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Inhibition of Cancer Cells Line by Biosurfactant Produced from *Leuconostocmesenteroides*ssp Cremoris

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ABSTRACT

Objective: This brief research was initiated to search for cytotoxic effect of bio surfactant produced by *Leuconostocmesenteroides*ssp. *cremoris*isolated from raw cow's milk.

Methods: Extraction of extracellular bio sufactant was completed and partially purified by cold acetone precipitation, bio surfactant were tested against two cell lines, AMN3 (cancer cell line of mice mammary gland) and REF (noon cancerous normal cell line transformed) specifically regarding cell viability and proliferation.

Results: Bio surfactant was found to decrease viability of AMN3 cancer cell line studied at concentrations 1:3,2:2 and 1:3 V/V (biosurfactant: free serum media), the inhibition percentage were 56.4%, 56.8% and 51.8% respectively, without affecting normal fibroblasts growth.

Conclusion: The results gathered in this work are very promising regarding the bio surfactant potential for cancer treatment and encourage further work with other cell lines.

INTRODUCTION

The probiotic bacteria has the ability to increase the efficiency of the useful enzymes like galactosidase and reduce the efficiency of the carcinogenic enzymes like nitroreductase enzyme,[1] also it destroys the toxins produced by the pathogenic bacteria and inhibit the inner toxins' action.[2]

Leuconostoc species are catalase-negative, Gram-positive microorganisms with coccoid morphology,[3] heterofermentative lactic acid bacteria that occur naturally in milk, herbage, grapes, grass and many vegetables.[4] Members of this group are used in dairy fermentations to produce aroma compounds.

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Bio surfactants are amphiphilic compounds produced by microbes, either on the cell surface or secreted extracellularly, they have anti-adhesive and antimicrobial properties[5] also they have antifungal and antiviral activity. Bio surfactants have potential application in pharmaceutical, food and cosmetic industries.[6]

Bio surfactants have recently emerged as promising molecules for their structural versatility, novelty and diverse properties that are potentially useful for many therapeutic applications. Mainly due to their surface activity, these molecules interact with cell membranes of several organisms and/or with the surrounding environments, and thus can be viewed as potential cancer therapeutics or as constituents of drug delivery systems.[7]

Cancer is a multiple complex disease that poses many complications in treatment owing to issues of drug efficacy and harmful side effects for normal cells as described by.[8] The urgent need to discovery novel

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cytotoxic compounds is very important for the development of anticancer treatments.[9]

For the discovery of new safe natural cytotoxic compound, this research describes the cytotoxic activity of bio surfactant produced by locally isolate *Leuconostocmesenteroides*ssp. *Cremoris* against AMN3 cells.

MATERIALS AND METHODS

Bacterial Isolate

Isolate of *Leuconostocmesenteroides ssp. Cremoris* was isolated from raw cow's milk, then identified throughout microscopical, cultural and biochemical test according to[10] and Vitek 2 system.

Extraction of Extracellular Bio Surfactant from *L. mesenteroides ssp. cremoris*

For crude bio surfactant production by *L.mesenteroides ssp. cremoris*, 600 ml of culture broth were inoculated with 12ml of an overnight subculture and incubated for 24 h at 30°C in aerobic condition. Briefly, culture broth was centrifuged at 10000rpm for 10 min at 4°C; the supernatant was filtered through a Millipore filter.[11]

Partial Purification of Bio Surfactant

Bio surfactant was partially purified by cold acetone precipitation. Three volumes of chilled acetone was added to the crude bio surfactant solution and allowed to stand for (15-20) h at 4°C. The precipitate was collected by centrifugation at 10,000rpm for 30 min and the resulting pellet served as partially purified bio surfactant it was further allowed to be evaporating to dryness to remove residual acetone after which it was dissolved in sterile water.[12]

Cell Lines used in this Study

Two types of cell lines have been used. Mammary adeno carcinoma cell line (AMN3) and fibroblastic and epithelial cells with normal chromosomal pictures (REF) as normal murine cell lines were used. Both of them are locally established in ICCMGR[13] and they are maintained for use.

The cells are cultured in $RPMI_{1640}$ media contains 10% fetal bovine serum, glutamine (2 mmol/L), streptomycin (100 U/ml) and penicillin (100 U/ml), then incubated in 5% CO_2 at 37ºC for 24 hour. In this time the cells will grow and become monolayer. Single cell then will use to determine the cytotoxicity of bio surfactant. The confluent monolayer cells treated with 1 ml of trypsin/versine to provide suspension of cells, then add 10 ml of prepared media. About 200 µl of the cells were culture on clean sterile 96- well microtiter plate then let the cells for 24 hr to make single monolayer to be ready to be treated with the bio surfactant.

Next day, random dilutions from immediate filtered stock bio surfactant was prepared as following, 3 ml from the bio surfactant + 1 ml from the free serum media to be the first concentration; then 2 ml from the bio surfactant + 2 ml from the free serum media to be the second concentration; finally 1 ml from the bio surfactant + 3 ml from the free serum media to be the last concentration.

Exposure day, decant the media from the cells and add 200 µl from bio surfactant concentrations. Each concentration was triplicated and returns the microtiter plates to the incubator. Leave wells contains only cells without treatment contains serum free media representing control cells. Three different exposure times of the cells were included in this research, 24, 48 and 72 hour. The protocol of handing and treating the cells was prepared as described by Butler, 2004.[14]

Cell Viability Assay

The cytotoxicity was determined after each exposure time using crystal violate. Decant the bio surfactant from the microtiter plate, add 200 µl of the crystal violate to the wells of the treated cells for 20 min. in the incubator 37ºC. The crystal violate will stain the nuclei of the viable cell and the color will be visible to the eye. Then the plates were read by ELISA reader at 495nm. And then the inhibition rate was calculated using the following equation:

%Growth Inhibition = (B– A)/A ×100 %

Were, A represent absorbance of control and B absorbance of sample

Statistical Analysis

In this study we used student t- test to determine the differences between the concentrations in each cell line and also to determine the differences between two cells in each exposure time. The probability p was determined to be $p>0.05$. Graph Pad Prism V6 was used to determine this statistical test. Excel 2010 sheet was used to draw the curves.

RESULTS AND DISCUSSION

Leuconostocmesenteroides ssp. cremoris showed ability to produce extracellular bio surfactant with production yield achieved 10.8 g/L of medium (MRS broth). Lactic acid bacteria have ability to produce biosurfactant as metabolic productsas described by the research of Rodrigues and his colleagues,[15] 2006. Acetone was tested to be a good purifying agent for proteins as also described by Nadrrmullah and Mukhtar, 2013,[16] and tested as well for biosurfactant purifying.[17]

The cytotoxicity of bio surfactant was determined toward mammary adenocarcinoma cell line (AMN3) and normal murine cell line (REF). Three concentrations of the bio

surfactant were tested against AMN3 and REF cell lines. Positive controls (without bio surfactant) were included and correspond to 100% cell viability. Negative controls, containing bio surfactant at each studied concentration to eliminate any possible interfere with the method.

Bio surfactant effect was shown to have a cytotoxic effect against AMN3 cancer cell line especially after 48 hour of exposure at two higher concentrations (3+1) and (2+2). These two concentration show inhibition rate toward cancer cells about 56.4% and 56.8%, even the lower dilution (1+3) show 51.8% inhibition of AMN3 cells during 48 hr exposure (Figure 1 and Table 1). While bio surfactant was save toward REF cells and the inhibition rate shown to be lower as presented in Figure 2, the lower concentration give 23.8% inhibition during 24 hr of exposure.

The results obtained clearly show an increase in the percentage of growth cell inhibition with bio surfactant concentrations and exposure times, suggesting its cytotoxic effect against the studied AMN3 cell line as presented in (Table 1 and Figure 1). However, for the highest bio surfactant concentration studied, a significant decrease of the total number of cells was observed, probably due to a prevalence of a detergent-like effect leading to cell membrane disruption as for this case the using of bio surfactant.[18]

While the treatment of REF cell line with bio surfactant as in (Figure 2 and Table 1), the conditions that promoted a more pronounced increase of cell viability without membrane disruption detectable by morphological visualization, were exposure durations to bio surfactant did not significantly make any changes to cell viability suggesting the safety of this bio surfactant toward REF cells.

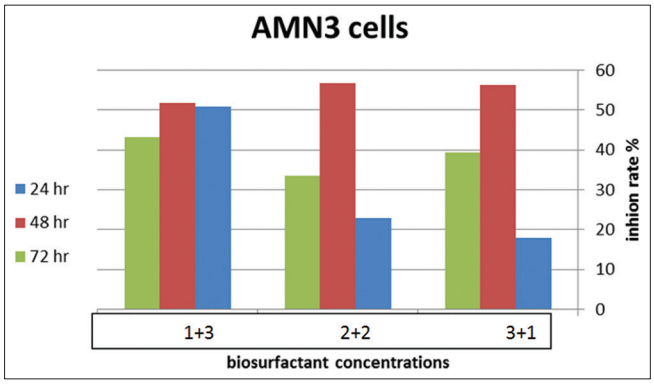

Figure 1: The inhibition rate % of different concentrations of Bio surfactant on AMN3 cell line at three different exposure times

In the last Figure 3, the comparisons between both cell lines and the cyto toxicity of three concentrations of bio surfactant in three exposure durations. There were significant differences between AMN3 and REF cell lines at 48 and 72 h in treatments concentrations. The figure presenting that all used bio surfactant concentrations at 48 h exposure time is the ideal duration to inhibit AMN3 cell viability 51.8-56.4 % inhibition rate. While at 72 h, the inhibition rate decreased to be 33.6-43.3% toward AMN3 cell growth. While REF cell shown to be not affected by the concentrations of bio surfactant at all duration times. Present study clarifies the safety of bio surfactant toward normal cells and toxic to ward cancer cell line.

Several studies were carried on different cancer cell lines and this study may be represent the first study which highlights the importance to carry out more and more researches to determine the inhibition of bio surfactant on locally established cancer cell lines.

In early study carried by Kim et al. 2007,[18] they found that both p21WAF1/Cip1 and p53 were strikingly induced by surfactin treatment, suggesting that surfactin-induced cell cycle arrest may be mediated by these proteins. Surfactin strongly inhibited cell proliferation and induced apoptosis of colon cancer LoVo cells.

Different lipopeptides produced by *Bacillus*, *Pseudomonas*, and *Serratia* species have exhibited antitumor activity against various human cancer cells.[19,20] Bio surfactants have been shown to have effects on cancer cells. For instance, the lipopeptidesurfactin was found to induce apoptosis in breast cancer cells.[21] Similarly, the glycolipids mannosylerythritol lipids (MELs) and succinoyltrehalose lipids (STLs) have been involved in growth arrest and apoptosis of tumor cells.[22,23]

In study of Cao et al. 2010[21] demonstrated that surfactin induces apoptosis in human breast cancer MCF7 cells

Table 1: Percentage of inhibition rate of different concentrations of bio surfactant towards AMN3 and REF cells at different exposure time

Concentrations exposure time (hr)	Inhibition rate %						P value
	AMN 3	REF	AMN 3	REF	AMN 3	REF	Differences between AMN3 & REF cells
	1+3 (1 bios.+3 FSM)	1+3 (1 bios.+3 FSM)	2+2 (2 bios.+2 FSM)	2+2 (2 bios.+2 FSM)	3+1 (3 bios.+1 FSM)	3+1 (3 bios.+1 FSM)	
24	17.9	9.7	22.9	20.8	51	23.8	0.3253
48	56.4	0	56.8	0	51.8	19.3	0.0018**
72	39.4	0	33.6	0	43.3	0	0.0002***

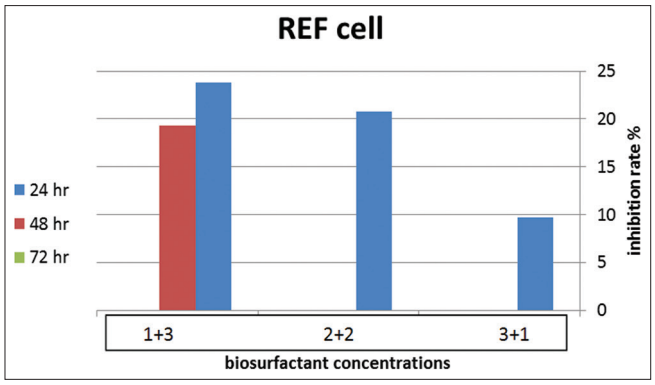

Figure 2: The inhibition rate % of different concentrations of bio surfactant on REF cell line at three different exposure times

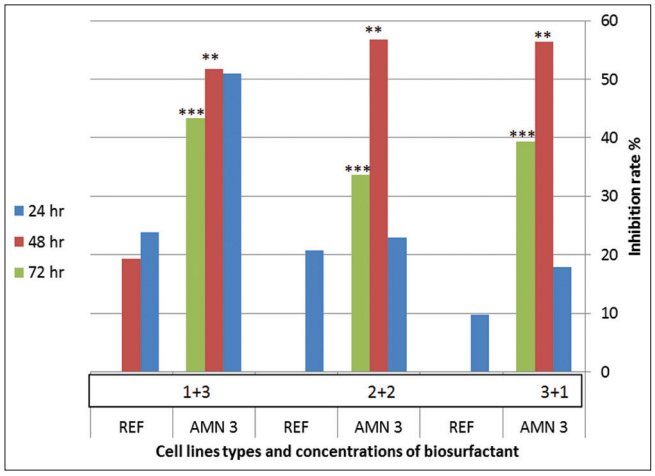

Figure 3: Inhibition rates % of different concentrations of biosurfactant when exposured to two types of cells AMN3 and REF cells at different exposure times. ** Mean signi f cant changes between AMN3 and REF cells at 48 hr of exposure. *** Mean signi f cant changes between AMN3 and REF cells at 72 hr of exposure

through a ROS/JNK-mediated mitochondrial/caspase pathway also demonstrated the cytotoxic effect of surfactinin a dose-dependent manner, against the human chronic myelogenous leukaemia cells K562 and the hepatic carcinoma cells BEL7402.

Abdul–Jalill etal. 2013[24] showed that *Enterococcus faecium* con centrated filtrate at the lowest concentrations (2 ng/ml) presentsignificance induction on cell viability of L20 B Cells.

The mechanism of bio surfactant was studied and presented simply by Wang et al. 2013,[25] suggested that Surfactant induced accumulation of the tumor suppressor p53 and cyclin kinase inhibitor p21waf1/cip1, and inhibited the activity of the cyclin B1/p34cdc2 and G2- specific kinase. These findings suggest that surfactin caused the G2/M arrest ofMCF7 cells through the regulation of their cell cycle factors, also demonstrated that surfactin induces apoptosis in HepG2 cells through ROS– endoplasmic reticulum stress –Ca^{2+}–extracellular signal-regulated protein kinase pathways.

Recently the anti-tumour activity of a surfactin produced by *Bacillus subtilis* 573 and a glycoprotein (BioEG) produced by *Lactobacillus paracasei* subsp. *paracasei*A20 was evaluated. Both bio surfactants were tested against two breast cancer cell lines, T47D and MDA-MB-231, and a non-tumour fibroblast cell line (MC-3 T3-E1), specifically regarding cell viability and proliferation Duarte et al. 2014.[26]

In recent study carried by Das et al. 2015[27] revealed the efficacy of a marine antimicrobial lipopeptide bio surfactant in blocking proliferation of breast cancer and colon cancer cell lines, the results of the *in vitro* studies displaying the cytotoxicity of this non-hemolytic marine bio surfactant product advocates for its exploitation as a potential drug candidate in anticancer chemotherapy.

CONCLUSION

In summary, the results gathered in this work are very encouraging regarding the bio surfactants potential for cancer cell line inhibition and even for other biomedical applications; nevertheless further research on their mechanisms of action is required.

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