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Determination of Hepatoprotective Effects of Vanillin Against Lipopolysaccharide-induced Acute Liver Injury In-Vivo Mice Model

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ABSTRACT

Background: Vanillin, a phenolic aldehyde extracted from Vanilla planifolia pods, has various biological activities, however, little is known about its hepatoprotective role. In this study, we developed an acute liver injury murine model using lipopolysaccharides (LPS) and elucidated the hepatoprotective activity of vanillin.

Methods: A pre-clinical study using a random sampling technique was conducted at Ziauddin University MDRL-1 and 2 labs, between March-December 2023. The inclusion criteria included 36 healthy male BALB/c. Six groups (n=6) of male BALB/c mice (25-30g) were used: Normal, LPS, Treatment groups (two different concentrations of Vanillin were intraperitoneally [I.P.] given to mice), toxicity, and positive control group respectively. The animals were pre-treated for four days and a single dose of LPS (2mg/kg) was I.P. administered one day before euthanizing animals. Using SPSS v.24, one-way ANOVA was performed followed by Tukey and Bonferroni's post hoc for results comparison between different groups with $p<0.05$ significant.

Results: The results indicated that vanillin had a markedly protective effect on acute liver injury induced by LPS in mice. Histopathological analysis showed that vanillin administration minimized liver injury levels. Also, the administration of vanillin inhibited the pro-inflammatory cytokine IL-6 growth in liver tissue. A significant elevation of liver enzymes in LPS-treated mice (2 mg/kg); AST and ALT activities were 360.63 ±14.56 U/L ($p<0.0001$) and 188.50 ±10.55 U/L versus 98.73 ±4.4 U/L and 36.53 ±3.56 U/L in the control group were seen.

Conclusion: : Our findings suggest that vanillin might be a promising candidate for LPS-induced acute liver injury via its regulating effects on inflammation in the hepatic cells.

Keywords: Lipopolysaccharide, Vanillin, Hepatoprotective, Acute Liver Injury, Inflammation.

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INTRODUCTION

The liver is an immunologically complex organ. It is involved in various physiological and pathophysiological functions such as immunity, metabolism, and detoxification. Due to these functions, the liver becomes susceptible to various microbial organisms during sepsis [1]. Sepsis leads to multiple organ failure and is characterized by severe inflammation and immune system dysregulation[2]. Acute liver injury (ALI) due to sepsis is one of the major causes of mortality in patients in intensive care units [3]. Sepsis is a critical condition which leads to disease progression and death. Despite massive global research efforts, there has been little effective target therapy for septic liver injury. Several risk factors contribute to acute liver injuries such as drug usage, viral infections, and inadequate nutrition[4].

LPS, a large molecule of glycolipid, belongs to the gram-negative bacteria and is present in the outermost layer of the bacteria cell wall. Many studies and experimental animal model studies supported that LPS behaves as an endotoxin-inducing liver sepsis which leads to liver dysfunction[4]. LPS activates the inflammatory macrophages that are identified as subpopulations in the Kupffer cells. Activation of Kupffer cells leads to massive production of inflammatory cytokine interleukin-6. LPS, acts as a potentially immunogenic molecule due to its conserved Lipid A region, jeopardizing the overall health of the liver. In our current research, the clinical implication of liver injury is a result of LPS which is used as a hepatotoxin. Therefore, promising preliminary studies from animal studies are urgently required and awaiting translation to human studies[5].

Thus LPS-induced hepatic insult in an animal model caters to a practical implication for the evaluation of natural compounds that interfere with liver immunology. IL-6 synthesis and secretion is increased in hepatocytes during inflammatory responses such as stimulation of Toll-like receptor 4 (TLR-4) by lipopolysaccharide. Moreover, persistent activation of IL-6 signaling pathways may lead to the development of various liver ailments. Intracellular signaling is induced when IL-6 binds to either soluble IL-6 receptors or signal transducing subunit gp 130 [6]. IL-6 belongs to a membrane of the cytokine family that is the main activator of liver physiopathology since it is the major inducer of the hepatic acute phase proteins. In our present study, we selected interleukin-6 (IL-6) as a major proinflammatory cytokine against LPS-induced ALI[7].

The use of natural compounds is used in many intervention studies to alleviate inflammatory diseases. Natural pharmacological compounds play an important role in medicine due to their safety and potency. Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a polyphenol with a molecular formula of $C_8H_8O_3$. It is abundant in beans and pods of perennial plant species, notably Vanilla planifolia [8]. Vanillin is a spice that is mostly used around the globe since it has fewer side effects, advantage of low cost, and is extracted from various sources. Vanillin has anti-inflammatory, anti-microbial, and anti-carcinogenic activities [9].

Based on the properties mentioned above, we anticipate that vanillin has broad pharmacological activities that can be used for the prevention of acute liver injury. There have been no studies that reported the hepatoprotective influence of vanillin in LPS-induced liver injury. In this study, we elucidated the role of vanillin in septic liver injury and its potential molecular mechanism with a focus on IL-6 in mice models.

METHODS

This is an in-vivo experimental study, and the random sampling technique was used. Before the conduct of the study, the study was approved by the Ziauddin University Animal Ethics Committee and a certificate was generated with an animal study protocol (ASP) number 2022-05/RK/FHS. The study was conducted in the MDRL-1 and 2 research labs, at Ziauddin University, Karachi between March-December 2023. The inclusion criteria included 36 healthy male BALB/c weighing around 20-30 grams, which were purchased from the University of Karachi, Karachi. The animals were kept at a 23°C controlled environment and a 12-h light-dark cycle and provided with rodent food and water ad libitum.

At the start of the experiment, the mice were randomly assigned to six groups (n=6). Control Group: Animals were administered with saline intraperitoneally for four days. Diseased Group: Mice were given saline for four days and then intraperitoneal injection of LPS (2 mg/kg body weight). Treatment group- Pure compound: Vanillin 50 (mg/kg) was injected into mice for four days simultaneously followed by LPS-2 (mg/kg) intraperitoneally. Treatment group- Pure compound: Vanillin 100 (mg/kg) was injected into mice for four days simultaneously followed by LPS 2 (mg/kg) I.P. Toxicity Testing Group: Animals will be given vanillin at 100mg/kg I.P. Positive Control Group: Animals will be given Silymarin 200mg/kg at standard dose P.O. for four days simultaneously and LPS-2 mg/kg I.P. Silymarin is the most convenient and highly compliant route for delivery since it has high oral bioavailability and solubility. Also, silymarin has been found to have a good safety profile without any adverse effects[10].

The pre-treatment study with vanillin and silymarin was continued for four days, on 5th day, animals were given an intraperitoneal injection of LPS

(except the animals in group 1 and in the toxicity groups, they were given normal saline instead of LPS via the same route. After 24 hours of LPS injection, all animals will be sacrificed. The blood was immediately isolated from the dissected mouse by a cardiac puncture in an anticoagulant tube and centrifuged immediately at 3000g to obtain serum and stored at −20◦C for biochemical analysis. The liver was also harvested and a portion of the liver sample was taken for histology and the remaining liver tissue was stored in an Eppendorf tube at –80◦C for ELISA. LPS from Escherichia coli (O55:B5), Catalog number: L2880, was purchased from Sigma Aldrich (St. Louis, MO, United States), Silymarin, (Catalog number S0292) was purchased from Sigma Life Sciences, vanillin (Catalog number: 10387020) was purchased from Fischer Scientific, US and Rat IL-6 (Interleukin-6) ELISA kit (Catalog number EH0201) was purchased from Wuhan Fine Biotech Ltd.

Liver tissue was fixed in formalin for 24hrs., and after tissue processing embedded in paraffin wax. The tissue was then sectioned at 5 µm thicknesses followed by staining with hematoxylin and eosin. The histological changes were observed on a Nikon Ts2R-FL Inverted research microscope at 200X magnification. A total of 80 mg of stored liver tissue sample was washed with distilled water to remove any residual blood. Lysis buffer was added to the liver tissue to make a homogenate with a homogenizer. The supernatant was collected by centrifugation to estimate the concentration of interleukin 6 (IL-6) by ELISA as per the kit protocol. Optical densities obtained were used to plot a standard curve. The extrapolated values were used to determine the concentrations in unknown samples. The obtained results were presented as pg/ml. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in the serum were determined by the kit method through Microlab 300 instrument (ELITech Group of companies).

For statistical analysis, data was analyzed by SPSS version 24 and presented as means ± standard deviation (n=6). To assess the statistical significance between different groups, one-way ANOVA was performed followed by Tukey and Bonferroni's post hoc for comparison between results of different groups. The normality of the distribution of individual variables was measured by applying the Shapiro-Wilk test. The graphs were made on GraphPad Prism 9.5.0 software. Statistical significance of a p-value less than 0.05 was considered significant.

RESULTS

Liver markers AST, ALT, and ALP were evaluated to see the preventive effects of vanillin in the experimental liver injury model. A significant elevation in the LPS group was observed when compared to the normal group, indicating liver injury as shown in Table 1. The treatment groups (Vanillin 100mg/kg and 50mg/kg) and positive control groups display a significant decline in the levels ($p < 0.001$). Positive control groups showed similar results in comparison with vanillin groups on AST, ALT, and ALP (n=6; $p<0.001$)

Table 1. Effect of vanillin on LPS-induced ALI on serum AST, ALT, and ALP.

Parameters	Control	LPS	Vanillin 100mg/kg	Vanillin 50mg/kg	Toxicity	Silymarin
AST (U/L)	98.73 ±4.4	360.63 ±14.56$^{\&}$	255.88 ±13.45***	278.20 ±11.90***	100.26 ±5.67***	292.15 ±14.21***
ALT (U/L)	36.53 ±3.56	188.50 ±10.55$^{\&}$	77.89 ±8.02***	111.24 ±12.84***	49.81 ±9.81***	170 ±2.45***
ALP (U/L)	39.83 ±5.40	452.24 ±10.68$^{\&}$	355.98 ±51.52***	180.67 ±37.83***	42.16 ±3.58***	259.67 ±157.82***

*AST: aspartate transaminase; ALT: alanine transaminase; ALP: alkaline phosphatase. Values are mean ±SD ! for six mice in each group. p<0.001 vs LPS group is represented as ***; p<0.0001 LPS group vs control group is represented as & is considered significant.*

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The liver in the control group demonstrated healthy hepatocyte architecture with no significant structural changes. Proper vasculature of the hepatocytes was observed as shown in Figure 2 A. While in the LPS model group, confluent necrosis, dissolving nucleus, focal and portal inflammation, and inflammatory infiltration were observed as shown in Figure 2 B. Eosinophilic cells with degenerated nuclei are shown. These changes result in lost homogeneity of hepatic lobules suggesting the development of a successful model of LPS-induced acute liver injury. Pretreatment with vanillin (50 mg/kg and 100 mg/kg) significantly resulted in improvement in the histopathology of the liver as shown in Figures 2 C and D. Vanillin at a high dose of 100 mg/kg, showed necrosis but to a lesser extent as shown in Figure 2 C. Whereas, in Figure 2 D, pretreatment with vanillin at low dose of 50 mg/kg resulted in less inflammation and necrosis with restoration of tissue towards the central area. Some of the hepatocytes were observed in the regeneration phase. Pretreatment with vanillin only showed normal histology of the liver as shown in figure 2 E. In addition to that, administration of silymarin at 200 mg/kg showed mild necrosis indicating significant improvement as shown in Figure 2 F. The histopathological evaluation was performed blindly as shown in Table 1. The necro inflammatory categories crosstabulation scored according to the HAI of the Ishak scoring system.

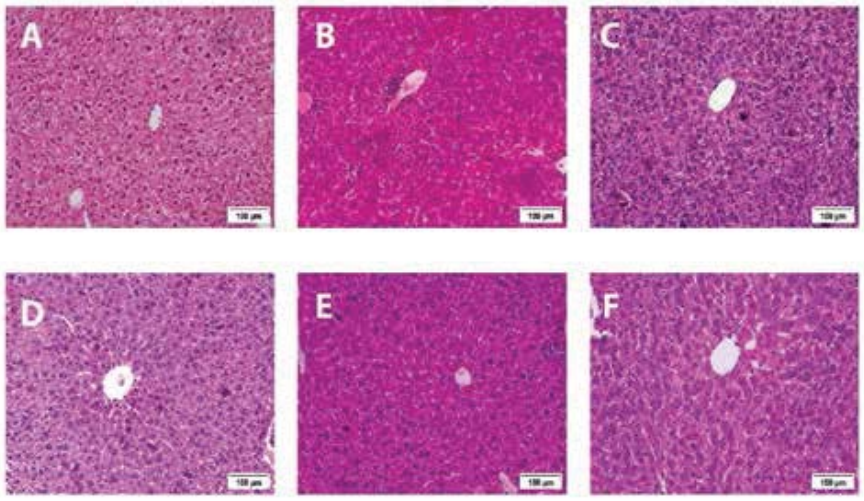

Figure 2. Effects of vanillin on histopathology of the liver. H&E stain is used for liver sections. The photomicrographs shown here represent liver sections from the six different mice groups. (A) Normal Group; (B) LPS Group; (C) Vanillin 100 mg/kg; (D) Vanillin 50 mg/kg; (E) Toxicity; (F) Silymarin 200 mg/kg (positive control group). (Original magnification at 200X). The arrows in Figure 2 B represent inflammatory reactive changes and spotty necrosis.

Table 2 shows the liver injury presented as necrosis and graded and expressed as minimal, mild, moderate, and severe according to HAI of the Ishak scoring system.

Table 2: **The necro inflammatory categories crosstabulation scored according to HAI of the Ishak scoring system**.

Groups	Minimal (-)	Mild (+)	Moderate (++)	Severe (+++)	p-value
Normal	100%	NA	NA	NA	< 0.001
LPS	NA	NA	50%	50%	< 0.001
Van 100mg/kg	NA	83.3%	16.7%	NA	< 0.001
Van 50mg/kg	NA	100%	NA	NA	< 0.001
Toxicity	66.7%	33.3%	NA	NA	< 0.001
Silymarin	100%	NA	NA	NA	< 0.001

Minimal (-), mild (+), moderate (++), and severe (+++)[11].

The severity of inflammation in LPS-induced ALI was positively correlated with proinflammatory cytokines IL-6 in the liver tissue [10]. The extent of IL-6 expression in the diseased (LPS) group was significantly increased when compared to normal or treatment groups as shown in Figure 3 ($P < 0.0001$). The intraperitoneal and oral administration of vanillin and silymarin significantly decreased the levels of IL-6 in the liver tissue of LPS-induced ALI mice ($p<0.0001$) as shown in Figure 3. In addition, Vanillin at a low dose of 50mg/kg had more effect on inhibiting the IL-6 level when compared to vanillin at 100 mg/kg, ($p < 0.001$). These data suggest that vanillin pretreatment inhibits inflammatory response in LPS-induced inflammatory mice.

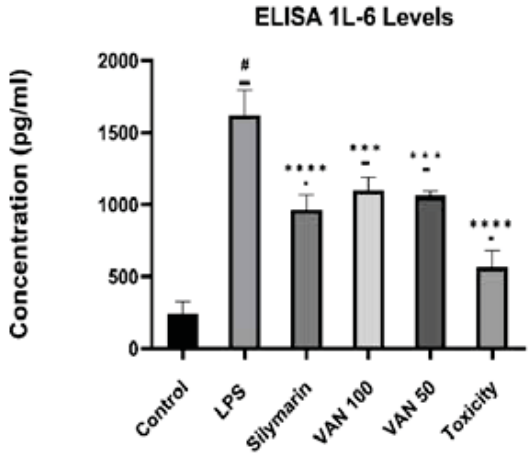

Figure 3: *In-Vivo* effect of vanillin on IL-6 expression level in liver tissues of all the experimental animals. Data is presented as mean ± SD (n = 6). ANOVA is used to obtain p values and Bonferroni's multiple comparison test. # $p < 0.0001$ vs normal group, *** $p < 0.001$, and **** $p < 0.0001$ vs LPS group.

DISCUSSION

The liver has a major role in metabolism and eliminating xenobiotics, making it a susceptible organ for injury leading to hepatotoxicity[11]. The progression of LPS-induced ALI is implicated by hepatic inflammation, oxidative stress, and tissue hypoperfusion[12]. The cells of the hepatic sinus cavity play an important role as defensive cells that eliminate any foreign bacteria and toxins, regulating the immune response in the body. Presently, the disease of ALI has detrimental effects on patient health and care causing high mortality due to poor diagnosis[13]. Liver injuries are associated with renal failure and systemic oxidative stress [13]. Therefore, a better understanding of the mechanism is needed for a better understanding of acute liver injury[14].

LPS-induced acute liver injury (ALI) is a well-defined animal model that mimics the same etiology as seen in human acute liver injury due to LPS toxins. In the current study, LPS is used to elevate serum liver markers and histological changes. In addition, LPS can further progress to chronic liver injury by generation of reactive oxygen and nitrogen species and hepatocyte apoptosis [15]. The natural phenolic aldehyde, vanillin possesses several beneficial effects for human health, mainly due to its strong antioxidant activity, in addition to its anti-inflammatory, To the best of our knowledge, this work constitutes a finding that explores the hepatoprotective role of vanillin, a polyphenolic agent, against LPS-induced acute liver injury (ALI) in mice in vivo.

Our research focused on early interventions to control overproduction of inflammation which is directly involved in the pathogenesis of ALI. Silymarin is polyphenolic and is sourced from a milk thistle plant Silybum marinum[15]. Silymarin is known as a hepatoprotective agent. The pharmacological effects of silymarin are mainly associated with anti-oxidation and anti-inflammatory effects[16]. Silymarin has been used medicinally to treat acute and chronic hepatic diseases[17]. A study by Zhao et al. stated that a high dose of silymarin (150mg/kg) body weight suppressed liver injury in Kunming mice. They stated that intragastric administration of silymarin resulted in the declined activity of liver biomarkers in their established animal model, along with reduced histopathological formations [17]. In our study, the positive control group silymarin indicates an anti-inflammatory effect by reducing the expression level of interleukin 6 (IL-6). A study conducted by Mohammadi et al showed that the administration of silymarin to fatty liver patients resulted in a decrease in AST, ALT, which is by our results. However, silymarin has poor bioavailability and absorbance, thereby limiting clinical applications [18,19]. Hence, drugs with fewer side effects and reliable

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treatments are urgently required for LPS-induced ALI.

Vanillin has been reported to have anti-inflammatory, antimutagenic, antioxidant properties [20-22]. A study by Sefi et al demonstrated that co-treatment by vanillin lowers the AST, and ALT concentrations compared to the mice treated with maneb which is a fungicide, indicating that vanillin has an important role in maintaining tissue integrity [23]. The results are by our findings as shown in Table 1, administration of vanillin at a low dose significantly decreased the serum biochemical markers. Another study by Guo et al in 2019 showed the anti-inflammatory activity of vanillin attenuating expression of IL-6 and TNF-alpha in RAW264.7 cell lines via ERK1/2, p38, and NF-κ B signaling and further validation by ELISA [24]. Our results show that interleukin 6 (IL-6) expression was elevated in the LPS-damaged group and decreased the production in the pre-treatment and positive control groups as seen in Figure 3.

In the present study, vanillin successfully reduced inflammation in vivo. However, our study has some limitations. We designed an experimental model of liver injury for a shorter duration. In consequence, to further elucidate the molecular mechanism concerning antioxidant parameters and additional inflammatory markers, long-term animal models need to be developed.

CONCLUSION

In this study, we provided evidence to characterize the ability of vanillin to reduce inflammation and decrease serum biochemical markers in acute hepatic injury. Vanillin may have important properties in liver disease. This research exhibits that vanillin pretreatment suppressed the expression of IL-6 in liver tissue lysate in vivo, reducing inflammation and improving liver injury. In the future, further studies are recommended to elucidate the detailed molecular mechanisms of vanillin in disease models.

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CONFLICTS OF INTEREST

The authors declared no conflict of interest.

ETHICAL APPROVAL

The Ziauddin University AEC (Animal Ethics Committee) gave ethical review approval with an animal study protocol (ASP) number 2022-05/RK/FHS.

AUTHORS CONTRIBUTION

All authors contributed equally.

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