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Full Length Research Paper

A new endophytic taxol- and baccatin III-producing fungus isolated from *Taxus chinensis* var. *mairei*

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120 endophytic fungi were isolated from the old inner bark of *Taxus chinensis* var. *mairei* and only a fungus was detected to produce Taxol and related taxanes in potato dextrose agar (PDA) medium. The presence of taxol and baccatin III was confirmed by high performance liquid chromatography combined with mass spectrometry (LC-MS) and competitive inhibition enzyme immunoassay (CIEIA). The fungal compound showed cytotoxic activity on liver cancer cell line BEL7402 *in vitro*. The strain was identified as one of *Didymostilbe* sp. (designated as DF110) according to its morphological characteristics. The isolation of such fungi provided a promising alternative approach to producing taxol in the near future.

Key words: Taxol, baccatin III, endophytic fungus, *Didymostilbe* sp.

INTRODUCTION

The tricyclic diterpene taxol, a highly effective anticancer drug originally isolated from the bark of *Taxus brevifolia* (Wani et al., 1971), has been employed for the treatment of a variety of cancers. With increasing applications in clinical use and scientific research, there is an urgent need of Taxol than ever. However, the isolation of taxol from the tree bark is limited on account of the relative scarcity of yew and extremely low content of taxol in the plant. To solve such a problem, other attempts such as tissue culture (Christen et al., 1989; Hu et al., 2003) and chemical synthesis (Baloglu and Kingston, 1999; Holton et al., 1995; Nicolaou et al., 1994) etc., have been made to produce the drug. In addition, a great deal of efforts have been focused on the isolation of endophytic taxol-producing fungi since an exciting progress of the first taxol-producing fungus, Taxomyces andreanae, had been discovered in *T. brevifolia* (Stierle et al., 1993). Subsequently, many other endophytic taxol-producing fungi were reported (Guo et al., 2006; Kumaran et al.,2011; Li et al., 1998, 1996; Liu et al., 2009; Metz et al., 2000; Shrestha et al., 2001; Soca-Chafre et al., 2011;Strobel et al., 1996; Wang et al., 2000, 2007; Zhao et al., 2009; Zhou et al., 2010). The isolation of such fungi bring a new promising way for the production of taxol by fermentation techniques, which have a lower cost compared to other methods.

In our previous report, we disclosed the discovery of an endophytic fungus BT2 being capable of producing taxol and taxane baccatin III from the old inner bark of *Taxus chinensis* var. *mairei* (Guo et al., 2006; Wang et al., 2007). In this study, we reported another newly isolated endophytic taxol- and baccatin III- producing fungus. Furthermore, its biological activity was also tested against liver cancer cell line.

MATERIALS AND METHODS

Isolation of endophytic fungus from *T. chinensis* var. *mairei* and its identification

The fungus used in this study was isolated from the old inner bark of *T. chinensis* var. *mairei*, which grows in Sichuan province, Southwest China. After the bark was cut into small pieces of about

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0.25 cm^2, these pieces were treated with 70% (v/v) ethanol for 5 min, and then rinsed three times with distilled water; the water was allowed to evaporate and then the outer black bark was removed off with a sterilized sharp blade. Small pieces of the inner bark were placed on the surface of water agar (2% g/v) in Petri plates and incubated at 25°C in the dark. After several days, fungi were observed growing from the inner bark fragments in the plates. Individual hyphal tips of the various fungi were transferred to new potato dextrose agar (PDA) medium and incubated at 25°C for one week. The same way was repeated 5 times for fungus purity. The endophytic fungus was identified according to its morphological characteristics (Barnett and Hunter, 1977).

Fungal culturing and taxane isolation

The endophytic fungus strain DF110 was grown in 1-L Erlenmeyer flasks containing 250 ml PDA liquid medium. The fungus was incubated at 25°C with shaking (140 rpm) for three weeks. Then the entire culture medium and mycelia were collected, respectively, through four layers of cheesecloth. The mycelia were re-suspended by 100 ml methanol and ultrasonicated for 15 to 20 min on ice, then centrifuged to collect the supernatant. The culture medium were blended well and extracted with equal volume of methylene chloride twice, and the organic phase was finally mixed with the mycelia supernatant. The mixtures were taken to dryness under reduced pressure at 50°C and the residue was dissolved in 1 ml methanol and the insoluble materials were removed off by centrifugation (12000 g) for 30 min at 4°C. The supernatant was filtered through a 0.2 µm polymeric filter prior to HPLC analysis.

LC-MS analyses

After purification, samples were analyzed by a Perkin-Elmer HPLC ISS 200 system combined with a Hewlett-Packard Series 1100 MSD system. The column was an Alltech Econosil C18. Mass spectra were acquired in positive ion mode. Samples in 10 µl of methanol were injected and eluted with 0.8 ml/min with a starting gradient from 40 : 60 (v/v) H_2O : methanol for 10 min, then eluted with 100% methanol for 20 min, and finally with 40:60 (v/v) H_2O : methanol for 10 min. A variable wavelength recorder set at 230 nm was used to detect taxol and baccatin III eluting from the column.

ELISA

A competitive inhibition enzyme immunoassay (CIEIA) kit (Hawaii Biotech Inc) was employed for the detection of taxol and baccatin III (Grothaus et al., 1993).The assay is sensitive to about 1 ng/ml. The assay was carried out using Taxane Immunoassay Kits (TA02, specific for Taxol; TA03, specific for baccatin III. Hawaii Biotech Inc) according to the procedure recommended by the suppliers.

Cytotoxicity study

The liver cancer cell line BEL7402 was used to evaluate the biological effect of the fungal crude extraction. The procedures were performed as follows: Cancer cells were placed in a 96-well plate of 3×10^4 per well and cultured at 37°C for 10 h, and then aliquots (100 µl) of the fungal extraction were added into the 96-well plate at 37°C for 48 h, meanwhile, aliquots of 50 µg/ml authentic taxol were added as a positive control. Another fungal extraction which did not produce taxol was also added as negative control. After incubation for 48 h, the activity of cancer cells was observed by using microscope.

RESULTS

Identification of the fungus

Colonies of strain DF110 grew and extended more rapidly on PDA medium under 25°C. The mycelium surface is approximately lanose and eggshell yellow when young and forms many yellow brown to dark green conidial areas when it matures; reverse of colonies becoming golden leaf's yellow. Conidiophores are commonly 125 to 375 µm in length and with diameter 2.8 to 3.0 µm, colorless, a part branched and some of them become light yellow in age. There are complex Penicilli on the top of conidiophores. Some of sterigmata show thin rod, commonly 7.5 to 25 µm by 1.5 to 2 µm. Conidia is usually elliptical or rod, a few oval, commonly 4 to 7.3 µm by 2.3 to 3.3 µm, usually forming a septum in age, and many of them are usually gathered into a drop of water. Some of conidiophores are usually gathered into synnemata. The strain DF110 was different from that of the endophytic taxol-producing fungi previously reported, and was identified as *Didymostilbe* sp. according to its morphological characteristics (Barnett and Hunter, 1977) and was named DF110 (Figure 1).

LC-MS identified taxol and baccatin III in the fungal compound

A total of 120 fungi were isolated from the old inner bark of *T. chinensis* var. *mairei*, but only strain DF110 was observed to produce taxol and baccatin III by LC-MS. The fungal compound produced a peak at 6.769 min when eluting from the C18 column, with approximate same retention time (6.798 min) as authentic baccatin III (Figure 2). However, the fungal compound has not appeared apparent peak at about the same retention time (15.2 min) as authentic taxol, probably due to the low level of taxol in this sample. So amplified 10-L fluid culture medium and mycelia were prepared and processed by methylene chloride and methanol by using the method described in material and methods. Finally, the residue was dissolved in 500 µl methanol and volume of 100 µl was used to detect the existence of taxol on HPLC and the rest for cytotoxicity assay. The results show that the fungal compound had a peak at 15.2 min corresponding to authentic taxol retention time (data no shown). Further convincing mass spectroscopic evidence for the identity of Taxol and baccatin III was obtained by mass spectroscopy (Figure 3). Characteristically, authentic taxol produced electrospray mass spectrum, with the major molecular ion being $(M+H)^+$ = 854, $(M+Na)^+$ = 876 and $(M+K)^+$ = 892, and authentic baccatin III yielded the major molecular ion being $(M+H)^+$ = 587, $(M+Na)^+$ = 609 and $(M+K)^+$ = 625 (Stierle et al., 1993). By comparison, the fungal compound also yielded a similar electrospray mass spectrum as authentic taxol and baccatin III, respectively. The content of baccatin III was calculated as about 8 to 15

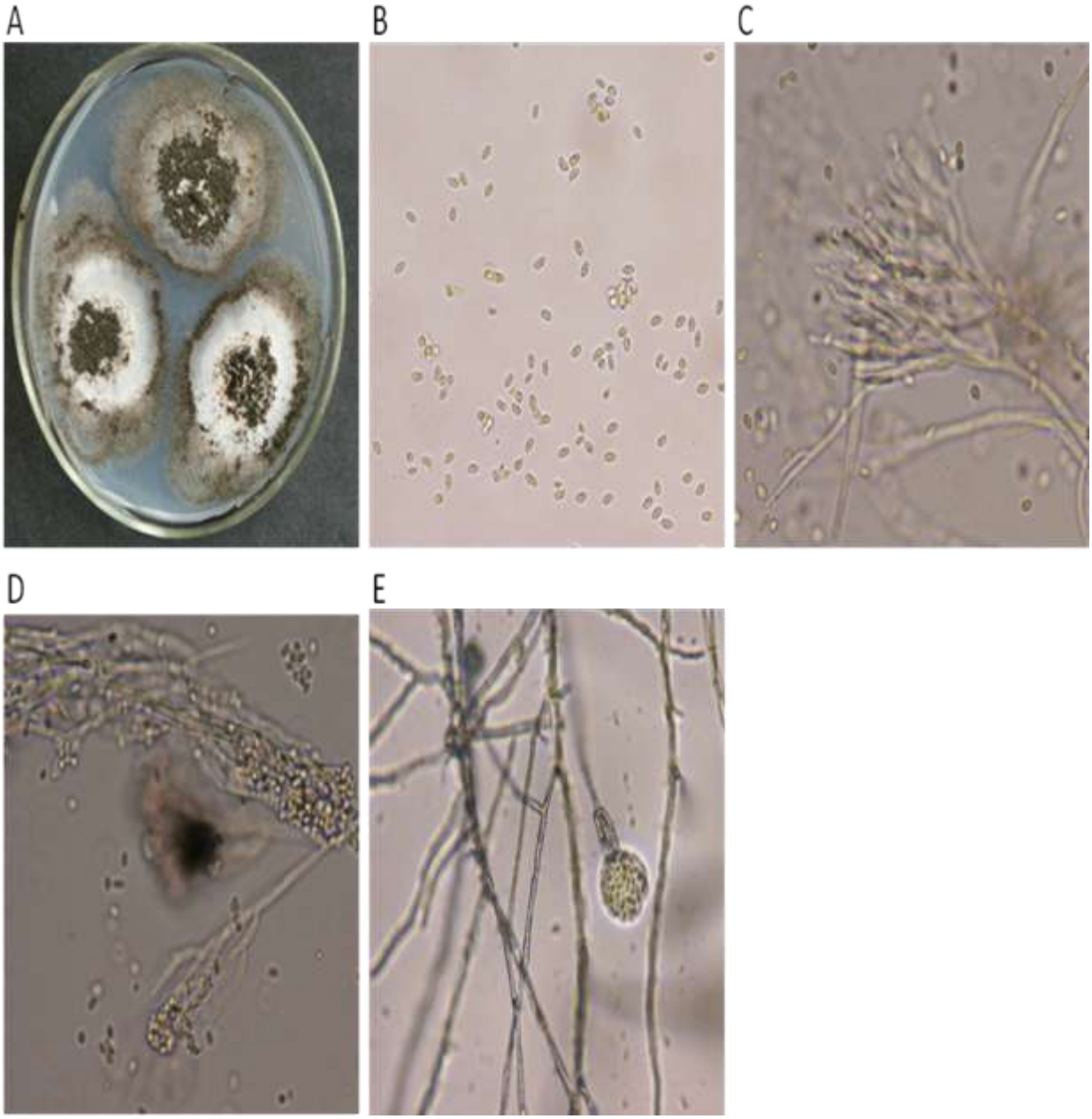

Figure 1. Morphological observation of *Didymostilbe* sp. strain DF110. A: Colonies of strain DF110 on PDA plate after 30-day incubation at 25°C; B: Conidia of strain DF110; C: Penicilli and conidiophore of strain DF110; D: Synnemata and Penicillus of strain DF110; E: The conidia of strain DF110 on the top of Penicillus.

µg per litre culture. However, the content of taxol was not precise quantified due to the low level of taxol under the HPLC quantification limit.

Taxol and baccatin III immunoassays

The competitive inhibition enzyme immunoassay (CIEIA) method was generally used to screen for the presence of taxanes in crude fungal extracts (Guillemard et al., 1999; Guo et al., 2006; Li et al., 1998; Stierle et al., 1993). In order to confirm reliability of the aforementioned results, taxol and baccatin III assays in the sample were carried out by using a taxane Immunoassay Kits. The test using specific monoclonal antibody TA02 and TA03 gave positive results, respectively.

The anticancer activities study

The cytotoxicity of the fungal compound was also studied, which showed that the fungal compound had an obvious

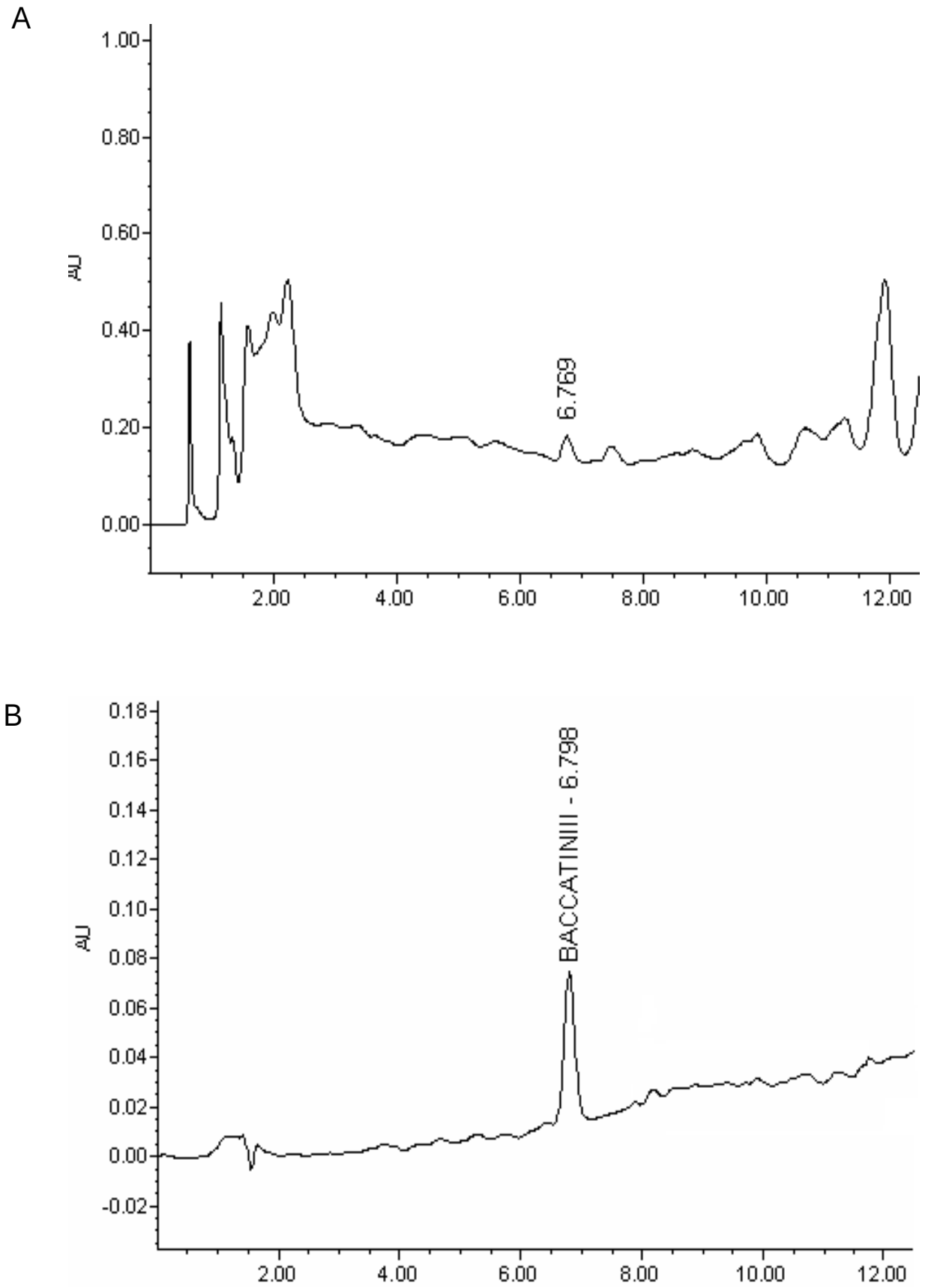

Figure 2. HPLC analysis of the fungus DF110 product (A) and of authentic baccatin III (retention time = 6.7 ± 0.1 min) (B).

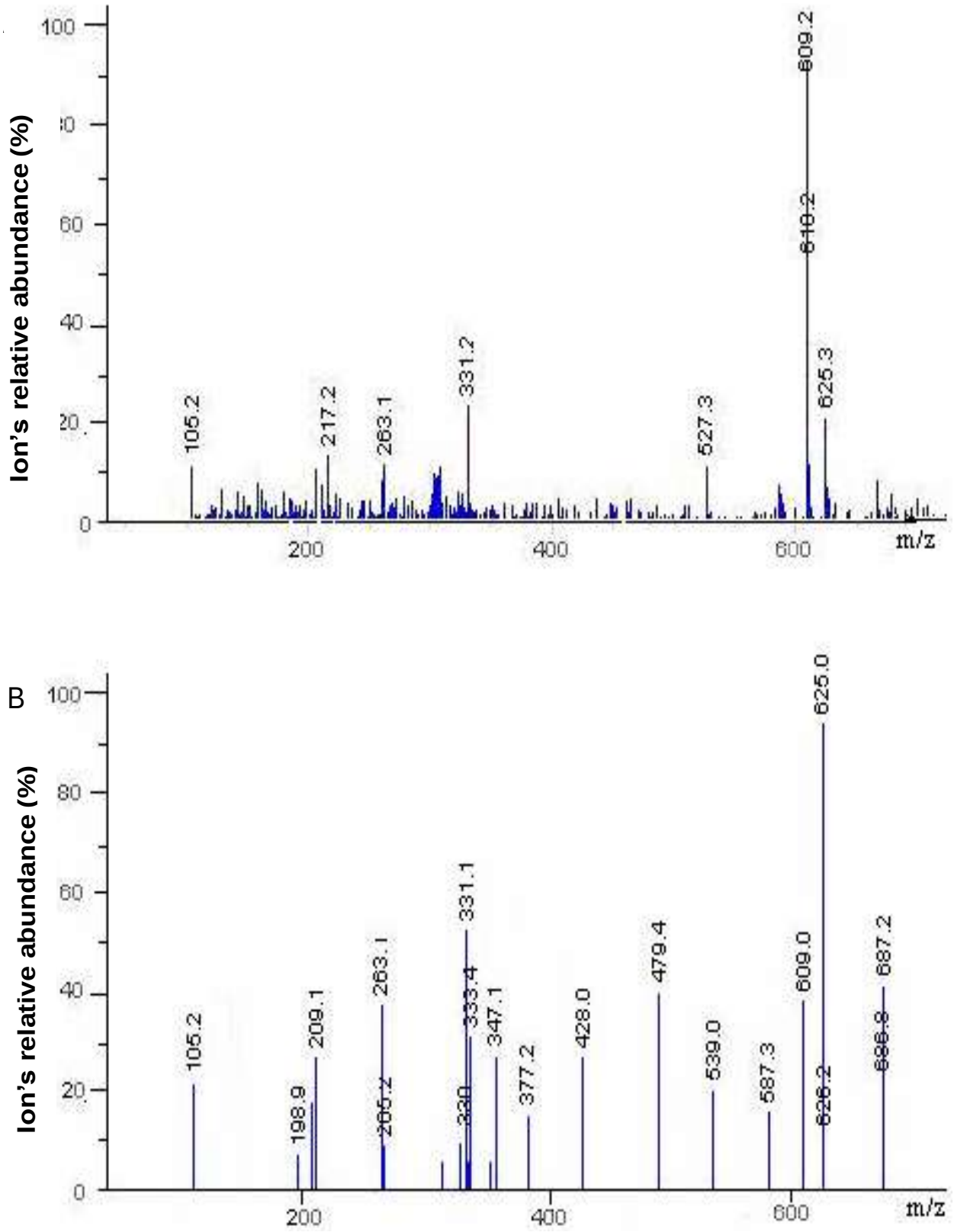

Figure 3. Combined reverse-phase HPLC-atmospheric pressure chemical ionization mass spectrum analysis of authentic baccatin III (retention time = 6.7 ± 0.1 min; (A) and of the fungal baccatin III (retention time = 6.7 ± 0.1 min; (B). The diagnostic mass spectral fragment ions are at m/z $(M+H)^+$ = 587, $(M+Na)^+$ = 609, and $(M+K)^+$ = 625; the mass spectra of authentic taxol (C) and fungal taxol (D) (retention time = 15.2 ± 0.1 min). The diagnostic mass spectral fragment ions are at m/z $(M+H)^+$ = 854, 609 $(M+Na)^+$ = 876, and $(M+K)^+$ = 892.

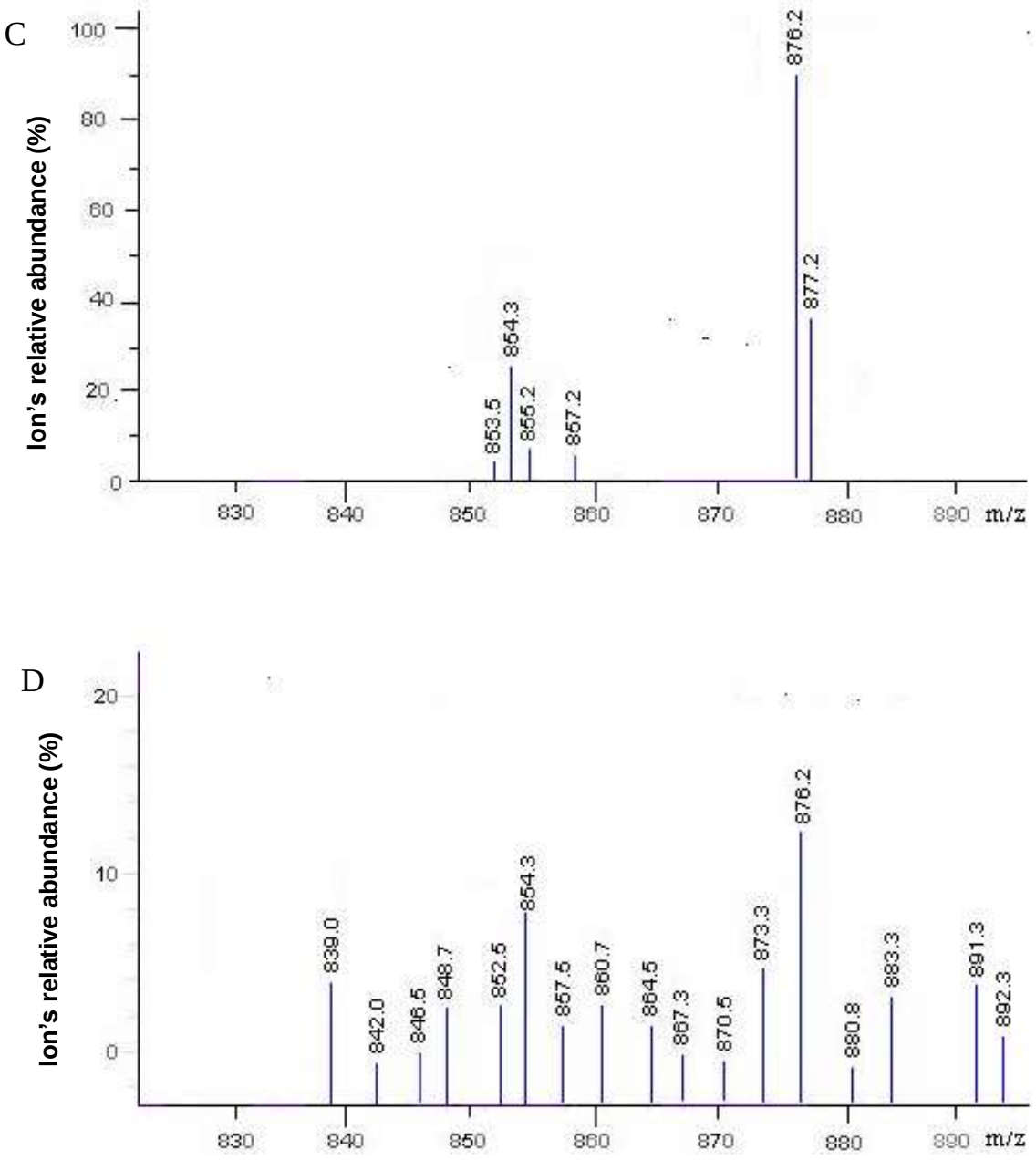

Figure 3. Contd.

cytotoxic effect on the liver cancer cell line BEL7402 (Figure 4). The negative control had a little cytotoxicity to cancer cell line BEL7402 because the sample used in this test was mixtures. We speculated that some components had a little cytotoxicity on the liver cancer cells BEL7402 in the negative control.

DISCUSSION

Since the first endophytic taxol-producing fungus was reported in 1993, great progresses on this field have been obtained. Although, the production of taxol by most endophytic fungi is relatively low compared with that of the taxus trees, the fungi have the short generation time and high growth rate; what's more, genetic manipulation of fungi is achieved more easily than that of plants, so it may be easier to improve the taxol production with the help of genetic engineering. Improving the culturing techniques and the application of genetic engineering may improve taxol and baccatin III production (Demain, 1981; Stierle et al., 1993). Strain DF110 is different from the previous

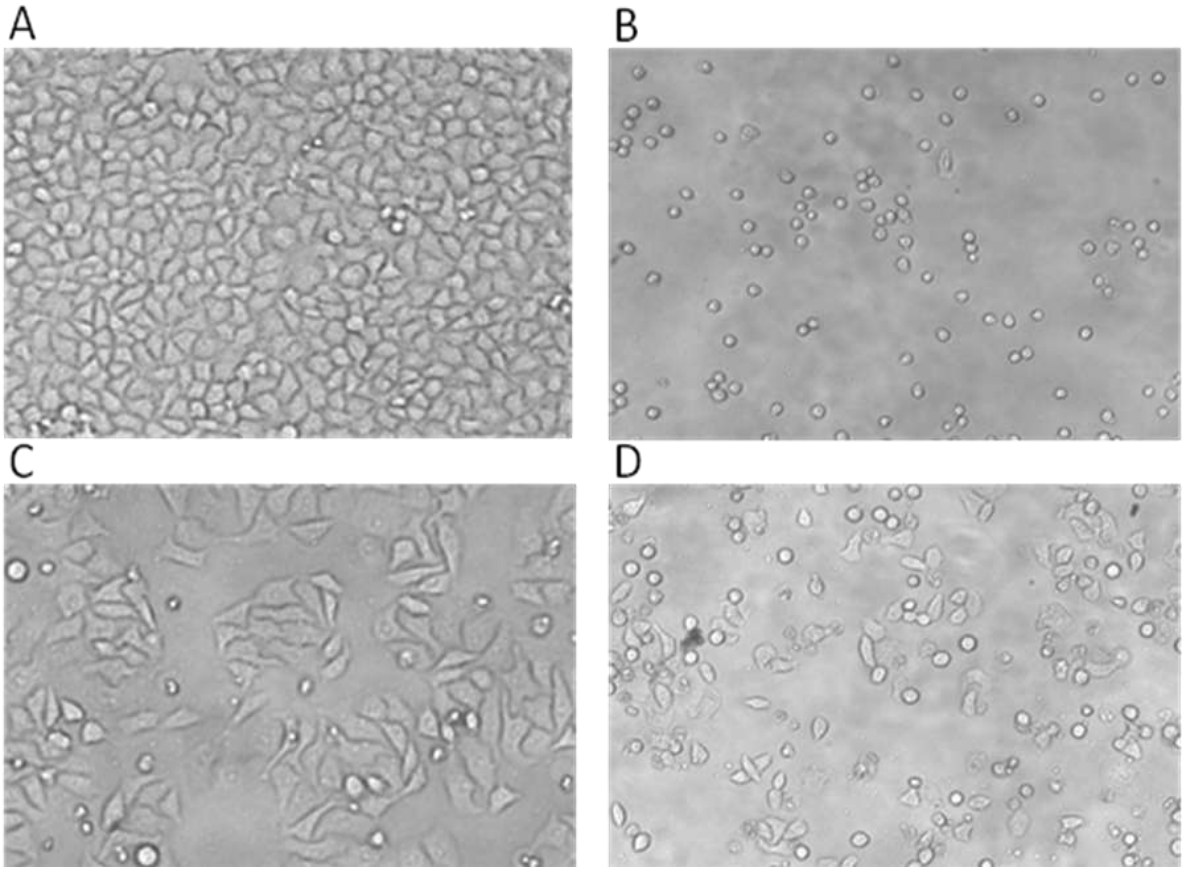

Figure 4. The microscope observation of the liver cancer cells BEL7402 treated with drug. (A) No addition. (B) 50 µg/ml authentic taxol. (C) No-taxol production fungal compound. (D) The fungus DF110 compound.

reported endophytic taxol-producing fungi. Although, the fungus produces low level of taxol, the amounts of baccatin III are about 8 to 15 µg/L culture. Baccatin III is an important staring material in taxol semi-synthesis (Baloglu and Kingston, 1999). Presently, most of the taxol for clinical use is produced by the chemical semi-synthetic approach (Holton et al., 1995). However, the extraction and isolation of the precursors is relatively complex and low yield from taxus tissues, depending on epigenetic and environmental factors (Vidensek et al., 1990; Wheeler et al., 1992). Therefore, this increases the urgent need for baccatin III as a starting material to synthesize taxol and taxotere, a synthetic analog with anticancer activity similar to taxol (Holton et al., 1995). Studies are in progress to transfer the key genes of taxol biosynthetic pathway into the fungus by restriction enzyme-mediated integration (REMI) technique, with an aim of obtaining stable fungal transformants with high productivity of taxol and baccatin III. UP to date, some fungal transformants have been obtained (unpublished). So the strain DF110 may be applied as staring material in taxol semi-synthesis and the fungus may be a candidate for exploring taxol biosynthetic pathway in fungi.

In conclusion, the combined LC-MS, immunochemical and cytotoxicity test suggest that the strain DF110 produces taxol and baccatin III. By optimizing culturing conditions and genetic manipulation, the fungus may be an alternative candidate for the production of taxol and baccatin III by fermentation technology under our efforts in the near future.

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