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Antioxidant, free radical scavenging, anti-inflammatory and hepatoprotective potential of the extract from *Parathelypteris nipponica* (Franch. et Sav.) Ching

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ABSTRACT

Aim of the study: The present study was conducted to evaluate the antioxidant, free radical scavenging, hepatoprotective and anti-inflammatory potential of *Parathelypteris nipponica* (Franch. et Sav.) Ching.
Methods and results: Antioxidant activity of the methanolic extract of *Parathelypteris nipponica* (Franch. et Sav.) Ching (TMPN) was studied using in vitro and in vivo models, total flavonoids content in TMPN was found to be 262 ± 5.6 mg/g (w/w). The TMPN exhibited strong antioxidant activity with EC_{50} values in reductive ability (0.18 ± 0.02 mg/ml) and ferric thiocyanate (FTC) assay (0.10 ± 0.01 mg/ml), strong free radical scavenging activity as evidenced by the low EC_{50} values in DPPH (1,1-diphenyl-2-picrylhydrazyl) (2.00 ± 0.02 mg/ml), superoxide anion (0.60 ± 0.05 mg/ml), OH radicals (0.26 ± 0.03 mg/ml), and hydrogen peroxide (0.45 ± 0.03 mg/ml) methods. Acute toxicity study revealed that the LD_{50} value of the extract was more than the dose 2000 mg/kg bodyweight of mice. Hepatoprotective activity of TMPN was determined by the carbon tetrachloride (CCl_4)-induced oxidative tissue injury in rat liver, the extract showed significant hepatoprotective activity that was evident by enzymatic examination and histopathological study. In assessing anti-inflammatory activity the carrageenan-induced rat paw oedema test was used, the extract reduced carrageenan-induced rat paw oedema in dose-dependent manner, achieving high degree of anti-inflammatory activity.
Conclusion: This study provides a scientific basis for the ethnomedical claims that *Parathelypteris nipponica* (Franch. et Sav.) Ching is effective against inflammation and liver injury.

1. Introduction

Parathelypteris nipponica (Franch. et Sav.) Ching is a member of the family Dryopteridaceae which distributed in the southern provinces of China. Its rhizomes have been used in folk medicine to treat inflammation, burn scald and acute icterus hepatitis (Editorial Board of China Herba, 1998). In spite of the very wide-spread use of *Parathelypteris nipponica* (Franch. et Sav.) Ching, few studies were carried out to support its ethnopharmacological use. In our previous work, the chemical constituents of this species were investigated and several flavonoids were isolated (Fu et al., 2010).

It is commonly accepted that in a situation of oxidative stress, reactive oxygen species (ROS) such as superoxide, hydroxyl and peroxyl radicals are generated. The ROS play an important role in the pathogenesis of various serious diseases, such as neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation (Aruoma, 1998; Kris-Etherton et al., 2004). Moreover, several studies suggest that antioxidant and anti-inflammatory agents could be beneficial in the prevention and treatment of these pathologies (Bauerova and Bezek, 1999; Rahman, 2002; Horton, 2003).

Likewise in liver injury, free radicals and lipid peroxidative metabolites also cause damages to hepatocytes leading to severe necrosis, sepsis or endotoxemia (Kono et al., 2003). Carbon tetrachloride (CCl_4) is a widely used hepatotoxin in rodents and its trichloromethyl radical (CCl_3)-induced toxicity in rat liver closely resembles to human cirrhosis and hence is an acceptable animal model for analyzing hepatoprotective agents.

The use of traditional medicine is wide-spread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several anti-inflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and radical scavenging mechanism as part of their

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activity (Perry et al., 1999; Lin and Huang, 2002; Repetto and Llesuy, 2002).

In the present study, we investigated the methanolic extract of *Parathelypteris nipponica* (Franch. et Sav.) Ching (TMPN) for its antioxidant, free radical scavenging, hepatoprotective and anti-inflammatory activities.

2. Methods and materials

2.1. Chemicals

Nitroblue tetrazolium (NBT), β-nicotinamide adenine dinucleotide reduced (β-NADH), horse radish peroxidase, phenol red, 2-deoxy-D-ribose, linoleic acid, ammonium thiocyanate, trolox, rutin, phenazine methosulphate (PMS), carrageenin, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), ethylenediamine tetraacetic acid (EDTA) were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Silymarin tablets was purchased from QianYuan Pharmaceutical Company Limited, China. Indometacin tablets was purchased from LinFen QiLin Pharmaceutical Company Limited, China.

2.2. Plant material

The whole plant of *Parathelypteris nipponica* (Franch. et Sav.) Ching were collected from Jiujiang city, Jiangxi province, China and authenticated by Prof. Ceming Tan, Jiujiang Forest Plants Specimen Mansion. A specimen (NO. JX060701) has been deposited in College of Pharmacy, Tongji Medical Center, Huazhong University of Science and Technology.

2.3. Preparation of extract

The air-dried and powdered whole plant of *Parathelypteris nipponica* (Franch. et Sav.) Ching (520 g) were extracted with 80% methanol at 65 °C for 3 h. The extract was concentrated to dryness under reduced pressure in a rotary evaporator to yield dried crude total extract (TMPN, 77 g).

For in vitro experiments, a weighed quantity of the TMPN was dissolved in distilled water and used, solutions of Trolox used as standard for these studies was prepared in distilled water. All these solutions were serially diluted with distilled water to get lower dilutions. For in vivo experiments the suspensions of TMPN was prepared in Na-CMC (0.3%, w/v) using distilled water.

2.4. Determination of total flavonoids content

Total flavonoids in the extract was estimated as rutin equivalent (Sakanaka et al., 2005). Briefly, 3.2 mg of the extracts were dissolved in 5 ml of 50% methanol, followed by addition of 1 ml of a 5% (w/v) sodium nitrite solution. After 6 min, 1 ml of a 10% (w/v) aluminium chloride solution was added and the mixture was allowed to stand for a further 5 min before 10 ml of a 10% (w/v) NaOH solution was added. The mixture was made up to 50 ml with distilled water and mixed well. The absorbance was measured at 450 nm with a spectrometer after 15 min. Using rutin, a standard curve was prepared. The linearity obtained was in the range of 5–55 μg/ml. On the basis of the standard curve, the total flavoniods content was calculated.

2.5. Antioxidant activity and radical scavenging activity

2.5.1. Reductive ability

The reducing power of TMPN was evaluated according to the method described by (Oyaizu, 1986). One milliliter of the plant extract (0.02–0.64 mg/ml) was mixed with 1 ml of 0.2 M phosphate buffer (pH 6.6) and 1.5 ml of 1% potassium ferricyanide, and then incubated at 50 °C for 20 min. 1 ml of 10% trichloroacetic acid was added to the mixture to stop the reaction, and then the mixture was centrifuged for 10 min at 3000 rpm. The supernatant (2.5 ml) was mixed with 2 ml distilled water and 0.5 ml of freshly prepared 0.1% $FeCl_3$ and then the absorbance was measured at 700 nm. Trolox was used as the standard. The reducing powers of the tested samples increased with the absorbance values. All tests were done in triplicate and averaged. EC_{50} value is the effective concentration at which the absorbance was 0.5.

2.5.2. Lipid peroxidation inhibition activity (ferric thiocyanate method)

This assay previously (Chang et al., 2002) was used to determine the amount of peroxide. Linoleic acid emulsion (0.02 M) was prepared with linoleic acid (0.28 g) and Tween 20 (0.28 g) in phosphate buffer (50 ml, 0.05 M, pH 7.4). A reaction solution containing the extract (0.1 ml, 0.02–5.00 mg/ml), linoleic acid emulsion (1.25 ml), and phosphate buffer (1.15 ml, 0.2 M, pH 7.0) was incubated at 40 °C in the dark, and the degree of oxidation was measured according to the thiocyanate method. To 0.1 ml of reaction mixture, 4.7 ml of 75% ethanol and 0.1 ml 30% ammonium thiocyanate were added. Exactly 3 min after the addition of 0.1 ml of 0.02 M $FeCl_2$ in 3.5% HCl to the reaction mixture, the absorbance was measured at 500 nm for every 24 h until the absorbance of the control reached maximum. The positive and negative controls were subjected to the same procedures as the sample, except that for the negative control, only the solvent was added, and for the positive control sample was replaced with Trolox. All measurements were made in triplicate and averaged. The inhibition percent of linoleic acid peroxidation was calculated as

$$\text{Inhibition } (\%) = \left(1 - \frac{\text{Absorbance of sample at } 500\,\text{nm}}{\text{Absorbance of control at } 500\,\text{nm}}\right) \times 100$$

EC_{50} value is the concentration of the sample required to inhibit 50% linoleic acid peroxidation.

2.5.3. DPPH radical scavenging activity

The antioxidant activity of the plant extract and the standard were assessed on the basis of the radical scavenging effect of the stable DPPH free radical (Blois, 1958). About 0.1 ml of the extract or standard (0.02–5.00 mg/ml) was added to 3.9 ml of DPPH in methanol solution (0.1 mM) in a test tube. After incubation at 37 °C for 30 min, the absorbance of each solution was determined at 517 nm using spectrophotometer. Ascorbic acid was used as the standard. All measurements were made in triplicate and averaged. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH radical scavenging activitye } (\%) = \left(1 - \frac{\text{Absorbance of sample at } 517\,\text{nm}}{\text{Absorbance of control at } 517\,\text{nm}}\right) \times 100$$

EC_{50} value is the concentration of the sample required to scavenge 50% DPPH free radical.

2.5.4. Superoxide anion radical scavenging activity

The effect of scavenging superoxide radical was determined by the nitroblue tetrazolium reduction method (Nishikimi et al., 1972). 1 ml of nitroblue tetrazolium (NBT) solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (468 μM NADH in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of the samples (0.02–5.00 mg/ml) were mixed. The reaction was started by adding 100 μl of phenazine methosulphate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured against blank samples, containing all the reagents except the PMS. The positive control (the sample

was replaced with Trolox) and the negative control (only the solvent was added) were subjected to the same procedures described above as the sample. All measurements were made in triplicate and averaged. The abilities to scavenge the superoxide radical were calculated using the following equation:

$$\text{Superoxide radical scavenging activity (\%)} = \left(1 - \frac{\text{Absorbance of sample at 560 nm}}{\text{Absorbance of control at 560 nm}}\right) \times 100$$

EC_{50} value (mg/ml) is the concentration at which the scavenging activity was 50%.

2.5.5. Hydroxyl radical scavenging activity

The effect of extract on hydroxyl radical was assessed by using the deoxyribose method (Aruoma and Halliwell, 1987). The reaction mixture contained 450 μl of 0.2 M sodium phosphate buffer (pH 7.0), 150 μl of 10 mM 2-deoxyribose, 150 μl of 10 mM $FeSO_4$–EDTA, 150 μl of 10 mM H_2O_2, 500 μl of H_2O, and 100 μl of sample solution (0.02–5.00 mg/ml). The reaction was started by the addition of H_2O_2. After incubation at 37 °C for 4 h, the reaction was stopped by adding 750 μl of 2.8% trichloroacetic acid and 750 μl of 1% thiobarbituric acid in 50 mM NaOH, the solution was boiled for 10 min, and then cooled in ice water. The absorbance of the solution was measured at 520 nm. Trolox was used as positive control. All measurements were made in triplicate and averaged. The ability to scavenge the hydroxyl radical was calculated using the following equation:

$$\text{Hydroxyl radical scavenging activity (\%)} = \left(1 - \frac{\text{Absorbance of sample at 520 nm}}{\text{Absorbance of control at 520 nm}}\right) \times 100$$

EC_{50} value (mg/ml) is the concentration at which the scavenging activity was 50%.

2.5.6. Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was measured using the modified method of peroxidase-dependent oxidation of phenol red (Ruch et al., 1989). Briefly, 0.1 ml of sample was first mixed with 400 μl of 4 mM H_2O_2 solution, and then the mixture was diluted to 1.5 ml with phosphate buffer (0.1 M, pH 7.4). After incubation for 20 min at 37 °C, and then 1 ml of horse radish peroxidase (HRPase)–phenol red solution (HRPase 24 μg/ml and phenol red 0.2 mg/ml in 100 mM, pH 7.4 phosphate buffer) were added. After another 10 min of incubation, 50 μl of 1 M NaOH was added to stop the reaction, the absorbance was measured at 610 nm. Two controls were used for this test, a negative control (containing all reagents except the test sample) and a positive control (Trolox). All measurements were made in triplicate and averaged. The scavenging effect was then calculated according to the following equation:

$$\text{Scavenging activity (\%)} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

EC_{50} value (mg/ml) is the concentration at which the scavenging activity was 50%.

2.6. Pharmacological procedures

2.6.1. Animals

Adult male and female Sprague–Dawley rats (180–220 g) and Swiss Albino mice (18–22 g) were purchased from Animal Center of Tongji Medical Center, Huazhong University of Science and Technology, Wuhan. Each animal was used only for one experiment. They were housed in acrylfiber cages (48 cm × 35 cm × 22 cm) at a controlled room (temperature 22 ± 3 °C and humidity 50 ± 10%) and were kept on a 12 h light:12 h dark cycle. They were fed with standard diet and water *ad libitum* and acclimated 7 days before they were used. In the case of oral administration, rats were fasted for 12 h before testing. All experiments were conducted in accordance with the European Community guidelines for the use of experimental animals and approved by the Huazhong University of Science and Technology Committee on Animal Care and Use.

2.6.2. Acute toxicity study

Swiss albino mice were divided in to test and control groups comprising of five animals in each group. The test was performed using increasing oral doses of TMPN in 0.3% Na-CMC (500, 1000, 1500 and 2000 mg/kg body weight), in 10 ml/kg volume to different test groups. Normal group received 0.3% Na-CMC (10 ml/kg). The mice were allowed for food and water *ad libitum*, kept observation for 24 h, for any mortality or behavioral changes (Rai et al., 2006).

2.6.3. CCl_4-induced hepatotoxicity in rats

The hepatoprotective effect was induced by CCl_4 according to methods described previously (Suzuki et al., 1990; Yoshitake et al., 1991).

Rats were divided into six groups ($n = 10$). Group I (normal control) animals were administered a single dose of water (25 ml/kg, p.o.) daily for 7 days and received olive oil (8 ml/kg, i.p.) on day 7. Group II (CCl_4 control) received water (25 ml/kg, p.o.) once daily for 7 days and received 0.2% CCl_4 in olive oil (8 ml/kg, i.p.) on day 7. Group III received standard drug silymarin (100 mg/kg, p.o.) once daily for 7 days. Groups IV–VI were administered orally a dose of 400, 800, 1600 mg/kg of the extract once daily for 7 days, respectively. The Groups III–VI animals were administered simultaneously 0.2% CCl_4 in olive oil (8 ml/kg, i.p.) on day 7 after 1 h of administration of the silymarin and the extract.

Animals were sacrificed 24 h after the treatment. Blood was collected, allowed to clot and serum was separated at 3500 rpm for 15 min and used for assessment of different enzyme activities. Liver tissue samples were taken from the left liver lobe, and cut into two pieces. One piece was fixed in formalin for pathological examination, the other piece was utilized for the following biological analyses.

2.6.4. Assessment of hepatoprotective activity

Hepatic enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were used as the biochemical markers of the hepatic damage and were assayed by the method of (Reitman and Frankel, 1957). Estimation of serum alkaline phosphatase (ALP) (King, 1965) and serum bilirubin (Malloy and Evelyn, 1937) were also carried out to assess the acute hepatic damage caused by CCl_4.

2.6.5. Assessment of antioxidant activity

The liver was perfused with 0.9% cold physiological saline to completely remove all the red blood cells. Then it was suspended in 10% (w/v) ice-cold 0.1 M phosphate buffer (pH 7.4) cut into small pieces, and the required quantity was weighed and homogenized using a homogenizer. The homogenate was centrifuged at 3500 rpm for 20 min, the supernatant was used for the estimation of superoxide dismutase (SOD), malondialdehyde (MDA) catalase (CAT) and glutathione peroxidase (GPx) (Beers and Sizer, 1952; Mc Cord and Fridovich, 1969; Hafemann et al., 1974). Protein content in the tissue was determined (Bradford, 1976) using bovine serum albumin as the standard.

2.6.6. Histopathological studies

The liver tissue was dissected out and fixed in 10% formalin for at least 24 h, Sections were prepared and then stained with hematoxylin and eosin (H–E) dye for photomicroscopic observation, including cell necrosis, fatty change, hyaline regeneration, ballooning degeneration.

Table 1
EC_{50} values of TMPN for reducing power, lipid peroxide, DPPH, superoxide anion, hydroxyl radical and hydrogen peroxide, trolox as reference.

Antioxidant activity	TMPN (mg/ml)	Trolox (mg/ml)
Reducing power	0.18 ± 0.02	0.21 ± 0.03
Lipid peroxide	0.10 ± 0.01*	0.14 ± 0.01
DPPH	2.00 ± 0.02	2.27 ± 0.04
Superoxide anion	0.60 ± 0.05	0.73 ± 0.08
Hydroxyl radical	0.26 ± 0.03*	0.18 ± 0.02
Hydrogen peroxide	0.45 ± 0.03	0.53 ± 0.05

Values are means ± S.D. ($n = 3$).
* $p < 0.05$ compared to the reference.

2.6.7. Anti-inflammatory activity

Paw edema was induced (Winter et al., 1962) by injecting 0.1 ml of 1% carrageenan in physiological saline into the subplantar tissues of the right hind paw of each rat. The extract (400, 800 and 1600 mg/kg) were administered orally 60 min prior to carrageenan administration. The paw edema was measured (cm) before and then at 1, 2, 4, 6 h, after carrageenan injection. Mean values of treated groups were compared with mean values of a control group and analyzed using statistical methods. Indomethacin (10 mg/kg) was used as the reference drug (Tunalier et al., 2007).

2.7. Statistical analysis

All values were expressed as means ± S.E.M. Results were analyzed statistically by one-way ANOVA followed by Tukey's multiple comparison using SPSS software (Student's version). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Total flavonoids content

The content of total flavonoids in TMPN was expressed as rutin equivalent in mg/g of extracts. The extract was found to contain 262 ± 5.6 mg/g total flavonoids. Since flavonoids are responsible for the antioxidant activity, the obtained amount of total flavonoids in the extract indicated the extract to possess a high antioxidant activity.

3.2. Reductive ability

Fig. 1A showed the dose–response curves for the reducing powers of the extract from *Parathelypteris nipponica* (Franch. et Sav.) Ching. The reducing power of the extract was compared with that of standard, Trolox. The increase in absorbance of the reaction mixture containing the extract showed increased reducing power with increase in concentration. The EC_{50} value for the extract was found to be 0.18 ± 0.02 mg/ml where as EC_{50} value for Trolox is 0.21 ± 0.03 mg/ml (Table 1).

3.3. Lipid peroxidation inhibition activity

When different concentrations of the extract (0.02–2.50 mg/ml) were added, a significant concentration-dependent inhibition of lipid peroxidation was observed. The autooxidation of linoleic acid in the negative control group increased rapidly at day 1, and reached maximum levels on day 4. The effects of different concentrations (0.02–1.25 mg/ml) of TMPN on lipid peroxidation of linoleic acid emulsion were showed in Fig. 1B, which indicated that TMPN showed good antioxidant activity. The EC_{50} values of TMPN and Trolox were 0.10 ± 0.01, 0.14 ± 0.01 mg/ml (Table 1). The EC_{50} of TMPN was significantly higher ($p < 0.05$) than Trolox.

3.4. DPPH radical scavenging activity

The radical scavenging activities of the extract were estimated by comparing the percentage inhibition of formation of DPPH radical by the extract with those of Trolox (Fig. 1C). The EC_{50} values of TMPN and Trolox were 2.00 ± 0.02, 2.27 ± 0.04 mg/ml (Table 1). The results indicated that TMPN has the same potent as Trolox.

3.5. Superoxide anion radical scavenging activity

As seen in Fig. 1D, superoxide anion radical scavenging activities of TMPN at the concentration of 2.50 mg/ml were 69.3 ± 4.36%. At the same concentration, Trolox exhibited 66.0 ± 3.25%. On the other hand, EC_{50} values of TMPN and Trolox were 0.60 ± 0.05 and 0.73 ± 0.08 mg/ml (Table 1). The results indicated that TMPN has the same potent as Trolox.

3.6. Hydroxyl radical scavenging activity

The scavenging abilities of TMPN on hydroxyl radical inhibition by the 2-deoxyribose oxidation method were showed in Fig. 1E. The EC_{50} values of TMPN and Trolox were 0.26 ± 0.03 and 0.18 ± 0.02 mg/ml (Table 1). The EC_{50} of TMPN was significantly higher ($p < 0.05$) than Trolox.

3.7. Hydrogen peroxide scavenging activity

As shown in Fig. 1F, hydrogen peroxide scavenging activity of TMPN and Trolox were all in concentration-dependent manner, and all of them manifested very strong ability for hydrogen peroxide. EC_{50} for TMPN and Trolox were 0.45 ± 0.03, 0.53 ± 0.05 mg/ml, respectively (Table 1). The results indicated that TMPN has the same potent as Trolox.

3.8. Acute toxicity study

Acute toxicity of the methanolic extract of *Parathelypteris nipponica* (Franch. et Sav.) Ching (TMPN) was evaluated in mice up to the dose of 2000 mg/kg body weight p.o. for 24 h. There were no changes in normal behaviour pattern and no signs and symptoms of toxicity and mortality were observed.

3.9. CCl_4-induced hepatotoxicity

3.9.1. Effects of extracts on AST, ALT, ALP and total bilirubin

The results of hepatoprotective effect of extracts on CCl_4-intoxicated rats were showed in Table 2. In the CCl_4-intoxicated group (II) serum AST, ALT, ALP and total bilirubin were increased to 163.40, 240.54 U/l, 111.65 IU/l and 2.11 mg/dl, respectively, whereas these values were showed 54.87, 95.25 U/l, 22.13 IU/l and 0.88 mg/dl in control group (I), respectively. The elevated levels of serum AST, ALT, ALP, and total bilirubin were significantly reduced in the animals groups treated with various concentration extract.

3.9.2. Effects of extracts on MDA, SOD, CAT, GPX levels

Results were cited in Table 3. Results of study clearly revealed increase in the levels of MDA in CCl_4-intoxicated rats compare to control group. Treatment with TMPN significantly prevented this raise in levels. GPX, SOD and CAT content have significantly increased in extract treated groups whereas CCl_4-intoxicated group has shown significant decrease in levels compare to control group. The TMPN (1600 mg/kg) treated group was superior to the others, and as effective as the silymarin.

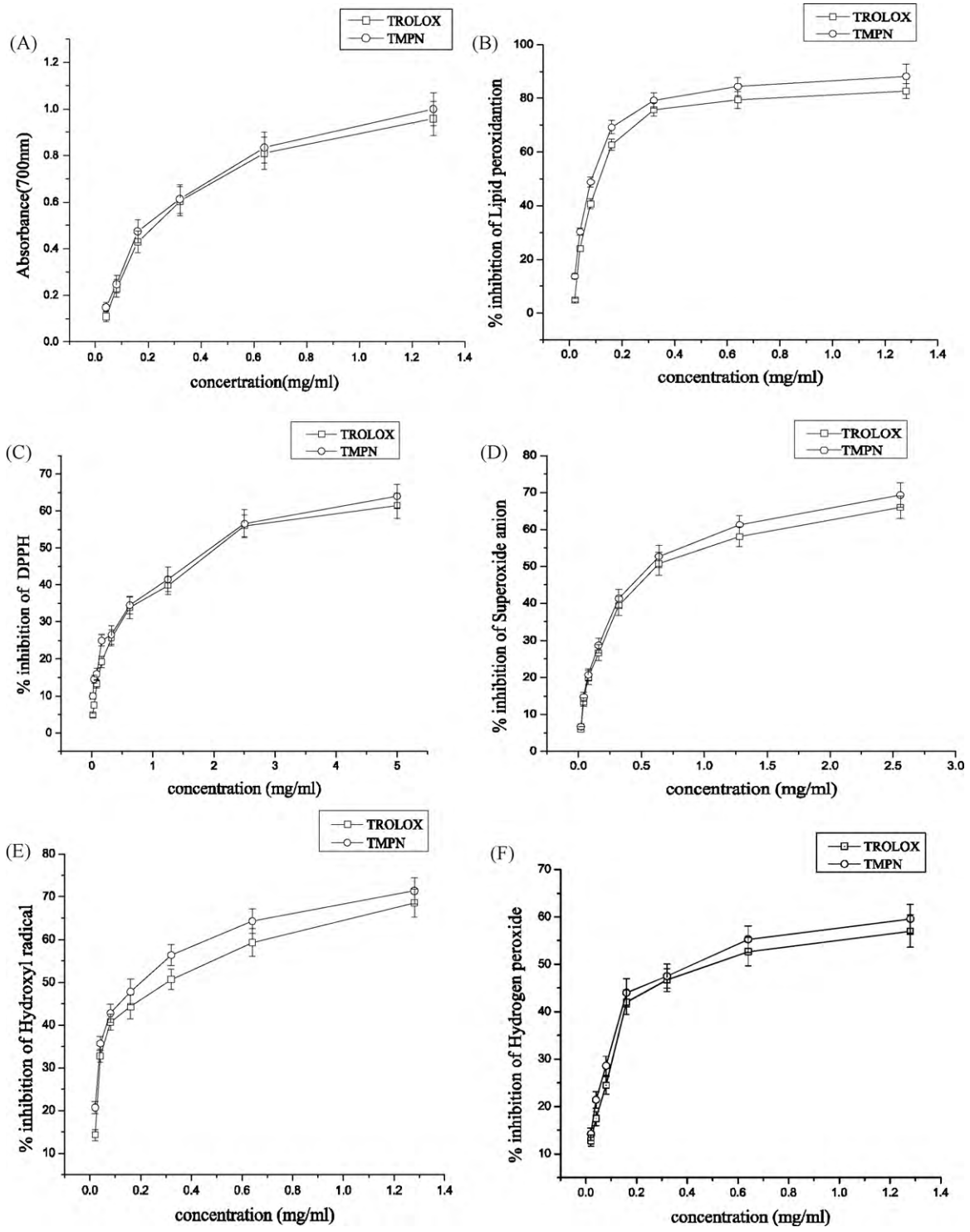

Fig. 1. In vitro antioxidant activities of TMPN, trolox was used as reference. (A) Reducing power. (B) Lipid peroxide inhibition activity. (C) DPPH radical scavenging activity. (D) Superoxide anion radical scavenging activity. (E) Hydroxyl radical scavenging activity. (F) Hydrogen peroxide scavenging activity. Values are means ± S.E.M. ($n = 3$).

3.9.3. Histopathological observations

Histology of the liver sections of control animals (Group I) showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus, nucleolus and visible central veins. The liver sections of CCl_4-intoxicated rats showed massive fatty changes, necrosis, ballooning degeneration and broad infiltration of the lymphocytes and the loss of cellular boundaries. The histological architecture of liver sections of the rats treated with ethanolic extracts showed more or less normal lobular pattern with a mild degree of fatty change, necrosis and lymphocyte infiltration almost comparable to the control and silymarin treated groups (Fig. 2).

3.10. Anti-inflammatory activity

Results on the topical anti-inflammatory activity of the plant extract were reported in Table 4. The rat's footpad became edematous soon after injection of carrageenan. Edema value of the injected footpad reached its peak at 4 h (the perimeter of paw

Table 2
Protective effect of TMPN and silymarin on carbon tetrachloride (CCl_4) induced elevation in aspartate aminotransferase (AST) and alanine aminotransferase (ALT), alkaline phosphatase (ALP) and total bilirubin levels.

Group	Treatment	AST (U/l)	ALT (U/l)	ALP (IU/l)	Total bilirubin (mg/dl)
I	Control	54.87 ± 1.91	95.25 ± 1.97	22.13 ± 1.14	0.88 ± 0.03
II	CCl_4 (10 ml/kg, i.p.)	163.40 ± 2.92[a]	240.54 ± 3.22[a]	111.65 ± 5.87[a]	2.11 ± 0.12[a]
III	CCl_4 + silymarin (100 mg/kg, p.o.)	66.56 ± 1.52[b]	108.73 ± 1.65[b]	41.56 ± 2.55[b]	1.23 ± 0.08[b]
IV	CCl_4 + TMPN (400 mg/kg, p.o.)	102.31 ± 1.83[b]	180.97 ± 1.84[b]	89.66 ± 3.21[b]	1.95 ± 0.06[b]
V	CCl_4 + TMPN (800 mg/kg, p.o.)	78.14 ± 2.55[b]	125.42 ± 1.93[b]	67.42 ± 2.74[b]	1.57 ± 0.07[b]
VI	CCl_4 + TMPN (1600 mg/kg, p.o.)	63.32 ± 1.94[b]	106.81 ± 1.72[b]	52.39 ± 1.68[b]	1.36 ± 0.07[b]

Values are the mean ± S.E.M. of six rats.
[a] Significance level: $p < 0.05$, compared to normal group.
[b] Significance level: $p < 0.05$, compared to CCl_4 group.

Table 3
Effect of TMPN and silymarin on rat liver malondidehyde(MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) in Carbon tetrachloride (CCl_4) induced hepatotoxicity in rats.

Group	Treatment	MDA (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	GPX (U/mg protein)
I	Control	2.41 ± 0.21	114.47 ± 6.24	296.82 ± 7.33	0.88 ± 0.06
II	CCl_4 (10 ml/kg, i.p.)	6.92 ± 0.34[a]	62.52 ± 3.45[a]	208.45 ± 3.45[a]	0.45 ± 0.02[a]
III	CCl_4 + silymarin (100 mg/kg, p.o.)	3.14 ± 0.52[b]	103.23 ± 5.25[b]	277.31 ± 2.14[b]	0.79 ± 0.03[b]
IV	CCl_4 + TMPN (400 mg/kg, p.o.)	5.86 ± 0.32[b]	74.86 ± 4.12[b]	233.28 ± 4.55[b]	0.54 ± 0.04[b]
V	CCl_4 + TMPN (800 mg/kg, p.o.)	4.68 ± 0.42[b]	85.12 ± 3.27[b]	251.51 ± 5.26[b]	0.63 ± 0.04[b]
VI	CCl_4 + TMPN (1600 mg/kg, p.o.)	3.52 ± 0.24[b]	92.65 ± 5.44[b]	268.83 ± 6.33[b]	0.77 ± 0.05[b]

Values are the mean ± S.E.M. of six rats.
[a] Significance level: $p < 0.05$, compared to normal group.
[b] Significance level: $p < 0.05$, compared to CCl_4 group.

increased by 0.74 cm). The highest and most significant anti-inflammatory activity of the extract was already obvious at 2 h after induction of inflammation, the most active was TMPN (1600 mg/kg), which induced 48.5% oedema inhibition. Those two lower doses induced oedema inhibitions between 47.1 and 38.2%. As reference, the non-steroidal anti-inflammatory drug indomethacin reduced the oedematous response by 69.1% at the dose of 10 mg/kg.

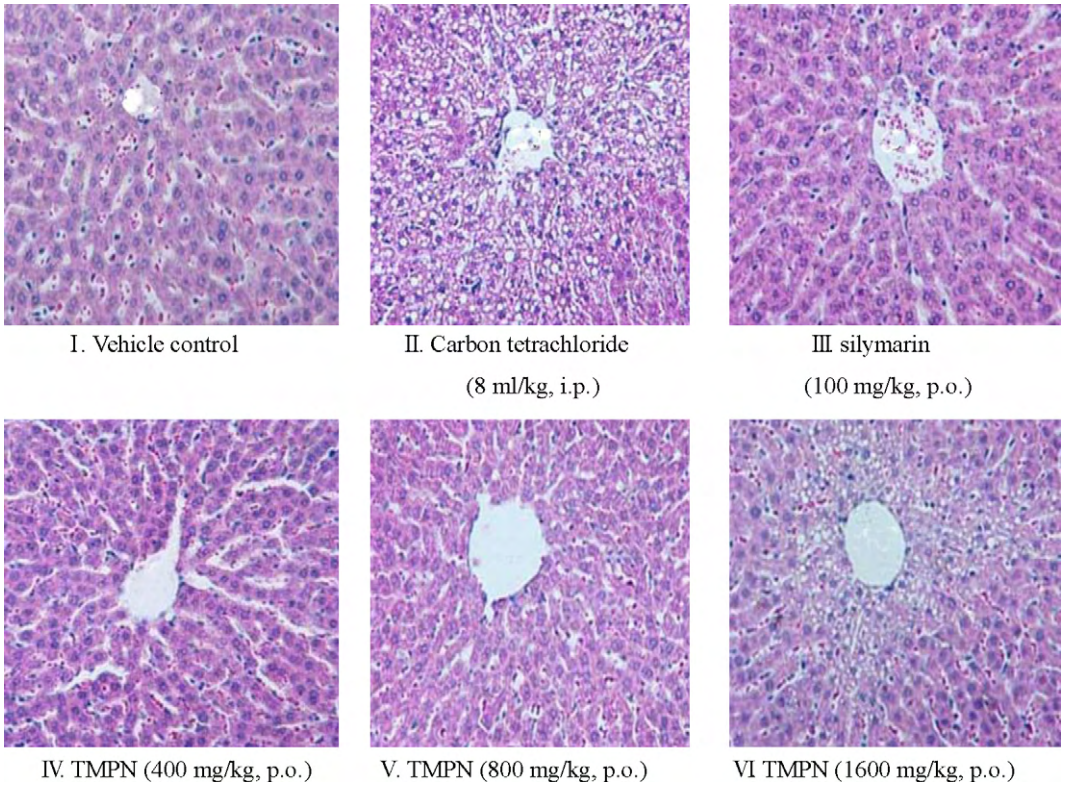

Fig. 2. Histopathology of liver tissues. (I) Section shows normal liver architecture. (II) Section shows ballooning degeneration, massive fatty changes, patches of liver cell necrosis with inflammatory collections. (III) Almost near normal. (IV) Hepatocytes with preserved cytoplasm and prominent nucleus. (V) Hepatic cell with well-preserved cytoplasm, prominent nucleus and nucleolus. (VI) Minimal inflammatory lymphocyte infiltration.

Table 4
Effects of TMPN against carrageenan-induced paw edema in rats.

Group	Dose (mg/kg, p.o.)	Edema value (cm) and inhibition rate (%)			
		1 h	2 h	4 h	6 h
Control (saline, 10 ml/kg, p.o.)	–	0.52 ± 0.12	0.68 ± 0.16	0.74 ± 0.15	0.72 ± 0.17
TMPN	400	0.38 ± 0.16* (26.9)	0.42 ± 0.16* (38.2)	0.46 ± 0.18* (37.8)	0.45 ± 0.13* (37.5)
TMPN	800	0.34 ± 0.14* (34.6)	0.36 ± 0.14** (47.1)	0.41 ± 0.17** (44.6)	0.40 ± 0.18** (44.4)
TMPN	1600	0.31 ± 0.12** (40.3)	0.35 ± 0.18** (48.5)	0.40 ± 0.15** (45.9)	0.39 ± 0.14** (45.8)
Indomethacin	10	0.18 ± 0.13** (65.4)	0.21 ± 0.13** (69.1)	0.24 ± 0.10** (67.5)	0.25 ± 0.15** (65.3)

Values represent the mean ± S.E.M. of 10 animals for each group. Values in parentheses indicate the percentage inhibition rate.
* Significant from control: $p < 0.05$.
** Significant from control: $p < 0.01$.

4. Discussion and conclusions

The present study reports for the first time the antioxidant, free radical scavenging, anti-inflammatory, anti-hepatotoxicity activities of the methanolic extract of *Parathelypteris nipponica* (Franch. et Sav.) Ching that was used in folk medicine to treat inflammation, burn scald and acute icterus hepatitis.

Firstly, the antioxidant activity of TMPN and the possible mechanisms had been investigated by assessing their roles on reducing power, lipid peroxidation inhibition, DPPH radical scavenging activity, superoxide anion removal, hydroxyl radical trapping potential and hydrogen peroxide quenching ability.

Reducing power is one mechanism for action of antioxidants and may serve as a significant indicator of potential antioxidant activity for antioxidants (Jayaprakasha et al., 2000). Several studies have indicated that the antioxidant effect is related to the development of reductones (Yen and Duh, 1993). Therefore, in this study, the antioxidant activity of an extract may be related to its reductive activity.

FTC method was used to determine the amount of peroxide generated at the initial stage of lipid peroxidation. During the linoleic acid oxidation, peroxides formed and these compounds oxidize Fe^{2+} to Fe^{3+}. The Fe^{3+} ions form complex with SCN−, which has a maximum absorbance at 500 nm. In this method, the concentration of peroxide decreases as the antioxidant activity increases.

Scavenging of DPPH radical is related to the inhibition of lipid peroxidation (Rekka and Kourounakis, 1991). DPPH radical involves a hydrogen atom transfer process (Kaviarasan et al., 2007). In this assay, the good antioxidant activity on DPPH radical of TMPN may be attributed to a direct role in trapping free radicals by donating hydrogen atom.

Superoxide anion is a relatively weak oxidant, but it can generate more dangerous species, including singlet oxygen and hydroxyl radicals, which could cause the tissue damage (Halliwell and Chirico, 1993). Hydroxyl radicals are highly strong reactive oxygen species, and there is no specific enzyme to defense against them in human body (Liu et al., 2005). Hydrogen peroxide itself is not very reactive, but it may induce hydroxyl radicals, which would result in great damage to cells (Halliwell, 1991). Therefore, it is important to discover some chemicals with good scavenging capacity on these reactive oxygen species. In this study, TMPN showed potent scavenging activity on the three reactive oxygen species, and the flavonoids as the characteristic constituents may play a significant role.

Since the distinct radical scavenging and anti-lipid properties of TMPN, the studies were further extended to in vivo conditions using CCl_4-induced hepatotoxicity in rats. The hepatic damage induced by CCl_4 is well known to be mediated by its free radical metabolites such as CCl_3 and Cl_3COO, which could readily interact with unsaturated membrane lipid to produce lipid peroxidation and/or with other critical cellular macromolecules leading to cell damage (Snyder and Andrews, 1996). Liver damage was assessed by biochemical studies (AST, ALT, ALP and total bilirubin) and by histopathological examination. This is also evidenced by the elevation of TBARS and decrease in the activity of free radical scavenging enzymes, SOD, CAT and glutathione peroxidase (GPx) in the CCl_4 treated animals. These enzymes constitute a mutually supportive team of defense mechanism against the harmful effects of the reactive oxygen species (ROS) and free radicals in biological systems (Halliwell and Gutteridge, 1989). Lipid peroxidation also yields a wide range of cytotoxic products most of which are aldehydes, as exemplified by MDA, which can be measured following the TBA method (Yagi and Rastogi, 1979). Moreover, the lipid peroxidation is accelerated when free radicals are formed as the results of losing a hydrogen atom from the double bond in the structure of unsaturated fatty acids. Scavenging of free radicals is one of the major antioxidation mechanism to inhibit the chain reaction of lipid peroxidation. The extract was evaluated for the hepatoprotective activity using hepatotoxicity induced by CCl_4 in rat model and the extract was observed to exhibit hepatoprotective effect as demonstrated by a significant decrease in AST, ALT, ALP, and total bilirubin concentrations, and by preventing liver histopathological changes in rats. Moreover, the extract enhanced the activities of antioxidant enzymes (SOD, CAT, GPx) and diminished the amount of lipid peroxide against the CCl_4-induced hepatotoxicity in these animals.

Finally, the anti-inflammatory activity of the extract was investigated by carrageenin-induced rat paw oedema. Carrageenin-induced oedema is commonly used as an experimental model for evaluation the anti-inflammatory potential of natural products (Winter et al., 1962). Carrageenin is able to induce inflammation of the rat paw reaching its peak activity at 4 h. In the present study, the highest and most significant anti-inflammatory activity of the extract was already obvious at 2 h after induction of inflammation. The anti-inflammatory activity of the extract, however, was not as strong as that of indomethacin.

It has been established that reactive oxygen species (ROS) are implicated in inflammation (Aruma, 1998). There exists a link of antioxidants with respect to scavenging ROS and anti-inflammatory effects and therefore play an important role in the treatment of inflammatory diseases (Conner and Grisham, 1996). The demonstration of both antioxidant and anti-inflammatory (carrageenin model) activities by TMPN may confirm this relationship. Hence, these activities may justify the ethnomedicinal use of the plant in treat inflammation, burn scald and acute icterus hepatitis which are inflammatory conditions occasioned by infection of the lungs and liver, respectively.

In conclusion, the present study revealed that complementary activities of antioxidant activities, anti-hepatotoxic and anti-inflammatory of the methanolic extract of *Parathelypteris nipponica* (Franch. et Sav.) Ching, may be related and mediated through its anti-inflammatory and antioxidant activities.

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