Title:

Betulin prevents high fat diet-induced non-alcoholic fatty liver disease by mitigating oxidative stress and upregulating Nrf2 and SIRT1 in rats

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a common chronic hepatic disorder characterized by hepatic lipid accumulation. This study explored the effect of betulin (BE), a terpenoid with promising antioxidant, anti-inflammatory and insulin sensitizing effects, on NAFLD induced by high fat diet (HFD). Rats received HFD and BE (15 and 30 mg/kg) for 12 weeks and blood and liver samples were collected for analyses. HFD caused hyperlipidemia, cholesterol and triglycerides accumulation in the liver, hepatocellular ballooning, fibrosis, insulin resistance (IR), lipid peroxidation (LPO), and NF-kB p65 upregulation. BE ameliorated serum and liver lipids, blood glucose, and insulin, liver LPO, prevented steatosis and fibrosis, suppressed NF-kB p65 and enhanced antioxidants in HFD-fed rats. BE downregulated ACC1 and FAS, and upregulated Nrf2, HO-1 and SIRT1 in the liver of HFD-fed rats. *In silico* investigations revealed the binding affinity of BE toward NF-kB, Keap1, HO-1 and SIRT1. In conclusion, BE attenuated HFD-induced NAFLD by ameliorating hyperlipidemia, IR, lipogenesis, liver lipid accumulation, and oxidative stress. The protective effect of BE was associated with enhanced Nrf2/HO-1 signaling and SIRT1.

Keywords: Steatosis; Oxidative stress; Dyslipidemia; Betulin; SIRT1.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a comm disorder that possesses a bidirectional association with the metabolic syndrome. Its global prevalence in adults is around 25% and might progress to liver cirrhosis and carcinoma [1, 2]. Steatosis in more than 5% of the hepatocytes without excessive alcohol consumption and in the presence of type 2 diabetes (T2D) or obesity are the characteristics of NAFLD [3]. Given this association with metabolic disorders, 80% of obese and 47. 3–63. 7% of T2D patients have NAFLD [4, 5]. The prevalence of NAFLD varies from 13.5% to 31.8 in African and Middle East countries, respectively [6].

This difference is attributed to different caloric intake, body fat distribution, genetic composition, physical activities, and others [1]. T2D patients are at a higher prevalence of NAFLD and can develop fibrosis, cirrhosis, and carcinoma, resulting in increased morbidity. Therefore, multidisciplinary management to address the complex link and the healthcare requirements for T2D and NAFLD is needed [7].

NAFLD is heterogenous and both the clinical outcomes and disease progression vary due to varying underlying mechanisms [8]. While the disease is stable or progresses slowly in most patients, it can lead to advanced fibrosis and increasing risk of liver failure in a small proportion [1]. The pathological findings of NAFLD include steatosis and hepatocyte ballooning with or without mild inflammation [2]. Overnutrition resulting in adipose tissue (AT) expansion and ectopic fat accumulation represents the primary cause of NAFLD. The infiltration of macrophages in visceral AT provokes a pro-inflammatory status and the development of insulin resistance (IR). Consequently, the rate of lipolysis increases resulting in unabated fatty acids delivery which in concert with the increased *de novo* lipogenesis overburdens the hepatic metabolic capacity. This imbalanced metabolism provokes the formation of lipotoxic molecules that promote oxidative stress (OS), inflammation and fibrogenesis [1, 9]. Reactive oxygen species (ROS) produce by various sources contribute to the progression of inflammation and fibrosis in NAFLD [10]. ROS and other pro-oxidant and lipotoxic species in NAFLD can saturate and exhaust the antioxidant system resulting in OS. Through direct and indirect mechanisms, OS contributes to chronic inflammatory response and the activation of stellate cells, hepatocyte apoptosis and fibrogenesis [11]. Given the role of OS and inflammatory cytokines in provoking IR and glucose intolerance [12] along with apoptosis and fibrogenesis, attenuation of these processes could be effective in preventing the progression of NAFLD. This notion is supported by evidence showing the ability of dietary antioxidants to reverse hepatic steatosis [13]. Recent studies have investigated the possible role of nuclear

factor erythroid 2–related factor 2 (Nrf2) in NAFLD and the possible ameliorating potential of Nrf2 activators [14]. Nrf2 is sequestered by Keap1 in the cytoplasm and translocate into the nucleus following exposure to electrophiles or ROS. Within the nucleus, Nrf2 binds ARE to promote the transcription of several cytodefenses, including heme oxygenase-1 (HO-1) [15]. Additionally, the activation of the histone deacetylase sirtuin1 (SIRT1) can confer protection against NAFLD and other liver diseases. It possesses beneficial role in lipid metabolism and suppressive effect on hepatic OS and inflammation. These beneficial effects are mediated via deacetylating NF-κB, p53 and other signaling mediators and transcription regulators [16, 17]. Therefore, Nrf2 and SIRT1 activators are of significant value for ameliorating OS, inflammation and altered lipid metabolism in several disorders.

Numerous compounds of plants origin demonstrated beneficial effects against hepatic OS and inflammatory responses, and ameliorated glucose intolerance, IR, and dyslipidaemias [12, 17-20]. Terpenoids are a large class of phytochemicals with promising beneficial anti-diabetic, antioxidant, and anti-inflammatory properties. The consumption of terpenoids ameliorated hyperglycemia, IR, and dyslipidemia and protected the liver against induced by OS and lipid accumulation in rats with T2D [21]. Betulin (BE) is a pentacyclic lupane-type terpenoid found in the birch tree bark and possesses multiple pharmacological properties, including anti-inflammatory, antioxidant and anticancer [22]. BE can specifically inhibit the maturation of SREBPs and decrease fatty acid levels and cholesterologenesis [23]. In a rodent model of diet-induced obesity (DIO), BE improved insulin sensitivity, decreased serum and tissue lipid accumulation and improved atherosclerotic plaque stability [23]. Despite these reported effects, the beneficial role of BE against NAFLD has not been explored. Given the beneficial effects of BE, this study evaluated its potential to ameliorate HFD-induced NAFLD in rats, emphasizing its effect on inflammation, OS, Nrf2 and SIRT1.

2. Materials and methods

2.1. Experimental design

Male Sprague-Dawley rats (200-220 g) were purchased from the Modern Veterinary Office for Laboratory Animals (Cairo, Egypt). The animals were maintained under standard conditions of temperature and humidity on a 12-h light/dark cycle with free access to food and water. The experimental protocol was approved by the ethics committee at Kafrelsheikh University (approval no. MKSU-50-2-2)

Forty rats were divided into five groups (n = 8) as follows:

Group I (Control): fed a normal chow diet (25.8% protein, 62.8% carbohydrates, and 11.4% fat).

Group II (BE): fed normal basal diet and received BE (30 mg/kg; Sigma, USA) [23].

Group III (HFD): fed a HFD (25% protein, 17% carbohydrates, and 58% fat) [24].

Group IV (HFD + 15 mg/kg BE): fed a HFD and received 15 mg/kg BE [23].

Group IV (HFD + 30 mg/kg BE): fed a HFD and received 30 mg/kg BE [23].

BE and diets were supplemented for 12 weeks. BE was administered daily via oral gavage. Blood was collected via cardiac puncture under the influence of thiopental (50 mg/kg; Eipico, Egypt) anesthesia for the separation of the serum. The liver samples were removed, washed, weighed, and homogenized in 10 mM ice-cold Tris–HCl buffer (pH 7.4). After centrifuging the homogenate at 6000 rpm for 10 min, the supernatant was collected and kept at -80°C. Other samples were fixed in 10% neutral-buffered formalin (NBF) and others in buffered glutaraldehyde (2.5%) whereas other samples were collected on RNALater and stored at -80°C.

2.2. Biochemical assays

Serum triglyceride (TG), total cholesterol (TC), glucose, LDL-C, HDL-C, ALT, AST, and total lipids were measured using Biomed Diagnostics (Egypt) kits. vLDL-C was calculated by subtracting both LDL-C and HDL-C from TC. Serum insulin was measured using Elabscience (China) ELISA kit. Malondialdehyde (MDA), a marker of lipid peroxidation (LPO), [25], and activities of superoxide dismutase (SOD) [26], catalase (CAT) [27], HO-1 [28], and glutathione peroxidase (GPx) [29] were measured in the supernatant of the hepatic homogenate using kits supplied by BioDiagnostics (Egypt) following the provided instructions. Lipids were extracted from the liver using chloroform/methanol (2:1) mixture [30] and cholesterol (CHOL) and TG were assayed using Biomed Diagnostics (Egypt) kits. HOMA-IR and QUICKI were calculated using the following equations:

$$HOMA - IR = Fasting\ insulin\ \left(\frac{\mu U}{ml}\right) - Fasting\ glucose\ (mmol/L)/22.5$$

$$QUICKI = 1/[\log fasting\ insulin\ \left(\frac{\mu U}{ml}\right) + \log fasting\ glucose\ (\frac{mg}{dl})]$$

2.3. Histopathology, electron microscopic (EM) and immunohistochemical (IHC) studies

Samples from the liver were fixed in 10% NBF, dehydrated, cleared, and embedded in paraffin wax. 5-μm sections were cut and processed for routine staining with hematoxylin and eosin (H&E) and Masson's trichrome (MT) and examined under light microscope. Steatosis grade was determined according to NASH clinical research network scoring system (0 = <5%, 1 = 5–33%, 2 = 34–66%, and 3 = >66%). Inflammation was classified according to the number of inflammatory foci/x20 field (0 = none, 1.0 = 1-2, 2 = 2-4 and 3 = >4). NAFLD activity score (NAS) was determined as previously reported [31]. Small tissue fragments were fixed in buffered glutaraldehyde (2.5%), dehydrated, and embedded in resin. 60–80 nm sections were stained with uranyl acetate and lead citrate and examined using JEOL JEM-2100, (Jeol Ltd, Tokyo, Japan).

For IHC staining of NF-κB p65 and SIRT1, sections from the NBF-fixed samples were dewaxed and treated with 0.05 M citrate buffer (pH 6.8) for antigen retrieval. The sections were then immersed in a 0.3% H_2O_2 solution for blocking the non-specific reaction and then probed with rabbit polyclonal anti-SIRT1 (GeneTex, Cat# GTX17532, 1:200 dilution) and anti-NF-κB p65 (Santa Cruz, Cat# (F-6): sc-8008, 1:100 dilution) overnight at 4°C. The slides were rinsed in PBS and then incubated with a secondary antibody (Cat# K4003, Dako) for 30 min. The tissues were visualized with a DAB kit and counterstained with Mayer's hematoxylin. The staining intensity was assessed by ImageJ (NIH, USA).

2.4. qRT-PCR

To determine the changes in mRNA of SIRT1, Nrf2, HO-1, NF-κB p65, FAS, and ACC1, RNA was isolated using RNA purification kit (Thermo Scientific, USA). The isolated RNA with OD260/280 ≥ 1.8 was reverse transcribed into cDNA with Intron-Power cDNA synthesis kit (Thermo Scientific, USA). cDNA amplification was carried out using SYBR green master mix (Thermo Scientific, USA) and the primer pairs in Table 1. The data was analyzed using the $2^{-\Delta\Delta Ct}$ method [32].

2.5. *In silico* molecular docking (MD)

The binding affinity of BE towards Keap1 (PDB ID 4L7B), SIRT1 (PDB ID 4ZZJ), NF-κB-DNA complex (PDB ID 1LE9), HO-1 (3HOK) and two FAS domains (KS (PDB ID:3HHD) and TE (PDB ID: 1XKT)) was modelled using AutoDock Vina and Autodock Tools (ADT) v1.5.6 as we previously reported [18, 33, 34].

2.6. Statistical analysis

The results are presented as mean ± SEM and all statistical comparisons were made by one-way ANOVA followed by Tukey's test on GraphPad Prism 8 software. P values < 0.05 were considered statistically significant.

3. Results

3.1. BE ameliorates dyslipidemia, liver lipid accumulation and NAS score in HFD-fed rats

HFD feeding resulted in dyslipidemia marked by elevated serum total lipids, TG, TG, LDL-C, and vLDL-C significantly when compared with the chow-fed rats ($P<0.001$; Fig. 1A-E). HDL-C was decreased significantly in HFD-fed rats as represented in Fig. 1F ($P<0.001$). BE (15 and 30 mg/kg) ameliorated all assayed lipids and enhanced HDL-C in HFD-fed rats. The effect of BE on total lipids, TC, TG, vLDL-C and HDL-C in HFD-fed rats was dose-dependent, and the 30 mg/kg dose didn't affect lipid homeostasis in chow-fed rats.

Liver TG (Fig. 1G) and CHOL (Fig. 1H) were elevated in HFD-fed rats ($P<0.001$). This hepatic lipid accumulation following HFD was confirmed by the histological findings (Fig. 2A) that revealed widespread vacuolated hepatocytes with peripherally located nuclei, dilated sinusoids and some vacuoles coalesce with each other. The TEM microscopy (Fig. 2B) added further support by showing numerous fat droplets in the hepatocytes of HFD-fed rats. Both doses of BE remarkably decreased liver TG and CHOL and lipid droplets in hepatocytes as shown by the histological and ultrastructural examinations. The high dose of BE didn't alter the normal content of liver TG and CHOL. BE suppressed steatosis grade (Fig. 2C), inflammation grade (Fig. 2D) and NAS score (Fig. 2E) and increased the number of mitochondria (2F).

3.2. BE attenuates fibrosis and hepatocyte injury in HFD-fed rats

Both the control and BE-supplemented rats showed a thin fibrous layer within the portal area whereas the HFD-fed rats exhibited marked perivascular and periductal fibrosis (Fig. 3A,B). Serum ALT (Fig. 3C) and AST (Fig. 3D) activities increased significantly in HFD-fed rats as compared to the control. BE significantly ameliorated liver fibrosis and serum transaminases in HFD-fed rats.

3.3. BE downregulates hepatic ACC1 and FAS in HFD-fed rats

The effect of BE on hepatic FAS and ACC1 was evaluated using qRT-PCR and MD (Fig. 4). The binding affinity of BE with FAS was explored using MD (Fig. 4A-B). The output revealed that BE forms hydrophobic interactions with the TE domain of FAS at the residues Ile2250, Ser2308, Glu2366, Ala2367, Phe2370, Phe2423, Leu2427, Glu2431 and Arg2482 (Fig. 4A) with no polar bonds. It forms a polar bond with Asp158 and hydrophobic interactions with Ser112, Thr159, Ala160, Phe200 and Phe395 of the KS domain (Fig. 4B). The analysis revealed -8.7 and -7.2 kcal/mol lowest binding energies with the TE and KS domains, respectively (Table 2). HFD upregulated the mRNA abundance of FAS (Fig. 4C) and ACC1 (Fig. 4D) in the liver of rats ($P<0.001$). BE dose-dependently downregulated ACC1 and FAS in HFD-fed rats.

3.4. BE prevents IR in HFD-fed rats

HFD induced IR characterized by elevated blood glucose (Fig. 5A), hyperinsulinemia (Fig. 5B), increased HOMA-IR (Fig. 5C) and decreased QUICKI (Fig. 5D) as compared to the chow-fed rats ($P<0.00$). BE (15 and 30 mg) effectively ameliorated blood glucose and insulin, and HOMA-IR and QUICKI values in HFD-fed rats.

3.5. BE attenuates hepatic OS in rats with NAFLD

The LPO marker MDA was elevated in rats that received HFD (Fig. 6A) and SOD (Fig. 6B), CAT (Fig. 6C) and GPx (Fig. 6D) were decreased as compared to the chow-fed rats ($P<0.001$). BE suppressed hepatic LPO and enhanced antioxidants in HFD-fed rats in a dose-dependent manner whereas had no effect on redox homeostasis in the chow-fed rats.

3.6. BE downregulates hepatic NF-kB in rats with NAFLD

MD simulations explored the possible binding of BE with NF-κB p50.p65 heterodimer (Fig. 7A and Table 2). While there was no polar bonding, BE forms hydrophobic interactions with the NF-κB p50.p65 heterodimer at the residues Arg54, Ser240, Lys241, Arg246, Lys272 and

Phe307 with -11.0 kcal/mol lowest binding energy. Changes in NF-kB p65 in the liver of rats were assayed using qRT-PCR (Fig. 7B) and IHC (Fig. 7C-D). The results showed a significant upregulation in rats with NAFLD ($P<0.001$). A remarkable and dose-dependent downregulation was observed in the liver of BE-treated rats.

3.7. BE upregulates hepatic Nrf2, HO-1 and SIRT1 in rats with NAFLD

MD simulations revealed polar bonds with Leu557 and Ile559 and hydrophobic interactions with Ala366, Gly367, Val418, Gly419, Val420, Val465, Ala510, Val512, Gly558, Val604, Val606, Ala607 and Val608 of Keap1 (Fig. 8A, Table 2) and polar bonds with Thr135, Arg136 and Asp140 and hydrophobic interactions with His25, Met34, Phe37, Gln38, Gly139, Gly143, Leu147, Phe207 and Asn210 of HO-1 (Fig. 8B, Table 2). HFD-fed rats showed a downregulation of Nrf2 (Fig. 8C) and HO-1 (Fig. 8D) mRNA, and HO-1 activity (Fig. 8E) as compared with the chow-fed rats. BE dose-dependently upregulated Nrf2 and HO-1 in the liver of rats.

The data represented in Figure 9 revealed downregulation of hepatic SIRT1 mRNA (Fig. 9A) and protein (Fig. 9B-C) in HFD-fed rats. Both doses of BE upregulated SIRT1 in the liver of HFD-fed rats. *In silico* investigation showed that BE forms polar bonds with Lys238, Glu477 and Asn648 and hydrophobic interactions with Lys235, Lys236, His473 and Arg649 of SIRT1 (Fig. 9D and Table 2).

4. Discussion

This study evaluated the effect of BE on dyslipidemia, inflammation, and OS in a rat model of NAFLD. Feeding a HFD resulted in increased TG and CHOL and decreased HDL-C as previously reported [35, 36]. Liver CHOL and TG were increased upon HFD feeding and the histological analysis revealed widespread vacuolated hepatocytes with peripherally located nuclei. In addition, the TEM data showed the presence of numerous fat droplets in hepatocytes

and steatosis and inflammatory grades along with NAS score were elevated in HFD-fed rats. Furthermore, marked perivascular and periductal collagen deposition was observed and serum transaminases were elevated in HFD-fed rats. These findings showed the development of the characteristic features of NAFLD following HFD feeding for 12 weeks. Hepatic lipid accumulation is primarily elicited by imbalanced TG and FAs acquisition and removal characterized by increased uptake and decreased export along with impaired β-oxidation and enhanced *de novo* lipogenesis [37]. Under HFD feeding, the lipid taken up entered the circulation as TG-rich chylomicrons and FFAs and 20% of them are taken by the liver. Within the liver, FFAs could be metabolized by β-oxidation, packaged with apolipoprotein B and secreted as VLDL to the blood or stored in lipid droplets following esterification to TG [38]. *de novo* lipogenesis, a source of hepatic TG deposition, is regulated by SREBP-1c and ChREBP in response to insulin signaling and glucose status. Both factors can upregulate many lipogenic genes, including FAS and ACC1 [39]. Our study revealed upregulated ACC1 and FAS expression in the liver of HFD-fed rats as previously reported [40], indicating the contribution of *de novo* lipogenesis to the developed NAFLD. Elevated levels of circulating transaminases (ALT and AST) denote hepatic injury and are involved in the progression of NAFLD as reported in previous studies [36, 41].

BE effectively decreased serum lipids and transaminases, prevented hepatic lipid accumulation, steatosis and fibrosis, and downregulated the lipogenic genes FAS and ACC1 in HFD-fed rats. The effective anti-hyperlipidemic effect of BE was previously reported in a model of DIO where it decreased serum and tissue lipids by downregulating the genes involved in CHOL and FAs biosynthesis [23]. The same study revealed the potency of BE to specifically inhibit the maturation of SREBPs and downregulate the mRNA abundance of SREBP-2, leading to suppressed cholesterologenesis [23]. BE downregulated the protein expression levels of SREBP-1 in ethanol-challenged LX-2 cells and in the liver of ethanol-induced mice,

effects that were accompanied with decreased serum aminotransferases and TG [42]. Our study added support to the potent anti-hyperlipidemic effect of BE. MD assessment revealed that the structure of BE occupied an active binding site on the enzyme surface of TE domain of FAS. Although no polar interacting residues were detected for this complex, large number of hydrophobic bonds were shown to bind BE to TE/FAS active site as commonly known for TE/FAS interactions. Two phenylalanine residues were involved in the binding interactions of this complex (Phe2370 and Phe2423). This suggests the high probability to exhibit a favorable thermodynamic π-π interactions. These interactions can compete successfully in the potential binding mechanism of BE to TE/FAS binding mechanism. In addition, docking with the KS domain of FAS showed that BE was located in the main active site on the enzyme surface with a large number of hydrophobic interactions and one polar bond. The binding energy of this complex showed a relatively stable BE-KS/FAS complex. As well, Phe200 and Phe395 were detected in the binding site and consequently suggested the probability of these residues to exhibit favorable π-π interactions which lead to energy minimization of this complex.

The ameliorative efficacy of BE on dyslipidemia and NAFLD could be related to its insulin sensitizing effects. IR plays a central role in NAFLD progression and 70-80% of the obese and T2D patients have NAFLD [43, 44]. IR can promote simple fatty liver progression to NASH [45]. AT and hepatic IR and reduced whole-body insulin sensitivity are strongly associated with NAFLD. Previous studies showed that 45-50% reductions in glucose disposal and impaired endogenous glucose production are indicative of whole-body insulin sensitivity and hepatic IR, respectively [46]. In this study, HFD-fed rats exhibited an increase in blood glucose, hyperinsulinemia, and IR. Impaired insulin's ability to suppress lipolysis and the increase in FFAs delivered to the liver contribute to the lipid deposition provoked by IR [46, 47]. BE ameliorated IR and this might contribute to its beneficial effect against NAFLD in this study.

This notion is supported by previous studies showing the role of BE in improving glucose tolerance in diabetic rats [48] and IR in a murine model of DIO [23].

Given the anti-inflammatory and antioxidant efficacies of BE [22, 48], and the role of OS and inflammation in the development and progression of NAFLD [49, 50], it was noteworthy assuming that BE mitigated NAFLD by attenuating these processes. HFD-fed rats in this study showed elevated hepatic MDA and declined antioxidants, demonstrating oxidative stress. Similar to our findings, rats with NAFLD induced by high carbohydrate diet [51] or HFD [35] showed an increase in MDA and decreased SOD, CAT and GPx. OS is a central contributor to hepatocyte injury via LPO, declined antioxidants, mitochondrial dysfunction and apoptosis [37]. Several studies pinpointed the role of OS characterized by elevated ROS and LPO and diminished antioxidants in NAFLD [10, 11]. Impaired mitochondrial function provoked by FFAs overload, lipotoxicity, and hypoxia, and activated NADPH oxidases are the main sources of ROS in NAFLD [11]. Dysfunctional electron transport and mitochondrial dynamics downregulate FA consumption and provoke fat accumulation, augmenting mitochondrial ROS generation [52]. Along with OS, a growing evidence links the initiation of hepatocyte injury and steatosis progression to inflammation [16]. Here, NAFLD was associated with the upregulation of NF-κB p65 both mRNA and protein. Increased ROS activates NF-κB and increases pro-inflammatory cytokines [53]. The released cytokines, in particular, TNF-α and IL-1β, impair insulin signaling, decrease peripheral glucose uptake and upregulate hepatic glucose production. Impaired insulin signaling provokes lipolysis and increased FFAs that directly provoke M1 polarization of macrophages resulting in a pro-inflammatory phenotype [54]. In addition, excessive FFAs are sensed by Kupffer cells (KCs) and impair β-oxidation and mitochondrial function and lipid accumulation is promoted [55]. Treatment with BE decreased hepatic MDA, downregulated NF-κB p65 and enhanced antioxidants in HFD-fed rats. These findings supported the previously reported antioxidant and anti-inflammatory

activities of BE [22, 48]. Given the implication of mitochondrial dysfunction in NAFLD, maintaining normal mitochondria is crucial for reducing lipid accumulation in hepatocytes. In this context, BE reverted the altered mitochondrial respiration and uncoupling and ameliorated the activity of complex I, II, and IV in rats with alcohol-induced steatohepatitis [56]. The suppressive effect of BE on NF-κB was further supported by MD to explore its binding affinity toward NF-κB p50.p65 heterodimer. The stability of BE/NF-κB complex was deduced from the existence of several hydrophobic interactions in the binding site as well as the involvement of different DNA units in the interactions at the active site and the low binding affinity. The active site interactions of this complex involved significant essential residues. The prospect of demonstrating substantial electrostatic interaction with DNA during NF-κB-stimulated gene expression is highlighted by this evidence and can limit gene expression.

To further explore the mechanism underlying the effect of BE on HFD-induced steatosis, we determined its effect on Nrf2, HO-1 and SIRT1 and conduced MD simulations to explore its binding affinity. HFD feeding downregulated Nrf2, HO-1 and SIRT1 whereas BE effectively upregulated their expression. BE has been previously shown to upregulate hippocampal Nrf2 and HO-1 in diabetic rats [48]. Given that OS is implicated in the pathogenesis of NAFLD [35, 51], Nrf2 upregulation participated in the ameliorative effect of BE on NAFLD. The role of Nrf2 activators in ameliorating Nrf2 has been pinpointed [14] and the study of Sugimoto et al showed that its deletion promoted a rapid progression of nutritional steatohepatitis in mice [57]. In contrast, hepatocyte-specific *Nrf2*-overexpression in normal chow- or methionine–choline-deficient (MCD) diet-fed mice resulted in upregulated GPx, NQO-1 and thioredoxin-1 and diminished oxidants [58]. The use of proteomics and *Nrf2*-deficient mice revealed the role of Nrf2 in regulating lipid metabolism [59]. It negatively regulates FABP1/4/5 and hence decreased FFAs uptake by hepatocytes [58]. Nrf2 can also downregulate SREBP-1c and ChREBP leading to the suppression of lipogenesis and improve mitochondrial function by

mitigating ROS [14]. Similar to our findings, BE has been previously reported to positively regulate SIRT1 in a mouse model of ethanol-induced liver injury [42]. The activation of SIRT1 can confer protection against NAFLD through its beneficial role in lipid metabolism by suppressing hepatic OS and inflammation and modulating various factors related to lipid metabolism. By deacetylating SREBP-1c at Lys-289 and Lys-309, SIRT1 downregulates its transcriptional activity on lipogenic genes and promotes its ubiquitination and proteasomal degradation [60]. By using adenovirus to overexpress SIRT1 in mice, the expression of SREBP-1c and lipogenic genes was suppressed, whereas its silencing or pharmacological inhibition increased SREBP-1c acetylation and upregulated lipogenesis [60]. The chemical activation of SIRT1 decreased SREBP-1c, FAS and ACC1 and protected the liver against steatosis induced by HFD [61]. In addition to SREBP-1c, deacetylation of ChREBP and preventing its transcriptional activity and lipogenesis seems to be controlled by SIRT1 [16]. SIRT1 can promote β-oxidation and prevent lipid accumulation within the liver by deacetylating PGC-1α and subsequently increasing PPARα transcriptional activity [62]. It can also confer protection against OS and inflammation in the context of NAFLD. SIRT1 activation deacetylated and activated FOXO1 and PGC-1α transcriptional activity under HFD feeding, effects that upregulated the target antioxidant genes [63]. SIRT1 can deacetylate and prevent the activity of NF-κB and the subsequent release of pro-inflammatory mediators. The beneficial role of SIRT1-mediated anti-inflammatory effect in NAFLD was supported by studies showing that its liver-specific knockout increased macrophage infiltration in response to HFD challenge [62, 64].

The effect of BE on Nrf2/HO-1 and SIRT1 was supported by MD simulations. The obtained low binding energy revealed the compatibility of BE to Keap1's binding pocket and showed that it occupies the main active site surrounded by three polar interacting residues with key residues [65]. A dense network of hydrophobic amino acids was detected in the binding pocket

of BE-Keap1 complex reflecting the bioavailability of BE as Keap1 inhibitor. In addition, the binding model of BE-HO-1 complex revealed several hydrophobic interactions along with two key polar interacting amino acid residues. The obtained low binding energy pinpointed the binding affinity of BE towards HO-1. Furthermore, the outputs of our MD uncovered the probable binding modes of BE with SIRT1. Three polar bonds and four hydrophobic interacting residues were included in the binding interaction of this complex. The estimated low binding affinity and the sufficient polar interacting residues are enough for the formation of BE-SIRT1 complex as compared to the reported binding energies in previous studies [33, 66].

5. Conclusion

The findings of this study confer new information on the protective efficacy of BE on HFD-induced NAFLD. BE ameliorated serum lipids, glucose and insulin and prevented lipid accumulation and biosynthesis in the liver of rats. BE attenuated hepatic OS and inflammation, and upregulated Nrf2, HO-1 and SIRT1 in HFD-fed rats. MD simulations revealed the binding affinity of BE towards NF-κB, Keap1, HO-1 and SIRT1. Therefore, BE is a promising candidate for preventing or resolving the development of NAFLD and could be employed in clinical settings, pending further studies to explore other mechanism(s) underlying its protective effect.

Declaration of Competing Interest

None.

Availability of data and materials

The manuscript contains all data supporting the reported results.

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Tables

Table 1. Primers used for qRT-PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Nrf2	TTGTAGATGACCATGAGTCGC	TGTCCTGCTGTATGCTGCTT
HO-1	GTAAATGCAGTGTTGGCCCC	ATGTGCCAGGCATCTCCTTC
SIRT1	TCTCCCAGATCCTCAAGCCAT	TTCCACTGCACAGGCACATA
FAS	GCCTAACACCTCTGTGCAGT	GGCAATACCCGTTCCCTGAA
ACC	TTGGTGCTTATATTGTGGATGG	ATGTGCCGAGGATTGATGG
NF-κB p65	TTCCCTGAAGTGGAGCTAGGA	CATGTCGAGGAAGACACTGGA
GAPDH	TGCTGGTGCTGAGTATGTCG	TTGAGAGCAATGCCAGCC

Table 2. Binding affinities, interacting polar residues, and hydrophobic interactions of BE with Keap1, NF-κB, HO-1, and FAS.

	Affinity (kcal/mol)	**Polar bonds**	**Hydrophobic interactions**
Keap1	-9.5	Leu557 and Ile559	Ala366, Gly367, Val418, Gly419, Val420, Val465, Ala510, Val512, Gly558, Val604, Val606, Ala607 and Val608
NF-κB	-11.0		Arg54, Ser240, Lys241, Arg246, Lys272 and Phe307
SIRT1	-6.4	Lys238, Glu477 and Asn648	Lys235, Lys236, His473 and Arg649
HO-1	-9.5	Thr135, Arg136 and Asp140	His25, Met34, Phe37, Gln38, Gly139, Gly143, Leu147, Phe207 and Asn210
FAS/TE	-8.7		Ile2250, Ser2308, Glu2366, Ala2367, Phe2370, Phe2423, Leu2427, Glu2431 and Arg2482
FAS/KS	-7.2	Asp158	Ser112, Thr159, Ala160, Phe200 and Phe395

Figures:

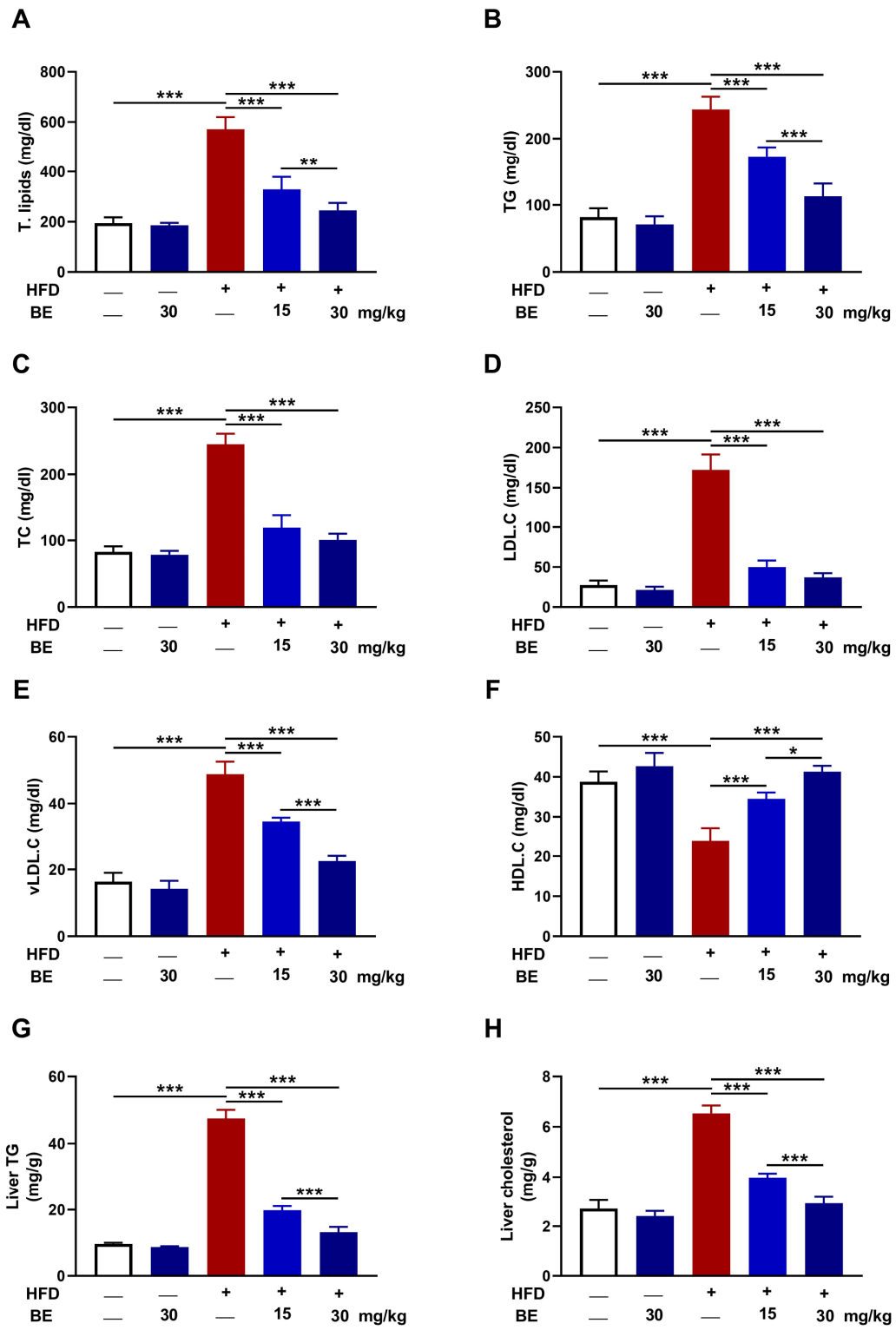

Fig. 1. BE ameliorated dyslipidemia in HFD-fed rats. BE decreased serum total lipids (A), triglycerides (B), total cholesterol (C), LDL-C (D), and vLDL-C (E), increased serum HDL-C (F), and decreased liver TG (G) and cholesterol (H) in HFD-fed rats. Data are Mean ± SEM, (n = 8). *P<0.05, **P<0.01, and ***P<0.001.

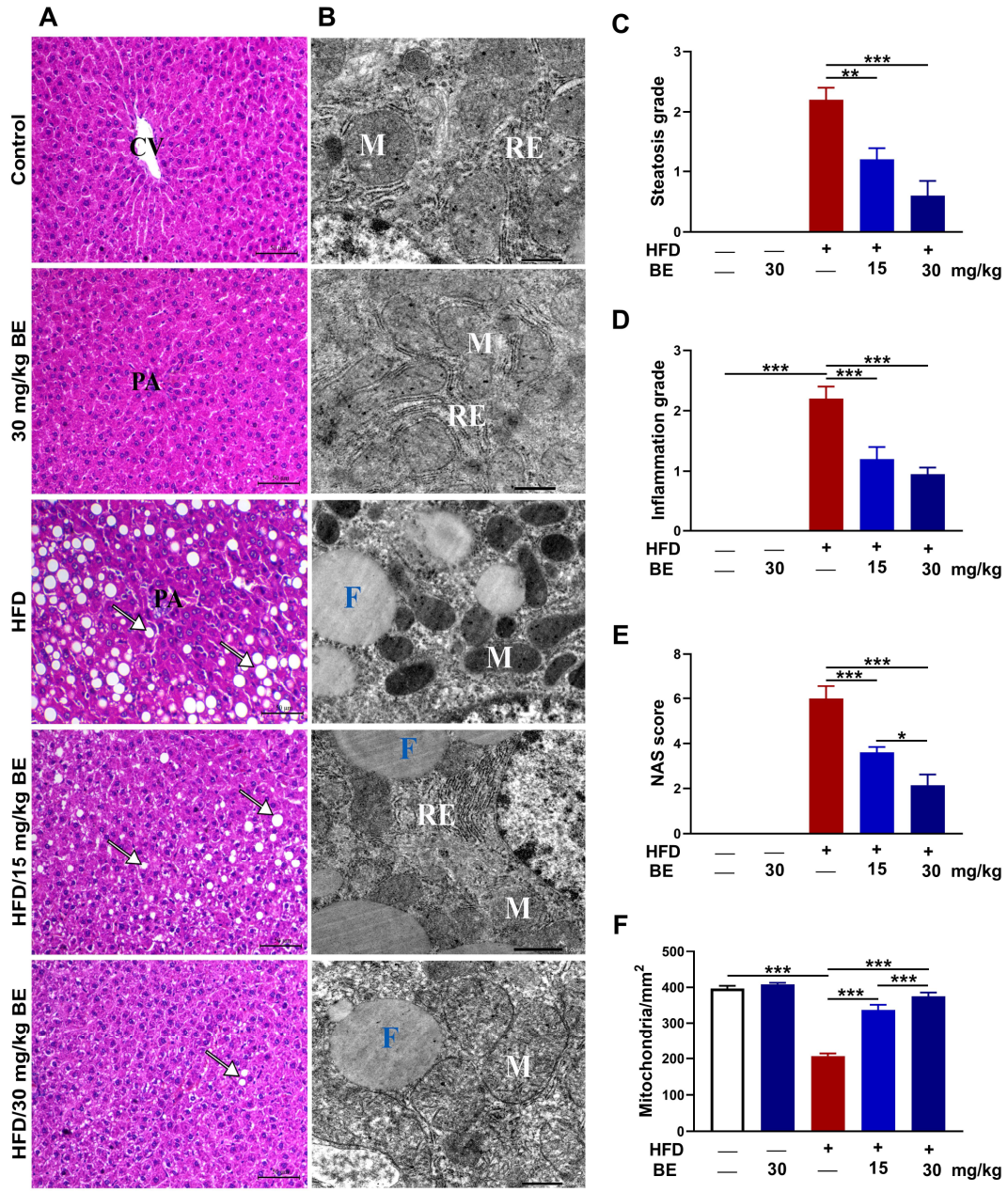

Fig. 2. (**A**) Photomicrograph of H&E-stained sections in control and BE-supplemented rats showing normal architectural pattern of hepatic lobule and hepatocytes with hepatocytes arranged in cords radiating from central vein (CV), normal portal area (PA) and hepatic sinusoids in between cords, HFD-fed rats showing widespread vacuolated hepatocytes with peripherally located nuclei (white arrows), some vacuoles coalesced with each other and dilated hepatic sinusoids, and HFD-fed rats treated with 15 and 30 mg/kg BE showing decreased number of vacuolated hepatocytes with very few vacuoles in the 30 mg/kg BE-treated group. (**B**) EM micrographs showing hepatocytes with an euchromatic nucleus (N), numerous mitochondria (M) and normal rough endoplasmic reticulum (RE) in control and BE-supplemented rats, numerous fat droplets in hepatocytes of HFD-fed rats, and a fewer number of fat droplets in the hepatocytes of 15 and 30 mg/kg BE-supplemented HFD-induced rats. (**C-E**) BE decreased steatosis grade (C), inflammation grade (D) and NAS score (E) in HFD-fed rats. (**F**) The number of mitochondria decreased in HFD-fed rats and increased following treatment with BE. Data are Mean ± SEM, (n = 8). ***P<0.001.

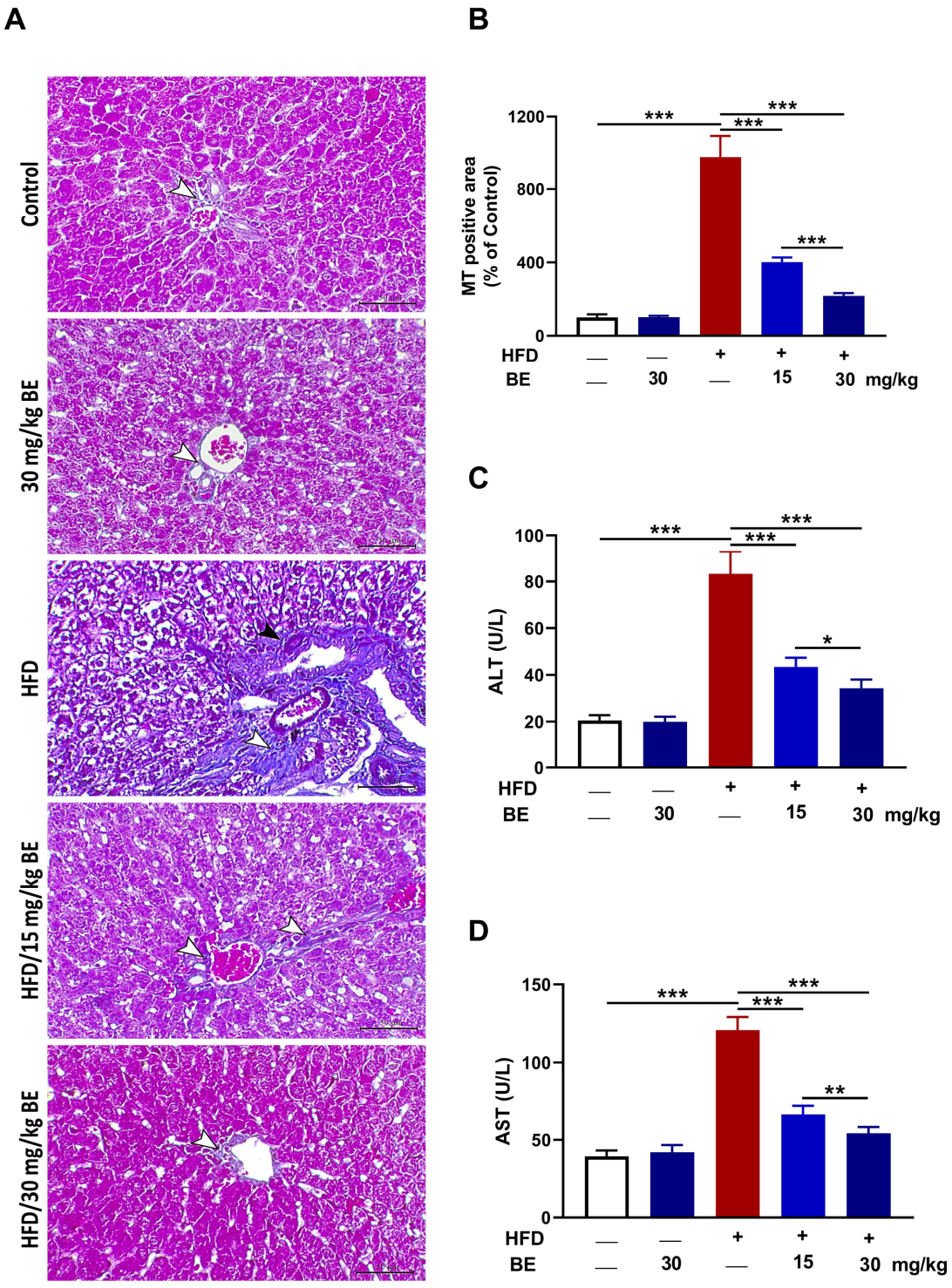

Fig. 3. BE attenuated fibrosis and hepatocyte injury in HFD-fed rats. (**A**) MT-stained sections in the liver showing a thin fibrous layer within the portal area (arrowheads) in Control and BE-supplemented rats, marked perivascular (black arrowhead) and periductal (white arrowhead) fibrosis in HFD-fed rats, and decreased periportal fibrosis (arrowheads) in HFD-fed rats treated with 15 and 30 mg/kg BE. (Scale bar = 50 µm). (**B**) Image analysis showing increased fibrosis in HFD-fed rats and its decrease in BE-treated groups. (**C**,**D**) BE decreased serum ALT (C) and AST (D) in HFD-fed rats. Data are Mean ± SEM, (n = 8). *P<0.05, **P<0.01 and ***P<0.001.

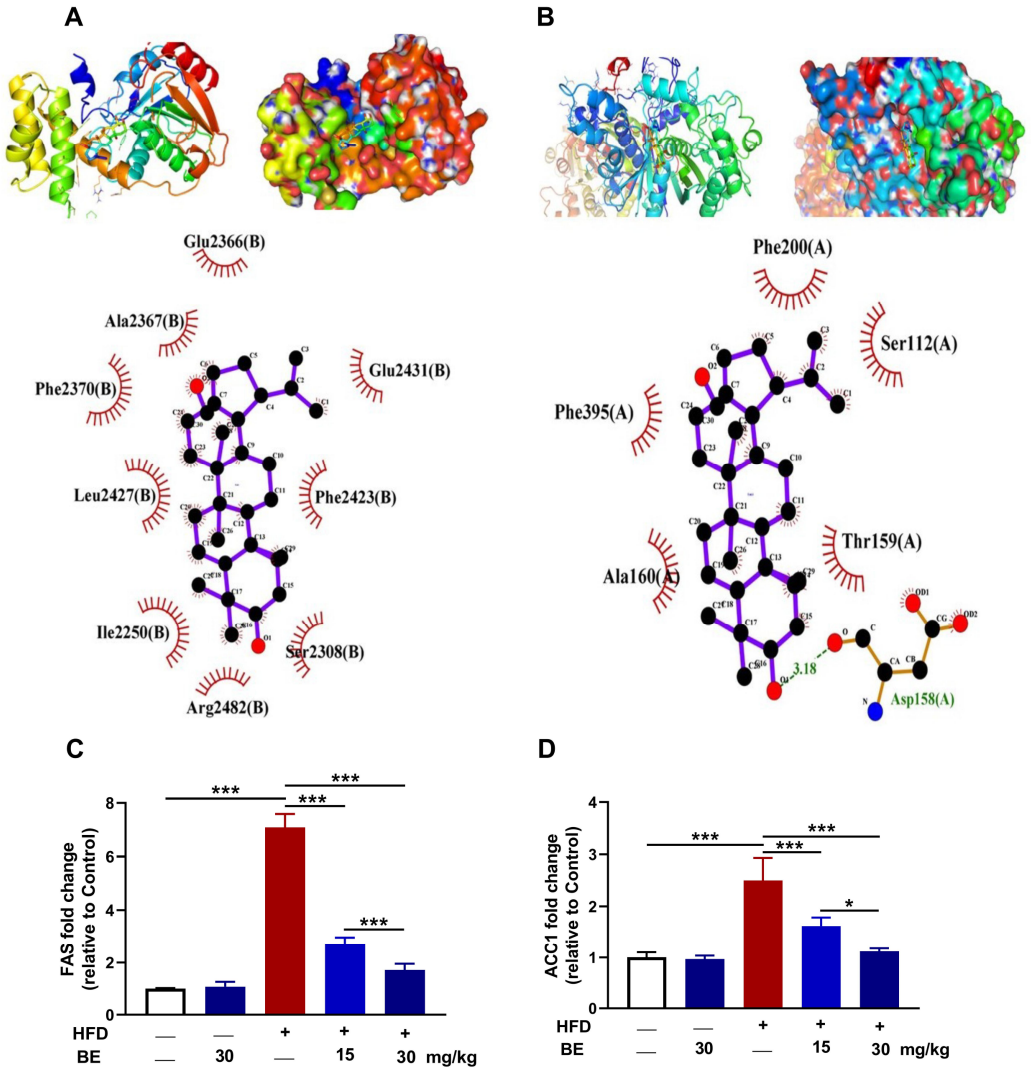

Fig. 4. BE downregulated FAS and ACC1 in HFD-fed rats. (**A** & **B**) Molecular docking simulation revealing the binding between BE and TE (**A**) and KS (**B**) domains of FAS. BE downregulated FAS (**C**) and ACC (**D**) mRNA abundance in the liver of HFD-fed rats. Data are Mean ± SEM, (n = 8). *P<0.05 and ***P<0.001.

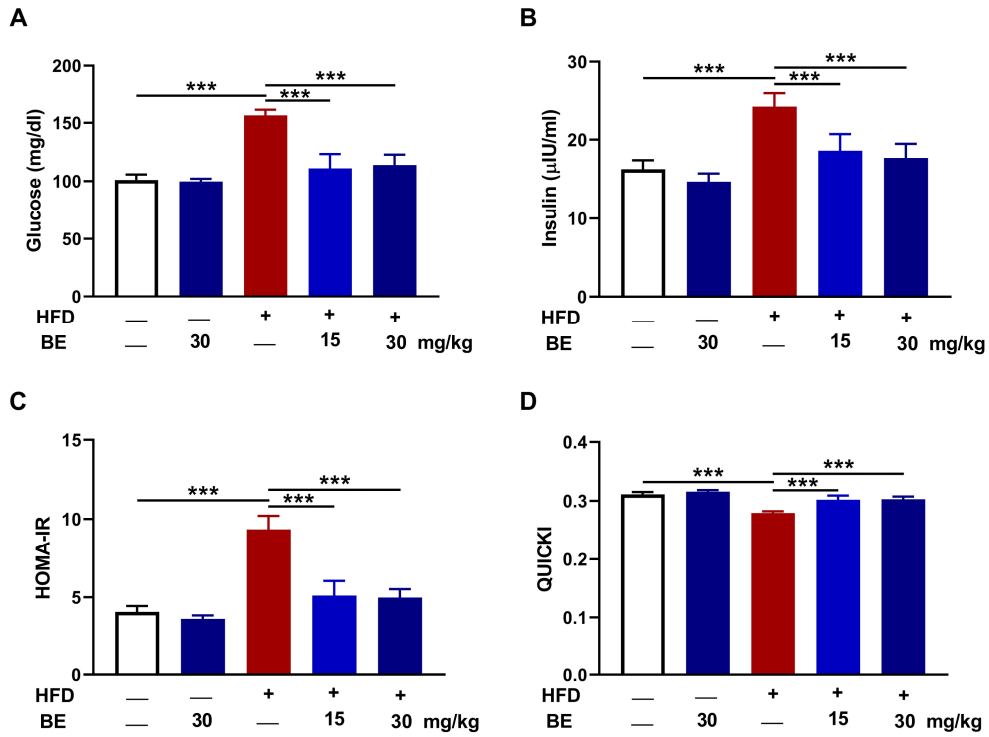

Fig. 5. BE prevented insulin resistance in HFD-fed rats. BE ameliorated blood glucose (**A**) and insulin (**B**), decreased HOMA-IR (**C**) and increased QUICKI (**D**) in HFD-fed rats. Data are Mean ± SEM, (*n* = 8). ***P<0.001.

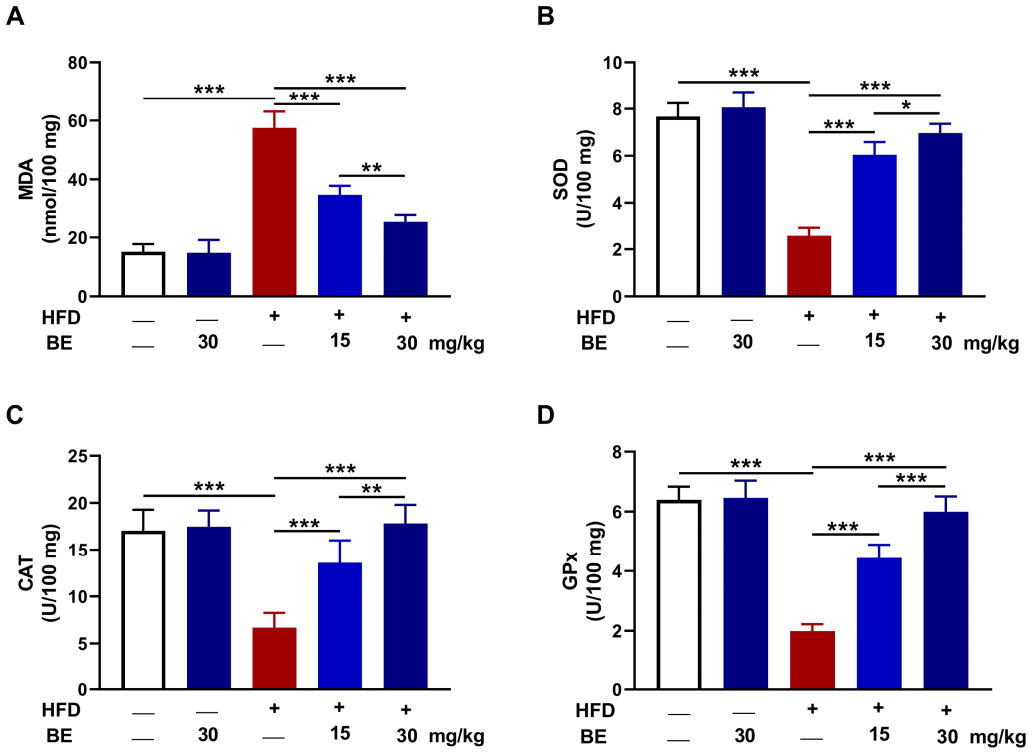

Fig. 6. BE attenuated oxidative stress in HFD-fed rats. BE decreased liver MDA (**A**), and increased SOD (**B**), CAT (**C**), and GPx (**D**) in HFD-fed rats. Data are Mean ± SEM, (*n* = 8). *P<0.05, **P<0.01, and ***P<0.001.

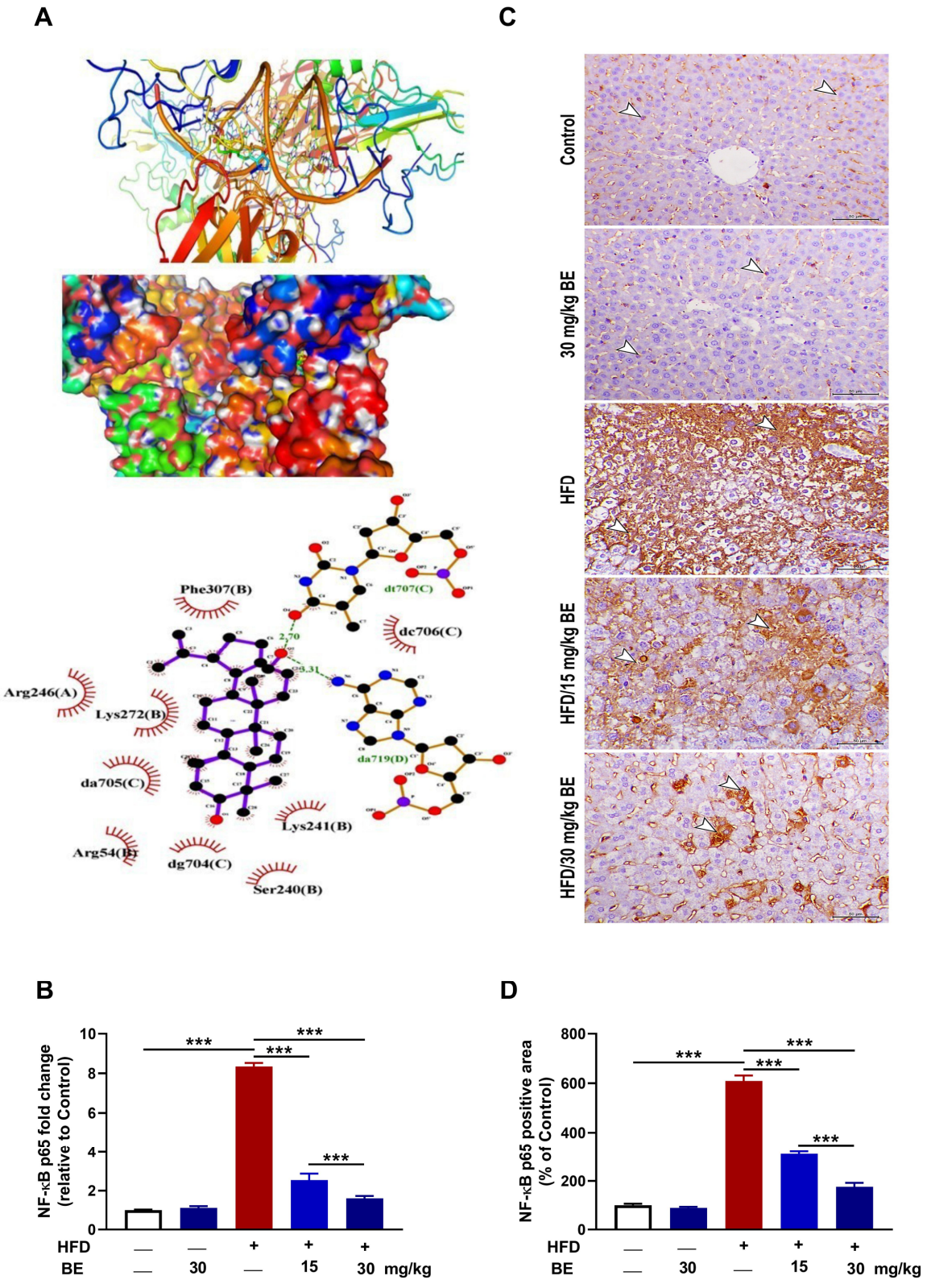

Fig. 7. BE suppressed inflammation in HFD-fed rats. (**A**) Molecular docking simulation revealing the binding between BE and NF-κB. (**B**) BE downregulated liver NF-κB p65 mRNA in HFD-fed rats. (**C**-**D**) Photomicrographs (**C**) and image analysis (**D**) showing increased NF-κB p65 immunostaining in the liver of HFD-fed rats and its decrease in BE-treated groups. Data are Mean ± SEM, (n = 8). ***P<0.001.

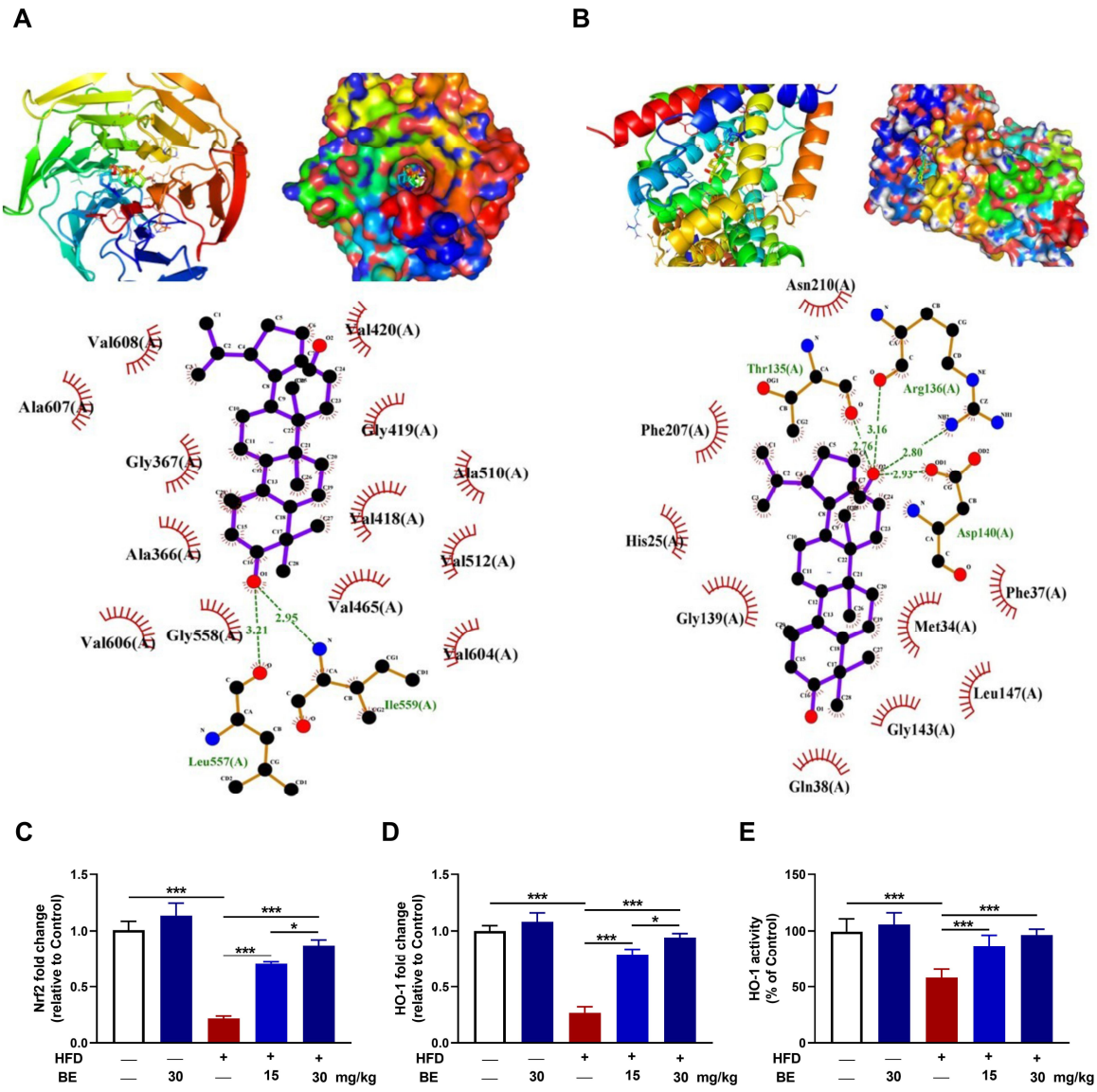

Fig. 8. BE upregulated Nrf2/HO-1 pathway in HFD-fed rats. (**A**-**B**) Molecular docking simulation revealing the binding between BE and Keap1 (**A**) and HO-1 (**B**). (**C**-**E**) BE upregulated liver Nrf2 (**C**) and HO-1 (**D**) mRNA and HO-1 activity (**E**) in HFD-fed rats. Data are Mean ± SEM, (n = 8). *P<0.05 and ***P<0.001.

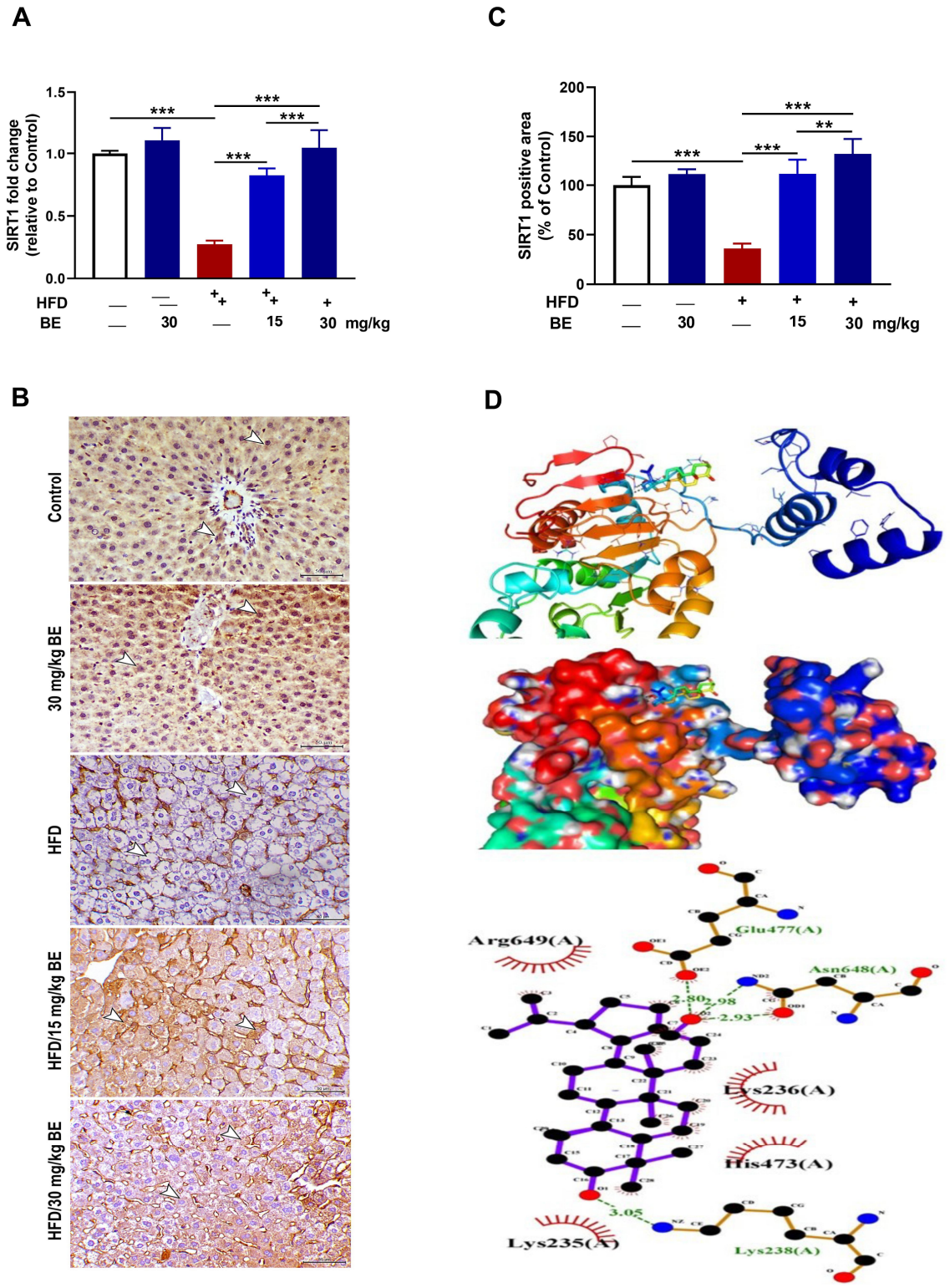

Fig. 9. BE upregulated SIRT1 in HFD-fed rats. (**A**) BE increased SIRT1 mRNA in liver of HFD-fed rats. (**B**-**C**) Photomicrographs (**B**) and image analysis (**C**) showing decreased SIRT1 immunostaining in the liver of HFD-fed rats and its increase in BE-treated groups. Data are Mean ± SEM, (*n* = 8). ***P<0.001. (**D**) Molecular docking simulation revealing the binding between BE and SIRT1.