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STUDY ON ENDOPHYTIC MYCOBACTERIUM SECONDARY METABOLITES AND ANTI - TUMOR ACTIVITY OF CERVICAL CANCER IN THREE STRAINS OF CRUDE EXTRACTS

Haidao Wang[1], Qing He[1,2,*], Tao Wang[1], Shuai Liu[1], Wei Wu[3], Zhenghua Li[2], Qiangcheng Zeng[1,2]

[1]Shandong key laboratory in University of Functional Bioresource Utilization, College of Life Sciences, Dezhou University, 566 University Road West, Dezhou 253023, Shandong Province, China
[2]Shandong Provincial Key Laboratory of Biophysics, Dezhou University, 566 University Road West, Dezhou 253023, Shandong Province, China
[3]College of Life Sciences, Shandong Normal University, Wenhua East Road 88, Jinan 250014, Shandong Province, China

ABSTRACT

To study the anti-cervical cancer activity of three secondary metabolites of endophytic fungi from Ginkgo biloba. The species of three strains were determined by the combination of colony morphology and 18 sRNA sequencing. Then the anti-cervical cancer activity of three strains of crude extracts was tested by MTT assay. Finally, the inhibitory effect Secondary metabolites of more than 50% of the isolates were isolated and the compound monomers with anti-tumor activity were screened. Colony morphology and 18 sRNA sequencing showed that strains J-1 and J-2 belong to the genus Fusarium and J-3 may belong to the genus Fusarium, but further validation is needed. MTT assay showed that the growth inhibition rates of crude extracts of strain J-1 and J-3 on Hela cells were 59.6% and 59.1%, respectively. Semi-preparative HPLC combined with MTT method to obtain four compounds with anti-cervical cancer activity of the monomer, the need for further structural identification. Two ginkgo endophytic fungi with strong anti-cervical cancer activity were obtained, indicating that endophytic fungi of Ginkgo biloba as a potential source of anti-tumor drugs deserve attention.

KEYWORDS:
Ginkgo biloba, endophytic fungi, secondary metabolites, anti-tumor activity

INTRODUCTION

Cervical cancer is the most common gynecological malignancy, and its incidence has a tendency of younger age in recent years. The development of new anti-tumor drugs has been the main aspect in the field of drug research [1]. Ginkgo biloba is a medicinal plant of our country. It has been found that Ginkgo biloba contained more than 100 kinds of chemical constituents. Its ginkgo biloba extract and polyprenol have anti-tumor effects [2]. However, Ginkgo biloba grown slowly, and it took more than 20 years from planting to fruiting under natural conditions, restricted the development of its medicinal efficacy. Therefore, ginkgo endophytic fungi have become a hot spot for research. Common ginkgo extracts include Ginkgo biloba extract (EGb), Ginkgo biloba polyglen alcohol (GP), Ginkgo biloba polysaccharide (GBLP), ginkgo exocarp polysaccharides (GBEP) four categories [3]. EGb is mainly flavonoids and terpenes. The study found that EGb on mouse sarcoma S180 and H22 cells in vitro inhibited both in vitro and in vivo [4]. The inhibitory rates of GP against human gastric cancer cell line SGC-7901, human colon adenocarcinoma cell line LOVO and human cervical cancer Hela cell line in vitro are 60%-80%, and inhibit the growth of transplanted tumor cells Heps, S180 and EC in vitro Tumor rate reached 50% to 65% [5]. GBEP could inhibit the growth of human hepatoma cell line BEL-7404, gastric adenocarcinoma cell line SGC-7901 and lung adenocarcinoma cell line SPC-A-1 in vitro for 24-72 h at doses of 10-320 μg / ml. Ginkgo biloba extract may have anti-tumor mechanisms: anti-oxidation and scavenging free radicals, affecting the proliferation and apoptosis of tumor cells, inhibiting the formation of tumor blood vessels, regulating the tumor and related genes, the cytotoxic effect on tumor cells [6]. In vivo anti-tumor study of endophytic fungi in Ginkgo biloba has not been reported at home and abroad.

According to the theory of endophytic symbionts in plants, it is likely that some endophytic fungi are present in Ginkgo biloba and produce the same or similar chemical composition as Ginkgo biloba [7]. 522 strains of endophytic fungi were isolated from Ginkgo biloba in Yangling of Shaanxi

Province by using the method of mycelium growth inhibition. The results showed that 50.7% of the strains had antibacterial activity. Miao Li et al [9] in vitro anti-tumor test showed that isolated from Fuyang, Anhui 19 strains of endophytic fungi, of which the most active strain YX5, the crude extract of the fermentation of tumor cells EC109, human nasopharyngeal carcinoma HONE1 and human cervical cancer HeLa IC50 were 18.3,3.6 and 6.5 μg / ml, respectively.

In this study, we first determined the species of the three strains by the combination of colony morphology and 18 sRNA sequencing. Then the anti-cervical cancer activity of three strains of crude extracts was tested by MTT assay. Finally, the semi-preparative HPLC and the secondary metabolites of the strains with the rate of more than 50% were isolated and the compound monomers with anti-tumor activity were screened.

MATERIALS AND METHODS

Material. Ginkgo biloba was collected from Linyi City, Shandong Province. The bark was taken from a trunk bark of about 2 cm from the ground with a thickness of about 3-8 mm. The ginkgo tree was about 30 years old with a trunk diameter of about 55 cm. After isolation and purification has been preserved in the laboratory -80 ℃ refrigerator. Tumor cells: Hela cervical cancer cells donated by the Department of Medicine. Thermo UltiMate 3000 high performance liquid chromatography, Heidolph rotary evaporator, autoclave, ultrasonic cleaning machines and other equipment were all in our laboratory. The instrument used in this study was the Agilent Technologies 6520 Accurate-Mass Q-TOF LC/MS. The mass spectrometry conditions of this test were electrospray ion source (ESI) mode detection, capillary voltage 3500V, drying gas temperature 325°C, drying gas flow rate 10L/min, atomizing gas pressure 30psig, fragmentation voltage 135V, 1.51 per 1s. In the subgraph, the mass scan range is 50-1600 m/z.

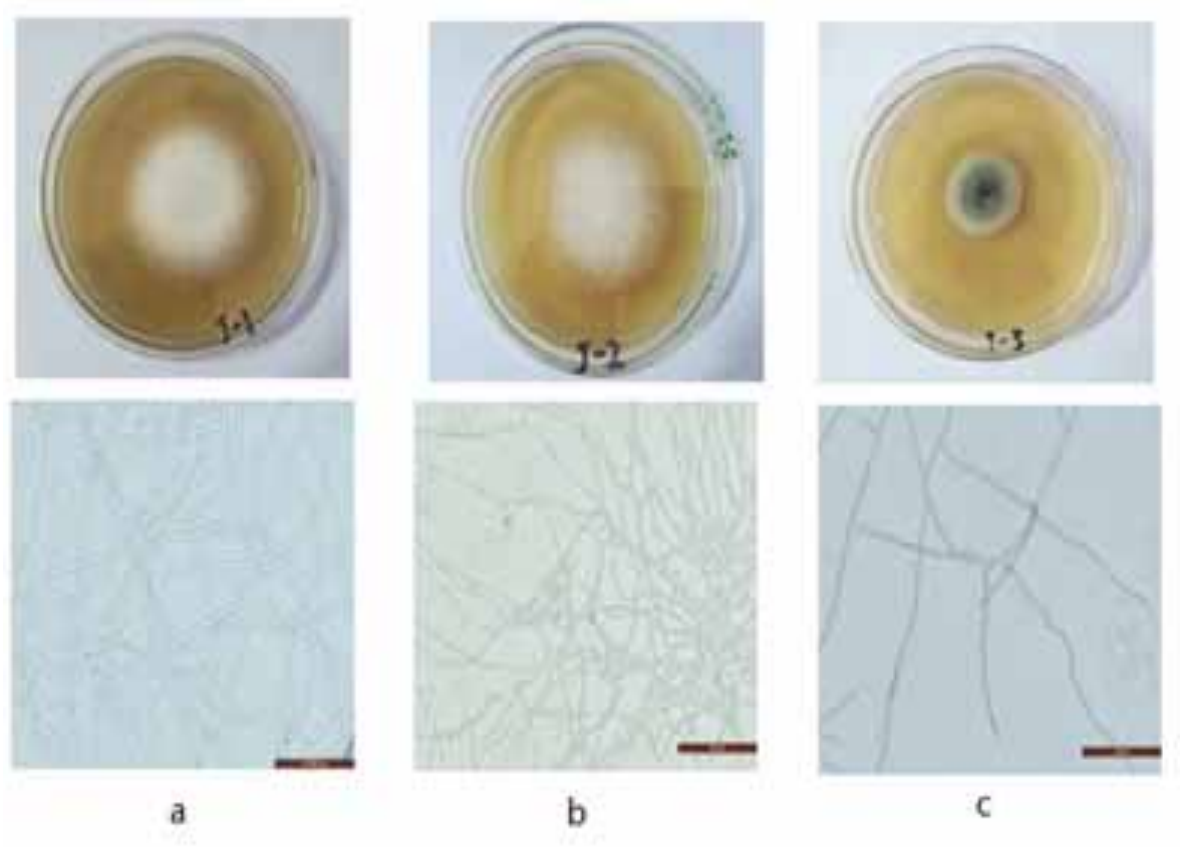

FIGURE 1
Colony and mycelial morphology of three ginkgo endophytic fungi
(a) Strain J-1; (b) Strain J-2; (c) Strain J-3

Identification of endophytic fungi. The three strains were respectively coated on PDA solid medium plate and incubated at 20 ° C for three days. The colony morphology of each strain was observed. Then the cells were respectively inoculated into PDA liquid medium and incubated at 20 ° C in a constant temperature shaker Seven days later, tablets were placed and the morphology of the mycelium was observed with an inverted microscope. Finally, 10 ml of the fermentation broth was taken from each strain and sent to Platinum Biotech (Shanghai) Co., Ltd. for 18 sRNA sequencing analysis.

Endophyte liquid fermentation culture and post-processing. Three strains of endophytic fungi cultured in PDA solid medium were harvested. The mycelium was inoculated into 250-ml PDA liquid flask and cultured at 20 ℃ for 120 days at 120 r / min. Ethyl acetate was then added to each flask at a volume ratio of 1: 1 and the cultivation continued for 4 days in a shaker. After the fermentation broth was filtered through 3-4 layers of gauze, the mycelium was removed and stratified by a separatory funnel to obtain an organic phase containing secondary metabolites of endophytic fungi from Ginkgo biloba. Most of the ethyl acetate in the organic phase is recovered using a rotary evaporator to give a concentrate containing secondary metabolites of endophytic fungi from Ginkgo biloba. Finally, the concentrate was completely dried using a vacuum-concentrated dryer and dissolved in DMSO to obtain crude bacterial extract.

HeLa cervical cancer cell culture and determination of endophytic fungi anti-tumor activity. Culture medium: 10% newborn calf serum, 89% RPMI 1640 complete medium, 1% double antibody (penicillin-streptomycin). Passaged was at 37 ° C with 5% CO_2 in a cell incubator. The crude extracts were tested for their anti-cervical cancer activity by MTT assay. Tumor cells in logarithmic growth phase were digested with trypsin and then made into a cell suspension in complete medium. The blood count plates were counted, followed by inoculation of 100 μl into 96-well plates, leaving two wells as a blank control. Placed in a 5% CO_2 incubator at 37 ℃ for 48 h, 20 μl of the sample diluted with complete medium (20 μl of the negative control group and 100 μl of the culture medium, 3 Repeat), continue to cultivate after 2 d, remove, aspirate medium lost, add 2.5 μg / μl MTT solution to each well 20 μl, 37 ° C for 4 h, each well plus 100 μl DMSO dissolved at 37 ℃ 30 min. The absorbance of each well was measured by microplate reader (measurement wavelength 570 nm respectively). Finally, the inhibition rate was calculated as follows: Inhibition rate = (negative control OD value - experimental OD value) / (negative control OD value - blank control OD value) X100%.

Semi-preparative HPLC analysis of endophytic ginkgo secondary metabolites. The column was a C18 column (250 mm x 10 mm, 5 μm) and the mobile phase was methanol-water. 20% methanol, volume flow 1.0 ml / min, detection wavelength 210 nm, column temperature 35 □, injection volume 100 μl. Depending on the peak shape of secondary metabolites of different strains, different substances are isolated. Then, each substance was co-cultured with cervical cancer HeLa cells, and the effective anti-tumor activity of cervical cancer was determined by MTT method.

RESULTS

Endophytic fungi colony morphology and species identification. Strains J-1 and J-2 were 99% and 98% similar to those of Fusarium spp., respectively. The results of the colonies combined with the results indicated that strains J-1 and J-2 belonged to Fusarium see Fig.1 and Fig.2. However, the similarity of 18SRNA sequence of J-3 with that of Fusarium was only 97%. Therefore, it was preliminarily supposed that strain J-3 might belong to Fusarium, but further observation of sporulation and other molecular biology means to confirm.

Endophytic fungus anti-cervical cancer activity. The inhibitory rates of the strains J-1 and J-3 on cervical cancer cells were 59.6% and 59.1%, respectively (Fig.3). J-1 and J-3 were shown a great inhibition rate of cervical cancer. While the inhibition rate of J-2 was only 13.3 %. Therefore, strains J-1 and J-3 were selected for further experiments. It was suggested that J-1 and J-3 may be play an important role on cervical cancer.

Ginkgo biloba endophytic fungi secondary metabolites of cervical cancer tumor active substance separation. The semi-preparative HPLC method was used to separate the secondary metabolites of strain J-1 and J-3. The separation results are as follows: from the semi-preparative HPLC peak (Fig.4), the secondary metabolites of strains J-1 and J-3 differed and contained many substances. Each compound represented by the peak shape was collected and MTT assay was performed to determine the monomeric compound having anti-cervical cancer activity.

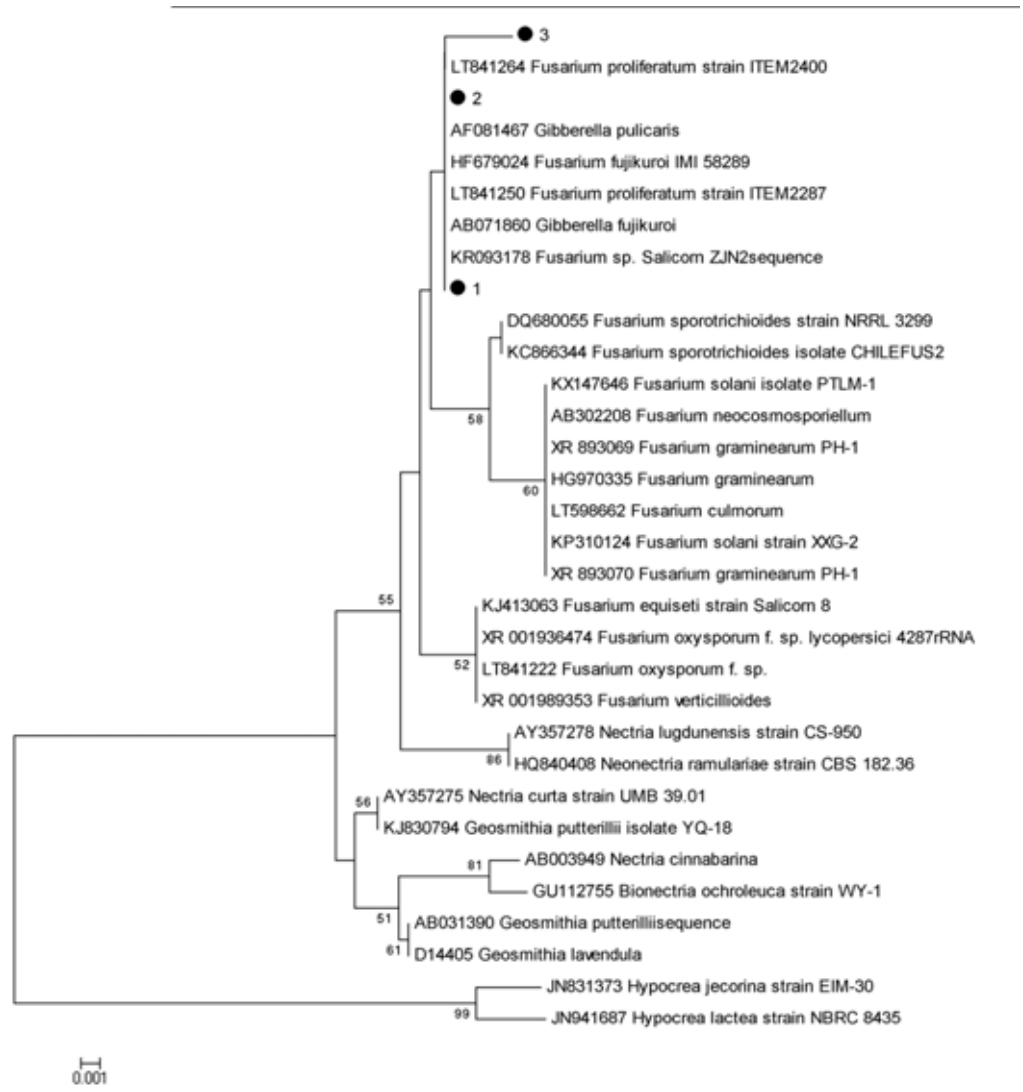

FIGURE 2
Phylogenetic tree of three ginkgo endophytic fungi
(1): strain J-1; (2): strain J-2; (3): strain J-3

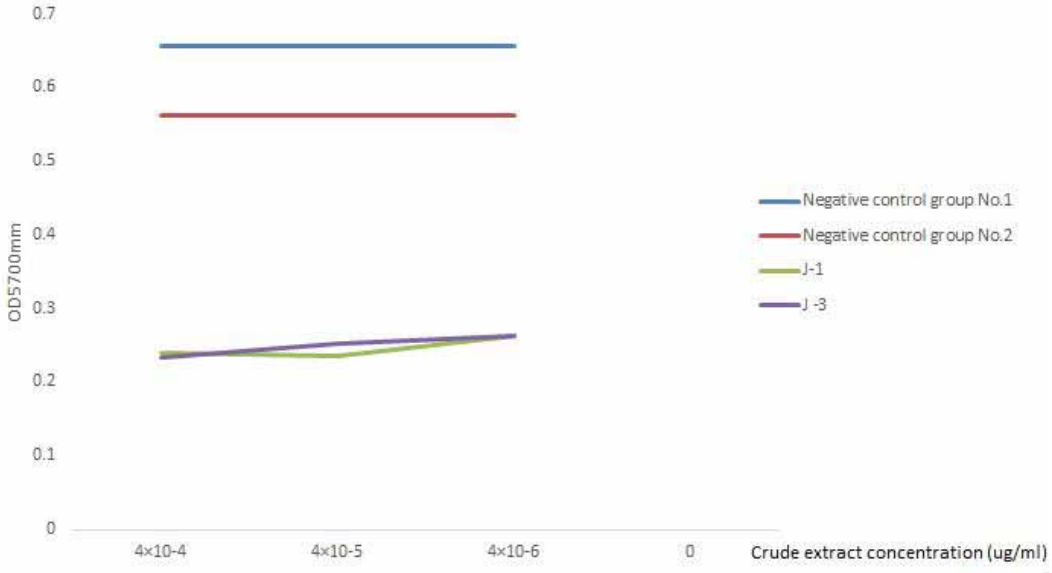

FIGURE 3
MTT assay for anti-cervical cancer activity of strains J-1 and J-3 (24 h treatment)

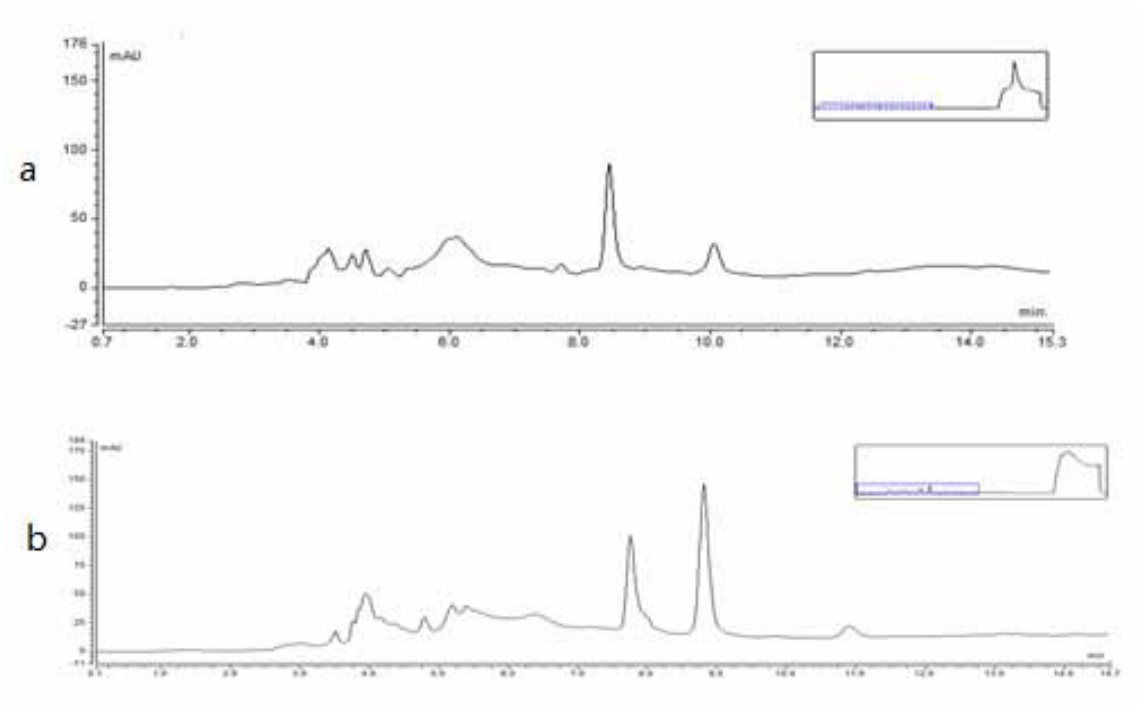

FIGURE 4

HPLC peaks of strains J-1 and J-3 (a) strain J-1; (b) strain J-3

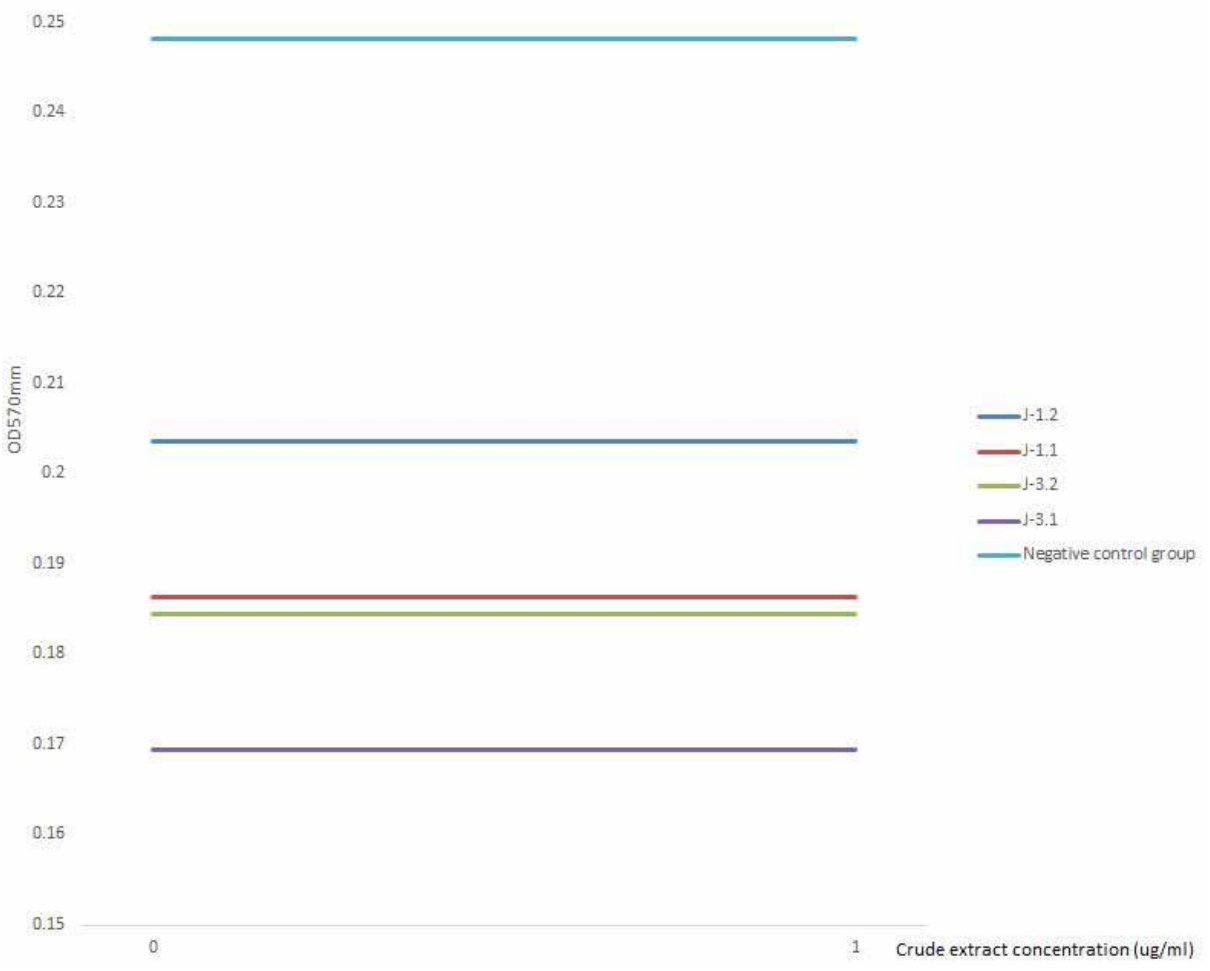

FIGURE 5

Anti-cervical cancer activity of the secondary metabolites of strains J-1 and J-3 (16 h treatment)

Crude extract components of cervical cancer activity test. Nine components of the secondary metabolites of strain J-1 (named J-1.1, J-1.2, J-1.3, respectively) and seven (Named as J-3.1, J-3.2 and J-3.3, respectively) for MTT assay. The results were shown in Fig. 5. The anti-cervical cancer activity of each component of secondary metabolites of strains J-1 and J-3 showed that the inhibitory rates of components J-1.1, J-1.2, J-3.1 and J-3.2 on cervical cancer cells were 25%, 18%, 32%, and 26%, while other components had no inhibitory effect on cervical cancer cells. It was confirmed that components J-1.1, J-1.2, J-3.1 and J-3.2 inhibited cervical cancer cells effect, the next step needs further structural identification.

DISCUSSION

In recent years, the field of medicine has generally accepted the way of obtaining natural active products from symbiotic microorganisms of plants and animals, and many reports on the research on endophyte anticancer drugs related to plants have been reported [10]. Endophytic fungi is a microbial resource with potential research value [11-12]. Not only can it promote the transformation and synthesis of secondary metabolites in the host plant, but also the secondary metabolites with unique chemical structures that are produced by the endophytic fungi themselves [13]. It has become an important new resource for finding new bioactive components, breaking through the limitations of increasingly depleted plant resources, and has great potential for development and application. This paper selected Ginkgo biloba, a unique plant resource of our country, to isolate its endophytic fungi from its healthy tissues. Ginkgo biloba drug research by the natural generation of ginkgo limited very serious, even in ginkgo endophytic fungi become a research hot spot, there are many people are actively looking for a new source of ginkgo drugs [14-16]. In fact, increasing research on medicinal endophyte resources, such as developing endophytic fungi resources of ginkgo, was of great significance.

Three strains of endophytic fungi isolated from Ginkgo biloba were identified in this study. Two strains could be identified as fusarium moniliforme. However, the other strain was difficult to determine its species by 18SRNA and colony morphology. Further identification was needed. In addition, MTT assay showed that the growth inhibition rates of crude extracts of strain J-1 and J-3 on Hela cells were 59.6% and 59.1%, respectively. Which showed that a strong antitumor activity. The strain J-2 Crude extract of cervical cancer cells Hela growth inhibition rate was only 13.3%. Therefore, J-1 and J-3 should be study in the next experimental study. Semi-preparative HPLC results showed that the secondary metabolites of strains J-1 and J-3 were quite different, and contained many substances. The compounds of each peak shape were collected and the results of MTT assay showed that the inhibitory rates of components J-1.1, J-1.2, J-3.1 and J-3.2 on cervical cancer cells were 25%, 18%, 32% and 26%, respectively, while the other components had no inhibitory effect on cervical cancer cells. Therefore, it was confirmed that components J-1.1, J-1.2, J-3.1 and J-3.2 inhibit cervical cancer cells and the next step required further structure identification.

With the deepening of research work on endophytes in plants, more and more substances with antitumor activity have been found. Which have provided great convenience [17-18] for the research and application of microbial drugs and laid a solid foundation for more in-depth study on endophytes foundation. However, there was also some limitation in this study. Firstly, the current endophytic fungi pay attention to the analysis of their species and metabolites, ignoring the study of the association between metabolites and components, and even less on plant endophytic fungi metabolites. The relationship between host components should be do in-depth study. Secondly, even if new components not found in host plants have been found, they have not been given any attention, and no further studies have been done. Moreover, no further studies on the pharmacodynamic effects of these new components and hosts have been conducted. Thirdly, under the current technical conditions, hundreds of endophytes can be isolated from medicinal plants once. If the endophytic fungi were subjected to component analysis and pharmacodynamic observation one by one, the workload was very large. And the efficiency is also very low. Therefore, research and application of efficient screening methods is imperative. In response to the above problems, the research on endophytic ginkgo endophyte should open up thinking, change research ideas, and strive to endophyte fungi to a high level.

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CORRESPONDING AUTHOR

He Qing
Shandong key laboratory
in University of Functional Bioresource Utilization,
College of Life Science,
Dezhou University,
566 University Road West,
Dezhou 253023, Shandong Province – China

e-mail: q_he18@outlook.com