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Antibacterial and Antioxidant Activities of Acetogenins from *Streptomyces* sp. VE2; An Endophyte in *Vernonia cinerea* (L.) Less.

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ABSTRACT

Strain VE2 was isolated from the stem tissue of *Vernonia cinerea* (L.) Less. and identified as *Streptomyces* sp. on the basis of morphology, chemotaxonomy and 16SrDNA sequencing. The fractionation of the crude ethyl acetate (CEA) extract from VE2 cultures led to the isolation of two acetogenins; squamocin and rollidecin B; these compounds and CEA extract had potential in antibacterial and antioxidant activities. The crude extract showed the highest activity against *Salmonella* Typhi ATCC19430 and *Bacillus cereus* ATCC7064, with MIC values of 32 µg/ml. Squamocin also showed the lowest MIC (32 µg/ml) and Minimum Bactericidal Concentration (MBC) (128 µg/ml) against *S. Typhi* and *B. cereus* with corresponding large diameter of the zone of inhibitions (27.5 and 28.2 mm, respectively). Rollidecin B showed the highest DPPH antioxidant activity with SC_{50} value of 58.92 µg/ml.

INTRODUCTION

Endophytes are microorganisms that colonize inside plant tissue. They live between the living cells of their respective host and cause no overt tissue damage (Azevedo *et al*., 2001; Zhang *et al*., 2006; Bandara *et al*., 2006). They would help to improve the host plant's growth and promotional activity against diseases caused by phytopathogenic fungi (Azevedo *et al*., 2001). Usually, fungi are the most commonly isolated endophytic microorganisms, but recently the endophytic actinomycetes were isolated from the tissues of healthy plants (Joseph *et al*., 2012). Some of endophytic actinomycetes can produce important compounds which exhibited various biological activities, such as antimicrobial activity, antioxidant activity, anti-inflammatory activity, etc (Strobel *et al*., 2003). Since bioactive compounds from endophytic *Streptomyces* have distinct chemical structures and some of them are novel compounds. In our previous studies, many endophytic *Streptomyces* were isolated from plant tissues, some of them produced the secondary metabolites against bacteria and phytopathogenic fungi (Taechowisan *et al*., 2005, 2008, 2012, 2014).

We report here the isolation of the stems of *Vernonia cinerea* (L.) Less. of another endophytic *Streptomyces* sp. VE2. The crude ethyl acetate (CEA) extract and purified compounds of *Streptomyces* sp. VE2 displayed strong antibacterial and antioxidant properties.

MATERIALS AND METHODS

Organisms and media

Streptomyces sp. VE2 was isolated from the stem tissues of *Vernonia cinerea* (L.) Less. by the surface-sterilization technique (Taechowisan *et al*., 2003). Identification of the isolate to species level was based on morphology, chemotaxonomy and also 16S rDNA sequencing as described in our previous study (Taechowisan and Lumyong, 2003).

Solid medium for sporulation used in this study was International *Streptomyces* Project Medium 4 (ISP-4) and the culture medium used for secondary metabolites production was ISP-2 (Shirling and Gottlieb, 1966).

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Preparation and fractionation of the crude extract

The 10-day-old cultures on ISP-2 agar (100 Petri dishes) were cut into small pieces and extracted three times with ethyl acetate. This organic solvent was pooled and then taken to dryness under flash evaporation to give a dark brown solid (734 mg). The solid was dissolved in 10 ml of chloroform and fractionated on column chromatography (Merck silica gel 60, 35-70 mesh) with hexane, diethyl ether and methanol. Fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol. The combined fractions eluted with 50% diethyl ether in hexane, 100% diethyl ether, and 5% methanol in diethyl ether (308 mg) were further separated by MPLC (400 x 40 mm column, Merck LiChroprep Si 60, 25-40 μm, UV-detection, 254 nm) to afford fr. A (42 mg), fr. B (68 mg) and fr. C (54 mg). The fr. A and C have no activity against tested microorganisms. Final purification of fr. B was achieved by prep TLC (Merck, Si gel 60, 0.5 mm; dichloromethane : diethyl ether = 75 : 25) to afford compounds **1** (13 mg) and **2** (17 mg).

Antimicrobial activity assay

An *in vitro* plate assay technique was used to test the inhibitory effects of *Streptomyces* sp. VE2 on the tested bacteria as described in the previous report (Taechowisan *et al*., 2008).

For screening of antibacterial activity of the endophytic actinomycetes, we used the solid media bioassay test against *Staphylococcus aureus* ATCC25932, *Bacillus cereus* ATCC7064, *Bacillus subtilis* ATCC6633, *Escherichia coli* ATCC10536, *Salmonella* Typhi ATCC19430 and *Pseudomonas aeruginosa* ATCC27853, these bacteria were cultured in ISP-2 broth at 37°C for 24 h. The cells were diluted to 10^5 cells/ml in soft agar and then were overlayed on 5 days pre-grown colony of endophytic actinomycetes on ISP-2 plates.

For antibacterial assays, the CEA extract and purified compounds were tested against the tested bacteria using the paper disk method (National Committee for Clinical Laboratory Standards (NCCLS), 1997). Two pieces of 8-mm sterile paper disks (Advantec, Toyo Roshi Kaisha, LTD., Japan) were respectively soaked in crude extract and purified compounds at the amount of 50 μg/disc. The air-dried discs were placed on ISP-2 plates. Each plate was then overlayed with top agar containing 10^5 cells/ml of bacteria strains. The plates were incubated at 37°C for 24 h. The width of inhibition zones was measured. Each treatment consisted of three replicates. The experiment was repeated twice. Ampicillin (30 Unit/disc) and Chloramphenicol (30 μg/disc) (Oxoid, UK) were used as references for antimicrobial activity.

Minimum inhibitory concentrations (MICs)

MICs of CEA extract and purified compounds were determined by NCCLS microbroth dilution methods (NCCLS, 2000). The agents were dissolved in dimethyl sulfoxide (DMSO). A 10 μl of bacterial suspension (10^5 cells/ml) was inoculated into each well of a 96-well microplate, each containing a different concentration of the test agents. We performed doubling dilutions of the test agents. Ampicillin and chloramphenicol were used as references for antibacterial activity. The range of sample dilutions was 512 to 1 μg/ml in nutrient broth supplement with 10% glucose (NBG) and a final concentration of test agent that inhibited bacterial growth, as indicated by the absence of turbidity. Test agent-free broth containing 5% DMSO was incubated as growth control. Minimum microbicidal concentration was determined by inoculating on to nutrient agar plates, a 10 μl of medium from each of the well from the MIC test which showed no turbidity. The plates were incubated at 37°C for 24 h. MMCs were defined as the lowest concentration of test agent where was no microbial growth on the plates.

Radical-scavenging activity - DPPH assay

The antioxidant activity of the CEA extract and purified compounds was evaluated by monitoring its ability in quenching the stable free radical DPPH, according to a slightly modified method (Choi *et al*., 2002). Spectrophotometric analysis was used to measure the free radical scavenging capacity and to determine the scavenging concentration (SC_{50}) of the CEA extract and purified compounds. The DPPH quenching ability was expressed as SC_{50} (the concentration required to inhibit radical formation by 50%). Six different ethanol dilutions of the CEA extract or each compound (2.5 ml), at 250, 125, 62.5, 31.25, 15.62 and 7.81 μg/ml were mixed with 1.0 ml of a 0.3 mM DPPH ethanol solution. Ethanol (1.0 ml) plus the CEA extract or each compound (2.5 ml) was used as a blank. The absorbance was measured at 518 nm by UV-VIS spectrophotometer after 30 min of reaction at room temperature. The radical was prepared daily and protected from light. Relative activities were calculated from the calibration curve of L-ascorbic acid standard solution working in the same experimental conditions. Scavenging capacity in percent (SC%) was calculated in following way, according to the equation:

$$SC\% = 100 - [(Abs_{sample} - Abs_{blank}) \times 100 / Abs_{control}]$$

Where Abs_{sample} is the absorbance of the test compound and $Abs_{control}$ is the absorbance of the control reaction (containing all reagents except the test agent). SC% was plotted against sample concentration, and a linear regression curve was established in order to calculate the SC_{50}. Tests were carried out in triplicate. Correlation coefficients were optimized.

RESULTS

An endophyte designated *Streptomyces* sp. VE2 was isolated from the stem tissues of *Vernonia cinerea* (L.) Less. This strain was of great interest, because of its potent antibacterial and antioxidant activities. Morphological observation of 21-day-old culture of VE2 grown on ISP-2 medium revealed that sporophores were straight to flexuous, producing oval-shaped spores (1x1.5 μm) with spin surfaces (Figure 1). The substrate mycelium was extensively branched with non-fragmenting hyphae. The aerial mycelium was white changing to brown with yellow soluble pigment occasionally discernible. From the chemotaxonomy study, this strain contained LL-type diaminopimelic acid in the whole-cell hydrolysates. Almost the complete 16S rDNA sequence was

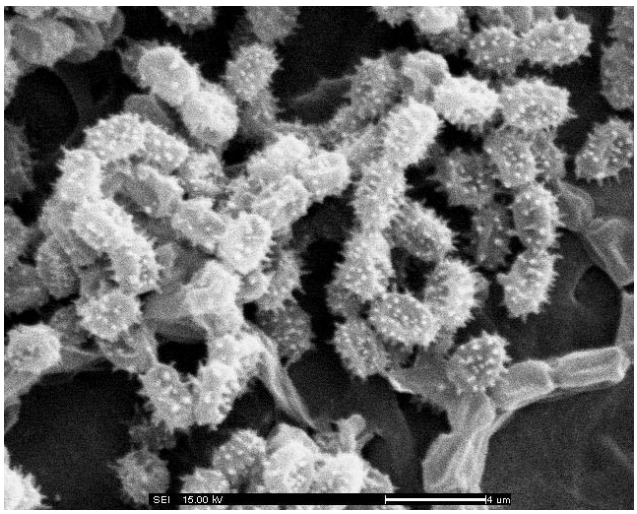


Fig. 1: Scanning electron micrograph showing spore chains and spore surface of Streptomyces sp. VE2. Bar, 4 µm.

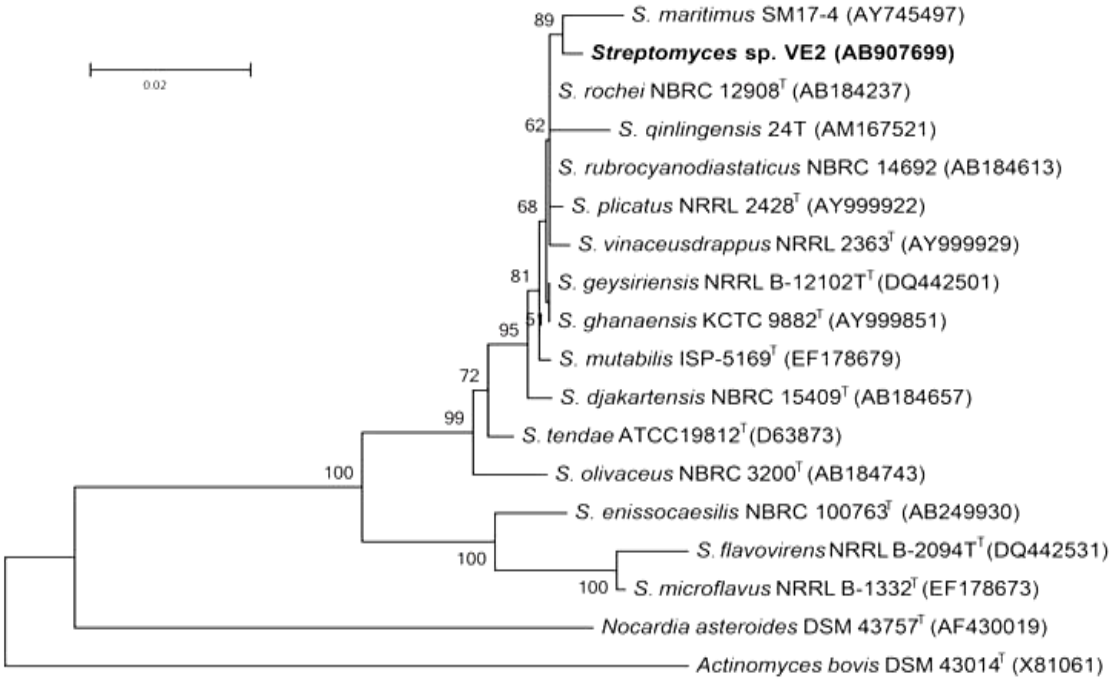
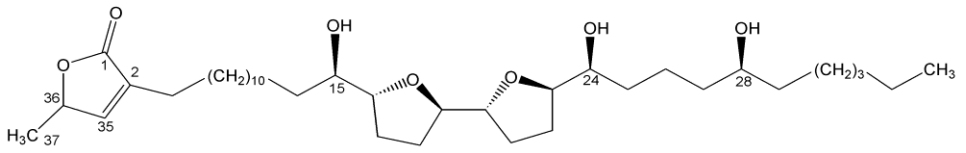


Fig. 2: Neighbor-joining phylogenetic tree of *Streptomyces* sp. VE2, including representatives of the most closely-related type strains which were retrieved from GenBank, and accession numbers appear in parentheses. Bootstrap (1,000 replicates) values are given in percentage. Bar, 0.01 substitutions per nucleotide.



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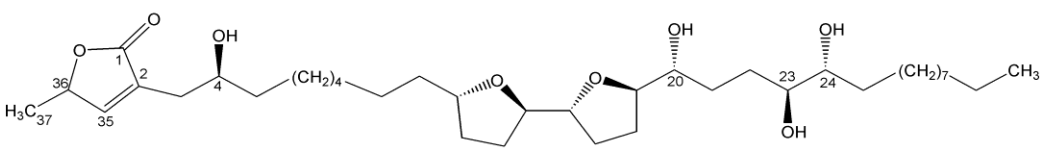

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Fig. 3: Chemical structures of Squamocin (1) and Rollidecin B (2).

determined for the endophytic *Streptomyces* sp. VE2 from position 27 to position 1423. BLAST search results for strain VE2 came from GenBank; when reference sequences were chosen. The BLAST search results and the phylogenetic tree generated from representative strains of the related genera showed that strain VE2 had high levels of sequence similarity to species of *Streptomyces maritimus* SM17-4 (accession number: AY745497) (Figure 2). 16S rDNA analysis revealed that strain VE2 is phylogenetically closely related to *Streptomyces maritimus* (the sequence similarity levels were 97.48%). The nucleotide sequence data reported in this paper appeared in the GenBank, EMBL and DDBJ databases with accession number AB907699.

Ethyl acetate extract from the strain VE2 was purified by column chromatography. In the active fraction, three compounds were isolated and identified as following.

Compound **1**; Squamocin (**1**): $C_{37}H_{66}O_7$; yellowish oil; $[\alpha]_D$: $+19^o$ (*c* 0.23, $CHCl_3$). UV (MeOH): λ_{max} (log ε) 210 (3.88); mp 190-192 ^oC; CIMS: *m/z* = 623 $[MH]^+$, 605 $[MH\text{-}H_2O]^+$, 587 $[MH\text{-}2H_2O]^+$, 569 $[MH\text{-}3H_2O]^+$, 519, 501, 483, 435, 417, 399, 347, 329, 295 (100), 267, 239, 169, 111, 97; EIMS: *m/z* = 417, 399, 347, 329, 295 (100), 267, 239, 169, 111, 97, 69; ^{1}H-NMR and ^{13}C-NMR (Fujimoto *et al*., 1994).

Compound **2**; Rollidecin B (**2**): $C_{37}H_{66}O_8$; yellowish oil; $[\alpha]_D$: $+19^o$ (*c* 0.23, $CHCl_3$). UV (MeOH): λ_{max} (log ε) 210 (3.88); mp 190-192 ^oC; CIMS: *m/z* = 639 $[MH]^+$ (100), 621 $[MH\text{-}H_2O]^+$, 603 $[MH\text{-}2H_2O]^+$, 585 $[MH\text{-}3H_2O]^+$, 567 $[MH\text{-}4H_2O]^+$, 449, 431, 413, 379, 361, 309, 299, 291, 281, 263, 247, 229, 211, 171, 153, 141, 123, 111, 97; EIMS: *m/z* = 449, 379, 309 (100), 211, 141, 97, 43; ^{1}H-NMR and ^{13}C-NMR (Shi *et al*., 1996). The chemical structures of compound **1** and **2** were shown in Figure 3.

The CEA extract from the culture of the strain VE2 showed the highest activity against *B. cereus* (24.6 mm) and against *S. Typhi* (25.5 mm) (Table 1). However, this CEA extract showed low activity against *E. coli* (16.5 mm) and *P. aeruginosa* (15.5 mm). The compound **1** showed higher activity than compound **2**, it also showed the highest activity against *B. cereus* (28.2 mm) and against *S. Typhi* (27.5 mm). The compound **2** and CEA extract showed activity against all the test microorganisms less than compound **1**.

A classification based on MIC values proposed by Algiannis *et al.* (2001), was used for this study. The extract or the compounds with MIC values up to <512 µg/ml were considered strong inhibitors, 512 µg/ml as moderate inhibitors and those above 512 µg/ml as weak inhibitors. The crude extract and all the compounds showed the MIC values less than 512 µg/ml (excepted compound **2** on *E. coli* and *P. aeruginosa*), therefore the crude extract and compound **1** were considered strong inhibitors against all the test microorganisms. The crude extract and compound **1** showed the lowest MIC (32 µg/ml) against *B. cereus* and *S. Typhi* (Table 2). These were followed by the MIC values (64 µg/ml) of the crude extract against *S. aureus* and *B. subtilis* and compound **1** against *S. aureus*. The compound **2** had high MIC values (512 µg/ml) against *E. coli* and *P. aeruginosa* and therefore considered moderate inhibitors. The compound **1** showed the lowest MBC (128 µg/ml) against *B. cereus* and *S. Typhi* (Table 3) whereas the crude extract had high MBC values (512 µg/ml) for all test microorganisms. The compound **2** had no activity in MBC for all test microorganisms.

The free radical scavenging capacity of the crude extract and purified compounds was assessed by the decolouration of the ethanolic solution of DPPH. In the presence of an active radical scavenger, the absorption vanishes and the resulting decolourization is stoichiometric at a selected range with respect to the degree of reduction. Ethanolic solutions of DPPH served as control and the calibration curve made with L-ascorbic acid was used to compare the activity, as positive control, since this standard antioxidant activity was well established.

Table 4 shows antioxidant activity of the crude extract and isolated compounds. The compound **2** is the most active compounds showing SC_{50} values of 58.92 µg/ml, which is comparable to that of a positive control, L-ascorbic acid showing SC_{50} values of 43.26 µg/ml. Comparing results from compound **1** and **2** indicated that a greater number of hydroxyl groups increased an antioxidant activity.

Table 1: Diameters of inhibition zones of the crude extract and purified compounds on the test organisms (mm).

Test agents	Microorganisms					
	S.a.[a]	B.c.	B.s.	E.c.	P.a.	S.T.
Crude extract	19.5 ± 1.28	24.6 ± 1.25	20.5 ± 1.43	18.5 ± 1.63	17.4 ± 1.58	25.5 ± 0.41
Compound 1	20.0 ± 1.55	28.2 ± 1.82	21.6 ± 1.58	22.8 ± 1.00	20.0 ± 1.24	27.5 ± 1.65
Compound 2	18.3 ± 1.20	19.2 ± 1.53	20.5 ± 1.44	15.0 ± 1.52	11.6 ± 1.84	17.5 ± 1.34
Ampicillin	31.3 ± 0.64	27.7 ± 0.55	26.4 ± 0.78	27.4 ± 0.83	24.2 ± 0.76	26.2 ± 0.62
Chloramphenicol	29.3 ± 0.82	27.3 ± 0.65	27.4 ± 0.88	26.5 ± 0.72	27.8 ± 0.44	27.2 ± 0.54

[a]S.a.; *Staphylococcus aureus* ATCC25932, B.c.; *Bacillus cereus* ATCC7064, B.s., *Bacillus subtilis* ATCC6633, E.c., *Escherichia coli* ATCC10536, P.a., *Pseudomonas aeruginosa* ATCC27853, and S.T., *Salmonella* Typhi ATCC19430.

Table 2: Minimum inhibitory concentrations (µg/ml) of the crude extract and isolated compounds.

Test agents	Microorganisms					
	S.a.[a]	B.c.	B.s.	E.c.	P.a.	S.T.
Crude extract	64	32	64	128	256	32
Compound 1	64	32	128	128	256	32
Compound 2	256	256	256	512	512	256
Ampicillin	4	4	4	4	4	4
Chloramphenicol	4	8	8	4	8	4

[a]S.a.; *Staphylococcus aureus* ATCC25932, B.c.; *Bacillus cereus* ATCC7064, B.s., *Bacillus subtilis* ATCC6633, E.c., *Escherichia coli* ATCC10536, P.a., *Pseudomonas aeruginosa* ATCC27853, and S.T., *Salmonella* Typhi ATCC19430.

Table 3: Minimum bactericidal concentrations (μg/ml) of the crude extract and isolated compounds.

Test agents	Microorganisms					
	S.a.[a]	B.c.	B.s.	E.c.	P.a.	S.T.
Crude extract	512	512	512	512	512	512
Compound 1	256	128	256	256	256	128
Compound 2	>512	>512	>512	>512	>512	>512
Ampicillin	16	16	16	32	32	32
Chloramphenicol	512	>512	>512	>512	>512	>512

[a]S.a.; *Staphylococcus aureus* ATCC25932, B.c.; *Bacillus cereus* ATCC7064, B.s., *Bacillus subtilis* ATCC6633, E.c., *Escherichia coli* ATCC10536, P.a., *Pseudomonas aeruginosa* ATCC27853, and S.T., *Salmonella* Typhi ATCC19430.

Table 4: Antioxidant activity of the crude extract and isolated compounds.

Test agents	SC50 (μg/ml)[a]	Standard deviation
Crude extract	124.62	9.54
Compound 1	86.76	8.25
Compound 2	58.92	6.70
L-ascorbic acid	43.26	3.68

[a]Concentration required to inhibit radical formation by 50%.

DISCUSSION

The strain VE2 was recovered from the stem tissues of *Vernonia cinerea* (L.) Less.; a medicinal plant contained several compounds for example: alkaloids, glycosides, steroids and triterpinoids (Misra *et al*., 1984; Haque *et al*., 2012; Dhanalakshmi *et al*., 2013). This bacterium produced yellow soluble pigment after inoculation on to ISP-2 medium for 5 days. Based on results in morphological observation as well as on the presence of LL-type diaminopimelic acid in the whole-cell extracts and 16S rDNA sequence, the endophytic actinomycetes VE2 was identified as belonging to the genus *Streptomyces*.

The high bioactivity of the CEA extract of the strain VE2 showed by its low MIC (32 μg/ml) against *B. cereus* and *S. Typhi*, followed by *S. aureus* and *B. subtitis* (64 μg/ml) and *E. coli* (128 μg/ml). Notably, antibacterial activity of the crude extract and compounds possessed antibacterial activity less than ampicillin and chloramphenicol.

Two compounds were isolated from the CEA extract of the strain VE2, those were classified as acetogenins. Normally, acetogenins have been isolated from several genera of Annonaceae plants for example *Annona squamosa* L. (Fujimoto *et al*., 1994), *Uvaria narum* Wall. (Hisham *et al*., 1991) and *Rollinia mucosa* (Jacq.) Baill. (Shi *et al*., 1996). Acetogenins have been described as antiprotozoal, insecticides, antimitotic, cytotoxic, fungicides and pesticides compounds (Gonzalez *et al*., 1997; Cavé *et al*., 1996). In our study, two acetogenins, Squamocin (compound **1**) and Rollidecin B (compound **2**) was obtained from the culture of *Streptomyces* sp. VE2, isolated from the stem tissue of *Vernonia cinerea* (L.) Less. It proved that Squamocin and Rollidecin B from *Streptomyces* sp. VE2 were all inhibitory to tested bacteria. In addition, Squamocin, the presence of free hydroxyl group at C-15 to be more essential for antibacterial activity than Rollidecin B. whereas Rollidecin B, the presence of four free hydroxyl group was associated with antioxidant activity of the compound. A previous study on the antioxidant activity of Acetogenins, also indicated that more hydroxyl group on the molecules was crucial for their antioxidant activity (Santos *et al*., 2010).

CONCLUSION

This work shows the antibacterial and antioxidant activities of Squamocin and Rollidecin B isolated from the culture of *Streptomyces* sp. VE2, an endophyte in *Vernonia cinerea* (L.) Less. Since Squamocin and Rollidecin B have antagonism against tested bacteria. They should be further studied in clinical isolates. The other biological activities of Squamocin and Rollidecin B should be studied as well.

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