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Antibacterial, antioxidant, cytotoxic effects and GC-MS analysis of mangrove-derived *Streptomyces achromogenes* TCH4 extract

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Abstract: The *in vitro* biological activities of the ethyl acetate extract of the culture filtrate from *Streptomyces achromogenes* TCH4 (TCH4 extract) were evaluated. The ethyl acetate extract of TCH4 produced a bacteriostatic effect against *Enterobacter cloacae*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Bacillus subtilis*, methicillin-resistant *S. aureus* (MRSA) and *Klebsiella pneumoniae*. The extract had bactericidal activity against *S. aureus*, *S. saprophyticus*, *S. aureus* (MRSA) and *K. pneumoniae* with minimum bactericidal concentration (MBC) values in the range of 500-1000 μg/mL. The total phenolic and flavonoid contents in TCH4 extract were 107.20±2.57 mg gallic acid equivalent (GAE)/g and 44.91±0.84 mg quercetin equivalent (QE)/g of dry extract. Antioxidant activity was determined by DPPH radical (IC_{50} 299.64±6.83 μg/mL) and ABTS radical scavenging (IC_{50} 65.53±0.95 μg/mL), and the ferric-reducing antioxidant power (FRAP) (822.76±9.12 mM $FeSO_4.7H_2O$/g dry extract) assays. TCH4 extract exhibited cytotoxic activity in the DU-145 cell line (IC_{50} 9.36±0.37 μg/mL). Analysis of extract constituents by GC-MS revealed pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) (36.85%), benzeneacetamide (23.76%), and deferoxamine (12.85%) as the major compounds, which have been reported to possess pharmaceutical properties. *S. achromogenes* TCH4 could be a potential source of bioactive metabolites with antibacterial, antioxidant and anticancer activities for pharmaceutical applications.

Keywords: mangrove; *Streptomyces achromogenes*; antibacterial; antioxidant; cytotoxic

INTRODUCTION

Mangrove ecosystems occur at the interface between terrestrial, fresh water and marine environments of the tropical and subtropical coastal zones of the world [1]. These systems provide large quantities of nutrient and organic matter in sediments that have come from diverse soil textures containing different proportions of sand, silt and clay. Mangrove sediment characteristics are attributed to high salinity, high humidity, high sulfide concentrations and low oxygen contents [2]. These ecosystems support a unique set of species of plants, animals and microbes adapted to living in extreme environmental conditions. Mangrove microbes play an important role as a potential producer of novel secondary metabolites [3].

Actinomycetes are a group of Gram-positive filamentous bacteria that are distributed in mangrove soil and sediments. Among actinomycetes, the genus *Streptomyces* in particular is responsible for the production of a wide range of bioactive metabolites including antibiotics, antitumor compounds, enzyme inhibitors and immunosuppressants [4-6]. Several studies have reported on the diversity, biological activities and bioactive metabolites of *Streptomyces* originating from mangrove sediments. Different mangrove sediment sites have been the source of the discovery

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of novel species of *Streptomyces*, such as *S. sanyensis* sp. nov. [7], *S. qinglanensis* sp. nov. [8], *S. pluripotens* sp. nov. [9], *S. mangrovisoli* sp. nov. [10], *S. malaysiense* sp. nov. [11] and *S. colonosanans* sp. nov. [12]. The mangrove-derived *Streptomyces* species have the ability to produce various bioactive metabolites. In [10] the detection of the antioxidant agent known as pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- in the extract of *S. mangrovisoli* sp. nov. using gas chromatography-mass spectrometry (GC-MS) was described. 2-Methylbutyl propyl phthalate was isolated from fermentation broth of *S. cheonanensis* VUK-A, and exhibits cytotoxicity against the MDA-MB-231, OAW-42, MCF-7 and HeLa cancer cell lines, as well as strong antimicrobial activity against *Proteus vulgaris*, *Candida albicans* and *Fusarium solani* [13].

In our previous study, we reported on the antimicrobial effects from the fermentation broth of *Streptomyces* species isolated from sediment samples collected from the mangrove forest areas in eastern Thailand [14]. The most active antimicrobial isolate was *Streptomyces* sp. strain TCH4 from mangrove sediments collected from the Mueang district, Chantaburi province, Thailand. Based on phenotypic, chemotaxonomic and 16S rRNA gene sequence analysis, the species was identified as *S. achromogenes*. The fermentation time course of this strain in ISP-2 broth medium supplemented with 3% sodium chloride (NaCl) was evaluated for antimicrobial activity; maximum antibiotic production of cell-free culture filtrate was noted between 21 and 28 days of cultivation. The present study was carried out to evaluate the antimicrobial, antioxidant and cytotoxic properties of crude extract from a fermentation broth of the *S. achromogenes* TCH4. The chemical constituents of the extracts were analyzed using GC-MS.

MATERIALS AND METHODS

Streptomyces strain

S. achromogenes TCH4 was subcultured and maintained in slant culture on yeast extract malt extract agar (ISP2) at 4°C and kept in 15% glycerol at -80°C as stock culture.

Fermentation process

Secondary metabolites from *S. achromogenes* TCH4 were produced in samples taken from stock culture grown on ISP2 broth with a 3% NaCl (w/v) agar slant for 7 days. The spores were harvested from culture slants with 0.1% Tween 80, counted under a microscope using a hemacytometer and adjusted to 1×10^6 spores/mL. A 1% (v/v) spore suspension was inoculated into a 500-mL flask containing 200 mL of ISP2 broth with 3% NaCl, and incubated on a rotary shaker incubator (150 rpm) at 30°C for 21 days. The fermentation broths were filtered through Whatman No.1 filter paper. The resulting filtrates were centrifuged at 5000 ×g for 15 min and the clear supernatants were collected for extraction.

Extraction

The cell-free fermentation broth was extracted twice with equal volumes of ethyl acetate (1:1, v/v) in a separating funnel. The organic phase was collected and dried using a rotary evaporator at 40°C under reduced pressure. The crude extract was weighed and stored at -20°C until further use.

Fermentation and extraction

S. achromogenes strain TCH4 was cultivated with agitation in ISP-2 liquid medium supplemented with 3% sodium chloride for 21 days. At the end of the fermentation process, small pellets formed in the clear liquid culture. The culture broth (30 L) was centrifuged to separate the pellet and the supernatant. The supernatant was extracted with ethyl acetate (1:1, v/v). After evaporation, the ethyl acetate extract had a sticky appearance with a dark brown color. The yield of ethyl acetate extract was 0.846 g.

Test organisms

The following bacterial strains were used in this study: *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *S. aureus* MRSA ATCC 43300, *Staphylococcus saprophyticus* ATCC 15305, *E. coli* ATCC 25922, *Salmonella typhimurium* ATCC 13311, *Enterobacter aerogenes* ATCC 13048, *Enterobacter cloacae* ATCC 23355, *Klebsiella pneumonia* ATCC

13883, and *Proteus mirabilis* DMST 8212. These organisms were obtained from the Department of Medical Science Thailand Culture Collection (DMST), Ministry of Public Health, Nonthaburi, Thailand. All the bacterial strains were maintained at 4°C on Mueller Hinton agar (MHA) and sub-cultured every 15 days.

Disc diffusion assay

The antibacterial activity of the crude extracts was determined using the disc diffusion method according to the standard protocol described by the National Committee of Clinical Laboratory Standards [15]. All bacteria were grown on a Mueller-Hinton agar (MHA) slant at 37°C for 18-24 h, and the bacterial suspension was prepared using a sterile normal saline solution (0.85% NaCl) equivalent to a 0.5 McFarland turbidity standard (corresponding to 1.5×10^8 CFU/mL). The bacterial suspension (100 µL) was spread onto MHA plates. A stock solution of extracts was prepared at a concentration of 10 mg/mL in absolute ethanol. The sterile 6-mm-diameter filter paper discs were impregnated with 10 µL of each extract solution (100 µg/disc), air-dried and placed on the inoculated agar. The treated plates were incubated at 37°C for 18-24 h. Antibacterial activity was measured based on the diameter expressed in mm of the clear zone on the disc. A sterile filter disc containing absolute ethanol without any extracts was used as a negative control. For standard antibiotics, penicillin G (10 U/disc) and chloramphenicol (30 µg/disc) were used as positive controls. Each experiment was carried out in triplicate and mean values were reported.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC values of the extracts were determined using a resazurin microtiter plate assay [16]. A bacterial inoculum was standardized at 0.5 McFarland and diluted 1:100 in normal saline to obtain a final concentration of 1×10^6 CFU/mL. The extracts were dissolved in 50% dimethyl sulfoxide (DMSO) to make a concentration of 10 mg/mL, and were then diluted by two-fold dilutions to obtain different concentrations ranging from 10-0.0195 mg/mL. To each well of a 96-well microtiter plate, 80 µL of Muller Hinton broth, 10 µL of diluted extract and 10 µL of bacterial suspension were added. The positive control drug was chloramphenicol, diluted in sterile distilled water; the concentrations tested ranged from 0.195-100 µg/mL, with 50% DMSO serving as the negative control. Microtiter plates were incubated at 37°C for 18-24 h. After incubation, 10 µL of aqueous resazurin solution (0.03%) was added to each well and incubated for 3 h to detect cell viability by converting from resazurin (blue and non-fluorescent) to resorufin (pink and highly fluorescent). The MIC of the extract was the lowest concentration at which no bacterial growth or no color change occurred. Determination of the MBC was carried out by streaking the mixture from each MIC assay negative well on an MHA plate and incubating at 37°C for 24 h. The lowest concentration that resulted in no visible cell growth was defined as the MBC value. All experiments were performed in triplicate.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Qualitative analysis

The antioxidant properties of the extract were analyzed by thin layer chromatography (TLC) bioautography, followed by the DPPH spray technique [17]. Each extract sample was applied on a TLC plate (silica gel 60 GF254, Merck). The plates were developed in 10% dichloromethane in methanol to separate different constituents of the extract. The developed chromatogram was sprayed with a solution of 0.2% DPPH in methanol and incubated for 30 min at room temperature. The active antioxidant compounds of the extract were detected as yellow bands against a purple background on the TLC plate.

Quantitative analysis

The DPPH radical scavenging capacity of the extract was evaluated as described [18], based on the electron transfer method that detects the electron-donating ability of antioxidant compounds for the conversion of a DPPH radical to a stable DPPH molecule [19]. The DPPH solution was freshly prepared by dissolving 2.4 mg of DPPH in 100 mL of ethanol. The extracts were

diluted in ethanol at concentrations ranging from 100-1,000 μg/mL (250 uL) mixed with DPPH solution (250 uL). The reaction mixtures were incubated in the dark for 30 min at room temperature, and the absorbance was measured at 520 nm. Butylated hydroxytoluene (BHT) at different concentrations was used as a reference standard. A negative control was prepared containing ethanol (250 μL) and DPPH solution (250 μL). The percentage inhibition of free radical DPPH was calculated according to the following equation:

DPPH radical scavenging activity(%)=$(A_0-A_1)/A_0\times100$,

where A_0 is the absorbance of the control and A_1 is the absorbance of the extract. The IC_{50} value (the concentration of extract required to scavenge 50% of the initial DPPH radicals) was calculated by plotting the percentage inhibition against extract concentration. BHT was used as the positive control. The DPPH radical scavenging assay was performed in three replicates in at least three independent experiments.

2,2'-Azino-bis[3-ethylbenzothiazoline-6-sulphonic acid] (ABTS) radical scavenging activity

The scavenging activity of extracts against the ABTS radical was carried out as described [18]. The ABTS assay measures the antioxidant capacity of compounds to reduce ABTS radical cations into the oxidized product via electron transfer (original ABTS structure) or hydrogen atom transfer (ABTSH) [20]. The $ABTS^{\bullet+}$stock solution was prepared by mixing 7 mM ABTS and 2.4 mM potassium persulfate in equal quantities. The mixture was kept in the dark at room temperature for 12-16 h until the radical solution turned an intense blue-green color. The $ABTS^{\bullet+}$stock solution was diluted with distilled water to an absorbance of 0.80±0.05 at 734 nm. Fifty μL of diluted extract were mixed with 450 μL of the $ABTS^{\bullet+}$ solution. After 1 min of bleaching of ABTS radicals, the absorbance was measured at 734 nm. Trolox was used as a standard antioxidant substance. The percentage ABTS radical inhibition and the IC_{50} value were calculated as mentioned for the DPPH radical scavenging activity. The ABTS scavenging activity was measured in triplicate and in at least three independent experiments.

FRAP assay

The ferric-reducing activity of the extract was determined as described [21] with some modifications. The FRAP assay is based on an electron transfer reaction that measures the capacity of the antioxidant in the reduction of the ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex into the ferrous tripyridyltriazine (Fe^{2+}-TPTZ) complex [22]. The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6) and 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the ratio 10:1:1 (v/v/v), and then incubating at 37°C before use. A 60-μL sample was mixed with 180 μL of distilled water and 1.8 mL of FRAP reagent. The reaction mixture was incubated at 37°C for 4 min and the absorbance was measured at 593 nm. The formation of a blue-colored complex was developed by reducing the Fe^{3+}-TPTZ complex to the Fe^{2+}-TPTZ complex. The standard curve was plotted using an aqueous solution of ferrous sulfate ($FeSO_4$ $7H_2O$) in the range 0.1-1 mM. The ferric-reducing activities were expressed as μM of $FeSO_4$ $7H_2O$ per g of extract. All experiments were performed at least three times and in triplicate.

Determination of the total phenolic content

The total phenolic content in the extract was analyzed using the Folin-Ciocalteu method [23] with slight modification. An aliquot of diluted extract or standard solution of gallic acid (20-200 μg/mL) was mixed with distilled water and Folin-Ciocalteu reagent. The reaction mixture was incubated for 30 min and sodium carbonate solution (7% w/v) was added with mixing and the volume was adjusted to 3.0 mL with distilled water and mixed thoroughly. When a blue color developed, the absorbance was measured at 756 nm. The total phenolic content was calculated from the linear equation based on the calibration curve using gallic acid as the standard. The standard calibration curve equation was y=0.0044x-0.0305, with a linear correlation coefficient (R^2) of 0.9960, where y is the absorbance of the sample and x is the concentration of gallic acid. The results were reported in terms of mg of gallic acid equivalent per g (mg GAE/g) of dry extract. The experiment was conducted in triplicate and values are presented as the mean±standard deviation (SD).

Determination of the total flavonoid content

The total flavonoid content of the extract was estimated using the aluminum chloride colorimetric method [23] with slight modification. In brief, the diluted extract (0.25-2 mg/mL) and the standard quercetin (10-100 µg/mL) were mixed with distilled water and a 5% (w/v) sodium nitrite solution. After incubation for 5 min, 10% (w/v) aluminum chloride was added and mixed with 1 M sodium hydroxide and distilled water. The absorbance of the reaction mixture was measured against a blank at 510 nm. The flavonoid content was calculated from the calibration curve using quercetin as standard. The standard calibration curve equation was y=0.0084x+0.0087, with a linear correlation coefficient (R^2) of 0.9994, where y is the absorbance of the sample and x is the concentration of quercetin. The results were expressed in mg of quercetin equivalent per g (mg QAE/g) of dry extract. All determinations were performed in triplicate and data are shown as mean±SD.

Cell culture

The human prostate adenocarcinoma cell line DU-145 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 unit/mL penicillin and 100 µg/mL streptomycin. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 until cell confluence reached 80-90%, and then passaged twice a week.

3-(4,5-Dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide (MTT) assay

Assessment of cytotoxicity of the extract on DU-145 cells was performed by the MTT assay as described [24]. The DU-145 cell lines were seeded in a 24-well plate at a density of 6×10^4 cells per well in serum containing medium, followed by incubation at 37°C under 5% CO_2 in an incubator for 24 h. The medium was discarded and the cells were treated with different concentrations of the extract (0.5, 1, 5, 10, 20, 30, 40 µg/mL) for 96 h. After incubation, the cells were rinsed with phosphate-buffer saline and 150 µL of 1 mg/mL MTT in serum-free DMEM was added to each well, followed by incubation for 4 h at 37°C. The medium was then removed and 150 µL of DMSO were added to each well to dissolve the formazan crystals. The plates were shaken for 30 min on a shaker and the absorbance of each well was measured at 570 nm using an ELISA reader (Biotex-synergy-HT, US). The assay was performed for at least 3 independent experiments with no less than 3 replicates for each concentration. The cytotoxic activity of the extract was expressed in terms of IC_{50} calculated using Microsoft Excel software and the results were expressed as the mean±SD.

Gas chromatography-mass spectrometry (GC-MS) analysis of extracts

The crude extract was analyzed using GC-MS in an Agilent Technologies (US) 6890N GC coupled to an Agilent 5973 inert mass selective detector. The GC capillary column was an HP-5MS (30 m × 0.25 mm × i.d., 0.25 µm film thickness 0.25 µm, Agilent Technologies). The column temperature was increased from 70-300°C at a rate of 4°C/min. The carrier gas was helium with a column flow rate of 1 mL/min. The injector was held at 240°C in splitless mode. The mass selective detector was operated in electron ionization mode at 70 eV with a mass range from 40-400 amu. The components were identified by comparing their spectra with those recorded in the Wiley7n mass spectral database [25].

Statistical analysis

The results for each assay were expressed as the mean±SD of 3 replicates. The cytotoxicity data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The results were considered significant when $P\leq0.05$. The IC_{50} values were calculated using linear regression analysis.

RESULTS

Antimicrobial activity of *S. achromogenes* TCH4 extract

The antimicrobial activity of the extract was evaluated against 10 pathogenic bacterial strains using the disc diffusion method. The ethyl acetate extract of *S. achromogenes* TCH4 (TCH4 extract) exhibited

Table 1. Antibacterial activity of the TCH4 extract based on disc diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays.

Bacteria	TCH4 extract			Pen G			Chloram		
	DIZ (mm)	MIC (µg/mL)	MBC (µg/mL)	DIZ (mm)	MIC (µg/mL)	MBC (µg/mL)	DIZ (mm)	MIC (µg/mL)	MBC (µg/mL)
B. subtilis	13.91 ± 0.35	125	>4,000	29.99±0.25	NT	NT	28.88±0.54	6.25	>200
S. aureus (MSSA)	18.22±0.51	500	500	36.23±0.39	NT	NT	19.55±0.31	50	100
S. aureus (MRSA)	13.39±0.42	500	1,000	9.45±0.29	NT	NT	20.62±0.36	100	>200
S. saprophyticus	14.59±0.34	250	500	40.97±0.57	NT	NT	25.65±1.29	6.25	100
E. coli	0.00	NT	NT	7.07±0.25	NT	NT	23.03±0.09	NT	NT
E. cloacae	24.95±2.47	125	>4,000	37.68±0.06	NT	NT	41.05±0.07	3.13	200
E. aerogenes	0.00	NT	NT	21.88±0.44	NT	NT	7.53±0.29	NT	NT
K. pneumoniae	12.32±0.59	125	1,000	35.45±0.29	NT	NT	21.12±0.38	6.25	200
P. mirabilis	0.00	NT	NT	22.37±1.55	NT	NT	12.67±0.25	NT	NT
S. typhimurium	0.00	NT	NT	22.83±0.11	NT	NT	28.40±0.86	NT	NT

Values are mean ± SD of three replications. DIZ – diameter of inhibition zone; MIC – minimum inhibition concentration; MBC – minimum bactericidal concentration; NT – not tested; PenG – penicillin G; Chloram – chloramphenicol

antibacterial activity on Gram-positive and Gram-negative bacteria at a concentration of 100 µg/disc compared to penicillin G (10 U/disc) and chloramphenicol (30 µg/disc). The results of the inhibition zone of the extract against various pathogens are shown in Table 1. TCH4 extract exhibited strong antibacterial activity against *E. cloacae* (24.95±2.47 mm) followed by *S. aureus* (MSSA) (18.22±0.51 mm), *S. saprophyticus* (14.59±0.34), *B. subtilis* (13.91±0.35 mm), *S. aureus* (MRSA) (13.39±0.42 mm) and *K. pneumoniae* (12.32±0.59 mm). No inhibitory activity was observed against *E. coli, S. typhimurium, E. aerogenes* or *P. mirabilis*. The antibacterial activity of TCH4 was quantitatively evaluated using the broth microdilution method against 6 selected bacteria (*E. cloacae*, *S. aureus* (MSSA), *S. saprophyticus*, *B. subtilis*, *S. aureus* (MRSA), *K. pneumoniae*). The MIC and MBC values of the extract are shown in Table 1.

Table 1 shows that TCH4 extract was effective against all tested bacterial species with MIC values ranging from 125-250 µg/mL and MBC values varying from 500 to >4000 µg/mL. The MIC values of the extract against *E. cloacae*, *K. pneumoniae*, and *B. subtilis* were 125 µg/mL, 250 µg/mL against *S. saprophyticus*, and 500 µg/mL against *S. aureus* (MSSA) and *S. aureus* (MRSA). The extract exhibited bactericidal activity, with MBC values of 500 µg/mL for *S. saprophyticus* and *S. aureus* (MSSA), 1000 µg/mL for *K. pneumoniae* and *S. aureus* (MRSA) and >4000 µg/mL for *B. subtilis* and *E. cloacae*.

Total phenolic and flavonoid contents

The total phenolic content in the TCH4 extract was 107.20±2.57 mg GAE/g, and the total flavonoid content was 44.91±0.84 mg QE/g extract.

DPPH radical scavenging activity

The results of the qualitative DPPH examination of the extract are shown in Supplementary Fig. S1. Results for the DPPH radical scavenging activity of the extract are presented in Table 2 and Fig. 1A. the TCH4 extract scavenged DPPH free radicals in a concentration-dependent manner. The percentages of scavenging activity at varying concentrations of the extract were in the range 29.60-74.85% for concentrations of 100-500 µg/mL. The standard antioxidant was BHT with concentrations in the range 20-100 µg/mL. The IC_{50} value in the DPPH assay of the TCH4 extract was 299.64±6.83 µg/mL while the value for BHT was 32.95±0.26 µg/mL.

Table 2. DPPH and ABTS radical scavenging activity and the ferric-reducing antioxidant power (FRAP) assay of the TCH4 extract.

Extract/ standard	IC_{50} (µg/mL)		FRAP (mM $FeSO_4.7H_2O$/gdw)
	DPPH	ABTS	
TCH4 extract	299.64±6.83	65.53±0.95	822.76±9.12
BHT	32.95±0.26	ND	ND
Trolox	ND	3.26±0.05	ND

ND – Not detectable

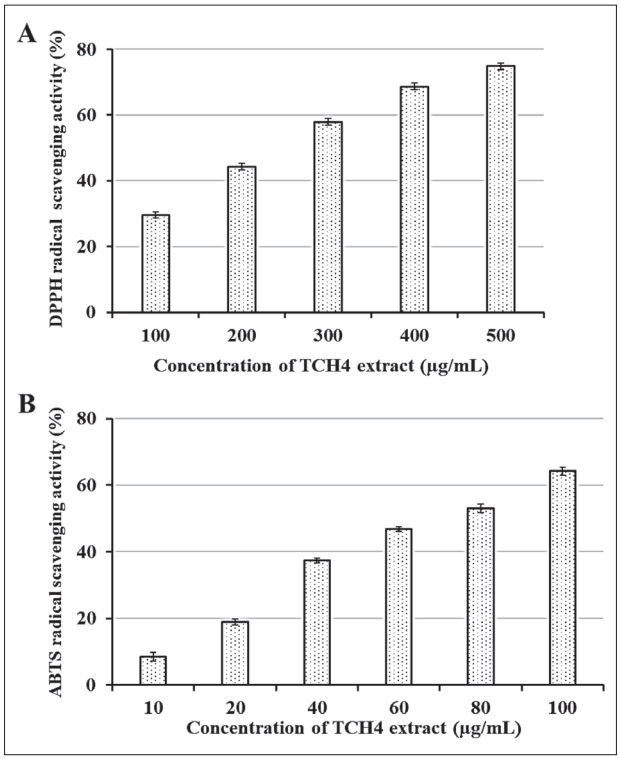

Fig. 1. Antioxidant activity of the TCH4 extract. **A** – DPPH radical scavenging activity; **B** – ABTS radical scavenging activity; error bars indicate ±SD.

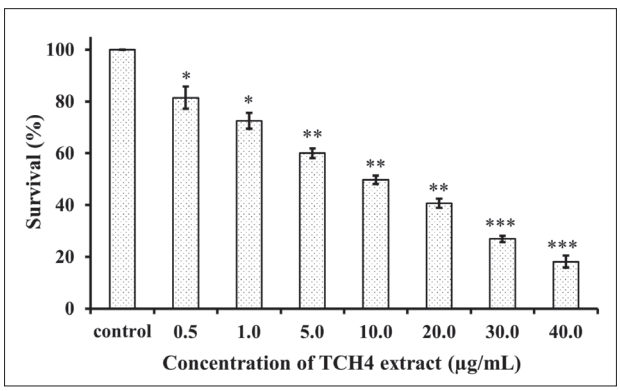

Fig. 2. Assessment of DU-145 cell viability using the MTT assay after exposure to different concentrations of the TCH4 extract for 96 h. Values are the mean±SD (n=4). All data were compared with the control (ANOVA test, *=P<0.05, **=P<0.01, ***=P<0.001).

ABTS radical scavenging activity

The results of ABTS assay were reported as percentages of inhibition against the ABTS radical (Fig. 1B) and as the IC_{50} value (Table 2). Fig. 1B shows that the extract scavenged ABTS radicals in a concentration-dependent manner. The percentage of inhibition in the extract increased from 8.39% at 10 µg/mL to 64.20% at 100 µg/mL, and its IC_{50} value was 65.53±0.95 µg/mL.

Ferric-reducing antioxidant power (FRAP)

The reducing power of the extract was evaluated from the standard linear curve of $FeSO_4.7H_2O$ and the FRAP value is shown in Table 3. The $FeSO_4.7H_2O$ calibration curve was used to obtain a regression equation ($y=0.3653x+0.052$, $R^2=0.9992$). The FRAP value of the extract calculated from the equation was 822.76±9.12 mM $FeSO_4.7H_2O$/g dry extract.

Cytotoxicity on DU-145 cell line

The cytotoxic effect of TCH4 extract was investigated on the DU-145 cell line by the MTT assay. Cells were treated with different concentrations of extract ranging from 0.5 to 40 µg/mL for 96 h (Fig. 2). The extract decreased cell viability to 81.42±4.25%, 72.43±3.07%, 60.03±1.89, 49.76±1.62, 40.68±1.83%, 26.85±1.26%, and 18.11±2.39% at concentrations of 0.5, 1, 5, 10, 20, 30 and 40 µg/mL, respectively. The results indicated that TCH4 significantly decreased ($P<0.05$) DU-145 cells compared to untreated cells in a dose-dependent manner. The IC_{50}value (calculated using regression analysis of the dose response curve) after 96 h was 9.36±0.37 µg/mL for the DU-145 cell line.

GC-MS analysis of volatile compounds in extract

The chemical constituents in the TCH4 extract were analyzed using GC-MS. In total, 24 compounds were identified representing 100% of total compounds in TCH4. The GC-MS chromatogram is shown in Fig. 3. The compounds corresponding to the peaks with their retention time (RT), molecular formula, molecular weight (MW) and concentration (peak area %) in the extract are listed in Table 3 (with the chemical structures of the main compounds (Supplementary Fig. S2)). Among the identified compounds, pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) (36.16%), benzeneacetamide (23.76%) and deferoxamine (12.85%) were the major compounds and 21 other minor compounds were obtained at low concentrations (comprising 0.31-4.82%).

Table 3. Chemical compounds detected in the TCH4 extract based on GC-MS analysis.

No.	Compound	Retention time	Area (%)	Formula	Molecular weight
1	2-Pentenoic acid, 3-methyl-, methyl ester	11.907	0.89	$C_7H_{12}O_2$	128
2	5-Thiazoleethanol, 4-methyl-	13.820	0.65	C_6H_9NOS	143
3	Indole	14.454	0.51	C_8H_7N	117
4	Benzeneacetamide	17.430	23.76	C_8H_9NO	135
5	2,5-Pyrrolidinedione, 1-butyl-	26.341	1.38	$C_8H_{13}NO_2$	155
6	2,4(1H,3H)-Pyrimidinedione, 1,3-dimethyl-	26.517	4.67	$C_6H_8N_2O_2$	140
7	3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione, *N*-acetyl-	26.587	1.19	$C_{10}H_{14}N_2O_3$	210
8	9H-Purin-6-amine, N,N,9-trimethyl-	26.698	0.49	$C_8H_{11}N_5$	177
9	Quinolin-2-ol, 4-amino-	26.951	4.82	$C_9H_8N_2O$	160
10	Gamma-Guanidinobutyric acid	27.127	0.83	$C_5H_{11}N_3O_2$	145
11	(3*S*,6*S*)-3-Butyl-6-methylpiperazine-2,5-dione	27.302	0.83	$C_9H_{16}N_2O_2$	184
12	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	29.335, 30.030, 31.777, 32.248, 32.481, 32.573	36.16	$C_{11}H_{18}N_2O_2$	210
13	Octadecanoic acid	29.945	0.38	$C_{18}H_{36}O_2$	284
14	Cyclooctasiloxane, hexadecamethyl-	30.529	0.31	$C_{16}H_{48}O_8Si_8$	592
15	9H-Pyrido[3,4-b]indole, 1-methyl-	33.161	0.56	$C_{12}H_{10}N_2$	182
16	Pyrimido[1,2-a]azepine, 2,3,4,6,7,8,9,10-octahydro-	33.478	0.37	$C_9H_{16}N_2$	152
17	Cyclononasiloxane, octadecamethyl-	34.196	0.33	$C_{18}H_{54}O_9Si_9$	666
18	*Z*-2-Amino-1-cyclohexanecarboxamide	35.357	1.32	$C_7H_{14}N_2O$	142
19	2-Methyl-3-amino-4,5-dihydroxymethylpyridine, triacetate	35.490	0.31	$C_{14}H_{18}N_2O_5$	294
20	Deferoxamine	36.160	12.85	$C_{25}H_{48}N_6O_8$	560
21	9-Octadecenoic acid, methyl ester, (*E*)-	36.398	0.65	$C_{19}H_{36}O_2$	296
22	*Z*-2-Amino-1-cyclohexanecarboxamide	37.796	1.42	$C_7H_{14}N_2O$	142
23	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	41.161, 42.123	3.68	$C_{14}H_{16}N_2O_2$	244
24	1-Oxa-3-azaspiro[4.5]decan-2-one, 4-hydroxy-3-[2-(5-methoxy-1H-indol-3-yl)ethyl]-4-methyl-	53.568	1.62	$C_{20}H_{26}N_2O_4$	358

DISCUSSION

Mangrove actinomycetes can produce a wide range of bioactive secondary metabolites containing diverse classes of organic compounds. Among the mangrove-derived actinomycetes, *Streptomyces* is the dominant genus in mangrove sediments [26-28]. In our previous studies, a mangrove-derived *S. achromogenes* strain TCH4 from cell free fermentation broth exhibited potent antimicrobial and cytotoxic activities [14]. In the current study, secondary metabolites were produced by culturing *S. achromogenes* strain TCH4 on ISP-2 broth medium supplemented with 3% NaCl for 21 days with agitation. The cell-free supernatant of this strain was extracted with ethyl acetate and evaluated by determining the chemical constituents and biological properties of the extract. The results from the disc diffusion assay of the TCH4 extract revealed a broad-spectrum inhibitory activity (based on the inhibition diameter) against Gram-positive and Gram-negative bacteria such as *E. cloacae* (24.95 mm) followed by *S. aureus* (MSSA) (18.22 mm), *S. saprophyticus* (14.59 mm), *B. subtilis* (13.91 mm), *S. aureus* (MRSA) (13.39 mm) and *K. pneumoniae* (12.32 mm), respectively. In the MIC and MBC assays, the TCH4 extract produced the lowest MIC value of 125 µg/mL against *E. cloacae*, *K. pneumoniae* and *B. subtilis*, followed by *S. saprophyticus* (MIC=250 µg/mL), while *S. aureus* (MSSA) and *S. aureus* (MRSA) had the highest MIC of 500 µg/mL. The lowest MBC was 500 µg/mL for *S. saprophyticus* and *S. aureus*

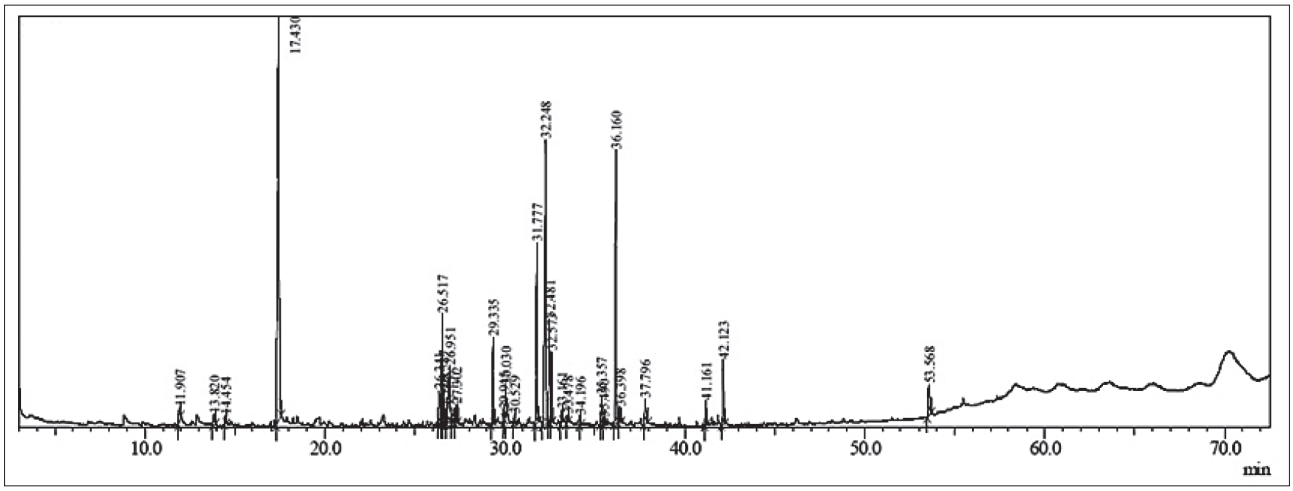

Fig. 3. GC-MS chromatogram of the TCH4 crude extract.

(MSSA). These results indicated that the TCH4 extract possessed bactericidal activity against *S. aureus* (MSSA) and *S. typhimurium* and bacteriostatic activity against *E. cloacae*, *K. pneumoniae*, *S. aureus* (MRSA) and *B. subtilis*. Other reports found that the ethyl acetate extract of *Streptomyces* sp. DOSMB-D105 from mangrove sediment had an inhibitory effect on *Pseudomonas* sp., *Proteus* sp., *Bacillus* sp., *E. coli*, *S. aureus*, *Lactococcus lactis*, *Salmonella infantis*, *K. pneumoniae*, *Vibrio cholerae* and *Citrobacter diserus* in the agar diffusion assay [29]. In another study, the methanolic extract of *Streptomyces* sp. strain MUSC 125 from mangrove soil inhibited the growth of *S. aureus* (MRSA) ATCC 43300 and *S. aureus* (MRSA) ATCC 33591 at MIC values of 12.5 and 25 mg/mL, respectively [30]. According to [31], the ethyl acetate extract of mangrove sediment-derived *Streptomyces* sp. strain ACTN 2 showed antibacterial property against *B. subtilis* and *P. aeruginosa* with MIC values of 0.1563 and 5.0 mg/mL, respectively.

Phenolic compounds, including phenolic acids, polyphenols and flavonoids, are the main source of antioxidant metabolites [32]. These compounds are responsible for scavenging free radicals, donating hydrogen atoms and chelating of metal cations [33]. In addition, phenolic compounds can promote health benefits and decrease the risk of chronic diseases, such as cardiovascular disease, cancer, diabetes and neurodegenerative diseases [34,35]. The current study results showed that the TCH4 extract had total phenolic and total flavonoid contents of 107.20±2.57 mg GAE/g dry extract and 44.91±0.84 mg QE/g dry extract, respectively. The total phenolic content of the ethyl acetate extract of mangrove-associated *Streptomyces olivaceus* (MSU3) contained 0.0421 mg GAE/g of dry weight of extract [36]. In addition, several studies showed a correlation between the antioxidant activity and the total phenolic content in the methanolic extract of different strains of mangrove-derived *Streptomyces* [30,37,38]. However, in some reports no flavonoids were detected in the methanolic extracts of mangrove *Streptomyces* [37,38]. The results of the current study showed that the TCH4 extract contained high amounts of phenolic compounds and flavonoids, which probably contributed to its antioxidant activity.

The antioxidant potential of the TCH4 extract was determined using different assays (DPPH radical scavenging activity, ABTS radical scavenging activity and FRAP). These assays assess different reaction mechanisms of the antioxidant compounds in the extract. The antioxidant potential in the current study showed that the TCH4 extract exhibited DPPH and ABTS radical scavenging activities in a dose-dependent manner with IC_{50} values of 299.64±6.83 µg/mL and 65.53±0.95 µg/mL, respectively, while the FRAP value was 822.76±9.12 mM $FeSO_4.7H_2O$/g dry extract. These results showed that the TCH4 extract had potential *in vitro* antioxidant activity. It was reported [36] that the *in vitro* antioxidant activity of the ethyl acetate extract of *S. olivaceus* (MSU3) isolated from mangrove sediment showed DPPH scavenging activity, a reducing power effect, hydroxyl radical scavenging activity

and nitric oxide activity, with IC_{50} values of 75.21, 39.75, 71.46 and 48.02 mg/mL, respectively. Similarly, the crude ethyl acetate extract of isolated *S. coelicoflavus* BC 01 from mangrove soil had a DPPH scavenging activity of 68.91%, a FRAP value of 78.00 μM/mL of ascorbic acid equivalent, and a total antioxidant capacity value of 93.33 μg/mL of ascorbic acid equivalent at 20 μg/mL concentration [39]. Previous reports demonstrated that the methanolic extracts from the different strains of mangrove-derived *Streptomyces* sp. exhibited antioxidant activities, including ABTS free radical scavenging activity, DPPH free radical scavenging activity, metal chelating activity and/or a superoxide dismutase-like activity [36-38,40]. These findings revealed that mangrove-derived *Streptomyces* can produce a diverse range of antioxidant metabolites via several chemical mechanisms.

The genus *Streptomyces* is an important source of various anticancer drugs such as doxorubicin, dactinomycin and bleomycin [41-43]. The current study showed that the TCH4 extract inhibited the proliferation of DU-145 cells in a dose-dependent manner, with an IC_{50} value of 9.36±0.37 μg/mL at 96 h, which points to the anticancer potential of this extract. Other studies have also reported the cytotoxic activity of different mangrove *Streptomyces* extracts against tested cancer cell lines. The methanolic extract of *S. pluripotens* MUSC 137 exhibited cytotoxicity against breast cancer MCF-7 cells, colon cancer HTC-116 cells, lung cancer A549 cells, cervical cancer CaSki cells and colon cancer HT-29 cells with IC_{50} values of 61.33, 83.72, 147.20, 300.50 and 300.98 μg/mL, respectively [44]. In addition, the crude methanolic extracts from three *Streptomyces* strains (*S. malaysiense* MUSC 136^T, *S. monashensis* MUSC $1J^T$, *S. gilvigriseus* MUSC 26^T) could contribute to the cytotoxic activity against HCT-116 colon cancer cells with cell viabilities of 82.30, 63.64 and 48.80%, respectively, when treated with 400 μg/mL of the extract [11,40,45].

In the current study, the GC-MS spectrum of TCH4 contained 3 major volatile compounds, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) (36.85%), benzeneacetamide (23.76%) and deferoxamine (12.85%). These compounds have been reported previously in the extract from mangrove-derived *Streptomyces*. The pyrrolopyrazine compound, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), was detected in extracts of mangrove strains of *Streptomyces* sp., including *S. malaysiense* MUSC 136^T, *S. colonosanans* MUSC $93J^T$ and *S.* sp. MUM 256 [11,12,46]. The compound pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) exhibits a wide range of biological activities such as antioxidant, antimicrobial and anticancer activities [47-50]. Benzeneacetamide was also produced by *S. omiyaensis* SCH2 and *S. pluripotens* MUSC137 from mangrove sediments [18,44]. Previous studies reported that benzeneacetamide exhibited significant antibacterial, antidepressant and anticonvulsant effects [51-52]. In addition, natural deferoxamine was detected in the crude extracts of *S. malaysiense* MUSC 136^T and *S. pluripotens* MUSC137 isolated from mangrove soil samples [11,44]. Deferoxamine was reported as an iron-chelating drug approved by the US Food and Drug Administration for the treatment of chronic iron overload and acute iron poisoning anemias [53]. This compound showed cytotoxic effects against human breast adenocarcinoma (MCF-7) and the human leukemia cell line (HL-60) and also demonstrated strong antioxidant activity, shown by the chelation of ferrous ions and the neutralization of reactive oxygen species such as hydroxyl, peroxyl, alkoxyl, and superoxide radicals [54-57]. It is possible that the major compounds in the TCH4 extract are responsible for exhibiting diverse biological activities.

In conclusion, the ethyl acetate extract from the culture filtrate of the *S. achromogenes* strain TCH4 showed antibacterial activity against tested bacterial pathogens. The extract also exhibited *in vitro* antioxidant activity that could be associated with the total phenolic and flavonoid contents. In addition, the TCH4 extract exhibited a cytotoxic effect against DU-145 cell lines in a dose-dependent manner. GC-MS profiling showed the presence of 3 major compounds, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) (36.85%), benzeneacetamide (23.76%) and deferoxamine (12.85%). Our results suggest that *S. achromogenes* strain TCH4 produces various biologically active secondary metabolites with antibacterial, antioxidant and anticancer properties with potential biomedical and pharmaceutical applications.

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Author contributions: JT and RS conceived and designed the experiment. JT, WP, WY performed the experiments. JT wrote the first draft of the manuscript. RT carried out the statistical analysis. All authors have read and approved the final manuscript.

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Supplementary Material

The Supplementary Material is available at: http://www.serbiosoc.org.rs/NewUploads/Uploads/Tangjitjaroenkun%20et%20al_6408_Supplementary%20Material.pdf