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RESEARCH ARTICLE

Antimicrobial Potential and Molecular Characterisation of Endophytic Fungi Isolated from *Conyza bonariensis* from Tanzania

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Abstract

Objective: The harnessing of medicinal plants for the treatment of infectious diseases has an environmental concern, and therefore environmentally friendly alternative sources like harnessing the potential of endophytes are of paramount importance. The present study aimed to evaluate chemical composition and antimicrobial activity of crude extracts of endophytic fungi isolated from *C. bonariensis* (L.) in Tanzania.

Materials and Methods: Initially, endophytic fungi were isolated from *C. bonariensis* (L.) and characterized, followed by mass cultivation and the harvesting of crude extracts. Then crude extracts were tested against selected microorganisms: Two Gram-positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis;* Two Gram-negative bacteria, *Escherichia coli* and *Salmonella typhi*, and a yeast *Candida albicans*. Gas Chromatography-Mass Spectrometry analyzed the chemical composition of crude extracts.

Results: Five endophytic fungi (*Talaromyces* sp., *Penicillium daleae*, *Neurospora crassa, Talaromyces radicus* 1 and *Talaromyces radicus* 2) were isolated. Furthermore, crude extract of *N. crassa* had the highest antimicrobial activity with the range of MIC found to be 1.5 µg/mL > MIC > 0.78 µg/mL for all tested microorganisms except for *S. aureus* where the range of MIC was 40 µg/mL>MIC>20 µg/mL. On the other hand, the crude extract of *Talaromyces radicus* 1 had the lowest MIC range (100 mg/mL>MIC>50 mg/mL). The GC-MS results demonstrated variation in chemical composition with proved various biological properties.

Conclusions: Ethyl acetate crude extracts of *N. crassa* demonstrated the highest antimicrobial activity, therefore warranting further exploration of the endophyte and their crude extracts for various medicinal and industrial applications. Further studies on the characterization of pure compounds, which may be responsible for the antimicrobial activity that was observed, are urgently needed.

Keywords: Endophytic fungi, Internal transcribed spacer, Minimum inhibitory concentration, Tanzania, Gas chromatography mass spectrometry, *Conyza bonariensis*

Introduction

Human beings have been interacting with their natural environment as a source of food, medicine, fertilizers, fragrances, and clothing (Fatemeh, 2018; Okot *et al*., 2020). Worldwide, medicinal plants continue to have the potential in producing secondary metabolites (Kianfé *et al*., 2020). Medicinal plants are plants that possess therapeutic or exert a beneficial pharmacological effect on the human or animal body (Kilonzo & Munisi, 2021). Indigenous people have been using the traditional medicinal plant for the treatment of various diseases. For example, medicinal plants *Achillea millefolium, Ipomoeae pandurata*, and *Hieracium pilosella* have been used as an antioxidant, antifungal and antimicrobial, respectively (Candan *et al*., 2003; Frey & Meyers, 2010; Abdollahi *et al*., 2012). However, the direct harnessing of medicinal plants for the treatment of infectious diseases has an environmental concern, and therefore an environmentally friendly alternative source like harnessing the potential of endophytes is of paramount importance (Ibrahim *et al*., 2021).

Endophytic microorganisms may be fungi or bacteria that live inside the tissue of plants for the whole or part of

their life cycle and have no negative effects on their host plants (Manyahi *et al*., 2014; Wu *et al*., 2020). Endophytes have been reported to produce more or less secondary metabolites as their host medicinal plants (Ancheeva *et al*., 2020; Ibrahim *et al*., 2021; Mwanga *et al*., 2019). Endophytic fungi crude extracts have been demonstrated to contain bioactive secondary metabolites such as alkaloids, phenols, flavonoids, tannins, terpenoids, and saponins, which have antimicrobial, antioxidant, anti-inflammatory, and anti-diabetic activity (Ancheeva *et al*., 2020; Patil *et al*., 2016). For example, a chemical analysis of a crude extract of *Nigrospora* sp. from *Moringa oleifera* demonstrated to contains griseofulvin with strong antifungal activities while dechlorogriseofulvin and mellein showed weak antifungal activity (Zhao *et al*., 2012). Similarly, crude extracts of *Nigrospora* sp., *Aspergillus fumigates Fusarium oxysporum* from *Leucas martinicensis, Sceletium tortuosum* and *Moringa oleifera*, were demonstrated to contain bioactive secondary metabolites with biological activities as their host plants (Manganyi *et al*., 2019).

In Tanzania, various communities including the Safwa people of the Mbeya region have been using *Conyza bonariensis* leaves and other plants for the treatment of fungal, bacterial, and inflammatory diseases (Araujo *et al*., 2013; Espinoza *et al*., 2020; Ghwanga & Chacha 2019). In Tanzania, antimicrobial activity of *C. bonariensis* has been tested experimentally, and the results were promising (Ghwanga & Chacha 2019). Although endophytic fungi isolated from *C. bonariensis* had been reported in some other parts of the world (Alsini *et al.,* 2022), such information remained to be evaluated in Tanzania taken into consideration that biological activity of crude extract of endophytic fungi may be influenced by geographical conditions like temperature, humidity and soil properties of a particular location (Langenfeld, 2013; Fang *et al*., 2019) where the host plant is found. Since the antimicrobial potential of the crude extract obtained from endophytic fungi isolated from *C. bonariensis* had not been assessed in Tanzaia, this study was conducted to investigate both the chemical composition and antimicrobial effects of these crude extracts derived from *C. bonariensis* endophytic fungi in Tanzani.

Materials and Methods

Sample collection

Fresh and healthy leaves of C*onyza bonariensis* were collected from the Mbeya region located between latitude 08°54′00″S and longitude 33°27′00″E by purposive random sampling for isolation of endophytic fungi. The voucher specimen sample was taken to the University of Dar Es Salaam at the Botany Department for identification and was provided with voucher specimen number FMM4139.

Test organisms

Five test microorganisms were involved in the present study as stipulated in Table 1. Four test microorganisms were bacteria and one was fungus. The test microorganisms were obtained from the microbial strains Library of the Department of Molecular Biology and Biotechnology (MBB), University of Dar es Salaam. The test microorganisms were sub-cultured and reconstituted as previously described (Mpinda *et al*., 2018).

Isolation of endophytic fungi from C. bonariensis

Dust and debris were removed from the leaves by washing with tap water and left to dry. The leaves were decontamination with 70% ethanol for 2 minutes, then dipped in 0.4% sodium hypochlorite (NaOCl) for 1 minute, followed by dipping in 70% ethanol for two minutes, and finally rinsed twice with sterile distilled water to remove ethanol leftover. Surface-sterilized leaves were placed on sterile aluminum foil and allowed to dry in a biosafety cabinet (Nxumalo *et al*., 2020). Leaves were then cut into small pieces of about 1 cm^2 by using a sterile surgical blade, and then placed in potato dextrose agar (PDA; containing potato 200 g/L), dextrose (20 g/L), and agar (15 g/L, pH 6.0) in petri dishes containing chloramphenicol (0.25 mg/mL) to prevent the growth of bacteria. Also, to test for the efficiency of surface sterilization, water from the last washing step was inoculated on PDA media with the same chemical composition. The inoculated petri-dished were incubated for 3-5 days at 30°C. After obtaining pure isolates, the colony diameter of each fungal endophyte was measured every day for five days. The colony diameter

Table 1. List of selected test microorganism used for screening of antimicrobial activity in the study

Serial number	Microorganism name	Culture number	Gram stain
1	*Staphylococcus aureus*	ATCC29213	Gram-negative
2	*Escherichia coli*	ATCC8736	Gram-negative
3	*Salmonella typhi*	ATCC 6539	Gram-positive
4	*Bacillus subtilis*	ATCC6051	Gram-positive
5	*Candida albicans*	DSM1665	-

information was used to establish the growth rate of each fungal endophyte. The fungal growth rate was calculated by dividing the growth difference between day 5 and day 4 to the past (day 4) growth as stipulated in the formula below:

$$\text{Fungal growth rate} = \frac{\text{Present growth (mm)} - \text{Past growth (mm)}}{\text{Past growth (mm)}}$$

Morphological and molecular characterization of endophytic fungi

Morphological characterization was performed by considering a variety of parameters such as the color of the colony (front and back view of the plate on PDA medium), texture, colony topology and spore formation of pure isolates (Paul *et al*., 2012).

Molecular characterization was performed by assessing genetic variation of the internal transcribed spacer (ITS) region of genomic DNA. Initially, genomic DNA was extracted by using a CTAB-based protocol as previously described by Umesha *et al*. (2016). Then, polymerase chain reaction (PCR) was done by using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC -3') primers as previously described by Mpenda & Mkangara (2022). The amplicons were then sent to Inqaba Biotech Company in South Africa for Sanger sequencing by using the same primers (ITS1 and ITS4).

Endophytic fungi mass cultivation and crude extracts harvesting

Endophytic fungi were cultivated in a 5000 mL conical flask containing 2000 mL of sterile malt extract broth (MEB) as previously described (Jaronski & Mascarin, 2016; Mwanga *et al*., 2019). Briefly, pure endophytic fungi mycelia were cut from PDA media and placed in a conical flask containing MEB broth and were left stationary at 30˚C for four weeks. After growth, fungal mycelia were filtered by using Whatman No. 1 filter paper with a pore size of 11 µm. The filtrate was mixed with an equal volume of ethyl acetate and shaken for 10 minutes to allow the absorption of secondary metabolites (Ancheeva *et al*., 2020). The top ethyl acetate solvent layer was collected by using a separating funnel and then was concentrated by using a rotary evaporator (Model No RE-501) at 40˚C at a pressure of 100 mbar. The crude extract was obtained by allowing evaporation of the remaining solvent from concentrate in a dark ventilated room. The crude extracts were preserved at 4˚C for further analysis (Santos *et al*., 2015).

Screening for antimicrobial activity

The test microorganisms described in Table 1 were grown on nutrient and potato dextrose agar for bacteria and fungus, respectively. The nutrient agar inoculated with bacteria was incubated at 37˚C for 24 hours; whereas, the PDA medium inoculated with fungus was incubated at 30˚C for 48 hours. Bacterial and fungal colonies were re-suspended in physiological saline solution, which was then compared with 0.5 McFarland standards (prepared by adding 0.05 mL of 1.175% $BaCl_2$ to 9.95 mL of 1% H_2SO_4).

Endophytic fungi crude extracts were screened by a disc diffusion method as previously described by Khaki *et al*. (2014). Briefly, a paper disc (5-mm-diameter) was soaked with 20 µL of 100 mg/mL for each crude extract. To ensure proper soaking before transferring to petri dishes, discs were kept in a refrigerator at 4°C for 30 minutes. Pre-soaked discs with a crude extract were placed on Petri dishes that had previously inoculated with test microorganism suspension using sterile cotton swabs. The incubation conditions were the same as discussed above. Also, chloramphenicol (0.25 mg/mL) and fluconazole (0.25 mg/mL) were employed as positive controls for bacteria and fungi, respectively and 10% dimethyl sulfoxide (DMSO) was utilized as a negative control. The experiment was performed in duplicate, and zones of growth inhibition were measured in mm after 24 hours of incubation for bacteria, and after 48 hours of incubation hours for fungi (Selvi, 2014).

Minimum inhibitory concentrations

Minimum inhibition concentration (MIC) was established for test organisms that were inhibited by endophytic fungi crude extracts by the disc diffusion method, as previously reported by Khaki *et al*. (2014). The ability of endophytic fungi crude extracts to suppress the growth of test organisms was evaluated for each of the two-fold serial dilution concentrations. Inoculum for the assays was prepared in 0.5 McFarland standards (approximately 1.5×10^8 CFU/mL). Minimum inhibition concentration in mg/mL was reported as a range between the last concentration that showed inhibition and the immediate subsequent concentration to that did not show inhibition.

Chemical analysis of endophytic fungi crude extract

Chemical analysis was performed by Gas Chromatography-Mass Spectrometry recorded in GCMS-2010 Shimadzu instrument operating in Electron Ionization (EI) mode (MS) at 70 ev, and Flame Ionization Detector (FID) for GC. A Restek-5MS column (30 m × 0.25 mm × 0.25 µm) was used. The oven temperature program was 90˚C to 280˚C

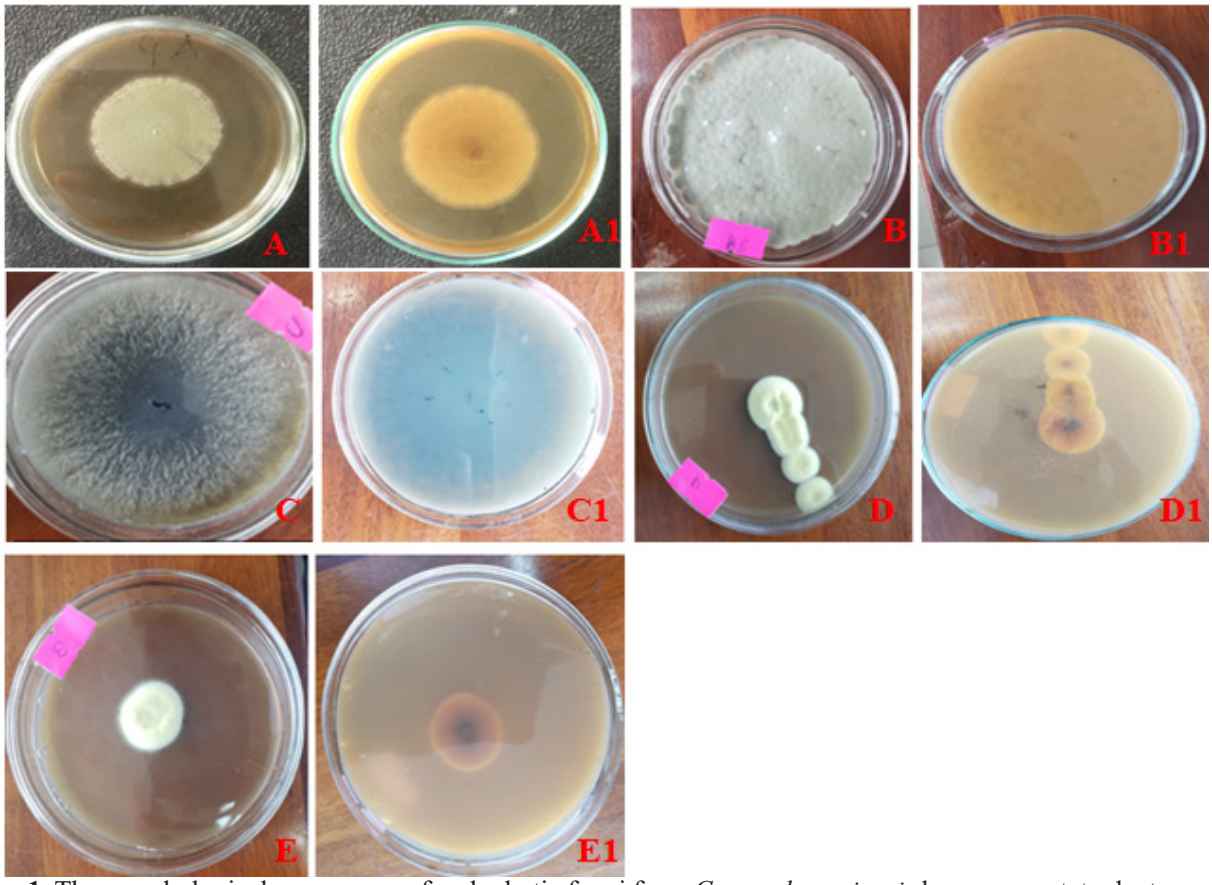

Figure 1. The morphological appearance of endophytic fungi from *Conyza bonariensis* leaves on potato dextrose agar: A and A1 is front and back view of *Talaromyces* sp. (ON394877) colony; B and B1 is front and back view of *Penicillium daleae* (ON394878) colony; C and C1 is the front and back view of *Neurospor acrassa* (ON394879) colony; D and D1 is the front and back view of *Talaromyces radicus* (ON394880) colony of isolate D; E and E1 is the front and back view of *Talaromyces radicus* (ON394881) colony of isolate E.

and held at 90˚C for two minutes. The temperature was increased to 28˚C for 10 minutes (hold time) at the rate of 15˚C per minute. The injection temperature was 250˚C with split injection mode. The flow rate of carrier gas helium was 1.21 mL min^{-1}. The ion source temperature and interface temperature in MS was 230˚C and 300˚C, respectively. The identification of chemicals in the extracts was done by scan method, which involves the use of Mass Spectral Library & Search Software (NIST 11). Quantification of chemicals in the extracts was done using the Peak Integration method (area normalization) whereby ion allowance was 20%, target ion and other five quantitation ions were used on quantitative analysis. 10 µL of the sample was dissolved in dichloromethane to make 1 mL and injected in GC (Da Silva *et al*., 2021), The results were reported as percentage compositions derived from the peak area of all scanned compounds in the extracts (Da Silva *et al*., 2021; Espinoza *et al*., 2020).

Data analysis

Descriptive and inferential statistics were performed using R software (Hackenberger, 2020). For descriptive statistics, bar graphs were used to summarize the statistics; for inferential statistics, two-way analysis of variance (ANOVA) was employed to tell whether there was a difference between the means of the zone of inhibition index of test organisms. Following ANOVA, we used Tukey HSD to perform a pairwise comparison, with a significant difference set at ($p<0.05$) (Gelfond *et al*., 2018; Leppink *et al*., 2017).

Raw DNA sequences were obtained electronically and by using Geneious (BIOMATTERS& geneious) bioinformatics software was use to generate consensus sequences. Similarity search was done using Basic Local Alignment Search Tool (BLAST) on National Center for Biotechnology Information (NCBI). Closely related sequences were retrieved from GenBank of NCBI, and multiple sequence alignment was performed by using MUSCLE algorithm on Molecular Evolution Genetic

Table 2. The morphological feature and growth rate of endophytic fungi isolated from *C. bonariensis* leaves. Number one to five in the figure represent days of endophytic fungi growth diameter (mm). The numbers in the growth rate column that bear different superscript letters (a, b, c) are significantly different ($p<0.05$).

Isolate	Texture	Front view	Back view	Spore	1	2	3	4	5	Growth rate (%)
Talaromyces sp.	Powder	White green	Yellow	Yes	1	3	6	12	15	25^{a}
Penicillin daleae	Powder	Greenish white	Yellow	No	1	2	4	5	6	20^{b}
Neurospora crassa	Cottony	Pale dark blue	Black	No	10	15	35	45	60	33^{c}
Talaromyces radicus	Velvety	Mint green	Pale yellow	Yes	1	2	4	5	6	20^{b}
Talaromyces radicus	Velvety	Mint green	Pale yellow	No	1	5	7	11	13	18^{b}

Table 3. Antimicrobial activities of endophytic fungi crude extracts from *Conyza bonariensis* leaves represented in activity indices and standard error of mean. Along the rows, the activity index values that bear different superscript letters are significantly different ($p<0.05$); whereas, across the column, the activity index values that bearing different superscript numbers are significantly different ($p<0.05$) The concentrations were 0.25 mg/mL and 100 mg/mL for positive control and crude extracts, respectively.

Isolate ID	Crude extract/ positive controls	Accession numbers	*E. coli*	*C. albicans*	*S. aureus*	*S. typhi*	*B. substilis*
			Mean values of activity indexes ± Standard error of mean				
-	Chloramphenicol / Fluconazole	-	$1.00\pm0.0^{a,1}$	$1.00\pm0.0^{a,1}$	$1.00\pm0.0^{a,1}$	$1.00\pm0.0^{a,1}$	$1.00\pm0.0^{a,1}$
A	*Talaromyces* sp.	ON394877	$0.47\pm0.0^{a,2}$	$0.46\pm0.0^{a,2}$	$0.46\pm0.0^{a,2}$	$0.47\pm0.0^{a,2}$	$0.4\pm0.0^{a,2}$
B	*Penicillin daleae*	ON394878	$0.55\pm0.0^{a,2}$	-	-	-	$0.4\pm0.0^{a,2}$
C	*Neurospora crassa*	ON394879	$0.76\pm0.0^{a,1}$	$1.19\pm0.0^{b,1}$	$0.76\pm0.1^{a,1}$	$1.28\pm0.0^{b,1}$	$0.98\pm0.0^{a,1}$
D	*Talaromyces radicus*	ON394880	$0.34\pm0.1^{a,2}$	-	$0.35\pm0.0^{a,2}$	$0.35\pm0.0^{a,2}$	$0.3\pm0.01^{a,2}$
E	*Talaromyces radicus*	ON394881	$0.48\pm0.1^{a,2}$	$0.48\pm0.1^{a,2}$	$0.63\pm0.0^{b,1}$	$0.54\pm0.0^{b,1}$	$0.52\pm0.0^{b,2}$

Analysis (MEGA X) software. Then, using aligned sequences, a UPGMA phylogenetic tree was constructed using MEGAX to depict genetic relatedness between isolated found in the present study and the previous one retrieved from GenBank of NCBI.

Results

Morphology features and growth rate of endophytic fungi

Five endophytic fungi were isolated and purified, and there was high morphological variation between isolates from *C. bonariensis* in the present study. The morphological features and growth rate of isolates are presented in Figure 1 and Table 2.

Identification of endophytic fungi

Isolates found in the present study were identified by a molecular-based approach in addition to morphological features. Following the submission of DNA sequence in GenBank of NCBI, the accession numbers of isolates were obtained and are presented in Table 3. Furthermore, the evolutionary relationship between isolates of the present study and previously reported isolates is portrayed in Figure 2.

Antimicrobial activity of endophytic fungi crude extracts

The antimicrobial activity of endophytic fungi crude extract from *Conyza bonariensis* leaves was investigated using the disc diffusion method. Figure 3 below displays zones of inhibition against tested microorganisms for some of the crude extracts (crude extracts of isolate A and C) evaluated in the present study.

Crude extract of endophytic fungi from *C. bonariensis* had antimicrobial activity against selected tested microorganisms (Fig. 3). In Table 4, the results clearly show that the antimicrobial activity of crude extract of endophytic fungi in the present study has significant antimicrobial activity. Specifically, *N. crassa* crude extract had the best antimicrobial activity as compared to other crude extracts (Table 3). Furthermore, the antimicrobial activity of crude extract of *N. crassa* had higher antimicrobial activity against some of the test microorganisms as compared to the positive control (chloramphenicol and fluconazole, 0.25 mg/mL) based on the activity indexes information (Table 2).

The minimum inhibition concentration

The MIC values for each of the test organisms as portrayed in Table 4. In summary, endophyte C crude extract had highest MIC range of 1.5 µg/mL>MIC>0.78 µg/mL in all (*B. subtilis, E. coli, S. typhi, C. albicans*) tested microorganisms with the exception of *S. aureus* (Table 4).

Chemical composition of endophytic fungi crude extracts

Chemical profiles of crude extracts of endophytic fungi isolated from *C. bonariensis* were established by

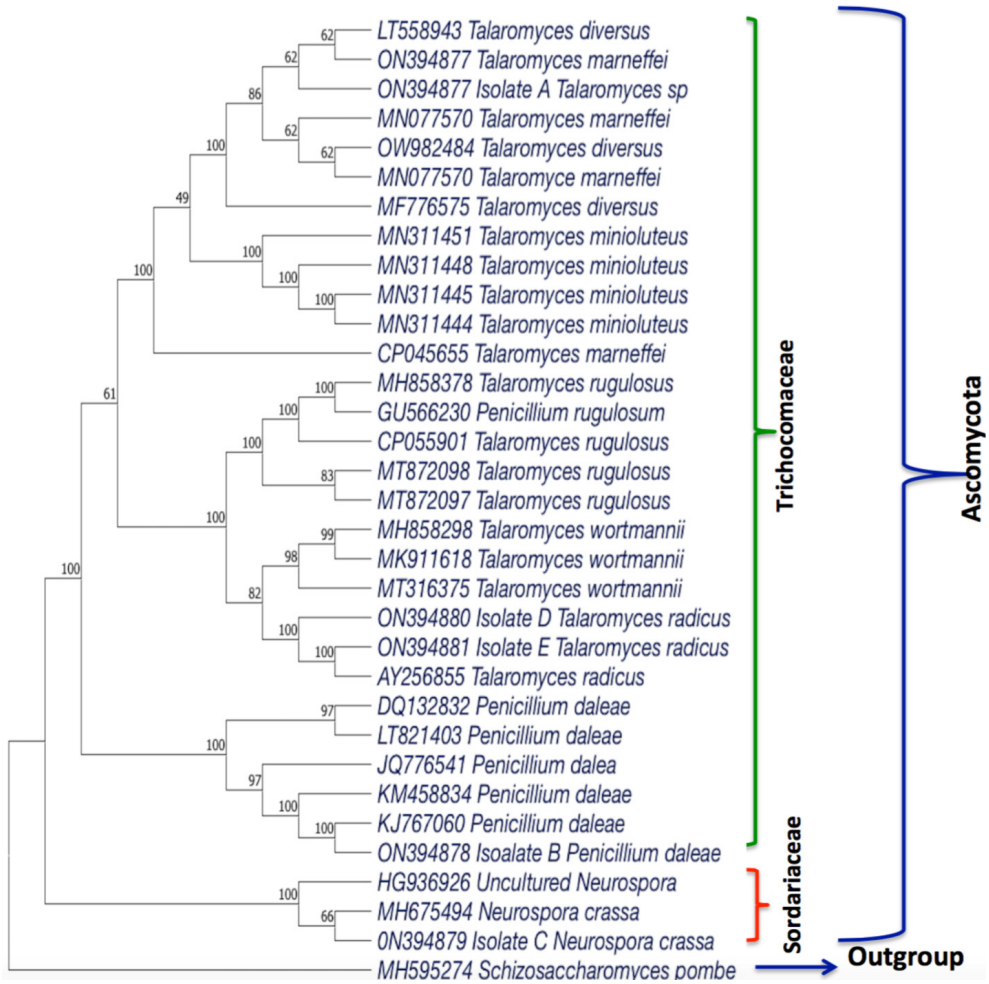

Figure 2. A UPGMA phylogenetic tree depicting the relationship of *Talaromyces* sp. (ON394877), *Talaromyces radicus* (ON394880), *Talaromyces radicus* (ON394881), *Penicillium daleae* (ON394878) and *N. crassa* (ON394878) with other related fungi obtained from GenBank. The *Schizosaccharomyces pombe (*MH595274.1) was used as an out-group. Numbers depicted on the branches represent values based on 1000 replications of Felsenstein's Bootstrap method.

performing chemical analysis using GC-MS. Table 5-9 display the percentage composition based on peak area of various compounds that were detected in the study. Also, biochemical information outlined in Table 5-9 is complimented by information found in the corresponding chromatograms (Fig. 4-8).

Chemical profiles of crude extracts were highly variable among endophytic fungi crude extracts (Fig. 4-8). Specifically, high (49%) composition of long-chain alkenes was observed in ethyl acetate crude extract of *Talaromyces* sp. (Table 5, Fig. 4). On the other hand, the high composition of fatty alcohols and alkenes was observed in crude extract of *Penicillium daleae* (Table 6, Fig. 5). In crude extract of *Penicillium daleae*, the 11-octadecenoicacid, methyl ester had a higher percentage composition (20.52%), and cis-10-nonadecenoic acid methyl ester had the lowest composition (3.61%) as shown in Table 6. Likewise, long-chain fatty alcohols and alkenes were predominated compounds in ethyl acetate crude extract of *N. crassa* (Table 7, Fig. 6). The percentage (24%) compositions of n-nonadecanol-1 was the highest; whereas, 11-octadecenoic methyl ester (3.8%) composition was the lowest (Table 7). However, some compounds had no match with the reference ion shown in Table 7 indicating the presence of novel compounds in crude extract of *N. crassa*.

Although the two extracts of *Talaromyces radicus* (*Talaromyces radicus* 1 and *Talaromyces radicus* 2*)* had antioxidant No. 33 compound (Table 8-9), but the chemical composition profiles of the two extracts were not similar (Fig. 7-8). The antioxidant No. 33 percentage composition was higher (45%) in *T. radicus* 2 as compared to percentage composition (33%) of *T. radicus* 1. For *T. radicus* 1 crude extracts, pentadecanoic acid was the second in composition proportion (33%); whereas, for *T. radicus* 2, Acetamide, 2,2-dichloro-N-[2-hydroxy-1-

Table 4. *Conyza bonariensis* endophytic fungi crude extracts minimum inhibitory concentrations (mg/mL) for all except C (µg/mL) observed in the present study (A: *Talaromyces* sp. (ON394877); B: *Penicillium daleae* (ON394878); C: *N. crassa* (ON394878); D: *T. radicus* (ON394880); E: *T. radicus* (ON39881)).

Crude extract	Test organism				
	S. aureus **(ATCC29213)**	***B. subtilis*** **(ATCC6051)**	***E. coli*** **(ATCC8736)**	***S. typhi*** **(ATCC 6539)**	***C. albicans*** **(DSM 1665)**
A	25>MIC>12.5	25>MIC>12.5	25>MIC>12.5	25>MIC>12.5	25>MIC>12.5
B	-	25>MIC>12.5	25>MIC>12.5	-	-
C	4010^{-3}>MIC>2010^{-3}	$15x10^{-4}$>MIC>7.810^{-4}	$15x10^{-4}$>MIC>7.810^{-4}	$15x10^{-4}$>MIC>7.810^{-4}	$15x10^{-4}$>MIC>7.810^{-4}
D	100>MIC>50	100>MIC>50	100>MIC>50	100>MIC>50	-
E	50>MIC>25	25>MIC>12.5	50>MIC>25	25>MIC>12.5	25>MIC>12.5

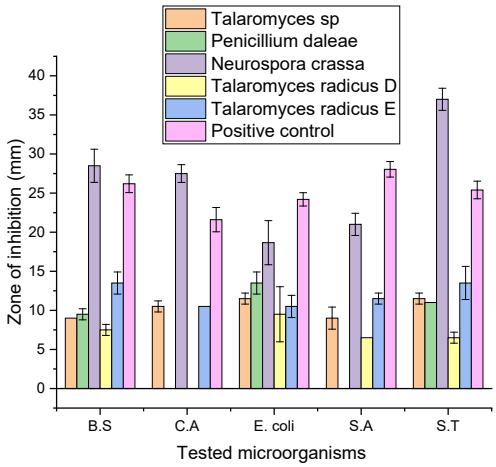

Figure 3. A bar graph portraying inhibition zones (mm) of endophytic fungi ethyl acetate crude extracts in the present study; error bars represent standard deviation; Positive controls were 0.25 mg/mL of chloramphenicol and fluconazole for bacteria and fungi, respectively**.**

(hydroxymethyl)- 2-(4-nitrophenyl)ethyl] was second in composition proportion (33%).

Discussion

Medicinal plants have been reported as the major source of secondary metabolites (Abdollahi *et al*., 2012; Amoateng *et al*., 2018; Frey & Meyers, 2010). Studies have been performed to identify novel compounds with biological potentials such as antimicrobial, antioxidant and anti-inflammatory (Kianfé *et al*., 2020). However, the direct harnessing of medicinal plants for the treatment of microbial infections has environmental implications. Therefore, environmentally friendly sources of secondary metabolites from endophytic fungi are highly desirable. In

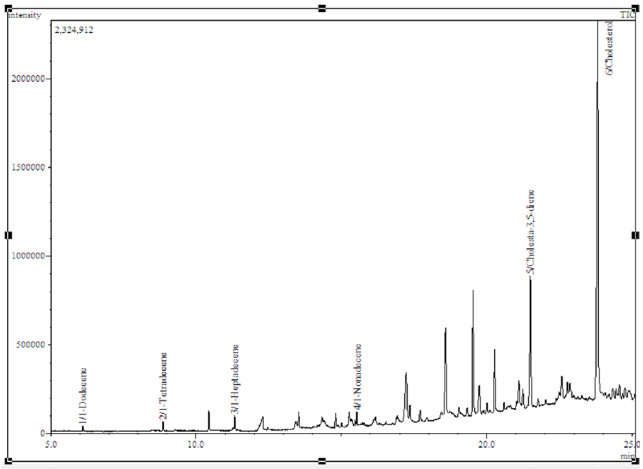

Figure 4. Chromatogram of isolate A, *Talaromyces* sp. (ON394877), ethyl acetate crude extract from *Conyza bonariensis* leaf.

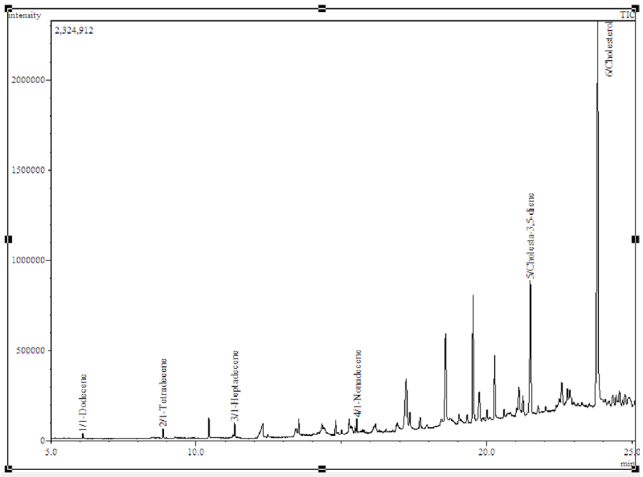

Figure 5. Chromatogram of crude extracts from isolate B, *Penicillin daleae* (ON394878), from *Conyza bonariensis* leaf

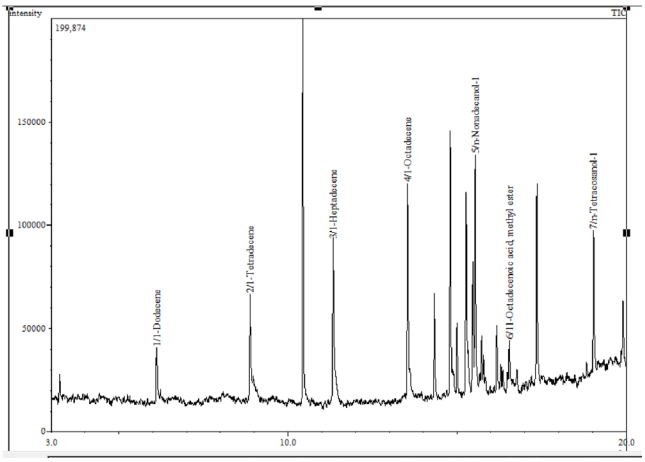

Figure 6. Chromatogram of crude extracts from isolate C, *Neurospora crassa* (ON394879) from *Conyza bonariensis* leaf.

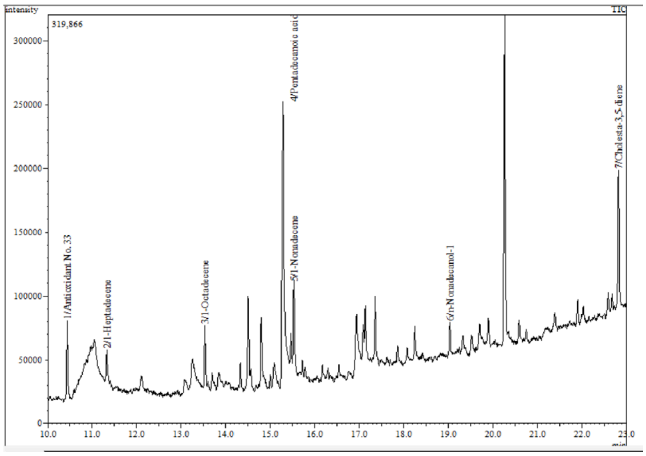

Figure 7. Chromatogram of ethyl acetate crude extracts of isolate D, *Talaromyces radicus* 1 (ON394880) from *Conyza bonariensis* leaf.

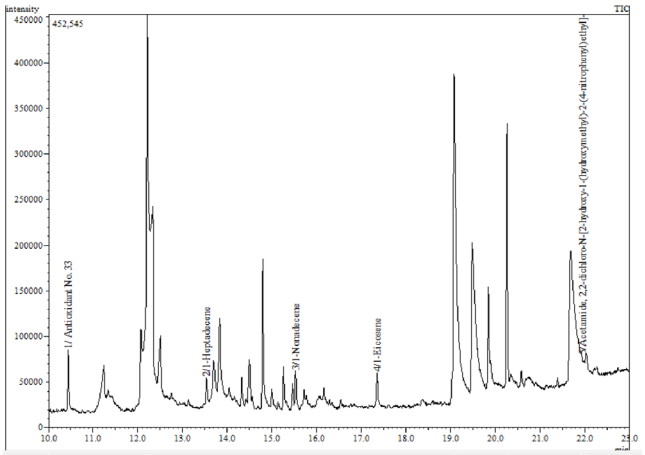

Figure 8. Chromatogram of crude extracts of isolate E, *Talaromyces radicus* (ON39488) from *Conyza bonariensis* leaf.

the present study, it was hypothesized that crude extract of endophytic fungi from *Conyza bonariensis* crude may have secondary metabolites with antimicrobial activity because the Safwa tribe of the Mbeya region of Tanzania has long traditionally used *C. bonariensis* leaves for treatment of microbial infections. Also, the same plant, experimentally, has been demonstrated to have antimicrobial activity (Araujo *et al*., 2013; Ghwanga & Chacha, 2019; Girma & Jiru, 2021). Indeed, results from the present study have supported this hypothesis because crude extracts of endophytic fungi from *C. bonariensis* had antimicrobial activity even higher compared to the antimicrobial activity of positive controls (Table 3, Fig. 3).

In this study, five endophytic fungi *(Talaromyces* sp., *Penicillum dalea*, *Neurospora crassa, Talaromyces radicus,* and *Talaromyces radicus*) were isolated. Based on the genetic relationship as depicted in Figure 2, all five isolates belong to the phylum Ascomycota. Furthermore, except for one isolate (*N. crassa*), which belongs to the family Sordariaceae, all other four isolates (*Talaromyces* sp., *Penicillum dalea*, *Talaromyces radicus* 1 and *Talaromyces radicus* 2*)* are members of the family Trichocomaceae (Fig. 2). Interestingly, all isolates are a member of the division Pezizomycotina of the Phylum Ascomycota (Amiri & Tibuhwa, 2020). Also, two isolates (D and E) were of the same species *(Talaromyces radicus),* which suggests that the two isolates may be of different strains, and because in the present study genotyping involved only the ITS region for identification of isolates, it is challenging to use the same information to decipher the two isolates to strain level. Furthermore, endophytic fungi isolated in the present study belong to the division Pezizomycotina not surprising because it is well known that most of the endophytes belong to the division Pezizomycotina (Amiri & Tibuhwa, 2020). In addition, the number of endophytes isolated from the present study is relatively low as compared to other studies (Mwanga *et al*., 2019; Yoon *et al*., 2018). This difference may be caused by the nature of the experiment. In the study, only *C. bonariensis* leaves were involved in the isolation of endophytes, and may be *C. bonariensis* leaves have a relatively lower richness of endophytic fungi.

In the present study antimicrobial activity of crude extracts of endophytic fungi was highly variable (Fig. 3). The variability in antimicrobial activity may be explained by the nature of endophytes isolated in the present study. The information generated in the present study indicates that endophytes were able to generate a variety of secondary metabolites with different levels of antimicrobial activity. Interestingly, taken as an example, the two species

Table 5. Chemical composition of isolate A, *Talaromyces* sp. (ON394877), crude extract from *Conyza bonariensis* leaves (R. time: Retention time, P. area: Peak area, P. height: Peak height).

Name of compounds	(% Composition P. area)	R. time	Chemical formula	Target peak	P. area	P. height	Activity
1-Dodecene	2.9206	6.107	$C_{12}H_{24}$	55.05	6723	3749	Antibacterial (Al-Abd *et al*., 2015; Khan *et al*., 2021; Xiong *et al*., 2013)
1-Tetradecene	4.90402	8.869	$C_{14}H_{28}$	55.1	11371	6295	Antimicrobial and antioxidant (Gautam *et al*., 2018)
1-Heptadecene	6.74488	11.329	$C_{17}H_{34}$	57.1	16273	8658	Antifungal (Mou *et al*., 2013)
1-Nonadecene	6.22916	15.533	$C_{19}H_{38}$	97.2	15001	7996	Anti-tuberculosis and antifungal (El-fayoumy *et al*., 2021)
Cholesta-3,5-diene	30.15565	21.505	$C_{27}H_{44}$	368.5	103942	38709	Wound healing (Bae *et al*., 2018)
Cholesterol	49.04568	23.813	$C_{27}H_{46}O$	386.5	210838	62957	Anticancer, antimicrobial, anti-psychotic, antioxidant (Albadawi *et al*., 2017)

Table 6. Chemical composition of crude extracts from isolate B, *Penicillin daleae* (ON394878), from *Conyza bonariensis* (R. time: Retention time, P. area: Peak area, P. height: Peak height).

Name of compounds	(% composition P. area)	R. time	Chemical formula	Target peak	P. area	P. height	Activity
1-Dodecene	3.61388	6.115	$C_{12}H_{24}$	55.1	9982	4083	Antibacterial (Al-Abd *et al*., 2015; Khan *et al*., 2021; Xiong *et al.,* 2013)
1-Tetradecene	6.5188	8.874	$C_{14}H_{28}$	55.1	16716	7365	Antimicrobial and antioxidant (Gautam *et al*., 2018)
1-Heptadecene	10.65312	11.332	$C_{17}H_{34}$	57.1	24598	12036	Antifungal
1-Octadecene	17.14182	13.535	$C_{18}H_{36}$	57.1	37753	19367	Antibacterial, antioxidant anticancer (Tonisi *et al*., 2020)
9-Hexadecenoic acid, methyl ester, (Z)-	5.62041	14.682	$C_{17}H_{32}O$	55.1	11971	6350	Antioxidant (Rahman *et al*., 2014)
11-Hexadecenoic acid, methyl ester	4.14494	14.729	$C_{17}H_{32}O_2$	55.1	8759	4683	Antioxidant, antimicrobial and ant-inflammatory (Yang *et al*., 1999)
n-Nonadecanol-1	19.11383	15.533	$C_{19}H_{40}O$	57.1	39401	21595	Antimicrobial and cytotoxicity (Begum *et al*., 2016)
11-Octadecenoic acid, methyl ester	20.52469	16.593	$C_{19}H_{36}O_2$	55.1	43122	23189	Antioxidant and antimicrobial (Rahman *et al*., 2014)
cis-10-Nonadecenoic acid, methyl ester	3.61211	17.551	$C_{20}H_{38}O$	55.1	7862	4081	Antibacterial and antioxidant (Gıdık, 2021)
n-Tetracosanol-1	9.05639	19.038	$C_{24}H_{50}O$	97.15	21446	10232	Antioxidant (Begum *et al*., 2016)

of *T. radicus*, which are said to might be different at the strain level, had different levels of antimicrobial activity, whereas *T. radicus* of isolate E had higher antimicrobial activity as compared to *T. radicus* isolate D (Table 3). A similar observation has been reported in other studies (Amiri & Tibuhwa, 2020; El-Said *et al*., 2016; Mwanga *et al*., 2019). The variation in antimicrobial activity may be explained by the difference in the chemical composition of secondary metabolites found in crude extract from particular endophyte. The GC-MS results from this study clearly show the difference in chemical composition and abundance of crude extracts (Table 5-9).

Table 7. Chemical composition of ethyl acetate crude extracts of isolate C, *Neurospora crassa* (ON394879), from *Conyza bonariensis* (R. time: Retention time, P. area: Peak area, P. height: Peak height).

Name of compounds	(% Composition P. area)	R. time	Chemical formula	Target peak	P. area	P. height	Activity
1-Dodecene	7.22334	6.12	$C_{12}H_{24}$	55.1	9695	3481	Antibacterial (Al-Abd *et al*. 2015; Khan *et al*., 2021; Xiong *et al*., 2013)
1-Tetradecene	11.83416	8.879	$C_{14}H_{28}$	55.1	15471	5703	Antimicrobial and antioxidant (Gautam *et al*., 2018)
1-Heptadecene	16.76869	11.338	$C_{17}H_{34}$	57.1	22560	8081	Antifungal (Mou *et al*., 2013)
1-Octadecene	22.28217	13.542	$C_{18}H_{36}$	57.1	25050	10738	Antibacterial, antioxidant anticancer (Tonisi *et al*., 2020)
9-Hexadecenoic acid, methyl ester, (Z)-	Ratio of reference ion does not match.	0	$C_{17}H_{32}O$	55.1	0	0	Antioxidant (Rahman *et al*., 2014)
11-Hexadecenoic acid, methyl ester	Ratio of reference ion does not match.	0	$C_{17}H_{32}O_2$	55.1	0	0	Antioxidant and antimicrobial (Rahman *et al.,* 2014)
n-Nonadecanol-1	24.00863	15.537	$C_{19}H_{40}O$	57.1	24599	11570	Antimicrobial and cytotoxicity (Begum *et al*., 2016)
11-Octadecenoic acid, methyl ester	3.84097	16.547	$C_{19}H_{36}O_2$	55.1	4433	1851	Antioxidant and antimicrobial (Mazumder *et al*., 2020)
cis-10-Nonadecenoic acid, methyl ester	Ratio of reference ion does not match.	0	$C_{20}H_{38}O_2$	55.1	0	0	Antibacterial and antioxidant (Gıdık, 2021)
n-Tetracosanol-1	14.04204	19.039	$C_{24}H_{50}O$	97.15	13554	6767	Antioxidant (Begum *et al*., 2016)

Table 8. Chemical composition of ethyl acetate crude extracts of isolate D, *Talaromyces radicus* (ON394880), from *Conyza bonariensis* (R. time: Retention time, P. area: Peak area, P. height: Peak height).

Name of compounds	(% composition P. area)	R. time	Chemical formula	Target peak	P. area	P. height	Activity
Antioxidant No. 33	32.64824	10.437	$C_{14}H_{22}O$	191.15	41231	22239	Antioxidant (Kahkönen & Heinonen, 2003)
1-Heptadecene	3.66428	11.33	$C_{17}H_{34}$	57.1	4922	2496	Antifungal (Mou *et al*., 2013)
1-Octadecene	6.872	13.533	$C_{18}H_{36}$	57.1	8420	4681	Antibacterial, antioxidant, anticancer (Tonisi *et al*., 2020)
Pentadecanoic acid	33.26189	15.289	$C_{15}H_{30}O_2$	73.05	69544	22657	Antibacterial and antifungal (Yoon *et al*., 2018)
1-Nonadecene	10.35424	15.532	$C_{19}H_{38}$	57.1	15721	7053	antimicrobial properties (El-fayoumy *et al*., 2021)
n-Nonadecanol-1	4.49814	19.039	$C_{19}H_{40}O$	57.1	6455	3064	Antimicrobial and cytotoxicity (Begum *et al*., 2016)
Cholesta-3,5-diene	8.70121	22.825	$C_{27}H_{44}$	368.35	11788	5927	Antimicrobial (Al-Hassan *et al*., 2020)

In the present study, *N. crassa* ethyl acetate crude extract shows the highest antimicrobial activities compared to other ethyl acetate crude extracts of endophytic fungi (*Talaromyces* sp., *Talaromyces radicus* 1, *Talaromyces radicus* 2, and *Penicillum dalea*) as shown in Figure 3 and Table 3. Furthermore, the MIC of *N. crassa* is highest compared to the positive control against several test microorganisms as shown in Figure 3. The differences in MIC among endophytic fungi crude extracts may be due to differences in chemical compositions (Santos *et al*., 2015; Selvi, 2014). The antimicrobial performance of crude extracts of *N. crassa* reported in the present study was even higher compared to the positive control (20 µL of 0.25 mg/mL of chloramphenicol). Furthermore, crude extracts of *N. crassa* had a higher performance as compared to previous studies (Ashok *et al*., 2015; Duhan *et al*., 2020). The variation in antimicrobial activity performance between studies exhibited by *N. crassa* has a multifaceted explanation. The genetic variability of *N. crass* in different studies is one of the possible explanations. Also, differences in tested organisms

Table 9. Chemical composition of ethyl acetate crude extracts of isolate E, *Talaromyces radicus* (ON39488), from *Conyza bonariensis* (R. time: Retention time, P. area: Peak area, P. height: Peak height).

Name of compounds	(% composition P. area)	R. time	Chemical formula	Target peak	P. area	P. height	Activity
Antioxidant No. 33	45.33531	10.442	$C_{14}H_{22}O$	191.15	48469	23850	Antioxidant (Kahkönen & Heinonen, 2003)
1-Heptadecene	6.52372	13.537	$C_{17}H_{34}$	57.1	8633	3432	Antifungal (Mou *et al.*, 2013)
1-Nonadecene	7.84862	15.534	$C_{19}H_{38}$	97.1	9012	4129	Antimicrobial (El-fayoumy *et al*., 2021)
1-Eicosene	6.94571	17.358	$C_{20}H_{40}$	57.1	7580	3654	Antioxidant and antimicrobial (Hsouna *et al*., 2011)
Acetamide, 2,2-dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]-	33.34664	21.693	$C_{11}H_{12}Cl_2N_2O_5$	153.05	107771	17543	Antimicrobial (Abu-Khadra *et al*., 2016)

and extracting solvents used in studies are other possible explanations for the observation. Furthermore, a growth difference in growth condition may be another possible reason for difference in antimicrobial activity between the present study and previous reports.

The results from this study have shown the potential use of endophytic fungi from *C. bonariensis* as an alternative source of bioactive secondary compounds with antimicrobial activity. Ethyl acetate crude extracts of *N. crassa* demonstrated the highest antimicrobial activity, therefore warranting further exploration of the endophyte and their crude extracts for various medicinal and industrial applications. Specifically, studies on the characterization of pure compound(s) that may be responsible for antimicrobial activity are urgently needed. This may result in the discovery of novel drugs.

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