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Identification of polyphenols in tobacco leaf and their antioxidant and antimicrobial activities

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Abstract

Crude polyphenols were extracted from tobacco leaf by 80% ethanol solution with ultrasonic treatment and then purified by a macroporous resin. The polyphenols from tobacco leaf (PTL) were subjected to analyses by reverse-phase high-performance liquid chromatography (RP-HPLC) and electrospray ionization mass spectrometry (ESI-MS). The dominant polyphenols in tobacco leaf were identified as chlorogenic acid and rutin. Furthermore, the antioxidant activities of PTL were investigated, including scavenging activities of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (5.02 μg/ml IC_{50} value), hydroxyl radicals (49.6 μg/ml IC50 value) and superoxide anion radicals (44.0 μg/ml IC_{50} value), inhibition activity of lipid peroxidation (132 μg/ml IC_{50} value) and reducing power. The proliferation inhibition activities on *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* were also measured for evaluating the antimicrobial activity of PTL. The diameters of inhibition zones were 20.23 ± 0.42, 17.66 ± 0.86 and 12.89 ± 0.29 mm, respectively. The results showed that PTL had great potential as antioxidant and antimicrobial agent.

Keywords: Tobacco; Polyphenol; Antioxidant activity; Antimicrobial activity; HPLC–MS

1. Introduction

Tobacco is a very important economic crop. In 2006, tobacco leaf production was about 500 million tons over the world, increasing by 0.69% compared with that in 2005. Furthermore, over 20% of tobacco resources are discarded as processing waste, which pollute the environment and cause a big waste. In fact, the discarded tobacco leaves are economically valuable because of abundant bioactive compounds in them. Therefore, it is important to investigate and utilize the resource of tobacco leaf.

Antioxidant activities of plant polyphenols have been claimed to have beneficial health functions for retarding aging and preventing cancer and cardiovascular diseases (Scalbert, Johnson, & Saltmarsh, 2005). The interest in polyphenol antioxidants has increased remarkably over the last decade because of their protective effects against different diseases, including cardiovascular, inflammatory and neurological diseases, as well as cancers (Lu & Foo, 1997). The generally accepted mechanism is that free radical-scavenging activity of polyphenols contributes to reduce the oxidative stress and to prevent the development of diseases (Huang, Chen, & Chen, 2001). Tobacco leaf is rich in polyphenols which possess various bioactivities (Ruiz et al., 1998). The presence of polyphenols affects the colour and quality of tobacco leaf (Bazinet, DeGrandpré, & Porter, 2005). However, from the literature available, no scientific evaluation of the antioxidant and antimicrobial activities of PTL has yet been done. Thus, the objectives of the present study were focussed on the antioxidant and antimicrobial activities of PTL. Major polyphenols in ethanolic extract of tobacco leaf were also identified by RP-HPLC and MS techniques.

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2. Materials and methods

2.1. Materials

The tobacco leaves (cultivar NC89) were donated from the China Tobacco Technology Training Center and ground into powder.

2.2. Chemicals

Nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), nicotinamide adenine dinucleotide (NADH), DPPH, α-tocopherol and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). S8 macroporous resin was purchased from the Chemical Company of Nankai University (Tianjin, China). All other chemicals and reagents used were of analytical grade.

2.3. Extraction and purification

The extraction of PTL was done according to the method of Lee and Wicker (1991), with some modifications. Tobacco leaf powder (20 g) was weighed and put into a 250 ml conical flask. Two hundred millilitres of 80% ethanol solution were added. The conical flask was placed in an ultrasonic cleaner (40 kHz, KQ-300DE, Sonicator Company, Kunshan, China) for 15 min at room temperature. The extract was centrifuged at 10 854*g* in a Kubota 6800 centrifuge (Kubota Co., Osaka, Japan) for 10 min at 4 °C. The supernatant was filtered using Whatman No. 1 filter paper, followed by a microbial filter with pore size of 0.45 μm (Sartorius minisart, Hannover, Germany). The filtrate was concentrated under reduced pressure at 40 °C by a rotary evaporator (RE52AA, Yarong Corporation, Shanghai, China). The crude polyphenols were obtained by lyophilization under vacuum. The crude polyphenols were dissolved in distilled water to a final concentration of 1 mg/ml. Then they were loaded onto a S8 macroporous resin column (1.6 × 60 cm). The desorption conditions were as follows: eluted by 300 ml of water, followed by 300 ml of ethanol/water (50:50, v/v), 1 ml/min of flow rate. The ethanol–water eluate was collected and lyophilized under vacuum. A yield of 537 mg was obtained. The purified polyphenols were stored at 4 °C for determination of polyphenol content, antioxidant and antimicrobial activities.

2.4. Total phenolic content

Total phenolic contents of PTL were determined by the Folin–Ciocaulteu assay, according to the method of Singleton, Orthofer, and Lamuela-Raventos (1999), with some modifications. Chlorogenic acid was employed as a standard reference and results were expressed as chlorogenic acid equivalents (CAE) (mg CAE/g of tobacco leaves on dry weight basis). All spectrophotometric data were acquired using an spectrumlab 52 UV spectrophotometer (Lengguang Tech Co., Shanghai, China).

2.5. RP-HPLC analysis

Analytical RP-HPLC analysis was conducted on a DIONEX summit liquid chromatograph (Dionex Corporation, Sunnyvale, USA), fitted with a C18 reverse-phase column (250 × 4.6 mm, Diamonsil, Dikma Technologies, Beijing, China), a Dionex PDA-100 photodiode array detector and Dionex P680 HPLC pump. PTL were dissolved in methanol and injected (20 μl) into the HPLC system. The mobile phase consisted of 1% aqueous acetic acid (solvent A) and methanol (solvent B). The elution was allowed to run with 90% A and 10% B for 2 min, then from 90% to 80% A and from 10% to 20% B for 10 min, from 80% to 50% A and from 20% to 50% B for 10 min, from 50% to 0% A and from 50% to 100% B for 5 min. The flow rate was 0.5 ml/min. Polyphenols in the eluate were detected at 345 nm with a diode array UV detector (Dionex PDA-100, Dionex Corporation, Sunnyvale, USA), whose wavelength was in the range 200–400 nm.

2.6. HPLC–MS analysis

HPLC–MS was conducted on a Waters 1525 HPLC (Waters, Milford, American), coupled with an ESI detector (Waters Micromass ZQ2000, Waters, Milford, America). Chromatographic separation of PTL was conducted using a C18 reverse-phase column (250 × 4 mm; Diamonsil, Dikma Technologies, Beijing, China). A mobile phase (A + B solvent gradient) was employed for the RP-HPLC analysis. Identification of polyphenols was done by means of UV absorbance at 345 nm. Mass spectra in the negative-ion mode were generated under the following conditions: fragmenter voltage = 100 V; voltage = 2800 V; nebulizer pressure = 25 psi; temperature = 100 °C; m/z range = 50–3000.

2.7. Antioxidant activity

2.7.1. Hydroxyl radical-scavenging activity

According to the method of Ghiselli, Nardini, Baldi, and Scaccini (1998), the measurement of hydroxyl radical-scavenging activity was carried out. 0.1 ml of 20, 40, 60, 80, 100, 120 or 140 μg/ml of PTL solution in methanol was mixed with 0.8 ml of reaction buffer (0.2 M KH_2PO_4 KOH buffer, pH 7.4, 1.75 μmol deoxyribose, 0.1 μmol iron ammonium sulphate and 0.1 μmol EDTA). 0.1 ml of 0.01 M H_2O_2 was then added to the reaction solution. The solution was incubated for 10 min at 37 °C prior to the addition of 0.5 ml of 1% thiobarbituric acid and 1 ml of 2.8% trichloroacetic acid. The mixture was boiled for 10 min and cooled rapidly. The absorbance of the mixture was measured at 532 nm. Vitamin C was used as a control. The blank was prepared using distilled water instead of sample. All the tests were performed in triplicate. The hydroxyl radical-scavenging activity was calculated as follows:

$$\text{Scavenging activity } (\%) = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100\% \quad (1)$$

where A_0 indicates the absorbance of blank; A_1 is the absorbance of the mixture in the presence of sample; A_2 is the absorbance of the mixture in the absence of sample. The plot of scavenging activity on hydroxyl radical was done and IC_{50} value (concentration of sample to scavenge 50% of the hydroxyl radicals) was calculated.

2.7.2. Superoxide anion radical-scavenging activity

The superoxide anion radical-scavenging activity was assessed by the method of Yu et al. (2006) with a slight modification. Superoxide anion radicals, generated in the phenazine methosulfate-reduced form of nicotinamide adenine dinucleotide (PMS-NADH) system by oxidation of NADH, were assayed by the reduction of NBT. In this experiment, superoxide anion radicals were generated in 1.25 ml of Tris–HCl buffer (16 mM, pH 8.0) containing 0.25 ml of NBT (300 μM), 0.25 ml of NADH (468 μM) and PTL solution (20, 40, 60, 80, 100 or 120 μg/ml). The reaction was started by adding 0.25 ml of PMS (60 μM) solution to the mixture. The reaction mixture was incubated at room temperature for 5 min and the absorbance was read at 560 nm by a spectrophotometer (UV-2100, Unico Corporation, Shanghai, China) against a blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion radical-scavenging activity. Vitamin C was used as a control. All the tests were performed in triplicate. The scavenging of the superoxide anion radicals was calculated by the following equation:

$$\text{Scavenging activity } (\%) = \frac{A_0 - A_1}{A_0} \times 100\% \quad (2)$$

where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance of the mixture containing sample. The plot of scavenging activity on superoxide anion radicals was done and IC_{50} value (concentration of sample to scavenge 50% of superoxide anion radicals) was obtained.

2.7.3. DPPH radical-scavenging activity

The DPPH radical-scavenging activity was determined according to the method of Shimada, Fujikawa, Yahara, and Nakamura (1992). The PTL extract was dissolved in methanol to prepare various concentrations of 5, 10, 15, 20, 25, 30 or 35 μg/ml. Two millilitres of PTL solution were mixed with 1 ml of 0.2 mM DPPH in methanol. The mixture was shaken vigorously and maintained for 30 min in the dark. The absorbance was measured at 517 nm. The absorbance of the control was obtained by replacing the sample with methanol. Vitamin C was used as a control. The scavenging activity was calculated using the following equation:

$$\text{Scavenging activity } (\%) = [(A_{517} \text{ of control} - A_{517} \text{ of sample})/ A_{517} \text{ of control}] \times 100. \quad (3)$$

The plot of scavenging activity on DPPH radical was done and IC_{50} value (concentration of sample to scavenge 50% of the DPPH radicals) was calculated.

2.7.4. Reducing power

The reducing power of PTL was determined according to the method of Ahmadi, Kadivar, and Shahedi (2007). Sample solution (0–50 μg/ml, 2 ml), phosphate buffer (2 ml, 0.2 M, pH 6.6) and potassium ferricyanide (10 mg/ml, 2 ml) were mixed, and then incubated at 50 °C for 20 min. Trichloroacetic acid (2 ml, 100 mg/ml) was added to the mixture. A volume of 2 ml from each of the above mixtures was mixed with 2 ml of distilled water and 0.4 ml of 0.1% (w/v) ferric chloride in a test tube. After incubation for 10 min, the absorbance was measured at 700 nm. The reducing power of vitamin C was also assayed as control. Distilled water was used as a blank. All the tests were carried out in triplicate. Increased absorbance indicated stronger reducing power.

2.7.5. Lipid peroxidation inhibition activity

The inhibition of lipid peroxidation was assayed by the method of Anup, Shereen, and Shivanandappa (2006) with some modifications. Five microgrammes of rat liver were homogenized in 20 ml of Tris–HCl buffer (40 mM, pH 7.0). The liver homogenate (0.1 ml) was incubated with sample (0–225 μg/ml, 0.2 ml), 30 mM KCl (100 μl), 0.16 mM $FeSO_4$ (100 μl) and 0.06 mM Vc (100 μl) at 37 °C for 1 h. TBA reagent (1 ml of 0.67% TBA and 1 ml of 15% TCA) was then added. The final solution was heated at 100 °C in a boiling water bath for 15 min, cooled by ice for 10 min, and then centrifuged at 5000*g* for 10 min. The absorbance of the supernatant was read at 532 nm, using a UV-754 spectrophotometer (Shanghai Jiangyi Instrument Co., Shanghai, China). The blank was performed by substituting Tris–HCl buffer (40 mM, pH 7.0) for sample. The inhibition percentage of the formation of TBA-reactive substances was calculated as $(A_{\text{blank}}\text{-}A_{\text{sample}})/A_{\text{blank}} \times 100$, where A_{blank} and A_{sample} are the absorbance values for the blank and sample, respectively. The plot of inhibition activity was done and IC_{50} value (concentration of sample to inhibit the formation of product of lipid peroxidation by 50%) was calculated.

2.8. Antimicrobial activity

Antimicrobial activities of PTL, using gentamycin as the standard reference, were tested against *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. Antimicrobial activity was determined by using the disc diffusion method (Bauer, Kirby, Sheriss, & Turck, 1966). The antimicrobial screening was performed using Mueller–Hinton agar. PTL were weighed under aseptic conditions in sterile volumetric flasks, and dissolved with 70% sterile ethanol to obtain a concentration of 0.1 mg/ml. Sterile discs (6 mm of diameter) were impregnated with 10 μl of PTL solution. One hundred microlitres (107 cells per ml) of suspension

were spread on the solid media plates and grown in agar media for 24 h, then spread over the surface of solid nutrient agar medium in 9 cm diameter Petri dishes. Filter paper discs (6 mm of diameter), loaded with samples and gentamycin, were placed on the surface of the nutrient agar, incubated at 37 °C for 24 h, and then the diameters of inhibition zones were measured in millimeters. All the determinations were done in triplicate.

2.9. Statistical analysis

All the tests were done in triplicate. Data were expressed as means ± standard errors. Statistical calculations by OriginPro Version 7.5 software (OriginLab Corporation, Northampton, USA) were carried out. One way analysis of variance was applied for determining significant difference at $P < 0.05$.

3. Results and discussion

3.1. Isolation and identification of polyphenols

Based on the absorbance values of the extracts reacted with Folin–Ciocalteu reagent and compared with the standard reference chlorogenic acid, the total yield of PTL was 23.6 ± 2.3 mg CAE/g.

HPLC, with reverse phase column technology, is the analytical technique that has dominated the separation and characterization of phenolic compounds (Robbins, 2003). In the present study, the major components in PTL were identified by RP-HPLC and MS analyses. The chromatogram of PTL was recorded at 345 nm and is shown in Fig. 1. The RP-HPLC profile of PTL indicated that the RP-HPLC system could separate easily the dominant polyphenols. By comparing with the commercial standards, two main peaks at 13.47 and 19.35 min were identified as chlorogenic acid and rutin, respectively. Their UV–visible scanning spectra, from the diode array detector over the range 200–400 nm, were also determined (Fig. 1). Chlorogenic acid had three absorbance peaks at 218.9, 244.1 and 328.7 nm, respectively, while rutin also had three absorbance peaks at 205.1, 257.8 and 356.8 nm, respectively. Two main compounds were further identified by HPLC-MS, as illustrated in Fig. 2. Identification of the tobacco polyphenols by HPLC-MS provided additional supporting evidence for the main polyphenols. As shown in the mass spectra of chlorogenic aicd (Fig. 2), [M-H] m/z 353.05 indicated the molecular weight of chlorogenic acid (Zhu et al., 2004), while m/z 191.10 meant the ion fraction after losing the 3,4-dihydroxycinnamoyl group. [M-H] m/z 609.00 indicated the molecular weight of rutin. The peak at m/z 300.07 was the ion fraction of rutin after losing the rutinoside linkage. The peak at m/z 178.97 confirmed the existence of glucose (Roesler, Catharino, Malta, Eberlin, & Pastore, 2007).

3.2. Antioxidant activity

3.2.1. Hydroxyl radical-scavenging activity

Hydroxyl radical is the most reactive free radical and it can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions, such as copper or iron. Hydroxyl radicals react with lipid, polypeptides, proteins and DNA, especially thiamine and guanosine. The resulting radical can undergo further reactions, such as reacting with oxygen to give peroxylradicals, or decomposing to phenoxyl-type radicals by water elimination (Kitada, Igarashi, Hirose, & Kitagawa, 1979). The scavenging activities of PTL on hydroxyl radicals are shown in Fig. 3. These were dose-dependent. Moreover, when the tested concentration was above 40 μg/ml, PTL showed higher scavenging activity than did vitamin C, which is considered to be a potent hydroxyl radical-scavenger.

3.2.2. Superoxide anion radical-scavenging activity

Superoxide anion is a reduced form of molecular oxygen, by receiving an electron. It is also an initial free radical formed from mitochondrial electron transport systems.

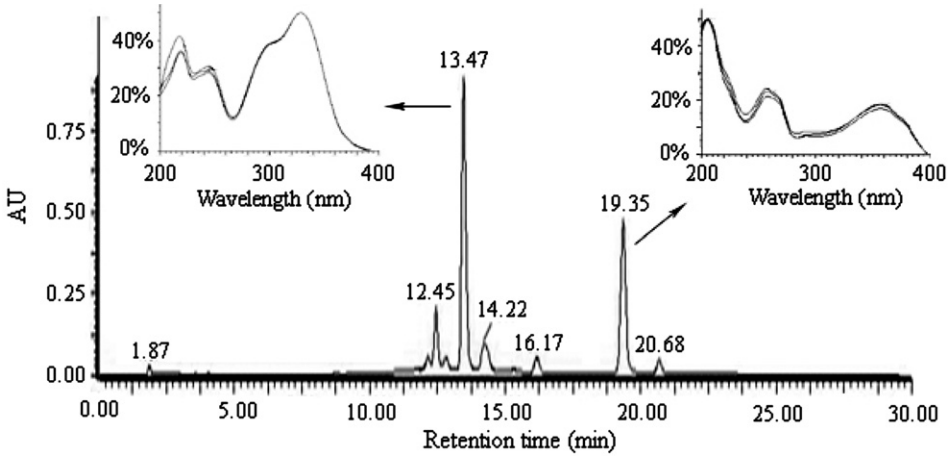

Fig. 1. HPLC chromatogram of PTL and UV–visible scanning spectra of chlorogenic acid and rutin. The peaks at retention times of 13.47 and 19.35 min were chlorogenic acid and rutin, respectively.

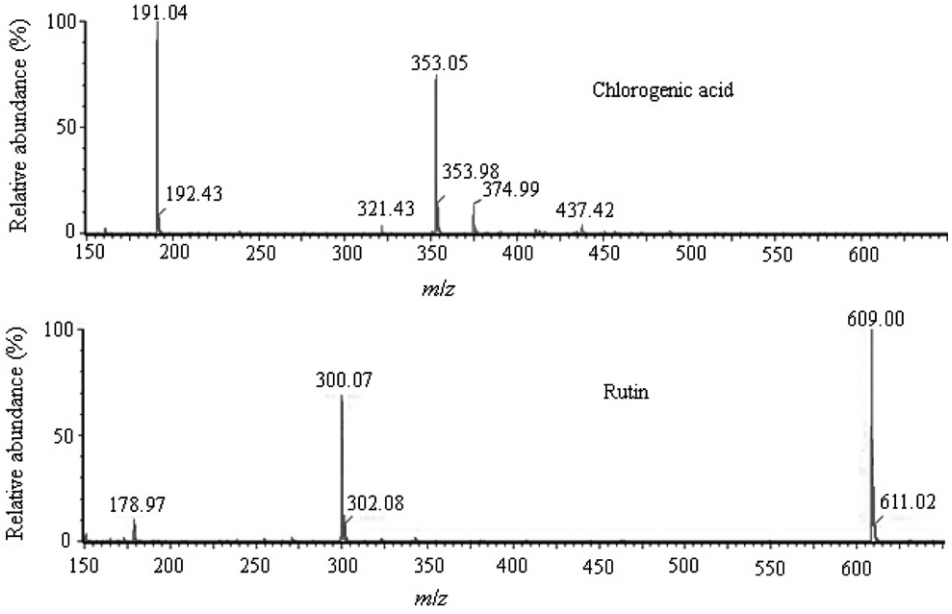

Fig. 2. Mass spectra of chlorogenic acid and rutin.

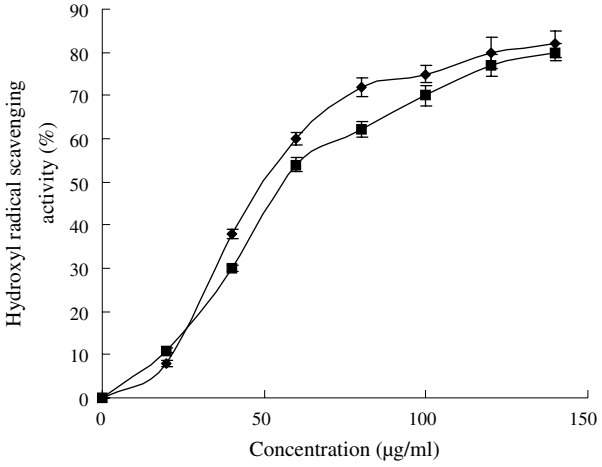

Fig. 3. Scavenging activities of PTL and vitamin C on hydroxyl radicals. ■, vitamin C; ♦, PTL.

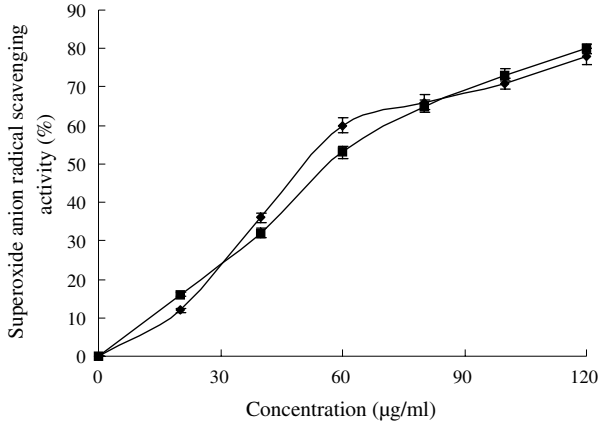

Fig. 4. Scavenging activities of PTL and vitamin C on superoxide anion radicals. ■, vitamin C; ♦, PTL.

Mitochondria generate energy using four electron chain reactions, reducing oxygen to water. Some of the electrons escaping from the chain reaction of mitochondria directly react with oxygen and form superoxide anion. It plays an important role in the formation of other reactive oxygen species, such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems (Kulisic, Radonic, & Katyalinic, 2005). The effects of PTL on superoxide anion radicals were determined and the results are shown in Fig. 4. PTL had a significant scavenging activity on the superoxide anion radicals in a dose-dependent manner. Compared with vitamin C, PTL showed insignificant difference ($P > 0.05$) with regard to superoxide anion radical-scavenging activity.

3.2.3. DPPH radical-scavenging activity

DPPH radical is commonly used as a substrate to evaluate antioxidant activity; it is a stable free radical that can accept an electron or hydrogen radical to become a stable molecule. The reduction of DPPH radical was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Fig. 5 shows the scavenging effect of PTL on DPPH free radicals. PTL exhibited a strong ability to quench DPPH radicals. The scavenging effect increased with increasing concentrations used in the test. The DPPH radical-scavenging activity of PTL at low concentration was significantly higher ($P < 0.05$) than that of vitamin C, a commercial antioxidant used in the food industry. This indicated that PTL was a good antioxidant with strong DPPH radical-scavenging activity.

3.2.4. Reducing power

Reducing power is often used as an indicator of electron-donating activity, which is an important mechanism for testing antioxidative action of phenolics (Yildirim,

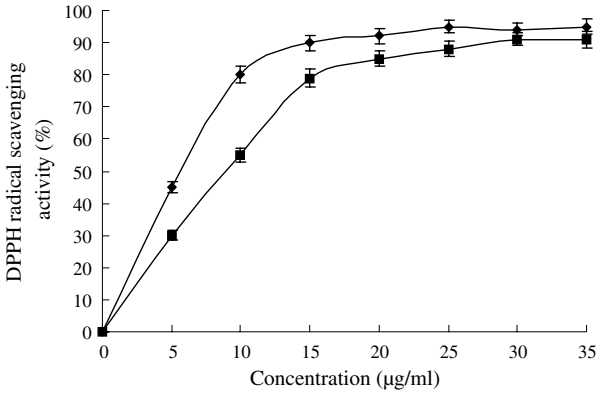

Fig. 5. Scavenging activities of PTL and vitamin C on DPPH radicals. ■, vitamin C; ♦, PTL.

Mavi, & Kara, 2001). The reducing power has also been used as an important test of antioxidant activity of medicinal herbs (Duh & Yen, 1997; Duh, Tu, & Yen, 1999). A good correlation between antioxidant activity and reducing power in some plant extracts has been established (Yen, Chen, & Peng, 2000). Therefore, reducing power may be used as an indicator of potential antioxidant activity. Fig. 6 presents the reducing power of PTL and vitamin C. It shows that PTL had a dose-dependent reducing power. When a relatively high concentration (no less than 25 μg/ml) was used, no significant difference ($P > 0.05$) was observed between the reducing power of PTL and vitamin C.

3.2.5. Inhibition of lipid peroxidation

Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in the cell membranes that generates a number of degradation products. Malonaldehyde, one of the products of lipid peroxidation, has been studied widely as an index of lipid peroxidation and a marker of oxidative stress (Janero, 1990). The lipid peroxidation inhibition activity of PTL was determined and compared with that of vitamin E. As shown in Fig. 7, the lipid peroxidation process was inhibited by the addition of PTL. PTL showed a dose-dependent relationship in inhibiting lipid peroxidation. At high concentration (no less than 125 μg/ml), the lipid peroxidation inhibition activity of PTL was not significantly different ($P > 0.05$) from that of vitamin E.

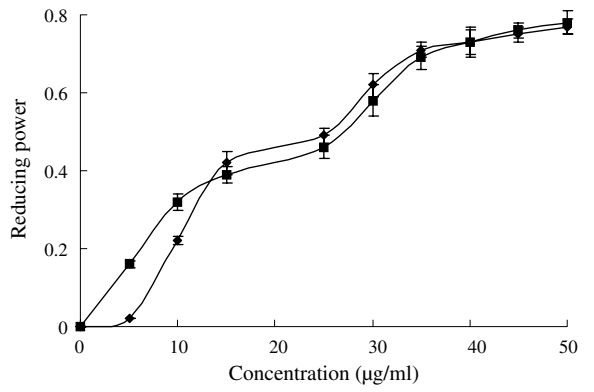

Fig. 6. Reducing powers of PTL and vitamin C. ■, vitamin C; ♦, PTL.

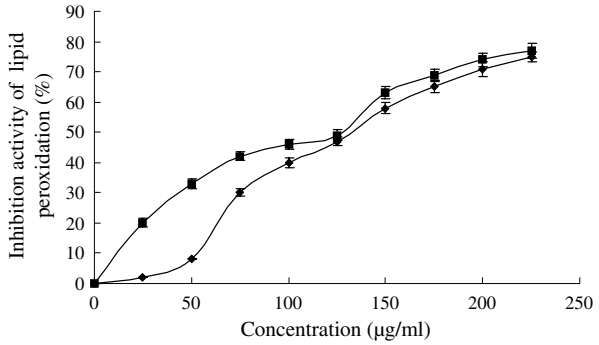

Fig. 7. Inhibition activities of PTL and vitamin E on lipid peroxidation. ■, vitamin C; ♦, PTL.

3.2.6. IC_{50} values for antioxidant activities

Concentration of sample at which the inhibition percentage reaches 50% is the IC_{50} value. IC_{50} value is negatively related to the antioxidant activity, as it expresses the amount of antioxidant needed to decrease the radical concentration by 50%. The lower the IC_{50} value, the higher is the antioxidant activity of the tested sample. The IC_{50} values of PTL for DPPH radical, hydroxyl radical and superoxide anion radical-scavenging activities, as well as lipid peroxidation inhibition activity, are summarized in Table 1. IC_{50} values of PTL for scavenging activities on hydroxyl radicals, superoxide anion radicals and DPPH radicals were 49.6, 44.0 and 5.02 μg/ml which were significantly lower ($P < 0.05$) than those of the control, vitamin C. The IC_{50} value of PTL (132 μg/ml) for lipid peroxidation was slightly higher than that of the control, vitamin E, possibly due to the weaker liposolubility of PTL than vitamin E. According to the results in Table 1, a conclusion could be drawn that PTL possessed strong antioxidant activity.

3.3. Antimicrobial activity

The disc diffusion method was used to determine the antimicrobial activity of PTL. The antimicrobial activity on pathogenic strains of Gram-positive (*S. aureus and B. subtilis*), Gram-negative (*Escherichia col*) bacteria of PTL was evaluated in the present study. Inhibition zones of bacteria by PTL and gentamycin were measured. Gentamycin is a well-known chemical with pronounced antimicrobial potential. According to the results in Table 2, diameters of the inhibition zone of gentamycin were 21.67 ± 0.91 mm for *E. coli*, 14.18 ± 0.75 mm for *B. subtilis* and 16.21 ± 0.48 mm for *S. aureus*. Gentamycin was used as a positive control because it has been commonly employed as the antibiotic for Gram-positive and Gram-negative bacteria. PTL showed good antimicrobial activity on *E. coli* (20.23 ± 0.42 mm), *B. subtilis* (12.89 ± 0.29 mm)

Table 1
Comparison of IC_{50} value of PTL and standard antioxidants

Samples	Hydroxyl radical-scavenging activity (μg/ml)	Superoxide anion radical-scavenging activity (μg/ml)	DPPH radical-scavenging activity (μg/ml)	Lipid peroxidation (μg/ml)
PTL	49.6	44.0	5.02	132
Vitamin C	53.3	46.1	9.38	
Vitamin E				130

Table 2
Comparison of antimicrobial activities of PTL and gentamycin*

Samples	Diameter of inbibition zone (mm)		
	Escherichia coli	*Bacillus subtilis*	*Staphylococcus aureus*
PTL	20.23 ± 0.42	12.89 ± 0.29	17.66 ± 0.86
Gentamycin	21.67 ± 0.91	14.18 ± 0.75	16.21 ± 0.48

and *S. aureus* (17.66 ± 0.86 mm). For *E. coli* and *S. aureus*, the inhibition zones of PTL were found to be not significantly different ($P > 0.05$) from those of Gentamycin. This result indicated that PTL was a good antimicrobial agent with strong antimicrobial activity.

4. Conclusions

The main polyphenols in PTL were identified as chlorogenic acid and rutin by analyses of RP-HPLC and MS. Strong scavenging activities on hydroxyl radicals, superoxide anion radicals and DPPH radicals were found for PTL. The analyses of lipid peroxidation inhibition activity and reducing power also indicated that PTL possessed good antioxidant activity. Meanwhile, the proliferation inhibition activity of PTL on *E. coli*, *S. aureus* and *B. subtilis* were investigated in this study. The results confirmed that it had good antimicrobial activity. From the above results, it appears important to develop natural antioxidants and bacterial inhibitors from tobacco leaf, and this may be a good way for extensively utilizing the tobacco resource.

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