Effects of Ethanol Extract of Stachytarpheta angustifolia (Mill.) Vahl Leaf on Lead Acetate-induced Reproductive Toxicity in Male Wistar Rats

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Abstract:
This study investigated the effects of ethanol extract of *Stachytarpheta angustifolia* (Mill.) Vahl leaf (EESA) on reproductive toxicity induced by lead acetate in malerats. Thirty sexually matured male Wistar rats were divided into 6 equal groups. Group I received 1 ml/kg distilled water, group II, 15 mg/kg lead acetate intraperitoneally; groups III and IV, 15 mg/kg lead acetate intraperitoneally, together with 500 and 1000 mg/kg of EESA orally respectively; groups V and VI, 500 and 1000 mg/kg of EESA orally respectively for 14 days. The results showed that lead acetate induced significant decrease in final body weight, sex organs relative weight, sperm concentration, motility and viability, serum testosterone concentration and glutathione (GSH) activity, with significant increase in sperm abnormalities. Moreover, lead acetate induced apparent alterations in the histological structure of the testis and epididymis. Treatment with EESA ameliorated the harmful effects of lead acetate, this was also proved histopathologically by the noticeable improvement in the histology of testis and epididymis. It may be concluded that EESA may be promising as a natural therapeutic agent in lead acetate-induced reproductive toxicity and oxidative stress in the male rat testes.

Keywords: *Stachytarpheta angustifolia*, lead acetate-induced reproductive toxicity, GSH

1. Introduction

Lead is considered as one of the hazardous pollutants and toxins that are found in the environment. Most of the lead in our environment comes from gasoline used in our cars, industrial emission, drinking water, petroleum refining, in construction, bullets of gun etc. The manipulation of lead for these uses has led to contamination of air, dust and soil by lead (Kiran *et al*. 2008). Under the influence of lead, onset of oxidative stress occurs on account of two different pathways operative simultaneously; first comes the generation of ROS, like hydroperoxides (HO2), singlet oxygen and hydrogen peroxide (H2O2), and second, the antioxidant reserves become depleted (Flora *et al*., 2012).

The exclusive use of herbal drugs, prepared and dispensed by unscientifically trained herbalists, for treatment of diseases is still very common in Nigerian communities, even the elites patronise the local herbal vendors for diverse concoctions. Among the numerous plants believed to be of medicinal importance is *Stachytarpheta angustifolia* (Mill.) Vahl (SA). It is called "Òpápará" or "Ìrùaláàngbá" in Yoruba language and "Tsarkiyarkusuu" in Hausa language. It is used in the treatment of many diseases, and its usefulness as a plant for treatment of male infertility especially in Yoruba land makes it very important. The leaves of the plant are commonly used. Fresh leaves are dried, ground to powder and soaked in alcoholic drinks - the commonest being Schnapps™. The patient is to take a tumbler of the concoction once or twice a day.SAhas many phytochemicals, which are distributed at varied concentrations in the parts; with the greatest levels detected in the leaf. SA synthesizes and accumulates a wide range of phytochemicals including alkaloids, phenol, flavonoids, saponins, tannins and glycosides. Alkaloid, flavonoid, saponins and tannin contents are highest in the leaf and least in the root. Greatest values of phytate and oxalate were found in the leaf (Ezeabara and Ezeh, 2015). Francis and co-workers (2010) reported that *Stachytarpheta angustifolia* is an active anti-oxidant. Therefore, the present study was designed to investigate the role of EESA on lead acetate- induced reproductive toxicity and oxidative stress in male rat.

2. Materials and methods

Chemical:

Lead acetate was obtained from BDH chemicals Ltd. Poole England (Prod 10142) (5927240C5009).

Preparation of stock solution of lead acetate

Three hundred (300 mg) of lead acetate was dispensed into a sterile universal bottle, 20 ml of distilled water was added resulting in a solution containing 15 mg/ml. The solution was thoroughly shaken. Fresh solution was prepared at three days interval. It was administered through intraperitoneal injection (Debosree*et al.,* 2013) depending on the body weight of each animal.

Animals:

Thirty male Wistar rats of ages 8-10 weeks weighing 170g-200g were obtained from the Animal Holding Unit, Obafemi Awolowo University, Ile-Ife and kept in well ventilated plastic rat cages for two weeks to acclimatize. They were given access to feed and water*ad libitum*.

Preparation of ethanol extract of *Stachytarpheta angustifolia*

The whole plant of *S.angustifolia*was obtained at Mabolaje area Oyo town in Oyo State and was identified in the Department of Botany Obafemi Awolowo University, Ile-Ife where a voucher number was obtained (IFE-17625), and a specimen was deposited in the herbarium. The leaves were separated from the plant, air dried, ground to powder using Warring blender and weighed. Seven hundred and fifteen (715 g) of the powder was macerated in5 litres of ethanol for 72 hours with regular shaking with an electric shaker. The resulting mixture was filtered with Whatmann No. 1 filter paper (0.2 mm). The filtrate was evaporated under reduced pressure using a Rotary evaporator, weighed and freeze dried in a lyophilizer (Ilshin Lab. Co. Ltd, Seoul, Republic of Korea) (*Azwanida,2015*). The extract obtained was kept at 4^{o}C in a refrigerator. The extract weight was 110 g which is equivalent to a yield of 15%. 20g of the extract was dispensed into sterile specimen bottle, 20 ml of distilled water was added to give a solution containing 1 g/ml (1000 mg/ml). The resultant solution was thoroughly shaken. Fresh solution was prepared at three days interval. EESA was administered orally using oral cannula.

Experimental design:

After the acclimatization period, rats were divided into six equal groups, each of five rats. Group I received 1 ml/kg distilled water (control), group II, 15 mg/kg lead acetate intraperitoneally; groups III and IV, 15 mg/kg lead acetate intraperitoneally, together with 500 and 1000 mg/kg of EESA orally respectively; groups V and VI, 500 and 1000 mg/kg of EESA orally respectively. All treatments were given for fourteen days.

Blood collection:

At the end of the experimental period, animals were fasted overnight, with free access clean water. The rats from each group were weighed and sacrificed by cervical dislocation. Blood samples were collected by cardiac puncture, transferred into separate cryovial plain bottles using 2ml syringes. The blood samples were centrifuged for 10minutes at 3000 rpm using a cold centrifuge to get the serum.

Sex organs weight:

The testes, epididymis and seminal vesicles were dissected out, trimmed off the attached fatty tissues and weighed.

Relative organ weight (R_w) was determined as follow;

$$\mathrm{Rw} = \frac{Organ\ wt\ (g)}{Body\ wt\ (g)} X\ 100$$

Sperm analysis:

Sperm analysis was carried out following the standard described by WHO (2009). The right testis was carefully excised through pelvic incision carefully trimming off the surrounding fats. The cauda epididymis was removed and minced in 2 ml of normal saline, a suspension was obtained and the following sperm parameters were measured. One drop of the suspension was smeared on a glass slide progressive motility was scored. New slide was smeared and stained by Eosin-nigrosin stain to determine the percentage of sperm cell viability (live-dead ratio). Morphological abnormalities were estimated using Walls and Ewas stain. Abnormal head and tails were evaluated accordingly.Sperm count was done with the aid of the improved Neubauer haemocytometer. The suspension was diluted with sodium bicarbonate-formalin in ratio 1: 19 (1 in 20). The improved Neubauer haemocytometer chamber was filled with well diluted sperm, then the sperm cells were counted in 2 square mm of Neubauer haemocytometer chamber. Sperm count was calculated in 1 ml of fluid by multiplying the number counted by 1,000,000 (*Rowe et al.,* 1993; Raji*et al.,* 2003;Sarkar *et al.,* 2006; Acharya*et al.,* 2008).

Hormonal and antioxidant assay

Serum luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone were quantified using Enzyme-linked Immunosorbent Assay (ELISA) method. Manufacturer's instructions were followed. Glutathione was quantified by the method of Beutler and Kelly (1963).

Histopathological examination:

Sections were taken from testis and epididymal tissues from different animals in each group immediately after sacrifice. The tissues were washed with the normal saline solution to remove blood, fixed in Bouin's fluid for a period of at least 24 hrs, dehydrated in different grades of alcohol, and processed for paraffin embedding. Sections of 5 μm thickness were cut using a rotary microtome. The sections were processed and passed through graded alcohol series, stained with Haematoxylin and Eosin, cleared in xylene and examined microscopically and photomicrographs were taken using Leica DM750 Camera Microscope.

Statistical analysis:

Data were analysed using one-way analysis of variance (ANOVA) and post hoc analysis was carried out using Student Neuman-Keuls test on Graph Pad 5.03 (Graph Pad Software Inc., CA, USA). P values less than 0.05 were considered to be significant.

3. Results

Body and sex organs weight:

Table 1: Effects of EESA on percentage weight change and relative weights of reproductive organs of male Wistar rats following lead acetate toxicity.

Groups	% Weight change	Testis	Epididymis	Seminal Vesicle
Control	15.44±2.49	0.55±0.03	0.22±0.01	0.37±0.09
Lead acetate 15 mg/kg	-1.7220±2.01$^{\#}$	0.38±0.05$^{\#}$	0.12±0.02$^{\#}$	0.11±0.03$^{\#}$
Pb+500 mg/kg EESA	14.42±5.07*	0.63±0.03*	0.36±0.06*	0.35±0.12*
Pb+1000 mg/kg EESA	10.00±1.49*	0.60±0.02*	0.25±0.01*	0.35±.0.07*
500 mg/kg EESA	18.64±4.31*	0.56±0.02*	0.26±0.01	0.35±0.14*
1000 mg/kg EESA	15.22±5.46*	0.57±0.01*	0.27±0.01	0.49±0.10*

Results are presented as Mean ± SEM, n = 5, # = significantly different from control * =significantly different from group II

Sperm characteristics:

Table 2: Effects of EESA on sperm parameters of rats following lead acetate-induced infertility

Groups	Sperm motility (%)	Sperm viability (%)	Sperm morphology (%)	Sperm Count (million/ml)
Control	93.00±1.22	96.80±0.73	11.18±2.91	138.60±5.58
Lead acetate 15 mg/kg	57.50±4.78#	88.25±4.15	15.22±0.13#	76.50±4.66#
Pb+500 mg EESA	76.00±2.44#*	93.60±2.22	13.01±0.51#*	122.40±4.65*
Pb+1000mg EESA	78.00±2.00#*	96.20±0.73	12.75±0.52#*	131.20±10.77*
500 mg EESA	84.80±3.08*	94.20±2.39	12.92±0.52*	133.84±3.48*
1000 mg ESA	89.20±2.48*	97.40±0.60	12.55±0.46*	139.20±2.63*

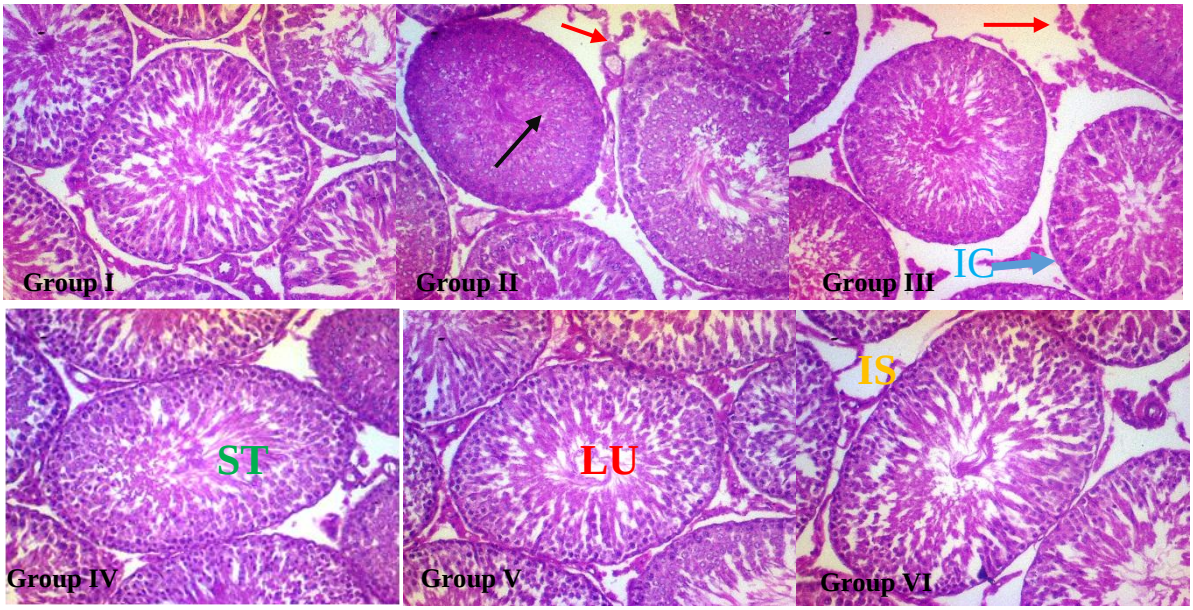
Results are presented as Mean ± SEM, n = 5, # = significantly different from control * =significantly different from group II

Table 3: Effects of EESA on serum hormonal profile of rats following lead acetate-inducedinfertility

Groups	LH (mIu/ml)	Testosterone(ng/ml)	FSH (mIu/ml)	GSH (mmol/mL)
Control	0.3100±0.008	0.5080±0.010	0.2600±0.011	1.60±0.04
Lead acetate 15 mg/kg	0.1250±0.013#	0.2350±0.015#	0.1250±0.012#	0.74±0.02#
Pb+500 mg EESA	0.2560±0.005#*	0.3980±0.009#*	0.2300±0.008*	1.47±0.03*
Pb+1000 mg EESA	0.2620±0.005#*	0.4000±0.015#*	0.2300±0.008*	1.41±0.09*
500 mg EESA	0.3100±0.005*	0.5100±0.015*	0.2520±0.015*	1.77±0.04#*
1000 mg ESA	0.3160±0.005*	0.5020±0.018*	0.2520±0.019*	1.86±0.07#*

Results are presented as Mean ± SEM, n = 5, # = significantly different from control * =significantly different from group II

Histopathological results:



KEY: ST = Seminiferous tubule, LU = Lumen, IS = Intestitial space, IC = Interstitial cells

PLATE 1: Photomicrographs of testes of treated rats (H & E × 400 Magnification).

The photomicrographs of Groups I, V, and VI show normal histoarchitecture. The seminiferous tubules appear normal. The interstitial cells are readily identifiable within the interstitial space. Group II revealed abortive/degenerative seminiferous tubules (black arrow) and widened interstitial space (red arrow) due to atrophy.

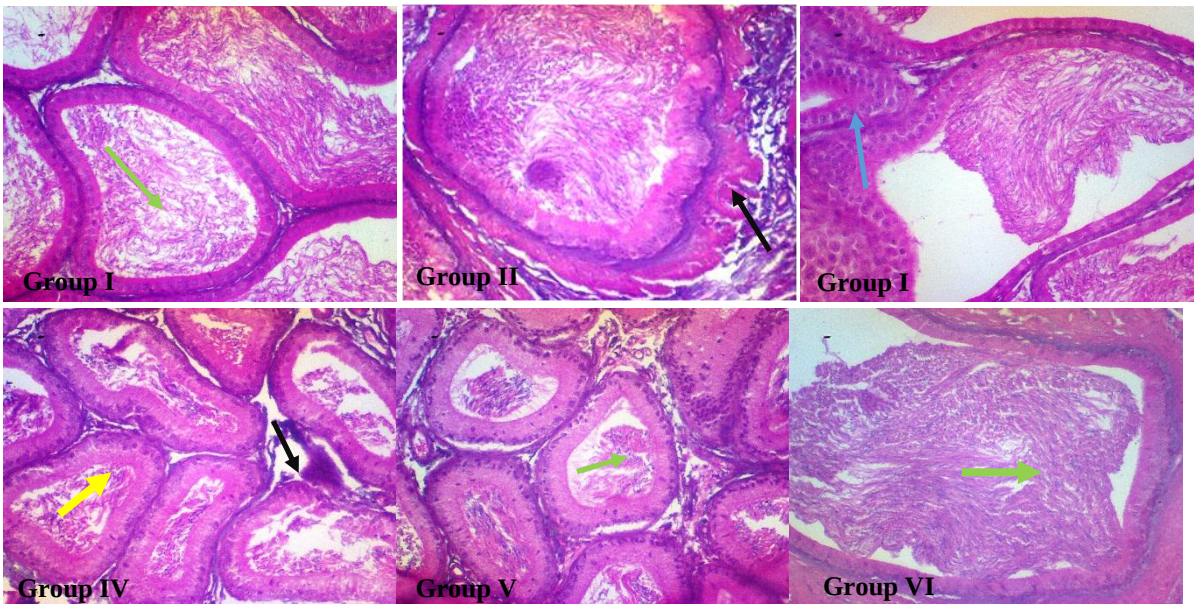


PLATE 2: Photomicrographs of epididymis of treated rats (H & E × 400 Magnification).

Photomicrographs of epididymis of Goups I, IV, and Vl show normal histoarchitecture, the lobules are filled with matured sperm cells (green arrow). In group II, the epithelia appear thickened with signs of atrophy (black arrow). Groups III and IV revealed abnormal cellularity resulting from hyperplasia (blue arrow).

4. Discussion:

There are recent reports of increased domestic and industrial exposure of humans to heavy metals, especially lead (El-Tohamy, and El-Nattat, 2010), in developing countries such as Nigeria as typified by massive lead poisoning following crude extraction of gold in Zamafara state and other states in northern Nigeria (Emokpae *et al.,* 2006). Similarly, there is a rising case of infertility of unknown causes where lead toxicity-induced infertility cannot be ruled out (Yamagochi *et al.,* 2007; Hussein *et al.,* 2011). This study investigated the effects of ethanol extract of Stachytarpheta angustifolia leaf (EESA) on lead acetate-induced infertility in male Wistar rats. The choice of this plant was predicated on the reported use of the plant for treatment of infertility in folklore medicine.This study reported a significant decline in body weight and weight of reproductive organs of the animals treated with lead acetate alone relative to control. The weight loss was accompanied by general weakness, lethargy, poverty of movement, pallor of the limbs and little resistance to handling. Similar effects of lead have been reported in previous studies (Ekeh *et al.,* 2015 and Ayoka *et al.,* 2016). The general weight loss can be attributed to noticeable reduced food intake in the group treated with lead acetate alone. The significant weight loss exhibited by lead acetate treated animals may also be accounted for by oxidative stress which usually accompanies lead toxicity (Vishavjeet and Madhu, 2013). EESA significantly reversed the body weight loss and reproductive organs weight loss. This can be attributed to appreciable content of proteins, fats, minerals and vitamins present in Stachytarpheta angustifolia as reported by Ezeabara and Ezeh (2015). Flavonoids found in *S. angustifolia* may have been an additional source of nutrients. Findings from this study revealed that lead acetate induced significant alterations in both the quantity and quality of sperm of rats. Previous studies have reported the reproductive toxicity of lead and its various compounds (Priya and Reddy 2012; Vishavjeet and Madhu, 2013). The percentage of sperms with abnormal morphology observed in lead acetate treated group was significantly increased. Sperms in group II presented with significantly increased number of sperms with double head, tailless heads, headless tails, bent tails, rudimentary tails, looped tails, curved midpiece and bent midpiece compared with control. This was also reported in previous studies (Fengyuan *et al.,* 2007; Wadi and Ahmad, 1999). The abnormal sperm characteristics, especially, sperm motility and morphology, coupled with the deleterious effects of lead on the seminiferous tubules, with widening of interstitial spaces due to testicular atrophy and widening of lumen as a result of erosion of the germinal epithelium as reported in this study established the toxicity of lead on male reproductive function. However, EESA significantly reversed the deleterious effect of lead acetate on the measured sperm parameters. This might be as a result of free radical scavenging activity of S. angustifolia reported by Francis and co-workers (2010). It was reported in this current study that there was no significant change in the live-dead ratio of sperm cells, (since life sperm whether morphologically viable or not were recorded to be alive), however, significant decrease recorded in other parameters including FSH, LH, testosterone and distorted histomorphological changes are pointers to a defective spermatogenesis resulting in reduced sperm viability in group II as typified also by increased in sperms with morphological abnormalities. The decreased sperm count observed in this study might be due to spermicidal effect of lead. Lead has been reported to be injurious to spermatogenic and Leydig cells (Imran *et al.,* 2003; Sokol *et al.,* 2006). Most of the testicular germ cells might have been destroyed due to membrane damage by reactive oxygen species (ROS) leading to a significant decline in sperm count and testicular weight loss. Hence, the reversal of lead acetate-induced changes by EESA through its antioxidant capacity as observed in this study. The presence of degenerated seminiferous tubules as evident in the photomicrographs of group II animals may also contribute to the low sperm count recorded in group II. Since spermatogenesis begins in the

seminiferous tubules, destruction of a large number of the tubules will affect sperm production, as degenerated tubules are incapable of producing healthy sperm cells. The alterations in sperm parameters induced by lead exposure in group II animals may also be a consequence of the lead toxicity-induced decrease in the weight of the seminal vesicle whose function is production of seminal fluid required for nurturing sperm cells since lead enters into the nucleus of cells and disrupts all activities regarding protein synthesis and DNA replication (Ishijima *et al.,* 1986). It is suggested that lead acetate-induced decrease in seminal vesicular weight resulted in decrease in production of seminal fluid with consequent adverse effect on sperm maturation (Varshini *et al.,* 2012), hence, the alterations in sperm parameters. The photomicrograph of testes of lead acetate treated group (group II) revealed widened interepithelial spaces as a result of atrophy, widening of lumen, severe degeneration of interstitial space and loss of interstitial cells. These degenerative changes in the seminiferous tubules must have contributed to the reduced weight of seminal vesicle, low sperm count and increased abnormal sperm cells noticed in group II. The photomicrographs of testes revealed that in Group II, the tunica albuginea appeared thickened. These structural changes in testes can be attributed to lead-induced toxicity on reproductive organs (Jegegde *et al.,* 2013). These testicular distortions exhibited by the lead treated group were however significantly reversed by EESA as seen in photomicrograph of groups treated with EESA (groups III and IV). This is probably due to the antioxidant effects of some phytochemicals such as flavonoids, terpenoids and tannins present in the extract of EESA reported by Francis and co-workers (2010). The group treated with lead acetate alone showed significant decrease in serum concentration of sex hormones including follicle stimulating hormone, luteinizing hormone and testosterone compared to control. Similar findings were reported by Ayoka and co-workers (2016). Sujatha and co-workers (2011) reported that lead has a toxic effect on the hypothalamic – pituitary-testicular axis. Lead has been reported to induce toxic effect mainly by oxidative stress mechanism through the generation of ROS (Ayoka *et al.,* 2016). It is proposed that the lead acetate-induced reduction in serum level of LH and FSH may be due to the toxic effect of lead acetate on the hypothalamic-pituitary axis as reported in this study. The reduction in sperm count may also be explained by the decrease in Leydig cells and decrease in serum testosterone in group II (Onyeka *et al.,* 2012). The common denominator of lead toxicity-induced gross anatomical and histomorphological changes of reproductive organs reported in this study is cell loss, as evident by significantly reduced cellularity in the reproductive organs and reduced serum level of male sex hormones. However, EESA significantly reversed the toxic effects of lead acetate on gross anatomy and histomorphology of reproductive organs and hormonal changes. It can therefore be deduced that EESA has protective effects on the reproductive toxicity of lead acetate. Some phytochemical contents like tannins and terpenoids present in EESA are suggested to be responsible for the reversal effect of EESA on the toxicity of lead acetate on the pituitary and the testes. Tannins, alkaloids and terpenoids that are abundantly present in EESA have been reported to have anti-inflammatory and antioxidant activities (Micheal 1998; Williams *et al.,* 2004; Ferrer *et al.,* 2008; Ferreyra *et al.,* 2010). Group II animals which were treated with lead acetate only had significantly low GSH compared to control. This could result from rapid conversion of GSH to its oxidized form - glutathione disulphide (GSSG) (also called L-(–)-glutathione), by lead through donation of a pair of electrons (Hultberg *et al.,* 2001; Patrick, 2006; Gagan *et al.,* 2012). EESA significantly reversed lead acetate-induced decrease in serum GSH. This suggests a potent anti-oxidant activity of EESA. In this study, the rats treated with EESA alone showed significantly higher serum GSH relative to the control group. This is suggestive that EESA has the ability to increase serum GSH. This is possible via the following mechanisms. Firstly, EESA may be blocking the oxidation potential of lead (i.e. preventing lead from donating a

pair of electrons to GSH) and or preventing deactivating effect of lead on enzymes such as δ-amino levulinic acid dehydratase (ALAD), glutathione reductase (GR), glutathione peroxidase (GPX) and glutathione-S-transferase responsible for GSH synthesis (Pigeolot *et al.*, 1990). Secondly, EESA may be rich in GSH (Pompella *et al.*, 2003), however, this is subjective to further investigations. Thirdly, it may be an enzyme inducer or activator of these enzymes (δ-amino levulinic acid dehydratase, glutathione reductase, glutathione peroxidase and glutathione-S-transferase). However, qualitative and quantitative studies are suggested to assess the mechanism(s) of the antioxidant potential of EESA.

Conclusion

The study concluded that at 500 and 1000 mg/kg, ethanol extract of *Stachytarpheta angustifolia* showed ameliorative effect on lead acetate-induced alterations in physical, histomorphological and hormonal indices of male reproductive function in male Wistar rats. These findings therefore provide some scientific rationale behind the choice of this plant by local herbal vendors in the treatment of male infertility.

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