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Determination of Antioxidant Activity of Some Commonly Consumed Leafy Vegetables in Thailand

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Abstract

Antioxidant activity of ten commonly consumed leafy vegetables in Thailand was determined using the β-carotene bleaching method. *Neptunia oleracea* exhibited the highest activity (13.1 mg BHA equivalent / g dry weight) followed by *Acacia pennata* and *Morinda citrifolia*. Nine vegetables out of ten showed a higher antioxidant activity than 25 mg BHA equivalent / 100 g fresh weight, while only one-third of the Japanese vegetables exhibited such a high activity. Extract from *Neptunia oleracea* gave five peaks (P-1, P-2, P-3, P-4 and P-5), which showed an antioxidant activity in HPLC analysis. It was suggested that P-3, P-4 and P-5 corresponded to the derivatives of apigenin based on the UV spectrum.

Key words: Antioxidant activity, β-carotene bleaching methods, vegetables, Thailand, *Neptunia oleracea* Lour.

Abbreviations:

HPLC: high performance liquid chromatography, UV: ultra-violet

Oxidative stress has been implicated in a variety of diseases, such as cardiovascular diseases and cancer [5, 12, 15, 16]. Reactive oxygen species (ROS), which induce the oxidative stress, are generated by the reaction of oxygen with radiation, various environmental chemicals, or produced in the course of metabolism by one-electron transfers to an unstable oxygen molecule. ROS react with most of the biological molecules and damage membranes, enzymes and genes [6, 8]. Since the generation of ROS can not be controlled, antioxidants and radical scavengers are important to suppress the oxidative stress [2, 4]. Many herbal diets contain antioxidative components, *e.g.* phenolics, terpenoides and tocopherols [3, 13, 21]. Thus, the consumption of fruits and vegetables could reduce the risk of chronic diseases. In fact, some epidemiological studies revealed that the incidence of heart diseases and some types of cancer in Mediterranean and Asian countries, where a large quantity of fruits and vegetables is consumed, is much lower than in northern Europe and North America [1, 18, 22].

The diet in the Southeast Asian countries is characterized by the intake of a wide variety of fruits, vegetables and spices[11]. In Thailand, several hundreds of wild/semi-wild plant species have been commonly consumed as diet and folk medicine for the treatment of diarrhea, constipation, inflammation, asthma, hypertension and as carminative agents over a long period of time[13,14]. Recently, several biochemical properties, *i.e.* antimutagenic activity and anti-tumor-promoting activity, of their constituents have been reported[9-11,17,20]. Active principles found in Thai plants were mostly phenol compounds, some of which show an antioxidant property. Therefore, it has been suggested that these plants could be a good source of dietary antioxidants, and that they play an important role in the prevention of diseases associated with oxidative stress. In this regard, the antioxidant activity of edible plants, in particular, commonly consumed leafy vegetables in Thailand, was determined using the β-carotene bleaching method, and fractionation of the extract of an active vegetable, *Neptunia oleracea* Lour., was carried out to investigate the chemical properties of active principles.

Materials and Methods

Materials

Ten leafy vegetables, *Acacia pennata* (L.) Willd. subsp. Insuavis Nielsen (common name in Thai: chaom), *Cassia siamea* Britt. (bai khee lek), *Ipomoea aquatica* Forsk. (pak bung cheen), *Melientha suavis* Pierre (pak wan), *Morinda citrifolia* (bai yor), *Neptunia oleracea* Lour. (pak krached), *Ocimum sanctum* Linn. (bai kraprao), *Ocimum basilicum* (bai horapa), *Ocimum canum* Sims. (bai mang luk), *Piper sarmentosum* Roxb. (bai chaplu), were obtained from retail markets, freeze-dried for further application and stored at -80 ℃ . Linoleic acid, β-carotene and Tween 40 (polyoxyethylenesorbitan monopalmitate) were purchased from Wako Pure Chemicals (Osaka, Japan). Apigenin and apigenin-7-glucoside were purchased from Funakoshi (Tokyo, Japan). All of the other reagents were of analytical grade.

Preparation of crude vegetable extracts

The freeze-dried vegetables (0.5 g) were homogenized and extracted twice with a total of 50 ml of 80% methanol. The insoluble solids were removed by centrifugation (TOMY MX-160, Japan) at 9,390 × g for 30 min. The obtained supernatants were used for determining the antioxidant activity.

Antioxidant activity assay

The antioxidant activity was determined by the β–carotene bleaching method at constant pH[19], which is based on the inhibition of the discoloration of β–carotene coupled with the auto-oxidation of linolate. The β–carotene-linolate solution contained β–carotene (5 mg/ml), linolate (400 mg/ml), Tween 40 (5 mg/ml) and 40 mM sodium phosphate buffer (pH 6.8). The reaction was initiated by mixing the vegetable extract (0.1 ml) and the β–carotene-linolate solution (4.9 ml) at 50° C. The decrease in absorbance at 470 nm, which was due to the decomposition of β–carotene, was recorded for 60 min with a Shimadzu UV-1200 spectrophotometer. The activity was estimated based on the decreasing rate of absorbance (15 - 45 min). Butylhydroxyanisol (BHA) was used as a standard antioxidant.

Fractionation of antioxidants

The crude extracts which showed a strong antioxidant activity were fractionated by reversed phase column chromatography. Concentrated extract was applied to a Wakogel LP-40 C18 column (diameter, 30 mm × 20 cm) equilibrated with water. The column was eluted by stepwise methanol gradients; 0% (100 ml), 15% (80 ml), 30% (100 ml), 50% (100 ml), 60% (100 ml), 70% (100 ml), 80% (100 ml) and 100% (100 ml). The absorbance at 280 nm and the antioxidant activity of the eluted fractions were measured.

High performance liquid chromatography analysis

HPLC analysis was performed using an automated liquid chromatography unit (Tosoh, Tokyo, Japan). The system consisted of a PX-8020 system controller, a CCPM-II pump, a PD-8020 photodiode array detector, a AS-8020 auto-injector and a CO-8020 column oven, and was equipped with a TSK gel super-ODS column (4.6 × 100 mm, Tosoh). The analysis was carried out at 40 ℃ .

Results and Discussion

Antioxidant activity of common vegetables in Thailand

The antioxidant activity (expressed as mg BHA equivalent) of the methanolic extracts is shown in Table 1. According to Tsushida *et al.*[19], extracts in 80% methanol showed a higher antioxidant activity than the extracts in several other solvents for most of the leafy vegetables in Japan. Thus, 80% methanol

Table1.Antioxidant activity of commonly consumed leafy vegetables in Thailand

Scientific name	Antioxidant activity (mg BHA equivalent/ g dry weight)	Antioxidant activity (mg BHA equivalent/ g fresh weight)
Neptunia oleracea	13.10	1.28
Insuavis Nielsen	7.86	1.44
Morinda citrifolia	7.08	1.30
Piper sarmentosum	5.24	0.93
Cassia siamea	4.84	1.36
Ocimum canum	4.46	0.54
Ipomoea aquatica	3.94	0.24
Ocimum sanctum	3.40	0.42
Ocimum basilicum	2.88	0.33
Melientha suavis	2.76	0.48

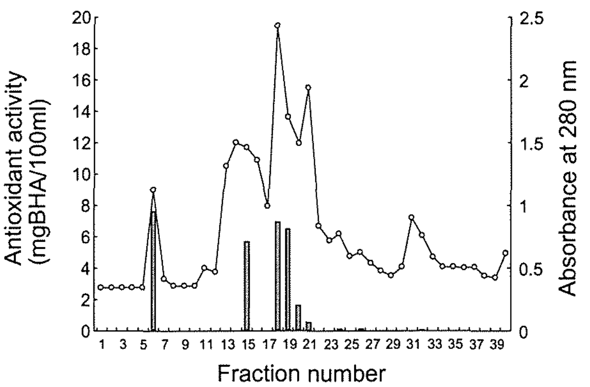

Fig.1. Reversed phase chromatogram of 80% methanol extract of *Neptunia oleracea*.
White circles and solid line: elution profile (absorbance at 280 nm); shadowed bars: antioxidant activity of each fraction.

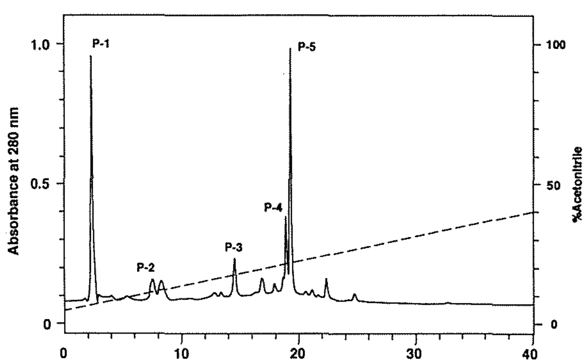

Fig.2. HPLC analysis of 80% methanol extract of *Neptunia oleracea*.
Solid line: elution profile (absorbance at 280 nm); dotted line: gradient curve.

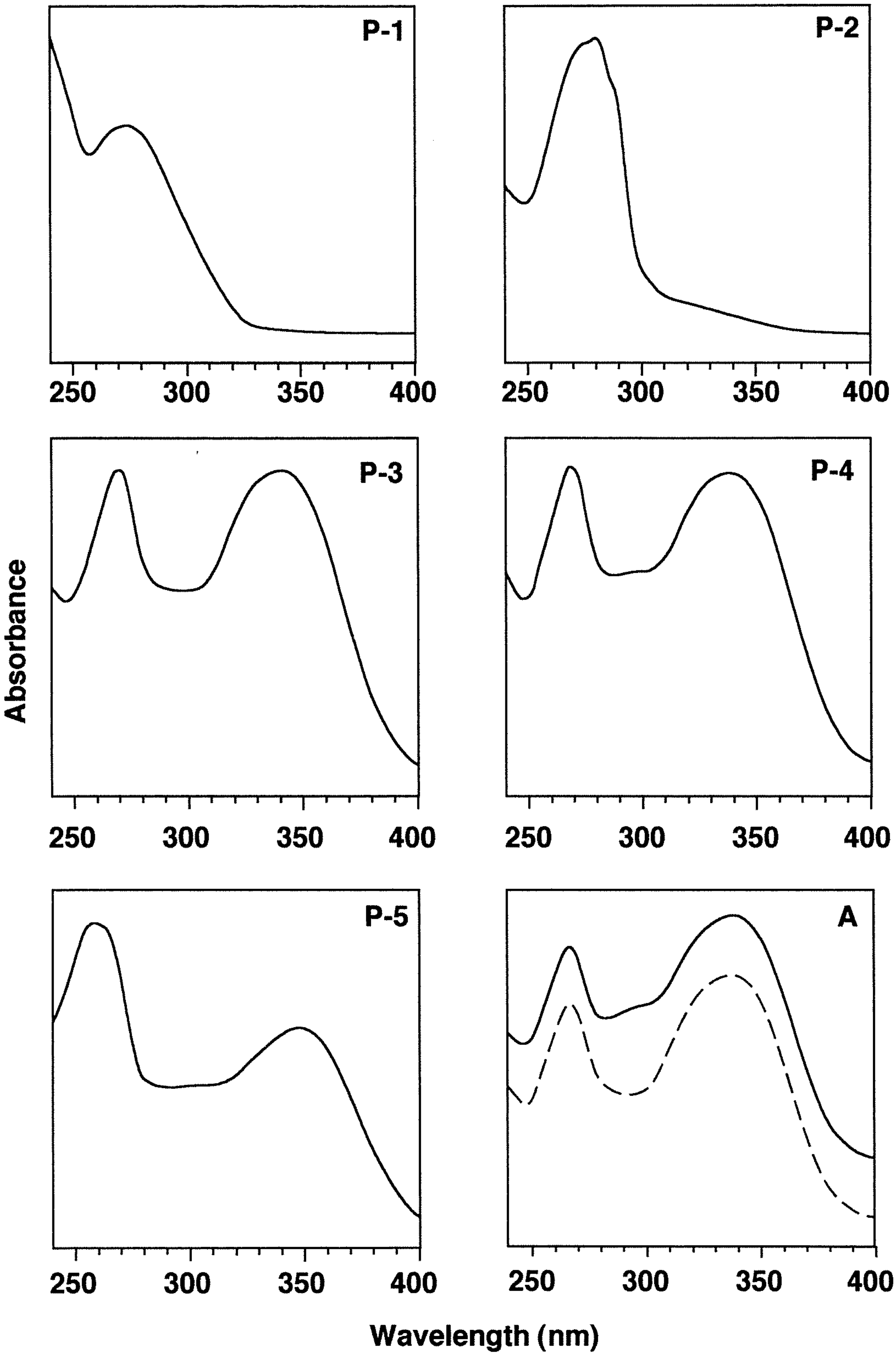

Fig. 3. UV spectra of authentic compounds and peaks obtained by HPLC analysis.
Panel A: UV spectra of authentic compounds. Solid line: apigenin; dotted line: apigenin-7-glucoside.

Table 2. Absorption maxima (λ_{max}) in UV spectra of fractions of *Neptunia oleracea*

peaks	λ_{max} (nm)
P-1	274
P-2	272s, 280, 287s
P-3	272, 335
P-4	268, 338
P-5	269, 339

was also used as the extractant in the current studies. The activity on a dry weight matter basis ranged from 2.76 to 13.1 mg BHA equivalent / g. Among ten kinds of vegetables, *Neptunia oleracea* exhibited the highest activity (13.1 mg BHA equivalent / g) followed by *Acacia pennata* (7.86) and *Morinda citrifolia* (7.08). On a fresh weight basis, *A. pennata* showed the highest activity (1.44 mg BHA equivalent /g fresh weight) followed by *Cassia siamea* (1.36), *M. citrifolia* (1.30) and *N. oleracea* (1.28). Among the vegetables examined in the present study which are commonly consumed, nine tested vegetables out of ten showed a higher antioxidant activity than 25 mg BHA equivalent / 100 g fresh weight, while only one-third of the Japanese vegetables exhibited such a high activity [19].

Fractionation of extract from Neptunia oleracea

Figure 1 shows the chromatogram of the crude extract of *Neptunia oleracea* obtained by reversed phase column chromatography. An antioxidant activity above 3.0 mg BHA equivalent/ 100 ml was detected in four fractions, fractions 6, 15, 18 and 19, which were eluted with an aqueous solution of methanol (less than 40%). These active fractions contained compounds which showed peaks designated as P-1, P-2, P-3, P-4 and P-5 by HPLC (Fig. 2). Peak P-1 was detected in fraction 6, P-2 in fraction 15, while P-3, P-4 and P-5 were found in three fractions 18, 19 and 20. The UV absorption spectra of P-1, P-2, P-3, P-4 and P-5 are shown in Fig. 3, and the λ_{max} values obtained are indicated in Table 2. The structure of the compound(s) in P-1 could not be determined only based on the data obtained in this study. Compound P-2 was ninhydrin-positive and showed the same retention time in the HPLC analysis as L-tryptophan. UV absorption spectrum of compound P-2 was also identical with that of L-tryptophan. Compounds in P-3, P-4 and P-5 showed similar UV spectra to that of apigenin, and the retention times in HPLC of these three peaks were shorter than those of apigenin and apigenin-7-glucoside. Compound P-3 was stable after treatment with 2N HCl at 100 ℃ for 30 min. Compounds P-4 and P-5 were converted to each other by this treatment, but apigenin was not observed. It is known that vitexin (apigenin-8-C-glycoside) and isovitexin (apigenin-6-C-glucoside) undergo isomerization during acid hydrolysis[7]. The behavior of P-4 and P-5 under the acid treatment was similar to the isomerization between vitexin and isovitexin. Thus, it was suggested that P-3, P-4 and P-5 are *C*-glycosyl derivatives of apigenin, although all of them exhibited different retention times on HPLC analysis from vitexin and isovitexin.

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タイにおける常食葉菜類の抗酸化活性

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摘　要

１０種類のタイの常食野菜（葉菜類）についてβ−カロチン退色法により抗酸化活性が測定された。*Naputunia oleracea* に最も高い活性（乾物重量１ｇ当たり 13.1 mg BHA 当量）が見られ，次いで *Acacia pennata* 及び *Morinda citrifolia* に高い活性が検出された。検討した１０種類の野菜のうち，９種類が鮮重量１００ｇ当たり 25 mg BHA 当量以上の高い活性を示した。日本の野菜では，このような高い活性はおよそ３分の１の野菜にしか見られない。*Naputunia oleracea* 抽出物は，HPLC 分析において５つの抗酸化成分のピークを示した（P-1, P-2, P-3, P-4 及び P-5）。これらのうち，P-3, P-4 及び P-5 は UV スペクトルから，アピゲニン誘導体であると考えられた。

キーワード：抗酸化活性、β−カロチン退色法、タイ野菜、*Naputunia oleracea*