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Natural Antioxidants. II.[1] Antioxidative Components Isolated from Seeds of *Plantago asiatica* LINNE

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Antioxidative components in the methanol extract of seeds of *Plantago asiatica* L. were investigated by using our test method based on the air oxidation of linoleic acid. Geniposidic acid, an iridoid glucoside, was found to be the most potent antioxidative component. The 50% inhibitory concentration (IC_{50}) of geniposidic acid was 1.56×10^{-2}% (thiobarbituric acid value), and this value was superior to that of *dl*-α-tocopherol (IC_{50}: 1.95×10^{-1}%). The antioxidative effects of other related iridoid glucosides (aucubin, geniposide, gardenoside) were also tested, but their activities were clearly weaker than that of geniposidic acid.

Keywords——*Plantago asiatica* seeds; antioxidant; linoleic acid air oxidation; geniposidic acid; iridoid glucoside; aucubin; geniposide; gardenoside; *dl*-α-tocopherol

Among the known antioxidants, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate, *dl*-α-tocopherol, *etc.* are widely used in Japan. Unfortunately, BHA was recently found to be carcinogenic,[2] and the use of these antioxidants is being reconsidered because of their toxicities.[3] Thus, new antioxidants, especially natural ones, are required. So far, natural antioxidants to have been identified include tocopherols in soybean oil,[4] flavonoids such as quercetin[5] and tannins such as catechin[6] in tea leaves, gossipol in cotton oil,[7] sesamol in sesame oil,[8] *etc.*

We also have been looking for new natural antioxidants by using our efficacy test method[9] based on the air oxidation of linoleic acid. In the preceding paper, we reported that *l*-epicatechin is the main antioxidative component in the leaves of green tea (*Thea sinensis* L.); its activity is comparable to that of BHA.[1] In addition, we recently reported that 20 kinds of methanol extracts obtained from 107 kinds of crude drugs which have been used in oriental medicine for a long time showed antioxidative activity against the air oxidation of linoleic acid.[10] In that report, we referred to the strong activity of the methanol extract of *Plantago asiatica* L. (Japanese name, "Shazenshi," Plantaginaceae), but no details have been reported.

In thie paper, we describe the isolation and antioxidative activity of geniposidic acid, a main antioxidative component of the methanol extract of seeds of *Plantago asiatica* L. The antioxidative activities of some compounds related to geniposidic acid were also investigated.

Experimental

The following instruments were used to obtain physical data. Infrared (IR) spectra were recorded on a JASCO IRA-202 infrared spectrophotometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were recorded on a JEOL LMN-900 FT spectrometer with tetramethylsilane (TMS) as an internal standard (δ value; s, singlet; d, doublet; t, triplet; m, multiplet). Thin-layer chromatography (TLC) was conducted on Kieselgel $60F_{254}$ (Merck). Column chromatography was carried out on silica gel (Kieselgel 60, 230—400 mesh, Merck). The developing solvent for TLC and the eluent for column chromatography were both chloroform–methanol–water (65 : 35 : 10, v/v, lower phase) mixture. The spots on TLC plates were detected by

spraying 50% H_2SO_4 followed by heating.

Reagents——BHA and BHT were purchased from Wako Pure Chemical Industries Co. *dl*-α-Tocopherol was obtained from Tokyo Kasei Co. Aucubin,[11] geniposide,[12] and gardenoside[12] were isolated by the reported methods and identified from the physical data. Other chemicals used were of special grade or equivalent quality.

Antioxidative Test——The antioxidative test was carried out under the conditions in the previous paper.[9]

Extraction and Separation——The dried seeds (500 g, Japanese commercial product) of *Plantago asiatica* L. were extracted three times with 3 l of hot methanol under reflux for 6 h. The extract was concentrated to dryness under reduced pressure. The resulting methanol extract (extract I, 80 g; yield 16.0%) was dissolved in water. The precipitate was removed by filtration. The filtrate was fractionated with *n*-hexane. *n*-Hexane extract (extract II, 4 g; yield 0.8%) and water extract (extract III, 7 g; yield 1.4%) were obtained. Extract III was dissolved in water again and fractionated with *n*-butanol. The water layer and *n*-butanol layer were each evaporated to dryness under reduced pressure to yield the water extract (extract IV, 3 g; yield 0.6%) and *n*-butanol extract (extract V, 2 g; yield 0.4%). The most active *n*-butanol extract, extract V, was repeatedly subjected to silica gel column chromatography. This separation was monitored by TLC. The substances corresponding to the major spots were isolated (S_I (Rf=0.18, 348 mg; yield 0.7%), S_{II} (Rf=0.24, 306 mg; yield 0.06%), S_{III} (Rf=0.30, 211 mg; yield 0.04%), S_{IV} (Rf=0.86, 140 mg; yield 0.03%)). S_{II}, the most potent antioxidant, was recrystallized with methanol. The substance obtained was identified as geniposidic acid by comparison of various physical data with those of authentic sample.

Geniposidic Acid——Colorless amorphous powder. TLC: Rf=0.24. IR ν_{max}^{KBr} cm^{-1}: 3300—3400, 1680, 1630. ^{1}H-NMR (D_2O) δ: 5.30 (d, J=7.0 Hz, C-1H), 5.96 (m, C-7H), 7.57 (s, C-3H). ^{13}C-NMR (D_2O) δ: 99.7 (d, C(1)), 153.0 (d, C(3)), 112.8 (s, C(4)), 35.2 (d, C(5)), 38.9 (t, C(6)), 129.6 (d, C(7)), 142.4 (s, C(8)), 46.6 (d, C(9)), 61.6 (t, C(10)), 171.8 (s, C(11)), 97.9 (d, C(1′)), 73.6 (d, C(2′), 76.5 (d, C(3′), 70.3 (d, C(4′)), 76.9 (d, C(5′), 60.5 (t, C(6′)). This sample was identical with an authentic sample of geniposidic acid (TLC, IR, ^{1}H-NMR, ^{13}C-NMR).[13]

Results and Discussion

The peroxide value (POV) and thiobarbituric acid value (TBAV) of linoleic acid after air oxidation were used as indexes of the antioxidant effects of test samples, as in the previous report.[9] The seeds of *Plantago asiatica* L. were extracted with hot methanol under reflux. The inhibitory ratio of the methanol extract (exctract I) added to linoleic acid at 0.1% concentration was high (100% for TBAV and 89% for POV). Thus, extract I was fractionated under the guidance of the efficacy test method[9] based on the air oxidation of linoleic acid. The water-soluble fraction of extract I was fractionated with *n*-hexane, yielding the *n*-hexane extract (extract II) and the water extract (extract III). The inhibitory ratio of extract II was low (37% for TBAV and 30% for POV) and that of extract III was high (100% for both TBAV and POV). Therefore, extract III was dissolved in water again and fractionated with *n*-butanol, yielding the water extract (extract IV) and the *n*-butanol (extract V) of extract III. The inhibitory ratio of extract IV was low (12% for TBAV and 6% for POV) and that of extract V was very high (100% for both POV and TBAV).

TABLE I. Effects of Various Fractions of Seeds of *Plantago asiatica* L. on the Air Oxidation of Linoleic Acid

Sample (0.1% added)	Inhibitory ratio	
	TBAV (%)	POV (%)
S_I	10	14
Geniposidic acid	100	97
S_{III}	9	10
S_{IV}	5	12
Aucubin	0	4
Geniposide	49	37
Gardenoside	0	0
BHA	100	100
BHT	100	100
dl-α-Tocopherol	17	20

The preparative TLC of extract V gave four main spots on a silica gel plate with the developing solvent of chloroform–methanol–water (65 : 35 : 10, v/v, lower phase). Thus, extract V was fractionated repeatedly by silica gel column chromatography with the same solvent. The antioxidative effects of the main substances (S_{I-IV}) on the air oxidation of linoleic acid are shown in Table I. Antioxidative activities were detected with $S_{I,III,IV}$, but the strongest antioxidative activity was observed with S_{II}. Thus, S_{II} was recrystallized from methanol. S_{II} was identified as geniposidic acid, an iridoid glucoside, by comparison with an authentic sample.

The relationship between inhibitory ratios and the added concentrations of tested samples was examined. All the active samples tested showed dose-dependent inhibitory effects on the air oxidation of linoleic acid. The 50% inhibitory concentration (IC_{50}) values were calculated from these results, and are listed in Table II.

The IC_{50} values of geniposidic acid were 1.82×10^{-2}% (POV) and 1.56×10^{-2}% (TBAV). Thus, geniposidic acid is a weaker antioxidant than BHA or BHT, but stronger than *dl*-α-tocopherol in terms of our evaluation method.

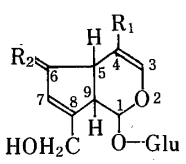
Inoue *et al.*[14] and Endo *et al.*[15] have already isolated geniposidic acid in the seeds of *Plantago asiatica* L. They identified it as geniposide pentaacetate, but did not refer to its antioxidative activities. The present finding is quite significant, since there is no previous report concerning antioxidant action of iridoid glucosides such as geniposidic acid.

We next examined the antioxidative effects of some iridoid glucosides related to geniposidic acid (aucubin, geniposide and gardenoside) by using the same test method. The results are shown in Table I; geniposide showed moderate antioxidative activity, but the other two showed no activity.

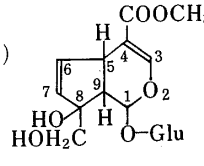
As shown in Chart 1, the difference between geniposidic acid (R_1=COOH) and geniposide (R_1=$COOCH_3$) lies only in the R_1 group. Thus, the proton in the R_1 group seems to be important for antioxidative activity. Aucubin (R_2= ) differs from geniposidic acid and geniposide in the R_2 group, and lacks the R_1 group. These data suggests that the R_1 group is very important for the antioxidative activity of geniposidic acid. On the other hand,

TABLE II. The 50% Inhibitory Concentration (IC_{50}) Values of Fractions of Seeds of *Plantago asiatica* L. for Inhibition of the Air Oxidation of Linoleic Acid

Sample	50% inhibitory concentration (IC_{50})	
	TBAV (%)	POV (%)
Extract I	5.93×10^{-2}	4.02×10^{-2}
Extract III	1.25×10^{-2}	1.21×10^{-2}
Extract V	1.23×10^{-2}	1.21×10^{-2}
Geniposidic acid	1.56×10^{-2}	1.82×10^{-2}
BHA	3.37×10^{-3}	3.75×10^{-3}
BHT	1.92×10^{-3}	2.24×10^{-3}
dl-α-Tocopherol	1.95×10^{-1}	2.48×10^{-1}



geniposidic acid (R_1=COOH, R_2=H_2)
aucubin (R_1=H, R_2= )
geniposide (R_1=$COOCH_3$, R_2=H_2)



gardenoside

Chart 1

gardenoside, whose chemical structure resembles that of geniposidic acid except in the position of a double bond and in the existence of a hydroxy group at C_8, showed no antioxidative activity. Further work on the structure–antioxidative activity correlation among iridoid glucosides is desirable

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