Evaluation of the Antimicrobial Activity of Extract from Buntan (Citrus grandis Osbeck) Fruit Peel

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Abstract: Antimicrobial substances were extracted from buntan (Citrus grandis Osbeck) fruit peel (flavedo and albedo) using various solvent such as n-hexane, ethyl acetate (EtOAc), butanol and methanol. The antimicrobial activity of the extracts was evaluated using the disc diffusion method, growth inhibition (%) with fungi growth and Minimum Inhibitory Concentration (MIC). Antimicrobial substance of EtOAc extracts of flavedo and albedo were fractionated using silica gel column chromatography (CC). Further purification to the albedo active fraction resulted in two active compounds, β-sitosterol (1) and oleic acid (2). Antibacterial activities of the compounds against Gram-negative and positive bacteria by using MIC was 270-350 ppm in β-sitosterol and 150-350 ppm in oleic acid. Their extracts could well be useful to prevent oxidation in fruit juices and essential oils of food products as well as for health supplements.

Key words: Buntan, fruit tissue extracts, antimicrobial, β-sitosterol, oleic acid, limonin

INTRODUCTION

Citrus essential oils are present in fruit tissues at various quantities. The antifungal efficacies of the oils were studied by Caccioni et al. it was determined that a positive correlation exists between monoterpenes and sesquiterpenes oil content and the pathogen fungi inhibition. The essential oils tested in vitro against the pathogenic fungi inhibited fungal growth. The citrus fruit tissues proved effective sources of inhibitory sources against cyclooxygenase and lipooxygenase.

Alzoreky and Nakahara demonstrated that extracts of acetone and buffered methanol (80% methanol and 20% of 5 mM sodium phosphate, pH 7.4) recorded significant antibacterial activity, as revealed by the disc assay. However, Dilika et al. recorded that fatty acids such as linoleic acid and oleic acid exhibited antibacterial activities. The acetone extract of root bark of Citrus grandis Osbeck also yielded flavone, coumarin and alkaloids Wu et al. which also exhibit antimicrobial properties. Antimicrobial activity was found to be present in multiple in Citrus grandis Osbeck (Buntan) fruit peel, (flavedo, albedo, juice sacs or membrane).

The goal of present research was to evaluate the antibacterial and antifungal activities of fractions and determine Minimum Inhibitory Concentration (MIC) for each compound isolated from buntan fruit tissues. The following illustrates research undertaken to report the isolation of β-sitosterol (1), oleic acid (2) and limonin (3) from albedo tissues of buntan fruits and their antimicrobial activities.

MATERIALS AND METHODS

Plant material and extraction methods: Buntan (Citrus grandis Osbeck) fruits were purchased in Kagoshima city for investigations. Fruits tissues (flavedo, albedo and segment membrane) were removed and dried separately at 45°C for 7 days and grinded in an electric blender, yielding fine dry samples: 700 g albedo, 500 g flavedo and 270 g segment membrane. Buntan fruit tissues were successively extracted using non-polar to polar solvents: n-hexane, ethyl acetate, n-butanol and methanol. Each sample was extracted three times at room temperatures (25°C) 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.

Microbial strains: Five species of bacteria were obtained from IFO Osaka Japan as follows: Bacillus cereus IFO 13597, Salmonella enteritidis IFO 3313, Escherichia coli IFO 13168, Bacillus subtilis IFO 3009, Staphylococcus

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_ aureus_ IFO 3761. Three species of moulds were obtained from the stock cultures at the Department of Plant Pathology Lab. of Kagoshima University and Ministry of Agriculture, Forestry and Fisheries, Japan as following: _Botrytis cinerea_ Persoon: Fr., _Rhizopus stolonifer_ (Ehrenb.) Vuill. and _Penicillium expansum_ Link.

**Bacteria culture media:** The bacteria stock cultures media were maintained on nutrient hard agar (peptone 1 g, meat extract 0.5 g, sodium chloride 0.25 g and agar 1 g/100 mL H₂O) slants which were stored at 4°C and subcultured every month. The bacterial culture medium was prepared as following: Nutrient Broth (NB) (peptone 0.5 g, meat extracts 0.25 g, sodium chloride 0.25 g/50 mL H₂O) and soft agar (peptone 0.5 g, meat extracts 0.25 g, sodium and chloride 0.125 g and agar 0.2 g/50 mL H₂O). However, the medium were adjusted to pH 6.6 and autoclaved at 121°C for 20 min. Other materials used in this experiment, such as paper discs, Petri dish microchop and pinset were sterilized at 100°C for 12 h.

To determine the antibacterial activity, describing growth conditions and preparation of spore suspensions were undertaken as following, the microorganisms were cultured in NB at 36°C overnight. After the growth conditions observed 3-5 µL of the inoculums (spores) NB medium was added to 3.5 mL soft nutrient agar and well shaken. The soft nutrient agar was then added onto a petri dish containing 15 mL hard agar. Filter paper discs (6 mm diameter), were impregnated with different extract concentrations (0.1 to 1 mg/discs) and allowed to dry completely for 10-15 min, then evenly placed on the surface of previously inoculated agar. The pure solvent of chloroform and methanol was added as a blank to the paper disc for comparison with inhibition zones of sample extracts. The petri dishes were incubated at 36°C for 24 h. Antibacterial activity was indicated by the appearance of clear inhibition zones around the discs. The inhibition zones mean that place around the paper discs were the bacteria inhibited by using extracted sample. Chloramphenicol was used as positive controls.

**Dilution method:** The Minimum Inhibitory Concentrations (MIC) were defined as the lowest concentration (mg L⁻¹) of the extract in agar plates showing no visible bacterial growth. The soft nutrient agar was then added onto a petri dish containing 15 mL hard agar as mentioned above. The samples (β-sitosterol, oleic acid and limonin) were dissolved in chloroform and methanol. The solutions were then individually added at different concentrations from 0 (control), to 1 mg mL⁻¹ to soft agar and mixed well before being poured into sterile Petri dishes containing 15 mL hard agar. The cultures (5 µL) were taken from nutrient broth (as mentioned above) and added to three places on the medium surface and incubated at 37°C up to 48 h for determination of MIC of the pure compounds isolated.

**Fungi culture media:** Inoculums were prepared by transferring spore from slant tubes of two-week old colonies onto petri dishes containing freshly prepared Potato Dextrose Agar (PDA) medium (hard agar: 200 g potato, 15 mL agar and 20 g dextrose). One milligram of extract was mixed with 3.5 mL soft agar (20 g potato, 0.2 agar and 2 g dextrose) at 50°C, then loaded onto petri dishes containing 15 mL hard agar. Hyphal tips were then harvested from the edge of 6-8 day old culture (6 mm diameter and 3 mm deep) by using a sterile cork borer onto the centre of the media. The petri dishes were inverted and incubated at 25°C until the control reached the edge of petri dishes. The colony diameters were recorded by a digital caliper and the data recorded as growth inhibition (％).

**Thin-layer chromatography analysis as chromatographic separation:** TLC analysis for fractions was performed on aluminium sheets (20x20 cm) silica gel 60 F₂₅₄ plate which were developed with appropriate solvent for each sample such as CHCl₃:MeOH:Hxane (5:1:0.5), CHCl₃:MeOH:H₂O (5:1:0.5) or EtOAc:MeOH:hexane (5:1:2). 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.

**General procedures:** Nuclear Magnetic Resonance (NMR) spectra were recorded on a JEOL FX-400 spectrometer operated at 400 MHz for ¹H-NMR and at 100 MHz for ¹³C-NMR. The spectra were observed on CDC₁₅ 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≤0.05). Each value is the mean of three replications.

**RESULTS AND DISCUSSION**

**Chromatographic separation:** Chromatographic separation of ethyl acetate extract of flavedo (4 g) was carried out on a silica gel 60 column chromatography. Column chromatography was eluted with benzene:acetone (Fig. 1) with increasing amount of acetone gradually to yielded six fractions 14:1, 9:1, 5:1, 1:1 and 0:1 (A,B, C, D and E), among of them two active fractions B and C were recorded. Fraction B was further chromatographed on a silica gel column and eluted hexane:EtOAc (2:1), yielding two active fractions (B.1 and
The fraction (B.1) was further subjected to preparative TLC (PTLC) plates (20x20 cm) silica gel 60 F254, Merck Ltd. Japan using EtOAc:hexane (1:1) as a solvent system. Compounds were scraped off and eluted with chloroform: methanol (1:1) to give β-sitosterol and compound (1) (20.3 mg). The fraction (C.2) was subjected to PTLC plates using ether:hexane (4:1), followed by PTLC using CH2Cl2: hexane (4:1) for more purity as a solvent system and a compound was scraped off and eluted with chloroform: methanol (1:1) yielding a compound under our investigation. The fraction (C) was subjected to a silica gel column and eluted with CHCl3:MeOH (9:1) to yield one active fraction (C.1) and followed by loaded in PTLC plates using hexane: EtOAc (2:1), followed by PTLC using ether:hexane:MeOH (5:1:0.1). A compound under our investigation was scraped off and eluted with chloroform: methanol (1:1).

Chromatographic separations of ethyl acetate extract (6.32 g) of albedo 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) (Fig. 2). Fraction (B) was eluted in silica gel column chromatography with hexane: EtOAc (2:1) followed by crystallization with CHCl3: MeOH (1:1) to produce a pure β-sitosterol compound (1). Fraction (C) was subjected to silica gel column chromatography with benzene:acetone (3:1) and CH2Cl2:MeOH (9:0.5), yielding two fractions (C.6 and C.4). Fraction (C.4) was crystallized with CHCl3: MeOH to produce a pure β-sitosterol (1). Fr. (C.6) was loaded in PTLC plates using CHCl3: MeOH: hexane (5:1:2) to produce one active fraction (C.22) followed by PTLC plates with mobile phase CHCl3: MeOH: water (5:1:0.1) yielding oleic acid compound (2). The fraction (D) was subjected to column chromatography with hexane: EtOAc (1:5) and (1:7) followed by crystallization with (MeOH: CHCl3) producing limonin compound (3) as determined by 1H and 13C-NMR spectra.

β-sitosterol (1), white needles; 1H and 13C-NMR spectra was identical to authentic β-sitosterol. Oleic acid (2) was a whitish powder, as described by Arnhold et al. Limonin compound (3) a white needle was investigated. Present finding of buntn fruit tissue extracts showed strong antimicrobial activity against several bacteria activities as determined by paper disc
Fig. 2: Separation scheme of the antioxidants and antimicrobial substances from albedo

Fig. 3: β-sitosterol, 1, oleic acid; 2 and limonin; 3 from albedo ethyl acetate extract
methods (Table 1). Among all extracts, ethyl acetate extracts of flavedo and albedo exhibited the most significant antibacterial activity. No considerable activity was recorded of EtOAc extracts of segment membranes and juice sacs was recorded. The ethyl acetate extracts of flavedo and albedo were tested for antifungal activities and the data demonstrated that the ethyl acetate extracts of flavedo at p=0.05 had significantly higher activity than albedo extracts under same concentrations 200 to 400 ppm.

The MIC was taken as the lowest concentration that inhibited growth after 48 h of incubation at 36°C. As shown in Table 3 the MIC of β-sitosterol and oleic acid was tested against B. cereus, B. subtilis, S. aureus, E. coli and S. enteritidis and inhibited by 300, 300, 270, 350 and 300 μg mL⁻¹, while the MIC of oleic acid was 250, 350, 230, 270 and 150 μg mL⁻¹, respectively. This data indicated that oleic acid exhibits strong antibacterial activity compared to β-sitosterol. Limonin however, did not show significant activity against bacteria strains tested (Table 3).

Synergistic effects of β-sitosterol and oleic acid could be partly responsible for the highest antimicrobial activity among the compounds isolated and identified from active fraction, which was isolated from EtOAc extracts of albedo in agreement with the results of Ajaiyewo et al.[11]. Oleic acid on the other hand was the most active extract against the gram positive and gram negative bacteria. The two main compounds present in the most active fraction were identified as β-sitosterol and oleic acid. Dilika et al.[13] also reports oleic acid to have antibacterial activity against Gram-positive bacteria tested. Identification of the responsible components for antimicrobial activities is underway.

Fruit tissues extracts showed to be effective sources of inhibitory against antibacterial strain tested. That fraction displays also potential antimicrobial benefit toward paper disc methods. However, the results of the present research indicate that the selective extraction of antioxidant and antimicrobial from natural sources by an appropriate solvent is very important in obtaining components with high antimicrobial activity. The results of the present study indicated that antimicrobial activity of ethyl acetate extracts of flavedo and albedo from buntan fruits showed a high activity toward paper disc methods and MIC. β-sitosterol and oleic acid extracted
from flavedo and albedo of buntan fruits showed higher antimicrobial activity than limonin.

REFERENCES


