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Effect of Aqueous Leaf Extract of *Ocimum gratissimum* on Antiretroviral Drug-Induced Hepatotoxicity in Rats

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Authors' contributions

This is a multidisciplinary research in which all the authors worked in close collaboration. Authors PU and PO conceptualized the research and wrote the proposal which was approved by all the authors. Author PU presented the proposal at the clinical conference of the College of Medicine, Chukwuemeka Odumegwu Ojukwu University. Authors CN and PU produced the aqueous extract and did the animal experimentation. Authors IN and CN did the biochemical tests. Author JO did the statistical analysis. The discussion of the results was done by authors PU and PO. The final report was read and approved by all the authors.

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ABSTRACT

Aim: Hepatotoxicity, among other adverse effects, constitutes one of the greatest impediments to successful antiretroviral drug therapy (ART) in HIV/AIDS patients. The main objective of the study was to determine if the aqueous leaf extract of *Ocimum gratissimum* has a protective effect on

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ART-induced hepatotoxicity in rats.
Place and Duration: Department of Pharmacology, Chukwuemeka Odumegwu University, Nigeria (six months duration).
Methodology: Twenty five (25) albino rats of both sexes were divided into 5 groups of 5 each and treated as follows: Group A (no antiretroviral drugs, no extract); group B (antiretroviral drugs alone); group C (extract alone); group D (antiretroviral drug plus 40 mg/kg extract); group E (antiretroviral drug plus 80 mg/kg extract). All treatment lasted for twenty eight days. Blood samples were collected and serum ALT and AST determined using UV-spectrophotometer. Thereafter, the animals were sacrificed and their livers harvested and examined histologically. The mean (± S.E.M) of data were calculated and further analyzed for statistical significance using graph Pad Prism 5.0.
Results: Mean serum ALT were 35.6±6.4, 54.0±9.4, 53.8±22.9, 90.5±21.9, 86.5±13.9 and that of AST were 143.8±19.7, 205.2±14.9, 58.0±27.9, 162.3±41.4, 150.5±44.8 for groups A, B, C, D, and E respectively. There was a statistically significant difference between the mean values of serum AST for group B and those for group C (p value of 0.016). However there was no statistically significant difference between the ALT values for the test and control groups of rats (p value > 0.999). Also, there was no statistically significant difference between the mean values of AST for group B and those of groups A, D, E (p value = 0.659). The histology report for the liver was normal for all groups.
Conclusions: This extract did not produce significant reduction of serum ALT and AST in ART-treated rats in this study.

Keywords: ALT; antiretroviral drugs; AST; hepatoprotective herbs; hepatotoxicity; Ocimum gratissimum; rats.

1. INTRODUCTION

Hepatotoxicity, defined as a rise in either (a) alanine transferase (ALT) level more than three times of upper limit of normal (ULN), (b) alkaline phosphatase (ALP) level more than twice ULN, or (c) total bilirubin level more than twice ULN when associated with increased ALT or ALP [1], is one of the most serious adverse effects of antiretroviral therapy (ART). This often results in discontinuation of treatment leading to worsening of the patient's condition [2]. Discontinuation of antiretroviral treatment worsens drug resistance which is also very common with these agents. It has also contributed to increased incidence of hospital admissions [3].

Though hepatotoxicity occurs with most antiretroviral regimen, it is more common with the older agents. In particular, studies show that protease inhibitors (PI) such as indinavir and ritonavir are more likely to produce liver toxicity compared to other agents [4].

Antiretroviral-induced hepatotoxicity occurs more in HIV patients co-infected with hepatitis B virus (HBV) or hepatitis C virus (HCV) as treatment for HBV and HCV improved tolerance to antiretroviral drugs [5]. Also, heavy alcohol intake, female gender, old age, high CD4+ count and previous exposure to ART are important risk factors for antiretroviral induced hepatotoxicity [6,7].

The prevalence of hepatotoxicity in HIV/AIDS patients starting new antiretroviral treatment ranges between 4.5% and 10% [8]. Moderate or severe hepatic injury is defined as 5-fold or 10-fold increase in plasma aminotransferases (AST, ALT) levels relative to pretreatment baseline.

Mechanisms of ART-induced liver damage include direct hepatic injury, immune reconstitution, hypersensitivity reaction, and mitochondrial injury [9,10,11].

There is documented evidence for the metabolism of antiretroviral drugs in rats. It has been documented that ritonavir, saquinavir, and efavirenz inhibited biliary excretion of taurocholic acid in human and rat hepatocytes [12]. It has also been demonstrated that efavirenz, abacavir, and lamivudine at dosages of 0.43 mg/kg, 0.43 mg/kg, and 0.21 mg/kg respectively independently elevated hepatic enzymes in albino rats after oral administrations for one week [13,14].

Certain therapeutic manipulations are reported to ameliorate ART-induce hepatic injury. In one study, it was found that procysteine, an intracellular glutathione stimulator, when combined with antiretrovirals reduced the toxicity

of the latter [15]. Also, magnesium dietary supplementation has been found to suppress anti-retroviral-induced oxidative stress in rat hepatocytes [16]. However, these orthodox therapeutic interventions present their own challenges. For example, interferon-alpha induces elevation of aminotransferases among other adverse effects [17]. There is, therefore, need to search for alternative agents such as herbal remedies that will ameliorate hepatotoxic effects of antiretroviral agents.

Some herbal remedies are reported to be hepatoprotective and have therefore found application in the management of liver disorders [18,19]. One of the medicinal plants credited with such hepatoprotective activity is *Ocimum gratissimum* [20]. This plant, also known as scent leaf (*'nchuanwu'* in Igbo) belongs to the family *Lamiaceaae.* It is commonly used as spice in foods and also in the treatment of fever, diarrhea, dysentery, pile, and convulsions [21,22]. Previous phytochemical studies on this plant revealed the presence of alkaloids, phytates, tannins, flavonoids, and oligosaccharides [23]. These constituents are credited with anti-inflammatory and ant-oxidant properties which may be important in the hepatoprotective effect of this plant [24].

This study is justified by the fact that there is high incidence and prevalence of anti-retroviral induced hepatotoxicity. For instance, the prevalence of hepatotoxicity in HIV/AIDS patients starting new antiretroviral treatment ranges between 4.5% and 10% [8]. Hepatotoxicity, when moderate or severe, may warrant discontinuation of antiretroviral therapy with the attendant increases in hospital stay, cost of treatment, and morbidity/mortality. The disruption of therapy also encourages the emergence of resistant strains of HIV which increases the prevalence of HIV/AIDS and the resultant public health concerns.

Orthodox drugs (e.g. procysteine, interferon-alpha) that may be used as adjuncts to reduce ART-induced hepatotoxicity [25,26] have not been effective clinically. Besides, they present their own problems of toxicity [17,26]. *Ocimum gratissimum* is a vegetable that is edible, so the chances of it being toxic are minimal. In fact, Fandohan et al. [27] demonstrated that its extract, when administered to Wistar rats for 14 days at a dose of 1500 mg/kg did not result in any toxic effect to the liver.

It is expected that the result of this research will help in mitigating this ART-induced hepatotoxicity. This will allow the continuation of initiated antiretroviral treatment programme, decrease the emergence of drug resistance, and reduce overall cost of treatment. In the long run, it will contribute to the control of the HIV/AIDS pandemic and also reduce the amount of money spent on antiretroviral drugs.

The main objective of the study was to determine if the aqueous leaf extract of *Ocimum gratissimum* has a protective effect on ART-induced hepatotoxicity in rats. Specific objectives included:

a. To determine the effect of aqueous leaf extract of *O. gratissimum* on serum ALT and AST in rats.
b. To determine the effect of aqueous leaf extract of *O. gratissimum* on serum ALT and AST following ART-induced hepatotoxicity in rats.
c. To determine the effect of increasing doses of aqueous leaf extract of *O. gratissimum* on ALT and AST following ART-induced hepatotoxicity in rats.

The Null hypothesis for this study stated that aqueous leaf extract of *O. gratissimum* does not decrease serum ALT and AST following the administration of ART in rats. The alternative hypothesis states that aqueous leaf extract of *O. gratissimum* decreases serum ALT and AST following the administration of ART in rats.

The null hypothesis was tested at a significant level (p value) of 0.05. It would be rejected if the p value is < 0.05 and accepted if p value > 0.05. At p value < 0.05, the null hypothesis would be rejected implying that aqueous leaf extract of *O. gratissimum* decreases serum ALT and AST following the administration of ART in rats.

2. MATERIALS AND METHODS

This study was conducted at the Pharmacology laboratory, Department of Pharmacology and Therapeutics, College of Medicine, Chukwuemeka Odumegwu Ojukwu University, Awka Campus. The procedures were in accordance with guidelines for the care and use of laboratory animals [28].

2.1 Calculation of Sample Size

Sample size of 25 rats at 95% power to detect a difference between means of 2.5 at a significant level (alpha) of 0.05 (two tailed) was chosen using the special formula for the calculation of

sample size for laboratory animals experiments [28]:

$$N = 1+2C[s/d]^2$$

Where, C = a constant (7.8) at 0.05 level of significance; s = 2.75 (standard deviation from a similar previous study) [29]; d = difference between means desired in present study.

2.2 Animal Source

Twenty five (25) rats of both sexes, 6-8 weeks old, were obtained from the animal house, Department of Pharmacology and Therapeutics, Chukwuemeka Odumegwu Ojukwu University, Awka Campus, Nigeria. The animals were certified healthy by a veterinarian. Each group of 5 rats was housed in a metal cage measuring 60 cm x 45 cm x 30 cm and was allowed free access to animal feeds (Growers, Top Feeds, Nigeria) and clean drinking water. Left over feeds and water were discarded and the cages cleaned with chlorhexidine antiseptic solution every 12 hours. Artificial light was provided by fluorescent lamps (Philips, Holland; 18 watts) and light-dark cycle of 12-12 hours maintained. The animals were maintained in this arrangement for two weeks for acclimatization.

2.3 Preparation of Plant Extracts

Two (2) kilogrammes of fresh leaves of *O. gratissimum* was collected, washed under running tap water, and air-dried at room temperature. Thereafter, the dried leaves were ground into fine powder and 50 grammes of the powder was extracted with 500 ml of distilled water using the Soxhlet method [30]. The filtrate was obtained by solvent evaporation.

2.4 Experimental Procedure

The rats were randomly divided into 5 groups of 5 each. Thereafter, we treated the animals with the following drugs by gastric gavage for 28 days [14] as follows:

Group A: Each rat was given normal feed (no ART, no extract)

Group B: Each rat was given efavirenz (Ranbaxy, India) 8.6 mg/kg, abacavir (Ranbaxy, India) 8.6 mg/kg, and lamivudine (Ranbaxy, India) 4.3 mg/kg [13].

Group C: Each rat was given 80 mg/kg aqueous leaf extract of *O. gratissimum*.

Group D: Each rat was given efavirenz 8.6 mg/kg, abacavir 8.6 mg/kg, and lamivudine 4.3 mg/kg, plus 40 mg/kg aqueous leaf extract of *O. gratissimum* [20].

Group E: Each rat was given efavirenz 8.6 mg/kg, abacavir 8.6 mg/kg, and lamivudine 4.3 mg/kg, plus 80 mg/kg aqueous leaf extract of *O. gratissimum*.

A treatment chart was maintained. After 28 days, blood samples were collected from each rat for the estimation of serum ALT and AST. The liver was also harvested for histology.

2.5 Collection of Blood Samples

The rats were anaesthetized, one at a time, using intramuscular ketamine (Nirma, India) and diazepam (Norris Medicals, India) 50 mg/kg and 5 mg/kg respectively [31]. Thereafter, blood samples were collected using the method described by Hoff [32]. Briefly, the skin over the jugular vein was cleaned with methylated spirit-soaked cotton wool and 1.0-1.5 mls of whole blood withdrawn through the jugular vein using a 25G hypodermic needle fitted unto a 2 ml syringe. The withdrawn blood samples was transferred gently into plain specimen bottles and allowed to clot naturally. Thereafter, the serum was separated into another specimen bottles using a micropipette and stored at 2-8℃ until use.

2.6 Harvesting of the Livers

Each rat was sacrificed by hitting the occiput on concrete slab and the liver harvested and stored in 10% buffered formalin until processed.

2.7 Determination of Serum ALT and AST

We determined the serum ALT and AST using AST and ALT kits (Randox, United Kingdom) and UV-VIS spectrophotometer (Spectrumlab 752S, New Life Medical, England) based on the principle described by Reitman and Frankel [33].

2.8 Histological Examination of the Liver

The livers were processed for histological analyses. The slides were prepared as described by Ochei and Kolhatkar [34].

2.9 Statistical Analysis

The mean values ± S.E.M. of AST and ALT were calculated. Then the data were tested for normality using D'Augostino and Pearson omnibus normality test. The data failed the D'Augostino and Pearson omnibus normality test. Therefore, values of serum ALT and AST obtained for the test and control groups of rats were tested for statistically significant differences using Mann Whitney test or Kruskal-Wallis test plus Dunn's multiple comparisons. There was no transformation of data. All the statistical tests were performed using Graph Pad Prism 6.0 and the results taken as statistically significant if the p value < 0.05.

3. RESULTS

Three rats (one from group C, one from group D, and one from group E) died during the course of the experiment. The cause of death was not known.

3.1 Yield of the Extract

Fifty (50) grammes of the powdered leaves yielded 22.5 grammes of extract giving a percentage yield of 45.

3.2 Serum Aminotransferases

The values of serum ALT and AST obtained were within the reference values. Contrary to expectations, these values did not change when increasing doses of the extract were added to the antiretroviral drugs.

The mean values (± S.E.M) of serum ALT are shown in Table 1. When subjected to further analysis, there was no statistically significant difference between the ALT values for the test and control groups of rats as shown in Fig. 1 (p value > 0.999).

The mean values (± S.E.M) of serum AST are shown in Table 2. Further analysis of the data revealed that there was a statistically significant difference between the mean values for group B (ART alone) and those for group C (extract alone) as shown in Figs. 2 and 4 (p value = 0.016).

However, there was no statistically significant difference between the mean values of serum AST for group B (ART alone) and those of group A (no extract, no ART), group D (ART + 40 mg/kg extract), or group E (ART + 80 mg/kg extract) as shown in Fig. 4.

Also, increasing the dose of the extract from 40 mg/kg (group D) to 80 mg/kg (group E) did not significantly affect the level of serum AST as shown in Fig. 3 (p value = 0.659).

3.3 Histology Report

Histologically, the livers appear normal for both the control and test groups. Even the group that received antiretroviral drugs alone did not show abnormal liver histology.

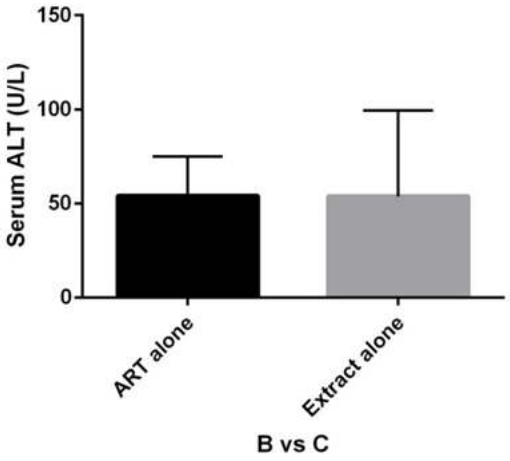


Table Analyzed	Data 1
Column B	Extract alone
vs.	vs.
Column A	ART alone
Mann Whitney test	
P value	> 0.9999
Exact or approximate P value?	Exact
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	25 , 20
Mann-Whitney U	10
Difference between medians	
Median of column A	56.00, n=5
Median of column B	52.50, n=4
Difference: Actual	-3.500
Difference: Hodges-Lehmann	-1.000

Fig. 1. Serum alanine aminotransferase (ALT) values of rats treated with antiretroviral drugs (ART) alone (group B) compared to those of rats treated with aqueous leaf extracts of *Ocimum gratissimum* alone (group C) using nonparametric Mann Whitney test

Table 1. Mean (± S.E.M) serum alanine aminotransferase (ALT) values (U/L) in the test and control groups of rats

Group A (A_1-A_5)	Group B (B_1-B_5)	Group C (C_1- C_4)	Group D (D_1-D_4)	Group E (E_1-E_4)
31	62	27	51	78
34	82	105	58	124
23	25	05	110	59
30	45	78	143	85
60	56	-	-	-
Mean 35.6±6.4	54.0±9.4	53.8±22.9	90.5±21.9	86.5±13.7

Table 2. Mean (± S.E.M) serum aspartate aminotransferase (AST) values (U/L) in the test and control groups of rats

Group A (A_1-A_5)	Group B (B_1-B_5)	Group C (C_1-C_4)	Group D (D_1-D_4)	Group E (E_1-E_4)
127	194	20	240	269
130	161	63	55	152
118	250	138	76	128
122	198	19	124	53
222	223	-	-	-
Mean 143.8±19.7	205.2±14.9	58.0±27.9	162.3±41	50.5±44.8

Table 3. AST/ALT ratios in the test and control groups of rats

Group	AST (U/L)	ALT (U/L)	AST/ALT ratio
A	35.6	143.8	0.25
B	54.0	205.2	0.26
C	53.8	58.0	0.92
D	90.5	162.3	0.56
E	86.5	150.3	0.58

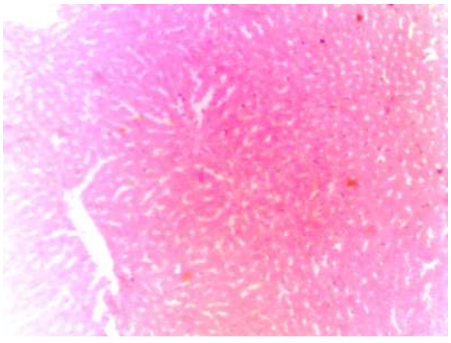


Plate 1. Photomicrograph (x 40) of the histology of the liver of a rat from group A (no extract, no ART) showing normal liver architecture

4. DISCUSSION

The mean values for serum ALT in group A (no ART, no extract) was 35.6±6.4 U/L which is within the reference range of 18-45 U/L [13]. Also, the mean value for serum AST in group A (no ART, no extract) was 143.8±19.7 U/L which is also within the reference range of 74-143 U/L. However, reference values for ALT and AST in rats vary widely from laboratory to laboratory and from region to region [35]. ALT is more specific for the liver, but AST is often used to monitor the response of the liver to drug and other toxic substances especially in rats [36].

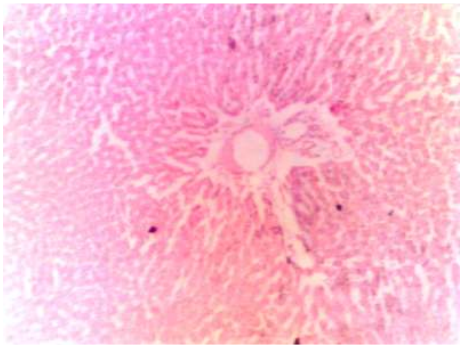


Plate 2. Photomicrograph (x 40) of the histology of the liver of a rat from group B (ART alone) showing normal liver architecture

The serum ALT and AST values in groups A, B, D, and E are slightly raised, but none is raised up to five times (5x) above normal value. Therefore, the animals sustained mild disturbances of liver function and not toxic liver damage [37]. The AST/ALT ratio is useful for categorizing liver damage. Ratios greater than 1 are suggestive of liver damage [38,39]. In contrast, Ajibade et al.

[40] orally administered high doses of the aqueous extract of this plant on adult Wistar rats and recorded abnormal liver enzymes values.

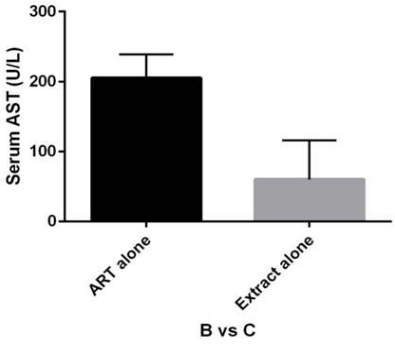


Table Analyzed	Data 1
Column B	Extract alone
vs.	vs.
Column A	ART alone
Mann Whitney test	
P value	0.0159
Exact or approximate P value?	Exact
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	35 , 10
Mann-Whitney U	0
Difference between medians	
Median of column A	198.0, n=5
Median of column B	41.50, n=4
Difference: Actual	-156.5
Difference: Hodges-Lehmann	-151.0

Fig. 2. Serum aspartate aminotransferase (AST) values of rats treated with antiretroviral drugs (ART) alone (group B) compared to those of rats treated with aqueous leaf extracts of *Ocimum gratissimum* alone (group C) using nonparametric Mann Whitney test

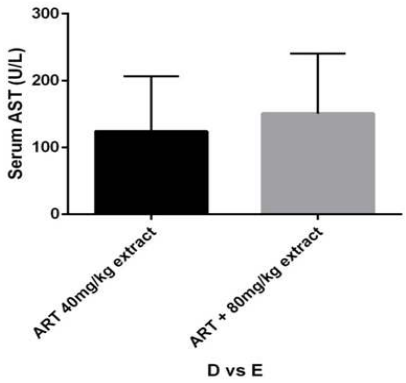


Table Analyzed	Data 1
Column B	ART + 80mg/kg extract
vs.	vs.
Column A	ART 40mg/kg extract
Mann Whitney test	
P value	0.6571
Exact or approximate P value?	Exact
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	16 , 20
Mann-Whitney U	6
Difference between medians	
Median of column A	100.0, n=4
Median of column B	140.0, n=4
Difference: Actual	40.00
Difference: Hodges-Lehmann	28.50

Fig. 3. Serum aspartate aminotransferase (AST) values of rats treated with antiretroviral drugs (ART) plus 40 mg/kg aqueous leaf extracts of *Ocimum gratissimum* (group D) compared to those of rats treated with (ART) plus 80 mg/kg aqueous leaf extracts of *Ocimum gratissimum* (group E) using nonparametric Mann Whitney test

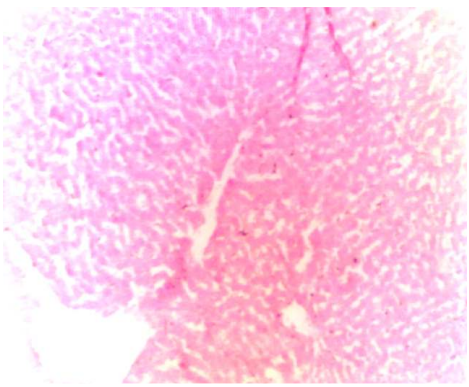


Plate 3. Photomicrograph (x 40) of the histology of the liver of a rat from group C (80 mg/kg extract of *Ocimum greatissimum*) showing normal liver architecture

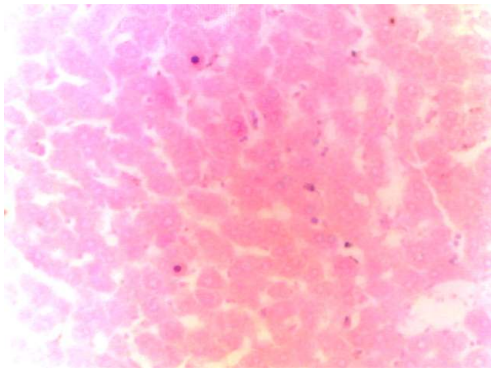
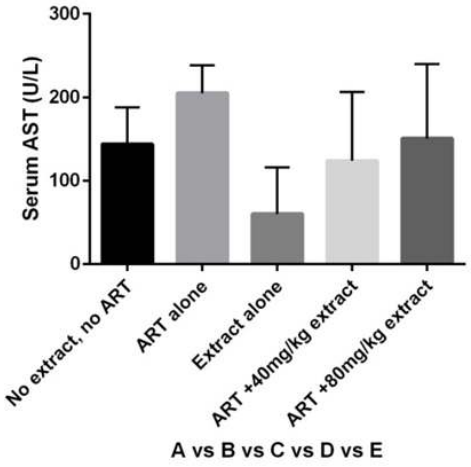


Plate 4. Photomicrograph (x 40) of the histology of the liver of a rat from group D (ART plus 40 mg/kg extract of *Ocimum gratissimum*) showing normal liver architecture



Number of families	1					
Number of comparisons per family	4					
Alpha	0.05					
Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary		B-?	
ART alone vs. No extract, no ART	6.600	No	ns		A	No extract, no ART
ART alone vs. Extract alone	12.35	Yes	*		C	Extract alone
ART alone vs. ART +40mg/kg extract	7.850	No	ns		D	ART +40mg/kg extract
ART alone vs. ART +80mg/kg extract	5.100	No	ns		E	ART +80mg/kg extract
Test details	Mean rank 1	Mean rank 2	Mean rank diff.	n1	n2	
ART alone vs. No extract, no ART	17.60	11.00	6.600	5	5	
ART alone vs. Extract alone	17.60	5.250	12.35	5	4	
ART alone vs. ART +40mg/kg extract	17.60	9.750	7.850	5	4	
ART alone vs. ART +80mg/kg extract	17.60	12.50	5.100	5	4	

Fig. 4. Serum aspartate aminotransferase (AST) values of rats treated with antiretroviral drugs (ART) alone (Group B) compared with those treated with a combination of ART and extract of *Ocimum gratissimum* using Kruskal-Wallis test followed by Dunn's multiple comparison test. [Group A (no extract, no ART), group C (extract alone), group D (ART + 40 mg/kg extract), group E (ART + 80 mg/kg extract)]

In this study, the AST/ALT ratios are less than 1, suggesting minimal disturbances of liver function. There was no statistically significant difference between the ALT values for the test and control groups of rats as shown in Fig. 1 (p value > 0.999), but there was a statistically significant reduction (P value = 0.0159) in AST values in group C (extract alone) compared to those in group B (ART alone). This finding which demonstrated the hepatoprotective of the extract is in line with some earlier studies [20]. However, the addition of the extract to the ART in groups D and E did not confer hepatoprotection on these groups as shown in Fig. 4. A possible explanation for this observation could be that the dose of extract added was suboptimal or that the duration of treatment was short. Usually, administration of hepatotoxic drugs to rats produces abnormal aminotransferases levels which peak within one week after initiation of treatment. This peak drops at four weeks and a steady level is maintained as long as ART treatment is continued [41]. Therefore, it seems that more time is needed for the hepatotoxicity to be reversed and the increased plasma aminotransferases to be restored to normal by the addition of the hepatoprotective extract. On the other hand, in some studies that produced hepatoprotection, the rats were pre-treated with the extracts before the hepatotoxic drugs were administered [42]. This pre-treatment with the extract probably stabilizes the hepatocytes before the administration of the hepatotoxic drugs since the mechanism of hepatoprotection by these herbs is known to be through the production of antioxidants and heat shock proteins which stabilize cell membranes [11,19]. Pre-treatment with the extract before the initiation of ART should be tried in subsequent studies.

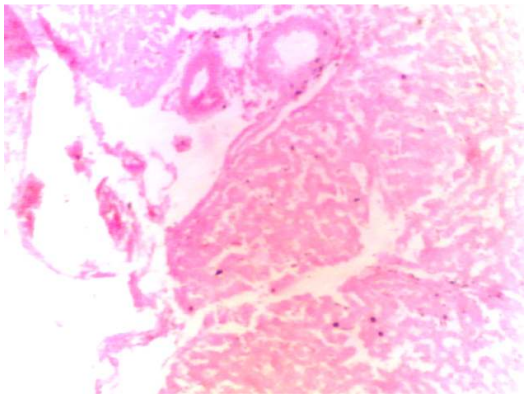


Plate 5. Photomicrograph (x 40) of the histology of the liver of a rat from group E (ART plus 80 mg/kg extract of *Ocimum gratissimum*) showing normal liver architecture

The findings in this study may not actually simulate what will happen when HIV/AIDS patients are used as subjects in this study. HIV/AIDS patients who usually have reduced plasma glutathione levels [43] are more likely to benefit from hepatoprotective herbs when on antiretroviral therapy. This is because such herbs are known to increase plasma glutathione level as already stated above. In addition to stabilizing the hepatocyte membrane, glutathione also boosts the immune system which is very important in hepatoprotection. It is obvious that the liver in such HIVAIDS patients will be more susceptible to hepatotoxic effects of antiretroviral drugs and will, therefore, benefit more when hepatoprotective herbs are used as therapeutic adjuncts in these patients.

The normal histological findings in the liver in this study are not surprising since the serum aminotransferases were not abnormally raised. This is in keeping with some findings that biochemical changes often occur earlier than histological changes in cases of liver disease [44].

5. CONCLUSION

This extract produced statistically significant reduction of serum aminotransferases in the group of rats that received extract alone. However, this reduction in serum aminotransferases was not observed in the groups that received antiretroviral drugs plus the extract. These statistical findings show that there was no statistically significant difference in serum ALT and AST between the exposed and control groups ($p > 0.05$). Consequently, the null hypothesis was accepted and the alternative hypothesis rejected. Therefore, it could be concluded that at the doses of ART and extract and length of exposure used in this study, aqueous leaf extract of *Ocimum gratissimum* did not reduce the serum level of ALT and AST in rats.

The reason for this could be suboptimal dose of extract or short duration of treatment. Perhaps, better results could be achieved if HIV/AIDS models of rats or HIV/AIDS patients are used as subjects in the study. Future studies should take these factors into consideration.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The study was approved by Institutional Ethical Committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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