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Antioxidant Activities of Water-soluble Polysaccharides from Buntan (*Citrus grandis* Osbeck) Fruit Flavedo Tissues

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Abstract: Buntan (*Citrus grandis* Osbeck) fruit flavedo tissues were extracted with hexane, ethyl acetate (EtOAc), *n*-butanol and methanol (MeOH). The highest concentration of antioxidant activity of the *n*-butanol extracts as evaluated using 2,2,-diphenyl-l-picrylhydrazyl radical (DPPH). After column chromatographic (CC) separation of this crude extract with silica gel column, three fractions (Fr. A, Fr. B and Fr. C) of the crude extract were obtained from the eluents of CH_2Cl_2:EtOAc:MeOH (0:9:1, 0:7-5:1 and 0:1:1), gradually. Among them, Fr. B and Fr. C showed better antioxidant activity by using DPPH antioxidant assay. Those fractions were further purified by preparative TLC (PTLC) and the main constituents were find to be glycoside, monosaccharide and total soluble sugar. It was interesting to find that the monosaccharide and glycosides exhibited exceptional DPPH free radical scavenging and β-carotene methods, whereas the identified sucrose, glucose and fructose possess less antioxidant activity. Present findings suggest that the brown materials present in Fr. B had a significant contribution to the antioxidant activity of the crude extract of glycoside.

Key words: Buntan fruit flavedo, glycoside, antioxidant, monosaccharide, sucrose, glucose, fructose

INTRODUCTION

Since people are particularly concerned about the quality and the safety of their food, only selected food additives are added for protection from off-flavour. Antioxidants are often used in oils and fatty foods to retard their autoxidation. The *Citrus* genus is characterized by accumulation of large quantities of glycosylated flavanones, which are the first intermediaries in the flavonoid biosynthetic pathway[1]. The levels of the flavanones, naringin, hesperidin and neohesperidin, found in the immature and mature fruits of *Citrus aurantium* and mainly synthesized during the early stages of fruit growth[2]. Researchers have investigated the biological activity of compounds isolated from plant species for the elimination of that antioxidant and antimicrobial activity. The growing interest in the substitution of synthetic food antioxidant by natural ones fostered research on vegetable sources and the screening of raw materials for identifying new antioxidants. During lipid oxidation, antioxidants act in various ways, binding metal ions, scavenging radicals and decomposing peroxides. Often, more than one mechanism is involved. Synergistic antioxidant effects between the compounds found in natural extracts are probably responsible for higher antioxidant activities observed for the crude extracts[3]. Many researchers studied the accumulation of glacoside flavanones in the citrus fruits occurring at specific stages of fruit growth. Some of these glycoside flavanones are of commercial interest because they are used in the pharmaceutical and food industries. The level of naringin in the grapefruit cultivar 'Issac' amounts to 88% in immature fruit and 22% in mature fruit[4]. This study reports the investigation of antioxidant activity of compounds isolated from *n*-butanol extract of buntan fruit tissues and fractionated using silica gel column and sephadex LH-20. The components responsible for the antioxidative activity were also investigated.

MATERIALS AND METHODS

Plant materials and extraction methods: Buntan (*Citrus grandis* Osbeck) fruit were purchased in Kagoshima. Flavedo (2.4 kg) of buntan fruits flavedo were separated and then dried at 45°C for 7 days and ground in an electric blender, yielding fine dry samples 500 g flavedo and was successively soaked in non-polar solvents to polar solvents, in order of *n*-hexane, ethyl acetate, *n*-butanol. Each solvent was extracted three times at room temperatures over a period of eight days. A period of 24 h was allowed for proper drying between each successive solvent. The extracts were dried under a vacuum on a rotary evaporator at 40°C to pursue further analysis. The crude *n*-butanol was fractionated by silica gel 60 column chromatography. The column chromatography was eluted with CH_2Cl_2:EtOAc:MeOH

with an increasing amount of MeOH gradually to yield three main fractions (A, B and C).

Determination of antioxidant activity using β-carotene linoleate model system: Antioxidant activity was measured using the methods of Mokbel and Hashinaga[5]. Briefly, a 3.34 mg mL^{-1} β-carotene solution in chloroform, 40 mg of linoleic acid and 400 mg of Tween-20 were mixed well. Chloroform was removed at 40°C under vacuum using a rotary evaporator. The resulting mixture was immediately diluted with 5-10 mL of triple distilled water and was mixed well. The emulsion was further made up to 100 mL with 0.01M hydrogen peroxide (H_2O_2). Aliquots (2 mL) of this emulsion were transferred into different test tubs containing 0.1 mL of test samples in methanol. In this experiment BHA was used for comparative purposes. A control containing 0.2 mL of methanol and 4 mL of the above emulsion was prepared. The tubes were placed at 50°C in a water bath. Absorbance's of all the samples at 470 nm were taken at zero time and every 20 min until the color of β-carotene disappeared in the control reaction, a mixture prepared same as above mentioned, but without β-carotene.

Free radical scavenging activity assay: This spectrophotometer assay uses the stable radical DPPH solution as a reagent. Fifty μL of various concentrations (0-1 mg mL^{-1}) of the extracts in methanol was added to 2.950 mL of a 1 mM methanol solution of DPPH solution at sample concentration. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. After 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm.

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the plot of inhibition percentage of DPPH radical against extract concentration. Fifty microliter of various concentrations of the extracts in methanol was added to 2.950 mL of a 1 mM DPPH solution. The mixture was shaken immediately after adding DPPH solution and allowed to stand at room temperature in the dark and the decrease in absorbance at 517 nm was measured after 30 min. These experiments were run in duplicate and calculated according to the formula described by Mokbel and Hashinaga[5].

TLC analysis of different extracts: Hexane, EtOAc, *n*-butanol and MeOH either crude, fractions, isolated compounds or corresponding control were performed on aluminium sheets of silica gel 60 F_{254} plate for determination of R*f* value for each sample. The spots were applied in as equal amounts as possible. The plates were developed with appropriate solvent for each sample such as $CHCl_3$:MeOH:hexane (5:1:2), $CHCl_3$:MeOH:H_2O (5:1:0.5), EtOAc:MeOH:hexane (5:1:2), $CHCl_3$:acetone:MeOH:H_2O (5:3:2:0.5), $CHCl_3$:acetone:MeOH:H_2O (5:3:3:1), butanol:acitic acid:H_2O:MeOH (2:1:1:0.1)). The resulting bands were located by both UV-light and 10% sulfuric acid followed by heating in the oven for about 5-10 min at 120°C.

NMR: Nuclear Magnetic Resonance (NMR) spectra were recorded on a JEOL ECA600 spectrometer operated at 600 MHZ for ^{1}H-NMR and at 150 MHZ for ^{13}C-NMR including HMBC, HMQC and ^{1}H-^{1}H Cosy. The spectra were observed on D_2O containing TMS as an internal standard.

Statistical analysis: Variance analysis of the results was taken using averages±SD or by two-way analysis of variance (ANOVA) with mean separation by Fisher PLSD test ($p \leq 0.05$). Each value is the mean of three replications.

RESULTS AND DISCUSSION

Chromatographing on silica gel column: *n*-butanol crude extract of flavedo tissue (500 g) was fractionated using silica gel column chromatography (CC) and purified using preparative TLC (PTLC). However, the column chromatography was eluted with CH_2Cl_2. FEtOAc:MeOH (Fig. 1) with an increasing amount of MeOH gradually to yield two main fractions 0:5:1 and 0:1:1 (CH_2Cl_2:EtOAc:MeOH). All two fractions recorded various antioxidant activities (B and C). Fraction B was further chromatographyed on a silica gel column and eluted with EtOAc:MeOH (9-5:0.5) more than two time and was followed by preparative TLC plates silica gel 60 F_{254} (Merch Ltd. Japan) using CH_2Cl_2:acetone:MeOH:H_2O (5:3:3:0.5) as a solvent system to give active antioxidant compounds glycoside compound (1) (50 mg/100 g dry weight extract) and monosaccharide compound (2) (62 mg/100 g dry weight extract). Fraction C was further chromatographed on silica gel column and eluted with EtOAc:MeOH (5-1:1), yielding total soluble sugar. Soluble fraction was subjected to sephadex LH-20, eluted with acetone:H_2O (9:1) and further purifications was subjected to preparative TLC using *n*-butanol:acetic acid:MeOH:H_2O (2:1:0.5:1) to give compounds 3, 4 and 5 sucrose, glucose and fractose) 562, 246 and 562 mg/100 g dry weight, respectively (Fig. 1). Most of isolated compounds are available commercially and identification was achieved by direct comparison with authentic samples.

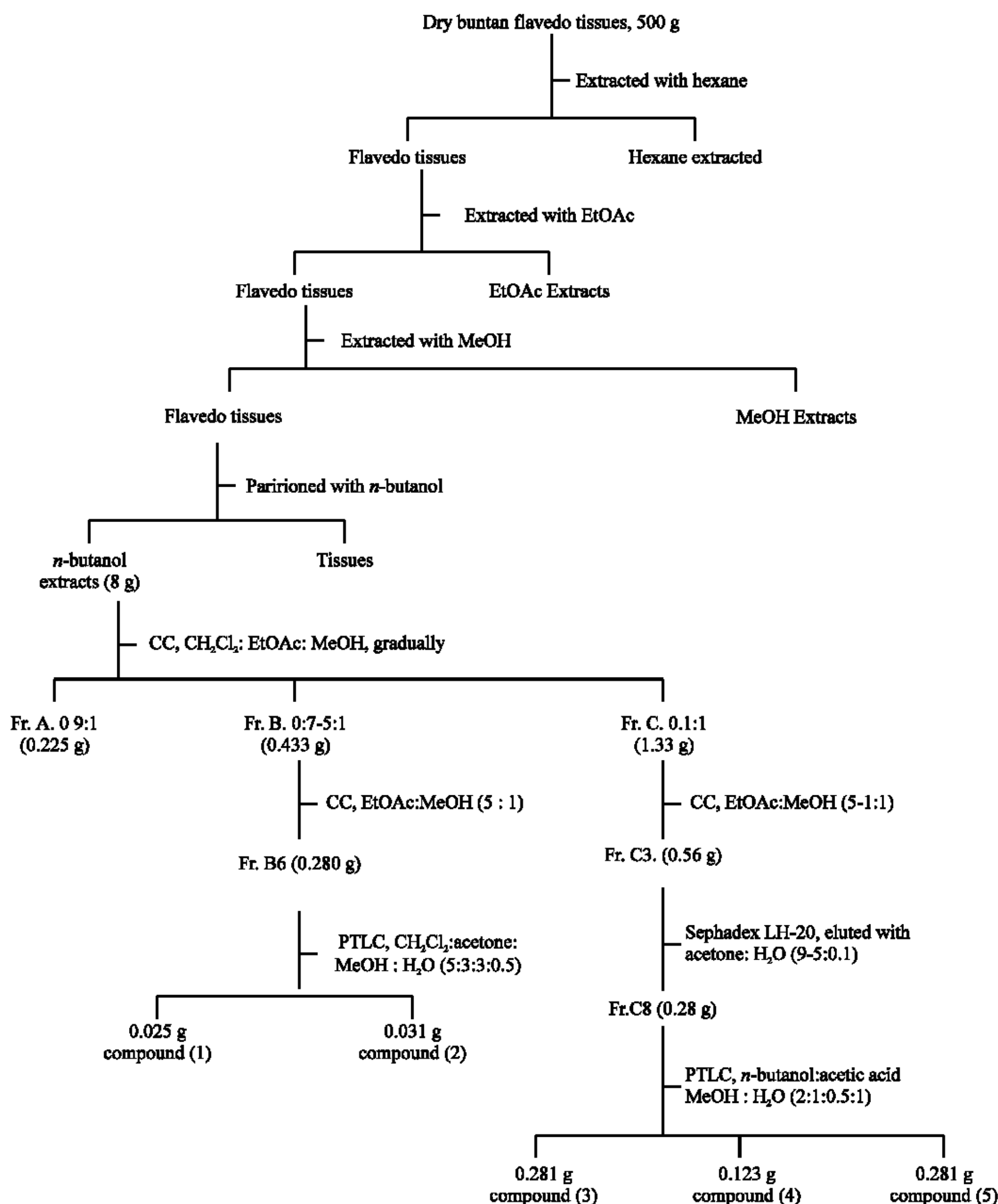

Fig. 1: Separation scheme of the antioxidants substances from flavedo

Fructose, glucose and sucrose were the major soluble sugars in soluble fractions of buntan fruit flavedo tissues as determined using ^{1}H and ^{13}C-NMR spectra. The doublet at 5.42 ppm is due to H-3' of sucrose; the triplet at 4.05 ppm is due to H-4' of sucrose; the resonance signals at 3.89, 3.82, 3.77, 3.68 and 3.56 ppm have also been assigned to sucrose. The doublets at 5.24 and 4.65 ppm are due to the anomeric protons of glucose, α-glucose H-1 and β-glucose H-1, respectively; the triplet at 3.25 ppm is due to the β-glucose H-2. The doublet at 4.12 ppm is the only resonance signal clearly assigned to fructose. The resonance signals in the region from 3.98 ppm are mainly due to overlapping resonances of ring protons of sucrose, fructose and glucose. These results corresponding with ^{1}H-NMR data of Raffo *et al.*[6]. However, sucrose, fructose and glucose showed insignificant antioxidant activities as measured by DPPH free radical.

The chemical structure of monosaccharide compound (2) was studied by ^{13}C-NMR spectroscopic analysis. The signal at 170.5 ppm is from C-6 of α-galacturonic acid and that at 100 ppm also corresponds to C-1 of α-galacturonic

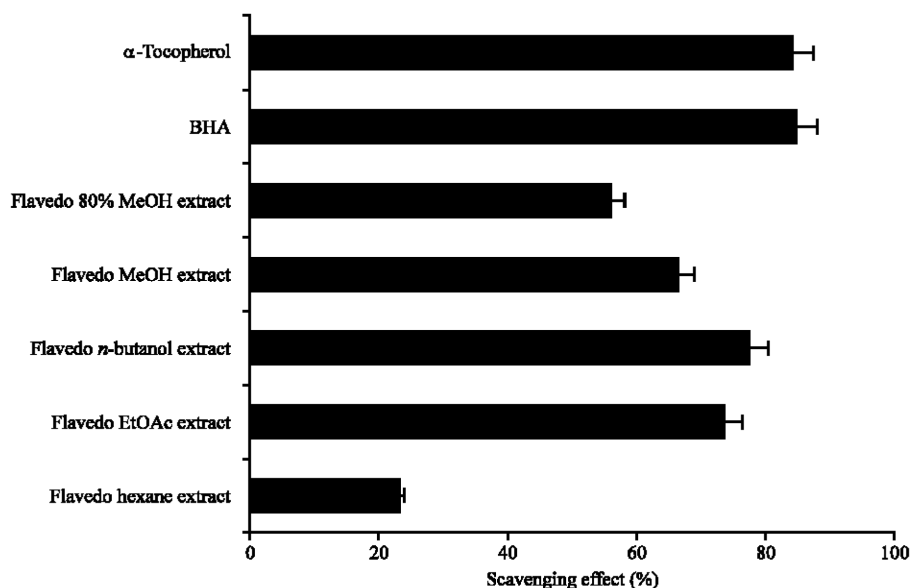

Fig. 2: Antioxidant activity of the extracts of buntan fruit tissues were added at 0.5 mg mL^{-1} as measured by free radical scavenging effects

acid. The signal in 52.8 ppm corresponds to *O*-methyl ester (OMe) groups at C-6. The evidence for starch in soluble fraction was characteristic signals in its ^{13}C spectrum at 100.4 ppm (C-1), 71.3 ppm (C-2), 74.1 ppm (C-3), 79.2 ppm (C-4), 72.3 ppm (C-5) and 61.6 ppm (C-6). Glucose was the major monosaccharide present in the soluble fraction. These results are in agreement with Iagher *et al.*[7]. From the results obtained by characterization of the neutral sugar composition of glucose, galacturonic acid unit and starch it became evident that the profile of monosaccharide could serve as a fingerprint.

Antioxidant activities: The antioxidant effects of crude extracts from various solvents of buntan fruit flavedo tissues was investigated and the results indicated that the *n*-butanol extract recorded significant antioxidant activities. However, our investigations for antioxidant activities were focused in *n*-butanol extracts of buntan fruit tissues due to displayed higher antioxidant activate using DPPH free radical (Fig. 2) and compared to BHA and α-Tocopherol at concentration 0.5 mg mL^{-1}. The results showed that the inhibitory activity of *n*-butanol extracts of buntan fruit flavedo using free radical method closed to the synthetic antioxidant reagent of BHA. Increasing the polarity throughout the extracts exhibited stronger activity, indicating that polyphenols or flavanones and flavonoids may also play important roles in the activities. These results were in agreement with Tepe *et al.*[8]. In all cases, the control without addition of antioxidant was oxidized more rapidly, while the sample elicited inhibition of bleaching of β-carotene. The free radical scavenging activity of *n*-butanol extract was superior to all other extracts, followed by EtOAc extracts and MeOH extract, while hexane extract showed little antioxidant activity. DPPH is a stable free radical that shows maximum absorption at 517 nm in methanol. The antioxidant activity of a substance can be expressed as its ability in scavenging the DPPH free radical. Free radical effects of each fraction were measured and the results showed that for fraction B (7-5:1) better antioxidative activity was recorded, data not shown. The antioxidant activity found in those fractions of Buntan fruits flavedo, should be attributed to the presence of flavonoids and other phenolic compounds. This information shows that *n*-butanol fraction of flavedo tissue waste can be used as antioxidant in food and medicinal preparations. Results showed that the butanolic fractions possessed significant radical scavenging activity approaching the activity of standards examined by the same tests.

Glycoside and monosaccharide components isolated and used as standard had a higher antioxidant activity than sucrose, glucose, fructose or control at concentration 100 mg mL^{-1}. The absorbance values for all the samples decreased with incubation time. The antioxidant activities of compounds isolated were

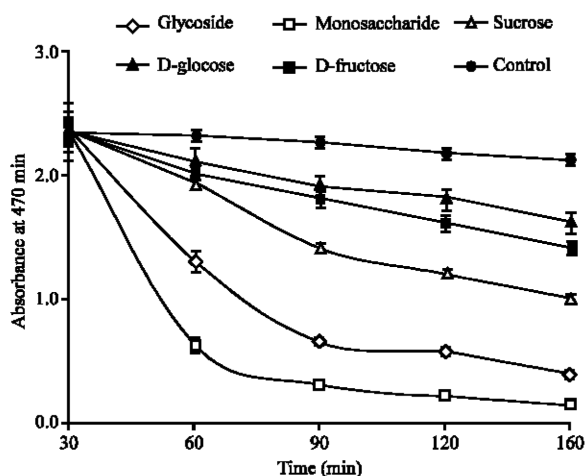

Fig. 3: Time course of absorbance reduction of isolated compound as measured by the decolorization reaction of β-carotene

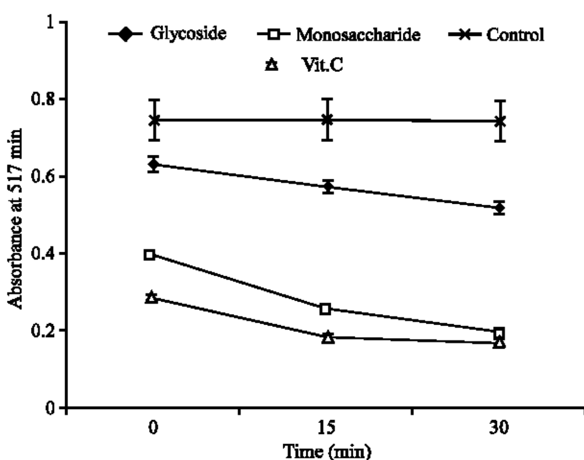

Fig. 4: Time course of absorbance reduction of isolated compounds and vitamin C using the decolorization reaction of free violet DPPH radicals from 0-30 min

evaluated by measuring their ability to scavenge the radical and the superoxide anion and to inhibit β-carotene oxidation in a lipid micelle system (Fig. 3). Glycosides and monosaccharide are important naturally occurring compounds specifically distributed in *Citrus* buntan fruit flavedo (Fig. 4). Due to their polyphenolic structure, these compounds possess health-related properties, which are based on their antioxidant activity. This result is in agreement with data of Claudia *et al.*[9]. Flavanone glycosides have been used as chemotaxonomic markers in quality control to identify adulterated processed foods. However, methanolic fruit extracts and crude polysaccharide extracts exhibited stronger antioxidant activity than purified polysaccharide fractions because crude extracts were identified to be rich in antioxidants (e.g., carotenoids, riboflavin, ascorbic acid, thiamine and naringen). This data is in agreement with data of Qiong *et al.*[10]. Results showed that flavedo extract exhibits interesting antioxidant properties, expressed by its capacity to scavenge DPPH and β-carotene bleaching. IC_{50} values (concentration of sample required to scavenge 50% of free radicals or to prevent lipid peroxidation by 50%) were calculated from the regression equations prepared from the concentrations of the extracts and percentage inhibition of free radical formation/percentage inhibition of lipid peroxidation in different systems of assay. IC_{50} activities of isolated compounds were, 50 μg mL^{-1} glycosides, 150 μg mL^{-1} monosaccharide, 800 μg mL^{-1} crude extract and 500 μg mL^{-1} total soluble sugar.

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