Esculetin Mitigates Ethanol and Lipopolysaccharide-Induced Hepatotoxicity in Male Wistar Rats: Anti-Apoptotic and Anti-Inflammatory Mechanisms

ABSTRACT

Ethanol-induced liver disease presents a significant global health concern. This study aimed to explore the protective effects of esculetin against ethanol and ethanol combined with lipopolysaccharide (LPS) induced hepatotoxicity in rats, focusing on anti-apoptotic and anti-inflammatory mechanisms. In this experimental investigation, thirty adult male Wistar rats were randomly divided into five groups (6 rats/group). The rats were exposed to ethanol (5 mL/kg b.w) for 10 days, followed by a single dose of LPS (5 mL/kg b.w) i.p on the 11th day. Esculetin was orally administered to rats for the same 11-day period at a dose of 50 mg/kg b.w, with a one-hour interval prior to inducer administration. Evaluation of hepatotoxicity at the experiment's conclusion involved biochemical, histopathological, immunohistochemical, and mRNA expression analyses. Biochemical analysis revealed significant elevations in bilirubin, creatinine, and phase II detoxification enzymes, alongside reductions in phase I detoxification enzymes in the ethanol and ethanol+LPS group. Furthermore, ethanol and ethanol+LPS administration upregulated the expression of Bax, caspase 3, TNF-alpha, NF-κB, and COX2, while downregulating the expression of Bcl2. However, treatment with esculetin mitigated these biochemical and molecular alterations, bringing them closer to normal levels compared to the ethanol and ethanol+LPS-induced groups. This study suggests that esculetin holds promise as a therapeutic agent against ethanol-induced liver injury, potentially through its anti-apoptotic and anti-inflammatory properties. Further exploration of this model may yield valuable insights into the mechanisms underlying alcoholic liver disease in humans.

Keywords: Apoptosis, detoxification, ethanol, esculetin, inflammatory response, lipopolysaccharide.

1.INTRODUCTION

Alcohol abuse and alcoholism pose significant contemporary challenges to global health and socioeconomic well-being. Despite notable advancements in research, finding effective medications for treating alcoholism remains a daunting task. Prolonged and heavy alcohol consumption commonly leads to Alcoholic Liver Disease (ALD), characterized by serious liver conditions such as fatty liver, hepatitis, and hepatic cirrhosis (Stickel et al. 2016). Ethanol, a fat-soluble non-electrolyte, is rapidly absorbed from the gastrointestinal tract, distributed uniformly throughout the body, and metabolized primarily through oxidative processes (Sathiavelu et al. 2009; Mueller et al. 2023)

Ethanol metabolism can induce oxidative stress in the liver, leading to lipid peroxidation and exacerbating alcoholic liver disease (Belia et al. 2014; Galicia-Moreno et al. 2014).

The liver plays a crucial role in detoxifying and metabolizing various exogenous and endogenous compounds, making them more water-soluble and less potent. These processes are performed by enzymes in hepatocytes, divided into Phase I and Phase II groups. Phase I enzymes, mainly from the P450 family, add polar groups to lipophilic molecules, increasing hydrophilicity. Phase II enzymes further modify these molecules by attaching water-soluble groups, like glucuronic acid or glutathione, reducing their reactivity.Phase II is impaired, excess reactive Phase I metabolites can accumulate, potentially causing hepatotoxicity (Grant, 1991).

Endotoxin (lipopolysaccharide; LPS), a component of gram-negative bacteria, is detoxified primarily by the reticuloendothelial system, especially Kupffer cells in the liver (Maldonado et al. 2016).Ethanol consumption elevates circulating LPS levels, exacerbating inflammatory responses and liver damage (Sandahl et al. 2014; Massey et al. 2015). Esculetin, a potent antioxidant, demonstrates anti-inflammatory effects and reduces oxidative stress, offering potential therapeutic benefits (Tien et al. 2009; Cai et al. 2023). Ethanol-induced toxicity affects various organs, including the liver and kidneys, as evidenced by disruptions in bilirubin metabolism and creatinine clearance (Conde de la Rosa et al. 2022; Epstein et al. 1997; Terg et al. 2019).

Ethanol-induced toxicity triggers complex cellular responses involving apoptosis and inflammatory cytokine pathways. Ethanol metabolism generates reactive oxygen species (ROS) and acetaldehyde, damaging cellular components (Le Dare et al. 2009).Ethanol disrupts mitochondrial function, activates caspases, and dysregulates apoptotic protein balance, promoting cell death (Karadayian et al.2024). Simultaneously, ethanol activates inflammatory cytokines like TNF-α and IL-1β through NF-κB activation, exacerbating tissue damage (Kawaratani et al. 2013) .Increased gut permeability due to ethanol allows bacterial endotoxins like LPS to enter circulation, triggering inflammation via TLR4 signaling (Yang et al. 2020).

Overall, ethanol-induced toxicity involves a cascade of events, including apoptosis and inflammation, leading to cellular damage and organ dysfunction, particularly in the liver. Understanding these pathways is crucial for developing therapeutic interventions to mitigate ethanol-induced harm.

2. MATERIALS AND METHODS

2.1. Experimental animals

Thirty healthy male Wistar rats weighing between 150 and 180 grams were sourced from Biogen Bangalore. These rats were accommodated in the Central Animal House at RMMC (Rajah Muthiah Medical College), Annamalai University. Before commencing the experiment, we ensured adherence to guidelines by obtaining approval from the Institutional Animal Ethics Committee for the Supervision and Administration of Experimental Animals (IAEC proposal No. AU—IAEC/1258/11/19). The rats were housed in specialized cages under a constant 12-hour light/12-hour dark cycle, with air conditioning maintaining a controlled temperature of 20–22°C and humidity at 60%. They were provided with standard rat pellet chow and had unrestricted access to tap water.

2.2. Experimental design

Thirtymale Wistar rats were sorted into five groups, with each group comprising six rats. Group 1 served as the control and received a normal pellet diet. Groups 2 were subjected to ethanol for 10 days at a dosage of 5 mL/kg body weight. Group 3 underwent the same ethanol treatment for 10 days (5 mL/kg b.w) and received a single dose of LPS (5 mL/kg b.w) orally on the 11th day. Group 4 received ethanol for 10 days (5 mL/kg b.w), a single dose of LPS (5 mL/kg b.w) intraperitoneal (i.p) on the 11th day, and was administered

esculetin orally for the same duration at a dose of 50 mg/kg b.w, with a one-hour interval before inducer administration. Group 5 received esculetin orally for 11 days at a dose of 50 mg/kg b.w. Esculetin was dissolved in warm water (60º C), cooled, and then administered orally to the rats.

Upon completion of the treatment protocol, the rats were anesthetized using an overdose of ketamine hydrochloride, and blood was collected via cardiac puncture for serum biochemical analysis following centrifugation. Subsequently, the livers were collected and washed with chilled saline solution. Liver samples from each group were preserved in 10% formalin for histopathological and immunohistochemical examinations.

2.3. Biochemical analysis

2.3.1. Estimation of serum bilirubin

The concentration of bilirubin in serum was assessed by combining absolute methanol (2.5 mL), 1.5% hydrochloric acid (0.5 mL), and a diazo reagent (0.5 mL) with serum (0.2 mL). After thorough mixing, the solution was left at room temperature for 30 minutes, and the absorbance was then measured at 540 nm (Malloy and Evelyn,1937).

2.3.2. Estimation of serum creatinine

Serum creatinine levels were assessed by combining 2 mL of alkaline picrate with 3 mL of deproteinized supernatant, obtained by centrifuging 0.2 mL of serum with 4.3 mL of 10% tricarboxylic acid cycle solution. The resulting mixture was incubated at 25°C for 30 minutes, followed by measuring the absorbance at 520 nm (Bonsnes et al.1945).

2.3.3. Assay of phase I enzymes

The determination of Cytochrome P450 and cytochrome b5 was conducted according to Omura and Sato's method in 1964. Cytochrome P450 was identified utilizing carbon monoxide (CO) difference spectra, with the absorbance of CO adducts formed by the reaction of reduced cytochrome P450 with CO being measured at 450 nm. The rapid reduction of cytochrome b5 was characterized by an increase in absorbance at 427 nm. The absorption coefficients used for determining Cytochrome P450 and b5 were 91 and 185 cm2 /M/m, respectively.

NADH-cytochrome P450 reductase (EC 1.6.2.2) activity was assessed following Mihara and Sato's (1972) method,with absorbance also measured at 450 nm. Enzyme activity

was calculated using an extinction coefficient of 1.02 m/M/cm, defining one unit of enzyme activity as the reduction of one mole of ferric cyanide per minute.

NADPH-cytochrome b5 reductase (EC 1.6.2.4) was assayed following Omura and Takesue's method in 1970,measuring the rate of NADPH oxidation at 340 nm. Enzyme activity was calculated using the extinction coefficient of 6.33 cm^2 /mM/cm, where one unit of enzyme activity corresponds to the oxidation of one mole of NADPH per minute.

2.3.4. Assay of phase II enzymes

UDP-glucuronyl transferase (UDP-GT) (EC 2.4.1.17) activity was assayed in liver tissue by monitoring p-nitrophenol disappearance (Isselbacher et al.1962). The reaction mixture (buffer, Triton X-100, $MgCl_2$, p-nitrophenol, water, enzyme) was incubated at 37°C for 2 minutes, followed by the addition of UDP-glucuronic acid. The reaction was stopped using TCA, centrifuged, and the supernatant was mixed with NaOH. Absorbance was measured at 450 nm, and activity was expressed as nmoles/min/mg protein.

The activity of DT-diaphorase (EC.1.6.9.92) was determined following the protocol outlined by Ernster et al. utilizing NADH as the electron donor and 2,6-dichlorophenol indophenol (DCPIP) as the electron acceptor, with absorbance measured at 600 nm. Reduction of DCPIP was quantified spectrophotometrically at 600 nm, employing an extinction coefficient of 21 m/M/cm(Ernster et al. 1962).

2.4. Mast cell staining

The staining for mast cells was done by the method of Khan et al. (2013). Staining with toluidine blue permits the identification of mast cells because mast cell granules stain metachromatically, resulting in deep purplish-blue granular cytoplasmic staining.

2.5. Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded liver sections following the protocol outlined by Elshopakey and Elazab (2021). After deparaffinization, antigen retrieval was carried out using potassium citrate (pH 6). The sections were then incubated overnight at 4˚C with a polyclonal primary antibody against rats. Following this, the sections were washed with TBS (tris buffer saline) and incubated with HRP (Horse radish peroxidase) conjugated donkey anti-mouse (1:1000) and goat anti-rabbit (1:1000) secondary antibodies corresponding to their primary antibodies for 1 hour at

room temperature. Development was initiated for 1.5 minutes using 3,3'-diaminobenzidine tetrahydrochloride (DAB) followed by counterstaining with hematoxylin and coverslipping. The tissues were then photographed (20X) using a light microscope (Axio Scope A1, Carl Zeiss, Jena, Germany).

2.6. Real-time quantitative polymerase chain reaction

Liver tissue was used to extract total RNA employing the TRIzol® reagent as per the manufacturer's instructions. Briefly, tissue lysate was incubated at room temperature for 5 minutes, followed by collection of the aqueous phase post-centrifugation at 15,000 g for 10 minutes at 4˚C into a new RNase-free tube, and addition of chloroform. Subsequent centrifugation at 15,000 g for 15 minutes at 4˚C led to the collection of the aqueous phase into another RNase-free tube, where isopropanol was added. The RNA pellet obtained was washed with 75% ethanol and air dried. DNase treatment was conducted following the manufacturer's instructions using the DNase I, RNase-Free kit. The cDNA synthesis was carried out using 2 µg of total RNA with the ReverAid First Strand cDNA Synthesis Kit (Thermo) according to the provided protocol. RT-qPCR was performed using Hieff® qPCR SYBR® Green Master mix (Yeasen) and LightCycler® 96 System (Roche). Each sample utilized 1.2 µL of cDNA as a template, with specific forward and reverse primers (0.2 µM each) added to the mix. The final volume of each sample was 20 µL. Cycling conditions comprised an initial denaturation step at 95˚C for 5 minutes, followed by 40 amplification cycles of 95˚C for 10 seconds and annealing/extension at 60˚C for 30 seconds. A melt curve analysis was performed from 65˚C to 97˚C with increments of 0.2˚C every second, following a step at 95˚C for 10 seconds and 65˚C for 60 seconds. All experiments, including negative controls lacking cDNA, were conducted in triplicate. RT-qPCR primer sequences were provided in Table 1. The 2–ΔΔCt method was employed to quantitatively analyze the expression levels of target genes, standardized by the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

The formula used was Ratio = 2−ΔΔCt, where ΔCt = Ct (target) − Ct (GAPDH), and ΔΔCt = ΔCt (experimental group) − ΔCt (control).

2.7. Statistical Analysis

The values are presented as the mean accompanied by the standard error of the mean. Statistical analysis was conducted using the SPSS for assessing statistical significance. Analysis of variance was carried out, followed by Duncan's Multiple Range Test (DMRT) to explore group differences. A significance level of $p \leq 0.05$ was deemed appropriate for establishing statistical significance.

3. RESULT

3.1. Effect of esculetin on serum bilirubin and creatinine levels

Table 2 illustrates the impact of esculetin on serum bilirubin and creatinine levels in both control and experimental rats. Significantly, heightened levels of serum bilirubin and creatinine were noted in rats administered ethanol and ethanol+LPS compared to control rats. However, upon supplementation of esculetin to ethanol+LPS administered rats, there was a significant reduction in serum bilirubin and creatinine levels compared to rats administered ethanol+LPS alone. Rats treated solely with esculetin did not display any statistically significant variance in serum bilirubin and creatinine levels compared to control rats.

3.2. Effect of esculetin on liver detoxification enzymes

Table 3 and 4 illustrates the enzymatic phase I and phase II detoxification activities observed in the liver tissues of both control and experimental groups of rats. Rats treated with ethanol and ethanol+LPS alone displayed notably elevated activity levels of phase I enzymes (including Cytochrome P450, Cytochrome b5, NADPH-cytochrome P450 reductase, and NADH-cytochrome b5 reductase) compared to the control rats. Esculetin supplementation decreased the activities of ethanol+LPS-metabolizing phase I enzymes. Additionally, compared to control rats, those treated with ethanol and ethanol+LPS alone exhibited a significant decrease in the enzyme activities of phase II (UDP-GT and DTD). Conversely, esculetin treatment notably enhanced the activities of phase II enzymes in rats treated with ethanol+LPS.

3.3. Effect of esculetin onmast cellspopulation

Figure 1 shows the histopathological analysis of Toluidine blue staining for mast cells. Ethanol and ethanol+LPS treated rats showed mast cell population accumulation were significantly increased when compared to control rats. The administration of esculetin to

ethanol+LPS treated rats showed mild expression of mast cell population accumulation near to control. However, the administration of esculetin to control rats did not show any significant changes when compared to control rats.

3.4. Effect of esculetin on apoptotic and inflammatory markers by immunohistochemistry

Figures 2 and 3 present the immunohistochemical staining of apoptotic and inflammatory proteins in liver tissue across all experimental groups. In rats treated with ethanol and ethanol+LPS, there was an observed increase in the expression of apoptotic markers Bax and Caspase-3, along with a decrease in Bcl-2 expression. Similarly, inflammatory markers NFKB, TNF-alpha, and COX2 were upregulated in these groups. In contrast, the ethanol+LPS group treated with esculetin showed a significant downregulation of Bax and Caspase-3, along with an increase in Bcl-2 expression. Additionally, the inflammatory markers NFKB, TNF-alpha, and COX2 were downregulated compared to the ethanol+LPS-induced group.

3.5. Effect of esculetin on mRNA expression of apoptotic and inflammatory markers by RT-qPCR

We performed RT-qPCR analysis to examine changes in apoptotic and inflammatory mRNA expression across control and experimental groups. As shown in Figures 4 and 5, rats treated with ethanol and ethanol+LPS displayed increased mRNA expression of Bax and Caspase-3, along with decreased Bcl2 mRNA expression. In terms of inflammatory markers, NFKB, TNF-alpha, and COX2 mRNA levels were upregulated. In contrast, rats supplemented with esculetin in addition to ethanol+LPS treatment demonstrated reduced mRNA expression of Bax and Caspase-3, along with an increase in Bcl2 mRNA expression. Furthermore, the inflammatory markers NFKB, TNF-alpha, and COX2 were downregulated in these groups.

4. DISCUSSION

Ethanol's pervasive influence on bodily organs stems from its capacity to penetrate all tissues, owing to its solubility in both water and fat. Operating through various pathways, ethanol affects the liver and other organs, thereby contributing to the onset of ALD (Rehmet al.2017). This study aimed to explore the impact of esculetin on ethanol+LPS-induced liver

toxicity in rats, with a primary focus on its potential in regulating apoptosis and inflammatory pathways.

Bilirubin, a yellowish pigment formed during the breakdown of red blood cells, undergoes processing in the liver and is expelled in bile (Kumari et al.2023). Damage to the liver induced by ethanol can disrupt the efficient processing of bilirubin, resulting in heightened levels of bilirubin in the bloodstream (Mo et al. 2020) .This elevation in bilirubin levels is commonly observed in conditions like alcoholic hepatitis or cirrhosis (Torkadi et al. 2014). Creatinine, a byproduct of muscle metabolism from the breakdown of creatine, is eliminated from the blood by the kidneys and excreted through urine (Kreider et al. 2022). Damage to the kidneys caused by ethanol can impede their filtration function, causing an increase in blood creatinine levels (Mo et al. 2020). Elevated creatinine levels typically signify decreased kidney function and are frequently associated with conditions such as acute kidney injury or chronic kidney disease (Kellum et al.2021). In our study, heightened levels of serum bilirubin and creatinine were observed in rats induced with ethanol and ethanol+LPS. However, supplementation with esculetin reduced the levels of serum bilirubin and creatinine in ethanol+LPS induced rats. Similarly, caffeic acid, a phenolic compound, decreased serum bilirubin and creatinine levels in rats induced with hyperammonemia (Pari et al. 2007).

Our findings indicate heightened activity of various enzymes including cytochrome P450, cytochrome b5, NADH-cytochrome b5 reductase, NADPH-cytochrome P450 reductase, and cytochrome P4502E1 in rats fed with ethanol and ethanol+LPS. Conversely, levels of UDP-GT and DT-diaphorase were observed to decline in the liver of ethanol and ethanol+LPS-fed rats, potentially attributed to the generation of reactive oxygen species (ROS) and ethanol-derived (hydroxyethyl) free radicals, which could instigate lipid peroxidation. Consistent with our findings, Jayaraman et al.(2021) documented increased activities of cytochrome b5, NADPH cytochrome P450 reductase, and NADH-cytochrome b5 reductase, alongside reduced DT-diaphorase activities in the livers of ethanol-fed rats. Furthermore, Zhao et al. (2021) similarly demonstrated elevated CYP2E1 activity in the liver following chronic ethanol consumption.

Supplementation of esculetin to rats fed ethanol and LPS resulted in decreased activities of various liver enzymes, including cytochrome P450, cytochrome b5, NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase, and cytochrome P4502E1.

This reduction in enzyme activity may stem from esculetin's ability to modulate cytochrome P450-dependent monooxygenases, which play a crucial role in metabolizing many xenobiotics (Esteves et al. 2021). Esculetin's impact on phase I and phase II enzymes likely occurs at different time points, offering significant protection against ethanol and LPS-induced toxicity. This protection could be attributed to esculetin's ability to mitigate the accumulation of free radicals produced during ethanol-induced lipid peroxidation.

UDP-GT plays a crucial role in phase II detoxification by catalyzing the conjugation of glucuronic acid to lipophilic substances, making them more water-soluble. This process enhances the excretion of toxins, drugs, and endogenous compounds like bilirubin. By facilitating glucuronidation, UDP-GT helps in the elimination of potentially harmful substances from the body (Sheehan et al. 2001). DT-diaphorase, also known as quinone reductase (QR), is an enzyme found in the hepatic cytosol that converts NADH to NAD+. Generally, DT-diaphorase is induced alongside other phase II detoxifying enzymes (Anbu et al. 2016). Supplementation with esculetin has been shown to increase the activities of DT-diaphorase and UDP-GT in rats subjected to ethanol and LPS feeding. In line with this, Chung et al.(1994) demonstrated that inducers of DT-diaphorase can boost NAD+ regeneration, thus enhancing ethanol metabolism in vivo and reducing hepatotoxicity.

Oxidation and inflammation are closely intertwined in biological systems, facilitating the progression of numerous diseases (Furnkranz et al.2005).The advancement of ethanol+LPS-induced liver damage involves parenchymal cells, with macrophages playing a role attributable to both the direct effects of alcohol and the indirect effects of its metabolites, oxidative stress, immunologic responses, and inflammatory processes. During the initial stages of inflammation, neutrophils are the first cells to migrate to inflammatory sites, regulated by molecules produced by rapidly responding macrophages and mast cells localized in tissues (Zhao et al.2021).The heightened presence of mast cells in ethanol+LPS-fed rats elucidates the severity of inflammation. However, supplementation with esculetin notably decreases mast cell numbers, demonstrating its anti-inflammatory properties.

The nuclear factor kappa B (NF-κB) serves as a pivotal transcription factor in the amplification of inflammatory cytokines. Its activation is triggered by oxidative stress across various cell types, instigating a detrimental cycle of inflammation (Mani et al. 2016). Upon exposure to various harmful inflammatory stimuli, activated NF-κB migrates to the nucleus, initiating the transcription of target genes, including cytokines, which may contribute to liver

injury. Ethanol is known to initially activate NF-κB through the generation of reactive oxygen species (ROS) and/or the release of proinflammatory cytokines (Liu et al. 2018). Additionally, ethanol promotes the nuclear translocation of NF-κB and accelerates the degradation of the inhibitor of NF-κB in hepatocytes (Songet al. 2018). Our research aligns with these findings, showing an elevated expression of NF-κB in the nucleus.

Conversely, supplementation with esculetin has been observed to mitigate ethanol+LPS-induced ROS production, thereby concurrently inhibiting the translocation of the proinflammatory transcription factor NF-κB into the nucleus while activating the anti-inflammatory transcription factor Nrf2. This results in a significant reduction in ethanol+LPS-induced inflammation. Anand et al. (2013) reported that esculetin downregulates NF-κB expression in benzo [a] pyrene-induced lung carcinogenesis in mice.

Tumor necrosis factor-alpha (TNF-α) plays a pivotal role in the progression of ethanol-induced hepatotoxicity, as highlighted by Rocha et al. (2012) The heightened release of these inflammatory factors, partly triggered by oxidative stress, can disrupt cytokine balance, impair immune function, and even induce liver cell apoptosis. TNF-α, a target gene within the NF-κB signaling pathway, operates as an autocrine/paracrine factor, promoting cytokine production and activating NF-κB signaling. Ethanol exposure significantly boosts TNF-α expression, particularly in necrotic areas and macrophages, as demonstrated by Jairaman et al. (2022). Furthermore, ethanol-induced NF-κB activation facilitates TNF-α transcription, along with other proinflammatory mediators, as discussed by Hayden and Ghosh (2004).

Our study corroborates these findings, revealing elevated TNF-α expression via immunohistochemical staining and RT-qPCR. This upregulation likely stems from inflammation, necrosis, and oxidative stress induced by ethanol and ethanol+LPS in rat models. Conversely, supplementation with esculetin markedly reduces TNF-α expression in the liver. The anti-inflammatory properties of esculetin observed in our study can be attributed to its phenolic nature, which potentially scavenges free radicals (Wang et al. 2022)These findings underscore the significant anti-inflammatory effects of esculetin in ethanol+LPS-induced hepatotoxicity.

However, COX-2 is recognized as one of the target genes regulated by NF-κB, with Kupffer cells primarily responsible for its expression. Increasing evidence underscores the pivotal role of COX-2 activity in various mechanisms of tissue injury through the synthesis of

vasoactive and proinflammatory compounds (Zhang et al. 2013). Moreover, it is established that endotoxin and lipid peroxides, implicated in ethanol-induced liver injury, induce COX-2 expression (Bianchi et al. 2010). In our investigation, the heightened expression of COX-2 observed in the livers of rats exposed to ethanol and ethanol+LPS may stem from diverse stimuli such as growth factors and cytokines, which are typically absent in healthy tissues. Notably, supplementation with esculetin reduced COX-2 expression, potentially exerting an inhibitory effect on other chemokines. These findings suggest that esculetin mitigates inflammation induced by ethanol+LPS. Notably, a derivative of esculetin, 4-Methylesculetin at 50 mg/kg, has been reported to alleviate inflammation by downregulating the expression of TNF-α, IL-1β, IL-6, COX-2, and PGE2, thereby directly inhibiting swelling and cartilage destruction (Hemshekhar et al. 2013).

Cell death in the liver primarily occurs through apoptosis or necrosis. Numerous studies have concentrated on unraveling the molecular mechanisms of apoptosis. The activation of Caspase is regarded as the pivotal event during apoptotic stimuli, responsible for cleaving various proteins. Caspase activation can occur via either the intrinsic mitochondrial pathway or the extrinsic death receptor-mediated pathway (Lossi et al. 2022). Conversely, Caspase activation is also implicated in necrosis. Bcl2 functions as an antioxidant and inhibits mitochondrial Caspase 3 activation. Bcl-2 regulates intracellular calcium levels in the mitochondria and protects the membrane from pro-apoptotic stimuli (Yee et al. 2016).

Liver cells, particularly hepatocytes, are highly susceptible to apoptosis mediated by death receptors. Guicciardi et al. (2016) reported an overall increase in the expression of the proapoptotic protein Bax in hepatitis. Deaciuc et al.(2000) demonstrated in their study that oxidative damage to mitochondria and mitochondrial dysfunction occur during ethanol+LPS-induced apoptosis, leading to the upregulation of apoptotic proteins Bax and Caspase 3, and the downregulation of the antiapoptotic protein Bcl-2. In our present study, we observed a significant increase in Bax and Caspase 3 proteins, and a decrease in Bcl-2 protein, in rats treated with ethanol and ethanol+LPS, indicating the susceptibility of hepatocytes to apoptosis. Apoptosis plays a crucial role in many liver diseases, and therapeutic intervention to modulate liver cell death shows promise. Treatment with esculetin downregulated the expression of Bax and Caspase 3, while upregulating the expression of Bcl-2. Jghef et al. (2023) have suggested that esculetin acts as a potent antioxidant in mitochondria, controlling the free radical security pathway and mitigating oxidative damage and apoptosis. Their study showed that esculetin increased the expression of Bcl-2 and decreased the expression of Bax

in isoproterenol-induced myocardial infarction in rats. Present study revealed that esculetin protects hepatocytes from apoptosis by increasing the expression of Bcl-2, thereby preventing mitochondrial Caspase activation. This may explain the decreased levels of Caspase 3 observed in the present study.

5. CONCLUSION

In conclusion, the findings of this study elucidate the potential beneficial effects of esculetin supplementation in mitigating liver dysfunction induced by ethanol and ethanol combined with lipopolysaccharide (LPS) in experimental rats. Esculetin administration demonstrated a significant reduction in serum bilirubin and creatinine levels, indicative of improved liver function and renal health. Additionally, esculetin supplementation attenuated the heightened activities of phase I detoxification enzymes induced by ethanol and ethanol+LPS, while concurrently enhancing the activities of phase II detoxification enzymes. Immunohistochemical and RT-qPCR analyses revealed that esculetin treatment effectively modulated the expression of apoptotic and inflammatory markers, restoring the balance between pro-apoptotic and anti-apoptotic proteins and reducing the inflammatory response within liver tissues. These findings underscore the potential therapeutic utility of esculetin in ameliorating liver dysfunction and inflammation associated with ethanol and LPS exposure, suggesting its promise as a candidate for further exploration in clinical settings.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

This work carried out after getting ethical approval from the Institutional Animal Ethics Committee for the Control and Supervision of Experimental Animals (IAEC proposal No. AU—IAEC/1258/11/19).

DISCLAIMER (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

ABBREVIATIONS

ALD: Alcoholic liver disease; **ANOVA:** Analysis of variance;**DAB:** 3,3’-diaminobenzidine tetrahydrochloride; **DTD:** DT-diaphorase; **DMRT:**Duncan’s multiple range test; **FFA:**Free fatty acids; **HRP:** Horse radish peroxidase; **GST:** Glutathione S-transferase; **LPS:** lipopolysaccharide; **NADH:** Nicotinamide-adenine dinucleotide; **NADPH:** Nicotinamide

adenine dinucleotide phosphate; **TBS:** Tris buffer saline; **QR:** Quinone reductase; **RMMC:** Rajah Muthiah Medical College; **ROS:** Reactive oxygen species; **UDP-GT:** UDP-glucuronyl transferase.

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Table 1: Apoptotic and inflammatory Primer

Markers	Forward primer	Reverse primer
NF-κB	ACAAATGGGCTACACCGAAG	ATGGGGCATTTTGTTGAGAG
TNF-α	AGCCCATGTTGTAGCAAACC	GCTGGTTATCTCTCAGCTCCA
COX-2	GAATCATTCACCAGGCAAATTG	TCTGTACTGCGGGTGGAACA
Bax	TGCTGATGGCAACTTCAACT	ATGATGGTTCTGATCAGCTCG
Bcl-2	GGTGGAGGAACTCTTCAGGGA	GGTTCAGGTACTCAGTCATCCA
Caspase-3	GAGCTTGGAACGCGAAGAAA	GCCCATTTCAGGGTAATCCA
GAPDH	GGGAAATCGTGCGTGACATT	GCGGCAGTGGCCATCTC

Table2:Effect esculetinonbilirubin and creatinineinliver tissue ofcontroland experimentalrats at 17th day of experimental perio

Groups	Bilirubin (mg/dL)	Creatinine (mg/dL)
Control	0.47±0.06^{a}	0.83±0.08^{a}
Ethanol (5mg/kg b.w)	0.79±0.05^{b}	1.44±0.11^{b}
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w)	0.83±0.06^{c}	1.53±0.09^{c}
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w) + Esculetin (50mg/kg b.w)	0.53±0.08^{d}	0.89±0.08^{d}
Esculetin (50mg/kg b.w)	0.44±0.07^{a}	0.79±0.06^{a}

Valuesaremeans ±SDof6 rats fromeachgroup. Values notsharingcommonalphabetsassuperscriptaresignificantlydifferentfromeach otheratthelevelofP≤0.05(ANOVAfollowed byDMRT).

Table3: Effect esculetinonphase I detoxification enzymesinliver tissue ofcontroland experimentalrats at 17th day of experimental period

Groups	**Cytochrome P450 [a]**	**Cytochrome b5 [b]**	**NADPH-cytochrome P450 reductase [c]**	**NADH-cytochrome b5 reductase [d]**
Control	7.12±0.91[a]	2.46±0.46[a]	59.61±4.68[a]	17.26±1.85[a]
Ethanol (5mg/kg b.w)	10.54±1.26[b]	4.31±0.41[b]	73.89±5.37[b]	28.46±1.99[b]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w)	11.68±1.31[c]	4.53±0.37[c]	77.68±4.90[c]	31.08±2.13[c]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w) + Esculetin (50mg/kg b.w)	7.98±0.99[d]	2.84±0.34[d]	60.72±4.87[d]	19.78±1.67[d]
Esculetin (50mg/kg b.w)	7.19±1.01[a]	2.43±0.45[a]	59.01±5.02[a]	17.32±1.83[a]

[a] µmol/mg protein

[b] µmoles/mg protein

[c] One unit of enzyme activity is defined as that causing the oxidation of 1 mole of NADPH/min/mg protein.

[d] One unit of enzyme activity is defined as that causing the reduction of 1 mole of ferriccyanide/min/mg/protein.

Valuesaremeans ±SDof6 rats fromeachgroup. Values notsharingcommonalphabetsassuperscriptaresignificantlydifferentfromeach otheratthelevelofP≤0.05(ANOVAfollowed byDMRT).

Table4: Effect esculetinonphase II detoxification enzymesinliver tissue ofcontroland experimentalrats at 17th day of experimental period

Groups	**UDP-GT [a]**	**DT-diaphorase [b]**
Control	4.92±0.31[a]	5.16±0.27[a]
Ethanol (5mg/kg b.w)	0.99±0.37[b]	1.92±0.31[b]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w)	0.85±0.42[c]	1.04±0.29[c]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w) + Esculetin (50mg/kg b.w)	4.75±0.39[d]	4.99±0.37[d]
Esculetin (50mg/kg b.w)	4.96±0.41[a]	5.19±0.28[a]

[a] nmoles/min/mg of protein

[b] µmoles of 2,6-dichlorophenolindophenol reduced/min/mg protein

Valuesaremeans ±SDof6 rats fromeachgroup. Values notsharingcommonalphabetsassuperscriptaresignificantlydifferentfromeach otheratthelevelofP≤0.05(ANOVAfollowed byDMRT).

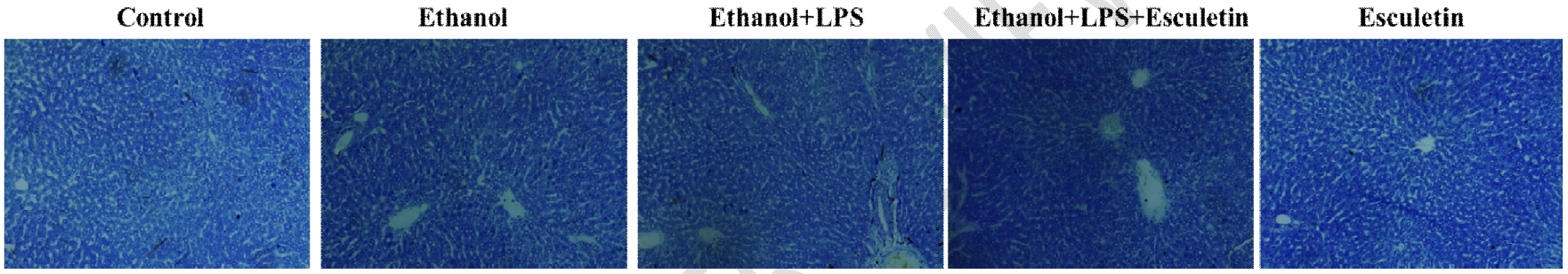

Figure 1:Photomicrographs depicting mast cell granules by toluidine blue staining. Original magnification: 20X.

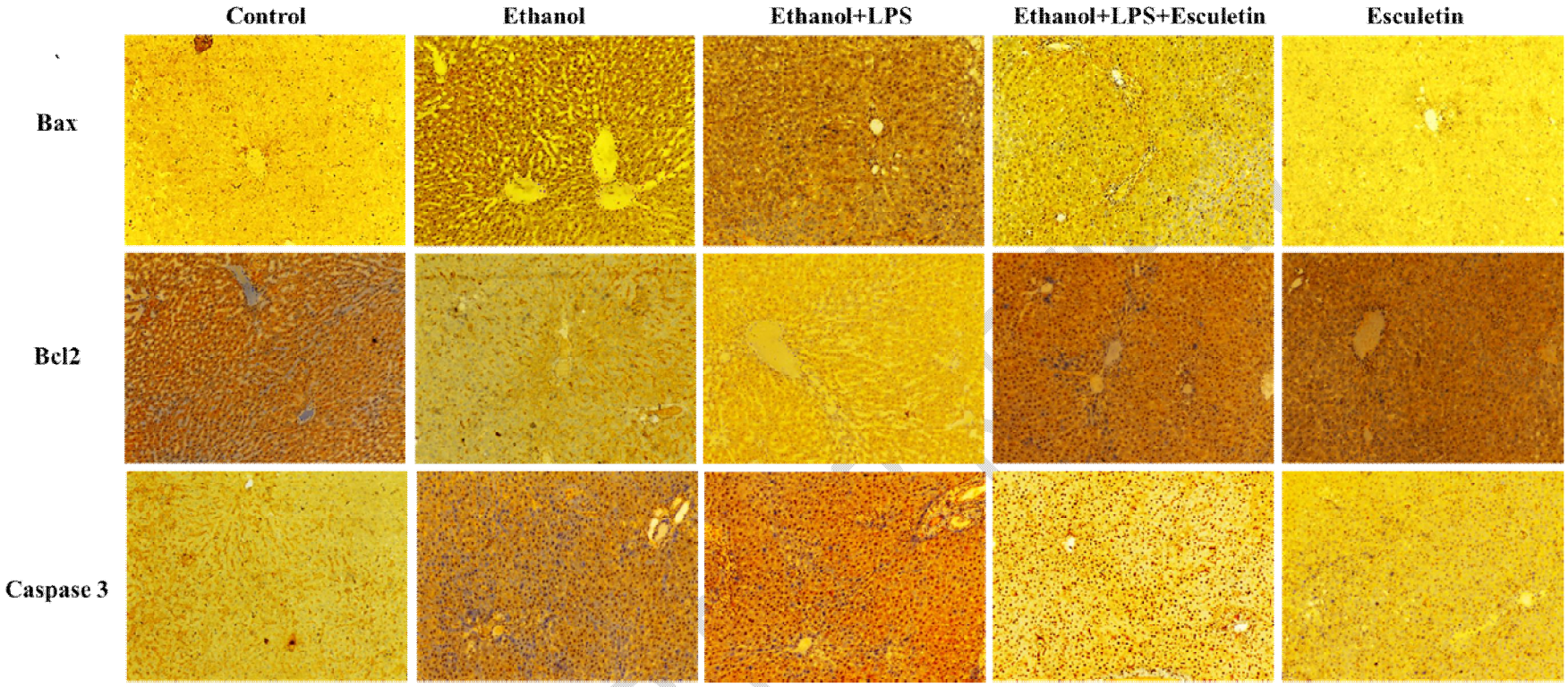

Figure 2: Effect of esculetin on apoptotic markers in the liver tissue ofcontroland experimentalrats. Immunohistochemical staining of Bax, Bcl2 and Caspase 3. Original magnification: 20X.

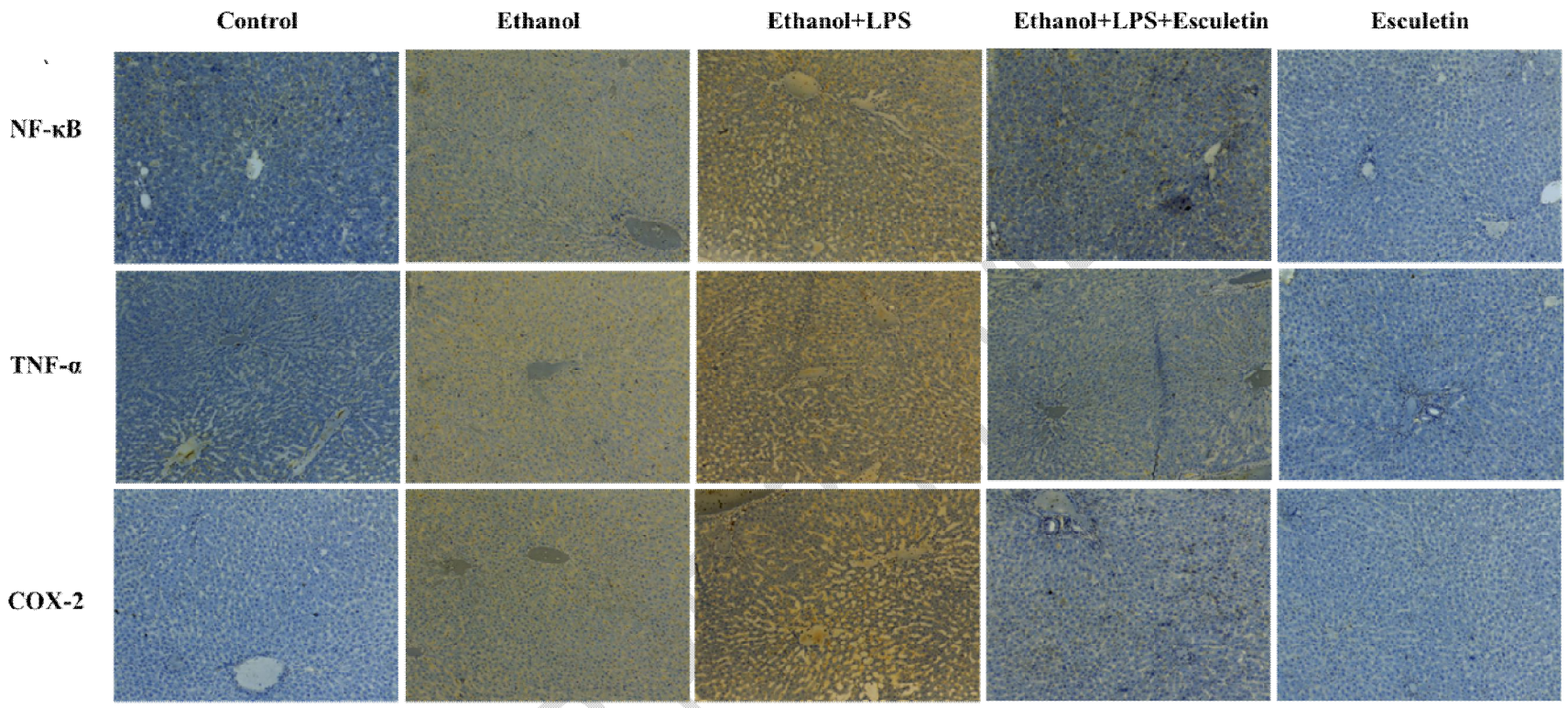

Figure 3: Effect of esculetin on inflammatory markers in the liver tissue ofcontroland experimentalrats. Immunohistochemical staining of NF-kB, TNF-α and COX-2. Original magnification: 20X.

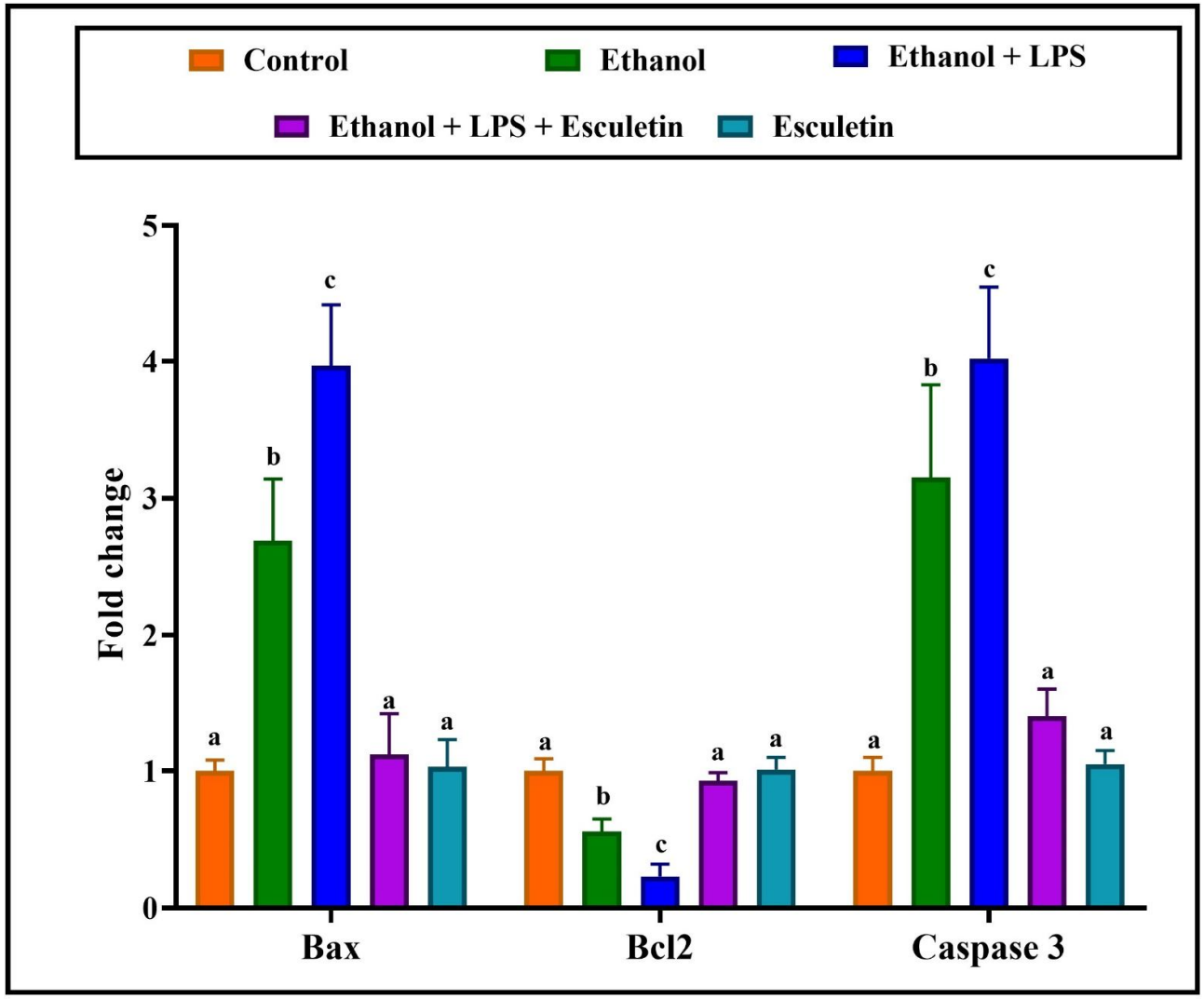

Figure 4: Effect of esculetin on apoptotic markers (Bax, Bcl2, caspase 3) in the liver tissue ofcontroland experimentalrats.Values not

sharing common alphabets as superscript are significantly different from each other at the level of P≤0.05 (ANOVA followed by DMRT).

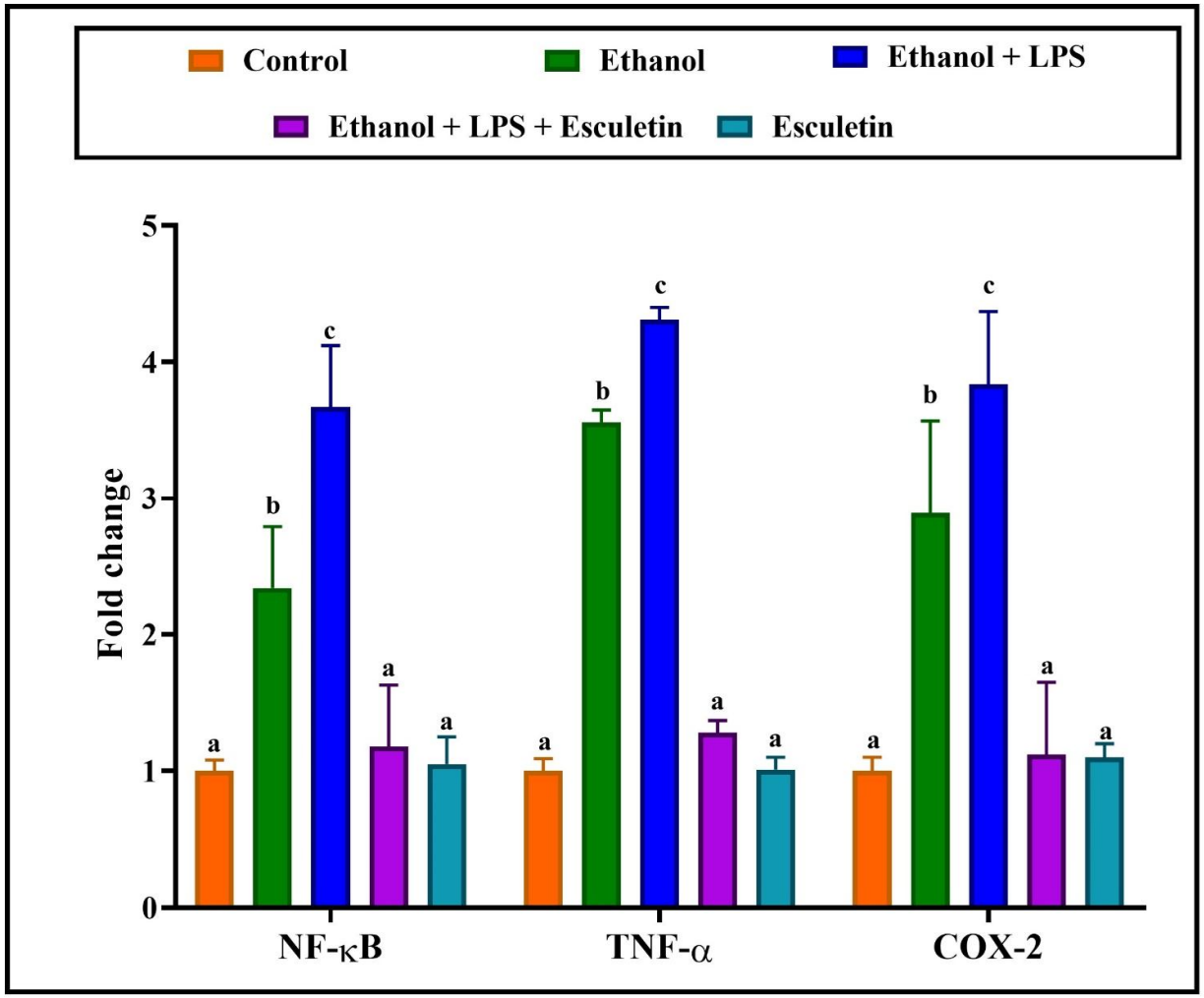

Figure 5: Effect of esculetin on inflammatory markers (NFKB, TNF-α, COX2) in the liver tissue ofcontroland experimentalrats.Values not sharing common alphabets as superscript are significantly different from each other at the level of P≤0.05 (ANOVA followed by DMRT).