

Full Length Research Paper

The antibacterial activity of bacterial endophytes isolated from *Combretum molle*

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Received 30 November, 2017; Accepted 26 January, 2018

Although intense research has gone into the exploration of various Combretaceae species towards the discovery of therapeutic relevant compounds, their endophytes have never been explored as potential repositories of alternative sources of novel and medically beneficial equivalents. In the present study, five bacterial endophytes (*Lysinibacillus*, *Staphylococcus*, *Enterobacter*, *Pseudomonas* and *Bacillus* species) were isolated from different parts (hard stem, leaves and soft stem) of *Combretum molle* and identified to species level using morphological data and sequencing of the 16S rRNA. Four of the five endophytes showed varying degrees of antimicrobial characteristics against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.

Key words: Bacterial endophytes, bioactive compounds, *Combretum molle*, phylogenetic analysis, medicinal plant.

INTRODUCTION

Antibacterial resistance of microbial pathogens remains a threat to public health worldwide (Costelloe et al., 2010). Infections are increasingly becoming a challenge and established antibiotics have become less effective against some common bacterial infections (Bhalodia and Shukla, 2011). Such challenges are often due to inappropriate use of antibiotics, large and increasing numbers of immunocompromised patients, delays in diagnosis of infection and poor hygiene (Santos et al., 2015). As a result, there is need for the search of new, diverse and efficacious antimicrobial compounds. Until this is accomplished, naturally derived products remain

an essential source for novel pharmaceuticals. A range of microorganisms termed endophytes have been shown to be a rich source of bioactive compounds that can be used in therapeutics (Ravnikar et al., 2015).

Endophytes are microorganisms that reside within plant tissues without causing any substantive harm (Kumar et al., 2015). Endophytes can either be fungal or bacterial in nature and are capable of producing biologically active compounds, some of which are used by the plant as part of its arsenal in its defence against pathogens, while some promote plant growth (Gonzalez-Teuber et al., 2014; Strobel and Daisy, 2013). Most of the bioactive

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compounds extracted from endophytes have shown a plethora of bioactivities including but not limited to antimicrobial, immunosuppressant and anticancer (Nair and Padmavathy, 2014).

Combretum molle is used as a remedy throughout Africa to cure various diseases such as infertility in women, malaria and microbial infections (Ademola and Eloff, 2010). *C. molle* leaves have been reported to possess analgesic, anti-inflammatory cardiovascular, antibacterial, antifungal, anti-malarial, antitrypanosomal and anthelmintic effects (Morais-Lima et al., 2012; Ojewole, 2009). To date, no endophytes studies have been carried out from *C. molle*, thus the aim of the present study was to isolate and identify endophytic bacteria from *C. molle* and further test their crude extracts on pathogenic microorganisms.

MATERIALS AND METHODS

Plant sample collection

The plant material was harvested from Lwamondo village in Venda (23°02'37.7"S 30°24'00.2"E), Limpopo province, South Africa. Healthy, disease free plant parts (stem and leaves) of *C. molle* were collected and placed in sterile polyethylene bags and transported to the laboratory at 4°C.

Identification of the plant

Plant material was identified at the University of Johannesburg herbarium (JRAU). The sample specimen was deposited in the herbarium and assigned voucher number Diale-Serepa-Dlamini 1 and species name *C. molle*.

Isolation of bacterial endophytes

Immediately after collection of the plant material in the laboratory, the endophytes were isolated from the plant (soft, hard stems and leaves) using a method described by Jasim et al. (2014). In brief, plant parts were thoroughly washed with tap water to remove dust and cut into small segments (1 to 3 cm long). Soil debris-free plant parts were subsequently treated with Tween 80 for 10 minutes with vigorous shaking followed by rinse with distilled water. The plant samples were further immersed in 70% ethanol for 1 min and then treated with 1% sodium hypochlorite (NaOCl) for 10 min. The samples were then rinsed five times with sterile distilled water and the final wash was spread on nutrient agar plates as controls.

For isolation of bacterial endophytes, the outer surface of the sterile plant parts was trimmed; the pieces were then macerated in phosphate buffered saline (PBS). Serial dilutions of up to 10^{-3} were prepared and 0.1 mL of the dilution was spread on nutrient agar plates. Plates (including the controls) were incubated at 30°C for 2 days. The plates were observed daily for bacterial colony growth. Isolated colonies were re-cultured on sterile Nutrient agar plates until pure colonies were obtained. Glycerol (30%, glycerol diluted in sterile distilled water) stocks of each bacterial isolate were prepared and stored at -80°C for future use.

Morphological identification of endophytic bacteria

Gram staining

Pure colonies were subjected to Gram staining as described by

Collins et al. (2004) to establish morphological characteristics such as shape and Gram stain reaction. Gram stain slides were observed using a compound bright-field microscope (OLYMPUS CH20BIMF200) with 100x magnification (Gupta et al., 2015).

Scanning electron microscope

Sample preparations for the Scanning Electron Microscope (SEM) were prepared using Golding et al. (2016) and Schadler et al. (2008) methods. In brief, bacterial strains were grown in 5 mL Luria broth overnight at 30°C, shaking at 150 rpm. The bacterial cultures were centrifuged at $1100 \times g$ for 10 min and the supernatant discarded. This was followed by brief rinse with distilled water and fixing the pellet with (1:1v/v) 1% formaldehyde and 2% glutaraldehyde for 1 h at room temperature (25°C). Samples were centrifuged at $1100 \times g$ for 10 min; the supernatant was removed and the pellet washed with 1000 μ L of sterile distilled water. For dehydration, bacterial cells were treated with different concentrations of ethanol (30, 50, 70, 90, 95 and 100%) with 10 min intervals. Samples were stored open at 4°C overnight. The dehydrated samples were mounted on SEM stubs, and coated with gold using emscope SC 500 and viewed with TESCAN VEGA SEM (VEGA 3 LHM, AVG9731276ZA) connected to a monitor.

Molecular identification of the bacterial endophytes using the 16S rRNA

Extraction of genomic DNA

Pure colonies of each bacterial isolate obtained from nutrient agar were inoculated into nutrient broth and grown overnight at 30°C. Cultures were centrifuged at $13000 \times g$ for 5 min and supernatants discarded. The DNA was extracted using ZR fungal or Bacterial DNA kit (Zymo Research, catalog No R2014) following manufacturer's protocol. The extracted DNA was quantified using the NanoDrop ND-2000 UV-Vis spectrophotometer (Thermo Fisher scientific, USA).

Polymerase chain reaction (PCR) amplification and sequencing

The 16S rRNA gene of each bacterial isolate was amplified by PCR following protocol and primers described by Yeates et al. (1997). The PCR products were cleaned with ExoSAP-it™ following manufacturer's recommendations and sequenced at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa.

Phylogenetic analysis

The obtained sequences were screened for chimeras using DECIPHER (Wright et al., 2012) and subjected to BLAST (v.2.6.0) analysis at NCBI against rRNA sequence database of bacteria and archaea to identify closest bacterial species. Only bacterial species with 99 to 100% similarity were selected for phylogenetic analysis. Alignments of nucleotide sequences (isolate and species obtained through BLAST) were performed using MUSCLE with default options (Liu et al., 2016). Phylogenetic trees were constructed using a Neighbor-Joining (NJ) method based on the Tamura-Nei model (Tamura and Nei, 1993). The positions with gaps and missing nucleotide data were eliminated. All evolutionary analyses were conducted in MEGA 7 (Kumar et al., 2016). The 16S rRNA gene sequences of bacterial isolates identified in the study were deposited in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) with the following accession numbers MF105747 *Enterobacter* species SSRP1, MF105748 *Lysinibacillus* species HSRN, MF105749

Table 1. Bacterial endophytes isolated from different part of *Combretum molle*.

Bacterial colony code	Plant part	Gram staining	Shape
HSRN	Hard stem	Gram positive	Rod
LCP	Leaves	Gram positive	Cocci
SSRP1	Soft stem	Gram negative	Rod
SSRN1	Soft stem	Gram negative	Rod
LRP	Leaves	Gram positive	Rod

Pseudomonas species SSRN1, MF105750 *Bacillus* species LRP, and MF105751 *Staphylococcus* species LCP. The assigned names of the bacterial isolates were based on the BLAST homology percentages as well as phylogenetic results.

Phytochemical analysis

Sample preparation

C. molle plant parts (stem, leaves and bulk) were dried at 27°C for 7 days and then they were blended into a fine powder.

Qualitative analysis of phytochemicals on *C. molle*

Phytochemical screening was conducted using Trease and Evans (1983) and Harbourne (1983) methods.

Phytochemicals analysis of endophytes crude extract

Phytochemical screening of endophytes crude extracts was conducted using the same methods (Trease and Evans, 1983; Harbourne, 1983) with some modifications.

Production of secondary metabolites from bacterial endophytes

Nutrient broth (8 L) was prepared in 2 L Erlenmeyer flasks and autoclaved at 121°C for 15 min. Each 2 L flask was inoculated with one of each endophytic bacteria and incubated at 30°C for 7 days (Sandhu et al., 2014). After 7 days of cultivation, sterilized XAD-7-HP resin (20 g/L) (SIGMA, South Africa, BCBR6696V) was added to the culture for 2 h shaking at 200 rpm. The resin was filtered using cheese cloth and eluted with acetone three times. Acetone was removed using a Rota evaporator. The remaining water was extracted with ethyl acetate three times and concentrated using a rotary evaporator (Maloney et al., 2009).

Antibacterial activity of the crude extracts from bacterial endophytes

Antibacterial tests were carried out by using a modified disc diffusion method described by Bauer et al. (1966). All pathogenic strains (Gram-negative strains: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella oxytoca* ATCC 13182; Gram-positive: *Staphylococcus aureus* NCTC 6571 and *Bacillus cereus* ATCC 10876) were grown overnight at 37°C in Muller-Hinton broth and adjusted using 0.5 McFarland standards such that the concentration was 10^7 to 10^8 colony forming unit (CFU/mL). Under sterile conditions, 0.1 mL of each pathogenic strain was spread on Muller-Hinton agar. Sterile circular paper discs

with a diameter of 6 mm were soaked in 10 µL of each bacterial endophyte crude. 10 µL of 1 mg/L streptomycin (SIGMA-ALDRICH, Switzerland, BCBP5897V) was used as positive control and loaded on the discs as described earlier. Six discs of different crude extracts including the control were placed on each spread plate inoculated with different pathogenic strains and incubated at 30°C for 72 h. Antimicrobial activity was observed daily by measuring the zone of inhibition (in mm). The antibacterial test was performed in triplicates.

RESULTS AND DISCUSSION

Isolation and identification of bacterial endophytes

Morphological identification

The surface sterilization of plant material is important for isolation and studying endophytes. The stems and leaves of *C. molle* were surface sterilized for isolation of bacterial endophytes. The surface sterilization method was satisfactory as no growth emerged on control plates. Thus, the isolated bacterial colonies can be considered true endophytes. Five bacterial colonies were isolated (Table 1). The colonies were differentiated based on their Gram reaction, colony colour and morphology. The Gram stain reaction results showed three of the bacterial isolates to be Gram-positive and two Gram-negative bacterium. The morphological shapes observed from Gram stain reaction were further confirmed using SEM results (Figure 1A to E) which had uniform cells indicating that the bacterial cultures were pure.

Phylogenetic relationship

The BLAST search results of the 16S rRNA gene sequences resulted in varying bacterial genera. Bacterial endophyte HSRN had maximum identity to *Lysinibacillus fusiformis* (100%), LCP had maximum identity to *Staphylococcus epidermis* (100%), SSRP1 had maximum identity to *Enterobacter cloacae* (99%), SSRN1 had maximum identity to *Pseudomonas fulva* (100%) and LRP had maximum identity *Bacillus subtilis* (99%), thus the isolated putative bacterial endophytes can be considered bacterial strains of *Lysinibacillus*, *Staphylococcus*, *Enterobacter*, *Pseudomonas* and

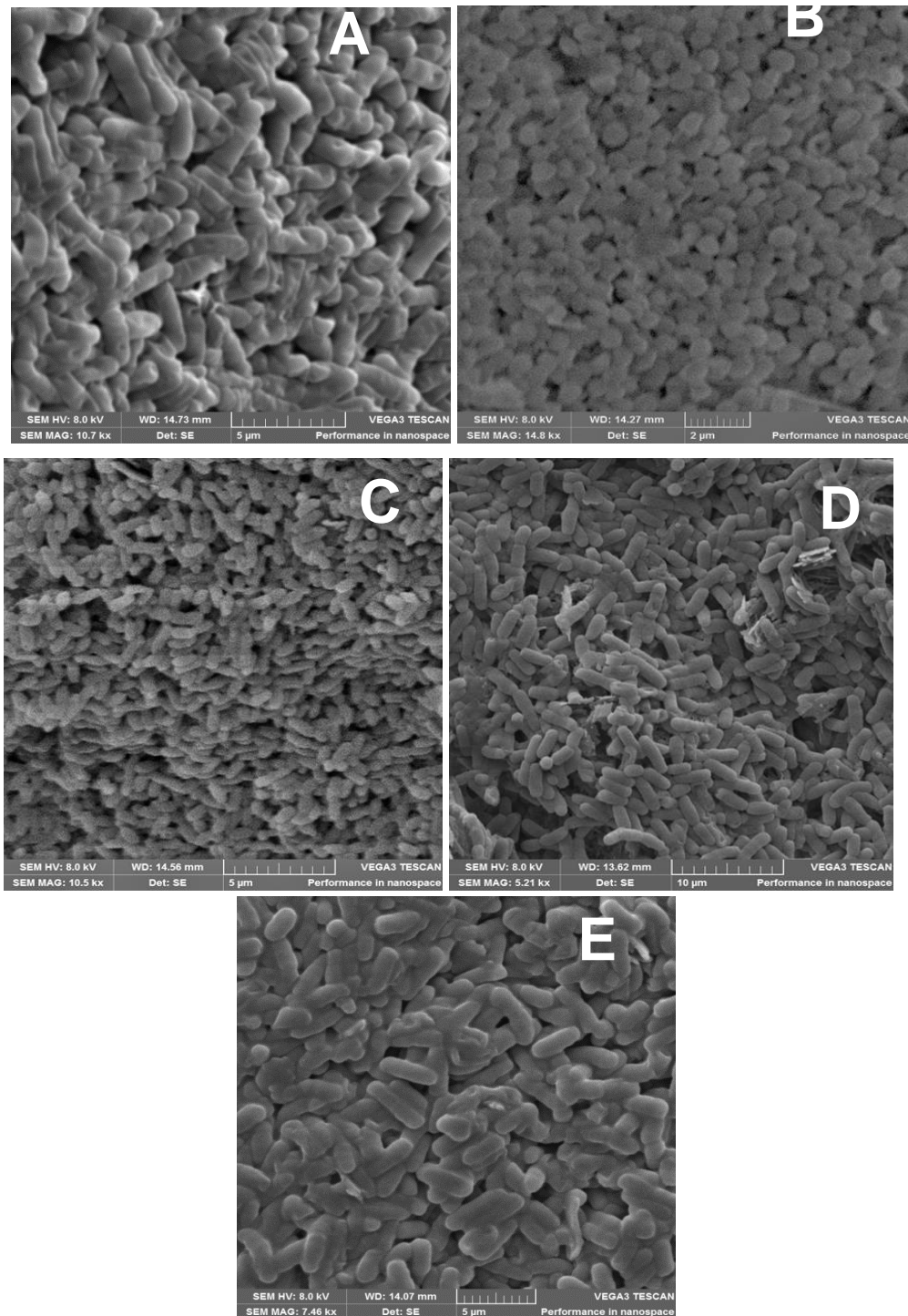


Figure 1. Scanning electron micrographs showing cell morphology of five endophytic bacteria isolated from *Combretum molle*. (a) *Lysinibacillus* spp. HSRN; (b) *Staphylococcus* spp. LCP; (c) *Enterobacter* spp. SSRP1; (d) *Pseudomonas* spp. SSRN1; (e) *Bacillus* spp. LRP.

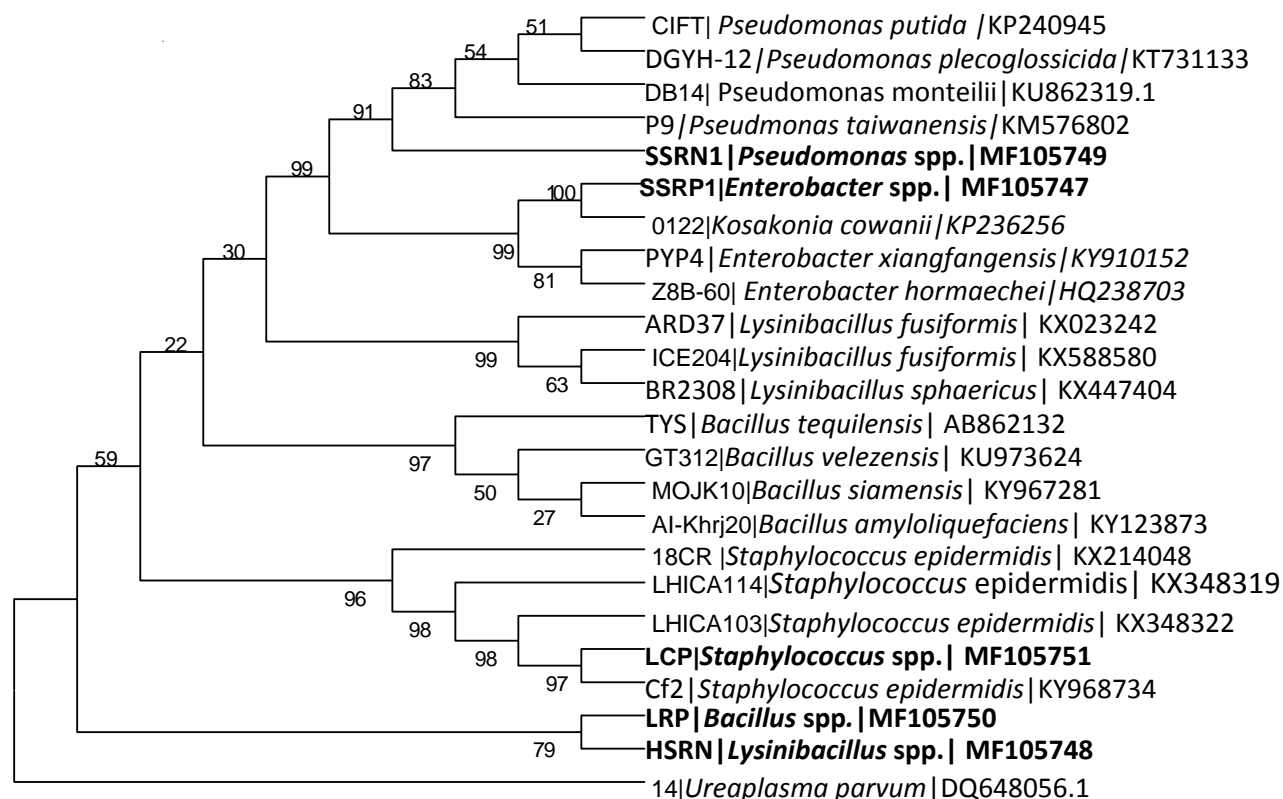
Bacillus spp. (Table 2). To our knowledge, this is the first study to report these bacterial endophytes from *C. molle*.

The phylogenetic analysis showed that the endophytic bacterial isolates are grouped with various closely related

bacterial species (Figure 2). From Figure 2, *Enterobacter* spp. SSRP1 MF105747 had a sister relationship with *Kosakonia cowanni* KP236256 a species isolated from a sea grass *Thalassia hemprichii* with a bootstrap of 100%.

Table 2. BLAST analysis of the 16S rRNA genes of bacterial endophytes from *C. molle*.

Bacterial colony code	Plant part	GenBank accession number	Assigned bacterial name	Closest NCBI related bacterial species with accession number
HSRN	Hard stem	MF105748	<i>Lysinibacillus</i> spp.	<i>Lysinibacillus fusiformis</i> KX867805
LCP	Leaves	MF105751	<i>Staphylococcus</i> spp.	<i>Staphylococcus epidermidis</i> MG027640
SSRP1	Soft stem	MF105747	<i>Enterobacter</i> spp.	<i>Enterobacter cloacae</i> CP022532
SSRN1	Soft stem	MF105749	<i>Pseudomonas</i> spp.	<i>Pseudomonas fulva</i> MF421780
LRP	Leaves	MF105750	<i>Bacillus</i> spp.	<i>Bacillus subtilis</i> MF187644



0.10

Figure 2. Neighbour joining tree based on 16S rRNA gene sequence of five endophytic bacteria isolated from *C. molle* and other similar species selected from GenBank.

K. cowanni belongs to the Enterobacteriaceae family. *Lysinibacillus* spp. HSRN MF105748 and *Bacillus* spp. LRP MF105750 also had a sister relationship with 75% bootstrap value. These two species are from the same family name Bacillaceae. *Staphylococcus* spp. LCP MF105751 had a sister relationship with *Staphylococcus epidermidis* KY968734 a species isolated from sphenoid sinus biopsy with a 97% bootstrap value. *Pseudomonas*

spp. SSRN1 MF105749 shared a common ancestor with *Pseudomonas taiwanensis* KM576802 isolated from rhizosphere soil with 91% bootstrap value.

All current bacterial endophytes strains were reported as endophytes from various plant species (Chaudhry and Patil, 2013; Christina et al., 2013; Mahummad et al., 2014; Zhao et al., 2015). Similar studies on isolation of endophytes have been reported by other researchers,

Table 3. Qualitative analysis of phytochemicals in leaf, stem, and bark of *Combretum molle*.

Test	Leaves	Stem	Bark
Alkaloids	-	-	-
Flavonoids	+	+	+
Steroids	-	+	+
Tannins	+	+	+
Saponins	-	-	-

Table 4. Qualitative analysis of phytochemicals of crude extracts of endophytes isolated from *Combretum molle*.

Test	<i>Lysinibacillus</i> spp. HSRN	<i>Staphylococcus</i> spp. LCP	<i>Pseudomonas</i> spp. SSRN1	<i>Enterobacter</i> spp. SSRP1	<i>Bacillus</i> spp. LRP
Alkaloids	-	-	-	-	-
Flavonoids	+	+	+	+	+
Steroids	-	-	-	-	-
Tannins	+	+	-	+	+
Saponins	-	-	-	-	-

where *Pseudomonas* and *Bacillus* spp. were isolated from *Echinacea* medicinal plant (Christina et al., 2013). *L. fusiformis* isolated from the medicinal plant *Panicum virgatum* and *S. epidermis* isolated from rice seeds have been reported to be endophytes (Ryan et al., 2008). The aforementioned indicates that these species are present as endophytes within a variety of plant species which makes them more interesting for further studies, such as plant growth promotion and their possible applications in drug discovery and agriculture.

The five-isolated putative bacterial endophytes represent five different genera, which indicate diverse bacterial endophytes present within *C. molle*. Endophytes are known to vary in diversity based on seasonal collection or sampling time, plant age, plant tissue type and environment (Jasim et al., 2013). In this study, it was strongly believed that *C. molle* is likely to be associated with other different types of bacterial endophytes.

Phytochemical analysis

Secondary metabolites studies of the leaves, stem and bark of *C. molle* showed the presence of tannins, flavonoids and steroids (Table 3). These secondary metabolites possess properties which are of great importance in the drug development (Joseph et al., 2013). Flavonoids are known to have antimicrobial, anti-cancer, anti-inflammatory and anti-viral properties (Kabera et al., 2014) and tannins have stringent properties and can be utilized for antibacterial, anti-diarrheal, haemostatic and anti-haemorrhoidal drugs (Ashok and Upadhyaya, 2012). All five endophytes crude extracts revealed the presence of flavonoids and tannins,

except *Pseudomonas* spp. SSRN1 showed absence of tannins as indicated in Table 4. Elof et al. (2008) reported that the family Combretaceae contains a wide variety of tannins and flavonoids. Presence of flavonoids was also detected in other plants of the same genera named *Combretum erythrophyllum* (Bhatnagar et al., 2012). Thus, presence of flavonoids and tannins in *C. molle* indicates therapeutic potential of the plant.

Antimicrobial activity

The crude extracts of the putative endophytic bacterial strains were assayed for antimicrobial activity against pathogenic strains (Gram-negative strains: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *K. oxytoca* ATCC 13182; Gram-positive: *S. aureus*, NCTC 6571 and *B. cereus*, ATCC 10876). Among the five endophytic bacteria, only four except *Staphylococcus* spp. LCP showed antimicrobial activity. *Pseudomonas* spp. SSRN1 and *Enterobacter* spp. SSRP1 were considered as the most active strains as they both had a moderate activity against *S. aureus*. High zone of inhibition was by *Pseudomonas* spp. SSRN1 and *Enterobacter* spp. SSRP1, followed by *Lysinibacillus* spp. HSRN, then lastly *Bacillus* spp. LRP (Table 5). Endophytic bacteria have potential to produce novel natural compounds with antibacterial and antifungal activity (Christina et al., 2013). Bacterial endophytes (*Pseudomonas* spp. and *Bacillus* spp.) isolated from *Plectranthus tenuiflorus* have shown great antimicrobial activity against some human pathogenic strains such as *Salmonella typhi*, *S. aureus*, *E. coli*, *Klebsiella pneumoniae*, *Streptococcus agalactiae*, *Proteus mirabilis*, *Candida albicans* (El-Deeb et al.,

Table 5. Antimicrobial activity of Endophytic bacteria from *C. molle*.

Pathogenic strains	<i>Staphylococcus</i> spp. LCP	<i>Bacillus</i> spp. LRP	<i>Pseudomonas</i> spp. SSRN1	<i>Enterobacter</i> spp. SSRP1	<i>Lysinibacillus</i> spp. HSRN	<i>Streptomycin</i> 1 mg/L
<i>Bacillus cereus</i> ATCC 10876	-	+	+	+	+	+++
<i>Staphylococcus aureus</i> NCTC 6571	-	-	++	++	+	+++
<i>Escherichia coli</i> ATCC 25922	-	+	+	+	+	+++
<i>Pseudomonas aeruginosa</i> ATCC 27853	-	+	+	+	+	+++
<i>Klebsiella oxytoca</i> ATCC 13182	-	-	-	-	-	++

+, Weak activity; ++, moderate activity; +++, Strong activity; -, No zone of inhibition.

2013). Furthermore, *Enterobacter* spp. isolated from *Raphanus sativus* L. also showed antibacterial activity against a few human pathogenic bacteria including *E. coli*, *Salmonella enteritidis*, *Shigella sonnei*, *Salmonella typhimurium*, *P. aeruginosa*, *Shigella flexneri* and *B. cereus* (Seo et al., 2010). *Pseudomonas* spp. have proven to possess antimicrobial compounds called ecomycins and pseudomycins (Christina et al., 2013). Secondary metabolites from *C. molle* were also reported to possess antimicrobial activity (Fankam et al., 2015; Kaleab et al., 2006). It is evident from the current study that the isolated bacterial endophytes also have antibacterial activity with a broad antibacterial spectrum. Thus, the bacterial endophytes with antibacterial activity from the current study can play a part in inhibiting plant pathogens growth. In addition, potential applications such as drug discovery and biocontrol use in agriculture can arise from these bacterial endophytes and necessitates further investigations.

Conclusion

This is the first study to report on bacterial endophytes occurrence in *C. molle*. The reported bacterial endophytes isolated have been shown to be a rich source of diverse bioactive compounds

with potential applications in drug discovery and agriculture. Studies are currently underway to ascertain if the bacterial endophytes produce the same or similar secondary metabolites as their plant host *C. molle*. Further studies can lead to the development of novel therapeutic drugs from secondary metabolites produced by these bacterial endophytes. Thus, in addition to the well-established photochemistry and bioactivity of *C. molle*, there is now evidence of an extended and potential alternative source of antimicrobials.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This work was funded by the National Research Foundation of South Africa and University of Johannesburg merit bursary for postgraduate students.

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