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Food Chemistry 94 (2006) 529–534
Food Chemistry
www.elsevier.com/locate/foodchem

Evaluation of the antioxidant activity of extracts from buntan (*Citrus grandis* Osbeck) fruit tissues

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Received 23 August 2004; received in revised form 8 November 2004; accepted 8 November 2004

Abstract

The goal of the present work was to evaluate the antioxidant properties of buntan (*Citrus grandis* Osbeck) using various solvents, such as *n*-hexane, ethyl acetate (EtOAc), butanol and methanol. The antioxidant activities of crude extracts were evaluated by using the free radical scavenging β-carotene assay and total polyphenol. Ethyl acetate extracts of falvedo exhibited high antioxidative activities, followed by albedo and segment membrane extracts. Chromatography separation of EtOAc extract of flavedo using a silica gel column, yielded six fractions (A, B, C, D, E and F) using gradient elution with benzene and acetone (19:1, 14:1, 9:1, 5:1, 1:1 and 0:l). Among them, two fractions (C and D) showed strong antioxidant activities using the free radical scavenging activity (DPPH) antioxidant assay. These two fractions were further purified using silica gel column chromatography and preparative TLC. Their extracts could well be useful to prevent oxidation in fruit juices and essential oil food products as well as for health supplements. Identification of the responsible components is underway.

Keywords: *Citrus grandis* Osbeck; Fruit tissue extracts; Antioxidant; β-sitosterol; Limonin

1. Introduction

Plants contain several compounds that have potent antioxidant activity. Antioxidant activity is not only produced by the plant but is also produced by microorganisms (Hirota, Morimitsu, & Hojo, 1997; Yen & Lee, 1996). The crude extracts of various parts of *Garcinia atroviridis* have antioxidant and also inhibited antibacterial activity (Mackeen et al., 2000). In a study of the total antioxidant activities of 12 fruits and 5 commercial fruit juices it has been recorded that strawberry has the highest antioxidant activity followed by plum, orange, red grape, kiwi fruit, pink grapefruit, white grape, banana, apple, tomato, pear and honeydew melon (Wang, Cao, & Rrior, 1996). The higher concentration of total polyphenols in *Gevuina avellana* and *Rosa rubiginosa* was recorded in the ethanolic extracts (Moure et al., 2001). Citrus seeds possessed greater antioxidant activity than peel (Bocco, Cuvelier, Richard, & Berset, 1998) and β-cryptoxanthin was also extracted from peel and flesh of citrus fruits (Kyoung-Cheol, Chan-Shick, Nam, Sam-pin, & Doo-Khil, 2000). Limonoid concentration profiles to differentiate citrus species limonin, nomilin, obacunone and deacetylnomilin, limonexic acid and deoxylimonin were found in varying amount of citrus species (Hashinaga & Itoo, 1981; Rouseff & Nagy, 1982). β-sitosterol is plant sterol found in almost all plants. β-sitosterol is already in our diet in common foods we eat every day, but only in small amounts and acts against human breast cancer cells (Awad, Roy, & Fink, 2003).

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doi:10.1016/j.foodchem.2004.11.042

Genus *Citrus*, with several species cultivated worldwide, produces a large amount of waste materials, including flavedo, albedo, segment membrane and seed waste. This study aims to evaluate the antioxidant activity of the *Citrus grandis* Osbeck fruit tissues. The following illustrates the research undertaken to report the isolation of β-sitosterol (**1**) and limonin (**2**) from flavedo and albedo tissues of *Citrus grandis* Osbeck and their antioxidant activities.

2. Material and methods

2.1. Plant material and extraction methods

Citrus grandis Osbeck (buntan) fruits were purchased in Kagoshima city for investigation. Buntan fruit tissues (flavedo, albedo and segment membrane) were removed and dried at 45 °C for 7 days, and ground in an electric blender, yielding fine dry samples: 700 g albedo, 500 g flavedo and 270 g segment membrane. Buntan fruit tissues were dried and finely ground tissues were successively treated using non-polar to polar solvents, *n*-hexane, ethyl acetate, *n*-butanol and methanol. 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.

2.2. Determination of antioxidant activity using free radical scavenging activity (DPPH)

The free radical scavenging activity of flavedo, albedo and segment membrane was measured as following: 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 (at concentrations 0.5–1 mg/ml) of an antioxidant extract solution was added to 2 ml acetate buffer, 1.9 ml methanol and 1 ml DPPH solution. Blanks contained 2 ml acetate buffer, 1.9 ml methanol and 0.1 ml of fruit tissue. While the control contained 2 ml acetate buffer, 1 ml DPPH and 2 ml methanol. The mixture was shaken immediately after adding DPPH and allowed to stand at room temperature in the dark, and the decrease in absorbance at 517 nm was measured after 30 min until the reaction reached a plateau. These experiments were run in duplicate. The inhibitory percentage of DPPH was calculated according to (Shyu & Hwang, 2002) as follows:

$$\text{Scavenging effect } (\%) = [(A_0 - (A - A_b))/A_0] \times 100\%,$$

where A_0 is the A_{517} of DPPH without sample (control), A is the A_{517} of sample and DPPH, and A_b is the A_{517} of sample without DPPH (blank).

2.3. Determination of antioxidant activity using β-carotene linoleate model system

Antioxidant activity was measured using the methods of Jayaprakasha, Singh, and Sakariah (2001) with slight modification. A 3.34 mg β-carotene solution in chloroform (1 ml), 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 0.01 M hydrogen peroxide (H_2O_2). Aliquots (2 ml) of this emulsion were transferred into different test tubes 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. Absorbances 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 blank mixture was prepared as above but without β-carotene.

2.4. Determination of total polyphenol

Total polyphenol of fruit tissue was measured using the method described by Yen and Hsieh (1998). The resulting solution (0.1 ml) was added to 2 ml of 2% Na_2CO_3. After 2 min 50% Folin–Ciocalteu reagent (0.1 ml) was added to the mixture and the absorbance was measured after 30 min at 750 nm using a spectrophotometer.

2.5. Thin-layer chromatography (TLC) analysis

TLC analysis for crude extracts or silica gel column chromatography fractions was performed on aluminium sheets (20 × 20 cm) of silica gel 60 F_{254} plate, which were developed with appropriate solvents for each sample such as ($CHCl_3$:MeOH:hexane (5:1:0.5), $CHCl_3$:MeOH:H_2O (5:1:0.5) or EtOAc:MeOH:hexane (5:1:2)). The resulting bands were located using UV-light and 10% sulfuric acid spray followed by heating in the oven for about 10 min at 120 °C.

2.6. 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 ^{13}C NMR. The spectra were observed on $CDCl_3$ containing TMS as an internal standard.

2.7. Chromatographic separation

Chromatographic separation of peel ethyl acetate extract (7.34 g) was chromatographed on a silica gel col-

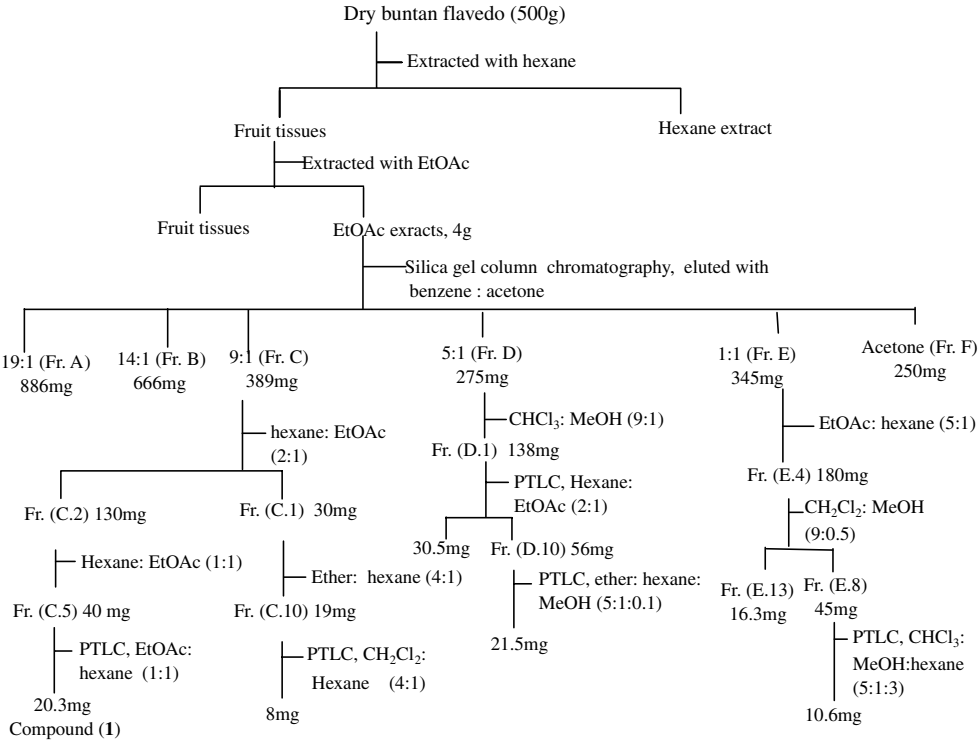


Fig. 1. Separation scheme of the antioxidants substance from flavedo.

umn chromatography. The column chromatography was eluted with benzene:acetone (Fig. 1) with an increasing amount of acetone gradually to yield six fractions 19:1, 14:1, 9:1, 5:1, 1:1 and 0:1 (A, B, C, D, E and F). Among them, two active fractions, C and D were recorded. Fraction C was further chromatographed on a silica gel column and eluted with hexane:EtOAc (2:1), yielding two active fractions (C.1 and C.2). The fraction (C.2) was further subjected to preparative TLC (PTLC) plates (20×20 cm) of silica gel 60 F_{254} (Merch 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 compound (**1**) (20.3 mg). The fraction (C.1) was subjected to PTLC plates using ether:hexane (4:1), followed by PTLC using CH_2CL_2: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 (D) was subjected to a silica gel column and eluted with $CHCl_3$:MeOH (9:1) to yield one active fraction (D.1), and followed by loading in PTLC plates using hexane:EtOAc (2:1), followed by PTLC using ether:hexane:MeOH (5:1:0.1). A compound was scraped off and eluted with chloroform: methanol (1:1). The active fractions were evaporated and monitored by aluminum thin-layer chromatography analysis (TLC).

Ethyl acetate extract of albedo (6.32 g) were subjected to silica gel column chromatography (Fig. 2) with hexane:ethyl acetate to gradually produce four fractions: 5:1, 1:1, 1:3 and 1:5 (A, B, C and D). Fraction (B) was eluted in a silica gel column with hexane:EtOAc (2:1) followed by crystallization with $CHCI_3$:MeOH (1:1) to produce a pure β-sitosterol compound (**1**). Fraction (C) was subjected to silica gel column chromatography with CH_2Cl_2:MeOH (9:0.5), yielding two fractions (C.6 and C.4). Fraction (C.4) was crystallized with $CHCl_3$:MeOH to give also pure β-sitosterol (1). While fraction (C.6) was loaded in PTLC plates using $CHCl_3$:MeOH:hexane (5:1:2) to produce one active fraction (C.22) followed by PTLC plates with mobile phase $CHCl_3$:MeOH:water (5:1:0.1) to give pure fraction under our investigation. The fraction (D) was subjected to column chromatograph with hexane:EtOAc (1:5) followed by crystallization with (MeOH:$CHCl_3$) producing limonin compound (**2**) as determined by ^1H and ^{13}C NMR spectra.

^1H and a ^{13}C NMR spectrum for β-sistosterol (**1**), were identical to authentic β-sitosterol (Castola et al., 2002). Limonin compound (**2**) has been previously isolated from citrus (Bennett & Hasegawa, 1982).

2.8. 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 ($P \leqslant 0.05$). Each value is the mean of three replications.

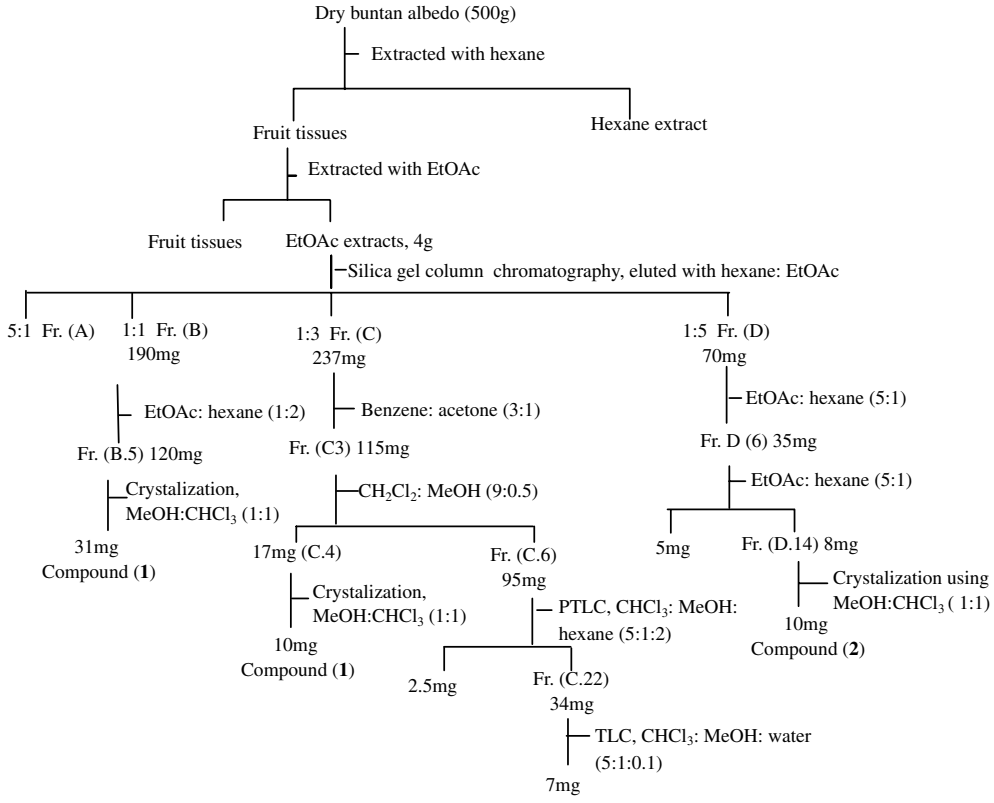


Fig. 2. Separation scheme of the antioxidants substance from albedo.

3. Results

Antioxidant rich fractions were extracted from *Citrus grandis* Osbeck (buntan) flavedo, albedo and segment membrane using various solvents, such as *n*-hexane, ethyl acetate, butanol and methanol. Each of the extracts were evaporated and the antioxidant activity was evaluated, using different antioxidant tests, including free radical scavenging, β-carotene and total polyphenol assays. The crude extracts did exhibit significant antioxidant activity being found in the ethyl acetate extracts followed by *n*-butanol and methanol extracts. Ethyl acetate extracts were fractionated by using silica gel chromatography.

Free radical of DPPH uses a stable free radical and is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Antioxidant activities of EtOAc extracts (0.5 mg/ml), as shown in Fig. 3, illustrated (at significantly $P \leqslant 0.05$) decrease in the concentration of DPPH radical, in the order of: BHA > α-tocopherol > flavedo EtOAc extracts > albedo EtOAc extracts > falvedo butanol extracts > flavedo MeOH extracts > segment membrane EtOAc extracts.

According to the β-carotene bleaching method the antioxidant activity of buntan extracts decreased in the order: BHA > flavedo > albedo > segment membrane. As shown in Fig. 4 the decrease in absorbance of β-carotene is statistically significant at $P \leqslant 0.05$ in the presence of different antioxidants. The total polyphenol content in dry flavedo, albedo and segment membrane, were 112.7, 89.6 and 43.6 mg/100 g of gallic acid equivalents, respectively. The different in the contents of total polyphenol in flavedo, albedo and segment membranes were statistically significant at $P \leqslant 0.05$. As shown in Fig. 5, the fractions obtained from flavedo extracts, 9:1 to 5:1 (benzene:acetone) exhibited highest antioxidant activity compared to other fractions, which indicated that these fractions contained compounds with

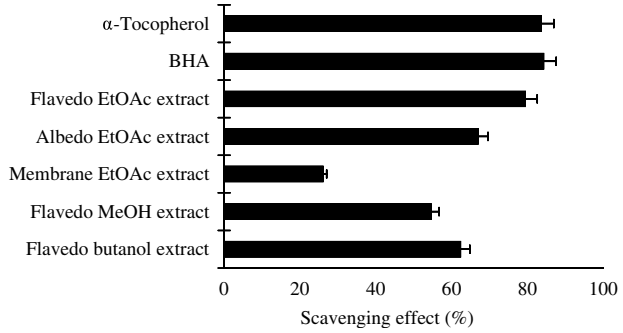


Fig. 3. Antioxidant activity of the extracts of buntan fruit tissues were added at 0.5 mg/ml as measured by free radical scavenging effects.

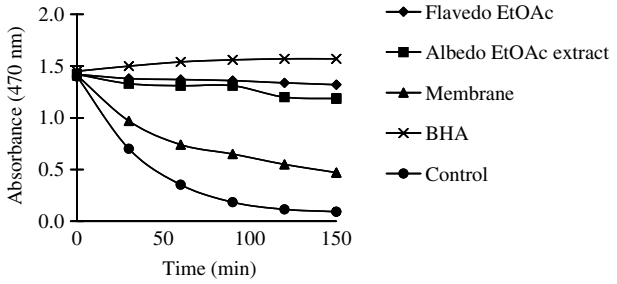


Fig. 4. Antioxidant activities of buntan fruit tissues were added at 0.5 mg/ml as measured by β-carotene bleaching method.

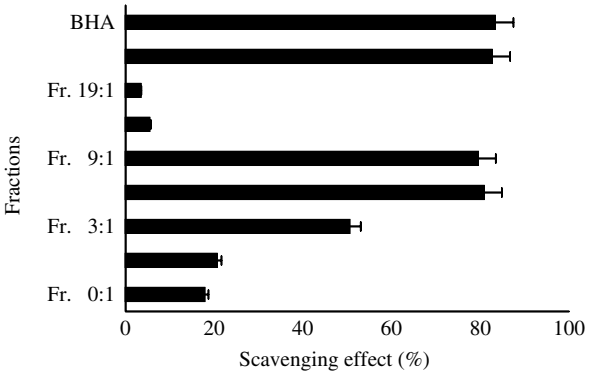


Fig. 5. Free radical scavenging effects of the EtOAc fractions of buntan flavedo extracts were added at 0.5 mg/ml eluted with benzene:acetone.

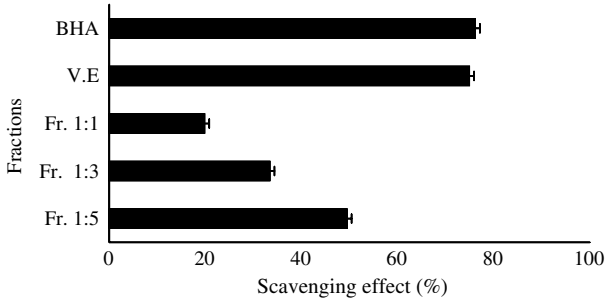


Fig. 6. Free radical scavenging effects of the fractions obtained from albedo extracts of buntan were added at 0.5 mg/ml eluted with EtOAc:hexane.

antioxidant activities. The more separation in silica gel column chromatography, then the higher activities were observed. As same as in albedo extracts (Fig. 6) indicated that fraction 1:5 (hexane:EtOAc) exhibited higher activities than others.

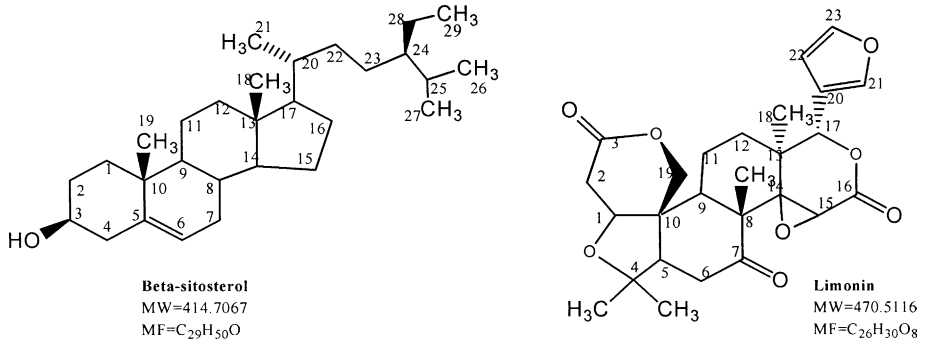
Antioxidative activity of β-sitosterol and limonin isolated from buntan fruit tissues EtOAc extract were introduced at a concentration of 0.1 mg/ml (Fig. 7). These compounds exhibited less antioxidant activity than did crude ethyl acetate extracts (were added at 1 mg/ml) and also in comparison with the standard α-tocopherol and BHA, which were introduced at a concentration of 0.1 mg/ml (Fig. 8).

4. Discussion

In nature, there are a large number of different types of antioxidant compounds that play an important role in blocking the generation of free radical chain reactions. Our data indicated that flavedo of buntan fruits are major sources of natural antioxidans. Antioxidative activities of various extracts, have been observed especially the ethyl acetate extract, which exhibits 93% inhibition of peroxidation of linoleic acid (Huang & Yen, 2001). In the present study, antioxidant activities of EtOAc extracts of buntan crude extracts exhibited stronger antioxidant activities in flavedo and albedo, while segment membrane extract elicited less activity as measured by DPPH scavenging, β-carotene, and total polyphenol activity. The polyphenol content of the dry fruit tissues (measured at between 4% and 11% by weight in this study) as a likely source of the antioxidant activity of the extracts.

The results of this study strongly showed that the extract of flavedo and albedo can be used as easily accessible source of natural antioxidant and as a possible food supplement or in pharmaceutical industry. The antioxidant activity of the ethyl acetate crude extract of broned yam flour showed a higher antioxidant activity than BHA (Farombi, Britton, & Emerole, 2000). Our data indicate that, we can isolate from these waste fruits compounds with high antioxidant activities to prevent oxidation in fruit juices and essential oils. Total antioxidant capacity varies considerably from one kind of fruit to another (Wang et al., 1996). Citrus fruits have been recorded as sources of pigment (Kyoung-Cheol et al., 2000). This indicated that citrus fruits (flavedo and albedo) contained various compounds, not only antioxidants, but also those that may have antibacterial effects. A possible way to valorize citrus flavedo and seeds, which are byproducts of the juice extraction industry, is to use them as natural antioxidants.

The antioxidative activity of ethyl acetate extract of buntan albedo tissue was much greater than that of β-sitosterol or limonin. The marked antioxidative activity of ethyl acetate extract of buntan albedo tissue might be attributable to minor components other than β-sitosterol and limonin present in the ethyl acetate extract, which had stronger antioxidative activity in the crude extracts. There does not appear to be any obvious organic functional groups on β-sitosterol or limonin which would generate antioxidant (radical scavenging) activity similar to that of the polyphenols. In addition, the minor components may exert a synergistic effect on β-sitesterol and fatty acids. Limonoids have attracted attention due to their insect antifeedant activity (Jayaprakasha, Singh, Pereira, & Sakariah, 1997).



Compound (1) Compound (2)

Fig. 7. β-sitosterol; 1, and limonin; 2 from flavedo and albedo extract.

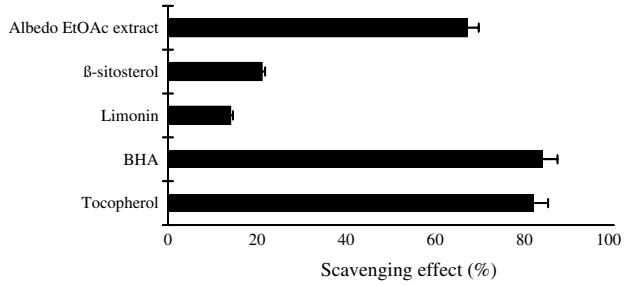


Fig. 8. Comparison of antioxidant activity of isolated compounds (β-sitosterol and limonin) from EtOAc extracts of flavedo and albedo tissues as measured by the DPPH radical. The concentration of the sample was 1 mg/ml and for all four standards were added at 0.1 mg/ml.

5. Conclusion

The results of the present work indicated that antioxidant activity of ethyl acetate extracts of flavedo and albedo from buntan fruits showed a high activity toward DPPH free radical scavenging effect and β-carotene. β-sitesterol and limonin extracted from flavedo and albedo of buntan fruits showed less antioxidant activity than crude ethyl acetate extracts.

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