Evaluation of random mutations in *Streptomyces peucetius* and their impact on the production of Daunorubicin

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The present study deals with evaluation of random mutations and their impact on the production of anti-tumor drug Daunorubicin. Daunorubicin is a secondary metabolite, produced by the strain *Streptomyces peucetius*. In our study, *S. peucetius* was irradiated with UV rays to create physical mutagenesis and was also subjected to chemical mutagenesis by Ethyl-methane sulfonate. Our study aims to develop a mutant and make a comparative study with the wild strain of *Streptomyces peucetius* in relation to the enhancement of the yield of Daunorubicin. The generated mutant was then set for fermentation in R2YE fermentation media at optimum conditions to authenticate enhanced yield. Further the anti-tumor activity of Daunorubicin produced from wild and mutant *Streptomyces peucetius* was measured using MTT cell proliferation assay followed by determination of IC_{50} values. The results obtained were promising that the anti-tumor activity and cell growth inhibition were found to be high in the Daunorubicin produced by the mutant strain than that of wild strain.

Keywords: Daunorubicin; *Streptomyces peucetius*; UV-radiation; Ethyl methane sulfonate; MTT assay.

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Introduction

Daunorubicin (DNR) is one of the most important anti-tumor anthracycline antibiotic synthesized by an actinobateria *Streptomyces peucetius* [1, 2]. It is used as a chemotherapeutic agent with Cerubidine as the trade name for treating acute leukemias [3, 4]. Doxorubicin is a hydroxylated form of DNR, which is also used as chemotherapeutic drug [5]. DNR, Doxorubicin, etc. (Anthracyclines) have the potential to show anti-tumor activities as single agent. But to improve their efficacy and reduce drug resistance and toxicity, they are now used in combination with other chemotherapeutic agents, such as tetrandrine [3, 6]. Anthracyclines show their mode of action primarily by forming complexes with DNA (intercalation), thereby inhibiting the activity of nuclear enzyme topoisomerase II [7]. Topoisomerase II is a key enzyme during DNA synthesis, which helps in condensation and de-condensation of DNA helix by producing nicks and re-ligating them [8]. DNR prevents the re-ligation of DNA by topoisomerase II after producing nicks in DNA strands. This results in accumulation of large number of DNA fragments, which gradually leads to cell apoptosis [9]. DNR also acts in the other ways such as it leads to the formation of free radicals within the cancer cells [10]. These free radicals damage the proteins, lipids, and cell membrane, increase cell cytotoxicity, and finally

leads to apoptosis [9].

One of the major side effects of using the DNR includes development of cardiac toxicity [11]. At present, DNR produced from *Streptococcus peucetius* by immobilization of wild strains [12]. There are several reports describing the synthesis of DNR in minute amount by wild strains. The present work deals with the improvement of such wild strain of *Streptomyces peucetius* for the enhanced production of DNR by creating random mutations using both physical and chemical mutagenesis. Further, the activity of DNR produced from the mutated strains was validated by performing MTT assay.

Materials and Methods

The freeze-dried culture of *Streptomyces peucetius* was obtained from National Chemical Laboratory (Pune, India). The freeze-dried culture was then revived in Yeast Maltose Extract (YME) media by incubating the inoculated cultures for 96 hours in orbital shaking incubator at 37°C. The YME media has the following composition of ingredients: Malt extract (3g/L), Yeast extract (3g/L), Glucose (10g/L), Peptone (5g/L), Agar (20g/L) as described in the literature [13]. The revived culture was sub-cultured in YME broth, YME agar plates, as well as in YME agar slants.

Creation of random mutations in *Streptomyces peucetius*

(1) Physical Mutagenesis

Ultraviolet rays are non-ionizing radiation with wavelength ranging from 10 nm to 400 nm in electromagnetic spectrum. These have the potential to produce thymidine dimers in DNA thereby inducing mutation in bacteria [14]. Therefore, UV irradiation is one of the most common methods employed to produce bacterial mutants. Initially for creation of mutants using UV radiation, a total of six YME agar plates of wild *Streptomyces peucetius* strains were prepared. Out of the six plates, three plates were exposed to UV radiation for 10 minutes by placing them 50 cm away from the UV light. Similarly, three other plates were exposed for 15 minutes by placing them at same distance from UV light.

Single isolated colonies from each plate which was set for UV treatment were picked and inoculated onto freshly prepared sterile YME agar plates. These mutants were allowed to multiply for 36 hours at 37°C in an incubator. These mutants were later sub-cultured in YME broth for further use.

(2) Chemical Mutagenesis

Random mutations were induced by using the chemical mutagen Ethyl methane sulfonate (EMS) [15]. It is an alkylating agent, which generally produces point mutations mostly by guanine alkylation [16]. In the present study, wild *S. peucetius* was mutated by Watson and Holloway method [17]. Briefly, a total of 0.03 ml of EMS was added to 5 ml of exponential phase culture, grown in YME broth and vortexed vigorously, and incubated at 37°C for 1 h without any disturbance. Then, the resultant culture was collected by centrifugation at 10,000 rpm for 10 min. To remove the traces of mutagen, cells were washed with saline water. The leftover culture which contained the mutated cells was then inoculated into 50 ml of YME broth and incubated at 30°C overnight in an orbital shaking incubator.

(3) Fermentation

Streptomyces peucetius being a gram positive actinobacteria shows good growth in media composed of a mixture of salts, buffers, carbon source, and trace elements under optimum conditions [18]. To obtain the optimum growth of *Streptomyces peucetius*, it was suggested to use R2YE media in earlier reports [19]. R2YE media is prepared using the following ingredients as described in earlier studies, which included Corn starch (46 g/L), NaCl (1 g/L), $(NH_4)_2SO_4$ (2 g/L), K_2HPO_4 (4.63 g/L), $CaCO_3$ (2 g/L), $MgSO_4$ (7.64 g/L), Tryptone (2 g/L), 1 ml of inorganic solution ($FeSO_4.7H_2O$ (1 mg/L),

$MgCl_2.6H_2O$ (1 mg/L), $ZnSO_4.7H_2O$ (1 mg/L), H_2O (1,000 ml)) [20].

All the strains including wild, UV mutants, and EMS mutants were fermented in 100 ml of R2YE media for 4 days at 29°C in an orbital shaker incubator at 200 rpm. Strains were inoculated at 10% (v/v) of media for proper growth. The cell growth was monitored by checking the optical density of culture at regular intervals of time using spectrophotometer.

Quantitative and qualitative analysis of Daunorubicin

(1) Sample Preparation

To perform quantitative and qualitative analysis, samples from fermented broth need to be prepared. Sample preparation was done under sterile conditions where 10ml of fermented broth was transferred into a dry and sterile 50 ml vial. To this, an equal amount of the mixture of 30% iso-propyl alcohol:HCl in the ratio of 50:1 was added and mixed well. The resultant mixture was then subjected to centrifugation at 15,000 rpm for 10 mins at room temperature. By doing so, all the cells and impure compounds got separated in the form of pellet which was discarded. The daunorubicin compound was suspended in supernatant and was collected for further analysis. For further purification of the daunorubicin, the supernatant which was obtained from the earlier centrifugation was filtered using 0.22 µm membrane filters. Obtained filtrate was used for quantitative and qualitative analysis as described in the literature [21].

(2) Quantitative Analysis of Daunorubicin

High performance liquid chromatography (HPLC) is one of the best and most common methods employed to detect the concentration of a desired compound present in a mixture solution [22]. In the present study, HPLC linearity graph was constructed by running standard DNR of different concentrations in Reverse phase C18 Column (250 mm X 4.6 mm, 5µm) with a flow rate of 1.0 ml/min. The standard DNR used here was purchased from Toronto Research Chemicals INC (Toronto, Canada). This was followed by running the prepared fermented samples in HPLC to detect the unknown concentration of DNR present in them. Mobile phase used is a mixture of methanol and acetone in 75:25 (v/v). The absorbance was observed at a wavelength of 228 nm [23].

(3) Qualitative Analysis by *in vitro* MTT assay

In general, the success of numerous *in vitro* assays which deal with cell response towards external factors mainly depends on the measurement of cell viability and proliferation. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely the reduction in cell viability when metabolic events lead to apoptosis or necrosis [24]. The cancer cell lines were purchased from National Centre for Cell Science (NCCS) (Pune, Maharashtra, India). The cells were maintained in Minimal Essential Medium (MEM) supplemented with 10 % Fetal bovine serum (FBS) and the antibiotics streptomycin (0.5 mg/mL), in an atmosphere of 5% CO_2 and 95% air at 37°C.

Cell viability was evaluated by the MTT Assay with independent experiments with six concentrations of compounds in triplicates. Cells were trypsinized and performed the trypan blue assay to know viable cells in cell suspension. Cells were counted by hemocytometer and seeded at density of 5.0 X 10^3 cells/well in 100 µl culture media in 96 well plate and incubated overnight at 37°C. After incubation, the old media was removed and fresh media of about 100 µl with different concentrations of test compound was added to the wells in 96 plates. After 48 hrs, the drug solution was discarded and the fresh media with MTT solution (0.5 mg/mL) was added to each well and plates were incubated at 37°C for 3 hrs. At the end of incubation time, precipitates were formed as a result of the reduction of the MTT salt to chromophore formazan crystals by the cells with metabolically active mitochondria. The optical density of solubilized crystals in Dimethyl sulfoxide (DMSO) was measured at 570 nm on a microplate reader. The percentage growth inhibition was calculated using the

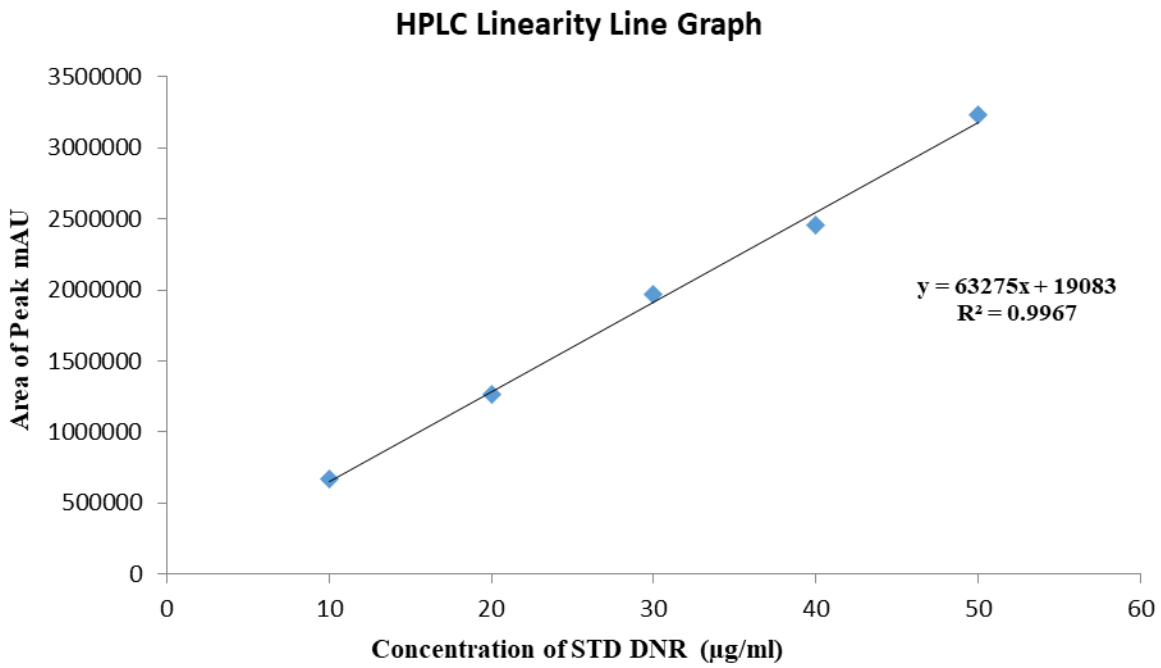

Figure 1. HPLC linearity line graph.

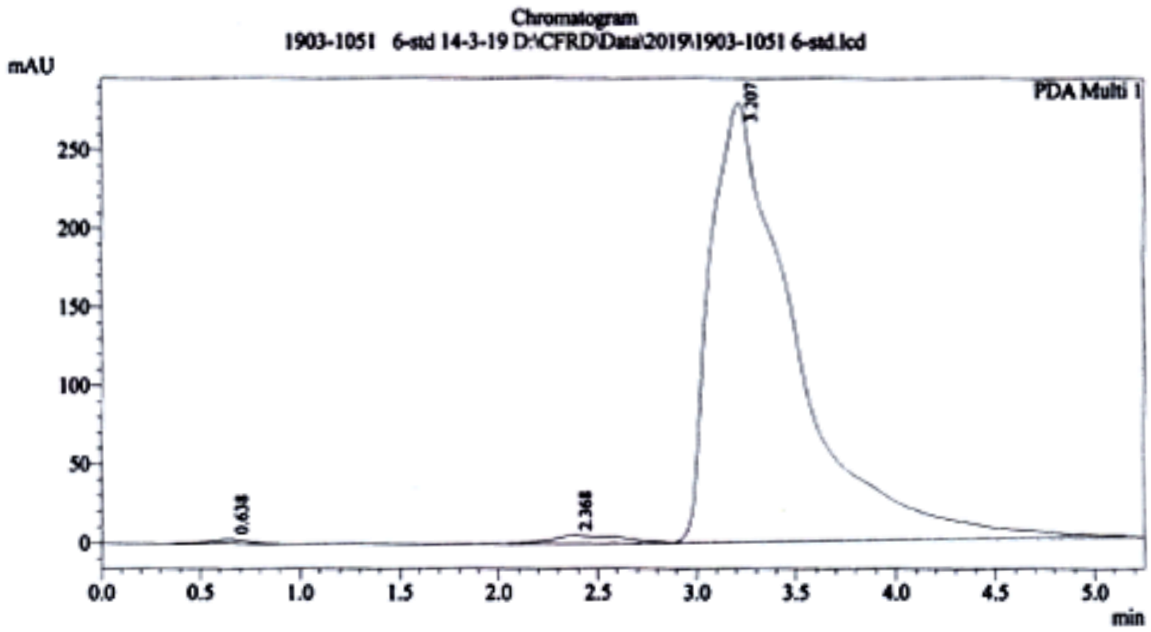

Figure 2. HPLC chromatogram of STD DNR (100 µg/ml).

following formula.

$$Inhibition\ (\%) = 100\ \frac{(\ Control - Treatment)}{(\ Control\ )}$$

The IC_{50} value was determined by using linear regression equation i.e. $Y = mX + c$ where $Y = 50$, m and c values were derived from the viability graph (% viability of cancer cells vs concentration

of daunorubicin. The lower of IC_{50} value the higher the drug's potential to inhibit cancer cell growth [25].

Results and Discussion

Wild *Streptomyces peucetius* showed good growth in yeast maltose extract (YME) broth within 24-48 hours of incubation while UV mutants and EMS mutants showed the similar growth pattern when incubated for 72-96 hours. Upon subjecting the strains to fermentation in R2YE media, there was noteworthy increase in turbidity of fermentation broth after 96 hours (4 days) of incubation in orbital shaker at 200 rpm, 29°C in all the fermented samples. After checking optical density of fermented media, samples were prepared as explained and subjected to quantitative and activity assays.

Quantitative analysis of daunorubicin

To calculate the unknown concentration of DNR in fermented samples, a HPLC linearity graph was constructed by plotting the area of chromatogram peaks (mAU) against concentrations of standard DNR (µg/ml). Figure 1 and 2 showed the linearity graph and chromatogram of standard DNR and Table 1 represented the area under the chromatogram peaks of the standard DNR solutions. The slope for this linearity line was calculated by substituting X and Y co-ordinates of graph in the formula $\left(\frac{Y_2-Y_1}{X_2-X_1}\right)$ and calculated *Y*-intercept by similar substitutions. The obtained slope and *Y*-intercept from calculations were 63,274.54 and 19,082.6 respectively. By substituting these values in $Y = mX + c$, the equation of linearity line becomes $Y = 63{,}274.54X + 19{,}082.6$

Using the obtained equation, the concentrations of DNR in the all the samples in all conditions were calculated. Table 2 represented the concentrations of DNA in wild strain, UV mutant, and EMS mutants. Figure 3 represented the chromatogram of the fermented samples of UV mutants.

Table 1. The areas of chromatogram peaks of standard DNR solutions.

No.	Concentration of standard DNR (µg/ml)	Area of peak (mAU)
1	10	665253
2	20	1265095
3	30	1965014
4	40	2459409
5	50	3231823
6	100	8351277

From these results, it can be concluded that the EMS mutants of *Streptomyces peucetius* produced 3 times more DNR than wild strains. UV mutants (15 mins) produced twice the amount of DNR than that in wild strains, while the UV mutants (10 mins) gave inconsistent yield of DNR.

Qualitative analysis by MTT assay

To evaluate the anti-tumor activity of produced DNR, MTT assay was performed. The optical density of solubilized crystals in DMSO was measured at 570 nm on a microplate reader. Table 3 and 4 showed the percentage of viability. By comparing the data obtained, it is clear that, as the concentration of DNR increases, the percentage of viability is found to be more in EMS mutant, which provide the hint that there is high anti-tumor activity in EMS mutant derived DNR than that of the wild strain (Table 3 and 4, Figures 4 and 5). Similarly, the IC_{50} was calculated by using the equations of lines as mentioned earlier. The significance of IC_{50} value is that the lower the IC_{50} value the higher the drug's potential to inhibit cell growth. From our data, the IC_{50} value of DNR from EMS mutants was 98.17 while that from wild strain was 112.4 (Table 3 and 4). These results confirm that the mutant *S. peucetius* produces higher amount of daunorubicin than that of wild strain.

Conclusion

From the above study, by observing the concentrations of DNR in the wild and mutant strains and also by the comparison of IC_{50} values of the wild and mutant strains, we can confirm

Table 2. HPLC data representing the area of peaks and the concentrations of DNR (µg/ml) the samples of wild, 10 minutes UV treated, 15 minutes UV treated, and EMS mutants, respectively.

Sample No.	Wild		UV-10min		UV-15 min		EMS Mutants	
	Area of peaks	Concentration of DNR (µg/ml)	Area of Peaks	Concentration of DNR (µg/ml)	Area of peaks	Concentration of DNR (µg/ml)	Area of peaks	Concentration of DNR (µg/ml)
1	505496	7.762	841619	13.056	999390	15.542	1551035	24.231
2	464693	7.119	1408906	21.992	978375	15.21	1382804	21.581
3	655578	10.126	465880	7.138	725745	11.231	1531056	23.916
Average		8.336		14.062		13.994		23.243
SD		1.583		7.478		2.399		1.448

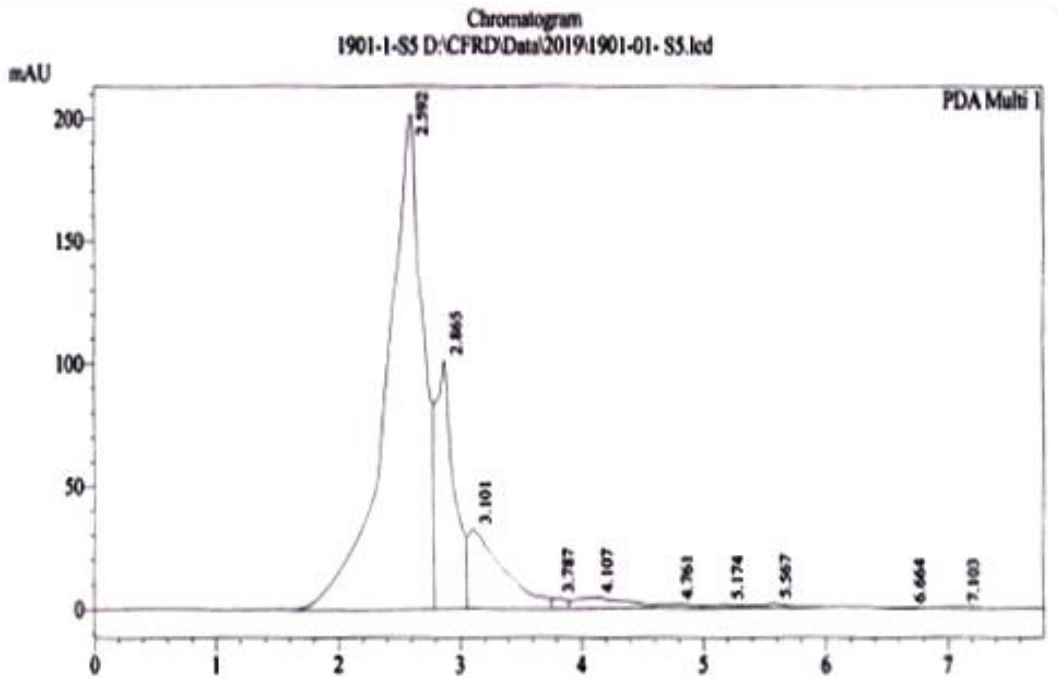

Figure 3. Chromatogram of a fermented sample.

Table 3. Anti-tumor activity of DNR from wild strain.

Amount of DNR (µg)	Absorbance at 570nm	% Inhibition	% Viability	IC_{50} (µg)
5	0.372	41.13	58.87	
10	0.256	59.49	40.51	
25	0.186	70.56	29.44	
50	0.203	67.87	32.13	
100	0.365	42.24	57.76	
Untreated	0.632	0	100	112.4
Blank	0	0	0	

that the wild strain which was subjected to mutagenesis had successfully mutated and lead for mutant of *S. peucetius*. Further from the above data also, it is crystal clear that the mutant *S. peucetius* has enhanced capability of producing DNR in a cost-effective way, which showed high anti-tumor activity than the DNR from wild strains.

Table 4. Anti-tumor activity of DNR from EMS mutants.

Amount of DNR (µg)	Absorbance at 570nm	% Inhibition	% Viability	IC_{50} (µg)
5	0.419	33.7	66.3	
10	0.371	41.29	58.71	
25	0.514	18.67	81.33	
50	0.542	14.24	85.76	
100	0.556	12.02	87.98	
Untreated	0.632	0	100	98.17
Blank	0	0	0	

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References

1. Thirumaran R, Prendergast GC, Gilman PB: Cytotoxic chemotherapy in clinical treatment of cancer. In Cancer Immunotherapy; 2007:101-116.
2. Gottlieb RA, Mehta PK: Cardio-Oncology: Principles, Prevention and Management. 2016. Academic Press.
3. Bertino JR: Encyclopedia of cancer. 2002. Academic Press.
4. Wojciech S, Irena O, Katarzyna C, Andrzej Z. 2001. The method of daunorubicin purification. Acta Polonia PharmaceuLitu-Drug Research. 58(4):263-268.
5. Hutchinson CR. 1997. Biosynthetic studies of daunorubicin and tetracenomycin C. Chemical reviews. 97(7):2525-2536.
6. Xu WL, Shen HL, Ao ZF, Chen BA, Xia W, Gao F, Zhang YN. 2006. Combination of tetrandrine as a potential-reversing agent with daunorubicin, etoposide and cytarabine for the treatment of refractory and relapsed acute myelogenous leukemia. Leukemia research. 30(4):407-413.
7. Joshua PG, Mary EK: Side effects of drugs annual: a worldwide yearly survey of new data in adverse drug reactions. Volume 37. Edited by Sidhartha D.R; 2015.
8. McClendon AK, Osheroff N. 2007. DNA topoisomerase II, genotoxicity, and cancer. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 623(1-2):83-97.
9. Bardal SK, Waechter JE, Martin DS: Applied pharmacology; 2011.
10. Bachur NR, Gee MV, Friedman RD. 1982. Nuclear catalyzed antibiotic free radical formation. Cancer research. 42(3):1078-1081.
11. Greaves P: Cardiovascular System in Histopathology of Preclinical Toxicity Studies; 2007.
12. Takashima Y, Nakajima H, Sonomoto K, Tanaka A. 1987. Production of daunorubicin by immobilized growing *Streptomyces peucetius* cells. Applied microbiology and biotechnology. 27(2):106-109.
13. Mendes-Ferreira A, Mendes-Faia A, Leao C. 2002. Survey of hydrogen sulfide production by wine yeasts. Journal of food protection. 65(6):1033-1037.
14. Harm W: Biological effects of ultraviolet radiation; 1980.
15. Barrios-Gonzalez J, Fernandez FJ, Tomasini A. 2003. Microbial secondary metabolites production and strain improvement. Indian journal of Biotechnology. 2(3):322-333.
16. Sega GA. 1984. A review of the genetic effects of ethyl methane sulfonate. Mutation Research/Reviews in Genetic Toxicology. 134(2-3):113-142.
17. Watson JM, Holloway BW. 1976. Suppressor mutations in *Pseudomonas aeruginosa*. Journal of bacteriology. 125(3):780-786.
18. Dekleva ML, Titus JA, Strohl WR. 1985. Nutrient effects on anthracycline production by *Streptomyces peucetius* in a defined medium. Canadian journal of microbiology. 31(3):287-294.
19. Shepherd MD, Kharel MK, Bosserman MA, Rohr J. 2010. Laboratory maintenance of *Streptomyces* species. Current protocols in microbiology. 18(1):10E-1.
20. Chi WJ, Song JH, Oh EA, Park SW, Chang YK, Kim ES, Hong SK. 2009. Medium optimization and application of affinity column chromatography for trypsin production from recombinant *Streptomyces griseus*. Journal of Microbiology and Biotechnology. 19(10):1191-1196.
21. Park HS, Kang SH, Park HJ, Kim ES. 2005. Doxorubicin productivity improvement by the recombinant *Streptomyces peucetius* with high-copy regulatory genes cultured in the optimized media composition. J Microbiol. Biotechnol. 15(1):66-71.
22. Adams MA, Nakanishi K. 1979. Selected uses of HPLC for the separation of natural products. Journal of Liquid Chromatography. 2(8):1097-1136.
23. Suman P, Siva Rao T, Krishna KVSR. 2017. Development and validation of a stability-indicating RC-HPLC method for estimation of Daunorubicin- A chemotherapeutic drug in bulk and pharmaceutical formulations. World Journal of Pharmaceutical Research. 6(7):1158-1174.
24. Van Meerloo J, Kaspers GJ, Cloos J: Cell sensitivity assays: the MTT assay. In Cancer cell culture. 2011:237-245.
25. Caldwell WG, Yan Z, Lang W, Masucci AJ. 2012. The IC_{50} concept revisited. Current topics in medicinal chemistry. 12(11):1282-1290.