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Proceeding Paper

EFFECTS OF *LAETIPORUS SULPHUREUS* ON VIABILITY OF HELA CELLS IN CO-CULTURE SYSTEM WITH *SACCHAROMYCES BOULARDII* †

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Abstract:

The aim of this study was to the evaluate impact of ethyl acetate extract of *Laetiporus sulphureus* on the viability of HeLa cells in 2D cell cultures and in co-culture system with *Saccharomyces boulardii*. Also, migratory potential of *S. boulardii* through agar in this co-culture system was investigated. Cell viability was assessed by trypan blue staining after 12 and 24 h. Tested extract had no cytotoxicity on HeLa cells in the 2D cell cultures. Our results indicate a potential cytotoxic effect of *S. boulardii* on HeLa cells which could be a consequence of physical contact between yeast and cancer cells, after migration of *S. boulardii* through agar toward cancer cells, or metabolic activity of *S. boulardii*. Also, *L. sulphureus* extract induced strong migration of yeast in co-culture after 12 h, compared to control. Further studies should be conducted regarding this mushroom in a co-culture system with *S. boulardii.*

Keywords: Co-cultures; ethyl acetate; extract; yeast; edible mushroom; cytotoxicity

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1. Introduction

Co-culture systems are widely used in biological research to study interactions between different cell populations. Recently, co-culture systems are increasingly used in the research of interactions between human cells and certain microorganisms. Many studies indicate the important role of microbiomes in the promotion and inhibition of tumor mass growth [1,2].

Laetiporus sulphureus (Bull.) Murrill is an edible mushroom with large sulfuric-yellow colored fruiting bodies and is a wood-rotting saprophyte, that prefers to grow in deciduous and evergreen forests all over the world [3]. This mushroom is known for its medical properties and proven antioxidant, antimicrobial, and antitumor properties [3]. Although possess confirmed anticancer effects [4], its cytotoxicity potential on HeLa cells in a co-culture system is insufficiently investigated.

Saccharomyces boulardii is a probiotic species used effectively in complementing the treatment of acute gastrointestinal diseases, such as diarrhea, or chronic diseases, such as inflammatory bowel disease [5]. *S. cerevisiae* can survive in a gastric-like environment and in an intestinal environment (bile salts, pancreatin, pH 7.0), which enables the application of this microorganism in extreme conditions, such as the cancer environment. Also, the advantage of *S. boulardii* is its very successful penetration through the mucin produced by the epithelial cells and adhesion to these cells. The probiotics express surface adhesins that mediate the attachment to the mucous layer by recognizing host molecules, such as transmembrane proteins (integrins or cadherins) and extracellular matrix components (collagen, fibronectin, laminin, elastin, etc). Also, a very common mechanism of attachment of *S. boulardii* to the host epithelium is *via* mannose residues in the cell wall [6].

Recently, studies showed that the cervical microbiome take part in inhibition and progressing of cancer. In accordance, the aim of this study was to develop and optimize a co-culture system using specific probiotic species and cancer cells. Furthermore, we examined the effect of ethyl acetate extract of *L. sulphureus* (EALS) in co-treatment with the probiotic species *S. boulardii* on the viability of the cervical adenocarcinoma cells (HeLa) cancer cell line in the co-culture system.

2. Materials and Methods

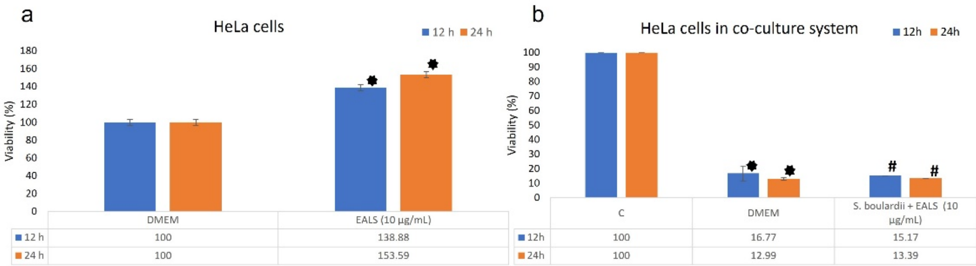
Laetiporus sulphureus was gathered from lying tree trunks of *Salix* sp. in the Šumadija area, Serbia, and extraction was performed with ethyl acetate solvent according to the standard procedure previously described [4]. HeLa cervical cancer cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), and antibiotics (100 U penicillin and 100 U/mL streptomycin). To determine cell viability in 2D culture, HeLa cells were seeded in a 6-well plate.
The 2D single cell culture treated with EALS at the concentration of 10 μg/mL. Untreated cells were used as control. The viability was determined with trypan blue staining 12 and 24 h after the EALS [7].

S. boulardii was provided by the Microbiology Laboratory, Institute for Information Technologies, University of Kragujevac, Serbia. The co-culture system was formed in 50 mL test tubes. A 40 μL of yeast suspension was added into 40 mL of sterile soft Sabouraud dextrose agar (0.7%, wt/vol) (Torlak, Belgrade, Serbia). The HeLa cells were seeded on the coverslips, and after incubation of 24 h were placed (up-side-down) on top of the agar and overlaid with 10 mL of DMEM (DMEM, 10% FBS, without antibiotics) or with 10 mL EALS of 10 μg/mL. and test tubes were placed in an incubator at 37°C, without CO_2 for 12 and 24 h of incubation. The HeLa cells viability was investigated: 1) The HeLa cells turned towards the surface of yeast medium (without *S. boulardii* inoculum) and overlaid with 10 mL of DMEM-represented negative control (C). 2) The HeLa cells on the surface and *S. boulardii* inoculated in agar and overlaid with 10 mL of DMEM; 3) The HeLa cells on the surface and *S. boulardii* inoculated in agar and overlaid with 10 mL of treatment. Also, the migratory potential and colony formation of yeast on the top of agar were evaluated. Migration of yeast through soft agar was measured using ImageJ software after 12 and 24 h of incubation. A thin layer on top of the agar indicates migration of *S. boulardii*, while a thicker layer most likely indicates the proliferation of *S. boulardii* and colony formation, considering that yeast cells double in number every 100 minutes [8].

3. Results and Discussion

Our results showed no cytotoxicity effect of EALS extract when it was tested outside the coculture system (control experiment). Moreover, the HeLa cell proliferation is statistically and significantly increased after 12 and 24 h of EALS treatment (Figure 2a). No significant cytotoxicity of EALS on HeLa cells was obtained in the research [9].

On the under hand, the *S. boulardii* significantly reduces the viability of the HeLa cells in the co-culture system compared to the control. Also, the EALS was shown no cytotoxicity effect on HeLa cells in the co-cultures with *S. boulardii*, as well as in the control experiment (Figure 2b). EALS has no negative effect on *S. bo*ulardii in the co-culture system, which is a positive result of this investigation. These results indicate a potential cytotoxic effect of *S. boulardii* on cervical adenocarcinoma cells (HeLa) (Figure 2b). The cytotoxicity could result from the metabolic activity of *S. boulardii* cells which seems to be further enhanced following the physical contact between yeast and cancer cells. The cytotoxic effect of *S. boulardii* was also shown on human breast cancer cells in a study by Jovanović et al. [10].

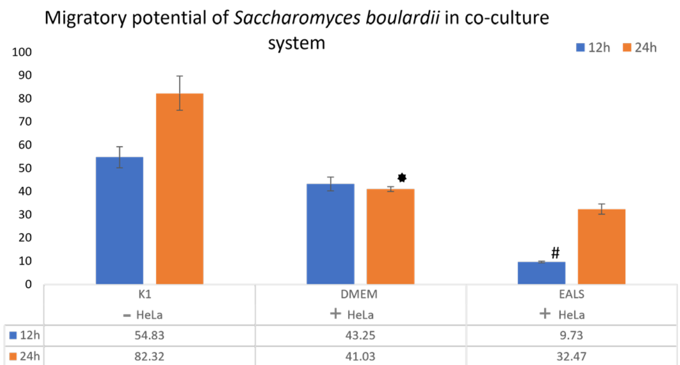


* A statistical significance difference in incubation between DMEM/EALS (a), and C/*S. boulardii* (b).
# A statistical significance difference in incubation between C/*S. boulardii* + EALS (10 µg/mL) (b).

Figure 2. The effect of EALS on HeLa cells without *S. boulardii* (a); HeLa cells in a co-culture system with *S. boulardii* (b). The viability of HeLa cells in control, without EALS and *S. boulardii* (negative control - C), The viability of HeLa cells with *S. boulardii*, without treatment (DMEM); and viability of HeLa cells with *S. boulardii* and EALS (*S. boulardii* + EALS). The viability of HeLa cells in both experiments is expressed as a percentage ± SE of 3 independent experiments.

In addition, our study aimed to evaluate the effect of treatment on the migration of *S. boulardii* in the co-culture system after 12 and 24 h of incubation. Results showed a statistically significant difference in yeast migration after the impact of EALS on 12 h of incubation, compared to DMEM (Figure 3). It is important to examine the influence of treatment on the migration of this yeast, given that physical contact is one of the potential mechanisms of the cytotoxic effect of *S. boulardii*. More investigations are recommended to be implemented to determine precise mechanisms of cytotoxicity of *S. boulardii*.

Also, the test tubes with *S. boulardii* were photographed after 12 and 24 h of incubation, and yeast migration and colony formation on top of the agar after the impact of EALS are shown visually (Figure 4). The colony formation is most probably a consequence of intensive yeast proliferation.



* A statistical significance difference between DMEM and K1 on 24 h of incubation.
# A statistical significance difference between EALS and DMEM on 12 h of incubation.

Figure 3. The *S. boulardii* migratory potential in the co-culture system after 12 and 24 h of incubation with 10 µg/mL of EALS. *S. boulardii* in a test tube without HeLa cells, only cover slip (K1), the *S. boulardii* and HeLa cells in a test tube without treatment (DMEM), and the *S. boulardii* and HeLa cells in a test tube with EALS.

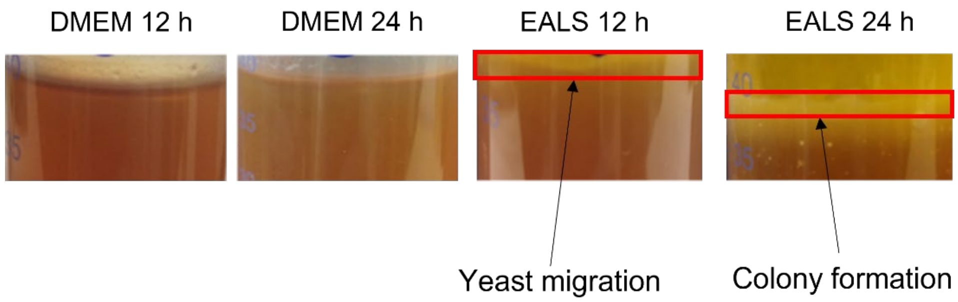


Figure 4. Visualization of *S. boulardii* in test tubes and on the cover slip after 12 and 24 h of incubation.

5. Conclusions

In this study, we demonstrated the impact of microbiota on the viability of HeLa cancer cells. Our results indicate a significant reduction in viability of cervical cancer cells (HeLa) in the co-culture system with the *S. boulardii*. Also, the results showed a positive effect of EALS extract on the migration of *S. boulardii* towards the HeLa cells. However, additional tests of the cytotoxic effect of this yeast on the viability of HeLa cells are recommended, because the physical contact, as well as metabolic activity, might be the cause of cytotoxicity.

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