Original article

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COMPARISON OF PICEATANNOL CONTENT IN SEED COAT AND EMBRYO OF PASSION FRUIT

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ABSTRACT

The aim of the present study was to investigate the use of seed coat of passion fruit seeds prepared by rinsing milled seeds in water for the extraction of piceatannol. The dry matter content of the embryo and the seed coat in the total seeds was 40% (w/w) and 60% (w/w), respectively. The content of piceatannol in the dried total seeds was 0.51% (w/w). It was found that piceatannol was contained substantially only in the seed coat at approximately 0.8% (w/w) of the dried seed coat, while piceatannol was not found in the embryo. The content and the recovery of piceatannol in the extracts prepared by the repeated batch extraction (round 1, 2, and 3) under the various extraction conditions from the total seeds, the seed coat, and the embryo were compared. The content of piceatannol in the extract of round 1, prepared by the extraction condition of refluxing the seed coat in 60% (v/v) ethanol, was 9.5% (w/w) at the maximal recovery of piceatannol (81.9%). By using the synthetic adsorbent and ethyl acetate/0.5% (w/v) sodium bicarbonate partition, the extract containing piceatannol (approximately 20-25%, w/w) was obtained. The present study demonstrated that the use of seed coat was efficient for preparing the extract containing higher amount of piceatannol.

Keywords: passion fruit, seeds, seed coat, embryo, piceatannol, extraction

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Introduction

The *Passifloraceae* family is composed of more than 500 species arranged in 18 genera, among which *Passiflora edulis* is very popular, not only because of its pulp, but also because of its leaves, which have been largely used in American and European countries as medicines such as sedative, tranquilizer, and anti-inflammatory drug.[1] *P. edulis* is native to southern Brazil through Paraguay to northern Argentina. It is cultivated commercially in tropical and subtropical areas for its sweet, seedy fruit. It is also abundantly distributed in Southeast Asia, and it is certainly a common fruit in Kingdom of Thailand.

The fruit of this plant is called passion fruit, and the pulp has been widely used for a whole fruit and juice. The seeds are often eaten together with the pulp.[2] The industrial scale production of passion fruit juice produces a large amount of seeds which are generally discarded as a waste although the seeds will be a source of oil production that has been explored. This oil can be utilized for food, cosmetics and pharmaceutical industries[3], and the chemical composition of seeds and kernel of passion fruit were reported.[4]

The seeds of *P. edulis* are of great interest and importance because they contain the beneficial biologically functional stilbene derivatives such as piceatannol, scirpusin B, and resveratrol. The chemical structure of piceatannol is shown in Figure 1.

Biological activity of piceatannol including antitumor, antioxidant, and anti-inflammatory activities was reviewed, suggesting that it might be a potentially useful nutritional and pharmacological compound.[5] Furthermore, the suppression of non-cancerous human breast epithelial cells MCF10A by piceatannol was reported.[6]

In addition, many studies have revealed that passion fruit seeds exhibit many biological functions. Those were the inhibition of melanogenesis and promotion of collagen synthesis by the extract of passion fruit seeds containing piceatannol and resveratrol[7], the vasorelaxing activity by scirpusin B from passion fruit seeds[2], the protective effect of piceatannol from passion fruit seeds on UVB-irradiated keratinocytes[8], the improvement of vascular function by up-regulating eNOS expression by piceatannol[9], the prevention of cardiac hypertrophy including in vivo study with a rat model by piceatannol[10], the inhibition of adipocyte lipogenic activity by piceatannol and resveratrol[11], α-glucosidase inhibitory effect of resveratrol and piceatannol[12], and the attenuation of the pathogenic inflammation by piceatannol[13], and the antiallergy effect of the extract of passion fruit seeds including in vivo study with a mouse model.[14]

The method for extracting piceatannol, resveratrol, and scirpusin B from passion fruit seeds have been reported. Those were the extraction from the total dried and milled seeds with 80% (v/v) ethanol by shaking at room temperature[7], the extraction from the total dried and milled seeds with 90% (v/v) ethanol by refluxing for 90 min[2], the extraction from the total dried and milled seeds with

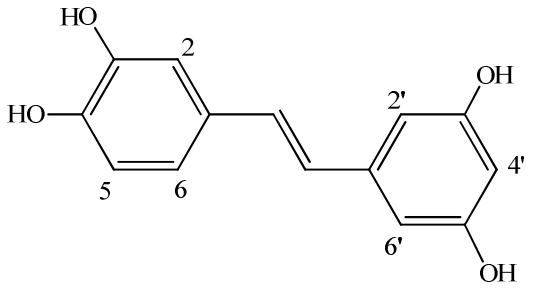

Figure 1 Chemical structure of piceatannol

1,3-butylene glycol[8], the extraction from the total dried and milled seeds with 80% (v/v) ethanol and ethanol at 4 °C for 20 h by stirring[14], and the extraction from the defatted passion fruit bagasse by the pressured liquid extraction method with aqueous ethanol.[15]

In all the studies described above, the total seeds and defatted bagasse of passion fruit for the source material and aqueous ethanol (and 1,3-butylene glycol) for the extraction solvent were used for preparing the extract containing piceatannol, scirpusin B, and resveratrol.

The aim of the present study was to investigate the use of the seed coat of passion fruit seeds prepared by rinsing the milled seeds in water for the extraction of piceatannol.

Materials and methods

Chemicals

Ethyl acetate (AcOEt), 95% (v/v) ethanol, acetone, chloroform, methanol, sodium bicarbonate of analytical grade, and acetonitrile of HPLC grade for the chromatographic analyses were purchased from RCl Labscan Limited (Pathumwan, Bangkok, Thailand). Water that was deionized and further filtered through the reverse osmosis membrane (0.0001 μ) was used for the present study including the extraction, HPLC analyses, and whatever. For the HPLC analyses, mobile phases were further filtered through whatman nylon membrane filters (0.45 μm, GE Healthcare companies, Germany). Piceatannol was purchased from Tokyo Chemical Industry, Co., Ltd. (Tokyo, Japan). Synthetic adsorbents (sepabeads HP20 and SP825) were purchased from Mitsubishi Chemical Corporation (Tokyo, Japan). Sephadex LH-20 was purchased from GE Healthcare Bio-Sciences, Uppsala, Sweden). The seeds of yellow passion fruit (*Passiflora edulis*) that were obtained from "The Royal Project Produce Center, Royal Project Foundation (Mae Hia, Chiang Mai, Thailand) in May, 2016 were used for the extraction in the present study.

Preparation of seed coat

The fresh seeds of passion fruit were placed on a sieve and washed by applying water and with the application of light frictional forces to eliminate the arils. The washed seeds were dried in an oven at 60°C for 20 h, and the dried seeds were then milled with grinding machine (Grain crusher, Henan Shuoman Machinery Co., Ltd., China). The embryo and the seed coat were prepared as shown in Figure 2. SC-1 and SC-2 were subjected to granulometric analysis of the solid particles using a set of standard sieves of the mesh numbers (No.10, No.40, No.60, No.80, No.100, No.120, No.170, and No.230).

Analysis of chemical composition of total seeds, embryo, and seed coat

The dried samples of total seeds, embryo (EM-1 and EM-2), and seed coat (SC-1 and SC-2) were characterized based on the analysis of protein, fiber, fat, ash, and moisture content according to the method as described by AOAC (2005).[16] The analyses were conducted at Central Laboratory, Faculty of Agriculture, Chiang Mai University, Thailand.

Single batch extraction from seed coat (SC-2) of passion fruit seeds and partial purification of extracts

Single batch extractions and partial purification by synthetic adsorbents were carried out as shown in Figure 3A and 3B, respectively.

EtOH/Refluxing/extracted solution was partially purified in the same manner as EtOH/extracted solution to give EtOH/Reflux/SP825/supernatant and EtOH/Reflux/SP825/E. Further purifications by AcOEt/water partition and AcOEt/0.5% (w/v) $NaHCO_3$ partition using the supernatants were carried out. Each of the supernatants (BW/HP20/supernatant, EtOH/SP825/supernatant, and EtOH/Reflux/SP825/supernatant) were separately partitioned with AcOEt three times. The combined AcOEt layer was evaporated under

reduced pressure. The resultant extracts named BW/HP20/AcOEt/E, EtOH/SP825/AcOEt/E, and EtOH/Reflux/SP825/AcOEt/E were prepared. After adding $NaHCO_3$ to each of the supernatants to make the supernatants alkaline, each of the supernatants were separately purified in the same manner as shown above. The resultant extracts named BW/HP20/AcOEt/$NaHCO_3$/E, EtOH/SP825/AcOEt/$NaHCO_3$/E, and EtOH/Reflux/SP825/AcOEt/$NaHCO_3$/E were prepared.

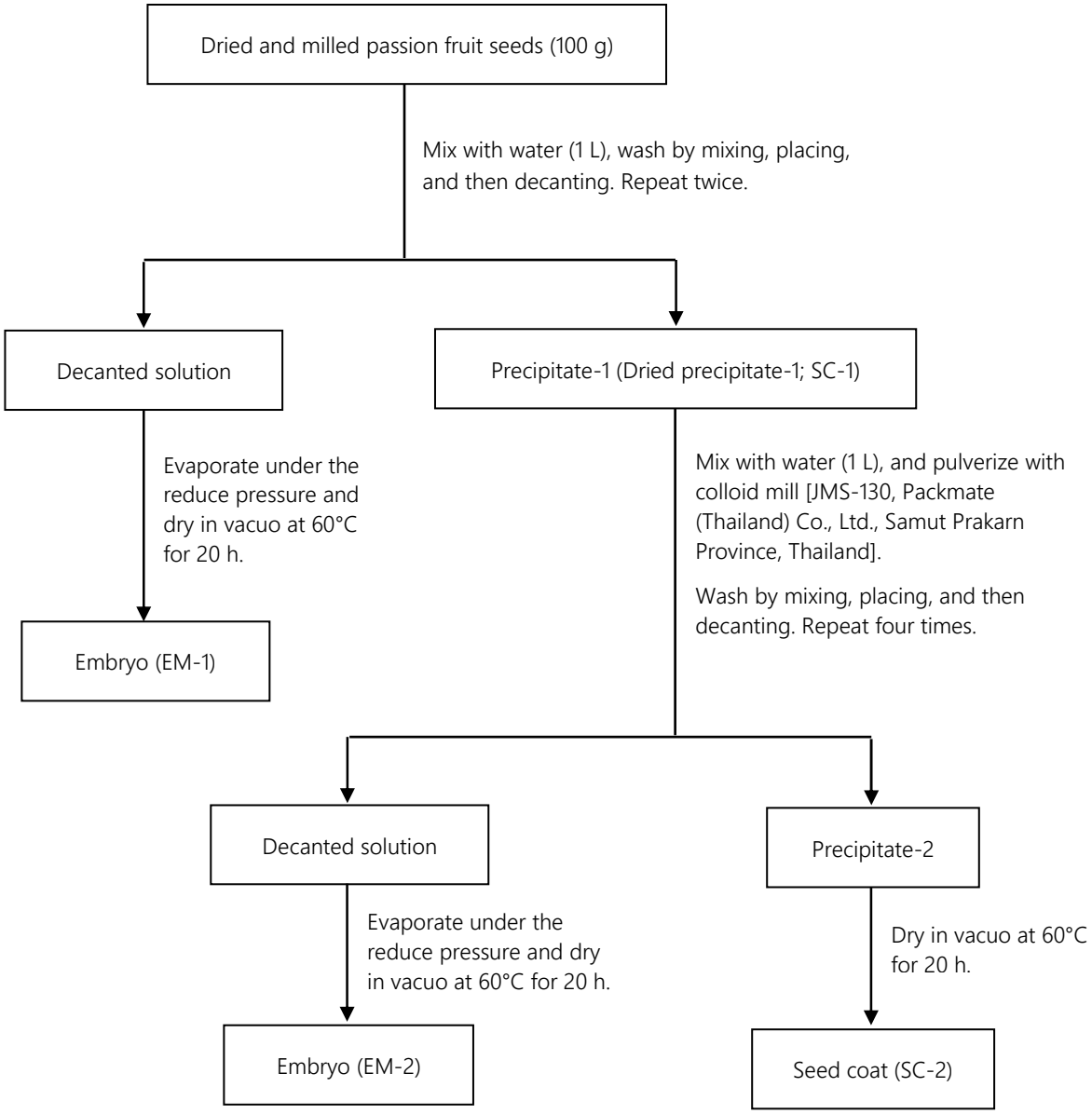

Figure 2 Separation of embryo and seed coat from total passion fruit seeds

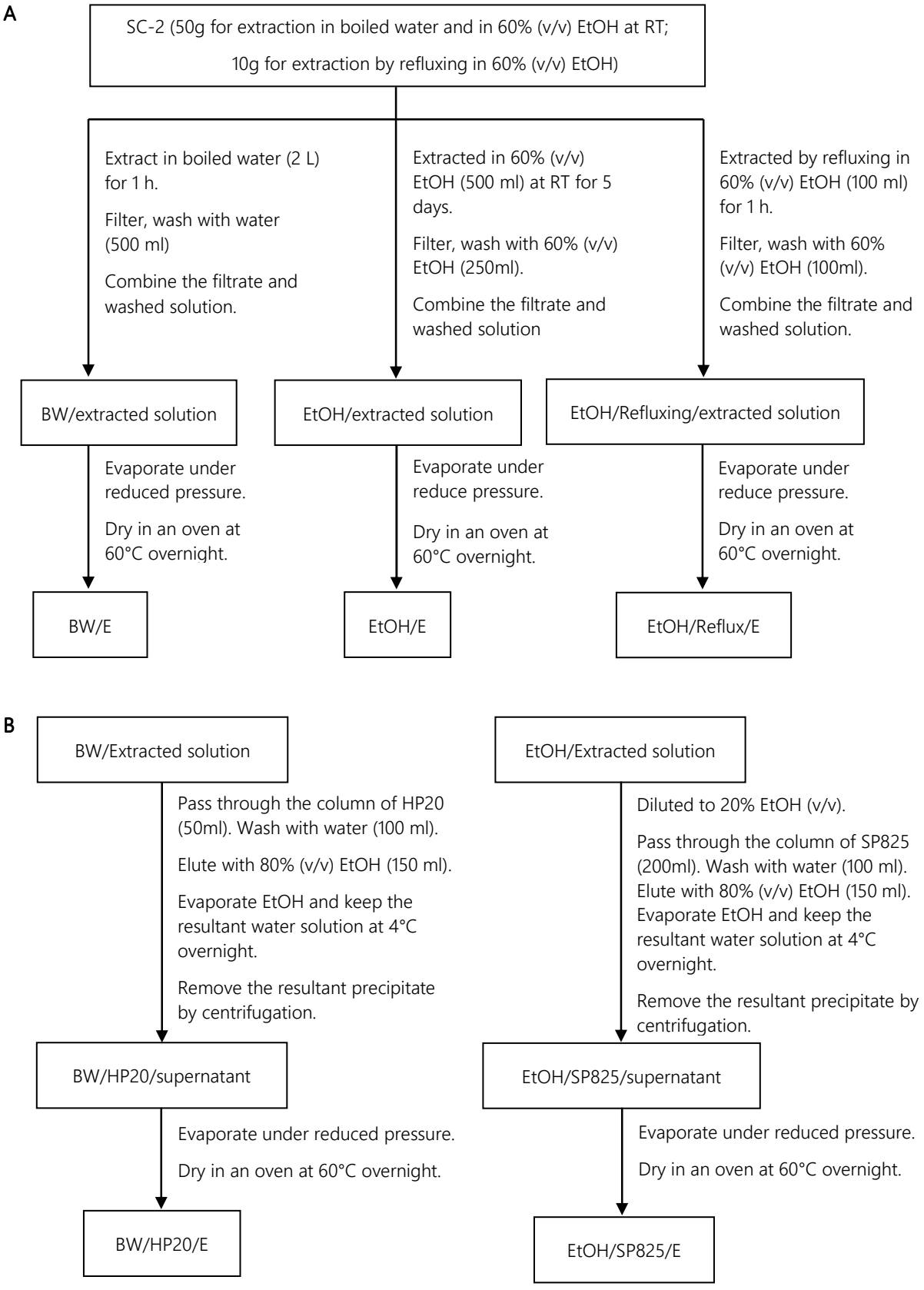

Figure 3 (A) Single batch extraction from seed coat (SC-2) of passion fruit seeds and (B) partial purification of extracts with synthetic adsorbents.

Isolation of piceatannol from extract of seed coat (SC-2) of passion fruit

The extract of EtOH/Reflux/SP825/AcOEt/$NaHCO_3$/E (2500mg) prepared from SC-2 as described above was subjected to the silica gel column chromatography (200g). The developing solvent was $CHCl_3$-MeOH (8:2). The fractions were monitored by TLC and the fractions which showed the same Rf spot as that of standard piceatannol were combined and evaporated under reduced pressure and dried in an oven at 60°C. Most of the strong blackish brown color was removed, and 833 mg of the yellow solid was obtained. About 208 mg of its yellow solid was dissolved in 80% (v/v) ethanol, and the solution was subjected to LH-20 column (50 ml) by developing with 80% (v/v) ethanol. The fractions containing the same Rf spot as that of standard piceatannol were dried in the same manner described above. The remaining yellow solid (625 mg) was subjected to LH-20 chromatography in the same manner. By the LH-20 chromatography, most of the strong yellowish color was removed, and the faint yellow solid (317mg) mainly consisting of the compound with the same Rf spot as that of standard piceatannol was obtained. The resultant faint yellow solid (317 mg) was further subjected to silica gel chromatography (50 g) by developing $CHCl_3$-MeOH (8:2). The fractions containing the same Rf spot as that of standard piceatannol were dried in the same manner described above. The resultant solid with a trace of yellow color was further subjected to silica gel chromatography (50 g) by developing $CHCl_3$-MeOH (9:1). The fractions containing the same Rf spot as that of standard piceatannol were dried in the same manner described above. Finally, 164 mg of the purified compound with the same Rf spot as that of standard piceatannol was obtained.

Repeated batch extraction of piceatannol from total seeds, seed coat (SC-1 and SC-2), and embryo (EM-2)

Each of the dried samples [10g, total seeds, seed coat (SC-1 and SC-2), embryo (EM-2)] was refluxed in 60% (v/v) ethanol (100 ml) for 1 h. The extract was separated by filtration, and the residue was washed with 60% (v/v) ethanol (100 ml). The combined solution of the extract and the washed solution was evaporated under reduced pressure and dried in an oven at 60 °C overnight. As for total seeds, the resultant powder was named Total/EtOH/reflux/Repeated-1/E. The washed residue was extracted further two times in the same manner. The resultant second powder and third powder were named Total/EtOH/reflux/Repeated-2/E and Total/EtOH/reflux/Repeated-3/E, respectively. As for SC-1, SC-2, and EM-2, the repeated batch extraction was carried out in the same manner, and the resultant powder was named SC-1/EtOH/reflux/Repeated-1/E, SC-1/EtOH/reflux/Repeated-2/E, and SC-1/EtOH/reflux/Repeated-3/E for SC-1, SC-2/EtOH/reflux/Repeated-1/E, SC-2/EtOH/reflux/Repeated-2/E, and SC-2/EtOH/reflux/Repeated-3/E for SC-2, EM-2/EtOH/reflux/Repeated-1/E, EM-2/EtOH/reflux/Repeated-2/E, and EM-2/EtOH/reflux/Repeated-3/E.

Determination of content of piceatannol in total seeds, embryo (EM-1 and EM-2), and seed coat (SC-1 and SC-2)

(1) Total seeds, SC-1, EM-2, and SC-2: The total amount of piceatannol obtained by the repeated batch extraction was regarded as the content of piceatannol in each seed part.

(2) EM-1: As for the content of piceatannol in EM-1, TLC method was applied because its content in EM-1 was very low. TLC condition was as follows. Silica gel 60 F254 plates (Merck) were used. The developing solvent was chloroform/methanol (8:2 v/v). TLC plate was examined under UV light [short-wave length (256 nm) and long-wave length (365 nm)]. The detection limit was confirmed to be approximately 0.4 µg by applying 2 µl of piceatannol solution with its various concentrations in 80% (v/v) ethanol and developing TLC as described above (data not shown). The embryo (EM-1) (~400 mg, weighed accurately to 0.1 mg) were mixed in 80% (v/v) ethanol (1 ml) and the mixture was warmed at 70 °C for 30 min, and its

extract (2 µl) was spotted on TLC. Presence or absence of piceatannol was observed. In the case that the piceatannol content was less than 0.05% (w/w), the piceatannol spot was not detected on TLC under the conditions described above.

HPLC analysis of piceatannol in extracts and partially purified samples

The extracted samples (~25 mg, weighed accurately to 0.1 mg) were dissolved in 80% (v/v) ethanol (10 ml), and the dissolved solutions were filtered with millipore filter. The content of piceatannol in the filtrates was analyzed by HPLC. A reverse phase HPLC analysis was performed on HPLC LC-20A (Shimadzu, Kyoto, Japan) by the analytical conditions reported by Matsui et al[7] with a slight modification. The HPLC column used in this study was Inertsil ODS-3 (250×4.6 mm i.d., 5 µm, GL Science, Tokyo, Japan) and a guard column (Inertsil ODS-3, 10×4.0 mm i.d., 5 µm). A linear gradient was carried out with the mobile phase consisted of (A) water and (B) acetonitrile (v/v) using an initial gradient elution of 10% B and a gradient of 10-45% B at 0-25 min. The column temperature was maintained at 45°C. All measurements were carried out at a flow rate of 0.8 ml/min using a detector wavelength of 280 nm.

Spectroscopic studies

UV spectra were recorded on a T60 UV/Vis spectrophotometer (PG Instruments Limited, United Kingdom). IR spectra (KBr pellet) were recorded on a Nicolet Nexus 470 FT-IR instrument (International Equipment Trading Ltd., USA). ^{1}H-NMR (400 MHz, acetone-d6) and ^{13}C-NMR (75 MHz, $CDCl_3$) were recorded on a Brucker Avance (Bruker Analytik GmbH, Germany) using TMS (^{13}C-NMR) as an internal standard.

Statistical analyses

The results were statistically evaluated by one-way analysis of variance (ANOVA), followed by the Duncan test, in which significant differences were set at level of 5%.

Results and discussion

Separation of seed coat and characterization of total seeds, embryo, and seed coat based on chemical composition and composition ratio of embryo and seed coat

First, it should be noted that the seed coat prepared and used in the present study was not the native seed coat but it was the seed coat prepared by rinsing with water. Many kinds of seed coat can be prepared depending on the rinsing conditions. Therefore, its chemical composition shown below does not correspond to the native seed coat.

Separation of the seed coat from the total seeds of passion fruit was carried out by a simple rinsing method. When the excess water was added to the dried milled seeds, and after gently mixing, the seed coat precipitates while standing still at an ambient room temperature. The rinsing was carried out in two steps as shown in Figure 2. Accordingly, total seeds (TS) =EM-1 + SC-1.

In the second step, the resultant wet precipitate (wet SC-1) prepared by the first step was further pulverized in the excess water by colloid mill. A narrow gap between a static cone (the stator) and a rapidly rotating cone (the rotor) was adjusted not to make the fine particle in order to bring about the rapid precipitation of the seed coat. Accordingly, SC-1 = EM-2 + SC-2 i.e., TS = EM-1 + EM-2 + SC-2.

The particle size of SC-1 was 2380 mµ ≥ d >250 mµ and the mean particle size [d (50)] was 1000 mµ, where d (50) was the mass medium diameter as it divided the sample equally by mass. The particle size of SC-2 was 1000 mµ ≥ d >177 mµ and d (50) was 700 mµ. The particle size of SC-2 was a little smaller than SC-1.

As for TS, EM-1, EM-2, SC-1, and SC-2, the chemical compositions and piceatannol were measured. The content of piceatannol in TS, SC-1, EM-2 and SC-2 in Table 1 and 2 was determined by the repeated (three times) batch extraction where single batch extraction was by refluxing the seed part in 60% (v/v) ethanol for 1 h. The results were shown in Table 1 and 2.

Table 1 Fractions of embryo (EM-1) and seed coat (SC-1) prepared by the first step rinsing from dried passion fruit seeds, their chemical composition, and piceatannol content

Seed part	Weight	Protein	Fat	Fiber	Ash	Moisture content	Pic[a]
Total seeds (TS)		11.0±0.1	22.3±0.4	59.1±0.7	1.6±0.0	3.5±0.1	0.51
Embryo (EM-1)	27.9±0.7	27.8±0.2	42.1±0.2	15.6±0.5	4.1±0.0	3.9±0.1	ND[b]
Seed coat (SC-1)	72.2±1.5	2.2±0.1	7.4±0.5	77.0±0.3	0.5±0.0	3.6±0.0	0.70

Values in the column of weight, protein, fat, fiber, ash, moisture content, and piceatannol are % (w/w); [a], Piceatannol content in seed part (%, w/w); [b], ND, not detected by TLC detection method (see "Materials and Methods").

Table 2 Fractions of embryo (EM-2) and seed coat (SC-2) prepared from wet seed coat (wet SC-1), their chemical composition, and content of piceatannol

Seed part	Weight	Protein	Fat	Fiber	Ash	Moisture content	Pic
Embryo (EM-2)	15.1±2.4	16.2±0.2	34.0±0.1	31.4±1.0	4.0±0.1	3.3±0.1	0.26
Seed coat (SC-2)	58.0±1.6	1.8±0.1	2.4±0.1	84.7±1.1	0.3±0.0	4.6±0.1	0.80

Values and other explanatory notes in Table 2 are the same as those in Table 1.

According to the study by Matsui et al., the content of piceatannol in the total seeds of fresh passion fruit seeds and dried passion fruit seeds was about 0.22 % (w/w) and 0.48 % (w/w), respectively.[7] Additionally, according to the study by Sano et al., the content of piceatannol in passion fruit seeds (dried) was about 0.57 % (w/w).[2] In the present study, the content of piceatannol in total seeds was 0.51% (w/w), which was consistent to the reported data.

Liu et al. reported the chemical composition (moisture, protein, fat, ash, fiber, and carbohydrate) in the seeds of "Tainung No.1" passion fruit which is a crossbreed developed in Taiwan.[4] The results (%, w/w) in their study were protein (10.80±0.60), fat (23.40±2.50) in the total seeds. Our results in the total seeds were consistent with those in Liu et al.

In EM-1, the content of fat and protein was remarkably high compared with those of SC-1, SC-2 and total seeds. The detection of piceatannol in EM-1 was carried out by TLC method because HPLC analysis by the present analytical conditions was considered to be unsuitable for analyzing the extracts in which piceatannol content was very low (data not shown), and piceatannol in EM-1 was not detected by TLC method. These facts indicated that chemical composition of EM-1 was close to that of the authentic embryo and that piceatannol was not contained substantially in embryo.

In SC-1, the content of fat, protein, and fiber was comparable with those of SC-2 though the content of protein and fat was a little higher and the content of fiber was a little lower, compared with SC-2, which suggested that SC-1 was mixed with a little embryo. The content of piceatannol in SC-1 was approximately 0.7% (w/w).

In EM-2, the content of fat and protein was smaller than those of EM-1 and remarkably higher than those of SC-2 and total seeds. In contrast, the content of fiber was remarkably higher than that of EM-1. These facts indicated that some part of SC-2 mixed into EM-2, which was considered to be caused by the excess rinsing during powdering with colloid mill. The mixing of some part of SC-2 to EM-2 resulted in some content of piceatannol in EM-2. In fact, approximately 7.7% (w/w) of total piceatannol was found in EM-2.

The total amount of piceatannol extracted by the repeated batch extraction was regarded as the total amount of piceatannol (see below). Then the

content of piceatannol in SC-2 was approximately 0.8% (w/w), which showed that SC-2 contained approximately 92.3% (w/w) of total piceatannol. The characteristic of SC-2 was a very high content of fiber, a very low content of protein, and a substantial content of piceatannol. Interestingly, SC-2 contained fat though its content was not so high. These facts suggest that the chemical composition of SC-2 will be close to that of authentic seed coat. Considering that the piceatannol content in EM-2 was due to the mixing of the piceatannol contained in SC-2, it was considered that almost all of piceatannol in TS was contained in the seed coat.

Considering that EM-1 was close to real embryo and SC-2 was close to real seed coat, the composition ratio of real embryo and real seed coat can be calculated by the chemical composition (fat, protein, and fiber content) of EM-1, SC-2, and TS. As a result of the calculation, the calculated SC and EM was approximately 60% (w/w) and 40% (w/w), respectively. In comparison of SC-1 (72.2%, w/w) and SC-2 (58.0%, w/w) suggested that SC-1 contained approximately 7% (w/w) of embryo and that SC-2 lost approximately 7% (w/w) of seed coat, which was mixed in EM-2. SC-1 and SC-2 were used for the investigation on piceatannol extraction and its partial purification.

As discussed above, the localization of piceatannol in seed coat was demonstrated as follows: (1) no detection of piceatannol in embryo (EM-1) by TLC detection method (2) the high content (0.8%, w/w) of piceatannol in seed coat (SC-2) (3) the consistency of piceatannol content in total seeds obtained by experimentation (0.51%, w/w) and piceatannol content (0.48%, w/w) obtained by the calculation of piceatannol content in SC-2 (0.8%, w/w) multiplied by composition ratio of seed coat in total seeds (60%, w/w).

Structure confirmation of isolated compound

HPLC of the isolated compound from SC-2 showed one peak with Rt (approximately 21.2 min), and TLC of the isolated compound, developed with $CHCl_3$-MeOH (8:2) showed one spot with Rf (0.44). Rt and Rf coincided with those of standard piceatannol.

The UV spectrum of the isolated compound and standard piceatannol (purity, > 98%, w/w) at 5 µg/ml in 80% (v/v) ethanol were almost identical (Figure 4), and the HPLC analyses of the isolated compound and standard piceatannol at the same concentration showed nearly the same HPLC area.

The UV spectrum, FT-IR, ^{1}H NMR, and ^{13}C NMR of peak 1 were measured. λ_{max} in 80% (v/v) ethanol = 220, 237, 303, and 325 nm. Main peaks of FT-IR were 822, 965, 1142, 1204, 1283, 1346, 1444, 1519, 1600, 3334, 3510 cm^{-1}. ^{1}H NMR (300 MHz, acetone-d6) δ 6.27 (1H, t, J = 2.1 Hz, 4'-H), 6.53 (2H, d, J = 1.8 Hz, 2',6'-H), 6.81 (1H, d, J = 16.2 Hz, olefinic H), 6.83 (1H, d, J = 9.0 Hz, 5-H), 6.90 (1H, dd, J = 8.4, 1.8 Hz, 6-H), 6.95 (1H, d, J = 16.5 Hz, olefinic H), 7.08 (1H, d, J = 1.8 Hz, 2-H), 8.00 (2H, br, OH) 8.20 (2H, s, OH); ^{13}C NMR (75 MHz, $CHCl_3$) δ 101.76 (C4), 104.81 (C2',6'), 112.96 (C2), 115.38 (C5), 119.14 (C6), 126.01 (olefinic), 128.50 (olefinic), 129.84 (C1), 139.97 (C1'), 145.19 (C4), 145.23 (C3), 158.66 (C3', 5').

All the data (^{1}H NMR, ^{13}C NMR, FT-IR, and UV) of the isolated compound in the present study were consistent with those of piceatannol reported in the past.[17,18]

Single batch extraction of piceatannol from seed coat of passion fruit and partial purification of extracts

In the present study, the extraction of piceatannol with boiled water was carried out because the extraction with boiled water will provide the significant advantage for the industrial scale production of piceatannol extract if it is feasible.

In the manufacturing processes which use a large volume of solvent, the application of the adsorption and desorption of piceatannol with the synthetic adsorbents will be very important process because the more efficient production of piceatannol will be conveniently achieved. At the same time, the process with the synthetic adsorbents will serve as the partially purifying process. Then, the use of the synthetic adsorbents (HP20 and SP825) was carried out.

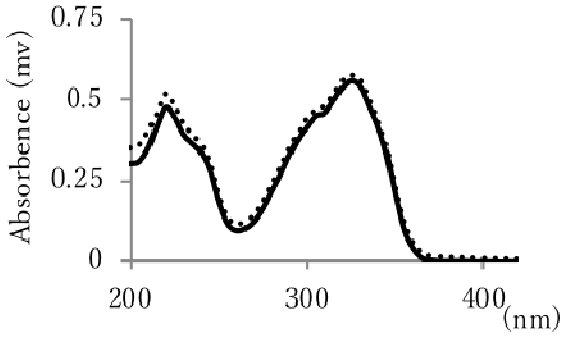

Figure 4 UV-spectrum of isolated compound and standard piceatannol (purity, > 98%, w/w) at 5 µg/ml in 80% (v/v) EtOH. Solid line, standard piceatannol; dotted line, isolated compound.

As for the boiled water extraction, the extract of water solution was directly passed through the column of HP20, and after washing the column with water, piceatannol was eluted by using 80% (v/v) ethanol.

As for the extraction with 60% (v/v) ethanol, two methods were carried out. One method was to pass the extract after diluting the extract in 60% (v/v) ethanol up to 20% (v/v) ethanol with water because the adsorption of piceatannol on the synthetic adsorbents (HP20 and SP825) in 20% (v/v) ethanol and the desorption with 80% (v/v) ethanol was confirmed by the preliminary test. By the preliminary test, the adsorption of piceatannol on SP825 in 20% (v/v) ethanol seemed stronger than HP20 (data not shown). Then, SP825 was used for this process. This method was applied for the extraction with 60% (v/v) ethanol at room temperature for 5 days.

As for the extraction with 60% (v/v) ethanol by refluxing for 1 h, ethanol was removed by evaporation, and the resultant water solution was passed through the column of HP20 and eluted with 80% (v/v) ethanol.

The color of the extract from the seed coat and total seeds was very deep blackish brown. For the further partial purification, AcOEt/water partition was carried out. AcOEt/water partition did not remove color but AcOEt/0.5% (w/v) $NaHCO_3$ partition remarkably removed the color though its removal was not complete.

From the point of view as described above, the single batch extraction and partial purification of piceatannol from seed coat (SC-2) under the various extraction conditions was carried out.

The maximum extraction of yield among the experimental conditions was obtained by refluxing with 60% (v/v) ethanol, then the estimated amount of piceatannol under various extraction conditions from SC-2 (100g), and the relative extraction efficiency (the ratio of the extracted piceatannol under various conditions to that under the maximum extraction) was calculated. The extraction of yield with boiled water and with 60% (v/v) ethanol at room temperature was not high (approximately 20-30% of the maximum extract).

According to Maruki-Uchida et al., the passion fruit seeds extract prepared by extracting with 30% (v/v) 1, 3-butylene glycol contained 3.7% (w/w) of piceatannol in the extract, and the extract was used for cell level assay.[8] The content of piceatannol in the simple extract (EtOH/Reflux/E) was about 2.6 times higher. The content of piceatannol in the extract treated with synthetic adsorbent (EtOH/Reflux/SP825/E) increased up to approximately 20% (w/w), and by AcOEt/$NaHCO_3$ partition, its content in the extract reached approximately 25% (w/w).

Table 3 Single batch extraction of piceatannol from seed coat (SC-2) and partial purification of extracts

Extraction	Extracts[a]	Seed coat[b] (g)	Wt[c] (mg)	Piceanannol[d] (%)	Piceatannol (mg) in SC-2 (100g)[e] and relative ext. efficiency (%)[f]
Extraction with boiled water[g]	BW/E	50	1527±212	5.4±1.4	165 mg (25%)
	BW/HP20/E	50	553±14	15.6±0.7	173 mg (26%)
	BW/HP20/AcOEt/E	50	368±45	20.0±1.6	147 mg (22%)
	BW/HP20/AcOEt/$NaHCO_3$/E	50	315±32	22.4±2.4	141 mg (21%)
Extraction with 60% (v/v) ethanol at RT[h]	EtOH/E	50	1513±50	4.5±0.5	136 mg (21%)
	EtOH/SP825/E	50	647±96	12.3±0.6	159 mg (24%)
	EtOH/SP825/AcOEt/E	50	626±85	16.0±0.2	200 mg (30%)
	EtOH/SP825/AcOEt/$NaHCO_3$ /E	50	523±48	20.2±2.3	211 mg (32%)
Extraction with 60% (v/v) ethanol by refluxing[i]	EtOH/reflux/E[j]	10	685±19	9.5±1.4	650 mg (98%)
	EtOH/reflux/SP825/E	10	312±10	21.2±0.6	661 mg (100%)
	EtOH/reflux/SP825/AcOEt/E	10	296±15	22.1±0.5	654 mg (99%)
	EtOH/reflux/SP825/AcOEt/ $NaHCO_3$/E	10	244±8	25.4±0.6	620mg (94%)

[a], Extracts and fractions partially purified from seed coat ; [b], Weight of seed coat used for extraction; [c], Weight of extracts; [d], Content of piceatannol (%, w/w) in the extracts; [e], Estimated amount of piceatannol in SC-2 (100g); [f], extraction efficiency = 100×(amount of piceatannol extracted under various conditions) / [amount of piceatannol extracted by 60% (v/v) ethanol/reflux/SP825/E]; [g], The seed coat was extracted in water for 1 h by boiling.; [h], The seed coat was extracted with 60% (v/v) ethanol for 5 days at room temperature.; [i], The seed coat was extracted in 60% (v/v) ethanol for 1 h by refluxing; [j], The results were taken from the repeated batch extraction (see below, Table 4).

Repeated batch extraction of piceatannol from total seeds, seed coat (SC-1 and SC-2), and embryo (EM-2) with 60% (v/v) ethanol by refluxing

The repeated batch extraction (three times) by refluxing the samples [10g, total seeds, seed coat (SC-1 and SC-2), and embryo (EM-2)] in 60% (v/v) ethanol (100 ml) for 1 h was carried out. The results are shown in Table 4.

The total amount of piceatannol was regarded as the content of each seed part, and used for calculating piceatannol content (%, w/w) in each part. The results are shown in Table 1 and 2.

In the case of total seeds, the recovery of piceatannol and its content in the extract were 72.7% (w/w) and 3.0% (w/w) by single extraction, respectively, and 96% (w/w) and 3.3% (w/w) by double extraction, respectively.

In the case of SC-1, the recovery of piceatannol and its content in the extract were 76.7% (w/w) and 8.4% (w/w) by single extraction, respectively, and 96% (w/w) and 8.2% (w/w) by double extraction, respectively.

In the case of SC-2, the recovery of piceatannol and its content in the extract were 81.9% (w/w) and 9.5% (w/w) by single extraction, respectively, and 96.4% (w/w) and 9.0% (w/w) by double extraction, respectively. SC-2 gave the results of the highest content of piceatannol, then the use of the well-rinsed seed coat will be useful for manufacturing the extract containing high amount of piceatannol.

Table 4 Repeated batch extraction of piceatannol from total seeds, seed coat, and embryo with 60% EtOH (v/v) by refluxing

Seed Part	Extracts	wt (mg)[a]	Piceatannol (%)[b]	Piceatannol (mg)[c]	Recovery of Piceatannol (%)[d]
Total seeds	Round 1	1234±6	3.0±0.1	37.0	72.7
	Round 2	225±9	4.6±0.4	10.4	20.4
	Round 3	109±5	3.2±0.4	3.5	6.9
	Total	1568±17	-	50.9[e]	-
SC-1	Round 1	635±28	8.4±0.6	53.3	76.7
	Round 2	183±8	7.5±0.3	13.4	19.3
	Round 3	109±4	2.8±0.1	3.1	4.5
	Total	927±32	-	69.5[e]	-
SC-2	Round 1	685±19	9.5±1.4	65.1	81.9
	Round 2	166±5	6.9±1.0	11.5	14.5
	Round 3	85±9	3.4±0.6	2.9	3.7
	Total	936±26	-	79.5[e]	-
EM-2	Round 1	692±20	3.3±0.2	22.8	88.4
	Round 2	197±7	1.4±0	2.8	10.9
	Round 3	79±9	0.3±0	0.2	0.8
	Total	912±30	-	25.8[e]	-

[a], Weight of extracts; [b], Content of piceatannol in the extracts (w/w); [c], Amount of extracted piceatannol; [d], 100 × (amount of piceatannol extracted) / (total amount of piceatannol in each sample); [e], Total piceatannol was regarded as the content of piceatannol in each seed part.

EM-2 contained piceatannol (0.26%, w/w), which was due to the mixing some part of SC-2. The results shown above indicated that the use of SC-2 was much better than that of total seeds for the extraction of piceatannol.

The use of the passion fruit seed coat for manufacturing the extract containing piceatannol was first reported in the present study. Therefore, the characterization of the seed coat prepared by a simple rinsing method was one of the aims of the present study. Then the rinsing for preparing the seed coat was carried out in two steps for the purpose of preparing the seed coat by weaker rinsing and by stronger rinsing conditions, and the properties of embryo and seed coat were investigated by analyzing their chemical compositions and piceatannol content. The content of piceatannol and impurities in seed coat varied depending on the rinsing conditions, and the composition of the prepared seed coat would affect the processes for the extraction and partial purification of piceatannol though the effects were limited. The optimization studies for manufacturing the extract containing a higher amount of piceatannol including scirpusin B and resveratrol from passion fruit seeds was not the aim of the present study. The optimization, however, is of critical importance and awaited.

Conclusions

This study provides a simple and efficient method for preparing seed coat of passion fruit seeds. This study demonstrated the chemical composition and composition ratio of embryo and seed coat of passion fruit seeds and indicated that almost all of piceatannol was contained in the seed coat and the application of the seed coat as the starting material for manufacturing the extract containing a higher amount of piceatannol was useful. This study also demonstrated the extraction of piceatannol under various conditions including extraction with boiled water and the comparison of extraction efficiency among the various extraction conditions, and it was indicated that the synthetic adsorbents and partition with AcOEt and $NaHCO_3$ were effective for partial purification of the extracts containing piceatannol.

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