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Biochemical Effect of Ethanolic Extract of *Phyllanthus amarus* (Euphorbiaceae) on Plasma Nitric Oxide and Penile Cyclic Guanosine Monophosphate (cGMP) in Mature Male Guinea Pigs

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ABSTRACT

The aim of this study was to investigate the probable mode of action of ethanolic extract of *Phyllanthus amarus* traditionally used to correct erectile dysfunction by monitoring the effect on the concentration of penile cyclic guanosine mono-phosphate (cGMP) and the concentration of plasma Nitric Oxide (NO) in male guinea pigs that were administered different doses (100, 200 and 400 mg kg⁻¹ body weight) of the plant extract using direct immunoassay kits. Physiological saline and sildenafil were given to animals that served as the controls. Phytochemical screening of the plant determined included alkaloids, flavonoids, terpenoids, saponins, tannins, steroids and cardiac glycosides. There was no statistical significant difference in the weight of the animals before and after the administration of the plant extract. The penile weight per body weight ratio showed a significant difference between groups ($p < 0.05$). There was a dose dependent increase in the level of cGMP in the different groups administered different doses of the extract, with the 400 mg kg⁻¹ group showing significant difference compared to lower-dose and saline groups ($p < 0.05$). Plasma nitric oxide concentration was not significantly different among the control and experimental groups. Conclusively, ethanol extract of the plant had a dose dependent increase on the level of cGMP which might account for the claim of the plant been use as a sexual stimulant and the increase level of the cGMP probably had a feedback effect on nitric oxide synthase which resulted to reduced concentration of NO in the plasma.

Key words: *Phyllanthus amarus*, neurotransmitter, cyclic guanosine monophosphate (cGMP), penile erection, phosphodiesterase-5 inhibitor

INTRODUCTION

Sexual function is an important component of quality of life and subjective well being in humans. Sexual problems are widespread and adversely affect mood, well being and interpersonal functioning (Laumann *et al.*, 1999). The main sexual problems are related to sexual desire and male sexual dysfunction. Successful treatment of sexual dysfunction may improve not only sexual relationship but also quality of life.

There have been a number of important approaches to restore sexual function. Plants are extensively used to manage sexual dysfunction (Adimoelja, 2000; Yakubu *et al.*, 2007).

Ginseng, for example, is an essential constituent in traditional Chinese medicine (Kim *et al.*, 1976) and at least 6 million Americans use the root of this slow-growing perennial plant (Nocerino *et al.*, 2000). *Phyllanthus amarus* is one of such plants claimed to possess aphrodisiac property. *P. amarus* (Euphorbiaceae) is a small annual herb that grows to a height of about 6-15 inches found throughout the tropics and subtropics. It is a broad-spectrum medicinal plant that has received worldwide recognition. It has recently attained the status of miracle plant because of its ability to cure several ailments as claimed by its proponents (Srividya and Periwal, 1995). It is locally called Iyin-olobe (Yoruba, south-west Nigeria) or kidney stone plant (Adeneye *et al.*, 2006). In traditional medicine, it is known for its hepatoprotective, antidiabetic, antihypertensive, analgesic, anti-inflammatory and antimicrobial properties (Adeneye *et al.*, 2006). The plant is also used in the treatment of stomach disorders, skin diseases and cold (Iwu, 1993). It has antidiarrheal effect (Odetola and Akojenu, 2000). Its anti-viral activity against hepatitis B virus (Thyagarajan *et al.*, 1988), anticarcinogenic and antimutagenic activities (Sripanidkulchai *et al.*, 2002) anti-nociceptive and anti-inflammatory activities (Kassuya *et al.*, 2006) have also been reported. The recognized active lignan in *Phyllanthus amarus* as reported by Sharma *et al.* (1993) are Phyllanthin and Hypophyllanthin and they were reported to exhibit anti-hepatotoxic property. These lignans comprises of lots of benzene rings and are bulky, showing similarities with known phosphodiesterase 5 (PDE 5) inhibitors, sildenafil, tadalafil, zaprinast and verdanafil. This might be an indication that the ethanol extract of *Phyllanthus amarus* is probably a PDE5 inhibitor.

Erectile dysfunction is defined as the inability to attain or maintain penile erection sufficient for sexual performance on at least two-thirds of occasions (Morales, 2003). An erect penis has always been a symbol of virility and fertility. The inability to obtain or maintain an erection sufficient for vaginal penetration is clinically known as erectile dysfunction and or impotence, which is a health problem of major concern among men from all geographical locations (Goldstein, 2000). Erection occurs when the smooth muscle of the corpora cavernosum relaxes, allowing pooling of blood. The relaxation of the smooth muscle is as a result of the autonomic nervous system through the action of Cyclic Guanosine Mono-Phosphate (cGMP) acting as a second messenger as a result of Nitric Oxide (NO) activating guanylyl cyclase. Guanylyl cyclase brings about increase in production of cGMP by converting Guanyl Triphosphate (GTP) to cGMP, while phosphodiesterase breaks it down (i.e., cGMP) to Guanosine Monophosphate (GMP). The most common treatment for erectile dysfunction is a phosphodiesterase-5 inhibitor. This works by blocking the breakdown of cyclic GMP that has been generated by nitric oxide. There are 3 phosphodiesterase inhibitors in general use viz; sildenafil, vardenafil and tadalafil. They prevent the breakdown of cGMP by inhibiting the action of phosphodiesterase and have gained worldwide fame for the treatment of male impotency (Goldstein, 2000).

The search for an effective, safe and easy to administer drug for use in erectile dysfunction, impotence and fertility has been a perennial pursuit of most societies from times immemorial throughout history. Treatments for these disorders have been the use of ginseng, rhinoceros horn and other dubious herbs to enhance potency. The discovery of testosterone in the late 1930s and its use to relieve the impotence in hypogonadal men was a major step forward in this field. Many substances used for recreational purposes (or sometimes abused) were also thought to have profound effects on sexual performance (Morton, 1992). A wide variety of drugs have been reported to possess erectolytic effects (Meinhardt *et al.*, 1997). *L-arginine* (nutrient) and *Yohimbe*, *Panax ginseng*, *Ginkgo biloba* and maca root (botanicals) are some products claimed to provide some benefits in correcting sexual dysfunction. Though little scientific reports are available on the ability

of *Phyllanthus amarus* to serve as an aphrodisiac. Obianime and Uche (2009) reported the use of the aerial part of this plant in improving libido and reproductive function in men, thus supporting the claims of traditional medicine practitioner of the plant being used as an aphrodisiac.

Impotence is not a disease but a secondary condition brought on by other, primary causes. These primary causes are mostly either due to the inhibition of the synthesis of Nitric Oxide (NO), or the fast action of phosphodiesterase (V) which breaks down cGMP (Merck, 1996). Since *P. amarus* is acclaimed to be used as an aphrodisiac there is the need to provide scientific information on its androgenic potentials and at the same time look at the possible mode of action of the plant extract. This could be done by determining its effect on nitric oxide synthesis and on phosphodiesterase activity via cGMP concentration.

The aim of this research was to determine the effect of ethanolic extract of *Phyllanthus amarus* on plasma Nitric Oxide (NO) and the concentration of cGMP in corpus cavernosum.

MATERIALS AND METHODS

Fresh *Phyllanthus amarus* plants were collected in August 2009 within the premises of the College of Medicine, University of Lagos Idi-araba. The plant was identified and authenticated at the Department of Botany, Faculty of Sciences, University of Lagos, Akoka, Lagos State. The whole plant was washed and oven dried at 40°C. The dried plant was pulverized with an electric blender (Blender/Miller III model MS-223, China). The powdered plant was then extracted with 96% (v/v) ethanol, by soaking 100 g of dried plant in 1 L of ethanol for 48 h at room temperature with constant shaking. The suspension was filtered with muslin cloth and resulting filtrate was then concentrated using a rotary evaporator (Stuart, RE300). The extract was finally concentrated in a thermo regulated water bath at 20°C for 24 h.

The concentrate was reconstituted in physiological saline to give different required doses of 100, 200 and 400 mg kg⁻¹ body weight. The reconstituted extract was then administered to all animals in different groups.

Phytochemical screening: Phytochemical tests were carried out on the ground plant using standard procedures to identify the constituents as described by Sofowara (1993).

Animal grouping and extract administration: A total of thirty (30) male Guinea pigs weighing between 220 and 600 g were obtained from University of Agriculture Abeokuta, Ogun State, Nigeria and were allowed to acclimatise for 30 days before the experiment. They were housed in aerated plastic cages of five animals per cage and were adequately fed normal animal chow throughout the experiment *ad libitum*. At the end of the acclimatisation period, only the twenty-five of the twenty-seven that survived were used.

The animals were randomly assigned into different groups; A, B, C, D and E of five animals each. Physiological saline, commercially acquired Sildenafil Citrate (HAB Pharmaceuticals and Research Ltd) which serve as controls and the reconstituted extract were administered orally using a rubber dropper as follow:

Group 1: Control (1mL physiological saline).

Group 2: 1 mL of sildenafil citrate corresponding to 100 mg kg⁻¹ body weight.

Group 3: 1 mL of the extract corresponding to 100 mg kg⁻¹ body weight.

Group 4: 1 mL of the extract corresponding to 200 mg kg⁻¹ body weight.

Group 5: 1 mL of the extract corresponding to 400 mg kg⁻¹ body weight.

Collection of samples: The animals were anaesthetized by injecting 5 mg kg⁻¹ body weight ketamin intraperitorially. The animals were quickly dissected and cardiac blood was collected into a heparinized bottle. The penile tissue was harvested and immediately frozen in liquid nitrogen and stored at -20°C pending cGMP analysis. The blood was transferred into centrifuge bottles and centrifuged at 400 x g for 15 min to get the plasma which was used for nitric oxide assay.

Determination of penile tissue (corpus cavernosum) cGMP: Frozen penile tissue samples were homogenized placed on an ice bath with 5 mL of 0.1 M HCl in a glass mortar. Centrifugation was done at 5,000 rpm for 10 min at room temperature and the supernatant was collected for quantitative immunoassay of cGMP concentration according to general principle of ELISA technique and the manufacturer's instructions (#k372-100, Biovision Research Products, USA). The kit utilizes the recombinant Protein G coated plate to anchor cGMP polyclonal antibody. cGMP-HRP conjugates directly competes with cGMP from the samples for binding to the cGMP specific antibody on the plate. After incubation and washing, the amount of cGMP-HRP bound to plate was determined by reading the optical density at 450 nm on a micorplate autoreader (Bio-Tek Instruments EL311). The absorbance is inversely proportional to the concentration of cGMP in the samples.

Determination of nitric oxide: Nitric Oxide (NO) is rapidly oxidized to nitrite and nitrate which are used to determine NO production. BioVision's Nitric Oxide Colorimetric Assay Kit (#K262-200, BioVision Research Products, USA) was used to measure the total nitrate/nitrite in the samples.

Statistical analysis: All data are presented as the Mean±SEM. Data were analysed by analysis of variance (ANOVA) followed by inspection of all differences by Tukey test for concentration of cGMP, NO and penile/body weight ratio. Mean weight were compared paired t-test. p>0.05 was considered statistically significant, statistical analysis was done using graph pad prism 5 statistical software.

RESULTS AND DISCUSSION

Phytochemical composition analysis of *Phyllanthus amarus* showed the presence of flavonoids, tannins, saponins, alkaloids, terpenoids, steroids and cardiac glycosides while resin was absent (Table 1).

Table 1: The phytochemical constituent of *Phyllanthus amarus*

Phytochemicals	<i>Phyllanthus amarus</i>
Alkaloids	+
Flavonoids	+
Terpenoids	+
Saponins	+
Tannins	+
Steroid	+
Resins	-
Cardiac glycosides	+

+: Presence of the phytochemical; -: Absence of the phytochemical

Table 2: Body weight of animals before and after administration of ethanol extract of *Phyllanthus amarus* and penile/body weight ratio after administration

Groupings	Weight before (g)	Weight after (g)	Penile/body weight ratio
Saline (group 1)	210.00±14.14	210.00±14.14	0.001550±0.000173 ^a
Sildenafil (group 2)	536.66±51.32	537.33±52.17	0.002300±0.000436 ^b
100 mg kg ⁻¹ body weight (group 3)	330.00±20.00	330.00±20.00	0.001280±0.000179 ^a
200 mg kg ⁻¹ body weight (group 4)	366.67±20.00	370.67±14.73	0.001560±0.000288 ^a
400 mg kg ⁻¹ body weight (group 5)	430.00±30.00	431.67±28.54	0.002240±0.000378 ^b

Value are Mean±SD. Values with same superscript are not significant within columns for penile/body weight ratio at p>0.05. Paired comparison was done for body weight

The mean weight difference of animals at day zero (before administration) and day ten (after administration) of plant extract and controls was not significant (p>0.05) (Table 2).

There was a significant difference (p<0.05) when the penile/body weight ratio of animals in groups 1 (saline), 3 (100 mg kg⁻¹) and 4 (200 mg kg⁻¹) were compared to groups 2 (Sildenafil) and 5 (400 mg kg⁻¹) but there was no difference (p>0.05) when group 1 was compared with groups 3 and 4 (Table 2).

Figure 1 shows a dose dependent increase in the concentration of cGMP in groups administered the ethanol plant extract. The statistical difference in the concentration of cGMP in animals administered lower doses (100 and 200 mg kg⁻¹) of the plant extract was not significant when compared with those administered physiological saline but significant compared with those administered sildenafil. Also, the difference in the level of cGMP was significant in the groups administered the lower doses and physiological saline when compared to the group administered the highest dose (400 mg kg⁻¹). However, the difference between the 400 mg kg⁻¹ group was significant compared to the sildenafil group.

The concentration of Nitric Oxide (NO) though highest in group 1, showed no significant difference when compared with all the other four groups. Also there was no significant difference when the level of NO was compared within groups.

The use of herbs is very common in developing countries, particularly in rural settings. However, during the last decade an increase in the use of plants has been observed in metropolitan areas of developed countries (Harnack *et al.*, 2001).

Sexual difficulties are extremely prevalent among both men and women. They are associated with a number of biological, medical and psychological risk factors and increase markedly with aging (Leiblum, 1999). The phytochemicals present in *Phyllanthus amarus* include: flavonoids, tannins, saponins, alkaloids, terpenoids, steroids and cardiac glycosides (Table 1). Flavonoids have been shown to possess many pharmacological properties such as anti-oxidant activities, anti-inflammatory activities, anti-cancer activities and anti-microbial effects hence, flavonoids may have a contributory effect to the fertility properties and other pharmacological effects of the plant (Adeneye *et al.*, 2006).

Etta (2008) reported a decrease in litter size and weight of albino rats intraperitoneally administered the ethanolic extract of the whole plant. This is in contrast with the result gotten from this research, which showed no significant difference in the weights of animals after 10 days of administration of the plant extract, (p>0.05) for all the treatment groups (Table 2). This is an indication that the ethanolic plant extract does not have any adverse effect on the weight of adult animals.

Improving sexual desire and function is possible using plant-based medicines that improve the activity of the male glandular system, improve the blood supply to erectile tissue and enhance the transmission or stimulation of the nerve signal. A number of natural agents occur to boost sexual function and could be appropriately described also as aphrodisiacs. Table 2 shows the body weight per penile weight ratio after administration of the plant extract. There was no significant difference in the penile weight per body weight ratio in animals administered physiological saline and those administered lower doses of the plant extract (100 and 200 mg kg⁻¹) but when compared with group2 (sildenafil) and group5 (400 mg kg⁻¹), the difference in body weight per penile weight ratio of animals in group1 was significant. Also, the body weight per penile weight ratio of animals in groups 3 and 4 were significant when compared to those in groups 2 and 4 (p<0.05). Preoccupation with penile size is a common condition in the male population. Penile length, circumference and/or geometry may also contribute to, or be a consequence of, medical treatment of erectile dysfunction (Shamloul, 2005). A higher penile/body weight ratio is an indication of the plant extract being a treatment for erectile dysfunction.

The discovery that penile erection is dependent on nitrergic neurons and subject to modulation by zaprinast (Rajfer *et al.*, 1992) promoted the development of selective PDE 5 inhibitors. At present selective inhibitors of PDE 5 (Sildenafil, Tadalafil and Vardenafil) is use for treatment of male erectile dysfunction and they do this by increasing cellular content of cGMP (Eardley and Cartledge, 2002; Francis and Corbin, 2003). Sildenafil citrate is a useful drug for the treatment of Erectile Dysfunction (ED) because it selectively inhibits phosphodiesterase type 5 (PDE-5) which inactivates cyclic guanosine monophosphate (cGMP), the mediator of smooth muscle relaxation in the corpus cavernosum. By selectively inhibiting cGMP catabolism in cavernosal smooth-muscle cells, sildenafil citrate can restore the natural erectile response to sexual stimulation without causing erections in the absence of such stimulation. Sildenafil citrate is rapidly absorbed, with maximal plasma concentrations occurring within 1hr after oral administration and a mean terminal half-life of 3 to 5 h (Goldstein, 1988).

There was a dose dependent increase in the concentration of cGMP in the three study groups fed with the extract. The levels of cGMP in animals in groups 3 and 4 though higher compared to the levels in group1, the difference was not significant (p>0.05). The cGMP concentration in the control (group 1) was significantly different (p<0.05) when compared with groups 2 and 5. Also the difference in the concentration of cGMP in animals fed with lower doses of *P. amarus* extract (groups 3 and 4) was significant when compared with groups 2 and 5. There was a significant difference in the cGMP concentration between the sildenafil group and 400 mg kg⁻¹ body weight group (group 5) (Fig. 1). This might be an indication that the extract has an effect directly or indirectly on muscle cGMP but the effect could only be noticed at higher concentration.

Hallen *et al.* (2001) reported that by quantifying the neuronal release of Nitric oxide from guinea pig colon and rabbit corpus cavernosum, the selective PDE 5 inhibitors altered the nerve-induced release of NO. On the other hand, Ibrahim *et al.* (2004), concluded that the PDE 5 inhibitor zaprinast has no regulatory effect on the NO-release in serum and aortic tissue though the mean arterial pressure was lowered.

The result showed the ethanolic plant extract did not have effect on the level of Nitric Oxide (NO). Although the concentration of NO was lower compared to the control, the differences were not significant (Fig. 2). PDE 5 inhibitors have been reported to have a great tendency to regulate the level of nitric oxide through its inhibitory action on nitric oxide synthase (NOs). Application of sildenafil, tadalafil and vardenafil all decreased the nerve-induced release of NO. This is an

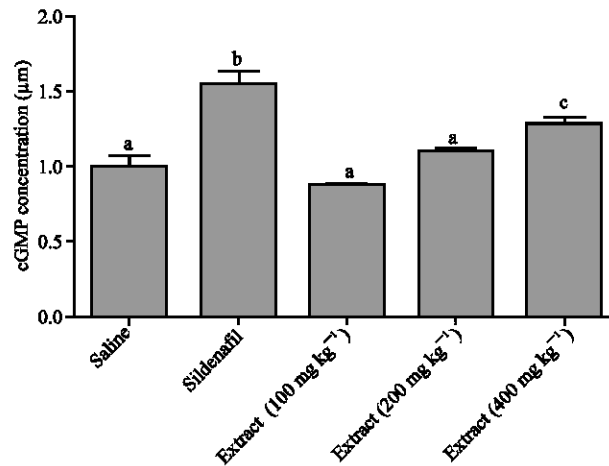


Fig. 1: Cyclic guanosine mono-phosphate (cGMP) concentrations in adult male guinea pigs administered *Phyllanthus amarus* ethanol extract. Control (physiological saline) (n = 5), sildenafil (n = 5), 100 mg kg⁻¹ (n = 5), 200 mg kg⁻¹ (n = 5), 400 mg kg⁻¹ (n = 5). Groups with same superscript are not statistically different.

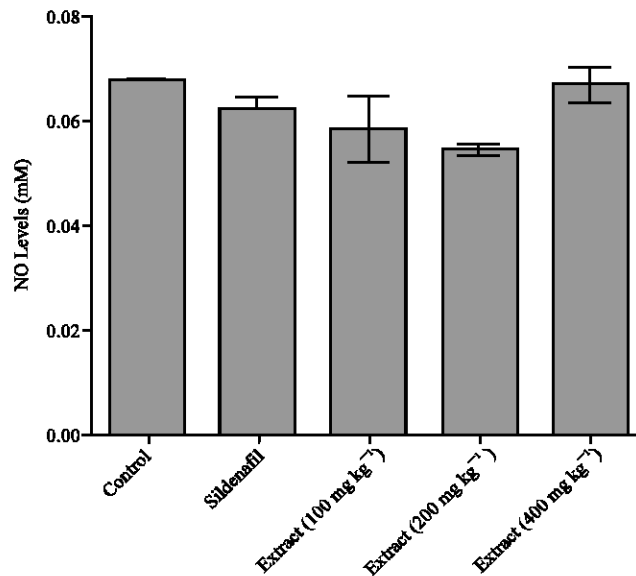


Fig. 2: Nitric Oxide (NO) concentrations in adult male guinea pigs. Control (physiological saline, n = 5), sildenafil (n = 5), 100 mg kg⁻¹ (n = 5), 200 mg kg⁻¹ (n = 5), 400 mg kg⁻¹ (n = 5)

indication that PDE 5 influences the formation and release of NO during stimulation in the cavernous tissue. It was suggested that accumulated cGMP reduces NO by phosphorylating nNOS by protein kinase A or protein kinase G (Dinerman *et al.*, 1994). Though present result shows no significant difference in concentration of NO in studied groups compared to the control, the concentration was still reduced by the plant extract. This is an indication that higher concentration of the extract might lead to accumulation of cGMP which will lead to reduction of NO.

The diminished release of NO suspected to be mediated by the plant extract might likely be of significant physiological and pharmacological importance. Burnett (2003) reported that if selective PDE 5 inhibitors increases endogenously formed NO, it would start a positive feedback circle, which might ultimately have induced priapism. However since the selective PDE 5 inhibitors diminished the formation of NO, NO on its own formation prevents priapism through negative feedback either via cGMP-dependent phosphorylation of NOs or via NO-inhibition of NOs.

CONCLUSION

Ethanol extracts of *Phyllanthus amarus* may have positive penile erection property as shown by its ability to increase the cGMP level in penile tissue of male guinea pigs. The increase in level of cGMP is dose dependent and the action of ethanol extract of the plant is probably through the inhibition of phosphodiesterase 5 an enzyme known to break down cGMP in corpus cavernosum in male penile tissue. The ethanol extract of the plant shows no significant effect on the level of plasma nitric oxide.

However, more research need to be carried out in confirming the plant as a source of a phosphodiesterase inhibitor, isolating the actual active ingredient (s) responsible for this action and determining its actual effect on nitric oxide.

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