Antioxidative Activity Estimation of Methanol Extracts of Crude Drugs by Electrochemical Detection–High Performance Liquid Chromatography (ECD-HPLC) and Correlation with 1,1-Diphenyl-2-picrylhydrasyl (DPPH) Radical Scavenging Activities

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As a method for estimation of the antioxidative activity of methanol extracts of crude drugs, cyclic voltammograms (CV), 1,1-diphenyl-2-picrylhydrasyl (DPPH) radical scavenging activities, and electrochemical detection (ECD)-HPLC were investigated. From a comparison of results, crude drugs which show oxidative peaks in the low potential region in CV, had strong and rapid radical scavenging activities. A correlation (r^2=0.76) between DPPH radical scavenging ability and total peak area detected by ECD-HPLC, was obtained. The ECD-HPLC method is therefore useful for evaluation of antioxidative activities, as well as a separational analysis method for antioxidative ingredients.

Key words crude drug; antioxidative activity; radical scavenging activity; electrochemical detection-HPLC; 1,1-diphenyl-2-picrylhydrasyl

Redox control disorder in humans is associated with many diseases.[1] For example, an increase of oxidized low density lipoprotein causes arteiosclerosis,[2] and an excess of constitutively produced active oxygen species causes cancer and rheumatism.[3] Therefore, the antioxidative properties of various kinds of drug substances and drug products have been investigated with keen interest.

Many evaluation methods for antioxidative activity have been developed. For example, the peroxide value method,[4] the thiobarbituric acid value method[4] (TBAV), 1,1-diphenyl-2-picrylhydrasyl (DPPH) radical scavenging activity determination,[5] the conjugated diene method[6] (inhibitory effect on lipid peroxidation induced by 2,2′-azobis(2-amidinopropane)dihydrochloride, 2,2′-azobis(2,4-dimethylvaleronitrile), Cu^{2+} or Fe^{3+}/ascorbic acid, active oxygen species (like O_2^-, OH, NO radicals) scavenging capability determination,[7] ESR techniques[7] using a spin trapping agent such as 5,5-dimethyl-1-pyrroline *N*-oxide, and a bioassay method[7] (*tert*-BuOO radical scavenging activity using a cultural broth of *Staphylococcus aureus* as a test sample).

Antioxidative activities of the antioxidants used in the food industry,[8] Chinese crude drugs used in medical treatment,[9] and so forth, have been investigated based on the above-mentioned analytical methods. Many investigations into the antioxidative activity of various kinds of crude drugs,[10] for instance, Mountan Radicis Cortex,[11] Rhei Rhizoma,[12] have also been described.

In this paper, we evaluate the antioxidative activity of methanol extracts of twelve crude drugs using cyclic voltammetry, a electrochemical detection (ECD)-HPLC system, and DPPH radical scavenging determination. The relationship between antioxidative activities determined with the ECD-HPLC system and DPPH radical scavenging activities is also discussed.

Experimental

Materials and Reagents The twelve crude drugs used in this study are summarized in Table 1. These crude drugs (three lots each) were all provided by Alps Pharmaceutical Inc. Co., Ltd. (Gifu, Japan).

Standard reagents, kaempherol (chemical grade), honokiol and magnolol were purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). Coptisine hydrochloride and eugenol were obtained from Nacalai Tesque, Inc., (Kyoto, Japan). Berberine hydrochloride was obtained from Sigma Chemical Co., Ltd.

HPLC grade acetonitrile was purchased from Katayama Chemical Industries, Ltd., (Osaka, Japan). Tetraethylammonium perchlorate (specially prepared for polarography) was obtained from Nacalai Tesque, Inc., (Kyoto, Japan). DPPH was obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). All other chemicals were of special grade.

Preparation of Methanol Extracts Methanol extracts of crude drugs were prepared according to the previous paper.[13] One gram of powder or a small portion of crude drug (Mountan Radicis Cortex, Coicis Semen were notched with a knife previously) was extracted with 90 ml of methanol under reflux at 90 °C for 1 h. After cooling to room temperature, the extract was filtered with a filter paper (Toyo Roshi Kaisya, Ltd.) and made up to 100 ml with methanol.

Measurement of the Cyclic Voltammogram of Methanol Extracts The methanol extract, containing 50 mM tetraethylammonium perchlorate as a supporting electrolyte, was used as the sample solution for a measurement of the voltammogram. Cyclic voltammetry was carried out using a three-electrode system: a working electrode made of glassy carbon (GC-P2, 3 mm d., disk type, Yanagimoto, Kyoto, Japan), a reference electrode of Ag/AgCl (RE-18, BAS Co., Ltd.) and a counter electrode of Pt wire (Kel-F, 23 cm, BAS Co., Ltd.) with a scan rate of 100 mV/s in the range −0.3 to +1.9 V. Before measurement, the working electrode was polished to a mirror-like finish with 0.05 μm alumina powder and washed for 30 s in distilled water in an ultrasonic cleaner (Type 3200, Yamato Scientific Co., Ltd., Tokyo, Japan). Measurement of the cyclic voltammogram was carried out using an electrochemical analyzer (CV-50W, BAS Co., Ltd.). The voltammogram at the third scan was adopted.

Chromatography of the Methanol Extracts The ECD-HPLC apparatus consisted of a pump (LC-6AD, Shimadzu), an autoinjector (SIL-6B, Shimadzu), a column heater (CTO-6A, Shimadzu), an electrochemical detector with a glassy carbon working electrode (Σ875, Irika Instruments Inc.), a system controller (SCL-6B, Shimadzu) and an integrator (C-R5A, Shimadzu). HPLC was performed with a reversed-phase column (Inertsil ODS-2, 150 mm×4.6 mm i.d.) with a linear gradient of aqueous acetonitrile (0%→50%, 0 min→60 min) and isocratic aqueous acetonitrile (50%, 60 min→75 min) containing 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. The column temperature was kept constant at 40 °C. The sample solution (20 μl) was injected by autoinjector. The applied potential of the ECD was set at every +0.1 V from +0.7 V to +1.1 V *vs.* Ag/AgCl.

DPPH Radical Scavenging Activity DPPH radical scavenging activity was determined according to the method of Blois.[5] A mixture of 4 ml of DPPH methanol solution (1.25 mM) and 4 ml of acetate buffer (0.1 M, pH 5.5) was made up to 10 ml with methanol to afford solution A. Solution A mixed with 0.1 ml of methanol gave a blank solution and solution A mixed

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with 0.1 ml of methanol extract gave solution B. After exactly 5 min, 1, 3, and 5 h, the absorbance at 523 nm of the blank solution and solution B was measured with a spectrophotometer (U-3300, Hitachi Ltd., Tokyo, Japan) to calculate the residual percentage. This method was not influenced by the coloring of the methanol extracts of crude drugs.

Results and Discussion

Cyclic Voltammetry of the Methanol Extracts Twelve crude drugs, each of which has been reported to exhibit strong antioxidative activities[14] based on inhibition of air oxidation of linoleic acid,[15] were selected as test samples. The cyclic voltammograms of methanol extracts of the crude drugs were measured to check electrochemical profiles. Representative voltammograms of the methanol extracts of crude drugs of lot A are shown in Fig. 1.

Caryophylli Flos (Fig. 1A) showed a sharp oxidative peak in the low potential region at about +0.8 V, assumed to be due to eugenol, one of the main components. Rhei Rhizoma (Fig. 1D) showed a broad oxidative peak in the low potential region at around +0.8 V, thought to be due to anthraquinone-like compounds *e.g.* aloe-emodine, catechol-like compounds, catechinic acid-like compounds and gallic acid-like compounds. Coptidis Rhizoma (Fig. 1B) showed oxidative peaks at +1.1 and +1.4 V. Magnoliae Cortex (Fig. 1C) showed many oxidative peaks in the high potential region above +0.8 V. Mountan Radicis Cortex (Fig. 1E) showed moderately strong oxidative peaks at +0.6 and +1.4 V. Zingiberis Rhizoma (Fig. 1G) showed weak oxidative peaks at +0.6 and +1.0 V. Cinnamonomi Cortex (Fig. 1I) showed a sharp oxidative peak in the high potential region at +1.4 V.

However, Astragali Radix, Bupleuri Radix and Coicis Semen had no outstanding oxidative peaks and little antioxidative activity. Therefore we decided to omit these three crude drugs from further experiments.

In the TBAV method, based on the inhibition of air oxidation of linoleic acid, Zingiberis Rhizoma and Mountan Radicis Cortex have strong antioxidative activities. On the other hand, with cyclic voltammetry, Caryophylli Flos and Rhei Rhizoma have strong antioxidative activities.[13] Therefore, no relationship between these two methods appears to exist.

ECD-HPLC Determination of the Methanol Extracts In order to clarify the electrochemically active components, ECD-HPLC was performed on the methanol extracts of crude drugs (3 lots each). Representative ECD-HPLC chromatograms of the methanol extracts (Caryophylli Flos, Magnoliae Cortex and Coptidis Rhizoma) are shown in Fig. 2. For Caryophylli Flos (Fig. 2A), many ECD active components were detected. Among them, peak 1 and peak 2 were identifed as kaempherol and eugenol, as judged by LC-MS analysis. In Magnoliae Cortex (Fig. 2C), magnocurarine, honokiol, and magnolol were identified in the same way (peak 1, peak 2, peak 3). In Coptidis Rhizoma (Fig. 2B), the

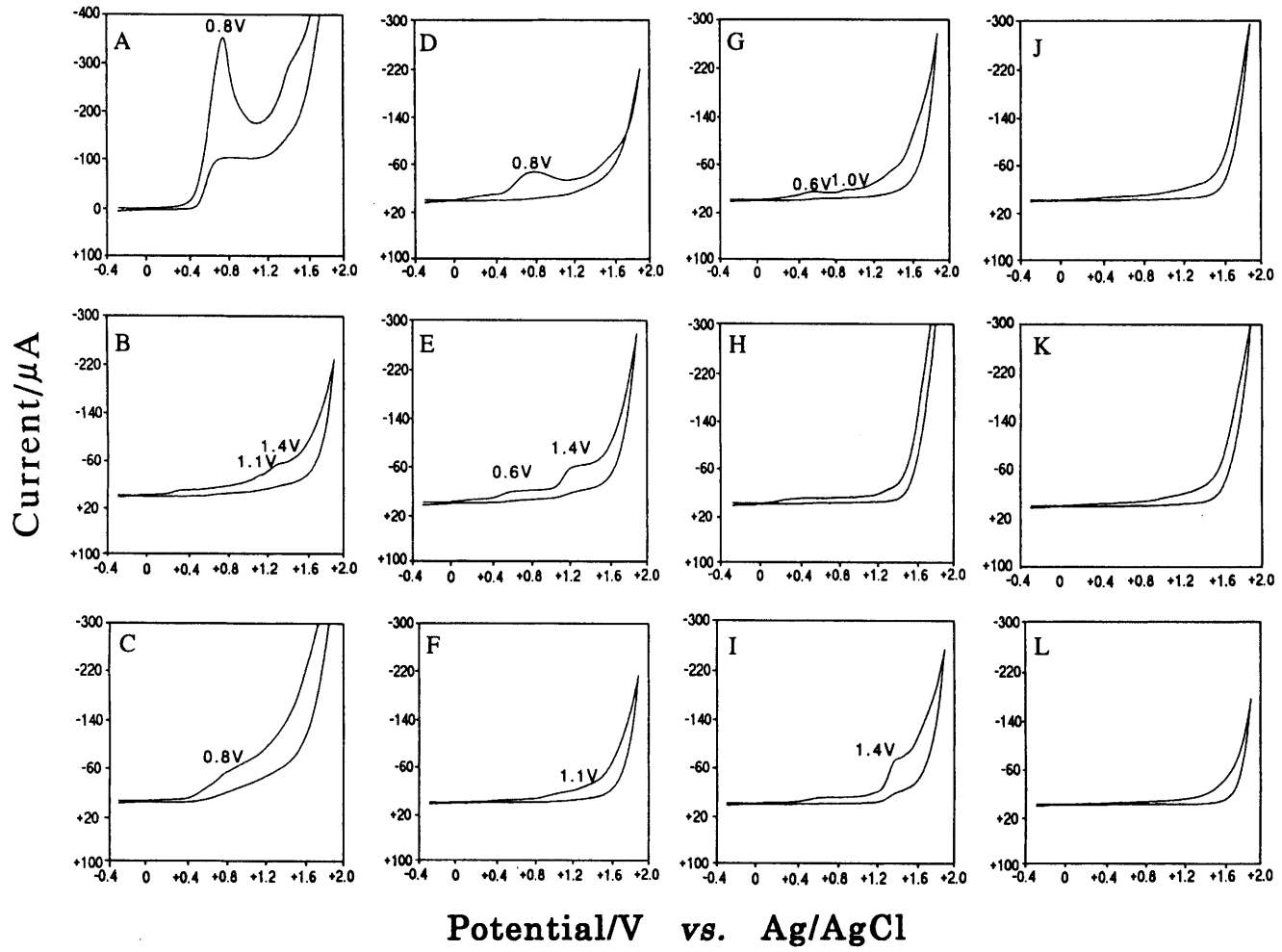

Fig. 1. Cyclic Voltammograms of Methanol Extracts of Crude Drugs

A, Caryophylli Flos; B, Coptidis Rhizoma; C, Magnoliae Cortex; D, Rhei Rhizoma; E, Mountan Radicis Cortex; F, Schizandrae Fructus; G, Zingiberis Rhizoma; H, Corni Fructus; I, Cinnamonomi Cortex; J, Astragali Radix; K, Bupleuri Radix; L, Coicis Semen.

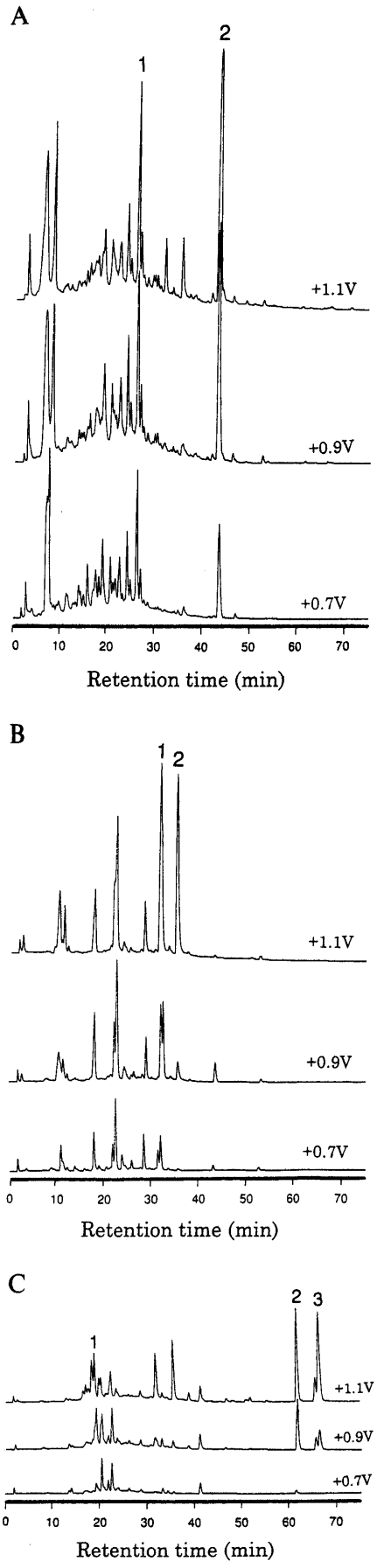

Fig. 2. ECD-HPLC Chromatograms of Methanol Extracts

A, Caryophylli Flos; B, Coptidis Rhizoma; C, Magnoliae Cortex.

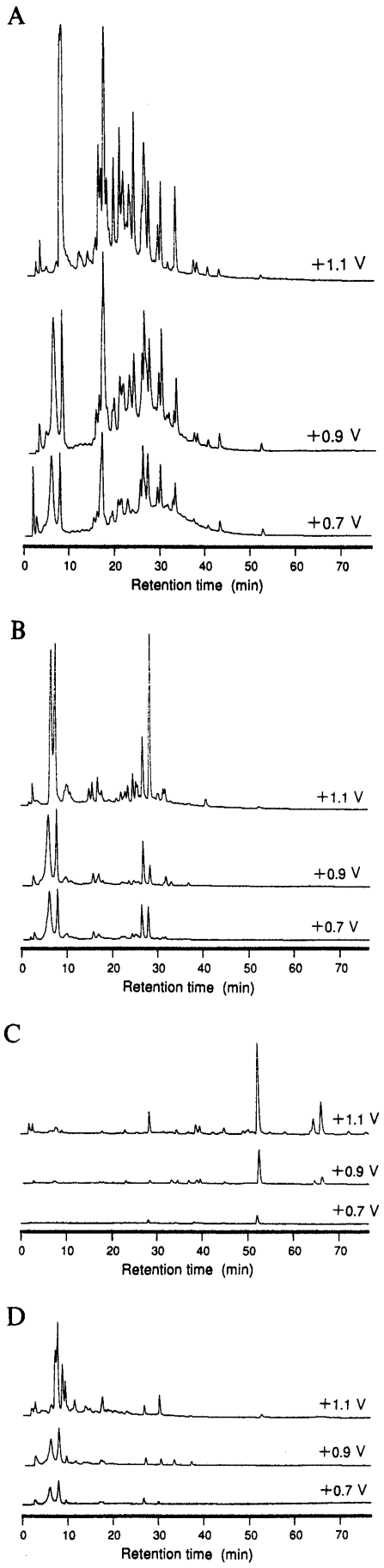

Fig. 3. ECD-HPLC Chromatograms of Other Four Methanol Extracts

A, Rhei Rhizoma; B, Mountan Radicis Cortex; C, Zingiberis Rhizoma; D, Corni Fructus.

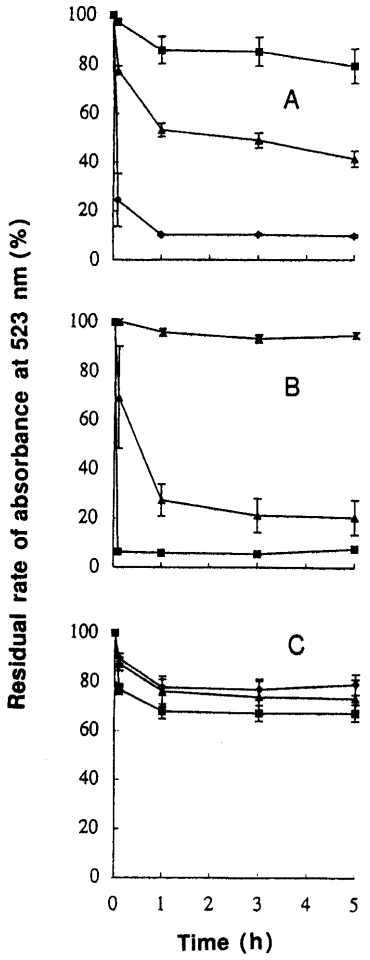

Fig. 4. DPPH Radical Scavenging Activities of Methanol Extracts

A, Caryophylli Flos (—◆—), Coptidis Rhizoma (—■—), Magnoliae Cortex (—▲—); B, Rhei Rhizoma (—■—), Mountan Radicis Cortex (—▲—), Schizandrae Fructus (—×—); C, Zingiberis Rhizoma (—◆—), Corni Fructus (—■—), Cinnamonomi Cortex (—▲—).

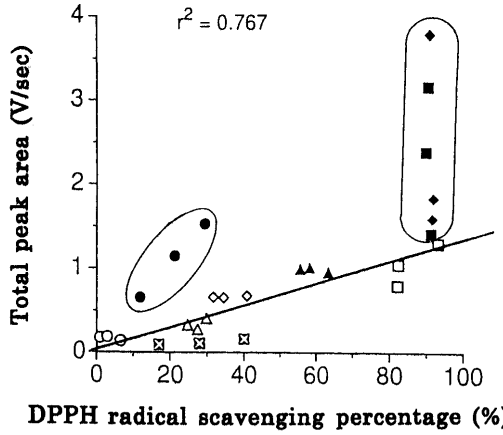

Fig. 5. The Relationships between DPPH Radical Scavenging Activities and the Total Peak Area of ECD-HPLC Active Ingredients

—■— Caryophylli Flos; —●—, Coptidis Rhizoma; —▲—, Magnoliae Cortex; —◆—, Rhei Rhizoma; —□—, Mountan Radicis Cortex; —○—, Schizandrae Fructus; —△—, Zingiberis Rhizoma; —◇—, Corni Fructus; —⊠—, Cinnamonomi Cortex.

Table 1. Crude Drugs Used in the Experiments

Crude drug	Japanese name	Origin
Caryophylli Flos	丁子	Zanzibar
Coptidis Rhizoma	黄蓮	China
Magnoliae Cortex	厚朴	Japan
Rhei Rhizoma	大黄	China
Mountan Radicis Cortex	牡丹皮	China
Schizandrae Fructus	五味子	China
Zingiberis Rhizoma	生姜	China
Corni Fructus	山茱萸	China
Cinnamonomi Cortex	桂皮	China
Astragali Radix	黄耆	China
Bupleuri Radix	柴胡	China
Coicis Semen	薏苡仁	China

alkaloid components, berberine and coptisine have been reported.[16] We assume that these compounds correspond to several of the peaks. Chromatograms of the other four crude drugs, except for Schizandrae Fructus and Cinnamonomi Cortex (since no outstanding peaks were detected from these two crude drugs), are shown in Fig. 3, although identification of each component was not successful.

In the case of Caryophylli Flos, eugenol represented around 30% of total activity (peak areas) in ECD. The eugenol contents in the three different lots A, B, and C were 19%, 35%, and 34% respectively, indicating that variation among the three lots was relatively large. In Magnoliae Cortex, magnocurarine content varied from 6% to 10%, honokiol content varied from 13% to 15% and magnolol content varied from 17% to 25%. Total ratios of these three components were about 50%. Deviation among lots was also found for other crude drugs. In Coptidis Rhizoma, the main components corresponding to peak 1 and peak 2 must be palmatine and berberine, judging from the previous paper.[16]

DPPH Radical Scavenging Activities of the Methanol Extracts DPPH radical scavenging activities of the methanol extracts of nine crude drugs (three lots each) were investigated according to the Blois method.[5] Results are shown in Fig 4. Caryophylli Flos and Rhei Rhizoma, which showed an outstanding oxidative peak, even in the low potential region at +0.8 V, trapped DPPH radicals within 5 min. Both of them showed the strongest radical scavenging activity among all the crude drug extracts. Magnoliae Cortex, which exhibited an oxidative peak over +0.8 V, and Mountan Radicis Cortex, which had a small peak in the low potential region at +0.6 V, also showed strong DPPH radical scavenging activity.

However, in Coptidis Rhizoma, which mainly contained alkaloids which have a quaternary nitrogen in their structures, radical scavenging activity was revealed to be weak, although strong enough for oxidative peaks to be seen. The large degree of oxidative capability was thus recognized in the cyclic voltammogram.

Relationship between ECD-HPLC and DPPH Radical Scavenging Activity as an Evaluation Method for Antioxidative Activity The relationship between the sum of all peak areas detected by the ECD-HPLC system and the DPPH radical scavenging rate was investigated. The result at +1.1 V is shown in Fig. 5. Except for Caryophylli Flos and Rhei Rhizoma, whose scavenging ratios were out of range (almost 100%), and Coptidis Rhizoma, owing to weak activities, a relationship between these two results was noted. That is, the sum of peak areas and the scavenging rates were proportional, with a correlation coefficient of 0.76. Additional investigation using one tenth diluted solutions, including the methanol extracts of Caryophylli Flos and Rhei Rhizoma,

also led to a correlation coefficient of 0.77 from all results (data not shown).

Concerning the applied voltage, it was found that the ratios of each component at +0.7, +0.9 and +1.1 V were different (data not shown). Some peak areas increased with an increase in the applied potential, and some decreased. Therefore, in the investigation of the correlation between the sum of all peak areas and the DPPH radical scavenging rate, the results at +1.1 V were used. Many types of antioxidative component, including weak ones with an oxidation potential above +1.0 V, will be detected at an applied voltage of +1.1 V. Even at +0.7 V, where only strong antioxidative components may be detected, there was a relationship between the two results with the same correlation coefficient of 0.76, as in the results at +1.1 V. However, with medium voltage (+0.9 V), a lower correlation coefficient value (r^2=0.62) was observed compared with the others. This may be caused by a large number of components present in the methanol extracts of crude drugs, whose oxidation potential is around +0.9 V (see Fig 1).

In conclusion, from the results above, it was found that crude drugs whose methanol extracts showed oxidative peaks in the low potential region, had strong and rapid radical scavenging activities. Furthermore, a relationship between the scavenging capability and peak areas detected by the ECD-HPLC system was found. The ECD-HPLC method should be useful for simple evaluation of antioxidative activities, as well as for separation analysis of antioxidative ingredients.

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