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PROTECTIVE ROLE OF ESCULETIN ON ETHANOL AND LIPOPOLYSACCHARIDE-INDUCED HEPATOTOXICITY IN MALE WISTAR RATS

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ABSTRACT: Alcohol-related liver diseases and their associated damage pose significant health concerns globally. Among potential remedies, esculetin, a saturated dicarboxylic acid known for its anti-inflammatory and antioxidant attributes, stands out. This study aims to explore the hepatoprotective effects of esculetin in rat models afflicted by liver damage induced by ethanol+ lipopolysaccharide (LPS). To emulate alcoholic liver damage, adult Wistar rats were subjected to 10 days of ethanol (5 mL/kg b.w) and a single dose of LPS (5 mL/kg b.w) orally on the 11th day. Esculetin was administered orally to rats for the same 11 days duration, at doses of 50 mg/kg b.w, with a one-hour interval before inducer administration. The results revealed a remarkable reduction in the elevated levels of serum enzymes AST, ALT, ALP, and GGT following esculetin treatment, effectively restoring them to normal levels. Moreover, esculetin exhibited a substantial decrease in liver lipid peroxidation levels compared to the ethanol and ethanol+LPS group, indicating its potent management of severe oxidative stress. The addition of esculetin to the ethanol and ethanol+LPS exposed rats significantly elevates both enzymatic and non-enzymatic antioxidant activities in the liver tissue. Furthermore, when administered alongside ethanol+LPS, esculetin restored the significant alterations in liver lipid levels in the rats. Histopathological analyses provided further evidence of esculetin hepatoprotective qualities, as the treated groups exhibited a restoration of normal hepatocellular architecture, unlike the group that solely received ethanol and ethanol+LPS. This study concludes that esculetin effectively shields the liver from ethanol+LPS-induced toxicity, showcasing its potential to mitigate alcohol-related liver harm.

INTRODUCTION: Excessive and chronic alcohol consumption poses a significant threat to health, leading to various metabolic abnormalities affecting both liver and non-liver diseases [1]. In cases of experimental liver damage, alcohol is a common hepatotoxin.

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While the precise cause of alcohol-induced liver disease remains partially unclear, evidence suggests that oxidative stress triggered by ethanol plays a pivotal role. This oxidative stress results in fibrosis, impaired liver function and other detrimental effects [2, 3].

Behavioural abnormalities of reduced central nervous system activity, sluggish motor coordination, and other signs of central nervous system intoxication further indicate the negative impact of excessive alcohol use [4]. Ethanol-induced liver damage exacerbates inflammation through

lipopolysaccharide (LPS) activation, leading to heightened oxidative stress and hepatocellular injury. This synergistic effect between ethanol and LPS promotes liver inflammation, impairing the organ's ability to detoxify and regenerate. The combined impact of ethanol and LPS contributes to the progression of alcoholic liver disease, highlighting the intricate interplay between alcohol consumption and endotoxin-mediated responses in the liver [5].

Ethanol-induced liver damage is characterized by alterations in various biochemical markers, reflecting the impact of chronic alcohol consumption on hepatic function and lipid metabolism [6]. Elevated levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are commonly observed, indicating hepatocellular injury. Alkaline phosphatase (ALP) levels may also increase, reflecting cholestasis and impaired bile flow. Gamma-glutamyl transferase (GGT) levels tend to rise, serving as an additional marker of liver dysfunction [7].

Frequent overindulgence in alcohol can result in physiological dysfunctions across multiple systems, including hepatic, gastrointestinal, neurological, and cardiovascular damage [8]. The cellular disruptions caused by alcohol abuse elevate the production of oxidative stress markers such as malondialdehyde (MDA), while simultaneously reducing levels of reduced glutathione and the activity of antioxidant enzymes [9]. This oxidative damage extends to biomolecules and various physiological organs, attributed to the actions of free radicals and reactive oxygen species (ROS). Studies have established connections between free radicals and numerous human diseases, including cardiovascular issues, pulmonary disorders, certain cancers, immune/autoimmune conditions, inflammation, diabetes, cataracts, and brain disorders like Parkinson's and Alzheimer's [10]. Yet, research has demonstrated that antioxidants, whether enzymatic or nonenzymatic, can counteract the harmful consequences of free radicals. The development of oxidative stress is linked to an imbalance between the generation of free radicals, specifically ROS, and the body's inherent antioxidant defense mechanisms. This imbalance leads to oxidative damage to a range of biomolecules, including lipids, proteins, and nucleic acids. Ultimately, this compromises the proper functioning of various tissues and organs [11]. Furthermore, ethanol abuse often leads to disturbances in lipid metabolism, manifesting as abnormal lipid profiles. Dyslipidemia, characterized by elevated triglycerides and decreased high-density lipoprotein cholesterol, is frequently observed in individuals with ethanol-induced liver damage [12].

Together, these biomarker alterations provide valuable insights into the severity and nature of liver injury caused by chronic alcohol consumption. Regular monitoring of these parameters is crucial for early detection and management of ethanol-induced liver damage [13]. In light of their medicinal attributes, phyto-chemicals hold significant importance, particularly as the medical field shifts from curative to preventative approaches. With increasing resistance to traditional curative drugs, there's a growing focus on finding hepatoprotective agents due to the liver's critical role in detoxification.

For instance, the inadequate treatment options for hepatitis within modern medicine have spurred interest in this area [14]. One such compound is esculetin, a saturated dicarboxylic acid found abundantly in grains like wheat, rye, barley, oats, and sorghum. Esculetin has been associated with antiproliferative, antidiabetic, and antileukemic activities [15]. However, to date, no studies have demonstrated the modulatory effects of esculetin on ethanol and LPS-induced liver damage. Consequently, the objective of this study was to investigate whether esculetin possesses hepatoprotective properties against acute ethanol and LPS-induced hepatotoxicityin Wistar rats.

MATERIALS AND METHODS:

Chemicals and Reagents: Esculetin, ethanol (with a purity of at least 99.8%), lipopolysaccharide (LPS) biochemical analysis kits, and haematoxylin and eosin (H&E) stain were procured from Sigma-Aldrich. All remaining chemicals and reagents utilized were of analytical grade and were sourced from Himedia Laboratory Ltd., Mumbai, India.

Animals: Male Wistar rats, obtained from Biogen Bangalore. These rats had a weight range of 150 to

180g. The ethical guidelines set forth by Annamalai University's ethical council for animal care and the Indian National Law on Animal Care were strictly adhered to in the handling and treatment of the rats. The study followed the principles outlined in the "Guide for the Care and Use of Laboratory Rats (IAEC proposal No. AU—IAEC/1258/11/19). "The rats were housed in plastic cages filled with rice husk bedding, maintaining a temperature of 27°C. The rats were subjected to a controlled environment with alternating 12-hour light and dark cycles. They were provided with stranded feed and water throughout the study.

Preparation of Esculetin, Ethanol and LPS: Esculetin was solubilized in warm water (60º C), subsequently cooled, and then orally administered to the experimental rats. The LPS was dissolved in distilled water. The dosage of Esculetin, ethanol and LPS was determined according to established literature [16, 17].

Experimental Protocol: Animals were divided into five groups of six animals each **Fig. 1**. Total experimental period was 17 days.

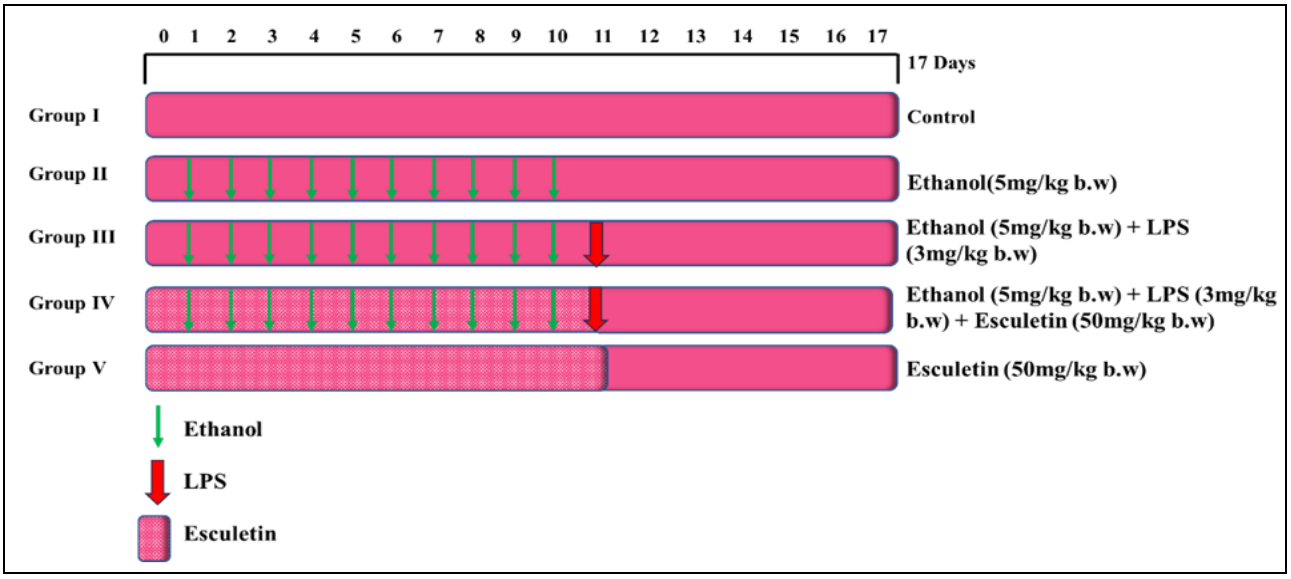

FIG. 1: EXPERIMENTAL DESIGN

The rats underwent a treatment involving ketamine hydrochloride and were euthanized at the end of the experimental period. Blood samples were collected at room temperature they were centrifuged at 3,000 rpm for 10 minutes to obtain serum. The liver was immediately rinsed with an ice-cold saline solution containing 0.9 percent sodium chloride. Afterwards, the liver was homogenized, and the resulting supernatant was utilized for performing biochemical analyses. To prepare for histopathological evaluations, segments of liver tissue were preserved in formaldehyde buffer solution (10%).

Activities of Hepatic Marker Enzymes in Serum: Utilizing commercially available diagnostic kits from Sigma Diagnostics (I) Pvt. Ltd. in Baroda, India, the levels of serum enzymes were evaluated using spectrophotometric methods following established protocols. Serum aspartate amino transferase (AST, E.C. 2.6.1.1), alanine amino transferase (ALT, E.C. 2.6.1.2), and alkaline phosphatase (ALP, E.C. 3.1.3.1) activities were determined using standard procedures.

For the measurement of gamma-glutamyl transferase (GGT, E.C. 2.3.2.2) enzyme activity, the method introduced by Rosalki *et al.* (1970) [18] was adopted, employing -glutamyl-p-nitroanilide as the substrate.

Estimation of Lipid Peroxidation: Hepatic lipid peroxidation was quantified using calorimetric techniques through the determination of thiobarbituric acid reactive substances (TBARS), as pioneered by Niehiaus and Samuelsson, 1968 [19]. Lipid Peroxidation (LPO) levels were evaluated following the approach outlined by Devasagayam and Tarachand, 1987 [20] and were presented as mmol per 100 g of tissue.

Assay of Enzymatic Antioxidant Enzymes: Superoxide dismutase (SOD) activity was evaluated following the protocol established by Kakkar *et al.*, 1984 [21]. This method involves measuring the inhibition of the NADPH-phenazine methosulphate nitroblue tetrazolium formazon formation using spectrophotometry at 560 nm. Catalase (CAT) activity was assessed through a colorimetric procedure outlined by Sinha, 1972, utilizing dichromate acetic acid reagent [22].

The activity of glutathione peroxidase (GPx) was determined using Rotruck *et al.*, 1973 [23] methods, based on the interaction between the residual glutathione after GPx action and 5,5-dithiobis-2-nitrobenzoic acid, resulting in the formation of a complex that exhibits maximum absorption at 412 nm. Glutathione S transferase (GST) activity was quantified spectrophotometrically by utilizing dichloro-2,4-dinitrobenzene as the substrate, following the approach outlined by Habig *et al.*, 1974 [24].

Determination of Non-enzymatic Antioxidants: The determination of reduced glutathione (GSH) followed Ellman's method (1959) [25], which relies on its reaction with Ellman's reagent (consisting of 19.8 mg of dithionitrobis benzoic acid dissolved in 100 ml of 0.1% sodium citrate solution). The concentrations of vitamin C and vitamin E were assessed using the techniques described by Omaye *et al.* (1979) [26] and Desai (1984) [27], respectively.

Assay of Lipid Profile: Liver lipids were isolated using the procedure outlined by Folch *et al.*, 1957 [28]. The quantification of total cholesterol, triglycerides, free fatty acids, and phospholipids was conducted utilizing the methodologies established by Siedel *et al.* 1983 [29], Foster and Dunn., 1973 [30], Falholt *et al.*, 1973 [31] and Zilversmit and Davis., 1950 [32].

Histopathology: The liver specimens preserved in a 10% formalin solution underwent dehydration by sequentially passing through a distinct mixture of ethyl alcohol and water. Subsequently, they were cleansed using xylene and encased in paraffin. Liver sections, measuring 3-5 µm in thickness, were then meticulously prepared and subjected to staining with hematoxylin and eosin dye.

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

RESULTS:

Effect of Esculetin on Body Weight: Table 1 presents the body weights of the rats, indicating that the initial body weight of each group ranged from 150 to 170g. During the experiment, the untreated rats exposed to ethanol and ethanol+LPS displayed a consistent decrease in body weight. However, both the control rats and the rats treated with esculetin exhibited a noteworthy increase in weight. Notably, the administration of esculetin led to a significant recovery in body weight.

TABLE 1: EFFECT OF ESCULETINON BODY WEIGHT AND LIVER WEIGHT OF CONTROL AND EXPERIMENTAL RATS AT 17TH DAY OF EXPERIMENTAL PERIOD

Groups	Initial body weight (g)	Final body weight (g)	Liver weight (g)
Control	163.58±3.53	228.12±2.56^{a}	5.32±6.52^{a}
Ethanol(5mg/kg b.w)	164.83±4.81	185.47±4.85^{b}	7.56±5.92^{b}
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w)	167.27±1.88	183.96±2.48^{c}	8.26±7.83^{c}
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w) + Esculetin (50mg/kg b.w)	159.99±1.78	210.73±1.79^{d}	5.82±2.72^{d}
Esculetin (50mg/kg b.w)	165.06±2.51	229.16±5.83^{a}	5.29±6.75^{a}

Values are means ±SD of 6 rats from each group. Values not sharing common alphabets as superscript are significantly different from each other at the level of P<0.05 (ANOVA followed by DMRT).

Effect of Esculetin on Liver Marker Enzyme: The concentrations of serum liver markers for both the experimental and control rats are outlined in **Table 2**. Oral ethanol and ethanol+LPS administration resulted in impaired liver function across all rats. Specifically, the rats subjected to ethanol and ethanol+LPS intoxication showed elevated levels of blood hepatospecific marker

enzymes including AST, ALT, ALP, and GGTlevels ($p < 0.05$). Conversely, in rats treated with both, ethanol+LPS and esculetin, the levels of serum AST, ALT, ALP, and GGT activity were significantly reduced ($p < 0.05$). This underscores the esculetin ability to mitigate the liver damage caused by ethanol+LPS.

TABLE 2: EFFECT OF ESCULETIN ON LIVER MARKERS ENZYME IN SERUM OF CONTROL AND EXPERIMENTAL RATS AT 17TH DAY OF EXPERIMENTAL PERIOD

Groups	AST(IU/L)	ALT(IU/L)	ALP(IU/L)	GGT(IU/L)
Control	79.31±4.58[a]	28.64±5.67[a]	95.92±3.14[a]	10.67±0.47[a]
Ethanol(5mg/kg b.w)	112.34±4.25[b]	64.82±6.48[b]	157.62±2.97[b]	24.71±0.91[b]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w)	117.95±6.73[c]	67.73±3.48[c]	161.50±3.46[c]	27.86±0.82[c]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w) + Esculetin (50mg/kg b.w)	83.48±3.91[d]	30.4.9±4.37[d]	99.78±3.78[d]	13.94±0.63[d]
Esculetin (50mg/kg b.w)	78.92±7.61[a]	28.38±5.97[a]	95.41±3.57[a]	10.39±0.23[a]

Values are means ±SD of 6 rats from each group. Values not sharing common alphabets as superscript are significantly different from each other at the level of P<0.05 (ANOVA followed by DMRT).

Effect of Esculetin on Lipid Peroxidation: Table 3 illustrates the disparities in liver lipid peroxidation markers between the experimental and control rats. Compared to the control rats, those exposed to ethanol and ethanol+LPS exhibited notably elevated levels of lipid peroxidation markers. However, the administration of esculetin to ethanol+LPS-exposed animals effectively lowered these markers. Interestingly, when comparing esculetin-treated control rats with untreated control rats, minimal differences were observed.

TABLE 3: EFFECT OF ESCULETIN ON LIPID PEROXIDATIVE MARKERS IN LIVER TISSUE OF CONTROL AND EXPERIMENTAL RATS AT 17TH DAY OF EXPERIMENTAL PERIOD

Groups	TBARS (mmol/mg of tissue)	LPO (mmol/mg of tissue)
Control	0.94±0.05[a]	5.64±0.21[a]
Ethanol(5mg/kg b.w)	1.63±0.03[b]	9.78±0.23[b]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w)	1.72±0.03[c]	10.08±0.31[c]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w) + Esculetin (50mg/kg b.w)	1.01±0.04[d]	6.31±0.29[d]
Esculetin (50mg/kg b.w)	0.93±0.06[a]	5.59±0.38[a]

Values are means ±SD of 6 rats from each group. Values not sharing common alphabets as superscript are significantly different from each other at the level of P<0.05 (ANOVA followed by DMRT).

Effect of Esculetin on Enzymatic and Non-Enzymatic Antioxidants: Tables 4 and **5** provide insights into the activities of enzymatic (SOD, CAT, GPx, and GST) and non-enzymatic (GSH, Vit C and Vit E) antioxidants. Ethanol and ethanol+LPS treatment led to a considerable decrease in antioxidant levels. Nevertheless, oral administration of esculetin in rats exposed to ethanol+LPS significantly altered the antioxidant levels.

TABLE 4: EFFECT ESCULETIN OF ENZYME ANTIOXIDANTS IN LIVER OF CONTROL AND EXPERIMENTAL RATS AT 17TH DAY OF EXPERIMENTAL PERIOD

Groups	SOD (units[#]/mgprotein)	CAT (units[#]/mgprotein)	GST (units[#]/mgprotein)	GP_X (units[#]/mgprotein)
Control	8.31±1.31[a]	79.66±3.47[a]	7.82±1.71[a]	16.02±2.64[a]
Ethanol(5mg/kg b.w)	3.67±1.47[b]	38.41±2.53[b]	3.12±1.67[b]	7.51±2.73[b]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w)	3.11±1.69[c]	33.62±3.14[c]	2.99±1.29[c]	7.20±3.19[c]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w) + Esculetin (50mg/kg b.w)	7.96±1.72[d]	73.45±4.07[d]	6.93±2.07[d]	15.87±2.49[d]
Esculetin (50mg/kg b.w)	8.39±1.24[a]	80.09±3.41[a]	7.81±1.62[a]	16.13±1.97[a]

Units of enzymes activities are expresses. SOD -one unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in one minute. CAT -µ moles of hydrogen peroxide consumed/minute. GST-µ moles of CDNB-GSH conjugate formed/minute. Values are means ±SD of 6 rats from each group. Values not sharing common alphabets as superscript are significantly different from each other at the level of P<0.05 (ANOVA followed by DMRT).

TABLE 5: EFFECT ESCULETIN OF NON-ENZYMATIC ANTIOXIDANTS IN LIVER OF CONTROL AND EXPERIMENTAL RATS AT 17TH DAY OF EXPERIMENTAL PERIOD

Groups	Vitamin C (mg/100 g of tissue)	Vitamin E (mg/100 g of tissue)	GSH (mg/100 g of tissue)
Control	5.08±2.45[a]	1.36±0.21[a]	18.13±2.51[a]
Ethanol(5mg/kg b.w)	2.64±1.97[b]	0.76±0.23[b]	8.59±3.01[b]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w)	2.43±1.96[c]	0.69±0.19[c]	8.09±2.83[c]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w) + Esculetin (50mg/kg b.w)	4.91±2.38[d]	1.24±0.25[d]	17.58±2.19[d]
Esculetin (50mg/kg b.w)	5.12±2.87[a]	1.35±0.34[a]	18.26±2.85[a]

Values are means ±SD of 6 rats from each group. Values not sharing common alphabets as superscript are significantly different from each other at the level of P<0.05 (ANOVA followed by DMRT).

TABLE 6: EFFECT OF ESCULETIN ON LIPIDS PROFILE IN LIVER TISSUE (MG/100G TISSUE) OF CONTROL AND EXPERIMENTAL RATS AT 17TH DAY OF EXPERIMENTAL PERIOD

Groups	TG (mg/100g tissue)	Cholesterol (mg/100g tissue)	FFA (mg/100g tissue)	Phospholipids (mg/100g tissue)
Control	54.62±4.82[a]	82.54±5.64[a]	77.64±3.16[a]	85.36±5.61[a]
Ethanol(5mg/kg b.w)	112.54±5.64[b]	120.05±4.62[b]	138.44±5.47[b]	129.45±5.12[b]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w)	120.53±4.91[c]	125.75±4.37[c]	142.46±3.48[c]	135.48±4.86[c]
Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w) + Esculetin (50mg/kg b.w)	70.60±4.62[d]	91.85±3.91[d]	86.48±4.23[d]	99.12±4.78[d]
Esculetin (50mg/kg b.w)	55.67±4.81[a]	82.19±6.71[a]	77.51±4.67[a]	86.93±6.15[a]

Values are means ±SD of 6 rats from each group. Values not sharing common alphabets as superscript are significantly different from each other at the level of P<0.05 (ANOVA followed by DMRT).

Effect of Esculetin on Lipid Profile: The levels of liver phospholipids (PL), free fatty acids (FFA), cholesterol, and triglycerides (TG) in both control and experimental rats are detailed in **Table 6**. Notably, the rats treated with esculetin following ethanol+LPS exposure experienced a marked reduction in cholesterol, TG, FFA, and PL levels. Conversely, these lipid levels were significantly higher in the group exposed only to ethanol and ethanol+LPS.

Histochemical Changes: In contrast to the histological appearance of a normal central vein, hepatocytes, and hepatic sinusoids shown in **Fig. 2 Fig. 2A** and **B**), exposure to ethanol and ethanol+LPS caused distinct alterations in the liver's tissue structure.

These alterations included parenchymal necrosis, lymphatic infiltration, enlarged sinusoids, cellular degeneration, intracellular vacuolation, and pyknotic nuclei **Fig. 2 C** and **D**. Following treatment with ethanol+LPS by esculetin, the hepatic histoarchitectural pattern appeared nearly normal, with minimal hepatic sinusoidal enlargement observed **Fig. 2 E.**

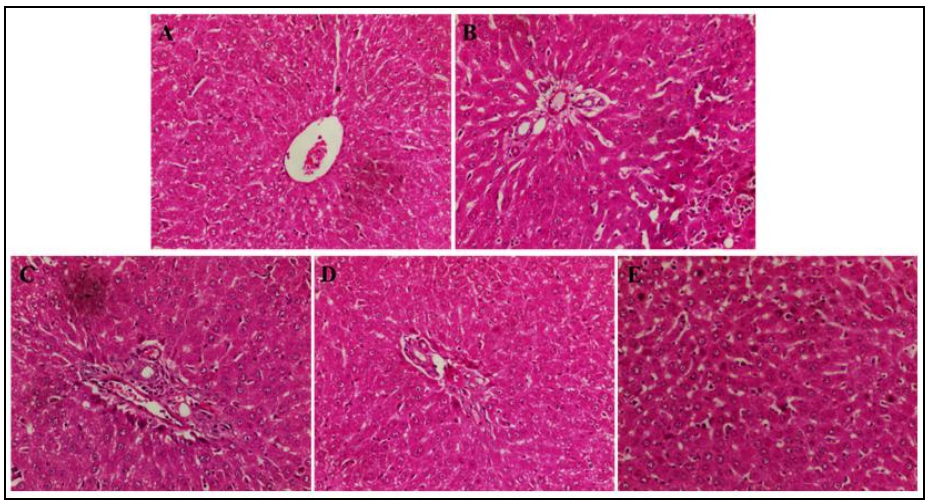

FIG. 2: HISTOPATHOLOGICAL CHANGES OF LIVER TISSUE OF CONTROL AND EXPERIMENTAL RATS. (A) Control, (B) Ethanol(5mg/kg b.w), (C) Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w), (D) Ethanol (5mg/kg b.w) + LPS (3mg/kg b.w) + Esculetin (50mg/kg b.w), (E) Esculetin (50mg/kg b.w).

DISCUSSION: The liver plays a critical role in numerous metabolic processes, with a particular emphasis on detoxifying toxins [33]. Recent studies have investigated the impact of free radicals on the toxicity induced by chemicals. Chronic alcohol consumption leads to various changes in the liver, including cirrhosis, alcoholic fibrosis, and alcoholic hepatitis [34]. Increasingly, research supports the idea that oxidative stress and nutritional deficiencies contribute to tissue damage caused by ethanol [35].

Ethanol, high in calories (7.1 Kcal per gram), lacks nutrients, and substituting it for carbohydrates at the same caloric level reduces weight gain. Furthermore, alcohol hinders nutrient absorption from the intestines, potentially causing reduced body weight due to secondary malnutrition related to alcoholism [36]. Given that body weight reflects health, rats coadministered with esculetin gained weight, suggesting protective effects against ethanol-induced liver damage. Prabakaran *et al.,* (2013) reported that esculetin increases the body weight in streptozotocin-induced diabetic rats [37].

In the present study, ethanol and ethanol+LPS consumption significantly elevated liver enzymes (AST, ALT, and ALP). Chronic alcohol use leads to various cellular and tissue abnormalities, including changes in liver-specific enzymes, indicating increased cell permeability, damage, or hepatocyte necrosis [38]. GGT elevation is common in regular drinkers due to alcohol's inducing effect on this enzyme. Elevated serum GGT levels are also observed in response to various substances and toxins. Notably, ethanol and ethanol+LPS administration considerably increased mean AST, ALT, ALP and GGT levels. Ethanol and ethanol+LPS-intoxicated rats showed higher levels of serum AST, ALT, ALP and GGT, well-known markers of tissue damage caused by toxic agents [39]. However, the administration of esculetin to ethanol and ethanol+LPS decreased the liver enzyme levels. Cai *et al.,* (2023) [40] also reported that esculetin reduces the liver enzyme levels in carbon tetrachloride-induced liver damage in Wistar rats. Lipid peroxidation is a significant factor in alcoholic liver disease. Ethanol's harmful impact on the liver is partly attributed to the generation of free radicals and lipid peroxidation products [41]. Our study revealed higher levels of TBARS and LPO in the livers of the ethanol and ethanol+LPS administered group. Lipid peroxidation, the oxidation of polyunsaturated fatty acids due to oxygen-derived free radicals, is a common process in living organisms, often occurring during mitochondrial electron transport [42]. Administration of esculetin decreased the lipid peroxidation markers in ethanol and ethanol+LPS-induced rats. Jiang *et al.,* (2021) [43] reported that esculetin reduced the lipid peroxidation markers such as TBARS and LOOH in 7, 12-dimethylbenz(a) anthracene-induced hamster buccal pouch carcinogenesis.

Ethanol administration leads to an overproduction of free radicals, including hydrogen peroxide, hydroxyl ethyl radical, superoxide radical, and hydroxyl radical (OH). These radicals can readily react with lipids, promoting LPO [44]. Ethanol consumption is recognized to elevate LPO, contributing to tissue damage and membrane dysfunction. Increased lipid peroxidation product accumulation in cells can lead to cellular dehydration, deformation and death [45]. Antioxidants are vital for maintaining metabolism against oxidative stress [46]. Cellular antioxidant defense systems counteract ROS. Enzymes such as SOD, CAT, GPX, GST, Vit C and Vit E play a role in this defense mechanism. Oxidative stress occurs when free radicals exceed the antioxidant system's capacity, leading to tissue damage [45]. SOD, CAT, GPX, and GST, as enzymatic antioxidants, play a vital role in scavenging ROS and inhibiting their production [47].

SOD neutralizes ROS by converting superoxide to H_2O_2, acting as the first line of defense against oxygen radicals [46]. Ethanol and ethanol+LPS significantly reduce SOD activity. In the present study, the increase in lipid peroxidation indices could be linked to suppressed SOD function, leading to increased superoxide levels. Catalase serves as a key antioxidant defense against LPO [44]. Rats treated with ethanol exhibited decreased catalase activity. Our investigation showed reduced non-enzymatic and enzymatic antioxidants due to alcohol-induced toxicity, consistent with earlier findings were observed. GPX collaborates with CAT to scavenge excess H_2O_2 and other free radicals in response to oxidative stress [46]. Maintaining the balance between these enzymes is crucial for the effective removal of oxidative stress

from organelles. Oxidative stress lowers GSH, Vit C and Vit E levels and diminishes GST activity. Vit-C and Vit-E donate electrons to ROS and free radicals, thereby neutralizing their damaging effects [48]. This is indicated by decreased SOD, CAT, GST, GPX, Vit C and Vit E activity in ethanol and ethanol+LPS-treated rats. Ethanol consumption negatively affects the antioxidant system, with the severity correlated to the amount consumed [49]. Esculetin, a natural antioxidant, may scavenge ethanol+LPS-generated free radicals, potentially explaining its increase in enzymatic and nonenzymatic antioxidant levels in rats treated with esculetin. Likely, esculetin increases both enzymatic and nonenzymatic antioxidant levels in carbon tetrachloride-induced toxicity in Male Sprague-Dawley rats [40].

Prolonged ethanol exposure affects membranes, causing fat accumulation in the liver. Elevated free fatty acids could enhance decreased NADPH (or NADH) production, activating NADPH-dependent microsomal peroxidation [50]. Phospholipids, vital components of PUFA-rich biomembranes, are susceptible to free radicals like O2- and OH-radicals [46]. Elevated liver cholesterol, FFAs, TGs, and PLs levels have been observed in tissues exposed to ethanol. Ethanol alters liver lipid levels and overall lipid balance, contributing to fatty liver, although the precise mechanism is not fully understood. Aldehyde dehydrogenase's role in ethanol metabolism leads to metabolic dysfunction and altered membrane function due to changes in reducing equivalents. Ethanol+LPS-treated rats exhibited notably higher levels of cholesterol, TG, FFA, and PL compared to the control group.

Oral administration of esculetin in ethanol+LPS-exposed rats lowered liver cholesterol, FFAs, TGs, and PLs supporting improved liver function. Esculetin potential to mitigate the adverse effects of elevated lipid levels induced by ethanol is evident. In this consent, Wang *et al.*, (2022) [51] reported that esculetin modulates the lipid profile (Serum total cholesterol (TC), triglyceride (TG), very low-density lipoprotein cholesterol (VLDL-C), and low-density lipoprotein cholesterol (LDL-C) in carbon tetrachloride exposed rats. Histopathology of liver tissue of ethanol and ethanol+LPS-induced rats exhibited parenchymal necrosis, lymphatic infiltration, enlarged sinusoids, cellular degeneration, intracellular vacuolation, and pyknotic nuclei. These results agree with Ali et al. (2021) [52]. On the contrary, rats treated with esculetin displayed minimal hepatic sinusoidal enlargement in the liver tissue. Therefore, those data suggest that esculetinhas the capability to provide a secure and efficient approach to treating liver damage with significant antioxidant activities. Similar findings were obtained in early research done by Pandey *et al.*, (2017) [53].

CONCLUSION: In conclusion, this research demonstrated the hepatoprotective effects of esculetin in rat models with ethanol and ethanol+LPS-induced liver damage. The study revealed a significant reduction in serum enzyme levels, lipid peroxidation, and restoration of antioxidant activities. Esculetin also influenced lipid metabolism and restored hepatocellular architecture. These findings highlight esculetin potential to combat ethanol+LPS-induced liver toxicity. Future research could delve deeper into the molecular mechanisms underlying esculetin effects, explore its applicability in different liver disease models, and consider potential human clinical trials.

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