Antioxidant and anticancer activities of organic extracts from \textit{Platycodon grandiflorum} A. De Candolle roots

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Abstract

This study examined the antioxidant and anticancer activities of the petroleum ether extracts from the roots of \textit{Platycodon grandiflorum} A. DC, which is a plant used as both a herbal medicine and food in Asia. Extracts from \textit{Platycodon grandiflorum} in petroleum ether were fractionated (fractions I–V) by silica gel column chromatography using gradient solvents (petroleum ether–ethyl ether, 9:1–5:5, v/v). The antioxidant activities of the fractions were evaluated in terms of their inhibition of lipid peroxidation as well as their free radical scavenging activity. Fraction II, which was extracted at an 8:2 mixture of petroleum ether and ethyl ether, exhibited the greatest antioxidant activity among the fractions. On the other hand, the cytotoxicity of each fraction, which was evaluated by the MTT assay using human cancer cell lines (HT-29, HRT-18 and HepG2), was greatest in fraction III, which was extracted with a 7:3 petroleum ether and ethyl ether mixture. Both fractions, II and III, were sub-fractionated by thin layer chromatography, and the sub-fractions each were screened for their antioxidant and anticancer activities. In addition, the antioxidant activity was closely related to the content of phenolic compounds, and the anticancer active fraction exhibited a typical UV absorbance spectrum of polyacetylene.

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1. Introduction

Antioxidants are added to a variety of foods to prevent or deter free radical-induced lipid oxidation, which is responsible for the development of off-flavors and the undesirable chemical compounds in food (Angelo, 1996). The free radicals can also be generated in biological systems in the form of reactive oxygen species (ROS), such as superoxide anion radicals (O$_2^•$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (OH•), and the singlet oxygen (1O$_2$) (Halliwell et al., 1995). These reactive ROS cause destructive and irreversible damage to the components of a cell, such as lipids, proteins and DNA (Lopacyrski and Zeisel, 2001). Although normal cells possess antioxidant defense systems against ROS, the continuous accumulation of damage to the cells induces diseases such as cancer and aging (Mateus and Sánchez-Jiménez, 2000). The continuous antioxidant dose also plays a preventive role against these diseases by removing the ROS in biological systems (Sgambato et al., 2001).

Anticancer agents, on the other hand, are mainly related to their curative role in a damaged system. Under normal conditions, the cells in which the DNA or other components are irreversibly damaged by various causes undergo apoptotic cell death, which is a self-destructive metabolism according to the genetically encoded cell death-signal (Korsmeyer, 1995; Hooper et al., 1999). However, cancer cells, which are already irreversibly developed, obtain the capability to evade apoptosis by various ways. The aim of anticancer agents is to trigger the apoptosis signaling system in these cancer cells whilst disturbing their proliferation (Bold et al., 1997).

Plants have many phytochemicals with various bioactivities, including antioxidant, anti-inflammatory and anticancer activities. For example, some studies have reported that extracts from natural products, such as fruits, vegetables and medicinal herbs, have positive effects against cancer, compared with chemotherapy or recent hormonal treatments (Pezzuto, 1997; Wu et al., 2002). Therefore, many plants have been examined to identify new and effective antioxidant and anticancer compounds, as well as to elucidate the mechanisms of cancer prevention and apoptosis (Puett et al., 1998; Kim et al., 1998; Swamy and Tan, 2000). In particu-
lar, oriental medicinal plants are considered to be one of the most promising sources due to their variety of species and applications. In addition, their therapeutic effect has been demonstrated by their clinical uses in Asia for many decades.

The roots of Platycodon grandiflorum A. De Candolle (Korean name, ‘Doraji’, Japanese name, ‘Kikyo’, and Chinese name, ‘Jiegeng’), which belongs to the Campanulaceae family, have been used as either a food material or a traditional oriental medicine. The extracts from Platycodon grandiflorum have been reported to have a wide range of health benefits. In particular, in Korea, the roots grown for 4 years have been used to treat bronchitis, asthma, pulmonary tuberculosis, diabetes and inflammatory diseases (Takagi and Lee, 1972; Lee, 1973). Recently, its immunopharmacological effects have been studied (Nagao et al., 1986), and some active compounds, such as triterpenoid (Nikaido et al., 1999) and saponin (Ishii et al., 1984) have been identified. Some studies even extended the cultivation period of Platycodon grandiflorum to 22 years using a patented method (Lee, 1991), and reported that its aqueous extracts were effective in preventing hypercholesterolemia, hyperlipidemia, and CCl4-induced hepatotoxicity (Lee and Jeong, 2002). However, there are few reports on the antioxidant and anticancer activity of the organic extract from Platycodon grandiflorum.

In a previous study, it was reported that the crude petroleum ether extract from Platycodon grandiflorum exhibited strong inhibitory activity against human cancer cell growth, and the activity of the organic extract was greater than that of the aqueous extract (Lee et al., 1998). In this study, the petroleum ether extract of Platycodon grandiflorum root was fractionated using gradient solvents, and their antioxidant activity was compared with commercial antioxidant agents. In addition, the anticancer activity of the fractions was also examined.

2. Materials and methods

2.1. Material

The chopped and dried roots of Platycodon grandiflorum A. De Candolle (Campanulaceae family) cultivated in Youngju, Korea, were purchased from the oriental herbal market, and the roots grown for 4 years were obtained from the American Type Culture Collection (Maryland, USA). Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum and trypsin-EDTA were acquired from Gibco BRL (Grand Island, NY, USA), and the culture supplies such as the 48-well plates were obtained from Nunclon Brand Products. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from the Sigma-Aldrich Company (St. Louis, MO, USA). Silicic acid (BIO-SIL, A 100–200 mesh) was a product from Bio-rad Lab. (Richmond, CA, USA), and the analytical and preparative thin layer chromatography (TLC) was performed using silica gel 60 F254 (Merck, Darmstadt, Germany).

2.2. Extraction and fractionation

The dried roots of Platycodon grandiflorum (600 g) were ground into powder, and extracted with petroleum ether (4 L) by shaking for 72 h at room temperature. After the powder particles had settled down, the clear yellow supernatant was filtered with a 0.22 μm pore size PTFE filter (Milipore Co., Billerica, USA), and concentrated (10.3 g, dry weight) by vacuum-evaporation. The concentrate was then fractionated (Fractions I–V) using a silica gel column with a solvent gradient of petroleum ether and ethyl ether (9:1–5:5), and the fractions were concentrated under reduced pressure.

2.3. Measurement of the antioxidant activity

2.3.1. Ferric thiocyanate test (FTC)

The antioxidant activity analysis using ferric thiocyanate was performed according to the method reported by Osaka and Namiki (1981). Six hundred micrograms of the dried solids from each fraction were dissolved in 0.12 mL of 98% ethanol, and 2.88 mL of a 2.51% linoleic acid solution in EtOH and 9 mL of a 40 mM phosphate buffer (pH 7.0) were added. The mixture was incubated at 40 °C in a dark screw-cap vial. During the incubation, a 0.1 mL aliquot was taken from the mixture, and diluted with 9.7 mL of 75% ethanol, followed by the addition of 0.1 mL of 30% ammonium thiocyanate. Precisely 3 min after adding the 0.1 mL of 20 mM ferrous chloride in 3.5% hydrochloric acid, the absorbance for the red color was measured at 500 nm. The level of lipid peroxidation inhibition by each fraction was calculated from the absorbance ratio to that of a blank without any sample.

2.3.2. Thiobarbituric acid test (TBA)

This assay was performed according to the method reported by Kikuzaki and Nakatani (1993). Two milliliters of 20% trichloroacetic acid and 2 mL of thiobarbituric acid solutions were added to 1 mL of the mixture solution containing linoleic acid, which was prepared according to the FTC procedure. The mixture was then placed in a boiling water bath for 10 min. After cooling, the mixture was centrifuged at 3000 rpm for 20 min, and the absorbance of the supernatant was measured at 532 nm.

2.3.3. DPPH radical scavenging test

This test was measured as described by Blois (1958). One milliliter of the fraction solutions (50, 100, and 200 μg/mL in ethanol) was added to 1 mL of a DPPH solution (0.2 mM in ethanol). After a 30 min of reaction at room temperature, the absorbance of the solution was measured at 517 nm.
The free radical scavenging activity of each fraction was determined by comparing its absorbance with that of a blank solution (no sample).

2.4. Cytotoxicity on cancer cells (MTT test)

This assay was performed according to a slight modification of the procedure reported by Mosmann (1983). In 48-well plates, a human cancer cell suspension (3 × 10^4 cells/well) was incubated for 24 h at 37 °C. The cells were then rinsed and grown in a new DMEM containing each fraction (300 μg/mL). After incubation for 24 or 48 h at 37 °C, the DMEM was removed, and the cells were again incubated again with 0.25 mL of DMEM and 0.05 mL of a MTT solution (0.5 μg/mL) for 4 h. 0.7 mL of the lysing buffer (20% sodium dodecyl sulfate in 50% N,N-dimethylformamide, pH 4.6) was then added to each well to dissolve the purple formazan produced by the MTT, and the cells were incubated for a further 2 h. The plate was read using a spectrophotometer (Beckman model DU-64) at a wavelength of 540 nm. The cytotoxicity was obtained by comparing the absorbance between the samples and the control.

2.5. Total phenolic content

The total phenolic content was determined using a Folin-Ciocalteu reagent according to the procedure reported by Singleton and Rossi (1965). The fractions (400 μg/mL in ethanol) were mixed with 0.75 mL of a Folin-Ciocalteu reagent, which was diluted 10-fold with distilled water immediately before use, which was followed by standing at room temperature for 5 min. Subsequently, 0.75 mL of a sodium bicarbonate solution (60 mg/mL) was added. This mixture was stored for 2 h at room temperature, and its absorbance was measured at 725 nm. The analysis was performed in triplicate, and the results are expressed as the ferulic acid equivalents.

2.6. Thin layer chromatography (TLC)

The fractions from the silica gel column were analyzed by TLC with a mobile phase of petroleum ether:chloroform:methanol (15:7:3, v/v/v) (A), or petroleum ether:dioxane:acetic acid (80:20:1, v/v/v) (B), according to a slight modification of the procedure reported by Amarowicz et al. (2000). The separated spots on the TLC plate were identified under UV lights of a short (254 nm) and a long (365 nm) wavelength, and also by spraying with H_2SO_4. The phenolic compounds could be visualized by spraying the plates with a 1% FeCl_3 solution in 1 M HCl (Rein, 1958). For the rapid detection of the antioxidant activity of the spots, the plates were stained with a DPPH solution (Soles-Rivas et al., 2000). After discerning the most active spot on the plates, a preparative TLC plate was used to scrape and collect—it’s a large amount for a quantitative DPPH assay of the antioxidant activity.

3. Results and discussion

3.1. Yield and total phenolic content

Fractions I–V were separated from the crude petroleum ether extract using a silica gel column and gradient solvents (9:1–5:5), yielding 0.02–0.33 g/g, based on the initial weight of the crude extract (Table 1). The total phenolic content in the fractions ranged from 1.66 to 4.80 mg/g, and fraction II, which was eluted with a 8:2 mixture of petroleum ether and ethyl ether, contained the highest level of the phenolic compounds.

3.2. Inhibition of lipid peroxidation

The antioxidant activity of the crude extract and its fractions were measured using ferric thiocyanate (FTC) and thio-barbituric acid (TBA) tests. In the FTC test, which determines the amount of peroxide produced at the initial stage of lipid peroxidation, a lower absorbance indicates a higher level of antioxidant activity. Fig. 1 shows the changes in the absorbance for each fraction during 3 days of incubation at 40 °C, in comparison with BHA and α-tocopherol. The absorbance of the control increased in proportion to the incubation time, and the absorbance of the other samples also increased with increasing incubation time. However, most samples, with the exception of fraction I, showed a significantly lower increment in the rate compared to the control after 48 h (P < 0.01). Although the differences between the samples were not so significant until 48 h, fraction II showed a slightly higher inhibition effect after 72 h than the others (P < 0.01). However, the differences in the absorbance between the fractions and commercial antioxidants, α-tocopherol and BHA, also increased as incubation continued.

Different from the FTC test, which is related to the peroxide formation in the initial stage of lipid oxidation, the TBA test measures the amount of malondialdehyde (MDA) produced after the decomposition of the lipid peroxide during the oxidation process. MDA is a very unstable compound causing mutagenic and cytotoxic events (Zin et al., 2002). At a low pH and high temperature (100 °C), MDA binds TBA to form a red complex that can be measured at

| Table 1 |
|-----------------|-----------------|-----------------|
| **Fraction**    | **Yields (g/g)**| **Total phenolic content (mg/g)** |
| I (9:1)         | 0.33            | 1.66 ± 0.42     |
| II (8:2)        | 0.11            | 4.80 ± 0.26     |
| III (7:3)       | 0.26            | 3.91 ± 0.24     |
| IV (6:4)        | 0.05            | 3.45 ± 0.17     |
| V (5:5)         | 0.02            | 3.79 ± 0.10     |

* g total phenolic/g fractions: Ferulic acid equivalents (mean ± S.D.; n = 3).
3.3. Scavenging activity of free radical

Free radicals are known to be a major factor in biological damages, and DPPH has been used to evaluate the free radical-scavenging activity of natural antioxidants (Yokozawa et al., 1998; Zhu et al., 2001). DPPH, which is a radical itself with a purple color, changes into a stable compound with a yellow color by reacting with an antioxidant, and the extent of the reaction depends on the hydrogen donating ability of the antioxidant (Bondent et al., 1997). Fig. 3A shows the DPPH free radical scavenging activity of each fraction at three different concentrations, 50, 100 and 200 μg/mL. All the fractions showed dose-dependent increase in activity. Fraction II exhibited the strongest antioxidant activity in accordance with the other tests (P < 0.01), and the activities decreased in the order of fraction III > crude > V > I > IV at a concentration of 200 μg/mL.

The IC50 of fraction II for the DPPH radical scavenging activity was calculated to be approximately 130 μg/mL.

Since fraction II had the highest antioxidant activity in the three antioxidant activity tests, it was further sub-fractionated by TLC. Five different spots from fraction II were detected under the short and long wavelength of UV light, and showed different colors by H2SO4: Rf = 0.45 (yellow-brown), 0.56 (violet), 0.64 (green-yellow), 0.68 (dark brown) and 0.81 (brown) for sub-fraction II-1, 2, 3, 4 and 5, respectively. From a rapid DPPH test on the TLC plate, all the sub-fractions, except for II-5, showed a bright yellow color, and in particular, sub-fraction II-2 had a relatively stronger free radical scavenging activity.
than the other sub-fractions. Since it was reported that the antioxidant activity of a plant extract often originates from phenolic compounds (Velioglu et al., 1998; Amarowicz et al., 2000), a phenolic spot test was performed directly on the TLC plate. Similar to the previous phenolic test results of fraction II, sub-fraction II-2 showed a strong blue color, indicating that it has a high concentration of phenolic compounds which are responsible for its high antioxidant activity.

According to the results of analytical TLC, all the five sub-fractions were obtained using preparative TLC, and the DPPH free radical scavenging activities of the sub-fractions were measured quantitatively (Fig. 3B). The radical scavenging activity of sub-fraction II-2 was significantly stronger than those of the other sub-fractions and α-tocopherol, but was still lower than that of BHA (P < 0.01). However, considering that the sub-fraction is not a pure compound, the active compound can be expected to be comparable to the strong antioxidant, BHA.

3.4. Cytotoxicity activity on cancer cells

The anticancer activities of the Platycodon grandiflorum extracts and its fractions were investigated using a MTT assay on three human cancer cell lines, HT-29, HepG2 and HRT-18. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells (Mosmann, 1983). Fig. 4A shows that fractions II, III and V significantly inhibited cancer cell growth at a concentration of 300 μg/mL (P < 0.01), and in particular fraction III exhibited the highest cytotoxicity (>50%) in all three cell lines. Among the cell lines, the cytotoxic susceptibility to fraction III was greater in the HepG2 (69.1 ± 0.52%) than in HT-29 (53.0 ± 2.22%) or HRT-18 (56.2 ± 0.50%) cells (P < 0.01), but this difference was not as significant for the other fractions. The significant negative activities (P < 0.01) of some fractions to specific cell lines means that they increased the growth rate of cancer cell, but more confirmative studies will be needed in the future to confirm this.

Fraction III was also examined by analytical TLC, and six sub-fractions were obtained by preparative TLC (III-1–III-6). The anticancer activities of the sub-fractions were assayed using one of the cell lines previously used, HT-29, at a concentration of 100 and 200 μg/mL after 24 h incubation. As shown in Fig. 4B, sub-fractions III-1 and III-2 had a significantly higher cytotoxicity on HT-29 than the other sub-fractions (P < 0.01), in a dose-dependent manner. The estimated IC50 for the cytotoxicity of sub-fractions III-1 and III-2 were approximately 340 and 390 μg/mL, respectively. The UV absorbance spectra of sub-fractions III-1 and III-2 revealed that they have a similar λmax at 238, 253, 268 and 283 nm, which is a typical spectrum for the diyn-ene chromophore of polyacetylene. Polyacetylenes
found in ginseng (Matsunaga et al., 1990) and other medicinal plants (Jung et al., 2002) have been reported to exhibit antioxidant activity. A few polyacetylene compounds have also been reported from the Platycodon grandiflorum organic extracts (Tada et al., 1995), but they are believed to be different from the polyacetylene in this study because of the different UV spectra.

4. Conclusion

The petroleum ether extract of Platycodon grandiflorum was confirmed to exhibit antioxidant and anticancer activities. The lipid peroxidation and free radical scavenging assays on the fractions from the silica gel column and TLC suggest that the antioxidant active and probably phenolic compound in this study has a high activity, which is comparable to BHA. Their MTT assay revealed that Platycodon grandiflorum also contains a strong polyacetylene anticancer compound, which exhibited cytotoxicity on the three human cancer cell lines. However, further studies will be needed to identify the exact molecular structures of both the antioxidant and anticancer compounds.

References


