International Food Research Journal 20(1): 313-317 (2013)
Journal homepage: *http://www.ifrj.upm.edu.my*

Determination of flavonoids in *Citrus grandis* (Pomelo) peels and their inhibition activity on lipid peroxidation in fish tissue

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Article history

Received: 7 March 2012
Received in revised form: 4 June 2012
Accepted:8 June 2012

Keywords

Flavonoids
Citrus grandis
lipid peroxidation
inhibition

Abstract

The peels of pomelo contribute 30% of the fruit weight and yet it has been dump without recognizing the possible nutritional value of the peels. Study has been carried out to identify flavonoid content of the peels and analysed the activity of the flavonoid towards inhibition of lipid peroxidation. Optimization of flavonoid extraction was conducted using aqueous solvent (methanol and ethanol), extraction time (1-3 h) and extraction temperature (50°C-80°) via water bath extraction. The total content of flavonoids was quantitatively determined by using coloration methods with chromogenic system of $NaNO_2$–Al $(NO_3)_3$–NaOH and and it was found that the extraction at 65ºC for 2 h in aqueous ethanol was the optimized condition for maximum flavonoids i.e. 190.42mg/L. A spectrophometric analysis was performed to evaluate flavonoid activity towards lipid peroxidation in the fish tissue. There was reduction in Peroxide value (PV) indicated the inhibition of lipid peroxidation in fish treated with pomelo peel as evidence of concurrency of positive flavonoid activity.

Introduction

Citrus is a common term and genus of flowering plants belonging to the family of *Rutaceae*, which originated in tropical and subtropical Southeast Asia. The most common citrus fruits are mandarin (*C. reticulate Blanco*), pomelo (*C.grandis Osbeck*), sour orange (*C. aurantium* L.), sweet orange (*C. sinensis* Osbeck), lime (*C. aurantifolia* Christm), citron (*C. medica* L.), lemon (*C. limon* L. Burn. f.) and grapefruit (*C. paradise Osbeck*). Citrus fruits are notable for their fragrance, partly due to flavonoids and limonoids (which in turn are terpenes) contained in the rind. Citrus fruits are recognized to be rich in certain phytonutrient that are vital in both health promotion and disease prevention (Okwu, 2004). Many researchers found out that the citrus plants contain a wide range of flavonoids constituents which is the secondary metabolites compound from plants. Although flavonoids glycosides can had a wide range of biological activities, the protective role of flavonoids in living systems was mostly due to their antioxidant potential, which is related to transfer of reactive oxygen species (ROS), chelation of metal catalysts, activation of antioxidants enzymes and inhibition of certain type of oxidases and colon cancer (Heim *et al*., 2002; Chidambara Murthy *et al*., 2012). The above considerations reflected the existence of clear scientific evidence that certain flavonoids possess antioxidant properties with synergistic and protective effects on vitamin C.

Flavonoids also have the potency to stimulate the immune system, induce protective enzymes in the liver or block damage to genetics materials. Flavonoids are very effective antioxidants. At present, there is overwhelming evidence to indicate that free radicals cause oxidative damage to lipids (Halliwell and Gutteridge, 1999), proteins, and nucleic acids (Berlett and Stadtman, 1997). Free radicals may lie at the heart of the etiology or natural history of a number of diseases including cancer and atherosclerosis (Hung *et al*., 2010). Therefore, antioxidants, which can neutralize free radicals become the central importance in the prevention of these diseases. Meanwhile, flavonoids can protect humans against cardiovascular diseases by reducing the oxidation of low-density lipoprotein (Kawaii *et al*., 1999). Plant flavonoids may also reduce the risk of thrombosis by inhibiting platelet aggregation and adhesion. A number of flavonoids may inhibit the enzymes implicated in the oxidation of polyunsaturated fatty acids. Flavonoids inhibit platelet aggregation by mediating in other enzyme systems. Their direct antioxidant properties also participate in their antithrombotic action (Hung *et al*., 2010).

Apart from the role of health benefactors,

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antioxidants are added in foods to prevent or delay oxidation of food, initiated by free radicals formed during their exposure to environmental factors such as air, light and temperature. At present most of the antioxidants are manufactured synthetically. They belong to the class of synthetic antioxidants. The main disadvantage with the synthetic antioxidants is the side effects when taken *in vivo*. Strict governmental rules regarding the safety of the food has necessitated the search for alternatives as food preservatives. Plants are the potential source of natural antioxidants. Natural antioxidants or phytochemical antioxidants are the secondary metabolites of plants. Carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols, tocotrienols, are some of the antioxidants produced by the plant for their sustenance. Beta-carotene, ascorbic acid and alpha tocopherol are the widely used antioxidants (Praveen and Awang, 2007). *Citrus grandis* contains a number of antioxidants such as beta-carotene, ascorbic acid, terpenoids, alkaloids, beta-sitosterol, carotene, polyphenols such as flavonoids, flavone glycosides, rutin. Easily cultivable *Citrus grandis* with its wide range of antioxidants can be a major source of natural or phytochemical antioxidants (Matook and Fumio, 2006). Researchers found that level of flavonoids were higher in the flavedos, the peels, than in the juices (Zhang *et al*., 2011) and therefore more study should be conducted on these materials commonly consider as wastes.

In order to extract the total content of flavonoids from the citrus peels there are various type of extraction techniques that have been revealed and can be used which included water bath extraction, maceration, supercritical fluid extraction (SFE), extraction using vapour, Soxhlet extraction, accelerated solvent extraction (ASE) and other methods (Naviglio *et al*., 2007). Water bath extraction is favourable due to low cost, easy to conduct and good in controlling the temperature.

This study mainly is to optimize extraction condition of total flavonoids by using water bath extraction and consequently to determine the extracted flavonoids. The extracts later were analysed for their inhibition activity on lipid peroxidation in fish tissues.

Materials and Methods

Collection of samples

The peels were collected either from the fruits stalls, neighbours, relatives or friends and kept it in the refrigerator at 4°C to maintain their freshness. The peels were allowed to be kept in the fridge for the duration only around 3-4 days to prevent the growth of fungal.

Samples preparation

The peels were divided into 2 parts, i.e. albedo (white color) and flavedo (green color). This was purposely done to ensure the moisture content in the peels were easily to evaporate. All of the samples were blended into powder (<90 μm) by a blender after being dried at 60°C for the period of 2 to 4 hours in an oven. The powders of the samples were kept in bottles.

Extraction of flavonoids for optimized condition

The flavonone compounds were extracted according to the method reported by previous researchers with slight modifications (Abeysinghe *et al*., 2007). The *Citrus grandis* peels were blended into powder by a blender and 10 g of the powder samples were accurately weighed and placed in a beaker and 100mL of ethanol-water (80:20, v/v) was added. The mixture was poured into test tubes which then covered with the aluminium foils and finally placed into water bath for extraction at temperature range of 50°C-80°C within 1-3 hours. The whole solution was filtered through the Whatman filter paper No.42 (125mm) and the filtrate was transferred into a crucible where the filtrate was allowed to evaporate into dryness in an oven at 60°C. Then, 0.05g of the extract was dissolved in the 10mL of solvent and the whole procedure was repeated using methanol instead of ethanol.

Determination of flavonoids

A 2 mL of the sample solution was accurately transferred into a 10mL volumetric flask and 5%, 0.6mL of sodium nitrite, $NaNO_2$ was added before the mixture was shaken and left for 6 min. Secondly, 0.5mL of the aluminium nitrate, $Al(NO_3)_3$ (10%) solution was added to the volumetric flask, shaken, and was left to stand for 6 min. Finally, 3.0mL of the sodium hydroxide, NaOH (4.3%) solution was added to the volumetric flask, followed by addition of water up to the scale, shaken, and left to stand for 15 min before determination. Using the sample solution without coloration as reference solution and 500 nm as determination wavelength, the coloration solution was used to determine the content of flavonoids in the sample by VersaMax Microplate Readers.

Preparation of standard solution

A standard solution/stock solution (1000ppm) of rutin (Sigma) was prepared as follows: rutin (0.01g) was accurately weighed and dissolved in 10mL of ethanol-water (80:20, v/v) and 10mL of methanol-

water (80:20, v/v) for each solution. Then, the dilution series of 30ppm, 60ppm, 90ppm, 120ppm and 150ppm was calibrated from the stock solution for standard curve generation.

Lipid peroxidation condition

The pomelo peel powder was extracted with ethanol-water mixture at 65°C in water bath for 2 hrs. Inhibition of lipid substrate was achieved by adding 10 mL of the peel extracts into 5g fish flesh and then the mixture was crunched by using mortar. Comparison was done with 0.02% synthetic antioxidant, butylated hydroxytoluene (BHT). Oxidation at 40°C was performed in the dark for the seven prepared samples at 1 hr interval from 0 to 6th hrs. The procedure for spectrophotometric determination of the peroxide value, PV was adapted from Shanta & Decker (1994).

Result and Discussion

According to the experiments, when the dried filtrate from the *Citrus grandis* peels were dissolved in the solvent and added with the $NaNO_2$– $Al(NO_3)_3$–NaOH, there exist a color reaction. This was because the flavonoids with 3′, 4′-dihydroxy-substituted structure could show a special color by reacting with the system of $NaNO_2$– $Al(NO_3)_3$–NaOH and most of the flavonoids in *Citrus grandis* peels possessed the 3′, 4′- dihydroxy-substituted structure as shown in Figure 1b. The color reaction of flavonoids and chromogenic system was presented in Figure 1c. As shown in Figure 3, this method was based on the reaction of aluminum ion with flavonoids at alkaline medium forming red chelates (Zhu *et al*., 2009). The red chelates indicated the presence of flavonoids therefore by measuring the absorption of such red chelates it could determine the total content of flavonoids in the *Citrus grandis* peels. The basic structure of flavonoids was presented in Figure 1a.

The color reaction of flavonoids and chromogenic systems was the qualitative method for the determination of the flavonoids compound contained in the *Citrus grandis* peels. In order to determine the total content of flavonoids which presented in the *Citrus grandis* peels precisely, the quantitative method had to be applied to obtain the actual amount of the total content of flavonoids from the *Citrus grandis* peels therefore a spectrophotometer, was used to identify the absorbance from the samples.

Since flavonoids owned the properties of natural antioxidant which enabled to bring many benefits to health, therefore it was inevitable to determine the most efficient extraction conditions in order to

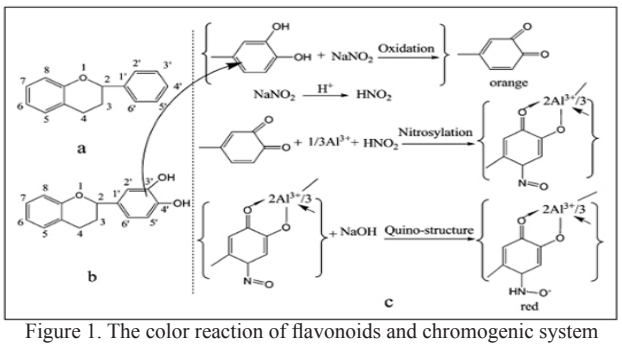

Figure 1. The color reaction of flavonoids and chromogenic system (Zhu *et al*., 2009)

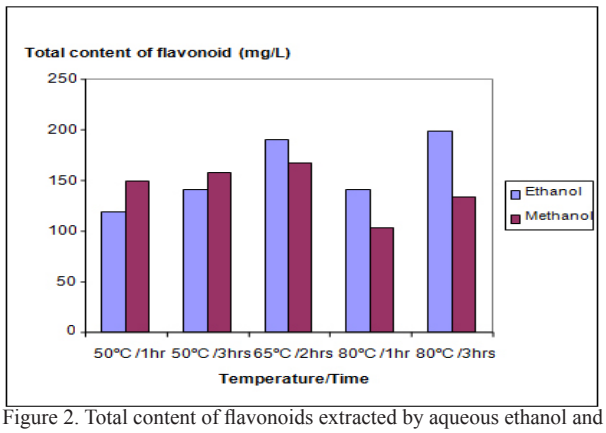

Figure 2. Total content of flavonoids extracted by aqueous ethanol and aqueous methanol from the *Citrus grandis* peels

enhance the yield of the total content of flavonoids which was extracted from the *Citrus grandis* peels. To achieve an effective extraction condition in this experiment, there were three critical parameters had been investigated which included the type of solvent used, temperature used for extraction and the time required for the extraction process in water bath.

Based on Figure 2, the extraction conditions at 50°C for 1 hour and 3 hours, the yield of the total content of flavonoids from the *Citrus grandis* peels extracted by aqueous methanol was 149.05mg/L and 158.09mg/L which yield higher than extracted by aqueous ethanol, 118.33mg/L and 141.25mg/L respectively. In contrast, under the extraction conditions at 80°C for 1 hour and 3 hours, the yield of the total content of flavonoids from the *Citrus grandis* peels extracted by aqueous ethanol was 141.67mg/L and 199.17mg/L which yield higher than extracted by aqueous methanol, 103.10mg/L and 133.34mg/L respectively. This was because the boiling point for methanol was 65°C while the boiling point for ethanol was 79°C. Hence, the total content of flavonoids extracted by aqueous ethanol at 80°C for 1 hour and 3 hours was much more higher if compared with the total flavonoids extracted by aqueous methanol at 50°C for 1 hour and 3 hours. Furthermore, under the moderate range of extraction conditions at 65°C for 2 hours, the yield of the total content of flavonoids

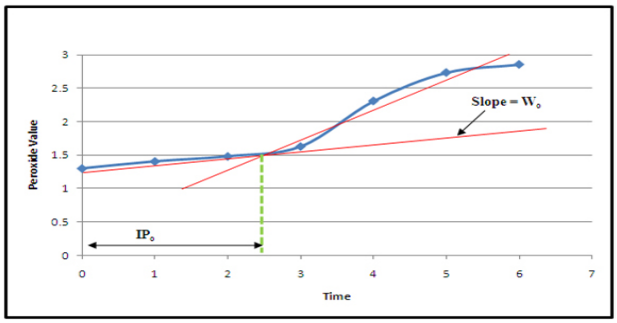

Figure 3. Graph of peroxide value against time for untreated sample

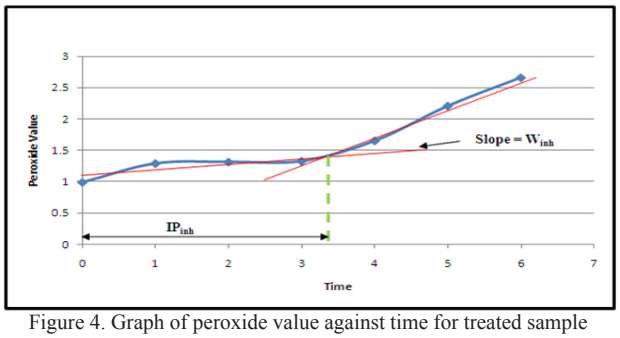

Figure 4. Graph of peroxide value against time for treated sample

Table 1. Results of peroxide value for control, sample and BHT

Time/hour	0	1	2	3	4	5	6
Untreated Sample (Control) (meq/kg)	1.3004	1.406	1.482	1.630	2.311	2.736	2.857
Treated Sample (meq/kg)	0.986	1.288	1.312	1.325	1.652	2.211	2.665
BHT(meq/kg)	0.894	1.147	1.209	1.232	1.529	1.881	2.140

from the *Citrus grandis* extracted by aqueous ethanol was 190.42mg/L while extracted by the aqueous methanol only yield 167.14mg/L. Even though 65°C is the boiling point of methanol, we assume that some of flavanoids have not extracted yet since the boiling point of flavonoid is relatively high, i.e. <165°C and therefore the total extracts were lower.

In overall, the yield of the total content of flavonoids from the *Citrus grandis* was more preferable and efficient when extracted using aqueous ethanol which was under the extraction condition of 65ºC for 2 hours. Moreover, the incubation of extract in water bath did improve the flavonoids extraction and heating might soften the plant tissue, weaken the cell wall integrity, hydrolyze the bonds, as well as enhance the solubility thus more flavonoids would distribute to the solvent.

The peroxide was a by product of oxidation process in lipid and the measured values had been used to show the effect of antioxidant such as flavonoid or their involvement in the process. Table 1 confirmed that there was a reduction in the PV value when treated with the extract of pomelo peel at optimized condition. The synthetic antioxidant, BHT showed higher inhibition activity at concentration of 0.02%, the concentration that was admitted and safe to be used in food. The one to one comparison cannot be made as the concentration is not equal, however, there is evidence that flavonoid did slow down the oxidation process with a theory that flovonoid (inhibitor, InH) introduced into the lipid oxidized system will change the mechanism of the kinetic process whereby the inhibitor reacted as the acceptor of the radicals (Farhoosh, 2005).

From both Figure 3 and Figure 4, values of W_o and W_{inh} can be obtained. W_{inh} oxidation rate in the presence of flavanoid and W_o is the initial oxidation rate of the control. These values are important in order to obtain the value of next parameter which is Oxidation Rate Ratio, (ORR). ORR is an inverse measure of the oxidation strength. The formula for ORR is:

$$ORR = W_{inh} / W_o \quad (1)$$

The value of W_o can be obtained from Figure 3, which is the slope value of the first curve of the graph, i.e. $W_o = 0.106$. Similarly, W_{inh} can be obtained from Figure 4, which is the slope value of the first curve of the graph i.e. $W_{inh} = 0.106$. The value of W_o and W_{inh} then were substituted into the Equation 1 and the ORR value is, 0.981. When ORR value is larger than 1, then the oxidation proceeds faster in the presence of an inhibitor than in its absence (Marinova & Yanishlieva, 1996). The lower the ORR, the stronger the inhibitor. Since the ORR value is less than 1, it shows that there is an antioxidant activity in the experiment.

Conclusion

In this study, the total content of flavonoids can be justified by quantitatively and qualitatively. The optimized extraction condition for maximum flavonoid yield using water bath extraction for pomelo peel are 65ºC for 2 hours extracted with aqueous ethanol. High in boiling point of both the solvent and the flavonoid, was suspected to play an important role in making ethanol is more suitable than methanol for the extraction. The reduction of peroxide value showed an antioxidant including flavonoid activities in the fish tissue and further evaluation has proven by the smaller value of ORR.

Acknowledgements

The authors gratefully acknowledge School of Bioprocess Engineering and particularly University Malaysia Perlis for their financial and facilities support.

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