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Isolation and identification of bacterial endophytes from *Crinum macowanii* Baker

Rebotiloe F. Morare[1*], Eunice Ubomba-Jaswa[1,2] and Mahloro H. Serepa-Dlamini[1]

[1]Department of Biotechnology and Food Technology, Faculty of Science, University of Johannesburg, Doornfontein Campus, P. O. Box 17011 Doornfontein 2028, Johannesburg, South Africa.
[2]Water Research Commission, Lynnwood Bridge Office Park, Bloukrans Building, 4 Daventry Street, Lynnwood Manor, Pretoria, South Africa.

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The widespread distribution of *Crinum macowanii* across the African continent has entrenched the plant's medicinal usage in treating diverse diseases. While its phytochemistry is well established, its microbial symbionts and their utility have not been described. As such, five bacterial endophytes, *viz. Staphylococcus* species C2, *Staphylococcus* species C3, *Bacillus* species C4, *Acinetobacter* species C5 and *Staphylococcus* species C6 were isolated from fresh *C. macowanii* bulb and their phenotypic and genotypic profiles verified by Gram staining and 16S rRNA gene sequencing; respectively. The latter was used to construct a phylogenetic tree that showed similarities (higher than 50 bootstrap values) among the endophytic bacterial isolates. Chemical analysis of bacterial endophytes was done by extracting the crude extracts of each endophyte. Antibacterial activity of each endophyte was performed against a few selected bacterial pathogenic strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Bacillus cereus*) using the disk diffusion method with Streptomycin used as a positive control. The crude extracts of all the endophytes showed no bioactivity against *K. pneumoniae*, though the inhibition was observed against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. cereus*. These results suggest that crude extracts of endophytic bacteria from *C. macowanii* have the potential to be used as antimicrobial agents.

Key words: Antibacterial activity, *Crinum macowanii*, endophytes, phylogentic analysis.

INTRODUCTION

Endophytes, fungi and bacteria are microbial symbionts that occupy internal tissues of plants such as leaves, stems, roots and flowers without causing diseases to their plant hosts (Alvin et al., 2014; Nisa et al., 2015; Wu et al., 2016). The plant-endophyte interaction is a mutual relationship (Pimentel et al., 2011; Ginting et al., 2013), with plants offering residence, nutrients and protection to the endophytes; whilst endophytes provide several benefits to improve growth and health of their plant hosts (Eljounaidi et al., 2016; Pereira et al., 2016). These

*Corresponding author. E-mail: rebotiloem@gmail.com.

microorganisms unlike pathogens serve as protective agent of plants by synthesizing secondary metabolites that protect their hosts against pathogens and insect attack (Ellouze et al., 2015). Another mechanism utilised by endophytes to prevent pathogenesis is by inhibition of pathogenic microorganisms (Wu et al., 2016). Endophytes also enhance plant growth by improving nutrient absorption, production of plant hormones and nitrogen fixation (Jin et al., 2014; Santoyo et al., 2016). This defence mechanism against pathogens and insects show potential of endophytes as bio-control agents in agricultural applications (Ryan et al., 2008). Secondary metabolites produced by endophytes have shown various biological activities such as anti-microbial, anti-oxidant, anti-cancer and anti-diabetic (Nair and Padmavathy, 2014). In this study, bacterial endophytes were isolated from medicinal plant, *Crinum macowanii* and tested for anti-bacterial activity against human pathogens.

C. macowanii Baker is a highly valued medicinal plant distributed in tropical areas of eastern and southern regions of Africa. It is found among the 130 species of genus *Crinum*, in a family of Amaryllideceae. Plant species in this family are known to produce alkaloids as a largest group of secondary metabolites (Elgorashi et al., 2003). Amaryllideceae alkaloids clinically approved are lycorine and galathamine, with anti-tumor and acetycholine esterase inhibitory activities, respectively (Acosta et al., 2014).

C. macowanii is mainly used as a healing agent for treatment of sexually transmitted diseases, backaches and is used to stimulate lactation in women and cows (Nair et al., 2000). Other medicinal applications of *C. macowanii* include treatment of kidney and bladder infections, tuberculosis, swelling of the body, scrofula, rheumatic fever, itchy rashes, sores, boils and acne, backache, and venereal disease (Maroyi, 2016). Due to the extensive medicinal application, following its slow reproduction system, this plant is gradually being an endangered species (Nair et al., 2000). With *C. macowanii* endangered, there is a need to explore the species endophytes and their biological activities. Thus, the aim of this study was to isolate, characterize and identify bacterial endophytes from *C. macowanii* and test their secondary metabolites on pathogenic bacterial species.

MATERIALS AND METHODS

Collection and identification of plant material

C. macowanii plant materials were collected in Makonde village with geographical co-ordinates 22°48'18.4"S 30°35'41.4"E, Thohoyandou, Limpopo province, South Africa. Disease free plant materials (leaves, bulbs) were collected and placed in sterile polyethylene bags and transported to the laboratory at 4°C. The plant material was identified at the herbarium of University of Johannesburg (JRAU). The identification of the plant was based on the plant parts collected. The plant specimen was deposited in the herbarium and assigned voucher number Morare-Serepa-Dlamini 1.

Isolation of bacterial endophytes

For isolation of bacterial endophytes, the bulb was the only part utilised by following a protocol by Jasim et al. (2014), which was slightly adjusted to achieve *C. macowanii* bulb surface sterilization. The first outer layer of the bulb (covered with heavy soil) was peeled off and the bulb washed several times with tap water to remove soil on the second layer. The bulb was treated with sufficient volume covering the whole bulb, of Tween 80 with vigorous shaking for 10 min. This was followed by several washes with sterile distilled water, after which the bulb was immersed in 70% ethanol for 1 min with shaking. The ethanol was rinsed off with sterile distilled water and the bulb further sterilized with 1% sodium hypochlorite (NaOCl) for 10 min. The sample was finally rinsed with sterilized distilled water 3 times. The last distilled water rinse was plated on nutrient agar plates as control.

The sterilized bulb outer surface was trimmed off (using sterile blades); the sample was cut into pieces, which were further macerated in phosphate buffered saline (PBS). Serial dilutions of up to 10^{-3} were done using the liquid from the macerated material. From the dilution, a volume of 0.1 mL was spread plated on nutrient agar plates. The control and experiment plates were incubated at 30°C for 2 days and observed daily for bacterial growth. Each bacterial isolate was transferred to sterile nutrient agar plates for purposes of obtaining pure cultures. Glycerol stock cultures for each bacterial isolate were prepared and stored at -80°C for future use.

Morphological identification of bacterial endophytes

Macroscopic methods were used for morphologically identifying endophytic bacteria, the colony shape, size and colour were the first characteristics used for identification. Endophytic bacterial isolates were further identified by traditional Gram stain reaction (Cruikshank et al., 1975) and viewed using a compound bright-field microscope (OLYMPUS CH20BIMF200) with 100x magnification.

Scanning electron microscopy (SEM)

SEM was used to confirm and study the putative bacterial endophyte isolates. This was done following the method reported by Golding et al. (2016). Bacterial endophytes were grown overnight in Luria-Bertani (LB) broth at 30°C in a shaking incubator at 150 rpm. The bacterial suspension of each isolate was centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the pellet cell were rinsed with sterile distilled water and chemically fixed by 1% formaldehyde and 2% of glutaraldehyde (1:1 volume) for 24 h at room temperature (25°C). The samples were serially dehydrated with different concentrations of ethanol (30, 50, 70, 90, 95 and 100%) at 10 min intervals for each concentration. The samples were left to dry overnight in Eppendorf tubes. Dried samples were sputter coated with gold using emscope SC 500 (Goulding et al., 2016), and viewed using the TESCAN VEGA SEM (VG9731276ZA) connected to a monitor.

Molecular identification of bacterial endophytes

Extraction of genomic DNA

Genomic DNA was extracted from obtained pure colonies of each bacterial endophyte isolate using the ZR fungal/bacterial DNA MiniPrep kit (Zymo Research, catalog No R2014) following manufacturer's protocol. The concentration of the extracted DNA was determined using a NanoDrop ND-2000 UV-Vis spectrophotometer (Thermo Fisher scientific, USA).

Table 1. Phytochemical tests for bacterial endophytes extracts.

Phytochemical test	Methods	Observations
Tannins	Add 2-3 drops of 10% $FeCl_3$ to 1 ml of endophytes extract	Blackish-blue or blackish-green colour
Alkaloids	Add few drops of Dragendroff's reagent to 1 ml of extract.	Turbidity or precipitation formation
Flavonoids	1 ml of extract + few drops of NaOH	Yellow formation
Saponins	Add few drops of olive oil to 5 ml of extract. shake vigorously	Froth formation
Steroids	1 ml extract + 1 ml of $CHCl_3$. add few drops of conc. H_2SO_4	Reddish brown ring

Polymerase chain reaction (PCR) of the 16S rRNA gene and sequencing experiments

The 16S rRNA gene of each bacterial endophyte was amplified following protocol described by Tsuchida et al. (2002). Briefly, the 16S rRNA gene was amplified using the primers (16S-27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 16S-1492R: 5'-CGGTTACCTTGTTACGACTT-3') with 2x PCR master mix with standard buffer. The PCR products and primers were sent for sequencing at Inqaba Biotechnical Company (Pty) Ltd, Pretoria, South Africa.

Phylogenetic analysis

Subsequent to sequencing, the obtained 16S rRNA gene sequences (base pairs) were screened for chimeras using DECIPHER (Wright et al*.,* 2012). The 16S rRNA gene sequences were subjected to BLAST (v.2.6.0) at NCBI to obtain closely related bacterial species. Highly similar sequences with a 96 to 100% identity were aligned with bacterial endophyte sequences isolate using MUSCLE (Edgar, 2004); phylogenetic trees were drawn using the Neighbour-Joining method based on Tamura-Nei model (Tamura and Nei, 1993). Bootstrap values of 1000 replicates were used to determine the tree strength (Pattengale et al., 2009). All evolutionary analysis was performed on MEGA 7 software (Kumar et al*.,* 2016). All obtained sequences of the bacterial endophytes were deposited into GenBank and assigned accession numbers: MF085046 *Staphylococcus* species C2, MF085048 *Staphylococcus* species C3, MF509594 *Bacillus* species C4, MF509593 *Acinetobacter* species C5 and MF509595 *Staphylococcus* species C6.

Production of secondary metabolites by bacterial endophytes

Bacterial endophytes were cultured in 10 Erlenmeyer flasks each containing 500 mL of sterile nutrient broth media, which was shaken at 200 rpm at 30°C for 7 days. An amount of 20 g of XAD-7-HP resin (SIGMA, South Africa, BCBR6698V) was added to the culture after 7 days. The resin was filtered through a cheesecloth, which was washed with deionized water and eluted with 100 ml acetone. The acetone-soluble fraction was dried using a rotary evaporator to yield a crude extract (Hu et al*.*, 2012).

Qualitative analysis of phytochemicals of *C. macowanii* and endophytes crude

Phytochemical screening of *C. macowanii* was adopted from (Trease and Evans, 1983; Harbourne, 1983). Same methods were followed for phytochemical screening of endophytes crude extracts (Trease and Evans, 1983; Harbourne, 1983) with some modifications shown in Table 1.

Antimicrobial activity of the crude extracts from bacterial endophytes

The disc diffusion method as described by Hoelzer et al. (2011) and Zhang et al*.* (2012) was carried out to evaluate the anti-bacterial nature of the bacterial endophytes' secondary metabolites crude extracts. Five pathogenic bacterial strains (Gram-negative strains *Escherichia coli*, ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 13182; Gram-positive strains *Staphylococcus aureus* NCTC 6571 and *Bacillus cereus* ATCC 10876), were grown overnight at 37°C on Mueller-Hinton (MH) broth which was adjusted to 0.5 McFarland standard. The cultures were further spread plated on MH agar plates and sterilized circular paper discs (6 mm) were placed on the MH agar containing bacterial lawn culture of the test pathogenic strains. The crude extract of endophytes was dissolved in ethyl acetate and a 10 µL of each endophyte crude extracts were aseptically placed on each disc. 10 µL of 1 mg/mL Streptomycin (Sigma, Aldrich, Switzerland, BCBP5897V) was also aseptically placed on one of the paper disk in each plate as a positive control. The plates were incubated at 37°C for 48 to 72 h and the anti-bacterial activity was assessed by measuring the diameter of the zone of inhibition in mm. The anti-bacterial tests were performed in triplicates.

Statistical analysis

The antibacterial data was reported as mean ± standard deviations (SD). Data obtained was analysed using Two-way analysis of variance (ANOVA). The analysis was carried out using the Microsoft Excel 2010 ANOVA. P values < 0.05 were considered statistically different.

RESULTS AND DISCUSSION

Morphological identification of endophytes from *C. macowanii*

C. macowanii bulb was surface-sterilized prior to isolation of bacterial endophytes. The surface sterilization method was adequate as all control plates did not have any microbial growth. Five putative bacterial endophytes were isolated and identified. The bulb was dominated with four Gram positive bacteria and one additional Gram negative bacterial endophyte. The Gram stain reaction further indicated that three of the bacterial endophytes were cocci shaped and the remaining two were rod shaped. The bacterial endophyte shapes were further confirmed by the SEM electron micrographs (results not shown). Bacterial endophytes have been previously reported in

Table 2. Relationship of endophytic bacteria isolates with highly similar genera found in NCBI.

Bacterial isolate codes	Bacterial name	Highly similar genus	NCBI blast homology % of dominant genus	Accession number	Size of 16S rRNA gene (basepairs)
C2	*Staphylococcus* spp.	*Staphylococcus*	99	MF085046	199
C3	*Staphylococcus* spp.	*Staphylococcus*	100	MF085048	500
C4	*Bacillus* spp.	*Bacillus*	100	MF509594	517
C5	*Acinetobacter* spp.	*Acinetobacter*	99	MF509593	551
C6	*Staphylococcus* spp.	*Staphylococcus*	99	MF509595	505

other medicinal plants including *Catharanthus roseus, Ocimum sanctum* and *Mentha arvensis* (Anjum and Chandra, 2015), *Lonicera japonica* (Zhao et al., 2015) and *Ferula songorica* (Liu et al., 2016); however, no bacterial endophytes have been reported in *C. macowanii*.

Medicinal plants including *C. macowanii* have been considered valuable sources of bioactive compounds for drug development. However, due to their destruction and loss, these can no longer be utilised for drug development as there is a high decline in plant populations caused by overexploitation in drug development and other industrial applications (Tomita, 2003). Because endophytes are known to produce similar secondary metabolites as their host plant, these can be isolated, identified and further investigations performed on their produced secondary metabolites for drug development. Even though *C. macowanii* bulbs had varying antimicrobial results (Sebola et al., 2016), it continues to be utilised as traditional medical plant. In order to explore the potential applications of the bacterial endophytes from *C. macowanii*, it was necessary to isolate and identify its bacterial endophytes.

Molecular identification and phylogenetic analysis

The 16S rRNA gene sequence results were used to confirm the bacterial endophyte identification. The BLAST search results indicate that bacterial isolate C2 was closely related to bacterial species belonging to *Staphylococcus* genus, C3 to *Staphylococcus* genus, C4 to *Bacillus*, C5 *Acinetobacter* and C6 *Staphylococcus* genus as indicated in Table 2. *Staphylococcus* was the dominant genus with three species, followed by one of *Bacillus* and *Acitenobacter*. The isolated putative bacterial endophytes grouped with closely related bacterial species on the delineated phylogenetic tree Figure 1. The two *Staphylococcus* C3 and C6 had a sister relationship with 100% bootstrap value. These two species also had a monophyletic relationship with the *Bacillus* C4 isolate. Another monophyletic relationship was observed between *Acinetobater* spp. C5 and *Acinetobacter johnsonii* PVB6L3 with the *Gamma proteobacterium* PM20.

Bacillus and *Acitenobacter* spp. are part of the 16 genera that have been identified as endophytes (Sekhar and Thomas, 2015; Gouda et al*.,* 2016). These species have been isolated from roots, stem and leaves of *L. japonica* (Zhao et al., 2015) and sterilized roots of *Beta vulgaris* (Shi et al., 2011). Although *Staphylococcus* spp. are established members of the human micro-flora, some species such as *Staphylococcus epidermidis* have been previously reported as plant endophytes (Berg et al., 2005; Kai et al., 2007, 2008; Vendan et al., 2010). Chaudhry and Patil (2016) have indicated that this *Staphylococcus* species are adapted in various hosts and have shown protection and development characteristics to its plant host. These are characteristics of endophytes within their plant hosts.

The bulb of *C. macowanii* was chosen for isolation of bacterial endophytes as it was expected to have more endophytes isolates than the above ground tissues. However, only five bacterial endophytes were isolated in this study, providing limited diversity of bacterial endophytes as compared to other similar studies. These results could be associated with factors other than the selected plant tissue. Jasim et al. (2014) stated that the number of endophytes isolates could differ due to the plant used, age, seasonal collection or the environment. The isolation method used also play a role in number of bacterial endophytes yielded. For this study a maceration of plant tissue was used. Huang et al. (2015) indicated that isolation method whereby plant material is cut into small pieces and placed on growth medium yield highly numerous endophytes.

Phytochemical analysis of *C. macowanii* and bacterial endophytes

The results of phytochemical screening showed that *C. macowanii* bulb constitutes alkaloids, saponins and tannins and contained no flavonoids and steroids. This plant is found in Amaryllideceae family which contain abundance of various alkaloid components (Tram et al., 2002). Numerous alkaloids were reported by Fennell and Staden (2001) in *Crinum* species. Maroyi (2016) further indicated alkaloids are abundant in bulbs of *Crinum* spp. Sebola et al. (2016) reported that *C. macowanii* bulb contains high amount of alkaloids which confirmed that

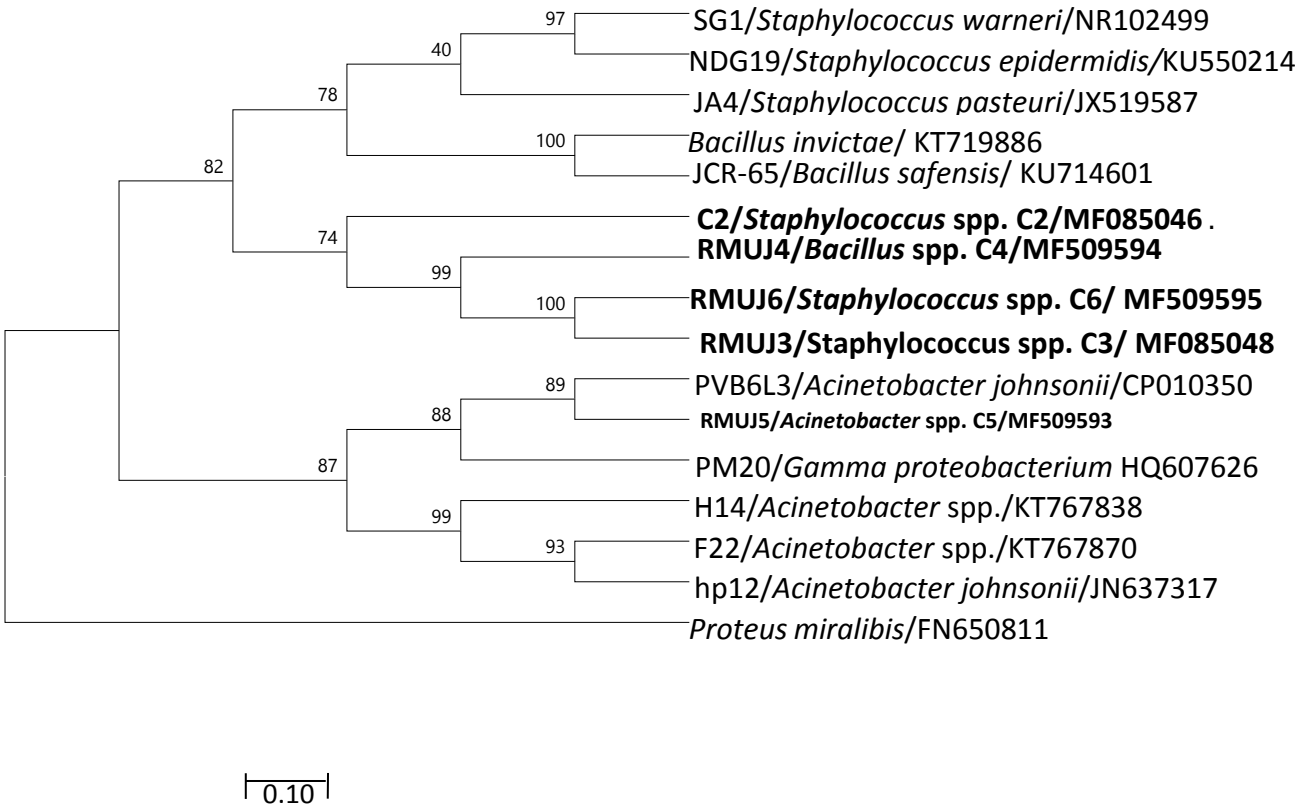

Figure 1. Phylogenetic tree analysis based on 16S rDNA sequences of five endophytic bacteria isolates, with *Bacillus* spp. C4 closely related to *Staphylococcus* species isolated and the *Acinetobacter* spp. C5 unrelated to the *Staphylococcus* species isolates by neighbour-joining method showing an ancestral group of *Proteus mirabilis*.

this species is rich with this kind of metabolites. The alkaloids in *Crinum* spp. have been reported to have therapeutic properties, hence *C. macowanii* and other *Crinum* spp. are used as medicine to treat illness (Fennell and Staden, 2001).

However, the bacterial endophytes isolated from *C. macowanii* have shown different chemical constituents to their host. Bacterial endophyte isolates, *Acinetobacter* spp. C5 and *Staphylococcus* spp. C6 indicated the presence of alkaloids, while *Staphylococcus* spp. C2 and C3 indicated the presence of flavonoids which was not found in *C. macowanii* bulb. *Bacillus* spp. C4 was the only species which contained tannins compounds. The saponins were not found in all the bacterial endophytes, but were present in the plant. There were no steroids in both the plant and their bacterial endophytes.

Antibacterial activity of endophytes extracts against pathogenic strains

The bacterial endophytes showed anti-bacterial activity against selected pathogenic strains as shown in Figure 2. All of the bacterial endophytes had antimicrobial activity against pathogenic strains (Gram-negative strains *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 13182; Gram-positive *S. aureus* NCTC 6571 and *B. cereus* ATCC 10876). *Bacillus* spp. C4 and *Acitenobacter* spp. C5 had the highest antibacterial activity against *S. aureus*, *E. coli* and *B. cereus*. None of the bacterial endophytes had antibacterial activity against *K. pneumoniae*; this could be due to the resistance mechanism by *K. pneumoniae* such as development of extended-spectrum β-lactamases (ESBLs) (Carlet, 2012; Lin et al., 2016). The ANOVA statistics analysis indicated that p value was less than 0.05 ($p < 0.05$), therefore there was a significant difference between all five bacterial endophytes antimicrobial activity.

Antibacterial activity of endophytes extracts have shown variation as compared to antibacterial activity of *C. macowanii* extracts reported by Sebola et al. (2016). The antibacterial activity of *C. macowanii* had broad inhibition spectrum against pathogenic strains such as *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus* and *S. epidermidis*. Therefore, there is a need to improve the extraction method of extracts from endophytes or increase the concentration of endophytes extracts in

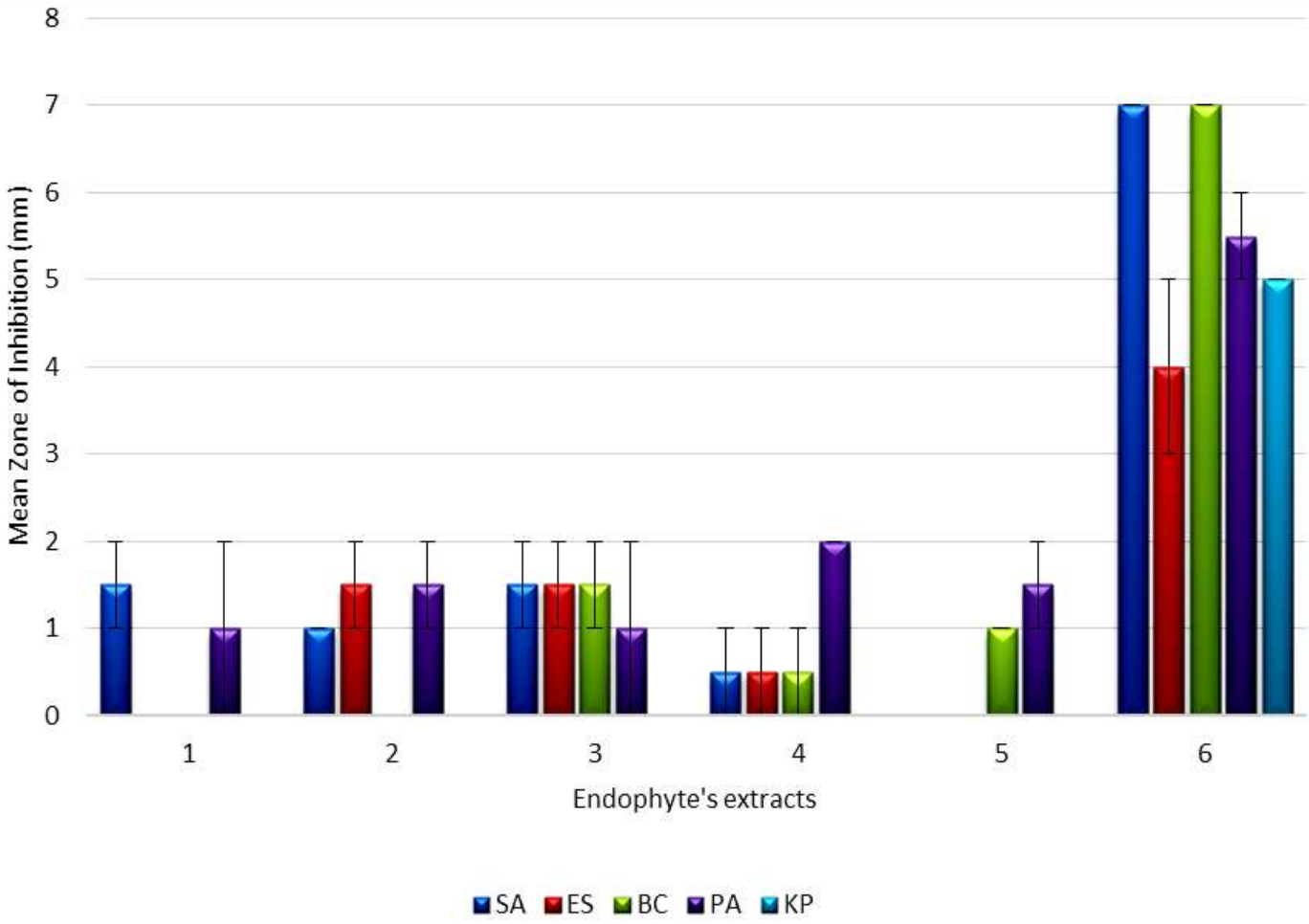

Figure 2. Antibacterial activity of endophytes extracts and streptomycin (positive control) against pathogenic strains. Antimicrobial activity is measured by zone of inhibition (mm) of the pathogenic strains. SA: *Staphylococcus aureus*, EC: *Escherichia coli*, BC: *Bacillus cereus*, PA: *Pseudomonas aeruginosa*, KP: *Klebsiella pneumoniae*. 1, *Staphylococcus* C2; 2, *Staphylococcus* C3; 3, *Bacillus* C4; 4, *Acinetobacter* C5; 5, *Staphylococcus* C6; 6, streptomycin.

antimicrobial activity.

According to Kumar et al. (2015), media composition and culture conditions enhance antibacterial producing ability of microorganisms. The production media must provide components which are source of energy for microorganisms to synthesize bioactive metabolites (Costa et al., 2002). In this study, Nutrient Broth (NB) was used as a growth media for endophytes and the antibacterial activity was low against the tested pathogenic strains. In comparison to a study conducted by Kumar et al. (2015), it was shown that extracts from microorganisms grown in Trypticase Soy Broth (TSB) had significant antibacterial activity, followed by Luria Broth (LB) based extracts with high activity and lastly extracts of microorganisms grown in Nutrient Broth (NB) had low activity. In another study by Malash et al. (2016), the extracts from *Bacillus* spp. grown in LB had higher antibacterial activity, while the extracts of the same species grown in NB had lower activity. It was also shown that *Pantoea agglomerans* grown in Nutrient Broth produced low amount of bioactive metabolites (Costa et al., 2002). It was stated that yeast extract is a good source of carbon and nitrogen for many microorganisms (Costa et al., 2002; Narayana and Vijayalakshmi, 2008); therefore, LB was seen as the best production medium since it contains yeast extract. The low antibacterial activity in this study was associated with the media used.

The potential of endophytes to inhibit growth of pathogenic strains have shown that these micro-organisms have potential in development of therapeutic drugs, furthermore Gram negative and Gram positive test microorganisms were both inhibited in the current study. Strobel and Daisy (2003) have indicated that endophytes are potential source for bioactive compounds which can be used in medical, agriculture and other industries. In the study reported by Sandhu et al. (2014), the significant number of endophytic bacteria isolated from medicinal plants is of great importance due to presence of bioactive

extracts which can be used against pathogenic strains.

Conclusion

The identified endophytes were the first endophytes isolated from *C. macowanii*. *C. macowanii* and its bacterial endophytes have shown slightly similar phytochemical analysis; only two species, *Acinetobacter* spp. C5 and *Staphylococcus* spp. C6 contains alkaloids constituents. The antimicrobial activity of endophytes has shown inhibition effects against the selected pathogenic strains. From these results, it can be concluded that the endophytic bacteria isolated from medicinal plant, *C. macowanii* produces potential bioactive compounds which can be explored further for other biological activities. For future purpose, this study or study similar to this can be improved by using alternative methods for isolation of bacterial endophytes to achieve numerous endophytes with great diversity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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