



Research Article

Exacerbative Effect of *Paullinia pinnata* Methanol Leaf Extract on Ethylene Glycol Monomethyl Ether-Induced Testicular Dysfunction in Male Wistar Rats

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Abstract

Paullinia pinnata (PP) is a medicinal plant whose parts are used for medicinal purposes traditionally in the treatment of various diseases including malaria, diarrhea and to help infertility which is currently a scourge globally. This study is designed to investigate the chemopreventive potential of *P. pinnata* leaves against gonadotoxicity using ethylene glycol monomethyl ether (EGME)-induced testicular dysfunction as the experimental model. Sixty adult male Wistar rats were weight-matched into six groups of ten animals each. All administrations were done orally daily for twenty-one consecutive days as follows: Group I (control) – distilled water; Group II – 1.5 ml/kg body weight of 10% dimethyl sulfoxide (vehicle); Group III- 200 mg/kg EGME; Group IV- 200 mg/kg PP; Group V- EGME (200 mg/kg) + PP (100 mg/kg) and Group VI- EGME (200 mg/kg) + PP (200 mg/kg). On day 22, blood was collected for the analysis of the reproductive hormones. The rats were euthanized and the brain, testes and epididymes were excised. The spermatozoa analysis, antioxidant and anti-inflammatory assays, and histological examination were carried out appropriately. The plasma concentration of luteinizing hormone was significantly increased in the co-administered groups while the plasma testosterone concentration was decreased. The spermatozoa parameters including testicular spermatozoa count were reduced in the EGME, EGME (200 mg/kg) + PP (100 mg/kg) and EGME (200 mg/kg) + PP (200 mg/kg) groups. Antioxidant parameters including catalase and glutathione-S-transferase were affected in the testes and brain in the EGME, EGME (200 mg/kg) + PP (100 mg/kg) and EGME (200 mg/kg) + PP (200 mg/kg) groups. The inflammatory markers were also elevated in the epididymis and testis of the EGME, EGME (200 mg/kg) + PP (100 mg/kg) and EGME (200 mg/kg) + PP (200 mg/kg) groups. The results were complemented by the histological observations. *Paullinia pinnata* leaves lack chemopreventive potential against Ethylene glycol monomethyl ether- induced gonadotoxicity rather, it exacerbates the deleterious effects.

Key Words: *Paullinia pinnata*, spermogram, antioxidant assay, reproductive hormones, ethylene glycol monomethyl ether

INTRODUCTION

Paullinia pinnata is a wood or sub-woody climber of the family Sapindaceae. It originates from tropical America and is now common in the savanna zones of tropical Africa and Madagascar. The common names are “bread and cheese plant”, and “sweet gum” (Burkill, 2000; Ikhane *et al.*, 2015). *P. pinnata* is used for medicinal purposes such as rickets, leprosy, fever debility, post-partum pain, localized pain, infectious diseases, diarrhea, cough, whooping cough, eye ailments and in complex treatments for jaundice and yellow fever. (Burkill, 2000; Osarenmwinda *et al.*, 2009).

Reports have been made about the folkloric use in the treatment of infertility and Zamble *et al.* (2006) have shown that using *in-vitro* experiment, extracts of the plant had the capacity to induce endothelium-dependent vaso-relaxation of bovine aortic endothelial cells through the nitric oxide pathway and inhibit endothelin-1 synthesis and therefore suggested that this could be the pharmacological mechanism responsible for the efficacy in unorthodox medicine for the treatment of impotence.

Furthermore, the extract has been shown to have antioxidant properties (Zamble *et al.*, 2006; Jimoh *et al.*, 2007; Tamokou *et al.*, 2013).

Infertility or sterility poses a global challenge arising at least in part, from gonadotoxic events and effective interventions are also being sought (Boivin *et al.*, 2007). There is the report that 15% of waiting couples are affected by infertility and the male factor infertility contributes to about 50% of the cases, 25% of which the cause is not known (Agarwal and Prabakaran, 2005; Parker and York, 2014). However, continuous exposure to gonadotoxicants in the environment and workplace can contribute or result in disruptions of the function of the reproductive system. Moreover, Sikka and Naz (2002) have explained that infertility can be as a result of oxidative stress amongst other factors.

We therefore hypothesize that *P. pinnata* may be able to help male infertility as a result of oxidative stress. Ethylene glycol monomethyl ether (EGME), an industrial toxicant, has been shown to elicit its gonadotoxic potential by altering the oxidative stress status in the male reproductive system (Malik and Gupta, 2013).

In the light of these, this study is designed to investigate the ability of the methanol leaf extract of *P.pinnata* to prevent oxidative stress induced by EGME as a means of assessing the ability of the plant to improve infertility.

MATERIALS AND METHODS

Reagent

EGME was a product of LobaChemie (Mumbai, India). All other reagents for the antioxidant assay and anti-inflammatory assays were either Sigma Aldrich, St. Louis, MO, U.S.A. or SureChem, U.K. products. DRG Diagnostics ELISA kit, Germany was employed for the reproductive hormone assay while DiaSorin ELISA kit, Italy was utilized for thyroxine and triiodothyronine plasma levels.

Collection of plant sample and preparation

The leaves of *P.pinnata* were collected from the Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria. The plant was authenticated at the same Institute and given the specimen voucher number FHI I06555. The leaves were air-dried, milled and extracted by cold maceration in absolute methanol and the recovered solvent was concentrated using a rotary evaporator (Heidolph HB, Germany) and a vacuum oven (Gallenhamp, England) at a temperature of 40-42 °C. A 14 % yield of the extract was realized (Adeyemo-Salami *et al.*, 2014).

Experimental Animals and Care

Ethical approval for the study was granted by the Animal Care and Use Research Ethics Committee of the University of Ibadan, Nigeria and the number UI-ACUREC/ APP/ 10/2016 /003 was assigned. Sixty male Wistar rats weighing 165- 190 g were obtained from the Primate Colony of the Department of Biochemistry, University of Ibadan and weight- matched into six groups of ten animals each. They were kept in appropriate laboratory cages and had free access to feed (Ladokun Feed, Nigeria) and laboratory water at the Animal house of the same Department, and were acclimatized for a week. The 12 hour light/dark cycle was maintained.

Experimental Design

The dose of 200 mg/kg body weight of EGME used in this study was determined from a preliminary study in our laboratory (Adeyemo-Salami and Farombi (2018). The doses of *P.pinnata* methanol leaf extract used were determined from previous work by Adeyemo-Salami and Makinde (2013). The treatment protocol was done orally once daily for twenty-one consecutive days as follows:

Group I was the normal rats which received 2 ml/kg of distilled water (Control 1)

Group II received the vehicle (10% dimethyl sulfoxide (DMSO) at 1.5 ml/kg (Control 2)

Group III received EGME only at 200 mg/kg dose

Group IV received *P.pinnata* extract only at 200 mg/kg body

Group V received EGME (200 mg/kg dose) + *P.pinnata* extract (100 mg/kg) (PP 100)

Group VI received EGME (200 mg/kg dose) + *P.pinnata* extract (200 mg/kg) (PP 200)

On the 22nd day, after an overnight fast, blood was collected by ocular puncture using heparinized capillary tubes into ethylene diamine tetra acetic acid (EDTA) specimen bottles

and plasma was separated. The animals were euthanized by cervical dislocation. The brain, seminal vesicles, epididymes, testes and prostate glands were excised and weighed. The left testes and epididymis, and the brain were homogenized and centrifuged to obtain the supernatant and used for biochemical analyses of antioxidant and inflammatory markers. The right testes and epididymes were used for spermatozoa analysis and fixed in Bouin's solution, processed, sectioned, mounted on slides and stained with haematoxylin and eosin for histological examination. The pituitary gland and the hypothalamus of the brain were carefully separated and also observed histologically. Plasma was separated from the blood by spinning at 2500 rpm in a centrifuge and used to determine the level of the male reproductive hormones which are luteinizing hormone, follicle stimulating hormone, testosterone, triiodothyronine and thyroxine using commercial enzyme immunoassay kits.

Spermatozoa Analysis

Assessment of the characteristics of epididymal sperm

The method of Zemjanis (1970) was employed to evaluate the motility of the sperm. Briefly, the cauda epididymal sperm was released on a sterile clean glass slide. 2.9 % sodium citrate dehydrate solution was added to the sperm and thoroughly mixed in order to evaluate the sperm progressive motility using a light microscope within 2- 4 mins of isolation. The data were expressed as percentages. The improved Neubauer chamber haemocytometer (Deep 1/10 m; LABART, Munich, Germany) was used to count the sperm according to Pant and Srivastava (2003). Employing the method of Wells and Awa (1970), part of the sperm suspension on a glass slide was made into a thin smear and then stained with Wells and Awa's stain (0.6 g fast green and 0.2 g eosin dissolved in distilled water and ethanol in a 2:1 ratio) for morphologic examination and also 5% nigrosin and 1 % eosin in 3 % sodium citrate dehydrate solution for viability. A total of 400 sperm/ rat were used for examination of the morphology.

Testicular sperm number (TSN) and Daily sperm production (DSP) determination

The method of Blazak *et al.* (1993) was employed. Briefly, frozen right testes from the control and treatment groups were decapsulated and homogenized in ice- cold physiological saline with 0.01% Triton X- 100. An aliquot of the homogenate was transferred to a glass vial and stored on ice. Aliquots of this stored homogenate were placed and counted twice on the improved Neubauer chamber haemocytometer at a magnification of x100 using a light microscope to determine the number of sperm heads (heads of spermatid at stage 19). Using these values, the total number of spermatids per testis and therefore the spermatids per gram of testis were obtained. To calculate the DSP, the number of spermatids at stage 19 was divided by 6.1 (6.1 is the period of seminiferous cycle in which the spermatids are present in the seminiferous epithelium).

Reproductive hormone assay

Determination of plasma testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH) concentrations

Commercial enzyme immunoassay kits (DRG Diagnostics, Germany) were used as per manufacturer's instructions to assay for the plasma concentrations of testosterone, LH and

FSH. The sensitivity of the testosterone assay was 0.08 ng/ml and with negligible cross reactivity with other androgen derivatives like androstenedione, 5 α -dihydrotestosterone and methyl testosterone.

Determination of plasma concentrations of triiodothyronine (T₃) and thyroxine (T₄)

Commercial enzyme immunoassay kits (DiaSorin, Italy) were used as per manufacturer's instructions to assay for the plasma concentrations of total T₃ and T₄. Inter assays coefficients of T₃ was 3.1- 8.9% and that of T₄ was 2.7- 3.8%. Sensitivity of the assays was 30 pg/ml for total T₄ and 700 pg/ml for total T₃. Plasma concentrations of total T₃ and T₄ were expressed as ng/ml and mg/dl respectively. All the samples were assayed on the same day to avoid the inter assay variation.

Biochemical analysis

The left testes and brain were homogenized in 4 volumes of tris-HCl/KCl buffer (pH 7.4) while the left epididymes were homogenized in 20 volumes of the same buffer. The homogenates were centrifuged at 10,000 g for 10 mins at 4 °C. The supernatant was removed and used for the antioxidant and anti-inflammatory assays. Protein determination was carried out using the method of Lowry *et al.* (1951).

Antioxidant assays

The activities of certain antioxidant enzymes were determined using the corresponding method in parenthesis and these are superoxide dismutase (SOD) (Misra and Fridovich, 1972); catalase (CAT) (Claiborne, 1985); glutathione-S-transferase (GST) (Habib *et al.*, 1974); glutathione peroxidase (GPx) (Rotruck *et al.*, 1973) and lactate dehydrogenase (LDH) (Vassault, 1983). The concentrations of reduced glutathione (GSH), hydrogen peroxide generated and ascorbic acid were determined using the methods of Beutler *et al.* (1963), Wolff (1994), and Jakota and Dani (1982) respectively. Lipid peroxidation was assessed by determining the concentration of malondialdehyde formed using the method of Varshney and Kale (1990).

Anti-inflammatory assays

Myeloperoxidase activity was determined using the method of Trush *et al.* (1994) and nitrite concentration was analysed using the method of Green *et al.* (1982).

Statistical Analysis

All data, as appropriate, were expressed as mean \pm standard error of mean (S.E.M). Statistical analyses were carried out using one- way analysis of variance (ANOVA). Values of $p < 0.05$ were considered to be significant and post- hoc tests were carried out using the least significant difference (LSD). The statistical package used was the International Business Machines (IBM) brand of the Statistical Package of the Social Science (SPSS) Version 23.

RESULTS

Change in body weight: Figure 1 shows the percentage change in body weight in the control and treatment groups. The groups that received co-administration of EGME with the extract showed weight loss ranging from 7.02% - 7.82% between day 16 to day 21 while the other groups showed increases in weight.

Effect on weight of organs

Table 1 shows that the weights of the testes (0.96 \pm 0.05; 0.95 \pm 0.06; 0.76 \pm 0.06 g) and epididymis decreased significantly ($p < 0.05$) compared to the control in the EGME only group and in the co-administered groups. The weight of the seminal vesicle and the prostate gland was also decreased compared to the control, but the decrease was significant ($p < 0.05$) in the EGME + PP (200 mg/kg) group (0.71 \pm 0.07 g and 0.15 \pm 0.02 g respectively). The relative weight of the testis (i.e. weight of testes per 100 g of body weight) decreased significantly ($p < 0.05$) compared to the control in the EGME only and the EGME + PP co-administered groups.

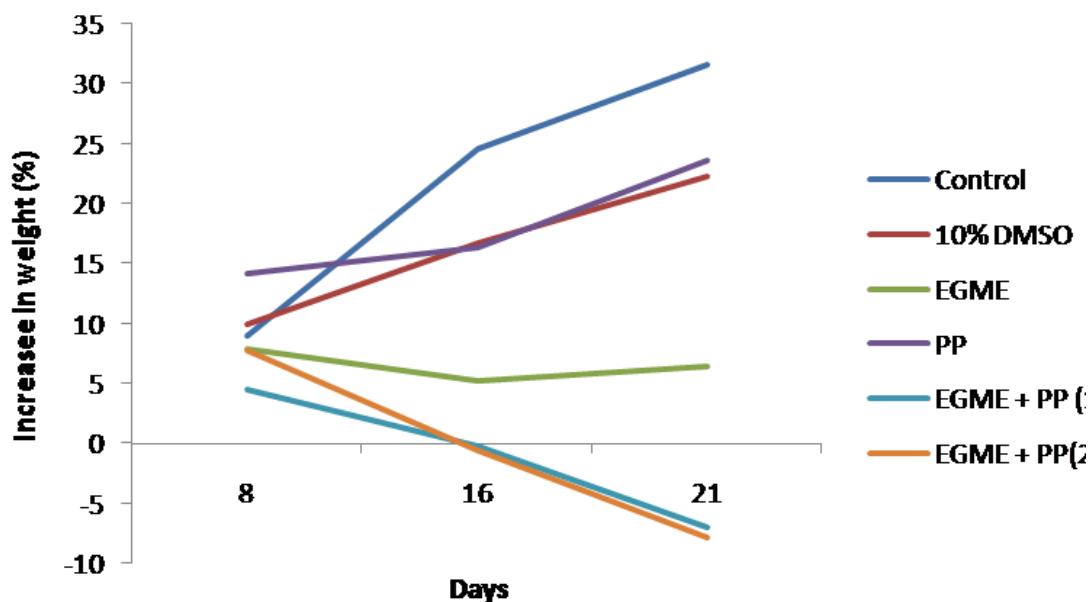


Figure 1: Percentage change in weight in the control and treatment groups

Table 1:

Weight and relative weight of organs after 21 day treatment of Ethylene glycol monomethyl ether (EGME) and *Paullinia pinnata* (PP) methanol leaf extract

Group	Weight of organs (g)					Relative weight of organs (%)				
	Testes	Epididymis	Seminal Vesicle	Prostate Gland	Brain	Testes	Epididymis	Seminal Vesicle	Prostate Gland	Brain
Control	2.46±0.11	0.57±0.02	0.94± 0.07	0.25± 0.03	1.77± 0.03	1.16± 0.06	0.25± 0.02	0.42± 0.03	0.11± 0.01	0.79± 0.04
10 % DMSO	2.24±0.06	0.52± 0.03	0.92± 0.05	0.28± 0.03	1.76± 0.03	1.11± 0.04	0.26± 0.02	0.44± 0.02	0.14± 0.02	0.82± 0.01
EGME	0.96±0.05*	0.47± 0.03*	0.94± 0.06	0.20± 0.03	1.73± 0.01	0.49± 0.02*	0.22± 0.02	0.46± 0.05	0.14± 0.02	0.83± 0.02
PP	2.36±0.11	0.54±0.03	1.01± 0.05	0.33± 0.02	1.75± 0.03	1.09± 0.05	0.24± 0.02	0.47± 0.03	0.14± 0.01	0.81± 0.03
EGME + PP(100)	0.95±0.06*	0.41± 0.03*	0.84± 0.11	0.20± 0.03	1.78± 0.03	0.55± 0.05*	0.23± 0.02	0.43± 0.04	0.12± 0.02	1.05± 0.06*
EGME + PP(200)	0.76±0.06*	0.34± 0.03*	0.71± 0.07*	0.15± 0.02*	1.71± 0.04	0.48± 0.05*	0.22± 0.02	0.38± 0.09	0.10± 0.02	1.07± 0.05*

Note: n = 10; *- significant at $p < 0.05$; values are mean±standard error of mean

Table 2:

Reproductive hormone profile after 21 day treatment of Ethylene glycol monomethyl ether (EGME) and *Paullinia pinnata* (PP) methanol leaf extract

Group	LH	FSH	TESTOSTERONE	T ₃	T ₄
Control	10.00± 0.00	8.50± 0.50	1.57± 0.12	1.37± 0.07	68.33± 1.76
10 % DMSO	11.67± 0.88	6.00± 2.65	1.50± 0.06	1.47± 0.07	75.50± 2.50
EGME	12.00± 1.00	9.50± 1.50	1.50± 0.10	1.50± 0.00	74.00± 4.00
PP	11.50± 0.50	9.67± 0.33	1.63± 0.09	1.50± 0.20	80.00± 1.00*
EGME + PP(100)	13.50± 1.50*	11.00± 2.00	1.15± 0.15*	1.77± 0.09*	87.67± 4.91*
EGME + PP(200)	14.67± 0.67*	11.67± 0.67	1.13± 0.07*	1.40± 0.10	87.00± 2.08*

Note: n = 10; *- significant at $p < 0.05$; values are mean±standard error of mean

KEY: Parameter (unit)

LH- Luteinizing Hormone (pg/ml); FSH- Follicle Stimulating Hormone (pg/ml) Testosterone (pg/ml); T₃- Triiodothyronine (pg/ml); T₄- Thyroxine (pg/ml)

Table 3:

Spermatozoa analysis after 21 day treatment of Ethylene glycol monomethyl ether (EGME) and *Paullinia pinnata* (PP) methanol leaf extract

Parameters	Control	10 % DMSO	EGME	PP	EGME + PP(100)	EGME + PP(200)
Abnormality (%)	11.94± 0.25	11.62± 0.22	13.01± 0.31*	12.79± 0.52	14.03± 0.37*	14.59± 0.14*
Motility (%)	67.50± 5.74	72.50± 4.96	12.50± 2.50*	80.00± 7.79	3.50± 1.50*	10.00± 2.89*
Viability (%)	70.00± 3.87	70.00± 5.88	11.75± 1.18*	82.86± 2.40*	11.00± 1.00*	17.50± 2.50*
Testicular Spermatozoa Number (TSN)	116.83±9.03	98.14± 6.14	86.83± 5.53*	105.00± 7.42	88.50± 0.50	93.00±5.00
Daily Sperm Production (DSP)	49.40± 0.22	40.90± 1.53	58.67± 1.29	45.73±4.75	67.24± 3.72*	101.00± 4.91*

Note: n = 10; *- significant at $p < 0.05$; values are mean±standard error of mean; KEY: Parameter (unit):- TSN ($\times 10^6$ / gm testis), DSP ($\times 10^6$ cells/ gm testis)

Effect on reproductive hormones

The reproductive hormone profile is presented in Table 2. The plasma levels of luteinizing hormone, follicle stimulating hormone, triiodothyronine and thyroxine were significantly ($p < 0.05$) increased compared to the control in the EGME + PP co-administered groups while the plasma testosterone level was significantly ($p < 0.05$) reduced (1.15 ± 0.15 ; 1.13 ± 0.07 pg/ml).

Effect on spermogram

The spermatozoa analysis (Table 3) shows that spermatozoa motility (12.50 ± 2.50 ; 3.50 ± 1.50 ; 10.00 ± 2.89 %), spermatozoa viability (11.75 ± 1.18 ; 11.00 ± 1.00 ; 17.50 ± 2.50 %) and testicular spermatozoa number were significantly ($p < 0.05$) reduced compared to the control in the EGME only and EGME + PP co-administered groups. The total sperm abnormality (13.01 ± 0.31 ; 14.03 ± 0.37 ; 14.59 ± 0.14 %) and daily sperm production (67.24 ± 3.72 ; $101.00 \pm 4.91 \times 10^6$ cells/gm testis) was significantly ($p < 0.05$) increased compared to the control in the EGME only and EGME + PP co-administered groups.

Effect on antioxidant parameters

The antioxidant profile in the epididymis (Table 4) shows that the levels of reduced glutathione (0.72 ± 0.13 ; 1.23 ± 0.12 μ M/g tissue) and vitamin C (6.12 ± 0.70 ; 3.60 ± 1.43 ; 3.00 ± 2.00 μ g/ml) were decreased significantly ($p < 0.05$) in the EGME only and EGME + PP co-administered groups while lipid peroxidation and the activities of catalase, glutathione-S-transferase and glutathione peroxidase were not significantly affected.

The antioxidant profile in the testis (Table 5) shows that the activities of glutathione-S-transferase (1.27 ± 0.04 ; 1.28 ± 0.07 ; 1.38 ± 0.05 IU), catalase (15.20 ± 1.33 ; 14.71 ± 0.71 ; 17.33 ± 1.23 μ moles H_2O_2 consumed/min/mg protein) and glutathione peroxidase (57.08 ± 3.08 ; 55.13 ± 3.64 mg/ml/mg protein) are increased while the activity of superoxide dismutase (5.00 ± 1.67 ; 2.38 ± 0.24 unit/ mg protein) and the level of vitamin C (23.67 ± 4.67 ; 20.25 ± 3.09 ; 17.00 ± 5.00 μ g/ml) are decreased significantly ($p < 0.05$) in the EGME only and EGME + PP co-administration groups compared to

the control. The elevations in lipid peroxidation is insignificant ($p < 0.05$) in the EGME only and EGME+ PP co-administered groups. Hydrogen peroxide generation (0.87 ± 0.04 ; 1.11 ± 0.09 ; 1.03 ± 0.09 μ M) increases significantly ($p < 0.05$) in the EGME only and EGME + PP co-administered groups compared to the control. The activity of lactate dehydrogenase was increased, though not significantly in the EGME only and EGME-PP co-administered groups. Reduced glutathione (2.32 ± 0.18 ; 2.43 ± 0.00 μ M/ g tissue) was significantly ($p < 0.05$) increased in the EGME only and EGME-PP co-administered groups compared to the control.

The antioxidant parameters in the brain (Table 6) shows that the activity of superoxide dismutase (7.14 ± 1.37 ; 4.76 ± 0.00 unit/ mg protein) was significantly ($p < 0.05$) reduced in the EGME + PP co-administered groups compared to the control. Also the glutathione-S-transferase activity (0.17 ± 0.01 ; 0.15 ± 0.02 IU) was increased significantly ($p < 0.05$) in the EGME only and the EGME + PP (100 mg/kg) groups compared to the control, and the activity of glutathione peroxidase (46.98 ± 0.78 mg/ml/mg protein) was increased significantly ($p < 0.05$) in the EGME + PP (200 mg/kg) group compared to the control. The level of vitamin C (5.50 ± 0.65 ; 3.00 ± 0.00 μ g/ml) was significantly ($p < 0.05$) reduced in the EGME-PP co-administered groups compared to the control.

Effect on markers of inflammation

Table 7 shows the effect of the treatments on the inflammatory markers in the epididymis, testis and brain. In the epididymis, nitric oxide concentration was not affected but the activity of myeloperoxidase (19.42 ± 1.98 ; 17.14 ± 1.91 ; 16.34 ± 1.24 IU/ mg protein) was significantly ($p < 0.05$) increased in the EGME only and EGME + PP co-administered groups compared to the control. In the testis, there were significant ($p < 0.05$) elevations in the activity of myeloperoxidase (18.32 ± 0.44 ; 19.04 ± 1.77 IU/ mg protein) and nitric oxide concentration (1.40 ± 0.10 ; 2.16 ± 0.18 ; 1.67 ± 0.24) in the EGME only and EGME + PP co-administered groups compared to the control. In the brain, there is no obvious effect on the myeloperoxidase activity and the nitric oxide concentration.

Table 4:

Antioxidant profile in the epididymis after 21 day treatment of EGME and *P. pinnata* (PP) methanol leaf extract

Group	SOD	CAT	GSH	LPO	GST	H ₂ O ₂	VIT. C	GPx
Control	9.63 ± 1.48	17.52 ± 1.23	2.04 ± 0.35	6.80 ± 0.81	0.17 ± 0.02	0.59 ± 0.04	14.00 ± 1.05	322.81 ± 24.51
10 % DMSO	7.56 ± 0.88	18.34 ± 1.37	1.47 ± 0.21	5.81 ± 0.48	0.13 ± 0.00	0.57 ± 0.03	11.00 ± 3.51	298.80 ± 7.02
EGME	2.22 ± 0.00	11.79 $\pm 1.40^*$	0.72 $\pm 0.13^*$	7.23 ± 0.14	0.23 ± 0.04	0.67 ± 0.12	6.12 $\pm 0.70^*$	281.86 ± 22.57
PP	13.33 ± 1.11	16.77 ± 1.47	1.48 ± 0.04	6.73 ± 0.75	0.18 ± 0.03	0.54 ± 0.05	13.40 ± 1.17	309.49 ± 20.99
EGME+ PP(100)	6.67 ± 0.00	15.86 ± 1.43	1.54 ± 0.24	7.35 ± 1.46	0.20 ± 0.01	0.58 ± 0.03	3.60 $\pm 1.43^*$	295.11 ± 5.05
EGME+ PP(200)	3.70 ± 1.48	13.67 ± 0.27	1.23 $\pm 0.12^*$	6.83 ± 0.74	0.18 ± 0.04	0.62 ± 0.05	3.00 $\pm 2.00^*$	273.60 $\pm 24.80^*$

Note: n = 10; *- significant at $p < 0.05$; values are mean \pm standard error of mean; KEY: Parameter (unit):- SOD- Superoxide dismutase (unit/ mg protein), CAT- Catalase (μ moles H_2O_2 consumed/min/mg protein), GSH- reduced glutathione (μ M/ g tissue), LPO- Lipid peroxidation (μ M malondialdehyde formed/ mg protein), GST- Glutathione-S-Transferase (IU), H₂O₂- Hydrogen peroxide generation (μ M), VIT. C- Ascorbic acid (μ g/ml), GPx- Glutathione peroxidase (mg/ml/mg protein)

Table 5:
Antioxidant profile in the testis after 21 day treatment of EGME and *P. pinnata* (PP) methanol leaf extract

Group	GSH	SOD	CAT	LPO	GST	H ₂ O ₂	VIT. C	GPx	LDH
Control	1.91± 0.08	9.52±0.00	9.81±0.82	2.06± 0.08	0.85± 0.02	0.59± 0.02	30.00± 3.81	43.86± 1.45	4.64± 1.58
10% DMSO	2.14±0.05	9.52± 0.00	9.05± 1.12	2.04± 0.11	0.77±0.02	0.62± 0.06	26.00± 1.53	40.67± 1.43	4.55± 1.10
EGME	2.32± 0.18*	7.14± 2.38	15.20± 1.33*	3.13± 0.11	1.27± 0.04*	0.87± 0.04*	23.67± 4.67*	48.07± 0.83	4.88± 1.44
PP	1.77± 0.10	11.11± 1.59	10.34± 0.66	1.99± 0.30	0.81± 0.06	0.76± 0.10	25.00± 2.20	46.07± 0.96	4.23± 1.02
EGME + PP(100)	2.00± 0.14	5.00± 1.67*	14.71± 0.71*	2.56± 0.37	1.28± 0.07*	1.11± 0.09*	20.25± 3.09*	57.08± 3.08*	4.76±0.14
EGME + PP(200)	2.43± 0.00*	2.38± 0.24*	17.33± 1.23*	2.58± 0.39	1.38± 0.05*	1.03± 0.09*	17.00± 5.00*	55.13± 3.64*	7.80± 0.72

Note: n = 10; *- significant at $p < 0.05$; KEY: Parameter (unit):- GSH- reduced glutathione ($\mu\text{M}/\text{g}$ tissue), SOD- Superoxide dismutase (unit/ mg protein), CAT- Catalase ($\mu\text{moles H}_2\text{O}_2$ consumed/min/mg protein), LPO- Lipid peroxidation (μM malondiald-ehyde formed/ mg protein), GST- Glutathione-S-Transferase (IU), H₂O₂- Hydrogen peroxide generation (μM), VIT. C- Ascorbic acid ($\mu\text{g}/\text{ml}$), GPx- Glutathione peroxidase (mg/ml/mg protein), LDH- Lactate dehydrogenase (unit/ mg protein)

Table 6:
Antioxidant profile in the brain after 21 day treatment of EGME and *P. pinnata* (PP) methanol leaf extract

Group	SOD	CAT	GSH	LPO	GST	H ₂ O ₂	VIT. C	GPx
Control	12.25± 1.11	3.89± 0.78	5.18± 0.74	1.61± 0.13	0.13± 0.01	0.53± 0.03	7.50± 0.56	40.44± 1.05
10 % DMSO	11.11± 0.00	5.08± 0.42	4.90± 0.80	1.61± 0.09	0.12± 0.01	0.56± 0.05	8.00± 0.71	42.41± 1.62
EGME	11.11± 0.00	3.68± 0.41	4.40± 0.35	1.44± 0.08	0.17± 0.01*	0.51± 0.06	6.33± 0.33	43.65± 1.13
PP	12.70± 1.59	4.51± 1.28	6.36± 0.50	1.63± 0.32	0.14± 0.01	0.54± 0.08	8.67± 0.67	39.72± 1.95
EGME + PP(100)	7.14± 1.37*	3.80± 0.65	6.93± 0.50	1.57± 0.09	0.15± 0.02*	0.54± 0.08	5.50± 0.65*	39.71± 0.91
EGME + PP(200)	4.76± 0.00*	2.46± 0.51	4.36± 0.07	1.25± 0.10	0.14± 0.01	0.54± 0.12	3.00± 0.00*	46.98± 0.78*

Note: n = 10; *- significant at $p < 0.05$; values are mean±standard error of mean

KEY: Parameter (unit)

SOD- Superoxide dismutase (unit/ mg protein); CAT- Catalase ($\mu\text{moles H}_2\text{O}_2$ consumed/min/mg protein) GSH- reduced glutathione ($\mu\text{M}/\text{g}$ tissue); LPO- Lipid peroxidation (μM malondialdehyde formed/ mg protein); GST- Glutathione-S-Transferase (IU); H₂O₂- Hydrogen peroxide generation (μM); VIT. C- Ascorbic acid ($\mu\text{g}/\text{ml}$); GPx- Glutathione peroxidase (mg/ml/mg protein)

Table 7:
Anti-inflammatory profile in the epididymis, testis and brain after 21 day treatment of Ethylene glycol monomethyl ether (EGME) and *Paullinia pinnata* (PP) methanol leaf extract

Group	Epididymis		Testis		Brain	
	MPO	NO	MPO	NO	MPO	NO
Control	8.97± 1.15	1.53± 0.21	13.83± 1.03	0.77± 0.08	12.31± 0.27	0.98± 0.10
10 % DMSO	8.36± 0.54	1.51± 0.17	13.61± 1.44	0.69± 0.09	12.72± 1.29	1.06± 0.05
EGME	19.42± 1.98*	1.35± 0.23	18.32± 0.44*	1.40± 0.10*	13.33± 3.13	1.20± 0.08
PP	12.00± 2.08	1.27± 0.69	14.53± 1.63	0.92± 0.06	15.67± 1.54	1.05± 0.09
EGME + PP(100)	17.14± 1.91*	1.01± 0.05	19.04± 1.77*	2.16± 0.18*	13.67± 1.85	1.15± 0.03
EGME + PP(200)	16.34± 1.24*	1.22± 0.07	17.27± 1.12	1.67± 0.24*	14.00± 1.41	0.91± 0.02

Note: n = 10; *- significant at $p < 0.05$; values are mean±standard error of mean; KEY: Parameter (unit):- MPO- myeloperoxidase (IU/ mg protein), NO- Nitric oxide (mM)

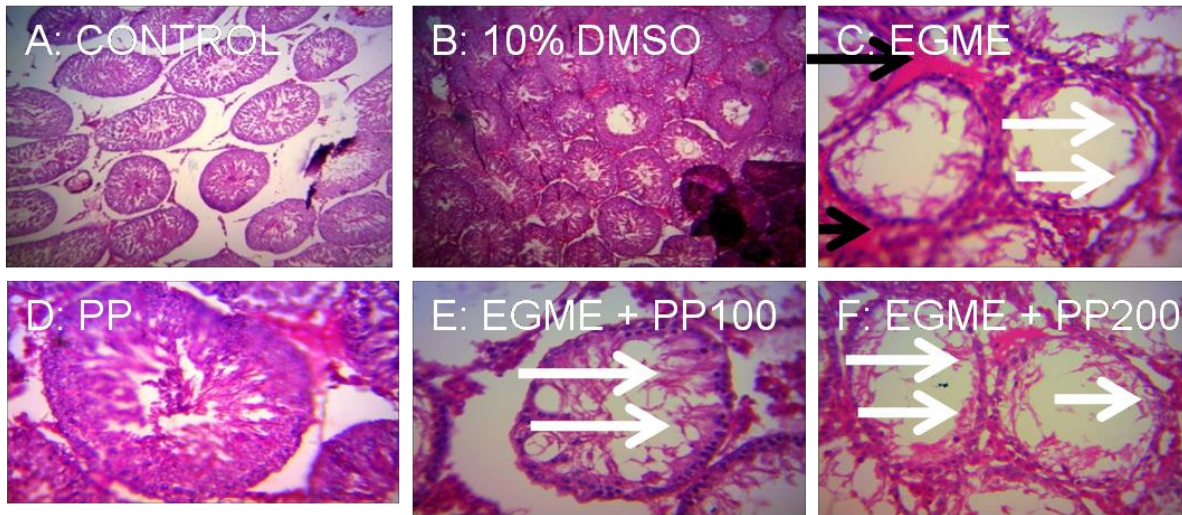


Plate 1:
Photomicrograph of the Testis in the study of *P.pinnata* in EGME-induced gonadotoxicity. H. & E., M. x 400
 A: No lesion; B: No lesion; C: Severe germinal erosion (white arrows) and severely congested interstitium (black arrows); D: No lesion; E: Severe germinal erosion; F: Severe germinal erosion.

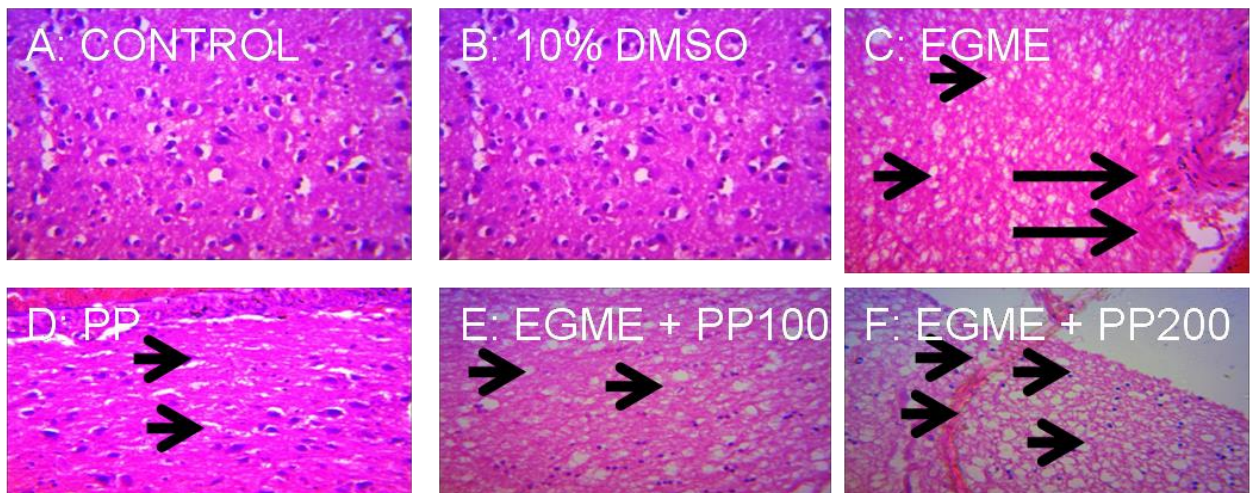


Plate 2:
Photomicrograph of the Hypothalamus in the study of *P.pinnata* in EGME-induced gonadotoxicity. H. & E., M x 400
 A: No lesion; B: No lesion; C: Moderate to severe diffuse spongiosis of the parenchyma (short arrow) and submeningeal portion is mildly haemorrhagic (long arrows); D: No lesion; E: Moderate spongiosis of the parenchyma; F: Severe spongiosis of the parenchyma

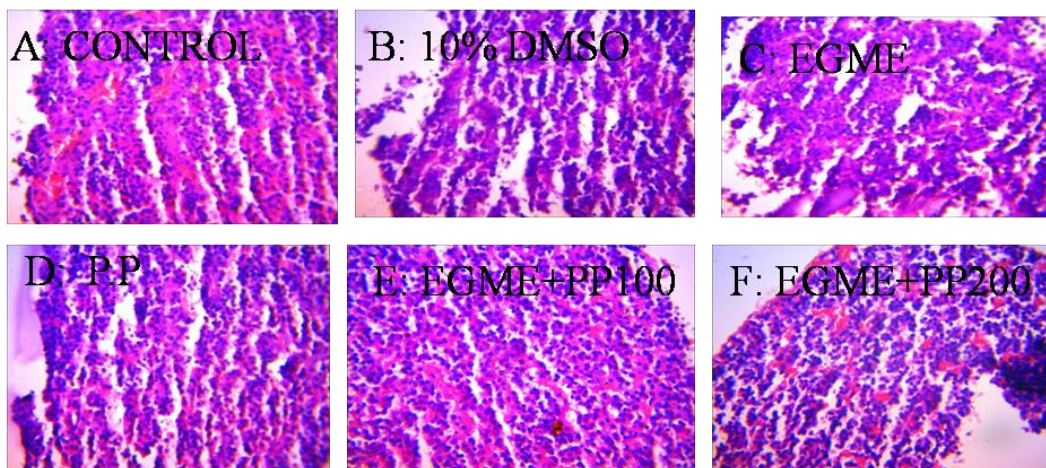


Figure 3: Photomicrograph of the Pituitary gland in the study of *P.pinnata* in EGME-induced gonadotoxicity. H. & E., M x 400. A-F: No Lesion.

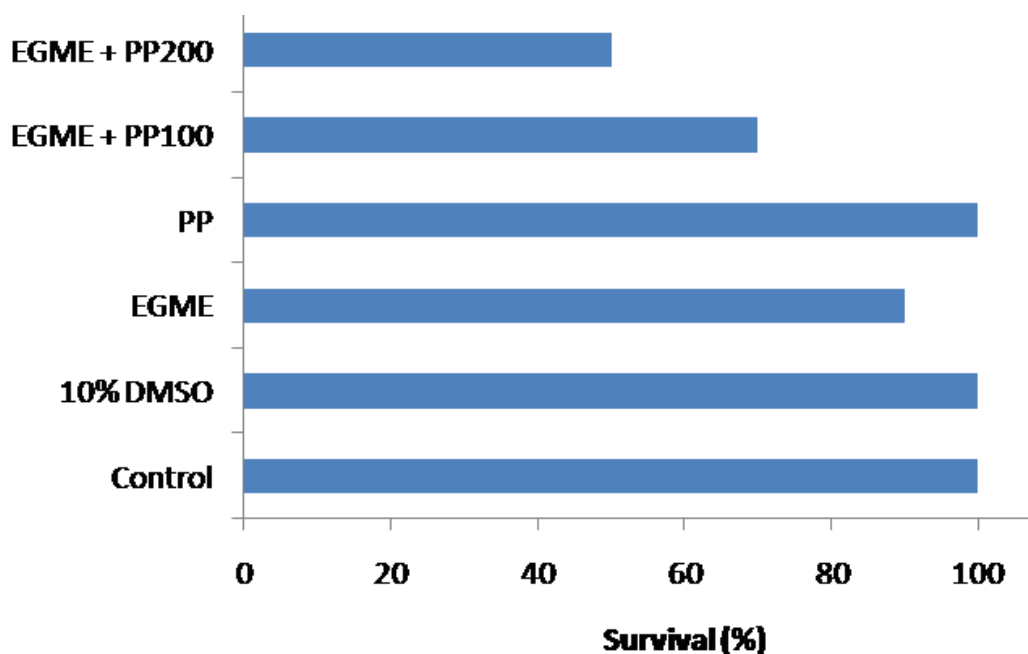


Figure 2:
Survival rate in the study of *P.pinnata* in EGME-induced gonadotoxicity

Histological examination

Plate 1 shows the histopathology of the testis. The control, 10 % DMSO and *P. pinnata* only groups showed no lesions (A, B & D). The EGME only group showed severe germinal erosion and severely congested interstitium (C). The EGME + PP co-administered groups showed severe germinal erosion (E & F).

Plate 2 shows the histopathology of the hypothalamus. Again, the control, 10 % DMSO and *P. pinnata* only groups showed no lesions (A, B & D). The EGME only group showed moderate to severe diffuse spongiosis of the parenchyma and the submeningeal portion was mildly hemorrhagic (C). The EGME + PP (200 mg/kg) group showed moderate spongiosis of the parenchyma (E) and the EGME + PP (200 mg/kg) showed severe spongiosis of the same (F).

In Plate 3, the histological examination of the pituitary gland shows that there were no lesions in the control and the treatment groups (A-F).

Survival rate

Figure 2 presents the survival rate and it shows that there was 100 % survival in the control, 10 % DMSO and *P. pinnata* only groups. The EGME only group showed 90 % survival while the EGME + PP (100 mg/kg) and EGME + PP (200 mg/kg) groups had 70 % and 50 % survival respectively.

DISCUSSION

This study suggests that *Paullinia pinnata* leaves do not have chemopreventive effect against gonadal oxidative stress as a result of exposure to ethylene glycol monomethyl ether. The observed body weight loss in the EGME-PP co-administered groups is a sign of toxicity. Moreover, the significant decrease in the weight of testes in the EGME and EGME- PP co-administered groups, which reflects in the relative weight of the organs, are manifestations of toxicity. In addition, the significant increase in the relative weight of the brain for the EGME- PP co-administered groups is as a result

of the significant loss in body weight of the animals which further buttresses the evidence of toxicity.

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are glycoprotein hormones released from the pituitary in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus. LH and FSH regulate gonadal function, including spermatogenesis and androgen biosynthesis (Sikka and Naz, 2002; O'Shaughnessy *et al*, 2010). Elevated plasma levels of LH and FSH in the EGME-PP co-administered groups, with that of LH being statistically significant, is an indication of the impairment in the function of the hypothalamus, which stimulates the pituitary through GnRH. Decreased plasma level of testosterone in the EGME-PP co-administered groups is an indication of improper function of the leydig cells of the testes because the leydig cells are the site of androgen production (Sikka and Naz, 2002).

Thyroxine (T_4) is the prohormone of triiodothyronine (T_3) and both are tyrosine-based hormones produced by the thyroid gland (Baldo, 2016). Their primary role is the regulation of metabolism. The pituitary, in response to stimulation by thyrotropin-releasing hormone (TRH) from the hypothalamus, releases thyroid-stimulating hormone (TSH) which stimulates the thyroid to secrete T_4 and then T_3 . Positive feedback to the hypothalamus and pituitary by T_4 and T_3 results in the stimulation or suppression of these organs depending on the plasma levels (Beckman Coulter, 2013). Significantly elevated plasma levels of T_3 and T_4 in the EGME-PP co-administered groups is an indication of over-stimulation of the thyroid gland by the pituitary in response to the TRH from the hypothalamus and may therefore suggest impairment in hypothalamus function. Thus resulting in hyperthyroidism, which may contribute to weight loss, which is one of the symptoms (Bowen, 2010).

The daily sperm production is significantly increased in the EGME- PP co-administered groups thus indicating over-stimulation of the seminiferous tubules. The significant decrease in motility and viability of the sperms with significant

increases in the percentage total sperm abnormality in the EGME only and the EGME-PP co-administered groups point to the existence of aberrations in the sperms of the animals in these groups and shows that the function of the Sertoli cells of the testes, which is to remove defective sperm cells, is impaired. This implies that co-administration of EGME with *P. pinnata* affects the proper function of the Sertoli cells and this can result in infertility. Since the sperms of the EGME-PP co-administered groups showed decreased viability and motility with increased abnormality, the ability of the sperms in these groups to fertilize the ovum of a female is minimized and therefore infertility is likely to be observed.

Oxidative stress is a condition induced by nitrogen-derived free radicals, oxygen and oxygen-derived free radicals known as reactive nitrogen species (RNS) and reactive oxygen species (ROS) (Lobo *et al.*, 2010). The presence of ROS in biological systems is as a result of normal physiological processes and cell activation (e.g. immunological responses and apoptosis) (Pham-Huy *et al.*, 2008). Also, exposure to many types of environmental contaminants, aging, chronic disease state or gonadal injury can induce oxidative stress associated with an increased rate of cellular damage that results in gonadotoxicity (Sikka and Naz, 2002). Enzymatic and non-enzymatic antioxidants in the gonads exist to combat these ROS (Adedara and Farombi, 2010). In the EGME only and EGME-PP co-administered groups, the activities of superoxide dismutase, catalase, glutathione peroxidase and levels of reduced glutathione and vitamin C were largely insignificantly reduced, and this had resulted in no observable lipid peroxidation which is reflected in no visible lesions in the epididymis. This shows that the oxidative stress status in the epididymis was not appreciably affected.

In the testes, activity of superoxide dismutase and vitamin C level were significantly reduced while that of catalase, glutathione-S-transferase, glutathione peroxidase, reduced glutathione and hydrogen peroxide were significantly increased in the EGME only and EGME-PP co-administered groups. This resulted in insignificant increases in lipid peroxidation thus suggesting minimal deleterious effect of the reactive oxygen species in these groups. The activity of lactate dehydrogenase (an indicator of tissue damage) increased insignificantly in the EGME only and EGME-PP co-administered groups. This suggests that there is minimal damage done to the testes but the histological examination shows that there are lesions in the EGME only and EGME-PP co-administered groups. This indicates that lactate dehydrogenase is probably being inhibited in these groups and since EGME is common to the groups, it may be that the metabolite(s) of EGME is/are responsible.

In the brain, there were no significant changes in the activity of catalase and levels of lipid peroxidation, reduced glutathione and hydrogen peroxide generation. However, there were significant decreases in the activity of superoxide dismutase and vitamin C level with significant increases in the activities of glutathione-S-transferase and glutathione peroxidase in the EGME-PP co-administered groups. This suggests that co-administration of EGME with *P. pinnata* facilitates the crossing of the blood-brain barrier.

Regarding the anti-inflammatory profile, the significantly elevated activity of myeloperoxidase in the epididymis of the EGME only and EGME-PP co-administration groups supports the observed decreased activity of superoxide dismutase. This is because when neutrophils are functioning, they generate free

radicals, especially superoxide radical, and this in turn is scavenged by superoxide dismutase forming hydrogen peroxide which is in turn cleaved by catalase and other peroxidases. When catalase is overwhelmed by the level of hydrogen peroxide, the hydrogen peroxide feed-back inhibits superoxide dismutase thus resulting in the decreased activity (Rahman, 2007; Lobo *et al.* 2010). In the testes, the elevated activity of myeloperoxidase and level of nitric oxide again supports the observed decreased activity of superoxide dismutase.

The pituitary gland and hypothalamus are parts of the brain that control the male reproductive system. Histopathology of the pituitary shows that there were no lesions in the control and treatment groups but the hypothalamus showed moderate to severe diffuse spongiosis of the parenchyma with the submeningeal portion being mildly haemorrhagic in the EGME only group while the EGME-PP co-administration groups showed moderate and severe spongiosis of the parenchyma. This corroborates the results of the analysis of the reproductive hormones and shows that the hypothalamus, and therefore its function, is affected in the EGME only and EGME-PP co-administered groups and this is reflected in the function of the pituitary gland.

The survival rate showed that co-administration of the methanol leaves extract of *Paullinia pinnata* with EGME exacerbated the effect of EGME. This is because there was 50 % mortality in the group administered with EGME +200 mg/kg of PP and the group that received EGME + 100 mg/kg of PP had 30 % mortality but the EGME only group had a mortality of 10 %.

In conclusion, although our hypothesis was not validated using ethylene glycol monomethyl ether-induced male reproductive dysfunction model, the study shows that *Paullinia pinnata* methanol leaf extract does not have chemopreventive effect against gonadotoxicity by ethylene glycol monomethyl ether and therefore may not help infertility as a result of exposure to similar gonadotoxicants.

Caution should be exercised when taking or ingesting *Paullinia pinnata* leaves or the preparations with drugs containing glycol ethers as excipients. Moreover, persons exposed regularly to Ethylene glycol monomethyl ether, especially at the work place, should avoid ingesting or taking preparations from the leaves of *Paullinia pinnata*.

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