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RESEARCH NOTE

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First Report: Diversity of Endophytic fungi Possessing Antifungal Activity Isolated from Native Kougoed (*Sceletium tortuosum* L.)

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ABSTRACT

Forty-three ($n = 43$) endophytic fungi with different morphologic characteristics were from a medicinal plant *Sceletium tortuosum,* were utilized to investigate their antifungal effectiveness against pathogenic fungi. All fungal isolates exhibited antifungal activity against one or more pathogens in the dual culture test whereas only 33 fungal culture filtrates (77%) showed decent antifungal effect. *Fusaria* and *Aspergillus* were the dominate genus that displayed significant antifungal activity. Isolates GG02, GG09, ND15, and ND17 showed the broadest spectrum of antifungal activity. Furthermore, culture filtrate of Fusarium sp. DR08 exhibited a broad range of antifungal activity against all the pathogens. The results suggest endophytic fungi isolated from medicinal plant might be a source of novel bioactive molecules. To the best our knowledge, this is the first report on endophytic fungi isolated from native kougoed exhibiting antifungal activity against plant fungal pathogens.

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Novel biotechnological approaches that focuses on the utilization of bioactive compounds isolated from endophytes for a broad spectrum of biological potentials, including antibiotics, antiviral, immunosuppressant, antiparasitic, and antifungal activities, have sparked tremendous interest in recent research [1]. Historically, ground breaking bioactive compounds isolated from endophytic fungi have been commercialized as pharmaceutical drugs from penicillin, camptothecin, podophyllotoxin to taxol [2]. Endophytic fungi live inside a plant tissue with no harmful symptoms observed. They are able to protect the plant from pathogens, to enhance their growth, and to avoid from herbivores [3]. It has been reported by various studies that plants colonized by endophytic fungi represent an essential source of fungal diversity and novel species [4].

The mutual co-exist in both the healing and unhealthy tissue highlights the uncertainty of boundaries in differentiating facultative pathogens, endophytes and latent pathogens. Fungi has a pathogenic nature that lead to plant decay and economic loss. A fungal outbreak can lead to a human disaster when it destroy the staple crops.

The survival spores, widespread by wind and massive abundance in soil, result in a rapid spread of fungal pathogens in nature. Plant pathogens not only affect the plant cultivation but also influence post-harvest storage and distribution before the consumption of agricultural products such as grains, roots, and fruits. Pathogenic fungi in the soil can result in difficulty for the same area to be used to grow vulnerable crops. They have caused terrible agricultural and economic impact. The aim of this present study is to investigate the antifungal potential of the endophytic fungal community isolated from native *Sceletium tortuosum* L. Bioactive compounds produced by endophytic fungi which are isolated from the host plant might be used for medicine and agriculture purposes [4]. In this study, a total of 43 endophytic fungi were isolated from healthy

S. tortuosum plants in South Africa and selected based on their biological activity. Deposit the ITS and EF1a DNA sequences to GenBank and provide GenBank accession numbers in Table 1.

The identities of the fungal isolates were determined through morphological and molecular identification using internal transcribed spacer (ITS) and elongation factor 1-alpha (EF-1α) primers as reported in a previous finding [3].

Endophytic fungi were subjected to fermentation process in order to produce the secondary metabolites. Each of the fungal isolate was placed in a 50 mL of malt extract broth, 250 mL of Erlenmeyer flasks. Rotary shaker (Labcon FSVE-Spo8, Gauteng, South Africa) was set at 150 rpm and fungal isolates were incubated (Labcon FSVE-Spo8) for 5 days at

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Table 1. Identification of endophytic fungi and activity against pathogenic fungi of plants according to the dual culture technique.

Strains	Distinguishing morphological characteristics on PDA	Identified as	Potential antifungal activity					
			A	B	C	D	E	F
GG01	Brown green colony that looks like an algae swamp, white on reverse	*Aspergillus* sp	+++	+++	+++	+++	+++	+++
GG02	Woolly growth resembling cotton candy. New growth is white in color but turns a grayish-brown with aging. The reverse is pale white	*Mucor circinelloides*	+++	+++	+++	+++	+++	+++
GG05	Colonies are brown greenish in color with white edges	*Fusarium solani*	++	++	+++	++	++	++
GG06	Pale white colony, very thin colony	*Ceratobasidium* sp	+++	+++	++	+++	++	++
GG09	Cream white with mix of slightly yellow colonies	*Neurospora* sp	+++	+++	+++	+++	+++	++
GG10	Powdery colonies with a characteristic buff or cinnamon-brown color on the surface and a yellow to beige-brown color on the reverse	*Aspergillus terreus*	+++	++	++	++	+++	++
GG11	White hairy cotton that grew up, fluffy like form	*Fusarium solani*	++	++	+++	++	++	++
GG12	White cottony colonies with the aerial mycelia becoming tinged in purple. The reverse was a rather non-descript pale to yellow	Fusarium oxysporum	+	++	+++	++	++	++
GG13	Powdery, showing various shades of green, blue-green to a grey-green with a narrow white border. The reverse is white to tan to pale yellowish	*Aspergillus fumigatus*	+	++	+++	++	++	++
GG14	White to yellowish felt-like mat of mycelia. Reverse is white to pale in color	*Aspergillus niger*	+	++	++	++	++	++
GG15.1	Brown green colony that looks like an algae swamp, white on reverse	*Aspergillus* sp	++	++	++	++	++	+
GG15.2	Brown green colony that looks like an algae swamp, white on reverse	*Aspergillus* sp	++	++	+++	++	++	++
GG16	Colony is brown/ green color with white and blue	*Aspergillus niger*	++	++	+++	+++	++	++
ND02	The surface of the colony is velvety, downy or powdery, having different shades of green, blue-green with a narrow white border. Reverse is white to pale yellow	*Aspergillus fumigatus*	+	++	+++	++	++	++
ND04	White mycelium cotton in the center of the colony but grows to become green.	*Penicillium echinulatum*	+	++	++	++	++	++
ND06	The colony is of a thin layer on the agar that is pale white	*Penicillium sp*	++	++	++	++	++	++
ND07	Pale white with a thin layer on the agar	*Geotrichum* sp	++	++	+++	+	+++	++
ND08	Grey to olive brown on the surface with short aerial hyphae, brown-black on reverse due to pigment production	*Alternaria* sp	+	++	+++	++	++	++
ND09	The colony has a white fur center that is surrounded by a blue-greenish part	*Aspergillus* sp	++	++	++	++	++	++
ND10	White center with yellow bubbles and grows to become green with white fur	*Alternaria* sp	+	++	++	+++	++	++
ND11	White mycelium cotton in the center of the colony but grows to become green		+	++	++	+	+	++
ND13	White fluffy cotton like colony with a pink center	*Fusarium oxysporum f.* sp. *ciceris*	++	+++	+++	+++	+++	+++
ND14	The colony is green brownish with a white fur layer above	*Aspergillus fumigatus*	+	++	+++	++	++	++
ND15	Black spores held by yellowish hyphae with a white surface	*Aspergillus niger*	+++	+++	+++	+++	++	+++
ND17	Yellow colony on the surface with black spores shooting up from the colony supported a very thin hyphae like	*Aspergillus* sp	+++	+++	+++	+++	+++	+++
ND19	The colony is green brownish with a white fur layer above	*Fusarium oxysporum f.* sp. *lycoperscici*	++	++	+++	++	++	++
DR03	White hairy cotton that has pink root like structure spreading out of it, in the surface with white reverse	*Coniothyrium aleuritis*	++	++	+++	+++	++	++
DR04	Green brownish colony with blue and white edges	*Fusarium oxysporum f.* sp. *ciceris*	+	++	+++	++	++	++
DR08	White hairy cotton that grew up, fluffy like form	*Fusarium* sp.	++	++	++	++	++	+
DR09	White thick cotton in the center and the edges of the cotton grew cream white like roots, growing out and long	*Fusarium equiseti*	++	++	++	++	++	+
DR10	Green brownish colony with blue and white edges	*Phomopsis columnaris*	+	++	+++	+	++	++
DR12	Green brown colonies with gold bubbles on top	*Pythium heterothallicum*	+	++	++	+	++	+++
DR14.1	Green brownish colony with blue and white edges	*Neonectria* sp.	+	++	+++	++	++	++
DR14.2	Green brown colonies with gold bubbles on top	*Neonectria* sp.	++	+++	+++	++	++	++
DR16	Green smooth colony with white fur at the edges	*Cladosporium* sp	+++	+++	+++	+++	++	++
DR17	White fluffy cotton like colony	*Fusarium solani*	++	++	++	+	++	++
DR19	Green brown colonies with gold bubbles on top	*Fusarium penzigii*	++	++	+	++	++	++
DR20	The colony is green brown, with white and blue ends	*Fusarium subglutinans*	+	++	+++	++	++	++
DR21	Thick white cotton with fur growing upwards and reverse is light yellow	*Fusarium equiseti*	++	++	++	+++	+	+
DR22	White, pale thin layer of cotton	*Fusarium* sp.	++	++	++	++	++	++
DR23	White cotton in the middle with cream looking for growing from it like roots	*Fusarium oxysporum*	++	++	+++	+++	++	++
DR24	White fluffy colony also in reverse	*Fusarium* sp.	++	++	++	++	++	++

Width of growth inhibition zone $T = 0$ mm; -, $0 < T \leq 3$ mm; +, $3 < T \leq 5$ ++, T > 5mm +++.
(A) 1 *Aspergillus*; (B) M *Fusarium*; (C) 2929 *Fusarium oxysporum*; (D) 13071 *Borytis cinerea*; (E) 10139 *Fusarium graminearum*; (F) 12517 *Colletotrichum gleosporioides*.

25 °C. The culture broth was filtered through a 0.45 μm sterile Acrodisc syringe filter (Pall Life Sciences, Ann Arbor, MI). In the dual culture method, the assay was conducted to check the antifungal activity of the endophytic fungi against selected pathogenic fungi. Potato dextrose agar (PDA; Merck Biolab, Gauteng, South Africa) media was used to perform a 3-point inoculation of 6 mm disks of the endophyte with a pathogenic fungus at the center. The plates were inoculated 5–8 days at 28 ± 10 °C. Incubation of the plates can result in the interference of the pathogen in the direction of endophytic fungal growth [5].

Fungal culture filtrates were subjected to antifungal screening against six selected pathogenic fungi. Thirty milliliter of PDA medium was poured into a sterilized 90 mm petri dishes and supplemented with 2 mL of the fungal filtrate

PDA only was then poured as a control. Upon solidification, plant pathogens were inoculated at the center of the plate and the growth was measured by mycelial growth inhibition and calculated according to the formula.

The activity of the culture filtrate was calculated using the formula given $\mu = \mu_1 + \mu_2/N$ where μ= mean, $\mu_{1 \text{ and } 2}$ = the measured diameter on each plate and N = the number of plates representing a particular isolate. The standard deviation of each isolate was then calculated using the formula

$$\sigma = \sqrt{\frac{\sum(x_1-\mu)^2+(x_2-\mu)^2}{N}}$$

where σ is the standard deviation, N is the number of plates, μ is the mean, and $x_{1 \text{ and } 2}$ is the measured diameter on each plate. The reason for using these equations was to tell how diameters of the plates are spread out from the average (mean) or expected diameter. A low standard deviation meant that the diameters are close to the average. A high standard deviation means that the diameters are more spread out.

The dual culture assay showed that all forty-three endophytic fungi were inhibitory against one or more pathogens (Table 1). *Aspergillus* sp. (ND17), and *A. niger* (ND15) exhibited a broad spectrum of activity for all six pathogens. *A. fumigatus* isolates, GG13, ND02, and ND14, exhibited similar activity pattern against the same pathogens (Table 1). Diversity data show that *Aspergillus* genus attributed to 43%, followed by *Fusaria* with 29%, then we have 14% *M. circinelloides* and *Neurospora* respectively. *Fusarium oxysporum* with 37%, followed by *Borytis cinerea* 17%, then we had *Fusarium* sp. at 14% and *F. graminearum* with 10% was the least inhibited. Table 2 represents the antifungal effect of the selected endophytic fungi using filtrate culture assay. From a total of forty-three ($n = 43$) tested endophytic fungi, 77% showed inhibition activity against one or more pathogens (Table 2). Culture filtrates of the endophytic fungi DR08 exhibited a broad range of antifungal activity against all the pathogens. Least activity was displayed by DR04, DR18, DR14.1, and DR20. The greatest antifungal activity in dual culture was displayed by the isolate GG09, however, that was not the case in the culture filtrate where it showed almost no activity for pathogen *Fusarium* 2929 while showing complete inhibition for *B. cinerea* 10139 (D), *F. graminearum* 12517 (E), and *Colletotrichum gleosporioides* (F). Additionally, the results revealed that majority of the endophytic fungi extracts from the medicinal plant have partial antifungal activity. None of the isolates tested were able to control all six pathogenic fungi. From the diversity of the antifungal activity, *Fusaria* was dominate with 32%, followed by *Aspergillus* genus with 21% and *Alternaria* with 11% (Figure 1(A)). Endophytic fungi (DR08, *Fusarium* sp.) showed the most activity followed *Fusarium* sp. (DR24). *Aspergillus* sp. (ND9) showed the least activity against the pathogens. Among all the six pathogens, *F. graminearum* (G) and *Colletotrichum gleosporioides* (H) were less resistant. *Aspergillus* sp. which was isolated from maize was the most resistant pathogen. Figure 1(B,C) illustrates the activity that occurred between the six pathogens and the filtrates tested against. *Aspergillus* was the most resistant pathogen out of the six tested with 50% and *F graminearum* was the most inhibited pathogen with 35%. From a total of forty-three ($n = 43$) tested endophytic fungi, all (100%) exhibited inhibitory activity against one or more pathogens using dual diffusion assay. *Fusarium* and *Aspergillus* were the dominate genus that showed significate antifungal effect. Seventy-seven (77%) displayed inhibition activity against one or more pathogens. Endophytic fungi (DR08, *Fusarium* sp.) was the most effective and *Aspergillus* sp. (ND9) was the least activity.

Current global trend is moving toward a more sustainable, improved, safer and eco-friendly alternatives to the conventional resistant antimicrobial drugs on the market. Medicinal plants have shown to possess several biological activities that has tremendous health benefits. This set a tone in the investigation of significantly important microbial community that has a symbiotic interactions with plants [6]. *S. tortuosum* L. commonly known as kougoed is a native medicinal plant that has been used in indigenous tribes for stress relief, depression, as a painkiller, alleviate hunger and overall mood-enhancer [7]. Hence, *S. tortuosum* L. was selected in the current study for the screening of

Table 2. Activity of culture filtrates of endophytic fungi against pathogenic fungi of plants.

	Colony growth (mm)					
Fungi	A	B	C	D	E	F
GG01	2.3 ± 0.10	3.65 ± 0.35	2.60 ± 0.20	4.00 ± 0.40	3.10 ± 0.14	4.00 ± 0.20
GG02	2.35 ± 0.25	3.35 ± 0.55	5.25 ± 1.15	4.70 ± 0.61	2.65 ± 0.25	3.35 ± 0.45
GG05	8.05 ± 0.05	3.95 ± 0.15	5.60 ± 0.30	3.15 ± 1.65	5.15 ± 0.21	4.95 ± 0.15
GG06	1.60 ± 0.40	1.70 ± 0.10	3.00 ± 0.20	3.85 ± 0.25	0.00 ± 0.00	0.00 ± 0.00
GG09	1.60 ± 0.80	6.75 ± 0.25	3.80 ± 0.70	0.20 ± 0.20	0.00 ± 0.00	0.00 ± 0.00
GG10	7.60 ± 1.00	5.85 ± 1.05	7.20 ± 0.00	4.10 ± 0.50	3.95 ± 0.21	3.55 ± 0.05
GG11	8.55 ± 0.05	3.55 ± 0.35	2.00 ± 0.10	4.35 ± 0.05	3.05 ± 0.21	2.00 ± 0.14
GG12	6.95 ± 1.65	3.65 ± 1.15	6.30 ± 0.10	2.30 ± 0.00	4.90 ± 0.28	1.95 ± 0.21
GG13	8.60 ± 0.10	1.20 ± 0.00	2.60 ± 0.00	5.50 ± 2.91	1.40 ± 0.14	1.00 ± 0.14
GG14	7.35 ± 0.75	6.85 ± 0.75	7.20 ± 0.40	4.65 ± 0.45	3.65 ± 0.25	4.45 ± 0.45
GG15	7.35 ± 1.25	4.30 ± 0.10	6.95 ± 0.05	2.65 ± 0.75	0.00 ± 0.00	0.00 ± 0.00
GG15.2	8.20 ± 0.10	8.35 ± 0.21	7.15 ± 0.05	5.40 ± 0.50	3.00 ± 0.10	2.95 ± 0.05
GG16	7.95 ± 0.15	8.50 ± 0.10	6.55 ± 0.05	1.25 ± 0.35	0.10 ± 0.14	0.10 ± 0.14
ND02	7.55 ± 0.35	7.25 ± 1.15	5.75 ± 0.25	8.70 ± 0.00	4.50 ± 0.40	5.95 ± 0.15
ND04	2.00 ± 0.40	2.05 ± 0.05	8.70 ± 0.00	3.80 ± 0.40	0.10 ± 0.14	0.00 ± 0.00
ND06	2.70 ± 0.28	3.90 ± 0.28	4.65 ± 0.21	3.80 ± 0.14	0.90 ± 0.80	0.40 ± 0.20
ND07	3.20 ± 0.14	3.95 ± 0.07	5.00 ± 0.05	4.40 ± 0.14	0.65 ± 0.45	0.65 ± 0.45
ND08	2.50 ± 0.60	3.45 ± 0.25	1.55 ± 0.35	3.50 ± 0.40	3.4 ± 0.14	0.95 ± 0.15
ND09	2.40 ± 0.10	8.70 ± 0.00	8.70 ± 0.00	3.35 ± 0.25	1.30 ± 0.10	2.95 ± 0.05
ND10	8.25 ± 0.35	7.30 ± 0.50	5.40 ± 0.50	0.80 ± 0.10	3.70 ± 0.10	3.80 ± 0.20
ND11	8.45 ± 0.15	4.20 ± 0.10	5.45 ± 1.25	2.40 ± 1.20	0.00 ± 0.00	2.70 ± 0.20
ND14	6.20 ± 0.00	8.60 ± 0.00	6.75 ± 0.15	0.85 ± 0.50	4.10 ± 0.20	3.65 ± 0.35
ND17	1.75 ± 0.35	1.70 ± 0.10	0.50 ± 0.50	2.15 ± 0.05	1.50 ± 0.10	0.00 ± 0.00
ND19	2.70 ± 0.10	3.30 ± 0.10	6.25 ± 0.65	2.50 ± 0.30	0.95 ± 0.05	2.30 ± 0.20
DR03	0.00 ± 0.00	0.50 ± 0.10	1.65 ± 0.15	1.75 ± 0.35	1.35 ± 0.05	0.60 ± 0.92
DR04	8.70 ± 0.00	6.80 ± 0.30	4.90 ± 0.10	7.40 ± 1.30	3.75 ± 0.15	5.80 ± 0.20
DR08	2.45 ± 0.25	0.45 ± 0.45	1.45 ± 0.15	0.00 ± 0.00	0.70 ± 0.20	0.40 ± 0.10
DR09	3.55 ± 0.45	2.3 ± 0.10	3.15 ± 0.15	4.40 ± 0.20	2.80 ± 0.10	1.85 ± 0.25
DR10	5.74 ± 0.15	5.40 ± 1.00	4.90 ± 0.50	7.15 ± 0.25	5.15 ± 0.05	5.80 ± 0.20
DR12	3.85 ± 0.25	7.05 ± 0.45	3.45 ± 0.45	2.90 ± 0.20	1.75 ± 0.35	2.00 ± 0.20
DR14.1	7.55 ± 0.25	8.40 ± 0.10	7.85 ± 0.05	6.55 ± 0.05	8.70 ± 0.00	6.65 ± 0.45
DR14.2	8.50 ± 0.20	2.45 ± 0.05	3.55 ± 0.45	5.45 ± 0.15	0.00 ± 0.00	0.00 ± 0.00
DR17	2.40 ± 0.20	5.45 ± 0.65	2.50 ± 0.30	3.00 ± 0.10	1.90 ± 0.00	1.50 ± 0.20
DR18	7.35 ± 0.53	7.60 ± 1.00	5.15 ± 0.05	4.7 ± 0.16	4.60 ± 0.50	2.10 ± 0.00
DR19	3.15 ± 0.75	0.60 ± 0.30	1.95 ± 0.05	1.00 ± 0.10	0.00 ± 0.00	1.60 ± 0.20
DR20	7.50 ± 0.30	7.35 ± 0.25	4.60 ± 0.70	7.50 ± 1.20	8.70 ± 0.00	5.95 ± 0.05
DR21	6.65 ± 1.05	3.00 ± 0.90	4.00 ± 0.20	4.80 ± 0.22	4.05 ± 0.07	3.75 ± 0.21
DR22	1.95 ± 0.15	1.50 ± 0.16	0.75 ± 0.15	4.15 ± 0.35	0.00 ± 0.00	0.00 ± 0.00
DR23	8.00 ± 0.60	1.75 ± 0.15	0.00 ± 0.00g	4.80 ± 0.40	0.00 ± 0.00	0.00 ± 0.00
DR24	0.85 ± 0.50	1.30 ± 0.20	0.00 ± 0.00	3.35 ± 0.15	0.00 ± 0.00	0.95 ± 0.95

(A) 1 *Aspergillus*; (B) M *Fusarium*; (C) 2929 *Fusarium oxysporum*; (D) 13071 *Borytis cinerea*; (E) 10139 *Fusarium graminearum*; (F) 12517 *Colletotrichum gleosporioides*.
0.99 ± 0.00 to 0.00 ± 0.00 = most active filtrate, 8.70 ± 0.20= least active filtrate.

endophytic fungi that exhibit antifungal activity against fungal pathogens. Fungal pathogens cause diseases that pose serious concern in the agricultural sector. Subsequently leading to pathogenic fungi infects agricultural crop resulting in considerable losses of yield and economic losses which will have an impact on the global crop prices. Recent fungicide used for agricultural commodities are posing more shortcomings such as causing cancer, toxicity, and resistant [8]. Hence, more and more people are questioning the utilization of the current fungicide on the market and are looking for a more health benefit and eco-friendly approach. It was reported that approximately 12% of endophytic fungi have shown to exhibit antifungal activity against four plant fungal pathogens (*B. cinerea, Sclerotinia sclerotiorum, Rhizoctonia solani* and *F. oxysporum*) using dual-culture method. In the same study, *Penicillium* sp. and *A. oryzae* demonstrated to be the most effective [9]. In the current study, 100% of the endophytic fungi displayed inhibitory activity against one or more pathogens. *Aspergillus* strains were dominate in the antifungal bioassay using dual method. *Fusaria* genus was predominate followed by *Aspergillus* genus. It is not surprising, *Fusaria* genus are considered as an enormous group inhibiting plants, animals and humans as saprophytic, opportunistic and even symbiotic association [10]. Several studies have demonstrated that endophytic fungi producing a wealth of bioactive compounds have the potential to be an outstanding antifungal agent and fungicide [11–13].

In this study, we established that endophytic fungi isolated from medicinal plant of South Africa possess excellent antifungal properties against pathogenic fungi. Endophytic fungi are untapped territories filled with effective, novel bioactive compounds [2] to control fungal pathogens responsible for the mortality rate and high crop losses (Figure 2). In conclusion, these endophytic fungi have potential to be used as a natural biocontrol agent in the agricultural sector; antifungal agents in the medical as well

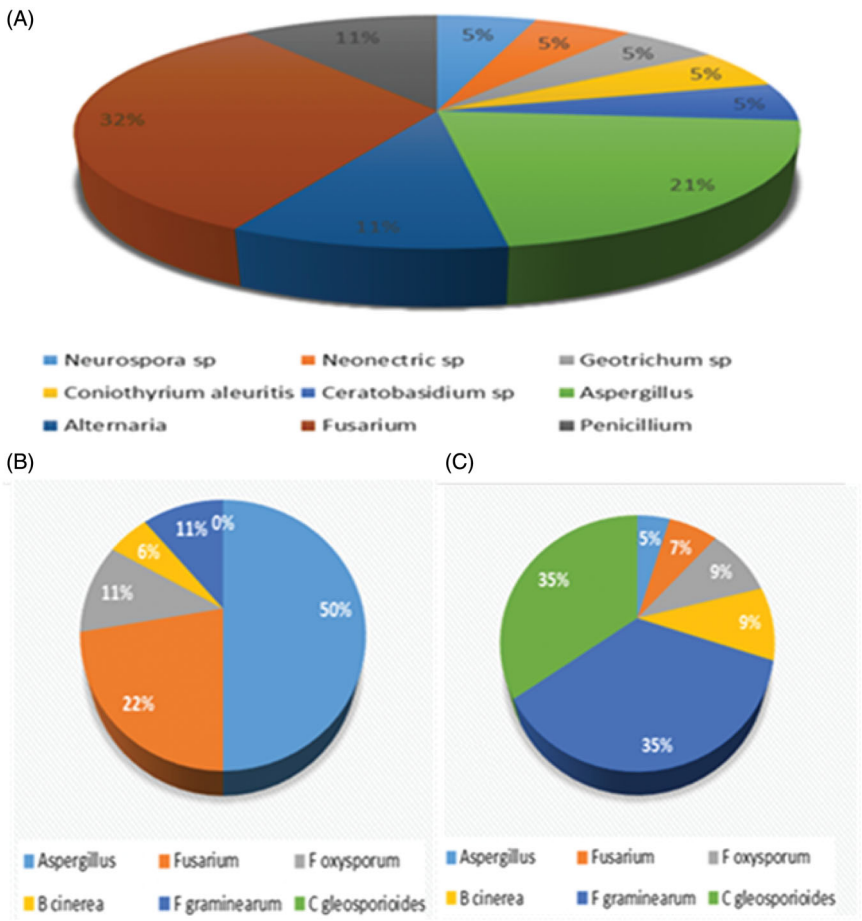

Figure 1. (A) Diversity of fungal extracts displaying antifungal activity, (B) resistant pathogens, and (C) least resistant pathogens; they were most inhibited by the filtrates.

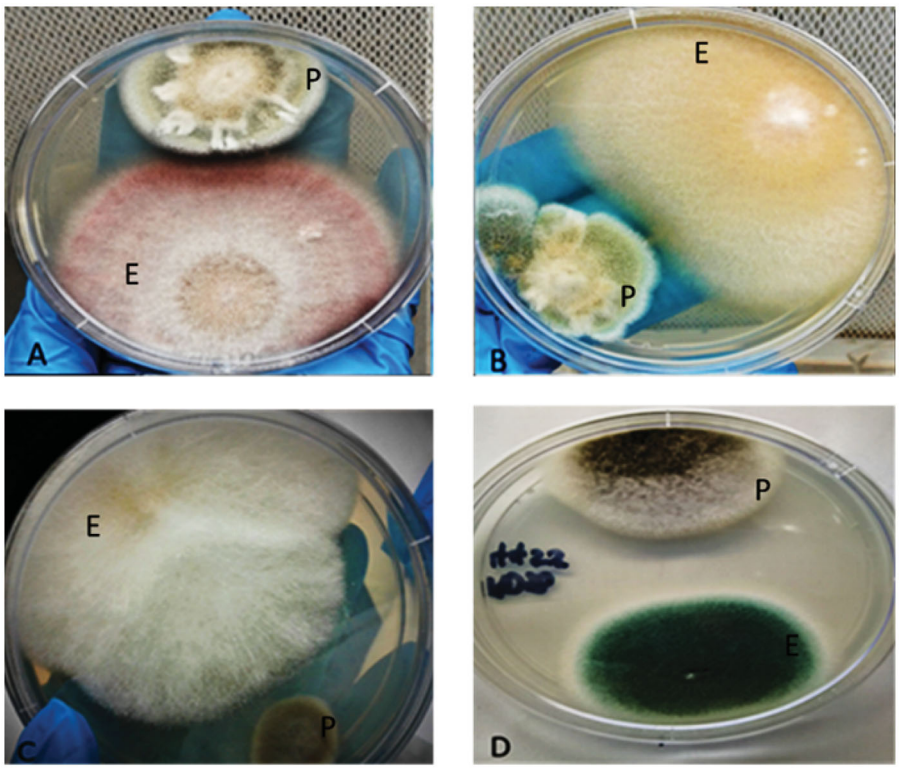

Figure 2. Endophytic fungi from *Sceletium tortuosum showing* activity in dual culture against fungal pathogens, E: endophytic fungi; P: pathogenic fungi.

as the pharmaceutical industries. To the best of our knowledge, this is the first report on antifungal activity of endophytic fungi isolated from *S. tortuosum* L. plants. Future research should focus on the isolation and characterization of these novel, lead bioactive compounds resulting in product development for a more efficacious, safer and eco-friendly fungicide and antifungal agent.

Disclosure statement

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