Investigating the Erectogenic Effects of γ-Aminobutyric Acid in Sildenafil: A Pharmacological Study

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ABSTRACT

Erectile dysfunction (ED) is a disorder of increasing socio-economic burden. Therapeutic drugs such as sildenafil have been in use for the treatment of ED, but with their associated side effects. γ-aminobutyric acid (GABA) is a neurotransmitter with possible vasodilatory properties. In this study, the effect of GABA on the erectogenic properties of sildenafil was investigated. Aqueous solution of GABA and sildenafil (1 mM) was separately prepared as well as the mixtures of both (75% GABA + 25% sildenafil; 50% GABA + 50% sildenafil; 25% GABA + 75% sildenafil). Thereafter, the *in vitro* effects of all the studied samples on the activities of arginase, angiotensin-I converting enzyme (ACE) and acetylcholinesterase (AChE) were investigated. The results revealed that all the samples inhibited arginase, ACE and AChE activities. Considering the various combinations, 25% GABA + 75% sildenafil had the highest arginase inhibitory effect, 50% GABA + 50% sildenafil showed the highest ACE inhibiting effect, while 25% GABA + 75% sildenafil exhibited the highest AChE inhibitory effect. Therefore, the observed enzyme inhibiting effect of sildenafil, GABA and their various combinations on rat penile arginase, ACE and AChE activities could be part of the mechanism by which they elicit their erectogenic properties. The various combinations could thus serve as therapeutic intervention for the management of ED with a possible reduction in the side effects associated with the use of sildenafil; nevertheless, the combination of 75% GABA with 25% sildenafil exhibited the highest erectogenic potential.

Keyword: Erectile dysfunction; γ-aminobutyric acid; sildenafil; arginase; ACE, AChE.

INTRODUCTION

Penile erection is a multifactorial process involving interactions between neural, psychological, vascular, and hormonal factors. The function of normal sexual activity in males consists of four stages: sexual desire, erection, ejaculation (i.e., orgasm), and detumescence (penile flaccidity)[1]. The process of erection cycle is initiated by sexual stimulation and subsides at ejaculation or cessation of sexual stimulation. In Western countries, at least one in ten men is affected by erectile dysfunction (ED), which is defined as the inability to maintain penile erection sufficient to permit satisfactory sexual intercourse[2]. It has been estimated that about 30 million men in the USA or half a million men in the UK have partial or complete ED[3]. The mechanism of penile erection involves responses to external sensory stimuli through parasympathetic activity, which leads to release of nitric oxide (NO) from nonadrenergic-non-cholinergic (NANC) cavernous (penile) nerve endings and the endothelium of the penile tissue. The decrease in NO bioavailability in endothelial dysfunction may be caused by reductions in the enzyme activity of the endothelial NO synthase (eNOS). This decline in eNOS activity may be as a result of lack of substrate (arginine), increase in arginase activity, **Author for Correspondence: saadefegha@futa.edu.ng* degradation of NO by reactive oxygen species (ROS), such as superoxide anion and/or alterations in intracellular signalling molecules such as acetylcholine (ACh), such that eNOS is not appropriately activated or uncoupled[4] It is feasible to regulate NO biosynthesis in both endothelial and smooth muscle cells of the penile tissues by controlling the availability of arginine for eNOS activity via regulation of arginase (arginine catabolizing enzyme) activity. Inhibition of arginase is associated with enhanced NANC and endotheliumdependent vaso-relaxation of human corpus cavernosum smooth muscle, suggesting that inhibition of arginase could increase NO biosynthesis through a NOS dependent manner[5].

Furthermore, accumulated evidences suggest that, in the ED victim, the presence of acetylcholinesterase (AChE) activity is higher than normal, which consequently generates ROS and causes lipid peroxidation in neuronal cell membranes[6]. Consequently, this brings about endothelial dysfunction, which is implicated in pathogenesis of ED. ACh is a pro-erectile neurotransmitter that stimulates endothelium-dependent relaxation of penile smooth muscles, thus favouring penile erection[7]. Therefore, inhibition of excessive AChE activity characteristic in ED can be a therapeutic target in managing this condition.

Sildenafil is a synthetic drug that use in protecting cyclic guanosine monophosphate (cGMP) from degradation by cGMP-specific phosphodiesterase type-5 (PDE-5) in the corpus cavernosum of the penile tissue. NO in the corpus cavernosum of the penile tissue binds to guanylate cyclase receptors, which results in increased levels of cGMP, leading to smooth muscle relaxation
(vasodilation) of the intimal cushions of the helicine arteries[8]. This smooth muscle relaxation leads to vasodilation and increased inflow of blood into the spongy

tissue of the penile tissue, causing an erection[8]. Sildenafil is a potent and selective inhibitor of cGMPspecific PDE-5, having a similar molecular structure with cGMP and acts as a competitive binding agent of PDE-5 in the corpus cavernosum, resulting in more cGMP concentration and better erections[9].

Gamma-aminobutyric-acid (GABA) is an inhibitory neurotransmitter; a four-carbon non-protein amino acid that was discovered about half a century ago in plants such as tomatoes[10-12], and was also found in great quantity in the brain. GABA receptors are found throughout the human body, including the brain and central nervous system, liver, sperm, testes etc.[13-15]. Although, the fact that only a small amount of GABA passes through the blood-brain barrier, still orally ingested GABA seems to bring many health benefits such as lowering hypertension, achieving relaxation and restful sleep, balance the brain and prevents nerve cell overfiring[16]. It is quite often useful in cases of sexual dysfunction via alleviation of anxiety that is often one of the real culprits in problems involving sexual performance[17]. GABA also helps increase the level of dopamine (feel good hormone) present in the system and encourages a feeling of euphoria[18]. Additionally, it has been discovered to increase sensitivity during sexual activity and to help men with such problems as ED and premature ejaculation[19,20].

ED is a health challenge with a high global socioeconomic burden that requires more scientific interventions. Sildenafil is a therapeutic drug that is being used for the treatment of ED; it exerts its erectogenic properties by inhibiting phosphodiesterase-5 and thus, potentiates the release of the vasodilator-nitric oxide. However, it comes with numerous side effects. GABA is an inhibitory neurotransmitter that is responsible for regulating sexual tone, reduces anxiety and improves sexual functioning. However, the interaction of GABA with major enzyme systems relevant to ED has been largely understudied; knowledge of these interactions could elucidate the role of GABA in promoting the proper sexual functioning and especially its erectogenic properties. This study investigated GABA activity on the arginase, angiotensin-I converting enzyme (ACE) and acetylcholinesterase inhibitory as well as antioxidant
properties of sildenafil

MATERIALS AND METHODS

Reagents

The sildenafil citrate tablet was sourced from Signature pharmaceutical limited, Mumbai-India. Gamma aminobutyric acid (GABA) was sourced from Sigma Al-drich Co. (St Louis, Missouri, USA). All chemicals used in this study were of analytical grade and glass-distilled water was used. *Preparation of samples*

One millimolar (1 mM) concentration of GABA and sildenafil were prepared separately. Different combinations of GABA and sildenafil were thereafter prepared thus:

Sample A – 100% GABA
Sample B – 75% GABA and 25% sildenafil
Sample C – 50% GABA and 50% sildenafil
Sample D – 25% GABA and 75% sildenafil
Sample E – 100% sildenafil

Experimental Animals

Twenty-five adult male Wistar rats weighing 180-190 g used for this experiment were purchased from the animal breeding colony of Animal production and health (APH) Department, Federal University of Technology, Akure, Nigeria and were handled in line with Guide for Care and Use of Laboratory Animals prepared by the National Academy of Science, USA. They were acclimatized for two weeks with free access to standard animal feed and water ad libitum and maintained at room temperature on a 12 h light/dark cycle with free access to food and water. They were acclimatized under this condition for two weeks prior to the commencement of the experiments.

Preparation of tissue homogenate

The rats were sacrificed by cervical dislocation and rapidly dissected. Penile tissue (corpora cavernosa) was removed and homogenized with an appropriate buffer solution. The supernatant was prepared by centrifuging the homogenate for about 20 min at 4,000 r.p.m in a Kenxin refrigerated centrifuge Model KX3400C (KENXIN Intl. Co., Hong Kong).

Arginase inhibition assay

Arginase inhibition was determined using the method of Kaysen and Strecker[21], by the measurement of urea produced by the reaction of Ehrlich's reagent. The reaction mixture contained in final concentration 1.0 mM Tris-HCl buffer, pH 9.5 containing 1.0 mM MnCl, 0.1 M arginine solution and 50 µL of the enzyme preparation in a final volume of 1.0 mL. The mixture was incubated for 10 min at 37°C. The reaction was terminated by the addition of 2.5 mL Erhlich reagent (2.0 g of p-dimethyl amino benzaldehyde in 20.0 mL of concentrated hydrochloric acid and made up to 100 mL with distilled water). The absorbance reading was taken after 20 min at 450 nm. The control experiment was performed without the test extracts and the arginase inhibitory activity was expressed as percentage inhibition.

$\%Inhibition = [(ABS_{ref} - ABS_{Samples})/ABS_{ref}] \times 100$ (1) where ABS_{ref} = Absorbance of reference; ABS_{sample}= Absorbance of sample

Angiotensin I converting enzyme (ACE) inhibition assay

ACE inhibition was determined by the method of Cushman and Cheung[22]. Appropriate dilution of the sample (50 µL) and ACE solution (50 µL, 4 mU) was incubated at 37°C for 15 min. The enzymatic reaction was initiated by adding 150 µL of 8.33 mM of the substrate (Bz-Gly-His-Leu) in 125 mM Tris-HCL buffer (pH 8.3) to the mixture. After incubation for 30 min, at 37°C, the action was arrested by adding 250 µL of 1 M
HCL. The Gly-His bond was then cleaved. And the BzGly produced by the reaction was extracted with 1.5 mL ethyl acetate. Thereafter, the mixture was centrifuged to separate the ethyl acetate layer; then the 1 mL of the acetate layer was transferred to a clean test tube and evaporated. The residue was re-dissolved in distilled water and its absorbance was measured at 228 nm. The ACE inhibitory activity was expressed as percentage inhibition (Eq. 1).

Acetylcholinesterase (AChE) Inhibition Assay

Inhibition of acetylcholinesterase (AChE) was assessed by a modified colorimetric method by Perry et al[23]. The AChE activity was determined in a reaction mixture containing 200 µL of AChE solution (EC 3.1.1.7) in 0.1 M phosphate buffer, pH 8.0, 100 µL of a solution of 5 ʾ5– dithio–bis-2-nitrobenzoicacid (DTNB) 3.3 mM in 0.1 M phosphate buffered solution, pH 7.0, containing NaHCO3 6 mM, extracts (0–100 µL) and 500 µL of phosphate buffered, pH 8.0. After incubation for 20 min at 25^0C acetylthiocholine iodide was added as the substrate, and AChE activity was determined by UV spectrophotometry from the absorbance changes at 412nm. The AChE inhibitory activity was expressed as percentage inhibition (calculation as shown in equation 1).

Lipid peroxidation and thiobarbituric acid reactions The lipid peroxidation assay was carried out using a modified method of Ademosun et al[24]. Briefly, 100 µL of the tissue was mixed with a reaction mixture containing 30 µL of 0.1 M Tris-HCL buffer (pH 7.4), samples (0100 µL) and 30 µl of 250 µM freshly prepared F_eso_4. The volume was made up to 300 µl with distilled water before incubation at 37°C for 1 h. Subsequently, 300 µL of 8.1% sodium dodecyl sulphate (SDS), 500 µL of acetic acid/HCL buffer (pH 3.4) and 500 µL of 0.8% thiobarbituric acid (TBA) were added to the reacting mixture. This mixture was incubated at 100°C for 1 h and thiobarbituric acid reactive species (TBARS) produced were measured at 532 nm using a spectrophotometer and TBARS produced was reported as MDA equivalent. *Fe^{2+} chelation assay*

The Fe^{2+} chelating ability of the samples was determined using a modified method of Adefegha et al[25]. Freshly prepared 500 µL 0.1 M Tris-HCL (pH 7.4), 218 µL saline and the samples (0 - 25 µL). The reaction mixture was incubated for 5 min, before the addition of 13 µL 0.25% 1, 10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in a spectrophotometer. The Fe^{2+} chelating abilities of the samples were subsequently calculated (calculation as shown in equation 1).

Data Analysis

Results of triplicate experiments were pooled and expressed as mean ± standard deviation (SD) and the mean was compared using a student T test and one-way analysis of variance (ANOVA) appropriately using statistical package for social science (SPSS) 16.0 for windows. The significance level was taken at $p < 0.05$ and the IC_{50} (sample concentration causing 50% enzyme inhibition) was determined using non-linear regression analysis[26].

RESULTS

The arginase inhibitory acts of GABA and sildenafil were investigated and the results are presented in Fig. 1, which shows that the samples inhibited arginase activity in a concentration dependent manner (0 - 30 µM). However, considering the IC_{50} (Table 1) values, GABA had a significantly higher ($P < 0.05$) arginase inhibiting activity (9.09 µM) than sildenafil (23.34 µM). Furthermore, the effect of GABA and Sildenafil combined in different proportions on the activity of arginase was also presented in Fig. 2. This showed that the different combinations of GABA and sildenafil inhibited the activity of arginase; nevertheless, the combination of 25% GABA and 75% sildenafil had the highest significant ($p < 0.05$) inhibitory effect.

Also, the ACE inhibitory effects of the sample were also studied and the result presented in Fig 3. This shows that both GABA and sildenafil inhibited ACE activity concentration dependently (0 - 30 µM). The IC_{50} (Table 2) revealed that GABA has a significantly higher ($p<0.05$) ACE inhibiting activity (18.56 µM) than sildenafil (30.16 µM). In addition, the effect of GABA and sildenafil combined in different proportions on the activity of arginase was also presented in Fig 4. This showed that the different combinations of GABA and sildenafil inhibited the activity of arginase; nevertheless, the combination of 50% GABA and 50% sildenafil had the highest significant ($P < 0.05$) inhibitory effect.

Furthermore, the AChE inhibitory effect of the sample was also studied and the result presented in Fig 5; both samples inhibited AChE activity in a concentration dependent manner (0 - 30 µM). However, judging by the IC_{50} (Table 1), GABA also has a significantly higher ($P < 0.05$) inhibitory effect (71.63 µM) than sildenafil (82.76 µM). The various combinations of GABA and sildenafil also showed AChE inhibitory effects (Fig 6); nonetheless, the combinations of 50% GABA and 50% sildenafil, as well as 25% GABA and 75% sildenafil showed the highest significance ($P < 0.05$) AChE inhibitory effects. Fig. 7 revealed the result of the Fe^{2+} chelating abilities of the samples. The result revealed that GABA, sildenafil and their various combinations exhibited Fe^{2+} chelating abilities. It could be observed from the result that 100% GABA had significantly higher ($P < 0.05$) chelating ability (36.99%) than 100% sildenafil (2.06%). Furthermore, considering the various combinations, 75% GABA combined with 25% sildenafil exhibited the highest significant ($P < 0.05$) chelating ability (37.00%) which was also not significantly higher ($P > 0.05$) than 100% GABA.

Incubation of Fe^{2+} with pancreas homogenate caused a significant ($P < 0.05$) increase (175%) in the malondialdehyde (MDA) content (Fig 8). However, the introduction of the samples inhibited lipid peroxidation in the penile tissue homogenate by causing a significant ($P < 0.05$) reduction in the MDA content; 100% GABA showed a significant ($P < 0.05$) higher inhibitory effect (75%) than 100% sildenafil (50%). However, considering the combinations, no significant difference ($P > 0.05$) was observed in their ability to cause reduction in the MDA content.

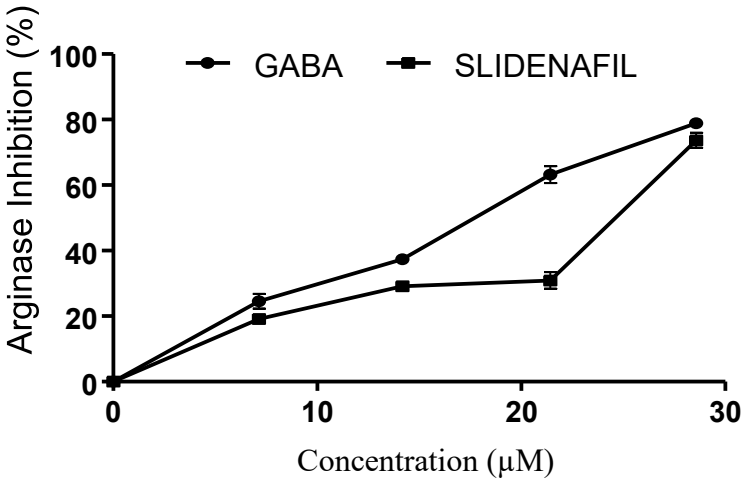

Figure 1: Arginase Inhibitory Effect (%) of GABA and Sildenafil.

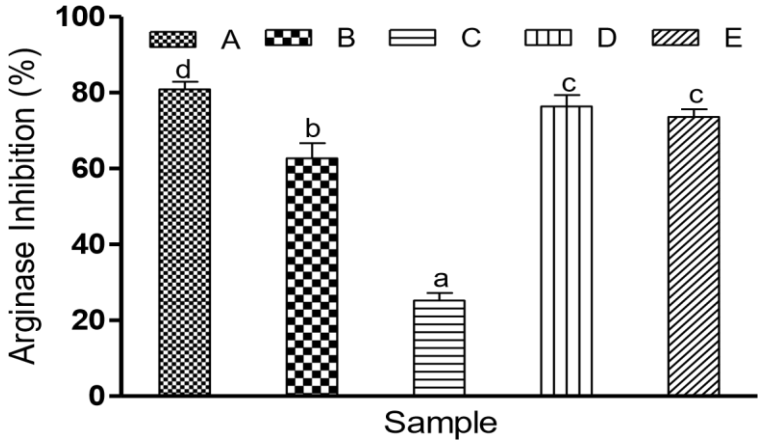

Figure 2: Effect of GABA on the Arginase Inhibitory Effect of Sildenafil

Bars represent mean ± standard deviation of duplicate readings. Bars with different letters are significantly different at $p<0.05$

KEY:

A= 100% GABA
B= 75% GABA + 25 % Sildenafil
C= 50% GABA + 50 % Sildenafil
D= 25% GABA + 75 % Sildenafil
E = 100% Sildenafil

DISCUSSIONS

This study showed that sildenafil, GABA and their various combinations exhibited arginase inhibitory properties. NO, which is synthesized by NO synthase (NOS) from L-arginine modulates penile erection[27]. However, arginase which is not only present in the liver, but also in human corpus cavernosum of the penile tissue, competes with NOS for L-arginine; and catalyses the breakdown of L-arginine to L-ornithine and urea[5,28]. Hence, the inhibition of arginase activity has been suggested as a practical therapeutic means for boosting Larginine content as a substrate for eNOS and ensuring its availability for eNOS. Therefore, the ability of sildenafil, GABA and their various combinations to inhibit arginase activity could be one of the mechanisms behind their erectogenic properties. Interestingly, of the various combinations understudied, the combination of 25% GABA and 75% sildenafil had the highest significant ($P < 0.05$) inhibitory effect.

Emerging evidence suggests that ED is a vascular disease in nature and several clinical reports have revealed a strong correlation between ED and cardiovascular disease

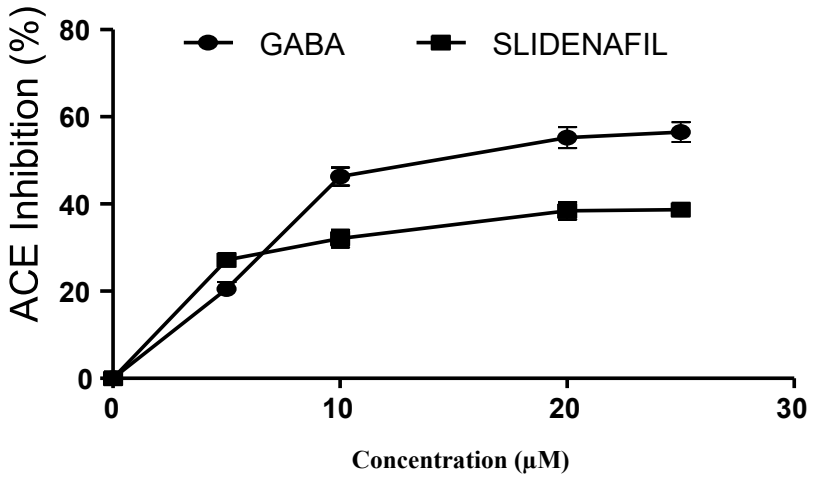

Figure 3: ACE Inhibitory effect (%) of GABA and Sildenafil.

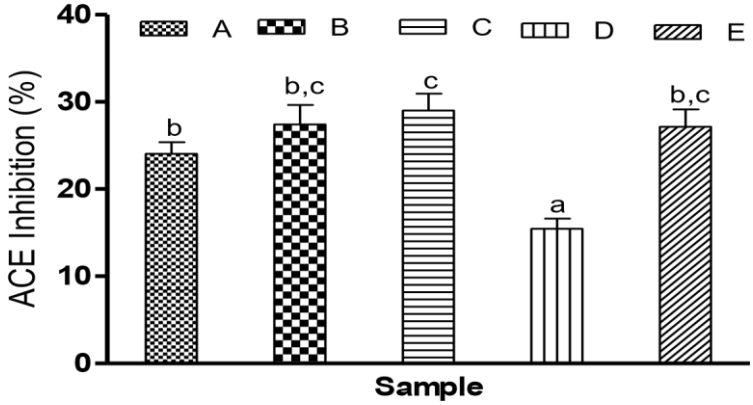

Figure 4: Effect of GABA on the ACE inhibitory effect of Sildenafil. Bars represent mean ± standard deviation of duplicate readings. Bars with different letters are significantly different at $p<0.05$.

KEY:
A= 100% GABA
B= 75% GABA + 25 % Sildenafil
C= 50% GABA + 50 % Sildenafil
D= 25% GABA + 75 % Sildenafil
E = 100% Sildenafil

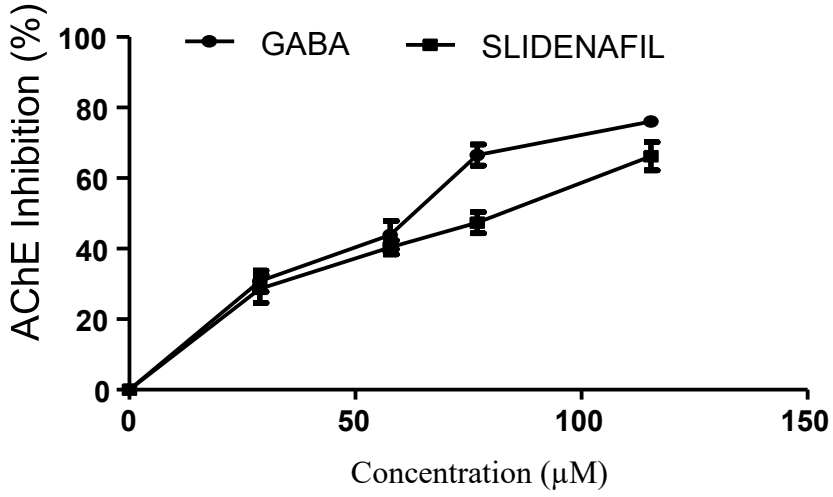

Figure 5: AChE inhibitory effect (%) of GABA and Sildenafil.

(CVD)[29]. CVDs are associated with possible dysfunction of the endothelial system; and dysfunctional endothelium is a critical factor in the development of ED[30]. Hence, mechanism of erectile function will require a sensitive balance between vasodilators and vasoconstrictors agents, and any modification or impairment in the endothelial function may contribute to ED. Critical to the regulation of vascular tone is the renin-angiotensin system (RAS) where production of angiotensin-II by angiotensin-I converting enzyme (ACE) elicits potent vasoconstrictory effect and there is evidence that a local RAS exists within the corpus cavernosum[31]. Thus, ACE inhibitors may improve erectile function by reducing angiotensin II production, attenuate the degradation of bradykinin; a known activator of NO release from NOS and subsequent relaxation of the corpus cavernosum[28]. A study has shown that captopril (a known ACE inhibitor) improved erectile function of hypertensive rats[31]. Hence, ACE inhibitors are expected to be beneficial on erectile function, based on their effects on the pathobiologic process of ED[33].

The result of this study showed that GABA, sildenafil and their various combinations inhibited ACE activity. The observed ACE inhibitory effect of these samples could be an indication of their modulatory effect on vascular tone which could be beneficial in ED management/treatment. In addition, of the different combinations of GABA and slinderfil understudied, the combination of 50% GABA and 50% sildenafil had the highest significant ($P < 0.05$) inhibitory effect. This could be as a result of interactions between these two therapeutic agents and could thus offer erectogenic properties while at the same time, possibly reducing the side effects of sildenafil.

The significance of parasympathetic nerves in maintaining penile tone is well established[34]. Studies have shown that penile tissues from humans and several animal species are rich in cholinergic nerves[35.36], where ACh released from these nerves acts as a neurotransmitter; ACh causes endothelium-dependent relaxation of the corpus cavernosum, penile arteries, and circumflex and dorsal veins in vitro[30]. Hence, the relaxation induced by ACh can be produced either by inhibition of the release of contraction factors such as noradrenaline and/or by the release of relaxation-producing factors (e.g., NO)[37]. It was observed in this study that the samples inhibited AChE activity in rat penile tissue homogenate. The role of ACh at central levels in the regulation of penile erection is mostly inferred from limited neuropharmacologic studies involving systemically and/or intracerebrally administered muscarinic agonists and antagonists and from lesioning studies in the brain[3841]. These studies have suggested that cholinergic mechanisms operating seemingly at the hippocampus

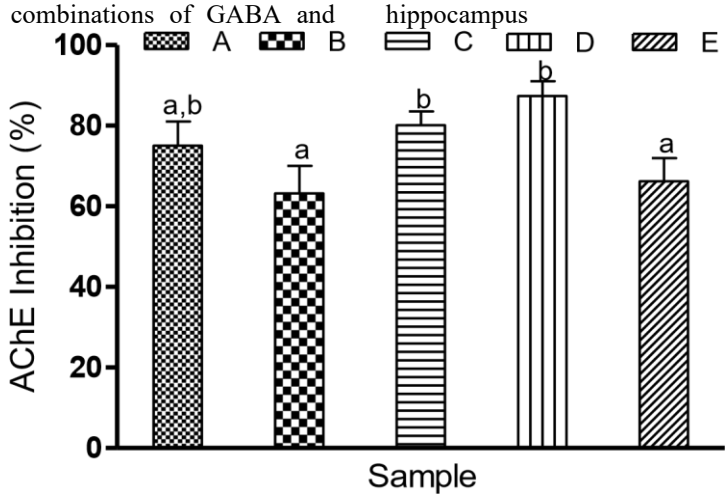

Figure 6: Effect of GABA on the AChE inhibitory effect of Sildenafil. Bars represent mean ± standard deviation of duplicate readings. Bars with different letters are significantly different at $P < 0.05$.

KEY:
A= 100% GABA
B= 75% GABA + 25 % Sildenafil
C= 50% GABA + 50 % Sildenafil
D= 25% GABA + 75 % Sildenafil
E = 100% Sildenafil

Table 1:IC_{50} values (µM) for the inhibitory effects of GABA and Sildenafil on arginase, ACE and AChE activities.

	Arginase	ACE	AChE
GABA	15.98±0.43[a]	16.14±1.66[a]	56.29±2.93[a]
Sildenafil	22.83±0.42[b]	88.17±4.19[b]	74.34±2.83[b]

Values represent mean ± standard deviation (n=3).
Values with different superscript letter are significantly different at $p<0.05$

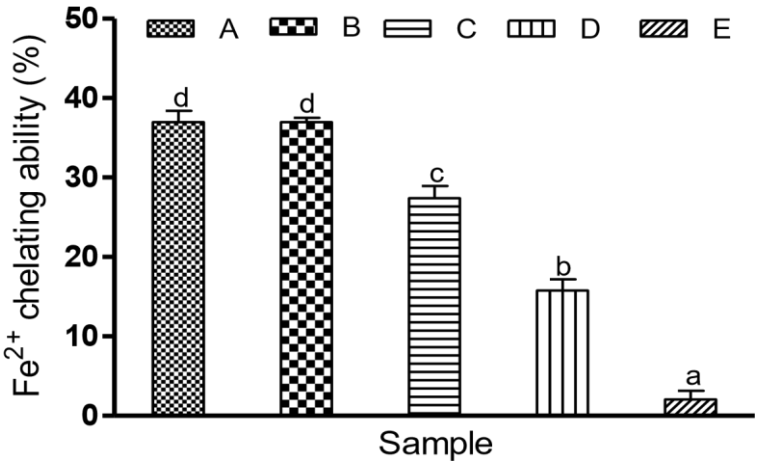

Figure 7: Effect of GABA on the Fe^{2+} chelating ability of Sildenafil
Bars represent mean ± standard deviation of duplicate readings. Bars with different letters are significantly different at $p<0.05$.

KEY:
A= 100% GABA
B= 75% GABA + 25 % Sildenafil
C= 50% GABA + 50 % Sildenafil
D= 25% GABA + 75 % Sildenafil
E = 100% Sildenafil

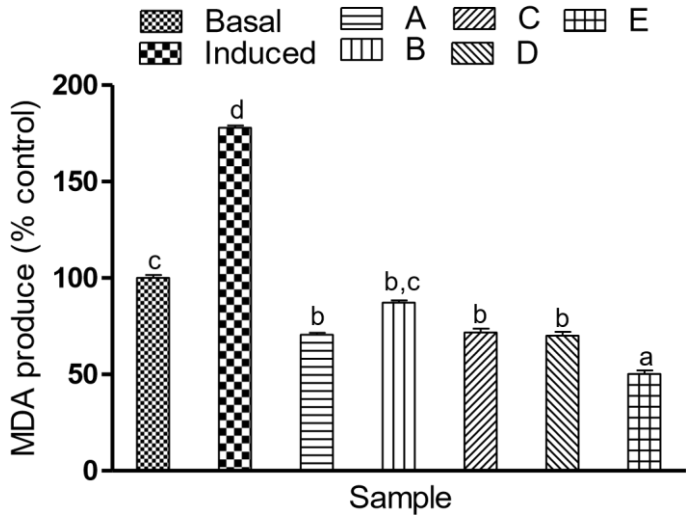

Figure 8: Effect of GABA on the inhibition of induced lipid peroxidation in rat penile tissue by Sildenafil
Bars represent mean ± standard deviation of duplicate readings Bars with different letters are significantly different at $p<0.05$

KEY:
A= 100% GABA
B= 75% GABA + 25 % Sildenafil
C= 50% GABA + 50 % Sildenafil
D= 25% GABA + 75 % Sildenafil
E = 100% Sildenafil

may have a regulatory role in erectile function. The ability of the combinations of both sildenafil and GABA to exhibit AChE inhibitory effects suggests that the combinations of both therapeutic agents could serve as AChE inhibitor and hence exhibit erectogenic properties; this could be as a result of interactions between both samples and could thus offer therapeutic potentials at treatment of ED while possibly helping to reduce the side effects of sildenafil.

Also, the samples showed Fe^{2+} chelating abilities, with the combinations of GABA and sildenafil in the ratio of 75:25 being more potent and not significantly different from 100% GABA'S chelating ability. Fe^{2+} catalyzes electron generating reactive oxygen species (ROS), such as the OH via fenton reaction, causes lipid peroxidation and also decomposes the lipid peroxides, which leads to the

generation of peroxyl and alkoxyl radicals which further favours the propagation of lipid peroxidation[42]. Therefore, it was also observed in this study that the incubation of isolated rat's penile homogenate in the presence of Fe^{2+} caused a significant ($P < 0.05$) increase in the MDA content. Previous studies have shown that incubation of rat penile tissues in the presence of 25 μm $FeSO_4$ solution caused a significant increase in their MDA content[43]. However, the introduction of GABA, sildenafil and their various combinations caused significant ($P < 0.05$) decrease in the MDA content of the incubated penile tissue homogenate. The possible mechanisms through which the sample protects against lipid peroxidation could be by Fe^{2+} chelation[44]. Therefore, the decrease in the penile MDA content by GABA, sildenafil and their various contributions could be attributed to the Fe^{2+} chelating properties. However, a combination of 50% GABA and 50% sildenafil had the highest inhibitory effect on MDA production.

CONCLUSION

This study has been able to show that sildenafil, GABA and their various combinations inhibited the activities of arginase, ACE and AChE in rat penile tissue (*in vitro*). In addition, these samples also chelated Fe^{2+} and inhibited Fe^{2+} lipid peroxidation in penile tissue homogenate. These observed biological activities could be part of the mechanism by which sildenafil and GABA exhibit erectogenic properties. Nevertheless, the ability of the various combinations of sildenafil and GABA to exhibit these erectogenic properties could be therapeutically important in the management of ED while at the same time, reducing the side effects of sildenafil. Therefore, the use of combinations of GABA with sildenafil could offer therapeutic properties essential for the management of ED while also offering the possible advantage of reducing the side effects associated with the use of synthetic drugs such as sildenafil

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