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Purification of the Antioxidant and Antimicrobial Substance of Ethyl Acetate Extracts from Buntan (*Citrus grandis* Osbeck) Fruit Peel

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Abstract: The extracts of buntan (*Citrus grandis* Osbeck) fruit peel were screened for antioxidant and antimicrobial activities using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging and paper disc diffusion methods. Non-polar extracts exhibited significant antimicrobial activity, while polar extracts exhibited significant antioxidative activity. The ethyl acetate (EtOAc) extract of flavedo were separated with silica gel column chromatography (CC) and four active fractions A, B, C and D (14:1, 9:1, 5:1 and 1:1) were obtained from the eluents of benzene:acetone. The inhibitory activity of isolated compounds using Minimum Inhibition Concentration (MIC) against Gram-negative and positive bacteria strong antimicrobial was recorded as follows oxypeucedanin hydrate (100-290 ppm), oleic acid (150-350 ppm), meranzin hydrate (620-850 ppm) and linoleic acid (700-950 ppm). The antioxidant inhibition concentration 50% (IC_{50}) for isolated components strongest activities were recorded as follows sitosterol linoleate described as a new isolated compound from the plants (95 ppm), meranzin hydrate, (160 ppm), isomeranzin (245 ppm) and oxypeucedanin hydrate (330 ppm) was recorded.

Key words: Buntan fruit peel, antimicrobial, antioxidant, β-sitosterol, linoleic acid, meranzin hydrate, isomeranzin, oxypeucedanin hydrate, sitosterol linoleate

INTRODUCTION

Citrus fruits have an antimicrobial activity and the isolated coumarins had high activity against the microorganisms[1]. Some of coumarins showed anti-HIV activity[2]. Furanocoumarins isolated from *Angelicae dahuricae* was investigated by Piao *et al.*[3] and these compounds contacting with hydroxyl group displayed potent antioxidant effects against the DPPH radical. It was found that the content of total polyphenols in the *Citrus* fruit peels was significantly higher than the other parts, which indicated that peels of fruits are major sources of natural antioxidant[4]. The coumarin glycosides present in *Citrus* peel oils have been investigated extensively and are useful taxonomic markers[5]. The essential oils showed antifungal activity as described by Zambonelli *et al.*[6]. The nonvolatile residue of *Citrus* essential oils greatly influences the olfactory properties of these oils. The oxygen heterocyclic compounds (coumarins, psoralens and polymethoxylated flavones) present in *Citrus* essential oils[7]. The acetone extract of root bark of *Citrus grandis* Osbeck also yielded flavone, coumarin and alkaloids[8,9] which also exhibit antimicrobial properties. Consequently, the development of natural antioxidants has become important in the field of food science. Dietary recommendations for healthy eating include the consumption of fruit juices whose beneficial health effects are mostly ascribed to natural antioxidants. As these compounds are predominantly found in most of fruit tissues, it would be worthwhile investigating the nature of polyphenols that are present in *Citrus* fruits.

So far, there is no much research work in screening antimicrobial and antioxidant activities in buntan peel waste. So, the present investigation was undertaken to evaluate the antioxidant and antibacterial power of buntan fruit peel, thereafter to isolate, identify and determined their antioxidant and antibacterial activities.

MATERIALS AND METHODS

Plant materials: *Citrus grandis* Osbeck (buntan) fruits were purchased in Kagoshima city in 2003. The flavedo (2.4 kg) and albedo (3 kg) of buntan fruits were separated and then dried at 45°C for 7 days and ground in an electric blender, yielding fine dry samples about 700 g albedo and 500 g flavedo. They were successively soaked *n*-hexane, EtOAc, *n*-butanol and MeOH. The extracts were dried under a vacuum using a rotary evaporator at 40°C to pursue further analysis.

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Antioxidant activities assay: The free radical of scavenging activity of flavedo was measured according to the method descried by Mokbel and Hashinaga[10]. Briefly, a 0.5 mM solution of DPPH (1,1-diphenyl-2-picrylhydrazol) in methanol and 0.05 M acetate buffer pH (5.5) was prepared. An aliquot of 0.1 mL (0.5-1 mg mL^{-1}) of an extract solution was added to a mixture of 2 mL acetate buffer, 1.9 mL methanol and 1 mL DPPH solution.

Extract providing inhibition concentration 50% (IC_{50}): This method was calculated from the plot of inhibition percentage of free radical scavenging to decrease by 50% against extract concentration. Fifty microliter of various concentrations of the extracts in methanol was added to 2.950 mL of a 1mM 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.

Aluminum thin-layer chromatography (TLC) assay: Was measured according to the method descried by Mokbel and Hashinaga[10], all fractions and isolated components were performed on aluminium sheets of silica gel 60 F_{254} plate for thin-layer chromatography.

Antimicrobial assay: The antimicrobial activity was measured according to the method descried by Mokbel and Hashinaga[11]. Briefly, the bacteria stock culture media were maintained on nutrient hard and soft agar medium. To determine the antibacterial activity the microorganisms were cultured in nutrient broth at 36°C overnight.

Determination of MIC: The Minimum Inhibitory Concentrations was measured according to the method descried by Mokbel and Hashinaga[11] and were defined as the lowest concentration (ppm) of the extract in agar plates showing no visible bacterial growth.

Gas chromatography/mass spectrometry: According to method descried by Mokbel and Hashinaga[12]. Molecular weight was determined using gas chromatography (Thermo Finnign Polaris Q) equipped with an EI ion source coupled to an MS (Polaris Q). Helium was used as gas carrier and the injector temperature was kept at 250°C.

Nuclear Magnetic Resonance (NMR) spectra: According to method descried by Mokbel and Hashinaga[11]. Data was recorded on a JEOL ECA600 spectrometer operated at 600 MHz for 1H NMR and at 150 MHz for ^{13}C NMR including HMBC, HMQC and ^{1}H-^{1}H Cosy.

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 ($p<0.05$). Each value is the mean of three replications.

Isolation and identification: The ethyl acetate extract of flavedo (7.34 g) was fractionated by silica gel 60 column chromatography. The column chromatography was carried out with benzene:acetone (Fig. 1) with an increasing amount of acetone gradually to yield four main fractions 14:1, 9:1, 5:1 and 1:1 (A, B, C and D). Among them, three fractions had antioxidant activity (A, B and C) and also three fractions antimicrobial activity (B, C and D) were recorded. Fraction A was further chromatographed on a silica gel column and eluted hexane: EtOAc (9:1) more than two time and was followed by preparative TLC (PTLC) plates silica gel 60 F254 (Merch Ltd. Japan) using chloroform:hexane (1:1) as a solvent system to give new active compound sitosterol linoleate compounds (1) (13.4 mg/100 g dry weight). Insignificant antimicrobial and antioxidant activities to methyl linoleate compounds (2) (8 mg/100 g dry weight) was recorded. Fraction B was further chromatographed on silica gel column and eluted with hexane:EtOAc (2:1), yield two active antimicrobial fractions (B.1 and B.2). Fraction (B.1) was further subjected to PTLC plates using CH_2Cl_2:hexane (4:1) and remarkable compounds were scraped off and eluted with chloroform:methanol (1:1) to give isomeranzin (7-methoxy-8-(2-oxo-3-methyl butyl)) compound (4) (2 mg/100 g dry wt.). Fraction (B.2) was subjected to a silica gel column and eluted with hexane:EtOAc (1:1) to yield fraction (B.5) and followed by loading in PTLC plat using EtOAc:hexane (1:1) to give β-sitosterol compound (3). Fraction (C) was subjected to a silica gel column and eluted with $CHCl_3$:MeOH (9:1) to yielded two active fractions (C.1 and C.2). Fraction (C.1) was subjected to a silica gel column and eluted with hexane:EtOAc (2:1) and followed by loading in PTLC plat using methyl ether:hexane:MeOH (5:1:0.1) to give linoleic acid (4.2 mg/100 g dry weight) compound (5). While, fraction (C.5) was subjected to a silica gel column and eluted with EtOAc:MeOH (9:0.5) to yield one active antioxidant and antimicrobial fraction (C.10) and followed by loaded PTLC using $CHCl_3$:MeOH:H_2O (5:1:0.1) to give meranzin hydrate (7-methoxy-8-(2',3'-dihydroxy-3'-methyl butyl)) compound (6) (8 mg/100 g dry weight). Fraction D was subjected to silica gel column eluted with EtOAc:hexane (5:1) and followed by CH_2Cl_2:MeOH (9:0.5) to yield one active fractions (D.8). Fraction (D.8) was loaded on PTLC plates using $CHCl_3$:MeOH:hexane (5:1:3) to give oxypeucedanin hydrate compound (7) (2 mg/100g dry weight). The fractions during purifications were monitored by TLC.

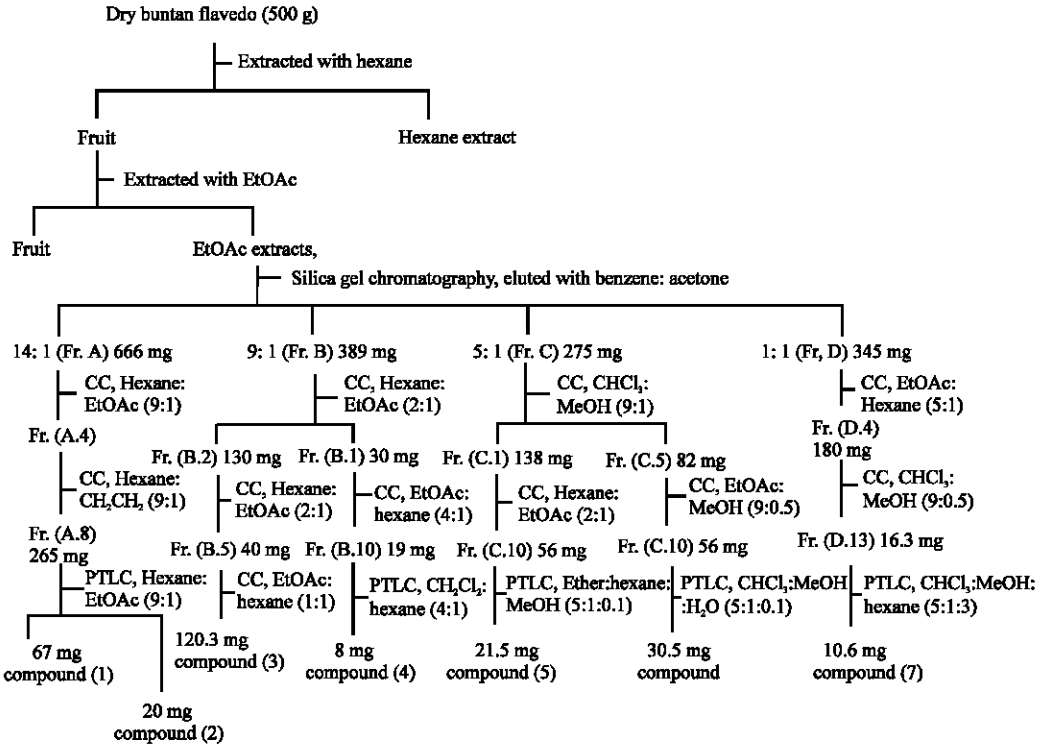

Fig. 1: Separation scheme of the antioxidants and antimicrobial substance from flavedo

Chromatographic separations of albedo ethyl acetate extract (6.32 g) were subjected to a silica gel column and eluted with hexane:ethyl acetate to gradually produce four fractions 5:1, 1:1, 1:3 and 1:5 (A, B, C and D). Fraction (C.1) was subjected to silica gel column and eluted with benzene:acetone (3:1) and followed by silica gel column eluted with $CHCl_2$:MeOH (9:0.5) to yield two active fractions (C.4 and C.6). Fraction (C.6) was further loaded on PTLC and plates using $CHCl_3$:MeOH:hexane (5:1:2) produced one active antimicrobial fraction (C.22) and followed by with mobile phase $CHCl_3$:MeOH:water (5:1:0.1) yielding oleic acid compound (8) (4.7 mg/100 g dry weight).

RESULTS AND DISCUSSION

The antimicrobial and antioxidant abilities of buntan fruit extracts were shown to be a particularly interesting field for applications on the food and cosmetics industries. Both the flavedo and albedo tissues were shown to be effective sources of inhibitory activity against bacterial strain tested. Some of isolated compounds are available commercially and identification was achieved by direct comparison with authentic samples. ^{1}H-,^{13}C-NMR, HMBC and HMQC of meranzin hydrate and isomeranzin spectra matched perfectly authentic of Dugo *et al.*[7] and Barik *et al.*[13]. As shown in Fig. 2, the ^{1}H- and ^{13}C-NMR spectrum of a new isolated compound (1) were matched perfectly authentic sitosterol linoleate. Oxypeucedanin hydrate showed as a yellowish color on TLC under UV light and the molecular formula was established as $C_{16}H_{17}O_6$. The ^{1}H-, ^{13}C-NMR, HMBC and HMQC spectra matched perfectly authentic Fujioka, *et al.*[14]. Oxypeucedanin hydrate isolated for the first time from buntan fruit peel. As shown in Table 1, EtOAc extracts of flavedo 9:1, 5:1 and 1:1 (benzene:acetone) exhibited higher antimicrobial activity compared to other fractions. The presented data indicated that the extracts of low polarity solvents exhibited strong antimicrobial activity, while antioxidant activities increased with increasing the polarity of the solvents. It seems that low polarity showed low antioxidant and high antimicrobial activities. However, fatty acid component isolated from EtOAc extracts of flavedo showed high yield. The yields and the antioxidant activities of extracts using different solvents, were significantly different ($p>0.05$). EtOAc extract of flavedo eluted on silica gel column chromatography with benzene:acetone displayed significantly antioxidant. Sitosterol linoleate acting as antioxidant substance as shown in Table 2. In fact, measuring the activity of linoleic acid and β-sitosterol separately non antioxidant activity was recorded.

Table 1: Antibacterial activity of buntan flavedo extracts using disc diffusion assay (1mg/disc)

	Extracts of flavedo				Eluate of benzene:acetone			
Bacteria	Hexane	EtOAc	Butanol	MeOH	14:1	9:1	5:1	1:1
Gram positive bacteria								
Staphylococcus aureus	–	+++	–	++	–	++	++	+++
Bacillus subtilis	–	++	–	++	–	++	+	+++
Bacillus cereus	–	+++	–	++	–	++	+	+++
Gram negative bacteria								
Salmonella enteritidis	–	++	–	+	–	+	+	++
Escherichia coli	–	++	–	+	–	+	+	++

Values are means ±SD of 9 paper disc for each three replications were used. Sterile paper discs (6 mm diameter) were impregnated with fruit tissue extracts (1mg mL^{-1}).: No antimicrobial activity, inhibition zone of sample <0.5 mm; +: Slight antimicrobial activity of sample 1-5 mm. ++: Clear antimicrobial activity, of sample 5-10 mm; +++: Strong antimicrobial activity, of 10-15mm

Table 2: DPPH scavenging activity of antioxidant constituents from buntan flavedo fractions and isolated compounds

	Crude extracts and fractions			Isolated compounds			
Buntan fruit tissues	Eluted solvent	Yield (g/100g dry wt.)	DPPH activities (%)	Compounds	Yield (mg/100g dry wt.)	DPPH activities (%)	IC_{50} ppm
Crude extracts	Hexane	0.04	24.1±0.7	β-sitosterol linoleate	13.4	82.5±0.7	110
	EtOAc	1.2	72.8±0.5	Meranzin hydrate	8.0	78.4±0.2	200
	Butanol	3.6	76.2±1.2	Isomeranzin	2.0	45.7±0.6	450
	MeOH	6.1	68.3±1.5	β-sitosterol	24.0	18.0±1.3	–
	70% MeOH	8.4	65.7±0.4	Oxypeucedanin hydrate	2.0	34.1±0.7	–
EtOAc extracts				Linoleic acid	4.2	–	–
Fraction A	Benzene: acetone (14:1)	0.133	56.4±0.8	Methyl linoleate	6.0	–	–
Fraction B	Benzene: acetone (9:1)	0.078	76.9± 1.1	Oleic acid	4.7	–	–
Fraction C	Benzene: acetone (5:1)	0.055	79.1±0.1	BHA		84.3±0.2	50
Fraction D	Benzene: acetone (1:1)	0.07	43.3±0.6	BHT		82.8±0.3	70
BHA			84.3±0.2				
BHT			82.8±0.3				

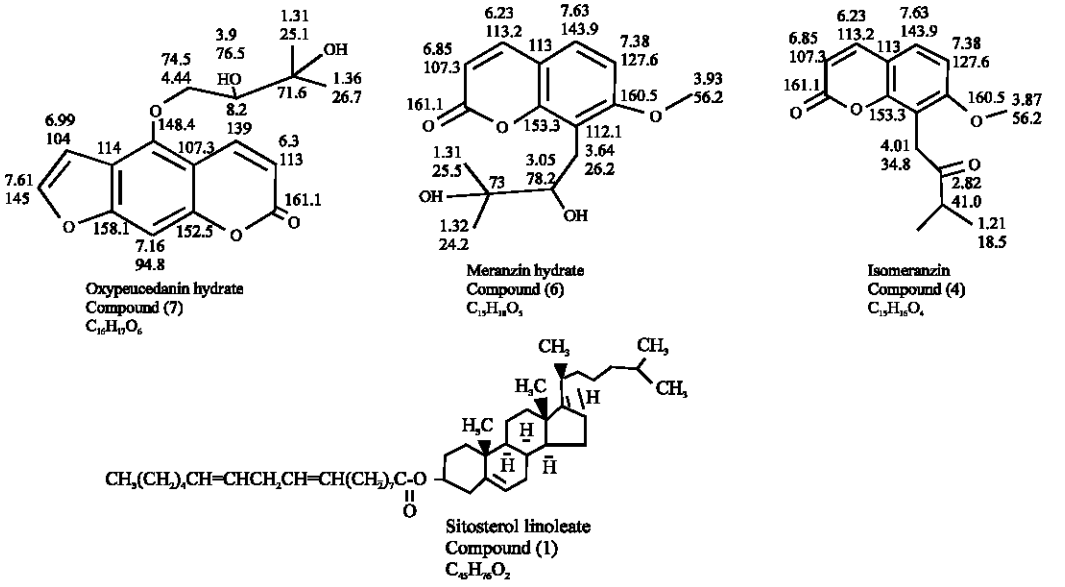

Fig. 2: Compounds isolated from flavedo of buntan fruit tissues

Normally in the nature linoleic acid in position COOH attached with β-sitosterol in the position of OH and changed to ester group with high antioxidant was recorded.

Antioxidant activities of isolated components were determined using IC_{50}, i.e., the concentration to inhibit free radical by 50%. Sitosterol linoleate showed strong antioxidant activity against DPPH free radical and the IC_{50} 95 ppm, meranzin hydrate 160 ppm, isomeranzin 245 ppm and oxypeucedanin hydrate 330 ppm was recorded. Those compounds with dihydroxy groups were more active in antioxidant and antimicrobial than compound with oxo and methoxy group[15]. Thus isolated components were tested against Gram-negative and

Table 3: Antibacterial activity of the isolated compounds from EtOAc extract of buntan flavedo using agar disc diffusion and MIC methods

	Isolated compounds										Standard antibiotics	
	Linoleic acid		*Oleic acid		Meranzin hydrate		Isomeranzin		Oxypeucedanin hydrate		chloramphenicol	
Microorganisms	DD[a]	MIC[b]	DD[a]	MIC[b]	DD[a]	MIC[b]	DD[a]	MIC[b]	DD[a]	MIC[b]	Dd[a]	MIC[b]
Gram positive bacteria												
Staphylococcus aureus	3.7±0.4	900	11.2±0.6	250	9.60±0.2	750	5.1±0.6	NT	12.1±0.3	240	25 ±0.5	5.5
Bacillus subtilis	3.2±0.3	800	10.5±0.4	350	10.3±0.3	680	5.3±0.3	NT	13.5±0.1	290	24 ±0.3	5.5
Bacillus cereus	4.1±0.1	700	10.9±0.3	250	8.70±0.1	620	4.8±0.5	NT	14.1±0.3	200	26 ±0.7	5.5
Gram negative bacteria												
Salmonella enteritidis	3.2±0.3	900	10.4±0.2	150	8.50±0.5	800	3.8±0.3	NT	11.8±0.5	100	20 ±0.5	5.5
Escherichia coli	2.9±0.4	950	9.8±0.2	270	7.90±0.3	850	3.1±0.4	NT	10.6±0.4	230	19 ±0.5	5.5

[a]DD, agar disc diffusion method. Sterile paper (6 mm diameter) were impregnated with fruit extracts (0.1mg/disc). Diameter of inhibition zone (mm) unincluding disk diameter of 6 mm. NT, not tested. [b]MIC, minimum inhibitory concentration; values given as ppm for the isolated compounds and chloramphenicol antibiotics as a standard. Values are means ± SD of 9 paper disc for each three replications were used.*Oleic acid was extract from buntan albedo tissue

positive bacteria. The present data Table 3 showed that, MIC of linoleic acid was 700-950 ppm, oleic acid 150-350 ppm, meranzin hydrate 620-850 ppm and oxypeucedanin 100-290 ppm. Oxypeucedanin hydrate used also against human gastric adenocarcinoma (MK-1) cell growth[14]. Fatty acid and essential oil (non-volatile) has antimicrobial effect as demonstrated by Dilika *et al.*[16,1,17]. However, buntan fruits have different levels of susceptibility to decay caused by Gram-negative and positive bacteria. EtOAc extracts from flavedo and albedo of buntan fruit displays potential antimicrobial and antioxidant activity. The results of the present study indicate that the selective extraction of antioxidant and antimicrobial from natural sources by an appropriate solvent is very important for obtaining components with high antioxidant and antimicrobial activity. Some isolated compounds have biological effects on the growth of bacteria and others have antioxidant effect. However, buntan fruits tissues contain groups of sitosterol, limonoid, fatty acid, coumarin, furanocoumarin and other essential oil (non-volatile fractions). Isolated compounds such as sitosterol linoleate showed to be a particularly interesting substances for applications on the food (*Citrus* and others fruits) and cosmetics industries. In fact, our data indicated that those components attached with ester, hydroxyl and/or carboxyl group have interesting biological effect on bacteria metabolism and antioxidant role.

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