

Electrochemical Method for Estimating the Antioxidative Effects of Methanol Extracts of Crude Drugs¹⁾

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The antioxidative effects of methanol extracts of crude drugs were estimated by an electrochemical method because there are many electrochemically-active substances in natural antioxidants. Twelve kinds of crude drugs, which had been reported to exhibit strong activity in an antioxidative test based on the air oxidation of linoleic acid, were studied. The oxidative capacity calculated from voltammograms of their methanol extracts were compared and examined together with data on their radical scavenging effects. The results showed that the electrochemical behavior in most cases correlated with the radical scavenging effect. Crude drugs which had clear oxidative peaks below +1.2 V and a large oxidative capacity were suggested to have strong radical scavenging effects. It was clear that substances oxidized at lower potentials had stronger radical scavenging effects than those oxidized at higher potentials. Therefore, this electrochemical method can be considered as a rapid and simple method for estimating the antioxidative effects as a radical scavenger.

Keywords antioxidative effect; crude drug; electrochemical method; voltammogram; radical scavenging effect

Recently, natural antioxidants have attracted attention because some synthetic antioxidants have been found to be carcinogenic and harmful to lungs and liver.^{2–8)} Estimation of antioxidative effects has been carried out, for example, by determining the inhibitory effect on the air oxidation of linoleic acid and the radical scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.⁸⁾ As part of our investigations on the analysis of constituents of crude drugs, we used an electrochemical method for the estimation of the antioxidative effect of methanol extracts of crude drugs because there are many electrochemically-active substances in natural antioxidants.

Experimental

Materials Corni Fructus and Astragali Radix were provided by Tenjin Li Sheng Pharmaceutical Factory, Tenjin, China. All other crude drugs were provided by Alps Pharmaceutical Inc. Co., Ltd., Gifu, 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 reagent grade.

Preparation of Methanol Extract One gram of powder or a small piece of crude drug was extracted with 90 ml of methanol under reflux for 1 h. After cooling, the extract was filtered and made up to 100 ml with methanol and gives a methanol extract.

Measurement of the Voltammogram of Methanol Extract The methanol extract containing 50 mM of tetraethylammonium perchlorate as a supporting electrolyte was used as the sample solution for recording the voltammogram. The methanol extract of *Coptidis Rhizoma* was diluted five-fold before use. Voltammetry was performed by using a three-electrode system. The working electrode was glassy carbon (GC-P2, 3 mm d., disc type, Yanagimoto, Kyoto, Japan), which was polished to a mirror-like finish with 0.05 μm alumina powder and washed for 10 s in distilled water in an ultrasonic cleaner (Type 3200, Yamato Scientific Co., Ltd., Tokyo, Japan). The reference electrode was Ag/AgCl (RE-18, BAS Co., Ltd., Tokyo, Japan). The counter electrode was Pt wire (Kel-F, 23 cm, BAS Co., Ltd.). Measurement of the voltammograms was carried out using a potentiostat (HA-501, Hokuto Denko, Ltd., Tokyo, Japan) with a function generator (HB-104, Hokuto Denko, Ltd.) and an X-Y recorder (Type 3086, Yokogawa Electric Corporation, Tokyo, Japan) from –0.3 to +1.9 V with a scan rate of 100 $\text{mV}\cdot\text{s}^{-1}$.

Estimation of the Radical Scavenging Effect of Methanol Extract on

DPPH Radical This was carried out according to the report of Uchiyama *et al.*⁹⁾ To 0.1 ml of methanol extract was added 4 ml DPPH ethanol solution (1.25×10^{-4} M), 4 ml acetate buffer (0.1 M, pH 5.5) and ethanol to a volume of 10 ml. After exactly 0.5, 1, 3, 5 and 7 h, the absorbance of this solution was determined at 524 nm using a spectrophotometer (U-3300, Hitachi Ltd., Tokyo, Japan) and the residual rate was calculated. It was confirmed that this method was not influenced by the coloring of the methanol extracts of *Coptidis Rhizoma*, *Cinnamonomi Cortex* *etc.*

Chromatography of the Methanol Extract of *Coptidis Rhizoma* The HPLC apparatus consisted of an injector (7125, Rheodyne, Cotati, CA, U.S.A.), a pump (LC-9A, Shimadzu, Kyoto, Japan), two reverse-phase columns connected in series (Nucleosil 5C18, 150 \times 4.6 mm i.d., Chemco Scientific Co., Ltd., Osaka, Japan), a column heater (CTO-6A, Shimadzu), an electrochemical detector (ECD) with a glassy carbon working electrode (Σ 875, Irika Instruments Inc.) and an integrator (C-R5A, Shimadzu). The mobile phase was consisted of a mixture of acetonitrile and 0.01 M dipotassium hydrogenphosphate (35:75), at a flow rate of 1.0 ml/min, the column temperature was 40 °C and the sample injection volume was 20 μl . The applied potential of the ECD was set at +0.7 or +1.6 V vs. Ag/AgCl.

Results and Discussion

Toda *et al.* carried out the antioxidative test based on the air oxidation of linoleic acid²⁾ using methanol extracts of 107 kinds of crude drugs to find new natural antioxidants and reported that 20 extracts exhibited a strong antioxidative effect.³⁾ Out of these 20 types of crude drug, 12 were available to us and were used in the present study. The oxidative capacity, calculated from the voltammograms of the methanol extracts of these 12 crude drugs, were examined and compared with the reported data of Toda *et al.* along with the results of the radical scavenging effects on DPPH radical. The voltammograms and oxidative capacity (subtracting the voltammogram area of the blank from the methanol extract) of the methanol extracts are shown in Fig. 1 and Table I.

Coptidis Rhizoma, *Magnoliae Cortex* and, especially, *Caryophylli Flos* had a extremely large oxidative capacity. On the other hand, the oxidative capacity of *Coicis Semen*, *Bupleuri Radix* and *Astragali Radix* was very limited. Of the 12 kinds of crude drugs reported by Toda *et al.*, the

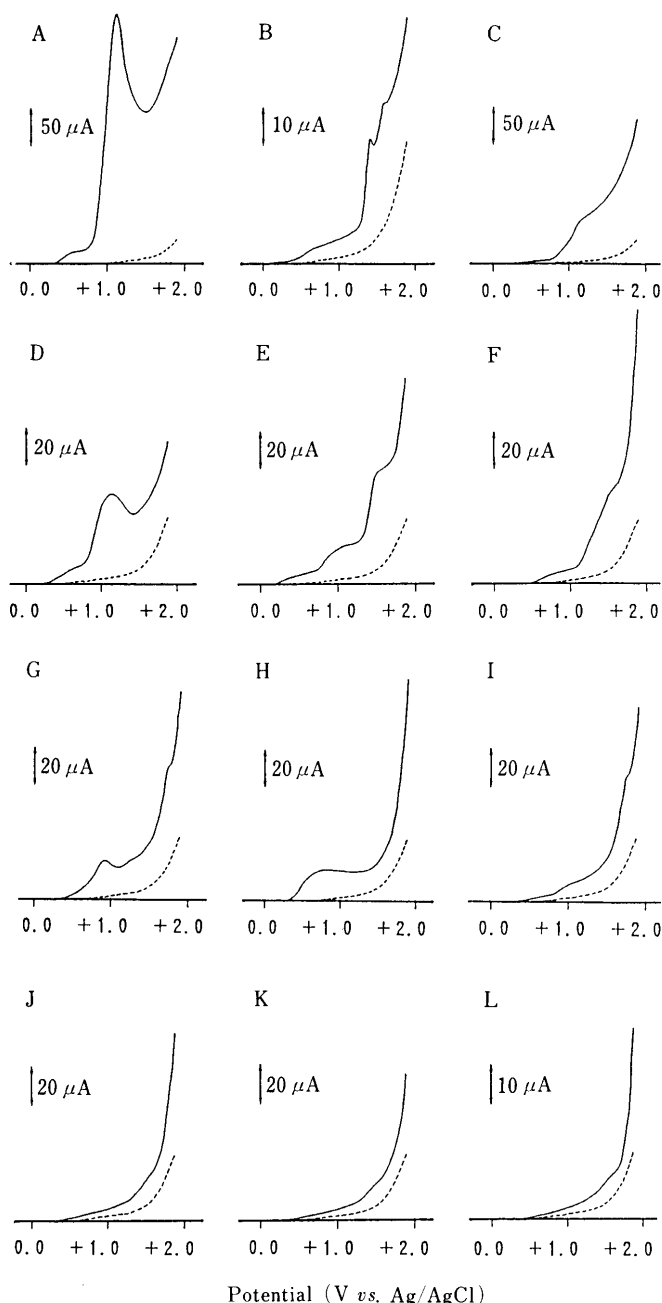


Fig. 1. Voltammograms of Methanol Extracts of Crude Drugs

A, Caryophylli Flos; B, Coptidis Rhizoma (diluted to five times); C, Magnoliae Cortex; D, Rhei Rhizoma; E, Moutan Radicis Cortex; F, Schizandrae Fructus; G, Zingiberis Rhizoma; H, Corni Fructus; I, Cinnamonomi Cortex; J, Astragali Radix; K, Bupleuri Radix; L, Coicis Semen. —, methanol extract; - - - -, blank (50 mM tetraethylammonium perchlorate).

antioxidative effect of Caryophylli Flos, Coptidis Rhizoma and Magnoliae Cortex was moderate and that of Coicis Semen, Bupleuri Radix and Astragali Radix was comparatively strong. These data were obtained by using the thiobarbituric acid and peroxide values as indexes of the antioxidative effect, so no correlation could be made between the data on the oxidative capacity and the report of Toda *et al.* Following the measurement of the cyclic voltammograms of the methanol extracts, all oxidative peaks obtained were found to be irreversible.

The DPPH stable radical loses its characteristic purple color when supplied electrons or hydrogen ions and the capacity of the tested substances to donate electrons can

TABLE I. Oxidative Capacity Calculated from the Voltammograms of Methanol Extracts of Crude Drugs and the Radical Scavenging Effect of Methanol Extracts of Crude Drugs on the DPPH Radical

Crude drugs	OC ^{a)} (μC)	[CV ^{b)} (%)]	RRA ^{c)} (%)
Caryophylli Flos	2348	[2.81]	9.3
Coptidis Rhizoma	984	[1.68]	71.9
Magnoliae Cortex	805	[1.40]	47.7
Rhei Rhizoma	390	[3.51]	9.4
Moutan Radicis Cortex	378	[2.06]	17.4
Schizandrae Fructus	324	[0.64]	93.3
Zingiberis Rhizoma	290	[1.70]	74.0
Corni Fructus	269	[3.17]	53.1
Cinnamonomi Cortex	195	[0.51]	79.8
Astragali Radix	140	[8.43]	95.8
Bupleuri Radix	104	[6.26]	92.3
Coicis Semen	53	[3.26]	99.5

a) Oxidative capacity. b) Coefficient of variation ($n=3$). c) Residual rate of absorbance at 524 nm after 30 min.

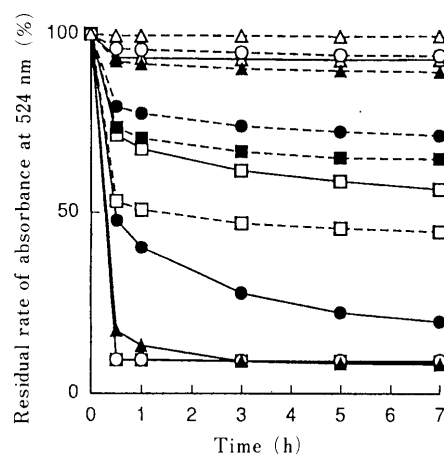


Fig. 2. Radical Scavenging Effect of Methanol Extracts of Crude Drugs on the DPPH Radical

—■—, Caryophylli Flos; —□—, Coptidis Rhizoma; —●—, Magnoliae Cortex; —○—, Rhei Rhizoma; —▲—, Moutan Radicis Cortex; —△—, Schizandrae Fructus; —■—, Zingiberis Rhizoma; —□—, Corni Fructus; —●—, Cinnamonomi Cortex; —○—, Astragali Radix; —▲—, Bupleuri Radix; —△—, Coicis Semen.

be estimated from the degree of loss of color.¹⁰⁾ The time course of fading of DPPH was examined in order to estimate the antioxidative effect of the methanol extracts as radical scavengers. The results are shown in Fig. 2 and residual absorbance at 524 nm after 30 min is shown in Table I.

Caryophylli Flos and Rhei Rhizoma reacted extremely rapidly with DPPH, followed by Moutan Radicis Cortex. Caryophylli Flos and Rhei Rhizoma scavenged all radical within 30 min, and so exhibited a strong radical scavenging effect. Clear oxidative peaks were observed from +1.1 to +1.2 V in the voltammograms of the methanol extracts of these three crude drugs. On the other hand, Astragali Radix, Bupleuri Radix and Coicis Semen, whose voltammograms exhibited no clear oxidative peak over the range studied had extremely weak or no radical scavenging effects. The data on the oxidative capacity correlated with the radical scavenging effect except in the case of Coptidis Rhizoma, Schizandrae Fructus and Corni Fructus. Actually, the radical scavenging effects of Coptidis Rhizoma and Schizandrae Fructus were weaker

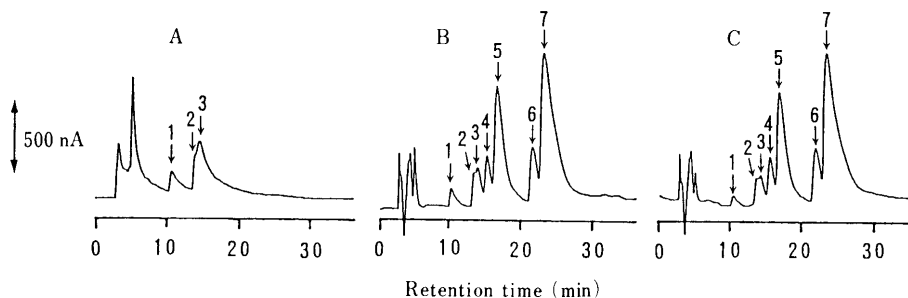


Fig. 3. Chromatograms of the Methanol Extract of Coptidis Rhizoma

A: methanol extract (2 ml) + ethanol (1 ml). B: methanol extract (2 ml) + ethanol (1 ml). C: methanol extract (2 ml) + DPPH ethanol solution (1 ml).

while that of Corni Fructus was stronger than would be expected from their oxidative capacity. Coptidis Rhizoma and Schizandrae Fructus had no clear oxidative peak below +1.2 V, while Corni Fructus had a clear oxidative peak at +0.8 V, as shown in Fig. 1. Accordingly, in addition to the earlier results, it can be presumed that a clear oxidative peak below +1.2 V is one of the signs that a methanol extract has a strong radical scavenging effect. The oxidative capacity of Coptidis Rhizoma shows itself mainly as oxidative peaks observed at about +1.4 and +1.6 V, *i.e.* the oxidative peaks of weakly oxidizable substances. On the other hand, the oxidative capacity of Coptidis Rhizoma was shown by mainly oxidative peaks observed at about +0.8 V, *i.e.* the oxidative peaks of readily oxidizable substances. So, it seems that substances oxidized at a lower potential have a stronger radical scavenging effect than substances oxidized at a higher potential. The behavior of each component of the methanol extract of Coptidis Rhizoma was investigated by HPLC with ECD in order to prove this assumption.

The chromatograms of the methanol extract of Coptidis Rhizoma obtained under the conditions described in the Experimental section are shown in Fig. 3.

A is a chromatogram of the mixture of 2 ml methanol extract and 1 ml ethanol determined at +0.7 V. B is a chromatogram of the same sample determined at +1.6 V. In addition to the peaks (1–3) oxidized at +0.7 V, 4 new peaks (4–7) appeared because of the rise in applied potential. C is a chromatogram of the mixture of 2 ml methanol extract and 1 ml 2.5×10^{-3} M DPPH in ethanol determined at +1.6 V after 30 min of mixing. The peak area of 4 peaks (4–7) oxidized at +1.6 V did not change, while that of 3 peaks (1–3) oxidized at +0.7 V decreased [The total peak area of 4 peaks (4–7) in C was 105% that in B, while the total peak area of 3 peaks (1–3) in C was 75% that in B]. Therefore, it was clear that the reaction was faster with radicals of the substances oxidized at a lower potential than with substances oxidized at a higher potential. In other words, substances oxidized at a lower potential had a stronger radical scavenging effect. This showed that our assumption was correct. Similar results were obtained in the experiment using Caryophylli Flos. The recording of voltammograms of 9 kinds of crude drugs, except for Astragali Radix, Bupleuri Radix and Coicis Semen, which showed clear oxidation peaks, was carried out changing the scan rate (v) from 10 to

100 $\text{mV} \cdot \text{s}^{-1}$. As the result, the plot of the maximum oxidation peak current (I_{max} : the peak current at +1.6 V in the case of Coptidis Rhizoma for example) against $v^{1/2}$ was linear and it is suggested that the current of these electrode reaction is governed by diffusion and that there is no kinetic current. I_{max} , measured after 24 h, did not change from its initial value and no chemical reaction among the components in the methanol extract was observed.

From the results mentioned above, we found that the electrochemical estimation of the antioxidative effect of methanol extracts of crude drugs was correlated in most cases with their radical scavenging effects. It is suggested that crude drugs which have a clear oxidative peak below +1.2 V and a large oxidative capacity have a strong radical scavenging effect. Substances oxidized at a lower potential have a stronger radical scavenging effect than substances oxidized at a higher potential. Antioxidants can be classified functionally into radical scavengers, peroxide decomposers, single oxygen quenchers, enzyme inhibitors *etc.* This electrochemical method can be considered as a rapid and simple method for estimating the antioxidative effects as a radical scavenger of not only crude drugs but also many other types of compound.

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References

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