A THESIS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Antioxidant and Antibacterial Activities of Citrus Press-Cake Dried by High Speed Drying and Far-infrared Radiation Drying

Mahinda Senevirathne

Department of Food Science and Engineering

GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

2009.02

Antioxidant and Antibacterial Activities of Citrus Press-Cake Dried by High Speed Drying and Far-infrared Radiation Drying

Mahinda Senevirathne

(Supervised by Professor Soo-Hyun Kim)

A thesis submitted in partial fulfillment of the requirement of the degree of

DOCTOR OF PHILOSOPHY

2009.02

The thesis has been examined and approved by

.....

Thesis director, Young-Hwan Ko, Professor of Food Science and Engineering

.....

Nak-Ju Sung, Professor of Food Science and Nutrition, Gyeongsang National University

.....

Sang-Bin Lim, Professor of Food Science and Engineering

.....

You-Jin Jeon, Professor of Marine Biotechnology

.....

Soo-Hyun Kim, Professor of Food Science and Engineering

2009-02

.....

Date

Department of Food Science and Engineering
GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

Content

국 문 요 약

감귤류는 천연 플라보노이드의 원천으로 전세계적으로 가장 많이 생산되는 과실 중 하나이다. 감귤 껍질은 과실보다 많은 양의 플라보노이드가 함유되어 있다. 감귤쥬스 공정 중에 많은 양의 감귤 부산물(감귤박)이 만들어지고 있으며, 이러한 감귤박 부산물에도 많은 양의 플라보노이드가 함유되어 있을 것이다. 그러나 감귤쥬즈 공정 후 생기는 감귤박은 산도가 아주 낮기 때문에 감귤 이외의 나무에는 비료로 사용하기 조차 힘들 뿐만 아니라, 수분함량이 90%이상으로 보관상의 어려움도 있어 전량 폐기되고 있는 실정이다. 가치는 있지만 폐자원인 감귤박을 효율적으로 이용하기 위해서는, 감귤박이 부패하기 전에 급속히 건조시키는 방안이 필요하다. 그러나, 현재 많이 사용되고 있는 건조방법은 비용이 비싸거나 건조하는데 시간이 오래 걸려 대량공정에 있어서 경제적으로 비생산적이다. 따라서, 이 연구에서는 건조 공정 효율을 높이기 위하여, 초고속건조(HSD), 원적외선 건조(FIR)공법을 이용하였다. 이 두 방법으로 건조한 감귤 부산물의 기능성을 검토하기 위하여 동결건조(FD)공법으로 제조한 시료와 함께 항산화 및 항균 활성을 측정하였다.

첫 번째 파트에서는 초고속 및 원적외선 건조를 한 감귤 부산물 추출물의 메탄올 추출물에 대한 항산화 활성을 검증하였다. 이에 일반적으로 시료 건조에 가장 이상적인 방법으로 많이 사용하는 동결 건조를 통해 건조한 감귤 부산물 추출물과 비교 검증하였다. 총폴리페놀 함량 및 총플라보노이드 함량은 발색법으로 측정하였다. HPLC 분석을 통하여 감귤 부산물 추출물에는 주로 flavone 과 polymethoxylated flavone 계열의 플라보노이가 주로 함유되어 있는 것을 확인 하였다. Hesperidin 은 감귤박의 주요 구성 플라보노이드 성분으로, 이 연구에서 HPLC 분석을 통하여 자유기 라디칼(ABTS·+) 소거율을 측정한 결과 flavone 계열의 hesperidin 이 주로 소거활성 능력을 나타내었고, 다른 종류의 플라보노이드 물질들도 우수한 소거활성을 나타낸 것을 확인 할 수 있었다. 따라서, 이러한 결과들은 감귤

부산물내에 존재하는 폴리페놀 및 플라보노이드 물질들에 의해 우수한 항산화 활성 보여주고 있다.

두 번째는, 초고속 건조 및 원적외선 건조를 한 감귤 부산물의 효소추출물을 제조하여 라디컬 소거 활성 및 DNA damage 보호효과 통하여 항산화 활성을 측정하였다. 총 6 개의 효소 추출물(AMG, Celluclast, Pectinex, Termamyl, Ultraflo 및 Viscozyme) 중에서 가장 우수한 항산화 활성을 보였던 AMG 효소 추출물은 우수한 라디칼 소거 활성, 지질과산화 생성 억제 활성 및 DNA 보호 효과를 나타내었다. 이러한 결과들은, 세포 실험에서도 감귤박 AMG 추출물에 의해 세포 생존률을 증가시켰고, 세포내 ROS 량도 감소시켰다. 뿐만 아니라 세포 수준에서 우수한 지질과산화 억제활성을 나타내기도 하였다. 이러한 결과는 AMG 효소 추출물의 항산화제로서의 가능성을 보여 주고 있다.

마지막으로, 감귤 부산물의 초고속건조 및 원적외선 건조물의 메탄올 추출물에 대한 항균 활성을 어병 세균과 식품 오염 미생물에 대하여 검증하였다. 그 결과 감귤 부산물의 원적외선 건조 추출물에서 대부분의 미생물에 대해 우수한 항균 활성을 나타내었을 뿐만 아니라 그람양성균의 생육도 억제 시킨 것을 확인 할 수 있었다. 이러한 결과는 감귤 부산물 유래 물질을 식품 보관에 사용시 천연 방부제로 사용이 가능 할 것으로 사료되며, 더 나아가서는 새로운 항생제 소재로 도출이 가능하리라 사료된다.

그러나 식품 안전성 확보를 위하여 향후 식품 산업공정에 대한 연구가 더 수행되어야 할 것이다.

결론적으로 초고속건조 공법은 감귤박을 건조하는데 시간을 감소시키고 하나의 공정과정으로 대량을 건조 시킬 수 있는 효율적이고 효과적인 건조 방법으로 간주 된다.

SUMMARY

Citrus is an important fruit with the largest production in the world and potentially it represents a rich source of natural flavonoids. Citrus peel shows higher amount of flavonoids than that in other parts of the fruit. The half of the fruit weight removed as waste. Therefore, citrus juice processing plants release large amount of press-cake and they may contain high amount of flavonoids as these press-cake mainly represent by the peel. To use this valuable product effectively, it is necessary to dry them quickly before they are subjected to spoilage. Though, there are several drying methods available at present, most of them are expensive and time consuming methods. Therefore, to transform this huge amount of agricultural product into dried from some efficient method is needed. Hence, in order to increase the efficacy in the drying process, Okadora Korea system was introduced in this study as the high speed drying (HSD) system. Existing drying methods such as far-infrared radiation drying and freeze drying were used as counterpart to the HSD method. Thereafter, the antioxidant activities and antibacterial activities of citrus press-cake (CPC) dried by HSD, far-infrared radiation dried (FIR) and freeze dried (FD) were investigated.

The antioxidant activities of methanolic extracts from CPC dried by HSD and FIR were evaluated for their antioxidant potential by various assays. Also, flavonoid content was determined by using HPLC, and they showed high amount of flavonone and polymethoxylated flavones. Hesperidin was found as the major compound available in CPC. Further, the extracts from CPC were evaluated by HPLC coupled on-line to an $ABTS^{+}$ radical scavenging detection system to determine and identify the antioxidant compounds at same time and results confirmed that hesperidin was the key compound in

CPC. The extracts from CPC showed strong antioxidant activities in all the assays tested. Hence, the results obtained in this study suggest that the total flavonoid and other polyphenolics in the CPC extracts could be good radical scavengers.

Then, the antioxidant activities of enzymatic digests from CPC were evaluated by different radical scavenging assays and comet assay. The enzymatic digests were prepared from the CPC by using six enzymes (AMG, Celluclast, Pectinex, Termamyl, Ultraflo and Viscozyme). As the AMG digest from CPC showed higher DPPH radical scavenging activity and total phenolic content at the preliminary investigations, it was selected for further antioxidant experiments. The AMG digest from CPC showed higher activities in the antioxidant assays tested. Further, digests showed strong lipid peroxidation inhibitory activities and remarkable inhibitory activities against H_2O_2-induced DNA damage.

Further, protective effect of the methanolic extracts from CPC against oxidative stress-induced damage in Vero cells was evaluated by different cell based assays. The results showed that the extracts from CPC could increase the cell viability and reduce the intracellular ROS level. Further, the extracts decreased the malondialdehyde levels in the oxidative stress-induced Vero cells and the fluorescence intensity in the comet tail of the cells pretreated with the extracts by enhancing the inhibitory effect in DNA damage. Based on the results, we can conclude that the extracts from CPC have potential ROS scavenging effects to inhibit, retard or prevent biological molecular damage of ROS in live cells.

The antibacterial activities of methanolic extracts from CPC against fish pathogenic and food born disease causing bacteria were evaluated in the final part of this study. Due to

the evolving resistance of microorganisms to existing antibiotics, there is an increasing need for new antibiotics not only in human but also in veterinary medicine. The extracts from CPC showed highest antibacterial activities in the assays such as disk diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. Also, scanning electron microscopic (SEM) results showed that the gram (+) bacteria was inhibited by the extracts. Therefore, the extracts from CPC have the potential as natural antibacterial agent for food preservation and medicinal use. However, it should be further researched for use in the food industries to improve food safety by the control or elimination of food born pathogenic bacteria in the tested samples. In conclusion, high speed drying method is an effective and efficient method to transform wet CPC into dried form considering its short drying time, the amount it transform at a single turn, and the antioxidant and antibacterial activities.

LIST OF FIGURES

μM H_2O_2 + 100 μg of extracts from CPC dried by FIR at 70°C, G: 700 μM H_2O_2 + 100 μg of extracts from CPC dried by FIR at 80°C, H: 700 μM H_2O_2 + 100 μg of extracts from CPC dried by FD.

Fig. 4-1. Scanning electron microscope observation of S.iniea treated with the CPC extracts. (A) control; (B) extracts from CPC dried by HSD; (C) extracts from CPC dried by FIR at 50°C (D) extracts from CPC dried by FD.

LIST OF TABLES

Table 4-8. Minimum bactericidal concentration (MBA) of methanolic extracts from CPC dried by HSD and FIR on fish pathogenic bacteria

BACKGROUND

Citrus is an important crop with production estimated around 80 million tonnes per year (Bocco et al., 1998). Jeju Island, the major citrus production area in Korea, has the unique conditions to cultivate citrus plants because of the exsisting subtropical climate. Other than that Jeju Island has proper soil conditions for citrus growing. Since the juice yield is about half of the fruit weight, large amounts of press-cake are generated from the juice processing plants. In the case of Korea, about 40,000 tons of citrus peel press-cake, out of 100,000 tons of citrus has been produced yearly. Citrus press-cake has been traditionally valorized as molasses for animal feed (Bocco et al., 1998), pectin production (Ros et al., 1996, Chou and Haung, 2003) and fuel production. However, a number of studies have been focused that some fruit or agricultural wastes could be a source of natural antioxidant, anticancer or anticoagulant agents in order to valorize these wastes, as these press-cake potentially represent a rich source of natural flavonoids owing to the large amount of peel produced.

Citrus flavonoids and distribution

Flavonoids are a group of pigments contained in plants and responsible for flower and fruit coloration (Macheix et al., 1990). The Citrus peel and seeds are very rich in phenolic compounds, such as phenolic acids and flavonoids. Peels are richer in flavonoids than are the seeds (Yusof et al., 1990).

Flavonoids are divided into six classes according to their molecular structures: flavones, flavanones, flavonols, isoflavones, anthocyanidins and flavanols (or catechins) (Fig. 1) (Peterson et al., 1998). Over 60 types of flavonoids have been identified in Citrus fruits and they belong to the five classes (Horowitz and Gentili, 1997): flavones, flavanones, flavonols, flavans and anthocyanins (the last only in blood oranges). Table 1 shows the main chemical structures of some flavonoids isolated from Citrus fruits.

Two types of citrus flavanones are present; glycoside and aglycone forms. Naringenin and hesperetin are the most important flavanones among the aglycone forms, (Table I). Neohesperidosides and rutinosides have been classified under glycoside form (Macheix et al., 1990). Flavanone neohesperidosides (naringin, neohesperidin and neoeriocitrin) consist of a flavanone with neohesperidose (rhamnosyl-α-1, 2 glucose) and they have bitter taste, while flavanone rutinosides (hesperidin, narirutin and didymin) have a flavanone and a disaccharide residue; rutinose (ramnosyl-α-1, 6 glucose) (table1). Flavanones are generally present as diglycoside form giving the typical taste to Citrus fruits (Macheix et al., 1990).

Phenolic andflavonoid compounds can be divided into two groups according to the retention time: the first eluted ompounds are flavanone glycosides while the second group is polymethoxylated flavones (Fig. 2) (Mouly et al., 1998).

Among flavonoids, anthocyanins are structurally derived from pyran or flavan and in particular, oxygen attributes a basic property to this molecule. They are avilable as aglycones (anthocyanidins metabolites of flavones) (Fig. 1).

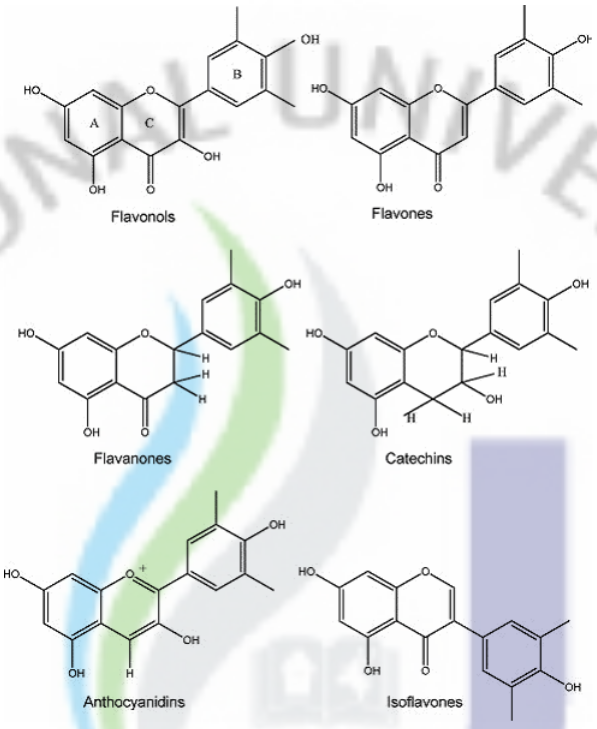
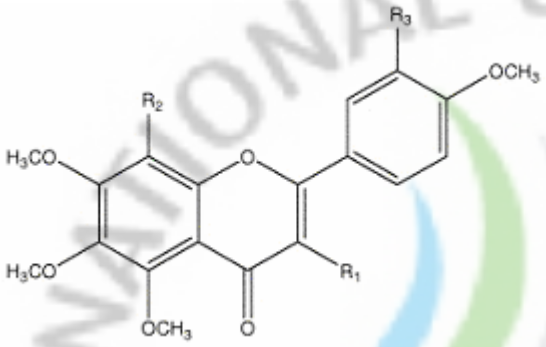


Fig. I. Molecular structures of flavonoids. The basic structure consists of the fused A and C ring, with the penyl ring B attached to through its 1' position to the 2-position of the C ring



R_1	R_2	R_3	
H	H	H	Scutellarein
H	H	OCH_3	Sinenseitin
H	OCH_3	H	Tangeretin
OCH_3	H	OCH_3	Hexamethoxyflavone
H	OCH_3	OCH_3	Nobiletin
OCH_3	OCH_3	OCH_3	Heptamethoxyflavone

Fig. II. Common citrus polymethoxylated flavones

Catechins, leucoanthocyanin and proanthocyanins are in the flavan group, as also are tannins. They can be found in monomer, dimer and polymer forms, respectively monoflavans, biflavans or triflavans (Cook and Samman, 1996).

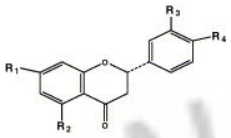
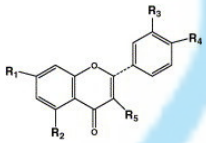
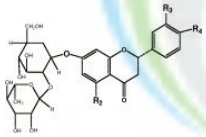
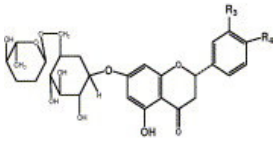
The 7-O-glycosylflavanones are the most abundant flavonoids in all Citrus fruits (Benavente-Garcia et al., 1995; Lewinsohn et al., 1989); for example, lemon peel is rich in glycosidic flavonoids (Park et al., 1983). Among the neohesperidoside flavanones, naringin, neohesperidin and neoeriocitrin, are mainly present in bergamot, grapefruit and bitter orange juices. Among rutinoside flavanones, hesperidin, narirutin and didymin are present in bergamot, orange, mandarin and lemon juices (Horowitz, 1986). The seed and peel compositions are not always same in Citrus fruits. For example, the lemon seed mainly contains eriocitrin and hesperidin, while the lemon peel is rich in neoeriocitrin, naringin and neohesperidin. Moreover, the glycosylated flavanone concentrations are different; neoeriocitrin and naringin have similar concentrations in peel while, in seed, eriocitrin is 40 times more abundant than is naringin (Bocco et al., 1998). Great amounts of neohesperidin, naringin and neoeriocitrin are extracted from peel. Bitter orange is a rich source of neohesperidin and naringin.The most important source of the glycosylated flavanones, naringin and neohesperidin are the seeds of bergamot, while the lemon is rich in eriocitrin and hesperidin. All other Citrus fruits have minor amounts of glycosylated naringin (Bocco et al., 1997; Yusof et al., 1990). Flavanone glycosyl compositions of peels and seeds are totally different from those of juices. Naringin has been found in lemon peel and seed and in mandarin seed, but it is not present in the juices of these fruits (Mouly et al., 1995; Ooghe and Detavernier, 1997). However, this glycosylated flavanone is never present in sweet orange juice (Mouly et al., 1994).

There is little difference in the glycosylated flavonoids amounts in the lemon juice extracted from several cultivars. Eriocitrin, 6, 8-di-C-β-glycosyldiosmin and 6-C-β-glycosyldiosmin are particularly abundant in lemon and lime, while they are almost absent in other Citrus fruits (Miyake et al., 1998). Anthocyanins are coloring compounds available in flowers and fruits, but sometimes in leaves, buds and roots (Herrman, 1976). They are in the epicarp, but they also colour the mesocarp of oranges.

Antioxidant and antibacterial activities of Citrus flavonoids

Free radicals are formed during normal metabolism in aerobic cells. The oxygen consumption inherent in cell growth leads to the generation of oxygen centered free radicals which are involved in the process known as oxidative stress. The interactions of theses radicals with lipid molecules produce new radicals; hydroperoxide and various peroxides. This group of radicals may interact with the biological systems in cytotoxic manner. Therefore, a great interest has paved to study the mechanism behind the free radical reactions. Phenolic compounds, particularly foavonolids, have been shown to posses an important antioxidant activity towards the free radicals which is based on their structural characteristics.

Table I. Structural characteristics and molecular weights of citrus flavonoids in the alglycone and glycoside forms

Compounds	Structural formula	Molecular weight	Molecular formula
Flavanone aglycone forms			
			
Naringenin	R_1 = OH; R_2 = OH; R_3 = H; R_4 = OH	271 Da	$C_{15}O_5H_{11}$
Hesperetin	R_1 = OH; R_2 = OH; R_3 = OH; R_4 = OCH3	288 Da	$C_{15}O_6H_{13}$
Sosakuranetin	R_1 = OH; R_2 = OH; R_3 = H; R_4 = OCH3	285 Da	$C_{16}O_5H_{13}$
Heridictyol	R_1 = OH; R_2 = OH; R_3 = OH; R_4 = OH	287 Da	$C_{15}O_6H_{11}$
Flavone and flavonol aglycone forms			
			
Apigenin	R_1 = OH; R_2 = OH; R_3 = H; R_4 = OH; R_5 = H	270 Da	$C_{15}O_5H_{10}$
Luteolin	R_1 = OH; R_2 = OH; R_3 = OH; R_4 = OH; R_5 = H	286 Da	$C_{15}O_6H_{10}$
Diosmetin	R_1 = OH; R_2 = OH; R_3 = OH; R_4 = OCH_3; R_5 = H	288 Da	$C_{15}O_6H_{12}$
Quercetin	R_1 = OH; R_2 = OH; R_3 = OH; R_4 = OH; R_5 = OH	302 Da	$C_{15}O_7H_{10}$
Kämpferol	R_1 = OH; R_2 = OH; R_3 = H; R_4 = OH; R_5 = OH	286 Da	$C_{15}O_6H_{10}$
Flavanone neohesperidoside forms			
			
Naringin	R_2 = OH; R_3 = H; R_4 = OH	604 Da	$C_{29}O_{14}H_{32}$
Neohesperidin	R_2 = OH; R_3 = OH; R_4 = OCH3	634 Da	$C_{30}O_{15}H_{34}$
Poncirin	R_2 = OH; R_3 = H; R_4 = OCH3	588 Da	$C_{29}O_{13}H_{32}$
Neoeriocitrin	R_2 = OH; R_3 = OH; R_4 = OH	620 Da	$C_{29}O_{15}H_{32}$
Flavanone rutinside forms			
			
Narirutin	R_3 = H; R_4 = OH	551 Da	$C_{29}O_{11}H_{27}$
Hesperidin	R_3 = OH; R_4 = OCH_3	583 Da	$C_{30}O_{12}H_{31}$
Didymin	R_3 = H; R_4 = OCH_3	567 Da	$C_{30}O_{11}H_{31}$
Eriocitrin	R_3 = OH; R_4 = OH	571 Da	$C_{29}O_{12}H_{31}$
Diosmin	R_3 = OH; R_4 = OCH_3	583 Da	$C_{30}O_{12}H_{31}$

Some studies have shown flavonoid antimicrobial activity (Huet, 1982) and their structure-activity relationship that influences antiviral activity (Kaul et al., 1985). The antiviral activity appears to be associated with non-glycosidic compounds, and hydroxylation at the 3-position is apparently a prerequisite for antiviral activity. Naturally occurring 4′-hydroxy-3-methoxyflavones possess antiviral activity against rhino- and poliomyelitis viruses (Middleton et al., 2000). The anti-picornavirus activity of the methoxyflavones was associated with the 4′-hydroxyl and 3-methoxyl groups. These methoxyflavones, poly-substituted in the A-ring, show a higher antiviral activity than do mono-substituted compounds (Meyer et al., 1991). Quercetin and hesperidin inhibit the infectivity and replication of herpes simplex, poliovirus, parainfluenzal virus and syncytial virus (Kaul et al., 1985). Several polymethoxylated flavones were found to strongly inhibit bacterial lipopolysaccharide-induced expression of TNF-α, whereas flavonoid glycosides were inactive (Manthey et al., 1999). In particular, hesperidin has significant inhibitory activities on inflammation, because it is able to reduce both LPS-elicited and infection-induced TNF-α production and inhibit infection-induced lethal shock, which resembles clinical cases. Hesperetin, the aglycone of hesperidin, has moderate antimicrobial activity against *Salmonella typhi* and *S. typhimurium* (Kawaguchi et al., 2004).

Formation of radicals involved in cellular oxidation process

Following are the radicals responsible for the cell oxidation processes; singlet oxygen, superoxide anion, hydroxyl radical and peroxyl radical. Singlet oxygen can be formed by photosensitizaton with several sensitizer compounds (Khan, 1985). These reactions

involve energy transfer from excited triplet state of sensitizer to molecular oxygen (Tournaire et al. 1993). The reactive oxygen species are formed by a sequential electron reduction mechanism. Molecular oxygen gives rise to superoxide radical, hydrogen peroxide and hydroxyl radical successively.

The electron supply mechanism may produce radicals to the medium by endogenous or exogenous reasons. The endogenous reasons may include the mitochondrial and microsomal electron transport chain, phagocytic cell action and autoxidation reactions of different compounds. The exogenous reasons are solar radiation, photosensitization, ionizing radiation, thermal shocks, environmental contaminants, and the induction of redox cycles.

The hydroxyl radicals are the most cytotoxic radical, and their formation from hydrogen peroxide may follow two mechanisms; the Haber-weiss or Fenton reactions.

The polyunsaturated fatty acids present in cell membranes are easily oxidized by both enzymatic and oxidative peroxidation via free radical chain reactions (Aust and Svigen, 1982). Speroxide, hydroxyl radicals or singlet oxygen can initiate fatty acid oxidation in the biological systems (Foote, 1976; Manson, 1982; Torel et al., 1986). It has been proven that lipid peroxidation can be inhibited by flavonoids acting as strong radical scavengers and singlet oxygen quenchers. Further, it has also been proposed that flavonoids react with peroxy radicals thus bringing about termination of radical reactions. The flavonoids may exercise their antioxidant actions by different meachanis (Bombard Elli and Morazzoni, 1993). Accordingly, they can act as antiradical agents (hydroxyl radical, superoxide), antilipoperoxidant agents (alkyl, peroxy, alcoxy), antioxygen agents (O_2, $^1O_2$) and metal chelating agents.

Structure-activity relationship of flavonoids

According to the kinetic studies of the aroxyl radical formation and decomposition reactions, the antioxidant capacity of a flavonoid is closely related to the particular structure of these polyphenolic compounds. Three structural groups are important for determining their radical scavenging/ antioxidant capacity (Bors et al., 1990 a, b). First one is the *O*-dihydroxy structure in the B-ring, which confers greater stability of aroxyl radicals possibly through the hydrogen bonding, and participating in electron delocalization. Second one is the 2, 3-double bonds in conjugation with 4-oxo function, which are responsible for electron dislocation from the B-ring, and the last one is the presence of both 3-(a) - and 5-(b) - hydroxyl groups for maximal radical scavenging capacity and the strongest radical absorption. The presence or absence of 5-hydroxyl group may be decisive factor on the flatness of the flavonoid, by introducing steroisomeric components into the electron dislocation capacity and thus into the stability of the flavonoid aroxyl radicals.

The antioxidant capacity of any flavonoid will be determined by a combination of these structural elements. However, the capacity will not be similar or show the same degree of effectiveness towards each of the above mentioned radicals, but depend on the different action of mechanisms which take place in each particular case. These mechanisms are influenced by the structural factors other than described, such as the presence or absence of glycosidic moieties in the flavonoid skeleton.

The superoxide radical scavenging activity of flavonoids has been studied using several methods. The activity is influenced significantly by the flavonoid concentration of the medium (Darmon et al., 1990). The dominant structural element is the C-ring

configuration, particularly the presence of 3-hydroxyl groups activating the 2, 3 double bond. When the reaction rate constant of superoxide anions is calculated (Bors et al., 1990 b), the negative influence of the catechol B-ring structure of the scavenging capacity becomes clear due to the generation of hydrogen peroxide in the reaction medium.

Flav-B-ringdiOH + $O_2^{\cdot-}$ → Flav-B-ring$OO^{\cdot-}$ + H_2O_2

Hydrogen peroxide is a strong oxidant which participates in propagation of the cell oxidation reactions. The absence of 3-hydroxyl group in the flavanones and flavones structures weaken their scavenging activity but some times increase the superoxide anion signals (Sichel et al., 1991).

Flavonoids are excellent hydroxyl radical scavengers (Pincemail et al., 1986) and they possess a degree of effectiveness depending on the structure. Also, the 3-O-glycosides are more active than their corresponding aglycones (Pincemail et al., 1986). The inhibition of hydroxyl radical generation is significantly reduced by increasing flavonoid concentration and such increases depends on the individual flavonoid in the reaction medium.

Flavonoids act as quenchers of singlet oxygen (Sorata et al., 1984). To establish a structure-activity relationship, the rate constant of the chemical reactions of these flavonoids with singlet oxygen and their rate constants of physical quenching were determined by Tournaire et al. (1993) by kinetic measurements at near-IR singlet oxygen luminescence.

PART I

Effective Drying of Citrus Press-Cake by High Speed Drying and Far-infrared Radiation Drying and Their Antioxidant Activities

ABSTRACT

The citrus press-cake (CPC) released from the citrus processing plants may contain high amount of potential bioactive compounds. These may includes limonoids, carotinoid and polyphenolic compounds. They may exhibit various biological activities. The aim of this study was to introduce a novel drying technique known as high speed drying (HSD) and to evaluate the antioxidant activities of CPC dried by HSD and far-infrared radiation drying (FIR). The extracts from CPC dried by HSD and FIR were analyzed by high performance liquid chromatography (HPLC) for flavonoids and showed high amount of flavonone (hesperidin and naritutin) and polymethoxylated flavones (heptamethoxyflavone and nobiletin). The antioxidant activities were evaluated by means of 1, 1 diphenyl-2-picrylhdrazyl (DPPH), superoxide, hydroxyl and alkyl radicals scavenging assays using electron spin resonance spectroscopy (ESR). Hydrogen peroxide, metal chelating, lipid peroxidation, total polyphenolic and total flavonoid contents were determined by colorimetric assays. The extract from CPC dried by HSD showed strong activities on radical scavenging (IC_{50} value of DPPH, superoxide, hydroxyl and alkyl radicals scavenging were 0.16, 0.28, 0.5, 0.017 mg/mL respectively). HSD showed higher activities in metal chelating and lipid peroxidation. Further, an HPLC coupled on-line to an $ABTS^{+}$ scavenging detection system was used to determine the antioxidant compounds in the extracts. The results revealed strong activity with this method too. Therefore, these results suggest that the flavanoids and other phenolic compounds in the extracts from CPC dried by HSD could be good antioxidants. Hence, HSD is more efficient method considering the drying time, the amount it tranasform into dried form and the antioxidant activities.

INTRODUCTION

Large amount of press-cake is formed from the citrus juice processing plants every year and those may contain high amount of potential bioactive compounds. Also, it is difficult to dispose those huge amounts as they have high biological oxygen demand. As the processing plants release huge amount of press-cake during the short harvesting period, storage and spoilage problems of these press-cake might be encountered. Therefore, it is necessary to dry this press-cake quickly before spoil to use them effectively. Though, there are several drying methods, some of them are high cost and time consuming. Hence, in order to increase the efficacy of drying process, high speed drying (HSD) was applied to transform wet CPC into dried form in this study. This method is more economical and less time consuming as it is able to convert large amount of press-cake into dried form in a single turn. We used Okadora (Incheon, Korea) as high speed drying system to convert CPC. Further, Okadora system is completely non-polluting, processing the wastes without producing any waste water or hazardous smells at all. Traditionally, waste materials are considered as no longer having any use, and treated them as a material to be disposed of. However, it is worth to use this valuable material in efficient manner as it contain high amount of bioactive compounds.

Since, the juice yield is about half of the fruit weight, very large amount of citrus peel is generated as press-cake from the juice industry. In the case of Korea, out of 100,000 tons of citrus, about 40,000 tons of citrus peel press-cake have been produced yearly in the citrus juice processing plants. Isolation of functional compounds from citrus peel can be of interest to the food industry as they can retard oxidative changes in food and thereby improve the quality and nutritional value of food.

Citrus peel is known to be rich in flavonoid compounds, with the majority (96%) of citrus fruits in major citrus producing countries converted into juice. Therefore, CPC released from processing plants represent rich source of naturally occurring flavonoids (Horowitz, 1961). The peel contains the highest amount of flavonoids than those of other parts (Manthey and Grohmann, 1996 and 2001) and those flavonoids belong to six peculiar classes according to their structure. They are; flavones, flavanones, flavonols, isoflavones, anthocyanidins and flavanols (Peterson et al., 1998).

Flavonoids exhibit their antioxidant activity in different ways (Bombardelli and Morazzoni, 1993); Antiradical ($^{\cdot}OH$), anti-lipoperoxidation ($R^{\cdot}$, $ROO^{\cdot}$, $RO^{\cdot}$), and metal chelating activities. Flavonoids are potential antioxidants against free radicals as they act as radical scavengers, and they show these activities due to their hydrogen–donating ability. The chemical nature of the flavonoids depends on structural classes, degree of hydroxylation, substitution and conjugation and degree of polymerization (Rice Evans et al., 1996). Three structural groups are important to evaluate the antioxidant capacity of flavonoids (Bors et al., 1990 a and b); First one is the ortho-dihydroxy structure in the B-ring which participate in hydrogen dislocation, second one is the 2, 3-double bond, in conjugation with a 4-oxo function, responsible for electron dislocation from the B-ring, and third one is 3 (a) and 5 (b)-hydroxy groups. The antiradical activity of several flavonoids on superoxide anion and hydroxyl radical has been studied by using several methods and they seem to have activity by depending on their structure (Darmon et al., 1990). The objectives of this study were to introduce a novel drying technique and to study the flavanoid content and potential antioxidant activities of the extract from CPC dried by HSD and FIR.

MATERIALS AND METHODS

Materials

CPC was obtained from Jeju provincial development Co. in Jeju Island, Korea. Authentic reference compounds Narirutin, Quercetagetin, Sinesetin, 3',4',7,8-Tetra methoxyflavone, 5,6,7,3',4',5'-Hexamethoxyflavone and Scutelarin tetramethyl ether were purchased from Extrasynthase (France). Hesperidin, Neohesperidin, DPPH, 5,5-Dimethyl-1-pyrolin N-oxide (DMPO), 2,2-azobis (2-amidinopropane) hydrochloride (AAPH) were purchased from Sigma Co. (St. Louis, USA). Nobiletin and Tangeritin were purchased from Wako pure chemicals (Japan). 3,5,6,7,8,3'4',heptamethoxyflavone was kindly provided by pharmacy department of Tokyo University. Ethylenediaminetetraacetic acid (EDTA), $FeSO_4.7H_2O$, H_2O_2 and deoxyribose were purchased from Fluka Co. (Buchs, Switzerland). All other chemicals used were of analytical grade supplied by Fluka or Sigma Co.

Principle of high speed drying process

High speed drying is a process which can be used to transform the large amount of agricultural or processing by-products into dried form within a short time as an alternative to existing drying systems, without destroying their bioactive compounds or their activities.

The loaded material is conducted upwards to the heat transmitting wall by the rotating cyclone fin and there, they are held against the heating wall in a thin film by centrifugal force. As a result, high heat increment can be maintained. The waste steam from the process is conducted to the super heat steam generator, where this waste steam is burnt at

temperature higher than 700°C and emitted under high temperature oxidization in an odorless condition. This waste steam, with high water content, is converted into super-heat steam, which has high energy, circulates inside the jacket of the super heat dryer, thereby heating it and obtaining high heat efficiency. As it expends energy, the steam reduces in temperature and when it reached at about 250°C, it release into the environment as a non-pollutant. The dried product can be discharged After 90 min (Fig. 1-1).

Principle of far-infrared radiation drying

FIR rays are defined as electromagnetic waves having a wavelength of longer than 4 μM but shorter than microwaves (λ>0.1 cm). According to the theory of FIR irradiation (Sandu, 1986), FIR energy from heaters suddenly impinges upon the surface of the product, and directly penetrates into the product, approximately 1 mm under the surface (Ginzburg, 1969; Nindo et al., 1995). Therefore, FIR energy is completely absorbed by the surface of the product into the depth of 1 mm, so called the penetrating layer. This layer is considered the location of the heat-conversion. The interior of the product from the depth of 1 mm to the core of the product is called the conductive layer, which heat is transferred by conduction. On the contrary, moisture inside the product is transferred from the core to the the surface of the product. Besides, heat and moisture at the surface of the product lose into the air within the irradiative chamber by natural convection.

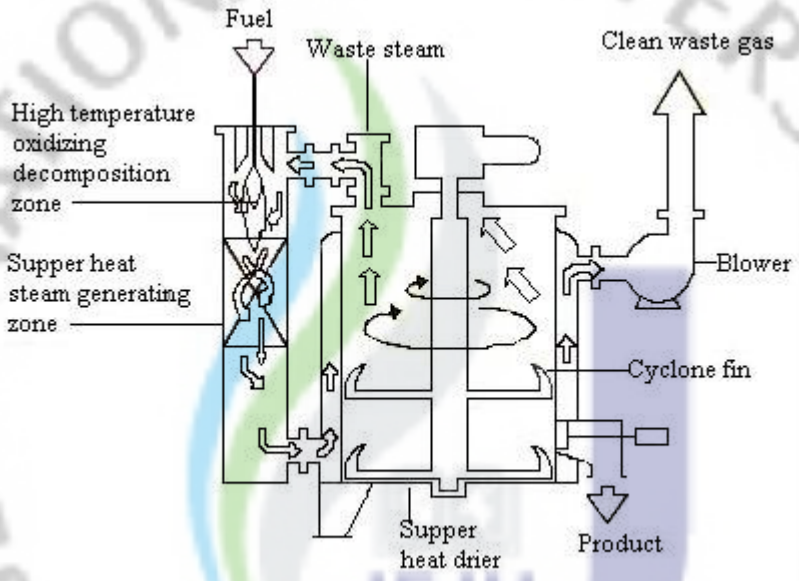


Fig. 1-1. Flow diagram of high speed drier (Model: Okadora Korea).

Principle of freeze drying process

Freeze drying is a dehydration technique, which products have previously been frozen to be dried under a vacuum. Generally, the freeze drying cycle is divided into three phases. First phase is an initial freezing process, which can carry out in the a way that the product exhibits the desired crystalline structure and the product is frozen below its eutectic temperature. Second phase is a primary drying (sublimation) phase and, during this phase, the partial pressure of the vapour surrounding the product must be lower than the pressure of the vapour from the ice, at the same temperature. During this phase the energy supplied in the form of heat must remain lower than the product's eutectic temperature. The last phase of the freeze drying cycle is the secondary drying and it is aimed at eliminating the final traces of water which remain due to absorption. The partial pressure of the vapour rising from the product will be at its lowest levels during this phase.

High speed drying, far-infrared radiation drying or freeze drying of CPC

CPC stored at -50°C were converted into dried form with high speed drier (Okadora Korea, Incheon, Korea). A 20 kg of wet CPC was loaded into the sample compartment of the drying system and dried within 90 min (Table 1-1). A high temperature was maintained within the sample compartment by steam supplied through the steam generator and the cyclone fin present in the compartment hold the sample against the wall of the compartment while rotating. Due to high temperature in this part, the moisture present in the sample is removed and converted into dried form. The dried sample was discharged from a slit available in the compartment. Then, the high speed dried CPC were pulverized into fine powder using a grinder (MF 10 basic mill, GMBH & Co.,

Staufen, Germany) and sieved through a 300 μm standard testing sieve (Fig. 1, 2, and Table 1).

CPC stored at -50°C was dried by FIR drying (Model TOURI-Q, Korea). FIR drying system operating conditions were given in Table 2. After drying, they were pulverized into fine powder using a grinder (MF 10 basic mill, GMBH & Co., Staufen, Germany) and sieved through a 300 μm standard testing sieve.

The wet CPC was dried separately using freeze drying method and CPC dried by FD was used as the counterpart to the CPC dried by HSD and FIR. The CPC was frozen before they are freeze drying. The freeze drying process was done in a lab scale freeze drier (Model: ilshin, ilshin Lab Co. Ltd., Korea) where the bottles were used to put the frozen samples and attached them to the main system. After 1-2 days, freeze dried CPC were taken out and pulverized into fine powder using a grinder (MF 10 basic mill, GMBH & Co., Staufen, Germany) and sieved through a 300 μm standard testing sieve.

Extraction of bioactive compounds from CPC

A 20 gram of ground CPC powder was mixed with 1000 mL of 100% methanol and kept the shaking incubator at 25°C for one day and filtered in a vacuum using Whatman No.1 filter paper. After that, the extracts were tested for ROS, together with lipid peroxidation. In each assay, all activities of HSD and FIR sample were compared with the values of FD.

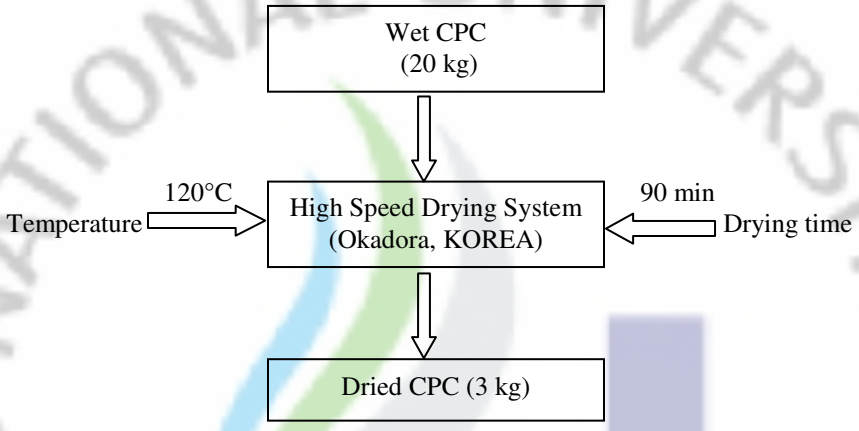


Fig. 1-2. Flow diagram of high speed drying process.

Table 1-1. Operating conditions of freez drier, high speed drier and far-infrared radiation drier

Components	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
Heating Tem. (℃)	-56	120	40	50	60	70	80
Drying time (hrs)	24	1.5	17	13	12	10	6.5
Dry weight (%)	6	17	15	15	14.9	14.8	14.7

Total phenolic content assay

Total phenolic content was determined according to the method described by Chandler and Dodds (1993). A 1mL of CPC extract was mixed in a test tube containing 1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% Foiln-Ciocalteu reagent .The resultant mixture was allowed to react for 5 min and 1 mL of 5% Na_2CO_3 was added. It was mixed thoroughly and placed in dark for 1 hr, and absorbance was recorded at 725 nm in the UV-VIS spectrophotometer (Opron 3000 Hansan Tech. Co.Ltd., Korea). A gallic acid standard curve was obtained for the calculation of phenolic content.

Total flavonoid content assay

The total flavonoid content of CPC was determined by the method described by Zhuang et al.(1992) with some modifications. A 0.5 mL aliquot of sample solution was mixed with 2 mL of distilled water, and with 0.15 mL of a 5% $NaNO_2$ solution. After 6 min, 0.15 mL of a 10% $AlCl_3$ solution was added and allowed to stand further 6 min. Thereafter, 2 mL of 4% NaOH solution was added to the mixture and distilled water was added immediately to bring the final volume to 5 mL. Then the mixture was properly mixed and allowed to stand for 15 min. Absorbance of the mixture was measured at 510 nm. Rutin was used as the standard for the quantification of total flavonoids.

HPLC determination of flavonoid constituents in CPC

The constituents and the amount of flavonoids in CPC were analyzed by SPD-M20A (Shimadzu Co., Japan). For identification of flavonoid constituents in CPC, the retention time was compared with authentic standards (11 flavonoids were used as standards).

A Sihm pack VP- ODS (C_{18}) column (250 mm x 4.6 mm i.d.) used for all chromatographic separation. The mobile phase was (A) water/acetic acid (99.5/0.5=v/v %): (B) acetonitrile/acetic acid (99.5/5.0= v/v %). The flow rate was 1.0 mL/min, injected volume was 10 µL and the room temperature was used as column temperature. The UV detector was set at 280nm.

DPPH radical scavenging assay

This assay is based on the scavenging ability of stable DPPH radicals by the radical scavenging constituents in the HSD and FD samples. Method of Nanjo et al. (1996) was used to investigate the free radical scavenging activity by electron spin resonance (ESR) spectrometer. DPPH in MeOH was prepared at the concentration of 60 µM. A 60 µL fraction of extract was added to the same volume of freshly prepared DPPH. Then the reactants were thoroughly mixed and transferred to 50 µL glass capillary tube and fitted into the ESR spectrometer. The spin adduct was measured after 2 min. The measurement conditions were as follows; central field 3475 G, modulation width 0.8 mT, amplitude 500 mT, microwave power 5 mW, scan width 10 mT and temperature 25°C.

Superoxide radical scavenging assay

Superoxide anion radicals generated by UV irradiated riboflavin/EDTA system (Guo et al., 1999) were detected by ESR spectrometry using DMPO as a spin trap. The reaction mixture contained 20 µL of 0.8 mM riboflavin, 20 µL of 1.3 mM EDTA, 20 µL of 0.8 mM DMPO and different concentrations of indicated samples. Riboflavin is oxidised spontaneously to a flavin radicals, with the formation of $O_2^{\cdot-}$ in the presence of oxygen (Hemmerich and Wessiak, 1976) via the following reaction: Flavin (red) + $2O_2$ → Flavin

(ox) + $O_2^{\cdot-}$ + $2H^+$. Then, the mixture was irradiated for 1 min under UV lamp at 365 nm and transferred to the capillary tube. Spin adduct was recorded with ESR spectrometer under the following operating conditions; central field 3475 G, modulation 0.1 mT, amplitude 200 mT, scan width 10 mT, microwave power 1 mW, temperature 25°C.

Hydroxyl radical scavenging assay

Ability of the extracts to scavenge the hydroxyl radical was investigated by the method described by Rosen and Rauckman (1980) by using ESR spectrometer. The Fenton reaction (Fe^{2}+ + H_2O_2----→ $^{\cdot}OH$ + OH^-), a well known and defined generator of $^{\cdot}OH$ radicals, was used to produce $^{\cdot}OH$, and the radicals produced were reacted rapidly with nitrone spin trap DMPO. The reaction mixture contained 20 µL extract, 20 µL of 0.3 M DMPO, 20 µL of $FeSO_4.7H_2O$ and 20 µL of 10 mM H_2O_2. The ESR spectrum of the resultant DMPO-OH adducts were investigated and recorded after 2.5 min. The measurement conditions were as follows; central field 3475 G, modulation width 0.2 mT, amplitude 100 mT, microwave power 1 mW, scan width 10 mT and temperature 25°C.

Alkyl radical scavenging assay

Alkyl radicals were generated by AAPH and their scavenging effects were investigated by the method described by Hiramoto et al. (1993). The reaction mixture contained 20 µL of distilled water, 20 µL of extract, 20 µL of 40 mM AAPH and 20 µL of 40 mM POBN, and the mixture was incubated at 37°C for 30 min. Then the reactants were transferred to 50 µL glass capillary tube and fitted into the ESR spectrometer. The measurement conditions were as follows; central field 3475 G, modulation width 0.2 mT, amplitude

500 mT, microwave power 8 mW, scan width 10 mT and temperature 25°C.

Hydrogen peroxide scavenging assay

This assay was carried out according to the method described by Muller (1995). Extract (80 μL) and 20 μL of 10 mM hydrogen peroxide were mixed with 100 μL of phosphate buffer (0.1 M, pH 5.0) in a 96-microwell plate and incubated at 37ºC for 5 min. Thereafter, 30 μL of freshly prepared 1.25 mM ABTS and 30 μL of peroxidase (1 U/mL) were mixed and incubated at 37º C for 10 min, and the absorbance was measured at 405 nm.

Metal chelating ability

Metal chelating ability was determined according to the method described by Decker and Welch (1990) with slight modifications. The extract (5 mL) was mixed with 0.1 mL of 2 mM $FeCl_2$. The reaction was started by the addition of 0.2 mL of 5 mM ferrozine, and reaction mixture was incubated for 10 min at room temperature under shaking condition. After incubation, absorbance was measured at 562 nm.

Lipid peroxidation inhibition by ferric thiocyanate (FTC) assay

The lipid peroxidation inhibitory activity of CPC was determined according to the method described by Larrauri et al. (1997) with slight modifications. Each extract (0.1 mL) was thoroughly mixed with 2 mL of 2.5% linoleic acid in ethanol, 4 mL of 0.05 M phosphate buffer (pH 7.0) and 2 mL of distilled water. The mixture was kept in the dark at 40°C and analyzed every 24 hr interval. FTC method is used to determine the

amount of peroxide at the initial stage of lipid peroxidation. Peroxide reacts with ferrous chloride to give a reddish ferric chloride dye. A 50 µL of incubated mixture was mixed with 9.7 mL of 75% ethanol and 0.1 mL of 30% thiocyanate. The mixture was allowed to stand for 5 min at room temperature before adding 0.1 mL of 20 mM ferrous chloride solution. The absorbance was recorded at 500 nm with an ELISA reader (Sunrise; Tecan Co., Austria)

HPLC coupled on-line to $ABTS^+$ assay for the rapid identification of antioxidants

The on-line radical scavenging activity of the extracts was determined by $ABTS^+$ assay modified by Stewart et al. (2005). A 2 mM $ABTS^+$ stock solution containing 3.5 mM potassium persulphate was prepared and kept to stand overnight at room temperature in the dark to allow radicals for stabilization. $ABTS^+$ reagent was prepared by diluting the stock solution by 8 fold in methanol. A 10 µL of the extract was injected, and separated using Agilent 1200 HPLC system, (Agilent Technologies, Santa Clare, USA) which equipped with binary pumps, a diode array detector (DAD), a UV/Vis detector, and an additional reagent pump. The analytical column was a reversed-phase Zorbax Eclipse XDB-C_{18}, 150 mm length, 4.6 i.d. and 5 uM particle size (Agilent Technologies). The mobile phase consisted of solvent A (acetonitrile) and solvent B (water with 0.1% of TFA v/v). A gradient elution was performed with solvent A and B as follows: 0–5 min, 10% A; 5–15 min,10- 15% A; 15–20 min,10- 15% A; 20–25 min,15- 18% A; 25–30 min,15- 18% A; 30–50 min,18- 35% A. The DAD was used in the 200-300 nm range and the chromatographic profile was recorded at 520 nm. A 10uL of extract was injected and flow rate was 0.5 mL/min. The analysis was performed at 40°C, and HPLC eluent from the

DAD then arrived at a “T” piece, where the $ABTS^+$ was added. The $ABTS^+$ flow rate was 0.2 mL/min delivered by an additional Agilent 1200 pump. After mixing, a 1 mL loop maintained at 40°C, and the absorbance was measured by a UV/Vis detector at 734 nm. Data were analyzed using ChemStation (Agilent Technologies).

Statistical analysis

All experiments were conducted in triplicate (n=3) and an ANOVA test (using SPSS 11.5 statistical software) was used to analyze the data. Significant differences between the means of parameters were determined by using the Duncan’s test ($p<0.05$).

RESULTS AND DISCUSSION

CPC dried by HSD or FIR drying were extracted with 100% MeOH and Fig.1-3 depicts the total soluble solid of each extract. Proximate chemical composition of CPC dried by HSD and FIR were determined according to the AOAC (1995) guidelines and presented in Table 1-2. Further, moisture content of the original wet citrus press-cake was 84.7%. Extract from CPC dried by HSD showed significantly higher ($p<0.05$) soluble solid content (43.5 g/100g), whereas the extracts from CPC dried by FIR at 50°C and 80°C showed lower soluble solid content (27 g/100g). Jayaprakasha and Patil (2007) and Singh et al. (2002) have reported that the extractable yield was highest in methanol extract from citrus and pomegranates in comparison with the solvents such as ethyl acetate and water.

Polyphenolic compounds are secondary metabolic products, which mainly include flavonoids (namely flavones, flavanones, flavonols, isoflavonoids, flavanols, and anthocyanidins), phenolic acids, coumarins, stilbenes and tannins (Liu, 2004). HPLC, has been reported to use to identify and quantify flavonoids in citrus using either normal or reversed-phase columns (Bronner and Beecher, 1995; Kanaze et.al 2003). Total flavonoid and total phenolic content were estimated by colorimetric assays and shown in Table 1-3. Significantly higher ($p<0.05$) total phenolic content was estimated as 4334.5 and 4280.2 mg gallic acid equivalents/100 g in CPC dried by HSD and FD, respectively. Total flavonoid content was 904.6 mg/100 g and 1001.9 mg/100 g in CPC dried by HSD and FD, respectively. Total flavonoid content ranged from 1300.2 to 1782.4 mg/100g (rutin equivalents) in CPC dried by FIR and significantly higher ($p<0.05$) levels was exhibited by CPC dried by FIR at 50°C followed by CPC dried by FIR at 70°C. Lee et al.

(2003) have reported that simple heat treatment could not cleave covalently bound phenolic compounds from the rice hull but far-infrared treatment could. Jeong et al. (2004) also have reported that new phenolic compounds have been formed when citrus peel heated at 150°C for 30 min. The extracts from CPC dried by FD, HSD and FIR were subjected to HPLC analysis using acetonitrile/acetic acid and acetic acid/water as mobile phase and the compounds were detected at 280 nm. Fig. 1-4 depicts the HPLC profiles of CPC dried by FD, HSD, and FIR. Table 1-4 showed the flavonoid constituent of each extracts. The major flavonoids found in the extracts were Narirutin, Hesperidin, Nobiletin, Heptamethoxyflavone by comparison of their relative retention times with authentic standards. Other than those mentioned above Quercertin, Neohesperidin Sinesitein, Tetramethoxyflavone, hexamethoxyflavone, scutelarin tetramethylether and Tangeritin were found in considerable amount. These constituents were almost same with the values obtained for CPC dried by FD.

Table 1-2. Proximate chemical composition of CPC dried by FD, HSD and FIR

Components	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
Moisture (%)	9.17±0.3^{e}	8.51±0.2^{d}	7.31±0.7^{c}	6.77±0.6^{b}	6.86±0.5^{b}	6.3±0.2^{a}	6.3±0.2^{a}
Ash (%)	3.75±0.07^{a}	3.8±0.05^{ab}	3.72±0.05^{a}	3.85±0.06^{ab}	3.90±0.07^{b}	4.10±0.08^{c}	4.07±0.07^{c}
Protein (%)	9.8±0.2^{d}	9.2±0.1^{ab}	9.87±0.2^{d}	9.17±0.1^{a}	9.31±0.1^{b}	9.55±0.2^{c}	9.45±0.2^{c}
Fat (%)	1.6±0.03^{a}	2.8±0.04^{d}	2.1±0.04^{b}	2.2±0.03^{b}	2.1±0.03^{b}	1.5±0.01^{a}	2.6±0.05^{c}
Carbohydrate (%)	75.68±2.4^{a}	75.69±2.1^{a}	77.0±1.8^{a}	78.01±1.9^{a}	77.83±2.3^{a}	78.55±2.5a	77.58±2.4^{a}

All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

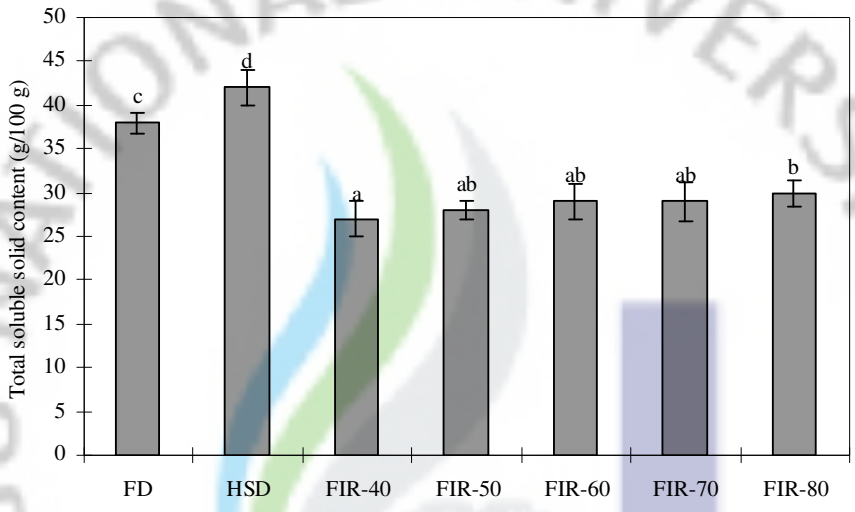


Fig. 1-3. Total soluble solid content of methanolic extracts from CPC dried by FD, HSD and FIR methods.All data are means of three determinations. Significant differences at $p<0.05$ indicated with different letters.

Table 1-3. Total polyphenolic and flavonoid contents of CPC dried by FD, HSD and FIR

Components	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
TPC (mg/100g)	4334.5±59.7^{g}	4280.2±53.6^{f}	3390.4±38.3^{e}	2351.8±29.1^{b}	2273.6±27.3^{a}	2643.6±31.9^{d}	2438.6±28.5^{c}
TFC (mg/100g)	1001.9±7.9^{b}	904.6±6.4^{a}	1364.5±5.4^{d}	1482.4±8.3^{f}	1430.0±6.1^{e}	1568.1±7.3^{g}	1300.2±5.2^{c}

TPC was expressed as gallic acid equivalent and TFC was expressed as rutin equivalents. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

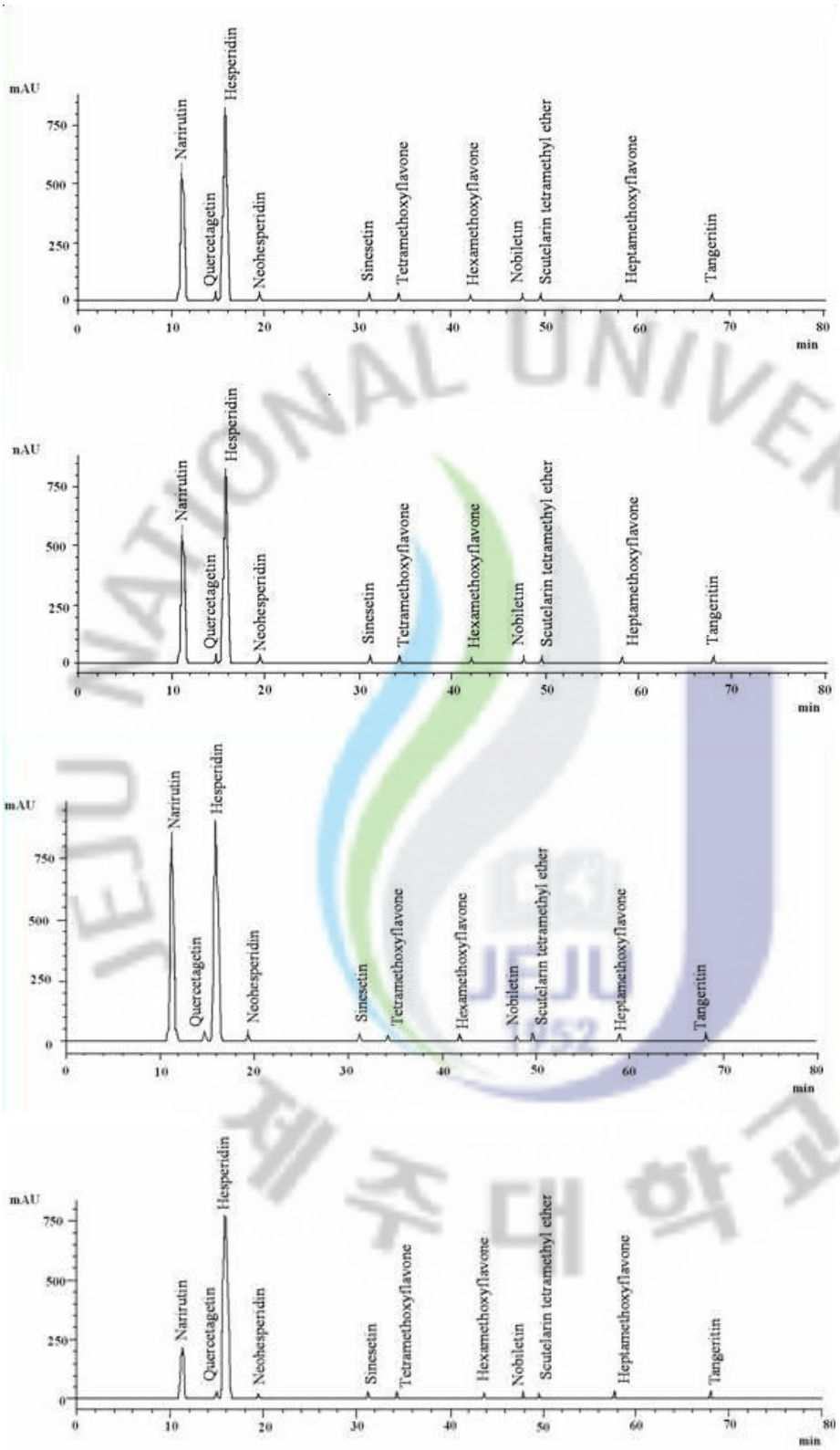


Fig. 1-4. HPLC profiles of the extracts from CPC dried by FD, HSD, FIR-40 and FIR-50, at 280 nm.

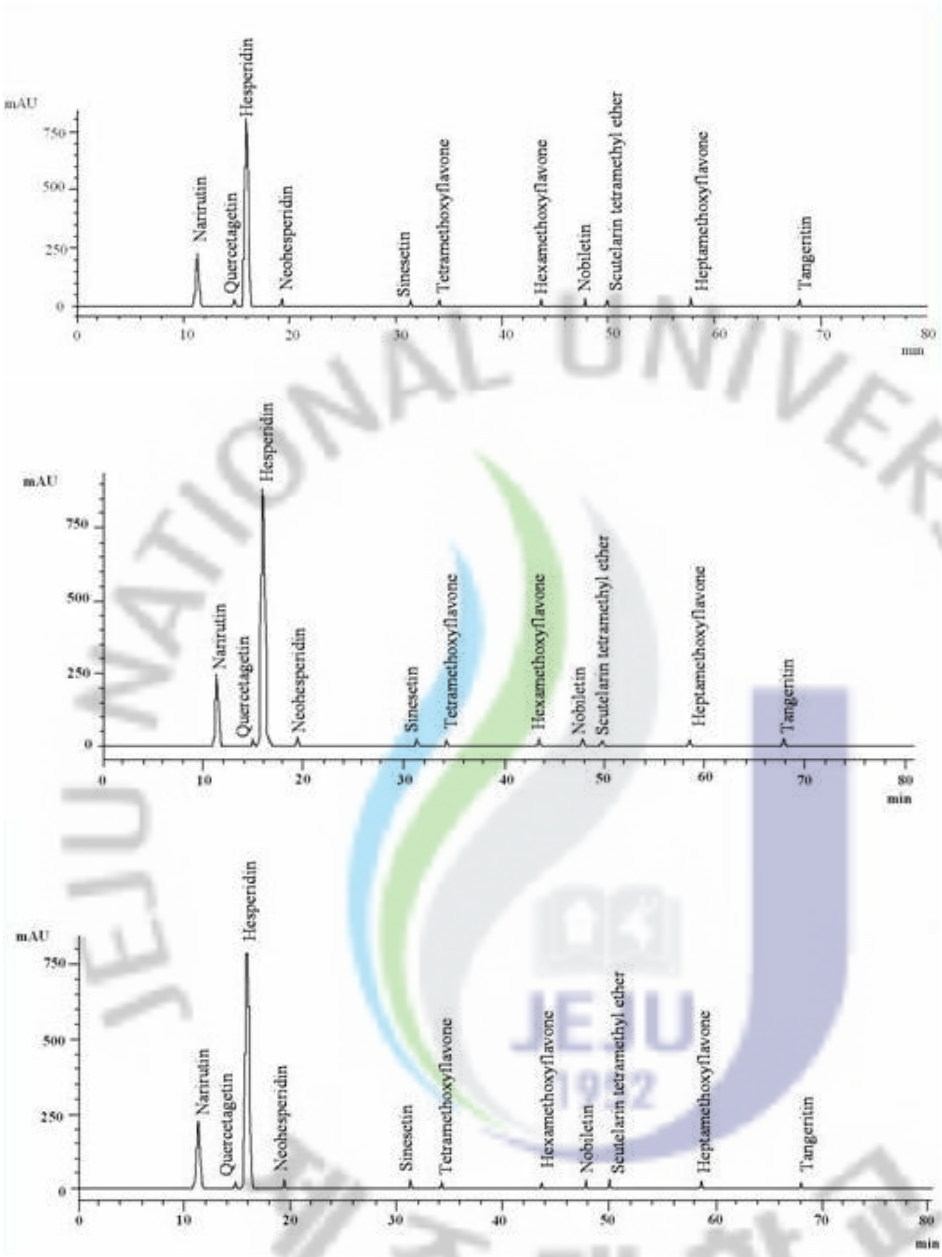


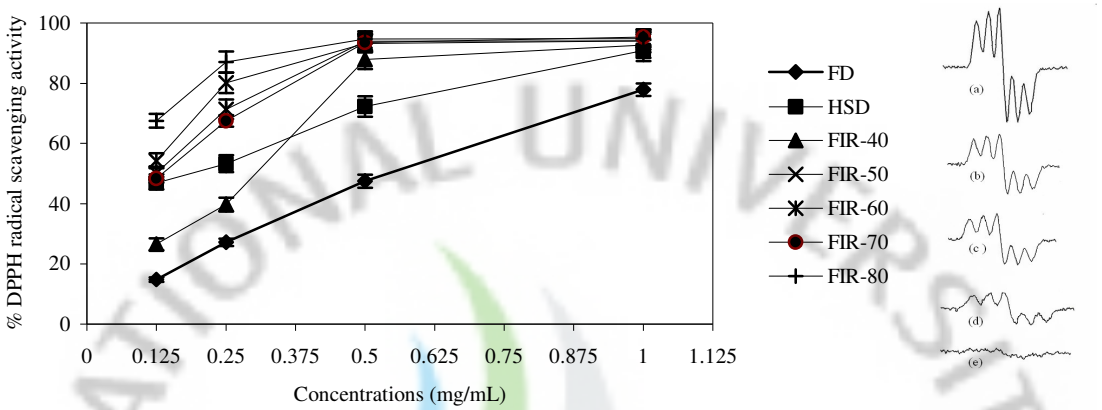
Fig. 1-4. Continued HPLC profiles of the extracts from CPC dried by FIR-60, FIR-70 and FIR-80, at 280 nm.

Table 1-4. Flavonoid composition of CPC dried by FD, HSD and FIR

Flavonoid	FD (mg/100g)	HSD (mg/100g)	FIR-40 (mg/100g)	FIR-50 (mg/100g)	FIR-60 (mg/100g)	FIR-70 (mg/100g)	FIR-80 (mg/100g)
Narirutin	109.24±0.83^{c}	85.54±4.91^{a}	82.75±2.10^{a}	99.15±1.01^{b}	98.7±1.03^{b}	109.50±0.79^{c}	97.35±1.82^{a}
Quercetagetin	6.02±0.59^{a}	8.62±0.81^{b}	8.90±0.03^{b}	14.27±0.07^{e}	12.67±0.70^{d}	13.45±0.36^{de}	11.45±0.21^{c}
Hesperidin	367.45±3.91^{d}	258.38±7.27^{a}	321.93±4.31^{c}	309.75±14.02^{b}	308.25±3.07^{b}	371.45±1.87^{d}	316.17±4.96^{bc}
Neohesperidin	12.62±0.08^{e}	18.17±0.74^{g}	5.57±0.38^{a}	6.75±0.07^{b}	9.63±0.15^{c}	11.25±.28^{d}	16.22±0.29^{f}
Sinesetin	7.80±0.27^{d}	5.55±0.74^{c}	3.47±0.41^{a}	3.10±0.04^{a}	3.13±0.23^{a}	2.27±0.27^{a}	3.03±0.22^{a}
3',4',7,8- tetramethoxy flavone	4.58±0.04^{e}	3.59±0.38^{d}	0.78±0.12^{a}	1.15±0.10^{b}	2.45±0.09^{f}	2.20±0.19^{c}	3.40±0.15^{d}
5,6,7,3',4',5'-hexamethoxy flavone	4.97±0.50^{cd}	15.86±0.62^{e}	2.49±1.26^{a}	2.97±0.02^{a}	5.17±0.13^{d}	4.15±0.08^{b}	4.42±0.12^{bc}
Nobiletin	58.20±0.88^{e}	38.10±0.55^{d}	19.85±0.59^{c}	11.65±0.03^{a}	13.67±0.41^{b}	11.97±0.02^{a}	13.85±0.22^{b}
Scutelarin tetramethyl ether	1.13±0.21^{cd}	1.06±0.01^{bcd}	0.61±0.03^{a}	0.97±0.02^{bc}	1.17±0.05^{d}	0.95±0.03^{b}	1.22±0.08^{d}
3,5,6,7,8,3',4'-heptamethoxy flavone	1.13±0.21^{a}	36.02±0.96^{f}	14.00±0.06^{e}	10.47±0.19^{b}	13.17±0.30^{d}	11.32±0.22^{c}	12.52±0.18^{d}
Tangeritin	8.13±0.57^{d}	6.52±0.01^{c}	2.81±0.32^{a}	2.77±0.03^{a}	2.97±0.09^{ab}	3.00±0.01^{ab}	3.4±0.04^{b}

All data are mean of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

The results of DPPH radical scavenging activities of different concentrations of the extracts from CPC dried by FD, HSD and FIR are depicted in Fig.1-5. Antioxidant components in the extracts react with DPPH radical, which is a nitrogen-centered radical and convert to 1, 1,-diphenyl-2-picryl hydrazine due to its hydrogen donating ability at very rapid rate (Jayaprakasha, et al., 2004). The extracts from CPC dried by FD, HSD and FIR showed dose dependent activity in DPPH radical scavenging. CPC dried by HSD showed IC_{50} as 0.16 mg/mL. The activities of the extracts from CPC dried by FIR ranged from IC_{50} 0.092 mg/mL to 0.31 mg/mL. Those activities were compared with that of CPC dried by FD. CPC dried by HSD showed significantly higher ($p<0.05$) activity at all the concentration compared with that of CPC dried by FD. The antioxidant activity of a sample is attributed to their hydrogen donating ability (Shimada et.al., 1992). The data obtained revealed that the flavonoid constituents in the extracts from CPC dried by HSD are free radical scavengers and primary antioxidants that react with DPPH radical, which may be attributed to its proton donating ability. It is generally accepted that antioxidant activity of flavonoids is mainly due to the presence of 2-3- double bond in conjugation with a 4-oxo function, and hydroxyl groups in positions 3',4' and 7. These sites can be considered as the active centers or the prerequisite factors for the scavenging of free radicals (Heimet et al., 2002). As CPC also contains several flavanone (Narirutin, Hesperidin and Neohesperidin) and flavone (Quercetagetin) which fulfill the above requirements, they might be encountered for the strong radical scavenging activity. Further, Parejo et al. (2005) have studied different quercetagetin and have exhibited that high radical scavenging activity. Other than the flavonoids, polyphenolic compounds also might be contributed to the scavenging activity.



Sample	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
IC_{50} (mg/mL)	0.18±0.004^{f}	0.16±0.005^{e}	0.31±0.01^{g}	0.11±0.005^{d}	0.12± 0.002^{c}	0.13±0.001^{d}	0.09±0.005^{a}

Fig. 1-5. DPPH radical scavenging activity of methanolic extracts from CPC dried by FD, HSD and FIR. ESR spectrums obtained for the extracts from CPC dried by FIR-50; (a) control; (b) 0.125 mg/mL; (c) 0.25 mg/mL, (d) 0.5 mg/mL; (e) 1.0 mg/mL. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

Superoxide radicals are generated in the biological systems during the normal catalytic functions of certain enzymes. These radicals are not harmful by themselves, but they can be converted into hydrogen peroxide via enzymatic or nonenzymatic pathways, leading to generation of cell-damaging hydroxyl radicals (Wyllie and Liehr, 1997). As illustrated in Fig. 1-6, all the extracts reduced the ESR signal intensity dose-dependent manner indicating their ability to scavenge the superoxide radicals. The extracts from CPC dried by FIR at 60°C exhibited the highest inhibitory activity against superoxide radical (IC_{50} 0.25 mg/mL). It was slightly higher than the IC_{50} value of the extracts from CPC dried by FD (0.26 mg/mL). The superoxide radical scavenging activity of extract from CPC dried by HSD showed significant activity (IC_{50} 0.28 mg/mL).

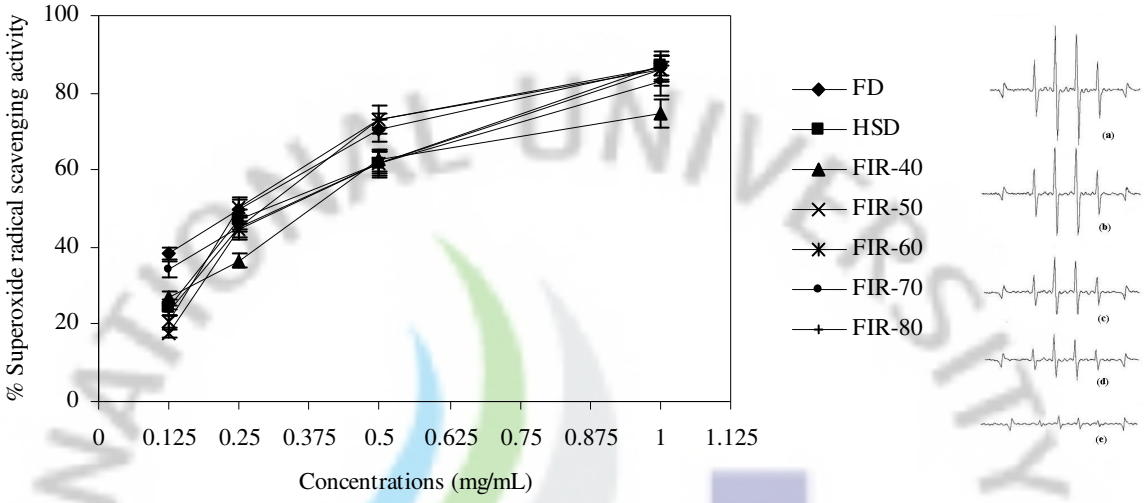
Hydroxyl radicals are highly reactive free radicals, which are capable of attacking biological substrates such as carbohydrates, DNA, polyunsaturated fatty acids and proteins. Prevention of such reactions is highly significant in terms of both human health and the shelf-life of food stuffs, cosmetics and pharmaceuticals (Sasaki, et al., 1996; Branen and Davidson, 1997). Hence, it is considered important to assess the protective ability of the extract from CPC dried by HSD and FIR against hydroxyl radicals. Flavonoids are considered as excellent hydroxyl radical scavengers and seem to possess a degree of effectiveness which depends on their structure according to the works of some researchers (Pincemail et al., 1986). The hydroxyl radicals generated in Fe^{2+}/H_2O_2 system trapped by DMPO forming spin adduct which could be detected by ESR spectrometer ($a^N = a^H = 14.9$). The relative amount of DMPO-OH adduct is represent by the height of the second peak of the ESR spectrum as seen in the Fig. 1-7. The decrease of the amount of DMPO-OH adduct is shown in the ESR spectrum after addition of the different

concentration of the extracts from CPC dried by HSD and FIR. The IC_{50} of hydroxyl radical scavenging activity of extracts from CPC dried by HSD was 0.5 mg/mL. The addition of extracts from CPC dried by FIR to the reaction mixture (from 0.25 to 2.0 mg/mL) resulted in a dose-dependent inhibition of the ESR signal intensity of DMPO-OH spin adduct. The extracts from CPC dried by FIR at 40 and 70°C exhibited significantly higher ($p<0.05$) activity (IC_{50} 0.48 mg/mL) than that of other FIR extracts. Further, antioxidant activity of CPC dried by HSD was higher than that of CPC dried by FD. Gao et.al. (1999) have shown strong hydroxyl radical scavenging activity for flavonoids with ESR spectrometry. Therefore, the strong hydroxyl radical scavenging activity of CPC dried by HSD demonstrate that great potential of flavonoid compounds present in the extract against a variety of complications of this extremely reactive radical.

The hyperfine coupling constants for the 4-POBN radical adducts were a^N =12.2 G and a^H =2.4 G. When AAPH was incubated with the spin trap 4-POBN at 37°C for 30 min, an alkyl radical adduct was observed (Fig. 1-8). The extract from CPC dried by HSD scavenged alkyl radical effectively and dose-dependently, the IC_{50} of the extracts was 0.17 mg/mL. With the addition of the extracts from CPC dried by FIR, a decreased ESR signals could be seen. The results showed that the extracts from CPC dried by FIR scavenged alkyl radical effectively and dose-dependently. However, there was no significant difference ($p<0.05$) in the activity between the extracts. CPC dried by FIR at 50°C extracts showed the highest activity (IC_{50}: 0.02 mg/mL) among all the extracts. Further, it showed higher activity than that of the extracts from CPC dried by FD. The results obtained for the alkyl radical scavenging showed potential to act against harmful alkyl radicals even at low concentrations. Alkyl radicals are directly produced by the

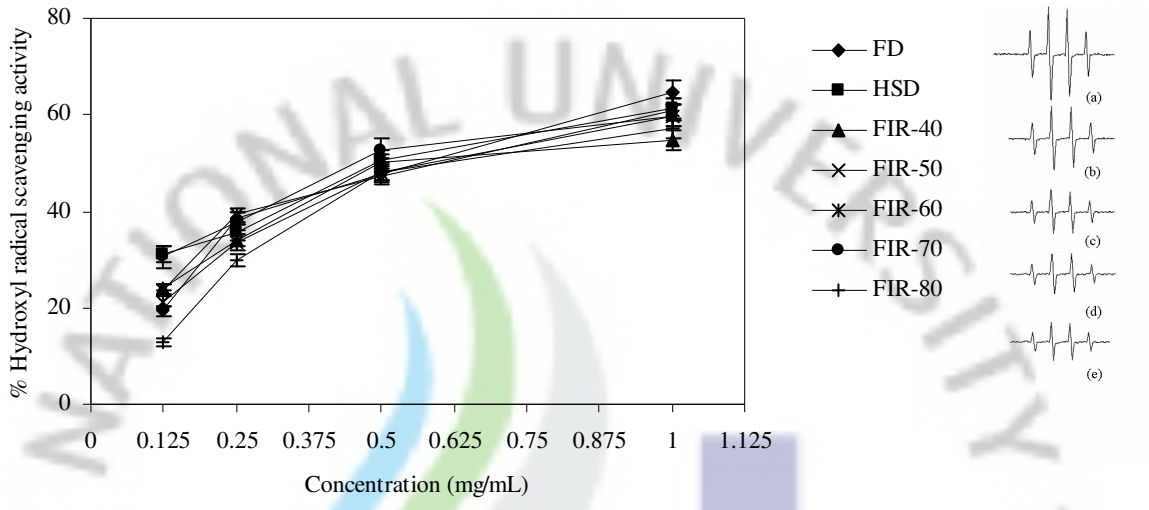
metal mediated free radical generation of unsaturated fatty acids. Therefore, different kinds of carbon centered alkyl radicals are produced during lipid peroxidation and the radicals formed react with variety of important biomolecules (Chamulitrat and Mason, 1990).

The extracts from CPC dried by FD showed significantly higher ($p<0.05$) hydrogen peroxide scavenging activity (IC_{50}: 0.22 mg/mL) than those of other extracts (Fig. 1-9). The extracts from CPC dried by FIR showed moderate activity against H_2O_2 scavenging. The extract from CPC dried by HSD scavenged hydrogen peroxide dose-dependently; the IC_{50} was 0.38 mg/mL. It is well established that hydrogen peroxide is not dangerous as it is, but it can be a deleterious because of its ability to form hydroxyl radicals, hence, it is important to eliminate hydrogen peroxide molecules from the medium. It has already been proven that dietary phenols protect mammalian and bacterial cells from cytotoxicity induced by H_2O_2 (Nakayama, 1994; Nakayama et al., 1993), indicating that the observed H_2O_2 scavenging activity of the extracts from CPC could be due to the presence of phenolic compounds.



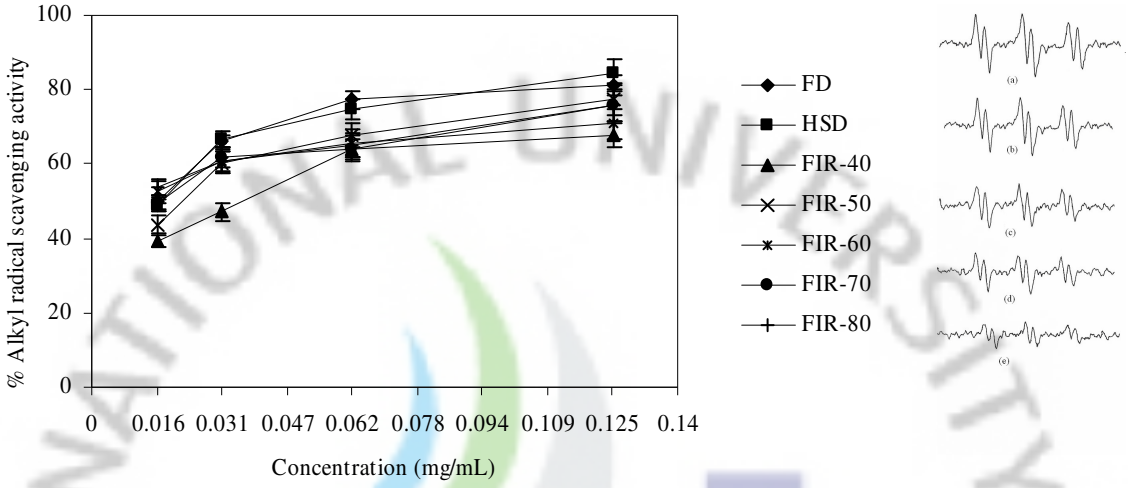
	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
IC_{50} mg/mL	0.26±0.005[a]	0.28±0.003[ab]	0.3± 0.005[bc]	0.32±0.006[c]	0.25±0.005[a]	0.36±0.002[d]	0.32±0.008[c]

Fig. 1-6. Superoxide radical scavenging activity of methanolic extract from CPC dried by FD, HSD and FIR. ESR spectrums obtained for the extracts from CPC dried by FIR-60; (a) control; (b) 0.125 mg/mL; (c) 0.25 mg/mL, (d) 0.5 mg/mL; (e) 1.0 mg/mL. All data are means of three determinations. Significant differences at $p<0.05$ indicated with different letters.



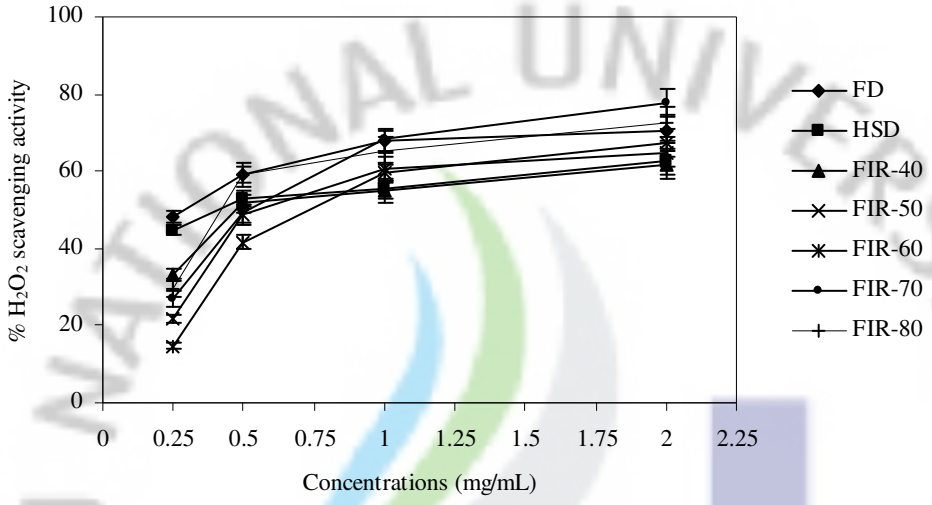
Sample	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
IC_{50} (mg/mL)	0.53 ± 0.01^{c}	0.5 ± 0.02^{ab}	0.49 ± 0.02^{a}	0.53 ± 0.01^{c}	0.52 ± 0.05^{bc}	0.48 ± 0.01^{a}	0.52 ± 0.01^{bc}

Fig. 1-7. Hydroxyl radical scavenging activity of methanolic extract from CPC dried by FD, HSD and FIR. ESR spectrums obtained for the extracts from CPC dried by FIR-70; (a) control; (b) 0.125 mg/mL; (c) 0.25 mg/mL, (d) 0.5 mg/mL; (e) 1.0 mg/mL. All data are means of three determinations. Significant differences at $p<0.05$ indicated with different letters.



Sample	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
IC_{50} (mg/mL)	0.016±0.0005[a]	0.017±0.0002[a]	0.04±0.0001[a]	0.02±0.0001[a]	0.014±0.0006[a]	0.015±0.0005[a]	0.014±0.002[a]

Fig. 1-8. Alkyl radical scavenging activity of methanolic extract from CPC dried by FD, HSD and FIR. HSD. ESR spectrums obtained for extracts from CPC dried by HSD extracts; (a) control; (b) 0.0156 mg/mL; (c) 0.0312 mg/mL, (d) 0.0625 mg/mL; e- 0.125 mg/mL. All data are means of three determinations. Significant differences at $p<0.05$ indicated with different letters.



Sample	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
IC_{50} mg/mL	0.22± 0.005[a]	0.38±0.01[b]	0.45±0.01[d]	0.55±0.02[e]	0.73±0.02[f]	0.55±0.01[e]	0.42±0.005[c]

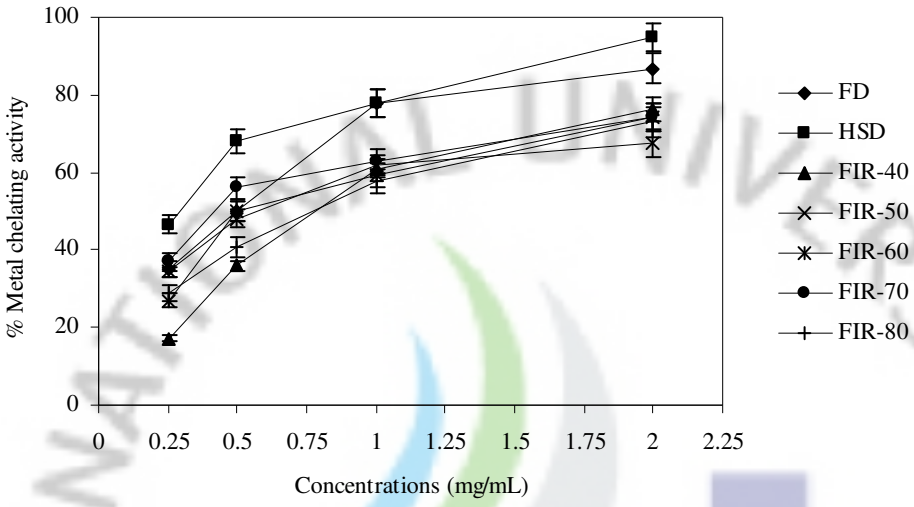
Fig. 1-9. Hydrogen peroxide scavenging activity of methanolic extract from CPC dried by FD, HSD and FIR. All data are means of three determinations. Significant differences at $p<0.05$ indicated with different letters.

The main mechanism of metal-chelating activity is the ability of compound/extracts to deactivate and/or chelate transition metals which can promote the Fenton reaction and hydroperoxide decomposition. Phenolic compounds mainly the flavonoids present in the extracts from CPC may be able to play a protective role against oxidative damage by sequestering ferrous ions that may otherwise catalyze Fenton-type reactions or participate in metal-catalyzed hydroperoxide decomposition reactions. The extracts from CPC dried by HSD showed significantly higher ($p<0.05$) activity (IC_{50}: 0.28) for metal chelating among all the extracts tested (Fig 1-10). Also, the extracts from CPC dried by FIR showed dose dependent metal chelating activity. The IC_{50} values of the extracts from CPC dried by FIR were 0.8, 0.62, 0.5, 0.45 and 0.9 mg/mL for the extracts from CPC dried by FIR at 40, 50, 60, 70 and 80°C, respectively. Accordingly, the extract from CPC dried by FIR at 70°C showed the highest activity on metal-chelating among the extracts from CPC dried by FIR.

Peroxidation is an important process in food deterioration and in the oxidative modification of biological molecules particularly lipids. Inhibition of lipid peroxidation by any external agent is often used to evaluate its antioxidant capacity. In the case of linoleic acid system, the extract from CPC seems to inhibit the oxidation of linoleic acid which is an important issue in food processing and preservation. As shown in the Fig. 1-11, the absorbance of linoleic acid emulsion without addition of the extracts (control) increased significantly. High absorbance is an indication of high concentration of peroxides. The extracts from CPC dried by HSD and FIR showed significantly higher ($p<0.05$) inhibitory activities compared with commercial antioxidants such as BHT and α-tocopherol. Also, its activity is almost same with the activity of the extracts from CPC

dried by FD. Antioxidants minimize the oxidation of lipid components in cell membranes or inhibit the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation that are known to be carcinogenic. Further, antioxidants are believed to intercept the free radical chain of reaction and to donate hydrogen from the phenolic hydroxyl groups, thereby forming stable end product, which does not initiate or propagate further oxidation of lipid (Sherwin, 1978).

The described on-line HPLC-$ABTS^+$ method can be used for a rapid assessment of antioxidative components in complex mixtures (Stewart et. al. 2005; Chandrasekar et al. 2006; Nuengchamnong et al. 2005; Perez-Bonilla et al. 2006). Simultaneously obtained UV (positive) and $ABTS^+$ (negative) radical quenching chromatograms using gradient elution of the crude extracts separated with acitonitrile, and water with TFA are presented in Fig. 1-12. HPLC elute was mixed with a stabilized solution of $ABTS^+$ radicals followed by HPLC separation and the separated fractions were directed to a UV/Vis detector monitoring absorbance at 734 nm. The absorbance of the $ABTS^+$ radical reaction mixture was decreased due to its reduction by the antioxidant compounds in the extracts and was indicated by the negative peak. Hence, $ABTS^+$-based antioxidant activity profile too showed that several active compounds are present in the extracts and those compounds exhibited strong antioxidant activity. Hence, again it was confirmed that flvonoids present in the extracts may be responsible for the antioxidative activities obtained in this study.



Sample	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
IC_{50} mg/mL	0.5 ± 0.01^{b}	0.28 ± 0.01^{a}	0.8 ± 0.05^{d}	0.62 ± 0.02^{c}	0.5 ± 0.02^{b}	0.45 ± 0.03^{b}	0.9 ± 0.03^{e}

Fig. 1-10. Ferrous ion chelating activity of methanolic extract from CPC dried by FD, HSD and FIR. All data are means of three determinations. Significant differences at $p<0.05$ indicated with different letters.

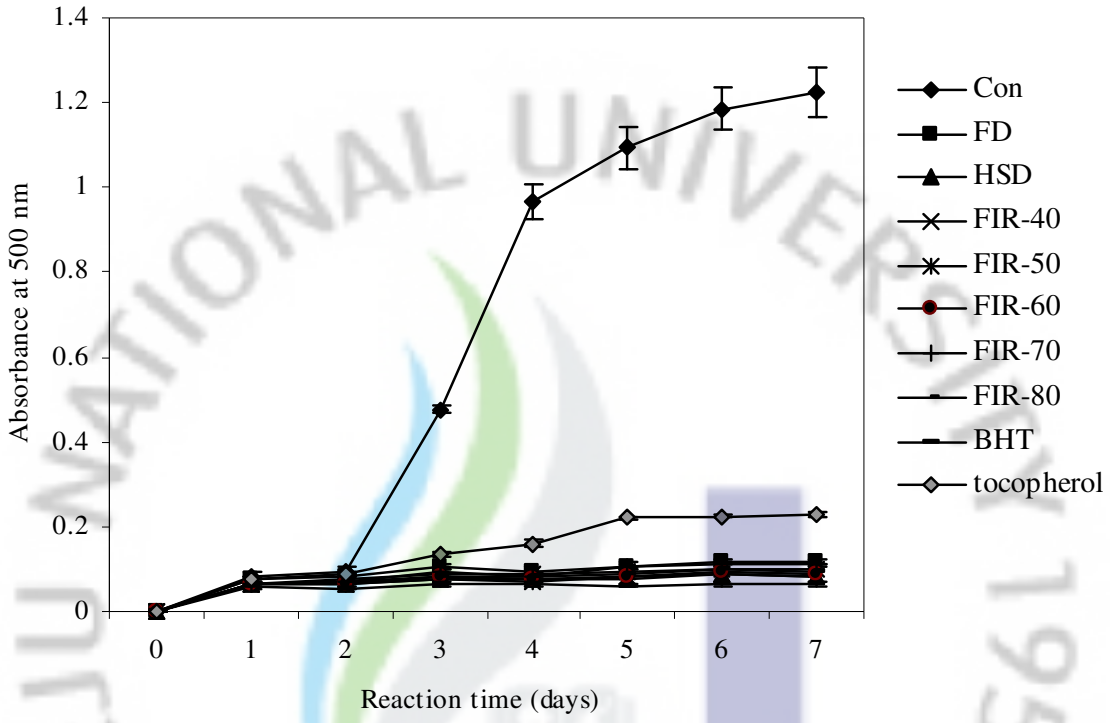


Fig. 1-11. Lipid peroxidation inhibitory activity of CPC dried by FD, HSD and FIR. Peroxidation was measured in linoleic acid emulsion system by ferric thiocyanate method. All data are means of three determinations.

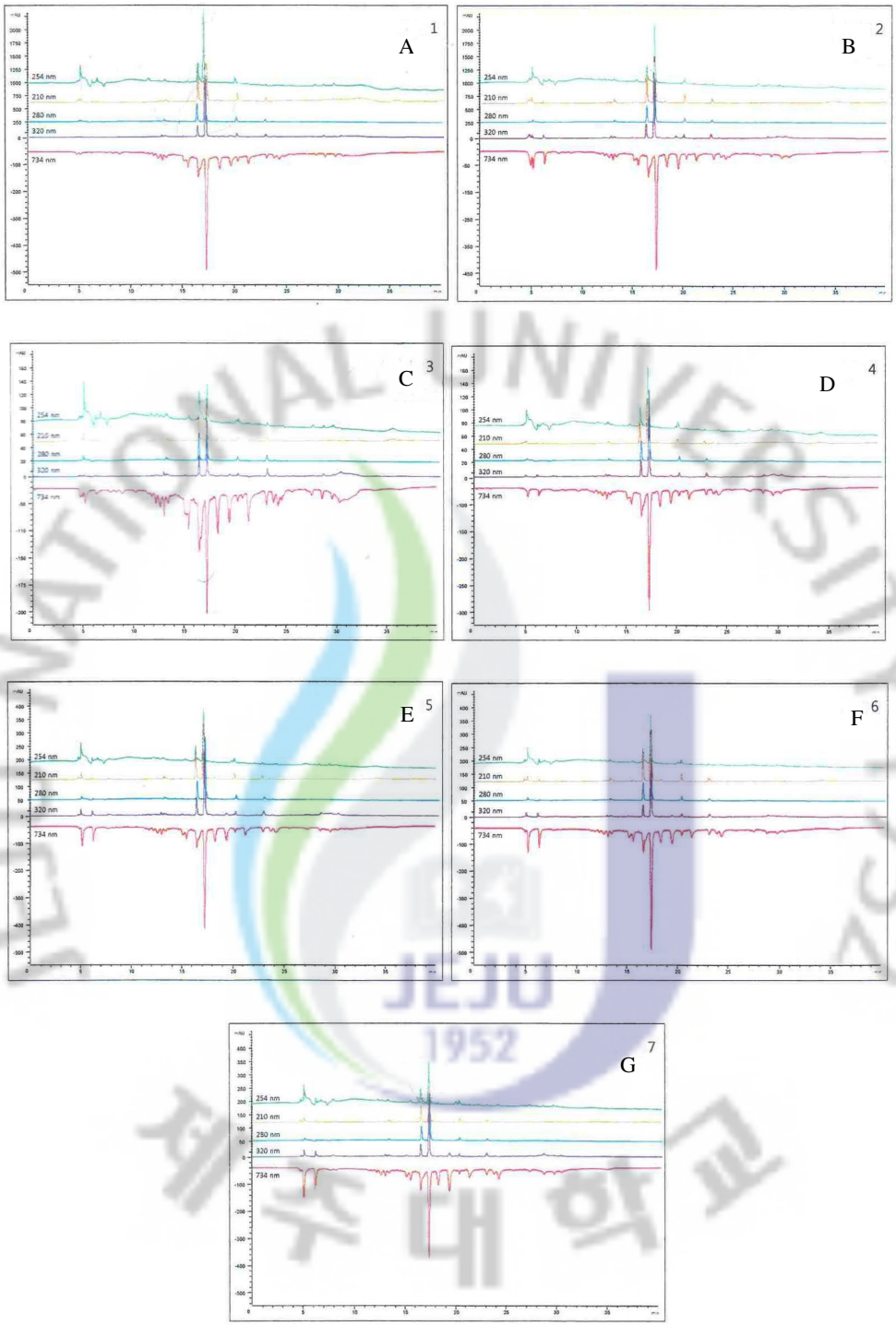


Fig. 1-13. On-line HPLC-ABTS^{+} analysis of methanolic extracts from CPC dried by FD, HSD and FIR. A 10 μL of aliquots were analysed by gradient reversed-phase HPLC with a DAD at 280 nm (positive trace) and and analysis of antioxidant potential at 734 nm (negative trace). A: FD, B: HSD, C: FIR-40, D: FIR-50, E: FIR-60, F: FIR-70, G: FIR-80

In conclusion, the DPPH, superoxide, hydroxyl and alkyl radical scavenging activities were higher in the the methanolic extracts from CPC dreid by FIR at 50, 60, 80°C, respectively. The extracts from CPC dried by HSD showed higher activity in metal chelating and lipid peroxidation while the extracts from CPC dried by FD showed higher activity in hydrogen peroxide scavenging. The bioactivities may increase in the extracts from CPC dried by FIR when the infrared intensity increased by increasing temperature, by liberating the bound compounds. However HSD extracts showed higher total phenolic content than that of the extracts from CPC dried by FIR. Also, the flavonoid content in the extracts from CPC dried by HSD and FIR were slightly higher than that of FD. The main constituents in the extracts from CPC dried by FD, HSD and FIR were hesperidin, narirutin and nobelitin according to HPLC analysis data. Functional properties of CPC were not adversely affected by the drying method. Therefore, HSD is a very efficient method compared with other two methods used in this study considering the drying time, the amount of press-cake transform at a time, antioxidant activities and flavonoid content. Therefore, HSD is and effective method to dry wet CPC into dried form and to determine their bioactivities.

Part II

Antioxidant Properties of Enzymatic Digests from Citrus Press-Cake Dried by High Speed Drying and Far-infrared Radiation Drying and Their Protective Effect on H_2O_2- induced DNA Damage

ABSTRACT

The antioxidant activieis of the enzymatic digests from citrus press-cake (CPC) were evaluated using different antioxidant assays. Freze drying (FD), High speed drying (HSD) and far-infrared radiation (FIR) drying methods were used to dry the CPC. Enzymatic digests were prepared from the CPC dried by FD, HSD and FIR by six enzymes (AMG, Celluclast, Pectinex, Termamyl, Ultraflo and Viscozyme). AMG extract was selected for further experiments depending on their total polyphenolic and DPPH radical scavenging activities. Antioxidant activities of AMG digests from CPC dried by HSD and FIR were evaluated by different in-vitro models such as DPPH, superoxide, hydroxyl, alkyl radicals, H_2O_2 scavenging, metal chelating, lipid peroxidation and comet assay. The AMG digests from CPC dried by FIR at 50°C showed strong antioxidant activities in DPPH, superoxide, alkyl and metal chelating assays while all the digests showed strong lipid peroxidation activities. CPC dried by HSD showed higher activity in hydrogen peroxide scavenging while the CPC dried by FIR at 40°C showed higher activity in hydroxyl radical scavenging. Further, enzymatic digests showed remarkable inhibitory activities against H_2O_2-induced DNA damage. Hence, the data obtained using different *in-vitro* models clearly established the antioxidant potential of enzymatic digests from CPC dried by HSD. Further, CPC can be used as source of potential natural antioxidant; hence, HSD is a choice of interest to transform wet CPC into dried form effectively.

INTRODUCTION

Oxidation is an essential process in living organisms to produce the necessary energy to fuel biological activities. However, over production of oxygen-centered free radicals and other reactive oxygen species (ROS) result in cell death and tissue damage. ROS has been implicated in several chronic diseases, such as aging, cancer, cardiovascular diseases, and diabetes (Halliwell and Gutteridge, 1999). Antioxidants are vital compounds which are capable of protecting the body from damage caused by free radicals induced oxidative stress.

Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are largely employed as preservatives in pharmaceutical, cosmetic and food industries. However, those synthetic antioxidants are believed to cause side effects on human (Silva et al., 2005). Hence, most consumers are concerned about the safety of their food, and about the detrimental effect of synthetic additives on their health. Therefore, the need of replacing synthetic antioxidants by naturally-occurring antioxidants, probably with safe antioxidants has initiated with the screening of plant sources, especially the inexpensive waste from agricultural industries (Moure et al., 2001).

Huge amount of CPC are formed every year from the citrus processing plants and they are excellent sources of different phytochemicals (Rio et al., 2004; Nam et al., 2002; Ko et al., 2000), most of them contain potent biological activities. As this press-cake may be deteriorated rapidly they need to be transformed into a dried form, if they are needed to use in effective manner. In order to increase the efficacy in drying process, HSD was applied to transform the CPC into a dried form in this study. This method is more

economical and less time consuming as it is able to transform large amount of press-cake into a dried form at a single turn. Also, this method can be used in a commercial scale economically the efficacy of HSD for CPC drying was examined by comparing with a conventional freeze drying because the final quality and potential activity of the dried products would be determined by the structural and compositional modifications which might have occurred during the drying treatment (Femenia et al., 2003). Further, far-infrared radiation (FIR) drying was applied separately to convert the wet CPC into dried form in order to increase the efficacy in the drying process. FIR drying provides many advantages over conventional drying methods. When FIR is used to dry some material, the radiation colloids on the surfaces of the exposed material and penetrates to create internal heating with molecular vibration of the material while FIR energy is converted into heat (Ginzburg, 1969). The depth of penetration depends on the composition and structure of the material and also on the wavelengths of FIR. It has been reported that the drying rate of FIR drying process is higher than the conventional hot-air drying, and increases with increased power supply to the far infrared emitter (Masamura et al., 1988). FIR is able to to liberate antioxidative polyphenolic compounds in plant material, which exist as covalently bound form with polymers (Niwa and Miyachi, 1986).

The extraction method affects the total polyphenolic contents and antioxidant capacities (Lee et al., 2003). Water, methanol, ethanol and ethyl acetate are commonly used to extract bioactive compounds from plant materials. Also, enzymes can macerate the tissue by breaking down of cell walls, and cellular components can be taken out of the cells. Therefore, in the present study, the efficiency of the enzymatic digestion of the CPC

dried by HSD, FIR and FD was evaluated, by comparing the antioxidant activities with that of the water extracts.

The enzymatic digestion is a choice of interest to get higher total soluble solid content of desired compounds from the natural resources while it is an easily accessible extraction and purification process in the industry (Fleurence, 1999; Jeon et al., 1999; Heo et al., 2003). During enzymatic digestion process, bioactive compounds are released from the complex interior storage materials. Furthermore, enzymatic digestion process possesses the number of advantages and characteristic features compared to conventional extraction procedures. Higher extraction efficacy, water solubility, variation of constituents, minimized environmental pollution and relatively less expensiveness are some of those advantages.

The aims of this study were to introduce an effective drying method to transform huge amount of wet CPC into dried form, and to determine antioxidative abilities to provide information on functional food additives.

MATERIALS AND METHODS

Materials

CPC was obtained from Jeju provincial development Co. in Jeju Island, Korea. DPPH, 5,5-Dimethyl-1-pyrrolin N-oxide (DMPO), 2,2-azobis (2-amidinopropane) hydrochloride (AAPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma Co. (St. Louis, USA). Viscozyme L (a multi-enzyme complex containing wide range of carbohydrases, including arabinase, cellulase, beta-glucanase, hemicellulase and xylanase), Celluclast 1.5 L FG (catalyzing the breakdown of cellulose into glucose, cellobiose and higher glucose polymers), AMG 300L (an exo l, 4-alpha-d-glucosidase), Termamyl 120L (a heat stable alpha amylase), Ultraflo L (a heat stable multi-active beta-glucanase) and Pectinex were purchased from Novo Co. (Novozyme Nordisk, Bagsvaed, Denmark). All other chemicals used were analytical grade supplied by Fluka or Sigma Co.

High speed drying or far-infrared radiation drying of CPC

CPC stored at -50°C was converted into drie form by high speed drier (Okadora Korea, Incheon, Korea) or far-infrared radiation drier (Model TOURI-Q, Korea). The operating conditions of the high speed dryer (Okadora Korea) were as follows: drying time 90 min, heating temperature 120°C and the amount of dried sample obtained from the drying process was 3 kg. The operating conditions of freeze dryer (Ilshin, Korea) were; drying time 3 days, heating temperature -56°C and the amount of dried sample obtained from the drying process about 600 g. Then, the dried CPC were pulverized into a fine powder

using a grinder (MF 10 basic mill, GMBH & Co., Staufen, Germany) and sieved through a 300 μm standard testing sieve.

Preparation of enzymatic digests

One gram of CPC dried by HSD or FIR was mixed with 100 mL of distilled water. The optimum pH was adjusted with 1M HCl / NaOH as described by Heo et al. (2003). Thereafter, the enzymatic digestion was performed for 24 hrs to reach an optimum degree of digestion. The resultant mixture was inactivated by keeping in a water bath at 100^{o}C for 10 min and filtered. Finally, each digest was adjusted to pH 7 with 1M HCl / NaOH.

Total Polyphenolic content assay

Total polyphenolic content was determined according to the method described by Chandler and Dodds (1983). One millilitre of the digest was mixed in a test tube containing 1 mL of 95% ethanol, 5 mL of distilled water and 0.5 mL of 50% Foiln-Ciocalteu reagent. The resultant mixture was allowed to react for 5 min and 1 mL of 5% Na_2CO_3 was added. It was mixed thoroughly and placed in a dark condition for 1 hr and absorbance was recorded at 725 nm in the UV-VIS spectrophotometer (Opron 3000 Hansan Tech. Co.Ltd., Korea). A gallic acid standard curve was obtained for the calculation of polyphenolic content.

Total flavonoid content assay

The total flavonoid content of the digest was determined by the method of Zhuang et al. (1992) with slight modifications. A 0.5 mL aliquot of the digest was mixed with 2 mL of

distilled water and subsequently with 0.15 mL of a 5% $NaNO_2$ solution. After 6 min, 0.15 mL of a 10% $AlCl_3$ solution was added and allowed to stand further 6 min, thereafter 2 mL of 4% NaOH solution was added to the mixture. Immediately, distilled water was added to bring the final volume to 5 mL. Then it was properly mixed and allowed to stand for 15 min. Absorbance of the mixture was measured at 510 nm. Rutin was used as standard for the quantification of total flavonoid.

DPPH radical scavenging assay

This assay is based on the scavenging ability of stable DPPH radicals by the radical scavenging constituents in the digest. Method described by Nanjo et al. (1996) was used to investigate the free radical scavenging activity in electron spin resonance (ESR) spectrometer. DPPH solution in MeOH was prepared at the concentration of 60 μM. A 60 μL fraction of the digest was added to the same volume of freshly prepared DPPH. Then the reactants were thoroughly mixed and transferred to 50 μL glass capillary tube and fitted into the ESR spectrometer. The spin adduct was measured after 2 min. The measurement conditions were as follows; central field 3475 G, modulation width 0.8 mT, amplitude 500 mT, microwave power 5 mW, scan width 10 mT and temperature 25°C.

Superoxide radical scavenging assay

Superoxide anion radicals generated by UV irradiated riboflavin/EDTA system (Hiramoto, 1993) were detected by ESR spectrometer using DMPO as a spin trap. The reaction mixture containing 20 μL of 0.8 mM riboflavin, 20 μL of 1.3 mM EDTA, 20 μL of 0.8 mM DMPO and different concentrations of indicated digest. Riboflavin is oxidized to a flavin radicals, with the formation of $O_2^{\cdot-}$ in the presence of oxygen (Muller, 1995) via the

following reaction: Flavin (red) + $2O_2$ → Flavin (ox) + $O_2^{\cdot-}$ + $2H^+$. Then, the mixture was irradiated for 1 min under UV lamp at 365 nm and transferred to the capillary tube. Spin adduct was recorded with ESR spectrometer under the following operating conditions; central field 3475 G, modulation 0.1 mT, amplitude 200 mT, scan width 10 mT, microwave power 1 mW, and temperature 25°C.

Hydroxyl radical scavenging assay

Ability of the enzymatic digest to scavenge the hydroxyl radical was investigated by the method described by Rosen and Rauckman (1980). The Fenton reaction (Fe^{2}+ + H_2O_2 → $^{\cdot}OH$ + OH^-), a well known and defined generator of $^{\cdot}OH$ radicals, was used to produce $^{\cdot}OH$ and the radicals produced were reacted rapidly with nitrone spin trap DMPO. The reaction mixture contained 20 μL sample, 20 μL of 0.3 M DMPO, 20 μL $FeSO_4{\cdot}7H_2O$ and 20 μL of 10 mM H_2O_2. The resultant DMPO-OH adducts was investigated and the ESR spectrum was recorded after 2.5 min. The measurement conditions were as follows; central field 3475 G, modulation width 0.2 mT, amplitude 100 mT, microwave power 1 mW, scan width 10 mT and temperature 25°C.

Alkyl radical scavenging assay

Alkyl radicals were generated by AAPH and the scavenging effects of the digests were investigated by the method described by Hiramoto et al. (1993). The reaction mixture containing 20 μL of distilled water, 20 μL of the digest, 20 μL of 40 mM AAPH and 20 μL of 40 mM POBN was incubated at 37°C for 30 min. Then the reactants were transferred to a 50 μL glass capillary tube and fitted into the ESR spectrometer. The

measurement conditions were as follows; central field 3475 G, modulation width 0.2 mT, amplitude 500 mT, microwave power 8 mW, scan width 10 mT and temperature 25°C.

Hydrogen peroxide scavenging assay

This assay was carried out according to the method described by Muller (1995). The digest (80 μL) and 20 μL of 10 mM hydrogen peroxide were mixed with 100 μL of phosphate buffer (0.1 M, pH 5.0) in a 96-microwell plate and incubated at 37ºC for 5 min. Thereafter, 30 μL of freshly prepared 1.25 mM ABTS and 30 μL of peroxidase (1 U/mL) were mixed and incubated at 37º C for 10 min. Then, absorbance was measured at 405 nm.

Metal chelating ability

Metal chelating ability was determined according to the method by Decker and Welch (1990) with slight modifications. The digest (5 mL) was added to a solution of 0.1 mL of 2 mM $FeCl_2$. The reaction was started by the addition of 0.2 mL of 5 mM ferrozine solution and reaction mixture was incubated for 10 min at room temperature under shaking condition. After incubation, the absorbance of the reaction mixture was measured at 562 nm.

Lipid peroxidation inhibition by ferric thiocyanate (FTC) method

The lipid peroxidation inhibitory effect of the digest was determined according to the method described by Osawa and Namiki (1985) with slight modifications. Each digest (2 mg) was thoroughly mixed with 5 mL of 2.5% linoleic acid in ethanol, 5 mL of 0.05 M

phosphate buffer (pH 7.0) and 2.5 mL of distilled water. The mixture was kept in the dark at 60°C and analyzed every 24 hr interval. Fifty microlitre of the incubated solution was mixed with 9.7 mL of 75% ethanol and 0.1 mL of 30% thiocyanate. The mixture was allowed to stand for 5 min at room temperature before adding 0.1 mL of 20 mM ferrous chloride in HCl. The absorbance was recorded at 500 nm with an ELISA reader (Sunrise; Tecan Co., Austria).

Determination of the DNA damage reduction (Comet assay) by CPC

Vero cell line used in the comet assay was cultured in DMEM medium containing 10% fetal calf serum, streptomycin (100 μg/mL) and penicillin (100 unit/mL) at 37°C under a humidified atmosphere of 5% CO_2 in air.

Each AMG digest was diluted in PBS to make the final concentration to 25, 50 and 100 μg/mL. Vero cells were treated in three different ways. First, Vero cells (4×10^4 cell/mL) were incubated without the digest for 30 min at 37°C in a dark incubator (I). Second, Vero cells (4×10^4 cell/mL) were incubated without the digest for 30 min at 37°C and damaged oxidatively with 100 μM H_2O_2 (final concentration) for 5 min on ice (II). Third, Vero cells (4×10^4 cell/mL) were incubated with the digest from CPC dried by HSD, FIR and FD for 30 min at 37°C in a dark incubator and then treated with 100 μM H_2O_2 for 5 min on ice (III). After each treatment, digests were centrifuged at 1500 rpm for 5 min and washed with PBS.

The alkaline comet assay was conducted according to the method described by Singh et al. (1988) with slight modifications. The cell suspensions made in previous steps were mixed with 100 μL of 0.7% low melting agarose (LMA), and added to the slides

precoated with 1.0% normal melting agarose. After solidification of the agarose, slides were covered with another 100 μL of 0.5% LMA and then immersed in lysis buffer (2.5 M NaCl, 500 mM EDTA, 1 M Tris, and 1% sodium laurylasarcosine, and 1% Triton X-100) for 90 min. Later, slides were transferred into the unwinding buffer for another 20 min for DNA unwinding. The slides were next placed in the electrophoresis tank containing 300 mM NaOH and 1 mM Na_2 EDTA (pH 13.0), and for electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 minutes. Afterthat, the slides were washed two times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 10 min, and treated with ethanol for another 5 min before staining with 40 μL of ethidium bromide (20 μL/mL). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, U.K) and fluorescence microscope (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

Statistical analysis

All experiments were conducted in triplicate (n=3) and an ANOVA test (using SPSS 11.5 statistical software) was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by using the Duncan's test ($p<0.05$).

RESULTS AND DISCUSSION

The soluble solid content of the digests from CPC dried by FD, HSD and FIR are shown in Fig. 2-1. The enzymatic digests showed significantly higher ($p<0.05$) soluble solid content than those of water extracts which was no treated with enzymes. However, the digest from CPC dried by HSD showed slightly lower soluble solid content than that of CPC dried by FD. The total polyphenolic content of the digests from CPC dried by HSD FIR and FD was determined using Folin–Ciocalteu reagent and the results are shown in Fig. 2-1. Viscozyme and AMG digest showed higher total phenolic content than other digest. Phenolic compounds are potential antioxidants and free radical scavengers. It is well-known that citrus fruit and peel may contain large amount of flavonoid. Flavonoid compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties, hence, the total flavonoid content CPC was determined and is given in Fig. 2-3. The water extract showed significantly higher ($p<0.05$) total flavonoid content. The digest from CPC dried by FIR at 80°C showed the highest total flavonoid content. Flavonoid content has increased with the increment of the temperature in the FIR chamber. Hence, we can expect higher active compounds at higher temperature. AMG digest was used for further antioxidant assays, considering the DPPH radical scavenging activity obtained from preliminary investigation conducted with all the enzymatic digestions (data not shown) and polyphenolic contents.

DPPH assay is known to give reliable information with regards the antioxidant potential of the tested compounds (Rice-Evans et al., 1997). Table 2-1 demonstrate the DPPH radical scavenging activities of enzymatic digests and water extracts from CPC dried by

FD, HSD and FIR. The AMG digest from CPC dried by FIR at 50°C showed significantly higher ($p<0.05$) DPPH radical scavenging activity (IC_{50}: 0.1 mg/mL) compared with the digest from CPC dried by FD or water extracts from CPC dried by HSD and FIR. Lower IC_{50} value reflects strong DPPH radical scavenging activity (Molyneux, 2004). The data obtained revealed that the both digests from CPC dried by HSD and FIR are free radical scavengers and primary antioxidants that react with DPPH radical effectively, which may be attributed to electron donating ability of polyphenolic compounds present in the digests. Kang et al. (2006) have reported that the DPPH activity of citrus peel but the activities were lower than the values reported in this study. Moreover, the data in this study were almost close with the data reported by Yi et al. (2008). The activities shown by the enzymatic digest from CPC dried by HSD and FIR may be due to the presence of variety of polyphenolic and flavonoid compounds.

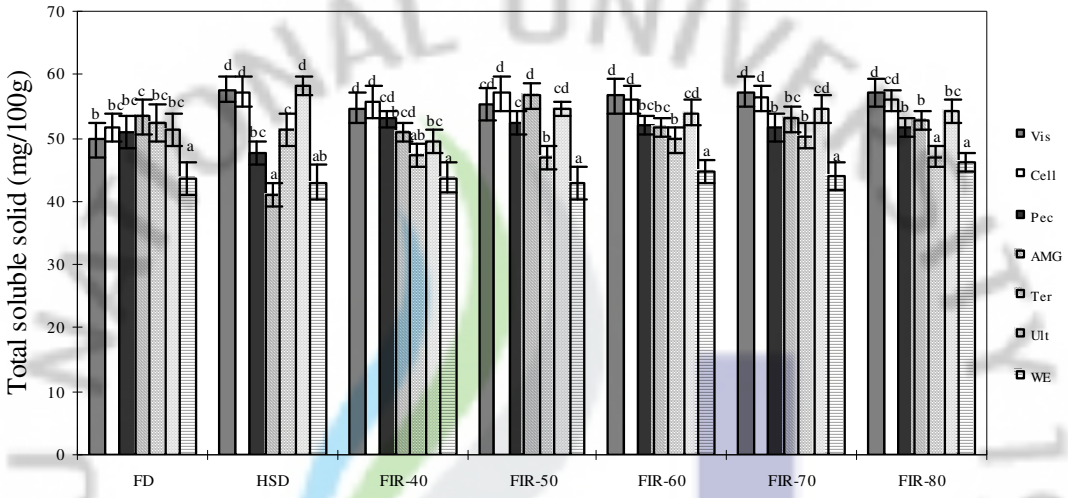


Fig. 2-1. Total soluble solid contents of the enzymatic digests and water extracts from CPC dried by FD, HSD and FIR. Vis: Viscozyme, Cell: Celluclast, Pec: Pectinex, AMG: Aminoglucozydase, Ter: Termamyl, Ult: Ultraflo, WE: water extract. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

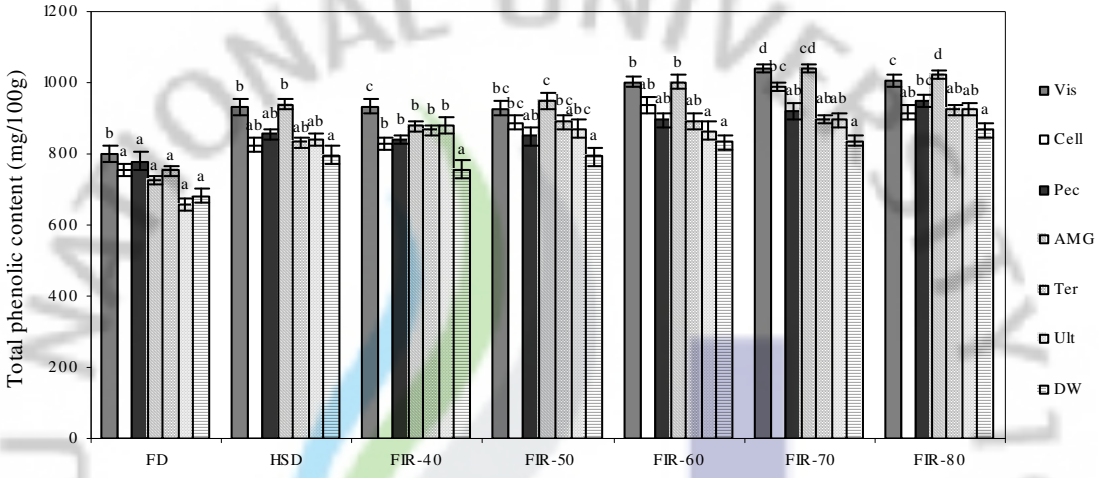


Fig. 2-2. Total phenolic contents of the enzymatic digests and water extracts from CPC dried by FD, HSD and FIR. Vis: Viscozyme, Cell: Celluclast, Pec: Pectinex, AMG: Aminoglucozydase, Ter: Termamyl, Ult: Ultraflo, WE: water extract. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

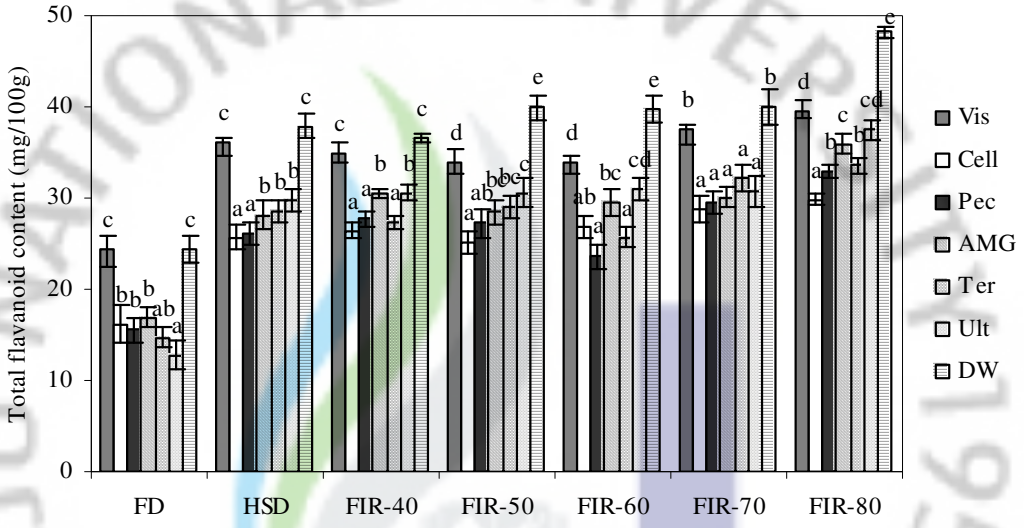


Fig. 2-3. Total flavonoid content of the enzymatic digests and water extracts from CPC dried by FD, HSD and FIR. Vis: Viscozyme, Cell: Celluclast, Pec: Pectinex, AMG: Aminoglucozydase, Ter: Termamyl, Ult: Ultraflo, WE: water extract. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

Superoxide radicals are generated in biological systems during the normal catalytic functions of certain enzymes. As illustrated in the Table 2-1, all the enzymatic digests from CPC dried by FD, HSD and FIR, and water extracts dose-dependently reduced the ESR signal intensity (ESR spectrum not shown) which indicates their ability to scavenge the superoxide radicals. The superoxide radical scavenging activity of the digest from CPC dried by FIR at 50°C was higher (IC_{50}: 0.28 mg/mL) than those of the digest from CPC dried by the FD (IC_{50}: 0.59 mg/mL) and HSD (IC_{50}: 0.43 mg/mL). The results did not showed significant difference ($p<0.05$). The superoxide radical scavenging activities of the digests from CPC dried by FIR at the different temperatures may be due to the presence of polyphenolic compounds and, the increase in the activity is due to increase in number of phenolic hydroxyl groups in the molecules. The reports about the superoxide radical scavenging activity of citrus peel or CPC are rare or not available; however, some workers have reported superoxide radical scavenging activities of citrus fruit juice (Jayaprakasha and Patil, 2007).

Hydrogen peroxide is not very reactive specie, but it can be toxic to cells, since hydrogen peroxide is capable of producing hydroxyl radicals inside the cell (Halliwell, 1991). Hence, removing of the excess hydrogen peroxide from the system is needed to protect the biomolecules from damaging. The AMG digests were capable of scavenging hydrogen peroxide in a dose-dependent manner and the results are shown in Table 2-1. AMG digest from CPC dried by HSD showed significantly higher ($p<0.05$) activity (IC_{50}: 0.57 mg/mL) in hydrogen peroxide scavenging than those of AMG digest from CPC dried by FD and FIR. Further, the digest from CPC dried by FIR at 50°C showed the strong (IC_{50}: 0.65 mg/mL) activity in H_2O_2 scavenging among the digests from CPC

dried by FIR at different temperatures. Yi et al. (2008) have reported the hydrogen peroxide scavenging activities of citrus peel extract and hesperidin. We are first reporting about the hydrogen peroxide activity of CPC. It has already been proven that dietary polyphenolic compounds protect the cells from cytotoxicity, induced by H_2O_2 (Nakayama et al.,1993; Nakayama,1994), indicating that the observed H_2O_2 scavenging activity of AMG digests could be due to the presence of phenolic compounds, specially the flavonoid compounds.

Hydroxyl radical is considered to be one of the quick initiators of the lipid peroxidation process by abstracting hydrogen atoms from unsaturated fatty acids. Hence, the hydroxyl radical scavenging potential of the enzymatic digests and water extracts from CPC dried by FD, HSD and FIR was evaluated and the results are depicted in the Table 2-1. The results obtained with the digest from CPC dried by HSD and FIR were compared with the results obtained for AMG digest from CPC dried by FD as well as water extracts from CPC dried by both HSD and FD. The AMG digest from CPC dried by FIR at 40°C showed higher activity (IC_{50} 0.29 mg/mL) than those of the AMG digest from CPC dried by FD or water extracts from the CPC dried by both HSD and FD however, it was not significantly higher ($p<0.05$) according to the statistical data. Anagnostopoulou et al. (2005) have reported strong hydroxyl radical scavenging activities for the different extracts, fractions and residues of citrus peel. Further, Yi et al. (2008) reported the hydroxyl radical scavenging activity of citrus and hesperidin, and indicated that those activities were due to the presence of multi-hydroxyl groups in the phenolic ring of the hesperidin molecule. The activities exhibited by the digests/extracts in this study, could be due to metal chelating activity, which retards the generation of hydroxyl radicals via

Fenton reaction other than the activities exerted by the flavonoids and other polyphenolic compounds present in the enzymatic digests or water extracts which were evaluated by colorimetric assays.

An alkyl radical spin adduct was observed (a^N=15.5 G, a^H=2.7 G) when AAPH was incubated with the spin trap 4-POBN at 37°C for 30 min. ESR signals were decreased with the addition of the tested digest (spectrum not shown). The results indicated in Table 2-1 showed that alkyl radical were scavenged effectively and dose-dependently by the enzymatic digests from CPC dried by HSD and FIR. Alkyl radical represent the direct radical scavenging activity. AMG digests from CPC dried by HSD and FD showed the same IC_{50} value (IC_{50}: 0.08 mg/mL) for alkyl radical scavenging. Also, the digest from CPC dried by FIR at 50°C showed the highest activity (IC_{50}: 0.02 mg/mL) for alkyl radical scavenging among all the digests. The inconsistency of these digests/extracts on free radical scavenging activity may come from the different inhibitory mechanism in different model systems.

Table: 2-1 Antioxidant activities of AMG digest and water extracts from CPC dried by FD, HSD and FIR

		IC_{50} (mg/mL)						
Assay	3D/E	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
DPPH	AMG	0.24 ± 0.008^{d}	0.18 ± 0.003^{c}	0.15 ± 0.006^{b}	0.10 ± 0.005^{a}	0.13 ± 0.004^{ab}	0.13 ± 0.005^{ab}	0.14 ± 0.003^{b}
	WE	0.42 ± 0.02^{d}	0.3 ± 0.02^{b}	0.29 ± 0.01^{b}	0.34 ± 0.02^{c}	0.40 ± 0.02^{d}	0.36 ± 0.01^{c}	0.22 ± 0.01^{a}
$O_2^{\cdot-}$	AMG	0.59 ± 0.02^{f}	0.43 ± 0.01^{d}	0.33 ± 0.01^{b}	0.28 ± 0.01^{a}	0.53 ± 0.02^{e}	0.43 ± 0.02^{d}	0.37 ± 0.01^{c}
	WE	0.40 ± 0.01^{c}	0.36 ± 0.02^{bc}	0.38 ± 0.01^{c}	0.33 ± 0.01^{b}	0.42 ± 0.02^{d}	0.44 ± 0.02^{d}	0.28 ± 0.01^{a}
H_2O_2	AMG	0.66 ± 0.02^{b}	0.57 ± 0.05^{a}	0.99 ± 0.05^{c}	0.65 ± 0.03^{b}	0.81 ± 0.04^{bc}	0.81 ± 0.04^{bc}	0.66 ± 0.03^{b}
	WE	0.62 ± 0.02^{b}	1.0 ± 0.03^{a}	0.99 ± 0.04^{ab}	0.62 ± 0.03^{ab}	0.82 ± 0.04^{ab}	0.82 ± 0.05^{a}	0.61 ± 0.03^{ab}
$^{\cdot}OH$	AMG	0.70 ± 0.04^{c}	0.44 ± 0.02^{ab}	0.29 ± 0.01^{a}	0.46 ± 0.03^{ab}	0.71 ± 0.05^{c}	0.66 ± 0.05^{c}	0.56 ± 0.03^{bc}
	WE	0.66 ± 0.05^{a}	0.63 ± 0.03^{a}	0.81 ± 0.06^{ab}	1.04 ± 0.04^{cd}	0.88 ± 0.03^{bc}	1.11 ± 0.06^{d}	0.99 ± 0.05^{bcd}
Alkyl	AMG	0.09 ± 0.003^{c}	0.08 ± 0.002^{c}	0.05 ± 0.002^{b}	0.02 ± 0.001^{a}	0.06 ± 0.002^{b}	0.05 ± 0.002^{b}	0.04 ± 0.001^{b}
	WE	0.16 ± 0.005^{d}	0.09 ± 0.004^{b}	0.06 ± 0.002^{a}	0.05 ± 0.002^{a}	0.11 ± 0.005^{c}	0.10 ± 0.004^{bc}	0.09 ± 0.004^{b}
MC	AMG	1.38 ± 0.07^{d}	0.41 ± 0.02^{a}	0.41 ± 0.02^{a}	0.40 ± 0.01^{a}	0.41 ± 0.02^{a}	0.83 ± 0.05^{c}	0.49 ± 0.03^{b}
	WE	0.95 ± 0.06^{f}	0.8 ± 0.04^{c}	0.80 ± 0.05^{c}	0.91 ± 0.06^{e}	0.85 ± 0.06^{d}	0.74 ± 0.03^{a}	0.77 ± 0.05^{b}

All data are mean of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

It is well-known that transition metal ions are involved in many oxidation reactions. Hence, the measurement of chelating ability is important for evaluating free radical scavenging activity of a compound (Halliwell and Gutteridge, 1990). In the present study, the metal chelating activities were determined by the measurement of the reduction rate of red color, which was quantitatively formed by ferrozine by reacting with ferrous ions. Table 2-1 shows the chelating activities of the enzymatic digests from CPC dried by FD, HSD and FIR on ferrous ions. The metal chelating activity of AMG digest from CPC dried by FIR at 50°C was higher (IC_{50}: 0.40 mg/mL) than that of its counterpart AMG digest from CPC dried by FD (IC_{50}: 1.38 mg/mL). However, there was no any significant difference ($p<0.05$) between data. The results obtained in this study revealed that enzymatic digests from CPC has potential effect on iron binding, suggesting that its action as peroxidation protector might be related to its iron-binding capacity. Lin et al. (2003) have reported the metal chelating activities with green tea and have analyzed catechin contents (flavonoid) in the extracts; however, those activities were lower than the activities reported in this study.

The ferric thiocyanate method was employed to determine the amount of peroxide at the initial stage of lipid peroxidation and it consists of a series of free radical-mediated chain reactions. When different digests were added, a significant inhibition of lipid peroxidation was observed (Fig. 2-4). The autooxidation of linoleic acid without adding digests was accompanied by a rapid increase of peroxide value. Each digests showed strong antioxidant activity in inhibition of linoleic acid peroxidation as compared with the control which was without digests. However, the inhibitory values of CPC dried by HSD and FIR were significantly ($p<0.05$) lower than that of BHT but significantly higher

($p<0.05$) than that of α-tocopherol. In FTC method, hydroperoxides generated from linoleic acid was added to the reaction mixture, and were indirectly measured. Ferrous chloride and thiocyanate react with each other to produce ferrous thiocyanate by means of hydroperoxides (Inatani et al., 1983); Kang et al. (2006); Rehman et al. (2006) have also reported the lipid peroxidation inhibitory activities of citrus peel using different model systems. Further, Manthey (2004) have reported that the antioxidant property observed in orange peel ultra filtered molasses due to the presence of polyphenolic compounds such as flavanones, flavone glycosides, polymethoxylated flavone, hydroxyl cinnamates and other various phenolic glycosides. The lipid peroxidation inhibitory activity shown by the AMG digest from CPC could be attributed to the presence of various polyphenolic compounds.

DNA, the genetic material and controling the cellular functions, can be damaged as a result of several factors such as reactive oxygen species, smoke, heat, toxic chemicals and ultraviolet light. The sequence of the DNA base pairs can be changed and leads errors/ disorders in replicating DNA if the damage could not be repaired by the existing DNA repair mechanisms. The DNA damage of cultured Vero cells was artificially induced by H_2O_2 and the ability of the enzymatic digests from CPC dried by FD, HSD and FIR to inhibit the damage was evaluated by the fluorescence intensity in the tail using comet assay. DNA damage is well known to be one of the most sensitive biological markers for evaluating oxidative stress caused by ROS (Kassie et al., 2000). As shown in Fig. 2-5, 2-6 and 2-7, the inhibition of DNA damage by enzymatic digests from CPC dried by HSD and FIR were investigated with three different concentrations. With the increased concentrations of the digest, increased inhibitory effect against H_2O_2-induced cell

damage was observed. The highest concentration (100 μg/mL) of the digests indicated the highest inhibitory effect against DNA damage. The H_2O_2-induced DNA damage was successfully overcomed/repaired by the enzymatic digests from CPC dried by FD, HSD and FIR at all concentrations. In the group treated with only hydrogen peroxide, the DNA was completely damaged but the addition of the enzymatic digests with hydrogen peroxide reduced the damage indicating that AMG digests have the ability to inhibit the DNA damage induced by hydrogen peroxide.

Antioxidant activities of enzymatic digests from CPC were evaluated using different invitro models in this study. The polyphenolic and total flavonoid contents were also evaluated and compared with the digests from CPC dried by FD as well as with the water extracts from CPC. All digests showed higher soluble solid content compared with water extracats. The total phenolic and flavonoid contents were lower than that of methanolic extracts. However, the digests showed higher antioxidant activities in the assays tested. The promising antioxidant activities of the digests from CPC suggest that they could be used as natural potential antioxidants agents. As the digests have high antioxidant activities, we can conclude that drying process has not affectd advesely on the activities though we used high temperature in the drying process. Hence, HSD drying systems can be used to transform wet CPC into dried form effectively and efficiently. However, further research works are needed to address the compounds present in the CPC which are responsible for those strong antioxidant activities.

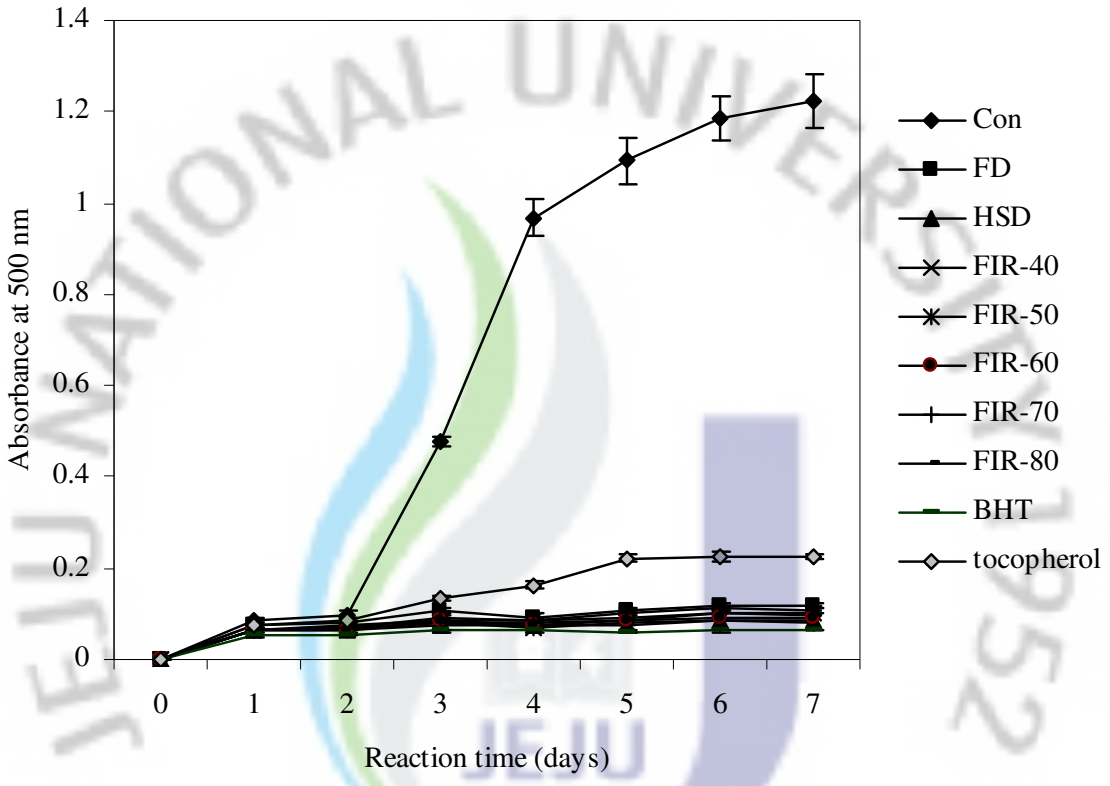


Fig. 2-4. Lipid peroxidation inhibitory activities of AMG digest from CPC dried by FD, HSD and FIR. Peroxidation was measured in linoleic acid emulsion system by ferric thiocyanate method.

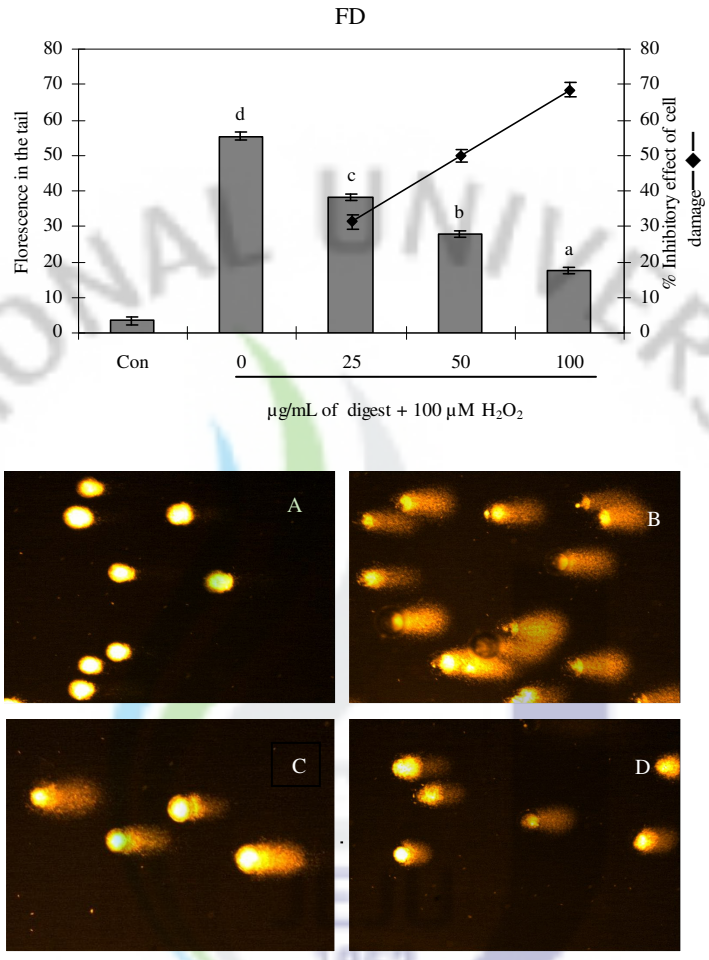


Fig. 2-5. Effect of AMG digest from CPC dried by FD on H_2O_2–induced DNA damage in Vero cells and their comet images. (A) negative control (B) Vero cells treated with 100 μM H_2O_2 (C) Vero cells treated with 50 μg/mL AMG digest + 100 μM H_2O_2 (D) Vero cells treated with 100 μg/mL AMG digest + 100 μM H_2O_2. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

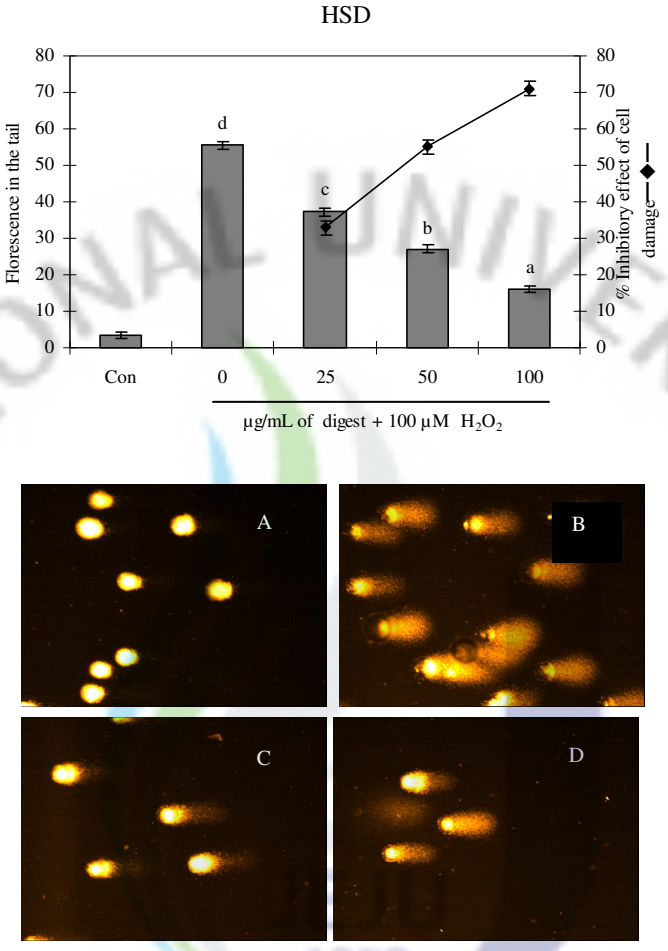
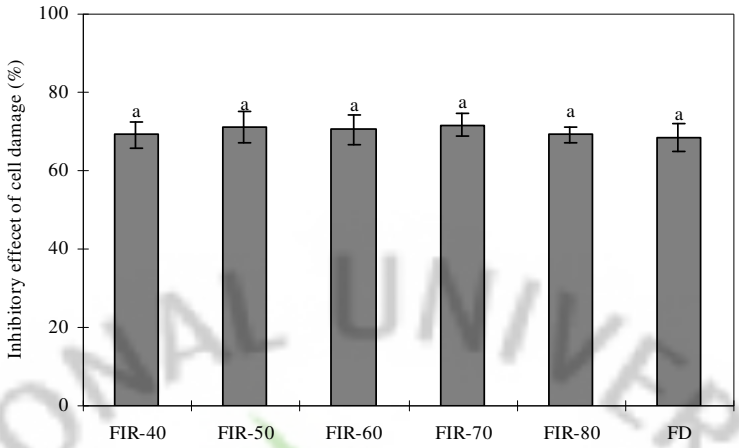


Fig. 2-6. Effect of AMG digest from CPC dried by HSD on H_2O_2–induced DNA damage in Vero cells and their comet images. (A) negative control (B) Vero cells treated with 100 μM H_2O_2 (C) Vero cells treated with 50 μg/mL AMG digest + 100 μM H_2O_2 (D) Vero cells treated with 100 μg/mL AMG digest + 100 μM H_2O_2. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.



.

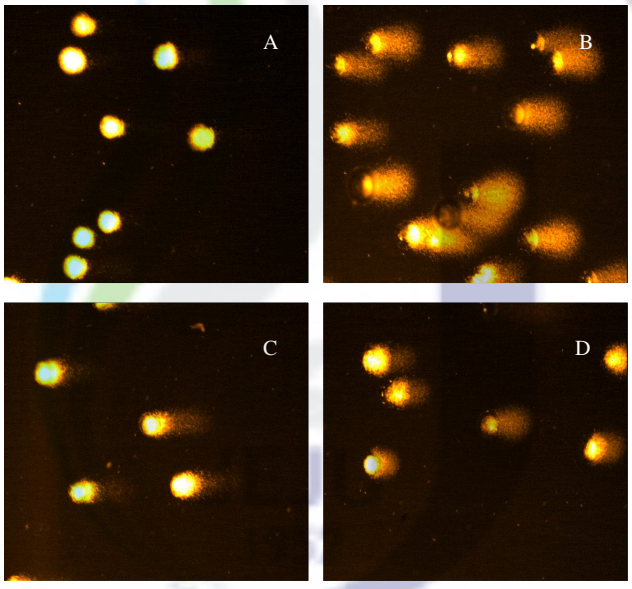


Fig. 2-7. Effect of AMG digests from CPC dried by FIR against H_2O_2–induced DNA damage in Vero cells and Photomicrograph of Vero cells obtained for AMG digest from CPC dried by FIR at 50°C. (A) negative control (B) Vero cells treated with 100 μM H_2O_2 (C) Vero cells treated with 50 μg/mL AMG digest from CPC dried by FIR at 50°C + 100 μM H_2O_2 (D) Vero cells treated with 100 μg/mL AMG digest from CPC dried by FIR at 50°C + 100 μM H_2O_2.

PART III

Protective Effect of Citrus Press-Cake Dried by High Speed Drying and Far-infrared Radiation Drying Against Oxidative Stress-induced Damage in Vero Cells

ABSTRACT

The protective effect of methanolic extracts from citrus press-cake (CPC) dried by high speed drying (HSD) and far-infrared radiation (FIR) drying against oxidative stress-induced damage in Vero cells was evaluated in this study. The intracellular inhibitory effect of the extracts against reactive oxygen species (ROS), cell viability, lipid peroxidation, DNA damage and apoptotic body formation were evaluated. The results showed that the extracts from CPC dried by HSD and FIR enhanced the cell viability and reduced the intracellular ROS level. Also, the extracts decreased the malondialdehyde levels and fluorescence intensity in the comet tail of the cells pretreated with the extracts by enhancing the inhibitory effect in the H_2O_2-induced Vero cells. Moreover, methanolic extracts from CPC showed higher inhibitory effects against H_2O_2 induced DNA damage. Hence, this study demonstrated that the extracts from CPC were able to relieve H_2O_2-induced oxidative stress and apoptosis in Vero cells by antioxidant mechanisms. The results obtained for the extracts from CPC dried by HSD and FIR was almost similar to those obtained from the extracts from CPC dried by FD. Therefore, these results suggest that extracts from CPC act as a potent antioxidant agent in live cells.

INTRODUCTION

Oxidative injury has been implicated in the pathogenesis of many neurological diseases, such as Parkinson's disease, Alzheimer's disease (Andersen, 2004) and stroke (Heistad, 2006). The excessive generation of reactive oxygen species (ROS) cause for the detrimental effects, including lipid peroxidation of cell membranes, enzyme inactivation and DNA breakage, alteration of lipid–protein interaction, and in the end, to cause cell injury, necrosis or apoptosis, which are attributed to inhibit uptake and inducing glutamate release (Mailly et al.,1999; Volterra et al., 1994), to enhance intracellular Ca^{2+} concentration (Hool and Corry, 2007) for the activation of neuroinflammatory reactions (Haorah et al., 2007) and apoptotic pathway. In these processes, large amount of hydrogen peroxide is generated by various types of cells including neurons and H_2O_2 thus formed is toxic to both producing cells and neighboring cells for its high cellular membrane permeability (Halliwell et al., 1992). Therefore, it is often used as a toxicant to mimic *in vitro* models of oxidative stress-induced injury.

The free radicals are produced in the human body and the scavenging of radicals by endogenous antioxidants is constant in normal situations. The continuous generation and removal of ROS is a general phenomenon in aerobic metabolism. The cells can normally deal with mild oxidative stress by upregulating the antioxidant defense mechanisms through the changes in gene expression. However, if there is an imbalance between free radicals and the antioxidant defense system, cell injury may occur because of the oxidative damage occurs to biomolecules such as DNA, proteins, lipids, and small compounds in living organisms, generating ROS-related diseases.

Polyphenolic antioxidants play an important role in the oxidation process by neutralizing the radicals. They can act as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelating agents due to their high redox potentials (Tsao and Deng, 2004).

The citrus juice processing plants generate large amount of press-cake every year and these may contain high amount of potential bioactive compounds. However, these by-products can be spoilage due to lack of enough storage facilities or processing treatments before using them. Therefore, it is necessary to dry these valuable product quickly to be used them effectively. Hence, in order to increase the efficacy in drying process, we used high speed drying system made in Okadora Korea (Incheon, Korea) to convert wet CPC into dried form. This method is more economical and less time consuming as it is able to convert large amount of press-cake into dried form at a single turn. The aim of this study was to evaluate the protective effect of CPC dried by HSD and FIR using several intracellular antioxidative models.

MATERIALS AND METHODS

Materials

CPC were obtained from Jeju provincial development Co. in Jeju Island of Korea. Fluorescence probes 2', 7'-dichlorodihydroflurescin diacetate (DCFH-DA) and 3-(4-5-dimethyl-2yl)-2-5-diphynyltetrasolium bromide (MTT), Folin Ciocalteu reagent was purchased from Sigma Co. (St. Louis, USA). DMEM, trypsin, Dulbeco phosphate buffered saline (PBS; pH 7.4) and fetal bovine serum (FBS) were from Gibco BRL, Paisley, UK. All other chemicals used were analytical grade supplied by Fluka or Sigma Co.

High speed drying or far-infrared radiation drying of CPC

CPC stored at -50°C were converted into dried form with high speed drier (Okadora Korea, Incheon, Korea) or far-infrared radiation drier (TOURI-Q, Korea).

A 20 kg of wet CPC were loaded into the sample compartment of HSD system and converted into dried form within 90 min. A high temperature is maintained within the sample compartment by steam supplied through the steam generator. Due to high temperature in this part, the moisture present in the samples is removed and converts them into dried form. Then, the HSD dried CPC were pulverized into fine powder using a grinder (MF 10 basic mill, GMBH & Co., Staufen, Germany) and sieved through a 300 μm standard testing sieve.

Also, CPC were dried using FIR drier (Model TOURI-Q, Korea). After drying, they were pulverized into fine powder using same grinder mentiond above and sieved through a 300 μm standard testing sieve.

Preparation of methanolic extracts from CPC

A 20-grams of ground CPC powder was mixed in 100% methanol (1000 mL) and kept in the shaking incubator at 25°C for one day and filtered in a vacuum using Whatman No.1 (Whatman Ltd., England) filter paper. Later, MeOH was evaporated and dissolve in DMSO.

Cell culture

The monkey kidney fibroblast cell line (Vero cell) was cultured in DMEM medium containing heat-inactivated 10% fetal bovine serum, streptomycin (100 µg/mL) and penicillin (100 unit/mL) at 37°C under a humidified atmosphere of 5% CO_2 in air.

Cell viability assay

The cell viability was estimated by MTT assay, which is a test of normal metabolic status of cells based on the assessment of mitochondrial activities (Hansen et al., 1989). It is a colorimetric assay which converts the yellow tetrazolium bromide to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells (Mosmann, 1983). Vero cells were seeded in 96-well plate at a concentration of 1.0×10^5 cells /mL. After 16 hrs, cells were treated with different concentrations of the methanolic extracts (25, 50 and 100 µg/mL), and incubated at 37°C in a humidified atmosphere for 1 hr. Then, 0.7 mM of H_2O_2 was added as final concentration and incubated for another 24 hrs at 37°C. Thereafter, MTT stock solution (50 µL; 2 mg/mL) was added and incubated for 4 hrs. Then, the plate was centrifuged at 2000 rpm for 5 min and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 µL of DMSO and

absorbance was measured using ELISA reader (Sunrise; Tecan Co. Ltd., Austria) at 540 nm. Relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The optical density of the formazan formed in the control cells was taken as 100% viability. Data were the mean percentages of viable cells versus the respective control.

Intracellular ROS determination by DCFH-DA

The oxidation-sensitive dye DCFH-DA was used to determine the formation of intracellular ROS according to the method described by Engelmann et al. (2005). The Vero cells were seeded in 96-well plate at a concentration of $1.0x10^5$ cells/mL. Aftrer 16 hrs, the cells were treated with different concentrations of extracts (25, 50 and 100 μg/mL) and incubated at 37°C for 30 min in a humidified atmosphere. A 0.7 mM H_2O_2 was added and the cells were incubated for an additional 30 min at 37°C. After that, 5 μg/mL DCFH-DA was added to the cells and detected at 485 nm excitation and at 535 nm emission using a PerkingElmer LS-5B spectrofluorometer.

Lipid peroxidation inhibitory assay

The lipid peroxidation was evaluated by measuring malondialdehyde by the method described by Ohkawa et al. (1979). Vero cells were seeded in a culture dish at a concentration of $1.0x10^5$ cells/mL and incubated at 37°C in a humidified atmosphere. After 16 hrs, cells were treated with different concentrations of the extracts (50 and 100 μg/mL). One hour later, 0.7 mM of H_2O_2 was added and cells were incubated for an additional hour. The cells were then harvested and washed with PBS and homogenized in

ice-cold 1.15% KCl. The extracts containing of 100 μL of the cell lysates were combined with 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid adjusted to pH 3.5 and 1.5 mL of 0.8% TBA. The mixture was brought to a final volume of 4.0 mL with distilled water and heated at 95°C for 2 hrs. After cooling to room temperature, 5.0 mL of a mixture of n-butanol and phyridine (15:1 v/v) was added to each mixture and vortex thoroughly. After centrifugation at 1500 rpm for 10 min, the supernatant was isolated and the absorbance was measured at 532 nm. Inhibitory activity was expressed as percentages of control samples.

Investigation on the reduction of DNA damage (Comet assay)

Each extract was diluted in PBS to make the final concentration of 25, 50 and 100 ug/mL. Vero cells were treated in three different ways. First, Vero cells (4×10^4 cell/mL) were incubated without the extract for 30 min at 37°C in a dark incubator (I). Second, Vero cells (4×10^4 cell/mL) were incubated without the extract for 30 min at 37°C and damaged oxidatively with 100 μM H_2O_2 for 5 min on ice (II). Third, Vero cells (4×10^4 cell/mL) were incubated with the extracts for 30 min at 37°C in a dark incubator and then treated with 100 μM H_2O_2 for 5 min on ice (III). After each treatment, extracts were centrifuged at 1500 rpm for 5 min and washed with PBS.

Then, alkaline comet assay was conducted according to the method described by Singh et al. (1988) with slight modifications. The cell suspensions made in previous steps were mixed with 100 μL of 0.7% low melting agarose (LMA), and added to the slides precoated with 1.0% normal melting agarose. After solidification of the agarose, slides were covered with another 100 μL of 0.7% LMA and then immersed in lysis buffer (2.5

M NaCl, 500 mM EDTA, 1 M Tris, and 1% sodium laurylasarcosine, and 1% Triton X-100) for 90 min. Later, slides were transferred into unwinding buffer for another 20 min for DNA unwinding. The slides were next placed in the electrophoresis tank containing 300 mM NaOH and 1 mM Na_2EDTA (pH 13.0), and for electrophoresis of the DNA, an electric current of 25 V/300 mA was applied for 20 minutes. After that the slides were washed two times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 10 min, and treated with ethanol for another 5 min before staining with 40 µL of ethidium bromide (20 µL/mL). Measurements were made by image analysis (Kinetic Imaging, Komet 5.0, U.K) and fluorescence microscope (LEICA DMLB, Germany), determining the percentage of fluorescence in the tail (tail intensity, TI; 50 cells from each of two replicate slides).

Nuclear staining with Hoechest 33342

The nuclear morphology of the cells was evaluated using the cell-permeable DNA dye, Hoechst 33342. The cells with homogeneously stained nuclei were considered viable, whereas the presence of chromatin condensation and/or fragmentation was indication of apoptosis (Gschwind and Huber, 1995; Lizard et al., 1995). Vero cells were seeded in 96-well plate at a concentration of 1.0 x 10^5 cells /mL. After 16 hrs, the cells were treated extracts of 100 µg/mL concentration, and incubated at 37°C in a humidified atmosphere. One hour later, 0.7 mM H_2O_2 was added and cells were incubated at 37°C. After 24 hrs, 1.5 µL of (stock 10 mg/mL), a DNA-specific Hoechst 33342 dye was added to each well, followed by 10 min of incubation at 37 °C. Then, stained cells were observed under a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera, in order to examine the degree of nuclear condensation.

Statistical analysis

All experiments were conducted in triplicate (n=3) and an ANOVA test (using SPSS 11.5 statistical software) was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by using the Duncan's test ($p<0.05$).

RESULTS AND DISCUSSION

The oxygen is reduced to superoxide radicals in aerobic metabolism and they are highly reactive radicals due to the presence of unpaired electrons in the shells of those small molecules. Therefore, superoxide radicals are needed an electron to become more stable, so they tend to obtain an electron from the nearest molecules such as proteins, lipid, DNA or non enzymatic antioxidants. Further, superoxide radicals can generate other reactive species, such as H_2O_2, by the addition of one or more electrons. H_2O_2-induced cell membrane lipid peroxidation is one of the most important lesions responsible for the loss of cell viability. Also, oxidative damage of amino acid residues in proteins results in the formation of carbonyl derivatives (Stadtman and Berlett, 1998). Other than that, oxidative damage of DNA involves both base modification and DNA strand breakage, which finally may lead to genetic modifications (Sankaranarayanan, 1991). A high level of radicals may cause the damage to the cells, and cells undergo apoptosis or programmed cell death. Those radicals are unwanted and toxic molecules which produced as by-products during the metabolism. However, they can be alleviated from the reaction system by various antioxidants, antioxidative enzymes or proteins (Kim et al., 2001; Jang and Surh, 2003).

The cell viability was measured by MTT assay. In order to select an optimal H_2O_2 concentration to induce an oxidative stress in Vero cells, cells were treated with different concentrations of H_2O_2 (0.4-1.0 mM) for 24 hrs. Consequently, 0.7 mM H_2O_2 was chosen for the experiments. Following incubation of the Vero cells with 0.7 μM H_2O_2 for 24 hrs, the cell viability was reduced to 40.2% of the control value. However, pretreatment of the

cells with extracts from CPC dried by HSD at different concentrations (25, 50 or 100 μg/mL), significantly increased ($p<0.05$) the cell viability by 45.6, 51.3 and 59% from the control value (Fig. 3-1). Significantly higher ($p<0.05$) cell viability was shown by the extracts from CPC dried by FIR at 60°C among the FIR extracts (Fig. 3-2) and the activity was dose dependent. The cell viability of 100 μg concentration of various FIR extracts were as follows (46.5, 47.5, 64.4, 59.8, and 51% in FIR-40, FIR-50, FIR-60, FIR-70 and FIR-80, respectively). The result showed that the extracts from CPC dried by HSD and FD moderately protect the Vero cells against H_2O_2-induced cell damage. In *in vitro* studies, H_2O_2 has been extensively used to induce oxidative stress in the cells (Dumont et al., 1999). The result showed that exposure of Vero cells to H_2O_2 significantly decreased the cell viability, but pretreatment of the cells with extracts from CPC dried by HSD and FD increased the cell viability. This implies that CPC dried by HSD and FD protects Vero cells from H_2O_2-induced cytotoxicity.

The scavenging effect of extracts from CPC dried by HSD and FIR on intracellular ROS was measured using 2′, 7′-dichlorodihydrofluorescin diacetate (DCFH-DA), an intracellular ROS-sensitive fluorescence probe. DCFH-DA penetrates into cells and is hydrolyzed by intracellular esterase to non-fluorescent DCFH. DCFH is oxidized to fluorescent 2′, 7′-dichlorofluorescein (DCF) by intracellular ROS (Lebel et al., 1992). The relative intracellular ROS level was determined as the total amount of the fluorescence of DCF by spectrofluorometer. The DCF fluorescence was highly increased in the cells treated only with H_2O_2 (Fig. 3-3 and 3-4). However, treatment with the extracts from CPC dried by HSD and FIR moderately decreased the fluorescence in the cells. When the concentration increased, the fluorescence intensity decreased gradually

indicating the activity is dose dependent. Further, several works have been done to evaluate the intracellular ROS scavenging activity using different cell lines (Ngo et al., 2008; Heo et al., 2008; Kim et al., 2006; Shin et al., 2008). Out of those works Heo et al. (2008) have shown strong activities for fucoxanthin against oxidative stress induced Vero cells. The results obtained in this assay suggested that the protective effect of the extracts from CPC is accompanied by the suppression of the intracellular ROS level.

The inhibitory effect of extracts from CPC dried by HSD or FD was investigated on lipid peroxidation in hydrogen peroxide induced Vero cells. Vero cells exposed to H_2O_2 showed an increased lipid peroxidation. However, the cells treated with extracts from CPC dried by HSD and FIR decreased lipid peroxidation (Fig. 3-5 and 3-6). The extract from CPC dried by HSD showed 51.1 % lipid peroxidation inhibitory activity at 100 μg/mL, which is almost close to the results obtained for the extracts from CPC dried by FD (56.2% at the 100 μg concentration). The extracts from CPC dried by FIR at 60°C showed significantly higher ($p<0.05$) inhibitory activity (64.4% at 100 μg/mL concentration) against lipid peroxidation among extracts from CPC dried by FIR. The generation of malondialdehyde, and also other related substances that react with thiobarbituric acid was substantially reduced by the extracts from CPC. Since the lipid peroxidation leads to a common cell death, the inhibition of lipid peroxidation is considered as the indication for antioxidative capacity and the extracts from CPC dried by HSD and FD showed the protective effect on lipid peroxidation that enhance the cell viability. Further, lipid peroxidation is directly related with cardiovascular diseases. Hence, inhibition of lipid peroxidation in cell membrane is a primary need to protect

them from prone to oxidation and to prevent the initiation of different degenerative diseases.

To evaluate the cytoprotective effect of methanolic extracts from CPC on apoptosis induced by H_2O_2, the nuclei of Vero cells were stained with Hoechst 33342 dye and assessed by microscopy. To stain the DNA, Hoechst 33342 dye is frequently used. Also it can be used to detect the nuclear fragmentation, chromatin condensation and the apoptotic bodies' formation (Kerr et al., 1995). The microscopic photograph shows that the control cells have intact nuclei (3-7a and 3-8a), while the H_2O_2 treated cells show significant nuclear fragmentation (3-7b and 3-8b), which is an indication of apoptosis. However, when the cells treated with methanolic extracts from CPC for 1 hr prior to H_2O_2 treatment, a significant decrease ($p<0.05$) in nuclear fragmentation was observed (3-7c, d and 3-8c, d, e, f). When, the cells pretreated with the extracts showed a reduced percentage of apoptotic bodys. These results suggest that the extracts inhibited H_2O_2 induced apoptosis by its ROS scavenging effect.

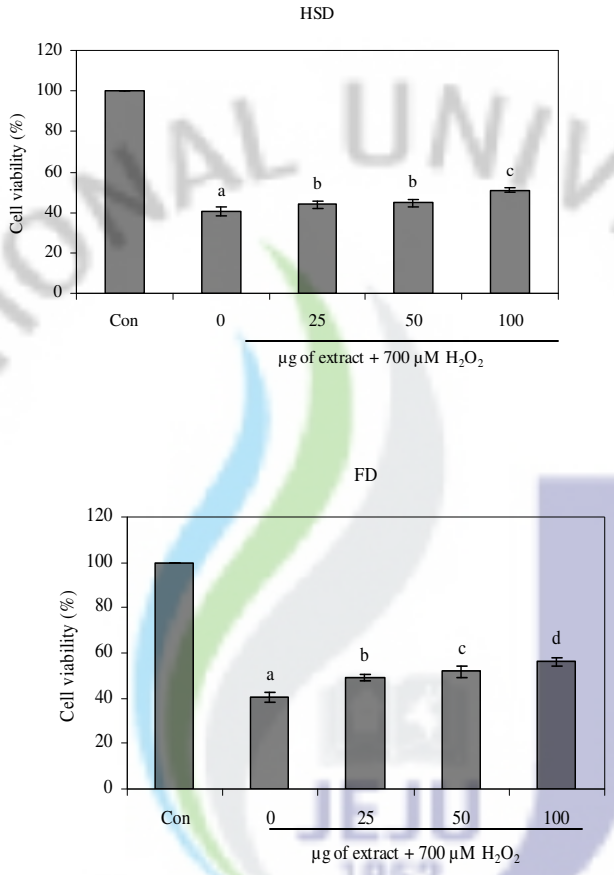


Fig. 3-1. Protective effect of methanolic extract from CPC dried by HSD against H_2O_2–induced oxidative damage on Vero cells. The viability of cells on H_2O_2 treatment was determined by MTT assay. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

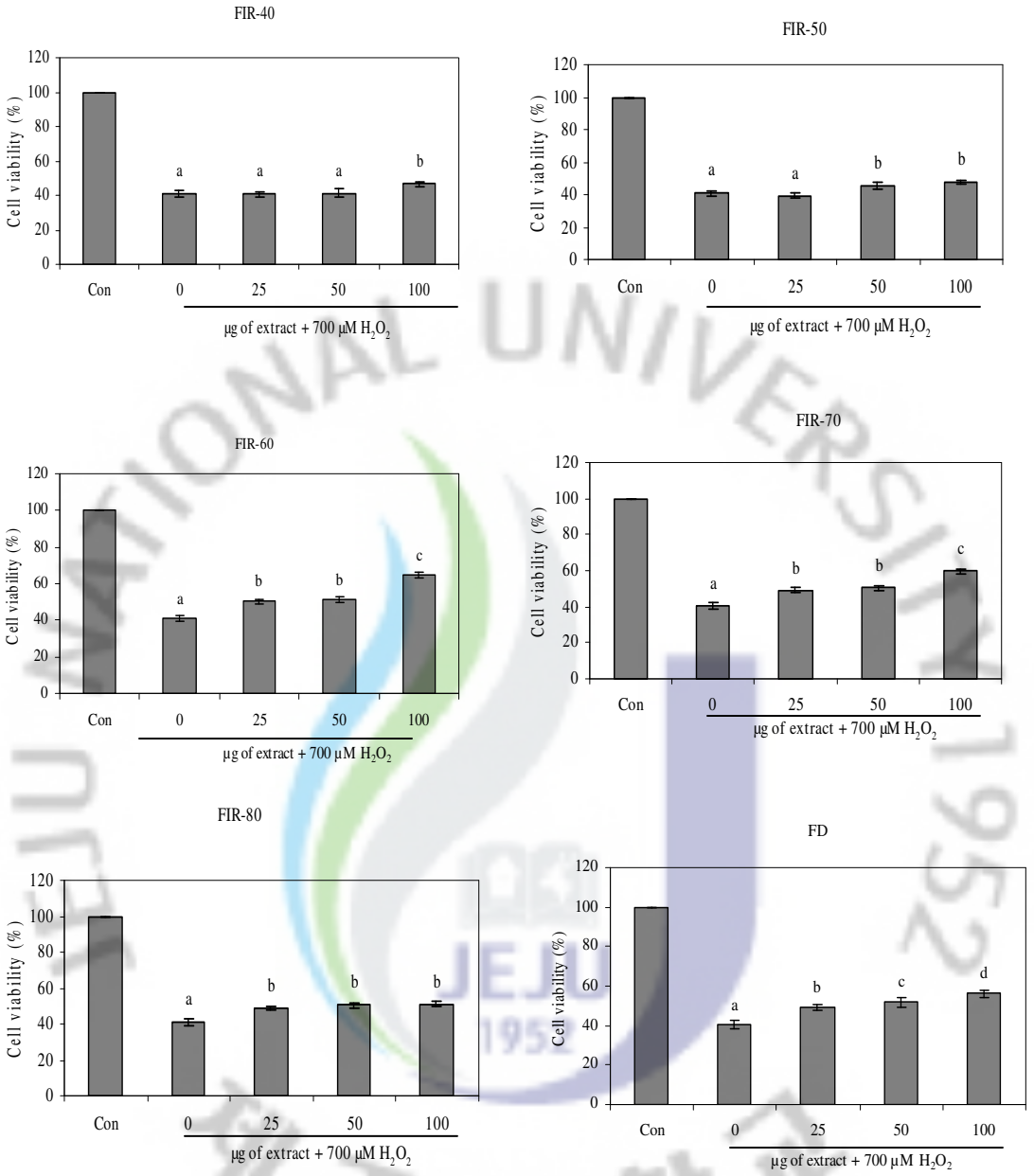


Fig. 3-2. Protective effect of methanolic extracts from CPC dried by FIR against H_2O_2–induced oxidative damage on Vero cells. The viability of cells on H_2O_2 treatment was determined by MTT assay. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

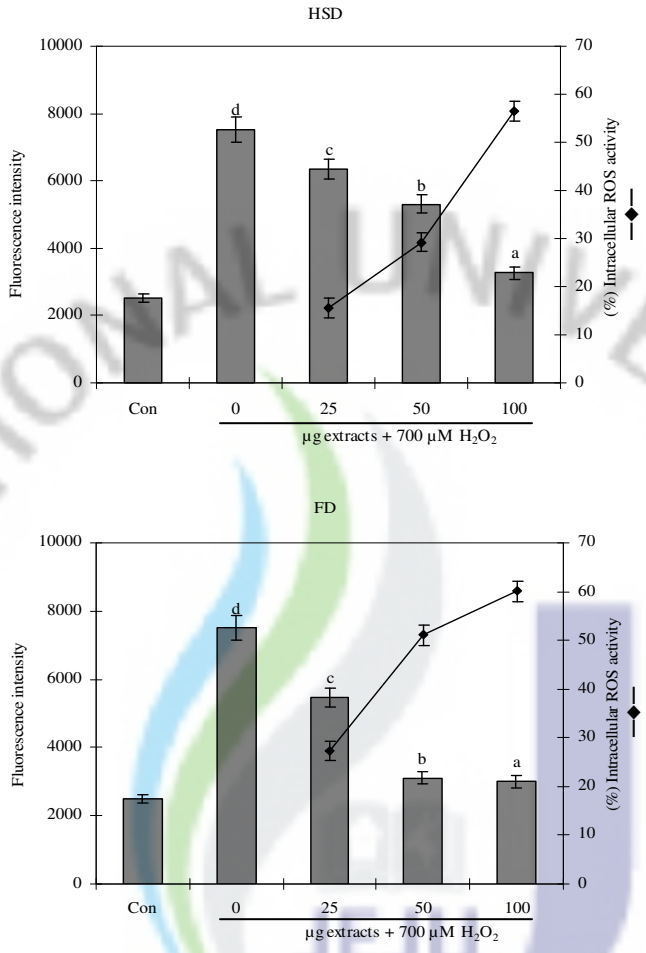


Fig. 3-3. Intracellular ROS scavenging effect of methanolic extract from CPC dried by HSD against H_2O_2–induced oxidative damage on Vero cells. The intracellular ROS generated was detected by DCFH-DA assay using spectroflurometry. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

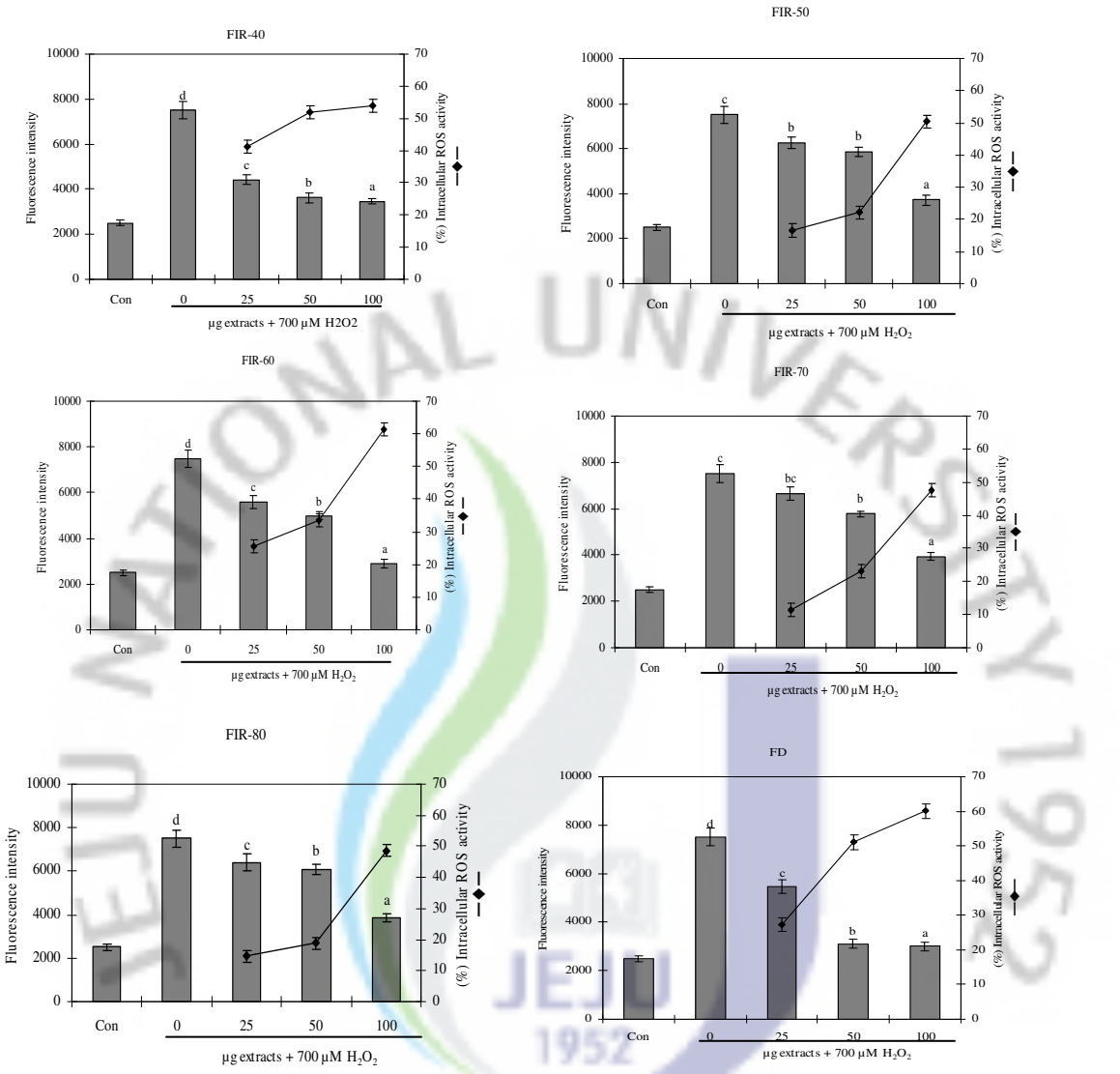


Fig. 3-4. Intracellular ROS scavenging effect of methanolic extracts from CPC dried by FIR against H_2O_2–induced oxidative damage on Vero cells. The intracellular ROS generated was detected by DCFH-DA assay using spectroflurometry. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

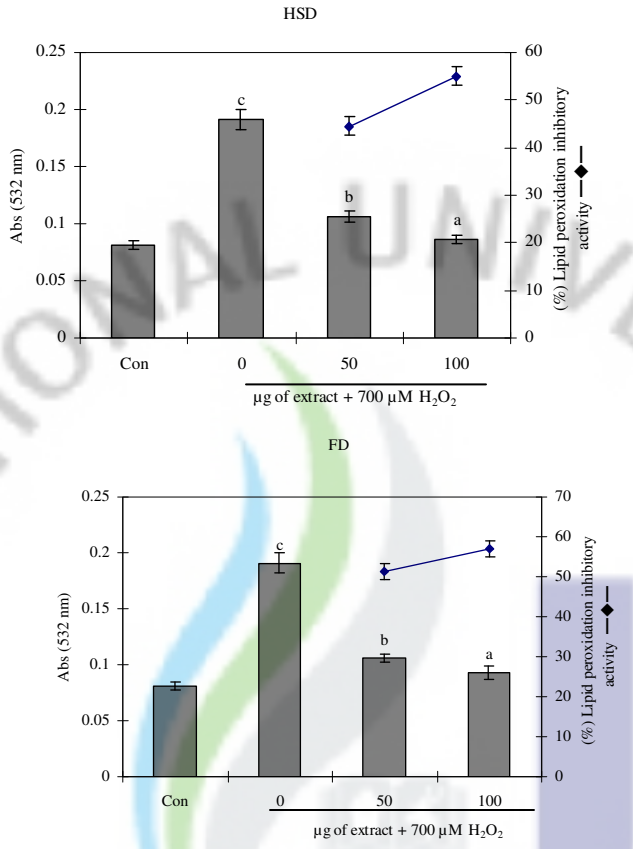


Fig. 3-5. Lipid peroxidation inhibitory activity of methanolic extract from CPC dried by FD and HSD against H_2O_2–induced oxidative damage on Vero cells. The vertical bar graph represents lipid peroxidation after the induction period of each extracts at 532 nm. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

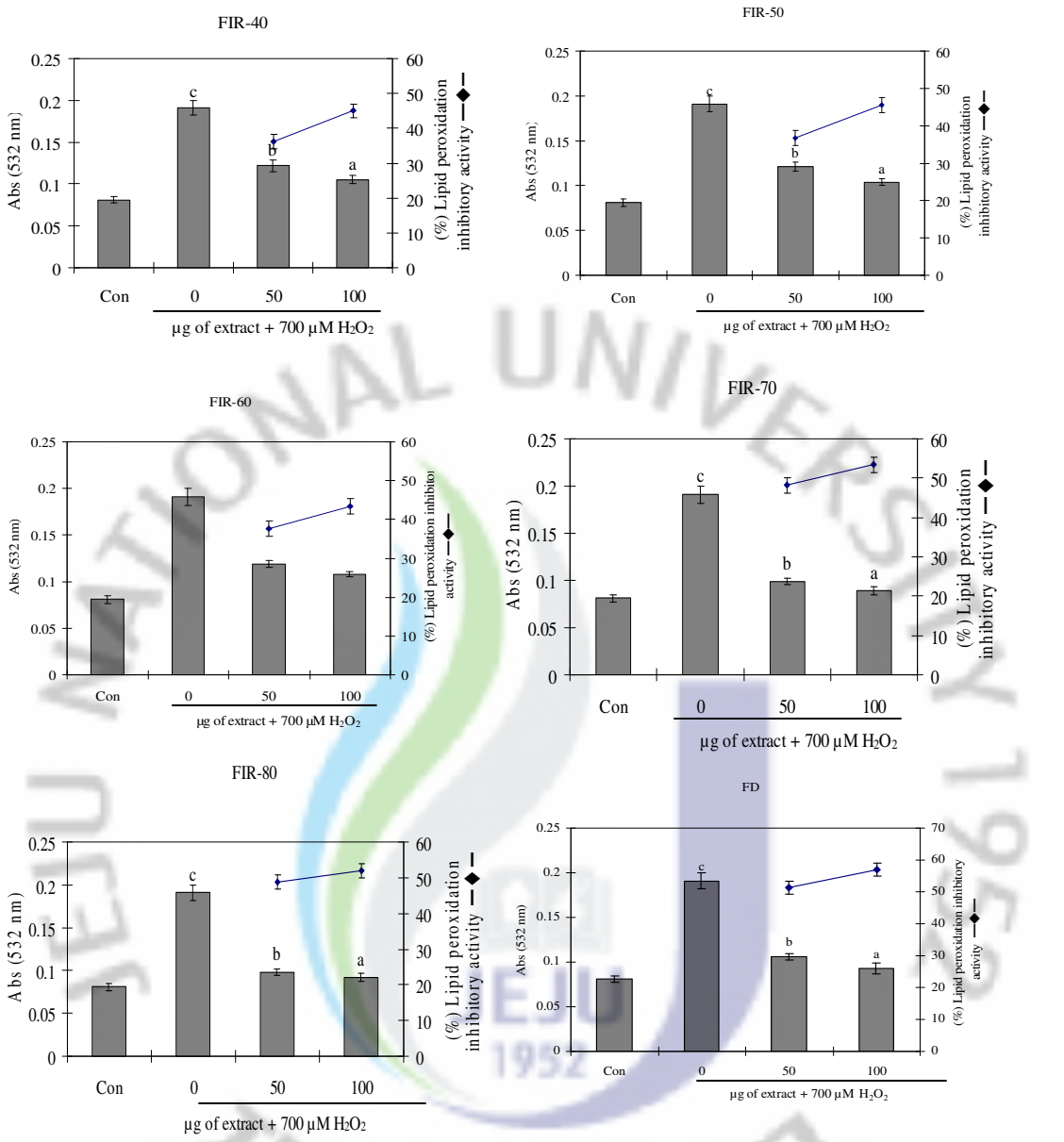


Fig. 3-6. Lipid peroxidation inhibitory activity of methanolic extracts from CPC dried by FIR against H_2O_2–induced oxidative damage on Vero cells. The vertical bar graph represents lipid peroxidation after the induction period of each extracts at 532 nm. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

Hydrogen peroxide is a known genotoxic agent that is believed to cause DNA strand breakage by the generation of the hydroxyl radical (·OH) via the Fenton reaction (Slupphaug et al., 2003). Exposure of DNA to oxidative stress leads to more than 20 different types of base damage, producing oxidized and ring-fragmented nitrogen bases (Slupphaug et al., 2003). Hydrogen peroxide is routinely used as positive control for comet assay analysis (Comet assay kit^{TM}, 2001). Single cell gel electrophoresis (comet assay) has been widely employed for the measurement of genotoxic and cytotoxic effects on animal and human cells by physical and chemical agents (Duthie and Collins, 1997). In the assay, under the alkaline conditions, DNA loops breaks, lose supercoiling, unwind, and released from the nucleus to form a comet-like image with a bright head containing fluorescent head and a tail streaming away from it when viewed by fluorescence microscope after gel electrophoresis (Fairbairn et al., 1995). DNA strand breaks are thus visualized by the comet assay and can be quantified by image intensification by computer analysis (Singh et al., 1998). Generally, cells with a high level of DNA damage exhibit increased comet parameters, which may be expressed as tail length, %DNA in the tail and tail moment (tail length × %DNA in the tail) or simply as high, medium and low damage (Faust et al., 2004). Our results demonstrated significant increases ($p<0.05$) in DNA strand breaks after introduction of hydrogen peroxide (Fig. 3-9 and 3-10). However with the addition of the extracts, significantly increased ($p<0.05$) inhibitory activity was observed by reduced fluorescence in the tail. The inhibitory effect of extracts from CPC dried by HSD on DNA damage was 25.9, 37.6, and 55.3% at the concentration of 25, 50 and 100 μg/mL respectively. The extract from CPC dried by FIR at 80°C showed significantly higher ($p<0.05$) inhibitory activity among extracts from CPC dried by FIR

and they were 22.16, 53.15 and 69.3% at the 25, 50 and 100 μg/mL concentrations, respectively. The extracts from CPC dried by HSD showed slightly lower activities than that of the extracts from CPC dried by FD (29.0, 48.8 and 64.8% at same concentration) but the extracts from CPC dried by FIR at 80°C showed slightly higher activities. Toxicity of H_2O_2 toward the Vero cells is more likely related to its effects on genotoxicity. Therefore, it can be suggested that the Vero cells, possibly by the activities of either catalase (Jeulin et al.,1989) or glutathione peroxidase (Alvarez and Storey,1989), contribute to the protection of DNA from oxidative stress.

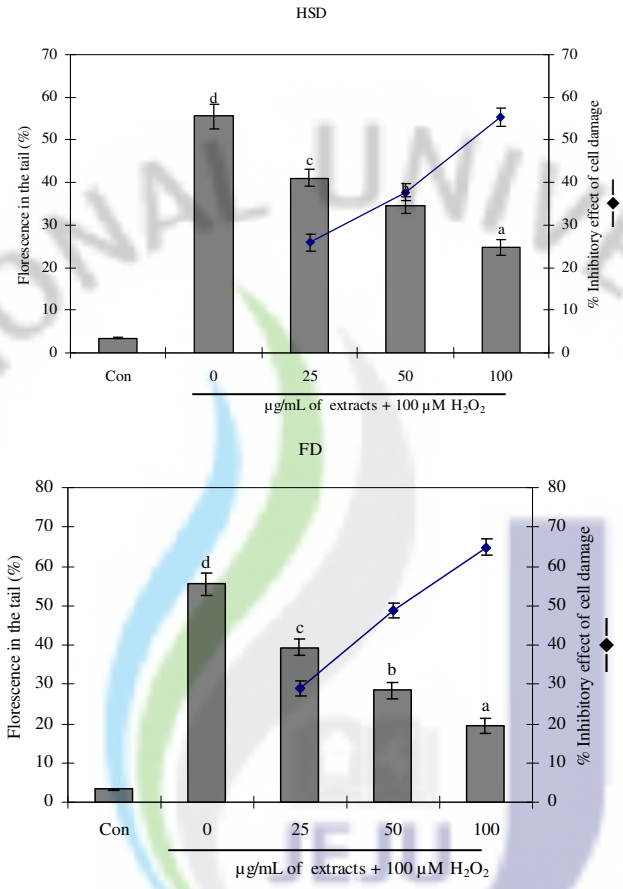


Fig. 3-7. Protective effect of methanolic extract from CPC dried by HSD on cellular DNA damage induced by H_2O_2. DNA damage was determined by alkaline comet assay. The vertical bar graph indicates the percentage fluorescence in tail. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

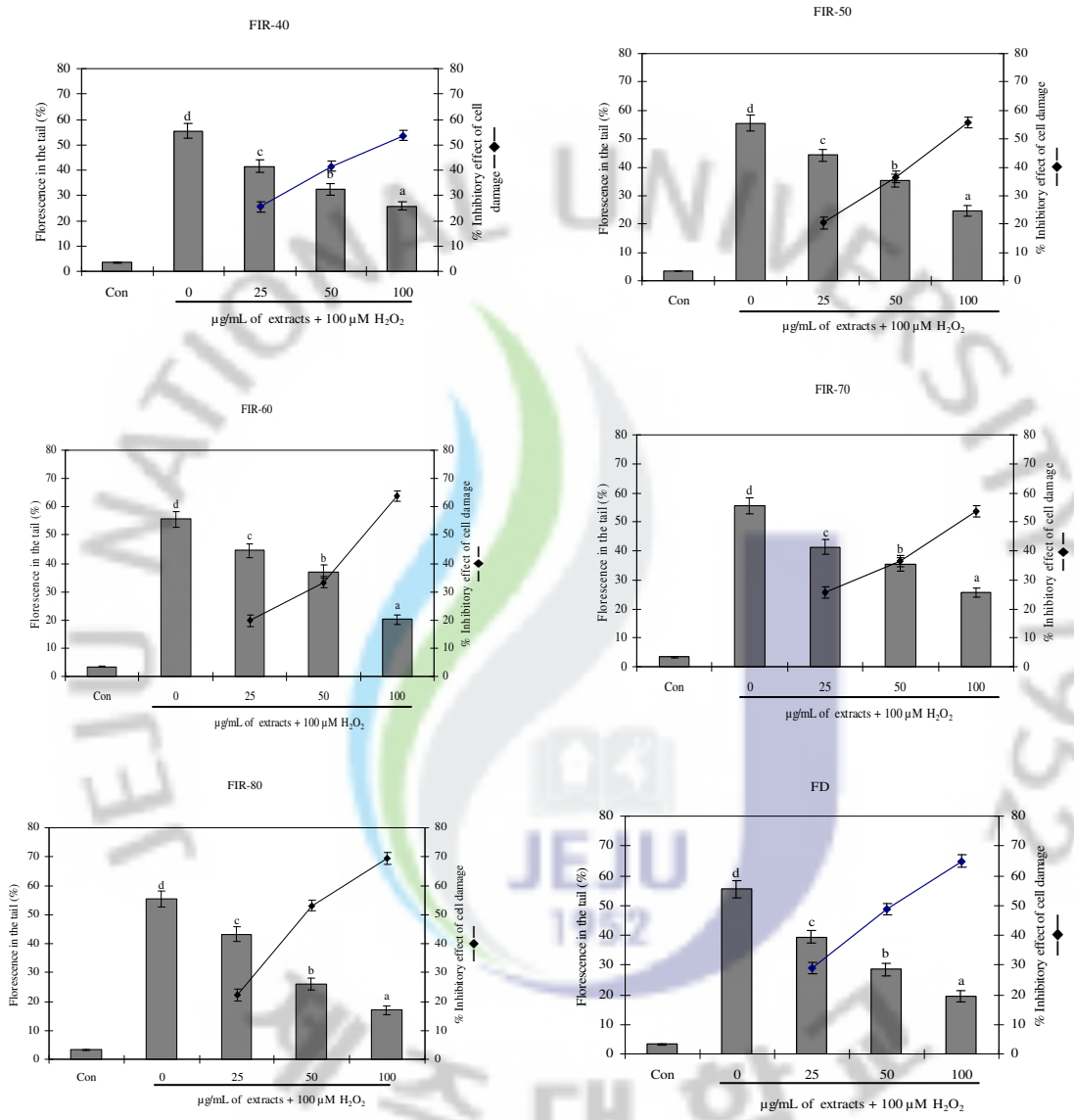


Fig. 3-8. Protective effect of methanolic extract from CPC dried by FIR on cellular DNA damage induced by H_2O_2. DNA damage was determined by alkaline comet assay. The vertical bar graph indicates the percentage fluorescence in tail. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

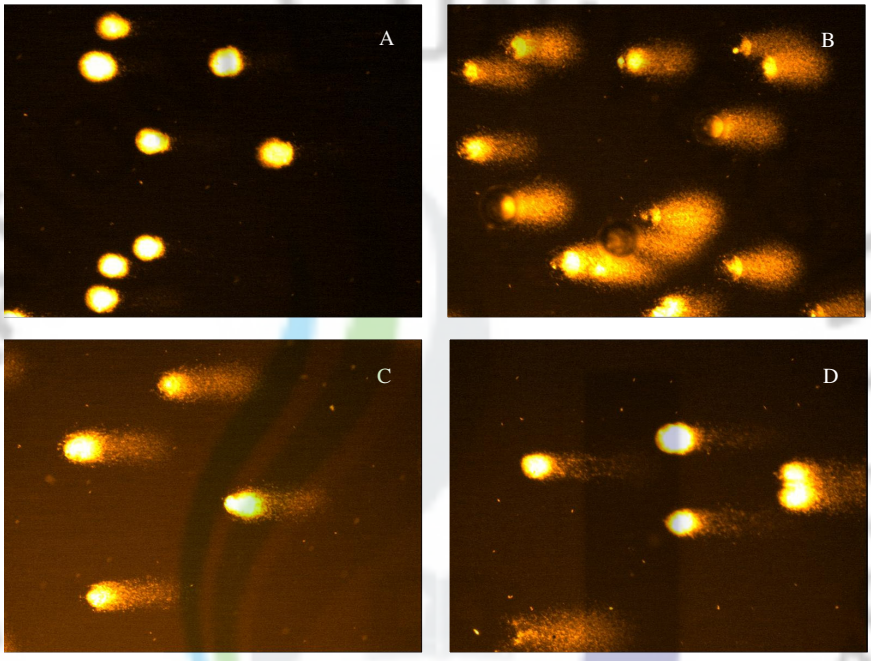


Fig. 3-9. Photomicrograph of comet from Vero cells for the extracts from CPC dried by HSD. A: negative control (untreated with extract), B: positive control (treated only with H_2O_2), C: 100 μM H_2O_2 + 50 μg of extracts from CPC dried by HSD, D: 100 μM H_2O_2 + 100 μg of extracts from CPC dried by HSD.

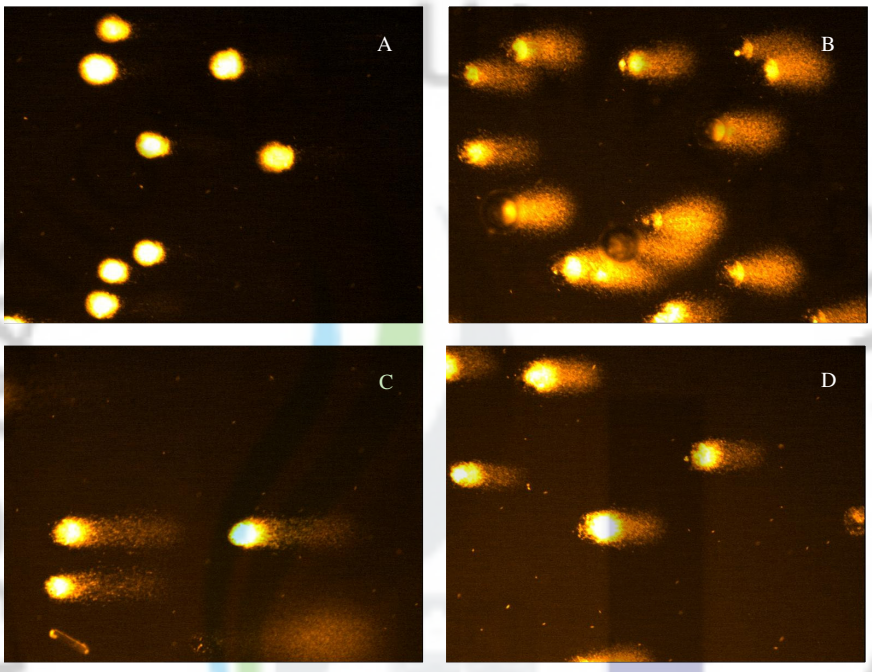


Fig. 3-10. Photomicrograph of comet from Vero cells for the extracts from CPC dried by FIR. A: negative control (untreated with extract), B: positive control (treated only with H_2O_2), C: 100 μM H_2O_2 + 50 μg of extracts from CPC dried by FIR at 50°C, D: 100 μM H_2O_2 + 100 μg of extracts from CPC dried by FIR at 50°C.

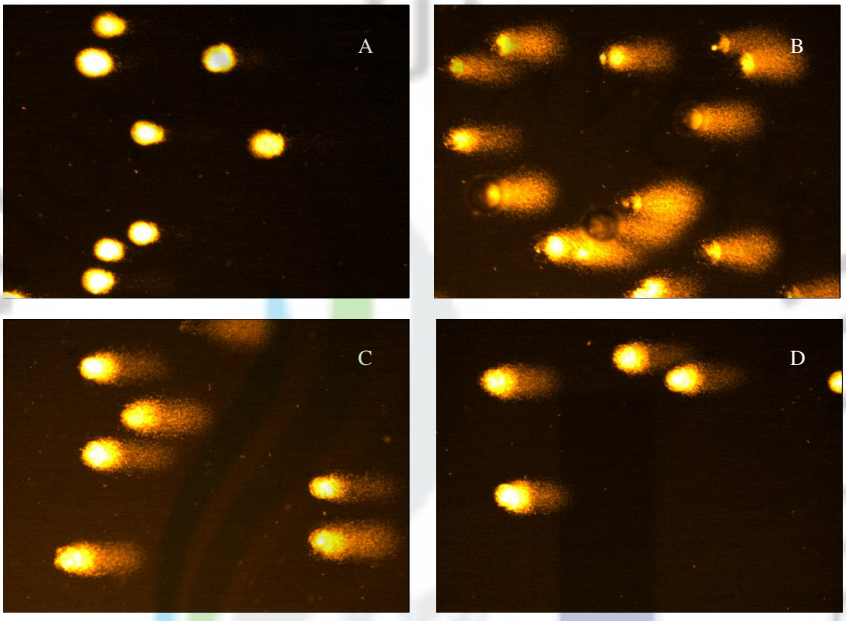


Fig. 3-11. Photomicrograph of comet from Vero cells for the extracts from CPC dried by FD. A: negative control (untreated with extract), B: positive control (treated with only H_2O_2), C: 100 μM H_2O_2 + 50 μg of extracts from CPC dried by FD, D: 100 μM H_2O_2 + 100 μg of extracts from CPC dried by FD.

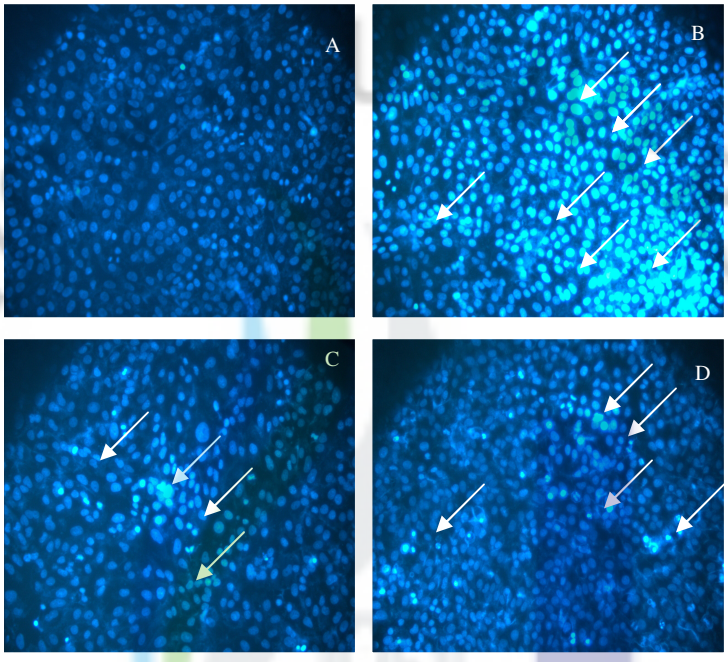


Fig. 3-12. The effect of methanolic extract from CPC dried by HSD and FD against H_2O_2-induced apoptosis on Vero cells. A: Negative control (untreated with extract), B: positive control (treated only with H_2O_2) C: 700 µM H_2O_2 + 100 µg of extracts from CPC dried by HSD, D: 700 µM H_2O_2 + 100 µg of extracts from CPC dried by FD.

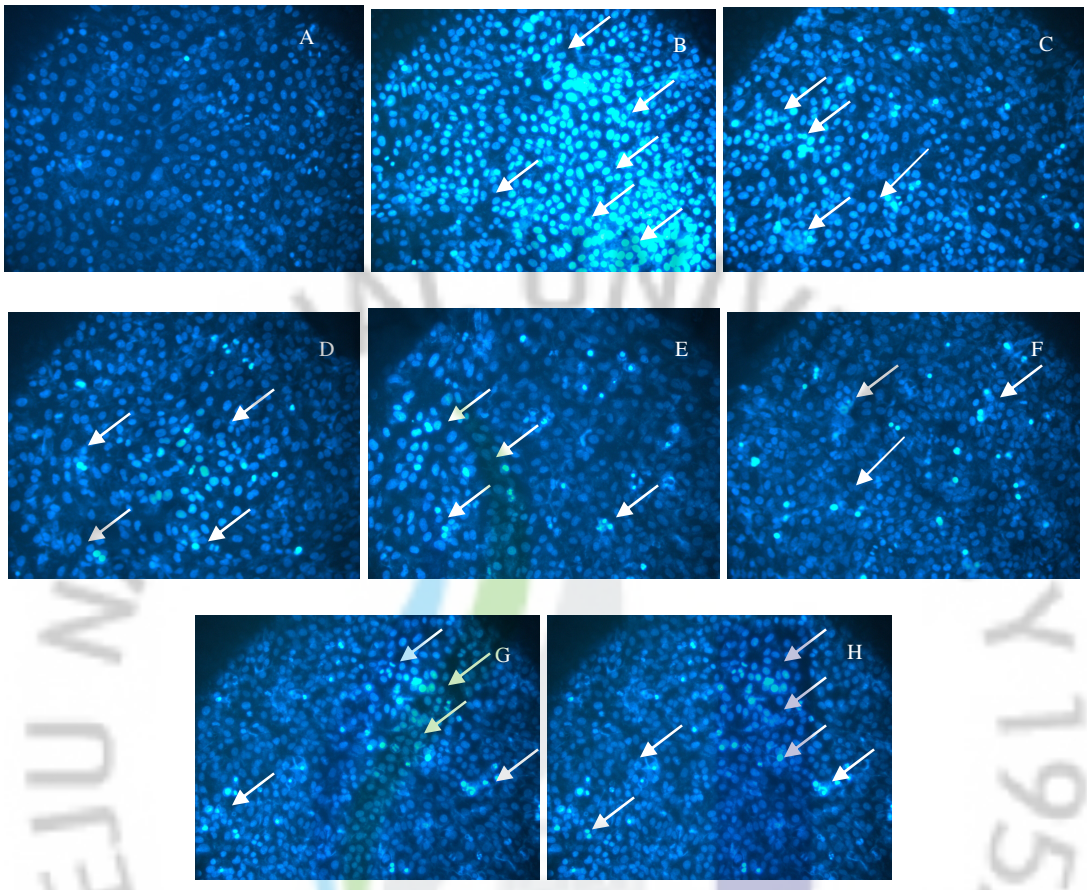


Fig. 3-13. The effect of methanolic extracts from CPC dried by FIR against H_2O_2-induced apoptosis on Vero cells. A: Negative control (untreated with extract), B: positive control (treated only with H_2O_2) C: 700 μM H_2O_2 + 100 μg of extracts from CPC dried by FIR at 40°C, D: 700 μM H_2O_2 + 100 μg of extracts from CPC dried by FIR at 50°C, E: 700 μM H_2O_2 + 100 μg of extracts from CPC dried by FIR at 60°C, F: 700 μM H_2O_2 + 100 μg of extracts from CPC dried by FIR at 70°C, G: 700 μM H_2O_2 + 100 μg of extracts from CPC dried by FIR at 80°C, H: 700 μM H_2O_2 + 100 μg of extracts from CPC dried by FD

Present study evaluated the ROS scavenging effects of methanolic extracts from CPC dried by HSD and FIR in cellular systems. The extracts from CPC exhibited the intacellular ROS scavenging abilities, which enhance the viability of Vero cells after exposure to hydrogen peroxide. The extracts showed strong inhibitory effects on lipid peroxidation. Since the lipid peroxidation leads to a common cell death, the inhibition of lipid peroxidation has been considered as an index of antioxidant capacity. Further, protective effect of the extracts from CPC on H_2O_2–induced apoptosis was strong and showed distinct morphological features consistent with apoptosis. Moreover, the extracts showed protective effect against H_2O_2-induced DNA damage indicating their effects in cells systems. Therefore, this study may contribute to the improvement of scientific understanding of functionality of the tested extracts in cell system and the extracts from CPC dried by HSD can be used as the natural antioxidant agent to mediate the oxidative stress.

PART IV

Antibacterial Activity of Citrus Press-Cake Dried by High Speed Drying and Far-infrared Radiation Drying Against Food-born and Fish Pathogenic Bacteria

ABSTRACT

Due to the evolving resistance of microorganisms to existing antibiotics, there is an increasing demand for new antibiotics not only in human but also in veterinary medicine. Competition for space and nutrients led to the evolution of antimicrobial defense strategies in the aquatic environment. Citrus fruits and their press-cake offer a particularly rich source of potential antimicrobial compounds. The aim of the present study was to identify the potentiality of citrus press-cake (CPC) for food born pathogenic and fish pathogenic microorganisms to be used them as an antibacterial agent. The extracts from CPC dried by high speed drying (HSD) and far-infrared radiation drying (FIR) were subjected to their antibacterial activities against fish pathogenic and food born diseases causing bacteria. Major bioactive compounds in CPC were identified as hesperidin, neohesperidin, nobelitin and heptamethoxyflavone. The methanolic extracts from CPC dried by HSD and FIR showed potent antibacterial properties. Also, scanning electron microscopy was used to observe the morphological changes of the bacteria treated with different CPC extracts. The results obtained in this study suggest that the extracts from CPC dried by HSD and FIR have potential for using them as natural antibacterial agent.

INTRODUCTION

There is an increasing epidemiological evidence for the beneficial health effects of regular intake of fruits and vegetables as part of a healthier diet (Dauchet et al. 2004). Polyphenols from fruits, vegetables and cereals, herbs and spices have been shown to have beneficial effects on human health. Further, some extracts from polyphenol-rich plants or agricultural products have been used as functional foods or supplements. Flavonoids are secondary metabolites which are known well for their biological effects among polyphenols and they show anticancer, antiviral, antimutagenic and anti-inflammatory activities (Benavente-Garcia et al. 1997; Vuorela et al. 2005). Many researchers have worked with flavonoids and proved that the dietary flavonoids can influence gastrointestinal bacterial populations. Further, considerable *in vitro* data on the direct and indirect activity of polyphenols, such as naringenin and hesperetin, against *Helicobacter pylori* (Puupponen-Pimia et al. 2001, 2006; Tombola et al. 2003; Funatogawa et al. 2004; Isobe et al. 2006). Flavonoids are generally present in glycosylated forms in plants, and their sugar moiety is an important factor determining their bioavailability.

Simple flavonoid glycosides can be taken up into cells, and aglycones are absorbed by passive diffusion, the small intestine is unable to absorb the rutinoside forms. Therefore, a full or partial deglycosylation step is critical for the absorption of flavonoids. Mandalari et al. (2007) have previously shown that in commercial enzyme preparations, Pectinase 62L and Pectinase 690L, can effectively deglycosylate bergamot flavonoids, improving their uptake and increasing the beneficial effects through greater bioavailability. It has been demonstrated that enzyme treatments of monosaccharidic and disaccharidic

flavonoids producing lipophilic derivatives increased both antimicrobial and antioxidant activities (Mellou et al. 2005).

Food processors and consumers are increasingly trying to avoid foods with chemical preservatives (Beuchat and Golden 1989; Gould 1996), and this is reflected by the interest in food industry in finding high quality products with natural compounds exhibiting antimicrobial activity. In addition, the replacement of synthetic colorants and chemicals with natural plant compounds is also being evaluated (e.g. the use of cactus pear betacyanins and various fruit anthocyanins for producing yellow-red-purple coloration in various foods: Castellar et al. 2003; Giusti and Wrolstad 2003). There is an increasing demand for natural and minimally processed ingredients that can effectively extend the shelf life of food products and guarantee a high degree of safety because of legislations governing the use of current preservatives. A number of aromatic oils isolated from plants which have antimicrobial activities have found to be used in industrial applications as preservatives of raw and processed foods (Lis-Balchim and Deans 1997; Hammer et al. 1999).

Still food borne diseases are a major problem and have a dramatic increase throughout the world in recent years (Mead et al., 1999). A variety of microorganisms may lead to food spoilage causing a threat for both consumers and the food industry. Some synthetic compounds have been developed to control the microbial growth and to reduce the incidence of food poisoning and spoilage. However, these synthetic compounds might be detrimental to human health even though these preservatives are effective in food industry. Consumers are much concerned about the safety of food which contains artificial preservatives. There has been a growing interest in new and effective

antimicrobial substances from natural sources like plants to reduce the cases of food borne diseases. Crude extracts of spices, herbs and medicinal plants rich in phenolic compounds are becoming increasingly important in food industry as preservatives because of their antimicrobial activity.

Antibiotic treatment for bacterial diseases in fish culture has been practiced for many years. The occurrence of antibiotic resistant bacteria associated with fish diseases has been worldwide problem in aquaculture, which has received considerable attention in last years and continues to increase due to the absence of more effective and safer antibiotics. The prevention and treatment of these infectious diseases by applying products from citrus appears as a possible alternative. Hence, the interest in citrus as a potential and promising source of pharmaceutical agent for microbial activity has increased during the last years (Ortuno et al., 2006; Yi et al., 2008; Jo et al., 2004; Kitzberger et al., 2007). Citrus varieties are considered as source of bioactive compounds as they are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities. Further, citrus by-products released from the juice processing factories might be a useful agent as those wastes mainly contained the citrus peel which constitutes flavonoids as the major compounds.

Therefore, the aim of the present study is to investigate the antimicrobial activity of extracts from CPC against fish pathogenic bacteria that are often the cause of bacterial diseases in aquaculture and food born pathogenic bacteria in human which cause serious problems. The possible use of active compounds from CPC for the prevention or treatment of the bacterial fish diseases or food born diseases should be discussed.

MATERIAL AND METHODS

Microorganisms and culture

CPC was obtained from Jeju provincial development Co.in Jeju Island of Korea. All fish pathogenic and food born disease causing bacteria were kindly provided by the Marine Microbes laboratory in the Department of Marine Biotechnology, Cheju National University, Korea. The bacterial strains were cultured at the appropriate temperature as given in the Table 4-1 and 4-2. Nutrient agar, nutrient broth, brain heart infusion agar or brain heart infusion were used for the relevant bacteria tested in this study. Cultures were appropriately diluted in respective sterile broth to obtain the cell suspension at $1x10^6$ CFU/mL.

High speed drying or far-infrared radiation drying of CPC

CPC stored at -50°C were converted into dried form by HSD drier or FIR drier (TOURI-Q, Korea).

A 20 kg of wet CPC were transferred into the sample compartment of HSD system and they can be converted into dried form within 90 min. A high temperature is maintained within the compartment by the steam supplied through the steam generator. Due to high temperature in this part, the moisture present in the samples is removed and converts them into dried condition. Then, the high speed dried CPC were pulverized into fine powder using a grinder (MF 10 basic mill, GMBH & Co., Staufen, Germany) and sieved through a 300 μm standard testing sieve

Also, CPC were dried using FIR drier. After drying, they were pulverized into fine powder using the same grinder as mentioned previously and sieved through a 300 μm

standard testing sieve.

Extraction of bioactive compounds from CPC

A 20 gram of ground CPC powder was mixed in 100% methanol (1000 mL) and kept in the shaking incubator at 25°C for one day and filtered in a vacuum using Whatman No.1 (Whatman Ltd., England) filter paper. After that, methanol were removed from the extracts and dissolved in 1% DMSO to be used for antimicrobial assays. In each assay, all activities of CPC dried by HSD and FIR were compared with the values of CPC dried by FD.

Antimicrobial assay (disk diffusion assay)

The extracts from CPC were screened for antimicrobial activity using the agar diffusion technique (Meena and Sethi, 1994) and (Rota et al., 2004) against 15 microorganisms which significantly important in fish diseases and food born diseases.

The bacterial strains used to assess the antibacterial properties of the test samples, seven gram-negative strains and three gram-positive strains as shown in the table 4-1. All strains were obtained from the Korean Collection for Type Cultures (KCTC) or KCCM and bacteria were grown in nutrient agar or brain heart infusion agar (Difco Laboratories, Detroit, MI).

Sterilized paper disks (Whatman No. 1, 6 mm diameter) containing 50 μL of each extracts were applied to the surface of agar plates that were previously seeded by spreading of 0.1 mL overnight culture. Amphicillin was used as the positive control and 1% DMSO was used as the negative control. The plates were incubated overnight at the

appropriate temperature, and the diameter of the resulting clear zone was measured in millimeters. The results indicated in the text represent the net clear zone including the diameter (6 mm) of the paper disk. The scale of measurement was the following (disk diameter included) : >20 mm clear zone of inhibition is strongly inhibitory activity; <20–12 mm clear zone is moderately/mildly inhibitory activity; and <12 mm is low inhibitory inhibitory.

Determination of minimum inhibitory concentration (MIC)

The antibacterial activity of the extracts was assessed by determining MIC values obtained by a modified microdilution broth method described by Cai and Wu (1996). Briefly, bacteria, from overnight cultures, were adjusted to 1-5x10^6 (CFU)/mL. The extracts from CPC were serially diluted with broth to give the concentrations of 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 mg/mL. A 100 μL of diluted CPC extracts were added into wells containing 100 μL of bacterial suspension. To adjust the interference of colour due to extracts themselves, a parallel series of mixtures, with uninoculated broth, was prepared. Triplicate samples were taken for each test concentration. After incubation for 24 hr at 30/37 °C under anaerobic conditions, bacterial growth was estimated spectrophotometrically, at 630 nm, using a microtitre plate reader (ELISA, Tecan). The MIC was defined as the minimum concentration of the test compound limiting turbidity to <0.05 absorbance units.

Table 4-1. Food-born disease causing bacteria used in this study

Bacteria	Strain	Gram stain	Shape	Incubation Temperature
Salmonella thyphimurium	KCCM 40253	(-)	Rod	30°C
Escherichia coli	KCCM 40880	(-)	Rod	37°C
Staphylococcus aureus	KCTC 1916	(+)	Round (coccui)	37°C
Listeria monocytogens	KCCM 40307	(+)	Bacilli	30°C

Table 4-2. Fish pathogenic bacteria used in this study

Bacteria	Strain	Gram stain	Shape	Temperature
Vibrio salmonicida	KCCM 41663	(-)	Rod	30°C
Vibrio vulnificus	KCTC 2662	(-)	Rod	30°C
Vibrio fluvialis	KCTC 2692	(-)	Rod	30°C
Vibrio alginolyticus	KCCM 40513	(-)	Rod	30°C
Streptococcus iniae	KCTC 3657	(+)	Coccui	25°C
Vibrio logei	KCTC 2721	(-)	Rod	25°C
Vibrio ichthyoenteri	KCCM 40870	(-)	Rod	26°C
Vibrio anguillarum	KCTC 2711	(-)	Rod	25°C
Vibrio ratiferianus	KCTC 12125	(-)	Rod	25°C
Streptococcus parauberis	KCTC 3651	(+)	Coccui	25°C
Vibrio parahemolyticus	KCCM 1965	(-)	Rod	30°C

Minimum bactericidal concentration (MBC) assay

A method in ASM Pocket Guide to Clinical Microbiology (Patrick, 1996) was slightly modified to determine MBC in this study. Briefly, 50 µL of the samples were taken from the wells of the MIC assays, where any visible turbidity (growth) was not observed, and spread on freshly prepared BHIA/NA plates. The plates were incubated at 30/37°C for 24 hrs so as to determine the MBC. The MBC was defined as the lowest concentration of the extract which allowed less than 0.1% of the original inoculum treated with the extracts to survive and grow on the surface of the medium used.

Scanning electron microscope (SEM) observation

The bacterial cells were suspended in nutrient/brain heart infusion broth and incubated overnight at 37°C/30°C. The suspension was divided equally into two tubes. Suitable concentrations of the crude extract from CPC were added to one of the tubes and incubated at 30°C for 4 hrs. The other tube was used as control. Later, the cells from both tubes were harvested by centrifugation (lowest rpm) and prefixed with a 2.5% glutaraldehyde solution for overnight at 4°C. The cells were collected by centrifugation and resuspended in 0.1 M Na-cacodylate buffer. The mixture was dehydrated rapidly with ethanol series (30%, 50%, 70%, 90% and 100%), and dried with liquid CO_2 at "critical point" (Balzers CPD 030) under 95 bar pressure and gold-covered by cathodic spraying (Edwards S 150 B). Finally, morphology of the bacterial cells was observed by scanning electronic microscope (JEOL, JSM 6700F).

Statistical analysis

All experiments were conducted in triplicate (n=3) and an ANOVA test (using SPSS 11.5 statistical software) was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by using the Duncan's test ($p<0.05$).

RESULTS AND DISCUSSION

A simple way of determining the susceptibility of a microorganism to an antimicrobial agent is to use a microbe-seeded agar plate and to allow the compound to diffuse into the agar medium, which is known as the Kirby-Bauer technique. A sterilized filter disk impregnated with the antibacterial compound is applied onto the seeded agar surface. The concentration decreases as a function of the square of the distance of diffusion, as the antibacterial substances diffuse from the sterilized filter paper into the agar. At some particular distance from each disk, the antimicrobial agent is diluted to a point that it no longer inhibits microbial growth. The effectiveness of a particular antimicrobial compound results in the production of growth-inhibition zones that appear as clear areas surrounding the disk from which the compound diffused. The diameter of the zones can be measured with a ruler and the results of such an experiment constitute an antibiogram (Atlas et al. 1995).

The agar diffusion test provides a rapid assessment of antimicrobial activity for a given compound. It is often used commercially to supply basic antimicrobial data during the manufacture, as well as, quality control assurance of the finished product (Ascenzi 1996; Block 1991; Paulson 1999). Antiseptics and disinfectants used in sanitation for food processing and the medical/surgical arena, antibiotics and pesticides for clinical and agricultural use, and preservatives for food, metals, cosmetics, medicine, and paints are all examples which can be tested by this procedure.

The results of antibacterial screening assay (disk assay) are shown in Table 4-3 and 4-4 respectively for the food-born diseases causing bacteria and fish pathogenic bacteria. All

the extracts showed that the antibacterial activity against the tested bacterial strains with various intensities. Some extracts showed strong antibacterial activities while others showed moderate or weak activities. In this study, we used 9 gram negative and two gram positive fish pathogenic bacteria. All extracts showed strong activities against *S. iniae, V. ichthyoenteri and S. parauberis*, and moderate activities against *V. alginolyticus, V. anguillarum, and V. ratiferianus* while weak activities against *V. salmonisida, V. vulnificus, V. fluuvialis and V. logei.*

The MICs of extracts from CPC dried by HSD and FIR to inhibit the growth of gram-positive and gram-negative bacteria are shown in the Table 4-5 and 4-6. The MIC for the methanolic extracts from CPC dried by HSD and FIR were lowest and confirmed that their activity against the bacteria tested in this study. Both extracts from CPC dried by HSD and FIR showed higher activities for all the gram positive and gram negative bacteria. The MIC vales ranged from 500- 4000 mg/mL depending on the extract. The MIC values for gram-negative bacteria were 1.0 -8.0 mg/mL. The reason for higher sensitivity of the gram-negative bacteria compared to positive bacteria could be ascribed to the differences between their cell wall compositions. The gram-positive bacteria contains an outer peptidoglycon layer which is an ineffective permeability barrier (Scherrer and Gerhardt, 1971), whereas in the case of gram-negative bacteria, outer phospholipidic membrane makes the cell wall impermeable to lipophollic solutes and porins constitutes acting as a selective barrier to the hydrophilic solutes (Nikaido and Vaara, 1985). In addition minimum bactericidal concentration for food-born disease causing bacteria and fish pathogenic bacteria are shown in Table 4-7 and 4-8.

Table 4-3. Growth inhibition zone (mm) showing antibacterial activity of CPC on food-born disease causing bacteria

Bacteria	Diameter of clear zone (mm)							
	Amp	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
Salmonella thyphimurium	32±2^{d}	16±1^{b}	14±0.5^{ab}	15±0.5^{bc}	15±1^{b}	15±1b^{c}	13±0.5^{a}	13±0.5^{a}
Escherichia coli	23±1.5^{b}	12±0.5^{a}	13±0.5^{a}	12± 0.5^{a}	13±0.5^{a}	13±0.5^{a}	13±0.5°	12±0.5^{a}
Staphylococcus aureus	47±3^{b}	11±0.5^{a}	11±0.5^{a}	11±0.5^{a}	11±0.5^{a}	11±0.5^{a}	10±0.5^{a}	11±0.5a
Listeria monocytogens	30±2^{e}	12± 0.5^{a}	13±0.5^{ab}	15±0.5^{c}	14± 0.5^{b}	17±1^{d}	14±0.5^{bc}	12±0.5^{a}

Diameter of inhibition zone including disc diameter of 6 mm. The concentration of the extract used 10 mg/disc. All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

Table 4-4. Growth inhibition zone (mm) showing antibacterial activity of CPC on fish pathogenic bacteria

Bacteria	Diameter of clear zone (mm)							
	Amp	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
Vibrio salmonicida	27±2^{c}	9±0.5^{a}	10±0.5^{ab}	11±1^{b}	10±0.5^{ab}	9±0.5^{a}	9±0.5^{a}	10±0.5^{ab}
Vibrio vulnificus	25±1.5^{e}	19±1^{d}	17±1^{c}	12±1^{ab}	12±1^{ab}	11±1^{a}	13±0.5^{b}	13±0.5^{b}
Vibrio fluvialis	15±1^{c}	15±1^{c}	14±1^{bc}	15±1^{c}	15±1^{c}	12±1^{a}	13±0.5^{ab}	12±0.5^{a}
Vibrio alginolyticus	-	15±1^{cd}	14±1^{bc}	16±1^{d}	15±1^{cd}	12±1^{a}	13±1^{ab}	13±0.5^{ab}
Streptococcus iniae	49±2^{d}	24±2^{b}	19±1^{a}	29±2^{c}	28±2^{c}	24±2^{b}	24±1^{b}	19±1^{a}
Vibrio logei	-	13±1^{c}	11±1^{ab}	12±1^{bc}	11±1^{ab}	10±1^{a}	10±0.5^{a}	12±1^{b}
Vibrio ichthyoenteri	24±1^{b}	22±1.5^{ab}	22±1.5^{ab}	23±1.5^{b}	22±1.5^{ab}	20±1.5^{a}	20±1^{a}	21±1^{a}
Vibrio anguillarum	24±2^{c}	17±1^{ab}	16±1^{a}	19±1^{b}	17±1^{ab}	15±1^{a}	16±1^{a}	16±1^{ab}
Vibrio ratiferianus	25±1.5^{d}	20±1.5^{c}	17±1^{b}	16±1^{b}	16±1^{b}	12±1^{a}	13±1^{a}	17±1^{b}
Streptococcus parauberis	46±2^{d}	27±2^{c}	20±1.5^{a}	21±1.5^{a}	21±1.5^{a}	18±1^{a}	20±1^{a}	24±2^{b}
Vibrio parahemolyticus	24±1.5^{b}	19±1.5^{a}	19±1^{a}	18±1^{a}	18±1^{a}	18±1^{a}	19±1^{a}	20±2^{a}

Diameter of inhibition zone including disc diameter of 6 mm. The concentration of the extract used 10 mg/disc All data are means of three determinations. Significant differences at $p<0.05$ were indicated with different letters.

Table 4-5. Minimum inhibitory concentration (MIC) of methanolic extracts from CPC dried by HSD and FIR on food-born disease causing bacteria

Bacteria	Minimum Inhibitory Concentration (mg/mL)							
	Amp[1]	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
Salmonella thyphimurium	40	4^{a}	4^{a}	4^{a}	4^{a}	8^{b}	4^{a}	8^{b}
Escherichia coli	40	4^{a}	4^{a}	8^{b}	8^{b}	8^{b}	8^{b}	8^{b}
Staphylococcus aureus	80	1^{ab}	2^{c}	2^{c}	0.5^{a}	1^{ab}	1^{ab}	1^{ab}
Listeria monocytogens	40	0.5^{a}	1^{b}	1^{b}	0.5^{a}	1^{b}	1^{b}	0.5^{a}

1. The concentrations of amphicillin was given in μg/mL.Significant differences at $p<0.05$ indicated with different letters.

Table 4-6. Minimum inhibitory concentration (MIC) of methanolic extracts from CPC dried by HSD and FIR on fish pathogenic bacteria

Bacteria	Minimum Inhibitory Concentration (mg/mL)							
	Amp[1]	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
Streptococcus iniae	160	2^a	2^a	4^b	2^a	4^b	2^a	2^a
Vibrio anguillarum	80	2^a	2^a	2^a	4^b	2^a	2^a	4^b
Streptococcus parauberis	80	2^a	4^b	4^b	4^b	4^b	4^b	4^b
Vibrio alginolyticus	-	8^a	8^a	8^a	8^a	8^a	8^a	8^a
Vibrio ichthyoenteri	160	8^a	8^a	8^a	8^a	8^a	8^a	8^a
Vibrio parahemolyticus	80	4^a	4^a	4^a	4^a	4^a	4^a	4^a

1. The concentrations of amphicillin was given in μg/mL.Significant differences at $p<0.05$ indicated with different letters.

Table 4-7. Minimum bactericidal concentration (MBA) of methanolic extracts from CPC dried by HSD and FIR on food-born disease causing bacteria

Bacteria	Minimum Bactericidal Concentration (mg/mL)							
	Amp[1]	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
Salmonella thyphimurium	80	4^{a}	4^{a}	4^{a}	4^{a}	8^{b}	4^{a}	8^{b}
Escherichia coli	80	4^{a}	4^{a}	8^{b}	8^{b}	8^{b}	4^{a}	8^{b}
Staphylococcus aureus	160	2^{ab}	4^{b}	4^{b}	1^{a}	2^{ab}	2^{ab}	2^{ab}
Listeria monocytogens	80	1^{a}	2^{b}	2^{b}	1^{a}	2^{b}	2^{b}	1^{a}

1. The concentrations of amphicillin was given in μg/mL.Significant differences at $p<0.05$ indicated with different letters.

Table 4-8. Minimum bactericidal concentration (MBA) of methanolic extracts from CPC dried by HSD and FIR on fish pathogenic bacteria

Bacteria	Minimum Bactericidal Concentration (mg/mL)							
	Amp[1]	FD	HSD	FIR-40	FIR-50	FIR-60	FIR-70	FIR-80
Streptococcus iniae	320	4^{a}	4^{a}	8^{b}	4^{a}	8^{b}	4^{a}	4^{a}
Vibrio anguillarum	160	4^{a}	4^{a}	4^{a}	4^{a}	4^{a}	4^{a}	8^{b}
Streptococcus parauberis	160	4^{a}	4^{a}	4^{a}	4^{a}	4^{a}	4^{a}	8^{b}
Vibrio alginolyticus	-	16^{a}	16^{a}	16^{a}	16^{a}	16^{a}	16^{a}	16^{a}
Vibrio ichthyoenteri	320	16^{a}	16^{a}	16^{a}	16^{a}	16^{a}	16^{a}	16^{a}
Vibrio parahemolyticus	160	4^{a}	4^{a}	4^{a}	4^{a}	4^{a}	4^{a}	4^{a}

1. The concentrations of amphicillin was given in μg/mL.Significant differences at $p<0.05$ indicated with different letters.

One selected bacteria was treated with the extract from CPC dried by HSD, FIR and FD, and observed by SEM to investigate any physical changes in the appearance of the cells. The SEM image of the treated specie is shown in Fig.4-1 and 4-2, and illustrates the destructive effect on the tested bacteria. The CPC extract could cause physical damage (considerable morphological alteration and damage) to the treated bacteria. In contrast to the control, many bacterial cells showed morphological changes in the surface. Non-treated bacterial cells (control) remained intact and showed a smooth surface Fig. 4-1a. For the treated bacterial cells, some cells presented damage as pores or deformities in the cell membranes.

The SEM observations proved that the crude extract from CPC could cause considerable morphological alteration and damage to the treated bacteria so as to exert their bacteriostatic or bactericidal effect. Several possible mechanisms of action were proposed. The active components in the crude extract from CPC bind to the cell surface, and then penetrate to the target sites, which would sensitize the phospholipid bilayer of the cytoplasmic membrane and inhibit membrane-bound enzymes. After combining to the target sites, the active components might inhibit proton motive force, the respiratory chain and electron transfer, substrate oxidation, and/or energy transport processes in the bacteria. As a result, such inhibitions could lead to the uncoupling of oxidative phosphorylation, restraining from active transport, loss of pool metabolites, and disruption of synthesis of DNA, RNA, protein, lipid, and polysaccharides (Denyer, 1990; Kim et al., 2004; Nychas, 1995).

The damage to the bacterial cell wall and cytoplasmic membrane might indicate loss in structural integrity and the membrane's ability to act as a permeability barrier (Billerbeck et al., 2001). The distortion of the cell physical structure would cause for expansion and destabilization of the membrane and increase membrane fluidity, which in turn would increase passive permeability and leak various vital intracellular constituents including ions, ATP, nucleic acids, and amino acids (Cox et al., 1998; Helander et al., 1998; Ultee et al., 2002). Cell death might be the result of the extensive loss of cell contents, the exit of critical molecules and ions or the initiation of autolytic processes (Denyer, 1990).

In addition, there may be relationships between the antibacterial activity and the chemical structures of the major phenolic compounds in the tested extract. The presence and position of the hydroxyl groups in phenolic compounds might influence their antibacterial effectiveness. Certain hydroxyl groups in the phenolic compounds might bind to the active site of enzymes and form hydrogen bonds with enzymes by altering their metabolism or lipid solubility while the degree of steric hindrance of the phenolic compounds might determine their antibacterial activity (Beuchat and Golden, 1989; Ceylan and Fung, 2004). Phenolic compounds might be involved in other modes of action for their antimicrobial activity. These compounds might interact with cytoplasmic membrane, thereby destroying its permeability and releasing intracellular constituents causing membrane dysfunction in respect of electron transport, nutrient uptake, nucleic acid synthesis, and ATPase activity (Denyer and Hugo, 1991; Rico-Munoz et al., 1987).

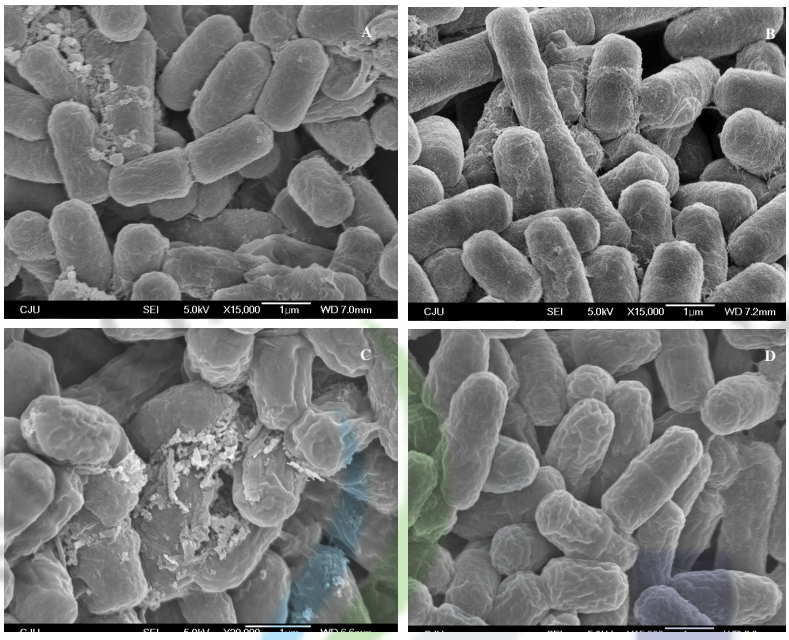


Fig. 4-1. Scanning electron microscope observation of *S.iniea* treated with the CPC extracts. (A) control; (B) extracts from CPC dried by HSD; (C) extracts from CPC dried by FIR at 50°C (D) extracts from CPC dried by FD.

The results of this study provided an insight into the antibacterial properties of the extracts from CPC on selected food-born disease causing bacteria and fish pathogenic bacteria. Results revelead that the extracts from CPC posseded strong antibacterial properties against both gram positive and negative bacterial straines. Hence, the major components in the CPC might have the potential to act as natural antibacterial agents for food preservation and medicinal use. It should be further investigate for the use in food sector to improve food safety.

REFERENCES

Alvarez JG, Storey BT. 1989. Role of gluthione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. Gamete Res. 23: 77-90.

Anagnostopoulou MA, Kefalas P, Papageorgiou VP, Assimopoulou AN, Boskou D. 2005. Radical scavenging activity of various extracts and fractions of sweet orange peel (citrus sinensis). Food Chem. 94: 19-25.

Andersen JK. 2004. Oxidative stress in neurodegeneration: cause or consequence. Nat. Med 10: 18-25.

Anonymous. Comet Assay TM. Reagent Kit for Single Cell Gel Electrophoresis Assay; 2001; Catalog #4250-050-K. Trevigen Inc., Gaithersburg, MD, USA.

AOAC 1995. Officical methods of analysis, 15 Ed. Association of Official Anlytical Chemist. Washinton, DC.

Ascenzi JM. 1996. Handbook of disinfectants and astiseptics. New York, NY: Marcel Dekker Inc.

Atlas RM, Brown AE, Parks LC. 1995. Laboratory manual of experimental microbiology. NewYork, NY, Mosby-Year Book, Inc.

Aust SD, Svingen BA, 1982. The role of iron in enzymeic lipid peroxidation. In ffree radicals in Biology, Pryor,W.A. Ed., Academic press. Newyork, pp1-28.

Benavente-Garcia O, Castillo J, Marin FR, Ortuno A, Del-Rio JA. 1997. Uses and properties of citrus flavonoids. J. Agric. Food Chem. 45: 4505-4515.

Beuchat LR, Golden DA. 1989. Antimicrobials occurring naturally in foods. Food Technol. 43: 134-142.

Billerbeck VGD, Roques CG, Bessiere JM, Fonvieille JL Dargent R. 2001. Effects of *Cymbopogon nardus* (L.) W. Watson essential oil on the growth and morphogenesis of *Aspergillus niger*. J. Microbiol. 47: 9-17.

Block SS. 1991. Disinfection, sterilization and Preservation. 4th ed, Malvern, PA: Lea & Febiger

Bocco A, Cuvelier ME, Richard H, Berset C. 1998. Antioxidant activity and phenolic composition of citrus peel and seed extracts. J. Agric. Food Chem. 46: 2123-2129.

Bocco A, Cuvelier ME, Richard H, Berset C. 1997.The antioxidant activity of phenolic compounds measured by an accelerated test based on citronellal oxidation. Sci. des Aliments 18: 13-24.

Bombardelli E, Morazzoni P. 1993. The flavonoids: New perspectives in biological activities and therapeutics. Chem. Today 11: 25-28.

Bors W, Heller W, Michel C, Saran M. 1990a. Radical chemistry of Flavonoids antioxidant. In Emerit (Ed.). *Antioxidants in therapy and preventive medicine* (Vol.1, pp.165-170). New York: Plenum press.

Bors W, Heller W, Michel C, Saran M. 1990b. Flavonoids as antioxidants: determination of radical scavenging efficiencies. In L. Packer & A. N. Glazer (Eds.).*Methods in enzymology* (Vol.186, pp. 343-356). San Diego, CA; Academic press.

Branen AL, Davidson PM. 1997. Use of antioxidants in self-preserving cosmetic and drug formulations. In: Preservative-Free and Self-Preserving cosmetics and Drugs. Cosmetic Sci.Technol. Series 16: 159-179.

Bronner WE, Beecher GR. 1995. Extraction and measurement of prominent flavonoids in orange and grapefruit juice concentrate. J. Chrom. A 705: 247-256.

Buyukokuroglu ME, Gulcin I, Oktay M, Kufrevioglu OI. 2001. *In vitro* antioxidant properties of dantrolene sodium. Pharmacol. Res. 44: 491-495.

Cai L, Wu D. 1996. Compounds from syzygium aromaticum possessing growth inhibitory activity against oral pathogens. J. Natl. Pro. 59:-987-990.

Castellar R, Obon JM, Alacid M, Fernandez-Lopez JA. 2003. Color properties and stability of betacyanins from *Opuntia* fruits. J. Agric. Food Chem. 51: 2772-2776.

Ceylan E, Fung DYC. 2004. Antimicrobial activity of spices. J. Rapid Mtds Automation Microbiol. 12: 1-55.

Chandler SF, Dodds JH. 1983. The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasidine in callus cultures of *Solanum laciniatum*. Plant cell reports 2: 105-110.

Chandrasekar D, Madhusudhana K, Ramakrishna S, Diwan PV. 2006. Determination of DPPH free radical scavenging activity by reversed-phase HPLC: A sensitive screening method for polyherbal. J. pharmacol. Biomed. Anal. 40: 460-464.

Chamulitrat W, Mason RP. 1990. Alkyl free radicals form the β–scission of fatty acid alkoxyl radicals as detected by spin trapping in a lipoxygenase system. Arch. Biochem. Biophy. 282: 65-69.

Chandler SF, Dodds JH. 1993. The effect of phosphate, nitrogen and sucrose on the production of phenolics and solasidine in callus cultures of Solanum laciniatum. Plant Cell Reports 2: 105-110.

Chou CF, Huang YL. 2003. Comparison of the chemical composition and physiochemical properties of different fiber prepared from the peel of citrus sinensis L. cv Liucheng. J. Agric. Food Chem. 51: 2615-2618.

Cook NC, Samman S. 1996. Flavonoids-chemistry, metabolism, cardioprotective effects and dietary sources. Nut. Biochem. 7: 66-76.

Cox SD, Gustafson JE, Mann CM, Markham JL, Liew YC, Hartland RP. 1998. Tea tree oil causes K^+ leakage and inhibits respiration in *Escherichia coli*. Lett. App. Microbiol. 26: 355-358.

Darmon N, Ferrandiz V, Canal MT, Mitjavilla S. 1990. Active antiradicallaire de flavonoides vis-à-vis de lanion superoxide et du radical hydroxyle. Liaison-grupe Pholyphenols Bulletin 15 : 158-162.

Dauchet L, Ferrières J, Arveiler D, Yarnell JW, Gey F, Ducimetière P, Ruidavets JB, Haas B. 2004. Frequency of fruit and vegetable consumption and coronary heart disease in France and Northern Ireland: the PRIME study. Br. J. Nutr. 92: 963-972.

Decker EA, Welch B. 1990. The role of ferritin as a lipid oxidation catalyst in muscle food. J. Agric. Food Chem. 38: 674-677.

Denyer SP. 1990. Mechanisms of action of biocides. Intl. Biodeterioration 26: 89-100.

Denyer SP, Hugo WB. 1991. Mechanisms of antibacterial action–a summary. In: S.P. Denyer and W.B. Hugo, Editors, *Mechanisms of action of chemical biocides*, Blackwell, Oxford, 331-334.

Dumont A, Hehner SP, Hofmann TG, Ueffing M, Droge W, Schmitz ML. 1999. Hydrogen peroxide-induced apoptosis is CD95-independent, requires the release of mitochondria-derived reactive oxygen species and the activation of NF-kappa B. Oncogene 18: 747-757.

Duthie SJ, Collins AR. 1997. The influence of cell growth, detoxifying enzymes and DNA repair on hydrogen peroxide-mediated DNA damage (measured using the comet assay) in human cells. Free Rad. Biol. Med. 22: 717-724.

Engelmann J, Volk J, Leyhausen G, Geurtsen W. 2005. ROS formation and glutathione levels in human oral fibroblasts exposed to TEGDMA and camphorquinone. J biomaterials Res. Part B: Appl. Biomat. 75: 272-276.

Fairbairn DW, Olive PL, O'Neill KL. 1995. The comet assay: A comprehensive review. Mut. Res. 339: 37-59.

Faust F, Kassie F, Knasmüller S, Bocdecker RH, Mann M, Mersch-Sundermann V. 2004. The use of the alkaline comet assay with lymphocytes in human biomonitoring studies. Mut. Res. 566: 209-229.

Femenia A, Pascual PG, Simal S, Rossello C. 2003. Effects of heat treatment and dehydration on bioactive polysaccharide acemannan and cell wall polymers from *Aloe barbadensis* Miller. Carbo. Polymers 51: 397-405.

Fleurence J. 1999. The enzymatic degradation of algal cell wall: a useful approach for improving protein accessibility. J. App. Phycol. 11: 313-314.

Foote CS. 1976. Photosensitized oxidation and singlet oxygen: Connsequences in biological systems. In *Free Radicals in Biology*, Pryor, W.A. Ed.; Academic Press: New York. Vol. 2, pp 85-133.

Funatogawa K, Hayashi S, Shimomura H, Yoshida T, Hatano T, Ito H, Hirai Y. 2004. Antibacterial activity of hydrolysable tannins derived from medicinal plants against *Helicobacter pylori*. Microbiol. Immunol. 48: 251-261.

Gao Z, Huang K, Yang X, Xu H. 1999. Free radical scavenging and antioxidant activities of flavonoids extracted from the radix of *Scutellaria baicalensis* Georgi. Biochemi. et Biophy. Acta 1472: 643-650.

Ginzburg AS. 1969. Application of Infrared Radiation in Food Processing, Chemical and Process Engineering Series, Leonard Hill, London.

Giusti MM, Wrolstad RE. 2003. Acylated anthocyanins from edible sources and their applications in food systems. Biochem. Eng. J. 14: 217-225.

Gould GW. 1996 Industry perspectives on the use of natural antimicrobials and inhibitors for food applications. J. Food Prot. (Suppl) 82-86.

Gschwind M, Huber G. 1995. Apoptotic cell death induced by β-amyloid 1-42 peptide is cell type dependent. J. Neurochem. 65: 292-300.

Halliwell B. 1991. The biological toxicity of free radicals and other reactive species. In: O.I. Aruoma and B. Halliwell, Editors, Free radicals and food additives, Taylor and Francis, London, p.41.

Halliwell B, Gutteridge JMC. 1999. Free radicals in biology and medicine. 2d ed. Oxford University Press, Oxford. pp.1-85.

Halliwell B, Gutteridge JMC. 1989. Free radicals in biology and medicine, 2nd Ed. Clarendon Press, Oxford, p305.

Halliwell B, Gutteridge JMC. 1990. Role of free radicals and catalytic metal ions in human disease: An overview. Mtds. Enzymol. 186: 1-85.

Halliwell B, Gutteridge JMC, Cross CE. 1992. Free radicals, antioxidants, and human disease: where are we now. J. Lab. Clinic. Med. 119: 598-620.

Hammer KA, Carson CF, Riley TV. 1999. Antimicrobial activity of essential oils and other plant extracts. J. Appl. Microbiol. 86: 985-990.

Hansen MB, Nielsen SE, Berg K. 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Mtds. 119: 203-210.

Haorah J, Ramirez SH, Schall K, Smith D, Pandya R, Persidsky Y. 2007. Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. J. Neurochem. 101: 566-576.

Heistad DD. 2006. Oxidative stress and vascular disease: 2005 Duff lecture, Arterioscler Thromb. Vasc. Biol. 26: 689-695.

Heim KE, Tagliaferro AR, Bobilya DJ, 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationship. J. Nutr. Biochem. 13: 572-584.

Helander IM, Alakomi HL, Latva-Kala K, Mattila-Sandholm T, Pol I, Smid EJ. 1998. Characterization of the action of selected essential oil components on gram-negative bacteria. J. Agric. Food Chem. 46: 3590-3595.

Hemmerich P, Wessiak A. 1976. The structural chemistry of flavin-dependent oxygen activation. In: T.P. Singer, Editor, *Proceedings of the fifth international symposium, flavins and flavoproteins*, Elsevier, Amsterdam pp. 9-22

Heo SJ, Ko SC, Kang SM, Kang HS, Kim JP, Kim SH, Lee KW, Cho MG, Jeon YJ. 2008. Cytoprotective effect of fucoxanthin isolated from brown algae Sardassum siliquastrum against H_2O_2-induced cell damage. Eur. Food Res. Technol. 228: 145-151

Heo SJ, Lee KW, Song CB, Jeon YJ. 2003. Antioxidant activity of enzymatic extracts from brown seaweeds. Algae 18: 71-81.

Herrman K. 1976. Flavonols and flavones in food plants: A review. J. Food Technol. 11: 433-448.

Hiramoto K, Johkoh H, Sako K, Kikugawa K. 1993. DNA breaking activity of the carbon-centered radical generated from 2, 2-azobis (2-amidinopropane) hydrochloride (AAPH). Free Radical Res. Commu. 19: 323-332.

Hool LC, Corry B. 2007. Redox control of calcium channels: from mechanisms to therapeutic opportunities. Antioxid. Redox Signal 9: 409-435.

Horowitz RM. 1986. Taste effects of Flavonoids. Progress Clinic.Biol. Res. 213: 163-175.

Horowitz RM. 1961. The citrus flavonoids. In: W.B. Sinclair, Editor, *The orange. Its biochemistry and physiology*, University of California, Division of Agricultural Science, Los Angeles, CA pp. 334-372.

Horowitz R, Gentili B. 1977. Flavonoids constituents of citrus. In: S. Nagy, P.E. Shaw and M.K. Vedhuis, Editors, *Citrus science and technology*, AVI Publishing, Westport, CT 397-426.

Huet R. 1982. Constituents of citrus fruits with pharmacodynamic effect: Citroflavonoids, Fruits 37: 267-271.

Inatani R, Nakatani N, Fuwa H. 1983. Antioxidative effect of the constituents of rosemary (*Rosemarinus officinalis* L.) and their derivatives. Agric. Biol. Chem. 47: 521-528.

Isobe T, Doe M, Morimoto Y, Nagata K, Ohsaki A. 2006. The anti-*Helicobacter pylori* flavones in a Brazilian plant, *Hyptis fasciculata*, and the activity of methoxyflavones. Biol. Pharm. Bull. 29: 1039-1041.

Jang HH, Surh YJ. 2003. Protective effects of resveratrol on β-amyloid induced oxidative PC12 cell death. Free Radic. Biol. Med. 34: 1100-1110.

Jayaprakasha GK, Jaganmohan RL, Sakariah KK. 2004. Antioxidant activities of flavidin in different in vitro model systems. Bio. Med. Chem. 12: 5141-5146.

Jayaprakasha GK, Patil BS. 2007. In vitro evaluation of the antioxidant activities in the fruit extracts from citron and blood orange. Food Chem. 101: 410-418.

Jeon YJ, Byun HG., Kim SK. 1999. Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. Process Biochem. 35: 471-478.

Jeong SM, Kim SY, Kim DR, Jo SC, Nam KC, Ahn DU, Lee, SC. 2004. Effect of heat treatment on the antioxidant activity of extracts from citrus peels. J. Agric. Food Chem. 51: 4400-4403.

Jeulin C, Soufir JC, Weber P, Laval-Martin D, Calvayrac R. 1989. Catalase activity in human spermatozoa and seminal plasma. Gamete Res. 24: 185-196.

Jo C, Park BJ, Chung SH, Kim CB, Cha BS, Byun MW. 2004. Antibacterial and Anti-fungal activity of citrus (*Citrus unshiu*) essential oil extracted from peel by- products. Food Sci. Biotechnol. 13: 384-386.

Kanaze FI, Gabrieli C, Kokkalou E, Georgarakis M, Niopas I. 2003. Simultaneous reversed-phase high-performance liquid chromatographic method for the determination of diosmin, naringin and hesperidin in different citrus fruit juices and pharmaceutical formulations. J. Pharma. Biomedical. Anal. 33: 243-249.

Kang HJ, Chawla SP, Jo C, Kwon JH, Byun MW. 2006. Studies on the development of functional powder from citrus peel. Biores. Technol. 97: 614-620.

Kassie F, Parzefall W, Knasmuller S. 2000. Single cell gel electrophoresis assay: a new technique for human biomonitoring studies. Mutat. Res./Rev. Mut. Res. 463: 13-31.

Kaul TN, Middleton E, Ogra PL. 1985. Antiviral effects of flavonoids on human viruses. J. Med. Viro. 15: 71-79.

Kawaguchi K, Kikuchi S, Hasunuma R, Maruyama H, Yoshikawa T, Kumazawa YA. 2004. Citrus flavonoid hesperidin suppresses infection-induced endotoxin shock in mice. Biol. Pharma. Bull. 27: 679-683.

Kerr JF, Gobe GC, Winterford CM, Harmon BV. 1995. Anatomical methods in cell death. Mtds cell boil. 46:1-27.

Khan AU. 1985. Singlet molecular oxygen spectroscopy. Chemical and photosensitized. In Singlet Oxygen, Frimer,A.A, Ed. CRC press. Boca Raton FL pp39-79.

Kim YS, Kim HH, Yoo MJ, Shin DH. 2004. Bactericidal effect of the extracts of *Polygonum cuspidatum* on *Bacillus cereus*. Food Sci. Biotechnol. 13: 430-433.

Kim HJ, So YJ, Jang JH, Lee JS, Oh YJ, Surh YJ. 2001. Differential cell death induced by salsolinol with and without copper: possible role of reactive oxygen species. Mol. Pharmacol. 60: 440-449.

Kim KN, Heo SJ, Song CB, Lee JH, Heo MS, Yeo IK, Kang KA, Hyun JW, Jeon YJ. 2006. Protective effect of Ecklonia cava enzymatic extracts on hydrogen peroxide-induced cell damage. Process Biochem. 41: 2393-2401.

Kitzberger CSG, Smania Jr A, Pedrosa RC, Ferreira SRS. 2007. Antioxidant and antimicrobial activities of shiitake (Lentinula edodes) extracts obtained by organic solvents and supercritical fluids. J. Food Eng. 80: 631-638.

Ko KC, Kim CH, Lee NH, Lee SP, Moon DK. 2000. Determination of ß-cryptoxanthin in peel and flesh of citrus fruits produced in Cheju Island. Food Sc. Biotechnol. 9: 288-291.

Larrauri JA, Ruperez P, Saura CF. 1997. Effect of drying temperature on the stability of polyphenols and antioxidant activity of red grape pomace peels. J. Agric. Food Chem. 45: 1390-1393.

Lebel CP, Ischiropoulos H, Bondy SC. 1992. Evaluation of the probe 2', 7'-dichlorofluorescin as an indicator and oxidative stress. Chem. Res. Toxicol. 5: 227-231.

Lee KW, Kim YJ, Lee HJ, Lee CY. 2003. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. J. Agric. Food Chem. 51: 7292-7295.

Lee SC, Kim JH, Jeong SM, Kim DR, Ha JU, Nam KC, Ahn DU. 2003. Effect of far-infrared radiation on the antioxidant activity of rice hulls. J. Agric. Food Chem. 51: 4400-4403.

Lin YJ, Tsai JS, Tsay, Lin JK. 2003. Factors affecting the levels of tea polyphenols and caffeine in tea leaves. J.Agric. Food Chem. 51: 1864-1873.

Lis-Balchim M, Deans SG. 1997. Bioactivity of selected plant essential oils against Listeria monocytogenes. J. Appl. Bacteriol. 82: 759-762.

Lizard G, Fournel S, Genestier L, Dhedin N, Chaput C, Flacher M. 1995. Kinetics of plasma membrane and mitochondrial alterations in the cells undergoing apoptosis. Cytometry 21: 275-283.

Liu RH. 2004. Potential synergy of phytochemicals in cancer prevention: Mechanism of action. J. Nut. 134: 479-3485.

Macheix JJ, Fleuriet A, Billot J. 1990. The main phenolics of fruits, *Fruit phenolics*, CRC Press, Boca Raton, FL. Pp 1-103.

Mailly F, Marin P, Israel M, Glowinski J, Premont J. 1999. Increase in external glutamate and NMDA receptor activation contribute to H_2O_2-induced neuronal apoptosis. J. Neurochem. 73: 1181-1188.

Mandalari G, Bennett RN, Bisignano G, Saija A, Dugo G, Curto RBL. 2007. Characterization of flavonoids and pectins from bergamot peel, a major byproduct of essential oil extraction. J. agric. Food Chem. 54: 197-203.

Manson RP, 1982. Free radical intermediates in the metabolism of toxic chemicals. In Free radicals in Biology, Pryor, WA., Ed.; Academic Press: New York, 1982; Vol5, pp 161-222.

Manthley JA. 2004. Fractionation of orange peel phenols in ultra filtered molasses and mass balance studies of their antioxidant levels. J. Agric. Food Chem. 52: 7586-7592.

Manthley JA, Grohmann K. 1996. Concentrations of hesperidin and other orange peel flavonoids in citrus processing byproducts. J. Agric. Food Chem. 44: 811-814.

Manthley JA, Grohmann K. 2001. Phenols in citrus peel byproducts. Concentrations of hydroxycinnamates and polymethoxylated flavones in citrus peel molasses. J Agric. Food Chem. 49: 3268-3273.

Manthey JA, Grohmann K, Montanari A, Ash K, Manthey CL. 1999. Polymethoxylated flavones derived from citrus suppress tumor necrosis factor-α expression by human monocytes. J. Natl. Pro. 62: 441-444.

Masamura A, Sado H, Honda T, Shimizu M, Nabethani H, Hakajima M, watanabe A. 1988. Drying of potato by far-infrared radiation. Nippon Shokuhin Kogyo Gakkaoshi 33: 309-314.

Mead PS, Slutsker L, Dietz V, McCaig JS, Bresee C Shapiro C. 1999. Food-related illness and death in the United States. Emerging Infectious Diseases 5: 607-625.

Meena MR, Sethi V. 1994. Antimicrobial activity of essential oils from spices. J. Food Sci. Technol. 31: 68-70.

Mellou F, Lazari D, Skaltsa H, Tselepis AD, Kolisis FN., Stamatis H. 2005. Biocatalytic preparation of acylated derivastives of flavonoid glycosides enhances their antioxidant and antimicrobial activity. J. Biotechnol. 116: 295-304.

Meyer ND, Haemers A, Mishra L, Pandey HK, Pieters JAC, Vanden DA, Berghe. 1991 .4′-Hydroxy-3-methoxyflavones with potent antipicornavirus activity. J. Med. Chem. 34: 736-746.

Middleton E, Kandaswami C, Theoharides T. 2000. The effects of plant flavonoids on mammalian cells: Implications for inflammation, heart disease, and cancer. Pharmaco. Rev. 52: 673-751.

Miyake Y, Yamamoto K, Morimitsu Y, Osawa T. 1998. Characteristics of antioxidative flavonoid glycosides in lemon fruit. Food Sci. Technol. Intl. 4: 48-53.

Molyneux P. 2004. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J. Sci. Technol. 26: 211-219.

Mouly P, Arzouyan CR, Gaydou EM, Estienne JM. 1995. Chromatographie des flavanonosides des jus de différentes variétes de pamplemousses. Différenciation par analyses statistiques multidimensionnelles (Differentiation of various grapefruit and pummelo juice varieties using liquid chromatography of flavanone glycosides and pattern recognition techniques). Analusis 23 : 336-341.

Mouly P, Arzouyan CR, Gaydou EM, Estienne JM. 1994. Differentiation of citrus juices by factorial discriminant analysis using liquid chromatography of flavanone glycosides. J. Agric. Food Chem. 42: 70-79.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Mtds. 65: 55-63.

Mouly P, Gaydou EM, Auffray A. 1998. Simultaneous separation of flavanone glycosides and polymethoxylated flavones in citrus juices using liquid chromatography. J. Chromato. A 800: 171-179.

Moure A, Cruz JM., Franco D, Dominguez JM, Sineiro J, Dominguez H, Nunez MJ, Parajo JC. 2001. Natural antioxidants from residual sources. Food Chem. 72: 145-171.

Muller HE. 1995. Detection of hydrogen peroxide produced by microorganism on ABTS peroxidase medium. *Zentralbl Bakterio*. Mikrobio. Hyg. 259: 151-158.

Nakayama T, Yamaden M, Osawa T, Kawakishi S. 1993. Suppression of active oxygen-induced cytotoxicity by flavonoids. Biochem. Pharma. 45: 265-267.

Nakayama T. 1994. Suppression of hydroxyperoxide-induced cytotoxicity by polyphenols. Cancer Res. 54: 1991-1993.

Nam TS, Lee SP, Kim CS. 2002. Determination of ß-cryptoxanthin in peel and flesh of domestic and foreign citrus fruits. Food Sci. Biotechnol. 11: 628-633.

Nanjo F, Goto K, Seto R, Suzuki H, Sakai M, Hara Y. 1996. Scavenging effects of tea catechins and their derivatives on 1, 1-diphenyl-2-picrylhydrazyl radical. Free radical Biol. Med. 21: 885-902.

Ngo DN, Kim MM, Kim SK. 2008. Chitin oligosaccharides inhibit oxidative stress in live cells. Carbo. Poly. 74: 228-234.

Nikaido H, Vaara M. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 1: 1-32.

Nindo CI, Kudo Y, Bekki E. 1995. Test model for studying sun drying of rough rice using far-infrared radiation. Drying Technol. Intl. J. 13: 225-238.

Niwa Y, Miyachi Y. 1986. antioxidant action of natural health products and chineese herbs. Inflam. 10: 79-91.

Nuengchamnong N, de Jong C.F, Bruyneel B, Niessen WMA, Irth H, Ingkaninan K. 2005. HPLC coupled on-line to ESI-MS and a DPPH-based assay for the rapid identification of antioxidant in Butea superba. Phytochem. Anal. 16: 422-428.

Nychas GJE. 1995. Natural antimicrobials from plants. In: G.W. Gould, Editor, *New methods of food preservation*, Blackie Academic and Professional, London, 58-89.

Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95: 351-358.

Ooghe WC and Detavernier CM. 1997. Detection of the addition of Citrus reticulata and hybrids to *Citrus sinensis* by flavonoids. J. Agric. Food Chem. 45: 1633-1637.

Ortuno A, Baidez A, Gomez P, Arcas MC, Porras I, Garcia-Lidon A, Del Rio JA. 2006. *Citrus paradisi* and *Citrus sinensis* Flavonoids: Their influence in the defence mechanism against *Penicillium digitatum*. Food Chem.. 98: 351-358.

Osawa T, Namiki M. 1985. Natural antioxidants isolated from eucalyptus leaf waxes. J. Agric. Food Chem. 33: 777-780.

Parejo I, Bastida J, Viladomat F, Codina C. 2005. Acylated quercetagetin glycosides with antioxidant activity from Tagetes maxima. Phytochem. 66: 2356 2362.

Park GL, Avery SM, Byers JL, Nelson DB. 1983 Identification of bioflavonoids from citrus. Food Technol. 37: 98-105.

Patrick R. 1996. Antimicrobial agents and susceptibility testing. Washington, DC: Murray.

Paulson DS. 1999. Tropical antimicrobial testing and evaluation. New York, NY: Marcel Dekker, Inc.

Peterson JMS, Dwyer J, Dsc RD. 1998. Flavanoids: Dietary occurance and biochemical activity. Nutr. Res. 18: 1995-2018.

Perez-Bonilla M, Salido S, Van Beek TA, Linares-Palomino PJ, Altarejos J, Nogueras M, Sanchez A. 2006. Isolation and identification of radical scavengers in olive tree (*Olea europaea*) wood. J. Chromato. A 1112:311-318.

Pincemail J, Deby C, Lion Y, Braguet P, Hans P, Drien K. 1986. Role of flavonoids in lipoperoxidation and radicalar reactions. In L. Farkas, M. Gabor, & F. Kallay (Eds.), *Flavonoids and bioflavonoids. Proceedings of the VIIth Hungarian bioflavonoid symposium. Szeged, Hundary* (pp. 423-436). New York: Elsevier Science.

Puupponen-Pimiä R, Nohynek L, Meier C, Kähkönen M, Heinonen M, Hopia A, Oksman-Caldentey KM. 2001. Antimicrobial properties of phenolic compounds in berries. J. Appl. Microbiol. 90: 494-507.

Puupponen-Pimiä R, Nohynek L, Alakomi HL, Oksman-Caldentey KM. 2006. The action of berry phenolics against human intestinal pathogens. Biofactors 23: 243-251.

Rehman ZU. 2006. Citrus peel extract: A natural source of antioxidant. Food Chem. 99: 450- 454.

Rice Evans C, Miller NJ, Paganga G. 1996. Structure-Antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biol. Med. 20: 933-956.

Rice-Evans C, Miller N, Paganga G. 1997. Antioxidant properties of phenolic compounds. Trends Plant Sci. 2: 152-159.

Rico-Munoz E, Bargiota EE, Davidson PM. 1987. Effect of selected phenolic compounds on the membrane-bound adenosine triphosphatase of *Staphylococcus aureus*. Food Microbiol. 4: 239-249.

Rio JAD, Fuster MD, Gomez P, Porras I, Lidon AG, Ortuno A. 2004. *Citrus limon*: a source of flavonoids of pharmaceutical interest. Food Chem. 84: 457-461.

Ros JM, Schols HA, Voragen AGJ. 1996. Extraction, characterization and enzymatic degradation of lemon peel pectins. Carbo. Res. 282: 271-284.

Rosen GM, Rauckman EJ. 1980. Spin trapping of the primary radical involved in the activation of the carcinogen N-hydroxy-2-acetylaminofluorene by cumene hydroperoxide hematin. Molec. Phamacol. 17: 233-238.

Rota C, Carramiñana JJ, Burillo J, Herrera A. 2004. In vitro antimicrobial activity of essential oils from aromatic plants against selected food borne pathogens. J. Food Protection 67: 1252-1256.

Sandu C. 1986. Infrared radiative drying in food engineering: a process analysis. Biotechnol. Progress 2: 109-119.

Sankaranarayanan K. 1991. Ionizing radiation and genetic risks. Nature of spontaneous and radiation-induced mutations in mammalian in vitro systems and mechanisms of induction of mutations by radiation. Mut. Res. 258: 75-97.

Sasaki S, Ohta T, Decker EA. 1996. Antioxidant activity of water-soluble fractions of salmon spermary tissue. J. Agric. Food Chem. 44:1682-1686.

Scherrer R, Gerhardt P. 1971. Molecular sieving by the Bacillus megaterium cell wall and protoplast. J. Bacteriol. 107:718-735.

Sherwin ER. 1978. Oxidation and antioxidants in fat and oil processing. J. American Oil Chem. Soc. 55: 809-814.

Sichel G, Corsaro C, Scalia M, Di Bilio AJ, Bonomo R. 1991. In vitro scavenger activity of some flavonoids and melanins against $O_2^{\cdot -}$. Free Rad. Biol. Med. 11: 1- 8.

Shimada KK, Fujikawa KY, Nakamura T. 1992. Antioxidative properties of xanthan on autoxidation of soybean oil in cyclodextrin. J. Agric. Food Chem. 40: 945-948.

Shin JS, Kang KA, Kim ES, Zhang R, Piao MJ, Ko DO, Wang ZH, Maeng YH, Eun SY, Chae SW, Chung HS, Hyun JW. 2008. Cytoprotective activity of annphenone against oxidative stress-induced apoptosis in V79-4 lung fibroblast cells. Cell Biol. Intl. 32: 1099-1107.

Silva BA, Ferreres F, Malva JO, Dias ACP. 2005. Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts. Food Chem. 90: 157-167.

Singh NP, McCoy MT, Tice RR, Schneider EL. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175: 184-191.

Singh RP, Chidamabara-Murthy KN, Jayaprakasha GK. 2002. Antioxidant activity of pomegranate (*Punica garanatum*) peel and seed extracts using in vitro models. J. Agric. Food Chem. 50: 81-86.

Slupphaug G, Kavli B, Krokan HE. 2003. The interacting pathways for prevention and repair of oxidative DNA damage. Mutat. Res-Fund Mol. Mech. Mutag. 531: 231-251.

Sorata Y, Takahama U, Kimura M. 1984. Protective effect of quercetin and rutin on photosensitized lysis of human erythrocytes in the precence of hematoporphyrin. Biochem. Biophys. Acta. 799: 313-317.

Stadman ER, Bertlett BS. 1998. Reactive oxygen-mediated protein oxidation in aging and disease. Drug Metab. Rev. 30: 225-243.

Stewart AJ, Mullen W, Crozier A. 2005. A on-line high performance liquid chromatography analysis of the antioxidant activity of phenolic compounds in green and black tea. Mol. Nut. Food Res. 49:52-60.

Tsao R, Deng Z. 2004. Separation procedures for naturally occurring antioxidant phytochemicals. J. Chromato. B 812: 85-99.

Tombola F, Campello S, De Luca L, Ruggiero P, Del Giudice G, Papini E, Zoratti M. 2003. Plant polyphenols inhibit VacA, a toxin secreted by the gastric pathogen *Helicobacter pylori*. FEBS Lett 543: 184-189.

Torel J, Cillard J, Cillard P. 1986. Antioxidant activity of flavonoids and reactivity with peroxy radical. Phytochem. 2: 383-385.

Tournaire C, Croux S, Maurette MT, Beck Y, Hocquaux M, Braun, AM, Oliveros E. 1993. antioxidant activity of flavonoids. Efficiency of singlet oxygen quenching. J. Photochem. Photobiol. B. 19: 205-215.

Ultee A, Bennik MHJ, Moezelaar R. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. Appl. Envir. Microbiol. 68: 1561-1568.

Volterra A, Trotti D, Tromba C, Floridi S. Racagni G. 1994. Glutamate uptake inhibition by oxygen free radicals in rat cortical astrocytes. J. Neurosci. 14: 2924-2932.

Vuorela S, Kreander K, Karonen M, Nieminen R, Hämäläinen M, Galkin A, Laitinen L, Salminen. 2005. Preclinical evaluation of rapeseed, raspberry, and pine bark phenolics for health related effects. J. Agric. Food Chem. 53: 5922-5931.

Wyllie AH, Liehr DS. 1997. Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: An overview, Cancer Metast. Rev. 11: 95-103.

Yi ZB, Yu Y, Liang YZ, Zeng B. 2008. In vitro antioxidant and antimicrobial activities of the extract of *pericarpium citri reticulatae* of a new citrus cultivar and its main flavonoids. Food Sci. Technol. 41: 597-603.

Yusof S, Ghazali MH, King GS. 1990. Naringin content in local citrus fruits. Food Chem. 37: 113-121.

Zhuang XP, Lu YY, Yang GS. 1992. Extraction and determination of flavonoid in ginkgo. Chineese Herb. Med. 23: 122-124.

ACKNOWLEDGEMENT

I am extremely grateful to my supervisor, prof. Soo-Hyun Kim and my co-supervisor prof. You-Jin Jeon for their continual advice, guidance and support. Their extensive comments and helpful discussions cause a direct impact on the final form of this thesis.

Also, I wish to offer my humble gratitude to all the staff members of the Department of Food Bioengineering, Prof. Dae-Jin Song, Prof. Yeung-Joo Kang, Prof. Jin-Hwan Ha, Prof. Young-Hwan Ko, Prof. Sang-Bin Lim and Prof. Kwang-Pyo Kim for teaching, kind support and advices. My special thanks go the Prof. Nak-Ju Sung from the Department of Food Science and Nutrition, Gyeongsang National University for being a committee member in my thesis defence. Further, I would like to extend my sincere gratitude to Prof. Sang-Chul Chung, Prof. Ki-Wan Lee, Prof. Myung-Cheol Oh, Prof. Chang-Kyung Oh and Dr. Albert Byung-Hun Um for their kind support and encouragement.

Special thanks go to Dr. Nalin Siriwardhana for guiding me to study in Korea and for giving much encouragement and invaluable support.

Many thanks to Yeong-Chan Kang, Yeon-Doek Kim, Yong-Seok Ahn, So-Kium Lee, Ho-Jung Kang, Jung-Lim Ko, Min-Ho Yang, Juo-Yoeng Lee, Yang-Jung Hwan, Tae-Hwan Kang, Jae-Sung Park, Tai-Hyun Kim, Shin-Woo Seok, Kim-Mi Bo and Hea-Won Lee of the Department of Food Science and Bioengineering for giving me the numerous support in my studies. Thank you very much Dr. Soo-Jin Heo, Kil-Nam Kim, Seon-Heui Cha, Ahn-Gin-Nae, Ji-Hyeok Lee, Seung-Hong Lee, Sung-Myeong Kang, Seok-Chun Ko, Areum-Daseul Kim, Chul-Hong Oh, Dr. Hyun-Sil Kang, Ho-Jin Park, of the Department of Marine Biotechnology for your support and kindness.

Also, I greatly acknowledge all my Sri Lankan friends Dr. Rohan, Dr. Prashani, Dr. Yasantha, Dr. Chamilani, Mahanama, Thushari, Nadun, and Herath for giving me great support and valuable suggestions during my study.

A huge thank-you to my parents, sisters and parents-in-law and brothers-in law for their enduring support and encouragement, and also to my dear wife Priyantha for her help and remarkable patience that has brought this study to its close.

I can't forget all my foreign friends, who are studying in CNU and those who have completed studies and left CNU, for their support during my study in CNU.

At last but not least I would like to thank all those whom I have not mentioned above but helped me in numerous ways to success my studies in CNU.