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In Vitro Free Radical Scavenging Activities of Dietary Supplements by Electron Spin Resonance

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Abstract

The scavenging effects of 10 dietary supplements including histidine dipeptide family (Carnosine = Car, Anserine = Ans, whale meat extract = WME containing 8.6% balenine = Bal), Twendee X (TwX), Supaliv (SuP), Twendee Mtcontrol (TwM), vitamin E family (α–tocopherol, Tocovid = Toco), and scallop and ascidian (Hoya) – derived plasmalogens (Plas) on methyl radical ($\cdot CH_3$), hydroxyl radical ($\cdot OH$), and superoxide anion (O_2^-) were examined using electron spin resonance (ESR) method. Car, Ans, and WME significantly suppressed $\cdot CH_3$, $\cdot OH$ and O_2^- in a dose-dependent manner. Compared with Car, Ans and WME have higher abilities of scavenging $\cdot CH_3$ and $\cdot OH$ in higher doses of 1000 mg/l and 10000 mg/l. TwM, TwX, and SuP significantly suppressed above three radicals. TwM and TwX have higher abilities of scavenging $\cdot OH$ and O_2^- in lower doses of 20 mg/l and 200 mg/l. Additionally, compared with α-tocopherol, a super mixed vitamin E, Toco has higher abilities of scavenging above 3 radicals. There is no big difference found between the same concentrations of scallop and Hoya-derived Plas in terms of scavenging above 3 radicals, although both have significant scavenging ability. These findings suggest that the intake of multiple dietary supplements is an alternative and promising way of scavenging various free radicals in order to provide us a healthier life.

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Abbreviations used: AD, Alzheimer's disease; Ans, Anserine; AsA, ascorbic acid; Bal, Balenine; Car, carnosine; CCH, chronic cerebral hypoperfusion; $\cdot CH_3$, methyl radical; DHA, docosahexaenoic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DMSO, dimethyl sulfoxide; EDA, edaravone; EPA, eicosapntemacnioc acid; ESR, electron spin resonance; H_2O_2, hydrogen peroxide; NaOH, sodium hydroxide; O_2^-, superoxide anion; $\cdot OH$, hydroxyl radical; Plas, plasmalogen; SuP, Supaliv; Toco, Tocovid; TwM, Twendee Mtcontrol; TwX, Twendee X; WME, whale meat extract.

Introduction

Free radicals play a pivotal role in neurological diseases such as stroke (Abe et al., 1988; Yamashita et al., 2009; Abe et al., 2017) and neurodegenerative diseases (Halliwell B, 2001). In order to prevent such free radicals-related diseases, the intake of nutrients consisting of effective free radical scavenging components has been dramatically regarded (Tadokoro et al., 2020), Based on previous reports, histidine dipeptide family, especially carnosine (Car), anserine (Ans), and balenine (Bal) have shown to possess antioxidant effects in stroke models and AD models (Herculano et al., 2013; Davis et al., 2016; Kaneko et al 2017; Kawahara et al., 2020). Moreover, a novel supplement, Twendee X (TwX) composed of multiple components with known antioxidant properties was shown to have a significant neuroprotective effect in an ischemic stroke mouse model (Kusaki et al., 2017), an Alzheimer's disease (AD) with chronic cerebral hypoperfusion (CCH) mouse model (Liu et al., 2019), and clinical patients with mild cognitive impairment by its anti-oxidative effect (Tadokoro et al., 2019). In addition to histidine dipeptide family and TwX, our research group also proved that a novel mix of vitamin E vitamers, Tocovid (Toco), majorly containing tocotrienols other than tocopherol could reasonably attenuate ischemic infarction in a stoke mouse model via reducing oxidative stress (Shang et al., 2018). Recent, increasing evidences suggest that plasmalogen (Plas), a subtype of phospholipids, has a potential in novel therapeutic approaches to AD pathology via its anti-oxidative effects (Fujino et al., 2017; Hossain et al., 2017; Su et al., 2019;). Although the above functional nutrients possess neuroprotective effects via reducing oxidative stress, the specific mechanisms by which these nutrients produce their anti-oxidative stress effect is still unclear.

Electron spin resonance (ESR) is frequently applied in investigating mechanism of free radical scavenging ability of antioxidants with obvious advantages such as higher specificity and effectiveness (Mitsuta et al., 1990; Ogawa et al., 1994). The H_2O_2/NaOH/DMSO system has already been developed for the evaluation of free radical scavenging ability of both water-soluble and oil-soluble antioxidants in ESR (Yoshimura et al., 1999). Additionally, superoxide anion (O_2^-), hydroxyl radical ($\cdot OH$), and methyl free radicals ($\cdot CH_3$) can be generated at the same time by using H_2O_2/NaOH/DMSO reaction system. Therefore, ESR-measurements coupled with the H_2O_2/NaOH/DMSO radical-generating system could be regarded as an optimum method for evaluation of the above three free radicals scavenging property of antioxidants.

Accordingly, in the present study, we investigated the free radical scavenging abilities of several nutrients including histidine dipeptide family (Car, Ans, whale meat extract = WME containing 8.6% Bal), TwX, Supaliv = SuP, Twendee Mtcontrol = TwM, Toco, and scallop and ascidian (Hoya) – derived plasmalogens (Plas) using the H_2O_2/NaOH/DMSO reaction system and ESR.

Materials and Methods

Nutrients

Edaravone (Eda) was obtained from Mitsubishi Tanabe Pharmaceutical Co. Ltd. SuP, TwX, and TwM were obtained from TIMA Japan Company. Toco was obtained from Hovid Bhd Company, Malaysia. α-Tocopherol (T3251), Car (C9625) and Ans (A1131) were obtained from Sigma-Aldrich. WME mainly containing 8.6% Bal was obtained from Japanese ship-fishing cooperation. Scallop-derived Plas was obtained from Dr. Fujino, Kyushu University, and ascidian (Hoya)-derived Plas was obtained from Sunsho Pharmaceutical Co. Ltd.

Reagents

5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO, 92688), docosahexaenoic acid (DMSO, D5879), 1 mol/L sodium hydroxide solution (NaOH, 28-3010-5), 30% hydrogen peroxide solution (H_2O_2, 13-1910-5) were obtained from Sigma-Aldrich. Ultrapure distilled water (10977-015) was purchased from Thermo Fisher Scientific.

Instruments

ESR spectra were recorded on a JEOL JES-FE3XG spectrometer using a quartz flat cell designed for aqueous solution.

ESR conditions

Conditions of ESR spectrometry were: magnetic field, 336.3 (5 mT; power, 8.0 mW; modulation frequency, 100 kHz; frequency, 9.425 GHz; modulation amplitude, 0.063 mT; gain, 500; time scan, 1 min; time constant, 0.03 s.

Experimental procedure

The proper doses used in the present study were determined by our preliminary experiments. Fifty microliters of DMSO, the same volume of 25 mM NaOH, and sample solution (aqueous, ultrapure distilled water) including Eda, TwX, SuP, TwM, Car,

Ans, WME, scallop-derived Plas, and ascidian-derived Plas were mixed in a disposable plastic tube, followed by the addition of 5 ul of DMPO and 50 ul of 30% H_2O_2. In the case of oil-soluble antioxidant including Toco and α-tocopherol, the sample was dissolved in 50 ul of DMSO and 50 ul of ultrapure distilled water and the same volume of 25 mM NaOH were mixed, followed by the addition of 5 ul of DMPO and 50 ul of 30% H_2O_2.

The reaction mixture was sucked into the quartz flat cell and set in the ESR apparatus; scanning was started at 10 min after the addition of H_2O_2. The signal intensities of $\cdot CH_3$, $\cdot OH$, and O_2^- species-DMPO adducts reached plateaus between 10 and 20 min after the start of the reaction.

Generation of free radicals

The signal height was calculated using a radical analyzer program attached to the ESR instrument. The calculation was done for the positive signal height of the $\cdot CH_3$-DMPO adduct and the $\cdot OH$-DMPO adduct and for the negative signal height of the O_2^--DMPO adduct in the lowest magnetic field. The signal heights were normalized to the Mn^{2+} resonance as a ratio of the height of each free radical to that of Mn^{2+} represented S (free radical) / M (Mn^{2+}) as shown in Figure 1.

Statistical analysis

All data were expressed as mean ± SD. Statistical analyses were performed using one way analysis of variance followed by a Tukey–Kramer post comparison test. Differences with a probability value of $p < 0.05$ were considered to be statistically significant.

Results

Typical ESR spectrum in the H_2O_2/NaOH/DMSO system

As shown in Fig. 1, $\cdot CH_3$, $\cdot OH$, and O_2^- derived signals were assigned. Signal heights were calculated at double-sided arrows in Fig. 1. Mn^{2+} as internal reference standard marker was observed on each side of the spectrum. The intensity of 3 free radical signals was respectively calculated as the ratio between positive signal height of $\cdot CH_3$-DMPO adduct in the lowest magnetic field and the positive and negative signal height of Mn^{2+} reference marker, the ratio between the positive signal height of the $\cdot OH$-DMPO adduct in the lowest magnetic field and the positive and negative signal height of Mn^{2+}, and the ratio between negative signal height of the O_2^--DMPO adduct in the lowest magnetic field and the positive and negative signal height of Mn^{2+}.

Free radical scavenging ability of Edaravone by ESR

Eda was added in the reaction system at 3 concentrations (3 mg/l, 6 mg/l, 12 mg/l) as described in Fig. 2A. Inhibition rate was defined as the following equation: 1-signal intensity (sample) / signal intensity (control). Eda significantly suppressed $\cdot OH$ and O_2^- in the dose-dependent manner among 3 above different concentrations. However, Eda did not significantly scavenge $\cdot CH_3$ in the above doses compared with control group (Fig. 2B, $*p<0.05$ VS Control, $**p<0.01$ VS Control; $\#p<0.05$ VS 3 mg/l Edaravone; $\&p<0.05$ VS 6 mg/l Edaravone).

Free radical scavenging ability of carnosine, anserine, and whale meat extract by ESR

Car, Ans, and WME were added in the reaction system at 4 concentrations (10 mg/l, 100 mg/l, 1000 mg/l, 10000 mg/l) as described in Fig. 3A. Car, Ans, and WME significantly suppressed $\cdot CH_3$, $\cdot OH$ and O_2^- starting from the dose of 10 mg/l in the dose-dependent manner compared with control group (Fig. 3C-3E, $*p<0.05$ VS Control, $*p<0.01$ VS Control; $^{\Omega}p<0.05$ VS 10 mg/l, $^{\Omega\Omega}p<0.01$ VS 10 mg/l; $^{\pi}p<0.05$ VS 100 mg/l, $^{\pi\pi}p<0.01$ VS 100 mg/l; $^{\theta}p<0.05$ VS 1000 mg/l.). Compared with Car, Ans and WME have higher abilities of scavenging $\cdot CH_3$ and $\cdot OH$ in higher doses of 1000 mg/l and 10000 mg/l (Fig. 3B, 3C, $\#p<0.05$ VS 1000 mg/l Car; $\&p<0.05$ VS 10000 mg/l Car).

Free radical scavenging ability of Twendee Mtcontrol, Twendee X, and Supaliv by ESR

TwM, TwX, and SuP were added in the reaction system at 3 concentrations (20 mg/l, 200 mg/l, 2000 mg/l) described in Fig. 4A. TwM, TwX, and SuP significantly suppressed $\cdot CH_3$, $\cdot OH$, and O_2^- in the dose of 20 mg/l, 200 mg/l, and 2000 mg/l compared with control group (Fig. 4B-4D, $*p<0.05$ VS Control, $**p<0.01$ VS Control). Compared with 200 mg/l, TwM, TwX, and SuP greatly reduced $\cdot CH_3$ in the dose of 2000 mg/l. However, there is no significant difference between 20 mg/l and 200mg/l (Fig. 4B, $^{\theta\theta}p<0.01$ VS 200 mg/l). As for reducing $\cdot OH$, there is significant difference between 20 mg/l and 200mg/l and a dramatically significant difference between 200 mg/l and 2000 mg/l, respectively in the TwM group and TwX group (Fig. 4C, $^{\pi}p<0.05$ VS 20 mg/l, $^{\theta\theta}p<0.01$ VS 200 mg/l). In the SuP group, no

significant difference was observed between 20 mg/l and 200 mg/l in terms of reducing · OH. However, a dramatically significant difference was found between 200 mg/l and 2000mg/l (Fig. 4C, $^{\theta\theta}p<0.01$ VS 200 mg/l). For scavenging O_2^-, obvious dose-dependent manner of TwM, TwX, and Sup was shown among 20 mg/l, 200 mg/l, and 2000 mg/l (Fig. 4D, $^{\pi}p<0.05$ VS 20 mg/l, $^{\pi\pi}p<0.01$ VS 20 mg/l; $^{\theta}p<0.05$ VS 200 mg/l $^{\theta\theta}p<0.01$ VS 200 mg/l). Compared with SuP, TwM and TwX have higher abilities of scavenging · OH and O_2^- in lower doses of 20 mg/l and 200 mg/l (Fig. 4C, 4D, #$p<0.05$ VS 20 mg/l SuP; &$p<0.05$ VS 200 mg/l SuP).

Free radical scavenging ability of α-tocopherol and tocovid by ESR

α-tocopherol and Toco were added in the reaction system at 3 concentrations (40 mg/l, 100 mg/l, 1000 mg/l) as described in Fig. 5A. α-tocopherol and Toco significantly suppressed above 3 radicals in the dose of 40 mg/l, 100 mg/l, and 1000 mg/l compared with control group (Fig. 5B-5D, *$p<0.05$ VS Control, **$p<0.01$ VS Control). Toco significantly scavenged above 3 free radicals as an obvious dose-dependent manner among above 3 concentrations (Fig. 5B-5D, $^{\pi}p<0.05$ VS 40 mg/l; $^{\theta}p<0.05$ VS 100 mg/l, $^{\theta\theta}p<0.01$ VS 100 mg/l). α-tocopherol also obviously reduced · CH_3 as an obvious dose-dependent manner among 3 concentrations (Fig. 5C, $^{\pi}p<0.05$ VS 40 mg/l; $^{\theta\theta}p<0.01$ VS 100 mg/l). However, there is no significant difference between 40 mg/l and 100mg/l in terms of suppressing · OH and O_2^-, and significant difference was just observed between 100 mg/l and 1000 mg/l (Fig. 5C, 5D, $^{\theta}p<0.05$ VS 100 mg/l, $^{\theta\theta}p<0.01$ VS 100 mg/l). The tendency of a dose-dependent manner of α-tocopherol exists in terms of reducing · OH and O_2^- (Fig. 5C, 5D, $^{\theta}p<0.05$ VS 100 mg/l, $^{\theta\theta}p<0.01$ VS 100 mg/l). Compared with α-tocopherol, Toco has higher abilities of scavenging above 3 radicals in 3 different doses. (Fig. 5B-5D, #$p<0.05$ VS 40 mg/l α-Tocopherol; &$p<0.05$ VS 100 mg/l α-Tocopherol; ∞$p<0.05$ VS 1000 mg/l α-Tocopherol).

Free radical scavenging ability of scallop and ascidian-derived (Hoya) plasmalogens by ESR

Scallop and ascidian-derived (Hoya) Plas were added in the reaction system at 4 concentrations (1 mg/l, 10 mg/l, 100 mg/l, 1000 mg/l) described in Fig. 6A. Scallop-derived Plas significantly suppressed · CH_3 in the dose of 100 mg/l and 1000 mg/l, · OH in the dose of 10mg/l, 100mg/l, and 1000 mg/l, and O_2^- in the dose of 100 mg/l and 1000 mg/l respectively, compared with control group (Fig. 6B-6D, *$p<0.05$ VS Control, **$p<0.01$ VS Control). Ascidian (Hoya)-derived Plas significantly scavenged · CH_3 in the dose of 10 mg/l, 100 mg/l, and 1000 mg/l, · OH in the dose of 1mg/l, 10mg/l, 100mg/l and 1000 mg/l, and O_2^- in the dose of 100 mg/l and 1000 mg/l, respectively, compared with control group (Fig. 6B-6D, *$p<0.05$ VS Control, **$p<0.01$ VS Control). A small dose-dependent manner was observed in terms of reducing 3 above free radicals in respectively 2 groups (Fig. 6B-6D). The remarkably significant difference between every two different concentrations in scallop or Hoya-derived Plas group was not observed (Fig. 6B-6D). Only in the scallop-derived Plas group, we observed that 1000 mg/l has a higher ability of reducing · CH_3 in relative to 1 mg/l, and for scavenging · OH, 10 mg/l, 100 mg/l, and 1000 mg/l have higher potential compared with 1 mg/l (Fig. 6B-6D, $^{\Omega}p<0.05$ VS 1 mg/l). Moreover, there is no big difference found between the same concentration of scallop-derived and ascidian-derived (Hoya) Plas in terms of scavenging above 3 radicals (Fig. 6B-6D).

Discussion

The typical signal heights of · CH_3, · OH, and O_2^- generated from the reaction system of H_2O_2/NaOH/DMSO are shown (Fig.1). The mechanisms of the present radical-generating system are as follows : OH and O_2^- are generated from the degradation of H_2O_2. Then, · OH can attack DMSO to generate · CH_3. Therefore, we chose the present reaction system to successfully evaluate the significant free radical scavenging ability of 10 types of nutrients by ESR.

As the positive control, Eda is a small synthetic compound with only 174.2 D in size. According to previous studies, Eda has been proved to be a potent · OH and O_2^- scavenger examined by ESR in a dose-dependent manner (Abe et al., 2004; Tokumaru et al., 2018) with no significant scavenging effect of · CH_3. Our present data are consistent to the previous reports (Fig. 2).

Car is a common histidine-derived imidazole dipeptide with roles of metal-ion chelation and antioxidant capacity (Alexander et al., 2013). Ans and Bal collectively contain a methylated variant of Car (Hipkiss et al., 2016). Previous studies showed that Car and Ans can scavenge · OH and O_2^- via interaction between radicals and their structure of histidine (Klebanov et al., 1997; Boldyrev et al., 2004). Our present data indicate that Car, Ans and WME containg 8.6% Bal not only suppress · OH and O_2^-, but also reduce · CH_3 in a dose-dependent manner,

probably relating to decreased amount of $\cdot OH$ or the formation of imidazole-DMSO adduct (Fig.3). Moreover, compared with Car, Ans and WME have higher abilities of scavenging $\cdot CH_3$ and $\cdot OH$ in higher doses of 1000 mg/l and 10000 mg/l (Fig. 3). To date, there is no corresponding conclusion about the rank of specific free radical scavenging ability among Car, Ans, and Bal. Therefore, our present data could offer benefit to some degree in terms of comparison among the three histidine dipeptides.

SuP, TwX, and TwM mainly consist of ascorbic acid, L-glutamine, L-cystine, coenzyme Q10, ascorbic acid (AsA), and riboflavin (Kusaki et al., 2017). TwM, TwX, and SuP significantly suppressed $\cdot CH_3$, $\cdot OH$, and O_2^- compared with control group (Fig. 4). Previous papers reported that higher concentration of AsA over 500 mg/l produces ascorbyl radical, and thus increases the signal height of $\cdot CH_3$ (Yamaguchi et al., 1999; Yoshihiro et al., 1999). However, such adverse phenomenon was not observed in the same ESR reaction system (Fig. 4), indicating that the mixed components into these supplements effectively inhibit the production of ascorbyl radical caused by higher concentration of AsA. Moreover, compared with SuP, both TwM and TwX have higher abilities of scavenging $\cdot OH$ and O_2^- in lower doses of 20 mg/l and 200 mg/l, probably owing to the improved components (Fig. 4).

Toco is a novel supplement containing a mixture of tocotrienol and tocopherol, which reasonably attenuated ischemic infarction in a stoke mouse model via reducing oxidative stress (Shang et al., 2018). Compared with single α-tocopherol, Toco has higher abilities of scavenging above 3 radicals in doses of 40 mg/l, 100 mg/l, and 1000 mg/l in a dose-dependent manner, evidencing a higher antioxidant potential than a single nutrient α-tocopherol (Fig. 5).

Plas are glycerophospholipids which are very sensitive to redox owing to the high reactivity to free radicals of their vinyl ether double bonds at the sn-1 position (Fujino et al., 2017). A polyunsaturated fatty acid is predominantly localized into sn-2 position of Plas, specifically docosahexaenoic acid (DHA; C22:6) or eicosapntemacnioc acid (EPA; C20:5) depending on the species derivation (Su et al., 2019). A previous paper reported that the amount of EPA and DHA in ascidian-derived (Viscera) (Hoya) Plas was respectively around 10 and 15 times higher than that in scallop-derived Plas (Viscera) in the case of the same amount of foodstuffs (umol/100g wet wt) (Yamashita et al., 2015). However, Yamashita's group did not conduct comparison between two species in the case of the same amount of total Plas. Therefore, more and further investigations are worthy of conducting for better understanding the variety of different species-derived Plas. In the present study, scallop and Hoya-derived Plas similarly suppressed 3 radicals compared with control group with a small dose-dependent manner (Fig. 6). The reason could be that vinyl ether may be more dominant to scavenge free radicals than polyunsaturated fatty acid, which also be proved by previous studies (Tobias et al., 2001; Broniec et al., 2011). And, we must clarify that H_2O_2/NaOH/DMSO is an alkaline environmental reaction system, not a physiological environment. Plas as a bioactive phospholipid act in the physiological condition with multiple mechanisms (Su et al., 2019). Therefore, we wonder that the viability of Plas probably was affected in the present alkaline system, which could partly explain why scallop and Hoya-derived Plas suppressed 3 radicals compared with control group with a small dose-dependent manner, and 50% inhibition rate was not observed even though in the highest-concentration groups. Hence, further studies about scavenging ability of district species-derived Plas are worth conducting in *in-vitro* and *in-vivo*, especially in physiological and various pathological conditions.

In summary, the present study reported the 3 free radical scavenging ability of 10 types of nutrients by ESR in a dose-dependent manner to some degree. Moreover, dietary supplements composed of different antioxidative spectrum could be more appropriately designed for preventing multiple free radicals and promoting health.

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Conflicts of Interest

The authors declare no potential conflicts of interest.

References

1. Abe, K., Yuki, S., Kogure, K., Strong attenuation of ischemic and postischemic brain edema in rats by a novel free radical scavenger. Stroke. 1988; 19, 480-485.
2. Yamashita, T., Kamiya, T., Deguchi, K., Inaba, T., Zhang, H., Shang, J., Miyazaki, K., Ohtsuka, A., Katayama, Y., Abe, K., Dissociation and Protection of the Neurovascular Unit after Thrombolysis and Reperfusion in Ischemic Rat Brain. Journal of Cerebral Blood Flow & Metabolism. 2009; 29, 715-725.
3. Abe, K., Aoki, M., Tsuji, S., Itoyama, Y., Sobue, G., Togo, M., Hamada, C., Tanaka, M., Akimoto, M., Nakamura, K., Takahashi, F., Kondo, K., Yoshino, H., Abe, K., Aoki, M., Tsuji, S., Itoyama, Y., Sobue, G., Togo, M., Hamada, C., Sasaki, H., Yabe, I., Doi, S., Warita, H., Imai, T., Ito, H., Fukuchi, M., Osumi, E., Wada, M., Nakano, I., Morita, M., Ogata, K., Maruki, Y., Ito, K., Kano, O., Yamazaki, M., Takahashi, Y., Ishiura, H., Ogino, M., Koike, R., Ishida, C., Uchiyama, T., Mizoguchi, K., Obi, T., Watanabe, H., Atsuta, N., Aiba, I., Taniguchi, A., Sawada, H., Hazama, T., Fujimura, H., Kusaka, H., Kunieda, T., Kikuchi, H., Matsuo, H., Ueyama, H., Uekawa, K., Tanaka, M., Akimoto, M., Ueda, M., Murakami, A., Sumii, R., Kudou, T., Nakamura, K., Morimoto, K., Yoneoka, T., Hirai, M., Sasaki, K., Terai, H., Natori, T., Matsui, H., Kotani, K., Yoshida, K., Iwasaki, T., Takahashi, F., Kondo, K., Yoshino, H., Safety and efficacy of edaravone in well defined patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled trial. The Lancet Neurology. 2017; 16, 505-512.
4. Halliwell, B., Role of Free Radicals in the Neurodegenerative Diseases. Drugs & Aging. 2001; 18, 685-716.
5. Tadokoro, K., Ohta, Y., Inufusa, H., Loon, A.F.N., Abe, K., Prevention of Cognitive Decline in Alzheimer's Disease by Novel Antioxidative Supplements. International journal of molecular sciences. 2020; 21, 1974.
6. Herculano, B., Tamura, M., Ohba, A., Shimatani, M., Kutsuna, N., Hisatsune, T., β-Alanyl-L-Histidine Rescues Cognitive Deficits Caused by Feeding a High Fat Diet in a Transgenic Mouse Model of Alzheimer's Disease. Journal of Alzheimer's Disease. 2013; 33, 983-997.
7. Davis, C.K., Laud, P.J., Bahor, Z., Rajanikant, G.K., Majid, A., Systematic review and stratified meta-analysis of the efficacy of carnosine in animal models of ischemic stroke. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2016; 36, 1686-1694.
8. Kaneko, J., Enya, A., Enomoto, K., Ding, Q., Hisatsune, T., Anserine (beta-alanyl-3-methyl-L-histidine) improves neurovascular-unit dysfunction and spatial memory in aged AβPPswe/PSEN1dE9 Alzheimer's-model mice. Scientific reports. 2017; 7, 12571-12571.
9. Kawahara, M., Tanaka, K.-I., Kato-Negishi, M., Zinc, Carnosine, and Neurodegenerative Diseases. Nutrients. 2018; 10, 147.
10. Kusaki, M., Ohta, Y., Inufusa, H., Yamashita, T., Morihara, R., Nakano, Y., Liu, X., Shang, J., Tian, F., Fukui, Y., Sato, K., Takemoto, M., Hishikawa, N., Abe, K., Neuroprotective Effects of a Novel Antioxidant Mixture Twendee X in Mouse Stroke Model. Journal of Stroke and Cerebrovascular Diseases. 2017; 26, 1191-1196.
11. Liu, X., Yamashita, T., Shang, J., Shi, X., Morihara, R., Huang, Y., Sato, K., Takemoto, M., Hishikawa, N., Ohta, Y., Abe, K., Clinical and Pathological Benefit of Twendee X in Alzheimer's Disease Transgenic Mice with Chronic Cerebral Hypoperfusion. Journal of Stroke and Cerebrovascular Diseases. 2019; 28, 1993-2002.
12. Tadokoro, K., Morihara, R., Ohta, Y., Hishikawa, N., Kawano, S., Sasaki, R., Matsumoto, N., Nomura, E., Nakano, Y., Takahashi, Y., Takemoto, M., Yamashita, T., Ueno, S., Wakutani, Y., Takao, Y., Morimoto, N., Kutoku, Y., Sunada, Y., Taomoto, K., Manabe, Y., Deguchi, K., Higashi, Y., Inufusa, H., You, F., Yoshikawa, T., von Greiffenclau, M.M., Abe, K., Clinical Benefits of Antioxidative Supplement Twendee X for Mild Cognitive Impairment: A Multicenter, Randomized, Double-Blind, and Placebo-Controlled Prospective Interventional Study. Journal of Alzheimer's Disease. 2019; 71, 1063-1069.
13. Shang, J., Yan, H., Jiao, Y., Ohta, Y., Liu, X., Li, X., Morihara, R., Nakano, Y., Fukui, Y., Shi, X., Huang, Y., Feng, T., Takemoto, M., Sato, K., Hishikawa, N., Yamashita, T., Abe, K., Therapeutic Effects of Pretreatment with Tocovid on Oxidative Stress in Postischemic Mice Brain. Journal of Stroke and Cerebrovascular Diseases. 2018; 27, 2096-2105.
14. Fujino, T., Yamada, T., Asada, T., Tsuboi, Y., Wakana, C., Mawatari, S., Kono, S., Efficacy and Blood Plasmalogen Changes by Oral Administration of Plasmalogen in Patients with Mild Alzheimer's Disease and Mild Cognitive Impairment: A Multicenter, Randomized, Double-blind, Placebo-controlled Trial. EBioMedicine. 2017; 17, 199-205.
15. Hossain, M.S., Abe, Y., Ali, F., Youssef, M., Honsho, M., Fujiki, Y., Katafuchi, T., Reduction of Ether-Type Glycerophospholipids, Plasmalogens, by NF-κB Signal Leading to Microglial Activation. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2017; 37, 4074-4092.
16. Su, X.Q., Wang, J., Sinclair, A.J., Plasmalogens and Alzheimer's disease: a review. Lipids in health and disease. 2019; 18, 100-100.
17. Mitsuta, K., Mizuta, Y., Kohno, M., Hiramatsu, M., Mori, A., The Application of ESR Spin-Trapping Technique to the Evaluation of SOD-like Activity of Biological Substances. Bulletin of the Chemical Society of Japan. 1990; 63, 187-191.
18. Ogawa, N., Tanaka, K.-i., Asanuma, M., Kawai, M., Masumizu, T., Kohno, M., Mori, A., Bromocriptine protects mice against 6-hydroxydopamine and scavenges hydroxyl free radicals in vitro. Brain Research. 1994; 657, 207-213.
19. Yoshimura, Y., Inomata, T., Nakazawa, H., Kubo, H., Yamaguchi, F., Ariga, T., Evaluation of Free Radical Scavenging Activities of Antioxidants with an H2O2/NaOH/DMSO System by Electron Spin Resonance. Journal of Agricultural and Food Chemistry. 1999; 47, 4653-4656.
20. Abe, S., Kirima, K., Tsuchiya, K., Okamoto, M., Hasegawa, T., Houchi, H., Yoshizumi, M., Tamaki, T., The Reaction Rate of Edaravone (3-Methyl-1-phenyl-2-pyrazolin-5-one (MCI-186)) with Hydroxyl Radical. Chemical and Pharmaceutical Bulletin. 2004; 52, 186-191.
21. Tokumaru, O., Shuto, Y., Ogata, K., Kamibayashi, M., Bacal, K., Takei, H., Yokoi, I., Kitano, T., Dose-dependency of multiple free radical-scavenging activity of edaravone. Journal of Surgical Research. 2018; 228, 147-153.
22. Boldyrev, A.A., Aldini, G., Derave, W., Physiology and Pathophysiology of Carnosine. Physiological Reviews. 2013; 93, 1803-1845.
23. Hipkiss, A.R., Baye, E., de Courten, B., Carnosine and the processes of ageing. Maturitas. 2016; 93, 28-33.
24. Klebanov, G.I., Yu. O., Teselkin, I.V., Babenkova, I.N., Popov, G., Levin, O.V., Tyulina., Boldyrev, A.A., Vladimirov, Yu.A., Evidence for a direct interaction of superoxide anion radical with carnosine. Biochemistry and Molecular Biology International. 1997; 43, 99-106.
25. Boldyrev, A., Bulygina, E., Leinsoo, T., Petrushanko, I., Tsubone, S., Abe, H., Protection of neuronal cells against reactive oxygen species by carnosine and related compounds. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2004; 137, 81-88.
26. Yamaguchi, F., Yoshimura, Y., Nakazawa, H., Ariga, T., Free Radical Scavenging Activity of Grape Seed Extract and Antioxidants by Electron Spin Resonance Spectrometry in an H2O2/NaOH/DMSO System. Journal of Agricultural and Food Chemistry. 1999; 47, 2544-2548.
27. Yamashita, S., Kanno, S., Honjo, A., Otoki, Y., Nakagawa, K., Kinoshita, M., Miyazawa, T., Analysis of Plasmalogen Species in Foodstuffs. Lipids. 2016; 51, 199-210.
28. Dansen, T.B., Wirtz, K.W.A., The Peroxisome in Oxidative Stress. IUBMB Life. 2001; 51, 223-230.
29. Broniec, A., Klosinski, R., Pawlak, A., Wrona-Krol, M., Thompson, D., Sarna, T., Interactions of plasmalogens and their diacyl analogs with singlet oxygen in selected model systems. Free radical biology & medicine. 2011; 50, 892-898.

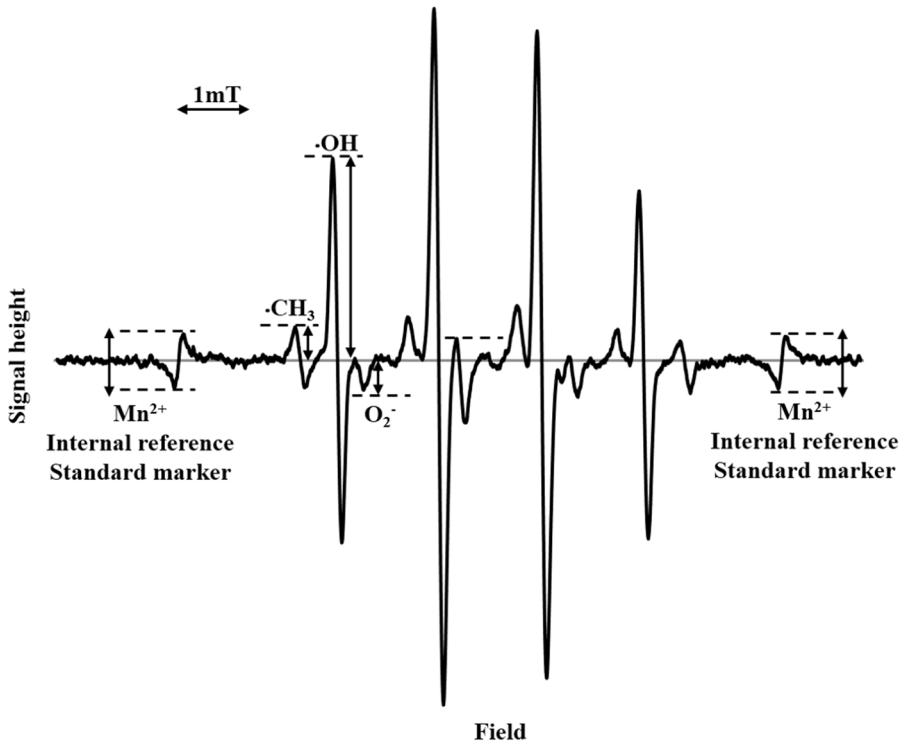

Fig. 1) Typical electron spin resonance (ESR) spectrum in the H_2O_2/NaOH/DMSO system. ESR conditions were described in the text. Each free radical derived signal was assigned, and signal heights were calculated at double-sided arrows. The amplification rate of the apparatus was adjusted to obtain the proper spectrum heights of the hydroxyl radical (. OH)-DMPO adduct as shown in Fig. 1 (1:2:2:1). Scale bar = 1 mT.

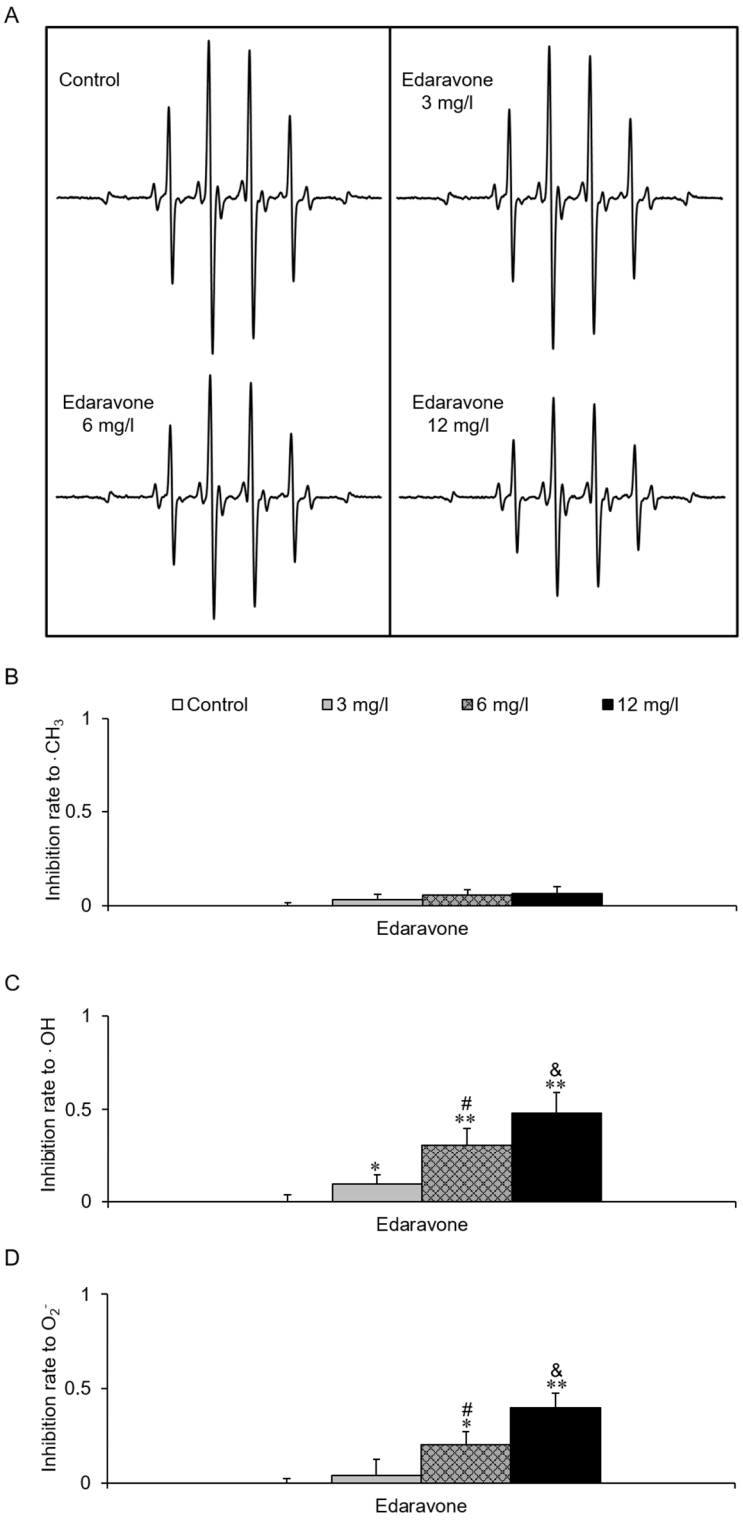

Fig. 2) Free radical scavenging property of a positive control Edaravone (Eda) by ESR. ESR spectra of free radical adducts under the condition of 3 concentrations of Eda (A). Inhibition rate = 1 – signal height (sample) / signal height (control). The signal heights were normalized to the Mn^{2+} resonance. Quantitative analysis of inhibition rate of Eda against methyl radical ($\cdot CH_3$), $\cdot OH$, and superoxide anion (O_2^-) (B-D, * p<0.05 VS Control, ** p<0.01 VS Control; # p<0.05 VS 3 mg/l Edaravone; & p<0.05 VS 6 mg/l Edaravone).

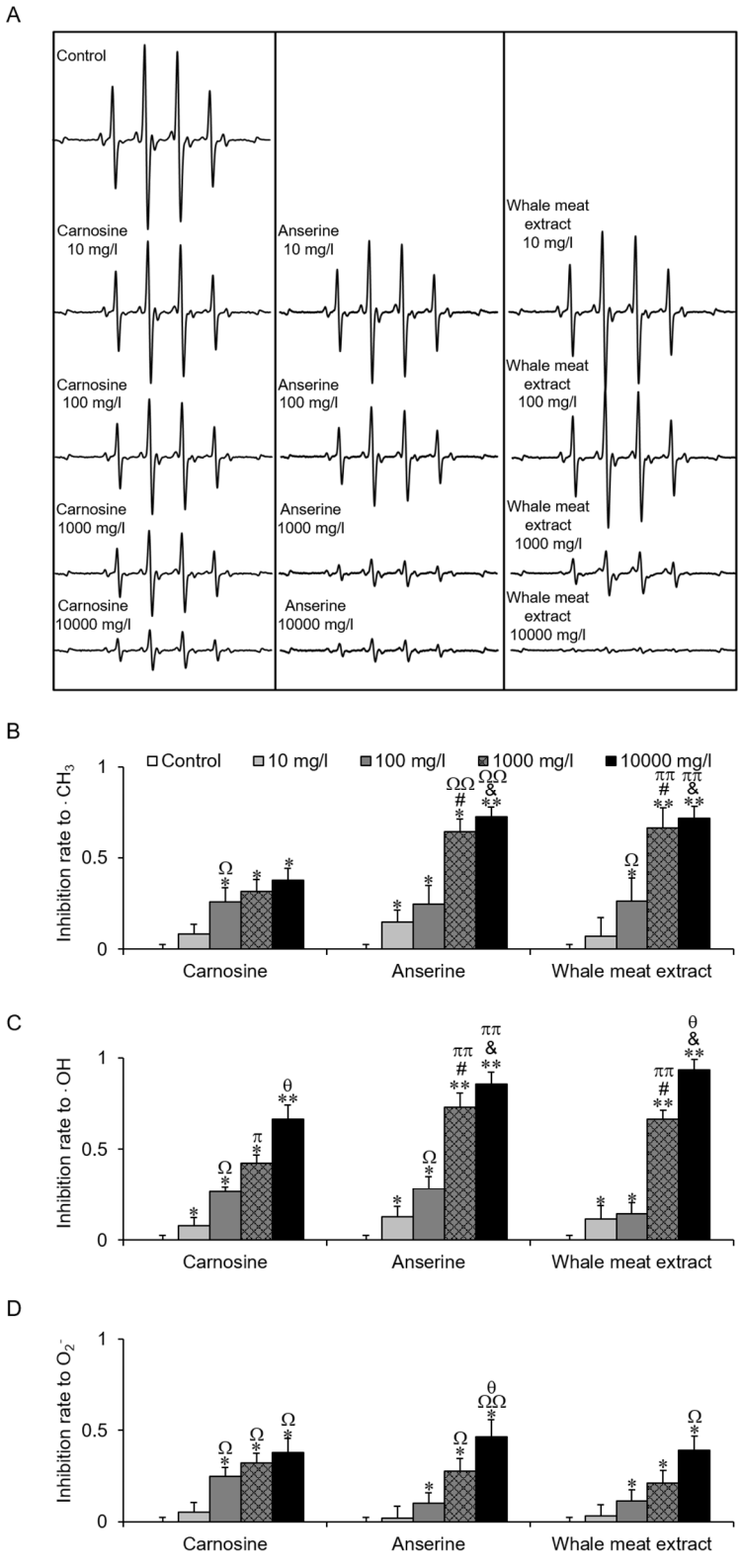

Fig. 3) Free radical scavenging property of carnosine (Car), anserine (Ans), and whale meat extract (WME) by ESR. ESR spectra of free radical adducts under the condition of 4 concentrations of Car, Ans, and WME (A). Quantitative analysis of inhibition rate of Car, Ans, and WME against $\cdot CH_3$, $\cdot OH$, and O_2^- (B-D, * $p<0.05$ VS Control, ** $p<0.01$ VS Control; $^{\Omega}p<0.05$ VS 10 mg/l, $^{\Omega\Omega}p<0.01$ VS 10 mg/l; $^{\pi}p<0.05$ VS 100 mg/l, $^{\pi\pi}p<0.01$ VS 100 mg/l; $^{\theta}p<0.05$ VS 1000 mg/l; # $p<0.05$ VS 1000 mg/l Carnosine; & $p<0.05$ VS 10000 mg/l Carnosine).

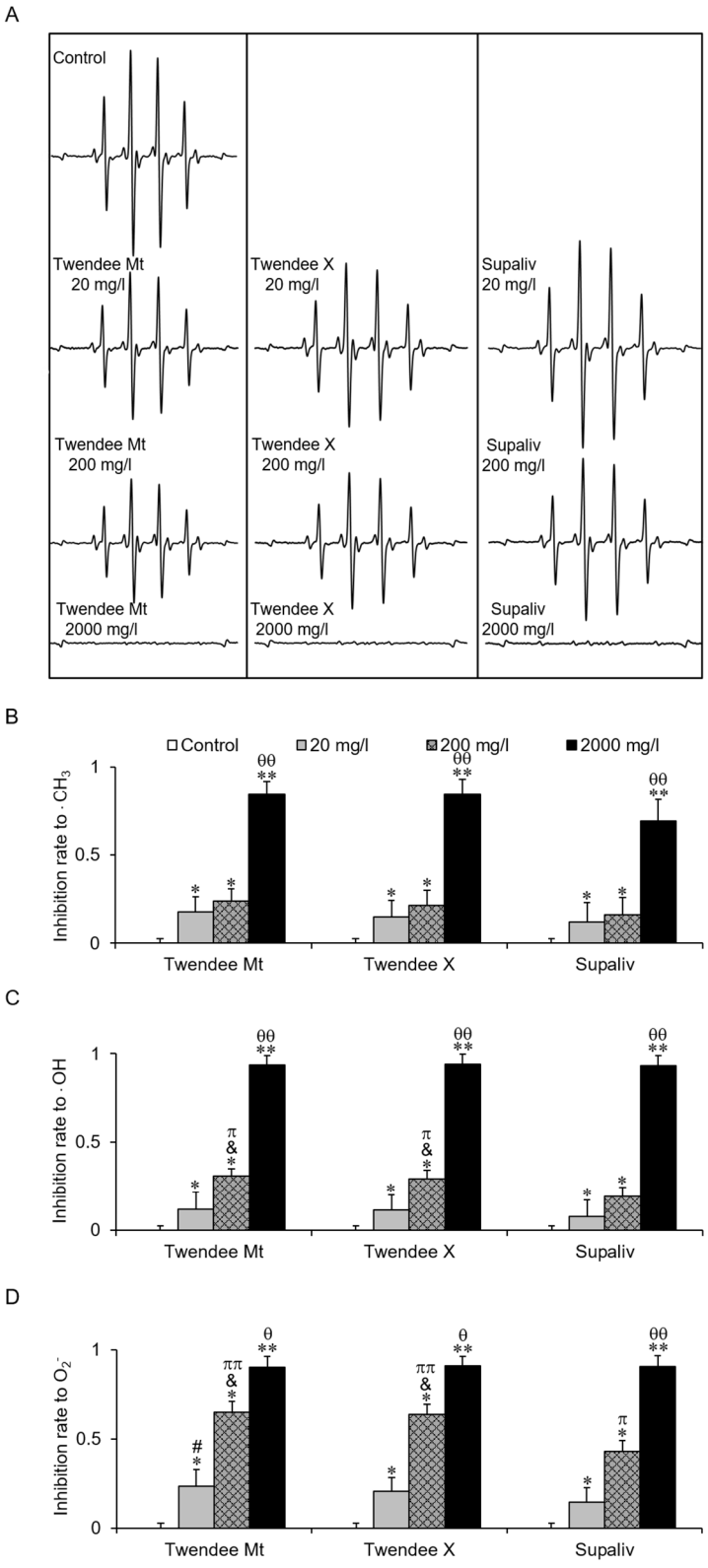

Fig. 4) Free radical scavenging property of Supaliv (SuP), Twendee X (TwX), and Twendee Mtcontrol (TwM) by ESR. ESR spectra of free radical adducts under the condition of 3 concentrations of SuP, TwX, and TwM (A). Quantitative analysis of inhibition rate of SuP, TwX, and TwM against $\cdot CH_3$, $\cdot OH$, and O_2^- (B-D, * $p<0.05$ VS Control, ** $p<0.01$ VS Control; $^{\pi}p<0.05$ VS 20 mg/l, $^{\pi\pi}p<0.01$ VS 20 mg/l; $^{\theta}p<0.05$ VS 200 mg/l, $^{\theta\theta}p<0.01$ VS 200 mg/l; # $p<0.05$ VS 20 mg/l Supaliv; & $p<0.05$ VS 200 mg/l Supaliv).

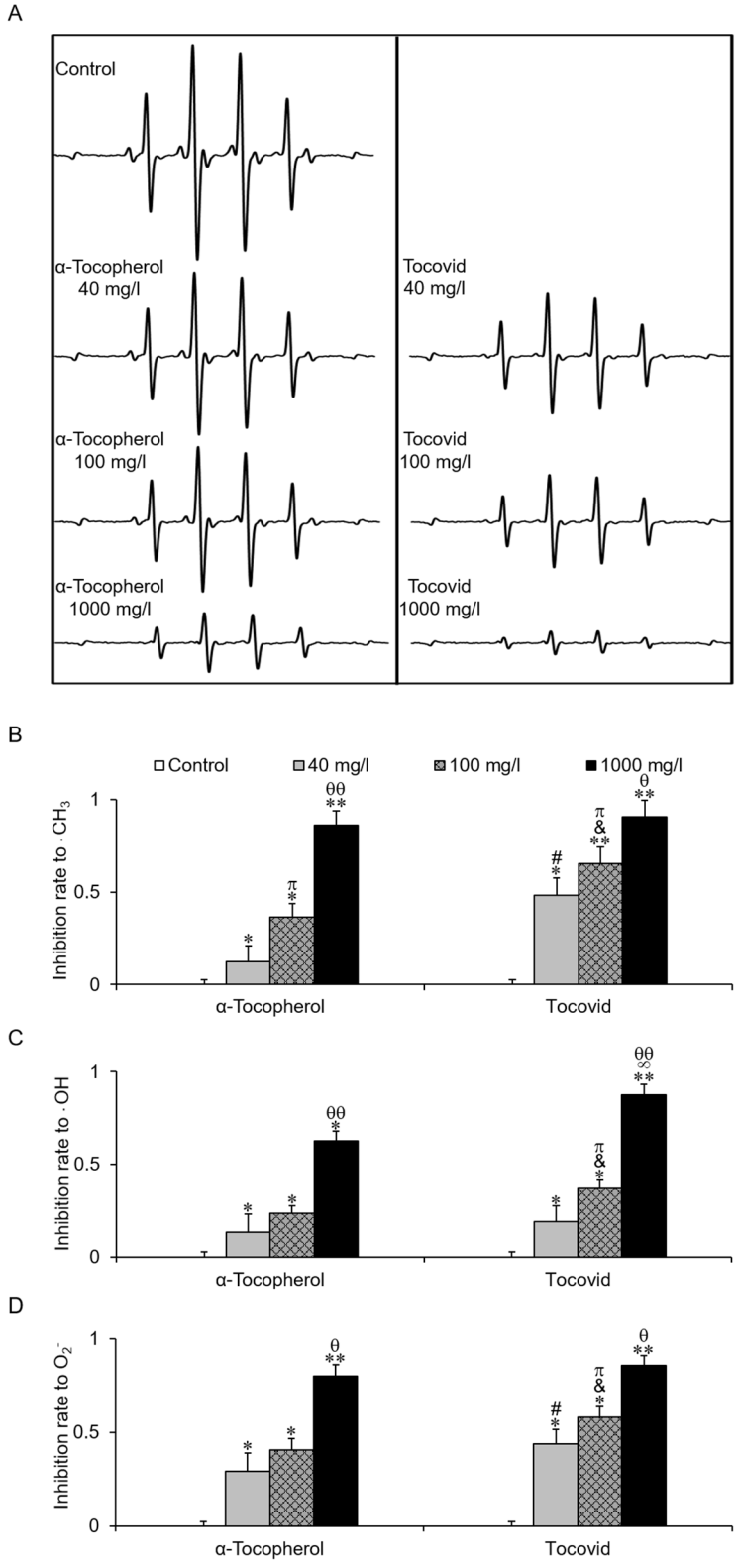

Fig. 5) Free radical scavenging property of α-tocopherol and tocovid (Toco) by ESR. ESR spectra of free radical adducts under the condition of 3 concentrations of α-tocopherol and Toco (A). Quantitative analysis of inhibition rate of α-tocopherol and Toco against $\cdot CH_3$, $\cdot OH$, O_2^- (B-D, * $p<0.05$ VS Control, ** $p<0.01$ VS Control; $^{\pi}p<0.05$ VS 40 mg/l; $^{\theta}p<0.05$ VS 100 mg/l, $^{\theta\theta}p<0.01$ VS 100 mg/l; # $p<0.05$ VS 40 mg/l α-Tocopherol & $p<0.05$ VS 100 mg/l α-Tocopherol; ∞ $p<0.05$ VS 1000 mg/l α-Tocopherol).

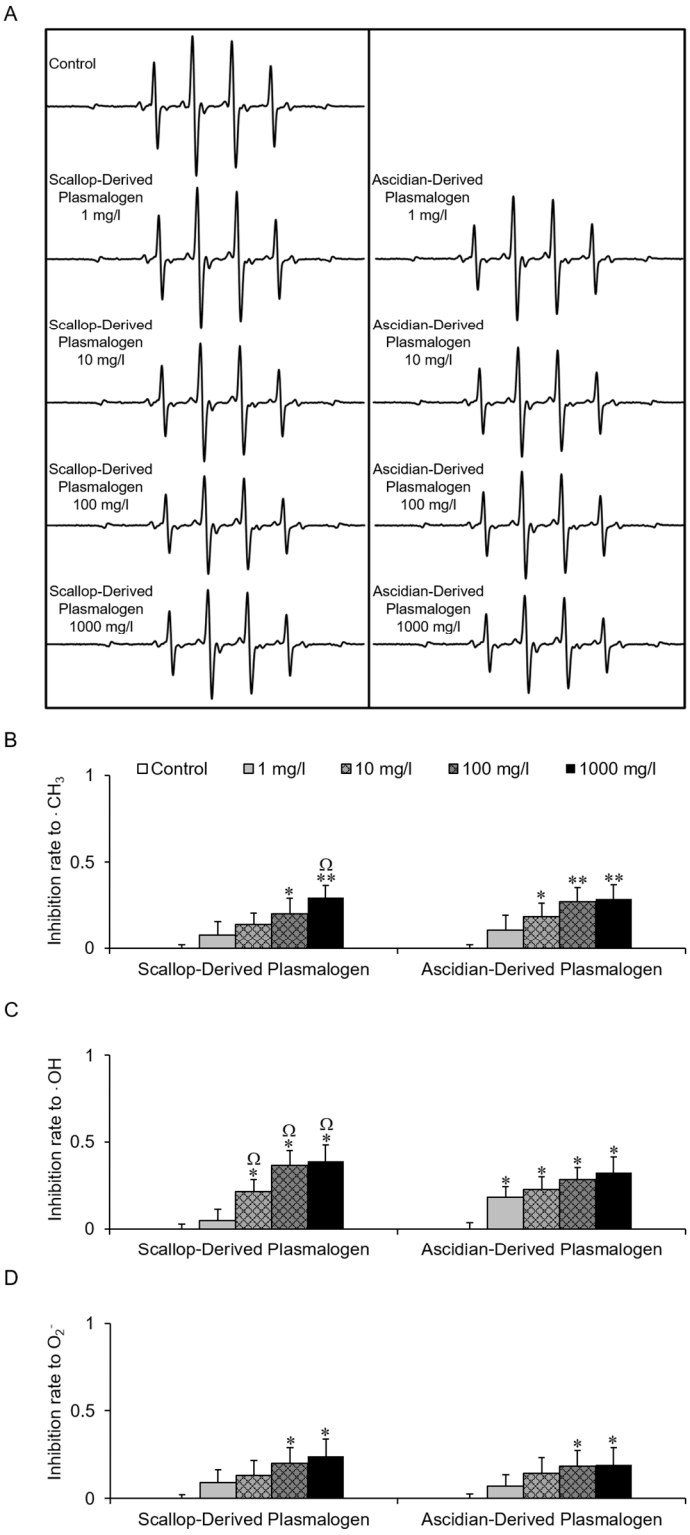

Fig. 6) Free radical scavenging property of scallop and ascidian (Hoya) – derived plasmalogens (Plas) by ESR. ESR spectra of free radical adducts under the condition of 4 concentrations of scallop and ascidian (Hoya) – derived Plas (A). Quantitative analysis of inhibition rate of scallop and ascidian (Hoya) – derived Plas against $\cdot CH_3$, $\cdot OH$, and O_2^- (B-D, * $p<0.05$ VS Control, ** $p<0.01$ VS Control; $^{\Omega}$ $p<0.05$ VS 1 mg/l).