

Resazurin based rapid screening and Spectrophotometric analysis of potent antimicrobials from *Streptomyces* isolated in Nepal

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Abstract

Background The rapid emergence of bacterial resistance and decreased efficacy of most antibiotics is a burning issue in the present medical world demanding swift development of novel classes of potent antibiotics. This study focuses on the robust assay of screening antimicrobials speeding up the antibiotics discovery process.

Results Putative *Streptomyces* designated as LAF4 isolated from a soil sample of Lamjung, mid-hills of Nepal, screened from 141 putative *Streptomyces* isolates had the highest inhibitory effect among all the strains that showed inhibition potential against two of the 'critical' WHO prioritized pathogens including *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The results from Resazurin based assays were all verified using broth assays for antimicrobial studies. The proposed method was efficient, easy to observe and gave results a lot faster than the conventional antimicrobial study. Also, a novel spectrophotometric analysis method has been proposed to access the potency of thus screened antimicrobials. GCMS analysis of LAF4 extract indicated the presence of 2,3-butanediol, Naphthalene, and Tributyl phosphate as major constituents exhibiting antimicrobial properties whereas Pyrrolo (1, 2- a) pyrazine -1,4-dione, hexahydro-3-(2-methylpropyl)- which is an antioxidant and an anticancer agent was also recorded.

Conclusion The present study validates and exhibits the time and cost effective resazurin based antimicrobial assay as the best one, for preliminary screening of antimicrobial producers compared to conventional disc/well diffusion assays since very small concentration of antimicrobials irrespective of their polarity can be detected unlike the latter methods.

Background

The "Patent Cliff" (1) experienced by pharma-industries have not been able to bring adequately new antibiotics (2). The continued overuse and misuse of antibiotics that resulted in resistance (3) is further aggravated by emergence of multi-drug resistant (MDR) bugs (4) even to different last line of drugs (5) (6) and collective resistance to these arsenals (7). Historical serendipitous discoveries was the milestone in treating infections (8) followed by incremental modifications of the chemical scaffolds of the drugs based on mechanisms of inhibition after the 70's (9) that now appears to have exhausted and natural products have regained momentum (10), but with limited success (11).

The excessive time and cost parameters required to develop a drug, emphasized the search of time and cost effective protocols for preliminary screening of antimicrobials. For this purpose, Resazurin based microtitre-plate assay as described by Drummond and Waigh in 2000 was modified to develop a simple, rapid, sensitive and cost-effective *in vitro* antimicrobial assay. This method uses Resazurin, an indicator which detects the microbial growth based on its reduction potential by the reductases produced by viable cells.

Resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) is a non-toxic, water soluble blue non-fluorescent dye which is converted into pink fluorescent resorufin (7-hydroxy-3H-phenoxazin-3-one) in

the presence of viable cells with active metabolism (12). Reductases of viable cells reduce resazurin resulting in the formation of its highly fluorescent metabolic product resorufin. This reduction is proportional to the number of metabolically active cells present (13).

The present study aims to screen potent antimicrobials from *Streptomyces* extracts isolated from various parts of Nepal based on Resazurin based antimicrobial assay and spectrophotometric analysis.

Results And Discussion

Screening of *Streptomyces* and antimicrobial potential of cell culture extracts

Putative *Streptomyces* were screened based on the typical morphology, chalky non-mucoid colonies (Figure 1A and 1B) as recommended by Shirling and Gottlieb (14) with varying aerial and substrate mycelia colors (data not shown). Using ISP media for *Streptomyces* (15), a total of 141 colonies were isolated. Out of these, 98 isolates were further pursued by screening out the look-alike isolates depending upon the culture and morphological characteristics (Figure 1C and 1D) including aerial and substrate mycelia colors, septa formation, pattern of growth and so on.

Most of the concentrated extracts did not show antimicrobial potential against *K. pneumonia* and *P. aeruginosa* in agar disc diffusion test (data not shown) whereas some did show insignificant inhibitory zones.

Upon accessing the antimicrobial properties of the 14 selected colored culture extracts against the test pathogens, all 14 (Supplementary Table 1) showed potential inhibition against them to certain extent indicating that there could be compounds with antimicrobial potential in all of these extracts.

Resazurin antimicrobial assay and confirmation of isolate

A new spectrophotometric analysis strategy has been proposed to access antimicrobial potency of compounds in the mixture based on oxidation of resazurin as described in materials and methods. Depending upon the absorbance at 550 nm, culture extracts of isolates LAF4, LAD1, LAE5 and HA5 showed relatively better antimicrobial potencies among the test strains. Extracts from LAF4 showed highest inhibitions against *K. pneumoniae* and *P. aeruginosa*. Also, LAF4, LAD1 LAE5 and HA5 showed similar or better antibacterial properties against these pathogens used at 20 mg/ml than Ampicillin and Kanamycin used at 50 mg/ml (Figure 2). Among the tested, the extracts of the isolate KH8 showed the least inhibition potential against these test organisms.

The isolated putative *Streptomyces* were confirmed by PCR amplification of the genomic DNA using StrepB/StrepE primer pairs designed specifically to identify *Streptomyces* (16) which gave bands corresponding to 520 bp indicating that the isolates were *Streptomyces* sp. Furthermore, partial

sequencing of the 16s rRNA genomic sequence of LAf4 and blast search among the Streptomyces revealed that the strain showed 87% identity out of 99% query cover with *Streptomyces flavoviridis*.

Gas chromatography–Mass spectrometry (GC-MS) analysis

The inhibitory potential of the LAf4 crude extract was further analyzed for the constituent compounds that could have potential antimicrobial properties by GCMS. The most probable compounds indicated through the NIST library revealed that major constituents were antimicrobials (Table 1).

Discussion

Streptomyces are known to produce metabolites that act as antimicrobials and they also produce indole derivatives as antimicrobials (17). An ecologically diverse country Nepal, 49th in the whole world (18), built through the rising of seabed from tectonic movement (19) could harbor different Streptomyces including marine which could produce potent secondary metabolites including antimicrobials. Thus, soil samples from 24 different parts of Nepal were collected for screening of *Streptomyces* species.

The isolates were cultured as described in materials and methods. The cell free culture media was further extracted using ethyl acetate as has been performed to extract metabolites produced by *Streptomyces* (20) to obtain polar and non-polar secondary metabolites and preferred also because of its minimal toxicity to test organisms (21). Ethyl acetate was evaporated and the extract was concentrated as described to test its anti-microbial potential.

The insignificant inhibitory zones during agar diffusion antimicrobial susceptibility tests could indicate that there were some antimicrobial compounds whose concentrations were either low or, the insolubility in agar due to polarity (22) could have hindered the movement and efficacy of the potential antibacterial molecules in the medium.

Moreover, in the present study the search of novel and effective antimicrobials were pursued based on color forming isolates because colored indole derivatives, indirubin and isatin, have been reported as the major constituents of plant and microbial extracts with potent antimicrobial properties (23,24,25).

Since the world is in desperate need of novel antibiotics (26), even the extracts with small amount of antimicrobials should be considered as the source of new drug candidate as these could be novel and could function against the emerging resistance. Thus, effective and robust assay system should be developed where factors like polarity of the compound should not be stumbling block in antimicrobial assay. A robust and faster screening protocol for detecting antimicrobials is thus of dire need for which Resazurin based microbial viability assay could be modified for antimicrobial screening.

During antimicrobial potency screening, the antioxidant property of the crude extract could be of concern. Since most antibiotics at present are known to produce reactive oxygen species (ROS) in killing the bacterial cells (27) (28) and if the mechanism of the inhibition could be through ROS then the antioxidant properties of the extract could hinder antimicrobial potential (29). Thus, antioxidant properties of these extracts were also investigated. Extracts from KH8 that had the lowest antimicrobial potential had the strongest antioxidant property among all the extracts tested (results not shown). Thus antimicrobial potency of the compound included in the extract could have been affected if the bactericidal effect was due to ROS formation. However this needs further validation. Moreover, in future screening of potential antimicrobials, the ROS quenching effect of the compound or extract should also be taken in consideration.

Some of the compounds reported in GCMS analysis were non-polar and their movement in agar could have been hindered (30). Thus, when screening for antimicrobials, one should consider the polarity of the compounds where resazurin based assay could be more potent. In addition, the assay could be done in 384 plates and robotics could be used to screen vast library of compounds or multiple extracts (31) (32).

Moreover, the extract contained a mixture of antioxidant compounds as well and could have impacted in antimicrobial potency of the compound(s) in the extract by mitigating ROS produced by the antimicrobials. In addition to other known antimicrobial compounds, the extract also contained pyrrolo (1, 2- a) pyrazine –1, 4-dione, hexahydro–3-(2-methylpropyl) (PPDHMP) that has not yet been reported as an antimicrobial agent to the best of our knowledge. The pyrrolo pyrazine scaffold in the ring structure (Figure 3) that could make it a probable antimicrobial agent by inhibiting the activities of S-adenosyl methionine utilizing enzymes (essential proteins) since it is an anticancer agent and most probable function could be due to the kinase inhibition and warrants further study. Furthermore, compound purification and antimicrobial tests against drug resistant strains could be done to access their inhibitory potential against increasing resistant strains.

Conclusion

The study describes the methods in improving the conventional methods involved in drug research and development mostly in terms of cost and time effectiveness. Thus, emphasis should be done on the more accurate, sensitive and simpler Resazurin based antimicrobial assays compared to conventional agar diffusion assays. The methods described, could drastically reduce the time and resource expenses involved in preliminary stages of drug development process.

Materials

Test tubes, Petriplates, Micropipettes and sterile tips of various sizes 10, 100, and 1000 μ l, 10 and 100 μ l multichannel pipettes, Incubator at 28 and 37 °C, Separating funnel, Rotavapor, Resazurin, Microtiter plates, Microtiter plate reader, ISP2, ISP4 and Mueller Hinton growth media, Antibiotics: Cycloheximide,

Ampicillin, Kanamycin; American type culture collection (ATCC) strains: *Klebsiella pneumoniae* ATCC BAA-1705, *Pseudomonas aeruginosa* ATCC 15442

Methods

Soil sample selection

A total of 24 different soil samples among all the samples, collected and stored in Central Department of Biotechnology (CDBT), Tribhuvan University (TU) was used for the isolation of Streptomycetes including those collected from river banks, forest areas, dumping site areas, varying altitudes, etc. such that it covered all the geographical regions of Nepal with variable environmental conditions.

Isolation

The air-dried soil samples were serially diluted in sterile distilled water and 100 µl of 10⁻⁴ dilutions was plated on ISP2 and ISP4 among all the International Streptomyces Project (ISP) medias recommended by Shirling and Gottlieb (Shirling and Gottlieb, 1996), using spread plating techniques. ISP2 is a general media with glucose as a common carbon source and yeast and malt extracts as other nutrient sources whereas ISP4 is more selective for *Streptomyces* screening. The agar plates were supplemented with 50µg/ml cycloheximide, an antifungal agent. The plates were incubated at 28°C for 2 weeks. The distinct strains were then selected based on their colony characteristics and subcultured on ISP2 agar plates for pure culture.

Secondary metabolites production

The putative Streptomycetes screened based on their colony characteristics were cultured in 50 ml ISP2 broths (pH=7) at 30°C for 7 days. The secondary metabolites containing media was then filtered out, separated using ethyl acetate in a separating funnel and concentrated using rotavapor at 38°C. All the crude extracts were dissolved in methanol such that their final concentration becomes 15 mg/ml and stored at -20°C until further use.

Preparation of bacterial culture / Standardization of inoculum

EUCAST guidelines (33) were considered for antimicrobial susceptibility testing. The test organisms were grown in Mueller Hinton broth overnight (16–24 hrs of incubation). The inoculums of test organisms were maintained to the density of a McFarland 0.5 standard, approximately corresponding to 1–2*10⁸ CFU/ml before the susceptibility tests to ensure the consistency of all the tests performed. McFarland 0.5

standard may not give a standardized number of colony forming units for all the strains however, this was not resolved since standardization was insignificant in this study.

Preparation of Resazurin solution

1% Resazurin solution was prepared in sterile distilled water and stored at 4°C until further use. The final concentration of the resazurin solution was made 0.02% during the assay.

Preparation of Resazurin assay microtitre-plates

In 96-welled microtiter plates, 83 µl of bacterial culture along with 15 µl extract (20 mg/ml) and 2 µl of 1% resazurin solution was kept in each well such that the final volume was 100 µl in each well and final resazurin concentration becomes 0.02%. Columns 9 and 11 contained Ampicillin (50 mg/ml) and Kanamycin (50 mg/ml) at the first well, with the respective two-fold dilutions on the corresponding wells below such that the concentrations of the antibiotics used from the 1st well to the 8th becomes 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, 1.56 mg/ml, 0.781 mg/ml and 0.39 mg/ml respectively. The wells containing only MHB broth and Resazurin (1st column) without the addition of bacteria were considered as positive controls for AST and the wells containing only resazurin and test organism without the addition of extracts (8th column) were considered negative controls for AST (no bacterial inhibition). Both controls were essential to validate the experiment. Columns 3, 6 and 10 were left blank to prevent probable cross-contamination and increase the accuracy of the test.

Spectrophotometric readings

The color change from violet resazurin to pink resorufin in negative controls was considered the end point of the assay and the spectrophotometric reading was taken in microtiter plate reader at 550 nm.

$$\% \text{ inhibition} = \frac{\text{O. D reading with test extract}}{(\text{Average of positive controls} - \text{Average of negative controls})} \times 100 \%$$

All the experiments were performed under strict aseptic conditions.

Molecular Identification and Confirmation of isolated putative *Streptomyces*

Genomic DNA was extracted from the putative *Streptomyces* strains by using Tiangen Kit and were used as templates for the 16S rRNA genomic DNA sequencing and polymerase chain reaction (PCR) amplification by *Streptomyces* specific StrepB/StrepE primer pairs.

Declarations

FDA- Food and Drug administration

GC-MS—Gas Chromatography Mass Spectroscopy

PPDHMP - Pyrrolo (1, 2- a) pyrazine –1, 4-dione, hexahydro–3-(2-methylpropyl)

ROS—Reactive Oxygen Species

WHO—World Health Organization

- Ethics approval and consent to participate—Not applicable
- Consent for publication—Not applicable
- Availability of data and materials—Not applicable
- Competing interests—“The authors declare that they have no competing interests”
- Funding—Research works have been funded by the University Grants Commission, Nepal.
- Author’s contributions

S.T and S.S did major works including screening of *Streptomyces*, secondary metabolite productions and antimicrobial susceptibility tests. S.T and P.A were major contributors in writing the manuscript. P.A, H. B. and R.M designed the overall outline of the whole project, R.M leading the project. Most of the results analysis parts were performed by S.T and P. A. S.G and S.M assisted during sample collection, PCR, antimicrobial assay, culture of test organisms and antimicrobial susceptibility testing of test pathogens.

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References

1. Calo-Fernández B, Martínez-Hurtado JL. Biosimilars: Company strategies to capture value from the biologics market. *Pharmaceuticals*. 2012;
2. Conly JM, Johnston BL. Where are all the new antibiotics? The new antibiotic paradox. *Canadian Journal of Infectious Diseases and Medical Microbiology*. 2005.
3. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. *P T A peer-reviewed J Formul Manag*. 2015;40(4):277–83.

4. Unemo M, Nicholas RA. Emergence of multidrug-resistant, extensively drug-resistant and untreatable gonorrhoea. *Future Microbiology*. 2012.
5. Morrill HJ, Pogue JM, Kaye KS, LaPlante KL. Treatment options for carbapenem-resistant Enterobacteriaceae infections. *Open Forum Infect Dis*. 2015;
6. Goli HR, Nahaei MR, Rezaee MA, Hasani A, Kafil HS, Aghazadeh M. Emergence of colistin resistant *Pseudomonas aeruginosa* at Tabriz hospitals, Iran. *Iran J Microbiol*. 2016;
7. Chen S, Hu F, Zhang X, Xu X, Liu Y, Zhu D, et al. Independent emergence of colistin-resistant Enterobacteriaceae clinical isolates without colistin treatment. *Journal of Clinical Microbiology*. 2011.
8. Ban TA. The role of serendipity in drug discovery. *Dialogues Clin Neurosci*. 2006;
9. Wright GD. The antibiotic resistome: The nexus of chemical and genetic diversity. *Nature Reviews Microbiology*. 2007.
10. Butler MS. The role of natural product chemistry in drug discovery. In: *Journal of Natural Products*. 2004.
11. Ganesan A. The impact of natural products upon modern drug discovery. *Curr Opin Chem Biol*. 2008;
12. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, et al. Cell Viability Assays. *Assay Guid Man [Internet]*. 2013;114(8):785–96.
13. Van den Driessche F, Rigole P, Brackman G, Coenye T. Optimization of resazurin-based viability staining for quantification of microbial biofilms. *J Microbiol Methods*. 2014;98(1):31–4.
14. Shirling EB, Gottlieb D. Methods for Characterization of *Streptomyces* species. *Int J Syst Bacteriol*. 1996;16(3):313–40.
15. Kim Y-P, Tomoda H, Iizima K, Fukuda T, Matsumoto A, Takahashi Y, et al. Takanawaenes, novel antifungal antibiotics produced by *Streptomyces* sp. K99–5278. I. Taxonomy, fermentation, isolation and biological properties. *J Antibiot (Tokyo)*. 2003;56(5):448–53.
16. Rintala H, Nevalainen A, Rönkä E, Suutari M. PCR primers targeting the 16S rRNA gene for the specific detection of streptomycetes. *Mol Cell Probes*. 2001;
17. Zhang J, Wang JD, Liu CX, Yuan JH, Wang XJ, Xiang WS. A new prenylated indole derivative from endophytic actinobacteria *Streptomyces* sp. neau-D50. *Nat Prod Res*. 2014;
18. Rhett A. Butler. The top 10 most biodiverse countries [Internet]. Mongabay. 2016 [cited 2018 Jan 18]. Available from: <https://news.mongabay.com/2016/05/top-10-biodiverse-countries/>
19. Lavé J, Avouac JP. Fluvial incision and tectonic uplift across the Himalayas of central Nepal. *J Geophys Res Solid Earth [Internet]*. 2001;106(B11):26561–91. Available from: <http://doi.wiley.com/10.1029/2001JB000359>
20. English AL, Boufridi A, Quinn RJ, Kurtböke DI. Evaluation of fermentation conditions triggering increased antibacterial activity from a near-shore marine intertidal environment-associated *Streptomyces* species. *Synth Syst Biotechnol*. 2017;

21. Wilbanks B, Trinh CT. Comprehensive characterization of toxicity of fermentative metabolites on microbial growth Mike Himmel. *Biotechnol Biofuels*. 2017;10(1).
22. Klančnik A, Piskernik S, Jeršek B, Možina SS. Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. *J Microbiol Methods*. 2010;81(2):121–6.
23. Krivogorsky B, Grundt P, Yolken R, Jones-Brando L. Inhibition of *Toxoplasma gondii* by indirubin and tryptanthrin analogs. *Antimicrob Agents Chemother*. 2008;52(12):4466–9.
24. Al-Dhabi NA, Balachandran C, Raj MK, Duraipandiyan V, Muthukumar C, Ignacimuthu S, et al. Antimicrobial, antimycobacterial and antibiofilm properties of *Couroupita guianensis* Aubl. fruit extract. *BMC Complement Altern Med*. 2012;12.
25. Fogaça MV, Cândido-Bacani P de M, Benicio LM, Zapata LM, Cardoso P de F, de Oliveira MT, et al. Effects of indirubin and isatin on cell viability, mutagenicity, genotoxicity and BAX/ERCC1 gene expression. *Pharm Biol*. 2017;55(1):2005–14.
26. Coates AR, Halls G, Hu Y. Novel classes of antibiotics or more of the same? *British Journal of Pharmacology*. 2011.
27. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. A Common Mechanism of Cellular Death Induced by Bactericidal Antibiotics. *Cell*. 2007;
28. Dwyer DJ, Kohanski MA, Collins JJ. Role of reactive oxygen species in antibiotic action and resistance. *Current Opinion in Microbiology*. 2009.
29. Vatansever F, de Melo WCMA, Avci P, Vecchio D, Sadasivam M, Gupta A, et al. Antimicrobial strategies centered around reactive oxygen species - bactericidal antibiotics, photodynamic therapy, and beyond. *FEMS Microbiology Reviews*. 2013.
30. Pimentel RB de Q, da Costa CA, Albuquerque PM, Junior SD. Antimicrobial activity and rutin identification of honey produced by the stingless bee *Melipona compressipes manausensis* and commercial honey. *BMC Complement Altern Med*. 2013;
31. Michael S, Auld D, Klumpp C, Jadhav A, Zheng W, Thorne N, et al. A Robotic Platform for Quantitative High-Throughput Screening. *Assay Drug Dev Technol*. 2008;
32. Forry SP, Madonna MC, López-Pérez D, Lin NJ, Pasco MD. Automation of antimicrobial activity screening. *AMB Express*. 2016;
33. Matuschek E, Brown DFJ, Kahlmeter G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clin Microbiol Infect*. 2014;20(4).

Tables

Table 1 GC-MS results of extract LAf4

S.N	Probable compound names	Retention time (min)	Area (%)	Quality (%)	Activity	Color and Polarity	Reference
1	2,3-butanediol	3.025	30.61	91	Antibacterial and Antiseptic	Colorless	(Ragasa <i>et al.</i> , 2004)
2	Naphthalene	9.566	0.42	96	Antibacterial	White, Non polar	(Rokade and Sayyed, 2009)
3	2,4-Di-tert-butylphenol	10.39	1.07	95	Antioxidant Quorum sensing inhibitor	Colorless, Non polar	(Yoon <i>et al.</i> , 2006) (Padmavathi <i>et al.</i> , 2014)
4	Tributyl phosphate	11.476	2.82	87	Antimicrobial agents	Colorless,	(Woolford, 1984)
5	pyrrolo (1, 2- a) pyrazine -1, 4-dione, hexahydro-3-(2-methylpropyl)	13.587	5.32	94	Anticancer activity Antioxidant	N/A	(Lalitha <i>et al.</i> , 2016) (Ser <i>et al.</i> , 2015)

Figures

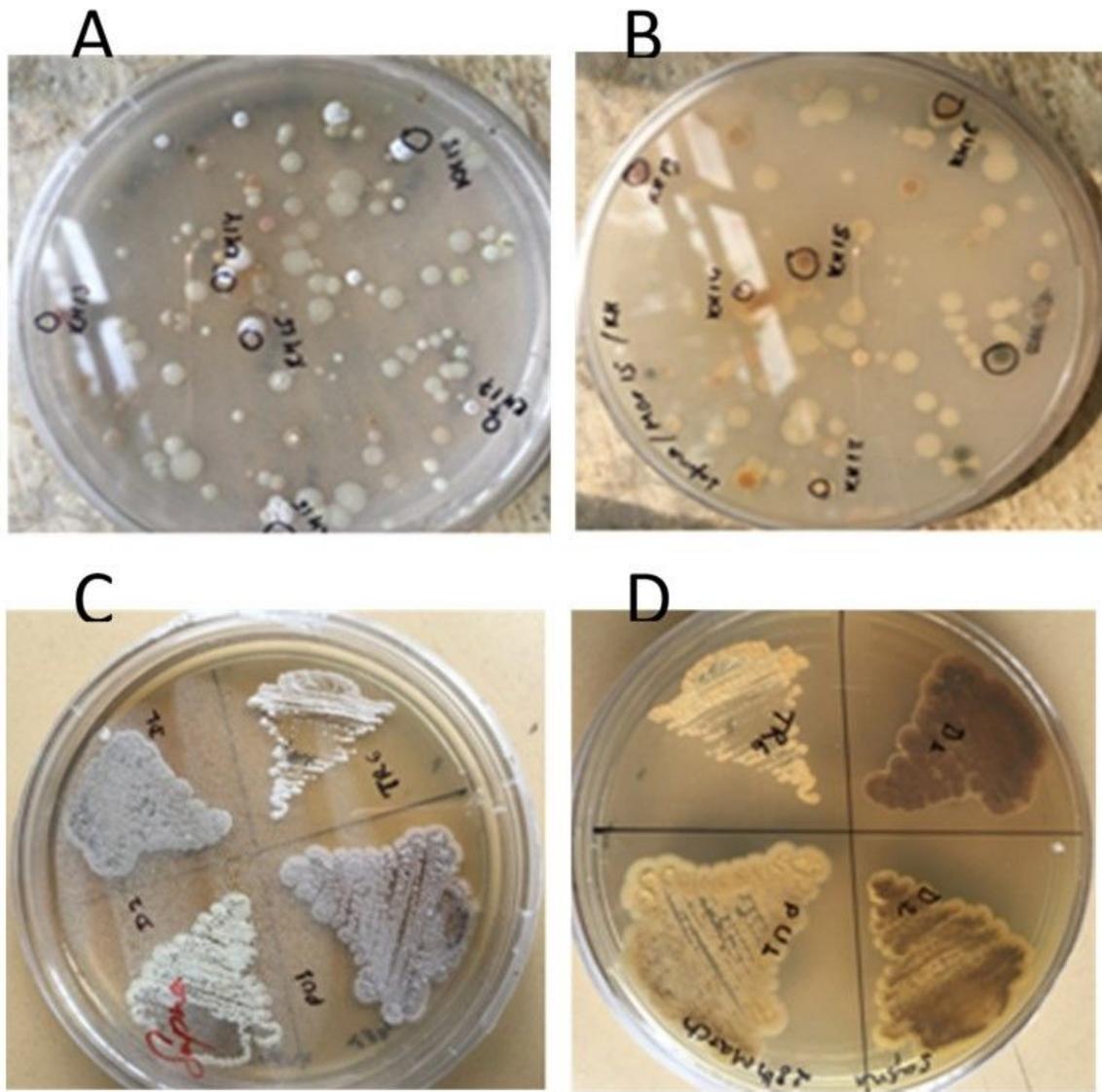


Figure 1

Morphology of screened *Streptomyces* colonies

Effect of different *Streptomyces* extracts on test pathogens.

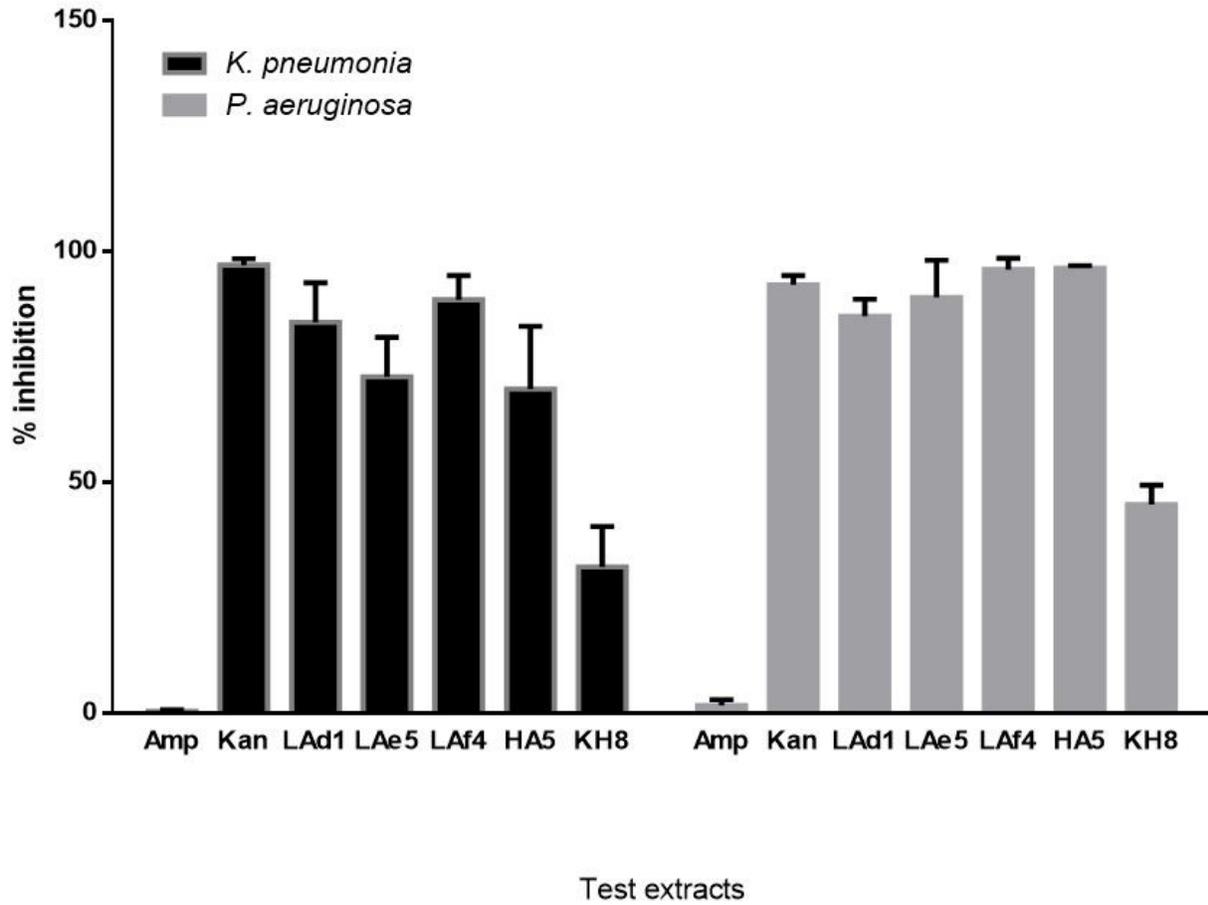


Figure 2

Resazurin antimicrobial assay of extracts against *Klebsiella pneumonia* and *Pseudomonas aeruginosa*. Amp – Ampicillin, Kan – Kanamycin

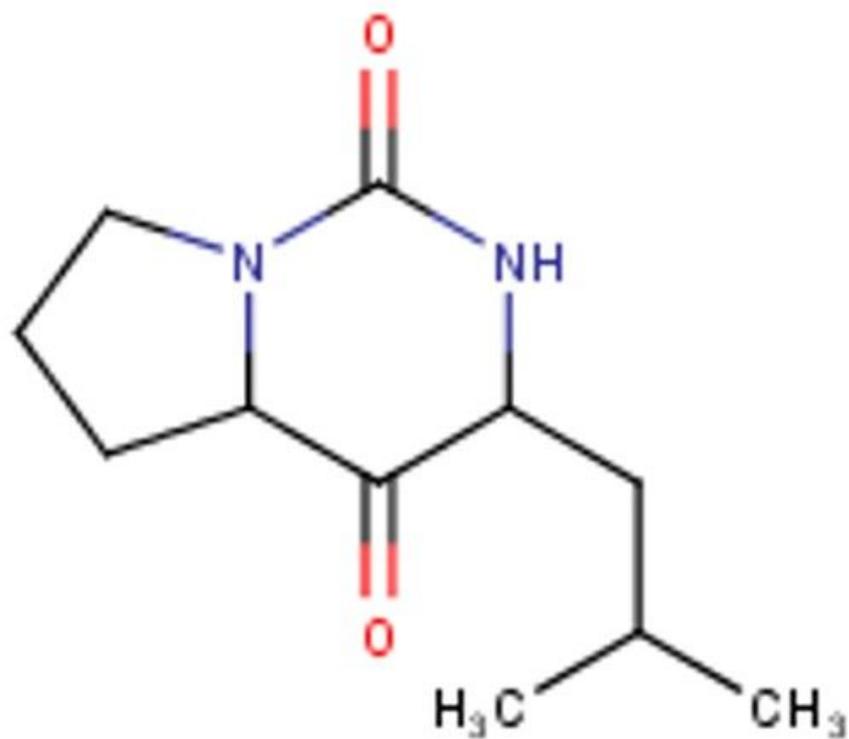


Figure 3

Structure of PPDHMP

Supplementary Files

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- [SupplementaryTable1.docx](#)