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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.

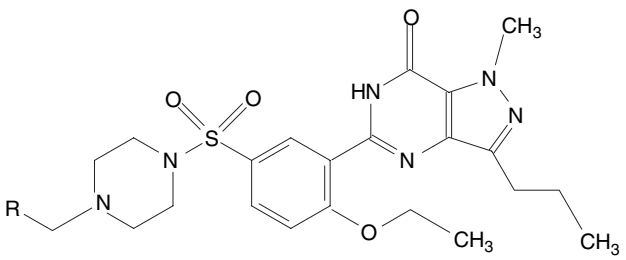
The ion source and desolvation temperature were set at 120°C and 350°C, respectively. The daughter ion scanning spectra was used as the identification of the adulterant. Table 1 listed the main tandem mass parameters used in the screening aphrodisiacs.

(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.

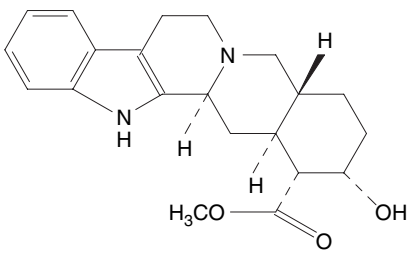
Table 1. Tandem mass parameters for the screening of sildenafil, yohimbine and a sildenafil analogue

	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,

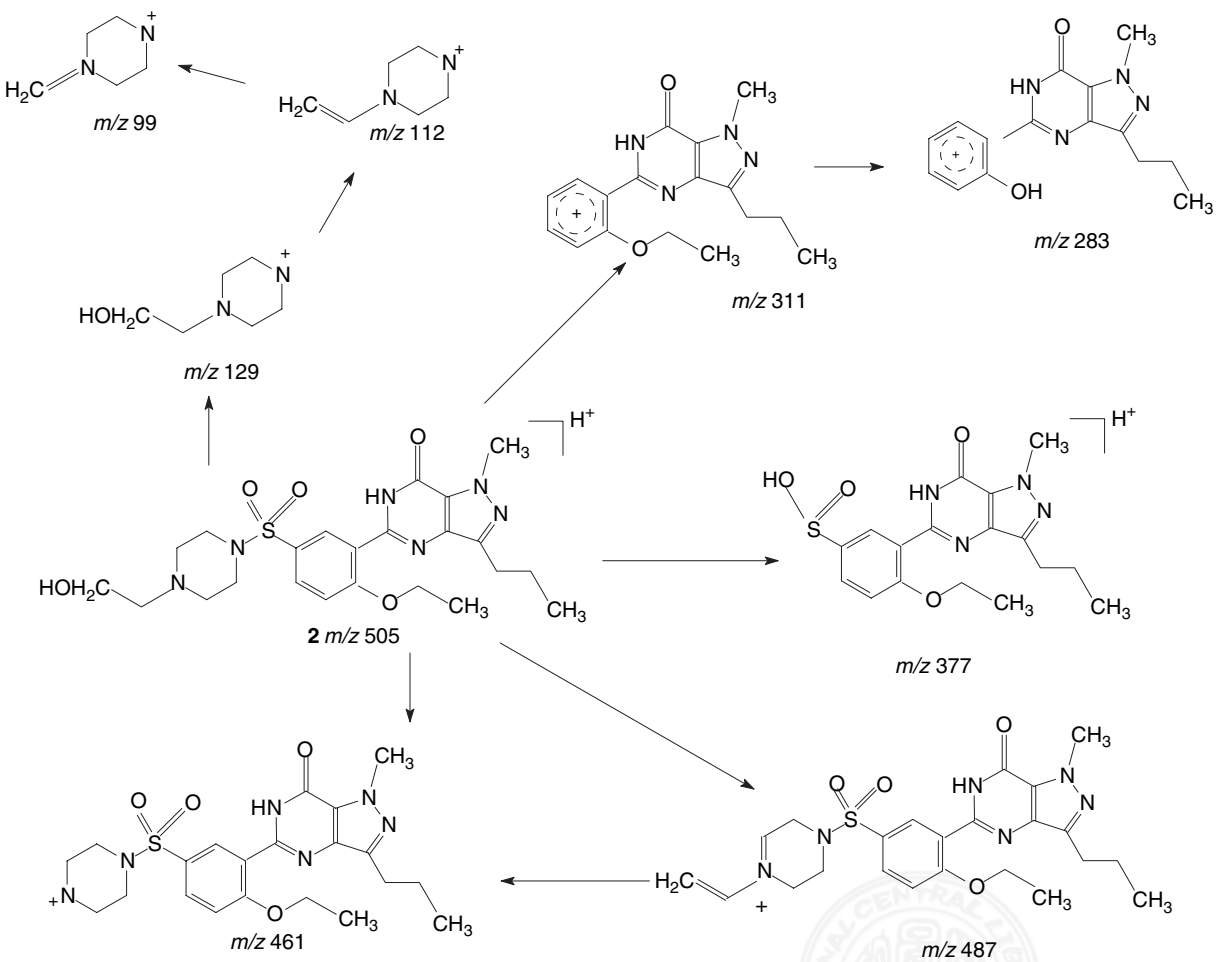


Figure 2. The detailed possible fragmentation pathways of the unknown compound at the LC/MS/MS electrospray positive (ESI+).

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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**.

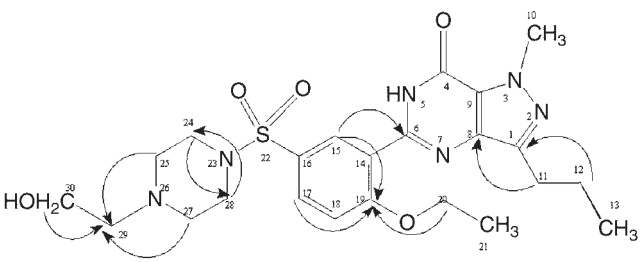


Figure 3. The major HMBC spectra of compound **2**.

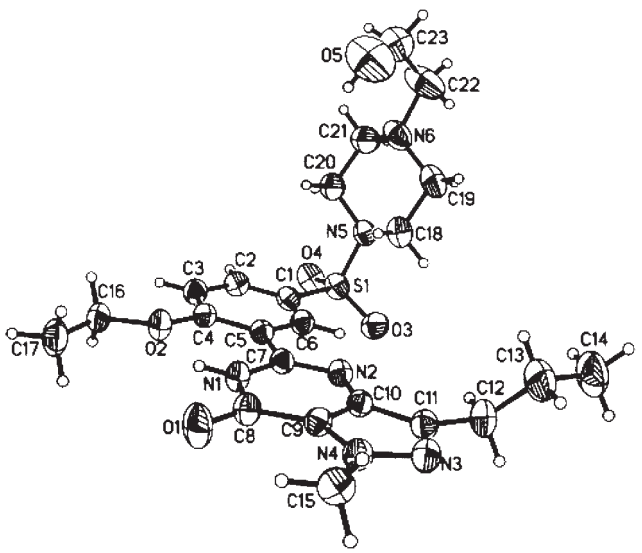


Figure 4. The X-ray diffractometeric picture of compound **2**.

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.

ACKNOWLEDGMENTS

We are grateful to Dr. Yun-Lian Lin, National Research Institute of Chinese Medicine and Dr. Shoei-Sheng Lee, School of Pharmacy, National Taiwan University, for kindness on NMR testing and assistance on structure determination.

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5

單劑量與多劑量柚皮苷及柚皮苷元於大白鼠之藥物動力學

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摘 要

柚皮苷（Naringin）及柚皮苷元（naringenin）爲柑橘屬植物中之主要黃酮類化合物。本研究以大白鼠探討柚皮苷及其苷元單劑量與多劑量之動力學。柚皮苷元分別以單劑量靜脈注射與口服，而柚皮苷則分別以單劑量與多劑量（每天二次共九天）口服投予大白鼠。血清檢品以sulfatase及β-glucuronidase分別水解前、後，以高效液相層析法定量柚皮苷元之濃度。柚皮苷元靜脈注射後，快速形成硫酸及葡萄糖醛酸結合態代謝物，其原形分子快速減少；而口服給予柚皮苷元及柚皮苷時，則幾無原形分子，只有結合態代謝物存在血循環中。柚皮苷多劑量投予後，第五天可達穩定狀態。口服投予柚皮苷與柚皮苷元後，柚皮苷元之硫酸結合代謝物之血中濃度皆遠高於葡萄糖醛酸結合態代謝物。總之，柚皮苷及柚皮苷元於大白鼠口服給藥後，血循環中皆以柚皮苷元之結合態代謝物存在。因此，有關柚皮苷及柚皮苷元之生物活性研究應著重其結合態代謝物，特別是硫酸結合代謝物。

關鍵詞：柚皮苷，柚皮苷元，藥動學，硫酸酯，葡萄糖醛酸苷

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利用液相層析質譜同時分析市售丹參多酚類及丹參酮類化學成分

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摘 要

中藥丹參係取材自丹參（*Salvia miltiorrhiza* Bunge）的根莖，廣泛的被用於治療心血管疾病、腎臟和肝臟疾病的傳統方劑中。以丹參酮類（tanshinones）和丹參酚酸類（salvianolic acids）兩類化合物同時作爲指標成分，分析台灣丹參市售品此兩類成分的含量。開發不同離子偵測模式的液相層析質譜分析，利用紫外光和質譜檢出器，藉由與標準品比較化合物的滯留時間（retention time），質譜（MS）及子代質譜（MS^2），同時分離及確認丹參六個主要多酚類組成分，caffeic acid（**1**），danshensu（**2**），lithospermic acid（**3**），rosmarinic acid（**4**），salvianolic acid B（**5**）和salvianolic acid A（**6**），及四個主要 abietane-type 二萜類（diterpenes），15,16-dihydrotanshinone I（**7**），cryptotanshinone（**8**），tanshinone I（**9**）和tanshinone IIA（**10**）。本研究選擇甲醇和0.25%的醋酸水溶液（用1N鹽酸調整pH至2.77）作爲移動相.使用LC-UV（290 nm）-MS方法可以容易且明確地偵測市售丹參所含的多酚類（**1-6**）和丹參酮類（**7-10**）的化學指紋圖譜，將有助於丹參的品質管制。

關鍵詞：丹參，*Salvia miltiorrhiza*，液相層析質譜，多酚類，丹參酮類，化學指紋圖譜

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保健食品中違法摻雜sildenafil類緣物之鑑別

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行政院衛生署藥物食品檢驗局

摘 要

隨著保健食品使用量日益增加，在維護民眾健康前提下，加強此類產品之監控是極爲必要。本報告係檢驗上市之兩種保健食品，檢出摻雜一種首次發現新成分爲sildenafil（商品名爲威而鋼）類緣物。該新成分係屬未知成分，經由核磁共振光譜、質譜、紅外線光譜及X射線繞射分析，才得以確認結構。其結構與sildenafil之主要基本結構相同，僅有一些些不同。即其側鏈之取代基由*N*-methylpiperazine 改爲*N*-hydroxyethylpiperazine。其名稱爲hydroxyhomosildenafil。經由文獻搜尋，此成分目前並未核准上市作爲藥品。

此類成分之檢出更值得吾人提高警覺，顯示保健食品中除可能違法摻雜已上市之藥品外，更有不肖業者，以已上市藥品之相關類緣物質摻雜其中，以規避檢驗及行政管理。而此種類緣物新成分的檢出，是檢驗工作上頗具挑戰性的一環。

關鍵詞：保健食品，核磁共振光譜，質譜，紅外線光譜，X射線繞射，sildenafil，hydoxyhomosildenafil

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溫度誘發霍亂弧菌O139進入活而不長狀態和其致病力

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摘 要

本研究測定溫度對致病性霍亂弧菌O139成爲活而不長狀態的影響；並進一步決定在可被培養、不可被培養和恢復生長三種不同狀態下，菌株的毒力和細胞蛋白質的表現。當O139菌株在含有0.5%食鹽的河水微環境中，4°C培養35-45天時，培養基的菌落已少於1 CFU/mL，而活菌計數的數值並無明顯變化。然而，培養在15°C或25°C下，並未偵測到菌株進入活而不長狀態。將4°C誘發的活而不長細菌樣本接種至含有分解H_2O_2化合物的培養基或直接轉到28°C下培養，活而不長的O139菌株均會回復生長。吸附實驗顯示，可被培養的O139細菌吸附到HeLa細胞株的數目明顯高於不被可培養的和恢復生長的細菌。細胞毒殺試驗顯示，可被培養的O139細菌會造成HeLa細胞全部殺死；恢復生長的細菌僅會使32.1 ± 4.6%標的細胞死亡；以不可被培養的細菌與無菌培養基分別培養後比較，標的細胞的死亡百分率並無差異。以可被培養的O139細菌攻擊小鼠後之致死率爲80%；但是以不可被培養的細菌和恢復生長的細菌攻擊後，所有的小鼠都活存下來。

關鍵詞：霍亂弧菌，活而不長，回復生長，致病力