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Cytotoxic potential of *Nephthea* sp.-derived actinomycetes supported by metabolomics analysis

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Abstract

Soft corals and associated microorganisms are known to produce leads for anticancer drugs. Keeping this in mind, *Nephthea* sp.; a Red Sea soft coral was investigated for the first time using the OSMAC approach. Two isolates, *Streptomyces* sp. UR63 and *Micrococcus* sp. UR67 were identified. Their extracts revealed the presence of alkaloids, macrolides, quinones, fatty acids, and terpenoids. Further comparison through a set of multivariate data analyses revealed their unique chemical profiles. The extracts displayed inhibitory potencies against HepG-2, Caco-2, and MCF-7 tumor cell lines with IC\textsubscript{50} values ranging from 11.4 to 38.7 μg/mL when compared with the positive control, doxorubicin. The study not only highlights the cytotoxic potential of soft coral-associated actinomycetes but also shows the advantage of using the OSMAC approach in this regard.

Keywords

Actinomycetes; Cytotoxic activity; Metabolic profiling; *Micrococcus*; *Nephthea*; OSMAC; *Streptomyces*.
1. Introduction

Marine environments are one of the most diverse ecosystems with a large assortment of different life forms (Huang et al., 2020). Various organisms occupying these environments have been appreciated as a prime source of valuable natural products with diverse structural features and therapeutic applications (Nie et al., 2018; Shaaban et al., 2021). Among them, soft corals belonging to the genus *Nephthea* have attracted considerable interest as a rich biochemical warehouse of several bioactive metabolites, e.g. steroids, terpenoids, quinones, nitrogenous compounds, and fatty acids that demonstrated a wide spectrum of biological properties, e.g. anti-inflammatory, anticancer, antimicrobial, anti-diabetic, and antifouling (Abdelhafez et al., 2019; 2020; 2021a,b). Coral-associated microbial aggregates are also another promising source of chemically varied compounds with antiviral, antibacterial, antiproliferative, and antifouling activities, among many others (Hou et al. 2019; Qin et al. 2021; Sang et al. 2019; Sharma et al., 2019). In this regard, research studies on different marine-derived *Streptomyces* species have reported the presence of a diverse pool of secondary metabolites of agricultural and medical value, e.g. antibacterial, antifungal, and antitumor effects (Cao et al., 2019; Luo et al., 2021; Peng et al., 2021; Yang et al., 2020). Likewise, marine bacteria of the genus *Micrococcus* have also provided a number of chemical entities with cytotoxic and antibacterial potential (Kumari et al., 2020). On the other hand, the one strain many compounds (OSMAC) approach has emerged as a privileged strategy for expanding drug discovery from microbial sources (Gamaleldin et al., 2020). This technique has been effectively applied to obtain a wide array of medicinally important microbial metabolites; allowing the opportunity to produce new chemical entities from a single microbial strain by culturing in different media under different culture conditions (Meng et al., 2017; Romano et al., 2018).

Cancer is ranked among the major life-threatening pathologies worldwide, with an increasing number of cases is reported annually as a result of environmental changes and life style modernization (Ferlay et al., 2021). Recent epidemiological studies have also indicated that lung and breast cancers are the most frequently diagnosed types and the leading triggers of cancer-related deaths in men and women, respectively (Johnson et al., 2021). To date, as potent marine supplies of anticancer leads, a large variety of natural metabolites with praiseworthy potential against different cancer cells has been reported from various *Nephthea* species (Abdelhafez et al., 2019); however, the chemical and biological traits of their associated bacteria has not been deliberated yet. Inspired by this, and as part of our ongoing research interest in the genus *Nephthea* (Abdelhafez et al., 2019; 2020; 2021a,b), the current study describes the isolation and identification of two actinomycetes
associated with the Red Sea soft coral *Nephthea* sp. for the first time, namely *Streptomyces* sp. UR63 and *Micrococcus* sp. UR67. The isolated bacterial strains were cultured using the OSMAC approach and their chemical profiles were explored by LC–MS-based metabolomics and compared by means of multivariate data analyses. The cytotoxic potential of the obtained bacterial extracts against a number of human cancer cells was also considered.

2. Results and Discussion

The fermentation of the two actinomycetes strains, *Streptomyces* sp. UR63 and *Micrococcus* UR67 was carried out using the solid approach on four different media (M1, ISP2, Malt, and agar with natural seawater), followed by extraction with ethyl acetate to afford eight different extracts. Various metabolites produced by the two strains under different culture conditions were investigated by LC–MS-based metabolomics, which showed the presence of diverse chemical classes like macrolides, alkaloids, quinones, fatty acids, and terpenoids (Table S1; Figures S1–S3). Different multivariate data analyses (MVDA) were then carried out using the raw MS data (Figures S4–S8), of which the Principal Component Analysis (PCA) was performed to highlight the differences and similarities of the resulting extracts (Figure S4). The obtained outliers were for the extracts of *Micrococcus* sp. on ISP2 and agar with natural seawater media (O3 and O4, respectively), indicating their unique chemical profiles. Therefore, the PCA scores plot revealed the variation of these culture extracts that was further confirmed by their unique patterns in the heat map plot (Fig. 1). Moreover, in the Partial Least Squares-Discriminant Analysis (PLS-DA), the Variable Importance in Projection (VIP) (Figure S8) demonstrated the metabolites responsible for such variations among these extracts; these compounds were further identified through the comparison with the MarinLit, DNP, and METLIN databases (Table S2; Figure S6). As a result, comparing different samples (O7–O10) of *Streptomyces* sp. UR63 indicated a high degree of similarity in the metabolic patterns of the samples O7, O9, and O10 (using agar and natural seawater, ISP2, and M1 media, respectively), whereas the extract O8 of *Streptomyces* sp. UR63 grown on Malt was the most different one. On the other hand, analysis of *Micrococcus* sp. UR67 extracts (O3–O6) showed the comparable metabolic profiles of O5 and O6 (using M1 and Malt media, respectively), while those of O3 and O4 (using ISP2 and agar with natural seawater media, respectively) were markedly different. Taken together, these findings highlighted the OSMAC approach as a powerful tool for the discovery of new microbial secondary metabolites.
On the other hand, investigating the cytotoxic potential of the resulting bacterial extracts against a number of tumor cell lines using the MTT viability assay showed that the crude extract of *Streptomyces* sp. UR63 cultured on agar with natural seawater exhibited potent inhibitory activities against MCF-7, Caco-2, and HepG-2 cells, with IC\(_{50}\) values of 11.4, 12.6, and 13.2 µg/mL, respectively, while the crude extract of *Micrococcus* sp. UR67 cultured on the ISP2 medium displayed moderate cytotoxic effects against the studied cell lines, showing IC\(_{50}\) values of 27.3, 20.4, and 17.5 µg/mL, respectively. However, the cytotoxic potential of different extracts was lower than that of the positive control, doxorubicin in terms of IC\(_{50}\) values (1.72, 2.1, and 1.3 µg/mL, respectively) (Table S3).

3. Conclusion

Metabolic profiling of the crude extracts of *Streptomyces* sp. UR63 and *Micrococcus* sp. UR67 derived from the soft coral *Nephthea* sp. showed their richness in several classes of metabolites, e.g. fatty acids, alkaloids, quinones, and terpenoids. Exploring the metabolic differences of these actinomycetes cultivated on different media revealed the evident potential of the applied OSMAC approach to induce the production of diverse microbial secondary metabolites. Besides, different extracts from both bacterial strains exhibited varying *in vitro* inhibitory potencies against HepG-2, Caco-2, and MCF-7 tumor cells, which also highlighted the importance of coral-associated actinomycetes as a prolific source of cytotoxic metabolites for the discovery of potential anticancer drug leads.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References


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Experimental

Soft coral collection

*Nephthea* sp. was collected by Prof. Safwat Ahmed, Professor of Pharmacognosy, Faculty of Pharmacy, Suez Canal University, Egypt using scuba diving from the Egyptian coasts of the Red Sea at Sharm El-Sheikh at a depth of 10 m and stored at −20 °C. The animal biomass was identified by Dr. Tarek Temraz, Marine Science Department, Faculty of Science, Suez Canal University, Ismailia, Egypt. A voucher specimen with the number SAA-26 was deposited in the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Suez Canal University.

Isolation of actinomycetes

The collected soft coral biomass was transferred to a plastic bag containing seawater in order to be transported to the laboratory. The soft coral specimens were then rinsed with sterile seawater, cut into pieces of *ca.* 1 cm³, and thoroughly homogenized in a sterile mortar with ten volumes of sterile seawater. The supernatant was diluted in ten-fold series (10⁻¹, 10⁻², and 10⁻³) and subsequently plated out on agar plates. Four different media were used for isolation of the actinobacteria, namely M1, ISP2, Oligotrophic medium (OLIGO), and Marine Agar (MA). All media were supplemented with 0.2 µm pore size filtered cycloheximide (100 µg/mL), nystatin (25 µg/mL), and nalidixic acid (25 µg/mL) to facilitate the isolation of slow-growing actinobacteria, where cycloheximide and nystatin inhibit fungal growth and nalidixic acid stops the growth of many of the fast-growing Gram-negative bacteria (Elsayed et al., 2018). All media contained Difco Bacto agar (18 g/L) and were prepared in 1 L of artificial sea water (NaCl 234.7 g, MgCl₂ • 6 H₂O 106.4 g, Na₂SO₄ 39.2 g, CaCl₂ 11.0 g, NaHCO₃ 1.92 g, KCl 6.64 g, KBr 0.96 g, H₃BO₃ 0.26 g, SrCl₂ 0.24 g, NaF 0.03 g, and dd H₂O to 10.0 L) (Shady et al., 2021). The inoculated plates were incubated at 30 °C for 6–8 weeks and distinct colony morphotypes were picked and re-streaked until visually free from contaminants. *Streptomyces* sp. UR63 and *Micrococcus* sp. UR67 were finally cultivated on the ISP2 medium and the isolates were maintained on plates for short-term storage and long-term strain collections in medium supplemented with 30% glycerol at −80 °C (Abdelmohsen et al., 2012).

Molecular identification and phylogenetic analysis

16s rRNA gene amplification, cloning and sequencing were performed according to Hentschel et al. (2001) using the universal primers 27F (Forward primer) and 1492R (Reversed primer). Chimeric sequences were identified by using the Pintail program. The genus-level affiliation of the sequences was confirmed using the Ribosomal Database Project Classifier. The genus-level identification of all the sequences was done with RDP Classifier (<g 16srrna, -f allrank>) and validated through the SILVA Incremental Aligner (SINA) (search and
classify options). An alignment was calculated again using the SINA web aligner (variability profile: bacteria). Gap-only positions were removed with trimAL (-noallgaps).

**Fermentation and extraction**

The two actinomycete strains, *Streptomyces* sp. UR63 and *Micrococcus* sp. UR67 were fermented using the solid approach. In the solid treatment, 150 μL of each strain were inoculated and streaked on ten solid plates of the following media: Malt (5 g peptone and 15 g agar), M1 (2 g peptone, 10 g dextrose, and 18 g agar), and ISP2 (4 g dextrose, 10 g Malt, and 18 g Agar). For each medium, the agar plates were cut into pieces and extracted with ethyl acetate (1L × 3) to afford four extracts for each strain, including M1 (extracts O10 and O5 for the strains UR63 and UR67, respectively), malt (extracts O8 and O6 for the strains UR63 and UR67, respectively), agar with natural seawater (extracts O7 and O4 for the strains UR63 and UR67, respectively), and ISP2 (extracts O9 and O3 for the strains UR63 and UR67, respectively).

**Metabolomics analysis**

Metabolomic profiling of the crude extracts of the two bacterial strains was carried out according to Abdelmohsen et al. (2014) using an Acquity Ultra Performance Liquid Chromatography system coupled to a Synapt G2 HDMS quadrupole time-of-flight hybrid mass spectrometer (Waters, Milford, USA). Ms converter software was used in order to convert the raw data into divided positive and negative ionization files. Additionally, the obtained files were then subjected to the data mining software MZmine 2.10 (Okinawa Institute of Science and Technology Graduate University, Japan) for deconvolution, peak picking, alignment, deisotoping, and formula prediction. The MarinLit (http://pubs.rsc.org/marinlit/), DNP (http://dnp.chemnetbase.com/faces/chemical/ChemicalSearch.xhtml), and METLIN (http://metlin.scripps.edu/index.php) databases were finally used for identification of the detected metabolites.

**Statistical and multivariate data analyses**

Statistical analyses were carried out by using MetaboAnalyst software on the different variables (Sayed et al., 2020). Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) were performed to reveal the differences in the metabolic composition of the examined samples. The peak intensity of all variables was log10 transformed. All variables were scaled to unit variance for PLS-DA derived from the LC-MS data sets. Each sample was measured in triplicate.

**Cytotoxic activity**

The cytotoxic activity of different extracts of *Streptomyces* sp. UR63 and *Micrococcus* sp. UR67 was tested against human hepatocellular carcinoma (HepG-2), breast cancer (MCF-7), and colon carcinoma (Caco-2) cell lines using MTT assay (Rasheed et al., 2017). Cell lines were obtained from the American Type Culture
Cells were cultured using DMEM (Invitrogen/Life Technologies, USA) supplemented with 10% FBS (HyClone, USA), 10 μg/mL of insulin (Sigma-Aldrich, Germany), and 1% penicillin-streptomycin. In 96-well plates, cells (at a density of 1.2–1.8 × 10,000 cells/well) were prepared in a volume of 100 µl/well of each of the complete growth medium and the tested sample at different concentrations (20, 30, 40, 50, and 60 μg/mL) for 24 h before testing. The MTT solution to be used was reconstituted with 3 mL of either the medium or balanced salt solution without phenol red and serum, and then added in an amount equal to 10% of the culture medium volume. Cultures were kept in an incubator for 2–4 hours depending on the cell type and maximum cell density (an incubation time of 2 h was mostly adequate, but was extended for low cell densities or cells with low metabolic activity). After incubation, cultures were removed from the incubator and the resulting formazan crystals were dissolved by adding an amount of DMSO equal to the original culture medium volume. The absorbance of each plate was then measured spectrophotometrically at 570 nm using an ELISA plate reader (Model 550, Bio-Rad, USA). Three independent experiments were carried out. IC$_{50}$ values were determined as the concentration that produces 50% inhibition of the growth of cells and were calculated by GraphPad Prism 5 (Version 5.01, GraphPad Software, San Diego, CA, USA). Doxorubicin (D1515, Sigma-Aldrich, Germany) was used as a positive control.

Results and discussion

In this work, two actinomycete strains, *Streptomyces* sp. UR63 and *Micrococcus* sp. UR67, were recovered from the Red Sea soft coral, *Nephthea* sp. and fermented on four different media (M1, ISP2, Malt, and agar with natural seawater), followed by extraction with ethyl acetate to provide eight different extracts. Different metabolites produced by the two strains under different culture conditions were explored by LC–MS-based metabolomics analysis as shown in the total ion chromatograms (Figures S3 and S4). The detected compounds (Table S1) were tentatively identified by employing macros and algorithms that coupled MZmine with online and in-house databases, e.g. MarinLit, DNP, and METLIN, in addition to the comparison with the reported literature data. As a result, several classes of metabolites (1–66) were characterized from the culture extracts of *Streptomyces* sp. UR 63, represented by 35 alkaloids and nitrogenous compounds, 14 macrolides, 7 terpenoids, 7 quinones and other oxygenated compounds, and 3 polyketides (Table S1; Figure S1). On the other hand, metabolic profiling of the crude extracts of *Micrococcus* sp. UR67 revealed a limited number of metabolites (Table S1; Figure S1), of which the mass ion peak at $m/z$ 239.164 was characterized as (6E,8Z)-5-oxo-6,8-tetradecadienoic acid (1a) with the molecular formula C$_{14}$H$_{22}$O$_{3}$. This unsaturated keto fatty acid was previously obtained from *Micrococcus* sp. associated with the coral *Catalaphyllia* sp. (Sharma et al., 2020). Nocarimidazole D (2a) was also dereplicated from the mass ion peak at $m/z$ 238.188 and the corresponding molecular formula C$_{13}$H$_{23}$N$_{3}$O. This alkanoylimidazole derivative (2a) was formerly reported from the coral *Mycedium* sp.-associated *Kocuria* sp. (Karim et al., 2020). The distribution pattern of the dereplicated
metabolites among different bacterial extracts was then compared by PCA (Figure S4), which was generated from five PCA components (PCs) and showed 93.4% of the total variation. Among them, the first and second PCs contributed separately to 83.3% of the total variation (PC1 and PC2 represented 69.6% and 13.7%, respectively), while the first, second, and third components accounted for 87.2% of the total variation (PC1, PC2 and PC3 represented 69.6% and 13.7%, and 3.9%, respectively). In the PCA loading plot (Figures S5 A and B), marked variations were observed among the extract samples where data were presented as distinct clusters. The differences and similarities among the ethyl acetate bacterial extracts were shown in the score scatter plot (Figure S5 C), in which the extracts of Micrococcus sp. on both ISP2 and agar with natural seawater media (O3 and O4, respectively) showed unique chemical profiles. Comparing the metabolic profiles of the obtained extracts revealed that all samples were positioned on the PC1 positive side except O3 and O4, confirming their distinct metabolic pools.

In the same manner, the Hierarchical Clustering Analysis (HCA) (Figure S7) showed the grouping of the studied samples into two main clusters, of which the first cluster of the dendogram proved the separation of the samples O3 and O4 from the other culture extracts, which is also in agreement with the results of the PCA scores plot. The first clustering was divided into two levels where O4 was at the first level, while O3 shared the second one. Moreover, the effect of the used culture media on the production of secondary metabolites was clearly demonstrated by the obtained dendrogram (Figure S7). In this respect, the samples O7, O9, and O10 (extracts of Streptomyces sp. UR 63 on agar with natural seawater, ISP2, and M1 media, respectively) were grouped together at the same level, suggesting their similar chemical profiles, whereas O8 (extract of Streptomyces sp. UR63 on Malt medium) occupied the second level, which denoted the distinctive metabolic pattern of that extract. On the other hand, regarding the samples of Micrococcus sp. UR67, the ethyl acetate extracts of Micrococcus sp. UR67 on M1 and Malt media displayed a large degree of metabolic similarity, which accounted for their grouping at the same level in the dendogram. In contrast, the samples O3 and O4 (extracts of Micrococcus sp. UR67 cultured on ISP2 and agar with natural seawater media, respectively) were markedly different. Results of the HCA can be visualized by colors using the heat map shown in Figure 1 that helps detect the highly, medium, and low expressed metabolites in different samples.

In the same framework, the PLS-DA was also carried out for further discrimination between the studied extracts (Figure S8), in which the model statistical parameters, the correlation coefficient R2, and the cross-validation correlation coefficient Q2 were higher than 0.8 using two components. As shown in the PLS-DA 2D scores plot (Figure S8), 82% of the total variation was explained by two PLS components (PCs), where the first and second PCs contributed to 55.5 and 26.5%, respectively. The PLS-DA derived heat map (Figure S8 B) highlighted the most 11 important features of the highest value identified by PLS-DA. The vertical axis showed the most 11 important features (m/z) in an ascending manner according to their scores that were plotted on the horizontal axis. The MarinLit, DNP, and METLIN databases were employed for annotation of the most important VIPs (Variable Importance in Projection) that were depicted in Table S2 and Figure S6.
The cytotoxic potential of the total ethyl acetate extracts of the two *Nephthea* sp. associated actinomycetes, *Streptomyces* sp. UR63 and *Micrococcus* sp. UR67 were evaluated against three different cell lines (HepG-2, Caco-2, and MCF-7) using the MTT cell viability assay. Overall, the tested samples revealed potent to moderate *in vitro* growth inhibitory potencies against HepG-2, Caco-2, and MCF-7 tumor cells, showing IC$_{50}$ values in the range of 11.4–38.6 µg/mL. As shown in Table S3, the total extract of *Streptomyces* sp. UR63 cultured on agar and natural seawater medium exhibited the highest cytotoxicity against HepG-2 cells, with IC$_{50}$ value of 13.2 µg/mL. Likewise, the total extracts of *Micrococcus* sp. UR67 and *Streptomyces* sp. UR63, both cultured on the ISP2 medium, showed moderate activities against the HepG-2 cell line, with IC$_{50}$ values of 17.5 and 19.4 µg/mL, respectively. In contrast, the extract of *Micrococcus* sp. UR67 cultured on agar with natural seawater exhibited weak cytotoxic effects against HepG-2 cells (IC$_{50}$ = 33.7 µg/mL), while the remaining extracts of both strains were inactive (IC$_{50}$ > 50 µg/mL). In the same connection, among the tested samples, the total extract of *Streptomyces* sp. UR63 using the agar and natural seawater medium exhibited the most potent cytotoxicity against MCF-7 cells, followed by that of *Streptomyces* sp. UR63 on ISP2, with IC$_{50}$ values of 11.4 and 23.6 µg/mL, respectively (Table S3). On the other hand, the total extracts of *Micrococcus* sp. UR67 grown on both ISP2 and agar with natural seawater media displayed moderate inhibition of MCF-7 cells, showing IC$_{50}$ values of 27.3 and 38.6 µg/mL, respectively. Additionally, the total extract of *Streptomyces* sp. UR63 using the agar and natural seawater medium exerted the highest cytotoxic effects against the Caco-2 cell line (IC$_{50}$ = 12.6 µg/mL). Correspondingly, the total extracts of *Micrococcus* sp. UR67 and *Streptomyces* sp. UR63 cultured on the ISP2 medium exhibited moderate activities against Caco-2 cells, with IC$_{50}$ values of 20.4 and 20.7 µg/mL, respectively (Table S3). On the contrary, the total extract of *Micrococcus* sp. UR67 cultured on agar with natural seawater showed weaker inhibitory potential against Caco-2 cells, with IC$_{50}$ value of 31.2 µg/mL, while the other extracts of both actinomycetes were inactive (IC$_{50}$ > 50 µg/mL). However, all the above-mentioned extracts showed lower cytotoxic potential as compared to the positive control, doxorubicin that revealed IC$_{50}$ values of 1.72, 2.10, and 1.30 µg/mL in the case of MCF-7, Caco-2, and HepG-2 cells, respectively (Table S3).

In the context of the anticancer potential of marine-derived actinomycetes, a number of metabolites with privileged cytotoxic potential were tentatively identified herein from the ethyl acetate extracts of *Streptomyces* sp. UR63 (as mentioned in Table S1), including 3-acetylamino-N-2-thienylpropanamide (3) that was dereplicated from the mass ion peak at *m/z* 213.065 in agreement with the molecular formula C$_3$H$_7$N$_2$O$_2$S. This compound was previously isolated from the mangrove soil derived *Streptomyces* sp. Q24 with antiproliferative properties against glioma cells (Ye et al., 2017). Moreover, the mass ion peak at *m/z* 225.148, consistent with the molecular formula C$_{13}$H$_{20}$O$_3$, was also identified as violapyrone B (4); an α-pyrone derivative earlier isolated from the marine-derived actinomycete, *Streptomyces* sp., which also revealed wide cytotoxic potential against a range of human cancer cell lines, namely HeLa, ACHN, HCT-15, HCT-116, MDA-MB-231, NCI-H23, NCI-H460, NUGC-3, HepG-2, and PC-3 (Shin et al., 2014). Similarly, usabamycin
A (20), which showed the molecular formula C_{16}H_{20}N_{2}O_{2} and was annotated from the mass ion peak at m/z 273.159, is an anthramycin-type analogue previously purified from the marine Streptomyces sp. NPS853 with weak growth inhibitory potential against HeLa cells (Sato et al., 2011). In the same vein, the mass ion peak at m/z 275.102 was annotated as venezueline B (21); a phenoxazine alkaloid formerly obtained from the marine bacterium, Streptomyces venezuelae. This metabolite was reported to exert moderate cytotoxic activities towards HCT-8, BGC-823, A549, A2780, and NIH3T3 tumor cells, as well as weak inhibitory potential against the human hepatoma Bel 7042 cells (Ren et al., 2013). Another antibiotic alkaloid with the molecular formula C_{19}H_{25}NO_{5} was characterized as kobutimycin A (33) based on the observed mass ion peak at m/z 348.180. This compound was also formerly identified from Streptomyces sp. TA3341 and showed notable cytotoxic effects against lymphoid leukemia L1210 cells (Kanbe et al., 1992). Likewise, the mass ion peak at m/z 443.097 was annotated as bireticulol (37); a dimeric isocoumarin with the molecular formula C_{22}H_{18}O_{10}, which was previously described from the terrestrial Streptomyces sp., exhibiting potent cytotoxic effects against KB and NCI-H187 cell lines (IC_{50}= 24.4 and 8.31 μg/mL, respectively) (Boonlarppradab et al., 2011). The mass ion peak at m/z 527.336 in consonance with the molecular formula C_{32}H_{46}O_{6} was also annotated as leptomycin A (46), which was formerly identified from Streptomyces sp. strain A92-30890 with antiproliferative potential against some human and mouse tumor cell lines. This compound was also reported to induce a reversible cell cycle arrest of rat 3Y1 fibroblasts in either the G1 or G2 phases (Wang et al., 1997). Additionally, clecarmycin C (49), dereplicated from the mass ion peak at m/z 551.154 and the corresponding molecular formula C_{29}H_{26}O_{11}, was also formerly isolated from the culture broth of Streptomyces sp., showing antiproliferative activities against human HeLa S3 cells (Fujii et al., 1995). The antibiotics clecarmycins were also reported to exhibit noteworthy antitumor potential against leukemia P388 and sarcoma 180 cells in mice (Fujii et al., 1995). Finally, the mass ion peak at m/z 559.275 was dereplicated as pactamycin (50) with the molecular formula C_{28}H_{38}N_{4}O_{8} that was earlier obtained as an antitumor and antibiotic metabolite from Streptomyces pactum (Bhuyan, 1962). The presence of such array of cytotoxic metabolites is therefore assumed to contribute to the observed antiproliferative potential of the studied extracts of Streptomyces sp. UR63 against MCF-7, Caco-2, and HepG-2 tumor cells. In view of that, the current findings highlight the capacity of the studied Nephthea sp.-derived actinomycetes, especially Streptomyces sp. UR63, as a promising source of alternative antitumor agents of natural origin.
Table S1. A list of the dereplicated metabolites from the investigated extracts of *Streptomyces* sp. UR 63 and *Micrococcus* sp. UR 67.

<table>
<thead>
<tr>
<th>No.</th>
<th>m/z</th>
<th>Exact mass</th>
<th>Molecular formula</th>
<th>Name</th>
<th>Source</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>205.086</td>
<td>204.078</td>
<td>C_{12}H_{12}O_{3}</td>
<td>7-Methoxy-2,3-dimethylchromone-4-one</td>
<td><em>S. rochei</em></td>
<td>Yu et al., 2019</td>
</tr>
<tr>
<td>2</td>
<td>208.097</td>
<td>207.089</td>
<td>C_{11}H_{10}NO_{3}</td>
<td>(R)-2-(1-Methyl-2-oxo-propylamino)benzoic acid</td>
<td><em>Streptomyces</em> sp.</td>
<td>Shaaban et al., 2017</td>
</tr>
<tr>
<td>3</td>
<td>213.065</td>
<td>212.061</td>
<td>C_{9}H_{12}N_{2}O_{2}S</td>
<td>3-Acetylamino-N-2-thienylpropanamide</td>
<td><em>Streptomyces</em> sp.</td>
<td>Ye et al., 2019</td>
</tr>
<tr>
<td>4</td>
<td>225.148</td>
<td>224.141</td>
<td>C_{13}H_{20}O_{3}</td>
<td>Violapyrone B</td>
<td><em>Streptomyces</em> sp.</td>
<td>Shin et al., 2014</td>
</tr>
<tr>
<td>5</td>
<td>227.117</td>
<td>226.110</td>
<td>C_{14}H_{12}N_{2}O</td>
<td>Prothracarcin</td>
<td><em>S. umbrosus</em></td>
<td>Shimizu et al., 1982</td>
</tr>
<tr>
<td>6</td>
<td>231.112</td>
<td>230.105</td>
<td>C_{13}H_{14}N_{2}O_{2}</td>
<td>Streptodiketopiperazine B</td>
<td><em>Streptomyces</em> sp.</td>
<td>Yi et al., 2020</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>11</td>
<td>247.132</td>
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<td><em>S. spinover-rucosus</em></td>
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<td>12</td>
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<tr>
<td>13</td>
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<td>Ding et al., 2012</td>
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<td>Huang et al., 2015</td>
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<td>34</td>
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<tr>
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<td>36</td>
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<td>38</td>
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<td>Merochlorin C</td>
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</table>

**Additional Information:**

- **S. spinoverrucosus** by Fu et al., 2016
- **Streptomyces sp.** by Sato et al., 2011
- **S. venezuelae** by Ren et al., 2013
- **Streptomyces sp.** by Lorente et al., 2010
- **S. lividans** by Wang et al., 2000
- **Streptomyces sp.** by Aoyagi et al., 1992
- **S. albus** by Vétesy et al., 1994
- **Streptomyces sp.** by Kobayashi et al., 1994
- **S. hygroscopicus** by Chen et al., 2015
- **Streptomyces sp.** by Arima et al., 1972
- **S. griseus** by Tang et al., 2000
- **S. scabrisporus** by Zhang et al., 2009
- **S. pulveraceus** by Kong et al., 2013
- **Streptomyces sp.** by Boonlappradab et al., 2011
- **S. tendae** by Bruntner et al., 1999
- **Streptomyces sp.** by Phillips et al., 1993
- **S. prunicolor** by Shin-ya et al., 1991
43  502.265  501.258  C\textsubscript{25}H\textsubscript{33}N\textsubscript{5}O\textsubscript{6}  Nobilamide F

44  518.260  517.253  C\textsubscript{25}H\textsubscript{35}N\textsubscript{5}O\textsubscript{7}  Plicacetin  \textit{S. plicatus}  Haskell et al., 1958

45  527.320  526.314  C\textsubscript{26}H\textsubscript{46}O\textsubscript{9}  Butyro lactol A  \textit{Streptomyces sp.}  Harunari et al., 2017

46  527.336  526.329  C\textsubscript{32}H\textsubscript{46}O\textsubscript{6}  Leptomycin A  \textit{Streptomyces sp.}  Wang et al., 1997

47  541.227  540.222  C\textsubscript{27}H\textsubscript{32}N\textsubscript{4}O\textsubscript{8}  Pyridomycin  \textit{S. pyridomyceticus}  Huang et al., 2011

48  550.229  549.222  C\textsubscript{28}H\textsubscript{46}O\textsubscript{9}  Butyrolactol A  \textit{Streptomyces sp.}  Harunari et al., 2017

49  551.154  550.148  C\textsubscript{29}H\textsubscript{26}O\textsubscript{11}  Clecarmycin C  \textit{Streptomyces sp. DO-114}  Fujii et al., 1995

50  559.275  558.268  C\textsubscript{28}H\textsubscript{38}N\textsubscript{4}O\textsubscript{8}  Pactamycin  \textit{S. pactum}  Bhuyan, 1962

51  564.245  563.238  C\textsubscript{32}H\textsubscript{46}O\textsubscript{9}  Saframycin Y3  \textit{S. lavendulae}  Arai et al., 1985

52  577.228  576.222  C\textsubscript{30}H\textsubscript{32}N\textsubscript{5}O\textsubscript{7}  Saframycin AD-1  \textit{S. lavendulae}  No. 314  Yazawa et al., 1986

53  578.260  577.253  C\textsubscript{30}H\textsubscript{35}N\textsubscript{5}O\textsubscript{7}  Saframycin YD-1  \textit{S. lavendulae}  No. 314  Yazawa et al., 1986

54  585.125  584.117  C\textsubscript{21}H\textsubscript{24}O\textsubscript{14}  Juglorescein  \textit{S. violaceus}  Hanada et al., 1991

55  639.408  638.403  C\textsubscript{35}H\textsubscript{58}O\textsubscript{10}  Kaimonolide B  \textit{Streptomyces sp.}  Hirona et al., 1990

56  651.411  650.403  C\textsubscript{36}H\textsubscript{58}O\textsubscript{10}  Flavofungin I  \textit{Streptomyces sp.}  Wang et al., 2017

57  685.451  684.445  C\textsubscript{37}H\textsubscript{60}O\textsubscript{11}  Monensin C  \textit{S. cinnamonomensis}  Liu and Reynolds, 1999

58  686.281  685.273  C\textsubscript{35}H\textsubscript{43}NO\textsubscript{13}  Auramycin C  \textit{Streptomyces sp.}  Kantola et al., 2000

59  688.428  687.420  C\textsubscript{35}H\textsubscript{61}NO\textsubscript{12}  Oleandomycin  \textit{Streptomyces sp.}  Vilches et al., 1990

60  689.387  688.382  C\textsubscript{36}H\textsubscript{56}O\textsubscript{11}  Reveromycin E  \textit{Streptomyces sp.}  Fremlin et al., 2011

61  700.427  699.420  C\textsubscript{37}H\textsubscript{57}N\textsubscript{5}O\textsubscript{8}  Tumescenamide A  \textit{S. tumescens}  Motohashi et al., 2010

62  701.372  700.367  C\textsubscript{35}H\textsubscript{60}O\textsubscript{14}  Chalcomycin  \textit{Streptomyces sp.}  Asolkar et al., 2002

63  720.292  719.286  C\textsubscript{40}H\textsubscript{46}ClNO\textsubscript{9}  Naphthomycin A  \textit{Streptomyces sp.}  Lu and Shen, 2007

64  836.444  835.447  C\textsubscript{43}H\textsubscript{61}N\textsubscript{5}O\textsubscript{10}  Nobilamide B  \textit{Streptomyces sp.}  Lin et al., 2011

65  821.452  820.460  C\textsubscript{44}H\textsubscript{60}O\textsubscript{14}  Tartrolon D  \textit{Streptomyces sp.}  Pérez et al., 2009
**Figure S1.** Chemical structures of the dereplicated metabolites from the extracts of *Streptomyces* sp. UR 63 and *Micrococcus* sp. UR 67.
Figure S1: Cont.
Figure S1: Cont.
Figure S1: Cont.

Figure S2. Total ion chromatogram of the crude extracts of *Streptomyces* sp. UR 63 cultured on different media.

A (O7): *Streptomyces* sp. on agar and seawater medium  
B (O8): *Streptomyces* sp. on Malt medium  
C (O9): *Streptomyces* sp. on ISP2 medium  
D (O10): *Streptomyces* sp. on M1 medium
Figure S3. Total ion chromatogram of the crude extracts of *Micrococcus* sp. UR 67 cultured on different media.

A (O4): *Micrococcus* sp. on agar and seawater medium
B (O6): *Micrococcus* sp. on Malt medium
C (O3): *Micrococcus* sp. on ISP2 medium
D (O5): *Micrococcus* sp. on M1 medium

Figure S4. PCA pairwise score plot of the unsupervised method (A) and PCA scree plot of the unsupervised method (B).
Figure S5. Metabolomics multivariate analysis: 2D PCA scores plot of the unsupervised method (A), 3D PCA scores plot of the unsupervised method (B), and PCA loading plot (C). (O1, O2, O11, and O12 represent the control media).
### Table S2. A list of the dereplicated outlier metabolites from the heat map.

<table>
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<tr>
<th>No.</th>
<th>m/z</th>
<th>Accurate mass</th>
<th>Molecular Formula</th>
<th>Name</th>
<th>Source</th>
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<td>1</td>
<td>313.107</td>
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<td>C$<em>{18}$H$</em>{16}$O$_5$</td>
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<td>2</td>
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<td>3',4',5,7-Tetrahydroxy isoflavone; 3'-O-α-L-rhamnopyranoside</td>
<td><em>S. diannanensis</em></td>
<td>Dong et al., 2005</td>
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<td>326.115</td>
<td>C$<em>{19}$H$</em>{18}$O$_5$</td>
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<td>Hori et al., 1993</td>
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<td>4</td>
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<td>309.168</td>
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<td><em>Streptomyces sp.</em></td>
<td>Bu et al., 2014</td>
</tr>
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<td><em>S. xiamenensis</em></td>
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<td>249.148</td>
<td>248.152</td>
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<td>Bohemamine H</td>
<td><em>S. spinoverrucosus</em></td>
<td>Fu et al., 2016</td>
</tr>
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<td>9</td>
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<td>229.110</td>
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<td>5-(3-Methyl-2-butenyl)-1H-indole-3-carboxylic acid</td>
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<td>Xiamycin B</td>
<td><em>Streptomyces sp.</em></td>
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<td>(-)-Biemamide D</td>
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Figure S6. Chemical structures of biomarker metabolites of different extracts of *Streptomyces* sp. UR 63 and *Micrococcus* sp. UR 67 on different culture media as obtained from the heat map.

Figure S7. HCA plot shown as a dendogram.
**Figure S8.** Metabolomics multivariate analysis: PLS-DA scores plot (A); VIP score plot of PLSDA (B).

**Table S3.** Cytotoxic activities of different ethyl acetate extracts of *Streptomyces* sp. UR 63 and *Micrococcus* sp. UR 67 and doxorubicin.

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<th>Extract</th>
<th>(the corresponding media)*</th>
<th>HepG-2 IC$_{50}$ (µg/mL)</th>
<th>Caco-2 IC$_{50}$ (µg/mL)</th>
<th>MCF-7 IC$_{50}$ (µg/mL)</th>
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<td>&gt; 50</td>
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<td>A+NS control</td>
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<td>&gt; 50</td>
<td>&gt; 50</td>
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<td></td>
<td>&gt; 50</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
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<tr>
<td><em>Streptomyces</em> sp. UR 63 (A+NS)</td>
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<td><strong>13.2</strong></td>
<td><strong>12.6</strong></td>
<td><strong>11.4</strong></td>
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<td><em>Streptomyces</em> sp. UR 63 (Malt)</td>
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<td>&gt; 50</td>
<td>&gt; 50</td>
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<tr>
<td><em>Streptomyces</em> sp. UR 63 (ISP2)</td>
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<td><strong>19.4</strong></td>
<td><strong>20.7</strong></td>
<td><strong>23.6</strong></td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. UR 63 (M1)</td>
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<td>&gt; 50</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
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<tr>
<td><em>Micrococcus</em> sp. UR 67 (ISP2)</td>
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<td><strong>17.5</strong></td>
<td><strong>20.4</strong></td>
<td><strong>27.3</strong></td>
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<tr>
<td><em>Micrococcus</em> sp. UR 67 (A+NS)</td>
<td></td>
<td><strong>33.7</strong></td>
<td><strong>31.2</strong></td>
<td><strong>38.6</strong></td>
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<tr>
<td><em>Micrococcus</em> sp. UR 67 (M1)</td>
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<td>&gt; 50</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
<tr>
<td><em>Micrococcus</em> sp. UR 67 (Malt)</td>
<td></td>
<td>&gt; 50</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
</tr>
<tr>
<td>Doxorubicin (positive control)</td>
<td></td>
<td>1.30</td>
<td>2.10</td>
<td>1.72</td>
</tr>
</tbody>
</table>

*(A+NS) refers to the medium containing agar with natural seawater.
References


