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Identification of a Sildenafil Analogue Adulterated in Two Herbal Food Supplements

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ABSTRACT

Consumption of herbal supplements becomes more and more popular in the world. For the sake of public health, enhanced supervision on those products is needed. A novel sildenafil analogue is detected from two herbal dietary supplements in our laboratory. Its structure has been identified by various NMR techniques, mass spectrum, IR and X-ray diffraction. This compound is slightly different from sildenafil (Viagra) in the piperazine moiety, where *N*-methyl group in sildenafil is replaced by *N*-hydroxyethyl group. Thereby this compound is named as hydroxyhomosildenafil. Noticeably, this compound has not been approved as medication. Therefore, it is necessary to monitor diverse analogues besides the commercial chemical compounds.

Key words: Herbal supplements, NMR, Mass Spectrum, IR, X-ray Diffraction, sildenafil, hydroxyhomosildenafil

INTRODUCTION

Synthetic chemical drugs adulterated in Traditional Chinese Medicine Preparations (TCMP) are prohibited by Law of Pharmaceutical Affairs in Taiwan. Through the alternative medicine progress, increasing herbal foods as dietary supplements have been distributed in the world market. Such kind of products is divided into two categories in Taiwan, i.e. health food, regulated by Health Food Control Act, and general food, regulated by Act Governing Food Sanitation[1]. The health care effect assessment report of health food should be reviewed by the central competent authority. The reasonable intake amount and chemical entities of them should be supported by relevant scientific literature. The Department of Health has published nine kinds of approved health care effects currently. Products claiming misrepresented or exaggerated health care effects will be inspected by competent authority. Because of firm law regulation on health food, the labels on so-called dietary supplement often claim that products are composed of natural herbs and good for health, in order to make people feel safe while using them and avoid the regulation of law. As a result, the global market of dietary supplements is increasing year by year. Recently, adulterated synthetic chemicals have been found in health food based on our survey[2] and those reported in literatures[3-7]. Therefore, continuous monitor on the quality of herbal food supplements is urgent for protecting public heath.

Two herbal supplements submitted by the local health bureau were suspected of illegal adulterants. Product labels indicated the herbal substance, serves as supplements of nutrition to enforce energy. Sample A is red capsule filled with brown powder and sample B is blue capsule filled with white powder. Through TLC screening, sildenafil (**1**) was identified from sample A by comparison with reference standard. Sildenafil, a phosphodiesterased-5 (PDE-5) inhibitor, was approved for the treatment of erectile dysfunction in 1998 by FDA; it shared the major market sales before two other PDE-5 inhibitors tadalafil and vardenafil, which were approved in 2002 and 2003, respectively. The three drugs can be obtained only with physician's prescription. Another compound (**2**) of the same UV spectrum as that of sildenafil was also detected. The following describes the structure determination of this unknown chemical.

MATERIALS AND METHODS

I. *Materials*

Sildenafil citrate from Pfizer Pharmaceutical (Taiwan) was provided for registration and inspection on Viagra and yohimbine was purchased from Sigma (St. Louis, MO, USA). Acetonitrile, methanol, chloroform and n-butanol of LC grade were purchased from Labscan (Dublin, Ireland). Glacial acetic acid, potassium bromide and dimethylsulfoxide-d_6 (DMSO-d_6) were purchased from E. Merck (Darmstadt, Germany). Ethanol (95%) was produced by Taiwan Tobacco and Liquor Corporation (Taipei, Taiwan).

II. *Methods*

(I) *Sample Preparation*

Sample powder was taken out from one capsule of

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sample A and B respectively and extracted with ethanol (5 mL) by ultrasonic shaking at room temperature for 30 minutes. After simple filtration, part of the filtrate was analyzed by thin layer chromatography (TLC), and the rest was filtered through 0.45 μm syringe filter before liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis.

(II) *TLC and UV Spectrophotometry*

TLC was performed on TLC plate (20 × 20 cm) coated with silica gel 60 F_{254} (E. Merck), using chloroform-ethanol (9:1) as a developing system, visualized at UV 254 nm and then sprayed with Dragendorff's reagent. The suspected spot on TLC was scrapped, extracted with ethanol by ultrasonication for 5 minutes. The supernatant was used for UV measurement scanned by a CARY 50 Conc (Varian Optical Spectroscopy Inc, Mulgrave, Victoria, Australia)

(III) *LC/MS/MS*

For LC/MS/MS experiments, the chromatographic system was conducted on a Waters 2690 Alliance LC & 996 photodiode detector (PDA) with an Automatic Liquid Sampler and an Injector (Milford, MA, U.S.A.). The separation was performed on a Cosmosil 5C18-AR (15 cm × 4.6 mm I.D., 5 μm) reverse phase column (Nacalai Tesque, Kyoto, Japan). The mobile phase was composed of acetonitrile-methanol-1% acetic acid with isocratic elution at 17: 25: 58 at a flow rate of 0.5 mL/min. The injection volume was 10 μL and the running time was 35 min. The LC chromatogram collected by a 996 PDA detector scanned the wavelength from 200 to 350 nm. The split ratio of column effluent into the PDA and mass was about 4:1. An approximately 0.1 mL column effluent was introduced into a tandem mass spectrometer (Quattro Ultima, Micromass, England) through the positive electrospray ionization interface. The control of MS/MS system and data acquiring was performed with MassLynx V4.0 (Micromass). NLFD3/LM mass spectrum data library used for identification of adulteration was created by our laboratory for no commercial LC/MS/MS database available.

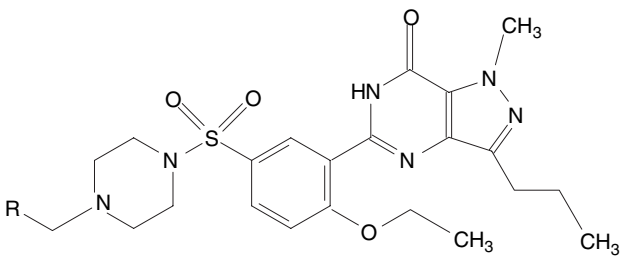
The ion source and desolvation temperature were set at 120°C and 350°C, respectively. The daughter ion scanning spectra was used as the identification of the adulterant. Table 1 listed the main tandem mass parameters used in the screening aphrodisiacs.

(IV) *Extraction and Separation of Sildenafil Analogue*

Sample B extract was stored at 4°C overnight and a white crystalline precipitated. After filtration and washing, the desired product was obtained and used to perform the following measurement to determine its structure. IR spectra were achieved on a Jasco FT/IR-480 plus Fourier Transform Infrared Spectrometer. Mass spectra were recorded on a VG Platform Electrospray ESI/MS and Finnigan MAT 95S mass spectrometer. NMR spectra were recorded on a Varian Unity Inova-500 spectrometer. X-ray diffraction study was carried out using a Nonious CAD4 Kappa Axis XRD diffractometer.

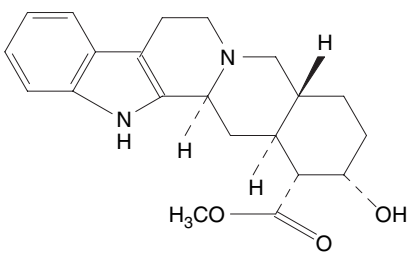
Table 1. Tandem mass parameters for the screening of sildenafil, yohimbine and a sildenafil analogue

	Sildenafil	Yohimbine	Sildenafil analogue
Daughter ion (m/z)	475	355	505
Capillary voltage (kV)	3	3	3
Cone voltage (V)	100	80	100
Collision energy (eV)	25	20	20



1. R=H ($C_{22}H_{30}N_6O_4S$, 474.6)

2. R=CH_2OH($C_{23}H_{32}N_6O_5S$, 504.6)



3. ($C_{22}H_{26}N_2O_3$, MW354.4)

Figure 1. Structures of sildenafil **(1)**, hydroxyhomosildenafil **(2)** and yohimbine **(3)**

RESULTS AND DISCUSSION

I. *Detection of Sildenafil and its Analogue with TLC and UV*

Samples were analyzed with TLC and the conditions were stated as above. After spraying with the Dragendorff's reagent, TLC of sample A showed an orange spot of the same R_f value as the sildenafil standard. Interestingly, another orange spot was shown in both sample A and B, and its R_f value was very close to that of sildenafil. Those suspected spots were further isolated

and scanned on the UV–Visible spectrometer. Sildenafil was easily identified from sample A, and the UV spectra of another spot isolated from sample A and B, are similar to sildenafil's UV spectrum. This analysis indicated the unknown adulterant might be structurally relevant to sildenafil and is a sildenafil analogue.

These samples were conducted on GC-MS, but little information was obtained.

II. *Identification of Sildenafil Analogue, Sildenafil and Yohimbine by LC/MS/MS*

To solve the increasing demand for detecting aphrodisiac adulterants in the herbal dietary supplements, we have established a LC/MS/MS simultaneous detection method. From TLC information, we expected the interference might be less in white-powder sample B, hence, sample B was chosen to conduct the test firstly. The positive and negative MS scanning spectra of sample B extract were obtained by direct injection. The major peaks were shown at m/z 505.6 ($[M^+H]^+$) and 503.6 ($[M\text{-}H]^-$), establishing a possible formula $C_{23}H_{32}N_6O_5S$, 30 amu, equivalent to CH_2O, more than that of sildenafil. A daughter ion obtained from positive collision appeared at m/z 487.5 [M+H-18], indicating a hydroxy group at the side chain. While a daughter ion from negative collision appeared at m/z 475.6 [M-H-28], attributed to the breakdown of the *O*-ethyl group; similar to that for sildenafil.

Detailed MS/MS analysis of crystalline **2** was obtained in order to confirm the above speculation. Daughter ion scanning set at m/z 505.5 showed the base peak at m/z 99 (*N*-methylpiperazine), major peaks at m/z 112 (*N*-ethylpiperazine), 487 ($[M+H\text{-}H_2O]^+$) and 461 ($[487\text{-}CH{=}CH]^+$). A fragment at m/z 129, equivalent to *N*-hydroxyethylpiperazine, is different from that of sildenafil. These data support **2** to be a sildenafil analogue with substitution of *N*-methypiperazine group. Parent ion chromatograms were obtained to confirm whether the daughter ion m/z 487, 461 and 377 were from m/z 505.5. The possible fragmentation pathways are shown in Figure 2.

Sample A extract was performed as above. Besides sildenafil and its analogue, another aphrodisiac, yohimbine (**3**, retention time 3.5 min.) was found in the total ion current chromatogram. Yohimbine possesses weak UV absorption and might be ignored by TLC screening. This prohibited drug in Taiwan was detected by LC/MS/MS,

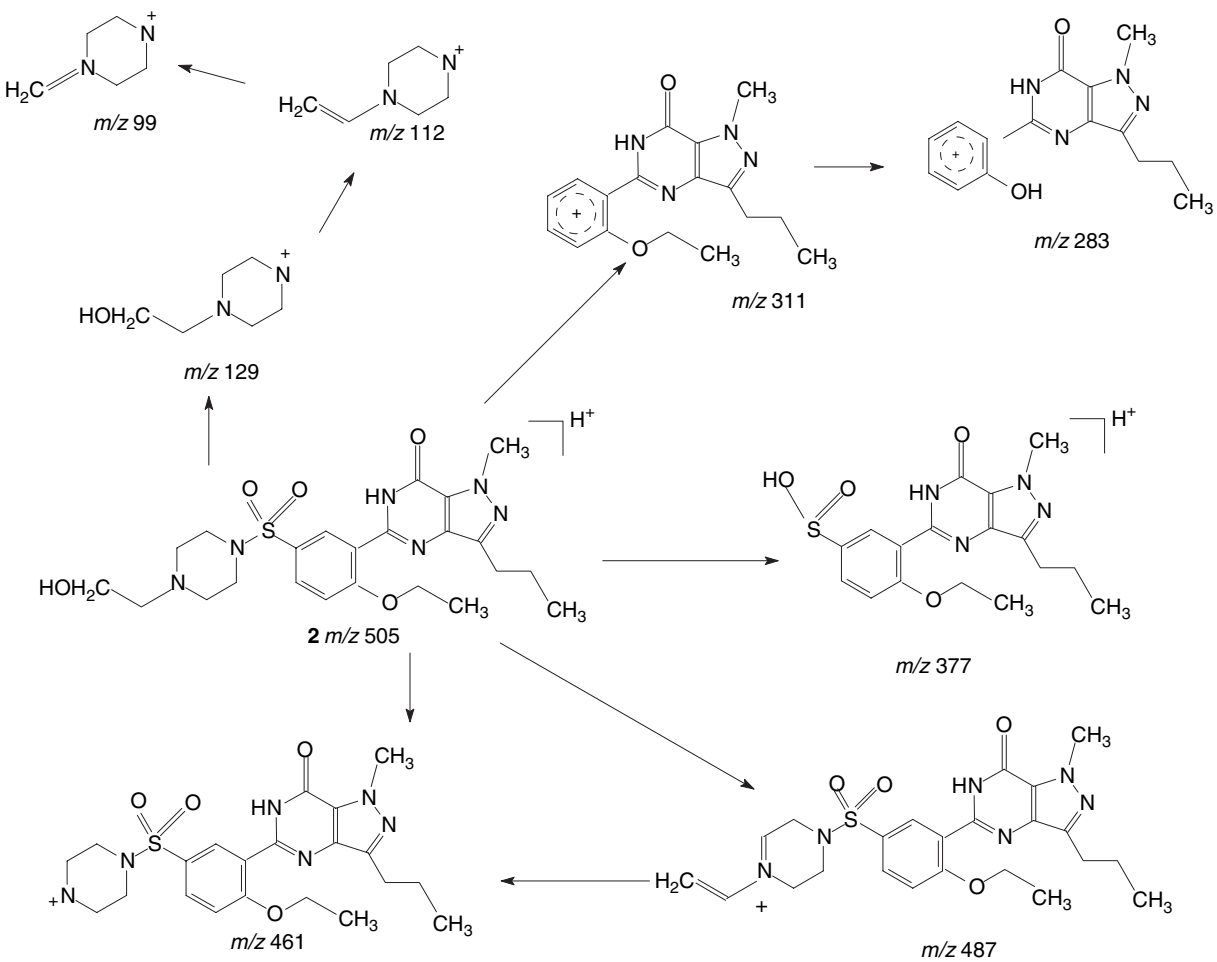


Figure 2. The detailed possible fragmentation pathways of the unknown compound at the LC/MS/MS electrospray positive (ESI+).

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accompanied with sildenafil and sildenafil analogues within one run.

III. *Structure Determination of Sildenafil Analogue* ***2***

The structure of **2** was identified by comparison of its data (^{1}H and ^{13}C NMR) with the NMR data of sildenafil (**1**). Basically, ^{1}H-NMR and ^{13}C-NMR of **2** were similar to that of **1**. The difference of ^{13}C-NMR spectra between **1** and **2** showed that one primary carbon signal at δ_C 44.7 of **1** disappeared and two other obvious secondary carbons at δ_C 59.2 and δ_C 60.3 of **2** appeared. The ^{1}H-NMR spectra indicated one singlet methyl group at δ_H 2.27 of **1** was replaced by two methylene protons at δ_H 2.37 and δ_H 3.41 ppm as well as one hydroxy proton at δ_H 4.37 ppm of **2**. These data would conclude **2** to possess *N*-hydroxyethyl substitution at the piperazyl moiety. The HMQC spectroscopy data of **2** showed two significant correlations of δ_C 59.2/δ_H 3.41(C30/H30) and δ_C 60.3/δ_H 2.37(C29/H29), and homo-COSY data showed δ_H 2.37/δ_H 3.41 (H29/H30) correlation. The HMBC (Figure 3) data also provided the correlation of δ_H 2.37/δ_C 59.2, 52.7 (H29/C30, C25, C27); δ_H 2.45/δ_C 60.3, 46.6 (H25, 27/C29, C24, C28); δ_H 3.41/δ_C 60.3 (H30/C29) and δ_H 4.37/δ_C 59.2 (HO/C30). Based on these 2D data, the structure of **2** was confirmed. Table 2 summarizes the NMR data of **2**.

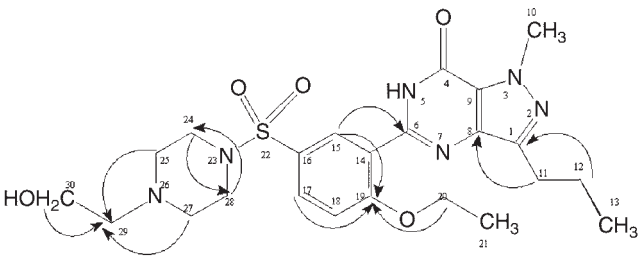

Figure 3. The major HMBC spectra of compound **2**.

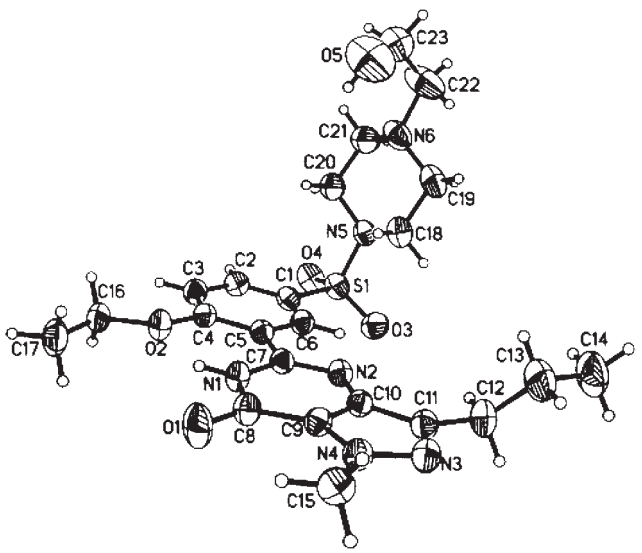

Figure 4. The X-ray diffractometeric picture of compound **2**.

Although **2** was not approved as a medicine, it had been found in the Netherlands[6] and Japan markets[7]. Blok-Tip *et al*., named as hydroxyhomosildenafil.

To elucidate the stereochemical structure, the crystal **2** was also performed on an X-ray diffractometer. Figure 4 shows the X-ray structure analysis of **2**. The crystal belongs to the triclinic system, space group Pī, and its unit cell dimension are as follows: a = 8.02350 Å, b = 11.3660 Å, c = 14.8470 Å, α = 74.9440, β = 83.8780, γ = 76.1770.

CONCLUSIONS

By application of NMR techniques and from MS spectra, IR spectra, this analogue was identified as hydoxyhomosildenafil. Because its structure is similar to that of sildenafil, it might present PDE-5 inhibition based on structure activity relationship. Without labels on the product and no relative toxicity were published, this illegal adulterant is dangerous to consumers if it has some side effect. We suspect that there is a tendency of synthesizing various sildenafil analogues for avoiding possible supervision around the world. Continuous monitoring

Table 2. NMR data of compound **2** (DMSO- d_6)

No.	^{1}H (ppm)	^{13}C (ppm)	COSY	HMBC
1		145.7		H-11/H-12
4		154.5		
5	12.20 (1H, *br.s*)			
6		148.9		H-15
8		138.5		H-11
9		125.1		H-10
10	4.17 (3H, *S*)	38.6		
11	2.77 (2H, *t*, J = 7.0 Hz)	27.9	H-12	H-12/H-13
12	1.75 (2H, *m*)	22.4	H-11/H-13	H-11/H-13
13	0.95 (3H, *t*, J= 7.03 Hz)	14.5	H-12	H-11/H-12
14		126.5		H-18
15	7.84 (1H, *d*, J=2 Hz)	130.7		H-17
16		124.6		H-15/H-17/ H-18
17	7.83 (1H, *dd*, J=8.5, 2 Hz)	132.2	H-18	H-15
18	7.38 (1H, *d*, J= 8.5 Hz)	113.9	H-17	
19		160.6		H-15/H-17/ H-18/H-20
20	4.21 (2H, *q*, J= 7 Hz)	65.6	H-21	H-21
21	1.34 (3H, *t*, J= 7 Hz)	15.0	H-20	H-20
24, 28	2.89 (4H, *br.s*)	46.6	H-25/H-27	H-25/ H-27
25, 27	2.45 (4H, *br*, *m*)	52.7	H-24, H-28	H-24/ H-28/H-29
29	2.37 (2H, *t*, J= 6.3 Hz)	60.3	H-30	H-25/H-27
30	3.41 (2H, *t*, J= 5.3 Hz)	59.2	H-29	H-29

and developing new methods to detect the diverse illegal compounds adulterated in herbal supplements are necessary and risk assessments of medicine analogues are proposed in the view of public health. Further quantitative analysis of this adulterant in sample A and B is in progress.

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