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Antioxidant and Anti-Inflammatory Activities of Ethyl Ether Extract from *Paeonia lactiflora* Pall. Flowers

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Paeonia lactiflora Pall. root is an important traditional Chinese medicine. In order to determine if the flower have any beneficial properties, we investigated antioxidant and anti-inflammatory activities of ethyl ether extract of *P. lactiflora* Pall. flowers. The results showed that the *P. lactiflora* Pall. flowers exhibited extremely high antioxidant properties, IC_{50} values of 1,1-diphenyl-2-pricylhydrazyl free radical scavenging activity was 7.31 mg/mL, which was lower than that of positive control butylated hydroxyanisole and it eliminated approximately 60.51 % of superoxide radicals at 400 µg/mL. Moreover, the *P. lactiflora* Pall. flowers showed strong total antioxidant activity and reducing power and provided protection against oxidative protein damage induced by hydroxyl radicals. Lipopolysaccharide-activated RAW264.7 cells were used to explore the modulatory role of *P. lactiflora* Pall. flowers on nitric oxide production and the activation of transcription factors using RT-PCR. *P. lactiflora* Pall. flowers diminished the production of nitric oxide and the mRNA expression of tumor necrosis factor-α, intedeukin 6, inducible nitric oxide synthase and cyclooxygenase-2.

Keywords: *Paeonia lactiflora* Pall. Flowers, Antioxidative, Anti-inflammatory, Free radicals, Flavonoid.

INTRODUCTION

In recent years, much attention has been focused on the biological properties of foods and natural herbs [1]. Many important bioactivities (such as antioxidant, anti-inflammatory, anti-diabetes, anticancer) have been investigated in plant [2]. Biological activities are correlated to the presence of certain compounds that may assist in predicting some traditional uses of medicinal plants [3]. Thus, it is assumed that some plants can be less toxic to humans and can be used as medicine for the treatment of diseases.

Paeonia lactiflora Pall. Ranunculaceae, a perennial root herbaceous plant, is grown on dry open stony slopes, riverbanks and sparse woodland edges. The dried root without bark of *P. lactiflora* is one of the most well-known herbs in East Asia with a long history. *P. lactiflora* not only has lots of pharmacological activities including anti-inflammatory, analgesic, antibacterial, antioxidant, anticancer, antidepressant and anti-hepatic fibrosis effects, but also has abilities to improve and treat autoimmune diseases, cardiovascular and cerebrovascular diseases as well as neurodegenerative diseases [4-6]. *P. lactiflora* is rich in different kinds of bioactive components, such as monoterpenes [7,8], flavonoids [9], stilbenes [10] and polyphenols [11,12], among which paeoniflorin a kind of monoterpene is the most studied medicinal compound [5,13,14]. Previous studies of *P. lactiflora* were mainly focused on the root [5,11,13,14], with monoterpenes as the main compounds. There were few reports about the aboveground parts of *P. lactiflora* Pall.

In the present study, we examined the bioactivity effect of *P. lactiflora* Pall. flowers extracts [2]. We further demonstrated that the antioxidant and anti-inflammatory activities of ethyl ether extract of *P. lactiflora* Pall. flower. These findings provide evidence that *P. lactiflora* Pall. flower may be a novel alternative therapeutic agent for the treatment of inflammatory.

EXPERIMENTAL

***P. lactiflora* Pall. flower extract preparation and HPLC analysis:** *P. lactiflora* Pall. flower were collected from Yangzhou and identified by Professor Jun Tao (College of Horticulture and Plant Protection, Yangzhou University, China). The extract was determined according to the method of Jin *et al.* [2]. The extract was then dried by vacuum concentration and then stored at -20 °C until used.

P. lactiflora Pall. flower extract were analyzed by a HPLC system (CBM-20A, Shimadzu Co. Ltd., Japan) with two

gradient pump systems (LC-20AT, Shimadzu), a UV-detector (SPD-10A, Shimadzu), an auto sample injector (SIL-20A, Shimadzu) and a column oven (CTO-20A, Shimadzu). Separation was achieved on a Gemini C_{18} column (4.6 × 100 mm, 3 μm, Phenomenex. Inc., Torrance, CA, USA) using a linear gradient elution program with a mobile phase containing solvent A (0.4 %, v/v, formic acid in distilled deionized water) and solvent B (acetonitrile). Initially started with a gradient of 18 % B changing to 32 % in 15 min and finally to 50 % in 40 min followed by washing for 25 min with a flow rate of 1.0 mL/min. Sample injection volume was 10 μL. Peaks were monitored at 280 nm. Paeoniflorin was used as reference compound for *P. lactiflora* Pall.

Antioxidant activity assay: The reducing power assay was determined according to the method of Hu *et al*. [15]. The free radical scavenging activities of the extracts, based on the scavenging activity of the stable DPPH free radical, were determined by Kilani *et al*. [16]. The effect of *P. lactiflora* Pall. flowers on protein oxidation was carried out according to the method of Hu *et al.* [15]. The superoxide anion scavenging activity was determined according to the method of Choi *et al*. [13]. Butylated hydroxyanisole was used as standards.

Cell lines and cell culture: RAW264.7 cell line were purchased from the Korean Cell Bank (Seoul, Korea) and grown in RPMI 1640 (Roswell Park Memorial Institute medium 1640), supplemented with 10 % fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were cultured in a humidified atmosphere and incubated at 37 °C in 5 % CO_2.

Nitric oxide assay and MTT assay: RAW264.7 cells (1×10^6 cells/mL) were cultured for 18 h, pretreated with *P. lactiflora* Pall. flowers for 0.5 h and further incubated with lipopolysaccharide (1 μg/mL) for 24 h. To check whether *P. lactiflora* Pall. flowers can directly inhibit nitric oxide release, *P. lactiflora* Pall. flowers was incubated with sodium nitroprusside (10 mM) in a microtube for 0.5 h. The inhibitory effect of *P. lactiflora* Pall. flowers on lipopolysaccharide-induced nitric oxide production was determined by analyzing the nitric oxide level using Griess reagent as previously described [17,18]. Subsequently, the mixture was incubated at room temperature for 10 min and the absorbance was measured at 550 nm.

The cytotoxicity of *P. lactiflora* Pall. flowers was decided by a conventional MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, as reported [17].

Determination of lipopolysaccharide-inducible inflammatory gene expression: For the evaluation of tumor necrosis factor-α, intedeukin-6, inducible nitric oxide synthase and cyclooxygenase-2 mRNA expression levels, the total RNA from the lipopolysaccharide treated-RAW264.7 cells (5×10^6 cells/mL) was prepared by adding TRIzol RNA isolation kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, as reported previously. Sequences for the specific primers used in the PCR were summarized in Table-1.

Statistical analysis: All tests were carried out independently in triplicate (n = 3). Data are expressed as the mean ± standard derivation (SD). The results were processed using Excel 2003 (Microsoft, Redmond, WA, USA).

RESULTS AND DISCUSSION

Antioxidant activity: To measure the reducing power activity, we investigated the Fe^{3+}–Fe^{2+} transformation in the presence of *P. lactiflora* Pall. flowers. As shown in Fig. 1, the reducing power of *P. lactiflora* Pall. flowers increased with increasing concentration and it was 0.13 at 25 μg/mL, 0.26 at 50 μg/mL, 0.46 at 100 μg/mL and 0.87 at 200 μg/mL. However, reducing power activity was 0.17 at 25 μg/mL, 0.41 at 50 μg/mL, 0.59 at 100 μg/mL and 1.17 at 200 μg/mL for butylated hydroxyanisole. These results indicate that *P. lactiflora* Pall. flowers might contain some reducing power compounds.

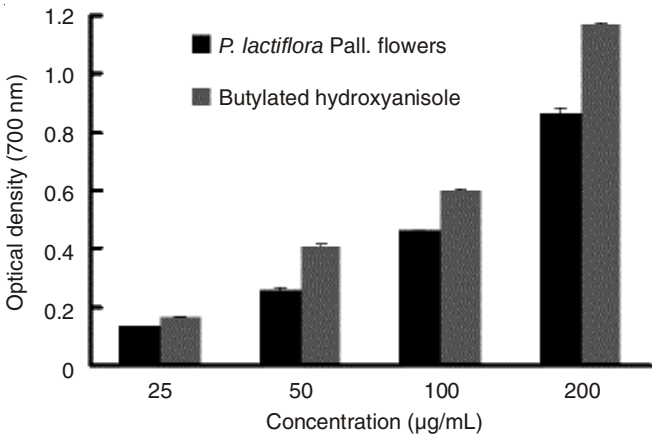

Fig. 1. Reducing power ability of *P. lactiflora* Pall. flowers. Data are expressed as means ± SD (n = 3). Butylated hydroxyanisole was used as a positive control

DPPH has been used extensively as a free radical to evaluate reducing substances in natural compounds [19]. The DPPH radical scavenging activity of butylated hydroxyanisole increased with increasing concentrations (Fig. 2), exhibiting 22.7, 39.5, 59.7 and 82.9 % scavenging activity for 2.5, 5, 10 and 20 μg/mL of butylated hydroxyanisole, respectively. Whereas, that of the *P. lactiflora* Pall. flowers sharply increased, demonstrating 23.2, 44.5, 75.9 and 95.3 % scavenging activity for 2.5, 5, 10 and 20 μg/mL of *P. lactiflora* Pall. flowers, respectively. This result suggests that the *P. lactiflora* Pall. flowers exhibited the stronger DPPH free radical scavenging activity than positive control butylated hydroxyanisole.

TABLE-1
PRIMERS AND AMPLIFICATION CONDITIONS USED FOR RT-PCR IN RAW264.7 CELLS

Primers	Sense	Antisense
Tumor necrosis factor-α	5'-TCTCATCAGTTCTATGGCCC-3'	5'-GGGAGTAGACAAGGTACAAC-3'
Intedeukin-6	5'-GTTCTCTGGGAAATCGTGGA-3'	5'-TGTACTCCAGGTAGCTATGG-3'
Inducible nitric oxide synthase	5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
Cyclooxygenase-2	5'-CACTACATCCTGACCCACTT-3'	5'-ATGCTCCTGCTTGAGTATGT-3'
GAPDH	5'-CACTCACGGCAAATTCAACGGCAC-3'	5'-GACTCCACGACATACTCAGCAC-3'

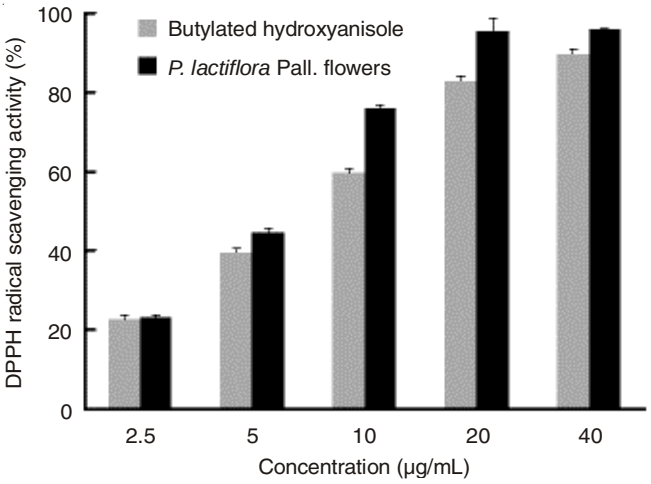

Fig. 2. DPPH radical scavenging activity of *P. lactiflora* Pall. flowers. Data are expressed as means ± SD (n = 3). Butylated hydroxyanisole was used as a positive control

Superoxide anion scavenging assay is commonly used to elucidate the mechanism of antioxidant activity. As shown in Fig. 3, the superoxide anion scavenging activity of *P. lactiflora* Pall. flowers was 15.80 % at 50 μg/mL, 33.18 % at 100 μg/mL, 43.60 % at 200 μg/mL, 60.51 % at 400 μg/mL and 70.62 % at 800 μg/mL. Butylated hydroxyanisole was used as the positive control, demonstrating 13.5, 16.6, 26.04, 40.20 and 73.06 % scavenging activity for 50, 100, 200, 400 and 800 μg/mL of butylated hydroxyanisole, respectively. These results suggests that *P. lactiflora* Pall. flowers had a notable effect on scavenging of superoxide anions.

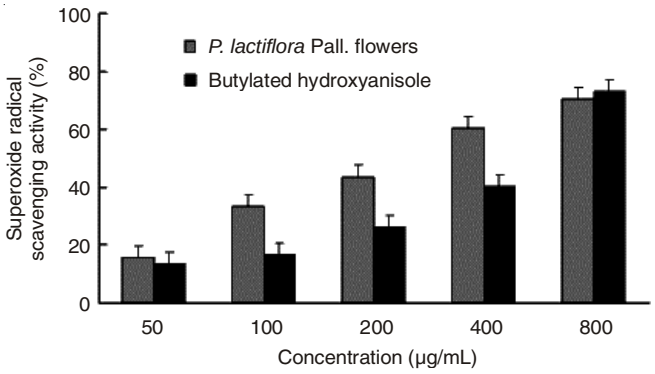

Fig. 3. Superoxide radical scavenging activity of *P. lactiflora* Pall. flowers. Data are expressed as means ± SD (n = 3). Butylated hydroxyanisole was used as a positive control

High absorbance value of an extract indicates high antioxidant activity. As shown in Fig. 4, the total antioxidant activity of *P. lactiflora* Pall. flowers increased with increasing concentration and it was 0.07 at 12.5 μg/mL, 0.13 at 25 μg/mL, 0.19 at 50 μg/mL and 0.37 at 100 μg/mL. Moreover, total antioxidant activity was 0.09 at 12.5 μg/mL, 0.16 at 25 μg/mL, 0.24 at 50 μg/mL and 0.58 at 100 μg/mL for butylated hydroxyanisole.

Proteins are major targets for oxidants due to their high abundance in biological systems and high rate constants for reaction of oxidants. Hydroxyl radical is recognized as a protein-damaging agent with physiological significance [20]. In the present study, protein damage was induced by the Fe^{3+}/H_2O_2/ascorbic acid system. The density of the BSA band decreased to than that of the control after incubation with the Fenton (Fe^{3+}/H_2O_2/ascorbic acid) system (Fig. 5). *P. lactiflora* Pall. flowers protected the cellular proteome (BSA) significantly at the concentration higher than 250 μg/mL. In comparison, 500 μg/mL of butylated hydroxyanisole produced a higher protective effect on the BSA. Results demonstrated that *P. lactiflora* Pall. flowers effectively inhibited BSA oxidation induced by the Fenton system.

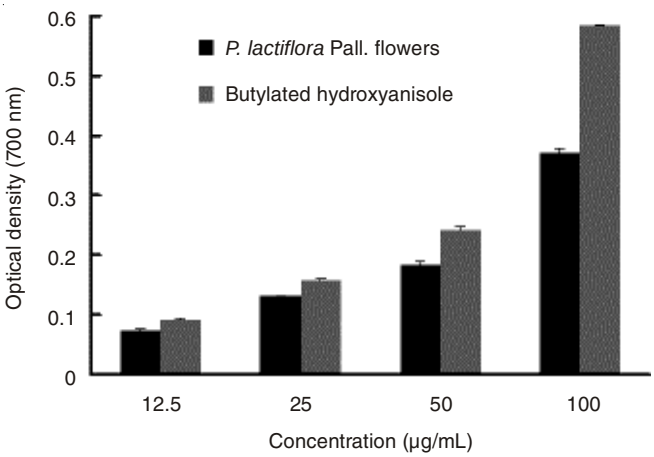

Fig. 4. Total antioxidant activity of *P. lactiflora* Pall. flowers. Data are expressed as means ± SD (n = 3). Butylated hydroxyanisole was used as a positive control

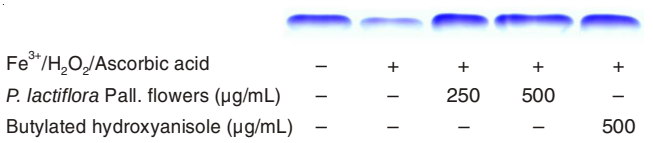

Fig. 5. Protection of BSA oxidative damage by *P. lactiflora* Pall. flowers. Butylated hydroxyanisole was used as a positive control

Anti-inflammatoty activity: Inflammation is a complex biological response to pathogens and damaged cells. Therapy of inflammatory diseases is usually directed at the inflammatory processes. Lipopolysaccharide can activate immune cells to upregulate inflammatory states. To determine whether *P. lactiflora* Pall. flowers is able to suppress the macrophage-mediated inflammatory responses, we first examined the inhibitory effect of *P. lactiflora* Pall. flowers on the production of nitric oxide in lipopolysaccharide-treated RAW264.7 cells. *P. lactiflora* Pall. flowers dose-dependently suppressed the production of nitric oxide (Fig. 6a) in macrophages under lipopolysaccharide stimulation, but did not affect cell viability (Fig. 6b). To understand the molecular mechanism of *P. lactiflora* Pall. flowers inhibition, we investigated its effects in terms of transcriptional control. To do this, we measured the mRNA levels of inflammatory genes using RT-PCR. As shown in Fig. 7, the lipopolysaccharide-induced upregulation of tumor necrosis factor-α, intedeukin-6, cyclooxygenase-2 and inducible nitric oxide synthase was strongly suppressed by treatment with *P. lactiflora* Pall. flowers for 6 h, indicating that the inhibition of inflammatory mediator production can be observed at the transcriptional level.

In China, the dried roots of *P. lactiflora* Pall. has been used in the treatment of rheumatoid arthritis, systemic lupus

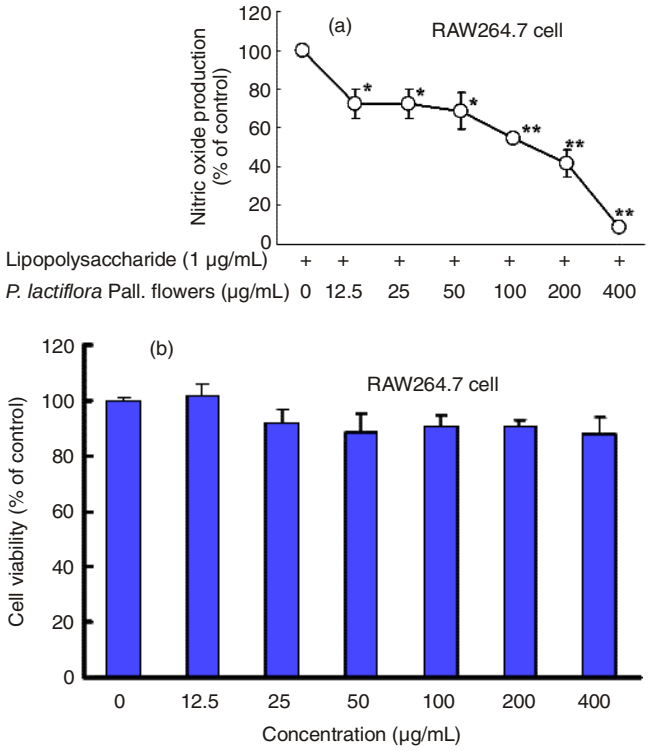

Fig. 6. Effect of *P. lactiflora* Pall. flowers on production of nitric oxide in lipopolysaccharide-activated RAW264.7 cells. (A) RAW264.7 cells (1×10^6 cells/mL) were incubated with *P. lactiflora* Pall. flowers in the presence of lipopolysaccharide (1 μg/mL) for 24 h. Culture supernatants were assayed for nitric oxide determination by Griess assay. (B) RAW264.7 cells (1×10^6 cells/mL) were incubated with *P. lactiflora* Pall. flowers for 24 h. Cell viability was determined by MTT assay. *: $P < 0.05$ and **: $P < 0.01$ compared to control

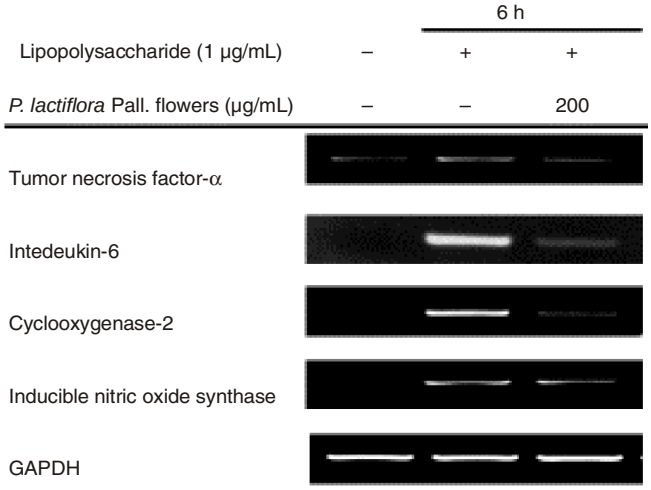

Fig. 7. Effect of *P. lactiflora* Pall. flowers on the mRNA expression of tumor necrosis factor-α, intedeukin-6, inducible nitric oxide synthase and cyclooxygenase-2 from lipopolysaccharide-activated RAW264.7 cells. RAW264.7 cells (5×10^6 cells/mL) were incubated with *P. lactiflora* Pall. flowers in the presence or absence of lipopolysaccharide (1 μg/mL) for 6 h

erythematosus, hepatitis, dysmenorrhea, muscle cramping and spasms and fever for more than 1200 years [21]. Previous studies of *P. lactiflora* Pall. were mainly focused on the root, with monoterpenoid glycosides as the main compounds. *P. lactiflora* flower including abundant nutrients is non-toxic, which makes it have a certain therapeutic effect. During the cultivation of medicinal *P. lactiflora* Pall., above ground biomass is often discarded as litter, resulting in enormous waste of resources. Recent studies indicated that the extracts of *P. lactiflora* flowers were had bioactivity [2]. However, *P. lactiflora* flowers have not been studied so far.

To determine whether the *P. lactiflora* Pall. flowers have antioxidant activity, we evaluated DPPH free radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical scavenging activity, total antioxidant activity and total reducing power of *P. lactiflora* Pall. flowers. In this study, the *P. lactiflora* Pall. flowers exhibited extremely high antioxidant properties, which were demonstrated by its ability to scavenge 50 % of DPPH free radicals at 7.31 μg/mL (Fig. 2) and it eliminated approximately 60.51 % of superoxide anion at 400 μg/mL (Fig. 3). In addition, the *P. lactiflora* Pall. flowers showed strong total antioxidant activity and reducing power and provided protection against oxidative protein damage induced by hydroxyl radicals. These results indicate that *P. lactiflora* Pall. flowers may have potential health benefits by reducing body oxidative stress.

Inflammation is a body defense system that protects us from exogenous pathogens, such as fungi, bacteria and viruses [22]. *P. lactiflora* roots, with immunomodulatory and anti-inflammatory effects, has been widely used as a component of traditional Chinese prescriptions [21]. Inflammation induces oxidative stress by producing oxidants like ROS and nitric oxide. Nitric oxide is also a potent mediator of inflammation. In this study, the *P. lactiflora* Pall. flowers was demonstrated to be effective in inhibiting the production of nitric oxide from lipopolysaccharide-stimulated RAW264.7 macrophages (Fig. 6a). Further experiments showed that *P. lactiflora* Pall. flower inhibited lipopolysaccharide-stimulated tumor necrosis factor-α and intedeukin-6 and inducible nitric oxide synthase and cyclooxygenase-2 release (Fig. 7). These data indicate that EEF anti-inflammatory effects by inhibiting the production of inflammatory mediators, such as nitric oxide and proinflammatory cytokines.

The main bioactive constituents were identified in the *P. lactiflora* Pall. flowers by high-performance liquid chromatography (HPLC). As shown in Fig. 8, the HPLC profile of *P. lactiflora* Pall. flowers was recorded at 280 nm, we observed paeoniflorin peak at 9.2 min. The main bioactive constituent was paeoniflorin in roots of *P. lactiflora* Pall., however our HPLC data showed that major compound of the *P. lactiflora* Pall. flowers is not paeoniflorin. Our data seem to suggest that paeoniflorin can not be bioactive compound of *P. lactiflora* Pall. flowers, other types of bioactive compounds could be present in this extract. Further phytochemical analysis needs to be performed to examine this possibility.

In conclusion, this *in vitro* study provides strong evidence that *P. lactiflora* flowers could be novel therapeutics for oxidative stress related diseases. The *P. lactiflora* Pall. flowers showed free radical scavenging and anti-inflammatory activities. In order to provide a better understanding of the antioxidant and anti-inflammatory function, the molecular mechanism and bioactive constituents of the anti-inflammatory effect elicited by *P. lactiflora* flowers will be investigated in further studies.

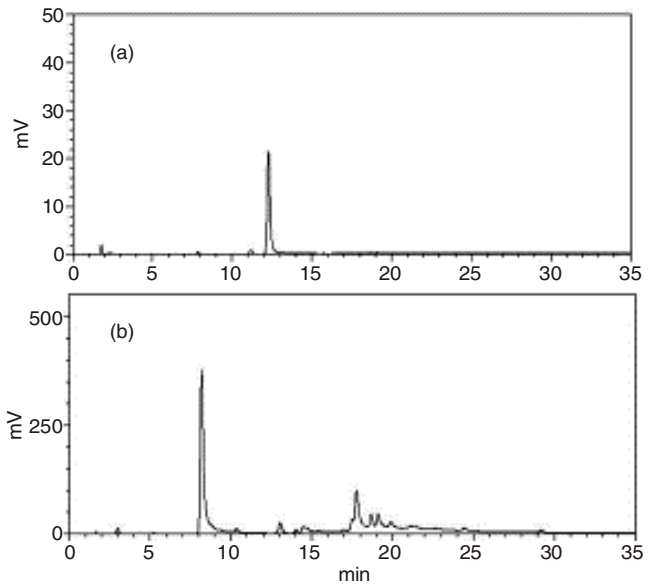

Fig. 8. HPLC chromatogram of paeoniflorin (a) and *P. lactiflora* Pall. flowers ethyl ether extract (b)

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