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PDE-5 inhibitors in selected herbal supplements from the Ghanaian market for better erectile function as tested by a bioassay

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ABSTRACT

Herbal supplements sold as 'all natural' on various markets in Accra (Ghana) and advertised as highly efficacious in treating erectile dysfunction (ED) were bought and analysed by a PDE-5 enzyme inhibition assay. The claimed efficacy of these products could be the result of inherent plant constituents, but also of intentionally added pharmaceuticals. Medically, ED is treated with potent inhibitors of the phosphodiesterase-5 (PDE-5) enzyme, as in the case of sildenafil. To test the efficacy of the Ghanaian supplements, extracts were made and tested using a PDE-Glo phosphodiesterase assay, a luminescent high-throughput screening (HTS) method.

Results revealed that about 90% of the selected samples were able to inhibit PDE-5 activity to a high extent. Estimated concentrations in sildenafil equivalents ranged from traces to very high, with 25 samples (62.5%) pointing at daily doses higher than 25 mg sildenafil equivalents and 9 (22.5%) of these at doses higher than the maximal recommended daily intake of 100 mg sildenafil equivalents. Further investigations are needed to confirm if the observed effects are due to inherent plant constituents or merely the result of added synthetic PDE-5 enzyme inhibitors, especially because doses above 100 mg sildenafil equivalents per day may result in severe health risks.

1. Introduction

Stimulation of sexual drive is initiated in the brain. Therefore activities that cause mental and emotional instability and certain chronic medical conditions (diabetes, hypertension), affect an individual's sexuality and overall sexual performance (Musicki et al. 2009). This may potentially result in sexual dysfunction. Aging is another factor contributing to the prevalence of sexual dysfunction (Burnett, 2006; Lewis et al. 2010) and this varies significantly between males and females (Laumann et al. 1999; Rosen 2000; Heiman 2002). Although dysfunction in sexual desire is less prevalent in men, erectile dysfunction (ED), which is the inability of a man to achieve and maintain an erection, has gained lots of attention during the past two decades. Solutions ranging from psychological therapies to prescriptive drugs have been approved for the treatment and management of ED since the early 1990s (Patel et al. 2014).

Currently, there are seven drugs approved for the treatment of ED. These include sildenafil citrate, sold as Viagra; tadalafil, sold as Cialis; vardenafil hydrochloride, sold as Levitra; udenafil, sold as Zydena; mirodenafil hydrochloride, sold as Mvix; iodenafil carbonate, sold as Helleva and avanafil, sold as Stendra (Patel et al. 2014). These drugs inhibit phosphodiesterase 5 (PDE-5) enzyme activity in the corpus cavernosum, resulting in the accumulation of cyclic guanosine monophosphate (cGMP), which helps the relaxation of the smooth-muscle cells and increases the blood flow to the penis, thus enhancing erection (Venhuis et al. 2008).

Studies in animals and human clinical trials suggest that certain plants and plant constituents, such as *Ginkgo biloba*, maca, red ginseng, and icariin, also possess intrinsic PDE-5 inhibition properties (Shamloul 2010; Singh et al. 2012; Kotta et al. 2013; Ongwisespaiboon and Jiraungkoorskul 2017). These plants may be used individually or combined with other plants to potentiate the expected outcome. Although they are often used in their raw state, some plants are (semi-)processed into powders or liquids or into finished products such as capsules, gels and ointment.

In most African countries, including Ghana, the majority of

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individuals experiencing ED do not consult qualified professionals to address their sexual related matters due to the high consultation charges, social stigma and possible embarrassment they may encounter. They resort to self-medication by employing available products from web shops, lorry stations, and open markets. The herbal supplements sold for the purposes of treatment and management of ED are much preferred because of the general perception of safety attached to natural products, the well-known side effects and possible drug-drug interaction of synthetic PDE-5 inhibitors (Lowe and Costabile, 2012; Ventimiglia et al. 2016), and the need of a formal prescription or supervision to purchase or use synthetic PDE-5 inhibitors. In addition, these herbal supplements have competitive selling advantages due to their relatively low prices compared to synthetic PDE-5 inhibitors.

The use of herbal supplements for the purposes of treating ED is not only limited to individuals experiencing ED. Healthy men who have no issues regarding erectile function also use these products for recreational purposes, e.g. to increase their penis size, boost sexual desire or extend their performance time during intercourse (Harte and Meston 2011); Danquah et al. 2011). Some of these supplements are imported from other countries like China, Canada and the USA, but the majority is produced locally and is advertised with attractive names such as, Be4be4, Touch Me and See, Hapimaan, Recharger, Alive Power, Bigger Longer More and Bazouka AK-47. The surge in market demand has resulted in their abundance on the open market with little to no attention paid to the quality or safety of the end-products, several of which are sold illegally.

Various concerns have been raised by Ghanaian stakeholders regarding the potency and efficacy of these herbal supplements as well as their safety for the health of consumers, especially when the youth is becoming overly dependent on these products. Currently, the purported potency and efficacy are based on claims by producers and sustained by consumers' testimonies which are being spread through aggressive media marketing, and are considered "successful" as far as demand is on the rise.

The purpose of the current study was to screen selected herbal supplements sold for the treatment / management of ED on markets and in pharmacies in Accra (Ghana) to determine their PDE-5 inhibition potential and estimate the levels of PDE-5 inhibitors expressed in sildenafil equivalents, in order to assess their possible risks. To achieve this, the PDE-Glo phosphodiesterase assay, a luminescent high-throughput (HT) screening method, which runs on the principle of competitive inhibition of enzyme-substrate (PDE-5-cGMP) complexes, was applied. The assay is based on the principle that an inhibitor reduces the degradation of cGMP by the PDE-5 enzyme. More cGMP means that more ATP will be expended in the phosphorylation process of protein kinase A (PKA) to protein kinase A substrate, and that less ATP remains after the phosphorylation process and is used to produce light by luciferase. The amount of light directly correlates to the PDE-5 enzyme activity and is inversely related to the concentration of inhibitor, i.e. more inhibitor results in less light. Application of this bioassay enabled estimation of the levels of PDE-5 inhibitors in the supplements expressed in sildenafil equivalents, enabling subsequent assessment of exposure and potential risks.

2. Materials and methods

2.1. Sample selection

Forty herbal supplements were collected based on pricing, popularity among users and recommendations by sellers. The majority of the samples were capsules ($n = 30$) and a few were powders ($n = 2$) and liquids ($n = 8$). Samples were kept in their original packaging at room temperature. Prior to analysis, each sample was assigned a sample identity (ID). Table S1 shows details of the products (sample ID, origin, product form, and instructions for use). Hereafter, products are referred to by their allocated ID.

2.2. Chemicals and reagents

Sildenafil citrate (CAS 171599–83-0) was purchased from Carbosynth (UK), acetonitrile (CAS 75–05-8) and methanol (CAS 67–56-1) from Biosolve Chemie SARL (Valkenswaard, The Netherlands) and dimethyl sulfoxide (CAS 67–68-5) was from Merck (Darmstadt, Germany). Water was prepared using a Milli-Q water purification system. The PDE-Glo phosphodiesterase assay kit (Promega, CAT No. V1361) was purchased from Fisher Scientific (Madison, WI, USA). The kit contained 5× PDE-Glo Reaction buffer, 5× PDE-Glo Detection buffer, Protein Kinase A, 5× PDE-Glo Termination buffer, 1 mM cGMP solution, 1 mM cAMP solution, Kinase-Glo substrate and Kinase-Glo buffer. Phosphodiesterase 5A1 human recombinant (CAT No. E9034) and 3-isobutyl-1-methylxanthine (IBMX) (CAT No. I5879) were purchased from Sigma-Aldrich (Saint Louis, USA). Coaster 96-well, flat bottom, non-treated, non-sterile white polystyrene assay plates were purchased from Corning (NY, USA).

2.3. Sample pre-treatment

Half the capsuled and powdered samples was emptied into clean beakers and mixed, whereas liquid samples were manually agitated for 1 min. Thereafter, 100 mg of the solid samples or 100 μL of liquid samples were aliquoted into 2.5 mL polypropylene vails with caps and 1 mL extraction solvent ACN/H_2O (80/20, *v*/v) was added to each sample. As controls, 100 mg of a known positive sample containing icariin (P) and of a negative sample (N) were treated in the same way as the samples, but both in duplicate, where one was spiked with 50 μL sildenafil stock solution (2 mg/mL = 4.2 mM). In addition, 1 mL ACN/H_2O (80/20, v/v) in duplicate was used as a chemical blank, where one was again spiked with 50 μL sildenafil stock solution (2 mg/mL). To ensure that all samples were of equal volume, 50 μL ACN/H_2O (80/20, v/v) was added to each sample that was not spiked with sildenafil. Next, mixtures were vortexed (Vortex-2 Gene) at speed 5 for one min then placed in a multi-tube vortex mixer (Heidoph Reax 2) for 30 min. Finally, samples were centrifuged (Eppendorf Centrifuge 5415 R) (985 *g*) for 5 min at 22 °C. Supernatants were collected and transferred into new vials. A 100 μL portion of the supernatant was aliquoted into a vial already containing 100 μL DMSO (as a keeper) and mixed. The ACN/H_2O was evaporated under a continuous stream of nitrogen at 60 °C for 40 min. Samples were further diluted, i.e. 10 fold, 100 fold, and further to 1000 and 10,000 fold with DMSO when necessary.

2.4. PDE-5 enzyme inhibition assay

The analysis of the bioassay was performed following the protocol by PROMEGA (PDE-Glo™ Phosphodiesterase Assay Technical Bulletin). Initially, a system suitability test was performed as recommended by the manufacturer to demonstrate the specificity of the enzyme assay. To this end a sildenafil concentration response curve, i.e. at 0 nM (DMSO), 0.5 nM, 50 nM, 500 nM and 50,000 nM, was made to determine the level of inhibition at increasing concentrations. Additionally, a chemical blank containing all reagents (minus sildenafil), a spiked chemical blank (plus sildenafil), a positive control (P) (sample containing icariin), and a botanical preparation previously tested as negative in the PDE-5 assay was used as negative control. The equation used to fit the data from the sildenafil response curve was ($y = a0 + a1*\exp.(-x/a2)$).

2.5. Analysis of samples using the PDE-Glo assay

An aliquot of 5 μL sample extract, 7.5 μL PDE-5 enzyme and 12 μL (20 μM) cGMP were pipetted into a Coaster 96-well plate, mixed for 5 min on a plate shaker and incubated in the dark for 90 min. The process was terminated by adding 12 μL termination solution (termination buffer +100 mM 3-isobutyl-1-methylxanthine). Next, 12 μL detection solution (detection buffer + protein kinase A) was added, mixed for 5

min and incubated for 20 min. Finally, 50 μL kinase glo reagent (kinase glo substrate + kinase glo buffer) was added and the plates were incubated for another 10 min. Luminescence signals (RLU) were measured using a Biotek Synergy HT (Vermont, USA). A schematic diagram of the assay protocol is presented in Fig. 1. Data analysis was performed with Microsoft Excel version 2016 and SlideWrite™ plus Version 6, and graphs were plotted using Microsoft Excel and GraphPad Prism 5.

2.6. Precision and repeatability

In order to test the reliability and repeatability of the assay protocol, three independent analyses were carried out on a selected number of samples (both liquid and capsules) four weeks after the initial analysis and one year after. Sample pre-treatment and analysis followed the same procedure as described above. The coefficient of variation (CV) from these three independent replicates was used to determine the levels of precision. The defined acceptance criterium was CV $\leq$ 20% among replicates.

2.7. Effects of matrix in sample extracts

Previously it was noticed that undiluted botanical sample extracts might lead to false positive and false negative screening outcomes in the PDE-5 enzyme inhibition assay due to matrix effects. Also the first pilot experiments in the present study showed matrix effects of some undiluted sample extracts. This could be the result of interferences between analytes of interest and other substances present in the matrix or the interaction of matrix constituents with enzymes or cofactors that form the bioassay, thereby influencing the measured light intensities. Matrix effects tended to disappear when sample extracts were diluted. To detect and overcome these matrix related effects, all samples were tested in a series of dilutions, i.e. undiluted, 10-fold, 100-fold, and in some cases 1000- and10,000-fold diluted sample extracts in DMSO.

3. Results and discussion

The purpose of this study was to determine the PDE-5 inhibition potential of herbal supplements sold on the Ghanaian market and to assess their possible health risk. To detect the potential presence of PDE-5 inhibitors, samples were tested with a PDE-5 enzyme inhibition assay including an estimation of the level of PDE-5 inhibitor expressed in sildenafil equivalents. Fig. 2 shows the concentration-response curve for sildenafil as obtained in the PDE-5 enzyme inhibition assay. Sildenafil results in a concentration dependent decrease in PDE-5 activity at a range of 0.5 to 50,000 nM. After mathematical curve fitting, an IC_{50} of 332 nM was calculated. It is important to stress that quantification of sildenafil equivalents in sample extracts showing responses in the range of the high and low plateau of the sildenafil standard curve is not accurate. In these parts it can only be expressed as smaller than (left side) or higher than (right side) a certain concentration. A more precise quantification of the response can only be performed in the steep part of the curve (roughly the part of the curve between 50 and 500 nM sildenafil). The concentration response curve obtained for sildenafil was in line with that obtained by Bovee et al. in another study (submitted for publication).

To determine whether the assay was fit for purpose, i.e. to test herbal extracts, a chemical blank, a known positive sample (containing icariin) and a negative herbal sample, all three with and without a sildenafil spike (1 mg/g) were tested in two independent studies (Fig. 3). As expected, the unspiked chemical blank did not result in any inhibition of the PDE-5 activity while the spiked chemical blank showed an inhibition. The negative control sample showed a slight inhibition when tested undiluted, resulting in a false positive screening outcome. However, no clear inhibition was observed with diluted extracts of the negative control sample. When spiked with sildenafil at a rather low level of 1 mg/g, a clear decrease in PDE-5 activity was observed in all dilutions. The positive control sample showed a clear inhibition at 10-fold dilution, while the undiluted sample extract did not. This was ascribed to matrix effects, resulting in a false negative screening outcome when testing the undiluted sample extract. These outcomes suggest that undiluted sample extracts may result in both false negative and false positive screening outcomes and that testing serial dilutions of sample extracts is essential to screen for the presence or absence of PDE-5 inhibitors. As expected, inhibition of PDE-5 activity by the spiked control samples decreased upon dilution, except for the 10,000-fold diluted extract of the spiked chemical blank. Using a fitted sildenafil curve and the amount spiked (1 mg/g), a recovery of 280% was calculated from the 100-fold diluted spiked chemical blank and 40% from the 100-fold diluted spiked negative control (responses in the steep part of the sildenafil curve).

Fig. 4 further demonstrates the matrix effect observed in positive sample extracts by showing the results of 6 of the selected samples, i.e. 3 positive solid samples (powders) and 3 positive liquid samples. The solid sample extracts were tested in higher dilutions based on the results from a pilot study. Matrix effects were observed for concentrated extracts of solid samples, i.e. undiluted and 10-fold diluted extracts of powdered

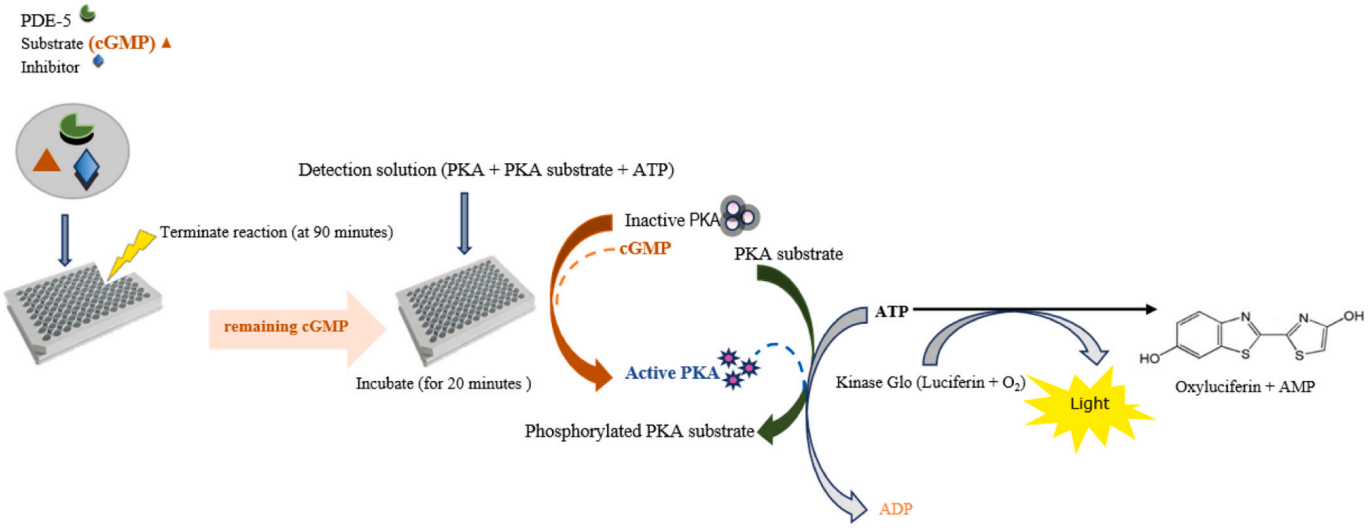

Fig. 1. Schematic diagram of the PDE-Glo Phosphodiesterase assay: a PDE-5 inhibitor reduces PDE-5 enzyme activity. Remaining ATP is measured as emitted light which directly correlates to enzyme activity and is inversely related to the concentration of inhibitor.

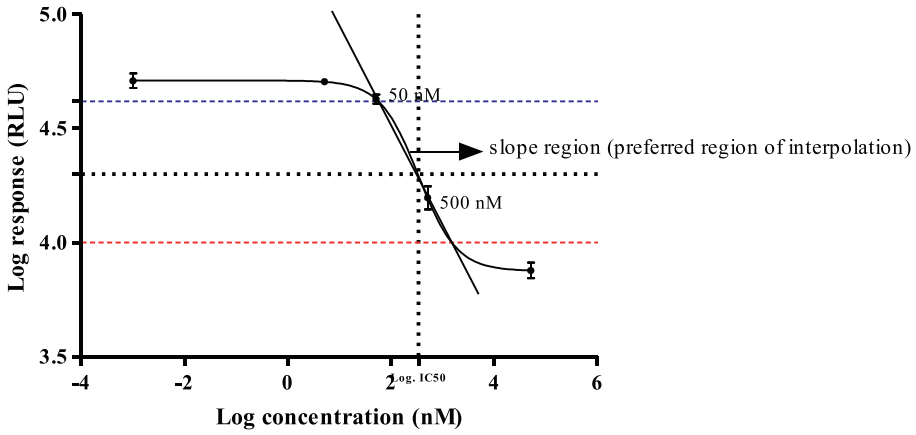

Fig. 2. Sildenafil-concentration dependent reduction in PDE-5 activity. An IC_{50} of 332 nM was calculated from the fitted dose-response curve. Response expressed as luminescent signal (RLUs mean ± SD). The blue and red dotted horizontal lines indicate the RLU boundaries above respectively below which quantification is not possible.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

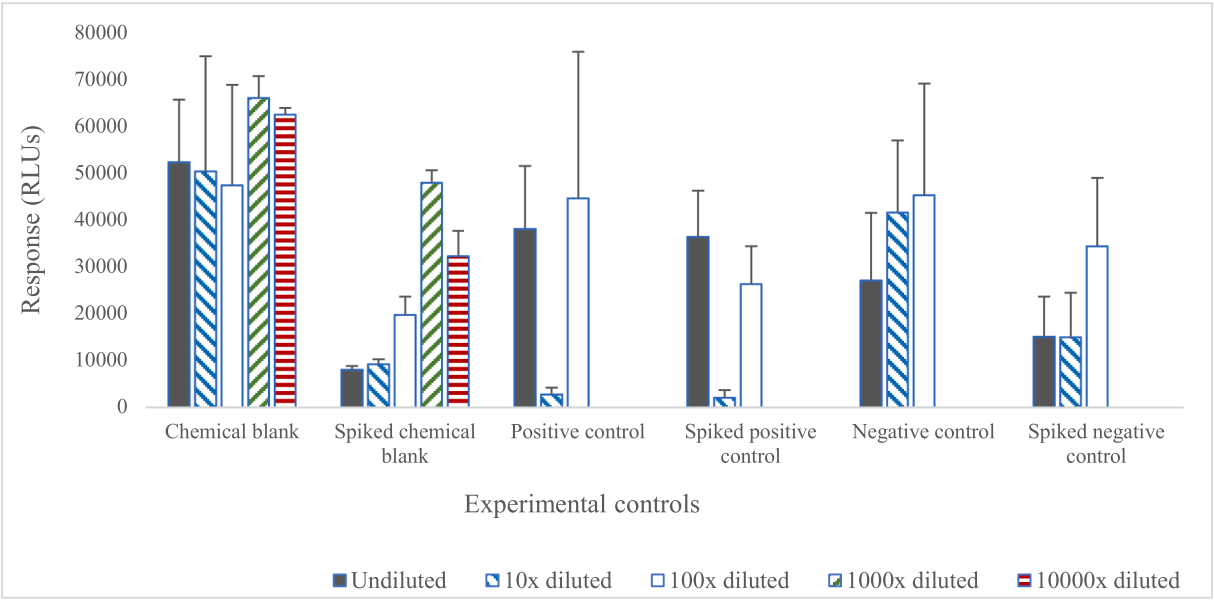

Fig. 3. PDE-Glo phosphodiesterase assay responses obtained for experimental controls with and without a sildenafil spike (1 mg/g). Response expressed as luminescent signal in relative light units (RLUs) (mean ± SD; for two independent clean-ups and subsequent PDE-5 assay testing).

samples, and thus the responses obtained with these dilutions are less reliable. As a result, the 100-fold diluted extracts of these solid samples show the highest inhibition. Further diluting resulted in less inhibition, as the compound(s) responsible for the observed inhibition are diluted and the measured response in RLUs should thus increase when there is no longer a matrix effect. The matrix effect from these solid samples would actually have resulted in a false negative screening outcome of 1 (S2) of these 3 solid samples when testing undiluted sample extracts only. No clear matrix effects were observed when testing extracts of positive liquid samples, i.e., as expected dilution resulted in less inhibition and an increase of the response as measured in RLUs when these extracts were diluted.

Fig. 5 shows the response obtained from the 100- and/or 1000-fold diluted extracts of the 40 supplements collected on the Ghanaian market. First, all sample extracts were tested undiluted, 10- and 100-fold diluted and based on these outcomes, most sample extracts were tested again with a 1000-fold diluted sample extract, and some were even diluted 10,000-fold. Overall, 36 (90%) of the 40 samples showed the ability to inhibit PDE-5 enzyme activity at 100- and 1000-fold dilutions. A complete list of results obtained for all the applied dilutions is presented in Fig. S1. The horizontal dotted lines in Fig. 5 indicate the steep part of the sildenafil calibration curve (Fig. 2) reflecting the range where conversion of the activity into sildenafil equivalents is feasible. It became evident that the undiluted and 10-fold diluted extracts were not suited to calculate sildenafil equivalent amounts in samples with high amounts of PDE-5 inhibitors, i.e. either due to maximal inhibition, responses were obtained in the lower asymptote of the sildenafil dose response curve, or due to matrix effects as discussed above. One must also be aware that quantitative results obtained with the 10,000-fold diluted sample extracts are less accurate, as a magnification of potential errors will occur (error multiplied by 10,000).

Responses between 16,000 and 43,000 RLU, the steep part of the sildenafil dose-response curve, were used to calculate the sample concentration in mg sildenafil equivalents per g supplement (Table S2). Sample dilutions with a response above 43,000 RLU and below 16,000 RLU, i.e. in both asymptotic ends of the sildenafil curve, were not used for calculations and are represented by less than (i.e. <0.07 and < 0.66 mg/g at 100- and 1000-fold dilutions, respectively) and greater than (i.

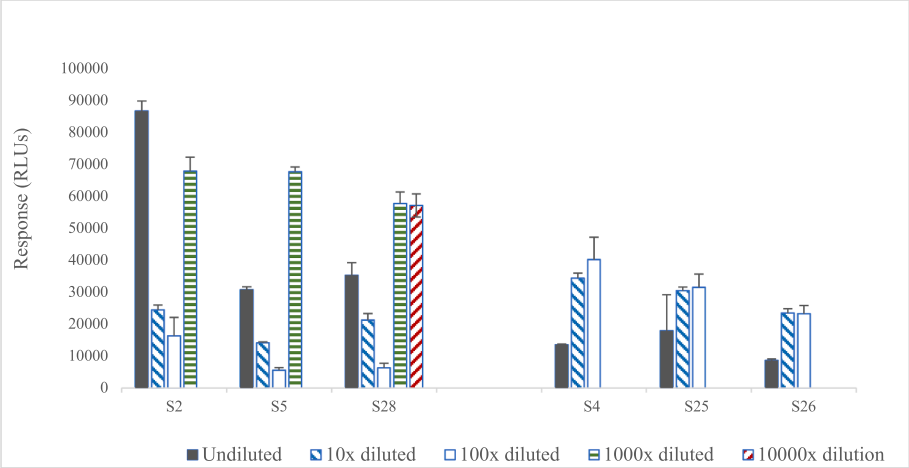

Fig. 4. PDE-Glo phosphodiesterase assay responses obtained with (un)diluted extracts of selected Ghanaian supplements including three solid (powdered) (S2, S5 and S28) and three liquid samples (S4, S25 and S26). Response expressed as luminescent signal in relative light units (RLUs mean ± SD).

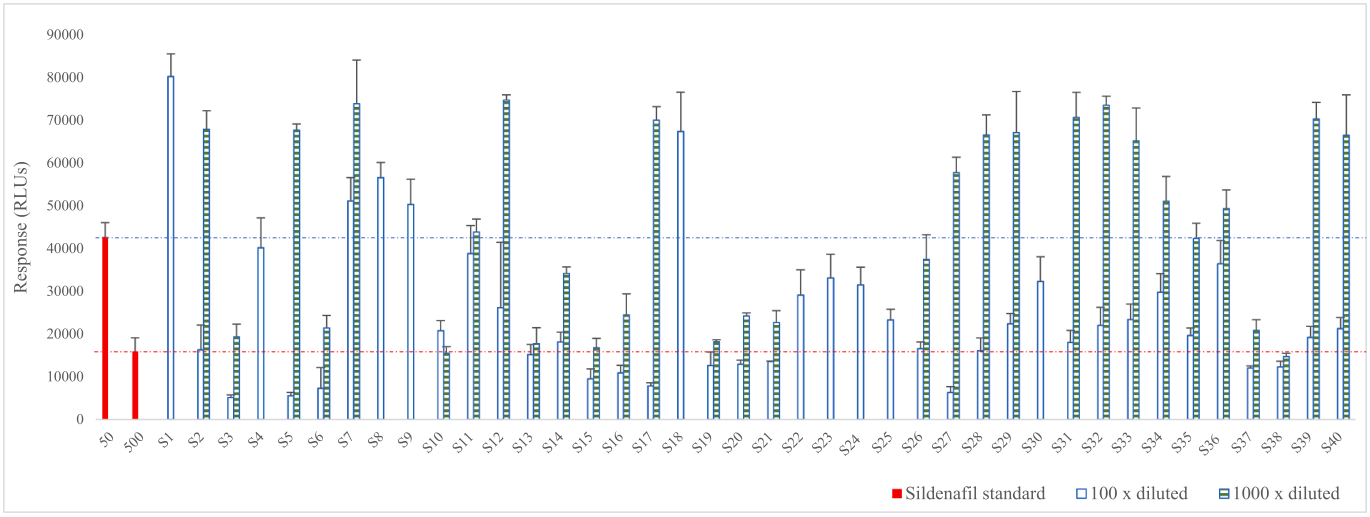

Fig. 5. PDE-Glo phosphodiesterase assay response (RLU) of extracts of the 40 Ghanaian supplements tested in 100- and 1000-fold dilutions. Based on the concentration response curve for sildenafil, responses between 16,000 RLUs (red dotted line) and 43,000 RLUs (blue dotted line) can be used for calculation of the amounts in sildenafil equivalents. Response in RLUs expressed as mean ± SD for 3 measurements of each dilution.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

e. ˃ 8.2 mg/g at 100-fold dilution). Table 1 shows the calculated sample concentrations for each sample when using the 100- and 1000-fold dilutions. Four supplements, i.e. S1, S7, S8, S18, showed high RLU values (i.e. no inhibition) for both the undiluted and diluted extracts, implying that no PDE-5 inhibitors were present in these samples. The other 36 samples showed a clear inhibition (i.e. low response) for the undiluted sample extracts which then often decreased (higher response) to some extent at higher fold dilutions. However, some samples, e.g. S3, S16, S19 and S20, still showed a near maximal inhibition at the higher dilutions, indicating the presence of high concentrations of PDE-5 inhibitors.

In a number of cases, e.g. S15, the response for the undiluted extract was high (implying a low concentration of inhibitors), but responses started to decrease at higher dilutions, showing that these samples do contain a substantial amount of PDE-5 inhibitors. For these samples the previously described matrix effect with undiluted sample extracts would actually have resulted in a false negative screening outcome when using the result from the undiluted and in some cases 10-fold diluted extract only.

It is evident from Table 1 (Table S2) that the calculated levels of inhibition in the supplements differ for each dilution (especially due to matrix effects in the concentrated extracts of solid samples), but the assay clearly identifies the samples with high levels of PDE-5 inhibitors. As a conservative approach, it may be best to base the final decision for follow-up on the highest estimated level. For the majority of the samples, more than one dilution gave a response within the range of the calibration curve enabling calculation of the sample concentration in sildenafil equivalents. These outcomes were eventually used to classify supplements as being negative (N), low (L), medium (M) and or highly (H) active.

To demonstrate the repeatability of the PDE-5 assay protocol, a number of samples was selected randomly and re-analysed 1 and 12 months after the initial experiment (Fig. S2a and S2b, respectively). The %CV within triplicates and between repeats was in general below 20% and resulted in the same classification of the supplements as being a positive or negative sample.

Next, the average weight of the recommended daily dose (g/day) of the supplement was multiplied by the estimated concentrations expressed in sildenafil equivalents (mg/g) to derive the intake per day expressed in mg sildenafil equivalents per day (Table 1). A complete list of the estimated amount per recommended daily intake derived from all

Table 1
Estimated concentration and estimated daily intake (EDI) of unknown PDE-5 inhibitors in 40 supplements, expressed in mg sildenafil equivalents. Quantification was performed only for dilutions that resulted in a PDE-5 inhibition response in the steep part of the sildenafil calibration curve. The levels for the other dilutions are expressed as lower or higher than based on the boundaries of the steep part (the upper and lower asymptote respectively).

Sample ID	Concentration (mg sildenafil-equivalents per gram)		Average weight of dose unit (g or mL)	No. of dose units per day	Estimated daily intake (mg per day)		Evaluation
	100-fold[a]	1000-fold[a]			100-fold[a]	1000-fold[a]	
S1	<0.07		1.07	4	<0.3		N
S2	4	<0.66	0.81	4	14	<2	L
S3	>8.2	36	1.01	2	> 17	72	M
S4 (l)	0.8		30	3	71		M
S5	>8.2	<0.66	1.01	4	> 36	<3	M
S6	>8.2	31	0.47	2	> 8	29	M
S7(l)	<0.07	<0.66	30	4	<8	<79	M
S8	<0.07		2.01	6	<1		N
S9	<0.07		0.51	2	<0.1		N
S10	3	47	0.46	2	3	43	M
S11	1	5	0.49	2	1	5	L
S12	2	<0.66	0.74	2	3	<1	L
S13	5	40	1.31	4	25	208	H
S14	4	1	1.16	4	18	62	M
S15	>8.2	43	0.36	6	>18	92	M
S16	7	26	0.65	2	9	34	M
S17	>8.2	<0.66	1.1	4	> 36	<3	M
S18	<0.07		0.65	4	<0.2		N
S19	6	39	0.43	2	5	33	M
S20 (l)	6	26	10	3	173	782	H
S21	6	29	0.97	4	21	112	H
S22 (l)	2		30	3	172		H
S23 (l)	1		20	1	29		M
S24 (l)	2		9	2	29		M
S25 (l)	3		60	1	167		H
S26	4	10	1.1	2	10	22	L
S27	>8.2	<0.66	1.67	4	> 55	<4	M
S28	5	<0.66	2.13	6	57	<8	M
S29	3	<0.66	1.09	4	13	<3	L
S30	2		30	4	185		H
S31	4	<0.66	1.35	4	21	<4	L
S32	3	<0.66	1.24	4	15	<3	L
S33	3	<0.66	1.53	4	17	<4	L
S34	2	<0.66	1.34	4	10	<4	L
S35	4	6	15	4	210	368	H
S36 (l)	1	1	0.96	2	2	2	L
S37	6	32	2.03	4	51	263	H
S38	6	50	1.47	4	36	292	H
S39	4	<0.66	1.7	4	25	<5	M
S40	4	<0.66	1.14	2	7	<2	L

(l): Represents liquid samples.
Evaluation: N: negative, L: <25 mg/d; M: 25–100 mg/d; H: >100 mg/d.
[a] Calculated from the response obtained with either the 100-fold or 1000-fold diluted sample extract.

dilutions is presented in Table S3. The daily use of the supplements based on the recommendation on the label and the resulting estimated daily intakes ranged from traces to levels far above 100 mg sildenafil equivalents per day. Based on the 100- and 1000-fold dilutions (most relevant dilutions), 4 (10%) out of the 40 samples were found to be negative in inhibiting PDE-5 activity whereas 36 (90%) resulted in detectable intake levels of PDE-5 inhibitors. Out of the 36 positive samples, 11 (31%) would result in a relatively low daily intake of less than 25 mg sildenafil equivalents, 16 (44%) would result in a daily intake between 25 and 100 mg, and 9 (25%) even in daily intake above 100 mg sildenafil equivalents (Table 1).

According to Pfizer, sildenafil citrate (Viagra) is available in three dosages, i.e. 25, 50 and 100 mg, of which the recommended dosage for an average patient is usually 50 mg. Based on the individual's circumstances and severity of ED, the recommended dosage may be downscaled to 25 mg or up-scaled to 100 mg once per day.[1] Comparing the estimated amounts (mg per day) taken when using the supplements to the recommended daily dosage with that for Viagra (25–100 mg), it was assumed that supplements resulting in daily doses below 25 mg may not have the desired effect and therefore will not potentially pose a risk to consumers, whereas samples resulting in daily doses of 25 to 100 mg sildenafil equivalents may cause an effect but not result in fatalities because they were within the recommended dose range. Although individual differences may play a role with regards to bioavailability, concentrations from 25 to 100 mg may thus be of less concern to health authorities, apart from the fact that they may have been deliberately adulterated with a pharmacologically active dose of sildenafil or analogues. However, supplements with concentrations above 100 mg sildenafil equivalents may be considered as high risk especially to vulnerable individuals. High amounts of sildenafil (above 100 mg) and other PDE-5 inhibitors may e.g. cause extreme relaxation of the smooth muscle cells which can inadvertently put the lives of unsuspecting consumers at unanticipated risks such as ischemic (low-flow) priapism which may lead to penile fibrosis of the corpus cavernosa and instead result in ED (Broderick et al. 2010; Zheng et al., 2013). There is also a risk for people using antihypertensives such as nitrates (e.g. nitroglycerine, doxazosin and terazosin) (Boden et al., 2012) and patients suffering from hypotension (Kloner, 2007) for whom use of PDE-5

[1] https://www.viagra.com/taking/finding-the-right-dose (accessed on 17th July 2020)

inhibitors is contra-indicated. The present data justify safety concerns raised by stakeholders and relevant authorities regarding the availability and ease in accessibility of these kind of supplements on the Ghanaian market.

4. Conclusions

This study revealed the potential efficacy and potency of herbal supplements sold for the purpose of treating or managing issues related to sexual dysfunction, especially ED, on markets and pharmacies in Accra (Ghana). Out of the 40 selected samples, 36 (90%) were positive in inhibiting PDE-5 activity. Twenty-five (62.5%) out of the 36 showed a response that implied a daily dose of over 25 mg sildenafil equivalents and 9 (22.5%) out of the 36 would result in a daily dose above 100 mg sildenafil equivalents, which is higher than the highest recommended daily dose of Viagra for treating ED in patients.

The PDE-Glo phosphodiesterase assay proved to be suitable for selecting samples that require further examination by LC/MS to disclose the identity of the compound(s) eliciting the observed inhibition of PDE-5 enzyme activity and to quantify the levels. These compounds may be natural plant constituents like icariin (as in the case of the positive control), but also illegally added (un)known synthetic PDE-5 inhibitors and their analogues (Singh et al. 2009; Patel et al. 2014; Rocha et al. 2016). In case of unknowns, subsequent analysis based on bioassay-guided fractionation combined with LC-(HR)MS(/MS) analysis could lead to the discovery of unknown active compounds responsible for the observed effect in the PDE-5 enzyme inhibition assay.

Collaboration

This project is a collaboration between the Dept. of Toxicology and Wageningen Food Safety Research at the Wageningen University and Research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2021.105130.

References

Boden, W.E., Finn, A.V., Patel, D., Frank Peacock, W., Thadani, U., Zimmerman, F.H., 2012. Nitrates as an integral part of optimal medical therapy and cardiac rehabilitation for stable angina: review of current concepts and therapeutics. Clin. Cardiol. 35 (5), 263–271.

Broderick, G.A., Kadioglu, A., Bivalacqua, T.J., Ghanem, H., Nehra, A., Shamloul, R., 2010. Priapism: pathogenesis, epidemiology, and management. J. Sex. Med. 7 (1), 476–500. https://doi.org/10.1111/j.1743-6109.2009.01625.x.

Burnett, A.L., 2006. The role of nitric oxide in erectile dysfunction: implications for medical therapy. J. Clin. Hypertens. 8, 53–62. https://doi.org/10.1111/j.1524-6175.2006.06026.x.

Danquah, C.A., Koffuor, G.A., Anto, B.P., Nimako, K.A., 2011. The indiscriminate use of sex enhancing products among Ghanaians: prevalence, and potential risk. Pelagia Research Library. Adv. Appl. Sci. Res. 2 (5), 350–359.

Harte, C.B., Meston, C.M., 2011. Recreational use of erectile dysfunction medications in undergraduate men in the United States: characteristics and associated risk factors. Arch. Sex. Behav. 40 (3), 597–606. https://doi.org/10.1007/s10508-010-9619-y.

Heiman, J.R., 2002. Sexual dysfunction: overview of prevalence, etiological factors, and treatments. J. Sex Res. 39 (1), 73–78. https://doi.org/10.1080/00224490209552124.

Kloner, R., 2007. Erectile dysfunction and hypertension. Int. J. Impot. Res. 19 (3), 296–302.

Kotta, S., Ansari, S.H., Ali, J., 2013. Exploring scientifically proven herbal aphrodisiacs. Pharm. Rev. 7 (13), 1. https://doi.org/10.4103/0973-7847.112832.

Laumann, E.O., Paik, A., Rosen, R.C., 1999. Sexual dysfunction in the United States: prevalence and predictors. JAMA 281 (6), 537–544. https://doi.org/10.1001/jama.281.6.537.

Lewis, R.W., Fugl-Meyer, K.S., Corona, G., Hayes, R.D., Laumann, E.O., Moreira Jr., E.D., Segraves, T., 2010. Definitions/epidemiology/risk factors for sexual dysfunction. J. Sex. Med. 7 (4), 1598–1607. https://doi.org/10.1111/j.1743-6109.2010.01778.x.

Lowe, G., Costabile, R.A., 2012. 10-year analysis of adverse event reports to the food and drug administration for phosphodiesterase type-5 inhibitors. J. Sex. Med. 9 (1), 265–270. https://doi.org/10.1111/j.1743-6109.2011.02537.x.

Musicki, B., Liu, T., Lagoda, G.A., Bivalacqua, T.J., Strong, T.D., Burnett, A.L., 2009. Endothelial nitric oxide synthase regulation in female genital tract structures. J. Sex. Med. 6, 247–253. https://doi.org/10.1111/j.1743-6109.2008.01122.x.

Ongwisespaiboon, O., Jiraungkoorskul, W., 2017. Fingerroot, *Boesenbergia rotunda* and its aphrodisiac activity. Pharm. Rev. 11 (21), 27. https://doi.org/10.4103/phrev.phrev_50_16.

Patel, D.N., Li, L., Kee, C.L., Ge, X., Low, M.Y., Koh, H.L., 2014. Screening of synthetic PDE-5 inhibitors and their analogues as adulterants: analytical techniques and challenges. J. Pharm. Biomed. Anal. 87, 176–190. https://doi.org/10.1016/j.jpba.2013.04.037.

Rocha, T., Amaral, J.S., Oliveira, M.B.P., 2016. Adulteration of dietary supplements by the illegal addition of synthetic drugs: a review. Compr. Rev. Food Sci. Food Saf. 15 (1), 43–62. https://doi.org/10.1111/1541-4337.12173.

Rosen, R.C., 2000. Prevalence and risk factors of sexual dysfunction in men and women. Curr. Psychiatry Rep. 2 (3), 189–195. https://doi.org/10.1007/s11920-996-0006-2.

Shamloul, R., 2010. Natural aphrodisiacs. J. Sex. Med. 7 (1), 39–49. https://doi.org/10.1111/j.1743-6109.2009.01521.x.

Singh, S., Prasad, B., Savaliya, A.A., Shah, R.P., Gohil, V.M., Kaur, A., 2009. Strategies for characterizing sildenafil, vardenafil, tadalafil and their analogues in herbal dietary supplements, and detecting counterfeit products containing these drugs. TrAC Trends Anal. Chem. 28 (1), 13–28. https://doi.org/10.1016/j.trac.2008.09.004.

Singh, R., Singh, S., Jeyabalan, G., Ali, A., 2012. An overview on traditional medicinal plants as aphrodisiac agent. J. Pharmacogn. Phytochem. 1 (4), 43–56.

Venhuis, B.J., Blok-Tip, L., de Kaste, D., 2008. Designer drugs in herbal aphrodisiacs. Forensic Sci. Int. 177 (2–3), e25–e27. https://doi.org/10.1016/j.forsciint.2007.11.007.

Ventimiglia, E., Capogrosso, P., Montorsi, F., Salonia, A., 2016. The safety of phosphodiesterase type 5 inhibitors for erectile dysfunction. Expert Opin. Drug Saf. 15 (2), 141–152. https://doi.org/10.1517/14740338.2016.1131818.

Zheng, D.C., Yao, H.J., Zhang, K., Xu, M.X., Chen, Q., Chen, Y.B., Wang, Z., 2013. Unsatisfactory outcomes of prolonged ischemic priapism without early surgical shunts: our clinical experience and a review of the literature. Asian J. Androl. 15 (1), 75. https://doi.org/10.1038/aja.2012.63.