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Antibacterial activity of new flavonoids from *Streptomyces* sp. BT01; an endophyte in *Boesenbergia rotunda* (L.) Mansf.

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ABSTRACT

Strain BT01 was isolated from the root tissue of *Boesenbergia rotunda* (L.) Mansf. and identified as *Streptomyces* sp. on the basis of morphology, chemotaxonomy and 16SrDNA sequencing. It was an antagonist of Gram positive bacteria; *Staphylococcus aureus* ATCC25932, *Bacillus cereus* ATCC7064 and *Bacillus subtilis* ATCC6633. The inhibitory effect of the crude extract from the strain BT01 was examined based on the paper disc diffusion method (5 mg per paper disc) with three replications. It has been shown to have antibacterial activity. The major active ingredients from the crude extract were purified by silica gel column chromatography, thin-layer chromatography and identified to be two new flavonoids, 7-methoxy-3, 3',4',6-tetrahydroxyflavone (**1**) and 2',7-dihydroxy-4',5'-dimethoxyisoflavone (**4**), together with four known compounds, fisetin (**2**), naringenin (**3**), 3'-hydroxydaidzein (**5**) and xenognosin B (**6**). Bioassay studies showed that these compounds had antibacterial activity with the minimum inhibitory concentrations within the range of 32 to 256 µg/ml.

INTRODUCTION

The symbiosis of endophytic microorganisms favours metabolic interactions with their host and their environment, thereby increasing the production of bioactive compounds (Bacon and White, 2000). Some of actinomycete could be isolated from the tissue of healthy plants which was called endophytic actinomycetes. Several reports refer to endophytic actinomycetes produced secondary metabolites against phytopathogenic fungi (Sardi *et al*., 1992; Shimizu *et al*., 2000, Taechowisan *et al*., 2003), and also produced the novel antibiotics for example: Munumbicins from *Streptomyces* sp. NRRL 30562, an endophyte of *Kennedia nigriscans* (Castillo *et al*., 2002), Kakadumycins from *Streptomyces* sp. NRRL 30566, an endophyte of *Grevillea pteridifolia* (Castillo *et al*., 2003), Coronamycins from *Streptomyces* sp. MSU-2110, an endophyte of *Monstera* sp. (Ezra *et al*., 2004). In our previous studies, many endophytic actinomycetes were isolated from plant tissues, some of them produced the secondary metabolites against bacteria and phytopathogenic fungi (Taechowisan *et al*., 2005, 2008, 2013). We report here the isolation of the roots of *Boesenbergia rotunda* (L.) Mansf. of another *Streptomyces* sp. BT01. Extraction of the culture medium of *Streptomyces* sp. BT01 afforded several flavonoids, which displayed strong antibacterial activity.

MATERIALS AND METHODS

Organisms and media

Streptomyces sp. BT01 was isolated from the root tissues of *Boesenbergia rotunda* (L.) Mansf. 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 by 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).

Preparation of the crude extract

A spore suspension of *Streptomyces* sp. BT01 was prepared in distilled water from cultures grown on ISP-4 medium at 30 °C for 10 days. The suspension, 10^8 spores per 100 ml of liquid medium, was added to ISP-2 broth in each 500-ml Erlenmeyer flask. Cultures were kept on a shaker at 120 rpm at 30 °C for 48 h and used as seed stocks.

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For large production of culture filtrates, the strain BT01 was grown in a modified 3000 ml glass container containing 1500 ml of ISP-2 broth, and incubated in an orbital shaker for 5 days in the same condition. The 5-day-old cultures were filtrated by Whatman paper No. 1 under vacuum. The mycelial mats were washed with distilled water and separated by centrifugation at 5000 rpm for 20 min. The culture filtrate and mycelial mats of the strain BT01 were extracted three times with 1/3 volumes of ethyl acetate. This organic solvent was pooled and then taken to dryness under flash evaporation at 40 ^{o}C. The yield of dry material per litre was about 753 mg.

Purification of the compounds

The residue of 753 mg 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. The combined fractions eluted with 50% diethyl ether in hexane, 100% diethyl ether, and 5% methanol in diethyl ether (286 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 (57 mg) and fr. B (104 mg). Final purification of fr. A and fr. B were achieved by prep TLC (Merck, Si gel 60, 0.5 mm; dichloromethane : diethyl ether = 75 : 25) to give 16 mg of compound **1**, 10 mg of compound **2** and 8 mg of compound **3** from fr. A and 28.5 mg of compound **4**, 33 mg of compound **5** and 25.5 mg of compound **6** from fr. B.

Structure elucidation of the compounds

The structures of purified compounds have been identified using NMR and mass spectral data. The melting point of the compounds was determined on a Buchi-540 melting point apparatus. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, IR spectra on a Perkin-Elmer 1 spectrometer, ^{1}H and ^{13}C NMR spectra on a Bruker DRX 500 spectrometer, and EI-MS and FAB-MS respectively on a Hewlett-Packard 5989 B and a Finnigan/Thermo Quest Mat 95 XL mass spectrometer.

Antagonism and antibacterial activity assay

An *in vitro* plate assay technique was used to test the inhibitory effects of *Streptomyces* sp. BT01 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 and *Pseudomonas aeruginosa* ATCC27853, the bacteria was cultured in ISP-2 broth at 37^{o}C for 24 h; the cells were diluted to 10^{5} cells/ml in soft agar and then were overlayed on pre-grown colony of endophytic actinomycetes on ISP-2 plates.

For antibacterial activity assay, the crude extract and purified compounds were tested for antibiosis against the tested bacteria using the paper disk method (Taechowisan *et al*., 2005). Two pieces of 8-mm sterile paper disks (Advantec, Toyo Roshi Kaisha, LTD., Japan) were respectively soaked in crude extract (5 mg/disc) and purified compound (50 µg/disc). The air-dried discs were places on ISP-2 plates. Each plate was then overlayed with top agar containing 10^{5} cells/ml of bacteria test strains. The plates were incubated at 37^{o}C for 24 h. The width of inhibition zones was measured. Each treatment consisted of three replicates. The experiment was repeated twice.

Minimum inhibitory concentrations (MICs)

MICs of crude extract and purified compounds were determined by NCCLS microbroth dilution methods (National Committee for Clinical Laboratory Standards, 1997). The crude extract and purified compounds were dissolved in DMSO. A dilution suspension of bacteria 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. The range of sample dilutions was 256 to 0.50 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 bactericidal concentration (MBC) 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. MBCs were defined as the lowest concentration of test agent where was no microbial growth on the plates.

RESULTS AND DISCUSSION

Identification of microorganism

An endophyte designated *Streptomyces* sp. BT01 was isolated from the root tissue of *Boesenbergia rotunda* (L.) Mansf. This strain was of great interest, because of its potent antibacterial activity. Morphological observation of 21-day-old culture of BT01 grown on ISP-2 medium revealed that sporophores to be monopodially branched, producing open spirals of oval-shaped spores (1x1.5 µm) with spiny surfaces (Figure 1).

The substrate mycelium was extensively branched with non-fragmenting hyphae. The aerial mycelium was orange changing to brown with yellow soluble pigment occasionally discernible. Based on results in morphological observation as well as on the presence of LL-type diaminopimelic acid in the whole-cell extracts, endophytic actinomycetes BT01 was identified as belonging to the genus *Streptomyces*. Almost the complete 16S rDNA sequence was determined for the endophytic *Streptomyces* sp. BT01 from position 25 to position 1425. BLAST search results for strain BT01 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 BT01 had high levels of sequence similarity to species of *Streptomyces emeiensis* DSM 41884 (accession number: DQ462649) (Figure 2). 16S rDNA analysis revealed that strain

BT01 is phylogenetically closely related to *Streptomyces emeiensis* (the sequence similarity levels were 98%). The nucleotide sequence data reported in this paper appeared in the GenBank, EMBL and DDBJ databases with accession number AB723804.

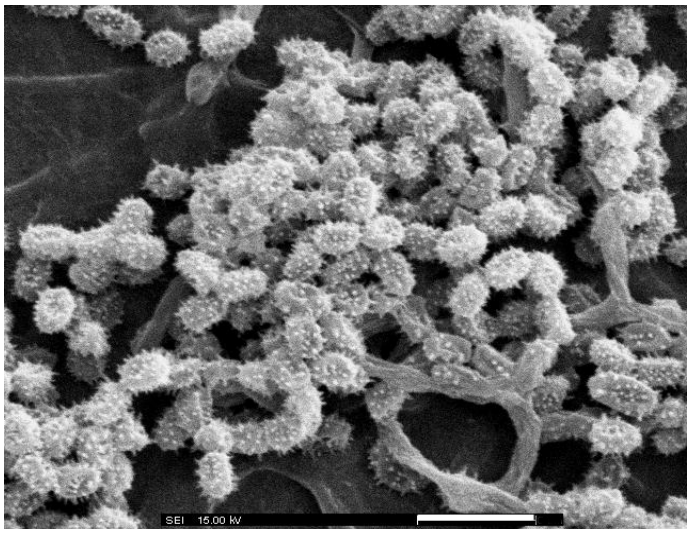


Fig. 1: Scanning electron micrograph showing spore chains and spore surface of *Streptomyces* sp. BT01 grown on ISP-2 at 30 ^{o}C for 21 days. Bar, 3 µm.

Structure elucidation of the compound and antibacterial activity

Ethyl acetate extract from the strain BT01 was evaluated against *S. aureus* ATCC25932, *B. cereus* ATCC7064, *B. subtilis* ATCC6633, *E. coli* ATCC10536 and *P. aeruginosa* ATCC27853. The extract obtained from the culture medium (blank) was tested in this assay and was not active, demonstrating that the positive results were because of the secondary metabolites produced by the strain BT01.

The extract showed strong antibacterial activity against *S. aureus* with MIC value of 32 µg/ml, *B. cereus* and *B. subtilis* with MIC value of 64 µg/ml, and showed weaker antibacterial activity against two Gram negative bacteria, *E. coli* and *P. aeruginosa* with MIC values of 128 and 256 µg/ml, respectively, as were presented in Table 1.

Ethyl acetate extract from the strain BT01 was purified by column chromatography. In the active fraction, six compounds were isolated and identified. The structures of these compounds were ascertained on the basis of their spectroscopic data and are in agreement with those reported previously for 3,3',4',7-tetrahydroxyflavone (fisetin **2**, Shafaghat and Salimi, 2008), 4',5,7-trihydroxyflavanone (naringenin **3**, Shafaghat and Salimi, 2008), 3'-hydroxydaidzein (**5**, Funayama, *et al*., 1989) and xenognosin B (**6**, Braz Fo, *et al*., 1977) and together with two new flavonoids, 7-methoxy-3, 3',4',6-tetrahydroxyflavone (**1**) and 2',7-dihydroxy-4',5'-dimethoxyisoflavone (**4**) (Figure 3).

The known compounds, Fisetin (**2**) has been previously isolated from a wide variety of plants for example: *Quebracho Colorado* and *Rhus cotinus* (Family Anacardiaceae) (Gábor and Eperjessy, 1966), *Dalbergia odorifera* (Family Leguminosae) (Chan *et al*., 1998), *Tanacetum parthenium* (Family Compositae) (Shafaghat and Salimi, 2008), *Acacia greggii* and *Acacia berlandieri* (Family Fabaceae) (Forbes and Clement, 2010). It was strongly active against Gram positive bacteria: *S. aureus*, *B. cereus* and *B. subtilis* with MIC value of 32 µg/ml, and was weakly active against Gram negative bacteria: *E. coli* and *P. aeruginosa* with MIC values of 128 and 256 µg/ml, respectively. Naringenin (**3**) has been previously isolated from a wide variety of plants for example: *Tanacetum parthenium* (Family Compositae) (Shafaghat and Salimi, 2008), *Choerospondias axillaries* (Family Anacardiaceae) (Lü *et al*., 1983), *Citrus aurantium* (Family Rutaceae) (Liu *et al*., 2008), *Mentha aquatica* (Family Lamiaceae) (Olsen et al., 2008). It had antibacterial activity less than fisetin (**2**) and 7-methoxy-3, 3',4',6-tetrahydroxyflavone (**1**). However it was active against Gram positive bacteria greater than Gram negative bacteria. 3'-hydroxydaidzein (**5**) has been previously isolated from plants: *Pueraria lobata* (Family Leguminosae) (Shi *et al*., 2012), and also has been reported to be a metabolite of daidzein in human urine (Kulling *et al*., 2001, Heinonen *et al*., 2004) and in the cultures of liver microsomes derived from an Aroclor-induced rat (Kulling *et al*., 2000) and has been isolated from dou-chi, a soybean food fermented by *Aspergillus oryzae* (Chen *et al*., 2005).

It has been reported to possess various biological activities, for example antioxidant activity (Shi et al., 2012), and anti-inflammatory activity, etc. (Jiang *et al*., 2005). In this study, 3'-hydroxydaidzein was active against Gram positive bacteria: *S. aureus*, *B. cereus* and *B. subtilis* with MIC value of 64 µg/ml, and was weakly active against Gram negative bacteria: *E. coli* and *P. aeruginosa* with MIC values of 256 and 512 µg/ml, respectively. Xenognosin (**6**) has been previously isolated from plants for example: *Astragalus membranaceus* (Family Fabaceae) (Du *et al*., 2006) and *Dalbergia odorifera* (Family Leguminosae) (Leung *et al*., 1991). It had antibacterial activity less than 3'-hydroxydaidzein (**5**) and 2',7-dihydroxy-4',5'-dimethoxyisoflavone (4). However it was active against Gram positive bacteria greater than Gram negative bacteria.

Table. 1: Antimicrobial activity (MIC, µg/ml) of the crude extract and isolated compounds.

Test agents	Microorganisms				
	S.a.[a]	B.c.	B.s.	E.c.	P.a.
Crude extract	32	64	64	128	256
Compound 1	32	64	64	128	256
Compound 2	32	32	32	128	256
Compound 3	32	64	64	256	512
Compound 4	64	128	128	256	>512
Compound 5	64	64	64	256	512
Compound 6	128	128	128	512	>512

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

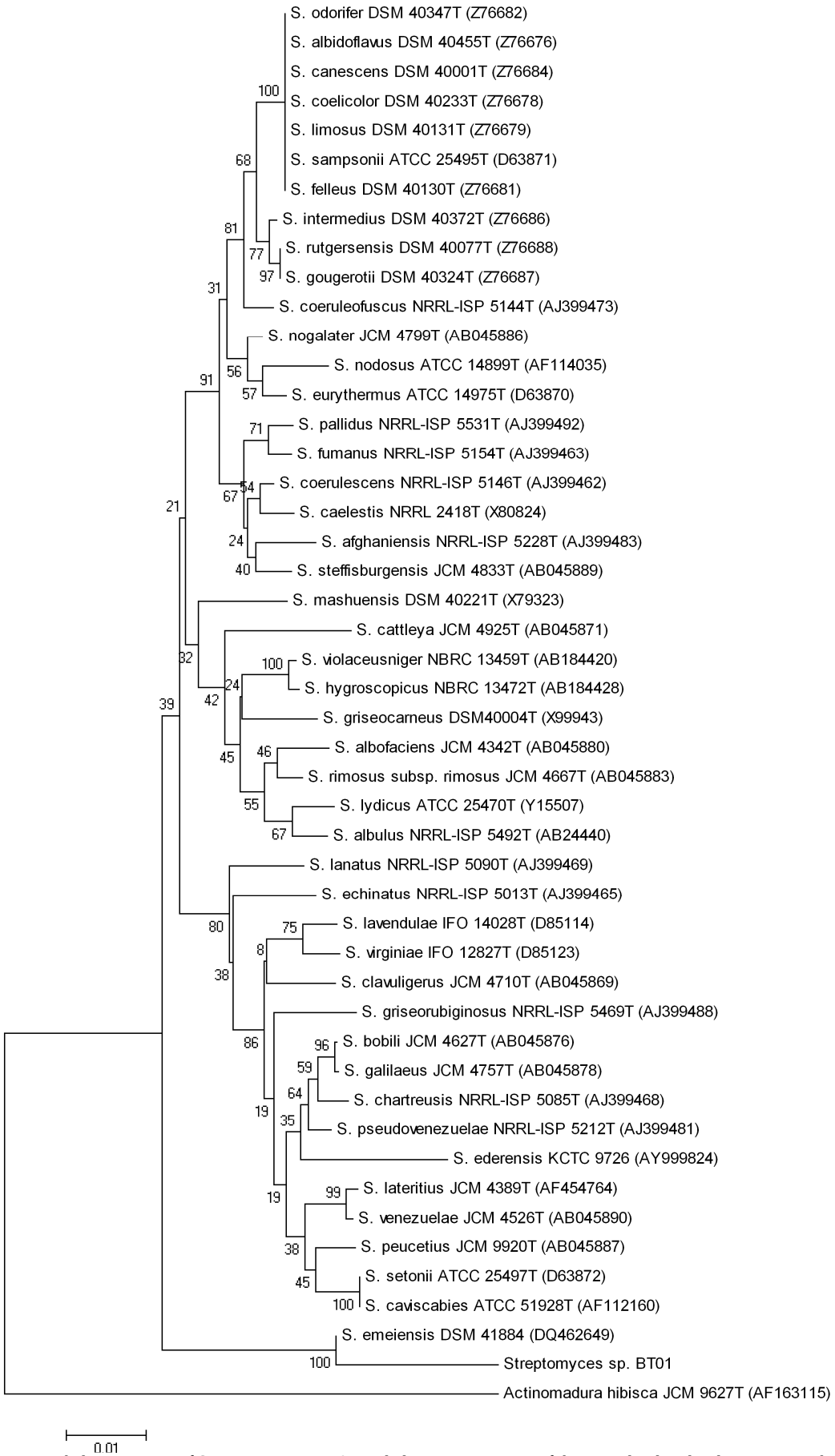
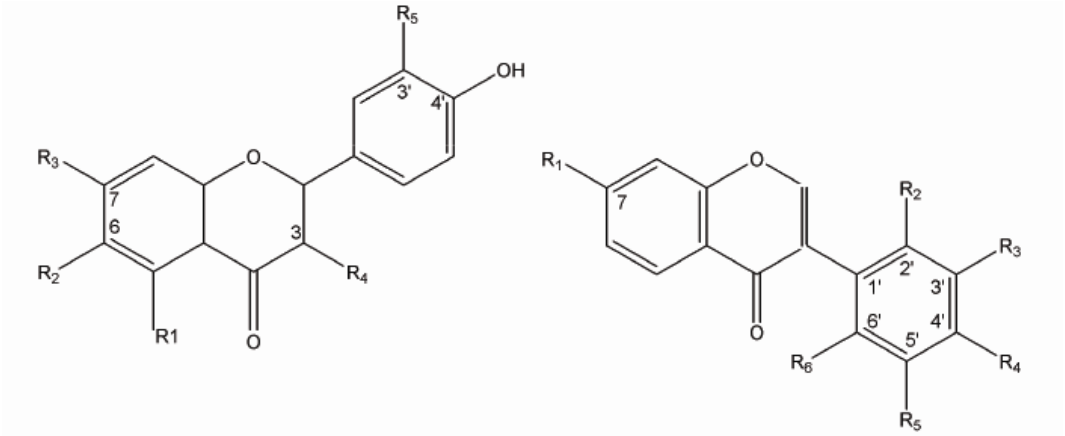


Fig. 2: Neighbor-joining phylogenetic tree of *Streptomyces* sp. BT01, including representatives of the most closely-related type strains which were retrieved from GenBank, and accession numbers appear in parentheses. Bootstrap (1,000 replicates) values are given in percentage. Bar, 0.01 substitutions per nucleotide.



1 R_1 = H, R_2 = R_4 = R_5 = OH, R_3 = OCH_3
2 R_1 = R_2 = H, R_3 = R_4 = R_5 = OH
3 R_1 = R_3 = OH, R_2 = R_4 = R_5 = H
4 R_1 = R_2 = OH, R_3 = R_6 = H, R_4 = R_5 = OCH_3
5 R_1 = R_3 = R_4 = OH, R_2 = R_5 = R_6 = H
6 R_1 = R_2 = OH, R_3 = R_5 = R_6 = H, R_4 = OCH_3

Fig. 3: Chemical structures of 7-methoxy-3, 3',4',6-tetrahydroxyflavone (**1**), 3,3',4',7-tetrahydroxyflavone (fisetin **2**), 4',5,7-trihydroxyflavanone (naringenin **3**), 2',7-dihydroxy-4',5'-dimethoxyisoflavone (**4**), 3'-hydroxydaidzein (**5**) and xenognosin B (**6**).

Two new compounds designated as compound **1** and compound **4**, their spectral data were reported as follows: Compound **1**, identified by NMR and mass spectral data as 3,3',4',6-tetrahydroxy-7-methoxyflavone ($C_{16}H_{12}O_7$), was yellow crystals having: mp 315-317 ^oC (from methanol), UV: λ_{max} nm (log ε) = 239 (4.290), 257 sh (4.262), 349 (4.454). λ_{max} nm (+ $AlCl_3$) (log ε) = 235 (4.586), 276 (4.179), 360 (3.93), 431 (4.394). λ_{max} nm (+ $AlCl_3$/HCl) (log ε) = 228 sh (4.269), 267 (4.257), 357 sh (3.973), 419 (4.454). IR ν_{max} cm^{-1}: 3596, 3511, 3333, 3117, 1636, 1609, 1551, 1508, 1497, 1435, 1393, 1289, 1223, 1169, 1123, 1038. EI-MS *m/z*: 316 (M^+, 100%), 301 (22), 273 (35), 167 (13), 150 (16), 149 (24), 137 (39), 135 (21), 128 (14), 123 (16), 120 (13), 95 (18), 69 (42), 63 (17), 53 (31), 51 (26). HR-MS: $C_{16}H_{12}O_7$, found: 316.0580, calcd: 316.0588. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 3.91 (3H, s, 7-OCH_3), 6.86 (1H, d, *J*=8.4 Hz, H-5'), 7.20 (1H, s, 8-H), 7.29 (1H, s, 5-H), 7.54 (1H, dd, *J*=8.4, 2.2Hz, 6'-H), 7.70 (1H, d, *J*=2.2 Hz, 2'-H), 9.00 (1H, s, 3-OH), 9.24 (1H, s, 3'-OH), 9.53 (1H, s, 4'-OH), 9.72 (1H, s, 6-OH). ^{13}C-NMR (DMSO-d_6, 75.4 MHz) δ: 56.54 (7-OCH_3), 100.46 (C-8), 106.9 (C-5), 115.00 (C-10), 115.33 (C-2'), 115.86 (C-5'), 119.94 (C-6'), 123.01 (C-1'), 137.39 (C-3), 145.04 (C-6), 145.36 (C-3'), 145.45 (C-2), 147.48 (C-4'), 150.22 (C-9), 153.96 (C-7), 171.91 (C-4).

Compound **4**, identified by NMR and mass spectral data as 2',7-dihydroxy-4',5'-dimethoxyisoflavone ($C_{17}H_{14}O_6$), was yellow crystals having: mp 237-239 ^oC (from methanol), UV: λ_{max} nm (log ε) = 248 sh (4.312), 264 sh (4.211), 301 (4.256). IR ν_{max} cm^{-1}: 3414, 2940, 1705, 1616, 1562, 1512, 1458, 1343, 1300, 1246, 1188, 1103. EI-MS *m/z*: 314 (M^+, 100%), 299 (85), 271 (19), 239 (22), 200 (20), 187 (28), 137 (30), 107 (24), 92 (22), 69 (57), 63 (36), 53 (25), 51 (33). HR-MS: $C_{17}H_{14}O_6$, found: 314.0799, calcd: 314.0730. ^{1}H-NMR (DMSO-d_6, 200 MHz) δ: 3.66 (3H, s, 5'-OCH_3), 3.72 (3H, s, 4'-OCH_3), 6.52 (1H, s, 3'-H), 6.81 (1H, s, 6'-H), 6.87 (1H, d, *J*=2.1 Hz, 8-H), 6.92 (1H, dd, *J*=8.7, 2.1 Hz, 6-H), 7.94 (1H, d, *J*=8.7 Hz, 5-H), 8.22 (1H, s, 2-H), 8.99 (1H, s, 7-OH), 10.80 (1H, br, 2'-OH). ^{13}C-NMR (DMSO-d_6, 50 MHz) δ: 55.65 (4'-OCH_3), 56.66 (5'- OCH_3), 101.53 (C-3'), 102.27 (C-8), 110.08 (C-1'), 115.42 (C-6), 116.02 (C-6'), 116.70 (C-10), 121.64 (C-3), 127.44 (C-5), 141.70 (C-5'), 149.81 (C-2'), 149.90 (C-4'), 155.24 (C-9), 157.64 (C-9), 162.79 (C-7), 175.42 (C-4).

Among 6 compounds tested, compounds **1**, **2** and **3** showed strong antibacterial activity greater than compounds **4**, **5** and **6**. By comparing results between compounds **1**, **2** and **3**, the overall order of potency is **2** > **1** > **3**, which indicated the presence of free phenolic OH group to be crucial for bioactivity. However, compounds **4**, **5** and **6**, although with free phenolic OH group, exhibited moderate to antibacterial activity. Compounds which were methylated on OH group, i.e. compounds **4** and **6**, showed weaker antibacterial activity in comparison to the unmethylated form, further indicating the essence of free phenolic hydroxyl group as an active group.

This study indicated that *Streptomyces* sp. BT01, an endophyte in *Boesenbergia rotunda* produces several antibacterial flavonoids. Since the flavonoids have been reported to have multiple biological effects including anti-inflammatory activity, antioxidant activity and anti-thrombotic activity, etc (Jiang *et al*., 2005, Shi *et al*., 2012), so it could be benefit in this point, we would like to further investigate of these compounds.

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