

Full Length Research Paper

## Antimicrobial activities of endophytic fungal isolates from medicinal herb *Orthosiphon stamineus* Benth

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**A total of 72 endophytic fungal isolates including 48 from leaves, 14 from stems, 6 from roots and 4 from flowers were obtained from medicinal herb, *Orthosiphon stamineus* Benth by adding host plant materials in isolation medium. Sixty-six (92%) of the 72 isolates exhibited significant inhibitory activity on at least one of the test microorganism. Methanolic extract, prepared from the fungal biomass, exhibited better antimicrobial activity compared with ethyl acetate extract from fermentative broth and volatile antimicrobial compounds. The antimicrobial activity of the isolates was affected by the culture medium. A better antimicrobial activity was observed in the yeast extract sucrose broth compared with malt extract broth. Significant improvements in the antimicrobial activity of the crude extract and volatile fungal metabolites were observed after addition of water extract of the host plant in the culture medium.**

**Key words:** *Orthosiphon stamineus* Benth, endophytic fungi, antimicrobial activity, host plant extract.

### INTRODUCTION

The mis-usage of antibiotics since the "Golden Age of Antibiotics" in 1950s had caused the threats of antibiotic-resistant "superbugs". Hence, today, many academic institutions are investing necessary resources to search for new natural products with significant pharmacological activity. Majority of the organisms containing new metabolites are disappearing rapidly and screening of the natural products has become costly and laborious process. Endophytes, which occupy a unique biotope with global estimation up to one million species, are a great choice to avoid replication in the study of natural products (Strobel and Daisy, 2003).

Endophytes are microorganisms which reside a whole or a part of its life-cycle asymptotically in the living plant tissues without causing any damage to the host plant (Tan and Zou, 2001; Strobel, 2003). In contrast,

they may contribute to the well-being of the host plant by producing bioactive secondary metabolites (Gao et al., 2010; Schulz et al., 2002). For centuries, drugs have been extracted from nature and more than 35,000 plant species have been utilised for medicinal purposes worldwide (Koshy, 2009). Furthermore, Schulz et al. (2002) concluded from their study that 51% of endophytic fungal-origin bioactive metabolites were previously unknown. The statistics show that endophytic fungi, that constitute a rich bio-resources from novel biotope, are a wise choice for bioprospecting.

Since the endophytes can be found in nearly all living plant species, a scientific basis in plant selection is necessary for the study of endophytes, to isolate microorganism with pharmaceutical potential. Prior history of the plant's extensive use in traditional medicines can be a criteria for plant selection (Strobel and Daisy, 2003). As part of our ongoing efforts towards finding new antimicrobial compounds, *Orthosiphon stamineus* Benth, commonly known as cat's whiskers or misai kucing in Malay, was used in this study. The

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flowers of this plant are white-bluish in color with long filaments, making the flowers look like cat's whiskers. This is the traditional medicinal herb in South East Asia, used extensively for the treatment of rheumatism, diabetes, hyper-tension, edema, eruptive fever, influenza, hepatitis, jaundice, biliary lithiasis and other kidney-related diseases (Banskota et al., 2003; Bayaty et al., 2010). The phytochemistry and pharmacological studies conducted on this medicinal herb have successfully isolated several natural products including diterpenes, monoterpenes, triterpenes, saponins, flavonoids hexoses, organic acids, rosmarinic acid, chromene and myo-inositol (Banskota et al., 2003; Amzad Hossain et al., 2008). However, therapeutic effect of this medicinal herb is due to the polyphenols.

Numerous reports are available on the pharmaceutical potentials of *O. stamineus* with wide range of biological activities including diuretic activity, antihypertensive, anti-inflammatory, antibacterial, antifungal, hepto-protective, antioxidant, antitumor, anti-allergic, and antimalarial activity (Banskota et al., 2003; Bayaty et al., 2010). However the medicinal values of their endophytes have not been investigated. The objective of this study was to isolate endophytic fungi from the herb and to study the antimicrobial activity of the isolates on various pathogenic microorganisms. In addition, for the first time, it was also aimed to ascertain the effects of plant extract in the culture media on the antimicrobial activity.

## MATERIALS AND METHODS

### Plant materials

Healthy and mature samples of *O. stamineus*, with no visible symptoms of any disease, were collected from Balik Pulau, Penang Island, Malaysia in March 2010. The pH value of the soil was 7.3. The studies were carried out for the entire plant components which included the leaves, stems, roots, and flowers. The reason for using these parts of the plant was because of their use in the traditional medicine. The collected plant materials were stored in separate ziplock plastic bags and processed within 4 h after the collection.

### Endophytic fungi isolation and storage

The isolation of fungi was performed following the process described by Okuda et al. (2005) with modifications. The samples were air-dried after cleaning thoroughly under running tap water. Acidic electrolyzed water was prepared by electrolyzing 1% sodium chloride solution until the pH of the solution at anode reached  $2.7 \pm 0.2$ . Intact plant samples were immersed in the freshly electrolyzed solution at different durations ranging from 0 to 60 min. Each immersion was separated from the other by 5 min interval. The efficacy of the procedure was examined by leaf-print method to optimize the time of immersion. The samples were cut aseptically after washing with sterile 0.5 g/L Tween 80 solution. The fragments were plated onto potato dextrose agar (PDA) plates (Merck) with the addition of powdered plant materials (10 g/L) and chloramphenicol (0.2 g/L) (Sigma). The plates were incubated at 30°C and the pure strains were obtained by repeated inoculation on the fresh medium. Stock fungal cultures were deposited at

Industrial Biotechnology Research Laboratory, University Sains Malaysia, Penang, Malaysia.

### Culture media

Two mycological media namely, yeast extract sucrose broth (Yeast extract, 20 g/L; sucrose 40 g/L; Magnesium sulfate 0.5 g/L) and malt extract broth (Oxoid), were used with and without water extract of *O. stamineus* to cultivate the endophytic isolates in a shake-flask system. The plant extracts were prepared by boiling 5 g of the dried plant materials in 500 ml distilled water for 30 min. The extracts were filtered and mixed with freshly prepared culture media and autoclaved at 121°C for 15 min.

### Cultivation and extraction

The inoculum was prepared by introducing two mycelial agar plugs into 250 ml Erlenmeyer flasks containing 100 ml of the broth medium. Both agar plugs were 1 cm in diameter and excised from the periphery of 7-days-old fungal culture. The cultures were cultivated at 30°C with rotational speed of 120 rpm. After 20 days of incubation, the fermented broth and fungal biomass were separated out by centrifugation at 5311 g (Sigma, Model 4K15). Freeze-dried fungal biomass was extracted by soaking in methanol (1:50, w/v) overnight. Supernatant was then extracted thrice with equal volume of ethyl acetate (1:1, v/v). The upper organic phase was concentrated to dryness under reduced pressure to obtain the crude broth paste. Due to the antimicrobial activities exhibited by *O. stamineus*, a control was included by extracting the sterile medium following exactly the same procedure as that for the endophytic cultures.

### Evaluation of antimicrobial activity

#### Test organisms

The test microorganisms used in the study included 4 Gram positive bacteria [*Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus subtilis*, and Methicilin-resistant *S. aureus* (MRSA)], 4 Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Shigella boydii*), 4 yeasts (*Candida albicans*, *Candida utilis*, *Saccharomyces cerevisiae*, and *Cryptococcus* sp.) and 4 fungi (*Aspergillus niger*, *Fusarium solani*, *Microsporium gypseum*, and *Trichophyton rubrum*). The cultures were provided by Industrial Biotechnology Research Laboratory and Plant Pathology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia. The bacterial cultures were subculturing every two weeks on fresh nutrient agar (NA) slants and incubated at 37°C, whereas the yeasts and fungal cultures were subculturing every four weeks on the fresh potato dextrose agar (PDA) slants and incubated at 37°C for yeasts and 30°C for fungi. All the cultures were then kept at 4°C until further use. The inoculum was prepared by adding 4 ml of sterile physiological saline to the agar slant, and shake vigorously to get the cell or spore suspension.

#### Disc diffusion assay

The assay was conducted as per the procedure defined by Jorgensen and Turnidge (2007). The crude extracts were dissolved in 50% dimethyl sulfoxide (DMSO). The test organisms with the inoculum size of  $10^5$  colony-forming units (CFU)/ mL for bacteria or  $4 \times 10^5$  CFU/mL for yeast cells or fungal spores were streaked on the surface of the media using sterile cotton swab. Muller-Hinton

agar (Hi-media) was used for test bacteria, whereas PDA was used for yeasts and fungi. Sterile Whatman antibiotic disc, impregnated with 20 µl of each extracts of 20 mg/ml concentration, were then placed on the surface of inoculated medium. Twenty percent DMSO was applied as a negative control to detect the solvent effects whereas 30 µg/ml chloramphenicol (Sigma) was used as the positive controls for bacteria, 30 µg/ml ketoconazole for fungi and yeasts, respectively. The plates were incubated at 30°C for 48 to 96 h for fungi, and at 37°C for 24 h for bacteria and yeasts. The diameter of the clear zones surrounding the disc were measured.

#### **Volatile antimicrobial assay**

Plate-to-plate method (Stinson et al., 2003) was performed to test the volatile antibiotic production. The assay was modified to test the volatile antibacterial and antiyeast compounds. Muller-Hinton agar plates were inoculated with 10 µl of bacterial inoculum size of  $10^5$  CFU/ mL or with the yeast cells of  $4 \times 10^5$  CFU/ ml on PDA plates. For test fungi, the agar plug with 1 cm diameter was placed at the middle of fresh PDA plates. Then 14-days-old endophytic fungal cultures, grown on different culture medium, were physically attached to the agar plates inoculated with test microorganisms. The two plates were sealed with two layers of Parafilm® and refrigerated at 4°C for 7 days to allow the complete fumigation of the volatile compounds. The plates were then incubated at 30°C for 48 to 96 h for fungi and at 37°C for 24 h for bacteria and yeasts. The diameter of test fungal cultures were measured, whereas the colony forming units for the yeast and bacterial cultures were counted. Data are presented as percentage of growth compared to untreated test microorganisms. Besides, the mycelia of the test fungi that showed positive result was transferred to fresh PDA plate to examine its viability.

## **RESULTS AND DISCUSSION**

### **Plant materials and isolation of endophytic fungi**

The selection of plant materials and sampling area were crucial, and they were the key determining factors for successful isolation of endophytes with pharmaceutical potentials. The maturity of the host plants, and environmental factors such as rainfall and atmospheric humidity may affect the diversity of the isolates (Chareprasert et al., 2006). In this study, only the plants of 3 or more years of age, and cultivated at areas free of fungicides, were selected to increase the diversity of the fungi isolated. Healthy plants, showing no disease symptoms, but surrounded by infected plants, are more likely to be selected (Yu et al., 2010).

In this work, a total of 72 fungal isolates including 48 from leaves, 14 from stems, 6 from roots and 4 from flowers were obtained. No data was available for comparison because of the lack of previous study reports on endophytic fungi from different tissues of *O. stamineus*. The endophytic fungi were most prevalent (67%) in the leaves and were least prevalent in the flowers. Beside thin cuticle layer, mature leaves growing to about 12 cm in length and about 7 cm in width, provide a large surface area for fungal penetration. The least number of isolates from flowers was thought to be due to

wilting of flowers within a few days, therefore it received lower amount of inoculum.

Surface disinfection can be performed using sodium hypochlorites, ethanol, formaldehyde, and hydrogen peroxide (Okuda et al., 2005). We used acidic electrolyzed water for surface disinfection in this study because Okuda et al. (2005) found a significance increase of true endophytes isolated from *Pieris japonica* when using acidic electrolyzed water compared to those obtained by using the conventional ethanol-sodium hypochlorite. Besides, the use of acidic electrolyzed water for surface disinfection minimized the occurrence of changes in color and texture of the plant samples. This is crucial especially for the surface sterilization of fragile samples such as leaves, roots, and flowers of *O. stamineus*. Some of the sterilants such as sodium hypochlorite and ethanol can cause the less robust samples, not accessible to the propagation of the microbial endophytes (Charepresert et al., 2006).

Since the study of endophytes is method-dependent with different hosts and plant tissues requiring different sterilization time, the immersion time of the samples in acidic electrolyzed water were optimized to ensure elimination of all the epiphytes. The optimized immersion time for leaves and stems were 20 and 30 min respectively, whereas the immersion time for both flowers and roots were 15 min.

Hyde and Soyong (2008) concluded that the isolation of endophytes are usually biased towards fast growing fungi on the isolation media. Hence, powdered plant materials were added to the isolation media to enhance the growth of the endophytic fungi as well as to minimize fungal contamination. This is due to the antifungal metabolites of the host which inhibits the growth of the non-endophytic fungi.

As per the data available from previous studies on various plant species, fungi with mycelia sterilia accounted for 24% of the total isolates. All of these isolates did not sporulate on basic mycological media such as PDA, malt extract agar, corn meal agar, Malt Czapek Dox agar and yeast extract sucrose agar, incubated in darkness. However, for two of the isolates, the production of conidia or other reproductive structures were observed on dried UV-sterilized host plant samples or carnation leaves plated on water agar with 0.5% potassium chloride after longer incubation period of 20 to 30 days.

### **Antimicrobial activity**

Figures 1 and 2 show the antimicrobial activity of crude extracts obtained from different culture medium. Sixty-six (92%) out of the 72 isolates, exhibited significant inhibitory activity at least against one test microorganism, with diameters of inhibition zones ranging from 9 to 26 mm for the test bacteria. However, only moderate

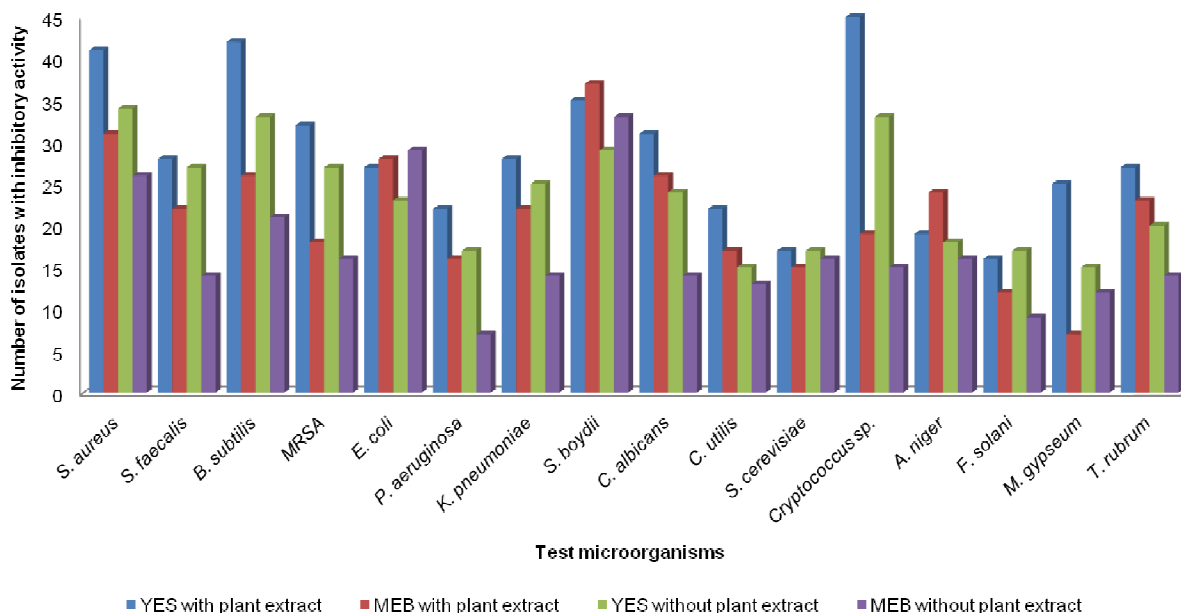


Figure 1. Antimicrobial activity of the methanolic extracts from fungal isolates cultivated on various medium.

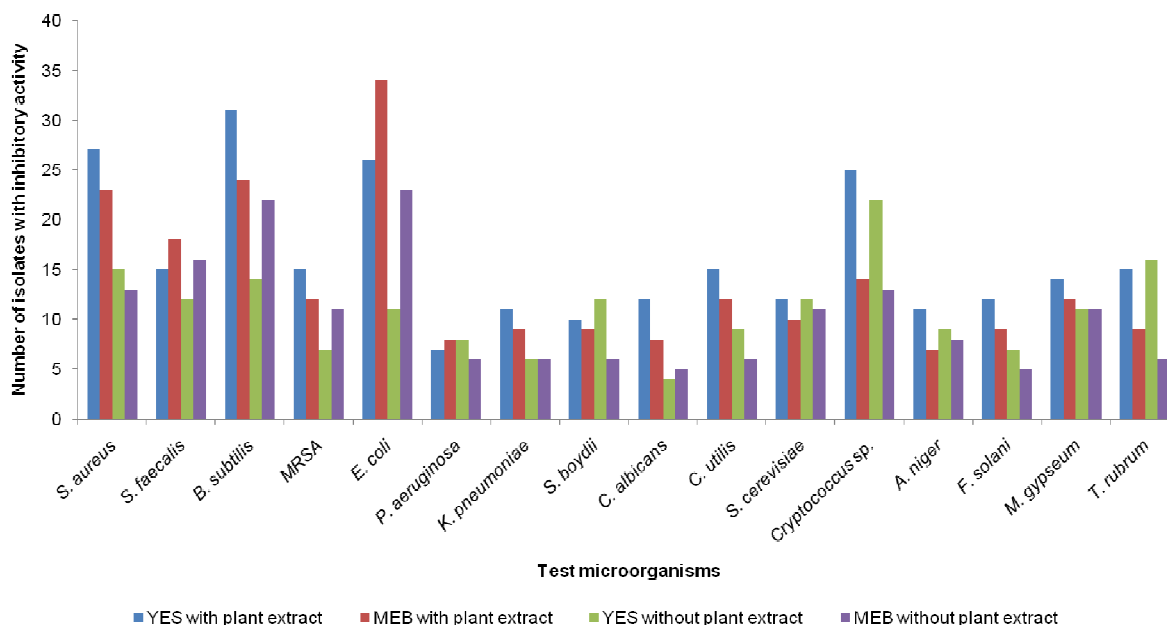


Figure 2. Antimicrobial activity of the ethyl acetate extracts from fungal isolates cultivated on various medium.

antiyeast and antifungal activities were observed for both methanolic and ethyl acetate extracts, with diameter of clear zone less than 16 mm on disc diffusion assay for all the endophytic isolates.

Two isolates, namely ED2 and ED24 demonstrated the most significant antimicrobial activity on the test organisms. Isolate ED2 with white-colored mycelia sterilia, produced greenish pigmentation on PDA culture

plates after 20 days of incubation. Molecular approach was required for identification of this isolate. Isolate ED24, with fan-shaped conidiophore under light microscope, produced red pigmentation on PDA culture plates after 7 days of incubation. This isolate was identified as a member of the genus *Penicillium* according to Alexopoulos et al. (2002). Both of the isolates exhibited broad-spectrum antibacterial activity on

**Table 1.** Antimicrobial activity of isolate ED16 and ED49 on volatile antimicrobial assay.

Test microorganisms	Inhibition percentage (%)	
	Isolate ED16	Isolate ED49
<i>E. coli</i>	97.8 ± 2.5	99.3 ± 1.7
<i>P. aeruginosa</i>	25.0 ± 3.8	-
<i>K. pneumoniae</i>	-	85.3 ± 2.0
<i>S. boydii</i>	53.0 ± 4.1	-
<i>M. gypseum</i>	-	47.7 ± 4.7
<i>T. rubrum</i>	-	53.1 ± 3.2

\* (-) = no inhibition.

both Gram positive and negative bacteria, but only isolate ED24 exhibited antifungal activity. They are potential candidates for further investigations.

The results also demonstrated better antimicrobial activity by methanolic extract from the fungal biomass compared with ethyl acetate extract from fermentative broth and volatile antimicrobial compounds. Only two isolates exhibited inhibitory activity on the volatile antimicrobial assays (Table 1). These results indicated that the main constituents of the antimicrobial compounds were produced intracellularly and they were associated with the mycelia.

Amzad Hossain et al. (2008) showed significant *in-vitro* antimicrobial activity of essential oil of *O. stamineus*. A bioassay device was modified to study the volatile antimicrobial compounds of their endophytic fungi. The percentage inhibition was calculated in term of reduction of colony forming units (CFU) for yeasts and bacteria compared to growth control and diameter of culture for test fungi. None of the isolates showed anti-candidal activity on this assay. Isolate ED16 and isolate ED49 showed antibacterial activity only on Gram negative bacteria. This was in contrast to the data of disc diffusion assay, where both isolates inhibited only Gram positive bacteria. Volatile compounds produced only by isolate ED49 killed *Microsporium gypseum* and *Trichophyton rubrum* because they did not survive in viability test.

Sieber et al. (1991) postulated that the fungus is more pathogenic if its incubation period is shorter. Endophytes isolated from their host with no apparent disease symptoms, are low virulence in term of pathogenicity. Hence, a longer incubation period was necessary for endophytic fungal cultivation especially for biosynthesis of secondary metabolites, which occurred in stationary growth phase, the third phase after inoculation.

The fungal extracts demonstrated more susceptibility to Gram positive bacteria compared with Gram negative bacteria. This is due to the more rigid and complex cell wall structures in Gram negative bacteria (Hugo, 1998). Only 32 isolates exhibited antiyeast activity and 27 isolates exhibited antifungal activity. A great number of active isolates exhibited antibacterial activity compared with the isolates exhibiting antiyeast and antifungal

activity. This may due to the similarities in eukaryotic characteristics between the endophytic fungi and the test organisms (Hugo, 1998). Some of the fungal extracts were found to be very active against 2 keratinolytic dermatophytes namely, *M. gypseum* and *T. rubrum*, which cause dermatophytosis.

#### Effects of culture medium on antimicrobial activity

The biosynthesis of secondary metabolites greatly affected by the medium composition. Highly different production of secondary metabolites of a target microorganisms can be observed with 2 different culture medium. For the methanolic extract, only the crude extracts for 12 isolates were found to exhibit the same activity in both culture medium. However, for ethyl acetate extract, only 7 isolates exhibited the similar activity. The activity, measured as diameter of clear zones, was different for all the isolates. The extract obtained from YES produced clear zones with larger diameter on disc diffusion assay. These findings were in agreement with the data available from the previous study by Wiyakrutta et al. (2004). The effect of the culture medium was more significant on the methanolic extract. This may be due to the chemical substances in the host extract that can stimulate the growth of the isolates and directly increase the biosynthesis of intracellular metabolites associated with their biomass. In contrast, no significant effect of the culture medium was observed on the antimicrobial activity for the volatile antimicrobial assays. This indicated that the production of volatile compounds were less dependent on the medium composition.

The mutual interactions between the host and the endophytes are beneficial not only to the host, but also to the endophytes by supplying nourishment to it (Tan and Zou, 2001). Hence, the extract of the host was utilised as the basal medium for cultivating the endophytic fungi in the study of Gogoi et al. (2008). It was demonstrated (Figures 1 and 2) that addition of water extract of host significantly increases the antimicrobial activity. A greater increment in antimicrobial activity was observed for ethyl

acetate extract (21.6%) compared with methanolic extract (18.2%). This increment may be due to certain chemicals from the host plant that are necessary for the biosynthesis of secondary metabolites in endophytic fungi. Endophytic fungi can produce antimicrobial metabolites with the supply of plant nutrients. It was also found that for the volatile antimicrobial assay, the addition of host extract in culture medium increase the inhibition percentage for almost all test microorganisms, for both endophytic isolates.

## Conclusion

Medicinal herb, *O. stamineus* harbors diverse species of endophytic fungi and some of these isolates exhibited significant inhibitory activity on pathogenic microorganisms. A significant enhancement in antimicrobial activity of the isolates was found on addition of the plant extracts to the culture medium. Further investigations on isolation of these antimicrobial compounds are crucial as an approach to search for novel natural products.

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## REFERENCES

- Alexopoulos CJ, Mims CW, Blackwell M (2002). Introductory Mycology. Wiley, India, Pp. 224-307.
- Amzad Hossain M, Zhari I, Atiqur R, Sun CK (2008). Chemical composition and anti-fungal properties of essential oils and crude extracts of *Orthosiphon stamineus* Benth. Ind. Crop. Prod., 27: 328-334.
- Banskota AH, Tezuka Y, Tran QL, Kadota S (2003). Chemical constituents and biological activities of Vietnamese medicinal plants. Curr. Top. Med. Chem., 3: 227-248.
- Bayaty FHA, Mohamed MAA, Hassan A, Hussein SF (2010). Malaysian medicinal plants on macrophage functions *in vitro* study. J. Med. Plants. Res., 4(14): 1459-1463.
- Chareprasert S, Piapukiew J, Thienirun S, Whalley AJS, Sihanonth P (2006). Endophytic fungi of teak leaves *Tectona grandis* L. and rain tree leaves *Samanea saman* Merr. World J. Microb. Biot., 22: 481-486.
- Gao F, Dai C, Liu X (2010). Mechanisms of fungal endophytes in plant protection against pathogens. Afr. J. Microbiol. Res., 4(13): 1346-1351.
- Gogoi DK, Mazumder S, Saikia R, Bora TC (2008). Impact of submerged culture conditions on growth and bioactive compound produced by endophyte *Hypocrea* spp. NSF-08 isolated from *Dillenia indica* Linn. in North-East India. J. Mycol. Med., 18: 1-9.
- Hugo WB (1998). Bacteria. In: Hugo WB, Russell AD (eds) Pharmaceutical Microbiology, sixth edition. Blackwell Science Ltd, United Kingdom, pp. 4-8.
- Hyde KD, Soyong K (2008). The fungal endophyte dilemma. Fungal Divers., 33: 163-173.
- Jorgensen JH, Turnidge JD (2007). Susceptibility test methods: dilution and disk diffusion methods. In: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA (eds) Manual of Clinical Microbiology. ASM Press, Washington, pp. 1152-1172.
- Koshy P, Sri Nurestri AM, Wirakarnain S, Sim KS, Saravana K, Hong SL, Lee GS, Syarifah NSAR (2009). Antimicrobial activity of some medicinal plants in Malaysia. Am. J. Appl. Sci., 6(8): 1613-1617.
- Okuda T, Ando K, Bills G (2005). Fungal germplasm for drug discovery and industrial applications. In: Bennett JW (ed) Handbook of Industrial Mycology. Marcel Dekker, New York, USA, Pp. 145-147.
- Schulz B, Boyle C, Draeger S, Rommert AK, Krohn K (2002). Endophytic fungi: a source of novel biologically active secondary metabolites. Mycol. Res., 106(9): 996-1004.
- Sieber TN, Canavesi SF, Dorwoth CE (1991). Endophytic fungi in red alder (*Alnus rubra*) leaves and twigs in British Columbia. Can. J. Bot., 69: 407-411.
- Stinson M, Ezra D, Hess WM, Sears J, Strobel G (2003). An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* producing selective volatile antimicrobial compounds. Plant Sci., 165: 913-922.
- Strobel GA (2003). Endophytes as sources of bioactive products. Microbes Infect., 5: 535-544.
- Strobel S, Daisy B (2003). Bioprospecting for microbial endophytes and their natural products. Microbiol. Mol. Biol. R., 67(4): 491-503.
- Tan RX, Zou WX (2001). Endophytes: a rich source of functional metabolites. Nat. Pro. Rep., 18: 448-459.
- Wiyakrutta S, Sriubolmas N, Panphut W, Thongon N, Danwisetkanjana K, Ruangrungsi N, Meevootisom V (2004). Endophytic fungi with antimicrobial, anti-cancer and anti-malarial activities isolated from Thai medicinal plants. World J. Microb. Biot., 20: 265-272.
- Yu H, Zhang L, Li L, Zheng C, Guo L, Li W, Sun P, Qin L (2010). Recent developments and future prospects of antimicrobial metabolites produced by endophytes. Microbiol. Res., 165: 437-449.