2; SOD – Super Oxide Dismutase, TFAM- Transcriptional Factor A (Mitochondrial)

Declarations

6.1. Ethics Approval:

All the animal experiments were approved by IAEC (IAEC No: 09/NIPER/CPCSEA/351) and performed according to CPCSEA and ARRIVE guidelines

6.2. Consent for Publication:

Not applicable

6.3. Availability of Data and Materials:

The data generated in this study is not publicly available but are available from the corresponding author on reasonable request

6.4. Competing Interests:

The authors declare that they have no competing interests

6.5. Funding:

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6.6. Author's Contribution:

Lingesh Allakonda: Designed the study and prepared manuscript

Ajay Godwin Potnuri: Data analysis and review of manuscript

Rakshit Ranjan: Phytochemical analysis of the extract

Gnana Bhaskar: LC-MS data analysis

Suramanyam Kurra: Performed the experiments in the study

Sudheer Kumar: Plant collection, preparation of extract and review of manuscript

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Table

Table1: Phytochemical analysis of Hydro Ethanolic Extract of Commelina benghalensis. The hydro ethanolic extract of Commelina benghalensis was subjected to phytochemical analysis by LCMS using C18 Accucore column.

S No.	Phytomolecules	Mol Wt.	Base/Fragment	Parent/Precursor m/z	Rt	Ionization	adduct
1	Campesterol	400.7	307	401	1.2	ES-	M+H
2	9,10 anthracenedione	208.21	209	209	1.23	ES+	M+H
3	Kaempferol	286.24	188	285	2.31	ES+	M-H
4	Stigmasterol	412.7	327	413	8.46	ES-	M+H
5	Tetratriacontane	478.9	329	477	8.95	ES-	M-H
6	n-triacontanol	438.8	314	439	9.7	ES+	M+H
7	Rutin	610.5	181	611	13.79	ES+	M+H
8	ferulic acid	194	277	194	16.48	ES+	M
9	1-hexadeconol	242.4	277	241	16.72	ES-	M-H
10	chlorogenic acid	354.31	277	353	16.93	ES+	M-H
11	Lutein	568.9	609	567	19.6	ES+	M-H
12	zeaxanthin	568.9	609	567	20.12	ES+	M-H
13	1,3-Docosenamide, (Z)	337.6	255	338	25.51	ES-	M+H
14	tetracosane	338.7	255	338	25.51	ES-	M
15	9-eicosene	280.5	281	282	26.3	ES-	M-H
16	Chlorophyll “b”,	907.5	269	908	26.91	ES-	M+H

Figures

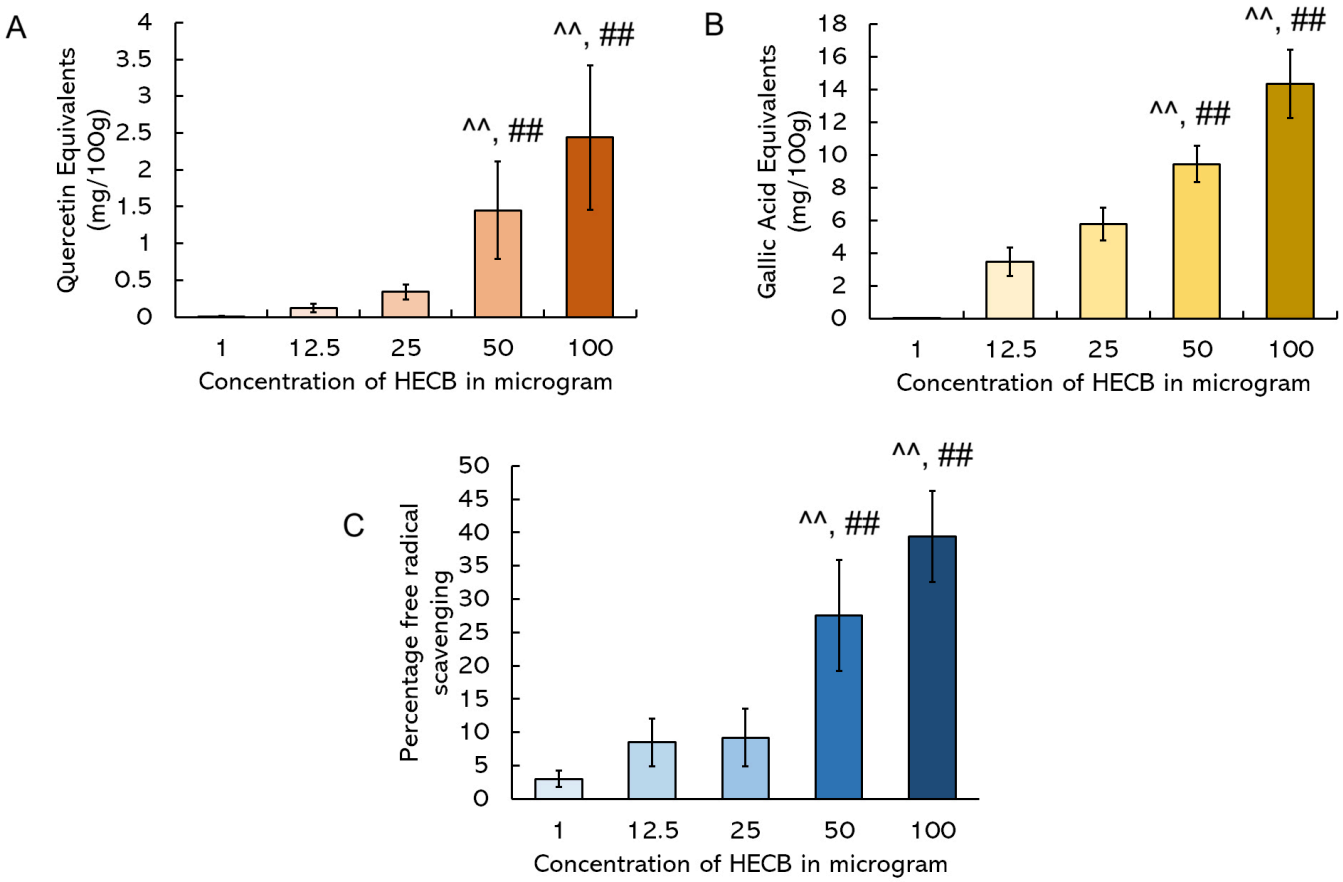

Figure 1

HECB contains Flavonoids, Phenolic compounds and possess antioxidant potency. Where ***A*** is the graphical representation of flavonoid content of HECB at different concentrations expressed as quercetin equivalents (mg/100g), ***B*** is the graphical representation of phenolic content of HECB at different concentrations expressed as gallic acid equivalents (mg/100g), ***C*** is the DPPH Free Radical Scavenging activity of different doses of HECB expressed as percentage free radical scavenging. Data was analysed by one-way ANOVA followed by Tukey HSD post hoc test and represented as mean ± SD. NS- Non-Significant; ^^ $p<0.01$ Vs 1µg HECB and ##$p<0.01$ Vs 12.5µg HECB (n=3).

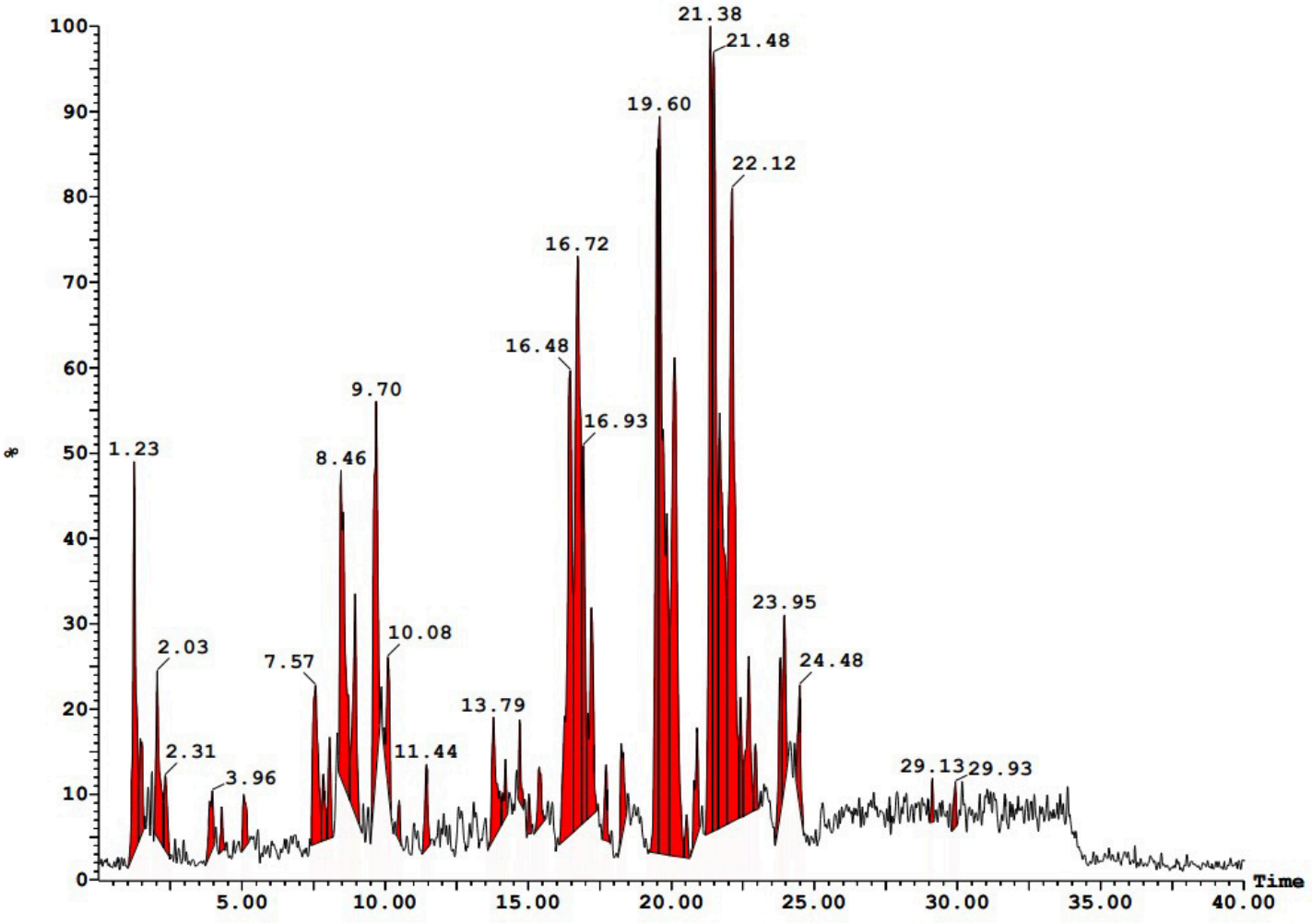

Figure 2

Total Ion Chromatogram of HECB LC-MS Analysis.

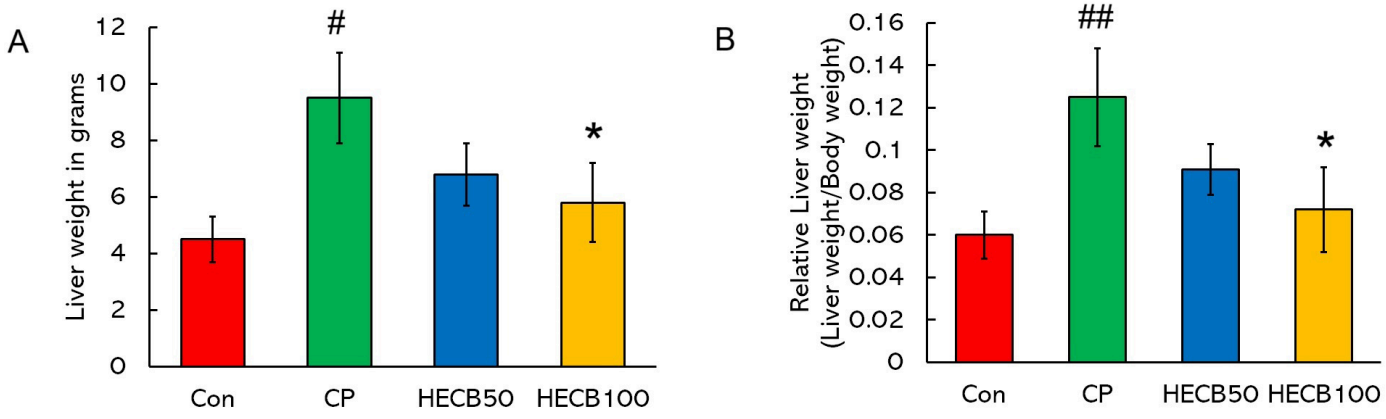

Figure 3

HECB preserves anthomorphometric indices of liver. Where ***A*** is the graphical representation of changes in the liver weight in response to various treatments represented as grams, ***B*** is the graphical representation of changes in the relative liver weight in response to various treatments expressed as percentage. Data was analysed by one-way ANOVA followed by Bonferroni post hoc test and represented as mean ± SD. #p<0.05 Vs Con; ## p<0.01 Vs Con and * p<0.05 Vs CP (n=6).

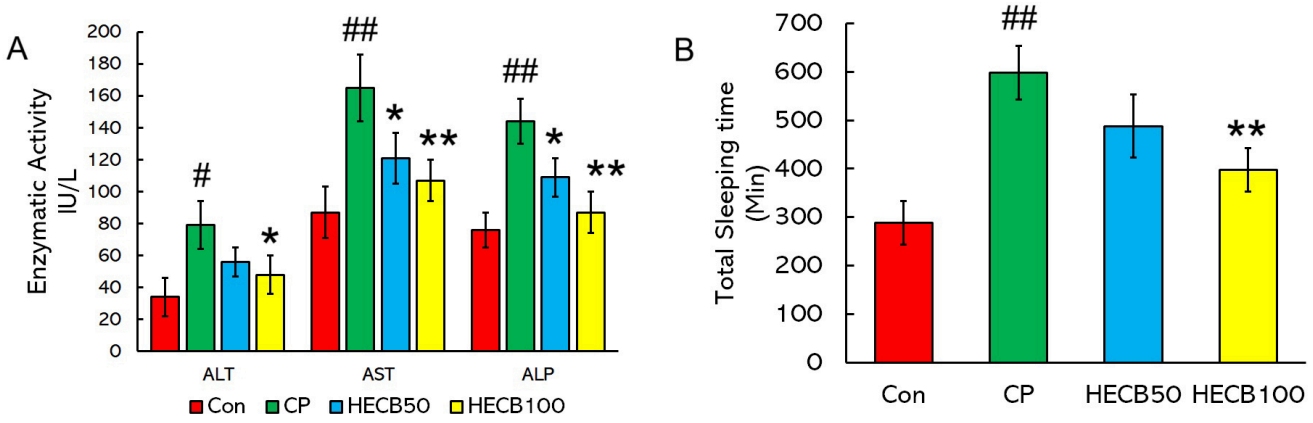

Figure 4

Improvement of Hepatic function upon treatment with HECB. Where ***A*** is the graphical representation of changes in the Hepatic enzymes in response to various treatments represented as IU/L, ***B*** is the graphical representation of changes in the Phenobarbitone induced sleeping time in response to various treatments expressed as min. Data was analysed by one-way ANOVA followed by Tukey HSD post hoc test and represented as mean ± SD. #p<0.05 Vs Con; ## p<0.01 Vs Con, * p<0.05 Vs CP and **p<0.01 Vs CP (n=6).

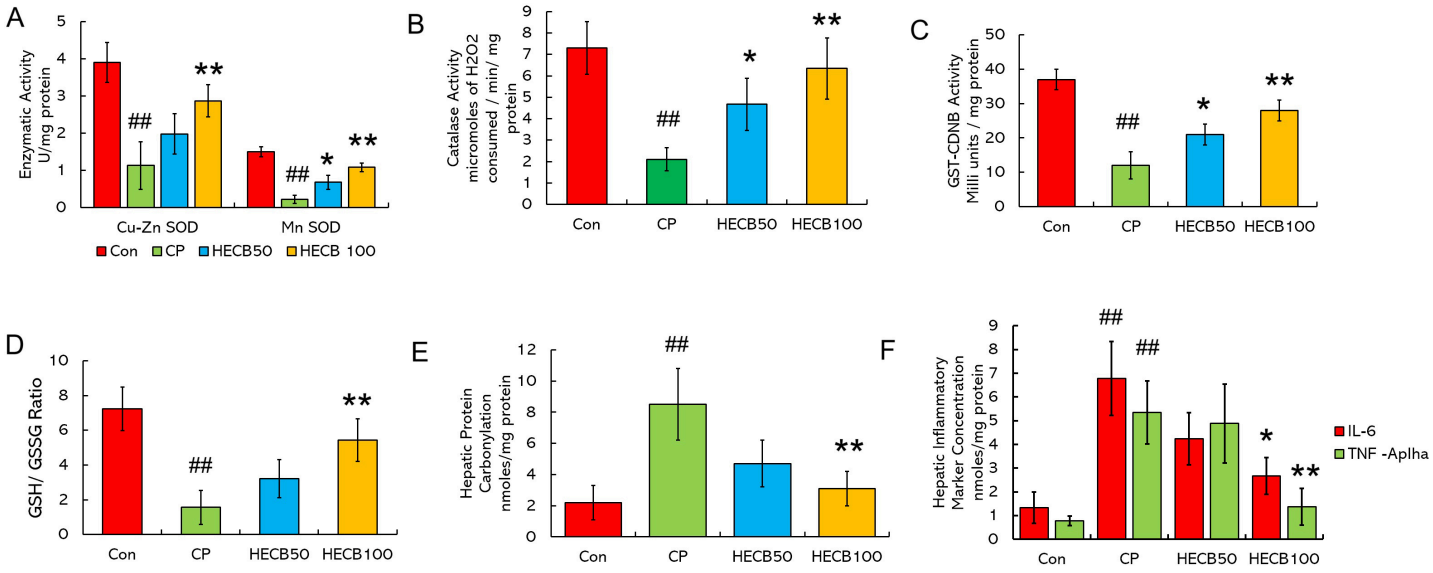

Figure 5

HECB preserves hepatic antioxidant status and reduces oxidative stress. Where ***A*** is the graphical representation of changes in the hepatic CU-Zn and Mn SOD enzyme activity in response to various treatments represented as U/mg protein, ***B*** is the graphical representation of changes in the hepatic catalase activity in response to various treatments expressed as micromoles of H_2O_2 consumed/min/mg protein, ***C*** is the graphical representation of changes in the hepatic GST-CDNB activity in response to various treatments expressed as milli units/mg protein, ***D*** is the graphical representation of changes in the hepatic GSH and GSSG levels in response to various treatments expressed as GSH/GSSG ratio, ***E*** is the graphical representation of changes in the hepatic protein carbonyl levels in response to various treatments expressed as nanomoles/mg protein and ***F*** is the graphical representation of changes in the hepatic IL-6 and c levels by CP and in response to various treatments expressed as nanomoles/mg protein. Data was analysed by one-way ANOVA followed by Tukey HSD post hoc test and represented as mean ± SD. ## p<0.01 Vs Con, * p<0.05 Vs CP and **p<0.01 Vs CP (n=6).

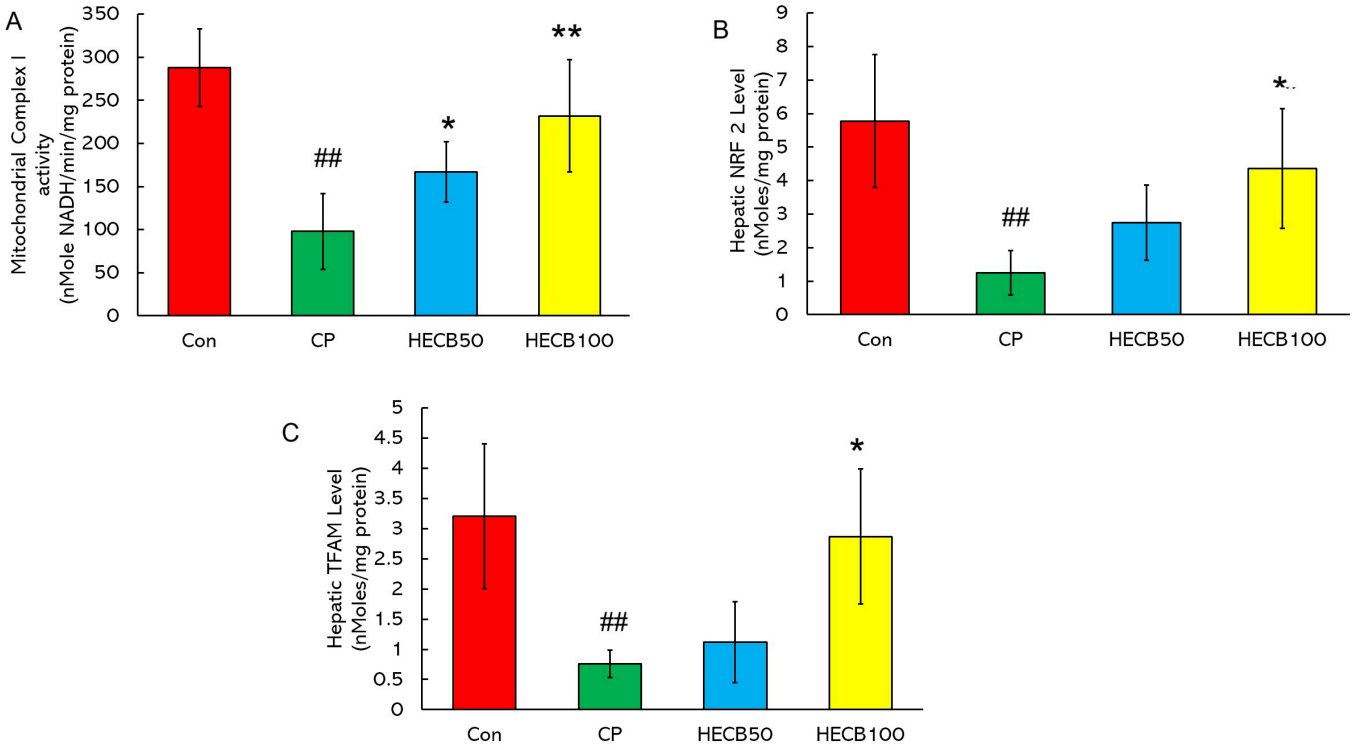

Figure 6

HECB preserves hepatic mitochondrial activity and upregulated Pro-Mitochondrial Proteins. Where ***A*** is the graphical representation of changes in the hepatic Mitochondrial Complex I activity in response to various treatments represented as nmole NADH /min/mg protein, ***B*** is the graphical representation of changes in the hepatic NRF2 levels in response to various treatments expressed as nmoles/mg protein, ***C*** is the graphical representation of changes in the hepatic TFAM levels in response to various treatments expressed as nanomoles/mg protein. Data was analysed by one-way ANOVA followed by Tukey HSD post hoc test and represented as mean ± SD. ## p<0.01 Vs Con, * p<0.05 Vs CP and **p<0.01 Vs CP (n=6).

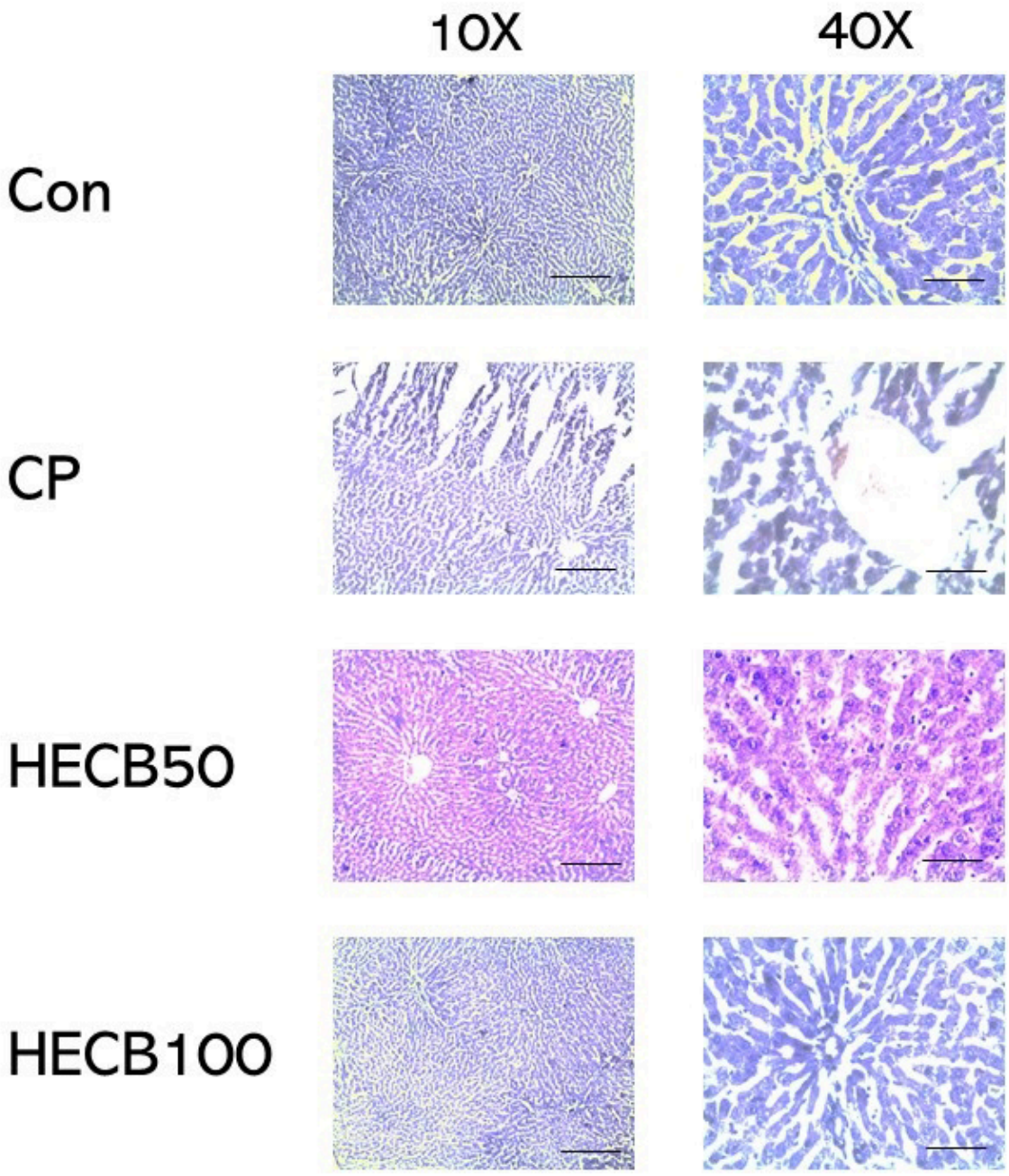

Figure 7

HECB preserves hepatic histological architecture. The histopathological analysis from H&E stained hepatic sections was carried out at both 10 and 40X magnifications. The scale bar in the 10X magnification images indicate 50μM while in the 40X magnification images indicate 10μM (n=3).