Exploring the biopotential of secondary metabolites derived from fungal endophytes of *Kirkia acuminata* stems for drug discovery

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Introduction

Antimicrobial resistance (AMR) is the ability of pathogens to resist contemporary antibiotics and leads to increased occurrences of infectious diseases, hospital stays and mortalities (van Elsland and Neefjes, 2018). Interestingly, prolonged AMR infections have been reported to induce cancer development in affected patients (van Elsland and Neefjes, 2018). Cancer results from the uncontrolled growth of oncogenic cells, leading to death in affected individuals and treatment failures as the available anticancer drugs are reportedly losing their efficacy and have severe side (Mongalo and Makhafola, 2018), highlighting the need for novel agents. Fungal endophytes from medicinal plants produce diverse and plant-similar bioactive metabolites with antimicrobial and anticancer potential (Rashid, 2021). However, there are limited studies on fungal endophytes from South African medicinal plants.

Kirkia acuminata Oliv. is an important medicinal plant used for its leaves, barks and roots to treat various ailments, such as thrush, cholera and diarrhea (Maroyi, 2017). However, owing to its therapeutic benefits, excessive harvesting has led to its decline in the wild (Mongalo and Makhafola, 2018). This highlights, the need to find other alternative sources for the plant's natural products, such as fungal endophytes. Thus, the current study aimed to isolate and screen the antimicrobial, cytotoxicity and anticancer activity of fungal endophytes from *Kirkia acuminata* Oliv. stems.

Methodology

Plant collection and isolation of endophytic fungi

- Healthy symptom-free stems of *K. acuminata* Oliv. were surface sterilised and plated on potato dextrose agar (PDA) plates at 28 °C for 5 - 7 d (Rashid, 2021).

Identification, fermentation and metabolite extraction

- Morphologically distinct pure isolates were identified through sequencing of the internal transcribed spacer (ITS) region.
- Hyphal plugs were fermented on potato dextrose broth (PDB) for 21 d at 25 °C (Rashid, 2021).
- Crude extracts were obtained by ethyl acetate extraction of filtered fermentation broth.

Antimicrobial screening

- The minimum inhibitory concentration (MIC) of crude extracts was assessed using broth microdilution method (Eloff, 1998).
- Microorganisms used: *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 15422), *Staphylococcus aureus* (ATCC 25923) and *Candida albicans* (ATCC 10231).

Cytotoxicity screening

- MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to assess viability of non-cancerous embryonic human kidney (HEK-293) cells treated with crude extracts (Hamid *et al*., 2004).
- Curcumin and DMSO were positive and negative controls, respectively.

Anticancer screening

- Alamar blue assay was used to assess antiproliferative activity of melanoma A375 and cervical cancer ME-180 cell lines treated with crude extracts (Hamid *et al*., 2004).
- Curcumin and DMSO were positive and negative controls, respectively.

Mycochemical analysis

- Folin–Ciocalteu reagent method was used to separately test the total phenolic and tannin contents, while the aluminium chloride method was used to test total flavonoid content of the crude extracts (Rashid, 2021).

References

- Eloff JN. (1998) 'A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria', *Planta Med*, 64:711–3.
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- Mongalo, N. I. and Makhafola, T. J. (2018) 'Ethnobotanical knowledge of the lay people of Blouberg area (Pedi tribe), Limpopo Province, South Africa', *Journal of Ethnobiology and Ethnomedicine*, 14(1), 46.
- Maroyi, A. (2017) '*Kirkia acuminata* Oliv.: A review of its ethnobotany and pharmacology', *African journal of traditional, complementary, and alternative medicines : AJTCAM*, 14(2), 217–226.
- van Elsland, D. and Neefjes, J. (2018) 'Bacterial infections and cancer', *EMBO reports*, 19(11), 46632.

Results

The ITS analysis of endophytic fungi

Table 1. Endophytic fungi isolated from host plant stem tissues

Isolate code	Endophytic fungi	NCBI Genbank accession number	Percent (%) ID
KaS-1	*Neofusicoccum parvum*	PQ867536	100
KaS-2	*Diaporthe* sp.	PQ867537	98.28
KaS-3	*Neofusicoccum parvum*	PQ867538	100
KaS-4	*Diaporthe macadamiae*	PQ867539	98.43
KaS-5	*Pseudofusicoccum olivaceum*	PQ867540	99.83
KaS-6	*Diaporthe neotheicola*	PQ867541	97.57
KaS-7	*Diaporthe* sp.	PQ867542	99.31
KaS-8	*Diaporthe parapterocarpi*	PQ867543	99.30
KaS-9	*Diaporthe arengae*	PQ867544	98.58
KaS-10	*Diaporthe* sp.	PQ867545	99.31
KaS-11	*Neofusicoccum parvum*	PQ867546	100
KaS-12	*Neofusicoccum parvum*	PQ867547	100
KaS-13	*Neofusicoccum parvum*	PQ867548	100
KaS-14	*Neofusicoccum parvum*	PQ867549	100
KaS-15	*Neofusicoccum parvum*	PQ867550	100
KaS-16	*Diaporthe vangueriae*	PQ867551	99.65
KaS-17	*Neofusicoccum parvum*	PQ867552	100
KaS-19	*Neofusicoccum kwambonambiense*	PQ867553	100

Cytotoxicity of crude extracts

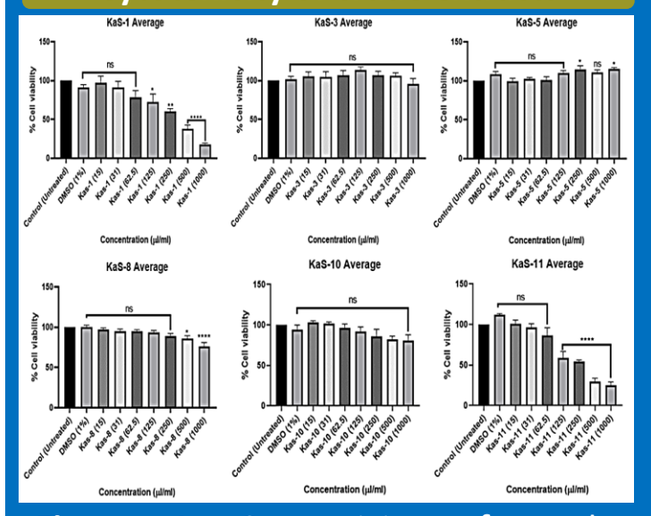

Figure 1. Cytotoxicity of crude extracts against HEK-293 cells.

Antimicrobial activity of extracts

Table 2. MIC of crude extracts (mg/mL) against clinical pathogens

Endophytic fungi	*S. aureus*	*E. coli*	*E. faecalis*	*P. aeruginosa*	*C. albicans*
Neofusicoccum parvum KaS-1	1.25	2.5	1.25	2.5	2.5
Diaporthe sp. KaS-2	1.25	2.5	1.25	2.5	2.5
Neofusicoccum parvum KaS-3	1.25	2.5	1.25	2.5	2.5
Diaporthe macadamiae KaS-4	1.25	2.5	1.25	2.5	2.5
Pseudofusicoccum olivaceum KaS-5	–	–	–	–	–
Diaporthe neotheicola KaS-6	1.25	1.25	1.25	1.25	1.25
Diaporthe sp. KaS-7	1.25	1.25	1.25	1.25	2.5
Diaporthe parapterocarpi KaS-8	1.25	1.25	1.25	1.25	1.25
Diaporthe arengae KaS-9	1.25	1.25	1.25	1.25	2.5
Diaporthe sp. KaS-10	1.25	2.5	1.25	1.25	2.5
Neofusicoccum parvum KaS-11	0.63	0.63	0.63	0.63	2.5
Neofusicoccum parvum KaS-12	1.25	0.63	1.25	1.25	2.5
Neofusicoccum parvum KaS-13	0.63	0.63	0.63	0.63	2.5
Neofusicoccum parvum KaS-14	0.63	0.63	0.63	0.63	2.5
Neofusicoccum parvum KaS-15	1.25	1.25	1.25	1.25	1.25
Diaporthe vangueriae KaS-16	1.25	1.25	1.25	1.25	1.25
Neofusicoccum parvum KaS-17	0.31	0.63	0.63	1.25	1.25
Neofusicoccum kwambonambiense KaS-19	1.25	1.25	1.25	1.25	1.25
Chloramphenicol	0.039	0.020	0.039	0.020	
Amphotericin B					0.020
DMSO	25%	25%	25%	25%	25%

Mycochemical analysis of extracts

Table 3. Mycochemical composition of non-toxic crude extracts.

Endophytic fungi	TPC (mgGAE/g)	TTC (mgTAE/g)	TFC (mgQE/g)
Neofusicoccum parvum KaS-3	14.18 ± 0.92 [a]	3.41 ± 0.23 [a]	2.11 ± 0.26 [a]
Diaporthe macadamiae KaS-4	12.71 ± 1.29 [a]	3.66 ± 0.32 [a]	1.30 ± 0.28 [a]
Pseudofusicoccum olivaceum KaS-5	63.59 ± 2.28 [a]	14.44 ± 0.87 [a]	4.28 ± 0.17 [a]
Diaporthe neotheicola KaS-6	26.56 ± 1.09 [a]	6.71 ± 0.22 [a]	2.06 ± 0.10 [a]

Anticancer activity of extracts

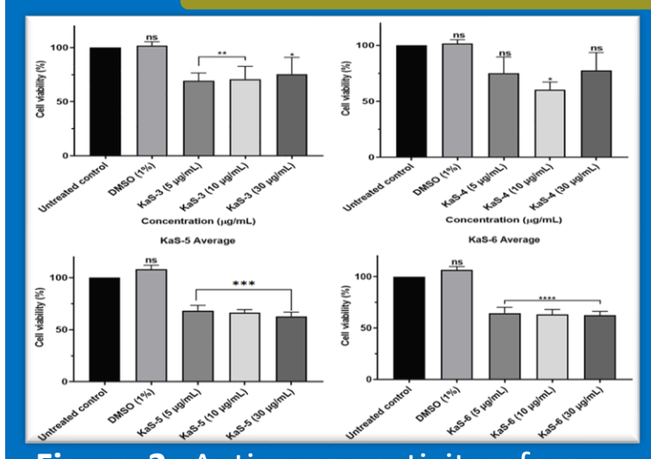

Figure 2. Anticancer activity of non-toxic crude extracts against ME-180 cells.

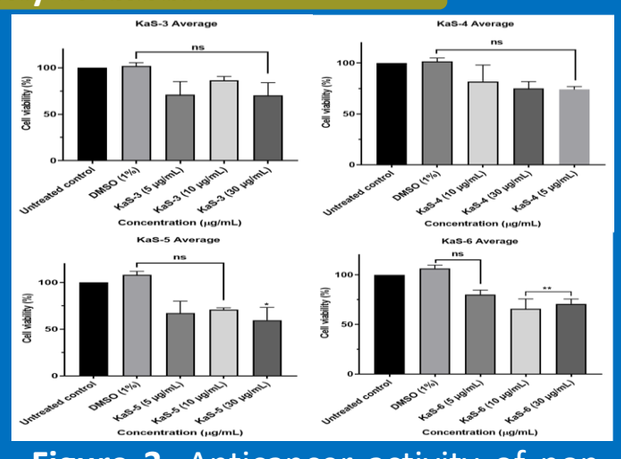

Figure 3. Anticancer activity of non-toxic crude extracts against A375 cells.

Discussion and Conclusion

Fungal endophytes produce various therapeutic agents that can curb AMR pathogens and cancer. In this study, 18 morphologically distinct ascomycetes isolates were obtained from *K. acuminata* Oliv. stems. All the crude extracts of the isolates, except for *Pseudofusicoccum olivaceum* KaS-5, exhibited inhibitory activity against all the tested pathogens. The MIC values ranged from 0.31 to 2.5 mg/mL and from 1.25 to 2.5 mg/mL against the bacterial pathogens and *C. albicans*, respectively. This highlighted the isolate's potential for antimicrobial production; However, the impact of their crude extracts on pathogen virulence factors is unknown and requires future work to assess their mechanisms of action. Interestingly, only the crude extracts of *Neofusicoccum parvum* KaS-3, *Diaporthe macadamiae* KaS-4, *P. olivaceum* KaS-5 and *D. neotheicola* KaS-6 showed promise as sources of safe anticancer agents as they exhibited no cytotoxicity towards non-cancerous HEK-2933 cells and moderate inhibitory activity against ME-180 and A375 cancerous cells. Furthermore, the high TPC contents in the non-toxic crude extracts of *N. parvum* KaS-3, *D. macadamiae* KaS-4 and *D. neotheicola* KaS-6 may play a role in their inhibitory activities; however, in the case of the *P. olivaceum* KaS-5 crude extract its high polyphenol content could mean it has a high concentration of antagonistic compounds that mask its biological activity. This suggests the need to further purify and identify the bioactive compounds within the crude extracts. Overall, the study highlights the potential of fungal endophytes associated with *K. acuminata* Oliv. as novel drug sources.

Acknowledgements

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