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Effect of microscopic fungi lysates on Ehrlich carcinoma growth

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A B S T R A C T

The article presents the influence of biologically active substances NL-51, T1 and K1 (supernatants, isolated from microscopic fungi collected from different regions of Georgia) on Ehrlich's carcinoma growth in mice. Investigations have shown that T1 and K1 samples reveal inhibitory activity on proteolytic enzymes that supposedly explains their anticancer effects (increased lifespan of treated carcinoma-bearing mice compared to control mice). The opposite effect was observed in case of NL-51 treated mice. NL-51 did not show inhibitory properties on proteolytic enzymes and the life span of treated animals was not prolonged compared to the control group animals.

***Key words*:** Cancer, Proteolytic enzyme, Enzyme inhibitors, Microscopic fungi.

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Introduction

Cancer is one of the leading causes of morbidity and mortality worldwide. According to the World Health Organization, the number of deaths from cancer in 2020 reached 10 million, and the number of new cases – 14 million. Annually increases the number of lung, liver, stomach, breast, and colon cancer cases [1].

At present, despite of wide range of anticancer treatment methods (surgical intervention, chemotherapy, radiotherapy, immunotherapy, hormone-based therapy, etc.), treatment still remains unsolved due to accompanying side effects (neuro-, hepato-, and cardiotoxicity, superinfections, etc.) [2-7].

For these reasons, scientists are actively involved in the development of anticancer drugs and methods that will be much more effective, safer, and less expensive. In this regard, the natural sources such as enzymes, their inhibitors, and biologically active compounds obtained from different microorganisms, supposedly having antitumor (tumor cell growth inhibition and/or destruction) activity, could be used against malignant growth [8-13].

Studies have shown that proteases – the representatives of the class of hydrolases, play an important role in vitally important biological processes. In particular, they can regulate cell proliferation and apoptosis, participate in the synthesis of new bioactive substances, regulation of intercellular signaling pathways, etc. [14-16]. However, in addition to a positive feature, the variability of their activity may dramatically affect the living organism [17].

According to recent studies, in some types of tumors at an early stage of development, the level of proteolytic enzymes involved in the processes of cancer development (proliferation, angiogenesis, metastasis) is sharply increased [18]. Therefore, their inhibitors could be considered as one of the most powerful anticancer treatment strategies [19].

Most of the studied and known inhibitors are proteins, peptides, polysaccharides, polyphenols, glycerolipids, triterpenes, and low molecular weight

non-protein compounds [20]. At present interest in the role of inhibitors of proteolytic enzymes isolated from microscopic fungi has increased [21]. Recently, inhibitors of serine, cysteine, and several aspartic proteases have been discovered and studies are underway to determine their antitumor potential [22].

Serine peptidase inhibitors have been found in various representatives of ascomycetes and basidiomycetes [23, 24]. Cysteine peptidase inhibitors are found in basidiomycetes. Aspartic protease inhibitors have been found in yeast. Low molecular weight inhibitors of serine and cysteine proteases have also been found in actinomycetes and streptomycetes [25–28].

Based on the above discussed, we think that, microscopic fungi could have anticancer treatment potential. **Aim:** determine the presence of inhibitors of proteases in intracellular biologically active substances (biomass/lysate) isolated from microscopic fungi through solid-phase fermentation and investigate their antitumor effects on Ehrlich carcinoma growth in lab mice.

Materials and Methods

Agarose, salts for buffer solution Na2HPO4, KH2PO4, NaCl, and KCl were purchased from Alfa Chemical (India). Skimmed milk powder was purchased from LTD Biologica. All chemicals were of analytical grade. As a source of commercial protease Chymoral, produced by Gelenikaa.d was used. Microscopic fungi's intracellular lysate, obtained through solid-phase fermentation was purchased from the scientific and Educational Center "Biomed", Technical University of Georgia.

The amount of total protein in the intracellular lysate was determined using the Bradford method [29]. 100 µl of analytical solutions were added to 1 ml of Bradford's reagent, the samples were placed for incubation in a thermostat at 25^0C for 10 minutes. After incubation, the samples were measured using a spectrometer at 578 nm against the reagent blank. The protein concentration was determined on a calibration curve constructed with standard solutions of bovine serum albumin (0.03, 0.062, 0.125, 0.25, 0.5, 1, 2 mg/ml).

The gel diffusion method with slight modifications was used to determine the inhibitory properties of culture fluids on proteolytic enzymes [30]. 1% skim milk powder was taken as a substrate, which was polymerized in 1% agarose gel (in Petri dishes). After the gel was solidified, rings with a diameter of 3 mm were cut and 20 µl samples were added (proteolytic enzyme was mixed to the test solutions in a ratio of 1:2. PBS and enzyme were taken in the same ratio as the control solution). Samples were incubated at 37°C for 18 hours. Inhibition can be visualized by the decrease in the diameter of the clear zone compared to the positive control generated diameter.

The Ehrlich ascites carcinoma (EAC) cells were provided by the Kavetsky Institute of Experimental Pathology, Oncology, and Radiobiology of the National Academy of Sciences of Ukraine, Department of Experimental Cell Systems, The Cell Line Bank (BCL) from Human and Animal Tissues (https://iepor.org.ua/www.onconet.kiev.ua).

The antitumor properties of biologically active compounds of lysates were tested on albino male mice with the weight range 20-25 g, purchased from the vivarium of the Alexander Natishvili Institute of Morphology (Tbilisi, Georgia. https://www.tsu.ge/en). After being placed in a laboratory (8 per cage) the animals were given a 7-day interval for acclimatization before the experiment. During this period, the animals were kept under constant environmental conditions with a light-dark cycle of 12/12 at a temperature of 23 ± 2 °C. They were fed a standard laboratory chow and given free access to water.

For in vivo modeling of the malignant tumor under brief ether anesthesia, each mouse was inoculated subcutaneously with a fixed number of viable cancer cells (2×10^6 cells/20 g body weight). Cells were counted by the hemocytometer. The viability of the EAC cells was 98% (by trypan blue exclusion assay) [31, 32].

For investigation of the effect of intracellular lysates on Ehrlich carcinoma growth the control group mice were injected with 100 µl of physiological solution, and the experimental groups were injected with 100 µl of the test solutions - T1, K1, NL-51 (protein concentration 2 mg/ml). The observation was carried out for 82 days.

SPSS (version 10.0) was used for analyzing data. Differences between tumor control and treated animals were determined by using the Independent-Samples T-test. The criterion for significance was set to $p<0.05$.

Results and discussion

The protein concentration in the research samples was calculated from the linear area of the calibration curve (Table.1). As part of the study, we wanted to determine the total protein amount of in-

tracellular lysates, to standardize injection solutions by protein. This was necessary to administer the same amount of protein to the test mice.

Research samples	Protein concentration mg/ml
T1	5± 0.1
K1	7.9± 0.14
NL-51	5.8± 0.11

Detection of protease inhibitors and observation of their influence on mice

Since some proteases may have tumor-promoting effects, one of the ways of suppressing malignant growth is the use of substances containing proteases inhibitors. In the study's framework, the test samples' inhibitory activity was determined by the gel-diffusion method. The presence of clear rings on agar is an indicator of proteolytic activity and the absence of rings indicates the presence of an inhibitor.

After 18 hours of incubation of the samples, the diameter of the control ring was compared with the rings of the test solutions (Diameter of the transparent circle (cm): Control - 1.3±0.05, T1 - 1±0.03, K1 - 0.6±0.01, NL-51 - 1.3±0.05). The presence of the inhibitor of proteolytic enzymes was clearly observed on the K1 sample, and weakly on the T1, as for the samples - NL-51 the presence of the inhibitor was not observed (Fig. 1).

Fig. 1 *Determination of inhibitors of proteolytic enzymes in research samples by gel diffusion method.*

Lifespan of Ehrlich's carcinoma bearing untreated and treated with K1, T1 and NL 51 mice.

After the injection of the test solutions in an in vivo model of Ehrlich's carcinoma, different results were obtained. As a result of exposure to K1 and T1 solutions, the viability of mice increased compared to the control group. The opposite effect was observed in the NL-51 sample (Fig. 2).

In T1 treated mice the lifespan was prolonged

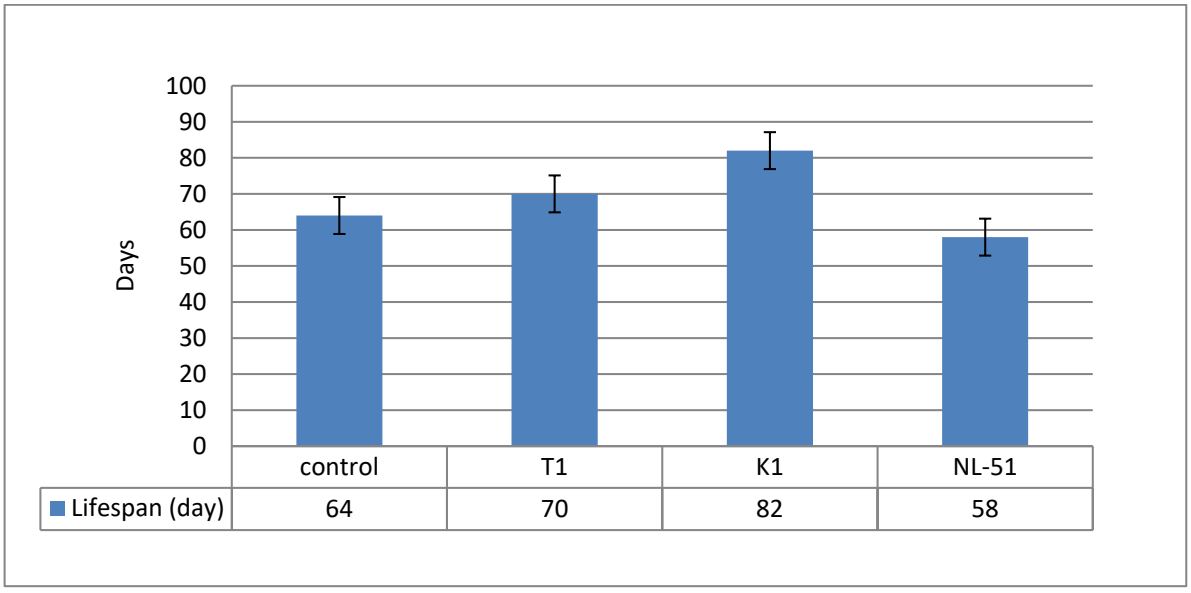

Fig. 2 *Lifespan of mice with Ehrlich carcinoma after exposure to K1, T1, and NL 51.*

by 11% ($p<0.01$), in K1 treated mice – by 28%, and in NL-51 treated mice, the lifespan was less by 9% compared to the control respectively (Fig. 3,4).

Conclusion

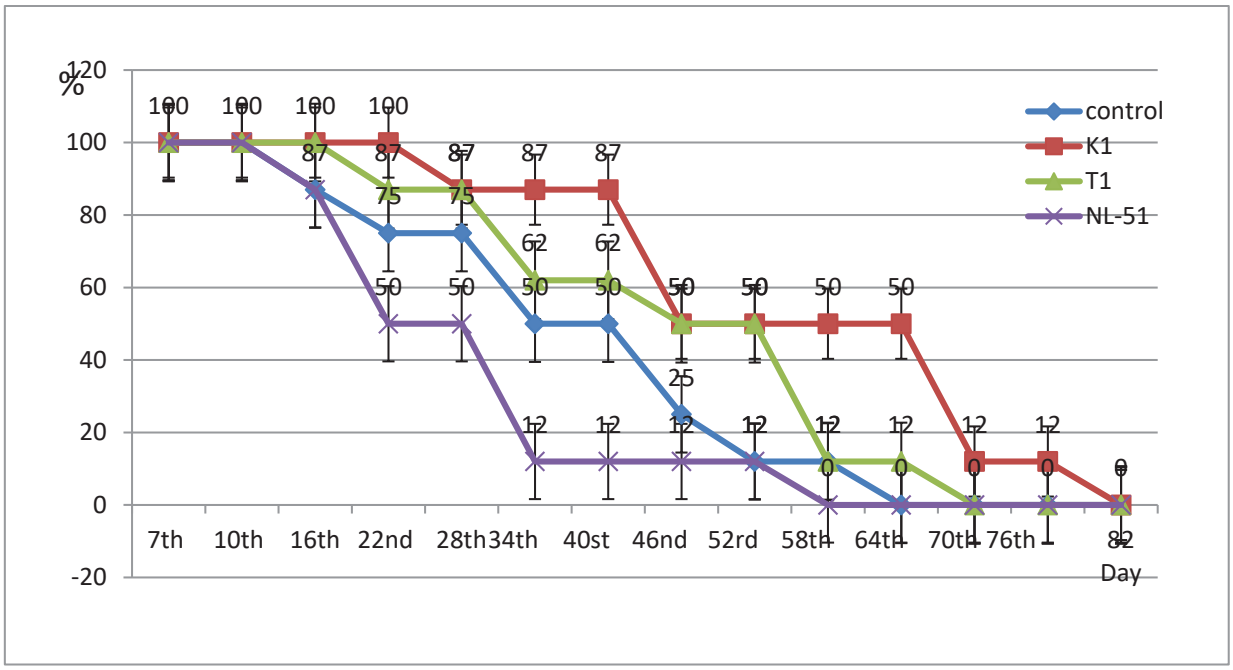

Fig. 3 *Lifespan and Percent of Survived mice with Ehrlich carcinoma (control) and after exposure to K1, T1, and NL 51.*

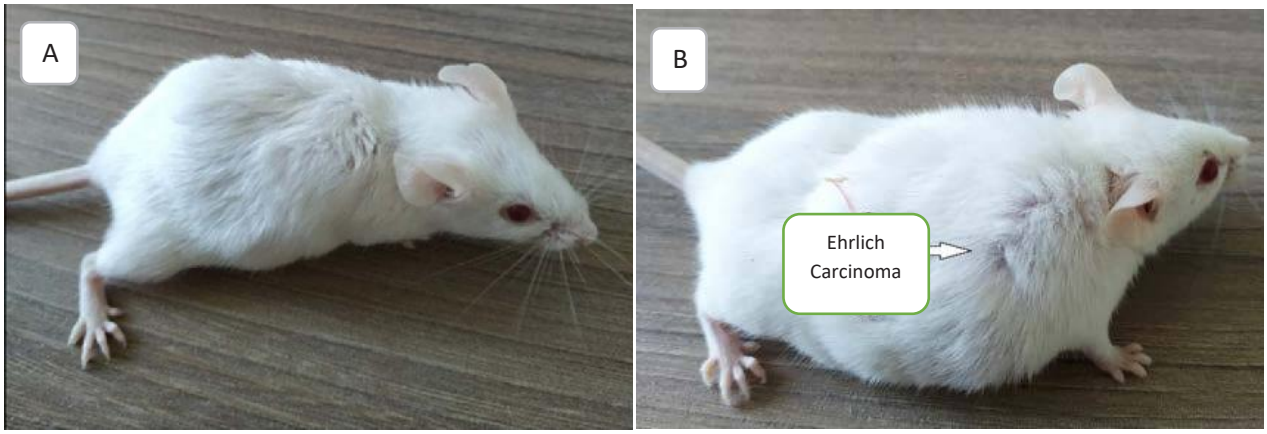

Fig. 4 *(A) mouse with Ehrlich's carcinoma after treatment with K1 (B) control mouse (untreated, cancer bearing) 58th day of Ehrlich carcinoma growth.*

Could be concluded that proteases inhibitors detected in K1 and T1 samples reveal antitumor effects. The study conducted provides preliminary evidence to support this claim. However, it is important to note that further research is needed to confirm the exact mechanism of action of the protease inhibitors and their potential as a therapeutic agent for cancer treatment. Therefore, it can be concluded that while the current study is promising, further investigation is necessary to fully understand the potential of protease inhibitors in the fight against cancer.

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References

[1] Ferlay, J., M. Ervik, F. Lam, M. Colombet, L. Mery, M. Piñeros, A. Znaor, I. Soerjomataram, and F. Bray. "Global Cancer Observatory: Cancer Today. Lyon: International Agency for Research on Cancer. IARC; 2018." (2020).

[2] Sudhakar, Akulapalli. "History of cancer, an-

cient and modern treatment methods." Journal of cancer science & therapy 1, no. 2 (2009): 1.

[3] DeVita Jr, Vincent T., and Edward Chu. "A history of cancer chemotherapy." Cancer research 68, no. 21 (2008): 8643-8653.

[4] Chan, Huan-Keat, and Sabrina Ismail. "Side effects of chemotherapy among cancer patients in a Malaysian General Hospital: experiences, perceptions and informational needs from clinical pharmacists." Asian Pacific Journal of Cancer Prevention 15, no. 13 (2014): 5305-5309.

[5] Baskar R et al. Cancer and radiation therapy: Current advances and future directions. International Journal of Medical Sciences. (2012); 9(3):193

[6] Dilalla V, Chaput G, Williams T, Sultanem K. Radiotherapy side effects: integrating a survivorshipclinical lens to better serve patients. Curr Oncol. (2020) Apr;27(2):107-112. doi: 10.3747/co.27.6233.

[7] Tohme, Samer, Richard L. Simmons, and Allan Tsung. "Surgery for cancer: a trigger for metastases." Cancer research 77, no. 7 (2017): 1548-1552.

[8] Siddiqui, Mustaqeem, and S. Vincent Rajkumar. "The high cost of cancer drugs and what we can do about it." In Mayo Clinic Proceedings, vol. 87, no. 10, pp. 935-943. Elsevier, 2012.

[9] Mohamed, Mona Mostafa, and Bonnie F. Sloane. "Multifunctional enzymes in cancer." Nature Reviews Cancer 6, no. 10 (2006): 764-775.

[10] Eatemadi, Ali; Aiyelabegan, Hammed T.; Negahdari, Babak; Mazlomi, Mohammad Ali; Daraee, Hadis;Daraee, Nasim; Eatemadi, Razieh; Sadroddiny, Esmaeil. Role of protease and protease inhibitors in cancerpathogenesis and treatment. Biomedicine & Pharmacotherapy, (2017), 86(), 221–231. doi:10.1016/j.

[11] Enzyme Inhibitors & Cancer Therapy, Oncology Times: October 10, (2003) - Volume 25 - Issue 19 - p 52doi: 10.1097/01.COT.0000290996.53543.74

[12] Rakashanda, Syed, Farukh Rana, Shaista Rafiq, Akbar Masood, and Shajrul Amin. "Role of proteases in cancer: A review." Biotechnol Mol Biol Rev 7, no. 4 (2012): 90-101.

[13] Shonia, Subramaniam, Rani Selvaduray Kanga, and Kutty Radhakrishnan Ammu. "Bioactive compounds: natural defense against cancer?." Biomolecules 9, no. 12 (2019).

[14] López-Otín, Carlos, and Judith S. Bond. "Proteases: multifunctional enzymes in life and disease." Journal of Biological Chemistry 283, no. 45 (2008): 30433-30437.

[15] R.G. Boy, M. Knapp, U. Eisenhut, W. Mier, Enzymes/transporters, Molecular Imaging II, Springer,(2008), pp. 131–143.

[16] López-Otín C, Matrisian LM. Emerging roles of proteases in tumour suppression. Nat Rev Cancer. (2007)Oct;7(10):800-8. doi: 10.1038/nrc2228. PMID: 17851543.

[17] Yang, Yunan, Hao Hong, Yin Zhang, and Weibo Cai. "Molecular imaging of proteases in cancer." Cancer growth and metastasis 2 (2009): CGM-S2814.

[18] S.D. Mason, J.A. Joyce, Proteolytic networks in cancer, Trends Cell Biol. (2011), 21 (4) 228–237.

[19] Pandey, Rachna, Nitin Patil, and Mala Rao. "Proteases and protease inhibitors: implications in antitumorigenesis and drug development." International Journal of Human Genetics 7, no. 1 (2007): 67-82.

[20] Doljak, Bojan, Francesca Cateni, Marko Anderluh, Giuseppe Procida, Jelena Zilic, and Marina Zacchigna. "Glycerolipids as selective thrombin inhibitors from the fungus Stereum hirsutum." Drug development and industrial pharmacy 32, no. 5 (2006): 635-643.

[21] Dunaevsky, Y.E.; Popova, V.V.; Semenova, T.A.; Beliakova, G.A.; Belozersky, M.A. (2014). Fungalinhibitors of proteolytic enzymes: Classification, properties, possible biological roles, and perspectives forpractical use. Biochimie, 2013, 101(), 10–20. doi:10.1016/j.biochi.

[22] Rawlings ND, Morton FR, Kok CY, Kong J, Barrett AJ. MEROPS: the peptidase database. Nucleic Acids Res. 2008 Jan;36(Database issue):D320-5. doi: 10.1093/nar/gkm954. Epub 2007 Nov 8. PMID: 17991683; PMCID: PMC2238837.

[23] Maier, Konrad, H. Müller, and Helmut Holzer. "Purification and molecular characterization of two inhibitors of yeast proteinase B." Journal of Biological Chemistry 254, no. 17 (1979): 8491-8497.

[24] K. Maier, H. Müller, R. Tesch, R. Trolp, I. Witt, H. Holzer, Primary structure of yeast proteinase Binhibitor 2, J. Biol. Chem. 254 (1979) 12555e12561.

[25] Maier, Konrad, H. Müller, Rudolf Tesch, Rainer Trolp, Irene Witt, and Helmut Holzer. "Primary structure of yeast proteinase B

inhibitor 2." Journal of Biological Chemistry 254, no. 24 (1979): 12555-12561.
[26] SAHEKI, Takeyori, Yoshiko MATSUDA, and Helmut HOLZER. "Purification and characterization of macromolecular inhibitors of proteinase A from yeast." European Journal of Biochemistry 47, no. 2 (1974): 325-332.
[27] Schu, Peter, and Dieter H. Wolf. "The proteinase yscA-inhibitor, IA 3, gene Studies of cytoplasmic proteinase inhibitor deficiency on yeast physiology." FEBS letters 283, no. 1 (1991): 78-84.
[28] Umezawa, H. A. M. A. O. "Chemistry of enzyme inhibitors of microbial origin." Pure and Applied Chemistry 33, no. 1 (1973): 129-144.
[29] Kielkopf, Clara L., William Bauer, and Ina L. Urbatsch. "Bradford assay for determining protein concentration." Cold Spring Harbor Protocols 2020, no. 4 (2020): pdb-prot102269..
[30] Mohan M, Kozhithodi S, Nayarisseri A, Elyas KK. Screening, Purification and Characterization of Protease Inhibitor from Capsicum frutescens. Bioinformation. 2018 Jun 30;14(6):285-293. doi: 10.6026/97320630014285. PMID: 30237674; PMCID: PMC6137568.
[31] Zitvogel, L., Pitt, J., Daillère, R. et al. Mouse models in oncoimmunology. Nat Rev Cancer 16, 759–773 (2016). https://doi.org/10.1038/nrc.2016.91
[32] Strober W. Trypan blue exclusion test of cell viability. CurrProtoc Immunol. 2001 May;Appendix 3:Appendix 3B. doi: 10.1002/0471142735.ima03bs21. PMID: 18432654.