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EVALUATION OF BIOLOGICAL EFFECTS OF SERICIN ON HUMAN LUNG CANCER CELL LINE

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ABSTRACT

Introduction: Non-small cell tumor is the most common type of lung carcinoma and the treatment involves surgery, chemotherapy, and radiation therapy. In this sense, the search for new chemical substances with characteristics of selectivity, effectiveness and low toxicity have been investigated in the treatment of cancer. Studies show that sericin, a protein extracted from silkworm cocoons, has antitumor and pro-apoptotic activity. **Objective**: To analyze the potential antitumor effect of sericin on non-small cell lung cancer cell line. **Materials and Methods**: The studies were conducted in culture, where the cell viability was evaluated through the neutral red assay, cytotoxicity with MTT; apoptotic potential, with annexin-5 and Alexa Fluor® and Propidium Iodide assays; and cell migration with the Wound Healing assay. **Results**: Low doses of sericin were able to increase lysosomal viability, reduce mitochondrial viability, increase apoptosis and cell migration, while high doses of sericin exponentially reduced cell migration and did not alter the rate of apoptosis / necrosis of cancer cells. **Conclusion**: Sericin is a biomaterial that causes biological effects on the cell line tested, and can be used to increase lysosomal viability, reduce mitochondrial function, increase apoptosis at low doses and inhibit high-dose cell migration.

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INTRODUCTION

According to data from the International Agency for Research on Cancer (IARC), lung cancer is the third most prevalent type of cancer. The expectation for the next five years is that the disease kills about 19 million people. The National Cancer Institute (INCA) estimates 28 thousand cases, with a mortality ratio of approximately 90% (FERLAY *et al., 2012;* MINISTÉRIO DA SAÚDE, 2016). The age with the highest incidence of lung cancer is over 55 years old and is the one with the highest number of deaths (FERLAY *et al., 2012)*. This fact is associated with the pattern of chronic exposure throughout life to environmental and occupational carcinogens (CONSONNI *et al.,* 2010*)*. In addition, hereditary factors such as mutations on chromosome 15q25 affect the interaction with nicotine and increase the possibility of cancer development (HUNG *et al.,* 2008*)*. The smoking habit is the main cause of lung cancer and accounts for about 85% of cases (PORTER *et al.,* 2011*)*.

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In addition, an association between the activation of transcriptase telomerase (TERT) during inflammatory processes and the occurrence of lung cancer, indicating a pro-oncogenic influence of this endogenous factor (BRENNER *et al.,* 2010). Of the existing lung cancers, the non-small cell type is the most prevalent accounting for 86% of the cases, and less responsive to surgical, chemotherapeutic and radiotherapeutic treatments (KUMAR *et al.,* 2014). The main chemotherapy regimen utilizes platinum derivatives (cisplatin and carboplatin), conjugated with the etoposide, and is frequently associated with radiotherapy (JUNIOR, 2012). It is important to emphasize that radiotherapy seems to shorten patients' survival (FRANÇA *et al.,* 2011). Scientific research in the treatment of cancer has aimed at the development of therapies focused on the receptors and enzymes that initiate or perpetuate the neoplastic process, aiming to restore metabolic pathways inhibited, such as apoptosis, or inhibit the activity of pro- oncogenic enzymes, such as tyrosinase. Surveys also include therapies with action on DNA and RNA (MOREIRA; ENG, 2014; ROSELL; KARACHALIOU, 2014). In this context, the sericin protein, extracted from the cocoon of the silkworm (*Bombyx mori*) presents some biological

characteristics of interest, such as: anti- thyrosinase activity ;antitumor and pro- apoptotic. In addition, it presents low cytotoxicity in normal cells, ability to regulate the intensity of inflammation and protective effect in the cell against lipid peroxidationand oxidative stress (KATO *et al.,* 1998; ZHAORIGETU *et al.,* 2001; DASH *et al*., 2008a; DASH *et al*., 2008b; CHLAPANIDAS *et al*., 2013).

Goal: To analyze the antitumorous effect of sericin on non-small cell lung cancer cell line.

MATERIALS AND METHODS

This study was developed in the Laboratories of Structural and Functional Biology and in the Molecular Pathology Laboratory of the State University of the West of Paraná (UNIOESTE).

Cell Culture: A549 human adenocarcinoma cell line was kindly donated by Dr. Ricardo Alexandre de Azevedo, from Alchemy, Research and Development Innovation. The cell line was cultured in RPMI 1640 medium, supplemented with 1% streptomycin and penicillin, and 10% Fetal Bovine Serum (FBS) in culture flasks, incubated at 37 ° C in a humid atmosphere of 5% CO2. Each time the culture reached 80% confluency, the cells were picked up until sufficient cell volume was obtained for each experiment.

Sericin Extraction: The sericin peptide was extracted from cocoons of the silkworm (*Bombyx mori),* obtained from the company BRATAC Silk Brazil with the extraction protocol proposed by Gimenes *et al.* (2014). The protein concentration measured by the Bradford method showed a concentration of 1395 µg / ml.

Doses of Sericin: The doses of 13.95, 34.88, 69.75, 104.625, 139.5, 697.5 and 1395 10-1 ng / µL were used for the MTT, Neutral Red and Cell Migration test. In the Apoptosis and Necrosis assay sericin was used in the doses of 13.95, 69.75, 10-1 139.5 and 1395 ng / ml.

Cell Viability Test by Exclusion of Tripan Blue: The cell viability test was used to quantify the number of viable cells from the samples used in the experiments. The resulting data were expressed as a percentage of viable cells, which were, for all trials, greater than ≥ 90%.

MTT Assay: This assay was performed in triplicate, as proposed by Mosmann (1983). Cell viability after exposure to different sericin concentrations was measured after 24 and 48 hours by spectrophotometer absorbance at the wavelength of 570 nM.

Cell Viability Assay with Neutral Red: This assay was performed in triplicate, as proposed by Repetto *et al.* (2008). Cell viability after exposure to different sericin concentrations was measured after 24 and 48 hours by spectrophotometer absorbance at 540 nM wavelength.

Apoptosis and Necrosis Assay with Alexa ® Fluor 488 Annexin V and Propidium Iodide: This test was carried out in triplicate according to the method proposed by the manufacturer [(22)] with the model of fixation proposed by Rieger *et al.* (2011). The number of cells in apoptosis, necrosis or viable, was measured after 24 hours under a fluorescence microscope, with a final total number of 300 cells per group. For the cellular counting procedure, the FIJI distribution of the ImageJ program was used.

Cell Migration Assay: The accomplishment of this test followed principles of the protocols proposed by Martinez-Mora *et al.* (2012) and Liang *et al*. (2007), with a total time of 46 hours. The images were obtained using an inverted microscope, and posteriorly measured in the FIJI distribution of the ImageJ program.

Statistical analysis

Was used the R statistical package, version 3.5.1. For the effect of comparison between the groups was used ANOVA Factorial test with Tukey post test, chi -square test for independence and multiple and polynomial regression. In all tests the level of significance was α = 5%.

RESULTS

MTT Assay: The result of the MTT assay is expressed in figure 1. In the 24 hour time it was verified that there was a decrease of cellular viability, except in the concentration of 13.95 x 10 $^{-1}$ ng / µL, wherethere was a slight increase in viability. No statistically significant difference (p = 0.6968) was found in the 24 hour time. In 48 hours there was a statistically significant reduction (p = 0.009) in the cellular viability of the sericin- treated groups, and in the comparison with the control, the 697.5 x 10 $^{-1}$ ng / µL was the only one that presented a significant difference (p = 0.0203243).

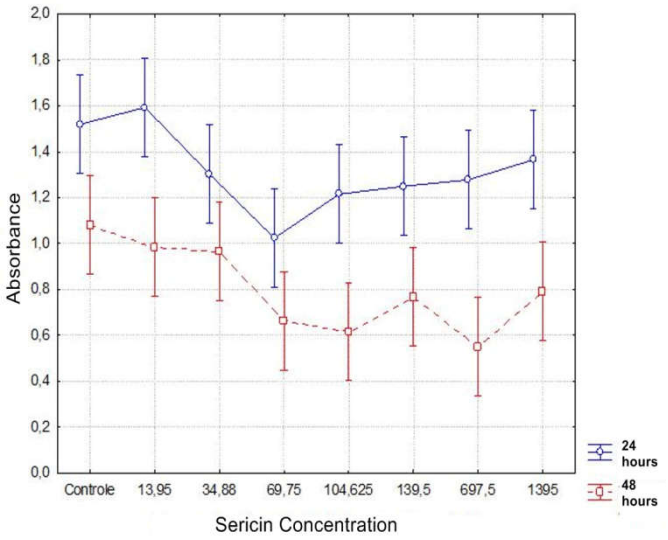

Figure 1. Result of the cell viability assay through the MTT assay showing each time (24, 48, 72 hours) the response of each dose of sericin used. The results are shown by mean and confidence interval (95%) according to the ANOVA test

Assay with Neutral Red (Figure 2): At the 24 hour time the doses of 69.75 and 104.625 (x 10 $^{-1}$ ng / µL) were statistically different in comparison with the control, indicating a significant increase in cell viability. At 48 hours the cellular viability adopted parabolic behavior, with increased viability at the lower doses and then decreased viability at higher doses. The doses of 34.88; 69.75 ; 104,625and 139.5 (x 10 $^{-1}$ ng / µL) showed a statistically significant increase in cell viability in comparison with the control group (Table 1).

Table 1. Result of comparison of the sericin- treated groups with the control group. Significant p-value values evidenced in bold

Doses (x 10 $^{-1}$ ng / µL)	Time	p - value
13.95	24 hours	p> 0.5
13.95	48 hours	p> 0.5
34.88	24 hours	p> 0.5
34.88	48 hours	p <0.001
69.75	24 hours	p <0.001
69.75	48 hours	p <0.001
104,625	24 hours	p <0.001
104,625	48 hours	p <0.05
139.5	24 hours	p> 0.5
139.5	48 hours	p <0.001
697.5	24 hours	p> 0.5
697.5	48 hours	p> 0.5
1395	24 hours	p> 0.5
1395	48 hours	p> 0.5

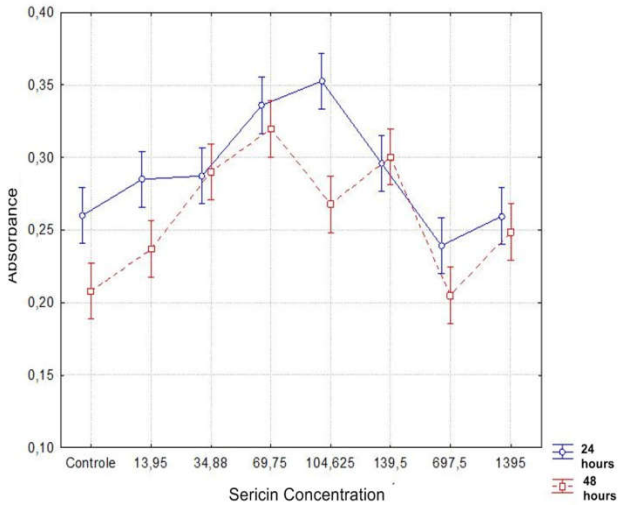

Figure 2. Result of the cell viability test with neutral red showing in values of absorbance, for each time (24, 48, 72 hours) the response of each dose of sericin used. The results are shown by mean and confidence interval (95%)

Apoptosisand Necrosis Assay: The results of the labeling of viable, apoptotic and necrotic A549 cells are shown in Figure 3, Figure 4 and Table 2.

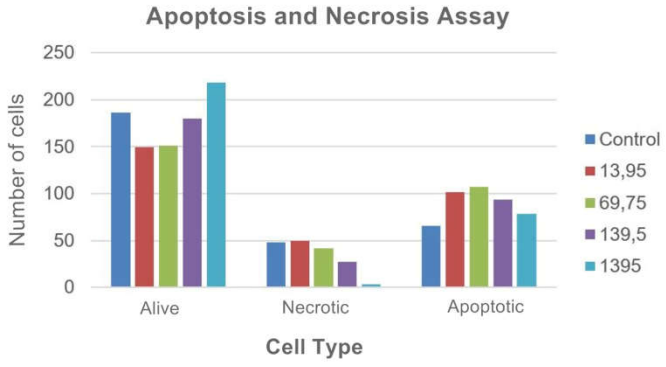

Figure 3. Number of cells: alive, necrotic and apoptotic found according to the dose of sericin

Table 2 shows the final count of each cell type for each dose of sericin tested, with the statistical differences indicated by letters. The number of viable cells was significantly higher at the dose of 1395 x 10-1 ng / L, whereas at the doses of 13.95 and 69.75 (x 10 $^{-1}$ ng / µL) was significantly lower. As for the number of necrotic cells, the 69.75 x 10-1 ng / µL showed significantly greater numbers and the group 1395 x 10-1 ng / µL at a significantly reduced number. The groups 13, 95 and 69,75 (x 10-1 ng / uL) had the greatest number of apoptotic cells, the group 69.75 x 10-1 ng / uL was statistically different from the control, which had the lowest rate of apoptosis (Table 2).

Table 2. Number of cell types found for each experimental group according to treatment used (doses in x 10-1 ng / µL)

	Control	13.95	69.75	139.5	1395
Alive	186 ^{A}	149 ^{B}	151 ^{B}	180 ^{A}	218 ^{C}
Necrotic	48	49 ^{B}	42 ^{C}	27 ^{C}	3 ^{D}
Apoptotic	66 ^{A}	102 ^{B}	107 ^{C}	93 ^{B}	79 ^{B}

* Different letters in each category (Living, Dead and Apoptotic) show significance in the intergroup comparison α = 5%

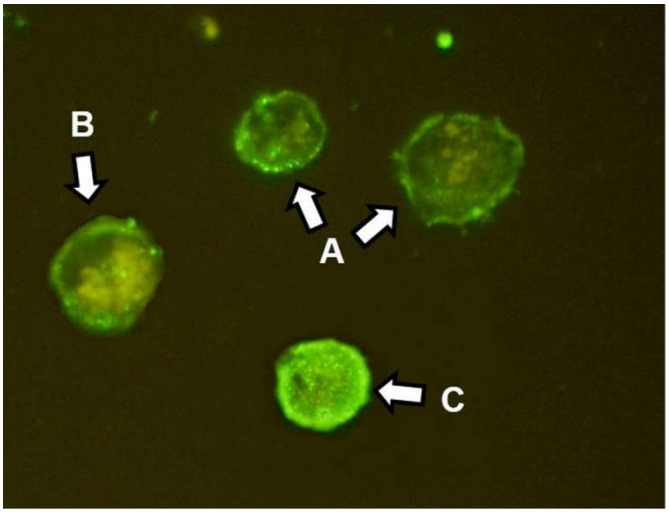

Figure 4. Photomicrographs of A549, alive (A), necrotic (B) and apoptotic (C) cells, as tested for apoptosis and necrosis with Alexa Fluor 488 Annexin V and Propidium Iodide

Cell Migration Assay: The area results were arranged as a polynomial multiple regression models plotted in Figure 5. In this test, lower doses of sericin caused an increase in cell migration and consequent decrease of the *scratch* area. This effect was reversed increasing sericin concentration, when the scratch area was higher and cell migration was lower (Figure 6). With 46 hours the scratch closed in our experiment, what can be related to the results found in the 48 hour time viability assays.

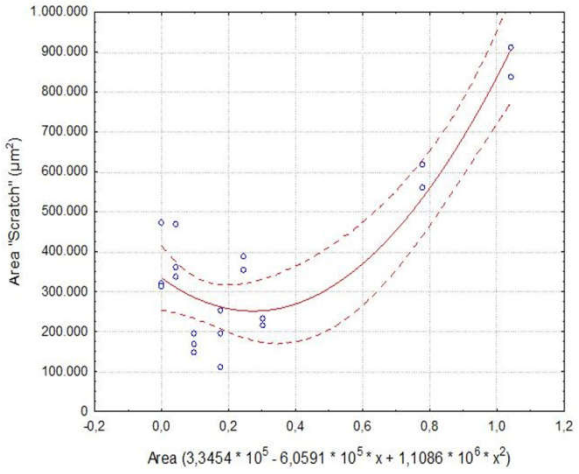

Figure 5. Result of the cellular migration test showing in the polynomial multiple regression curves the values of scratch area evaluated. The results are expressed by the scratch area in µm2 depending on the constructed model, expressed by the y-axis function

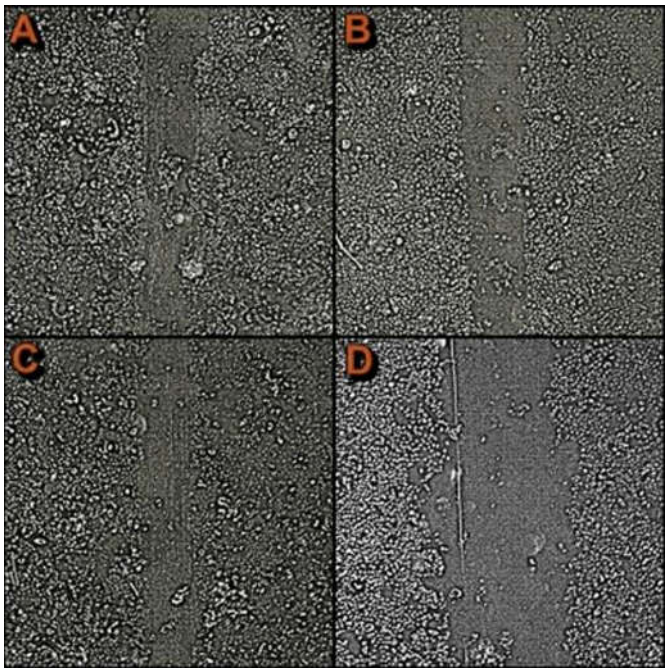

Figure 6. Photomicrographs of A549 cells during Wound Scratch Assay, the cell migration assay. In A, control group; B, dose of 13.95* 10-1 ng/µL; C, dose of 104.625* 10-1 ng/µL; D, dose of 1395* 10-1 ng/µL

DISCUSSION

Non-small cell lung cancer is what kills most among lung cancer types. The search for new therapies for this type of cancer that uses different or complementary pathways to commonly used chemotherapy has been verified in the literature. Studies describing the action of herbal medicines in the treatment of this type of cancer have revealed that some natural compounds have actions of interest because they are more harmful to cancer cells than to normal cells. In one of these studies, Wu *et al*. (2016) using theobromine from green tea and A549 lineage of non-small cell human lung cancer cells, observed decreased expression of human anti–apoptotic enzyme, increased expression of apoptotic enzymes, increased p53 gene action and apoptosis trigger mainly induced by caspases. The effect increased as concentration and time increased. Still using A549 cell lines from non-small cell lung cancer, Lin *et al*. (2017) concluded that exposure of this strain to a type of marine microalgae, common in eastern cuisine, increases BAX expression and decreases Bcl-2 expression. In addition, there was also an increase in the active forms of caspases 3 and 9. According to the authors, the compound used was efficient in producing apoptosis in the A549 line through the mitochondrial pathway. Kaewkorn *et al*. (2012) when using low molecular weightsericin (61-132 kDa), reported increased apoptosis inhuman colorectal cancer, with a significant reduction in the expression of pro-apoptotic enzymes and increased expression of caspase 3 and BAX enzymes. This study showed a tendency to reduce the mortality and apoptosis of A549 lineage cells with increasing dose in 24 hours, and the most significant results of apoptosis were found in the lowest doses used. In addition to the apoptotic potential, other effects were reported using natural compounds, such as decreased metastatic potential. In a study using Cinamaldehyde, a strong pro- apoptotic action was also found as reduced epithelial transformation to the mesenchymal tissue, which reduces the invasive and migratory potential of cancer cells (WU *et al*., 2017). In contrast, studies such as Martinez-Mora *et al*., 2012 showed that sericin has the potential to increase cell migration in vitro. Using a human keratinocyteline and a dose of 0.05%sericin, equivalent to half the lowest dose used in this study (13.95 10-1 ng/µL), the authors achieved a statistically significant increase in cell migration. This fact was confirmed in the data obtained, where small amounts of sericin produced a decrease in scratch area, indicating cell migration. Similarly, in a study with cell lines and a non-living model of myocardial infarction, the sericin was able to decrease post-infarction scar area, increase neovascularization, reduce apoptopse and inflammation, promotes cell migration and transformation (SONG *et al*., 2016). The amount of sericin used in this experiment, 2%, is equivalent to the 139.5 x 10-1 ng / µL dose used in this study, which obtained similar results in cell migration analysis and number of cells under apoptosis. Aramwit *et al*. (2016) using sericin in a system of extended release microspheres corroborated the effects of sericin on the fibroblast cell line of L929 mice, already reported by Cao *et al*. (2015). The authors also investigated the effect of sericin on hybridoma lines, hepatic cancer cell lines, breast cancer; and A549 strain, who presented in this study a good acceptance of the replacement of fetal bovine serum by sericin. The amounts of 5 and 10% better growth sericin are similar to the doses of 697.5 x 10-1ng/µL and 1395 x 10-1ng/µL used herein. Although this study did not replace serine with fetal serum but added sericin edosages, the viability results were different from those found byCao*et al*. (2015) when compared to the same viability test. In general, the viability of the A549 lineage showed a tendency to decrease with the use of these dosages of sericin at the times used. If, however, the lysosomal function is considered, the dose of 697.5 x 10-1ng/µL, equivalent to a dosage of 5% of the study of Cao *et al*. (2015) represents an exception, since at this dose in the referred study and in the present study, there was an increase in viability above the values of the control group. One of the great difficulties in the treatment is theinespecificaction of the therapy, which ends up provoking side effects. Among the strategies used for this purpose are target therapies with chemotherapeutic binders. Huang *et al. (*2016) used doxorubicin - loaded conjugated sericin-folate nanoparticles in the treatment of human cervical cancer cell line HeLa, which have high expression of folate receptors. The authors testified of the efficacy of this therapy and alluded that this strategy could be used in the therapy of other cancers with the high expression of folate receptors, with high affinity and low side effects. In O'Shanessy's *et al.* (2012) study, 74%ofadenocarcinomas had high expression of folate receptors. The study showed that groups treated with low doses of sericin had increased lysosomal viability in comparison with the control group, which corroborates the treatment with sericin using lysosomal routes. Moreover, the decrease of mitochondrial viability in these low dosages means further advantage is the use of sericin. According to Okon and Zou (2015) production of reactive oxygen species by mitochondria may also play a role in promoting oncogenesis and producing resistance to chemotherapeutic agents, especially when the target of the therapy is EGFR, such as non-small cell lung cancer.

Conclusion

Sericin has the ability to influence citophysiologic functions of the human lung cancer cell line, A549. These results suggest that sericin does not have a defined action of its own, acting as a promoter, or as an inhibitor of the growth and proliferation of

cancer cells. However, sericin shows potential for use in non-small cell lung cancer, since its results show that it can influence the function of two important cellular organelles, the mitochondria and the lysosome.

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