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Bio-prospecting of Root Endophytic Aquatic Fungus *Cylindrocarpon aquaticum* as Antibacterial Potential

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Root endophytic freshwater hyphomycetes *Cylindrocarpon aquaticum* Nils. was evaluated for its antibiotic potential against some Gram-positive (*Bacillus subtilis*) and Gram-negative (*Agrobacterium tumefaciens*, *Escherichia coli*, *Erwinia chrysanthemum*, *Xanthomonas pseudomonas*) bacterial strains. Antimicrobial activity was assessed with preliminary and secondary antibiotic assays. The results were found positive with the applied fungus by producing significant zone of inhibition was observed for all tested bacterial strains. In primary antimicrobial assay maximum zone of inhibition was counted against Gram-positive bacterium *Bacillus subtilis* (26 mm) followed by Gram-negative bacteria *Xanthomonas phaseoli* (24 mm) and *Agrobacterium tumefaciens* (23 mm) while minimum zone of inhibition was observed against *Erwinia chrysanthemi* and *Escherichia coli* (22 mm, each). Whereas, in secondary antimicrobial assay the maximum zone of inhibition was recorded against human pathogenic bacterial strain *Escherichia coli* (16 mm) followed by *Bacillus subtilis* (15 mm) and minimum inhibition was found against *Agrobacterium tumefaciens* (10 mm).

Key words: *Cylindrocarpon aquaticum*, root endophyte, anti-pathogenic, antibiotic assay.

Microorganisms represent the largest reservoir of undescribed biodiversity and possess the greatest potential for the discovery of new natural products. Bio-prospecting of microorganisms may open the door for new or better bio-products from biological sources. Many pathogenic microorganisms have developed resistance towards current antibiotics and this trend has become more serious (Cassell and Mekalanos, 2001) and such problems demand more novel chemical compounds as antimicrobial. In same sequence many pharmaceutical industries have become interested in screening of fungi for their secondary metabolites (Shu, 1998). Endophytic aquatic fungi have drawn more interest in the search for novel antimicrobial bioactive compounds and recently some valuable novel compounds have been discovered from aquatic fungi. Fisher et al. (1988) discovered a new antibiotic, Quinaphthin, from *Helicoon richonis*. Harrigan et al. (1995) described a new anti-fungal metabolite, Anguillosporal, from *Anguillospora longissima* and Poch et al. (1992) discovered several new bioactive metabolites from *Kirschsteiniothelia* sp.

Aquatic hyphomycetes are specific group of conidial fungi that are abundant in almost all running freshwater bodies throughout the world (Webster and Descals, 1981; Ingold, 1975; Maranova and Barlocher, 1988; Sati et al., 2002; Sati and Tiwari, 1997). Occurrence of these fungi as root endophytes in healthy plants were also reported (Waid, 1954; Sati and Belwal, 2005),

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indicates that they are biologically important (Bills and Polishook, 1992). The antimicrobial potential of some freshwater fungi have been studied earlier (Fisher and Anson, 1983; Shearer and Zare-Maivan, 1988; Platas, et al., 1998; Gulis and Stephanovich, 1999; Arya and Sati, 2012) and now they are gaining an importance, as they are producing bioactive compounds of potential agriculture and medicinal importance (Petrini 1991; Petrini, et al., 1992; Dreyfuss and Chapela, 1992; Bills and Polishook, 1992). It is suggested that due to the specific habitat aquatic fungi may have biosynthesis capabilities different from terrestrial fungi (Gulis and Stephanovich, 1999).

The bio-prospecting of fungi for their natural products are continues but less information, regarding antimicrobial potential of aquatic hyphomycetous fungi, is available. The objectives of present investigation were the isolation and bio-prospection of *Cylindrocarpon aquaticum*, a root endophytic freshwater hyphomycetous fungus, against some Gram-positive and Gram-negative bacterial strains.

MATERIALS AND METHODS

Sample Collection and Strain Isolation

The samples were collected from the different riparian areas of Nainital, (Kumaun Himalaya), Uttarakhand, India. The fresh and healthy roots segments were took from the living plants *in situ* and put into sterile polybags and then taken back to laboratory. Root samples were washed with running tap water (2-4 hrs), then with sterile water (5-7 minutes) and finally washed with 2 % sodium hypochlorite solution (2-5 minutes) to remove temporarily and loosely adhering free living microbes. The samples were then cut into small pieces (1-2 cm) using a sterilized knife and transferred into Petri dishes containing approx. 20 ml sterile water. These dishes were incubated at 22 ±2°C for 3-7 days to observe the emerging mycelium. Initial observations were made under the low power of compound microscope and then studied with high power for detail study of mycelium growth and conidia respectively. Floating conidia were picked up by sterile needle and place onto glass slide for studied further by preparing semi-permanent slides. Pure cultures were prepared by transferring the conidia aseptically, condition, onto the 2 % malt extract agar. The pure cultures were also inoculated onto agar slants and preserved at 4°C.

Preliminary Antimicrobial Assay

For antimicrobial assay, *Bacillus subtilis* MTCC 121 was used as Gram-positive and *Agrobacterium tumefaciens* MTCC 609, *Escherichia coli* MTCC 40, *Erwinia chrysanthemum*, *Xanthomonas pseudomonas*, were adapted as the Gram negative test bacteria. The specific medium for test bacteria was poured into Petri dishes and inoculated with 200 ¼L of the bacterial suspension (after incubation for 24-48 h at 37°C). Then the inoculum was spread by a sterile glass rod (L-shaped) on the surface of the medium. The Nutrient Agar medium (Peptic – 5gm, Beef extract – 1.5g, Yeast extract – 1.5, Sodium chloride – 5gm, Agar – 15gm/litre, pH – 7.4) was used for cultivation of the test bacteria, and the Malt Extract Agar medium was used for cultivation of *Cylindrocarpon aquaticum*. Discs (5 mm diameter) of the isolate fungal culture (7 days old culture, grown on MEA medium plate at 25°C) were cut using a sterile borer and picked up to place on the surface of the above medium seeded with test organisms (Malibari, 1991). The plates were refrigerated at 4°C overnight for complete diffusion of antibiotics, thereafter they were incubated at 37°C for 16 h. The diameter of the inhibition zone was measured and the average of three replicate agar disks was taken to assess the strength of antimicrobial activity.

Secondary Antimicrobial Assay

Fungal strain was inoculated into 250 mL conical flasks containing 50 mL of ME broth medium and were shaken on a thermostatic shaker with the rotary speed of 180rpm at 20 ±2°C. After 7 days, the cultures were centrifuged at 5000 rpm at 4°C to isolate the mycelia and the broths. The mycelia were extracted with 100 mL of methanol overnight and the broths were extracted with 150 mL of ethyl acetate to yield the methanol extract and the ethyl acetate extract, respectively, then the two extracts were filtered, combined, and evaporated to dryness. The resulting crude extract was finally dissolved in 2.5 mL of methanol for assay. In a clean air bench, extract was added using a pipette to a sterile paper disc (5 mm diameter, Whatman No.1), which was air dried and placed on the surface of the medium seeded with test

organisms (Eleeyinmi, 2007). Likewise, the Petri dishes were incubated and measured for inhibition zones. The average of three repeated paper discs was taken to evaluate the activity. The broad spectrum antibacterial agent Gentamycin (dose: 30 mcg/disc) and Ampicillin (10 mcg/disc) were used as positive control while methanol was used as negative control. All experiments were done in triplicate manner and average values with standard error of mean were recorded for the assessment.

Statistical Analysis

The statistical analysis was conducted for all experiments and standard deviation (SD) was calculated, and given as mean ± SD values in representation (Mead and Currow, 1983). All experiments were done in triplicate manner under identical conditions.

RESULTS

Sample Collection and Strain Isolation

Periodic sampling were carried out to enhance the probability of isolate and disregarding age and size, plants were selected randomly for their living roots samples. The fungal isolation from the collected plants resulted in *Cylindrocarpon aquaticum*, a root endophytic aquatic conidial fungus, isolated from the living roots of a riparian plant *Eupatorium adenophorum*.

Morphology and Taxonomy of Isolated Endophyte Hyphae hyaline well developed, septate. Conidiophores long and septate. The distance between two septa of conidium is about 6.6 µm. Conidia hyaline cylinder shaped, septate and up to 29.7-42.9 µm long and 4.2-6.6 µm wide. Cylinder-shaped conidia are characteristic of isolated fungus. So, on the basis of morphological analyses, the isolated fungus was identified as *Cylindrocarpon aquaticum* (Nils.) Marvanova and Descals. (Figure 1AB)

Preliminary Antimicrobial Assay

Fungal discs of isolated root endophytic fungus, *Cylindrocarpon aquaticum* (7 – days old cultures, grown on 2% MEA), were screened and found positive for antimicrobial activity against five bacterial strains spread on specific medium plates (Figure 2). The results showed that the isolated fungus *C. aquaticum* the root endophyte aquatic conidial fungus possessed strong inhibitory activities against the all test bacterial strains. The maximum zone of inhibition was observed against Gram-positive bacteria (*Bacillus subtilis*, 29 mm), while the minimum inhibition was observed against Gram-negative human pathogenic bacterial strain (*Escherichia coli*, 25 mm). The applied fungus has shown a significant inhibitory activity against Gram-positive and Gram-negative bacterial strains (Table 1).

Secondary Antimicrobial Assay

Seven days old cultures of isolated root endophytic fungus, grown in MEB (malt extract broth), were harvested and the ethyl acetate-methanol extracts was re-screened for antimicrobial activity using paper disc agar diffusion method. Results obtained are also summarized in table 1. As evident by the results presented in the table 1, the isolated fungus *Cylindrocarpon aquaticum* is found able to produce antimicrobial substances against the all the test bacteria. For secondary antimicrobial assay, the maximum zone of inhibition was recorded against human pathogenic bacterium *E. coli* (16 mm), followed by plant pathogenic bacteria *B. subtilis* (15 mm) and *X. pseudomonas* (12 mm). The results are found interesting as a major inhibitory activity was found against human pathogenic bacterium *E. coli* while comparatively

Table 1. Average zone of inhibition (ZOI) by *C. aquaticum* against five test bacteria during preliminary and secondary antibiotics assay

Bacterial Strains	Preliminary Antibiotics Bioassay ZOI (mm)*	Secondary Antibiotics Bioassay ZOI (mm)*	Gentamycin ZOI (mm)*
Agrobacterium tumefaciens	23 (±0.6)	10 (±2.0)	28 (±1.0)
Bacillus subtilis	26 (±0.3)	15 (±1.5)	30 (±1.4)
Erwinia chrysanthemum	22 (±0.8)	11 (±1.1)	29 (±1.3)
Escherichia coli	22 (±0.6)	16 (±2.0)	29 (±1.0)
Xanthomonas pseudomonas	24 (±1.3)	12 (±0.3)	30 (±0.3)

* All the values are mean ± Standard Error of Mean of three replicates

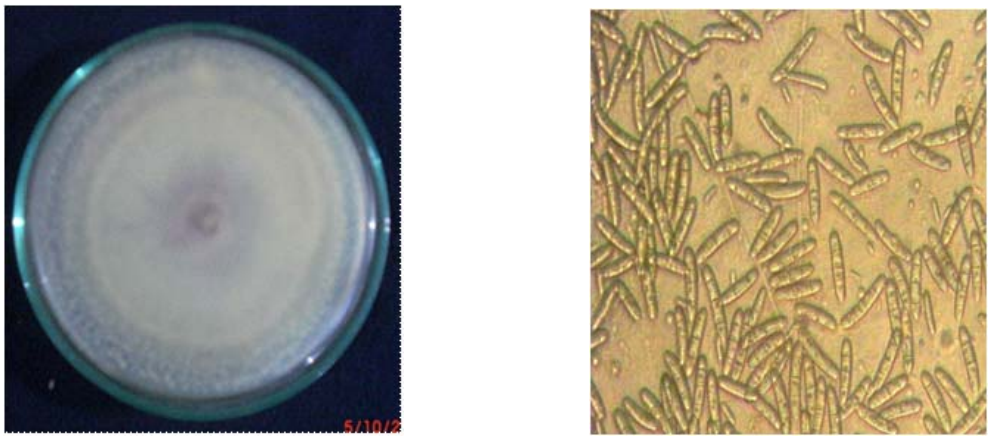

Fig. 1. *Cylindrocarpon aquaticum* Nils. (A) Culture B) Floating conidia

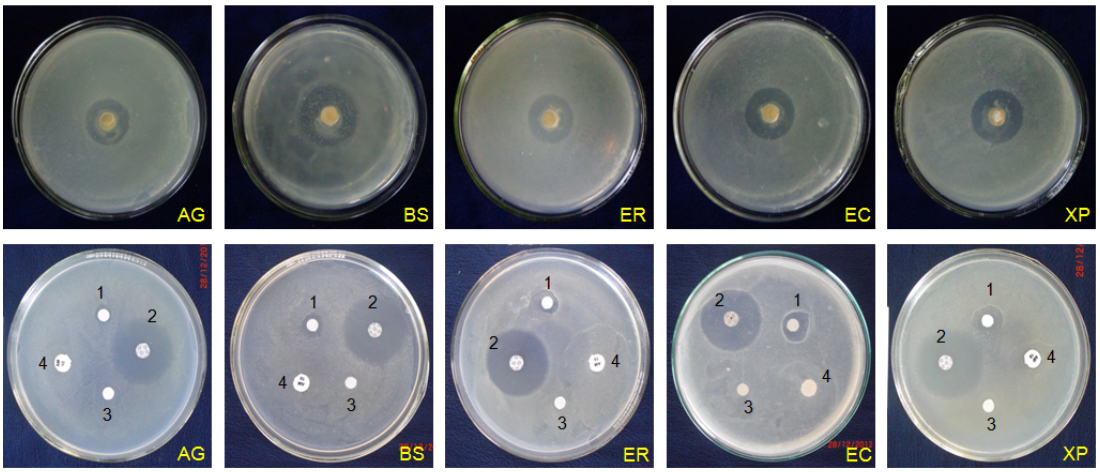

Fig. 2. Zone of Inhibition by the isolated root endophytic fungus against five test bacterial strains in preliminary (first row) and secondary (second row) antimicrobial assay. AG- *Agrobacterium tumefaciens*, BS- *Bacillus subtilis*, ER- *Erwinia chrysanthemi*, EC- *Escherichia coli* and XP- *Xanthomonas phaseoli*, 1- fungal extract, 2&4- positive controls (Gentamycin & Ampicillin), 4- negative control (methanol)

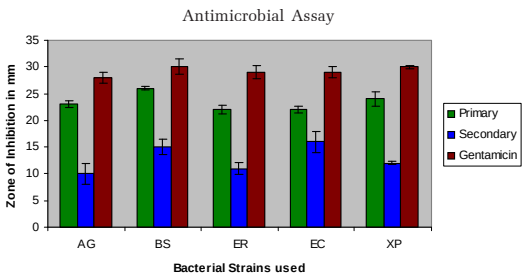

Fig. 3. The antimicrobial activity of isolated root endophytic fungus *C. aquaticum* against five pathogenic bacteria in preliminary and secondary antimicrobial assay. AG- *Agrobacterium tumefaciens*, BS- *Bacillus subtilis*, ER- *Erwinia chrysanthemi*, EC- *Escherichia coli* and XP- *Xanthomonas phaseoli*

a lower inhibition was recorded for the same bacterium in preliminary antibacterial assay (Table 1).

DISCUSSION

The existence of antimicrobial endophytes was probably originated from nutritional competition between endophytes and other organisms, or was due to their participation in the host's chemical defensive mechanisms (Bugni and Ireland, 2004). In the present study, the antimicrobial substances secreted by the applied fungus could be small molecular secondary metabolites, i.e. antibiotic chemical or large

molecules such as polysaccharides, peptides, enzymes, etc. To determine the ability of the above active strain as antibiotic producer, the isolate (*Cylindrocarpon aquaticum*), was tested with relatively broader antimicrobial spectrum by using five bacterial strains in preliminary assay, followed by secondary assay.

It was noteworthy that isolated endophytic fungus not only exhibited broad spectrum activity but also was potent enough to compare with the positive control Ampicillin (as no inhibition was observed). The diameters of inhibition zones against specific test organisms were countable or even larger than those of the positive control (Ampicillin). Fungal isolate crude extracts, showed considerable inhibitory activity against all five tested bacterial strains (Table 1). In secondary antimicrobial assay the human pathogenic bacterial strain *E. coli* had its maximum inhibition (Fig. 3). Gloer (1995) suggested that freshwater fungi, due to their specific habitat, might have biosynthetic capabilities then those of terrestrial fungi. In this investigation, the inhibition in bacterial growth by the applied endophytic fungus might be due the production of some antimicrobial chemicals. Aquatic endophytes, due to their specific habitat, might be capable to produce antimicrobial agents for their self defense against harmful organisms. Endophytic fungi are well known for their metabolites but only few reports are present for the metabolites and antimicrobial potential of freshwater endophytes. Sati and Arya (2010) reported inhibitory potential of some freshwater hyphomycetes against some pathogenic fungi as well as bacteria (Arya and Sati, 2012). Similarly, Xiao et al. (2013) isolated some antimicrobial compounds from a freshwater fungus *Pestalotiopsis photiniae*. Fisher and Anson (1983) and Platas et al. (1998) in their investigations found that *Massarina aquatica* is capable to inhibit the growth of the yeasts *Candida albicans* and *Sporobolomyces roseusi*. Gulis and Stephanovich (1999) reported growth inhibition of *Candida utilis* and others microorganism (including; bacteria) by *C. aquaticum*, isolated from grasses, while no inhibition was recorded against some species of *Bacillus*, *Escherichia* and *Xanthomonas*. In the present study, growth inhibition in all five tested bacterial strains was recorded. It is also quite clear that a variation in culture and morphological characters has been observed in the fungus isolated from different habitats and hosts. Therefore, there is a probability to produce more or less potent antimicrobial compounds for self defense. In this study the fungus was isolated from *Eupatorium adenophorum* and found more potent to inhibit the growth. Relying upon the results present study supports the earlier findings and proves antimicrobial potential of root endophytic freshwater fungus *C. aquaticum*, by preliminary and secondary antibacterial assays.

CONCLUSION

The endophytic conidial aquatic fungus *Cylindrocarpon aquaticum* was isolated from the riparian plant roots from Nainital, Uttarakhand, India. The bioassay data showed globally that root endophytic fungus is capable to inhibiting the test organisms. There is, therefore, a high possibility of obtaining new antibiotic metabolites of medicinal and agricultural importance from the freshwater endophytic fungi. In promoting our understanding of aquatic hyphomycetes in a freshwater ecosystem it is required to determine the actual chemistry of the metabolites produced by these endophytic fungi.

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REFERENCES

1. Arya, P., Sati, S.C. Evaluation of endophytic hyphomycetes for their antagonistic activity against pathogenic bacteria. *Int. J. Microbiol*., 2012; **2**(9): 343 – 347.
2. Bills, G.E., Polishook, J.D. Recovery of endophytic fungi from *Chamaecyparis thyoides*. *Sydowia*, 1992; **44**: 1-12.
3. Bugni, T.S., Ireland, C.M. Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Nat. Prod. Rep.*, 2004; **21**: 143-63.
4. Cassell, G.H., Mekalanos, J. Development of

antimicrobial agents in the era of new and reemerging infectious diseases and increasing antibiotic resistance. *J Am Med Assoc.*, 2001; 285: 601-605.

5. Daszak , P., Cunningham A.A., Hyatt, A.D. Emerging infectious diseases of wildlife-threats to biodiversity and human health. *Science*, 2000; 287: 443-449.
6. Dreyfuss, M.M., Chapela, I.H. The potential of fungi in discovery of novel, low molecular weight pharmaceuticals. In: Discovery of novel natural products with therapeutic potential. London: Butterworth Publications, Biotechnology Series. 1992
7. Eleeyinmi, A.F. Chemical composition and antibacterial activity of *gongronema latifolium. J. Zhejiang. Univ.*, 2007; **8**: 352-358.
8. Fisher, P.J., Anson, A.E., Webster, J., Adriaenssens, P., Whitehurst, J.S. Quinaphthin, a new antibiotic, produced by *Helicoon richonis. Trans. Brit. Mycol. Soc.*, 1988; **90**: 499-502.
9. Fisher, P.J., Anson, A.E. Antifungal effects of *Massarina aquatica* growing on oak wood. *Trans. Brit. Mycol. Soc.* 1983; **81**: 523-528.
10. Gloer, J.B. The chemistry of fungal antagonism and defense. *Can. J. Bot.*, 1995*;* **73**(1): 265-274.
11. Gulis, V.I., Stephanovich, A.I. Antibiotic effects of some aquatic hyphomycetes. *Mycol. Res.*, 1999; **103**: 111-115.
12. Harrigan, G.G., Armentrout, B.L., Gloer, J.B., Shearer, C.A. Anguillosporal, a new antibacterial and antifungal metabolite from the freshwater fungus *Anguillospora longissima*. *J. Nat. Prod.*, 1995; **58**: 1467-1469.
13. Hyde, K.D. Increasing the likelihood of novel compound discovery from filamentous fungi. In: *Bio-exploitation of Filamentous Fungi* (Pointing SB, Hyde KD eds). Fungal Diversity Research Series 6, Fungal Diversity Press, Hong Kong, 2001; pp 77-91.
14. Ingold, C.T. An illustrated guide to aquatic and waterborne hyphomycetes (Fungi Imperfecti) with notes on their biology. *Fresh. Biol. Assoc. Sci.*, 1975; **30**: 1-96.
15. Malibari, A.A. Isolation and screening of antibiotics producing streptomycetes from western region soils of Saudi Arabia. J. King Abdulaziz Univ. Sci., 1991; **3**: 31-42.
16. Maranova, L., Barlocher, F. Hyphomycetes from Canadian streams. I. Basidiomycetous anamorphs. *Mycotaxon*, 1988; **32**: 339-351.
17. Mead, R., Currow, R.N. (ed): Statistical methods in agricultural and experimental biology. Chapman and Hall, London UK, 1983; pp 335.
18. Petrini, O. Fungal endophytes of tree leaves. In: *Microbial Ecology of Leaves* (Andrews JA, Hirano SS eds). Springer, New York, 1991; pp 179-197.
19. Petrini, O., Sieber, T.N., Toti, L., Viret, O. Ecology, metabolite production and substrate utilization in endophytic fungi. *Natural Toxins*, 1992; **1**:185-196.
20. Platas, G., Pelaez, F., Collado, J., Villuendas, G., Diez, M.T. Screening of antimicrobial activities by aquatic hyphomycetes cultivated on various nutrient sources. Cryptogamie *Mycologie*, 1998; **19**: 33-43.
21. Poch, G.K., Gloer, J.B., Shearer, C.A. New bioactive metabolites from a freshwater isolate of the fungus *Kirschsteiniothelia* sp. *J. Nat. Prod.*, 1992; **55**: 1093-1099.
22. Sati, S.C., Arya, P. Antagonism of some aquatic hyphomycetes against plant pathogenic fungi. *The Scient. World J.*, 2010; **10**: 760-765.
23. Sati, S.C., Belwal, M. Aquatic hyphomycetes as endophytes of riparian plant roots. *Mycologia*, 2005; **97**(1): 45-49.
24. Sati, S.C., Pant, N., Belwal, M. Conidial aquatic fungi of Kumaun Himalaya, India. *Mycotaxon*, 2002; **80**: 445-455.
25. Sati, S.C., Tiwari, N. Glimpses of conidial aquatic fungi in Kumaun Himalaya. In: *Recent Researchers in Ecology, Environment and Pollution* (Sati SC, Saxena J, Dubey RC eds). Today and Tomorrow printers and Publishers, New Delhi, India, 1997; **10**: 17-37.
26. Shearer, C.A., Zare-Maivan, H. *In vitro* hyphal interactions among wood- and leaf inhabiting ascomycetes and fungi imperfecti from freshwater habitats. *Mycologia*, 1988; **80**: 31-37.
27. Shu, Y.Z. Recent natural products based drug development: a pharmaceutical industry perspective. *J. Nat. Prod.*, 1998; 61: 1053-1071.
28. Waid, J.S. Occurrence of aquatic hyphpomycetes upon the roots surface of beech grown in woodland soil. *Trans. Brit. Mycol. Soc.*, 1954; **37**: 420-421.
29. Webster, J., Descals, E. Morphology, distribution and ecology of conidial fungi in freshwater habitats. In: *Biology of Conidial Fungi* (Cole GT, Kendrick B eds). Academic Press, New York, 1981; **1**: 295-355.
30. Xiao, L.Y., Zhuang, Z.L., Hai, Y.L., Zhi, Q.L. Antifungal metabolites from the fermentation broth of plant endophytic fungus *Pestalotiopsis photiniae*. *Afri. J. Microbiol. Res.*, 2013; **7**(27): 3563-3565.