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Research Article
Effect of *Vernonia amygdalina* Leaves Extract on Testicular Functions of D-Galactose Induced Ageing Male Albino Rats

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Abstract

Background and Objective: Developing countries, particularly in Africa are affected by the ageing population's challenges because of the expensive health care provision. An inexpensive and safe medicinal plant could be an option. This present study investigated the anti-ageing effects of Methanolic Extract of *Vernonia amygdalina* (MEVA) leaves on the testis of D-galactose induced ageing male Albino rats. **Materials and Methods:** Four groups containing eight rats each were randomly selected. Group I (control) rats were given 1 mL of normal saline, group II rats were treated with D-galactose of 300 mg kg^{-1} b.wt., only, group III rats were treated with 300 mg kg^{-1} b.wt., of D-galactose and 200 mg kg^{-1} b.wt., of MEVA while group IV received 300 mg kg^{-1} b.wt., of D-galactose and 300 mg kg^{-1} b.wt., of MEVA for 28 days orally. Quantitative analysis of phytochemicals and antioxidant constituents of the leaves were carried out. Catalase, superoxide dismutase, reduced glutathione and malondialdehyde were assayed in the testis homogenate using spectrophotometry. Testosterone, luteinizing hormone and follicle-stimulating hormone were measured in plasma using enzyme-linked immunoassay methods (ELISA). Semen analysis was carried out on the caudal epididymis and histology of the testis was done by the H and E staining method. **Results:** The results showed a significant reduction ($p<0.05$) in antioxidants and hormonal levels in the D-galactose group compared to the control. In contrast, treatment with 300 mg kg^{-1} of MEVA significantly ($p<0.05$) improved sperm properties, testosterone level and testicular redox state compared with the D-galactose group. **Conclusion:** This study suggests that methanolic extract of *Vernonia amygdalina* at a dose of 300 mg kg^{-1} may reduce the possibilities of male infertility associated with ageing via oxidative stress mechanism.

Key words: Ageing male, antioxidant action, phytochemicals, testis, herb medicine, male infertility, D-galactose, alkaloids

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Several consequences accompany ageing in males and one significant effect is the decline of testicular function[1,2]. The decline in Leydig cell number and the reduction in the activities of enzymes required for testosterone's secretion results in a reduced level of testosterone[3]. This decline in testosterone level can reduce sexual function and fertility[4]. Reactive Oxygen Species (ROS) plays a significant role in the ageing process[5-8]. In the biological function of ageing, increasing evidence is suggesting that a central role is being performed by oxidative stress in this process[9,10]. Ageing can be induced in an animal model by the chronic administration of D-galactose[11-13]. The high concentration of D-galactose in the body leads to its conversion to D-galactose-hexodialdose and hydrogen peroxide by galactose oxidase. The transformation of D-galactose-hexodialdose to galactitol by aldose reductase enzyme will occur[11]. The accumulation of the obtained products in the cells may result in osmotic damage and oxidative stress that accelerates ageing[14]. D-galactose can also be used to induce senescence due to its high tendency to increase the generation of oxidants, alteration in antioxidant enzymes actions and accumulation of oxidative damage[12,15]. An increase in MDA production and altered antioxidant capacity as observed in studies related to D-galactose induced ageing might be due to these processes[16-18].

Antioxidants' ability to donate an electron neutralizes the damaging effect of free radicals, reducing oxidative stress and slowing down the process of ageing[19]. Food and medicinal plant are good sources of antioxidants[20]. The aqueous extract of *Vernonia amygdalina* has anti-oxidative stress properties[21]. *Vernonia amygdalina*, a member of the Asteraceae family, is a valuable medicinal plant that is common in West Africa, with a characteristic bitter taste which is due to its anti-nutritional contents such as alkaloids, saponins, tannins and glycosides[22]. It is a vegetable consumed in various West and Central Africa communities[23]. *Vernonia amygdalina* has multiple health benefits such as anticancer[24], antitumor[25,26], anti-diabetic[27-29], antiviral[30], antioxidant[31-33], anti-malarial[34,35] and anti-trypanosomal[36]. Previous studies reveal that the extracts of *Vernonia amygdalina* has an analgesic effect and can reduce fever, with its infusion taken in Nigeria as an anti-helminthic for the treatment of enteritis control rheumatism[37].

The previous study by Oyedeji *et al.*[38], reported the positive effect of methanolic extract of *Vernonia amygdalina* on reproductive parameters. There is an established relationship between male infertility and oxidative stress[39-41]. A proxy measure of male fertility is semen quality. After exposure to agents which are toxic or to host factors such as age, notable changes in semen quality is observed[42,43]. Therefore, ageing is an essential physiological factor to consider in the treatment of male infertility. This is corroborated by continued age-related infertility that has continued to be an issue because of the quest for better life expectancy and the need for an alternative therapy that is cheap and safe.

This study thus investigated the anti-ageing potentials of methanolic extract of *Vernonia amygdalina* leaves on the testicular function of D-galactose induced ageing male Albino rats.

MATERIALS AND METHODS

Study area: The study was performed at the Department of Animal Holdings Physiology, Ladoke Akintola University of Technology, Ogbomoso, Nigeria, from October, 2020-July, 2021.

Experimental animals: Thirty-two adult male Albino rats weighing between 140-160g from the Animal House of Physiology Department, Basic Medical Sciences Faculty, Ladoke Akintola University of Technology (LAUTECH), Ogbomoso, were used for this study. The rats were subjected to standard laboratory conditions such as ambient temperature with a 12 hrs daylight cycle and had free access to feed and water *ad libitum*. The acclimatization of the rats to the laboratory conditions took 15 days before the start of the experiments. The rats received care according to the guidelines for the use and maintenance of the animal in research by the Helsinki declaration[44]. The Research Ethics Committee, Faculty of Basic Medical Sciences, Ladoke Akintola University of Technology, Ogbomoso, Nigeria, gave the ethical research approval. All chemicals used in this experiment were of analytical grade and from Sigma-Aldrich Corporation (USA).

Plant collection and authentication: The samples were fresh leaves of *Vernonia amygdalina* from the LAUTECH school farm. The plant's identification and authentication were made by Professor A.T.J. Ogunkunle, A Professor of Plant Taxonomy in the Environmental Biology Unit of the Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso. Nigeria. The plant sample was then deposited in the University Herbarium with Voucher Number: LHO 537.

Methanolic extraction of *Vernonia amygdalina*: Methanolic Extract of *Vernonia amygdalina* (MEVA) preparation was according to the method previously described by Oyedeji *et al.*[38] fresh specimens of the plant *V. amygdala*,

which was collected, were washed thoroughly to ensure they are free of soil and debris and then air-dried for 4 weeks. The dried samples were pulverized into a dry powder by the use of mortar and pestle. A total of 556 g of the pulverized specimen was macerated in 70% of methanol (1:2 wt./vol.) for 72 hrs at room temperature and the solution produced was filtered using Whatman No 1 filter paper, with the residue rinsed several times to obtain a bright phase. The filtrate's evaporation took place in a rotary evaporator set at 60 to get the crude extract. The evaporated sample yielded 10.24% of the starting material. A total of 10 g of the MEVA was dissolved in 100 mL of distilled water to prepare a concentration of 0.1 g mL^{-1}. The extract dosages administered to the animals in this study were according to that of Adefisayo *et al.*[45].

Qualitative and quantitative analysis of phytochemicals of *Vernonia amygdalina*

Test for tannins: A total of 50 mg of MEVA was weighed, boiled in 20 mL of distilled water and then filtered. Drops of 0.1% $FeCl_3$ were added to the filtrate and colour change was observed, a blue-black or brownish-green or colour indicates tannins' presence[46].

Test for saponins: This screening utilizes saponins ability to generate emulsion with oil[46]. A total of 20 mg of MEVA was boiled in 20 mL of distilled water in a water bath for 5 min and then filtered. Subsequently, 10 mL of the filtrate was added to 5 mL of distilled water and mixed vigorously to form froth. Three drops of olive oil were then added to the froth, which was shaken vigorously and examined for the formation of an emulsion.

Determination of total polyphenols: Phenolics that were bound, free and accessible biologically were determined by making use of Folin-Ciocalteu's reagent with the aid of a microplate reader according to the method of Rocchetti *et al.*[47]. Plant sample of 25 µL in quantity was mixed with 125 µL 0.2 M Folin-Ciocalteu reagent in a 96-well microplate covered with aluminium foil. This was incubated for 10 min at 37. At the end of the incubation, 125 µL of 7.5% sodium carbonate solution was added to each well. The microplate was shaken for 40 sec and then incubated for 40 min at 37 before reading the absorbance at 765 nm using a multimode microplate reader (Tecan SPARK 10M, V1.2.20, Austria). The standard curve plotted against gallic acid was used to evaluate the concentration of polyphenols.

Test for alkaloids: MEVA (0.4 g) was mixed with 8 mL of 1% HCl and the resultant mixture was warmed and filtered[48]. Total 2 mL of the filtrate was treated separately with (a) With few drops of potassium mercuric iodide (Mayer's reagent) and (b) Potassium bismuth (Dragendorff's reagent). Turbidity or precipitation with any of (a) and (b) indicates the presence of alkaloids.

Test for terpenoids: The presence of terpenoids in MEVA was detected by measuring 5 mL (1 mg mL^{-1}) of MEVA and mixing it with 2 mL of chloroform, then mixed with 3 mL of concentrated H_2SO_4. Change in the interface's colorations to reddish-brown indicted the terpenoids presence[49].

Test for flavonoid and determination of total flavonoid content: Total 50 mg of MEVA was mixed with 100 mL of distilled water and then filtrated. About 5 mL of dilute ammonia solution was added to 10 mL of the filtrate, then a few drops of concentrated H_2SO_4. Yellow coloration confirmed flavonoids presence[49]. Total flavonoid was determined using quercetin as the standard calibration curve. The method described by Marinova *et al.*[50] was followed. The absorbance of the reaction mixtures was measured against blank at 420 nm wavelength with a varian UV-Vis spectrophotometer. All the measurements were carried out in triplicate.

Determination of total phenolic content, β-carotenoid and antioxidant DPPH assays

Total phenolic content: The total phenolics of MEVA was determined using the Folin-Ciocalteu's reagent (Sigma Aldrich, St. Louis, MO, USA), according to the procedure described by the previous researchers[51]. Sample and standard readings were made using a spectrophotometer (Cary 50 Bio UV-Vis spectrophotometer, varian) at 765 nm against the reagent blank.

2 β-carotene assay: The β-carotene assay was carried out by procedure described by Mohamad[52]. MEVA was applied on Thin Layer Chromatography (TLC) plate silica gel 60 F254 plate (20×20 cm). The plate emerged from hexane: Acetone (1:1). The blot was scraped and diluted back to the extraction solvent. The absorbance was taken at 451 nm. β-carotene content was assayed as mg of β-carotene equivalent using an equation derived from the standard curve of β-carotene.

2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay: The method described by Cheng *et al.*[53] was employed for this assay.

Summarily 100 µL of 500 µg mL^{-1} of extract and 100 µL of prepared stock of DPPH (200 M) were mixed and incubated for 30 min at 37. The absorbance was taken at 515 nm with gallic acid as a positive control. Percent radical scavenging activity was equal to:

$$\{1\text{-}(\text{sample}-\text{blank})\ (\text{control}-\text{blank})\}\times 100$$

Determination of vitamin contents

Vitamin e assay: Vitamin E assayed making use of the method described previously[54]. About 1 g of the plant sample was soaked in 20 mL of ethanol and subsequently filtered. Then 0.2% ferric chloride in ethanol with 1 mL of 0.5% α-α-dipyridine was added to 1 mL of the filtrate. The resultant solution was diluted with 5 mL of distilled water. Absorbance reading was taken at 520 nm. The same procedure was used in preparing the standard solutions and the concentration of vitamin E was extrapolated from the standard curve.

Vitamins B_1 and B_2 assay: Thiamine (vitamin B_1) and Riboflavin (vitamin B_2) were estimated using the method of Okwu and Josiah[55].

Vitamin B_1 assay: Ethanol sodium hydroxide (50 mL) together with plant extract (5 g) were homogenized and filtered into a flask, after which 10 mL of potassium dichromate was added to 10 mL of the filtrate. The absorbance of the solution was taken at 360 nm.

Determination of vitamin B_2: About 5 g of the plant sample and 100 mL of 50% ethanol were extracted. This solution was shaken for 60 min and this was followed by filtration in a 100 mL flask. About 10 mL of the extract was pipetted out into a 50 mL of volumetric flask. To this, 10 mL of 5% potassium permanganate and 10 mL of 30% hydrogen peroxide were added. This was allowed to stand for 30 min on a hot water bath. Then 2 mL of 40% sodium sulphate was added and the solution was made up to 50 mL in a standard flask, aliquots were prepared. The absorbance was measured at 510 nm using a spectrophotometer (Cary 50 Bio UV-Vis spectrophotometer, varian).

Estimation of ascorbic acid (vitamin C): Ascorbic acid was assayed using the method of Lalitha and Vijayalakshmi[56]. A test tube made up of 0.5 mL of the plant sample, 0.2 mL of distilled water and 0.5 mL of N-(Dithiocarboxy) sarcosine (DTCS) reagent were taken. The tubes were incubated at room temperature for 3 hrs. Then, 1.5 mL of ice-cold 65% Sulphuric acid was added. The contents of the tube were mixed well and incubated at 37°C for 30 min. The colour developed was measured as absorbance at 520 nm.

Experimental design: Thirty-two adult male Albino rats were randomly separated into 4 groups of 8 animals each received the following treatment:

- Group I known as control animals, received 1 mL of normal saline as a placebo
- Group II animals received 300 mg kg^{-1} b.wt., of D-galactose only
- Group III animals received 300 mg kg^{-1} b.wt., of D-galactose with 200 mg kg^{-1} b.wt., of MEVA
- Group IV animals received 300 mg kg^{-1} b.wt., of D-galactose with 300 mg kg^{-1} b.wt., of MEVA

All treatments mentioned above lasted for 28 days.

Sample collection: All the animals have sacrificed 24 hrs after the last dosing. Blood samples were collected through the cardiac puncture into the heparinized bottle and centrifuged at 3000 rpm for 15 min to obtain plasma used for hormones assay. The testes and epididymis were excised and trimmed of fat.

Epididymal sperm collection followed the previous method[57]. The epididymis was separated from the left testis and used for epididymal sperm analysis. The epididymis' caudal part was that blotted with filter papers and lacerated to collect the sperm in a petri-dish to determine the sperm motility, viability, count and morphology. The right testis hereafter preserved in Bouin's solution for histological studies.

Hormonal assay: The plasma, testosterone, Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH) concentration measurement followed the Enzyme-Linked Immunosorbent Assay (ELISA) technique with ELISA kit purchased from Monobind Inc. the USA, following the manufacturer's guide.

Semen analysis

Sperm count: The caudal epididymis of the right testes was removed and blotted with filter paper. It was then immersed in 5 mL of formal-saline in a graduated test tube, while the volume of displaced fluid was equal to the amount of the epididymis. The caudal part of the epididymis was blended in the normal saline to form a solution used to measure sperm count by the improved Neubauer Chamber method (LAB ART,

Germany). The sperm count was examined under the light microscope[58] at a magnification of ×40 while evaluating different fields. The expression of sperm count was in a million per µL of suspension.

Sperm motility: Two drops of warm 2.9% sodium citrate were added to sperms milked on a pre-warmed microscope slide from incised caudal epididymis and then covered with coverslip before observing it under the microscope at ×400 magnification. Ten sperms from 10 fields of the lens were randomly assessed for different categories of motility according to the previous method[59].

Sperm viability: Sperm viability was carried out using the Eosin/Nigrosin stain method to differentiate between the living and dead sperms. The count and percentages of the stained and unstained sperm were calculated according to the Laing[60] technique described by Ajayi and Akhigbe[57].

Sperm morphology: The sperm morphology evaluation was done with the aid of a light microscope at ×400 magnification. Two drops of moderately hot Walls and Ewas stain was added to the epididymal fluid on a pre-warmed slide and a uniform smear made from it and air-dried. The stained slide was then examined under the microscope[60]. Five fields of the lens randomly selected were considered for the types and number of abnormal spermatozoa. Documentation of the number of sperms with normal morphology defected head, middle piece and tail took place.

Preparation of tissues homogenates: The already separated testes were rinsed in Potassium Chloride (KCl) washing buffer and homogenized with an appropriate volume of chilled Tris-HCl buffer with pH 7.4 using a mortar and pestle.

Assay for oxidative stress indices

Determination of superoxide dismutase (SOD) activity: Determination of SOD activity applied methods previously described[61,62]. Briefly, a ratio of 1:10 dilutions was made from 1 mL of the sample and 9 mL of distilled water. An aliquot of the diluted sample was added to 2.5 mL of 0.05 M carbonate buffer pH 10.2 and the required reaction was started by adding 0.3 mL of adrenaline. The change in absorbance was monitored with a spectrophotometer (Cary 50 Bio UV-Vis spectrophotometer, varian) at 480 nm for 5 min.

Assessment of lipid peroxidation (MDA): This was carried out based on the method described in previous studies[63,64]. Estimation of oxidative degradation of lipid as a result of the reaction of Malondialdehyde (MDA) with 2-Thiobarbituric Acid (TBA) forming an MDA-TBAR adduct that absorbs strongly at 532 nm occurred. About 0.4 mL of the reaction mixture, i.e., sample already quenched with 0.5 mL of 30% TCA was added to 1.6 mL of Tris HCl pH 7.4. The addition of 0.5 mL of 8% TBA and incubation for 45 min at 800 produced pink coloured reaction mixtures centrifuged at 14000 rpm for 15 min. The absorbance of clear pink supernatant gave the TBARS measurement of lipid peroxidation readings at 532 nm.

Determination of catalase (CAT) activity: CAT activity determination followed the method described in earlier studies[64,65]. About 0.1 mL of the sample was pipetted into a cuvette containing 1.9 mL of 50 mM Phosphate buffer, pH 7.0. Then the reaction was launched by the addition of 1.0 mL of freshly prepared 30% (w/v) hydrogen peroxide (H_2O_2). The amount of decomposition of H_2O_2 was measured using a spectrophotometer with a change in absorbance at 240 nm. The enzyme activity expression was in µmol min^{-1}.

Determination of reduced glutathione (GSH): GSH concentration applied previous methods[66,67]. Total 10% tissue homogenate was mixed with 4% Sulphur-salicylic acid (w/v) in a 1:1 ratio (w/v). The specimens were incubated at 4°C for 1 hr and then centrifuged at 1200×g for 20 min at 4°C. The assay mixture contained 0.1 mL of supernatant, 1.0 mM DTNB and 0.8 µL phosphate buffer. The developed yellow colour was read promptly at 412 nm in a spectrophotometer. The GSH absorbance was extrapolated from the GSH standard curve and expressed in µg mL^{-1}.

Testicular histology

Tissue processing: The histology assessment of the testes used the methods previously described[57,68]. Testicular tissues were fixed in Bouin's fluid for 6 hrs and afterwards transferred into 10% formalin and then dehydrated with varying percentages of ethanol. Sections were after that cleared in xylene and embedded in molten wax. Sections of 5 µm were cut and stained with hematoxylin and eosin. Observation of the stained slides followed under the microscope for morphological changes.

Statistical analysis: Statistical tests were performed using GraphPad prism 5. Application of One-way analysis of variance (ANOVA) with Turkey's HSD posthoc comparisons. Expression of values was in Mean±SEM and the significance of values was set at $p<0.05$.

RESULTS

Phytochemicals, antioxidant and vitamin assays: In Table 1 the phytochemical screening of the leaves of *Vernonia amygdalina* showed the following: Tannins, saponin, polyphenols, alkaloids, terpenoids and flavonoids. Polyphenols had the highest quantity, followed by alkaloids and flavonoids. The total phenolic content, β-carotenoid and antioxidant DPPH assays of *Vernonia amygdalina* leaves are shown in Table 2. At the same time, in Table 3 the quantity of some vitamins found in *Vernonia amygdalina* leaves such as vitamin B_1 (0.55±0.01 mg/100 g), vitamin B_2 (0.88±0.01 mg/100g), vitamin C (42.44±0.02 mg/100 g), vitamin E (6.35±0.03 mg/100 g) were displayed.

Effect of methanolic extract of *Vernonia amygdalina* leaves on D-galactose induced oxidative stress: The result in Table 4 below shows the effect of methanolic extract of *Vernonia amygdalina* leaves on D-galactose induced oxidative stress on oxidative stress indices in the testes of male Albino rats. The result indicates that MDA was significantly ($p<0.05$) higher in the D-galactose induced group when compared with the control group. Treatment with 300 mg kg^{-1} of the extract significantly ($p<0.05$) reduced MDA concentration when compared with the D-galactose induced group and a non-significant difference in the value when compared with the control group. Also noted is a dose-related difference between the assayed values of the groups administered with 200 and 300 mg kg^{-1} b.wt., which was also statistically significant. The concentration of GSH decreased significantly ($p<0.05$) in the D-galactose induced group compared with the control group. SOD and CAT were significantly ($p<0.05$) lower in the D-galactose induced group when compared with the control group and treatment with 300 mg kg^{-1} of *Vernonia amygdalina* extract significantly ($p<0.05$) increased the activity of SOD when compared with both the control and D-galactose induced group. The group treated with 200 mg kg^{-1} of the extract demonstrated a non-significant increase in SOD concentration.

Effect of methanolic extract of *Vernonia amygdalina* leaves on semen analysis of D-galactose induced ageing male Albino rats: In Table 5, treatment with D-galactose significantly reduced sperm count when compared to control. The group of rats treated with 200 mg kg^{-1} of the extract had a significant ($p<0.05$) decrease in sperm count compared to control, while the group of rats treated with 300 mg kg^{-1} had a significant ($p<0.05$) increase in sperm count compared to the D-galactose group. D-galactose treatment led to a significant ($p<0.05$) decline in both rapid and slow progressive motility in comparison with control. In contrast, administration of 300 mg kg^{-1} of the extract led to a significant ($p<0.05$) increase in slow progressive motility. A significant ($p<0.05$) increase in non-motile sperm cells in the D-galactose group compared to the control ensued. Treatment with 300 mg kg^{-1} led to a significant ($p<0.05$) decrease in the number of non-motile sperm cells when compared with the D-galactose group.

Table 1: Qualitative and quantitative analysis of phytochemicals of *Vernonia amygdalina* leaves

Qualitative analysis of phytochemicals	Quantitative analysis of phytochemicals (mg g^{-1})
Tannins + +	0.03±0.00
Saponin + + +	1.43±0.01
Polyphenols + + +	6.77±0.02
Alkaloids+ + +	6.67±0.01
Terpenoids + +	0.04±0.00
Flavonoids + +	3.88±0.01

+++: Indicates the presence of phytochemicals

Table 2: Total phenolic content, β-carotenoid and antioxidant DPPH assays of *Vernonia amygdalina* leaves

Assay	Content
Total phenolic content (mg GAE g^{-1} dry weight)	15.55±0.01
β-Carotenoid (mg g^{-1})	0.68±0.00
DPPH (μmol trolox g^{-1})	14.45±0.02

Table 3: Quantitative analysis of some vitamins of *Vernonia amygdalina* leaves

Vitamins	Quantity (mg/100 g)
Vitamin B_1	0.55±0.01
Vitamin B_2	0.88±0.01
Vitamin C	42.44±0.02
Vitamin E	6.35±0.03

Table 4: Oxidative stress indices in the testis of D-galactose induced ageing male rats treated with MEVA

	Groups			
Variables	I (control)	II (D-Gal only)	III (D-Gal+E-200 mg)	IV (D-parameters Gal+E-300 mg)
MDA (nmol g^{-1} tissue)	8.26±0.79	21.93±0.61[a]	17.38±1.32	9.66±0.47[b]
GSH (μg mL^{-1})	42.90±1.09	27.00±1.88[a]	34.18±0.74	41.10±2.56
SOD (μmol min^{-1})	50.00±1.76	31.74±1.17[a]	36.85±1.15[a,b]	43.22±2.05[a,b]
CAT (μmol min^{-1})	34.03±3.26	13.68±0.78[a]	16.03±0.78	21.08±0.93

[a]Represent a significant difference at $p<0.05$ when compared with group I (control), [b]Represents a significant difference at $p<0.05$ when compared with group II (D-galactose only)

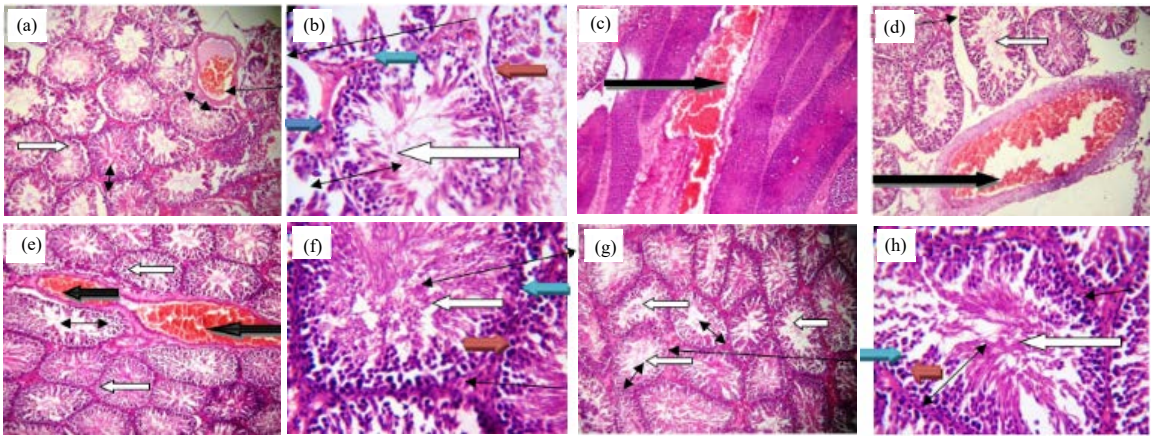

Fig. 1(a-h): Histology of the testis D-galactose induced ageing male rats treated with MEVA, (a) Photomicrograph of group I rat testes stained with H and E. ×100, (b) Photomicrograph of group I rat testes stained with H and E. ×400, (c) Photomicrograph of group II rat testes stained with H and E. ×100, (d) Photomicrograph of group II rat testes stained with H and E. ×400, (e) Photomicrograph of group III rat testes stained with H and E. ×100, (f) Photomicrograph of group III rat testes stained with H and E. ×400, (g) Photomicrograph of group IV rat testes stained with H and E. ×100, (h) Photomicrograph of group IV rat testes stained with H and E. ×400

Blue arrow: Seminiferous tubules with spermatogonia cells, Red arrow: Sertoli cells, spanned arrow: Germ cell layer, White arrow: Lumen showing the presence of spermatozoa, Slender arrow: Interstitial spaces and leydig cells, Black arrow: Vascular congestion noted involving the tunica albuginea

Table 5: Semen analysis of d-galactose induced ageing male rats treated with MEVA

Variables	Group I (control)	Group II (D-Gal only)	Group III (D-Gal+E-200 mg)	Group IV (D-Gal+E-300 mg)
Microscopic sperm count ($\times 10^6$ cells L^{-1})	51.90±7.097	18.64±3.759[a]	19.12±2.140[a]	38.54 ±4.961[b]
Rapid progressive motility	30.00±3.162	14.00±2.449[a]	18.00±2.000	20.00±4.472
Slow progressive motility	24.00±4.472	12.00±2.000[a]	16.00±2.449	22.00±2.449[b]
Non-motile sperms cells	24.00±2.449	38.00±2.000[a]	32.00±2.000	28.00±3.742[b]
Dead sperm cells (sperm viability)	18.00±3.742	36.00±2.449[a]	26.00±2.449	22.00±2.000[a]
Normal morphology	50.00±4.472	32.00±2.000[a]	34.00±2.449[a]	34.00±2.449[a]
Sperm with defect of the head	38.00±3.741	53.00±2.000[a]	46.00±2.449	35.00±3.742[a]
Sperm with middle piece defect	14.00±9.000	7.000±1.225	6.000±1.000	6.000±1.000
Sperm with tail defect	5.000±0.000	5.000±0.000	5.000±0.000	6.000±1.000

[a]Represent a significant difference at $p<0.05$ when compared with Group I (control), [b]Represents a significant difference at $p<0.05$ when compared with group II (D-Galactose only)

This Table 5 also displayed that there was a significant ($p<0.05$) increase in the number of dead sperm cells in the D-galactose group compared with the control values. However, this was reversed significantly in the group treated with 300 mg kg^{-1} of bitter leaf extract compared to the control. The group of rats treated with D-galactose had a significant ($p<0.05$) decrease in the number of sperms with normal morphology and a significant ($p<0.05$) increase in the value of sperms with the defected head when compared with the control group values. Treatment with 300 mg kg^{-1} significantly ($p<0.05$) increased the normal morphology and decreased sperm with the defected head when compared with the D-galactose groups. Sperm with the middle piece and tail defects were only comparable among the groups.

The effect of methanolic extract of *Vernonia amygdalina* leaves on reproductive hormones of D-galactose induced ageing male Albino rats.

In Table 6 there is a significant decrease ($p<0.05$) in testosterone and luteinizing hormone levels in all the groups compared with control. The group of rats treated with D-galactose had a significant ($p<0.05$) decrease in FSH level but treatment with 200 and 300 mg kg^{-1} did not produce any significant difference in FSH level compared to the control. Nevertheless, testosterone and luteinizing hormone levels significantly increased with 300 mg kg^{-1} of extract compared to the D-galactose group.

Histology of the testis D-galactose induced ageing male rats treated with MEVA: The result of Fig. 1a-h shows the

Table 6: Reproductive hormones concentration of D-galactose induced ageing male rats treated with MEVA

Variables	Group I (control)	Group II (D-Gal only)	Group III (D-Gal+E-200 mg)	Group IV (D-Gal+E-300 mg)
Testosterone (ng mL^{-1})	4.7±0.13	1.20±0.26[a]	2.15±0.16[ab]	3.29±0.40[ab]
Luteinizing hormone (Mlu mL^{-1})	2.79±0.31	1.07±0.19[a]	1.69±0.08[ab]	1.86±0.13[ab]
Follicle stimulating hormone (Mlu mL^{-1})	4.02±0.12	2.97±0.32[a]	4.18±0.09[b]	3.42±0.30

Values in this study are expressed as the Mean±SEM (Standard error of the mean) using the student t-test, D-Gal only: D-Galactose induced only, D-Gal+E-200 mg: D-Galactose+200 mg kg^{-1} b.wt., of extract, D-Gal+E-300 mg: D-Galactose+300 mg kg^{-1} b.wt., of extract, [a]Represent a significant difference at $p<0.05$ when compared with group I (control), [b]Represents a significant difference at $p<0.05$ when compared with group II (D-Galactose only)

photomicrograph of testicular sections stained by haematoxylin and eosin. In Fig. 1a-b, the tunica albuginea appears normal without vascular congestion in the control group. Figure 1c-d, vascular congestion involving the tunica albuginea was observed in the D-galactose group. Figure 1e-f, Treatment with 200 mg kg^{-1} b.wt., of *Vernonia amygdalina* reduced the vascular congestion in tunica albuginea, while in (Fig. 1g-h) the vascular congestion were absent as the tunica albuginea appeared normal following the administration of 300 mg kg^{-1} of the extract.

DISCUSSION

Administration of D-galactose in this study induced ageing which is demonstrated by the increased oxidative stress parameters and reduced antioxidant enzymes. The results showed a significant increase in MDA concentration in the D-gal group compared to the control. D-galactose administration is a reliable and stable model for the induction of ageing in rats and resembles natural ageing in man, which is characterized by reduced life span, cognitive function and neuronal function[69,70]. There is usually accompanied by a marked increase in oxidative stress, the generation of the advanced glycation end product with changes in gene transcription which affects normal body physiology[15,71]. However, administration of 300 mg kg^{-1} of methanolic extract of *V. amygdalina* (MEVA) resulted in a significant reduction in MDA concentration compared to the control. The result, therefore, indicates that MEVA contains anti-oxidative and anti-ageing properties, particularly at a specific level. Lolodi and Eriyanremu[72] documented a similar report. A biomarker of oxidative stress is Malondialdehyde (MDA), a by-product of lipid peroxidation induced by free radicals and a biomarker of oxidative stress[73], Chen *et al.*[74] reported D-galactose's peroxidative effect in mice. An increase in total lipid peroxidation may be due to polyunsaturated fatty acids present in testis[75].

Results from this study also revealed that D-galactose treatment led to a significant decrease in antioxidant enzyme levels of GSH, CAT and SOD. However, the activities of CAT and GSH were improved significantly after treatment with MEVA in the group treated with 300 mg kg^{-1} b.wt., of the extract than the group that received 200 mg kg^{-1} b.wt., of the extract when juxtaposing with the control. One of the cells' measures to counter the deleterious effects of oxidative degradation of lipid is by increasing the activity of endogenous antioxidant enzymes such as CAT, peroxidases and SOD[76]. An earlier report on *V. amygdalina* showed its unique nutritional and phytochemical properties with several biochemical, physiological and morphological benefits[77]. Therefore, the results indicated that *V. amygdalina* has anti-oxidative properties that conform to the work of Farombi and Owoeye[21]. The presence of polyphenols, alkaloids and flavonoids from the phytochemical screening in this study points to compounds that could be responsible for this plant's anti-oxidative stress potential. These compounds are essential in the regulation of oxidative stress, inflammation and advance glycation end-product formation. They are non-enzymatic antioxidants that augment antioxidant enzymes' activities in living cells[72]. The presence of these metabolites in methanol extract of *Vernonia amygdalina* could be responsible for the amelioration of oxidative stress induced by D-gal. These findings agree with several earlier studies[72,78,79]. Therefore, this vegetable's constant consumption is important for healthy living due to the anti-oxidative characteristics inherent in it[77,80]. The antioxidant properties of the plant were further confirmed by the results obtained for the free radical scavenging activities of the plant by the DPPH activities, the phenolic content β-carotenoid and vitamin C assay, which all suggested that the plant is a good antioxidant candidate commonly used as an anti-ageing agent and also has potentials to promote reproductive functions[51].

A significant decrease in sperm function was caused by D-gal, which is evident in the significant decline in sperm motility, sperm count, normal morphology and sperm viability, even though the number of sperms with the middle piece and the tail defect was not significantly different from the control. The result agrees with the work of Dunson *et al.*[81] which found a decrease in semen quality with advancing age and subsequent infertility. The treatment of rats with 200 and 300 mg kg^{-1} b.wt., of MEVA in this study significantly improved sperm qualities as reflected by an increase in sperm

count, sperm motility and sperm viability which is significant and observed in MEVA treated groups when compared with D-gal induced group. A significant improvement in sperm parameters has been previously reported by Temitope *et al.*[82], Oyeyemi *et al.*[83] and Saalu *et al.*[77] in rats treated with MEVA. Sperm count is essential in assessing the effects of chemicals on spermatogenesis[84]. Increasing glucose metabolism, thereby producing pyruvate, which is necessary for basic activities and survival of sperm cells, might be the mechanism through which this extract improves the quality of sperm[85].

Furthermore, D-gal caused a significant decrease in the levels of testosterone LH and FSH. Whereas treatment with 200 and 300 mg kg^{-1} b.wt., of the extract significantly increased testosterone, LH and FSH levels compared to the D-gal group. The reduction in the hormonal levels in the D-gal group may be because semen quality and reproductive hormones decrease with advancing age which may be due to a higher level of ROS and low level of total antioxidative capacity in old age[42,81]. In support of this present study, reports had shown that bitter leaf extract activates the secretion of hormones of the hypothalamus, which may be responsible for the improved process of sperm formation seen. It does this by stimulating the process of sperm formation in the tubules of the testis, functions of epididymis or activities of testosterone on the nervous system releasing factors and anterior pituitary secretion of LH and FSH, which may result in improved sperm formation[86,87]. As antioxidants, these extracts also enhance the formation of steroids by enhancing the endocrine function of Leydig cells, which increased the production and circulation of testosterone and resulted in stimulation of spermatogenesis[88]. Therefore, *Vernonia amygdalina* extract could reverse the reduced sperm and testicular production of testosterone associated with ageing,

Analysis of the testis' histology revealed the presence of vascular congestion involving the tunica albuginea in the D-gal group compared to the control group. Treatment with 200 mg kg^{-1} b.wt., of *Vernonia amygdalina* reduced the congestion that disappeared following 300 mg kg^{-1} of the extract. Structural deformities occurred with several vessels appearing dilated and moderately congested in the D-gal group and showing disrupted Leydig cells that spread all over the intercellular spaces.

Nevertheless, the administration of *Vernonia amygdalina* extract reversed all the abnormalities in the germ cell layer. The result obtained in the D-gal group may be due to arrested germ cell differentiation associated with regressed reproductive organs functions during ageing[89]. This result is consistent with previous studies on D-galactose[83,89,90].

The observed effect on the testis showed that *Vernonia amygdalina* extract reverses D-galactose's impact on the testis by increasing the Leydig cell and improving the endocrine functions of the testis through the increased secretion of testosterone which usually decreases with age[91]. This mechanism is via the hypothalamic-pituitary-testicular axis in which the testosterone level has an inverse relationship with luteinizing hormone during ageing and oxidative stress plays an important role[90,92]. This implies that administration of *Vernonia amygdalina* extract prevents decline in testicular functions associated with ageing and this plant extract is recommended as a supplement in preventing reproductive dysfunction in adult males, further studies to identify the bioactive component responsible for this potential is also required.

CONCLUSION

Findings from this study show that induction of ageing with D-gal caused oxidative stress damages and affected the testis' integrity and functions. However, the methanolic extract of *Vernonia amygdalina* leaves prevents this damage via an oxidative stress-dependent mechanism in which the antioxidant properties of the metabolites such as polyphenol and alkaloids, flavonoids, β-carotenoid and vitamin C found in the plant may be responsible. This study suggests that MEVA at a 300 mg kg^{-1} dose may reduce the possibilities of male infertility associated with ageing.

SIGNIFICANCE STATEMENT

This study discovers the possible role of methanolic extract of *Vernonia amygdalina* leaves in preventing testicular damage caused by oxidative stress this can be beneficial for D-galactose induced ageing in male Albino rats. This study will help the researcher to uncover the critical area of preventing a decline in reproductive function associated with ageing that many researchers were not able to explore. Thus, a new theory on antioxidant properties of the metabolites of the plant such as polyphenol and alkaloids, flavonoids, β-carotenoid and vitamin C in reproductive function, may be arrived at.

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