International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 10, Issue 9, 2018

Original Article

IDENTIFICATION OF SILDENAFIL CITRATE AS AN ADULTERANT IN HERBAL PRODUCTS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOTODIODE ARRAY DETECTOR

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Received: 03 Apr 2018 Revised and Accepted: 24 Jul 2018

ABSTRACT

Objective: Investigation of sildenafil citrate as an adulterant in the traditional liquid herbal products of the local market in Bangladesh.

Methods: A reversed-phase high-performance liquid chromatographic (HPLC) method with photodiode array (PDA) detector system has been developed and validated for investigating the presence of synthetic phosphodiesterase 5 (PDE-5) enzyme inhibitor as an adulterant in the traditional herbal products. Nine of the liquid preparations (syrup), Balarista (A), Jinsant (B), Jernide (C), Bolarist (D), Sree Gopal Oil (E), Menostroge (F), Enerton (G), Ginseng (H) and Ginsin Plus (I), of six companies form local market of Bangladesh were investigated.

Results: All the products (A–I) were found to contain sildenafil citrate as an adulterant. HPLC peak of the adulterant was confirmed by comparing retention time, UV spectra generated by PDA detector and peak spiking with the authentic sample of sildenafil citrate. The quantity of sildenafil citrate in A, B, C, D, E, F, G, H and I syrups were found to be 17, 22, 26, 25, 10, 24, 29, 22 and 17 mg/100 ml, respectively.

Conclusion: The study indicated that all tested liquid herbal products contain sildenafil citrate as an adulterant. As PDE-5 inhibitors have severe side effects, possess drug-drug interaction and highly recommended to prescribe by registered physicians, the regulatory agency of Bangladesh should take necessary action to minimize the risk of patients.

Keywords: PDE-5 inhibitor, Herbal, Adulterant, Male erectile dysfunction (ED), HPLC

DOI: http://dx.doi.org/10.22159/ijpps.2018v10i9.26425

INTRODUCTION

PDE is a ubiquitous enzyme in the human cell that is the essential regulators of cyclic nucleotide signalling with diverse physiological functions. So far 11 subtypes of PDE (1–11) were identified. These enzymes are involved in various regulatory processes like ion-channel functions, cell differentiation, apoptosis, muscle contraction etc. [1]. Nowadays, PDE is considered as an important molecular target in drug discovery. Pfizer initiated the medicinal chemistry research program to develop selective PDE-5 inhibitors for the treatment of hypertension and other cardiovascular diseases in 1985. Sildenafil was synthesized in that project as an active drug [2]. During the clinical trial, sildenafil serendipitously found to have an effect in penile engorgement. This drug was eventually approved by Food and Drug Administration (FDA) of USA in1998 for the treatment of ED. Sildenafil is selective to PDE–5 compared to PDE1–4, but it is less selective to PDE–6. As PDE–6 is found in the retinal cell, use of sildenafil is associated with visual side effects [2]. Moreover, sildenafil also showed several drug-drug interactions and contraindicated to organic nitrates. Concomitant use of sildenafil and organic nitrates might cause severe and fatal hypotension [3].

Though sildenafil is approved as the prescription drug, it has great abuse potential for recreational purpose. Recently in the literature, it was reported that sildenafil is used as an adulterant in several traditional Chinese medicines that were marketed in Singapore and Denmark [4]. In the USA, the presence of sildenafil was reported in dietary supplements and bulk herbal products [5]. In Singapore, cases of 22 fatalities were reported during 1998–2009 due to the adverse reactions of adulterated herbal drugs and sildenafil was detected as one of the adulterants in those herbal products [6]. In India, one out of tested 85 Ayurvedic preparations from the local market was found to be adulterated with sildenafil [7]. Recently, In Bangladesh, a study was conducted using 35 traditional medicines and dietary supplements. The result indicated that 20 % traditional medicines and 70 % of the dietary supplements were found to contain sildenafil citrate as an adulterant. They also analysed two liquid dosages form among 35 products and found that one liquid dose of traditional medicines contains the high concentration of sildenafil citrate as an adulterant. They concluded that more study is essential for the falsified products available in Bangladesh to observe the trends and find out the risk [8]. As traditional liquid medicines contain the high concentration of sildenafil citrate and there are many popular liquid herbal products available in the market as the sole remedies for physical and sexual weakness, it's important to conduct the study in those products to protect the people from deadly effect.

Many people of Bangladesh prefer to use liquid herbal products from their common believes that these products might have fewer side effects with maximum therapeutic benefit. However, if a patient with diabetes or ischemic heart diseases or related diseases uses sildenafil adulterated herbal products, it might possess a great health threat [3, 6, 9]. Therefore, monitoring for synthetic adulterants in marketed liquid herbal products which are most commonly used as strength and energy booster throughout Bangladesh is highly recommended for the safety of local people who solely depend on herbal remedies. In this study, we investigated the presence of specific PDE-5 inhibitor in the most popular marketed liquid herbal products in Bangladesh by HPLC system to confirm their safe use.

MATERIALS AND METHODS

Reagents and materials

Sildenafil citrate INN as a standard PDE-5 inhibitor was purchased from Pol Pharma (Poland, Batch No. 101121128, Potency 99.99%). Potassium dihydrogen phosphate and phosphoric acid (85%) were purchased from Merck (Germany), water (HPLC grade) and acetonitrile (HPLC grade) from Active Fine Chemicals Ltd. (Dhaka,

Bangladesh). The Broncochin used for the treatment of respiratory tract diseases was purchased from Unani Herbal Ltd (Bangladesh) as the blank herbal product. The herbal products (A) Balarista syrup 450 ml (Batch No. 071), (B) Jinsant syrup 450 ml (Batch No. 036) and (C) Jernide Syrup 100 ml (Batch No. 005) of Hamdard Laboratories (Bangladesh), (D) Bolarist Syrup 400 ml (Batch No. 11), and (E) Sree Gopal Oil 50 ml (Batch No. 02) of Modern Herbal Research Garden (Bangladesh), (F) Menostroge syrup 400 ml (Batch No. 12) of MXN Homoeo Laboratory (Bangladesh), (G) Enerton syrup 200 ml (Batch No. 307003) of Square Herbal and Nutraceuticals Ltd. (Bangladesh), (H) Ginseng syrup 100 ml (Batch No. 03/12) of MaxFair and Company Ltd. (Bangladesh) and (I) Ginsin Plus Syrup 450 ml (Batch No. 001) of Muslim Pharmaceuticals (Unani, Rajshahi, Bangladesh) were purchased from local market of Bangladesh.

Standard preparation

The stock solution of standard sildenafil citrate was prepared by dissolving 1.5 mg/ml in methanol based on the solubility of the synthetic drug and stored in a refrigerator and brought to room temperature prior to use. The working solution was prepared by making a serial dilution of the stock solution with methanol to about 0.0467 mg/ml. The injection volume was 20 µl for each working solution and the procedures were done for triplicate.

Samples preparation

One ml of each liquid herbal formulation was put into a 50 ml volumetric flask and taken to the volume with methanol. The flask was shaken vigorously and sonicated for 20 min at 40˚C temperature. The solutions were filtrated by filter paper to remove the unwanted materials, and the supernatant was again filtrated by a membrane filter to make a clear liquid solution of each herbal product. Then 1 ml of the clear solution of each formulation was taken in the HPLC vial and 20 µl of solution was injected.

HPLC method development

A Shimadzu SIL–20AHT prominence HPLC system controlled by LCsolution LC–Assist Software, version 2.1., with a Shimadzu SPD-M20A prominence PDA detector (Shimadzu Corporation, Kyoto, Japan) was used. Phenomenex® Luna Analytical column (particle size: 5µ, stationary phase: C18 (ODS), pore size: 100 Å) was used under the isothermal condition at 40˚C. The system was pumped at a flow rate of 1.0 ml/min and full UV spectra were recorded on-line during the 30 min chromatographic run. The selection of the mobile phase was carried out in accordance with published literature [11] and consisted of (A) acetonitrile: 50 mmol potassium dihydrogen phosphate (adjust to pH 2.5 with phosphoric acid) (10:90, v/v) and (B) acetonitrile: 50 mmol potassium dihydrogen phosphate (adjust to pH 2.5 with phosphoric acid) (70:30, v/v); which were used in a gradient mode at a flow rate of 1.0 ml/min. The gradients system was: Tmin/A: B (V/V); T 0.01-5/100:0.00, T15/40:60, T20/0.00: 100, T25/100:0.00, T30/100:0.00.

HPLC method validation

The validation of HPLC method was performed based on the published literature [10] with slight modification. Initially, the separation of sildenafil citrate was achieved by changing the ratio of mobile phase A and B. The best separation of the two drugs was achieved using gradient system and by retaining the pH 2.5 of buffer solution. The injection volume was 20 µl, and wavelength range was maintained at 190–400 nm using a PDA detector.

The method was validated based on the linearity, accuracy, and precision. For the linearity, solutions of sildenafil citrate were prepared in the range of 0.75–0.0467 mg/ml and the method were performed in triplicate. Using these concentrations of standard drugs, the calibration lines were generated, and regression parameters were established. The concentrations of the standards and samples were evaluated by the established calibration lines.

The percentage recovery, accuracy and precision of spiked sildenafil citrate at different concentration ranges were also evaluated. The concentration of spiked standards was also evaluated by using calibration lines. The limit of detection (LOD) and limit of quantification (LOQ) were also determined. For LOD and LOQ, the sample showing no signals at the retention time (RT) was considered as the blank and spiked sample that gave three times higher response in blank was taken as LOD and spiked sample that gave ten times response in blank was considered as LOQ.

Spiking studies of herbal products (A–I) with standard

For the confirmation of synthetic sildenafil citrate, all herbal products were spiked with 10 µg of standard sildenafil citrate in compliance with published literature [7]. In briefly, 1 mg of the standard was dissolved to make 1 mg/ml stock solution. The stock solution was diluted 10 times to make a solution of 100 µg/ml, then 100 µl of this stock solution was put in a HPLC vial containing 900 µl solution of herbal product to make a working solution of 10µg/ml standard sildenafil citrate. The working solutions were injected into HPLC system for the confirmation of standard sildenafil citrate in nine herbal products (A–I).

Quantification of sildenafil citrate in herbal products (A-I)

For the quantification of the amount of sildenafil citrate in herbal products (A–I), all products were injected in triplicate under the developed operating condition and the mean amount of the three injections was recorded as the total amount of standard sildenafil citrate in herbal products as an adulterant.

RESULTS

For standard sildenafil citrate, three calibration standards were prepared to evaluate the relationship between the area under the curve and the concentration. The linearity of the relationship was determined for standard sildenafil citrate in a concentration range of 0.75–0.047 mg/ml. The calibration curves were obtained using linear regression and were confirmed with the R^2 values. Table 1 showed the mean R^2values and demonstrated that the standard calibration curves for sildenafil citrate are linear within the selected concentration ranges. To evaluate the LOD and LOQ, the different concentrations of the standard were mixed in blank herbal products (herbal product without PDE–5 inhibitors). Table 1 showed the values of LOD and LOQ. The results indicated that the LOD was 0.02 µg and LOQ 0.07 µg/ml.

Table 1: Linearity, LOD, LOQ

Compound	R^2	LOD (µg)	LOQ (µg)
Sildenafil citrate	0.9997±0.0005	0.02	0.07

The results are mean±standard deviation for n= 3 determinations with detection at 293 nm

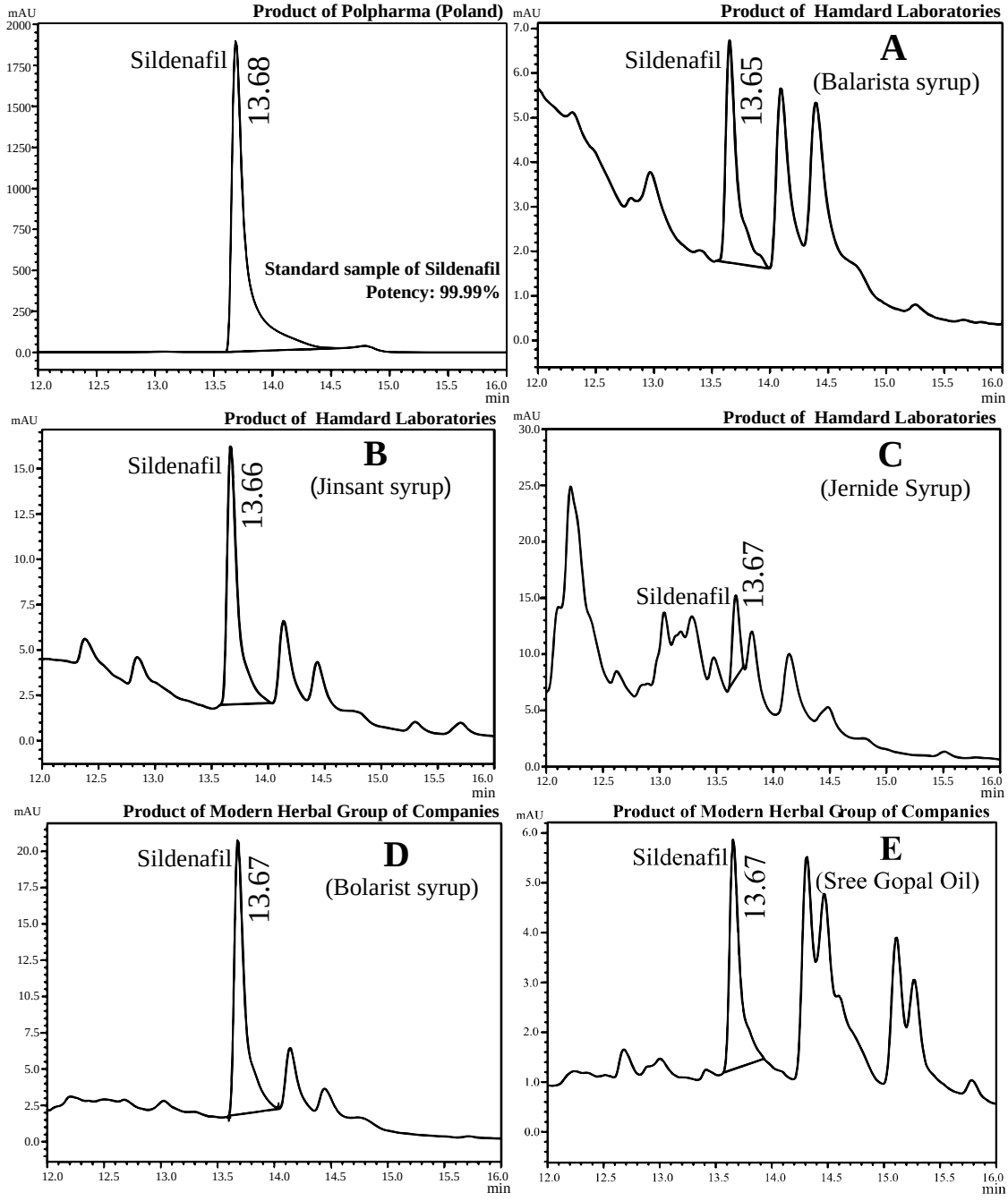
The recovery study of the standard was determined using four concentration ranges. It was determined by different spiked concentrations of standard sildenafil citrate in the blank solvent. The calculation for the recovery studies was done using standard calibration lines. The result (table 2) indicated that the mean recoveries of sildenafil citrate were ranging from 92.4–105% and ensured that the method was suitable for analysis. From the recovery studies, accuracy and precision were also evaluated and shown in table 2. The limits of accuracy were between (-5.0)–7.60% and the limits of precision were in a range of 0.94–1.95%. The results of recovery studies, accuracy and precision indicated that the method was suitable for analysis. As all the results for validation of methods were suitable for analysis, therefore the analysis of herbal products was conducted in accordance with this method.

Table 2: Recovery, accuracy and precision studies

Sample	Spiked concentration (µg/ml)	Calculated spiked concentration (µg/ml)	Recovery (%)	Precision (%RSD)	Accuracy
Sildenafil citrate	40	42.0±0.82,	105.0	1.95	-5.0
	250	231±2.16	92.4	0.94	7.6
	800	748.25±8.14	93.5	1.09	6.47

The results are mean±standard deviation for n= 4 determinations with detection at 293 nm

Fig. 1 showed the HPLC chromatogram of standard sildenafil citrate and herbal products (A–I). The chromatogram indicated the RT of standard sildenafil citrate at 13.6 min. The HPLC chromatogram also indicated that all herbal products (A–I) showed a large peak at 13.6 min. The results revealed that all herbal products might contain sildenafil citrate. Although the sildenafil citrate and herbal product showed the similar peak at 13.6 min, sometimes different compounds can show the same retention time in HPLC analysis. Therefore, the ultraviolet spectrums (UV max) of identified peaks at 13.6 min. were also evaluated.



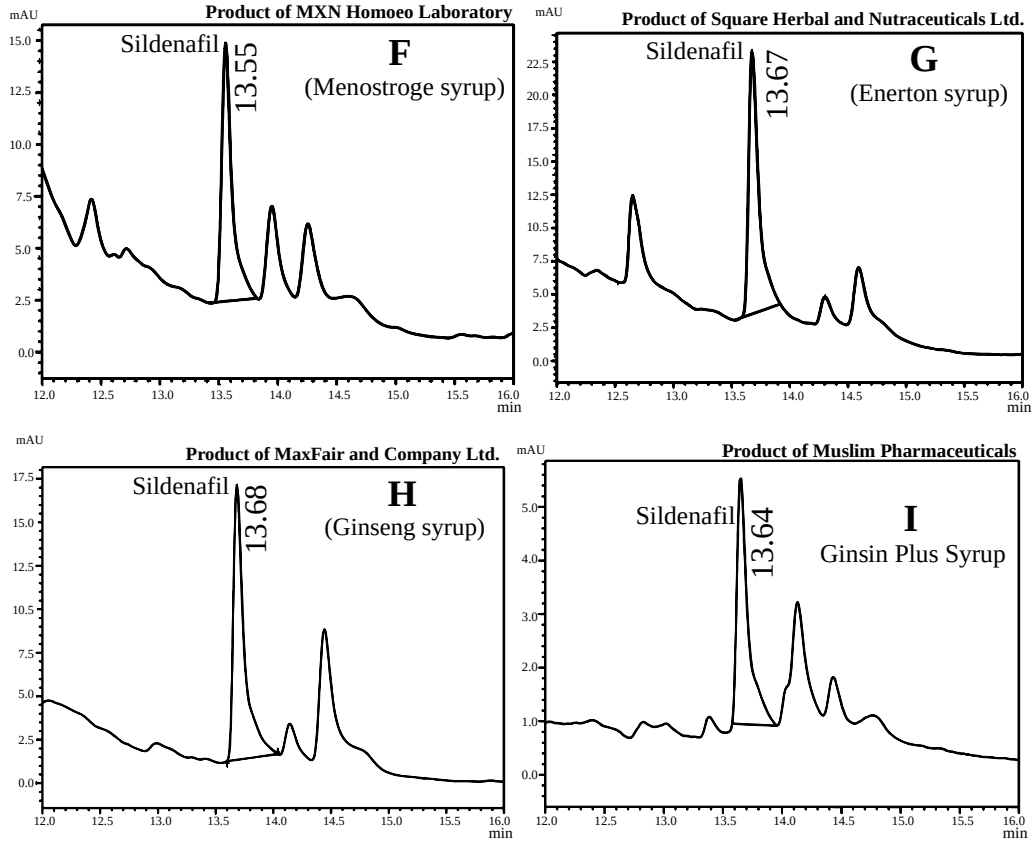


Fig. 1: HPLC chromatograms of standard sildenafil citrate and herbal products (A-I)

Fig. 2 showed the HPLC chromatograms of A (1)–I (1) of herbal products and A (2)–I (2) of sildenafil citrate spiked in herbal products (A–I). The results indicated that the retention time of peaks of herbal products and the retention time of sildenafil citrate spiked in herbal products were similar. Moreover, the UV spectrums of these peaks were also similar in both herbal product and spiked herbal products. These results indicated that the retention time of peaks of herbal products at 13.6 min was due to the presence of sildenafil citrate. Based on the above analytical parameters, it was confirmed that all herbal products contained PDE-5 inhibitor such as sildenafil citrate. Therefore, the quantity of sildenafil citrate in each herbal product was also evaluated.

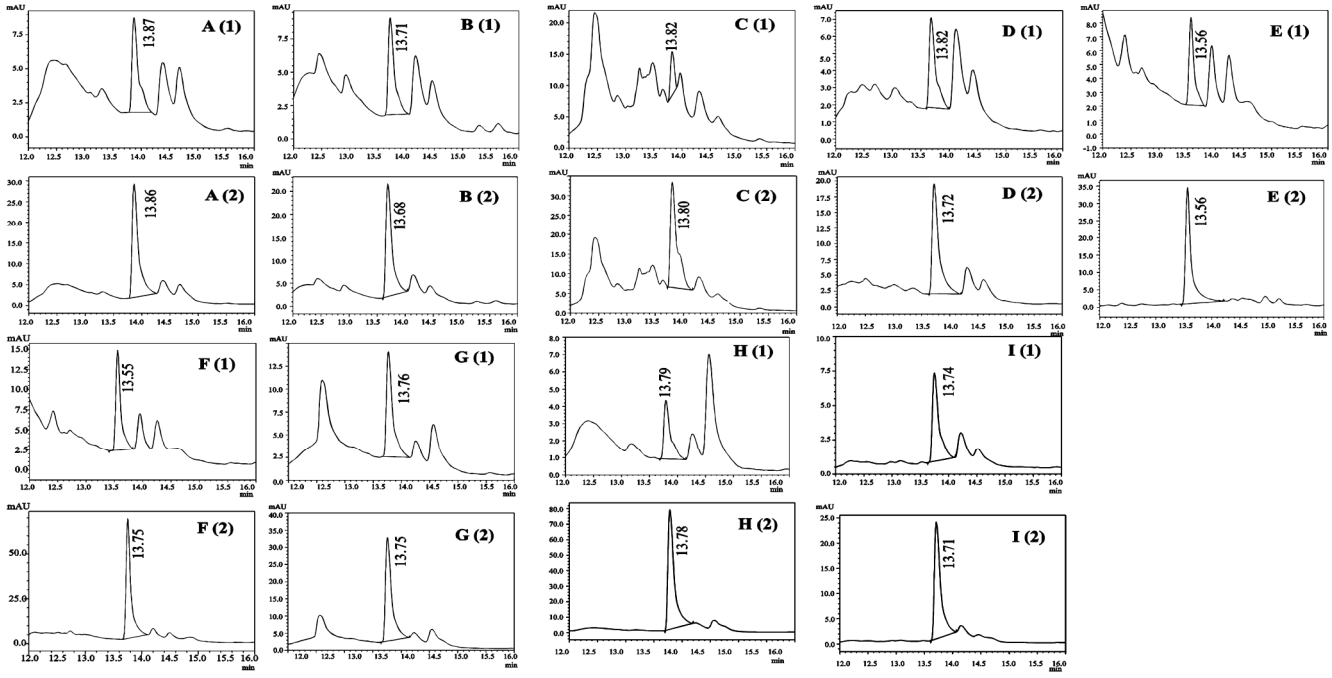


Fig. 2: HPLC chromatograms of (1) herbal products (A-I), and (2) sildenafil citrate spiked in the herbal products (A-I)

Table 3 showed the quantity of sildenafil citrate as an adulterant in herbal products. The results indicated that the herbal products Balarista (A), Jinsant (B) and Jernide (C) of Hamdard Laboratory contained 17, 22 and 26 mg, respectively, Bolarist (D) and Sree Gopal Oil (E) of Modern Herbal contained 25 and 10 mg, respectively; Enerton (G) of Square Herbals contained 29 mg, Mengstroge (F) of MXN Homoeo contained 24 mg, Ginseng (H) of MaxFair contained 22 mg, and Ginsin Plus (I) of Muslim Pharmaceuticals contained 17 mg of sildenafil citrate in 100 ml syrup.

Table 3: Quantity of sildenafil citrate as an adulterant in herbal products (A-I)

Sample	Dosage	Sildenafil citrate (mg/100 ml of syrup)
A	Liquid	17±1.5
B	Liquid	22±2.1
C	Liquid	26±1.8
D	Liquid	25±2.7
E	Liquid	10±1.1
F	Liquid	20±3.1
G	Liquid	29±2.8
H	Liquid	22±1.6
I	Liquid	17±1.1

The results are mean±standard deviation for n= 3 determinations

DISCUSSION

The results indicated that the herbal companies of Bangladesh use the large amount of synthetic PDE–5 inhibitors such as sildenafil citrate in their formulations. Some products even contain the high amount of sildenafil citrate compared to others. However, according to Pfizer labs, the recommended dose is 50 mg per day with a maximum dose must not exceed 100 mg [12]. So, the local people are unaware of their doses regimen as they buy these products from local market without knowing the presence of synthetic sildenafil citrate which possesses fatal effect on the human health with the high dose.

Oral PDE-5 inhibitors are the treatment for the ED patients. The mechanism involves the release of nitrite oxide in the corpus cavernosum during the physical relationship. Then, Nitric oxide activates the enzyme guanylate cyclase, which increases the levels of cyclic guanosine monophosphate (cGMP), leading to smooth muscle relaxation in blood vessels and allowing blood flow in the corpus cavernosum. Based on the literature, this mode of action only happens in sexual intimation. FDA recommended sildenafil citrate for ED patients. However, it is suggested that the patients must take sildenafil citrate by consulting with the registered physician for the safe use. This drug must not be used without medical examination and prescription by the registered physician [2,13]. In Bangladesh, patients are buying these liquid herbal products without physical examination and prescription by the registered physician. The use of these herbal products might possess a serious unwanted effect on patient's health.

In addition, the sildenafil citrate also increases the risk of many cardiovascular diseases including heart attack, myocardial infarction, and sudden death. It's also possible that the medication could interact with other drugs and may lead to fatal effect, for example, sildenafil citrate causes the synergic effect with alpha-blockers which could lead to the dangerous health issue [13].

It is also recommended that sildenafil citrate must not buy from any nonstandard sources including online sources and products without labeling of sildenafil citrate. In this study, we found that all liquid herbal products mention that they use traditional medicines in their formulations. They also have no labeling about synthetic sildenafil citrate in their formulation. Moreover, it is also prohibited for the herbal companies to use any synthetic product in their formulation as herbal formulation must contain traditional medicines and use of synthetic medicines could cause counter interaction with natural medicines.

However, In Bangladesh, most of the well reputed herbal companies are using synthetic sildenafil citrate as an adulterant in their liquid formulations. As the PDE-5 inhibitors cause the serious health problem, the use of these herbal products by patients may also cause fatal effect as the patients received these products without proper guidelines. Therefore, the government and the people of Bangladesh should be aware of these herbal products because the use of these products might cause patient death.

CONCLUSION

The study presents that all herbal products (A-I) were found to contain a synthetic PDE–5 inhibitors "sildenafil citrate". The results suggested that as the synthetic PDE-5 inhibitors have several side effects, possesses severe drug-drug interactions and highly recommended to prescribe with caution for patients with various health problems, the use of synthetic agent in herbal products for the purposes of ED, physical and sexual weakness, and fatigue and as tonic might cause fatal effects. Therefore, the drug regulatory agency of Bangladesh should take necessary action urgently to minimize the future risk in patients.

AUTHORS CONTRIBUTIONS

All authors equally contributed to drafting the paper. All authors have read and approved the final manuscript.

CONFLICTS OF INTERESTS

All authors declare no conflicts of interest

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