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# Metabolites Profiling of Soil Actinomycetes with Antimicrobial and Anticancer Activities

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## Abstract

Metabolic profiling of actinomycetes isolated from Nepalese soil has uncovered promising candidates for the development of new antimicrobial and anticancer agents. This research examines the potential of actinobacteria isolated from soil, namely strains PY40, PY118, and PY122, as promising sources for biological applications. These strains were identified using 16S rRNA gene sequencing as *Streptomyces* sp. PY40, *Streptomyces* sp. PY118, and *Amycolatopsis* sp. PY122. Comprehensive bioassays were carried out on ethyl acetate (EA) extract originating from the fermentation broths of these species to assess their antimicrobial and anticancer activities. The results showed noteworthy antifungal and anticancer properties of *Streptomyces* sp. PY40 extract, thereby indicating its possible use in the synthesis of pharmaceuticals. Furthermore, potent antifungal activity was observed with an extract from *Amycolatopsis* sp. PY122. These isolates showed strong inhibition against several multi-drug-resistant strains of bacteria, including *Shigella sonnei* ATCC 25931, *Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* ATCC 25922, and, *Staphylococcus aureus* ATCC 43300, suggesting significant antibacterial activity of the extracts. Gas Choursomatography-Mass Spectrometry (GC-MS) analysis revealed particularly volatile chemical constituents in the extracts. Antibiotic agents such as diketopiperazines and hexahydropyrrolo[1,2-a]pyrazine-1,4-dione were prevalent in the extracts of these isolates. This study enhances our comprehension of the bioactive potential of these microorganisms and underscores their value as a source for creating novel therapeutics to address multi-resistant infections and cancer demands. These findings show that actinomycetes from Nepalese soil have high prospects for biological applications.

**Keywords**: *Streptomyces* species; multiple drug resistance; antimicrobial agents; volatile compounds; gas chromatography; therapeutics agents

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## Introduction

The genera *Streptomyces* and *Amycolatopsis* belong to the phylum Actinomycetota, Gram-positive filamentous bacteria that are eminent producers of bioactive metabolites. Actinomycetes have produced approximately 50% of the explored bioactive secondary metabolites, including antibiotics, antitumor drugs, anti-inflammatory agents, and enzymes [1,2]. Within actinomycetes, the genus *Streptomyces* is a prolific producer of antibiotics, responsible for producing approximately 70 to 80% of all-natural bioactive metabolites [1,3]. Another genus, *Amycolatopsis*, is a significant reservoir of bioactive secondary metabolites, including remarkable antibiotics such as rifamycin and vancomycin [4,5]. The secondary metabolites derived from these genera are used to control multidrug-resistant (MDR) pathogens and cancers [2].

New antimicrobial treatments are urgently needed due to the rise in MDR organisms, [3,6] affecting the clinical outcomes of people in various healthcare settings. Factors such as horizontal gene transfer, enzymatic degradation or modification of drugs like beta-lactamase production by bacteria, chromosomal abnormalities, and evasion of host immunological responses are some causes of MDR in pathogenic bacteria and fungi [7]. MDR bacteria, such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae*, are acquired by intrinsic processes and mobile genetic elements [8]. Previous studies have highlighted that MDR issues impose a higher risk of sepsis development during hospital stay, high rates of mortality, and re-hospitalization within 30 days [9]. *Streptomyces* and *Amycolatopsis* species are valuable sources of bioactive compounds that show promise in treating MDR pathogens and cancer [10,11].

Actinomycetes show great promise in producing bioactive secondary metabolites, especially in the soils of Nepal [12,13]. An abundance of actinomycetes thrive in Nepal's varied landscape, and the country's higher

elevations provide ideal conditions for discovering novel antibacterial chemicals [14]. Various natural environments have been found to have actinomycetes, which may include bioactive compounds that have not yet been discovered [15]. Novel metabolites from rare actinomycetes were sought after by researchers against MDR pathogens and cancer [5]. Researchers have found microbes in unexplored soil that may produce bioactive chemicals [3]. Technologies like GC-MS have opened up new possibilities in metabolomics for identifying and characterizing thermolabile volatile metabolites [16]. Soil bacteria produce a wide range of strain-specific volatile organic compounds (VOCs), significantly impacting microbial development and showing promise as pathogen control techniques [17]. This study recognizes bioactive compounds through computational molecular annotation and profiling by employing GC-MS technique.

# Materials and methods

## Isolation of Actinomycetes and Morphological Characterization

To isolate actinomycetes from high altitude soil samples collected from Lamachaur, Dolpa (PY40), Namche Bazar, Solukhumbu (PY118), and Chandragiri, Kathmandu (PY122), a three-fold serial dilution of soil samples was performed. Then, diluted soil suspension was spread on the ISP4 plate (supplemented with cycloheximide and nalidixic acid) with a sterile glass rod. Finally, the colony of bacteria was observed after incubating the plate at 28°C for 5–8 days [18]. Gram-staining was performed to characterize the morphology of the isolated strains.

## Fermentation and extraction of metabolites

Bacterial strains were cultured in 20 mL of Tryptone Soya Broth (TSB) medium with glass beads at 28°C in a shaking incubator (180 rpm) for 3 days. Subsequently, 1 mL of seed culture was transferred to a 100 mL fermentation medium (TSB) and incubated under the same conditions for 7 days [19]. 100mL Ethyl acetate (EA) was mixed with fermentation broth to extract metabolites as it effectively dissolves a wide range of bioactive compounds including both polar and nonpolar rather than other solvents dissolve metabolites based on polarity, and the mixture was agitated overnight in a shaking incubator to isolate metabolites. The EA extract was separated using a separating funnel and dried in a water bath at 37°C for further use.

## Extraction of genomic DNA and PCR

Actinomycetes PY40, PY118, and PY122 were cultured in TSB medium at 28°C at 180 rpm for 4 days. Then, 15 mL of each broth was centrifuged at 4000 rpm for 10 min to harvest cells. Pellets were first washed with lysis buffer (10% sucrose) with vertexing, and 1 mg/mL lysozyme was added. Then, it was incubated at 37°C until complete lysis of cells (about an hour), followed by adding 250 $\mu$L of 0.5M EDTA and proteinase K, and again incubated for 5 min at the same temperature. Further, 200 $\mu$L of 10% sodium dodecyl sulfate (SDS) was added and incubated at 70°C for 10 min, then 500 $\mu$L of 5 M potassium acetate solution was added and stored in an ice bath. Cells were then mixed with phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged. The supernatant was treated with chloroform and centrifuged, then 40 $\mu$L RNAse was added and incubated at 37°C for an hour. One volume of DNA was precipitated with isopropanol and ethanol in a 1:2 ratio, centrifuged, washed with 70% ethanol, and dried. Finally, DNA was dissolved in 20 $\mu$L Tris-EDTA (TE) buffer.

16S rRNA gene amplification from genomic DNA (PY40, PY118, and PY122) was performed using universal primers (27F:5`-AGAGTTTG ATCCTGGCTCAG-3` and 1492R: 5`-GGTTACCTTG TTACGACTT-3`). The reaction mixture in 10 $\mu$L included 0.4 $\mu$L genomic DNA, 0.3 $\mu$L of 100 pM of each primer in nuclease-free water, and 5X *Taq* polymerase PCR premix. PCR conditions comprised an initial denaturation at 98°C for 5 min, followed by 29 cycles of denaturation at 98°C for 10 sec, 54°C for 10 sec, and extension at 72°C for 2 min, with final extension steps at 72°C for 7 min. Using a PCR Purification Kit (Bio Basic Inc., Amherst, NY, USA), the PCR products were purified and sequenced at GenoTech Inc. Daejeon, Republic of Korea. The NCBI Blast search (http://www.ncbi.nlm.nih.gov/blast/) assessed sequence similarity against reference antimicrobial strains. The partial 16S rRNA gene sequence of these actinomycetes was aligned with all known actinomycetes using ClustalW, and a phylogenic tree was constructed using the multiple alignment and Neighbour-joining method in MEGA-11 software [20] to determine the evolutionary relationship between the isolated actinomycetes and the putative common ancestor.

## Antifungal assay

The antifungal activity of EA extracts of actinomycetes was evaluated against *Aspergillus niger* and *Saccharomyces cerevisiae* [21] using the agar well diffusion method [22]. Briefly, 6 mm diameter wells were created in the potato dextrose agar (PDA) plates. Then, 80 $\mu$L of EA extracts dissolved in 50% DMSO with 2 mg/mL concentrations in each sample and 1 mg/L cycloheximide were applied as a positive control to each well. Plates were incubated at

28°C for 24 hours for *A. niger* and incubated at 30°C for 72 hours for *S. cerevisiae* in the dark to ensure optimal growth conditions and accurate observation of microbial activity.

## Antibacterial activity

The perpendicular streak plating method on Mueller Hinton Agar (MHA) was used for pure culture of actinomycetes to perform primary screening for antibacterial activity. The actinomycetes extracts were then examined for antibacterial potential using the agar well diffusion method following a protocol described in a previous study [19]. Bacterial suspensions of test organisms were cultured in Mueller Hinton Broth (MHB) at 37°C for 24 hours and adjusted to $1.5\times10^8$ CFU. Extracts, along with neomycin (positive control) and 50% DMSO (negative control), were tested against *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, and *Shigella sonnei* ATCC 25931. After creating 6mm wells, 80 $\mu$L of 2.2 mg/mL each extract was added to each well on MHA plates. After 24 hours of incubation at 37°C, inhibition zones (in mm) were measured to evaluate antibacterial efficacy.

## Determination of MIC and MBC

The MIC and MBC of the crude extracts were determined using the broth microdilution method as per CLSI guidelines [23]. A two-fold dilution of extracts was prepared in 96 well plates with MHB and a bacterial inoculum (final concentration of $10^6$ CFU/mL). 50 mg/L neomycin was used as a positive control. Plates were incubated at 37°C for 24 hours, followed by resazurin addition to determine MIC. Moreover, MBC was determined by streaking well contents onto NA (nutrient agar) plates and incubated at 37°C for 18 hours to examine bacterial growth [24].

## Anticancer activity

The anticancer activity of actinomycetes isolates was evaluated using the MTT assay on MCF-7 (breast cancer) and HeLa (cervical cancer) cell lines obtained from PGIMER, Chandigarh, India, and Shikhar Biotech, Lalitpur, Nepal, respectively, as per NCBI guidelines [25]. MCF-7 and HeLa cells were cultured in DMEM (GIBCO Laboratories, Green Island, NY) supplemented with 10% FBS, 7.5% sodium bicarbonate, 200 mM L-glutamine, 100 mM sodium pyruvate, and 1% penicillin-streptomycin (GIBCO Laboratories, Green Island, NY) at 37°C with 5% $CO_2$ and 95% humidity. Cells were seeded in 96-well plates at a density of $3.5\times10^3$ cells/well for HeLa and $4\times10^3$ cells/well for MCF-7 and allowed to adhere for 16 hours in the $CO_2$ incubator. After 24 hours, cells were treated with 0, 10, 25, 50, and 100 $\mu$g/mL concentrations of each extract and 0.5% DMSO as negative control for 48 hours. For post-treatment, 20 $\mu$L of MTT (HiMedia Laboratories, India) in 1X PBS (Thermo-Fisher Scientific Inc., Waltham, MA, USA), solution (5 mg/mL) was added to each well and incubated for 4 hours. The resulting formazan crystals were dissolved in 100 $\mu$L of DMSO, and the absorbance was measured at 570 nm using a microplate reader (BMG Lab, Ortenberg, Germany). The percentage of cell viability was calculated, and $IC_{50}$ values were determined by using the following formula:

$$IC50 = \frac{(0.5-C)}{M}$$

The equation $IC_{50}$ = (0.5 - C)/M determines the half-maximal inhibitory concentration of the anticancer substance. The value of $IC_{50}$ is the concentration required to inhibit cell growth by 50%. The concentration of the drug (X-axis) is graphed against the cell viability (Y-axis) to get the dose-response curve, and M is the slope of this curve. C is the observed absorbance or cell viability at a specific concentration. At the halfway point of the dose-response curve, defined as the concentration that lowers cell viability by 50%, we get the $IC_{50}$ value. All experiments were performed in triplicate, and data were statistically analyzed and plotted using GraphPad Prism software (version 8.0.1). The *p*-value was determined using the R studio software (version 4.3.1).

## Gas Chromatography-Mass Spectrometric Analysis

GC-MS analysis was conducted using an Agilent 8890 GC system and an Agilent 5977 Mass Selective Detector (MSD) at SAIF IIT Madras, India. The chromatographic separation was achieved on an HP-5ms Ultra Inert column (30 m × 250 µm × 0.25 µm) with helium as the carrier gas, maintaining a constant flow rate of 1.2 mL/min. The oven temperature was programmed to start at 75°C, held for 0.5 min, then ramped at the rate of 5 °C/min to 180°C (held for 3 min), and further ramped at 5°C/min to 300°C (held for 5 min), with a total run time of 53.5 min. The split inlet mode was set to a ratio of 15:1 at 250°C. A 1 $\mu$L injection volume was used, with sample and solvent washes programmed for optimal injection precision. The MSD operated in electron ionization (EI) mode with an ion source temperature of 230°C and a quadrupole temperature of 150°C, scanning a mass range of 50–600 Da with a fixed electron energy of 70 eV. The solvent delay was set to 1.5 min, and real-time total ion current plots were enabled for continuous monitoring.

The data acquisition parameters were rigorously controlled to ensure the accuracy and reproducibility of the results. We employed the NIST (2017) Mass Spectral Library search for compound identification.

# Results

## Isolation and morphological characterization of actinomycetes

These actinomycetes PY40, PY118, and PY122 were obtained from soil specimens from different parts of Nepal. The first characteristic that identified these isolates was the colonial morphology, rough, tough, dry, and raised colonies, as shown in **Figure S1**. Notably, all these morphological characters are used to make the preliminary identification of actinomycetes out of other bacteria isolated from the soil. After culturing, colonies of these strains became light yellowish-white, typical of many actinomycetes species. The colony was subcultured onto an agar plate followed by Gram-staining at a magnification of 100 x under the microscope. Hair-like mycelia are observed which is a characteristic feature of Gram-positive actinomycetes.

Strain PY40 developed rough, tough, and dry colonies with raised structures. These features were congruent with the texture and structure of the colonial nature of actinomycetes. The colonial morphology of actinomycetes was smooth which they ordinarily produced. Strain PY118 was as morphologically similar to PY40, but a slight difference in texture differentiates between the two. As for strain PY122, it has maintained the rough and dry colony morphology with high and wide elevations and well-developed colony structure, which point strongly to its belonging to the actinomycete group of bacteria.

## Molecular identification and phylogenetic analysis

The genomics DNA of these active isolates was extracted by phenol-chloroform isoamyl alcohol (PCI) reagent, and then extracted DNA was electrophoresed in 0.4% agarose stained with ethidium bromide. PCR product of the culture in agarose gel (0.4%) predicted

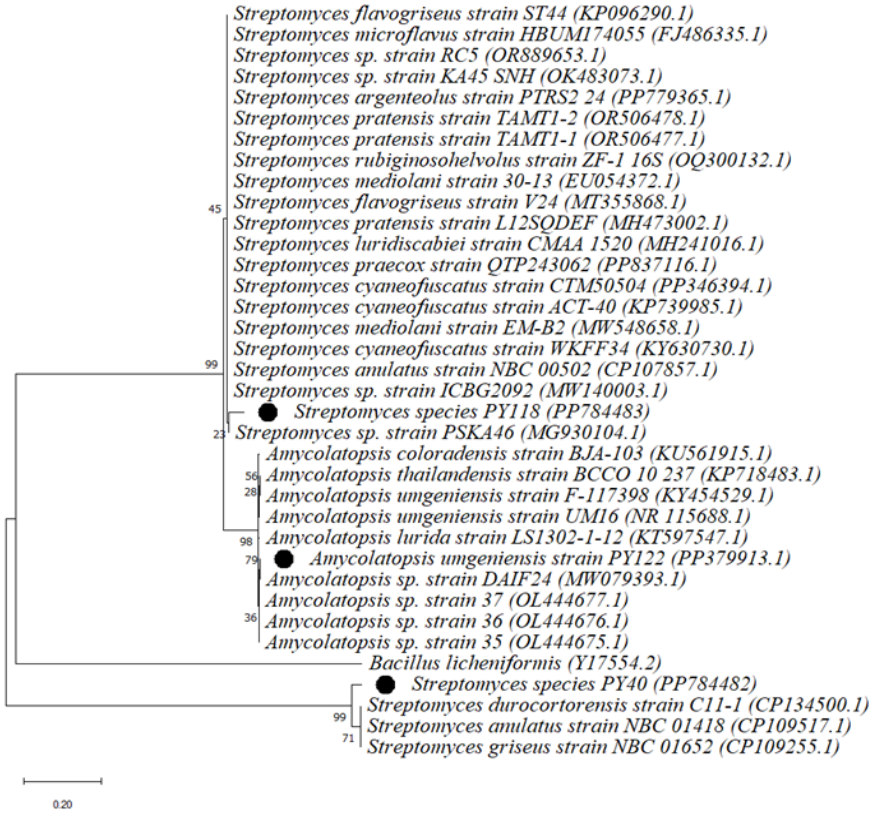



**Figure 1**. Phylogenetic tree of 16S rRNA of *Streptomyces* strains PY40 and PY118, and *Amycolatopsis ungeniesis* strain PY122 constructed using Neighbour-joining statistical method showing their evolutionary relationship to closet identified taxa. The bar 0.20 indicates substitutions per nucleotide position, as determined using 1000 bootstrap replications. GenBank accession numbers are shown in parentheses. *Bacillus licheniformis* acts as an outgroup.

**Table 1**. MIC and MBC values of *Streptomyces* sp. PY40, PY118, and *Amycolaptosis* sp.PY122 against tested bacterial strains.

| Tested Bacterial Strains | *Streptomyces* sp. PY40 | | *Streptomyces* sp. PY118 | | *Amycolatopsis* species PY122 | | Positive Control | |
|---|---|---|---|---|---|---|---|---|
| | MIC (µg/mL) | MBC (µg/mL) | MIC (µg/mL) | MBC (mg/mL) | MIC (µg/mL) | MBC (µg/mL) | MIC (µg/mL) | MBC (µg/mL) |
| *Staphylococcus aureus* ATCC 43300 | 179.25 | 385.50 | 650 | 1.3 | 155.5 | 155.50 | 31.85 | 31.85 |
| *Klebsiella pneumoniae* ATCC 700603 | 358.5 | 717 | 650 | 1.3 | 155.5 | 311 | 15.92 | 31.85 |

their size as about 1500 bp. Based on BLAST analysis of partial 16S rRNA gene sequence, actinomycetes PY40, and PY118 were found to be *Streptomyces* species, while actinomycetes PY122 was found to be an *Amycolatopsis* species. Thus, these isolates are named *Streptomyces* sp. PY40, *Streptomyces* sp. PY118, and *Amycolatopsis* sp. PY122 respectively and their partial 16S rRNA sequences were deposited accordingly. A phylogenetic tree (**Figure 1**) showed their evolutionary relationship to closet identified taxa.

The blast analysis showed *Streptomyces* sp. PY40 (accession number PP784482) and *Streptomyces* sp. PY118 (accession number PP784483) was closely associated with *Streptomyces durocortorensis* strain C11-1 (94%) and *Streptomyces cyaneofuscatus* strain WKFF34 (94%) while *Amycolatopsis* sp. PY122 (accession number PP379913) showed the closest relatedness with *Amycolatopsis umgeniensis* strain UM16 (99%) .

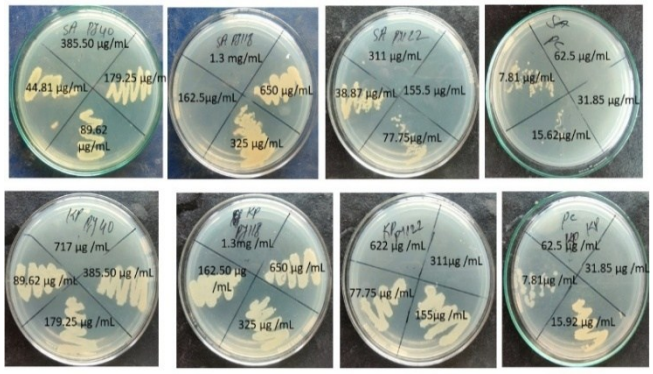



**Figure 2.** Minimum inhibitory concentration (MIC) values of secondary metabolites from *Streptomyces* strains PY40 and PY118 and *Amycolatopsis* strain PY122 against *Klebsiella pneumoniae* ATCC 700603 and *Staphylococcus aureus* ATCC 43300 pathogen.

## Antibacterial assay

The antimicrobial activity of the EA extracts from these isolates is shown in **Table S1 and Figure S2**. All three isolates showed strong inhibition against tested pathogens. The extracts of *Streptomyces* sp. PY40, *Streptomyces* sp. PY118, *and Amycolatopsis* sp. PY122 significantly inhibited *S. sonnei*, *S. aureus*, and *K. pneumoniae*. *Amycolatopsis* sp. PY122 exhibited stronger inhibition against these pathogens than that of *Streptomyces sp. PY40* and *Streptomyces sp. PY118.* The zone of inhibition displayed by extracts was comparable to that exhibited by positive control, neomycin.

## MIC and MBC

The results of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of EA extract are shown in **Figures 2 and 3** and tabulated in **Table 1**. The *Streptomyces* sp. PY40 exhibited moderate inhibitory activity against *S. aureus* bacteria, with a MIC of 650.00 $\mu$g/mL, as represented graphically in **Figure S3.**

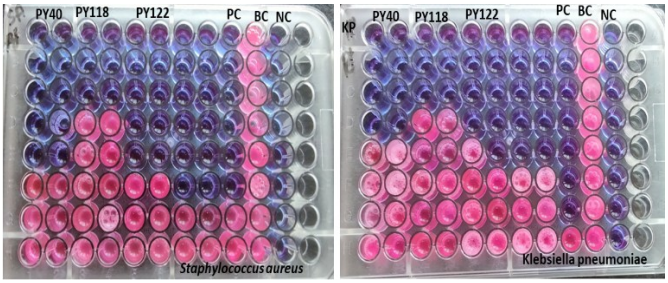



**Figure 3**. Minimum bactericidal concentration (MBC) values of secondary metabolites from *Streptomyces* strains PY40 and PY118 and *Amycolatopsis* strain PY122 against *Klebsiella pneumoniae* ATCC 700603 and *Staphylococcus aureus* ATCC 43300 pathogen.

In contrast, *Streptomyces* sp. PY118 and *Amycolatopsis* sp. PY122 revealed significant inhibitory effects, with MIC values of 179.25 µg/mL and 155.50 µg/mL, respectively. The positive control (PC) exhibited significant inhibition, with a MIC value of 31.85 µg/mL. The inhibitory effect of *Streptomyces* sp. PY40's extract against *K. pneumonia* was considerable, as shown by its MIC value of 650.00 $\mu$g/mL. However, both isolates PY122 and PY118 showed significant suppression of *K. pneumoniae* bacteria, with MIC values of 155.50 $\mu$g/mL and 358.50 $\mu$g/mL, respectively. The positive control showed a highly suppressive effect, with a MIC value of 15.92 $\mu$g/mL. On the other hand, the lowest MBC value of 155.50 $\mu$g/mL was demonstrated by *Amycolatopsis* sp PY122 against

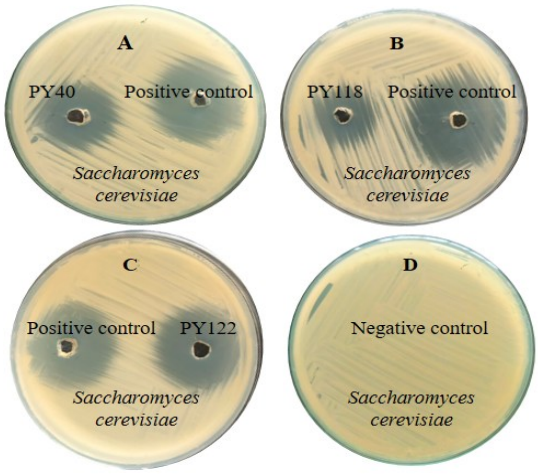
*Staphylococcus aureus*, and that of positive control was 31.85 $\mu$g/mL.

However, EA extracts of isolate PY118 displayed bactericidal effects only in a concentration of 1300.00 $\mu$g/mL for both tested pathogens, which is very high compared to that of EA extracts of isolates PY122 and PY40. Hence, the examined EA extracts of all isolates have shown considerable antibacterial activities compared to positive control neomycin.

## Antifungal assay

The antifungal potential of EA extracts of the isolates was evaluated using the agar well diffusion method. By measuring the zones of inhibition (ZOIs) (**Table S3**), the antifungal capability was examined against two fungi species *Saccharomyces cerevisiae* and *Aspergillus niger*, as shown in **Figure 4.**

**Figure 4**. Antifungal activity against *Saccharomyces cerevisiae* (A)




*Saccharomyces cerevisiae* on *Streptomyces* sp. PY40, (B) *Saccharomyces cerevisiae* on *Streptomyces* sp. PY118, (C) *Saccharomyces cerevisiae* on *Amycolaptosis* sp. PY122, (D) Negative control.

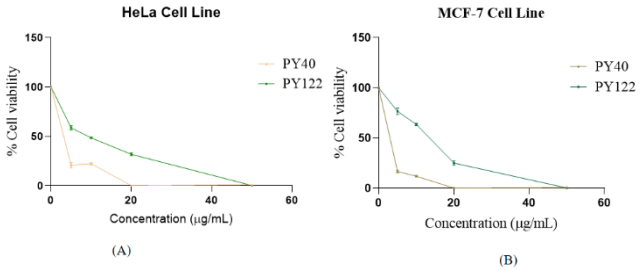
Regarding the efficiency of isolates on *Aspergillus niger*, isolate PY122 also showed strong antifungal activity with a ZOI of 16 mm (**Figure S4**), which is slightly lower than that of the positive control (18 mm). Thus, it confirmed the efficiency of EA extracts of isolate PY122 against *Aspergillus niger*. The PY40 isolate displayed a ZOI of 15 mm, indicating a moderate level of antifungal activity. The PY118 isolate exhibited the lowest antifungal activity against *Aspergillus niger*, with a ZOI of only 7 mm.

Among the isolates tested, the EA extract of isolate PY122 had significant antifungal activity against *Saccharomyces cerevisiae*, with a ZOI of 27 mm, which was only slightly lower than the ZOI observed for the positive control (31 mm), as shown graphically in **Figure 5S**. While the antifungal activity of EA extracts of PY40 and PY118 strains was not as pronounced as that of PY122, they nevertheless exhibited some degree of antifungal activity. This indicates a significant potential of the PY122 strain to inhibit the growth of *Saccharomyces cerevisiae*.

## Anticancer properties

The MTT assay was used to evaluate the anticancer potential of EA extract with negative control (DMSO) that did not show any noticeable cytotoxic effect, confirming that the EA extracts genuinely produced the observed effects. The half-maximal inhibitory concentration ($IC_{50}$) values for both extracts were calculated, which is the extract concentration needed to block 50% of cell viability. The cytotoxic effects of these extracts on cancer cells are further supported by the dose-dependent reduction in cell viability, as depicted in **Figure 5**.

**Figure 5**. Anticancer activity of EA extracts from *Streptomyces* species PY40 and *Amycolaptosis* PY122 against (A) HeLa cell line and (B) MCF-7 cell line.




The PY40 strain displayed a somewhat lower $IC_{50}$ value of 4.18 $\mu$g/mL for the MCF-7 cell line than the PY122 strain, which had an $IC_{50}$ value of 5.10 $\mu$g/mL. This result indicates that the PY40 strain is more effective than the PY122 strain in preventing the proliferation of MCH-7 cells. Similarly, the PY40 strain had an $IC_{50}$ value of 4.30 $\mu$g/mL in the HeLa cell line, but PY122 had a value of 5.09 $\mu$g/mL. These findings show that both extracts have significant anticancer potential; in particular, PY40's extract was more effective than PY122's extract in lowering cell viability in the two cell lines under investigation.

## Statistical Analysis

The cytotoxic activity, expressed as a percentage of cell viability across various sample concentrations, was analyzed using one-way ANOVA followed by Tukey's post-hoc test. The analysis was performed in triplicate, and the results are expressed as mean ± standard deviation (SD). A p-value ≤ 0.05 was considered statistically significant.

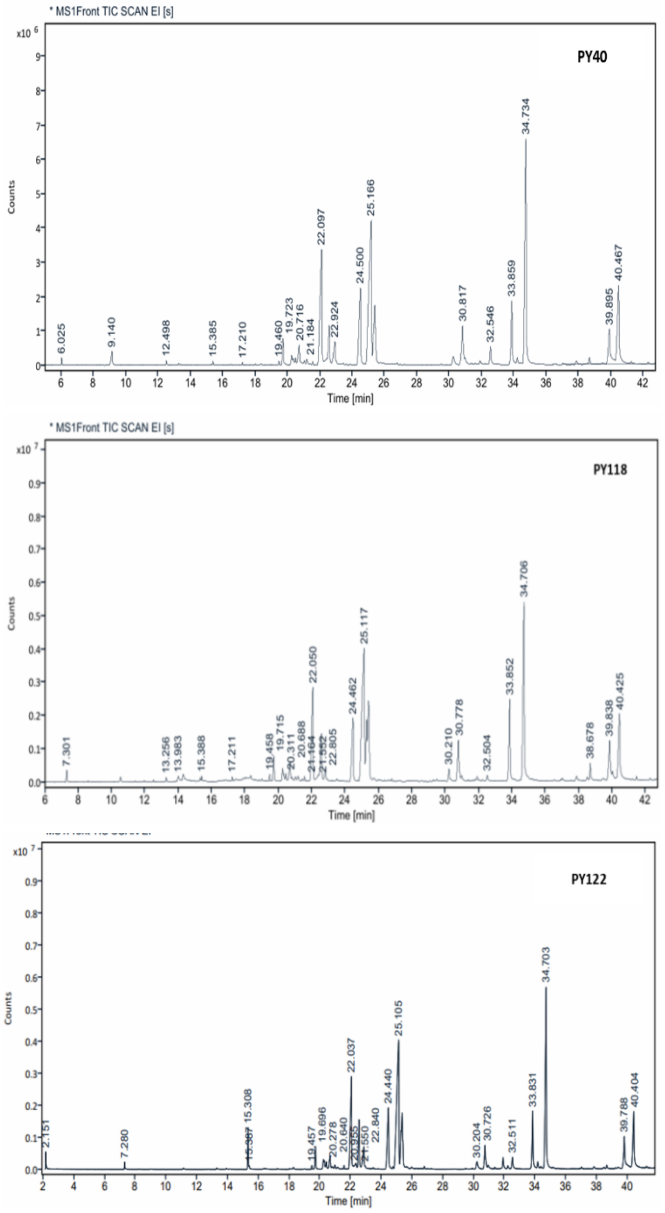



**Figure 6**. GC-MS chromatograms of EA extracts of *Streptomyces* species PY40, PY118, and *Amycolaptosis* sp. PY122 with retention time

## Identification of volatile metabolites using GC-MS analysis

The EA extracts' GC-MS chromatograms showed several peaks indicative of possible bioactive metabolites (**Figure 6**). We identified 30 volatile metabolites in all samples, as depicted in **Table 2**. Cyclo(L-prolyl-L-valine) was identified as the most abundant metabolite in all samples. Other metabolites such as pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)- and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- were also detected as the major metabolites.

The identified compounds included diketopiperazines ( 1,4-diazabicyclo[4.3.0]nonan-2,5-dione, 3-methyl, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro, cyclo(L-prolyl-L-valine), pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, etc), alcohol (phenylethyl alcohol, 1- hexadecanol), amide (benzeneacetamide, acetamide, *N*-(2-phenylethyl)-), alkene (cetene, 3-eicosene, (*E*)-), ester derivatives (formic acid, (2-fluoro-5-nitrophenyl) methyl ester, 2-propenoic acid, pentadecyl ester) and, etc. To the best of our knowledge, among the identified compounds, 14 compounds were bioactive, and the biological activities of the remaining 16 compounds have not been reported till today. Furthermore, it is possible that the synergistic action of secondary metabolites in the EA extract of these isolates was responsible for all the observed biological activities. The chemical structures of identified metabolites are presented in **Figure 7**, and each metabolite's MS profiles are displayed in **Figure S6–S34**

## Discussion

Multidrug-resistant organisms (MDROs) and cancer are major worldwide health issues, with increasing infections and treatment failures caused by resistance mechanisms such as enzymatic degradation and drug outflow. Recent research has shown an alarming rise in MDR bacteria such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, resulting in increased mortality and illness[43]. Similarly, cancer remains a significant concern despite advances in therapy. To overcome these difficulties, we have looked to natural sources, such as soil-derived actinomycetes, to uncover new bioactive compounds [44].The high-altitude actinomycetes investigated in this work revealed promising antibacterial and anticancer capabilities, indicating that these microorganisms, especially from underexplored places like the Nepalese Mountain, may give vital answers for generating novel therapeutic medicines [45]. Natural products, especially those derived from actinomycetes, provide attractive treatment possibilities for multidrug-resistant organisms (MDROs) and cancers. Actinomycetes generate a large number of bioactive metabolites, including antibiotics and anticancer drugs, many of which remain undiscovered[15]. In this work, strains obtained from Nepal's mountain areas, such as *Streptomyces* sp. PY40, PY118, and *Amycolatopsis* sp. PY122 has shown the ability to create new bioactive chemicals [46]. These harsh conditions, characterized by freezing temperatures and high UV radiation, promote the emergence of novel metabolites.

**Table 2**. List of volatile compounds identified putatively in EA extracts of *Streptomyces* sp. PY40, PY118, and *Amycolaptosis* sp.PY122.

| S.N | Annotated molecules | Retention time (min) | Molecular formula | Probability % | % Area | Source | Biological activity |
|---|---|---|---|---|---|---|---|
| 1 | Phenylethyl alcohol | 6.02 | $C_8H_{10}O$ | 82.8 | 0.30 | PY40 | Antimicrobial[26] |
| 2 | Benzene acetic acid | 9.14 | $C_8H_8O_2$ | 37.63 | 1.29 | PY40 | Antifungal, antibacterial activity [27] |
| 3 | Benzeneacetamide | 12.49 | $C_8H_9NO$ | 29.08 | 0.23 | PY40 | Not reported |
| 4 | 2,4-Di-tert-butylphenol | 15.38 | $C_{14}H_{22}O$ | 53.42 | 0.11 | PY40,PY122 | Antimicrobial [28] |
| 5 | Cetene | 17.21 | $C_{16}H_{32}$ | 8.11 | 0.11 | PY40, PY118 | Not reported |
| 6 | Dodecyl acrylate | 19.46 | $C_{15}H_{28}O_2$ | 22.03 | 0.18 | PY40,PY122 | Antimicrobial[29] |
| 7 | 1,4-diazabicyclo [4.3.0]nonan-2,5-dione, 3-methyl | 19.72 | $C_8H_{12}N_2O_2$ | 97.04 | 1.66 | PY40,PY118, PY122 | Antimicrobial [30,31]. |
| 8 | Pyrrolo[1,2-a] pyrazine - 1,4-dione, hexahydro | 20.71 | $C_7H_{10}N_2O_2$ | 76.9 | 1.57 | PY40,PY118 PY122 | Antimicrobial [32] |
| 9 | Furegrelate | 21.18 | $C_{15}H_{11}NO_3$ | 62.29 | 0.23 | PY40,PY118 | Not reported |
| 10 | Cyclo(L-prolyl-L-valine) | 22.09 | $C_{10}H_{16}N_2O_2$ | 97.75 | 14.40 | PY40,PY118, PY122 | Antibacterial [33] |
| 11 | 3,6-Diisopropyl piperazin-2,5-dione | 22.92 | $C_{10}H_{18}N_2O_2$ | 18.33 | 2.06 | PY40 | Not reported |
| 12 | Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- | 25.16 | $C_{11}H_{18}N_2O_2$ | 89.75 | 25.01 | PY40,PY118, PY122 | Anticancer [34] |
| 13 | 2,5-Piperazinedione, 3,6-bis(2-methyl propyl)- | 30.81 | $C_{12}H_{22}N_2O_2$ | 74.08 | 3.75 | PY40,PY118, PY122 | Antimicrobial, Antifungal [35,36] |
| 14 | 2,5-Piperazinedione, 3-benzyl-6-isopropyl | 32.54 | $C_{14}H_{18}N_2O_2$ | 93.87 | 1.56 | PY40,PY118, PY122 | Antinematode [37] |
| 15 | Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- | 34.73 | $C_{14}H_{16}N_2O_2$ | 88.84 | 23.15 | PY40,PY118 | Antifungal, anticancer [38] |
| 16 | Quinine di-N-oxide | 39.89 | $C_{20}H_{24}N_2O_4$ | 48.11 | 3.37 | PY40,PY118, PY122 | Not reported |
| 17 | Formic acid, (2-fluoro-5-nitrophenyl) methyl ester | 40.46 | $C_8H_6FNO_4$ | 16.33 | 7.32 | PY40,PY118, PY122 | Not reported |
| 18 | 2-Piperidinone | 7.30 | $C_5H_9NO$ | 80.64 | 2.02 | PY118,PY122 | Antimicrobial [39] |
| 19 | Benzene, (1,2-dibromoethyl)- | 13.25 | $C_8H_8Br_2$ | 29.33 | 0.75 | PY118 | Not reported |
| 20 | Pyrrolizin-1-one, 7-propyl | 13.98 | $C_{10}H_{17}NO$ | 24.92 | 1.35 | PY118 | Not reported |
| 21 | 1- Hexadecanol | 17.21 | $C_{16}H_{34}O$ | 5.05 | 0.57 | PY40, | Anticancer and antimicrobial [40] |
| 22 | 2-Propenoic acid, pentadecyl ester | 19.45 | $C_{18}H_{34}O_2$ | 8.34 | 0.96 | PY118, | Anticancer activity[41] |
| 23 | *dl*-Alanyl-l-leucine | 20.31 | $C_9H_{18}N_2O_3$ | 78.45 | 0.92 | PY118,PY122 | Anticancer[42] |
| 24 | Acetic acid, chloro-, octadecyl ester | 21.54 | $C_{20}H_{39}ClO_2$ | 6.69 | 0.66 | PY118 | Not reported |
| 25 | 7-Ethyl-4,6-pentadecandione | 22.80 | $C_{17}H_{32}O_2$ | 31.27 | 2.10 | PY118,PY122 | Not reported |
| 26 | Acetamide, N-(2-phenylethyl)- | 15.30 | $C_{10}H_{13}NO$ | 89.66 | 2.81 | PY122 | Not Reported |
| 27 | 2,5-Piperazinedione, 3-methyl-6-(phenyl methyl)- | 30.21 | $C_{12}H_{14}N_2O_2$ | 49.74 | 2.63 | PY118,PY122 | Not reported |
| 28 | 3-Isobutyl-2,5-piperazinedione | 20.955 | $C_8H_{14}N_2O_2$ | 54.71 | 0.33 | PY122 | Not reported |
| 29 | 3-Eicosene, (E)- | 21.550 | $C_{20}H_{40}$ | 4.75 | 0.26 | PY122 | Antimicrobial [29] |

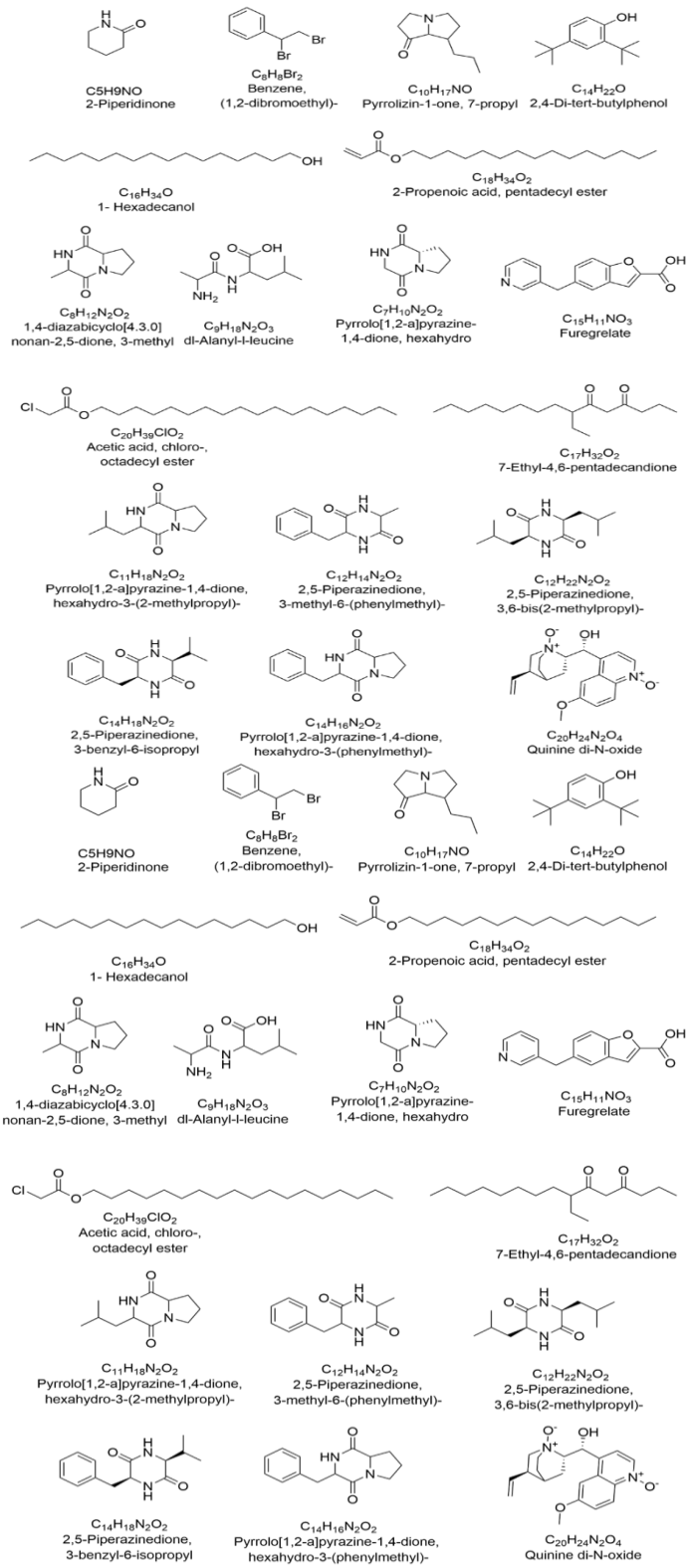



**Figure 7**. Chemical structures of putative compounds in *Streptomyces* species PY40, PY118, and *Amycolaptosis* sp.PY122

These isolates' bioassays demonstrated strong antibacterial and anticancer capabilities, indicating that high-altitude actinomycetes from Nepal's various habitats might be excellent sources for creating novel therapeutics to attack MDROs and malignancies [47].

The actinomycetes PY40, PY118, and PY122 were identified using 16S rRNA gene sequencing and phylogenetic analysis, which revealed that they are closely related to *Streptomyces durocortorensis* (94% similarity), *Streptomyces cyaneofuscatus* (94%), and *Amycolatopsis umgeniensis* (99%), respectively. These findings suggest that the isolates from Nepal's high-altitude locations may represent new actinomycete strains, particularly those with less than 97% similarity. *Amycolatopsis* sp. PY122 has the highest antibacterial activity against bacterial pathogens including *Shigella sonnei*, *Escherichia coli*, and *Staphylococcus aureus*, according to the bioassay data. *Streptomyces* sp. PY118 has the lowest antibacterial activity. The observed differences in antibacterial activity highlight the necessity for strain-specific study since various actinomycetes strains have distinct metabolic capacities. These results indicate the possibility that high-altitude Nepalese soils have microbial species capable of producing bioactive chemicals with medicinal uses. The isolated strains in this investigation displayed substantial antibacterial, antifungal, and anticancer properties, underlining their potential as viable candidates for innovative drug development.

A GC-MS analysis of EA extracts from *Streptomyces* isolates PY40, PY118, and *Amycolaptosis* sp. PY122 included a broad range of bioactive chemicals with high biological activity. Cyclo(L-prolyl-L-valine), a diketopiperazine recognized for its antibacterial properties, was one of the most prevalent metabolites. The study found that cyclo(L-prolyl-L-valine) may impair quorum sensing processes and inhibit virulence-associated genes in pathogens such as *Pseudomonas aeruginosa*. This implies that it might be used as a substitute or combined with standard antibiotics like streptomycin and penicillin[33]. Among the other vital compounds discovered was pyrrole[1,2-a] pyrazine-1,4-dione hexahydro, which has shown promise in treating MRSA by interfering with cell integrity and metabolic activities[48]. Despite their efficiency, traditional antibiotics are confronting resistance issues, necessitating the development of new antimicrobial substances.

These isolates included 1-hexadecanol, a long-chain fatty alcohol with antibacterial and cancer-treatment properties. It benefits integrated therapeutic strategies since it can potentially lower cancer cell growth and treat infections common in immunized cancer patients [49,50]. These actinomycete-derived compounds are therapeutically relevant because they include additional

natural product metabolites, such as benzene acetic acid and phenylethyl alcohol, which have antibacterial and antifungal properties[26,27]. Studies have indicated that pyrrolo[1,2-a]pyrazine-1,4-dione and its derivatives may improve chemotherapy efficacy, reduce adverse effects, and combat drug resistance [51–53].

Another antibacterial molecule with a distinct bicyclic structure, 1,4-Diazabicyclo[4.3.0]nonan-2,5-dione, 3-methyl, is also available, increasing the prospect of discovering novel antibiotics[30]. *Streptomyces* sp. PY40 and *Amycolaptosis* sp. PY122 produces compounds such as 2,4-Di-tert-butylphenol, exhibiting strong antifungal and antibacterial properties against *Staphylococcus aureus* and *Escherichia coli* [54,55]. The presence of another molecule, 2,5-piperazinedione, 3,6-bis(2-methylpropyl)-, in all thoursee isolates indicates that it may be effective in infection treatment owing to its broad-spectrum bioactivity including nematode [36].

Several of these compounds have shown interesting anticancer properties, including pyrrolo[1,2-a]pyrazine-1,4-dione hexahydro-3-(phenylmethyl). Because of its complex structure and the fact that it has been demonstrated to work against cancer cell lines such as MCF-7 and HeLa [38] , this metabolite is an appealing target for future study. Among the chemicals recovered from PY118, 1-hexadecanol had considerable anticancer activity in the EA extract, but 2-propenoic acid pentadecyl ester effectively inhibited the growth of HeLa and MCF-7 cells [40,41] . These findings highlight the potential of natural compounds derived from actinomycetes as novel cancer therapies.

This study highlights the necessity of discovering novel bioactive chemicals in high-altitude Nepalese actinomycetes such as *Streptomyces* sp. PY40, PY118, and *Amycolaptosis* sp. PY122. These strains' antibacterial, antifungal, and anticancer activities were attributed to pyrrolo[1,2-a]pyrazine-1,4-dione and cyclo(L-prolyl-L-valine). The findings indicate the efficacy of natural therapies in the battle against cancer and multidrug-resistant organisms. Future studies should concentrate on isolating and purifying these compounds and researching their synergistic effects to develop innovative medicines to address global health concerns.

## Conclusion

Actinomycetes isolates PY40, PY118, and PY122 create secondary metabolites with remarkable antifungal, anticancer, and antibacterial capabilities; this work highlights their potential. Except *Amycolatopsis* sp. PY122, the EA extract of these isolates, demonstrated antibacterial activity against drug-resistant bacterial strains additionally, several isolates, especially *Amycolatopsis* sp. PY122 suppressed fungal development. *Streptomyces* sp. PY40 was the most potent anticancer agent in experiments conducted on MCF-7 and HeLa cell lines, suggesting that it might be used to develop new cancer treatments. Furthermore, the diverse bioactive chemicals identified by GC-MS in these extracts lend credence to the notion that their biological actions result from a synergistic interaction. Our research shows that actinomycetes contain various chemicals and that we should look for new bioactive substances in natural settings like the Nepalese highlands. The discovery and isolation of these chemicals, followed by *in vivo* studies, should form the backbone of future research into their medicinal potential.

## Author's Contribution:

R.P.Y. performed microbiological experiments; R.P.Y., B.R.B., and R.B. drafted the manuscript; P.B. analyzed GC-MS data; M.R. performed anticancer assays; R.T.M. performed PCR and assisted with DNA sequencing; B.B.T. collected soil samples and reviewed literature; A.D.M. reviewed the manuscript; N.P. supervised the project and edited the manuscript.

## Competing Interests

The authors declare that there is no competing interest

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## Ethical Approval and Consent

We confirm that all microbial-related experiments were conducted as per established biosafety and biosecurity guidelines. Proper containment measures were implemented to ensure personnel safety and the environment.

## Data Availability

Data from this work will be provided to the corresponding author upon request

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# Supplementary Materials

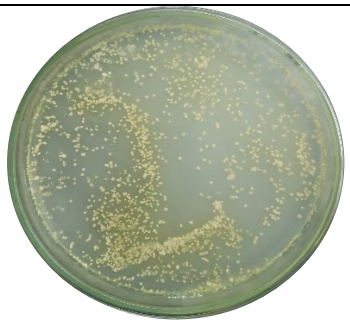
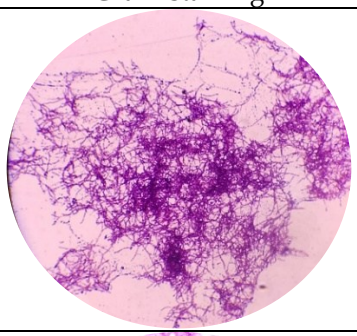
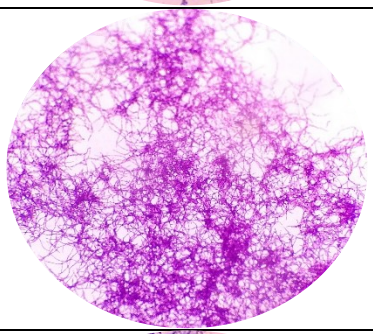
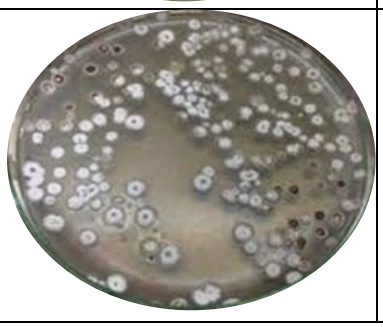
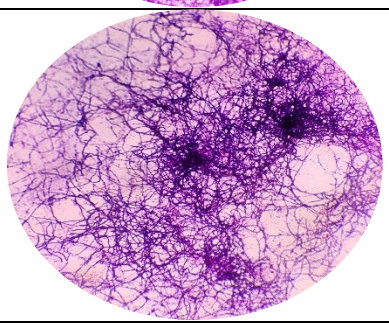
| Samples | Isolation | Subculture | Gram-staining |
|---|---|---|---|
| PY40 |  |  |  |
| PY118 |  |  |  |
| PY122 |  |  |  |

**Figure S1**. (A)Isolation of soil sample by using ISP4 media after a week of incubation, which represents the morphology of colonies (white or greyish white) (B) Subculture of those isolates by picking up the immersed colony for pure culture on the same media, (C) respective microscopic feature of mycelia at 100X oil immersion of four representative genera of actinobacteria isolates (PY40, PY118 and PY122)

## Antimicrobial Susceptibility Test

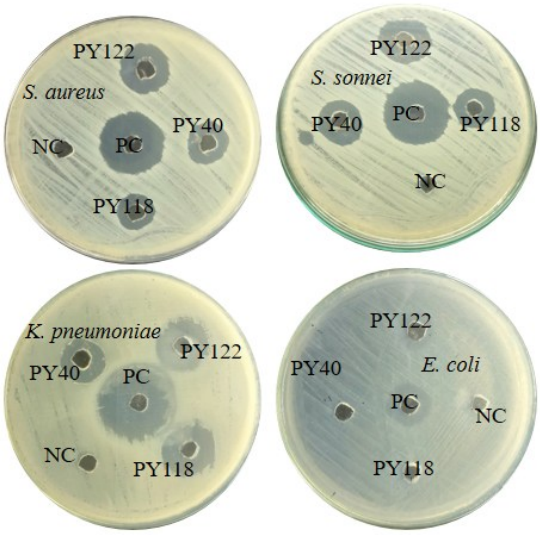



**Figure S2.** Antimicrobial assay of Actinobacteria PY40, PY118, and PY122 against *E. coli*, *Salmonella shigella*, *Staphylococcus aureus*, and *Klebsiella pneumonia*.

**Table S1**. Zone of inhibition of Actinobacteria PY40, PY118, and PY122 against tested pathogenic bacteria.

| Samples | Zone of Inhibition (in mm) | | | |
|---|---|---|---|---|
| | *Escherichia coli* ATCC 2591 | *Shigella sonnei* ATCC 25931 | *Staphylococcus aureus* ATCC 43300 | *Klebsiella pneumoniae* ATCC 700603 |
| *Streptomyces* sp. PY40 | 10 | 15 | 17 | 18 |
| *Streptomyces* sp. PY118 | 9 | 15 | 14 | 17 |
| *Amycolatopsis* sp. PY122 | 11 | 20 | 18 | 20 |
| Neomycin | 13 | 23 | 22 | 28 |

## Minimum Inhibitory Concentration (MIC) and MBC (Minimum Bactericidal Concentration)

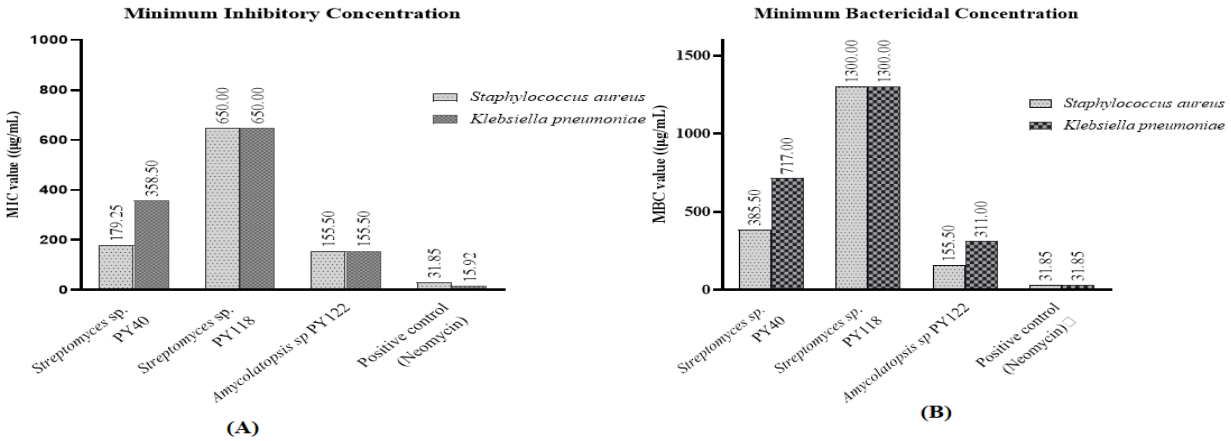



**Figure S3**. (A) MIC values and (B) MBC values of *Streptomyces* sp. PY40, PY118, and PY122 against tested bacterial strains.

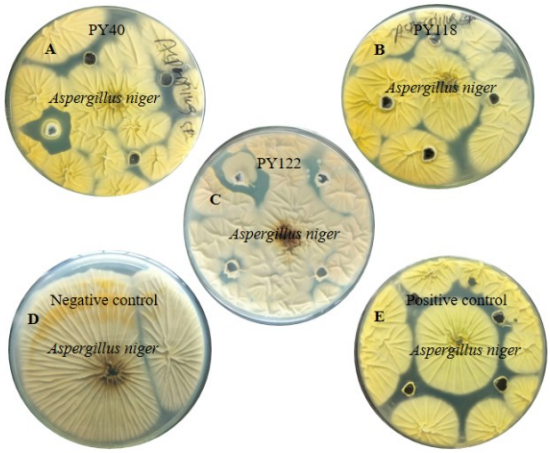



**Figure S4.** Antifungal activity against *Aspergillus niger* (A) *Aspergillus niger* on *Streptomyces* sp. PY40, (B) *Aspergillus niger* on *Streptomyces* sp. PY118, (C) *Aspergillus niger* on *Amycolaptosis* sp. PY122, (D) *Aspergillus niger* on cycloheximide (positive control), and (E) Negative control.

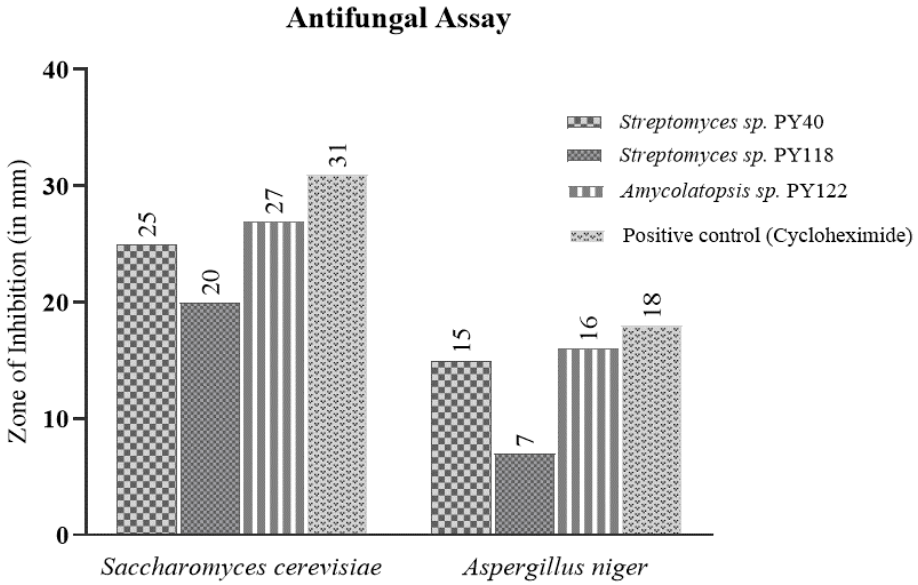



**Figure S5.** Antifungal activity of actinomycetes PY40, PY118, and PY122.

**Table S2.** MIC and MBC values of Actinobacteria PY40, PY118, and PY122 against tested bacterial strains.

| Tested bacterial strains | *Streptomyces* sp. PY40 | | *Streptomyces* sp. PY118 | | *Amycolatopsis* species PY122 | | Positive Control | |
|---|---|---|---|---|---|---|---|---|
| | MIC (µg/mL) | MBC (µg/mL) | MIC (µg/mL) | MBC (mg/mL) | MIC (µg/mL) | MBC (µg/mL) | MIC (µg/mL) | MBC (µg/mL) |
| *Staphylococcus aureus* ATCC 43300 | 179.25 | 385.50 | 650 | 1.3 | 155.5 | 155.50 | 31.85 | 31.85 |
| *Klebsiella pneumoniae* ATCC 700603 | 358.5 | 717 | 650 | 1.3 | 155.5 | 311 | 15.92 | 31.85 |

**Table S3.** Antifungal activity of Actinobacteria PY40, PY118, and PY122.

| Fungal mycelium | Zone of Inhibition (in mm) | | | |
|---|---|---|---|---|
| | *Streptomyces* sp. PY40 | *Streptomyces* sp. PY118 | *Amycolatopsis* species PY122 | Positive control ( cycloheximide) |
| *Saccharomyces cerevisiae* | 25 | 20 | 27 | 31 |
| *Aspergillus niger* | 15 | 7 | 16 | 18 |

Peak @ 6.025 Area 540978.847 Area % 0.30

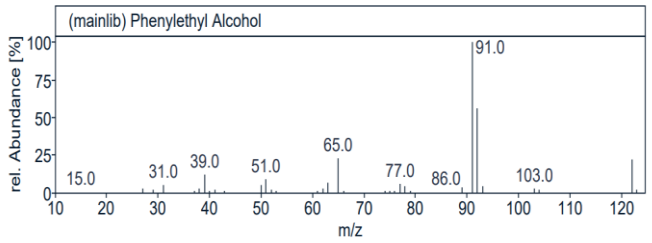



**Figure S6.** GC-MS profile of Phenylethyl alcohol

Peak @ 9.140 Area 2298849.491 Area % 1.29

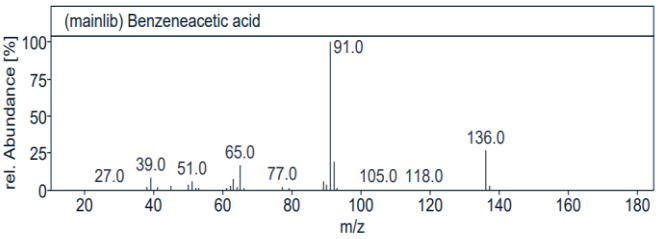



**Figure S7.** GC-MS profile of Benzeneacetic acid

Peak @ 12.498 Area 410746.888 Area % 0.23

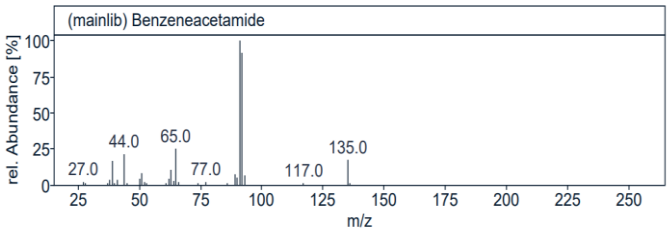



**Figure S8.** GC-MS profile of Benzeneacetamide

Peak @ 15.385 Area 199697.078 Area % 0.11

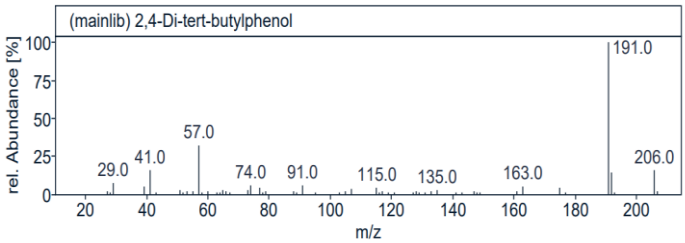



**Figure S9.** GC-MS profile of 2, 4 -Di-tert-butylphenol

Peak @ 17.210 Area 194978.971 Area % 0.11

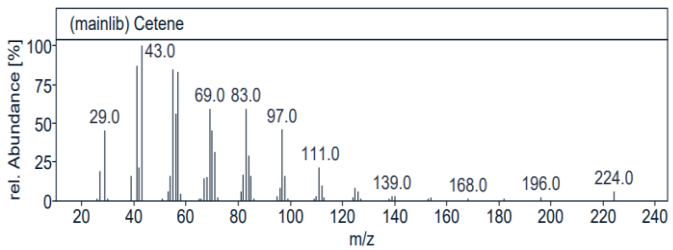



**Figure S10.** GC-MS profile of Cetene

Peak @ 19.460 Area 313383.541 Area % 0.18

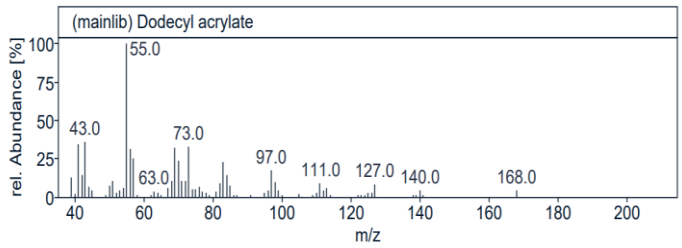



**Figure S11.** GC-MS profile of Dodecyl acrylate

Peak @ 19.723 Area 2950052.462 Area % 1.66

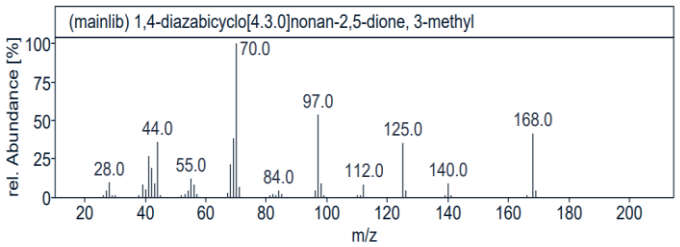



**Figure S12.** GC-MS profile of 1, 4-diazabicyclo [4.3.0]nonan-2, 5-dione, 3-methyl

Peak @ 20.716 Area 2785453.082 Area % 1.57

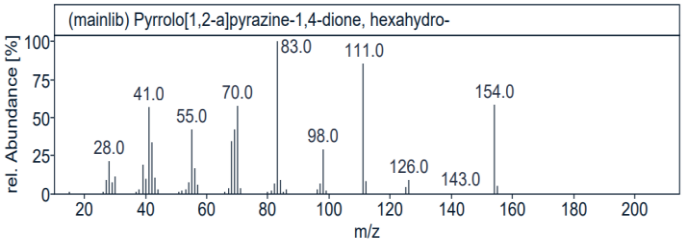



**Figure S13.** GC-MS profile of Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro

Peak @ 21.184 Area 407222.769 Area % 0.23

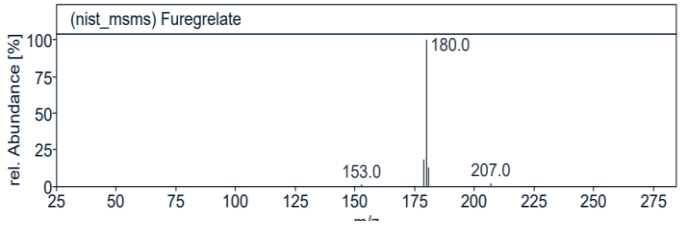



**Figure S14.** GC-MS profile of Furegrelate

Peak @ 22.924 Area 3669047.544 Area % 2.06

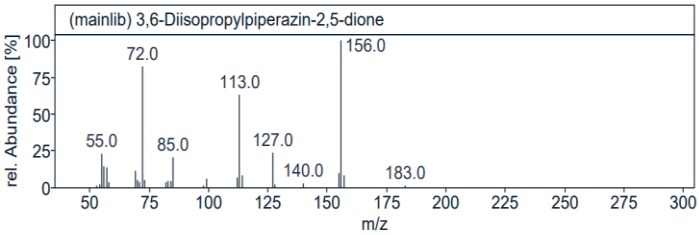



**Figure S15.** GC-MS profile of 3,6-Diisopropylpiperazin-2,5-dione

Peak @ 22.097 Area 25629534.838 Area % 14.40

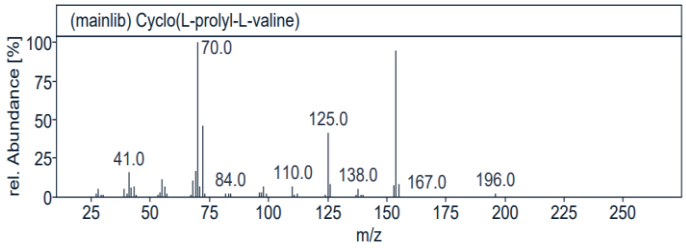



**Figure S16.** GC-MS profile of Cyclo(L-prolyl-L-valine)

Peak @ 30.817 Area 6677033.448 Area % 3.75

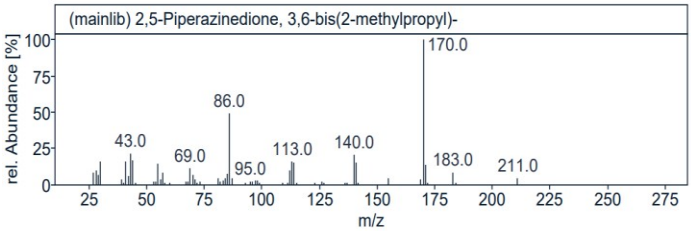



**Figure S17.** GC-MS profile of 2, 5-Piperazinedione, 3,6-bis(2-methylpropyl)-

Peak @ 32.546 Area 2779844.968 Area % 1.56

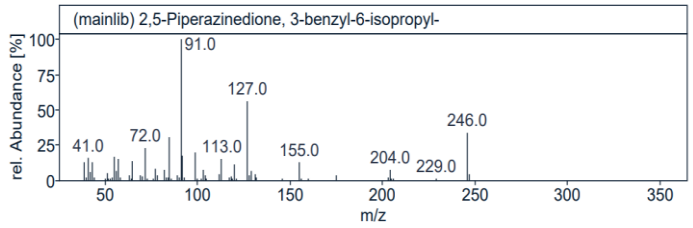



**Figure S18.** GC-MS profile of 2, 5-Piperazinedione, 3-benzyl-6-isopropyl

Peak @ 25.166 Area 44518396.091 Area % 25.01

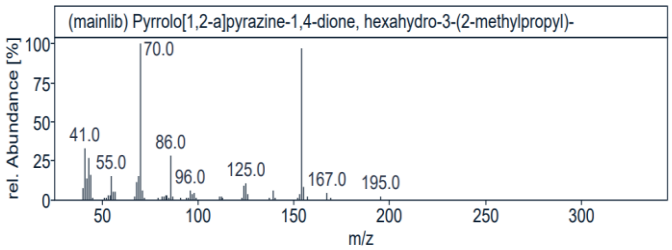



**Figure S19.** GC-MS profile of Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-

Peak @ 34.734 Area 41202678.091 Area % 23.15

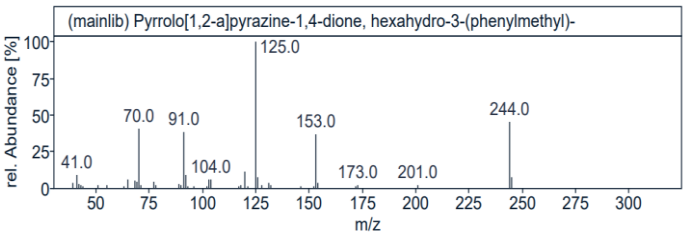



**Figure S20.** GC-MS profile of Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)-

Peak @ 39.895 Area 5998779.668 Area % 3.37

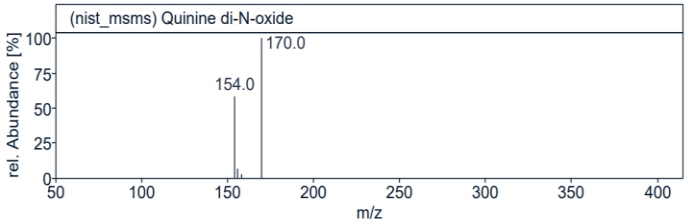



**Figure S21.** GC-MS profile of Quinine di-N-oxide

Peak @ 40.467 Area 13025265.133 Area % 7.32

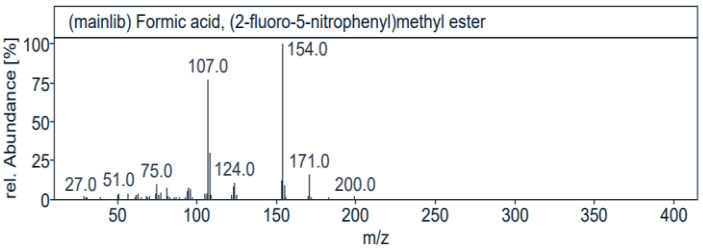



**Figure S22.** GC-MS profile of Formic acid, (2-fluoro-5-nitrophenyl) methyl ester

Peak @ 7.301 Area 1146733.053 Area % 2.02

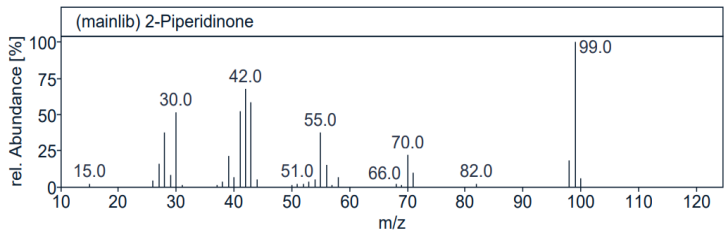



**Figure S23.** GC-MS profile of 2-Piperidinone\

Peak @ 13.256 Area 428193.401 Area % 0.75

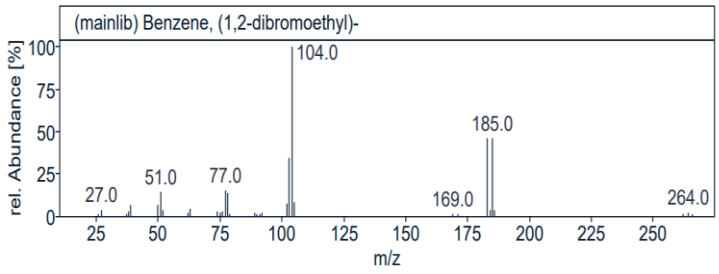



**Figure S24.** GC-MS profile of Benzene, (1, 2-dibromoethyl)-

Peak @ 13.983 Area 765329.201 Area % 1.35

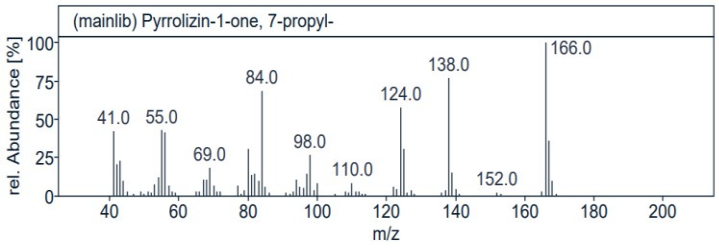



**Figure S25.** GC-MS profile of Pyrrolizin-1-one, 7-propyl

Peak @ 17.211 Area 322630.119 Area % 0.57

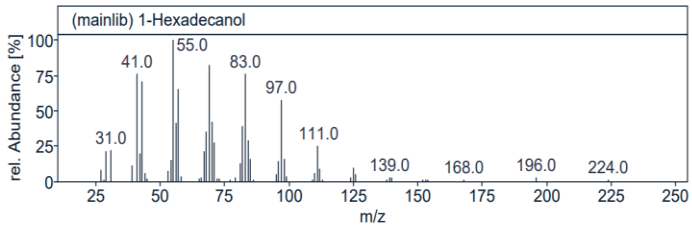



**Figure S26.** GC-MS profile of 1-Hexadecanol

Peak @ 19.458 Area 544569.873 Area % 0.96

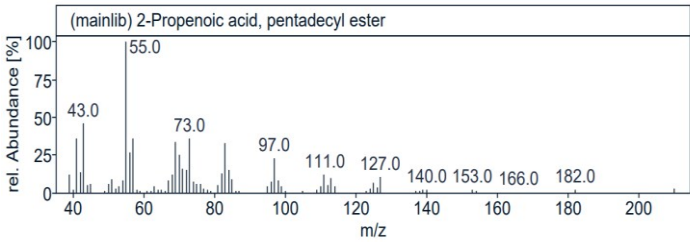



**Figure S27.** GC-MS profile of 2-Propenoic acid, pentadecyl ester

Peak @ 20.311 Area 520106.942 Area % 0.92

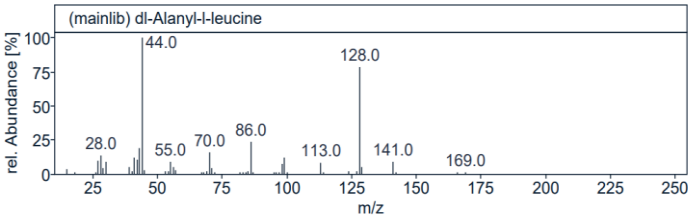



**Figure S28.** GC-MS profile of dl-Alanyl-l-leucine

Peak @ 21.552 Area 372836.655 Area % 0.66

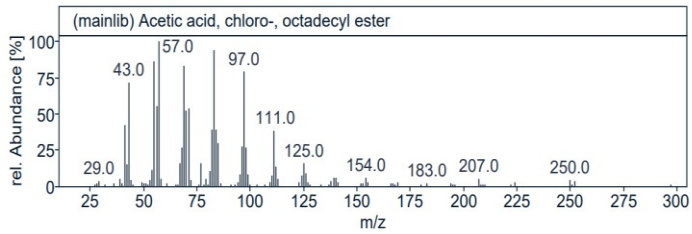



**Figure S29.** GC-MS profile of Acetic acid, chloro-, octadecyl ester

Peak @ 22.805 Area 1193124.171 Area % 2.10

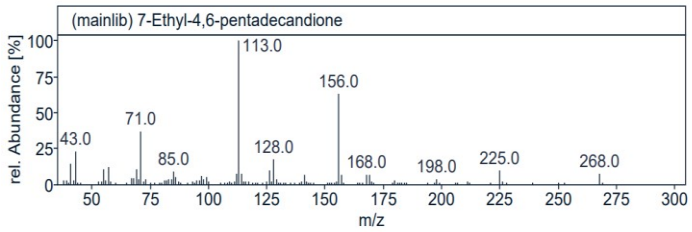



**Figure S30.** GC-MS profile of 7-Ethyl-4, 6-pentadecandione

Peak @ 30.210 Area 1493260.246 Area % 2.63

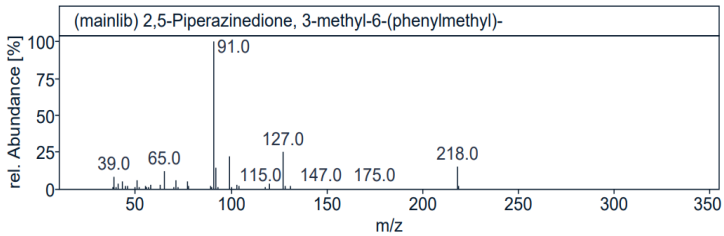



**Figure S31.** GC-MS profile of 2, 5-Piperazinedione, 3-methyl-6-(phenylmethyl)-

Peak @ 15.308 Area 3340786.126 Area % 2.81

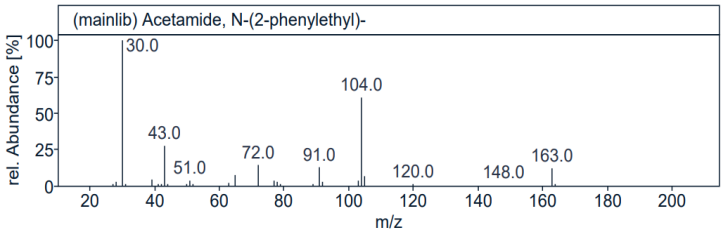



**Figure S32.** GC-MS profile of Acetamide, N-(2-phenylethyl)-

Peak @ 20.955 Area 391064.628 Area % 0.33

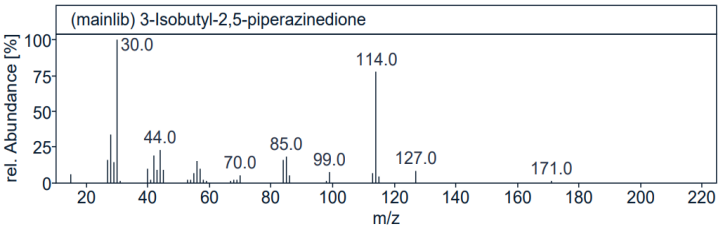



**Figure S33.** GC-MS profile of 3-Isobutyl-2, 5-piperazinedione

Peak @ 21.550 Area 304092.771 Area % 0.26

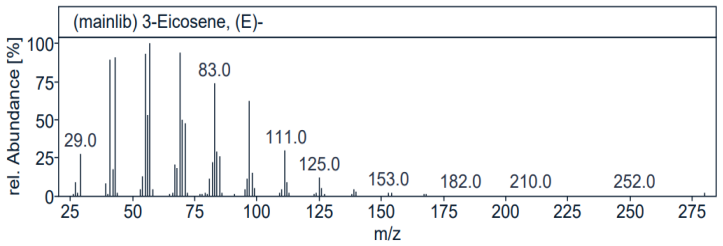



**Figure S34.** GC-MS profile of 3-Eicosene, (E)-