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Histomorphological effects of ß,Ɛ-Carotene-3,3'-diol on the liver following indomethacin-induced hepatotoxicity in adult male Wistar rats

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ABSTRACT

Medicinal plants are globally used as food and in the management of different ailments based on their peculiar phytochemical contents. This study aimed to evaluate the histomorphological effects of ß,Ɛ-Carotene-3,3'-diol on the liver following indomethacin-induced hepatotoxicity in adult male Wistar rats. This is to determine the ameliorative effect of graded doses of ß,Ɛ-Carotene-3,3'-diol. A total of twenty-five adult Wistar rats were randomly divided into five groups (n=5). Liver injury was induced by oral administration of 20 mg/kg b.w of indomethacin after 24 hours fast to animals in groups B-E. Twenty four hours after the induction, animals in groups C-E were given different doses of ß,Ɛ-Carotene-3,3'-diol. At the end of the experimentation, the animals were sacrificed and the livers were collected and fixed for histopathological and histomorphological analyses. The results showed that indomethacin increased the activities of AST, ALT and ALP and also induced histopathological changes. Treatment with ß,Ɛ-Carotene-3,3'-diol was able to attenuate liver injury caused by indomethacin in a dose dependent manner as evidenced in the histoarchitectural changes. This was also corroborated by the histomorphometric analyses. In conclusion, the results suggested the positive influence of ß,Ɛ-Carotene-3,3'-diol in ameliorating liver injury caused by indomethacin and by extension, may be adopted in the management of liver related injury.

KEYWORDS: ß,Ɛ-Carotene-3,3'-diol, Liver, Indomethacin, Histopathology, Medicinal plants

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INTRODUCTION

Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) used to treat and manage acute and chronic pain just like other NSAIDs. The application of NSAIDs in the management of ailments such as arthritis, inflammation, musculoskeletal disorders, and edema dated back to 1963 (Herminghaus *et al.*, 2020). Despite this huge success, indomethacin has been documented to be implicated in multisystem lesions in viscera such as lungs, brain, liver, blood vessels, glands, intestines and kidneys thereby causing degenerative and or necrotic changes in experimental animals (Khan *et al.*, 2019). For instance, Rudnick *et al.* (2006) revealed that PiZ mice exposed to indomethacin caused severe hepatic injury due to increased hepatocellular proliferation and increased activation of caspase 9. Also, indomethacin was reported by Mahmoud *et al.* (2021) to increase liver enzymes and induce alterations in the histoarchitecture of the liver of adult male Wistar rats. NSAIDs have been documented to inhibit COX- 1 leading to deleterious effects due to the reduction in prostaglandin (PG) synthesis (Gunaydin & Bilge, 2018; Drożdżal *et al.*, 2021) while inhibition of COX-2 is associated with their anti-inflammatory effect (Sohail *et al.*, 2023).

About two million deaths annually are attributable to liver disease and this account for 4% of all deaths (Devarbhavi *et al.*, 2023). These deaths are majorly related to complications associated with cirrhosis and hepatocellular carcinoma. Cirrhosis occurs due to viral hepatitis, alcohol, and non-alcoholic fatty liver disease. Though acute hepatitis is sometimes caused by hepatotropic viruses, drug-induced liver injury also accounts for a significant proportion of cases (Devarbhavi *et al.*, 2023). Most times, acute liver failure occurs in relation to hepatic injury, hepatic encephalopathy and an elevated prothrombin time/international normalized ratio (Goldberg *et al.*, 2018). According to Mehta (2022), more than 900 toxins and drugs

have been known to cause liver related injury. This has led to the withdrawal of one-third of approved drugs from circulation. About 75% of the idiosyncratic drug reactions lead to liver transplantation or death. As a result of this, liver injury should be urgently attended to because of its life threatening capacity. Over the years, medicinal plants have been a source of relief to so many medical complications due to their numerous health benefits and availability (Farzaneh & Carvalho, 2015; Goldberg *et al*., 2018). Apart from being sources of food, medicinal plants are also very useful in the management of ailments globally due to their phytochemical contents (Owojuyigbe *et al*., 2020). Phytochemicals such as phenol, carotenoid, vitamins and flavonoids, are responsible for most of the protective potentials of some of these herbal therapies (Vardi *et al*., 2008; Amen *et al*., 2020) As a result of this, the consumption of leafy vegetables and fruits have been advocated by several authors (Vardi *et al*., 2008; Randhawa *et al*., 2015; Zerfu *et al*., 2018). According to Bhattacharyya *et al*. (2010), ß,Ɛ-Carotene-3,3'-diol is mostly found in leafy vegetables and member of the Xanthophyll family; it is a non-provitamin type of carotenoid. Previous studies have confirmed the health benefits of ß,Ɛ-Carotene-3,3'-diol to include but not limited to anti-inflammatory, antioxidant, anti-cancer, anti-hypertensive and anti-diabetic effects (Johnson, 2014; Erdman *et al*., 2015). ß carotene, also a form of carotenoid, is known to be a powerful free radical scavenger and a chain breaking antioxidant thereby offering great protection on viscera such as the heart and preventing it against heart attack. A diet rich in beta carotene prevent osteoarthritis among many other health benefits (Kumar *et al*., 2025). Beta carotene is still the most potent of the over 600 compounds that have been characterized so far (Vardi *et al*., 2010) and exhibits antioxidant activity primarily by directly reacting with peroxy radicals, suppressing singlet oxygen and scavenging peroxide radicals thereby stabilizing membrane lipids from free radical attack (Vardi *et al*., 2010).

This study therefore hypothesized that ß,Ɛ-Carotene-3,3'-diol could attenuate indomethacin-induced hepatotoxicity in adult male Wistar rats.

MATERIALS AND METHODS

Chemicals and Reagents

Indomethacin was procured from a certified pharmaceutical store at the premises of Obafemi Awolowo University, Nigeria, ß,Ɛ-Carotene-3,3'-diol was procured from Ambeed Inc, United States of America.

Experimental Animal

This research was executed following the approval from the Health Research Ethics Committee (HREC) of the Obafemi Awolowo University, Ile-Ife, Nigeria. The HREC number for the study is IPH/OAU/2032. Twenty-five adult presumably healthy Wistar rats were procured from the Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife, Nigeria. The animals were randomised into five groups (n=5).

Study Design

Twenty-five adult male Wistar rats were used for the study.

The rats were randomly assigned into 6 groups with five animals each: A, B, C, D and E

Group A: Control group (1 mL/kg normal saline + 1 mL/kg 20% tween 80, 12 hourly)
Group B: Indomethacin + 1 mL/kg b.w 20% tween 80, 12 hourly
Group C: Indomethacin + 20 mg/kg b.w of ß,Ɛ-Carotene-3,3'-diol, 12 hourly
Group D: Indomethacin + 40 mg/kg b.w of ß,Ɛ-Carotene-3,3'-diol, 12 hourly
Group E: Indomethacin + 80 mg/kg b.w of ß,Ɛ-Carotene-3,3'-diol, 12hourly

The administration went on for 2 weeks.

Induction of Liver Injury

Twenty-four hours after the animals were fasted, liver injury was effected for rats in groups B-E by a single oral administration of 20 mg/kg b.w of indomethacin (Khan *et al*., 2019) while animals in group A received an equivalent volume of normal saline. Twenty-four hours after liver injury induction, the animals in each group were subjected to the treatments outlined in the study design.

Animal Sacrifice

The animals were sacrificed 24 hrs after the last administration under chloroform anesthesia. The animals were pinned on a dissecting board to achieve a midline incision and have unhindered access to the organ of interest. Blood samples were collected through a syringe from the left ventricles while the livers were excised rinsed in normal saline, blotted dry on filter paper and fixed in 10% formol saline.

Determination of Liver Function Test

The liver function test was carried out on the plasma of each of the rats. The procedure for the assessments of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) was in accordance with the protocol manuals that accompanied the Randox standard laboratory kits.

Histological Procedures/Morphometric Analyses

The fixed tissues were processed for routine paraffin embedding and thereafter, subjected to H & E staining for routine histological demonstration as well as Gordon & Sweet for reticular fibre demonstration. All images were archived using Leica a DM 750 with an ICC50 camera attached. Photomicrographs from slides stained with Gordon & Sweet were imported into image J software for the quantification of reticular fibre deposit in each of the groups by measuring the stained area per 1600 mm^2 on five random spots on each

slide in a group. The values generated which represented the staining intensity were inputted through a statistical package (SPSS 22) for analysis.

Statistical Analysis

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

RESULTS

Assessment of Liver Function Test

The result of the liver function tests showed a significant increase ($p<0.05$) in the AST, ALT and ALP in the negative control (Group B) when compared with the control and the ß,Ɛ-Carotene-3,3'-diol treated groups. There was a dose dependent reduction in AST, ALT and ALP activities when treated with ß,Ɛ-Carotene-3,3'-diol. The highest dose of ß,Ɛ-Carotene-3,3'-diol (80 mg/kg b.w) was observed to be close to normal control as shown in Table 1.

Histopathology-H&E Staining

The result of rats in group A showed a normal histoarchitectural outline with radially arranged hepatocytes around the central vein when compared with a negative control group (Group B) which was characterised by fat droplets, neutrophils infiltration, steatosis and microvesicular stenosis as shown in Figure 1. There were also signs of degeneration and inflammation of most of the hepatocytes of animals in group B rats; the photomicrographs of the liver revealed congested blood vessels and dilated congested intervening blood sinusoids. The histoarchitectural organization of ß,Ɛ-Carotene-3,3'-diol treated groups showed a mild degeneration of the hepatocytes and little or absence of microvesicular stenosis. These observations were dose dependent as shown in Figure 1.

Histopathology-Gordon & Sweet Staining

Gordon and Sweet stains are specific for reticular fibres. There was sparse distribution of reticular fibres in group B (negative control) when compared with the vehicle control group (Figure 2) as evidenced by weak staining intensity. This was underscored by the low density of reticular fibres observed in the negative control group (Table 1). The reticular fibre density in the ß,Ɛ-Carotene-3,3'-diol treated groups were observed to be significantly ($P<0.05$) higher in a dose dependent manner when compared to the negative control group (Figure 2 & Table 2).

DISCUSSION

The long term usage of NSAIDs leading to organ toxicity both in animals and humans has been well documented despite the anti-inflammatory, analgesic and antipyretic potentials (Khan *et al.*, 2019). Indomethacin exposure in previous animal experimentations has led to significant morphological alterations (Ejaz *et al.*, 2004; Taiwo & Contah, 2008; Khan *et al.*, 2019) most especially in viscera (Basivireddy *et al.*, 2004; Perron *et al.*, 2013; Chen *et al.*, 2020). The indiscriminate usage of NSAIDs may be attributed to their availability either through prescription or over-the-counter (Chen *et al.*, 2020). Devarbhavi *et al.* (2023) posited that drug-induced liver toxicity has increased over the years and is responsible for 4% of about two million liver-related deaths annually. The susceptibility

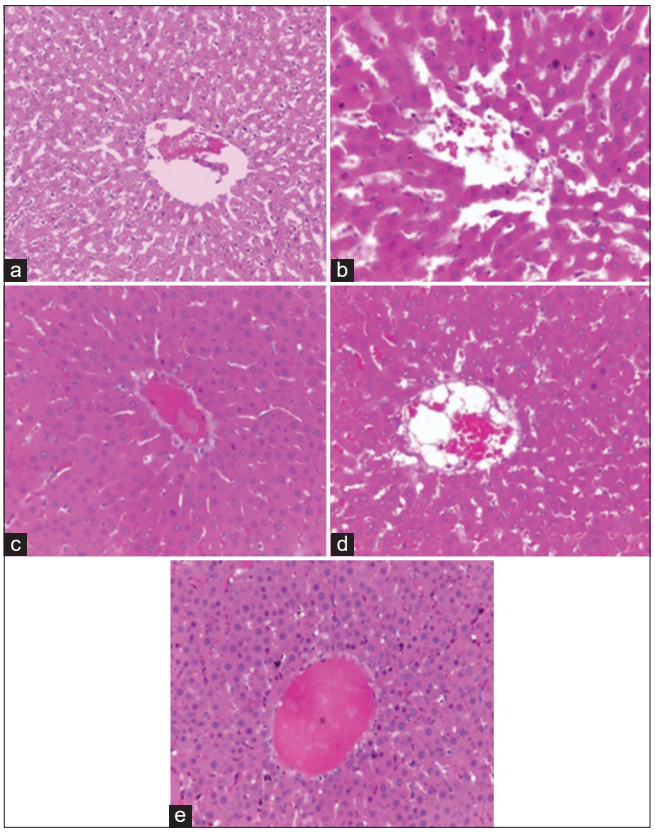

Figure 1: Photomicrographs of the liver in a) Group A: Normal control, b) Group B: Negative control (note the disruption in the hepatic parenchyma), c) Group C: Treated with 20 mg/kg b.w of ß,Ɛ-Carotene-3,3'-diol, d) Group D: Treated with 40 mg/kg b.w of ß,Ɛ-Carotene-3,3'-diol and e) Group E: Treated with 80 mg/kg b.w of ß,Ɛ-Carotene-3,3'-diol (note the similarity with group A)

Table 1: Effects of ß,Ɛ-Carotene-3,3'-diol on the Plasma Levels of AST, ALT and ALP following indomethacin-induced hepatotoxicity in adult male Wistar rats

Groups	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Group A	61.80 ± 3.35^{a}	39.40 ± 3.14^{a}	91.20 ± 6.47^{a}
Group B	167.20 ± 12.24^{d}	84.40 ± 6.88^{c}	145.00 ± 9.35^{c}
Group C	130.00 ± 2.73^{c}	72.40 ± 3.04^{bc}	130.20 ± 1.59^{bc}
Group D	89.00 ± 4.00^{b}	64.20 ± 2.20^{b}	125.40 ± 3.02^{b}
Group E	70.20 ± 2.74^{a}	47.60 ± 5.76^{a}	100.00 ± 1.58^{a}

Values are given as Mean±SEM, means with different letters within the column differs significantly at $p<0.05$ while means with the same letters does not differ significantly at $p<0.05$, Group A: Normal control, Group B: Negative control, Group C: Treated with 20 mg/kg b.w of ß,Ɛ-Carotene-3,3'-diol, Group D: Treated with 40 mg/kg b.w of ß,Ɛ-Carotene-3,3'-diol, Group E Treated with 80 mg/kg b.w of ß,Ɛ-Carotene-3,3'-diol

Table 2: Effects of ß, Ɛ-Carotene-3, 3′-diol on Mean reticular fibre density per 1600 mm² following indomethacin-induced hepatotoxicity in adult male Wistar rats

Groups	Group A M±SEM	Group B M±SEM	Group C M±SEM	Group D M±SEM	Group E M±SEM
Reticular fibre density/1600 mm²	153.40±3.25^{d}	69.40±1.47^{a}	100.00±2.63^{b}	121.00±3.32^{c}	147.00±2.55^{d}

Values are given as Mean±SEM, means with different letters differs significantly at $p<0.05$ while means with the same letters does not differ significantly at $p<0.05$, Group A: Normal control, Group B: Negative control, Group C: Treated with 20 mg/kg b.w of ß,Ɛ-Carotene-3,3′-diol, Group D: Treated with 40 mg/kg b.w of ß,Ɛ-Carotene-3,3′-diol, Group E: Treated with 80 mg/kg b.w of ß,Ɛ-Carotene-3,3′-diol

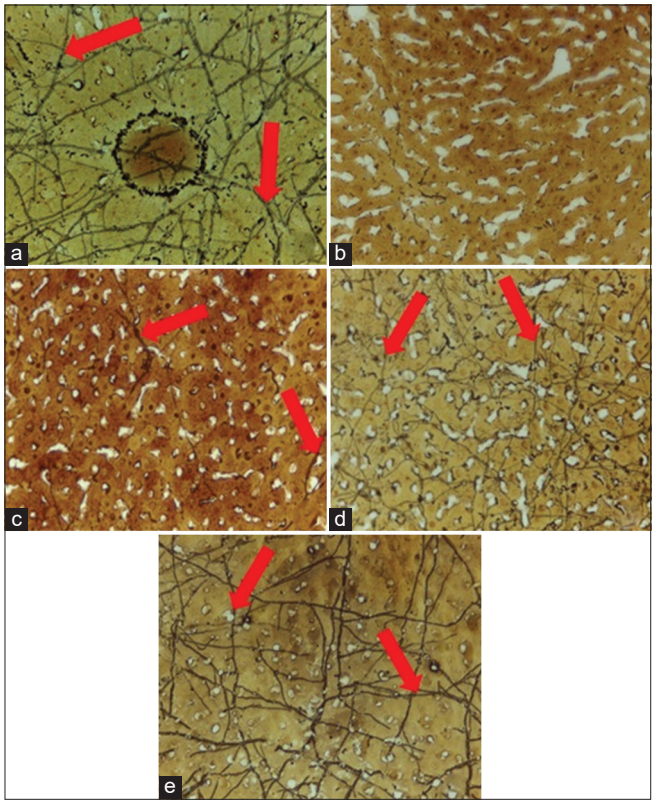

Figure 2: Photomicrographs of the liver in a) Group A: Normal control (note the depletion of reticular fibres), b) Group B: Negative control (note the abundant reticular fibres), c) Group C: Treated with 20 mg/kg b.w of ß,Ɛ-Carotene-3,3'-diol (note the gradual restoration of reticular fibres), d) Group D: Treated with 40 mg/kg b.w of ß,Ɛ-Carotene-3,3'-diol (note the gradual restoration of reticular fibres) and e) Group E: Treated with 80 mg/kg b.w of ß,Ɛ-Carotene-3,3'-diol (note the outline of reticular fibres formation vis-à-vis group A). Gordon & Sweet 400× Red arrow = Reticular fibre

of the liver to drug-related injury was attributed to its high metabolic capacity and high concentration of many xenobiotics (Woolbright & Jaeschke, 2017). This study examined the effects of ß,Ɛ-Carotene-3,3'-diol on the liver following indomethacin-induced hepatotoxicity in adult male Wistar to determine the hepatotoxic effects of indomethacin and the ameliorative potential of ß,Ɛ-Carotene-3,3'-diol.

The administration of indomethacin in this study caused toxicity in the liver of animals in groups B to E in line with the report of (Cherkas *et al.*, 2016). According to Khan *et al.* (2019) indomethacin exposure result in multiple organ toxicity with the liver, intestinal mucosa and kidney being the most significant. The mechanism by which indomethacin causes cellular toxicity is still shrouded in misery, but some authors (Khan *et al.*, 2019; Basivireddy *et al.*, 2004) posited that, it might be connected with an increase in oxidative stress, DNA damage and inhibition of cellular respiration and prostaglandins synthesis. Reactive oxygen species (ROS) and oxidative stress have been documented to be implicated in drug related toxicity (Sabiu *et al.*, 2015; Cherkas *et al.*, 2016).

The administration of indomethacin significantly increased the AST, ALT and ALP activities when compared with the control group. These liver enzymes are always elevated in liver injury as seen in this study (Oke *et al.*, 2019). Indomethacin may have damaged hepatocytes and reduced the integrity of the cell membrane leading to the leakage of transaminase enzymes (Mansourian *et al.*, 2020). This observation indicates successful induction of liver injury. Studies have shown that AST is dominant in the mitochondria while ALT is restricted to hepatocyte cytoplasm; therefore, an increment in the ALT is indicative of an increase in AST level (Oke *et al.*, 2019; Mansourian *et al.*, 2020). There was a significant reduction in the activities of AST, ALT and ALP in the groups treated with ß,Ɛ-Carotene-3,3'-diol when compared with the negative control which is indicative of liver tissue regeneration and the ability of ß,Ɛ-Carotene-3,3'-diol to inhibits cellular activities that enhances cholestasis (Oke *et al.*, 2019).

The hepatotoxic effect of indomethacin on the histoarchitecture of the liver was observed to be more pronounced in group B that was not treated with ß,Ɛ-Carotene-3,3'-diol. There were observable features of fat droplets, neutrophils infiltration and steatosis in group B. All these histopathological features were abated in the treatment groups as evidenced in varying degrees of remediation/recovery in animals administered with ß,Ɛ-Carotene-3,3'-diol. The recovery process was observed to be proportional to the dose of the ß,Ɛ-Carotene-3,3'-diol administered to the animals in the treatment groups. For instance, the microanatomy of the liver of the animals administered with the highest dose of ß,Ɛ-Carotene-3,3'-diol, showed a near-normal histological outline when compared to the control group (group A); this may be due to the antioxidant and anti-inflammatory properties of carotenoid (Vardi *et al.*, 2010; Kawata *et al.*, 2018). Wu *et al.* (2023) confirms the ability of carotenoid (β-carotene) to reduce inflammatory processes and also inhibits the reactive oxygen species by decreasing the expression of the NF-kB p65 protein. The high density of conjugated dienes in carotenoids has been linked to their antioxidant activities (Kawata *et al.*, 2018).

There was a significant increment in the reticular fibres in the liver of ß,Ɛ-Carotene-3,3'-diol treated groups when compared

with the negative control (group B). Reticular fibres which form the basis for the structural integrity of most viscera, especially liver, can easily become fibrotic in case of toxicity according to Ofusori *et al.* (2023). Tissue fibrosis has been documented by Antar *et al.* (2023) to be associated with inflammation, metabolic homeostasis, and transforming growth factor-β1 (TGF-β1) signaling, where the balance between the oxidant and antioxidant systems appears to be a key modulator in managing these processes. Depletion of reticular fibres in the liver as observed in the negative control (group B) is one of the pathological features of hepatic fibrosis (Oke *et al.*, 2019). Elicitation of reactive oxygen species has been implicated in hepatic fibrosis (Kawata *et al.*, 2018); this is one of the mechanisms by which indomethacin induces cellular toxicity as earlier hypothesised by Basivireddy *et al.* (2004). It is believed that indomethacin through documented evidences from this study caused hepatic fibrosis in animals in groups B to E which were ameliorated in a dose dependent manner in animals treated with ß,Ɛ-Carotene-3,3'-diol.

In conclusion, it is evident from this study that ß,Ɛ-Carotene-3,3'-diol is capable of restoring compromised histological alterations following hepatotoxicity induced by indomethacin. The observed evidences are believed to be connected with the antioxidant and anti-inflammatory properties of ß,Ɛ-Carotene-3,3'-diol. More findings are encouraged to corroborate these findings.

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