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Research Article

The HPLC Fingerprint and Isovanillin Content of *Benincasa hispida* Seeds

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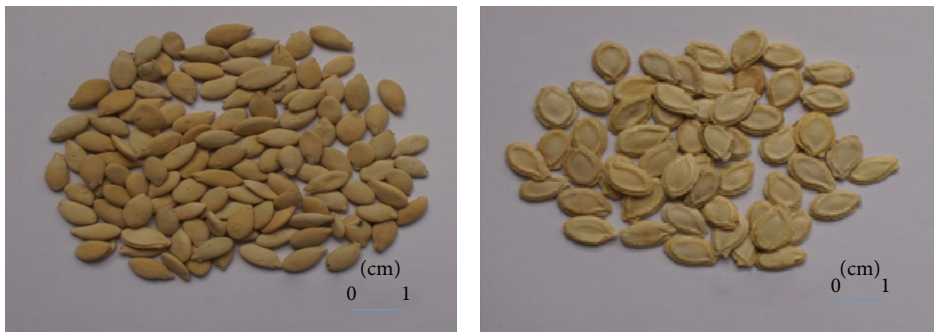
The *Benincasa hispida* seed is used as traditional Chinese medicine (TCM). However, there is still a lack of medicinal quality control of *B. hispida* seeds. The seeds may contain isovanillin, but this finding remains to be confirmed and quantified. The current study aimed to confirm the existence of isovanillin and then preliminarily establish medicinal quality standards for *B. hispida* seeds. Fourteen batches of unilateral and bilateral *B. hispida* seeds were purchased from 7 different producers in China. Semipreparative high-performance liquid chromatography (HPLC) was used to isolate and purify the isovanillin from *B. hispida* seeds. Its chemical structure was elucidated by UV, ^{1}H-NMR, IR spectroscopy, and GC-MS. The *B. hispida* seed fingerprint and isovanillin determination were performed on an HPLC instrument. Data obtained from the unilateral and bilateral specimens were analyzed with a similarity evaluation system. The HPLC fingerprint showed 19 characteristic peaks with high similarity between the unilateral and bilateral *B. hispida* seeds. The isovanillin content among the fourteen batches ranged from 13.46 to 46.80 μg/g. The results of this study may provide a preliminary reference for the quality control of *B. hispida* seeds.

1. Introduction

Benincasa hispida, a member of the family Cucurbitaceae, is a well-known crop that is grown primarily for its fruits, which possess nutritional and medicinal properties, especially in Asian countries such as India and China. Its dried ripe seed is often called "dong gua zi" in China. There are two types of *B. hispida* seed commonly sold at market: unilateral and bilateral *Benincasa hispida* seeds (Figure 1). These seeds differ in their shape and properties, with unilateral *B. hispida* seeds having smooth edges and bilateral *B. hispida* seeds having an edge "ring pattern" on both sides [1]. Although the seeds are derived from different cultivars of the same original plant, bilateral *B. hispida* seeds are more expensive at market because of their low yield.

B. hispida seeds have long been used as traditional Chinese medicine (TCM) to reduce fevers, eliminate phlegm, discharge pus, and expel dampness, and it has been used in China for the treatment of conditions such as cough accompanied by phlegm and fever, pulmonary abscesses, periappendicular abscesses, gonorrhea, spermatorrhea, and edema [1, 2]. Modern studies have demonstrated that many pharmacological effects, including anti-inflammatory, analgesic, antioxidant, antiangiogenic, and antidiabetic activities, have been associated with *B. hispida* seeds [3–8].

The *B. hispida* seed has become more appreciated because of the increasing demand for Chinese medicine throughout the world combined with its high-quality and low-cost benefits, both food-related and medicinal, leading to broad potential applications. However, few studies on the seeds have been conducted. To date, we know that the seeds are rich in fatty acids, sterols, triterpenes, alkaloids, and mineral elements [1, 9]. In our previous study, we found that *B. hispida* may contain isovanillin [10]. The chemical name of isovanillin is 3-hydroxy-4-methoxybenzaldehyde, and it is mainly used as a spice, flavoring agent, food-grade cosmetic additive, plant growth regulator, and drug intermediate. There is a huge market potential for isovanillin as the starting material for



(a) (b)

Figure 1: *Benincasa hispida* seeds. (a) Unilateral. (b) Bilateral (much more expensive).

the synthesis of the next-generation anticancer substance CA-4, which targets the tumor vasculature and is of high efficacy and low toxicity, through a 6-step reaction [11]. However, the content of isovanillin has not been confirmed and quantified. Additionally, the current medicinal quality standards for *B. hispida* seeds are quite simple, but studies concerning *B. hispida* seed quality standards (utilizing microscopic characteristics and thin-layer chromatography) cannot ensure their efficacy and safety for clinical use [12]. Therefore, improving the quality standards for their medicinal purposes is necessary to improve quality control and ensure clinical efficacy and safety. TCM fingerprint technology is a comprehensive and quantitative analysis that reflects the chemical information contained in TCM and has been recognized by the modern global society. Chemical TCM fingerprints display the different chemical components and their quantities contained in medicines, but the fingerprint of *B. hispida* seeds has not yet been reported.

Based on the aforementioned context, we extracted and quantified the isovanillin content and established the chemical fingerprints of *B. hispida* seeds. The aim of this study was to preliminarily establish quality standards for the *B. hispida* seed with regard to its medicinal application.

2. Materials and Methods

2.1. Materials. Fourteen batches of unilateral and bilateral *B. hispida* seeds were collected from different regions of China and were authenticated by Yanqing Zheng, the chief pharmacist of the Dezhou Institute for Drug Control (Dezhou, China). One specimen (DGZ-1) was deposited into the Specimen Room of the Dezhou Institute for Drug Control. Isovanillin control product was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

2.2. Sample Extraction and Separation. A total of 500 g of pulverized *B. hispida* seeds was extracted twice, first for 1.5 h and then for 1 h, with methanol (1000 mL) using a Soxhlet extractor. The combined methanol extract was evaporated under reduced pressure (yield 11.3%, w/w). The extract was fractioned through a silica gel chromatography column using gradient elution with mixtures of petroleum ether and acetone in increasing proportions. The fraction containing 10 : 1 petroleum ether : acetone was collected and evaporated to dryness. The residue was dissolved in methanol and filtered through a 0.45 μm pore size filter prior to isolation and purification on an Agilent 1100 semipreparative system together with a UV detector and YMC-C18 column (250 mm × 10 mm, 5 μm). The mobile phase was a mixture of methanol and 0.4% aqueous acetic acid (65 : 35), and the flow rate was 3.0 mL min^{-1}. The detection wavelength and column temperature were 280 nm and 30°C, respectively.

2.3. Structural Identification of Isovanillin. One of the fractions collected was evaporated under reduced pressure to produce 8.6 mg of residue. The material was a white powder and was analyzed by ^{1}H-NMR (JEOL JNM-ECP 600, Tokyo, Japan) with $CDCl_3$ as the solvent; the chemical shift δ values were as follows: 9.84 (1H, s, CHO), 7.43–7.45 (2H, m, H-2, H-6), 7.06 (1H, d, J = 8.6 Hz, H-5), and 3.98 (3H, s, -OCH_3). This substance was formulated with methanol as the solvent into an approximately 10 ppm solution and measured by UV (PGeneral TU-1901, Beijing, China) with a scanning range of 200–400 nm and an observed UV λ_{max} of 280 nm. Then, the above solution was analyzed by GC-MS (Agilent 7890A-5975, USA). The MS conditions were a GC-MS interface temperature of 280°C, an ion source temperature of 230°C, a quadrupole temperature of 150°C, an electron energy of 70 ev, and a scanning mode of Scan, which resulted in an EI-MS m/z ratio of 151 [M-H$^-$]. Additionally, IR was used to analyze isovanillin (BRUKER TENSOR 27, Germany), and we found IR (KBr) ν_{max} results of 3228, 3031, 2936, 2847, 1672, 1578, 1512, 1445, 1247, 1168, 1121, 1022, 866, and 831 cm^{-1}; these data are consistent with previous isovanillin data [13]. Therefore, this compound was identified as isovanillin.

2.4. Preparation of Sample and Standard Solutions. The dried powder (2.0 g) of *B. hispida* seeds from 14 different regions was accurately weighed, dissolved in 20.0 mL methanol, and then placed into a sealed Erlenmeyer flask and weighed. After ultrasonic extraction for 30 min, the extract solution was cooled down to room temperature (~25°C) and weighed again to replenish the lost weight. The solution was filtered through a 0.45 μm membrane before HPLC injection.

The isovanillin control product was accurately weighed and dissolved in methanol, and then a standard solution was prepared at a concentration of 0.266 mg/mL. Meanwhile, methanol was injected as a negative control.

2.5. HPLC Conditions for B. hispida Seed Fingerprinting and Isovanillin Measurements. HPLC analyses were performed on a Shimadzu LC-20A HPLC instrument (Shimadzu, Tokyo, Japan) that was equipped with a diode array detector (DAD) and a Syncronis-C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of a mixture of acetonitrile (A; Tedia Reagent Co., USA) and a 0.1% phosphoric acid aqueous solution (B) using a linear gradient program: 0 min, 10 : 90 (v/v) A : B, 0–35 min, linear change to 35 : 65 (v/v) A : B. The flow rate was 1 mL/min, and the injection volume was 20 μL. The detection wavelength and column temperature were 280 nm and 30°C, respectively.

2.6. Standard Curve. A set of linear test solutions for isovanillin was prepared by diluting the standard solution to yield five concentrations of 1.33, 6.65, 13.30, 26.60, and 53.20 μg mL^{-1}. The equation of the standard curve for isovanillin was determined using a 1/C/C weighted linear least squares regression model (y = 86905x+9804.1; y = peak area and x = concentration). A linear response was obtained over a range of 1.33–53.20 μg mL^{-1} (correlation coefficient, 1.000).

2.7. Method Validation

2.7.1. Method Validation for B. hispida Seed Fingerprinting. A validation study was conducted to establish precision, stability, and repeatability. The apparatus precision was evaluated by the analysis of six successive injections of the same sample solution. The repeatability was evaluated by the analysis of six injections of six sample solutions prepared by the same method. Using the peaks of isovanillin as the reference peak, the RRT (relative retention time, RRT = retention time of characteristic peak/retention time of marker peak) and RPA (relative peak area, RPA = peak area of characteristic peak/peak area of marker peak) of the other common peaks were calculated. Meanwhile, the relative standard deviations (RSDs) of the RRTs and RPAs were also calculated.

2.7.2. Method Validation for Determining Isovanillin Content. The validation of this analytical method was performed in accordance with the International Conference on Harmonization (ICH) guidelines. The method was validated in terms of linearity, limit of detection (LOD) and limit of quantification (LOQ), precision, stability, repeatability, and accuracy. The LOD and LOQ were determined by the signal to noise (S/N) ratio method. They were estimated as the minimum concentration of analyte providing S/N ratios of 3 : 1 and 10 : 1, respectively, by injecting a series of diluted solutions with known concentrations. The accuracy of the assay method was evaluated using six S1 sample solutions that were prepared using the same method, with the same volume of standard solution added. The percent recoveries were calculated from the slope and y-intercept of the calibration curve.

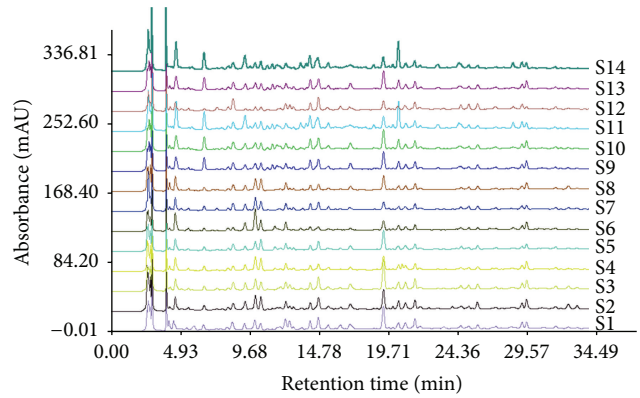


Figure 2: HPLC chromatograms from 14 batches of *Benincasa hispida* seeds.

2.8. Data Analysis. Data analysis was performed using LC Solution software (Shimadzu Corporation, Japan). As recommended by the State Food and Drug Administration, the similarity evaluation system for chromatographic fingerprint of traditional Chinese medicine (SES, Version 2004A) was used for evaluating the similarities between different samples.

3. Results

3.1. B. hispida Seed Fingerprinting. Fourteen batches of *B. hispida* seeds collected from various locations were analyzed under optimal conditions. As shown in Figure 2, all samples exhibited similar chromatographic profiles. Comparing the retention times of the same peaks from each sample, we obtained a standardized chromatographic fingerprint using the software "similarity evaluation system for chromatographic fingerprint of traditional Chinese medicine" (v.2004A). The standardized chromatographic fingerprint (Figure 3) contained nineteen peaks. The common peaks were further quantitatively expressed in terms of RRT. Peak 12 (isovanillin) was selected as the marker peak. The results indicated that the RRTs of the nineteen common peaks were invariable between samples, which demonstrated that the present HPLC method was valid for *B. hispida* seed fingerprint analysis.

A similarity analysis was performed using the similarity evaluation system. The results are shown in Table 1. The closer the similarity value is to 1, the more similar the two chromatograms are. It was obvious that most similarity values of the fourteen samples from different locations were greater than 0.90 with the exceptions of unilateral *B. hispida* seed S6 and bilateral *B. hispida* seeds S11 and S12. The results showed high similarity between the different *B. hispida* seed batches (including unilateral and bilateral *B. hispida* seeds), suggesting that the established *B. hispida* seed fingerprint has high specificity and that the chemical composition observed in the unilateral and bilateral *B. hispida* seed fingerprints was generally similar.

Table 1: Similarities in isovanillin content in 14 batches of *Benincasa hispida* seeds.

Sample number	Collection location	Cultivar	Similarity	Content (μg/g)
S1	Heze, Shandong province	Unilateral	0.948	38.15
S2	Bozhou, Anhui province	Unilateral	0.963	32.86
S3	Anguo, Hebei province	Unilateral	0.951	46.80
S4	Chengdu, Sichuan province	Unilateral	0.928	20.42
S5	Nanjing, Jiangsu province	Unilateral	0.944	30.30
S6	Kunming, Yunnan province	Unilateral	0.838	14.98
S7	Jiaozuo, Henan province	Unilateral	0.932	13.46
S8	Anyang, Henan province	Bilateral	0.933	21.65
S9	Heze, Shandong province	Bilateral	0.954	29.23
S10	Bozhou, Anhui province	Bilateral	0.954	32.22
S11	Chengdu, Sichuan province	Bilateral	0.874	23.87
S12	Kunming, Yunnan province	Bilateral	0.869	14.65
S13	Anguo, Hebei Province	Bilateral	0.946	30.52
S14	Nanjing, Jiangsu province	Bilateral	0.901	23.33

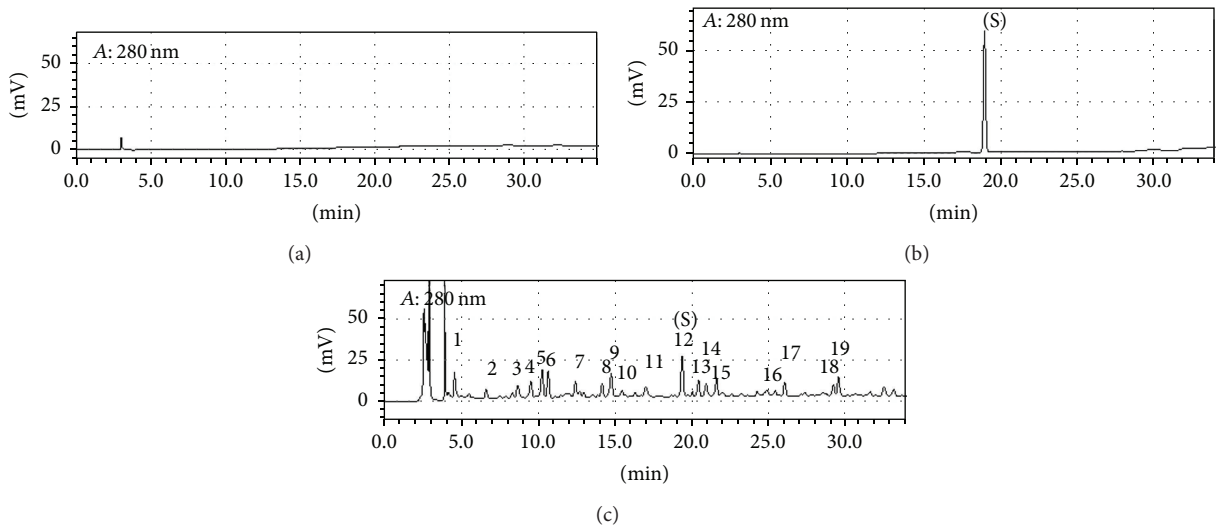


Figure 3: Standardized HPLC chromatographic fingerprints. (a) Negative control. (b) Isovanillin control. (c) Experimental samples.

3.2. Methodological Study of the B. hispida Seed Fingerprint. In terms of precision, the RSDs of the RRTs and RPAs of the abovementioned 19 characteristic peaks were found to be less than 0.02 and 1.90%, respectively. These data show that the precision of the apparatus was satisfactory.

The stability was evaluated by the analysis of six injections of the same solution over a time period of 24 h, and the RSDs of the RRTs and RPAs were less than 0.34 and 2.10%, respectively. These data indicate that the stability of the solution was excellent.

The repeatability measurement showed that the RSD values of the RRTs and RPAs of other common peaks were less than 0.04 and 2.97%, respectively, indicating that the repeatability of the analytical method was acceptable.

3.3. Isovanillin Content Measurements. Fourteen batches of *B. hispida* seeds collected from various locations were analyzed under the HPLC conditions described in Section 2.4, and the isovanillin contents were calculated as shown in Table 1. The content of isovanillin ranged from 13.46 μg/g to 46.80 μg/g.

3.4. Validation of Isovanillin Measurements. The method validation of the isovanillin determination for precision, stability, and repeatability was conducted simultaneously with the methodology study ofthe *B. hispida* seed fingerprint. In the precision test, the RSD value of the isovanillin areas of the six injections was 0.41%. In the stability and repeatability tests, the RSD values of the isovanillin contents were 0.42% and 4.00%, respectively.

As shown in Table 2, the average percent recovery for the isovanillin determination was calculated as 97.0%. Using this method, the LOD and LOQ were 0.07 ng and 0.23 ng, respectively.

From the above results, the validation data indicate that the analytical method was specific and sensitive and could be used for the quantification of isovanillin in *B. hispida* seeds.

Table 2: Percent recovery of isovanillin.

Weight of sample (g)	Isovanillin amount in sample (μg)	Amount added (μg)	Amount measured (μg)	Percent recovery (%)	Average percent recovery (%)	RSD[a] (%)
1.0001	38.15	33.25	31.26	94.0	97.0	4.9
1.0015	38.21	33.25	30.54	91.8		
1.0003	38.16	33.25	30.98	93.2		
1.0009	38.18	33.25	32.75	98.5		
1.0012	38.20	33.25	34.22	102.9		
1.0007	38.18	33.25	33.85	101.8		

[a]RSD: relative standard deviation.

4. Discussion

Presently, the quality standards for *B. hispida* seeds are not stringent enough; therefore, meeting the quality control demands for *B. hispida* seeds is difficult. In this study, one compound was extracted and isolated from *B. hispida* seeds, which was identified as isovanillin through structural measurements. By optimizing the chromatographic conditions and system suitability tests and using isovanillin as a reference, this *B. hispida* seed fingerprinting study (including unilateral and bilateral *B. hispida* seeds from different sources) was conducted to provide a basis and method for improving *B. hispida* seed quality standards.

During the optimization of the chromatographic conditions, three types of reverse-phase columns, Agilent Zorbax-C18, Kromasil-C18, and Syncronis-C18, were investigated. The Syncronis-C18 column was most suitable for the separation of plant constituents. To obtain good separation, methanol-water, acetonitrile-water, and acetonitrile-water containing acid were investigated as mobile phases. More compounds were separated using acetonitrile-water containing 0.1% phosphoric acid, and the chromatographic peaks exhibited better shapes. The UV spectrum of each peak in the chromatogram was obtained using a DAD detector. The optimal detection wavelength was determined to be 280 nm because most peaks showed maximum absorption at this wavelength. According to the optimum chromatographic conditions described above, the HPLC fingerprint of *B. hispida* seeds was established according to the analysis of 14 samples from different sources. There were 19 peaks common to all fingerprints, showing high similarity among the different sources of the *B. hispida* seeds, and the methodological results showed good system adaptability.

Traditional methods for determining the authenticity of herbs are mainly characteristic and microscopic identification and determining component contents. We cannot fully control the quality of Chinese herbal medicines because of the complex nature of their chemical compositions. However, chromatographic fingerprint analysis, which can reflect the chemical TCM profiles, has the advantages of integrity and unambiguity and has been widely accepted for quality evaluation and species differentiation [14, 15]. Fingerprinting will likely become the basis for future developments in Chinese herbal medicine quality control. Li and Zhao performed fingerprint analysis on 15 batches of the *Pericarpium Citri Reticulatae Viride* medicine from different sources and found that all similarities were greater than 0.9, which provides a basis for the quality control and evaluation of *Pericarpium Citri Reticulatae Viride* [16]. Cao et al. established the HPLC fingerprint of young horn of *Cervus nippon Temminck* and conducted pattern recognition for fingerprints of young horns of *Cervus nippon Temminck* and young horns of *Cervus elaphus*, providing a reference for the quality control methods and various identifications of pilose antlers [17]. The 2010 edition of "Chinese Pharmacopoeia" first recorded the characteristic HPLC spectrum inspection methods, such as those methods for the hawthorn leaf extract, *Panax notoginseng* saponins, and other varieties [18]. This study is the first to examine the HPLC fingerprint of *B. hispida* seeds.

Here, isovanillin was isolated from *B. hispida* seeds, and its quantity was assessed. The isovanillin content from 14 samples from different sources was 13.46–46.80 μg/g. No great difference in the isovanillin content from unilateral or bilateral *B. hispida* seeds was observed. The isovanillin content results between the unilateral and bilateral *B. hispida* seeds agreed with the considerable similarities between the two fingerprints and with our previous work that showed no significant differences in the types and amounts of amino acids, fatty acids, and inorganic elements between these seeds [10]. To the best of our knowledge, this report is the first examining isovanillin in *B. hispida*. These results preliminarily explain why there is no observable distinction between the two species of *B. hispida* seeds in their clinical application, despite the significant differences in their characteristics and market prices.

As an important pharmaceutical intermediate, isovanillin has been used in many research institutes in recent years to synthesize a new generation of antibiotics and anticancer drugs of high potency and low toxicity [19]; additionally, the high response of isovanillin in the liquid chromatogram of *B. hispida* seeds, good separation, and easy extraction make it scientific, simple, and feasible for *B. hispida* seed quality control through establishing *B. hispida* seed fingerprinting, which is achieved by setting isovanillin as the index peak to measure its content. Saponins and phenolic compounds in *B. hispida* seeds have been reported to be closely related to their expectorant, antipyretic efficacy, and antioxidant properties [20, 21]. However, at present, few studies have examined the

pharmacological action of individual chemical components of *B. hispida* seeds, and the active ingredients are still unclear.

There were some limitations to this study. Our investigation into the effective chemical constituents of *B. hispida* seeds was still not deep enough, as only the isovanillin peak was identified in the fingerprint. We failed to both identify more indicator peaks related to the efficacy of *B. hispida* seeds and determine their contents. A deeper and systematic future investigation is needed to study the chemical constituents of *B. hispida* seeds, to establish methods for the content measurement and multi-indexing of the active ingredients, and to use the scientific method to fully control the medicinal quality of *B. hispida* seeds.

This study describes a simple, reproducible, and accurate chromatographic method for medicinal quality standards of the *B. hispida* seed, both qualitatively and quantitatively, and is significant because it ensures *B. hispida* seed safety for clinical use.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

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