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Review

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Flavonoids with Potent Antioxidant Activity Found in Young Green Barley Leaves

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ABSTRACT: Saponarin, a flavonoid found in young green barley leaves, possesses potent antioxidant activities, which are determined by its inhibition of malonaldehyde (MA) formation from various lipids oxidized by UV light or Fenton's reagent. Lipids used were squalene, ethyl linoleate, ethyl linolenate, ethyl arachidonate, octadecatetraenoic acid (ODTA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), cod liver oil, lecithin I, lecithin II, and blood plasma. The addition of saponarin inhibited the formation of MA from squalene upon UV irradiation at the level of 2 μmol/mL by almost 100%, whereas BHT inhibited its formation by 75% at the same level. Saponarin showed potent antioxidant activity toward fatty acid ethyl esters at levels >100 μg/mL. Saponarin inhibited MA formation in ODTA by 60%, in EPA by 50%, and in DHA by 43% at the level of 15 μmol/mL. Saponarin exhibited strong antioxidant activities with dose−response levels toward cod liver oil and lipoproteins (lecithins I and II), higher than those of α-tocopherol. A mixture of saponarin/lutonarin (4.5:1, w/w) inhibited MA formation appreciably from all lipids tested with dose response. This mixture exhibited highest effect toward cod liver oil (86%), followed by DHA, lecithin II, blood plasma, EPA, and lecithin I. Supplementation of young green barley leaves containing saponarin should be beneficial to health and may prevent diseases caused by oxidative damage such as various cancers, inflammations, and cardiovascular diseases.

KEYWORDS: *antioxidant, flavonoid, green barley leaves, malonaldehyde, ω-3 polyunsaturated fatty acids, saponarin*

INTRODUCTION

Barley is a major cereal grain and has been cultivated since ancient times, especially as feed for livestock. Although barley grain and leaf are not major sources of livestock fodder today, a few locations in the United States, such as Montana, produce fodder with barley forage. However, since the beneficial effect of barley leaves was determined through the presence of potent antioxidants, barley has begun to receive much attention as possible feed or food supplement for animals and humans to prevent various diseases.

In the case of livestock health issues, recent outbreaks of mad cow disease or bovine spongiform encephalopathy (BSE) have been an extremely serious problem, especially in terms of the risks to human health.[1] It was found that free radicals and oxidative stress were factors in the outbreak of BSE.[2] Over the past three decades, artificial feeds, such as feed prepared from citrus peels, have become widely used to replace natural feeds because they are easier to source and contain high nutrient value. However, a major drawback of using artificial feed is the lack of antioxidants. Barley leaves, which are one of the main conventional natural feed ingredients, have been discovered to contain high levels of the potent antioxidants saponarin and lutonarin.[3−5] Therefore, the outbreak of BSE may have occurred because of the lack of these antioxidants in artificial feeds. A case study reported that antioxidant therapies may prevent the occurrence of BSE.[2] Another study demonstrated a decline in mortality in a herd of cows fed grass pasture after a BSE outbreak.[6] As a result, there was a pressing desire for animals to return to natural diets to protect them from BSE and other diseases. In addition, various animal studies demonstrated that barley leaves possess certain bioactive chemicals that promote health-beneficial properties, including demonstrating antiulcer,[7] antioxidant and hypolipidemic,[8] microbial biomass production,[9] antidepressant,[10] and antidiabetic [11] potential.

There are several reports on the biological activities of young green barley essences, in particular, on the presence of potent antioxidants and flavonoids. Polyphenols, including flavonoids, are well-known antioxidants, which prevent various diseases.[12,13] There are comprehensive reviews on flavonoids found in fruits and vegetable as a possible cancer-preventive agents.[14,15] Quercetin, a typical botanical flavonoid, can be considered the prototype of a naturally occurring chemopreventive agent.[16] Flavonoids present in herbs can potentially offer cardiac protection against ischemic heart disease.[17] Another review describes the anticarcinogenic properties of plant polyphenols/flavonoids.[18] In the central nervous system, some flavones bind to the benzodiazepine site on the GAVA(A)-receptor, resulting in sedative, anxiolytic, or anticonvulsive effects.[19] One of the critical reviews on antioxidants reported neuroprotective properties of polyphenols/flavonoids present in blueberry.[20] An in vivo study demonstrated the antibacterial properties of flavonoids.[21]

Because of these various biological activities, polyphenols/flavonoids found in plants have been widely known as natural antioxidants, which can be used for food supplement to prevent various diseases. In particular, the "French Paradox" suggests that the drinking of red wine is linked to the low incidence of coronary heart disease (CHD) in France.[22] The antioxidant activity of wine phenolic compounds was confirmed,[23] and

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possible mechanisms for the protective role of antioxidants, including flavonoids, phenolic compounds, and other phytochemicals, in wine and plant foods were summarized.[24] A recent review also describes wine pigments, anthocyanins, as having a preventive effect toward cardiovascular disease.[25] Use of the waste left after winemaking may be a good antioxidant source for feed supplements. In addition, wastes from coffee and tofu (soybean curd) preparations can be excellent antioxidant sources for feed supplements because of their potent flavonoid-antioxidant content[26] and high levels of isoflavonoids.[27] Numerous studies indicate that natural plant flavonoids are important polyphenolic antioxidants, and they are widely used in health food today. In particular, many health food supplements prepared from green barley leaves are available in world markets. Therefore, this review focuses on the potent antioxidants found in young green barley leaves.

ISOLATION AND PURIFICATION OF A FLAVONOID WITH POTENT ANTIOXIDANT FROM YOUNG GREEN BARLEY LEAVES

Barley leaves (*Hordeum vulgare* L. var. nudum Hook) were grown in a greenhouse and harvested two weeks after germination. The overall isolation procedure for flavonoids reported previously is shown in Figure 1.[28] The framed samples

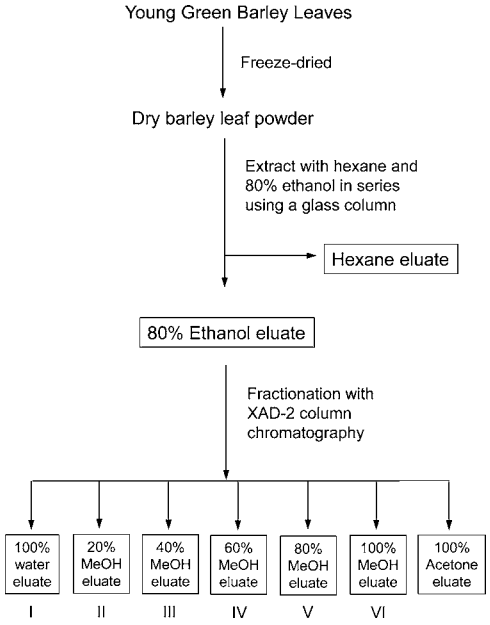

Figure 1. Isolation procedure for flavonoids from young green barley leaves. The framed flavonoids were tested for antioxidant activity.

were tested for antioxidant activity later. The barley leaves were freeze-dried for 3 days and then ground into a fine and uniform powder. The barley powder was placed in an empty glass column and eluted with a 500 mL portion of *n*-hexane twice to remove chlorophyll and then with a 500 mL portion of 85% ethanol twice. The solvents were removed using a rotary-flash evaporator under reduced pressure (95 mmHg) at room temperature for *n*-hexane and 55 °C for ethanol. The ethanol eluate was fractionated using column chromatography (Amberlite XAD-2 nonionic polymeric absorbent) with methanol solutions (0, 20, 40, 60, 80, and 100%) and acetone (100%) in series. After removal of the solvent, each fraction was examined for antioxidant activity using a thiobarbituric acid (TBA) assay to find the fractions with antioxidants.

Figure 2 shows the results of TBA assay on the fractions. The results indicated that methanol fractions contained antiox-

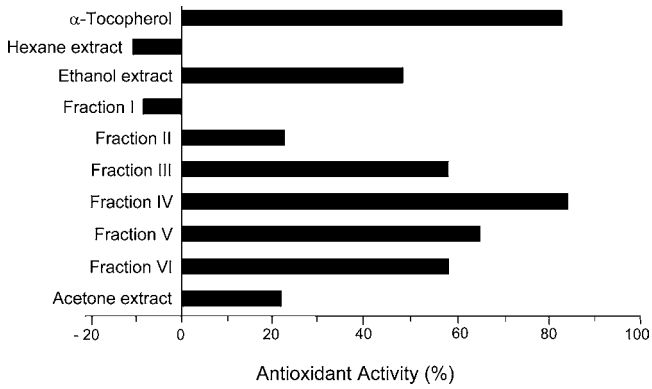

Figure 2. Results of TBA assay on the fractions from young green barley leaves. Fraction numbers refer to Figure 1.

idant(s), particularly fraction IV (60% methanol eluate), which resulted in 84.9% activity, slightly higher than that of α-tocopherol (83.8%). A brown paste material in fraction IV was recrystallized with 100% methanol twice. A light yellow powder obtained was further purified using a preparative HPLC. The structure of the purified compound was tentatively identified as 2′-*O*-glycosylisovitexin by ^{13}C NMR. Later, the structure of this compound was confirmed as 7-*O*-glycosylisovitexin (GIV) or saponarin.[29] Therefore, this compound will be discussed as saponarin in this review. Later, the isolation method was improved by changing the conditions of column chromatography by eluting with 20, 30, 40, and 50% methanol solution in series.[30] Saponarin was rich in the 50% methanol eluate. In addition, another flavonoid, lutonarin, was rich in the 20% methanol eluate in this method. The structures of saponarin and lutonarin are shown in Figure 3.

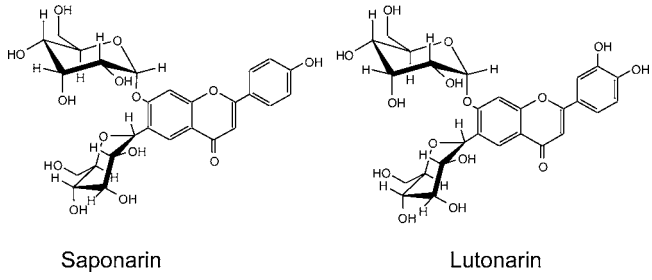

Figure 3. Structures of saponarin and lutonarin.

METHOD USED TO DETERMINE ANTIOXIDANT ACTIVITY OF A FLAVONOID FOUND IN YOUNG GREEN BARLEY LEAVES

The main assay used to determine antioxidant activity of extracts from young green barley leaves has been malonaldehyde/gas chromatography (MA/GC) assay, which involves analysis of MA formed from oxidized lipids.[31] Once lipid peroxidation occurs, various so-called secondary oxidation products, including formaldehyde, acetaldehyde, acrolein, glyoxal, methylglyoxal, and MA, are formed. Some of these products, in particular MA, have been used as a marker of lipid

peroxidation to investigate antioxidant activities of various substances.[32−35] Lipids, which are commonly used for this assay, include unsaturated fatty acids, such as linoleic acid, linolenic acid, arachidonic acid, and various ω-3 fatty acids, as well as cod liver oil.[36−39]

MA, which is a useful biomarker to investigate the final stage of lipid peroxidation, is extremely difficult to analyze because it is very reactive and highly soluble in water. Therefore, preparation of a stable derivative is required for MA analysis. Figure 4 shows two commonly used derivatives for MA

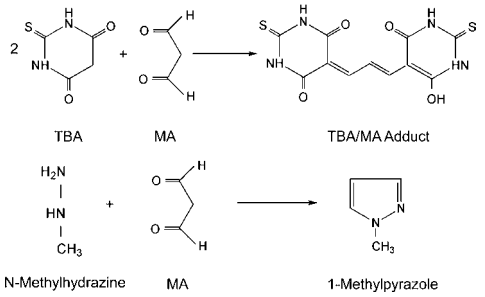

Figure 4. Derivatives used for MA analysis.

analysis. The preparation of an adduct between MA and TBA followed by monitoring of the formed adduct by spectrophotometry has been the most commonly and widely used assay to evaluate antioxidant activities of various natural products.[31]

MA is also analyzed by gas chromatography after derivatization into 1-methylpyrazole with *N*-methylhydrazine (Figure 4). This method was first developed to analyze volatile carbonyl compounds, including MA, formed by lipid peroxidation.[40]

ANTIOXIDANT ACTIVITY OF SAPONARIN ISOLATED FROM YOUNG GREEN BARLEY LEAVES

Saponarin isolated from young green barley leaves was tested for antioxidant activity toward various lipids.

Inhibition of a Skin Lipid, Squalene, Oxidation Induced by UV Irradiation. It has been reported that exposure to ultraviolet (UV) radiation from stratospheric ozone depletion in countries in the Southern Hemisphere has increased skin cancer incidences significantly in the past three decades. The skin directly suffers from the UV irradiation[41,42] and produces reactive oxygen species (ROS) from skin lipids, such as squalene.[43] Because mutagenic MA is formed from squalene by ROS upon UV irradiation,[44] the series of pathologically damaging events including photosensitivity, toxicity, and carcinogenicity associated with UV-exposed skin have been reported.[45,46] As the major component of human sebum, squalene has focused on oxidation due to its high degree of unsaturation and possible role in promoting oxidative skin damage.[47] MA, a secondary product of lipid oxidation, is commonly used as a biomarker of lipid peroxidation. Because MA reacts with DNA to form adducts,[48] it provides another means of instigating DNA oxidation. Therefore, there is a pressing need to find preventive and therapeutic agents that can protect skin surface lipids from UV-induced oxidative injury.

Saponarin (Figure 3), α-tocopherol, and ethyl BHT were tested for their inhibitory effect toward squalene oxidation upon UV irradiation. An ethanol solution of squalene was irradiated with various amounts of these three chemicals for 6 h. The antioxidant activity of these chemicals was determined for monitoring the amount of MA formed from squalene upon UV irradiation.[2] Figure 5 shows the results of the antioxidant

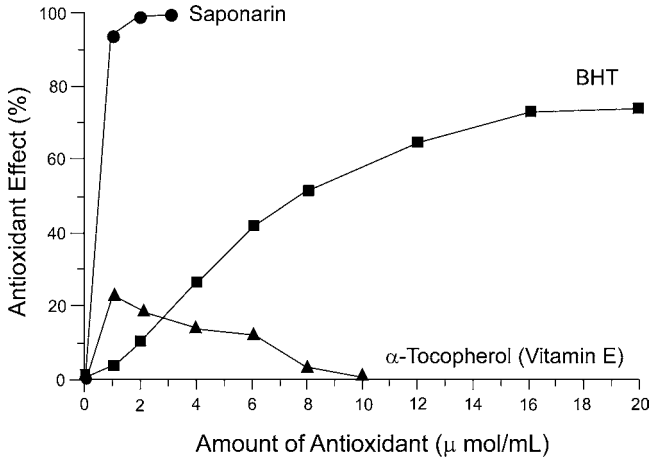

Figure 5. Results of antioxidant effect of saponarin, α-tocopherol, and BHT toward squalene oxidized by UV irradiation.

effect of saponarin, α-tocopherol, and BHT toward squalene oxidized by UV irradiation. Potent antioxidant activity was exhibited by saponarin. The addition of 2 μmol/mL of saponarin inhibited the formation of MA from squalene by almost 100%, whereas BHT required 16 μmol/mL to obtain a 75% effect. α-Tocopherol did not show any appreciable antioxidant activity in this system.

Antioxidant activities of saponarin and selected essentials oils and their components tested by the more simulated skin system were also reported.[49−51] Squalene mixed with various amounts of antioxidants was coated on the inside wall of a test tube and then irradiated by UV light (λ = 300 nm) for 12 h. Figure 6 shows the results of antioxidant activity of saponarin, essential oils, and their components from these experiments. Saponarin exhibited >90% antioxidant activity at all levels tested, which was comparable to that of BHT at the level of 500 μg/mL. Among the essential oils reported, thyme oils showed the highest antioxidant activity, followed by parsley seed oil, clove leaf oil, rose oil, and cinnamon leaf oil. However, parsley seed oil was the only one that exhibited dose−response activity. For example, thyme oil showed >80% activity at the level of 50 μg/mL, whereas it exhibited only 53% activity at the level of 100 μg/mL. The potent antioxidant activity of thyme oil and clove leaf oil may be revealed by the presence of thymol and eugenol, respectively, because they showed strong antioxidant activity with dose response as shown in Figure 6.

These reports indicate that some naturally occurring antioxidant can prevent the skin diseases associated with oxidative damages caused by UV irradiation. Also, they can be used in skin care products as a medicinal supplement.

Inhibition of Oxidation toward Various Lipids. It is well-known that lipids are degraded into various toxic chemicals, such as low molecular weight carbonyl compounds, upon oxidation. Once a lipid molecule is activated by ROS, including superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), triplet oxygen ($^3O_2$), hydroxy radical ($^{\bullet}OH$), alkoxy radical ($RO^{\bullet}$), and peroxy radical ($ROO^{\bullet}$), degradation occurs.[52] The basic mechanisms of lipid peroxidation have been advanced by many researchers.[53−56] An ROS abstracts a hydrogen atom from a methylene group of an unsaturated fatty acid and subsequently forms free radicals such as a peroxy radical.[57] Once these free

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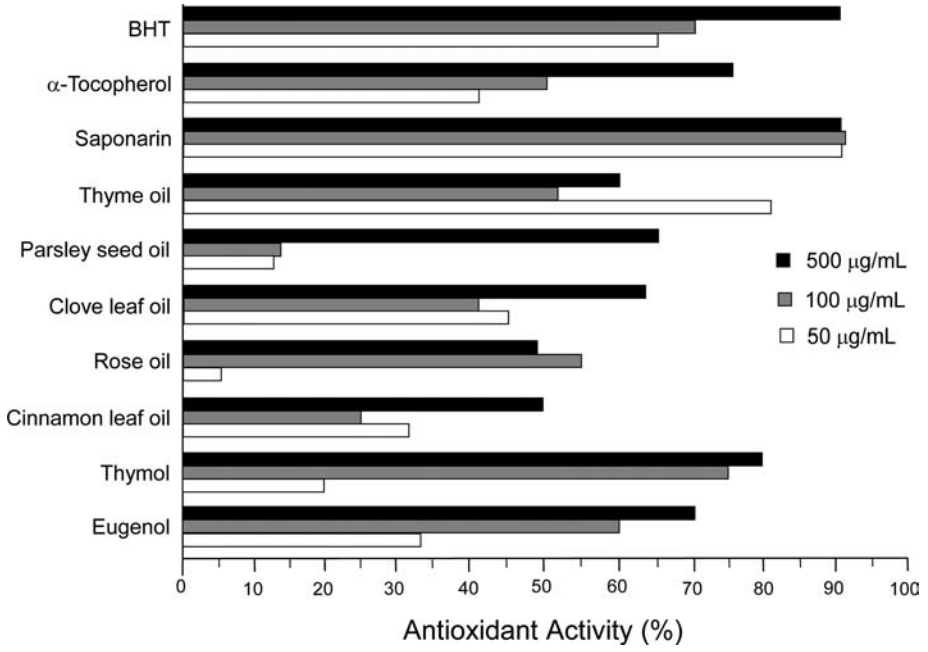

Figure 6. Results of antioxidant activity test on saponarin, essential oils, and their components.

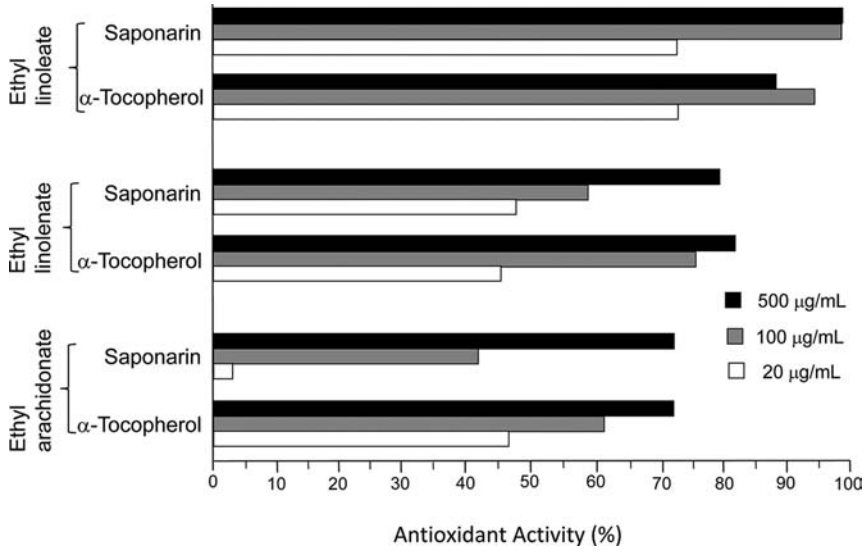

Figure 7. Inhibitory effects of saponarin and α-tocopherol toward fatty acid ethyl esters oxidized by Fenton's reagent.

radicals are formed, lipid peroxidation progresses and, consequently, many secondary oxidation products are formed.

As mentioned earlier, MA may be used most widely as a biomarker for various studies associated with lipid peroxidation. However, its toxicity has not yet been well established. The fact that MA reacts with DNA to form adducts to deoxyguanosine and deoxyadenosine subsequently implicates it in mutagenicity and carcinogenicity.[58] Therefore, monitoring MA formation is one avenue to investigate the role of antioxidant in lipid peroxidation for disease prevention.

Fatty Acid Ethyl Esters. Figure 7 shows inhibitory effects of saponarin toward fatty acid ethyl esters oxidized by Fenton's reagent along with those of a standard antioxidant, α-tocopherol (prepared on the basis of results reported previously[2]). Saponarin inhibited ethyl linoleate oxidation by almost 100% at the level of 100 μg/mL, which was approximately 5% higher than the inhibition shown by α-tocopherol. Overall, the inhibitory effect of saponarin was higher than that of α-tocopherol. Generally, the lipids with a greater number of unsaturations exhibited the higher resistance toward antioxidant inhibition. Both antioxidants showed highest antioxidant activity toward ethyl linoleate (two unsaturations), followed by ethyl linonenate (three unsaturations) and ethyl arahidonate (four unsaturations). The results indicate that saponarin has appreciable antioxidant activity toward fatty acid ethyl esters at levels >100 μg/mL. These tests were performed at pH 7.4, which is a simulated biological system. Another study reported that both antioxidants exhibited much higher antioxidant activity toward ethyl linoneate and ethyl linonenate at pH 7.4 than at pH 3.5 or 11, indicating that these antioxidants work best under the biological condition.[59]

ω-3 Polyunsaturated Fatty Acids. ω-3 polyunsaturated fatty acids, including octadecatetraenoic acid (ODA), eicosapentaenoic acid (EPA), and docosahexaenoicc acid (DHA), are

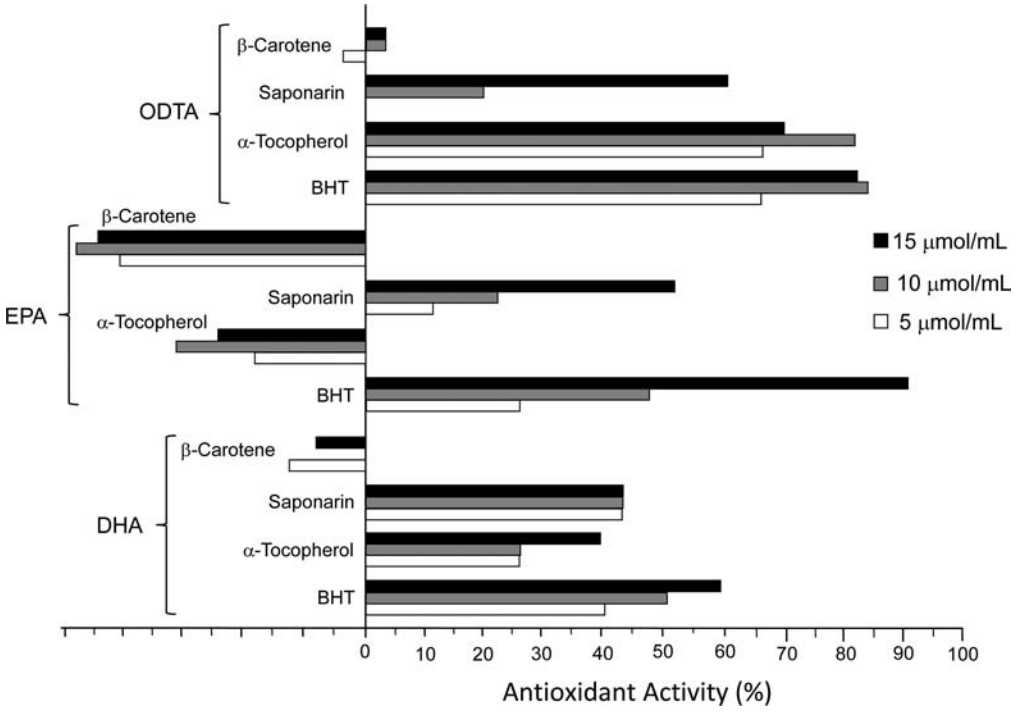

Figure 8. Inhibitory effect of saponarin, β-carotene, α-tocopherol, and BHT toward ω-3 polyunsaturated fatty acids.

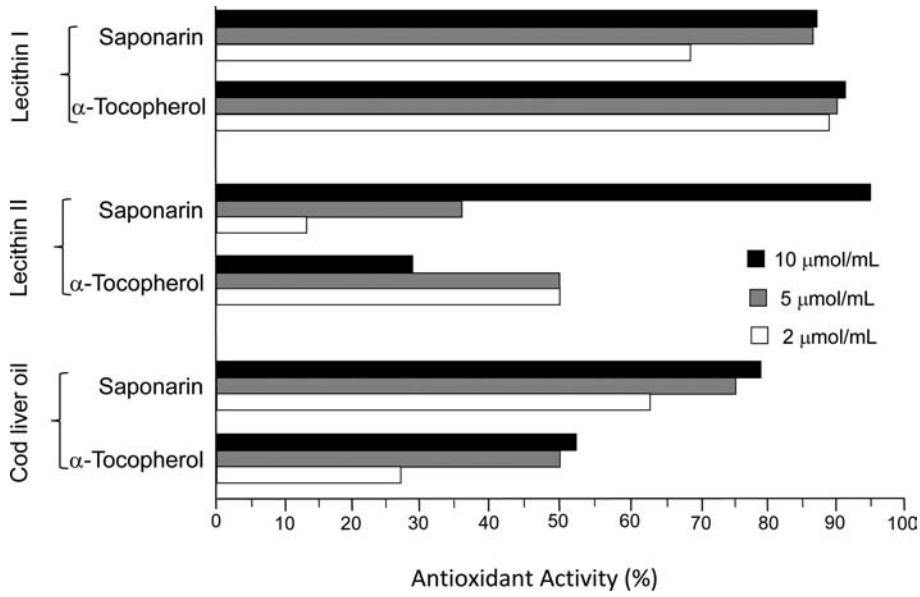

Figure 9. Antioxidant activity of saponarin and α-tocopherol toward lipoproteins and cod liver oil.

known to possess effects on the reduction of coronary heart disease and hypertension.[60,61] Recent reviews have reported biological effects of these ω-3 polyunsaturated fatty acids on Alzheimer's disease,[62] various cancers,[63] inflammation,[63,64] cardiovascular disease,[65] HIV,[66] depression,[67] and aging.[68] As mentioned above, most of these diseases are directly or indirectly associated with oxidative damages, and ω-3 polyunsaturated fatty acids are the lipids most susceptible to oxidation.[69]

Figure 8 shows the inhibitory effects of saponarin, β-carotene, α-tocopherol, and BHT toward three ω-3 polyunsaturated fatty acids (prepared from the data reported previously[70]). Saponarin exhibited dose−response antioxidant activity toward all three ω-3 polyunsaturated fatty acids. It inhibited MA formation in ODTA by 60%, in EPA by 50%, and in DHA by 43% at the level of 15 μmol/mL. Those activities were 30−40% lower than those of BHT in the three ω-3 polyunsaturated fatty acids. However, its activity was higher than that of α-tocopherol at all levels in DHA. β-Carotene, the antioxidant activity of which has been reported,[71,72] did not exhibit any inhibitory effects with all three ω-3 polyunsaturated fatty acids. Moreover, it showed some pro-oxidative activity with EPA and DHA. This may be because certain amounts of MA were formed from β-carotene itself by Fenton's reagent. These results suggest that saponarin prevents the oxidation of ω-3 polyunsaturated fatty acids and the formation of toxic MA.

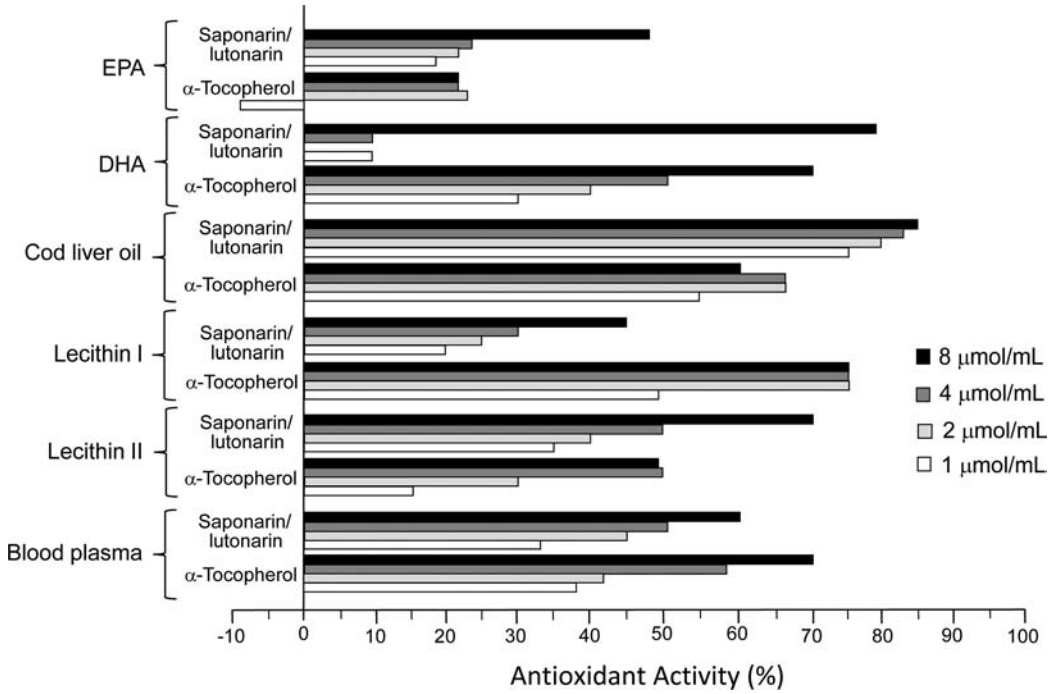

Figure 10. Antioxidant activities of a mixture of saponarin (S) and lutonarin (L) (S/L = 4.5:1, w/w).

Lipoproteins and Cod Liver Oil. Recent papers have indicated that oxidized lipoprotein is strongly associated with arteriosclerosis[73] as well as oxidized ω-3 polyunsaturated fatty acids mentioned above. Therefore, it is important to inhibit the oxidation of lipoprotein to prevent blood-related diseases. Figure 9, prepared with data published previously,[3] shows the antioxidant activity of saponarin toward lipoproteins and cod liver oil along with that of a standard natural antioxidant, α-tocopherol. Saponarin exhibited strong antioxidant activities with dose response in the three samples, which were higher than those of α-tocopherol. In particular, it inhibited MA formation from lecithin II by >95% at the level of 10 μmol/mL, whereas that of α-tocopherol was 30% at the same level. Another paper demonstrated that saponarin inhibited MA formation by 60% in blood plasma, which contains lipoproteins, at the level of 100 nmol/mL.[74]

Cod liver oil contains high levels of ω-3 polyunsaturated fatty acids: 10% EPA and 30−33% DHA.[75] Fish oils have been used for various health therapies, such as cardiovascular disease.[65] However, massive doses of fish oil as a treatment for diseases may cause biological complications due to the formation of toxic carbonyl compounds including glyoxal, malonaldehyde, and diacetyl.[3] The results of this study indicate that saponarin effectively stabilizes lipoproteins and fish oils.

ANTIOXIDANT ACTIVITY OF A SAPONARIN/LUTONARIN MIXTURE ISOLATED FROM YOUNG GREEN BARLEY LEAVES

Young green barley leaves contain lutonarin in addition to saponarin. However, lutonarin content is much less than that of saponarin. Therefore, the flavonoid fraction obtained from freeze-dried young green barley was also examined for antioxidant activity.

An aqueous solution of freeze-dried barley leaves was boiled for 1 h in water and then filtered. The filtrate was poured onto a glass column packed with XAD-2 resin. The column was eluted with 500 mL each of aqueous methanol solution (10, 20, 30, 40, and 50%) in series. The fractions eluted with 30, 40, and 50% methanol solutions were combined. After centrifugation, the solid layer was recrystallized with methanol. A light yellow powder containing saponarin (81.72%) and lutonarin (18.28%) was tested for antioxidant activity using the MA/GC assay.

Figure 10 shows the antioxidant activities of a mixture of saponarin (S) and lutonarin (L) (S/L = 4.5:1, w/w) (prepared from the data published previously[4]). A S/L mixture inhibited MA formation appreciably from all lipids tested with dose response. It exhibited the highest effect toward cod liver oil, followed by DHA, lecithin II, blood plasma, EPA, and lecithin I. Its effect toward cod liver oil was 86.0% at the level of 8 μmol/mL, whereas that of α-tocopherol was 57.5% at the same level. It exhibited higher activity toward cod liver oil and lecithin II than α-tocopherol did at all levels tested. On the other hand, it showed slightly less activity toward lecithin I and blood plasma than α-tocopherol did at all levels tested. Overall, a S/L mixture showed, however, antioxidant activities comparable to those of α-tocopherol.

CONCLUSION

In particular, people in developed countries, such as the United States and Japan, tend to eat more lipid-rich foods and consequently increase their risk of disease incidences caused by oxidative damage, such as atherosclerosis. It is well-known that antioxidants are beneficial to human health because they inhibit oxidative damage. Therefore, antioxidants, such as vitamins E and C, have been used to treat various diseases associated with oxidative damage. However, use of synthetic antioxidants, such as BHT, in human foods has been restricted because of their possible chronic toxicity.

The papers discussed in this review presented potent antioxidant activity of a flavonoid, saponarin, found in young green barley leaves. There have also been many reports on the antioxidant activity of flavonoids present in natural plants. Therefore, supplementation with young green barley leaves

containing saponarin should be beneficial to health and may prevent the diseases mentioned above.

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Notes

The authors declare no competing financial interest.

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