Chapter 3
Materials and Methods

3.1. Collection and authentication of plant material

Fresh and disease free leaves *C. bonplandianus* plant were collected from the garden of University of North Bengal (26.71°N, 88.35°S), West Bengal, India.

The plant material was identified and authenticated by Prof. Abhaya Prasad Das, senior Taxonomist of Department of Botany, University of North Bengal. A voucher specimen was stored at the herbarium of Department of Botany, University of North Bengal with an Accession no. 09870.

3.2. Preparation of plant extract

Leaves of *C. bonplandianus* (CBL) were separated and washed thrice with distilled water to remove dirt and dried at 50°C for two hours to eliminate moisture. Dried leaves were then milled with a grinder (Maharani, India, Model–Sujata Dynamix). A fine powdered leaf was stored in a refrigerator at −20°C. One hundred gm of the dried powder was stirred in 1 L of 70% methanol for 10 hours. The mixture was refluxed for 2 hours in soxhlet apparatus and centrifuged at 8000 rpm for 15 minutes. Supernatant was collected and concentrated by Rotary evaporator (45°C) and finally freeze dried. Extract was stored in air-tight vessel at −20°C for further studies.

3.3. Ethical statement

Croton bonplandianus (accession number-09870) was collected from the university campus area. These places are not under a National Park/Reserve Forest/Govt. protected area. All the experiments using animals were reviewed and approved by the Animal Ethical Committee of Department of Zoology, University of North Bengal (Permit No. 840/ac/04/ CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals). The experiments with animals were performed in accordance with the legislation for the protection of animals used for scientific purposes.

3.4. Animal maintenance

Swiss albino mice (6 male/group) were used for hepatoprotective analyses. All the mice were maintained in side cage bins (Tarson, India) wise rice husk bedding in the animal house of the department of Zoology, University of North Bengal at a constant 12 hour photoperiod (temperature

25±2 °C; humidity 55±5 %) with food and water ad libitum. Blood for immunization purpose was collected from sheep by puncturing the jugular vein with a sterile syringe. The blood was diluted with equal volume of Alsever's solution (114 mM dextrose, 27 mM sodium citrate, 71 mM NaCl, pH 6.1) and stored at 4°C as sheep RBC solution (sRBC) until further use. Blood was collected from a guinea pig by puncturing the heart using a sterile needle and allowed to clot at 4°C for 30 min to separate the serum. The blood was then centrifuged at 1000 rpm for 5 min and the clear supernatant serum was collected. This pooled serum was used as guinea pig complement and stored at 0°C to minimize the complement activity. All surgical procedures were performed following standard procedures according to Reeves and Reeves (2001).

3.5. Acute toxicity study

OECD guidelines (test 423: Acute oral toxicity – Acute toxic class method; 2002) were followed to study the acute toxicity of CBL extract on animal model (OECDi Library, 2002). Mice were divided into different groups (n=6) and kept on fast for overnight prior to the experiment. The plant extract was administered orally in an increasing dose upto 2000 mg/kg body weight (BW) and observed carefully for the development of clinical or toxicological symptoms at 30 min and then 2, 4, 8, 24 and 48 h. No mortality was observed in the experimental mice at 2000 mg/kg dose. Therefore, 1/40th, 1/20th and 1/8th of the maximum dose was considered for the in vivo studies.

3.6. Collection of brain samples and blood

Blood sample needed for hemolytic assay and erythrocyte membrane stabilizing activity was collected by puncturing the heart of Swiss albino mice under proper anesthesia and collected in an EDTA containing tube. The brain of the same mouse was used for lipid peroxidation assay.

3.7. Phytochemical analysis

The phytochemical constituents in *C. bonplandianus* were studied by the following methods**:**

3.7.1. Sample preparation for preliminary phytochemical analysis

Disease free fresh leaves, stem and root were collected from a mature *C. bonplandianus* plant. The parts were washed properly first with tap water and then with double distilled water to remove dirt. The parts were then shade dried at room temperature for 14 days and grinded to powder using a blender **(**Lords® Hummer 1100**)**. The powder was then passed through a 0.5 mm metallic mesh. Aqueous and methanolic fractions were prepared to perform the qualitative test.

Aqueous extract: The crude dried powder (10 g) was taken in a 250 ml conical flask and 100 ml of double distilled water was added to it. The mixture was stirred on a

magnetic stirrer for 10 h and then filtered through Whatman filter paper number 1 (150 mm). The filtrate was used for the following phytochemical tests.

Methanolic extract: 10 g of crude dried plant powder was taken with 100 ml of 70% methanol in a 250 ml conical flask. The mixture was mixed in a magnetic stirrer for 10 h in room temperature and filtered through Whatman filter paper number 1. The resultant extracts were used for the preliminary phytochemical investigations according to the standard chemical tests (Brain and Turner, 1975; The Indian Pharmacopoeia, 1996; Khandelwal, 2008; Gokhale and Kokate, 2008).

3.7.2. Qualitative tests

3.7.2.1. Tannin

The aqueous extract (10 ml) was mixed with few drops of 0.1% $FeCl_3$ solution. Formation of blue-black precipitate indicated the presence of tannin.

3.7.2.2. Phlobatannin

The aqueous extract (10 ml) was taken in a boiling tube and 2 ml of concentrated HCl was added to it. The mixture was boiled for 1 minute. Deposition of red precipitate indicated the presence of phlobatannins.

3.7.2.3. Carbohydrate

The aqueous extract (2 ml) was mixed with 2 ml of Molish's reagent (5% α-napthol in absolute ethanol) and shaken vigorously. Concentrated H_2SO_4 (2 ml) was slowly added along the wall of the test tube. Formation of reddish-violet ring at the junction of two liquids indicated the presence of carbohydrates.

3.7.2.4. Proteins

The aqueous solution (2 ml) was mixed with 1 ml of 40% NaOH solution. Few drops of $CuSO_4$ solution was added to it. Change of colour of the solution into violet indicated the presence of proteins.

3.7.2.5. Terpenoid

The methanolic extract (5 ml) was mixed with 2 ml of chloroform. Concentrated H_2SO_4 (3 ml) was added slowly along the wall of the test tube. Development of reddish-brown colour at the junction of two liquid phases indicated the presence of terpenoids.

3.7.2.6. Glycoside

The methanol extract (5 ml) was mixed with 2 ml of glacial acetic acid containing 2% $FeCl_3$ solution. Concentrated H_2SO_4 (1 ml) was added slowly along the walls of the test tube. Formation of a brown ring at the interphase of two liquid notified the presence of glycoside.

3.7.2.7. Steroid

The methanol extract (5 ml) was treated with 0.5 ml of anhydrous CH_3COOH and was cooled on an ice bath for 15 min. Then chloroform (0.5 ml) was added to the solution and 1 ml of concentrated H_2SO_4 was poured along the walls of test tube. Formation of a reddish-brown ring at the separation level of two liquids was an indication of the presence of steroids.

3.7.2.8. Cholesterol

The methanolic extract (2 ml) was mixed

with 2 ml of chloroform followed by addition of 10-12 drops of acetic acid anhydride. Then, few drops of concentrated H_2SO_4 was added to it. Change of reddish-brown color to blue-green on addition of H_2SO_4 indicated the presence of cholesterol.

3.7.2.9. Alkaloid

The methanolic extract (2 ml) was taken in a test tube and 2 ml of 2N HCl was added to it. The solution was shaken vigorously to mix and incubated for 5 min at room temperature. The aqueous phase formed was separated from the two liquid phases and few drops of Mayer's reagent ($HgCl_2$ + KI in water) was added to it and shaken. Generation of creamy coloured precipitate indicate the presence of alkaloids.

3.7.2.10. Phenolics

The methanolic extract (10 ml) was treated with 4-5 drops of 2% $FeCl_3$ solution. Change of coloration of the solution indicates presence of phenolics.

3.7.2.11. Flavonoid

The crude powdered plant sample (2 g) was heated with 10 ml of ethyl acetate over a water bath for 5 minutes. The solution was filtered through Whatman filter paper number 1. The filtrate (2 ml) was mixed with dilute ammonia solution (10%) and shaken vigorously. Yellow coloration of the solution indicates the presence of flavonoids.

3.7.2.12. Anthraquinone

The crude plant powder (500 mg) was mixed with 20 ml of benzene and stirred in a magnetic stirrer for 4 h and filtered. The filtrate (10 ml) was mixed with 0.5 ml ammonia solution and mixed properly. Presence of violet colour at the layer phase indicates presence of anthraquinones.

3.7.2.13. Saponin

The powdered plant material (500 mg) was boiled with 15 ml of double distilled water in a boiling water bath. Formation of intensive froth is the indication of the presence of saponin.

3.7.3. Quantitative tests

The quantitative estimation of different phytochemicals were performed according to various standard methods with minor modifications.

3.7.3.1. Alkaloid

The total alkaloid content was estimated according to the methods with minor modifications (Obadoni and Ochuko, 2001; Harborne, 1983). In brief, 5 g of powdered sample was mixed with 20% CH_3COOH in ethanol. The mixture was shaken on a magnetic stirrer for 10 h and filtered. The filtrate was placed on a hot water bath (60 °C) until the volume turns ¼th of its initial volume. Concentrated NH4OH was added drop wise till the saturation point which gave rise thick precipitate. The whole solution was allowed to settle down. The precipitate was collected by filtration, dried in an oven and weighed.

3.7.3.2. Flavonoid

A standard method (Boham and Kocipai DC, 1994) was followed with slight

modifications to quantify the total flavonoid content. The powered sample was mixed with 100 ml of 70% methanol and was stirred using a magnetic stirrer for 3 hours and filtered. The remaining powdered material was re-extracted once again with 70% methanol and filtered as previous. Filtrates of both the phases were mixed and transferred into a crucible and evaporated to dryness over a water bath of 60°C and weighed.

3.7.3.3. Saponin

Total saponin content was estimated according to a slightly modified standard method (Edeoga *et al*., 2005). The powdered plant material (10 g) was mixed with 100 ml of 20% ethanol and heated over a hot water bath of 55°C for a period of 5 h with stirring. The mixture was filtered and the supernatant liquid was separated. The residue was again reextracted with 20% ethanol as previous. The supernatant liquids of both phases were mixed and placed on a hot water bath of 90°C and heated till the volume of the extract was reduced to 20% of its initial volume. Then 10 ml of diethyl ether was added to it and shaken vigorously. After the solution settles down the aqueous layer was separated carefully into another flask and the ether layer was discarded. The purification process was repeated. Then 60 ml n-butanol extracts were washed twice with 10 ml of 5% aqueous NaCl solution. The remaining solution was heated in a water bath at 50°C until the solvent evaporates and the solution turns to semi dried form. The sample was then dried in an oven into a constant weight. The saponin content was calculated by the following equation: Content of saponin was measured by the following equation: Amount of saponin = (WEP / WS) × 100, where, WEP = weight of oven dried end product and WS = weight of powdered sample.

3.7.3.4. Tannin

The assay was performed according to a previously described standard method with slight modifications (Van-Burden and Robinton, 1969). The crude powder sample (1 g) was mixed with 50 ml of double distilled water and shaken on a magnetic stirrer for 10 h at room temperature. The mixture was filtered and made up to 50 ml using distilled water. The solution (5 ml) was pipetted out in a test tube and 0.008 M K_4[Fe(CN)6] and 0.1 M $FeCl_3$ in 0.1 N HCl was added to it. The absorbance was measured in spectrophotometer at 605 nm within 10 minutes. A blank was prepared and read at the same wavelength. Tannic acid was used to prepare standard curve.

3.7.3.5. Riboflavin

The test was performed according to a standard method (Abe and Yamauchi, 1992) with slight modifications. The dry powder (10 g) was mixed with 100 ml 50% ethanol and stirred for 10 h on a magnetic stirrer at room temperature. The solution was filtered and 25 ml of 5%

$KMnO_4$ solution was added to it. The mixture was stirred continuously while 25 ml of 30% H_2O_2 was added to it. This was placed on a 80°C water bath for 30 min. Then, 5 ml 40% Na_2SO_4 was added to it and the absorbance was measured at 510 nm against a suitable blank. The riboflavin content was calculated from a riboflavin standard curve.

3.7.3.6. Thiamine

A previously standard method was followed with slight modifications (Poornima and Rai, 2009) to quantify the thiamine content. The dried plant powder (50 g) was dispersed in 50 ml ethanolic NaOH (20%) and stirred over a magnetic stirrer for 3 h at room temperature and then filtered. Then, 10 ml of the filtrate was mixed with 10 ml of 2% potassium dichromate solution. The absorbance was read at 360 nm against a suitable blank. The thiamine content was calculated from a thiamine standard curve.

3.7.3.7. Ascorbic acid

Slightly modified method of Barakat *et al*., (1993) was followed to estimate the quantity of ascorbic acid. The dried powder sample (5 g) was mixed with 100 ml extraction mixture (TCA: EDTA at 2**:**1) and stirred on a magnetic stirrer for 3 h at room temperature. This was centrifuged at 2000 rpm for 30 min. After centrifugation the supernatant liquid was filtered and 2-3 drops of 1% starch indicator was added to it and was titrated against 20% $CuSO_4$ solution until a dark end point is reached.

3.7.3.8. Phenols

To estimate total phenol (Obadoni and Ochuko, 2001) content the test sample needed to be fat free. The crude plant powder (5 g) was mixed with 100 ml n-hexane and defatted using a soxlet apparatus for 2 h. The resultant was used for determination of total phenols. The fat free sample was boiled with 50 ml ether for 15 min. The resultant was filtered and 5 ml of the filtrate was mixed with 10 ml of double distilled water. Then, 2 ml of NH_4OH solution and 5 ml of concentrated amyl alcohol was added to the solution with constant stirring. The solution was incubated at room temperature for 30 min for colour development and the absorbance was read at 550 nm against a suitable blank. The phenolic content was evaluated from a gallic acid standard curve.

3.7.3.9. Protein

Total protein was estimated according to the method proposed by Lowry *et al*. (1951) with slight modifications. Known concentrations of bovine serum albumin was taken as standard and the OD was read at 750 nm using a suitable blank.

3.7.3.10. Lipid content

The assay was performed according to a standard method (Jayaraman, 2011) with slight modifications. The dried sample (1 g) was macerated with 10 ml distilled water. To this, 30 ml of chloroform-methanol (2**:**1 v/v) was mixed thoroughly and the mixture was left overnight at room

temperature. Then, 20 ml chloroform and equal volume of distilled water was added and centrifuged at 1000 rpm for 10 min. After centrifugation three layers were formed, out of which the lower layer was collected which contained chloroform containing lipid. The mixture was kept in an oven for 60 min at 50°C to evaporate the chloroform. Weight of the remaining was calculated.

3.7.3.11. Total sugar

The total sugar content was determined according to DuBois *et al*. (1951) with slight modifications. The powdered sample (50 g) was macerated in a pestle and mortar with 20 ml of ethanol and kept for incubation at 30°C for 10 h. The mixture was centrifuged at 1500 rpm for 20 min and the supernatant was collected separately. To the resultant 1 ml of alcoholic extract, 1 ml 5% phenol solution was added and mixed thoroughly. Then, 5 ml of concentrated H_2SO_4 was added rapidly with constant stirring. This was allowed to stand for 30 min at room temperature. Absorbance was measured at 490 nm against a blank.

3.7.3.12. Moisture and ash content

Moisture and ash content of different parts of the plant was estimated by subjecting specific amount of sample to 90°C for 12 h in an oven and at 400°-450°C in a furnace for 5 min, respectively. The resultant weight was calculated for moisture and ash content estimation respectively.

3.8. Fourier Transform Infrared Spectroscopy analysis

FTIR spectrophotometry was used to identify the characteristic functional groups in CBLE. Small quantity (<10 mg) of the extracts were taken in CaF_2 vessel and placed in a sample cup of a diffuse reflectance accessory. The IR spectrum was obtained using Shimadzu 8300 FT-IR spectrophotometer at ambient temperature. Background correction was made by taking IR spectrum of de-ionized water as the reference in identical condition. The sample was scanned from 400 to 4000 cm^{-1} for 16 times to increase the signal to noise ratio.

3.9. Gas chromatography-mass spectrometry analysis

CBLE was separately dissolved in dichloromethane and n-hexane and the mixtures was centrifuged thrice at 12,000 rpm for 15 min. The clear supernatant was used for GC-MS analysis. Agilent 5975C GC-MS system (Agilent Technologies, USA) attached with HP-5ms Capillary Column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) and equipped with inert MSD triple axis mass detector conditioned at ion trap 200 °C, transfer line 280 °C, electron energy 70 eV (vacuum pressure-2.21e - 0.5 torr) was used for analysis. The carrier gas was helium at a flow rate of 1 ml/min. The sample (2 µl) was injected in a splitless mode. The column temperature was set at 60 ºC for 1 min. followed by 5 ºC/ min upto 250 °C. The major and essential compounds in NOLE were

identified by their retention times and mass fragmentation patterns using Agilent Chem Station integrator and the database of National Institute Standard and Technology (NIST) with a MS library version 2010.

3.10. Immunomodulatory Activity

Both *in-vivo* and *in-vitro* experiments were performed to investigate the immunomodulatory activities of *C. bonplandianus*.

3.10.1. *In-vivo* experiments

3. 10.1.1. Doses

Swiss albino mice (male) were randomly divided into four groups (n=6) and following treatments were done once per day for 21 consecutive days: Control group received normal saline; low dose extract (CBLL) group received 50 mg/kg BW, medium dose extract (CBLM) group received 100 mg/kg BW and high dose extract (CBLH) group received 200 mg/kg BW of *Croton bonplandianus* leaf extract respectively.

Measurement of the body and organ weight and counting of splenocyte and leukocyte at day 21, 24 h after the last dose, all the animals were sacrificed under proper anesthetic condition and relative organ weight (organ weight/100 g of BW) of liver, spleen and the percentage body weight gain/loss were measured. Single cell suspensions of spleen, (prepared in RPMI containing 5 % FBS) and total leukocyte were prepared (using Leishman's stain) to count the cellularity by haemocytometer (Harisha, 2007).

3.10.1.2. Plaque forming cell (PFC) assay

The plaque forming cell assay (PFC), the key experiment to determine the effect of immunomodulation was done according to standard method (Raisuddin *et al.,* 1991) with slight modifications. On day 17, mice were immunized with 0.1 ml of 25 % sRBC intravenously through tail vein and after 4 days of immunization whole blood was collected from the heart and stored for further use. Single cell suspension (2×10^6 cells/ml) of the spleen was prepared for the dettermination of PFC assay. Guinea pig complement (50 µl) and 50 µl of 25% sRBC (prepared in PBS) were mixed with 100 µl of the cell suspension of spleen. The whole nmixture was then charged into Cuningham chambers, prepared using glass slide and double gum tape and then sealed with sealing material prepared by mixing paraffin and petroleum jelly at 1:1 ratio. All the chembers were incubated at 37^0C for 3-4 h. The plaques were counted after incubation under a binocular and the activity was expressed as PFC/10^6 spleen cells.

3.10.1.3. Estimation of total IgM

Blood samples were collected from the immunised mice of the PFC assay and kept at room temperature for 60 min for separation of serum. After collection, serum was diluted 10,000 fold for the estimation of total IgM level using a commercially available kit (My Biosource) according to the manufacture's instruction.

3.10.1.4. Hemagglutination (HA) titre assay

HA titre assay was performed according to a standard method (Karthikumar *et al.,* 2011). Briefly, Swiss albino mice were fed with different doses of CBL extract for 20 days. All the experimental mice were immunized with sRBC as was done in the PFC assay. On day 4 of the immunization, serum was collected and kept at 56 ^{0}C for 45 min in a water bath to inactivate the complement activity. Khan tubes were used for determination of HA titre. Serum (0.1 ml) and PBS (0.9 ml) was added in the first Khan tube. In the remaining Khan tubes 0.5 ml of PBS was added. Then 0.5 ml of mixture was added into the second khan tube from the first one and 0.5 ml of mixture was simultaneously added into the third tube from the second one. In the same manner, eight such dilutions were prepared as double fold dilution; 0.5ml of the solution was thrown away from the last tube, thus yielding a serial dilution of 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640 and 1/1280. 0.1ml of 10% sRBC was added into each tube. Then all the khan tubes were incubated at 37^{0}C for 12 h in a humidified atmosphere and after incubation, visible hemagglutination was observed and the degree of agglutination was also noted.

3.10.1.5. Counting of Peritoneal Macrophages

Peritoneal macrophages were enumerated according to the standard protocol (Chakraborty and Chakravarty, 1984). 0.5 ml of Freund's incomplete adjuvant was injected in the peritoneum, one day prior to the experiment at day 20, to count the peritoneal macrophages. The peritoneal exudates were collected by washing the peritonium with PBS. The peritoneal exudate cells were collected after a spin for 5 min at 1,000 rpm in a centrifuge followed by washing two times in PBS; the remaining pellet was resuspended in PBS. The total mixture was incubated for 45 min at 37^0 C in a petridish. After incubation, the petridish was washed with 2% EDTA in chilled PBS and centrifuged for 5 min at 1,000 rpm. After centrifugation, the pellet was mixed in PBS. Neutral red, a vital dye, was added to the cell suspension and the macrophages were counted using haemocytometer under the phase contrast microscope.

3.10.1.6. Assessment of Phagocytic Activity of Macrophages

Stimulation of phagocytic activity of macrophages was performed according to a standard method with little modifications (Lin *et al*., 1995). The procedure of macrophage collection was described previously. After the collection of macrophages from the *C. bonplandianus* treated mice (50 mg/kg BW; 100 mg/kg BW and 250 mg/kg BW respectively for 20 days), cell suspension was prepared in a way that the density became 5×10^6 cells/ ml. Then 0.1 ml cell suspension, 0.1 ml of 20 % FBS and heat-treated yeast cell

suspension (100×10^6 cells/ml) were mixed and incubated at 37°C for 1 h with occasional shaking. After incubation, the 50 µl mixture was smeared on glass slide and stained with Wright-Giemsa. The stained slide was observed and 500 cells were counted under light microscope. The phagocytic activity was expressed as phagocytic capacity (PC). Phagocytic index (PI) was calculated using the following formula:

PI = AB

Where A = percentage of yeast-ingesting phagocytes and B = number of yeast-ingested per phagocytes; PC = mean percentage of cells that engulfed ≥ 4 yeast cells.

3.10.1.7. Estimation of Total Serum Protein, Albumin and Globulin

Serum was collected on 21st day from the tail vein of mouse for the estimation of protein, albumin and globulin. Commercially available kits (Crest Biosystems, India) were used to determine the total serum protein, albumin and globulin.

3.10.2. *In vitro* experiments

3.10.2.1. Hemolytic assay

Blood samples were collected from Swiss albino mice (25±2 g) followed by three times washing of blood cells with 20Mm Tris-HCl containing 144mM NaCl (pH 7.4). 2% erythrocyte suspension was prepared for hemolytic assay (Yeap *et al*., 2011). The experiment was done in a 96 well plate and 100 µl of 0.85% NaCl solution containing 10mM $CaCl_2$ was added in each well. The first well containing only solvent served as control and from the second well onwards, 100 µl of plant extract was added with various concentrations (0-200 µg/ml). Triton X (0-200 µg/ml) was added in the last well which served as positive control. Then 2% erythrocyte suspension was added in each well and incubated at room temperature for 30 minutes. After incubation, the suspension was centrifuged and the supernatant was collected. Then the supernatant was used to measure the absorbance of liberated haemoglobin at 540 nm. The hemolytic activity was calculated using the following formula:

$$\% \text{ of hemolytic activity} = \frac{A0 - A1}{A0} \text{ X } 100$$

Where, H_0 is the absorbance of the blank and H_1 is the absorbance of sample and standard (Titron X).

3.10.2.2. In vitro Cell Adhesion Assay

Cell adhesion assay was performed according to the standard method with slight modifications (Malagoli, 2007). 0.5 ml Freund's incomplete adjuvant was injected intra peritoneally one day prior to the experiment. For the collection of macrophages, a midline incision was made in the abdomen of mice with proper anesthesia and the peritoneum was carefully washed with RPMI-1640 on the 4th day. Then the peritoneal exudates cells

were collected and centrifuged at1000 rpm at 4 ^{0}C for 10 min. The supernatant was discarded after centrifugation and the remaining pellet was resuspended in RPMI -1640. The number of cell was adjusted to 5×10^6cells/ml and seeded in 96 wells plate with different concentrations like 0–200 µg/ml of *C. bonplandianus* extract. Then the total experimental set-up was incubated for 60 min and after incubation cells were gently washed with RPMI 1640. After washing, 100 µl crystal violet (0.5% dissolved in 25% of methanol) and 10% ethanol were added to each well and incubated for 4 h at 37ºC under humidified condition. At the end of the incubation, 100 µl of 1% SDS was added and the absorbance was measured at 570 nm. Adherence property was measured using the following formula:

$$\% \text{ increase of adherence } = \frac{A0 - A1}{A0} \text{ X } 100$$

Where, A_0 =absorbance of the control (cells without treated plant extract) and A_1 = absorbance of the test sample.

3.10.2.3. Respiratory burst activity

The assay was performed according to standard protocol with some modifications (Cook *et al*., 2001). Murine peritoneal macrophages were collected in RPMI-1640 as previously described and seeded into 96 wells plate. The plate was pre-coated with 0.2% poly-L-lysine along with various concentrations (0-200 µg/ml) of *C. bonplandianus* extracts. Zymosan (0.1%) was added and the plate was incubated for 30 min at 37°C under humidified condition. After incubation cells were washed thrice with RPMI-1640 followed by staining with 100 µl NBT (0.3%) at RT. NBT solution was discarded after 30 min and the reaction was stopped by addition of 100 µl absolute methanol. The formazan which was generated was dissolved in 120 µl of 2M KOH and 140 µl of DMSO and the absorbance was immediately taken at 630 nm.

3.10.2.4. Myeloperoxidase (MPO) release assay

The assay was performed according to standard protocol with slight modifications (Sengupta *et al*., 2011). Murine peritoneal macrophages (2×10^6 cells/ml) were seeded into 96 wells culture plate. To this, 100 ng/ml LPS and varying concentrations (0-200 µg/ml) of plant extracts were added. The cells were then incubated at 37°C under humidified condition for 60 min and after incubation; the solutions from each well were centrifuged for 13,000 rpm for 10 min. After centrifugation the supernatants were removed and 0.01% SDS (dissolved in RPMI-1640) was added to lyse the cells. The solution was centrifuged at 13,000 rpm for 10 min and supernatant (100 µl) was collected from each group were mixed with 100 µl substrate buffer (ortho-phenylenediamine). After 20 min, the reaction was stopped using 100 µl of 2N H_2SO_4 and the absorbance was read at 492 nm.

3.10.2.5. Inhibition of Lipopolysaccharide (LPS) Induced Nitric Oxide Production

Inhibition of nitric oxide (NO) production was done according to the standard methods with slight modification (Choi and Oh, 1999). Griess reagent method was applied to estimate the NO level. Macrophage was collected intra peritoneally by the method as described previously. 200 µl of cell suspension (2× 10^6cells/ml) was added in each well of 96 well plate with the addition of 50 U/ml penicillin, 50 U/ml streptomycin, 50 U/ml nystatin, 10 % FBS, 100 µl of plant extract of different concentrations (0–200 µg/ml) and LPS suspension (20 µg/ml). After addition, the total reaction mixture was incubated for 24 h under 5 % CO_2 in humidified atmosphere of 90% air at 37^0C temperature. After incubation, centrifugation was done at 5,000 rpm for 5 min.50 µl of the supernatant was mixed with 200 µl of Griess reagent in each well of 96-well plate and incubated for 20 min at room temperature. Purple azo-dye was formed after incubation and the dye was measured at 540 nm. The nitric oxide level was measured using the following formula:

$$\% \text{ inhibition of nitric oxide level} = \frac{A0 - A1}{A0} \text{ X } 100$$

Where, A_0= absorbance of the control (cells without treated plant extract) and A_1= absorbance of the test sample.

3.10.2.6. Carbon Clearance test

The test was performed according to a standard method (Gonda *et al.,* 1990) with minor modifications. Different doses (50 and 200 mg/kg) of CBL were administered orally for 14 days to Swiss albino mouse and a control group received water as previously described. On 16^{th} day (48 h after the last dose), 0.1 ml of Indian ink was injected in tail vein and then, 25 µl blood samples were drawn from the orbital vein at 0, 5, 10 and 15 min after injection and mixed with 2 ml of 0.1% Na_2CO_3. The absorbance was read at 650 nm to estimate the extent of carbon clearance or in other words the rate of the elimination of carbon from the blood.

3.10.2.7. Cell proliferation assay

Cell proliferation assay was performed using EZcountTM MTT Cell Assay Kit (HiMedia) according to the manufacturer's instructions. In brief, murine splenocytes were cultured in 96 well plate for 48 h accordingt to previously described methods. After incubation 10 µl of MTT (5 mg/ml) solution was added to each well and incubated for 4 h. After incubation 100 µl solubilizing reagent was added and the absorbance was measured at 570 nm using Bio-Rad (Hercules, CA, USA) iMarkTM microplate absorbance reader.

3.10.3. Statistical analysis

All data are reported as the mean ± SD of six measurements. One way analyses of variance (ANOVA) test was done to compare between the control and the test group using KyPlot version 2.0 beta 15 (32 bit) for windows. $P<0.05$ was considered

significant. Percentage of inhibition/ scavenging was calculated by the following formula: (X0-X1)/X0×100, where X0= absorbance of control and X1= absorbance in the presence of the samples or standard. The IC_{50} (half maximal inhibitory concentration) values were calculated by the following formula: Y = A1/(X+A1)×100, where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The linear correlation analysis was performed by Graph Pad Prism V6.0.

3.11. Anti-inflammatory activity

3.11.1. Culture of splenocytes

Spleen was removed aseptically under proper anaesthesia from an untreated Swiss albino mouse (not fed with CBLE). Splenocyte suspension was prepared in RPMI-1640 using a tissue grinder. and washed thrice using RPMI-1640 (1000 rpm) for 10 min. After proper washing the pellete resuspended in 1 N NH4Cl to lyse RBCs. Splenocytes were adjusted as 2×10^6 cells/ml with RPMI- 1640 (supplemented with 50 U/ml penicillin, 50 lg/ml streptomycin, 50 U/ml nystatin and 10 % FBS). Splenocytes with all culture medium were seeded into six well culture plates. Concanavalin A (con A) was added to get a final concentration of 5 µg/ml and 100 µl of different concentrations of CBLE (0, 10, 30, 50, 80 µg/ml) was then added to the wells. The plates were then incubated under 5 % CO_2 and humidified atmosphere of 90 % air at 37^0C temperature for 48 h.

3. 11.2. Measurement of NO inhibition

Murine splenocytes were incubated with different concentrations of CBLE (0–80 µg/ml) for 48 h. Then the culture solutions from each group were centrifuged at 2,850×g for 5 min and the extent of inhibition of NO was estimated from the supernatant using the Griess reagent method (Hibbs *et al.,* 1988) with slight modifications. Culture supernatant (50 µl) was mixed with 200 µl of Griess reagent (1% sulfanilamide and 0.1 % N-(1-naphthyl) ethylenediamine hydrochloride in 2.5 % H_3PO_4) and incubated at 37 ^{0}C for 20 min. After incubation the absorbance was measured at 540 nm.

3.11.3. Estimation of cytokine expression

Mouse splenocytes were cultured with different concentrations (0–80 lg/ml) of CBLE according to previously described methods. After 48 h of culture cell suspension was collected from the each well and centrifuged 2,850×g for 5 min. After centrifugation culture supernantents were collected to estimate the levels of IL-2, IFN-γ, IL-4, IL-10 and TNF-a using RayBio ELISA kits according to the manufacturer's instructions. Precisely, 100 µl culture supernatant from different experimental groups were added to the anti -mouse cytokine (IL-2, IFN-c, IL-4, IL-10, TNF-a) coated 96 well ELISA plate. The plate was incubated at room temperature for 2.5 h. After incubation all the wells

were washed 4 times with 300 µl assay buffer. After proper washing 100 µl biotinylated anti-mouse cytokine (IL-2, IFN-c, IL-4, IL-10, TNF-a) antibody was added to each well. Then the plate was incubated at room temperature with continuous shaking for 1 h and washed with assay buffer as previously. Horseradish peroxidase (HRP) streptavidin conjugate solution (100 µl) was added into the culture plate. After adding HRP solution the plate was incubated for 45 min at room temperature with gentle shaking. 100 µl of 3, 3, 5, 5′- tetramethylbenzidine (TMB) was added followed by 30 min incubation at room temperature in the dark and after 30 min 50 µl of 0.2 M sulphuric acid was added to each well to stop the reaction. Absorbance was taken at 450 nm using Bio-Rad iMarkTM microplate ELISA reader.

3.11.4. Measurement of COX activities

COX activity assay kit (Cayman Chemicals Company) was used to measure COX 1 and COX 2 activities by ELISA method. Briefly, as previously described methods mouse splenocytes were cultured with varying concentrations (0–80 lg/ml) of CBLE for 48 h. After incubation, the cell suspensions were centrifuged at 2,850×g for 5 min. After centrifugation cell pellets were homogenized in cold buffer consisting of 0.1 M Tris- HCl in 1 mM EDTA (pH 7.8) and the homoginate solution was centrifuged at 10,000×g for 15 min. The resultant supernatants were used for the measurement of COX activities. Assay buffer, heme reagent, test samples and background samples were added according to the manufacturer's instructions. The plate was incubated at 25^0C for 5 min after adding DuP-697 (COX 2 selective inhibitor) and SC-560 (COX 1 selective inhibitor) to the respective inhibitor wells. 20 µl N,N,N′,N′-tetramethyl-p phenylenediamine (TMPD) and Arachidonic acid (20 µl) were added to each well to start the reaction. Then plate was incubated at 25^0C for 5 min. After incubation absorbance was measured at 590 nm. COX activity was calculated using the following formula:

COX activity (nmol/min/ml) = (ΔA_{590} per 5 min ÷ 0.00826 μM^{-1}) × (0.21 ÷ 0.04) ÷ 2

Where, 2 molecules of TMPD were required to reduce prostaglandin G_2 (PGG_2) to prostaglandin H_2 (PGH_2). Percentage inhibition of COX activity was calculated by the following formula:

Percentage of inhibition = [(TCA_S – TCA_1 or TCA_2) ÷ TCA_s] × 100

Where, TCAS = Total COX activity (COX 1 + COX 2) of sample; TCA1 = COX 1 activity (DuP-697 treated sample); TCA2 = COX 2 activity (SC-560 treated sample).

3.11.5. Measurement of Prostaglandin E2 Level

Prostaglandin E_2 was measured by PGE_2 EIA Kit (Cayman) according to the manufacturer's instructions. In brief, 50 µl cell culture supernatants were added to the respective wells of a 96-well plate

according to previously described methods. Then the cell culture supernatants were precoated with goat polyclonal anti-mouse IgG, followed by 50 µL of PGE_2-AChE tracer and 50 µL PGE_2 monoclonal antibody and incubated for 18 h at 4°C. After incubation the culture plate was washed five times with wash buffer. Ellman's reagent (200 µl) was added after proper washing and the culture was incubated in dark for 60 min with constant mild shaking. Absorbance was taken at 415 nm using Bio-Rad iMark™ microplate absorbance reader.

3.13.5. Molecular Docking

Proteins were chosen based on literature survey, having functional implications in immunomodulatory activity. The X-ray structures of the proteins available in the Protein Data Bank (http://www.rcsb.org) were used. Molecular docking was conducted using Auto Dock Vina (Trott and Olson, 2010). The receptor structures were defined as rigid, and the grid dimensions were 100, 100 and 100 for the X, Y, and Z axes for proteins having PDB ID's 1cqe, 6cox, 1tnf. On the other hand for proteins with PDB ID's 1hik and 2h24 grid dimension were 80, 80, 80 for X, Y and Z axes respectively. Gasteiger charges were assigned for all the compounds, and nonpolar hydrogen atoms were merged. All torsions of the ligand were allowed to rotate during docking. The value for the exhaustiveness of the search was 8. All graphic manipulations and visualizations were performed using the AutoDock Tools and ligand docking with Autodock Vina.

3.11.6. Statistical analysis

All data are reported as the mean ± SD of six measurements. One way analyses of variance (ANOVA) test was done to compare between the control and the test group using KyPlot version 2.0 beta 15 (32 bit) for windows. $P<0.05$ was considered significant. Percentage of inhibition/ scavenging was calculated by the following formula: (X0-X1)/X0×100, where X0= absorbance of control and X1= absorbance in the presence of the samples or standard. The IC_{50} (half maximal inhibitory concentration) values were calculated by the following formula: Y = A1/(X+A1)×100, where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The linear correlation analysis was performed by Graph Pad Prism V6.0.

3.12. Antioxidant assays

A number of antioxidant assays were done to determine the antioxidant activity of *C. bonplandianus* against ROS.

3.12.1. DPPH radical scavenging assay

The DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay was done following the method of Saha *et al*., (2016) with slight modifications. Bio-Rad micro plate reader was used to measure optical density (OD) at 517 nm and compared with ascorbic acid standard. The following equation was used to calculate the percent of scavenging:

$$Percentage of scavenging = \frac{A_0 - A_1}{A_0} \times 100$$

Here, A_1= absorbance in the presence of samples and standardandA_0= absorbance of the control.

3.12.2. Nitric oxide (NO) radical scavenging assay

GriessI-llosvoy reaction (Garratt, 1964) was used to perform Nitric oxide radical (NO) scavenging assay followed by slight modification of Dey et al. (2012).Aqueous sodium nitroprusside (SNP) and oxygen react to generate NO. The diazotization of nitrite ions with sulphanilamideand coupling with N-(1-naphthyl) ethylenediaminedihydrochloride (NED) generate pink azo dye. Here, curcumin was used as the standard. Above mentioned formula was used to calculate the percentage of inhibition.

3.12.3. Superoxide radical scavenging assay

The method of Fontana *et al*., (2001) with slight modifications was used to perform superoxide radical scavenging assay. Non-enzymatic combination of reduced nicotinamide adenine dinucleotide (NADH) andphenazinemethosulfate (PMS) produces superoxide radical ($O_2^{\bullet-}$), that further reduces nitro blue tetrazolium (NBT) to purple-colored formazan. Quercetin was used as standard.

3.12.4. Hydroxyl radical scavenging assay

The method of Elizabeth and Rao, (1990) with slight modifications followed by Saha *et al.,* (2016) was used to perform hydroxyl radical scavenging activity. Through Fenton reaction hydroxyl radical ($OH^{\bullet}$) was generated and the OD was measured at 532 nm using Bio-Rad micro plate reader. Mannitol was used as standard. Again equation I was used to calculate the percent of inhibition.

3.12.5. Hydrogen peroxide scavenging assay

The assay was performed according to a previously described method (Long *et al.*, 1999) with minor modifications. An aliquot of 50 mM H_2O_2 and various concentrations (0-2 mg/ml) of samples were mixed (1**:**1 v/v) and incubated for 30 min at room temperature. After incubation, 90 µl of the H_2O_2-sample solution was mixed with 10 µl HPLC-grade methanol and 0.9 ml FOX reagent was added (9 volumes of 4.4 mM BHT in HPLC-grade methanol with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25M H_2SO_4). The reaction mixture was vortexed and incubated at room temperature for 30 min. The absorbance was measured at 560 nm. Sodium pyruvate was used as the reference compound.

3.12.6. Total antioxidant activity (TAA)

Total antioxidant activity (TAA) of the specimens was evaluated following the modified method of Prietto *et al*., (1999). The activity was measured observing the reduction of Mo^{6+} to Mo^{5+}. In this purpose, ascorbic acid standard was used.

3.12.7. Hypochlorous acid scavenging assay

The method of Aruoma and Halliwell (1987) was used for this assay with minor modifications by monitoring the absorbance decrease of catalase. At pH 6.2 NaOCl and H_2SO_4 were mixed to prepare Hypochlorous acid (HOCl). As the standard, ascorbic acid was used.

3.12.8. Measurement of reducing power

The method described by Oyaizu, (1986) was followed with slight modification to evaluate the total reducing power of the CBL extracts. Different concentrations (0-1 mg/ml) of extracts (0.5 ml) were mixed with 0.5 ml phosphate buffer (pH 6.6) and 0.5 ml 0.1% potassium hexacyanoferrate. The solutions were incubated at 50 °C in a water bath for 20 min. Then, 0.5 ml of 10% TCA was added in each tube to terminate the reactions. The upper portion of the solutions (1 ml) was mixed with 1 ml distilled water, and 0.1 ml $FeCl_3$ solution (0.01%) was added. The reaction mixtures were left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. A higher absorbance of the reaction mixture indicated greater reducing power. Butylated hydroxytoluene (BHT) was used as a positive control.

3.12.9. Iron chelation assay

Slight modified method of Haro-Vicente *et al*., (2006) was used to evaluate iron chelation assay. This activity was determined through the measurement of the intensity decrease of violet complex which is generated by coupling of ferrozineandFe^{2+}. As standard, EDTA was used.

3.12.10. Lipid peroxidation inhibition assay

The method of Kizil *et al*., (2008) followed by minor modifications of Saha *et al.,* (2016) was used to assay lipid peroxidation inhibition. The activity of the both fruit extracts against lipid peroxidation was measured in the mice brain sample by the inhibition of OH^- catalyzed malondialdehyde (MDA) production from the polyunsaturated fatty acid (PUFA). Both the fruit extracts (RLE and RTE) were compared with the standard Trolox.

3.12.11. Peroxynitrite scavenging activity

A previously described standard method (Beckman *et al.,* 1994) was followed to synthesize peroxynitrite ($ONOO^-$). In brief, 5 ml 0.6 M KNO_2 was mixed with an acidic solution (0.6 M HCl) of 5 ml H_2O_2 (0.7 M) on ice bath and 5 ml of ice-cold 1.2 M NaOH was added to it. The solution was subjected to treatment with granular MnO_2 prewashed with 1.2 M NaOH to remove the excess H_2O_2. The reaction mixture was left overnight at -20°C. Peroxynitrite solution was collected from the frozen mixture and the concentration was measured at 302 nm (ε = 1670 M-1 cm-1). The reaction mixture contained 50 mM phosphate buffer (pH 7.4), 0.1 mM

DTPA, 90 mM NaCl, 5 mM KCl, 12.5 µM Evans Blue, various doses of plant extract (0- 200 µg/ml) and 1 mM peroxynitrite in a final volume of 1 ml. The mixture was incubated at 25 °C for 30 min and the absorbance was measured at 611 nm. Gallic acid was used as the reference compound.

3.12.12. Singlet oxygen scavenging assay

The method of Pedraza-Chaverrí *et al*., (2004) with some modifications was used for this assay. The reaction between H_2O_2 and sodium hypochlorite (NaOCl) generates singlet oxygen ($_1O^2$). This assay was performed by monitoring the bleaching of N, N-dimethyl-4-nitrosoaniline (RNO) by singlet oxygen ($_1O^2$). Lipoic acid was used as standard. Equation I was again used to calculate singlet oxygen scavenging assay.

3.12.13. Erythrocyte membrane stabilizing activity (EMSA)

Erythrocyte membrane stabilizing activity of CBL extract was performed by standard method as described by Dey *et al*., 2012. Briefly, varying concentrations of CBL extract (0–200 µg/ml) was added to the mixture of 50 mM phosphate buffer (0.5 ml; pH 7.2), distilled water (1 ml), 10% RBC suspension (0.25 ml PBS), 12 mM EDTA (100 µl), NBT (150 µl of 1% solution), and riboflavin (100 µl). The solution then kept under bright light for 30 sec and incubated for 30 min at 50 °C followed by centrifugation at 1000 rpm for 10 min. The absorbance of the supernatant was measured at 562 nm and compared with the standard compound, quercetin.

3.12.14. Quantification of total flavonoid and phenolic content

Total flavonoid and phenolic content of CBL were estimated by standardized method of Dey *et al*., (2012) at 510 nm and 725 nm respectively. The phenolic content was calculated from gallic acid (GA; R2 =0.9708) standard curve and flavonoid content was calculated from quercetin (QC; R2 =0.9891) standard curve.

3.12.15. Statistical analysis

All data are reported as the mean ± SD of six measurements. One way analyses of variance (ANOVA) test was done to compare between the control and the test group using KyPlot version 2.0 beta 15 (32 bit) for windows. $P<0.05$ was considered significant. Percentage of inhibition/ scavenging was calculated by the following formula: (X0-X1)/X0×100, where X0= absorbance of control and X1= absorbance in the presence of the samples or standard. The IC_{50} (half maximal inhibitory concentration) values were calculated by the following formula: Y = A1/(X+A1)×100, where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The linear correlation analysis was performed by Graph Pad Prism V6.0.

3.13. Hepatoprotective activity

3.13.1. Experimental design: *in vivo*

3.13.1.1. Doses

Swiss albino mice were randomly divided into six groups (n=6) and following treatments were done once per day for 21 consecutive days: Control group received normal saline; CCl_4 group received 1:1 (v/v) CCl_4 in olive oil; Silymarin group received 1:1 (v/v) CCl_4 in olive oil and 100 mg/kg BW silymarin; low dose extract (CBLL) group received 1:1 (v/v) CCl_4 in olive oil and 50 mg/kg, medium dose extract (CBLM)) group received 1:1 (v/v) CCl_4 in olive oil and 100 mg/kg and high dose extract (CBLH) groups received 1:1 (v/v) CCl_4 in olive oil and 250 mg/kg bw respectively.

On 22nd day i.e. 24 h after the last dose, blood was collected by cardiac puncture under anesthesia and finally the animals were sacrificed with proper care. Blood was allowed to clot for 60 min at room temperature (20°C). Then serum was separated by centrifuging at 1000 rpm for 5 min from the clotted blood. The straw colored serum was used to study *in vivo* liver marker enzymes. Liver was separated from diaphragm by cutting the falciform and coronary ligaments. The liver was washed with phosphate buffer saline to remove blood. Isolated liver was homogenized and centrifuged. After centrifugation the supernatant was collected and used for *in vivo* antioxidant enzymatic assays. Liver tissue was chopped and preserved in 10% formaldehyde solution for histological study.

3.13.1.2. Liver function test: in vivo

Serum samples from each group were used to study several liver function tests like ACP, albumin, globulin, glucose, ALP, bilirubin, cholesterol, LDH, GGT, AST, ALT, total protein, urea and urea N_2 levels using commercially available kits (Crest Biosystems, India).

3.13.1.3. Estimation of peroxidase activity

Peroxidase activity was estimated by measuring the oxidation of guiacol in the liver of treated mice according to a standard method (Sadasivam and Manickam, 2008). 50 mg of tissue samples were homogenized in 0.1M ice cold phosphate buffer (pH 7.0) and centrifuged at 3000 rpm for 15 min for the study of peroxidase activity. The supernatant (100 µl) was mixed with 20 mM guiacol. Time was recorded for the increase of absorbance by 0.1 at 436 nm in presence of 300 µl H_2SO_4 (12.3 mM).

3.13.1.4. Estimation of catalase activity

Catalase activity was assessed by the standard protocol of (Luck and Bergmeyer, 1971) with some modifications, wherein degradation of substrate H_2O_2 by catalase in the liver tissue samples was measured. 50 mg of tissue samples were homogenized in 0.05 M of 1 ml Tris-HCl buffer (pH 7.0) and centrifuged at 10,000 rpm for 10 min at 4°C for the study of catalase activity. The supernatant was

collected. In a spectrophotometric cuvette, 500 µl of 0.34 mM H_2O_2, 2.5 ml H_2O and 40 µl supernatant were added and change in absorbance was noted six times at 30 sec intervals at 240 nm.

3.13.1.5. Estimation of reduced glutathione (GSH)

Reduced glutathione activity was measured according to the standard protocol (Ellman, 1959). An aliquot of 1 ml liver tissue supernatant was treated with 0.5 of Elman reagent (19.8 mg DTMB dissolved in 100 ml of 0.1 % sodium nitrate). After the treatment with Elman reagent, 3 ml of phosphate buffer was added and the absorbance was measured at 412 nm.

3.13.1.6. Estimation of superoxide dismutase (SOD)

For the estimation of superoxide dismutase, standard method was followed with minor modifications (Mishra and Fridorich, 1972). Reaction mixture was prepared using 1 ml of 50 mM sodium carbonate, 0.4 ml of 25 µM nitroblue tetrazolium and 0.2 ml of 0.1 mM freshly prepared hydroxylamine hydrochloride. Clear supernatant of liver homogenate (0.1 ml, 1:10 w/v) was added to the reaction mixture. The changes in absorbance of the sample were recorded at 560 nm.

3.13.2. Experimental design: *in vitro*

In vitro hepatoprotective potentiality of *C. bonplandianus* extracts was studied according to the previously described standardized protocol with some modifications (Dey *et al*., 2015; Freshney, 2005; Mishra *et al*., 2011). Different experimental groups of primary explant culture of mice hepatocytes were prepared in RPMI-1640 medium containing 50 U/ml penicillin, 50 U/ml streptomycin and 50 U/ml nystatin supplemented with 10% fetal bovine serum (FBS) for *in vitro* experimentation. Following treatments were done after 48 h of incubation: Control had no separate treatment; CCl_4 group received 25 µl/ml CCl_4; Silymarin group received 25 µl/ml CCl_4 and 100 µg/ml silymarin; low dose extract group (CBLL) received 25 µl/ml CCl_4 and 25 µg/ml CBL; medium dose extract group (CBLM) received 25 µl/ml CCl_4 and 50 µg/ml CBL; high dose extract groups received 25 µl/ml CCl_4 and 100 µg/ml CBL extract. The plates were incubated for 2 h and centrifuged at 5000 rpm for 10 min. After centrifugation culture supernatant was collected for further experiments.

3.13.2.1. Liver function test: in vitro

Culture supernatant from the experimental groups were analysed for ACP, ALP, bilirubin, LDH, AST, ALT and total protein levels using commercially available kits (Crest Biosystems, India).

3.13.2.2. Measurement of lipid peroxidation

Estimation of lipid peroxidation or MDA content was done using TBARS assay kit (Cayman, USA) according to the manufacturer's instructions. Supernatant

was measured at 340 nm.

3.13.2.3. Measurement of TNF-α

TNF-α released in culture supernatants of the experimental mice were measured using TNF-α ELISA kit (Ray Bio, USA) according to the manufacturer's instructions. Absorbance of the sample was immediately measured after the assay at 450 nm using Bio-Rad iMark™ microplate absorbance reader.

3.13.2.4. Measurement of inhibition of NO

Nitric oxide level was determined using the Griess reagent method (Hibbs *et al*., 1988) with some modifications. Culture supernatants of the experimental groups were taken to quantify the NO level. Briefly, 60 µl culture supernatant from each group was mixed with 240 µl of Griess reagent [(1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine hydrochloride in 2.5% H_3PO_4)] in a 96-well plate. Then the plate was incubated for 20 min at room temperature for the development of purple azo-dye. The dye was detected at 540 nm.

3.13.2.5. MTT cytotoxicity assay

Carbon tetrachloride (CCl_4) creates necrosis in hepatocytes. Therefore, MTT cytotoxicity assay was performed in six sets using EZcountTM MTT Cell Assay Kit (HiMedia) according to the manufacturer's instructions, to examine the prevention rendered by CBL extract against CCl_4 mediated toxicity.

3.13.3. Histopathological studies

Livers were removed from the experimental mice, cut into small pieces and fixed in 10% formaldehyde solution for overnight followed by dehydration. Dehydrated tissues were embedded in paraffin. 4 µm sections were cut using microtome. Then liver sections were dewaxed in xylene, rehydrated in a series of different grades of alcohol and then washed with distilled water for 5 min. The liver sections were stained with basic stain haematoxylin for 40 sec and counterstained with acidic stain eosin for 20 sec. After proper staining the slides were observed (100X and 400X) using Nikon ECLIPS E200 microscope to identify the damages like necrosis, portal inflammation, vascular congestion, fatty infiltration, vacuolar degeneration, leukocyte infiltration, loss of structure of hepatic nodules and so forth (Knodell *et al*., 1981; Ruwart *et al*., 1989). Fibrosis was also observed in the CCl_4 intoxicated group.

3.13.4. Detection of intracellular ROS generation

H_2DCFDA (2', 7'-dichlorodihydrofluorescein diacetate), a hydrogen peroxide detecting probe, is used for the detection of hydrogen peroxide production in intact cells. Standard protocol (Cui *et al*., 2016) was followed with some modification for the measurement of intracellular ROS. The oxidation of 2-7 dichlorofluorescin

(H_2DCF) to 2-7 dichlorofluorescein (DCF) can be used for the quantization of H_2O_2. Oxidation of H_2DCF by ROS converts the molecule to 2, 7 dichlorodihydrofluorescein (DCF), which is highly fluorescent and the change in fluorescence intensity can be used to measure the intracellular production of ROS. Human hepatic cell line (WRL-68) was grown on coverslip in 35mm Petri-plate culture dishes and incubated for 24 hours at 37°C with 5% CO_2 in N-biotech incubator. Then cells were treated with different experimental concentrations of extract (50, 80, 100, 150 and 200µg/ml) with CCl_4 and two plates were kept without treatment for control. After 23hrs of incubation, in one untreated plate H_2O_2 (0.03%) was added again and kept for 1hr. All plates were washed twice with PBS and fresh serum media was added with 20µM 2,7-dichlorofluorescein diacetate and incubated for 30 min at 37 °C in the CO_2 incubator. Immediately after the incubation, cells were washed thrice with serum free media and glass slides were prepared by inverting coverslips on the slide in 20% glycerine/PBS solution. Cells were observed under LED-based fluorescence microscope, Magnus MLXi microscope. The cells were excited at 480 nm using LED cassettes and emission was collected using a long pass filter. Cells were observed at10X magnification and images were captured by digital SLR Olympus camera mounted on the head for high resolution image.

3.13.5. Molecular Docking

Proteins were chosen based on literature survey, having functional implications in hepatotoxic activity. The X-ray structures of the proteins available in the Protein Data Bank (http://www.rcsb.org) were used. Molecular docking was conducted using Auto Dock Vina (Trott and Olson, 2010). The receptor structures were defined as rigid, and the grid dimensions were 100, 100 and 100 for the X, Y, and Z axes for proteins having PDB ID's 1nfi, 1vkx, 2jod. On the other hand for proteins with PDB ID's 1ilg, 1n3u, 3i7h, 7api grid dimension were 80, 80, 80 for X, Y and Z axes respectively. Gasteiger charges were assigned for all the compounds, and nonpolar hydrogen atoms were merged. All torsions of the ligand were allowed to rotate during docking. The value for the exhaustiveness of the search was 8. All graphic manipulations and visualizations were performed using the AutoDock Tools and ligand docking with Autodock Vina.

3.13.6. Statistical analysis

All data are reported as the mean ± SD of six measurements. One way analyses of variance (ANOVA) test was done to compare between the control and the test group using KyPlot version 2.0 beta 15 (32 bit) for windows and Graph Pad Prism V6.0. $P<0.05$ was considered significant. Percentage of inhibition/scavenging was calculated by the following formula: (X0-X1)/X0×100, where X0= absorbance of

control and X1= absorbance in the presence of the samples or standard. The IC_{50} (half maximal inhibitory concentration) values were calculated by the following formula: Y = A1/(X+A1) ×100, where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration.

3.14. Neuromodulatory activity

3.14.1. Experimental Design (*In-vitro*)

3.14.1.1. Acetylcholinesterase (AChE) inhibitory activity

Acetylcholinesterase inhibiting activity of *C. bonplandianus* leaf extract was carried out based on Ellman *et al*., (1961) method with concise modification. Briefly, a reaction mixture was prepared containing sodium phosphate buffer (0.1 mM), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB; 0.1 mM), various concentration of plant extract (0-200 µg/ml) and acetylcholinesterase (2U/ml) in a 96 well micro plate and incubated for 15 min at 25^0 C. Following incubation, acetylcholin iodide (0.05 mM) was added as substrate in the reaction mixture and the enzyme activity was measured immediately after 3 min in a Bio-Rad iMark^{TM} microplate absorbance reader at 412 nm. Eserine was used as positive control. Percentage of inhibition was calculated in terms of percentage by dividing the difference of sample absorbance from control with control absorbance × 100.

3.14.2. Experimental Design (*In-vivo*)

3.14.2.1. Memory-Enhancing Behavioral Study

3.14.2.1.1. Experimental design and drug administration

A total of six group of mice, six in each group received the following treatment schedule:

Group I: normal control, received normal saline for 20 days; **Group II:** Scopolamine treated mice (1 mg/kg BW; dissolved in Mili-Q water), received normal saline for 20 days; **Group III:** Scopolamine-treated mice, received standard drug, Donepzil 1 mg/kg BW per day (dissolved in Mili-Q water) for 20 days; **Group IV:** Scopolamine-treated mice, received low dose CBL extract (dissolved in Mili-Q water) at 50 mg/kg BW per day for 20 days; **Group V:** Scopolamine-treated mice, received medium dose CBL extract (dissolved in Mili-Q water) at 100 mg/kg BW per day for 20 days; **Group VI:** Scopolamine-treated mice, received high dose CBL extract (dissolved in Mili-Q water) at 250 mg/kg BW per day for 20 days.

On the 21^{th} day, plant extract/standard drug/normal saline was injected after 90 min. of scopolamine treatment followed by behavioral test performed after 45 min of injection. The reaction of learned task was examined after 24 hour of last injection (22^{nd} day). All the trial and examining sessions were carried out at night, preferably between 19:00 and 23:00 h due to restless movement of the mice during night.

3.14.2.1.2. Step-through passive Avoidance task

The task was carried out as per previously described methods of Reddy, (1997), using a wooden two compartment passive avoidance apparatus (dark and night chamber, partitioned by a wall with an open able door in the middle part), with slight alterations of the time intervals. After compliance of the trial, on the 20th, the animal groups were kept in the experimental room. One hour leter, each mouse was placed in the light chamber for the attainment trial and was left to familiarize to the apparatus. After 100 s, the middle door was opened, and the mouse was allowed to enter the dark compartment. The latency time with which the animal crossed into the dark compartment was recorded. Animals that fail to enter the dark compartment within 100s were removed from the experiments. Once the mouse entered with all its four paws to the dark compartment, the middle door was closed and the animal was transferred into its home cage. The same trial was again repeated after 30 min of the first test, but the door was opened after 5s. Once the animal reached to the dark compartment, the door was closed and a mild foot shock (25V, AC, 5s) was immediately transferred to its home cage. In this test, the initial latency (IL) period of entrance into the dark chamber was noted with in 120s (selected as maximum time). A retention test was performed to determine short time memory of mice after 24 h of last training (i.e., 21st day). Briefly, each animal was placed in the light compartment for 20s and the middle door was opened. The step through latency (STL) was measured for entering into the dark compartment and the test session ended when the animal reached the dark chamber or waited in the light compartment for 300s. During these sessions, no electric shock was applied.

3.14.2.1.3. Preparation of brain tissue sample

After behavioral evaluations, the animals were anesthetized and sacrificed. The brain of the sacrificed mice of each group were removed quickly from the skull and washed consciously in ice cold normal saline followed by homogenization in 1X K_H2PO_4 saline (10%, w/v, pH8), to which BHT (0.004%, w/v) was added to prevent autoxidation of the samples. Subsequently, the homogenate was centrifuged at 10,000 × g for 30 minutes at 4^{0}C and an aliquot of supernatant was separated for further biochemical studies.

3.14.2.1.4. Determination of AchE activity in the brain tissue

The brain AchE activity was measured as per Ellman's methods (1961) using DTNB (5, 5'-dithiobis-2-nitrobenzoic acid). The absorbance was measured at 412 nm.

3.14.2.1.5. Estimation of peroxidase activity

Peroxidase activity was estimated by measuring the oxidation of guiacol in the

brain of treated mice according to a standard method (Sadasivam and Manickam, 2008). 50 mg of tissue samples were homogenized in 0.1M ice cold phosphate buffer (pH 7.0) and centrifuged at 3000 rpm for 15 min for the study of peroxidase activity. The supernatant (100 µl) was mixed with 20 mM guiacol. Time was recorded for the increase of absorbance by 0.1 at 436 nm in presence of 300 µl H_2SO_4 (12.3 mM).

3.14.2.1.6. Estimation of catalase activity

Catalase activity was assessed by the standard protocol of Luck (Luck and Bergmeyer, 1971) with some modifications, wherein degradation of substrate H_2O_2 by catalase in the brain tissue samples was measured. 50 mg of tissue samples were homogenized in 0.05 M of 1 ml Tris-HCl buffer (pH 7.0) and centrifuged at 10,000 rpm for 10 min at 4° C for the study of catalase activity. The supernatant was collected. In a spectrophotometric cuvette, 500 µl of 0.34 mM H_2O_2, 2.5 ml H_2O and 40 µl supernatant were added and change in absorbance was noted six times at 30 sec intervals at 240 nm.

3.14.2.1.7. Estimation of reduced glutathione (GSH)

Reduced glutathione activity was measured according to the standard protocol (Ellman, 1959). An aliquot of 1 ml brain tissue supernatant was treated with 0.5 of Elman reagent (19.8 mg DTMB dissolved in 100 ml of 0.1 % sodium nitrate). After the treatment with Elman reagent, 3 ml of phosphate buffer was added and the absorbance was measured at 412 nm.

3.14.2.1.8. Estimation of superoxide dismutase (SOD)

For the estimation of superoxide dismutase, standard method was followed with minor modifications (Mishra and Fridorich, 1972). Reaction mixture was prepared using 1 ml of 50 mM sodium carbonate, 0.4 ml of 25 µM nitroblue tetrazolium and 0.2 ml of 0.1 mM freshly prepared hydroxylamine hydrochloride. Clear supernatant of brain homogenate (0.1 ml, 1:10 w/v) was added to the reaction mixture. The changes in absorbance of the sample were recorded at 560 nm.

3.14.3. Histopathological studies

Brains were removed from the experimental mice, cut into small pieces and fixed in 10% formaldehyde solution for overnight followed by dehydration. Dehydrated tissues were embedded in paraffin. 4 µm sections were cut using microtome. Then brain sections were dewaxed in xylene, rehydrated in a series of different grades of alcohol and then washed with distilled water for 5 min. The brain sections were stained with basic stain haematoxylin for 40 sec and counterstained with acidic stain eosin for 20 sec. After proper staining the slides were observed (100X and 400X) using Nikon ECLIPS E200 microscope to

identify the damages.

3.14.4. Statistical analysis

All data are reported as the mean ± SD of six measurements. One way analyses of variance (ANOVA) test was done to compare between the control and the test group using KyPlot version 2.0 beta 15 (32 bit) for windows and Graph Pad Prism V6.0. $P<0.05$ was considered significant. The IC_{50} (half maximal inhibitory concentration) values were calculated by the following formula: $Y = A1/(X+A1) \times 100$, where $A1 = IC_{50}$, Y = response (Y = 100% when X = 0), X = inhibitory concentration.