Alcumena
Pismo Interdyscyplinarne
Interdisciplinary Journal

Nr 2(14)/2023

DOI: 10.34813/psc.2.2023.7
www.alcumena.fundacjapsc.pl

Investigating the anti-cancer properties of *Trametes gibbosa* extract on human colon cancer cells

Badanie antykancerogennych właściwości ekstraktu z wrośniaka garbatego (*Trametes gibbosa*)

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Trametes gibbosa, also referred to as lumpy bracket, is a fungus belonging to the *Polyporaceae* with natural bioactive compounds that have aroused great interest for their potential benefits in human health. This pilot study aimed to investigate the anti-cancer effect of *Trametes gibbosa* extract against the HCT-116 cell line (human colorectal cancer cells). The extract was obtained by macerating dry biomass with 3 solvents: acetone, isopropyl alcohol and DCM, isolating established anticancer agents – polysaccharide complexes (PSK and PSP) from the extract, and then being dissolved. Cellular toxicity was assessed by trypan blue staining, determining the effect of 1- and 24-hour continuous exposure to the extract of varying concentrations: 100 µl/mL, 200 µl/mL, 500 µl/mL, 1000 µl/ mL. Results have demonstrated a cytotoxic effect in time- and dose-dependent manners. The proposed cellular mechanisms behind the results are most likely apoptosis followed by secondary necrotic cell death, as the literature suggests, although that remains unclear and requires further experiments. Within the course of consecutive studies, it can be established whether the extract or the compounds contained may hold promise for clinical use, e.g., for adjuvant therapy.

Key words: *Trametes gibbosa*, colon cancer, cancer treatment, fungi.

Acknowledgements:
I would like to thank Marta Polańska, PhD, from the Faculty of Biology of the University of Warsaw for giving access to laboratory equipment for the research. Maciej Uranowski has created "Extract preparation protocol" and was responsible for preparation of *Trametes gibbosa* dried mass.

Introduction

Cancer is notoriously recognized as one of the leading causes of death globally. Although there are plenty of treatment methods, they all work differently on each type of cancer, and concerns about their efficiency and worries about the ill and their families cause an emotional distress among many. That is why there are still people who neglect common therapies and turn to alternative or folk medicine for help. Being surprised how widespread the use of folk medicine is instead of regular therapies, an investigation as to whether some of the elements may actually boast about their prospectively beneficial clinical use has begun. That is why I decided to take an innovative approach and check whether alternative and traditional medicine may actually intersect later and be adjusted in accordance with common standards for the greater benefit of medical science. There were plentiful documentaries of varying degrees of credibility pinpointing the beneficial uses of fungi; hence, the kingdom of nature may seem to have a widely unused potential for evidence-based medical sciences.

Theoretical background

Cancer

Cancer is a common term for a group of diseases affecting different areas (tissues or organs) of the human or animal body that arise from the malfunctioning of one's own cells.

The main characteristic features of cancer cells are:

- Cell division proceeds at a very high, abnormal rate,
- Genomic alternations occur leading to abnormal cell structure, often characterized as a large nucleus, an irregular cell shape,
- Invasive growth and ability to metastasize,
- Escaping apoptosis,
- Insensitivity to anti-growth signals and self-sufficiency in growth signals (Hanahan, 2022),

- Limitless replication potential (Hanahan, Weinberg, 2000),
- When a tumor is formed, neo-angiogenesis takes place (Hanahan, Weinberg, 2011),

among others mentioned in Figure 1, below.

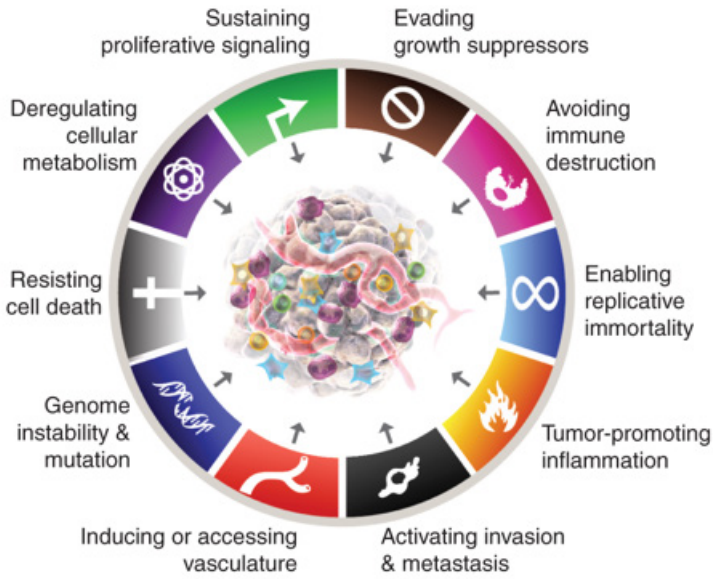

Figure 1. The other characteristics of cancer cells

Source: Hanahan, D. (2022). Hallmarks of Cancer: New Dimensions, *Cancer Discovery, 12(1),* pp. 31–46.

Cancer Treatment and Anticancer Activity

However, with the advances in medicine, a variety of new techniques aimed at treating cancer were discovered or are still being researched. One of the important treatment methods is chemotherapy, which uses drugs known for their cytotoxic effects, especially on fast-growing and dividing targeted cancer cells. Cytotoxic agents are referred to as "substances that kill cells, including cancer cells. These agents may stop cancer cells from dividing and growing and may cause tumors to shrink in size" (NCI, n.d.). Another use of drugs is complementary treatment, e.g., adjuvant therapy, which aims at boosting the effects of primary therapies (radiation, chemotherapy, surgical intervention) and preventing consecutive tumors (NCI, n.d.).

Fungi with anticancer activity

Having seen many different scientific papers on the effectiveness of mushroom-derived chemical substances and knowing many wood mushrooms used for healing purposes, my idea has come up to examine the effect of a commonly occurring in Pomeranian area fungus - *Trametes gibbosa*. It is a polypore, white rot causing fungus that is often found on deadwood or abandoned beech stumpts (Gierczyk et al., 2017).

Table 1: Taxonomy of *Trametes gibbosa*

Kingdom	Fungi
Division	Basidiomycota
Class	Agaricomycetes
Order	Poryporales
Family	Polyporaceae
Genus	*Trametes*
Species	*Trametes gibbosa*

Figure 2: *Trametes gibbosa* in the wild.

As this species of the *Trametes* genus contains polysaccharide-K complex (PSK) – an unhomogeneous substance that is composed of a main chain beta-(1,4) glucan with beta-(1,3)– and beta-(1,6)–linked side chains. Small amounts of galactose, mannose, and arabinose have also been detected in the hydrolysate as suggested by professor Tsukagoshi (Tsukagoshi et al., 1984). Moreover, it is very likely to be cytotoxic and has become used for clinical purposes in the form of a drug used in supplementary therapy called Krestin (Hayakawa et al., 1993). However, it contains polar proteoglucans, terpenoids and phenol that can contribute to cytotoxic activity (Hsieh et al., 2002). Previous research of *Trametes* genus has shown cytotoxic activity towards the other types of cancer cell lines, and the following explanations were proposed:

- Cell cycle arrest in G0/G1 or S phase (Hsieh et al., 2002)
- Apoptosis followed by secondary necrotic cell death (Knežević et al., 2018)
- Activation of caspase-3 inducing apoptosis (Hirahara et al., 2011)

Basing on the available literature concerning research on *Trametes* genus, the extract might be likely to induce cytotoxic effect against cells.

Research question

Research question: How do concentrations of 100 microliters/millilitre, 200 microliters/millilitre, 500 microliters/millilitre and 1000 microliters/millilitre of *Trametes gibbosa* extract influence its cytotoxic activity against human colon cancer cells (HCT116) with 24 hour and 1 hour time exposures measured by trypan blue staining and cell counting?

Method development and procedures

Methods

There are numerous methods used to assess anticancer activity, as they target specific traits of the cell or cellular mechanisms. However, a huge obstacle is most often the availability and affordability of the equipment needed for each method.

Trypan blue staining determines the cell viability by a dint of the stain, and it is based on the assumption that in dead or dying cells that are in late apoptosis or necrosis, cell membrane integrity is damaged, thus stain is introduced to the cell and color change appropriate to stain type is visible (Strober, 2015).

The cell viability was assessed manually under the microscope in the process of counting cells by a dint of the hemocytometer. The blurry or colored with all the shades of navy cells represent dead cells; the remaining ones are viable.

The chosen cancerous cell line is the human colon cancer cell line (HCT-116). The reason behind choosing this kind of cell was the World Health Organization statistics – colorectal cancer being notoriously reported to be ranked in the top 3 in terms of most common cases of cancer yearly as well as the most common cause of cancer death (WHO, 2022; Cancer.Net, 2023).

Procedures

1. Extract preparation

Trametes gibbosa specimens were collected from the same forest in the southeastern Pomeranian district of Poland. 329 grams of the fresh fruiting body were cut into irregular cubes, which were then dried for two days at 40°C. The retrieved 147 g of dried mass was milled in an electric mill. The raw material received was transported into a SIMAX cylinder and flooded with a mixture of hot solvents (Acetone:DCM:isopropanol in a 1:1:1 ratio of 300 ml each. Received 900 ml solvent was transported for heating, then poured into the raw material.) Maceration lasted three days being shaken and heated. The dissolved extract was filtered through tissue filters and

cotton wool. The cleared filtrate was distilled under vacuum on a rotary evaporator. To remove most solvents, about 50 ml of the extract was evaporated on a concentrator to obtain 9 ml. The rest was left to evaporation, and what was obtained was gently heated to get rid of residual solvents and run the formation of a brown, pure extract devoid of them. After cooling, the extract was placed in a cabinet desiccator and dried for two days. 10 mg of dried mass was dissolved in 25 ml of DMSO.

2. Cell culture

HCT-116 is an adherent human cell line: epithelial colorectal carcinoma purchased from the American Type Culture Collection. Cells were cultured with the use of McCoy 5A + 10% Fetal Bovine Serum solution, in constant 37 degrees Celsius temperature and 5% CO_2 concentration.

3. Trypan blue staining

Trypan blue staining protocol was taken from the Creative Bioarray Technical Bulletin (Creative Bioarray, n.d.) and followed. Steps included: centrifugation of the sample for 5 minutes at 100*g, supernatant was discarded, resuspension in 1 mL of PBS followed, 0.4% trypan blue in the buffered isotonic salt solution was added. After an incubation for 2 minutes, the trypan/cell mixture was placed on the hemacytometer.

List of variables

Independent variables

1. **Concentration of extract solution [microlitres/millilitres]** – varying concentrations should have varying impact on cell viability based on the differences in the content of probably anticarcinogenic agents. It is controlled by dissolving stock concentration to the wanted ones. The concentrations of 100, 200, 500, and 1000 were chosen to show if there is a progression in the cytotoxicity once the previous concentration is being doubled (in a dose-dependent manner).
2. **Time exposure [hours of extract incubation in a sample]** - this variable determines whether only varying concentrations of extract solutions influence cell viability. It is controlled by setting a timer.

Dependent variables

1. **Cell viability [measured in the percentage of all cells]** - enables to assess whether the independent variables influence the proliferation of cells or cell cycle by establishing how many cells are still alive, therefore how many cells are not as well. Moreover, it provides a reliable prediction for bigger samples by not putting a clear value, but a trend measured in percentage that could be used easily. Recognized by cell staining.

Results

Sample group/ extract concentration [uL/mL]	% of dead cells [uncertainty 5%]			
	trial 1	trial 2	trial 3	trial 4
Control - medium only	0,00	0,00	0,00	0,00
Control - DMSO c. 750uL/mL	0,00	9,72	6,45	3,51
100	5,88	0,00	5,00	25,64
200	26,09	32,10	56,31	40,26
500	42,86	43,26	63,33	84,00
1000	75,93	56,94	75,47	84,38

Table 2. Percentage of dead cells for each extract concentrations shown for 4 trials after 24-hour incubation

Sample group/ extract concentration [uL/mL]	% of dead cells [uncertainty 5%]			
	trial 1	trial 2	trial 3	trial 4
Control - medium only*	0,00	0,00	7,14	5,65
100 uL	8,16	6,98	20,83	10,20
200 uL	15,38	14,29	44,44	30,00
500 uL	47,06	45,00	55,00	48,39
1000 uL	70,37	61,11	50,00	58,51

Table 3. Percentage of dead cells for each extract concentrations shown for 4 trials after 1 hour incubation. DMSO control was not continued, as in the 24-hour incubation time it did not distort cytotoxicity measurements.

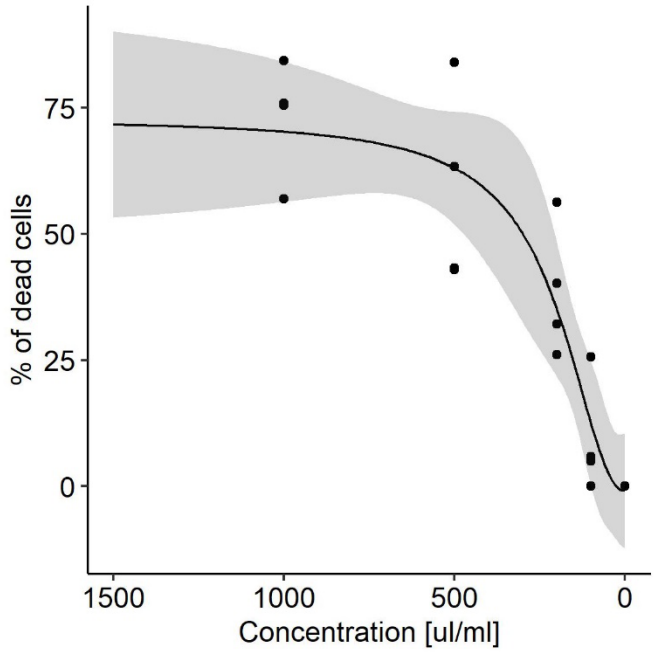

Figure 3. The extract concentration impact on cytotoxicity against the HCT-116 cell line is expressed by the percentage of unviable cells.

It was shown that *Trametes gibbosa* extract is characterized by cytotoxic activity against human colon cancer cells. A relationship between dose (concentration) and cell viability may be sought, as the highest concentration of the extract (1000 µl) contributed to the death of more cells (75%). According to the hypothesis, a significant difference between the effect of the extract over time periods was observed – greater efficacy was measured at daily exposure, but at one-hour exposure we also noted a significant anti-carcinogenic effect. It is clearly depicted that there are remarkable differences in the percentage of dead cells. A visible trend is that with higher extract concentration the percentage of dead cells seems to increase. However, in figure 6 control groups with 0% of extract concentration have corresponding low percentage of dead cells. Therefore, to examine if hypotheses based on both figures are intact, statistical analyses will be performed.

Data processing and analysis

The extract concentration versus cytotoxicity

To analyse the effectiveness of each concentration solution sample, a two-way ANOVA was performed.

Three null hypotheses were proposed:

H_0: Concentration does not differentiate between the samples.

H_1: Time exposure does not differentiate between the samples.

H_2: Time exposure and concentration do not differentiate between the samples.

The calculated value F indicates the ratio of explained variance to unexplained variance. In this case F_0=560.5382, F_1=2.5936, F_2=124.1548 which compared to the F at 0.05 level of significance F_0=2.014, F_1=2.427,F_2=2.054 of research with this number of samples, shown that $H_{0,1 \text{ and } 2}$ are rejected, thus differing concentrations, timespans and their interaction influence the cytotoxic effect against HCT116.

Solvent-cytotoxicity interactions

T-test between controls and the highest extract concentration of each trial was performed to check and eliminate the negative impact of the medium or the solvent in the 24-hour time exposition. The t for control with medium only was 56.5291, whereas for the water of concentration equivalent to the solution with 1000ul concentration (+medium) was 55.9099 that compared to the t critical value for this size 6.314 shown that indeed there is a significant difference between the effect of medium or water and the highest concentrated extract solution; p values based on the 2 separate t-test were less than 0.0001, meaning an extreme statistical difference.

Conclusions

Every *Trametes gibbosa* extract concentration shows cytotoxic properties; however, with increasing its concentration, the cell viability decreases. In a 1-hour time exposure the cytotoxicity against human colon cancer cells was significantly smaller compared to the longer exposure. Interaction between especially high concentration and long-time exposure boosts the cytotoxic effect. This result seems to follow trends in the literature about the *Trametes* genus (Hsieh et al., 2002), although which active substances: proteoglucans, terpenoids or phenols, are mostly responsible for cytotoxicity, remains unclear. The effects of solvent or medium contamination were cancelled due to statistical analyses.

Our research sheds new light on how to deal with health problems: the cytotoxicity of HCT-116 suggests that, with further research, the extract or its compounds could be used as a potential anticancer drug. DMSO was excluded as the sole cause of cytotoxicity by statistical analysis (t-test). As this experiment has revealed an interesting trend and holds promise for possible future clinical use, especially as complementary therapies, follow-up experiments should be conducted. To examine if its prospective clinical use will be intact, there are some measures necessary to take up to examine the effect of the extract on an organism as a whole, e.g., measuring hepatotoxicity in varying timespans. It is reasonable to repeat this experiment on healthy cells or establish stability in human plasma and the overall half-life of the extract. Chosen organic substances could be extracted from the extract, and their cytotoxic efficiency, along with differing concentrations and time exposures, could be compared with that of the extract. In that way, an evidence-based answer could be provided to the question of what makes the extract effective.

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