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Cytotoxicity Analysis of the Effects of Heterobasidion Annosum Mycelia and Cisplatin on Colon Adenocarcinoma (CACO-2) Cell Line

Çiğdem Sevim [1,a,*], Sabri Ünal [2,b], Temel Kan Bakır [3,c], Mertcan Karadeniz [2,d], Ali Taghizadeh [4,e]

[1] *Department of Medical Pharmacology, Faculty of Medicine, Kastamonu University, Kastamonu, Türkiye.*
[2] *Department of Forest Engineering, Faculty of Forestry, Kastamonu University, Kastamonu, Türkiye.*
[3] *Department of Chemistry, Faculty of Science, Kastamonu University, Kastamonu, Türkiye.*
[4] *Deparment of Medical Pharmacology, Faculty of Medicine, University of Bilecik Şeyh Edebali, Bilecik, Türkiye.*
**Corresponding author*

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ABSTRACT

Colorectal cancer ranks as the third most prevalent form of cancer and stands as the second leading cause of mortality. Both environmental and genetic risk factors contribute to its manifestation. Presently, 5-fluoruracil/leucovorin (5-FU/LV) remains the recommended course for adjuvant therapy in addressing this condition. Conversely, mushrooms, celebrated for their biologically active constituents, including valuable enzymes, have emerged as a captivating subject in diverse medical disciplines, particularly within the realm of cancer therapy, due to their promising therapeutic properties. This specific investigation aimed to conduct in vitro cytotoxic experiments using extracts obtained from *Heterobasidion annosum* micelles cultivated in a liquid malt extract medium. The pulverized extracts were dissolved in Dulbecco's Modified Eagle Medium (DMEM) at varied concentrations ranging from 25ng/mL to 200ng/mL and subsequently administered to colon adenocarcinoma (Caco-2) cells. The cytotoxic effects of both the fungus and cisplatin, a well-known anticarcinogenic agent, were examined at intervals of 24, 48, and 72 hours. The findings indicated a significant inhibition of cancer cell development within this timeframe. Moreover, a noteworthy discovery emerged, revealing that cisplatin, known for its efficacy in various cancer studies, substantially diminished the viability of cancer cells after 72 hours in comparison to the control group.

Keywords: Cisplatin, Colon adenocarcinoma, *Heterobasidion annosum.*

[a] *cigdemsevim@kastamonu.edu.tr* *https://orcid.org/0000-0002-0575-3090*
[b] *sabriunal@kastamonu.edu.tr* *https://orcid.org/0000-0002-3026-0597*
[c] *temelkan@kastamonu.edu.tr* *https://orcid.org/0000-0002-7447-1468*
[d] *mkaradeniz@kastamonu.edu.tr* *https://orcid.org/0000-0002-3627-9424*
ali.tgzd@bilecik.edu.tr *https://orcid.org/0000-0002-3506-0324*

Introduction

Colorectal cancer is the third most commonly diagnosed and second most lethal type of malignancy in which environmental and genetic risk factors play a role [1]. Adenocarcinomas account for more than 90% of this cancer type. Currently, the main treatment principle for non-metastatic stage colon cancer is surgical resection. Although neoadjuvant treatment is not standard, if surgery is planned in advanced disease, adjuvant treatment in stage 3 (node-positive) patients and surgery in combination with chemotherapy in oligo-metastatic lung and liver disease provide curative benefit [1–3]. Adjuvant fluoruracil (5-FU)-based chemotherapy has become the standard for stage 3 colon cancer. 6 months of adjuvant therapy with the combination of leucovorin (LV), 5-FU has been shown to improve 5-year disease-free survival. In a study conducted in 2004 by adding drugs such as oxaliplatin, capecitabine, and irinotecan, the effect of adjuvant 5-FU/LV on survival times was investigated [1]. Dichlorodiammineplatinum II (also known as cisplatin) is the initial platinum compound that has been approved by the Food and Drug Administration (FDA) for treating cancer. It has demonstrated anti-cancer properties in a range of tumors, including ovarian, testicular, as well as head and neck tumors [4]. Studies have shown that cisplatin, a heavy metal complex, shows significant activity in human colorectal carcinoma cell lines. The primary organs typically impacted by cisplatin toxicity include the liver, heart, kidney, and auditory system. However, given the nature of cell damage induced by cisplatin, it is plausible that all organs could be potentially affected. As an illustration, to mitigate cisplatin-induced kidney damage, outpatient treatment often includes strategies such as maintaining hydration in all patients and administering magnesium supplements along with forced diuresis using mannitol [5,6]. At this juncture, the inclusion of various natural agents with anticarcinogenic properties and minimal adverse effects alongside chemotherapy hints at the potential for mitigating these side effects. Otherwise, there is a strong relationship between colorectal cancer patients and fungal microbiota profiles. Therefore, therapeutic methods used to alter the composition and activity of the gut microbiota have been investigated and, although controversial, effective results have been found [7]. At this point, the question of to what extent we can

benefit from fungi with high environmental contributions that are not present in the microbiota comes to the fore.

Recently, there has been an increase in the desire for safer and more effective therapeutic agents for the chemoprevention of cancer. As a result, natural ingredients including fruits, vegetables, plant extracts, and herbs are essential to the chemotherapeutic treatment of cancer [8,9]. This demonstrates how natural products will significantly impact cancer chemotherapy in the future.

Therapeutic effects with their biological contents such as high amount of protein, vitamins, minerals, essential unsaturated fatty acids, aromatic phenols, terpenoids and steroids [10]. It can be used in the treatment of fungi, many mutagenic and oxidant diseases with the benzoic acid, riboflavin and other B group vitamins, phenolic substances such as quercetin, catechin and crissula (Papaspiride) and flavonoids they contain [11,12]. Most mushroom extracts contain proteins, phenolics, tocopherols, carotenoids, and flavonoids that inhibit the growth of blood vessels, as well as bioactive compounds that can restrict the growth of abnormal cells [13]. Therefore, mushrooms have been utilized in traditional medicine for an extended period to treat various illnesses, including several forms of cancer.

Heterobasidion species are fungi that cause white rot with the enzymes they secrete on the trunk and roots of living and dead trees, infecting the newly cut stumps of coniferous and broad-leaved trees, wounds and even through root fusion [14–16]. Of these species, *H. annosum* is known as one of the most serious pathogens that cause root and bottom rot in forest trees in the northern hemisphere of the world and has made it the subject of many studies in recent years [17–19].

This fungus, which is known to cause damage to many tree species in the world, has also been the focus of interest in anti-cancer studies today. Sadowska et al. (2020) showed that it has beneficial potential against colon cancer as a cytotoxic agent or adjuvant anticancer therapy in their study with *H. annosum* sporocarps [20]. Extracts from the fungus, which was previously investigated as a remedy against various ailments, including cancer, are usually obtained from sporacarps [21,22]. Unlike other studies, this study was carried out with extracts obtained from mushroom micelles instead of sporocarps. For this purpose, in this study, the effect of methanolic extracts isolated from *H. annosum* micelles. This research assessed the efficacy of cisplatin and Heterobasidion micelles, both individually and in combination, on human colon adenocarcinoma cells. Additionally, we explored how the impact of cisplatin on cancer cell proliferation would be altered when combined with Heterobasidion micelles.

Materials and Methods

Research Design

This is an in vitro analysis (cell culture) study.

Research Location and Time

This study was conducted in the laboratories of Kastamonu University and Erzurum Atatürk University.

Study Design

Preparation of mushroom mycelial extracts

H. annosum micelles with the code Tr501 used in the study were obtained from Isparta University of Applied Sciences, Faculty of Forestry, Dendrochronology Laboratory. These mycelles were allowed to grow in liquid malt extract medium at 23°C for one week. For the preparation of micelial extracts, Sadowska et al. (2020)'s method was used with minor modifications [14]. Developed micelles were taken from Erlenmayer flask and washed with distilled water for extraction. Then it was filtered with filter paper and weighed (Wet weight: 0.2893 gr). The micelles were then dried in a room dryer (NUVE KD 400, Turkey) at 45°C for 1 hour. The micelle sample dried on the filter paper was weighed again (Dry weight: 0.0837 g). Dry micelle samples were extracted with 8.37 mL of 99.8% methanol, anhydrous. It was mixed in the homogenizer. It was kept in the mixer for 24 hours. It's been filtered. The filtrate was evaporated in the evaporator. The obtained isolate was homogeneously powdered for use in cytotoxic analyses. In this study, all chemicals supplied from Merck KGaA, Darmstadt, Germany were of analytical purity and deionized water was used in each step.

In vitro Analyses

Cytotoxicity Analysis of Heterobasidion Annosum

Caco-2 Cell Culture

Caco-2 cells (HTB-37™) were obtained from the Department of Medical Pharmacology at Ataturk University in Erzurum, Turkey. Upon thawing, the cells underwent a brief centrifugation process to generate a pellet. Cells were cultured in DMEM (1% antibiotics—amphotericin B, penicillin, and streptomycin—and 10% FBS) and maintained under optimal conditions (37 °C; 5% CO_2). Once they achieved 85% confluency, the cells were harvested and seeded into 48-well plates (Corning, Corning, NY, USA) at a concentration of 1×10^5 cells/mL per well.

Cell Treatments

Subjected to various concentrations of *H. annosum* (25–200 ng/ml) and 15 µg/ml cisplatin, the cells were incubated for 24 hours under conditions of 5% CO_2 and 37 °C. The cells underwent exposure to different treatments for durations of 24, 48, or 72 hours. 3-(4,5-

Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Cell Viability Assay

For the assessment of cell viability, 5,000 cells per well were seeded in a 96-well plate. Following a growth period of 24, 48, and 72 hours, the cells were exposed to varying concentrations of Heterobasidion annosum (25–200 ng/ml) and 15 µg/ml cisplatin, excluding the control cells.

The MTT kit, in accordance with the manufacturer's guidelines (Cayman Chemical, MI, USA), was employed to determine cell viability after 24, 48, and 72-hour intervals. The stock MTT solution, prepared in sterile PBS, was added to the 96-well plates at a concentration of 10%, depending on the desired time point. Subsequent to a 4-hour incubation at 37 °C in an environment with 5% CO2, 100 µL of DMSO was introduced to dissolve the formazan crystals. Formazan absorbance was evaluated using an ELISA reader (Thermo Scientific, Canada) at a wavelength of 570 nm [23].

Statistical Analysis

For the comparison of MTT data among various groups, we employed one-way analysis of variance (ANOVA) with the statistical software IBM SPSS 20.0. To examine the uniformity of variances within the groups, Levene's test was utilized, and the normal distribution within each group was assessed using the Shapiro–Wilk test. Distinctions between groups were determined through the application of one-way ANOVA, followed by a Duncan multiple range test (DMRT) ($p < 0.05$). The outcomes for each group are presented as the mean ± SD.

Results

There was no statistical significance between the 24-hour cisplatin-treated control group and the untreated group, a decrease in cancer cell viability was observed. A significant difference was found between Cisplatin-control (Cis-control) and Cisplatin + *Heterobasidion* (Cis+H) 100 ng/mL dose group ($p<0.010$) and Cisplatin + *Heterobasidion* 200 ng/mL dose group ($p<0.004$) (Figure 1).

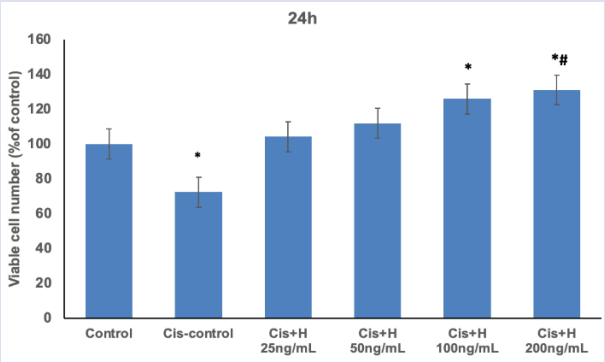

Figure 1. MTT assay in a human colon adenocarcinoma cell line called Caco-2. The cells were treated with cisplatin and Heterobasidium annosum for a duration of 24 hours. The viability of the cells was determined using the MTT assay. The results were expressed as the mean and standard deviation. Values with * shows significantly different to the control group; # shows $p<0.005$ shows significantly different to the cisplatin control group. (Cis: Cisplatin; H: Heterobasidium annosum; each group was studied in triplicate.)

Although there was no statistical significance between the 48-hour cisplatin-treated control group and the untreated group, a decrease in cancer cell viability was observed. A significant difference was found between Cis-control and Cis+H 25 ng/mL dose group ($p<0.041$) and Cis+H 200 ng/mL dose group ($p<0.001$) (Figure 2).

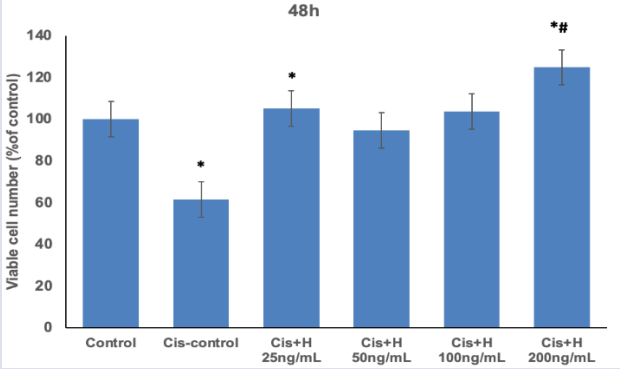

Figure 2. MTT assay in a human colon adenocarcinoma cell line called Caco-2. The cells were treated with cisplatin and Heterobasidium annosum for a duration of 48 hours. The viability of the cells was determined using the MTT assay. The results were expressed as the mean and standard deviation. Values with * shows significantly different to the control group; # shows $p<0.005$ shows significantly different to the cisplatin control group. (Cis: Cisplatin; H: Heterobasidium annosum; each group was studied in triplicate.)

A notable reduction in the survival of cancer cells was observed when comparing the 72-hour cisplatin-treated control group with the group that received no treatment ($p<0.000$). There was also a significant difference between the Cis-control group and the Cis+H group at doses of 25 ng/mL ($p<0.000$), 50 ng/mL ($p<0.000$), 100 ng/mL ($p<0.000$), and 200 ng/mL ($p<0.000$) (see Figure 3).

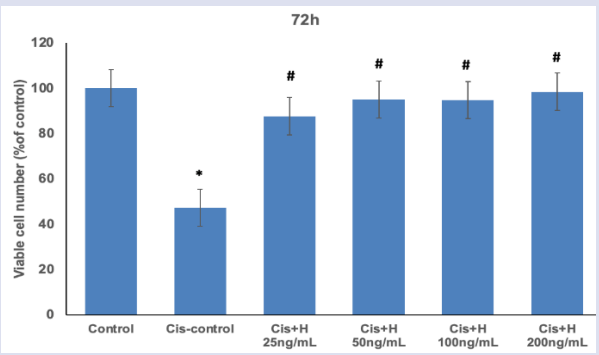

Figure 3. MTT assay in a human colon adenocarcinoma cell line called Caco-2. The cells were treated with cisplatin and Heterobasidium annosum for a duration of 72 hours. The viability of the cells was determined using the MTT assay. The results were expressed as the mean and standard deviation. Values with * shows significantly different to the control group; # shows $p<0.005$ shows significantly different to the cisplatin control group. (Cis: Cisplatin; H: Heterobasidium annosum; each group was studied in triplicate.)

Discussion

Colorectal cancer is one of the most common malignancies, however its morbidity and mortality rates vary [24]. Even though chemotherapy holds a crucial role in cancer therapies, there has been a growing emphasis on the efficacy and synergistic potential of natural product molecules in cancer treatment in recent years. The principal objective of chemotherapy is to provoke the demise of cancer cells while causing minimal or no damage to non-cancerous cells or tissues. Cisplatin stands as a chemotherapy agent employed in treating various tumors, such as colon cancer and multiple other cancer types. The prevalence of side effects such as mucositis, cardiotoxicity, and myelosuppression constrains the range of treatment indications. Consequently, researchers aim to harness the positive impact of the drug while mitigating its adverse side effects [25–27]. Upon examining the literature, indications propose that certain mushroom extracts may possess anticancer attributes and offer therapeutic benefits against diverse types of cancers. The presence of bioactive compounds, including lectins, terpenoids, and secondary metabolites, contributes to these therapeutic effects. Typically, their mechanisms involve the initiation of apoptotic pathways and the induction of DNA damage in cancer cells [25–28].

In a 2020 investigation, Sadowska and her team explored the efficacy of HA extract in treating colorectal cancer in mice. The reference drug for the treatment was 5-fluorouracil (5FU), and the study delved into the effectiveness of combining HA extract with 5FU. The findings indicated that the control group with cancer exhibited the largest tumor volume, while the group treated with 5FU and/or HA extract demonstrated the smallest tumor volume. In the combination treatment, there was an increase in caspase 8 and p53 protein concentrations compared to the patient control group, accompanied by a decrease in survivin and Bcl-2 levels. This research furnished insights into the antitumor activity of HA extract and its interaction with anticancer chemotherapeutic agents [20].

Fungal species complex of *Heterobasidion annosum* s.l. There are five phylogenetically distinct species. Two of these five members are *H. annosum* s.s., which causes infection in pine. and *H. irregulare*, and the other three are *H. parviporum*, *H. abietinum* and *H. occidentale*, which cause infection in non-pine species [29,30]. *H. annosum* s.l. It produces toxins that inhibit plant growth and these substances cause physiological disorders in the plant [31]. Fomannosine, which causes stem wounds in *Pinus taeda* seedlings, and fomannoxin, which has a much more toxic effect on plant cells, are some of these substances [32].

Unlike other studies, in this study, the mycelium of *H. annosum* was used instead of its fruit bodies. The mycelium of the mushroom has been cultivated and isolated in liquid Malt Extract medium. Upon reviewing the literature, no applications utilizing the mycelia of this mushroom have been identified. Therefore, the results of this study are considered to possess originality, as no prior applications utilizing the mycelia of this mushroom have been found in the literature. It is believed that these findings will contribute to the existing body of knowledge. Since the anticancer effects of mushrooms are known, whether the mycelium also possesses this effect and its synergistic effect with cisplatin were investigated on cancer cell lines at three different time intervals. The similarity between the Cis + H dose group and the control group indicates that they did not contribute to the development of tumor cells. Previously, in a study conducted by Sadowska et al., the methanolic extract of *H. annosum* was analyzed for its active components, and their effects on colorectal cancer were investigated both in vitro and in vivo. It has been found that the extract of *H. annosum* significantly reduces the viability and proliferation of DLD-1 cells in a concentration-dependent manner. Additionally, in the group of mice receiving the extract, tumor growth was reduced. However, it exhibited mild to moderate toxicity [20].

David Hansson et al.'s (2014) study on pine-infecting and non-pine-infecting Heterobasidion annosum s.l. In their study, which included secondary metabolite comparison of the species within the and H. irregulare samples were found to form more clumps. Additionally, in the same study, in the H. annosum s.s species, (S)-2-(2-hydroxypropan-2-yl)-2,3-dihydrobenzofuran-5-carbaldehyde,2-(2-hydroxypropan-2-yl)-benzofuran-5 carbaldehyde; 2-(1,2-dihydroxy-2-propanyl)- 2,3-dihydrobenzofuran-5-carbaldehyde; 3-hydroxy-2-(prop-1-en-2-yl)-2,3-dihydrobenzofuran5-carbaldehyde; 5-formyl-2-hydroxybenzoic acid; Fomajorin D; Amino acids such as Epoxydrimenol and Tryptophan were detected [33].

The lack of an increase in the viability of tumor cells obtained in our study raises the question of whether the tumor cells can continue their development at this point. The answer to this question is difficult to assess in detail solely through cytotoxicity analyses. This significant finding reveals that cisplatin, which has been shown to be effective in many cancer studies, significantly reduces the viability of cancer cells compared to the control group after 72 hours when administered alone. The effect of the combination therapy group has shown that it is not as effective as the group receiving cisplatin alone. However, it raises the possibility that further studies on cancer cell lines using *H. annosum* mycelia could shed light on potential applications.

Conclusion and Suggestions

With this analysis in terms of cytotoxicity, it is not possible to say that the combination of cisplatin and mushrooms is effective in this dose range. Different doses of mushrooms may have an effect in different areas by evaluating their efficacy alone, as well as by looking at their oxidant capacity. Apart from these missing points, it should not be overlooked that they do not stimulate the growth of cancer cells. We have focused our attention on the fact that the combination groups showed similar

characteristics to the control group and that cisplatin reduced cancer cell stimulation compared to the control group at 72 hours. The literature information which were reviewed did not provide any information on both dosing and combination therapy and from this point of view, it was conducted to provide data for the in vivo study. It is essential to determine effective dose ranges before proceeding with animal experiments. When looking at the literature, the dose ranges of mushroom extracts can be found, but it could not come across studies with the mycelia of this mushroom. With this study, on which will be added many more studies, it can touch the missing parts in the literature to some extent.

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Conflict of Interest

Authors declare no conflict of interest.

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