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ORIGINAL ARTICLE

Heat Stress Resistance Effect of Flavonoids from *Penthorum chinense* Pursh on *Caenorhabditis elegans*

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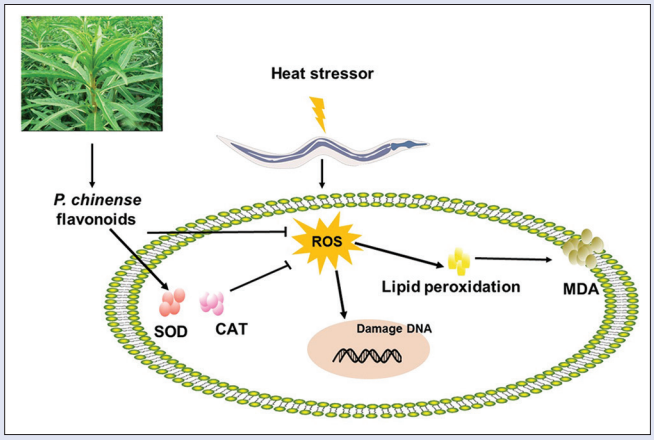
ABSTRACT

Background: Excessive reactive oxygen species (ROS) may overwhelm antioxidative defense, which is implicated in the onset and progression of various diseases. Flavonoids extracted from *Penthorum chinense* Pursh have a remarkable spectrum of bioactivities, while these pharmacological activities of flavonoids are at least partially due to their scavenging ROS and antioxidant ability. Therefore, we used the multicellular *Caenorhabditis elegans* as a model animal to investigate their antioxidant property and the possible molecular mechanisms in this study. **Materials and Methods:** The chemical compositions in *P. chinense* flavonoids were identified by ultra-performance liquid chromatography/time-of-flight mass spectrometry. Their anticancer and antioxidant ability *in vitro* were evaluated by inhibitory rate of HeLa cells, free radical scavenging, and iron-chelating ability. *In vivo*, synchronized L4 larvae *C. elegans* were treated by *P. chinense* flavonoids for 48 h. The survival rates of *C. elegans* were measured under heat stress condition. The antioxidant effect of *P. chinense* flavonoids on *C. elegans* was assessed by superoxide dismutase (SOD) and catalase (CAT) activities and malondialdehyde content. **Results:** *P. chinense* flavonoids was composed of six quercetin derivatives, two kaempferol derivatives, six pinocembrin derivatives, thonningianins A, and vanillic acid glucoside. *P. chinense* flavonoids not only showed a significantly inhibitory rate of HeLa cells but also exerted iron chelation and free radical scavenging ability. Furthermore, *P. chinense* flavonoids could extend the mean lifespan of *C. elegans* by approximately 17% under heat stress, which might be due to the increase of SOD and CAT activities. **Conclusion:** The study demonstrated that *P. chinense* flavonoids might be developed as a promising natural agent against environmental stress.

Key words: Antioxidant enzymes, *Caenorhabditis elegans*, flavonoids, heat stress resistance, *Penthorum chinense* Pursh

SUMMARY

- The *Penthorum chinense* flavonoids significantly inhibited the growth of HeLa cell lines and had high antioxidant abilities *in vitro*
- The *P. chinense* flavonoids could significantly increase the heat stress resistance of *C. elegans* through increasing antioxidant activities.



Abbreviation used: ROS: Reactive oxygen species; SOD: Superoxide dismutase; CAT: Catalase; MDA: Malondialdehyde; Vc: Ascorbic acid; UPLC-Q-TOF-MS: Ultra-performance liquid chromatography/time-of-flight mass spectrometry; PCF: *P. chinense* total flavonoids; PCF1: *P. chinense* purified flavonoids; ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); NGM: Nematode growth medium.

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INTRODUCTION

Reactive oxygen species (ROS) within certain boundaries is viewed as specific signaling molecules to prevent infection or regulate proliferation in cells.[1] When ROS homeostasis is perturbed by endogenously metabolic stress or exogenously environmental stress like ultraviolet, drugs, or heat, excess ROS may overwhelm the antioxidant defense system, cause oxidative stress, and subsequently damage cellular proteins, nucleic acid, and lipids. Oxidative stress is implicated in the aging process and a diverse array of chronic degenerative diseases, such as diabetes, neurodegenerative diseases, cardiovascular diseases, and cancers.[2] For example, oxidative stress is regarded to implicate in the aggregation of pathological proteins including α-synuclein and amyloid β proteins.[3] DNA mutations and chromosomal aberrations result from oxidative stress, which is crucial factors of tumor onset.[4,5] The mounting evidence showed a positive correlation between flavonoids-rich plants' intake and postponement of onset and progression of various chronic degenerative diseases.[6] It was reported that flavonoids from *Saraca asoca* bark and fresh plums were remarkable free radical scavengers *in vitro*, and the scavenging abilities were positively related to the concentration of flavonoids in an extract.[7,8] Flavonoids from cocoa could inhibit low-density lipoprotein oxidation, demonstrating their cardioprotective

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potential.[9] Likewise, excessive studies demonstrated anticancer abilities of plant extracts wherein one of the representative fraction was that flavonoids.[10] Flavonoids extract from *Crataegus monogyna* could combat the growth of HeLa, hepG2, and MCF-7.[11] Apart from above all, some flavonoid-rich plant extracts exerted neuroprotective properties and anti-neuroinflammation involving in age-related neurodegenerative diseases, such as Alzheimer' disease, Parkinson disease, and multiple sclerosis.[12] As an example, *Rhus verniciflua* flavonoids protected HT22 neuronal cells against glutamate-induced neurotoxicity.[13] Evidence indicated the widespread pharmacological activities of flavonoids was at least partially due to their scavenging ROS or antioxidant abilities. Therefore, it is essential to first investigate antioxidant and free radical scavenging potent of the flavonoids from plant extracts before developing nutraceutical or new drugs.

Penthorum chinense Pursh, belonging to genus *Penthorum* in family *Penthoraceae*, is a perennial plant native to East Asia, including China, Korea, Japan, Russia, and Thailand. *P. chinense* has been historically served as a folk medicine to treat adiposis, hepatica, infectious hepatitis, cholecystitis, edema, traumatic injury, and jaundice.[14] Recent advances in the last decade, *P. chinense* was extensively developed as health products and functional foods due to the rich source of flavonoids. In China, approximately 80 nutraceuticals with *P. chinense* extracts were used to protect liver function or prevent liver diseases.[15] Our previous studies exhibited that the total flavonoids extract from *P. chinense* had antioxidant abilities in comparison to ascorbic acid (Vc) *in vitro*. Given that the bioactivities of *P. chinense* extract is, at least partially, positively associated with the amounts of flavonoids in the extract, our laboratory used macroporous resin to purify further total flavonoids extract. The total flavonoids were loaded on DM130 macroporous resin with 70% ethanol elution, and then, the purity of flavonoids was increased by 23.9%.[16] In the present study, we selected *Caenorhabditis elegans* as an animal model to investigate the protective effect of purified flavonoids and shed light on underlying molecular mechanisms. Up to now, *C. elegans* is still the extremely popular animal model to screen biochemical drugs and bioactive natural products. This is possible because about 60%–80% of *C. elegans* genes is homologs to human.[17] In addition, the genome of *C. elegans* has been fully sequenced which displays a clear genetic background. In light of the above reasons, the investigation of bioactive compounds using *C. elegans* is more likely to elucidate their mechanisms of actions.

In the present study, free radical scavenging abilities and inhibition of HeLa cells viability of the purified flavonoids from *P. chinense* were detected. The antioxidant mechanism of *P. chinense* flavonoids was explored in worms. The major bioactive compounds in *P. chinense* were also identified by ultra-performance liquid chromatography/time-of-flight mass spectrometry (UPLC-Q-TOF-MS).

MATERIALS AND METHODS

Preparation of *Penthorum chinense* flavonoids

P. chinense flavonoids were previously purified from the total flavonoids by our lab.[16] Briefly, dried *P. chinense* powder was extracted by 68% alcohol for 2.2 h at 82°C. The alcohol extracts were concentrated and dried under vacuum to get the *P. chinense* total flavonoids (PCF). PCF with 1.0 mg/mL (110 mL) was then loaded onto DM130 macroporous resins column eluted with 70% ethanol at 1.0 mL/min and the purify of flavonoids was increased by 23.9%. *P. chinense* purified flavonoids (PCF1) were used for the following experiments.

DM130 macroporous resins were purchased from the Anhui Sanxing Resin Technology Co., Ltd., (Anhui, China). 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferrozine were purchased from Sigma Chemical Company (St. Louis, USA). The assay kits of malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) were purchased from Nanjing Jiancheng Technology Co., Ltd., (Nanjing, China).

UPLC-Q-TOF-MS analyzed the chemical compounds in *Penthorum chinense* flavonoids

PCF1 was dissolved in methanol to get the final concentration of 5 mg/mL solution. The sample solution was filtrated through a 0.22-μm filter membrane before determination of the flavonoids composition by UPLC-Q-TOF-MS.

The UPLC separation was performed on Hypersil Gold C_{18} (50 × 2.1 mm, 1.9 μm), and the column temperature was kept at 22°C. The mobile phase was composed of 0.1% formic acid in water (solvent A) and methanol (solvent B). The gradient elution conditions were as follows: 0–4 min, 30% B; 4–6 min, 30%–50% B; 6–9 min, 50% B; 9–12 min, 50%–90% B; 12–14 min, 90% B; and 14–15 min, 30% B. The flow rate was 0.3 mL/min. The injection volume of the sample solution was 5 μL.

For the mass analysis, the ion source was HESI ionization source carrying positive/negative ion scan modes. The capillary and cone voltage were +3.5 kV and −3.0 kV, respectively. The electrospray pressure was set at 13 psi. The dry gas flow rate was set at 35 L/min at the temperature of 300°C. The scan range was from 100 to 1000 m/z. The mass resolution was set at 70,000 at m/z 200.

Cell culture

The cervical cancer cells HeLa was gifted from West China School of Medicine, Sichuan University (Sichuan, China). HeLa cells were maintained in RPMI 1640 medium in the presence of 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in 37°C CO_2 incubator. The adherent cells were detached by trypsin EDTA into a 96 well plate at 70%–80% confluence for the following assay.

Cell viability assay

The anticancer effect of PCF1 on HeLa cancer cells was evaluated by MTT colorimetric method with slight modifications.[18] HeLa cells were seeded in 96 well plates and incubated for 24 h with a density of 1 × 10^5 cells/well. HeLa cells were treated with or without PCF1 at the indicated time interval. Subsequently, 1 mg/mL MTT was added to RPM11640 medium to prepare to determine cell viability. After 4 h of exposure to MTT, the supernatant was removed, and the DMSO was added to well to dissolve the formazan crystals. The absorbance at 570 nm was quantified by a microplate reader (SpectraMax M5, Molecular Devices, USA). The inhibitory rate of tumor cells was calculated as follows:

$$\text{Inhibitory rate}(\%)=\frac{A1-A0}{A2-A0}\times 100$$

whereas *A0* is the absorbance of blank control; *A1* is the absorbance of the PCF1 group; and *A2* is the absorbance of negative control group (DMSO instead of PCF1).

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay

The scavenging ability of PCF1 against ABTS·+ was evaluated as the previous description with slight modifications.[19] Briefly, the potassium persulfate (2.45 mM) was combined with ABTS solution (7 mM) in the dark at 25°C for 12 h to get the ABTS·+ stock solution. ABTS·+ stock solution was diluted with distilled water to obtain ABTS·+ working solution ($OD_{734\ nm}$ = 0.70 ± 0.02). 20 μL PCF1 solutions with different concentrations were mixed with 2 mL ABTS·+ working solution and

then incubated 37°C for 6 min before reading at 734 nm. Vc was used as a positive control. 70% alcohol was as blank control. The scavenging ability of ABTS free radical was quantified as follows:

$$\text{ABTS free radicalscavenging ability}(\%)=(1-\frac{A1-A2}{A0})\times 100$$

whereas *A0* is the absorbance of the blank control; *A1* is the absorbance of the mixture solution of PCF1 solution and ABTS·+ working solution; and *A2* is the absorbance of the mixture solution of distilled water and ABTS·+ working solution.

Hydroxyl radical scavenging assay

The scavenging ability of PCF1 against hydroxyl radical was carried out according to previous description with slight modifications.[20] Briefly, 50 μL PCF1 solution was reacted with a series of equal volumes reagents including 9 mM sodium salicylate, 9 mM $FeSO_4$, and 0.025% H_2O_2 (w/v) for 30 min at 37°C. Vc was used as a positive control. 70% alcohol was as blank control. The absorbance of working solution at 510 nm was measured. The scavenging ability of hydroxyl radical was calculated as follows:

$$\text{Hydroxyl radical scavenging ability}(\%)=(1-\frac{A1-A2}{A0})\times 100$$

whereas *A0* represents the absorbance of the blank control; *A1* represents the absorbance of the PCF1 solution; and *A2* represents the absorbance of the PCF1 solution in the absence of hydroxyl radical generating system solution.

Iron-chelating assay

The chelating effect of PCF1 on iron was quantified as described previously with minor modifications.[21] Briefly, 50 μL PCF1 solutions were mixed with 100 μL 0.125 mM ferrous sulfate solution. 50 μL 1.0 mM ferrozine was added to the mixture to start the chelating reaction. After 10 min, the absorbance of reaction mixture at 562 nm was monitored. EDTA-Na_2 was served as a positive control. 70% alcohol was as blank control. The iron-chelating ability was quantified as follows:

$$\text{Iron chelating ability}(\%)=(1-\frac{A1-A2}{A0})\times 100$$

whereas *A0* is the absorbance of the blank control; *A1* is the absorbance of the PCF1 solution; and *A2* is the absorbance of the PCF1 solution in the absence of ferrozine.

Caenorhabditis elegans maintenance

The wild-type Bristol N2 *C. elegans* and *Escherichia coli* OP50 were provided by Caenorhabditis Genetics Center (CGC), University of Minnesota, Twin Cities. Wild-type N2 worms were maintained at 20°C on nematode growth medium (NGM) plates seeded with a lawn of *E. coli* OP50. Synchronous worms were obtained by egg-laying assay. Briefly, approximately 20 gravid adults were transferred to fresh NGM plate to lay eggs for 2 h. Synchronized hatching eggs grew to L4 stage for the following experiments.[22]

Food clearance assay

The optimal concentration of PCF1 for *C. elegans* was identified according to the previous description with minor modifications.[23] Approximately 20–30 synchronized L4 worms were transferred to 96 well plates with a series of dose dilution of PCF1 (0.0, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/mL) and *E. coli* OP50 as food sources. The 96 well plates containing worms were incubated at 20°C. The absorbance of liquid media with *E. coli* OP50 and worms was read daily at 595 nm by a microplate reader (SpectraMax M5, Molecular Devices, USA).

Heat shock assay

The protective effect of PCF1 on *C. elegans* under heat stress was measured as the previous description with slight modifications.[24] Synchronized L4 worms were maintained on NGM containing 0.1 mg/mL PCF1. After 48 h of exposure to PCF1, worms were transferred to a 35°C incubator to assess their stress resistance ability. The survival rate of worms was checked every 2 h. Worms were identified as dead when they failed to respond to the platinum wire gentle touch. 0.05% DMSO was used as a blank control.

Antioxidant enzyme activities

SOD and CAT activities of worms were followed from a previous description with modifications.[25] Briefly, synchronized L4 worms were treated in the presence of and in the absence of 0.1 mg/mL PCF1 for 48 h. After 3 h of exposure to 35°C, approximately 2000 worms were washed and homogenized. SOD and CAT activities were measured according to the instruction of respective assay kits.

Malondialdehyde content

MDA content of worms was followed from a previous description with slight modifications.[22] Briefly, synchronized L4 larvae were treated with or without 0.1 mg/mL PCF1 for 48 h. After 3 h of exposure to 35°C, approximately 2000 worms were washed and homogenized. MDA content was measured according to the instruction of MDA assay kit. MDA content was normalized by protein content and expressed as mmol/mg protein.

Statistical analysis

All assays were the replica in three independent experiments. Statistical analyses were carried out by SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Data were statistically analyzed by Student's *t*-test or one-way analysis of variance. All results were presented as the mean standard deviation of the mean. Survival analyses were performed by the Kaplan–Meier method and the log-rank test. $P < 0.05$ or less was considered as statistically significant between means.

RESULTS

Identification of flavonoids compounds in PCF1

Generally, flavonoids extract from plants has several different chemical compounds. In the context, we determined that the chemical compositions of purified flavonoids extract. [Figure 1] exhibited total ion chromatogram of the chemical components in *P. chinense* flavonoids. A total of 19 chemicals, including two unknown chemicals, were identified by UPLC-Q-TOF-MS. The detected compounds were consisted of six quercetin derivatives, two kaempferol derivatives, six pinocembrin derivatives, thonningianins A, and vanillic acid glucoside [Table 1]. These individual flavonoids were determined by comparing with previous studies.[26-30] Studies indicated that flavonoids extract exerted a broad range of biological activities which was due to their various chemical compounds.[31] However, it is difficult to simplify purify and fast isolate the individual compound from flavonoids' extract. Therefore, it has gained increasing attention to use the synergic efficacy of mixed chemical components without separation to evaluate the biological activities of flavonoids.

PCF1 inhibited HeLa cell viability *in vitro*

To investigate the anticancer effect of PCF1 on cervical cancer HeLa cell lines, a series of concentrations of PCF1 treated the cancer cell for 12, 24, and 48 h separately. As shown in Figure 2, PCF1 appeared a time-dependent growth-inhibition effect on HeLa cells from 12

Table 1: Identification and speculation of the chemical compounds in *Penthorum chinense* flavonoids by ultra-performance liquid chromatography/time-of-flight mass spectrometry

n[a]	Flavonoids compounds	Retention time (min)	MS (m/z)[b] [M-H]-	Fragment ions
1	Vanillic acid glucoside	1.77	329	167, 329
2	Quercetin-di-glucoside	2.02	625	301, 463, 625, 647
3	Quercetin 3-O-ruitinoside	3.74	301	301
4	Quercetin-3-O- glucoside	4.2	463	301, 463
5	Quercetin-3-O-xyloside	5.87	433	149, 300, 301,433
6	Quercetin-3-O-rhamnoside	6.16	447	301, 447
7	Quercetin-3-O-rhamnoside	6.68	447	301, 447
8	Kaempferol	6.88	285	285
9	Kaempferol-3-O-rhamnoside	7.09	431	285, 431
10	Quercetin	7.26	301	301
11	Pinocembrin-7-O -glucoside	7.45	417	255, 417
12	Pinocermbrin-7-O-galloy-D-glucoside isomer	8.05	569	255, 419,569
13	Pinocembrin-7-O-[4″,6″-HHDP]-glucoside	8.45	719	255, 275, 301, 719
14	Pinocembrin-7-O-[3″-O-galloyl-4″,6″-HHDP]-glucoside	9.14	871	255, 301, 871
15	No identification	10.65	-	257, 417, 707
16	No identification	10.82	-	257, 571
17	Pinocembrin dihydrochalcone-7-O-[4″, 6″- HHDP]-glucoside	11.22	721	257, 277, 301, 721
18	Pinocembrin dihydrochalcone -7-O-[3″-O-galloyl-4″, 6″-HHDP]-glucoside	11.36	873	257, 301, 873
19	Thonningianins A	11.61	873	873

[a]The number for peaks refers to the total ion chromatogram of the flavonoids' profile in *Penthorum chinense* Pursh, [b]Fragment ions detected in negative ion. MS: Mass spectrometry

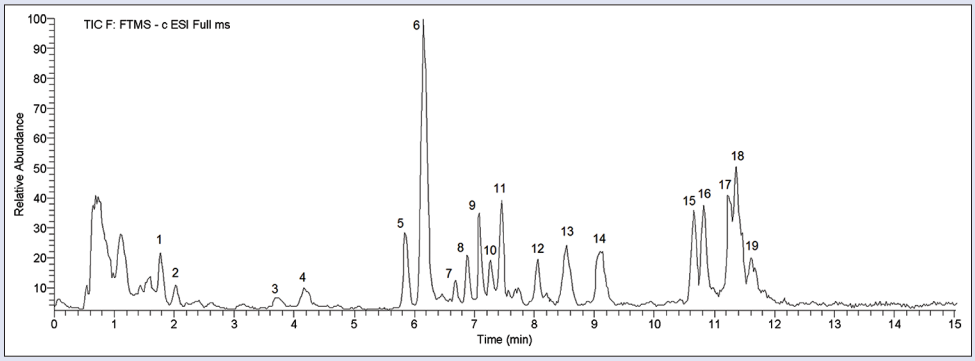

Figure 1: Total ion chromatogram of the flavonoids' profile in *Penthorum chinense* Pursh

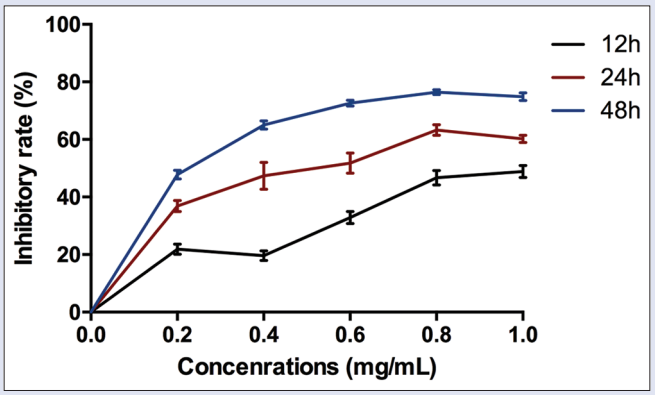

Figure 2: Effect of *Penthorum chinense* flavonoids against on HeLa cell viability *in vitro*

to 48 h. After 48 of treatment to PCF1, the growth of HeLa cell line was significantly inhibited in comparison to treatment 12 and 24 h. The IC_{50} values (the concentration of PCF1 that inhibited 50% of cancer cell growth) of 12, 24, and 48 h treatment group were 1.07, 0.46, and 0.21 mg/mL, respectively. In addition, PCF1 also exhibited a concentration-dependent growth-inhibition effect on HeLa cells within 0.05–1.0 mg/mL. At 1.0 mg/mL, the inhibitory rates of 12, 24, and 48 h treatment group were 28.31% ± 7.52%, 43.26% ± 9.47%, and 56.12% ± 12.02%, respectively. These results indicated that PCF1 possessed a potential against cervical cancer cells.

PCF1 significantly scavenged 2,2′22vengedntlyn-dependent growth-inhibition ic acid) radicals *in vitro*

Accumulating evidence implicated that flavonoids-rich extracts had strong anticancer activities on account of the free radical scavenging ability.[10] Therefore, we used ABTS assay to evaluate the free radical scavenging property of PCF1. The results showed that PCF1 exhibited significantly total free radical scavenging ability with an IC_{50} of 0.21 mg/mL [Figure 3a]. Vc, as a positive control, displayed extremely similar antioxidant activity with an IC_{50} of 0.22 mg/mL as compared to PCF1

PCF1 scavenged hydroxyl radicals *in vitro*

Hydroxyl radical, a highest reactive radical, is the primary initiator of lipoperoxidation. Thus, we also detected the hydroxyl radical

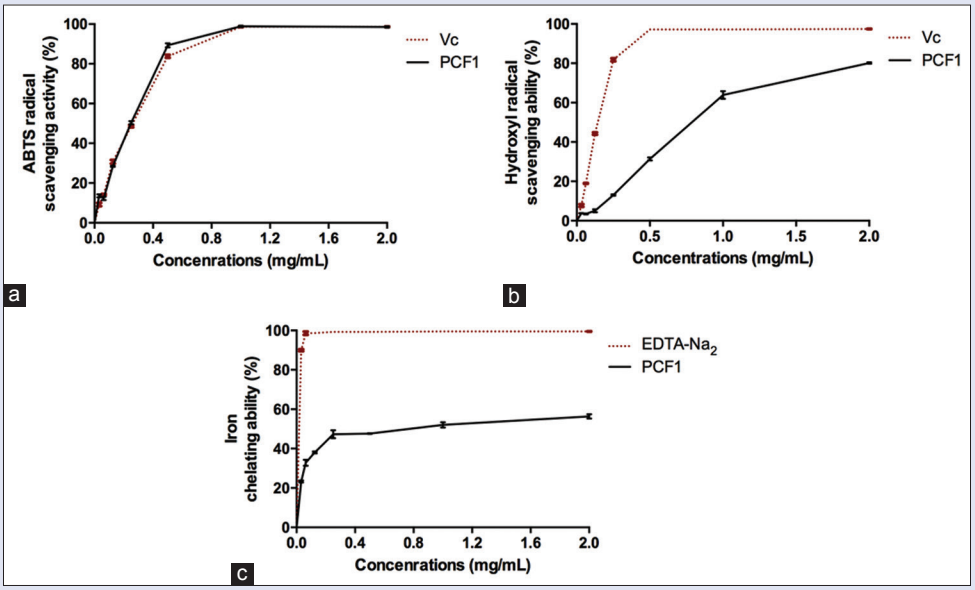

Figure 3: Effects of *Penthorum chinense* flavonoids on free radical scavenging abilities *in vitro.* (a) Effect of PCF1 on 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radicals' scavenging ability; (b) effect of PCF1 on hydroxyl radicals' scavenging ability; (c) effect of PCF1 on iron-chelating ability

scavenging ability of PCF1 in this study. As shown in Figure 3b, PCF1 exerted a dose-dependent manner on hydroxyl radical scavenging activity with an IC_{50} value of 0.77 mg/mL. At the concentration of 2.0 mg/mL, PCF1 showed a maximum hydroxyl radical scavenging ability of 80.10% ± 0.55%. Compared to PCF1, Vc showed much higher antioxidant activity with an IC_{50} value of 0.13 mg/mL. It reached the maximum hydroxyl radical scavenging ability of 97.18% ± 0.04% at the concentration of 0.5 mg/mL.

PCF1 increased iron-chelating ability *in vitro*

Iron could oxidize the free radicals to generate stronger free radicals that damage cellular components. Hence, the iron-chelating potential is commonly used to evaluate to free radical scavenging ability of natural antioxidant. As shown in Figure 3c, at the 0.25 mg/mL, the chelating ability of PCF1 against iron almost reached to the maximum values of 47.30% ± 3.50% (IC_{50} = 0.68 mg/mL). However, DETA-Na_2, performed as a positive control, existed more chelating effect on iron than PCF1. At 0.06 mg/mL, the iron-chelating ability of DETA-Na_2 was up to 98.63% ± 1.42% (IC_{50} = 0.01 mg/mL).

The proper PCF1 concentration for screening in *Caenorhabditis elegans*

Because the flavonoids are able to work as antioxidants or a pro-oxidants to modulate the redox (reduction/oxidation) cellular signaling according to their different concentrations.[32] The higher concentrations of flavonoids might be toxic, thereby cause an adverse effect. Therefore, the proper PCF1 concentration *in vivo* is important for the following assays. Given that the strong ability of *C. elegans* to reproduce, the ability of strong growth, and the 3-day life cycle of *C. elegans*, they could rapidly consume *E. coli* OP50 supplement in limited resources space. We first screened the proper concentration of *P. chinense* flavonoids to treat *C. elegans* using the food clearance assay. As shown in Figure 4, the absorbance of wells in the absence of PCF1 decreased after 3 days. After exposure to 1.0 or 2.0 mg/mL of PCF1, food clearance rates of 96 wells were delayed which was in good agreement with body sizes decline of *C. elegans* (data not shown). Any compounds, within certain boundaries, could reduce *C. elegans* size,

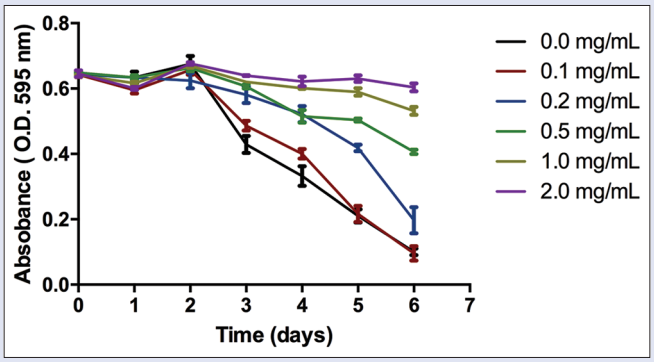

Figure 4: Treatment concentration of *Penthorum chinense* flavonoids was screened by food clearance assay

fecundity, and survival rate with the decline of food consumption in limited spaces.[23] At the concentration of 0.2 and 0.5 mg/mL, the food consumption rates were slower than that of 0.1 mg/mL PCF1 group. *C. elegans* were cultured at 0.1 mg/mL PCF1 displaying similar rate of food consumption in comparison to group without PCF1. The findings suggested that proper concentrations for *C. elegans* were in range from 0.1 to 0.2 mg/mL. Thus, the concentration of 0.1 mg/mL PCF1 was selected for the following assays.

PCF1 improved heat stress resistance of *Caenorhabditis elegans*

To measure whether *P. chinense* flavonoids had the antioxidant ability *in vivo*, N2 *C. elegans* pretreated with PCF1 for 48 h were exposed to 35°C and then their survival rates were quantified [Figure 5]. After 5 h of heat shock, *C. elegans* in both groups started to die, whereas the survival rate of *C. elegans* in the PCF1-pretreatement group was significantly higher than the control group without PCF1 in whole experiment process ($P < 0.001$). The median lifespan of PCF1 treated *C. elegans* was extended by approximately 17%, indicating that *P. chinense* flavonoids could protect *C. elegans* against heat stress.

PCF1 significantly increased superoxide dismutase and catalase activities in *Caenorhabditis elegans*

Heat stress is commonly considered to lead to large amounts of ROS accumulation and cause oxidative stress in cells. As noted above, PCF1 had strong ROS scavenging ability *in vitro* and increased heat stress resistance of worms [Figures 3 and 5]; it is more likely that the protective mechanism of PFC1 has been involved in antioxidant defense system in *C. elegans*. Therefore, we investigated whether PCF1 activated antioxidant enzymes of *C. elegans* under thermal stress. As expected, SOD and CAT activities in the PCF1-pretreatment group were enhanced by 15.17 and 15.73%, respectively [Table 2]. The results indicated that *P. chinense* flavonoids might increase heat stress tolerance of *C. elegans* through promoting the activities of antioxidant enzymes.

PCF1 significantly decreased malondialdehyde content in *Caenorhabditis elegans*

The high levels of ROS can cause oxidative deterioration of membrane lipids. MDA is commonly the product of lipid deterioration. In this study, we investigated whether PCF1 mitigated lipid peroxidation induced by the toxicity of heat stress. After exposure to 35°C, MDA content in the PCF1 group was markedly reduced by 60% ($P < 0.05$) in comparison to control group without PCF1, suggesting *P. chinense* flavonoids could reduce lipid peroxidation in *C. elegans* [Table 2].

DISCUSSION

Vegetables and fruits intake have a positive association with health promotion and various diseases precaution wherein one of these beneficial effects is attributable to flavonoids constituents.[33,34] Since the 1990s, there has been an increase in people that interested in dietary flavonoids in light of their extensive bioactives including antioxidant, anti-inflammatory, anti-aging, and decline risk of cancers. Accumulating studies have attributed widely health benefits of flavonoids to their antioxidant and metal-chelating abilities, while the most antioxidant activities are owing to free radical scavenging mechanisms.[35-37] Free radicals are groups of unstable molecules carrying one or more unpaired electrons. The high level of free radicals can attack the cellular genetic material causing mutation, which may play a role in the development of cancer.

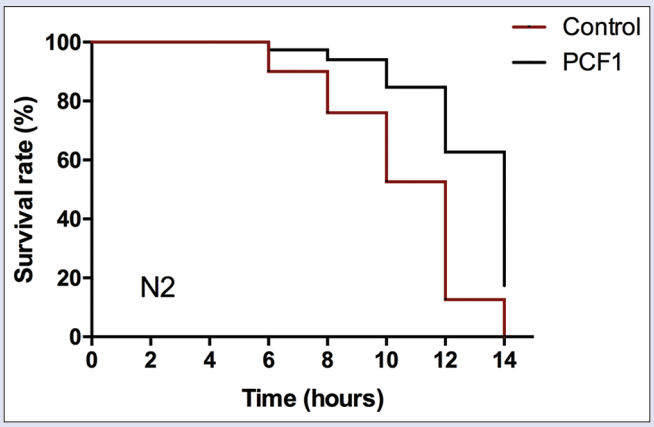

Figure 5: Effect of *Penthorum chinense* flavonoids on heat stress resistance of *Caenorhabditis elegans*

Table 2: Effects of *Penthorum chinense* flavonoids on antioxidant enzymes activities and malondialdehyde content in *Caenorhabditis elegans*

Treatment	Antioxidant enzymes activities		MDA content (nmol/mg prot)
	SOD (U/mg prot)	CAT (U/mg prot)	
Control	25.37±0.86[b]	20.21±0.63[b]	3.94±0.52[a]
PCF1	40.56±3.12[a]	35.94±2.89[a]	1.58±0.09[b]

[a,b]Mean with different letters in the same column are significantly different at P=0.05 level. MDA: Malondialdehyde; SOD: Superoxide dismutase; CAT: Catalase; PCF1: *P. chinense* purified flavonoids

Given the above statement, we examined the anticancer effect of flavonoids extract from *P. chinense* on HeLa cells in the present study. PCF1 dramatically inhibited the growth of cancer cells in a time- and dose-dependent manner, indicating that *P. chinense* flavonoids could prevent cancer cells growth. Numerous flavonoids extracts from plants, such as *Tragopogon porrifolius* and *Abrus Precatorius* L, exerted their anticancer activities.[38,39] Moreover, the anticancer activity of *T. porrifolius* flavonoids was due to their free radical scavenging and metal-chelating abilities. We also observed PCF1 could highly get rid of free radicals, and the antioxidant property of PCF1 was slightly higher than Vc in ABTS assay. Similar results also were reported by Zeng *et al*. The flavonoids extracts from *P. chinense* had highly scavenging free radical ability *in vitro*.[40] Apart from free radical scavenging capability, PCF1 also exhibited its antioxidant property by iron ion-chelating ability. Iron ions are able to catalyze free radicals to generate higher reactivity of free radicals formation in organisms. *P. chinense* flavonoids may donate hydrogen to iron ion to lighten the toxicity of free radicals. In the context, *P. chinense* flavonoids suppressed the growth of cervical cancer HeLa cells which might be correlated with the strong antioxidant ability.

Till now, *C. elegans* is used to evaluate the protective effect of natural compounds and their underlying molecular mechanisms.[41] The possible factor was the evolutionary conservation of *C. elegans*, or explored experiments could be performed on a whole organism with different tissues in contrast to mammalian cell system *in vitro*.[17] Alternatively, *C. elegans* has a mean lifespan of approximately 18–20 days at 20°C, which shortens the whole experimental time to screen bioactive natural compounds.[24] Recent advances in the last decades, the majority of pharmacological activities of *P. chinense* flavonoids were assessed *in vitro* systems, such as mammalian cell cultures or free radicals scavenging assays *in vitro*. In this study, we first used *C. elegans* as an animal model to investigate the protective activities of *P. chinense* flavonoids *in vivo*.

As is well known, any compounds, within certain boundaries, could impact *C. elegans* development and survival rate. Thus, we screened the proper concentration of flavonoids (0.1 mg/mL) to treat *C. elegans* before carrying out the *in vivo* assays. The antioxidant effect of *P. chinense* flavonoids on *C. elegans* was determined by heat stress resistance. PCF1 significantly increased the survival rate of *C. elegans* under 35°C. Heat stressor could induce free radical accumulation rapidly in *C. elegans*.[42] SOD is able to convert the dangerous superoxide anion to hydrogen peroxide (H_2O_2), and CAT catalyzes H_2O_2 to decompose into water and oxygen. Given the high ability of free radical scavenging *in vitro*, we further verified the possible mechanism of *P. chinense* flavonoids by assessing the SOD and CAT activities of *C. elegans* under heat stress condition. PCF1 vigorously enhanced the contents of endogenous SOD and CAT in comparison to the untreated group. After 3 h of exposure to 35°C, the stressor caused the rapid accumulation of ROS in *C. elegans*, while excess ROS might cause oxidative damage to increase the mortality rate of *C. elegans*. However, due to *C. elegans* were pretreated with PCF1 for 48 h, *P. chinense* might activate antioxidant enzymes system of *C. elegans*. The triggered endogenous antioxidant enzymes were more sensitive to ROS and could fast response to oxidative stress to increase the survival rate of *C. elegans*. In addition, PCF1 also diminished the level of MDA, a hallmark of oxidative damage, indicating that *P. chinense* flavonoids had high antioxidant potential *in vivo*. Previous studies also showed that flavonoids-rich extracts exerted a protective effect on *C.*

elegans.[43,44] For example, *Ginkgo biloba* extract (EGb761) increased thermal stress resistance of *C. elegans* by 25%.[45] Taken together, the present study indicated that PCF1 could be further developed as functional foods or a promising natural antioxidant.

CONCLUSION

In this context, *P. chinense* flavonoids significantly inhibited the growth of cervical cancer HeLa cell lines and exerted high free radical scavenging and iron ion-chelation ability *in vitro*. In addition, *P. chinense* flavonoids could increase SOD and CAT activities, as well as reduce the oxidative damage marker MDA level in *C. elegans*, suggesting a protective action of *P. chinense* flavonoids against heat stress. Comprehensively, *P. chinense* flavonoids exhibited beneficial effects and could be as a promising natural antioxidant or studied as a functional food.

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Conflicts of interest

There are no conflicts of interest.

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