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Research Article

QUALITATIVE AND QUANTITATIVE ANALYSIS OF SILDENAFIL IN TRADITIONAL MEDICINES AND DIETARY SUPPLEMENTS

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ABSTRACT

Objective: Use of traditional medicines as aphrodisiac and sex stimulant products is a common phenomenon worldwide as well as in our country. In addition to that, in the name of dietary supplements, a number of urban people are using these items as the source of their sex potential. These products ought to be responsible for raising several sexual violence and perversion in the society. That's why these products need to be tested whether they contain only natural ingredients or any other allopathic active pharmaceutical ingredients (APIs).

Methods: High performance liquid chromatography was carried out using a C_{18} column with a mobile phase composed of (diluted sulfuric acid: acetonitrile=60:40) and detected at the wavelength of 290nm. Presence of sildenafil in the identified products was further confirmed by infrared (IR) spectroscopy.

Results: In the study, found that 5 out of 25 traditional medicines and 7 out of 10 dietary supplements contain sildenafil. In addition to that 4 out of total 35 samples were found to contain sildenafil that was exceeding the highest dose (100mg per dosage unit) recommended by the manufacturer (Pfizer, UK) of the brand product Viagra™. Notably none of the samples were labelled to contain sildenafil.

Conclusion: About 35% of the market samples were adulterated with sildenafil. The developed method is simple, precise, specific, accurate, robust, and cost-effective. Hence it can be used for qualitative and quantitative analysis of sildenafil in falsified market products.

Keywords: Traditional medicines, Dietary supplements, Sildenafil, IR, HPLC and Validation.

INTRODUCTION

Traditional medicines (Unani and Ayurvedic systems) are very popular in our country. Though modern healthcare (allopathic) facilities are now readily available in the neighborhood, a good proportion of our people particularly in the rural and semi-urban areas still desire to use this system for their usual ailments such as common cold, cough, fever, headache, diarrhea, itching, constipation, jaundice, piles and etc. [1]. There is a common belief that these products utilize natural ingredients to produce their medicinal preparations and people have a general perception that natural equates to safe. So, they are free from the potential of side effects. These products are advertised as "all natural" but the present study had revealed that many of these products contain synthetic phosphodiesterase type-5 (PDE-5) inhibitors like sildenafil. Manufacturers of these medicines also promote this belief. Taking the advantage of general people's psychology and due to the inherent property of natural products to show their efficacy slowly, some companies are illegally incorporating pharmaceutical active substances and their structurally related analogues with these products to win a fast business in the competitive market. No toxicological study or clinical trials have been carried out on them, thus they can be very dangerous. Besides traditional medicine, use of dietary supplements is becoming popular among the people of the urban areas along with modern medicine now-a-days from a belief that these products contain very necessary ingredients that are lacking in the normal diet. But if the manufacturers deliberately add APIs in these products for getting specific therapeutic benefits, this is again illegal and deceptive to the consumers. On the other hand, if these products contain any APIs that are potentially harmful for public health, this secret incorporation of APIs in food supplements can be considered as a crime.

Bangladesh government has published separate formularies for all the components of Traditional Medicine (e.g. Unani Formulary, Ayurvedic Formulary, Homeopathic Formulary etc.) to help manufacturers to make products as per the guidelines specific for their respective systems. Use of allopathic ingredients (APIs) in either of the Unani, Ayurvedic or Homeopathic systems is unlawful because of these mandatory guidelines. Considering the severe adverse effects of the overdose of some APIs, it is a serious potential hazard for the user of traditional medicines as they might be even life-threatening. Regulations for dietary supplements (including herbal products) are not the same as those for prescription or over-the-counter drugs. In general, the regulations for dietary supplements are less strict as they require no prescription to buy.

It had been reported in various journals [2,3,4,5] and newspapers[6,7] that some of the allopathic active ingredients (APIs) are being used in traditional products unlawfully & unethically putting the public health in a state of serious threat. One investigation of 2,600 samples by E. Ernst revealed that 24% of Chinese herbal remedies in Taiwan were adulterated with at least one synthetic medicine [2]. In another survey conducted in USA, showed that 7% of their herbal products were adulterated with at least one synthetic medicine [3].

It is now well established clinically that PDE–5 inhibitors are the effective oral treatment of choice for male erectile dysfunction (ED) [8,9]. Among the three most popular PDE-5 inhibitors (Sildenafil, Tadalafil and Verdenafil) the first developed and consequently the most famous and relatively cheap PDE-5 inhibitor is sildenafil, approved by the Food and Drug Administration (FDA) in early April, 1998.The drug was the first oral treatment for men suffering from ED. Though ED is uncommon among young men but the indication of this drug has raised its recreational use among them over the years [10,11,12]. The USFDA had also warned their consumers that there is possibility of containing sildenafil or its analogues in any sexual enhancement product that claims to work as like as prescription drugs [13].

Sildenafil Citrate (SC) chemically,1-[4-ethoxy-3-(6, 7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo [4,3d] pyrimidin-5-yl)phenylsulfonyl]-4-methylpiperazine citrate[14], as shown in fig. 1, is the active ingredient of the brand product VIAGRA™, is a prescription drug for ED patients but not for all [15]. It gives action by inhibiting cGMP-specific phosphodiesterase type-5, an enzyme that causes degradation of cGMP, which controls blood flow in the

penis [8]. Its recommended doses are either 25mg, 50mg or 100mg not more than once daily. The drug has a broad profile of side effects and adverse effects along with its efficacy like irregular heartbeat, shortness of breath, angina, myocardial infarction, stroke, priapism, sudden hearing loss and blindness etc. [8].

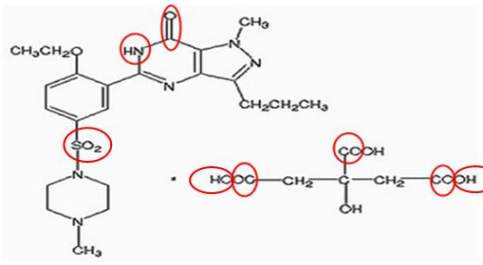


Fig. 1: Structural formula of SC

If this drug is intentionally incorporated in traditional medicines and dietary supplements without any labelling unethically, it will put public health in a state of serious threat.

So, in this study an initiative to investigate on this type of falsifications in our country has been taken. The objectives of the study are:

- To get an idea on the unethical use of SC in the name of traditional medicines and dietary supplements without labelling.
- To identify the products that exceeding the highest dose recommended by brand manufacturer Pfizer.

MATERIALS AND METHODS

Reagents and Materials

SC working standard (Source: SMS Pharmaceuticals Limited, India) was obtained from ACI Limited, Narayanganj-1400. A total of 35 market samples were collected randomly from different retail stores of Dhaka considering the popularity in the local market in April, 2013. Among them 25 were traditional medicines and 10 were dietary supplements. Out of total 35 samples 18 were tablets/pills, 15 were capsules and 2 were syrups. All the samples were claimed to contain natural ingredients as per their respective formularies. The samples were randomly numbered from 1 to 35. All reagents like methanol, acetonitrile and water used were of HPLC grade and sulfuric acid used were of analytical grade.

Methods

Various methods were applied to detect and estimate SC in pharmaceutical falsifications. Infrared Spectroscopy [16], Raman Spectroscopy [17], HPLC [14, 18, 19], LC-MS [4, 5] techniques were developed over the years for this purpose. Among the above methods HPLC and IR are the more convenient and cost-effective methods for qualitative and quantitative analysis. That's why this study had been designed to develop a simple, precise, specific, accurate, robust, cost-effective and validated RP-HPLC method according to USP and ICH guidelines [20, 21] to determine the presence and amount of SC in traditional products and dietary supplements. After that presence of sildenafil in the identified products was further confirmed by infrared (IR) spectroscopy.

Chromatographic Conditions

Chromatographic Parameters

A Shimadzu HPLC - Prominence integrated with photo diode array detector and degasser was employed to carry out the investigation. The chromatographic separation was performed using a Prontosil Kromaplus C_{18}, 250 mm length × 4.6 mm internal diameter with 5µ or equivalent particle size column. The mobile phase composed of diluted sulfuric acid**:** acetonitrile (60**:**40, v/v) was pumped at a flow rate of 0.5 $mLmin^{-1}$. The wavelength of detection was set at 290 nm. The column temperature was maintained at 30°C. The injection volume was set at 10µL. Mobile phase was used as diluting solution to prepare both of the standard and test sample preparations.

Preparation of Mobile Phase

34 mL of 2N sulfuric acid was taken to a 1000 mL volumetric flask containing 566 mL of distilled water. The solution was mixed with 400 mL of HPLC grade acetonitrile and then sonicated for 15 min followed by filtering using 0.2 µm membrane filter.

Preparation of stock solution of standard

100 mg working standard of SC was dissolved into 100mL mobile phase to give a concentration of 1 $mgmL^{-1}$.

Preparation of sample solution

Approximately 30 mg of sample (enough to give response within the linearity range of concentration) was weighed and taken into a clean and dry 25 mL volumetric flask. About 15 mL of diluting solution was added to it and shaken vigorously to extract the drug from the ingredients of the sample. The resulting solution was sonicated for 5 min. After cooling to room temperature its volume was adjusted up to the mark with the same diluting solution. Then different sample solutions were injected consecutively in triplicate into the HPLC machine and the chromatograms were recorded. Different analytical parameters such as linearity, accuracy, specificity, precision, robustness, sensitivity (limit of detection and limit of quantification) and system suitability were determined to validate the proposed method according to ICH guidelines [21].

Chromatographic Method Validation Parameters

Validation of the proposed method was performed [22] using standard SC and test sample no. 34.

System Suitability

To determine system suitability of the proposed method, at first retention time, peak area (%RSD NMT 2.0), theoretical plate (NLT 3000) and tailing factor (NMT 1.5) of six replicate injections of standard SC of 250 μgmL^{-1} (nominal concentration) were determined. Then the percentage of relative standard deviation (%RSD) values were determined for each case.

Linearity

To determine linearity of the proposed method, five different concentrations of SC i.e. 80%, 90%, 100%, 110% and 120% of nominal concentration ranging from 200 to 300 μgmL^{-1} (by diluting appropriate amounts of stock solution) were prepared. A calibration curve was constructed in the specified concentration range by plotting peak area versus concentration. The linear relationship was evaluated using the least square method within Microsoft Excel® program.

Specificity

The specificity of the proposed method was determined by comparing the chromatograms of both standard and sample to investigate whether there is any interference of unknown excipients present in the market samples with the same retention time of SC peak. A blank sample was also run to check any interference of it. The peak purity tool was utilized to check the purity of both standard and sample solution.

Accuracy

To determine accuracy of the proposed method, recovery experiments were performed by the standard addition method. The study was performed by spiking a known concentration of the test sample with appropriate concentrations of standard solution to produce 80%, 100% and 120% of nominal standard concentration. Samples were prepared in triplicate at each levels and the recovery percentage was determined.

Precision

To analyze precision of the proposed method, intraday precision (repeatability) and interday precision (intermediate precision) was measured in terms of %RSD of the measurements for both standard and market sample solutions in nominal concentration. The analysis

was repeated six times on the same day for intra-day precision and on two different days for inter-day precision.

Sensitivity

Following ICH Q2 (R1) recommendations based on signal to noise approach the limit of detection (LOD) was determined for which a signal height to noise ratio of 3 was obtained and for limit of quantitation (LOQ), a signal height to noise ratio of 10 was obtained.

Ruggedness

To determine ruggedness of the proposed method, test sample solution was analyzed in six replicates comparing %RSD of the measurements by two analysts in the same laboratory.

Robustness

To determine robustness of the proposed method, six test sample preparations were prepared and analyzed by varying most critical analytical parameters while keeping the other parameters unchanged. Such as the composition of mobile phase (±5%), flow rate (±0.2 mL/min), detection wavelength (±5 nm), column temperature (±5°C).

Analysis by HPLC

Qualitative Analysis

Sildenafil appeared as a single peak with a retention time of approximately 7 min. It showed maximum absorption of UV light at 294 nm (from spectrum view extracted from that peak). Qualification of sildenafil was performed by comparing the retention times of SC single peak of both standard and sample preparations.

However there is a possibility of appearing a peak for a totally different compound with the same retention time like sildenafil due to having the same elution rate as sildenafil. To eliminate this type of uncertainty, spectrum views (extracted from that peak) in ultraviolet-visible (UV) region (200nm - 800 nm) by means of PDA detector for both standard and sample preparation were also compared in this study.

Quantitative Analysis

The quantity of sildenafil in the identified samples was determined as concentration (μgmL^{-1}) from the calibration curve, substituting the respective peak areas (SC single peak) obtained for the identified sample preparations.

Analysis by IR Spectroscopy

To assist in the identification of the falsified products IR spectrum of sildenafil positive (by HPLC) products were analyzed. IR spectroscopy is concerned with the interaction between a molecule and radiation from the IR region of the EM spectrum (4000 – 400 cm^{-1}). IR spectrum can be divided into two approximate regions:

- Functional group region (4000-1500 cm^{-1}), valuable information are obtained from this region to interpret any IR spectrum.
- Fingerprint region (< 1500 cm^{-1}), usually consists of a very complicated series of absorptions that are characteristic for a particular compound [23].

FT-IR is a very fast technique considering that no further sample preparation is needed and spectrum acquisition requires only a few seconds. By an accurate comparison between the IR spectrum of any sample with that of the original drug it is possible to determine whether they have the same composition or not, thus it permits to ascertain definitively if the investigated sample is falsified or not.

In this study, a Shimadzu IR Prestige-21 fourier transformation infrared (FT-IR) spectrophotometer was employed for qualitative analysis of sildenafil in the identified (by HPLC) market samples. Spectra were recorded with 30 scans and a resolution of $2cm^{-1}$.The approach [24] was:

Firstly, the IR bands distinct for the reference substance due to its functional groups were identified. Each substance has a unique IR spectrum.

Secondly, presence of these distinct bands was searched in the IR spectrum of the samples.

RESULTS AND DISCUSSION

Chromatographic Method Validation

System Suitability

The results are shown in Table 1, indicating good performance of the system.

Table 1: Results of system suitability study for SC

Chromatographic Parameters	Value (Mean ± %RSD)
Retention time (min)	(7.154±0.075)
Tailing factor	(1.387±0.537)
Theoretical Plate	(9486±1.582)
Peak area (mAU)	(5901368±0.273)

Linearity

A linear correlation was found between the peak areas and concentrations of SC in the concentration range of 200 to 300 μgmL^{-1} and the correlation coefficient (R^2 = 0.9991) was found highly significant. The results of regression analysis were presented in Table 2.

Table 2: Results of linearity study for SC

Regression Parameters	Values
Regression coefficient (R^2)	0.9991
Slope	24337
Intercept	-139508
Concentration range(μgmL^{-1})	200 - 300
Number of points	5

Specificity

HPLC chromatogram of standard SC and sample is showed in fig. 2. Standard SC appeared as a single peak with a retention time of approximately 7 min. It was found that unknown excipients were not interfering with the developed method as no peaks were revealed around retention time 7 min and no impurities were detected. Moreover the peak purity was 99.99 % which indicates that SC is clearly separated from other unknown ingredients of the falsified market preparations.

Accuracy

The results were shown in table 3, in terms of %RSD of recovery of the added standard, indicating good accuracy of the proposed method.

Precision

The results were presented in terms of %RSD of the measurements for intraday and interday variations in table 4, indicating good precision of the proposed method.

Sensitivity

The values of LOD and LOQ of SC were presented in in table 5 and their chromatograms were shown in fig. 3, indicating high sensitivity of the proposed method.

Ruggedness

The results were shown in table 6, in terms of %RSD of the measurements.

Robustness

The results were shown in table 7, the proposed method was found to be robust with respect of changes in mobile phase composition, flow rate, detection wavelength and column temperature.

Chromatographic Analysis

Qualitative Analysis

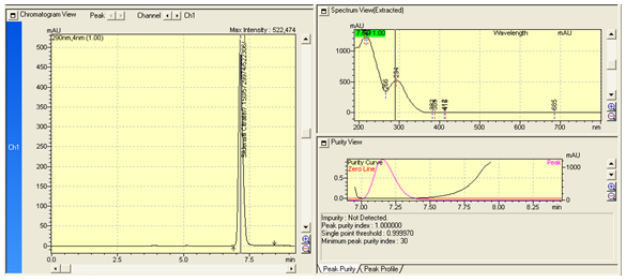
12 out of total 35 samples were found to give response in a zone similar to that of standard. The identified samples not only showed a peak of same retention time as that of standard but also the same pattern in the spectrum view extracted from that peak similar to that of standard as represented in the fig. 2.

Quantitative Analysis

SC content per dosage unit in the identified samples was determined. The results were shown in table 8.

IR Spectroscopic Analysis

Comparing the IR spectrum (Fig. 4) with the structure of standard SC (Fig. 1), 7 functional groups were identified which were summarized in the table 9.



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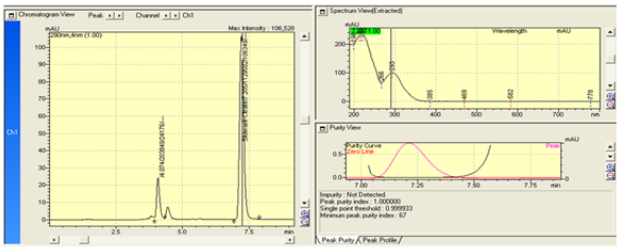

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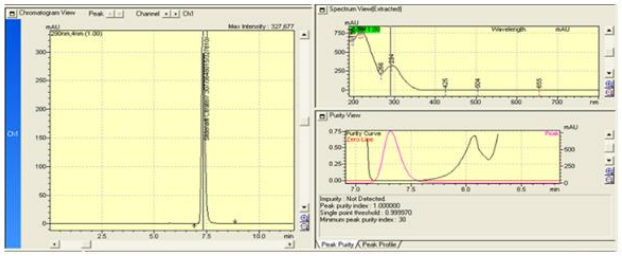


(c)

Fig.2: Chromatogram of (a) standard SC, (b) Sample no. 9, (c) Sample no. 34

Among the identified groups, the ketonic carbonyl group and the secondary amine group produced intense bands in the spectrum at 1702.21 cm^{-1} and 3299.3 cm^{-1} regions respectively. In this study presence of these distinct bands was searched in the IR spectra of the samples. Result of IR analysis of the suspected samples was found congruent with HPLC analysis.

Table 3: Results of Accuracy study for SC

Recovery level	Amount of standard added (µgmL^{-1})	Total amount (µgmL^{-1})	Amount found (µgmL^{-1})	Amount of standard recovered (µgmL^{-1})	% Recovery	Mean of %Recovery ± %RSD*
80%	100	201.5	201.35	99.85	99.85	99.97 ± 0.157
	100	201.5	201.42	99.92	99.92	
	100	201.5	201.65	100.15	100.15	
100%	150	251.5	252.02	150.52	100.35	100.04 ± 0.287
	150	251.5	251.48	149.98	99.99	
	150	251.5	251.17	149.67	99.78	
120%	200	301.5	301.53	200.03	100.02	100.00 ± 0.148
	200	301.5	301.78	200.28	100.14	
	200	301.5	301.19	199.69	99.85	

***Acceptance criteria < 2.0, Fixed sample concentration = 101.5 µgmL-1**

Table 4: Results of intraday and interday precision study for SC

S. No.	Concentration (µg/mL)	Intra-day precision		Inter-day precision	
		Standard*	Sample*	Standard*	Sample*
1	250	5895276	5909364	5895346	5909868
2	250	5915384	5937751	5915874	5937964
3	250	5906275	5915483	5896753	5915297
4	250	5935336	5896594	5935381	5895674
5	250	5909247	5936754	5909349	5936374
6	250	5927321	5895997	5927194	5895564
Avg.		5914807	5915324	5913316	5915124
%RSD		0.247	0.314	0.273	0.317

***Acceptance criteria for RSD of Peak Area < 2.0**

Table 5: Results of sensitivity study for SC

Parameters	Signal height	Noise	Conc. (µgmL^{-1})
LOD	116	38.82	0.0178
LOQ	388		0.0535

Table 6: Results of Ruggedness study for SC

Analyst-1		Analyst-2	
Sample No.	**Peak area***	**Sample No.**	**Peak Area***
1	5908946	1	5908873
2	5938872	2	5928422
3	5916983	3	5976938
4	5893535	4	5892553
5	5934771	5	5937417
6	5895979	6	5895779
Avg.	5914848	Avg.	5923330
%RSD	0.323	%RSD	0.535

***Acceptance criteria < 2.0**

Table 7: Results of Robustness study for SC

Parameters		Retention Time	Tailing factor	%RSD of Peak Area
Mobile Phase Composition	Acid : ACN = 62:38	7.097	1.325	0.594
	Acid : ACN = 58:42	7.208	1.384	0.582
Flow rate	0.3	11.834	1.372	0.387
	0.7	5.089	1.404	0.603
Detection wavelength	285	7.157	1.356	0.375
	295	7.151	1.368	0.353
Column temperature	25°C	7.216	1.349	0.437
	35°C	7.024	1..361	0.386

***Acceptance criteria < 2.0**

Table 8: Quantitation of SC

Sam. No.	Type of dosage form	SC content per dosage unit (mg) ± SD #
8	Tablet	15.86 ± 0.1
9	Capsule	165.87 ± 0.073
11	Capsule	20.52 ± 0.179
12	Capsule	26.36 ± 0.172
13	Syrup	99.59 ± 0.274
26	Capsule	90.54 ± 0.252
28	Capsule	151.74 ± 0.161
29	Capsule	137.04 ± 0.117
31	Capsule	131.4 ± 0.158
33	Capsule	63.28 ± 0.133
34	Tablet	67.7 ± 0.113
35	Tablet	96.54 ± 0.154

 # Mean ± Standard deviation of 6 samples

Table 9: Result of IR spectrum analysis of SC

S. No.	Functional group(s)	Characteristic Absorption(s) range (cm^{-1})	Identified in this study (cm^{-1})
1	SO_2 stretch	1200 - 1100	1172.74
2	Aromatic C=C bond	1700 – 1500	1582.62
3	C=O stretch	1750 – 1680	1702.21
4	Saturated C–H stretch	2950 – 2850	2962.71
5	Unsaturated C–H stretch	3100 - 3010	3029.26
6	Secondary N-H stretch	3500 – 3300	3299.3
7	O-H stretch	3550 – 3200	3617

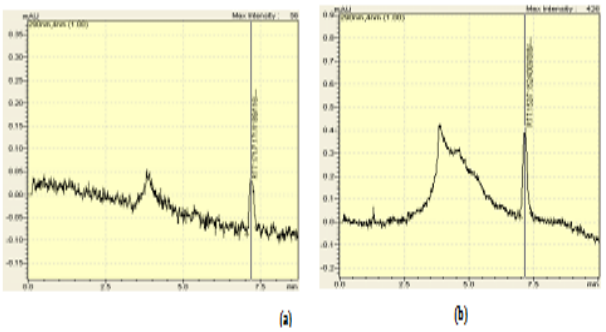


Fig. 3: HPLC chromatogram of (a) LOD of SC and (b) LOQ of SC.

CONCLUSION

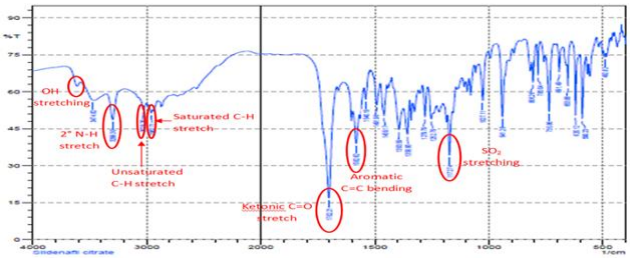
About 35% of the total collected market samples were found to contain sildenafil without any labelling, which may be responsible for some of the reported benefits of these products. 20% (Sam. no. 8, 9, 11, 12 and 13) of the traditional medicines and 70% (Sam. no. 26, 28, 29, 31, 33, 34, and 35) of the dietary supplements were SC positive which indicates that the condition is even worse for dietary supplements. From the collected market samples around 11% of the samples (Sam. no. 9, 28, 29 and 31) were found that exceed the highest dose as per recommended.

As this analytical work was conducted on a limited number of samples, this may not be an adequate reflection of the nature & number of falsifications actually prevailing in the market. Thus, this survey can be referred as an indicative or pilot study. The real situation might be more dangerous and thus further more analysis

will be required. More such analysis on different types of falsified products available in the market (such as drugs for obesity, weight gain, asthma etc.) is important to observe the trends and find out the risks.

Government should take necessary steps to make people aware about these falsified products and formulate appropriate regulations to stop this type of unethical use of APIs. All the dietary supplements should be brought under the regulation of Drugs Administration and any kind of unjustified advertisements of traditional medicines and dietary supplements in mass media (newspaper, TV) should be brought under regulation like prescriptional drugs.

All these measures are to be taken immediately to save public health, because these falsifications and hiding of facts is dangerous for the consumers due to the inherent serious adverse effects of the undisclosed ingredients used in these products.



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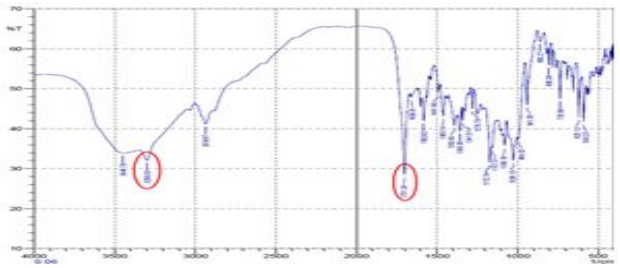

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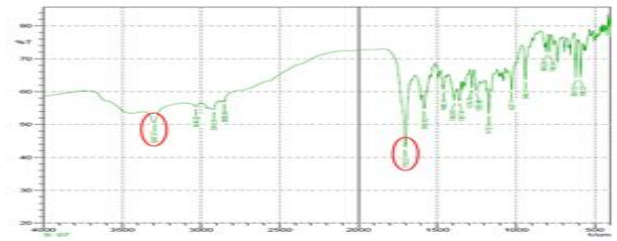

(c)

Fig. 4: IR spectrum of (a) standard SC, (b) sample no. 26, (c) sample no. 31

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