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Development of a Method for Comprehensive and Quantitative Analysis of Yohimbine in *Pausinystalia yohimbe* by Liquid Chromatography-Ion Trap Mass Spectrometry

Q. CHEN, P. LI*, B. LI, F. YUAN, X. LI, AND J. ZHU

Department of Pharmacy, Affiliated Dongfeng Hospital, Hubei University of Medicine, Hubei 442008, People's Republic of China
E-mail: cqh77@126.com

Summary. A rapid and sensitive method for the identification and quantification of yohimbine in *Pausinystalia yohimbe* is described. The method used is liquid chromatography–quadrupole ion trap mass spectrometry (LC–QIT/MS). The yohimbine standard solution was directly infused into the ion trap mass spectrometers (IT/MS) for collecting the MS^n spectra. The major fragment ions of yohimbine were confirmed by MS^n at *m/z* 355, 224, 212, and 144, in the positive-ion mode. The possible main fragment ion cleavage pathway was studied. Yohimbine provided good signals corresponding to the protonated molecular ion $[M + H]^+$. The method is reliable and reproducible, and the detection limit is 0.1 ng mL^{-1}. The method was validated in the concentration range 0.1–50 μg mL^{-1}; the intra- and interday precision ranged from 1.36% to 2.73%; and the accuracy was 96.5–108.2%. The mean recovery of yohimbine was 97.1–101% with a relative standard deviation (RSD) <1.93%. The LC–IT/MS method was successfully applied to determine the yohimbine in *P. yohimbe*.

Key Words: liquid chromatography–ion trap mass spectrometry, yohimbine, *Pausinystalia yohimbe*

Introduction

Yohimbine, the dried bark of *Pausinystalia yohimbe* (Rubiaceae), which is a tree native to tropical West Africa, has long been considered as an aphrodisiac and is extensively used by local populations as part of traditional health care systems. The name is used to label both the medicinal plants and the group of alkaloids contained in their extracts; the chemical structures of these alkaloids are very variable, as well as their pharmacological properties. In the recent years, attention has been focused on the biological activity of yohimbine [1–3], which is a selective α-2-adrenergic antagonist effective in the treatment of erectile functional disturbance and is an anxiogenic [4, 5].

Several chromatographic and electrophoretic methods have been reported for the analysis of yohimbine. Previously, we had developed a gas chromatography–mass spectrometry (GC–MS) to determine the content of

yohimbine in the dried bark of *P. yohimbe* [1]. But methods based on liquid-phase separation techniques have been often used. Nonaqueous capillary electrophoresis (NACE) [1,6] and micellar electrokinetic chromatography (MEKC) [7] have been investigated for the analysis of yohimbine in plants and pharmaceuticals. Compared with capillary electrophoresis (CE), high-performance liquid chromatography (HPLC) can afford better analytical precision and higher sample loading capacity. HPLC coupled to UV [8–11] and MS detection [12] has been applied in the determination of the yohimbine. HPLC in combination with tandem mass spectrometry (MS/MS) appears to be a suitable technique for the screening of yohimbine in plant samples, especially in biological fluids, in terms of sensitivity and selectivity. Zanolari et al. have developed an HPLC–UV–API/MS method for the separation and determination of yohimbine in the selected-ion monitoring (rSIM) mode with the molecular ion $[M + H]^+$ at m/z 355 [12].

In recent years, analytical techniques have made great advances. Liquid chromatography–mass spectrometry (LC–MS) and LC–tandem MS (LC–MS/MS) have played a crucial role in plant analysis and mainly focus on hybrid mass spectrometers such as quadrupole time-of-flight (Qq-TOF) [13] and quadrupole-linear ion trap (Qq-LIT) [14]. The application of ion trap mass spectrometers (IT/MS) in structure elucidation analysis and identification of unknowns in complex matrices is well established [15]. However, the aforementioned data show that IT/MS can also be very useful for quantitative analysis [16, 17]. By MS, yohimbine can be studied in the positive-ion mode because of its structure formula. More importantly, HPLC–MS^n (n = 2, 3) can afford much more confidence for compound identification and much lower noise levels for quantification. The fragmentation map of a target analyte is useful in performing MS^n for either qualitative or quantitative analysis. Therefore, it is significant to investigate the fragmentation pathways of the compounds of interest.

To date, however, there is no comprehensive electrospray ionization (ESI)–MS fragmentation study of yohimbine. In this work, we describe a new method of structure elucidation based on MS^n. Direct infusion was used to collect the MS^1 profile and targeted MS^2 and MS^3 data of selected ions (yohimbine) in positive-ion modes. Meanwhile, we developed a simple and sensitive HPLC–MS method for the determination of yohimbine in *P. yohimbe* in the positive-ion mode with the precursor ion m/z 355→144.

Experimental

Chemicals and Materials

Yohimbine hydrochloride was purchased from Sigma (Sigma-Aldrich, USA); HPLC grade methanol and formic acid were obtained from Wuhan Analytical reagent company (Wuhan, China); deionized water was purified using a Milli-Q system (Millipore, Bedford, MA, USA); helium (purity, 99.999%) and liquid nitrogen were obtained from Wuhan Analytical Instrument Factory (Wuhan, China); other reagents used in the experiment were of analytical grade from commercial sources. Bark of *P. yohimbe* was purchased from a traditional Chinese medicine store (Wuhan, China).

Standard and Sample Preparation

The stock solution was prepared by dissolving 10.0 mg of yohimbine in 10 mL methanol. The stock solution was diluted with methanol to obtain 100 μg mL^{-1} as the working standard solution; the solutions were kept at 4°C before use. Standard solutions of yohimbine at various concentrations were prepared by diluting the working solution with methanol.

The barks of *P. yohimbe* were crushed, and dried in an oven at 60°C for 24 h. Aliquots of 100 mg of the powdered bark were extracted in a volumetric flask with 25.0 mL of methanol under ultrasonication at 50°C for 0.5 h. The extraction was repeated three times. The extracted solutions were added together in the volumetric flask and diluted to 100 mL with methanol. The solution was then filtered through a 0.22-μm membrane before injection.

MS^n Analysis of the Reference Compound Solution

An LC/MSD Trap SL Plus spectrometer (Agilent Technologies, Waldbronn, Germany) equipped with an ESI interface and an IT mass analyzer was used for the MS and multistage mass spectrometry (MS^n) analysis. System control and data analysis were provided by the Agilent LC Chemstation and by Bruker Daltonics Trap Control and QuantAnalysis. A syringe pump was used for the direct loop injections of the reference compound working solutions, and the flow rate was set at 0.5 mL h^{-1}. The ESI source was used and operated in the positive-ion mode. Typical operating conditions were as follows: 350°C drying gas (N_2) temperature; 10 L min^{-1} drying gas flow;

50-psi nebulizer gas (N_2) pressure; and 4500-V capillary voltage. Data were acquired with a smart target of 20,000 and a maximum accumulation time of 200 ms. First, full-scan MS spectra were obtained by scanning from 50 to 500 *m/z*. MS^2 acquisition of the most abundant ions in the full-scan MS mode was then carried out. Finally, MS^3 acquisition was used to confirm the identity of the analytes.

HPLC–MS Conditions and Instrumentation

HPLC analyses were performed on an Agilent 1100 HPLC instrument (Agilent Technologies, Waldbronn, USA) coupled to a binary pump, a UV detection system, an autosampler, and a column thermostat. The sample was separated on a DL-C_{18} column (5.0 μm, 150 × 4.6 mm; Japan). The mobile phase consisted of methanol and 10 mM formic acid in water (90:10). The flow rate was 0.5 mL min^{-1} and the column temperature was set at 30°C. The injection volume was 10 μL.

The Agilent 1100 HPLC system was coupled online to an LC/MSD Trap SL Plus spectrometer (Agilent Corp, Waldbronn, America) equipped with an ESI source. The AutoMS operation parameters were as follows: positive-ion mode (ESI^+); nitrogen drying gas flow, 10 L min^{-1}; nebulizer pressure, 50 psi; gas temperature, 350°C; compound stability, 80%; mass range, 50–500 *m/z*. Detection of yohimbine was performed in the selected-ion monitoring (rSIM) mode with $(m/z)^+$ 355→144.

Results and Discussion

Identification and MS^n Behavior of the Reference Compounds

Yohimbine reference compounds were dissolved in methanol and analyzed by ESI–MS^n in the positive-ion mode. The molecular ion of $[M + H]^+$ and major fragments ions were observed in the full-scan MS and MS^n spectra in the positive-ion mode. *Fig. 1* shows the molecular ion and chemical structure obtained by the ESI–MS. The MS spectra from $[M + H]^+$ of yohimbine contained the molecular ion and exhibited the highest intensity. We did not find the molecular ion of $[M + NH_4]^+$, $[M + Na]^+$, or $[M + K]^+$. It shows that the molecular ion of yohimbine is $[M + H]^+$ and is very stable. *Fig. 2* shows the fragment ions at *m/z* 224, 212, 194, 180,162, 144, 134, and 117 by ESI–MS^2. The major fragment ions at *m/z* 224, 212, and 144 exhibit higher in

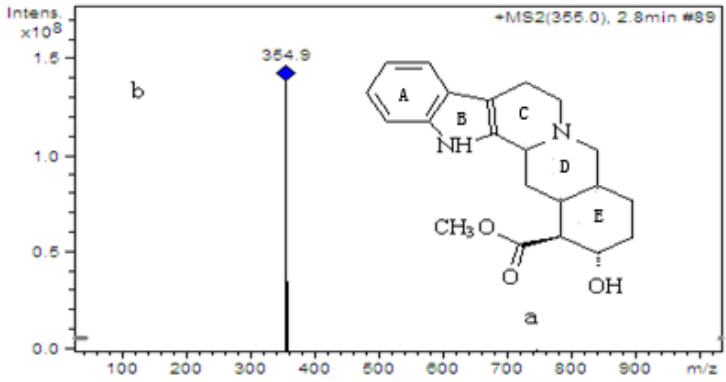

Fig. 1. Chemical structure (a) and molecular ion (b) by full-scan ESI–MS spectra of yohimbine in the positive-ion mode

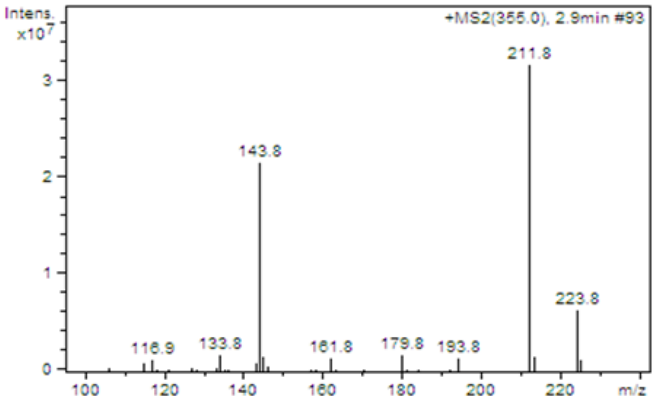

Fig. 2. Full-scan ESI–MS^2 spectra at m/z 355 of yohimbine in the positive-ion mode

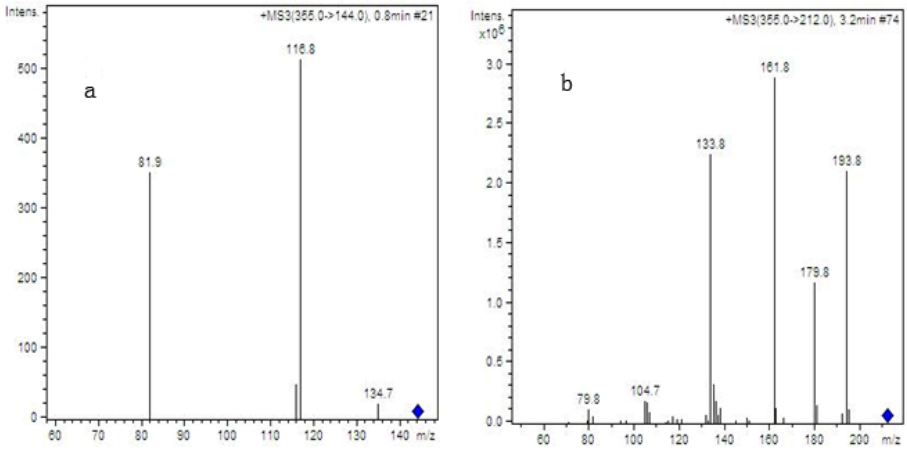

Fig. 3. Full-scan ESI–MS^3 spectra at m/z 144 (a) and 212 (b) of yohimbine in the positive-ion mode

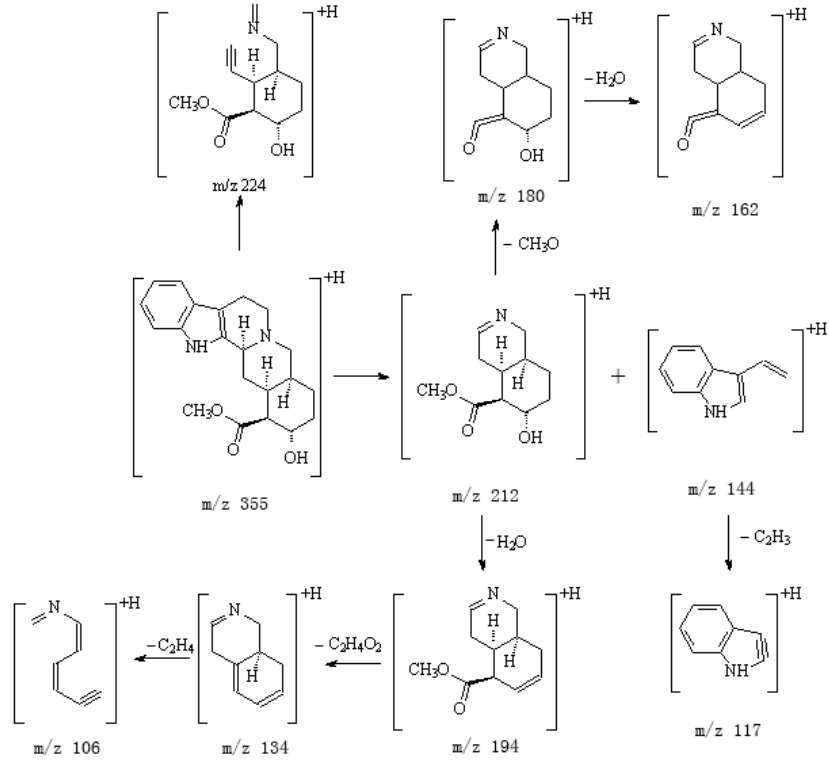

Fig. 4. Proposed fragmentation pathways of main fragment ions for yohimbine in the positive-ion mode

tensity. The molecular ion $[M + H]^+$ may be split in ring C and give the three major fragment ions. The major fragment ions at m/z 212 and 144 are related. *Fig. 3a* shows the fragment ions at m/z 117 by ESI–MS^3 spectra from m/z 144 ($[M - 212]^+$). The ESI–MS^3 spectra from m/z 212 ($[M - 144]^+$) contains ions at m/z 194, 180, 162, 134, and 106, as shown in *Fig. 3b*. The fragment ions at m/z 144 lose ethinyl ($-C_2H_3$) to form the ion at m/z 117. The fragment ions at m/z 212 lose methoxyl ($-CH_3O$) and H_2O to form the ions at m/z 180 and 194, respectively. The fragment ions at m/z 180 lose H_2O to form the ions at m/z 162. The fragment ions at m/z 194 first lose acetic acid ($C_2H_4O_2$) and further lose ethylene to form the ions at m/z 134 and 106. So, the main cleavage pattern was a protonated molecular ion, which first forms the ions at m/z 224, 212, and 144; then the major fragment ion at m/z 212 forms the ions at m/z 194, 180, 162, 134, and 106; and the major fragment ion at m/z 144 forms m/z 117. The possible main fragment ion cleavage pathway is shown in *Fig. 4*.

Quantification of Yohimbine by HPLC–MS

An HPLC–MS method was developed by optimizing the LC separation and ionization and ion transport conditions. A mobile phase composition must be selected to meet the requirement of mass spectrometric detection. The selection of the mobile phase components is a critical factor to obtain good chromatographic peak shape and resolution. In order to enhance the ESI response, formic acid is usually used in the mobile phase of the HPLC. The effect of the formic acid concentration on the separation was studied in the range from 5 to 30 mM. Acceptable retention and separation of analytes was obtained by using as the mobile phase a solution of methanol–water containing 10 mM formic acid. *Fig. 5* shows a typical extracted ion chromatogram and mass spectra of yohimbine in the positive-ion mode by SIM. The intensity of peak at m/z 212 and 144 was higher than that at m/z 224, and the signal-to-noise ratio (SNR) at m/z 144 was more than that at m/z 212 and 224. Therefore, detection of yohimbine was performed in selected ion monitoring (rSIM) mode with $(m/z)^+$ 355→144. *Fig. 6* shows typical HPLC–MS profiles of the standard and the sample. There were no peaks due to foreign matter that could interfere with the analyte and a stable baseline was maintained throughout.

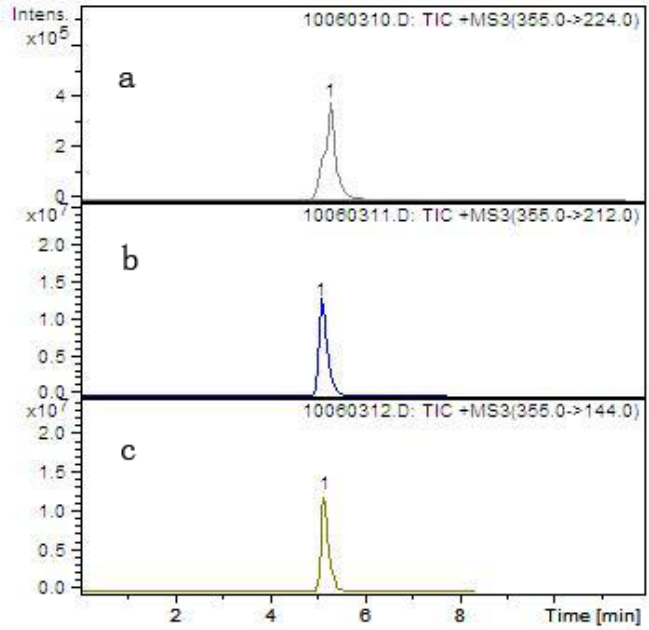

Fig. 5. Typical extracted ion chromatograms of yohimbine by SIM at m/z 224 (a), 212 (b), and 144 (c)

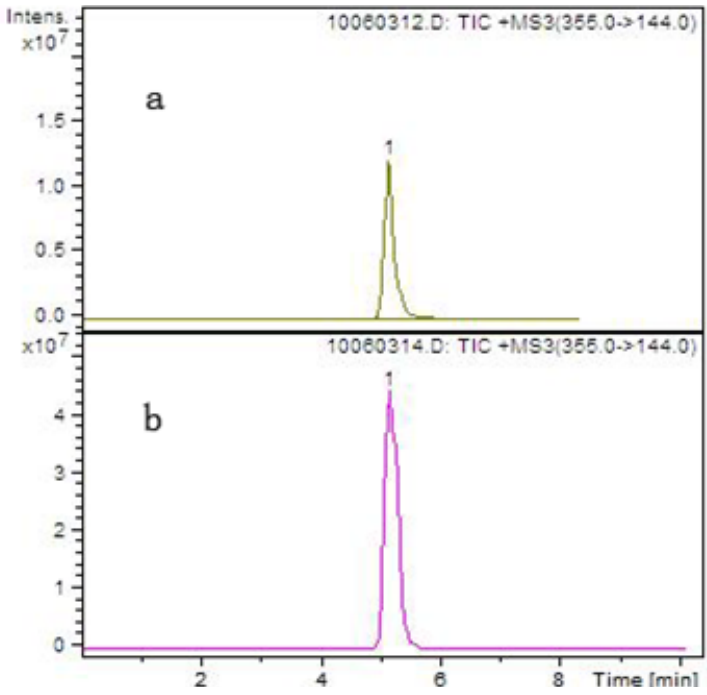

Fig. 6. Typical HPLC–MS profiles of yohimbine standard (a) and sample (b) by SIM

Method Validation

In order to increase the sensitivity of the developed method, quantitative determination was performed by the SIM of ions at m/z 355→144 for yohimbine. The calibration curves were established by injecting six concentration levels of yohimbine (each in triplicate) in the concentration range 0.1–50 μg mL^{-1}. Linear regression was calculated by the peak areas of yohimbine as a function of concentration. The regression equation and its coefficient are $Y = (2 \times 10^6)X + 1 \times 10^7$ and $R^2 = 0.9962$, respectively, for yohimbine by LC–MS. As can be seen, the proposed method presents excellent correlation coefficients and sensitivity.

The limit of detection (LOD) and limit of quantification (LOQ) for the yohimbine standard were determined on the basis of the yohimbine/baseline SNR. A standard stock solution of yohimbine was initially prepared. Dilutions and injections of this standard were then made until HPLC–MS showed that the yohimbine peak height reached an SNR of approximately 10:1 and 3:1 for the LOQ and LOD solutions, respectively. Five injections of the LOQ and three injections of the LOD solutions were made and the relative standard deviation (RSD) for the LOQ solution was determined.

The LOD and LOQ were determined at the same levels. The LOD of yohimbine was determined to be 0.1 ng mL^{-1}. The LOQ was 1.0 ng mL^{-1} with 3.7% RSD for five consecutive injections.

The reproducibility of the method was proved by analyzing samples of yohimbine at three concentration levels: 0.1, 1.0, and 50 μg mL^{-1}. RSDs (n = 5) were 1.7–3.1%.

The precision of injection was evaluated by repeated injection of the sample solution six times. The RSD values of the peak areas for yohimbine were better than 2.73%. The acceptable intra- and interday precisions (%RSD) and accuracy (relative error, %RD) were set as <5% and between −5 and 5%, respectively. Intra- and interday variabilities were determined by analysis of the average amount of standards in quality control samples prepared by standard solutions at low, medium, and high concentrations on three different days. The quality control samples were prepared as a single batch on the same day at each concentration, and then divided into aliquots that were stored at 4°C until required for analysis. The calculated RSDs from repeated measurements are summarized in *Table I*. The assay precision ranged from 1.36% to 2.73%, and the accuracy was 96.5–108.2%.

Table I. Intra- and interday precision and accuracy of yohimbine

Analyte	Actual concentration (μg mL^{-1})	Intraday		Interday	
		RSD (%)	Accuracy	RSD (%)	Accuracy
Yohimbine	0.1	1.43	101.6	2.73	108.2
	1.0	1.78	97.8	1.36	96.5
	50	1.87	99.5	2.16	98.7

Table II. Recoveries of yohimbine in *Pausinystalia yohimbe*

Analyte	Content of sample (μg)	Spiked amount (μg)	Found amount (μg)	Recoveries	
				Mean	RSD (%)
Yohimbine	4.75	1	5.76 ± 0.11	101	1.86
	4.75	5	9.61 ± 0.19	97.1	1.93
	4.75	25	29.53 ± 0.42	99.1	1.41

Method recovery was calculated by spiking five samples with the standard solution at low, medium, and high concentrations of the analyte. Two injections of each preparation were made, and the theoretical amount of

analyte in the sample preparations and the average percentage analyte recovered in the spiked solutions were calculated. The mean recovery for yohimbine was 97.1–101% with an RSD <1.93%. The results are shown in *Table II*.

Applications

Analysis of real samples was performed on dried powdered barks (100 mg) of *P. yohimbe* extracted with methanol (100 mL) under the above conditions. The quantification of yohimbine was performed by HPLC–MS, and the content of the analyte in *P. yohimbe* was found to be 0.95 mg/100 mg (RSD% = 2.01, n = 3). The results obtained by HPLC–IT/MS were compared with those of GC–MS, NACE, and HPLC–UV–API/MS as estimated by the Student's t-test at 95% confidence level, which indicated no significant difference between the methods [1, 12].

Conclusions

A novel HPLC–IT/MS method was developed to identify and quantify yohimbine in *P. yohimbe*. The method provided acceptable figures of merit. It has been demonstrated by MS^n spectra that there are major fragment ions at m/z 355, 224, 212, 194, 180, 162, 144, 134, and 117 in the positive-ion mode. The possible main fragment ion cleavage pathway was discussed. According to the ion peak intensity, we could choose the molecular ion at m/z 355→144 in the positive mode as monitoring ions by SIM. Subsequently, we developed a simple and sensitive HPLC–MS method for the determination of yohimbine in *P. yohimbe* in the positive-ion mode with m/z 355→144. The LOD and LOQ were up to 0.1 and 1.0 ng mL^{-1}, respectively. The method showed good linearity, reproducibility, precision, accuracy, and recovery, and could be used for the quantitative analysis of yohimbine in *Pausinystalia yohimbe*.

Acknowledgments

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