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行政院國家科學委員會專題研究計畫成果報告

計畫編號：NSC 94-2313-B-038-004
執行期限：94 年 08 月 01 日至 95 年 07 月 31 日
主持人：侯文琪　執行機構名稱：台北醫學大學生藥所

一、中文摘要

由臺灣產台農一號山藥、台農二號山藥與名間長紅山藥塊莖抽取其粗黏質多醣與經部分純化後之黏質多醣進行抗氧化活性分析比較。利用分光光度計方法分析 DPPH 自由基，氫氧自由基與超氧自由基之清除實驗；也利用電子自旋共振儀分析氫氧自由基之清除實驗。以 50%清除濃度 (IC_{50}) 顯示，台農一號山藥、台農二號山藥與名間長紅山藥多醣在清除DPPH 自由基而言，純化前後分別為 0.329、0.279; 0.547、0.653 和 0.847、0.631 mg/ml。在清除氫氧自由基而言，純化前後分別為 0.668、1.146; 1.461、1.096 和 0.946、1.554 mg/ml。在清除超氧自由基而言，純化前後分別為 0.802、0.368; 0.681、0.258; 和 0.086、0.148 mg/ml。利用電子自旋共振儀分析氫氧自由基清除實驗，純化之台農一號山藥、台農二號山藥與名間長紅山藥多醣之 50%清除濃度為 0.083、0.47、0.004 mg/ml。以上結果顯示栽培種之間與純化前後之多醣有不同抗氧化活性。

關鍵詞：DPPH 自由基; 電子自旋共振儀; 氫氧自由基; 黏質多醣;超氧自由基;山藥

Abstract

Crude mucilages (CM) and partially purified mucilages (PPM) from three different Taiwanese yam cultivars, including *Dioscorea alata* L. cv.Tainong 1 (TN1), *Dioscorea alata* L. cv.Tainong 2 (TN2), and *D. alata* L. var. *purpurea* (Roxb.) Ming-Jen (MJ), were used for evaluating the antioxidant effects, including 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, hydroxyl radical, superoxide radical scavenging activities, and by electron spin resonance (ESR) spectrometry for hydroxyl radical scavenging activities. The IC_{50} stands for the concentration required for 50 % scavenging activity. The IC_{50} of CM and PPM against DPPH radical were 0.329, 0.279; 0.547, 0.653; and 0.847, 0.631 mg/ml, respectively, for TN1, TN2 and MJ. The IC_{50} of CM and PPM against hydroxyl radical by spectrophotometry were 0.668, 1.146; 1.461, 1.096; and 0.946, 1.554 mg/ml, respectively, for TN1, TN2 and MJ. The IC50 of CM and PPM against superoxide radical were 0.802, 0.368; 0.681, 0.258; and 0.086, 0.148 mg/ml, respectively, for TN1, TN2 and MJ. Using ESR to detect hydroxyl radicals, the IC_{50} of PPM against hydroxyl radical were 0.083, 0.47, and 0.004 mg/ml, respectively, for TN1, TN2 and MJ. The results demonstrated that different cultivars of yams exhibited different antioxidant ability, and the purification process could partially increase the antioxidant activity of the mucilage polysaccharide. Taken together, these results suggest that mucilage polysaccharides of the yam tuber might play an important role on antiradicals and antioxidants.

Keywords: 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical; electron spin resonance (ESR); hydroxyl radical; mucilage; superoxide radical; yam

二、緣由與目的

Active (or reactive) oxygen species and free radical-mediated reactions have involved in degenerative or pathological processes such as aging (Harman, 1995), cancer, coronary heart disease and Alzheimer′s disease (Ames, 1983; Smith et al., 1996; Diaz et al., 1997). Meanwhile there are many epidemiological results revealing an association between a diet rich in fresh fruit and vegetable and a decrease in the risk of cardiovascular diseases and certain forms of cancer (Salah et al. 1995) in humans. Several reports were concerned natural compounds in fruit and vegetable for their antioxidant activities, such as phenolic compounds (Rice-Evans et al., 1997), anthocyanin (Espin et al., 2000), echinacoside in Echinaceae root (Hu and Kitts, 2000), methanolic and hot-water extracts of Liriope spicata L. (Hou et al., 2004), water and ethanolic extracts of different sweet potato organs (Huang et al., 2004), the storage proteins of sweet potato root (Hou et al., 2001a), yam tuber (Hou et al., 2001b) and potato tuber (Liu et al., 2003). In cells, there are normally metabolic pathways to degrade free radicals. If the generation rates of free radicals are faster than those of degradation under environmental stresses, cells suffer oxidative stresses. Two distinct pathways, nonenzymatic or enzymatic, were found in plant cells as routes of free radical scavengers. The former included ascorbate (Njus and Kelley, 1993) or chlorogenic acids (Kono et al., 1998) or vitamin E (Halliwell, 1999); the latter included different forms of superoxide dismutase to metabolize superoxide free radical to hydrogen peroxide (Bowler et al., 1992; Lin et al., 1993, Hou et al., 2003). The hydrogen peroxide produced was further metabolized either by catalase or different forms of peroxidase such as glutathione peroxidase (EC 1.11.1.9).

Yam (*Dioscorea* species) is a member of the monocotyledonous family Dioscoreaceae and is a staple food in West Africa, Southeast Asia and Caribbean (Akoruda, 1984). Yam was recognized as herbal plants since the tuber dried slices were freqently used as Chinese herbal medicines. The tuber storage proteins of yam, dioscorin, exhibited carbonic anhydrase, trypsin inhibitor activities (Hou et al., 1999a) and both of dehydroascorbate reductase and monodehydroascorbate reductase activities (Hou et al., 1999b). Yam tuber contains mucilages which were reported to be a mannan-protein complex (Misaki et al., 1972; Tsai and Tsai, 1984). Recently, we reported that yam tuber mucilage exhibited angiotensin converting enzyme inhibitory activities (Lee et al., 2003). In this work we reported that crude mucilages (CM) and partially purified mucilages (PPM) from three different Taiwanese yam cultivars, including *Dioscorea alata* L. cv.Tainong 1 (TN1), *Dioscorea alata* L. cv.Tainong 2 (TN2), and *D. alata* L. var. *purpurea* (Roxb.) Ming-Jen (MJ), were used for evaluating the antioxidant effects of scavenging DPPH radical, hydroxyl radical, superoxide radical by spectrophotometry, and hydroxyl radical scavenging activity assay by electron spin resonance (ESR) spectrometry.

Materials and Methods

Materials

1,1-diphenyl-2-picrylhydrazyl (DPPH), NADH, 5,5-dimethyl-1- pyrroline-*N*-oxide (DMPO), ferrous sulfate and phenazine methosulfate (PMS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (33%) was from Wako Pure Chemical Industry (Osaka, Japan). Other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

Extraction and Purificaction of Mucilage from Yam Tuber

Fresh yam tubers of TN1, TN2, and MJ were purchased from a wholesaler. After washing and peeling, the tubers were cut into strips for mucilage extractions and purifications according to the methods of Lee et al. (2003) with some modifications. Yam tuber was homogenized with four volumes (W/V) of 50 mM Tris-HCl buffer (pH 8.3) containing 1% vitamin C. After centrifugation at 14,000 ×g for 30 min, the supernatants were

mixed with isopropanol to a final concentration of 70%, and stirred quickly at 4 ºC overnight. The precipitates were filtrated and dehydrated with 100% isopropanol, then, rinsed with acetone. After drying at 40 ºC oven, the crude mucilage (CM) was grinded and collected for further purifications by both SDS and heating procedures. About 1.0 g CM powder was dissloved in 200 ml distilled water and kept in a 50 ºC water bath. Forty ml of 5 % SDS solution (dissloved in 45 % ethanol) was added to the CM solution. The mixture was kept with gentle stirring at 50 ºC for 30 min, then, at room temperature for another 2 hours. After that, the mucilage solution was placed in an ice bath to quickly lower down the temperature in order to precipitate the SDS-protein complex. After centrifugation at 14,000 ×g at 0 ºC for 30 min, the supernatants were precipitated with isopropanol and dried at 40 ºC oven as described earlier. The mucilage was again grinded, dissolved, and then heated at boiling water for 20 min. After centrifugation at 14,000 ×g at 0 ºC for 30 min, the supernatants were mixed with isopropanol to a final concentration of 70%. The partially purified mucilage (PPM) was filtrated, dehydrated, rinsed with acetone, dried, and then collected for further uses.

Scavenging activitiy against 1,1-dipheny-2-picrylhydrazyl (DPPH) radical analyzed by spectrophotometry

The scavenging activity of CM and PPM from TN1, TN2, and MJ cultivars against DPPH radical was measured according to the method of Hou et al. (2001a, b). Each 0.3 ml of CM (0.25, 0.5, 1.0 and 1.5 mg/ml) and PPM (0.1, 0.15, 0.3, 0.5 and 1.0 mg/ml) solution was added to 0.1 ml of 1 M Tris-HCl buffer (pH 7.9), and then mixed with 0.6 ml of 100 μM DPPH in methanol for 20 min under light protection at room temperature. After brief centrifugation at 12,000 ×g for 10 min, the absorbance at 517 nm was measured. Deionized water was used as a blank. The scavenging activity of DPPH radicals (%) was calculated following the equation : $(A517_{blank} - A517_{sample}) \div A517_{blank} \times 100$ %. The IC_{50} stands for the concentration required for 50 % scavenging activity and was calculated from the above equation.

Scavenging activity of CM and PPM against metal ion-dependent hydroxyl radicals

The hydroxyl radical was determined by the deoxyribose method (Halliwell et al., 1987). Every 0.5 ml sample containing different amounts of CM (0.375 , 0.75, and 1.5 mg/ml) and PPM (0.4, 0.8, and 1.6 mg/ml) from TN1, TN2, and MJ cultivars were added to 1.0 ml solution of 20 mM potassium phosphate buffer (pH 7.4), 2.8 mM 2-deoxy-ribose, 104 μM EDTA, 100 μM $FeCl_3$, 100 μM ascorbate and 1mM hydrogen peroxide. The mixtures were incubated for 1 h at 37 ºC. After incubation, equal volume of 0.5% thiobarbituric acid in 10% trichloroacetic acid was added and the mixtures were boiled at 100 ºC for 15 min. Deionized water was used as a blank experiment. The absorbance at 532 nm was measured. The scavenging activity of hydroxyl radicals (%) was calculated with the equation: $(A532_{blank} - A532_{sample}) \div A532_{blank} \times 100$ %. The IC_{50} stands for the concentration required for 50 % scavenging activity and was calculated from the above equation.

Scavenging Activity against Superoxide Radicals analyzed by Spectrophotometry

The superoxide radical was generated by the PMS-NADH system (Lai et al., 2001). Every 0.2 ml sample containing different amounts of CM (0.125, 0.25, 0.5, and 1.0 mg/ml) and PPM (0.125, 0.5, 1.0 mg/ml) solution from TN1, TN2, and MJ cultivars was added in sequence to 0.2 ml of 630 μM nitroblue tetrazolium, 0.2 ml of 33 μM PMS and 0.2 ml of 156 μM NADH in 100 mM phosphate buffer (pH 7.4). Means of triplicates were measured. Deionized water was used as a blank experiment. The changes of absorbance at 560

nm were recorded during 1 min and expressed as △A560nm/min. The scavenging activity against superoxide radicals was calculated as following: ($\triangle A560nm/min_{blank} - \triangle A560nm/min_{sample}$) ÷ $\triangle A560nm/min_{blank}$ × 100 %. The IC_{50} stands for the concentration of 50% scavenging activity.

Scavenging activities against hydroxyl radical by electron spin resonance spectrometry

The hydroxyl radical was generated by Fenton reaction according to the method of Kohno et al. (1991). The total 500-μl mixture contained different concentrations of PPM solution of TN1 (0.031, 0.062, 0.1, and 0.2 mg/ml), TN2 (0.12, 0.25, 0.5, and 1 mg/ml), and MJ (0.0004, 0.004, and 0.007 mg/ml) cultivars, 5 mM 5,5-dimethyl-1- pyrroline-*N*-oxide (DMPO), and 0.05 mM ferrous sulfate. After mixing, the solution was transferred to an ESR quartz cell and placed at the cavity of the ESR spectrometer, and then hydrogen peroxide was added to a final concentration of 0.25 mM. Deionized water was used instead of sample solution for blank experiments. After 40 s, the relative intensity of the signal of the DMPO-OH spin adducts was measured. All ESR spectra were recorded at the ambient temperature (298 K) on a Bruker EMX-6/1 EPR spectrometer equipped with WIN-EPR SimFonia software version 1.2. The conditions of ESR spectrometry were as follows: center field, 345.4 (5.0 mT; microwave power, 8 mW (9.416 GHz); modulation amplitude, 5 G; modulation frequency, 100 kHz; time constant, 0.6 s; scan time,1.5 min.

Results and Discussion

Yam (*Dioscorea* species) is a member of the monocotyledonous family Dioscoreaceae and is a staple food in West Africa, Southeast Asia and Caribbean (Akoruda, 1984). Yam was recognized as herbal plants since the tuber dried slices were freqently used as Chinese herbal medicines. In this study, three native cultivars of Taiwanese yam were used for mucilage isolation and purification, and then for antioxidant activity assay.

DPPH radicals were widely used in the model system to investigate the scavenging activities of several natural compounds. When DPPH radical was scavenged, the color of the reaction mixture changes from purple to yellow with decreasing of absorbance at wavelength 517 nm. Figure 1A shows the scavenging activity against DPPH radicals of CM from TN1, TN2 ,and MJ yam cultivars. It was found the dose-dependent DPPH radical scavenging activities of CM from three native yam cultivars. The order of DPPH scavenging activity was TN1 > TN2 > MJ. The IC_{50} of CM against DPPH radical were 0.329, 0.547, and 0.847 mg/ml, respectively, for TN1, TN2 and MJ cultivars. After being purified with SDS and heated at boiling water, the PPM of three yam cultivars was again assayed for antioxidant activity. Figure 1B shows the scavenging activity against DPPH radicals of PPM from TN1, TN2 ,and MJ cultivars. It was found that the DPPH radical scavenging activities of PPM was better than those of CM from three native yam. The IC_{50} of PPM against DPPH radical were 0.279, 0.653, and 0.631 mg/ml, respectively, for TN1, TN2 and MJ cultivars. Our previous study reported (Hou et al., 2002) that PPM from Japanese yam also exhibited DPPH scavenging activity, and the IC_{50} of PPM against DPPH radical was 0.86 mg/ml which was similar to the report of Lai et al. (2001) for Hsian-tsao leaf gum and higher than those of PPM from TN1, TN2 ,and MJ cultivars.

Figure 2 showed the scavenging activity against hydroxyl radical from CM (A) and PPM (B) of TN1, TN2, and MJ yam cultivars. Similar to the results of Figure 1, it was found the dose-dependent hydroxyl radical scavenging activities of CM from three native yam cultivars. The order of hydroxyl radical scavenging activity was TN1 > MJ > TN2 (Figure 2A). The IC_{50} of CM against hydroxyl radical were 0.668, 1.461, and 0.946 mg/ml, respectively, for TN1, TN2 and MJ cultivars. Figure 2B shows the scavenging activity against hydroxyl radicals of PPM from TN1, TN2 ,and MJ cultivars. The lower hydroxyl radical scavenging activities of PPM was found than those CM from three native yam. The IC_{50} of PPM against hydroxyl radical were 1.146, 1.096, and 1.554 mg/ml, respectively, for TN1, TN2 and MJ cultivars. Previuosly, we reported that the tuber storage protein, dioscorin, exhibited hydroxyl radical scavenging activity (Hou et al., 2001b). The dioscorin should be removed during SDS and heating treatments and resulted in lesser hydroxyl radical scavenging activity of PPM in three native yam cultivars.

Figure 3 showed the scavenging activity against superoxide radical from CM (A) and PPM (B) of TN1, TN2, and MJ yam cultivars. Similar to the results of Figures 1 and 2, it was found the dose-dependent superoxide radical scavenging activities of CM from three native yam cultivars. The order of superoxide radical scavenging activity was MJ > TN2 > TN1 (Figure 3A). The IC_{50} of CM against superoxide radical were 0.802, 0.681, and 0.086 mg/ml, respectively, for TN1, TN2 and MJ cultivars.

The hydroxyl radical was generated by Fenton reaction and was trapped by DMPO to form DMPO-OH adduct. The intensities of DMPO-OH spin signal in ESR spectrometry were used to evaluate the scavenging activity of PPM of TN1, TN2, and MJ yam cultivars against hydroxyl radical. Figure 4 shows scavenging activities against hydroxyl radicals of (A) TN1 (0.031, 0.062, 0.1, and 0.2 mg/ml), (B) TN2 (0.12, 0.25, 0.5, and 1 mg/ml), and (C) MJ (0.0004, 0.004, and 0.007 mg/ml) cultivars. From the results of Figure 4A, the scavenging activities against hydroxyl radical were 1, 26, 69.6, and 81% at 0.031, 0.062, 0.1, and 0.2 mg/ml, respectively, of PPM from TN1 cultivars. From the results of Figure 4B, the scavenging activities against hydroxyl radical were 13, 31.5, 51.9, and 72.2% at 0.12, 0.25, 0.5, and 1 mg/ml, respectively, of PPM from TN2 cultivars. From the results of Figure 4C, the scavenging activities against hydroxyl radical were 5.5, 50, and 51.8% at 0.0004, 0.004, and 0.007 mg/ml, respectively, of PPM from MJ cultivars. Using ESR to detect hydroxyl radicals, the IC_{50} of PPM against hydroxyl radical were 0.083, 0.47, and 0.004 mg/ml, respectively, for TN1, TN2 and MJ yam cultivars.

In conclusion, mucilages from three Taiwanese yams exhibited different antioxidant activities against DPPH radicals (Figure 1), hydroxyl radicals (Figure 2, Figure 4), and superoxide radicals (Figure 3) and the purification process could partially increase the antioxidant activity of the mucilage polysaccharide. Table 1 shows the comparison of the antioxidant activity (IC_{50}) of mucilages from TN1, TN2, and MJ yam cultivars before (crude mucilage, CM) and after purification (partially purified mucilage, PPM) against DPPH, hydroxyl and superoxide radicals. Taken together, these results suggest that mucilage polysaccharides of the yam tuber might play an important role on antiradicals and antioxidants.

Acknowledgments. The authors want to thank the financial support (SKH-TMU-94-03) from 新光醫院。

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Figure 1

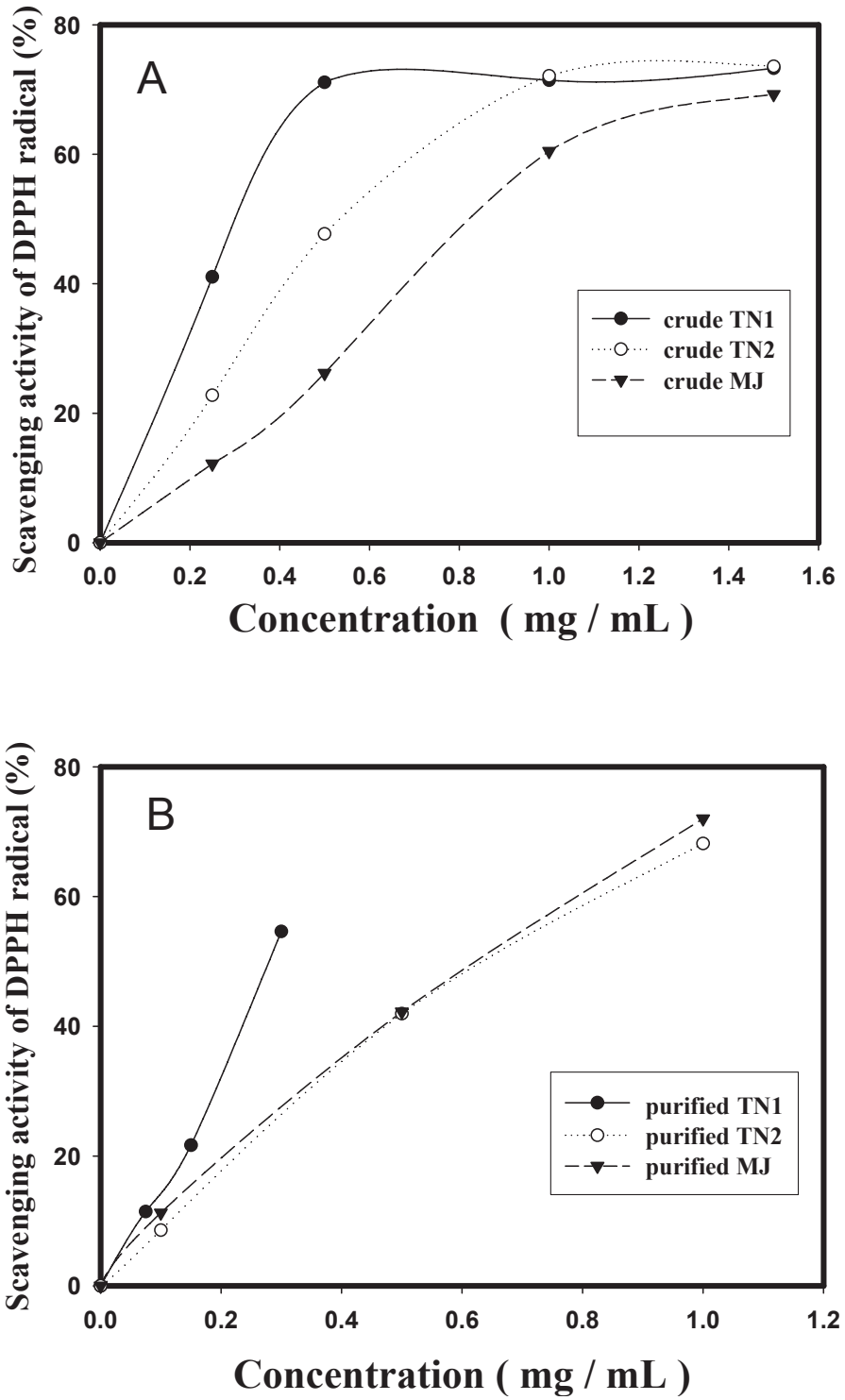


Figure 1. The scavenging activity of crude mucilage (CM) (A) and partially purified mucilage (PPM) (B) of TN1, TN2, and MJ yam cultivars against DPPH radical. Means of triplicates were measured. Deionized water was used as a blank experiment. The scavenging activity of DPPH radical (%) was calculated according to the following equation: $(A517_{blank} - A517_{sample}) \div A517_{blank} \times 100$ %.

Figure 2

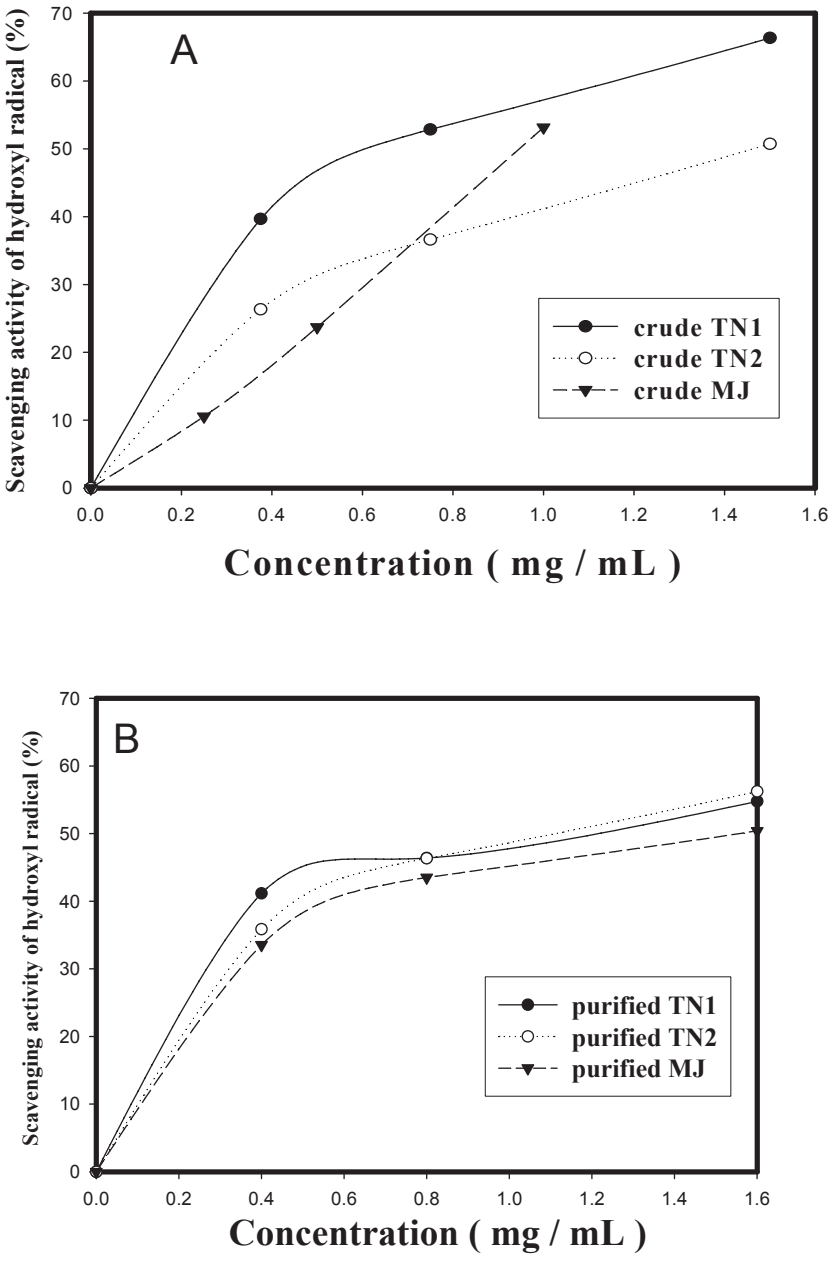


Figure 2. The scavenging activity of crude mucilage (CM) (A) and partially purified mucilage (PPM) (B) of TN1, TN2, and MJ yam cultivars against hydroxyl radical. Means of triplicates were measured. Deionized water was used as a blank experiment. The absorbance at 532 nm was measured. The scavenging activity of hydroxyl radicals (%) was calculated with the equation: $(A532_{blank} - A532_{sample}) \div A532_{blank} \times 100$ %.

Figure 3

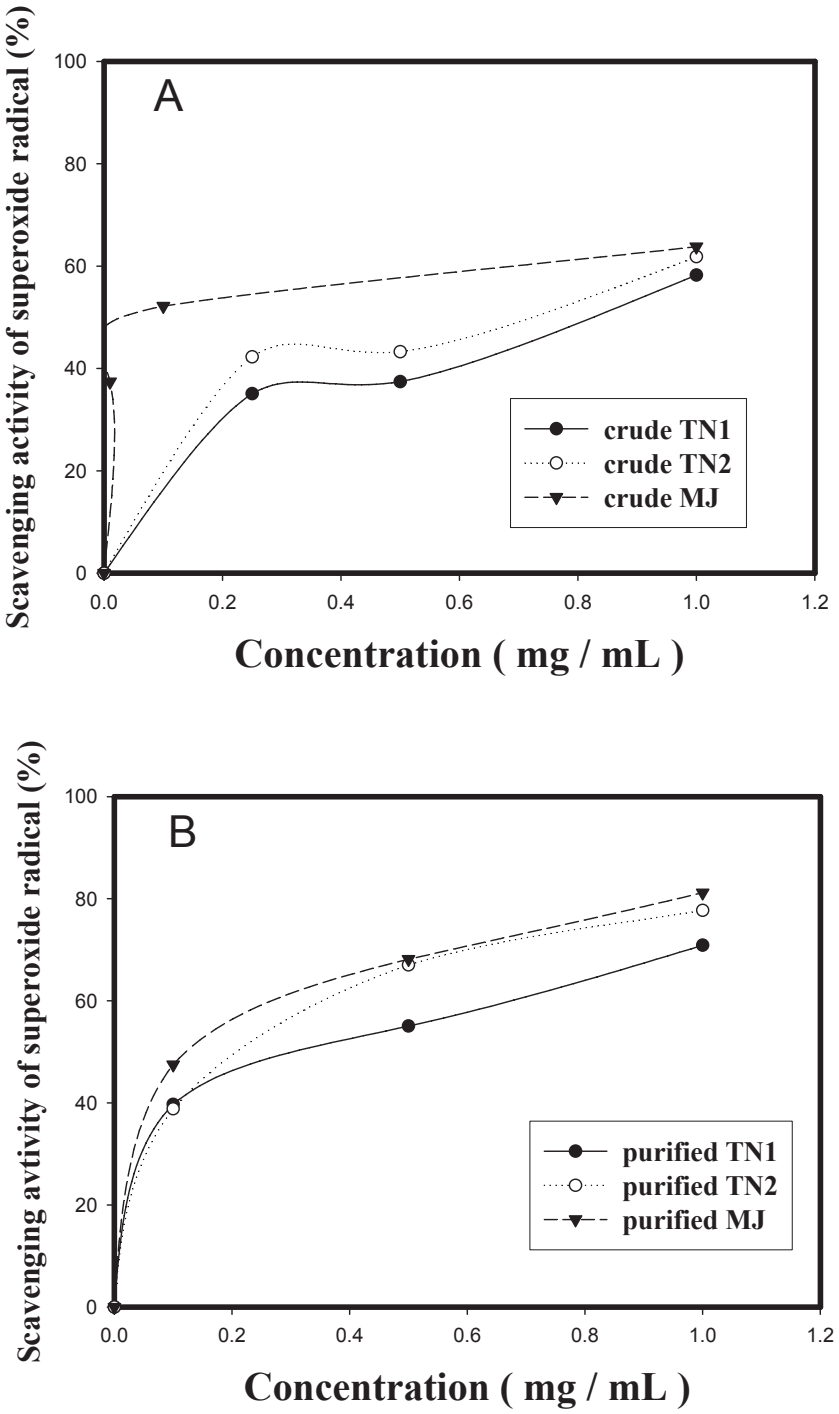


Figure 3. The scavenging activity of crude mucilage (CM) (A) and partially purified mucilage (PPM) (B) of TN1, TN2, and MJ yam cultivars against superoxide radical. Means of triplicates were measured. Deionized water was used as a blank experiment. The changes of absorbance at 560 nm were recorded during 1 min and expressed as △A560nm/min. The scavenging activity of the superoxide radical was calculated as following: ($\triangle$ A560nm/min$_{blank}$ − $\triangle$ A560nm/min$_{sample}$) ÷ $\triangle$ A560nm/min$_{blank}$ × 100 %.

Figure 4A

Figure 4. The scavenging activity of partially purified mucilage (PPM) of (A) TN1 (0.031, 0.062, 0.1, and 0.2 mg/ml), (B) TN2 (0.12, 0.25, 0.5, and 1 mg/ml), and (C) MJ (0.0004, 0.004, and 0.007 mg/ml) cultivars against hydroxyl radical. The signal intensities of DMPO-OH adduct were determined by electron spin resonance spectrometry. The scavenging activity against hydroxyl radical was also showed in the figure.

（1）blank

（2）0.031 mg/ml　(1%)

（3）0.062 mg/ml (26%)

（4）0.1 mg/ml (69.6%)

（5）0.2 mg/ml (81%)

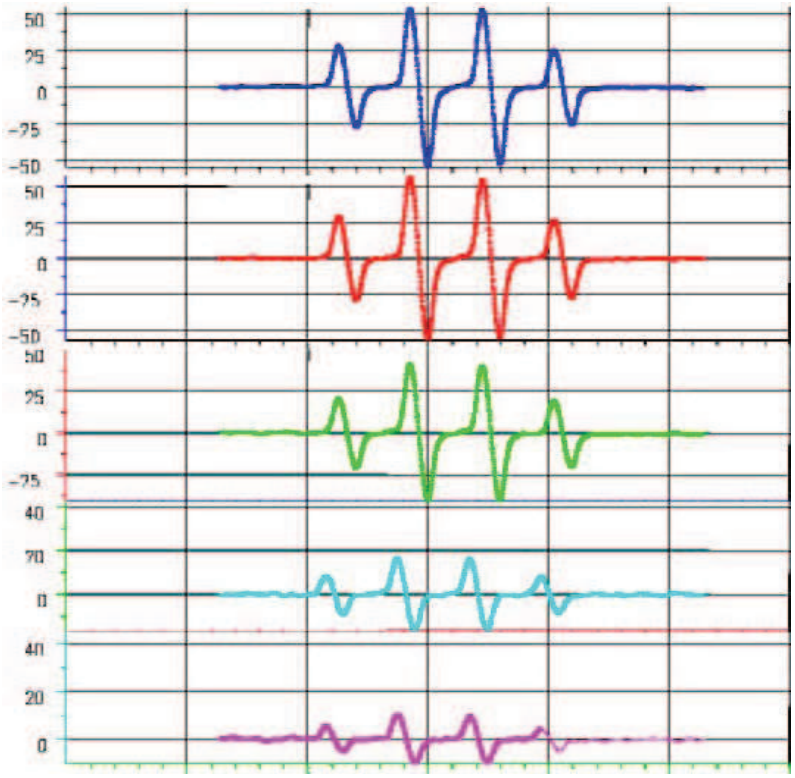


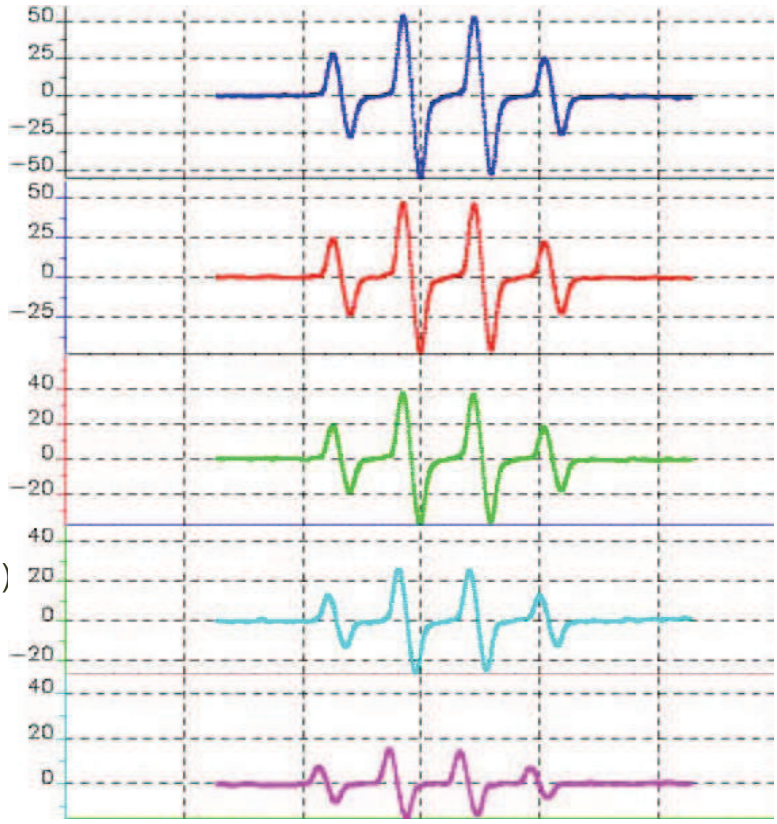
Figure 4B

（1）blank

（2）0.12 mg/ml (13%)

（3）0.25 mg/ml (31.5%)

（4）0.5 mg/ml (51.9%)



（5）1 mg/ml (72.2%)

Figure 4C

(1) blank

（2）0.0004 mg/ml (5.5%)

（3）0.004 mg/ml（50%）

（4）0.007 mg/ml (51.8%)

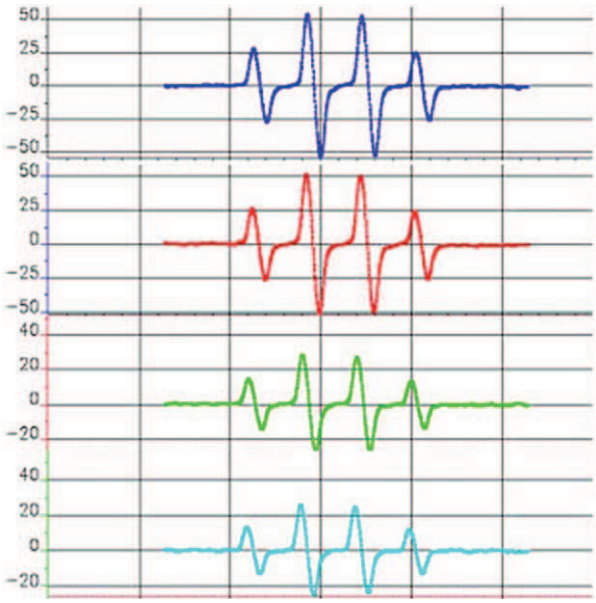


Table 1. Comparison of the antioxidant activity of mucilages from TN1, TN2, and MJ yam cultivars before (crude mucilage, CM) and after purification (partially purified mucilage, PPM) against DPPH, hydroxyl and superoxide radicals

Yam cultivars	DPPH radical (mg/ml)[a]		Hydroxyl radical (mg/ml)[a]		Superoxide radical (mg/ml)[a]	
	CM	PPM	CM	PPM	CM	PPM
TN1	0.329	0.279	0.668	1.146	0.802	0.368
TN2	0.547	0.653	1.461	1.096	0.681	0.258
MJ	0.847	0.631	0.946	1.554	0.086	0.148

[a] expressed as the IC_{50} value