RESEARCH ARTICLE

Editorial Process: Submission:09/12/2024 Acceptance:01/18/2025

Investigating the Anticancer Properties of Bacterial Toxoid in Combination Vaccines

Alyaa Jabbar Qasim, Alyaa Kareem Abood, Ahmed Majeed Al-Shammari*

Abstract

Background: The use of bacterial vaccines as a potential Bacterial-Based Cancer Therapy (BBCT) presents an innovative approach, transforming these vaccines into multifunctional tools capable of serving dual roles in medicine. **Materials and methods:** This study aimed to conduct in vitro, immunity-independent experiments to investigate the anticancer properties of vaccine-derived bacterial toxoids on various cancer cell lines. Six concentrations of the DTP vaccine (5×10^{-4}, 25×10^{-5}, 125×10^{-6}, 625×10^{-7}, 312×10^{-7}, and 15×10^{-6} µg/ml) were tested on two cancer cell lines (SKG and HCAM) and a normal Rat Embryonic Fibroblast (REF) cell line. The cytotoxic effects were evaluated using the Crystal Violet assay to determine the percentage of cell death for each toxoid concentration, leading to the calculation of IC_{50} values. Apoptotic effects and other cytopathological changes were observed under an inverted microscope. **Results:** The findings revealed significant toxic effects of the bacterial toxoid on the SKG and HCAM cancer cell lines ($p < 0.001$). In contrast, the toxic effects on the normal REF cell line were evident only at the highest toxoid concentrations. Microscopic analysis showed marked cytological changes in cancer cells treated with the toxoid, with minimal impact on normal cells.

Keywords: Bacterial toxoid- SKG- REF- HCAM cell line- Bacterial vaccines

Asian Pac J Cancer Prev, **26 (1)**, 233-238

Introduction

Cancer is a global health problem responsible for one in six deaths worldwide and is also the main cause of death of approximately 10 million people each year [1]. Surgery, radiation therapy, and chemotherapy as single treatment or in combination are the most known options for cancer treatment for many decades as conventional treatment approaches in addition to the significant advances including stem cell therapy, targeted therapy, nanoparticles, natural antioxidants [2]. Each treatment has drawbacks, either requiring additional treatment or lacking target specificity, resulting in additional side effects on the patient. For example, chemotherapy and radiation therapy have low specificity toward cancer cells, destroying healthy and diseased cells with detrimental side effects [3].

The limitations of current cancer treatments call for alternative interventions, as cancer cells continue to evolve and build resistance against existing chemotherapeutic agents [4]. Thus, there is a dire need for new molecules with higher selectivity and specificity against cancer cells. One of the promising novel therapeutic candidates against cancer is microbial-based cancer therapy. Microorganisms, particularly bacteria, possess a broad range of proteins and peptides with antitumoral properties, such as toxins, immunotoxins, enzymes, and others [5].Toxins produced by pathogenic bacteria interfere with the physiological functions of cells and are sometimes lethal. They act on the cell membrane or on some intracellular target. Occasionally, they are the sole cause of the disease; in most cases, they act in concert with other virulence factors which allow bacteria to establish themselves in the host and resist or escape its defensive mechanism [5]. Detoxification is the process by which bacterial toxins can be transformed by several and different procedures into toxoids which are not only nonpathogenic bacterial agents, but also very vital tools for extremely important medical use, including vaccination [6]. Vaccines can be highly effective tools in combating antimicrobial resistance as they reduce infections caused by antibiotic-resistant bacteria and the consumption of antibiotics associated with disease. The protection effect of bacterial vaccines depends on the immune response of the host to different antigenic components of the bacteria [7].

One of the oldest vaccines used in humans and the most important and essential vaccines in the universal childhood vaccination program is the triple vaccine or Diphtheria-Tetanus-Pertussis (DTP) vaccine that prevents three serious, potentially fatal diseases that affect children and adults, including diphtheria, tetanus, and Pertussis that are caused by Corynebacterium diphtheriae, Clostridium tetani, and Bordetella pertussis respectively

*Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetic Research. Mustansiriyah University, Baghdad, Iraq. *For Correspondence: alshammari.am@uomustansiriyah.edu.iq*

[8]. Diphtheria toxoid combined with tetanus toxoid and whole cell pertussis are the basic components of the diphtheria–tetanus–pertussis (DTP) vaccine [9]. The use of bacterial routine vaccines as a Bacterial-Based Cancer Therapy could be regarded as a sophisticated and innovative approach that turns vaccines into multipurpose instruments with two distinct but equally important roles in medicine. In this regard, various works tackled bacterial-based cancer immunotherapy; current work seeks to try the Invitro immuno-independent experiments to explore the anticancer potential of vaccine–derived bacterial toxoids in different cancer cell lines.

Materials and Methods

Cell Lines

Two different cancer cell lines SKG (esophagus cancer) and HCAM (Hepatocellular carcinoma cell line) in addition to the transformed Rat Embryonic fibroblast cell line (REF), were provided by the Iraqi Center for Cancer and Medical Genetics Research, Mustansiriyah University, cell bank unit. Cell lines were kept in RPMI-1640 solution medium that had been supplemented with 10% bovine embryo serum and 100 µg/ml of streptomycin antibiotic and 100 units/ml of penicillin antibiotic [10].

DTP vaccine serial dilution preparation

Six concentrations of DTP vaccine (5×10^{-4}, 25×10^{-5}, 125×10^{-6}, 625×10^{-7}, 312×10^{-7} and 15×10^{-6} µg /ml) were prepared by half dilutions assay through addition of 500 µl of the stock solution (0.5 µg /ml of vaccine) into 500 µl of free serum-RPMI solution medium to make the first concentration 5×10^{-4} µg /ml and sequentially the other concentrations until reach the lowest concentration of vaccine, 15×10^{-6} µg /ml.

Cell Culture

Cells were grown in 96-well plates (10000 cell/well) and treated with the six subjects of the vaccine labeled as shown in the vaccine preparation. Negative control treated with serum-free medium was included to ensure that the test was valid for each experiment. Cells were incubated at 37^{O}C for 48 hours in a humidified environment with 5% CO_2.

Cytotoxicity test of the DTP vaccine

The toxic effect of the DTP vaccine on different cell lines was evaluated by assessing light absorption by treated cells that is reflected by optical density (OD) using crystal violation-dependent Elisa technique by adding 200 µg/ml of crystal violation solution for approximately 20 minutes at 37^{O}C with gentle stirring of the cells that were incubated for 48 hours after vaccine exposure.

Killing percentage determination

Spectrophotometric analysis was used to determine the ex-inclusion values at 485 nm, and the following formula was used to calculate the rate of growth inhibition or cytotoxicity/ killing percentage as below:

Killing percentage = A – B / A * 100 %

The A term refers to the optical density of control wells, while B refers to the optical density of treated wells.

Growth inhibition rates and IC_{50}*% determination*

The growth inhibition rates and the inhibitory concentration to eliminate 50% of infected cells or what is commonly known as IC_{50} were determined in the GraphPad prism 7.0 version 2016 program.

Morphological Study

An inverted microscope supported with a photographic program that provide accurate photo for each field within the wells of the plate was facilitate the assessment of Cytopathological effects include apoptotic induction, damaged cell membrane, cell shrinkage, loss of shape, and the formation of spherical bodies.

Statistical Analysis

For all used for triplicate measurements, the data are reported as mean ± standard deviation (SD). Multiple comparisons of the one-way ANOVA test were used to elucidate differences between groups based on statistical significance estimation (GraphPad Prism version 7.0 for Windows, GraphPad Software, San Diego, CA, USA) which also normalization and nonlinear regression to obtain the p value, R square and identity the IC_{50}% of vaccine on SKJ, HCAM and REF cell lines in vitro. Statistical significance was defined as $p < 0.05$

Results

Study of the anti-tumor activity of DTP vaccine

The antitumor activity of the DTP vaccine was studied by testing its ability to inhibit the proliferation of SKG, HCAM and REF cell lines that are treated with a serial dilution of the vaccine (5×10^{-4}, 25×10^{-5}, 125×10^{-6}, 625×10^{-7}, 312×10^{-7} and 15×10^{-6} µg /ml). Growth inhibition and IC_{50}% were calculated as treatment with respect to control cells that had not been exposed to the vaccine, growth inhibition was dose dependent. Table 1 explains that the highest percentage of death from the DTP vaccine was observed in the HCAM cell line (86.4%) followed by SKG (65.6%) then the REF cell line (59.8%). while IC_{50}% of the vaccine on the three cell lines HCAM, SKG and REF cell line was 0.0000566, 0.000289, 0.00022 µg/ml respectively.

Furthermore, inhibition of cancer cell growth by

Table 1. Cytotoxic Effect of Toxoid on Different Cell Lines

Cell Line		The highest death%	IC_{50}% (µg/ml)	S.D.	P-value	R^2
1	HC	86.4	0.0000566	0.23	<0.0001	0.92
2	SKG	65.6	0.000289	0.42	<0.0001	0.58
3	REF	59.8	0.00022	0.94	0.005	0.71

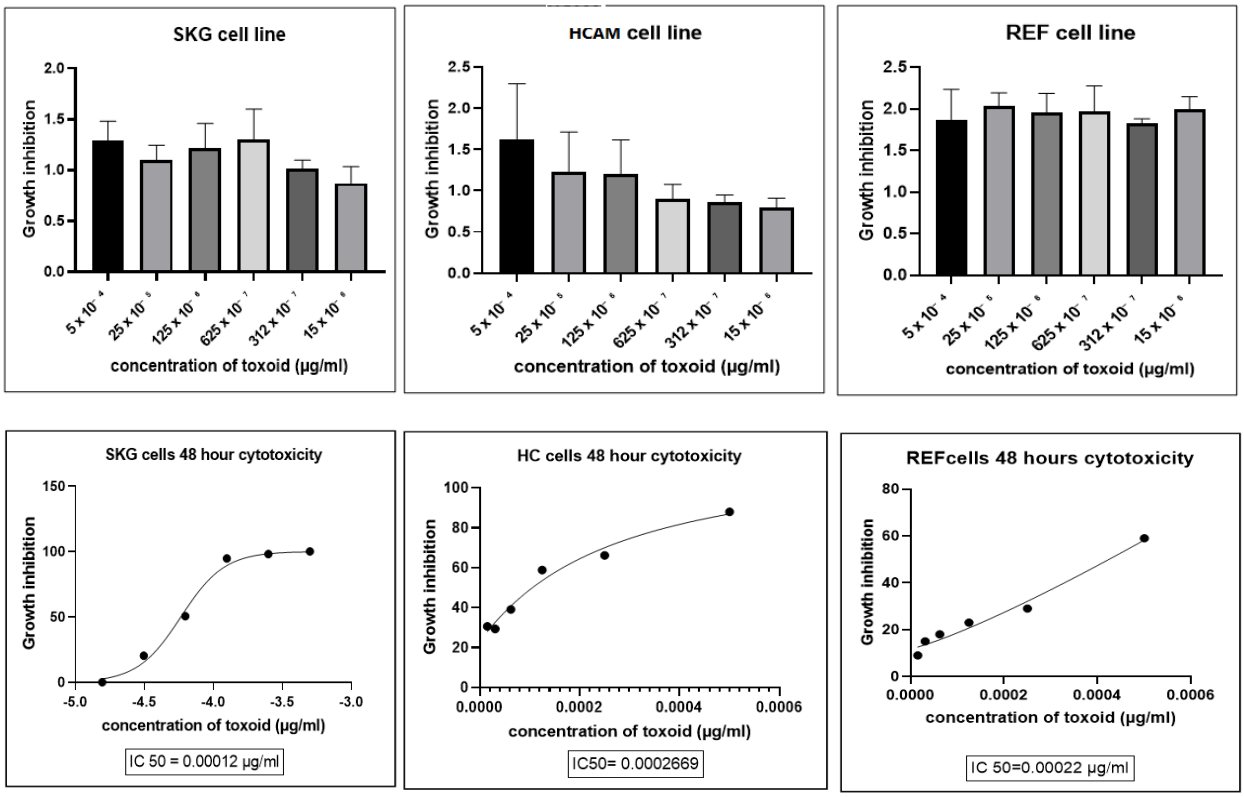

Figure 1. IC_{50}% of the DTP Vaccine in SKJ, HCAM, REF Cell Lines and Determined. Values are mean and SD for three replicates from three experiments.

the DTP vaccine appeared to be dose dependent, with $R^2 > 0.97$. Normal cell lines showed minimal growth inhibition, while cancer cell lines showed significant growth suppression ($p < 0.05$), Figure 1.

Morphological Study

Based on crystal violate staining, the morphology of treated and untreated cells was observed in photographic images prepared with inverted microscope (Figure 2). The cellular features of the untreated cells were similar to those of the origin cell line. Damaged cell membranes and shrinking cells were hallmarks of the morphological alterations in treated cells. Cell damage leads to the loss of shape and the formation of spherical bodies, both characteristics of apoptosis.

Discussion

Cancer is one of the main causes of morbidity and mortality in the world, while becoming a major concern for human health in the 21st century. In 2018, about 17.0 million new cases of cancer were reported, along with 9.5 million deaths worldwide due to cancer [11].

The most prevalent form of cancers (according to estimated new cases in 2018) are breast cancer, lung and bronchus cancer, prostate cancer, colon and rectum cancer, skin melanoma, bladder cancer, non-Hodgkin lymphoma, kidney and renal pelvis cancer, endometrial cancer, leukemia, pancreatic cancer, thyroid cancer, and liver cancer [11].

Furthermore, the rapid emergence of cancer cell resistance against chemotherapeutic agents made the situation alarming and generated the urgent need for new alternatives/therapeutic molecules for cancer treatment. However, enormous investments and sustained efforts have been made in the last few decades in cancer research, but notable success has not yet been achieved [12]. Recently, bacteria and substances produced by bacteria deserve serious consideration as cancer therapeutic agents [13].

However, the uses of bacteria and their products for cancer treatment have first been reported by William Coley who used culture supernatants of Streptococcus pyogenes and Serratia marcescens (Coley's toxins) to treat patients with unresected tumors. Interestingly, patients treated with Coley's toxins showed tumor regression and began to recover [14]. Tumor necrosis factor (TNF-a) secretion was increased behind the therapeutic effect of Coley's toxin. Microbial infections activate macrophages and lymphocytes, which induces the production of cytotoxic substances such as TNF-a and helped in tumor regression [15].

Recently, bacteria alone and bacterial produce substances characterized by potential anticancer activities. Interestingly, bacteria alone can act as a potential antitumor agent by targeting hypoxic areas of solid tumors. Furthermore, bacteria efficiently targeted cancerous tissues employing different strategies, such as the secretion of toxins, enzymes, proteases, and lipases, for further tests. Salmonella sp.p, Clostridium spp., Bifidobacterium spp., Lactobacillus spp., Escherichia spp., Pseudomonas spp., Caulobacter spp., Listeria spp.,

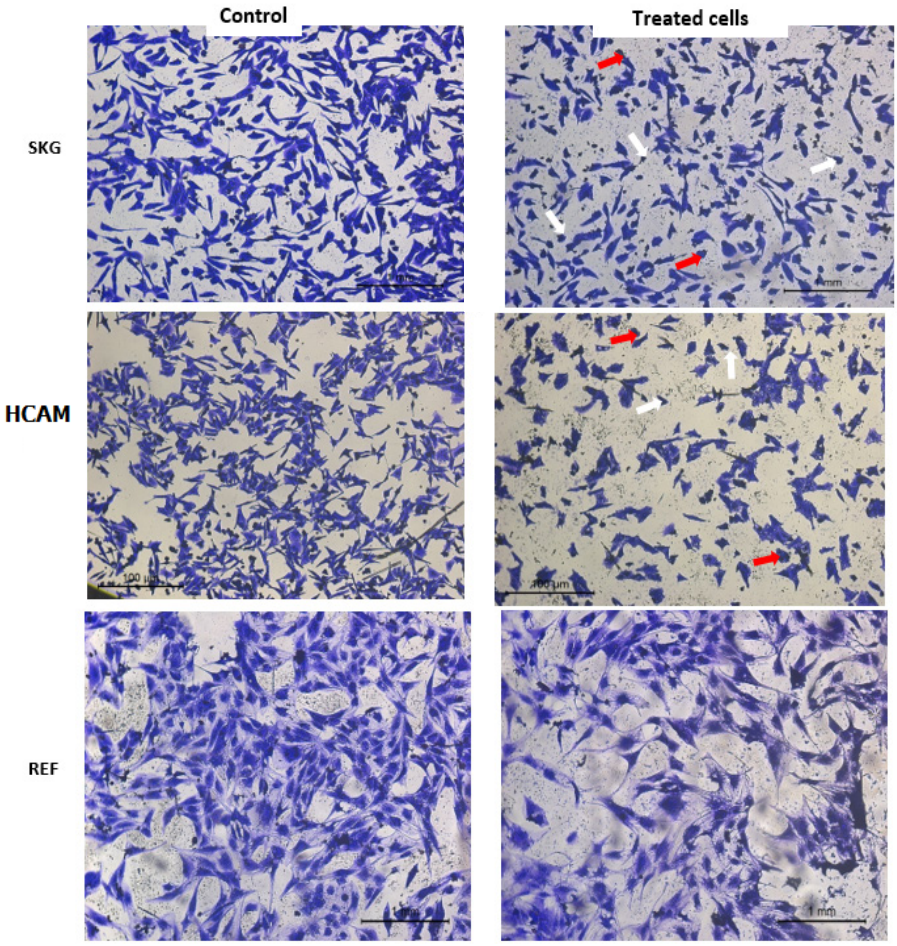

Figure 2. Cytological Examination for the Treated Cells versus Non Treated, the Cytopathic Effect of Microbial Toxoid on Cancer Cell Lines (SKT4 and HCAM), showing presence of necrotic cells characterized by cell rounding and nucleus condensation (white arrows), and apoptotic cells featured apoptotic bodies and blebs (red arrows). Cell stained with crystal violate.

Proteus spp. and Streptococcus spp. are the bacterial genera that have been already reported for anticancer potential [16]. The hallmark interest of the current study is the use of a combination of bacterial products that are commonly found in a form of a well-known vaccine. The DTP vaccine or the triple vaccine is the vaccine of choice in this exploration as a new anticancer agent, according to the in vitro results indicating effective killing against three different cell lines [6].

Three main components are found within the DTP vaccine, including the diphtheria toxoid of the Corynebacterium diphtheria strain CN2000, the tetanus toxoid of the Clostridium tetani Harvard strain 49205, and the Bordetella pertussis whole cell, strain 134 and strain 509 [17].

In the normal state, most bacterial toxins have pathogenic and toxic effects on human cells, as several of them have a unique ability to enter cells and mediate quite a lot of mechanisms leading to cell lysis and death. Although detoxification reduces most of the pathogenic powerful of the toxin, other than numerous toxoids [18].

Detoxification is the process by which bacterial toxins can be transformed by several and different procedures into toxoids which are not only nonpathogenic bacterial agents but also very vital tools for extremely important medical use including vaccination [17]. The diphtheria toxin is an exotoxin produced by Corynebacterium diphtheria and is a polypeptide chain consisting of two subunits A and B. Subunit B binds first to the surface of the host cell and facilitates the penetration of subunit A into the host cell [17].

The diphtheria toxin shows anticancer properties along with side effects; thus, it is used in cancer therapeutics in combination with other agents or in non-toxic mutant forms. CRM197 is a non-toxic diphtheria toxin mutant that is a specific inhibitor of hairpin binding epidermal growth factor (HB-EGF), belongs to the EGF family. EGF growth factor is involved in oral cancer cell progression, proliferation, and metastasis. CRM197 showed potent cytotoxicity to oral squamous carcinoma cells (HSC3 and SAS), both in vitro and in vivo [18]. In another study, CRM197 reported inhibiting human adrenocortical carcinoma (AC) cells implanted in nude mice. CRM197 inhibited AC cell invasion and migration along with reduced angiogenesis and apoptosis induction [19]. The less recombinant receptor diphtheria toxin, DT385, was shown to inhibit tumor growth (HEp3 and Lewis lung carcinoma), both in vitro and in vivo, by inhibiting protein synthesis and induction of apoptosis [20]. In a similar study, DTAT is another diphtherial derived in

a form of immunotoxin anticancer agent that showed potent cytotoxicity for glioblastoma cell lines, U118MG, U373MG, and U87MG [21].

The cytotoxicity effects of the DTP vaccine on three cell lines (SKJ, HCAM) as well as (REF) were investigated. The results indicated a dose-dependent increase in cell killing percentage at all cell lines. The morphological changes in the cancer cell lines after vaccine treatment indicated the cytopathic effects observed in infected cells 48 h later. Many confined cells showed granulation and shrinking, thus resulting in opaque foci due to increased cell granulation. Several studies have shown that the pertusis toxin inhibits the functions of antioxidant enzymes, including peroxidase, catalase, and glutathione [21]. Apoptosis is a well-organized process in which the genome of a cell is broken into smaller fragments and consumed by neighboring (phagocytic cells). If apoptosis does not occur, these damaged cells may survive and grow into cancer cells. When cells are not connected to other cells or the extracellular matrix, they 'self-destruct'. Pro-death proteins (BH3) accumulate in malignant cells, but are not sufficient to counteract the increase in antiapoptotic proteins (Bcl-2). Medications that mimic BH3 proteins can also boost pro-death signals, thus triggering the apoptosis pathway forward and triggering apoptosis [22].

In conclusions, as a result of the rapid development of resistance to chemotherapeutic drugs, the search for effective novel drugs remains a paramount concern in cancer treatment. Moreover, many chemotherapy drugs typically have high toxicity and adverse side effects, which necessitates the need to develop antitumor drugs that can be employed to treat deadly tumors with fewer negative effects on health and better efficacy. Isolation of several chemotherapeutic drugs from a wide range of natural sources has been performed, which include plants, microbes, fungi, and marine microorganisms. Considering the trends of previous decades, microbial diversity has grown to play a significant role in the formulation of pharmaceuticals and drugs, especially antibiotics and anticancer medications. Our observations suggest that DTP vaccine affects cancer cell proliferation through cell death, which can make DTP vaccine a promising treatment for different cancers with high clinical potential and safety. It is important to consider conducting more in-depth molecular studies to better understand the mechanism of action within the cell lines. Additionally, further research is recommended using different types of cancer cells or vaccines.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

authors would like to thank Experimental therapy department, Iraqi center for Cancer and Medical Genetic Research, Mustansiriyah university, Baghdad, Iraq for their support during the work.

References

1. Debela DT, Muzazu SG, Heraro KD, Ndalama MT, Mesele BW, Haile DC, et al. New approaches and procedures for cancer treatment: Current perspectives. SAGE Open Med. 2021;9:20503121211034366. https://doi.org/10.1177/20503121211034366.
2. Surgery GCaNIfHRGHRUoG. Global variation in postoperative mortality and complications after cancer surgery: A multicentre, prospective cohort study in 82 countries. Lancet. 2021;397(10272):387-97. https://doi.org/10.1016/s0140-6736(21)00001-5.
3. Pucci C, Martinelli C, Ciofani G. Innovative approaches for cancer treatment: Current perspectives and new challenges. Ecancermedicalscience. 2019;13:961. https://doi.org/10.3332/ecancer.2019.961.
4. Al-Madboly LA, El-Deeb NM, Kabbash A, Nael MA, Kenawy AM, Ragab AE. Purification, characterization, identification, and anticancer activity of a circular bacteriocin from enterococcus thailandicus. Front Bioeng Biotechnol. 2020;8:450. https://doi.org/10.3389/fbioe.2020.00450.
5. Chauhan S, Dhawan DK, Saini A, Preet S. Antimicrobial peptides against colorectal cancer-a focused review. Pharmacol Res. 2021;167:105529. https://doi.org/10.1016/j.phrs.2021.105529.
6. Molujin AM, Abbasiliasi S, Nurdin A, Lee PC, Gansau JA, Jawan R. Bacteriocins as potential therapeutic approaches in the treatment of various cancers: A review of in vitro studies. Cancers (Basel). 2022;14(19). https://doi.org/10.3390/cancers14194758.
7. Frost I, Sati H, Garcia-Vello P, Hasso-Agopsowicz M, Lienhardt C, Gigante V, et al. The role of bacterial vaccines in the fight against antimicrobial resistance: An analysis of the preclinical and clinical development pipeline. Lancet Microbe. 2023;4(2):e113-e25. https://doi.org/10.1016/s2666-5247(22)00303-2.
8. Prygiel M, Mosiej E, Górska P, Zasada AA. Diphtheria-tetanus-pertussis vaccine: Past, current & future. Future Microbiol. 2022;17:185-97. https://doi.org/10.2217/fmb-2021-0167.
9. Spiller V, Barnes JM, Holt LB, Cullington DE. Immunization against diphtheria and whooping-cough; combined v. Separate inoculations. Br Med J. 1955;2(4940):639-43. https://doi.org/10.1136/bmj.2.4940.639.
10. Ammar E, Al-Mudallal N, Abbas SM. Cytotoxic effect of stenotrophomonas maltophilia isolated from prostate and bladder cancer patients in iraq. Asian Pac J Cancer Prev. 2024;25(10):3701-5. https://doi.org/10.31557/apjcp.2024.25.10.3701.
11. Baerheim A, Sandvik H. Effect of ale, garlic, and soured cream on the appetite of leeches. Bmj. 1994 dec 24;309(6970):1689. Https://doi.Org/10.1136/bmj.309.6970.1689.
12. Park NH, Cheng W, Lai F, Yang C, Florez de Sessions P, Periaswamy B, et al. Addressing drug resistance in cancer with macromolecular chemotherapeutic agents. J Am Chem Soc. 2018;140(12):4244-52. https://doi.org/10.1021/jacs.7b11468.
13. Baindara P, Korpole S, Grover V. Bacteriocins: Perspective for the development of novel anticancer drugs. Appl Microbiol Biotechnol. 2018;102(24):10393-408. https://doi.org/10.1007/s00253-018-9420-8.
14. Wiemann B, Starnes CO. Coley's toxins, tumor necrosis factor and cancer research: A historical perspective. Pharmacol Ther. 1994;64(3):529-64. https://doi.org/10.1016/0163-7258(94)90023-x.
15. Baindara P. Host-directed therapies to combat tuberculosis and associated non-communicable diseases. Microb

Pathog. 2019;130:156-68. https://doi.org/10.1016/j.micpath.2019.03.003.
16. Song S, Vuai MS, Zhong M. The role of bacteria in cancer therapy - enemies in the past, but allies at present. Infect Agent Cancer. 2018;13:9. https://doi.org/10.1186/s13027-018-0180-y.
17. Collier RJ. Diphtheria toxin: Mode of action and structure. Bacteriol Rev. 1975;39(1):54-85. https://doi.org/10.1128/br.39.1.54-85.1975.
18. Dateoka S, Ohnishi Y, Kakudo K. Effects of crm197, a specific inhibitor of hb-egf, in oral cancer. Med Mol Morphol. 2012;45(2):91-7. https://doi.org/10.1007/s00795-011-0543-6.
19. Martarelli D, Pompei P, Mazzoni G. Inhibition of adrenocortical carcinoma by diphtheria toxin mutant crm197. Chemotherapy. 2009;55(6):425-32. https://doi.org/10.1159/000264689.
20. Zhang Y, Schulte W, Pink D, Phipps K, Zijlstra A, Lewis JD, et al. Sensitivity of cancer cells to truncated diphtheria toxin. PLoS One. 2010;5(5):e10498. https://doi.org/10.1371/journal.pone.0010498.
21. Rustamzadeh E, Li C, Doumbia S, Hall WA, Vallera DA. Targeting the over-expressed urokinase-type plasminogen activator receptor on glioblastoma multiforme. J Neurooncol. 2003;65(1):63-75. https://doi.org/10.1023/a:1026238331739.
22. Kapoor R, Saini A, Sharma D. Indispensable role of microbes in anticancer drugs and discovery trends. Appl Microbiol Biotechnol. 2022;106(13-16):4885-906. https://doi.org/10.1007/s00253-022-12046-2.