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ORIGINAL ARTICLE

Antitumor Potential of Peptides Isolated from *Brucea javanica* Globulin Fraction on MCF-7 Cells

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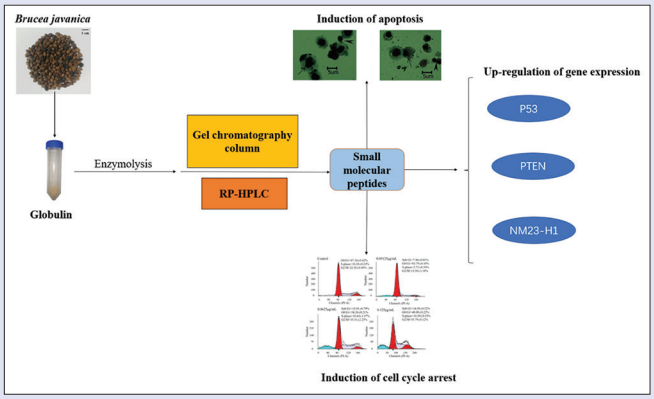
ABSTRACT

Background: Breast cancer is the most common malignancy in women worldwide, so it is imperative to find new medications for this disease. The dry seeds of *Brucea javanica* have been extensively used in Traditional Chinese Medicine (TCM) for the treatment of tumor. **Objectives:** To isolate antitumor peptides from *B. javanica* seeds and research their antitumor activity with MCF-7 cells. **Materials and Methods:** Globulin of *B. javanica* was extracted and enzymatically hydrolyzed by pepsin. Then ultrafiltration, gel filtration chromatography (GFC), and reverse-phase high-performance liquid chromatography (RP-HPLC) were employed to purify the peptides. Anticancer activity was studied in humans in human breast cancer MCF-7 cells, MTT (3- [4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)-assessed cell viability, Giemmsa staining determined whether the cells should undergo apoptosis, and apoptosis and cycle assays assessed the antitumor mechanism of the cells. Real time quantitative polymerase chain reaction (RT-qPCR) was used to study the alterations of oncogenes and cancer metastasis suppressor genes in MCF-7 cells treated with peptide extracts. **Results:** The fraction F9-9 extracted from *B. javanica* significantly inhibited the proliferation of MCF-7 cells with the IC_{50} of 0.124 ± 0.004 μg/mL. The possible mechanisms of this fraction were further explored. F9-9 obviously induced cell cycle arrest at G0/G1 phase and promoted apoptosis of MCF-7 cells. The underlying molecular mechanisms were explored by RT-qPCR and results showed that in *B. javanica* small molecular peptides upregulated the expression of tumor suppressor genes P53 and PTEN as well as tumor metastasis suppressor gene NM23H-1. **Conclusion:** This study suggests that *B. javanica* peptides have therapeutic activity and mechanisms for antibreast cancer, encouraging further isolation and purification to obtain monomeric peptides of this extract for anticancer use.

Key words: Apoptosis, biopeptide, breast cancer, *Brucea javanica*, enzymatic hydrolysis, globulin fraction

SUMMARY

- Dried fruits of *Brucea javanica* are commonly used int TCM. Antituomor is the most outstanding pharmacological function of this herb. In this study, globulin of *B. javanica* was extracted and enzymatically hydrolyzed by pepsin. Then ultrafiltration, gel filtration chromatography (GFC), and reverse phase high-performance liquid chromatography (RP-HPLC) were employed to purify the peptides. we found that the isolated peptide fraction of *B. javanica* significantly reduced the viability of MCF-7 cells. In addition, this fraction induced apoptosis in MCF-7 cells at high concentrations and G0/G1 phase cell cycle arrest at low concentrations, and it induced upregulation of the expression of tumor suppressor genes P53 and PTEN as well as tumor metastasis suppressor gene NM23-H1. Therefore, the small molecular biopeptide of *B. javanica* would be a promising candidate bioactive agent for melanoma treatment.



Abbreviations used: TCM: Traditional Chinese Medicine; GFC: Gel Filtration Chromatography; RP-HPLC: Reverse-Phase High-Performance Liquid Chromatography; RT-qPCR: Real Time Quantitative Polymerase Chain Reaction; ER: Estrogen Receptor; PR: Progesterone Receptor; HER2: Human Epidermal Growth Factor Receptor-2.

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INTRODUCTION

Bioactive peptides are peptide sequences within proteins that have a positive impact on human health.[1] They play crucial roles in human physiological regulation including antimicrobial, antithrombotic, antihypertensive, antitumor, opioid, immunomodulatory, and antioxidative. Moreover, the high selectivity, safety, efficacy, and bioavailability make them better alternatives compared with therapeutic classes. Peptides are also easily optimized and evaluated as potential therapeutics for many diseases.[2] Given their well-tolerated properties compared with classical chemotherapies, peptides thus became a novel therapeutic strategy and have been paid more scientific attraction.[3,4] The sources of obtaining peptides include natural products, proteolysis, chemical synthesis, and genetic engineering etc.[5,6] Among them, peptides originated from natural proteins have given more attention, due to the enzymatic treatment of the interested protein at specific pH

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and temperature. This approach takes the advantage of easy scale-up, short reaction times, and access to sequences that may not be available during normal digestion.[1] Because plant seeds have more proteins, they are selected for isolation and extraction of bioactive peptides. Until now, various studies have reported the extraction of bioactive peptides from plant seeds. For example, bioactive peptides isolated from sesame have been reported to have *in vitro* antioxidant and antihypertensive potential.[7] Lunasin, a peptide derived from soybeans, has been shown to be chemopreventive against oncogenes and chemical carcinogens in mammalian cells and in the skin cancer mouse model.[8] Medicinal herbs of Traditional Chinese medicine (TCM) are now recognized as an effective complementary and alternative medicine modality global wide because of their fewer side effects.[9] Therefore, the herbal and traditional remedies for the cancer's treatment attract more and more attention.

Breast cancer is the most common cancer and has the second-highest cancer-related morbidity in women worldwide.[10] Based on the molecular markers, breast cancer is clarified into three major subtypes: hormone receptor-positive/ERBB2 negative, ERBB2 positive, and triple negative. The typical remedies are tamoxifen, letrozole, trastuzumab, pertuzumab, docetaxel, adriamycin, and so on.[11] However, all these drugs have certain side effects. For example, in 20%–30% of patients with estrogen receptor-positive breast cancer, tamoxifen therapy fails due to the presence or development of resistance.[12] At present, the modern medical emphasis is on the prevention, and treatment of cancer with naturally nutritional components.[13]

Brucea javanica (L.) Merr. an evergreen shrub of Simarouaceae family, is widely distributed in the tropical and subtropical area of China, including Guangxi, Guangdong, Yunnan, Fujian, and Taiwan.[14] The fruits of *B. javanica* named as Ya-dan-zi in TCM are bitter in taste and cold in nature and has been used for the treatment of various types of diseases.[15] *B. javanica* has demonstrated a variety of pharmacological properties, for instance antitumor, antimalaria, and anti-inflammation, antiphytovirus, and antifeedant.[14-16] The most remarkable biological effect of *B. javanica* is the antitumor and the corresponding constituents included nigakilactones, alkaloids, triterpenoids, flavonoids, and fatty acids.[17] *B. javanica* and *B. javanica* oil, which is a complex mixture of fatty acids and their derivatives have been applied in-clinic treatment of various cancers, commonly administrated alone or in combination with chemotherapy or radiotherapy.[18] Quassinoids, as the main component also have significant antitumor effects. The study has shown that quassinoids have a significant inhibitory effect on nonsmall cell lung cancer cell lines *in vitro*.[19] Our previous study showed that enzymatic hydrolysates of *B. javanica* globulins inhibited the proliferation of breast cancer cells MCF-7 at the different extent and pepsin hydrolysate manifested the strongest effect among all tested enzymes. Hence, the intention of this study was designed to 1) obtain highly active peptide fractions from the *B. javanica* hydrolysate of globulin fraction that was prepared by enzymatic digestion and then purified by various methods and 2) investigate the *in vitro* antiproliferative mechanism of the biopeptides on human breast cancer cells MCF-7 by cell cycle arrest, apoptosis promotion, and the tumor inhibition-related gene expression at mRNA level.

MATERIALS AND METHODS

Preparation of small molecular peptides (≤3 KD) from *B. javanica* globulin fraction

The dry seeds of *B. javanica* [Figure 1] (Guangxi, Lot No. 20160325) were bought from Beijing Tongrentang (Beijing, China) and were identified by Prof. Chunsheng Liu of Beijing University of Chinese Medicine. The voucher specimen has been deposited in the School of Life Sciences, Beijing University of Chinese Medicine. Globulin fraction was prepared according to the method described by Basu.[20] Briefly,

Figure 1: The dry seeds of *B. javanica*

defatted meals were solubilized in six times the volume of distilled water. After incubation with agitation at 4°C for 30 min, the sample was centrifuged for 20 min at 10,000 rpm, and the supernatant (albumin fraction) was discarded followed by the second repetition. After then, globulins were extracted from the pellet, in the same manner, using 5% NaCl and the supernatant was carefully collected. Then the globulins solution proceeded to dialysis (MWCO: 3 kDa) against distilled water at 4°C for 24 h with the water changed every 4 h, and stored at −80°C after freeze-drying. The freeze-dried globulin fraction was dissolved in 0.01 M of HCl and digested by pepsin with a protein substrate concentration of 1%, a substrate-enzyme ratio of 10:1 for 48 h at 37°C. The hydrolysis was stopped through thermal treatment at 95°C water for 10 min. The supernatants were collected after centrifuge at 10000 rpm and 4°C for 20 min. Then the filtrate was passed through an ultrafiltration tube (Millipore) with a molecular weight cut-off value of 3 kDa, and the fraction less than 3 kDa was collected and freeze-dried.

Purification of *B. javanica* globulin fraction hydrolysate

B. javanica globulin fraction hydrolysate was further separated by gel filtration chromatography. The sample was dissolved with distilled water and loaded onto a Sephadex G-10 gel (Biodee, Beijing, China) chromatography column (1.6 × 60 cm), which was previously equilibrated with distilled water. Then the sample was eluted with distilled buffer at a flow rate of 0.8 mL/min and the absorbance was detected at 220 nm. The aliquots were manually collected every 10 min and lyophilized for analysis. The isolated fraction with highest antiproliferation activity was further separated using RP-HPLC analysis (2545, Waters, America) with a Symmetry Prep™ C_{18} column (7 μm, 7.8 × 300 mm) equipped with UV/Visible detector. The freeze-dried fractions were dissolved in double-distilled water and filtered through 0.45 μm membrane. The injection volume was 100 μL. The gradient elution was 5%–10% A (0–10 min), 10%–25% A (10–20 min), and 25%–50% A (20–50 min) using acetonitrile with 0.1% TFA as mobile phase A and distilled water with 0.1% TFA as mobile phase B at the flow rate of 2 mL/min. The column temperature was 30°C and the elution of peptide fractions was monitored at the absorbance of 220 nm. Major eluting peaks were collected and lyophilized for analysis.

Determining the concentration of peptides

The peptides concentration in the lyophilized material was estimated by BCA assay (Biodee, Beijing, China). The sample was dissolved in deionized

water and then mixed with BCA reagent A and B in a 50:1 ratio and incubated at 37°C for 30 min in the dark. The optical density of the purple product was assessed by an enzymatic calibrator (Anthros, Durham, NC) at 562 nm. Peptides concentration was calculated from a calibration curve of bovine serum albumin (BSA) in the range of 0–10 μg/μL.

Cells culture

MCF-7, human breast cancer cell line was kindly provided by Prof. Zhenxiao Sun (Beijing University of Chinese Medicine), which was purchased from the Cell Bank of the Chinese Academic of Science (Shanghai, China). The MCF-7 cells were cultured in RPMI-1640 culture medium (Gibco, USA) with 10% fetal bovine serum (Tianhang Biological Technology, China), 100 U/mL penicillin and 100 U/mL streptomycin (Gibco, USA) at 37°C with 5% CO_2.

Cell viability assay

The viability of MCF-7 cells was evaluated through MTT (3- [4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Briefly, the cells in logarithmic growth phase were plated in 96-well plates at the density of 2×10^3 cells/100 μL. After incubation overnight, cells were washed twice with PBS and then treated with different concentrations of peptides dissolved in the medium for 24, 48, and 72 h where 5-fluorouracil (50 μg/mL) was a positive control. Then 20 μL of MTT was added to each well and incubated in the dark for 4 h. After incubation, the supernatant was removed and 150 μL of DMSO was added to the wells. Finally, the absorbance was recorded at a wavelength of 570 nm using a microplate reader (BioTek, China). All experiments were performed at least three times. The potency of cell growth inhibition was expressed in survival rate, which was calculated by the following formula:

Inhibition rate (%) = (control group OD − experimental group OD)/(control group OD − blank group OD) × 100%

The values of the half-maximum inhibitory concentration (IC_{50}) were obtained by non-linear regression.

Cell culture and treatment

Briefly, 2×10^5 cells per well were seeded into the six-well plates. After 12 h, the supernatant was discarded and the cells were treated with various concentrations of F9-9 (0.03125, 0.0625, and 0.125 μg/mL) for 48 h. After the treatment, the medium was discarded, and the cells were digested with trypsin. Cells were collected by centrifuging with 1000 g for 5 min at room temperature. The collected cells were washed with PBS for three times. All experiments were repeated at least three times.

Cell morphology analysis by Giemsa staining

The morphological changes of the cells treated by F9-9 were determined by Giemsa staining using optical microscopy (TE2000-S, Nikon, Japan). Cells collected were adjusted to 1×10^4 cells/mL. Smear preparations were made by centrifuging at 1000 g for 7 min. The slides were fixed with methanol for 5 min and then stained with Giemsa (Sigma Aldrich, USA) for 10 min. After being washed with distilled water, the slides were air-dried and visualized.

Measurement of cell cycle

The cell cycle assay kit (Keygen Biotech, China) was used to measure cell cycle distribution. Cells were inoculated in six-well culture plates with the density 3×10^5 cells/well and maintained overnight. The collected cells were fixed in 70% cold ethanol at 4°C for 12 h, followed by three washes with PBS, after which they were stained with a PI solution in the dark for 30 min. Cell cycle assays were performed for each concentration of F9-9 in MCF-9 cells with three replicates. The fluorescence signal at 488 nm was detected by flow cytometry (BD FacsCanto II, USA) and analyzed using ModFit LT 3.1 software.

Measurement of cell apoptosis

To assess the mechanism of MCF-7 cell death induced by *B. javanica* peptide fraction F9-9, the apoptotic/necrosis assays were conducted by Annexin V/PI kit according to the manufacturer's recommendation (Keygen Biotech, China). Briefly, MCF-7 cells were cultured overnight in six-well plate (3×10^5 cells/well) followed by F9-9 treatment for 48 h at indicated concentrations of 0.03125, 0.0625, and 0.125 μg/mL. The cells were then collected and suspended in a binding buffer. Annexin V-FITC and PI were sequentially added to the samples, followed by incubation at room temperature for 15 min in the dark. Samples were analyzed with a flow cytometer (BD FacsCanto II, USA) with cells treated with fresh medium considered as negative controls. Membrane-linked protein V+/PI- and membrane-linked protein V+/PI+ cells were considered as early and late apoptotic cells.

RNA extraction and qRT-PCR analysis

The mRNA expressions of P53, PTEN, and NM23-H1 in MCF-7 cells under the action of F9-9 were quantified by RT-PCR. After the cells were treated with various concentrations of F9-9 (0.125, 0.25, and 0.5 μg/mL) for 24 h, total RNA was isolated with RN01-TRIpure Reagent (Aidlab Biotechnologies Co., Ltd, China). RNA concentration and purity were assessed microplate reader (Thermo, USA) and agarose gel electrophoresis. Complementary DNA was synthesized from total RNA using PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian), and the transcript expression of the gene was amplified using SYBR Green Master Mix (Bio-Rad) in the CFX96 fluorescent qPCR instrument (Bio-Rad, USA). The following amplification conditions were used: 30 s 95°C followed by 40 cycles of 5 s 95°C and 30 s 60°C. After the cycles were finished, the melting curves were detected at15 s 95°C, 60 s 60°C, and 15 s 95°C. β-actin was used as an internal reference. The results were analyzed using the Ct (ΔΔCt) method to calculate the relative change in gene expression. Oligonucleotide primers were designed based on the cDNA sequences reported in the GenBank database. The sequences of the primers were listed in Table 1.

Statistical analysis

All experiments were repeated at least three times. All data were presented as means ± SD. The statistical analysis was performed using SAS 9.3 statistical software for one-way analysis of variance and $P < 0.05$ was considered as statistically significant.

Table 1: Sequences of oligonucleotides used as primers

Target gene	Sequence (5′ to 3′)	Length
β-actin		
Sense	TGAGCGCAAGTACTCTGTGTGGAT	24
Antisense	TAGAAGCATTTGAGGTGCACGATG	24
P53		
Sense	GAGAGCTGAATGAGGCCTTG	20
Antisense	TTATGGCGGGAGGTAGACTG	20
PTEN		
Sense	CCGTTACCTGTGTGTGGTGA	20
Antisense	AGGTTTCCTCTGGTCCTGGT	20
NM23-H1		
Sense	GAGACCAACCCTGCAGACTC	20
Antisense	CAAGCCGATCTCCTTCTCTG	20

RESULTS

MCF-7 cells growth inhibition by *B. javanica* globulin fraction hydrolysate *in vitro*

After enzymatic hydrolyzation of *B. javanica* globulins by pepsin for 48 h, the hydrolysate was ultrafiltered and fractions ≤3 kDa were collected. MCF-7 cells were then treated with small molecular hydrolysate. The cell morphology and growth rate were changed obviously compared with the control. The treated cells showed obviously lower growth rate with rounded or even broken cell morphology. After 48 h treatment, according to statistical analysis, the IC_{50} value was 2.05 ± 0.04 μg/mL, which demonstrated that this fraction possessed a strong cytotoxic effect on MCF-7 cells. Therefore, the small molecular hydrolysate was further separated to obtain peptides with greater antitumor potential.

Separation and purification of small molecular peptides hydrolysate

The ≤3 kDa fraction was further separated by Sephadex G-10 column chromatography, and nine fractions were obtained, which were named as F1 ~ F9 [Figure 2a]. As shown in Table 2, faction F9 had the highest inhibitory activity with the IC_{50} value of 0.25 μg/mL (72 h), which was 10 times higher than that of the former fraction. Therefore, F9 was selected for further separation by RP-HPLC. The chromatogram of fraction F9 separated by RP-HPLC was shown in Figure 2b. Totally, fractions were collected (F9-1 ~ F9-9). MTT assay was conducted with the concentration of 0.25 μg/mL for each component, the effects of the fractions on MCF-7 cell proliferation were significantly different [Figure 2c]. The strongest fraction F9-9 showed an inhibition rate of 62.74%, and the IC_{50} values of 0.12 μg/mL. Compared with the prior fraction F9 the antitumor activity of F9-9 increased by more than two times. The F9-9 was selected for underlying antitumor mechanism research.

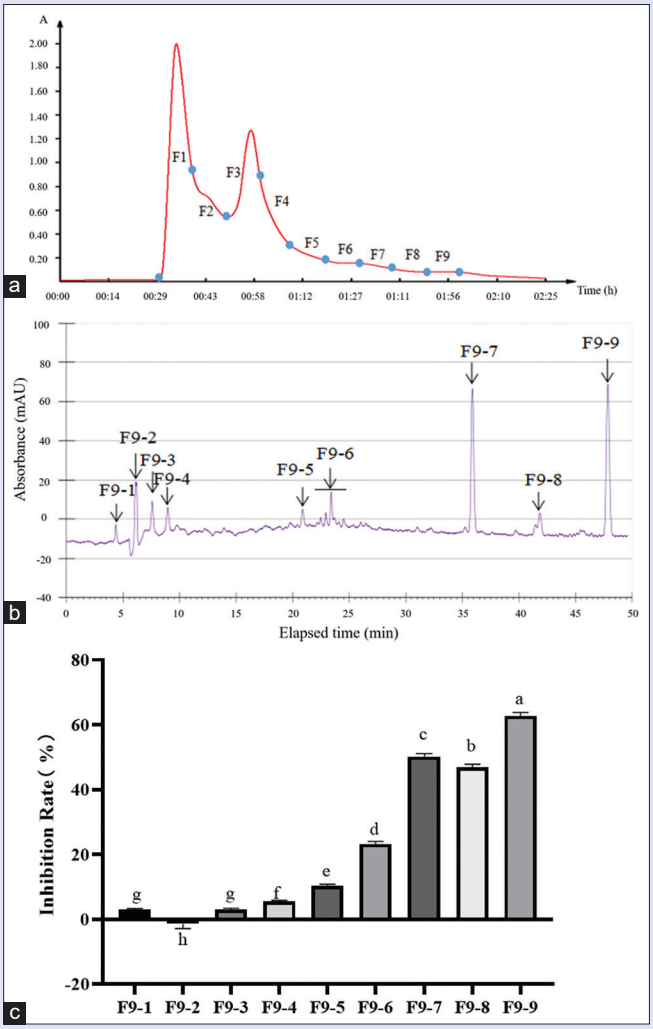

Figure 2: Separation and purification of small molecular peptides. (a) Gel filtration chromatogram of *B. javanica* globulin fraction hydrolysate; (b) RP-HPLC separation pattern of fraction F9; (c) MTT assay was performed to detect the proliferation inhibition rate of isolated fractions at concentrations of 0.25 μg/mL (F9-1 ~ F9-9). Different letters represented differences at 0.05 level

In vitro morphology assessment of MCF-7 treated with F9-9 by Giemsa staining

Cell morphological changes induced by fraction F9-9 were examined by Giemsa staining, which could conveniently distinguish normal cells from apoptotic cells in an optical microscope. Figure 3 showed that the negative control cells manifested normal morphology. Fraction F9-9 caused obviously morphological alterations of the cells including nucleus condensing, nucleus fragmenting, and apoptosis bodies [Figure 3. b, c, and d, arrow indicated]. In addition, the number of apoptotic cells increased in a dose-dependent manner.

Table 2: Proliferation inhibiting rate of gel filtration separation fractions

Fractions	IC_{50} (μg/mL)
F1	33.68±0.03^{b}
F2	69.87±0.04^{a}
F3	32.60±0.04^{c}
F4	2.03±0.04^{e}
F5	2.44±0.02^{d}
F6	0.47±0.02^{f}
F7	0.42±0.03^{f}
F8	0.30±0.02^{g}
F9	0.25±0.02^{g}

Different letters represent differences at 0.05 level

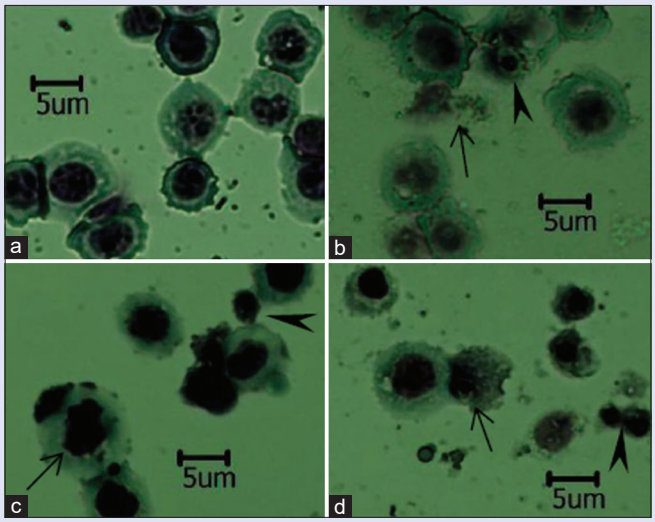

Figure 3: Morphological detection of MCF-7 cells with Giemsa staining. (a) Control group; (b) The cells treated with 0.03125 μg/mL F9-9 for 48 h; (c) The cells treated with 0.0625 μg/mL F9-9 for 48 h; (d) The cells treated with 0.0125 μg/mL F9-9 for 48 h. Arrows showed nucleus condensing and nucleus fragmenting; arrow heads indicated apoptosis bodies

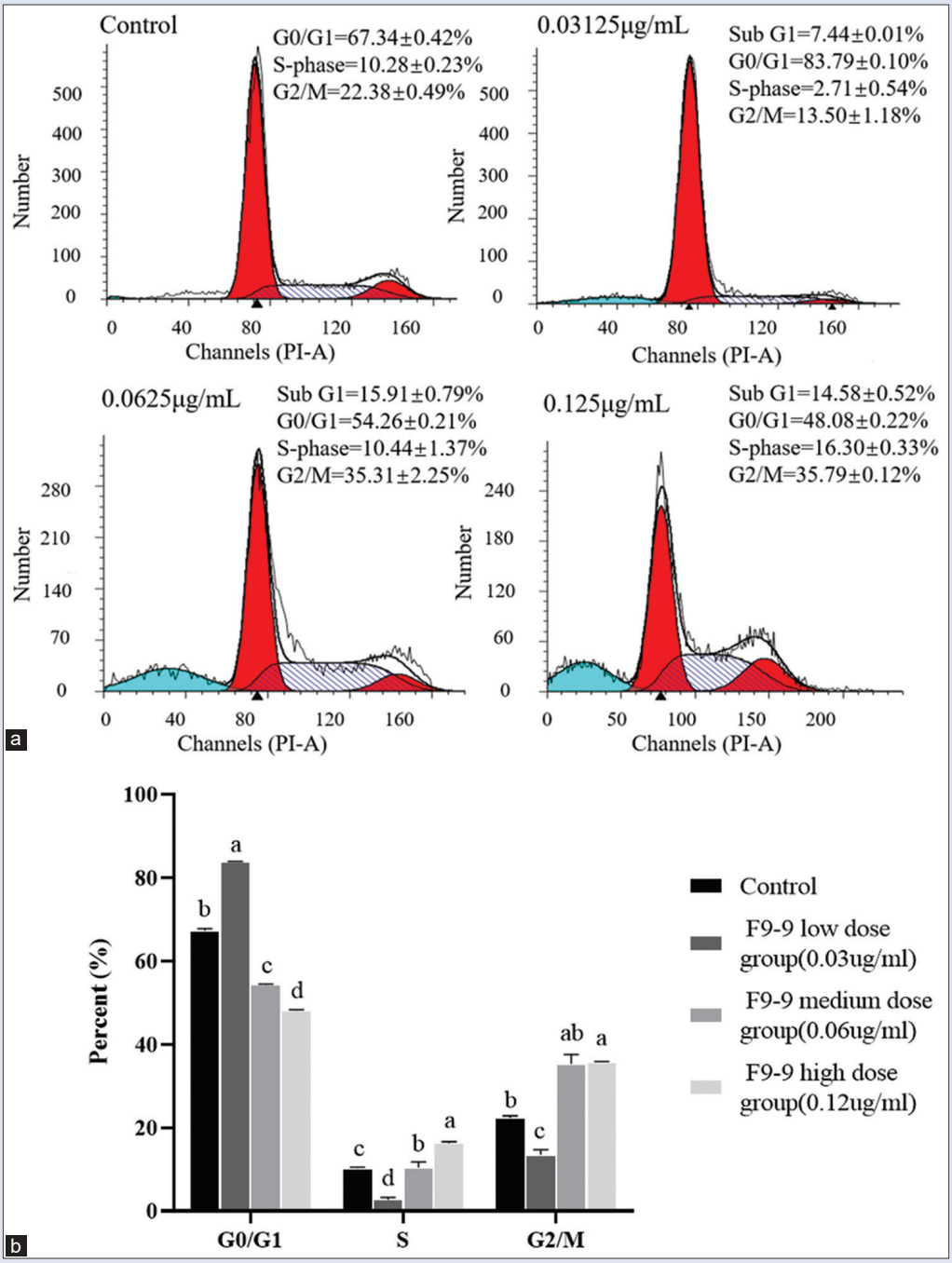

Figure 4: Fraction F9-9 blocked MCF-7 cells in G0/G1 phase. (a) Cell cycle of F9-9-treated MCF-7 cells was assessed by PI staining. (b) Statistical analysis of the cycle distribution of MCF-7 cells after F9-9 treatment. All data are expressed as the means ± SD of three independent experiments. Different letters represented differences at 0.05 level

G0/G1 cell cycle arrest induced by F9-9

To explore the internal mechanism by which *B. javanica* biopeptide inhibited the proliferation of MCF-7 cells, the cell cycle distribution was analyzed. As shown in Figure 4, for the low dose group, the proportion of G0/G1 was 83.79%, which was significantly higher than the control group. For the high dose group, the proportions of S and G2/M phase were significantly increased (16.30% and 35.79%, respectively). Moreover, samples treated with the increased amount of F9-9 showed more obvious apoptosis peaks.

Apoptosis induced by F9-9 in MCF-7 cells

The effect of F9-9 on the apoptosis rate of MCF-7 cells was estimated by Annexin V-FITC/PI double staining and flow cytometry. As depicted in Figure 5, most of the cells for the negative control group showed normal cell viability. While medium concentration treatment (0.0625 μg/mL) of F9-9 for 48 h resulted in an increased number of necrotic cells (3.3%, $P < 0.05$), late apoptotic (17.03%, $P < 0.05$), and early apoptotic (21.90%, $P < 0.05$) cells. In the high dose group (0.125 μg/mL), the percentage of early apoptosis and late apoptosis was 25.40% and 25.25%, respectively.

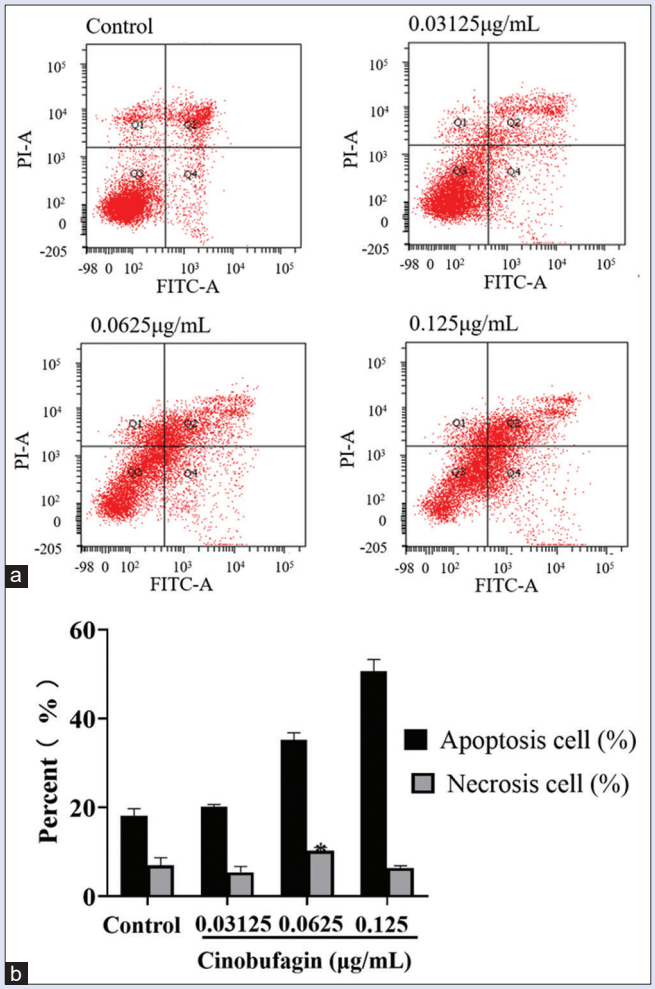

Figure 5: Fraction F9-9 induced apoptosis in MCF-7 cells. (a) Apoptosis rate of F9-9-treated MCF-7 cells was assessed by Annexin V-FITC/PI double staining. (b) Statistical analysis of apoptosis rate and necrosis rate of MCF-7 cells after F9-9 treatment. All data are expressed as the means ± SD of three independent

It suggested that *B. javanica* small molecular peptide effectively induced apoptosis in MCF-7 cells in a dose-dependent manner.

The mRNA expression of key genes involved in breast cancer regulation in MCF-7 cells under F9-9 treatment

The integrity of purified RNA is critical for all gene expression analysis techniques. To assess the quality of the extracted RNA, the samples were verified by 1% agarose gel electrophoresis. Two bands of 18S RNA and 28S RNA were clearly visible in each group, which means a qualified RNA sample [Figure 6a]. The proto-oncogenes P53 and PTEN, and the tumor metastasis suppressor gene NM23-H1 are closely related to the occurrence and development of breast cancer. The influences on these gene transcription were measured in real-time-PCR. The expression of P53, PTEN, and NM23-H1 was significantly increased in F9-9-treated MCF-7 cells compared with a control group ($P < 0.05$). The P53 [Figure 6b] expression level increased by 73.07% at the low dose (0.125 μg/mL), 95.19% at the medium dose (0.25 μg/mL), and 76.92% at high dose (0.5 μg/mL), respectively ($P < 0.05$). Similar trend was detected for PTEN and NM23-H1, in which the relative expression level was significantly promoted [Figure 6c and d].

DISCUSSION

Despite continuous improvements in cancer treatment technology, breast cancer is still considered a difficult disease to treat due to drug resistance and severe side effects.[21] MCF-7 cells are commonly used in the study of estrogen receptor (ER) positive breast cancer cell experiments, which are easily cultured. Meanwhile, both progesterone receptor (PR) and epidermal growth factor receptor-2 (HER2) are present on MCF-7 cells.[22] Moreover, PR, ER, and HER2-negative (triple-negative) sublines would be generated from ER-positive MCF-7 cell lines by continuous culture.[23] The variants of a single cancer cell might be able to encapsulate the development of multiple phenotypes in clinical cancers,[24] Therefore, MCF-7 was selected as the suitable model cell in this investigation.

Meanwhile, the use of plant extracts for research and development of effective anticancer drugs is becoming mainstream. Curcumin, ursolic acid, flavopiridol, paclitaxel, and α-lycopene, which are isolated from natural sources, have significant antitumor effects.[25] Peptides have high affinity, high target specificity, low toxicity, and good tissue penetration compared with chemotherapeutic antibodies due to their small size.[26] Therefore, it is an encouraging strategy to explore novel agents from peptides. Venom-derived peptides ICD-8 inhibited proliferation of MCF-7 cells with an IC_{50} of 36.45 ± 0.38 μg/mL. A peptide named Ruviprase, isolated from Daboia russelii russelii venom, inhibited the proliferation of MCF-7 cells with an IC_{50} value of 4.0 μg/mL.[27] In this research, F9-9 showed high cytotoxicity in MCF-7 with the IC_{50} of 0.124 μg/mL, which was obviously lower than those of synthesized chalcones.[28]

Generally, an important manifestation of tumor cells is the uncontrolled cell cycle, which often leads to abnormal proliferation as well as the appearance of genomic changes.[21] Our study showed that the cell cycle was significantly stalled at the G0/G1 phase due to drug treatment and that this effect was more pronounced at low dose points in MCF-7 cells. Moreover, significant apoptosis at high dose points was also detected. p53 is the most commonly mutated gene associated with cancer, binding to DNA in a sequence-specific manner to induce cell cycle checkpoint activation and apoptosis. The expression of hundreds of genes is directly regulated by p53.[29] As a tumor suppressor gene, the expression of PTEN in MCF-7 cells can activate MEK1/2/ERK1/2 signaling, and regulate caspase-3/Bcl-2 signaling to achieve inhibited the proliferation and migration, and promote the apoptosis of MCF-7 cells.[30] Thus, it may be a suitable target for breast cancer gene therapy. The expression of NM23-H1, a key tumor metastasis suppressor gene, could inhibit the migration of MCF-7 breast cancer cells.[31] The data presented in this study indicate that the transcription levels of P53, PTEN, and NM23-H1 were markedly increased after F9-9 treatment, which revealed the fundamental anticancer mechanism of F9-9.

Although biopeptides possess more satisfactory antitumor application prospects than other compounds, there are still additional problems to conquer, such as instability during circulation, enzymatic degradation, immunogenicity, short half-life, and inability to cross cell membranes.[26] Therefore, more superior materials should be developed to protect protein or peptide drugs from degradation while allowing them to target tumor cells, improve the transmembrane efficiency of the drug and control its release at the targeted target.

CONCLUSION

This study manifested the vital role of the *B. javanica* peptides from globulin fraction in the inhibition of breast cancer. Exposure of MCF-7 cells to *B. javanica* biopeptide led to a decreased proliferative activity, triggered apoptosis as well as G0/G1 phase cycle arrest, and an

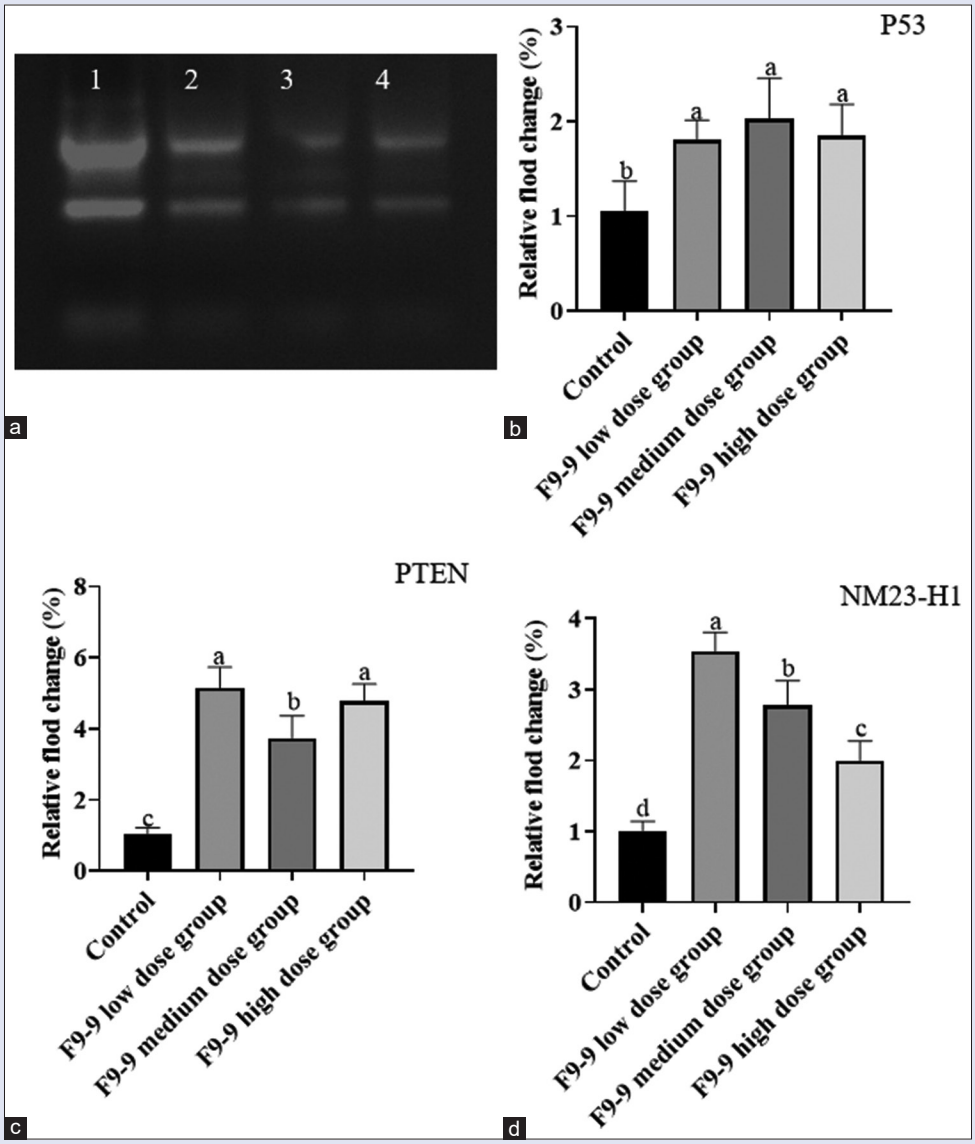

Figure 6: Fraction F9-9 induced elevated mRNA expression of key genes regulating breast cancer in MCF-7 cells. (a) RNA electropherogram (1) Blank. (2) F9-9 low dose group (0.125 μg/mL). (3) F9-9 medium dose group (0.25 μg/mL). (4) F9-9 high dose group (0.5 μg/mL). (b) Influence of F9-9 on P53 mRNA express in MCF-7 cells. (c) Influence of F9-9 on PTEN mRNA expression in MCF-7. (d) Influence of F9-9 on NM23-H1 mRNA expression in MCF-7. Different letters represented differences at 0.05 level

increased tumor suppressor gene expression. Thus, *B. javanica* peptide fraction could be used as a potential anticancer component in the near future.

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Authors' contributions

All authors participated in the design, interpretation of the studies, the analysis of the data and review of the manuscript. HYS wrote the first draft of the paper and made subsequent revisions; HFJ, HYS, FZZ, YYY and YZ conducted the experiments; FJS and LZW gave comments and suggestions during the writing of the paper and made amendments to the pre-final draft. All authors reviewed the final draft of the manuscript before submission.

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Conflicts of interest

There are no conflicts of interest.

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