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Natural Antioxidants. I. Antioxidative Components of Tea Leaf (*Thea sinensis* L.)[1)]

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Antioxidative components in the leaves of green tea (*Thea sinensis* L.) were examined by using our evalution method based on the air oxidation of linoleic acid. *l*-Epicatechin showed the strongest activity, its 50% inhibitory concentration (IC_{50}: 3.54×10^{-3}%) was nearly equal to that of butylated hydroxyanisole (BHA).

Keywords——antioxidant; air oxidation; linoleic acid; *Thea sinensis* L.; *l*-epicatechin; *d*-catechin

In the previous paper, we reported a new and simple method[2)] for the evaluation of antioxidative substances. We are seeking natural antioxidants which are superior to *dl*-α-tocopherol, a typical natural antioxidant, and in the present work, we investigated the leaves of green tea (*Thea sinensis* L.). It is well known that tea leaf contains amino acids, saponins, tannins, caffeines, *etc.* However little is known about the antioxidative components in tea leaf, though Kajimoto identified epigallocatechin gallate and gallic acid by paper chromatography in the alcohol and water extract of tea leaf as antioxidative components.[3)] However, he did not use an antioxidative activity assay during the isolation of components of tea leaf.

In the paper, we describe the isolation and identification of antioxidative components in tea leaf.

Results and Discussion

The separation of antioxidative components in tea leaves was carried out according to the method described in the experimental section. The antioxidative effect of the fractions added to linoleic acid at 0.1% concentration is shown in Table I.

Extract III (*n*-butanol phase extract) and extract IV (acetone-soluble part of extract III) exerted the strongest inhibitory effect on the air oxidation of linoleic acid. Thus, extract IV was analyzed by thin layer chromatography (TLC) and four spots were detected on the TLC plate. Column chromatography of extract IV on silica gel yielded four substances (S_{I-IV}). Stronger antioxidative effects were observed with S_I and S_{II} (Table I). S_I was identified as caffeine by direct comparison with an authentic sample (infrared (IR) spectrum, mixed melting point and TLC). S_{II} appeared to be a mixture of *d*-catechin and *l*-epicatechin by comparing its IR spectrum and gas liquid chromatogram with those of authentic *d*-catechin and *l*-epicatechin. However, the separation of *d*-catechin and *l*-epicatechin in S_{II} was very difficult by usual methods. Thus, commercial *d*-catechin and *l*-epicatechin were used in tests of

TABLE I. Effects of Tea Leaf Fractions on the Oxidation of Linoleic Acid

Fraction (0.1% added)	Inhibitory ratio (I.R.)	
	TBAV (%)	POV (%)
Extract I	38	19
Extract II	42	18
Extract III	100	92
Extract IV	100	100
ppt I	44	13
S_I	97	75
S_{II}	100	100
S_{III}	37	9
S_{IV}	8	0
BHA	100	100
BHT	100	100
dl-α-Tocopherol	17	20

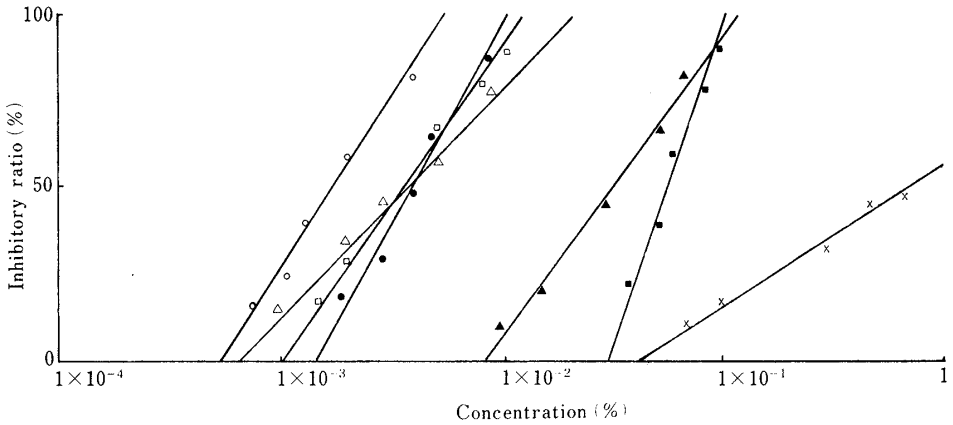

Fig. 1a. Relationship between I.R. and Added Concentration of Tea Leaf Components on the Oxidation of Linoleic Acid (TBAV)

▲—▲, caffeine; △—△, S_{II}; ■—■, *d*-catechin; □—□, *l*-epicatechin; ●—●, BHA; ○—○, BHT; ×—×, *dl*-α-tocopherol.

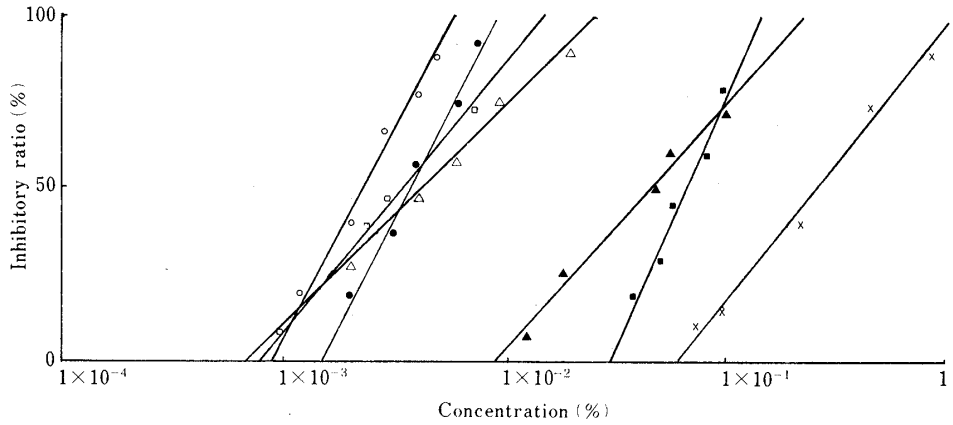

Fig. 1b. Relationship between I.R. and Added Concentration of Tea Leaf Components on the Oxidation of Linoleic Acid (POV)

▲—▲, caffeine; △—△, S_{II}; ■—■, *d*-catechin; □—□, *l*-epicatechin; ●—●, BHA; ○—○, BHT; ×—×, *dl*-α-tocopherol.

TABLE II. The 50% Inhibitory Concentration (IC_{50}) Values of S_{II}, Caffeine, *d*-Catechin and *l*-Epicatechin on the Oxidation of Linoleic Acid

Sample	50% inhibitory concentration (IC_{50})	
	TBAV (%)	POV (%)
S_{II}	3.62×10^{-3}	4.30×10^{-3}
Caffeine	2.93×10^{-2}	5.42×10^{-2}
d-Catechin	5.52×10^{-2}	6.63×10^{-3}
l-Epicatechin	3.54×10^{-3}	3.41×10^{-3}
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}

antioxidative effect.

The effects of caffeine, *d*-catechin, *l*-epicatechin and known antioxidants were examined. The relationship between inhibitory ratio (I.R.) and added concentration of each sample is shown in Fig. 1a (POV: peroxide value) and 1b (TBAV: thiobarbituric acid value).

All samples tested showed concentration-dependent inhibitory effects on the air oxidation of linoleic acid. The 50% inhibitory concentration (IC_{50}) values are listed in Table II. The IC_{50} of *l*-epicatechin was 3.54×10^{-3} (in TBAV), nearly equal to that of BHA. Thus, *l*-epicatechin was concluded to be the main antioxidative component of tea leaf in terms of effect on the air oxidation of linoleic acid.

Our result differs from Kajimoto's results.[3] He reported that epigallocatechin was the strongest component. This may be due to the differences in principle and method for finding active components. In any case, it is interesting that both our and his results indicated tannins to be the antioxidative components of tea leaf. We also found that caffeine inhibited the oxidation of linoleic acid (Table II), in contrast to Kajimoto's report.[3] Our result is consistent with that of Fujio *et al.*,[4] who first reported the antioxidative efficacy of caffeine.

Experimental

Material and Reagents——Tea leaves (green tea) grown in Shizuoka prefecture were obtained from a commercial source. Caffeine, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *dl*-α-tocopherol were purchased from Wako Pure Chemical Ind., Osaka, Japan. *d*-Catechin was obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan and *l*-epicatechin was a product of Aldrich Co., Ltd., U.S.A. Other chemicals used were of special reagent grade or equivalent.

General Methods——Melting points were determined with a Yanagimoto micro melting point apparatus and are uncorrected. Gas liquid chromatography (GLC) was carried out with a Shimadzu GC-6A chromatograph using 1.5% SE-30 on Chromosorb W (60—80 mesh) in a 1 m × 3 mm i.d. glass column with a flame ionization detector. Nitrogen was used as the carrier gas at a flow rate of 40 ml/min, and the oven temperature was 250 °C. TLC was performed on Merck precoated Silica gel 60 plates. Column chromatography was carried out on silica gel (Kieselgel 60, 70—230 mesh, Merck).

Antioxidative Test——The antioxidative test was carried out by our method[2] based on the air oxidation of linoleic acid. The effect of a test substance expressed in terms of inhibitory ratio (I.R., %) and 50% inhibitory concentration (IC_{50}). These indices were calculated in the same manner as in the previous paper.[2]

Separation of Antioxidative Components in Tea Leaf——Commercial powdered tea leaves (2.0 kg) were extracted repeatedly with boiling water and the extract was concentrated to dryness under reduced pressure. The resulting water extract (extract I, 728 g, yield 36.4%) was dissolved in water and the solution was then extracted with *n*-butanol. The water layer was concentrated to dryness (extract II, 470 g, yield 23.5%) and the *n*-butanol layer was also concentrated to dryness (extract III, 230 g, yield 11.5%). All of extract III was dissolved in 500 ml of methanol and this solution was added to 5 l of acetone. The precipitate (ppt I, 16 g, yield 0.8%) and extract IV (acetone-soluble part, 200 g, yield 10.0%) were obtained.

Isolation of Active Substances in Extract IV——Extract IV was developed on a silica gel plate (solvent system, chloroform–methanol–water (65 : 35 : 10, lower phase); detecting reagent, 1% $Ce(SO_4)_2$ in 10% H_2SO_4 solution) and found to consist of four substances (S_I $Rf=0.90$, S_{II} $Rf=0.71$, S_{III} $Rf=0.52$, S_{IV} $Rf=0.45$). These four substances were chromatographed on a silica gel column using chloroform–methanol–water (7 : 3 : 1, lower phase) as the eluent. The products were purified again by extraction with methanol (S_I) and by silica gel column chromatography using *n*-butanol–ethyl acetate–water (1 : 1 : 1, upper phase) as the eluent. The amounts of S_I—S_{IV} were 7.62 g (yield: 15.2%), 4.04 g (8.1%), 7.42 g (14.8%) and 6.52 g (13.6%), respectively.

S_I——Colorless needles, mp: 235—237 °C. IR ν_{max}^{KBr} cm^{-1}: 1696, 1652 (C=O), 1247 (C–N). This product was identified as caffeine by direct comparisons (mp, TLC and IR) with an authentic sample.

S_{II}——IR ν_{max}^{KBr} cm^{-1}: 1610 (C=O), 3400 (OH). The IR spectrum coincided with that of *l*-epicatechin of *d*-catechin, which have identical IR spectra.

GLC of S_{II}——Five mg of S_{II} or *d*-catechin or *l*-epicatechin was added to 0.2 ml of pyridine and then 0.2 ml of *N*-trimethylsilylimidazole was added. After degassing of this mixture, it was heated at 60 °C for 1 h. For GLC analysis, 0.1 μl of the reaction mixture was used. The retention time of authentic *l*-epicatechin was 15.47 min and that of authentic *d*-catechin was 16.90 min. The gas liquid chromatogram of S_{II} showed two peaks which coincided with *l*-epicatechin and *d*-catechin in retention times. Therefore, S_{II} was considered to be a mixture of *l*-epicatechin and *d*-catechin.

References and Notes

1) A part of this study was presented at the 103rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1983.
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