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Antioxidant and Free Radical Scavenging Activity of *Camellia nitidissima* Chi

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The antioxidant and free radical scavenging activity of various fractions of the ethanol extract of *Camellia nitidissima* Chi leaves were evaluated *in vitro* using DPPH, $ABTS^+$ radical scavenging activity and phosphomolybdenum method. The DNA damage and human umbilical vein endothelial cell (HUVEC) (CRL-1730) damage were also applied to assess the antioxidant capacity of the *n*-butanol fraction, which showed the highest antioxidant activity in all of the test fractions. The total phenolic contents were assessed in order to evaluate the relationship of total phenolic contents with antioxidant and free radical scavenging capability. Results showed that *n*-butanol fraction possess the highest free radical scavenging activities with the EC_{50} of 37.64 μg/mL for DPPH and 14.74 μg/mL for $ABTS^+$. Furthermore, it demonstrated powerful capacity to protect the DNA damage induced by AAPH at 1 mg/mL and protect the CRL-1730 cell damage induced by hydrogen peroxide (H_2O_2) (EC_{50}, 9.85 μg/mL). Maximum amounts of total phenolic contents were found in the *n*-butanol fraction which correlates well with the antioxidant and free radical scavenging capacities.

Key Words: *Camellia nitidissima* Chi, Antioxidant activity, DNA damage, HUVEC damage, Total phenolic contents, Free radical scavenging activity.

INTRODUCTION

Reactive free radicals, including superoxide, hydroxyl radical and peroxyl radical, generally result in degradation of protein, lipid peroxidation and oxidation of DNA, which have been considered to be linked with many chronic diseases such as diabetes, cancers and atherosclerosis[1,2]. Antioxidants are compounds that inhibit or terminate the oxidation process by removing free radical intermediates and inhibit other oxidation reactions. In recent years, more and more plants have been shown to have antioxidant and free radical scavenging capacities, polyphenols are the major antioxidant constituents isolated from many medicine and edible plants[3].

Camellia nitidissima Chi. (Theaceae) is an evergreen shrub with a natural distribution limited in Guangxi Province in China and neighboring regions of North Vietnam. *Camellia nitidissima* Chi is a group of rare and endangered plants in the word and it is honored as "the Queen of Camellia" and "the panda of the plants"[4]. The genus of *Camellia* comprise of about 258 species mainly found in the Guangxi Province. Many plants were widely used by the local people for the treatment of some diseases, including Nephritis, edema, urinary tract infection, sore throat, dysentery, jaundice, hepatic cirrhosis, hypertension, ulcers, preventing cancer[5]. The researches on the chemistry and pharmacology of *Camellia nitidissima* Chi were not seen as a result of its distribution predominantly local to southwest China, especially in the Guangxi province. Ethanol extract of *Camellia nitidissima* Chi leaves has been found to exhibit many activities, including antiallergic[6], antiatherosclerosis[7], antihepatoma[8] and scavenging hydroxyl radical, superoxide, hydrogen peroxide[9]; ethanol extract of its seeds also demonstrated antileukemie activity[10]. The chemical constituents of *Camellia nitidissima* Chi include flavonols, phenolic acids, polysaccharides, saponin and volatile[11-13].

Literature survey showed that studies have not yet been conducted on free radical scavenging activity of *Camellia nitidissima* Chi. A large number of various methods are used to evaluate antioxidant activities of natural compounds and extracts from the medicines plants or foods. DPPH and $ABTS^+$ were the two methods used extensively in estimation the antioxidant capacities of pure compounds and plant extracts. Therefore we carried out a comprehensive evaluation of antioxidant and free radical scavenging activities of the leaves of the plant in various solvents (chloroform, ethyl acetate, *n*-butanol) using the common two methods coupled with three other methods.

EXPERIMENTAL

Gallic acid, trolox, quercetin and 1,1-diphenyl-2-picryl-hydrazyl (DPPH), Folin-Ciocalteu reagent, agarose, 2,2'-azo*bis*(2-methylpropionamidine)dihydrochloride (AAPH), were purchased from sigma chemical company (St. Louis MO, USA); 2,2'-azino-*bis*-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), was obtained from TCI-SU (Tokyo Japan), pBR322 DNA were purchased from MBI company, acrylamide, N,N'-methylene-*bis*-acrylamide and guanidine HCl, potassium persulfate, sodium dodecyl sulfate (SDS) and all solvents used were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China); visible spectra measurements were done using UV-2450 spectrophotometer (Shimadzu, Japan).

Solvent extraction: The leaves of *Camellia nitidissima* Chi (Theaceae) were collected in October 2009 from the Fangchenggang country, Guangxi province, China and identified by a taxonomist of Jiangxi University of traditional Chinese medicine, China. The leaves were washed in water and dried at 40 ºC in an air oven, finally ground to fine powder. The ground plant material (1 g) was extracted with 40 mL of each of the solvent system (10 % ethanol, 30 % ethanol, 50 % ethanol, 70 % ethanol and 90 % ethanol) in 250 mL conical flask and refluxed at 80 ºC for 1 h. The extracts were centrifuged at 10000 g (Allegra-64R, Beckman) and the supernatants were used for testing the total phenolic contents to optimize the extraction solvent system. Extraction was carried out by added 10 g of the powered leaves with 400 mL 50 % ethanol (v/v) into a 1 L conical flask and refluxed at 80 ºC for 1 h. The extracts were concentrated *in vacuo* at 45 ºC, using a rotary evaporator (EYELA, N-1000, Rikakikai Co. Ltd. Tokyo, Japan). The dried extracts were suspended in 100 mL water and subjected to sequential liquid-liquid extraction with the equal volumn chloroform (CF), ethyl acetate (EF) and *n*-butanol (BF). The three organic fractions and water fraction (WF) were obtained by removed the solvent. All these four fractions were weighted and calculated the yield rate.

Determination of total phenolic contents (TPC): The total phenolic contents (TPC) were determined by using Folin-Ciocalteu reagent and external calibration with gallic acid (GA)[14]. Briefly, 1 mL of extract solution in a test tube and 5 mL of Folin-Ciocalteu reagent (diluted 10 times) were added and the contents mixed thoroughly. After 4 min, 4 mL of sodium carbonate (7.5 % w/v) was added with mixture. The mixed solution was then immediately diluted to volume (25 mL) with deionized distilled water and mixed thoroughly. The tubes were allowed to stand for 90 min before absorbance at 760 nm was measured by using UV-2450 spectrophotometer (UV-2450, Shimadzu, Japan). The TPC were calculated by using GA calibration curve within range of 50-250 mg/L. The calibration equation for gallic acid was Y = 0.07411X + 0.0589 (R^2 = 0.9977). The results were expressed as gallic acid equivalents GAE mg/g dry extracts.

DPPH free radical-scavenging capacity: The free radical-scavenging activity of the extracts was evaluated by DPPH using the method of Sajjad with slightly modifications[15]. Briefly, DPPH solution (0.6 mM) was prepared in ethanol and to 0.5 mL of this solution was mixed with 0.5 mL of extract samples (from 120-600 µg/mL). The volume of the solution was adjusted with ethanol to a final volume of 5 mL. After incubation in a dark place for 0.5 h at room temperature, the absorbance of the mixture was measured at 515 nm against ethanol as blank using UV-2450 spectrophotometer (Shimadzu, Japan). Trolox and Quercetin were used as positive control. Each sample was measured in triplicate and averaged. The activity was calculated according to the formula:

$$\text{DPPH scavenging activity } (\%) = \left[\frac{(A_C - A_S)}{A_C}\right] \times 100$$

where A_C = absorbance value of the control and A_S = absorbance value of the added test samples solution. Extract concentration providing 50 % inhibition (EC_{50}) was calculated from the plot of inhibition percentage against extract concentration.

$ABTS^+$ cation free radical-scavenging activity: For ABTS assay, the procedure followed the method of Pellegrini with some modifications[16]. ABTS was dissolved in water to make a concentration of 7 mM/L. $ABTS^+$ was produced by reacting the ABTS stock solution with 2.45 mM/L potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. For the test of samples, the $ABTS^+$ stock solution was diluted with 80 % methanol to give an absorbance of 0.70 ± 0.02 at 734 nm. After the addition of 4.75 mL of diluted $ABTS^+$ to 0.25 mL of extract samples (from 72-480 µg/mL), the absorbance reading was taken 6 min after the initial mixing. Trolox and quercetin were used as positive control. Each sample was measured in triplicate and averaged. This activity is given as percentage $ABTS^+$ scavenging that is calculated by the same formula with DPPH.

Phosphomolybdenum method: The total antioxidant capacity of the extract samples were evaluated according to the method described by Prieto[17]. An aliquot of 0.5 mL of extract samples solution (from 120-600 µg/mL) were combined with 4.5 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In case of blank, 0.5 mL of ethanol was used in place of samples. The tubes were incubated in a boiling water bath at 95 ºC for 90 min. After the samples were cooled to room temperature, the absorbance of the aqueous solution of each samples were measured at 695 nm against blank in UV-2450 spectrophotometer. The total antioxidant activity was expressed as the absorbance of the sample at 695 nm. The higher absorbance value indicated higher antioxidant activity[18].

Determination of oxidative DNA strand breakage: Conversion of the supercoiled form of plasmid DNA to the open-circular and further linear forms has been used as an index of DNA damage[19]. To assay DNA strand breakage induced by peroxyl radical and inhibited by *n*-butanol fraction, 5 µL supercoiled pBR 322 plasmid DNA (100 ng) was incubated with 5 µL various concentrations (0.1-100 mg/mL) of the *n*-butanol extract and 15 µL 10 mM AAPH in PBS for 1 h at 37 ºC, 2 µL of 6 × loading buffer (0.05 % xylene cyanol FF, 0.05 % bromophenol blue, 30 mM EDTA and 36 % (v/v) glycerol) was added and the resulting mixtures were subjected to 1 % agarose gel stained with ethidium bromide (EB). Then, the samples (10 µL) were electrophoresed in a horizontal slab gel

apparatus in TAE buffer (40 mM *tris*, 20 mM sodium acetate and 2 mM EDTA) at 100 V for 0.5 h. The gel was photographed under a transilluminator (GelDoc2000, Bio-Rad, USA).

CRL-1730 cell culture and treatment: CRL-1730 cells (from ATCC Global Bioresource Center) were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum. These cells were incubated in a humidified atmosphere containing 5 % CO_2 at 37 ºC. Cells were counted and seeded into 96-well culture plates at a density of 1×10^4 cells/well. After incubation together with 50 µg/mL of the *n*-butanol extract for 4 h, each well was washed twice by PBS. Then, for each well H_2O_2 (final concentration 0.4 mM) was added. After continued incubation for 20 h, cell viability in every well was assayed by MTT method[20]. The cell viability rate is calculated as following formula:

$$\text{Cell viability rate } (\%) = \left[\frac{(OD_{sample} - OD_{model})}{(OD_{mormal} - OD_{model})}\right] \times 100$$

where OD_{sample} = absorbance value of the added sample test solution and H_2O_2, OD_{model} = absorbance value of the added H_2O_2 but not sample test solution and OD_{normal} = absorbance value of the absence of H_2O_2 and sample test solution. If the cell viability surpassed 50 %, the various concentrations were tested for calculated 50 % inhibition (EC_{50}).

Statistical analysis: Results were given as mean ± standard deviation of three replicates. Experimental results were analyzed by SPSS version 16.0 (SPSS Inc. Chicago, IL). Differences between means were determined using one-way ANOVA and Duncan's test. The level of statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Total phenolic contents and percentage yields: Phenolic compounds are a large group constituents exist in many medicine plants, fruits and vegetables, which are characterized by having at least one aromatic ring with one or more hydroxyl groups attached[21]. The phenolic compounds may contribute directly to the antioxidant action due to the presence of hydroxyl groups which are potent hydrogen donors[22]. Total phenolic contents of *Camellia nitidissima* Chi extracts were determined by Folin-Ciocalteu method which was used very extensively in the total phenolic contents test due to its sensitivity, simple and lower interference. The total phenolic contents of the extracts obtained from different ethanolic extract range from 7.29 ± 0.08-12.53 ± 0.23 GAE mg/g dry material (Fig. 1). Maximum amount of total phenolic contents (12.53 ± 0.23 mg/g) was observed with 50 % ethanolic extract. The effect of ethanol concentration on total phenolic contents was significant ($p < 0.05$). So the next step extraction was carried out by 50 % ethanol concentration. Four fractions were obtained by liquid-liquid extraction with chloroform, ethyl acetate and *n*-butanol. The highest total phenolic contents were detected in *n*-butanol fraction (125.31 ± 0.71 mg/g) and the lowest in aqueous after partition extract (43.79 ± 0.99 mg/g). Chloroform and ethyl acetate fractions show comparable values (Table-1). It is different from many reports that the ethyl acetate fraction possessed of the highest total phenolic contents and displayed strongest antioxidant activity[23], while it had been reported that the *n*-butanol extract of *Pterodon emarginatus vogel* seeds manifested the stronger antioxidant activity than the ethyl acetate and hexane extracts[24]. The phenolic constituents from the genus of *Camellia maybe* were polar molecules, so most of which were obtained by the polar solvent.

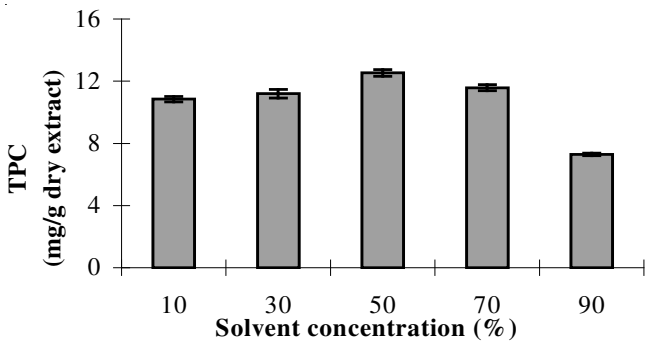

Fig. 1. Total phenolic contents of *Camellia nitidissima* Chi extracts with different ethanol concentrations

DPPH free radical-scavenging capacity: DPPH has been widely used in the evaluation of antioxidant activity of pure compounds and plant extracts, the presence of antioxidant compounds in the extracts lead to the decrease or disappearance of DPPH free radical chromogens which can be detected spectrophotometrically at 515 nm. Fig. 2 show the antioxidant activity of the four fractions of *Camellia nitidissima* Chi extracts in the reaction with DPPH radical, the EC_{50} values are show in Table-1, lower the EC_{50} value of sample, more ability it will be for scavenging of DPPH free radical. The EC_{50} value of *n*-butanol fraction was lowest 37.64 µg/mL, while other three fractions were all more than 60 µg/mL. The ability of scavenging DPPH radical of the four fractions were in order of *n*-butanol fraction > ethyl acetate fraction > chloroform fraction > water fraction, which was consistent with the total phenolic contents of the four fractions. Many literatures had demons-

TABLE-1
Yield (%), TOTAL PHENOLIC CONTENTS AND EC_{50} VALUES OF THE EXTRACT FRACTIONS

Fractions	Yield (%)	Total phenolic contents (mg/g of dry extract)	DPPH free radical scavenging (EC_{50}, µg/mL)	($ABTS^+$ cation free radical scavenging (EC_{50} µg/mL)
CF	0.65[a]	80.57 ± 0.99[a]	>60[a]	>24[a]
EF	5.13[b]	83.99 ± 2.55[a]	>60[a]	>24[a]
BF	5.27[b]	125.31 ± 0.71[b]	37.64[b]	14.74[b]
WF	15.61[c]	43.79 ± 0.99[c]	>60[a]	>24[a]
Quercetin	–	–	2.37[c]	1.08[c]
Trolox	–	–	2.76[c]	2.05[c]

Note: Values are means of 3 replicates ± SD, different letters within the same column indicate significant difference at $p < 0.05$ by Duncan's test.

trated that extracts with high phenolic content showed the high radical scavenging and antioxidant activity[25].

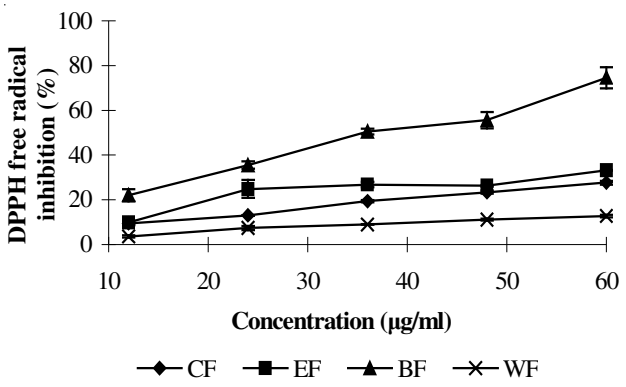

Fig. 2. DPPH free radical scavenging activity of four fractions from *Camellia nitidissima* Chi leaves extract

ABTS$^+$ cation free radical-scavenging activity: ABTS assay is based on the antioxidant ability to react with ABTS$^+$ generated by potassium persulfate oxygenization the ABTS. This method is widely used to evaluate antioxidant activity in foods and medicines systems. ABTS$^+$ cation radical have a maximum of absorbance at 734 nm, extracts contain antioxidant components will decrease the absorption values of ABTS$^+$ at 734 nm by redox reaction directly scavenging the ABTS$^+$ cation radical. Fig. 3 show the antioxidant activity of the four fractions of *Camellia nitidissima* Chi extracts in the reaction with ABTS$^+$ cation radical, the EC_{50} values are show in Table-1. The inhibition rate at the test concentrations range only *n*-butanol fraction surpassed 50 % ($p < 0.05$), the EC_{50} value of *n*-butanol fraction was 14.74 μg/mL. The ability of scavenging ABTS$^+$ cation radical of the four fractions were in order of *n*-butanol fraction > ethyl acetate fraction > chloroform fraction > water fraction, which was consistent with the total phenolic contents and the DPPH radical scavenging activity of the four fractions.

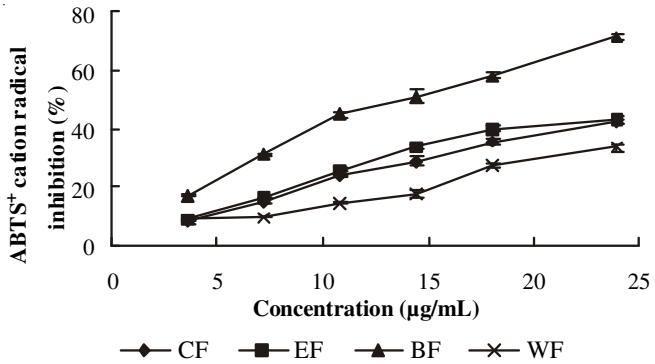

Fig. 3. ABTS$^+$ cation radical scavenging activity of four fractions from *Camellia nitidissima* Chi leaves extract

Total antioxidant capacity: The phosphomolybdate method has been routinely used to evaluate the total antioxidant capacity of pure compounds and plant extracts. In the presence of antioxidants, Mo(VI) is reduced to Mo(V) and forms a green coloured phosphomolybdenum(V) complex, which shows a maximum absorbance at 695 nm. Fig. 4 shows that the antioxidant capacity of four fractions of *Camellia nitidissima* Chi leaves can be ranked in the order of *n*-butanol fraction > ethyl acetate fraction > chloroform fraction > water fraction, the *n*-butanol fraction showed the highest total antioxidant activity, however, the values were not significantly different from that ($p > 0.05$) of chloroform fraction and ethyl acetate fraction, which were significantly different from ($p < 0.05$) of the water fraction.

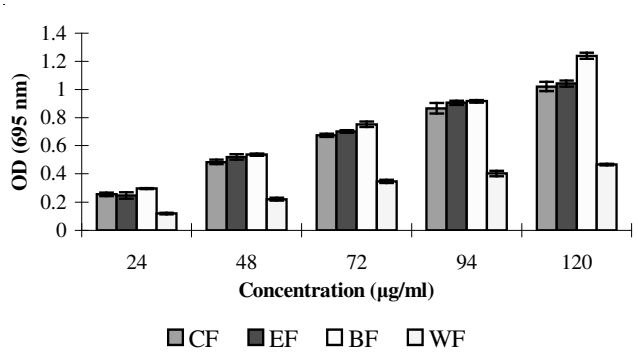

Fig. 4. Total antioxidant activity of four fractions from *Camellia nitidissima* Chi leaves extract

Effect of *n*-butanol fraction on oxidative DNA strand breakage: DNA damage alters replication and transcription, causes cell death or leads to mutations. Oxidative modifications of DNA have been suggested to contribute to aging and various diseases including diabetes, cancers and atherosclerosis. To assay the potential of *n*-butanol fraction to prevent DNA damage, oxidative DNA strand breakage induced by AAPH was measured with pBR322 DNA. As showed in Fig. 5 the plasmid DNA was mainly of the supercoil form in the absence of AAPH (Fig. 5 lane 1). With the addition of 10 mM AAPH the DNA supercoil form disappeared and converted into the linear form (Fig. 5 lane 2). Addition of various concentration of *n*-butanol fraction (from 100-1 mg/mL) significantly inhibited the increasing of linear form of pBR322 DNA, while at the concentration of 0.1 mg/mL there were no activity.

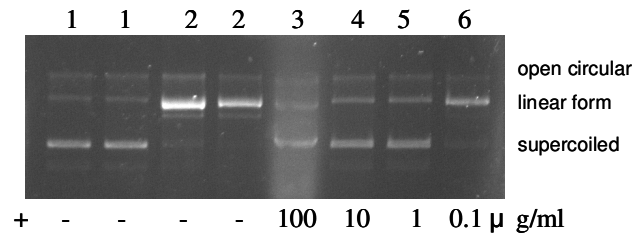

Fig. 5. Protection of *n*-butanol fraction to 10 mM AAPH induced pBR322 DNA strand breakage

Effect of *n*-butanol fraction on CRL-1730 cell damaged by H_2O_2: To evaluate the *n*-butanol fraction protects the HUVEC from oxidative stress, preliminary assay was carried out by the National Center of Pharmaceutical Screening of China (Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College). CRL-1730 cells were pretreated with 50 μg/mL *n*-butanol fraction for 4 h and followed by exposing to 0.4 mM H_2O_2 (final concentration) for 20 h. After then, cell viability was measured by MTT assay. At the concentration of 50 μg/mL, the cell survival rate was 66.15 %; then the serial concentrations of sample

were test in order to determine the EC_{50} of *n*-butanol fraction protects the CRL-1730 cell damaged by H_2O_2, the results show that *n*-butanol fraction had significantly capacity of protecting CRL-1730 cell damaged by H_2O_2 with the EC_{50} at 9.85 μg/mL.

Conclusion

This paper provides the antioxidant and radical-scavenging capacities of *Camellia nitidissima* Chi extracts as well as the effect of the *n*-butanol fraction on protection of pBR322 DNA damage induced by AAPH and HUVEC cell damage induced by H_2O_2. The total phenolic contents in the four fractions of *Camellia nitidissima* Chi extracts were determined by the classical Folin-Ciocalteu colorimetric assay. The highest total phenolic contents was found in the *n*-butanol fraction, which showed the powerful activities in all test methods. The preliminary phytochemical screening of the *n*-butanol fraction showed that the phenolic acids and saponins were the two major groups compounds in the fraction. This evaluation may throw the light on a better understanding on the potential of *Camellia nitidissima* Chi as a functional antioxidant or a nourishment for their high antioxidant activity. Total phenolic acids and total saponins of the *n*-butanol fraction were chosen for further investigation of its antioxidant activities and mechanisms *in vivo* in our future work.

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