J. Phytol. Res. **38**(1): 61-67, 2025 **ISSN 0970-576**

SUPPRESSION OF TUMOR BY AN ENDOPHYTIC BACTERIUM, *BREVUNDIMONAS VESICULARIS* JAP ISOLATED FROM *MORINDA CITRIFOLIA* Linn.

NEENU A SANTHOSH[1*] AND ANTO PV[2]
[1]Department of Botany, Mercy College, Palakkad, Kerala, India 678006
[2]Department of Botany, St. Thomas College, Thrissur, Kerala, India 680001
[*]**Corresponding Author's Email:** neenus007@gmail.com

The present investigation was focused on the active compounds present in the endophytic bacterium *Brevundimonas vesicularis* strain JAP that has been isolated from the fresh and healthy leaves of the plant *Morinda citrifolia* L. Since this plant is well-known for anticancer activity, endophytic bacteria in it may have active compounds showing similar property. Extraction of the metabolites from the extracellular culture supernatant in ethyl acetate solvent was evaporated and reconstituted in ethyl alcohol and 1% propylene glycol for invitro and in vivo studies respectively. The active compounds in it were evaluated for in vivo and in vitro antitumor property. In vitro cytotoxicity study was performed by dissolving the extract in Dimethyl Sulphoxide (DMSO). The method adopted were trypan blue dye exclusion technique using DLA (Dalton Lymphoma Ascites) tumour cell of mice. In addition, the cytotoxic potential of the extract against breast cancer cell line MCF-7 was tested using MTT (3-(4,5-dimethythiazol-2yl)-2,5-diphenyl tetrazolium bromide) assay. EAC (Erhlich Ascites Carcinoma) induced in vivo antitumor study reports that there is an increase in life span of drug treated mice with higher dose followed by lower dose compared to standard cyclophosphamide. The findings suggest that the bioactive compounds in the bacterial sample labeled dose dependent reduction in tumor size, justifying the chemical constituents present in the endophytic bacteria, may in future satisfies the criteria for the drug discovery.

Keywords: Antitumor, Dalton Lymphoma Ascites, Erhlich Ascites Carcinoma, Endophytic bacteria, *Morinda citrifolia*, MTT assay

Introduction

Endophytic bacteria have been identified as depots of novel secondary metabolites for immense therapeutic use[1]. Bacteria produce a wide array of volatile organic compounds, which develop metabolic products or by-products such as hydrocarbons, aliphatic alcohols, ketones, and indole[2]. To extract intracellular and extracellular secondary metabolites, we can use organic solvents like chloroform, ethyl acetate, and ethanol to add solvent to the culture medium[3]. From the microbial cells, extracellular metabolites can be separated using simple techniques like filtration or centrifugation, whereas intracellular metabolites achieved by breaking the cell, which is a complex process[4, 5]. Various analytical techniques are introduced to identify and quantify exometabolome present in the spent microbial culture media. These metabolites formed due to secretion in different growth phases during cell uptake of nutrients from the external culture medium[6]. Gas chromatography-mass spectrometry (GC-MS) is the most appropriate technique for finding several components from a complex mixture of volatile active compounds[7].

Received: 5 Dec,2024 *Revised: 10 July, 2025* *Accepted: 15 July,2025*

Microbial-based cancer therapy is one of the innovations in treating cancer diseases. Advancement in the study of bacterial by-products like enzymes, proteins, immunotoxins, fatty acids, and secondary metabolites have made to target cancer cells or to regress tumor via cell growth inhibition, inducing apoptosis or arresting cell cycle stages [8, 9, 10]. Microbial drug discovery is paying an inevitable influence on cancer chemotherapy. For more than a century, bacteria to reduce the growth rate or size of certain forms of cancer have been screened[11]. Research on identifying several anaerobic bacterial species and facultative anaerobic bacteria for their ability to regress or target tumors in experimental animals has been studied[12]. Studies on the application of bacteria as antitumor agents are an emerging invention in cancer therapy[13].

Brevundimonas vesicularis belongs to phylum proteobacteria is a gram-negative, non-fermenting, aerobic bacilli. Various studies on acute and chronic inflammation in mice have been reported in many plants and fungi extracts. But a handful of literature was found on bacterial components showing anti-inflammatory activity. There are no reports on the findings of an in-vivo anti-inflammatory study of phlogistic agents present in the isolated endophytic bacteria *Brevundimonas vesicularis*. Also, the in vivo tumor and antioxidant study have not yet be conducted on this particular endophyte which pay way to our work to point out these activities.

Material and Methods

Plant Material:

The leaves of medicinal plant *Morinda citrifolia* L. were collected from Thrissur district (Lat 10°37'06"N and Lon 76°12'17"E), Kerala, India. A voucher specimen of *M. citrifolia* (15785) was maintained in Kerala Forest Research Institute, Peechi, Thrissur.

Endophytic Bacteria Isolation:

The leaves of the plant were employed as the source material for the isolation of endophytic bacteria. Fresh and healthy leaves were surface sterilized in 1 % sodium hypochlorite (10 min), 70 % ethanol (1 min), and washed thrice in sterile double distilled water. With a clean and sterile mortar and pestle, the material was macerated in 0.85% NaCl and serially diluted (10^{-1} to 10^{-7} dilution factors). 100 µL of it spread plated onto nutrient agar (HiMedia) incubated at 37°C for five days and observed periodically for bacterial growth[14].

Molecular Identification by 16S rDNA Sequencing:

The 16S rRNA gene of the isolate was amplified using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3')[15]. The genomic DNA of the strain was amplified using a PCR machine (Thermo fisher scientific, U.S.A). 50 µL of reaction mixture containing 1 µL genomic DNA, 1 µL forward and reverse primer each, 38 µL sterile water, 1 µL Taq DNA polymerase (HiMedia), 5 µL 10X assay buffer, and 3 µL 10mM dNTP mix was used. The DNA amplicon was sequenced using BigDyeTM Terminator v3.1 Cycle sequencing kit on ABI 3730 XL cycle Sequencer. The data was used to carry out BLAST with the NCBI GenBank non-redundant (nr) database[16].

Extraction of the Culture Filtrate:

The bacterial isolates were inoculated into the LB broth separately and incubated at 37 °C for ten days in an orbital shaker. After incubation, centrifuge the culture broth at 3000 rpm for 10 min, and the cell-free supernatant was collected. The culture filtrate (100 mL) was extracted twice with ethyl

acetate solvent in the ratio of 1:1 (v/v) using a separating funnel. The ethyl acetate fraction was concentrated using a lyophilizer, and the residue was dissolved in an ethyl alcohol solvent[17]. For in vivo studies, ethyl alcohol extract was again evaporated and reconstituted in 1% propylene glycol.

Experimental Animals:

Male Swiss Albino mice weighing 26 ± 5.2gm (Mean ± S.E) were used in this study. Animal studies were conducted following Institutional Animal Ethics Committee (IAEC) regulations approved by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) (ACRC/IAEC/20(1)-P13) and conducted humanely. The mice were obtained from Small Animal Breeding Station (IAEC Reg. no. 328/PO/c/01/CPCSEA), Kerala Veterinary and Animal Science University (KVASU), Mannuthy, Thrissur, Kerala.

Acute Toxicity Study:

For toxicity study, two groups of male Swiss albino mice (6 mice/group) were taken. First group was kept as control, and Group II was administered with a single dose drug (300 mg/Kg b.wt) orally as per the OECD (Organisation for Economic Co-operation and Development) guidelines. The experimental animals were monitored for 14 days, and the mortality rate of the mice was noted. After completion of in vivo studies, carbon dioxide inhalation method of euthanasia is adopted to kill the mice taken for study.

In vitro Cytotoxicity Studies:

Trypan Blue Exclusion Test of Cell Viability:

Cytotoxic activity of the endophytic bacterial extract was studied using the trypan blue dye exclusion technique by analyzing the percentage of viability of Daltons Lymphoma Ascites (DLA) cells. DLA cells were grown in the peritoneal cavity of mice weighing 25-30 g by injecting cell suspension ($1x10^6$ cells/mL). On the 15^{th} day, tumor cells were aspired from the peritoneal cavity of the mice and centrifuged for 15 min at 1500 rpm after washing with PBS (Phosphate-Buffered Saline) (0.2 M, pH 7.4). The pellet was again dissolved in PBS, and the process was repeated thrice. At last, the cells were suspended in a known quantity of PBS, and the cell count was adjusted to $1x10^6$ cells/ mL. Next, cell suspension of 0.1 mL was dispensed in 0.8 mL of phosphate buffer and incubated with different concentrations (10-200 µg/mL) of samples for 3 h at 37 ºC. After 3 h, add 0.1 mL trypan blue dye (1%), and cells were examined in a compound microscope using a hemocytometer. The IC_{50} value was calculated[18].

MTT Assay:

The cytotoxic potential of sample extract against breast cancer cell line MCF-7 was tested using MTT (3-(4,5-dimethythiazol-2yl)-2,5-diphenyl tetrazolium bromide) assay[19]. The monolayer cell culture was trypsinized (0.25% Trypsin and 0.03% EDTA), and the cell count was adjusted to $1.0x10^6$ cells/mL in the Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal bovine serum (FBS). About $1x10^6$ cells were seeded in each well of the 96 well plates. When a partial monolayer was formed after 24 h, the supernatant was flicked off, and 100 µL of test extracts (12.5, 25, 50, 75, and 100 µg/mL) were added to the cells in microtiter plates incubated at 37ºC in an atmosphere of 5% CO_2 for 24 h. Cells incubated in a complete medium without test extract were kept as control. After incubation, the medium was discarded, and 20 µL MTT (5 mg/mL) was pipetted, and the cells were again incubated for 3 h. After the incubation, the MTT solution covering the cells was removed using a micropipette. 100 µl of dimethyl sulphoxide was added to the wells

of the microtitre plate. The absorbance were noted in a microplate reader (ELISA Reader, Biotek) at 570 nm to determine cell viability. The experiment was repeated thrice. The percentage of cell viability and IC_{50} value of the test extract was calculated using the formula-

$$\%\ of\ Cell\ Viability\ =$$

$$\left(\frac{Mean\ OD\ of\ experimental\ wells}{Mean\ OD\ of\ control\ wells}\right) \times 100$$

In vivo antitumor activity

EAC Induced Ascites Tumour Model:

Male Swiss albino mice were grouped into five comprising of six animals each. All the groups were injected with EAC cells (0.1 ml of 1×10^6 cells/mouse). Group I was kept as control without drug treatment and group II was treated with vehicle control 1% propylene glycol. Similarly standard drug cyclophosphamide (10 mg/kg), drug extract at different doses (25 and 50 mg/kg b. wt.) was administered in group III, IV, V respectively. The percentage increase in life span was calculated- % ILS = (T-C/C) × 100 where 'T' and 'C' are mean survival of treated and control mice, respectively[20].

Statistical analysis

All the experiments were performed in triplicates (n=3), and the values were expressed in Mean ± SE. The data were checked for statistical significance ($P<0.001$, $P<0.01$, and $P<0.05$) using one-way analysis of variance (ANOVA) followed by an appropriate post hoc test (Tukey's multiple comparison test) using Prism 9.0 GraphPad software Inc. USA.

Results and Discussion

The isolated endophytic bacterium was identified as *Brevundimonas vesicularis* strain JAP and the sample code given was NMC15. The sequence was deposited in NCBI GenBank and got accession number MK646066. Phylogenetic tree was constructed in determining proper phylogenetic position of isolated strain as in Fig. 1.

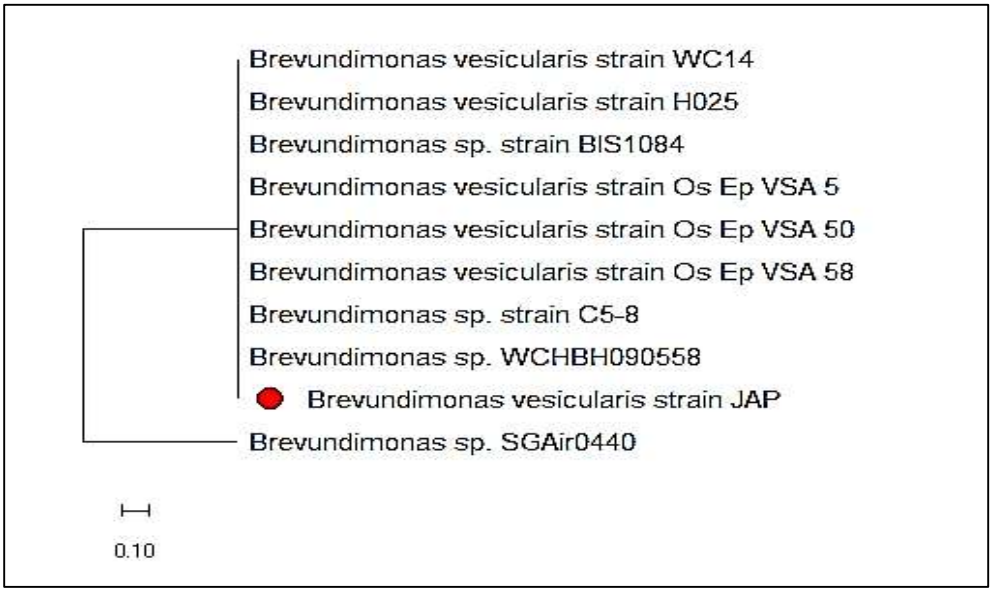

Figure 1: Phylogenetic position of isolated endophytic bacterial strain.

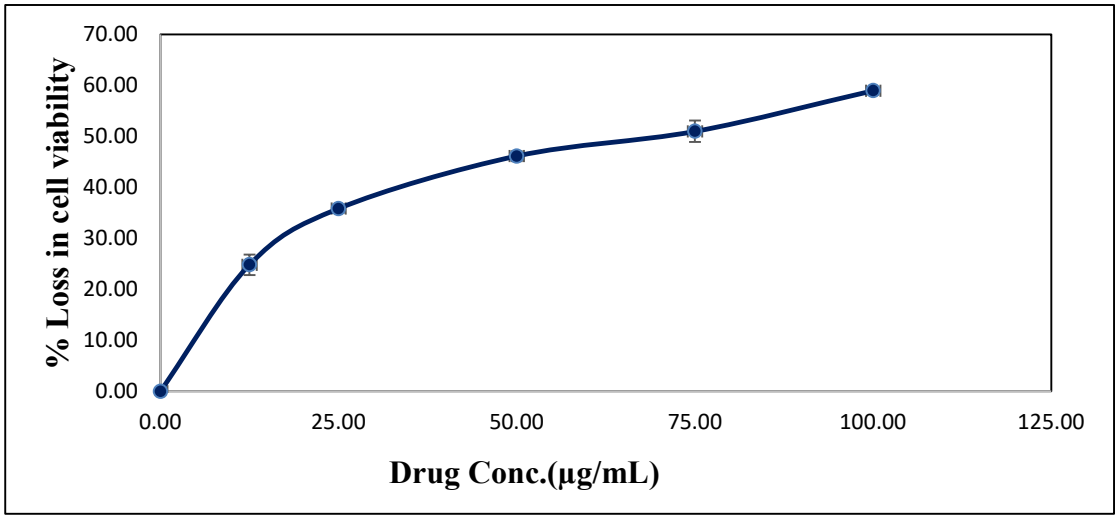

Figure 2: Cell viability profile of NMC15 extract against MCF-7 breast cancer cell lines.

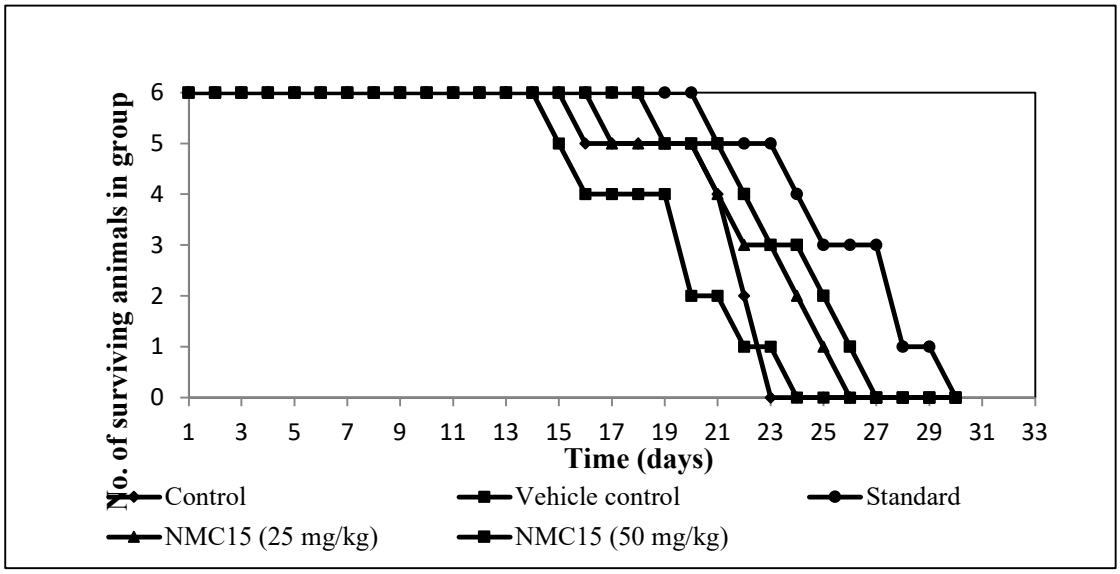

Figure 3: Effect of sample extract on mean survival days of EAC bearing mice.

The administration of propylene glycol extract of the sample (single dose) in mice reported no observable toxicity such as weight loss, hair loss, skin color change, food, and water intake difference. Also, no mortality was recorded, assuming the drug is safe for in vivo studies. The short-term in vitro activity studied using DLA cells of mice showed IC_{50} 39.0 ±0.627 µg/mL. Anti-proliferative activity of the sample extract analysed by MTT assay towards MCF-7 cell line showed IC_{50} 79.365 ±1.02 µg/mL (Fig.2.). An increase in life span of drug treated mice of higher dose (48.83%), followed by lower dose (22.09 %) compared to cyclophosphamide (60.46%). Thereby, ensuring the compounds present in the bacterial extract have anticancer. The in vivo anticancer study of the bacterial extract was depicted in Table 1 and Fig. 3.

Table 1: Showing mean survival days and percent increase in lifespan of EAC bearing mice.

Groups	Mean Survival Days	% ILS
Control	17.2 ±2.16	……..
Vehicle control	18.0 ±1.67^{ns}	4.65
Standard	27.6 ±2.06***	60.46
NMC15 (25 mg/kg body wt.)	21.0 ±1.22***	22.09
NMC15 (50 mg/kg body wt.)	25.6 ±2.03***	48.83

Values are expressed as mean ± SD for 6 animals in each group. P<0.001*, P<0.01**, ns – non-significant.*

Conclusion

Endophytic bacteria produce innumerable bioactive compounds relative to the secondary metabolites of the host plant they inhabit. In the current study, the bacteria *Brevibacterium vesicularis* JAP strain isolated from an important medicinal plant *Morinda citrifolia* L. disclose the in vitro and in vivo antitumor activity that marked these compounds can be used in microbiology pharmaceuticals. This work represents an essential primary step in understanding the endophytic bacterial compounds and their biological activity to benefit the medical field in further investigation.

Ethics approval and consent to participate:

This research involves animal studies which were approved by Institutional Animal Ethics Committee (IAEC) following regulations of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) under reference number (ACRC/IAEC/20(1)-P13). The experiment was conducted in biochemistry department, Amala Cancer Research Centre, Thrissur, Kerala, India.

Conflicts of interest:

The authors declare that we have no conflict of interest.

Funding

This work was supported by the grants given to Neenu A Santhosh by CSIR-UGC, New Delhi, India and DST-FIST, St. Thomas College (autonomous), Thrissur, Kerala, India.

Acknowledgements

The authors would like to thank Dr. Ramdas Kuttan, Director and Dr. Achuthan C Raghavamenon, Scientist, Amala Cancer Research Institute, Thrissur, for their assistance and valuable guidance.

References:

1. Tan RX and Zou WX 2001, Endophytes: A rich source of functional metabolites. ***Nat Prod Rep*. 18**(4) 448-459.
2. Audrain B, Farag MA, Ryu CM and Ghigo JM 2015, Role of bacterial volatile compounds in bacterial biology. ***FEMS Microbiol Rev*. 39**(2) 222-233.
3. Khanna M, Solanki R and Lal R 2011, Selective isolation of rare Actinomycetes producing novel antimicrobial compounds. ***Int J Adv Biotechnol Res*. 2**(3) 357-375.
4. Villas-Boas SG and Bruheim P 2007, The potential of metabolomics tools in bioremediation studies. ***Omics*. 11**(3) 305-313.

5. Tredwell GD, Edwards-Jones B, Leak DJ and Bundy JG 2011, The development of metabolomic sampling procedures for pichia pastoris, and baseline metabolomedata. ***PLoS One*. 6**(1) 16286.
6. Villas-Boas SG, Roessner U, Hansen MAE, Smedsgaard J and Nielsen J 2007, *Metabolome Analysis: An Introduction*. (1 Ed.) Willey-Interscience.
7. E. E and Ren J (2012) GC-MS Analysis of Volatile Plant Secondary Metabolites. Gas Chromatography in Plant Science, Wine Technology, Toxicology and Some Specific Applications.
8. Frankel D, Mehindate K and Schipper HM 2000, Role of heme oxygenase-1 in the regulation of manganese superoxide dismutase gene expression in oxidatively-challenged astroglia. ***J Cell Physiol*. 185**(1) 80-86.
9. Wei HC, Rollins J and Fabian L 2008, Depletion of plasma membrane PtdIns(4,5) P2 reveals essential roles for phosphoinositides in flagellar biogenesis. ***J Cell Sci*. 121**(7) 1076-1084.
10. Bernardes N, Seruca R, Chakrabarty AM and Fialho AM 2010, Microbial-based therapy of cancer: current progress and future prospects. ***Bioeng Bugs*. 1**(3) 178-190.
11. Chakrabarty AM 2003, Microorganisms and cancer: quest for a therapy. ***J Bacteriol*. 185**(9) 2683-2686.
12. Gill GV and Holden A 1996, A malignant pleural effusion infected with *Salmonella enteritidis*. ***Thorax*. 51**(1) 104-105.
13. Kalia VC, Patel SKS, Cho BK, Wood TK and Lee JK 2022, Emerging applications of bacteria as antitumor agents. ***Semin Cancer Biol.* 86**(Pt 2):1014-1025.
14. Fahy PC and Persley GJ 1983, Plant Bacterial Diseases, a Diagnostic Guide. In: Fahy, P.C and Haywad, A.C., Eds., Media and Methods for Isolation and Diagnostic Tests, Acadamic Press, New York, 369-370.
15. Jose PA, Santhi VS and Jebakumar SR 2011, Phylogenetic-affiliation, antimicrobial potential and PKS gene sequence analysis of moderately halophilic Streptomyces sp. inhabiting an Indian saltpan. ***J Basic Microbiol*. 51**(4) 348-356.
16. Zhang Z, Schwartz S, Wagner L and Miller WA 2000, Greedy algorithm for aligning DNA sequences. ***J Comput Biol*. 7**(1-2) 203-214.
17. Ahmed AA 2007, Production of antimicrobial agent by *Streptomyces violachromogenes*. ***Saudi J Biol Sci*. 14** 7-16.
18. Moldeus P, Hogberg J, Orrenius, S 1978, Isolation and use of liver cells. ***Methods in Enzymology*. 52** 60–71.
19. Mosmann T 1983, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. ***Journal of Immunological Methods*. 65**(1–2) 55–63.
20. Chihara G, Hamuro J, Maeda Y, Arai Y and Fukuoka F 1970, Fractionation and purification of the polysaccharides with marked antitumor activity, especially lentinan, from Lentinus edodes (Berk.) Sing. (an edible mushroom). *Cancer* ***Res*. 30**(11) 2776-2781.