Anti-stress and Antioxidant Effects of Non-Centrifuged Cane Sugar, Kokuto, in Restraint-Stressed Mice

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Abstract: Stress is a part of everyday life, but excessive stress can be related to diverse diseases. Recently, oral intake of a non-centrifuged cane sugar, Kokuto, was reported to produce potential anti-stress effects in humans. However, the molecular components associated with the anti-stress property of Kokuto remain mostly unknown. Therefore, we focused on the non-sugar component (NSC) fractions of Kokuto, and investigated how serum corticosterone level (used as a stress marker) and antioxidant activity were affected in restraint-stressed mice treated with NSC fractions obtained from the elusion on HP-20 resin with 25%, 50%, 75%, and 100% aqueous methanol (MeOH) solutions. Among the four NSC fractions, the 50% MeOH fraction showed a high content of phenolic compounds and high antioxidant activity. Moreover, oral administration of the 50% MeOH fraction suppressed both corticosterone secretion into the serum and reduction of antioxidant activity in serum and liver in restraint-stressed mice. Component analysis of the 50% MeOH fraction identified five antioxidative phenolic compounds: p-hydroxybenzaldehyde, p-hydroxyacetophenone, schaftoside, isoschaftoside, and p-coumaric acid. Phenolic compounds detected in the NSC fractions of Kokuto might contribute to the anti-stress property of Kokuto. In addition, this research provides more understanding of potential health benefits offered by the constituents of Kokuto.

Key words: non-centrifuged cane sugar, serum corticosterone, antioxidant activity, p-coumaric acid, p-hydroxybenzaldehyde

1 INTRODUCTION

People today are increasingly facing stressful situations in their daily life. Stress disrupts the normal physiological condition, which results in a state of threatened homeostasis, and excessive stress can be related to the pathogenesis of diverse diseases such as depression, high blood pressure, and immunosuppression-related disorders¹⁻³. Moreover, stress increases the energy requirement of organisms, and thus an elevated amount of reactive oxygen species (ROS) is generated during stress. ROS generation can trigger protein carbonylation, DNA damage, and lipid peroxidation and thereby cause a range of chronic health problems, such as aging, cancer, Alzheimer’s disease, and Parkinson’s disease⁴⁻⁵. Stress-induced responses can be suppressed or even prevented using effective anti-stress agents derived from plants such as Bacopa monniera and Ginkgo biloba, and the use of dietary antioxidants holds the potential to become a key strategy for maintaining a healthy life⁶⁻⁷.

Non-centrifuged cane sugar is produced from sugarcane (Saccharum officinarum L.) juice and is widely recognized worldwide under different names such as Panela (Latin America), Jaggery (South Asia), and Kokuto (Japan). In previous work, Kokuto, which contains various components such as phenolic compounds and Maillard reaction products⁸⁻¹⁰, has been shown to exert functional effects such as anti-cariogenic, antitoxic, cytoprotective, anticarcinogenic, and antioxidant effects¹⁰⁻¹¹. Focusing on the potential anti-stress property of Kokuto, Takahashi et al.¹² investigated the effects of oral Kokuto intake on...
mental stress: before and after subjecting study participants to a stress task, salivary stress markers were measured and the participants’ mental state was rated subjectively. When the participants consumed Kokuto before the stress task, the secretion of salivary stress markers such as chromogranin A and α-amylase was suppressed; by contrast, Kokuto consumption after the stress task produced no such effect. Takahashi et al. further suggested that the suppressive effects of Kokuto might be related to mood improvement. Initially, the sugars in Kokuto were considered to be related to the observed suppressive effects. The anti-stress effect of sugars on the participants’ performance appeared to be based on the rate and extent of the postprandial rise and fall in blood glucose levels, measured as the glycemic index (GI). This notion was supported by the work of Benton et al., who investigated memory performance in humans and measured a superior performance within 150–220 min after intake of low-GI sugars as compared with that after intake of high-GI sugars. However, the suppressive effects of Kokuto were relatively more rapid and were observed within 15 min after intake. These findings highlighted the importance of examining the non-sugar components (NSCs) in Kokuto that contribute to the effects of Kokuto.

Here, to identify the molecular components associated with the Kokuto anti-stress property, we investigated the effects of the NSC fractions of Kokuto on stress induced response in mice. We assessed serum stress-marker levels and antioxidative activity in restraint-stressed mice treated with NSC fractions of different polarity, and we performed HPLC analyses to identify the constituents related to the anti-stress property of the NSC fractions.

2 MATERIALS AND METHODS

2.1 Chemicals

The following reagents were from commercial sources. Gallic acid monohydrate (AAPH), 2,2′-azobis(2-aminopropane) dihydrochloride (trole), 2,2′-azobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and p-hydroxybenzaldehyde were purchased from Wako Pure Chemical Industries (Osaka, Japan). Folin-Ciocalteu reagent, sodium carbonate, and p-hydroxyacetophene were purchased from Nacalai Tesque (Kyoto, Japan). Fluorescein sodium salt and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO., U.S.A.). p-Coumaric acid was purchased from Tokyo Chemical Industries (Tokyo, Japan). Schaftoside and isoschaftoside were isolated from sugarcane products and identified based on NMR and UV-Vis spectra previously. All other reagents were from Wako Pure Chemical Industries.

2.2 Fractionation of NSCs from Kokuto

Kokuto, processed from sugarcane in 2014 at Iheyajima, was obtained from Okinawa Brown Sugar Cooperative Association (Okinawa, Japan). The Kokuto sample (3 kg) was dissolved in distilled water (15 L) and centrifuged at 1690 × g for 15 min (10 °C) in a refrigerated centrifuge (CR20G, Hitachi, Tokyo, Japan), after which the supernatant was filtered under suction and 3 kg of Diaion® HP-20 resin (Mitsubishi Chemical Co., Tokyo, Japan) was mixed with the filtrate. After shaking the mixture for 60 min and rinsing with water (10 L), the adsorbed resin was packed into a glass column that was filled with cotton wool at the bottom. The column was successively eluted with 5 L each of aqueous methanol (MeOH) solutions (25%, 50%, 75%, and 100%, v/v), and the obtained eluates were concentrated under reduced pressure by using a rotary vacuum evaporator, and the concentrates were then freeze-dried using an FDU2000 freeze dryer (EYELA, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The freeze-dried products were regarded as the NSC fractions eluting at 25%, 50%, 75%, and 100% MeOH, respectively (Fig. 1, Phase I), and the four fractions were used as samples in measurements of total phenolic content (TPC) and antioxidative activity and in the stress test on mice.

2.3 TPC of NSC fraction

Samples were prepared by dissolving 10 mg of each NSC fraction in 50% aqueous MeOH, and the TPC of each fraction was measured by using the method of Payet et al. with modifications. Briefly, 20-μL samples were mixed with 40 μL of distilled water, 20 μL of MeOH, and 15 μL of Folin-Ciocalteu reagent (diluted two-fold with distilled water) in 96-well microplates (PerkinElmer, Inc., Waltham, MA, USA). After 5 min, 75 μL of 2% sodium carbonate was added into each well and the microplates were incubated

Fig. 1 Fractionation of non-sugar components (NSCs) in Kokuto. Each fractionation yield is expressed as the percentage of the solid content. Fraction 1 (Fr. 1) was obtained from internal fractions 1–28, Fr. 2 from fractions 29–55, Fr. 3 from fractions 56–74, Fr. 4 from fractions 75–95, and Fr. 5 from fractions 96–120.
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2.4 Antioxidant activity of NSC fractions

The antioxidant activity of each NSC fraction was determined based on oxygen radical absorbance capacity (ORAC), trolox equivalent antioxidant capacity (TEAC), and DPPH-assay results. All determinations were performed using the same sample as in the TPC measurements, and all samples were analyzed in triplicate.

The ORAC values of NSC fractions were determined as described by Prior et al. with minor modifications. Briefly, 25 μL of each sample was mixed with 150 μL of 90 mmol/L fluorescein solution in the inner wells of a black 96-well microplate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). The outer wells of the microplate were filled with 200 μL of water and the plate was placed for 10 min in a microplate reader (Synergy HT, BioTek) that had been prewarmed to 37°C. Subsequently, 31.7 mmol/L AAPH solution (75 μL) was added into the inner wells and the samples were monitored every minute for 60 min by using excitation and emission wavelengths of 485 and 535 nm, respectively. The ORAC value was determined using a calibration curve of trolox standard (5–40 μmol/L) and is expressed as nmol trolox equivalents (TE)/kg NSC fraction.

The TEAC assay was performed as described by Miller et al. An ABTS radical solution was produced by reacting 5 mmol/L ABTS aqueous solution (20 mL) with solid manganese dioxide (0.5 g) for 30 min and then passing the ABTS radical solution through a Whatman No. 1 filter paper (Advantec Tokyo Roshi Kaisha, Ltd., Tokyo, Japan) and a 0.2 μm syringe filter (DISMIC-13CP, Advantec Tokyo Roshi Kaisha, Ltd.) to remove excess manganese dioxide. Before analysis, the solution was diluted with 75 mmol/L phosphate-buffered saline (PBS, pH 7.2) to a final absorbance of 0.70 ± 0.02 at 734 nm (measured using the microplate reader). In the assay, 20 μL of the sample was mixed with 200 μL of the ABTS radical solution (75 mmol/L phosphate buffer was used in the blank) in 96-well microplates and then incubated for 60 min at room temperature. Subsequently, the solutions in the microplates were mixed well and the 734-nm absorbance at 30°C was measured using the microplate reader. The ABTS radical-scavenging activity was calculated using a calibration curve of trolox standard (0–5 μmol/L) and is expressed as nmol TE/kg NSC fraction.

The DPPH assay was performed according to the method of Takahashi et al. Samples or standards (50 μL) in 96-well microplates were thoroughly mixed with 50 μL of 200 mmol/L MES buffer (pH 6.0) and 50 μL of 0.4 mmol/L DPPH in ethanol (EtOH) solution (or EtOH, in the case of blank wells), and after incubation for 20 min at room temperature, the absorbance was measured at 520 nm. The DPPH radical-scavenging activity was calculated using a calibration curve of trolox standard (0–10 μmol/L) and is expressed as nmol TE/kg NSC fraction.

2.5 Restraint-stress tests on mice

Male BALB/c mice (7 weeks old, weighing 23.2 ± 1.1 g) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The mice were housed at room temperature (23°C ± 2°C) under 50%–60% humidity and a 12/12 h light/dark cycle, and allowed free access to water and feed. Mice were weighed and divided into the following six groups according to average body weight (n = 5 per group): blank, control, and 25%, 50%, 75%, and 100% MeOH fraction groups. After a 5-day adaptation period, mice in the blank group (no stress treatment) and control group (subjected to stress treatment) were orally administered PBS (150 μL) once daily for 7 days, whereas mice in the four NSC fraction groups (subjected to stress treatment) were administered 25%, 50%, 75%, and 100% MeOH fractions dissolved in PBS, respectively. The NSC fraction dose in all samples was fixed at 200 mg/kg body weight. On the last day of the treatment period, all groups except the blank group were rested for 60 min after oral administration and then subjected to restraint stress by using a custom-built restrainer trap (16 × 11 cm) for 60 min. Mice were rested for 30 min after the restraint-stress treatment and then sacrificed by decapitation. Blood and liver were collected and assayed (as described below) for corticosterone level and antioxidant activity. This study was conducted in accordance with the applicable guidelines from the University of the Ryukyus Policy on Animal Care and Use after approval by the Animal Care and Use Committee of the University of the Ryukyus (Okinawa, Japan).

2.6 Preparation of serum and liver homogenates

Blood was harvested through decapitation of mice, collected in microtubes, and then allowed to stand overnight at 6°C. On the next day, the blood samples were centrifuged at 1000 × g for 15 min (4°C) in a CT15RE centrifuge (Hitachi), and the supernatants were centrifuged again at 3440 × g for 5 min (4°C). The final supernatants were used as serum samples, which were collected in microtubes and stored at −30°C until analysis. Livers were excised and, after weighing, were frozen instantly with liquid nitrogen and stored at −80°C. Subsequently, 200 mg liver samples were homogenized using a Handy microhomogenizer PHICOSUTRON NS-310EIII (Microtec Co., Ltd., Chiba, Japan) in five volumes of PBS (100 mmol/L, pH 7.4). The homogenates were centrifuged at 3000 × g for 15 min at 4°C, and the supernatants were collected in microtubes and stored.
at −80°C until antioxidant activity analysis.

### 2.7 Measurement of serum corticosterone level

The serum corticosterone level, a stress marker, was determined using an enzyme immunoassay kit (DetectX®, Corticosterone Enzyme Immunoassay Kit, Arbor Assays, Ann Arbor, MI, USA), according to manufacturer guidelines.

### 2.8 Antioxidant activity in serum and liver

The ABTS radical-scavenging activity in liver and serum was measured by performing the TEAC assay as described under the section 2.4. Liver protein content was measured using the Bradford method, with a commercially available kit (Protein Quantification Kit-Rapid, Dojindo Molecular Technologies, Inc., Kumamoto, Japan), and then the activities in both serum and liver were expressed as nmol TE/mL of serum and nmol TE/mg of protein in liver, respectively. Superoxide dismutase (SOD) activity in the liver was measured using an SOD Assay Kit-WST (Dojindo Molecular Technologies, Inc.). According to the protocol provided with the kit, 1 U of SOD was defined as the amount of enzyme in the liver protein extract (20 μL) that inhibited the reduction reaction of WST-1 with superoxide anion by 50%. The result is expressed as U/mg of protein in liver.

### 2.9 Fractionation of 50% MeOH fraction

The 50% MeOH fraction (200 mg) was fractionated by using Toyopearl® HW-40C gel (Tosoh Co. Ltd., Tokyo, Japan) successively with aqueous MeOH solutions (20%, 40%, and 60%, v/v) (Fig. 1, Phase II). The eluate was collected using a fraction collector, CHF161RA (Advantec Toyo, Tokyo, Japan), and absorbance was measured at 240, 270, and 340 nm by using a Spectrophotometer UV-1800 (Shimadzu, Kyoto, Japan). The collected eluate was divided into five fractions (Fr. 1–5) according to the characteristic spectral profiles. Each fraction was concentrated using a rotary vacuum evaporator, freeze-dried, and stored in a vacuum desiccator until further analysis.

### 2.10 TPC and DPPH radical-scavenging activity of fractions separated from the 50% MeOH fraction

The TPC and the DPPH radical-scavenging activity of the five fractions separated from the 50% MeOH fraction were measured according to the methods described earlier in this section.

### 2.11 HPLC-based identification of phenolic constituents

Phenolic constituents in the 50% MeOH fraction were identified through HPLC analysis performed using the following conditions. The column temperature was 40°C, the injection volume was 1 μL, and the column used was a Develosil® ODS-MG-3 (3.0 × 150 mm). The mobile phase comprised purified water containing 0.1% (v/v) acetic acid (solvent A) and acetonitrile (solvent B), and was delivered at a flow rate of 0.4 mL/min. The gradient involved an increase from 10% B to 60% B over 25 min, after which 60% B was held for 5 min. After 30 min, the mobile-phase concentration was returned to 10% B and held for 10 min for column equilibration. A UV detector was used with a measurement wavelength of 280 nm. Phenolic constituents were identified based on the UV spectrum of the external standard and on the retention time in the chromatography.

### 2.12 Statistical analysis

Experimental results are expressed as means ± SD. Data were analyzed statistically by performing one-way analysis of variance (ANOVA) and the Tukey-Kramer test by using BellCurve for Excel 2012 (Social Survey Research Information Co. Ltd., Tokyo, Japan). Differences were considered statistically significant at p < 0.05.

### 3 RESULTS

#### 3.1 TPC and antioxidant activity of NSC fractions

Table 1 shows the TPC and the antioxidant activity (ORAC value and ABTS and DPPH radical-scavenging activity) of the NSC fractions obtained using 25%, 50%,

| Fraction   | Total phenolic content (g GAE/kg) | ORAC value (mmol TE/kg) | ABTS radical-scavenging activity (mmol TE/kg) | DPPH radical-scavenging activity (mmol TE/kg) |
|------------|-----------------------------------|-------------------------|-----------------------------------------------|-----------------------------------------------|
| 25% MeOH   | 113.8 ± 13.3ab                   | 2594.2 ± 21.2ab         | 1459.0 ± 48.3ab                               | 204.5 ± 26.9ab                                |
| 50% MeOH   | 159.4 ± 15.8ab                   | 4386.2 ± 175.8ab        | 2032.0 ± 226.4ab                              | 295.2 ± 28.0ab                                |
| 75% MeOH   | 140.1 ± 16.6ab                   | 4741.3 ± 498.2ab        | 1862.8 ± 147.6ab                              | 233.5 ± 23.3ab                                |
| 100% MeOH  | 89.8 ± 12.9ab                    | 2474.9 ± 305.5ab        | 1344.9 ± 56.0ab                               | 217.4 ± 42.4ab                                |

GAE, gallic acid equivalents; TE, trolox equivalents; ORAC, oxygen radical absorbance capacity

Values are means ± SD (n = 3). Different letters indicate significant differences between samples (p < 0.05).
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75%, and 100% MeOH. The TPC of the 50% and 75% MeOH fractions was significantly higher \((p<0.05)\) than that of the 25% and 100% MeOH fractions. Moreover, the antioxidant activity of the 50% and 75% MeOH fractions was higher \((p<0.05)\) than that of the 25% and 100% MeOH fractions in terms of ORAC levels and ABTS radical-scavenging activity, and the DPPH radical-scavenging activity was highest in the 50% MeOH fraction, followed by that in the 75%, 100%, and 25% MeOH fractions. Therefore, the TPC and the antioxidant activity were both highest in the 50% MeOH fraction.

3.2 Serum corticosterone level
Measurement of serum corticosterone in restraint-stressed mice (Fig. 2) revealed that the levels of this stress marker were significantly higher \((p<0.01)\) in the control group than in the blank group, and that the levels in the groups treated with the NSC fractions tended to be lower than that in the control group. Notably, the corticosterone level in the 50% MeOH group did not differ significantly from that in the blank group but was significantly lower \((p<0.05)\) than the level in the control group.

3.3 Antioxidant activity in serum and liver
The ABTS radical-scavenging activity in serum and liver in the control group was significantly lower \((p<0.05)\) than that in the blank group (Fig. 3A and 3B). In the groups treated with the NSC fractions, serum antioxidant activity tended to be higher than that measured in the control group, although the difference was not statistically significant (Fig. 3A), whereas the activity in the liver was significantly higher \((p<0.05)\) than the corresponding activity in the control group (Fig. 3B). However, the ABTS radical-scavenging activity (both in serum and liver) did not differ significantly between the groups treated with the distinct NSC fractions (Fig. 3A and 3B). Moreover, SOD activity in the liver was not significantly different between the blank and control groups, although the activity in the control group tended to be lower than that in the blank group, as in the case of the activity against ABTS. By contrast, SOD activity in the group treated with the NSC 50% MeOH fraction was significantly higher \((p<0.05)\) than that in the control group (Fig. 3C).

3.4 Phenolic constituents in 50% MeOH fraction
Table 2 shows the TPC and the antioxidant activity of the five fractions (Fr. 1–5) separated from the 50% MeOH fraction. The highest TPC measured was in Fr. 5, followed by that in Fr. 4, Fr. 3, Fr. 2, and Fr. 1. Conversely, the highest DPPH radical-scavenging activity was in Fr. 4, followed by that in Fr. 5, Fr. 3, Fr. 2, and Fr. 1. Considering the high TPC and antioxidant activity of Fr. 3, Fr. 4, and Fr. 5, we analyzed the phenolic constituents of these three
fractions by using HPLC. In Fr. 3 and Fr. 4, we identified five phenolic compounds (Fig. 4), but the phenolic compounds in Fr. 5 could not be precisely determined due to overlapping peaks in the chromatogram. In Fr. 3, we detected four phenolic compounds, p-hydroxybenzaldehyde (peak a), p-hydroxyacetophenone (peak b), schaftoside (peak c), and isoschaftoside (peak d), and in Fr. 4, we identified the phenolic compound p-coumaric acid (peak e).

4 DISCUSSION

In this study, we investigated the effects of NSC fractions of Kokuto on stress-induced response, including serum corticosterone level as a stress marker and antioxidant activity, in restraint-stressed mice. Stress, particularly anxiety-associated stress, is followed by the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which is the main part of the stress physiological response. If the HPA axis is activated by physiological and psychological stress stimuli, glucocorticoids (cortisol in humans, corticosterone in rodents) are secreted from adrenocortical cells. We found that the serum corticosterone level was significantly higher in the control group (subjected to stress treatment) than in the blank group (no stress treatment) (Fig. 2). Therefore, our restraint-stress method, applied using a custom-built restrainer trap for 1 h, might have triggered glucocorticoid secretion by activating the HPA axis. Conversely, the serum corticosterone level in the groups treated with the NSC fractions tended to be lower than that in the control group, and oral administration of the 50% MeOH fraction in particular strongly suppressed the elevation of serum corticosterone levels. In the 50% MeOH fraction, both TPC and antioxidant activity were high (Table 1). Stress-induced response has been reported to be suppressed by various phenolic compounds, such as resveratrol, a natural phenolic compound enriched in Polygonum cuspidatum, and flavonoids isolated from buck-

Table 2  Total phenolic content and DPPH radical-scavenging activity of five fractions separated from the 50% MeOH fraction.

| Fraction | Total phenolic content (g GAE/kg) | DPPH radical-scavenging activity (mmol TE/kg) |
|----------|----------------------------------|---------------------------------------------|
| Fr. 1    | 120.6 ± 14.9                    | 338.9 ± 14.5                                |
| Fr. 2    | 191.8 ± 10.6                    | 578.2 ± 5.6                                 |
| Fr. 3    | 200.0 ± 13.4                    | 685.4 ± 55.7                                |
| Fr. 4    | 230.5 ± 15.1                    | 859.1 ± 42.7                                |
| Fr. 5    | 242.7 ± 15.1                    | 696.8 ± 14.5                                |

GAE, gallic acid equivalents; TE, trolox equivalents

Value are means ± SD (n = 3). Different letters indicate significant differences between samples (p < 0.05).

Fig. 4  Phenolic constituent profiles of fractions 3 and 4 (Fr. 3 and Fr. 4) separated from the 50% MeOH fraction, as identified in HPLC analyses performed using a detection wavelength of 280 nm. The identified phenolic compounds (peaks (a)–(e)) were as follows: (a)p-hydroxybenzaldehyde, (b) p-hydroxyacetophenone, (c)schaftoside, (d) isoschaftoside, and (e)p-coumaric acid.
wheat sprouts. These phenolic compounds suppressed the increase in secretion of corticosterone into the blood in stressed rodents. Therefore, the phenolic compounds in the 50% MeOH fraction might be involved in producing the suppressive effects recorded here. By contrast, the 75% MeOH fraction also showed high TPC and antioxidant activity (Table 1) but was only weakly effective in normalizing the serum corticosterone level (Fig. 2). Our results agree with the work of Mendes et al., who reported that despite containing polyphenols and exhibiting antioxidant activity, the extracts of Baccharis trimera and Davilla rugosa exerted no effect on plasma corticosterone levels in restraint- and cold-stressed rats.

Stress is recognized to lead to an increase in ROS. Here, ABTS radical-scavenging activities in both serum and liver were significantly lower in the control group than in the blank group (Fig. 3A and 3B). Similarly, liver SOD activity was lower in the control group than in the blank group (Fig. 3C). Li et al. reported that the ORAC level and SOD activity in the plasma and liver of mice were markedly decreased by acute restraint stress. Therefore, we suggest that the ROS production elicited by acute restraint stress is induced by the decline of in vivo antioxidant activity. Conversely, the groups treated with the NSC fractions indicated a significantly increase of ABTS radical-scavenging activity in the liver compared with the control group (Fig. 3A), and a comparative increase of the activity can also be observed in the serum (Fig. 3B). Significant differences were detected among the antioxidant activities of the NSC fractions (Table 1), but the ABTS radical-scavenging activity in the serum and liver did not differ between the groups treated with the distinct NSC fractions. This difference in the results of our in vivo and in vitro assays was likely due to the distinct pharmacokinetics of antioxidative phenolic compounds: Phenolic compounds are reported to be widely metabolized in vivo, primarily by colonic microflora during transfer across the small intestine and in the liver after absorption. Therefore, the entire human metabolism contributes substantially to the antioxidant potential of various compounds, for instance by converting the compounds into their active forms, linking them to various biomolesules that can alter their original properties, or causing the inactivation of the compounds. We suggest that the same reasons might also underlie the different effects of the NSC fractions measured in the serum and liver. Furthermore, variations were also observed in liver SOD activity among the groups treated with NSC fractions (Fig. 3C), particularly in the case of the group treated with the 50% MeOH fraction, in which the activity was significantly different from that in the control group. SOD, an antioxidant enzyme, functions as a catalyst in the dismutation of superoxide anions to oxygen and hydrogen peroxide. Liu et al. reported that quercetin, a phenolic compound, markedly upregulated mRNA expression levels of antioxidant enzymes, including SOD, in the liver in a rat model of lead-induced hepatic injury. Here, we found that stress induced a reduction in antioxidant activity in vivo (Fig. 3). Therefore, the 50% MeOH fraction might contain phenolic compounds that enhance SOD activity. Overall, among the NSC fractions, the 50% MeOH fraction showed both a high content of phenolic compounds and high antioxidant activity, and the 50% MeOH fraction also showed both a high content of phenolic compounds and high antioxidant activity, and the 50% MeOH fraction also showed both a high content of phenolic compounds and high antioxidant activity, and the 50% MeOH fraction also showed both a high content of phenolic compounds and high antioxidant activity.

To identify the phenolic compounds in the NSC 50% MeOH fraction that might contribute to the observed suppressive effects on stress-induced response in mice, we further examined the fraction and detected five antioxidants: p-hydroxybenzaldehyde, p-hydroxyacetophenone, schaftoside, isoschaftoside, and p-coumaric acid (Fig. 4). The antioxidative phenolic compound p-hydroxybenzaldehyde was previously detected in sugarcane molasses extract. Intriguingly, Ha et al. reported that p-hydroxybenzaldehyde exerted an inhibitory effect on γ-aminobutyric acid (GABA) transaminase, which functions as a GABA-degrading enzyme; GABA is a major inhibitory neurotransmitter in the mammalian brain and plays a key role in the inhibitory regulation of HPA axis activation. Previously, p-hydroxyacetophenone was reported to be present in several brown cane sugar products and to possess antioxidant and anticholinesterase activities, and schaftoside and isoschaftoside were found to account for the DPPH radical-scavenging activity in extracted cane juice. Lastly, Fr. 4 contained p-coumaric acid, which was reported to be present not only in sugarcane molasses, but also in sugary products such as massecuite and in very-high-polarization sugars, and p-coumaric acid was found to exhibit in vitro and in vivo antioxidant activity and other biofunctions such as tyrosinase inhibitory activity and antimelanogenic activity. Furthermore, p-coumaric acid can potentially be included as an orally active nutritional supplement or functional food to reduce the symptoms of mild to moderate stress and anxiety; this is because p-coumaric acid exhibited considerable GABAergic activity, and oral administration of p-coumaric acid produced a strong anti-anxiety effect in rats, as measured using the elevated plus-maze paradigm, which agrees with the anxiety-reducing effects of orally administered diazepam.

As noted in the preceding paragraph, among the five phenolic compounds, p-hydroxybenzaldehyde and p-coumaric acid have been linked to anti-stress effects through their association with GABA signaling; thus, the p-hydroxybenzaldehyde and p-coumaric acid detected here in the 50% MeOH fraction might be related to the suppressive effects on stress-induced response. However, the 50% MeOH fraction also contained three other phenolic compounds: p-hydroxyacetophenone, schaftoside, and isoschaftoside; these phenolic compounds have been reported...
to exhibit antioxidant activity, but no study has previously described their anti-stress property. Therefore, further investigation is required to evaluate the suppressive effects of not only p-hydroxybenzaldehyde and p-coumaric acid in the NSC fractions, but also p-hydroxyacetophenone, schaftoside, and isoschaftoside.

5 CONCLUSION
We investigated the effects of four NSC fractions, obtained from the elution with 25%, 50%, 75%, and 100% MeOH on HP-20 resin, on the stress-induced response of mice, and found that oral administration of the NSC 50% MeOH fraction suppressed not only the secretion of corticosterone into the serum, but also the reduction of in vivo antioxidant activity induced by restraint stress. These suppressive effects might be related to five phenolic compounds detected in the NSC fraction, although further studies should be performed to clarify the amounts of these phenolic compounds. Our results provide evidence supporting the previous report regarding the anti-stress property of Kokuto.

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