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Antibacterial Activities of Endophytic Fungi from *Capsicum annuum* L. (Siling labuyo) leaves and fruits

Eliza Ann A. Arena, Mae Breech R. Bernal, and Jazzlyn T. Imperial*
Bicol University College of Science, Legazpi City
*Corresponding author: jtimperial@bicol-u.edu.ph

Abstract

Endophytic fungi were isolated from *Capsicum annuum* (siling labuyo) from three ecological zones in the province of Albay, Philippines and were identified through morpho-cultural characteristics and by molecular analysis of the internal transcribed spacer (ITS) region of the 18S ribosomal DNA. Eight fungal endophytes were isolated from the leaves and fruits. Seven were identified as *Colletotrichum gloeosporioides, Lasiodiplodia pseudotheobromae*, *Nigrospora sphaerica, Guignardia mangiferae, Coprinopsis cinerea, Colletotrichum siamense and Colletotrichum truncatum* while one remained unidentified. The extracted metabolites from the isolates were then screened for their antibacterial activities at 1,000 μg/mL. Among the fungal isolates, *C. cinerea* was found to be the most active against *Staphylococcus aureus* and *Escherichia coli* with zones of inhibition (ZOI) of 16.00 ± 1.00 and 17.00 ± 1.53 mm, respectively. Phytochemical screening showed that *C. cinerea* produced a variety of secondary metabolites such as flavonoids, alkaloids, terpenoids and quinones. The other fungal endophytes were also found to synthesize various secondary compounds that may be responsible for their antibacterial activities.

Keywords: *antimicrobial, Bicol, Escherichia coli, mycology, sili, Staphylococcus aureus*

Introduction

Antibiotic resistance is the ability of microorganisms to modify itself to become resistant or unaffected when continually exposed to antimicrobial drugs. The upsurge of antibiotic resistance threatens public health on a global scale as it lessens the effectiveness of available treatments. This brings to the forefront the need to search for new antibiotics (Matjaz *et al.*, 2015).

Traditionally, microorganisms that were investigated for novel therapeutic agents were isolated from soil. However, scientists had recently shifted their attention to less investigated ecological niches such as fungal endophytes which are considered as a possible source of bioactive natural products because of the variety of secondary metabolites with remarkably complex molecular scaffolds they produce (Pelo *et al.*, 2020). Moreover, they have shown to yield important compounds of pharmaceutical and commercial interest (Strobel, 2018). This leads to notable researches on fungi from plants as sources of bioactive metabolites with potential use in the health sector and in drug discovery (Torres & Dela Cruz, 2015).

Endophytes are highly diverse microorganisms that live within plant tissues without causing disease (Jia *et al.*, 2016). They act as reservoirs of novel bioactive secondary metabolites, such as alkaloids, phenolic acids, quinones, steroids, saponins, tannins, and terpenoids which serve as a latent candidate for antimicrobial, anti-insect, and anticancer agents, among others (Gouda *et al.*, 2016). In addition to this, metabolites that are obtained from fungal endophytes from several medicinal plants are identified to be good sources of pharmaceutical leads (Guerrero *et al.*, 2019). Hence, they are promising sources of new bioactive products that are clinically and biotechnologically relevant (Ding *et al.*, 2010).

The Philippines is recognized as a biodiversity hotspot with over 9,000 plant species inhabiting the archipelago (Conservation International, 2007). However, amidst the country's floral diversity, there are limited studies regarding the associated mycoflora of Philippine plants. Fungal endophytes from *Pandanus amaryllifolius* (Bungihan *et al.*, 2013), *Ipomea batatas* (Hipol, 2012), *Musa* spp. (Dagamac *et al.*, 2010) and *Canarium ovatum* (Torres & Dela Cruz, 2015; Guerrero & Dalisay, 2018) were previously studied due to their

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economic significance. One of the plants that is yet to be explored for endophytic fungal communities is the *C. annuum* (siling labuyo), an important crop in the Bicol Region and is often used in promoting Bicolano culture through cuisine and herbal medicine. Being a medicinal plant, it has been used to aid problems with digestion like upset stomach, intestinal gas, stomach pain, diarrhea, and cramps. It is also used to treat cardiovascular conditions that include poor circulation, excessive blood clotting and high cholesterol (Gururaj *et al.*, 2004).

There is a possibility that the medicinal properties of *C. annuum* be mirrored by its fungal endophytes. The fungi, even without the plant can be used to harness and produce the same benefits similar to its host (Guerrero & Dalisay, 2018). Thus, this inspired the current study to isolate the endophytic fungi from *C. annuum* and screen their secondary compounds against test microorganisms.

Materials and Methods

Sampling sites and collection of plant samples

Three ecological zones were chosen as sampling sites of *C. annuum* leaves and fruits: the East Washington Drive Legazpi City (13.145865° N, 123.734674° E; E: 13.81 masl) as lowland, Bonot Legazpi City (13.158089° N, 123.7471° E; E: 5.63 masl) as coastland and Maslog Legazpi City (13.107071° N, 123.768533° E; E: 79.35 masl) as upland. The plant sample collection was carried out by randomly collecting ten pieces of mature healthy plant leaves and fruits from each of the chosen sampling sites. The collected specimens were ensured to have no visual disease symptoms such as necrosis, chlorosis, deformities and insect foraging. Mature leaves were those that were darker in color and had bigger blade while mature fruits were those that were reddish and shinier in color and had plump shape. Complete leaf blades and fruits were collected and placed in sterile polyethylene bags in preparation for endophytic fungal isolation. The plant samples gathered were authenticated by the National Museum Botany Division, Manila with control specimen number of 17-08-1464.

Isolation of endophytic fungi

Endophytic fungi were isolated from the leaves and fruits of *C. annuum* following the protocol of Torres and Dela Cruz (2015) with some modifications. The plant samples were rinsed gently in running water to remove dust and debris. After washing, a metallic one-hole puncher with 6 mm diameter hole was used to punch out two leaf explants under aseptic conditions, from each sampling sites. For the fruit tissues, a flame-sterilized scalpel was used to cut 2 pcs of 5x3x2 mm fruit tissue (Hipol *et al.*, 2014). Afterwards, all explants and tissues were subjected to surface-sterilization. Explants and tissues were sterilized using sequential immersion in 95% ethanol for 1 min and NaOCl for 3 min. After immersing into the chemicals, both leaf explants and fruit tissues were rinsed with distilled water 3 times for 3 min each and were airdried under sterile conditions. Tissue printing was also done wherein samples were touched onto uninoculated Potato Dextrose Agar (PDA) plates for possible epiphytic growth. In addition, open plate method was done by opening 4 uninoculated PDA plates while transferring surface sterilized explants and tissues to check for possible contaminations. Two leaf explants and fruit tissues were inoculated per petri dish in triplicates thus having 6 segments per ecological zone.

The surface-sterilized explants and tissues were plated immediately with the use of flame-sterilized forceps. Chloramphenicol at 150 mg/L was added to prevent the growth of bacterial contaminants. Inoculated plates were incubated at room temperature for 3-7 days, and observed for growth every after two days. Emerging hyphae from the edge of the explants were isolated in freshly prepared PDA slants to obtain pure cultures.

Identification and phylogenetic analysis

After obtaining pure cultures, identification of the isolated fungal endophytes were performed by comparing their cultural and morphological characteristics with published literatures (Abass & Hussein, 2014; Badalyan *et al.*, 2011; Baldassari *et al.*, 2008; Intan Sakinah *et al.*, 2014; Munirah *et al.*, 2017). Accession numbers were also assigned to each morphospecies. Species having distinct morphocultural characteristics from the rest of the cultured endophytic fungi were sent to Macrogen Inc. (South Korea) for molecular identification. The genomic DNA from selected endophytes were amplified and sequenced using the primer pair ITS1F (5' TCCGTAGGTGAACCTGCGG 3') and ITS4R (5' TCCTCCGCTTATTGATATGC 3'). DNA sequences were then compared with the National Center for Biotechnology Information (NCBI) database with the use of BLASTN algorithm. Sequences of identified species were aligned using CLUSTAL W in MEGA 6.

The evolutionary distances were computed using the p-distance method followed by the construction of a phylogenetic tree to provide a graphical representation of divergence among isolated fungal endophytes. The following fungi served as outgroup taxa: *Colletotrichum orbiculare* (KT454388.1), *Colletotrichum acutatum* (AF090853.1), *Colletotrichum dematium* (KJ425529.1), *Lasiodiplodia citricola* (KM675758.1), *Nigrospora musae* (KX986076.1), *Pilobolus longipes* (AH006442.5), *Guignardia* sp. isolate 10 (AF374362.1), and *Coprinopsis* sp. CAL2 (JF681946.1).

Fermentation and extraction of fungal metabolites

Fungal metabolites were extracted as described by Torres and Dela Cruz (2015) with modifications. Eight fungal isolates were subjected to liquid surface fermentation for the production of secondary metabolites. Fungal mycelia from the different isolates were inoculated using flame sterilized cork borer (6 mm) in ten Erlenmeyer flasks (250 mL) containing 100 mL sterile Potato Dextrose Broth (PDB). After inoculation, all flasks were incubated for 35 days at room temperature under stationary conditions. Afterwards, 100 mL of ethyl acetate (EtOAc) was added to every broth culture and left to soak overnight. After soaking, the broth of the individual strains were mixed for 2 hrs at 200 rpm and then filtered using Whatman no. 1 filter paper. The solvent with the putative metabolites were separated from the broth using separatory funnel. The separated mixture was concentrated and evaporated to dryness at reduced pressure using rotary evaporator (BIOBASE RE100-Pro) at 65 ℃.

Phytochemical screening

The standard protocol for determination of the phytochemical constituents was adapted from Kala and co-workers (2012) and Tiwari and co-workers (2011) as shown in Table 1. Being a colorimetric analysis, color intensity was used as basis for measuring the amount or presence (++ = abundant, + = present, - = absent) of a metabolite.

Table 1. Standard protocol for phytochemical screening

Phytochemical	Reagents used /test performed	Observation showing positive result
Alkaloids	Dragendorff's test	Formation of orange red precipitate
Tannins	Ferric Chloride	Presence of deep blue-black color
Flavonoids	Alkaline reagent test	Formation of an intense yellow color
Terpenoids	Salkowski's test	Reddish-brown coloration of the interface
Phenols	Ferric Chloride test	Presence of blue or green color
Quinones	Concentrated H2SO4	Formation of red color

Antibacterial assay

Antibacterial activity of crude extracts derived from selected fungal isolates was assessed in triplicates using the disc diffusion method (Torres & Dela Cruz, 2015). The human pathogenic bacteria *Staphylococcus aureus* BIOTECH1823 and *Escherichia coli* BIOTECH1825 were procured from the Bicol University Research Development Center (Legazpi City) and maintained on Nutrient Agar (NA) medium at 4℃. Bacterial suspension prepared from 24h-old bacterial cultures were standardized by 0.5 McFarland to contain approximately 1.5 x 10^8 cells/mL. Five discs of sterile filter paper Whatman no. 1 (6 mm) containing the fungal extracts were inoculated equidistantly to the NA plates using various concentrations (10, 100 and 1000 ug/mL) of fungal crude extracts. Paper discs inoculated with EtOAc served as negative control while streptomycin (10 mg/mL) served as the positive control. The inoculated petri dishes were subsequently incubated at room temperature for 24 h. Following incubation, the diameter of the zone of inhibition (ZOI) were measured and interpreted following the categories used by Torres & Dela Cruz (2015): >19 mm ZOI (very active), 14 - 19 mm ZOI (active), 10 – 13 mm ZOI (partially active), and <10 mm ZOI (inactive).

Results and Discussion

Isolation of endophytic fungi from C. annuum

A total of eight endophytic fungi were isolated from *C. annuum* leaves and fruits from the three ecological

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zones. The fungal isolates were members of divisions Ascomycota (6) and Basidiomycota (1) with one unidentified morphospecies. The isolates were classified into 6 genera shown in Table 2 with *Colletotrichum* being isolated in high frequency and abundance from two ecological zones. The genus *Lasiodiplodia* was an isolate only found at the upland ecological zone. The remaining genera, namely *Nigrospora, Phyllosticta and Coprinopsis* (including the unidentified morphospecies), were isolated from the coastland ecological zone. Identities of the isolates are summarized in Table 3 and their phylogenetic relationships in Figure 1.

Qualitative determination of phytochemical constituents

The phytochemical constituents found in the fungal crude extracts are shown in Table 4. The phytochemicals detected were flavonoids, alkaloids, terpenoids and quinones. *C. cinerea*, in particular, was found to have an abundance of flavonoids, alkaloids, terpenoids and quinones among the other fungal isolates.

Antibacterial activity of fungal endophytes

Antibacterial screening by disc diffusion method was carried out with the fungal crude extracts against the Gram-positive *S. aureus* and Gram-negative *E. coli* as presented in Table 5. Of the eight isolates, only seven were tested since the metabolite yield of the ethyl acetate extract from *Nigrospora sphaerica* was not enough for the assay.

Antibacterial activities were evident at 1000 µg/mL concentration. *C. cinerea* fungal crude extract was notably the most active with 16.00 ± 1.00 and 17.00 ± 1.53 mm ZOI against *S. aureus* and *E.coli*, respectively (Figure 2). The other fungal extracts were partially active, except for *G. mangiferae,* which showed bioactivity only against *S. aureus* with a ZOI of 10.67 ± 0.88 mm. Fungal endophyte isolate 544 was also noted to have no antibacterial activity against *E. coli.*

C. annuum is part of Bicolano culture most commonly used in cuisines as well as herbal medicine. There are, however, limited studies regarding its fungal mycoflora. The results of this study showed that *C. annuum* is a good source of fungal endophytes. It showed that the leaf explant harbor more endophytic communities than the fruit tissue in all ecological zones. Only the plated segments from the coastal area (Bonot) were found to be colonized by at least one endophyte. Moreover, this study also revealed that a high diversity of endophytic fungi was mostly isolated at the coastland ecological zone, bearing the highest latitude and the lowest altitude. Though there is limited information regarding the endophytes associated with plants in high-latitude sites or in relatively extreme environments, the high diversity of endophytes may be due to the plant's ability to resist stress. The endophytes may have conveniently transferred and conferred to plants the ability to resist stress, as well as coping with multiple adversities, as endophytes are also mediators of stress tolerance (Li *et al*., 2019). Jalgaonwala and co-workers (2011) also stated that host-endophyte relationships vary from host to host, which also depends on the environmental conditions. In addition to this, the number and diversity of endophytic fungi found in host plants can be due to various factors such as humidity, altitude, precipitation, host plant species, communities, and temperature whereas its colonization rate may be affected by the genotype, growth stage, physiological status of the plant, type of plant tissues, environmental condition of the soil in which it is grown, sampling season, surface sterility, selective media and culture conditions as well as different agricultural practices (Zhou *et al*., 2015; Gaiero *et al.*, 2013).

The majority of the identified fungal genera belonged to the division Ascomycota, with only one species belonging to Basidiomycota which was also in line with recent endophytic studies (Khiralla *et al*., 2016; Tibpromma *et al*., 2018). It was on record that the genera that had the most occurrence among the three ecological zones was *Colletotrichum*, which was in congruence with the study of Paul and co-workers (2012), wherein it was the dominant species that were isolated frequently during the fruiting stage of *C. annuum*.

The generated Neighbor Joining (NJ) tree (Figure 3) has shown that Ascomycota division was composed of three identified species of *Colletotrichum* (*C. gloeosporioides, C. siamense* and *C. truncatum*), one unidentified morphospecies (Fungal endophyte isolate 544), one species of *Guignardia* (*G. mangiferae*), and one species each of *Lasiodiplodia* and *Nigrospora*. The unidentified morphospecies (Fungal endophyte isolate 544) formed a clade with *Colletotrichum* sp. indicating that it was more likely to be in close relationship with the said species as supported by high bootstrap value of 100%.

Endophytic fungi are known as a source of novel secondary metabolites, some of which have beneficial biological activities (Strobel & Daisy, 2003). This is supported by the results of the phytochemical screening

Table 2. Fungal endophytes from *C. annuum* leaves and fruits explants from three ecological zones

Divisions	Genus	Ecological Zones[a]		
		Upland (Maslog)	Coastland (Bonot)	Lowland (Albay)
Ascomycota	*Colletotrichum*	+	-	+
Ascomycota	*Lasiodiplodia*	+	-	-
Unidentified	Fungal endophyte isolate 544	-	+	-
Ascomycota	*Nigrospora*	-	+	-
Ascomycota	*Guignardia*	-	+	-
Basidiomycota	*Coprinopsis*	-	+	-

[a]*Presence of fungal species (+); absence of fungal species (-)*

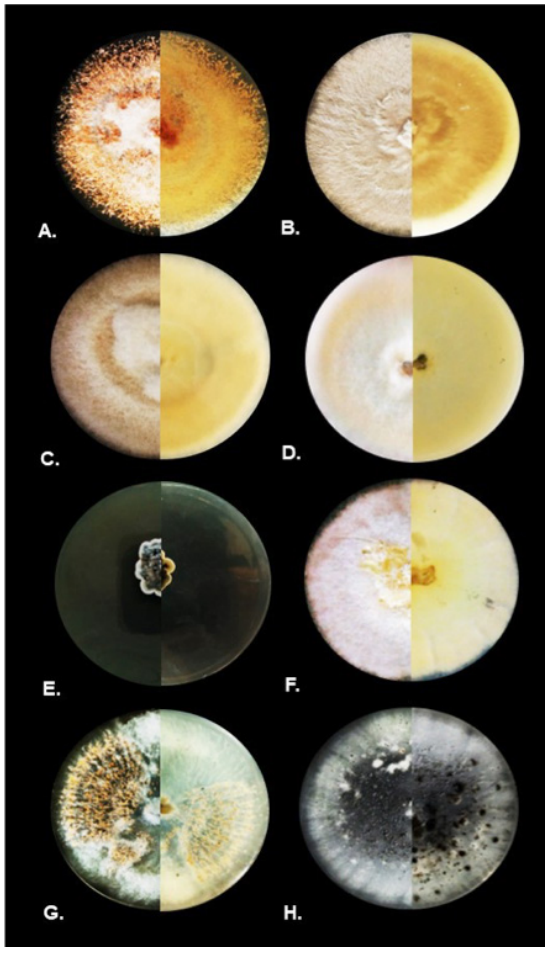

Figure 1. Seven-day old purified fungal cultures from *C. annuum* leaves and fruits cultivated in Potato Dextrose Agar (PDA). Images are presented such that both the surface and opposite views of the plates are shown: (A) *Colletotrichum gloeosporioides*, (B) La*siodiplodia pseudotheobromae*, (C) Fungal endophyte isolate 544, (D) *Nigrospora sphaerica*, (E) *Guignardia mangiferae*, (F) *Coprinopsis cinerea*, (G) *Colletotrichum siamense*, and (H) *Colletotrichum truncatum*.

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Table 3. Sequence similarity (94-100%) of isolated fungal endophytes from *C. annuum* leaves and fruits based on DNA analysis of 18S internal transcribed spacer (ITS) region. The closest relatives in GenBank according to BLAST search were also presented.

Fungal Isolates	Tissue	Closest related species	Genbank Accession No.	% Similarity
ML1	Leaf	*Colletotrichum gloeosporioides*	KX620309.1	94
MF1	Fruit	*Lasiodiplodia pseudotheobromae*	FJ904913.1	99
BL1	Leaf	Fungal endophyte isolate 544	EU687131.1	99
BL2	Leaf	*Nigrospora sphaerica*	KM893076.1	100
BF1	Fruit	*Guignardia mangiferae*	EU747726.1	100
BF2	Fruit	*Coprinopsis cinerea*	KC881188.1	100
AL1	Leaf	*Colletotrichum siamense*	KY471303.1	99
AF1	Fruit	*Colletotrichum truncatum*	KU571506.1	99

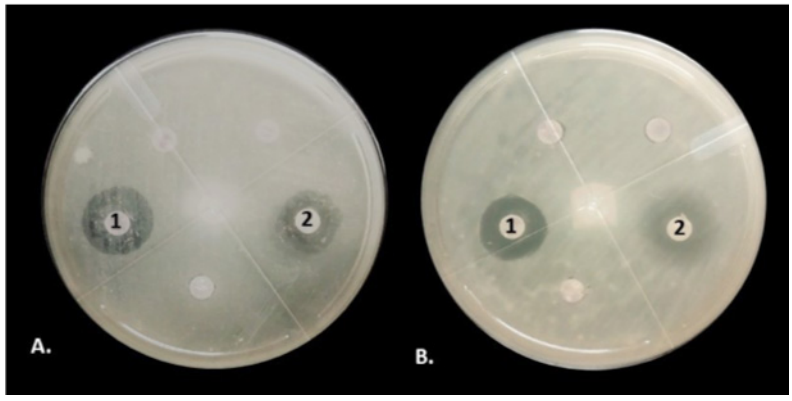

Figure 2. Antibacterial activities of fungal crude extracts from *C. cinerea* (1) and streptomycin (2) against *E. coli* (A) and *S. aureus* (B)

Table 4. Phytochemical screening of fungal crude extracts

Fungal crude extracts	Phytochemical constituents[a]					
	Flavonoids	Alkaloids	Terpenoids	Quinones	Phenols	Tannins
Colletotrichum gloeosporioides	+	-	+	+	-	-
Lasiodiplodia pseudotheobromae	+	++	++	++	-	-
Fungal endophyte isolate 544	++	+	+	+	-	-
Nigrospora sphaerica	+	-	+	+	-	-
Guignardia mangiferae	+	-	-	+	-	-
Coprinopsis cinerea	++	++	++	++	-	-
Colletotrichum siamense	+	++	++	+	-	-
Colletotrichum truncatum	+	-	+	++	-	-

[a]*Abundance of phytochemical (++); Presence of phytochemical (+); absence of phytochemical (–)*

Table 5. Antibacterial activity of fungal crude extracts against *E. coli* and *S. aureus*

Fungal Isolates	Zone of inhibition after 24 hrs (mm)[a]									
	E. coli					*S. aureus*				
	10 ug/mL	100 ug/mL	1000 ug/mL	EtOAc	Strepto-mycin (10 mg/mL)	10 ug/mL	100 ug/mL	1000 ug/mL	EtOAc	Strepto-mycin (10 mg/mL)
Colletotrichum gloeosporioides	-	-	+	-	+	-	-	+	-	+++
Lasiodiplodia pseudotheobromae	-	-	+	-	+	-	-	+	-	+
Fungal endophyte isolate 544	-	-	-	-	++	-	-	+	-	+
Guignardia mangiferae	-	-	+	-	++	-	-	+	-	+
Coprinopsis cinerea	-	-	++	-	++	-	-	++	-	+
Colletotrichum siamense	-	-	+	-	++	-	-	+	-	++
Colletotrichum truncatum	-	-	+	-	++	-	-	+	-	++

[a]*ZOI < 10 mm (no activity) (-); ZOI from 10 - 13 mm (partially active) (+); ZOI from 14 - 19 mm (active) (++); ZOI above 19 mm (very active) (+++)*

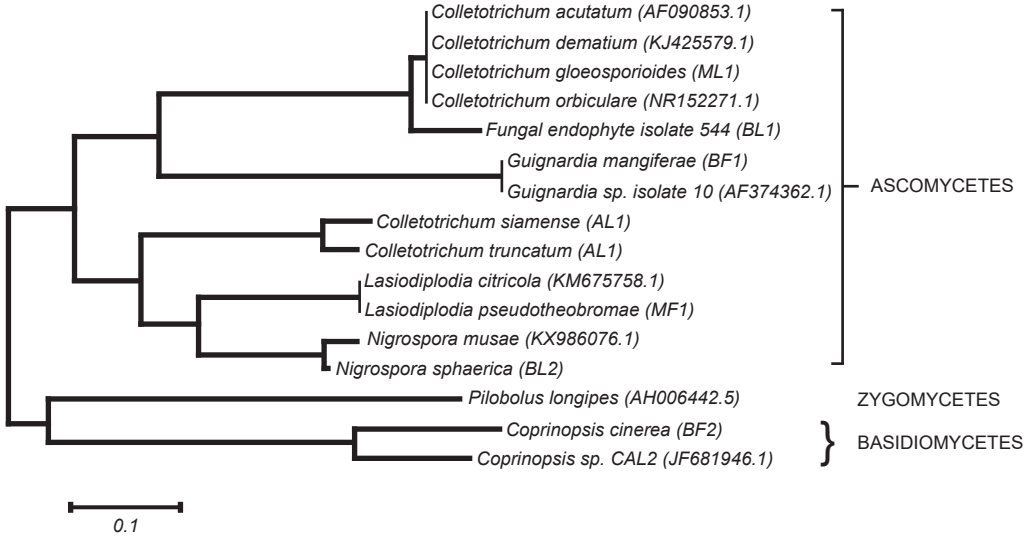

Figure 3. Unrooted neighbor joining tree of internal transcribed spacer 1 (18S) partial sequence compared with GenBank-accessed sequences with their accession numbers given. The ITS sequences obtained in this work were highlighted. The evolutionary distances were computed using the p-distance method. The percentage of replicate (1000) trees in which the associated taxa clustered together in the bootstrap test (1000) are shown next to the branches. Scale bar represents two nucleotide substitutions for every 70 nucleotides. Evolutionary analyses were conducted in MEGA 6.

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wherein the secondary metabolites detected in the fungal crude extracts correlated to its antibacterial activities. As shown in Table 4, all the fungal crude extracts contained flavonoids, which belongs to the group of polyphenolic compounds that are commonly known for its health-promoting abilities, such as antimicrobial, anticancer, antioxidant, and anti-allergic properties (Aiyelaagbe & Osamudiamen, 2009). Correspondingly, most of the fungal crude extracts also tested positive for the presence of alkaloids which have been reported to be analgesic, antispasmodic, bactericidal, antimalarial, and analgesic (Okwu & Okwu, 2004). Almost all the fungal crude extracts were also positive for the presence of terpenoids, except *G. mangiferae*. Pharmaceutical industries noted that terpenoids like triterpenes and diterpenes are known to be antibiotics, insecticidal, anthelmintic, and antiseptic (Duke, 1992). Quinones, considered to have a wide range of potential antimicrobial effects, were reported in all crude extracts of the fungi. The said metabolite targets microbial cells surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes and often inactivates proteins leading to loss of function that makes quinones as a possible antimicrobial source (Stern *et al*., 1996).

Evaluation of the antibacterial activity showed that the fungal crude extracts were more effective against Gram-positive bacteria compared to Gram-negative bacteria. The result of this study was in congruence with other studies showing the consistent sensitivity of Gram-positive bacteria against fungal extracts as compared to Gram-negative ones (Koohsari *et al*., 2015). Gram-negative bacteria are known to be more resistant to antibiotics, so they are difficult to control.

Most of the species under division Ascomycota showed partially active antibacterial activities. However, these were not as active as *C. cinerea*, which is from the division Basidiomycota. Basidiomycetes are a class of higher fungi that have adapted to various climates and habitats. *C. cinerea* crude extract displayed an active ZOI against both bacteria. Based on the study of Suay and colleagues (2000), Basidiomycetes have a more favorable source of biologically active natural products than Ascomycetes supporting the high antibacterial properties of *C. cinerea*.

Colletotrichum species have been described as prolific endophytic bioactive fungi with antimicrobial (Liu *et al*., 2003) and cytotoxic (Rosa *et al*., 2010) activities. In this study, the extracts from *C. gloeosporioides, C. siamense,* and *C. truncatum* were partially active against both test bacteria. Flavonoids, terpenoids as well as quinones were identified to be present in *C. gloeosporioides* ethyl acetate extract. Earlier findings show the crude extracts of *C. gloeosporioides* have antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi, as well as being a prolific producer of a plethora of antimicrobial compounds, such as colletotric acid and colletoic acid (Zou *et al*., 2000), diketopiperazines (Trigos *et al*., 1997), artemisinin (Wang *et al*., 2006), phillyrin compounds (Zhang *et al*., 2012) and piperine (Chithra *et al*., 2014).

Conclusion

The increasing resistance to existing antibiotics is a "never-ending war" which has prompted an intensive search for newer and more effective types. This has triggered immense interest in the search for new antibacterial drugs of endophytic origin. This study showed that seven out of the eight isolated fungal endophytes possess antibacterial properties against the two bacterial pathogens. The fungal crude extract showing the most active ZOI was *C. cinerea* against both test bacteria. Fungal endophyte isolate 544 only inhibited the growth of *E. coli.* Results of phytochemical screening indicated the species as good sources of phytochemicals specifically flavonoids, alkaloids, terpenoids, and quinones thus signifiying their potential antibacterial activities. This study recommends the identification of novel compounds from the said endophytes.

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