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Virgin coconut oil mitigates the hepatotoxic effect of atrazine and diabetes on liver enzymes in male Wistar rats

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Abstract

To assess the hepatoprotective action of Virgin Coconut Oil(VCO) in atrazine administered rats. Adult male Wistar rats weighing 180-200 g body weight were separated into two major experimental groups. 35 rats in the test group were divided into five sub-groups of 7rats: Subgroup (SG)1,2 and 3 received 10 ml/kg body weight of distilled water, 10 ml/kg VCO and 123 mg/kg Atrazine (ATZ) respectively, SG 4 was diabetic control group and SG 5 was diabetic group treated with 10 ml/kg of VCO for 2weeks, after which the animals were sacrificed and blood collected for analysis. 35 rats for the recovery group were also divided into 5 subgroups of 7 rats; SG1 and 2 received 10 ml/kg body weight of distilled water and 10 ml/kg of VCO, SG 3, 4 and 5 received 123 mg/kg of ATZ for 2 weeks. After the first 2 weeks, SG 1,2,3 continued the initial treatment while the rats in SG4 and SG5 were fed with 10 ml/kg of VCO and10 ml/kg of distilled water respectively. After 2 weeks, all the animals were sacrificed and blood collected for analysis. We observed a significant increase in the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels of the diabetes control and diabetes treated with VCO groups compared to the normal control. In the groups administered with atrazine, the result showed elevations in serum AST and ALT. With VCO,a significant decrease in ALT levels when compared to the ATZ continued group was observed, further potentiating the hepatoprotective ability of VCO.

Keywords: Atrazine; AST; ALT; Diabetes; Liver enzymes; Virgin Coconut Oil

1. Introduction

The liver plays a major role in the regulation of carbohydrate homeostasis. It plays an important role in maintenance of normal glucose levels during fasting as well as in the postprandial period [1]. This function is deranged in association with liver enzymes abnormality in type 2 diabetes mellitus and in obese individuals. Indeed, hepatic dysfunction resulting from insulin-resistance syndrome may lead to development of type 2 diabetes [2]. The liver enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyltransferase (GGT), are routinely used in evaluation of liver function. Aspartate aminotransferase (AST) and ALT are considered markers of hepatocellular health, whereas GGT also indicates biliary tract function. Although AST is a marker of hepatocellular health, it is a less specific marker of liver of liver pathology related to development of type II diabetes than ALT [3]. Some prospective studies [4, 5] have reported that higher ALT concentrations predicted development of type 2 diabetes while less correlation between AST and DM has also been reported [5, 6]. Alanine aminotransferase (ALT) is the most specific marker of liver pathology and is found primarily in this organ [7]. AST and GGT are also found in other tissues

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and are therefore less specific markers of liver function [8]. Although GGT is a less specific marker of liver function, higher GGT levels have been found to be an independent predictor of the incidence of type 2 diabetes.

Type 2 Diabetes Mellitus (DM) is usually associated with chronic mild elevation of transaminases and downfall in hepatic functions [9]. Moreover, accumulation of intracellular glycogen in hepatocytes lead to liver injury showing typical biochemical findings of mild to moderate rise in ALT, AST and normal synthetic function with or without ALP elevation [10]. One of the hepatic manifestations of diabetes mellitus with metabolic syndrome is Non Alcoholic Fatty Liver Disease (NAFLD) and more specifically ALT has been used as a marker of NAFLD [11].

Humans are regularly exposed to a raft of Persistent Organic persistent (POPs), which now must also be taken into account as serious risk factors for the development of diabetes [12]. ATZ has been reported to disrupt insulin homeostasis and this could ultimately lead to diabetes. Treatment of DM mainly include: administration of insulin and oral hypoglycaemic agents; with dietary counselling and life style modifications which may not effectively managed the situation as long as we are still exposed to some of these pollutants. It is the therefore expedient to take another approach in the management of diabetes by looking at how ATZ can cause metabolic derangement and also if reduced exposure to ATZ could reduce the observed metabolic disruption.

VCO has been reported to have hypoglycemic actions, enhance insulin secretion and also ameliorate oxidative stress [13]. VCO has also been found to possess antioxidative, antimicrobial, antiviral, antihypercholesterolic and antithrombotic effects [14]. The present study was designed to investigate if there is any mitigating effect of VCO on atrazine toxicity in rats.

2. Materials and methods

2.1. Experimental animals

Adult male albino Wistar rats (180-200 g body weight) were purchased and maintained at the animal house Unit of the Department of Physiology, Faculty of Basic Medical Sciences, University of Calabar. The animals were kept in a well ventilated space to acclimatize for two weeks. The animals were fed with rat chow and allowed drinking water ad libitum. After the acclimatization period, the animals were weighed, their fasting blood glucose level were measured and reassigned before the commencement of the experimental treatment. The cages were cleared and kept clean throughout the period of the experiment.

2.2. Experimental design and treatment of animals

The rats were randomly separated into two major experimental groups (the test and recovery groups) of 35 rats in each major group. Experiment for the test group lasted for two weeks while experiment for the recovery group lasted for four weeks.

Thirty-five (35) rats in the test group were randomly divided into five sub-groups of 7 rats each (n=7) and were oral gavaged and treated thus: Sub-group SG1 **(Normal control+H_2O)** served as normal control and received 10ml/kg body weight of distilled water, SG 2 **(Normal control +VCO),** received 10ml/kg of Virgin Coconut Oil (VCO) SG 3 **(ATZ treated),** received 123 mg/kg (20% of lethal dose) of Atrazine SG 4 **(Diabetic Control)** were left untreated and SG 5 **(Diabetes+VCO)** was the diabetic group that were treated with 10 ml/kg of VCO. Treatment in the test group lasted for 2 weeks, after which the animals were sacrificed and blood collected for analysis. This is shown in Table 1.

During these 2 weeks' period, thirty-five rats for the recovery group were also divided into 5 sub-groups (SG) of 7 rats per sub-group (n = 7) and were treated as follows: SG 1 **(Normal control+H_2O)** served as normal control and received 10 ml/kg body weight of distilled water, SG 2 **(Normal control +VCO)** received 10 ml/kg of Virgin Coconut Oil (VCO), SG 3 **(ATZ continued)**, 4 **(VCO after ATZ)** and 5 **(Untreated after ATZ)** received 123 mg/kg of ATZ. After 2 weeks, the animals were re-treated for recovery and were treated thus: (SG) 1 served as normal control and received 10 ml/kg body weight of distilled water, SG 2 received 10 ml/kg of Virgin Coconut Oil (VCO), SG 3 received 123 mg/kg of ATZ, SG 4 was treated with 10 ml/kg of VCO and SG 5 was given 10 ml/kg of distilled water. Treatment for recovery also lasted for 2 weeks, after which the animals were sacrificed and blood collected for analysis. The groupings are shown in Table 2.

2.2.1. Experimental grouping and treatment

Table 1 Test group (2 weeks)

Groups	Treatment
Normal Control + H_20 (SG 1)	10 ml/kg of distilled water (H_2O)
Normal Control + VCO (SG 2)	10 ml/kg of Virgin Coconut Oil (VCO)
Atrazine Treated (SG 3)	123 mg/kg (20% of lethal dose) of Atrazine
Diabetic Control (SG 4)	10 ml/kg of distilled H_2O
Diabetes +VCO (SG 5)	10 ml/kg of VCO

Table 2 Recovery group (4 weeks)

Groups	Treatment (1st 2 weeks)	Treatment (2nd 2 weeks)
Normal Control + H_20 (SG 1)	10 ml/kg of distilled water (H_2O)	10 ml/kg of distilled H_2O
Normal Control + VCO (SG 2)	10 ml/kg of Virgin Coconut Oil (VCO)	10 ml/kg of VCO
Atrazine Treated (SG 3)	123 mg/kg (20% of lethal dose) of Atrazine	123 mg/kg of Atrazine
VCO after ATZ (SG 4)	123 mg/kg of Atrazine	10 ml/kg of VCO
Untreated after ATZ (SG 5)	123 mg/kg of Atrazine	10 ml/kg of distilled H_2O

2.3. Induction of Diabetes mellitus (DM)

Diabetes was induced intraperitoneally using 150 mg/kg body weight of alloxan monohydrate [15, 16]. The diabetic state was observed from about 48 hours by the symptoms of polyuria and glucosuria. After 72 hours, DM was confirmed with blood glucose level of 180-200 mg/dL and above [13] using a glucometer (ACCU-CHECK Active) and ACCU-CHECK compatible glucose test strips.

2.4. Preparation of Virgin Coconut Oil (VCO)

Mature dried coconuts were purchased from the local market and VCO was extracted using the modified wet extraction method [17]. The solid endosperm of mature coconut was crushed and made into thick slurry. About 500 ml of water was added to the slurry obtained and squeezed through a fine sieve to obtain coconut milk. The resultant coconut milk was left for about 18 hours to facilitate the gravitational separation of the emulsion. Demulsification produced layers of an aqueous phase (water) at the bottom, an oil phase in the middle layer and emulsion phase (cream) on top. The cream on top was removed and the oil was scooped and warmed for about 5 minutes to remove moisture. The oil obtained was then filtered and stored at room temperature.

2.5. Median Lethal Dose (LD_{50})

The toxicity level of atrazine was assessed using the Lorke's method [18] which involves administration of the chemical to the animals and observation of mortality amongst the animals within a period of 24 hours. This was done as follows:

- 12 Swiss albino mice were fasted and weighed
- The animals were sub-grouped into four for graded intra-peritoneal doses of 1250 mg/kg (1.25 g/kg), 750 mg/kg (0.75 g/kg), 1000 mg/kg (1.00 g/kg) and 500 mg/kg (0.50 g/kg)
- The administration followed was based on their body weight and the experimental mice were examined 24hours after dosage administration.
- There were physical signs of toxicity in the groups administered with doses 1250 mg/kg, 750 mg/kg and 1000 mg/kg and mortality was recorded.
- But no physical signs of toxicity and mortality were recorded in the group administered with the 500mg/kg dosage. As shown in Table 3

Table 3 Dosages for Lethal concentration

Dose-1250 mg/kg		
Mice	**Body weight**	**Dosage**
T_1	25.60 g	0.64 ml
T_2	25.23 g	0.63 ml
T_3	26.04 g	0.66 ml
Dose-1000mg/kg		
Mice	Body weight	Dosage
T_4	28.18 g	0.56 ml
T_5	27.63 g	0.55 ml
T_6	24.45 g	0.49 ml
Dose-750mg/kg		
Mice	Body weight	Dosage
T_7	26.37 g	0.40 ml
T_8	27.53 g	0.41 ml
T_9	22.90 g	0.34 ml
Dose-500mg/kg		
Mice	**Body weight**	**Dosage**
T_{10}	19.70 g	0.20 ml
T_{11}	21.40 g	0.21 ml
T_{12}	27.60 g	0.28 ml

2.6. Evaluation of Liver enzymes

2.6.1. Estimation of aspartate aminotransferase (AST) activity

Randox kit (UK) for quantitative measurement of aspartate aminotransferase was used as employed by Ahur et al, (2012) [19].

Assay Procedure

The working reagents were brought to the reaction temperature of 30^{o}C. 0.5 mL of solution 1 (L-aspartate) was pipetted into a well labeled test tube. 0.1 mL of the sample was pipetted into a test tube labeled sample and 0.1 mL of distilled water was pipetted into test tubes labeled sample, reagent and blank respectively. The mixture in the test tubes were mixed and incubated for 30 minutes at 37°C. After which 0.5 ml of solution 2 (2,4-dinitrophenyldrazine) was then pipette into the test tubes, mixed and allowed to stand for 20 minutes at 20-25°C, 5.0 ml of sodium hydroxide solution was then pipetted into the test tubes. The absorbance of sample was read against the reagent blank at wavelength of 546 nm

2.6.2. Estimation of Alanine Aminotransferase (ALT) activity

Randox kit (UK) for quantitative measurement of alanine aminotransferase was used as employed by Ahur *et al*, (2012) [19].

Assay Procedure

0.5 mL of solution 1 (L-alanine) was pipetted into a well labeled test tube. 0.1 mL of the sample was pipetted into a test tube labeled sample and 0.1mL of distilled water was pipetted into test tubes labeled sample, reagent and blank

respectively. The mixture in the test tubes were mixed and incubated for 30 minutes at 37°C. After which 0.5 ml of solution 2 (2,4-dinitrophenyldrazine) was then pipette into the test tubes, mixed and allowed to stand for 20 minutes at 20-25°C, 5.0 ml of sodium hydroxide solution was then pipetted into the test tubes. The absorbance of sample was read against the reagent blank at wavelength of 546 nm

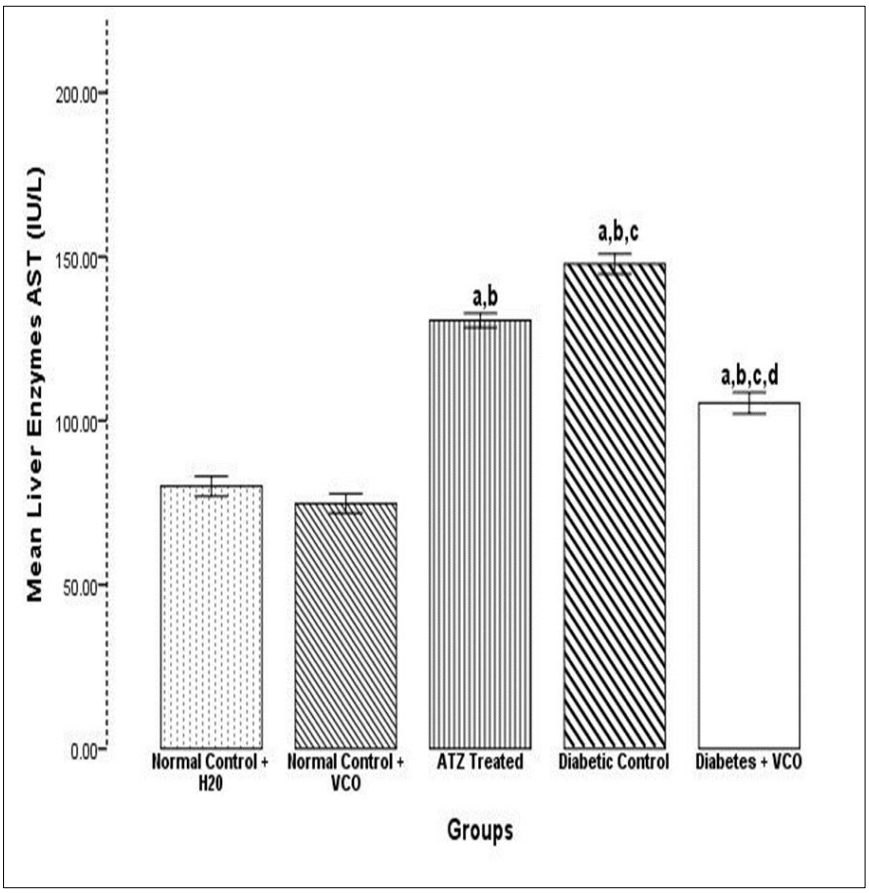
2.7. Statistical analysis

Statistical analysis was carried out using windows SPSS package (SPSS 20.0). Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. Data were expressed as mean ± standard error of mean (Mean±SEM). Results with values of $p<0.05$ were considered significant.

3. Results

3.1. AST (IU/L) levels in Normal control+H_2O (NC + H_2O), Normal Control + VCO (NC + VCO), ATZ treated, diabetic control and diabetes treated groups

AST levels in the ATZ treated (130.60±1.08 IU/L) and diabetic control (147.80±1.53 IU/L) groups were significantly ($p<0.05$) higher when compared with NC + H_2O (80.00±1.52 IU/L) and NC + VCO (74.80±1.49 IU/L) groups. The AST level in the diabetic control group was significantly ($p<0.05$) higher than ATZ treated group. Diabetic + VCO (105.40±1.63 IU/L) group showed a significant ($p<0.05$) decrease in AST level when compared with the ATZ treated and diabetic control groups but was significantly ($p<0.05$) higher than NC + H_2O and NC + VCO groups (Figure 1)

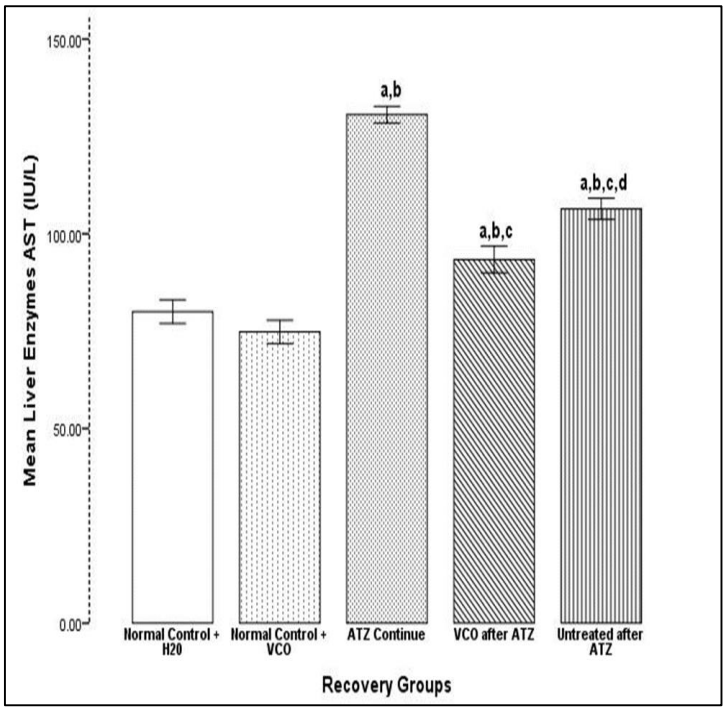


Values are mean ± SEM. n=7. a = $p < 0.05$ vs NC, b = $p < 0.05$ vs NC + VCO; c = $p < 0.05$ vs ATZ, d = $p < 0.05$ vs Diabetic control

Figure 1 Comparison of AST levels in Normal control+H_2O (NC + H_2O), Normal Control + VCO (NC + VCO), ATZ treated, diabetic control and diabetes treated groups

3.2. AST (IU/L) levels in Normal control+H_2O (NC + H_2O), Normal Control +VCO (NC + VCO), ATZ continued, VCO after ATZ and untreated after ATZ groups

The mean values for AST in the recovery group are 81.20±0.62 IU/L, 74.28±1.68 IU/L, 135.50±1.68 IU/L, 93.40±1.72 IU/L and 106.40±1.36 IU/L for NC + H_2O, NC + VCO, ATZ continued, VCO after ATZ and untreated after ATZ groups respectively. AST level was significantly higher in the ATZ continued group when compared with the NC + H_2O and NC + VCO groups. VCO administration significantly ($p<0.05$) reduced AST levels in the VCO recovery group when compared with the ATZ continued group but significantly ($p<0.05$) higher than the NC + H_2O and NC + VCO groups. There was a significant decrease in AST levels in the ATZ untreated group when compared with the ATZ continued group (Figure 2)

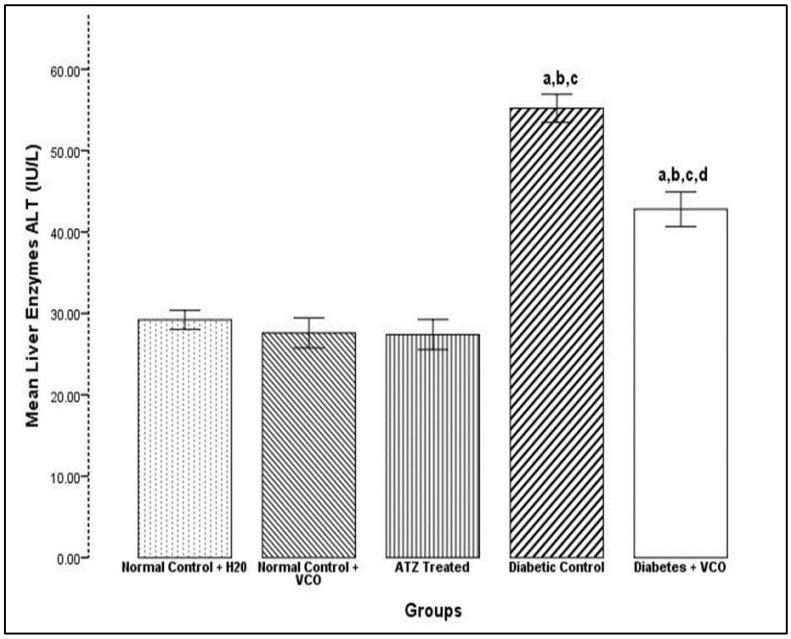


Values are mean ± SEM. n=7. a = $p < 0.05$ vs NC, b = $p < 0.05$ vs NC + VCO, c = $p < 0.05$ vs ATZ continued, d = $p < 0.05$ vs VCO after ATZ

Figure 2 Comparison of AST levels in Normal control+H_2O (NC + H_2O), Normal Control +VCO (NC + VCO), ATZ continued, VCO after ATZ and untreated after ATZ groups

3.3. ALT (IU/L) levels in Normal control+H_2O (NC + H_2O), Normal Control + VCO (NC + VCO), ATZ treated, diabetic control and diabetes treated groups

There was no significant ($p<0.05$) difference in ALT level in the NC + H_2O (29.20±0.58 IU/L), NC + VCO (27.60±0.93 IU/L) and ATZ treated (27.40±0.64 IU/L) groups. ALT level was significantly ($p<0.05$) increased in the diabetic control (55.20±0.86 IU/L) when compared with the NC + H_2O, NC + VCO and ATZ treated groups. VCO treatment significantly ($p<0.05$) reduced ALT level in the diabetic + VCO group (42.80±1.07 IU/L) when compared with the diabetic control group but still significantly ($p<0.05$) higher than NC + H_2O, NC + VCO and ATZ treated groups (Figure 3).

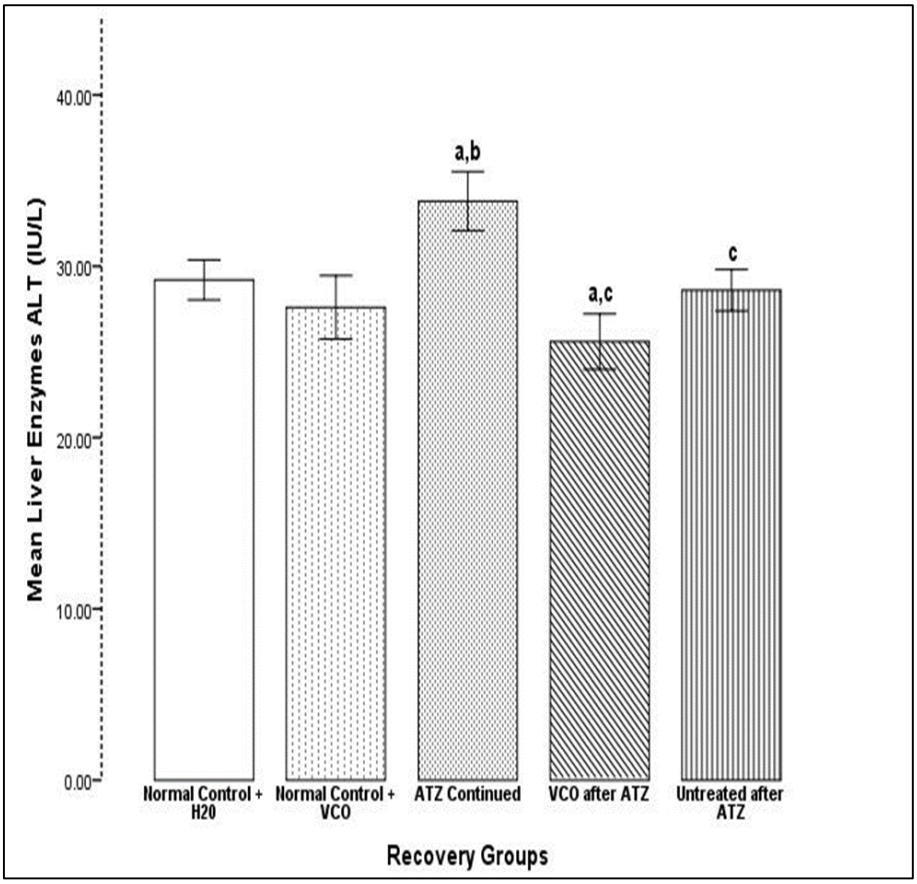


Values are mean ± SEM. n=7. a = p < 0.05 vs NC, b = p < 0.05 vs NC + VCO, c = **p < 0.05 vs ATZ, d = p < 0.05 vs Diabetic control**

Figure 3 Comparison of ALT levels in Normal control+H_2O (NC + H_2O), Normal Control + VCO (NC + VCO), ATZ treated, diabetic control and diabetes treated groups

3.4. ALT (IU/L) levels in Normal control+H_2O (NC + H_2O), Normal Control +VCO (NC + VCO), ATZ continued, VCO after ATZ and untreated after ATZ groups

Figure 4 showed that with continuous ATZ use, the ALT level in the ATZ treated group was significantly ($p<0.05$) higher than NC + H_2O (29.60±0.82 IU/L) and NC + VCO (28.20±0.52 IU/L) groups. ALT level in the VCO recovered group (25.60±0.81 IU/L) was significantly ($p<0.05$) lower than the NC + H_2O and ATZ continued (33.80±0.86 IU/L) groups but not significantly ($p<0.05$) different from the NC + VCO. The ATZ untreated group (28.60±0.60 IU/L) showed a significant ($p<0.05$) decrease in ALT level when compared with ATZ continued group but not significantly ($p<0.05$) different from NC + H_2O, NC + VCO and VCO recovered groups.



Values are mean ± SEM. n=7. a = p < 0.05 vs NC, b = p < 0.05 vs NC + VCO; c = p < 0.05 vs ATZ continued, d = p < 0.05 vs VCO after ATZ

Figure 4 Comparison of ALT levels in Normal control+H_2O (NC + H_2O), Normal Control +VCO (NC + VCO), ATZ continued, VCO after ATZ and untreated after ATZ groups

4. Discussion

Aminotransferases, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), measure the concentration of intracellular hepatic enzymes that have leaked into the circulation and serve as a marker of hepatocyte injury [20]. Increased activity of these markers is associated with insulin resistance [2], metabolic syndrome, and type 2 diabetes [21, 22]. In the present study, it was observed that there was no significant difference in the AST and ALT levels between the normal control and the normal control +VCO group "(shown in Fig. 1)". This supports the study by Eleazu *et al.*, (2019) [23] who also observed a non-significant difference in these liver enzymes. The non-significant effect administration of VCO had on the AST and ALT activities of the normal rats further affirm the non-hepatotoxic action of VCO. This is in contrast with the report by Famurewa *et al.*, (2017) [24] who observed a significant increase in AST, ALP levels and a significant decrease in ALT levels; they concluded that the underlying mechanism for the observed contrasting liver enzyme activities is unclear. Narayanankutty *et al*., (2017) [25] reported that the AST activity was only found to be significantly higher in animals fed with refined coconut oil (RCO). The report of Otuechere *et al*., (2014) [26] indicated a marginal reduction in AST activity and significant depression in ALP activity following VCO supplementation. Because of the contrasting reports, there may be a need for further investigations on the role of VCO supplementation in hepatic enzyme regulation. Result of this study showed a significant increase in the AST and ALT levels of the diabetes control and diabetes treated with virgin coconut oil (VCO) groups compared to the normal control and normal control given VCO groups. This result corresponds with the reports of Harris, 2005 and Everhart, 1995 [9, 27] where elevated serum activity of AST occurs more in diabetics than in the general population and mild chronic elevation of transferases often reflects underlying insulin resistance. Aspartate aminotransferase (AST) measure the concentration of intracellular hepatic enzymes that have leaked into the circulation and serve as a marker of hepatocyte injury [20]. Therefore, the elevated AST levels in diabetes could be attributed to hepatocyte injury. It was observed that the groups administered with VCO (Normal control + VCO) and diabetes+VCO showed a significant decrease in AST and ALT levels when compared to Normal control+H_2O and diabetes control groups respectively. This could be as a result of the increased polyphenolic and antioxidant contents present in VCO. This in concordance with the study by Margata *et al.,* (2018) [28], who observed an improvement in liver of dyslipidemic rats treated with virgin coconut oil (VCO) and

hydrolyzed VCO (HVCO). AST is a mitochondrial enzyme found in the heart, liver, skeletal muscle, and kidney and is normally present in plasma [29]. In the groups administered with atrazine, the result showed elevations in serum AST. This result is in agreement with other studies which also observed an elevated AST following atrazine administration [30, 31]. Atrazine has been reported to induce oxidative stress in the liver of mice [32]. The elevated serum AST is apparently due to mitochondrial damage, elevation in intracellular Ca^{2+} generation of ROS induced by atrazine which represent the cytotoxic mechanism caused by atrazine [33]. In the recovery group (shown in Fig. 2), we observed a significant reduction in AST levels in the group recovered with VCO but there was no significant change between the ATZ continued group and the group left untreated. This further consolidates the hepatoprotective effects of VCO. Zakaria *et al.*, (2011) [34] induced liver injury in rats with paracetamol (PCM or acetaminophen); they observed that VCO was able to reverse the induced liver injury. It is believed the liver is frequently exposed to similar injury from environmental pollutants such as ATZ, therefore our result further ascertains their findings. They also reported that hepatoprotection was only observed at a concentration of 10 ml/kg and not at lower doses. VCO has also been observed to possess the ability to restore glucose and oestradiol metabolism [35]; GLUT4 and insulin metabolism [36] in both diabetic and atrazine rat model.

The most common abnormality of liver function test seen in type 2 diabetes mellitus is elevated alanine aminotransferase (ALT) [2]. It is also hypothesized that the elevated ALT, agluconeogenic enzyme whose gene transcription is suppressed by insulin, could indicate impairment in insulin signalling rather than purely hepatocyte injury [20]. As shown in [36], Atrazine administration negatively affect insulin levels. As shown in Figures 3, we observed a non-significant increase in the ALT levels in the atrazine administered groups when compared with the normal control groups (NC+H_2O and NC+VCO), this result is similar to a study by Hussain *et al.*, (2012) [37] which reported that rats treated with atrazine for 14 consecutive days resulted in non-significant elevation in serum ALT enzyme, though an increase was observed from day 30. Therefore, the non-significant result in this study could also be due to the period of the experiment. Though, this in contrast with other studies which have reported an increase in ALT levels [30, 38]. In the recovery group (shown in Fig. 4), we observed a significant increase in ALT levels in the ATZ continued when compared to the VCO after ATZ and the Untreated after ATZ group further consolidating the report of Hussain *et al.*, (2012) [37] that the period of exposure could affect the extent of the liver damage. Although AST is a marker of hepatocellular health, it is a less specific marker of liver of liver pathology related to development of type II diabetes than ALT [8]. Alanine aminotransferase (ALT) is the most specific marker of liver pathology and is found primarily in this organ [7] while AST is found in other organs like heart, liver and skeletal muscle [29]. We therefore infer that ATZ probably did not have a significant effect on the liver in the first two weeks as observed in the unchanged ALT levels, despite the increase serum levels of AST but with continuous use in the subsequent two weeks, the probable liver injury became more pronounced as a significant increase in ALT levels was observed. With VCO administration for recovery, a significant decrease in ALT levels when compared to the ATZ continued group was observed, further potentiating the hepatoprotective ability of VCO against environmental pollutant. Otuechere *et al.*, in 2013 [26] reported that the active components of coconut oil had protective effects against the toxic effects induced by Trimethoprim-sulfamethoxazole (TMP-SMX) administration, especially in the liver of rats. It has also been reported that atrazine induced increase in AST and ALT enzymes were restored by treatment with clomiphene citrate [30, 38]. As shown in [39], VCO was able to modulate atrazine induced oxidative stress by increasing the Superoxide Dismutase, Catalase and glutathione (GSH) levels which were previously depressed by atrazine administration.

5. Conclusion

Conclusively, atrazine administration for a short period did not significantly disrupt the ALT levels which is a more specific marker for liver damage but with continuous use, a probable liver damage was observed due to the increased ALT levels in the recovery groups. With VCO administration for recovery, a significant decrease in ALT levels when compared to the ATZ continued group was observed, further potentiating the hepatoprotective ability of VCO against environmental pollutant.

Compliance with ethical standards

Disclosure of conflict of interest

All authors declared no conflict of interest.

Statement of ethical approval

Maintenance and care of all animals were carried out in accordance with directives for animal experiments. Guide for the care and use of Laboratory Animals, were strictly adhered to. This study protocol was reviewed and approved by

Faculty Animal Research Ethics Committee, Faculty of Basic Medical Sciences, University of Calabar, Calabar, Cross Rivers State, with approval number [022PY30417]."

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