EFECTO CITOTÓXICO DE EXTRACTOS ANTIFÚNGICOS DE ORIGEN BIOLÓGICO SOBRE LÍNEAS CELULARES

CYTOTOXIC EFFECT OF ANTIFUNGAL EXTRACTS OF BIOLOGICAL ORIGIN ON CELL LINES

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RESUMEN

Dentro de la industria alimenticia, el uso de compuestos químicos sintéticos para su conservación y protección de plagas es muy común. Sin embargo, una de las características de plaguicidas químicos puede generar enfermedades al consumidor final a largo plazo. Por ello, la búsqueda de alternativas para dejar de utilizar estos químicos ha llevado a la investigación de fuentes biológicas con los mismos efectos benéficos para la industria. El objetivo de este trabajo fue analizar la citotoxicidad de un extracto bacteriano de *Bacillus amyloliquefaciens* (EB) y uno vegetal de *Equisetum arvense* (EV) *in vitro*. En una microplaca se colocaron cultivos celulares 1132SK correspondiente a fibroblastos y macrófagos murinos RAW 264.7, incubados por 24 h a 37 °C. Se retiró el medio de cultivo y a cada pozo se agregaron 100 µL de los tratamientos diluidos en el medio de cultivo con las concentraciones definidas (0.1, 0.25, 0.5 y 1 %, v/v). Se incubaron las placas por 72 h a 37 °C. Al término del periodo de incubación se realizó una revisión mediante microscopio. Se añadieron 50 µL de solución XTT/PMS, dejando incubada la placa por 4 h a 37 °C. Al término se agitó la placa y se transfirieron 100 µL de cada muestra a otra placa, midiendo la absorbancia a 450 nm. Como un control negativo se utilizó DMSO 20% y como control positivo medio de cultivo sin tratamiento. Los resultados obtenidos demostraron que la viabilidad de las células 1132SK se mantuvo sin diferencia significativa en comparación con el control al aplicar ambos extractos a las diferentes concentraciones. Sin embargo, en el caso de las células RAW 264.7 la viabilidad fue afectada a partir de 0.5 % principalmente por EB. El uso de estos extractos a concentraciones bajas puede ser una alternativa con bajo

riesgo de daño celular.

Palabras clave: *Bacillus amyloliquefaciens;* citotoxicidad; *Equisetum arvense;* viabilidad celular.

ABSTRACT

In the food industry, chemical products are used to conserve and protect against different pests. However, one of the principal characteristics of chemical pesticides is that they can lead to the development of customer diseases in the long term. For this reason, searching for new alternatives to replace chemical products has led to investigating biological sources with similar pesticide activities but without all the risks. The main objective of this study was to analyze the *in vitro* cytotoxic effect of two extracts, one from *Bacillus amyloliquefaciens* and another from *Equisetum arvense*. In a 96-well microplate were placed two cell lines, 1132 SK of fibroblast family and RAW 264.7 of murine macrophages. The plates were incubated at 37 °C for 24 h. The cell medium was withdrawn, and 100 µL of each extract at different concentrations was added to each well (0.1, 0.25, 0.5, 1% v/v). The plates were incubated at 37 °C for 72 h. After the incubation, 50 µL of XTT/PMS solution was added to each well and incubated at 37 °C for a maximum of four hours;100 µL was transferred to a new microplate and measured by spectrophotometry at 450 nm. The results obtained showed the viability of the 1132 SK cells was not affected by the concentration of the extracts. However, the RAW 264.7 cells decreased viability with the 0.5% concentration from *B. amyloliquefaciens.* These extracts' low damage showed the possible applications as an alternative to the low cell damage shown.

***Keywords**: Bacillus amyloliquefaciens;* cytotoxicity; *Equisetum arvense;* cell viability.

INTRODUCTION

The food industry is essential for daily life, and the production of different crops is one of humans' basic activities. Crops have different challenges since the seed is planted; one of the challenges to achieve

is to reduce or eliminate pests. Pests affect crops in different forms and can produced by different microorganisms, insects, or plants. In microorganisms, the principal agents that cause diseases and losses in crops are fungi. They affect them since the seed is planted and starts to germinate, the plant formation and maturation, and in many cases, the diseases are produced after the harvest of the products. At the same time, they are transported or stored (Sawicka and Egbuna, 2020).

Some chemical pesticides have been used to control pests and diseases. Many of these pesticides are used over the soil, and they protect it from pathogens. These remnants continue the crop cycle production all the time. People are involved in the crop's management until the production cycle's end. The remnants interact with skin, eyes, and mouth and sometimes are indirectly ingested. These interactions can cause long-term health damage due to the day-by-day contact or ingestion; on the other hand, the interaction or ingestion can happen after the harvest, when the customer buys a fruit with remnants of the pesticides and eats it, causing immediate health damage or long term diseases developing some cancer (Singh y col., 2018).

To reduce the risk of developing diseases caused by pesticides, the search for biological sources like microorganisms or plant extracts is an alternative. Some microorganisms can produce metabolites that can be used for different pests, such as the case of some antibiotic compounds to inhibit fungi growth. Some microorganisms can produce metabolites with pesticide activity, and some can also degrade chemical pesticides by producing exo-enzymes (Książek-Trela y Szpyrka, 2022). In the case of plant extracts, some have been reported to have insecticide activity and pest control in different crops, which is a part of innovative agriculture. They are more environment-friendly than chemical pesticides due to their minimal residue activity and low activity to non-target insects (Meena y Mishra, 2020).

This study aims to perform the cytotoxic tests of two biological extracts from different sources, one from bacteria and one from plants, for which, in previous research, antifungal activity against post-harvest pathogens was demonstrated. The cytotoxic evaluation is important to determine the possible use as an antifungal product and to ensure any health risks they may develop.

Material and Methods

Extracts production

The aqueous *Equisetum arvense* extract was provided by Greencorp Biorganiks S.A. de C.V.; the extraction methodology is an industrial secret.

The bacterial extract from *Bacillus amyloliquefaciens* was produced in a modified TSB (Tryptic Soy broth), 25 g/L starch was added as a carbon source, and 5 g/L yeast extract was added as a nitrogen source. The rest of the compounds in the medium were 3 g/L soy peptone, 5 g/L NaCl, and 2.5 g/L K_2HPO_4. The extract was produced under 150 rpm agitation at 37 °C for 42 h with an initial inoculum of $1x10^7$ cells/ mL. The extract was centrifugated at 10,000 rpm for 10 min and filtered through a 0.2 µm nylon filter to eliminate the bacteria cell presence (Zalila-Kolsi y col., 2022).

XTT/PMS cytotoxic assay

The cytotoxic assay was performed on two different cellular lines, 1132 SK human fibroblast and RAW 264.7 murine macrophages (American Type Culture Collection (ATCC), Manassas, VA, USA). DMEM-F12 medium (Eagle modified Dulbecco-F12, Corning, Manassas, VA, USA), 10% fetal bovine serum (FBS), and the mixture of antibiotic-antimycotic 1X (Sigma Aldrich, St. Louis, MO, USA) were used for this assay. The cellular lines were collected by enzymatic digestion (Trypsin/EDTA). Immediately, culture medium and FBS relation 4:1 v/v were added and centrifugated at 150 rpm/ 5 min. The supernatant was withdrawn, and the precipitate was resuspended in 1 mL PBS; in each microplate well was added 150 µL with 7500 cell concentration. The microplate was incubated for 24 h at 37 °C (5% CO_2, >90% humidity) and observed at microscope after the incubation to verify the correct cellular growth. The medium of each well was withdrawn from the microplate, and 100 µL of the medium was added with the different treatment concentrations (0%, 0.1%, 0.25%, 0.5%, 1% of each extract, and 20% Dimethyl sulfoxide (DMSO) as negative control), after the medium addition the microplate was incubated for 72 h at 37 °C (5% CO_2, >90% humidity). After the 72 h incubation, 50 µL XTT/PMS [2,3-

bis (2 metoxi-4-nitro-5-sulfofenil)-2H-tetrazolio-5-carboxianilina)/ Phenazine methosulfate 25 µM] was added to each well and incubated for 3-4 h at 37°C. The microplate was shaken softly, and 100 µL was taken and placed in another microplate. Finally, the optic density of the microplate was read at 450 nm (Coecke y col., 2005; Health, 2001; ISO-10993-5; Stevens y Olsen, 1993). To calculate the viability percentage, the next equation was used:

$$\%\ Viability = \frac{OD_{treatment}\ x\ 100}{OD_{Control}}$$

Statistical Analysis

All experiments were carried out in triplicate. A comparison of means was performed to analyze the data. The variance analysis was performed using the Infostat software 2020l version using Tukey's range procedure.

Results

The extracts evaluated in the study showed similar results while evaluating the different cell lines. Figure 1 shows the viability of the 1132 SK cell line of fibroblasts. Applying both extracts at different concentrations did not affect the cell line. There was no significant difference compared to the control group. The lower percentage viability values were obtained with the lowest concentration of the treatments (0.1%), 84.99% from *Bacillus* extract and 95.81% from *Equisetum* extract, and increases the viability as the concentration increases, compared to the negative control (DMSO), which reduce the viability of the cell lines to 11.51%.

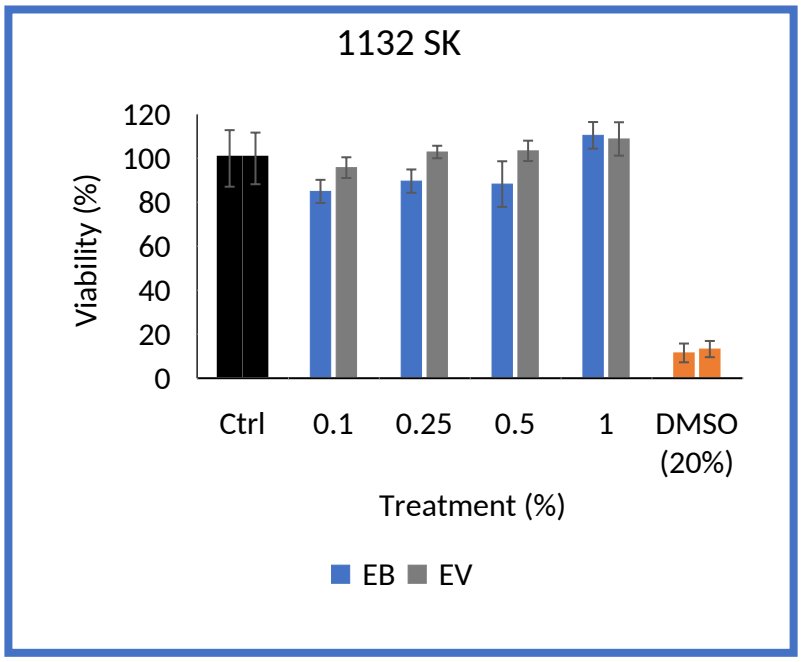

Figure 1. Viability of 1132 SK fibroblast cell line after 72 h incubation with the applied treatments: EB= *Bacillus amyloliquefaciens* extract, EV= *Equisetum arvense* extract.

Figure 2 shows the RAW 264.7 murine macrophage cell line's viability after incubation for 72 h with treatments. The viability was affected by both extracts at certain concentrations. The *Bacillus* extract started to affect the viability of the cell line at 0.5% concentration with significant difference compared to the control, the viability of the cell dropped to 71.97 % and continue decreasing at 1% treatment with a viability of 67.96 %, showing the tendency to continue decreasing. The *Equisetum* extract showed similar affection to the viability as the *Bacillus* extract. It started to decrease after the 0.5% treatment. However, the statistical analysis showed no significant difference compared to the control.

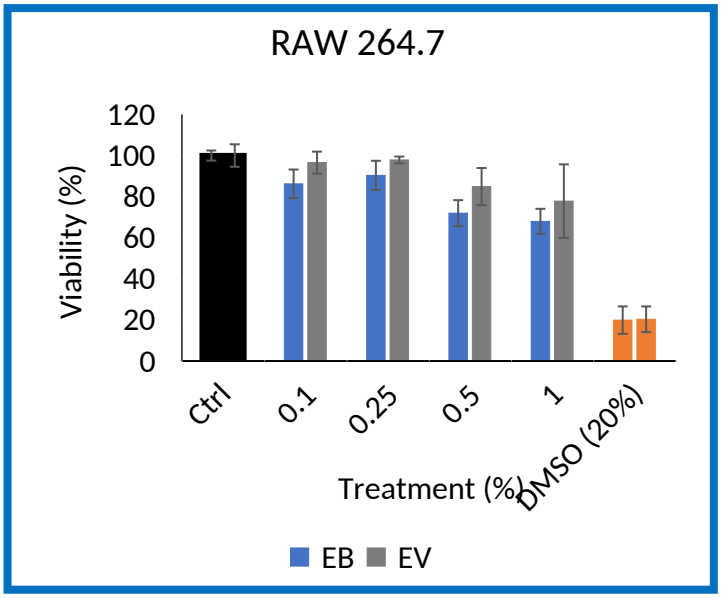

Figure 2. Figure 2. Viability of RAW 264.7 murine macrophage cell line after 72h incubation with the treatments applied. EB= *Bacillus amyloliquefaciens* extract, EV= *Equisetum arvense* extract.

DISCUSSION

The viability of the cell lines was affected by both evaluated extracts, each cell line with a different response. While the 1132 SK cell line was unaffected in negative tendency, the RAW 264.7 cell line was affected. The biologically active compounds of both used extracts were not purified. It could be the main reason why they could affect each case differently. Also, the *Equisetum* extract has been used as an artisanal beverage to reduce some health issues, such as kidney problems as a diuretic (Carneiro y col., 2019). The way this extract interacts with both cell lines shows us that it can be used in low concentrations, and in some cases, as in the 1132 SK, it can promote the viability of the cell lines as its concentration increases.

Moreover, the *Bacillus* extract showed a positive interaction with the 1132 SK cell lines, as the *Equisetum* extract promotes the viability of this cell line at 1% with an increasing tendency, which could mean that the compounds the bacteria produce help to proliferation of the cell line. However, the RAW 264.7 cell line was affected differently. After interacting with a 0.5% concentration of the bacteria extract, the cell

line reduced its viability by almost 30%. The higher concentrations of the extract decreased cell viability which could mean that this extract could be toxic at certain concentrations. Still, the assay was led to a final time of 72 h, and the interaction of the extract with the cell line could be less toxic at lower interaction times.

CONCLUSIONS

The extracts evaluated in this study showed they can be harmless to the used cell lines at low concentrations. The application of these extracts in the food industry can be viable if they are used at low concentrations to reduce any damage they can develop in the organism. More studies must be conducted to identify the negative risks to human health.

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