# Constituents of *Amomum tsao-ko* and Their Radical Scavenging and Antioxidant Activities

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**ABSTRACT:** Constituents of the fruit of *Amomum tsao-ko* were investigated following a preliminary screening of the antioxidant activity of several extracts of the fruit of this plant that showed that the dichloromethane extract and the ethyl acetate-soluble and water-soluble fractions of the 70% aqueous acetone extract had higher activity than α-tocopherol and butylated hydroxytoluene (BHT). Eleven compounds were isolated from the ethyl acetate-soluble fraction, and their structures were elucidated as (+)-hannokinol (**1**), *meso*-hannokinol (**2**), (+)-epicatechin (**3**), (–)-catechin (**4**), β-sitosterol (**5**), β-sitosterol 3-*O*-glucoside (**6**), 2,6-dimethoxyphenol (**7**), protocatechualdehyde (**8**), protocatechuic acid (**9**), vanillic acid (**10**), and *p*-hydroxybenzoic acid (**11**) based on mass and various nuclear magnetic resonance (NMR) spectroscopic techniques. This is the first isolation of epicatechin and catechin from the genus *Amomum*. The radical scavenging activity of the isolated compounds was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and colorimetric and electron spin resonance (ESR) analyses. The antioxidant activity of the compounds was also determined based on the oxidative stability index (OSI). The catechins and catechol derivatives showed strong activities in both the DPPH radical scavenging activity and antioxidant activity assays.

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**KEY WORDS**: *Amomum tsao-ko,* antioxidant, BHT, catechin, DPPH, epicatechin, ESR, oxidative stability index (OSI), radical scavenging, α-tocopherol.

There is currently a strong interest in natural antioxidants, especially those from edible plants, as evidenced by the escalating amount of published research on natural antioxidants. Such interest is triggered by the knowledge that antioxidants play an important role not only in the food industry, increasing the shelf life of food products and improving the stability of lipid and lipid-containing foods, but also in human health, preventing radical-induced diseases such as cancer and atherosclerosis.

As part of our research to discover new natural antioxidants from spices and herbs that are beneficial for human health (1), we have been investigating some species of cardamom (family Zingiberaceae). The fruits of these plants are used worldwide as spices and are one of the most ancient spices in the world and is one of the most highly valued. In addition to their importance as highly regarded spice, these are also used in traditional medicine for the treatment of several ailments such as stomach disorders, liver abscess, and infection of the throat (2,3). Preliminary screening of the antioxidant activity of these plants showed that, for *Amomum tsao-ko* Crevostet et Lamarié, its dichloromethane extract and the ethyl acetate-soluble and water-soluble fractions of its 70% aqueous acetone extract had higher activity than α-tocopherol and as much activity as butylated hydroxytoluene (BHT). This prompted us to undertake further study on the constituents of the fruit of *A. tsao-ko*. The present paper reports on the isolation of the constituents of the fruits of this plant and the radical-scavenging and antioxidant effects of the isolated compounds.

## EXPERIMENTAL PROCEDURES

*General procedures*. Nuclear magnetic resonance (NMR) ($^1$H at 500 MHz and $^{13}$C at 125 MHz) spectra were recorded with a Varian Unity 500 instrument (Palo Alto, CA) using tetramethylsilane (TMS) as internal standard. Electron impact–mass spectrometry (EI–MS) was done with a Hitachi M-2000 (Tokyo, Japan). Column chromatography (CC) was performed using Merck silica gel 60 (70–230 mesh; Darmstadt, Germany), Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), Chromatorex octadecyl silica gel (ODS) DM1020T (100–200 mesh) (Fuji Sylisia Chemical, Ltd., Kasugai, Japan), and MCI-Gel CHP20P (75–150 μ) (Mitsubishi Chemical Corp., Tokyo, Japan). Thin-layer chromatography (TLC) was run using Merck silica gel GF-254 and Merck RP-18 $F_{2545}$. High-performance liquid chromatography (HPLC) was done on a Hitachi L-6250 Intelligent Pump equipped with a Hitachi L-4200 UV-VIS detector using an ODS-5 Develosil Pack Column and an ODS-HG-5 Develosil Pack Column (Nomura Chemical Co., Ltd., Japan). Optical rotation was measured using a Union PM-101 automatic digital polarimeter (Uniongiken, Osaka, Japan). For measuring the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, a UV-2500PC UV-VIS spectrometer (Shimadzu, Kyoto, Japan) and a JEOL (Tokyo, Japan) JES-RE1X ESR (electron spin resonance) spectrometer were employed. An omnion oxidative stability instrument (Omnion, Inc., Rockland, MA) was used for the oxidative stability index (OSI) measurement.

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*Reagents*. DPPH, α-tocopherol, and BHT were bought from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Silicone Oil TSF 451-100 was obtained from Toshiba Co. (Tokyo, Japan). Methyl linoleate was obtained from Tokyo Kasei Co. (Tokyo, Japan).

*Plant material*. Dried fruit of *A. tsao-ko* was bought in Singapore in January 1998 and in Thailand in September 1998. Voucher specimens are deposited at the Department of Food and Nutrition, Faculty of Human Life Science, Osaka City University, Japan.

*Extraction, isolation, and identification of the constituents of the fruit of A. tsao-ko*. The constituents of the dried, ground fruit of the Singaporean *A. tsao-ko* (850 g) were extracted with dichloromethane followed by 70% acetone. The organic solvent in 70% acetone fraction (fr.) was evaporated *in vacuo* to give the aqueous fr., which was extracted with ethyl acetate. The ethyl acetate fr. was evaporated and the resulting extract was suspended in 2% methanol in dichloromethane. The soluble part was evaporated and labeled as fr. ATEF (2.1 g) and the insoluble part was labeled as fr. ATER (7.2 g). Fr. ATER was subjected to Sephadex LH-20 CC using isopropanol to give frs. 1–21. Fr. 2 was further chromatographed over silica gel with $CH_2Cl_2/CH_3OH$ (98:2) as the eluting solvent followed by repeated HPLC on ODS using different proportions of $H_2O$-$CH_3OH$ as eluting solvents to give **1** (10 mg), **2** (17 mg), and **11** (1.9 mg). Fr. 8 afforded **9** (10 mg), after subjecting it to silica gel CC [$CH_2Cl_2/CH_3OH$ (95:5)] then Sephadex LH-20 ($CH_3OH$). Compounds **3** and **4** (181 mg) were obtained as a mixture from fr. 14 after CC over silica gel using $CH_2Cl_2/CH_3OH$ (9:1). Fr. ATEF was fractionated into 13 fractions, frs. 1–13, over silica gel CC using $CH_2Cl_2/CH_3OH$ (50:1) as the eluting solvent. Fr. 3 gave **5** (22 mg) as a white precipitate while fr. 9 afforded **6** (17 mg), also as a precipitate. Fr. 3 was subjected to silica gel CC [$C_6H_6/CH_3COCH_3$ (50:1)] followed by Sephadex LH-20 [$(CH_3)_2CHOH$] to yield **7** (5 mg). Frs. 8 and 9 were separately chromatographed over Sephadex LH-20 [$CH_3COCH_3$ and $(CH_3)_2CHOH$, respectively] followed by silica gel [$C_6H_6/CH_3COCH_3$ (9:1)] to afford **8** (1 mg). Fr. 10 yielded **10** (1.2 mg) after chromatography over Sephadex LH-20 ($CH_3COCH_3$) followed by HPLC-ODS using 40% $CH_3OH$.

The constituents of the dried, ground fruit of *A. tsao-ko* (960 g) obtained from Thailand were extracted following the procedure described earlier to give the dichloromethane, aqueous, and ethyl acetate extracts. These extracts showed the same TLC behavior when compared with the extracts obtained from the Singaporean plant. The ethyl acetate extract (22.0 g) from the Thai plant was subjected to Sephadex LH-20 CC using $(CH_3)_2CHOH$ to give 15 frs. Fr. 8 was further subjected to repeated CC over Sephadex LH-20 CC [$(CH_3)_2CHOH$], silica gel [$CH_2Cl_2/CH_3OH$ (98:2)], and MCI-Gel CHP20P (30% $CH_3OH$) to give **3** (30 mg) and **4** (4 mg).

*(+)-Hannokinol* (**1**). White powder. $[\alpha]_D$ +11.4° (MeOH, *c* = 0.38). $^1$H NMR (acetone-$d_6$) δ: 2.55 (2H, *ddd*, *J* = 6.84, 9.34, 13.73 Hz, H-1a, 7a), 2.67 (2H, *ddd*, *J* = 5.62, 9.77, 13.73 Hz, H-1b, 7b), 1.68 (4H, *m*, H-2ab, 6ab), 3.88 (2H, *m*, H-3, 5), 1.57 (2H, *t*, *J* = 6.35 Hz, H-4ab), 7.02 (4H, *d*, *J* = 8.54 Hz, H-3′, 5′, 3″, 5″), 6.73 (4H, *d*, *J* = 8.54 Hz H-2′, 6′, 2″, 6″). $^{13}$C NMR (acetone-$d_6$) δ: 31.8 (C-1, 7), 40.0 (C-2, 6), 68.2 (C-3, 5), 44.8 (C-4), 134.1 (C-1′, 1″), 130.0 (C-2′, 2″, 6′, 6″), 115.8 (C-3′, 3″, 5′, 5″), 156.1 (C-4′, 4″).

*Meso-hannokinol* (**2**). Colorless oil. $[\alpha]_D$ 0° (MeOH, *c* = 0.7). $^1$H NMR (acetone-$d_6$) δ: 2.56 (2H, *td*, *J* = 7.81, 9.34, 13.92 Hz, H-1a, 7a), 2.66 (2H, *td*, *J* = 7.81, 9.77, 13.92 Hz, H-1b, 7b), 1.68 (4H, *q*, *J* = 8.06 H-2ab, 6ab), 3.80 (2H, *m*, H-3, 5), 1.52 (1H, *td*, *J* = 9.52, 14.16 Hz, H-4a), 1.64 (1H, *td*, *J* = 2.93, 14.16 Hz, H-4b), 7.02 (4H, *d*, *J* = 8.54 Hz, H-3′, 5′, 3″, 5″), 6.73 (4H, *d*, *J* = 8.54 Hz H-2′, 6′, 2″, 6″). $^{13}$C NMR (acetone-$d_6$) δ: 31.4 (C-1, 7), 41.1 (C-2, 6), 71.6 (C-3, 5), 44.2 (C-4), 134.0 (C-1′, 1″), 130.0 (C-2′, 2″, 6′, 6″), 115.8 (C-3′, 3″, 5′, 5″), 156.1 (C-4′, 4″).

*(+)-Epicatechin* (**3**). Brownish-white powder. $[\alpha]_D$ +57.8° (MeOH, *c* = 0.5). EI–MS *m/z*: 290 $[C_{15}H_{13}O_6]^+$ $^1$H NMR and $^{13}$C NMR: see Table 1.

*(–)-Catechin* (**4**). Brownish-white powder. $[\alpha]_D$ −4.54° (MeOH, *c* = 0.22). EI–MS *m/z*: 290 $[C_{15}H_{13}O_6]^+$ $^1$H NMR and $^{13}$C NMR: see Table 1.

*β-Sitosterol* (**5**). White needles. Comparison of its $^1$H NMR spectrum with that presented in the literature for β-sitosterol (4) showed that the two spectra are superimposable.

*β-Sitosterol 3*-O-*glucoside* (**6**). White powder. $^1$H NMR spectrum is identical with **5** with additional signals of the glucose moiety. $^{13}$C NMR (pyridine-$d_5$) δ: 37.4 (C-1), 30.2 (C-2), 78.0 (C-3), 39.3 (C-4), 140.8 (C-5), 129.9 (C-6), 32.0 (C-7), 32.1 (C-8), 50.3 (C-9), 36.9 (C-10), 21.2 (C-11), 39.9 (C-12), 42.4 (C-13), 56.8 (C-14), 24.5 (C-15), 28.5 (C-16), 56.2 (C-17), 12.1 (C-18), 19.2 (C-19), 36.3 (C-20), 19.0 (C-21), 34.1 (C-22), 26.3 (C-23), 46.0 (C-24), 29.5 (C-25), 19.4 (C-26), 20.0 (C-27), 23.3 (C-28), 11.9 (C-29), 102.5 (C-1′), 75.0 (C-2′), 78.6 (C-3′), 71.6 (C-4′), 78.4 (C-5′), 62.8 (C-6′).

*2,6-Dimethoxyphenol* (**7**). Brownish-white powder. EI–MS *m/z*: 154 $[C_8H_{10}O_3]^+$; $^1$H NMR ($CDCl_3$) δ: 6.80 (1H, *dd*, *J* = 8.54, 8.54 Hz, H-4), 6.59 (2H, *d*, *J* = 8.54 Hz H-3, 5), 3.89 (3H, s). $^{13}$C NMR ($CDCl_3$) δ: 147.2 (C-2, 6), 134.8 (C-1), 119.1 (C-4), 104.8 (C-3, 5), 56.3 (2-$OCH_3$).

*Protocatechualdehyde* (**8**). White powder. EI–MS *m/z*: 138 $[C_7H_{10}O_3]^+$; $^1$H NMR (acetone-$d_6$) δ: 9.78 (1H, *s*), 7.36 (1H, *d*, *J* = 1.95 Hz, H-2), 7.32 (1H, *dd*, *J* = 1.95, 7.81 Hz, H-6), 7.00 (1H, *d*, *J* = 7.81 Hz, H-5). TLC comparison of **8** with authentic protocatechualdehyde sample showed that they were the same.

*Protocatechuic acid* (**9**). Brownish white powder. $^1$H NMR (DMSO-$d_6$) δ: 7.73 (1H, *d*, *J* = 1.95 Hz, H-2), 7.19 (1H, *dd*, *J* = 1.95, 8.06 Hz, H-6), 6.60 (1H, *d*, *J* = 8.06 Hz, H-5). $^{13}$C NMR (DMSO-$d_6$) δ: 171.1 (COOH), 129.9 (C-1), 117.6 (C-2), 147.7 (C-3), 144.9 (C-4), 114.8 (C-5), 121.0 (C-6). TLC comparison of **9** with authentic protocatechuic acid sample showed that they were the same.

*Vanillic acid* (**10**). Yellowish-white powder. EI–MS *m/z*: 168 $[C_8H_8O_4]^+$; $^1$H NMR ($CD_3OD$) δ: 7.54 (1H, *d*, *J* = 1.95 Hz, H-2), 7.54 (1H, *dd*, *J* = 1.95, 8.79 Hz, H-6), 6.82 (1H, *d*, *J* = 8.79 Hz, H-5). $^{13}$C NMR ($CD_3OD$) δ: 170.0 (COOH),

125.2 (C-1), 115.8 (C-2), 152.6 (C-3), 148.6 (C-4), 113.7 (C-5), 123.0 (C-6), 56.3 ($OCH_3$).

*p-Hydroxybenzoic acid* (**11**). White powder. $^1$H NMR ($CD_3OD$) δ: 7.44 (2H, *d*, *J* = 8.79 Hz, H-2, 6), 6.79 (2H, *d*, *J* = 8.79 Hz, H-3, 5). TLC comparison of **11** with authentic *p*-hydroxybenzoic acid sample showed that they were the same.

*Evaluation of the antioxidant activity of the isolated compounds by the DPPH radical-scavenging method. (i) Colorimetric analysis.* The compound to be tested was added to a 1 mM ethanol solution of DPPH to make a final concentration of 20 μM (final DPPH concentration was 100 μM). The mixture was shaken vigorously on a vortex stirrer and then incubated for 30 min in a water bath at 20°C after which the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The scavenging activity of the tested compound was measured as the decrease in absorbance of the DPPH expressed as a percentage of the absorbance of a control DPPH solution without test compounds (5–7). All analyses were carried out in duplicate. In another set of test compounds, a 150 μM ethanol solution of DPPH was added to the compound to be tested to make a final concentration of 25 μM and 75 μM of the compound and DPPH, respectively. The mixture was shaken vigorously on a vortex stirrer then incubated in a water bath at 20°C. The absorbance of the remaining DPPH was determined colorimetrically at 517 nm at exactly 1 min and then 30 min after the addition of the DPPH solution. All analyses were carried out in triplicate. The scavenging activity of the tested compound was determined as above.

*(ii) ESR analysis*. One hundred microliters of 150 μM DPPH in ethanol was added to a solution of 50 μL water and 50 μL of the compound to be tested to make a final concentration of 25 μM. The solution was stirred on a vortex stirrer for 10 s then transferred into a flat quartz cell (60 × 10 × 0.31 nm inner size), and the amount of DPPH radicals generated was measured by the ESR spectrometer exactly 60 s after the addition of the DPPH solution. The DPPH signal intensities were evaluated by the peak height of the third of five signals against a control. The conditions used were as follows: measurement at room temperature (20°C) with manganese oxide as marker; magnetic field 336 ± 10 mT; microwave frequency 9.76 GHz; modulation frequency 100 kHz; power 8.0 mW; field modulation width 0.32 mT; sweep time 2.0 min; receiver gain 10 × 10; and time constant 2.0 s. All analyses were carried out in three or nine replicates. For the determination of the concentration at which the scavenging activity showed 50% of control ($IC_{50}$), four different concentrations (6.25, 12.5, 25.0, and 50.0 μM) of some of the isolated compounds were prepared and then measured as described earlier. The $IC_{50}$ was obtained by extrapolation from linear regression analysis (8).

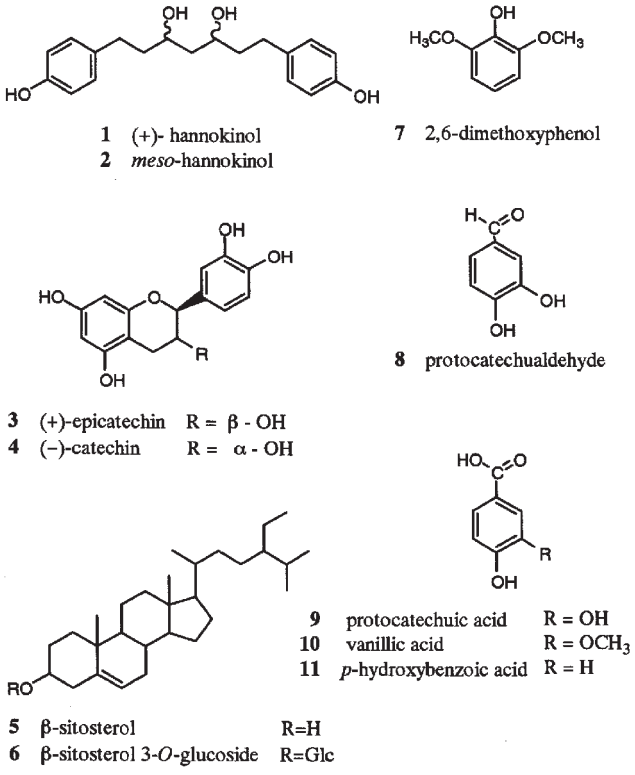
*Evaluation of the antioxidant activity of the isolated compounds by the OSI method.* The oil sample was prepared by mixing 10% methyl linoleate and 90% silicone oil. To 5 ± 0.02 g of this oil sample, 1 μmol in 100 μL of methanol of the isolated compound was mixed in a glass cylinder. The induction period for oxidation of the oil sample as affected by the isolated compound was measured with the Omnion oxidative stability instrument, which is equipped with an on-line computer that monitored the conductivity vs. time and automatically plotted the inflection point or induction period in hours, according to AOCS Official Method Cd 12b-92 (9–10). Oxidation was carried out at 90°C. All tests were run in triplicate.

*Statistical analysis*. One-way analysis of variance (ANOVA) based on the data collected was carried out. Significance was established at $p < 0.05$.

## RESULTS AND DISCUSSION

*Isolation and identification of the constituents of* A. tsao-ko. The constituents of the dried powdered fruit of *A. tsao-ko* were extracted with dichloromethane followed by 70% aqueous acetone. The acetone was evaporated and the resulting aqueous solution was fractionated into the ethyl acetate-soluble and water-soluble fractions. The ethyl acetate-soluble fraction was subjected to repeated column chromatography using silica gel, Sephadex LH-20, Chromatotrex ODS, and MCI-Gel CHP-20P and to repeated HPLC-ODS to afford 11 compounds.

Identification of the isolated compounds was based on mass and various NMR spectroscopic techniques, by TLC comparison with authentic samples, and/or by comparison of the spectral data with those reported in the literature. Compounds **5–11** were identified as β-sitosterol (**5**) (4), β-sitosterol 3-*O*-glucoside (**6**) (11), 2,6-dimethoxyphenol (**7**), protocatechualdehyde (**8**), protocatechuic acid (**9**), vanillic acid (**10**), and *p*-hydroxybenzoic acid (**11**) (Scheme 1).




**SCHEME 1**

Compounds **1** and **2** were identified as (+)-hannokinol and *meso*-hannokinol, respectively. Nomura and coworkers (12) previously reported the isolation of hannokinol and its *meso*-structure from *Alnus japonica*. However, they did not indicate the optical rotation of this compound. In another paper, Alegrio and coworkers reported the isolation of hannokinol having an $[\alpha]_D$ of –19° from *Centrolobium sclerophyllum*, establishing it as (–)-hannokinol (13). The optical rotation obtained for **1** in the present paper was $[\alpha]_D$ +11.4° (MeOH, *c* = 0.38), establishing **1** to be (+)-hannokinol.

The EI–MS of compound **3** gave a molecular ion peak $[M^+]$ at *m/z* 290 consistent with the molecular formula $C_{15}H_{13}O_6$. Close examination of its NMR spectra showed that it was a flavan-3-ol compound. The $^1H$ and $^{13}C$ NMR data (Table 1) of **3** established it to be epicatechin having the 2,3-*cis* configuration as indicated by the coupling constant of *J* = 0.73 Hz of H-2 with H-3 and the resonance position of C-2 at 79.3 ppm. The optical rotation of **3** was determined to be $[\alpha]_D$ +57.8° (MeOH, *c* = 0.5) establishing **3** to be (+)-epicatechin (14–16).

Compound **4** also gave a $[M^+]$ at *m/z* 290, and its NMR spectra is almost identical with those of **3** except for the following signals (Table 1): Carbon-2 resonated at 82.7 ppm and H-2 was observed at 4.55 ppm, which coupled with H-3 with a coupling constant of *J* = 7.81 Hz, indicating a 2,3-*trans* configuration. Thus, **4** was revealed to be catechin. Its $[\alpha]_D$ was determined to be –4.54° (MeOH, *c* = 0.22), establishing it as (–)-catechin (14–16).

Reported investigations on the constituents of *A. tsao-ko* are still very few. Thus, except for (+)-hannokinol (**1**) and *meso*-hannokinol (**2**), the isolation of the compounds from this plant was done for the first time. What is more noteworthy is that this is the first isolation of epicatechin and catechin from the genus *Amomum*. Whereas (+)-catechin and (–)-epicatechin occur frequently in nature, (–)-catechin and (+)-epicatechin are rare compounds (15).

*Antioxidant activity of the compounds isolated based on the DPPH radical scavenging method*. Colorimetry with the stable DPPH radical has been reported as a simple method for evaluation of the radical scavenging activity of a given substance. Absorbance decreases as a result of a color change from violet to yellow as the radical is scavenged by antioxidants through donation of hydrogen to form the stable DPPH-H. The more rapidly the absorbance decreases, the more potent the antioxidant activity of the compounds in terms of hydrogen donating ability. (7–8,17). The antioxidant activity of the compounds isolated from the fruit of *A. tsao-ko* measured in terms of their hydrogen donating or radical scavenging activity using DPPH is shown in Table 2. At a concentration of 20 μM and at a reaction time of 30 min, epicatechin exhibited the strongest activity, stronger than α-tocopherol and BHT. The activity of the compounds decreased in the order epicatechin ≅ protocatechualdehyde > catechin ≅ 2,6-dimethoxyphenol ≅ α-tocopherol ≅ protocatechuic acid > BHT > vanillic acid ≅ *p*-hydroxybenzoic acid. *Meso*-hannokinol and (+)-hannokinol did not show any activity. At a concentration of 25 μM and a reaction time of 30 min, protocatechualdehyde exhibited the strongest activity, although the activity was not significantly different from that of epicatechin. The activity of the compounds decreased in the order protocatechualdehyde ≅ epicatechin > α-tocopherol ≅ catechin > 2,6-dimethoxyphenol > protocatechuic acid. For the test with a 1-min reaction time, the activity decreased in the order protocatechualdehyde > epicatechin > α-tocopherol ≅ catechin > 2,6-dimethoxyphenol ≅ protocatechuic acid.

The DPPH radical scavenging activity of the compounds isolated was also evaluated by direct ESR measurements. At a concentration of 25 μM, epicatechin showed the strongest scavenging activity among the isolates as shown in Table 3. The activity of the compounds decreased in the order α-tocopherol ≅ epicatechin ≅ catechin ≅ protocatechualdehyde ≅ 2,6-dimethoxyphenol > protocatechuic acid > BHT > vanillic

**TABLE 1**
**$^1H$- and $^{13}C$-NMR Spectral Data for (+)-Epicatechin (3) and (–)-Catechin (4) (δ, ppm, acetone-$d_6$)**

| | (+)-Epicatechin (**3**) | | (–)-Catechin (**4**) | |
|---|---|---|---|---|
| Position | $^{13}C$ | $^1H$ | $^{13}C$ | $^1H$ |
| 2 | 79.3 | 4.88 (1H, *t*, *J* = 0.73 Hz) | 82.7 | 4.55 (1H, *d*, *J* = 7.81 Hz) |
| 3 | 66.8 | 4.19 (1H, *m*) | 68.3 | 3.97 (1H, *m*) |
| 4 | 29.0 | 2.74 (1H, *dd*, *J* = 2.93, 16.60 Hz) | 28.8 | 2.52 (1H, *dd*, *J* = 8.56, 16.0 Hz) |
| | | 2.87 (1H, *dd*, *J* = 4.63, 16.60 Hz) | | 2.91 (1H, *dd*, *J* = 7.81, 16.0 Hz) |
| 5 | 157.5 | | 157.2 | |
| 6 | 96.1 | 6.02 (1H, *d*, *J* = 2.20 Hz) | 96.0 | 6.02 (1H, *d*, *J* = 2.20 Hz) |
| 7 | 157.4 | | 156.9 | |
| 8 | 95.6 | 5.92 (1H, *d*, *J* = 2.20 Hz) | 95.4 | 5.87 (1H, *d*, *J* = 2.20 Hz) |
| 9 | 157.0 | | 157.7 | |
| 10 | 99.7 | | 100.6 | |
| 1′ | 132.1 | | 132.1 | |
| 2′ | 115.1 | 7.05 (1H, *d*, *J* = 1.95 Hz) | 115.2 | 6.89 (1H, *d*, *J* = 1.95 Hz) |
| 3′ | 145.3 | | 145.6 | |
| 4′ | 145.2 | | 145.6 | |
| 5′ | 115.4 | 6.78 (1H, *d*, *J* = 8.06 Hz) | 115.6 | 6.79 (1H, *d*, *J* = 8.06 Hz) |
| 6′ | 119.2 | 6.83 (1H, *d*, *J* = 1.95, 8.06 Hz) | 120.0 | 6.75 (1H, *d*, *J* = 1.95, 8.06 Hz) |

**TABLE 2**
**DPPH[a] Radical Scavenging Activity of the Compounds Isolated Based on the Colorimetric Method[b]**

| Compound | 20 μM (30 min)[c] | 25 μM 1 min[a] | 25 μM 30 min[c] |
|---|---|---|---|
| **1** (+)-Hannokinol | 0.36 ± 1.32 | | |
| **2** *meso*-Hannokinol | 0.96 ± 4.66 | | |
| **3** (+)-Epicatechin | 89.96 ± 0.79 | 55.98 ± 0.83 | 91.53 ± 0.85 |
| **4** (–)-Catechin | 50.05 ± 7.68 | 39.13 ± 1.81 | 75.12 ± 0.40 |
| **7** 2,6-Dimethoxyphenol | 40.79 ± 2.41 | 19.74 ± 0.21 | 62.19 ± 1.54 |
| **8** Protocatechualdehyde | 85.51 ± 1.04 | 71.80 ± 2.24 | 93.92 ± 0.16 |
| **9** Protocatechuic acid | 32.84 ± 4.20 | 17.42 ± 1.53 | 53.19 ± 1.90 |
| **10** Vanillic acid | 14.10 ± 0.77 | | |
| **11** *p*-Hydroxybenzoic acid | 9.41 ± 0.94 | | |
| BHT[d] | 32.27 ± 0.07 | | |
| α-Tocopherol | 38.98 ± 3.55 | 39.54 ± 0.56 | 76.33 ± 3.58 |

[a]1,1-Diphenyl-2-picrylhydrazyl.
[b]Each value is the mean ± SD of two or three replicates.
[c]Reaction time.
[d]Butylated hydroxytoluene.

acid ≅ *p*-hydroxybenzoic acid. The activity of *meso*-hannokinol and (+)-hannokinol did not show any significant difference against the control. The activity of α-tocopherol, epicatechin, catechin, protocatechualdehyde, and 2,6-dimethoxyphenol did not show any significant difference from each other. The concentration at which the scavenging activity of the tested compound showed 50% of control ($IC_{50}$) was determined for epicatechin, catechin, 2,6-dimethoxyphenol, protocatechualdehyde, protocatechuic acid, and α-tocopherol. The results are shown in Table 3. Except for protocatechuic acid, the $IC_{50}$ of the tested compounds are almost the same.

Studies have shown in a consistent manner that the antioxidant activity of phenolic compounds is reasonably related to their structure, namely, the substitutions on the aromatic rings and the structure of the side chain (18). All flavonoids with the 3′,4′-dihydroxy configuration possess marked antioxidant activity. This activity increases with the number of hydroxyl groups substituted on the A and B rings (19). The presence of a second hydroxyl group in the *ortho* or *para* position is known to increase antioxidant activity due to additional resonance stabilization and *o*-quinone or *p*-quinone formation (5). The presence of phenolic hydroxyl groups increases the antioxidant activity of phenolic acids, while methoxylation of the hydroxyl groups causes a decrease in activity (19). The difference in the ability of (+)-epicatechin and (–)-catechin to scavenge the DPPH radical suggests that the difference between the steric structures of these compounds may play an important role in their abilities to scavenge the DPPH radical (20). For protocatechualdehyde and protocatechuic acid, a probable explanation for the difference in their activity is the fact that carboxylic acids are capable of intermolecular hydrogen bonding while aldehydes are not. Because of this bonding, the electron-withdrawing capacity of protocatechuic acid is decreased. This makes protocatechuic acid less active toward the DPPH radical. In a similar study, protocatechualdehyde showed more than 18-fold the antioxidant activity of protocathechuic acid (21).

**TABLE 3**
**DPPH[a] Radical Scavenging Activity of the Compounds Isolated Based on ESR[b] Analysis**

| Compound | Relative peak height/conc. in μM 0[c] | 6.25[c] | 12.5[c] | 25[d] | 50[c] | $IC_{50}$[e] |
|---|---|---|---|---|---|---|
| **1** (+)-Hannokinol | 4.79 | | | 4.726 | | |
| **2** *meso*-Hannokinol | 4.79 | | | 4.748 | | |
| **3** (+)-Epicatechin | 5.15 | 3.798 | 2.753 | 1.107 | 0.068 | 12.55 ± 0.65 |
| **4** (–)-Catechin | 5.15 | 3.946 | 2.864 | 1.226 | 0.092 | 12.66 ± 0.49 |
| **7** 2,6-Dimethoxyphenol | 5.15 | 4.190 | 3.316 | 2.027 | 0.865 | 15.89 ± 2.07 |
| **8** Protocatechualdehyde | 5.15 | 3.516 | 2.641 | 1.691 | 0.731 | 14.39 ± 3.31 |
| **9** Protocatechuic acid | 5.15 | 4.266 | 3.906 | 3.446 | 2.865 | 55.18 ± 3.76 |
| **10** Vanillic acid | 4.79 | | | 4.444 | | |
| **11** *p*-Hydroxybenzoic acid | 4.79 | | | 4.478 | | |
| BHT[a] | 4.79 | | | 4.061 | | |
| α-Tocopherol | 5.15 | 3.970 | 2.961 | 1.059 | 0.145 | 12.57 ± 0.62 |

[a]See Table 2 for definitions.
[b]Electron spin resonance.
[c]Each value is the mean of three replicates.
[d]Each value is the mean of nine replicates.
[e]Each value is the mean ± SD of three replicates.

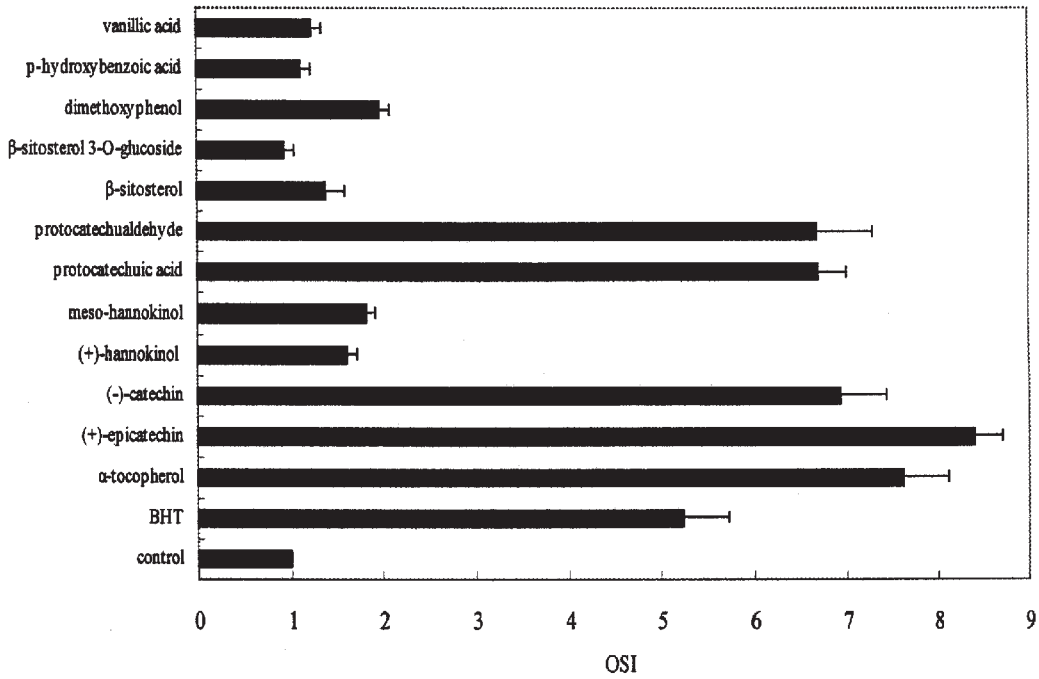



**FIG. 1.** Antioxidant activity of the compounds isolated from *Amomum tsao-ko* based on the oxidative stability index (OSI) (90°C, 1 μmol/5 g oil).

The difference between the results obtained in the DPPH-ESR method and the DPPH-colorimetric method cannot be discussed at this time. Further experiments are necessary to address this.

*Antioxidant activity of the compounds isolated as measured by the OSI method.* The antioxidant activity of the compounds isolated from *A. tsao-ko* was determined by the OSI method. This method measured the induction period for the onset of rancidity of oil at 90°C. The longer the induction period of the oil with the isolated compound added compared to a control, the stronger the antioxidant activity of that compound. Figure 1 shows the result of this determination. Epicatechin exhibited the longest induction period and, thus, the strongest activity. The activity decreased in the order epicatechin ≅ α-tocopherol, followed by catechin ≅ protocatechuic acid ≅ protocatechualdehyde, then BHT followed by the rest of the compounds, which showed no significant differences from each other based on statistical analysis. Although protocatechualdehyde and protocatechuic acid showed different abilities to scavenge DPPH free radicals in an alcoholic system, both inhibited the oxidation of oil sample at a similar rate. Such results may imply that the antioxidant effect of a given compound appears to be dependent on the method or system used for evaluation (22).

At this point, it is clear that the antioxidant activity of the ethyl acetate-soluble fraction of the 70% aqueous acetone extract of the fruit of *A. tsao-ko* is a contribution of several active components forerunnered by the catechins and catechol derivatives, the catechins also being two of the major constituents of this extract.

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