Int J Clin Exp Med 2019;12(10):12236-12242
www.ijcem.com /ISSN:1940-5901/IJCEM0095119

Original Article

Anti-bacterial and anti-cervical cancer tumor properties of secondary metabolites of endophytic fungi from *Ginkgo biloba*

Shuai Liu[1], Qing He[1], Yibo Shao[1], Jianzhou Meng[2], Fang Song[1], Wei Liu[1], Tianjiao Li[1], Qiangcheng Zeng[1]

[1]*Shandong Key Laboratory in University of Functional Bioresource Utilization, College of Life Sciences, Dezhou University, Dezhou 253023, Shandong Province, China;* [2]*Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China*

Received April 9, 2019; Accepted September 5, 2019; Epub October 15, 2019; Published October 30, 2019

Abstract: Objective: To evaluate the anti-bacterial and anti-tumor activities of the secondary metabolites of endophytic fungi derived from *Ginkgo biloba*. Methods: MTT assay was utilized to detect the effect of secondary metabolites on inhibiting cervical cancer. A cylinder plate method was adopted to assess the anti-bacterial activity of the secondary metabolites. Liquid chromatography-mass spectrometry was performed to separate and identify the substances in the secondary metabolites. Flow cytometry was utilized for preliminarily analysis on the mechanism underlying the anti-cancer effect of the secondary metabolites. Results: MTT assay demonstrated that the crude extracts from the J-1 and J-3 strains yielded a proliferation-inhibiting effect of 76.2% and 65% on the cervical cancer cell line HeLa, whereas they exerted no significant inhibitory effects on proliferation in the lung cancer cell line A549. The J-3 strain could significantly suppress *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*. The J-1 strain exerted certain inhibitory effects on *Staphylococcus aureus* and *Escherichia coli*, whereas it did not affect *Bacillus subtilis*. The J-2 strain exerted certain inhibitory effects on *Staphylococcus aureus*. Flow cytometry indicated that J-1 and J-3 strains affected apoptosis in cervical cancer cells, and crude extracts of the J-2 strain reduced the proportion of HeLa cells in the S-phase. Liquid chromatography-mass spectrometry combined with a literature search showed that resveratrol, scopoletin, rosmarinic acid and taxifolin are the secondary metabolites of endophytic fungi from *Ginkgo biloba* that exerted an anti-tumor effect. Conclusion: J-1 and J-3 strains can exert potent anti-cervical cancer and anti-bacterial effects, which deserves widespread investigation and application in clinical practice.

Keywords: Endophytic fungi of *Ginkgo biloba L.*, secondary metabolite, anti-tumor activity, mechanism

Introduction

Cervical cancer is the most common gynecologic malignant tumor. In recent years, the age of onset of cervical cancer has tended to be younger. The development of new anti-tumor drugs has been a hot topic in the field of drug research [1]. *Ginkgo biloba* is a native medicinal plant in China. Up to now, *Ginkgo biloba* leaves have been proven to contain more than 100 types of chemical components. Ginkgo flavin and polyprenol, as the extracts of *Ginkgo biloba,* exert anti-tumor effects [2]. However, the long period of growth of *Ginkgo biloba* up to 20 years significantly constrains the application in drug research. Consequently, endophytic fungi of *Ginkgo biloba* have become a hot topic in this field.

Common extracts of *Ginkgo biloba* mainly include *Ginkgo biloba* compound extract (EGb), *Ginkgo biloba* polyprenol (GP), *Ginkgo biloba* polysaccharide compounds (GBLP) and *Ginkgo biloba* exocarp polysaccharide (GBEP) [3]. EGb mainly consists of flavonoids and terpenes. Studies have demonstrated that EGb exerts inhibitory effects on the transplanted sarcoma S180 and liver cancer H22 cells in mice both *in vivo* and *in vitro* [4]. The inhibitory rate of GP on human gastric cancer cell line SGC-7901, human colon adenocarcinoma cell line LOVO and human cervical cancer HeLa cell line *in*

vitro, ranged from 60% to 80%; and 50%-65% for the transplanted tumor cells Heps, S180 and EC *in vivo* [5]. GBEP exerts inhibitory effects on human hepatoma cell line BEL-7404, gastric gland cancer cell line SGC-7901 and lung adenocarcinoma cell line SPC-A-1, at the dose of 10-320 μg/ml for 24-72 h *in vitro*. The possible mechanisms of anti-tumor effects of *Ginkgo biloba* extracts include antioxidant and scavenging free radicals, affecting proliferation and inducing apoptosis of tumor cells, inhibiting formation of tumor vessels, regulating tumors and related genes, and cytotoxic effects on tumor cells [6]. Nevertheless, *in vivo* anti-tumor studies of endophytic fungi from *Ginkgo biloba* have not been reported.

According to the symbiosis theory of plant endophytic fungi, endophytic fungi probably exist in *Ginkgo biloba*, which can produce the identical or similar chemical components as *Ginkgo biloba* [7]. Guo *et al*. [8] have isolated 522 endophytic fungi from *Ginkgo biloba* in Yangling of Shaanxi Province and demonstrated that 50.7% of the strains exert antibacterial activity. Miao *et al*. [9] have isolated 19 endophytic fungi from *Ginkgo biloba* in Fuyang of Anhui Province, among which YX5 is the most active strain. The IC50 of crude extracts of YX5 is 18.3, 3.6 and 6.5 μg/ml for the inhibition of tumor cell line EC109, human nasopharyngeal carcinoma cell line HONE1, and human cervical carcinoma cell line HeLa.

In this investigation, MTT assay was adopted to detect the anti-cervical cancer activity of crude extracts from *Ginkgo biloba*. Cylinder plate method was utilized to evaluate the anti-bacterial activity of the secondary metabolites. Liquid chromatography-mass spectrometry was performed to separate and identify the substances in the secondary metabolites. Flow cytometry was conducted to preliminarily analyze the mechanism underlying the anti-cervical cancer effect of the secondary metabolites.

Materials and methods

Materials

Ginkgo biloba was collected from the trunk bark of a 30-year Ginkgo biloba with a diameter of roughly 55 cm approximately 2 cm from the ground, from Linyi, Shandong Province. After separation and purification, the Ginkgo biloba was preserved in the -80°C freezer in our laboratory. ANNEXIN V-FITC/PI apoptosis detection kit (Solarbio Life Sciences, Beijing, China), cell cycle and apoptosis detection kit C1052 (Beyotime Biotechnology, Shanghai, China) were used. The human cervical cancer cell line HeLa was donated by the Department of Medicine in our university. Self-controlled CO_2 incubation box (model: 2300 Shellab Company), ultraclean bench (model: YJ-450. Suzhou Purification Equipment Factory, China), electrothermal thermostatic water bath box (model: BS2 Beijing Medical Equipment General Factory, China), flow cytometer (model: FACScan Becton-Dickinson Company), Heidolph rotary evaporator, high pressure steam sterilization pot and other instruments were provided by the laboratory.

Culture and post-treatment of endophytic fungi

Three strains of ginkgo endophytic fungi cultured in PDA solid medium were obtained, and the mycelial masses were inoculated into a triangular flask containing 250 ml PDA liquid medium, and cultured in a shaker at 20°C for 7 d at 120 r/min. Then, ethyl acetate was supplemented to each flask at a volume ratio of 1:1, and the flask was further cultured in a shaker for 4 d. The fermentation liquid was filtered through 3-4 layers of gauze, and mycelium was removed, and the separation funnel was used for layering to obtain an organic phase containing secondary metabolites of ginkgo endophytic fungi. Most ethyl acetate in the organic phase was recovered by a rotary evaporator to obtain a concentrated solution containing secondary metabolites of ginkgo endophytic fungi. Finally, the concentrated solution was completely dried by a vacuum concentration dryer and dissolved in DMSO to obtain crude thallus extract.

Culture of HeLa cells and determination of anti-tumor activity of crude extracts from endophytic fungi

Culture medium for cell culture consisted of 10% fetal bovine serum, 89% DMEM complete culture medium, 1% double antibody (penicillin-streptomycin). The cells were cultured and passaged in a cell incubator with 5% CO_2 at 37°C. MTT assay was utilized to determine the anti-cervical cancer tumor activity of crude extracts: tumor cells in logarithmic growth phase were collected, digested with trypsin, and the cell

suspension was incubated in complete culture medium, cell counting was conducted with blood cell count plate and 100 μl of the solution was inoculated into 96-well plate. The blank control and negative control were established. After being placed in a 5% CO_2 incubator for 24 h at 37°C, 100 l of the sample diluted with the complete culture solution was added and collected after continuous culture for 24 h. The culture medium in the suction well was discarded, 2.5 μg/μl of MTT solution 20 μl was added to each well, after reaction at 37°C for 4 h, 100 μl DMSO was supplemented to each well, and dissolved at 37°C for 30 min. The absorbance values of each well were measured by microplate reader at a wavelength of 570 nm. Finally, the inhibition rate was calculated according to the following formula: inhibition rate = (negative control OD value - experimental group OD value)/(negative control OD value - blank control OD value) ×100%

Antimicrobial activities of secondary metabolites of three endophytic fungi from Ginkgo biloba

Escherichia coli strain was inoculated into LB liquid culture medium in advance, shaken for 10-12 h at 37°C. LB solid culture medium was established, sterilized, cooled and supplemented with bacterial liquid in ultraclean bench (100 ml bacterial solution per 100 μl solid culture medium), shaken and poured onto the flat plate. On the back of the solidified plate, three to four regions were divided and the samples were added to each corresponding region. In each region, an Oxford cup was firmly placed in the central part, gently pressed to ensure that the cup was not inserted into the flat plate. Subsequently, 100-200 μl of samples were added into each cup and transferred in an incubator at 37°C. The experimental procedures of *Staphylococcus aureus* and *Bacillus subtilis* were identical to those of *Escherichia coli*.

Cell culture

The cell suspension was added into a 24-well plate and cultured in a 5% CO_2 incubator at 37°C for 45 min. After the cells adhered to the wall, the culture medium was discarded. RPMI1640 medium, a determined dose of drug and an equivalent quantity of blank solution were added to each well in a 5% CO_2 incubator at 37°C for 24 h.

Flow cytometry

The cells were collected, centrifuged at 1000 *r*/min for 10 min, washed with pre-cooled PBS and centrifuged. The supernatant was removed, fixed with 1 ml of pre-cooled 70% ethanol while shaking, and put into -20°C overnight. The cell suspension was washed with PBS to remove the fixative before staining, then 100 μl RNAse was supplemented in a water bath at 37°C for 30 min, and then supplemented with 400 μl pyridine iodide staining solution. The cell suspension was stained for 30 min at 4°C in the dark. The apoptosis rate and cell cycle distribution of HeLa cells were detected on a flow cytometer. According to the percentage of G0G1, S and G2M phases obtained by Modfit analysis software, intra- and inter-group horizontal comparisons were performed.

Isolation and identification of active compounds from crude extracts

The analytical instrument in this experiment was Q Exactive plus (Thermo), and the ion source was ESI. Chromatographic conditions: The chromatographic column was Waters Atlantis T3 (100×3 mm, 1.8 mm), the column temperature was 35°C, and the flow rate was 0.500 ml/min. Mobile phase: A. Equate = "0.1% CH_3COOH-H_2O"; D. Equate = "acetonitrile". Mass spectrum conditions: the scan range was m/z 80-1200; Resolution: 70,000; Spectrum data type: Profile; Capillary voltage: 4000 V (positive) and 3500 V (negative); Capillary Temperature: 350°C.

Statistical analysis

All data analyses were performed by using SPSS statistical software (SPSS Inc., Chicago, IL). The data obtained before and after corresponding treatment were statistically compared by using the paired *t*-test. A *P* value of less than 0.05 was considered as statistical significance.

Results

Anti-tumor activity of crude extracts from fermentation broth

As illustrated in **Figure 1**, the inhibition rates of J-1 and J-3 strains on cervical cancer cells were calculated as 76.2% and 65%, whereas the

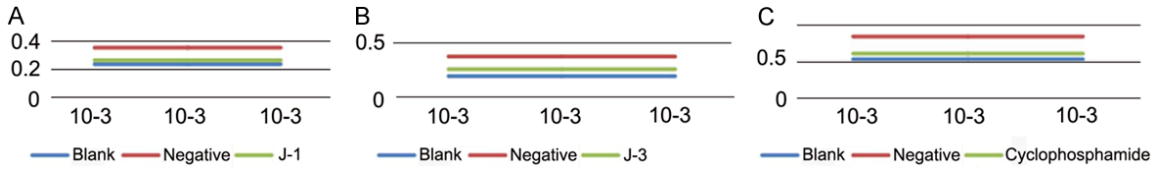

Figure 1. Detection of anti-cervical cancer activity of crude extracts of fermentation broth after 24-h treatment. A. J-1 fermentation broth; B. J-3 fermentation broth; C. Cyclophosphamide.

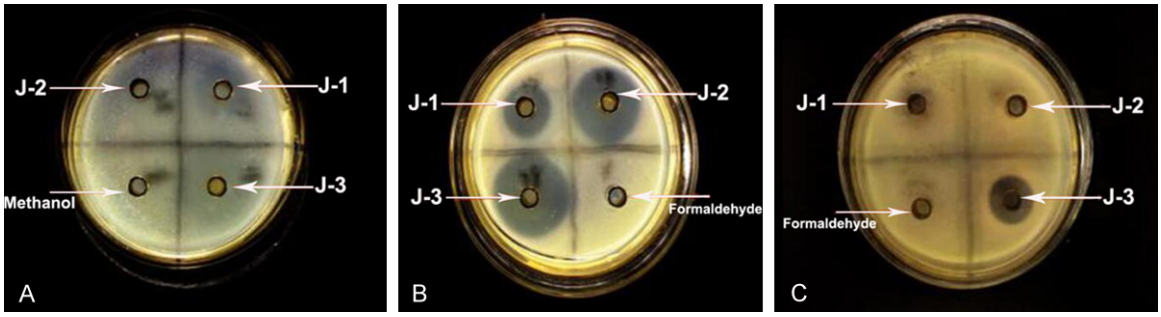

Figure 2. Detection of antibacterial activity of crude extracts of fermentation broth against Escherichia coli (A), Staphylococcus aureus (B) and Bacillus subtilis (C).

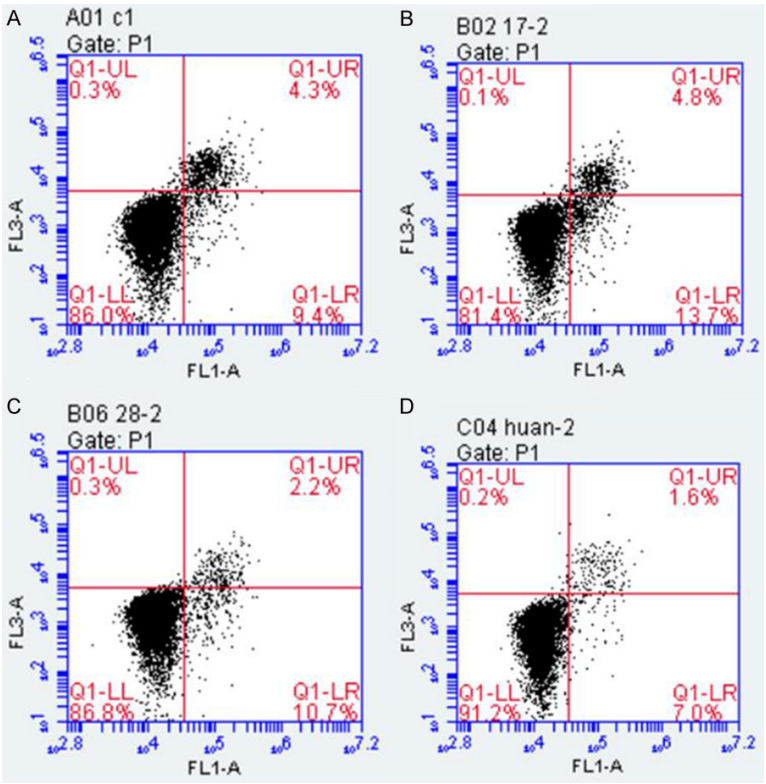

Figure 3. Flow cytometry for the detection of the anti-cervical cancer activity of cyclophosphamide (A); J-1 strain (B), J-3 strain (C) and blank control (D).

inhibition rate of J-2 strain was merely 23%. The inhibition rate of positive drug cyclophosphamide on the cervical cancer cells was up to 75%. In addition, MTT assay demonstrated that the crude extracts of fermentation broth of J-1, J-2 and J-3 strains possessed the inhibitory rates of 19.2%, 25.5% and 16.0%, respectively on the lung cancer A549 cells, which preliminarily indicated that these strains exerted no significant inhibitory effect on lung cancer A549 cells.

Antimicrobial activity of crude extracts from fermentation broth

The J-3 strain exerted effective antimicrobial effect on *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* with a diameter of the inhibition zone of 3.1 cm and 3.2 cm and 1.8 cm, respectively. The J-1 strain exerted certain inhibitory effect on *Staphylococcus aureus* and *Escherichia coli* and the diameter of the inhibition zone was measured as 2.0 cm and 2.0 cm,

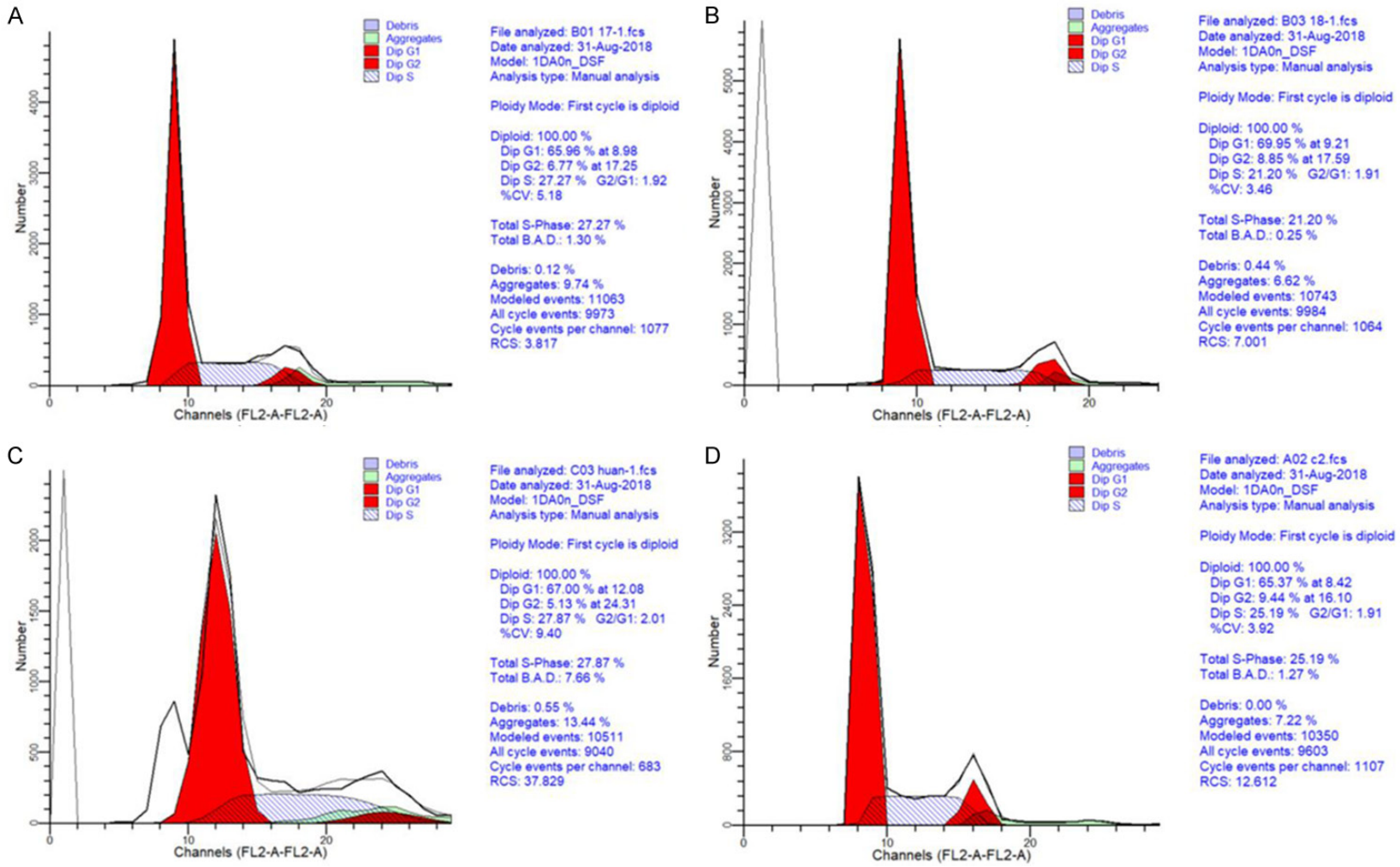

Figure 4. Effect of J-1 strain (A); J-2 strain (B), (C) cyclophosphamide and blank control (D) on apoptosis rate and cell cycle distribution of HeLa cells.

Table 1. Active substances of crude extracts of fermentation broth of three strains of endophytic fungi from *Ginkgo biloba*

Substances	Anti-tumor activity	Anti-bacterial activity	Anti-oxidant activity	Anti-viral activity
Quercetin	+	+	+	+
Resveratrol	+	+	+	+
Naringin	+	+	+	+
Rosmarinic acid	+	+	+	+
Methyl jasmonate	+		+	
Taxifolin	+		+	

whereas it had no significant inhibitory effect on *Bacillus subtilis*. However, J-2 strain exerted certain inhibitory effects on *Staphylococcus aureus* with a diameter of inhibition zone of 2.9 cm, while had no inhibitory effect on *Escherichia coli* and *Bacillus subtilis* (**Figure 2**).

Tumor cell apoptosis by flow cytometry

As illustrated in **Figure 3**, the crude extracts of the fermentation broth of J-1 and J-3 strains exerted effective inhibitory effects upon the cervical cancer cells, especially the inhibitory effect of the J-1 strain was almost identical to that of cyclophosphamide. The inhibitory effect of J-2 strain on the cervical cancer cells was not obvious, which was consistent with the detection results of MTT assay.

Apoptosis cycle of tumor cells by flow cytometry

As demonstrated in **Figure 4**, J-1 and J-3 strains exerted no significant effect on the cell cycle of cervical cancer cells, whereas the crude extracts of the fermentation broth of J-2 strain could reduce the proportion of HeLa cells in S phase.

Liquid chromatography-mass spectrometry detection

The results of liquid chromatography-mass spectrometry revealed that more than 180 compounds were detected in the crude extracts of three strains of endophytic fungi from *Ginkgo biloba*. Through the literature search, the substances have been proven to have definite anti-tumor activity are illustrated in **Table 1**. In addition, the crude extracts of the fermentation broth also contained a variety of active substances, such as antibacterial, antioxidant and antiviral substances.

Discussion

In recent years, the application of natural active products from symbiotic microorganisms of animals and plants has captivated widespread attention. Anticancer drugs derived from endophytic bacteria in plants have been reported [10]. Previous study [11] has investigated the effect of *Ginkgo biloba* exocarp extracts upon Lewis lung cancer cells and demonstrated that *Ginkgo biloba* exocarp extracts exert the anti-tumor and anti-metastatic effects depending upon the suppression of tumor angiogenesis, and such inhibitory effect is intimately correlated with the blockage of the Wnt/β-catenin-VEGF signaling pathway in Lewis lung cancer.

Oxidative stress has been regarded as a major risk factor for a variety of human diseases from inflammation to cancer. Emerging evidence have demonstrated the active substances extracted from natural plants, such as *Ginseng*, *Zizyphus Jujuba*, *Astragalus lentiginosus* and *Ginkgo biloba* can play a key role in the management of multiple diseases [12]. GA has been proven to exert a potential inhibitory effect on human larynx cancer and tongue squamous carcinoma cells and exerts no cytotoxic effects on the non-tumorigenic cells. In addition, *Ginkgo biloba* reduced the viability of multiple types of cancer cells in a manner of inhibiting division, preventing the progression of cell cycle and inducing cell apoptosis without compromising the viability of non-tumorigenic cells [13]. Ma *et al*. [14] have demonstrated that *Ginkgo biloba* can serve as a safe and potent anti-tumor agent against pancreatic cancer through regulating the signaling pathway and genes driving lipogenesis in cancer cells. However, whether other mechanisms are involved in the anti-tumor effects of *Ginkgo biloba* and whether *Ginkgo biloba* exerts a synergistic effect with other chemotherapy drugs remains to be elucidated.

In this investigation, of the three endophytic fungi in *Ginkgo biloba*, two can be identified as *Fusarium proliferatum*. However, it is challenging to distinguish the species and genus of the remaining strain merely depending on the 18 sRNA and colony morphology, which requires

further identification. In addition, the crude extracts from the fermentation broth of J-1 and J-3 strains possess an inhibition rate of 76.2% and 65% on the cervical cancer HeLa cells and have strong anti-cervical cancer activity, whereas they possess no activity on the lung cancer A549 cells. The J-3 strain exerts excellent antibacterial effect on *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*, whereas J-1 strain has certain inhibitory effect on *Staphylococcus aureus* and *Escherichia coli*.

The results of flow cytometry have demonstrated that the crude extracts from the fermentation broth of J-1 and J-3 strains exert effective inhibitory effects upon the cervical cancer cells, especially the inhibitory effect of J-1 strain is almost identical that of cyclophosphamide. Nevertheless, the inhibitory effect of J-2 strain on the cervical cancer cells is not obvious, which is consistent with the results of MTT assay. In addition, the crude extracts of the fermentation broth, such as resveratrol, scopoletin and taxifolin are promising substances applied in the treatment of multiple diseases. Therefore, it is an urgent task to enhance the yield, optimize the culture conditions and increase the purity of the above substances. Taken together, J-1 and J-3 strains possess high anti-cervical cancer and anti-bacterial activities, which deserve further development and widespread application.

Acknowledgements

This study is supported by Shandong Provincial Natural Science Foundation of China (ZR2017-BH081).

Disclosure of conflict of interest

None.

Address correspondence to: Qing He, Shandong Key Laboratory in University of Functional Bioresource Utilization, College of Life Sciences, Dezhou University, 566 Univeristy Road West, Dezhou 253023, Shandong Province, China. Tel: +86-0534-898-5840; E-mail: heqing@yandex.com

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