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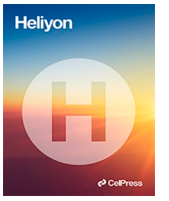

Isolation and identification of a novel vardenafil analogue, propoxy-vardenafil, found as an adulterant in a health supplement

Jian Sun, Hong Yu, Jingxian Zhang, Yingying Ran, Yushuang Zhao, Shen Ji *, Qing Hu **

NMPA Key Laboratory for Quality Control of Traditional Chinese Medicine, Shanghai Institute for Food and Drug Control, 1500 Zhangheng Road, Shanghai 201203, China

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ABSTRACT

Background: A novel vardenafil analogue was detected from a health wine claimed to be anti-impotence during a special inspection in an online store.
Methods: The unknown compound was found by using ultra-high performance liquid chromatography coupled to quadrupole time of flight mass spectrometry (UHPLC/Q-TOF MS). The characteristic product ions were similar to those of vardenafil. The UV spectrum of the compound closely mirrored that of vardenafil. The analogue underwent purification by semi-preparative HPLC and structurally identified by FT-IR and NMR analysis.
Results: Based on the data, The structure of the analogue was characterized as 2-[2-propyloxy-5-(4-ethylpiperazin-1-yl)sulfonylphenyl]-5-methyl-7-propyl-3H-imidazo [5,15,1-f] [1,2,4]triazin-4-one, simplified as propoxy-vardenafil.
Conclusion: To the best of our knowledge, the analogue has not been reported and is even only ninth vardenafil analogue, which was confirmed that a n-propyloxy group had replaced the ethoxy group on the aromatic ring of vardenafil. Therefore, It is essential to pay more attention to vardenafil analogues in the routine inspection of health supplements.

1. Introduction

Several phosphodiesterase type 5 (PDE-5) inhibitors, including sildenafil, tadalafil, vardenafil, avanafil, udenafil, mirodenail, and lodenafil carbonate, have gained approval as therapeutic agents for patients with erectile dysfunction in numerous countries [1–4]. It is crucial to underscore that these drugs should only be administered under medical supervision due to their potential adverse effects. However, these PDE-5 inhibitors have been identified in dietary supplements that are labeled as “safe” and “natural” [5].

Since homosildenafil was discovered in 2003, more and more PDE-5 inhibitor analogues have been adulterated to hamper target detection due to the modification of chemical structures [6]. These analogues may exhibit similar pharmaceutical activity to their parent molecules, but the toxicity of these analogues was often not studied, which could pose a severe and unknown risk to public health [7]. The sildenafil and tadalafil analogues comprised more than 90% of the total PDE-5 inhibitors identified in dietary supplements. Interestingly , the number of vardenafil analogues reported was fewer than sildenafil and tadalafil. There were several modification moieties of the vardenafil structure, such as the N-ethylpiperazine ring, the sulfo group and the oxo group.

* Corresponding author.
** Corresponding author.
E-mail addresses: jishen2013@163.com (S. Ji), huqingyjs@163.com (Q. Hu).

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Pseudovardenfil [8], hydroxyvardenafil [9], N-desethylvardenafil [6], acetylvardenafil [10], hydroxythiovardenafil [11], norneovardenafil [6], desulfovardenafil [12], morphardenafil [13] had been found.

In this paper, a novel vardenafil analogue was isolated from a health wine. Its structure was elucidated using a combination of ultra-high-performance liquid chromatography coupled to quadrupole time of flight mass spectrometry (UHPLC/Q-TOF MS), infrared (IR) and nuclear magnetic resonance (NMR). The structural analysis identified the compound as propoxy-vardenafil, of which the n-propyloxy group replaced the ethoxy group on the aromatic ring. Previous reports on vardenafil analogues had been limited, which may have led to less attention in the detection. However, the discovery of this compound as an adulterant in dietary supplements for the first time highlights a new trend in the structural modification of PDE-5 inhibitors.

2. Experimental

2.1. Materials and chemicals

Vardenafil was obtained from TLC Pharmaceutical Standards Ltd. (Aurora, Ontario, Canada). LC/MS-grade solvents including acetonitrile, methanol, and water were purchased from Merck (Darmstadt, Germany). Formic acid sourced from Sigma-Aldrich (MO, USA) was also LC/MS-grade. Dimethyl sulfoxide-d_6 was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). All other chemicals utilized in the study were of analytical grade. A health wine that was received as a yellow liquid claimed to be anti-impotence, was obtained during a special inspection in an online store.

2.2. UHPLC/Q-TOF MS screening

In the non-target screening procedure, the liquid dietary supplement was mixed equally and measured 1 mL, dissolved in 50 mL 50:50 (*v/v*) MeOH: H_2O, and sonicated for 15 min. The resulting extracts were filtered via 0.22 μm PTFE-membrane filters. The reference standard (vardenafil, 10 mg) was dissolved in methanol (50 mL). The standard solution of vardenafil was further diluted to 1 μg/mL using 50:50 (*v/v*) MeOH: H_2O for direct infusion.

High-resolution mass (HR-MS) analysis was conducted on the 6530 quadrupole time of flight mass spectrometry (Q-TOF MS, Agilent Technologies, Santa Clara, CA, USA) coupled to an ultra-high performance liquid chromatography (UHPLC, Agilent Technologies, Santa Clara, CA, USA), employing an electrospray ionization (ESI) source. A Poroshell 120 SB-C18 column (2.7 μm, 3.0 × 75 mm, Agilent) was used for the separation of analytes. Chromatographic conditions were composed of solvents A (0.1% formic acid in water) and B (acetonitrile). The gradient conditions were as follows: 0–15 min (5–98% B), 15–17 min (98% B), 17–17.5 min (98-5% B), and 17.5–20 min (5% B). The column temperature was maintained at 30 °C, and the flow rate was set at 0.4 mL/min, with an injection volume of 1 μL.

The mass conditions were acquired in positive mode with auto MS/MS mode. Mass spectrometry parameters were set as follows: Gas temperature 325 °C; Gas flow 12 L/min; Nebulizer 50 psi; Sheath gas temperature 375 °C; Sheath gas Flow 12 L/min; Capillary 5000 V. Full MS scan data acquisition was conducted as follows: mass range 100–1200 *m/z*, acquisition rate 5 spectra/s. The following settings for the full MS/MS scan data acquisition were used: mass range 50–1200 *m/z*, acquisition rate 10 spectra/s, isolation width 4 *m/z*, with fixed collision energy of 10, 20, 40 V.

2.3. HPLC-DAD detection

The sample extracts were inspected by a 1290 ultra-high performance liquid chromatography equipped with a diode array detector (UHPLC, Agilent Technologies, Santa Clara, CA, USA). The separation process was performed using an Agilent Eclipse Plus C18 （3.0 × 150 mm， 1.8 μm）column，which was maintained at a temperature of 35 °C. The mobile phase system consisted of 0.1% formic acid in water (A) and methanol (B). The elution process proceeded according to the following gradient program: 0–1 min, 10%–50% B; 1–16 min, 50%–64% B; 16–19 min, 65%–98% B; 19–22 min, 98% B. The injection volume was set at 1 μL, and the flow rate at 0.4 mL/min. UV spectra were obtained with the DAD detector across a range from 200 to 400 nm, with the UV signal specifically monitored at 254 nm.

2.4. Sample isolation

The isolation experiment of the liquid was conducted on a 1260 infinity II semi-preparative high-performance liquid chromatography (prep-HPLC, Agilent Technologies, Santa Clara, CA, USA). The sample of 24 mL was divided into eight equal parts. Each 3 mL was injected into the prep-HPLC and separated using a Shim-pack PREP-ODS column (20 × 250 mm，15 μm) maintained at 30 °C and a flow rate of 10 mL·min−1. The mobile phase consisted of 0.1% trifluoroacetic acid solution and acetonitrile (35:65, *v/v*). To determine the component most closely related to vardenafil, the spectrum of the unknown compound's chromatographic peak was compared with the spectrum obtained from a vardenafil reference standard. The collected fractions were consolidated, concentrated by rotary evaporation, and dried in a vacuum. The unknown compound as yellow oil was afforded, which weighed 10 mg.

2.5. Fourier transform infrared (FTIR)

The infrared (IR) spectra of the isolated compound were recorded on a Thermo Nicolet iS50 FT-IR spectrometer (Thermo Nicolet,

Vernon Hills, Illinois, USA) in the 4000–650 cm^{-1} range.

2.6. NMR analysis

The unknown compound of 10 mg was dissolved in DMSO-d_6 for NMR spectroscopic analysis. Proton NMR (^{1}H NMR), carbon-13 NMR (^{13}C NMR), correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC), and heteronuclear multiple quantum correlation (HSQC) spectra were recorded on a Bruker AVANCE III 600 MHz FT-NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) using Me4Si as the internal standard in DMSO-d_6. Chemical shift (δ) values are in ppm (part per million) and coupling constants (J) are in Hz (hertz).

3. Results and discussion

3.1. UHPLC/Q-TOF MS analysis

The molecular formula of the isolated compound was deduced as $C_{24}H_{34}N_6O_4S$ from the HR-MS data (*m/z* 503.24537 $[M + H]^+$). Fragmentation of the precursor ion yielded several characteristic product ions (*m/z* 376.10509, 299.11327, 256.09469, 151.08630, 113.10743 and 72.08113), which were similar to those of vardenafil. Furthermore, the fragment ion of *m/z* 326.17356 ($[C_{18}H_{22}N_4O_2]^+$) from the unknown compound and *m/z* 312.15880 ($[C_{17}H_{20}N_4O_2]^+$) from vardenafil were distinctive, which suggested the modification of the ethoxy group with the propyloxy group on the aromatic ring. Fig. 1A and B and Scheme 1 illustrate the mass spectra and deduced fragmentation pathways of the unknown compound and vardenafil, respectively.

3.2. HPLC-DAD analysis

The chromatogram of the sample exhibited a significant peak at 7.410 min, differing from the retention time of vardenafil, which appeared at 5.861 min (see Fig. 2A and B). The UV spectrum of the unknown compound, with peak absorbances at 216 nm, closely mirrored that of vardenafil (Fig. 2C).

3.3. FTIR analysis

The infrared (IR) spectrum of the unknown compound (Fig. 3) indicated the presence of alkyl aromatic ether group (1270, 1024 cm^{-1}), carbonyl (1685 cm^{-1}) and aromatic ring (1621, 1595 and 1482 cm^{-1}).

3.4. NMR analysis

The ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, HMBC and HSQC data of the unknown compound and the ^{1}H and ^{13}C NMR data of vardenafil [14] for comparison were compiled in Table 1. The NMR signals of the unknown compound closely resemble those of vardenafil, except for an additional methyl group signal at C-31 in both ^{1}H and ^{13}C NMR spectra, indicating that the unknown compound was

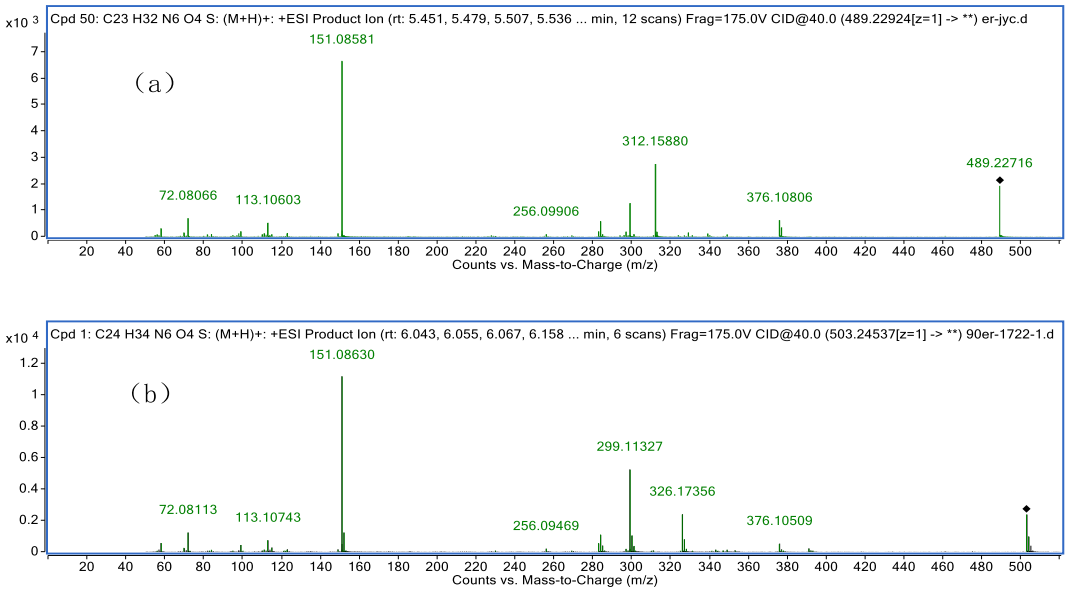
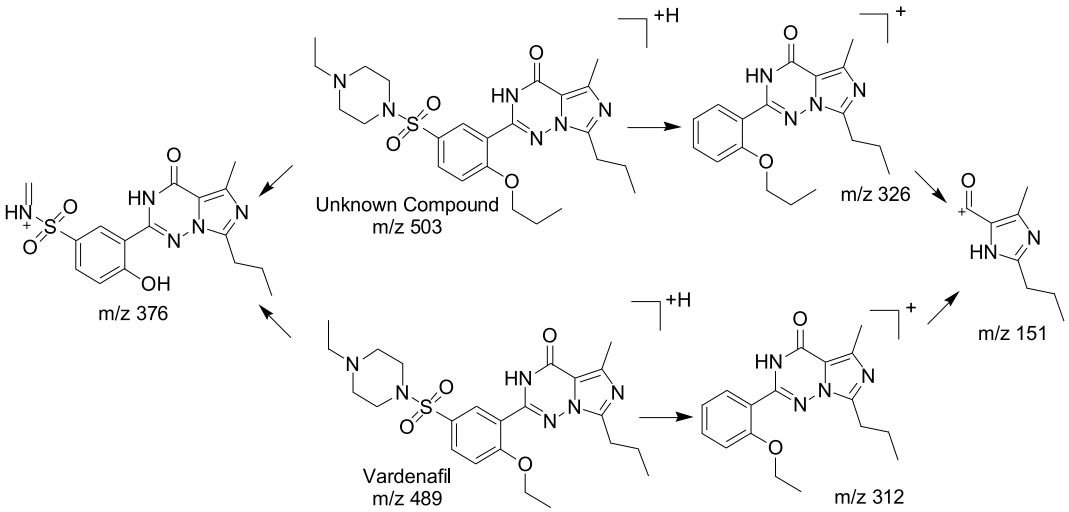


Fig. 1. UHPLC/Q-TOF MS spectra of (a) vardenafil with the parent ion at *m/z* 489, and (b) the unknown compound with the parent ion at *m/z* 503.



Scheme 1. MS fragmentation patterns of the unknown compound and vardenafil.

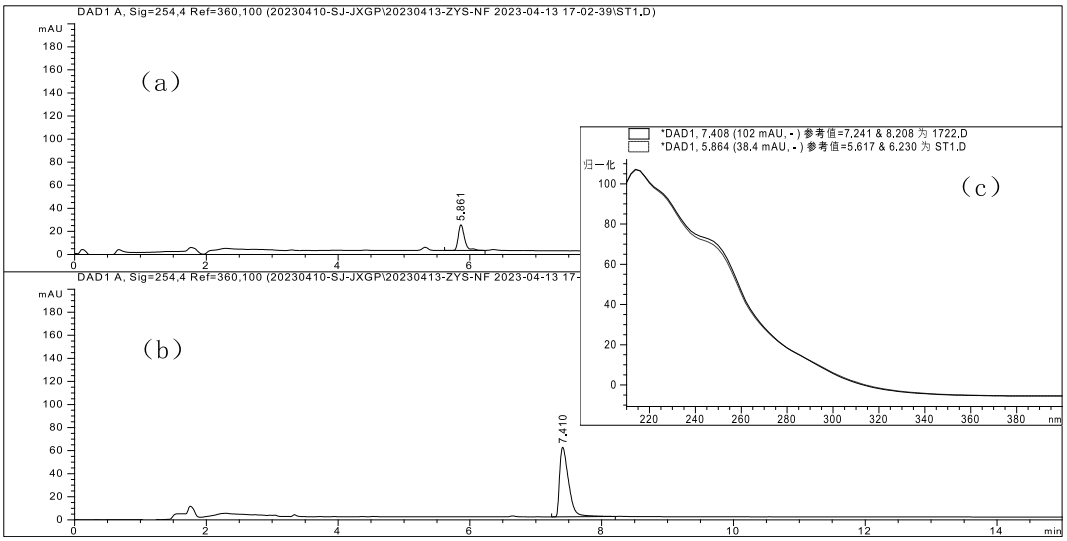


Fig. 2. HPLC chromatogram of vardenafil (a) and the unknown compound (b) at 254 nm, and compared UV spectra (c).

structurally related to vardenafil. Notably, due to the chair conformation of the hexatomic ring in solution DMSO-d_6, the four protons on the piperazine ring were in different chemical environments. In the ^{1}H NMR spectrum of the unknown compound, there were geminal and vicinal couplings between these protons at C-24, C-28 (δ_H 3.85–3.80, m, 2H; 3.07–2.96, m, 2H) and C-25, C-27 (δ_H 3.61–3.55, m, 2H; 3.07–2.96, m, 2H), exhibiting four sets of multiple peaks.

While the proton signal of the methyl group (C-21) was a triplet in vardenafil, the corresponding proton signal of the unknown compound was observed as a multiple peak (δ_H 1.97–1.85, m, 2H), and an additional signal of methyl group (C-31) was observed as a triplet (δ_H 1.08, t, J = 7.4 Hz, 3H). In ^{13}C NMR spectra, C-21 was a methylene carbon (δ_C 22.1), and C-31 was an additional methyl carbon (δ_C 10.4). The HMBC correlations of C-31 and H-20,21 and correlations of ^{1}H–^{1}H COSY between H-21 and H-31 showed that the C-31 methyl group was attached to C-21. Besides, the modification of the ethoxy group with the n-propyloxy group on the aromatic ring was further confirmed by the HSQC spectra. The structures of vardenafil and the unknown compound are shown in Fig. 4.

4. Conclusion

In this study, a novel vardenafil analogue was detected by employing UHPLC/Q-TOF MS and HPLC-DAD in a special inspection sample. The compound was isolated using preparative-LC, and its structure was identified as propoxy-vardenafil based on spectrographic data from LC-HRMS, DAD, IR, and NMR. All these experiments confirmed that a n-propyloxy group had replaced the ethoxy group on the aromatic ring of vardenafil.

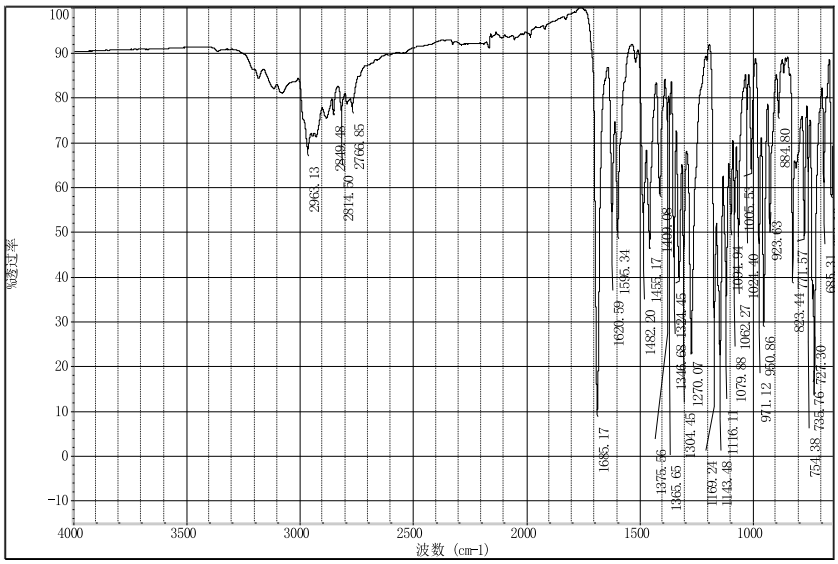


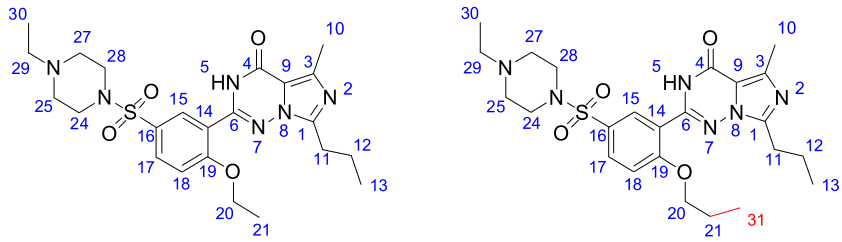
Fig. 3. IR spectrum of the unknown compound.

Table 1
NMR data of vardenafil and the unknown compound in DMSO-d_6 (δ in ppm, J in Hz).

Position	Vardenafil		Unknown compound				
	δ_H	δ_C	δ_H	δ_C	COSY	HMBC	HSQC
1	–	144.4		144.1		H-11,H-12	
3	–	137.6		134.1		H-10	
4	–	155.0		153.4		H-10	
5	11.82 (1H,br,s)	–	11.21 (s, 1H)				
6	–	146.1		149.3		H-15,H-18	
9	–	113.7		114.9		H-10	
10	2.50 (3H,s)	14.1	2.79 (s, 3H)	11.2			H-10
11	2.86 (2H,t,J = 7.4)	27.1	3.21 (t, J = 7.6, 2H)	25.9	H-12	H-12,H-13	H-11
12	1.76 (2H,qt)	20.2	1.97–1.85 (m, 2H)	20.6	H-11,H-13	H-11,H-13	H-12
13	0.94 (3H,t,J = 7.4)	13.7	1.03 (t, J = 7.4, 3H)	13.6	H-12	H-11,H-12	H-13
14	–	126.4		125.5		H-15,H-18	
15	7.97 (1H,d,J = 2.4)	130.3	8.05 (d, J = 2.3, 1H)	130.2	H17	H-17	H-15
16	–	120.9		119.3		H-18	
17	7.95 (1H,dd)	132.2	7.90 (dd, J = 8.8, 2.3, 1H)	133.3	H-18 , H15	H-15	H-17
18	7.45 (1H,d,J = 8.8)	113.6	7.20 (d, J = 8.9, 1H)	113.2	H-17		H-18
19	–	160.7		161.2		H-15,H-17,H-18,H-20	
20	4.24 (2H,q,J = 6.9)	65.1	4.18 (t, J = 6.4, 2H)	71.7	H-21	H-21,H-31	H-20
21	1.35 (3H,t,J = 6.9)	14.3	1.97–1.85 (m, 2H)	22.1	H-20,H-31	H-20,H-31	H-21
24,28	3.50 (2H,br,m)	43.0	3.85–3.80 (m, 2H)	43.2	H-24,H-25.H-27, H-28		H-24, H-28
	3.81 (2H,br,m)		3.07–2.96 (m, 2H)				
25,27	3.10 (4H,br,m)	49.4	3.61–3.55 (m, 2H)	50.8	H-24,H-25.H-27, H-28	H-29	H-25, H-27
			3.07–2.96 (m, 2H)				
29	2.90 (2H,q)	50.6	3.12 (q, J = 7.3, 2H)	52.3	H-30	H-30,H-25,H-27	H-29
30	1.23 (3H,t,J = 7.2)	8.7	1.38 (t, J = 7.3, 3H)	9.2	H-29	H-29	H-30
31			1.08 (t, J = 7.4, 3H)	10.4	H-21	H-20,H-21	H-31

Consequently, the structure of the adulterant was characterized as 2-[2-propyloxy-5-(4-ethylpiperazin-1-yl)sulfonylphenyl]-5-methyl-7-propyl-3H-imidazo [5,1-f] [1,2,4]triazin-4-one, simplified as propoxy-vardenafil.

To the best of our knowledge, it has not been reported and is even only ninth vardenafil analogue. Although the modification may be small, its potential toxicity need to be further evaluated and studied like other PDE-5 inhibitor analogues. As detection for sildenafil and tadalafil analogues becomes more stringent, there may be an increase in vardenafil analogues. Therefore, it is essential to pay more attention to vardenafil analogues in the routine inspection of health supplements, such as focusing on mass spectrometry diagnostic ions 151 and 376. In addition, food safety regulation and public education are also necessary to improve the safety and quality of dietary supplements.



Vardenafil Unknown compound

Fig. 4. The structure of vardenafil and the unknown compound.

Author contribution statement

Jian Sun: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Hong Yu, Jingxian Zhang: Performed the experiments; Analyzed and interpreted the data.

Yingying Ran, Yushuang Zhao: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Shen Ji, Qing Hu: Conceived and designed the experiments; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

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

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