Component Analysis of Wasabi Leaves and an Evaluation of their Anti-inflammatory Activity

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The methanol extracts of wasabi leaves (Wasabia japonica Matsumura) from Shizuoka, Japan have been found to inhibit the production of nitric oxide (NO) in an in vitro assay using murine macrophage J774.1 cells stimulated with lipopolysaccharide. Fourteen known compounds, including five phenylpropanoid glycosides (1 – 4 and 9), three phenylpropanoids (5 – 7), a phenolic glycoside (8), two flavonoid glycosides (10 and 11), two terpenoids (12 and 13) and a carotenoid (14), were isolated from wasabi leaves collected from Shizuoka and their structures elucidated using spectroscopic methods. This study therefore represents the first reported isolation of compounds 8, 9, 12, 13 and 14 from wasabi leaves. 5-Hydroxy ferulic acid methyl ester (5) and all-trans-lutein (14) were found to inhibit NO production in J774.1 cells with IC₅₀ values of 22 and 25 µM, respectively. The results therefore suggested that these compounds are the active components of wasabi leaves.

Keywords: Wasabia japonica Matsumura, leaves, components analysis, anti-inflammatory, J774.1 cells

Introduction

Wasabia japonica Matsumura (sawa-wasabi in Japanese) is an edible plant belonging to the Cruciferae family and its roots and rhizomes are widely used in Japanese cuisine as the pungent spice wasabi (“Japanese horseradish”), which is used to garnish traditional dishes such as sushi and sashimi. The strong pungent smell associated with the roots of wasabi is derived directly from volatile allyl isothiocyanates via their reaction with myrosinase (Kojima et al., 1973). Several allyl isothiocyanate derivatives from wasabi have been reported to exhibit a variety of interesting biological properties, including antimicrobial (Inoue et al., 1983; Goi et al., 1985; Ono et al., 1998), antimitogenic (Morimitsu et al., 2000; Kinae et al., 2000), antiplatelet (Morimitsu et al., 2000) and apoptosis-inducing (Watanabe et al., 2003) activities. Notably, however, wasabi leaves are boiled or fried when they are used in foods, with the majority of the leaf being discarded. Although several studies have been reported in the literature concerning the potential health benefits of the extracts of wasabi leaves, including their anti-oxidative (Hosoya et al., 2008) and anti-obesity (Ogawa et al., 2010) activities, there have been few studies aimed at identifying the active components of these extracts. Previous studies have reported the isolation of phenylpropanoid and flavonoid glycosides from wasabi leaves cultivated in Okutama, Tokyo, Japan (Hosoya et al., 2005 and 2008). Although several studies have reported interesting bioactivities for wasabi leaf extracts, their active components remain unclear and further research is therefore required to elucidate the structural and biochemical properties of these components.

As part of our ongoing screening program identifying bioactive components from the agricultural products found in Shizuoka, Japan, we recently found that methanol extracts of wasabi leaves from this area showed anti-inflammatory activities in an in vitro
assay using murine macrophage J774.1 cells, which were stimulated with lipopolysaccharide (LPS). The methanol extract of these leaves was subjected to chemical analysis, and the results demonstrated that the compounds contained in the extract exhibited anti-inflammatory activity.

**Material and Methods**

**General experimental procedure**  
$^1$H (400 MHz) and $^{13}$C NMR (100 MHz) spectra, as well as all 2D NMR spectra, were recorded on a Bruker AVANCE III 400 spectrometer (Bruker BioSpin, Billerica, MA, USA). Standard plus sequences and parameters were used for the experiments. The chemical shift values ($\delta$) have been reported in ppm, and the coupling constants ($J$) have been reported in Hz. The chemical shifts in the $^1$H and $^{13}$C NMR spectra have been corrected using the residual solvent signals of methanol-$d_4$ ($\delta_H$ 3.31, $\delta_C$ 49.0), chloroform-$d_3$ ($\delta_H$ 7.24, $\delta_C$ 77.0) and dimethylsulfoxide-$d_6$ ($\delta_H$ 2.50, $\delta_C$ 39.5). High-resolution electrospray ionization mass spectra (HR-ESIMS) were recorded using an Accela LC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a quadrupole mass spectrometer, Q-Exactive (Thermo Fisher Scientific). Xcalibur software was used for system control and data analysis. HPLC analyses were carried out on a Tosoh CCPD system (Tosoh Co., Inc., Tokyo, Japan) equipped with a Tosoh UV-8010 and a Jasco PU-2086 Plus equipped with a Jasco UV-2075 Plus detector ($\lambda$ 254 nm) and a Michiyuki RP- 18GP column ($5 \mu m$, $\phi 20 \times 250 mm$). The column was subjected to gradient elution with H$_2$O (solvent A) and MeCN (solvent B) ($0$ to $20$ min $10 - 30$% solvent B) to give compound (1.6 mg) and a mixed fraction. The mixed fraction was further purified on the same system using a mobile phase composed of $18$% (v/v) MeCN to give compound (2.8 mg) and a mixed residue, which was purified with $8$% MeCN to give compound (1.1 mg).

**Anti-inflammatory assay**  
Murine macrophage J774.1 cells were cultured in RPMI-1640 medium (Nacalai Tesque Co., Inc., Kyoto, Japan) supplemented with $10$% fetal bovine serum and penicillin ($10$ U/mL)/streptomycin ($100$ µg/mL). J774.1 cells were seeded into a 96-well microplate at $1 \times 10^5$ cells in $100 \muL$ per well, and preincubated for $12$ h at $37^\circ C$ in a humidified atmosphere containing $5$% CO$_2$. The cells were cultured in the medium containing LPS ($5$ µg/mL) with or without various concentrations of the test samples dissolved in DMSO for $24$ h. The concentration of DMSO in the medium was $0.1$%. A Griess assay was used to determine the production of nitric oxide (NO). The supernatant ($100 \muL$) was transferred to a 96-well microtiter plate at $1 \times 10^5$ cells in $100 \muL$ per well, and the formazan in the cells was determined from the difference in the absorbance values of the samples and the controls ($0.1$% DMSO). 

**Plant material**  
The *Wasabia japonica* Matsumura leaves used in this study were cultivated in Shizuoka, Japan in April, 2012.

**Isolation of compounds**  
Fresh wasabi leaves (6 kg) were lyophilized and the lyophilizate (840 g) was extracted with methanol (MeOH) ($2 \times 25 \ L$, 3 days each) at room temperature. The combined extracts were then filtered and concentrated in vacuo to a residue ($267.4 g$). The residue was dissolved in water, and the resulting solution was sequentially extracted with n-hexane and ethyl acetate (EtOAc). The respective layers were then concentrated in vacuo to give the following extracts: n-hexane (79.7 g), EtOAc (5.2 g) and water (186.7 g). The water extract was concentrated in vacuo to give a residue ($321 g$). The residue was dissolved in DMSO, and the absorbance of the resulting mixture on the same system using a mobile phase composed of $90$% (v/v) MeCN in H$_2$O to give compounds (7.3 mg), $6$ (3.5 mg), $7$ (0.5 mg) and $12$ (1.3 mg). Subsequent elution of the column with MeOH, followed by purification of the resulting mixture on the same system using a mobile phase composed of $30$% (v/v) MeCN in H$_2$O to give compounds (7.3 mg), $6$ (3.5 mg), $7$ (0.5 mg) and $12$ (1.3 mg). Subsequent elution of the column with MeOH, followed by purification of the resulting mixture on the same system using a mobile phase composed of $90$% (v/v) MeCN in H$_2$O to give compound (14 (5.4 mg)). Three hundred milligrams of the seventh fraction (350.3 mg) was purified by HPLC using a Quaternary Gradient Pump Jasco PU-2089 system equipped with a Jasco UV-2075 Plus detector ($\lambda$ 254 nm) and a Michiyuki RP-18GP column ($5 \mu m$, $\phi 20 \times 250 mm$). The column was subjected to gradient elution with H$_2$O (solvent A) and MeCN (solvent B) ($0$ to $20$ min $10 - 30$% solvent B) to give compound (1.6 mg) and a mixed fraction. The mixed fraction was further purified on the same system using a mobile phase composed of $18$% (v/v) MeCN to give compound (2.8 mg) and a mixed residue, which was purified with $8$% MeCN to give compound (1.1 mg).

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**Cell viability assay**  
Murine macrophage J774.1 cells were cultured in RPMI-1640 medium (Nacalai Tesque Co., Inc., Kyoto, Japan) supplemented with $10$% fetal bovine serum and penicillin ($10$ U/mL)/streptomycin ($100$ µg/mL). J774.1 cells were seeded into a 96-well microplate at $1 \times 10^5$ cells in $100 \muL$ per well, and the formazan in the cells was determined from the difference in the absorbance values of the samples and the controls ($0.1$% DMSO). (L-Nξ-monomethyl 1-L-arginine acetate NMMA) (98%) was used as a positive control ([IC$_{50}$ = 22 µg/mL]).
Spectra Max 190 microplate reader. All experiments were performed in triplicate for each sample. The ratio of living cells was determined from the difference in the absorbance values of the samples and the controls (0.1% DMSO).

Real time PCR for measuring iNOS gene expression  J774.1 cells were seeded into 24-well plates at $5 \times 10^5$ cells in 0.5 mL per well. After an overnight pre-incubation, the cells were treated with different concentrations of the test compounds containing LPS, and the resulting mixtures were incubated at 37°C for 24 h. The total RNA was then extracted from each cell pellet using NucleoSpin RNA II (Takara Bio., Shiga, Japan) according to the manufacturer’s instructions, and reverse-transcribed into cDNA.

Fig. 1. Chemical structures of the compounds isolated from wasabi leaves collected in Shizuoka, Japan.
Fig. 2. Suppressive effects of compounds 5 (A) and 14 (B) on the LPS-induced NO production in J774.1 cells and effect on J774.1 cell viability. Values of NO production rate (solid column) and cell viability (solid curves) are given as the mean of triplicate analysis. Bars, s.d.

using a PrimeScript RT reagent kit (Takara Bio.). The resulting cDNA was subjected to PCR using SYBR Premix Ex Tag II (Takara Bio.) and primers. The murine iNOS primers (i.e., forward 5′-CCGATTTAGCTTGGTGAAATG-3′ and reverse 5′-CTGACCCCTGCAAGCCATGA-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (forward 5′-CGTGTTCCTACCCCCAATGT-3′ and reverse 5′-ATGTCCATCACATCTGGCAAGTTTCT-3′) were purchased from Hokkaido System Science Co., Ltd. (Tokyo, Japan). PCR amplification experiments were carried out on a Thermal Cycler Dice Real Time System II (Takara Bio.) under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s and dissociation. The data were collected and analyzed using the Takara Dice Real Time Single software.

Results and Discussion

Isolation of compounds from wasabi leaves  The column chromatography and preparative-HPLC processes described in the experimental section led to the isolation of five phenylpropanoid glycosides (1–4 and 9), three phenylpropanoids (5–7), a phenolic glycoside (8), two flavonoid glycosides (10 and 11), two terpenoids (12 and 13) and a carotenoid (14) from the methanol extracts of the wasabi leaves cultivated in Shizuoka. The structures of these compounds were determined spectroscopically using NMR and MS analyses.

Determination of phenylpropanoids, their glycosides and a phenolic glycoside  Compounds 1–4 were analyzed by 1H and 13C NMR spectroscopy in CD3OD; the resulting spectra were found to be very similar and implied that these compounds consisted of two phenylpropanoid and two sugar moieties. One of these groups was determined to be a sinapoyl group in all four compounds, whereas the other groups in compounds 1–4 were 5-hydroxyferuloyl, caffeoyl, sinapoyl and feruloyl groups, respectively. Consideration of the 1H NMR data for these compounds suggested that the olefinic protons were in a trans-configuration based on the large coupling constant between the protons (i.e., J = 16 Hz). The NMR data for the sugar moiety of each compound was identical to that of gentiobiose, which consists of two glucose units linked through a 6→1′ glycosidic bond. HMBC correlations between H-1/C-9, H-6/C-1′ and H-2′/C-9″ suggested that there was an acyl group attached to the C-1 position of one of the two glucose units, and that the other sinapoyl group was attached to the C-2′ position of the other glucose unit through an ester bond. The molecular formulae of compounds 1–4 were determined to be C39H38O13 (m/z 739.2075 [M–H]−) and C41H32O11 (m/z 709.1968 [M–H]−) for C39H38O13, 709.1974, C41H32O11 (m/z 735.2229 [M–H]−) for C39H38O13, 753.2237) and C43H40O13 (m/z 723.2126 [M–H]−) for C39H38O13, 723.2131), respectively. Taken together, these data suggested that the structures of compounds 1–4 were 1-O-trans-5′-hydroxy feruloyl-2′-O-trans-sinapoylgentiobiose (1), 1-O-trans-caffeoyl-2′-O-trans-sinapoylgentiobiose (2), 1,2′-O-trans-disinapoylgentiobiose (3) and 1-O-trans-feruloyl-2′-O-trans-sinapoylgentiobiose (4) (Hosoya et al., 2008). Spectroscopic analysis also revealed that compounds 8 and 9 were glycosides bearing a single acyl group. Furthermore, NMR analysis of compounds 8 and 9 implied that these acyl groups were 4-hydroxybenzoic acid and trans-sinapic acid, respectively. HMBC spectroscopy also revealed that the glucose units of these compounds were connected to the carbonyl groups of their respective acyl units at their C1 position. Based on these data, compounds 8 and 9 were determined to be 1-O-(4-hydroxybenzoyl)-β-D-glucopyranoside (Klick and Herrmann, 1987) and 1-O-trans-sinapoyl-β-D-glucopyranoside (Miyake et al., 2007).

Compounds 5–7 were determined to be trans-5-hydroxy ferulic acid methyl ester, trans-sinapic acid methyl ester and trans-ferulic acid methyl ester, respectively, through a comparison of their spectroscopic data with those of the authentic samples.

Determination of flavonoids  Compounds 10 and 11 were obtained as amorphous yellow solids. The molecular formulae of these compounds were determined to be C39H38O13 and C41H32O11, respectively, by HRESIMS (m/z 593.1498 [M–H]−) for C39H38O13, 593.1501 and m/z 447.0918 [M–H]−) for C39H38O13, 447.0922, respectively) and NMR spectroscopy. The 1H and 13C NMR spectra of compound 10 implied the presence of C-6-substituted apigenin-type flavone bearing two sugars units. The chemical shift of the anomic carbon of compound 10 (δc 73.1)
suggested the presence of a C-glucoside linkage at C6, which was confirmed by HMBC correlations between H-1 and C-5/C-6/C-7. Furthermore, the second sugar unit of this compound was determined to be attached to the flavone moiety through its C-4′ position based on a HMBC correlation between H-1 of the sugar and C-4′ of the B-ring. Taken together, these data suggested that compound 10 was isosaponarin (Dubois et al., 1985). NMR analysis of compound 11 revealed that it also contained a C-6-substituted flavone, although this particular flavone was a luteolin-type rather than an apigenin-type flavone. Based on the analyses, compound 11 was identified as isoorietin (Hörhammer et al., 1958).

**Determination of terpenoids** Compound 12 was obtained as a colorless oil, and its molecular formula was determined to be C₁₃H₂₀O₂ by HR-ESIMS (m/z 223.1328 [M+H]+) and NMR analysis. The ¹H NMR spectrum of compound 12 in CD₃OD contained two olefinic methane signals at δ₈ 6.43 (1H, d, J = 15.6 Hz) and δ₈ 7.00 (1H, d, J = 15.6 Hz), which suggested that the double bond was in the trans configuration. The ¹H NMR spectrum also contained four methyl signals, two of which were revealed to be allyl methyl proton signals. The ¹³C NMR spectrum of compound 12 contained numerous carbon signals, including signals consistent with two ketones, a quaternary olefin moiety and three olefinic methane carbons. Two-dimensional NMR analysis of compound 12 revealed several correlations that were consistent with those of dehydrovomifoliol. Compound 12 was therefore confirmed to be dehydrovomifoliol based on a comparison of its ¹H and ¹³C NMR data with the literature (Park et al., 2011).

Compound 13 was isolated as a colorless oil, and its molecular formula was determined to be C₁₀H₁₆O₅ by HR-ESIMS (m/z 387.2002 [M+H]+) and NMR spectroscopy. The ¹H and ¹³C NMR data of compound 13 were similar to those of compound 12, except for the signals belonging to the sugar moiety. HMBC analysis of compound 13 showed a correlation between H-1′ (δ₈ 4.34) and C-9 (δ₁₃C 77.3), which revealed the position of the sugar connection. Several other correlations were also observed in the HMBC spectrum of compound 13, which were consistent with those of cis-roseoside. Compound 13 was therefore identified as cis-roseoside through a comparison of its spectroscopic data with the literature (Mohamed et al., 1999; Tsopmo and Muir, 2010). This study therefore represents the first account of the isolation of compounds 12 and 13 from wasabi leaves.

**Determination of all-trans lutein** Compound 14 was obtained as a yellow oil. Analysis of this compound by ¹H and ¹³C NMR spectroscopy revealed it to be a carotenoid-type compound. Compound 14 was determined to be a mixture of geometric isomers, which were characterized based on their spectroscopic data, including their ¹³C and 2D-NMR, UV and MS data. The ¹H and ¹³C NMR data of the β- and ε-ends of compound 14 corresponded well with those reported in the literature for all-trans-lutein (Englert, 1995; Moss, 1976), and the correlations for this compound were also confirmed to be the same as those of all-trans-lutein by HMBC analysis. HR-APCIMS analysis of compound 14 gave a signal with an m/z value of 551.4239 for [M+H-H₂O]+ (Goupy, 2013). Furthermore, the UV absorption spectrum of compound 14 in methanol showed peaks at 335, 443 and 470 nm, which indicated that all of the double bonds in compound 14 were in the (E)-configuration (Takaichi, 2009). Taken together, the results of these analyses revealed that compound 14 was all-trans-lutein. This report therefore represents the first reported isolation of all-trans-lutein from wasabi leaves.

**Evaluation of anti-inflammatory effect** The methanol extract of wasabi leaves cultivated in Shizuoka, Japan showed inhibitory activity towards the NO production in J774.1 cells stimulated by LPS. The n-hexane, EtOAc and water extracts after partition gave an inhibition ratio of 21, 52 and 12% at 50 μg/mL, respectively. Furthermore, none of the extracts exhibited any cytotoxicity towards J774.1 cells at any of the tested concentrations; cell viability was greater than 95% in all cases. The inhibitory activities of isolated compounds 1–14 towards NO production were measured in an *in vitro* cellular assay at the concentrations of 100, 50, 25 and 12.5 μM. As shown in Table 1, compounds 5 and 14 exhibited potent inhibitory activities towards the production of NO in J774.1 cells without cytotoxicity. Furthermore, compounds 5, 6 and 14 inhibited NO production in a dose-dependent manner. The IC₅₀ values of these compounds were determined to be 22, 82 and 25 μM, respectively. The inhibitory activities of compounds 5–7 were assessed at 100 and 50 μM, and were determined to be in the rank order of 5 > 6 > 7. The structure of compound 5 contained an ortho-dihydroxyl moiety. This trend in NO inhibitory activity (5 > 6 > 7) is consistent with the reported potencies for radical scavenging activity of phenylpropanoids (Hosoya et al., 2008).

LPS-stimulation of J774.1 cells induces the overexpression of inducible nitric oxide synthase (iNOS). The overexpression of iNOS in this way leads to the synthesis of NO, which can result in numerous physiological reactions, including inflammation and mutagenesis. Real-time PCR experiments were used to evaluate the effect of these compounds on the expression of iNOS in LPS-stimulated J774.1 cells. In the current study, LPS-stimulation of J774.1 cells led to the overexpression of iNOS and the subsequent overproduction of NO. Compound 5 inhibited the expression of iNOS at 40 and 20 μM (58.2 and 29.3%, respectively). Treatment of J774.1 cells with compound 5 therefore demonstrated that inhibition of iNOS expression resulted in reduced NO production in LPS-stimulated J774.1 cells. Compound 14 (all-trans-lutein) has been reported to inhibit the production of NO in RAW 264.7 mouse macrophage cells (Rafi and Shafaei, 2007). The inhibitory mechanism of all-trans-lutein was demonstrated by a decrease in the expression of iNOS at the mRNA and protein levels in RAW 264.7 mouse macrophage cells. The mechanistic action of
Table 1. NO production rates (%) and IC\textsubscript{50} values for compounds 1–14

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<th>Compound</th>
<th>Concentration (µM)</th>
<th>IC\textsubscript{50} (µM)</th>
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\textsuperscript{a}Mean ± s.d. of triplicate analysis

compounds 5 and 14 most likely involves inhibition of iNOS expression at the mRNA level. The inhibition efficiency against NO production was higher than that of iNOS expression. It is presumed that these compounds scavenged NO radicals directly, in addition to inhibiting iNOS expression. (Hosoya et al., 2008; Fritz et al., 2012; Eliseu et al., 2012).

In summary, we isolated 14 compounds from the methanol extract of wasabi leaves collected from Shizuoka, Japan. The structures of these compounds were elucidated based on their spectroscopic data, and revealed to be five phenylpropanoid glycosides (1–4 and 9), three phenylpropanoids (5–7), a phenolic glycoside (8), two flavonoid glycosides (10 and 11), two terpenoids (12 and 13) and a carotenoid (14). Furthermore, the methanol extract of the wasabi leaves exhibited inhibitory activity towards NO production in our screening program. The active components of this extract were subsequently determined and their mechanism of inhibitory activity revealed.

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References


