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Research Paper

Anti-inflammatory and antitumour activity of various extracts and compounds from the fruits of *Piper longum* L.

Ziyan Guo[a,*], Jie Xu[a,*], Jianhua Xia[a], Zi Wu[a], Jiachuan Lei[b] and Jianqing Yu[a]

[a]Key Laboratory of Combinatorial Biosynthesis and Drug Discovery (Wuhan University), Ministry of Education, Wuhan University School of Pharmaceutical Sciences, and [b]Renmin Hospital, Wuhan University, Wuhan, China

Keywords
active compound; anti-inflammatory; antitumour; extraction; *Piper longum*

Correspondence
Jianqing Yu, School of Pharmaceutical Sciences, Wuhan University, 185 Donghu Road, Wuchang District, Wuhan 430071, China.
E-mail: jqyu@whu.edu.cn

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*Ziyan Guo and Jie Xu contributed equally to this work.

Abstract

Objectives To explore effective extraction method and to find active constituents, we investigated the biological activity of three extracts and isolated active compounds from the fruits of *Piper longum* L.

Methods Three extracts from the fruits were obtained by reflux, ultrasonic and supercritical fluid extraction, respectively. Active compounds were isolated by the bioassay-guided method. The anti-inflammatory activity, antiproliferation activity and cytotoxicity were evaluated. The apoptosis was detected by Hoechst 33258 staining assay. The relevant proteins were investigated by Western blot assay.

Key findings The anti-inflammatory activity and cytotoxicity of supercritical fluid extract (SE) were stronger than those of the other two extracts. Among all isolated compounds, the anti-inflammatory activity of eight compounds was stronger than that of indomethacin, and compounds **8**, **9**, **11**, **14** and **15** were found to possess anti-inflammatory effect for the first time. Compounds **1**, **2**, **3** and **14** exhibited significant cytotoxicity against cancer cells. SE and piperine were found to reduce colony formation, inhibit cell migration and promote apoptosis through increasing cleaved PARP and the ratio of Bax/Bcl-2.

Conclusions The anti-inflammatory and antitumour effects of SE were better than those of the other two extracts. The compounds responsible for the activity were elucidated. SE and piperine inhibit cell growth through apoptosis.

Introduction

Natural products, originating from bacterial, fungal, plant and marine animal sources, are widely used in agriculture, human diseases and veterinary medicine. Currently, lots of natural products and natural product-inspired compounds are in clinical trials as anti-inflammatory drugs, anticancer drugs or other pharmaceutical agents.[1] *Piper longum* L. belonging to the *Piperaceae* family is commonly known as 'long pepper' and widely distributed in Indonesia, Sri Lanka, Vietnam, Philippine, the America and China.[2] The excellence of abundant resources, low price and wide application of *P. longum* L. reveal the favourable prospects of development and application. The fruits of *P. longum* L. have a pungent taste and are used as spice and in pickles.[3] *P. longum* L. was used as a component of medicines in the treatment of gonorrhoea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gut related pain and arthritic conditions.[4]

It was reported that water extracts of *P. longum* L. showed good antioxidant activity and its amide alkaloids showed significant scavenging effects on 1,1-Diphenyl-2-picrylhydrazyl radical, 2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) and superoxide radical.[5,6] 95% ethanol extract and hexane fraction showed antifertility effect in female rats.[7] The extract of root had the similar antiamoebic activity to its extract of fruit.[8] Moreover, previous studies have shown that *P. longum* contained a variety of chemical constituents, primarily including alkaloids, saponins, amygdalin and essential oils.[9] Piperine (PIP) was the first amide isolated from *Piper* species and considered as the main active ingredient,[10] which was found to produce cytotoxic towards Dalton's lymphoma ascites cells and Ehrlich ascites carcinoma cells.[4] It exhibited

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antidiarrhoeal and antispasmodic activity by mediating calcium channel, and could modulate permeability characteristics of intestine by inducing alterations in membrane dynamic.[11,12] PIP also possessed bronchodilatory activity and could treat airways disorders.[13] Pipernonaline had the antiproliferation effect, antioxidant activity and antihyperlipidaemic activity, and it could improve cognitive function in a murine model of Alzheimer's disease.[14–17] However, the effects of extraction methods on biological activity and some active ingredients of *P. longum* were still unclear.

There are many methods for the extraction of bioactive compounds from plant including solvent extraction, ultrasonic extraction, microwave-assisted extraction, supercritical fluid extraction, etc. As a kind of solvent extraction, heat reflux extraction needs long extraction times, large quantities of solvents and high temperature,[18] while ultrasonic extraction could reduce the handling time and use of solvent to obtain extraction content.[18] Due to the low operated temperatures, ultrasonic extraction reduces the heat loss caused by high temperatures. Supercritical fluid extraction is a newer environment-friendly method and plays an important role in drug discovery from natural sources. It has the advantages of small amount of solvent, rapid extraction and steady thermally labile compounds.[19]

The active ingredients of extracts from *P. longum* were not very clear. In this work, three extraction methods of *P. longum* were conducted including supercritical fluid extraction, reflux extraction and ultrasonic extraction. The constituents of three extracts were analysed by HPLC, and the chemical composition of SE was further analysed by GC/MS. Cytotoxicity and anti-inflammatory activity of three extracts were investigated. Compounds were isolated from the ethanol extract using a bioassay-guided method, and their biological activity was also evaluated. Compounds **8**, **9**, **11**, **14** and **15** were found to inhibit inflammation for the first time. Then, the mechanism of antitumour activity of extracts and compounds was studied *in vitro*.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI), Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DME/F-12) and fetal bovine serum (FBS) were obtained from Gibco-BRL (Grand Island, NY, USA). PIP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lipopolysaccharide (LPS) and the Griess reagent were purchased from the Sigma-Aldrich (Saint Louis, MO, USA). Indomethacin and cisplatin (CDDP) were obtained from National Institutes for Food and Drug Control (Beijing, China). Primary antibodies against PARP, Bax, Bcl-2 and GAPDH, and horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from ProteinTech Group, Inc. (Rosemont, IL, USA). Polyvinylidene fluoride membrane (PVDF) and Luminata Forte Western HRP Substrate were purchased from Millipore Corp. (Billerica, MA, USA). All other chemicals and solvents were of analytical grade.

Cell culture

Three human cancer cell lines and a macrophage cell line were purchased from China Center for Type Culture Collection (Wuhan, Hubei, China). Cells were maintained in DMEM (HeLa, HepG2 and RAW 264.7) or DME/F-12 (SKOV-3), supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were incubated under an atmosphere of 37°C, 5% CO_2.

Plant material and preparation of extracts

Piper longum was collected from Pingnan County, Guangxi Zhuang Autonomous Region, and identified by Professor Jianqing Yu (School of Pharmaceutical Sciences, Wuhan University, China). A voucher specimen was preserved at the Herbarium of School of Pharmaceutical Sciences, Wuhan University, China.

The fruits of *P. longum* were air-dried and powdered at room temperature. Equal amounts of dried powder (40 g) were respectively extracted three times by reflux (each 3 h) and ultrasonic (each 0.5 h) extraction with 95% ethanol at 55°C. The solvent was evaporated under vacuum subsequently to obtain the reflux extract (RE) and ultrasonic extract (UE), and both the yields of RE and UE were approximately 12%. The supercritical fluid extraction was achieved at the temperature of 55°C and the pressure of 20 MPa for 1 h, and the yield was 10%.

HPLC analysis of extracts

Chromatographic analysis of the SE, UE and RE was achieved using a LC-20AT prominence liquid chromatograph with the SPD-M20A PDA detector (SHIMADZU). A Sepax GP-18 column (5 μm, 2.1 × 100 mm i.d.) was used. The mobile phase consisted of water (solvent A) and methanol (solvent B). Elution was carried out with a linear gradient from 60% to 100% of B in 40 min. The flow rate was 0.5 ml/min, and the sample injection volume was 5.0 μl. The UV spectra were registered in 254 nm.

GC/MS analysis

The determination of chemical constituents of SE was achieved using a GC/MS-TQ8040 system (SHIMADZU). The SH-Rxi-5Sil ms column (30 m × 0.25 mm × 0.25 μm)

was used. The column temperature was initially 60°C for 1 min, then increased to 150°C at 30°C/min, kept there for 5 min, and further increased to 260°C at 5°C/min, kept there for 10 min, and finally increased to 300 at 3°C/min.

Isolation of compounds

The dried and powdered fruits of *P. longum* (4.4 kg) were extracted three times with 95% ethanol (25L) under reflux at 65°C. Then, the crude extract (544.7 g) was dissolved in deionized water and partitioned with ethyl acetate. The resulting fraction was subjected to silica gel (100–200 mesh) column (50 × 10 cm) chromatography, eluted with petroleum ether/ethyl acetate (from 20 : 1 to 1 : 1) mixtures, analysed by thin-layer chromatography, then purified by Sephadex LH-20 gel column to obtain fifteen compounds. All these compounds were identified by spectroscopic methods.

Anti-inflammatory activity assay

RAW 264.7 murine macrophage cell proliferation was measured by MTT assay. The anti-inflammatory assay was performed with the concentration of the extracts or compounds without cytotoxic activity. RAW 264.7 cells were treated with samples and 1 μg/ml LPS for 24 h. The concentration of nitric oxide (NO) in cell culture medium was detected by a Griess assay as previously described.[20] Dimethyl sulfoxide (DMSO, 0.5%) was used as vehicle control, and indomethacin was used as a positive control.

Cytotoxicity assay

The cytotoxic activity of extracts and compounds was tested on various human cancer cells. Cells were treated with samples for 48 or 72 h. MTT assay was performed to measure cytotoxicity as described previously with minor modifications.[21] DMSO (0.5%) was used as vehicle control, and CDDP was used as a positive control. Finally, the absorbance was read at 570 nm.

Colony formation assay

Colony formation assay was performed on SKOV-3 cells as described previously with some modifications.[22] Cells were treated with samples for 24 h. Then, fresh drug-free medium was replaced for additional 10 days. Cells were fixed with methanol and stained with 0.1% crystal violet. Then, the colonies were scored.

Wound healing assay

Wound healing assay was performed on SKOV-3 cells as previously described.[23] SKOV-3 cells were seeded in a 6-well plate and incubated until wells were covered completely. The wound on cells was inflicted, and cells were treated with samples for 24 h. Width of the wound was measured by Photoshop software.

Hoechst 33258 staining assay

Hoechst 33258 staining assay was performed on SKOV-3 cells as previously described.[24] Cells were treated with samples for 24 h, washed with PBS and fixed with 4% methanol for 10 min, and stained with Hoechst 33258 for 30 min. The photographs were taken by an inversion fluorescence microscope.

Western blot assay

The cells were harvested after treatment with samples for 24 h. The proteins were separated by 12% SDS–PAGE and transferred onto PVDF membranes. After the blocking with 5% nonfat milk for 1 h, the membranes were incubated overnight with antibodies against the following primary antibodies: PARP (1 : 500), Bax (1 : 1000), Bcl-2 (1 : 1000) and GAPDH (1 : 6500). Membranes were incubated with secondary antibodies (1 : 10 000) for 1 h and treated with the Luminata Forte Western HRP Substrate.[25] The bands were quantified by ImageJ software.

Statistical analysis

All experiments were repeated at least three times. All data were expressed as means ± SD. Statistical significance was evaluated by an ANOVA. Differences were considered to be significant when a $*P$ value was less than 0.05 and a $**P$ value was less than 0.01.

Results

Chemical analysis of three extracts from the fruits of *Piper longum*

Three extracts had similar major constituents according to their HPLC chromatograms (Figure S1). Chemical composition of SE was analysed by GC/MS (Figure 1), and the detailed component analysis of SE was shown in Table S1. Forty-six compounds were identified through GC/MS analysis. The most abundant component was piperine with the content of 37.71%, and the major phytochemicals of SE also included (2E,4E,14E)-*N*-(2-methylpropyl)-2,4,14-eicosatrienamide (9.75%), piperanine (8.73%) and pipereicosalidine (8.58%). These compounds represented more than 60% contents of SE. Other minor components were also detected.

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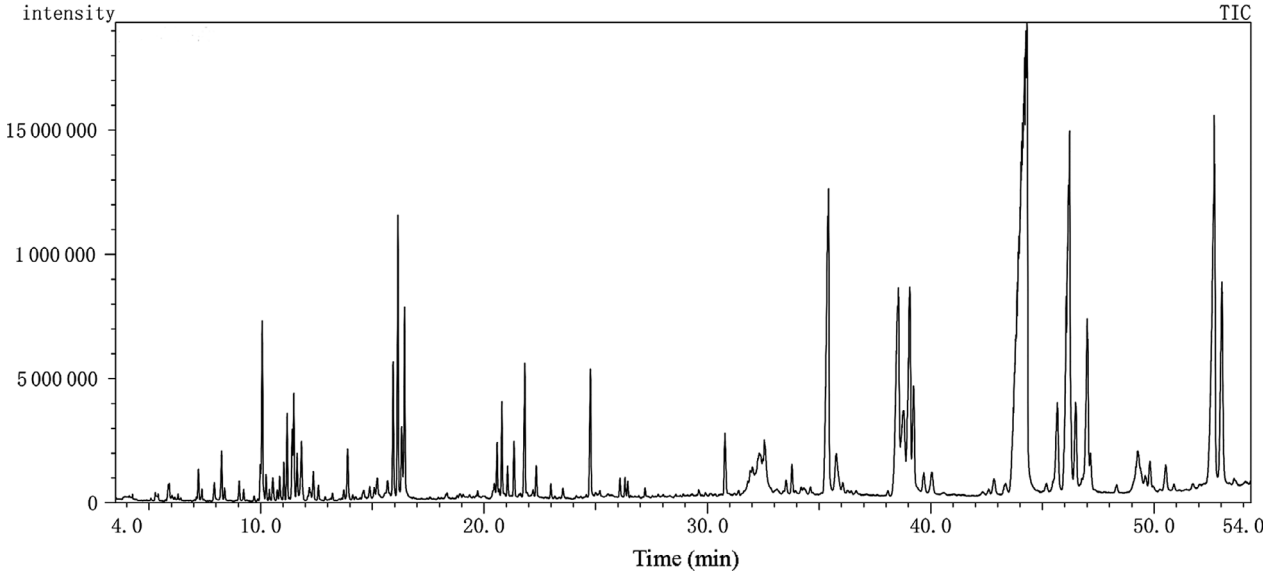

Figure 1 Total ion chromatogram of SE from the fruits of *Piper longum* L.

Compounds isolated from *Piper longum*

Fifteen compounds (Figure 2) were obtained from the ethanol extract and identified by comparison of spectroscopic characteristics with literature.[9,26–31] Fifteen compounds included were as follows: piperine (**1**) (630 mg), guineensine (**2**) (17 mg), pipercide (**3**) (44 mg), dihydropiperlonguminine (**4**) (14 mg), piperlonguminine (**5**) (12 mg), piperchabamide B (**6**) (43 mg), methyl piperate (**7**) (16 mg), 1,2-dihydroxybisabola-3,10-diene (**8**) (19 mg), (2E,4E)-*N*-Isobutyl-2,4-octadecadienamide (**9**) (22 mg), (2E,4E,14Z)-*N*-Isobutyldocosa-2,4,14-trienamide (**10**) (19 mg), (2E,4E,14Z)-*N*-Isobutyleicosa-2,4,14-trienamide (**11**) (19 mg), piperonal (**12**) (30 mg), pellitorine (**13**) (24 mg), pipernonaline (**14**) (23 mg) and piperanine (**15**) (36 mg). The data of ^{1}H-NMR and ^{13}C-NMR are shown in Appendix S1.

Extracts and compounds on the inhibitory of NO

Nitric oxide is regarded as a pro-inflammatory marker, and LPS could stimulate RAW 264.7 macrophage cells and increase the level of NO in culture supernatant.[32] Three extracts and fifteen compounds were evaluated for their anti-inflammatory activity by detecting the inhibitory effect of NO. The anti-inflammatory activity of all extracts was stronger than that of indomethacin, and supercritical fluid extract (SE) exhibited the strongest activity with the IC_{50} value of 4.19 ± 0.38 μg/ml (Table 1). Eight compounds exhibited more effective anti-inflammatory activity compared with indomethacin. Compounds **14** and **15** showed the strongest anti-inflammatory activity with the IC_{50} values of 2.48 ± 0.17 and 3.02 ± 0.06 μM, and compounds **1**, **6**, **8**, **9**, **11** and **13** also exhibited strong anti-inflammatory activity (Table 2). Of which, compounds **8**, **9**, **11**, **14** and **15** were found to inhibit inflammation for the first time. The data suggested that *P. longum* contained various anti-inflammatory compounds and the anti-inflammatory activity of extracts might be attributed to these compounds.

Cytotoxicity of extracts and compounds

Extracts were evaluated for their cytotoxicity against HepG2, HeLa and SKOV-3 cancer cell lines for 48 h. All extracts exhibited cytotoxicity against three cell lines in a dose-dependent manner, and SE showed prominent cytotoxicity compared with other extracts (Table 3). The cytotoxicity of fifteen isolated compounds was also evaluated on these cell lines for 72 h. Compound **2** showed the strongest cytotoxicity with the IC_{50} values of 14.90 ± 0.62, 17.13 ± 1.06 and 17.96 ± 0.39 μM against HepG2, HeLa and SKOV-3, respectively. Compounds **1**, **3** and **14** also exhibited strong cytotoxic effect (Table 4). Other compounds did not show obvious cytotoxicity with the IC_{50} values of >100 μM.

Effects of SE and PIP on colony formation

Since the biological activity SE exhibited was slightly stronger than other two extracts and PIP was the main ingredient of *P. longum*, further investigations were focused on the mechanism of antitumour effect of SE and PIP in SKOV-3 ovarian cancer cells. Colony formation assay was

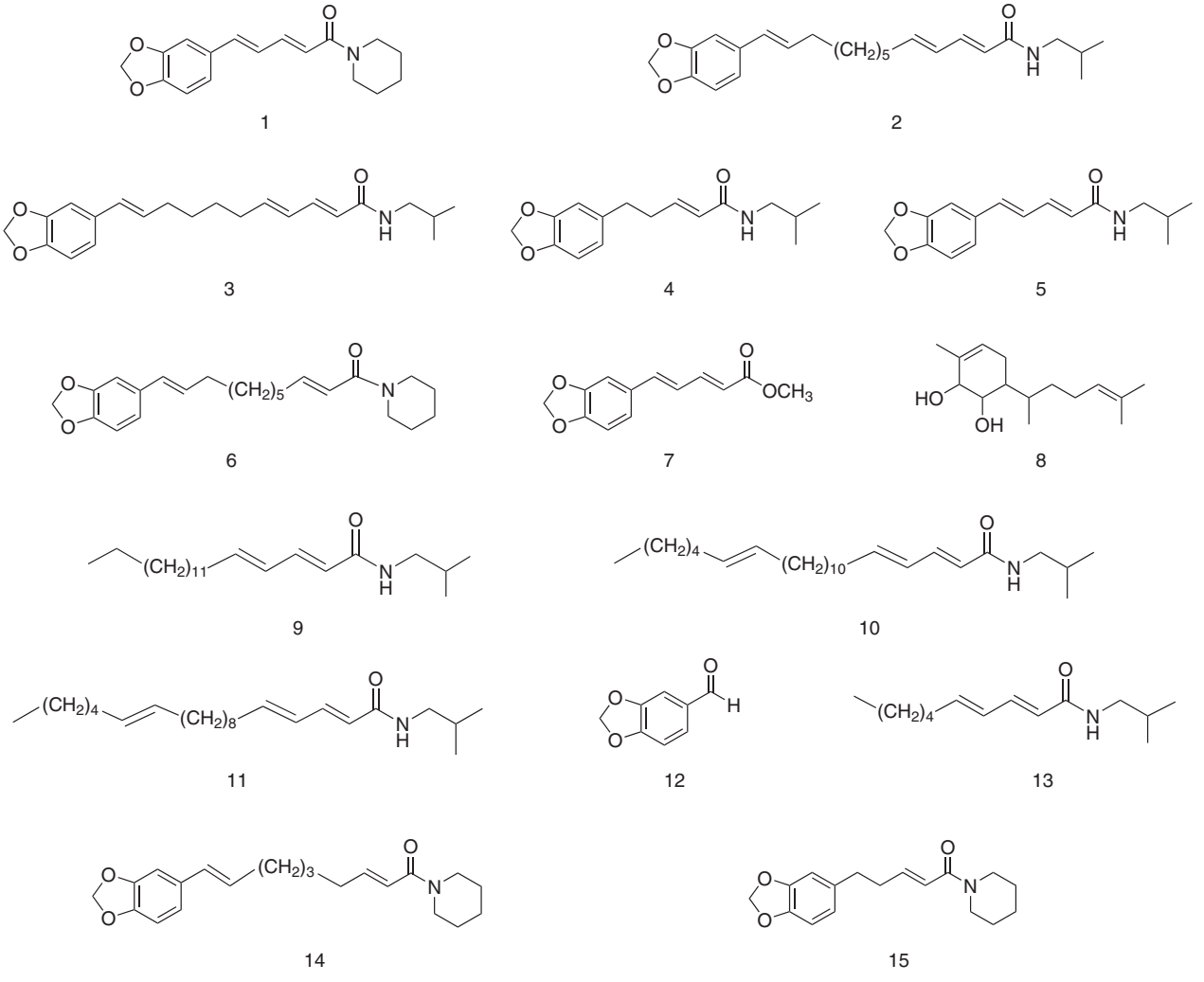

Figure 2 Chemical structures of the compounds **1-15** isolated from *Piper longum.*

Table 1 NO inhibition of three extracts from *Piper longum* in LPS-stimulated RAW264.7 cells[a]

Extract	IC_{50} (μg/ml)
RE	6.15 ± 0.78
UE	7.13 ± 1.16
SE	4.19 ± 0.38
Indomethacin	15.40 ± 3.35

[a]Data are described as means ± SD, $n = 3$.

used to evaluate the long-term effect of SE or PIP treatment on SKOV-3 cell growth, and colonies with ≥50 cells after treatment were counted as positive. After treatment with different concentrations of SE, the colony information efficiency was reduced by approximately 43% at 80 μg/ml and 63% at 100 μg/ml (Figure 3a). After treatment with PIP, the efficiency was reduced by approximately 55% at 80 μg/ml and 81% at 100 μg/ml (Figure 3b).

Effects of SE and PIP on cell migration

The migration effects of SE and PIP on SKOV-3 cells were evaluated by wound healing assay. The migration rate was 62% at 60 μg/ml, 35% at 80 μg/ml and 22% at 100 μg/ml after treatment with SE for 24 h (Figure 4a). The migration rates were around 48%, 27% and 22% in the presence of 60, 80 and 100 μg/ml of PIP, respectively (Figure 4b). The results demonstrated that both SE and PIP inhibited migration of SKOV-3 cells in a dose-dependent manner. By comparing two groups of photographs, PIP showed stronger inhibitory effect than SE.

SE and PIP induced cell apoptosis

Apoptotic SKOV-3 cells were detected by Hoechst 33258 staining. Condensation of chromatin, fragmentation of

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Table 2 Nitric oxide inhibition of fifteen compounds from *Piper longum* in LPS-stimulated RAW264.7 cells[a]

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1	31.36 ± 2.13	**6**	38.86 ± 9.79	**11**	26.26 ± 6.06
2	ND[b]	**7**	>100	**12**	>100
3	ND[b]	**8**	30.75 ± 4.86	**13**	15.40 ± 5.99
4	93.44 ± 1.77	**9**	7.21 ± 1.87	**14**	2.48 ± 0.17
5	>100	**10**	59.12 ± 15.15	**15**	3.02 ± 0.06

[a]Data are described as means ± SD, $n = 3$. [b]The IC_{50} values were not determined for their limited solubility (<25 μM) in medium.

Table 3 Cytotoxicity of three extracts against three cancer cell lines[a]

	IC_{50} (μg/ml)		
Extract	HepG2	HeLa	SKOV-3
RE	25.34 ± 0.45	58.10 ± 0.26	74.38 ± 2.42
UE	25.10 ± 0.25	39.31 ± 1.58	84.97 ± 8.40
SE	21.91 ± 1.44	30.44 ± 1.48	68.92 ± 2.25
CDDP	2.75 ± 0.09	6.37 ± 0.18	9.09 ± 0.26

[a]Data are described as means ± SD, $n = 3$.

Table 4 Cytotoxicity of compounds against various cancer cell lines[a]

	IC_{50} (μM)		
Compound	HepG2	HeLa	SKOV-3
1	43.94 ± 2.80	75.52 ± 2.52	56.91 ± 0.21
2	14.90 ± 0.62	17.13 ± 1.06	17.96 ± 0.39
3	27.74 ± 1.72	39.27 ± 7.79	23.46 ± 0.06
14	53.27 ± 2.02	60.30 ± 3.77	60.36 ± 0.20
CDDP	6.59 ± 0.16	13.63 ± 1.06	4.66 ± 0.16

[a]Data are described as means ± SD, $n = 3$.

nucleus and formation of apoptotic bodies were mainly morphologic characteristics of apoptosis.[33] These morphologic changes were clearly observed after SE or PIP treatment at the concentrations of 80 and 100 μg/mL, but no characteristic of apoptosis presented in control group (Figure 5).

SE and PIP regulated expression of apoptosis-related genes

Most anticancer strategies which induced cell death were associated with the activation of apoptosis.[34] Apoptosis-related genes were detected in our study, including PARP, Bcl-2 and Bax. PARP protein is an important biomarker of apoptosis.[35] Bcl-2 family of proteins are key regulators of apoptosis, Bcl-2 protein promoted cell survival, and Bax promoted cell death; therefore, the ratio of Bax/Bcl-2 is always considered as a crucial factor in determining susceptibility to apoptosis.[36]

After treatment of SKOV-3 cells with SE, the levels of cleaved PARP and Bax were upregulated and Bcl-2 was downregulated (Figure 6a). After treatment with PIP, the level of cleaved PARP was upregulated; meanwhile, the level of Bcl-2 was significantly downregulated (Figure 6b). Both the expression of cleaved PARP and the ratio of Bax/Bcl-2 were increased after treatment with SE and PIP. These results were in accordance with the apoptotic results of the Hoechst 33258 staining and demonstrated that both SE and PIP induced apoptosis by increasing the protein of cleaved PARP and the ratio of Bax/Bcl-2 on SKOV-3 cells.

Discussion

Piper longum L. were used as both a traditional medicine and a seasoning in many countries due to its biological activity and flavour. Owing to the characteristics of the high production and the wide range of application, *P. longum* L. has broad development prospects in the field of food and pharmacological industries. This herbal drug could be used to treat gonorrhoea, respiratory tract infections, tuberculosis analgesic, anxiolytic and arthritis.[37] In practical use, the key purpose of plant material extraction is to obtain large amounts of extract rich in the desired active compounds in a time-sensitive and cost-effective manner. Three extracts from the dried fruits of *P. longum* L. were obtained by reflux, ultrasonic and supercritical fluid CO_2 extraction, respectively. Since three extracts had similar principal components and the bioactivity of SE was better, the chemical constituents of SE were further analysed by GC/MS. The chemical composition of SE included amide, terpenes, olefins and ester.

Inflammation is involved in many complex diseases and disorders including autoimmune diseases, metabolic syndrome, neurodegenerative diseases, cancers and cardiovascular diseases.[38] In this study, murine RAW 264.7 macrophage cell line was stimulated to produce NO by LPS. And the anti-inflammatory activity was evaluated by the inhibition rate of NO release after the treatment of three extracts and fifteen active compounds. Indomethacin is a member of the nonsteroidal anti-inflammatory drug class with strong effects. Therefore, we chose the indomethacin as a positive control. Our study demonstrated that the anti-inflammatory activity of the three extracts was stronger than indomethacin, and SE showed the strongest anti-inflammatory activity. Bioassay-guided separation is a rapid and effective method for isolation of compounds with bioactivity.[39] The ethyl acetate fraction exhibited strong activity, and fifteen compounds were isolated from

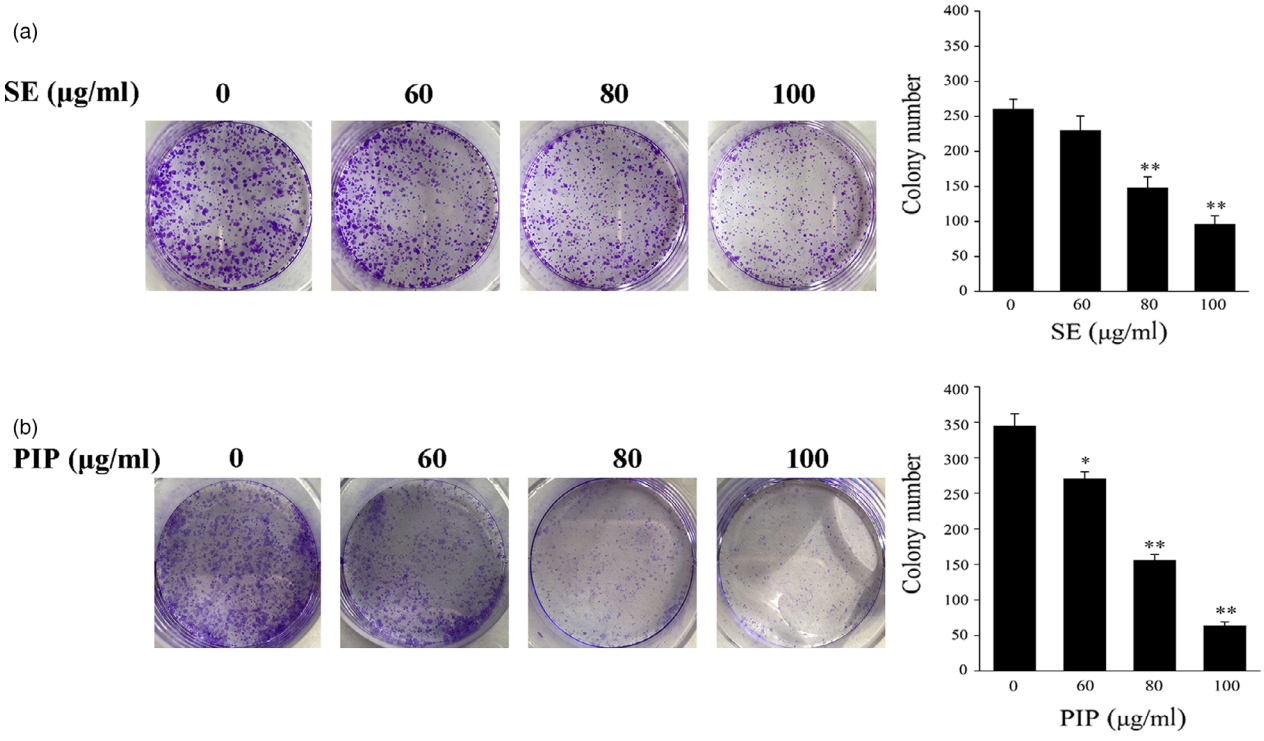

Figure 3 Colony formation of SKOV-3 cells treated with serial concentrations of SE (a) and PIP (b) for 24 h. The results are from three independent experiments (n = 3). Values are the means ± SD. of triplicate experiments. *P < 0.05 and **P < 0.01 for the comparison with control. PIP, piperine. [Colour figure can be viewed at wileyonlinelibrary.com]

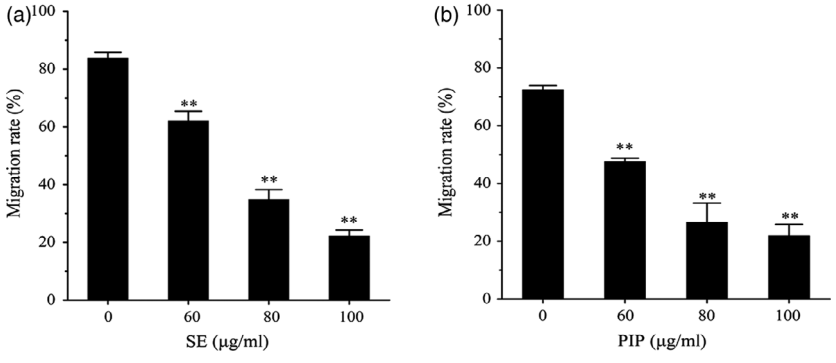

Figure 4 Wound healing of SKOV-3 cells treated with serial concentrations of SE (a) and PIP (b) for 24 h. The results are from three independent experiments (n = 3). Values are the means ± SD of triplicate experiments. *P < 0.05 and **P < 0.01 for the comparison with control. PIP, piperine.

this fraction by column chromatography. Among the fifteen compounds, the anti-inflammatory activity of compounds **14** and **15** was strongest. Moreover, we demonstrated that compounds **8**, **9**, **11**, **14** and **15** had anti-inflammatory effect for the first time.

Three human cancer cells including cervical cancer HeLa cells, hepatic cancer HepG2 cells and ovarian cancer SKOV-3 cells were used to evaluate the cytotoxicity of three extracts and fifteen ingredients. We found that all three extracts inhibited the growth of these three tumour cells, and the activity of SE was stronger than the other two extracts in HepG2, HeLa and SKOV-3 cells. In addition, compound **2** among fifteen ingredients showed the strongest cytotoxicity against three humour tumour cell lines *in vitro*.

Piperine was the main ingredient of *P. longum* fruits which determines the unique flavour of *Piper* species. Supercritical fluid extract showed the best activity among the three extracts. Therefore, the mechanisms of antitumour effect of SE and PIP were further investigated in SKOV-3 cells. The ability to inhibit cell proliferation and migration was evaluated by cell colony formation assay and wound healing assay. The results showed that both SE

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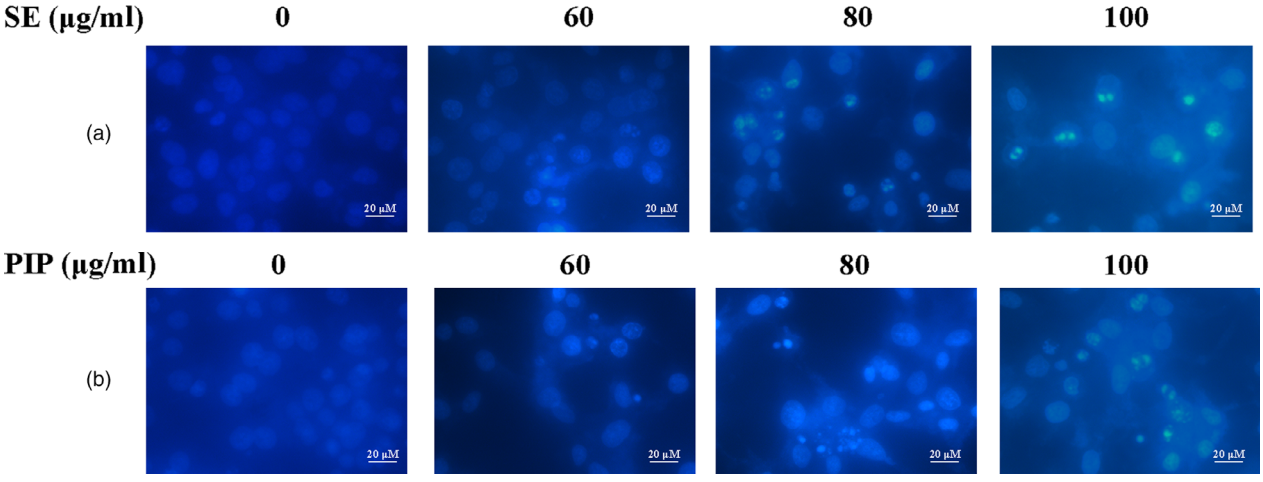

Figure 5 SKOV-3 cells were treated with SE (a) or PIP (b) for 24 h, and their morphology was analysed with Hoechst 33258 staining under a fluorescent microscope. The morphological changes in cell apoptosis such as condensation of chromatin and nuclear fragmentations were seen clearly after the treatment with SE or PIP. PIP, piperine. [Colour figure can be viewed at wileyonlinelibrary.com]

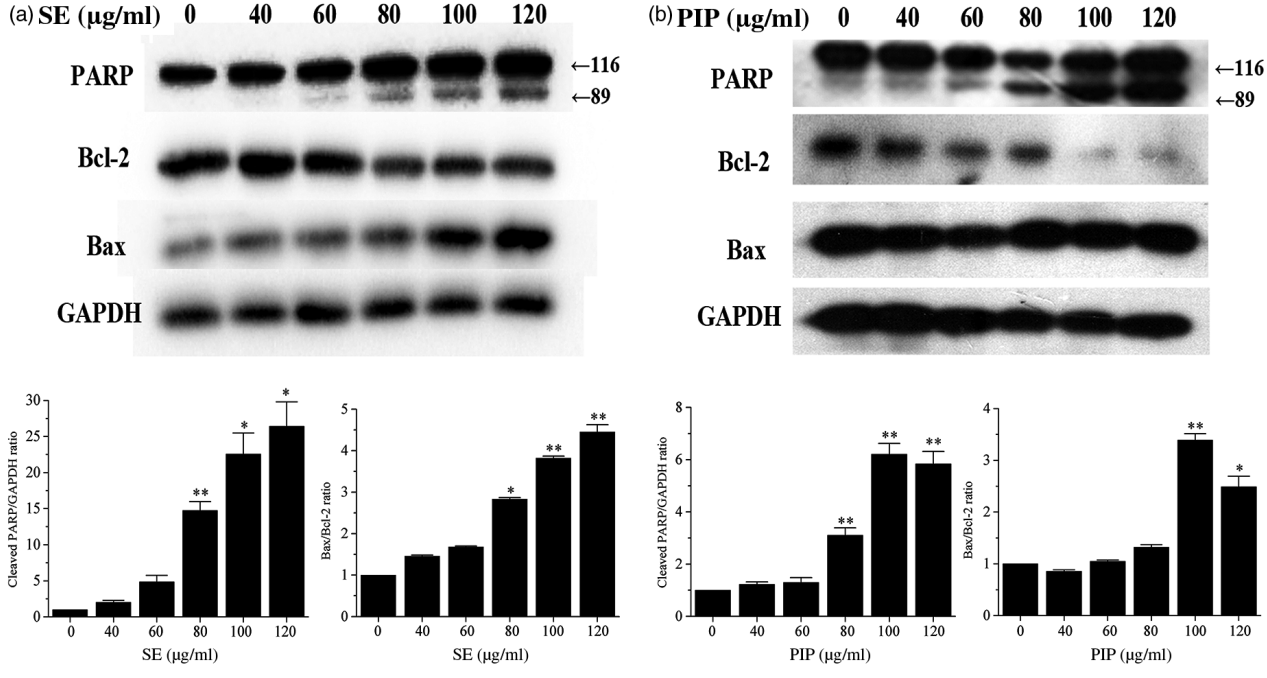

Figure 6 The treatment effects of SE and PIP on protein levels of apoptosis-related genes. (a) The changes in expression levels of PARP, Bax and Bcl-2 after the treatment with SE. (b) The changes in expression levels of PARP, Bax and Bcl-2 after the treatment with PIP. Data are presented as mean ± SD of three independent experiments. The asterisks indicate a significant difference (*$P < 0.05$ and **$P < 0.001$). PIP, piperine.

and PIP could inhibit the proliferation and migration of SKOV-3 cells in a dose-dependent manner. And the inhibitory effect of PIP is slightly stronger than that of SE.

There are numerous methods to detect the apoptosis through the morphological, biochemical and molecular changes undergoing in a cell during this process.[40] Hoechst 33258 fluorescent staining is a simple and reliable method to detect the apoptosis through observing apoptotic cells morphologically. Known as bisbenzimides, the Hoechst stains are a family of fluorescent dyes largely employed to stain the DNA molecule in molecular biology applications, allowing one to visualize the DNA with fluorescence microscopy.[41] After stained with Hoechst 33258, cell shrinkage, nucleus chromatin condensation and the formation of apoptotic bodies were observed under microscopes, suggesting that SE and PIP inhibited the growth of

SKOV-3 cells by inducing apoptosis. This speculation was further validated by Western blot assay through the detection of the expression level of apoptosis-related proteins. PARP is a cleavage substrate of the caspase-3. After the SKOV-3 cells were treated with SE or PIP drugs, the cleavage of PARP was observed in the process of apoptosis. At the same time, the expression level of Bax was increased by SE or PIP treatment, while the expression level of Bcl-2 decreased. Therefore, the ratio of Bax/Bcl-2 gradually increased. The pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins play an important role in regulating the apoptosis.[42] These results together proved that SE and PIP inhibit cell growth by inducing apoptosis, which indicated their potential antitumour activity.

There is increasing evidence that inflammatory cells have powerful effects on tumour development. These cells are powerful tumour promoters, producing an attractive environment for tumour growth, facilitating genomic instability and promoting angiogenesis in the early neoplastic process.[43] And neoplastic cells also divert inflammatory mechanisms to favour neoplastic spread and metastasis in advanced neoplasia.[43] Inflammation substantially contributes to the development and progression of cancer. However, the inflammatory cells may also suppress tumour growth. The association between the inflammation and cancer about these extracts or compounds could be further investigated in future.

Conclusions

Three extracts with different extraction methods and fifteen compounds were obtained from the fruits of *P. longum*, and their biological activity was evaluated in this study. Three extracts had similar major constituents. The anti-inflammatory activity and cytotoxicity of SE were stronger than other two extracts. Compared with two traditional extraction methods, supercritical fluid CO_2 extraction could obtain the larger amounts of extract rich in the desired active compounds. Among fifteen compounds, four compounds showed cytotoxic activity, and five compounds were found to inhibit inflammation for the first time, especially eight compounds with stronger anti-inflammatory activity than indomethacin. Furthermore, SE and PIP were found to have the antitumour effect through inhibiting cell proliferation and migration. The characteristics of apoptosis were detected, and the cleaved PARP and the ratio of Bax/Bcl-2 were significantly increased after the treatment with SE and PIP. Our study demonstrated SE and PIP induced apoptosis by increasing the protein levels of cleaved PARP and the ratio of Bax/Bcl-2, resulting in cell death of cancer cells. These results from the current study showed that *P. longum* contained various active ingredients.

Declarations

Conflict of interest

The Authors declare that there are no conflicts of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. HPLC chromatograms of three extracts from the fruits of *Piper longum* L.(a) SE, (b) UE, (c) RE.

Table S1. Detailed component analysis of SE by GC/MS.

Appendix S1. The Data of ^{1}H-NMR and ^{13}C-NMR of compounds **1–15**.