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BACILLUS SP RM3 ISOLATED FROM MANGROVE FOREST HAS POTENTIAL AS ANTIBACTERIAL ACTIVITY AND CYTOTOXICITY

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Abstract

Background: Antibacterial resistance is a critical health concern, driving the search for new compounds from natural sources such as mangrove-associated bacteria, which produce diverse secondary metabolites.
Objective: This research focused on studying the bioactivity of the extract of *Bacillus* sp. RM3, previously isolated from mangrove forests through an extraction method using ethyl acetate solvent.
Methods: The *Bacillus* sp. RM3 isolate was fermented in ISP-2 media and extracted with ethyl acetate. The resulting crude extract was tested for antibacterial activity using the Kirby-Bauer method against *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* FNCC 0405 (Gram-positive), *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 700603 (Gram-negative). Cytotoxic effects were assessed via the Brine Shrimp Lethality Assay (BSLT).
Results: The ethyl acetate extract displayed moderate antibacterial activity, evidenced by inhibition zone diameters between 5 to 10 mm. Triplicate measurements revealed the following average inhibition zone diameters: *E. coli* (9.65±1.62 mm), *K. pneumoniae* (7.88±1.92 mm), *S. aureus* (7.77±1.26 mm), and *S. mutans* (7.03±0.99 mm). Furthermore, the BSLT assay revealed an LC50 value of 504.586 ppm, which indicates the extract was toxic (LC50 < 1000 ppm).
Conclusions: Overall, these results indicate the potential of *Bacillus* sp. RM3 isolate as a producer of antibacterial and cytotoxic compounds

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INTRODUCTION

Infectious diseases resulting from antibiotic resistance have become a global public health concern, including in Indonesia. The development of antibiotic resistance is driven by the uncontrolled use of antibiotics, as observed in pathogens such as *Staphylococcus aureus* and *Escherichia coli*, which can elevate morbidity and mortality rates (1). Furthermore, the significant increase in cancer cases underscores the urgency to identify more effective alternative therapies. Mangrove plants, like *Rhizophora mucronata*, have attracted interest because they could be a valuable reservoir of substances that combat cancer (2-4).

Microorganisms in mangrove sediments, such as bacteria and fungi, play a crucial role in the decomposition of complex organic matter into simpler nutrients, which is essential for the mangrove ecosystem (5). The detritus formed from this process is rich in vital elements such as enzymes, protein nitrogen, phosphorus, and organic carbon, supporting the growth of other organisms within the ecosystem (6, 7). Mangrove species such as *Rhizophora apiculata*, *Rhizophora mucronata*, *Avicennia marina*, and *Sonneratia alba* are known to harbor bacteria in their sediments, indicating a significant role in the biological cycles of the mangrove ecosystem (8).

The unique ecological niche of mangrove ecosystems fosters a diverse microbial community that has evolved sophisticated mechanisms for survival, including the production of a wide array of secondary metabolites (8-10). These compounds often exhibit potent biological activities, representing a largely untapped resource for novel pharmaceuticals. Endophytic bacteria, residing within plant tissues, are of particular interest as they can produce bioactive compounds that may contribute to the host plant's defense mechanisms or adaptation to its environment (7). Isolating and characterizing these endophytic bacteria from mangrove plants like *R. mucronata* could yield novel antibacterial and cytotoxic agents with potential applications in combating antibiotic-resistant infections and cancer (11).

Our previous study reported the isolation of bacterial isolate encoded as *Bacillus* sp. RM3 from mangrove soil of the Ngurah Rai mangrove forest in Bali, in which the isolate showed potential antibacterial activity based on perpendicular streak (12). Designated as RM3, this code represents the third bacterial isolate previously recovered from *R. mucronata* soil. Therefore, this study aimed to investigate the bioactivity of a bacterial isolate, *Bacillus* sp. RM3, obtained from a mangrove forest environment. Specifically, the research focused on screening the ethyl acetate extract of this isolate for its in vitro antibacterial activity against a panel of clinically relevant Gram-positive and Gram-negative bacteria (*S. aureus*, *Streptococcus mutans*, *E. coli*, and *Klebsiella pneumoniae*) and its cytotoxic potential using the Brine Shrimp Lethality Assay (BSLT). The findings of this screening are expected to provide preliminary insights into the potential of mangrove-associated *Bacillus* sp. RM3 as a source of novel antibacterial and cytotoxic compounds, thereby contributing to the ongoing efforts to address antibiotic resistance and the need for new anticancer therapies.

MATERIALS AND METHODS

Extraction of *Bacillus* sp. RM3

The isolate was revitalized by transferring one loop of *Bacillus* sp. RM3 into 10 mL of sterile liquid ISP-2 medium and incubating it for 2 days at room temperature on a shaker at 150 rpm. The 2-day-old *Bacillus* sp. RM3 culture was then transferred into 90 mL of liquid ISP-2 medium and again shaken at 150 rpm for 7 days at room temperature. The supernatant from the pure culture of *Bacillus* sp. RM3 isolate was separated from the cell mass through filtration using Whatman no. 1 filter paper. The resulting filtrate, a viscous extract, was subsequently subjected to an extraction process using ethyl acetate as the solvent. The extraction was performed twice, yielding an organic phase and an aqueous phase. The organic phase containing the extracted compounds was then evaporated using a rotary evaporator until a dry extract was obtained.

Gram staining

Gram staining was performed to classify bacteria as Gram-positive, characterized by a purple color, or Gram-negative, characterized by a red color. The staining procedure involved sequential application of crystal violet solution for 1 minute, iodine for 1 minute, ethanol for 30 seconds, and safranin for 1 minute on Zobell marine agar media. Each bacterial colony was stained following the Gram staining procedure. Moreover, cell morphology was observed by staining under a light microscope (Leica, Germany) with 1000 times magnification.

Antibacterial screening

The Kirby-Bauer method was employed in this study to assess antibacterial activity. Prior to the antibacterial activity assay, each test bacterium was revitalized, and 200 µL of each bacterial culture, namely *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Staphylococcus aureus* ATCC 25923, and *Streptococcus mutans* FNCC 0405, was spread onto Luria-Bertani (LB) agar. The bacterial isolate to be tested was streaked perpendicularly on LB agar using a sterile cotton swab and incubated at 37^{o}C for 24 hours until sufficient colony growth was observed. Subsequently, 20 µL of the *Bacillus* sp. RM3 extract was applied to three 6 mm paper discs, which were then placed on the pre-incubated LB agar plates. These plates were then re-incubated at 37^{o}C for 24 hours. The interpretation of the antibacterial assay results was categorized into four groups based on the diameter of the inhibition zone: weak (0-5 mm), moderate (5-10 mm), strong (10-20 mm), and very strong (>20 mm).

Brine Shrimp Lethality Test (BSLT)

The Brine Shrimp Lethality Test (BSLT) was conducted using ten newly hatched Artemia salina larvae. The tested extract comprised seven different concentrations: 2000, 1000, 500, 250, 100, 50, and 25 ppm, with each concentration performed in duplicate. For each extract concentration, ten larvae were introduced and allowed to stand for 24 hours. Following the 24-hour period, the surviving *Artemia salina* were counted, and the lethal concentration was analyzed. The toxic effect was determined by calculating the percentage of *A. salina* mortality in each vial after 24 hours. Potassium dichromate, at concentrations ranging from 0.1 to 0.9 mg/mL with the same number of replicates as the treatment, was used as a positive control. Conversely, seawater without extract addition served as the negative control. Mortality was calculated by dividing the number of dead *A. salina* by the

initial number and multiplying by 100%. The Lethal Concentration 50 (LC50) value was calculated using probit analysis with the Statistical Package for the Social Sciences (SPSS) version 27. An extract was considered to exhibit a toxic effect if its LC50 value was less than 1000 ppm.

RESULTS AND DISCUSSIONS

The results of this study indicated that the *Bacillus* sp. RM3 bacterial isolate exhibited Gram-positive characteristics, as evidenced by the purple staining of cells following the Gram staining procedure. Microscopic observations revealed rod-shaped cells that formed colonies (Figure 1).

The Gram reaction of a bacterium is closely associated with the physical and chemical properties of its cell wall. Gram-positive bacteria are characterized by a thick peptidoglycan layer, whereas Gram-negative bacteria possess a thin peptidoglycan layer enveloped by an outer membrane rich in lipopolysaccharides (13). This difference in cell wall structure results in a high affinity of Gram-positive bacteria for crystal violet and a low affinity in Gram-negative bacteria. Gram-positive bacteria are able to form a crystal violet-iodine complex; subsequent addition of an alcohol-based decolorizing agent causes cell dehydration, leading to pore shrinkage and reduced permeability of the cell wall (14). Consequently, the crystal violet-iodine complex is trapped within the cell, resulting in the persistent purple coloration.

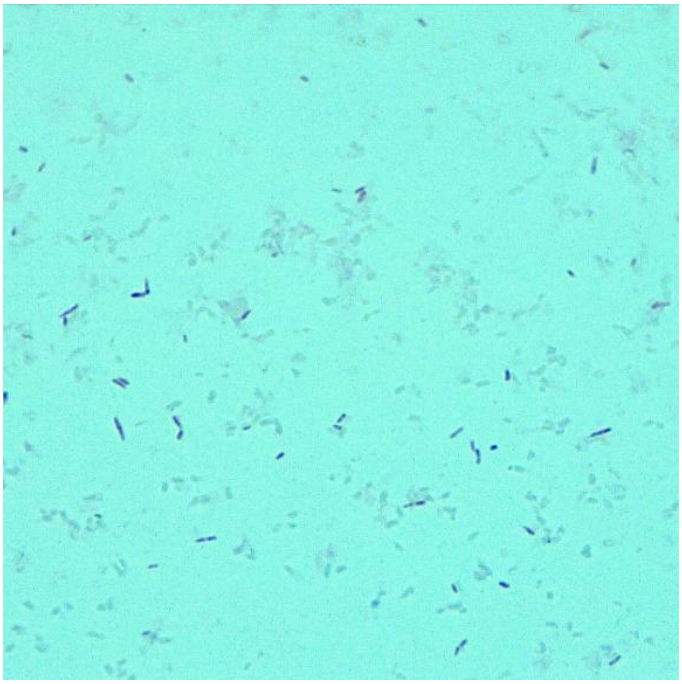

Figure 1. Gram staining of *Bacillus* sp. RM3 observation was performed using a light microscope at 1000x magnification

The inhibition zone data for the *Bacillus* sp. RM3 extract revealed the largest zone observed for *E. coli* ATCC 25922 (9.65±1.62 mm) and the smallest for *S. mutans* FNCC 0405 (7.03±0.99 mm), as depicted in Figure 2. Comparison with the positive control, levofloxacin, demonstrated very strong antibacterial activity (>20 mm, Table 1), whereas the negative control with ethyl acetate showed no inhibition zone (Figure 3).

However, the inhibition zones produced were smaller compared to the positive control antibiotic, levofloxacin, likely due to the suboptimal quantity and quality of secondary metabolites in the *Bacillus* sp. RM3 isolate. This finding demonstrates less potent antibacterial activity compared to prior research, which reported the most potent activity (evidenced by an inhibition zone of 17.60±3.73 mm) from an ethyl acetate extract of an endophytic *Bacillus* sp. isolate sourced from *R. apiculata* mangrove leaves (15).

Table 1. Inhibition Zone Diameters of Ethyl Acetate Extract of *Bacillus* sp. RM3 against Test Bacteria

Treatments		Diameter zone of inhibition (mm) ± SD	Category
S. aureus ATCC 25923	Extract of *Bacillus sp.* RM3	7.77±1.26	Moderate
	Levofloxacin (K+)	24.68±1.29	Very Strong
	Ethyl acetate (K-)	0±0	-
S. mutans FNCC 0405	Extract of *Bacillus sp.* RM3	7.03±0.99	Moderate
	Levofloxacin (K+)	22.94±0.26	Very Strong
	Ethyl acetate (K-)	0±0	-
E. coli ATCC 25922	Extract of *Bacillus sp.* RM3	9.65±1.62	Moderate
	Levofloxacin (K+)	25.60±0.82	Very Strong
	Ethyl acetate (K-)	0±0	-
K. pneumoniae ATCC 700603	Extract of *Bacillus sp.* RM3	7.88±1.92	Moderate
	Levofloxacin (K+)	23.47±0.80	Very Strong
	Ethyl acetate (K-)	0±0	-

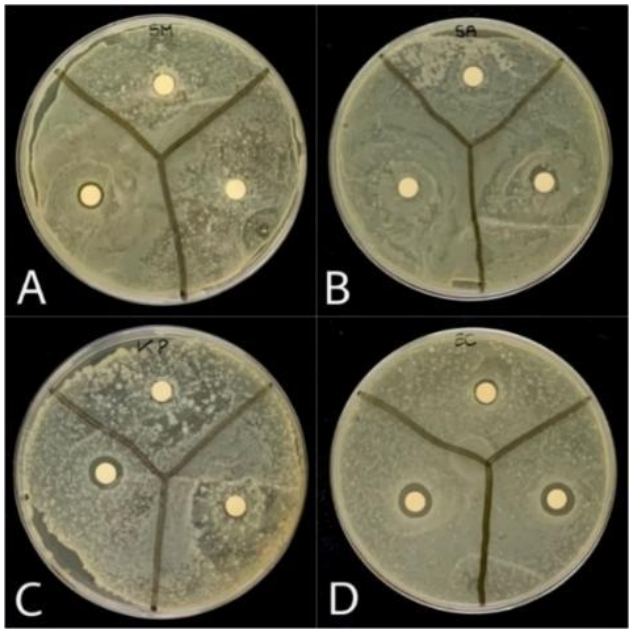

Figure 2. Inhibition zones formed by the ethyl acetate extract from *Bacillus* sp. RM3 isolate. A. *S. mutans* FNCC 0405, B. *S. aureus* ATCC 25923, C. *K. pneumoniae* ATCC 700603, D. *E. coli* ATCC 25922.

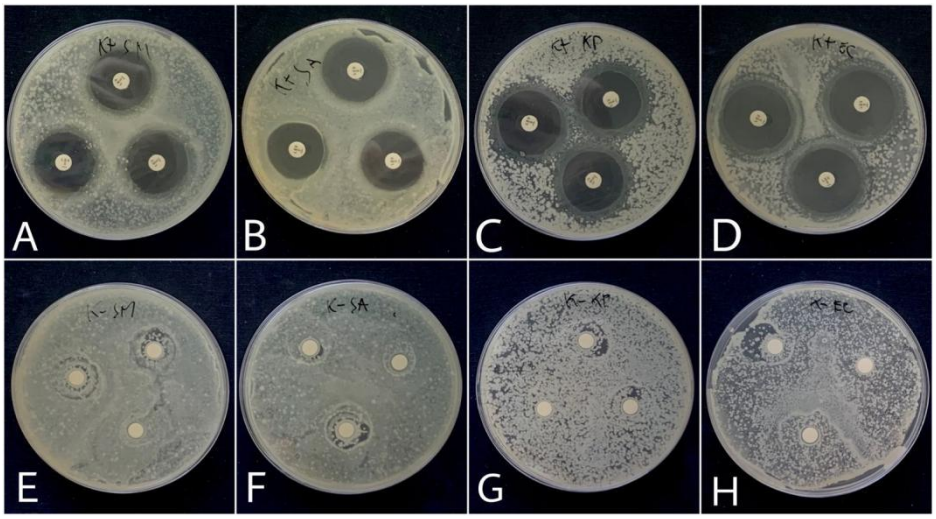

Figure 3. Antibacterial Assay of Levofloxacin (K+) and Ethyl Acetate (K-). A. (K+) against *S. mutans* FNCC 0405, B. (K+) against *S. aureus* ATCC 25923, C. (K+) against *K. pneumoniae* ATCC 700603, D. (K+) against *E. coli* ATCC 25922, E. (K-) against *S. mutans* FNCC 0405, F. (K-) against *S. aureus* ATCC 25923, G. (K-) against *K. pneumoniae* ATCC 700603, H. (K-) against *E. coli* ATCC 25922.

The results of the BSLT assay are presented across concentrations of 2000, 1000, 500, 250, 100, 50, and 25 ppm. The mortality rate of brine shrimp larvae in the positive control, using a mixture of seawater and potassium dichromate, yielded an LC50 value of 10.55 ppm, categorized as very toxic. Conversely, no larval mortality was observed in the negative control, which used only seawater. The BSLT assay revealed an LC50 value of 504.59 ppm, which falls into the toxic category (16). The toxicity categories of substances based on LC50 values are defined as follows: very toxic (LC50 < 30

ppm), toxic (LC50 30-1000 ppm), and non-toxic (LC50 > 1000 ppm) (17). The data from the BSLT assay are summarized in Table 2 and Table 3.

Previous studies on bioactive compounds produced by *Bacillus* sp., such as flavonoids and terpenoids, provide a basis for the hypothesis that the *Bacillus* sp. RM3 isolate may also produce these compounds (18, 19). The observed capability of *Bacillus* sp. RM3 to synthesize various secondary metabolites, including those with antibacterial and cytotoxic properties, may be attributed to its ecological niche within mangrove ecosystems, which are defined by fluctuating salinity, anoxic or hypoxic conditions, elevated UV radiation, and significant inter-microbial competition (7). To survive and thrive in such harsh environments, *Bacillus* species adapt by producing a wide range of secondary metabolites that serve as defence mechanisms, competitive tools, or signalling molecules (20). Therefore, identifying the active compounds in the Bacillus sp. RM3 extract using analytical techniques like GC/MS or LC/MS will be a crucial next step to fully understand its antibacterial and cytotoxic mechanisms.

Table 2. Cytotoxicity Assay Results of Ethyl Acetate Extract from *Bacillus* sp. RM3

Concentration (ppm)	x Log (ppm)	Number of larvae	Death larvae I	Death larvae II	Average	Death Percentage	Probit value
2000	3.30	10	8	8	8.0	80	5.84
1000	3.00	10	8	7	7.5	75	5.67
500	2.70	10	3	8	5.5	55	5.13
250	2.40	10	3	3	3.0	30	4.48
100	2.00	10	2	2	2.0	20	4.16
50	1.70	10	1	1	1.0	10	3.72
25	1.40	10	0	0	0	0	0.00
LC 50 ethyl acetate extract = 504.586 ppm (toxic)							

Table 3. Cytotoxicity Assay Results using BSLT

Extract/treatment	LC50 (ppm)	Category
ethyl acetate extract	504.59	Toxic
positive control (Potassium dichromate)	10.55	Very toxic
negative control (Seawater)	0	Non toxic

CLINICAL IMPLICATION

The finding that *Bacillus* sp. RM3 produces compounds with modest efficacy against relevant bacterial targets is significant. It opens up a promising new direction for developing antibacterial agents, which are urgently needed to combat the growing problem of antibiotic resistance. This research could be a crucial step in discovering novel drugs to overcome

some of our toughest bacterial threats. Furthermore, the observed cytotoxic activity of the extract warrants further investigation into its potential as a source of anticancer compounds. Isolating and characterizing the specific bioactive compounds, alongside conducting in vivo studies and toxicity assessments, will be crucial future research steps that could enable the development of novel therapeutic strategies for bacterial infections and cancer.

LIMITATIONS

The study's findings are limited by its reliance on in vitro antibacterial testing, which may not reflect in vivo efficacy. Furthermore, the use of a crude extract prevented the identification of specific bioactive compounds and their mechanisms of action. Finally, the preliminary cytotoxicity assessment using the BSLT assay necessitates further validation with mammalian cell lines and in vivo models to confirm anticancer potential. These shortcomings mean further studies are essential to completely uncover the therapeutic capabilities of *Bacillus* sp. RM3.

CONCLUSIONS

The ethyl acetate extract from *Bacillus* sp. RM3 showed promising antibacterial and cytotoxic properties. This suggests that *Bacillus* sp. RM3 could be a valuable source of compounds for therapeutic applications. Future studies should focus on identifying the specific chemical constituents responsible for the observed bioactivities. Further research is also warranted to explore the anticancer potential of the extract in relevant models and to optimize extraction strategies for maximizing the recovery of these valuable compounds and fully understanding the spectrum of their biological activities.

CONFLICT OF INTEREST

The authors have no financial or commercial ties that could present a conflict of interest with this research.

AUTOR CONTRIBUTIONS

The experiments and initial manuscript draft were completed by Ida Ayu Kartika Maheswari. Anak Agung Gede Indraningrat designed and supervised the experiments, also providing necessary resources. Anak Agung Ayu Lila Paramasatiari supervised the experiments and analyzed the antibacterial data. Daegeun Choe contributed to data analysis, formatting, and proofreading of the manuscript.

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REFERENCES

1. Bottery MJ, Pitchford JW, Friman V-P. Ecology and evolution of antimicrobial resistance in bacterial communities. The ISME Journal. 2021;15(4):939-48.
2. Arnold M, Morgan E, Rumgay H, Mafra A, Singh D, Laversanne M, Vignat J, Gralow JR, Cardoso F, Siesling S, Soerjomataram I. Current and future burden of breast cancer: Global statistics for 2020 and 2040. The Breast. 2022;66:15-23.
3. Mardela AP, Maneewat K, Sangchan H. Breast cancer awareness among Indonesian women at moderate-to-high risk. Nursing & Health Sciences. 2017;19(3):301-6.
4. WHO. Global Cancer Observatory. World Health Organization.; 2019.
5. Ancheeva E, Daletos G, Proksch P. Lead Compounds from Mangrove-Associated Microorganisms. Mar Drugs. 2018;16(9):319.
6. Chen Q, Zhao Q, Li J, Jian S, Ren H. Mangrove succession enriches the sediment microbial community in South China. Sci Rep. 2016;6:27468.
7. Nabeelah Bibi S, Fawzi MM, Gokhan Z, Rajesh J, Nadeem N, Kannan RRR, R DDGA, Pandian SK. Ethnopharmacology, Phytochemistry, and Global Distribution of Mangroves-A Comprehensive Review. Mar Drugs. 2019;17(4).
8. Dahibhate NL, Saddhe AA, Kumar K. Mangrove Plants as a Source of Bioactive Compounds: A Review. The Natural Products Journal. 2019.
9. Friess DA. Ecosystem Services and Disservices of Mangrove Forests: Insights from Historical Colonial Observations. Forests. 2016;7(9):183.
10. Wijaya MD, Indraningrat AAG. Antibacterial Activity of Mangrove Root Extracts from Ngurah Rai Mangrove Forest, Denpasar-Bali. Biology, Medicine, Natural Product Chemistry. 2021;10(2):117-21.
11. Mubaraq A, Sembiring M, Widiastuti E, Basyuni M. Bioprospection of Endophytic Bacteria Isolated from Mangrove Ecosystems and their Potential Biotechnological Applications. OnLine Journal of Biological Sciences. 2024;24:836-47.
12. Indraningrat AAG, Wijaya MD, Suryanditha PA, Siskayani AS, Janurianti NMD. Antibacterial Screening of Bacterial Isolates Associated with Mangrove Soil from the Ngurah Rai Mangrove Forest Bali. Biology, Medicine, Natural Product Chemistry 2021;10(2).
13. Paray AA, Singh M, Mir MA, kaur D. Gram Staining: A Brief Review. International Journal of Research and Review. 2023;10:336-41.
14. Mai-Prochnow A, Clauson M, Hong J, Murphy AB. Gram positive and Gram negative bacteria differ in their sensitivity to cold plasma. Scientific Reports. 2016;6(1):38610.

15. Sormin RBD, Nendissa DM, Mailoa MN, Rieuwpassa F, Wenno MR. Antibacterial activity of Rhizophora apiculata extract originated from Inner Ambon Bay against selected pathogen bacteria. IOP Conference Series: Earth and Environmental Science. 2021;797(1):012017.
16. Banti CN, Hadjikakou SK. Evaluation of Toxicity with Brine Shrimp Assay. Bio Protoc. 2021;11(2):e3895.
17. Waghulde S, Kale MK, Patil V. Brine Shrimp Lethality Assay of the Aqueous and Ethanolic Extracts of the Selected Species of Medicinal Plants. Proceedings. 2019;41(1):47.
18. Zhao H, Yan L, Xu X, Jiang C, Shi J, Zhang Y, Liu L, Lei S, Shao D, Huang Q. Potential of Bacillus subtilis lipopeptides in anti-cancer I: induction of apoptosis and paraptosis and inhibition of autophagy in K562 cells. AMB Express. 2018;8(1):78.
19. Dan AK, Manna A, Ghosh S, Sikdar S, Sahu R, Parhi PK, Parida S. Molecular mechanisms of the lipopeptides from Bacillus subtilis in the apoptosis of cancer cells - A review on its Current Status in different cancer cell lines. Advances in Cancer Biology - Metastasis. 2021;3:100019.
20. Rajan L, Chakraborty K, Chakraborty RD. Pharmacological properties of some mangrove sediment-associated bacillus isolates. Arch Microbiol. 2021;203(1):67-76.