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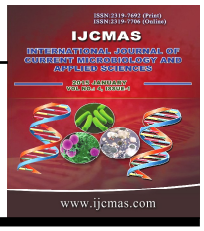

Original Research Article

Evaluation of Phytotoxic and Bioactive Potential of Paclitaxel from *Fusarium solani*

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K e y w o r d s

Fusarium solani, Paclitaxel, Phytotoxic, Beetle leaf

A B S T R A C T

The genus *Fusarium* was comprised of a large, complex group of fungi and contains numerous species that produce noxious secondary metabolites and cause serious plant diseases. The *Fusarium solani* was screened for their bioactive and ability to produce toxic secondary metabolites. The toxin production by Fusarium *solani* was studied under five different cultivation media, different pH and different incubation periods. The secondary metabolite detected by TLC showed the Rf value of 0.6 may be paclitaxel derivatives. UV Spectrum data shows the peak value at 200 to 500nm, which indicate it may be paclitaxel. The HPLC data had the retention time of 4.473 indicates that it may be paclitaxel.NMR studies and molecular modeling were also used in determining the three-dimensional structure of paclitaxel and correlating the conformation of paclitaxel with its microtubules-binding ability. So further investigation is needed for the complete structural elucidation and High throughput screening against the infectious pathogen, plant derived pathogens and the cancerous cells.

Introduction

The genus Fusarium occurs widely in nature as saprophytes in soil and decaying vegetables, some species are plant parasites, where specialized patho types may cause vascular wilt, stem rot, ear diseases and damping off (Booth, 1971; Domch *et al.*, 1980). Additionally, several species of Fusarium have been associated with foot rot and root rots of wheat (Fouly *et al.*, 1996; Parry, 1990; Pettitt *et al.*, 1996; Smiley and Patterson, 1996).

Fusarium solani was most frequently isolated from warmer and wetter soils (Summerell *et al.*, 1993).

The microscopic morphology of *Fusarium solani* hyphae are septate and hyaline conidiophores are simple (non-branched) or branched monophialides (phialides with a single opening). Macroconidia are moderately curved, stout, thick-walled, usually 3–5 septate, measures 4–6 × up to

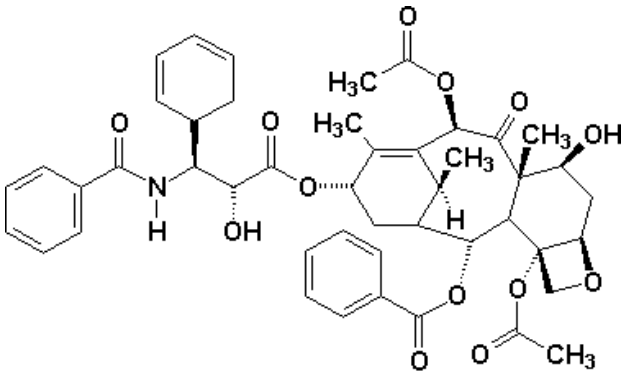
65µm long and are borne on short conidiophores that soon form sporodochia. Microconidia are borne from long monophialides and occur in false heads only (in clusters of conidia at the tip of the phialide). Chlamydoconidia are present (some-time profuse) and occur both singly and in pairs (Sutton *et al.,* 1998). On Potato Dextrose Agar (PDA) *Fusarium solani* colonies are white to cream in colour, some have a slight green or blue pigmentation.

A number of napthaquinone type pigments including fusarubin, anhydro-fusarubin, javanicin, methyl ether- fusarubin, marticin, isomarticin lactone are ethyl ether fusarubin, solaniol, nectriafusarubin and dihydro-fusarubin lactone are known to produce by *Fusarium solani*. Some napthaquinone have antimicrobial activity and nematicidal properties (Hameed *et al.,* 2001).

Paclitaxel, a diterpene compound, has been used alone or in combination with other chemotherapeutic agents for the treatment of a variety of cancers, as well as AIDS-related Kaposi sarcoma (Croom, 1995; Brown, 2003). It was first isolated from the bark of the pacific yew tree, *Taxus brevifolia* (Strobel *et al.,* 1993, 1996).

Paclitaxel is a hydrophobic compound with tremendous potential in proliferation vascular disease and its ultimate clinical use may depend on through characterization of mechanisms (Axel *et al.,* 1997; Sollot *et al.,* 1995; Heldman *et al.,* 1997; Farb *et al.,* 1997; Heller, 1998). Structure determination of paclitaxel was achieved after a year in 1968 through a combination of spectroscopic techniques, chemical degradation and X-ray crystallography of derivatives of degraded moieties. It was evident that paclitaxel had the empirical formula of $C_{47}H_{51}NO_{14}$ with a molecular weight of 853.9 and it was comprised of a taxone nucleus to which a rare four-membered oxetane ring was linked to C-4 and C-5 and an ester was attached at the C-13 position (Wani*et al.,* 1971). The complete IUPAC name for paclitaxel is tax-11-en-9-one-5β, 20-epoxyl-1.2α, 4, 7 β, 13 α-hexahydroxy-4, 10-diactate-2-benzoate, 13- β-benzoyl-amino- α-hudroxybenzenepropionate.

Chemical structure of paclitaxel



Paclitaxel is a potent chemotherapeutic agent originally extracted from the bark and leaves of the Western yew *Taxusbrevifolia*. Paclitaxel's primarymechanism of antieoplastic activity derives from its capacity of half cell-cycle progression by inhibiting dynamic depolymerization of α/β tubulin polymers, leading to rapid neoplastic cell death and inhibition of tumor progression (Donaldson *et al.,* 1994). Paclitaxel has been approved to treat breast, ovarian and lung cancer as well as AIDS-related kaposi's sarcoma (Wiseman and Spencer, 1998).

The present investigation is aimed to evaluate the bioactive and phytotoxic potential of secondary metabolite from *Fusarium solani* through screening theirtoxicological effect on *Piper betel* leaves and screening against human bacterial pathogens.

Materials and Methods

Fungal strain

Fusarium solani MTCC-4289 used in this present investigation was obtained from IMTECH- Chandigarh and maintained as pure culture in Potato Dextrose Agar (PDA) slants for further experimental purpose.

Morphological studies

The Morphological characteristics were studied based on the Alexopolus *et al.* (1996). The fungal strain was streaked on PDA slant and inoculated in to PDA plates, then incubated at 37°C for 3 to 4 days. The slide culture technique was also adopted. The mycelium and spores of the fungal species were observed under microscope (40X) and confirmed as *Fusarium solani.*

Culture maintenance

Fungal culture was maintained on PDA (Potato Dextrose Agar) slopes at 28ºC and stored at 8ºC. It was then subcultured for every 4 to 6 weeks.

Growth on different cultivation media

The five different culture media Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), CzapekDox Agar (CDA), Sabouraud Dextrose Agar (SDA) and Oat Meal Agar (OMA) were prepared and inoculated with the mycelial plug taken from the PDA plate. It was then incubated for seven days and then colony diameter was measured.

Physiological studies

Effect of temperature on growth of *Fusarium solani*

The inoculated plates were placed in sterile plastic bags and incubated at 10°, 15°, 20°, 30° and 35°C. After a period of 5 days, the colony diameter was measured. Daily radial growth rate was calculated.

Effect of water potential on growth of *Fusarium solani*

Five different culture media with mycelium growth with potassium chloride at various concentration (-1, -2, -3, -4, -5 and -6 MPa.) were prepared. The inoculated plates were placed in sterile plastic bags and incubated at 20°C. After a period of five days, the colony diameter was measured. Daily radial growth rate was calculated.

Effect of pHon growth of *Fusarium solani*

The five different sterile culture media were mixed with an equal volume of the buffer to give the desired concentration of medium. The pH was obtained over the range from 3 to 8 with citrate phosphate and trisaminomethane buffers respectively. The plates were inoculated with mycelial plugs taken from PDA and incubated at 20°C. After a period of five days, the colony diameter was measured. Daily radial growth rate was calculated.

Extraction of paclitaxel

The *Fusarium solani* was grown in 500 ml of potato dextrose liquid medium in 1000 ml Erlenmeyer flasks and incubated for 7 days at 25°C. The mycelium growth on liquid medium was extracted twice with an equal volume of methylene chloride (CH_2Cl_2) (Strobel *et al.,* 1996). The organic phase was taken to dryness under reduced pressure by using rotary vacuum evaporator at 40°C.The residue was redissolved in l ml methanol. Then it was subjected for thin layer Chromatography analysis

Toxicity studies of paclitaxel from *Fusarium solani* on *Piper bête l*eaves

Effect of different culture media on toxicity of *Piper betel* leaves

The toxic metabolites of *Fusarium solani* cultured on five different culture media, pH

adjusted to 5.8 by adding drops of 1M NaOH or 1M CH_3COOH solution. After incubation at 25^0C for 4 days, the liquid cultures were harvested, filtered and diluted to 1%, 10%, 20% and 50% concentration.

Detached leaves of *Piper betel* were immersed in these solutions and control was maintained. After 24hrs toxicity were observed and wilt formation were measured (Table 2).

Effect of different pH on toxicity of *Piper betel* leaves

The effect of pH on *Fusarium solani* toxin production was studied after growing the isolates in 250 ml Potato Dextrose (PD) medium with pH adjusted to 3.5, 4.5, 5.5, 6, 6.5 and 7.5.v.

Then the culture was incubated at 25°C for 4 days, the liquid cultures were harvested, filtered and diluted to 10%, 20% and 50% concentration. Detached leaves of *Piper betel* were immersed in these solutions and distilled water (pH 4.7) was used as control. After 24 hrs toxicity was observed and measured.

Effect of different incubation time on toxicity of *Piper betel* leaves

Fusarium solani toxin production was studied by growing the isolates in conical flasks and incubated at 25°C, liquid culture were harvested at 10, 15, 20, and 30 days and repeatedly filtered.

The culture filtrates of different incubation time was harvested and diluted to 10%, 20% and 50% concentration. Detached leaves of *Piper betel* were immersed in these solutions and control was maintained. After 24hrs toxicity was observed and measured.

Separation and Characterization of paclitaxel

Detection of paclitaxel using TLC

The fungal filtrate was extracted with an equal volume of methylene chloride, evaporated and dissolved in methanol. The separation was carried out in acetone-methylene chloride in a ratio 20:80. After a final drying step, plates were sprayed with a mixture of 0.5g vanillin in 100ml sulphuric acid in the ratio of 40:10 ethanol and heated gently for 2 to 5minsat 40°C (Cardellina, 1991). The bands were observed and the results were recorded.

UV spectrometry analysis

The UV- Spectrometry analysis was carried out for the culture supernatant. The UV-Spectrometer Shimadzu (UV- 1650 PC) was done between 200 to 500nm. The UV spectra were taken in Science Instrumentation Centre, ANJA College, Sivakasi.

HPLC analysis

HPLC analysis was carried out by the spot eluted from the TLC plate. It was performed on Schimadzu, Spinchrom HPLC-530 available in science Instrumentation Centre, ANJA College, Sivakasi.

FTIR analysis

FTIR spectral analysis was carried out for the culture supernatant to detect the functional groups of the secondary metabolite paclitaxel. It was performed on Schimadzu FTIR 8400s instrument with resolution of 4cm, available in Science Instrumentation Centre, ANJA College, Sivakasi. The electromagnetic spectrum was

recorded from Infra red region of the electromagnetic spectrum.

NMR analysis

The NMR analysis was carried out for the culture supernatant. The ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) Spectra of the complexes in DMSO were recorded on a Brucker instrument operating at 100 and 300.1312 MHz using TMS as internal standard. The NMR spectra were taken at Department of Organic Chemistry, Madurai Kamaraj University, Madurai.

Result and Discussion

The morphology of mycelium and spores of the fungi were observed. In solid media culture, such as Potato Dextrose agar plate, the *Fusarium solani* colonies are white to cream in colour. In Sabouraud Dextrose agar, the mycelium first appears white and then change to light pink colour. In Malt Extract Agar (MEA) and Czapek Dox Agar (CDA) the mycelium appears white in colour. In Oat Meal Agar (OMA), the mycelium appears dirty white in colour (Table 1). The mass culture was done, to measure the diameter of *Fusarium solani* on five different cultivation media. The diameter was high (8.3cm) in Potato dextrose agar and 7.6cm in Sabouraud dextrose agar and low (4.0cm) in Oat meal agar (Fig. 1).

The rate of mycelial growth of *Fusarium solani* on five different media in response to changes in temperature was noted. The rate on mycelial growth was increased as temperature increased up to 25°C and then decreased. Mycelial growth was relatively low at 10°C with an average of 1.25 cm. The mycelial growth was high at 25°C with an average of 6cm. Among the media used radial growth rate at all temperature was observed high in Potato dextrose agar media with 4.5cm. The radial growth was low in Oat meal agar with 1.75cm (Fig. 2).

The optimum toxin production at pH 5.5 and infection in leaves is high at 50% concentration (5.3 cm) at pH 5.5. The infection rate was low at 50% concentration (3.02 cm) at pH 7.5 (Table 3) (Plate 1). The rate of infection in *Piper betel* leaves was high at 50% concentration with 4.226 cm at 25 days. Significant difference was observed among filtrates harvested at 20, 25 and 30 days when compared with 10 and 25, 30 days (Table 4) (Plate 5).

The UV Spectrum analysis of paclitaxel of *Fusarium solani* was provided in figure 3. The data for the present study showed the peak at 200nm to 250nm which indicates the presence of paclitaxel derived secondary metabolite. The thin layer chromatography analysis of paclitaxel production of *Fusarium solani* MTCC- 4289 revealed that the bluish colour spot was observed after spraying the reagent. The calculated Rf value was 0.6, which indicates the presence of paclitaxel derived secondary metabolite.

The HPLC analysis of paclitaxel production *by Fusarium solani* was provided in figure 4. The HPLC data for the present study showed the peak at various retention times such as 3.677 and 4.473. The peak obtained at the retention of 4.473 indicated the presence of paclitaxel derived secondary metabolite in the culture supernatant.

The FTIR spectral analysis of the secondary metabolite of *Fusarium solani* was depicted in figure 5. It reveals that FTIR data showed that the peak values at 3780.60, 3404.47, 2926.11, 2854.74, 2174.45, 1735.99, 1656.91, 1411.94, 1383.01, 1261.49, 1076.32, 1043.52, 885.36 and 802.41. The spectrum of compound showed a band in the

region of 3780.60 and 3404.47 assignable to OH group.

H NMR spectral studies were also been carried out for the secondary metabolite of *Fusarium solani*. It was revealed that the compound with empirical formula of $C_{47}H_{51}NO_{14}$ with reference to the peak obtained at (ppm value) was indicated the paclitaxel residues (Fig. 6).

Table.1 Colony Morphology of *Fusarium solani* on different cultivation media

Cultivation Media	Growth	Colony Morphology
SDA	+++	White to Pink
PDA	+	White to cream colour
OMA	++	Dirty white colour
MEA	+++	White colour
CDA	++	White Colour

+++ - Confluent growth, ++ - Medium growth, + - Moderate growth
SDA - Sabouraud Dextrose Agar, MEA - Malt Extract Agar, PDA - Potato Dextrose Agar, CDA - Czapek - Dox Agar, OMA - Oat Meal Agar

Table.2 Effect of *Fusarium solani* culture filtrate at five different culture media on toxicity of *Piper betel* leaves after 48 hours inoculation

Culture medium	**Diluted culture filtrate and wilt formation(cm)**			
	1%	**10%**	**20%**	**50%**
PDA	1.00 ± 0.005	5.083 ± 0.101	5.153 ± 0.213	6.086 ± 0.098
SDA	1.00 ± 0.005	2.01 ± 0.015	4.183 ± 0.274	5.016 ± 0.015
MEA	1.21 ± 0.0337	2.04 ± 0.052	2.076 ± 0.106	4.15 ± 0.216
CDA	1.00 ± 0.005	2.013 ± 0.015	2.29 ± 0.441	3.223 ± 0.326
OMA	1.00 ± 0.005	1.00 ± 0.005	1.143 ± 0.222	3.013 ± 0.015
Control	1.00 ± 0.005	1.00 ± 0.005	1.00 ± 0.005	1.00 ± 0.005

± - Standard error of triplicates

Table.3 Effect of *Fusarium solani* culture filtrate at different pH on toxicity of detached *Piper betel* leaves after 48 hours inoculation

pH of culture media	**pH of culture filtrate**	**Diluted culture filtrate and wilt formation (cm)**		
		10%	**20%**	**50%**
3.5	4	1.00 ± 0.005	1.073 ± 0.109	3.083 ± 0.101
4.5	5	2.316 ± 0.505	3.283 ± 0.447	4.09 ± 0.095
5.5	5.5	3.32 ± 0.502	5.123 ± 0.153	5.3 ± 0.433
6	5.5	1.276 ± 0.453	3.18 ± 0.277	4.153 ± 0.213
6.5	6	1.25 ± 0.389	3.043 ± 0.049	4.113 ± 0.161
7.5	6	1.21 ± 0.337	2.27 ± 0.456	3.02 ± 0.02
Control	1.0	1.00 ± 0.005	1.00 ± 0.005	1.00 ± 0.005

± - Standard error of triplicates

Fig.1 Mycelial growth of *Fusarium solani* on five different cultivation media

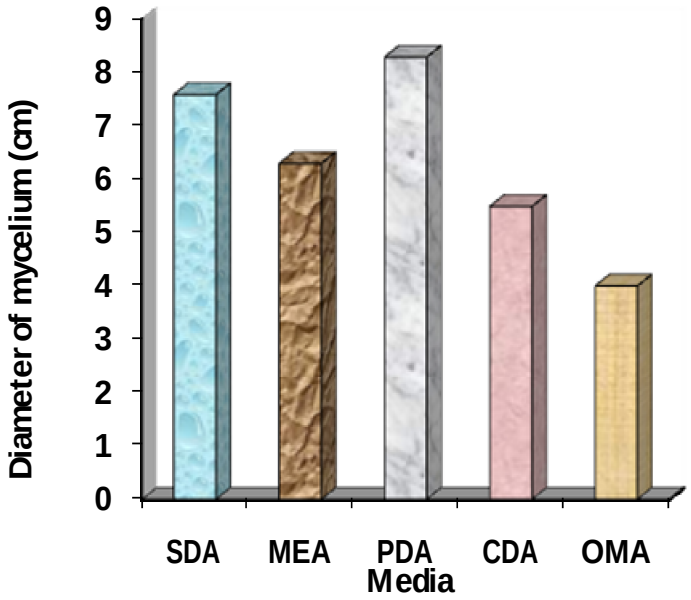


Fig.2 Effect of temperature on radial mycelial growth of *Fusarium solani*

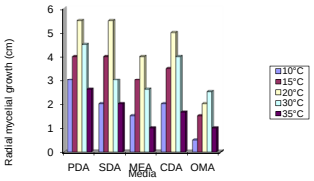


Plate.1 Toxic effect of different concentration of Paclitaxel from *Fusarium solani* on wilt infection of betel (*Piper betel*) with different pH ranges

pH - 3.5

pH - 4.5

pH - 5.5

pH - 6

pH – 6.5

pH – 7.5

% = Concentration; C = control

Fig.3 UV analysis of paclitaxel

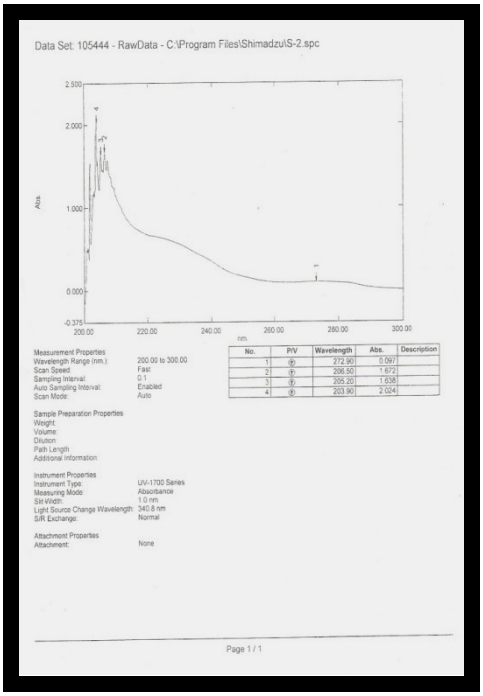


Fig.4 HPLC analysis of paclitaxel

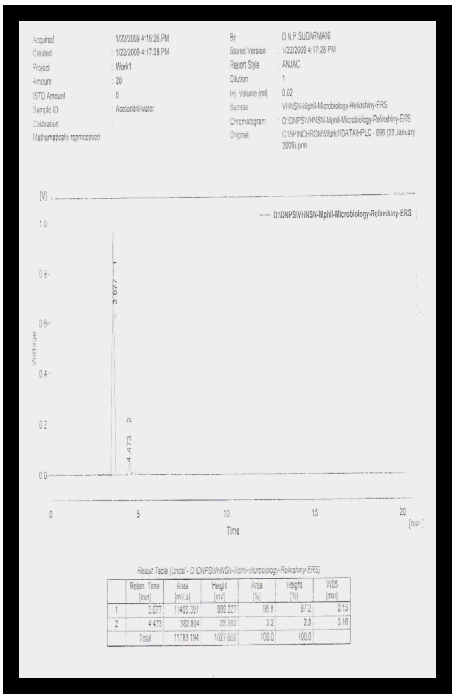


Fig.5 FTIR analysis of paclitaxel

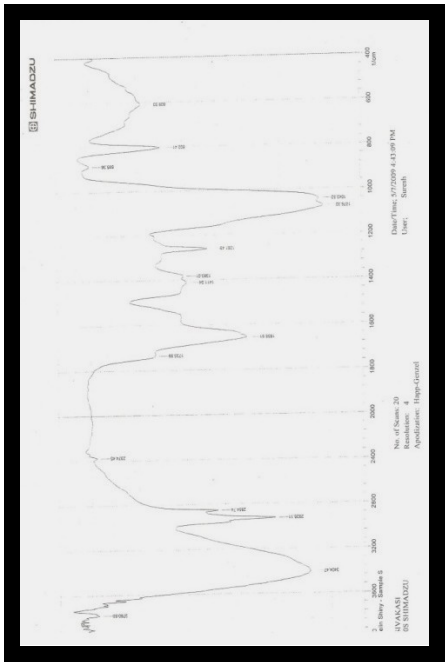
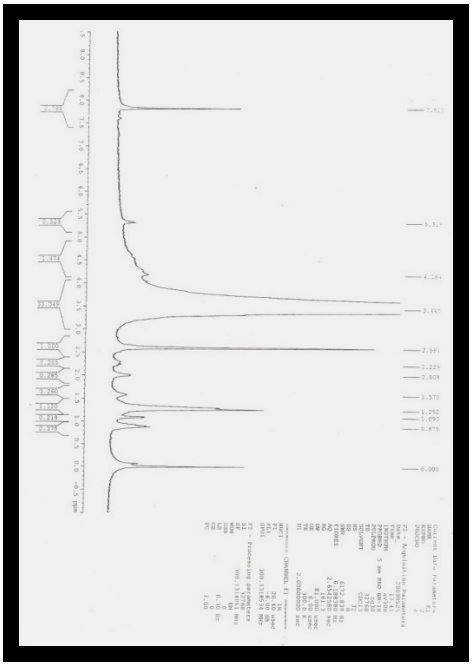


Fig.6 NMR analysis of paclitaxel



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