ORIGINAL ARTICLE

Bioactivity of Pyrrolo[1,2-a] pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)- Extracted from *Streptomyces* sp. VITPK9 Isolated from the Salt Spring Habitat of Manipur, India

Pratibha Sanjenbam, Krishnan Kannabiran

Department of Biomedical Sciences, School of Biosciences and Technology, VIT University, Vellore, Tamil Nadu, India

Abstract

Aim: The aim of the present study was to evaluate the biological activity of pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)- (PPDHP) extracted from *Streptomyces* sp. VITPK9, isolated from the brine spring of Thoubal district, Manipur, India. **Materials and Methods:** Hemolytic effect of the lead compound was determined, and human erythrocytes (red blood cells) were collected and used within 24 h. For genotoxicity study, chromosome preparations were obtained from phytohemagglutinin-stimulated peripheral blood lymphocytes. The viability of cells was evaluated by 3-(4,5-dimethythiazol-.2-yl)-2,5-diphenyl tetrazolium bromide assay on RAW 264.7 cell lines. **Results and Discussion:** The antifungal compound PPDHP showed mild hemolytic activity with effective concentration 50% value of 115.5 µg/mL on human erythrocytes. Cytotoxicity study on normal cell-line RAW 264 exhibited a moderate toxicity with 50% inhibitory concentration value of 500 µg/mL. Genotoxicity study revealed that the compound showed minimal chromosomal aberrations when compared to streptomycin. **Conclusion:** The results of the study suggest that the diketopiperazine type of compounds extracted from *Streptomyces* sp. VITPK9 can be used as the potential antifungal compound.

Key words: Cytotoxicity, genotoxicity activity, hemolytic activity, pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)-, *Streptomyces* sp. VITPK9

INTRODUCTION

Natural products are wide range of entities such as drugs, enzymes, catalyst, bio-control agents, and immunosuppressants. They are mainly derived from natural sources such as microbes, plants, and animals as a result of their primary or secondary metabolism. They are the most successful source for the exploration of novel drugs.[1] Natural products have higher chemical novelty than that of synthetic drugs. Microorganisms are explored continuously for drugs and serve as the richest source of drugs.[2] Demain and Sanchez reported that out of the 22,500 bioactive compounds extracted from microbes, 45% are from actinobacteria, 38% are from fungi, and 17% are from bacteria.[3] Schmitt *et al*. stated that natural products serve as a catalyst for innovation in pharmaceutical industries.[4] An untold number of useful principles are being exploited from microorganisms. The bioactive microbial compounds are small molecules and can be grouped as primary metabolites (required for growth) and secondary metabolites (not required for growth). Most of the useful bioactive molecules belong to secondary metabolites, and one such compound is antibiotics. The awareness about this precious organism began with the discovery of streptomycin from *Streptomyces griseus* by Selman Waksman in the year 1940s.[5] They are Gram-positive, filamentous bacteria with

Address for correspondence:
Dr. Krishnan Kannabiran,Department of Biomedical Sciences, School of Biosciences and Technology, VIT University, Vellore - 630 214, Tamil Nadu, India. Tel.: +91-416-2243091/93.
Fax: +91-416-2243092/91-416-2240411.
E-mail: kkb.biomol@gmail.com

Received: 25-07-2016
Revised: 12-08-2016
Accepted: 21-08-2016

high G + C content (60-70%) in DNA. Actinomycetes that are being extensively studied over a decade still continue to be the best natural source for the discovery of novel bioactive compounds.[6] They are regarded as a treasure box for discovery of "wonder drugs." It was already well-known that apart from a terrestrial source, actinomycetes can be isolated from marine environment. Pathom-Aree *et al*. reported the isolation of actinomycetes from the ocean trenches.[7] They are unique bacteria which produces a wide range of biologically important compounds. Of late, scientists have been looking for new habitats. The genus *Salinospora* was first isolated from the marine environment and produced a novel antibiotic salinosporamide. Similarly, a new class of antibiotics, abyssomicins have been obtained from *Verrucosispora*, a novel genus of marine actinomycetes.[8] Researches have shown that the genus *Streptomyces* is a potential and promising organism for the extraction of diverse principles. Suthindhiran and Kannabiran reported the antiviral activity of *Streptomyces* sp. VITSDK1 isolated from Marakkanam, Puducherry, India.[9] Saurav and Kannabiran reported the anti-*Aspergillus* activity of *Streptomyces* sp. VITSVK5 isolated from Puducherry.[10] Deepika and Kannabiran reported *Streptomyces* spp. VITDDK3 isolated from Ennore saltpan, Tamil Nadu, India, with antidermatophytic activity.[11] Although most of the findings are on discovery of new drugs, there are reports on the extraction of enzymes, inhibitors, and immunosuppressant too. Kavitha and Vijayalakshmi isolated *Streptomyces tendae* TK-VL 333 with L-asparaginase activity.[12] Sanjenbam *et al*. reported the glycolytic enzyme inhibitory activity of *Streptomyces* species isolated from a brine spring of Manipur, India.[13] Microorganisms are playing a greater role in the expansion of drug development pipeline. There is evidence that several biomolecules in the pharmaceutical product development are produced by microbes.

In this study, the pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)- (PPDHP) was assessed for hemolytic activity on red blood cells (RBCs), cytotoxic effect on normal cell line and assay of genotoxicity on human chromosomes.

MATERIALS AND METHODS

Actinomycetes isolate and the lead compound

Streptomyces sp. VITPK9 was isolated from the salt spring of Manipur, India, its isolation and characterization of was previously reported.[14] The anticandidal activity of PPDHP ($C_{14}H_{16}N_2O_2$) against *Candida albicans*, *Candida kefyr*, and *Candida tropicalis* was already reported.[15]

Assay of hemolytic activity

Hemolytic activity of the lead compound was determined as described by Saurav and Kannabiran.[16] Erythrocytes (RBCs) were separated from blood samples collected from human volunteers and used within 24 h. It was washed three times with sterile saline (0.85%) solution. The cells were centrifuged for 10 min at 8000 rpm, and the pellet was collected. It was diluted in sterile 0.85% NaCl solution (1:9 v/v), and then, in sterile Dulbecco's phosphate buffer saline (1:24) v/v, pH 7.0 containing 0.5 mM boric acid, and 1 mM calcium chloride. The activity of the lead compound was tested in 96 well titer plates. Each well received 100 µl of 0.85% NaCl solution containing 10 mM $CaCl_2$. The first well served as the positive control containing 20 µl of 0.1% Triton X-100 in 0.85% saline. Different concentrations (100-6.25 µg/mL) of lead compound were added to the subsequent wells. Each well then received 100 µl of 2% suspension of human erythrocytes in 0.85% saline containing 10 mM $CaCl_2$. After 30 min incubation at room temperature, cells were centrifuged and the supernatant was used to measure the absorbance of the liberated hemoglobin at 540 nm. The average value was calculated from triplicate assay.[17] The effect of the lead compound on hemolysis of RBC was determined.

Cytotoxicity assay

The lead compound was dissolved in 10% dimethyl sulfoxide (DMSO) to give a final concentration of DMSO <0.5% which does not affect the cells. The viability of cells was evaluated by 3-(4,5-dimethythiazol-.2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay on RAW 264.7 cell lines.[18] The RAW 264.7 cells were plated separately in 96 well plates at a concentration of 1×10^5 cells/well. After 24 h, cells were washed twice with 100 µl of serum-free medium and starved for an hour at 37°C. After starvation, cells were treated with different concentrations of test compound (10-500 µg/mL) for 24 h. At the end of the treatment period, the medium was aspirated and serum free medium containing MTT (0.5 mg/mL) was added and incubated for 4 h at 37°C in a CO_2 incubator. The 50% inhibitory concentration value (IC_{50}) of the crude extracts was identified for normal fibroblast cell line.

The MTT containing medium was then discarded, and the cells were washed with PBS (200 µl). The crystals were then dissolved by adding 100 µl of DMSO, and this was mixed properly by pipetting up and down. Spectrophotometric absorbance of the purple, blue formazan dye was measured in a microplate reader at 570 nm (Biorad 680). Cytotoxicity was determined using GraphPad Prism 5 software.[19]

Genotoxicity assay

Chromosome preparations were obtained from phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes following the modified method of Moorhead *et al*.[20] About 2 ml of venous blood sample was collected in a sterile heparinized syringe. 0.5 ml of the blood was inoculated into the vials containing 5 ml of RPMI 1640 medium containing 1 ml of fetal bovine serum and 0.2 ml of PHA

under aseptic condition. The culture vials were then placed in an incubator at 37°C. The cultures were shaken periodically, and carbon dioxide was released once every day. Various sets of experiments were conducted. Streptomycin was taken as positive control. At the 71st h of incubation, the culture was treated with 10 µg/mL of test compound for 1 h. After 1 h of incubation, the culture was thoroughly washed by centrifuging the content at 1000 rpm for 10 min, and supernatant was discarded carefully, and 6 ml of fresh RPMI 1640 medium was added to the pellet and mixed well with Pasteur pipette. After washing process, the dividing cells were arrested at metaphase by adding 2 drops of 0.001% colchicine solution to each culture vial. The cultures were incubated further for 20 min at 37°C. The contents of the vials were then transferred to 15 ml centrifuge tubes and centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and the cells were resuspended in a small amount of solution left behind by gently tapping the cell button. 6 ml of prewarmed (37°C) hypotonic solution (0.075 M KCl) was added to the tubes, and the contents were mixed gently using a Pasteur pipette. It was then incubated for 5 min at 37°C after which it was centrifuged for 6 min. The supernatant was carefully removed, and the cells were fixed with 6 ml of filtered Carnoy's fixative (3:1 methanol:acetic acid). The tubes were left at room temperature for 2 h. One change of fixative was given prior to slide preparation. The cell button was suspended in a small quantity of freshly prepared fixative. A test slide was prepared by gently placing a drop of the cell suspension on a well-cleaned glass slide and dried immediately on a hot plate (40°C). The test slide was examined under the microscope for cell density and metaphase spreads. Other slides were prepared after suitable modifications. The slides were stained in 4% Giemsa solution for 4 min and washed in distilled water for 1 min and then air-dried. Scoring of well-spread and stained cells was performed under oil immersion objective (×100 lens) of the light microscope (Leica ATC 2000). Well-spread metaphases were photographed under oil immersion objective lens (×100) of Olympus microscope with camera.

Statistical analysis

All the experiments were carried out in triplicates, and the data obtained were evaluated as mean ± standard error. $P < 0.05$ was considered as statistically significant. Using the Probit analysis, Chi-square values calculated. The IC_{50} and minimum inhibitory concentration values and their respective 95% confidence intervals (95% CI) were evaluated by nonlinear regression analysis using the data analysis software (Prism).

RESULTS AND DISCUSSION

Hemolytic assay

The hemolytic activity of the pure compound PPDHP [Figure 1] extracted from *Streptomyces* sp. VITPK9 was carried out to study the effect of the compound on membrane stability. PPDHP exhibited a weak membranolytic activity on erythrocyte membrane with effective concentration 50% (EC_{50}) value of 115.5 µg/mL [Figure 2]. The complete hemolysis was achieved with 20 µL of 0.1% Triton X-100 (positive control) after 1 h of incubation. The EC_{50} value and 95% CI were evaluated by nonlinear regression analysis. The results obtained from the present study revealed that the compound PPDHP is nonhemolytic at low concentrations of <100 µg/mL. Evaluation of membrane stability for the exposed drug plays a very important role to understand the toxicity of the drug to erythrocytes. Erythrocytes serve as a good model for the toxicity study of new compounds/drugs. The effect of bioactive compounds on the erythrocytic membrane stability serves as an index for the assessment of membrane stability.[16]

Cytotoxicity assay

The cytotoxic effect of different concentrations of PPDHP (12.5-250 µg/mL) on RAW 264 cells was studied and presented in Figure 3. The cytotoxic effect of the compound was determined by MTT assay in 96 well plates. The compound PPDHP showed concentration-dependent inhibition on

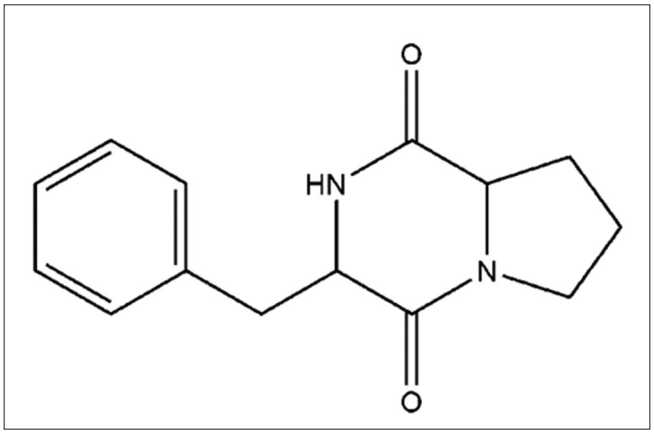

Figure 1: Structure of pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)- extracted from *Streptomyces* sp.VITPK9

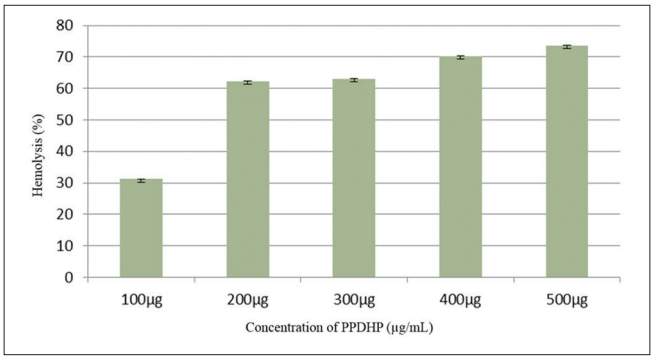

Figure 2: Hemolytic activity of pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)- on erythrocytes

RAW 264 cells. The effect of different concentration of PPDHP (12.5-250 µg/mL) on RAW-264 cells after 24 h of incubation is shown in Figure 4. Nearly, 80% of cells are viable after 24 h of incubation with 250 µg/mL of PPDHP. The present study indicated that PPDHP was less toxic to the normal cell. Numerous reports are available on cytotoxicity of compounds isolated from *Streptomyces* species. The antifungal compound 5-(2, 4-dimethylbenzyl) pyrrolidin-2-one extracted from *Streptomyces* sp.VITSVK5 exhibited cytotoxicity against normal Vero cell line (IC_{50} value of 22.6 µg/mL).[16] Wang *et al*. reported the cytotoxic activity (IC_{50} value of 18.2 µg/mL) of novel furan derivative isolated from *Streptomyces* spp. HS-HY-071.[21]

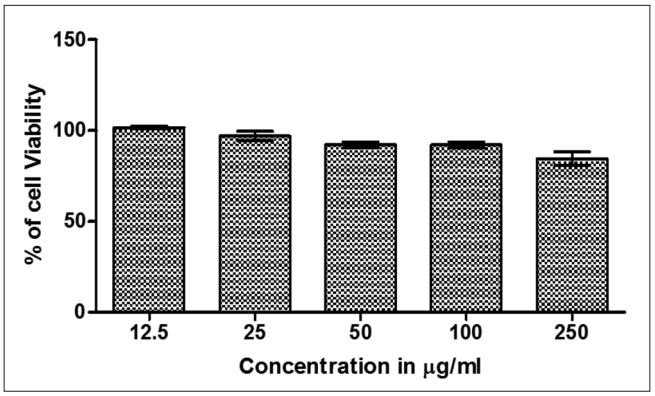

Figure 3: Cytotoxic study of pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)- against normal cell-line RAW-264

Genotoxicity assay

The effect of PPDHP on chromosomes was studied at different concentrations ranging from 15 to 60 µg/mL. The positive control showed more chromosomal breaks (2.75 ± 1.71) and chromatids breaks (2.25 ± 0.96). The test compound showed the lesser percentage of chromosomal aberrations that include chromosomal breaks (2.45 ± 1.50) and chromatid breaks (2.0 ± 0.90) [Table 1]. The number of chromosome breaks or chromosomal aberrations was significantly low as compared to the positive control [Figure 5].

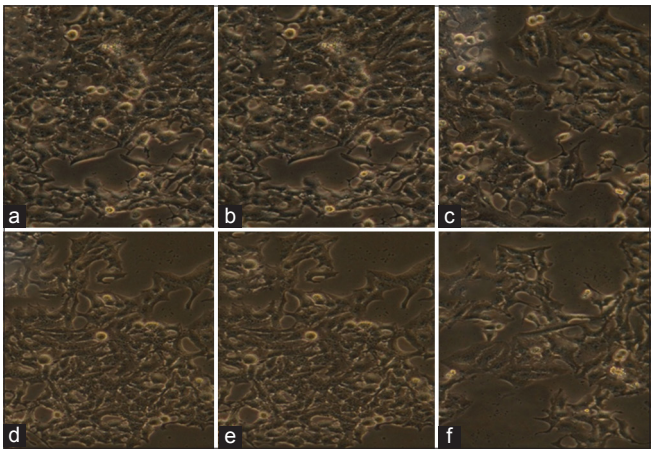

Figure 4: Cytotoxic effect of pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)- on RAW-264 cell line after 24 h of incubation, (a) Control, (b) treated with 12.5 µg/mL, (c) treated with 25 µg/mL, (d) treated with 50 µg/mL, (e) treated 100 µg/mL, (f) treated with 250 µg/mL

Antibiotic resistance is growing worldwide. Recently, the rise in new forms of antibiotic resistance is a growing menace that highlights the need for effective drugs.[22] Modern pharmaceutical industries are no longer fulfilling the growing need, and hence, the resistance remains as one of the major problems in human health care. To overcome these new drugs has to be discovered on war footing. Traditionally, the discovery of natural products was limited only to plants.[23] The application of combinatorial genetics has made possible to insert the desired gene from plants to bacteria like *Streptomyces* species for production of more secondary metabolites.[24] To facilitate the production of numerous bioactive metabolites, high-throughput system-based mechanisms are available. A decade ago, there were a lot of difficulties in separation of the mixtures of natural origin but the unprecedented analytical techniques of the 21st century made it overcome the obstacles.[23] Recent studies have shown that marine environment serves as a novel niches for exploration of new leads.[25,26] About 70% of the earth's surface is covered with water called oceans, and deep sea bacteria remains to be unexplored for novel products. Marine actinomycetes have been reported as a source of novel secondary metabolites.[27] With the advancement of combinatorial studies, it is possible to develop many novel drugs which can be exploited for the treatment of human diseases.[28] It is evident that based on several reports that continuous screening of *Streptomyces* may lead to the identification of numerous leads with wide pharmaceutical applications. The exploration of actinomycetes from various niches is a promising area in terms of drug discovery.[29]

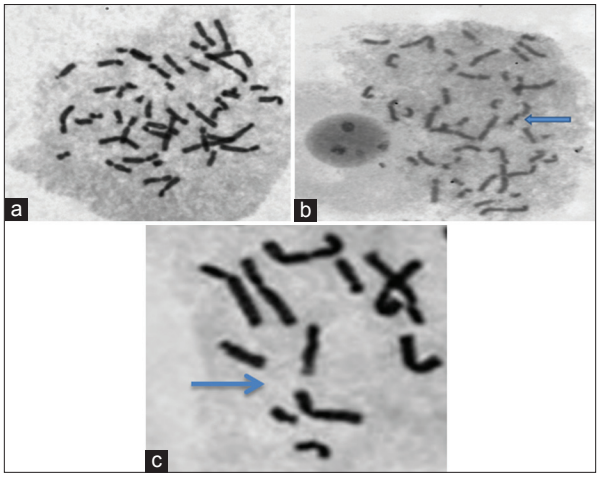

Figure 5: Effect of pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenylmethyl)- (PPDHP) on human chromosome, (a) Untreated sample, (b) positive control (streptomycin), (c) treated with the compound PPDHP (60 µg/mL)

Table 1: Genotoxicity of PPDHP

Control	Total metaphase	Chromosome break	Chromatid break	Total chromosome aberration	%	Number of aberration per cell
Untreated 01	100	3	2	4	4	0.04
Total	100	3	2	4	4	0.04
Concentrations (µg/mL) positive control-streptomycin						
15	100	1	1	2	2	0.02
30	100	2	2	4	4	0.03
45	100	3	3	6	6	0.07
60	100	5	3	8	8	0.08
Total	400	11	9	20	5	0.05
Mean±SD		2.75±1.71	2.25±0.96			
Concentrations (ng/mL) PPDHP						
15	100	1	1	2	2	0.02
30	100	2	2	4	4	0.03
45	100	3	3	6	6	0.07
60	100	4	2	7	8	0.08
Total	400	10	8	19	5	0.05
Mean±SD		2.45±1.50	2.0±0.90			

PPDHP: Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-, SD: Standard deviation

CONCLUSION

The study concludes that the compound isolated from *Streptomyces* sp. VITPK9 is less toxic to normal human cell line and chromosomes. Therefore, the bioactive potency of the compound could be studied further by *in vivo* studies.

ACKNOWLEDGMENT

The authors wish to thank the management of VIT University, for providing the facilities to carry out this study.

REFERENCES

1. Bull AT, Stach JE. Marine actinobacteria: New opportunities for natural product search and discovery. Trends Microbiol 2007;15:491-9.
2. Handelsman J, Rondon MR, Brady SF, Clardy J, Goodman RM. Molecular biological access to the chemistry of unknown soil microbes: A new frontier for natural products. Chem Biol 1998;5:R245-9.
3. Demain AL, Sanchez S. Microbial drug discovery: 80 years of progress. J Antibiot (Tokyo) 2009;62:5-16.
4. Schmitt EK, Moore CM, Krastel P, Petersen F. Natural products as catalysts for innovation: A pharmaceutical industry perspective. Curr Opin Chem Biol 2011;15:497-504.
5. Waksman SA, Bugie E, Schatz A. Isolation of antibiotic substances from soil microorganisms with special reference to streptothricin and streptomycin. In: Proceeding Staff Meetings Mayo Clinic. 1944. p. 537-48.
6. Fenical W, Jensen PR. Developing a new resource for drug discovery: Marine actinomycete bacteria. Nat Chem Biol 2006;2:666-73.
7. Pathom-Aree W, Stach JE, Ward AC, Horikoshi K, Bull AT, Goodfellow M. Diversity of actinomycetes isolated from Challenger Deep sediment (10,898 m) from the Mariana Trench. Extremophiles 2006;10:181-9.
8. Jensen PR, Williams PG, Oh DC, Zeigler L, Fenical W. Species-specific secondary metabolite production in marine actinomycetes of the genus Salinispora. Appl Environ Microbiol 2007;73:1146-52.
9. Suthindhiran K, Kannabiran K. Diversity and exploration of bioactive marine actinomycetes in the Bay of Bengal of the Puducherry coast of India. Indian J Microbiol 2010;50:76-82.
10. Saurav K, Kannabiran K. Antifungal activity of *Streptomyces* VITSVK5 spp. Against drug resistant *Aspergillus* clinical isolates from pulmonary tuberculosis patients. J Mycol Méd 2010;20:101-7.
11. Deepika TL, Kannabiran K. Isolation of a Novel Compound from Marine *Streptomyces* and Some of its Biological Activities. Ph. D Thesis Submitted to VIT University, Vellore, India; 2011.
12. Kavitha A, Vijayalakshmi M. Optimization and purification of L-asparaginase produced by *Streptomyces tendae* TK-VL_333. Z Naturforsch C 2010;65:528-31.
13. Sanjenbam P, Thenmozhi M, Kannabiran K. Screening

of glycolytic enzyme inhibitory activity of *Streptomyces* isolates from brine spring and marinesediments of India. Int J Pharm Res Rev 2013;2:5-11.
14. Sanjenbam P, Kannabiran K. Antimicrobial and larvicidal activity of *Streptomyces* sp. VITPK9 isolated from a brine spring habitat of Manipur, India. Der Pharm Lett 2013;5:65-70.
15. Sanjenbam P, Gopal JV. KannabiranK.Isolation and identification of anticandidal compound from *Streptomyces* sp. VITPK9. J Appl Biochem Microbiol 2014;50:492-9.
16. Saurav K, Kannabiran K. Cytotoxicity and antioxidant activityof5-(2,4-dimethylbenzyl)pyrrolidin-2- extracted from marine *Streptomyces* VITSVK spp. Saudi J Biol Sci 2012;19:81-6.
17. Van Zyl RL, Viljoen AM. *In vitro* activity of *Aloe* extracts against *Plasmodium falciparium*. South Afr J Bot 2002;68:106-10.
18. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;5:55-63.
19. Suthindhiran K, Kannabiran K. Probing the mechanism of cytotoxic furan-2yl acetate *in-vitro* and *in-silico* analysis pharmacological study. J Pharm Toxicol 2013;8:1-8.
20. Moorhead PS, Nowell PC, Mellman WJ, Battips DM, Hungerford DA. Chromosome preparations of leukocytes cultured from human peripheral blood. Exp Cell Res 1960;20:613-6.
21. Wang J, Zhang H, Yang X, Zhou Y, Wang H, Bai H. HS071, a new furan-type cytotoxic metabolite from *Streptomyces* sp. HS-HY-071. J Antibiot (Tokyo) 2008;61:623-6.
22. Bush K. Why it is important to continue antibacterial drug discovery? ASM News 2004;70:282-6.
23. Tulp M, Bohlin L. Unconventional natural sources for future drug discovery. Drug Discov Today 2004;9:450-8.
24. Roessner CA, Scott AI. Genetically engineered synthesis of natural products: From alkaloids to corrins. Annu Rev Microbiol 1996;50:467-90.
25. Jha RK, Zi-Rong X. Biomedical compounds from marine organisms. Mar Drugs 2004;2:123-46.
26. Subramani R, Aalbersberg W. Marine actinomycetes: An ongoing source of novel bioactive metabolites. Microbiol Res 2012;167:571-80.
27. Fiedler HP, Bruntner C, Bull AT, Ward AC, Goodfellow M, Potterat O, *et al.* Marine actinomycetes as a source of novel secondary metabolites. Antonie Van Leeuwenhoek 2005;87:37-42.
28. Floss HG. Combinatorial biosynthesis – Potential and problems. J Biotechnol 2006;124:242-57.
29. Singh SB, Pelaez F. Biodiversity, chemical diversity and drug discovery. Prog Drug Res 2008;65:141, 143-74.

Source of Support: Nil. **Conflict of Interest:** None declared.