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In vitro Anticancer Activity Effect of Extracellular Metabolites of Some Bacterial Species on HeLa Cell Line.

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Abstract:

Background: Cancer is still one of the most serious problems that affect human health. Despite the intense efforts to develop treatments, effective agents are still not available. In some cases, conventional therapy could be harmful or fail because of emerging drug resistance. Therefore, the development of novel therapies against cancer is of utmost importance. Assessment of anticancer effects of bacterial metabolites on cancer cells may help in the process of finding new cheap, reliable, contentious and safe cancer therapy.

Objective: To determine the anticancer effect of the extracellular metabolites of eight bacterial species on HeLa cell line.

Methodology: Extracellular metabolites were prepared by isolating and culturing eight bacterial species (Escherichia coli, Staphylococcus aureus, Micrococcus, Pseudomonas aeruginosa, Lactic acid bacteria, Klebsiella, Proteus and E. coli with its phage) in liquid media. Tubes were incubated overnight and centrifuged. Supernatant was filtered and concentrated using Infra-Red concentrator. Different concentrations were prepared and their anticancer effect were evaluated using MTT cell proliferation assay.

Results: Results showed variation among the eight bacteria concerning proliferation inhibition against HeLa cells in a time and concentration dependent manner. Pseudomonas and E. coli with its phage revealed considerable anticancer activity with 63% and 86% inhibitory effects (both at 1000 µg\ml) and IC50 of 301 and 1395 µg/dl at 24h respectively. While Proteus and Micrococcus showed low inhibitory effects and S. aureus enhanced the proliferation of HeLa cells at low concentrations.

Conclusion: Among the tested bacteria, Pseudomonas and E. coli and its phage gave the best anticancer inhibitory effects against HeLa cells. Further studies using purified components of effective bacteria are recommended.

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Bacterial metabolites, Cancer, HeLa cells and Phage.

1. Introduction:

Cancer is still one of the most serious problems that affect human health. Globally is considered as the second leading cause of death with nearly 8.8 million deaths in 2015. Nearly 1 in 6 deaths is due to cancer (WHO, 2017). Cancer is an uncontrolled growth of cells that in some cases can metastasize through lymph or blood (Ravuri and Kumari, 2013). Estimated annual incidence for 2018 had to be 40,000 cases or more (National cancer institute, 2018).

Cervical cancer was found to be the fourth most commonly diagnosed cancer in women in 2012, with an estimated 527,600 new cases worldwide (American cancer society, 2017). In Gaza, 4.6% of female cancers was cervical cancer (Palestinian Ministry of Health, 2015).

The cancer-curative methods are surgery, immunotherapy, radiotherapy and the popular one is chemotherapy (Gillet, et al., 2007). These techniques are useful in certain cases but could be harmful, failure sometimes due to chemotherapy-induced resistance.

Despite the intense efforts to develop treatments, effective agents are still not available. There upon, natural products extracts seem to be the most promising source of new drugs for cancer (El-Gendy et al., 2008).

Some microorganisms such bacteria can be a source of bioactive natural products with the advantage of sustainable production of secondary metabolites (ER et al., 2015). Bacteria have unique capabilities that make them well-suited as 'perfect' anticancer agents. In recent studies they showed that they could control tumor growth (Forbes, 2010). For example, Lactic acid bacteria (LAB) have been reported to possess certain anticancer properties (Kim et al., 2002). The therapeutic effects of LAB include improvement of lactose intolerance, prevention of intestinal infection, decrease in serum cholesterol, increase in immune response, anti-carcinogenic activity and antioxidative effects (Lin and Chang, 2000).

One reason for choosing bacterial extracts is that the side effects of chemotherapy and drug resistance of some cancer types belong to the significant current therapeutic problems. Hence, searching for new anticancer substances and medicines are very important. Among them, bacterial proteins and peptides are a promising group of bioactive compounds and potential anticancer drugs (Karpiński & Adamczak, 2018).

And since the world is facing an increasing cancer rates, an important global attention for health care organizations is needed; as it performs a great load for government healthcare systems. Therefore, the development of therapies against cancer is a significance strategy for healthcare research. One common feature of the disruption of metabolic pathways observed in cancer cell growth is metabolic product pathways; as a novel approach to manage cancer. Therefore, assessment of anticancer effects of bacterial metabolic extract on cancer cells will help in the process of finding new cheap, reliable, contentious and safe cancer therapy.

2. Materials and Methods

2.1 Extraction of metabolic products of bacteria

Lactic acid bacteria, *Pseudomonas aeruginosa*., *Proteus, Klebsiella, Staphylococcus aureus, Micrococcus* and *E. coli* isolates were sub-cultured in Nutrient broth media (HiMedia, India) at 37 °C for 24–48 hrs. For the *in vitro* preparation of the cell-free filtrate, cultured bacterial cells were centrifuged at 4600 rpm for 20-30 min. The supernatants were filtered using a 0.22 µm syringe filter. Adjusting the pH of the supernatants to 7 with NaOH and storing them at 4 °C (ER et al., 2015).

2.2 HeLa cells culture

HeLa cell line was used and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator.

2.3 MTT Assay

HeLa cells were plated and allowed to reach approximately 70% - 80% confluence where a 100 µl of cells were added into each well and incubated overnight. On the next day: Cells were treated with different concentrations of the extracts starting from 12.5 to 1000 µg/ml. Final volume should be 100 µl per well.

On the third day, 100 µl of 5 mg/ml MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) was added to each well. Considering one set of wells as control (MTT with cells but no extract). After 3 hours of incubation at 37 °C, 100 µL of dimethylsulfoxide (DMSO) replaced the MTT solution to dissolve the blue crystals then the absorbance was read at 550 nm using Multiskan FC (Republic of Korea) ELISA reader (van Meerloo et al., 2011).

3. RESULTS AND DISCUSSION

The effect of extracellular extracts on the viability of cancer cells was determined by MTT assay on HeLa cells, a routine technique, which was used to determine the cytotoxic effect of Lactobacillus *plantarum* 5BL on different cancer cells, including HeLa, Michigan Cancer Foundation-7 (MCF-7), adenocarcinoma gastric cell line (AGS) and human colonic adenocarcinoma cell line (HT-29) (Er, et al., 2015). This technique is based on the ability of cells to metabolize the yellow tetrazolium salt MTT to a blue crystalline formazan product (Er, et al., 2015). The manner in which a cell dies determines the nature of the response by the surrounding tissue. Death by necrosis acts by inducing oxidative stress and production of numerous pro-inflammatory cytokines (Er, et al., 2015).

In this study, eight bacterial strains were isolated from various sources, including clinical samples and from isolates from the Microbiology laboratory at Islamic University of Gaza. The anticancer activity of the microbial extracellular metabolites was determined against one established cancer cell lines; HeLa cells (table 1).

Table 1 ***The inhibitory effect of bacterial extract at 24 on Hela cells***

	Inhibitory effect % (24 h)							
Conc µg/ml	***E. coli* & Phage**	***Proteus***	***S. aureus***	***P. aeruginosa***	***Micrococcus spp.***	***Klebsiella***	***E. coli***	**LAB**
12.5	20.0	-26.5	3.8	33.1	3.6	NT	NT	NT
25	17.9	11.2	-5.3	17.6	14.0	-4.6	19.1	-19.9
50	21.9	15.8	3.9	29.5	24.1	6.0	32.1	-11.5
100	20.1	10.8	-22.3	16.8	21.1	24.9	37.7	-7.3
200	31.0	18.5	-18.6	28.7	18.5	9.2	36.6	-3.6
400	24.8	18.5	3.8	24.6	28.4	-5.0	39.2	17.1
800	79.0	25.7	58.2	58.1	34.8	-16.2	37.9	-11.6
1000	85.7	27.1	NT	62.7	30.4	-16.8	41.0	12.5

NT=Not Tested. (-) Indicates enhancing effect, LAB= Lactic Acid Bacteria

Upon using the HeLa cell line, the crude extracellular metabolites of the tested bacteria (*E. coli* with Phage, *P. aeruginosa, E. coli, Micrococcus* spp., and *Proteus*) showed 85.7% 62.7%, 41%, 30.4%, and 27.1% inhibition, respectively, after 24h by using the MTT assay.

The inhibitory effect % were determined by exposing cells to various concentrations of the crude extracellular metabolites (12.5, 25, 50, 100, 200, 400, 800, and 1000 µg/ml) for 24 h. Results indicated that the crude extract of *E. coli* with its phage had promising anticancer activity against HeLa cell lines, and exhibited a reasonable degree of anticancer activity, causing 85.7% inhibition after 24 h.

The inhibitory effect of different concentrations of *E. coli* with its phage crude extract at different time points on HeLa cells was studied. It increased the inhibitory effect in dose dependent manner. The inhibitory effect increased from 20% to 85.7% as the concentration extract increased from 12.5 to 1000µg/ml for 24 h (Table 1).

Colicins A, E1 and E3 are produced by *E. coli* and have molecular sizes: more than 20, 57 and 9.8 kDa, respectively (Kaur, et al., 2015). Colicin E1 and A inhibited 10 cell lines: breast carcinoma (MCF7, ZR75, BT549, BT474, MDA-MB-231, SKBR3 and T47D), osteosarcoma (HOS), leiomyosarcoma (SKUT-1) and fibrosarcoma (HS913T). Only the colon carcinoma line (HT29) was insensitive to colicin E1. Colicin E1 showed 50% inhibition of fibrosarcoma (HS913T) and 17–40% inhibition of other cancer cell lines. Colicin A showed 16 to 56% inhibition of cancer cell lines and 36% inhibition of normal diploid fibroblasts with wild-type p53 (MRC5). Colicin E3 demonstrated no significant inhibition activity against tested cancer cells (Chumchalová and Šmarda, 2003).

Our results showed that *Proteus* spp. crude extracellular metabolites enhanced the proliferation of HeLa cells at 12.5 µg/ml concentrations. In contrast, the inhibitory effect slightly increased from 11.2% to 27.1% as the concentration of the crude extract was increased from 25 to 1000 µg/ml. As shown in Table 1, the inhibitory effect of *P. aeruginosa* on HeLa cells increased from 58.1% to 62.7% as the concentration increased from 800 to 1000 µg/mL

P. aeruginosa PAO1, a potential pathogen of plants and animals, produces the cyclodipeptides (CDPs) cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Phe), and cyclo (L-Pro-L-Val) (PAO1-CDPs), whose effects have been implicated in inhibition of human tumor cell line proliferation (Hernández-Padilla, et al., 2017). The crude *P.*

aeruginosa PAO1-CDPs mixture promoted cell death in HeLa cells in a dose-dependent manner, showing efficacy similar to that of isolated *P. aeruginosa* PAO1-CDPs (LD50 of 60 to 250 mM) and inducing apoptosis with effective concentration 50 (EC50) between 0.6 and 3.0 mM (Hernández-Padilla, et al., 2017).

P. aeruginosa is a pathogenic and opportunistic bacterium that produces a large number of virulence factors. CDPs can be considered the molecules that can regulate the production of virulence factors in a quorum sensing-dependent manner in this microorganism (Campbell, et al., 2009, Galloway, et al., 2010, Rojas et al., 2015 & Dandekar et al., 2013).

In the context of antiproliferative properties attributed to CDPs, a mixture of CDPs composed of cyclo(L-Pro-L-Tyr), cyclo(L-Pro-L-Val), and cyclo(L-Pro-L-Phe) isolated from the *P. aeruginosa* PAO1 strain can inhibit the proliferation of human tumor cell lines: HeLa and CaCo-2. In addition, it has been reported that a crude PAO1-CDP mixture showed Inhibitory concentration (IC50) of 0.53 mg/ml (Vázquez-Rivera, et al., 2015).

The probable reason for the observed effects is that molecules isolated from living entities such as *P. aeruginosa* are produced with chiral specificity، ensuring stereochemical specificity and therefore strong activity (Brauns, et al., 2004).

The results of this study demonstrated cancer enhancing activity of *Klebsiella* spp. This enhancing activity fluctuated with varying concentration (25 to 1000 µg/ml).

Microcin E492 (M-E492) is a bacteriocin produced by *K. pneumoniae* RYC492 and it has a molecular mass of 7.9 kDa. The cytotoxicity of M-E492 was detected in the case of various malignant human cell lines, including cervical adenocarcinoma (HeLa), acute T cell leukaemia (Jurkat), B cell line originated from Burkitt's lymphoma (Ramos) and B-lymphoblastoid cell lines transformed by infection with Epstein-Barr virus (RJ2.25, a variant of the Raji B-LCL) (Joo, Ritchie, Kamarajan, Miao, & Kapila, 2012).

In our study, the *Lactobacillus* spp. Extracellular metabolite, enhanced the proliferation of HeLa cells differently at different concentrations (25 to 1000 µg/mL) for 24 h. The maximum enhancing effect was 19.9% at 25 µg/ml.

Contrasting to our results, most probiotic microorganisms are lactic acid bacteria (LAB) such as *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp. LAB have been reported to possess certain anticancer properties (Er, et al., 2015).

Plantaricin A is a bacteriocin of *L. plantarum* C11 and its molecular weight reaches 2.4 kDa. In the case of artificially synthesized plantaricin A, the cytotoxicity against the human T cell leukaemia was determined *in vitro* (Zhao, et al., 2006).

A study has shown that *P. pentosaceus*, *L. plantarum*, and *W. confusa* have the potential to inhibit the proliferation of Caco-2 cells. *L. plantarum* showed the strongest inhibitory effect. Although there are anticancer studies with *L. plantarum* (Kim, et al., 2002), there are not adequate data regarding the anticancer effects of *P. pentosaceus* and *W. confusa*. By using the

MTT assay, Patel et al., (2010) reported that dextran isolated from *P. pentosaceus* has no cytotoxic effect on HeLa cells (Patel, et al., 2010).

The inhibitory effect of different concentrations of *E. coli* extracellular metabolites at different time points on HeLa cells was studied. As shown in Table 1. The extracellular metabolites decreased the proliferation of cells at concentrations 25–1000 µg/ml at 24 h. As the concentration was increased from 25 to 1000 µg/ml the inhibitory effect increased from 19.1% – 41%.

A related study, reported that the crude ethyl acetate extract of *Aspergillus* sp isolate showed promising results by MTT assay with IC50 as low as 20.37±0.36 µg/mL on HeLa, and 44.75±0.81 µg/mL on MCF-7 cells (Thomas, et al., 2011).

In another study by Adnyana et al., in 2007, they found that the ethyl acetate extract of *S. galbus* TP2 strain shows a potential anticancer (T47D cell line) activities (Andayani, et al., 2015).

In a study, Vijayabharathi et al., reported that *Sterptomyces* strain, isolated from humus soils in the Western Ghats, has an anticancer activity against HepG2 (hepatic carcinoma) and HeLa (cervical carcinoma) *in vitro* (Vijayabharathi, et al., 2011).

A study performed by WAHYUDI et al., in (2006) showed that the best toxicity and anticancer activity were performed by *B. subtilis* crude extract with LC50 and IC50 were 378 µg/ml and 132.877 µg/ml, respectively (Safari, et al., 2015).

Microbes are the large repositories of bioactive compounds with different structural and functional forms, namely; sugars, acids, esters, ethers, terpenoids, peptides, proteins, and nucleopeptides, which are meant for the immense applications in the treatment of the wide range of disease types, such as anticancer, anti-inflammatory, antimicrobial, antiviral, and antifungal (Kumavath, 2015).

This study suggests that *E. coli* with its phage and *P. aeruginosa* may considered as potential candidates for anticancer use (as illustrated in Figure 1). More work is needed in order to identify and purify the active metabolites from those crude extracts and then to be tested on HeLa cell lines and other available tumor cell lines.

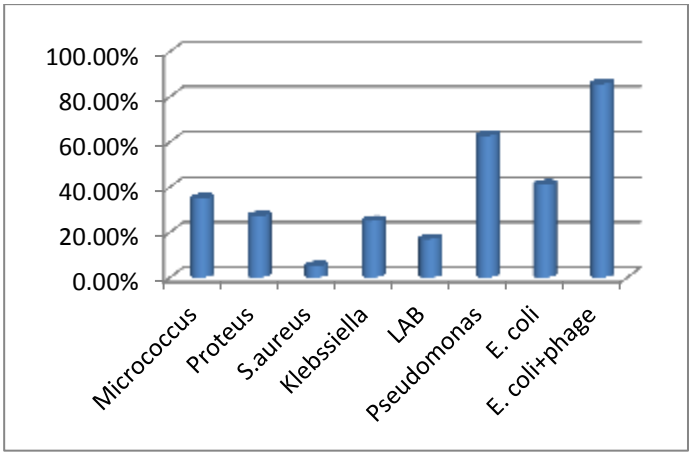

Figure 1 ***Anti-proliferative effect of all tested microorganisms at 1000µg/ml***

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