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Research Article

# SCREENING AND IDENTIFICATION OF POTENTIAL COELOMYCETOUS STRAIN(S) FOR THE PRODUCTION OF EXTRACELLULAR ANTIMICROBIAL METABOLITES

**SUBBAN KAMALRAJ AND JOHNPAUL MUTHUMARY***

**Center for Advanced Studies in Botany, University of Madras, Maraimalai Campus, Guindy, Chennai - 600 025, Tamil Nadu, India.**
**E-mail:** mycolkamal@gmail.com, mm_j@rediffmail.com

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**ABSTRACT**

In the search for new fungal derived antibiotics, 112 endophytic coelomycetous fungi were isolated from the inner tissue of different tropical and subtropical plants. Through preliminary and secondary screening, 25 of them were found to be able to produce broad-spectrum antimicrobial metabolites. Among the isolates five active strains were characterized for secondary metabolites such as *Pestalotiopsis mangiferae*, *Bartalinia robillardoides*, *Seimatosporium mariae*, *Truncatella angustata* and *Pestalotia circularis* which showed good antimicrobial activity in six different media. Among them, the *Pestalotiopsis mangiferae* exhibited excellent antimicrobial activity against human pathogenic bacteria and yeast. Based on the activity and morphological methods these isolates were subjected to molecular taxonomy studies.

**Keywords:** Coelomycetous fungi, antimicrobial, *Pestalotiopsis* spp., Amphisphaeria.

## INTRODUCTION

Ever since the introduction of large-scale screenings for antibiotics, natural products have been playing essential roles as active principles themselves, as lead structures for the development of drugs as tools for the characterization of new and selective targets. Although numerous antimicrobial compounds have been discovered in the course of screening efforts as well as chemical synthesis programmes, new products applicable to human therapy are rare and urgently needed, owing to a drastic increase in the number of immunocompromised patients coupled with the development of resistance among clinically relevant fungi against several of the limited number of antimycotics in current use. Recent estimates have put the worldwide number of fungal species at 1.5 million [1]. Of these, only about 10% have been discovered and described as yet and barely 1% examined for their spectrum of secondary metabolites. A major contribution to the uncharacterized fungal biodiversity is thought to be made by endophytes, i.e. fungi spending a substantial part of their life-cycle as symptomless commensals or symbionts in living plant tissues [2]. Most of them were anamorphic fungi belonging to some common endophytic genera such as *Colletotrichum*, *Pestalotiopsis, Phomopsis* and *Phoma*. The coelomycetous fungi have high potential to produce various bioactive metabolites [3-5]. The ascomycetous fungus, *Leptosphaeria maculans* Ces. et de Not. (asexual stage, *Phoma lingam* (Tode) Desm.) produced Sirodesmin PL, a phytotoxin and mycotoxin, which exhibited antibacterial activity against gram-positive bacteria particularly against *Bacillus subtilis* [6]. Terrain was a compound isolated from fungi *Phoma* by bioassay guided fractionation [7, 8]. The aim of this work was first to evaluate the biotechnological potential of 112 Indian endophytic coelomycetous species from Tamil Nadu, as source of antimicrobial compounds that could be used as lead compounds to develop new antibiotics for clinical or therapeutic use.

## MATERIALS AND METHODS

### Study area

The present study 112 coelomycetous fungi were isolated from *Rhododendron arboretum* Sm., *Taxodium distichum* Rich. and *T. mucronatum* Ten. collected from the forest area of Tamil Nadu botanical garden (2623 m above sea level) in Tamil Nadu National Park, Ooty, Nilgiri district of Tamil Nadu, South India (Lat.10° 38´ 11.49´´; Long. 76° 0´ 77.15´´) and also *Mangifera indica* Linn., collected from Botany field lab, University of Madras, Maduravoyal, Chennai, Tamil Nadu.

### Isolation of Endophytic fungi

The collected samples were washed thoroughly by running tap water. From 300 segments (approx. 0.5 $cm^2$) of each leaves, Stem and bark tissues from each plant were surface sterilized and placed on PDA medium poured petriplates for the study of fungal endophytes[9-11]. The Petri plates were incubated in a light chamber for a period of 3-4 weeks[12] at 23 ± 2°C. The light regime was 12 h light followed by 12 h darkness (24 h/day). The Petri dishes were observed at regular intervals starting from the second day onwards for the fungal growth. The emerging fungal propagules were isolated, purified and maintained by subsequent subculturing [13].

### Primary screening of antimicrobial activity

All the Coelomycetous fungi were preliminary screened for antimicrobial activity against human pathogens such as *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96), *Micrococcus luteus* (MTCC 1541), *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 424) and *Candida albicans* (MTCC 227) using plug agar method [14-17].

Discs (9 mm) from 12 days old culture of the fungal isolates maintained in Potato dextrose agar medium were picked up by using a sterile cork borer and then the agar plug with mycelia was placed on the surface of Nutrient agar medium seeded with test organisms separately. The plates were refrigerated at 5°C overnight for complete diffusion of metabolites from the agar plug, after that it was incubated for 2-5 days at 37°C. The seeded NA plates received similar volume of respective media and agar plug served as the control. The diameter of the inhibition zone was measured and the average of three replicate agar discs was taken to assess the strength of antimicrobial activity.

### Secondary screening for antimicrobial assay

Based on the preliminary screening of antimicrobial activity, among them 25 coelomycetous fungi were selected for secondary screening using agar well diffusion method [14]. The mycelial discs about 9 mm cut from the mother culture of selected coelomycetous fungi were inoculated in 100 ml saline bottle containing 20 ml PDB with 1 disc (9 mm) of inoculums separately and incubated at 23 ± 2°C under static condition (surface culture) for 25 days.

NA plates were inoculated with test organisms. The plates were evenly spread out. Then well were prepared in the plates with a cork borer. Each well was loaded with 0.1ml of each culture filtrate of 25 isolates. The well that received similar volume of respective medium served as control. The plates were incubated for 48h at 37°C. The development of inhibition zone around the well was measured and recorded [18].

### Growth and antimicrobial activity of *Pestalotiopsis* sp. MUBL1002 and other fungi on different media

Among the 25 coelomycetous fungi, five were selected for further studies such as *Pestalotiopsis* sp. MUBL1002, *Bartalinia* sp.

MUBL1096, *Seimatosporium* sp. MUBL1107, *Truncatella* sp. MUBL1109 and *Pestalotia* sp. MUBL1067. About 50 ml of PDB, PDYEB, M-1DB, YPSsB, MEB and CDB were prepared and dispensed in 250 ml Erlenmeyer flask and sterilized at 121°C for 15 min at 15 psi. Mycelial discs (9 mm) were cut from the mother culture of five coelomycetous fungi were inoculated on the respective broth. The flasks were incubated under static condition at 23 ± 2°C for 25 days. After incubation the growth of fungi was measured by means of mycelial dry weight.

The culture filtrates were collected by passing through double layer of cheese cloth in a glass funnel and about 19-26 ml of culture filtrate were collected from each strain. The NA plates were swabbed separately and activity tested following methods [18].

**Extraction of antimicrobial metabolites**

Based on the growth and antimicrobial activity on different medium, *Pestalotiopsis* sp. MUBL1002 was selected for extraction of secondary metabolites using ethyl acetate. Double volume of ethylacetate was added to culture filtrate (liquid-liquid) and kept in shaking condition for 12 h at 25 ± 2°C. The organic layer was then separated by using separating funnel and was evaporated under reduced pressure at 50°C by rotary evaporator. All the four crude extract of different solvent was introduced to antimicrobial activity against test pathogens at 50 µg /well dissolved in 10% DMSO.

**TLC and bio-autography**

The *Pestalotiopsis* sp. MUBL1002 of PDB culture filtrate of ethyl acetate crude extract was detected using TLC bio-autography overlay assay [19] against *C. albicans*.

**Isolation, PCR optimization and phylogenetic analysis of fungal genomic DNA**

The *Pestalotiopsis* sp. MUBL1002, *Bartalinia* sp. MUBL1096, *Seimatosporium* sp. MUBL1107, *Truncatella* sp. MUBL1109 and *Pestalotia* sp. MUBL1067 were grown on potato-dextrose agar (PDA) for 10-20 days[19, 20] and total genomic DNA was extracted from the mycelium using a modification of the protocol described by Möller *et al*. [21]. The commercially available Polymerase chain reaction (PCR) ready mix, primers such as ITS 1 (5'-TCC GTA GGT GAA CCT GCC G-3') and ITS 4 (5'- TCC TCC GCT TAT TGA TAT GC-3') used in this study were purchased from Chromous Biotech. Pvt. Ltd. Bangalore, India[21]. PCR was performed using standard procedures according to the manufacturer's protocol were used to amplify part of the nuclear rDNA spanning the 3'end of the 18S rDNA, the internal transcribed spacers, the 5.8S rDNA and a part of the 5' end of the 28S rDNA. The primers LR0R and LR7 were used to amplify part of the large subunit nuclear rDNA [22]. The DNA was then purified and amplified products were purified using a modified PEG method [23]. The sequence data of ITS region was aligned using multalign online programme [24].

**Statistical analysis**

The data were subjected to One-way Analysis of Variance (ANOVA) to determine the significance of individual differences at $p < 0.05$ level. Significant means were compared by the Duncan's multiple range test. All statistical analyses were carried out using SPSS statistical software package (SPSS, Version 10.0, Chicago, USA).

**RESULTS**

**Coelomycetous fungi**

The present study reported that 2,644 endophytic isolates was recorded; only 112 different morphospecies of coelomycetes were isolated and identified from 14,400 segments of healthy tissues such as leaves (4,800), stem (4,800) and bark (4,800) were isolated from *Rhododendron arboretum*, *Taxodium distichum*, *Mangifera indica* and *T. mucronatum*.

**Preliminary screening of coelomycetes for antimicrobial activity**

The isolated coelomycetes were screened by plug agar diffusion assay against human pathogens such as *B. subtilis*, *S. aureus*, *M. luteus*, *E. coli*, *P. aeruginosa, K. pneumoniae* and *C. albicans*. The isolates such as MUBL1037, MUBL1053, MUBL1052, MUBL1111 and MUBL1108 did not show significant antimicrobial activity against human pathogens. However, the rest of 107 coelomycetous fungi exhibited remarkable antimicrobial activity against human pathogens. A total of 25 isolates exhibited moderate antimicrobial activity against 7 tested pathogens. Among the 25 isolates, 5 isolates showed good antimicrobial activity against tested pathogens compared to others such as *Pestalotiopsis* sp. MUBL1002 and other genera like *Bartalinia* sp. MUBL1096, *Seimatosporium* sp. MUBL1107, *Truncatella* sp. MUBL1109 and *Pestalotia* sp. MUBL1067 showed significant antimicrobial activity (Fig. 1). Interestingly, *Pestalotiopsis* sp. MUBL1002 showed excellent antimicrobial activity against *B. subtilis* (14 ± 0.26 mm); *M. luteus* (16 ± 0.32 mm); *E. coli* (20 ± 0.40 mm); *P. aeruginosa* (10 ± 0.20 mm); *K. pneumoniae* (11 ± 0.22 mm) and *C. albicans* (13 ± 0.26 mm) (Table 1).

**Table 1: Preliminary screening of coelomycetes for antimicrobial activity against human pathogens**

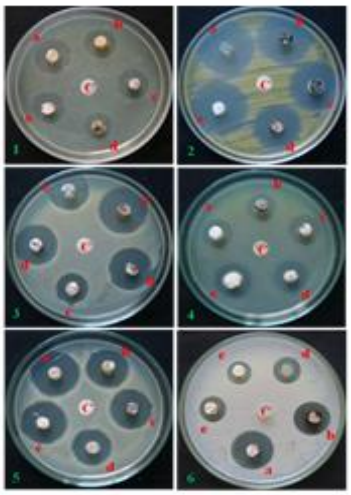
| S. No | ISOLATED STRAINS | ZONE OF INHIBITION (MM) | | | | | |
|---|---|---|---|---|---|---|---|
| | | *B. subtilis* | *E. coli* | *M. luteus* | *P. aeruginosa* | *K. pneumonia* | *C. albicans* |
| **1** | MUBL1000 | – | 11±0.22$^{j}$ | 11±0.22$^{k}$ | – | 11±0.22$^{i}$ | 11±0.22$^{f}$ |
| **2** | MUBL1001 | 13±0.26$^{i}$ | 11±0.22$^{j}$ | 12±0.24$^{j}$ | 12±0.24$^{g}$ | – | – |
| **3** | MUBL1002* | 14±0.26$^{h}$ | 20±0.40$^{b}$ | 16±0.32$^{f}$ | 10±0.20$^{i}$ | 11±0.22$^{i}$ | 13±0.26$^{d}$ |
| **4** | MUBL1003 | 14±0.28$^{h}$ | 15±0.30$^{f}$ | 11±0.22$^{k}$ | 10±0.20$^{i}$ | – | – |
| **5** | MUBL1004* | 10±0.20$^{l}$ | 12±0.24$^{i}$ | 13±0.26$^{i}$ | – | 12±0.24$^{h}$ | 15±0.30$^{c}$ |
| **6** | MUBL1005* | 15±0.30$^{g}$ | 17±0.34$^{d}$ | 13±0.26$^{i}$ | – | 10±0.20$^{j}$ | 12±0.24$^{e}$ |
| **7** | MUBL1006* | 15±0.30$^{g}$ | 13±0.26$^{h}$ | 15±0.30$^{g}$ | – | 10±0.20$^{j}$ | 10±0.20$^{g}$ |
| **8** | MUBL1007* | 14±0.28$^{h}$ | 15±0.30$^{f}$ | 12±0.20$^{j}$ | – | 11±0.22$^{i}$ | 10±0.20$^{g}$ |
| **9** | MUBL1008* | 16±0.32$^{f}$ | – | 15±0.30$^{g}$ | 11±0.22$^{h}$ | 10±0.20$^{j}$ | 10±0.20$^{g}$ |
| **10** | MUBL1009 | 13±0.26$^{i}$ | – | 10±0.20$^{f}$ | 10±0.20$^{i}$ | – | – |
| **11** | MUBL1010 | 11±0.22$^{k}$ | – | 10±0.20$^{j}$ | 11±0.22$^{h}$ | – | 21±0.42$^{a}$ |
| **12** | MUBL1011 | 13±0.26$^{i}$ | – | 16±0.32$^{f}$ | – | 15±0.30$^{e}$ | 10±0.20$^{g}$ |
| **13** | MUBL1012* | 14±0.28$^{h}$ | 13±0.26$^{h}$ | 12±0.24$^{j}$ | 11±0.22$^{h}$ | – | 10±0.20$^{g}$ |
| **14** | MUBL1013* | – | 10±0.20$^{k}$ | 11±0.22$^{k}$ | 11±0.22$^{h}$ | 10±0.20$^{j}$ | 10±0.20$^{g}$ |
| **15** | MUBL1014 | 12±0.24$^{j}$ | 11±0.22$^{j}$ | 10±0.20$^{l}$ | – | 10±0.20$^{j}$ | 13±0.26$^{d}$ |
| **16** | MUBL1015 | 10±0.20$^{l}$ | 11±0.22$^{j}$ | 14±0.28$^{h}$ | – | – | 11±0.22$^{f}$ |
| **17** | MUBL1016 | – | – | 11±0.22$^{k}$ | – | – | 11±0.22$^{f}$ |
| **18** | MUBL1017 | 10±0.20$^{l}$ | 12±0.24$^{i}$ | 15±0.28$^{g}$ | – | 10±0.20$^{j}$ | 13±0.26$^{d}$ |
| **19** | MUBL1018 | 10±0.20$^{l}$ | 10±0.20$^{k}$ | – | – | 10±0.20$^{j}$ | 10±0.20$^{g}$ |
| **20** | MUBL1019* | 12±0.24$^{j}$ | 14±0.28$^{g}$ | – | 10±0.20$^{i}$ | 11±0.22$^{i}$ | 12±0.24$^{e}$ |
| **21** | MUBL1020* | 12±0.24$^{j}$ | 10±0.20$^{k}$ | – | 10±0.20$^{i}$ | 10±0.20$^{j}$ | 12±0.24$^{e}$ |
| **22** | MUBL1021* | 13±0.26$^{i}$ | 10±0.20$^{k}$ | – | 11±0.22$^{h}$ | – | 13±0.26$^{d}$ |
| **23** | MUBL1022 | – | 11±0.22$^{j}$ | – | 10±0.20$^{i}$ | – | – |
| **24** | MUBL1023 | – | 12±0.24$^{i}$ | – | – | – | – |
| **25** | MUBL1024* | 14±0.28$^{h}$ | 13±0.26$^{h}$ | 12±0.24$^{j}$ | 10±0.20$^{i}$ | 19±0.38$^{a}$ | – |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **26** | MUBL1025 | 13±0.26$^{i}$ | 11±0.22$^{j}$ | – | – | 16±0.32$^{d}$ | – |
| **27** | MUBL1026* | 11±0.22$^{k}$ | 14±0.28$^{g}$ | 17±0.34$^{e}$ | – | 19±0.38$^{a}$ | 10±0.20$^{g}$ |
| **28** | MUBL1027 | – | 11±0.22$^{h}$ | – | – | – | 11±0.22$^{f}$ |
| **29** | MUBL1028 | 10±0.20$^{l}$ | – | 10±0.20$^{l}$ | – | 10±0.20$^{j}$ | 11±0.22$^{f}$ |
| **30** | MUBL1029 | 10±0.20$^{l}$ | – | – | – | 19±0.38$^{a}$ | 12±0.24$^{e}$ |
| **31** | MUBL1030 | – | – | – | – | 11±0.22$^{i}$ | 12±0.24$^{e}$ |
| **32** | MUBL1031 | 10±0.20$^{l}$ | 11±0.22$^{j}$ | – | 13±0.26$^{f}$ | – | 10±0.20$^{g}$ |
| **33** | MUBL1032 | 10±0.20$^{l}$ | 11±0.22$^{j}$ | – | 14±0.28$^{e}$ | – | – |
| **34** | MUBL1033 | 10±0.20$^{l}$ | 12±0.24$^{i}$ | – | – | 14±0.28$^{f}$ | – |
| **35** | MUBL1034 | 12±0.24$^{j}$ | – | 10±0.20$^{l}$ | – | 15±0.30$^{e}$ | 12±0.24$^{e}$ |
| **36** | MUBL1035 | – | – | – | 13±0.26$^{f}$ | 13±0.26$^{g}$ | 12±0.24$^{e}$ |
| **37** | MUBL1036 | – | – | – | – | 18±0.36$^{b}$ | 12±0.24$^{e}$ |
| **38** | MUBL1037 | – | – | – | – | – | – |
| **39** | MUBL1038 | 16±0.32$^{f}$ | – | – | – | – | 11±0.22$^{f}$ |
| **40** | MUBL1039 | 10±0.20$^{l}$ | 12±0.24$^{i}$ | – | – | 11±0.22$^{i}$ | 13±0.26$^{d}$ |
| **41** | MUBL1040 | 20±0.40$^{b}$ | – | – | – | 10±0.20$^{j}$ | 10±0.20$^{g}$ |
| **42** | MUBL1041 | 18±0.36$^{d}$ | – | – | – | 12±0.24$^{h}$ | 12±0.24$^{e}$ |
| **43** | MUBL1042 | 14±0.28$^{h}$ | – | – | – | 11±0.22$^{i}$ | 15±0.30$^{c}$ |
| **44** | MUBL1043 | 12±0.24$^{j}$ | – | – | – | – | 11±0.22$^{f}$ |
| **45** | MUBL1044 | 13±0.26$^{i}$ | 24±0.48$^{a}$ | – | – | 14±0.28$^{f}$ | 11±0.22$^{f}$ |
| **46** | MUBL1045 | 16±0.32$^{f}$ | – | – | – | 16±0.32$^{d}$ | 13±0.26$^{d}$ |
| **47** | MUBL1046 | 15±0.81$^{g}$ | 11±0.22$^{j}$ | – | – | 13±0.77$^{g}$ | 11±0.22$^{f}$ |
| **48** | MUBL1047 | 14±0.28$^{h}$ | 16±0.72$^{e}$ | – | – | 17±0.34$^{c}$ | 10±0.20$^{g}$ |
| **49** | MUBL1048 | 20±0.40$^{b}$ | – | – | – | 12±0.24$^{h}$ | 12±0.24$^{c}$ |
| **50** | MUBL1049 | 10±0.20$^{l}$ | – | – | – | 16±0.83$^{d}$ | 13±0.26$^{d}$ |
| **51** | MUBL1050 | 19±0.38$^{c}$ | 15±0.30$^{f}$ | – | – | 11±0.22$^{i}$ | – |
| **52** | MUBL1051 | 16±0.32$^{f}$ | – | – | – | 13±0.26$^{g}$ | – |
| **53** | MUBL1052 | – | – | – | – | – | 15±0.30$^{c}$ |
| **54** | MUBL1053 | – | – | – | – | – | 10±0.20$^{g}$ |
| **55** | MUBL1054 | – | 12±0.24$^{i}$ | – | – | – | 12±0.24$^{e}$ |
| **56** | MUBL1055 | – | – | – | – | – | 10±0.20$^{g}$ |
| **57** | MUBL1056 | – | – | – | – | – | 12±0.24$^{e}$ |
| **58** | MUBL1057 | – | – | – | – | – | 12±0.24$^{e}$ |
| **59** | MUBL1058 | – | – | – | – | – | 10±0.20$^{g}$ |
| **60** | MUBL1059 | – | 24±0.48$^{a}$ | – | – | – | 11±0.22$^{f}$ |
| **61** | MUBL1060 | – | – | – | – | – | 12±0.75$^{e}$ |
| **62** | MUBL1061 | – | 11±0.73$^{j}$ | – | – | – | 11±0.73$^{f}$ |
| **63** | MUBL1062 | – | 16±0.32$^{e}$ | – | – | – | 10±0.20$^{g}$ |
| **64** | MUBL1063 | – | – | 10±0.20$^{l}$ | – | – | – |
| **65** | MUBL1064 | – | 10±0.20$^{k}$ | 10±0.20$^{l}$ | 21±0.52$^{b}$ | 11±0.22$^{i}$ | – |
| **66** | MUBL1065* | – | 14±0.28$^{g}$ | 14±0.28$^{h}$ | 10±0.20$^{i}$ | 12±0.24$^{h}$ | 13±0.26$^{d}$ |
| **67** | MUBL1066* | – | 10±0.20$^{k}$ | 19±0.38$^{d}$ | 12±0.24$^{g}$ | 10±0.20$^{j}$ | 10±0.20$^{g}$ |
| **68** | MUBL1067* | 16±0.32$^{f}$ | 19±0.38$^{c}$ | 20±0.40$^{c}$ | 25±0.50$^{a}$ | 10±0.20$^{j}$ | 12±0.24$^{e}$ |
| **69** | MUBL1068 | – | 10±0.20$^{k}$ | 10±0.20$^{l}$ | – | – | 12±0.24$^{e}$ |
| **70** | MUBL1069 | 17±0.34$^{e}$ | 10±0.20$^{k}$ | – | – | – | – |
| **71** | MUBL1070 | – | 14±0.28$^{g}$ | – | – | – | – |
| **72** | MUBL1071 | – | 17±0.34$^{d}$ | – | 15±0.30$^{d}$ | – | 10±0.20$^{g}$ |
| **73** | MUBL1072 | – | 14±0.28$^{g}$ | – | 13±0.26$^{f}$ | – | 11±0.22$^{f}$ |
| **74** | MUBL1073 | – | 12±0.24$^{i}$ | 19±0.38$^{d}$ | – | – | – |
| **75** | MUBL1074 | – | 10±0.20$^{k}$ | 17±0.34$^{e}$ | 11±0.22$^{h}$ | – | 11±0.22$^{f}$ |
| **76** | MUBL1075* | 10±0.20$^{l}$ | 14±0.28$^{g}$ | 10±0.20$^{l}$ | – | 14±0.28$^{f}$ | 12±0.24$^{e}$ |
| **77** | MUBL1076 | 13±0.26$^{i}$ | 12±0.24$^{i}$ | – | – | 11±0.22$^{i}$ | 11±0.22$^{f}$ |
| **78** | MUBL1077 | – | 11±0.22$^{j}$ | 10±0.20$^{l}$ | – | 11±0.22$^{i}$ | 16±0.32$^{b}$ |
| **79** | MUBL1078 | 10±0.20$^{l}$ | 13±0.26$^{h}$ | – | – | 14±0.28$^{f}$ | 11±0.22$^{f}$ |
| **80** | MUBL1079 | 13±0.26$^{i}$ | 15±0.30$^{f}$ | – | – | 15±0.30$^{e}$ | 11±0.22$^{f}$ |
| **81** | MUBL1080* | 12±0.24$^{j}$ | 12±0.24$^{i}$ | 16±0.32$^{f}$ | – | 12±0.24$^{h}$ | 10±0.20$^{g}$ |
| **82** | MUBL1081 | 13±0.26$^{i}$ | 11±0.22$^{j}$ | – | – | – | 12±0.24$^{e}$ |
| **83** | MUBL1082 | – | 12±0.24$^{i}$ | 26±0.52$^{a}$ | – | – | – |
| **84** | MUBL1083 | – | – | 12±0.24$^{j}$ | – | 14±0.28$^{f}$ | 10±0.20$^{g}$ |
| **85** | MUBL1084 | 10±0.20$^{l}$ | 13±0.26$^{h}$ | – | – | 10±0.20$^{j}$ | 11±0.22$^{f}$ |
| **86** | MUBL1085* | 21±0.42$^{a}$ | 11±0.22$^{j}$ | 13±0.26$^{i}$ | – | 17±0.34$^{c}$ | 13±0.26$^{d}$ |
| **87** | MUBL1086 | 15±0.30$^{g}$ | – | 16±0.32$^{f}$ | 15±0.30$^{d}$ | – | 12±0.24$^{e}$ |
| **88** | MUBL1087 | 11±0.22$^{k}$ | – | 15±0.28$^{g}$ | – | – | 11±0.22$^{f}$ |
| **89** | MUBL1088 | 12±0.24$^{j}$ | – | – | – | – | 11±0.22$^{f}$ |
| **90** | MUBL1089 | 11±0.22$^{k}$ | 14±0.28$^{g}$ | 10±0.20$^{l}$ | – | – | 10±0.20$^{g}$ |
| **91** | MUBL1090 | – | – | – | – | 10±0.20$^{j}$ | 11±0.22$^{f}$ |
| **92** | MUBL1091* | 12±0.24$^{j}$ | 16±0.32$^{e}$ | – | 10±0.20$^{i}$ | 15±0.30$^{e}$ | 11±0.22$^{f}$ |
| **93** | MUBL1092 | – | – | – | 15±0.30$^{d}$ | 11±0.22$^{i}$ | – |
| **94** | MUBL1093* | 11±0.22$^{k}$ | – | 20±0.40$^{c}$ | 11±0.22$^{h}$ | 13±0.26$^{g}$ | 11±0.22$^{f}$ |
| **95** | MUBL1094 | – | 11±0.22$^{j}$ | – | – | – | – |
| **96** | MUBL1095 | – | – | – | 16±0.30$^{c}$ | – | – |
| **97** | MUBL1096* | 17±0.34$^{e}$ | 13±0.26$^{h}$ | 16±0.32$^{f}$ | 13±0.26$^{e}$ | 14±0.28$^{f}$ | 15±0.30$^{c}$ |
| **98** | MUBL1097 | 11±0.22$^{k}$ | 10±0.20$^{k}$ | – | 12±0.24$^{g}$ | – | 12±0.24$^{e}$ |
| **99** | MUBL1098 | – | 10±0.20$^{k}$ | 10±0.20$^{l}$ | – | – | – |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **100** | MUBL1099 | – | – | – | – | 11±0.22$^{i}$ | – |
| **101** | MUBL1100 | – | – | – | – | – | – |
| **102** | MUBL1101* | – | 11±0.22$^{j}$ | 10±0.20$^{l}$ | 11±0.22$^{h}$ | 11±0.22$^{i}$ | 10±0.20$^{g}$ |
| **103** | MUBL1102 | – | 11±0.22$^{j}$ | 11±0.22$^{j}$ | – | 10±0.20$^{j}$ | – |
| **104** | MUBL1103 | – | – | 13±0.26$^{i}$ | – | – | 11±0.22$^{f}$ |
| **105** | MUBL1104 | – | 12±0.24$^{i}$ | – | – | – | – |
| **106** | MUBL1105 | – | 14±0.28$^{g}$ | 11±0.22$^{k}$ | – | – | – |
| **107** | MUBL1106 | – | – | – | – | – | – |
| **108** | MUBL1107* | 14±0.28$^{h}$ | 15±0.30$^{f}$ | 21±0.42$^{b}$ | 14±0.28$^{e}$ | 13±0.26$^{g}$ | 15±0.30$^{c}$ |
| **109** | MUBL1108 | – | – | – | – | – | – |
| **110** | MUBL1109* | 16±0.32$^{f}$ | 14±0.28$^{g}$ | 21±0.42$^{b}$ | 13±0.26$^{f}$ | 16±0.32$^{d}$ | 12±0.24$^{e}$ |
| **111** | MUBL1110 | – | – | 10±0.20$^{l}$ | 11±0.22$^{h}$ | 11±0.22$^{i}$ | 10±0.20$^{g}$ |
| **112** | MUBL1111 | – | – | – | – | – | – |

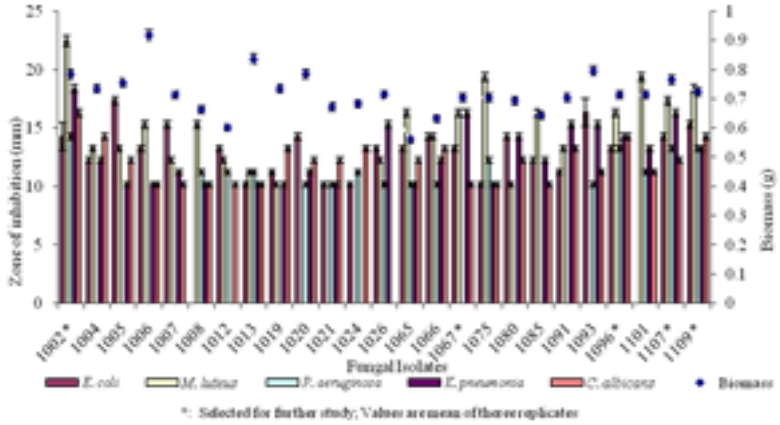
***Isolates subjected for secondary screening**
**- No activity**

**Figure 1.** Screening of coelomycetous fungi for antimicrobial activity using plug agar method against human pathogens



1. *E.coli* ; 2. *M. luteus*; 3. *S. aureus*; 4. *P. aeruginosa*; 5. *K. pneumoniae*; 6. *C. albicans*
a. *Pestalotiopsis sp.* MUBL1002; b. *Bartalinia sp.* MUBL1096; c. *Truncatella sp.* MUBL1109;
d. *Seimatosporium sp.* MUBL1107; e. *Pestalotia sp.* MUBL1087; C. Control

**Figure 2.** The antimicrobial activities and biomass of the fungal isolates against five test microorganisms in the secondary assay




*: Selected for further study; Values are means of three replicates

**Secondary screening of coelomycetes for antimicrobial assay**

The culture filtrates of selected 25 fungi were used for bioassay against human pathogens by agar well diffusion assay. *Pestalotiopsis* sp. MUBL1002, *Bartalinia* sp. MUBL1096, *Seimatosporium* sp. MUBL1107, *Truncatella* sp. MUBL1109 and *Pestalotia* sp. MUBL1067 showed strong antimicrobial activity against the test pathogens. Of these, *Pestalotiopsis* MUBL1002 showed highest antimicrobial activity against *C. albicans* (16 ± 0.2 mm); *E. coli* (16 ± 0.62 mm); *M. luteus* (22 ± 0.6 mm); *P. aeruginosa* (14 ± 0.54 mm) and *K. pneumoniae* (18 ± 0.44 mm) in PDB, respectively. However, the remaining four isolates MUBL1096, MUBL1107, MUBL1109 and MUBL1067 showed significant antimicrobial activity against the tested human pathogens (Fig. 2).

**Growth and antimicrobial activity of *Pestalotiopsis* sp. MUBL1002 and other genera on different media**

Generally, PDB medium supported the maximum growth and production of antimicrobial substances in *Pestalotiopsis* and other genera followed by PDYEB, M-1DB, YESsB, MEB and CDB. However, PDB medium supported growth and antimicrobial activity in all the isolated cultures. Among them, *Pestalotiopsis* sp. MUBL1002 showed good growth and antimicrobial activity followed by *Bartalinia* sp. MUBL1096, *Seimatosporium* sp. MUBL1107 and *Truncatella* sp. MUBL1109 and *Pestalotia* sp. MUBL1067 and remaining strains moderately support the growth in synthetic media such as M-1DB, YPSs, MEB and CDB. It was observed that *Pestalotiopsis* sp. MUBL1002 showed highest growth and antimicrobial activity against *C. albicans* (11 ± 0.38 mm), *E. coli* (25 ± 0.5 mm), *M. luteus* (26 ± 0.52 mm), *P. aeruginosa* (11 ± 0.22 mm) and *K. pneumoniae* (17 ± 0.34 mm) in PDYEB followed by M-1DB showing antimicrobial activity against *C. albicans* (21 ± 0.32 mm), *E. coli* (16 ± 0.32 mm), *M. luteus* (32 ± 0.64 mm), *P. aeruginosa* (24 ± 0.48 mm) and *K. pneumoniae* (21 ± 0.42 mm). Rest of the three media (YPSsB, MEB and CDB) moderately supported the growth compared to PDB, PDYEB and M-1DB. However, *Bartalinia* sp. MUBL1096, *Seimatosporium* sp. MUBL1107, *Truncatella* sp. MUBL1109 and *Pestalotia* sp. MUBL1067 showed minimum activity against human pathogens. Among the six media, PDB supported highest growth and metabolites production in *Pestalotiopsis* sp. MUBL1002 followed by *Bartalinia* sp. MUBL1096, *Seimatosporium* sp. MUBL1107, *Truncatella* sp. MUBL1109 and *Pestalotia* sp. MUBL1067 (Table 2).

**Table 2: Growth and antimicrobial activity of *Pestalotiopsis* sp. MUBL1002 and other genera on different liquid media against human pathogens**

| ***Pestalotiopsis* sp. MUBL1002** | | | | | | |
|---|---|---|---|---|---|---|
| **Media** | **Biomass** | ***C. albicans*** | ***E. coli*** | ***M. luteus*** | ***P. aeruginosa*** | ***K. pneumoniae*** |
| **PDYEB** | 1.13±0.02[a] | 11±0.38[d] | 25±0.50[a] | 26±0.52[b] | 11±0.22[b] | 17±0.34[c] |
| **M1DB** | 0.99±0.19[b] | 21±0.42[a] | 16±0.32[c] | 25±0.64[c] | 11±0.48[b] | 15±0.34[d] |
| **YPSsB** | 0.63±0.02[e] | 22±0.44[b] | – | 34±0.68[a] | 25±0.50[a] | 24±0.48[a] |
| **MEB** | 0.77±0.05[c] | 18±0.36[c] | – | 22±0.44[d] | 11±0.22[b] | 18±0.36[b] |
| **CDB** | 0.71±0.01[d] | 10±0.20[e] | 20±0.40[b] | 18±0.36[e] | – | – |
| ***Bartalinia* sp. MUBL1096** | | | | | | |
| **Media** | **Biomass** | ***C. albicans*** | ***E. coli*** | ***M. luteus*** | ***P. aeruginosa*** | ***K. pneumoniae*** |
| **PDYEB** | 0.87±0.07[a] | 22±0.44[a] | 24±0.48[b] | – | – | – |
| **M1DB** | 0.82±0.06[b] | 16±0.32[c] | 25±0.50[a] | 27±0.54[a] | 16±0.32[a] | 13±0.26[a] |
| **YPSsB** | 0.80±0.05[c] | 12±0.24[d] | – | 21±0.42[b] | 11±0.22[b] | 10±0.26[b] |
| **MEB** | 0.70±0.03[d] | 17±0.34[b] | – | – | – | – |
| **CDB** | 0.63±0.02[e] | 10±0.20[e] | – | 17±0.34c | 11±0.22[b] | – |
| ***Seimatosporium* sp. MUBL1107** | | | | | | |
| **Media** | **Biomass** | ***C. albicans*** | ***E. coli*** | ***M. luteus*** | ***P. aeruginosa*** | ***K. pneumoniae*** |
| **PDYEB** | 0.90±0.07[a] | 14±0.28[e] | – | 33±0.66[a] | 22±0.44[a] | 21±0.42[a] |
| **M1DB** | 0.80±0.05[b] | 20±0.40[a] | – | – | – | – |
| **YPSsB** | 0.72±0.04[c] | 17±0.34[b] | – | – | – | – |
| **MEB** | 0.71±0.01[d] | 15±0.30[d] | – | 12±0.24[c] | – | – |
| **CDB** | 0.69±0.03[e] | 16±0.32[c] | – | 24±0.48[b] | 13±0.26[b] | 19±0.38[b] |
| ***Truncatella* sp. MUBL1109** | | | | | | |
| **Media** | **Biomass** | ***C. albicans*** | ***E. coli*** | ***M. luteus*** | ***P. aeruginosa*** | ***K. pneumoniae*** |
| **PDYEB** | 0.91±0.01[a] | 10±0.20[d] | 24±0.48[a] | 17±0.34[b] | 12±0.24[a] | 16±0.32[a] |
| **M1DB** | 0.81±0.06[b] | 19±0.38[a] | 12±0.24[b] | 19±0.38[a] | 10±0.20[c] | 14±0.28[b] |
| **YPSsB** | 0.77±0.05[c] | 12±0.24[c] | – | – | – | – |
| **MEB** | 0.61±0.01[d] | 14±0.28[b] | – | – | – | – |
| **CDB** | 0.61±0.01[d] | 19±0.38[a] | – | 19±0.33[a] | 11±0.22[b] | – |
| ***Pestalotia* sp. MUBL1067** | | | | | | |
| **Media** | **Biomass** | ***C. albicans*** | ***E. coli*** | ***M. luteus*** | ***P. aeruginosa*** | ***K. pneumoniae*** |
| **PDYEB** | 0.86±0.06[a] | 11±0.22[d] | – | 24±0.48[a] | – | 15±0.30[a] |
| **M1DB** | 0.93±0.08[b] | 18±0.36[c] | 14±0.28[a] | 23±0.46[b] | 10±0.20[b] | 13±0.26[b] |
| **YPSsB** | 0.83±0.06[c] | 19±0.38[b] | – | 23±0.46[b] | 13±0.26[a] | 13±0.26[b] |
| **MEB** | 0.70±0.03[d] | 10±0.20[e] | – | – | – | – |
| **CDB** | 0.66±0.06[e] | 22±0.44[a] | – | 10±0.20[c] | – | – |

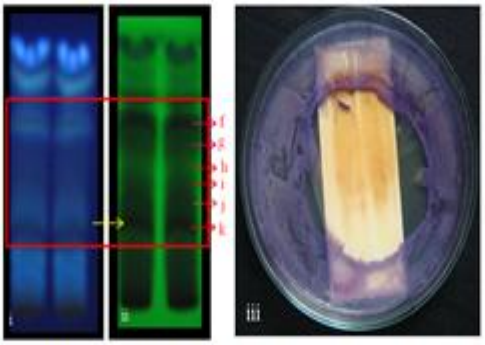
**- No activity ;**

**PDYEB-Potato Dextrose Yeast Extract Broth; M1DB-Modified medium-1 Broth; YPSsB-Emerson's Yeast Phosphate Soluble starch Broth; MEB-Malt Extract Broth; CDB-Czapex-Dox- Broth**

**TLC Bio-autography**

50 µl of crude ethyl acetate sample was used for bioautography assay. It is a very sensitive assay and gives accurate localization of active compounds. This technique was used to track the active compound in crude extract which exhibited $R_f$ values 0.78, 0.70, 0.69, 0.63, 0.62 and 0.57 with good inhibition zone around the mentioned spots. 6 bands with active molecules showed good antimicrobial activity and hence, taken for further study (Fig. 3).

**Figure 3.** Detection of active principle using TLC bioautographic overlay assay from ethyl acetate extract of *Pestalotiopsis* sp. MUBL1002



i- UV (254 nm), ii-UV (365 nm) iii-Bioautographic overlay assay of *C. albicans*

**Sequence arrangements of purified PCR product**

The purified PCR product was analyzed for sequence arrangement with the primers ITS1 and ITS 4 in forward and reverse direction. The sequence results showed that the purified PCR product was 526, 527, 565, 569, 534, 582 and 527 bp respectively in *Pestalotiopsis mangiferae* (MUBL1002), *Pestalotia circularis* (MUBL1067), *Bartalinia robillardoides* (MUBL1096), *Seimatosporium mariae* (MUBL1107) and *Truncatella angustata* (MUBL1109). The sequence data obtained in the present study was deposited in the GenBank database (Maryland, USA) with the accession number HM802301, HM802303, HM802304, HM802306 and HM802307.

The aligned sequences were again scrutinized for evolutionary relationships using the software MEGA version 4. The analyses were performed by obtaining closely related taxa of *Pestalotiopsis* spp. and other genera collected from Genbank database. In order to reveal the conserved regions in the ITS sequence of *Pestalotiopsis* spp. and other genera, and an out group of *Amanita muscaria* AF438561.2 were acquired from database and subjected to multiple sequence alignment. Even though the isolates *C. circularis* was misrepresented by NCBI, it exists within *Pestalotiopsis* spp. and other genera. *C. circularis* exhibits 100% similarity of *S. mariae.*

**Evolutionary trees**

The data was analyzed using Maximum Parsimony method and tree building method. Complete deletion and pairwise deletion was investigated by Close Neighborhood Interchange (CNI) method. The consensus trees produced by both models had the same topology but differed in the statistical support of internal branches. The strict consensus trees derived using pairwise deletion provides better statistical support than complete deletion. Therefore, pairwise deletion phylogenies are presented in this investigation. The evolutionary trees created from combined data set of ITS I and ITS II are shown in Figure 6. Interestingly, clustering different species of *Pestalotiopsis* spp. and other genera were observed in 7% and 18% of bootstrap value in phylogram. The result of the present investigation genetically supports the isolated strains as *P. mangiferae* (Fig. 4 and 5).

**Figure 4.** Cladogram strongly supports with classical taxonomical hypothesis

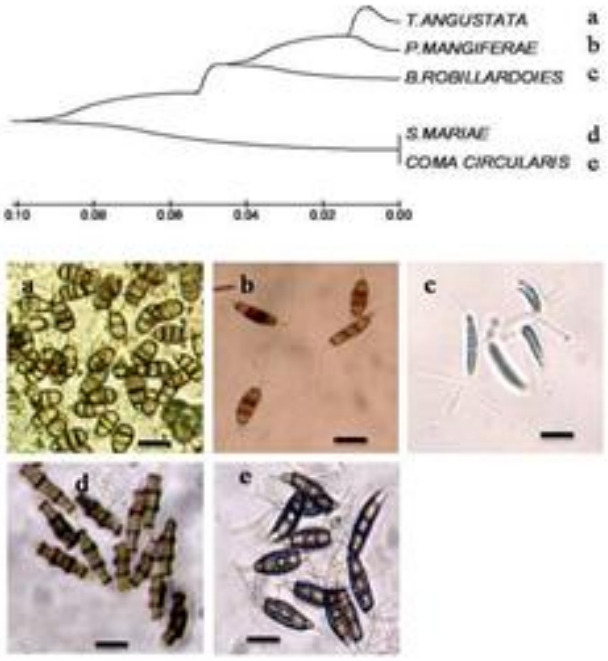


**Figure 5.** Maximum parsimony phylogenetic dendrogram of *Pestalotiopsis* spp. and other genera

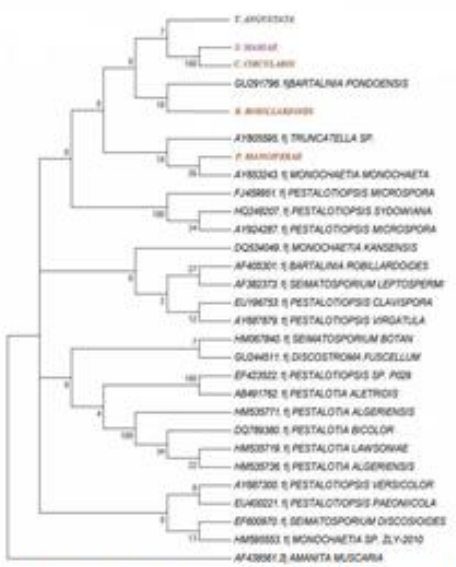



**Figure 5.** Maximum parsimony phylogenetic dendrogram of *Pestalotiopsis* spp. and other genera with species inferred from nucleotide sequences of the ITS I and ITS II. Out group for the analysis was *A. muscaria* AF438561.2 Numerial values on branches are the bootsrap values as percentage bootstrap replication from 100 replicate analysis with 20 random addition sequences

**DISCUSSION**

During the past two decades, over 100 endophytic microorganisms have been cultured and subjected to detailed investigations leading to the chemical characterization and biological evaluation of a large number of natural products. Many of which have been shown to have novel structures and interesting biological activities [25]. Thus the results were discussed with screening the fungi for antimicrobial activity from 112 isolates against human pathogens. The results of the present study clearly revealed that an intensive search for newer and more effective agents must be carried out to detain potentially useful bioactive compounds from fungi.

*Pestalotiopsis* species have been reported to secrete immunosuppressive compounds in culture broth [26] and 0.01ml culture filtrate of ME broth were pipetted into the well (7 mm) [27] for antimicrobial activity. In the present work, 0.1 ml culture filtrate of 25 isolates grown in PDB broth were pipetted into the well (9 mm) exhibited strong antimicrobial activity only for 5 isolates such as *Pestalotiopsis* sp. MUBL1002, *Bartalinia* sp. MUBL1096, *Seimatosporium* sp. MUBL1107, *Truncatella* sp. MUBL1109 and *Pestalotia* sp. MUBL1067 against the tested human pathogens. Moreover, among the six media, PDB supported highest growth and metabolites production in *Pestalotiopsis* sp. MUBL1002 followed by other four isolates.

The metabolites produced by the strain *Pestalotiopsis* sp. MUBL1002 were separated and extracted using organic solvent extraction procedure and purified by thin layer chromatography (TLC) methods as described by Yao *et al*. [28]. The culture filtrate of *Penicillium* sp. and *Streptomyces* sp. were harvested after 72 h, the filtered broths were extracted with EtOAc [29-31] crude extract. The metabolite profile of the ethyl acetate extract was carried out by using TLC. TLC is one of the simplest methods for detection of metabolite constituents [32, 33]. In the present study, the mobile phase for ethyl acetate crude extract on TLC using chloroform: ethyl acetate: methanol at 6:4.05:0.5 (v/v/v) ratio exhibited 14 visible spots under UV with 254 and 365 nm.

This statement was strongly supported by the findings of the present study, where 50 µl of crude ethyl acetate sample subjected to bioautography assay holds accurate localization of active compounds. However, based on the above data, *Pestalotiopsis* and other genera such as *Pestalotiopsis* sp. MUBL1002, *Pestalotia* sp. MUBL1067, *Bartalinia* sp. MUBL1096, *Seimatosporium* sp. MUBL1107 and *Truncatella* sp. MUBL1109 were subjected to molecular taxonomy.

The phylogenetic analyses with molecular sequences of rDNA are generally in agreement with morphological hypothesis proposed by Steyeart [20] and Nag Raj [34].

However, the molecular study provides good support for the classification established by Steyeart [20] who erected the genus *Truncatella* to accommodate species with two median cells. The morphological based hypotheses states that *Truncatella* is related to *Pestalotiopsis*[35] and closed clusters were strongly suggested the same in *P. mangiferae* with *T. angustata* and *Truncatella* sp. AY805595. The results indicated that *P. mangiferae* with *T. angustata* and *Truncatella* sp. AY805595 were genetically and morphologically strong by 18S rDNA sequences in maximum parsimony (MP).

In the present study, the molecular results are also informative for clarifying the relationship at species level. *P. mangiferae* was clustered away from *P. microspora* in which, two median cells of both the species differ in terms of appendage characters. In addition, they possess basal appendages; another important conclusion from our molecular analysis is that the wide generic concept of *Pestalotiopsis* proposed by Nag Raj [34].

The pleomorphic anamorphs are increasingly being classified as holomorphic taxa based on taxonomic analysis of DNA, morphological and other available data for e.g. *Brachysporium* anamorphs of holomorphic genus was *Cryptadelphia* reported by Reblova and Seifert [36]. The data obtained in this study, together with previous results, suggest that the classical taxonomy hypothesis was not related in molecular level. Notably, results of molecular taxonomy suggested that the sequence of *Pestalotia circularis* was not matched with 100% *Pestalotia* sp. but accurately matched with 100% *Coma circularis*, but in case of classical taxonomy *Pestalotiopsis circularis* was matched with *Pestalozzilla circularis* (anamorphic) or *Pestalotia* sp. [20, 35], hence the results of NCBI/BLAST data was not correlated with the previous classical taxonomy data. This result strongly indicates that the genetic ranges were changed during the sexual reproduction from anamorphic stage of

coelomycetes which is more notorious in molecular taxonomy. Our current result showed that species of *P. mangiferae, T. angustata, S. mariae* and *Coma circularis* or *Pestalotia circularis* were closely related as they cluster together in a single monophyletic clade with high bootstrap support.

The previous study was not supported by the taxonomic treatment of Guba[35]. Phylogenetic analyses of the rDNA sequences are generally in agreement with the morphological hypothesis proposed by Steyeart[20] and Nag Raj[34]. To gain some insight on the phylogenetic relationships of *Pestalotiopsis* and its allied genera, the large subunit (28 S) of ribosomal DNA region was examined and compared with existing morphological information. The Phylogenetic analyses were conducted using parsimony, distance and maxiumum likelihood criteria. Results of these analyses showed that *Bartalinia*, *Pestalotiopsis*, *Seimatosporium* and *Seiridium* represent distinct monophyletic groups with high bootstrap support, indicating that the genus *Truncatella* is paraphyletic with *Bartalinia* sharing a common ancestor[37]. However, the present study of the phylogenetic analyses showed that *P. mangiferae, T. angustata, S. mariae* and *Coma circularis* or *Pestalotia circularis* represent distinct monophyletic groups with fairly supported bootstrap values. Until now, no suitable 28S sequence for phylogenetic analysis of *Pestalotiopsis* and other genera was found but there are few reports indicating that rDNA sequences was generally in agreement with the morpholgical hypotheses proposed by Steyeart[20] and Nag Raj[34]. Similarly, meager report was found for 18S rDNA in *Pestalotiopsis* and other genera with morpholgical hypotheses. The present molecular study also supportive for clarifying relationship at the species level by rDNA sequences for a wide variety of taxonomic levels from the kingdom to genera with morphological hyphothesis using universial primers ITS 1 (5´-TCC GTA GGT GAA CCT GCC G-3´) and ITS 4 (5´-TCC TCC GCT TAT TGA TAT GC-3´). They also pointed out that the internal transcribed spacer (ITS) regions of the nuclear ribosomal repeat *Pestalotiopsis mangiferae*, *Bartalinia robillardoides*, *Seimatosporium mariae*, *Truncatella angustata* and *Pestalotia circularis* that the potentially useful regions for the closely related species and currently an out group value is the keystone tool for understanding broad fungal relationships.

## CONCLUDING REMARKS

To conclude, the present study clearly revealed that the coelomycetes of five active strains were characterized to be *Pestalotiopsis mangiferae*, *Bartalinia robillardoides*, *Seimatosporium mariae*, *Truncatella angustata* and *Pestalotia circularis* are promising antimicrobial metabolites. The present investigation proofed its potential in wide range of antimicrobial principles. However, further research is needed in the purified compounds for treatment of patients with malignant diseases.

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