Development of a Genus-Universal Nucleotide Signature for the Identification and Supervision of Ephedra-Containing Products

Gang Wang, Xuanjiao Bai, Xiaochen Chen, Ying Ren and Jianping Han *

Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100193, China; wanggang@implad.ac.cn (G.W.); baixjz@163.com (X.B.); chloe.chen0802@gmail.com (X.C.); renying@implad.ac.cn (Y.R.)
* Correspondence: happymyra2007@163.com

Abstract: Ephedra plants generally contain ephedrine alkaloids, which are the critical precursor compounds of methamphetamine (METH). METH could cause serious physical and mental damage, and therefore Ephedra materials are strictly in supervision internationally. However, unlawful utilization of Ephedra herbs and its products still exist. Thus, it is imperative to establish a universal method for monitoring Ephedra ingredients in complex mixtures and processed products. In this study, 224 ITS2 sequences representing 59 taxa within Ephedra were collected, and a 23-bp genus-level nucleotide signature (GTCCGGTCCGCCTCGGCGGTGCG) was developed for the identification of the whole genus. The specific primers MH-1F/1R were designed, and 125 individuals of twelve Ephedra species/varieties were gathered for applicability verification of the nucleotide signature. Additionally, seven batches of Chinese patent medicines containing Ephedra herbs were used to test the application of the nucleotide signature in complex and highly processed materials. The results demonstrated that the 23-bp molecular marker was unique to Ephedra and conserved within the genus. It can be successfully utilized for the detection of Ephedra components in complex preparations and processed products with severe DNA degradation. The method developed in this study could undoubtedly serve as a strong support for the supervision of illegal circulation of Ephedra-containing products.

Keywords: Ephedra; ephedrine alkaloids; methamphetamine; supervision; nucleotide signature; DNA barcoding

1. Introduction

Ephedra L. (Ephedraceae) contains approximately 67 species, mainly distributed in arid and desert regions of Asia, America, Europe, and Northern Africa [1]. In China, there are about 15 species and 4 varieties, some of which have been used as Chinese traditional medicine for a long history [2]. Ephedra herbs show satisfactory pharmacological activities in the treatment of cold, fever, asthma, flu, chills, headache, nasal congestion, and cough. Generally they are called “Ma Huang” and commonly found existing in Chinese classical prescriptions [3]. Modern pharmacological studies have also indicated that the crude extracts and isolated compounds from Ephedra have the effects of anti-inflammatory, antibacterial, antioxidant, anticancer, and hepatoprotection [4,5]. Classified as herbal supplements in the Dietary Supplement Health and Education Act of 1994, ephedra-containing products were largely unregulated without standardization in strength or purity. Between the 1990s and early 2000s, Ephedra ingredients were prevalent in dietary supplements and drugs for weight loss and physical performance enhancement [6]. It can accelerate metabolism and fatigue remission in bodies and can alter the intestinal flora of obese people [7]. However, afterwards, high-dose and long-term use of Ephedra materials was reported to cause a series of adverse effects due to the content of ephedrine-type alkaloids [8]. Ephedrine alkaloids, such as l-ephedrine, d-pseudoephedrine, l-methylephedrine,
and l-norephedrine, are considered as the main active chemical constituents of *Ephedra* herb, but the misuse and abuse may induce the occurrence of hypertension, insomnia, anxiety, palpitations, arrhythmia, and even sudden death [9,10]. The data from the National Poison Data System (NPDS) of the United States showed that in 2002 alone, the number of the cases of ephedra poisoning reached up to 10,326, including 108 major effects and 3 deaths [11]. Thus, in 2004, the U.S. Food and Drug Administration (FDA) announced the prohibition of the sale of *Ephedra* and ephedrine-containing products, and the ban is still upheld [12]. However, the investigation of 105 weight loss products sold online in 2018 revealed that nearly 20% of the samples contained ephedrine alkaloids or *Ephedra* herb, which violated the 2004 ban of FDA and posed health and safety risks to individuals [13].

More worryingly, *Ephedra* species generally contain ephedrine alkaloids, which are the pivotal chemical precursors for the synthesis of methamphetamine, commonly known as “ice drug”. *Ephedra* herbs and related compound preparations have become the main raw materials for methamphetamine production by criminals [14]. The report on the Drug Situation in China [15], released by the Office of National Narcotics Control Commission, pointed out that methamphetamine was the most commonly abused drug in China, causing extremely serious damage to human’s physical and mental health. The government has imposed strict controls on the production, processing, circulation, and sale of *Ephedra* herb and its products. Nevertheless, illicit trading activities in the market still exist, with illegal elements extracting ephedrine from herb or compound preparations for the production of methamphetamine [16]. It is known that about 0.9 g of pseudoephedrine can be extracted from a box of New Contac capsules, and a bottle (100 mL) of Compound Furacin Nasal Drops contains 0.1 g of ephedrine, while 0.75 g methamphetamine can be produced from 1 g ephedrine [17]. At present, pharmacies within China have set a purchase quantity limit of ephedrine-containing compound preparations and also require real-name registration of customers. However, to avoid inspection authorities, drug makers often prepare *Ephedra* materials in the form of powder, mixture, and crude extract, which leads to difficulties in their identification and brings challenges to customs inspection and market circulation supervision [18]. Therefore, it is necessary to develop a method for the accurate detection of mixture samples, compound preparations, and highly processed products containing *Ephedra* herb.

HPLC (high performance liquid chromatography) has been used in ephedrine alkaloids analysis, and SDS (sodium dodecyl sulfate) is often applied into the mobile phase to improve the resolution. However, it also brings more difficulties to separate the amphiphilic compounds. Lacking a specific and strong chromophore for chemical detection is also a challenge for conventional HPLC-UV detectors. The GC (gas chromatography) method is considered as the most popular technique for the quantitation of ephedrine analogs. For samples with complex composition, to enhance the sensitivity and remove interference compounds, tedious cleanup procedures and precolumn derivatization are required, resulting in pretty time-consuming protocols. Therefore, other more feasible and rapid methods may be needed for the detection of ephedrine or *Ephedra* herb-containing products. DNA barcoding has been recognized as a relatively simple and universal tool for species identification [19]. Chen et al. proposed the preliminary system of DNA barcoding for herbal materials based on the combination of internal transcribed spacer 2 (ITS2) and *psbA-trnH* barcodes, and it has been widely used in the authentication of medical plants [20]. However, the universal barcode sequences are often unsuitable for complex materials or deeply processed products due to severe DNA degradation. Shaw et al. found that short DNA fragments (88–121 bp) could be successfully amplified from pulverized samples that had been boiled for 60 min, while the longer sequences failed [21]. Meusnier et al. presented a “mini-barcode” to well address this problem. It was indicated that the full-length *CO1* with 650 bp could identify 97% of the tested species, while the identification success rates using the shorter sequences of 100 bp and 150 bp within *CO1* region also achieved 90% and 95%, respectively [22]. Furthermore, Han et al. put forward the nucleotide signature, generally 20–50 bp, for the determination of specific species in extracts, decoctions, and
Chinese patent medicines [23–25]. Ephedra genus is the representative group containing ephedrine alkaloids and is the mono-genus belonging to Ephedraceae. Thus, it might be also feasible to develop a genus-level molecular marker for Ephedra L.

In the study, we aimed to develop a short molecular marker for the identification of Ephedra herb and its processed products. A unique 23-bp nucleotide signature (GTC-CGTCGCGCTCGGCGGTGCG) was found highly-conserved and universal within the whole Ephedra genus. Further, the genus-universal nucleotide signature was successfully applied to the determination of Ephedra component in compound preparations with severe DNA degradation. The method developed in our study can also serve as an adjunct to chemical analysis. It can help trace the source of ephedrine in ephedrine-containing products especially in forensic science and determine whether it is caused by the presence of Ephedra herbs. This study will undoubtedly provide a strong support for the supervision of Ephedra-containing products.

2. Materials and Methods

2.1. Collection of Materials

A total of 224 sequences of internal transcribed spacer 2 (ITS2) representing 59 taxa in Ephedra were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (accessed on 19 August 2021) to develop the nucleotide signature. The accession numbers of downloaded sequences were shown in Table S1. To further verify the nucleotide signature, 125 individuals of 12 Ephedra species and varieties were collected from all over China (Table 1). The voucher specimens were deposited in the Herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing, China. To evaluate the detection ability of this method, seven batches of Chinese patent medicines containing Ephedra herb (called Ephedrae Herba in formulas) were purchased from drug stores in Beijing, Heilongjiang, Shandong, and Guangdong provinces, in the forms of tablets, honeyed pills, watered pills, and concentrated pills (Table 2).

| No. | Species       | Voucher No. | Collection Site | GenBank Accession |
|-----|---------------|-------------|----------------|--------------------|
| 1   | E. equisetina | IMD0001954  | Hebei          | OL456774           |
| 2   | E. equisetina | IMD0001953  | Shaanxi        | OL456770           |
| 3   | E. equisetina | IMD0001955  | Shanxi         | OL456771           |
| 4   | E. equisetina | IMD0001952  | Hebei          | OL456772           |
| 5   | E. equisetina | IMD0001951  | Xinjiang       | OL456773           |
| 6   | E. equisetina | IMD0001950  | Xinjiang       | OL456775           |
| 7   | E. equisetina | IMD0001949  | Xinjiang       | OL456776           |
| 8   | E. equisetina | IMD0001946  | Xinjiang       | OL456777           |
| 9   | E. equisetina | IMD0001964  | Beijing        | OL456763           |
| 10  | E. equisetina | IMD0001963  | Beijing        | OL456764           |
| 11  | E. equisetina | IMD0001966  | Xinjiang       | OL456765           |
| 12  | E. equisetina | IMD0001961  | Hebei          | OL456766           |
| 13  | E. equisetina | IMD0001958  | Jiangsu        | OL456767           |
| 14  | E. equisetina | IMD0001957  | Beijing        | OL456768           |
| 15  | E. equisetina | IMD0001956  | Beijing        | OL456769           |
| 16  | E. fedtschenkoae | IMD0001967 | Qinghai       | OL456778           |
| 17  | E. gerardiana | IMD0001970  | Xizang         | OL456779           |
| 18  | E. gerardiana | IMD0001969  | Xizang         | OL456780           |
| 19  | E. gerardiana | IMD0001968  | Xizang         | OL456781           |
| 20  | E. gerardiana | IMD0001971  | Xizang         | OL456782           |
| 21  | E. gerardiana | IMD0001972  | Xizang         | OL456783           |
| 22  | E. intermedia | IMD0001980  | Qinghai       | OL456784           |
| 23  | E. intermedia | IMD0001978  | Qinghai       | OL456785           |
| 24  | E. intermedia | IMD0001976  | Xinjiang       | OL456786           |
| No. | Species            | Voucher No. | Collection Site | GenBank Accession |
|-----|--------------------|-------------|-----------------|-------------------|
| 25  | *E. intermedia*    | IMD0001975  | Qinghai         | OL456787          |
| 26  | *E. intermedia*    | IMD0001974  | Xinjiang        | OL456788          |
| 27  | *E. intermedia*    | IMD0001995  | Sichuan         | OL456789          |
| 28  | *E. intermedia*    | IMD0001994  | Neimenggu       | OL456790          |
| 29  | *E. intermedia*    | IMD0001992  | Xinjiang        | OL456791          |
| 30  | *E. intermedia*    | IMD0001991  | Xinjiang        | OL456792          |
| 31  | *E. intermedia*    | IMD0001989  | Xinjiang        | OL456793          |
| 32  | *E. intermedia*    | IMD0001988  | Hebei           | OL456794          |
| 33  | *E. intermedia*    | IMD0001996  | Neimenggu       | OL456795          |
| 34  | *E. intermedia*    | IMD0001986  | Xinjiang        | OL456796          |
| 35  | *E. intermedia*    | IMD0001984  | Gansu           | OL456797          |
| 36  | *E. intermedia*    | IMD0001983  | Xinjiang        | OL456798          |
| 37  | *E. intermedia*    | IMD0001981  | Gansu           | OL456799          |
| 38  | *E. intermedia*    | IMD0001985  | Sichuan         | OL456800          |
| 39  | *E. intermedia*    | IMD0001987  | Xinjiang        | OL456801          |
| 40  | *E. intermedia* var. tibetica | IMD0001982 | Xizang          | OL456802          |
| 41  | *E. intermedia* var. tibetica | IMD0002003 | Xizang          | OL456803          |
| 42  | *E. intermedia* var. tibetica | IMD0002002 | Xizang          | OL456804          |
| 43  | *E. intermedia* var. tibetica | IMD0002001 | Xizang          | OL456805          |
| 44  | *E. intermedia* var. tibetica | IMD0002000 | Xizang          | OL456806          |
| 45  | *E. intermedia* var. tibetica | IMD0001999 | Xizang          | OL456807          |
| 46  | *E. intermedia* var. tibetica | IMD0001998 | Xizang          | OL456808          |
| 47  | *E. intermedia* var. tibetica | IMD0001997 | Xizang          | OL456809          |
| 48  | *E. intermedia* var. tibetica | IMD0002016 | Xizang          | OL456810          |
| 49  | *E. intermedia* var. tibetica | IMD0002014 | Xizang          | OL456811          |
| 50  | *E. intermedia* var. tibetica | IMD0002013 | Xizang          | OL456812          |
| 51  | *E. intermedia* var. tibetica | IMD0002012 | Xizang          | OL456813          |
| 52  | *E. intermedia* var. tibetica | IMD0002011 | Xizang          | OL456814          |
| 53  | *E. intermedia* var. tibetica | IMD0002010 | Xizang          | OL456815          |
| 54  | *E. intermedia* var. tibetica | IMD0002009 | Xizang          | OL456816          |
| 55  | *E. intermedia* var. tibetica | IMD0002007 | Xizang          | OL456817          |
| 56  | *E. intermedia* var. tibetica | IMD0002006 | Xizang          | OL456818          |
| 57  | *E. intermedia* var. tibetica | IMD0002005 | Xizang          | OL456819          |
| 58  | *E. intermedia* var. tibetica | IMD0002008 | Xizang          | OL456820          |
| 59  | *E. intermedia* var. tibetica | IMD0002021 | Xizang          | OL456821          |
| 60  | *E. intermedia* var. tibetica | IMD0002017 | Xizang          | OL456822          |
| 61  | *E. intermedia* var. tibetica | IMD0002019 | Xizang          | OL456823          |
| 62  | *E. intermedia* var. tibetica | IMD0002020 | Xizang          | OL456824          |
| 63  | *E. intermedia* var. tibetica | IMD0002018 | Xizang          | OL456825          |
| 64  | *E. intermedia* var. tibetica | IMD0002024 | Xizang          | OL456826          |
| 65  | *E. intermedia* var. tibetica | IMD0002023 | Xizang          | OL456827          |
| 66  | *E. intermedia* var. tibetica | IMD0002022 | Xizang          | OL456828          |
| 67  | *E. likiangensis*   | IMD0002027  | Yunnan          | OL456829          |
| 68  | *E. likiangensis*   | IMD0002026  | Sichuan         | OL456830          |
| 69  | *E. likiangensis*   | IMD0002025  | Yunnan          | OL456831          |
| 70  | *E. minuta*         | IMD0002029  | Xizang          | OL456832          |
| 71  | *E. minuta*         | IMD0002030  | Xizang          | OL456833          |
| 72  | *E. minuta*         | IMD0002028  | Sichuan         | OL456834          |
| 73  | *E. monosperma*     | IMD0002037  | Qinghai         | OL456835          |
| 74  | *E. monosperma*     | IMD0002043  | Xizang          | OL456836          |
| 75  | *E. monosperma*     | IMD0002044  | Xizang          | OL456837          |
| 76  | *E. przewalskii*    | IMD0002060  | Gansu           | OL456841          |
| 77  | *E. przewalskii*    | IMD0002059  | Qinghai         | OL456842          |
| 78  | *E. przewalskii*    | IMD0002057  | Gansu           | OL456843          |
| 79  | *E. przewalskii*    | IMD0002056  | Xinjiang        | OL456844          |
| 80  | *E. przewalskii*    | IMD0002055  | Qinghai         | OL456845          |
| 81  | *E. przewalskii*    | IMD0002063  | Gansu           | OL456846          |
Table 1. Cont.

| No. | Species       | Voucher No. | Collection Site | GenBank Accession |
|-----|---------------|-------------|-----------------|-------------------|
| 82  | E. przewalskii | IMD0002062  | Gansu           | OL456838          |
| 83  | E. przewalskii | IMD0002061  | Gansu           | OL456839          |
| 84  | E. przewalskii | IMD0002051  | Xinjiang        | OL456840          |
| 85  | E. regeliana   | IMD0002066  | Qinghai         | OL456850          |
| 86  | E. regeliana   | IMD0002064  | Xinjiang        | OL456847          |
| 87  | E. regeliana   | IMD0002067  | Qinghai         | OL456848          |
| 88  | E. regeliana   | IMD0002070  | Xinjiang        | OL456849          |
| 89  | E. saxatilis   | IMD0002071  | Xizang          | OL456851          |
| 90  | E. saxatilis   | IMD0002078  | Xizang          | OL456852          |
| 91  | E. saxatilis   | IMD0002076  | Xizang          | OL456853          |
| 92  | E. saxatilis   | IMD0002075  | Xizang          | OL456854          |
| 93  | E. saxatilis   | IMD0002077  | Sichuan         | OL456855          |
| 94  | E. saxatilis   | IMD0002081  | Xizang          | OL456856          |
| 95  | E. saxatilis   | IMD0002080  | Xizang          | OL456857          |
| 96  | E. saxatilis   | IMD0002079  | Xizang          | OL456858          |
| 97  | E. saxatilis   | IMD0002090  | Xizang          | OL456859          |
| 98  | E. saxatilis   | IMD0002089  | Xizang          | OL456860          |
| 99  | E. saxatilis   | IMD0002088  | Xizang          | OL456861          |
| 100 | E. saxatilis   | IMD0002087  | Xizang          | OL456862          |
| 101 | E. sinica      | IMD0002098  | Hebei           | OL456887          |
| 102 | E. sinica      | IMD0002099  | Hebei           | OL456863          |
| 103 | E. sinica      | IMD0002097  | Hebei           | OL456864          |
| 104 | E. sinica      | IMD0002095  | Shanxi          | OL456865          |
| 105 | E. sinica      | IMD0002106  | Hebei           | OL456866          |
| 106 | E. sinica      | IMD0002105  | Neimenggu       | OL456867          |
| 107 | E. sinica      | IMD0002104  | Hebei           | OL456868          |
| 108 | E. sinica      | IMD0002103  | Hebei           | OL456869          |
| 109 | E. sinica      | IMD0002101  | Shanxi          | OL456870          |
| 110 | E. sinica      | IMD0002109  | Neimenggu       | OL456871          |
| 111 | E. sinica      | IMD0002108  | Hebei           | OL456872          |
| 112 | E. sinica      | IMD0002118  | Neimenggu       | OL456873          |
| 113 | E. sinica      | IMD0002117  | Neimenggu       | OL456874          |
| 114 | E. sinica      | IMD0002115  | Neimenggu       | OL456875          |
| 115 | E. sinica      | IMD0002112  | Neimenggu       | OL456876          |
| 116 | E. sinica      | IMD0002111  | Jilin           | OL456877          |
| 117 | E. sinica      | IMD0002125  | Neimenggu       | OL456878          |
| 118 | E. sinica      | IMD0002122  | Neimenggu       | OL456879          |
| 119 | E. sinica      | IMD0002123  | Neimenggu       | OL456880          |
| 120 | E. sinica      | IMD0002121  | Neimenggu       | OL456881          |
| 121 | E. sinica      | IMD0002119  | Hebei           | OL456882          |
| 122 | E. sinica      | IMD0002136  | Neimenggu       | OL456883          |
| 123 | E. sinica      | IMD0002135  | Neimenggu       | OL456884          |
| 124 | E. sinica      | IMD0002134  | Neimenggu       | OL456885          |
| 125 | E. sinica      | IMD0002132  | Neimenggu       | OL456886          |

Table 2. Detailed information of the seven batches of Chinese patent medicine.

| No.     | Sample Name              | Type          | Collection Site | Number of Ingredients | Ingredients on Label                                                                 |
|---------|--------------------------|---------------|-----------------|-----------------------|-------------------------------------------------------------------------------------|
| ZCY-01  | Mahuang Fuzi Xsinsou     | Concentrated pills | Guangdong       | 3                     | Ephedrae Herba, Aconiti Lateralis Radix Praeparata, Asari Radix Et Rhizoma             |
| ZCY-02  | Maxing Zhike Tablets     | Tablets        | Jilin           | 4                     | Ephedrae Herba, Armeniacae semen Amarum, Gypsum Fibrosum, Gycyrrhziae Radix Et Rhizoma Praeparata Cum Melle |
| ZCY-03  | Mahuang Zhisou Pills     | Watered pills  | Shandong        | 7                     | Citri Exocarpium Rubrum, Ephedrae Herba, Platycodonis Radix, Fritillariae Cirrhosa Bulbus, Schisandrae Chinensis Fructus, Poria, Asari Radix Et Rhizoma |
Table 2. Cont.

| No.  | Sample Name         | Type            | Collection Site | Number of Ingredients | Ingredients on Label                                                                 |
|------|---------------------|-----------------|-----------------|-----------------------|-------------------------------------------------------------------------------------|
| ZCY-04 | Tongxuan Lifei Pills | Honeyed pills   | Beijing         | 11                    | Perillae Folium, Peucedani Radix, Platycodonis Radix, Armoniacae Semen Amarum, Ephedrae Herba, Glycyrrhizae Radix Et Rhizoma, Cinn. Reticulatae Pericarpium, Pinelliae Rhizoma, Poria, Aurantii Fructus, Scutellariae Radix |
| ZCY-05 | Xiaoer Feire Kechuan Granules | Granules        | Heilongjiang    | 11                    | Ephedrae Herba, Armoniacae Semen Amarum, Gypsym Fibrosus, Glycyrrhizae Radix Et Rhizoma, Lonicerae Japonicas Flos, Forsythiae Fructus, Anemarrhenae Rhizoma, Scutellariae Radix, Isatisidis Radix, Ophiopogonis Radix, Houttuyniae Herba |
| ZCY-06 | Zhengtian Pills     | Watered pills   | Guangdong       | 15                    | Uncariae Ramulus Cum Uncis, Paeoniae Radix Alba, Chaunxiong Rhizoma, Angelicae Sinensis Radix, Rehmanniae Radix, Angelicae Dahuricae Radix, Saponashkoviae Radix, Štotepteryyi Rhizoma Et Radix, Persicae Semen, Canthami Flos, Asari Radix Et Rhizoma, Angelicae Pubescentis Radix, Ephedrae Herba, Aconiti Lateralis Radix Praeparata, Spaltholobi Caulis |
| ZCY-07 | Qiguanyan Pills     | Watered pills   | Beijing         | 31                    | Ephedrae Herba, Armoniacae Semen Amarum, Gypsym Fibrosus, Glycyrrhizae Radix Et Rhizoma, Paeonlai Radix, Cynanchi Stauntoni Rhizoma Et Radix, Stemonae Radix, Asteris Radix Et Rhizoma, Farfarae Flos, Meretricis Concha Cyclinae Concha, Descurainiae Semon Lepidii Semon, Cetri Grandis Exsorciarum, Platycodonis Radix, Poria, Pinelliae Rhizoma, Polygalae Radix, Irrulae Flos, Pumice, Perillae Fructus, Codonopsis Radix, Rijzuiji Fructus, Schisandrae Chinensis Fructus, Cinnamomi Ramulus, Alli Macrostemonius Bulbus, Paeoniae Radix Alba, Mori Folium, Belamcandae Rhizoma, Scutellariae Radix, Indigo Naturalis, Taraxaci Herba, Eriobotryae Folium |

2.2. DNA Extraction

Voucher samples: The surface of specimen samples was cleaned up with 75% ethanol, and then approximately 20 mg of each sample was added to a centrifuge tube for DNA extraction. The samples were ground into fine powder with a ball-milling instrument (Retsch Co., Shanghai, China) at a frequency of 30 Hz for 2 min. Then the genomic DNA was extracted using a Plant Genomic DNA Extraction Kit (Tiangen Biotech Beijing Co., Beijing, China) according to manufacturer’s instructions.

Chinese patent medicines: Approximately 50 mg of the Chinese patent medicines was added to a centrifuge tube, and six parallel tubes of each preparation were prepared. Seven-hundred microliters of the prewash buffer (700 mM NaCl; 100 mM Tris-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 2% PVP-40; and 0.4% β-mercaptoethanol) was added to the tubes to wash the powder several times until the supernatant was colorless and transparent, and then the mixture was centrifuged at 7500 × g for 5 min. The genomic DNA was subsequently extracted from the precipitate via Plant Genomic DNA Extraction Kit (Tiangen Biotech Beijing Co.) in accordance with manufacturer’s instructions. Finally, the DNA in six replicates of the same batch was eluted with 50 µL of double-distilled water into a single tube. The DNA quality of voucher samples and Chinese patent medicines was examined via Nanodrop 2000 (Thermo Scientific, Waltham, Massachusetts, USA). The value of A260/280 was 1.8–2.0, and the DNA concentration ranged from 60 ng/µL to 80 ng/µL, which met the requirements of subsequent PCR reaction.

2.3. Primer Design, PCR Amplification and Sequencing

The primer pair MH-1F (5’TACATCGAGTCTTTGAACGC3’)/MH-1R (5’ATGCCAA GGTCCTCCTT-T3’) was designed using Primer Premier 6.0 software (Premier Co., Palo Alto, CA, USA) for the amplification of the short DNA fragments (~150 bp) containing the nucleotide signature of Ephedra L. Polymerase chain reactions (PCR) were performed in a 25-µL system consisting of 12.5 µL of 2 × PCR Master Mix (Aidlab Biotechnologies Co., Beijing, China), 1.0 µL of forward/reverse primers (MH-1F/MH-1R, 2.5 µM), and 2.0 µL of
DNA templates and filled with double-distilled water. The reactions were then performed as follows: 94 °C for 5 min; followed by 35 cycles of 94 °C for 45 s, 56 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR products were examined via 1% (w/v) agarose gel electrophoresis that had been prestained by GelRed (Mei5 Biotechnology Co., Beijing, China) and bidirectionally sequenced using an ABI 3730XL sequencer (Applied Biosystems Co., Foster City, CA, USA) at the Major Engineering laboratory of Chinese Academy of Agricultural Sciences (Beijing, China).

2.4. Sequence Analysis

The relatively conserved regions, screened out from downloaded ITS2 sequences of *Ephedra* L., were selected as the candidates of the nucleotide signature. BLAST analysis was carried out in the website of National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) (accessed on 19 August 202) for the validation of the intragenus conservation and intergeneric specificity of the nucleotide signature. The sequencing data of voucher specimens and Chinese patent medicine were edited and assembled via CodonCode Aligner 5.2.0 (CodonCode Co., Centerville, MA, USA). The sequences were then aligned by MEGA-X software. All the experimental sequences of the 125 specimens were submitted to GenBank database, with accession numbers listed in Table 1.

3. Results

3.1. Development of the Genus-Universal Nucleotide Signature for *Ephedra* L.

Through sequence screening of 224 ITS2 sequences representing 59 taxa downloaded from GenBank, a 55-bp relatively conserved fragment was obtained as the candidate of the nucleotide signature of *Ephedra* L. The regions with different lengths within the selected fragment were intercepted and BLAST analysis was performed to determine the optimal sequence size of the nucleotide signature. It was indicated that longer DNA segments were less conserved in genus level, and a single sequence cannot cover the entire *Ephedra* L. (Figures S1–S5). However, too short sequences lost their intergeneric specificity and matched the species outside *Ephedra* genus (Figure S6). Finally, a 23-bp fragment (GTCCGGTCCGGTCCGGGTGGCGGTGG) was identified as the unique nucleotide signature for *Ephedra* (Table 3). This sequence was highly conservative in *Ephedra* L., and no mutation sites were found in the nucleotide signature region of the 224 downloaded ITS2 sequences. BLAST results in NCBI also showed that the 23-bp nucleotide signature was specific to *Ephedra* genus, and there was at least one variation in this region between *Ephedra* and non-*Ephedra* (Figure S7). Therefore, the 23-bp nucleotide signature can be considered as a genus-universal molecular marker for *Ephedra* L.

| No. | Sequences (5′-3′) | Length/bp | Intergeneric Specificity | Intragenus Conservation |
|-----|------------------|-----------|-------------------------|-------------------------|
| S1  | TCGGGGGGACGGCCTTGACCGTCCGGTCCGGTCGGGTGGTGAAT | 55        | √                       | ×                       |
| S2  | GGGGGACGGCCTTGACCGTCCGGTCCGGTCGGGTGGT   | 45        | √                       | ×                       |
| S3  | GCCCTGACGGCCTCGGGTCGGGTGGGTGGT   | 35        | √                       | ×                       |
| S4  | GACCGTCCGGTCCGGTCCGGTCCGGTCCGGT   | 30        | √                       | ×                       |
| S5  | CCGGTTCGGTCCGGTCCGGTCCGGTCCGGT   | 27        | √                       | ×                       |
| S6  | GTCCGGTCCGGTCCGGTCCGGTCCGGT   | 23        | √                       | √                       |
| S7  | CCGGTTCGGTCCGGTCCGGTCCGGT   | 20        | √                       | √                       |

3.2. Verification of the Nucleotide Signature in the Species and Varieties within *Ephedra* L.

To further test the reliability and universality of the nucleotide signature in *Ephedra* L., 125 individuals covering 12 species and varieties were collected. The primer pair MH-1F/MH-1R was designed to amplify the short DNA fragments containing the nucleotide signature. The results showed that all specimens were successfully amplified, indicating a good applicability of the primers (Figure 1). Finally, 125 sequences of 152–153 bp were obtained from specimen samples, which were subsequently aligned with the 224 ITS2
sequences of *Ephedra* L. downloaded from GenBank. The alignment of the total 349 ITS2 regions demonstrated that the 23-bp nucleotide signature existed in all the sequences without any variations. In conclusion, the developed nucleotide signature was universal within *Ephedra* L. and could be amplified successfully from specimens by using the primers MH-1F/MH-1R.

**Figure 1.** Amplification of 12 species and varieties in *Ephedra* genus with the primers MH-1F/MH-1R. M. DNA marker; CK. Negative control; 1–3. *E. equisetina*; 4. *E. fedtschenkoae*; 5–6. *E. gerardiana*; 7–9. *E. intermedia*; 10–13. *E. intermedia var. tibetica*; 14. *E. likiangensis*; 15. *E. minuta*; 16. *E. monosperma*; 17–18. *E. przewalskii*; 19. *E. regeliana*; 20–21. *E. saxatilis*; 22–24. *E. sinica*.

### 3.3. Application of the Nucleotide Signature on the Detection of Ephedra Herb in Compound Preparations

To validate the application of this method on complex and processed products, a total of seven batches of commercial Chinese patent medicines containing *Ephedra* herb were gathered from pharmacies in China, i.e., Mahuang Fuzi Xixin Soup, Maxing Zhike Tablets, Mahuang Zhisou Pills, Tongxuan Lifei Pills, Xiaoer Feire Kechuan Granules, Zhengtian Pills, and Qiguanyan Pills. The formulations of these preparations consisted of 3 (Mahuang Fuzi Xixin Soup) to 31 (Qiguanyan Pills) ingredients, respectively (Table 2). All the compound preparations were successfully amplified and sequenced using the designed primers MH-1F/MH-1R. It was showed that the *Ephedra* herb listed on label could be detected in the seven Chinese patent medicine samples by retrieving the nucleotide signature in sequencing results. Moreover, the direct sequencing of the PCR products presented very clean traces (Figure 2), which illustrated that the MH-1F/1R primer pair could specifically amplify the nucleotide signature of *Ephedra* in a complex system. To sum up, the nucleotide signature together with specific primers was suitable for the inspection of *Ephedra* ingredients in compound preparations and other deeply processed products.
4. Discussion

4.1. Significance of the Development of a Nucleotide Signature for Ephedra

DNA barcoding is a powerful tool for species authentication based on species-specific differences of a standard and short DNA sequence. It can help achieve the rapid, accurate, and automated identification of plants and animals [26,27]. At present, a universal standard as well as the databases for DNA barcoding have been established and widely applied [28]. It was also suggested as an efficient tool for the supervision of herbal markets. Han et al. investigated 1436 samples representing 295 medicinal species from seven herb markets in China, and found that 1260 samples were successfully identified by ITS2 barcodes, among which approximately 4.2% were adulterants [29]. The survey of species authenticity for herbal products using DNA barcoding showed that only 62% of the 21 samples labeled as “Nan-she-teng (Celastrus orbiculatus)” and 31% of the 26 samples labeled as “Lei-gong-teng (Tripterygium wilfordii)” were genuine [30]. However, in view of the strict regulation, to avoid inspection authorities, the Ephedra raw materials are often transported and traded by lawbreakers in the forms of powder, crude extract, compound preparations, and other processed products. In this circumstance, conventional DNA barcodes are no longer applicable due to severe DNA degradation. Liu et al. found that complete ITS2 region could not be amplified from herbal decoction using universal primers 2F/3R [23]. Song et al. also indicated that the five primary barcode loci (ITS2, psbA-trnH, rbcL, matK, and trnL) could be successfully amplified in only 8.89%–20% of processed samples, while the amplification rate of the short trnL (UAA) intron P6 loop was up to 75.56% [31]. Thus, in this study, we aimed to develop a short nucleotide signature to more effectively identify Ephedra-containing products.

An ideal nucleotide signature at genus level should meet the qualifications of both intragenus conservation and intergenus specificity. In this study, 224 ITS2 sequences...
of 59 taxa in *Ephedra* L. were downloaded from GenBank to develop a 23-bp nucleotide signature. Then 125 individuals representing 12 *Ephedra* species and varieties were collected together to test the nucleotide signature. In total, nearly 90% of the taxa within *Ephedra* genus were involved. Considering the possible errors in published data, firstly we verified the accuracy of the sequences downloaded from GenBank by BLAST analysis to ensure their reliability. Finally, it was proved that the 23-bp nucleotide signature universally indeed existed in *Ephedra* L. The 125 experiment specimens, collected from different areas in China, were identified by professional taxonomists, and the nucleotide signature was found to exist in all the samples without any mutations. It can be inferred that the nucleotide signature is ubiquitous and highly conserved in *Ephedra* genus. In addition, the BLAST results in NCBI also showed that the nucleotide signature was unique to *Ephedra* L. Therefore, we believe that currently available data is sufficient to support that the 23-bp DNA fragment can serve as a molecular tag for the identification of *Ephedra*-containing products. Additionally, compared to species-specific molecular markers, the genus-level nucleotide signature can effectively avoid the complex work of developing multiple detection methods for different taxa in *Ephedra*.

### 4.2. Supervision of Ephedra-Containing Products with the Genus-Universal Nucleotide Signature

The genus *Ephedra* contains about 67 species widely distributed around the world. The chemical constituents in *Ephedra* generally include alkaloids, flavonoids, tannins, polysaccharides, phenolics, etc [32], among which ephedrine-type alkaloids are regarded as the main active ingredients to exert pharmacological effects [33]. *Ephedra* herb has been used in traditional Chinese medicine with a long history, which was originally recorded in the ancient book of *Shennong’s Classic of Materia Medica*. More than 60 classical prescriptions containing *Ephedra* herb are recorded in the Chinese Pharmacopoeia (2020 edition). Besides official value, *Ephedra* materials were also supplemented into dietary, health care products, and cosmetics for weight loss and energy enhancement [34]. However, under the unrestricted and controlled use, more and more side effects on people appeared continuously [35]. The subsequent studies found that long-term or high-dose use of ephedrine-containing products can lead to hypertension, arrhythmia, stroke, cardiac arrest, and even sudden death [36]. Though the government and regulatory authorities have strictly controlled *Ephedra* and ephedrine products, its illegal production and sales still exist. Worryingly, ephedrine is also an important raw material for the production of methamphetamine. Ephedrine obtained by criminals from *Ephedra* herbs, crude extracts, and compound preparations for the synthesis of methamphetamine have caused great damage to society and human’s physical and mental health [18]. In a survey of 592 drug users, more than 70% claimed to have injected methamphetamine, and the proportion of the participants reporting methamphetamine as their most frequently injected drug increased from 2.1% in 2005 to 29.6% in 2015 [37]. Although chemical methods such as HPLC and GC have been widely used for the detection of ephedrine and *Ephedra* herbs, for processed samples with complex components, the interfering components will affect the analysis results, and the tedious pre-treatment process will greatly increase the detection time. Thus, it is urgent to establish other more effective methods for the supervision of *Ephedra* and its deeply processed products.

In this study, a 23-bp nucleotide signature was developed for the detection of *Ephedra* herb and its products with severely degraded DNA, which has been proved to be conserved within *Ephedra* genus and divergent with other genera. Furthermore, the method was applied to the determination of seven batches of Chinese patent medicines that contained *Ephedra* ingredients. The results showed that *Ephedra* herb could be successfully detected in all the samples by seeking the nucleotide signature in sequencing results. It is worth emphasizing that in the proprietary Chinese patent medicine Qiguanyan Pills, in addition to Ephedrae Herba, there are an additional 30 kinds of other herbs, including Armeniaceae Semen Amarum (Kuxingren), Gypsum Fibrosum (Shigao), Glycyrrhizae Radix Et Rhizoma (Gancao), Peucedani Radix (Qianhu), Cynanchi Stauntonii Rhizoma Et Radix
(Baiqian), Stemonae Radix (Baibu), etc. The nucleotide signature could be still amplified and sequenced successfully with the newly designed primers MH-1F/MH-1R. It is suggested that even if the ingredients are complex and diverse, the Ephedra herb can still be detected, which simultaneously reflects the high sensitivity of this method. In conclusion, the genus-universal nucleotide signature proposed in this study can be considered as a powerful tool for the supervision of Ephedra and ephedrine-containing products.

5. Conclusions

In this study, a 23-bp genus-specific nucleotide signature was developed for the detection of Ephedra herb and its highly processed products. The nucleotide signature was verified to be unique and highly conserved within Ephedra, which could be successfully amplified and sequenced from processed products containing Ephedra ingredients using the primer pair MH-1F/MH-1R. This method can also serve as an adjunct to chemical analysis to help trace the source of ephedrine in ephedrine-containing products especially in forensic science. Besides, the genus-level molecular marker effectively avoids the tedious work of establishing various identification/detection methods for different species in Ephedra. Our study will undoubtedly provide a strong support for the identification and regulation of Ephedra-containing products to prevent illegal supplement and methamphetamine production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27072342/s1, Table S1: Information of the 224 ITS2 sequences of Ephedra downloaded from GenBank; Figure S1: BLAST results in NCBI of the sequence S1; Figure S2: BLAST results in NCBI of the sequence S2; Figure S3: BLAST results in NCBI of the sequence S3; Figure S4: BLAST results in NCBI of the sequence S4; Figure S5: BLAST results in NCBI of the sequence S5; Figure S6: BLAST results in NCBI of the sequence S7; Figure S7: BLAST results in NCBI of the nucleotide signature of Ephedra L.

Author Contributions: Conceptualization, J.H.; Data curation, G.W. and X.B.; Investigation, G.W.; Resources, G.W., X.B. and Y.R.; Writing—original draft, G.W.; Writing—review & editing, X.C. and J.H. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Key Research and Development Program of China (2019YFC1604701).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are openly available in https://www.ncbi.nlm.nih.gov/ with the GenBank accession numbers in Table 1.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bánki, O.; Roskov, Y.; Vandeputte, L.; DeWalt, R.E.; Remsen, D.; Schalk, P.; Orrell, T.; Keping, M.; Müller, J.; Aalbu, R.; et al. Catalogue of Life Checklist; Catalogue of Life: Leiden, The Netherlands, 2021. [CrossRef]
2. González-Juárez, D.E.; Escobedo-Moratilla, A.; Flores, J.; Hidalgo-Figueroa, S.; Martínez-Tagüeña, N.; Morales-Jiménez, J.; Muñiz-Ramírez, A.; Pastor-Palacios, G.; Pérez-Miranda, S.; Ramírez-Hernández, A.; et al. A Review of the Ephedra genus: Distribution, Ecology, Ethnobotany, Phytochemistry and Pharmacological Properties. Molecules 2020, 25, 3283. [CrossRef] [PubMed]
3. Miao, S.-M.; Zhang, Q.; Bi, X.-B.; Cui, J.-L.; Wang, M.-L. A review of the phytochemistry and pharmacological activities of Ephedra herb. Chin. J. Nat. Med. 2020, 18, 321–344. [CrossRef]
4. Seif, M.; Deabes, M.; El-Askary, A.; El-Kott, A.F.; Albadrani, G.M.; Seif, A.; Wang, Z. Ephedra sinica mitigates hepatic oxidative stress and inflammation via suppressing the TLR4/MyD88/NF-κB pathway in fipronil-treated rats. Environ. Sci. Pollut. Res. 2021, 28, 62943–62958. [CrossRef] [PubMed]
5. Khalil, M.; Khalifeh, H.; Saad, F.; Serale, N.; Salis, A.; Damonte, G.; Lupidi, G.; Daher, A.; Vergani, L. Protective effects of extracts from Ephedra foeminea Forssk fruits against oxidative injury in human endothelial cells. J. Ethnopharmacol. 2020, 260, 112976. [CrossRef]
6. Palamar, J. How ephedrine escaped regulation in the United States: A historical review of misuse and associated policy. Health Policy 2011, 99, 1–9. [CrossRef]
7. Kim, B.-S.; Song, M.-Y.; Kim, H. The anti-obesity effect of Ephedra sinica through modulation of gut microbiota in obese Korean women. *J. Ethnopharmacol.* 2014, 152, 532–539. [CrossRef]

8. Munafò, A.; Frara, S.; Perico, N.; Di Mauro, R.; Cortinovis, M.; Burgaletto, C.; Cantarella, G.; Remuzzi, G.; Giustina, A.; Bernardini, R. In search of an ideal drug for safer treatment of obesity: The false promise of pseudoephedrine. *Rev. Endocr. Metab. Disord.* 2021, 22, 1013–1025. [CrossRef]

9. Duan, S.; Xie, L.; Zheng, L.; Huang, J.; Guo, R.; Sun, Z.; Xie, Y.; Lv, J.; Lin, Z.; Ma, S. Long-term exposure to ephedrine leads to neurotoxicity and neurobehavioral disorders accompanied by up-regulation of CRF in prefrontal cortex and hippocampus in rhesus macaques. *Behav. Brain Res.* 2020, 393, 112796. [CrossRef]

10. Takemoto, H.; Takahashi, J.; Hyuga, S.; Odaguchi, H.; Uchiyama, N.; Maruyama, T.; Yamashita, T.; Hyuga, M.; Oshima, N.; Amakura, Y.; et al. Ephedrine Alkaloids-Free Ephedra Herb Extract, EFE, Has No Adverse Effects Such as Excitation, Insomnia, and Arrhythmias. *Biol. Pharm. Bull.* 2018, 41, 247–253. [CrossRef]

11. Zell-Kanter, M.; Quigley, M.A.; Leikin, J.B. Reduction in Ephedra Poisonings after FDA Ban. *N. Engl. J. Med.* 2015, 372, 2172–2174. [CrossRef]

12. Food and Drug Administration. Final Rule Declaring Dietary Supplements Containing Ephedrine Alkaloids Adulterated Because They Present an Unreasonable Risk; Final Rule. *Fed. Regist.* 2004, 69, 6788–6834. Available online: https://www.govinfo.gov/content/pkg/FR-2004-02-11/pdf/04-2912.pdf (accessed on 5 February 2022).

13. Lai, S.; Yu, C.; Dennehy, C.E.; Tsourounis, C.; Lee, K.P. Online Marketing of Ephedra Weight Loss Supplements: Labeling and Marketing Compliance with the U.S. Food and Drug Administration Ban on Ephedra. *J. Altern. Complement. Med.* 2021, 27, 796–802. [CrossRef]

14. Glowacka, K.; Wielu-Hojeńska, A. Pseudoephedrine—Benefits and Risks. *Int. J. Mol. Sci.* 2021, 22, 5146. [CrossRef]

15. China National Narcotics Control Commission. Drug Situation in China (2019). Available online: http://www.nncc626.com/2019-06-25/c_1210675877.htm (accessed on 5 February 2022).

16. Yu, W.-N.; Wang, L.-H.; Cheng, H.-W. Regulatory analysis on the medical use of ephedrine-related products in Taiwan. *J. Food Drug Anal.* 2018, 26, 481–486. [CrossRef]

17. Shi, L.; Wang, Z.; Xin, Z.; Ren, Z. Problems in Supervision of Ephedrine Compound Prescription Preparation. *Chin. J. Pharmacovigil.* 2010, 7, 673–675.

18. Zhu, B.; Conlan, X.; Cao, J.; Meng, L.; Zheng, K.; Yang, D.; Yang, W. Case studies on illegal production of ephedrine/pseudoephedrine within Fujian China. *Forensic Sci. Int.* 2020, 312, 110326. [CrossRef]

19. CBOL Plant Working Group; Hollingsworth, P. M.; Forrest, L.L.; Spouge, J.L.; Hajibabaei, M.; Ratnasingham, S.; van der Bank, M.; Chase, M.W.; Cowan, R.S.; Erickson, D.L.; et al. A DNA barcode for land plants. *Proc. Natl. Acad. Sci. USA* 2009, 106, 12794–12797. [CrossRef]

20. Chen, S.; Pang, X.; Song, J.; Shi, L.; Yao, H.; Han, J.; Leon, C. A renaissance in herbal medicine identification: From morphology to DNA. *Biotechnol. Adv.* 2014, 32, 1237–1244. [CrossRef]

21. Lo, Y.-T.; Li, M.; Shaw, P.-C. Identification of constituent herbs in ginseng decoctions by DNA markers. *Chin. Med.* 2015, 10, 1–7. [CrossRef]

22. Meusnier, I.; Singer, G.A.; Landry, J.-F.; Hickey, D.A.; Hebert, P.D.; Hajibabaei, M. A universal DNA mini-barcode for biodiversity analysis. * BMC Genom.* 2008, 9, 214. [CrossRef]

23. Liu, Y.; Wang, X.; Wang, L.; Chen, X.; Pang, X.; Han, J. A Nucleotide Signature for the Identification of American Ginseng and Its Products. *Front. Plant Sci.* 2016, 7, 319. [CrossRef] [PubMed]

24. Wang, X.; Liu, Y.; Wang, L.; Han, J.; Chen, S. A Nucleotide Signature for the Identification of Angelicae Sinensis Radix (Danggui) and Its Products. *Sci. Rep.* 2016, 6, 34940. [CrossRef] [PubMed]

25. Guo, M.; Jiang, W.; Yu, J.; Pang, X. Investigating the authenticity of Ophiopogonis Radix and its Chinese patent medicines by using a nucleotide signature. *J. Ethnopharmacol.* 2020, 261, 113134. [CrossRef] [PubMed]

26. De Boer, H.J.; Ichim, M.C.; Newmaster, S.G. DNA Barcoding and Pharmacovigilance of Herbal Medicines. *Drug Saf.* 2015, 38, 611–620. [CrossRef]

27. Matthes, N.; Pietsch, K.; Rullmann, A.; Näämann, G.; Popping, B.; Szabo, K. The Barcoding Table of Animal Species (BaTAnS): A new tool to select appropriate methods for animal species identification using DNA barcoding. *Mol. Biol.Rep.* 2020, 47, 6457–6461. [CrossRef]

28. Tnah, L.; Lee, S.; Tan, A.; Lee, C.T.; Ng, K.K.S.; Ng, C.; Farhanah, Z.N. DNA barcode database of common herbal plants in the tropics: A resource for herbal product authentication. *Food Control* 2018, 95, 318–326. [CrossRef]

29. Han, J.; Pang, X.; Liao, B.; Yao, H.; Song, J.; Chen, S. An authenticity survey of herbal medicines from markets in China using DNA barcoding. *Sci. Rep.* 2016, 6, 18723. [CrossRef]

30. Zhang, J.; Hu, X.; Wang, P.; Huang, B.; Sun, W.; Xiong, C.; Hu, Z.; Chen, S. Investigation on Species Authenticity for Herbal Products of Celastrus Orbiculatus and Tripterygium Wilfordii from Markets Using ITS2 Barcoding. *Molecules* 2018, 23, 967. [CrossRef]

31. Song, M.; Dong, G.-Q.; Zhang, Y.-Q.; Liu, X.; Sun, W. Identification of processed Chinese medicinal materials using DNA mini-barcoding. *Chin. J. Nat. Med.* 2017, 15, 481–486. [CrossRef]

32. Ibragic, S.; Sofic, E. Chemical composition of various Ephedra species. *Bosn. J. Basic Med. Sci.* 2015, 15, 21. [CrossRef]
33. Yun, N.; Kim, H.J.; Park, S.C.; Park, G.; Kim, M.K.; Choi, Y.H.; Jang, Y.P. Localization of Major Ephedra Alkaloids in Whole Aerial Parts of Ephedrae Herba Using Direct Analysis in Real Time-Time of Flight-Mass Spectrometry. *Molecules* 2021, 26, 580. [CrossRef]

34. Pawar, R.S.; Grundel, E. Overview of regulation of dietary supplements in the USA and issues of adulteration with phenethylamines (PEAs). *Drug Test. Anal.* 2017, 9, 500–517. [CrossRef]

35. European Food Safety Authority. Scientific Opinion on safety evaluation of Ephedra species for use in food. *EFSA J.* 2013, 11, 3467.

36. Gardner, S.F.; Franks, A.; Gurley, B.J.; Haller, C.A.; Singh, B.K.; Mehta, J.L. Effect of a multicomponent, ephedra-containing dietary supplement (Metabolife 356) on Holter monitoring and hemostatic parameters in healthy volunteers. *Am. J. Cardiol.* 2003, 91, 1510–1513. [CrossRef]

37. Al-Tayyib, A.; Koester, S.; Langegger, S.; Raville, L. Heroin and Methamphetamine Injection: An Emerging Drug Use Pattern. *Subst. Use Misuse* 2017, 52, 1051–1058. [CrossRef]