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# Bitter leaf (*Vernonia amygdalina Del*) extract potentiates testicular metabolic stress induced by petroleum-tainted diets in rats

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**ABSTRACT**

The effect of hydrocarbon adulterated diets on male fertility has been of grave concern to researchers. This study evaluated the effects of bitter leaf methanol extract on petroleum-induced testicular damage. *Rattus norvegicus* species of albino rats were allotted into six sets. Each set composed six rats. The sets were defined as follows: Set A was rats fed Feed without any treatment; Sets B and C were given feed without treatment and measured doses of bitter leaf extract; Set D was fed unrefined petroleum tainted feed; Sets E and F were given tainted feed and doses of bitter leaf extract used in sets B and C, respectively. After the exposure period, testes were removed from sacrificed animals and clinical investigation carried out. Analysis of variance (ANOVA) was employed for data comparison. The results revealed that unrefined petroleum tainted diets meaningfully ($P < 0.05$) prompted testicular metabolic stress indicators, while significantly ($P < 0.05$) plummeting the antioxidant defense markers compared to control. Administration of bitter leaf extract was not able to ameliorate altered indices of stress but contributed to increase in testicular metabolic stress. The study concluded that bitter leaf enhanced testicular damage imposed by unrefined petroleum adulterated feed and may possibly have a noxious effect on the testes.

**Key Words**: Bitter leaf, testes, metabolic stress, petroleum, tainted diets

## INTRODUCTION

The biosphere in which we animate has increasingly been overwhelmed with numerous types of environmental toxicants which have the tendencies of causing injuries and metabolic derangement to plants, animals and humans alike (Ita et al., 2018 ). Several industrial pollutants which include unrefined petroleum and its allied products such as kerosene, flared gases, premium motor spirit, diesel, 1,3-dinitrobenzene or nonylphenol, methanoxyethanol, glycol ether and brake oils are known to exert testicular metabolic derangement and atrophy (Samanta et al., 1997; Han et al., 2004; Gonzalez-Fletcha, 2004;Chitra et al., 2008; Adesanya et al., 2009). One significant danger of exposure to environmental toxicants is the increased risk of infertility (Zegers et al., 2009; WHO, 2010). Ample evidences from studies reveal that most male infertility are a result of testicular oxidative derangement which has been reported to affect seminal plasma antioxidants (Tremellen, 2008) increased lipid peroxidation; alteration of sperm morphology (Henkel, 2011), impaired sperm motility (Macleod, 1943; Plante et al., 2011; Wright et al., 2014) and reduced sperm concentration due to DNA damage (Schutle et al., 2010; Zribi et al 2011; Ita et al., 2018).The defense against oxidative stress depends on the ability of the body to the boost inherent antioxidants in clearing of oxidative radicals generated from various metabolic processes and toxicants. Today, *Vernonia amygdalina* which is a well-known vegetable common to many tribes of Nigeria has been elucidated for its antioxidant buffering capacities (Fraga, 2007; Oboh et al., 2008; Ohigashi et al., 1991; Fasakin et al., 2011; Egharevba et al., 2014).*Vernonia amygdalina* is well known for its use as an alternative regimen for malaria (Challand and Wilcox, 2009; Masaba, 2000). It has been used severally as a protective and ameliorative agent for the deleterious effects of many toxicants such as cyanide, carbon tetrachloride, unrefined Petroleum and cycasin (Adaramoye et al., 2008; Adesannye and Farombi, 2010; Lolodi and Eriyamremu, 2013; Kadiri, 2017, Achuba, 2018a).

In fact, there is ample evidences on the ability of unrefined petroleum to induce various forms of metabolic oxidative stress, there exist little evidence on the possible role of unrefined oil intoxication to induce testicular damage occasioned by unrefined petroleum adulterated feeds as well as the ability of *Vernonia amygdalina* to induce the possible restoration or control of activated metabolic stress parameters. This study therefore was carried out to cover these existing gaps. (NRC, 2011). Analytical reagents were employed for the biochemical assays

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## MATERIALS AND METHODS

Matured bitter leaves (Vernonia amygdalina Del) were obtained and identified as reported previously by Achuba (2018). Male albino rats (Rattus norvegicus), thirty six, an average weight of 166 g were acquired as earlier reported Achuba (2018). The rats were accommodated in a wooden cage and allowed to acclimatize for one week on grower's mash (a product of Rainbow Feed Limited). The component of the feed as declared by the manufacturer was previously published by Achuba (2018a). National research council guide for animal care was adopted during the investigation ( NRC, 2011).. Analytical reagents were employed for the biochemical assays.

### Bitter Leaf Extract Preparation

The bitter leaves were first washed and subsequently dried for one week at room temperature in the laboratory. After drying, the bitter leaves were separated from the stalk and ground with a warren blender to a smooth dry powder. The bitter leaf extract was prepared using methanol as reported by Yin *et al*. (2013). One hundred (100 g) of the powdered leaf was dissolved in methanol (400 mL) through sonication for 10 min, then filtered with WhatmanNo.1 using vacuum pump. The extract obtained concentrated and stored in line with Achuba (2018).

### Experimental Design and Treatment

The experimental design followed the description done by Achuba (2018) thus: Set A were rats given untreated feed; Sets B were rats given untreated feed and 100 mg for each kilogram body weight of extract; Set C wer rats given untreated feed and 200 mg for each kilogram body weight of extract; Set D were rats given unrefined petroleum-tainted feed; Set E and F were rats given adulterated feed and 100 mg and 200 mg for each kilogram body weight of extract, respectively. The bitter leaf extract used was prepared daily. To do this, 20 g of the ground leaf was dissolved in 100 ml of distilled water to generate 200 $mgml^{-1}$ out of which aliquots of the freshly dissolved extract was administered by gavage according to the rat's body weight once a day. The rats in sets A and D were not placed on the extracts. Rats in all sets had free access to water. All the treatments lasted for 30 days.

### Sample Collection

The rats were sacrificed on the thirty first day without feeding them the previous night. The testes were collected into pre-chilled labelled sample containers. The testes from each set were homogenised and stored as stated in Achuba (2018)

### Biochemical Analysis

Standard methods were employed for the assay of level of lipid peroxidation (MDA) as described by Gutteridge and Wilkins ( 1982) and enzymatic oxidative stress markers as follows; aldehyde oxidase (AO) (Omarov et al. 1998), sulphite oxidase (SO) (Macleod et al. 1961); monoamine oxidase (MO) and xanthine oxidase (XO) McEwen ( 1971). Assay for the non-enzymatic antioxidant profile determined using the methods of Ellman ( 1959) for reduced glutathione while Assay for vitamin C employed the methods reported by Achuba ( 2008). Assay for specific activities of enzymatic antioxidants were carried out employing the methods of Misra and Fredorich (1972) for total superoxide dismutase (SOD), Crapo et al.(1972) for Cu/ZnSOD and MnSOD, Cohen et al., (1972) for Catalase, Habig et al. (1974) for gluthatione-s-transferases (GSTs) and Khan et al. (2009) for glutathione peroxidase (GPx). The routine of Lowry et al. (1951) was used to evaluate for protein.

### Histological Examination

A known portion of the testes, of each rat was taken and subjected to the procedure described by Achuba ( 2018). Thin sections staining with haematoxylin and eosin (H and E) was carried out using the technique of Odoula *et al* ( 2009), examined and filmed via a light microscope.

### Statistical Analysis

Statistical Package for Social Sciences (SPSS 17) was used for data analysis. Post hoc analysis (comparisons across Sets) was done using Bonferroni setting significant level at $P < 0.05$.

## RESULT

Result presented (Table 1) revealed a significant rise in thiobarbituric acid reactive substance, stated as MDA in rats given different doses of bitter leaf extract without tainted diets (B and C) relative to positive control (A) which was fed normal diets. However, not significantly different with rat fed tainted diets without treatment (sets D) and rats fed tainted diets and given different doses of the bitter leaf extract (sets E and F). Also, the activities of aldehyde oxidase (AO), sulphite oxidase (SO), monoamine oxidase ( MO) and xanthine oxidase (XO) of rats given the different doses of bitter leaf extract in sets B and C increased relative to rat fed with untreated feed (set A) but reduced relative to rats fed with unrefined petroleum tainted feed (set

D). When the respective doses of bitter leaf extract were given to rats fed with unrefined petroleum tainted feed (sets E and F), the activities of the oxidases (AO, SO, MO and XO) increased relative to the rat fed with untreated feed (set A) and rats fed with unrefined petroleum tainted feed (set D). However, no significant changes between sets E and F when taken together.

Table 1: Effect of bitter leaf extract on testicular lipid peroxidation and oxidative enzyme activities

| Sets | MDA $\mu mol g^{-1}$tissue | AO Unitsg$^{-1}$tissue | SO Unitsg$^{-1}$tissue | MO Unitsg$^{-1}$tissue | XO Unitsg$^{-1}$tissue |
|---|---|---|---|---|---|
| **A** | 743.04±10.18$^{a}$ | 65.25±2.99$^{a}$ | 612.50±11.75$^{a}$ | 133.00±5.60$^{a}$ | 47.00±2.58$^{a}$ |
| **B** | 812.68±8.57$^{b}$ | 69.25±2.62$^{ab}$ | 639.00±4.16$^{b}$ | 140.50±5.51$^{a}$ | 62.00±4.32$^{b}$ |
| **C** | 828.57±25.00$^{b}$ | 74.25±2.06$^{b}$ | 652.00±6.37$^{b}$ | 151.00±3.56$^{b}$ | 57.75±6.95$^{ab}$ |
| **D** | 820.54±27.14$^{b}$ | 81.50±1.29$^{c}$ | 672.25±4.92$^{c}$ | 159.00±5.29$^{bc}$ | 66.25±4.99$^{b}$ |
| **E** | 833.93±10.00$^{b}$ | 88.75±2.22$^{d}$ | 688.75±7.36$^{d}$ | 166.25±1.71$^{cd}$ | 67.75±9.60$^{b}$ |
| **F** | 799.46±7.68$^{b}$ | 92.00±2.71$^{d}$ | 697.25±2.98$^{d}$ | 170.50±2.08$^{d}$ | 64.25±5.79$^{b}$ |

MDA = Malondialdehyde level; AO=Aldehyde oxidase activity; SO= sulphite oxidase activity; MO= Monoamine oxidase activity; XO = Xanthine oxidase activity. All values are expressed as Mean±SD values followed by different alphabet superscript in the same column indicates a significant different.

As shown (Fig 1), the actions of CuZnSOD did not differ meaningfully in rats given 100 mg of bitter leaf extract (sets B) from rats given untreated diets. However, rats given 200 mg exhibited elevated action likened to control. Also, the CuZnSOD actions in rats given respective doses were meaningfully elevated in the rats given petroleum adulterated diets without treatment and rats fed tainted diets and treated with the respective doses of bitter leaf extract. The actions of MnSOD did not change in rats in sets A and B but elevated meaningfully when rats in set C and set A are matched. The actions of MnSOD meaningfully reduced in rats fed with petroleum adulterated diets and rats fed normal diets and treated with respective doses of bitter leaf extract in sets B and C. Treatment of rats fed tainted diets with 100mg and 200 mg of bitter leaf extract showed no noteworthy difference relative to the untreated rats in set D. Total SOD actions showed no significant difference across sets A-E but meaningfully reduced in set F which was exposed to tainted diets and treated with 200 mg of extract compared to sets A-D.

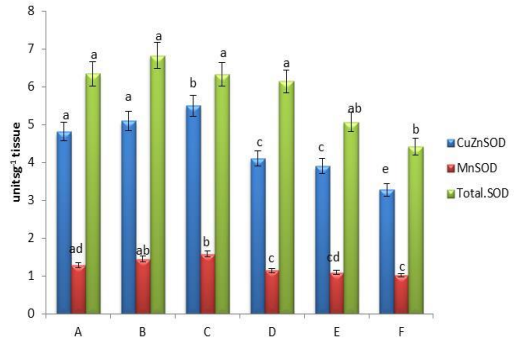


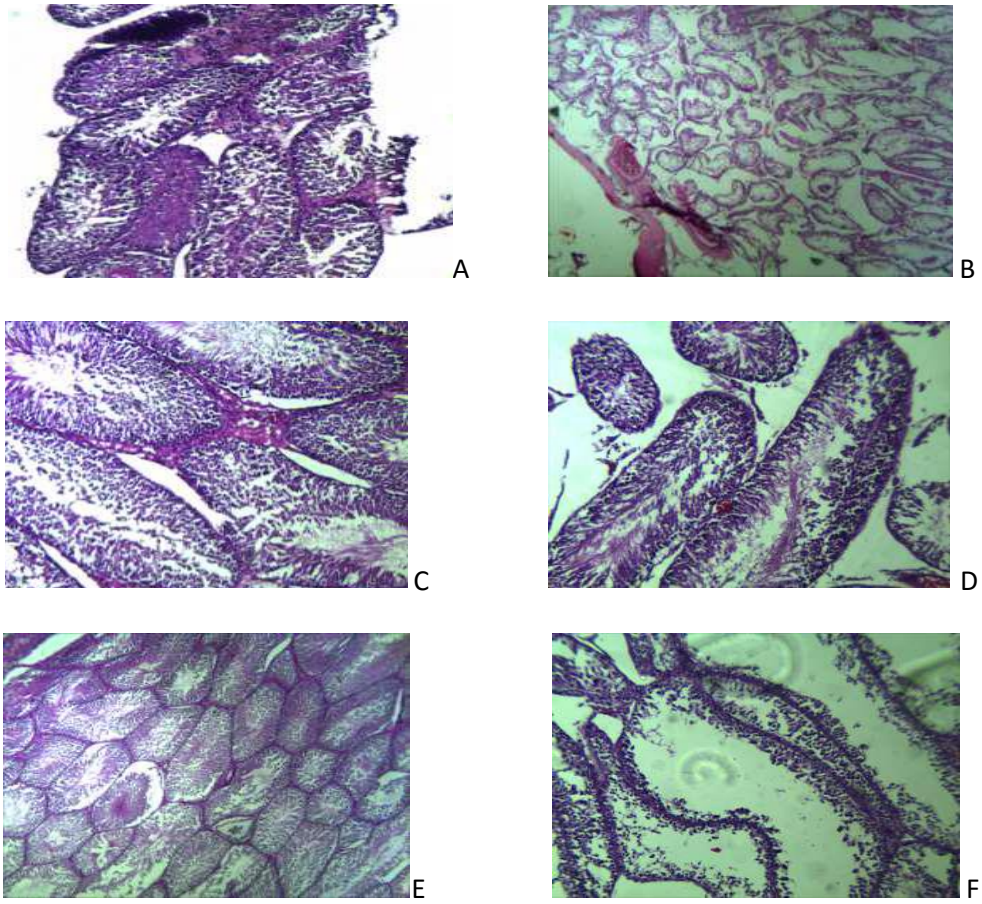

Figure 1: Effect of bitter leaf extract on testicular CuSOD, MnSOD and total SOD activities of rats Bars of same colour with different alphabet superscript indicate a significant difference.

Results presented (Table 2) reveal that levels of Vitamin C did not change substantially across all sets. GSH levels were observed to have no noteworthy change in rats placed on 100 mg and 200 mg of bitter leaf extract (B and C) comparative to set A but meaningfully elevated in relation to rats fed petroleum adulterated diets. Rats placed on 100 mg of bitter leaf extract increased GSH levels comparative to those fed only tainted diets but reduced relative to normal control (set A). Those fed adulterated diets and placed on 200 mg of bitter leaf extract (set E) remained unchanged comparative to group D but reduced in relation to all other sets. The action of the antioxidant enzyme, catalase was meaningfully elevated in rats placed on 100 mg but not with 200 mg comparative to control set A. This was however improved meaningfully for both doses comparative to rats in group D fed tainted diets without management with bitter leaf extract. Management with the respective doses led to a further reduction in catalase actions comparative to sets A and D. GPX and GSTs actions were observed to have no substantial change for rats place on 100 mg without contamination relative to control (set A) and reduced meaningfully for GPX while increasing for GSTs relative to rats fed only polluted diets (group D). Management with respective doses of bitter leaf extract meaningfully reduced in GSTS actions comparative to the control set A while GPX actions were only meaningfully reduced for the 200 mg dose. Comparative to set D however, it was observed that GSTs actions remained meaningfully unchanged both doses (100 mg and 200 mg) .GPX on the other hand reduced meaningfully for both doses.

Table 2: Effect of bitter leaf extract on some enzymatic and non-enzymatic antioxidant profiles in testes

| | **Vitamin C mgg$^{-1}$Fwt** | **GSH µmolmg$^{-1}$ protein** | **CAT *nmol*mg$^{-1}$ protein** | **GPx µmolmg$^{-1}$ protein** | **GSTs µmolmg$^{-1}$ protein** |
|---|---|---|---|---|---|
| **A** | 3.52±0.72$^{a}$ | 0.28±0.05$^{a}$ | 211.57±1.04$^{a}$ | 0.51±0.09$^{ac}$ | 378.05±28.92$^{a}$ |
| **B** | 3.75±0.76$^{a}$ | 0.32±0.07$^{a}$ | 229.34±13.03$^{b}$ | 0.50±0.02$^{ac}$ | 389.05±28.03$^{ab}$ |
| **C** | 3.78±1.24$^{a}$ | 0.30±0.03$^{a}$ | 213.33±4.05$^{a}$ | 0.56±0.09$^{ab}$ | 423.05±19.18$^{b}$ |
| **D** | 2.60±0.41$^{a}$ | 0.16±0.02$^{b}$ | 183.43±1.07$^{c}$ | 0.69±0.53$^{b}$ | 316.54±20.09$^{c}$ |
| **E** | 3.45±0.31$^{ab}$ | 0.22±0.01$^{c}$ | 164.66±0.86$^{d}$ | 0.57±0.046$^{a}$ | 339.00±12.08$^{cd}$ |
| **F** | 2.32±0.96$^{a}$ | 0.19±0.00$^{b}$ | 153.27±2.16$^{d}$ | 0.36±0.06$^{c}$ | 305.50±14.10$^{c}$ |

GSH= Reduced glutathione level; CAT = Catalase activity; GPx = Glutathione peroxidase activity; GSTs = Glutathione – S- transferase activity
All values are expressed as Mean ± SEM values followed by different alphabet superscript in the same column indicates a significant difference




Key: A= Control diet, showing normal architecture; B= diet + 100mg of extract, showing mild distorted architecture; C= diet+ 200mg of extract, showing normal architecture; D = diet+ 4 mL crude petroleum, showing severe distortion of architecture; E = diet+ 4mL crude petroleum+100mg of extract normal testicular architecture; F = diet+ 4 mL crude petroleum + 200mg of extract, showing distorted architecture**.** Haematoxylin and eosin x 100 magnifications

Fig. 2A-F: Photomicrograph of rat testes fed petroleum contaminated diets administered methanol extract of bitter leaf

Figures, A, C and E show normal testicular architecture indicating very clear seminiferous tubules and different stages of spermatogenesis and spermatozoa while photomicrograph B indicates early stages of germinal infiltration and inflammation of the seminerous tubules containing depleted germ cells. Photomicrograph D shows early stages of inflammation, well established seminiferous tubules and early stages of germ cell depletion. Photomicrograph F show established inflammation and distorted seminiferous tubules with depleted germ cells.

**DISCUSSION**

Unrefined petroleum contamination is a noteworthy contributor to numerous endocrine induced metabolic stress and malfunction (Kisin *et al.,* 2015; Ebokaiwe and Farombi., 2016). In the same vein, oxidative stress is involved in the many cases of infertility world over (Agarwe and Said, 2005; Aitken and Roman, 2008). The result presented in this study (Table 1) revealed enhanced MDA and the actions of the oxidative enzymes (AO, SO, MO and XO) in the testes of rats fed hydrocarbon tainted diets comparative to normal control. Rise in MDA is widely documented as a potent marker for the negative affronts of consuming unrefined petroleum diets and other refined oil allied exposures (Okpoghono *et al.,* 2018a, Achuba, 2018c; Ita *et al.,* 2018). Petroleum induced rise in peroxidation of tissues are said to go concurrently with eventual rise in oxidative enzymes which are needed to initiate eventual clearance of the peroxides and super oxides generated by petroleum contamination (Achuba, 2018b, Okpoghono *et al.,* 2018b). It is key to state that centred on the biological position and disposition of the testes, it is said to be highly vulnerable to toxins hence there exist an inbuilt enhanced antioxidant shielding due to the existence of an array of non-enzymatic and enzymatic antioxidants (Peltola *et al.,* 1992; Paolicchi *et al.,* 1996; Kaur *et al.,* 2006;; Sonmez *et al*., 2005; Maneesh *et al*., 2005). Therefore, for oxidative damage to occur, there must be evidence established overwhelming of the antioxidant defence capacities of the tissues and biological organs involved (Asagba, 2010; Achuba *et al.,* 2018). The observed MDA and the oxidative enzymes in the testes of rats fed petroleum polluted diets without management with bitter leaf extract agree with the observed reduced levels of the antioxidant defensive markers (Fig 1) and (Table 2). These results agree with earlier observations made by Achuba *et al.,* (2018); Achuba (2018a) and Ita *et al.,* (2018). The management of these rats with bitter leaf extract was not able to reverse to a comparable status the levels of these non-enzymatic and enzymatic antioxidants relative to the control which was not fed with petroleum tainted diets. These observations are not in line with earlier submissions made by Ita *et al,* (2018), Achuba, (2018a) and Okpoghono *et al.,* (2018a) who reported the abilities of *Ageratum conyzoides,Vernonia amygdalina* and *Monodora myristica* to successfully mitigate the rising metabolic stress in the testis, kidney and liver of rats fed petroleum tainted diets respectively. This observed trend thus indicates the inability of the bitter leaf extract to control the oxidative and metabolic balance of the testes in the presence of petroleum hydrocarbons.

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This study established a connection between rise in testicular MDA, the antioxidant system, and enzymes involved in oxidative reactions in rats managed with the respective doses of the bitter leaf extract without exposure to unrefined oil polluted diets. This observation also further substantiates the earlier claim of the possible noxiousness of bitter leaves on the testes which has been previously implicated to have a possible anti spermatogenic effect, functional characteristics of the testes, reduction in testosterone level and increased toxicological profile of the testes (Oyedeji *et al*., 2013; Salau *et al.,* 2013).The possible rationalization of this observation may be associated to adverse interactions that occur during drug and xenobiotic metabolism (Barnerjee *et al.,* 2016; Gandhi *et al.,* 2012). This is predicated on ample evidence that submits that in the course of drug and xenobiotic biotransformation, that certain enzymes such as the lipoxygenases, cyclooxygenase and the oxidases have the capacities of developing oxidative radicals which in turn contributes to the pool of ROS present in the tissues that eventually shuts down their antioxidant defence systems by the depletion of these enzymes and other antioxidant enzymes (Asagba and Eriyamremu, 2007;Asagba, 2010; De Faria *et al.,* 2015; Hirota, 2015). There is no doubt that findings in this study indicated testicular hypertrophy and autophagy in the rats. The observed distortion in testicular architecture are similar to those reported by Salau *et al.,* (2013) and Oyedeji *et al.,* (2013) thus substantiating further the possible contribution of bitter leaf to all the observed metabolic stress reported in this study.

**CONCLUSION**

The current study established that the consumption of petroleum tainted diets contributed to the induction of testicular metabolic stress in experimental rats.

However, treatment with bitter leaf methanolic extracts could not reverse the observed metabolic stress but contributed to the damage in the testes. This thus gives an insight into the possible toxic effect of bitter leaf on the ability to induce infertility in males. Based on this therefore, it is submitted that there is need for further research to understand the possible mechanism and molecular bases for the observed alteration of theses metabolic stress in the testis owing to the proven records of the antioxidant buffering capacity of bitter leaf in other tissues.

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