附件一

行政院國家科學委員會補助專題研究計畫

■成果報告 □期中進度報告

（計畫名稱）

山藥水萃物銅離子螯合活性有效分子確認及對銅離子代謝異常相關疾病保健功能之評估

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共同主持人：
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執行單位：中山醫學大學 生命科學系

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中文摘要：

山藥已廣泛使用於傳統醫藥及保健食品，許多種療效，包括止咳抗糖尿止瀉及抗癌都歸功於山藥。雖然薯芋皂素(saponin)解釋了一些山藥上述的生化活性，然而有關山藥所含具生物活性有效成分的資訊仍非常少。本研究中，我們發現台農二號山藥(*Dioscorea alata* L.)塊根肉水萃取物(YAE)能有效抑制 H_2O_2-CuSO 在牛胸腺 DNA 所造成的傷害；在人類淋巴母細胞株(lymphoblastoid cells)也明顯降低二價銅離子所造成的核 DNA 的股斷裂(strand breaks)。YAE 波長 260-262 nm 有明顯的吸收光值，加入二價銅離子時，此特殊吸收峰將由原先的 260-262 nm 轉移至 236 nm，該 236 nm 吸收峰的轉移可被金屬螯合劑(EDTA)逆轉；YAE 也可明顯解開銅離子指示劑(tetramethyl murexide)與銅離的結合。利用薄層色層分析法(thin layer chromatography)分離 YAE 中之主要成分，發現含有一明顯及數種可能為皂素的分子，相較於皂素，YAE 中含酚類化合物(phenolic compound)的量則很低。根據這些結果，我們提出：(1)YAE 所具有的銅離子螯合活性可能是山藥重要生化活性重要之作用機轉；(2)水溶性薯芋皂素而非酚類化合物可能是 YAE 中具銅離子螯合活性的有效分子，並發揮保護銅離子造成 DNA 傷害的活性。未來有必要進一步鑑定 YAE 中主要皂素的確實結構及在動物模式下之保健功能。

關鑑字： 台農二號山藥 (*Dioscorea alata* L.)；Fenton 氏反應；金屬螯合活性；核酸傷害保護活性；抗氧化；皂素

英文摘要：

Dioscorea plants have been widely used as traditional medicine and food for health benefits. Several therapeutic properties such as anti-cough, anti-diabetic, anti-diarrhoea, and anti-cancer have been attributed to these plants. Although the steroidal saponins (such as diosgenin) account for some of Dioscorea's activity, there is little information on the effective components. We show here that aqueous extract of *Dioscorea alata* L. (YAE) inhibited the H_2O_2-$CuSO_4$ induced damage of calf thymus DNA, and protected cultured human lymphoblastoid cells from $CuSO_4$-induced DNA damage. This aqueous extract exhibited a major absorption peak at 260-262 nm. This absorption peak was shifted to 236 nm upon the addition of $CuSO_4$, and the 236 nm peak was sensitive to the divalent ion chelator EDTA. This aqueous extract was also capable of dissociating the complex formation of Cu(II) with tetramethyl murexide (a copper indicator). Separation of YAE by TLC indicated that it contains one major and several minor types of saponin but contains less or no phenolic compounds. We conclude that water-soluble saponin(s) within YAE are the potential effective compound which carries copper-chelating activity, and protects against H_2O_2-$CuSO_4$-induced DNA damage. Further studies will be necessary to elucidate the effective compound amongst the water-soluble saponin(s) within this extract.

Keywords: Yam (*Dioscorea alata* L.); Fenton reaction; metal chelating activity; DNA damage protection; antioxidant; saponin

INTRODUCTION

The metabolism of oxygen and many toxic agents is accompanied by the formation of reactive oxygen species.[1-4] Reactive oxygen species, such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$), are formed through a process of one-electron reduction of molecular oxygen (O_2). They are generated by a process known as redox cycling, and are catalyzed by transition metals such iron and copper ions to cause DNA damage, thiol and lipid oxidation, and cell apoptosis.[5]

Reactive oxygen species have been implicated in over a hundred human disorders, including arthritis, haemorrhagic shock, arteriosclerosis, advancing aging, ischemia and reperfusion injury of many organs, cardiovascular disorders, Alzheimer's and Parkinson's diseases, gastrointestinal dysfunctions, cancers and AIDS.[6-8] To minimize potential oxidative damage *in vivo*, antioxidants from various dietary sources have been studied extensively. Accumulating evidence has shown that dietary intake of phytochemicals, including α-tocopherol, ascorbic acid and the flavonoids, have been linked to the maintenance of health and protection from diseases.[9,10]

The tubers of some *Dioscorea* species have been used in traditional medicine in China and Zimbabwe for diarrhoea, diabetes, skin problems, rheumatism, and tonic for the spleen, stomach, lung and kidneys.[11-15] Many of the *Dioscorea* species are a very important sources of secondary metabolites used in the pharmaceutical industry and general medicine. Diosgenin and related steroidal saponins which provide the steroid building-blocks for developing human sex hormones are obtained commercially from the tubers of various *Dioscorea* species.[16] The major biological functions of *Dioscorea* extracts include anti-tumour/anti-cancer activity,[17-26] anti-microbial activity,[27,28] anti-diabetic activity,[12,13,29] anti-hypertension,[30] anti-fatigue,[15] anti-oxidant,[31-34] and reno- & hepato-protection[35] according to the clinical supplement trial and animal/cell model system test. These functions may correlate with the extract's important components such as saponins,[17-26] polyphenols,[32] storage protein,[33] and mucilage[34] but the molecular mechanisms of its biological functions are still unclear. Furthermore, the really effective components and their structures have still not been identified yet.

The present study was designed to investigate the protective effect of an aqueous extract of yam (*Dioscorea alata* L.), YAE on catalytic transition metal-driven oxidative DNA damage. The results show that YAE acts as a transition metal-chelator acting against copper-mediated DNA damage *in vitro*.

MATERIALS AND METHODS

Chemicals

Amplex red was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were analytical grade and obtained from Sigma (St. Louis, MO) and Merck (Darmstadt, Germany).

Preparation of *Dioscorea* aqueous extracts (YAE)

Tubers of yam (*Dioscorea alata* L., Tainong No. 2) were collected from local cultivation in Taichung, Taiwan. *Dioscorea* tubers were peeled off and cut into small pieces and stored at -80 °C until the extract was prepared. The small pieces were placed in sterilized MilliQ water (1:4 w/v) and homogenized in a blender (Osterizer, Delray Beach, Florida, USA). The homogenate was centrifuged at 5,000 rpm for 30 min to remove the undissolved materials. The residue was discarded and the supernatant was freeze-dried (ZiRBUS vacoII, Bad Grund, Germany). The freeze-dried extract was stored at –20 °C until used for the determination of anti-oxidant activity. Ten per cent of the freeze-dried extract powder dissolved in sterilized MilliQ water served as a stock solution.

Cell culture

Human peripheral blood samples were drawn from healthy volunteers with

heparinized syringes. Lymphocytes were isolated by centrifugation in a Ficoll-Paque cushion (Pharmacia Biotech, Sweden), washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium containing 10% fetal calf serum (HyClone, Logan, UT, USA) and 1% PSN antibiotic mixture. The lymphocytes were transformed into immortal lymphoblastoid cell lines using Esptein-Barr virus. The cell cultures were grown in a humidified atmosphere with 5% CO_2 in air at 37 °C. The reagents for cell culture were obtained from Gibco BRL (NY, USA).

Ethidium bromide fluorescence binding assay

The ethidium bromide (EtBr) binding assay, based on the formation of a fluorescent complex between double-stranded DNA and EtBr, was used to measure DNA damage[36]. H_2O_2-$CuSO_4$ damages DNA and inhibits the binding of EtBr to DNA, resulting in a decrease in intensity of fluorescence. Several forms of DNA damage, including strand scission, base oxidation and base liberation contribute to the loss of fluorescence. Hence, the assay detects a broad range of different DNA lesions. A 2 ml standard reaction mixture contained 20 mM phosphate buffer (pH 7.0), 100 µg/ml calf thymus DNA, 50 µM $CuSO_4$, and 25 mM H_2O_2. To measure the protective effect of *Dioscorea* aqueous extracts on DNA damage, varying amounts of *Dioscorea* extract were added to the reaction mixture after the addition of $CuSO_4$ and before the addition of H_2O_2. The reactions were carried out at 37 °C for 10 min and terminated by the addition of a stock solution of 0.5 M EDTA, to a final concentration of 10 mM. Four µl of a 1 mM EtBr solution was added and fluorescence intensity at 590 nm was measured with a Hitachi spectrofluorometer with excitation at 510 nm. The reduction in fluorescence was used as a measure of DNA damage.

Hydrogen peroxide decomposition assay

The H_2O_2 decomposing activity of AE was assessed by Amplex Red, as described by Zhou et al. [37] Because Amplex Red is a colourless and non-fluorescent derivative of resorufin, but the substrate produces a highly fluorescent product in response to H_2O_2 upon the action of horseradish peroxidase (HRP), the H_2O_2 decomposing activity of extracts can be determined. Briefly, different doses of YAE (0.1-100 µg/ml) were incubated with 1 µM H_2O_2 for 5 min at 37 °C in 10 mM Tris-HCl buffer, pH 7.0, and then 40 µl 100 µM Amplex Red and 10 µl 100 µU/ml HRP were added to achieve final concentrations of 4 µM and 1 µU/ml, respectively. Following another 5-min incubation at 37 °C, the fluorescence intensity of Amplex red was measured using a Hitachi spectrofluorimeter at an excitation wavelength of 563 nm and an emission wavelength of 584 nm.

The direct quenching effect of AE on the fluorescent product of Amplex Red was also determined by the addition of YAE after a 5-min incubation of H_2O_2, Amplex Red and HRP at 37 °C.

UV/VIS spectral analysis

YAE dissolved in sterilized MilliQ water was subjected to UV/VIS scanning using a spectrophotometer (JASCO V-530). The copper and iron chelating capacities of the YAE were measured with $CuSO_4$ and $FeSO_4$. YAE, 0.5 mg/ml, was either mixed with 50 µM $CuSO_4$ or $FeSO_4$, or left without, and incubated at 37 °C for 10 min. After incubation, the absorbance spectra from 200 to 700 nm were recorded and compared on a UV/VIS spectrophotometer. When spectral changes were detectable, 125 µM EDTA was added in order to test the reversibility of the chelation.

Assay of copper/iron chelating capacity

The copper- and iron-chelating capacity of *Dioscorea* extracts were measured in a hexamine buffer with tetramethyl murexide (TMM).[38] TMM has a maximal absorption at 530 nm, and will complex with free Fe(II) or Cu(II) to give a maximal absorption at 485 nm. To measure the chelating effect of *Dioscorea* extracts, 0.5 ml *Dioscorea* extract and 0.2 ml 1 mM TMM were added to a 0.5

ml mixture containing 30 mM hexamine, 30 mM potassium chloride and 3 mM $FeSO_4$ or $CuSO_4$. After 3 min incubation at room temperature, the absorbance ratio at 485 vs. 530 nm of the mixture was determined on a Jasco UV-visible spectromotometer. The lower the absorbance ratio for 485 vs. 530 nm, the higher the chelating power.

DNA nicking assay

For assay of the hydroxyl radical scavenging ability of *Dioscorea* extract, plasmid DNA pGL3 (a kind gift from Dr. K.Y. Jan, Academia Sinica, Taipei, Taiwan) was diluted, dispensed in Eppendorf tubes (1 μg per tube) and mixed with increasing concentrations (1-5 mg/ml) of extracts in sterilized MilliQ water. The mixture of plasmid and extracts was exposed to 6 Gy X-rays at a dose rate of 0.114 Gy/s using an X-ray inspection system (Torrex 150D, Pantak Inc.USA) on ice. The supercoiled (SC) and open circular (OC) forms of DNA were separated on a 0.8% agarose gel with 0.5 TBE buffer, at pH 8.3. After staining with ethidium bromide, the DNA bands were visualized, photographed and analyzed with the Gel Doc 2000 system (Bio Rad, Hercules, CA, USA). The level of DNA damage was expressed as $OC\% = [\ OC_{light\ density}/(OC_{light\ density} + SC_{light\ density}) \times 100\%]$. The higher the OC percentage, the more the DNA damage.

Alkaline comet assay

The method of Singh[39] was adopted with some modifications, as described previously.[40]

Thin layer chromatography

YAE and reference standards were applied to a TLC plate (Merck plastic sheets, 20 x 20 cm, Silica gel 60) in bands of 1 cm width. The plates were developed to about 9.0 cm. The solvent system was as follows: ethyl acetate:formic acid:water (65:15:20, v/v). Detection was done by evaluation under white (normal) light, or UV light, before or after staining with 10% H_2SO_4 in ethanol for general organic compounds, 1% $FeCl_3$ in 50% methanol for phenolic compounds, and several spray reagents for saponins, including Liberman-Buchard (5 ml concentrated H_2SO_4, 5 ml glacial acetic acid in 50 ml ethanol) , anisaldehyde-sulphuric acid (5 ml anisaldehyde, 1 ml sulfuric acid, and 50 ml glacial acetic acid), antimony trichloride ($CHCl_3$-saturated antimony trichloride solution), and vanillin-sulphuric acid (15 g vanillin, 2.5 ml sulfuric acid, and 250 ml ethanol). Individual R_f for each spot visualized on the TLC plate was measured. A phenolics mixture, including caffeic acid, chlorogenic acid and tannic acid and a saponin mixture from quillaja bark (saponin content > 25%, Sigma) were used as reference standards.

Statistical evaluation

Results are expressed as mean ± SE. Comparisons were made using Student's t test. Differences were considered to be significant at $p<0.05$.

RESULTS

Protection of metal-mediated DNA damage by YAE

YAE at 0.1 mg/ml protected the damage to calf thymus DNA induced by H_2O_2-$CuSO_4$, but not that induced by H_2O_2-$FeSO_4$ (Fig. 1A). YAE inhibited the H_2O_2-$CuSO_4$-induced DNA damage in a dose-dependent manner and the 50% inhibitory activity was estimated to be at a concentration of 0.22 mg/ml (Fig. 1B). To confirm the protective effect of YAE against DNA damage, we performed experiments using human lymphoblastoid cells. A 2 h treatment with 25 μM $CuSO_4$ or 300 μM $FeSO_4$ induced significant DNA damage in these cells, but a 2 h treatment with 0.5 mg/ml YAE alone did not induce DNA strand breaks (Fig. 2). A concomitant treatment of YAE with $CuSO_4$ or $FeSO_4$ decreased DNA damage by 70% and 40%, respectively.

Metal-chelating activity of YAE

We performed a UV/visible absorption spectral analysis to examine whether or not YAE binds transition metal ions. YAE exhibited a major absorption peak at 260-262

nm. When 100 μM $CuSO_4$, and $FeSO_4$, were added to 0.5 mg/ml YAE, apparent modification of the absorption spectrum was detected between 200 and 700 nm (Fig. 3), but this spectral modification was not detected for $NiSO_4$ and $CoSO_4$ (Fig. 3). The absorption peak of YAE at 260-262 nm was shifted to 236 nm in the presence of $CuSO_4$ (Fig. 4A), and the absorption at 236 nm increased with increasing concentration of $CuSO_4$ (Fig. 4A insert). The formation of copper-YAE complexes as detected at the 236 nm peak was completely inhibited by an equimolar ratio of the divalent ion chelator EDTA (Fig. 3 and Fig. 5). YAE also reduced the complex formation of tetramethyl murexide (a copper and iron ion indicator) with Cu(II) and Fe(II) (Fig. 4B).

Hydrogen peroxide-degrading activity of YAE

Apart from a transition metal chelating activity, the protective inhibition effect of YAE on copper-mediated DNA damage *in vitro* may also result from its hydroxyl radical and hydrogen peroxide scavenging activities. The direct scavenging of YAE on H_2O_2 was studied in a cell-free system. The assay is based on the oxidation of Amplex Red to its fluorescent products in the presence of H_2O_2 and horseradish peroxidase. The presence of substances with H_2O_2-decomposing activity prevents the oxidation of Amplex Red by removing H_2O_2, and causes a decrease in fluorescence that is proportional to the antioxidant activity. YAE decreased the fluorescence intensity of activated Amplex Red in a dose-dependent manner (Fig. 6). However, YAE also quenched the fluorescence intensity of activated fluorescent products of Amplex Red to the same extent. Therefore, YAE was not responsible for decomposing H_2O_2.

Protection of X-ray-induced DNA damage by YAE

The hydroxyl radical scavenging ability of YAE was also investigated by its modulation of the relaxation (induction of the first single-strand break) of supercoiled plasmid DNA on X-ray irradiation. In general, it is considered that the ionizing radiation-induced scission of DNA takes place by attack of hydroxyl radicals produced from radiolysis of water.[41] When a hydroxyl radical scavenger is added to the assay system, ionizing radiation-induced DNA scission is retarded.[42-44] The supercoiled pGL3 plasmid DNA (originally containing more than 90% of the DNA in supercoiled form) exposed to 6 Gy X-rays was induced to about 30% relaxation as open-form plasmid DNA, and the addition of YAE did not affect the extent of DNA relaxation (Fig. 7). These results indicate that YAE-protected inhibition of H_2O_2-$CuSO_4$-induced DNA damage *in vitro* was due to its copper chelating activity rather than the scavenging activity of H_2O_2 or hydroxyl radicals.

TLC chromatograms of YAE

Table 1 and Figure 8 show the *Rf* values and colours of spots on the TLC plate of YAE under white (normal) or UV light or after staining with spraying agents for general organic compounds, phenolic compounds, and saponins. YAE extracts yielded only one fluorescent spot with Rf values of 0.1 under UV light, but the fluorescence intensity was higher under UV light at 365 nm than at 254 nm. Chromatograms sprayed with H_2SO_4 showed that YAE had only one grey-black spot with *Rf* values of 0.1 under white light and had four blue fluorescent spots with *Rf* values of 0.1, 0.19, 0.60, 0.71, and 0.80. Except for Liberman-Burchard spray agent, chromatograms sprayed with anisaldehyde-sulphuric acid, vanillin-sulphuric acid, and antimony tricholoride showed that YAE also yield only one spot with *Rf* of 0.09-0.1 under white light and two to six blue fluorescent spots with *Rf* values of 0.09-0.1, 0.17-0.19, 0.25-0.28, 0.63 and 0.69. However, chromatograms sprayed with $FeCl_3$ showed that YAE did not yield any spot under white light and UV light. These results showed that YAE had a major saponin spot with *Rf* value

of 0.1 and had less, or no, phenolic compounds.

DISCUSSION

Protection mode of YAE

Recently, many reports have shown that redox imbalance might act as a common human pathological risk. Oxygen-derived species are generated in living cells by cellular oxygen metabolism. Of these species, H_2O_2 and $O_2^{\cdot-}$ do not cause damage to DNA unless they are converted into more reactive species in reactions with transition metal ions such as copper and iron.[45,46,our present data] Iron and copper have been shown to produce typical hydroxyl radical-induced base modification in isolated DNA and mammalian chromatin in the presence of H_2O_2.[47] The availability of iron and copper ions for generation of free radicals is very limited in living organisms due to sequestration of these elements in transport and storage proteins.[48] However, metal ions may be released from their storage sites by oxidative stress and subsequently bind to DNA, giving rise to the generation of free radicals. There appears to be a causal relationship between iron and copper ions and the risk of cancer, arteriosclerosis, and neurodegeneration in humans.[49-52] Recently, many studies arising from the search for natural anti-oxidants involved in health promotion from food or herbs have shown that their anti-oxidant activity mostly depends on their free radical scavenging and/or transition metal chelating properties.[53-55] However, recent results also strongly suggest that not all of the transition metal chelating anti-oxidants are always safe in metal ion-mediated cellular damage. It has been shown that flavonoids including flavanones, flavones, and isoflavones act as pro-oxidants in the presence of transition metals.[56-58] In this study, we found that YAE could significantly protect both copper Fenton and copper ion induced DNA damage *in vitro*, but could not or could only slightly protect ferrous Fenton and ferrous ion-induced DNA damage *in vitro* (Fig. 1 and Fig. 2). These differences were not due to the chelating activity with copper and iron, because both UV-visible spectral analysis and metal chelating assay by tetramethyl murexide have shown that YAE can complex with both Fe(II) and Cu(II) to a similar extent (Fig. 3 and Fig. 4). This may be due to the difference in redox activity of YAE-copper and YAE-iron complexes. Thus, YAE may form a redox-active complex with iron but it forms a non-active redox complex with copper for free radical generation. There is plenty of evidence of polyphenols or metal chelators complexing with copper/iron and forming redox-active polyphenol-metal complexes to induce/enhance oxidative damage in different biomolecules.[59-61] *In vitro*, YAE could not protect plasmid DNA from X-ray irradiation-induced DNA strand breaks and had no H_2O_2-degrading activity. These results suggest that the protection of plasmid DNA against H_2O_2-$CuSO_4$-induced DNA strand breaks by YAE comes from compound(s) which have copper-chelating activity specifically.

Potentially effective compound(s) in YAE

The UV/visible spectral data appearing in Figure 4 showed maximum absorption of YAE in the 240-300 nm range. This may be due to the presence of phenolic compounds in the YAE. Phenolic substances are aromatic and therefore show intense absorption in the UV region of the spectrum. Most of the benzoic acid derivatives display their maxima at 246-262 nm, with a shoulder at 290-315 nm.[62,63] Low levels of polyphenol in YAE could be detected by a Folin-Ciocalteau reagent as 5.0 mg equivalents to tannic acid (TAE)/g-lyophilized powder (data not shown). Various phenolic compounds such as catechin, epicatechin, anthocyanin and the flavonol quercetin have been isolated from *Dioscorea*.[25,64] Furthermore, depending on the structural characteristics and transitional-binding potential, we first considered that polyphenol might be an interesting active component to match the copper-binding activity in YAE.

Using the TLC technique to characterize the possible composition, we found that YAE

extracts yield no spot on TLC plates sprayed with $FeCl_3$ (a popular spray reagent for detecting phenolic compounds). However, YAE yielded a major spot with *Rf* value of 0.1 under white light and several minor spots including the major spot under UV light at 254 nm, when sprayed with reagents for saponin (Table 1; Fig. 8). Although we did not know the exact compound responsible for copper-chelating activity in YAE, the water-soluble saponin(s) are a candidate active compound. In fact, many recent reports have shown that several new saponins have been isolated and characterized from different yam (*Dioscorea*) species and found that saponin(s) also expressed strong copper-binding activity. *Dioscorea* saponin extracts have also been used for the removal of heavy metals including copper from solid waste.[65] Furthermore, research has shown that Panax quinquefolium saponins protect low density lipoproteins from copper-meidate oxidation[66]. These results also support our TLC finding that saponin but not polypheneols was a potential effective compound in YAE, and contributes to its protective role on H_2O_2-$CuSO_4$-induced DNA strand breaks *in vitro* and at the cellular level.

Health benefit and therapeutic potential of YAE

Although *Dioscorea* plants have varied health benefits, there is little functional evidence to support these. This is the first time that a hypothesis involving the relationship between water-soluble saponin, copper chelating ability, and inhibition of copper catalyst Fenton reaction-induced DNA damage in aqueous extracts (YAE) of yam (*Dioscorea alata* L.) has been put forward. Furthermore, in transgenic model animal studies, more recent reports have demonstrated that a copper chelator supplement could (1) markedly inhibit β-amyloid accumulation in Alzheimer's disease,[67] (2) delay onset of disease and extend survival of familial amyotrophic lateral scelerosis[68] and (3) act as therapy in Wilson and Menkes' diseases[69,70]. Based on these findings, this study also opens newer vistas on the potential efficacy of the *Dioscorea alata* L. aqueous extracts (YAE) in the management of copper-mediated oxidative disorders, such as Willson/Menkes diseases, neurodgenerative diseases and diabetes. Further work on the purification and full structural characterization of the copper chelating component of YAE, especially the water-soluble saponins, is in progress in our laboratory.

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FIGURE LEGENDS

Figure 1 (A). Effect of YAE on $CuSO_4$–H_2O_2- and $FeSO_4$ –H_2O_2-induced DNA damage. Calf thymus DNA was incubated with 50 μM $CuSO_4$ or 1 mM $FeSO_4$ in the presence of 25 mM H_2O_2 with or without YAE (0.5 mg/ml). The extent of DNA damage was evaluated by ethidium bromide fluorescence binding assay (see Materials and Methods). Values are means ± S.E. (n=3). The asterisk indicates a significant difference between the groups with and without AE (*$P < 0.05$). (B). Effect of YAE on H_2O_2-$CuSO_4$-induced DNA damage. In the absence of $CuSO_4$, DNA-ethidium bromide gave a measurable maximum amount of fluorescence (control), which is defined as 100%. Fifty mM $CuSO_4$ plus 25 mM H_2O_2 in 50 mM phosphate buffer, pH 7.2, incubated with DNA for 10 min caused loss of ethidium bromide-induced DNA fluorescence. The degree of fluorescence loss is expressed as percentage of the control and indicates the extent of DNA damage. Values are means ± S.E. (n=3).

Figure 2. Protective effect of YAE on $CuSO_4$ and $FeSO_4$-induced DNA damage of human lymphoblastoid cells in the comet assay. Cells were co-treated with YAE (0/5 mg/ml) and 25 μM $CuSO_4$ or 300 μM $FeSO_4$ in HBSS buffer for 2 hours. After treatment, the cells were washed with phosphate buffered saline and harvested, and then the DNA damage was determined by comet assay. Values are means ± S.E. (N=2). *: $P < 0.05$ refers to the differences between metal-treated cells and lymphoblastoid cells either treated with AE or untreated.

Figure 3. Absorption spectra of YAE: effect of copper (A), iron (B), nickel (C), and cobalt (D). The spectra of 0.5 mg/ml YAE (—), YAE plus 100 μM metal ions (---), and AE + metal ions + EDTA (…) were recorded with water, 100 μM metal ions, or 100 μM metal ions + 100 μM EDTA, respectively, at room temperature.

Figure 4. (A). Spectra of the complexes formed between 0.5 mg/ml YAE and several concentrations of $CuSO_4$ in water. The spectra of the complexes were recorded with 0.5 mg/ml YAE as the blank. Samples were immediately subjected to spectral analysis after mixing. The spectra shown are representative of three independent determinations. The inset shows the interdependence of the YAE-copper complex formation at 236 nm and the concentration of $CuSO_4$. (B). Chelating effect of YAE on $CuSO_4$ and $FeSO_4$. $CuSO_4$ and $FeSO_4$ (3 mM) were mixed with different concentrations of YAE (0.3125 - 0.5 mg/ml) and the ratio of absorbance at 485 to that at 530 nm was determined by spectrometric assay. The sample (1-ml) has transition metal-binding ability when the absorbance ratio of the reaction mixture at 485 nm vs. 530 nm is lower than that of the control, which does not contain metal ion-binding substance. The absorbance ratio of the control was 2.34 ± 0.02 for $CuSO_4$ and 1.26 ± 0.03 for $FeSO_4$, respectively

Figure 5. Effect of EDTA concentration on Cu(II)-YAE complex dissociation. YAE (0.5 mg/ml) was incubated with 400 μM $CuSO_4$. After 5 min, different doses (50, 100, 200, 400 or 500 μM) of EDTA were added to the YAE and $CuSO_4$ mixture, and the spectra were recorded at room temperature against mixture with no AE.

Figure 6. H_2O_2-decomposing activity in YAE. Different doses of YAE were incubated with 1 μM H_2O_2 in 10 mM Tris, pH 7.0, at 37 °C for 5 min. After incubation, Amplex Red and horseradish peroxidase were added to make a final volume of 1 ml consisting of 4 μM Amplex red and 1 mU/ml horseradish peroxidase, and the mixture was incubated at 37 °C for another 5 min. The change in fluorescence intensity of Amplex Red was determined by spectrofluorimetric analysis as described in Materials and Methods.

Figure 7. Effect of YAE on X-ray-induced DNA nicking. (A) Agarose gel

electrophoresis pattern showing effect of AE on X-ray induced DNA nicking in plasmid PGL2 DNA. YAE was added at concentrations of 0, 0.5, 1, and 2.5 mg/ml prior to exposure to 6 Gy of X-rays. Unirradiated and irradiated DNA was analyzed by DNA nicking analysis, as described in Materials and Methods. CC and OC are supercoiled and open circular forms of DNA, respectively. (B) Densitometric analysis of the bands corresponding to CC and OC forms of plasmid pGL2 DNA from the gel reported in (A) was performed with the Gel Doc 2000 gel documentation system (Bio-Rad).

Figure 8. Typical TLC chromatograms of phenolic compounds, quillaja saponin standards and YAE. YAE and reference standards were analyzed by TLC and the separated spots were visualized by spraying with (A) H_2SO_4, (B) anisaldehyde, and (C) $FeCl_3$, as described in Materials and Methods. The TLC chromatograms were photographed by a digital camera (Nikon collpix 990) under white light. 1: standard phenolic compounds (a. caffeic acid, b. tannic acid, c. chlorogenic acid), 2: Standard quillaja saponins, 3: YAE.

成果自評：

本研究確立省產重要山藥品系台農二號山藥水萃取物(YAE)的銅離子螯合活性及在試管及細胞中對對銅離子所造成核DNA傷害的保護活性。利用分析及製備級TLC也已分離出在YAE中一主要可能有效成分，該成分經測試發現兼具有銅離子螯合活性及抑制H2O2-CuSO4在牛胸腺DNA造成之傷害，經特殊性染色初步認定此有效成分極可能為皂素，確實結構正以更精確之方式判讀中，本研究成果及所發現之有效成分對山藥保健功能作用之探討重要，成果良好。