MC-Transaction on Biotechnology, 2012, Vol. 4, No. 1, e5

Effects of *Clinacanthus nutans* (Burm.f) Lindau leaf extracts on protection of plasmid DNA from riboflavin photoreaction

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Abstract

Clinacanthus nutans (Burm.f) (CN) Lindau is widely grown in tropical Asia and is an important herbal medicine in China, Malaysia and Thailand. This study inspects the antioxidant activity and protective effects of *Clinacanthus nutans* extracts on plasmid DNA integrity in *E. coli*. The antioxidant activities of *Clinacanthus nutans* are lower compared with green tea. The superoxide dismutase (SOD) activity and total phenolic contents of green tea are almost 30- and 10-fold higher than that of C*linacanthus nutans* leaves, respectively. However, *Clinacanthus nutans* leaf extract's retention of the integrity of super-coiled plasmid DNA under riboflavin photochemical treatment was shown to be better in comparison with extracts of green tea.

Keywords: *Clinacanthus nutans,* antioxidant, super-coiled plasmid DNA, riboflavin photochemical treatment
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Received 25 August 2012/Revised 10 September 2012/Accepted 12 September 2012

Introduction

Clinacanthus nutans (Burm.f) (CN) Lindau is widely grown in tropical Asia and is an important herbal medicine in China, Malaysia and Thailand, being used as an anti-hepatitis and anti-herpes agent[1]. In Thailand, an alcoholic extract of the fresh leaves of *Clinacanthus nutans* is used externally for treatment of skin rashes, snake and insect bites, herpes simplex virus (HSV), and varicella-zoster virus (VZV)

lesions[2].

Green tea leaves (*Camellia sinensis* L.) contain 10–30% (dry leaf weight) of polyphenols. Tea polyphenols are natural antioxidants[3] and considered to be responsible for the anticarcinogenic and antimutagenic properties of tea in addition to their protective action against cardiovascular diseases[4].

Reactive oxygen species (ROS) are generally reactive molecules or radical species, including hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\bullet}OH$), the superoxide anion radical ($O_2^{\bullet-}$), and peroxyl radical ($ROO^{\bullet}$), which consequently may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative diseases.

One of the main characteristics of an antioxidant is its ability to trap free radicals. Extracts of plant foods have been shown to exhibit antioxidant and free radical scavenging capacities against external and endogenous agents in the treatment of human health problems[5-8]. It also has been reported, for the antioxidant activity of phenolic compounds used in traditional medicinal plants, a positive correlation was observed between high phenolic content and strong antioxidant activity[9]. Antioxidant activity can be measured by radical scavenging ability and reducing power. The reducing power of a compound is expressed as the capability of donating electrons to react with free radical species, convert them into more stable metabolites, and terminate the radical chain reaction[10].

$O_2^{\bullet-}$ is the intermediate product generated during oxidation and reduction, and can be formed from the hydroxyl radical and hydroperoxide compounds, causing cell damage, inflammation, atherosclerosis and aging[11, 12]. Superoxide dismutase (SOD) is a metalloenzyme widely found in organisms that can convert $O_2^{\bullet-}$ into H_2O_2 and O_2. By scavenging superoxide radicals, oxidation of lipid membranes can be prevented[13].

Degradation of 2′-deoxy-D-ribose has been used to quantify hydroxyl radicals as described previously[11]. Induction of a single-strand breakage of supercoiled plasmid DNA leads to the formation of relaxed open circular DNA. The produced ROS in riboflavin photochemical treatment in this study was evaluated by its influence on the integrity of plasmid DNA transformed within *E. coli*.

Clinacanthus nutans is a medicinal plant used in Thailand and Malaysia in recent years as a folk medicine for cancer treatment. Green tea leaves contain heigh amount of polyphenols and the phenolics are also well-known natural antioxidants[3]

and as a control in this study. The aim of this study was to inspect the antioxidant activity of the ethanol extract of *Clinacanthus nutans* and green tea by examining their scavenging ability against 1-diphenyl-2-picrylhydrazyl (DPPH) radical, reducing power, SOD activity, total phenolic contents and protective effects on the integrity of plasmid DNA in *E. coli*.

Materials and Methods

Chemicals

DPPH, ferric chloride, Folin–Ciocalteu reagent, L-methionine, monopotassium phosphate, potassium ferricyanide, potassium dihydrogen phosphate, riboflavin, sodium carbonate, trichloroacetic acid and 2'-thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, MO). The reagent used for staining DNA was HealthView Nucleic Acid Stain purchased from Genomics (New Taipei City, Taiwan). Nitro blue tetrazolium (NBT) was purchased from Bio Basic Inc. (Markham Ontario, Canada). Ultra pure deionized water from the Milli-Q system was used as a solvent in this study.

Plant extracts

Leaves of *Clinacanthus nutans* were collected from Taiping, Perak, Malaysia. Green tea (Pi Lo Chun) was purchased from Ten-Ren Tea Co. (Taipei, Taiwan). The leaves were lyophilized and ground through a 60-mesh sieve. One gram of leaf and 5 ml 70% ethanol (pH 3.0) were put into a centrifuge tube and sonicated for 20 min. The samples were centrifuged at 4°C, and 3,000 rpm for 10 min, and the supernatant was collected. The process was repeated for the precipitate and the combined extracts were quantitated to 10 ml and stored at 4°C.

Determination of total phenolics

The total phenolic content of each extract was determined by a modified Folin–Ciocalteu method[14]. The extract solution (250 µl) was mixed with 1 N Folin–Ciocalteu reagent (250 µl), 500 µl of 20% sodium carbonate (Na_2CO_3) and 4ml of water and incubated for 25 min at room temperature. The mixture was centrifuged at 4°C and 5,000 rpm for 10 min. The supernatant was measured at 730 nm (PerkinElmer Lambda35 UV/Vis spectrometer). The amount of total phenolics was expressed as a gallic acid equivalent (GAE) in mg per gram of dry plant extract.

Reducing power activity

The reducing power of the extracts was determined by the method of Oyaizu[15].

The extract solution (200 µl) was mixed with of 0.2 M phosphate buffer (200 µl, pH 6.6), 1% potassium ferricyanide (200 µl). The mixture was incubated at 50°C for 20 min. Ten-percent trichloroacetic acid (200 µl) was added to the mixture, followed by centrifugation at 3,000 rpm for 10 min. The supernatant (500 µl) was mixed with ultra pure water (500 µl) and 0.1% ferric chloride (100 µl), and incubated for an additional 10 min. The supernatant was then measured at 730 nm. Higher absorbance of the reaction mixture indicated greater reducing powers.

Capacity of scavenging DPPH radical

Each extract sample was mixed with 160 µM DPPH in methanol. After incubation for 20 min at room temperature in the dark, the absorbance was read at 517 nm[16]. The inhibitory percentage of DPPH (% scavenging activity) was calculated according to the equation shown as follows.

[(absorbance of the control – absorbance of the sample)/ absorbance of the control]×100%

Superoxide radical scavenging activity

The $O_2^{\bullet-}$ scavenging activity of the extracts was determined by the method developed by Beauchamp and Fridovich with minor modifications[17]. The photo-induced reactions were performed in a plastic box with a 30W fluorescent lamp that contained white cardboard and an outer surface covered with black cloth. The distance between the reactant and the lamp was fixed, and the voltage was adjusted by the regulator until the intensity of illumination reached 4,000 Lux. All solutions were 50 mM in phosphate buffer (pH 7.8). The total volume of reactant was 3 ml and the concentrations of riboflavin, methionine and nitro blue tetrazolium (NBT) were 2.4×10^{-6} M, 0.01 M and 1.6×10^{-4} M, respectively. The reactant was illuminated for 20 min. The photochemically-reduced riboflavins generated $O_2^{\bullet-}$ which reduced NBT to form blue formazan that can be detected at 560 nm. The unilluminated reaction mixture was used as a blank (A_0). Extracts of *Clinacanthus nutans* (or green tea) leaves (50 µl) were added to the reaction mixture, in which $O_2^{\bullet-}$ was scavenged, thereby inhibiting the NBT reduction. Absorbance (A_1) was measured and the decrease in $O_2^{\bullet-}$ was represented by $A_0–A_1$. The degree of scavenging was calculated by the following equation:

Scavenging (%) = ($A_0 - A_1$)/A_0×100%

SOD activity (U/g) = [Scavenging (%)/50%] × [Dilution factor/Extract solution (50 µl)]

DNA integrity assay

Jian *et al.*[18] studied the effects of luminance and light quality on the photo-decomposition of riboflavin and production of ROS. Ohara *et al.*[19] demonstrated the anticancer effects of blue light and riboflavin in cultured cancer cells and tumor-bearing animals. Degradation of 2′-deoxy-D-ribose has been used to quantify hydroxyl radicals as described previously[11]. DNA integrity assay was performed using supercoiled plasmid DNA as the target molecule. Plasmid DNA, pGEM-7Zf(-), was transformed into *E. coli,* DH5α strain, and grown overnight in LB broth at 37°C. The culture was then harvested and the DNA was purified using a Plasmid Miniprep kit (BioKit, Miaoli, Taiwan). After purification, 120 µl plasmid DNA dissolved in ultra pure water was added to the bottom of a glass tube of 6mm in diameter and 100 mm in height. Then, solutions of 160 µl riboflavin (120 µM) and 4.67 µl *Clinacanthus nutans* (or green tea) extracts (10,000, 1,000, 100, 10 and 0 µg/ml) were added to the wall of the tube. Each reaction was started by illumination of LED chips and a power supplier was used to control the voltage and current at 1.5 mW/cm^2. After 10-60 min, the 10 µl reaction solution was taken out and quenched by adding 2 µl of loading dye (0.25% bromophenol blue and 40% sucrose) prior to electrophoresis in a 1.2% agarose gel. Plasmid DNA was visualized by internally staining the gel with HealthView Nucleic Acid Stain.

Results

Antioxidant activities of extract

The antioxidant activities of extracts of *Clinacanthus nutans* and green tea were investigated according to their DPPH radical scavenging activity, reducing power activity, SOD activity and total phenolic contents. Fig. 1 shows the dose-response curves for the DPPH radical-scavenging activity of the extracts of *Clinacanthus nutans* and green tea. The IC_{50} of green tea was 163.3 µg/ml, while the IC_{50} of *Clinacanthus nutans* cannot be determined under current conditions.

In the reducing power assay, the antioxidant activity of the samples was measured by formation of ferrous products at 700 nm with increased absorbance, indicating a stronger reducing power. As shown in Fig. 2, the green tea extracts appeared to contain more active ingredients that could donate electrons and react with free radicals in a dose-dependent manner.

The total phenolic contents were expressed as gallic acid equivalents (GAE). In Fig. 3, the amount of GAE of green tea and *Clinacanthus nutans* leaf exyracts were 227.1

and 23.5 mg/g dry material, respectively. The total phenolics may play a role in the antioxidant activity, since the phenolic content in green tea is almost 10-fold higher than that of *Clinacanthus nutans* leaves.

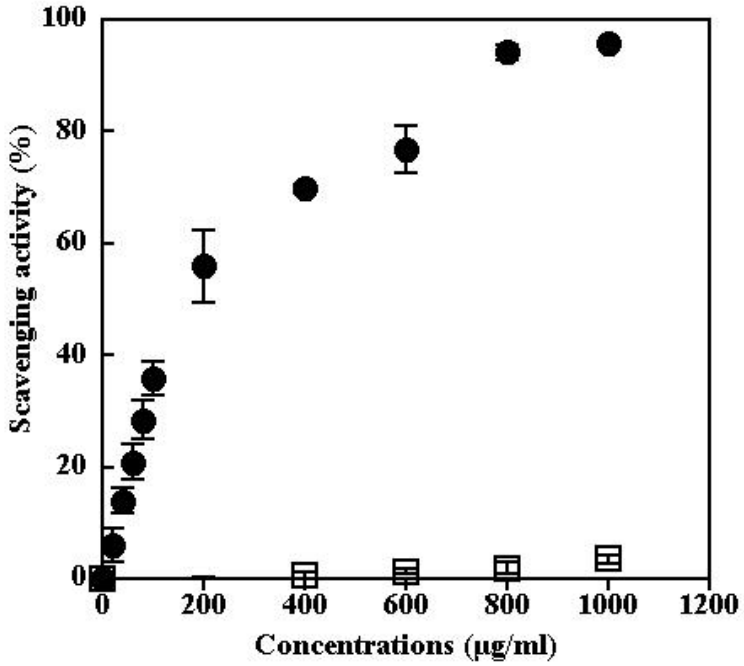

Figure 1. The capacity of scavenging DPPH radical of (●) green tea and **(**□**)** *Clinacanthus nutans* leaves.

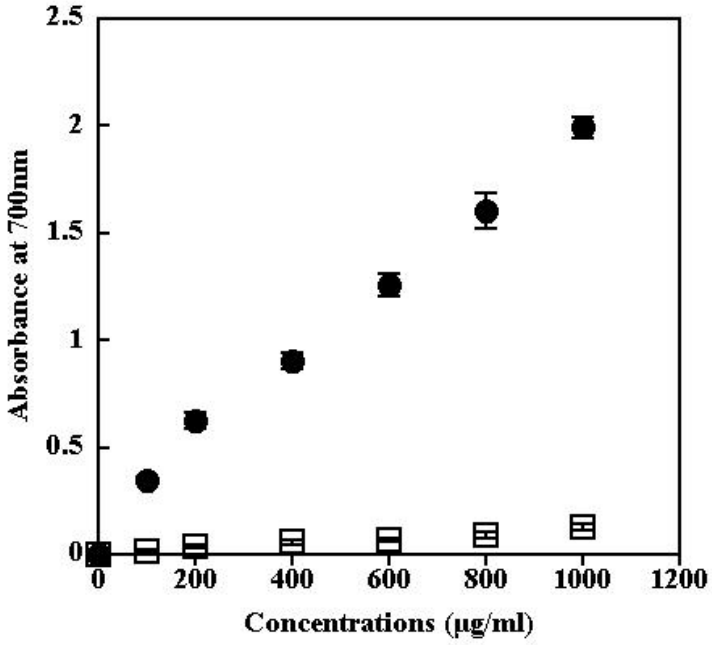

Figure 2. The reducing power of (●) green tea and (□) *Clinacanthus nutan* leaves.

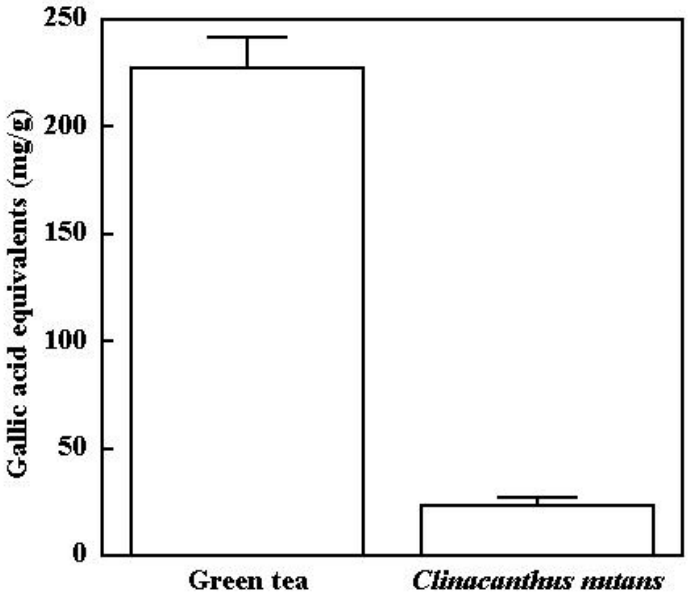

Figure 3. The total phenolic contents of green tea and *Clinacanthus nutans* leaves.

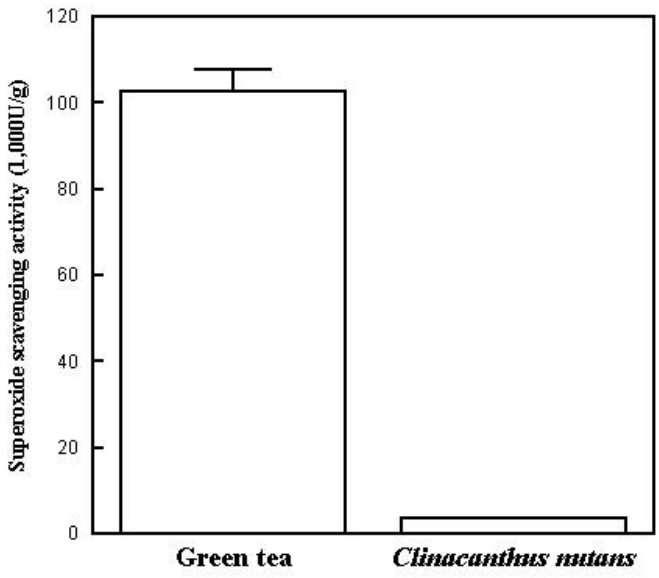

Figure 4. The $O_2^{\bullet-}$ scavenging activity of green tea and *Clinacanthus nutans* leaves.

The scavenging effects from *Clinacanthus nutans* and green tea on $O_2^{\bullet-}$ were determined by the NBT method. The results (Fig. 4) indicate the SOD activity (in U/g) of green tea and *Clinacanthus nutans* are 102,817.3 and 3,651.5, respectively. The SOD activity of green tea is almost 30-fold higher than C*linacanthus nutans* leaves.

DNA integrity assay

The super-coiled plasmid DNA was allowed to react with $O_2^{\bullet-}$ generated via a riboflavin photoreaction treatment. The results of DNA integrity assays by riboflavin photochemical treatments in the presence of extracts of green tea and *Clinacanthus nutans* leaves are shown in Fig. 5. As shown in lane B, super-coiled plasmid DNA was observed. In lanes Ⅰ-Ⅴ, plasmid DNA treated with riboflavin at 1.5 mW/cm^2 by blue LED illumination in the presence of 10,000, 1,000, 100, 10 and 0 μg/ml green tea and *Clinacanthus nutans* leaf extracts, respectively. The plasmid DNA bands were all retained, although mostly in linear forms rather than super-coiled forms with the riboflavin photoreaction. The results presented here suggest riboflavin photoreactions generating $O_2^{\bullet-}$ enhanced the levels of DNA cleavage, but the green tea and *Clinacanthus nutans* leaf extracts at 10,000 μg/ml treatments can scavenge the $O_2^{\bullet-}$ and in turn protect the plasmid DNA.

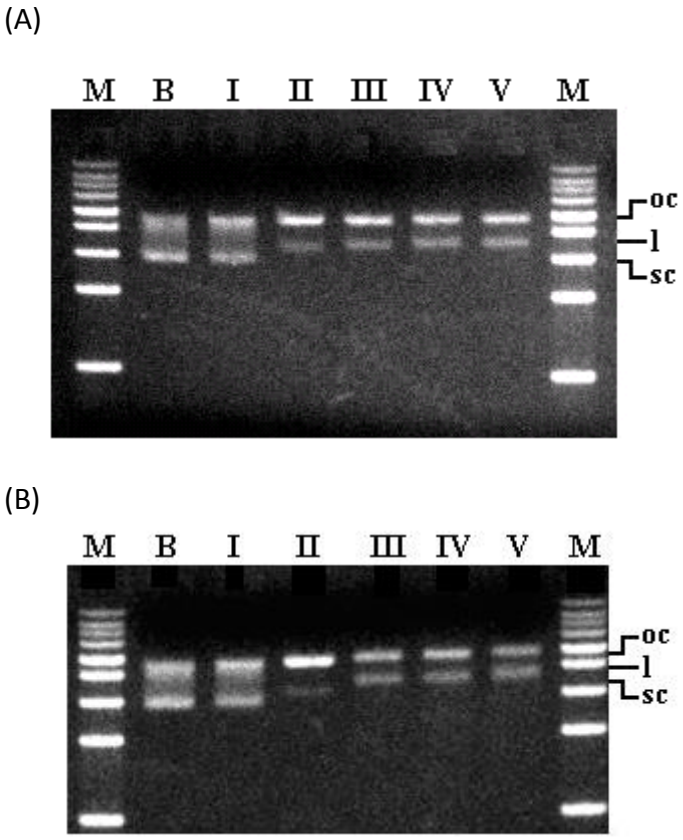

Figure 5. The concentration effects of the green tea (A) and *Clinacanthus nutans* leaf (B) extracts on DNA integrity assay under the riboflavin photochemical treatment. Lane M, 1 kb DNA marker. Lane B, plasmid DNA alone. Lanes I-V, plasmid DNA treated with riboflavin at 1.5 mW/cm^2 by blue LED illumination for adding 10,000, 1,000, 100, 10 and 0 μg/ml of botanical extracts, respectively. The notions, -oc, -l, and -sc, stand for open-circular, linear, and super-coiled plasmid DNA, respectively.

In lanes 10-60 of Fig. 6, the integrity assay showed plasmid DNA treated with

riboflavin and 10,000 μg/ml green tea and *Clinacanthus nutans* extracts at 1.5 mW/cm^2 blue LED illumination for 10, 20, 30, 40, 50 and 60 min, respectively. In Fig. 6, the plasmid DNA bands were all retained, although partly in linear forms rather than super-coiled forms with the riboflavin photoreaction. While under the same conditions, green tea extracts exhibited protective effects for only 30 min, as shown in Fig. 6(A), and the integrity assay of *Clinacanthus nutans* leaf extracts showed the super-coiled plasmid DNA under riboflavin photochemical treatment were all retained for up to 50 min (Fig. 6(B)).

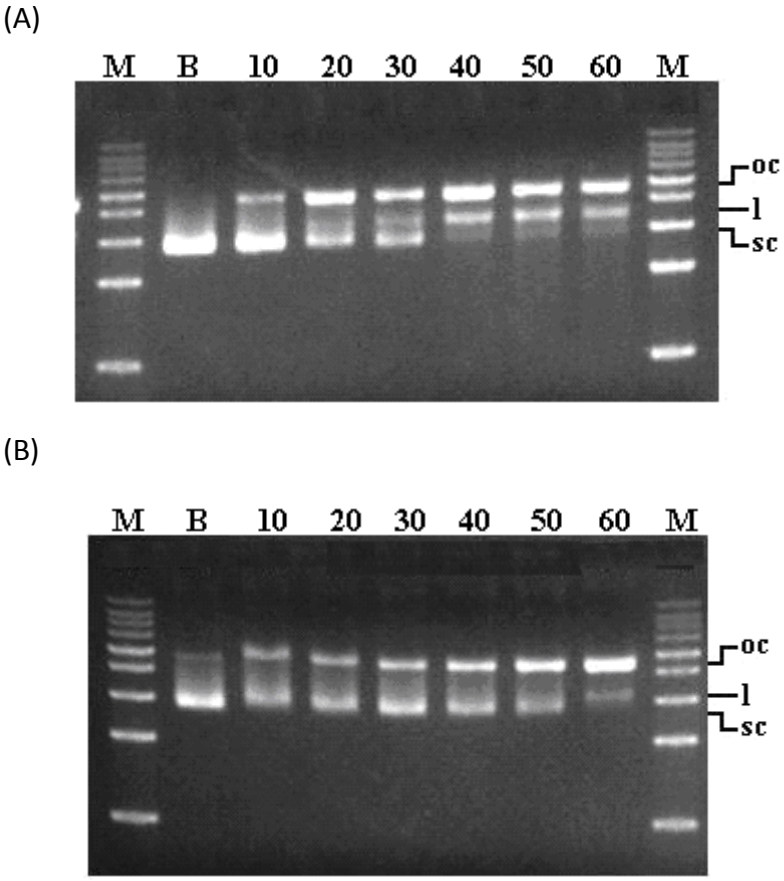

Figure 6. DNA integrity assay to the different illumination doses in the riboflavin photochemical treatment for the green tea (A) and the *Clinacanthus nutans* leaf (B) extracts. Lane M, 1 kb DNA marker. Lane B, plasmid DNA alone. Lanes 10-60, plasmid DNA treated with riboflavin and 10,000 μg/ml green tea extracts at 1.5 mW/cm^2 blue LED illumination for 10, 20, 30, 40, 50 and 60 min, respectively. The notions are the same as Figure 5.

Discussion

Catechins are the largest part of the polypenols found in green tea and are effective free radical scavengers[20]. Tea polyphenols are considered to be responsible for the

anticarcinogenic and antimutagenic properties of tea. It was reported the ethanolic extract of *Clinacanthus nutans* had shown antioxidant activity and a protective effect against free-radical-induced hemolysis[21]. *C*-glycosyl flavones, vitexin, isovitexin, shaftoside, isomollupentin 7-*O*-β-glucopyranoside, orientin, isoorientin and sulfur containing glucosides have been isolated from butanol and water-soluble portions of the methanol extract of the stem and leaves of *Clinacanthus nutans*[22], but the constituents of *Clinacanthus nutans* extracts possessing free radical scavenging activity are not well established[21]. In this study, the antioxidant activities, DPPH radical scavenging activity, reducing power activity, SOD activity, and total phenolic contents of *Clinacanthus nutans* were examined and found to be lower compared to that of green tea, suggesting some of the traditional efficiency of *Clinacanthus nutans* are not as potent as those of green tea.

The $O_2^{\bullet-}$ produced from photo-decomposition of riboflavin was shown to enhance the levels of DNA cleavage. Gutteridge[23] showed the hypoxanthine/xanthine oxidase system generated $O_2^{\bullet-}$ that was able to degrade deoxyribose with the formation of TBA-reactive materials. The effects of degradation suggest this is due to $O_2^{\bullet-}$ reducing the Fe^{3+}, a contaminant of reagents, and making it more redox-active [23]. In DNA integrity assay, the $O_2^{\bullet-}$ was produced from photo-decomposition of riboflavin and the redox-active effects occurred by reducing Fe^{3+} to Fe^{2+} in a Fenton's reaction to increase the levels of $^{\bullet}OH$. ROS may also occur by redox reactions of $O_2^{\bullet-}$. In DNA integrity assay, green tea and *Clinacanthus nutans* leaf extracts decreased the levels of DNA cleavages with the extracts of *Clinacanthus nutans* retaining high levels of super-coiled plasmid DNA compared to those of green tea extracts. While the constituents of *Clinacanthus nutans* extracts that decreased the levels of DNA cleavages are not identified at this stage, their protective effects can be attributed to three factors described as follows. First, an inhibitory effect is exerted on $O_2^{\bullet-}$ production. Second, a Fenton's reaction can be inhibited. Third, constituents possess direct free-radical-trapping ability.

Acknowledgments

We would like to thank the Department of Biotechnology at Ming Chuan University. We are grateful to Mr. Teik Khan Tan for kindly providing the *Clinacanthus nutans* (Burn f.) Lindau from Taiping, Perak, Malaysia. Finally, we are grateful to Mr. Simon R. Jacques, who proof-read the manuscript of this paper. This study was supported by the Graduate Student Research Fund in the Department of Biotechnology at Ming Chuan University.

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核黃素光化學法檢測優遁草葉子萃出物對質體DNA的保護作用

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中文摘要

優遁草廣泛生長於亞洲熱帶地區，且是中國、馬來西亞、泰國重要的草藥。本實驗以綠茶作為對照，比較優遁草葉子萃出物的抗氧化能力及保護大腸桿菌的質體DNA 的完整性。優遁草萃出物的抗氧化能力都較綠茶為低。綠茶的總多酚、SOD 活性高於優遁草的 30 倍及 10 倍。但是，以核黃素光化學法檢測保護質體超螺旋DNA 完整性的效果上，優遁草萃出物優於綠茶。

關鍵字：優遁草、抗氧化、超螺旋質體 DNA、核黃素光化學法
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收稿：2012-8-25 修改：2012-9-10 接受：2012-9-12

MC-Transaction on Biotechnology, 2012, Vol. 4, No. 1, e5