Research Journal of Microbiology

ISSN 1816-4935

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS Research Journal of Microbiology

ISSN 1816-4935
DOI: 10.3923/jm.2020.15.21

Research Article
Novel Endophytic *Penicillium chrysogenum* Strains Isolated from *Plectranthus amboinicus* L. of West Malaysia

Kartikeya Tiwari and Manjuu Ganesen

Department of Microbiology, International Medical School, Management and Science University, Shah Alam, 40100 Selangor, Malaysia

Abstract

Background and Objective: In nature, plants incorporate a wide scope of restoratively esteemed bioactive compounds. The fungal endophytes are the organisms which grow inside the plant parts such as roots, stems, leaves and flowers and produce similar bioactive compounds of medicinal significance. The fungal endophytes from the plant *Plectranthus amboinicus* were not well explored in West Malaysia. Therefore, this study was designed with the objectives to harness novel endophytic bioactive compound producing fungal strains from this plant. **Materials and Methods:** Screening for fungal endophytes was performed by using surface sterilisation method. Sterile explants were placed on a potato dextrose agar (PDA) plates and incubated under 28°C. These fungal endophytes were identified microscopically by lactophenol-cotton blue staining protocol and molecular characterization was performed by amplification of randomly amplified polymorphic DNA tool. **Results:** A total of ten fungal endophytes were isolated and identified. The prevalent endophytic fungal strain found was of *Penicillium chrysogenum* in comparison with *Aspergillus niger*, *Fusarium oxysporum* and *Alternaria solani*. Therefore, these strains selected for assessment of genetic diversity. **Conclusion:** The most prevalent bioactive compound producing fungal strain found was of *Penicillium chrysogenum* associated endophytically with the plant *Plectranthus amboinicus.*

Key words: *Plectranthus amboinicus*, fungal endophytes, colonization frequency, *Penicillium chrysogenum, Pestalotiopsis* sp., potato dextrose agar medium, randomly amplified polymorphic DNA

Citation: Kartikeya Tiwari and Manjuu Ganesen, 2020. Novel endophytic *Penicillium chrysogenum* strains isolated from *Plectranthus amboinicus* L. of West Malaysia. Res. J. Microbiol., 15: 15-21.

Corresponding Author: Kartikeya Tiwari, Department of Microbiology, International Medical School, Management and Science University, Shah Alam, 40100 Selangor, Malaysia Tel: +60149294304

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Emergence of pathogenic strains is a growing concern all over the world. Drug resistant pathogens or super bugs are using new methods for survival. These pathogens are having drug resistance genes either on plasmids or integrated with chromosomes[1]. Recently it was found that these pathogens also shifted the cell wall synthesis precursor molecules to escape the target action from drugs acting on cell wall[1]. A large number of side effects were seen in patients due to overuse of synthetic drugs[2]. Therefore, the search for new bioactive compounds from natural resources is increasing year by year.

Large community of medicinal plants identified all over the world as potentialistic drug producer in natural manner. Herbal medicines are used all over the world for health care management. *Plectranthus amboinicus* is a perennial herb and possesses antimicrobial and antioxidant properties[1]. It is traditionally used to treat conditions like coughing, skin infections and fever. The leaves of this herb are used for flavoring and in preparation of traditional food[2]. The earlier research data discloses that this herb contains 70 volatile and 30 non-volatile compounds of phytochemicals with significant pharmacological properties[3].

Endophytes are organisms that colonize in healthy tissues of plants and produce metabolites of pharmaceutical significance. During growth and development of medicinal plants, these endophytes colonize naturally in various plant parts and maintain healthy relationship. These endophytic organisms produce various bioactive compounds, which help the plant to metabolize certain nutrients, in return the plant facilitate the colonization and survival of these organisms. *Pestalotiopsis* sp., *Taxomyces* sp. and *Penicillium* species are some important genera associated endophytically with various medicinal plants and producing unique bioactive compounds of medicinal importance. Research studies have shown, that the endophytic *Penicillium* species produce unique bioactive compounds such as terminatone, ergosterol, 4-hydroxybenzaldehyde and 4-hydroxy-hexadec-6-6-enoic acid methyl ester[4]. These compounds possess antimicrobial and antioxidant activity. Toghueo and Boyom[5], discussed in their comprehensive review about various potentialistic fungal endophytes isolated from *Terminalia* species.

Maheshwari *et al.*[6] explored endophytic mycoflora and its biological activities from *Terminalia arjuna* medicinal plant of Indian region.

Similarly, Rajeswari *et al.*[7] found out various significant endophytic fungi from *Terminalia chebula* plant of the Eastern Ghats, Tamil Nadu, India.

Kim *et al.*[8] discovered two species of *Penicillium* from Korea in their research work. These *Penicillium* species were significant bioactive compound producers.

In India, diverse endophytic fungal communities explored for the discovery of novel bioactive compounds at molecular level by Verma *et al.*[9] and Tiwari[10]. Therefore, in the present work, novel fungal endophytic strains from the herb *Plectranthus amboinicus* were isolated, identified and molecularly characterized in the search of novel unique bioactive compounds.

MATERIALS AND METHODS

Research duration: The research was conducted in Department of Microbiology, Management and Science University, Shah Alam, Malaysia for duration of 6 months (April-October, 2019).

Sample processing: The samples were collected from different regions of Selangor, Malaysia. This herb is grown easily in pods. The healthy plant parts (Leaves and roots) were collected and transferred in sterilized poly bags. The plant parts were processed in MSU microbiology laboratory by standard protocol.

Isolation and identification of fungal endophytes: Isolation of fungal endophytes was carried out by standard protocol given by Tiwari and Chittora[11]. The isolated fungal endophytes were identified on the basis of macroscopic and microscopic characteristics.

Colonization frequency: The colonization frequency was calculated by using the formula given below[11]:

$$\text{Colonization frequency (\%)} = \frac{\text{Number of explants with endophytic fungal growth}}{\text{Total number of explants}} \times 100$$

Cultivation of *P. chrysogenum* strains: Initially numbering was given to all endophytic fungal strains isolated. The *P. chrysogenum* strains were named as *P. chrysogenum*-I, *P. chrysogenum*-II, *P. chrysogenum*-III, *P. chrysogenum*-IV, *P. chrysogenum*-V and *P. chrysogenum*-VI. All 6 novel endophytic fungal strains of *P. chrysogenum* (I-VI) were grown in potato dextrose agar medium, were transferred to czapek yeast autolysate (CYA) agar medium containing plates and slants by maintaining all aseptic conditions. After 7 days incubation at 25°C, the phialides, conidiophore and conidia of

pure cultures were examined for the identification by lactophenol cotton blue staining protocol. For the cultivation of these strains, an agar cube of equal size was cut (Same size, Cork borer) from the pure culture PDA plate on laminar air flow by maintaining all aseptic conditions. This cube was transferred to Czapek Yeast Autolysate (CYA) broth for penicillin production and incubated in shaker incubator at 25°C, 100 rpm (rotation per minute) for 10 days in dark[12]. Shake flask culturing steps were performed in 500 mL Erlenmeyer flasks containing 100 mL growth medium protocol standardized by Chen *et al.*[12].

Penicillin extraction: The 4 step extraction method was used for the extraction of penicillin from these novel strains of *P. chrysogenum* as described by Rafi[13].

Sensitivity estimation: The penicillin extract poured in wells of Mueller-Hinton agar medium containing plates flooded with *Staphylococcus aureus* clinical isolate. After two days of incubation, the diameter of zone of inhibition was measured[14].

Assessment of genetic diversity: Genomic DNA from six novel *Penicillium chrysogenum* strains was isolated[15]. Assessment of genetic diversity among six endophytic fungal strains of *P. chrysogenum* isolated from *Plectranthus amboinicus* were carried out using PCR based RAPD primers and 32 random decamer primers were used. Out of 32 primers screened, 8 primers amplified and produce results. Percentage of polymorphism was calculated on the basis of number of polymorphic bands/number of total bands. The clade and dendrogram constructed by unweighted pair group method based on Jaccard distance[15].

Statistical analysis: The prevalence of fungal endophytes was calculated by colonization frequency (%) and the graph was constructed in Microsoft excel by number of identified endophytic fungal genera obtained during this research study.

RESULTS

Colonization of endophytic fungal genera in *Plectranthus amboinicus*: The healthy plant *Plectranthus amboinicus* collected for processing was shown in Fig. 1. After five days of incubation, the sporulating fungal endophytes started growing on potato dextrose agar medium from leafy and nodal explants, which were presented in Fig. 2a, b. These fungal endophytes were transferred carefully to another set of

Fig. 1: Healthy *Plectranthus amboinicus* plant

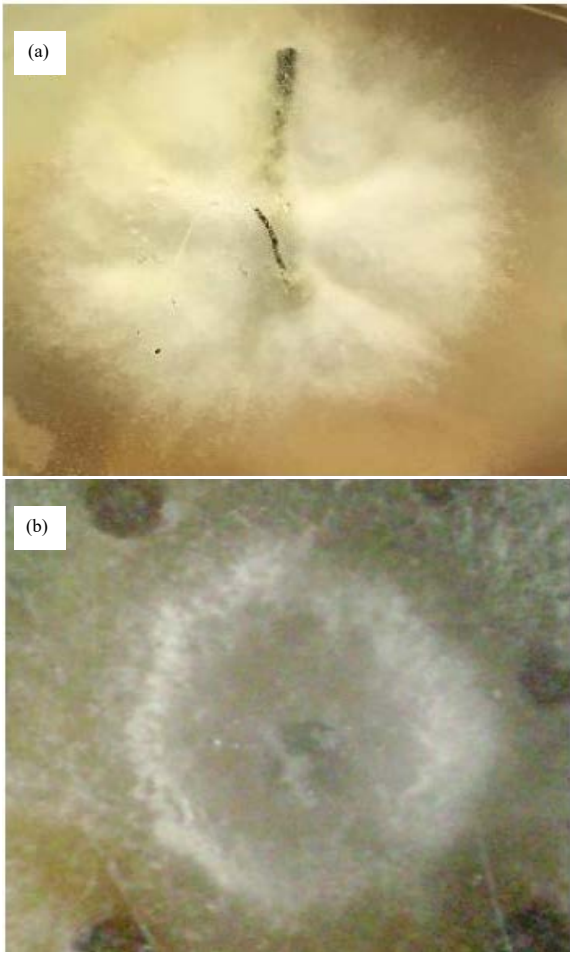

Fig. 2(a-b): Sporulating fungal endophytes from nodal and leafy explants of *Plectranthus amboinicus* L.

Fig. 3: Pure culture of *Penicillium chrysogenum* strain after 2 days of incubation

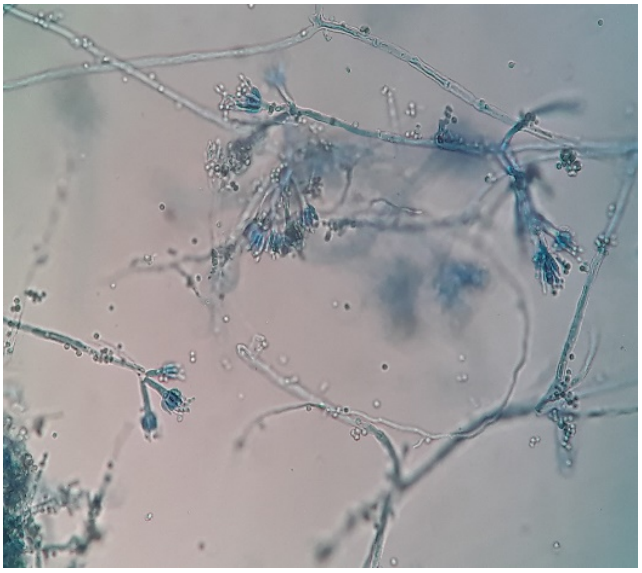

Fig. 4: Microscopic view of *Penicillium chrysogenum* strain at 40× magnification showing broom shape structure and conidia

potato dextrose agar plates for the maintenance of pure culture and further investigation. The pure culture of *P. chrysogenum* strain on potato dextrose agar medium after 2 days of incubation is shown in Fig. 3. The microscopic view of *P. chrysogenum* strain showing phialides and conidia (Broom shaped structure) by lactophenol cotton blue staining presented in Fig. 4.

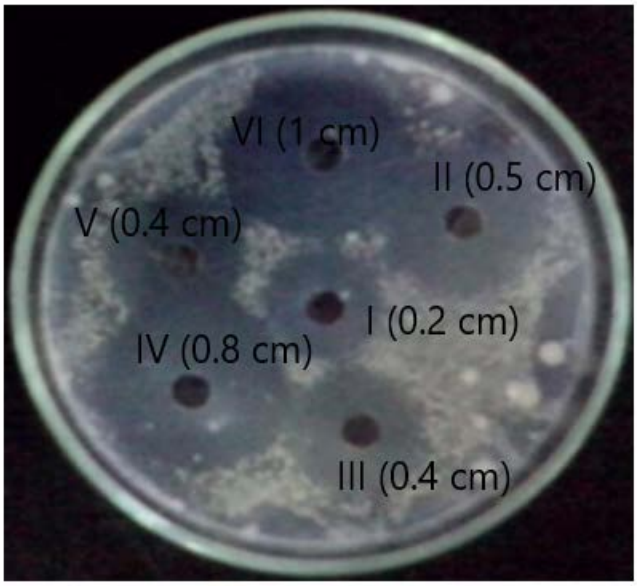

Fig. 5: Aqueous extract (1 mg mL^{-1}) of novel *P. chrysogenum* strains (I-VI).
P. chrysogenum strain-VI has shown maximum zone of inhibition (1 cm) against methicillin resistant *Staphylococcus aureus* (MRSA) clinical isolate

Measurement of zone of inhibition and sensitivity test: The aqueous extract of *Penicillium chrysogenum* strains (I-VI) shown zone of inhibition against methicillin resistant *Staphylococcus aureus* (MRSA) clinical isolate. The maximum zone of inhibition found was of 1 cm with *P. chrysogenum* -VI strain (1 cm) in comparison with other strains (I, II, III, IV and V) of P. chrysogenum which has shown zone of inhibition of 0.2-1 cm (Fig. 5). Therefore, the VI strain of *P. chrysogenum* was found as maximum penicillin producer at batch level.

Prevalence: A total of ten novel endophytic fungal strains were isolated and identified from the plant *Plectranthus amboinicus*. The most prevalent endophytic fungal species found was *Penicillium chrysogenum* in comparison with *Aspergillus niger*, *Fusarium oxysporum* and *Alternaria solani* (Fig. 6).

Genetic diversity assessment: The genetic diversity assessment was performed by randomly amplified polymorphic DNA (RAPD) tool. A total of 39 loci were amplified. Out of 39 bands, 21 (53.84%) were found to be polymorphic and 18 (46.15%) were found to be monomorphic bands.

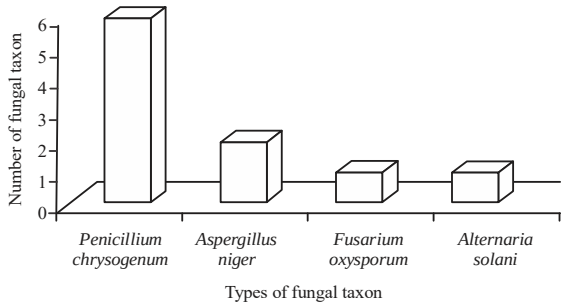

Fig. 6: Prevalence of novel endophytic fungal strains isolated from *Plectranthus amboinicus*

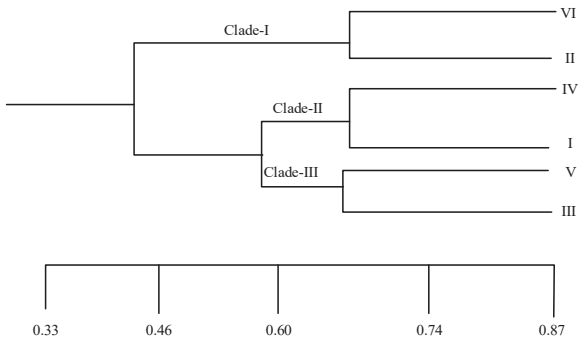

Fig. 7: Dendrogram showing genetic diversity among 6 *Penicillium chrysogenum* strains

Dendrogram: Dendrogram was constructed by unweighted pair group method based on Jaccard distance. Six novel strains of *P. chrysogenum* aliened into three large clades (I, II and III). Each clade contains two strains which are the closest to each other. Clade I contains VI and II, clade II contains IV and I and clade III contains V and III. These strains are the closest match with each other (Fig. 7).

DISCUSSION

In the present study, results confirmed that the *P. chrysogenum* (Fig. 6) is most prevalent endophytic fungal strain associated with the plants of *Plectranthus amboinicus* in West Malaysia. These *P. chrysogenum* strains are symbiotic and naturally selected by the plant during development. Surprisingly, these strains are producing penicillin in small scale.

The significant *P. chrysogenum* fungal strain was VI (Clade I) in comparison with other strains (I, II, III, IV and V). This strain significantly inhibited the growth of *Staphylococcus aureus* clinical isolate with zone of inhibition of 1 centimeter. These strains were assessed for genetic diversity by randomly amplified polymorphic DNA (RAPD) tool and shown closest match between VI and II strain in clade I. Various researchers used RAPD tool for the genetic diversity assessment of endophytic fungal strains and found it significant.

Similar results were found by Astuti *et al.*[16] in which, potentialistic bioactive compound producing fungal endophytes isolated from this plant. These endophytes also shown antimicrobial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and other drug resistant pathogens.

Gaikwad *et al.*[17] also studied diverse endophytes from this plant and found *Aspergillus flavus* most prevalent in comparison with other endophytes.

Rajendran *et al.*[18] isolated novel fungal endophyte *Pestalotiopsis microspora* EF01 from the plant *Plectranthus amboinicus*. This fungus was producing taxol (an anticancerous drug) and shown significant anticancerous activity against human liver carcinoma cell lines.

From the present work, it was found that the endophytic *P. chrysogenum* strains were significant penicillin and other bioactive compound producer. Similar results were found by Korejo *et al.*[19] in which the endophytic *Penicillium* species had shown antibacterial and antifungal activity.

In Philippines, Campos *et al.*[20] reported myco-pharmacological properties of endophytic fungi from the *Plectranthus amboinicus*. The major fungal strains isolated in this research work, were *A. tamarii*, *A. terreus* and *A. niger*. These strains were producing saponins, tannins, phenols, quinones, sterols and terpenoids. Moreover, these strains were significantly inhibiting the growth of *Staphylococcus aureus* and *Escherichia coli* bacterial pathogens *In vitro*. Contradictory, in the present work, *P. chrysogenum* endophytic fungal strains were found prevalent and potentialistic penicillin producer at batch level.

In India, Sukrutha *et al.*[21] carried out the research work and found that, isolated endophytic fungal strains were producing polyunsaturated fatty acids in significant manner. Although these strains were isolated from various medicinal plants including the plant *P. amboinicus*.

In Pakistan, Urooj *et al.*[22] reported that the endophytic *Penicillium* species is involved and suppressing the growth of soil borne root rotting fungi *M. phaseolina*, *R. solani*, *F. solani* and *F. oxysporum In vitro*. It proved that the endophytic *Penicillium* species is producing distinguished secondary metabolites in association with medicinal plants.

Furthermore, in Brazil, leishmanicidal and fungicidal activity of lipases obtained from endophytic fungal extracts were demonstrated *In vitro* by Alves *et al.*[23]. This shows that a

large number of secondary metabolites are produced by the fungal endophytes in association with medicinal plants of unique environmental niches.

Houbraken *et al*.[24] discovered new penicillin producing *Penicillium* species. The present work also confirms that the *Penicillium* species has enormous potential for the production of penicillin and other related bioactive compounds.

CONCLUSION

This study concluded that *Plectranthus amboinicus* L. harbors a large number of fungal endophytes of medicinal importance. The novel *Penicillium chrysogenum* strains isolated from this plant are significant penicillin producer and RAPD tool is effective for the genetic diversity assessment of these novel strains. Further research work is in progress for the purification of penicillin and other related bioactive compounds from these novel *P. chrysogenum* strains.

SIGNIFICANCE STATEMENT

This study discovered that the *P. chrysogenum* strains were dominantly colonized in *Plectranthus amboinicus* L. plants of West Malaysia. This study will help the researchers to uncover the critical areas of plant and fungal endophyte interaction that many researchers were not able to explore. Thus, a new theory on unique endophytic interaction between *P. chrysogenum* and *Plectranthus amboinicus* L. plants may be arrived at.

ACKNOWLEDGMENT

This study has been carried out as a partial fulfillment of bachelor's degree (Bachelor of Medical Sciences, International Medical School) in Management and Science University. Therefore, authors would like to thank microbiology laboratory, Management and Science University for providing laboratory facilities.

REFERENCES

1. Svara, F. and R.J. Rank, 2011. The evolution of plasmid-carried antibiotic resistance. BMC Evol. Biol., Vol. 11. 10.1186/1471-2148-11-130.
2. Arnold, C., 2013. The new danger of synthetic drugs. Lancet, 382: 15-16.
3. Arumugam, G., M.K. Swamy and U.R. Sinniah, 2016. *Plectranthus amboinicus* (Lour.) Spreng: Botanical, phytochemical, pharmacological and nutritional significance. Molecules, Vol. 21. 10.3390/molecules21040369.
4. Shoeb, M., M. Haque and N. Nahar, 2014. Bioactive compounds from endophytic fungus *Penicillium thiomii* isolated from *Terminalia chebula* Retz. J. Nat. Prod. Plant Resour., 4: 65-70.
5. Toghueo, R.M.K. and F.F. Boyom, 2019. Endophytic fungi from *Terminalia* species: A comprehensive review. J. Fungi, Vol. 5, No. 2. 10.3390/jof5020043.
6. Maheshwari, V.L., M.P. Patil, R.H. Patil and S.G. Patil, 2014. Endophytic mycoflora of Indian medicinal plant, *Terminalia arjuna* and their biological activities. Int. J. Biotechnol. Wellness Ind., 3: 53-61.
7. Rajeswari, S., R. Revathi and A.D. Prasanth, 2017. Isolation and identification of endophytic fungi from *Terminalia chebula* of Eastern Ghats, Tamilnadu. Asian J. Pharmaceut. Clin. Res., 10: 21-23.
8. Kim, C.S., M.S. Park and S.H. Yu, 2008. Two species of endophytic *Penicillium* from *Pinus rigida* in Korea. Mycobiology, 36: 222-227.
9. Verma, V.C., S.K. Gond, A. Kumar, R.N. Kharwar and G. Strobel, 2007. The endophytic mycoflora of bark, leaf and stem tissues of *Azadirachta indica* A. Juss (neem) from Varanasi (India). Microb. Ecol., 54: 119-125.
10. Tiwari, K., 2013. Frequency distribution and assessment of genetic diversity of novel endophyte *Alternaria alternata* accessions isolated from *Pongamia pinnata* L. Pak. J. Biol. Sci., 16: 1004-1009.
11. Tiwari, K. and M. Chittora, 2013. Assessment of genetic diversity and distribution of endophytic fungal communities of *Alternaria solani* isolates associated with the dominant Karanja plants in Sanganer region of Rajasthan. SpringerPlus, Vol. 2. 10.1186/2193-1801-2-313.
12. Chen, Z.W., C.H. Ku, C.J. Weng and T.L. Chen, 1997. Effect of thickening agents on the penicillin fermentation. Applied Biochem. Biotechnol., 67: 249-258.
13. Rafi, M., 2001. Studies on comparative penicillin production potential in native isolates of *Penicillium chrysogenum*. M.Sc. Thesis, University of Agriculture, Faisalabad, Pakistan.
14. Rahman, S.U., M.H. Rasool and M. Rafi, 2012. Penicillin production by wild isolates of *Penicillium chrysogenum* in Pakistan. Braz. J. Microbiol., 43: 476-481.
15. Guo, L.D., G.R. Huang, Y. Wang, W.H. He, W.H. Zheng and K.D. Hyde, 2003. Molecular identification of white morphotype strains of endophytic fungi from *Pinus tabulaeformis*. Mycol. Res., 107: 680-688.
16. Astuti, P., S. Sudarsono, K. Nisak and G.W. Nugroho, 2014. Endophytic fungi isolated from *Coleus amboinicus* lour exhibited antimicrobial activity. Adv. Pharmaceut. Bull., 4: 599-605.
17. Gaikwad, M.N., S.S. Bodke and L.H. Kamble, 2017. Biodiversity of fungal endopyhtes from *Coleus aromaticus* (Benth.). Int. J. Bot. Stud., 2: 64-67.

18. Rajendran, L., K. Rajagopal, K. Subbarayan, K. Ulagappan, A. Sampath and G. Karthik, 2013. Efficiency of fungal taxol on human liver carcinoma cell lines. Am. J. Res. Commun., 1: 112-121.
19. Korejo, F., A.S. Ali, A.H. Shafique, V. Sultana, J. Ara and S. Ehteshamul-Haque, 2014. Antifungal and antibacterial activity of endophytic *Penicillium* species isolated from *Salvadora* species. Pak. J. Bot., 46: 2313-2318.
20. Campos, P.R.C., K.J.S. Jacob, H.C. Ramos and F.B. Temanel, 2019. Mycopharmacological properties of endophytic fungi isolated from cuban oregano (*Plectranthus amboinicus* Lour.) leaves. Asian J. Biol. Life Sci., 8: 103-110.
21. Sukrutha, S.K., Z. Adamechova, B.K. Rachana, S. Janakiraman and M. Certik, 2012. Endophytic fungi: Are they potential candidates for the production of polyunsaturated fatty acids? Int. J. Applied Microbiol. Sci., 1: 44-49.
22. Urooj, F., H. Farhat, S.A. Ali, M. Ahmed and V. Sultana *et al*., 2018. Role of endophytic *Penicillium* species in suppressing the root rotting fungi of sunflower. Pak. J. Bot., 50: 1621-1628.
23. Alves, D.R., S.M. de Morais, F. Tomiotto-Pellissier, F.R. Vasconcelos and F.D.C.O. Freire *et al*., 2018. Leishmanicidal and fungicidal activity of lipases obtained from endophytic fungi extracts. PLoS ONE, Vol. 13. 10.1371/journal.pone.0196796.
24. Houbraken, J., J.C. Frisvad, K.A. Seifert, D.P. Overy, D.M. Tuthill, J.G. Valdez and R.A. Samson, 2012. New penicillin-producing *Penicillium* species and an overview of section *Chrysogena*. Persoonia, 29: 78-100.