J. Biomol. Toxicol., 2(1), 01-10, 2026
https://doi.org/10.56717/jbt.2026.v02i01.06
ISSN(Print): 3134-6537, ISSN(Online): 3134-6545

Journal of Biomolecular Toxicology

Research Article

The histoarchitectural and histomorphometric effects of ß, Ɛ -carotene-3,3′-diol on ethanol-induced hepatotoxicity in adult male Wistar rats

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Abstract

Liver injury is one of the most frequent life-threatening injuries in humans, caused by several factors such as viral agents, ethanol or drugs. The consumption of ethanol is globally, making it one of the leading risk factors for liver injury. This study aimed to evaluate the ameliorative effects of ß, Ɛ- carotene- 3, 3′-diol on ethanol-induced hepatotoxicity in rats. Forty-eight adult male Wistar rats (190 - 220 g) were randomly assigned into six groups (A-F) of eight rats each. Liver injury was induced in rats in groups B-F by the oral administration of 2 mL/kg b.w of 40% ethanol, once daily, for 21 days. After the last ethanol administration, the animals were fasted for twenty-four hours for gastric emptying. Thereafter, the animals in groups C-E were subjected to oral administration of ß, Ɛ-carotene-3,3′-diol, one dose every 12 h, for 21 days. Group F (positive control) rats were treated with oral administration of silymarin, at a dose of at a dose of 200 mg/bw in every 12 h, for 21 days. At the end of the experiment, the animals were sacrificed, the livers were excised and fixed for on histopathological and histomorphological analyses. The results showed that ethanol induced liver injury, characterized by the presence of pathological cell degeneration. Ethanol also reduced the number of intact hepatocytes and the percentage area of reticulin fibers. We conclude that treatment with ß, Ɛ-carotene-3,3′-diol mitigated ethanol-induced liver injury in a dose-dependent manner. This result highlights its ability to ameliorate ethanol-induced liver injury.

Article Information
Received: 27 July 2025
Revised: 20 September 2025
Accepted: 22 September 2025
Published: 19 January 2026

Academic Editor
Prof. Dr. Raffaele Capasso

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Keywords
Hepatotoxicity, ß, Ɛ-carotene-3,3′-diol, ethanol, pyknosis, karyorrhexis, karyolysis.

1. Introduction

Liver injury is a serious pathological condition that is commonly found in many liver diseases. It ranges from mild to severe fatty liver diseases and hepatitis. If the condition worsens, it can lead to liver fibrosis, and cirrhosis, ultimately resulting in liver failure or liver cancer [1]. Liver injury is one of the most frequent life-threatening injuries in humans [2] and it can occur due to several factors, however, the major causes are viral agents, ethanol or drugs [3]. The use of ethanol for recreational purposes dates back to ancient times, with evidence suggesting intentional ethanol production as early as 9000 years ago. Specifically, traces of fermented beverages made from rice, honey, and fruit have been found in China and Georgia dating back to 6000 and 7000 BC respectively [4]. Statistically, it has been documented that ethanol

is accepted in society and culture, statistics reported that 84.9% of adults who are 18 years or older consumed alcohol at least once in their lifetime [5]. When consumed, it causes toxicity to several organs, such as the brain, heart, gastrointestinal tract, and liver, and is related to habitual ethanol consumption [6-8]. The liver is susceptible to damage because it is the primary site of ethanol metabolism in the body due to the high levels of ethanol metabolizing enzymes [9].

About two million deaths are attributed to liver disease annually accounting for 4% of all deaths [10]. This makes it the third leading risk factor for disease and disability globally, due to its high consumption rate [3, 11]. These deaths are primarily related to complications associated with cirrhosis and hepatocellular carcinoma [12]. In Sub-Saharan African countries, approximately 18 % of liver injuries were attributed to ethanol consumption [13]. According to report by Nigerian hospitals, liver diseases accounted for 7.9% of medical admissions, indicated that ethanol consumption was the main risk factor, responsible for 52.1 % of these medical admissions [14].

Ethanol-induced liver injury mainly involves structural damage to liver cells through oxidative stress, lipid accumulation, and inflammation [15]. Also, several authors have posited that ethanol causes severe alterations in liver histoarchitecture [16, 17] through elevated alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) levels.

One of the factors contributing to liver injury is drug use [3], for instance, more than 900 toxins and drugs have been known to cause liver related injury [18], and the side effects of some of the drugs used in the management of liver related diseases cause adverse effects like jaundice, neuropsychological events, nausea, diarrhea, skin reactions, and gastrointestinal problems [19]. This has led to the withdrawal of one-third of the approved drugs from circulation. Consequently, researchers have recently shifted their focus to plant nutrients as alternative treatments for ethanol-induced hepatotoxicity. One of such plant nutrient is ß, Ɛ-carotene-3, 3'-diol, a natural source of antioxidant that-is mostly found in carrots, peas, and spinach. Due to its availability in fruits and vegetables, it is often used as a food supplement, providing prophylactic effects against eye-related diseases, such as cataracts, and has prophylactic effects against health-related diseases [20, 21]. ß, Ɛ-Carotene-3, 3' diol is different from other carotenoids in that it has hydroxyl groups at both ends and conjugated double bonds, giving its anti-inflammatory and anti-oxidant properties, [22] making it more efficacious, compared with other forms of carotenoids. Therefore, this study aimed to investigate the histological effects of ß, Ɛ-carotene-3,3'-diol on the histoarchitecture and histomorphometry of the liver in ethanol-induced hepatotoxicity in adult male Wistar rats.

2. Materials and methods

2.1. Chemicals and reagents

ß, Ɛ-Carotene-3,3'-diol was procured from Ambeed Inc., United States of America, Silymarin tablets (140 mg) were obtained from a pharmaceutical outlet at the Obafemi Awolowo University teaching hospital complex, Ile-Ife Osun state, Nigeria. Ethanol was procured from SIGMA Aldrich, USA.

2.2. Experimental animal

Forty-eight presumably healthy Wistar rats were procured from the Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife, Nigeria.

2.3. Study design

The rats were randomly divided into six groups (n=8). The rats in group A received 2 mL/kg b.w. of distilled water. Liver injury was induced in the animals in groups B-F by the oral administration of 2 mL/kg b.w of 40% ethanol, once daily, for 21 days [23]. After the last ethanol administration, the rats were fasted for 24 h [24] for gastric emptying. The rats in groups C-E were subjected to oral administration of ß, Ɛ-carotene-3,3'-diol at 10, 20, and 40 mg/kg b.w., respectively, one dose every 12 h, for 21 days. Group F (positive control) rats were treated orally with silymarin at 200 mg, one dose every 12 h, for 21 days.

2.4. Animal sacrifice and analyses

The rats were sacrificed 24 h after the last administration under ketamine anesthesia. The livers were excised and then fixed. After fixation, the tissues were processed for histological demonstrations. For the assessment of liver function, blood samples were

https://doi.org/10.56717/jbt.2026.v02i01.06

obtained, the serum samples were used to measure the chemical integrity of the liver by evaluating the enzymatic activity of alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

2.5. Histopathology

The hepatocyte counts were carried out on H & E-stained sections to quantify the percentage area of intact and necrotic hepatocytes following ethanol-induced hepatotoxicity. A calibration of 480 x 640 pixels was set, the desired photomicrograph was opened in Image J, and a grid (4000 μm^2 area per point) was applied across the image. To quantify hepatotoxicity, hepatocytes were counted in 7 high power fields (400x) taking account of the following morphological criteria: increased eosinophilia, cell swelling and lysis. The total numbers of individual cells in the photomicrograph was obtained, number was summed, and each was expressed as percentage. Percentage of intact hepatocytes was calculated as:

$$\frac{\text{Intact hepatocytes}}{\text{Total hepatocytes}} \text{x} 100$$

Percentage of necrotic hepatocytes was calculated as:

$$\frac{\text{Necrotic hepatocytes}}{\text{Total hepatocytes}} \text{x} 100$$

Gordon and Sweet's stains are used specifically for the demonstration of reticular fibers. Sections were then mounted for microscopic examination. Image J software was used to determine Reticulin Fiber Morphometry in each group, as shown in Fig. 4.

The percentage area of reticulin fiber was calculated using the following formula.

$$\frac{\text{Mean of total area captured}}{\text{Area covered by reticular fibers}} \text{x} 100$$

All images were archived using a Leica DM 750 microscope with an ICC50 camera attached.

2.6. Statistical analysis

Data were expressed as Mean ± SEM. Statistical significance between groups was determined by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Body weight change

There was a non-significant decrease ($p > 0.05$) in the body weight of the rats in Group B compared to group A. However, there was a dose-dependent significant increase ($p < 0.05$) in the body weight of the rats treated with ß, Ɛ-carotene-3, 3'-diol, as shown in Fig. 1. A non-significant increase in body weight was observed in the body weight of rats in group E when compared to group A.

The administration of ethanol significantly increased ALT, AST, and ALP activities compared to the control group, as shown in Table 1. However, a dose dependent reduction in AST, ALT and ALP activities was observed in the groups treated with ß, Ɛ-carotene-3,3'-diol. There was also a non-significant difference ($p > 0.05$) in the ALT and AST levels in group A when compared to group E, however, there was a significant difference ($p<0.05$) between group E (high dose) and F (positive control) in ALT level and a non-significant difference ($p > 0.05$) in both groups in the AST levels. A significant difference ($p < 0.05$) was also observed in the ALP level in group E compared to group A.

Photomicrographs from group A rats showed a normal histoarchitectural outline with radially apparent sinusoids, linearly arranged hepatocytes and a prominent central vein (Fig. 2). In the negative control (Group B), features of cellular degeneration characterized by pyknosis, karyorrhexis, and inflammation of hepatocytes were observed. Signs of blood sinusoid dilatation and hemorrhagic congestion in the central vein were also observed. However, in groups C, D, and E, there was a dose-dependent restoration of histoarchitectural arrangements characterized by a marked reduction in cellular degeneration, vacuolation, less congestion of the central vein and sinusoidal dilatation, especially in group E.

The results of hepatocytes count in the liver sections following ethanol-induced liver injury and the effects of ß, Ɛ-carotene-3,3'-diol and silymarin (positive control) treatments are shown in Figs. 3a and 3b. This highlights the percentage of intact and necrotic hepatocytes based on cellular degeneration such as pyknosis, karyorrhexis, karyolysis, and necrosis, in the six experimental groups. The effects of ß, Ɛ-carotene-3,3'-diol were dose dependent with little to

https://doi.org/10.56717/jbt.2026.v02i01.06

Table 1. Effects of ß, Ɛ-carotene-3,3′-diol on the serum level of AST, ALT and ALP following ethanol-induced hepatotoxicity in adult male Wistar rats.

Groups	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
Group A	108 ± 2.89	147 ± 8.85	85.0 ± 1.41
Group B	501 ± 23.5 $^{\alpha}$	556 ± 3.70 $^{\alpha}$	214.0 ± 3.28 $^{\alpha}$
Group C	421 ± 9.67 $^{\alpha\beta}$	422 ± 3.12 $^{\alpha\beta}$	126.0 ± 4.83 $^{\alpha\beta}$
Group D	342 ± 4.85 $^{\alpha\beta}$	335 ± 1.73 $^{\alpha\beta}$	121.0 ± 6.54 $^{\alpha\beta}$
Group E	121 ± 8.80*$^{\beta}$	197 ± 2.54 $^{\alpha\beta}$	103 ± 3.38 $^{\alpha\beta}$
Group F	131 ± 14.4*$^{\beta}$	200 ± 2.01 $^{\alpha\beta}$	94.9 ± 0.85*$^{\beta}$

Values are given as Mean ± SEM, means with α differs significantly at P<0.05 to group A while ✱ does not differ significantly at P>0.05 to group A. β differs significantly at P<0.05 to group B.
Group A: Normal control, Group B: Negative control, Group C: Treated with 10 mg/ kg b.w of ß, Ɛ-carotene-3,3′-diol, Group D: Treated with 20 mg/kg b.w of ß, Ɛ-carotene-3,3′-diol, Group E: Treated with 40 mg/kg b.w of ß, Ɛ-carotene-3,3′-diol, Group F (positive control): Treated with 200 mg/kg b.w of Silymarin.

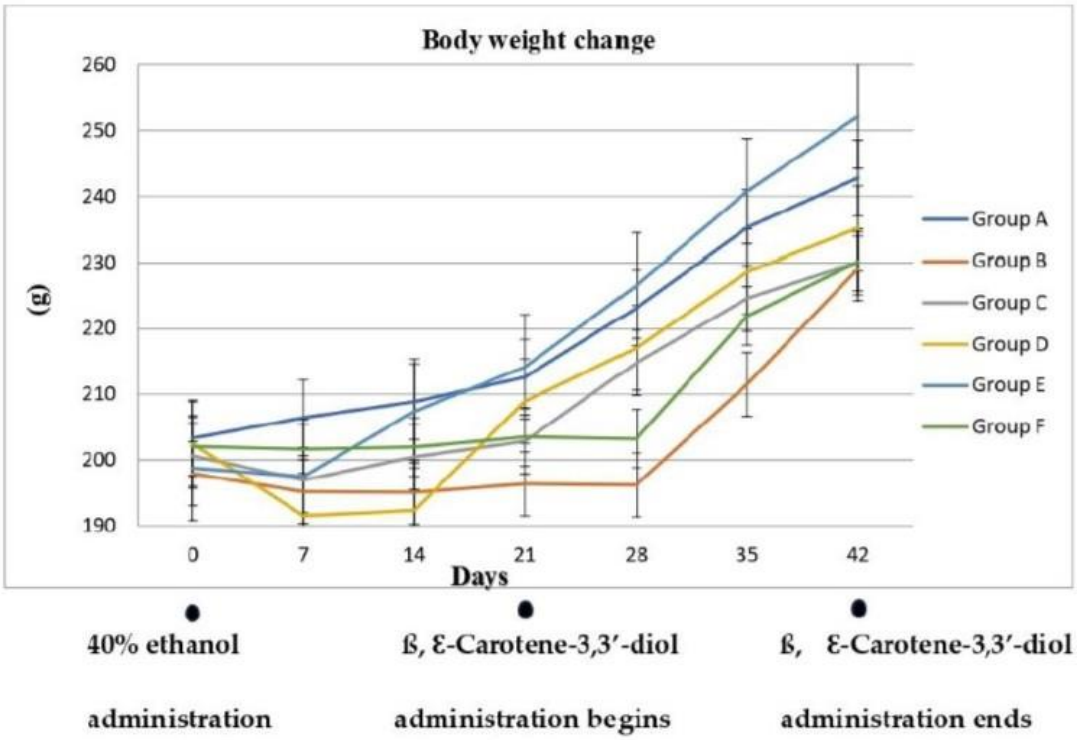

Figure 1. Effects of ß, Ɛ-carotene-3, 3′- and silymarin on body weight of Wistar rats following ethanol-induced hepatotoxicity.

no cellular degeneration in groups D and E.

The result showed a significant total depletion of reticular fibers in group B compared with group A. Treatment with ß, Ɛ-carotene-3,3′-diol helped restore the reticulin framework. This was underscored by weak staining intensity, which was significantly lower ($p < 0.05$) in groups C, and D, compared to normal control, as shown in Figs 4 and 5. However, the reticular fiber density in group E (high dose ß, Ɛ-carotene-3,3′-diol treated groups) and group F (silymarin-treated) were observed to be non-significant ($P > 0.05$) that compared to the normal control group.

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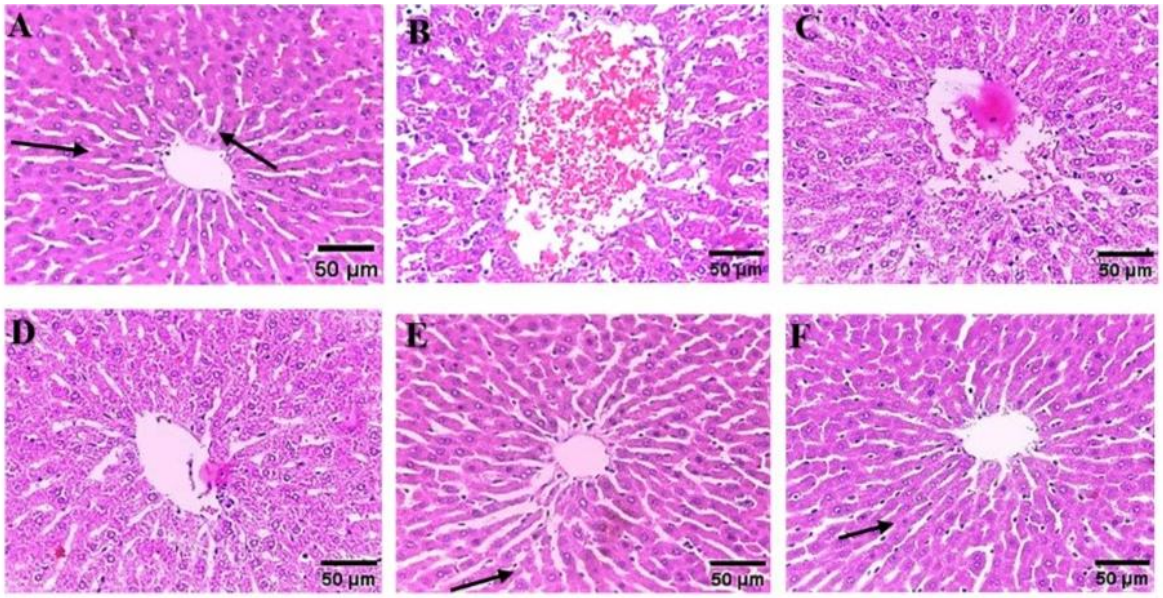

Figure 2. Light micrographs of the liver subjected to ethanol-induced hepatotoxicity then treated with ß, Ɛ-Carotene-3,3′diol and silymarin. H&E x400. A: Normal control, B: Ethanol treated (note the distortion of histoarchitectural appearance), C: Treated with 10 mg/kg b.w of ß, Ɛ- carotene-3,3′-diol, D: Treated with 20 mg/kg b.w of ß, Ɛ-carotene-3,3′diol, E: Treated with 40 mg/kg b.w of ß, Ɛ- carotene-3,3′-diol and F: Treated with 200 mg/kg b.w of silymarin.

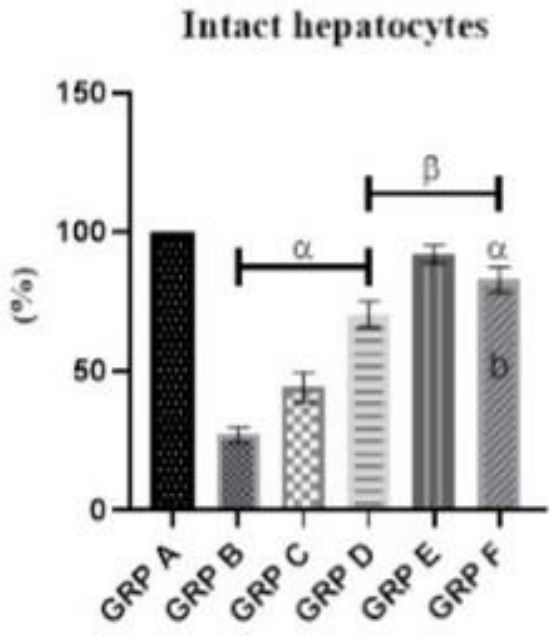

Figure 3a. Effects of ß, Ɛ-carotene-3, 3′-diol and silymarin on percentage of intact hepatocyte in Wistar rats following ethanol-induced hepatotoxicity, n = 5. Mean ± SEM, ANOVA, $p < 0.05$. Group A = Control; Group B = treated with 40% ethanol; Group C = treated with 10 mg/kg b.w of ß, Ɛ- carotene -3, 3′-diol; Group D = treated with 20 mg/kg b.w ß, Ɛ- carotene - 3, 3′-diol; Group E = treated with 40 mg/kg b.w ß, Ɛ- carotene -3, 3′-diol; Group F = treated with 200 mg/kg b.w of silymarin.

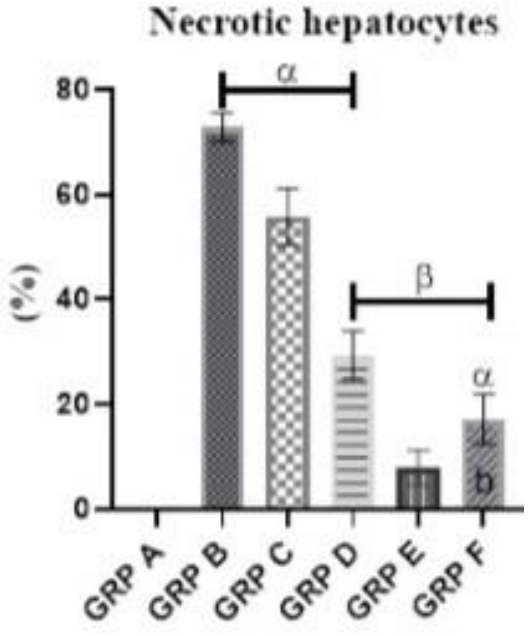

Figure 3b. Shows the effects of ß, Ɛ- carotene-3, 3′ diol and silymarin on percentage necrotic hepatocyte of Wistar rats following ethanol-induced hepatotoxicity, n = 5. Mean ± SEM, ANOVA, $p < 0.05$. Group A = Control; Group B = treated with 40% ethanol; Group C = treated with 10 mg/kg b.w of ß, Ɛ- carotene -3, 3′-diol; Group D = treated with 20 mg/kg b.w ß, Ɛ- carotene - 3, 3′-diol; Group E = treated with 40 mg/kg b.w ß, Ɛ- carotene -3, 3′-diol; Group F = treated with 200 mg/kg b.w silymarin.

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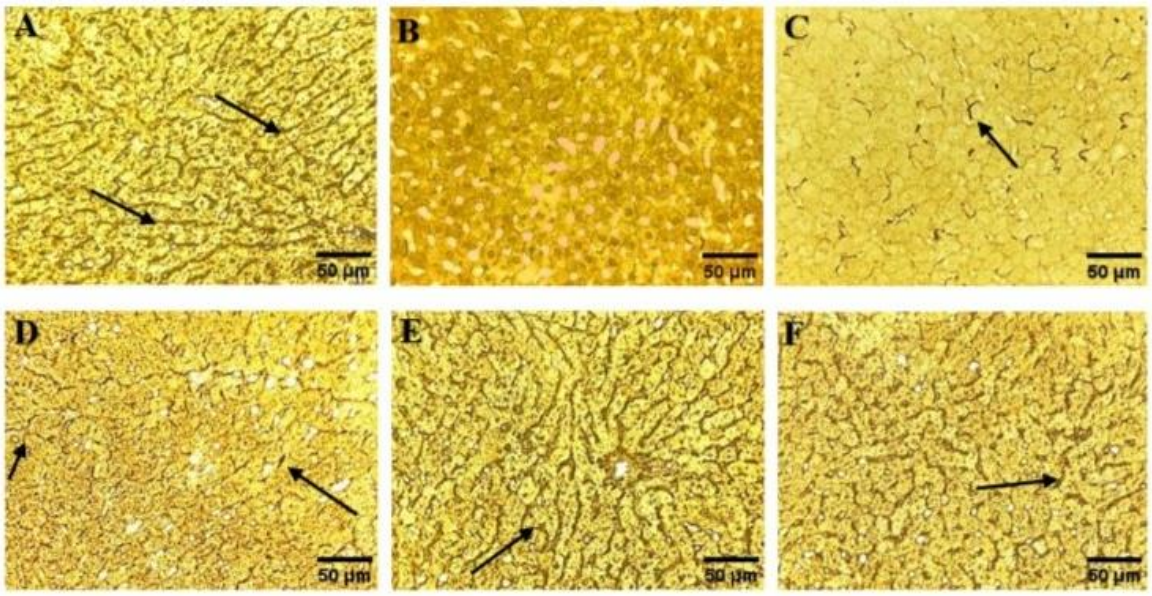

Figure 4. Light micrographs of the liver subjected to ethanol-induced hepatotoxicity then treated with ß, ε-carotene-3,3′diol and silymarin. Gordon and sweet x400. A: Normal control, B: Negative control (note the depletion of reticular fibers), C: Treated with 10 mg/kg b.w of ß, Ɛ- carotene-3,3′-diol, D: Treated with 20 mg/kg b.w of ß, Ɛ-carotene-3,3′diol, E: Treated with 40 mg/kg b.w of ß, ε-carotene-3,3′-diol and F: Treated with 200 mg/kg b.w of silymarin.

The result showed a significant total depletion of reticular fibers in group B compared with group A. Treatment with ß, Ɛ-carotene-3,3′-diol helped restore the reticulin framework. This was underscored by weak staining intensity, which was significantly lower ($p < 0.05$) in groups C, and D, compared to normal control, as shown in Figs 4 and 5. However, the reticular fiber density in group E (high dose ß, Ɛ-carotene-3,3′-diol treated groups) and group F (silymarin-treated) were observed to be non-significant ($P > 0.05$) that compared to the normal control group.

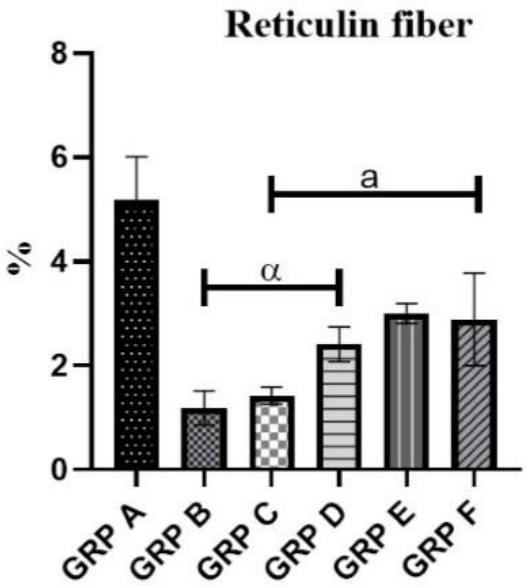

Figure 5. Effects of ß, Ɛ- carotene-3, 3′-diol and silymarin on the percentage area of reticular fibers following ethanol-induced hepatotoxicity, n = 5. Mean ± SEM, ANOVA, $p < 0.05$. Group A = Control; Group B = treated with 40% ethanol; Group C = treated with 10 mg/kg b.w of ß, Ɛ-carotene -3, 3′-diol; Group D = treated with 20 mg/kg b.w ß, Ɛ- carotene - 3, 3′-diol; Group E = treated with 40 mg/kg b.w ß, Ɛ- carotene -3, 3′-diol; Group F = treated with 200 mg/kg b.w. of silymarin.

4. Discussion

The acute and chronic usages of alcohol leading to liver toxicity in both animals and humans has been well documented. These effects are due to several mechanisms by which ethanol induces hepatotoxicity [14, 25-28]. One week after ethanol administration, there was a reduction in the body weight of the animals in groups B to F, compared to that in group A (control). The loss of body weight observed in this study may be due to the effect of acute ethanol administration, which causes alterations in gastric acid secretion, leading to gastric injury. This agrees

https://doi.org/10.56717/jbt.2026.v02i01.06

with previous study [29] that reported that a discontinuation in the lining of the gastrointestinal tract due to gastric injury can cause a reduction of appetite, and consequently low food intake, hence the loss of body weight. Ethanol also causes the disruption of hunger hormones like leptin and ghrelin, leading to a false feeling of fullness, corroborating a study that reported that a reduction in food intake due to the disruption of these hormones caused loss of body weight [30]. This may be responsible for the loss of body weight observed in this study. However, the administration of ß, Ɛ-carotene-3,3'- diol caused an increase in body weight, which may be a result of its ability to support gastric healing by inhibiting inflammation and oxidative stress in gastric tissues. It also helps to boost digestive functions and the recovery process within the gastric mucosa, in agreement with a recent study [30] that highlighted its ability to heal gastric injury caused by ethanol.

In addition, the restoration and/ or regulation of hunger and satiety hormones by ß-carotene, a precursor to vitamin A, might be linked to its ability to promote fatty acid oxidation that was otherwise reduced by ethanol. This underscores the ability of ß, Ɛ-carotene-3,3'- diol to regulate long term energy balance, and control short term appetite, thereby leading to an increase in body weight.

A normal liver histoarchitecture is essential for optimal liver function, metabolism, detoxification, and synthesis of vital proteins and enzymes [31, 32]. Liver enzymes are proteins that speed up chemical reactions in the liver [33], and they include ALT, AST, and ALP, which are measured to determine the liver health [34]. Liver disease is usually associated with elevated levels of these enzymes [35, 36] following ethanol consumption, because acetaldehyde increases oxidative stress by causing an imbalance between free radicals and antioxidants in the body. This leads to inflammation and injury to liver cells, and when liver cells are damaged, they leak their internal components, including liver enzymes into the bloodstream. This agrees with recent studies [36, 37] that similarly reported that an increase in these serum markers is an indication of liver damage. Ethanol can also initiate the loss of hepatocytes through mitochondrial-mediated apoptosis, which contributes to elevated liver enzyme levels. The pattern of elevated ALT, AST, and ALP levels in ethanol-treated rats aligns also with previous studies that documented the hepatotoxic effects of ethanol [18, 38].

However, the ß, Ɛ-carotene-3,3'-diol treated groups showed a dose-dependent restoration of the optimum levels of hepatic function markers, where the highest dose of ß, Ɛ-carotene-3,3'diol (40 mg/kg b.w) was observed to be of prominent efficacy. This is because ß, Ɛ- carotene-3,3'diol acts primarily through its antioxidant actions that combat oxidative stress by protecting the liver from ethanol induced injury. Also, ß, Ɛ-carotene-3,3'diol rich fruits like carrots, spinach, broccoli, etc. have been shown to inhibit mitochondrial-mediated apoptosis in the liver. They also help to safeguard the liver by lowering the elevated enzyme levels, thus restoring and protecting the liver from further damage.

Similarly, ß, Ɛ-carotene-3,3'diol is rich in naturally occurring phytochemical compounds like saponins, flavonoids and polyphenols. These bioactive compounds exhibit protective properties and contribute to its health and therapeutic benefits by scavenging free radicals and reactive oxygen species which are known to damage liver cells and contribute to elevated liver enzyme levels in restoring the elevated of liver enzymes. On the other hand, in comparing ß, Ɛ-carotene-3,3'diol-treated group with the silymarin-treated group, a result almost similar to the highest treated group was observed. This indicates that ß, Ɛ-carotene-3,3'diol at a high dose might offer a somewhat superior restorative effect than silymarin.

Histologically, the administration of ethanol in this study caused some histoarchitectural distortions characterized by hemorrhagic congestion of the central vein, apoptosis, necrosis, pyknosis, karyorrhexis, karyolysis and hepatocellular vacuolation. Ethanol metabolism leads to hepatocyte damage and triggers inflammatory mediators like IL-6 and TNF-α that damage sinusoidal endothelial cells. This damage causes sinusoidal hemorrhage and dilatation, which is visible as hemorrhagic congestion around the central vein, a key feature of ethanol-induced liver injury. This corroborates several studies which reported that ethanol causes disruptions such

https://doi.org/10.56717/jbt.2026.v02i01.06

as sinusoidal dilatations, hemorrhagic congestion of the central vein, hepatocellular vacuolation and pathologic cell injury [5, 12, 39, 40] in ethanol-fed rats. Treatment with ß, Ɛ-carotene-3,3′-diol, especially in the high-dose group showed histoarchitectural restoration characterized by a prominent central vein, normal hepatocytes, and significant restoration of radially arranged sinusoids. This may be due to its anti-inflammatory mechanism, which is vital for promoting cell viability and survival. The dose-dependent ameliorative effects observed in this study indicate that higher doses provide greater protection [30] due to increased association in lowering the inflammatory mediators. Silymarin treatment also elicited a similar response as a curative agent.

In terms of liver framework and integrity, reticular fibers, composed of collagen type III provide a scaffold to maintain liver architecture. The total distortion of the reticular network observed in the liver parenchymal cells in the ethanol-treated group might be due to the effect of ethanol, which causes dilatation and collapse of the sinusoid. This corroborates a study that reported that the liver's structural integrity can easily be compromised in cases of toxicity [41]. In addition, it was also reported that the weakening of the reticulin framework is due to toxicity that makes sinusoids prone to collapse causing a depletion of reticular fibers, and overall disruption of structural integrity [42]. However, the administration of graded doses of ß, Ɛ-carotene-3,3′-diol countered the effect of ethanol in a dose dependent manner by restoring the reticular framework. This is in line with a report that underscores the ability of ß, Ɛ-carotene-3,3′- diol to restore reticulin fiber integrity [43].

5. Conclusions

The results of this study revealed the ameliorative abilities of ß, Ɛ-carotene-3,3′-diol in compromised structures on the liver following ethanol-induced hepatotoxicity. While the silymarin-treated group also exhibited ameliorative properties, the effect of ß, Ɛ-carotene 3,3′-diol, especially at a high dose, is of notable importance. These results were mostly similar to those of control, an effect believed to be connected to its anti-inflammatory and antioxidant properties. These potentials can be harnessed in the quest for alternative therapy for the management of liver injury in Wistar rats.

Disclaimer (artificial intelligence)

Author(s) hereby state that no generative AI tools such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators were utilized in the preparation or editing of this manuscript.

Ethical statements

The study was reviewed by the Health Research and Ethics Committee (HREC) of Obafemi Awolowo University (OAU). The study was conducted according to the guidelines of the declarations of OAU, approved by HREC of the Institute of Public Health (IPH), Obafemi Awolowo University of Ile-Ife, Nigeria. An approval number IPH/OAU/2655 was obtained.

Authors' contributions

Conceptualization, funding acquisition, methodology, T.V.O., D.A.O.; data curation, formal analysis, project administration, writing (original draft), T.V.O.; Investigation, resources, T.V.O., V.O.A., D.A.O.; supervision, validation, D.A.O.; visualization, writing (review and editing), V.O.A., D.A.O.

Acknowledgements

The authors would like to acknowledge Mr. M.S. Ige of the Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife, Osun state for his histopathological expertise during the course of this research.

Funding

This research received no specific grant from any funding agency.

Availability of data and materials

All data will be made available on request according to the journal policy.

Conflicts of interest

The authors declare no conflict of interest.

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