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Antioxidant Activity of Flavonoids Isolated from Young Green Barley Leaves toward Biological Lipid Samples

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Natural plant flavonoids, saponarin/lutonarin = 4.5/1, isolated from young green barley leaves were examined for their antioxidant activity using cod liver oil, ω-3 fatty acids, phospholipids, and blood plasma. The saponarin/lutonarin (S/L) mixture inhibited malonaldehyde (MA) formation from cod liver oil by 76.47 ± 0.11% at a level of 1 μmol and 85.88 ± 0.12% at a level of 8 μmol. The S/L mixture inhibited MA formation from the ω-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by 45.60 ± 1.08 and 69.24 ± 0.24%, respectively, at a level of 8 μmol. The S/L mixture inhibited MA formation from the phospholipids lecithin I and II by 43.08 ± 0.72 and 69.16 ± 2.92%, respectively, at a level of 8 μmol. It also inhibited MA formation from blood plasma by 62.20 ± 0.11% at a level of 8 μmol. The antioxidant activities obtained from the S/L mixture were comparable to those obtained from α-tocopherol and butylated hydroxy toluene (BHT) in all lipids tested.

KEYWORDS: Antioxidant; blood plasma; cod liver oil; lutonarin; malonaldehyde; saponarin

INTRODUCTION

The association between lipid peroxidation and illness, such as atherosclerosis (*1*, *2*), diabetes (*3*), Alzheimer's (*4*), cancer (*5*, *6*), and AIDS (*7*), is well-known (*8*). Therefore, antioxidants, in particular natural antioxidants, have received much attention as chemicals useful in preventing lipid peroxidation associated with the various diseases mentioned above.

Flavonoids, which are naturally occurring polyphenolic compounds, have been found in fruits and vegetables, and their dietary intake is quite high compared to other dietary antioxidants such as vitamins C and E (*9*). To date, more than 4000 different flavonoids have been identified (*9*). In plants, these compounds are known to protect against damage caused by ultraviolet radiation, pathogens, and herbivores (*10*). In mammals, studies on flavonoids have shown a range of beneficiary actions, such as antiviral, anti-inflammatory, antiallergic properties (*11*), cardioprotective effects (*12*), and anti-carcinogenic effects (*13*, *14*, *15*). Most of these beneficiary effects are due to the antioxidant and chelating abilities of flavonoids. Flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and other free radicals produced by lipid peroxidation (*16*−*18*).

Lipid peroxidation is initiated with a reactive oxygen species such as a hydroxyl radical to form a hydroperoxide followed by production of many reactive secondary oxidized products, including formaldehyde, acetaldehyde, malonaldehyde (MA), and glyoxal (*19*). There have been many reports on the formation of these reactive secondary oxidized products from lipids, such as lanoline acid, arachidonic acid, and various ω-3 fatty acids, (*19*, *20*−*22*). The biological consequences are caused by the interaction of these secondary products with proteins, such as DNA and RNA (*23*). Therefore, monitoring the formation of these secondary lipid peroxidation products is a convenient method for investigating diseases caused by oxidative damage.

MA has been widely utilized as a biomarker for various studies associated with oxidative damage because MA is one of the final products of lipid peroxidation (*19*, *20*, *24*). A specific gas chromatographic method to determine MA (MA/GC assay) has been developed (*25*−*29*). This method involves derivatization of MA to stable, nitrogen containing 1-methyl pyrazole, which is subsequently analyzed by a gas chromatograph with a nitrogen−phosphorus detector. Many studies on the antioxidant activity of natural products have been conducted using this MA/GC assay to monitor the amount of MA formed in various lipid peroxidation model systems, including fatty acids (*30*), phospholipids (*31*), ω-3 fatty acids (*32*), blood plasma (*33*), and DNA (*34*).

In the present study, the antioxidant activity of flavonoids isolated from young green barley leaves was investigated in various biological matrixes using an MA/GC assay.

MATERIALS AND METHODS

Chemicals and Materials. Cod liver oil, trizma HCl, trizma base, *N*-methylhydrazine, and 2-methylpyrazine were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Butylated hydroxy toluene (BHT), sodium dodecyl sulfate (SDS), hydrogen peroxide, sodium sulfate, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), dimethyl sulfoxide (DMSO), and HPLC-grade methanol were bought from Fisher Scientific (Fairlawn, NJ). 1,2-Dilinoleoyl-*sn*-glycero-3-

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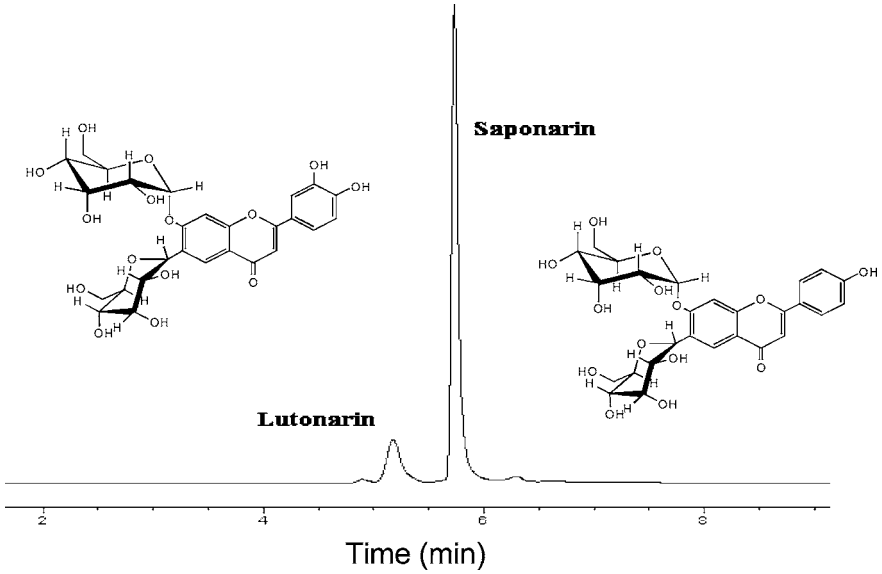

Figure 1. Sample high performance liquid chromatogram of the flavonoid mixture.

phosphocholine (lecithin I) and 1,2-dilinolenoyl-*sn*-glycero-3-phosphocholine (lecithin II) were purchased from Avanti Polar Lipids (Alabaster, AL). Donor horse serum and donor bovine serum were purchased from Atlanta Biologicals (Lawrenceville, GA). Bond Elut C18 SPE cartridges were purchased from Varian, Inc. (Chicago, IL).

Standard stock solutions of the testing antioxidants were prepared as follows: a 0.005 M flavonoid mixture (saponarin/lutonarin = 4.5/1) solution was prepared by dissolving 28.88 mg of the complex in 10 mL of DMSO, a 0.01 M α-tocopherol solution was prepared by dissolving 107.7 mg in 25 mL of DMSO, and a 0.01 M BHT solution was prepared by dissolving 220.4 mg in 100 mL of DMSO.

A standard stock solution for antioxidant testing was prepared by dissolving 0.25 mmol of phosphate buffer (pH 7.4), 1 μmol of $FeCl_2$, 0.5 μmol of H_2O_2, 0.75 mmol of KCl, and SDS (0.2% in the stock solution) into 5 mL of deionized water.

Isolation and Purification of Flavonoids from Young Green Barley Leaves. Flavonoids were isolated from young green barley leaves (*Hordium vulgare L. var. nudum Hook*) harvested 2 weeks after germination by a previously reported method (*35*). The freeze-dried barley leaves (300 g) were dispersed into 1500 mL of deionized water. After the solution was boiled for 1 h and cooled to room temperature, it was poured onto a glass column (45 cm × 4.5 cm, i.d.) packed with XAD-2 resin. After the solvent was eluted, 2 L of deionized water was poured through the column. Subsequently, 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 the combined eluate was allowed to stand overnight at 5 °C in a refrigerator, the solution was centrifuged. The solid layer was recrystalized with methanol. Approximately 1 g of a light yellow powder, a saponarin (81.72%) and lutonarin (18.28%) mixture, was obtained.

The main flavonoid with antioxidant activity found previously in green barley leaves was tentatively identified as 2″-*O*-glycosylisovitexin (*35*). Later, this main flavonoid from green barley leaves was characterized as saponarin and not 2″-*O*-glycosylisovitexin (*36*). In the present study, saponarin was the main flavonoid in green barley leaves as reported in this article.

An Agilent model 1100 high performance liquid chromatograph (HPLC) equipped with a model G1365B multiple wavelength detector and a 250 mm × 4.6 mm i.d. C_{18} Alltech Econosil column was used for analysis of the saponarin and lutonarin mixture. The structural confirmation of saponarin and lutonarin was conducted by an Agilent model 1100 HPLC interfaced to an Applied Biosystems API 2000 MS/MS via an atmospheric pressure chemical ionization (APCI) source operating in the positive ion mode at 475 °C with nitrogen gas. An HPLC chromatogram of these flavonoids is shown in **Figure 1** along with their structure.

Sample Preparations of Lipids for MA/GC Assay. Cod liver oil (10 μL), lecithin I (5 mg), lecithin II (5 mg), EPA (10 mg), DHA (10 mg), or blood plasma (50 μL) was added to the antioxidant testing solution prepared above (5 mL). Subsequently, 1, 2, 4, and 8 μmol each of standard stock solution of antioxidants was added to each lipid solution. An aqueous solution (5 mL) containing just a lipid and SDS was prepared as a blank sample. A control sample was prepared for each lipid using the same method but without the addition of antioxidants. Each solution was incubated at 37.1 °C for 16 h in a water bath. While incubating, the samples were covered in aluminum foil to prevent any influence of light on the peroxidation system. After incubation, oxidation was stopped by adding 50 μL of 4% BHT according to a previous report (*37*).

Analysis of MA. The MA formed in each sample was analyzed after it was derivatized into 1-methylpyrazole (1-MP) by a previously reported method (*34*). *N*-Methylhydrazine (30 μL) was added to each incubated sample solution and constantly stirred with a magnetic stirrer at room temperature for 1 h. The reaction solution was placed in a C_{18} SPE cartridge (Varian, Inc., Chicago, IL) and then eluted with 5 mL of ethyl acetate under reduced pressure. The SPE cartridge was preconditioned by rinsing with one volume each of ethyl acetate, methanol, and deionized water, in series, prior to use. The eluent was concentrated under a purified nitrogen stream to 0.5 mL in volume. The volume of the ethyl acetate eluent was brought to 5 mL with ethyl acetate, and then, 10 μL of 2-methylpyrazine solution (10 mg/mL ethyl acetate) was added as a GC internal standard. MA was analyzed as 1-methylpyrazole by a GC with a nitrogen−phosphorus detector (NPD). An Agilent 6890 gas chromatograph equipped with a DB-5 fused-silica capillary column (30 m × 0.25 mm × 2.5 μm) (J&W Scientific, Folsom, CA), and an NPD was used for quantification of 1-methylpyrazole. The initial oven temperature was 40 °C, held for 1 min and programmed to 90 °C at 10 °C/min. The injector temperature was 200 °C, and the detector temperature was 325 °C. The helium carrier gas flow rate was 1 mL/min.

RESULTS AND DISCUSSION

The amount of MA formed from each lipid sample without an antioxidant (control) was 31.9 ± 0.0 μg from 10 μL of cod liver oil, 7.6 ± 1.2 μg from 5 mg of lecithin I, 6.6 ± 0.6 μg from 5 mg of lecithin II, 5.8 ± 0.3 μg from 10 mg of EPA, 62.6 ± 1.5 μg from 10 mg of DHA, and 61.6 ± 1.5 μg from 50 μL of blood plasma. Values are mean ± standard error (SE) (n = 3), and blank values (less than 0.1 μg from all samples) were subtracted. **Figure 2** shows the inhibitory effect of antioxidants toward cod liver oxidation. The values are mean

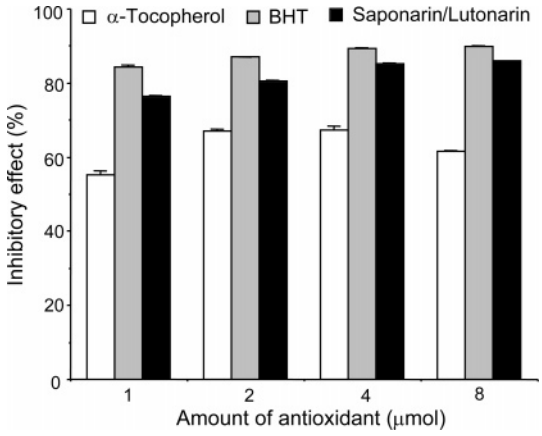

Figure 2. Inhibitory effect of antioxidants toward MA formation from cod liver oil oxidized with Fenton's reagent.

± standard deviation (SD) ($n = 3$). The inhibitory effect was calculated using the following equation:

$$\text{Inhibitory effect (\%)} = \frac{\text{MA formed in control} - \text{MA formed in sample with antioxidant}}{\text{MA formed in control}} \times 100$$

α-Tocopherol and BHT were used as a standard natural antioxidant and a standard synthetic antioxidant, respectively. The saponarin/lutonarin (S/L) mixture and BHT showed a dose response effect. Both samples exhibited high inhibitory effect at a low level of 1 μmol, where the S/L mixture inhibited MA formation by 76.47 ± 0.11%. At the highest level tested (8 μmol), the S/L mixture inhibited MA formation by 85.88 ± 0.12%. The highest inhibition (89.95 ± 0.20%) was obtained from BHT at a level of 8 μmol. α-Tocopherol exhibited the lowest effect among the three samples. α-Tocopherol inhibited MA formation by 55.10 ± 1.34% at a level of 1 μmol, and its inhibitory effect did not change significantly when the level was increased.

α-Tocopherol inhibited the formation of secondary oxidation products, such as low molecular weight aldehydes, in the oxidation of cod liver oil (*38*). Caffeic acid also inhibited cod liver oil oxidation induced by heat (100 °C). It inhibited diene conjugation and reduced losses of ω-3 polyunsaturated fatty acids (*39*). A previous study of MA formation by a flavonoid found in young green barley leaves (*40*) was consistent with the results obtained in the present study. Cod liver oil is often used in an in vitro model system to investigate the role of lipid peroxidation in cardiovascular disease (*41*) because it contains high levels of ω-3 fatty acids (*42*).

Figure 3 shows the inhibitory effects of antioxidants toward MA formation from EPA. The values are mean ± SD ($n = 3$). At the lowest level of 1 μmol, α-tocopherol showed prooxidant effects, increasing MA formation by 7.17%. Once the level was increased, α-tocopherol exhibited antioxidant activity. However, its effect was just moderate (23.5−26.4%). The S/L mixture also exhibited moderate effects. The S/L mixture inhibited MA formation by 45.60 ± 1.08% at a level of 8 μmol. Even BHT inhibited MA formation by only 52.69 ± 2.73% at the highest level of 8 μmol, suggesting that EPA oxidation is hard to prevent.

Figure 4 shows the inhibitory effects of antioxidants toward MA formation from DHA. The values are mean ± SD ($n = 3$). α-Tocopherol and BHT showed similar inhibitory effects with clear dose response toward MA formation from DHA. On the other hand, the S/L mixture did not exhibit significant effects at levels of 1, 2, and 4 μmol toward MA formation from DHA. It does, however, show a high inhibitory effect at a level of 8 μmol. It inhibited MA formation from DHA by 69.24 ± 0.24%, which was higher than those of BHT (65.23 ± 0.41%) and α-tocopherol (60.00 ± 0.73%) at the same level, suggesting that the S/L mixture is an excellent antioxidant for DHA oxidation at higher levels.

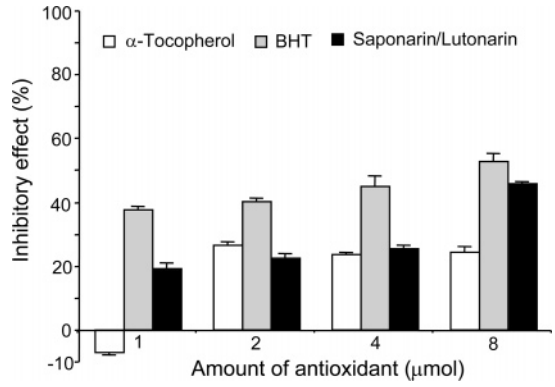

Figure 3. Inhibitory effect of antioxidants toward MA formation from EPA oxidized with Fenton's reagent.

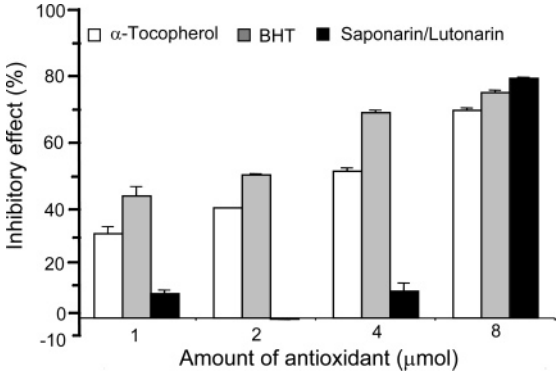

Figure 4. Inhibitory effect of antioxidants toward MA formation from DHA oxidized with Fenton's reagent.

Fish oil supplements, containing ω-3 fatty acids, reportedly had a beneficial effect on blood pressure and serum triacylglycerols in patients treated for coronary artery disease (*41*). However, it is well-known that polyunsaturated ω-3 fatty acids are extremely susceptible to oxidative deterioration. Therefore, much research has been conducted to prevent lipid peroxidation occurring in ω-3 fatty acids. For example, extracts from grapefruit seed, astaxanthin, soybean lecithin, and green tea stabilized the shelf life of anchovy oil containing high levels of ω-3 fatty acids, whereas α-tocopherol did not stabilize the same sample (*43*). BHT, vitamin C, and vitamin E inhibited lipid peroxidation of ω-3 fatty acids prepared from cod liver oil.

Figure 5 shows the inhibitory effects of antioxidants toward MA formation from lecithin I. The values are mean ± SD ($n = 3$). α-Tocopherol showed the greatest inhibitory effects at all levels, compared to BHT and S/L. α-Tocopherol inhibited MA formation by 74.82 ± 0.90% at a level of 2 μmol, whereas BHT and the S/L mixture inhibited only 38.29 ± 0.33 and 26.76 ± 0.13%, respectively, at the same level. A clear dose response effect was exhibited by BHT. The highest effect among the three antioxidants was obtained from BHT (84.71 ± 0.14% at a level of 8 μmol). Compared with the two standard antioxidants, the S/L mixture exhibited only moderate effects of 43.08 ± 0.72% at a level of 8 μmol.

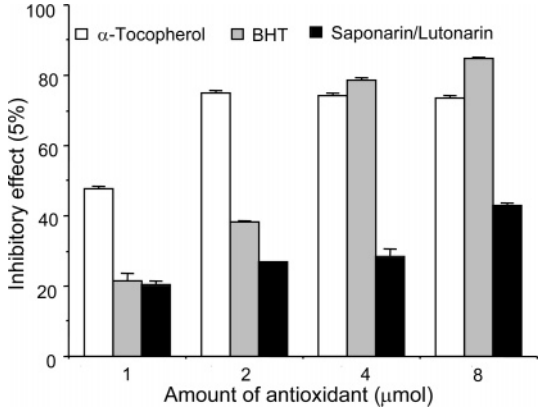

Figure 5. Inhibitory effect of antioxidants toward MA formation from lecithin I oxidized with Fenton's reagent.

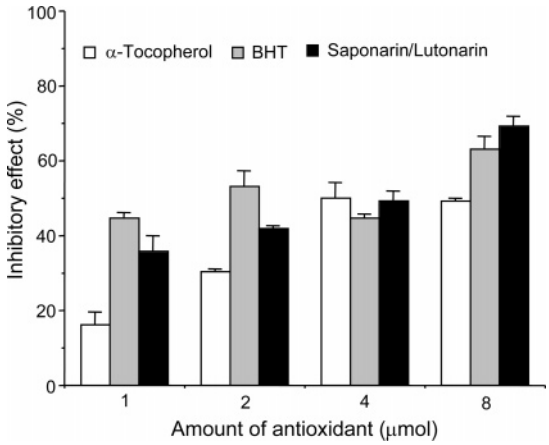

Figure 6. Inhibitory effect of antioxidants toward MA formation from lecithin II oxidized with Fenton's reagent.

Figure 6 shows the inhibitory effects of antioxidants toward MA formation from lecithin II. The values are mean ± SD ($n = 3$). All three antioxidants exhibited similar effects. At a level of 4 μmol, all three antioxidants showed inhibitory effects of 45−50%. The greatest effect toward lecithin II oxidation was obtained from the S/L mixture, which inhibited MA formation by 69.16 ± 2.92% at a level of 8 μmol.

Phospholipids or phosphatide, including lecithin I and II, are characteristic major components of cell membranes. Phospholipids are also known to be susceptible to oxidation and form toxic secondary oxidation products including glyoxal and MA (*40*). Therefore, there have been many reports on the inhibitory effects of certain chemical(s) to lecithin oxidation. For example, melanins inhibited lecithin photooxidation and the extent of this effect strongly depended on the type and concentration of melanin (*44*). Flavonoids isolated from various plants including hawthorn fruits, grape seed, Japanese quince fruits, and *Rosa rugosa* hips exhibited potent antioxidant activity to the oxidation of lecithin liposomes (*45*). Some Mediterranean herbs also exhibited antioxidant activity toward iron promoted oxidation of phospholipids (*46*). The results obtained from the present study are consistent with those previous reports.

Figure 7 shows the inhibitory effects of antioxidants toward MA formation from blood plasma. The values are mean ± SD ($n = 3$). α-Tocopherol and the S/L mixture exhibited similar inhibitory effects on horse blood plasma. Both samples showed dose response activity. The S/L mixture had a moderate effect at low levels (33.46 ± 0.27) at 1 μmol. α-Tocopherol and the S/L mixture inhibited MA formation by 71.77 ± 0.04 and 62.20 ± 0.11% at a level of 8 μmol, respectively. On the other hand,

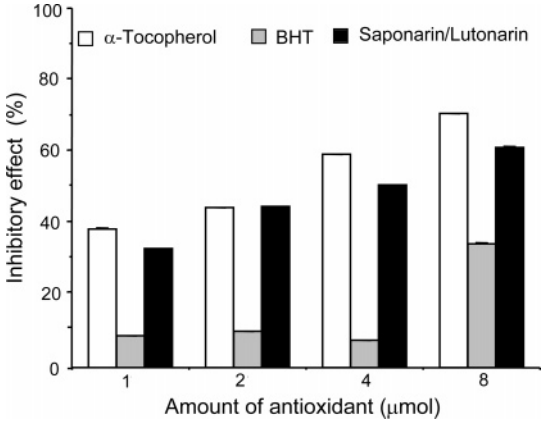

Figure 7. Inhibitory effect of antioxidants toward MA formation from blood plasma oxidized with Fenton's reagent.

BHT exhibited no appreciable effect at the lower levels of 1, 2, and 4 μmol. Even at a level of 8 μmol, BHT inhibited MA formation by 34.91 ± 0.20%, which is only half of that of α-tocopherol. It is difficult to explain this result, but BHT might be consumed to inhibit the oxidation of some unknown water soluble substrate in the sample.

In another report, flavonoid and other naturally occurring compounds exhibited a clear inhibitory effect toward MA formation from horse blood plasma (*33*). Aroma extracts from eucalyptus and clove also inhibited MA formation from horse blood plasma by 23 and 48%, respectively (*47*). There are also many reports on investigations of blood plasma oxidation using low density lipoprotein (LDL) prepared from blood plasma. A complex of antioxidant vitamins (C and E) inhibited LDL phospholipids in blood plasma (*48*). Phenolic constituents isolated from safflower seed inhibited in vitro LDL oxidation, and these phenolic compounds were found as both intact and conjugated metabolites in the blood plasma of experimental mice fed a safflower seed diet (*49*). These reports suggest that naturally occurring compounds including flavonoids have protective effects against atherosclerosis caused by lipoprotein oxidation in blood plasma.

There have been several reports on the biological activities of young green barley leaf extract based on studies using experimental animals and humans. When a male New Zealand white rabbit (1−1.5 kg bw) was fed with a feed containing 1% young barley leaf extract for 12 weeks, the plasma levels of triacylglycerol, total cholesterol, and LDL-C decreased significantly (*50*). A study using type 2 diabetes patients suggested that supplementation with green barley leaf extract protected against vascular diseases in diabetes patients (*51*). It was also found that barley leaf extract possessed certain positive effects toward plasma lipids and LDL oxidation in hyperlipidemic smokers (*52*). All of these reports suggest that barley leaf extract contains some biologically active components, particularly antioxidants. The present study suggests that these biological activities of green barley leaf extract are due to the antioxidant activity of flavonoids including saponarin and lutonarin. An essence prepared from young green barley leaves is being consumed already in the U.S., Japan, and Europe. Barley leaves can be one of the dietary sources of naturally occurring antioxidants.

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