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Biological activity of folkloric plants used in the treatment of '*u wela*' against pathogens

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Background: '*U wela*' also known as '*Divhu*' in '*Tshivenda*' is a sexually transmitted infection caused by a combination of fungal and bacterial microorganisms that affects males because of unprotected sexual encounters with a woman who has had an abortion or miscarriage.

Aim: The study aimed to investigate medicinal plants used to treat 'u wela' and determine their biological activity against *Neisseria gonorrhoeae* and *Candida albicans*.

Setting: Eight plant species (*Elaeodendron transvaalense*[Burtt Davy] R.H. Archer, *Albizia versicolor* Welw. ex Oliv, *Xanthocercis zambesiaca* Baker, *Cassia abbreviata* subsp. beareana [Holmes] Brenan, *Anthocleista grandiflora* Gilg, *Myrothamnus flabellifolius* Welw., *Mimusops zeyheri* Sond, and *Capparis tomentosa* Lam.) used to combat 'u wela' were selected from the Ethnomedicinal plant's database of over 300 medicinal plants used for medicinal purposes in humans, in the Vhembe district, Limpopo province, South Africa.

Methods: The antimicrobial activity of the plant extracts was investigated against *Candida albicans* and *Neisseria gonorrhoeae* using serial dilution and bioautography assays.

Results: The plant extracts of *A. versicolor* and *C. abbreviata* had excellent activity with a low minimum inhibitory concentration (MIC). value of 0.02 and 0.07 mg/mL, respectively. In bioautograms developed in benzene/ethanol/ammonia hydroxide (BEA), active compounds were visible in the extracts of *A. versicolor*.

Conclusion: *A. versicolor* had excellent antimicrobial activity and may be used in traditional therapy to combat 'u wela'.

Contribution: The study has demonstrated that *A. versicolor* is a promising plant species that could lead to the discovery of novel drugs to combat 'u wela'.

Keywords: *U wela*; gonorrhoea; antimicrobial activity; bioautography assay; *Candida albicans; Neisseria gonorrhoeae*.

Introduction

Infectious diseases caused by antimicrobial-resistant microbes are a major concern worldwide (Gyles 2011; Srivastava et al. 2014). Among the various diseases, sexually transmitted infections (STIs) are pervasive. These infections are caused by microorganisms that reside on the skin or close to the mucous membrane of the genital region. Furthermore, STIs are quite expensive to treat and may cause health complications (Cavanaugh et al. 2011; Li & Webster 2018). Among the STIs, 'u wela' (also known as 'makgoma') is a common disease caused by a combination of fungal and bacterial microorganisms that infect males following unsafe sexual encounters with a woman who has had an abortion or miscarriage (Mulaudzi 2001; Ramavhale & Mahlo 2019). 'U wela' has been reported mostly in Vhembe District, and it causes morbidity in males. However, it is difficult to treat with conventional Western medicine (Muswede, Tshivhase & Mavhandu-Mudzusi 2021; Shirindi & Makofane 2015). The symptoms of 'u wela' include weight loss, dry mouth, a distended vein appearing on the forehead, and swelling of the genital parts (Mulaudzi & Makhubela-Nkondo 2006). Some of the symptoms have previously been reported to be comparable to those associated with human immunodeficiency virus and/or acquired immunodeficiency syndrome (HIV and/or AIDS) (Ramavhale & Mahlo 2019). However, information on the therapy of 'u wela' is not well documented and little has been reported on medicinal plants used to combat the disease in the Vhembe district, Limpopo province, South Africa. Males infected with the disease are required to perform a

cleansing ritual to remove contaminants in their bodies (Niehaus 2013). Traditional health practitioners prefer medicinal remedies for the treatment of '*u wela*'. This is because plants possess secondary metabolites, some of which have antifungal and antibacterial properties (Gunatilaka 2006). Therefore, screening of medicinal plants could result in the development and detection of novel drugs that are cheap, effective, and less toxic.

Escherichia coli (E. coli) is associated with *Chlamydia trachomatis*, and *Trichomonas vaginalis* infections (Chiu et al. 2021), and causes diarrhoeal as well as urinary tract infections (UTIs). *Candida albicans*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, and *Treponema pallidum* have been reported by the World Health Organization (WHO 2017) as pathogens causing STIs. *Neisseria gonorrhoeae*, the Gram-negative bacteria causing STIs remains one of the most harmful microorganisms. Infections that are not medicated can result in major complications such as pelvic inflammatory disease (PID) and ectopic pregnancy (Rizzo et al. 2015). Azithromycin is used to treat genital infections caused by *C. trachomatis* such as cervicitis, urethritis, and proctitis (Ruddock et al. 2011). *Chlamydia trachomatis* is a human-adapted microorganism with over 14 different serovars that cause trachoma, which is the main cause of STIs (Chiarelli et al. 2020). *Candida albicans* causes candidiasis in humans. This fungal pathogen is resistant to antifungal agents such as caspofungin, micafungin, and anidulafungin (Tóth et al. 2019). Antifungal drugs such as itraconazole econazole, amphotericin B, fluconazole, and ketoconazole are ineffective against some fungal pathogens (Homa et al. 2018). However, some of these drugs such as ciprofloxacin and fluconazole are expensive, and are not freely available (Sanguinetti, Posteraro & Lass–Flörl 2015).

Validation and acceptance of therapeutic plants to treat diseases induced by pathogens can be of considerable benefit to all citizens in rural areas who have minimal exposure to essential, lifesaving, and sometimes expensive modern medicine (Patwardhan & Patwardhan 2005). More than 100 000 licensed traditional health practitioners in South Africa are serving more than 20 million patients (Street 2016). Therefore, ethnobotanical surveys, as well as screening, are critical and essential for recording valuable plant species that may contribute to new antimicrobial compounds in the development of novel drugs. In this article, we investigate medicinal plants used in Limpopo province for the treatment of '*u wela*' and to evaluate the activity of plant extracts against pathogens.

Research methods and design

Plant identification and selection

Plant materials were collected in January 2022 from Nzhelele in the Vhembe district, Makhado Local Municipality, Limpopo province (Figure 1). The plants were stored in open mesh orange bags at room temperature of 25 °C to ensure efficient drying of the material. Plants were identified at the University of Limpopo herbarium. Voucher specimens were prepared and deposited at the Larry Leach Herbarium. Plant materials such as leaves, stems, bark, and roots were allowed to dry at room temperature of 25 °C for 3–5 weeks and ground to a fine powder.

Plant collection

Eight plant species, namely *Elaeodendron transvaalense*, *Albizia versicolor*, *Xanthocercis zambesiaca*, *Cassia abbreviata*, *Anthocleista grandiflora*, *Myrothamnus flabellifolius*, *Mimusops zeyheri*, and *Capparis tomentosa* were selected from the ethnomedicinal plant database of over 300 medicinal plants used for therapeutic purposes in Limpopo province. Sixty plant species used to treat 'u wela' were recorded in the database. Furthermore, eight plant species used to combat 'u wela' were selected based on ethnobotanical data and the availability of the plant species.

Ethical considerations

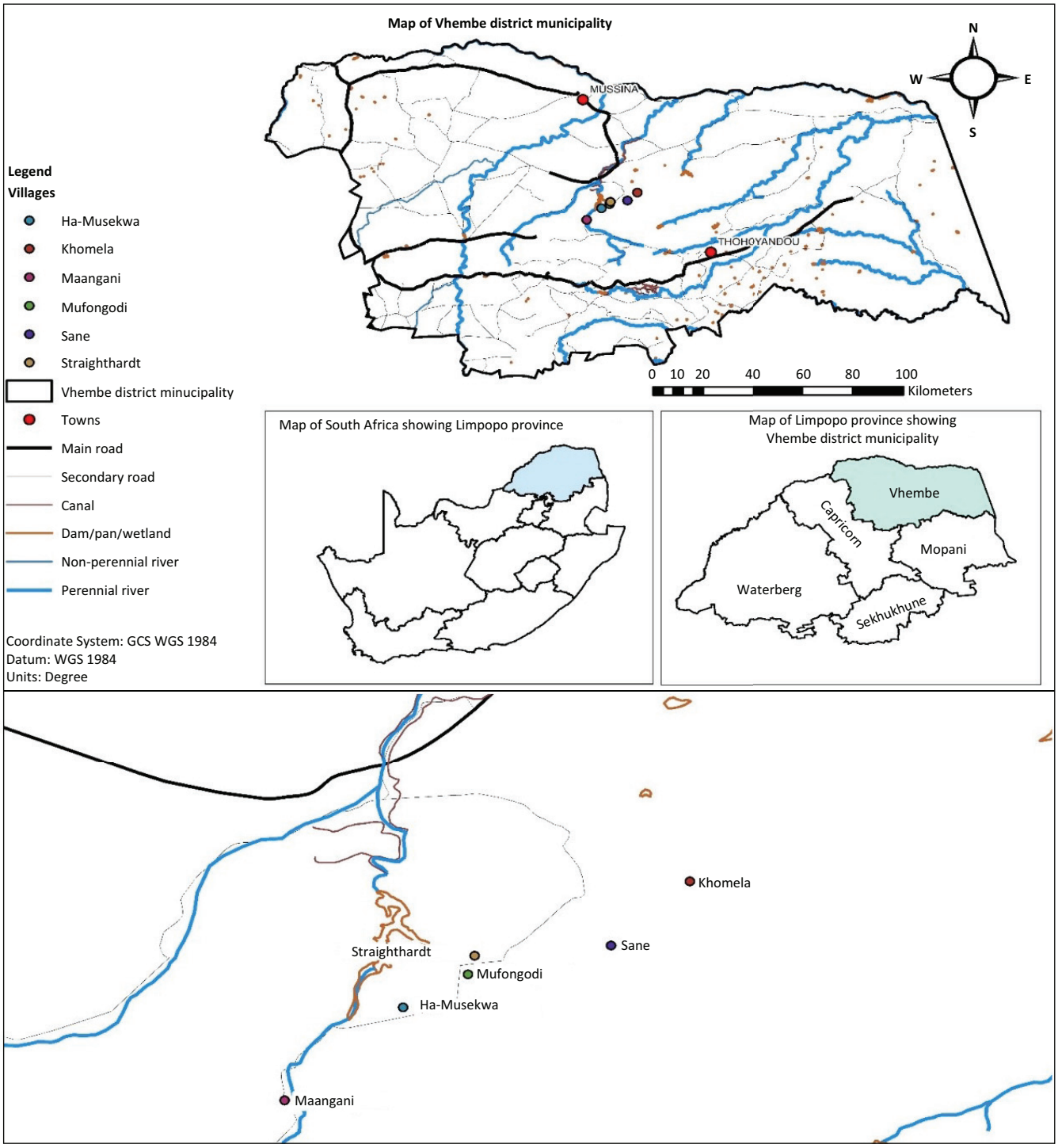
The research was conducted in Vhembe district, Limpopo province. The study was approved by the University of Limpopo Research Committee, (project number: TREC/67/2023: PG)

Plant extraction

Plant parts such as roots, leaves, and bark were cut into small pieces and allowed to dry at room temperature (25 °C) for approximately 3–4 weeks or even more until the plant parts were completely dry. Dried finely ground 5 g of plant material was then extracted with 50 mL of various solvents such as hexane, dichloromethane (DCM), acetone, methanol, ethyl acetate, and water in polyester plastic tubes. The plant extracts were shaken vigorously for 3 min–5 min on an orbital shaker at 150 rpm. The plant material was centrifuged at 3500 rpm for 5 min and filtered using Whatman No.1 filter paper. The supernatants were decanted into weighed vials. The process was repeated in triplicate and the extracts were combined. The solvent was removed under a stream of cold air at room temperature.

Traditional method

Five grams of finely ground plant material was weighed using an analytical balance. The plant material was then transferred into a 100 mL beaker. Subsequently, 50 mL of water was added to the beaker, ensuring complete immersion of the plant material. Traditional health practitioners prefer water to make their decoction. The mixture was gradually boiled for 20 min–40 min using a hot plate. After boiling, the mixture was allowed to cool at room temperature, 25 °C. The aqueous extract was then filtered using a Whatman No. 1 filter paper. The process was repeated in triplicate and the extracts were combined. A freeze dryer was then used to remove water from the aqueous extracts.



Source: Ramavhale, T.T., 2024, 'Isolation, structure elucidation, antimicrobial activity, and effects of plant extracts used for the treatment of "u wela"', PhD thesis, University of Limpopo

FIGURE 1: A map showing study areas in Vhembe district, Makhado Local Municipality.

Fungal strains and inoculum quantification

Candida albicans (ATCC 10231) was obtained from the Department of Veterinary Tropical Diseases at the University of Pretoria. For the quantification of fungi, the Neubauer haemocytometer cell-counting method was used for counting the number of cells for each fungal culture (Aberkane et al. 2002). The inoculum of each isolate was prepared by growing the fungus on Sabouraud dextrose agar for 7 days at 35 °C. The final inoculum concentration was adjusted to approximately 1.0×10^6 cells/mL.

Bacterial strains and inoculum quantification

The *N. gonorrhoeae* (ATTC 49226) strain was purchased from the KwikStik company. The isolates were maintained on Mueller Hinton chocolate agar for 24 h at 35 °C and incubated under 5% CO_2 atmosphere. Before the bacterial cultures were used, they were diluted with sterile Muller Hinton

broth to a turbidity that matches 0.5 McFarland standard (1.0×10^6 Colony Forming Unit [CFU]/mL^{-1}) (Mulu, Tessema & Derbie 2004).

Antioxidant activity

Qualitative DPPH radical-scavenging assay

The qualitative screening for antioxidant activity was determined using DPPH (1,1-diphenyl-2-picrylhydrazine) assays with some minor modifications (Braca et al. 2002). Thin layer chromatography (TLC) plates were loaded with 10 µL of plant extract and dried before being developed in three eluent solvent systems (benzene/ethanol/ammonia hydroxide [BEA], chloroform/ethyl acetate/formic acid [CEF], and ethyl acetate/methanol/water [EMW]). The plates were sprayed with the solution of DPPH (0.2%) in methanol. The R_f values were determined by dividing the distance travelled by the antioxidant compound by the distance travelled by the solvent front. Yellow bands with radical scavenger capacity on a purple background suggested the presence of antioxidant compounds.

Quantitative DPPH free radical-scavenging assay

The DPPH (1,1-diphenyl-2-picrylhydrazine.) free radical-scavenging assay was performed with various modifications, as previously described by Ammar et al. (2009). The crude extracts isolated from *A. versicolor*, *M. flabellifolius*, and *E. transvaalense* at various concentrations (15, 30, 60, 120, and 250 µg /mL) were blended with a 0.2 mM of DPPH solution in methanol and the centrifuge tubes were vigorously shaken. The centrifuge tubes were incubated in the dark for 30 min at room temperature, and the absorbance at 517 nm was measured using a spectrophotometer. Ascorbic acid and methanol hydroxide solution were used as positive and negative controls, respectively. The test solutions were generated in triplicates. The degree of discolouration (from purple to yellow) showed the compounds' free-radical scavenging effectiveness. The antioxidant activity of the extracts was measured using the formula below, where Ab = absorbance of the blank and Ac= absorbance of the samples:

$$\% \text{ free radical scavenging activity} = \frac{Ab - Ac}{Ab} \times 100 \quad \text{[Eqn 1]}$$

Determination of antifungal activity

Micro-dilution assay

The micro-plate method was used to determine the antifungal activity of plant extracts (Masoko & Eloff 2005). The plant extracts were serially diluted (50%) with water in 96-well microtiter plates. Acetone was used as a negative control and Amphotericin B was used as a reference anti-fungicide. About 100 µL of fungal culture was added to each well in the microplate and incubated for 24 h as an indicator of growth, and 40µL of 0.2 mg/mL p-iodonitrotetrazolium violet (INT) dissolved in water was added to the microplate. Microplates were incubated for 3–5 days at 35 °C at 100% relative humidity. The minimum inhibitory concentration (mg/mL) was recorded as the lowest concentration that inhibits the growth of fungi.

Bioautography assay

The TLC plates were loaded with each plant's extracts and developed using different eluent solvent systems such as Chloroform: Ethyl-acetate: formic acid (CEF), Benzene: ethanol: ammonia hydroxide (BEA), and Ethyl-acetate: methanol: water (EMW). Furthermore, the TLC plates were allowed to dry under a stream of cold air to remove all the solvents. The developed TLC plates were sprayed with an overnight culture of *C. albicans* and *N. gonorrhoeae* each until wet. The plates were incubated separately overnight, and sprayed with 2 mg/mL solution of p-iodonitrotetrazolium violet, and further incubated overnight at 35 °C in a chamber at 100% relative humidity in the dark. The white areas in the plates indicated the inhibition of fungal growth (Begue & Kline 1972).

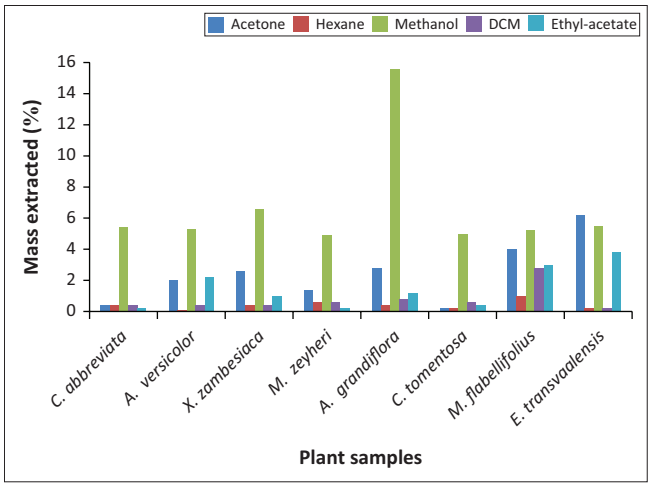
Data analysis

Descriptive and inferential statistics such as graphs, percentages, and frequencies were used to analyse the data.

Results and discussion

Extraction of plant materials using different solvents

Methanol extracted the highest quantity of plant material from the bark of *A. grandiflora* (15.6%), followed by acetone (6.2%) and ethyl-acetate (5%) bark extract from *E. transvaalense* (Figure 2). Methanol is a polar solvent and has the ability to extract both lipophilic and hydrophilic compounds from plants because of its high volatility (Selvaraj et al. 2020). Similarly, in a study conducted by Malada, Mogashoa and Masoko (2022), methanol extracted a large quantity of plant material from an *Mystroxylon aethiopicum* extract compared to other solvents. Ethyl-acetate extracted a wider range of



Source: Ramavhale, T.T., 2024, 'Isolation, structure elucidation, antimicrobial activity, and effects of plant extracts used for the treatment of "u wela"', PhD thesis, University of Limpopo

FIGURE 2: Percentage mass of material extracted from 4 g of powdered plant material.

compounds of polar alkaloids, non-polar lipids, as well as essential oils. Acetone isolates non-polar fats, oils, and specific non-polar secondary metabolites (Bulugahapitiya 2013). Other researchers found that acetone can dissolve both polar and non-polar compounds (Maulidiyah et al. 2023).

Antifungal activity of the plant extracts against Candida albicans

The antifungal activity of plant extracts was determined against *C. albicans* using serial dilution assay. The plant extracts of *A. versicolor* and *C. abbreviata* had an excellent antifungal activity with a low minimum inhibitory concentration (MIC). value of 0.02 mg/mL – 0.03 mg/mL (Table 1). Plant extracts with low MIC values could be potential candidates for developing plant natural products with antimicrobial properties. Noteworthy anti-candida activity was observed in aqueous extracts of *E. transvaalense, A. versicolor, X. zambesiaca, M. zeyheri,* and *C. abbreviata* with MIC values of 0.02 mg/mL. These results support the use of water for the preparation of traditional medicine by traditional health practitioners. Methanol bark extracts of *X. zambesiaca* were not active with the highest MIC value of 1.25 mg/mL. Similar results were obtained with methanol stem extracts of *X. zambesiaca* with low activity against *C. albicans* (Ngobeni 2016). Notably, dichloromethane (DCM) (root) and acetone (bark) extracts of *C. tomentosa* and *E. transvaalense* exhibited poor antifungal activity against the tested fungal pathogen. Contrastingly, it was found that the antimicrobial activities of ethanol extracts of aerial leaves and stem of *C. tomentosa* inhibited the growth of *C. albicans* (Gebrehiwot & Chaithanya 2020). Excellent antifungal activity was observed in the root extracts of *C. abbreviata* against *C. albicans* with low MIC values of 0.02 mg/mL.

Antibacterial activity of plant extracts against N. gonorrhoeae

The antibacterial activity of the plant extracts was determined using the micro-dilution assay. The hexane, aqueous, and decoction extracts of *C. abbreviata*, *M. zeyheri*, *A. grandiflora, A. versicolor*, *X. zambesiaca*, *M. flabellifolius*, and *C. tomentosa* showed promising antibacterial activity against *N. gonorrhoeae* with low MIC values ranging from 0.02 mg/mL – 0.03 mg/mL (Table 2). Similar results were obtained from water extracts of *C. abbreviata* against *N. gonorrhoeae* (Mulubwa & Prakash 2015). Previously, it was reported that *C. tomentosa* exerted a substantial *in vitro* antibacterial efficacy against *Staphylococcus aureus* and *Bacillus cereus* (Gebrehiwot & Chaithanya 2020). Ethanol root extracts of *C. abbreviata* were reported to possess good activity against gonorrhoea and syphilis (Prinsloo, Marokane & Street 2018). Poor activity was observed in the methanol, DCM, and acetone extracts of *M. zeyheri, A. grandiflora,* and *M. flabellifolius* against *N. gonorrhoeae* with MIC values ranging from 1.25 mg/mL – 2.5 mg/mL. However, other researchers found that *M. flabellifolius* possessed good activity against bacterial pathogens (Van Vuuren 2008). The decoction extracts exhibited strong antimicrobial activity against the sexually transmitted bacteria. These findings support the use of water by traditional health practitioners.

TABLE 1a: Minimum inhibitory concentrations (mg/mL) of selected plant species against *Candida albicans*.

Plant species		*Elaeodendron transvaalense*							*Albizia versicolor*							*Xanthocercis zambesiaca*							Amp B
Fungi	Time (hours.)	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	
Candida *albicans*	24	0.02†	0.02	0.02	1.25	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.02	0.02	1.25	1.25	1.25	1.25	0.02	0.02	0.02	0.03	< 0.02
	48	0.02	0.02	0.02	1.25	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.02	0.02	1.25	1.25	1.25	1.25	0.02	0.02	0.02	0.03	< 0.02
	72	0.02	0.02	0.02	2.5	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.02	0.02	2.5	1.25	1.25	1.25	0.02	0.02	0.02	0.03	< 0.02

Source: Ramavhale, T.T., 2024, 'Isolation, structure elucidation, antimicrobial activity, and effects of plant extracts used for the treatment of "u wela"', PhD thesis, University of Limpopo
Amphotericin-B was used as a positive control.
A, acetone; H, hexane; D, dichloromethane; M, methanol; E, ethyl-acetate; Dh2o, Distilled aqueous; Th2o, Decoction mixture.
†, The lowest MIC value of 0.02 mg/ml indicate that the plant extract had excellent activity against the tested fungal pathogen.

TABLE 1b: Minimum inhibitory concentrations (mg/mL) of selected plant species against *Candida albicans.*

Plant species		*Cassia abbreviata*							*Anthocleista grandiflora*							*Capparis tomentosa*							Amp B
Fungi	Time (hours.)	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	
Candida *albicans*	24	0.02	0.02	0.02	0.02	0.02	0.02	0.02	1.25	0.02	0.02	0.02	1.25	0.62	2.5	2.5	0.02	0.02	0.02	0.02	0.15	0.31	< 0.02
	48	0.02	0.02	0.02	0.02	0.02	0.02	0.02	1.25	0.02	0.02	0.02	1.25	0.62	2.5	2.5	0.02	0.02	0.02	0.02	0.15	0.31	< 0.02
	72	0.02	0.02	0.02	0.02	0.02	0.02	0.02	2.5	0.02	0.02	0.02	1.25	0.62	2.5	2.5	0.02	0.02	0.02	0.02	0.15	0.31	< 0.02

Source: Ramavhale, T.T., 2024, 'Isolation, structure elucidation, antimicrobial activity, and effects of plant extracts used for the treatment of "u wela"', PhD thesis, University of Limpopo
Amphotericin-B was used as a positive control.
A, acetone; H, hexane; D, dichloromethane; M, methanol; E, ethyl-acetate; Dh2o, Distilled aqueous; Th2o, Decoction mixture.

TABLE 1c: Minimum inhibitory concentrations (mg/mL) of selected plant species against *Candida albicans.*

Plant species		*Mimusops zeyheri*							*Myrothamnus flabellifolius*							Amp B
Fungi	Time (hours.)	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	
Candida *albicans*	24	1.25	0.02	0.02	0.02	0.02	0.02	0.15	0.31	0.31	0.03	0.31	0.31	0.15	0.03	< 0.02
	48	1.25	0.02	0.02	0.02	0.02	0.02	0.15	0.31	0.31	0.03	0.31	0.31	0.15	0.03	< 0.02
	72	1.25	0.15	0.02	0.02	0.02	0.02	0.15	0.31	0.31	0.03	0.31	0.31	0.15	0.03	< 0.02

Source: Ramavhale, T.T., 2024, 'Isolation, structure elucidation, antimicrobial activity, and effects of plant extracts used for the treatment of "u wela"', PhD thesis, University of Limpopo
Amphotericin-B was used as a positive control.
A, acetone; H, hexane; D, dichloromethane; M, methanol; E, ethyl-acetate; Dh2o, Distilled aqueous; Th2o, Decoction mixture.

TABLE 2a: Minimum inhibitory concentrations (mg/mL) of selected plant species against *Neisseria gonorrhoeae.*

Plant species		*Elaeodendron transvaalense*							*Albizia versicolor*							*Xanthocercis zambesiaca*							Gent
Bacteria	Time (hr)	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	
N. g	24	0.02	0.02	0.02	0.02	0.03	0.62	0.31	0.02	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	< 0.02
	48	0.02	0.02	0.02	0.02	0.03	0.62	0.31	0.02	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.15	0.03	0.02	0.02	< 0.02
	72	0.03	0.03	0.02	0.02	0.03	2.5	2.5	0.02	0.02	0.03	0.07	0.03	0.02	0.02	0.02	0.03	0.02	0.15	0.03	0.02	0.03	< 0.02

Source: Ramavhale, T.T., 2024, 'Isolation, structure elucidation, antimicrobial activity, and effects of plant extracts used for the treatment of "u wela"', PhD thesis, University of Limpopo
A, acetone; H, hexane; D, dichloromethane; M, methanol; E, ethyl-acetate; Dh2o, Distilled aqueous; Th2o, Decoction mixture; *N. g*, *Neisseria gonorrhoeae*; Gent, Gentamicin.
Gentamicin was used as a positive control.

TABLE 2b: Minimum inhibitory concentrations (mg/mL) of selected plant species against *Neisseria gonorrhoeae.*

Plant species		*Cassia abbreviata*							*Anthocleista grandiflora*							*Capparis tomentosa*							Gent
Bacteria	Time (hr)	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	
N. g	24	0.07	0.02	0.62	0.07	0.02	0.02	0.02	1.25	0.02	0.02	0.62	1.25	0.02	0.02	0.02	0.02	0.02	0.31	0.02	0.03	0.03	< 0.02
	48	0.07	0.02	0.62	0.07	0.02	0.02	0.02	1.25	0.02	0.02	0.62	1.25	0.03	0.02	0.02	0.02	0.02	0.31	0.02	0.03	0.03	< 0.02
	72	0.07	0.02	2.5	1.25	0.02	0.02	0.02	1.25	0.02	0.02	1.25	1.25	0.03	0.02	0.07	0.15	0.03	0.31	0.02	0.03	0.03	< 0.02

Source: Ramavhale, T.T., 2024, 'Isolation, structure elucidation, antimicrobial activity, and effects of plant extracts used for the treatment of "u wela"', PhD thesis, University of Limpopo
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Gentamicin was used as a positive control.

TABLE 2c: Minimum inhibitory concentrations (mg/mL) of selected plant species against *Neisseria gonorrhoeae.*

Plant species		*Mimusops zeyheri*							*Myrothamnus flabellifolius*							Gent
Bacteria	Time (hr)	A	H	M	D	E	D H_2O	T H_2O	A	H	M	D	E	D H_2O	T H_2O	
N. g	24	0.02	0.02	2.5	1.25	0.02	0.02	0.02	1.25	0.02	1.25	0.62	0.02	0.02	0.03	< 0.02
	48	0.07	0.02	2.5	1.25	0.02	0.02	0.02	1.25	0.02	1.25	0.62	0.02	0.03	0.03	< 0.02
	72	0.07	0.02	2.5	2.5	0.07	0.02	0.02	0.31	0.03	2.5	2.5	0.02	0.03	0.03	< 0.02

Source: Ramavhale, T.T., 2024, 'Isolation, structure elucidation, antimicrobial activity, and effects of plant extracts used for the treatment of "u wela"', PhD thesis, University of Limpopo
A, acetone; H, hexane; D, dichloromethane; M, methanol; E, ethyl-acetate; Dh2o, Distilled aqueous; Th2o, Decoction mixture; *N. g*, *Neisseria gonorrhoeae*; Gent, Gentamicin.
Gentamicin was used as a positive control.

TABLE 3: Plant extracts with excellent antibacterial activity (0.02 mg/mL) against *Neisseria gonorrhoeae*.

Extractants	Plant species								
	E. transvaalense	*A. versicolor*	*X. zambesiaca*	*M. zeyheri*	*C. abbreviata*	*A. grandiflora*	*C. tomentosa*	*M. flabellifolius*	Average
A	0	1	1	0	0	0	0	0	2
H	0	1	0	1	1	1	0	0	4
M	1	0	1	0	0	1	0	0	3
D	1	0	0	0	0	0	0	0	1
E	0	0	0	0	1	0	1	1	3
D H_2O	0	1	1	1	1	0	0	0	4
T H_2O	0	1	0	1	1	1	0	0	4
Total	**2**	**4**	**3**	**3**	**4**	**3**	**1**	**1**	**21**

Source: Ramavhale, T.T., 2024, 'Isolation, structure elucidation, antimicrobial activity, and effects of plant extracts used for the treatment of "u wela"', PhD thesis, University of Limpopo
A, acetone; H, hexane; D, dichloromethane; M, methanol; E, ethyl-acetate; Dh2o, Distilled water; Th2o, Decoction mixture.

Plant extracts with excellent antibacterial activity

Albizia versicolor and *Cassia abbreviata* had more plant extracts with good antibacterial activity containing MIC values of 0.02 mg/mL (Table 3). Traditionally, *A. versicolor* has been reported to possess several therapeutic applications. The roots and bark are used to cure anaemia and STIs, and are used as an anthelmintic (Ramavhale et al. 2021; Rukunga & Waterman 1996). The bark is used to treat flu and body aches (Rukunga & Waterman 2001) as well as malaria (Bapela, Meyer & Kaiser 2014).

The *Albizia* species have been reported to contain a variety of phytochemicals, most notably triterpenoids, saponins, and lignanoids (He et al. 2020). In a study conducted by Chisamile, Sonibare and Kamanula (2023), it was revealed that *C. abbreviata* root and stem decoction are used in Tanzania and Mozambique to cure diarrhoea, stomach pains, and syphilis. The leaves, and barks have been used to cure earache, and fruits can be used to treat eye infections (Osunga et al. 2023). Three plant extracts with 0.02 mg/mL MIC values were found in *X. zambesiaca*, *M. zeyheri*, and *A. grandiflora*, followed by *E. transvaalense* with two plant extracts with excellent antibacterial activity. Traditional health practitioners employ the boiled stem and roots of *X. zambesiaca* to cure stomach symptoms and '*Nyoko*', a gall bladder dysfunction (Ngobeni 2016). Furthermore, it is used in traditional remedies to treat diabetes and gastrointestinal problems. The root and bark decoction is used to treat colds and snakebites. In traditional medicine, a decoction of *M. zeyheri's* bark and leaves is used to cure wounds and ulcers. The root is used as an infusion to treat candidiasis (Omotayo et al. 2020). *C. tomentosa* and *M. flabellifolius* had the lowest number of

extracts with good activity. Previously, it was reported that the acetone extract of *M. flabellifolius* was active against *C. albicans* with MIC value of 6 mg/mL (Gufe et al. 2023).

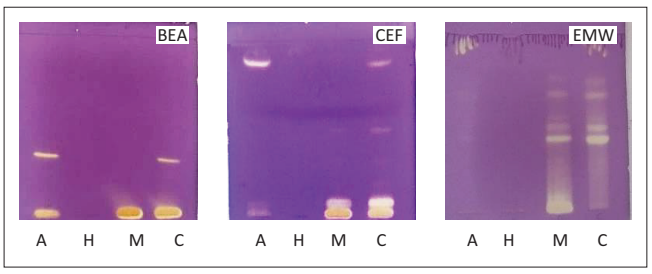
The bark of *C. tomentosa* is traditionally used to treat wounds, such as leprosy as well as tuberculosis, and gonorrhoea (Gebrehiwot & Chaithanya 2020). *Myrothamnus flabellifolius* is used in traditional medicine to treat respiratory problems, inflammation, wounds, heart problems, and renal problems. It is also used as a tonic and to moisturise the skin, as well as to cure chest problems, epilepsy, and mental illnesses (Marks et al. 2022).

Bioautography assay against the fungal pathogens

Antifungal compounds were observed in decoction extract and aqueous extracts of *C. abbreviata*, *A. versicolor*, and *E. transvaalense* against *C. albicans* with R_f values ranging from 0.55–0.90. Similar active compounds with an R_f value of 0.85 were observed in bioautograms developed in CEF against *N. gonorrhoeae*. No antifungal compounds were observed in bioautograms separated with EMW and BEA, the possible reason may be synergy between the compounds found in the plant extract. In addition, some of the active compounds may have evaporated during the drying period of the TLC plates. Based on the literature, there is limited information on the antibacterial activity of *A. versicolor* (Bapela et al. 2014). Therefore, there is a need to explore the biological activity of these plant species against several sexually transmitted pathogens.

Qualitative DPPH free radical scavenging activity assay on thin layer chromatography plates

The DPPH free radical scavenging technique is frequently used to assess the antioxidant properties of an extract. This technique is a quick, easy, and frequently used approach for testing antioxidant activity (Le et al. 2019). The plant extract's antioxidant activity was measured using a spectrophotometer, adopting the 1,1-diphenyl-2-picrylhydrazy (DPPH) free radical scavenging assay. In the presence of antioxidants, DPPH changes colour from purple to light yellow, indicating that it has decreased (Zamani, Delfani & Jabbari 2018).



Ramavhale, T.T., 2024, 'Isolation, structure elucidation, antimicrobial activity, and effects of plant extracts used for the treatment of "u wela"', PhD thesis, University of Limpopo

BEA, Benzene: ethanol: ammonia hydroxide; CEF, Chloroform: Ethyl-acetate: formic acid; EMW, Ethyl-acetate: methanol: water.

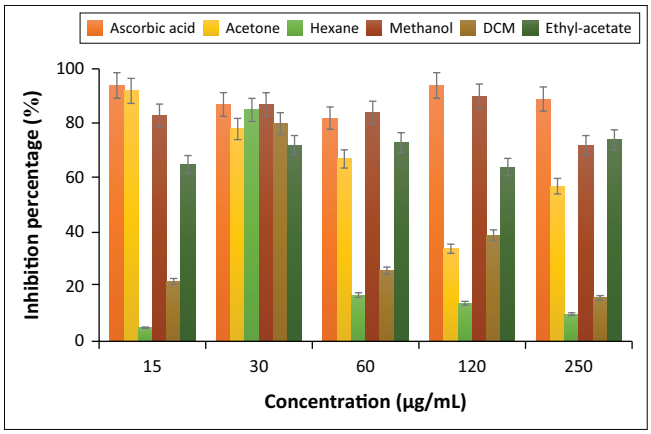
FIGURE 3: Thin layer chromatography chromatograms of *A. versicolor* extracted with acetone, hexane, methanol (MeOH) and chloroform (left to right), developed in benzene ethanol ammonia hydroxide, chloroform ethyl acetate formic acid and ethyl acetate methanol water, and sprayed with 0.2% DPPH solution.

The results in Figure 3 depict the antioxidant activity of *A. versicolor* bark extracts using the DPPH solution. The TLC chromatograms separated with EMW displayed excellent separation when compared to CEF and BEA. The antioxidant compounds are represented by the yellow bands against the purple background. In TLC chromatograms separated with BEA, similar antioxidant compounds were visible in the acetone extract with the same R_f values of 0.36 each. Antioxidant compounds were visible in the acetone, methanol, and chloroform extracts with R_f values ranging from 0.07–0.87 in TLC chromatograms developed in CEF. Noticeably, more compounds were observed in the methanol and chloroform extracts with the same R_f values ranging from 0.40–0.75 in the TLC chromatograms developed in EMW. Antioxidant activity was observed in the chloroform extracts, hence they possessed strong antioxidant activity compared to other solvents. However, no antioxidant compound was present in the hexane extract. Previously, it was reported that the hexane was unable to separate antioxidant compounds from *Coffea arabica* leaf extracts (Marcheafave et al. 2019). Based on our findings, it is possible that methanol and chloroform extracts possess therapeutic qualities and require further investigation.

Quantitative antioxidant activity assay

The antioxidant activity of the plant extracts was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) reduction with comparison to the ascorbic acid, as shown in Figure 4 to Figure 6. The antioxidant activity of the plant extracts was represented as a percentage inhibition and the values used were mean of triplicates ± standard deviation (Figure 4, Figure 5, and Figure 6).

The methanol extracts of *A. versicolor* (Figure 4) demonstrated the overall highest antioxidant activity, whereas the hexane extract had the lowest. In a study conducted by Johari and Khong (2019), the polar methanol extract of *Pereskia bleo*



Source: Ramavhale, T.T., 2024, 'Isolation, structure elucidation, antimicrobial activity, and effects of plant extracts used for the treatment of "u wela"', PhD thesis, University of Limpopo

Note: Data is expressed as the mean of triplicate ± standard deviation.

FIGURE 4: The percentage free radical (DPPH) inhibition of *A. versicolor* at different concentrations. Lanes from left to right: ascorbic acid, acetone, hexane, methanol, dichloromethane and ethyl acetate.

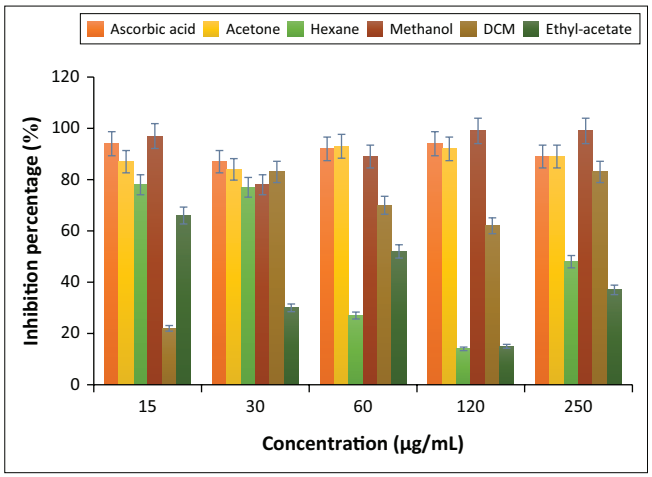


FIGURE 5: The percentage free radical (DPPH) inhibition of *M. flabellifolius* at different concentrations. Lanes from left to right: ascorbic acid, acetone, hexane, methanol, dichloromethane and ethyl acetate.

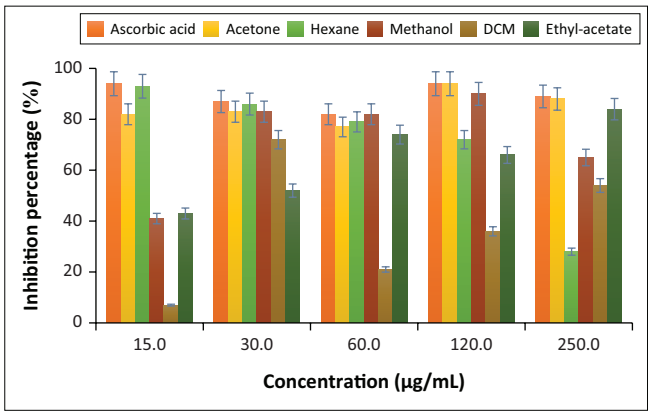


FIGURE 6: The percentage free radical (DPPH) inhibition of *E. transvaalense* at different concentrations. Lanes from left to right: ascorbic acid, acetone, hexane, methanol, dichloromethane and ethyl acetate.

displayed greater antioxidant activity when contrasted to the hexane extracts, hence these authors reported similar results to the current study. This further emphasises that methanol extracts potentially possess greater potential to scavenge free radicals than other extracts. Under the 30 µg/ml concentration, all the extracts (acetone, hexane, methanol, chloroform, and ethyl-acetate extracts) showed significant antioxidant activity. A high quantity of ascorbic acid was found in the extract of *A. versicolor*, *M. flabellifolius* and *E. transvaalense* at concentration of 120 µg/mL. Other researchers reported that the quantitative determination of ascorbic acid in plant extracts displays that they are good sources of ascorbic acid (Veeru, Kishor & Meenakshi 2009). The roots and bark of *A. versicolor* are used for several ailments such as anaemia, enlarged glands, and disorders caused by sexual activity and backaches (Fern 2022).

In the current study, the methanol extract of *M. flabellifolius* (Figure 5) demonstrated the highest activity under 15, 120, and 250 µg/ml concentrations, while the ethyl-acetate extract had the lowest activity under 30, 120, and 250 µg/mL concentrations. These findings indicate that *M. flabellifolius* is efficiently extracted by polar solvents when compared to solvents that are moderately polar or non-polar. Similar findings were reported by Ezez and Tefera (2021), who discovered that ethyl-acetate was less effective than methanol in separating ginger extracts. In serial dilution assay, the extracts of *A. versicolor* and *M. flabellifolius* were active against *N. gonorrheae* with MIC values ranging between 0.02 mg/mL – 0.03 mg/mL. These bacteria cause STIs in humans. The presence of antioxidants in plants prevents free radicals from causing chronic diseases in humans by inhibiting the oxidation of free radicals at the cellular level. As such, plant species containing antioxidant compounds could be used more widely than just in antifungal agents because many secondary metabolites have antimicrobial and antioxidant activity. *M. flabellifolius* leaf decoction and infusions have been used to treat individuals with immunological deficiencies. Asthma, infectious illnesses, respiratory diseases, inflammation, and epilepsy are among some of the medical applications (Nantapo & Marume 2022).

The acetone extract of *E. transvaalense* (Figure 6) demonstrated the highest activity under the 120 µg/mL concentration, whereas the dichloromethane extract had the lowest activity under the 15 and 60 µg/mL concentrations. Acetone was the best solvent because it extracted most of the polar compounds. Based on the literature, there is limited information on the antioxidant activity of *A. versicolor*, *M. flabellifolius* and *E. transvaalense*. The South African Red Data list classifies *E. transvaalense* as near threatened (Rasethe & Semenya 2019). The stem bark of *E. transvaalense* is widely used in traditional medicine in Southern Africa, mostly for gastrointestinal tract diseases and skin illnesses (Khumalo et al. 2019). *Elaeodendron transvaalense* is harvested by the local people and traditional health practitioners in the Vhembe district for different medicinal purposes including 'u wela', leading to population decline. More importantly, it will affect the plant and its ability to reproduce which may contribute to its threatened status. Therefore, the community must be educated on conservation measures and sustainability of these plant species to avoid overexploitation and extinction.

The results of this study indicate that the extract can be used as a readily available source of natural antioxidants.

Conclusion

The plant extracts were active against the tested fungal pathogens with MIC values ranging between 0.02 and 2.5 mg/mL. The aqueous and decoction extracts possessed strong antibacterial activity against the bacterial pathogens. The plant extracts were active against the tested pathogens and showed a degree of excellent activity with MIC values ranging from 0.02 mg/mL – 0.03 mg/mL. The bioautography assay showed more active compounds via TLC bioautograms separated with BEA compared to CEF and EMW eluent solvent systems. No active compounds were observed in TLC bioautograms developed in CEF. This study revealed that some of the plants used for the treatment of '*u wela*' can be utilised for medicinal purposes against *N. gonorrhoeae*.

The aqueous and decoction extracts exerted excellent activity against the tested microorganisms. These findings confirm the effectiveness of the use of water by traditional health practitioners and the local people to prepare their medications. *A. versicolor* possessed good antimicrobial activity against the tested microorganisms. The bark extracts of *A. versicolor* showed strong antioxidant activity by inhibiting DPPH. The chloroform extracts possess strong antioxidants in the qualitative assay as compared to other solvents. In the quantitative assay, methanol possessed the highest antioxidant activity followed by hexane. As a result, further biological investigations and isolation of these antioxidant molecules are necessary before they may be utilised as natural antioxidant supplements. The local community need to be taught conservation interventions to avoid extinction and over-exploitation of endangered plant species such as *A. versicolor* and *E. transvaalense*, as these plant species might be a potential primary source of treatment against 'u wela' and gonorrhoea.

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Competing interests

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

Authors' contributions

S.M.M., and T.T.R. designed the project. T.T.R. conducted experiments under the supervision of S.M.M. and J.N.E. T.T.R. wrote the manuscript; S.M.M. and J.N.E. reviewed and edited the article.

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Data availability

The data used to support the findings of this study may be released upon application to the corresponding author, S.M.M.

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