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BLACK CUMIN SEED OIL AS A MUTUAL INHIBITOR OF CYCLOOXYGENASE-2 AND 5-LIPOXYGENASE IN LPS-INDUCED ACUTE LIVER INJURY IN MICE

Rehab Hosni [1], Hesham Haffez [2,3*] and Hatem K. Amin[2,4]

[1]*El Sisi Comprehensive clinic, General Authority for Health Insurance-Giza, 12556, Giza, Egypt.*

[2]*Biochemistry and Molecular Biology Department, Faculty of Pharmacy, Helwan University 11795, Cairo, Egypt.*

[3]*Center of Scientific Excellence "Helwan Structural Biology Research, (HSBR)", Helwan University, 11795, Cairo, Egypt.*

[4]*Biochemistry Department, Faculty of Pharmacy, Galala University*

Abstract

Acute hepatitis is characterized by acute hepatic parenchymal inflammation, raising liver function indices, and offering little chance of successful treatment. Consequently, there is a lot of curiosity in the discovery of natural products as valuable alternatives to traditional anti-inflammatory agents with inhibitory effect, especially on cyclooxygenase-2 (COX-2) and *5*-lipoxygenase (5-LO). Black cumin seed oil (BCSO) possesses antioxidant and anti-inflammatory properties due to the constituent of thymoquinone (TQ). The protective anti-inflammatory properties of BCSO and its nanoemulsion formulation were investigated in mice that had been given lipopolysaccharide (LPS) to cause liver injury by hepatic inflammatory markers assessment on both gene and protein levels, molecular docking, and histological examination. Results showed that BCSO is a promising potent COX-2 inhibitor. The nanoemulsion was shown to enhance the anti-inflammatory effect of BCSO compared to Indomethacin as standard anti-inflammatory drug. In order to reduce these side effects, combinatorial therapy of BCSO with Indomethacin at comparatively lower doses of Indomethacin showed a potentiated anti-inflammatory effect. TQ showed enhanced binding affinity interaction to (5-LO) that may explain its potential inhibitory activity to COX-2. The current presented data suggests the potential anti-inflammatory effect of both BCSO and its nanoemulsion for wide scale clinical applications.

Keywords: BCSO; Liver inflammation; LPS; COX-2; 5-LO; TQ; Indomethacin.

1. Introduction

Inflammation is intricate biological reaction of body tissues to damaging stimuli, as infections, injured cells, or irritants[1]. It falls into one of two categories: acute or chronic [1]. Acute inflammation results from the body's early reaction to detrimental stimuli, which is the enhanced migration of blood leukocytes and plasma into the injured tissues [2]. The inflammatory response is initiated and developed by a sequence of biochemical processes that include the immune system, the local vascular system, and different cells in the inflamed tissues [3]. Persistent inflammation, defined as chronic inflammation [4]. The liver is essential for the elimination of toxins, the storage of glucose, immunological homeostasis, and metabolism; however, these vital processes are typically compromised by inflammatory reactions occurring in acute liver injury [5]. Bacterial lipopolysaccharides (LPS) is a lipid and polysaccharide-containing compounds known as endotoxin,, comprise the majority of the outermost layer of Gram-negative bacteria [6]. For molecular pathology study, LPS-induced hepatic damage in mice has served as a model, mimicking the progression of liver damage and failure in acute liver injury [7].

Arachidonic acid (AA) and its byproducts (prostaglandins and leukotrienes) are significant mediators which have an crucial role for the regulation of pain and inflammation pathways [8]. AA is metabolized by COX-2 and 5-LO for prostaglandins and leukotrienes (LTs) biosynthesis, respectively [9]. The conversion of AA to prostaglandins by COX-2 is implicated in fever, pain, swelling, and inflammation [10]. While 5-LO catalyzes the conversion of AA to 5-hydroperoxyeicosatetratenoic acid (5-HPETE), which is then transformed into LTs that act as inflammatory mediators and regulate the innate immune response [11]. Successful inflammation management protocol is focusing on many arachidonic acid pathways with preserving typical physiological processes [11]. Thus, it is useful to simultaneously inhibit COX-2 and 5-LO in order to develop possible and effective anti-inflammatory medications with less adverse effects [12].

Non-steroidal anti-inflammatory medications (NSAIDs) are commonly employed to treat disorders associated with

*Corresponding author e-mail: hesham.haffez@pharm.helwan.edu.eg.; (Hesham Haffez)

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inflammation by acting as an analgesic and anti-inflammatory [13]. However, gastrointestinal bleeding, ulceration, and perforation are linked to long-term use of conventional NSAIDs including Indomethacin, diclofenac, and naproxen [14]. Consequently, a number of strategies have been explored to specifically target COX-2 and 5-LO to prevent the production of prostaglandins and LTs that are produced during inflammation [7, 11, 15]. Therefore, using natural products with multiple anti-inflammatory targets is a popular technique in drug discovery, offering a better safety profile with less negative effects on the cardiovascular, renal, and gastrointestinal systems.

Black cumin seed oil (BCSO) is a natural herbal oil that has been extensively used as medicinal supplement for range of medical ailments [16]. Numerous studies have demonstrated diverse biological effects of BCSO or its active ingredients, such as their anti-tussive, analgesic, antioxidant, and anti-cancer properties [17]. One of the main bioactive components of BCSO is thymoquinone (TQ) which has potent anti-inflammatory properties as prevent the generation of inflammatory cytokines like TNF-α and interleukins [18, 19]. Additionally, it has been demonstrated that BCSO reduces the symptoms of a variety of illnesses, including long-term conditions like diabetes, dyslipidemia, and hypertension [17]. Previous research had examined that BCSO's primary mechanisms for managing inflammation include blocking the generation of histamine, leukotriene, and immunomodulatory effects [17].

Furthermore, applying nanotechnology could lead to new opportunities for improving BCSO's bioavailability parameters and consequently, its effectiveness and efficiency. As a result, nanoemulsions will offer many benefits, including improved kinetics, increased solubilization potential, and improved drug delivery [20].

In order to alleviate LPS-induced inflammation in mice's livers, the current study compared and assessed the anti-inflammatory capacities of BCSO and its nanoemulsion formulation in liver induced injury. We explored the synergistic boost for therapeutic benefit of Indomethacin at lower dose to minimize side effects, so the combinatorial effect of Indomethacin with BCSO was also be examined.

2. Materials and Methods

2.1. Animal grouping and applied treatment

42 mice of 4 weeks old weighted 30 g were purchased from Egyptian Company for Production of Vaccines and Sera (VACSERA), Cairo, Egypt. Experiments were performed in the animal shelter of the Faculty of Pharmacy, Helwan University. The Helwan University Faculty of Pharmacy's Scientific Research Ethics Committee accepted all procedures, which complied with the Declaration of Helsinki (Ethical committee approval number: 01A2020). The authors attested to the fact that every animal got human care and that all procedures involving animals were carried out in compliance with applicable laws and regulations. BCSO pure oil was 100% pure and obtained from Wilko company, UK. Pilot study showed optimum dose of BCSO preparation or BCSO nanoemulsion treatment (1 mL/kg) that equivalents to (0.2 mL/kg) of pure oil once daily for 14 days before LPS- induction which matched with previously mentioned in literature [21, 22] and Indomethacin in dose of (5 mg/kg/day) [23, 24] for three days prior to LPS- induction once daily. The dose of Indomethacin in combination with BCSO was reduced to 2.5 mg/kg/day. LPS dose (2 mg/kg) was selected for induction of acute inflammation as previously mentioned in literature with lowest mortality rate [25, 26]. Six hours after LPS treatment, the animals were sacrificed.

Six groups of mice were randomly assigned: negative control group received tween 20 (1 mL /kg/day), LPS-induced group, LPS- induced group pre-treated with BCSO, LPS-induced group pre-treated with nanoemulsion form of BCSO, LPS-induced group pre-treated with Indomethacin and LPS-induced group pre-treated with BCSO for 11 days followed by reduced dose of Indomethacin for three days.

Following cervical dislocation, the livers were removed and divided into two sections. One section was preserved for histopathological analysis in 10% buffered neutral formalin, while the other half was rinsed in cold saline and the samples were quickly preserved at -80 C for additional biochemical assessment.

2.2. GC-MS assessment of pure BCSO

The GC-MS system was employed at the Central Laboratories Network, National Research Center in Cairo, Egypt using mass spectrometer detector (5977A) and gas chromatography system (7890B) from Agilent Technologies. Helium was used as the carrier gas in the analysis, with a split ratio of 1:20, a flow rate of 3.0 ml/min, an injection volume of 1 µl, and a temperature program of 40 °C for one minute, rising at a rate of 7 °C per minute to 250 °C, and holding for five minutes. At 250 °C, the injector and detector were maintained. Using a spectral range of m/z 30-440 and a solvent delay of six minutes, mass spectra were obtained by electron ionization (EI) at 70 eV. Identification of the oil constituents was performed by comparing the spectrum fragmentation pattern with those found in Wiley and NIST Mass Spectral Library data.

2.3. BCSO nanoemulsion formulation preparation

2.3.1. Nanoemulsion formulation preparation

The BCSO is prepared as an oil in water (O/W) nanoemulsion using the method described by *Gumus et al* [27]

2.3.2. Droplet size, PDI and Zeta potential

The mean droplet size, droplet size distribution as expressed by polydispersity index (PDI), as well as the surface charge expressed by zeta potential (ZP) were measured using dynamic light scattering technique by Malvern Zetasizer (nano ZS, Zetasizer, Malvern Instruments Ltd., UK).

2.3.3. Morphological analysis

The size and shape of the droplets of BCSO in water nanoemulsion were observed with transmission electron microscopy (TEM) using JTEM-1010 microscope (JEOL, Tokyo, Japan) by the application of negative staining technique. Briefly, onto a copper grid coating that was coated with carbon, one drop of the nanoemulsion was applied. Filter paper was then used to carefully remove the extra liquid droplets. One drop of uranyl acetate solution (2% w/v) was then put onto the grids after waiting 5 minutes. After that, the sample was air dried at room temperature, and an 80 kV examination was performed.

2.4. Determination of COX-2 protein using ELISA kits

All groups' weighted liver tissue sections were gathered, homogenized, and centrifuged for 15 minutes at 5000 rpm after being washed in 0.01M PBS. Following the removal of the supernatants, COX-2 concentrations were quantified using ELISA kits (Cat. No. MBS269104) [28] from *My BioSource company*, USA, Science and Technology Centre, Egypt.

2.5. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed as previously described [7]. Triazole reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract total RNA from liver tissues. cDNA was synthesized using Revert Aid First Strand cDNA Synthesis kit (Cat. No. #K1622) from *Thermofisher Scientific Company, USA, Analysis for Life, Egypt*. RT-PCR reactions were performed with HERA plus qPCR SYBR® Green master mix kit (Cat No. WF10306001) from *Willow fort company, UK, Sigma Scientific Service, Egypt* was used for quantitative PCR reaction. As an internal control, the mRNA levels of β-actin were utilized to normalize the expression of mRNAs. COX-2 primer sequence was sense, 5'- ACA CAC TCT ATC ACT GGC ACC-3' and antisense, 5'- TTC AGG GAG AAG CGT TTG C-3'[29]. Then $2^{-\Delta\Delta Ct}$ method was used to analyze the relative quantification of mRNA values [30]

2.6. Hematoxylin-Eosin Staining

Mice's liver tissues were preserved in 10% formalin, and the preserved samples were paraffin-blocked, sectioned (5 µm), and stained with Hematoxylin-eosin (H&E) for histological examination in accordance with standard procedures [31]. The sections in this study were blindly observed.

2.7. In-silico molecular screening study

As a model for targeting 5-Lipooxygenase (5-LO), the crystal structure with PDB code of 6N2W was used to perform molecular docking. Also, the Co-Crystallized Ligand (CCL) of 6N2W (namely Nordihydroguaiaretic Acid, shortly NDGA) which code is 30Z was considered the reference for inhibitory activity essential interactions. Next, docking of TQ on target protein was processed by AutoDock Vina [32]. Docking parameters of Vina were set to 32 and 9 for exhaustiveness and number of modes respectively while all remaining parameters were set to default values. At last, binding poses and affinities were evaluated and reported.

2.8. Statistical Analysis

The One-way ANOVA test was utilized to evaluate several independent groups using GraphPad Prism 7 software. The statistical results were given as the mean ± SEM with plotting figures. P-value < 0.05 was regarded statistically significant.

3. Results and Discussion

3.1. Nanoemulsion formulation

3.1.1. Droplet size, PDI and Zeta potential

The mean droplet size obtained by the formulation is 60.44 ± 4.05 nm, while the Polydispersity Index (PDI) was 0.24 ± 0.03. The zeta potential (ZP) value was – 32.0 ± 2.17. From the obtained results, the droplet size obtained was small and a moderately narrow droplet size distribution was obtained.

3.1.2. Morphological analysis

The Transmission electron microscopy (TEM) micrograph of the BCSO nanoemulsion droplet is shown in **Figure 1** The negatively stained droplets were spherical in shape, relatively homogenous. The droplet size was well correlated with the results from particle size analysis using DLS technique and there was no signs of aggregation or coalescence.

3.2. BCSO GC-MS analysis

Table 1 and **suppl. Data 1** displays the sequence of elution of the 11 primary active components based on percentage weights, which were identified and quantified in 1 mL of BCSO pure oil used in this experiment. The main components with highest peak area were P-Cymene (35.65%) and Thymoquinone (TQ) (18.63%).

The primary compounds identified in BCSO did not differ considerably from the spectra data with constituents and percents for the oil extracted or commercially used [33-35].

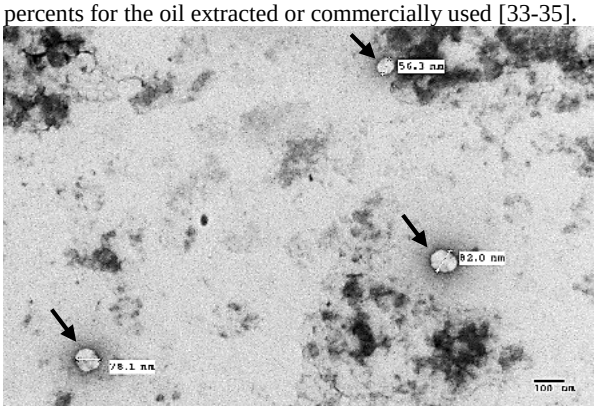

Figure 1: TEM micrograph of BCSO nanoemulsion formulation. Black arrows showed the average particle size of BCSO nanoemulsion.

Table 1: GC-MS analysis of BCSO

Peak	Retention time (RT) (Min.)	Name of the constituent	Formula	Area Sum %
1	10.34	Bicyclo- [3.1.0] hex-2-ene, 2-methyl-5-(1-methylethyl)-	$C_{10}H_{16}$	14.66
2	10.54	alpha-Pinene	$C_{10}H_{16}$	4.28
3	11.44	Bicyclo- [3.1.0] hexane, 4-methylene-1-(1-methylethyl)-	$C_{10}H_{16}$	0.1
4	11.416	beta-Pinene	$C_{10}H_{16}$	1.86
5	12.655	D-Limonene	$C_{10}H_{16}$	2.46
6	12.781	p-Cymene	$C_{10}H_{14}$	35.65
7	15.525	cis-4-methoxy thujane	$C_{11}H_{20}O$	5.89
8	18.487	Thymoquinone	$C_{10}H_{12}O_2$	18.63
9	20.141	Phenol, 2-methyl-5-(1-methylethyl)-	$C_{10}H_{14}O$	0.52
10	21.23	Longifolene	$C_{15}H_{24}$	1.7
11	25.598	p-Cymene-2,5-diol	$C_{10}H_{14}O_2$	14.25

3.3. Induction of liver injury using LPS-induced mice model

LPS has been extensively utilized to study the therapeutic benefits of several drugs on acute liver damage as well as the pathologic mechanisms behind it [36-39]. LPS is a component of gram negative bacteria, triggers intracellular signalling cascades by activating Toll-like receptor 4 (TLR4), which in turn stimulates nuclear factor-κB (NFκB) and mitogen-activated protein kinase (MAPK) pathways [40], these signalling pathways enhance production of cytokines, chemokines, and the production of a large class of inflammatory mediators [41].

Arachidonic acid is released from membrane phospholipids during the inflammatory process due to the activation of phospholipase A2 (PLA2), AA can then be metabolized by cyclooxygenases (COX) (COX-1 and COX-2) and lipoxygenases to form products including prostaglandins (PGs) and LTs respectively [12].

COX-2 is a potent enzyme involved in the initiation and progression of liver fibrosis [42], it has the ability to boost prostaglandin synthesis and start an inflammatory response [42]. Inflammatory marker COX-2 was measured for liver injury assessment in LPS-treated and control groups on protein and gene levels. LPS-induced group showed significant increase in hepatic COX-2 proteins (21.67 pg/g ±1.43, *P <0.0001*) compared to control healthy group (1.633 pg/g ±0.1022) as shown at **Figure 2A**. Furthermore, LPS-induced group showed significant over-expression of COX-2 gene (41.29-fold ±12.62, *P< 0.0001*) when compared to healthy control group as shown at **Figure 2B**. Gene expression analysis was presented as fold–change normalized to the control group.

3.4. Effect of BCSO, its nanoemulsion formulation and Indomethacin on LPS-induced liver injury

Inflammatory marker COX-2 was measured for liver injury assessment in all LPS treated groups on protein and gene levels. Pre-treatment with BCSO in LPS-induced group showed significant down-regulation of hepatic COX-2 (11.98pg/g ±0.7181, *P <0.0001*) protein levels compared to LPS-induced group (21.67pg/g ±1.43), Indomethacin pre-treatment dose (5mg/kg) was able to significantly decrease the hepatic COX-2 protein (5.48pg/g ±0.5903, *P< 0.0001*). While BCSO nanoemulsion group showed preferential significant down-regulation than BCSO group for hepatic COX-2 (2.883pg/g ±0.1579, *P<0.0001*) protein levels. Moreover, when compared to Indomethacin-group, BCSO/Indomethacin therapy resulted in a synergistic reduction of LPS-induced hepatic COX-2 (3.433±0.2186, *P<0.0001*) proteins compared to Indomethacin and LPS-induced groups as shown at **Figure 2C**.

We examined the gene expression of COX-2 genes which were upregulated in LPS-induced group.

Results showed that BCSO was able significantly to down-regulate COX-2 (9.226-fold ±2.428, *P=0.0009*) compared to LPS group (41.29-fold ±12.62). Indomethacin pre-treatment dose (5mg/kg) was able to down-regulate COX-2 gene expression (19.86-fold ±4.719, *P=0.0207*), while BCSO nanoemulsion showed better down-regulation of COX-2 gene (7.146-fold ±0.2646, *P=0.0003*) compared to LPS and BCSO groups in away supporting the preferential results on inflammatory markers. BCSO/Indomethacin therapy resulted in a significant down-regulation of COX-2 gene (1.649± 0.4614, *P*

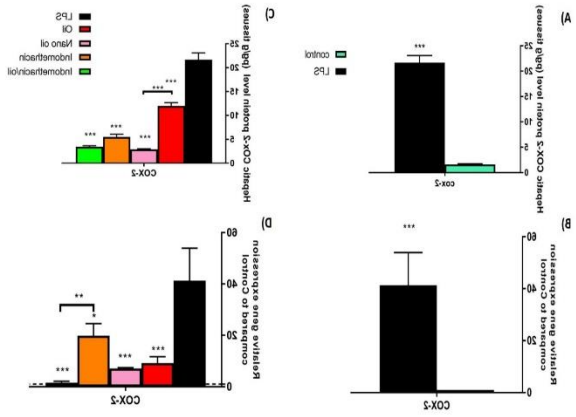

Figure 2*:* Evaluation of key inflammatory marker COX-2 after induction of liver injury using LPS-induced mice model compared to healthy control treated with tween 20 (1ml/kg). A) COX-2 hepatic protein level in LPS animal group compared to control group, B) COX-2 gene level in LPS animal group compared to control group, C) COX-2 hepatic protein level in LPS animal group compared to all LPS treated groups and D) COX-2 gene level in LPS animal group compared to all LPS treated groups. The quantification of target mRNA after LPS treatment was relative to control group and normalized to the internal reference gene β-actin. The $2^{-\Delta\Delta ct}$ technique was used to quantify relative gene expression, which was then presented as the mean of three separate experiments. Data are demonstrated as means ± SEM (n = 6). The values are considered statically significant compared to LPS group at P*<0.05, P** <0.01, P*** < 0.001<*0.0001*) when compared to Indomethacin and LPS-induced groups as shown at **Figure 2D**. Pre-treatment with BCSO has been shown to attenuate the effect of LPS-induced inflammation through downregulation of hepatic COX-2 on protein and gene level. BCSO nanoemulsion formulation was used to enhance oil kinetic stability, solubilization capacity and drug delivery [43-47]. Consequently, nanoemulsion formulation findings showed preferential down-regulation of hepatic COX-2 protein and gene level compared to BCSO or Indomethacin groups.

Indomethacin is utilized in different inflammatory diseases [48, 49]. However, Indomethacin has numerous side effects on cardiovascular [50], renal [50] and GIT systems [51]. Literature showed that prolonged use of Indomethacin cause liver toxicity [52]. So, we aim to compare anti-inflammatory effect of BCSO with that of Indomethacin to limit the use of Indomethacin and counteract its side effects. Our results showed that BCSO and its nanoemulsion formulation have preferential inhibitory action on hepatic COX-2 protein and gene levels than Indomethacin, which proved that BCSO and its nanoemulsion formulation have more powerful anti-inflammatory properties with high safety margin than Indomethacin. Furthermore, combined therapy of Indomethacin/BCSO was also able to enhance COX-2 inhibitory action of Indomethacin alone suggesting that BCSO is promising adjuvant therapy with anti-inflammatory chemical drugs.

3.5. Histopathological examination of BCSO, its nanoemulsion formulation and Indomethacin on LPS-induced liver injury tissues compared to LPS and control mice groups

Normal parenchymal hepatocyte and central vein (CV) histological architecture were shown by histopathology analysis of control, healthy liver tissues. These findings matched those previously reported in the literature [53] as shown at **Figure 3A**. However, LPS-mice model's liver had pronounced hepatocellular vacuolar degeneration. Portal area in liver showed infiltrated leukocytic inflammatory cells with early thrombus formation in portal vein as shown at **Figure 3B**. The liver of BCSO pre-treatment mice's groups showed that liver tissue injury was greatly improved from cell infiltration caused by inflammation as well as mild increase in bi-nucleated cells and activated Kupffer cells as shown at **Figure 3C**. The liver of mice group treated with nanoemulsion formulation of BCSO showed good restoration of the hepatic parenchyma as shown at **Figure 3D**.

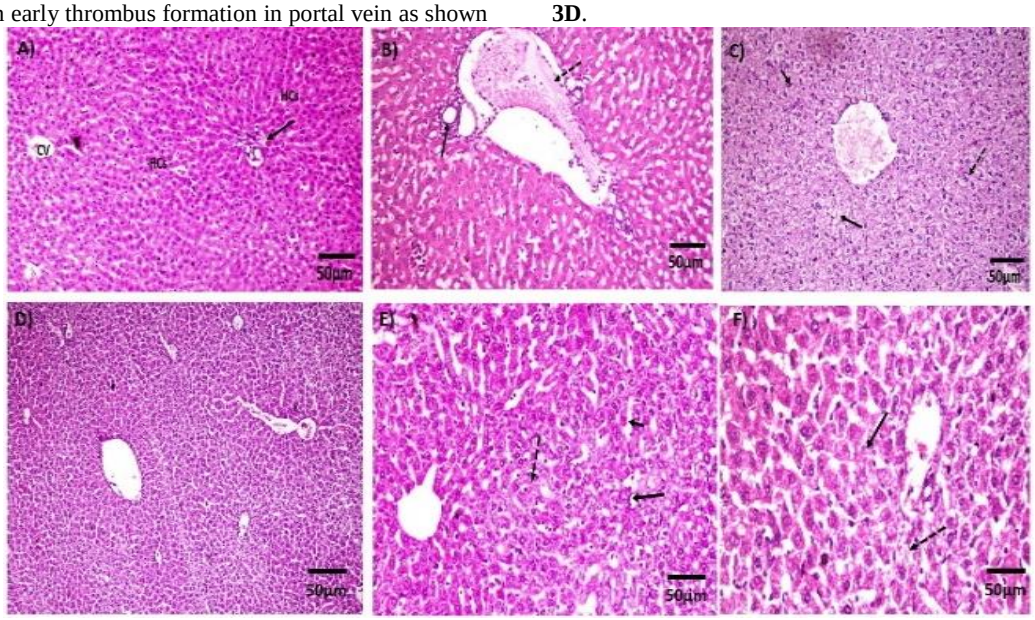

Figure 3: Histopathological examination of liver tissues of control and LPS treated mice-groups; A) Liver of control mice treated with tween, B) Liver of LPS-induced mice model, C) Liver of BCSO pretreatment mice model, D) Liver of nanoemulsion formulation of BCSO pretreatment mice model, E) liver of Indomethacin pretreatment mice model F) liver of BCSO and Indomethacin combination pretreatment mice model.

Moreover, livers of Indomethacin treated mice showed mild hepatocellular vacuolation and scattered necrotic cells with few dilated sinusoids as shown at **Figure 3E**. Liver of mice group treated with BCSO/Indomethacin showed few scattered degenerate and necrotic cells as shown at **Figure 3F**. The presented results showed that LPS induction for 6 hours cause overexpression of pro-inflammatory cytokine COX-2 on protein and gene level in hepatic tissues which agrees with our histological examination results that showed hepatic infiltrated leukocytic inflammatory cells with early thrombus formation in portal vein of liver. These results indicated the success of acute liver injury induction.

Our histological examination results showed that liver cell infiltration caused by inflammation was improved in BCSO pretreated LPS-induced models while BCSO nanoemulsion formulation showed preferential curative effects and good restoration of the hepatic parenchyma. Moreover, livers of Indomethacin treated mice showed mild hepatocellular vacuolation and scattered necrotic cells, while liver of mice group treated with BCSO/Indomethacin showed fewer scattered degenerate and necrotic cells that demonstrates more anti-inflammatory action of BCSO/Indomethacin combination than Indomethacin alone.

3.6. In-silico molecular screening study for potential 5-LO inhibition

Validation of molecular docking was tested using super imposition and Root-Mean-Square Deviation (RMSD) of original CCL over target CCL of 5-LO as shown in **Figure 1 suppl**. Calculated RMSD score of docked CCL over original CCL for 5-LO (PDB: 6N2W) was found to be 1.4Å which is in the acceptable range (<2Å). 5-LO inhibition is illustrated at **Figure 4**

Calculated affinity values for docked CCL and Thymoquinone to 5-LO are relatively close. Docked CCL shows an affinity to 5-LO of -5.75kcal/mol while Thymoquinone shows an affinity value of -5.24kcal/mol. Also, both CCL and Thymoquinone shows successful hydrophilic binding to an activity-essential residue Arg596 with two bidirectional hydrophilic bonds ranging from 2.1 to 2.3Å. However, Thymoquinone lack the hydrophilic interaction with His372 which is not essential for inhibitory activity yet favourable for sustained duration of action on 5-LO.

Lipoxygenases; a class of oxidative enzymes containing a non-heme iron atom in their active site, control inflammatory reactions by producing pro-inflammatory mediators called LTs [54]. 5-LO serves as a rate-limiting enzyme responsible for the biosynthesis of LTs [11]. LTs are the primary mediators of inflammation and are ultimately responsible for a number of human disorders, including diabetes[55], Alzheimer's disease [56], atherosclerosis [57], asthma [58] and cancer [59] . Previous research proved that 5-LO overexpression exacerbates memory loss in a rat model of Alzheimer's disease [60] ,while 5-LO deficient was reported to enhance cognitive recovery [60], this fact provides concrete proof of the critical function 5-LO in the inflammation.

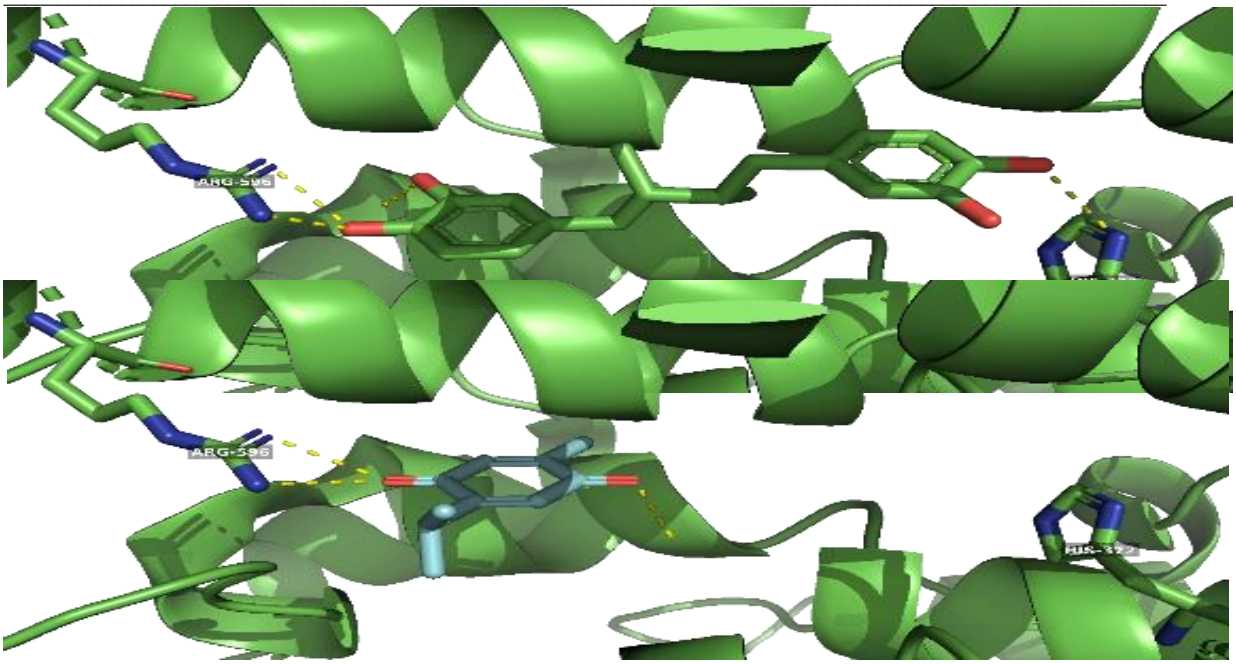

Figure 4: Intramolecular interactions between 5-LO Co-Crystallized Ligand (30Z) and 5-LO "Top" Compared to Interactions of TQ "Bottom" and 5-LO. Visualized by PYMOL v2. 5.0 (open-source)

Table 2: Biological roles of BCSO in several studies

Biological role	Mechanism of action	Reference
Anti-inflammatory in collagen-induced arthritis	Oral administration of TQ (5 mg/kg) daily in rats for 21 days suppressed the increase in interleukin IL-1β, IL-6, TNF-α, PGE2 and COX-2 protein levels.	[64]
Anti-inflammatory in blood cells model	TQ at 3, 10, 30 and 100mM doses is inhibiting by dose-dependently the synthesis of leukotrienes on 5-lipoxygenase and leukotriene C4 (LTC4) synthase activities in human blood cells.	[65]
Anti-inflammatory in airway inflammation	TQ in dose of 3 mg/kg relieve the inflammatory response in BALB/c mice by suppressing the expression of COX-2 protein and the generation of Prostaglandin D2 (PGD2) in the lung.	[66]
Anti-inflammatory in mouse microglia cell line model	TQ at 10 μM concentration treatment targets the antioxidant route in LPS-triggered BV2 mouse microglia cell line inflammation that activates Nrf2 (NF-E2-related factor 2) /ARE (antioxidant responsive element) signalling to suppress NF-κB dependent neuroinflammation in BV2 microglia.	[67]
Anti-inflammatory in carrageenan-induced model	TQ was administered orally 20 mg/kg, and it exhibits strong anti-inflammatory properties through AMP-activated protein kinase (AMPK) and sirtuin1 activation.	[68], [69]
Anti-inflammatory in hepatitis model	Oral administration of TQ (25 mg/kg) in LPS/D-GalN-induced hepatitis rats caused significant reduction in ALT and AST.	
Anti-inflammatory in pristane-induced arthritis	TQ in dose of (2 mg/ kg) in rats caused significant increase in body-weight, decrease in clinical score of inflammation and leukocyte count (TLC), and normalization of differential leukocyte count (DLC).	[70]

Also in our study we focused on BCSO effect on 5-LO activity as it is an crucial inflammatory agent engaged in the regulation of inflammatory responses in acute liver injury [61, 62]. TQ is the main therapeutic ingredient extracted from BCSO with wide range of medical applications [17]. It was evaluated in silico with docking with 5-LO as the enzymatic activity of 5-LO vanishes by mutating Arg596 [63], we can conclude that inhibition, silencing, or binding to Arg596 will render the enzyme inactive. Hence, the interaction between TQ and 5-LO may result in inhibition of the enzyme as TQ (despite its small size) has a descent calculated affinity compared to the co-crystallized ligand of 5-LO. In addition, TQ manages to satisfy the essential interaction for enzymatic inhibition by binding to Arg596 [63]

Although its minimal pocket occupation. Focusing on AA pathways will offer efficient anti-inflammatory therapy and preserving regular physiological processes. Thus, it makes sense to simultaneously inhibit COX-2 and 5-LO in order to develop possible anti-inflammatory medications with fewer adverse effects [11].

4. Conclusion

Acute liver damage is a hazardous clinical syndrome known for its high morbidity and fatality rate. It caused by various factors including toxins, drugs, and viruses. Therefore, the purpose of this study was to assess the therapeutic effect of BCSO pre-treatment on inflammation and acute liver damage in mice treated with lipopolysaccharide (LPS) as well as the synergistic impact of BCSO with reduced dosage of Indomethacin. Current investigation showed that BCSO has a potent anti-inflammatory impact on acute liver injury, and it is a promising molecule as dual COX-2/5-LO inhibitor. The nanoemulsion formulation showed enhanced anti-inflammatory activity over either BCSO or Indomethacin. Additionally, the combination therapy of BCSO/Indomethacin showed improved activity compared to Indomethacin alone. According to the current findings, BCSO can be used as a natural anti-inflammatory medication and as adjuvant therapy with Indomethacin to protect the liver against acute liver injury. BCSO's nanoemulsion formulation has demonstrated a significant improvement in therapeutic impact. Our results are coincided with other previous studies that were summarized in **Table 2**.

5. Declaration

The authors state that their work has not been published elsewhere.

6. Authors' contributions

HKA and HH designed the study and supervised the project. HH and RH performed the experiments and analysed the data. HH improved the protocol. HH and RH wrote the draft. HKA, HH and RH also worked on the data analysis and final manuscript preparation. The authors reviewed and approved the published version of the manuscript

7. Ethics approval

The Helwan University Faculty of Pharmacy's Scientific Research Ethics Committee accepted all methods, which complied with the Declaration of Helsinki (Approval number 01A2020). The study was carried out in compliance with the globally recognized guidelines for the use and care of laboratory animals, as outlined in Animal Research: Reporting of *In-vivo* Experiments.

8. Consent for publication

Not applicable

9. Availability of data and materials

The article contains the datasets used to support the results.

10. Funding

No funding was received.

11. Author details

[1]El Sisi Comprehensive clinic, General Authority for Health Insurance-Giza, 12556, Giza, Egypt.

[2]Biochemistry and Molecular Biology Department, Faculty of Pharmacy, Helwan University 11795, Cairo, Egypt.

[3]Center of Scientific Excellence "Helwan Structural Biology Research, (HSBR)", Helwan University, 11795, Cairo, Egypt.

[4]Biochemistry Department, Faculty of Pharmacy, Galala University

12. Competing interests

The authors claim to have no conflicting interests.

13. Acknowledgements

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14. References

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15. List of abbreviations

Cyclooxygenase-2	COX-2
5-lipoxygenase	5-LO
Black cumin seed oil	BCSO
Thymoquinone	TQ
Lipopolysaccharide	LPS
Arachidonic acid	AA
Leukotrienes	LTs
5-hydroperoxyeicosatetratenoic acid	5-HPETE
Non-steroidal anti-inflammatory drugs	NSAIDs
Oil in water	O/W
Polydispersity index	PDI
Zeta potential	ZP
Transmission electron microscopy	TEM
Hematoxylin-eosin	H&E
Co-Crystallized Ligand	CCL
Nordihydroguaiaretic Acid	NDGA
Standard error of mean	SEM
Central vein	CV
Root-Mean-Square Deviation	RMSD
Toll-like receptor 4	TLR4
Nuclear factor-κB	NFκB
Mitogen-activated protein kinase	MAPK
Phospholipase A2	PLA2
Prostaglandins	PGs
AMP-activated protein kinase	AMPK
NF-E2-related factor 2	Nrf2
antioxidant responsive element	ARE
Leukocyte count	TLC
differential leukocyte count	DLC
Tumor necrosis factor alpha	TNFα
Interlukin	IL
Prostaglandin D2	PGD2