Original Article

Acupuncture and Herbal Medicine

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Oxalic acid in ginger specifically denatures the acrid raphides in the unprocessed dried tuber of *Pinellia ternata*

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Abstract

Objective: Pinellia Tuber, the dried tuber of *Pinellia ternata*, is widely used in Japanese Kampo medicines and traditional Chinese medicines. The unprocessed Pinellia Tuber is known to cause very strong acrid irritation at oral and laryngopharynx mucosa. Recent studies have shown that the sharp needle-like crystals called raphides, that are composed of calcium oxalate and proteins, are the main causative substances of the irritation. Ginger, the rhizome of *Zingiber officinale*, has been used in the processing to reduce the acridity of Pinellia Tuber since before the sixth century, however, the mechanisms of reducing acridity have not been scientifically proved yet.

Methods: We developed the raphides denaturation assay (RDA) to quantify the degree of denaturation in the raphides to cause irritation. By their lipophilic characters, the raphides could be extracted in petroleum ether (PE) layer from powdered Pinellia Tuber suspended in water, and the contents of the raphides in PE layer were measured by the absorbance. By this assay, we conducted the activity-guided fractionation from the boiling water extract of ginger to find the ingredients to denature the raphides. We also conducted the gustatory tests to detect the change of the irritation of the denatured raphides.

Results: The treatment of powdered Pinellia Tuber suspension with ginger extract reduced the distribution of raphides in PE layer in RDA in a concentration-dependent manner. The activity-guided fractionation using RDA revealed that oxalic acid was the main active ingredient in ginger extract to denature the raphides of Pinellia Tuber. Oxalic acid reduced the lipophilicity of the raphides in the thermo-, time-, and concentration-dependent manners, and its activity was affected by pH. The treatment of powdered Pinellia Tuber suspension with oxalic acid significantly reduced its acrid irritation in gustatory test in human.

Conclusions: We found that oxalic acid is the main active ingredient in ginger to reduce the acrid irritation of Pinellia Tuber.

Keywords: Acridity, Ginger, *Pinellia ternata*, Pinellia Tuber, Processing, Raphide

Graphical abstract: http://links.lww.com/AHM/A10

Introduction

Pinellia Tuber, the dried tuber of *Pinellia ternata*, is the crude drug registered in the 18th Edition of Japanese Pharmacopoeia[1]. Pinellia Tuber is mostly used as an unprocessed cut form to prepare the decoctions of Japanese Kampo formula, such as shosaikoto, hangeshashinto, and hangekobokuto[2]. The unprocessed Pinellia Tuber is known to cause very strong acrid irritation at oral and laryngopharynx mucosa when taken orally. The past studies had enumerated 3,4-dihydroxybenzaldehyde, homogentisic acid, and calcium oxalate as the candidates of the cause of acridity[3]. However, the recent studies have shown that the acridity is caused by the insoluble needle-like crystals, called raphide, which is composed of calcium oxalate and proteins[4–5]. It has been shown that the protein isolated from the raphides could induce eye inflammation in rabbits, and that ethanol treatment destroyed the raphides to lose their points of needles as well as their activity to cause inflammation[4,6]. In taro, the rhizome of *Colocasisa* species, it was proposed that the raphides could play a role in penetration and carrying the acrid factor, which could be a 26 kDa protein deposited on the surface and groove of the raphides[7].

The acridity of unprocessed Pinellia Tuber is known to be disappeared by boiling, and it is recommended that the Kampo formulas containing Pinellia Tuber should be decocted sufficiently to remove its acridity in Japanese Kampo medicine[8]. On the other hand, Pinellia Tuber is mainly used as the processed form to avoid its toxicity beforehand in traditional Chinese medicine[9]. The

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Acupuncture and Herbal Medicine (2022) 2:1

Received 2 December 2021 / Accepted 28 February 2022

http://dx.doi.org/10.1097/HM9.0000000000000025

Chinese Pharmacopeia lists three kinds of the processed Pinellia Tuber whose names and preparation methods are as follows: "*Fabanxia*: Prepared Pinellia Tuber", the tuber of *P. ternata* is soaked in the mixture of the licorice decoction and the lime solution before dried; "*Qingbanxia*: Pinellia Tuber prepared with alum," the tuber of *P. ternata* is soaked in an 8% solution of alum before dried; and "*Jiangbanxia*: Pinellia Tuber prepared with ginger," the tuber of *P. ternata* is boiled in the decoction of the slices of the fresh rhizome of *Zingiber officinale* (ginger) with alumen before dried[10]. These processing methods are known to reduce the acridity of Pinellia Tuber, however, except for the solubilization of calcium oxalate by the aluminum ion of alum to destroy the needle crystal[11], the mechanism of how these processing techniques reduce the acridity still remains unknown. Ginger has been used to reduce the toxicity of Pinellia Tuber since before the sixth century[12]. The acrid irritation caused by unprocessed Pinellia Tuber is said to be relieved by taking a bite of fresh ginger or drinking ginger decoction in oral traditions, and also it is recommended that Pinellia Tuber is decocted preferably with ginger in Japan[13]. Nevertheless, the mechanism or even the activity of ginger to reduce the acridity has not been scientifically proved yet. Besides, recent studies have found no evidence that ginger extract interacts with the irritative protein isolated from Pinellia Tuber[14].

Against these backgrounds, we have previously reported that the raphides of Pinellia Tuber have lipophilic character, and the treatments such as boiling, the incubation with methanol, or ginger boiling water extract denature the raphides to decrease their lipophilicity. It was also suggested that the acridity of the raphides coordinated with the lipophilicity of them which could be shown as the dispersion of them in the petroleum ether (PE) layer in water/PE partition[15].

The aim of the present study is to find the active ingredients in ginger extract on denaturing the raphides of Pinellia Tuber. We fractionated ginger extract and investigated the features of the interaction between them using raphides denaturation assay (RDA) newly developed.

Materials and methods

Materials

All crude drugs used were under the quality control of the 18th edition of the *Japanese Pharmacopoeia*[1]. The dried sliced form (approximately 2 mm width) of Pinellia Tuber (lot number, #009120002) and the dried rhizome of *Z. officinale* (dried ginger, lot number, #005819004) in cut-form with about 5 mm pieces were purchased from Tochimototenkaido (Osaka, Japan). Acetic acid, citric acid, and oxalic acid were obtained from Kanto Chemical (Tokyo, Japan). Maleic acid and DL-malic acid were purchased from Sigma-Aldrich (St. Louis., MO, USA). L-(+)-tartric acid and succinic acid were bought from Nacalai Tesque (Kyoto, Japan).

Preparation of low starch Pinellia Tuber suspension (LSPS)

The sliced dried Pinellia Tuber (50 g) was milled using an electric mill (Y-308B, Yamamoto Denki, Fukushima, Japan), and was filtered through 150 μm mesh. The powdered Pinellia Tuber was mixed with H_2O (350 mL), and kept at room temperature for 30 min. Then, the mixture was vortexed vigorously to obtain thick suspension. The suspension was centrifuged at $18 \times g$ for 10 min to precipitate the bulk of starch, and the supernatant containing the raphides was separated. H_2O (300 mL) was added to the precipitate, and the suspension was vortexed vigorously. After centrifugation at $18 \times g$ for 10 min, the second supernatant was separated and merged with the first one. The merged supernatants were centrifuged at $1{,}000 \times g$ for 10 min to precipitate the residue containing raphides. After the removal of the supernatant, the residues were washed with H_2O twice, and re-suspended in H_2O (50 mL) to prepare LSPS for the subsequent use in the denaturation assay. LSPS was stored at −20°C before use.

Preparation of dried ginger decoction

Dried ginger (4.0 g) was mixed with H_2O (32 mL) in sealed plastic centrifuge tube (50 mL), and the tube was incubated in boiling water for 30 min. Then, the suspension was filtered through cotton gauze, and the filtrate was centrifuged $1{,}000 \times g$ for 10 min. The supernatant was collected and stored as dried ginger decoction at −20°C before use. A part of the decoction was lyophilized to yield the extract, and the concentration of this decoction was 25 mg/mL. The extract (0.76 mg) was dissolved in methanol, and used for fingerprint analysis shown in Supplementary Figure 1A, http://links.lww.com/AHM/A9.

The dried ginger decoction and the aqueous solutions of sodium oxalate (Nacalai) (40 μg/mL, 0.20 mg/mL, and 1.0 mg/mL as oxalic acid) were centrifuged at 12,000×g for 5 min, and the supernatants (5 μL) were injected into HPLC system (Shimadzu LC-10A$_{VP}$, Kyoto, Japan) as following: column, Cosmosil HILIC (250 mm × 4.6 mm i.d., Nacalai); mobile phase, 10 mM phosphate buffer (pH 7.0)/acetonitrile (70:30) 1.0 mL/min; column temperature, 40°C; detector, UV at 203 nm. Oxalate was eluted at 9.2 min (Supplementary Figure 1B, http://links.lww.com/AHM/A9), and linear regression of the concentration range of oxalate by the peak-area was calibrated with the least-squares method ($r^2 = 0.999$), and the concentration of oxalate in the dried ginger decoction was calculated by this regression formula. Then, the concentration of oxalic acid in the decoction was determined as 0.48 mg/mL as oxalic acid (5.4 mM).

The contents of other organic acids in the dried ginger decoction were analyzed. The extract of dried ginger (0.25 mg) in H_2O (10 μL) was injected to HPLC with the following conditions: column, Cosmosil 5C$_{18}$-PAQ (250 mm × 4.6 mm i.d., Nacalai); mobile phase, 20 mM phosphate buffer (pH 2.5) 1.0 mL/min; column temperature, 30°C; detector, UV at 210 nm. In this condition. tartaric acid (2.4 min), malic acid (2.8 min), acetic acid (4.1 min), citric acid (4.6 min), succinic acid. (4.6 min), and maleic acid (5.0 min) can be detected. Among these organic acids, only tartaric acid was detected (Supplementary Figure 1C, http://links.lww.com/AHM/A9).

Assays of the denaturation activity against the raphides of Pinellia Tuber

Dried ginger decoction, its fractions, organic acids, and calcium chloride were diluted or dissolved in H_2O, the pH of the organic acid solutions were adjusted using 1 M

NaOH to prepare the aqueous sample solutions. Aqueous sample solutions (3.5 mL) and LSPS (0.50 mL) were mixed and pre-incubated for 30, 45, or 90 min at 4°C or 40°C in sealed plastic centrifuge tubes. Then, PE (3.0 mL) was added and vortexed vigorously (Supplementary Figure 2A, http://links.lww.com/AHM/A9). The mixtures were incubated at room temperature by standing for 15 min at room temperature until upper, middle, and lower layers appeared (Supplementary Figure 2B, http://links.lww.com/AHM/A9). Then, the tubes were gently shaken for 10 s to raise the cloud of raphides at the middle layer into the upper layer. The upper PE layer containing the raphides cloud was collected, and the turbidity was measured immediately as the optical densities at 660 nm (OD_{660}) were measured (Supplementary Figure 2C, http://links.lww.com/AHM/A9). The procedure after adding PE was named Raphides Denaturation Assay (RDA). When the raphides of Pinellia Tuber are denatured by samples, their lipophilicities are decreased[15], therefore, the OD_{660} of PE layer is decreased. This effect of the sample is named as raphides denaturing activity. In other experiments, the suspension of raphides in PE was properly diluted by PE, and the suspension was dried under vacuum to measure the dried weight of raphides to calculate the concentration of the suspension. The concentrations of raphides (μg/mL) were calibrated as $270 \times OD_{660}$, and the detectable range was 0 to 810 μg/mL. Data were shown as this concentration.

Fractionation of the decoction of dried ginger

Dried ginger decoction (20 mL) was sequentially extracted with 8 mL each of PE, ethyl acetate, and water-saturated *n*-butanol to obtain each fraction. The final water layer was evaporated and suspended in 10 mL of methanol, and the soluble and insoluble fractions were separated. Each fraction was suspended in 20 mL of H_2O, and each sample (3.5 mL) was mixed with LSPS (0.50 mL) in sealed plastic centrifuge tube, and incubated for 60 min at 40°C. Then, RDA was conducted.

Gustatory test

A gustatory test was performed in three healthy volunteers. The procedures were approved by the ethical committee in Toho University, School of Medicine with permission code #A19081. Written informed consent was obtained from all individual participants included in the study.

5 mL of citric acid solution in H_2O (0.2 M, pH 4.6) or oxalic acid solution in H_2O (0.2 M, pH 4.6) was mixed with LSPS (5.0 mL), then incubated at 40°C for 120 min. The aliquot of the mixture (0.60 mL) was collected, and mixed with H_2O (2.0 mL) in sealed plastic centrifuge tube, and PE (3 mL) was added to conduct RDA. The aliquot of the mixture (8.0 mL) was centrifuged at $1,000 \times g$ for 10 min. The precipitates were washed with H_2O twice, and finally re-suspended in H_2O (8.0 mL) to prepare the sample suspensions for the gustatory test. The sample suspensions (1.0 mL) were kept in the mouth of three blindfolded healthy volunteers for 1 min before being expelled. The acridity was determined after 5 min based on the irritation.

The PE extraction (PEX) of the raphides of Pinellia Tuber

The powdered dried Pinellia Tuber (30 g) was suspended in H_2O (120 mL), and was kept standing at room temperature for 60 min. Then, PE (40 mL) was added, and mixed vigorously. The suspension was kept standing at 4°C for 90 min, and three layers appeared. Then, it was shaken gently to raise the raphides from the middle layer to upper PE layer in the same way as RDA. The cloudy upper layer was immediately collected avoiding the contamination of middle layer carefully. This collecting procedure was repeated three times with fresh PE (40 mL), and all PE layers were merged and centrifuged $1,000 \times g$ for 5 min. The supernatant was removed, and the precipitated raphides were washed with fresh PE (20 mL) three times, and then H_2O (10 mL) three times. The raphides were finally suspended in PE (7.0 mL), which was named PEX raphides suspension, and stored at −20°C in glass vials. An aliquot of the suspension was diluted with PE, and OD_{660} was measured. In another aliquot of the suspension was lyophilized and the dried weight of the raphides was precisely measured.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Bonferroni's multiple tests and multiple-way ANOVA was used to compare multiple data. All analyses were conducted using Mac Statistical Analysis Ver 3.0 (Esumi, Tokyo, Japan). Data are expressed as mean ± standard deviation (SD), while $P < 0.05$ were considered significant.

Results

Dried ginger decoction was diluted with H_2O, pre-incubated with LSPS for 90 min at 40°C, and then RDA was conducted. Dried ginger extract exhibited the decrease of OD_{660} of the PE layer in RDA in a concentration dependent manner to show the raphides denaturing activity, and the half-maximal (50%) inhibitory concentration (IC_{50}) was 15 mg/mL (Figure 1A).

In order to identify the active ingredients in ginger decoction, we prepared its fractions by participating with organic solvents, mixed these fractions with LSPS, and conducted RDA. Among the fractions of ginger decoction, only the final water layer methanol soluble fraction exhibited the significant raphides denaturing activity (data not shown).

Since it is predicted that this layer contains small-molecular-weight water-soluble organic compounds, cations, and anions, we investigated the effects of organic acids contained in general plants including acetic acid, citric acid, maleic acid, malic acid, oxalic acid, tartaric acid, and succinic acid at pH 5.0, because the pH of the aqueous solution of the final water layer methanol soluble fraction was 5.0. As shown in Figure 2A, all organic acids tested exhibited significant raphides denaturing activities, and oxalic acid was found to have the strongest activity among them.

Subsequently, we evaluated the effect of pH in the solution of organic acids using acetic acid, citric acid, and oxalic acid. The raphides denaturing activity of citric acid exhibited no pH dependency, and those of acetic acid and oxalic acid were found to become weaker monotonously in accordance with the increase of the pH, and exhibited significant pH dependencies (Figure 3).

Since oxalic acid exhibited the strongest raphides denaturing activity among organic acids evaluated, we further evaluated its features of raphides denaturing

activity. At first, oxalic acid exhibited concentration-dependent raphides denaturing activity at pH 5.0, and its IC_{50} values was 6.9 mM (Figure 1B). The effects of temperature and incubating time length at the pre-incubation phase mixing oxalic acid solution with LSPS before RDA were investigated, and the results were shown in Figure 4. Multiple-way ANOVA indicated the statistically significant effect between data obtained at 45 and 90 min ($F_{1,16}=62$, $P<0.001$), between control and oxalic acid groups ($F_{1,16}=421$, $P<0.001$), between data obtained at 4°C and 40°C ($F_{1,16}=88$, $P<0.001$), the interaction between the time and the sample treatment ($F_{1,16}=6.7$, $P<0.05$), and between the sample treatment and the temperature ($F_{1,16}=52$, $P<0.001$). The raphides denaturing activity of oxalic acid was observed in incubation-time-length-dependent manners, and that appeared at 40°C was significantly stronger than that appeared at 4°C.

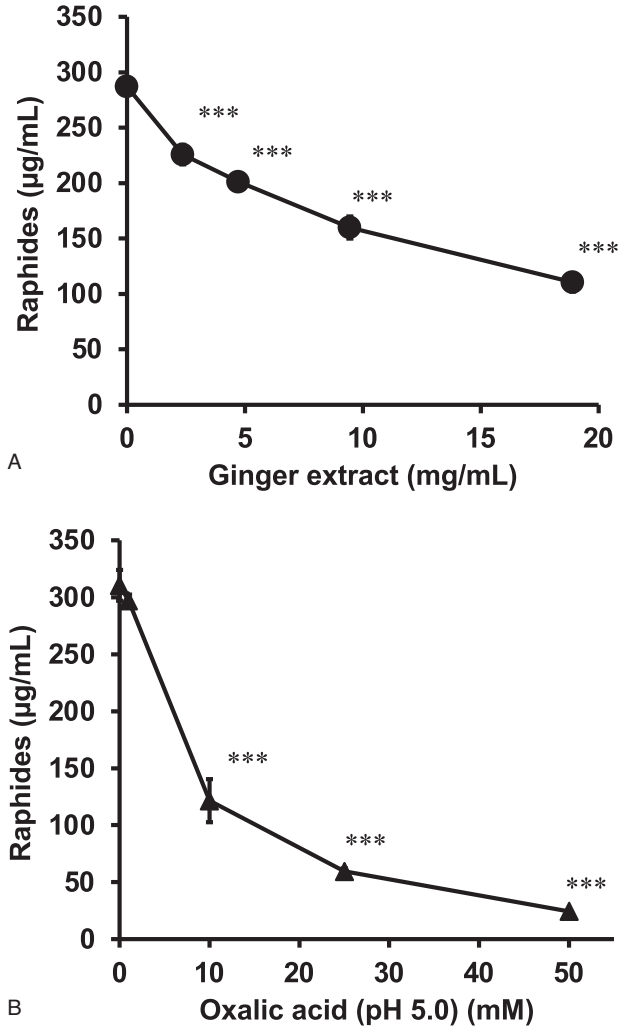

Figure 1. The raphides denaturing activity of ginger extract and oxalic acid. (A) Dried ginger decoction was diluted with H_2O, subsequently, each sample solution (3.5 mL) was mixed with low starch Pinellia Tuber suspension (LSPS, 0.50 mL), and pre-incubated at 40°C for 90 min. (B) Oxalic acid aqueous solution (20 mM) that pH was adjusted at 5.0 using 1.0 M NaOH (0.20 mL, 2.0 mL) or oxalic acid aqueous solution (0.10 M) that pH was adjusted at 5.0 using 1.0 M NaOH (1.0 mL, 2.0 mL) was mixed with LSPS (0.50 mL) and H_2O to make the final volume of the mixture as 4.0 mL, and pre-incubated at 40°C for 30 min. Then, raphides denaturation assay (RDA) was conducted. Data are expressed as mean ± SD ($n=3$). $^{***}P<0.001$, statistically significant differences compared with the group without ginger extract of oxalic acid evaluated by one-way ANOVA followed by Bonferroni's multiple comparison test.

Calcium chloride was added to dried ginger decoction at the concentrations of 0 to 20 mM before mixing with LSPS, pre-incubated for 90 min at 40°C, and the RDA was conducted. The denaturing activities of the dried ginger decoction were significantly suppressed by the addition of calcium chloride in a concentration-dependent manner. The concentration of calcium chloride at 7.5 mM reached the maximum, and the effect exhibited plateau at more concentrations (Figure 5). Subsequently, we prepared 5.4 mM oxalic acid solution in acetate buffer (0.06 M, pH 5.4), whose concentration of oxalic acid and pH were equal to those of dried ginger decoction. After calcium chloride was added into this solution instead of dried ginger decoction in the same way, the solution was mixed with LSPS, pre-incubated for 90 min at 40°C, and then, the RDA was conducted. The concentration-dependent curve of OD_{660} values to calcium chloride in oxalic acid solution exhibited quite similar to that in dried ginger decoction (Figure 5). Two-way ANOVA indicated a no significant effect between ginger decoction and oxalic acid solution ($F_{1,20}=1.1$, $P=0.30$) and the interaction between the sample treatment and their concentrations ($F_{4,20}=1.7$, $P=0.18$), but significant effects among the concentrations ($F_{4,20}=226$, $P<0.001$).

Subsequently, LSPS was treated with either oxalic acid or citric acid to prepare the samples for the gustatory tests. Although citric acid significantly denatured the raphide, the gustatory test showed no improvement on the acrid irritation compared with the raphides without the treatment, while the treatment with oxalic acid denatured the raphides and reduced the acridity significantly in gustatory test (Table 1).

Finally, we investigated the raphides denaturing activity of organic acids against raphides extracted by PEX. PEX raphides were found to be denatured by 50 mM organic acid solutions at pH 5.0. While slight but significant differences were found in the groups treated with citric acid and acetic acid compared to the control, the raphides denaturing activity of oxalic acid was significantly higher than those two groups and the control (Figure 2B).

Discussion

One of the largest difficulties in the investigation on the effect of the processing Pinellia Tuber on its acridity was the objective and quantitative evaluations, since the index for its acrid irritation could be evaluated only by the scores of gustatory tests using human sense, which was very difficult to be precisely quantified. In our previous study, we found that the interaction between Pinellia Tuber powder and dried ginger decoction resulted in the denaturation of the raphides in time-dependent manners, which was observed by the microscopic image analysis, and that the denaturation of the raphides could be used for the objective index of acrid irritation[15]. In the present study, we newly developed a quantitative analytical method named RDA to evaluate the degrees of the raphides' denaturation. The intact raphides have the affinities with low-polarity organic solvents by their hydrophobicity, which is decreased by the denaturation, and disperse in the PE layer in PE/water partition[15]. Therefore, the degree of the raphides' denaturation can be evaluated by the decrease of the numbers of the raphides dispersing in PE layer, that is, the decrease of the turbidity

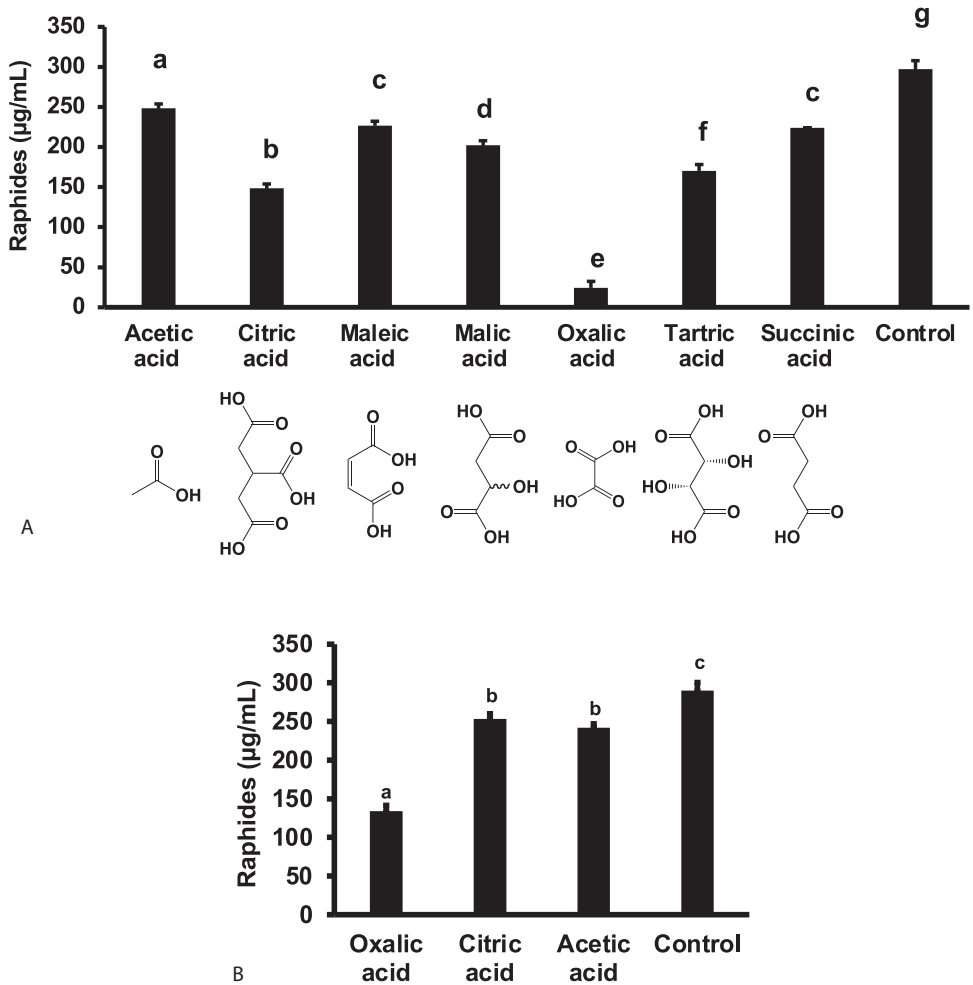

Figure 2. The raphides denaturing activity of organic acid against low starch Pinellia Tuber suspension (LSPS) or PEX raphides. (A) pH values of aqueous sample solutions of organic acids (0.1 M) were adjusted at 5.0 using 1.0 M NaOH solution. Each organic acid solution (2.0 mL) was mixed with LSPS (0.50 mL) and H_2O (1.5 mL), and pre-incubated at 40°C for 30 min. (B) PEX raphides suspension in PE (0.18 mL) was dried in a plastic tube, then H_2O (2.0 mL) was added and vortexed vigorously to prepare the homogeneous aqueous suspension. Each 2.0 mL of aqueous solutions of oxalic acid, citric acid, and acetic acid (0.10 M), that pH were adjusted by 5.0 using 1.0 M NaOH, or of H_2O was added to the PEX raphides aqueous suspension, and incubated for 30 min at 40°C. Then, raphides denaturation assay (RDA) was conducted. Data are expressed as mean ± SD ($n = 3$). Different alphabetical letters (a–g) over the columns indicate statistically significant differences at $P < 0.05$ evaluated by one-way ANOVA followed by Bonferroni's multiple comparison test. PE: Petroleum ether; PEX: PE extraction.

of the PE layer, which is measured and quantified as OD_{660}. In fact, dried ginger extract exhibited the decrease of OD_{660} of PE layer in a concentration-dependent manner to show the raphides denaturing activity in this study, and the IC_{50} value was 14.5 mg/mL. The results from RDA were highly reproductive and shown to be in accordance with the sensitivity of acrid irritation in the gustatory tests, as it had been shown in the previous study[15]. RDA is expected to be a powerful tool in the investigation on the acridity of Pinellia Tuber.

In the present study, we tried to identify the active ingredients in dried ginger decoction. We had speculated that the active ingredients might be hydrophobic essential oil constituent, since our previous study showed that the raphides denaturing ingredients in dried ginger decoction were soluble in PE[15]. However, the general partition fractioning technique revealed that the denaturing activities in dried ginger decoction transferred into the final water layer methanol-soluble fraction, and it was considered that this fraction contained small molecular hydrophilic organic compounds, anions, and cations.

Among these small molecular hydrophilic organic compounds, we firstly focused on the major organic acids in plants. All organic acids evaluated were revealed to have significant raphide denaturing activities, and the activity of oxalic acid was found to be by far the highest. Subsequently, we chose oxalic acid, citric acid, and acetic acid as the major organic acids, and compared their pH dependencies in their activities. Oxalic acid showed the strongest denaturing activity in the range of pH 4.0 to 6.0, which was a common range in the decoctions of crude drugs[16–17]. It was notable that the raphide denaturation activities of acetic acid and oxalic acid were affected by the pH of the reaction mixtures, while no significant pH dependency was shown in citric acid. The mechanism of the denaturation by citric acid may not be same as those of acetic acid or oxalic acid. For example, citrate ion was reported to bind on the surface of the calcium oxalate crystal which resulted in the modification of crystal surface structure[18]. The modification of the crystal surface may be involved in the raphides denaturing process by citrate.

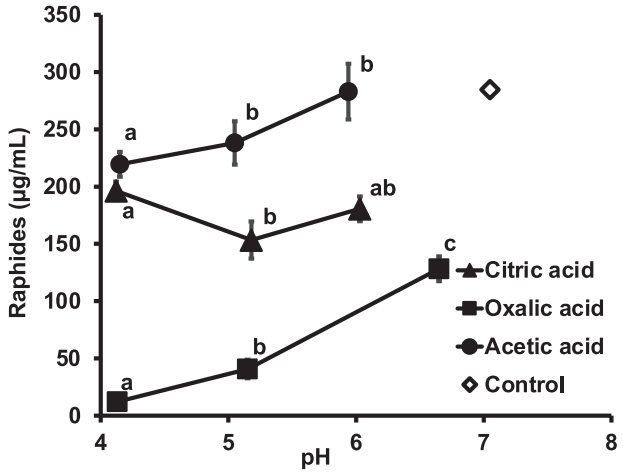

Figure 3. The effect of the pH on the raphides denaturing activity in the solutions of acetic acid, citric acid, and oxalic acid. pH values of aqueous sample solutions of acetic acid, citric acid, and oxalic acid (100 mM) were adjusted at 4.0, 5.0, and 6.0, respectively, using 1 mol/L NaOH. Each organic acid solution (2.0 mL) was mixed with low starch Pinellia Tuber suspension (LSPS, 0.50 mL) and H_2O (1.5 mL), then pre-incubated at 40°C for 30 min. The final pH value of each solution was measured, and raphides denaturation assay (RDA) was subsequently conducted. OD_{660} of the control group was 1.1 ± 0.0. Data are expressed as mean ± SD ($n = 3$). Different alphabetical letters (a–c) at the symbols indicate statistically significant differences at $P < 0.05$ among three groups of one organic acid sample evaluated by one-way ANOVA followed by Bonferroni's multiple comparison test.

Our previous study showed that the raphides denaturing activity of dried ginger decoction evaluated by microscopic observation did not appear at 4°C, and that the appearance of this activity required for more than 30 min incubation time length[15]. We confirmed the dependencies of temperature and incubation time length of oxalic acid on the raphides denaturation activity, and the results were consistent with the previous observations using dried ginger decoction.

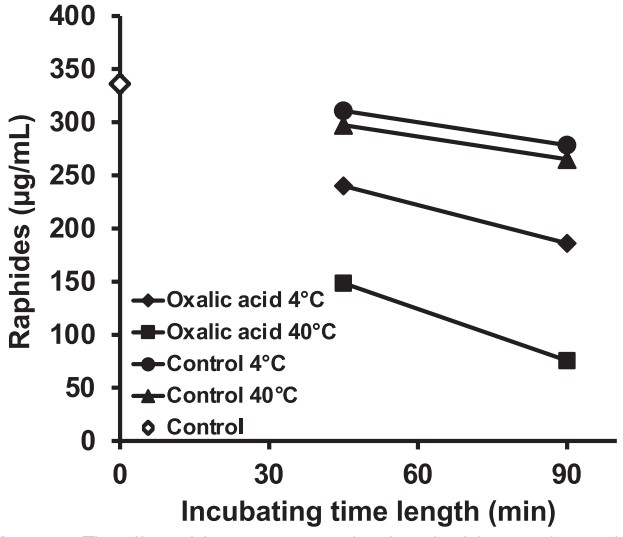

Figure 4. The effect of the temperature time length of the reaction on the raphides denaturation activity of oxalate. Oxalic acid aqueous solution (20 mM) that pH was adjusted at 5.0 using 1.0 M NaOH (2.0 mL) was mixed with low starch Pinellia Tuber suspension (LSPS, 0.50 mL) and H_2O (1.5 mL), and pre-incubated for 0, 45, or 90 min at 4°C or 40°C. Then, raphides denaturation assay (RDA) was conducted. Control is shown on *Y* axis. Data are expressed as mean ± SD ($n = 3$).

Oxalic acid in aqueous solution can be removed by adding calcium ion to precipitate it as calcium oxalate. In fact, the raphides denaturation activities of both oxalic acid and dried ginger decoction were suppressed by the addition of calcium chloride into the reaction mixture in a concentration dependent manner. We prepared the aqueous solution of 5.4 mM oxalic acid in 50 mM acetate buffer (pH 5.4), which concentration and pH were adjusted to those of the dried ginger decoction, and compared the suppressive profiles of the raphides denaturation activities by the addition of calcium chloride. Consequently, the profiles of oxalic acid aqueous solution were identical to those of dried ginger decoction, suggesting that oxalic acid mainly contributed to the raphides denaturing activity of the dried ginger extract.

The treatment of LSPS with oxalic acid exhibited not only the significant reduction of the raphides denaturing activity by RDA, but also the significant reduction of their acrid irritation taste in the gustatory test. Citric acid also significantly reduced the raphides denaturing activity, but this reduction seemed not to be enough to exhibit the significant improvement in the acrid irritation in gustatory test. Moreover, the purified PEX raphides from Pinellia Tuber were also denatured by oxalic acid. These results also support the hypothesis that the denaturation results from the direct interaction between raphides and oxalic acid, and that the main constituents to denature the raphides in dried ginger decoction would be oxalic acid.

In traditional Chinese medicine, the processing of raw Pinellia Tuber using ginger had first been seen in the period of the Northern and Southern dynasties. *Liu Juanzi's Ghost-Bequeathed Prescriptions* (Liú Juān Zǐ Guǐ Yí Fāng) published in 499 described that the raw tuber of *P. ternata* should be washed seven times with hot water, and then soaked in ginger juice for a half-day before use[12]. The concentration of soluble forms of oxalic acid in fresh ginger

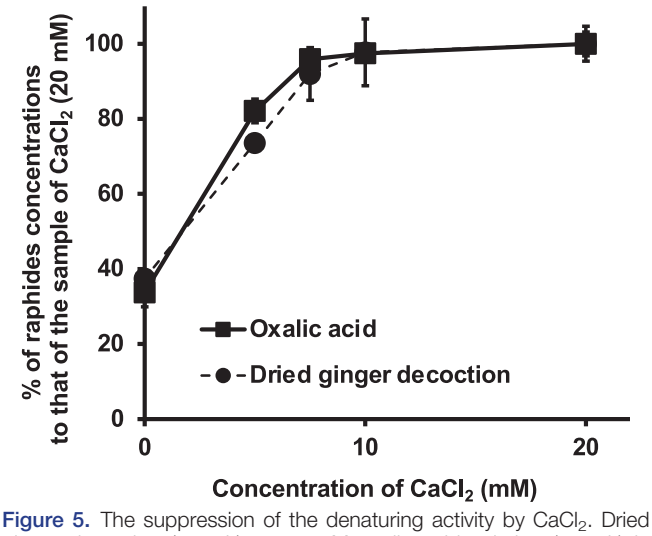

Figure 5. The suppression of the denaturing activity by $CaCl_2$. Dried ginger decoction (3.3 mL) or 5.4 mM oxalic acid solution (3.3 mL) in acetate buffer (0.06 M, pH 5.4) were mixed with 400 mM $CaCl_2$ aqueous solution (0.20 mL, 0.10 mL, 75 μL, and 50 μL), and H_2O were added to make the final volume of the mixture at 3.5 mL, then pre-incubated for 10 min at room temperature. Then, low starch Pinellia Tuber suspension (LSPS, 0.50 mL) was added, and incubated for 90 min at 40°C. Then, raphides denaturation assay (RDA) was conducted. The results of each sample were normalized against the mean value of raphide concentrations of the sample of $CaCl_2$ (20 mM).

Table 1

The gustatory test of the raphides treated with oxalic acid or citric acid

Sample	Raphides (μg/mL) on RDA	Gustatory test		
		+	±	−
Oxalic acid treated raphides	0.0 ± 0.0^{a}	−	1	2
Citric acid treated raphides	62.1 ± 5.4^{b}	3	−	−
Control (no treatment)	89.1 ± 2.7^{c}	3	−	−

Different alphabetical letters (a, b, and c) at the upper right of the numbers in the column of the concentrations of the raphides on raphides denaturation assay (RDA) indicate statistically significant differences at $P < 0.05$ among three groups of one organic acid sample evaluated by one-way ANOVA followed by Bonferroni's multiple comparison test. The numbers in the columns of gustatory test represent the number of subjects who report the following taste: +, acrid irritation on the oral mucosa was sensed as clear and intense; ±, acridity was sensed very slightly; −, no taste. Kruskal-Wallis test for the result of gustatory test indicated that there were significant differences among the groups ($P < 0.05$)

has been reported to be approximately 2.0×10^2 mg in 1.0×10^2 g fresh weight[19–20]. We prepared fresh ginger juice, and measured its pH value at 6.0. It took more than 90 min to completely denature the raphides in LSPS at 40°C in 25 mM oxalic acid solution (pH 5.0). Therefore, it would be reasonable to soak the raw tuber of *P. ternata* in ginger juice for such a relatively long time as a half-day described in *Liu Juanzi's Ghost-Bequeathed Prescriptions* to remove the acridity. On the other hand, it would be difficult to relieve the acrid irritative pain caused by Pinellia Tuber with drinking ginger juice, just a recommendation passed down as the legend[13]. Hence, time would not be enough for oxalic acid to interact with raphides in this case. Plants contain oxalic acid as the insoluble form of calcium oxalate and soluble forms of other oxalate salts, and the total contents and the balance between soluble and insoluble forms of oxalic acid usually vary in a wide range among plant species[19,21]. While the contents of soluble forms of oxalic acid have not been reported on many kinds of crude drugs yet, the reports on the spices or vegetables imply that ginger specifically contains a high proportion of soluble oxalate salts[19,22]. It might be one of the reasons why ginger had been chosen to process the raw tuber of *P. ternata* in ancient China.

The mechanism of the denaturation by oxalic acid still remains unclear yet. The optical microscopic observation showed no clear differences between the images of intact raphides and those denatured by oxalic acid (data not shown), suggesting that the denaturation by oxalic acid was unlikely to be accompanied by the morphological changes of the raphides crystals such as the aggregation or the decomposition. It had been reported that the raphides of Pinellia Tuber contained calcium oxalate and proteins[4,5,12]. In fact, we have isolated lipophilic protein from the raphides in our preliminary investigation, which could be related to the lipophilicity of the intact raphides as well as their acridity. Oxalic acid may interact with the protein to lose its lipophilicity, and the pH dependencies on the raphides denaturation activity could have resulted from the conformational change of the protein. Further investigations are needed.

In conclusion, we developed RDA to quantify the degree of the raphides' denaturation of Pinellia Tuber precisely, and the results of RDA were related to the degree of the acrid irritation. We found oxalic acid as the major raphides-denaturing ingredient in ginger decoction, and that oxalic acid denatures the raphides in thermo-, time-, and concentration-dependent manners. We provide scientific evidence in the meaning of the processing of Pinellia Tuber with ginger.

Conflict of interest statement

Toshiaki Makino received grant support from Tsumura & Co., Kracie Pharmaceuticals, and JPS Pharmaceuticals.

Funding

This study was sponsored by the grant from Oriental Medicine Research Foundation in 2020.

Author contributions

Tsukasa Fueki, Takao Namiki, and Toshiaki Makino designed the whole research. Koichiro Tanaka designed the clinical part of this research. Tsukasa Fueki and Itsuki Nose performed the experiments. Yan Liu investigated the history of Pinellia Tuber processing. Tsukasa Fueki wrote the draft article, and Toshiaki Makino finalized the article. All authors read and approved the final manuscript. All data were generated in-house and no paper mill was used. All authors agreed to be accountable for all aspects of the work and ensure its integrity and accuracy.

Ethical approval of studies and informed consent

The procedures were approved by the ethical committee in Toho University, School of Medicine with permission code #A19081. Written informed consent was obtained from all individual participants included in the study.

Acknowledgments

We are grateful for Oriental Medicine Research Foundation to support our research.

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How to cite this article: Fueki T, Nose I, Liu Y, Tanaka K, Namiki T, Makino T. Oxalic acid in ginger specifically denatures the acrid raphides in the unprocessed dried tuber of *Pinellia ternata*. Acupunct Herb Med 2022;2 (1):33–40. doi: 10.1097/HM9.0000000000000025