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Antioxidant Activities of *Chrysanthemum coronarium* L. Fractions on the Liposomal Phospholipid Membrane

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Abstract

This experiment was designed to investigate the antioxidant effects of *Chrysanthemum coronarium* L. (CC) fractions on the liposomal phospholipid membranes. The sample CC was extracted and fractionated into five different types, methanol (CCMM), hexane (CCMH), ethylacetate (CCMEA), butanol (CCMB), and aqueous (CCMA) fractions. The antioxidant activities of CC fractions in oxidized dilinoleoylphosphatidylcholine (DLPC) liposomes were examined by spectrophotometry measuring conjugated dienes. The oxidation indices of five CC fractions exhibited weaker antioxidant activities than that of BHT in oxidized DLPC liposomes, however, showing much similar antioxidant activities of α-tocopherol in the oxidized DLPC liposomes, which is known as a potent antioxidant. Among CC fractions, CCMM and CCMA in oxidized DLPC liposomes showed rather effective than α-tocopherol after 2 h. These results strongly indicate that bioactive substances in CC fractions have a kind of function as potent antioxidants against biomembrane oxidation.

Key words – *Chrysanthemum coronarium* L., antioxidant activity, DLPC, liposome

Introduction

Chrysanthemum coronarium L. (CC), namely crown daisy which is one of the *compositae* plants, has been widely cultivated in Korea as a vegetable for years. It originated in the Mediterranean Sea, and came to Korea through China long time ago. This has a favorable scent to use as an appetizer for dishes, helps intestinal functions to use for constipation as well [9,10]. The composition compounds of CC consist of water (93.5 g%), protein (2.5 g%), lipid (0.4 g%), carbohydrate (3.1 g%), calcium (74 mg%), phosphorus (29 mg%), iron (4.2 mg%), vit.A (4950 IU), thiamine (0.15 mg%), riboflavin (0.30 mg%), and vit.C (45 mg%) [22]. Especially, it is rich in mineral and vitamin compared to other vegetables [10,22].

Recently, in Asian countries, various researchers have studied for the screening of bioactive substances from natural products or food materials such as *Artemisia iwayomogi* (Mugwort) [15], *Allium cepa* L. (Onion) [13], *Ixeris sonchifolia* H. (Godulbaegi) [1], and *Angelica radix* (Danggui) [8]. These studies suggest that the natural products applied in the experiments have similar effects as drug materials having therapeutic efficacy of cancer. In fact, there is extensive evidence suggesting the protective role of fruits and vegetables against chemical induced carcinogenesis. According to Kim *et al.* [12], quinone reductase-inducing activity was found mostly in hexane and ethylacetate fractions of MeOH extract of CC while it was not detected in n-butanol and water fractions, confirming the presence of potent QR inducer(s) in CC. Including the report, there are several studies of

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Chrysanthemum coronarium L. (CC) [11,9,10,23], however, few is known about the interaction of CC with liposomal phospholipid bilayers.

It is well recognized that biological membranes are vulnerable to peroxidation reactions. Antioxidants are of continuing interest for their ability to protect the unsaturated lipids of biological membranes from oxidative damage, and thus have been the subject of many scientific papers and review [2,3,14,16,17]. Without protection, such oxidative damage can lead to tissue damage and various pathological events [18,21]. That is, membrane oxidation causes damage to the membrane fluidity, and it brings further destruction of the sustenance of biological homeostasis.

Model membrane systems are commonly used to study antioxidant behavior preliminary to monitoring their behavior in a native membrane, where the protein and the variety of lipid types in the membrane have complicate composition for the study. Uninhibited autoxidation of phospholipid membranes follows the free radical mechanism presented in Fig. 1 Lipid peroxidation is initiated by free radicals originating mainly from oxygen and oxygen-derived species. When a phenolic antioxidant is added to a system undergoing autoxidation, the antioxidant molecules (AH) can trap peroxyl radicals (LOO ·) formed during the propagation step.

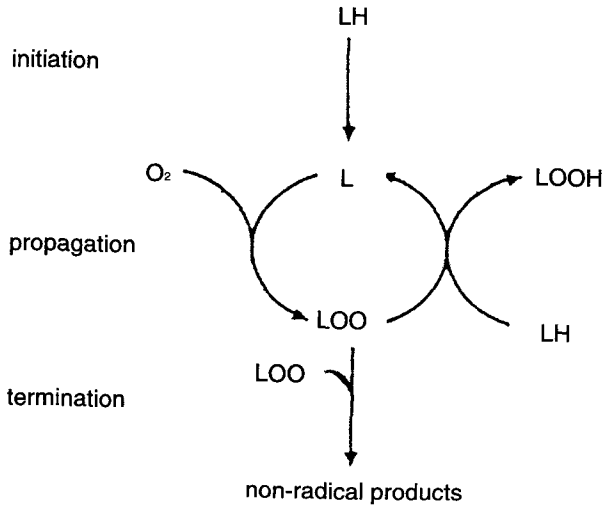

Fig. 1. General scheme for the oxidation of lipids (LH) by a free radical chain mechanism.

$$LOO\cdot + AH \rightarrow LOOH + A\cdot$$

$$LOO\cdot + A\cdot \rightarrow LOOA$$

Biological membranes are mainly protected against lipid peroxidation by the fat-soluble antioxidant vitamin E. Numerous other food constituents such as carotenes [20], flavonoids [19], phenoles [4], and quinones [5] may also protect against lipid peroxidation. In the previous report, [12] the finding of the presence of potent QR inducer in CC seems to be associated with putative antioxidant properties of the CC. It would be necessary to examine the antioxidant activity of *Chrysanthemum coronarium* L. (CC) in biological membrane peroxidation.

Phospholipids have a UV absorbance peak at a very short wavelength (200-205 nm). The presence of conjugated diene in oxidized lipid is indicated by the appearance of a peak at 234 nm.

The methods most frequently used to assess the extent of lipid oxidation are based on the spectroscopic measurement of conjugated diene formation [9]. The measurement of conjugated diene value is one of the simple method used for detecting lipid oxidation, showing absorbance peak at 234 nm.

Materials and Methods

Materials and reagents

Chrysanthemum coronarium L. (CC) was purchased from a local market in Pusan, Korea. L-α-dilinoleoylphosphatidylcholine (DLPC), α-tocopherol, and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and benzoyl peroxide from Polysciences Inc. (Warrington, PA), and these were used without further purification. All other chemicals were of reagent grade.

Extraction and fractionation of *Chrysanthemum coronarium* L.

Dried and chopped *Chrysanthemum coronarium* L. (CC)

was extracted three times with methanol (1.5). The methanol (MeOH) was removed by the evaporation under reduced pressure, and the resulting insoluble material was filtered off. The MeOH extract was fractionated into n-hexane (CCMH), methanol (CCMM), ethylacetate (CCMEA), butanol (CCMB), and aqueous (CCMA) layers. Each partition layer was evaporated, and freeze-dried for the samples (Fig. 2).

Preparation of multilamellar liposomes (MLVs)

Appropriate amounts of stock solutions of DLPC in methanol and CC fractions in methanol/chloroform (1:1) were mixed to provide the desired concentration ($\mu g\ m\ell^{-1}$) for making multilamellar liposomes (MLVs). The organic solvents were then evaporated under a stream of dry N_2 to make a thin film of the lipid, and the last traces of solvents were completely removed by a further evaporation under high vacuum for 3 hours. The dried thin film was suspended in phosphate buffered saline (PBS) at pH 7.4 by mixing on a vortex mixer for 1 min, and then left in a thermobath at a temperature above their phase transition temperature for 2 min.

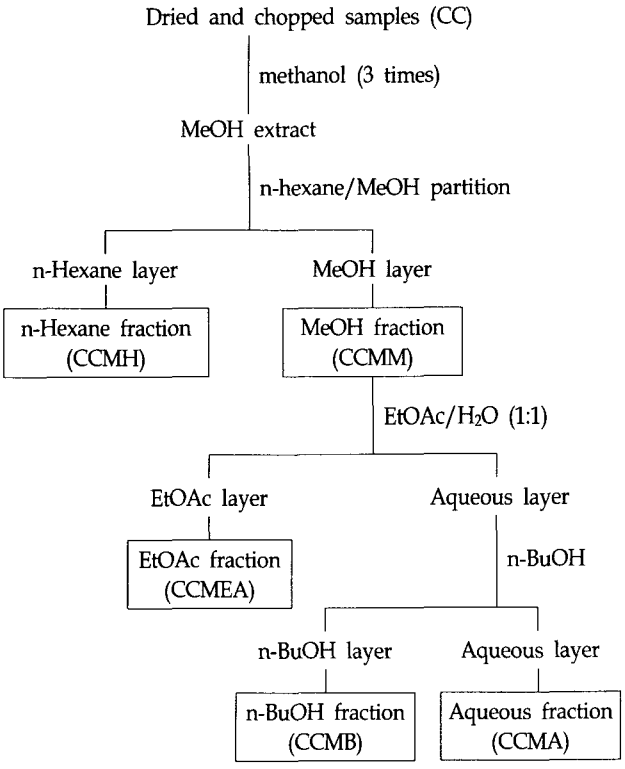

Fig. 2. Fractionation procedure of *Chrysanthemum coronarium* L.

Antioxidant activity of *Chrysanthemum coronarium* L. (CC) fractions in liposomes

1) Liposomal oxidations

Peroxidation was initiated in liposomal suspensions by addition of benzoyl peroxide and incubated at 37℃ for 4 hours. The final concentration of DLPC, antioxidants, and oxidant was 10 $\mu g\ m\ell^{-1}$, 2 $\mu g\ m\ell^{-1}$, and 7 $\mu g\ m\ell^{-1}$, respectively.

2) Measurement of conjugated dienes

The major products formed from linoleate oxidation are conjugated hydroperoxides formed by oxygen addition at the 9 and 13 positions of the carbon chain, 9- and 13-hydroperoxides in the trans-cis and trans-trans isomeric forms (Fig. 3). Changes in the UV absorbance of lipids are the first sign of the occurrence of radical chain reactions which can lead to oxidation. Phospholipids have a UV absorbance peak at a very short wavelength (200-205 nm). The presence of conjugated diene in oxidized lipid is indicated by the appearance of a peak at 234 nm. In this study, DLPC had a UV absorbance peak at 202 nm, so its oxidation index was calculated by the following equation [10], at different times 0, 1, 2, 3 and 4 h during incubation.

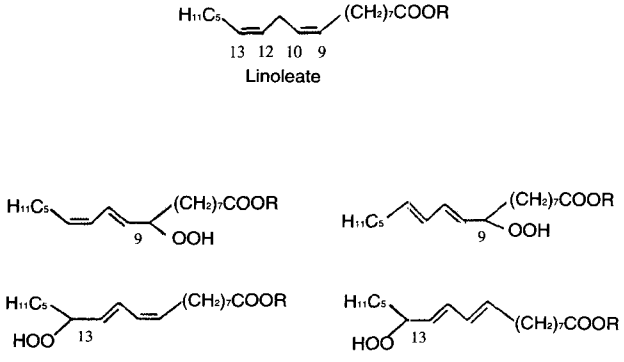

Fig. 3. Geometrical hydroperoxide isomers in linoleate oxidation.

Oxidation Index = $A_{234\ nm}$ / $A_{202\ nm}$

This measurement was carried out with a Uvikon 860 spectrophotometer (USA) and a KI-310 incubator (Kum Hwa Industrial Co., Korea).

Results and Discussion

Fig. 4 is depicting the representative UV spectra of oxidized dilinoleoylphosphatidylcholine (DLPC) liposomes. Non-oxidized DLPC had a UV absorbance peak at a very short wavelength at 202 nm. As the lipid oxidation was initiated by 7 $\mu g\ m\ell^{-1}$ benzoyl peroxide, the appearance of conjugated dienes showed an increase of absorption at 234 nm. 10 $\mu g\ m\ell^{-1}$ DLPC liposome alone was heavily oxidized by benzoyl peroxide. Addition of 2 $\mu g\ m\ell^{-1}$ CCMM and BHT exhibited spectra of moderately and lightly peroxidized DLPC liposomes.

The inhibitory effect of CC fractions on the peroxidation of DLPC liposomal bilayers induced by benzoyl peroxide is shown in Fig. 5. DLPC liposome alone was rapidly oxidized by the addition of benzoyl peroxide, so its oxidation index reached about 0.37 up to 0.6 at 1 h and around 0.8 at 4 h. The peroxidation of DLPC was inhibited by the addition of CC fractions. Five CC fractions incorporated into DLPC liposomes decreased the oxidation index of the peroxidized phospholipids. Their inhibitory effect on the liposomal oxidations was very similar, being difficult to discriminate which of the five CC fractions was considerably effective in the peroxidation systems.

The antioxidant activity of CC fractions in oxidized DLPC liposomes was compared with two well-known antioxidants, α-tocopherol and BHT. Representatively, the antioxidant activity of CCMEA and CCMA compared

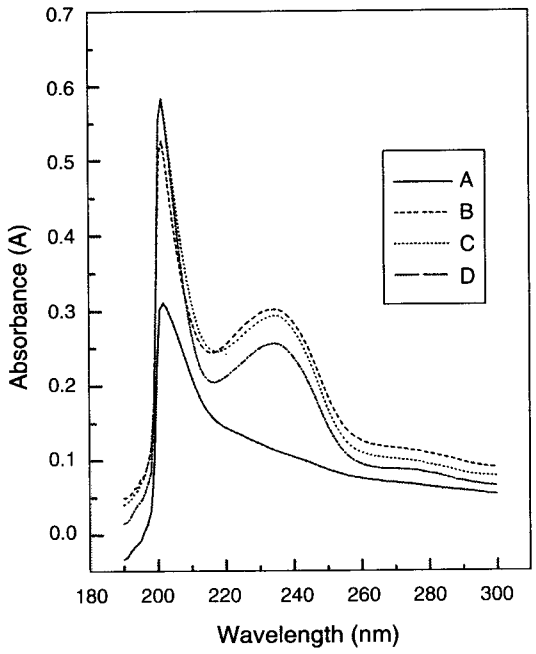

Fig. 4. The representative UV spectra of the DLPC iposomes (A), the oxidized DLPC liposomes (B), and the oxidized liposomes incorporated with CCMM (C) and BHT (D).

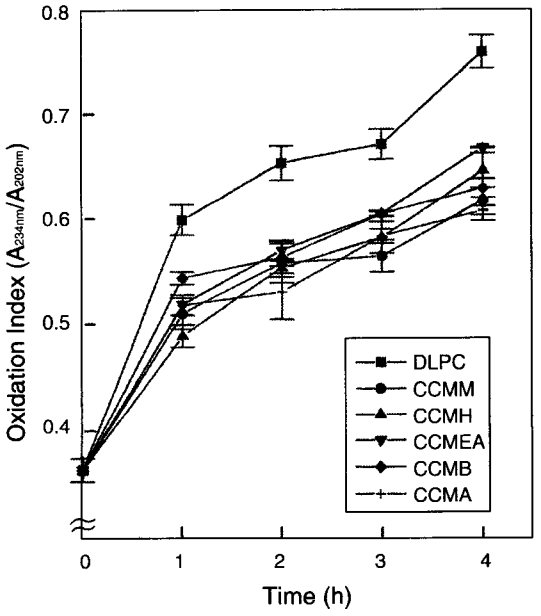

Fig. 5. The antioxidant effects of CCMM, CCMH, CCMEA, CCMB and CCMA on the benzoyl peroxide catalyzed DLPC liposomes. Reported values are means ±S.D.(n=3)
CCMM; methanol fraction of CC
CCMH; hexane fraction of CC
CCMEA; ethylacetate fraction of CC
CCMB; buthanol fraction of CC
CCMA; aquous fraction of CC

with α-tocopherol and BHT are shown in Fig. 6 and 7. BHT exhibited the strongest antioxidant activity in the

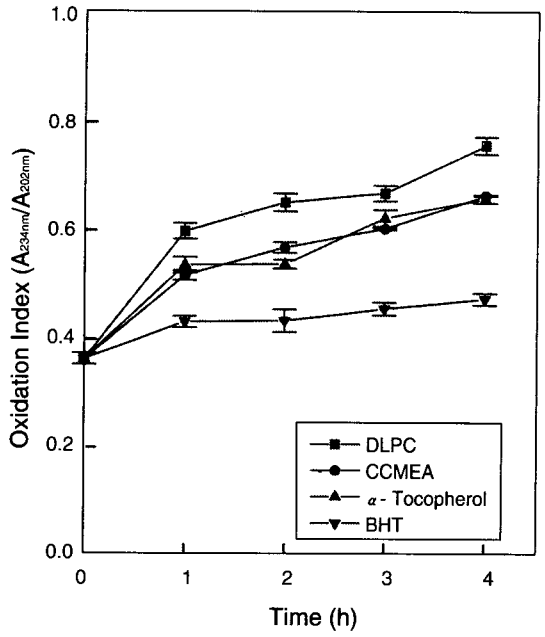

Fig. 6. The antioxidant effects of CCMEA, a-tocopherol and BHT on the benzoyl peroxide-catalyzed DLPC liposomes. Reported values are means±S.D.(n=3)

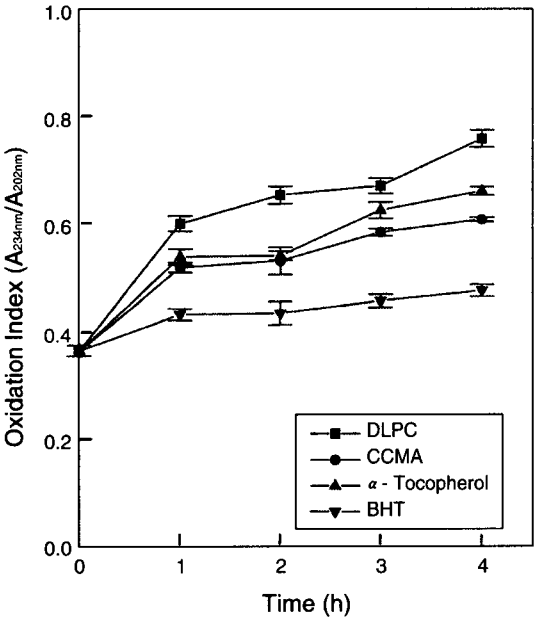

Fig. 7. The antioxidant effects of CCMA, a-tocopherol and BHT on the benzoyl-peroxide catalyzed DLPC liposomes. Reported values are means±S.D.(n=3)

oxidized liposomes ; its oxidation index showed only an increase of 0.5 after an incubation during 4 h at 37℃. α-tocopherol in the systems protected the oxidation of DLPC liposomes initiated by benzoyl peroxide, but being much less effective than BHT. The inhibitory effects of CC fractions on the lipid peroxidations could be significantly comparable with the effect of α-tocopherol on the oxidized DLPC liposomes. The antioxidant activities of the five CC fractions in the oxidized DLPC liposomes were much similar to the activity of α-tocopherol in the oxidized DLPC liposomes, which has known as a potent antioxidant. However, CCMM and CCMA of the CC fractions in oxidized DLPC liposomes showed rather effective than α-tocopherol after 2 h. The results might explain that certain substances in CC fractions have a kind of biological function as potent antioxidants in oxidized biomembranes.

Antioxidants such as BHT and α-tocopherol exhibit their antioxidant activity by rapid donation of hydrogen atoms to lipid radicals to break the chain reaction of lipid. After lipid peroxidation of liposomes, hydroperoxides present in the phospholipids will increase the degree of disorder in the bilayer and affect the permeability and fluidity behavior of the vesicles. The aspect of a change in membrane permeability of the liposomes may be of importance especially when these vesicles are meant to be used as carriers for drugs or other substances. From all of these results, more studies should be done to elucidate the antioxidative effect of CC fractions in oxidized liposomes, specially the relationship between the antioxidant activity and the location of CC fractions in the bilayer.

Conclusions

Biological membranes are vulnerable to peroxidation reactions. Membrane oxidation causes damage to the membrane fluidity and even destruction of the sustenance of biological homeostasis. The peroxidation of DLPC liposomes initiated by benzoyl peroxide was inhibited by

five *Chrysanthemum coronarium* L. (CC) fractions, α-tocopherol, and BHT. All CC fractions on the peroxidation of DLPC bilayers exhibited weaker antioxidant activity than BHT in oxidized DLPC liposomes, but showing the potent antioxidant activity of α-tocopherol in the oxidized DLPC lposomes which has known as a potent antioxidant. Therefore, based on these results, we strongly suggest that *Chrysanthemum coronarium* L. fractions may be developed into a potentially useful antioxidant agent among the other food materials.

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초록 : 인지질막 liposome에 미치는 쑥갓 분획물의 항산화 효과

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쑥갓(*Chrysanthemum coronarium* L., CC)은 국화과(Compositae)에 속하는 일년생 초본식물로 독특한 향기를 가진 지중해 원산 식물이다. 우리 나라에는 중국을 거쳐 조선초기에 전해졌으며 시기용으로 널리 애용되고 있다.

본 연구는 쑥갓의 메타놀 분획물(CCMM), 헥산분획물(CCMH), 에칠아세테이트 분획물(CCMEA), 부타놀 분획물 (CCMB) 및 수층인 CCMA 분획물을 얻고 각각 일정량을 불포화인지질인 L-α-dilinoleoylphosphatidylcholine(DLPC) liposome에 가하고 benzoyl peroxide를 가하여 산화시켜 분광광도법에 의하여 산화지수(A_{234nm} / A_{202nm})를 측정 비교하였다. 그 결과 쑥갓의 각 분획물 일정량을 가한 경우의 DLPC lipcsome의 산화지수는 시료를 가하지 않은 경우보다 낮아 높은 항산화력을 나타내었으나 강한 항산화제인 BHT를 가한 경우보다는 항산화 정도가 낮았다. 그러나 특히 본 연구에서 알려진 결과로는 쑥갓 분획물들의 항산화력이 널리 알려진 항산화제인 α-tocopherol을 가한 경우와 그 효과가 비슷하여 식용으로 애용되고 있는 쑥갓의 항산화적 생리활성이 본 연구에서 입증되었으며 이 효과를 기초로하여 식물 속의 항산화성을 이용한 식품의 개발이 기대된다.