Metabolomic profiling and biological evaluations of *Spongia irregularis*-associated actinomycetes supported by multivariate statistical analysis

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Running head: *Spongia irregularis*-associated actinomycetes.

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Abstract:

Aim: Metabolomics analysis using LC-HRESIMS of twelve extracts of *Spongia irregularis* associated actinomycetes for dereplication purposes in addition to evaluation of cytotoxic and antiviral activities of the extracts.

Methods and Results: In the present study, three actinomycetes belonging to the genera *Micromonospora*, *Streptomyces*, and *Rhodococcus* were recovered from the marine sponge *Spongia irregularis*. Applying OSMAC approach, each strain was fermented on four different media resulting in twelve extracts. All extracts were subjected to metabolomics analysis using LC-HRESIMS for dereplication purposes. Multivariate data statistical analysis was carried out for the differentiation between extracts. Additionally, the cytotoxic and anti-HCV potentials of extracts were evaluated. Most of extracts showed strong to moderate cytotoxicity effects against HepG-2, CACO-2 and MCF-7 cell lines with general IC_{50} range of 2.8-8.9 µg/mL. Moreover, the extracts of *Micromonospora* sp. UR44 using ISP2 and OLIGO media and *Streptomyces* sp. UR32 using ISP2 medium exhibited anti-HCV activity with IC_{50} of 4.5±0.22, 3.8±0.18 and 5.7±0.15 µM, respectively.

Conclusion: Metabolomic analysis of twelve extracts of *Spongia irregularis* associated actinomycetes led to identification of large number of secondary metabolites. Morever, investigation of cytotoxic and antiviral activities of the extracts revealed that only three extracts exhibited antiviral activity and 7 extracts exhibited cytotoxic activity.

Significance and impact of the study: Different biological responses of the extracts are attributed to the different composition of media constituents which seriously affect

the production of the bioactive metabolites. This can be considered as an efficient approach for microbial strain selection for further investigations and drug discovery.

Keywords: Sponges, Actinomycetes, Metabolomics, PCA, Hierarchical Clustering, Cytotoxic, Antiviral.

Introduction:

Sponges are considered as hosts for wide varieties of microorganisms, which represent a significant percentage of the biomass of the sponge body. The sponge-associated microorganisms could in many cases contribute in the biosynthesis of sponge-derived bioactive metabolites (Kiran et al. 2018). Some of these metabolites contribute to the chemical defence against predation in their habitat, overgrowth by fouling organisms or competition for space. Additionally, many of them have exhibited multiple biological activities (Máximo et al. 2016).

The isolation of different genera of actinomycetes was previously reported from various marine environmental forms, but the majority was isolated from sponges (Abdelmohsen et al. 2014). The Secondary metabolites produced from actinomycetes were most reported for their structural diversity and novelty. Additionally, they exhibited different biological activities (Juliana et al. 2014).

Metabolomics is considered a highly valuable tool for analysis of the natural products under definite conditions. This technique aimed for the rapid identification of known metabolites in samples using gas or liquid chromatography combined with HRESIMS or NMR spectroscopic methods

(Macintyre et al. 2014). Additional metabolomics statistical analysis using MetaboAnalyst 4.0 web server can enrich results, support batch processing to provide improved pathway interpretation and facilitate detection of variations between samples (Chong et al. 2018).

Sponge-associated actinomycetes were reported as a rich source of bioactive compounds with antitumor properties from different structural classes. Different mechanisms were reported for their cytotoxic action such as directly targetting DNA, inducing cell cycle arrest, inducing apoptosis and other mechanisms (Kowalski et al. 2020).

In addition, hepatitis C virus (HCV) infection is a challenging public health problem in Egypt. Chronic HCV infection can develop several complications such as cirrhosis, hepatocellular carcinoma and may lead to mortality. Many strategies were applied in order to achieve the target of elimination of HCV infections in Egypt by 2030 (El-Ghitany 2019). In the current study, three marine sponge-associated actinomycetes were isolated, grown on different media using OSMAC approach and exposed to metabolomics analysis using LC-HRESIMS and multivariate data statistical analysis. Additionally, cytotoxic and anti-HCV activities were evaluated.

Materials and Methods:

Bacterial isolation, fermentation and extract preparation:

Strain isolation:

The marine sponge *Spongia irregularis* was collected at a depth of 10 m in the Red Sea (Ras Mohamed, Sinai, Egypt; GPS: 27˚47.655 N; 34˚12.904 W). Sponge specimens were

rinsed in sterile seawater, cut into pieces of ca. 1 cm^3, and then thoroughly homogenized in a sterile mortar with 10 volumes of sterile seawater. The supernatant was diluted in ten-fold series (10^{-1}, 10^{-2}, 10^{-3}) and subsequently plated out on agar plates. Four different media [M1 (Mincer et al. 2005), ISP medium 2 (Shirling and Gottlieb 1966), oligotrophic medium (OLIGO) (Olson et al. 2000), and marine agar (MA) (Reasoner and Geldreich 1985) were used for the isolation of actinobacteria. 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. Cycloheximide and nystatin inhibit fungal growth, while nalidixic acid inhibits many fast-growing Gram-negative bacteria (Webster et al. 2001). All media contained Difco Bacto agar (18 g/L) and were prepared in 1 L artificial sea water (NaCl 234.7 g, $MgCl_2.6\ H_2O$ 106.4 g, Na_2SO_4 39.2 g, $CaCl_2$ 11.0 g, $NaHCO_3$ 1.92 g, KCl 6.64 g, KBr 0.96 g, H_3BO_3 0.26 g, $SrCl_2$ 0,24 g, NaF 0.03 g and ddH_2O to 10.0 L) (Lyman and Fleming 1940). The inoculated plates were incubated at 30°C for 6–8 weeks. Distinct colony morphotypes were picked and re-streaked until visually free of contaminants. *Rhodococcus* sp. UR21, *Streptomyces* sp. UR32 and *Micromonospora* sp. UR44 were cultivated on ISP2 medium. The isolates were maintained on plates for short-term storage and long-term strain collections were set up in medium supplemented with 30% glycerol at −80°C.

Molecular Identification and Phylogenetic Analysis:

16SrRNA gene amplification, cloning and sequencing were performed according to (Hentschel et al. 2001) using the universal primers 27F and 1492R (Lane 1991). Chimeric sequences were identified by using the Pintail program (Ashelford et al. 2005). The genus-level affiliation of the sequence was validated using the Ribosomal Database Project

Classifier (Wang et al. 2007). The genus-level identification of all the sequences was done with RDP Classifier (-g 16SrRNA, -f allrank) and validated with the SILVA Incremental Aligner (SINA) (search and classify option).

Extract Preparation:

The three actinobacterial strains, *Rhodococcus* sp. UR21, *Streptomyces* sp. UR32 and *Micromonospora* sp. UR44, were cultured in 250 mL Erlenmeyer flasks containing 150 mL of 4 different production media (M1, ISP2, OLIGO, MA). The liquid cultures were grown for 10 days at 30 °C, while shaking at 150 rpm. The culture was then filtered, and supernatant was extracted with ethyl acetate while the cells and mycelia were extracted by shaking with methanol for 4 hours. The ethyl acetate extract (about 20 mg, for each medium) and methanolic extract were stored at 4°C.

LC-HRMS Metabolomic Analysis and Dereplication:

The previously prepared extracts of actinomycetes were subjected to LC-HRESIMS metabolomics analysis as the following; the actinomycetes samples (1 mg/mL in MeOH) were analysed by an Accela HPLC (Thermo Fisher Scientific, Bremen, Germany) using an ACE C_{18}, 75 mm × 3.0 mm, 5 μm column (Hichrom Limited, Reading, UK) attached to Accela UV-visible and Exactive (Orbitrap) mass spectrometer (Thermo Fisher Scientific). The gradient elution technique was employed at 300 μL/min using purified water [total organic carbon (TOC) was 20 ppb] and acetonitrile, each containing 0.1% formic acid. The elution was started with 10% acetonitrile, and then gradually increased to 100% acetonitrile within 30 min, followed by an isocratic period of 5 min before a gradient decrease to 10%

acetonitrile for 1 min. The injection volume was 10 µL and the column was set at 20 °C. HRESIMS was provided in both negative and positive ionization modes with a spray voltage of 4.5 kV and capillary temperature of 320 °C. The ESI-MS mass range was assigned at *m/z* 100–2000 for ESI-MS using in-source CID (collision-induced dissociation) mechanism and *m/z* 50–1000 for MS/MS using untargeted HCD (high-energy collision dissociation). For differentiation of the HRMS data, the raw data were imported and analyzed in MZmine 2.12. Excel macros were also employed to dereplicate each *m/z* ion peak with metabolites in the METLIN and DNP databases, using retention time and m/z threshold of ±5 ppm, to achieve the putative identification of compounds.

Multivariate and statistical analysis:

MetaboAnalyst 4.0 is a web-based tool suite applied to perform downstream statistical analysis of metabolomic LC-MS data.(Chong et al. 2018). This exploration requires an input file contains a table with sample name, peak list (m/z) and peak intensities exported as comma-separated values (.csv). MS peak list and intensities data should be uploaded as one zip file containing the (.csv) files. The data quality was checked and normalized by sum, log transformation and auto scaling. Then, statistical analysis was performed using unsupervised principal components analysis (PCA) as a multivariate method of analysis and hierarchical cluster analysis (HCA) methods (Chong et al. 2018) and (Demarque et al. 2020).

Cytotoxic activity:

The cytotoxic activities of the total extracts of *S. irregularis*-associated actinomycetes were evaluated against CACO-2 (colon carcinoma), HepG-2 (hepatocellular carcinoma) and MCF-7 (breast carcinoma) cell lines. All cytotoxic activities were evaluated using the MTT

cell viability assay (Mosmann 1983). Cells were cultured in DMEM high glucose with 15% FBS as background medium at 5% CO_2 and 37 °C. Cells were plated in 96-well plate with cell density 22 x 10^4cell/cm^2 overnight. The total extracts of *S. irregularis*-associated actinomycetes were dissolved in DMSO and then added to the cells at 20, 30, 40, 50 and 60 µg/mL. After 24 h, cell viability was assessed using MTT assay. Briefly, cells were incubated with 150 µL 0.5 mg/mL MTT per well for 3 h at 37 °C, then formed crystals were dissolved by incubation with 150 µL DMSO per well at 37°C for one hour. Absorbance was measured using a microplate reader at 570 nm. Viability baseline was adjusted using DMSO then dose response curve was established and percent inhibition and IC_{50} were calculated for each sample for the different three cell lines compared to the standard drug (Doxorubicin).

Anti-HCV activity:

HCV replicon were inoculated at 26x10^4 cells (per well in a 48-well plate) 24 h prior to the experiment (Reblikon, Mainz, Germany). The total extracts of *S. irregularis* associated actinomycetes were added to the culture broth at different concentrations (1-200 µM). After 72 h, the treated cells were harvested and lysed in cell culture lysis reagent. Luciferase activity was evaluated with a luciferase assay system and the resulting luminescence was detected by the luminescence plate reader (PerkinElmer) and corresponded to the expression level of the HCV replicon (Montefiori 2004). To monitor the cytotoxicity which could be attributed to some compounds in the samples, the cytotoxicity was measured by MTS assay (Hwang et al. 2004).

Results:

Metabolomic profiles of the cultures' extracts:

The bioactive metabolites produced by the symbiotic microorganisms are seriously affected by the environmental conditions, symbiotic interaction between sponges and associated microbial organisms and their functional genes (Kiran et al. 2018) and (Batista et al. 2018). Metabolomics is considered as an important field of research that is used as a characterization tool of small molecule metabolites in biological samples. Metabolomic studies were classified either as non-targeted or targeted approaches. The non-targeted approach was widely applied in fingerprinting of many medicinal and food plants (Wishart 2008).

In this context, the marine sponge-associated actinomycetes, *Rhodococcus* sp. UR21, *Streptomyces* sp. UR32 and *Micromonospora* sp. UR44, were allowed to grow on four different media (M1, ISP2, OLIGO and MA). The metabolomics profiling of the ethyl acetate extracts of the marine derived actinomycetes using LC–HRESIMS for dereplication purposes resulted in the characterization of a wide range of metabolites of different chemical classes. The detected phytochemicals were tentatively identified by searching the Dictionary of Natural Products (DNP), METLIN and Marinlit databases. In addition, they were also compared with the reported literatures. The tentatively identified secondary metabolites from the marine sponge-derived actinomycetes (*Rhodococcus* sp. UR21, *Streptomyces* sp. UR32 and *Micromonospora* sp. UR44) exhibited a great structural diversity which belong to polyketides, alkaloids, peptides, and terpenoids as demonstrated in **tables S1**, **S2** and **S3**, respectively. In addition, examples of these secondary metabolites are summarized in **Figures 1**, **2** and **3**, respectively.

Multivariate data analysis:

Worth mentioning that the codes of 12 extracts were labelled as (En 1-12) as the following; the actinomycete *Micromonospora* sp. UR44 (samples En 1-4) using those different media media (M1, ISP2, OLIGO, MA), respectively and this was the same for the actinomycete *Rhodococcus* sp. UR21 (samples En 9-12) and the actinomycete *Streptomyces* sp. UR32 samples (En 5-8)).

Principal Component Analysis (PCA):

Principal Component Analysis (PCA) is considered as an unsupervised method of analysis to explain the variance within a dataset by a smaller number of principal components (PCs). All of the PCs are constructed such that they are pairwise orthogonal (decorrelated) to each other and ordered according to the amount of variance they explain. The data are summarized into much fewer variables called scores which are weighted average of the original variables. The weighting profiles are called loadings, which contains the weights for the original variables to transform them into the scores. The main advantage of this method is its applicability of dimensional reduction (Bartel et al. 2013).

In the current metabolomics data analysis, 5 Principal-components (PCs) explained 88.5% of the total variation, in which the first and second PCs separately contributed 55.5% and 14%, respectively explaining the variance of the two main axes (**Figure 4**). The different extracts were mainly restricted to different areas, which indicated statistically significant differences between the samples.

By comparing the metabolite profiles of the samples, all extracts were observed to be positioned on the PC1 positive side except En 1 and En 7. Considering the main component PC2, En 2, En 4, En 5, En 8, En 10 and En 11 extracts were on the positive side while other

extracts were positioned on the negative side (**Figure 5A**). In PCA loading plot (**Figure 5B** and **5C).**

Hierarchical Clustering Analysis (HCA):

Hierarchical cluster analysis, each sample begins as a separate cluster and the algorithm proceeds to combine them until all samples belong to one cluster. Hierarchical clustering was performed considering two parameters using Euclidean distance, and ward clustering algorithm (Bouhmala 2016). Results of hierarchical clustering can be visualized by colors using the heat map (**Figure 6**). The significance of the heat map is to detect which metabolites are highly, medium or low expressed in certain samples specially the most expressed mass peaks. The clustering results of the heat map can also be translated to the dendrogram to make data clearer and ease detection of the variations between samples (**Figure 7**).

Investigation of biological activities of extracts of sponge associated actinomycetes:

Investigation of cytotoxic activity:

Concerning the cytotoxic activity of the 12 total extracts of marine sponge associated actinomycetes were evaluated against three different cell lines (HepG-2, CACO-2 and MCF-7 cell lines) using the MTT cell viability assay. Results summarized in **table 1** showed that the total extract of *Micromonospora* sp. UR44 using ISP2 medium (En2) exhibited high cytotoxicity against HepG-2, CACO-2 and MCF-7 cell lines with IC_{50} of 5.6, 7.9 and 8.9 µg/mL, respectively (**Figure 8A**) while the extracts of the same strains using M1, OLIGO or MA media were inactive.

Concerning *Streptomyces* sp. UR32, the total extract using M1 medium was inactive while extracts of other media showed different cytotoxicities against the three cell lines. It was notable that the extract of MA medium showed lowest values of IC_{50} of 6.3, 6.2 and 4.1 µg/mL against HepG-2, CACO-2 and MCF-7 cell lines, respectively (**Figure 8B**). Additionally, the total extract of *Rhodococcus* sp UR21 using M1 medium was inactive while extracts using other media showed high cytotoxicities against the three cell lines with IC_{50} values ranging from 2.8 to 5.8 µg/mL (**Figure 8C**). The various results for each strain could referred to using different media which consequently induced production of different metabolites. This was demonstrated in (**Tables S1-S3).**

Investigation of anti-HCV activity:

By investigating the anti-HCV activities of the total extracts of the marine sponge associated actinomycetes, the results showed that the total extract of *Micromonospora* sp. UR44 using ISP2 and OLIGO media exhibited anti-HCV activity with IC_{50} of 4.5 ± 0.22 µM and 3.8 ± 0.18 µM, respectively. Also, the total extracts of *Streptomyces* sp. UR32 using ISP2 medium exhibited anti-HCV activity with IC_{50} of 5.7 ± 0.15 µM. On the other hand, the other nine extracts were inactive against HCV where the IC_{50} values of the extracts exceeded 100 µM using cyclosporine as a positive control for inhibition of replication of hepatitis C virus.

Discussion:

Secondary metabolites derived from sponge associated actinomycetes were reported for both cytotoxic and antiviral activities. In the context of cytotoxic metabolites isolated from

actinomycetes, several cytotoxic metabolites were reported from the genus *Micromonospora* such as sibanomicin which showed significant in vivo antitumor activity in mice bearing leukemia P388 cells. Also, two indolocarbazole derivatives, 4'-N-methyl-5'-hydroxystaurosporine and 5'-hydroxystaurosporine showed a potent in vitro antiproliferative activity against a panel of human cancer cell lines as a result of inhibition of the protein kinase C (Hifnawy et al. 2020).

A review published by Jakubiec-Krzesniak *et al* reported a large number of cytotoxic metabolites, from which gilvocarcin HE isolated from the ethyl acetate extract of *Streptomyces* sp. QD01-2 showed a moderate cytotoxicity against MCF-7, K562 and P388 cell lines. Also, fradimycins A and B (new modified anthraquinones) from marine *Streptomyces fradiae* PTZ0025 displayed cytotoxicity towards human cancer cell lines. Citreamicin θA and citreamicin θB were reported from a marine *Streptomyces caelestis* strain and showed cytotoxicity against HeLa cells. This activity was correlated to the five-member nitrogen heterocycle in their structures (Jakubiec-Krzesniak et al. 2018).

Additionally, the extract of *Rhodococcus* sp. Lut0910 exhibited *in vivo* and *in vitro* antitumor activity. It showed cytotoxic effect against two human cancer cell lines, hepatocellular carcinoma HepG2 and cervical carcinoma Hela cells. Also, the oral administration of the extract to the mice with a solid tumor resulted in the inhibition of tumor growth compared to the control group (Zhang et al. 2017). Hifnawy *et al* published a review paper discussing biological activities of metabolites from the genus *Micromonospora* form which megalomicin C1 was reported for its antiviral activity against swine fever virus and herpes simplex virus type 1 (HSV-1) (Hifnawy et al. 2020). Also, metabolites derived from actinomycetes (especially *Streptomyces*) from

different sources were reported to exhibit antiviral activities against the Western equine encephalitis virus, porcine epidemic diarrhea virus, HIV-1 and other viruses (Jakubiec-Krzesniak et al. 2018). Additionally, a novel human adenoviral vector vaccine for the pathogenic *Rhodococcus equi* was developed and tested in the mouse model in order to prevent *R. equi* disease in foals (Giles et al. 2016).

The bioactive metabolites produced by the symbiotic microorganisms are seriously affected by the environmental conditions, symbiotic interaction between sponges and associated microbial organisms and their functional genes (Kiran et al. 2018) and (Batista et al. 2018). Metabolomics is considered as an important field of research that is used as a characterization tool of small molecule metabolites in biological samples. In addition, MetaboAnalyst 4.0 is a comprehensive web-based tool suite applied to enable users to easily perform downstream statistical analysis, visualization, and functional interpretation of metabolomic LC-MS data which were obtained using MZmine 2.12 as mentioned above. Also, it enables users to improve data quality and to detect variations between samples (Chong et al. 2018).

In this context, metabolomics profiling of the extracts of *Spongia irregularis* associated actinomycetes using LC–HRESIMS resulted in the characterization of large number of metabolites from different chemical classes. It was interesting that some metabolites were produced in extracts of different growth media where others were selectively produced using definite medium rather than other ones. As examples, the M1 medium of *Micromonospora* sp. UR44 could induce the production of 6,6'-dihydroxy-α,α'-diisocyano-[1,1'-biphenyl]-3,3'-dipropanamide and antibiotic SB 219383, where phallusialide A was selectively produced using MA medium. Also, 3-trehalosamine, aurachin *Q* and

leucylblasticidin S were selectively produced by *Rhodococcus* sp. UR21 using ISP2 medium, where the M1 medium could induce the formation of spirocardin A and nocazine A. In addition, allowing *Streptomyces* sp. UR32 to grow on M1 medium resulted in production of JBIR-57 and phenazostatin B, which were not expressed using other media. Using OLIGO medium revealed selective production of several metabolites such as sinefungin, cervicarcin, divergolide N and nitricquinomycin C. Additionally, parimycin, microtermolide A, kanamycin B, antibiotic FR 900109 and maltophilin were selectively produced using MA medium. This can support and confirm the role of growth media on the induction of the production of secondary metabolites through activation of the expression of their genes.

From PCA, It could be clearly demonstrated that there are variations between the extract samples where data were represented as distinct clusters. This is because some metabolites could act as chemotaxonomic markers for definite sample extracts. Thus, they are responsible for the variations among the samples. There are variations between the extract samples where data were represented as distinct clusters except some exact masses such as 280.263, 282.275, 294.154 and 999.785, which were not identified in the used databases, were significantly differed than other clusters. The variations between the extract samples were because some metabolites could act as chemotaxonomic markers for definite sample extracts. Thus, they are responsible for the variations among the samples.

According to the HCA dendrogram (**Figure 7**), it can be concluded that 12 extracts of actinomycetes are classified to two main clusters. One cluster contains only the sample En 1 (Extract of *Micromonospora* sp. UR44 using M1

338 medium) and all other samples in the second cluster. Thus, the extract En 1 is the
339 most different sample compared to all other ones. The effect of media on the
340 production of metabolites can be observed in the dendrogram. Considering the
341 actinomycete *Micromonospora* sp. UR44 (samples En 1-4), extracts EN 1 & 2
342 (using M1 & ISP2 media, respectively) are highly different where the extracts
343 En 3 & 4 (using OLIGO & MA media, respectively) are closely related to each
344 other.
345 Comparison of *Streptomyces* sp. UR32 samples (En 5-8) showed that there is a
346 high degree of similarity of metabolites of samples En 5, 7 & 8 (using M1,
347 OLIGO & MA media, respectively). On the other hand, the extract En 6 using
348 ISP2 medium was the most different one among the four extracts of
349 *Streptomyces* sp. UR32.
350 Considering *Rhodococcus* sp. UR21 (samples En 9-12), the four extracts could
351 be divided into two couples. The first couple contains the samples En 9 & 12
352 (using M1 & MA media, respectively) and the second one contains the samples
353 En 10 & 11 (using ISP2 & OLIGO media, respectively). Each couple of samples
354 showed high degree of similarity of their metabolites forming one cluster. The
355 variations between extracts explain the great effect of growth media in induction
356 of variable metabolites; hence highlight the power of OSMAC in the discovery
357 of microbial secondary metabolites.
358 Different biological responses of the extracts justify the role of media in elicitation of
359 different secondary bioactive metabolites. In our experiments, this can be clearly
360 observed where the ISP2 medium is the only one which induces the production of

cytotoxic metabolites in case of *Micromonospora* sp. UR44. On the other hand, the extracts of actinomycetes which were grown on M1 medium did not exhibit any cytotoxic activity. Also, ISP2 and OLIGO media can induce the production of anti-HCV secondary metabolites for the extracts of *Micromonospora* sp. UR44. Also, this could be achieved in case of *Streptomyces* sp. UR32 extracts using ISP2 medium.

Based on the above, marine sponge associated actinomycetes are rich sources for the production of new or novel bioactive metabolites. The biosynthesis of these metabolites depends on several factors such as environmental conditions, symbiotic association with their host sponges and media used for fermentation of isolated actinomycetes. All these factors can induce expression of certain genes and consequently production of definite metabolites. Our study demonstrated the utilization of metabolomics tools and statistical analysis using MetaboAnalyst 4.0 to perform screening and dereplication of known metabolites of 12 extracts of sponge-associated actinomycetes and differentiation between extracts. Also, cytotoxic and antiviral activities were discussed. It was notable that only three extracts exhibited antiviral activity and 7 extracts exhibited cytotoxic activity. Different biological responses of the extracts of certain actinomycete strain using different media can be attributed to the different composition of media constituents which seriously affect the biosynthesis and production of the bioactive metabolites. This can be considered as an efficient approach for microbial strain selection for further investigations and drug discovery.

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Conflict of interest

Authors declare no conflict of interest.

Author Contributions

Conceptualization, U.R.A., M.N.S. and S.Y.D.; methodology, E.R.A., M.N.S., M.E.R., O.M.H. and U.R.A.; data curation, E.R.A., M.N.S., U.R.A.; writing original draft preparation, E.R.A., M.N.S, M.E.R., O.M.H. and U.R.A.; Manuscript editing, U.R.A., S.Y.D.. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The research data are available.

Figure quality

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Figure legends:

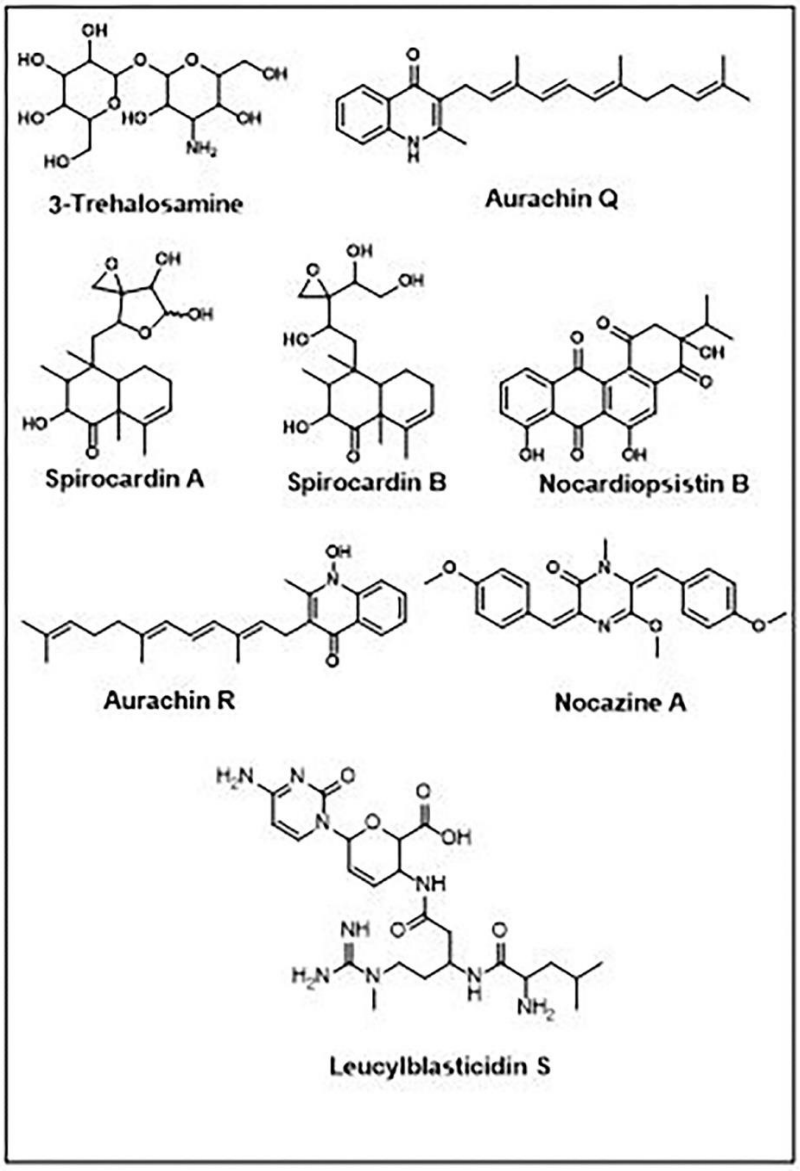
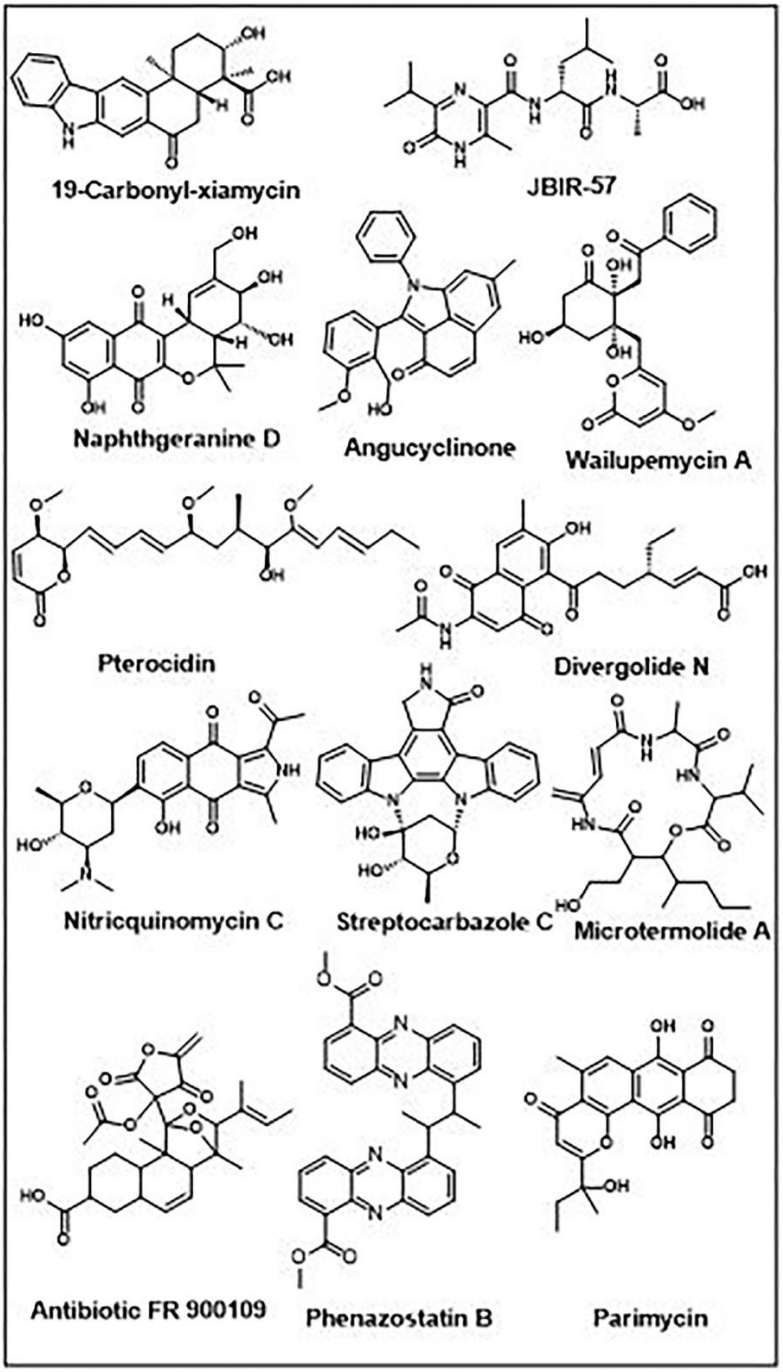


Figure 1: Examples of secondary metabolites identified in the total extract of *Rhodococcus* sp. UR21.



490

491 **Figure 2**: Examples of secondary metabolites identified in the total extract of *Streptomyces* sp.

492 UR32.

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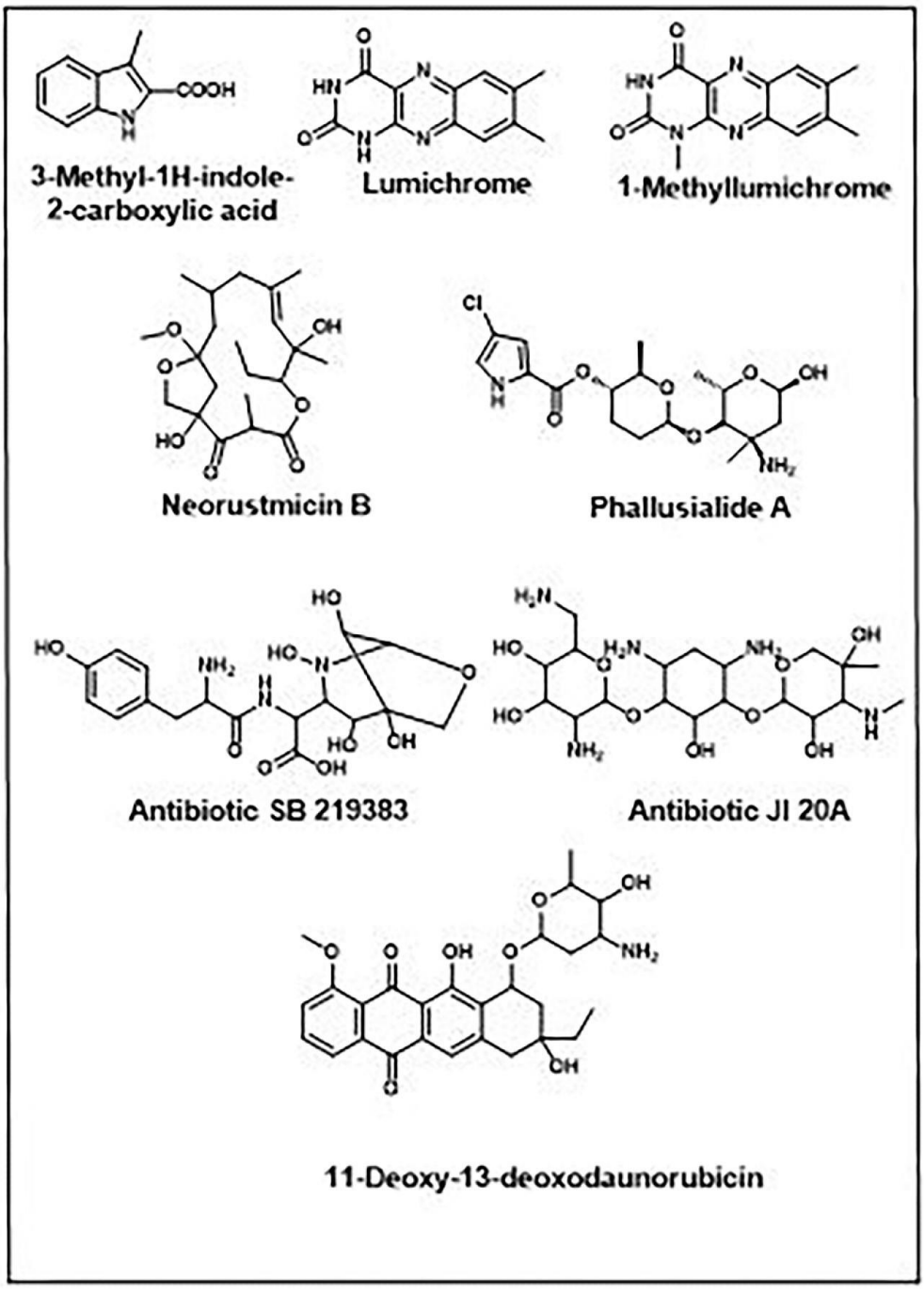

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495 **Figure 3**: Examples of secondary metabolites identified in the total extract of *Micromonospora*

496 sp. UR44.

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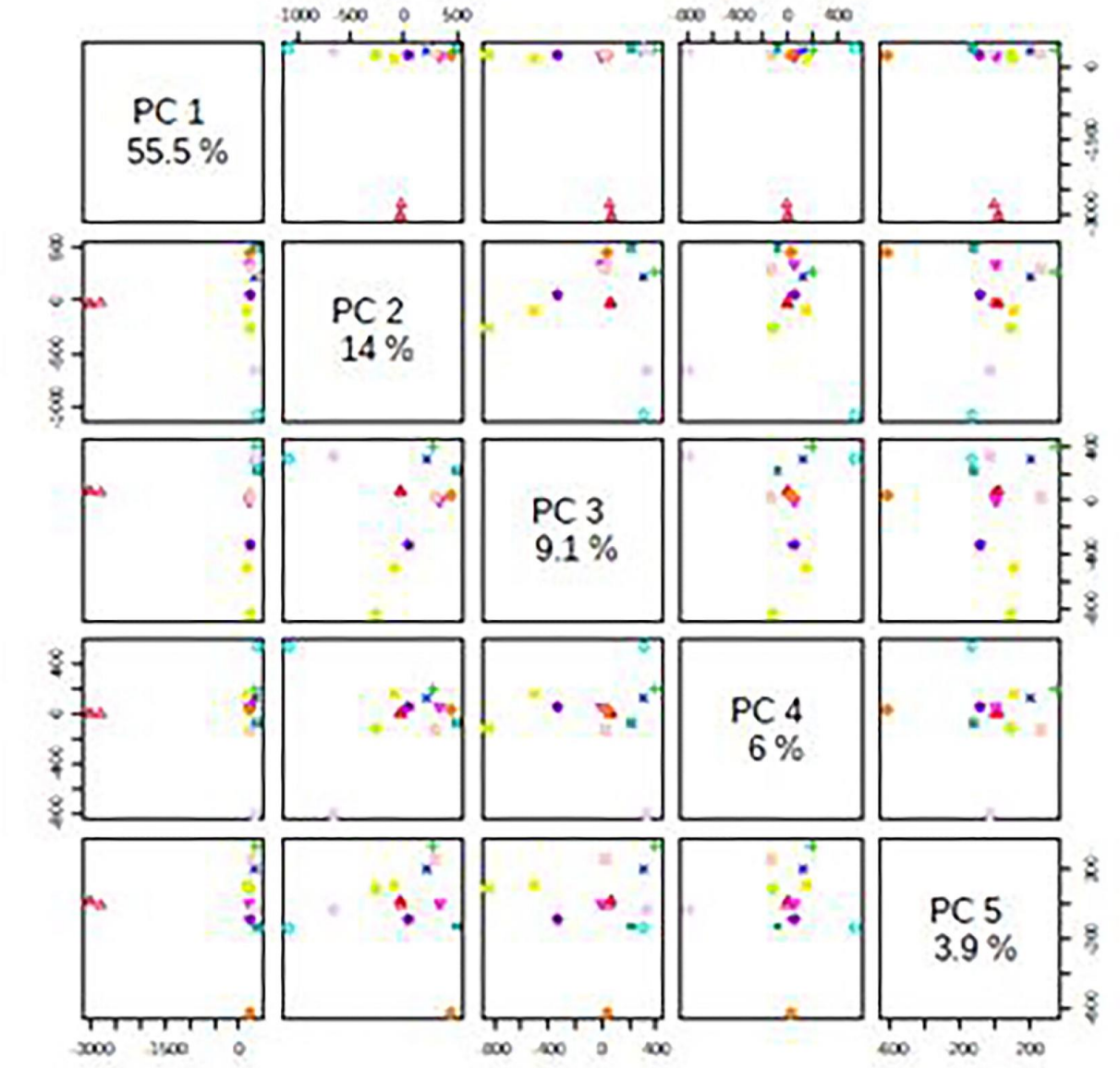

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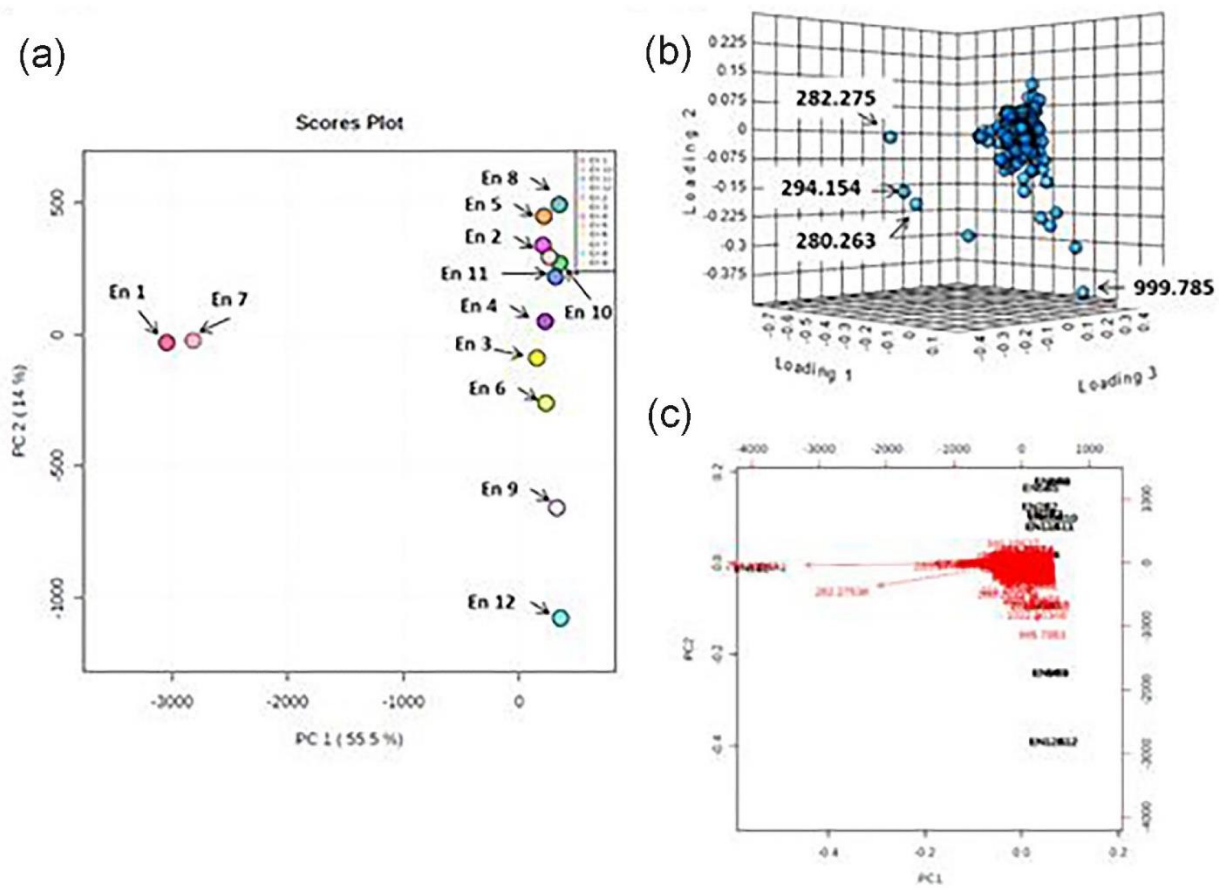
499 **Figure 4**: Pairwise score plots between the selected PCs. The explained variance of each PC is

500 shown in the corresponding diagonal cell.

501



502

503 **Figure 5**: (A) Scores plot between the selected PCs. (B) Three dimensional Loadings plot for the

504 selected PCs. (C) PCA biplot between the selected PCs.

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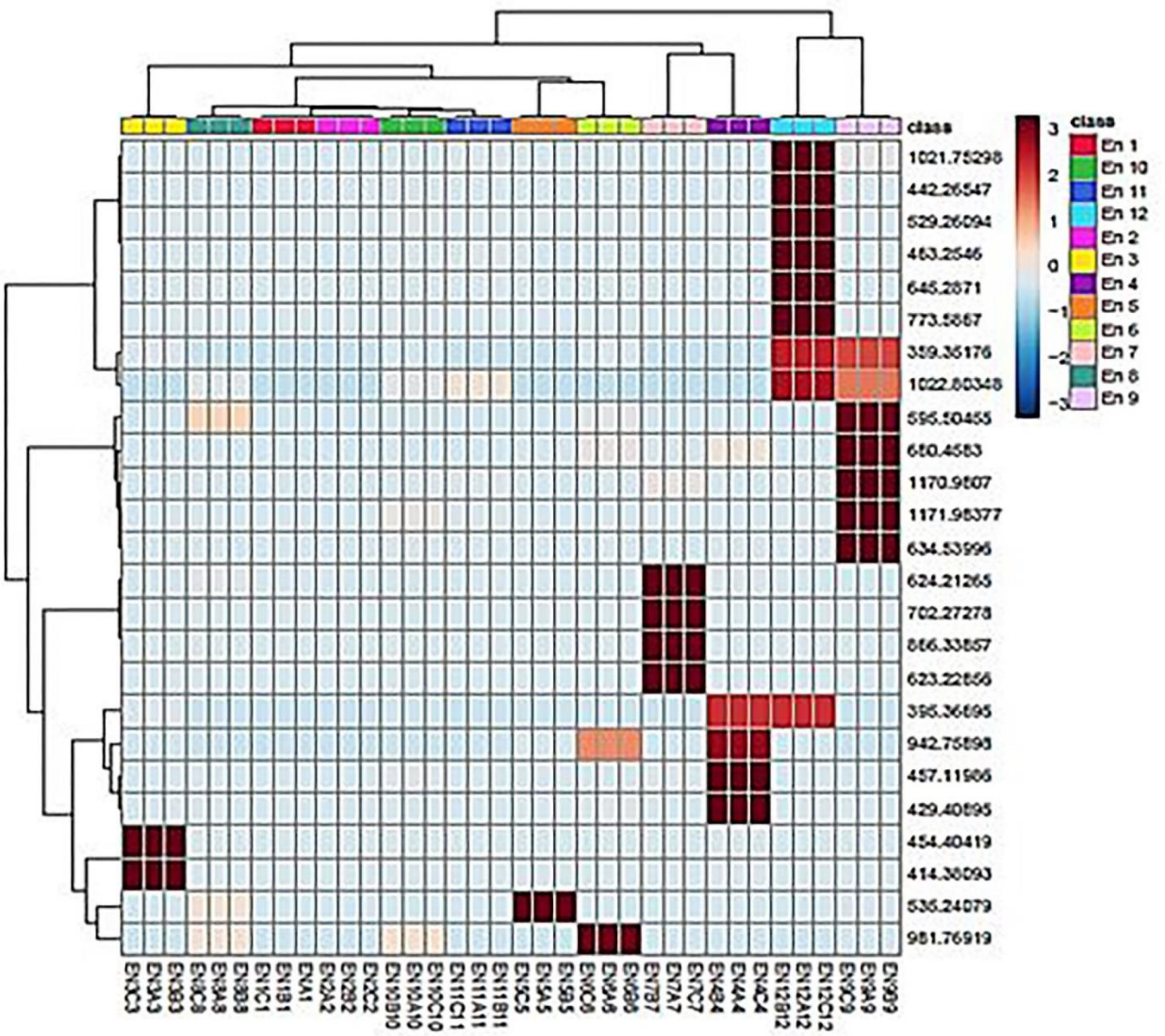

506

507 **Figure 6**: Hierarchical clustering results shown as heat map.

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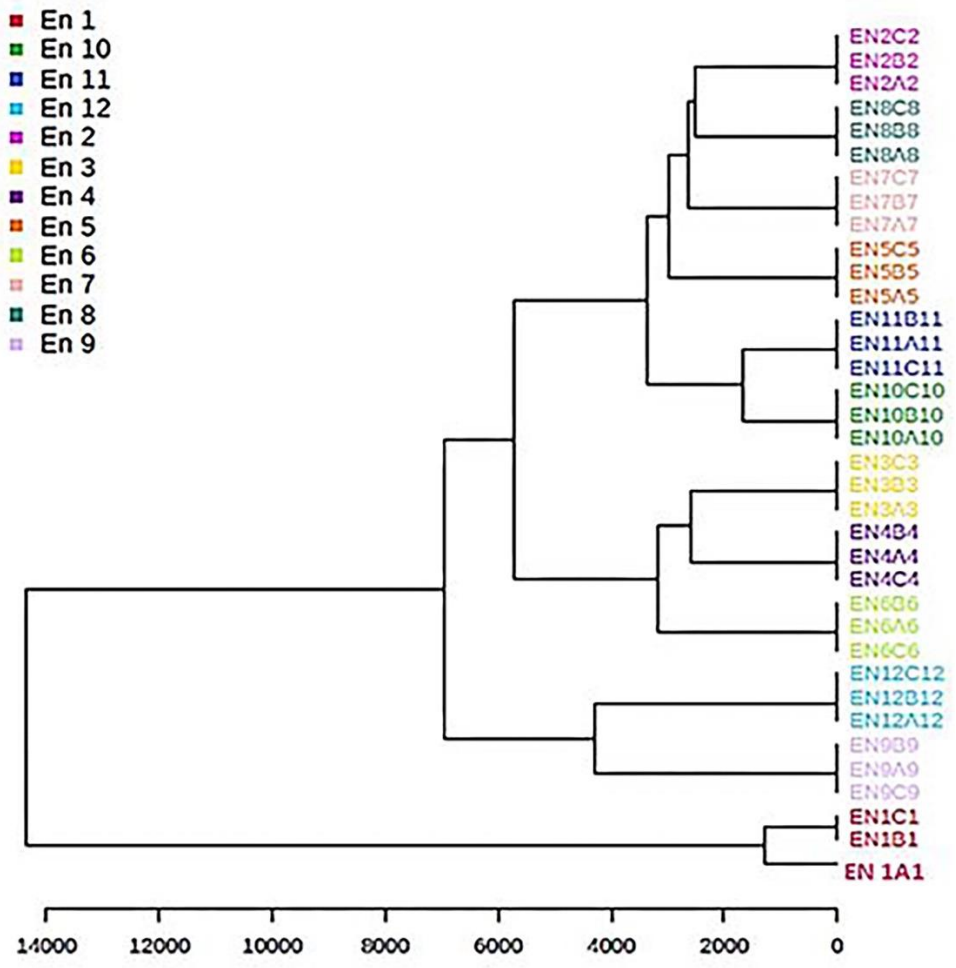

509

510 **Figure 7**: Hierarchical clustering results shown as dendrogram.

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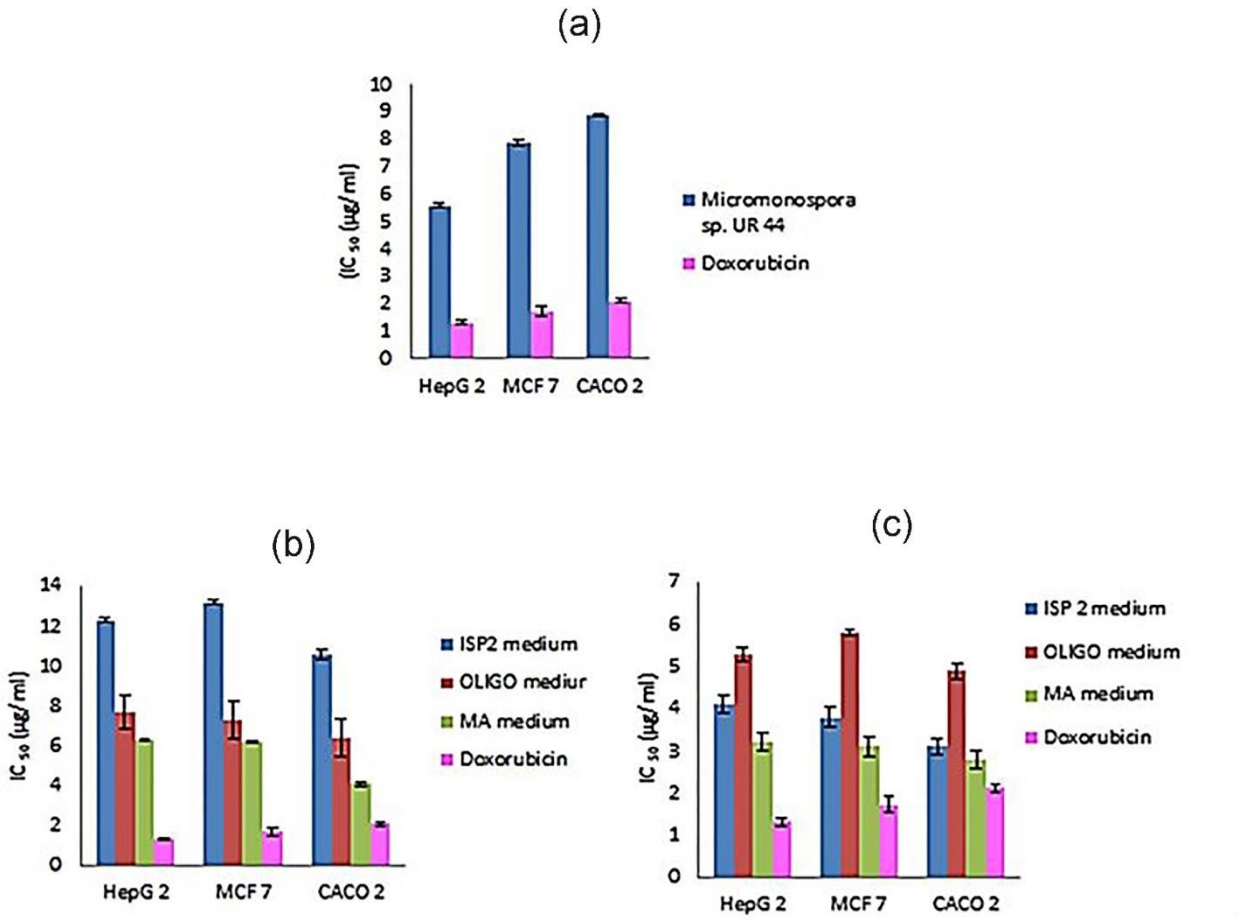

512

513 **Figure 8**: (A) IC_{50} values of the total extract of *Micromonospora* sp. UR44 using ISP2 medium.

514 (B) IC_{50} values of the total extracts of *Streptomyces* sp. UR32 using different media. (C) IC_{50}

515 values of the total extracts of *Rhodococcus* sp UR21 using different media.

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521 **Table 1**: Results of in vitro cytotoxic activity of the total extracts of *S. irregularis* associated

522 actinomycetes using MTT assay.

523

Actinomycete strain	IC $_{50}$ (µg/ml)			
	Cell lines / Media	HepG 2	MCF 7	CACO 2
Micromonospora sp. UR44	M1	inactive	Inactive	inactive
	ISP2	5.6 ± 0.09	7.9 ± 0.12	8.9 ± 0.01
	OLIGO	inactive	Inactive	inactive
	MA	inactive	Inactive	inactive
Streptomyces sp. UR32	M1	inactive	Inactive	inactive
	ISP2	12.3 ± 0.11	13.2 ± 0.09	10.6 ± 0.23
	OLIGO	7.7 ± 0.87	7.3 ± 0.9	6.4 ± 0.99
	MA	6.3 ± 0.09	6.2 ± 0.07	4.1 ± 0.1
Rhodococcus sp UR21	M1	inactive	Inactive	inactive
	ISP2	4.1 ± 0.20	3.8 ± 0.23	3.1 ± 0.18
	OLIGO	5.3 ± 0.17	5.8 ± 0.09	4.9 ± 0.19
	MA	3.2 ± 0.21	3.1 ± 0.23	2.8 ± 0.2
Doxorubicin (10 µM)		1.32 ± 0.08	1.72 ± 0.19	2.12 ± 0.09