Identification and Analysis of Adulterants in Aphrodisiac Herbal Medicines Sold by Private Herbal Clinics, Pharmacies and Herbal Drug Shops in Kampala, Uganda

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High performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC), column chromatography and Fourier transform infrared spectrophotometer (FTIR) were used for the identification and analysis of three phosphodiesterase type-5 (PDE-5) inhibitors in 50 randomly selected aphrodisiac herbal samples. Twenty-seven samples were found to contain one or two or all three synthetic PDE-5 inhibitors representing 54% of the analyzed samples. The FTIR spectral characteristics obtained from the isolated compounds were found to be in conformity with those of sildenafil, tadalafil and vardenafil reference standards. Patients who use these herbal sexual enhancers with the notion that they are safe and natural are likely to be exposed to serious health risks related to safety and quality of the herbal products. There is need for additional effort to effectively regulate herbal medicines in order to protect the consumers from the threat of adulteration.

Key words: Aphrodisiac herbal medicines, herbal sexual enhancers, sildenafil citrate, tadalafil, vardenafil hydrochloride, erectile dysfunction.

INTRODUCTION

There is an emerging popularity in the use of herbal medicines worldwide, with 80% of the population in developing countries reported to use herbal remedies for their healthcare needs [1]. Studies show that 70-80% of the Ugandan population uses traditional medicines for day-to-day health care [2]. The popularity of these remedies is well evidenced by the increased herbal adverts in the various media platforms. These products are advertised widely with exaggerated names and claims and sold to the public by unscrupulous manufacturers. Remedies for erectile dysfunction among men seem to be of most interest. Research indicates that the proportion of men suffering from various forms of erectile dysfunction is expected to double by 2025, with men in developing countries of Africa and Asia most affected [3]. There has been an increasing demand for these herbal medicines premised on their perceived minimal side effects and good natural attributes [4, 5].

Traditional treatment of erectile dysfunction includes the use of medicinal plants such as ginseng root (*Panax ginseng* C.A. Mey), *Mondia whytei* [6], *Citropsis articulata*, *Cannabis sativa*, *Cleome gynandra* and *Cola acuminata* [2]. Several pure compounds derived from natural products such as yohimbine, citrulline, pyrano-isoflavones, berberine and forskolin have been reported to improve sexual performance [7]. Modern medication of erectile dysfunction includes oral medication with phosphodiesterase type 5 (PDE-5) inhibitors, mainly sildenafil citrate (Viagra), tadalafil (Cialis) and vardenafil hydrochloride (Levitra). However, taking these drugs can be dangerous to those who suffer from conditions such as hypertension, heart disease and diabetes mellitus or those who take nitrates and blood thinning medication [8, 9].

Although these drugs were approved for treatment of erectile dysfunction, it should be noted that sildenafil citrate, vardenafil hydrochloride and tadalafil are prescription medicines, recommended for use after a thorough

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evaluation of the patient by a medical practitioner [10]. These drugs can potentiate the hypotensive effects of medicines used in treating heart disease causing a sudden and dangerous drop in blood pressure [8]. As a result, many men seek alternative medicines, in the form of herbal sexual enhancers, as a therapy for erectile dysfunction with the belief that these products are safe [11]. Unfortunately, these products have often been reported to be adulterated with phosphodiesterase type-5 (PDE-5) inhibitors [12, 13]. In order to safeguard consumers from the emerging threat of adulteration of herbal medicines with synthetic drugs, there was an urgent need for identification and analysis of adulterants in aphrodisiac herbal medicines sold on the Ugandan market.

EXPERIMENTAL

Study area and sample collection

A study of herbal products claimed to enhance sexual activity was carried out in the central business district of Kampala (Central Division). This area was selected because it has the highest exposure due to its high population (268,659 according to the Uganda Bureau of Statistics, National population and housing census 2014). In addition, there are many aphrodisiac herbal medicine brands freely dispensed in pharmacies, herbal drug shops and herbal clinics without prescription. Samples were randomly obtained from pharmacies, herbal drug shops and herbal clinics, markets and from hawkers using the covert method of sampling (mystery buyer). Both locally manufactured and imported herbal products were considered and different dosage forms were sampled including powders, liquids, capsules and tablets.

Chemicals, solvents and reagents

Reagents used included HPLC grade methanol (Sigma Aldrich, St Louis, MO, USA, 99.9%; lot No. STBG2524Y), tert-butyl methyl ether-99.5% (Sigma Aldrich Chemie, St Louis, MO, USA, lot No. STBG9211), HPLC grade acetonitrile (Sigma Aldrich, Burlington, USA, 99.9%; lot No. I1084330014) and analytical grade ammonium hydroxide (Acros Organics, New Jersey, USA, lot. 2672). Glass plates precoated with HPTLC Silca gel 60 F_{254} (Merck, Darmstadt, Germany, lot: HX71850142) were used as the chromatographic plates. Sildenafil citrate (Sigma Aldrich, St Louis, MO, USA, lot No. LRAA9454), tadalafil (Sigma Aldrich, St Louis, MO, USA, lot No.: LRAA8681) and vardenafil hydrochloride (United states Phamacopea, Rockville, Germany, lot No.: F018G0) were used as reference standards. Purified water was prepared in the laboratory using a Millipore- Elix 70 water system (Merck, Molsheim, France). Silica gel-high purity grade, pore size 60A, 35-60 mesh (Sigma Aldrich, St Louis, MO, USA, lot No. MKCD4689) and Glass wool (Supelco-Sigma Aldrich, St Louis, MO, USA, lot No. 95853) were used for packing of the chromatographic column.

Identification of adulterants using HPTLC

The analytical method was adopted from United States Phamacopeia [14]. Samples were prepared by weighing 0.6 g of the sample into a 25 mL volumetric flask, making the volume up to mark with methanol followed by ultrasonication for 30 minutes.The resultant solution was filtered through a 0.45 µm syringe filter. Reference standard solutions were prepared as a mixture of 0.2 mg/mL of each of sildenafil citrate, tadalafil and vardenafil hydrochloride reference standards using methanol as the diluent.

HPTLC System

The HPTLC system (CAMAG, Muttenz, Switzerland) consiting of an automatic TLC sampler, an automatic development chamber, TLC visualizer, TLC scanner and supported by the visionCATS software was used for the identification and isolation of adulterants. A 3 µL aliquot of sample was applied as 8-mm bands. The developing solvent was a mixture of *tert*-butyl methyl ether, methanol and 28.0% (w/w) ammonium hydroxide in the ratio (20:2:1), The chromatographic plates were saturated for 20 minutes at ambient temperature and relative humidity of 33% using magnesium chloride and developing distance was 6 cm. Two methods of detection were used. The first method involved visual detection under illumination with 254 nm

and 365 nm UV light. In the second method, UV-visible spectrometry (scanning densitometer) over a scan range of 190-550 nm was employed. During analysis, reference standards were ran alongside samples.

Identification and analysis of adulterants using HPLC-DAD

The analysis method was adopted from the United States Phamacopea [14]. The HPLC analytical method was validated to ensure that it was fit for purpose [15]. Sample solutions were prepared by weighing and transferring 0.6 g of the sample into a 25 mL volumetric flask, making the volume up to the mark with 50% acetonitrile in water followed by vortex mixing and ultra-sonication for 30 minutes. The resultant solution was filtered through a 0.45 µm syringe filter. The reference standard stock solution was prepared by preparing a mixture of 1 mg/mL of each of sildenafil citrate, tadalafil and vardenafil hydrochloride reference standards. Different concentrations were prepared by diluting the stock standard solution in diluent for constructing the standard curve.

HPLC system

An integrated Agilent 1260 infinity series HPLC system (Agilent Technologies, Germany) supported by Open LAB software was used for all chromatographic experiments. An Agilent eclipse XDB-C18, 5 µm chromatography column, of dimensions 150 × 4.6 mm ID (Agilent technologies, Germany) was used as the stationary phase with the column oven maintained 40 °C. The injection volume was 5 µL while the flow rate was 0.8 mL/min. The mobile phase comprised a mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile run in a gradient mode. The eluents were monitored by means of UV detection at 290 nm using a photodiode array detector.

Sample isolation and purification using column chromatography

Positive quantifiable samples were extracted and purified using column chromatography. The glass column was packed with silica gel in a mixture of CH_2Cl_2: CH_3OH (95:5). Extracted sample solutions were loaded on the column and eluted using a mixture of CH_2Cl_2: CH_3OH (100:1.5) whereby 10 mL fractions were collected at different intervals and monitored by HPTLC [16]. Sample fractions with more than one component were combined and further purified on a chromatographic column packed with Sephadex-LH 20 and eluted with 0.1% formic acid in water [17]. Fractions with spots of the same retardation factor (R_f) were combined. The solvent was then dried off in vacuo.

Identification of isolated adulterants using FTIR

The isolated compounds obtained after evaporation of the solvent were analyzed on a Nicolet 6700 FTIR spectrometer (Thermo Scientific, USA). The attenuated total reflection (ATR) method was utilized during analysis by scanning between 4000 to 400 cm^{-1} in transmittance mode. The individual reference standards of sildenafil citrate, vardenafil hydrochloride and tadalafil were also analyzed on the FTIR machine.

RESULTS AND DISCUSSION

In spite of the guidelines on market authorization of herbal medicines which spell out the labeling requirements for these products [18, 19], most of the herbal samples did not meet these labeling requirements. Sixty percent of the sample labels were observed to be devoid of composition and amount of the different ingredients in the products, manufacturer's address, dosage manufacturing and expiry dates. Some samples had simple labels with indications, side effects and dosage. No information on drug interactions and toxicity was provided. No sample bore information advising consumers to consult with a physician before taking the products.

The packaging of most of the samples was poor with stocks of herbal medicines maintained in sacks, jerrycans, mineral water bottles raising concerns about the conditions of hygiene within which these products are manufactured. Although herbal medicines are expected to exert their effects gradually [13], most of the products

were claimed to work instantly which raised suspicions about adulteration of these herbal medicines. Notably, some herbal products were labeled with widely exaggerated names, claims and pictures all intended to attract patients. The verbal instructions given during sampling raised a lot of safety concerns. In most instances tough instructions were given to ensure that the partner is available and has accepted to play sex before use of the herbal medicine. It is therefore important that the public exercises caution while using herbal sexual enhancers. There is need to always seek health care support to ensure appropriateness and safety.

HPTLC Analysis Results

The HPTLC analytical method was found to be specific at retardation factors of about 0.26 for sildenafil citrate, 0.32 for vardenafil hydrochloride and 0.47 for tadalafil (figure 1). Different samples exhibited chromatographic spots corresponding in terms of shape, intensity, color and retardation factors as principle spots obtained in the reference standard solution despite the manufacturer's claim that their products contained only the extracts of the plants mentioned on the label. The chromatographic spots appeared as dark bands against the fluorescent background at 254 nm and typically exhibited different shades of blue fluorescence at 365 nm. Evaluation of the chromatographic spots using the TLC scanner (scanning densitometry) resulted in densitograms with densitometric peaks at the same retardation factors as obtained in reference standard solution. Figure 1(a) shows the densitogram for a reference standard solution with three densitometric peaks at retardation factors of 0.25, 0.31 and 0.46 corresponding to sildenafil, vardenafil and tadalafil, respectively. A densitogram for sample QC1200-17/18 is shown in figure 1 (b) with a densitometric peak at a retardation factor of 0.25 corresponding to that of sildenafil in the standard solution. Other samples desitograms are shown in figure 1 (c) and (d).

The complex nature of herbal medicines (they contain a series of bioactive components) makes analysis of adulterants in the mixtures challenging because of matrix effects [11].There may be possibilities of chromatographic spots from these phytochemical constituents in the herbal sample appearing at the same retardation factors as those of PDE-5 inhibitors (sildenafil citrate, vardenafil hydrochloride and tadalafil) in the standard solution. It was therefore necessary to perform densitometric scanning of the chromatographic spots obtained. This was carried out between 190 nm and 560 nm which resulted into UV spectra. The UV spectra obtained from both sample and reference standard chromatographic spots were inspected for their shape and absorption maxima at 295 nm for sildenafil citrate [20], 270 nm for vardenafil hydrochloride [21] and 220, 280 and 290 nm for tadalafil [22].

High performance thin layer chromatography (HPTLC) provided a chromatographic separation step prior to analyte detection/identification and this enabled the separation of several phytochemicals from the herbal sample matrix that could interfere with or suppress the signals of the compounds of interest [23]. Therefore, synthetic adulterants were isolated from the complex herbal mixture on the chromatographic plate before their identification. Using densitometric scanning the isolated chromatographic spots were successfully evaluated to obtain chromatographic peaks and UV spectra. The UV spectra obtained from the standard solutions were in conformity with those obtained from the samples facilitating identification of adulterants in the complex herbal mixtures.

HPLC analysis results

The method was found to be specific for identification of PDE-5 inhibitors at retention times of about 7.6 minutes for vardenafil hydrochloride, 8.4 minutes for sildenafil citrate and 10.9 minutes for tadalafil. HPLC results on sample analysis showed that different herbal samples exhibited chromatographic peaks at the same retention time as the principal peaks of sildenafil citrate, vardenafil hydrochloride and tadalafil, respectively, obtained in the reference standard solution. Figure 2 shows the HPLC chromatogram for reference standards with chromatographic peaks at retention times of

7.697, 8.499 and 10.997 minutes corresponding to vardenafil hydrochloride, sildenafil citrate and tadalafil respectively. The HPLC chromatogram for sample QC1318-17/18 is shown in figure 3 with chromatographic peaks at 8.459 minutes and 10.995 minutes similar to retention times for sildenafil citrate and tadalafil, respectively, in the reference standard chromatogram. Other samples are shown in figures 4, 5, 6 and 7 respectively.

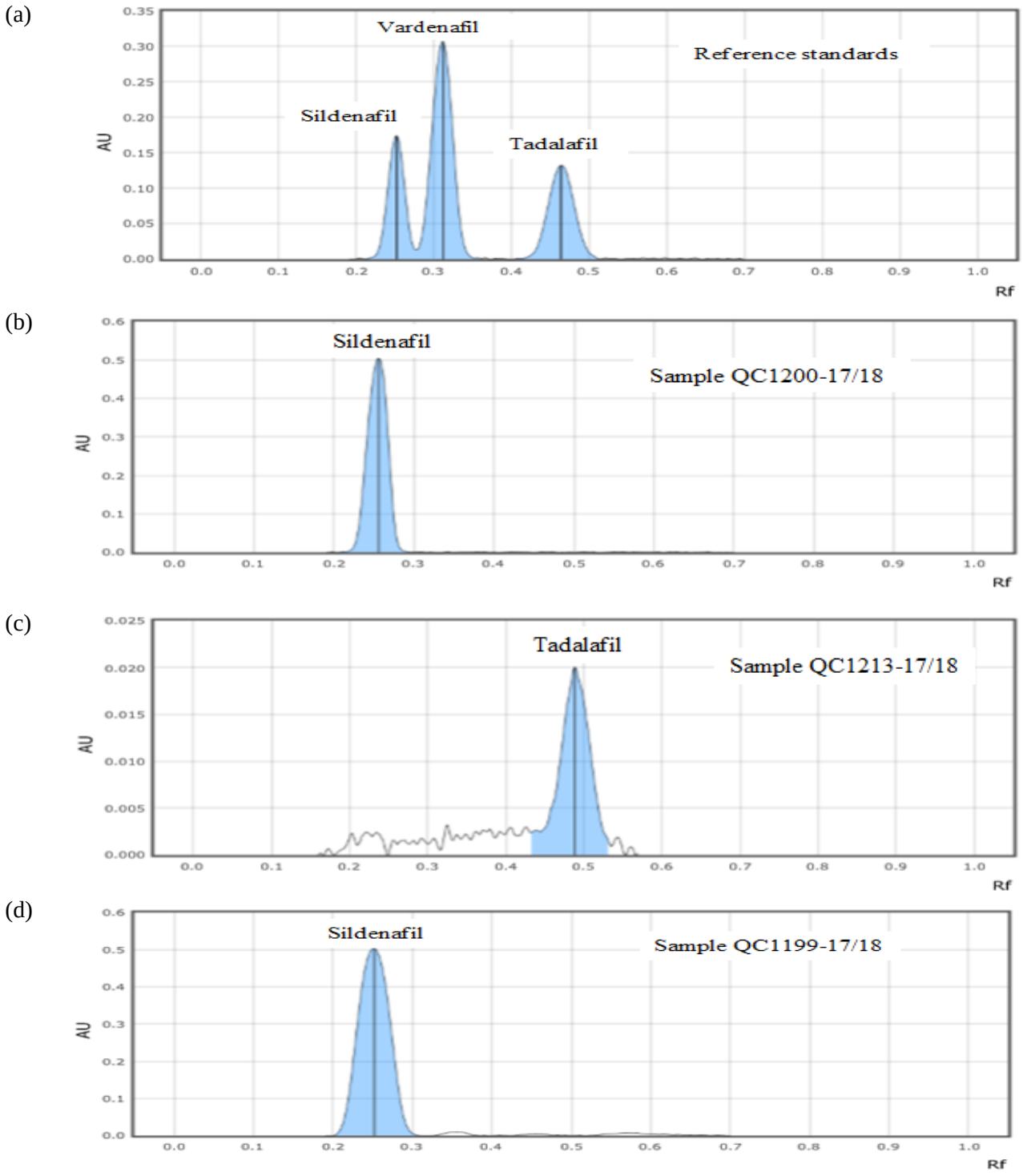

Figure 1: HPTLC densitograms for (a) reference standard solution, (b) sample QC1200-17/18, (c) sample QC1213-18/19 and (d) sample QC1199-17/18

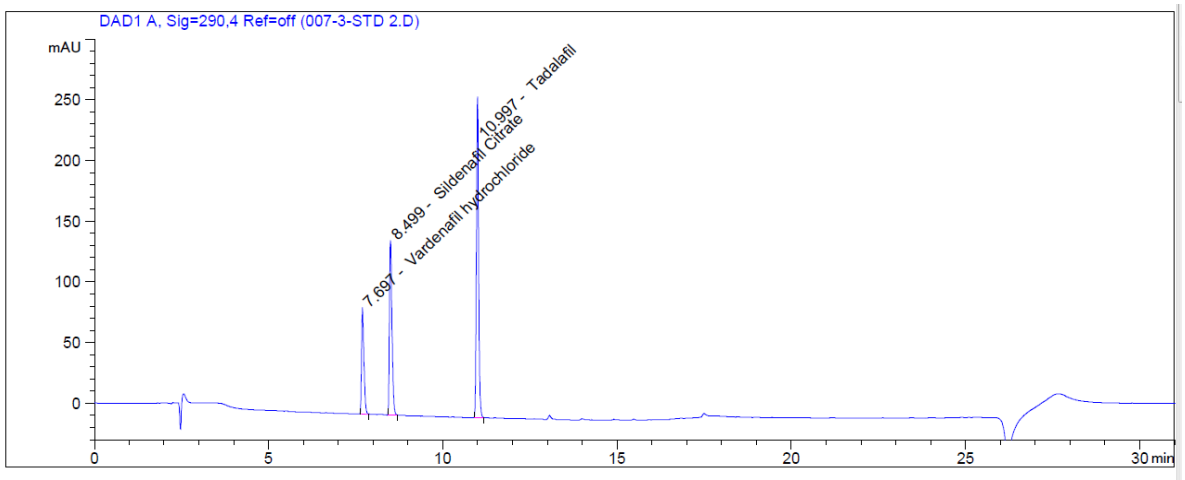

Figure 2: HPLC chromatogram for reference standard showing chromatographic peaks at a retention times of 7.697 min, 8.499 min and 10.997 min corresponding to vardenafil hydrochloride, sildenafil citrate and tadalafil respectively.

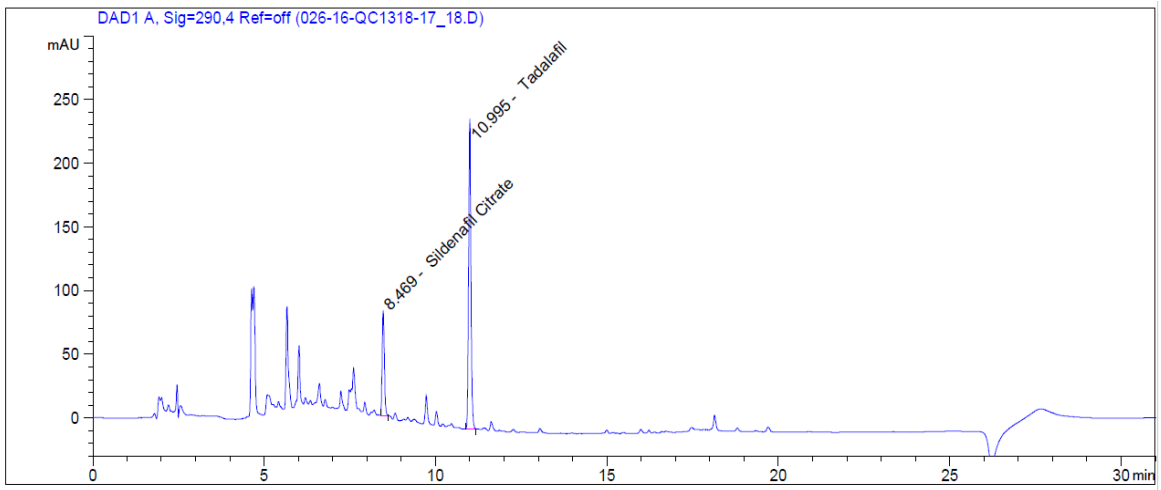

Figure 3: HPLC chromatogram for sample QC1318-17/18 with chromatographic peaks at retention times of 8.469 min and 10.995 min corresponding to sildenafil and tadalafil

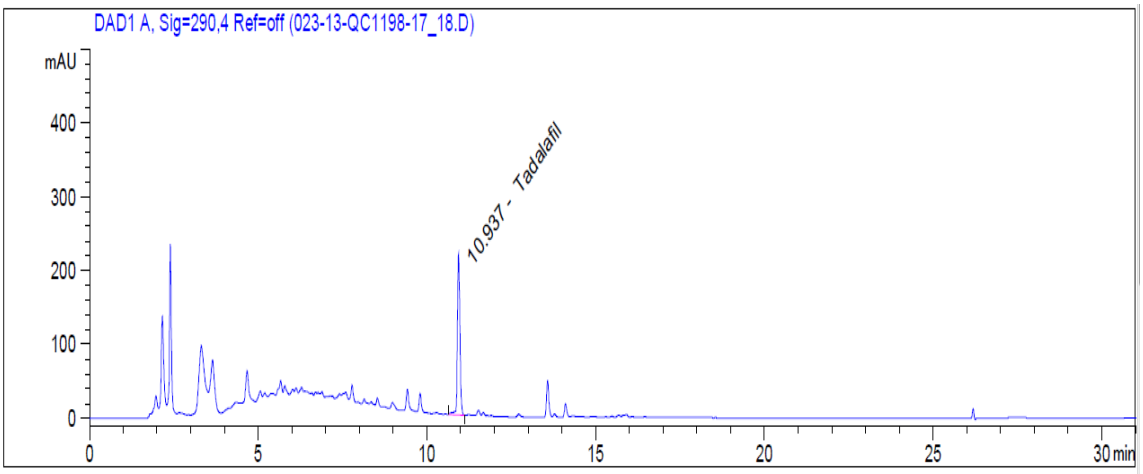

Figure 4: HPLC chromatogram for sample QC1198-17/18 with a chromatographic peak at 10.937 min corresponding to tadalafil

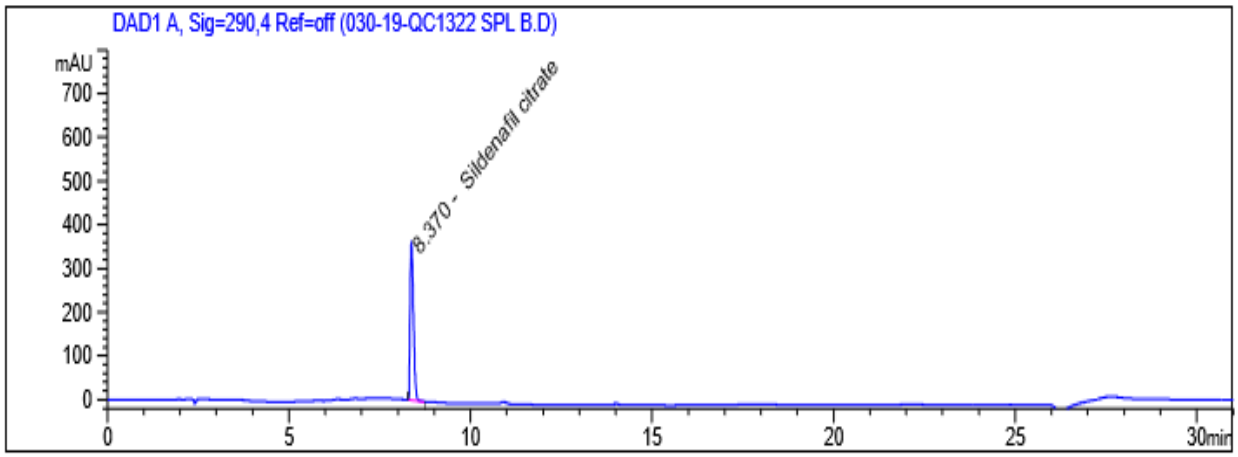

Figure 5: HPLC chromatogram for sample QC1322-17/18 with a chromatographic peak at 8.370 min corresponding to sildenafil citrate

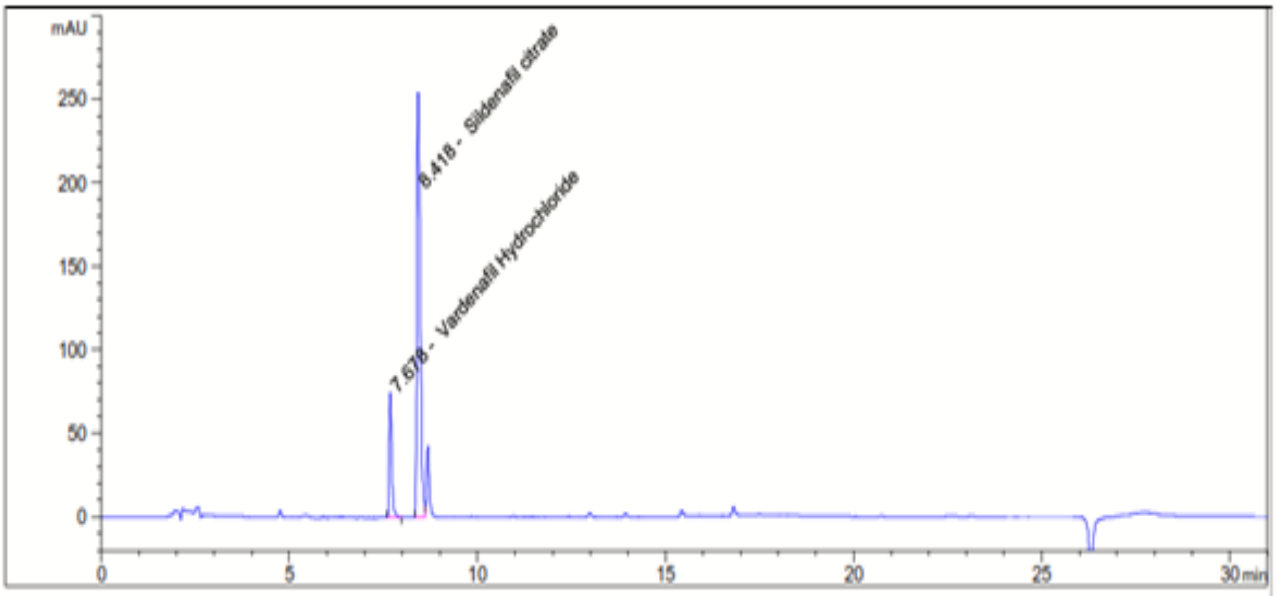

Figure 6: HPLC chromatogram for sample QC1309-17/18 with a chromatographic peak at 7.578 min and 8.418 min corresponding to vardenafil hydrochloride and sildenafil citrate

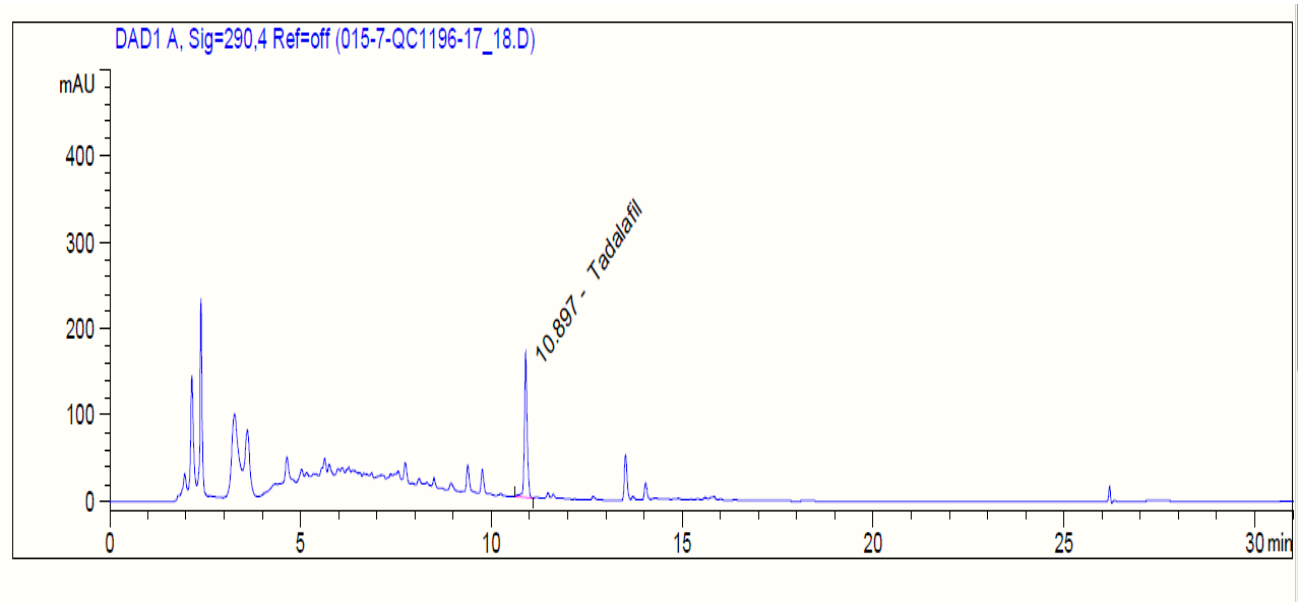

Figure 7: HPLC chromatogram for sample QC1196-17/18 with a chromatographic peak at 10.897 min corresponding to tadalafil

Tables 1 and 2 below show locally manufactured and imported aphrodisiac herbal medicine samples, respectively, that were found in this study to be adulterated with the three PDE-5 inhibitors. The adulterant concentration is presented in mg/g of sample.

Table 1: Local aphrodisiac herbal medicines adulterated with PDE-5 inhibitors

Sr. No.	QC Number	Concentration in mg/g of sample		
		Sildenafil citrate	Tadalafil	Vardenafil hydrochloride
1.	QC1307-17/18	0.16	-	-
2.	QC1306-17/18	0.21	0.32	-
3.	QC1322-17/18	13.61	0.14	-
4.	QC1318-17/18	0.96	2.10	-
5.	QC1309-17/18	0.67	-	0.18
6.	QC1213-17/18	0.12	0.08	-
7.	QC1211-17/18	1.11	1.87	1.18
8.	QC1212-17/18	0.43	-	0.53
9.	QC1192-17/18	11.38	5.97	2.14
10.	QC1202-17/18	-	0.43	0.53
11.	QC1314-17/18	0.13	-	0.83
12.	QC1319-17/18	0.16	0.69	1.50
13.	QC1190-17/18	61.66	-	-
14.	QC1203-17/18	-	0.86	-
15.	QC1320-17/18	10.69	1.61	-
16.	QC1210-17/18	1.15	-	0.11
17.	QC1315-17/18	12.41	0.64	-
18.	QC1317-17/18	0.52	-	-

Table 2: Imported aphrodisiac herbal medicines adulterated with PDE-5 inhibitors

Sr. No.	QC Number	Concentration in mg/g of sample		
		Sildenafil citrate	Tadalafil	Vardenafil hydrochloride
1.	QC1379-17/18	80.75	-	-
2.	QC1199-17/18	8.16	0.43	0.83
3.	QC1200-17/18	78.29	-	-
4.	QC1378-17/18	22.4	0.26	-
5.	QC1381-17/18	-	0.18	-
6.	QC1194-17/18	0.12	-	-
7.	QC1196-17/18	-	1.5	-
8.	QC1197-17/18	-	4.10	-
9.	QC1198-17/18	-	93.70	

HPLC permitted identification and quantification of adulterants within each sample in mg/g of sample. Twenty-seven samples (54%) were adulterated with synthetic PDE-5 inhibitors. Notably, 69.2% (9 out of 13) of the imported products and 48.6% (18 out of 37) of the locally manufactured samples were adulterated as shown in tables 1 and 2 above. The amounts of sildenafil citrate, vardenafil hydrochloride and tadalafil found, allowed comparisons to be made with therapeutic doses of sildenafil citrate in Viagra®, vardenafil hydrochloride in Levitra® and tadalafil in Cialis®. Herbal samples were found to be adulterated with vardenafil hydrochloride, sildenafil citrate and/or tadalafil at levels that ranged between 0.11-2.14 mg/g, 0.12-80.75 mg/g and 0.14-93.70 mg/g, respectively. The maximum daily doses of these molecules are 20 mg of vardenafil hydrochloride, 100 mg of sildenafil citrate and 20 mg of tadalafil recommended for Levitra® [24], Viagra® [25, 26] and Cialis® [27], respectively.

Sample QC1192-17/18 formulated as a capsule contained tadalafil at a concentration of 93.70 mg/g. The prescription on this sample was one capsule per day; the sample had an average weight of 1.4325 g per capsule. This implied that patients were unknowingly being exposed to 65.41 mg of tadalafil. This was over three times the maximum daily dose of tadalafil [27]. Our results were in agreement with Ahmed and co-workers (2015) who reported adulteration of herbal preparations, with sildenafil detected at 165 mg and tadalafil detected at 60 mg contrary to the maximum recommended doses of 100 mg for sildenafil and 20 mg for tadalafil [28]. This is extremely dangerous as it can result in priapism [29, 30]. Priapism is a medical emergency that if not treated quickly enough can lead to permanent damage of the penis.

Although the concentration of PDE-5 inhibitors in most of the investigated herbal aphrodisiac samples were in sub-therapeutic ranges, their regular use can cause serious adverse effect particularly with nitrates [13]. This is particularly the case when patients believe they are consuming harmless herbal products. The easy accessibility and lower pricing of the sildenafil citrate and tadalafil raw materials from illegal sources and the easy accessibility and low pricing of Viagra tablets on the market [31] may in part explain the high incidence of adulteration with sildenafil citrate. Vardenafil hydrochloride was comparatively less encountered as an adulterant.

FTIR confirmation of presence of sildenafil in sample QC1379-17/18

To further confirm the presence of the PDE-5 inhibitors, FTIR analysis was performed on compounds isolated from samples. The overlay FTIR spectrum of the isolated compound obtained from sample QC1379-17/18 and sildenafil reference standard is shown in figure 8. The spectrum exhibited an absorption band at 3305 cm^{-1} which is attributed to the stretching vibrations of the N-H secondary amides. Band at 1583 attributed to the N-H bending vibrations. The C-H stretching in the alkanes was observed at 2870 and 2960 cm^{-1} [21, 32]. A strong intensity

characteristic band was observed at 1689 cm^{-1} attributed to C=O. The aromatic hydrocarbons showed absorption bands in the regions of 1600–1585 cm^{-1} and 1500–1400 cm^{-1} due to carbon–carbon stretching vibrations in the aromatic rings. An asymmetric stretch of the S=O occurred at 1350 cm^{-1} and there was a S=O symmetrical stretching at 1170 cm^{-1} [33, 34]. The medium intensity characteristic for the C-N stretching modes occurred around 1300–1000 $cm^{-1}$and overlapped with the aromatic amines and sulfones. The weak bands observed at wave numbers of 1200–1000 cm^{-1} are assigned to in-plane C-H deformations [34, 35]. The spectrum obtained from the isolated compound was found to be in conformity with that of sildenafil citrate reference standard and this confirmed the presence of sildenafil in sample QC1379-17/18.

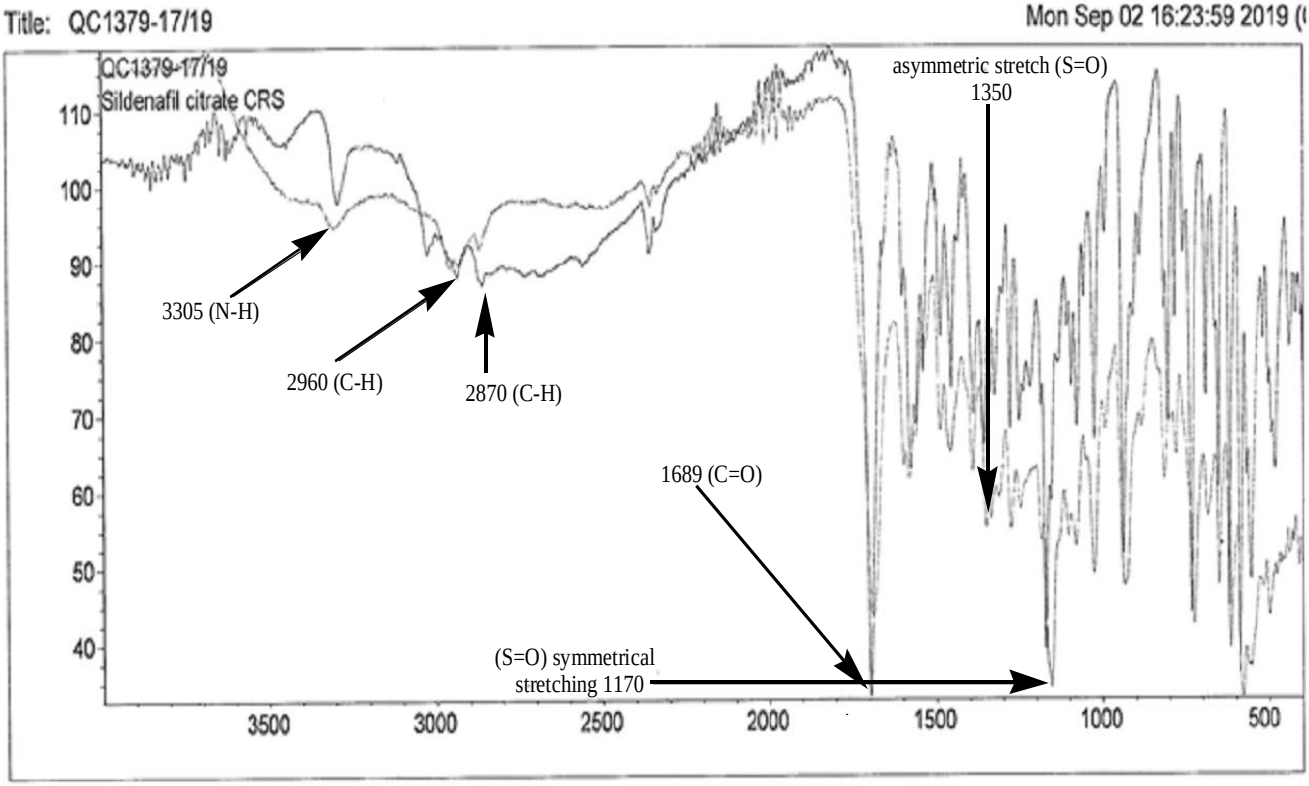

Figure 8: Overlay FTIR spectrum of isolated compound obtained from sample QC1379-17/18 and that of sildenafil citrate reference standard.

A number of samples were found to contain unknown chromatographic spots and chromatographic peaks at different retardation factors and retention times. These could be attributed to the various herbal matrices in the samples or unidentified adulterants. For instance, in addition to the three conventional phosphodiesterase type-5 inhibitors (sildenafil citrate, vardenafil hydrochloride and tadalafil), there are over 50 potentially dangerous unapproved structurally modified analogues of prescription PDE-inhibitors which are normally used as adulterants [14].

The consequences of adulteration may be severe, hinged on the unverifiable quality of the added medicines, unpredictable dosing and exposure of patients to drugs that may be harmful to them by virtue of their health status. The safety and toxicity profile of these adulterated products is often not known and hence consumers of such products are exposed to higher health risks [8, 36]. A high tendency towards these herbal products is due to the unanimous belief that they are safe and as such they are freely dispensed in pharmacies, herbal drug shops and herbal clinics without prescription. Hence, adulteration of

herbal medicines with PDE-5 inhibitors, should be strongly prohibited.

CONCLUSION

This study revealed that herbal products used to treat erectile dysfunction are adulterated with synthetic phosphodiesterase type-5 inhibitors (sildenafil citrate, tadalafil and vardenafil hydrochloride). Thus, patients who use these herbal sexual enhancers with the notion that they are safe and natural are likely to be exposed to serious health risks related to safety and quality of the herbal products. There is therefore need for additional effort to effectively regulate the herbal medicines in order to protect the consumers from emerging threat of adulteration.

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