EXTRACTION AND CHARACTERIZATION OF TAXOL, AN ANTICANCER DRUG FROM LEAF SPOT FUNGUS *PHOMA MORICOLA*

Synopsis

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B.CHITRA KANI

Reg. No. F9298

Department of Molecular Microbiology
School of Biotechnology
MADURAI KAMARAJ UNIVERSITY
(University with potential for Excellence)
MADURAI-625 021
Tamil Nadu
India

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Name	: B. Chitra Kani
PhD registration number	: F9298
Date of Registration	: 12-04-2014
Name of the Department	: Department of Moelcular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai – 21.
Name and Designation of the Supervisor	: Dr. M. Pandi, Assistant Professor Department of Moelcular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai – 21.
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SYNOPSIS

INTRODUCTION

Fungi are one of the most various life forms on plant, estimating the number of fungal species which is considered important with mycologists. There are 1.5 million species of fungi predicted Hawksworth (1991, 2001a). Fungi are an essential part of the life in the biosphere and they have enormous role in ecosystems. They are the major components of soil biomass, accelerate biological decaying and they interact with other living organisms during many different kinds of associations. Most of the fungal species reported are found to be saprobes or pathogenic on higher plants.

The Plant pathogenic fungi extensively contribute to the overall defeat in crop yield. Generally, leaf spots are considered to be more artistic than life threatening trouble. Leaf spot diseases are mainly caused by fungi, although other organisms such as nematodes and bacteria can also be connected with leaf spots. The Leaf spot diseases are possibly the most general type of plant diseases. These diseases are mainly widespread after moderately cool, spring, wet, weather, since water on leaf outside is usually needed for infection (Douglas, 2012).

Secondary metabolites are produced by almost all types of living organisms, including bacteria and fungi. Most studies have reported the activities for their secondary metabolites against the growth of postharvest fungal pathogens in citrus (Singh, 1984), in peach (Pusey *et al*., 1984) and in apple and pear (Janisiewcz *et al*., 1988). Among the microscopic fungi, the ability of imperfect fungi, ascomycetes and several other filamentous endophytic fungal species to produce bioactive products is the most significant. The total number of bioactive fungal compounds is approximately 8,600, representing 38% of all microbial products. Around 950, 900, and 350 compounds have been isolated from the most common ascomycetes, such as *Aspergillus*, *Penicillium*, and

Fusarium species, respectively (Berdy, 2005) and some of them are bioactive against some phytopathogens (Okeke, *et al.*, 1994).

Fungi have a tremendous source in producing novel bioactive compounds and many of these currently have important therapeutic and other uses. In 1995, six of the top twenty best-selling drugs were originated from fungus. They are Ausmentin, Cyclosporine, Ceffriazone, Lorastatin, Pravastatin and Simvastatin (Concepcion *et al*., 2001). In fungi, every year enormous new species were described (Hawksworth 1991 and Kirk *et al*., 2001).

Fungi are the most frequently encountered endophytes and plant pathogens that form a multifarious group of microbes, for which they have a potential to synthesize several bioactive compounds (Bills, 1996, Kusari, 2012, Kumaran *et al*., 2008 & 2009). They are considered as an extra supply of new bioactive compounds, with reported antimicrobial, anticancer, antioxidant, insecticidal, antiparasitic, antiviral, antitubercular and immunomodu-latory activities having wide scope in pharmaceutical and agrochemical industries (Kaul, 2012, Chen *et al*., 2014). Many antimicrobial compounds such as phomenone, trichodermin, cryptocin, altenusin, dihydroxycadalene, ambuicacid and nodulosporins from fungal endophytes have been reported to protect plants against phytopathogens (Deshmukh *et al*., 2015, Mousa, 2013). Sporadically, endophytic fungi and pathogenic fungi also produce host plant secondary metabolites having therapeutic potential like paclitaxel, camptothecin, podophyllotoxin, hypericin and azadirachtin (Kusari *et al*., 2012, Bhalkar *et al.*, 2016, Kumaran *et al*., 2008 & 2009).

Among the therapeutic compounds, Paclitaxel (Taxol) is the generally effective antitumor drug developed in the past three decades. It has been used for effective handling of variety of cancers with refractory ovarian cancer, non-small cell lung cancer, breast cancer, head and neck carcinoma, AIDS related Kaposi's sarcoma and other cancers (Wani *et al*., 1971; Croom, 1995). Taxol inhibits cell propagation in promoting the stabilization of microtubules at the G2-M phase of the cell cycle, by which depolymerisation of microtubules to soluble tubulin is blocked (Stierle *et al.*, 1993; Strobel *et al*., 1996).

Taxol was initially isolated from the barks of pacific yew trees (Wani *et al.*, 1971). Approximately, for obtaining 1 kg of taxol 25,000 pounds of *T. brevifolia* barks were needed (Cragg *et al.*, 1990), which affects the environmental sustainability and the economy. The use of *taxus* species as a source of taxol is ecologically unsuitable because of the low abundance, slow growth of the plant and less yield of the product (Frense, 2007).

On the other hand, demand for the drug has increased considerably due to the expansion of clinical trials and treatments, which leads to investigation of other renewable sources to defeat the essential like total compound mixture (Holton *et al*., 1994a & b; Nicolaou *et al*., 1994), semi-synthesis from its precursor (Holton *et al*., 1995) and plant tissue culture (Zhong, 2002) but none could meet the high demand for taxol production (Sreekanth *et al*., 2011). To overcome these problems, scientists searched for an alternative source for taxol production.

Since, the first report of taxol production from endophytic fungi *Taxomycess andreanae and Taxus brevifolia* (Stierle *et al*., 1993). There has been a phenomenal increase in studying fungal endophytes as potential producers of antimicrobial, insecticidal, cytotoxic and anticancer compound (Zhao *et al*., 2010). Some of the endophytic fungi isolated other than *Taxus* sp., could also produce taxol reported by various scientists (Strobel *et al*., 1996, Liu et al., 2009 and Zhou *et al.*, 2007 & 2009). Taxol producing fungi having potential advantages such as cheap, fast growing at high cell density cultivation, easy genetic manipulation, and the possibility of scale-up on an industrial level with less side effects (Pandi *et al*., 2011; Merlin *et al*., 2012; Kathiravan *et al*., 2013).

The leaf spot fungus *Phyllosticta citricarpa*, isolated from the diseased leaves of *Citrus medica*, showed the production of taxol (Kumaran *et al*., 2008). The presence was confirmed by chromatographic and spectroscopic methods. They furthermore recorded the maximum amount of taxol production in the fungus grown on M1D medium (265 µg/l) followed by PDB medium (137 µg/l). The production rate was increased to 5.3 ×103 fold than in when compared to an earlier reported fungus, *Taxomyces andreanae.*

Kumaran *et al*., 2009 reported the maximum presence of taxol production was recorded in *Phyllosticta tabernaemontanae* is a good source for taxol production. The quantity of taxol was confirmed in chromatographic and spectroscopic analysis. In addition the fungal taxol showed strong cytotoxic activity against cancer cell line. However, the literature survey reveals that only a few studies are reported on fungal Taxol from pathogenic fungi and their efficacy on anticancer activities. Therefore, more studies are needed in this direction.

Format of thesis

The thesis will have Introduction part as it introduces the thesis with an emphasis on its key components, followed by objectives. The Review of Literature will give an idea about the basics of research work carried out earlier in this area and the Materials and Methods elaborate the methodology followed to complete the objectives. The results and discussion will answer the objectives undertaken in the research work which was discussed in four chapters. Each chapter will be described independent of each other followed by supporting references. Summary and conclusion part holds the concluding comments and report the conclusions that have resulted from this study along with future prospective.

OVERALL RESULTS REPORTED IN THESIS

CHAPTER-I

Isolation and morphological identification of leaf spot fungi from different plants

The present study was carried out at the Madurai Kamaraj University (MKU) campus, to isolate and identify the fungi from 16 different plant infected leaf samples. About 18 leaf spot fungi were isolated and evaluated for their pathogenicity test. Further all eighteen positive isolates identified based on morphological features were chosen for further characterization and bioprospecting studies.

CHAPTER-II

Molecular Identification of leaf spot fungi based on ITS sequence & ITS-2 sequence secondary structure

Internal transcribed spacer is a universal marker for fungal identification. The isolates ascertained as pathogens were identified using ITS based phylogenetic analysis. Six genera were recognized among the 18 isolates. They were *Alternaria, Colletotrichum, Guignardia, Phoma, Nigrospora and Diaporthe / Phomopsis* accounting for 1, 10, 1, 1, 3 and 2 isolates respectively. Based on the ITS2 sequence secondary structure phylogeny, the leaf spot fungal isolates were further characterized into 10 different species belonging to the above 6 genera. They were *Alternaria alternata, Colletotrichum gloeosporioides, Colletotrichum karstii, Colletotrichum truncatum, Guignardia mangiferae, Phoma moricola, Nigrospora spherica, Nigrospora oryzae, Diaporthe pseudomangiferae,* and *Phomopsis tersa.* Three distinct species were observed in *Colletotrichum* and it was the most dominant genera. *Nigrospora* sp., were represented by 2 distinct species and one species were recognized in each of the following genera: *Guignardia, Phoma, Alternaria, Diaporthe* and *Phomopsis*. The ITS2 sequence secondary structure based analyses were valuable in distinguishing closely related species. The ITS sequences generated for the isolates in this study were submitted in NCBI database for global access.

CHAPTER-III

Extraction and Characterization of Taxol from Leaf Spot Fungi using Spectral and Analytical Methods

All the eighteen fungal pathogens were chosen for extraction and characterization of taxol, a diterpenoid with potential anticancer activity. All 18 isolates were preliminarily screened for the taxol production based on thin layer Chromatography (TLC). Among the 18 isolates, 3 were found to be positive for Taxol production, in which two leaf spot fungi were already reported for taxol production i.e., *Colletotrichum gleosporioides*, *Colletotrichum truncatum* . The production of taxol from *Phoma moricola* was not reported elsewhere, hence this isolate was further characterized by ultra-violet spectroscopy (UV), high-performance liquid chromatography (HPLC),

fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance spectroscopy (^{1}H NMR) and liquid chromatography-mass spectrometry (LC-MS) analysis.

CHAPTER-IV

Efficacy of Taxol- Extracted from *Phoma moricola* Against Human Lung Cancer Cell Line - A549

The fungal taxol, confirmed using spectral and analytical methods was further analysed for its anticancer activity against human Lung cancer cell line A549. The *in-vitro* cytotoxicity effect of fungal taxol was analysed through 3- (4, 5- dimethylthiazol -2-yl)-2, 5- diphenyltetrazolium bromide (MTT) assay in 10-100µg at 24hrs and 48 hrs. Inhibition of A549 cell proliferation by fungal taxol was observed at an IC_{50} value of 51µg/ml after 24 h incubation. The fungal taxol exerts anti-proliferative effect on human lung cancer cell line by arresting the cell cycle. Morphology and nuclear changes of A549 cells after treatment with fungal taxol at 24hrs was also observed. The cell shrinkage increased progressively when compared to control cells.

SUMMARY

- In the present investigation, over all 18 leaf spot fungi were isolated, and all are spore producing fungi.
- The pathogenicity of the isolates was evaluated through detached leaf assay.
- All the fungi were subjected to morphological identification using light microscope & Scanning electron microscope level.
- Further all the 18 isolates were identified at genus level based on ITS sequence based phylogenetic analysis and at species level using ITS-2 sequence secondary structure based phylogenetic analysis.
- Based on the molecular analysis, the fungi were observed to belong to 6 genus & 10 different species of Ascomycetes. They are: *Colletotrichum gloeosporioides, Colletotrichum karstii, Guignardia mangiferae, Phoma moricola, Nigrospora*

sphaerica, Alternaria alternata, Nigrospora oryzae Diaporthe pseudomangiferae, Colletotrichum truncatum, Phomopsis tersa.

- All the 18 isolates were subjected to TLC based screening for taxol. Among them, following three fungi *Colletotrichum gloeosporioides* Ls-08*, Phoma moricola* Ls-12, *Colletotrichum truncatum* Ls-18 have potential to produce taxol.

- The UV spectra of both fungal and authentic taxol showed similar absorption spectra with characteristic peaks at 220 and 273 nm.

- The fungal taxol was further subjected to IR spectroscopy and spectrum of the fungal taxol was similar to the authentic taxol.

- All the three fungal isolates produced taxol. Among them *Phoma moricola* fungus is not yet reported for taxol production till date. So this culture is chosen for further studies.

- To confirm the presence and quantification of Taxol, the *Phoma moricola* fungal extract was subjected to high performance liquid chromatography (HPLC). The fungal sample gave a peak with similar retention time as that of standard Taxol. The quantity of Taxol produced by the fungus was calculated and it was estimated to be 302µg/L.

- The fungal compound produced an identical mass spectrum as that of authentic paclitaxel. Characteristically, authentic taxol yielded both an $(M + H)^+$ peak at 854 and an $(M + Na)^+$ peak at 876. By comparison, fungal taxol also yielded an $(M + H)^+$ peak at 854 and an $(M + Na)^+$ peak at 876.

- The identity of fungal Taxol was further confirmed by mass spectrum and ^{1}H-NMR spectra spectroscopy. The mass spectrum and ^{1}HNMR spectrum of isolated Taxol was identical in all respects to that of the authentic Taxol.

- The cytotoxic effect of fungal Taxol was evaluated in A549 cell line by the MTT assay. The IC_{50} value of fungal Taxol was calculated as 51µg/ml.

- ❖ Morphology and nuclear changes of A549 cells after treatment with fungal Taxol at 24h was observed. The cell shrinkage increased progressively when compared to control cells.

CONCLUSION

This study was carried out to isolate and bioprospect the leaf spot pathogenic fungi from 16 different plants growing in the Madurai Kamaraj University (MKU) campus. About 18 fungal isolates were recovered from them. Pathogenicity of the isolates was verified through detached leaf assay and they were identified based on ITS2 sequence-secondary structure based phylogenetic analysis. The presence of fungal taxol was screened using chromatographic and spectral methods. Three fungal isolates namely *Phoma moricola* Ls-12, *C gloeosporioides* Ls-08 and *C. truncatum* Ls-18 were observed to produce taxol. Among these, taxol from *Phoma moricola* Ls-12 was evaluated for anticancer activity against A549 cancer cells. Effective inhibition of cell proliferation and induction of cell death was visualised. Based on the above results, the fungal Taxol extracted from the leaf spot fungus *Phoma moricola* could serve as an alternative source of this novel therapeutic drug.

PUBLICATIONS

- ➢ Bose Chitrakani, Senthuran Sureshkumar, Mohan Pandi (2017). Screening and Characterization of Fungal Taxol from Leaf Spot Fungi. *American Journal of Bioscience and Bioengineering* 2017; 5(6): 113-120. http://www.sciencepublishinggroup.com/j/bio doi: 10.11648/j.bio.20170506.11 ISSN: 2328-5885 (Print); ISSN: 2328-5893.

- ➢ Bose Chitrakani, Senthuran Sureshkumar, Pandy Rajapriya, Mohan Pandi (2019). Molecular characterization of leaf spot fungi using internal transcribed spacer (ITS) based phylogenetic inference. *Bioinformation* 15(1): 46-54 (2019). ISSN 0973-2063 (online) 0973-8894 (print).

REFERENCES

Berdy, J. 2005. Bioactive microbial metabolites. *J. Antibiot*. (Tokyo) 58:1–26.

Bhalkar BN, Patil SM, Govindwar SP. Camptothecine production by mixed fermentation of two endophytic fungi from *Nothapodytes nimmoniana. Fungal Biol.* 2016; 120: 873–883.

Bills GF. Isolation and analysis of endophytic fungal communities from wood plants. In: Redlin SC, Car-ris LM, editors. Endophytic fungi in grasses and woody plants: systematics, ecology, and evolution. St. Paul: APS Press; 1996. pp. 31–65.

Chen L, Zhang QY, Jia M, Ming QL, Yue W, Rahman K, et al. Endophytic fungi with antitumor activities:their occurrence and anticancer compounds. *Crit Rev Microbiol.* 2014; 42: 454–473.

Concepcion, G.P., J.E. Lazaro and K.D. Hyde. 2001. Screening for bioactive novel compounds. In Bio-Exploitation of filamentous fungi. Eds. Pointing, S.B. and K.D. Hyde. *Fungal diversity Research series*, **6**: 93-129.

Cragg, G.M. and Snader, K.M. 1990. Isolation of anticancer drugs from plant sources. *Canc. Cells*, (3) 233.

Croom, E.M. 1995. *Taxus* for taxol and taxoids. *Stuffness MTaxol: science and applications, Boca Raton*, 37-70.

Deshmukh SK, Verekar SA, Bhave SV. Endophytic fungi: a reservoir of antibacterials. *Front Microbiol*.2015; 5: 1–43. https://doi.org/10.3389/fmicb.2014.00715 PMID: 25620957

Douglas, S. M. 2012 Leaf spot diseases of ornamental trees and shrubs. *The connecticut agricultural experiment station. www.ct.gov/caes.*

Frense, D. 2007. Taxanes: perspectives for biotechnological production. *Appl. Microbiol. Biotechnol*, (73): 1233-40.

Hawksworth 1991. Kevin D. Hyde, Saisamorn Lumyong, E.H.C. McKenzie and E.B. Gareth Jones. Occurrence of fungi on tissues of the peat swamp palm *Licuala longicalycata. Fungal Diversity* 25: 157-173.

Hawksworth, D.L., 2001a. Occurrence of fungi on tissues of the peat swamp palm *Licuala longicalycata. Fungal Diversity* 25: 157-173.

Holton RA, Biediger RJ & Boatman PD (1995) Semisynthesis of taxol and taxotere. Taxol: *Science and Application* (Suffness M, ed), pp. 97–121.

Holton, R.A., Kim, H.B., Somoza, C., Liang, F., Biediger, R.J., Boatman, P.D., Shindo, M., Smith, C.C., Kim, S., Nadizadeh, H., Suzuki, Y., Tao, C., Vu, P., Tang, S., Zhang, P., Murthi, K.K, Gentile, L.N. and Liu, J.H. 1994b. First total synthesis of taxol. 2. Completion of the C and D rings. *J. Am. Chem. SocI*, (116): 1599–1600.

Holton, R.A., Somoza, C., Kim, H., Liang, F., Biediger, R.J., Boatman, P.D., Shindo, M., Smith, C.C., Kim, S., Nadizadeh, H., Suzuki, Y., Tao, C., Vu, P., Tang, S., Zhang, P., Murthi, K.K., Gentile, L.N. and Liu, J.H. 1994a. First total synthesis of taxol. 1. Functionalization of the B ring. *J. Am. Chem. Soc*, (116): 1597–1598.

Janisiewcz, W., and J. Roitman. 1988. Biological control of blue mold and grey mold on apple and pear with Pseudomonas cepacia. *J. Phytophathol*. 78:1697–1700.

Kathiravan, G., Sureban, S. M., Sree, H.N., Bhuvaneswari, V. and Kramony, E. 2013. Isolation of anticancer drug taxol from *Pestalotiopsis breviseta* with apoptopsis and B - cell lymphoma protein docking studies. *J. basic clin. Pharm*, (1): 4.

Kaul S, Gupta S, Ahmed M, Dhar MK. Endophytic fungi from medicinal plants: a treasure hunt for bioactive metabolites. *Phytochem. Rev*. 2012; 11: 487–505.

Kirk, P.M., Cannon, P.F., David, J.C., and Stalpers, J.A., 2001. *Ainsworth and Bisby's Dictionary of the fungi 9th ed*., CABI Biosciences, U.K. 655.

Kumaran, R.S., Muthumary, J., and Hur B.K. 2008.Isolation and identification of taxol, an anticancer drug from *Phyllosticta melochiae* yates, an endophytic fungus of *Melochia corchorifolia* L. *Food Sci. Biotechnol.*17(6), 1246-1253.

Kumaran, R.S., Muthumary, J., Eun- Ki Kim and Byung-Ki Hur. 2009. Production of Taxol from *Phyllosticta dioscoreae*, a leaf spot fungus isolated from *Hibiscus Rosa Sinensis*. *Biotechnology and Bioprocess Engineering*. (14):76-83.

Kusari S, Hertweck C, Spiteller M. Chemical ecology of endophytic fungi: origins of secondary metabolites. *Chem. Biol*. 2012; 19: 792–798.

Liu, K., Ding, X., Deng, B., Chen, W. 2009. Isolation and characterization of endophytic taxol-producing fungi from *Taxus chinensis. J. Ind. Microbiol. Biotechnol*, (36): 1171–1177.

Merlin, J.N., Nirmal Christhudas, I.V.S., Kumar, P., Kumar, M. and Agastian, P. 2012. Taxol production by endophytic *Fusarium solani* LCPANCF01 from *Tylophora indica. J. Acad. Indus.* (5): 1.

Mousa WK, Raizada MN. The diversity of anti-microbial secondary metabolites produced by fungal endophytes: an interdisciplinary perspective. *Front Microbiol*. 2013; 4: 1–18.

Nicolaou, K.C., Yang, Z., Liu, J.J., Ueno, H., Nantermet, P.G., Guy, R.K., Claiborne, C.F., Renaund, J., Couladouros, E.A., Paulvannan, K. and Sorensen, E.J. 1994. Total synthesis of taxol. *Nature*, (367): 630– 634.

Okeke, B., F. Seigle-Murandi, R. Steiman, and N. B. De Gusmao. 1994. Fungal metabolite extracts active against phytopathogens. *Sci. Total Environ*. 155:125–130.

Pandi, M., Kumaran, S.R., Choi, Y.K., Kim, H.J. and Muthumary, J. 2011. Isolation and detection of taxol, an anticancer drug produced from *Lasiodiplodia theobromae*, an endophytic fungus of the medicinal plant *Morinda citrifolia*. *Afri J. Biotechnol.* **10**(8): 1428-1435.

Pusey, P. L., and C. L. Wilson. 1984. Postharvest biological control of stone fruit brown rot by *Bacillus subtilis*. *Plant Dis*. 68:753–756

Singh, V., and B. J. Deverall. 1984. Bacillus subtilis as a control against fungal pathogens of citrus fruit. *Trans. Br. Mycol. Soc*. 83: 487–490.

Sreekanth, D, G. K. Sushim, A. Syed, B. M. Khan, and A. Ahmad. 2011. Molecular and Morphological Characterization of a Taxol-Producing Endophytic Fungus, *Gliocladium* sp., from *Taxus baccata***.** *Mycobiology*. 2011 Sep; 39(3): 151–157**.**

Stierle, A., Strobel, G.A. and Stierle, D. 1993. Taxol and taxane production by *Taxomyces andreanae,* an endophytic fungus of pacific yew. *Science*, (260): 214–216.

Strobel, G. A., Yang, X., Sears, J., Kramer, R., Sidhu, R.S. and Hess, W.M. 1996. Taxol from *Pestalotiopsis micro*spo*ra*, an endophytic fungus of *Taxus wallachiana*. *Microbiol*. (142): 435–440.

Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P. and McPhail, A.T. 1971. Plant antitumor agents. VI The isolation and structure of Taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *Journal of American Chemical Society*, **93**: 2325-2327.

Zhao, J., Zhou, L., Wang, J., Shan, T., Zhong, L., Liu, X. and Gao, X. Endophytic fungi for producing bioactive compounds originally from their host plants. 2010. *Curr. Res. Technol. Edu. Top. Appl. Microbiol. Microbial Technol*, (1): 567-576.

Zhong, J. J. 2002. Plant cell culture for production of paclitaxel and other taxanes. *J. Biosci. Bioeng*, (94): 591–599.

Zhou, X., Wang, Z., Jiang, K., Wei, Y., Lin, J., Sun, X. and Tang, K. 2007. Screening of taxol producing endophytic fungi from *Taxus chinensis*. *Appl. Biochem. Microbiol*, (43): 490–494.

Zhou, X., Zheng, W., Zhu, H. and Tang, K. 2009. Identification of a taxol- producing endophytic fungus EFY 36. *Afr. J. Biotechnol*, (8): 2623-2625.