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Differences between the chemical constituents of tincture and *Arishta*, an ayurvedic herbal liquor prepared from ginger, long pepper, and jujube

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Abstract

In Ayurveda, practitioners use a herbal liquor called *Arishta* to treat patients suffering with emaciation or indigestion. *Arishta* is produced by decocting various herbs and then subjecting the resultant liquid to alcoholic fermentation. During the alcoholic fermentation stage, the constituents of *Arishta* change chemically, which would also alter the medicinal effects of *Arishta*. In order to characterize the components of *Arishta*, we investigated the differences between the compositions of *Arishta* and a tincture (an alcohol-soaked herbal liquor). First, we attempted to prepare *Arishta* in our laboratory from ginger, jujube, and long pepper and compared the piperine and [6]-gingerol contents of *Arishta* with those of the abovementioned tincture using HPLC. The alcohol content of the laboratory-made *Arishta* (LM-*Arishta*) was 15%, which was almost the same as that of the *Arishta* sold in Sri Lanka. The piperine content of the LM-*Arishta* was 14.6 mg/l, which was slightly higher than that of the tincture. It seems that decoction is more effective way to extract piperine than ethanol immersion. However, [6]-gingerol content of the LM-*Arishta* was 11.3 mg/l, which was less than 50% of that of the tincture. Subsequently, we incubated [6]-gingerol with yeast in culture medium to search [6]-gingerol metabolites. LC-MS/MS analysis suggested that [6]-gingerol is transformed to 4 metabolites such as [6]-shogaol and 3 or 5 acetoxy-[6]-gingerdiol during alcoholic fermentation. These metabolites were also detected in LM-*Arishta* and thus, it was suggested that [6]-gingerol was metabolized during LM-*Arishta* making.

Key words Ayurveda, Arishta, alcoholic fermentation, [6]-gingerol, [6]-shogaol.

Introduction

Ayurveda originated in India several thousands years ago, making it one of the world's oldest medical systems. Even today, Ayurveda continues to be practiced in South Asia including India and Sri Lanka, where about 80% of the population uses Ayurveda alone or combination with conventional (Western) medicine.[1)] As Ayurveda is the only medicinal system that aims to promote wellness, it has attracted worldwide attention as a form of complementary/alternative medicine. According to the 2007 National Health Interview Survey, more than 200,000 U.S. adults have used ayurvedic medicine.[1)]

In ayurvedic medicine, various types of medicine are used depending on each patient's individual requirements. For example, in addition to common forms of medicine such as herbal teas, infusions, decoctions, tinctures, and ointments, indigenous forms of medicine such as ash prepared from plant material (*Kshara*), infused oils (*Taila*), and herbal liquor (*Arishta* and *Asava*) are

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also employed in ayurvedic medicine.[2] *Arishta* and *Asava* are considered to be valuable therapeutics in ayurvedic medicine and are mainly used to treat patients suffering with indigestion or emaciation. *Arishta* is made by decocting herbs in boiling water and then subjecting the resultant mixture to alcoholic fermentation, while *Asava* is prepared by subjecting a mixture of fresh herbal juices to alcoholic fermentation.[3] There are various types of *Arishta* or *Asava* such as Ashokarishta, *Dasamoolarishtaya, Aravindasava,* and *Pippalyadiasava* that contain various crude drugs.

Traditional fermented liquors such as wine, *sake*, and beer have attracted a lot of attention in the medicinal field because they contain different functional nutrients than non-fermented food and drink. For instance, ethyl α-D-glucoside, which is produced during the brewing of *sake*, has a hepatoprotective effect and enhances the differentiation of keratinocytes.[4-6] In addition, Chari *et al*. reported that both beer and fermented glucose stimulated pancreatic enzymes and suggested that the active components of these substances are biosynthesized during the alcoholic fermentation process.[7] Therefore, components that arise during the fermentation process are considered to be responsible for the medicinal effects of *Asava* and *Arishta*. However, very few studies have examined the components of *Asava* or *Arishta*.

In a previous study, we compared the volatile compound profile of *Asava* with that of a tincture (an alcohol soaked herbal liquor) prepared from ginger and jujube using GC-MS. The volatile compound profile of *Asava* differed from that of the tincture, and it was suggested that glycosides were hydrolyzed to volatile compounds during the fermentation process.[8] However, no comparison between *Arishta* and herbal tinctures has been performed to date, and the bioconversion of the constituents of *Arishta* during its fermentation remains to be examined.

In this study, in order to characterize the components of *Arishta* we elucidated the differences between the components of *Arishta* and those of a tincture prepared from ginger, jujube, and long pepper. We adopted these herbs because they were described as ingredients of *Arishta* in Sushruta Samhita, one of bibles of Ayurveda. First, we investigated the preparing procedure of *Arishta* generally adopt in Sri Lanka. Then, based on the results of our field investigation, we attempted to prepare *Arishta* in our laboratory. Subsequently, we compared the piperine and [6]-gingerol contents of laboratory-made *Arishta* (LM-*Arishta*) with those of a tincture prepared from the same materials. Moreover, we attempted to identify the [6]-gingerol metabolites produced during fermentation with yeast.

Materials and Methods

Crude drug materials: The following crude drugs were used in this study:

Small pieces (3-10 mm) of ginger (dried rhizomes of *Zingiber officinale* Rosc., lot No.: 78J1112) and Jujube (dried fruits of *Zizyphus jujuba* Mill. var. *inermis* Rehd., lot No.: 7901152) were purchased from Uchida Wakanyaku Co, Ltd. (Tokyo, Japan). These crude drugs were compliant with JP 15. Long pepper (dried fruits of *Piper longum* Linn., lot No.: VVCMQ), Maritta-maru (dried flower of *Woodfordia floribunda* Salisb. (= *W. fruticosa* Kurz.), Voucher No.: KANP06756), and brown sugar were purchased from a local market. These materials were identified by the authors and stored in the laboratory of Herbal Medicines and Natural Resources, School of Pharmacy, Kanazawa University.

Dried yeast: Dried yeast (*Saccharomyces cerevisiae* UCD522, Lot No.: 962112) purchased from Mauri Yeast Australia Pty Ltd (NSW, Australia) was used to prepare LM-*Arishta*. The yeast strain (*Saccharomyces cerevisiae* Kagoshima-5) supplied by the Kagoshima prefectural brewing association (Kagoshima, Japan) was used for the study of [6]-gingerol metabolites.

Chemicals: Acetic acid, bromothymol blue, Ethanol, formic acid, [6]-gingerol, glucose, methanol, neutral red, piperine, potassium hydrogen phthalate, and yeast extract were purchased from Nacalai Tesque (Kyoto, Japan). Acetonitrile and sodium hydroxide were purchased from Kanto chemical Co. Inc. (Tokyo, Japan). Phenolphthalein was purchased from Kishida Chemical Co. Ltd. (Tokyo, Japan). Peptone was purchased from Becton, Dickinson and Company (New Jersey, USA).

Preparation of *Arishta:* LM-*Arishta* was prepared in

accordance with the procedure used by Pilapitiya Ayurveda Products (Fig. 1), with minor modifications.

1. Ginger (40 g), jujube (40 g), long pepper (40 g), and distilled water (920 ml) were mixed, boiled, and concentrated to 230 ml.
2. After subjecting the resultant liquid to filtration through a mesh, brown sugar (90.7 g) was added to the decoction, which was then cooled to room temperature.
3. Maritta-maru (20 g) and dried yeast (0.1 g) were added to the decoction.
4. The decoction was incubated at 25 C for 15 days in an incubator (LPH 200 - RDSMP, Nippon Medical & Chemical Instruments CO., LTD., Osaka, Japan).

The preparation of LM-*Arishta* was repeated three times, and analytical data were obtained from three separate experiments.

Preparation of the tincture: Ginger (40 g), jujube (40 g), and long pepper (40 g) were soaked in 14.0% (v/v) ethanol (230 ml), and then the mixture was stored at 25 ℃ for 15 days in an incubator.

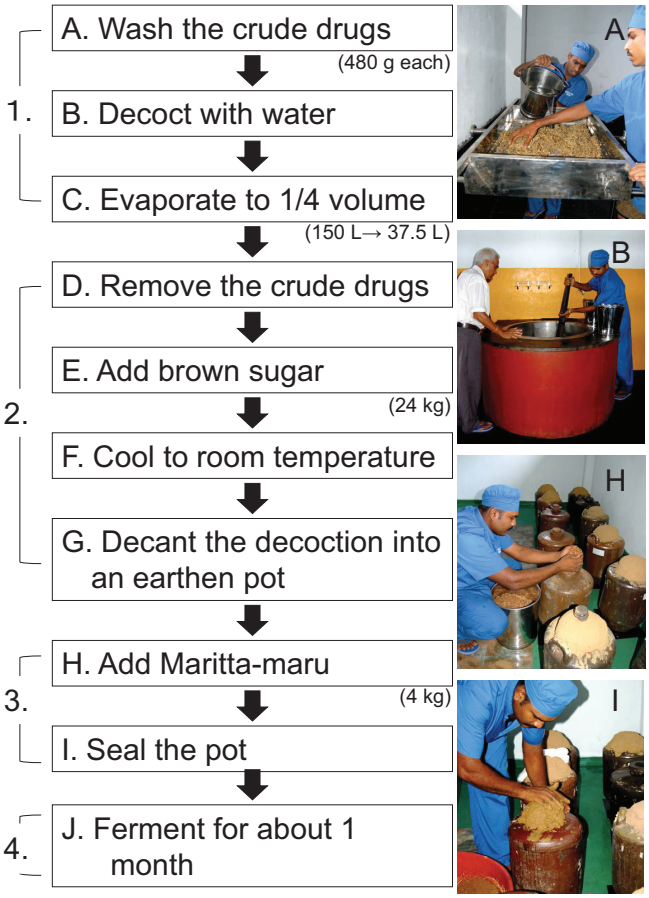

Fig. 1 The preparing procedure of *Arishta* generally adopt in Sri Lanka.

Examination of sugar content and pH: We measured sugar content using a Pocket PAL-1 refractometer (measurement range: Brix 0.0-43.0%, resolution: Brix 0.1%, measurement accuracy: Brix 0.2%, measurement temperature: 10℃-60℃) and monitored pH using a Horiba B211 twin pH meter on days 0, 1, 2, 3, 4, 5, 10, and 15.

Examination of alcohol content: We measured alcohol content according to the method described by the National Tax Agency of Japan.[9)]

1. An aliquot of 100 ml of the LM-*Arishta* or tincture was directly distilled until 70 ml of the distilled liquid had been collected.
2. Distilled water was added to the distilled liquid to give a total volume of 100 ml.
3. The distilled liquid was poured into a glass cylinder, and an alcohol meter (range: 0 - 30, minimum value: 1; Yokota Seisakusho, Tokyo, Japan) was floated on the surface of the distilled liquid.

Quantification of piperine and [6]-gingerol: LM-*Arishta* and tincture (1 ml) were filtered through a 0.45 μm cellulose acetate membrane filter, and 10 μl of each sample were injected into a HPLC system (L-2130, Hitachi, Tokyo, Japan). The separation was performed with an Inertsil HPLC column (4.6 mm id×250 mm, GL Sciences Inc., Tokyo, Japan). The elution was performed with 0.5% (v/v) acetic acid : acetonitrile (61 : 39), and the chromatography was performed at room temperature at a flow rate of 1 ml/min. An ultraviolet (UV) detector (L-2400, Hitachi, Tokyo, Japan) was used for the UV detection at 280 nm.

Analysis of [6]-gingerol metabolites using *Saccharomyces cerevisiae*:

~Culturing of *Saccharomyces cerevisiae* in the presence of [6]-gingerol~

Yeast extract peptone dextrose (YPD) medium (2% glucose, 2% peptone, and 1% yeast extract) was used as a basal medium, and 20 ppm of [6]-gingerol was added. *S. cerevisiae* was added to a test tube containing 10 ml of sterilized medium and cultured for 3 days at 30℃.

YPD medium containing [6]-gingerol but not *S. cerevisiae* was used as a control.

~HPLC-MS/MS conditions~

The chromatographic analysis was performed with a Shimadzu HPLC system containing a Shimadzu autosampler (SOL-20ACHT), a binary pump (LC-20AD), a column oven (CTO-20A), and a UV/visible spectrophotometer (SPD-20A) (Shimadzu, Kyoto, Japan). The system was interfaced with a 3200QTrap™ LC-MS/MS system (Absciex, CA, USA) using a Turbo VTM interface equipped with a heated nebulizer and turbo ion spray probes. The separation was performed with a Waters Cosmosil column 5C18-MS Ⅱ (4.6 mm id×150 mm, Nacalai Tesque, Kyoto, Japan). Gradient elution was performed with a mobile phase of 0.2% (v/v) formic acid (A) and acetonitrile (B) under the following conditions: 0 min, 40% solvent B; 5 min, 40% solvent B; 35 min, 100% solvent B; 40 min, 100% solvent B; and 52 min, 40% solvent B. The chromatography was performed at 40℃ at a flow rate of 0.3 ml/min. The injection volume was 10 μl, and the autosampler was thermostated at 4℃. The interface and instrumental parameters were set as follows: positive polarity, ion source mode: turbo ion spray mode; curtain gas pressure: 10 psi, ion source gas pressure: 30 psi, turbo gas pressure: 70 psi, desolvation temperature: 700℃, collision activated dissociation gas: 3.0 a.u., and ion spray voltage: 5500 V. Precursor ion (PI) scans (product of m/z; 137.3) was performed to detect the [6]-gingerol metabolites and enhanced product ion (EPI) scans were performed to obtain their MS/MS spectra. To detect these metabolites in LM-*Arishta*, multiple reaction monitoring (MRM) was performed (Q1 filtering were 295, 277, 298, and 339, Q3 filtering was 137).

Results and Discussion

In this study, we investigated the differences between the components of LM-*Arishta* (a fermented herbal liquor) and a tincture derived from the same ingredients (an alcohol-soaked herbal liquor). First, we investigated the preparing procedure of *Arishta* at a factory that produces ayurvedic medicines (Pilapitiya Ayurveda Products, Kesbewa, Piliyandala, Sri Lanka) from 4th to 17th December 2006. As shown in steps E and H of Figure 1, considerable amounts of sugar and Maritta-maru were added to the mash to avoid contamination and accelerate fermentation in the factory. Subsequently, we attempted to prepare *Arishta* in our laboratory using the same protocol; however, the mash did not ferment. As we had successfully prepared *Asava* according to the protocol practiced in Sri Lanka in a previous study, it seems that *Arishta* is more easily contaminated by airborne bacteria than *Asava* due to its complicated production process. In *Arishta* preparation, the mash was cooled to room temperature after the addition of brown sugar, as shown in step F of Figure 1 and exposed to airborne bacteria for a prolonged period. Although these conditions were amenable to bacterial contamination, the alcoholic fermentation of *Arishta* was successful at the Sri Lankan factory. It seems that alcoholic fermentation yeast lives as the predominant microorganism at the factory during the production of *Arishta* for years. Thus, it was necessary to modify the procedure we used to produce *Arishta* at our laboratory in order to promote the alcoholic fermentation without contamination.

We added dried yeast to the mash during the initial step to accelerate fermentation and avoid bacterial contamination. As a result, the alcohol content of the mash reached 15.2%, and gas formation was observed. The sugar content of the mash decreased to 20% (w/v), and its pH shifted to 4.3 within 10 days and had almost plateaued by 10-15 days (Fig. 2). The sugar content, pH,

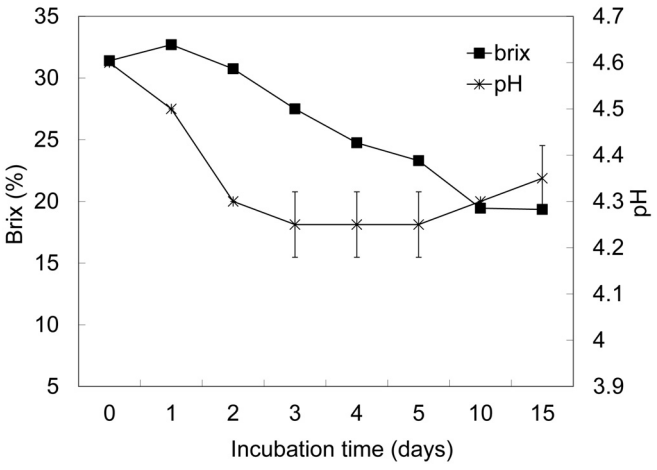

Fig. 2 The time course of the Brix and pH values of the LM-*Arishta*.
LM-*Arishta* was prepared by the addition of dried yeast and incubated at 25℃ for 15 days. The bars indicate standard deviation values obtained from 3 independent experiments.

and alcohol content of the mash were almost equivalent to those of the commercial *Arishta* by day 15.[10] Thus, we employed the LM-*Arishta* preparation method described in the Materials and method section to prepare *Arishta* in our laboratory.

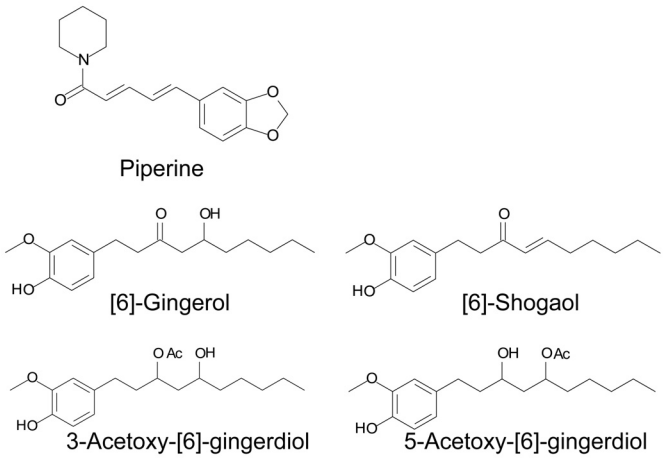

Fig. 3 The chemical structures of piperine and [6]-gingerol-related compounds.

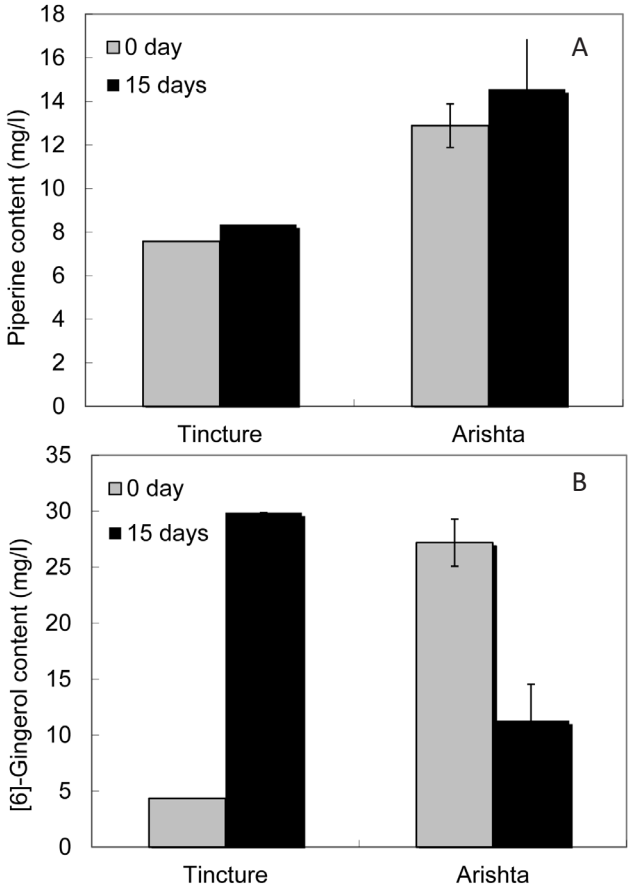

Fig. 4 The differences in the piperine (A) and [6]-gingerol (B) contents of LM-*Arishta* and the tincture.
The contents of piperine and [6]-gingerol were quantitated after 0 and 15 days by HPLC. The bars indicate standard deviation values obtained from 3 independent experiments.

The piperine and [6]-gingerol contents of LM-*Arishta* were compared with those of a tincture prepared from same amounts of herbs and sugar. In *Arishta* making, the herbal decoction was used as initial mash and thus, these contents in LM-*Arishta* at 0 day in Figure 4 correspond to those in decoction. As shown in Figure 4-A, the piperine content of LM-*Arishta* was higher than that of the tincture, and the piperine contents of both products increased slightly during the production process. It was reported that reflux extraction was highly effective at recovering piperine from peppercorn materials.[11] It seems that hot water is more effective at extracting piperine than ethanol. Piperine, the principle pungent compound of long pepper, was recently shown to some medicinal properties.[12,13] Therefore, *Arishta*, which was found to contain more piperine than the tincture, might have different effects from those of tincture.

Figure 4-B shows the [6]-gingerol contents of LM-*Arishta* and the tincture. The [6]-gingerol content of the tincture increased during the immersion period; however, the [6]-gingerol content of LM-*Arishta* significantly decreased during fermentation and was less than 50% of that of the tincture. To investigate the effect yeast has on [6]-gingerol content, we incubated [6]-gingerol with yeast in culture medium and quantified [6]-gingerol in the medium. As shown in Figure 5, the

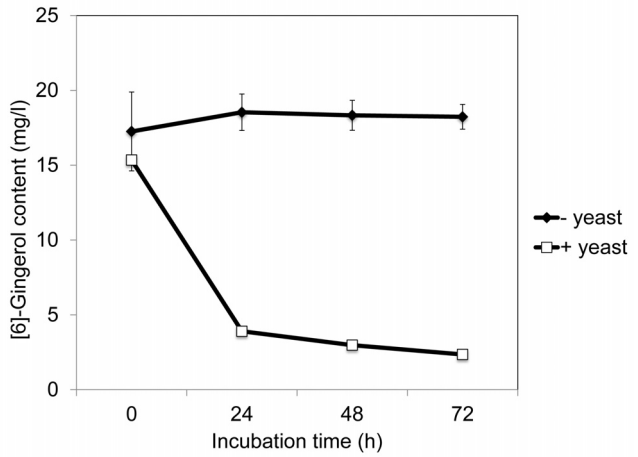

Fig. 5 Degradation of [6]-gingerol by incubation with *Saccharomyces cerevisiae.*
[6]-Gingerol was incubated in YPD medium with or without *Saccharomyces cerevisiae* for up to 72 hr. The content of [6]-gingerol was assessed after 0, 24, 48, and 72 h using HPLC. The bars indicate standard deviation values obtained from 3 independent experiments.

amount of [6]-gingerol remained stable when [6]-gingerol was incubated without yeast, whereas in the presence of yeast the amount of [6]-gingerol decreased significantly from 24 h, and gas formation was also observed. Therefore, it was suggested that [6]-gingerol is metabolized by yeast during alcoholic fermentation.

Since it was suggested that [6]-gingerol is metabolized to other compounds, we attempted to identify the metabolites of [6]-gingerol using LC-MS/MS. First, PI scans were performed. In PI mode, MS was only performed when the detected compound had a specific product ion. As metabolites share structures with their parent compounds, they often have the same product ions as their parent compounds. Therefore, PI scans are an effective way of searching for specific metabolites.[14] We set the target product ion mass at m/z 137 after analyzing a [6]-gingerol standard. As a result, fermented medium contained four metabolites in addition to [6]-gingerol. The Precursor ions of metabolite 1 (retention time; 18.81 min), metabolite 2 (19.91), metabolite 3 (26.11), and metabolite 4 (27.16) were m/z 298, m/z 298, m/z 339, and m/z 277, respectively. Figure 6 shows HPLC-UV chromatograms of the [6]-gingerol-containing medium obtained at 0 and 48 h. As shown in Figure 6-B, four metabolites were found to be produced as the [6]-gingerol content of the medium decreased. Subsequently, we acquired MS/MS spectra using the EPI mode to estimate these compounds. In EPI scans, MS/MS spectra were only acquired when the relevant precursor ions were detected.[14] We compared the acquired MS/MS spectra with those reported in related articles and predicted the metabolites we had detected. The combination of PI and EPI analysis demonstrated that 3 or 5-acetoxy-[6]-gingerdiol and [6]-shogaol were candidates of metabolite 3 and 4, respectively (Fig. 7).[15] It was reported that heating, storage, and processing of ginger converted [6]-gingerol to 3 or 5-acetoxy-[6]-gingerdiol or [6]-Shogaol.[16] In addition, it was recently shown that [6]-gingerol was converted to [6]-shogaol when ginger was incubated with particular brewing microorganisms.[17] Both [6]-gingerol and [6]-shogaol are pungent compounds, but [6]-shogaol has been reported to have different medicinal property from [6]-gingerol.[18-21] Although, there are no reports about pharmacological activity of 3 or 5-acetoxy-[6]-gingerdiol, it would have different medicinal property from [6]-gingerol due to acetoxylation.

We attempted to analyze these [6]-gingerol metabolites in LM-*Arishta*. Unfortunately, they were not be detected because of impurities by HPLC-UV, thus, we

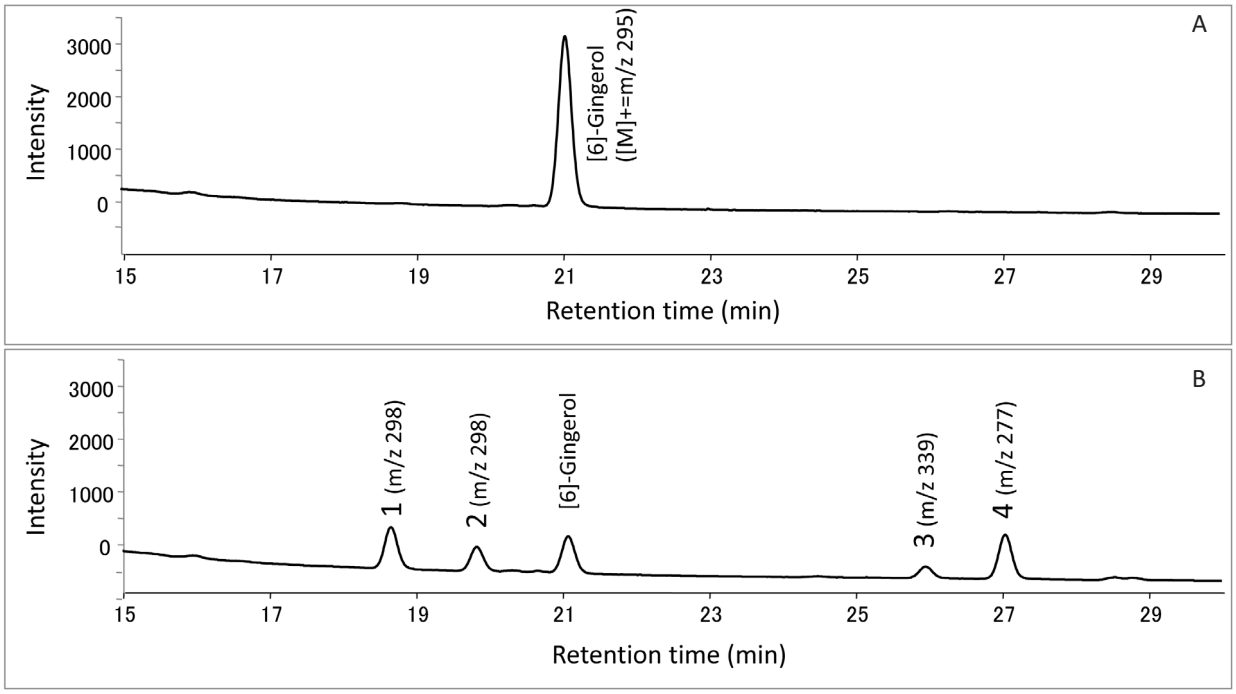

Fig. 6 HPLC-UV chromatograms of YPD medium containing [6]-gingerol.
A and B indicate the chromatograms obtained at 0 and 48 h, respectively.

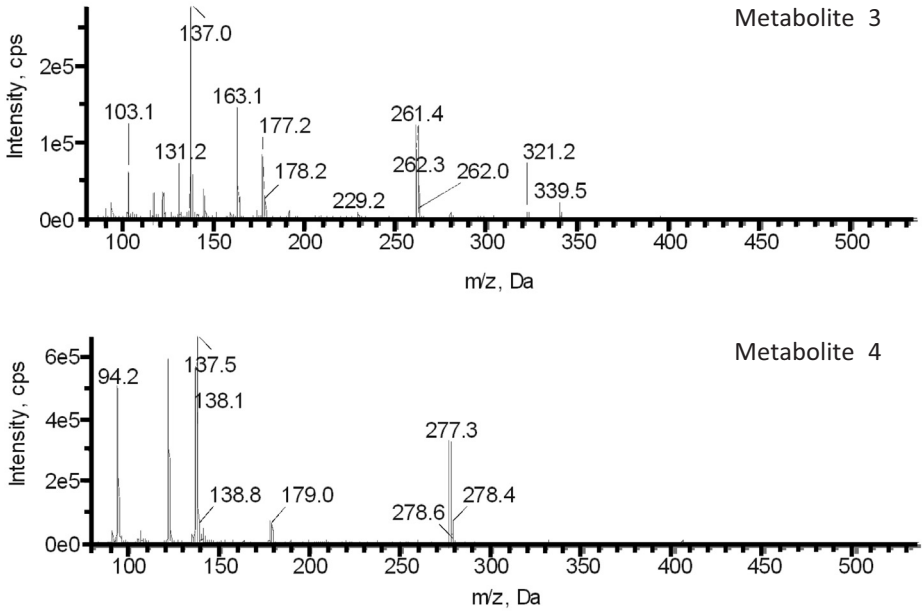

Fig. 7 LC-ESI-MS/MS spectra of the [6]-gingerol metabolites produced by *Saccharomyces cerevisiae*. Metabolites 3 and 4 were prospected 3 or 5 acetoxy-[6]-gingerdiol and [6]-shogaol, respectively.

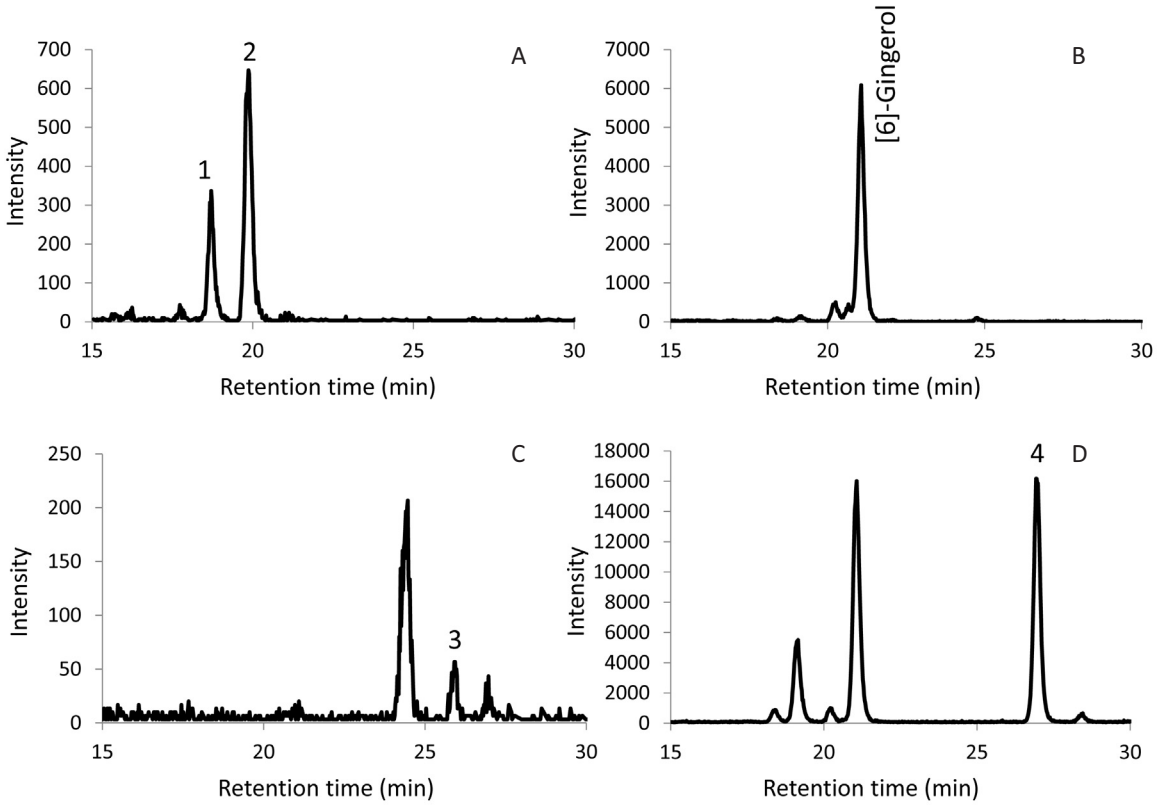

Fig. 8 MRM chromatograms of [6]-gingerol metabolites in LM-*Arishta*. Extracted ion (Q1/Q3) of A, B, C, and D were 298/137, 295/137, 339/137, and 277/137, respectively.

used LC-MS/MS with MRM that can detect exclusive compounds by setting specific mass ion for each compounds. Consequently, all four metabolites were also detected in LM-*Arishta* (Fig. 8). Therefore, these gingerol metabolites shown in this study would effect to the medicinal property of LM-*Arishta*. In the future, it would be useful to identify these compounds by structure analysis using NMR and compare their medicinal properties.

In Japan, herbal liquors are often consumed by

elderly individuals with indigestion. Due to the trend towards personalized medicine, aging societies such as Japan require different types of herbal liquor that have different effects and tastes from tinctures. However, tinctures, in which all of the constituents are uniformly extracted, are the only type of herbal liquor that is currently available in Japan. In the present study, we found evidence that some active constituents of herbs are enriched or depleted during the production of *Arishta*. If *Arishta* became available in Japan as a new type of herbal liquor, then patients would be able to choose herbal liquors that suited their symptoms. In Japan, a wide variety of fermented foods have been manufactured since ancient times; therefore, Japanese fermentation technology is well developed. Thus, it would be possible to develop Japanized *Arishta* containing Japanese herbs and brewing microorganisms.

Conclusion

In the present study, we revealed the differences between the chemical constituents of LM-*Arishta* and a tincture prepared from ginger, jujube, and long pepper. The piperine content of LM-*Arishta* was higher than that of the tincture, but its [6]-gingerol content was less than 50% of that of the tincture. LC-MS/MS analysis of [6]-gingerol-containing yeast culture medium and LM-*Arishta* suggested that [6]-gingerol is transformed to four metabolites such as [6]-shogaol and 3 or 5 acetoxy-[6]-gingerdiol during alcoholic fermentation. Due to the differences in their piperine and [6]-gingerol contents, LM-*Arishta* and tinctures are expected to have different medicinal properties.

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