Amelioration of CCl₄ induced liver injury in swiss albino mice by antioxidant rich leaf extract of Croton bonplandianus Baill.

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Abstract

The progress in industrialization has blessed mankind with a technologically superior lifestyle but poor management of industrial waste has in turn poisoned nature. One such chemical is carbon tetra chloride (CCl₄), which is a potent environmental toxin emitted from chemical industries and its presence in the atmosphere is increasing at an alarming rate. Presence of CCl₄ in human body is reported to cause liver damage through free radical mediated inflammatory processes. Kupffer cells present in the liver are potentially more sensitive to oxidative stress than hepatocytes. Kuffer cells produced tumor necrosis factor-α (TNF-α) in response to reactive oxygen species (ROS), that might further cause inflammation or apoptosis. In this study hepatoprotective capacity of antioxidant rich extract of Croton bonplandianus Baill. (CBL) was evaluated on CCl₄ induced acute hepatotoxicity in murine model. Hydro-methanolic extract of C. bonplandianus leaf was used for evaluation of free radical scavenging activity. Liver cells of experimental mice were damaged using CCl₄ and subsequently hepatoprotective potential of the plant extract was evaluated using series of in-vivo and in-vitro studies. In the hepatoprotective study, silymarin was used as a positive control. Antioxidant enzymes, pro-inflammatory markers, liver enzymatic and biochemical parameters were studied to evaluate hepatoprotective activity of Croton bonplandianus leaf extract. Free radical scavenging activity of CBL extract was also observed in WRL-68 cell line. The phytochemicals identified by GCMS analysis were scrutinized using in-silico molecular docking procedure. The results showed that CBL extract have potent free radical scavenging capacity. The biochemical parameters were over expressed due to CCl₄ administration, which were significantly normalized by CBL extract treatment. This finding was also supported by histopathological evidences showing less hepatocellular necrosis, inflammation and fibrosis in CBL and silymarin treated group, compared to CCl₄ group. ROS generated due to H₂O₂ in WRL-68 cell line were normalize in the highest group (200 μg/ml) when compared with control and negative control (CCl₄) group. After molecular docking analysis, it was observed that the compound α-amyrin present in the leaf extract of C.
bonplandianus has better potentiality to protect hepatocellular damages than the standard drug Silymarin. The present study provided supportive evidence that CBL extract possesses potent hepatoprotective capacity by ameliorating haloalkane induced liver injury in the murine model. The antioxidant and anti-inflammatory activities also affirm the same. The synergistic effects of the phytochemicals present in CBL are to be credited for all the hepatoprotective activity claimed above.

Introduction

In aerobic system, oxygen and nitrogen are abundantly found. These molecules take part in various physiological and metabolic processes and undergo changes [1]. The molecules in turn gets transformed to unpaired moieties which are readily reactive, example singlet oxygen, superoxide, nitric oxide etc. [2]. The oxygen free radicals and nitrogen free radicals are such reactive moieties. Later it was found that apart from unpaired free radicals certain paired non-radical compounds like hydrogen peroxide, ozone and peroxynitrite are equally harmful [3]. So, the terms Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) were coined to include all these reactive radical and non-radical molecules which are produced by different endogenous and exogenous sources inside living system [4]. Although, ROS and RNS have certain beneficial role like signal transduction, bodies defence mechanisms against microbes etc., and are required in trace amount inside our body. But, due to their chemical nature they helps lipid peroxidation causes DNA damage and oxidize several molecules including cellular membrane and causes injury [5]. Inspite of having a strong antioxidant mechanism (natural antioxidant in body), cell damage from ROS/ RNS is pervasive. Oxidative/nitrosative stress is an essential phenomenon associated in this regard. It occurs due to an imbalance between the production of this oxygen free radical (oxidants) and their elimination by defence mechanisms referred to as antioxidant. Similarly, nitrosative stress is also equally dangerous and arises mainly due to nitrogen free radical [6].

Hepatic cells are equally if not more vulnerable to such stresses [7]. Oxidative stress is a commonly used term that refers to a state when the cellular redox balance is altered [8]. The parenchyma and the non-parenchyma cells of liver are equally affected by oxidative stress, although both show different response. The mitochondrion microsome and peroxisome in parenchyma cells can produce ROS, which triggers the regulation of PPARα gene. The PPAR α, δ, γ are involved in kitogenesis. Although parenchyma cells consists of 70–80% of liver volume and covers bulk of the liver constitution, the non-parenchyma cells also plays a huge role on oxidative stress. The changes in composition of parenchyma cells as well as extra cellular matrix of liver due to oxidative and nitrosative stress modulates immune system. Immune and inflammatory cells get recruited at the site of injury and this activates non-parenchyma cells like hepatic stellate cells and kupffer cells [9]. There is a substantial increase in levels of growth hormone, cytokines and chemokines which leads to fibrosis and chronic liver diseases. Liver fibrosis is the gateway to several liver-related ailments and among all the reasons for liver dysfunctions, the exposure to toxic reagents and drug cannot be ignored. Carbon tetrachloride (CCL₄) has been widely used for liver studies for long now [10].

Croton bonplandianus Baill. (C. bonplandianus) (Euphorbiaceae) is an exotic weed commonly found in wastelands which possess medicinal properties [11]. Leaf of C. bonplandianus is used in the treatment of skin diseases and applied traditionally in external cuts and wounds [12]. Leaf of C. bonplandianus contains diterpene resins which are reported to have antitumor
activity [13]. *C. bonplandianus* is reported to possess potent hepatoprotective, anti-helminthic activity and also used for controlling high blood pressure [14–16]. Inspite of having diverse medicinal properties ethnobotanically, their proper pharmacognostic evidence is largely obscure. The present study aims to investigate the hepatoprotective potential of *C. bonplandianus* in CCl₄ induced murine model.

**Materials and methods**

**Preparation of plant extract**

Leaf of *Croton bonplandianus* was collected during April and May, 2016, from the garden of medicinal plants of Department of Botany, University of North Bengal (26.7072° N; 88.3558° E). The plant was identified by plant taxonomist of Department of Botany, University of North Bengal. The specimen (Accession number-09870) was deposited at the Herbarium of the same department. 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.

**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.

**Animal maintenance**

Swiss albino mice (6 male/group) were used for hepatoprotective analyses. Male Swiss albino mice were used for all the experiments. Mice (6 male/group, n = 6) were kept in polypropylene cages (Tarson, India), with paddy husk as bedding material. The experimental mice were maintained in the animal house of the Department of Zoology, University of North Bengal with sufficient food and water *ad-libitum* under a constant 12 hour dark/light cycle at an environmental temperature of 25°C.

**Determination of in vitro antioxidant activity**

Total antioxidant, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging, hydroxyl radical scavenging, superoxide radical scavenging, nitric acid radical scavenging, singlet oxygen scavenging, reducing power, Fe²⁺ chelation, peroxynitrite scavenging and hypochlorous acid scavenging activities were determined by following the previously reported methods with minor modification for the evaluation of free radical scavenging properties of CBL extract [17,18].
**Determination of erythrocyte-membrane stabilizing activity**

Erythrocyte membrane stabilizing activity of CBL extract was performed by standard method as described by Dey et al. [19]. 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.

**Determination of total phenolic and flavonoid content**

The total phenolic and flavonoid content of CBL extract was determined using standard protocol [17]. A standard curve prepared with known quantities of gallic acid (R² = 0.9468) and quercetin (R² = 0.9947) were used to measure the phenolic and flavonoid content respectively.

**Detection of intracellular ROS generation**

A colorimetric procedure has been used to generally quantify H₂O₂ by oxidation of 2’-7’ dichlorofluorescin (H₂DCF) to 2’-7’dichlorofluorescein (DCF). Originally, DCF was thought to be specific for hydrogen peroxide, but recent evidence has shown that other ROS/RNS such as nitrate and hypochlorous acid can oxidize H₂DCF [20, 21]. 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% CO₂ in N-biotech incubator. Then cells were treated with different experimental concentrations of extract (50, 80, 100, 150 and 200μg/ml) with CCl₄ and two plates were kept without treatment for control. After 23hrs of incubation, in one untreated plate H₂O₂ (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 CO₂ incubator. Immediately after the incubation, cells were washed thrice with serum free media and glass slides were prepared by inverting cover slips on the slide in 20% glycerine/PBS solution. Cells were observed under LED-based fluorescence microscope, Magnus MLXi microscope. The cells were excited at 480nm 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.

**Experimental design: In vivo hepatoprotective activity**

**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 (OECDiLibrary, 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.

**Doses.** Swiss albino mice (36 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; CCl₄ group received 1:1 (v/v) CCl₄ in olive oil; Silymarin group received 1:1 (v/v) CCl₄ in olive oil and 100 mg/kg BW silymarin; low dose extract (CBLL) group received 1:1 (v/v) CCl₄ in olive oil and 50 mg/kg, medium dose extract (CBLM) group received 1:1 (v/v)
CCl₄ in olive oil and 100 mg/kg and high dose extract (CBLH) groups received 1:1 (v/v) CCl₄ in olive oil and 250 mg/kg BW respectively.

On 22nd day i.e. 24 h after the last dose, under proper anesthesia (2% ether) all the animal were sacrificed by cervical dislocation to alleviate suffering. 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.

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 N₂ levels using commercially available kits (Crest Biosystems, India).

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 [22]. 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 H₂SO₄ (12.3 mM).

Estimation of catalase activity. Catalase activity was assessed by the standard protocol of Luck [23] with some modifications, wherein degradation of substrate H₂O₂ 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 H₂O₂, 2.5 ml H₂O and 40 μl supernatant were added and change in absorbance was noted six times at 30 sec intervals at 240 nm.

Estimation of reduced glutathione (GSH). Reduced glutathione activity was measured according to the standard protocol [24]. An aliquot of 1 ml liver tissue supernatant was treated with 0.5 of Elman reagent (19.8 mg DTNB 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.

Estimation of superoxide dismutase (SOD). For the estimation of superoxide dismutase, standard method was followed with minor modifications [25]. 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.

Experimental design: In vitro. In vitro hepatoprotective potentiality of C. bonplandianus extracts was studied according to the previously described standardized protocol with some modifications [10, 26, 27]. 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; CCl₄ group received 25 μl/ml CCl₄; Silymarin group received 25 μl/ml CCl₄ and 100 μg/ml silymarin; low dose extract group (CBLL) received 25 μl/ml CCl₄ and 25 μg/ml CBL; medium dose extract group (CBLM) received 25 μl/ml CCl₄ and 50 μg/ml CBL; high dose extract groups received 25 μl/ml CCl₄ 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.

**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).

**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.

**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.

**Measurement of inhibition of NO.** Nitric oxide level was determined using the Griess reagent method [28] 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% H₃PO₄)] 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.

**MTT cytotoxicity assay.** Carbon tetrachloride (CCl₄) creates necrosis in hepatocytes. Therefore, MTT cytotoxicity assay was performed in six sets using EZcount™ MTT Cell Assay Kit (HiMedia) according to the manufacturer’s instructions, to examine the prevention rendered by CBL extract against CCl₄ mediated toxicity.

**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 [29,30]. Fibrosis was also observed in the CCl₄ intoxicated group.

**GC–MS analysis**

CBL extract was dissolved in n-hexane and the mixture was centrifuged thrice at 12,000 rpm for 15 min for GC–MS analyses. Supernatant was collected and used for GC–MS analysis. Agilent 5975 GCMS system (Agilent Technologies, USA) attached with HP 5 ms Capillary Column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) and equipped with inert MSD triple axis mass detector condition edation trap 200˚C, transfer line 280˚C, electronenergy70eV (vacuum pressure-2.21e0.5 Torr) was used to identify the bio active compounds present in CBL extract. Helium was used as a carrier gas at a flow rate of 1 ml/min and 2 ml sample was injected in a split less mode. The column temperature was set at 60˚C for 1 min followed by 5˚C/min up to 250˚C and the essential compounds in CBL were identified by the retention times and mass fragmentation patterns using Agilent Chem Station integrator and the database of National Institute of Standard and Technology (NIST) with a MS library version2011.
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 AutoDock Vina [31]. 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 (IkappaBalpha/NF-kappaB complex), 1vkx (Crystal structure of p50/p65 heterodimer), 2jod (adenylate cyclase). On the other hand for proteins with PDB ID’s 1ilg (Human Pregnane X Receptor), 1n3u (oxygenase-1), 3i7h (Crystal Structure of DDB1), 7api (human alpha1-antitrypsin) 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.

Statistical analysis

All data were analysed post-blank normalization and quantitative data are reported as the mean ± SD of six measurements. Statistical analysis was performed by paired t-tests using KyPlotV5.0 (32 bit) 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. Extent of dose-dependent activity was calculated by pair wise linear correlation analysis between group mean percent of inhibition Vs respective concentrations.

Results

In vitro antioxidant activity

The free radical scavenging activity of CBL in dose dependent manner and the differences in the activities compared with standard compound. Half maximal inhibitory concentration (IC$_{50}$) of CBL and corresponding references are shown in the S1 Table.

Leaf extract of CBL showed lower IC$_{50}$ value than ascorbic acid, mannitol, sodium pyruvate, curcumin, quercetin in DPPH, hydroxyl radical, H$_2$O$_2$, nitric oxide, superoxide anion, hypochlorous acid scavenging assay and found comparable with peroxinitrate shown by gallic acid and lipid peroxidation shown by trolox. On the other hand, CBL showed higher IC$_{50}$ value than lipoic acid and EDTA in singlet oxygen and iron chelation scavenging assay. In case of total antioxidant activity, CBL showed better scavenging activity than the standard ascorbic acid. The reducing power of CBL was also determined and found that the reducing capacity of CBL extract was increased in a dose dependent manner comparable to the reference compound ascorbic acid (Figs 1–6).

Differential effects on the individual components of Haber–Weiss reaction (Fenton chemistry)

The Fenton chemistry as a part of the Haber-Weiss reactions is considered central to the intracellular free radical formation cascade. *C. bonplandianus* extract was evaluated for their capacity to directly affect the individual components of the Haber–Weiss reaction. *C. bonplandianus* demonstrated significantly H$_2$O$_2$ neutralizing activity (Fig 2A and 2B). The ferric iron (Fe$^{3+}$) chelation capacity of *C. bonplandianus* at 200 μg/mL was 59.36 ± 1.81% (Fig 4A and 4B). EDTA, however demonstrated significantly (P<0.001) superior ferric chelation capacity as demonstrated by its
low IC_{50} value of 10.23±0.89 μg/ml. In case of direct scavenging of OH•, *C. bonplandianus* demonstrated superior activity than that of the standard mannitol (Fig 1C and 1D).

**Inhibition of OH•: Bivariate analysis**

Dose-dependent bivariate correlation analysis of Fe^{2+} chelation and H_{2}O_{2} inhibition were performed vs inhibition of OH• in order to reflect how individually *C. bonplandianus* may inhibit the formation of OH•. Fe^{2+} chelation activity by the inhibition of OH• is higher in the presence of *C. bonplandianus* (r = 0.8925 and r^2 = 0.7966). The correlation between the inhibitions of OH• and H_{2}O_{2} were much more comparable, with minor trend towards H_{2}O_{2} mediated effect (r = 0.9825 and r^2 = 0.9652) (S1 Fig).

**Determination of phenol and flavonoid content in CBL extract**

Total amount of phenolic content present in the hydromethanolic extract of CBL was found to be 75.06 ± 2.33 mg/ml gallic acid equivalent per 100 mg plant extract and the total flavonoid content of CBL extract was 52.17 ± 4.36 mg/ml quercetin equivalent per 100 mg plant extract.

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Fig 1. Antioxidant activity of *Croton bonplandianus*. (A)&(B) DPPH scavenging activity; (C) % of hydroxyl radical (OH•) scavenging Vs standard mannitol; (D) depicts remaining unneutralized OH•. Data expressed as mean ± S.D (n = 6). *α* p<0.05; *β* p<0.01; *γ* p<0.001; NS-Non significant when compared with standard.

https://doi.org/10.1371/journal.pone.0196411.g001
Detection of intracellular ROS generation

Human hepatocell line WRL-68 was used to examine the effects of CBL under oxidative stress. CCl₄ increases oxidative stress levels in the liver tissue, and based on that study, it is speculated that CCl₄ may induce the oxidative stress in WRL-68 cells. Therefore, WRL-68 cells were treated with CCl₄ for 0–24 h, and intracellular oxidative levels were measured using the dichlorofluorescein assay. Fig 7 demonstrated that cells exposed to CCl₄ exhibited significantly increase in ROS levels. Tremendous decrease in fluorescence was detected at higher doses of CBL (200 μg/ml) at 24h post exposure compared to the CCl₄ (Fig 7). The resulting change in fluorescence intensity gives strength to the hypothesis that CBL affects in the production of intracellular ROS.

Hepatoprotective activity

Acute toxicity study. CBL extract was administered to the experimental animals up to 2,000 mg/kg body weight. However, at the 2000 mg/kg body weight dose, no sign of mortality
and physiological deformation were observed in the experimental animals. Therefore, 50 mg/kg BW, 100 mg/kg BW and 250 mg/kg BW doses were selected as a low, medium and high dose in the in vivo hepatoprotective experiments.

Body and liver weight. Significant body weight changes were observed in CCl₄, CBLM and silymarin groups shown in Table 1. Final body weight was decreased only in CCl₄ groups (12.53 ± 1.39). On the other hand liver weight of CCl₄ group (5.16 ± 0.15) resulted in the highest relative liver weight (26.37 ± 1.40) among all the groups. Interestingly only high dose group (CBLH) prevented utmost percent of body weight changes. The relative liver weight of all the groups were closes another, except CCl₄ group.

In vivo liver marker enzymes and biochemical parameters. The effect of CCl₄ and the subsequent administration of silymarin and CBL on the various serum enzymatic and biochemical parameters are shown in Table 2. All the in vivo experimental parameters were increased in case of CCl₄ group and subsequently decreased with silymarin and CBL treatment except protein and albumin.
Estimation of hepatic antioxidative enzymes: Catalase, peroxidase, superoxide dismutase and reduced glutathione. Significant inhibition of enzymatic catalase and SOD (superoxide dismutase) and non-enzymatic reduced glutathione by CBL extract occurred in CCl₄ intoxicated mice when compared with the control (Fig 8). CBL treatment enabled significant increase in the enzyme activity of catalase and reduced glutathione when compared with CCl₄ treated groups. On the other hand silymarin treatment significantly increase the percent of inhibition compared with the CCl₄ treated mice. On the other hand the activity of peroxidase enzyme in hepatic tissue is significantly lowered as a result of CCl₄ treatment (Fig 8). The peroxidase activity in the control group was 14.79 unit/mg tissues which were lowered 7.43 unit/mg tissues due to CCl₄ administration. The lowered peroxidase activity was significantly elevated by CBLH (12.74 unit/mg tissue) when compared with the standard silymarin treated group (12.07 unit/mg tissue).
In vitro liver marker enzymes and biochemical parameters. The hepatoprotective potential of CBL extract was reflected through in vitro liver marker enzymes and biochemical parameters. The results were compared with the standard drug silymarin as shown in Table 3. The CCl₄ group showed the higher toxicity than the other groups.

Lipid peroxidation (MDA level). Lipid peroxidation or MDA level in the treated groups are illustrated in (Fig 9A). The MDA content was elevated from 8.99 μM/litre in control to 19.27 μM/litre in CCl₄ group. Significant results found when the elevated MDA level was lowered to 10.60 μM/litre after CBLH administration.

MTT cytotoxic effect. Viability of cells were decreased in the CCl₄ group significantly (p<0.01) compared to the control group (Fig 9C). The viability of the cells in CCl₄ group was only (43.67 ± 6.11), where in the standard silymarin group the percent of viability of cells was (87.00 ± 5.29) which was very close to the control group. On the other hand, in the experimental groups, the cell viability was increased gradually. Percentage of cell viability in CBLH group (70.33 ± 8.02) was very close to the standard group.

Fig 5. Antioxidant activity of Croton bonplandianus. (A) % inhibition of Singlet O₂ (¹O₂) Vs standard lipoic acid; (B) depicts unneutralized Singlet O₂ (¹O₂); (C) % inhibition of Nitric oxide (NO) Vs standard curcumin; (D) depicts unneutralized nitric oxide (NO). Data expressed as mean ± S.D (n = 6). α p<0.05; β p<0.01; γ p<0.001; NS-Non significant when compared with standard.

https://doi.org/10.1371/journal.pone.0196411.g005
Measurement of the release of TNF-α. Measurement of TNF-α release are demonstrated in the Fig 9B. The level of TNF-α in control group was 120.32 ± 8.04 pg/ml, which was increased 1259.20 ± 96.96 pg/ml due to CCl₄ toxicity. However the TNF-α level decreased better by CBLH group (716.66 ± 73.06 pg/ml) when compared with the standard silymarin group (786.22 ± 49.70 pg/ml).

Inhibition of nitric oxide (NO). CCl₄ toxicity resulted increases in NO release when compared to the control (Fig 9D). However, significant (p < 0.001) lowering of NO level was observed in the treated groups. The NO level in silymarin and CBLH groups were 141.33 ± 12.70 and 175.33 ± 12.50% respectively, when NO release of control was considered as 100%.

Histopathological examination

There are several histological parameters showed the injury level of experimental groups as enlisted in S2 Table. The haematoxilin-eosin staining of hepatocytes displayed clearly the well...
maintained hepatocellular integrity, healthy cellular architecture, and clear cytoplasm with prominent nucleus in the control group. But in the CCl$_4$ group, several damages have been observed. Hepatocytes of the CCl$_4$ groups showed hepatocellular necrosis, bile duct proliferation, sinusoidal dilatation, inflammation (leukocyte infiltration), vascular congestion, loss of structure of hepatic nodules, fatty infiltration, vascular degeneration and calcification. Most strikingly fibrosis, the thickening and scoring of connective tissue, as a result of injury was notified in the CCl$_4$ group (Figs 10 and 11; S2 Fig). The injury level found in the CCl$_4$ group was down regulated by the administration of standard drug silymarin. Interestingly, in the

Fig 7. Effects of CBL in depletion of intracellular ROS production generated by CCl$_4$ in WRL-68 cells. Production of ROS was measured by cleavage of acetate group of non-fluorescent H$_2$DCFDA (2',7'-dichlorodihydrofluorescein diacetate) which convert into DCF (2',7'-dichlorofluorescein) highly fluorescent. Cells were exposed to CCl$_4$ before treatment with CBL 50, 80, 100, 150 and 200 μg/ml for 24 h. The ROS production displays the intensity of fluorescence through the images of WRL-68 cells treated with different concentration of CBL (D-H), CCl$_4$ (C), H$_2$O$_2$ (B) and control (A).

Table 1. Changes of body weight (g) and liver weight (g) in different experimental groups. Data represented as mean ± SD of six observations.

| Group          | Initial body weight | Final body weight | % body weight change | Liver weight | Relative liver weight |
|----------------|---------------------|-------------------|----------------------|--------------|-----------------------|
| Control        | 22.82 ± 0.65        | 25.84 ± 0.18 *    | 11.69 ± 2.63 ▲      | 4.70 ± 0.16  | 18.21 ± 0.68          |
| CCl$_4$        | 22.06 ± 0.29        | 19.61 ± 0.50 **   | 12.53 ± 1.39 ▼      | 5.16 ± 0.15  | 26.37 ± 1.40          |
| Silymarin      | 22.71 ± 0.54        | 24.67 ± 0.85 *    | 7.92 ± 1.24 ▲       | 4.52 ± 0.21  | 18.33 ± 0.57          |
| CBL (50 mg/kg BW) | 23.22 ± 0.41      | 23.87 ± 0.17 *    | 2.72 ± 1.08 ▲       | 5.07 ± 0.12  | 21.24 ± 0.42          |
| CBL (100 mg/kg BW) | 22.05 ± 0.52     | 22.99 ± 0.48 **   | 4.06 ± 0.32 ▲       | 4.89 ± 0.25  | 20.88 ± 0.60          |
| CBL (250 mg/kg BW) | 22.73 ± 0.76      | 24.59 ± 0.41 *    | 7.57 ± 2.72 ▲       | 4.63 ± 0.22  | 18.83 ± 1.12          |

NS p > 0.05  
*p < 0.05  **p < 0.01.

Final body weight was compared with initial body weight of corresponding group and liver weight of treated groups was compared with liver weight of control group. ▲ represents increase and ▼ represents decrease.

https://doi.org/10.1371/journal.pone.0196411.t001
Table 2. Describes the levels of various enzymatic and biochemical parameters in the serum of six (n = 6) treated groups. The data represented as mean ± SD of six observations.

| Parameters (units) | Control | CCl4 | Silymarin | CBL (50 mg/kg BW) | CBL (100 mg/kg BW) | CBL (250 mg/kg BW) |
|--------------------|---------|------|-----------|------------------|-------------------|-------------------|
| ACP (K.A.)         | 3.81 ± 0.04 | 13.13 ± 0.63** | 6.02 ± 0.48<sup>b</sup> | 11.21 ± 0.23<sup>**</sup>d | 9.88 ± 0.73<sup>**</sup>c | 8.21 ± 0.22<sup>b</sup> |
| ALP (K.A.)         | 13.09 ± 0.30 | 31.16 ± 0.25** | 16.23 ± 0.35*** | 29.30 ± 0.48<sup>**</sup>d | 28.24 ± 0.35<sup>**</sup>b | 23.07 ± 0.66<sup>**</sup>c |
| AST (u/ml)         | 63.34 ± 0.59 | 142.19 ± 0.66** | 82.66 ± 0.94<sup>**</sup>c | 141.70 ± 0.81<sup>**</sup>d | 127.72 ± 0.91<sup>b</sup> | 108.49 ± 0.11<sup>c</sup> |
| ALT (u/ml)         | 47.94 ± 0.65 | 137.39 ± 0.61<sup>c</sup> | 56.53 ± 0.87<sup>c</sup> | 123.18 ± 0.39<sup>c</sup> | 105.72 ± 1.11<sup>c</sup> | 77.20 ± 0.32<sup>c</sup> |
| GGT (u/l)          | 3.76 ± 0.11 | 8.26 ± 0.52** | 4.90 ± 0.20<sup>b</sup> | 7.06 ± 0.33<sup>d</sup>d | 6.32 ± 0.26<sup>d</sup>d | 5.60 ± 0.32<sup>b</sup> |
| Glucose (mg/dl)    | 57.04 ± 1.53 | 85.18 ± 1.19** | 63.85 ± 1.32<sup>b</sup> | 81.72 ± 2.29<sup>d</sup>d | 72.15 ± 0.65<sup>c</sup>c | 67.41± 0.91<sup>b</sup> |
| Protein (g/dl)     | 5.93 ± 0.06 | 4.07 ± 0.05*** | 5.72 ± 0.07<sup>NSb</sup> | 4.11 ± 0.06<sup>**</sup>d | 4.26 ± 0.09<sup>d</sup>d | 5.12 ± 0.31<sup>a</sup> |
| Albumin (g/dl)     | 4.54 ± 0.33 | 2.27 ± 0.20<sup>b</sup> | 3.43 ± 0.17<sup>b</sup> | 2.52 ± 0.13<sup>d</sup>d | 2.87 ± 0.10<sup>d</sup>d | 3.03 ± 0.16<sup>d</sup> |
| Globulin (g/dl)    | 2.19 ± 0.02 | 0.84 ± 0.03*** | 1.99 ± 0.03<sup>c</sup> | 0.99 ± 0.03<sup>b</sup>b | 1.16 ± 0.03<sup>c</sup>c | 1.72 ± 0.04<sup>c</sup> |
| Bilirubin (mg/dl)  | 0.81 ± 0.04 | 2.04 ± 0.07** | 1.05 ± 0.05<sup>b</sup>b | 1.88 ± 0.09<sup>d</sup>d | 1.71 ± 0.04<sup>b</sup>b | 1.33 ± 0.04<sup>b</sup> |
| Urea (mg/dl)       | 35.43 ± 3.94 | 128.76 ± 6.38** | 58.16 ± 4.76<sup>b</sup> | 116.46 ± 4.29<sup>**</sup>d | 100.51 ± 0.68<sup>b</sup>b | 78.24 ± 3.04<sup>b</sup> |
| Urea N<sub>e</sub> (mg/dl) | 12.69 ± 0.96 | 72.55 ± 1.91** | 30.65 ± 0.91<sup>b</sup>b | 68.05 ± 1.38<sup>c</sup>c | 53.70 ± 1.45<sup>c</sup>c | 40.61 ± 1.24<sup>b</sup> |
| LDH (u/l)          | 240.73 ± 2.89 | 571.83 ± 4.11*** | 296.01 ± 2.15*** | 548.55 ± 4.45*** | 502.66 ± 6.04<sup>b</sup>b | 340.50 ± 6.7<sup>c</sup>c |
| Cholesterol (mg/dl)| 68.66 ± 1.51 | 132.17 ± .38*** | 90.37 ± 2.05<sup>**</sup>c | 113.38 ± 3.55<sup>b</sup>b | 101.35 ± 2.19<sup>b</sup>b | 93.22 ± 1.67<sup>b</sup> |

<sup>NS</sup> = non significant (p> 0.05)
<sup>*</sup>P < 0.05
<sup>**</sup>P < 0.01 and
<sup>***</sup>P < 0.001 vs control group; whereas
<sup>#</sup>p = non significant (p> 0.05)
<sup>1</sup>P < 0.05
<sup>2</sup>P < 0.01 and
<sup>3</sup>P < 0.001 vs CCl<sub>4</sub> group.

During the present study it was observed that high dose of plant extract (CBLH) down regulates the injury better or similar compared to the standard silymarin.

**GC-MS analysis**

GC-MS analysis showed the chemical fingerprint of the CBL extract (Fig 12 and S3 Table). The GC-MS data reported that the presences of several bioactive compounds, of which many of them are documented, possess distinct and definitive pharmacological activities.

**Molecular docking**

The bioactive compounds of *Croton bonplandianus* was checked for possible interactions with several proteins playing the essential role in different metabolic pathways of humans and other major vertebrates. The proteins were chosen those have relationship with the health of the liver. These proteins acted as receptors required for molecular docking experiments. The ligands required to conduct the experiment are the compounds identified my GC-MS analysis of the plant extract. Upon a series of receptor-ligand interaction study, it was identified that each of the ligands has different binding affinity with the selected proteins. It is seen that α-amyrin has the highest interaction with all the receptors on an average followed by Campes-tanol and Ethyl iso-allocholate (Fig 13). On the other hand 1-Octacosanol has the least binding affinity with the receptors. For the comparative analysis a standard was used. Silymarin a proven drug against hepatotoxicity was used as a standard in this regard. One of the phyto-chemical α-amyrin had a binding affinity better than silymarin with all the receptors on average. The highest binding affinity was found between α-amyrin and a protein with PDB ID
NFκB protein and Campesterol also has good binding affinity and as seen in the molecular surface view of the protein moiety the ligand binds nicely inside a cavity in the protein surface (S3 Fig).

**Discussion**

The demand of late medicine or health supplement of natural origin has increased many folds because of their potential to prevent and reduce the risk of several oxidative damage with minimal side effects [32]. Antioxidant property covers a broad spectrum of chemical phenomenon and definite antioxidant activity should not be concluded based on a single experimental model. Therefore, in practice several in vitro antioxidants or free radical scavenging activities were carried out with our sample of interest. In the present antioxidant profiling, *C. bonplandianus* leaf extract showed potential free radical scavenging activities. The molecule DPPH is a free radical that can accept an electron or hydrogen radical to become stable and reacts with...
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Table 3. Changes in the levels of various enzymatic and biochemical parameters of the culture supernatants of the experimental groups. Data represented as mean ± SD of six observations.

| Parameters (units) | Control | CCl₄ | Silymarin | CBL (50 mg/kg BW) | CBL (100 mg/kg BW) | CBL (250 mg/kg BW) |
|--------------------|---------|------|----------|-------------------|-------------------|-------------------|
| ACP (K.A.)         | 0.80 ± 0.04 | 1.95 ± 0.04** | 1.21 ± 0.03**b | 1.72 ± 0.03***a | 1.58 ± 0.04**a | 1.36 ± 0.05**b |
| ALP (K.A.)         | 3.38 ± 0.05 | 8.06 ± 0.07*** | 4.92 ± 0.23***c | 6.83 ± 0.14***b | 6.22 ± 0.12**b | 5.45 ± 0.23**b |
| AST (u/ml)         | 15.54 ± 0.66 | 54.63 ± 0.75*** | 20.32 ± 0.94**c | 43.98 ± 0.52***d | 40.30 ± 1.1***b | 28.52 ± 0.85**b |
| ALT (u/ml)         | 7.71 ± 0.34 | 34.57 ± 1.64*** | 13.95 ± 0.29*** | 29.29 ± 0.77***a | 26.28 ± 0.80***a | 18.42 ± 1.00***c |
| GGT (u/l)          | 0.64 ± 0.03 | 1.16 ± 0.03*** | 0.79 ± 0.04**b | 1.08 ± 0.03***d | 1.03 ± 0.01***a | 0.913 ± 0.02***b |
| Bilirubin (mg/dl)  | 0.21 ± 0.02 | 0.67 ± 0.02*** | 0.316 ± 0.02**c | 0.64 ± 0.03***d | 0.54 ± 0.02**c | 0.41 ± 0.02**c |
| Protein (g/dl)     | 7.49 ± 0.04 | 5.69 ± 0.27*** | 7.05 ± 0.06***a | 5.99 ± 0.03***d | 6.63 ± 0.27**a | 6.91 ± 0.16**b |
| LDH (u/l)          | 42.59 ± 1.10 | 211.01 ± 2.59*** | 121.18 ± 1.36*** | 188.94 ± 2.78***a | 167.48 ± 3.04***b | 135.20 ± 1.49***c |

NSP = non significant (p > 0.05)
*P < 0.05
**P < 0.01 and
***P < 0.001 vs control group; whereas
语言为英文，是一页文档的自然语言文本。
proxynitrate, hydroxyl radical, superoxide, singlet oxygen and other free radicals that cause the harmful effect in our biological system. Thus, CBL extract might prove to be a key component in prevention of various diseases related to oxidative stress and free radical generation. Keeping in mind the crucial role played by oxidative stress in liver disease, medicinal plant derived antioxidant can clearly be considered as a good therapeutic strategy. In the present study it is tried to establish how antioxidants are linked with hepatic damage or disorder. For this above mentioned study CCl$_4$ (Haloalkane) was chosen to induce hepatic damage in murine model amelioration by the leaf extract of *C. bonplandianus* was investigated through antioxidant and anti-inflammatory activities. The toxicity profile of CCl$_4$ is well established worldwide [34–36]. Extensive usage of CCl$_4$ in industrial sectors has a rich history of environmental toxicity and occupational hazards. This had lead to awareness in the industrial and domestic use of CCl$_4$ from the early 70’s, leading to the production and import of CCl$_4$ [37]. Multiple sources for generation of reactive oxygen species (ROS) have been identified; among them CCl$_4$ was used.
Even though the extract treated groups possessed injury marks however, the extent of signs of injury were much lower in the extract treated groups compared to CCl₄ group. (A) Control group liver demonstrated normal liver architecture with normal sinusoids (NS), hepatocytes with intact nucleus (IN), un-inflamed portal vein (PV); (B) CCl₄ group liver demonstrated significant loss of hepatocellular architecture with extensive fatty infiltration (FI) leading to steatosis, bile duct proliferation (BdP), vascular congestion (VC) and haemorrhagic necrosis (HN) around portal vein. Loss of hepatic nodular structure and disorganized hepatocytes marked the CCl₄ induced liver damage; (C) Silymarin group demonstrated hepatoprotective activity by substantial amendment of proliferated bile duct (Bd) with normal sinusoids (NS) and intact portal veins (PV); (D) CBLL group was marked by less leukocyte infiltrations (LI), sinusoidal dilations (SD) and bile duct proliferation (BdP); (E) CBLM group reflected comparatively less haemorrhagic necrosis (HN) and fatty infiltrations (FI); (F) CBLH group demonstrated lowering of most of the injury signs however, leukocyte infiltrations (LI) could be identified in the liver samples.

https://doi.org/10.1371/journal.pone.0196411.g010
Fig 11. Photomicrographs (400×) of the histopathological examinations of the liver samples of different groups. (A) Control group liver sample possessed well packed hepatocytes with intact nuclei (IN) and normal sinusoids (NS); (B) CCL₄ group liver possessed extensive fatty infiltrations (FI), Necrotic hepatocytes (N), prominent signs of inflammation with leukocyte infiltrations (LI), prominent calcification (C) around the congested vesicles (VC) with bile duct proliferations (BdP); (C) Silymarin group liver samples were characterized with normal sinusoids (NS) and intact nuclei (IN) containing healthy hepatocytes; (D) CBLL group demonstrated lower fatty infiltrations (FI), sinusoidal dilations (SD) and leukocyte infiltrations (LI); (E) CBLM group resulted in renewal of normal hepatic architecture with several hepatocytes with intact nuclei (IN) and lowered sinusoidal dilations (SD); (F) CBLH group showed near to normal hepatic architecture with predominantly intact nuclei (IN) containing normal hepatocytes and undiluted normal sinusoids (NS).

https://doi.org/10.1371/journal.pone.0196411.g011
in the present study as a source for intracellular production of ROS. Hydrogen peroxide (H$_2$O$_2$) is a stable free radical having important role in signalling pathways [38]. Increased levels of ROS productions are associated with oxidative stress in cell. H$_2$DCFDA was used to detect the production of intracellular ROS generation. H$_2$DCFDA detects hydrogen peroxide by exhibiting fluorescence on WRL-68 cell line exposed to H$_2$O$_2$, suggesting a H$_2$O$_2$ induced oxidative stress. Generally, CCl$_4$ contribute to increase in ROS level. However, a substantial reduction in fluorescence intensity was seen with the increase in concentration of CBL from 50–200 $\mu$g/ml. This suggests that under the influence of CBL, CCl$_4$ induced ROS was diminished proportionately. It can be inferred that CBL plays an important role in reducing the impact of CCl$_4$ on normal intracellular function.

Carbon tetrachloride (CCl$_4$) induced hepatoxicity is caused to some extent by the partial pressure of reactive oxygen in tissues. Low partial pressure of oxygen results in the formation of CCl$_3^-$ and CHCl$_2^-$ radicals [39,40]. Metabolism of lipid is hampered by CCl$_4$ and cause steatosis or fatty liver. On the other hand, high partial pressure of oxygen shifts CCl$_4$ metabolisms towards the formation of CCl$_3$.OO$^-$ radical with consequent lipid peroxidation and lead the cells from steatosis into apoptosis [39,41]. In CCl$_4$ induced liver injury model, oxidative stress can provoke and promote lipid peroxidation that damage the hepatocellular membrane [39].

Fig 12. Gas Chromatography Mass Spectrometry (GC-MS) analysis of Croton bonplandianus.

https://doi.org/10.1371/journal.pone.0196411.g012
This hepatocellular damage is followed by the release of pro-inflammatory chemokines and cytokines (Fig 15) [42].

Now a day’s CCl₄ is required for the synthesis of chlorofluorocarbons (CFCs) that are used as heat transfer agents in refrigerating equipments and as aerosol propellants. In United States, CCl₄ has been widely used for industrial and domestic cleaning and sterilisation. There are many cells like kupffer cells, hepatic stellate cells and endothelial cells those are more sensitive to oxidative stress related molecules. TNF-α can be produced in kupffer cells by oxidative stress, which might increase inflammation and apoptosis (Fig 15). In studied animal model significant (P < 0.001) loss of body weight and relative liver weight have occurred after CCl₄ toxicity. After the treatment with CBL extract, the changes in the final body weight were much less compared to control and sylimarin group. This result indicates CBL extract has the potentiality in restricting drastic body weight changes through anti-hyperlipidimic activity. Bio-marker of hepatotoxicity represents the altered levels of hepatobillary enzymes transaminase and phosphatase. Whereas normalization of these enzymatic parameter represent the improvement of normal liver function [43–45]. Significant elevation of ACP, ALP, AST, ALT, GGT, LDH, glucose, urea, globulin, bilirubin and cholesterol levels and subsequent liver injury are caused due to CCl₄ toxicity. All the parameters were subsequently normalized to certain extent due to the sylimarin extract and CBL extract administration. Cultured liver cells can serve as a model for evaluation of in vitro hepatotoxicity because of its similarity between intact hepatic systems [46]. The in vitro enzymatic result also supports the hepatoprotective
Fig 14. Molecular docking (secondary structure view) between Hepatitis BX protein and α-amyrin.

https://doi.org/10.1371/journal.pone.0196411.g014
potentialities of the plant extract. CCl₄ is biotransformed by CYP2E1. CYP2E1 is a member of cytochrome P₄₅₀ mixed function oxidase system. CYP2E1 is involved in the metabolism of xenobiotics in the body to produce CCl₃ and CCl₃OO·, and as a result of that tremendous hepatocellular necrosis is caused. Zonal haemorrhagic necrosis around the portal veins in the CCl₄ group demonstrated the hepatocellular injury. Hepatic injury was also supported by MTT cell viability assay which showed loss of cell viability due to CCl₄ toxicity. However significant improvement was observed after the treatment with CBL extract. Generation of oxidative stress due to CCl₄, deactivates the cellular antioxidative enzymes [47]. Peroxidase, catalase and superoxide dismutase are the major anti-oxidative enzymes responsible for the neutralization of free radicals. Hydrogen peroxide and lipid peroxides converts into non reactive species by the action of peroxidase enzyme. On the other hand catalase prevents the formation of highly reactive OH· by scavenging H₂O₂, the key molecule of fenton reaction. SOD (superoxide dismutase) alternatively catalyzes the dismutation of superoxide radicals into ordinary molecular oxygen or hydrogen peroxide. Glutathione is a major anti-oxidant enzyme that can also serve as a redox or cell signaling regulator and guard the cells against oxidative injury by reducing H₂O₂ and scavenging reactive oxygen and nitrogen radicals. CCl₄ derived...
trichloromethyl peroxy radicals (CCl₃OO•) accept the proton from polyunsaturated fatty acid in the biological membrane and cause lipid peroxidation and inhibition of oxidative enzymes. By the inhibition of anti-oxidative enzymes, there is accumulation of O₂•⁻ and H₂O₂ which is cascade phenomenon of free radical formation and cause hepatic damage [47]. Due to high levels of polyunsaturated fatty acid and transition metals, lipid membranes are vulnerable to oxidative stress and nitrosative stress and this transition metals such as iron are capable of damaging nuclear protein, DNA, inhibit enzymes and degrade lipid membrane through oxidative Haber-Weiss reaction [48–50]. CCl₄ toxicity markedly increases oxidative stress, lowering liver anti-oxidative enzymes. In this study it is established that the diminished catalase, peroxidase and superoxide dismutase levels and elevated MDA levels were subsequently normalize by CBL administration.

Liver disease/failure is accompanied by the up and down inflammatory conditions. TNF-α and NO plays a major role as pro-inflammatory mediators during oxidative stress related liver injury which leads towards the apoptotic cell death and fibrosis [46,51]. Kupffer cells secret a vast array of cytokines (TNF-α, IL-1, IL-6, IL-8), chemokines (KC/GRO, IP-109, MIP-2, MCP-1) and pro-inflammatory mediators like NO which initiates hepatic inflammation and toxicity under such xenobiotic induced hepatotoxic condition. Reactions of the hepatotoxicity and fibrinogenesis are initiated by the action of TNF-α and the overproduction of NO resulting in endotoxin shock and inflammatory hepatic injury [51–53]. Excess amount of NO couples with O₂•⁻ to generate highly reactive ONOO⁻ in the liver mitochondria. In the present study CCl₄ toxicity resulted significant (P<0.001) increase in TNF-α and NO levels, those were significantly lowered by the administration of CBL extract. These results proved that leaf extract of C. bonplandianus exhibited potent anti-inflammatory activities through the suppression of pro-inflammatory mediators of chronic hepatotoxicity.

A key aspect of liver injury is the role of GSH (reduced glutathione) in response to exogenously and endogenously imposed stress by redox reaction. This stress activates various signal transduction and transcriptional pathways. TNF-α is a crucial cytokine which mediates liver injury (Fig 15). The binding of soluble TNF-α to TNFR1 on the plasma membrane of hepatocytes trigger the exposure of cytoplasmic death domain of TNFR1 to form complex 1, which activates NF-κβ, JNK and P53 cascade to propagate inflammation and survival signaling (Fig 15). GSH is depleted due to CCl₄ toxicity after the susceptibility of hepatocytes by TNF-α [54, 55]. The altered GSH level due to CCl₄ toxicity was subsequently controlled by the administration of CBL extract. The hepatoprotective potentialities of leaf extract of C. bonplandianus were further established by detailed histopathological study. The results clearly demonstrated that due to CCl₄ toxicity hepatic architectures were deformed and subsequently attenuated by CBL extract. CCl₄ toxicity initiates tremendous hepatocellular damages like hepatocellular necrosis, bile duct proliferation, leukocytes infiltration (inflammation), vascular congestion, loss of structure of hepatic nodules, hepatocellular fibrosis, fatty acid infiltration, vascular degeneration and calcification. All these were normalized by the action of CBL extract. Phytochemical constituents were further identified using FTIR and GC-MS analyses for the potent hepatoprotective potentialities.

The constituents of the plant extract were detected by GC-MS and FTIR. In biochemical terms these phytochemicals are the constituents of CBL extract, responsible for all the exciting results obtained so far in this study. This result suggests the presence of active biochemicals in the plant extract. To further understand these bioactive molecules on a molecular level in silico methods like Molecular Docking experiments were carried out. The bioactive chemicals treated as ligand showed overall good binding affinity with the proteins taken as receptors for the molecular docking experiments (Fig 13). Among the receptors, Hepatitis BX (PDB ID 3I7H) protein showed best binding affinity with the phytochemicals (Fig 13). Hepatitis BX may act as the precursor for Hepatocellular carcinoma (HCC). Hepatitis BX promotes the expression
of insulin-like growth factor (IGF) in HCC [56]. Thus blocking this protein with this phytochemical can reduce the chances of development of HCC in case of liver diseases. The protein with second highest binding affinity is of Human Cytochrome p450 3A4. Cytochrome p450 3A4 is the major isozyme in the human liver. Proteins like pregnane X and NF-κB also showed good interactions with these phytochemicals. Pregnan X whose primary function is to sense the presence of alien toxic substances and in response up-regulate the expression of proteins involved in the detoxification and clearance of such substances from the body. NF-κB controls cytokine production and cell survival, but in certain cases its regulation is related to cancer, inflammation and autoimmune diseases. Phytochemicals from the CBL extract act as suitable ligand for all these receptors. So whether it is because of the individual bioactive phytochemical or the result of synergistic effects of all the biochemicals, the plant can be considered to have medicinal benefits against hepatotoxicity. Silymarin, a potent antioxidant and hepatoprotective agent, is used as standard for molecular docking. It is seen that a compound named α-Amyrin is present in CBL extract as detected by GC-MS procedure; α-Amyrin has best binding affinity with all our receptors. Even it showed better results than silymarin. So, even for those who does not agree with synergistic effect of compounds in herbal medicine, α-Amyrin has a point to prove as it shows better molecular binding affinity than a already established drug.

Conclusion

The current study reports the first ever detailed anti-oxidant and hepatoprotective evaluation of ethnomedicinal plant *Croton bonplandianus*. Oxidative stress can arise from overproduction of ROS by metabolic reaction. Overproduction of ROS uses oxygen and shifts the balance between oxidant/anti-oxidant status. In recent years free radicals such as NO, ONOO−, H2O2, O2− and OH− are the prime that mediate oxidative stress emerge as the corner-stone or precursor of several harmful diseases. These free radicals take part in immune reaction during chronic pro-inflammatory response, causing the tissue damage. On the other hand overproduction of these reactive oxygen and nitrogen species are involved in liver damage. CCl4 induced liver toxicity is a multidimensional phenomenon predominantly governed by free radicals and inflammatory related responses. Inflammation and oxidative stress are closely linked with each other and one of them may appear before or after the other. But when one of them appears the other, one is most likely to appear and take part in the pathogenesis of many chronic diseases including liver damage. In the present study it is evident that CBL extract aided in the recovery of oxidative stress, liver enzymatic levels, normalize hepatic anti-oxidative liver enzymatic levels, inhibited lipid peroxidation and cell death. On the basis of these facts, antioxidant therapy by CBL extract alone or in combination with other pharmacological strategies appear as the most reasonable treatment of CCl4 induced hepatic damage and provides a better means of treating various hepatic complications in future.

Supporting information

S1 Fig. Pairwise correlation of H2O2 inhibition and Fe2+-chelation Vs OH• scavenging for *C. bonplandianus* represented in section (A) & (B), respectively. All data are expressed as mean ± S.D. (n = 6). r = Pearson’s correlation coefficient, r² = coefficient of determination and P = significance value.
(TIF)

S2 Fig. Photomicrographs (400×) of the histopathological examinations of the liver samples of CCl4 group showing fibrosis.
(TIF)
S3 Fig. Molecular docking image. Molecular docking (molecular surface view) between NFκB protein and Campesterol.

(TIF)

S1 Table. IC_{50} values of Croton bonplandianus (CBL) and standard for different antioxidant and free radical scavenging assays.

(DOC)

S2 Table. Describe the effect of CBL on liver histology parameters of the CCl_4 induced injured liver.

(DOC)

S3 Table. Chemical fingerprint of CBL extract revealed by GC-MS analyses.

(DOCX)

Acknowledgments

Authors are thankful to Mrs. Jolly Roy, Assistant Teacher of English, Jorepatki High School, Mathabhanga, West Bengal, India, for her generous help in proofreading the manuscript.

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