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Immunomodulatory Activity of an Endophytic Fungus Isolated from *Tripterygium wilfordii*

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1. Background

Endophytic fungi from medicinal plants are potential sources of a diverse array of bioactive compounds which may have potential for therapeutic purposes (1). Evidences have been established with the discovery of Taxol (anticancer drug) producing fungi which live inside the plant of *Taxus brevifolia* (2). This has triggered hopes to search other plant-associated fungi that may produce substances of pharmaceutical values. In this study, a Chinese medicinal plant *Tripterygium wilfordii* (Lei Gong Teng) was chosen and screened for presence of immunosuppressive substances such as triptolide, triptodiolide, triptonide and other derivatives. Our studies demonstrate that fungi associated with the plant could be the alternative source of biologically active compounds. Using fermentation technique, pilot scale production of the compounds is feasible.

2. Objectives

1. To carry out the anti-proliferation and cytotoxic assays for fungal extracts in order to find the immunomodulatory fungal isolates.
2. To screen and bioassay of immunomodulatory substances purified from extract of *Pestalotiopsis leucothës* an active endophytic fungus among the total screened fungal isolates

3. Materials and Methods

3.1 *Endophytes of Tripterygium wilfordii*

The plant samples were surface sterilized by dipping in 75% ethanol for 1 min, then in a solution of Chlorox (3.25%) for 30 seconds, and finally into 75% ethanol for 30 seconds (3). Surface sterilized samples were washed with three changes of sterile distilled water and blotted with sterile tissue paper.

The plant segments were then transferred to Potato Dextrose Agar (PDA, Oxoid) plate amended with 1% streptomycin to inhibit bacterial growth. Plates were labeled accordingly and incubated at 24°C with 12 h cycle of dark and light. The growing edges of colonies from the plant segments were transferred to PDA plates by hyphal tipping. Continuous transfer of fungi was carried out as new colonies continued to appear for up to two or three weeks. Plates were then incubated and periodically ascertained for purity by hyphal tipping. The cultures failed to sporulate within 2-4 months of incubation were designated as "Mycelia sterilia" according to cultural characteristics. The fungal isolates were numbered and submitted to the Hong Kong University Culture collection (HKUCC).

3.2 *Cultivation and extraction of selected fungal isolates*

The selected endophytic isolates were inoculated on three 250mL flasks of Potato Dextrose (Difco, pH 5.5) broth (100mL) with 12 hrs cycle of light at temperatures between 22°C and 25°C for 21 days. After fermentation, the entire culture was blended and extracted with ethyl acetate (3×50mL) and the organic phase was dried over Na_2SO_4 for 10 min and filtered using the silicon coated Whatmann filter paper and concentrated *in vacuo* at 35°C. Thus crude extracts obtained were stored in -20°C until assayed.

3.3 *Purification of active crude extracts of Pestalotiopsis leucothës by chromatography*

The ethyl acetate extracts of mycelia and broth were purified by flash chromatography using Merck silica gel 60 (size: 230-400 mesh) with two different solvents system Hexane: EtoAc and Benzene (C_6H_6) : Chloroform ($CHCl_3$). Fractions were collected and then analyzed by means of thin layer chromatography (TLC) on Si-gel plates (Merk Silica F_{254} plates) 20 × 20 cm. Two solvent system were used; i.e. Hexane: EtoAC (1:1) and Benzene: Chloroform (4:1). Fraction profiles (spots) were visualized by following spraying reagent Phenyl mercuric acetate (PMA). Kedde reagent was used to identity the unsaturated lactone ring by producing a purple color as an indicator (4).

3.4 *Extract preparation for the bioassays*

All extracts were dissolved in absolute ethanol and sterilized by filtration in Nalgene filters (0.22µm pore size). A stock solution of the extracts was prepared by adding an appropriate volume of RPMI1640 medium to the filtrate. Concentration of ethanol in the final solution was always less than 0.1% (v/v); which had no measurable effect on growth of test culture. 200µl of drug solution was added to each well of a 96-well titer micro-plate. Two fold dilutions were then made across the plate in each column in order to produce various concentrations of drugs from 125 to 0.105µg/mL.

3.5 *Proliferation and viability assay of Peripheral Blood Mononuclear Cells (PBMC)*

Lymphocyte proliferation assay was performed as previously described by (5). Briefly, PBMC were cultured at a density of 1×10^5cells/well in 50µl of RPMI 1640 complete medium on 96 well flat-bottomed microplates with or without various concentrations of fungal extracts. 7.5µg/mL of PHA-M in 50µl of RPMI-1640 was added to induce the proliferation of lymphocyte and the culture plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 3 days. Four hours before the end of the incubation, 0.5µCi [^{3}H] –thymidine in 20µl of RPMI-1640 was added to each well. The cells were harvested onto glass fiber filter papers with an automated Matrix96™ cell harvester (Packard). Incorporation of [^{3}H] –thymidine was measured as count per minute (CPM) was monitored by liquid scintillation counter. Viability of PBMC was determined by standard tryphan blue exclusion test. 50% inhibition concentration (IC_{50}) was recorded to compare the extent of inhibition on cell proliferation by each extract.

3.6 *In vitro Ig production*

PBMC were incubated in the presence or absence of varying concentrations of active fractions for 72 hours. IgG and IgM levels in the culture supernatants were measured by ELISA as described by (5).

3.7 *Immunophenotyping by means of flow cytometry*

Expression of CD3, CD4 and CD8 on cellular surface was evaluated by monitoring the fluorimetric changes of FITC/PE conjugated monoclonal antibody (Becton-Dickinson) with a flow cytometer. 2×10^6/mL of treated and untreated PBMC were cenrtrifuged at 2000 rpm and pellet was dissolved in 1X PBS and divided in to two tubes respectively for CD4 and CD8 marker analysis. PBMC were again washed with 300µL of 1X PBS and centrifuged at 2000 for 3 mins. The supernatant was discarded and tubes were blocked with 1mL of PBS blocking buffer containing1%BSA and 0.1%NaN_3 and centrifuged again. Finally 100 µL of blocking buffer containing 5 µL of FITC conjugated anti-human CD3 was added to each tubes and followed by 2mL of PE anti-human CD4 and CD8 marker were added to the respective tubes and incubated for 2 hours at 4C. Analysis was carried out in FACScan using Cell Quest software.

3.8 *Data analysis*

All experimental data were the average of triplicate experiments with mean ± SD indicated. Dunnets test was used to analyze the different variables in the same subject and P values less than 0.05 taken as significant. The IC_{50} values with 95% confidence intervals were calculated by inverse prediction and probit analysis. Student T-test was employed to compare the mean values of percentage viability.

4. Results

4.1 *Anti-proliferative fungal extracts*

Anti-proliferative activity was found from the culture extracts of 11 fungal species (17.7%) of the 60 selected endophytic isolates. Among these fungal extracts, *Pestalotiopsis leucothës*, *Mucor* sp., *Verticillium* sp. and *Pestalotiopsis disseminata* inhibited the proliferation in a dose-dependent manner at doses between 0.12 to 500µ g/ml ($P<0.001$-0.05) (Table 1). The IC_{50} values of these four fungal extracts are in the range of 0.75-0.8±0.12µg/ml. To ascertain whether these inhibitory activities of these fungal extracts were not a consequence of cytotoxicity, viability of PBMC was examined after treated with various concentrations (0.1-500µg/ml); however the cytotoxicty was examined at 125µg/ml to avoid the unspecific killing of the high concentrated fungal extracts. The results indicated that these fungal extracts had no cytotoxicity because the viability of stimulated cells was not significantly reduced after treated with these fungal extracts for 3 days as compared with control cells with or without PHA in 1% ethanol medium (89-92±1.5-1.8% vs. 95±1.8%)($P> 0.05$).

Table 1. Influence of endophytic strains on the proliferation and viability assay of PBMC.

Species	Fungal code	Inhibition of Proliferation (IC_{50}±C.I in µg/ml)	Viability [a,b] (%Viability at 125µg/ml)
Pestalotiopsis leucothës	RA4a	0.75 ±0.11	92± 1.5
Mucor sp.	S7a	0.79 ±0.12	89±1.7
Verticillium sp.	S4b	0.82±0.12	91±1.5
Pestalotiopsis disseminata	S7c	0.82±0.12	89±1.8
Phomopsis sp. B	S3a	1.81±0.12	89±1.5
Pestalotiopsis visimiae	B5b	2.12±0.2	88±2.7
Mycelia sterilia sp.4	RA3	0.66±0.12	40±2.3*
Mycelia Sterilia sp.15	r6a	1.75±0.16	20±2.4*
Acremonium sp.1	S5b	1.14±0.12	25±3.6*
Pestalotiopsis suffocata	B10a	1.4±0.14	12±1.4*
Acremonium sp.4	R4c	0.43±0.09	13±1.5*

a. %Viability results are expressed as the average of three experiments ± SD.
b. Viabilty of unstimulated and stimulated cultures in 1% ethanol in medium = 95±1.8%.
*Statistical significance vs. control: $P<0.005$.

4.2 *Fractionation of crude extracts of P. leucothës*

Gradient elution of the extracts by column chromatography showed high activity in 100% EtoAc portion. The 50% inhibition concentration (IC_{50}) value of this fungal extract is 0.75-±0.12 µg/ml with no cytotxicity at 125 µg/ml. This active portion was further purified by a mixed solvent system composed C_6H_6 of and $CHCl_3$. Altogether, 50 subfractions were obtained separately from the mycelial and broth crude extracts and grouped according to their TLC profiles.

4.3 *Suppressive of lymphoproliferation by the eluted fractions*

To study the effects on PBMC proliferation, PBMC were treated with various concentrations of fraction's crude extracts of *P. leucothës* with or without PHA-M for 3 days. Table 2. shows the active fractions of both mycelial and broth extract. Among these active fractions PM-34-38 and PB-33-36 (highlighted) shows high suppressive effect with no cytotoxity.

Table 2. Influence of active fractions on the proliferation and viability assay of PBMC

Active fractions	Eluting solvent (v/v)	IC_{50}±C.I (µg/mL)[a]	% Viability at 125µg/mL[b]
PM-19-23	C_6H_6 - $CHCl_3$ (2:1)	0.9±0.13	83±2.1
PM-31-33	C_6H_6 - $CHCl_3$ (1:3)	0.8±0.17	89±1.4
PM-34-38	C_6H_6 - $CHCl_3$ (1:4)	0.45±0.09	85±1.6
PM-39-41	100% $CHCl_3$	1.3±0.16	28±1.2*
PM-42-45	$CHCl_3$ -Methanol (99:1)	1.8±0.19	20±2.5*
PB-33-36	C_6H_6 - $CHCl_3$ (1:4)	0.32±0.14	92±1.1
PB-37-40	100% $CHCl_3$	0.8±0.13	82±1.5
PB-41-45	100% $CHCl_3$	0.56±0.07	23±2.5*

[a] IC_{50} value expressed with confidence interval of 3 different experiments.
[b] %Viability results are expressed as the average of three experiments ± SD. Viability of unstimulated and stimulated cultures in 1% ethanol in medium = 95±1.8%.
*Statistical significance vs. control: $P<0.005$ by student t test.

4.4 *Flow cytometry analysis for $CD3^+/CD4^+$ and $CD3^+/CD8^+$ markers*

In view of significant activity of fractions PM-34-38 and PB-33-36, we investigated here, in which PBMC subpopulation was the target of these fractions. PM- 34-38 in various concentrations significantly modified the number of T-helper and T-suppressor's surface markers (Table 3). The presence of PB-33-36 in the culture medium did not significantly modify the T cell subpopulations (P> 0.05).

Table 3. Effect of PM-34-38 and PB-33-36 on the T-cell subpopulation in PBMC [a]

Subjects	T-helper (CD3+/CD4+)	T-suppressor (CD3+/CD8+)
Control	10.4 ± 1.2	17.5 ± 1.2
Concentration of PM-34-38		
125µg/mL	0.44 ± 0.09*	1.95 ± 0.12*
62.5µg/mL	1.1 ± 0.12*	5.9 ± 0.84*
31.6µg/mL	1.4 ± 0.14*	10.93 ± 1.32*
15.6µg/mL	1.8 ± 0.2*	11.95 ± 1.22*
Concentration of PB-33-36		
125µg/mL	8.7 ± 1.5	14.6 ± 0.95
62.5µg/mL	9.7 ± 1.4	17.2 ± 1.5

[a] Each data represents the mean value ± S.D of 3 different individual's PBMC
* P values less than 0.05 taken as significant by Dunnets test.

4.4 *In vitro Immunoglobulin production*

The incubation of PM-34-38 and PB-33-36 with PHA stimulated PBMC shows concentration dependent suppression of both IgG and IgM ($P<0.05$)(Table 4) Suppression was more marked for IgG production than for IgM production. PM-34-38 was found to be highly suppressive than PB-33-36.

Table 4. Effect of PM-34-38 and PB-33-36 on Ig (IgG and IgM) production by PBMC

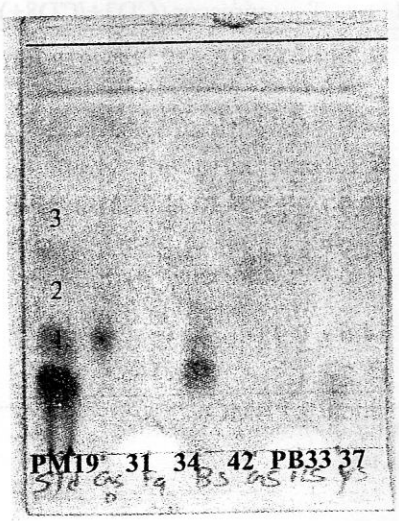
Concentrations (µg/mL)	*PM-34-38 (IgG) ng/mL	*PB-33-36 (IgG) ng/mL	*PM-34-38 (IgM) ng/mL	*PB-33-36 (IgM) ng/mL
0	298 ± 4.2	298 ± 4.2	309.5 ± 5.5	309.5 ± 5.5
0.98	31.23 ± 2.8	42.34 ± 3.6	87.45 ± 2.5	120.56 ± 2.7
2	16.95 ± 2.2	24.54 ± 2.7	65.76 ± 2.4	98.65 ± 2.1
3.9	14.29 ± 2.2	18.97 ± 3.4	54.86 ± 1.9	78.87 ± 2.5
7.8	10.46 ± 1.1	16.97 ± 1.8	45.89 ± 1.5	64.76 ± 1.5
15.6	8.38 ± 0.8	14.4 ± 1.2	36.76 ± 1.5	54.87± 1.5
31.3	7.34 ± 0.5	10.17 ± 0.8	23.65 ± 1.5	42.67± 1.5
62.5	6.69 ± 0.4	9.54 ± 0.6	18.87± 1.5	37.86 ± 1.5
125	5.73 ± 1.2	8.34 ± 1.2	15.98 ± 1.5	29.66 ± 1.5

Each data represents the mean value ± S.D of 3 different individual's PBMC
P values less than 0.05 taken as significant by Dunnets test.

4.5 *Analysis of PM-34-38 and PB-33-36 fractions*

The column fractions of this fungal extract were analyzed by thin layer chromatography for the content of triptolide, triptonide and triptophenolide by comparing the bands corresponding to these standards. Two bands from eluted column fractions appeared showed similar R_f value to triptolide and triptonide respectively (Fig 1). However, none of these fractions developed purple color in Kedde reagent and it seems to be that some other metabolites, which may have response for the immunomodulatory activities.

Fig 1. TLC profiles of active fractions with comparison of standards using anisaldehyde as a spraying reagent. Standard numbers. 1: Triptolide, 2: Triptonide, 3: Triptophenolide.



5.Conclusion

In this present study, we found selected fungal extracts had anti-lymphocyte proliferative effects. These inhibitory activities were not due to direct toxicity to PBMC because there was no significant cell death after PBMC were treated with fungal extracts at 125 µg/mL. Among these fungal extract, *P. leucothës* extract shows profound immunomodulatory activity which was revealed by their partial purified fractions PM-34-38 and PB-33-36 of *P. leucothës* extract suppress the various

activities of PBMC (proliferation, surface markers and Ig production) with out changing its viability. A literature search indicates that there was no other report of immunomodulatory substances from this fungal taxa. Hence, further purification and investigation of these fractions are being done to characterize the immunomodulatory constituents. This may also have implications on immunosuppressive therapy.

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7. References

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