

In vitro production of antioxidants of naringin and hesperidin and their activity in unsaturated fatty acid for oil preparation pharmaceuticals

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ABSTRACT

*The use of natural antioxidants in pharmaceuticals has been restricted by the limited natural resources available. Plants contribute as the main source of natural resources but provide inconsistent supply due to global climate changes and utilization of land for urbanization. To produce in vitro antioxidant compounds is making possible with the emergence of tissue culture technique. Research on young pomelo (*Citrus grandis*) fruit has successfully produced callus tissues that contain the antioxidant compounds. Analysis by HPLC found that naringin and hesperidin are the major composition of total flavonoids which detected as 250.6 mg/l and 2.7 mg/l respectively when the callus was grown on media supplemented with 2250 mg/l nitrate, 535 mg/l phosphate and 35 g/l sucrose for 7 days. Tested on fish oil proved that antioxidants compound in the callus did has an antioxidant activity whereby the induction period (IP) has been delayed. Therefore callus from pomelo may be applied in oil preparation of pharmaceutical products as alternative for plant origin antioxidants.*

Keywords - Antioxidant; Callus; *Citrus grandis*; Hesperidin; Naringin.

INTRODUCTION

An extensive research has been done on the potential of using natural plant metabolites to replace synthetic additives in human food, pharmaceuticals, cosmetics and medicine. However, plants need to grow for several years before they are ready for harvesting and some other problems will occur with the productions of these natural products. Most of the time, natural compounds are achieved through extraction from the raw sources that produced it. However depletion of raw sources is one of the major concerns in using this technique without producing enough based material. Furthermore, secondary metabolites isolation has proved to has a lot of disadvantageous due to the variability in result

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production from the differentiation of plant age, maturity and other environmental and physiological factors [1].

Due to these circumstances, biotechnology methods such as tissue and suspension culture have been chosen as a reliable solution and have a lot of interest by researchers in applying it to optimize the production of secondary metabolites from plants. Therefore, cultured cells are rather better than plants as a possible alternative production method. Production of secondary metabolites via tissue culture techniques have been reported in both callus and cell suspension cultures. In previous study, polysaccharides production in callus cultures of *Silene vulgaris* were reported will be optimized when supplied with 60 μM nitrogen and 3.75 μM of phosphate [2]. Application of plant cell culture has been verified to accumulate several products at higher level through medium modification and their cultural condition. Therefore, extraction is the most important part and as the availability of phenolic compounds in the pomelo peels has been ensured, the suitable and high efficiency extraction process must be attained. A group of factors that influence the quantification of phenolic compounds has been identified. The extraction method applied, the assay method and their chemical nature were some of those factors [3].

One example of potential plant for producing natural antioxidants through in vitro technique is *Citrus grandis* (Osbeck) or pomelo. It is one of important crop in Malaysia with production estimated around six to seven ton per year [4]. It normally consumed as fresh fruits when matured and for quality purpose, the younger fruits are often pruned, only one or two fruits will be allowed to develop in a bunch. The wasted younger pomelo contains various useful compounds as they are still in their growth stage and due to their strong antioxidants activities and their ability to scavenge the free radicals, they may create one excellent product for human being. Young tissues which contain young cells are very active in dividing new cell and studies have shown that the youngest part of plants always generates active cell division proliferation [5].

Flavonoids are polyphenols with diphenylpropane ($\text{C}_6\text{C}_3\text{C}_6$) skeletons. They are considered as the largest group of naturally occurring phenol and it is estimated that 2% of all the carbon photosynthesized by plants is converted into flavonoids [6]. Flavonone occur predominantly and exist as the major class of flavonoids present in citrus [7]. Furthermore, although flavonoid are abundant elsewhere in the plant kingdom, there are several compounds such as flavonone, flavanone glycosides and polymethoxylated flavones are unique to citrus fruits, therefore the young pomelo peels can act as one useful byproduct. Researchers also found that the level of flavanones, naringin, hesperidin and neohesperidin can be found in immature fruits of *Citrus aurantium* that is mainly synthesized during the early stages of fruits growth [8].

Oil is used as a carrier for injected medicine and vaccines in the production of a variety of pharmaceutical products. Lipid peroxidation occurs through a free

radical mediated chain reaction and can cause a change in the organoleptic and technological properties of oils and fats, reducing their shelf life. Fish oil is preferred over vegetable oil for its effect on reducing the formation of chemicals called prostanoids, which, when produced in excess, increase inflammation in various tissues and organs [9]. Traditionally, synthetic phenolic compounds, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), are used as antioxidants in fat-containing formulations; however, their harmlessness is at controversial point [10].

The main objectives of this study were (i) to determine the production of naringin and hesperidin are chosen as flavonone compounds, (ii) to detect total flavonoids accumulated and (iii) to determine the induction period (IP) using peroxide value analysis of an oil system. The production of naringin and hesperidin are chosen as flavonone compounds in suspension cultures and existing of those compounds were detected by High Performance Liquid Chromatography (HPLC) and all other method were analyze by using UV-Vis Spectrophotometer.

METHODOLOGY

Chemicals

Chemicals for the Murashige and Skoog (MS) medium preparation were purchased from Sigma-Aldrich Chemical Co. (St. Louis, United States), Merck, and Fluka, Japan. Hormone such as 2,4-dichlorophenoxyacetic acid (2,4-D), 6-furfurylaminopurine (kinetin), abscisic acid (ABA) were obtained from Fluka, Japan. Naringin and hesperidin were purchased from Sigma-Aldrich, United States. The HPLC grade solvents, methanol were purchase from Merck and all chemicals and reagents used were of analytical grade. The fish oil (Bio Life, Malaysia) was purchased from local pharmacy.

Plant Material Sterilization

Young pomelo fruits were collected at established plantation in northern area of Malaysia. The chosen young pomelo were about five to seven weeks after pollination and it should be uniformly shape, free from insect attacks, blemished, disease infections and any symptom of necrosis. The explants were sterilized and rinsed in running tap water for 5 minutes to remove dust, dirt and small insect that might have been attached. The fruits collected were washed with tap water for about 30 minutes and then held in distilled water mix with 20% Chlorox® solution for 1 hour. After the treatment, the solution was decanted and the fruit rinsed three times in sterile distilled water and cultured on the day in which they were collected.

Culture Conditions

Fruits cutting began by removing about 1 cm of the outer part of the fruit. The albedo part that used in this study was cut into smaller pieces (about 1 cm × 1 cm × 0.5 cm) before transferring onto prepared MS medium in the disposable petri dishes. Cultures were maintained in Murashige and Skoog (MS) medium with addition of 30 g/l sucrose and 7 g/l agar. The medium adjusted to pH 5.7 and sterilized at 121 °C for 15 minutes and distributed in petri dishes (15 ml medium). Cultured were maintained in dark at 25 ± 2°C until callus induction was observed on the surface at the cut edges sections within 30 days weeks of incubation. After the callus became 1.0 – 1.5 cm in diameter, they were transferred onto fresh media. The age of four-week-old callus was calculated from each sub culture interval.

Extraction and HPLC Analysis of Naringin and Hesperidin

The flavonone compounds were extracted according to the method reported by previous researchers with slight modifications [11]. The four-week-old callus cultures were used in this study. The fresh callus (FC), oven dried callus (ODC, dried at 60 °C for 1 hour) and freeze dried callus (FDC, freeze dried for 1 day) were prepared separately and grounded into small dices. 1 g of each sample was extracted with 10 ml of 80% methanol/water and vortexes for 1 min in every 30 minutes. Samples were filtered through a 0.2 µm nylon filter and inject to HPLC system (Shimadzu SPD-20A model). HPLC system consist a reverse-phase column coupled to a UV/VIS detector from Japan with auto-injector. This HPLC was equipped with LC Solution software for their computer operating. The chromatographic separation was performed on C18 Hypersil GOLD capillary column (250 × 4.6 mm) from THERMO Scientific (Waltham, USA). 10 µl of samples were analyze by a mobile phase containing 75 mM citric acid and 25 mM ammonium acetate in distilled water (A) and methanol (B) with a ratio 30:70 (v/v). The flow rate was 1 ml/min and the detection was carried out at 282 nm. The retention times and spectra were compared to those of authentic standards. System was left to allow for equilibrium at state within 20 minutes before samples was injected and the operating temperature was maintained at room temperature. The retention times and spectra were compared to those authentic standards. A calibration curve was prepared using a standard solution of naringin (50, 100, 150, 200 mg/l, $r^2 = 0.999$). The contents of naringin and hesperidin were expressed in mgDW⁻¹.

Determination of Total Flavonoids

Total flavonoids (TF) contents of callus extracts were determined according to the calorimetric assay developed by previous research [12]. One ml of callus extract was mixed with 4 ml of distilled water. At zero time, 0.3 ml of (5% w/v) NaNO₂ was added. After 5 min, 0.3 ml of (10% w/v) AlCl₃ was added. At 6 min, 2 ml of 1 M solution of NaOH were added. After that, the volume was made up to 10 ml,

immediately by the addition of 2.4 ml of distilled water. The mixture was shaken vigorously and the absorbance of the mixture was read at 510 nm by using UV-Vis spectrophotometer. A calibration curve was prepared using a standard solution of catechin (20, 40, 60, 80 and 100 mg/l, $r^2 = 0.996$). The results were also expressed on a fresh weight basis as mg catechin equivalents (CEQ) / 100 g of sample.

Methods of Assessment of Oil Oxidation and Stability

The peroxide value (PV) was determined using the American Oil Chemists' Society official method [13] with some adaptation upon adding 0.02 g of callus in 2g fish oil and varying the keeping time. These sample then were dissolved in 30 ml of chloroform: glacial acetic acid (3:2, v/v). Then 0.5 ml saturated solution of KI was added. The mixture was shaken by hand for 1 min and 30 ml of deionized water added and mixed again for 1 min. After incubating the flask for 5 min, 10 ml of the upper aqueous solution from each flask was dispensed in falcon tubes. The procedure continued by the addition of 0.5 ml of 1% starch indicator (with 0.3% chloroform) and mixing for 5 seconds. After that 4ml of mixture was dispense in assay tube and the absorbance of samples was immediately measured at 576 nm. Analyses were conducted at 3 days intervals until day fifteen. Samples were kept in dark at 60°C for 15 days.

PV value can be calculated base on iodine equation from standard curve (With $R^2=0.99$)

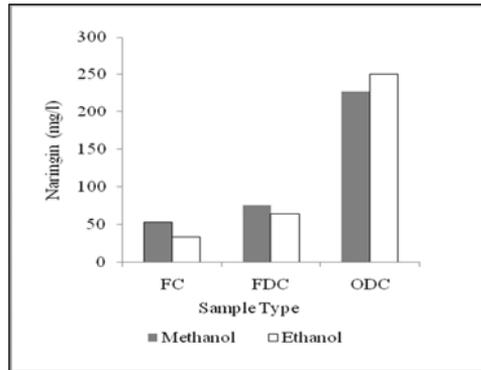
$$SPV = [(OD \text{ value} + 0.400)/0.908] \times 2 \text{ meq} \times (48 \text{ ml}/4 \text{ ml}) / 2 \text{ g}$$

RESULT & DISCUSSION

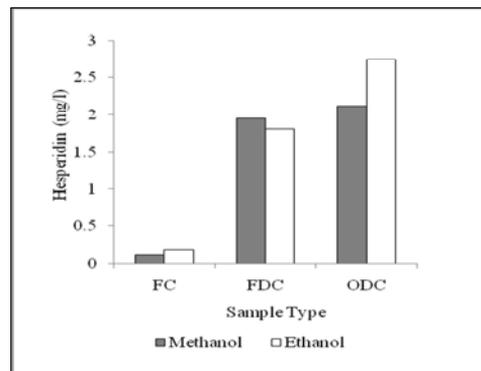
Effect of Solvent and Sample Preparation Type on Extraction of Naringin, Hesperidin and Total Flavonoids Accumulation

Solvent type is the most critical factor that affected on food samples as it will determine the amount and type of phenolic compounds being extracted. Aqueous alcohols such as methanol, acetone and ethanol are most commonly used in phenolic compounds extraction from natural products materials [14]. The recovery of polyphenols from plant materials is influenced by the solubility of the phenolic compounds in the solvents used. Therefore solvent polarity will play a key role in increasing phenolic compounds solubility [15]. Figure 1 (a) showed that the amount of naringin collected by oven dried callus (ODC) with ethanol (250.859 mg/l) used as the extraction solvent was slightly higher compared to aqueous methanol (227.47 mg/l). Hesperidin (2.749 mg/l) and TF (242.671 mg/l) was accumulated after extracted with ethanol shown in Figure 1 (b) and (c). Although methanol was least polar compared to ethanol, ethanol and water mixture are more commonly used for the extraction of phenol from plant materials because is

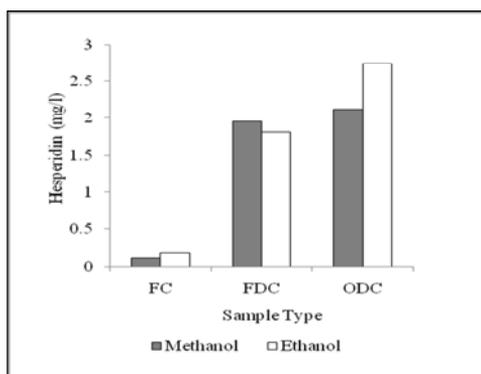
categorized under GRAS (Generally Recognized as Safe) chemical that would be preferable in the application of food sample analysis [16]. Therefore ethanol and oven dried sample type was chosen for the next experiments compared to freeze dried callus (FDC) and fresh callus (FC). Indirectly, high accumulation of flavanones and TC in ODC samples indicate that those compounds are stabilized in cultured system even at high temperature and this would be beneficial for storage purposes. Furthermore, preparation of ODC was rather cheaper compared to FDC in terms of machinery requirements.



(a)



(b)



(c)

Figure 1: Effect of solvent type and sample preparation type on naringin (a), hesperidin (b) and total flavonoids (c) accumulation from callus culture of *C. grandis*. *FC = Fresh Callus, FDC = Fresh Dried Callus & ODC = Oven Dried Callus

Effect of Ethanol Concentration on Extraction of Naringin, Hesperidin and Total Flavonoids Accumulation

The naringin, hesperidin and total TF content of callus extracts was determined by differentiated ethanol concentration. The naringin, hesperidin and TF accumulation was affected by the increasing of ethanol concentration up to 60% (naringin = 197.568 mg/l, hesperidin = 1.57 mg/l and TF = 197.5 mg/l) and then reduced by the increasing of the concentration as showed in Figure 2. Chan *et al.* (2009) also found that increment of solvent concentration on extraction of phenolic compounds from *limau purut* (*Citrus hystrix*) peels will reduce the phenolic compounds accumulation [17]. Nepote *et al.* also reported that the increasing of ethanol concentration beyond 70% will dramatically reduced the amount of phenolic extracted from peanut skins [18]. This finding showed that absolute solvent do not ensure a good recovery of phenolic compounds as compared to aqueous ethanol. Therefore, moderate ethanol concentration can act as an appropriate solvent mixture to extract flavonone from citrus samples.

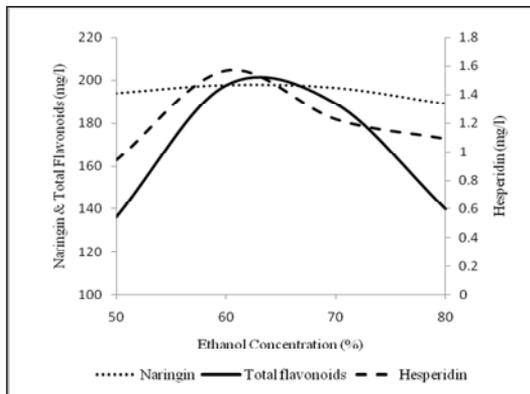


Figure 2: Effect of ethanol concentration on naringin, hesperidin and total flavonoids accumulation from callus culture of *C. grandis*

Effect of Extraction Time on Naringin, Hesperidin and Total Flavonoids Accumulation

Time is another factor that gives tremendous impact in extraction procedure. The extraction time tested from one hour to four hours and is shown in Figure 3. The best extraction duration for naringin, hesperidin and TF extraction from callus culture of young pomelo is two hours and it decreased when the extraction time increased. The extraction time of each type of sample will be varied as short as few minutes or very long up to 24 hours [19]. It depends on what type or extraction equipment and other parameters such as solvent concentration, temperature, time and pressure used. Furthermore, prolonged extraction process might lead to phenolic oxidation due to light and oxygen exposure [17], hence an excessive time was not useful to extract more phenolics antioxidants because it can increased the extraction cost as many power supply and solvent will be use [20].

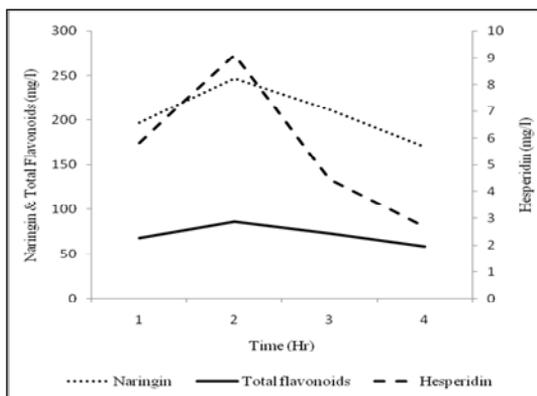


Figure 3: Effect of extraction time on naringin, hesperidin and total flavonoids accumulation from callus culture of *C. grandis*

Effect of Extraction Temperature on Naringin, Hesperidin and Total Flavonoids Accumulation

Appropriate extraction temperature was another important factor that can give effect on compounds accumulated after those treatment. In this research, the extraction of naringin, hesperidin and TF was slightly decreased from 50°C to 80°C as showed in Figure 4. By this result we can conclude that by increasing the temperature beyond certain value may promote possible concurrent decomposition of phenolics compounds which already immobilized at lower temperature or even the breakdown of phenolics that are still remained in the plant matrix [21]. High temperature also can give effects on increasing the extraction cost and also may encourage to solvent loss through evaporation. Therefore for extraction of naringin and hesperidin, lower temperature setting should be used to increase their accumulation.

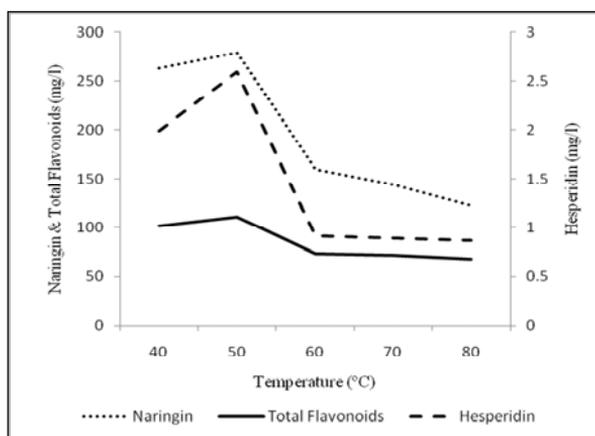


Figure 4: Effect of extraction temperature on naringin, hesperidin and total flavonoids accumulation from callus culture of *C. grandis*

Effect of Callus Powder on Induction Period of Fish Oil

The data for fish oil autooxidation, measured as a change of PV, at 60°C after addition of callus powder are presented in Table 1. The amount of callus in oil, calculated on a dry weight basis is 1% (w/w). It is evident that callus powder showed some oil stabilizing effect even though slightly lower than butylated hydroxytoluene (BHT) at the same amount. This finding lends further support that callus culture of pomelo contains strong antioxidative substances which earlier has been identified as naringin and hesperidin. For instance, PV of fish oil with 0.02g of callus powder after 3 days of storage was approximately 20 meq kg⁻¹, whereas in blank sample is increased to 42.831 meq kg⁻¹. Having in mind that BHT is a pure compound while the callus powder are complex mixtures containing ineffective substances in terms of their antioxidative activity or even some amount

of pro-oxidant compounds it could be supposed that callus powder contains very strong constituents retarding lipid peroxidation.

Table 1: Effect of callus powder on the formation of peroxides in fish oil at 60°C

Additive	Concentration (%)	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Control		9.066	19.410	42.831	45.846	49.533	52.097
Callus	0.02	7.348	14.722	35.670	36.436	40.718	45.079
BHT	0.02	6.660	12.449	15.912	33.965	37.057	44.683

The relative efficiencies of callus powder are compared to control and BHT as shown in Figure 5 where an induction period (IP) of fish oil after the addition of callus powder is presented. Experimental data showed that the IP of fish oil with callus powder was extended this is positive indicator to illustrate effectiveness and stability of antioxidants [22]. This result is comparable to BHT, a commercial antioxidant where about 24 hrs were delayed from the control.

For this study, the whole callus not the extract were analysed for their antioxidant activity. Without concentrated antioxidant compounds, the callus still have the capability to show their antioxidant properties. This is one advantage of using cultured tissue whereby extraction is not necessary as the tissues are constructed from homogenous cells and definitely almost residue free. Extraction process in many manufacturing of getting pure compounds always imposed higher cost and introduced hazardous chemical to the environment.

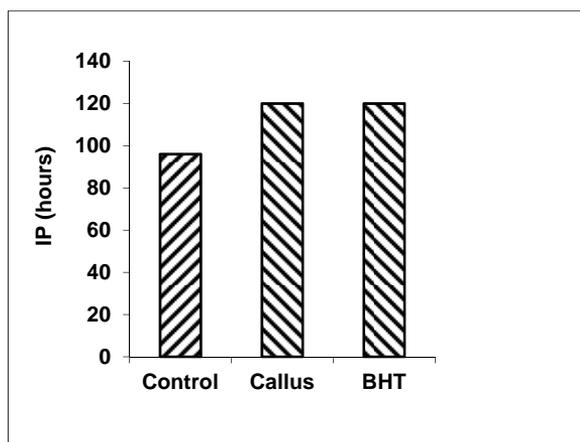


Figure 5: Changes of the induction period (IP) of fish oil after the addition of callus and BHT at concentration of 0.02%

CONCLUSION

This study successfully revealed the suitable value for chosen parameters to extract naringin, hesperidin and total flavonoids in callus culture of young *C. grandis*. The result showed that those compounds accumulated after extraction processes were most affected by extraction time (2 hours) and temperature (50°C) with ethanol as the solvent. At the amount indicated by the extraction procedure, the studied flavanone had illustrated antioxidant property to inhibit reaction towards auto oxidation.

Whole callus preparation without extraction has showed a ability to inhibit for autooxidation process in oil-based pharmaceutical products. Report on similar study on antioxidant activity in tissues produced via in vitro system is very scarce whereas this kind of study is important for consistent quality of antioxidant material for the protection of oil against oxidation.

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