Environment and Natural Resources Journal

Volume 22
Issue 6
2024

Antioxidant Activity and Suppression of Intracellular Radical Generation of *Streptomyces* Strains and Genome Analysis of Strain ET3-23

Khaing Zar Wai[1], Nantiya Bunbamrung[2], Pattama Pittayakhajonwut[2], Nisachon Tedsree[3], Somboon Tanasupawat[4], and Rataya Luechapudiporn[5,6*]

[1]*Pharmaceutical Sciences and Technology Program, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*
[2]*National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand Science Park, Pathum Thani 12120, Thailand*
[3]*Faculty of Science and Arts, Burapha University, Chanthaburi Campus, Chanthaburi 22170, Thailand*
[4]*Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*
[5]*Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*
[6]*Center of Excellence for Ageing and Chronic Diseases, Chulalongkorn University, Bangkok 10330, Thailand*

ARTICLE INFO

Received: 26 Feb 2024
Received in revised: 30 Aug 2024
Accepted: 17 Sep 2024
Published online: 5 Nov 2024
DOI: 10.32526/ennrj/22/20240047

Keywords:
Antioxidant activity/ Genome analysis/ RAW264.7 macrophage cells/ *Streptomyces*/ Soil

*** Corresponding author:**
E-mail:
rataya.L@pharm.chula.ac.th

ABSTRACT

Actinomycetes, predominantly found in soil, represent a remarkable source of natural products. The natural antioxidants obtained from them have been employed for human infectious diseases. In particular, *Streptomyces* strains serve as significant sources of natural antioxidants with demonstrated health benefits. The *in vitro* antioxidant activity and the inhibition of intracellular radical generation by crude extracts from various *Streptomyces* strains were evaluated and the genome sequence of selected strains was analyzed. Strains CT2-10, NE1-12, and ET3-23 showed 16S rRNA gene sequence similarity to *Streptomyces capoamus* JCM 4734^T (98.94%), *Streptomyces nigra* 452^T (99.78%), and *Streptomyces morookaense* LMG 20074^T (99.49%), respectively . The genome analysis of strain ET3-23 had 106 contigs, with a total length of 8121874 bp and an average G+C content of 71.46%. Ethyl acetate extracts of strains CT2-10, ET3-23, and NE1-12 exhibited, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity with IC_{50} 555.9 µg/mL, 66.5 µg/mL, and 463.3 µg/mL and showed NO scavenging activity, except for CT2-10. Strain NE1-12 significantly inhibited ROS production induced by hydrogen peroxide on macrophage cells, while strains NE1-12 and CT2-10 inhibited NO production induced by lipopolysaccharides with IC_{50} 82.4 µg/mL and 2.3 µg/mL, respectively. These findings suggest their ability to modulate NO production which is crucial in inflammatory responses and tissue injury. The antioxidant activities of *Streptomyces* strains indicate their potential as valuable sources of bioactive natural products and effective antioxidant agents, warranting further investigation for therapeutic applications.

1. INTRODUCTION

Soil is a complex habitat with a diverse range of organisms (EL-Kamali et al., 2017). Among these, microbes have been extensively explored as sources of bioactive natural products, essential for defense and survival in harsh environments. More than 95% of Actinomycetes strains have been isolated from soil, with *Streptomyces* being the dominant genus (Rammali et al., 2022).

Citation: Wai KZ, Bunbamrung N, Pittayakhajonwut P, Tedsree N, Tanasupawat S, Luechapudiporn R. Antioxidant activity and suppression of intracellular radical generation of *Streptomyces* strains and genome analysis of strain ET3-23. Environ. Nat. Resour. J. 2024;22(6):510-524. (https://doi.org/10.32526/ennrj/22/20240047)

Actinobacteria are Gram-positive bacteria with high G+C content, and the largest genus of actinobacteria is *Streptomyces*, belonging to the family *Streptomycetaceae* (Kämpfer, 2006). *Streptomyces* strains are renowned for producing numerous bioactive secondary metabolites such as antibiotic and antitumor agents (Hasani et al., 2014). Over 23,000 bioactive secondary metabolites have been isolated from microorganisms, and more than 10,000 of these compounds are from Actinomycetes, with nearly 7,600 produced by *Streptomyces* strains (Bérdy 2005).

In recent years, there has been increasing interest in the potential of *Streptomyces* strains as a source of natural antioxidants, which may have potential health benefits by preventing or reducing oxidative stress, a factor implicated in various diseases. Several studies have reported that extracts of the *Streptomyces* strain MUM292 from mangrove soil exhibited DPPH radical scavenging activities, highlighting its potential as an antioxidant agent (Tan et al., 2018). Actinomycin C2, benthocyanins, and carquinostatin isolated from *Streptomyces lavendulae* SCA5, *Streptomyces prunicolor*, and *Streptomyces exfoliates*, respectively have shown antioxidant activities (Karthik et al., 2013). Neocarazostatin A, B, and C from the culture of *Streptomyces* strain GP38 demonstrated inhibitory effects on lipid peroxidation induced by free radicals in rat brain homogenates (Kato et al., 1991) while pyrrolopyrazine-1,4-dione, hexahydro- from the extract of the novel *Streptomyces* strain MUSC149^{T} isolated from mangrove soil exhibited strong antioxidant activity by scavenging or reducing free radicals using the reducing power assay (Ser et al., 2015).

Nowadays, natural products from microorganisms have become a focus of modern research for safe therapeutics (Kim et al., 2014). Screening of natural products has concentrated on discovering and identifying bioactive metabolites based on genetic information to evaluate their biosynthetic potential (Ayuso-Sacido and Genilloud, 2005). This study investigated the antioxidant activity and intracellular radical suppression of various *Streptomyces* strains, with particular focus on the genome analysis of ET3-23. This research contributes to the identification of novel natural antioxidants and the development of therapeutic agents for oxidative stress-related diseases. The outcomes provide insights into the antioxidant potential of *Streptomyces* strains, their phenotypic characteristics, chemotaxonomic features for *Streptomycetes* classification, and their genetic features. The sequencing of specific genes also provides a deeper understanding of the phylogenetic relationships of these prokaryotes at the genus, species and subspecies level.

2. METHODOLOGY

2.1 Sample collection and isolation of actinomycetes

Soil samples were obtained from Nong Khai, Phichit, and Chachoengsao Provinces for actinomycete isolation. The collection involved sampling soil from a 5-10 cm depth beneath the surface. To facilitate the isolation process, the soil samples were air-dried, heated to 100°C, and treated with 1.5% phenol (Vargas Gil et al., 2009). Subsequently, serial dilutions (10^{-1}-10^{-4}) of these soil samples were prepared and spread plated onto a medium supplemented with humic acid and vitamins. Nalidixic acid and cycloheximide were included in the medium to prevent the growth of Gram- negative bacteria and fungi, respectively. The plates were then incubated at 30°C for 14 days to facilitate the growth and isolation of actinomycetes.

2.2 Identification methods of actinomycetes

Phenotypic characteristics were studied according to Shirling and Gottlieb (1966). Morphological characteristics were examined after cultivation on ISP 2 medium at 30°C for 7-14 days (Shirling and Gottlieb, 1966). The cell morphology of the selected isolate was observed using a scanning electron microscope (model JEOL JSM-IT500HR) after cultivation on ISP 3 medium at 30°C for 14 days. The colors of aerial and substrate mycelia and soluble pigments were determined using the NBS/ISCC color system (Kelly et al., 1965). Physiological characteristics were evaluated for the growth response of actinobacteria on ISP 2 medium at temperatures (28°C and 45°C), pH values (5, 7, and 10) and tolerance of NaCl concentration (2-6%) (Shirling and Gottlieb, 1966). Biochemical characteristics included starch hydrolysis, milk peptonization, milk coagulation, nitrate reduction, and gelatin liquefaction (Gordon, et al., 1974). The ability to produce acid from various carbon sources (L-arabinose, D-cellobiose, D-galactose, lactose, inositol, D-mannitol, D-maltose, D-raffinose, D-sorbitol, and D-xylose) were determined using standard methods (Shirling and Gottlieb, 1966).

Chemotaxonomic features were based on the diamino acid components in cell wall peptidoglycan. Cell wall type is the primary chemotaxonomic for identifying a strain to the genus level (Wai et al., 2022).

The diamino acid components as diaminopimelic acid (DAP) isomers in cell wall peptidoglycan were determined using the standard thin layer chromatography (TLC) method as described by Staneck and Roberts (1974). Dried cells were obtained from cultures grown in ISP 2 broth on an orbital shaker (180 rpm) at 30°C for 3-5 days. The cultured mycelium was washed with distilled water and then air-dried.

The genomic DNA was extracted from a pure culture inoculated in fresh ISP 2 broth at 30°C, 200 rpm for 2 days, and evaluated using physical and chemical combination methods (Tamaoka and Komagata 1984). The 16S ribosomal DNA was amplified from the extracted genomic DNA by sequencing with the universal primers 1492R (5´ACGGCTACC TTGTTACGACTT 3´) and 27F (5´AGTTTGATCCTGGCTCAG 3´) directions. The amplified PCR product was visualized by gel electrophoresis and purified and the obtained sequences were analyzed (Macrogen, Seoul, Korea) using EzTaxon-e server BLASTn searching program (http://www.ezbiocloud.net/eztaxon) and aligned against the selected type strain sequences received from GenBank using Bioedit software (Yoon et al., 2017).

2.3 Genome analysis

Genomic DNA was extracted using a PureLink™ Genomic DNA mini kit (Invitrogen; USA) and sequenced using the MiSeq sequencing system (Illumina; USA). The bioinformatics data of the genome were analyzed using the bacterial bioinformatics database and analysis resource (Seemann, 2014). The biosynthetic gene cluster in the genome was determined using the antiSMASH database (Bankevich et al., 2012). A phylogenetic tree based on genome data was constructed using the TYGS web server (Meier-Kolthoff and Göker, 2019).

2.4 Fermentation and preparation of ethyl acetate extracts

The spore suspensions of actinomycetes were inoculated into seed culture broth (100 mL) for 3 days and cultivated into ISP 2 broth (1,000 mL) at 37°C for 14 days. These fermentation broths were extracted with two volumes of ethyl acetate and cell suspension (2:1 v/v). The ethyl acetate extracts were evaporated to dryness before testing various biological activities (Janardhan et al., 2014).

2.5 Determination of total phenolic content

The total phenolic content (TPC) was determined through a reaction in which a phenol molecule released an H^+ ion, forming a phenolate ion capable of reducing the Folin-Ciocalteu (FC) reagent. In the assay, ethyl acetate extract, FC reagent, sodium bicarbonate, and DI water were sequentially added to a 96-well plate, incubated for 30 min at room temperature and measured with a microplate reader at 765 nm (Govindan and Muthukrishnan, 2013). Gallic acid was used as a positive control to establish a standard curve. The TPC was calculated and expressed as µg/gallic acid equivalent (GAE)/mg of extract.

2.6 Determination of ferric reducing antioxidant potency

The Fe^{3+}-reducing potential was determined by the reduction of ferric iron (Fe^{3+}) and 2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride (TPTZ) to the ferrous form (Fe^{2+}) at low pH 3.6, following the method described by Benzie and Strain (Sarma et al., 2010). Ethyl acetate extracts were incubated with the FRAP reagent at room temperature for 15 min and then measured at 593 nm. Ascorbic acid was used as a positive control. FRAP values were expressed as µg of ascorbic acid equivalent (AAE)/mg of extract (Bajpai et al., 2017).

2.7 DPPH radical scavenging activity assay

The scavenging activity against the 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical was assessed following the method outlined by Brand-Williams et al. (1995) with slight modifications (Kawahara et al., 2012). Briefly, various concentrations (50- 1,000 µg/mL) of ethyl acetate extracts (100 µL) were mixed with 100 µL ethanolic DPPH solution (50 µM) and incubated at room temperature in the dark for 20 min. The absorbance was measured at 550 nm to calculate the DPPH radical scavenging ability.

2.8 Determination of nitric oxide (NO) scavenging

Nitric oxide generated from sodium nitroprusside (SNP) reacts with oxygen to produce nitrite ions, forming a purple chromophore through the Griess reaction (Hazra et al., 2009). Different concentrations (50- 1,000 µg/mL) of ethyl acetate extracts were incubated with sodium nitroprusside (10 mM) in phosphate buffer saline (pH 7.4) at 30°C for 2 h. Griess reagent was added and incubated for 15 min. The absorbance of the resulting purple azo chromophore was measured at 542 nm and then the NO

scavenging activity was calculated. Ascorbic acid was used as a positive control.

2.9 Cell cytotoxicity assay

RAW264.7 macrophage cells (ATCC TIB71) were seeded at a density of 2×10^5 cells per well and incubated for 24 h to allow cell attachment and growth. Ethyl acetate extracts at concentrations ranging from 50 to 1,000 µg/mL were added to the cells and further incubated for 24 h. The MTT reagent was then added and incubated for 3 h, followed by solubilization of the purple formazan crystals with DMSO (Chen et al., 2011). Absorbance was measured at 540 nm. All experiments were conducted in triplicate.

To induce intracellular radical generation, 100 µM hydrogen peroxide (H_2O_2) was utilized (Jung et al., 2006). Cells were treated with non-toxic concentrations (10-200 µg/mL) and (1-20 µg/mL) of ethyl acetate extracts and incubated for 24 h. Subsequently, the cells were incubated with 100 µM H_2O_2 for 30 min. After incubation, the MTT assay was performed as described earlier.

2.10 Determination of intracellular ROS generation in RAW264.7 macrophage cells

Intracellular reactive oxygen species (ROS) levels were assessed by monitoring the fluorescent signal generated from the oxidation of non-fluorescent 2′,7′-dichlorofluorescein diacetate (DCFH-DA) inside the cells. DCFH-DA is cleaved by esterase to form DCFH, which is then oxidized by ROS to produce the fluorescent compound DCF (Aranda et al., 2013). RAW264.7 macrophage cells were seeded at a density of 2×10^5 cells/mL in a black 96-well plate and incubated at 37°C for 24 h. Different concentrations (0.1-200 µg/mL) and (0.1-10 µg/mL) of ethyl acetate extracts in serum-free medium were added to the cells and incubated for 24 h. Then, 10 µM DCFH-DA in serum-free medium was added and incubated for 30 min. The cells were washed with PBS before intracellular ROS induction by adding 100 µM H_2O_2 for 30 min. Fluorescence intensities were measured using a fluorescence microplate reader at excitation and emission wavelengths of 485 nm and 530 nm, respectively (Chansriniyom et al., 2018).

2.11 Determination of nitric oxide production in RAW264.7 macrophage cells

Nitric oxide (NO) production was assessed by measuring nitrite levels using the Griess reagent reaction. RAW264.7 macrophage cells were treated with different concentrations (0.1-200 µg/mL) and (0.1-10 µg/mL) of ethyl acetate extracts in serum-free medium, along with lipopolysaccharide (LPS) at 100 ng/mL for 24 h. After incubation, the cell culture supernatant was mixed with Griess reagent and incubated for 15 min at room temperature. The absorbance was then measured at 540 nm (Jung et al., 2014). A standard curve using sodium nitrite was used to quantify NO production.

2.12 Chemical profile analysis of the crude extracts

The chemical profile of the crude extract was analyzed using High-Performance Liquid Chromatography (HPLC) equipped with a C-18 column and a UV/UVVIS detector. A linear gradient system was employed during the analysis. The HPLC chromatograms were compared with an in-house database to identify the components present in the extract.

The LC-MS/MS system, equipped with an Inertsil ODS-4 column, was utilized to measure the LC-ESI-MS spectra. This allowed for a more detailed analysis of the chemical composition. The chemical profile including retention time, UV absorbance, and pseudomolecular ion was compared with the reported chemical profiles in the in-house database and the Dictionary of Natural Products database. This comparison helped to identify and characterize the compounds present in the crude extract.

3. RESULTS AND DISCUSSION

3.1 Identification of actinomycetes

Based on the phenotypic and chemotaxonomic characteristics, these three strains belonged to the *Streptomyces* genus. Through analysis of the 16S rRNA gene sequence similarity, strain CT2-10 (accession number LC635735) exhibited a close relationship to *Streptomyces capoamus* JCM 4734^T (98.94%), strain ET3-23 (accession number LC635739) showed similarity to *Streptomyces morookaense* LMG 20074^T (99.49%), and strain NE1-12 (accession number LC635728) displayed high similarity to *Streptomyces nigra* 452^T (99.78%). The morphological characteristics of strain ET3-23 are shown in Figure 1(a) and 1(b). These three isolates contained LL-DAP in the cell wall peptidoglycan. Detailed information regarding the cultural, genotypic, and phenotypic characteristics of the strains is shown in Table 1.

(a) (b)

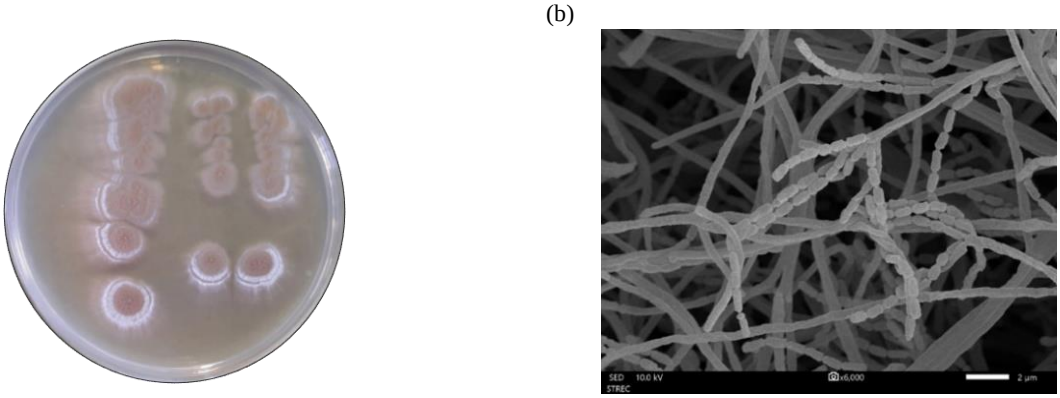

Figure 1. Colonial appearance (a) and scanning electron micrograph (b) of *Streptomyces* strain ET3-23 grown on ISP 3 agar at 30°C for 14 days.

Table 1. Characteristics of *Streptomyces* strains

Characteristics	Isolate No.		
	CT2-10	ET3-23	NE1-12
Cultural characteristics			
Upper surface color	Greyish white	Light yellowish brown	Grey greenish yellow
Reverse surface color	Moderate yellow	Pale yellow	Moderate greenish yellow
Genotypic characteristics			
Similarity (%)	98.94	99.49	99.78
Accession No.	LC635735	LC635739	LC635728
Nearest type strain	*Streptomyces capoamus* JCM 4734^T	*Streptomyces morookaense* LMG 20074^T	*Streptomyces nigra* 452^T
Phenotypic characteristics			
Growth on 2-6% NaCl	+	+	+
Growth at 28 °C	+	+	+
Growth at 45 °C	-	-	+
Growth at pH 5, 7, 10	+	+	+
Nitrate reduction	+	+	+
Hydrolysis of starch	-	+	-
Gelatinization	-	+	-
Milk coagulation	+	-	+
Milk peptonization	+	-	-
Acid production from:			
L-Arabinose	+	w	+
D-Cellobiose	+	w	+
D-Galactose	+	+	+
Lactose	+	w	+
Inositol	+	+	+
D-Mannitol	+	+	w
D-Maltose	+	+	+
D-Raffinose	+	-	w
D-Sorbitol	+	-	-
D-Xylose	+	+	+

Note: +, positive reaction; w, weak positive reaction; -, negative reaction.

3.2 Genome analysis

The draft genome of strain ET3-23 was submitted to GenBank. The accession number was JAJEJY000000000, and the assembled genome consisted of 106 contigs, with a total length of 8,121,874 base pairs, and an average G+C content of

71.46%. It contained 7,718 protein-coding sequences (CDS), along with 79 transfer RNA (tRNA) and 3 ribosomal RNA (rRNA) genes, as detailed in Supplementary Table S1.

The analysis of the biosynthesis gene clusters of secondary metabolites using antiSMASH revealed that strain ET3-23 harbored genes associated with the production of terpene, lanthipeptide, and thiopeptide, as indicated in Table 2. Notably, geosmin and neocarazostatin A exhibited a 100% similarity value. Neocarazostatin A1, derived from *Streptomyces* sp. MA37, demonstrated potent free radical scavenging properties, effectively protecting cells against damage (Liu et al., 2019). Neocarazostatins A, B, and C from *Streptomyces* sp. GP38 also exhibited strong inhibitory effects on free radical-induced lipid peroxidation in rat brain homogenate (Kato et al., 1991). Furthermore, carquinostatins A and B, lavanduquinocin, neocarazostatins A-C, carbazoquinocins A-F, and carbazomycins A-H showed promising antioxidant potential in protecting neuronal cells against oxidative damage caused by free radicals. This study reported the biological activities and provided insights into the genome sequence of the type strain of *Streptomyces morookaense* LMG 20074^T.

Table 2. Distribution of biosynthetic gene clusters in strain ET3-23 with similarity values greater than 20%

Cluster	Type	Most similar known cluster (class)	Similarity (%)
1	T2PKS	Spore pigment (Polyketide)	66
2	NRPS, T1PKS	Divergolide (Polyketide)	100
3	Terpene	Geosmin (Terpene)	100
4	Amglyccycl	B-D galactosylvalidoxylamine (Saccharide)	22
7	Lanthipeptide	SBI-06990 A1, SBI-06989 A2 (Lanthipeptide)	75
11	Siderophore	Desferrioxamine (Other)	83
13	NRPS	Coelichelin (NRP)	100
16	Terpene	Isorenieratene (Terpene)	75
19	NRPS, Nucleoside	Nogalamycin (Polyketide)	40
22	T3PKS	Germicidin (Other)	100
25	T1PKS, T3PKS, NRPS, Oligosaccharide	Lobophorin A (Polyketide)	73
26	Terpene, NRPS	Hopene (Terpene)	92
27	Ecotine	Ecotine (Other)	50
37	T1PKS	X 14547 (Polyketide)	21
38	T1PKS, Other	Lobophorin A (Polyketide)	26
43	T1PKS	Sceliphrolactam (Polyketide)	24

3.3 Total phenolic content

Strain NE1-12 exhibited the highest phenolic content (21.2±1.13 µg GAE/mg), followed by CT2-10 (16.8±0.66µg GAE/mg), while ET3-23 showed a lower phenolic content (9.1±0.54 µg GAE/mg). Notably, strain NE1-12 demonstrated significant levels of phenolic compounds, particularly those containing phenolic hydroxyl groups, indicating its strong antioxidant potential in relation to scavenging activities against DPPH radicals and NO.

Studies have reported the potential benefits of phenolic compounds in the prevention of inflammation, neurodegenerative, cardiovascular diseases and even cancer (Aleixandre-Tudo and Du Toit, 2019). Lee et al. (2014) found that the ethyl acetate extract of *Streptomyces* sp. strain MJM10778 had a total phenolic content of 8.8±0.2 µg GAE/g dry weight (Lee et al., 2014). In the case of *Streptomyces cavouresis* KUV39, the ethyl acetate extract exhibited a total phenolic content of 20.24 µg GAE/mg of the extract (Narendhran et al., 2014).

3.4 Ferric reducing antioxidant potential (FRAP)

Among the tested strains, ET3-23 exhibited the highest antioxidant capacity in terms of FRAP, with a value of 95.0±3.14 µgAAE/mg while strains CT2-10 and NE1-12 showed a moderate antioxidant capacity of 31.4±3.84 µgAAE/mg and 33.9±0.55 µgAAE/mg, respectively.

The significant antioxidant capacity displayed by strain ET3-23 suggested its potential as a strong antioxidant, with ferric reducing capacity indicating its ability to donate electrons, an important characteristic of antioxidants. This property enabled the strain to act

as a free radical inhibitor or scavenger, effectively neutralizing harmful radicals and serving as a primary antioxidant. The proton-donating ability of ET3-23 further contributed to its overall antioxidant activity.

These findings highlighted the potential of strain ET3-23 as a valuable source of natural antioxidants with significant free radical scavenging capabilities and the ability to protect against oxidative stress.

3.5 DPPH radical scavenging activity

Vitamin C demonstrated potent DPPH radical scavenging activity with a concentration-dependent response and an IC_{50} value of 5.7 µg/mL (Figure 2(a)). Strain ET3-23 exhibited DPPH radical scavenging with an IC_{50} value of 66.5 µg/mL. At 200 µg/mL concentration, it achieved a maximum activity of 77.7±0.98% (Figure 2(c)). The strains CT2-10 and NE1-12 also exhibited DPPH radical scavenging with IC_{50} values of 555.9 µg/mL and 463.3 µg/mL, respectively (Figure 2(b) and (d)). Among the strains tested, ET3-23 displayed more effective DPPH radical scavenging activity, with a lower IC_{50} value than NE1-12 and CT2-10. The observed DPPH radical scavenging activity of ET3-23 was attributed to its high phenol content and FRAP value. The strain reduced stable DPPH free radicals and exhibited characteristics of a hydroxyl radical scavenger by donating hydrogen atoms to free radicals and neutralizing their reactivity by removing odd electrons.

Similar studies have reported the DPPH radical scavenging activity of ethyl acetate extracts from other *Streptomyces* strains. For instance, ethyl acetate crude extracts from *Streptomyces olivaceus* (MSU3) demonstrated increasing inhibition at higher concentrations, with an IC_{50} value of 75.21 µg/mL (Sanjivkumar et al., 2016). *Streptomyces* sp. R56-07 strains JBIR-94 and JBIR-125 exhibited DPPH radical scavenging activity with IC_{50} values 11.4 µM and 35.1 µM, respectively (Taechowisan et al., 2017). An ethyl acetate extract of *Streptomyces* sp. AM-S1 culture demonstrated strong DPPH radical scavenging activity with an IC_{50} value of 68.4 µg/mL. Its mechanism of antioxidant action involves acting as a hydrogen donor, terminating the oxidation process by converting free radicals into stable forms (Sowndhararajan and Kang, 2013).

3.6 Nitric oxide radical scavenging activity

Vitamin C demonstrated potent activity, with NO scavenging activity of 85% and IC50 value 405.6 µg/mL in a concentration-dependent manner (Figure 3A). Strains NE1-12 and ET3-23 showed NO scavenging of 44.1±3.69% and 45.0±0.56%, respectively at 400 µg/mL. As the concentration increased, the NO scavenging activity decreased, indicating a relatively low capacity for NO scavenging (Figure 3(b) and 3(c)). These strains exhibited concentration-dependent scavenging of the nitrite radical, reaching a plateau where further increases in concentration did not result in increased quenching of the nitrite radical.

Lee et al. (2014) reported that the extract of *Streptomyces* strain MJM10778 displayed NO scavenging activities at increasing concentration, with highest scavenging rates of 95.4±0.1% and IC50 value 0.02 µg/mL. The soil-borne actinobacteria exhibited potential antioxidant activity against NO radicals (Lee et al., 2014).

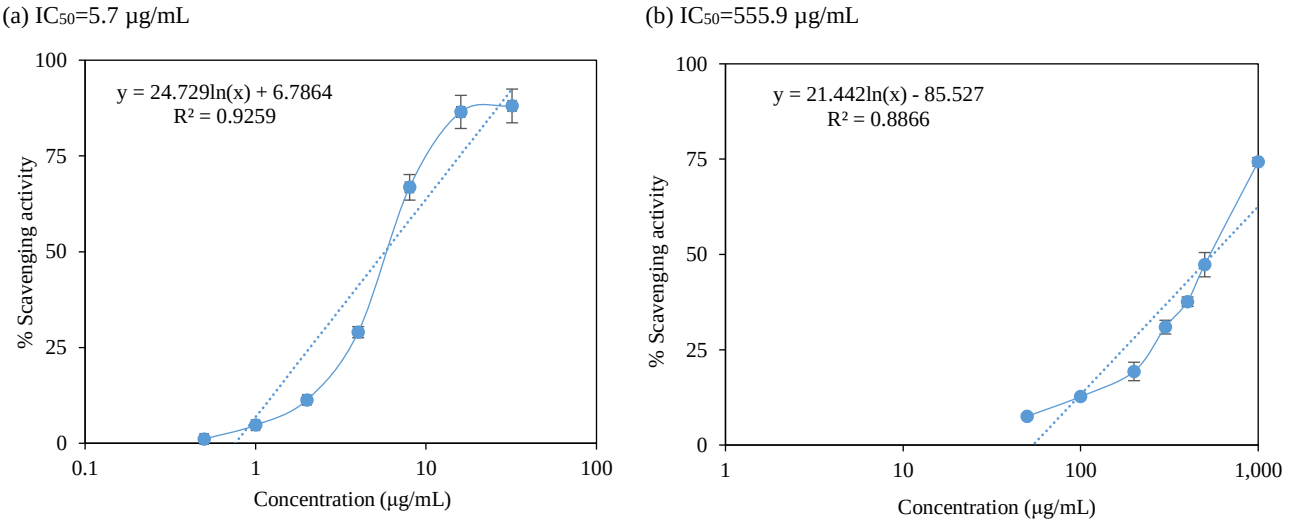

Figure 2. IC_{50} values of vitamin C (a) and ethyl acetate extracts of CT2-10 (b), ET3-23 (c) and NE1-12 (d) for DPPH radical scavenging activity. Data are expressed as mean±SEM (n=3).

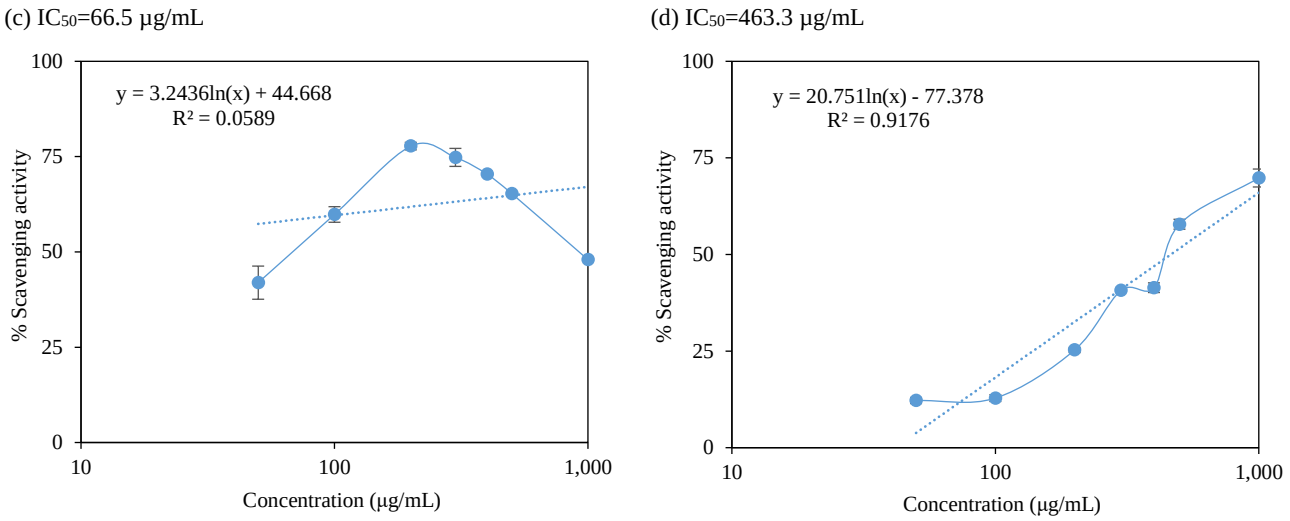

Figure 2. IC_{50} values of vitamin C (a) and ethyl acetate extracts of CT2-10 (b), ET3-23 (c) and NE1-12 (d) for DPPH radical scavenging activity. Data are expressed as mean±SEM (n=3) (cont.).

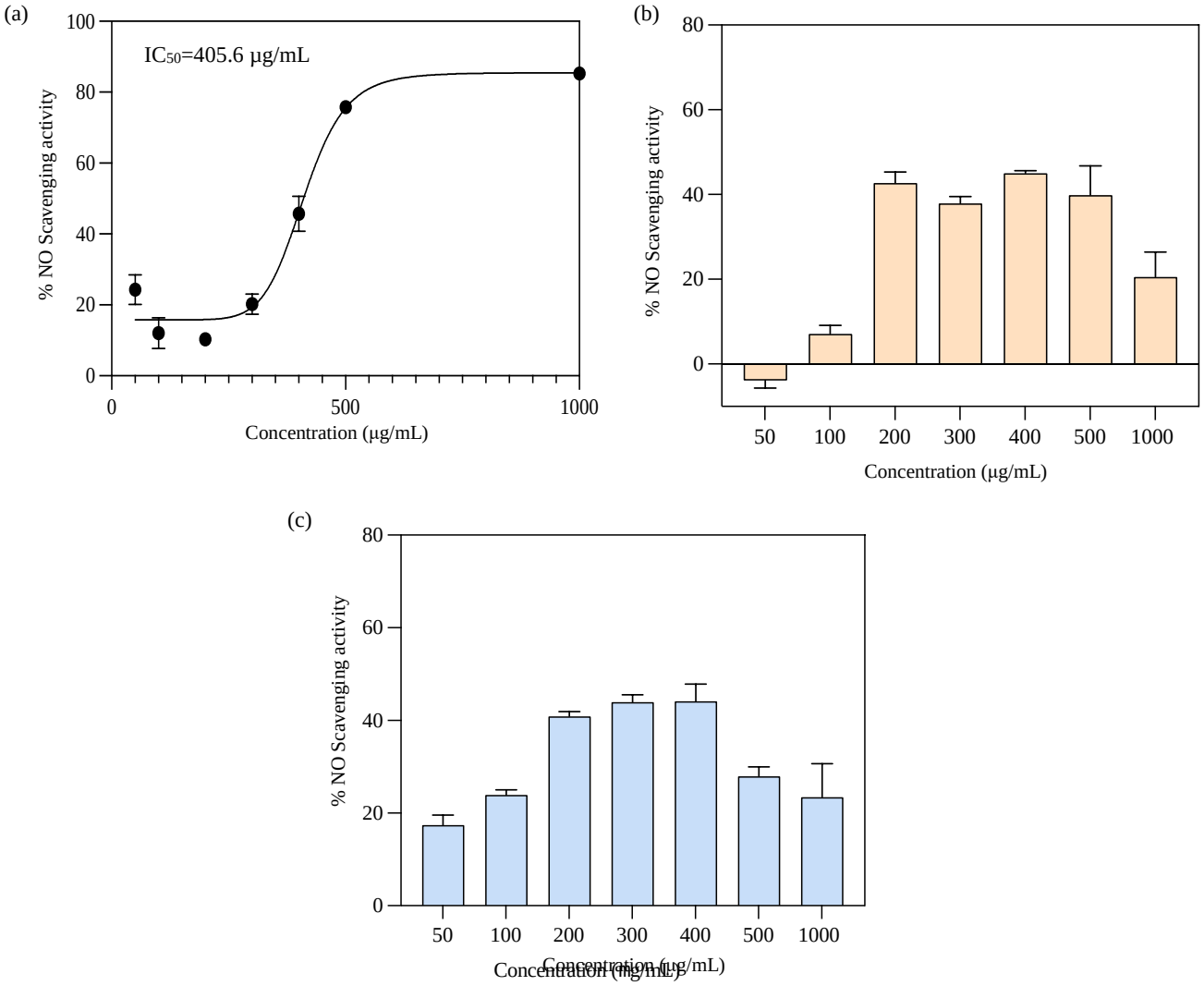

Figure 3. IC_{50} values of vitamin C (a) and NO scavenging activities of ethyl acetate extracts of ET3-23 (b) and NE1-12 (c). Data are expressed as mean±SEM (n=3).

3.7 Cytotoxic effects of Streptomyces strains

The cytotoxic effects of *Streptomyces* strains ET3-23, NE1-12, and CT2-10 were evaluated using the MTT assay on RAW264.7 macrophage cells. The ethyl acetate extracts of ET3-23 and NE1-12 showed no cytotoxicity, with high cell viability across concentrations of 10-200 µg/mL and 10-1,000 µg/mL, respectively (Figure 4(b) and (c)).

The cell viability of CT2-10 was 90% at 10 µg/mL. However, at a higher concentration of 50 µg/mL, the viability dropped to 46%. Further increasing the concentration from 100 µg/mL to 1,000 µg/mL resulted in consistently low cell viability, with values of 0-2%. (Figure 4(a)). These findings suggested that CT2-10 exhibited a concentration-dependent cytotoxic effect on the tested cells. Notably, the cytotoxicity of CT2-10 on macrophage cells suggested its potential as a chemotherapeutic drug, with the ability to selectively target and impact the viability of cancer cells.

Based on these results, non-toxic concentrations of the crude extracts were selected for further investigation of intracellular ROS and NO production in macrophage cells stimulated by hydrogen peroxide and lipopolysaccharide. ET3-23 and NE1-12 were studied in the 10-200 µg/mL range, while CT2-10 was studied in the 1-20 µg/mL range. The low cytotoxicity of the extracts suggested their potential as therapeutic agents for use in clinical settings for oxygen radical diseases.

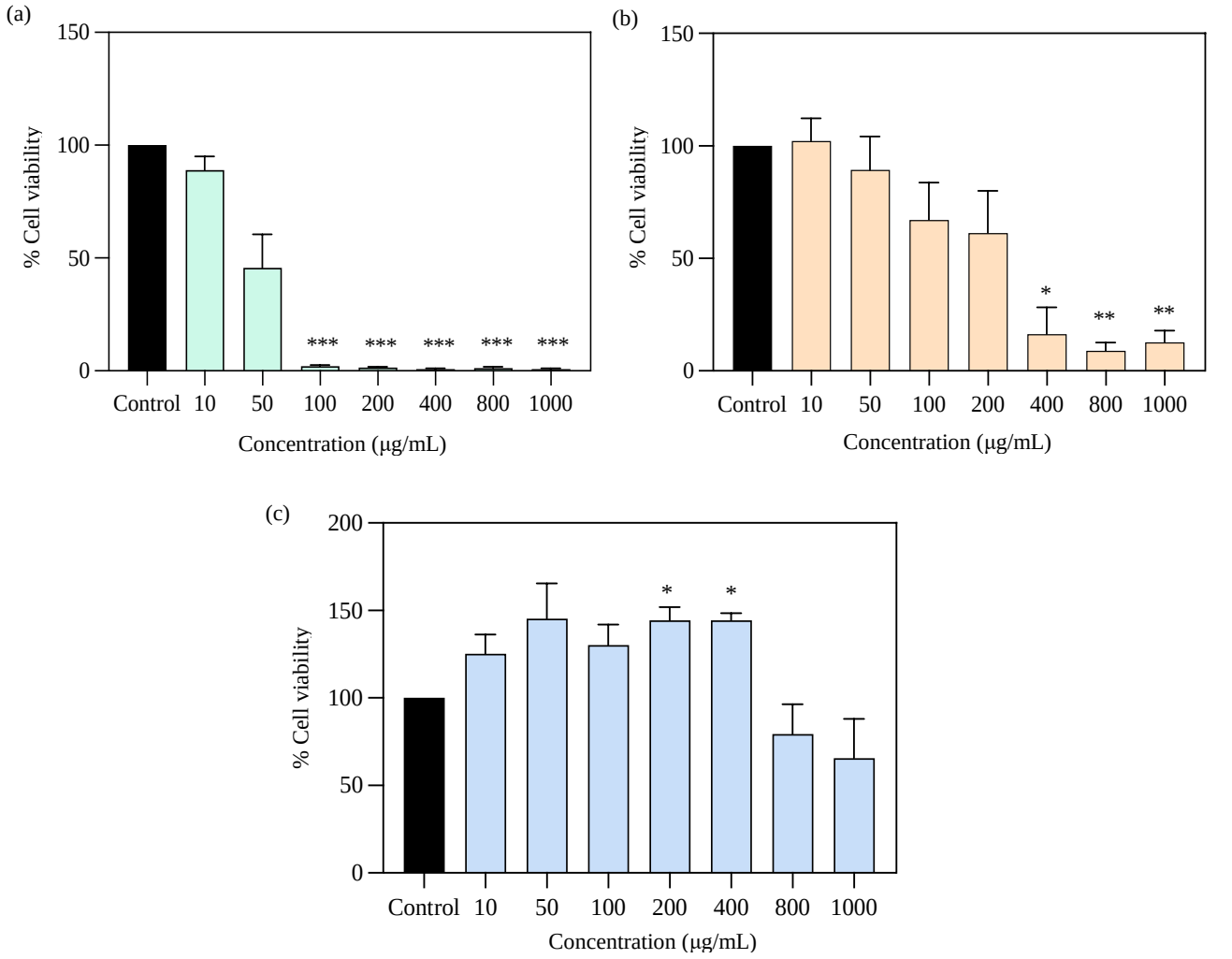

Figure 4. Cell viability of ethyl acetate extracts of CT2-10 (a), ET3-23 (b) and NE1-12 (c) on RAW264.7 macrophage cells. Data are presented as mean ± SEM (n=3). *p<0.05, **p<0.01, ***p<0.001 compared to the control (DMSO).

3.8 Inhibition of intracellular ROS generation

CT2-10 displayed ROS production ranging from 99% to 88% at concentrations of 0.1 µg/mL to 10 µg/mL (Figure 5(a)). Strain NE1-12 demonstrated a significant reduction in ROS production, ranging from 91% to 38% across a concentration range of 0.1 µg/mL

to 200 µg/mL (Figure 5(a)). Strain NE1-12 showed 74% inhibition of ROS production induced by hydrogen peroxide, with an IC_{50} value of 1.64 µg/mL (Figure 5(b)). Similarly, ET3-23 exhibited 67% inhibition of ROS production, with IC_{50} value 1.41 µg/mL (Figure 5(b)). ET3-23 efficiently suppressed H_2O_2-induced ROS production, as indicated by lower DCF fluorescence intensity. The reduction in ROS production observed in ET3-23 suggested its antioxidant potential in inhibiting radical release, possibly attributed to phenolic compounds isolated by ethyl acetate solvent.

When exposed to H_2O_2, elevated ROS levels reached 119±2.7% compared to the control cells. Leirós et al. (2014) found that compounds B and E from a *Streptomyces* strain exhibited significant reductions in ROS levels at a concentration of 1 µM in the presence of H_2O_2. Furthermore, compounds A, B, and C at a concentration of 0.1 µM noticeably decreased H_2O_2-induced ROS production while compound F, at a concentration of 0.01 µM, exhibited inhibition of H_2O_2-induced ROS production. These findings highlighted the potential of these compounds derived from *Streptomyces* to attenuate ROS generation under oxidative stress conditions (Leirós et al., 2014).

(a) Intracellular ROS production

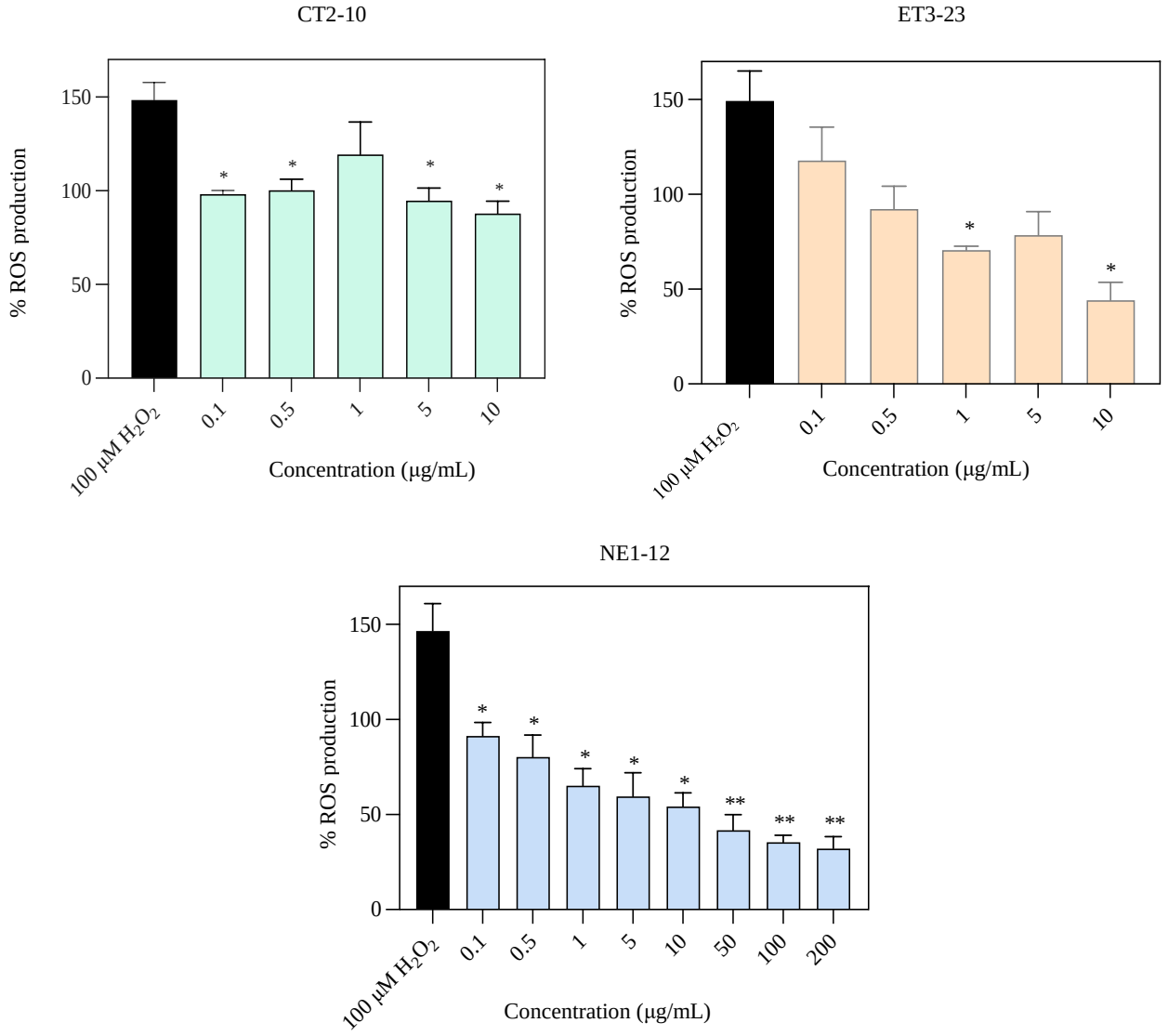

Figure 5. Effect of strains CT2-10, ET3-23 and NE1-12 on intracellular ROS production (a) and percentage inhibition (b) in H_2O_2 induced macrophage cells. Data are presented as mean±SEM (n=3). *p<0.05, **p<0.01 compared to the control (H_2O_2).

(b) Percentage inhibition of ROS production

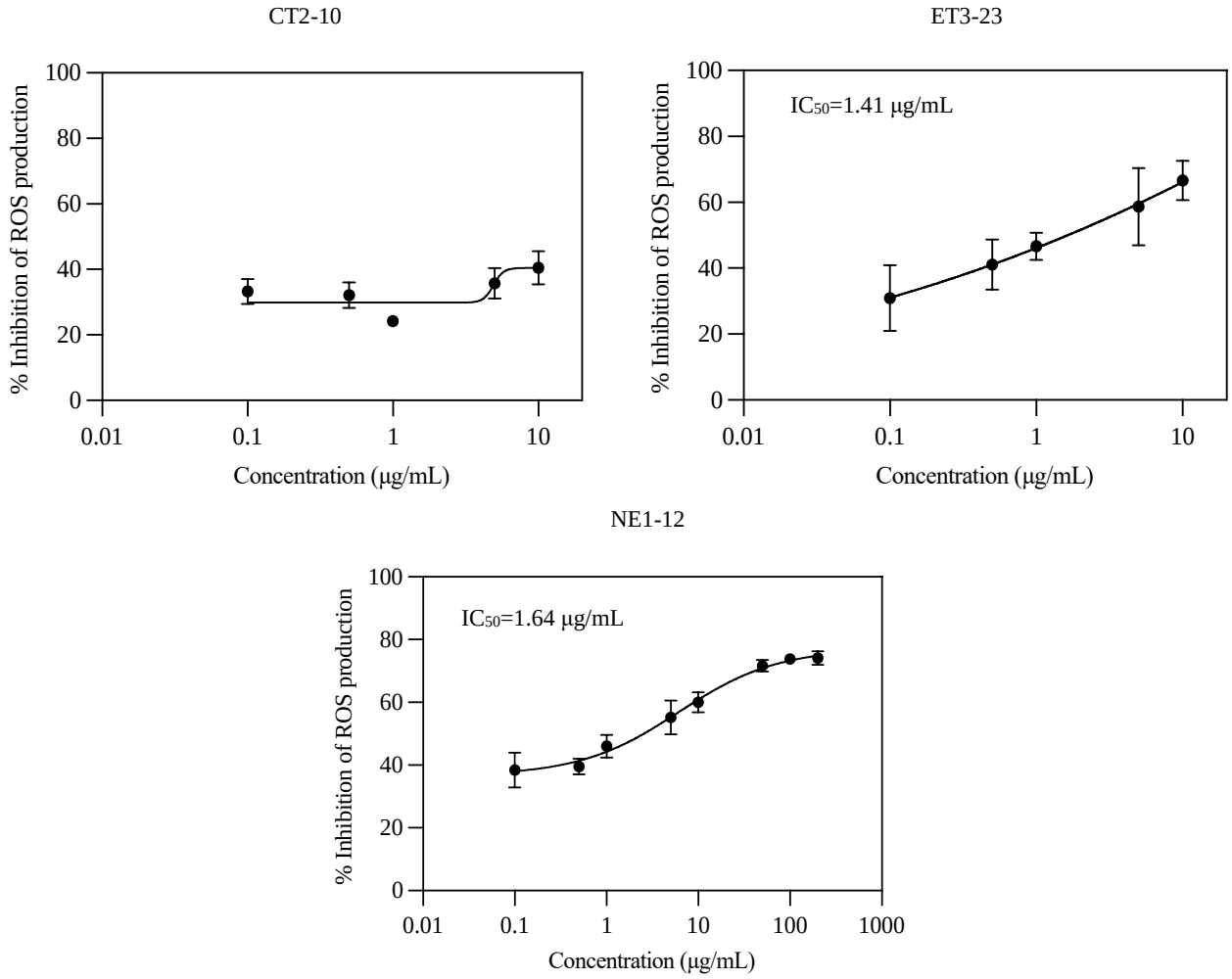

Figure 5. Effect of strains CT2-10, ET3-23 and NE1-12 on intracellular ROS production (a) and percentage inhibition (b) in H_2O_2 induced macrophage cells. Data are presented as mean±SEM (n=3). *p<0.05, **p<0.01 compared to the control (H_2O_2).

3.9 Inhibition of NO generation

Strains CT2-10 and NE1-12 reduced NO production across a range of concentrations (Figure 6A) and demonstrated IC50 values of 2.3 µg/mL and 82.4 µg/mL, respectively (Figure 6B). ET3-23 also showed 70% inhibition of NO production at 0.01 µg/mL. This reduction in NO generation was attributed to the antioxidant properties of these extracts which contributed to the decrease in nitrite levels. Notably, the low IC50 value of CT2-10 and ET3-23 suggested that some compounds within these extracts played a significant role in inhibiting oxygen radicals, indicating the potential of *Streptomyces*-derived compounds to treat diseases associated with excessive free radicals.

Nitric oxide is an important chemical mediator generated by various cell types including endothelial cells, macrophages, and neurons while NO plays a significant role in physiological processes such as vasodilation, neurotransmission, and immune response. However, excessive production of NO is associated with inflammatory reactions and can lead to cellular and tissue injury. In another study, geldanamycin from *Streptomyces* sp. W14 isolated from the rhizome tissue of *Zingiber zerumbet* (L.) Smith, inhibited the production of NO in LPS-induced RAW264.7 cells in a dose-dependent manner at concentrations of 1-5 µg/mL (Taechowisan et al., 2019). Similarly, Park et al. (2012) observed that dechlorothienodolin and thienodolin, isolated from *Streptomyces* sp. CNY325, inhibited NO production in LPS-stimulated RAW264.7 cells, suggesting their potential as anti-inflammatory and cancer chemoprevention agents (Park et al., 2012). These findings highlighted the ability of *Streptomyces*-derived compounds to modulate NO production which is crucial in inflammatory responses and tissue injury.

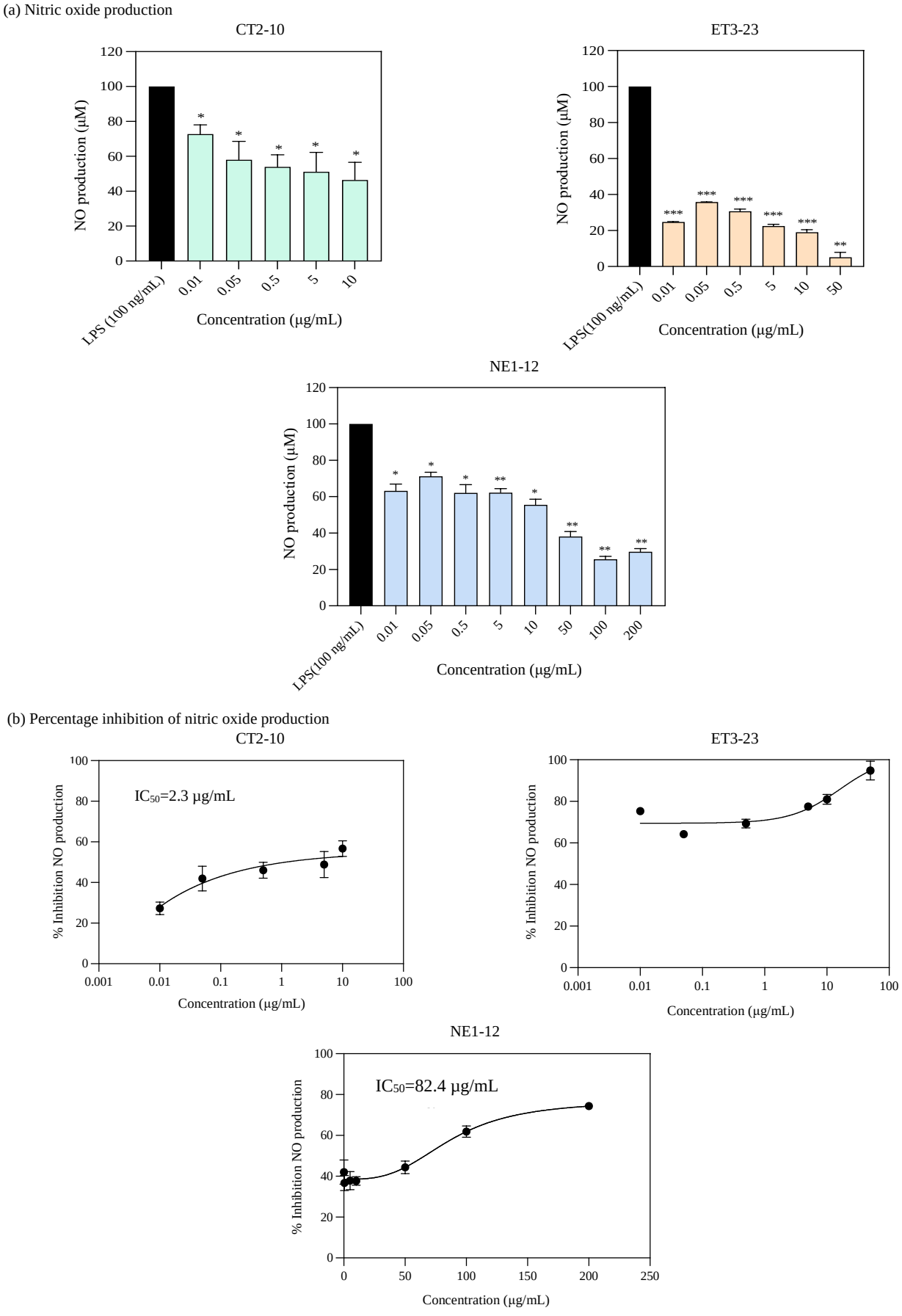

Figure 6. Effect of strains CT2-10, ET3-23 and NE1-12 on nitric oxide (NO) production (a) and percentage inhibition (b) in lipopolysaccharide induced RAW264.7 macrophage cells. Data are presented as mean±SEM (n=3). *p<0.05, **p<0.01 ***p<0.001 compared to the positive control (LPS).

3.10 Chemical profile analysis of secondary metabolites of *Streptomyces* strains

Chemical profile analysis of secondary metabolites from *Streptomyces* strains provides valuable information about the presence and quantity of various compounds, including phenolic compounds. UV-visible spectroscopy is a reliable method for quantifying phenolic compounds, renowned for their antioxidant properties. Different sub-families of phenolics are identified by their absorption maxima at specific wavelengths based on their ability to absorb UV light (Supplementary, Table S2). The absorbance at 280 nm is a key indicator for flavanol monomers, polymers, and some phenolic acids due to the phenolic ring's ability to absorb UV light, while hydroxycinnamic acids are quantified at 320 nm, flavonols at 360 nm, and anthocyanins at 520 nm (Aleixandre-Tudo and Du Toit 2019).

The chemical profiles of the secondary metabolites from *Streptomyces* strains were analyzed using UV-visible spectroscopy, with results presented in Figures S1, S2, and S3. These profiles provided insights into the presence and abundance of various compounds, particularly phenolic compounds. The presence of these compounds suggested that the crude extracts from *Streptomyces* strains contained potential antioxidant compounds. However, the compounds could not be identified using the in-house database. Thus, isolation and purification are necessary to identify the bioactive ingredient(s).

The effective antioxidant activity exhibited by *Streptomyces* strains highlights their potential as a valuable microbial source for drug discovery, particularly for developing effective antioxidant agents. Our findings provide insight into developing suitable candidates for pharmaceutical and bioactive natural products. Nevertheless, further investigation is needed to purify and determine the structures of the active components in the extracts.

4. CONCLUSION

This study evaluated the potential of soil-dwelling *Streptomyces* strains as natural sources of antioxidants. The strains CT2-10, NE1-12, and ET3-23 were identified and showed similarity to *Streptomyces capoamus* JCM 4734^T, *Streptomyces nigra* 452^T, and *Streptomyces morookaense* LMG 20074^T, respectively. For in vitro antioxidant activity, ET3-23 exhibited notable radical scavenging activity with an IC50 value of 66.5 µg/mL. ET3-23 and NE1-12 significantly reduced the generation of ROS in macrophage cells induced by hydrogen peroxide, while CT2-10, with an IC50 value of 2.3 µg/mL, effectively reduced lipopolysaccharide-induced NO generation. These findings suggested that *Streptomyces* strains possessed promising antioxidant properties, making them potential sources for bioactive natural products and the development of powerful antioxidants.

ACKNOWLEDGEMENTS

This study was supported by the 90th Anniversary of Chulalongkorn University Fund, Graduate School, Chulalongkorn University. The authors would like to thank the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University for providing research facilities.

REFERENCES

Aleixandre-Tudo JL, du Toit W. The Role of UV-Visible Spectroscopy for Phenolic Compounds Quantification in Winemaking [Internet]. 2019 [cited 2024 April 28]. Available from: http://dx.doi.org/10.5772/intechopen.79550.

Aranda A, Sequedo L, Tolosa L, Quintas G, Burello E, Castell JV, et al. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay: A quantitative method for oxidative stress assessment of nanoparticle-treated cells. Toxicology In Vitro 2013; 27(2):954-63.

Ayuso-Sacido A, Genilloud O. New PCR primers for the screening of NRPS and PKS-I systems in Actinomycetes: Detection and distribution of these biosynthetic gene sequences in major taxonomic groups. Microbial Ecology 2005;49(1):10-24.

Bajpai VK, Baek KH, Kang SC. Antioxidant and free radical scavenging activities of taxoquinone, a diterpenoid isolated from *Metasequoia glyptostroboides*. South African Journal of Botany 2017;111:93-8.

Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. Journal of Computational Biology 2012;19(5):455-77.

Bérdy J. Bioactive microbial metabolites. Journal of Antibiotics 2005;58(1):1-26.

Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT-Food Science and Technology 1995;28(1):25-30.

Chansriniyom C, Bunwatcharaphansakun P, Eaknai W, Nalinratana N, Ratanawong A, Khongkow M, et al. A synergistic combination of *Phyllanthus emblica* and *Alpinia galanga* against H_2O_2-induced oxidative stress and lipid peroxidation in human ECV304 cells. Journal of Functional Foods 2018;43:44-54.

Chen B, Liu Y, Song WM, Hayashi Y, Ding XC, Li WH. In vitro evaluation of cytotoxicity and oxidative stress induced by multiwalled carbon nanotubes in murine RAW 264.7 macrophages and human A549 lung cells. Biomedical and Environmental Sciences 2011;24(6):593-601.

EL-Kamali HH, Hassan HI, EL-Kheir MSM. Effect of soil physico-chemical properties and plant species on bacterial

diversity in semi-arid parts in Central Sudan. Part III: AL-Gaeli Region, Khartoun North. Open Access Library Journal.2017;4(3):1-11.

Gordon RE, Barnett DA, Handerhan JE, Pang CH-N. Nocardia coeliaca, Nocardia autotrophica, and the Nocardin strain. International Journal of Systematic Bacteriology 1974; 24(1):54-63.

Govindan P, Muthukrishnan S. Evaluation of total phenolic content and free radical scavenging activity of *Boerhavia erecta*. Journal of Acute Medicine 2013;3(3):103-9.

Hasani A, Kariminik A, Issazadeh K. *Streptomycetes*: Characteristics and their antimicrobial activities. International Journal of Advanced Biological and Biomedical Research 2014;2(1):63-75.

Hazra B, Sarkar R, Mandal S, Biswas S, Mandal N. Studies on antioxidant and antiradical activities of Dolichos biflorus seed extract. African Journal of Biotechnology 2009;8:3927-33.

Janardhan A, Kumar AP, Viswanath B, Saigopal DVR, Narasimha G. Production of bioactive compounds by Actinomycetes and their antioxidant properties. Biotechnology Research International 2014;2014:Article No. 217030.

Jung CH, Jun CY, Lee S, Park CH, Cho K, Ko SG. *Rhus verniciflua* stokes extract: Radical scavenging activities and protective effects on H_2O_2-induced cytotoxicity in macrophage RAW 264.7 cell lines. Biological and Pharmaceutical Bulletin 2006;29(8):1603-7.

Jung H, Kwak H-K, Hwang KT. Antioxidant and antiinflammatory activities of cyanidin-3-glucoside and cyanidin-3-rutinoside in hydrogen peroxide and lipopolysaccharide-treated RAW264.7 cells. Food Science and Biotechnology 2014;23(6):2053-62.

Kämpfer P. The family Streptomycetaceae, part I: Taxonomy. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-L, Stackebrandt E, editors. The Prokaryotes. Volume 3: Archaea Bacteria: Firmicutes, Actinomycetes. New York: Springer; 2006. p. 538-604.

Karthik L, Kumar G, Rao KVB. Antioxidant activity of newly discovered lineage of marine actinobacteria. Asian Pacific Journal of Tropical Medicine 2013;6(4):325-32.

Kato S, Shindo K, Kataoka Y, Yamagishi Y, Mochizuki J. Studies on free radical scavenging substances from microorganisms. II. Neocarazostatins A, B and C, novel free radical scavengers. Journal of Antibiotics 1991;44(8):903-7.

Kawahara T, Izumikawa M, Otoguro M, Yamamura H, Hayakawa M, Takagi M, et al. JBIR-94 and JBIR-125, Antioxidative phenolic compounds from *Streptomyces* sp. R56-07. Journal of Natural Products 2012;75(1):107-10.

Kelly KL, Judd DB. Inter-society color council. ISCC-NBS Color-name Charts Illustrated with Centroid Colors. Washington, DC: U.S. National Bureau of Standards; 1965.

Kim MC, Lee J, Kim DH, Son HJ, Heo MS. Isolation and identification of antioxidant producing marine-source actinomycetes and optimal medium conditions. Food Science and Biotechnology 2014;23(5):1629-35.

Lee DR, Lee SK, Choi BK, Cheng J, Lee YS, Yang SH, et al. Antioxidant activity and free radical scavenging activities of *Streptomyces* sp. strain MJM 10778. Asian Pacific Journal of Tropical Medicine 2014;7(12):962-7.

Leirós M, Alonso E, Sanchez JA, Rateb ME, Ebel R, Houssen WE, et al. Mitigation of ROS insults by *Streptomyces* secondary metabolites in primary cortical neurons. ACS Chemical Neuroscience 2014;5(1):71-80.

Liu Y, Su L, Fang Q, Tabudravu J, Yang X, Rickaby K, et al. Enzymatic reconstitution and biosynthetic investigation of the bacterial carbazole Neocarazostatin A. Journal of Organic Chemistry 2019;84(24):16323-8.

Meier-Kolthoff JP, Göker M. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. Nature Communications 2019;10(1):Article No. 2182.

Narendhran S, Rajiv P, Vanathi P, Sivaraj R. Spectroscopic analysis of bioactive compounds from *Streptomyces cavouresis* KUV39: Evaluation of antioxidant and cytotoxicity activity. International Journal of Pharmacy and Pharmaceutical Sciences 2014;6(7):319-22.

Park EJ, Pezzuto JM, Jang KH, Nam SJ, Bucarey SA, Fenical W. Suppression of nitric oxide synthase by thienodolin in lipopolysaccharide-stimulated RAW 264.7 murine macrophage cells. Natural Product Communications 2012;7(6):789-94.

Rammali S, Hilali L, Dari K, Bencharki B, Rahim A, Timinouni M, et al. Antimicrobial and antioxidant activities of *Streptomyces* species from soils of three different cold sites in the Fez-Meknes region Morocco. Scientific Reports 2022;12(1):Article No. 17233.

Sanjivkumar M, Babu DR, Suganya AM, Silambarasan T, Balagurunathan R, Immanuel G. Investigation on pharmacological activities of secondary metabolite extracted from a mangrove associated actinobacterium *Streptomyces olivaceus* (MSU3). Biocatalysis and Agricultural Biotechnology 2016;6:82-90.

Sarma AD, Mallick AR, Ghosh AK. Free radicals and their role in different clinical conditions: An overview. International Journal of Pharma Sciences and Research 2010;1(3):185-92.

Seemann T. Prokka: Rapid prokaryotic genome annotation. Bioinformatics 2014;30(14):2068-9.

Ser HL, Palanisamy UD, Yin WF, Abd Malek SN, Chan KG, Goh BH, et al. Presence of antioxidative agent, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- in newly isolated *Streptomyces mangrovisoli* sp. nov. Frontiers in Microbiology 2015;6:Article No. 854.

Shirling EB, Gottlieb D. Methods for characterization of *Streptomyces* species. International Journal of Systematic Bacteriology 1966;16:313-40.

Sowndhararajan K, Kang SC. Evaluation of in vitro free radical scavenging potential of *Streptomyces* sp. AM-S1 culture filtrate. Saudi Journal of Biological Sciences 2013;20(3):227-33.

Staneck JL, Roberts GD. Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. Applied Microbiology 1974;28(2):226-31.

Taechowisan T, Chaisaeng S, Phutdhawong WS. Antibacterial, antioxidant and anticancer activities of biphenyls from *Streptomyces* sp. BO-07: An endophyte in *Boesenbergia rotunda* (L.) Mansf A. Food and Agricultural Immunology 2017;28(6):1330-46.

Taechowisan T, Puckdee W, Phutdhawong W. Streptomyces zerumbet, a novel species from *Zingiber zerumbet* (L.) Smith and isolation of its bioactive compounds. Advances in Microbiology 2019;09:194-219.

Tamaoka J, Komagata K. Determination of DNA base composition by reversed-phase high-performance liquid chromatography. FEMS Microbiology Letters 1984;25(1):125-8.

Tan LT, Chan KG, Chan CK, Khan TM, Lee LH, Goh BH. Antioxidative potential of a *Streptomyces* sp. MUM292

isolated from mangrove soil. Biomed Research International 2018;2018:Article No. 4823126.

Vargas Gil S, Pastor S, March GJ. Quantitative isolation of biocontrol agents *Trichoderma* spp., *Gliocladium* spp. and actinomycetes from soil with culture media. Microbiological Research 2009;164(2):196-205.

Wai KZ, Luechapudiporn R, Tedsree N, Phongsopitanun W, Malisorn K, Tanasupawat S. *Streptomyces barringtoniae* sp. nov., isolated from rhizosphere of plant with antioxidative potential. International Journal of Systematic and Evolutionary Microbiology 2022;72(5):Article No. 005364.

Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, et al. Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. International Journal of Systematic and Evolutionary Microbiology 2017;67(5):1613-7.