Detecting phytoestrogens and mammalian estrogens in blackcurrants (Ribes nigrum L.)

Naoki Nanashima

n_nanashima@auhw.ac.jp

Aomori University of Health and Welfare: Aomori Kenritsu Hoken Daigaku https://orcid.org/0000-0002-4729-3432

Kayo Horie

Hirosaki University: Hirosaki Daigaku

Hayato Maeda

Hirosaki University: Hirosaki Daigaku

Yoji Kato

Hirosaki University: Hirosaki Daigaku

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Abstract

Phytoestrogens are estrogen-like molecules found in various plants. Menopausal women experience various menopausal symptoms due to reduced estrogen secretion. The ingestion of phytoestrogens is known to alleviate these symptoms. Blackcurrant (*Ribes nigrum* L.) is a fruit rich in phytochemicals, including anthocyanins. Blackcurrant extract (BCE) and anthocyanins have phytoestrogenic effects. Although BCE has stronger phytoestrogenic activity than blackcurrant anthocyanins, this aspect has not been explored. BCE likely contains phytoestrogens other than anthocyanins. In this study, the components of blackcurrant fruits were comprehensively investigated through metabolome analysis. In addition to phytoestrogens, such as naringenin, daizein, and resveratrol, mammalian estrogens, such as estrone and β-estradiol, were detected in blackcurrant fruits. An ultrasensitive estrogen measurement analysis revealed that BCE contains approximately 40.2 and 24.5 pg/g of estrone and β-estradiol, respectively, corresponding to approximately 100 times the estrogen content in blackcurrant fruits. We report for the first time that blackcurrant contains phytoestrogens and mammalian estrogens.

Introduction

Estrogen is a steroid hormone and is of three types: estrone, β-estradiol, and estriol. Among these, β-estradiol is the most active (Denver et al. 2019). Women experience reduced estrogen levels during menopause, aggravating various menopausal symptoms, such as hot flashes and osteoporosis (Sassarini and Lumsden 2015). Many plants contain phytoestrogens, such as isoflavones and resveratrol, which function similarly to estrogen, and their intake is known to reduce menopausal symptoms (Patra et al. 2023). In addition, mammalian estrogens have been detected in plants (Janeczko 2021; Janeczko and Skoczowski 2005).

Blackcurrant (*Ribes nigrum* L.) is a small fruit grown in cool regions, such as Poland, and is known to improve vision and blood flow (Cortez and Gonzalez de Mejia 2019). In addition, blackcurrant extract (BCE) and four anthocyanins exhibit phytoestrogenic activity (Nanashima et al. 2017; Nanashima et al. 2015). Our previous research indicated that BCE alleviates menopausal symptoms in menopausal model rats (Horie et al. 2021; Nanashima et al. 2020). Furthermore, it was found that the phytoestrogen effect of blackcurrant is stronger with BCE than with anthocyanin alone (Nanashima et al. 2015); however, it is challenging to determine the cause. Therefore, we hypothesized that BCE contains phytoestrogens other than anthocyanins.

Metabolome analysis is a tool for comprehensively identifying various molecules in serums, urine, and food. In this study, we investigated the phytoestrogens and estrogen-like molecules other than anthocyanins in BCE using a metabolomic analysis and quantified them using ultrasensitive estrogen measurement analysis.

Materials and Methods

Materials

The Tsema and Aomori cultivars were collected at Fujisaki Farm, Faculty of Agriculture and Life Science, Hirosaki University. The blueberries were collected from YoKa Food Science Laboratory Inc. (Hirosaki, Japan). The blackcurrant extract (BCE) powder, i.e., CaNZac-35, was purchased from Koyo Mercantile Co. (Tokyo, Japan). BCE contains high concentrations of anthocyanins (approximately 38%) (Nanashima et al. 2015).

Metabolome analysis: capillary electrophoresis (CE)-time-of-flight mass spectrometry (TOFMS) measurement

All metabolome analyses were performed at Human Metabolome Technologies (HMT; Tsuruoka, Japan). The blackcurrant fruit sample was added to a methanol solution containing an internal standard substance and crushed under cooling. Subsequently, chloroform and Milli-Q water were added, followed by stirring and centrifugation (2300 × g, 4°C, 5 min). After the centrifugation, the aqueous layer was transferred to an ultrafiltration tube (Ultrafree MC-PLHCC, HMT, centrifugal filter unit, 5 kDa) and centrifuged (9,100 × g, 4°C, 120 min). The filtrate was dried, dissolved in Milli-Q water, and used for measurement. CE-TOFMS measurements in the cation and anion modes were performed under the conditions presented in Table S1.

Metabolome analysis: liquid chromatography (LC)-TOFMS measurement

The blackcurrant fruit was crushed at 4°C in a 1% formic acid-acetonitrile solution containing 20 μM of the internal standard substance. Next, Milli-Q water was added, and the mixture was further crushed. Centrifugation was performed (2300 × g, 4°C, 5 min), and the supernatant was collected. Further, 1% formic acid-acetonitrile and Milli-Q water were added to the precipitate and stirred. After centrifugation, the supernatants were collected and mixed, and the mixture was subjected to ultrafiltration using an ultrafiltration tube (Nanosep 3 K Omega; Pall, Port Washington, NY, USA). The phospholipids were removed via solid-phase extraction. The filtrate was dried and dissolved in 50% isopropanol aqueous solution (v/v) for measurement. Positive- and negative-mode measurements were performed under the conditions presented in Table S2.

Data processing

The detected peaks were automatically extracted using the automatic integration software MasterHands ver. 2.17.1.11 (developed by Keio University (Sugimoto et al. 2010)). Peaks with a signal/noise ratio of ≥ 3 were automatically extracted. The mass-to-charge ratio (*m/z*), peak area values, migration time (MT) for CE-TOFMS, and retention time (RT) for LC-TOFMS were recorded. The peak areas were converted into relative areas using the following formula: relative area = area of target peak/area of internal standard or sample

Candidate metabolite search

The detected peaks were compared and searched against all substances registered in the HMT metabolite library and the known–unknown library according to *m/z* and MT or RT values. The tolerances for the search were ± 0.5 min for MT, ± 0.3 min for RT, ± 10 ppm (CE-TOFMS), and ± 25 ppm (LC-TOFMS) for *m/z*.

Ultrasensitive estrogen quantitative analysis

All ultrasensitive estrogen quantitative analyses were performed using liquid chromatography–mass spectrometry (LC-MS/MS) at ASKA Pharma Medical Co., Ltd. (Fujisawa, Japan) (Ishihara et al. 2017).

Result and Discussion

Phytoestrogens have been detected in various plants, e.g., naringenin and daidzein in soybeans and resveratrol in grape skins (Lephart 2021). However, there have been no reports on detecting these phytoestrogens in blackcurrants. In this study, Tsema and Aomori cultivars were examined for the two varieties of blackcurrants via metabolome analysis. Naringenin and resveratrol were detected in both varieties as a molecule known as phytoestrogen (Lephart 2021), and daidzein was detected in Aomori (Fig. 1) alone. Estriol and β-estradiol, along with the primary metabolite of β-estradiol, i.e., estrone-3-glucuronide, were detected in both varieties (Fig. 1). We attempted to quantify these phytoestrogens using high-performance liquid chromatography but were unable to detect them below the detection limit (data not shown). Hence, in the future, they must be quantified using a highly sensitive method. The results also suggested that the type of phytoestrogen depends on the variety of blackcurrant. Furthermore, we compared blueberries and blackcurrant fruits. Naringenin and β-estradiol were detected in blackcurrants and blueberries; however, β-estradiol 17-glucuronide—a metabolic product of β-estradiol—was detected only in blackcurrants (Fig. S1).

Mammalian estrogen has been detected in plants (Janeczko 2021; Janeczko and Skoczowski 2005), but its presence in blackcurrants has not been reported. We quantified samples of the blackcurrant fruits and BCE using the ultrasensitive estrogen measurement method. The fruits and BCE could quantify estrone and β-estradiol. The samples were more concentrated in the BCE than in the fruit; 40.2 pg/g dry weight (D.W.) of estrone—approximately 100 times higher than the concentration in the fruit—and 24.5 pg/g D.W. of β-estradiol were detected (Table 1) in the BCE.

The blackcurrant estrogen concentration is lower than the estrogen concentration in other plants reported to date (Janeczko 2021; Janeczko and Skoczowski 2005); however, our research indicated BCE estrogen activity. Molecules other than anthocyanins have also been claimed to exhibit estrogen-like effects. Furthermore, pollens of kiwifruit contain relatively high concentrations of β-estradiol (Janeczko 2021). Because the concentration is expected to vary depending on the part or variety of the blackcurrant, it is necessary to investigate each part and variety.

Conclusions

In summary, blackcurrant anthocyanins exhibited phytoestrogenic activity; however, we could not detect the presence of molecules that show estrogen activity in addition to anthocyanins. This study revealed that in addition to phytoestrogens, blackcurrants contain mammalian estrogens.

Declarations

Ethics Approval

Not applicable

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

N.N., K.H., H.M., and Y.K. designed the study; N.N., K.H., and Y.K. performed the experiments and analyzed the data; N.N. and H.M. wrote the original manuscript. All authors have reviewed the final manuscript and approved it for publication.

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Data Availability

The data generated in this study are available from the corresponding author upon request.

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Tables

Table 1. Quantification of estrogens

Sample	Estrone	β-estradiol
Whole fruit (pg/g F.W.)	0.55±0.2	0.25±0.1
BCE powder (pg/g D.W.)	40.2±5.1	24.5±2.0

Data represent the mean± standard deviation of triplicate experiments.

F.W.-fresh weight; D.W.-dry weight

Figures

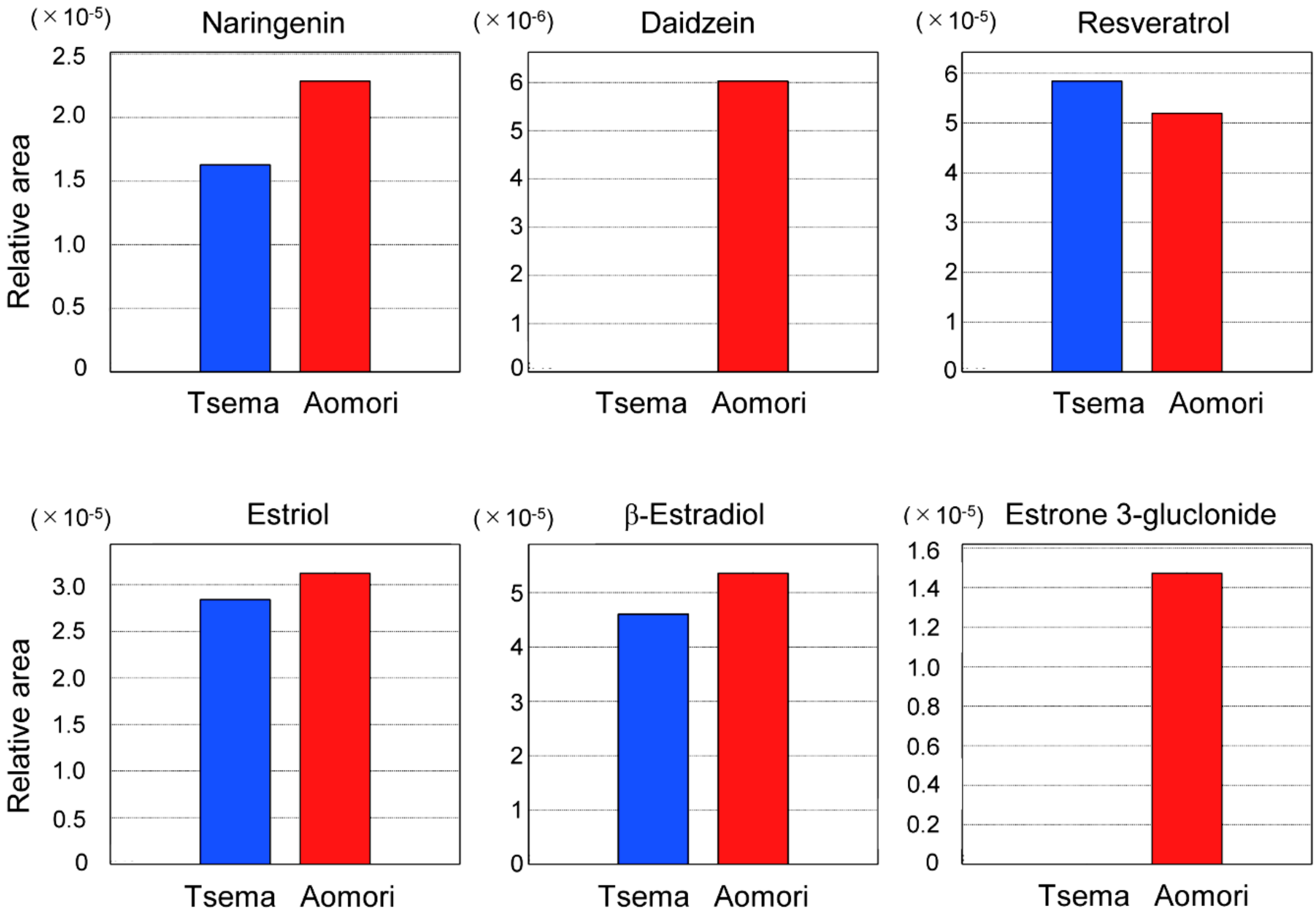

Figure 1

Metabolome analysis of blackcurrants. The bars indicate the relative area of each molecule in Tsema and Aomori. The steps for calculating the relative area are presented in the Supplementary Information.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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