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ORIGINAL ARTICLE

Evaluation of secondary metabolites from mangrove associated fungi *Meyerozyma guilliermondii*

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KEYWORDS

Marine fungi;
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Abstract *Background:* Mangrove associated fungi are the second most diverse organisms that remain less explored. Cytotoxic and antibacterial activities of foliar fungus *Meyerozyma guilliermondii* were investigated.

Methods: Foliar fungus was isolated from the leaves of *Rhizophora mucronata* collected from Pichavaram mangrove forest, Tamilnadu. Extracts from liquid state culture were tested for cytotoxicity against two cancer cell lines using the MTT assay. Antibacterial activity was determined using the well diffusion method. The DNA was isolated from the fungi, and the ITS region of 5.8s RNA was sequenced. The spectral properties (GC/MS) of the purified compounds were determined.

Results: The ethyl acetate extracts showed potent cytotoxicity against Hep2 and human breast adenocarcinoma (MCF-7) cell lines with IC_{50} values of 1.25 and 0.625 μg/ml, respectively. Antibacterial activity of the fungal extract was demonstrated against five test organisms. The fungus was found to be a new strain based on ITS sequence and database in NCBI Blast tool.

Conclusion: Results indicate the potential for production of bioactive agents from mangrove foliar fungi.

1. Introduction

Bioactive natural compounds produced by microorganisms have been promising potential usefulness in safety and human health concerns, although there is still a significant demand of drug industry for synthetic products due to economic and time-consuming reasons. Recent review by Newman and Cragg, provides us a list of all approved agents from 1981 to 2006, from which a significant number of natural drugs are produced by microbes.[1] Marine microorganisms have been an important source of pharmacologically active metabolites.[2] Considering that only a small amount of mangrove fungi have been studied, recently, several research groups have been motivated to evaluate and elucidate the potential of these microorganisms applied on biotechnological processes focusing on the production of anti-cancer compounds. It is fascinating to discover how simple fungi could hold the key to powerful new approaches to treat cancer. Mangrove associated fungi provide a broad variety of bioactive secondary metabolites with unique structure,

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including alkaloids, benzopyranones, chinones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones, and others. There are some evidences that bioactive compounds produced by fungi associated with plants could be alternative approaches for discovery of novel drugs, since many natural products from plants, microorganisms, and marine sources were identified as anticancer agents.[2] Cancer is a group of diseases characterized by unregulated growth and spread of abnormal cells, which can result in death if not controlled.[3] It has been considered one of the major causes of death worldwide: 7.4 million (about 13% of all deaths) in 2004.[4] The anticancer drugs show nonspecific toxicity to proliferating normal cells, possess enormous side effects, and are not effective against many forms of cancer.[5,6] Thus, the cure of cancer has been enhanced mainly due to diagnosis improvements, which allow earlier and more precise treatments.

The importance of compounds bearing antioxidant activity lies in the fact that they are highly effective against damage caused by reactive oxygen species (ROSs) and oxygen-derived free radicals, which contribute to a variety of pathological effects, for instance, DNA damages, carcinogenesis, and cellular degeneration.[7,8] These free radicals occur in the body during an imbalance between ROSs (Reactive Oxygen Species) and antioxidants. Hence, the role of antioxidant is necessary and important to balance the antioxidant status that would reduce the pathological conditions induced by free radicals. Fungi are remarkably a diverse group including approximately 1.5 million species, which can potentially provide a wide variety of metabolites such as alkaloids, benzoquinones, flavonoids, phenols, steroids, terpenoids, tetralones, and, xanthones.[9] Fungal extracts of mangroves are being researched in this regard owing to their multipurpose applications and also because fungi have emerged as the new sources of antioxidants in the form of their secondary metabolites.

2. Methodology

2.1. Isolation of fungus and extraction of metabolite

The leaves of the collected mangrove plants were washed, surface sterilized and inoculated in potato dextrose agar. The pure culture isolated by the above method was grown in Sabouraud's dextrose broth. The flasks were incubated in the shaker – incubator at 200 rpm for 5 days. The filtrate was separated and subjected to solvent extraction. The filtrate was extracted several times with ethyl acetate, petroleum ether and ethanol (v/v) in a separating funnel and evaporated under vaccum at 50 °C till dryness.

2.2. Antibacterial assay

Antibacterial activity was carried out against *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25,923), *Escherichia coli* (ATCC 25,922), *Pseudomonas aeruginosa* (ATCC 27,853) and *Proteus vulgaris* (ATCC 29,905) by agar well diffusion method. Pre-warmed Mueller–Hinton agar (MHA) plates were seeded with 10^7–10^8 cfu suspension of test bacteria. Gentamicin sulfate (10 μg) was used as the positive control. Plates were incubated at 37 °C for 48 h. Antibacterial activity was expressed as the diameter of the inhibition zone (mm) produced by the extracts. Then different concentrations were tested for ethyl acetate which showed maximum activity against all the bacterial pathogens.

2.3. Cytotoxic activity (MTT assay)

Cytotoxicity of extracts at various concentrations (15–1000 μg/ml) was assessed for Hep2 and MCF-7 using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) but with minor modification,[10] following 72 h of incubation. Assay plates were read using a spectrophotometer at 520 nm. Data generated were used to plot a dose–response curve of which the concentration of extract required to kill 50% of cell population (IC_{50}) was determined.

$$\text{Cell viability}(\%) = \text{Mean OD}/\text{control OD} \times 100$$

2.4. GC–MS analysis

The crude extract was quantified using a gas chromatograph (Shimadzu QP2010) equipped with a VF-5 ms column (diameter 0.25 mm, length 30.0 m, film thickness 0.25 μm) mass spectrometer (ion source 200 °C; EI −70 eV), programed at temperature 40–650 °C with a rate of 4 °C/min. Injector flow rate was 200 °C; carrier gas was He 99.9995% purity, column flow rate1.51 ml/min, injection mode-split.

2.5. Fungal isolation, identification

The total deoxyribonucleic acid (DNA) of marine-derived fungus was extracted by CTAB method. The internal transcribed spacers (ITS) of ribosomal DNA (rDNA) were amplified employing the combination of a conserved forward primer ITS1 (5′TCCGTAGGTGAACCTGCGG3′) and reverse primer ITS4 (5′TCCTCCGCTTA TTGATATGC3′). The polymerase chain reaction product is about 0.7 kb. The purified ITS rDNA were sequenced.

3. Results and discussion

Antimicrobial properties of mangrove fungi are being increasingly reported from various parts of the world. Fig. 1 shows the pure culture of the fungus isolated. The isolated fungi were cultured in Sabaroud's broth for 5 days. The broth was filtered

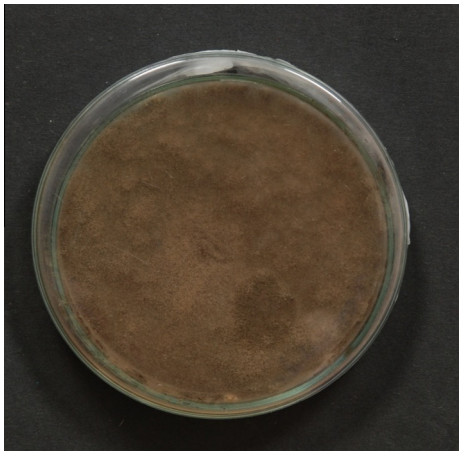

Figure 1 *Meyerozyma guilliermondii*.

Table 1 Antibacterial activity of *Meyerozyma guilliermondii* – different solvents.

Bacterial pathogens	Zone of inhibition in mm					
	Ethyl acetate	Positive control	Petroleum ether	Positive control	Ethanol	Positive control
E. coli	15	10	–	12	15	10
Pseudomonas	14	15	–	15	–	13
Enterococcus	15	12	–	–	12	–
Staphylococcus	15	15	–	10	15	12
Bacillus	14	12	–	15	15	13

Table 2 Antibacterial activity of *Meyerozyma guilliermondii* at different concentrations using ethyl acetate extract.

Pathogens	Zone of inhibition in mm				
	std	25 µl	50 µl	75 µl	100 µl
Proteus vulgaris (ATCC 29,905),	16	13	18	22	25
Bacillus subtilis (ATCC 6633)	17	15	20	22	23
Pseudomonas aeruginosa (ATCC 27,853)	18	12	20	24	25
Escherichia coli (ATCC 25,922)	16	10	17	21	26
Staphylococcus aureus (ATCC 25,923)	14	16	17	18	23

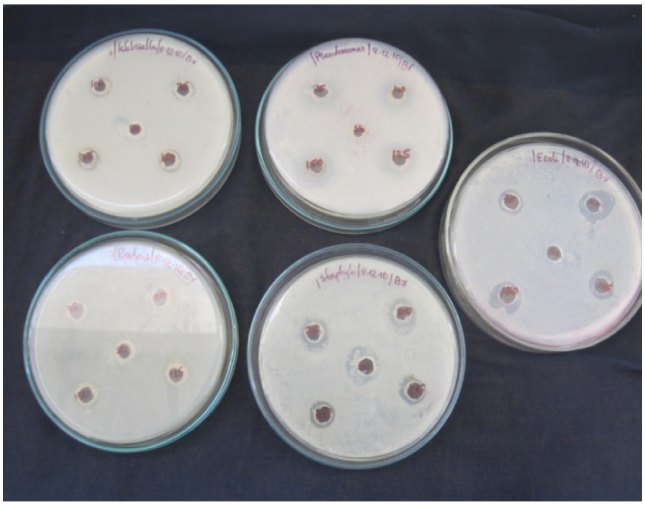

Figure 2 Antibacterial activity.

Table 3 Anticancer activity of *Meyerozyma guilliermondi* against Hep2 cell line.

Sl. no	Concentrations (µg/ml)	Dilutions	Absorbance	Cell viability
1	5	Neat	0.11	23.40
2	2.5	1:1	0.14	29.78
3	1.25	1:2	0.23	48.93
4	0.625	1:4	0.27	57.44
5	0.3125	1:8	0.34	72.34
6	0.156	1:16	0.35	74.46
7	0.078	1:32	0.41	87.23
8	0.039	1:64	0.45	95.74
9	Cell control	–	0.47	100

and the filtrate was taken since the aim of the present study is to check the extracellular metabolites produced by this isolated fungi. The antimicrobial properties of the extract isolated with three different solvents are shown in Table 1. Secondary metabolites extracted with ethyl acetate showed appreciable activity against the entire panel of test organisms. Petroleum ether showed nil activity suggesting a bad proportion of secondary metabolites. The ethanol extract was active against

Table 4 Anticancer activity of *Meyerozyma guilliermondi* on MCF 7 cell line.

Sl. no	Concentrations (µg/ml)	Dilutions	Absorbance	Cell viability
1	5	Neat	0.02	4.87
2	2.5	1:1	0.06	14.63
3	1.25	1:2	0.15	36.58
4	0.625	1:4	0.19	46.34
5	0.3125	1:8	0.24	58.53
6	0.156	1:16	0.29	70.73
7	0.078	1:32	0.33	80.48
8	0.039	1:64	0.38	92.68
9	Cell control	–	0.41	100

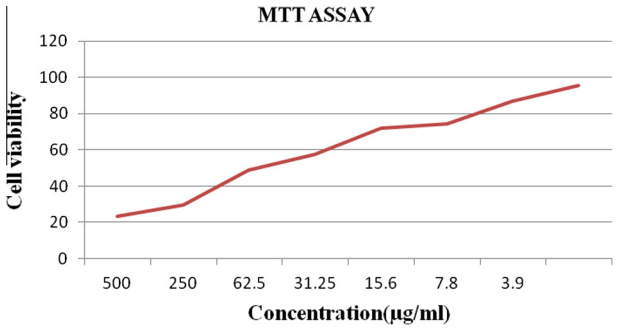

Figure 3 MTT assay of Hep2 cell line.

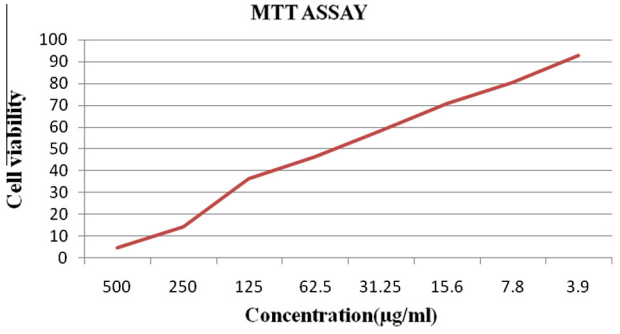

Figure 4 MTT assay of MCF 7 cell line.

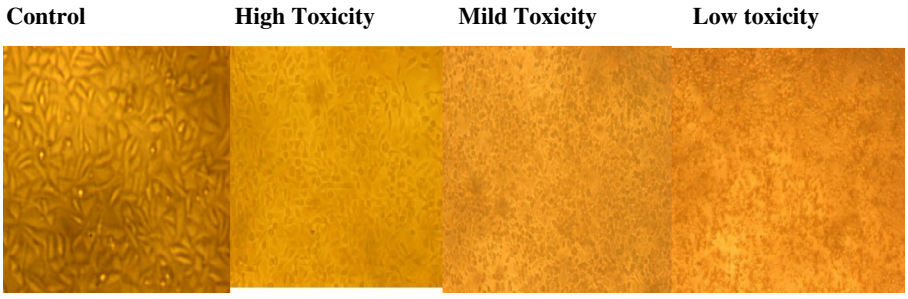

Figure 5 Hep2 cell line treated with crude extract of *Meyerozyma guilliermondii*.

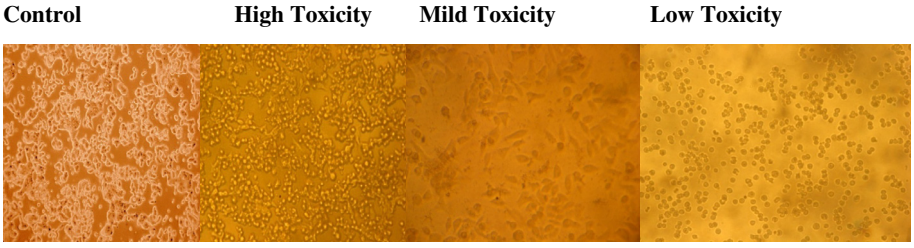

Figure 6 MCF7 cell line treated with crude extract of *Meyerozyma guilliermondii*.

Table 5 Compounds present in GC MS spectrum.

Activities	RT	Area	Compound names
Antibacterial compounds	5.907	0.19	Isohexyl neopentyl ester
	11.870	0.44	Pentadecane
	34.221	1.48	1-Nonadecanol
Anticancer compounds	8.602	0.37	Tridecane
	18.736	0.70	Isobutyl undecyl ester
	31.844	21.14	9-Octadecenoic acid

all the organisms except *Pseudomonas*. The ethyl acetate extract of the fungus at different concentrations (25–100 µl) shown in Table 2, was found to be potent against *P. vulgaris* (ATCC 29,905), *B. subtilis* (ATCC 6633), *P. aeruginosa* (ATCC 27,853), *E. coli* (ATCC 25,922) and *S. aureus* (ATCC 25,923). The rate of inhibition was found to be directly proportional to the concentration of the extract with inhibition zones ranging from 23 to 26 mm at 100 µl as seen in Fig. 2. *E. coli* was found to be the most inhibited. Antibacterial activity may be due to active components, which are present in fungal extracts and the solubility of those active compounds in the solvent used (ethylacetate) as against the positive control gentamicin sulfate that displayed inhibitory activity against all pathogens. This inhibitory effect draws attention to the use of mangrove fungal extracts as a new generation of antimicrobial agents. These results are in sync with reported papers on bioactive compounds isolated from culture broth in several reviews.[11–14] The effects of Mangrove extracts on some microorganisms including *Shigella sp., Staphylococcus* sp., *Pseudomonas* sp. have been reported in some studies in the area of pharmacology. Also different types of solvents including ethanol, chloroform and ethyl acetate have been used for extraction.[11]

Cytotoxicity of the extract against Hep2 and MCF-7 cell lines is shown in Tables 3 and 4. At IC_{50} level of 1.25 µg/ml, the extracts were active against Hep2 cell line with a cell viability of 48.93% at an absorbance of 0.23. Appreciable activity against MCF-7 cell line is confirmed with an IC_{50} level of 0.625 µg/ml, which showed cell viability of 46.34 at 0.19 absorbance. In both cases the absorbance values are lower than the negative controls, which suggest a reduction in the overall cell viability. Results are also shown as a dose–effect curve in Figs. 3 and 4. Quantitative efficacy of the extract to kill half the tested Hep2 cell lines is found to be 1.25 µg/ml as in Fig. 3. The fact that the extract is found to be extremely potent against the MCF-7 cell line is established by the steeper curve in Fig. 4. The appearance of Hep2 and MCF-7 cell lines in chamber slides at different stages of high, mild and low toxicity in comparison to the standard is represented in Figs. 5 and 6. As per GC MS analysis (Table 5 and Fig. 7) the compounds present in the extract were found to be Isohexyl neopentyl ester, Pentadecane and 1-Nonadecanol, Tridecane, Isobutyl undecyl ester and 9-Octadecenoic acid with RT values 5.907, 11.870 and 34.221, 8.602, 18.736 and 5.907, respectively.

In the present study, the fungus isolated was identified by internal transcribed spacer. Since the ITS region is the most widely sequenced DNA region in all fungi, it has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). The DNA was isolated and the ITS region of 5.8s rRNA was

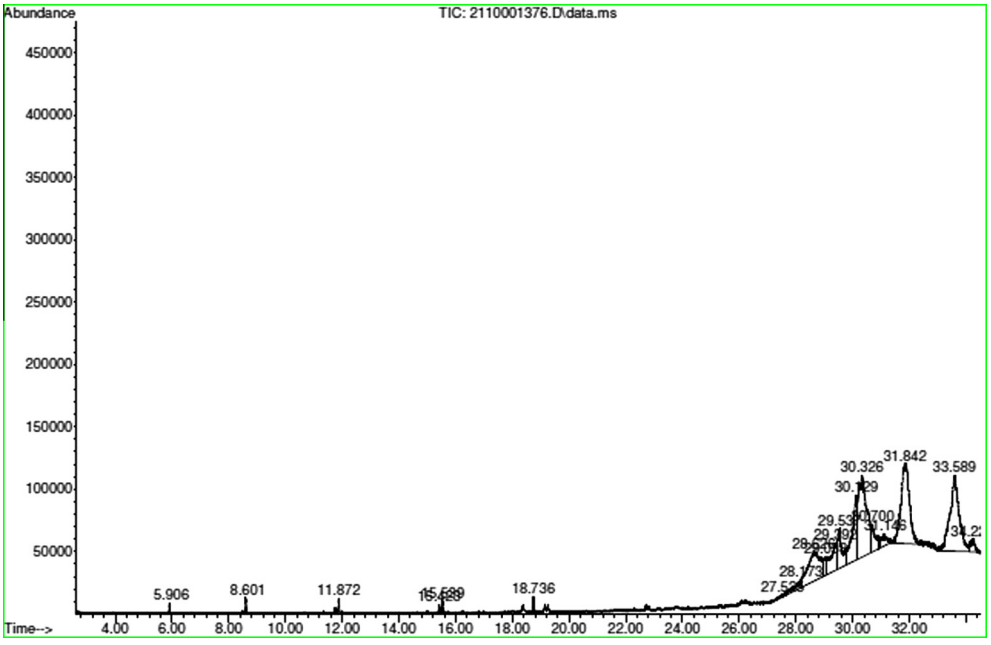

Figure 7 GC MS spectrum analysis.

amplified using specific primers ITS1 and ITS4 and sequence was determined using automated sequencers. The blast search sequence similarity found against the existing non redundant nucleotide sequence database identified the percentage of similarity between the fungus and database. Thus it was confirmed as a novel strain, named *Meyerozyma guilliermondii* VB7 and assigned an accession number JF730118 in Genbank http://www.ncbi.nlm.nih.gov/nuccore/JF730118.1.

Not only mangroves,[15,16] but also various other sources of plant fungi are found to be potent sources of anti-cancer compounds. "Podophyllotoxin" ($C_{22}H_{22}O_8$) and analogs are clinically relevant mainly due to their cytotoxicity and are valued as the precursor to useful anticancer drugs like etoposide, teniposide, and etopophos phosphate.[17,18] Novel microbial sources of Podophyllotoxin were reported from the fungi *Aspergillus fumigatus* Fresenius isolated from *Juniperus communis* L. Horstmann, *Phialocephala fortinii* isolated from *Podophyllum peltatum*[19] and *Fusarium oxysporum* from *Juniperus recurva*. Also, extract of a fungal strain (*Aspergillus ustus*), isolated from the rhizosphere soil of the mangrove *Acrostichum aureum* grown in Guangxi Province of China, showed cytotoxic activity against P388 (mice lymphocytic leukemia) cell line.[20]

As there is no previous work, both chemically and pharmacologically, on the fungus mentioned, further investigations to exploit the potential of this fungus are needed. Future studies involve the elucidation of the structures of the compounds by Nuclear Magnetic Resonance (NMR) spectral analysis.

4. Conclusion

In conclusion, this preliminary screening of the foliar fungus revealed its potential to yield potent bioactive compounds for drug discovery programmes. Extract of JF730118 *M. guilliermondii* showed very potent cytotoxic effect indicating its possible potential for development as an anticancer drug and warrants further investigation. Fungal extracts have great potential as antimicrobial compounds against microorganisms. Thus, they can also be used in the treatment of infectious diseases caused by resistant microbes.

References

1. Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. *J Nat Prod* 2007;**70**(3):461–77.
2. Firáková S, Šturdíková M, Múčková M. Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia* 2007;**62**(3):251–7.
3. American Cancer Society. *Cancer facts & figures 2009*. Atlanta, Ga, USA: American Cancer Society; 2009, http://www.cancer.org/acs/groups/content/@nho/documents/document/500809webpdf.pdf. p. 221–82.
4. WHO Mortality Database. Fact sheet No. 297. 2009. p. 87–93. <http://www.who.int/whosis/mort/download/en/>.
5. Gangadevi V, Muthumary J. Taxol, an anticancer drug produced by an endophytic fungus Bartalinia robillardoides Tassi, isolated from a medicinal plant. Aegle marmelos Correa ex Roxb. *World J Microbiol Biol* 2008;**24**(5):717–24.
6. Pasut G, Veronese FM. PEG conjugates in clinical development or use as anticancer agents: an overview. *Adv Drug Deliv Rev* 2009;**61**(13):1177–88.
7. Huang Y, Cai Z, Xing J, Corke H, Sun M. A potential antioxidant resource: endophytic fungi from medicinal plants. *Econ Bot* 2007;**61**(1):14–30.
8. Seifried, Anderson DE, Fisher EI, Milner JA. A review of the interaction among dietary antioxidants and reactive oxygen species. *J Nutr Biochem* 2007;**18**(9):567–79.
9. Arora DS, Chandra P. Antioxidant activity of fungi isolated from soil. *Braz J Microb* 2010;**41**:465–77.
10. Grever MR, Shepartz SA, Chabner BA. *Semin Oncol* 1992;**19**:1117–20.
11. Debbab K, Aly A, Lin WH, Proksch P. Bioactive compounds from marine bacteria and fungi. *Microb Biotechnol* 2010;**3**(5):544–63.

12. Ebel SR, Mander L, Liu H, Eds W. Natural product diversity from marine fungi. *Compr Nat Prod II Chem Biol (Oxford, Elsevier)* 2010;**2**:223–62.
13. Valentin Bhimba B, Agnel Defora Franco DA, Jibi Merin Jose, Geena Mary Jose, Elsa Lycias Joel, Thangaraj M. Anticancer and antimicrobial activity of mangrove derived fungi *Hypocrea lixii* VB1. *Chin J Nat Med* 2012;**10**(1):0077–80.
14. Valentin Bhimba B, Agnel Defora Franco DA, Jose GM, Jibi Merin Mathew, Elsa Lycias Joel. Characterization of cytotoxic compound from Mangrove derived fungi *Irpex hydnoides* VB4. *Asian Pac J Trop BioMed* 2011;**3**(8):223–6.
15. Elsa Lycias Joel, Valentin Bhimba B. Isolation and characterization of secondary metabolites from the mangrove plant *Rhizophora mucronata*. *Asian Pac J Trop Med* 2010;**3**(8):602–4.
16. Valentin Bhimba B, Meenupriya J, Elsa Lycias Joel, Edaya Naveena D, Suman Kumar, Thangaraj M. Antibacterial activity and characterization of secondary metabolites isolated from mangrove plant *Avicennia officinalis*J. *Asian Pac J Trop Med* 2010;**3**(7):544–6.
17. Kusari S, Lamshöft M, Spiteller M. *Aspergillus fumigatus* Fresenius, an endophytic fungus from *Juniperus communis* L. Horstmann as a novel source of the anticancer pro-drug deoxypodophyllotoxin. *J Appl Microb* 2009;**107**(3):1019–30.
18. Kour A, Shawl AS, Rehman S. Isolation and identification of an endophytic strain of *Fusarium oxysporum* producing podophyllotoxin from *Juniperus recurva*. *World J Microb Biotechnol* 2008;**24**(7):1115–21.
19. Eyberger AL, Dondapati R, Porter JR. Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. *J Nat Prod* 2006;**69**(8):1121–4.
20. Huinan ZHOU, Tianjiao ZHU, Shengxin CAI, Qianqun GU, Dehai LI. *Drimane sesquiterpenoids* from the Mangrove-derived Fungus *Aspergillus ustus*. *Chem Pharm Bull* 2011;**59**(6):762–6.