

Multimechanistic Antifibrotic Effect of Biochanin A in Rats: Implications of Proinflammatory and Profibrogenic Mediators

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Abstract

Objective: Biochanin A (BCA) is an isoflavone found in red clover and peanuts. Recently, it drew much attention as a promising anticancer and antioxidant. Due to its diversity in pharmacological actions, we were encouraged to investigate its potential as an antifibrotic, elucidating the different molecular mechanisms involved.

Design: Rats were pretreated with BCA, then injected with carbon tetrachloride (CCl₄) for 6 weeks. Changes in liver weight and histology were examined and levels of aspartate and alanine aminotransferases, cholesterol, triglycerides, alkaline phosphatase and total bilirubin measured. To assess hepatic efficiency, indocyanine green was injected and its clearance calculated and albumin, total proteins and insulin-like growth factor-1 expression were measured. Cytochrome P4502E1 activity, cytochrome P4501A1 expression, in addition to sulfotransferase1A1 expression were determined to deduce the effect of BCA on hepatic metabolism. As oxidative stress markers, lipid peroxides levels, reduced glutathione, superoxide dismutase and catalase activities, as well as the total antioxidant capacity, were assessed. Nitric oxide, inducible nitric oxide synthase and cyclooxygenase-2 were used as indicators of the inflammatory response. Signaling pathways involving tumor necrosis factor- α , nuclear factor- κ B, transforming growth factor- β 1, matrix metalloproteinase-9 and α -smooth muscle actin were investigated accordingly. Extent of fibrosis was examined by Masson's stain and measuring hydroxyproline levels.

Results: BCA pretreatment significantly protected against the chronic damage of CCl₄. Liver injury, oxidative stress, inflammation and fibrosis markers decreased, while hepatic efficiency improved.

Conclusion: Our findings suggested that BCA administration protects against fibrotic complications, a property that can be contributed to the multimechanistic approach of the drug.

Citation: Breikaa RM, Algandaby MM, El-Demerdash E, Abdel-Naim AB (2013) Multimechanistic Antifibrotic Effect of Biochanin A in Rats: Implications of Proinflammatory and Profibrogenic Mediators. PLoS ONE 8(7): e69276. doi:10.1371/journal.pone.0069276

Editor: Partha Mukhopadhyay, National Institutes of Health, United States of America

Received: May 11, 2013; **Accepted:** June 12, 2013; **Published:** July 16, 2013

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Funding: The authors have no funding or support to report.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Chronic liver diseases represent a major public health problem worldwide. Their prognosis greatly depends on the extent of progression leading to serious complications such as fibrosis [1]. Hepatic fibrosis is a dynamic process characterized by imbalance in production and degradation of extracellular matrix (ECM) components in the liver. The fibrotic process involves various cells and factors that lead to disruption of intercellular contacts, extracellular matrix composition and liver ability to regenerate [2]. Because of significant progress in uncovering its pathogenetic mechanisms, combined with a growing realization that effective antifibrotic therapies may soon alter the history of chronic liver diseases, many candidates are now being investigated as potential therapies for fibrosis [3]. However, a major obstacle in the development of new antifibrotic drugs is the long-term use of the drugs which dictates absence of toxicities associated with the

medications. This property gives natural compounds a profound advantage [4].

Flavonoids are phenolic compounds that represent vital constituents of the human diet. With their hydroxyl groups, they possess strong antioxidative activity, making them perfect candidates to protect against various diseases [5]. Their importance is further substantiated by the fact that they do not act as conventional hydrogen-donating antioxidants but may exert modulatory actions in cells through actions at various signaling pathways [6]. A clear understanding of their mechanisms of action is key to the evaluation of these potent biomolecules in treatment and prophylaxis.

BCA is an O-methylated isoflavone. It is a natural compound found in red clover, soy, alfalfa sprouts, peanuts and chickpea. A broad spectrum of biological benefits has been reported, including anti-inflammatory, antioxidative and antineoplastic effects [7].

Based on the ever-increasing list of the beneficial effects of flavonoids, and the present day need for an effective, economical and simple strategy for reversing liver injury, we planned the present study. The potential of BCA to act as antifibrotic was assessed by tackling different molecular mechanisms including effects on tumor necrosis factor- α (TNF- α), nuclear factor-kappa B (NF- κ B) and transforming growth factor-beta1 (TGF- β 1) which are attractive targets for therapeutic interventions as they represent cross-talks between cells [8].

Materials and Methods

Chemicals

BCA, CCl₄, dimethylsulfoxide (DMSO), p-nitrophenol, p-nitrocatechol, Ellman's reagent, chloramine-T, p-dimethylaminobenzaldehyde, indocyanine green (ICG) and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co., St Louis, MO, USA. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was supplied by Sorachim Chemicals, Lausanne, Switzerland. All other chemicals used were of highest grade commercially available.

Animals and Experimental Protocol

The study was conducted on 200–250 g male Wistar rats, supplied by the Animal Breeding Laboratory, Helwan, Egypt. All conditions were in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, developed by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and approved by the ethical committee of Ain Shams University, Egypt.

The animals were randomly divided into four groups of eight rats each. Group A served as control, taking DMSO and corn oil on alternative days and group B as the hepatotoxic model, taking intraperitoneal (IP) CCl₄ (1 ml/kg, 1:1 mixture with corn oil) twice weekly. Group C was given both CCl₄ and BCA (50 mg/kg, IP dissolved in DMSO) three times per week on alternating days with CCl₄. The dose was selected on the basis of preliminary studies, including determination of the median lethal dose (LD₅₀) and the least effective hepatoprotective dose [9]. Group D was given BCA alone. All groups were injected over a period of six weeks, then blood samples were collected from retro-orbital plexus and serum was separated by centrifugation at 1000 g for 10 min and was used for the assessment of liver functions. Rats were sacrificed by decapitation 24 h after the last injection and livers were removed and weighed, then divided into three parts: one for homogenization in saline, one put in formalin for immunohistochemical and histopathological analysis, and the last part kept as such and put together with the 20% homogenate in -80°C until needed.

Hepatic Integrity Markers and Liver Index

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), triglycerides (TG), alkaline phosphatase (ALP) and total bilirubin were estimated colorimetrically using available commercial kits (Spectrum diagnostics, Cairo, Egypt). Liver index was calculated according to the formula: (liver weight/body weight) \times 100.

Assessment of Hepatic Efficiency

Blood flow. At the end of the six weeks, six animals per group were injected with a single dose of 3 mg/kg ICG, IP, dissolved in saline. Blood samples were collected after 10 min, 30 min, 1 h and 2 h. The plasma was separated and its optical density measured spectrophotometrically at 805 nm. The elimination rate constant (β), clearance (Cl), half-life ($t_{1/2}$), area under the curve (AUC) and

volume of distribution (Vd) were calculated. Serial dilutions of ICG (from 2.5–50 $\mu\text{g}/\text{ml}$) were prepared to construct a standard calibration curve.

Synthetic capacity. Serum albumin and total proteins (TP) were assessed colorimetrically using commercial kits (Spectrum diagnostics, Cairo, Egypt), while insulin-like growth factor-1 (IGF-1) was assessed in homogenate using enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems, USA.

Metabolic capacity. Cytochrome P450 2E1 (CYP2E1) activity was assessed in homogenate colorimetrically by monitoring the formation of p-nitrocatechol from p-nitrophenol by CYP2E1. The enzymatic product, p-nitrocatechol, was assayed at 535 nm after acidification of the reaction mixture with trichloroacetic acid (TCA) followed by neutralization using 2 N NaOH [10]. Assessment of cytochrome P450 1A1 (CYP1A1) expression in homogenate was done using ELISA kits from Cusabio Biotech., China. Sulfotransferase 1A1 (SULT1A1) expression was estimated by real time-polymerase chain reaction (RT-PCR). RNA was extracted from liver according to manufacturer's protocol (Qiagen, Hilden, Germany), then reverse transcribed and RT-PCR was performed using SYBR Green. Beta-Actin (β -Actin) served as internal control. Primers used were: *ratACTBF320:5'-AGGCCCTCTGAACCC TAAG-3'*, *ratACTBR435:5'-AGAGGCATACAGGGACAACACA-3'*; *rSULT1A1-F530:5'-AGCTGAGACACACTCACCCGTGT-3'*, *rSULT1A1-R651:5'-ATC-CACAGTCTCCTCGGGTAGA-3'* [11].

Assessment of Oxidative Stress

Lipid peroxidation was determined in homogenate by estimating level of thiobarbituric acid reactive substances (TBARS) measured as malondialdehyde (MDA), according to the method of Mihara [12]. To determine reduced glutathione (GSH), 0.5 ml homogenate was added to 0.5 ml of 10% TCA. The tubes were shaken intermittently for 15 min, followed by centrifugation at 1000 g for 10 min. An aliquot of the resulting supernatant (0.2 ml) was added to a tube containing 1.7 ml phosphate buffer and 0.1 ml Ellman's reagent then the absorbance was read at 412 nm [13]. Superoxide dismutase (SOD) and catalase (CAT) activities were determined in homogenate using commercial kits (Biodiagnostics, Cairo, Egypt). In addition, the total antioxidant capacity (TAC) was measured by providing a specific amount of exogenous H₂O₂ which is then reduced by antioxidants in the sample. The residual H₂O₂ was determined colorimetrically.

Assessment of Inflammatory Response

TNF- α expression was assessed using ELISA kit from RayBiotech, Inc., USA. Total nitric oxide (NO) content was estimated in homogenate spectrophotometrically by Miranda's method [14] and results were expressed as $\mu\text{mol}/\text{g}$ wet tissue. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions were examined immunohistochemically. Liver sections of 4 μm were cut, then after preparing the slides, one of the following ready-to-use primary antibodies was applied: rabbit polyclonal antibody to rat iNOS (Thermoscientific, iNOS Cat#RB-9242-R7) or rabbit polyclonal anti-rat COX-2 (Thermoscientific, COX-2 Cat#RB-9072-R7). Examination was done with a light microscope.

Assessment of NF- κ B

NF- κ B expression was examined immunohistochemically using rabbit polyclonal IgG to rat NF- κ B p65 (SantaCruz Biotech, Cat No. sc-372).

Assessment of Fibrosis Markers

TGF- β 1 was assessed using ELISA kit from R&D Systems, USA. Matrix metalloproteinase-9 (MMP-9) and alpha-smooth muscle actin (α -SMA) were examined immunohistochemically using goat polyclonal IgG to rat MMP-9 (SantaCruz Biotech, MMP-9 Cat No. sc-6840) and mouse monoclonal to rat α -SMA (SantaCruz Biotech, Cat No. sc-53142). To examine the extent of fibrosis, hydroxyproline was estimated in the liver using the simplified method of Reddy [15]. In addition, Masson's trichrome stain was used for collagen fiber detection.

Histopathologic Examination

Liver sections were stained with hematoxylin and eosin (H&E) and examined by light microscope.

Statistical Analysis

Data is presented as mean \pm standard deviation (SD). Multiple comparisons were performed using one-way ANOVA followed by Tukey-Kramer as a post-hoc test. Differences between groups were considered significant at $p < 0.05$. All statistical analyses were performed using Instat software package (version 3.06). Graphs were sketched using GraphPad Prism software (version 5).

Results

Hepatic Integrity and Liver Index

AST and ALT increased after chronic CCl₄ challenge by 1.8 and 1.5, respectively, compared to control group. TC and TG doubled, while levels of ALP and total bilirubin increased by 1.7 and 1.5 folds (Table 1). Pretreatment with BCA normalized these levels, while BCA alone showed levels below the control group. The liver index increased in CCl₄ group by 1.5 and decreased significantly by BCA pretreatment (Table 1).

Hepatic Efficiency

Blood flow. ICG elimination rate decreased to the half by CCl₄ challenge, while the clearance decreased by 23%, compared to control group. Its $t_{1/2}$, AUC and Vd significantly increased by about 4 folds, 1.4 folds and 3.2 folds, respectively (Table 2). BCA pretreatment, however, increased the elimination and the clearance by 1.5 folds and 1.2 folds, as compared to CCl₄ group. $T_{1/2}$, AUC and Vd decreased with BCA by 3, 1.3 and 2.7 folds in comparison to CCl₄ group.

Synthetic capacity. Albumin, TP and IGF-1 were depleted by chronic CCl₄ intoxication, where TP reached half and IGF-1 1/16 of the control levels. BCA pretreatment significantly prevented their depletion (Table 3).

Metabolic capacity. The activity of CYP2E1 increased in CCl₄ group by 10%, as compared to control (Fig. 1). The pretreatment with BCA significantly reduced the activity even below levels of control.

In addition, the expression of CYP1A1 was significantly higher after chronic CCl₄ challenge, while it approached normal levels by BCA pretreatment (Fig. 1). The group treated with BCA alone showed levels that reach one third of control group. RT-PCR results showed increased expression of SULT1A1 in all groups compared to control (Fig. 1). However, increases in CCl₄ and pretreated group were higher than BCA alone suggesting that not only BCA increased the expression, but also the CCl₄ challenge did.

Oxidative Stress Markers

CCl₄ increased MDA level by 62%, while GSH was severely depleted, reaching about 11% of the control level. The TAC decreased by 41%, as compared to control group (Table 4). Animals pretreated with BCA showed near normal MDA and TAC levels. The dramatic depletion of GSH was reversed and even increased by 19% above control, while BCA alone increased GSH level by 31%. Furthermore, SOD and CAT activities were suppressed by CCl₄ and returned to normal levels by BCA (Table 4).

Inflammation Markers

TNF- α and total NO in CCl₄ group reached 138% and 163% of control group, respectively. BCA restored their levels (Fig. 2 A & B). Immunohistochemical staining showed extensive expression of COX-2 and iNOS in CCl₄ group, while the pretreated group showed marked reduction (Fig. 3 A & B). The control and BCA model group showed minimal expression.

Effect on NF- κ B

Control rats showed minimal immunostaining (Fig. 3 C), while CCl₄ group showed significant expression. Pretreatment with BCA significantly inhibited its expression. BCA alone showed expression levels below control group.

Fibrosis Markers

Fibrosis was first evaluated by measuring TGF- β 1 expression which increased by 1.8 folds in CCl₄ group, while levels decreased by 30% by BCA pretreatment, compared to the CCl₄ group (Fig. 2 C). Immunohistochemistry revealed high MMP-9 (Fig. 3 D) and α -SMA (Fig. 3 E) in CCl₄ group. Furthermore, high hydroxyproline levels were detected with dense blue color in Masson's slides around and inbetween portal tracts and central

Table 1. Effect of BCA on hepatic integrity markers and liver index.

Group	AST (U/L)	ALT (U/L)	TC (mg/dl)	TG (mg/dl)	ALP (U/l)	Total bilirubin (mg/dl)	Liver index (%)
Control	54.3 \pm 9.29***	52.4 \pm 3.73***	59.5 \pm 7.58***	52.7 \pm 10.13***	106.7 \pm 5.22***	2.6 \pm 0.21**	2.9 \pm 0.1***
CCl ₄	80.4 \pm 11.93	95 \pm 26.47	108.2 \pm 22.78	112.6 \pm 38.55	181.1 \pm 42.8	3.8 \pm 0.95	4.3 \pm 0.41
BCA+CCl ₄	48 \pm 10.26***	69.2 \pm 7.27**	69.4 \pm 10.95***	73 \pm 17.48**	107.7 \pm 11.88***	2.6 \pm 0.41**	3.8 \pm 0.19**
BCA	42.1 \pm 4.55***	50 \pm 8.07***	59.7 \pm 11.31***	43.7 \pm 14.5***	101.5 \pm 9.31***	2.5 \pm 0.58***	2.9 \pm 0.29***

n = 8.

* $p < 0.05$ compared to CCl₄ group.

** $p < 0.01$ compared to CCl₄ group.

*** $p < 0.001$ compared to CCl₄ group.

doi:10.1371/journal.pone.0069276.t001

Table 2. Effect of BCA on ICG pharmacokinetics.

Group	β (hr ⁻¹)	$t_{1/2}$ (hr)	AUC (hr· μ g/ml)	Cl (ml/kg/hr)	Vd (ml/kg)
Control	39±5.4***	0.026±0.008***	209.2±24.75**	15.3±0.97***	0.6±0.21***
CCl ₄	18.7±2.34	0.101±0.023	291.1±41.6	11.8±0.39	1.9±0.62
BCA+CCl ₄	27.5±6.1	0.032±0.007***	223.3±36.82*	14.6±2**	0.7±0.19***
BCA	32.4±9.93**	0.029±0.008***	227±28.93*	14.2±1.43*	0.5±0.13***

β (elimination rate constant), Cl (clearance), $t_{1/2}$ (elimination half-life) and AUC (area under the curve).

n = 6.

*p<0.05 compared to CCl₄ group.

**p<0.01 compared to CCl₄ group.

***p<0.001 compared to CCl₄ group.

doi:10.1371/journal.pone.0069276.t002

veins, indicating pseudolobules formation (**Fig. 3 F**). Significantly lower levels were found in the other groups.

Histopathology

CCl₄ caused marked necrosis with fatty deposits and ballooning degeneration, associated with extensive fibrosis and inflammatory cells infiltration (**Fig. 4**). BCA significantly ameliorated these changes. Sections from control and BCA model group showed normal hepatic architecture with polyhedral hepatocytes and prominent nuclei. The histopathological findings were scored by a histopathologist.

Discussion

Hepatic fibrosis is the consequence of wound-healing response of the liver to repeated injury resulting in accumulation of tough, fibrous scar tissue [16]. It develops with different spatial patterns according to causes of the parenchymal damage. Key events are the propagation of oxidative and inflammatory responses with subsequent transformation of quiescent hepatic stellate cells (HSCs) into myofibroblast-like cells [17].

Flavonoids are phenolic compounds that represent vital constituents of the human diet. With their hydroxyl groups, they possess strong antioxidative activity, making them perfect candidates to protect against various diseases [5]. Their importance is further substantiated by the fact that they do not act as conventional hydrogen-donating antioxidants but may exert modulatory actions in cells through actions at various signaling pathways [6]. A clear understanding of their mechanisms of action is key to the evaluation of these potent biomolecules in treatment and prophylaxis. BCA is a natural isoflavone with multiple pharmacological activities, including antihyperglycemic, anti-inflammatory and antioxidative effects [18,19]. A study by Badria

et al has shown that BCA inhibits HSC proliferation, thus pointing to a potential antifibrotic activity [20].

Events of hepatic fibrosis were simulated in this study using a CCl₄-induced fibrosis model. First, to ensure development of fibrosis by CCl₄ and its decrease by BCA, collagen deposition was determined both biochemically and histologically by visualizing the blue color of collagen fibers using Masson's stain. Both confirmed fibrotic lesions after CCl₄ challenge and minimal deposition with BCA. In addition, the fact that BCA was able to decrease α -SMA, a marker of activated HSCs, confirms its antifibrotic effect [21].

The mechanisms underlying this antifibrotic activity were then examined thoroughly. High AST, ALT, TC and TG levels confirmed CCl₄-induced hepatocellular damage. High ALP and total bilirubin indicated dysfunction of hepatocytes and increased biliary pressure. The reversal of these alterations by BCA is a clear indication of the improvement of the functional status of hepatocytes with preservation of cellular architecture [22].

In an attempt to investigate possible changes in liver efficiency, hepatic blood flow, synthetic and metabolic capacities were examined. Effect on blood flow was investigated by assessing changes in ICG pharmacokinetics which is exclusively eliminated by the liver without metabolic modifications or enterohepatic recirculation. Furthermore, its presence in the blood can be accurately assessed in presence of hyperbilirubinemia [23]. Thus, the rate limiting step for ICG elimination is the hepatic blood flow [24]. CCl₄ clearly decreased the elimination of ICG, reflecting compromised blood flow which can be attributed to the necrotic effect of CCl₄ causing irreversible damage of microvascular circulation, in addition to excessive deposition of collagen fibers in extracellular spaces, causing hindered blood infusion [25]. BCA successively improved the blood flow. The hepatic synthetic capacity was evaluated by measuring albumin, TP and IGF-1, all of which are synthesized in the liver. Chronic CCl₄ challenge caused their severe depletion, while BCA restored their levels. Owing to its unique vascular and metabolic features, the liver is exposed to many xenobiotics, which are either activated or detoxified by drug-metabolizing enzymes [26]. CYP2E1 is one principal member of the phase I metabolizing enzymes, the cytochrome P450 (CYP450) family, responsible for bioactivation of many xenobiotics, including CCl₄ [27]. BCA was found to affect the activity of CYP2E1 and even inhibit it to levels lower than those of the control group, thus, allowing BCA to suppress the bioactivation of various xenobiotics. In addition to CYP2E1, CYP1A1 is another important P450 enzyme causing propagation of liver injury, with marked role in oxidative stress by excessive generation of reactive oxygen species (ROS) [28,29]. SULT1A1 was chosen as a phase II enzyme helping the liver to get rid of

Table 3. Effect of BCA on hepatic synthetic capacity.

Group	Albumin (g/dl)	TP (g/dl)	IGF-1 (pg/mg protein)
Control	3.1±0.52	6.6±1.06***	2674.5±319.87***
CCl ₄	2.4±0.43	3.4±0.99	161.4±53.51
BCA+CCl ₄	3.3±0.91*	5.4±0.81***	1053.8±144.63***
BCA	3.4±0.31*	5.6±0.81***	2583.9±331.63***

n = 8.

*p<0.05 compared to CCl₄ group.

**p<0.01 compared to CCl₄ group.

***p<0.001 compared to CCl₄ group.

doi:10.1371/journal.pone.0069276.t003

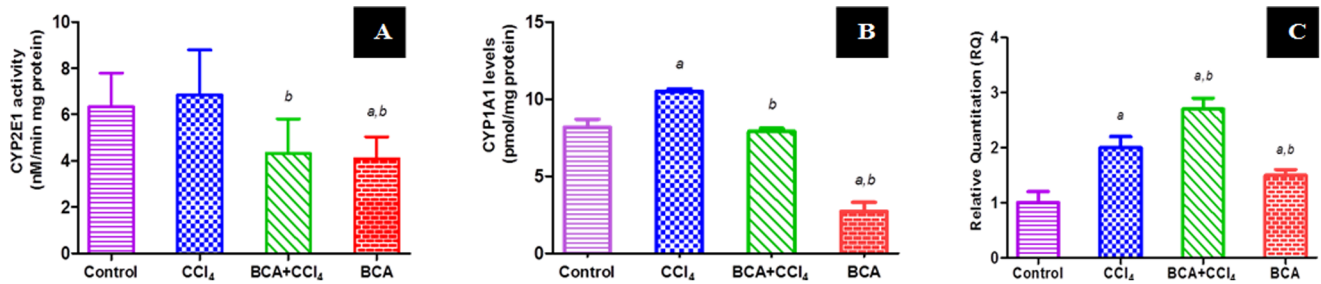


Figure 1. Effect of BCA on hepatic metabolic capacity. (A) CYP2E1 activity levels; (B) CYP1A1 expression levels; (C) SULT1A1 relative quantitation (RQ) versus group mean plot. *a*: significantly different from control gp. *b*: significantly different from CCl₄ gp. doi:10.1371/journal.pone.0069276.g001

xenobiotics [30]. Our study revealed increase of CYP1A1 expression by chronic CCl₄ injection associated with decrease by BCA, while SULT1A1 increased both by BCA and CCl₄, suggesting that sulfotransferases can act as defense against a xenobiotic challenge as CCl₄.

Oxidative stress produced by free radicals is the main and primary step in CCl₄ toxicity contributing to both onset and progression of fibrosis [31]. This was evidenced by enhanced lipid peroxidation, associated with low levels of GSH which represents the non-enzymatic part of the antioxidant defense of the body [32]. The enzymatic part was represented in our study with SOD and CAT. The activities of both enzymes were reduced by CCl₄. Since it became clear that the cooperation among different antioxidants provides greater protection against attack by ROS and free radicals than any single compound alone, the overall antioxidant capacity may provide more accurate information compared to that obtained by the measurement of individual components, as it considers the cumulative effect of all antioxidants present in the liver [33]. As expected, CCl₄ markedly decreased the TAC. Flavonoids are potent molecules that donate a hydrogen atom from an aromatic hydroxyl group to a free radical, yielding a stable phenolic radical. Furthermore, their amphiphilicity enhances their ability to trap chain-initiating radicals at the interface of the membranes, preventing progression of the radical chain reaction [34]. BCA indeed showed superior antioxidant activity evident by TAC levels above control group.

Another pathway involved in fibrosis is the inflammatory process that is initiated by activation of Kupffer cells, which further release a number of proinflammatory mediators like TNF- α [35]. The heightened inflammatory response following CCl₄ injection was evident by increased levels of TNF- α , which was significantly attenuated by BCA. NO plays crucial roles in inflammation and liver injury [36]. It is produced in large

quantities by Kupffer cells, endothelial cells and the hepatocytes themselves in response to tissue damage and inflammation induced by various xenobiotics including CCl₄. In addition, its role in oxidative stress cannot be neglected, since high levels of NO have been associated with oxidative injury via lipid peroxide.

production and antioxidant consumption [37]. Our findings confirmed elevated NO in the CCl₄ group, while BCA caused decreased levels. Two enzymes responsible for initiation and propagation of the inflammatory cascade are iNOS [38] and COX-2 which accounts for the increased production of PGs in response to proinflammatory stimuli [39]. Inhibition of iNOS and COX-2 expressions were found to be two mechanisms by which BCA can act as an anti-inflammatory, thereby protecting the liver. Furthermore, sustained hepatic inflammation provoked by long-term intoxication with CCl₄ is believed to be through NF- κ B pathway [40,41]. NF- κ B is a pleiotropic protein with pivotal role in controlling cell signaling under certain physiological and pathological conditions [42]. In this context, activation of many kinases involved in NF- κ B pathway is shown to be dependent on oxidative stress [43], where ROS have been shown to cause prolonged NF- κ B DNA binding activity [44,45]. Further, Kupffer cells display powerful NF- κ B activation in response to liver injury by CCl₄, resulting in production and secretion of proinflammatory cytokines and profibrogenic mediators such as TGF- β 1 [35]. TGF- β 1 is a multifunctional growth factor stimulating synthesis and deposition of ECM components, in addition to favoring transition of hepatocytes to myofibroblast-like cells [46]. Thus, in our study, both NF- κ B and TGF- β 1 were found upregulated in fibrotic lesions, while BCA decreased their expression.

Quantitative and qualitative changes in matrix degrading activity play an important role in ECM remodeling and accumulation of ECM components accompanying liver fibrosis [47].

Table 4. Effect of BCA on oxidative stress markers.

Group	MDA (nmol/g)	GSH (μ mol/g)	TAC (μ mol/g)	SOD (U/mg)	CAT (U/g)
Control	11 \pm 1.32***	6 \pm 2.1***	0.0032 \pm 0.0005***	55.7 \pm 11.71***	898.9 \pm 56.55***
CCl ₄	17.7 \pm 2.81	0.7 \pm 0.23	0.0019 \pm 0.0006	21 \pm 6.31	640 \pm 73.72
BCA+CCl ₄	11.6 \pm 0.96***	7.2 \pm 2***	0.0033 \pm 0.0002***	51.5 \pm 7.8***	899.1 \pm 85.63***
BCA	11.5 \pm 0.74***	7.9 \pm 1.97***	0.0035 \pm 0.0002***	51.4 \pm 6.3***	896.7 \pm 40.95***

n = 8.
 *p < 0.05 compared to CCl₄ group.
 **p < 0.01 compared to CCl₄ group.
 ***p < 0.001 compared to CCl₄ group.
 doi:10.1371/journal.pone.0069276.t004

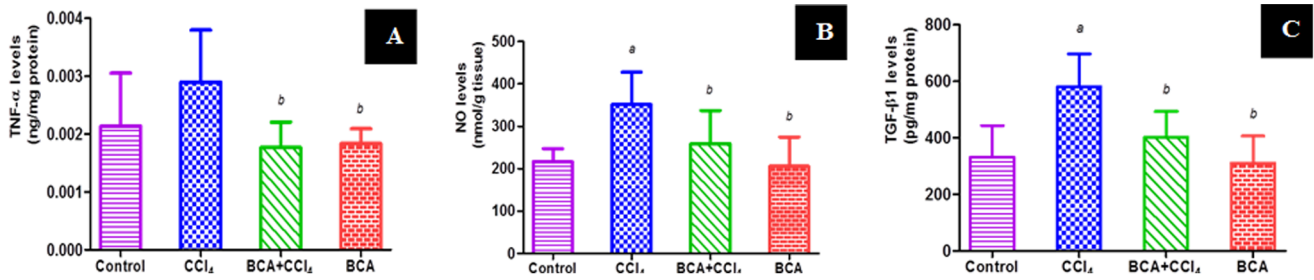


Figure 2. Effect of BCA on TNF- α , NO and TGF- β 1 levels. (A) TNF- α expression levels; (B) NO levels; (C) TGF- β 1 expression levels. *a*: significantly different from control gp. *b*: significantly different from CCl₄ gp. doi:10.1371/journal.pone.0069276.g002

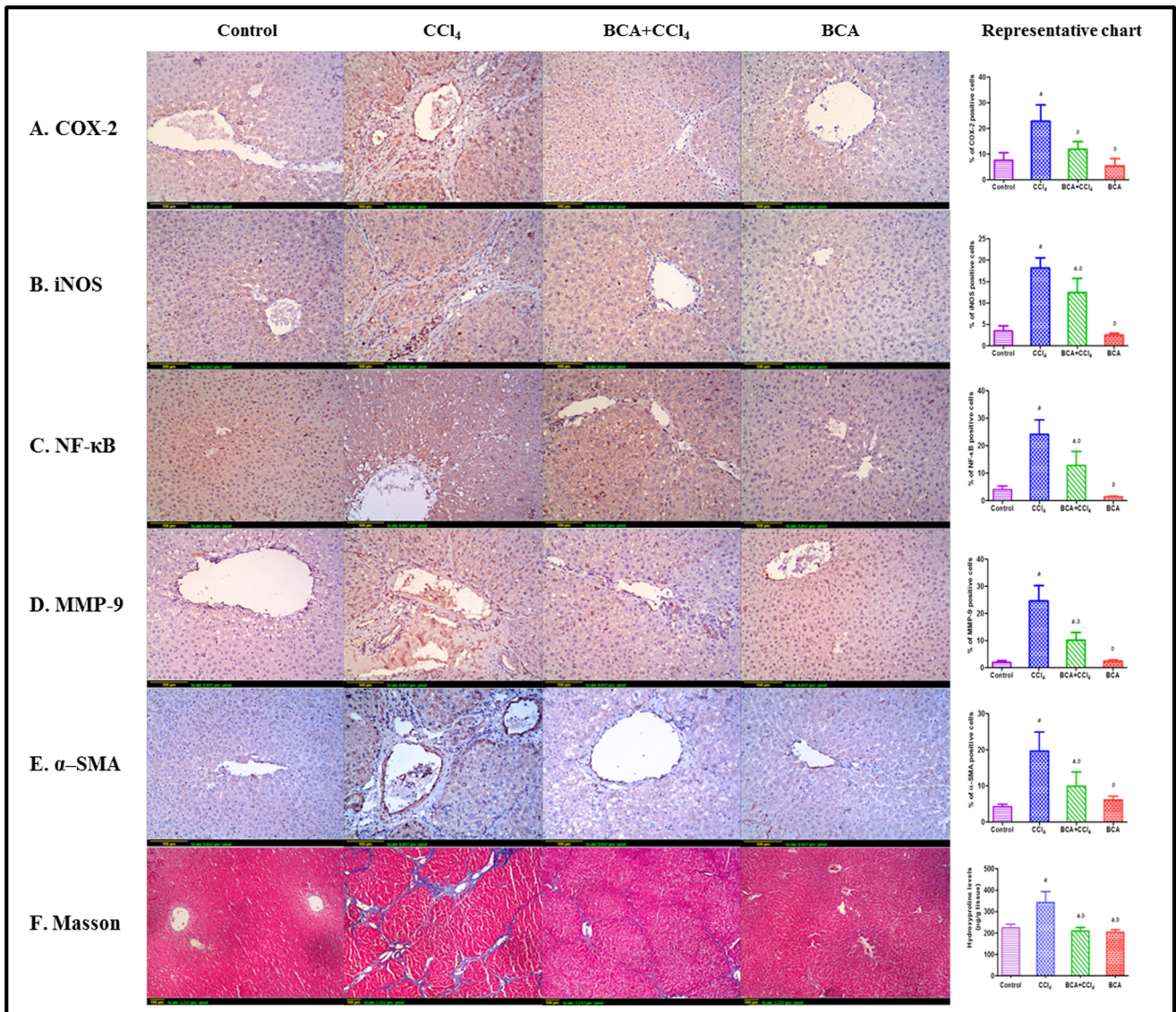


Figure 3. Representative images of liver sections of different experiments. (A–E) Immunohistochemical detection of COX-2, iNOS, NF- κ B, MMP-9 and α -SMA (x100). Control gp: minimal expression; CCl₄ gp: extensive expression; BCA+CCl₄ gp: less than CCl₄ gp; BCA gp: minimal expression. Image analysis was performed by examining 6 fields/slide. (F) Masson trichrome stain (x40). Control gp: shows absence of collagen fibers (stained blue) between hepatic lobules; CCl₄ gp: extensive fibers deposition with pseudoblobules formation (bridging fibrosis); BCA+CCl₄ gp: less fibers than CCl₄ gp; BCA gp: absence of fibers. The bar chart represents levels of hydroxyproline expressed as μ g/gm of wet tissue measured by Reddy's method. *a*: significantly different from control gp. *b*: significantly different from CCl₄ gp. doi:10.1371/journal.pone.0069276.g003

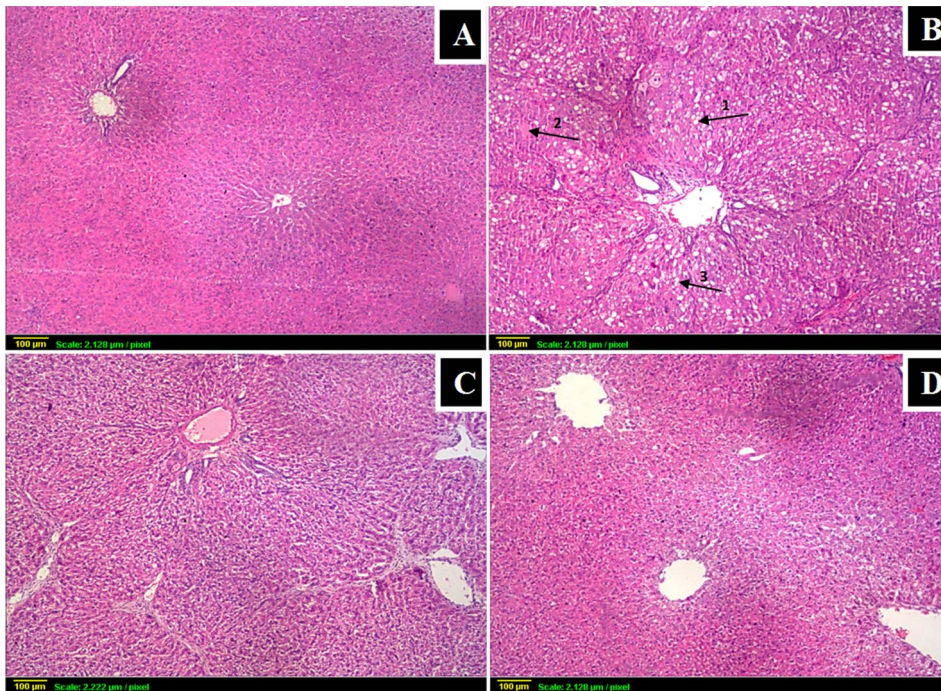


Figure 4. Histopathological findings followed by grading of liver damage. (x40) (A) control: normal histological structure of portal area and surrounding hepatocytes; (B) CCl₄: loss of architecture with severe ballooning degeneration (arrow1), necrosis (arrow2) and fatty changes (arrow3) accompanied with fibrosis; (C) BCA+CCl₄: much less damage than in CCl₄ gp (D) BCA alone: similar to control gp.
doi:10.1371/journal.pone.0069276.g004

A large family of MMPs has been characterized that is responsible for degrading collagens [48]. However, high levels of MMPs cause excessive degradation of normal subendothelial ECM hastening its replacement by fibrous collagen, which further activates stellate cells in a positive feedback loop [49]. Furthermore, MMP-induced degradation of the ECM releases previously sequestered growth factors that can induce a variety of biological responses including inflammation [50]. One member of this family is MMP-9 which is considered a hallmark of fibrosis [51] and whose expression increases by TNF- α and TGF- β 1 during the onset of liver fibrogenesis [52]. MMP-9 increased by CCl₄, which could be one of the reasons for aggravating its hepatotoxicity.

In light of all the previous findings, the present study provides evidence for the hepatoprotective and antifibrotic effects of BCA. The mechanisms underlying these promising effects involve attenuating oxidative stress, as well as decreasing the expression of NF- κ B and the subsequent inflammatory cascade and the

production of profibrogenic factors. The net effect is, thus, preservation of the hepatocellular integrity and the hepatic efficiency.

Acknowledgments

The authors would like to thank Prof. Adel Kholoussy and dr. Abeer Shaalan, Pathology department, Cairo University, for their help in histopathological and immunohistochemical examination of specimens.

Author Contributions

Conceived and designed the experiments: RMB. Performed the experiments: RMB MMA. Analyzed the data: RMB EED. Contributed reagents/materials/analysis tools: MMA ABAN. Wrote the paper: RMB. Critical revision of the manuscript and supervision of the whole work: EED ABAN.

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