Acta Scientific Agriculture (ISSN: 2581-365X)

Volume 2 Issue 5 May 2018

Research Article

Anti-Tumor Cell Growth by Extracts of Two Wild-Collected Mushrooms

Kit L Chin[1], Santosh Khanal[2], Gerald R Hankins[2]*, Venu Perla[2] and Yadong Qi[1]

[1]SUAREC, Southern University Land Grant Campus, Baton Rouge, Louisiana, USA

[2]Department of Biology, West Virginia State University, Institute, Wes Virginia, USA

***Corresponding Author:** Gerald R Hankins, Department of Biology, West Virginia State University, Institute, Wes Virginia, USA.

Received: March 13, 2018; **Published**: April 24, 2018

Abstract

Phytochemicals of many edible mushrooms are known to exhibit anti-tumor activities. The extracts of two wild-collected mushrooms, *Cantharellus cibarius* and *Boletus edulis*, known to contain a variety of phytochemicals with a diverse spectrum of biological activities were used for the evaluation of their effects on cell viability of four cancer cell lines (A172 glioblastoma, U87 glioblastoma, CH157-MN meningioma and PAN-C1 pancreatic) and one NIH3T3 fibroblast cell line. The WST-8, Crystal Violet and CyQUANT assays were used to in-vitro evaluate the cell viability of the respective cell line plated onto 96 well plates followed by the mushroom extract treatments. The combined methanol, neutral saline and hot water extracts of *C. cibarius*, and the neutral saline and hot water extracts of *B. edulis*, respectively were used in the assays. The assay was conducted using five concentrations of the mushroom extracts, namely 0, 10, 100, 1,000 and 2,000 ug/mL over three days. While there were some variant results, treatments with high concentrations of *C. cibarius* extracts resulted in significantly reduced number of viable cancer cells for all cell lines tested. Treatments with high concentrations of *B. edulis* extracts of neutral saline and hot water, respectively resulted in significantly reduced number of viable cells. Caspase activation is known as a signature of Apoptosis. Three assay methods produced similar results on cancer cell viability. The FAM-FLICA caspase activation assay was used to determine the effectiveness of the *C. cibarius* mushroom extract in inciting apoptosis. The caspase activation assay results indicated that mushroom extracts did likely account to some extent, for reduced viability of cancer cells.

Keywords: Anti-tumor; Cantharellus cibarius; Boletus edulis; Wild Mushroom; In Vitro; Bioactivity

Abbreviations

DMEM: Dulbecco's Modified Eagle Medium; PBS: Phosphate-buffered Saline; FAM FLICA: Fluorochrome Inhibitor of Caspases; SDS: A detergent present in SDS-PAGE sample buffer.

Introduction

The number of mushroom species on Earth is estimated to be around 140,000 with only about 10% of them are known species [1]. A variety of mushrooms have been used traditionally in many different cultures as food and flavoring materials, for the maintenance of health and in the prevention and treatment of various diseases [2]. Some have been used as food due to their high amount of proteins, carbohydrates, fibers, low fat, and significant level of vitamins (thiamine, riboflavin, ascorbic acid, Vitamin D2) and minerals (USDA NIFA mushroom update), some are used as a food flavoring material due to their unique flavors. There are about 126 medicinal functions are thought to be produced by medicinal mushrooms and fungi including antitumor, immunomodulating, antioxidant, radical scavenging, cardiovascular, anti-hypercholesterolemia, antiviral, antibacterial, antiparasitic, antifungal, detoxification, hepatoprotective and anti-diabetic effects [2]. While the focus has been on medicinal properties of grown edible and medicinal mushrooms, many wildly grown mushrooms are collected, consumed and believed to have medicinal properties. Little research has been done to investigate their potentially beneficial bioactivities. This paper consists of a preliminary study on the effect of extracts of two wild-collected mushrooms (*Cantharellus cibarius* and *Boletus edulis*) on anti-tumor cell growth.

Materials and Methods

Two wild-collected mushroom species

Cantharellus cibarius (known as the Chanterelle, or Girolle) is a difficult to cultivate mushroom species that is found in North America, Europe and Asia. It was collected in the wooded area in Institute, West Virginia (38.38380 N and 81.76580 W) and *Boletus edulis* (known as King bolete, Porcini or Steinpilz) was collected at the base of oak tree grown in West Virginia State University at Institute, West Virginia. Chemical analysis of *Cantharellus cibarius* Kolundzic., *et al.* [3] showed the presence of linoleic acid, cis-vaccenic acid, oleic acid, sterols, β-glucans, and polyphenolic compounds.

Boletus edulis (known as King Bolete, Porcini or Steinpilz) produced a variety of organic compounds with a diverse spectrum of biological activities such as steroid derivative ergosterol, a sugar binding protein, antiviral compounds, antioxidants, and phytochelatins which help build resistance to toxic heavy metals with a diverse spectrum of biological activity [4].

Preparation of mushroom extracts

The mushroom fruiting bodies were air-dried and then pulverized using a mortar and pestle. Two hundred grams of pulverized *C. cibarius* and *B. edulis*, respectively were extracted with 3,000 mL of 80% methanol and neutral saline (0.9% NaCl) with stirring. Hot water extract was prepared by boiling 200g of pulverized mushroom sample in 3,000 mL deionized water at 1000C for 3 hours. Final methanol, saline and hot water extracts were prepared as described by Cha., *et al* [5]. Supernatant and ethanol precipitates were rotary- evaporatively dried at 400C. The dried samples were reconstituted with distilled water at the rate of 10, 100, 1,000 and 2,000 ug/mL, respectively as extract concentration treatments.

Cells and cell culture

Four cancer cell lines namely U87 glioblastoma, A172 glioblastoma, CH157-MN meningioma and PAN-C1 pancreatic cancer and one NIH3T3 fibroblast cell line were obtained from commercial source and used for the cell viability and proliferation assays. The cells were maintained in 1X high glucose DMEM (obtained from Life Technologies) supplemented with 5% fetal bovine serum(obtained from Atlas Biologicals) for CH157-MN and 10% fetal bovine serum for all other cell lines and 1X anti-biotic/anti-mycotic (obtained from Corning Celgro).

WST-8 assay, a modification of MTT assay, is colorimetric, non-radioactive assay for assessing cell viability and proliferation (Sigma-Aldrich Manual and Protocol). WST-8 assay kits are available commercially. It is used to determine mitochondrial dehydrogenase activities in living cells (CK40). Tetrazolium salt (WST-8), is reduced by NADH produced in the mitochondria to a soluble purple formazan which dissolved directly into the culture medium. Cells were plated into 96 well plates and allowed to attach overnight before adding the extract. Cells were incubated with the extracts for one, two or three days and assays were performed according to the supplier's (Dojindo Molecular Technologies) instructions. Pilot studies indicated that the extracts themselves had reducing activity, so immediately before adding the substrate the media with extract was replaced with fresh media. The optical absorbance was measured at 450 nm on a BioTek Synergy microplate reader (BioTek, Winooski, VT). Relative live cells were tabulated from the absorbance data [6].

Crystal violet assay (BioVision Manual and Protocol) was used to determine the viability of the cultured cells basing on the staining of the nuclei of viable. Crystal violet assay kits are available commercially. Cells that undergo cell death lose their adherence and are subsequently lost from the population of cells, reducing the amount of crystal violet staining in a culture. The attached cells are stained with crystal violet dye, which binds to proteins and DNA [7]. Crystal violet staining was carried out on the same plates for which WST-8 assay was done. Crystal violet stain was made by adding 5g crystal violet (Thermo-Fisher Scientific) to 50 mL 95% EtOH and then diluting to 1000 mL using distilled water. A 1% glutaraldehyde was made from concentrated glutaraldehyde (Ladd) diluted with 1X PBS (Life Technologies). Following the WST-8 assay, culture medium was removed and cells were fixed with 100 µL/well 1% glutaraldehyde for 15 minutes. The glutaraldehyde was removed and 100 µL of crystal violet was added and allowed to stain for 15 minutes. The crystal violet solution was removed by aspirating. The plates were then rinsed by submerging under running water until residual stain was removed then allowed to dry upside down overnight. Once the plates were dried, 200 µL of 1% SDS was added to each well to solubilize the crystal violet. The optical density was read at 590 nm on a Synergy HT plate reader (BioTek) and then converted into relative number of live cells.

CyQUANT cell proliferation assay (Thermo-Fisher Scientific Manual and protocol) is based on the staining of DNA content which remains constant for a given cell line or cell type. CyQUANT cell proliferation assay kits are available commercially (Thermo-Fisher Scientific). Cell proliferation assays were preformed according to the manufacturer's (Life Technologies) protocol to quantify cells and examine effects on cell proliferation and cytotoxicity through a highly sensitive fluorescence based method. DNA content can be directly quantified between 50 and 50,000 cells per well without relying on metabolic activity that may be influenced by factors besides cell numbers. The major component of the CyQUANT cell proliferation kit is a proprietary dye that has strong fluorescence enhancement when bound to nucleic acids. Fluorescence at excitation 480 nm and emission 520 nm was measured on a BioTek Synergy microplate reader. Fluorescence data were converted to relative number of live cells.

Polycaspase Assay is used for determining apoptosis. Polycaspase assay kits are available commercially. Apoptosis is an evolutionarily conserved form of cell suicide which is mediated by events of proteolytic enzymes called caspases. Pro-apoptotic signal triggers the caspase enzymatic cascade leading in the cleavage of protein substrates, which results to the disassembly of the cells. Apoptosis was detected with the FAM FLICA Polycaspase assay kit (ImmunoChemistry Technologies, Bloomington, MN) with the green fluorescent inhibitor probe FAM-VAD-FMK that labels active caspase enzymes in living cells. Cells were cultured on phenol red-free media (Corning Cellgro DMEM) in T25 flasks. Cells at about 90% confluence were tested with various treatments. Staurosporine (6 µM) (ImmunoChemistry Technologies) was used as a positive control. At 0.5, 1, 2 and 4 HAT, floating cells with media were collected in a 15-mL disposable centrifuge tube and centrifuged at 5000g for 5 minutes at room temperature. After discarding the supernatant, cells were mixed with 600 µL of 1x apoptosis wash buffer. Remaining adhered cells on the flask were lifted with trypsin, centrifuged, and mixed with the previously collected cell suspension after discarding the supernatant. FAM-FLICA poly caspase inhibitor reagent (1x) was mixed with 500 µL cell suspension and incubated at 37°C for 1h with intermittent shaking. During this incubation period, a portion of the remaining cells was used for counting the cells with a hemocytometer with trypan blue (0.04%). After incubation, 2 mL wash buffer was added to cells, centrifuged, and supernatant was discarded. Again 2 mL of wash buffer was added to cells and incubated at 37°C for 10 minutes to remove excess FAM-FLICA reagent. Cells were centrifuged and the supernatant was discarded. Finally, cells were suspended in 500

µL wash buffer and kept on ice. In total, 100 µL cell suspension was used for estimating poly-caspase activity in 96-well flat black-bottom plates. Fluorescence was measured in a BioTek Synergy HT microplate reader at 488/520 nm (ex/em), and the measured RFU values were normalized to total number of cells.

Results and Discussion

Figures 1-5 show the effect of combined methanol/hot and saline water extracts od *Cantharellus cibarius* on four human cancer cell lines (A172 Glioblastoma, U87 glioblastoma, Pan-C1 and CH157-MN meningioma) and one mouse fibroblast cell line (NIH3T3) using WST-8 cell proliferation assay. Error bars show standard errors (n = 8). There were some variant results. Overall, the number of viable cells decreased with increasing doses of extract as the treatment time increased. Higher concentrations, particularly at 1,000 and 2,000 ug/mL of *Cantharellus cibarius* resulted in significant reduction of viable cells for all cell lines tested. Such reduction could be attributed to the presence of beta-glucans and phenolic compounds [3] which exhibited anti-oxidative activity [8] and the suppression of angiogenesis [9]. The cell viability assay results were similar to that of the crystal violet assay (data not shown) except that the effects were not apparent after one day of treatment.

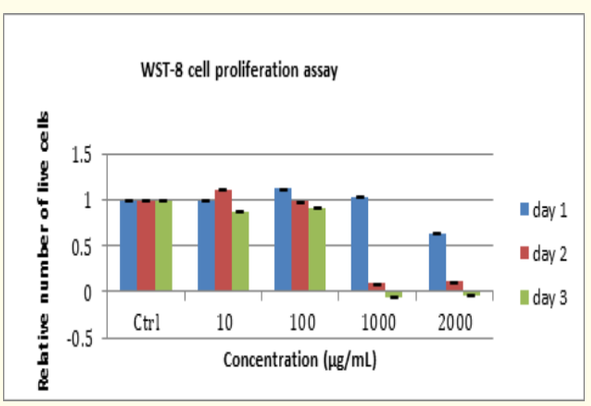

Figure 1: Effect of *C. cibarius* extracts on cell viability of A172 glioblastoma cells.

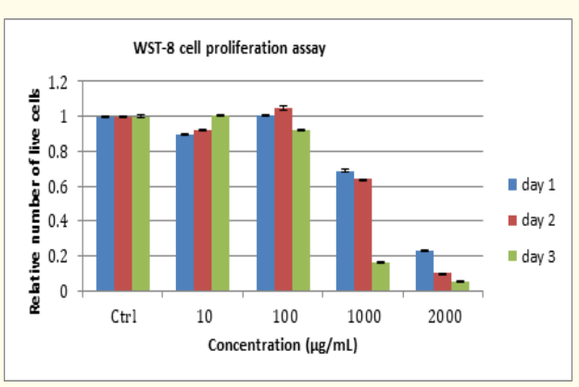

Figure 2: Effect of *C. cibarius* extracts on cell viability of U87 glioblastoma cells.

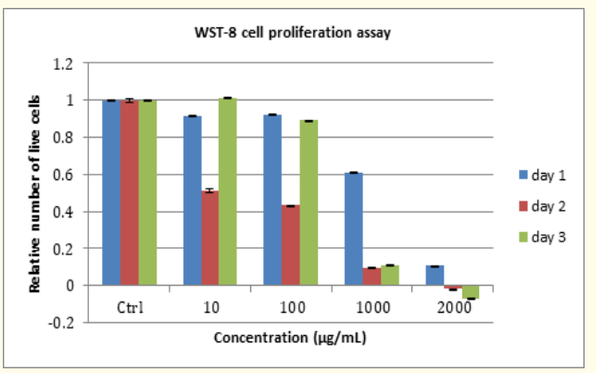

Figure 3: Effect of *C. cibarius* extract on cell viability of PAN-C1 pancreatic cells.

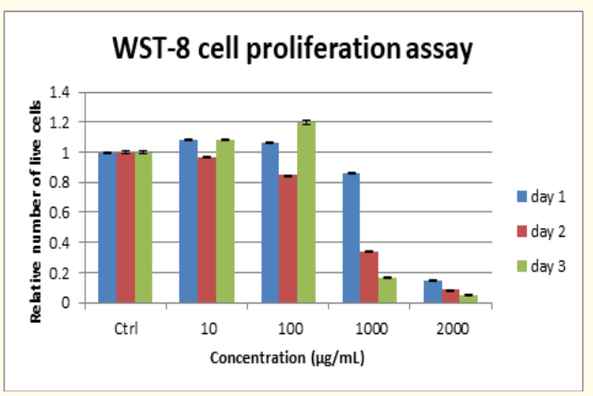

Figure 4: Effect of *C. cibarius* extracts on cell viability of CH157-MN meningioma cells.

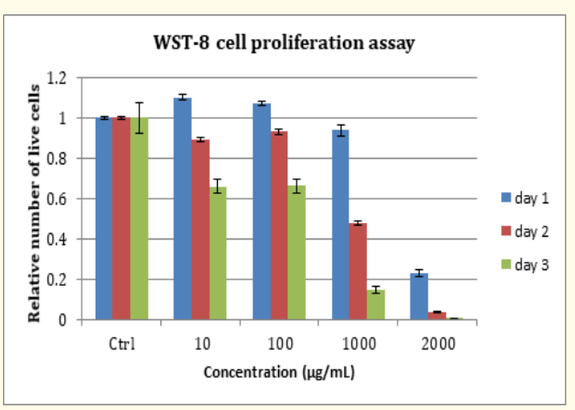

Figure 5: Effect of *C. cibarius* extracts on cell viability of NIH3T3 fibroblast cells.

WST-8 cell proliferation assay was used for assessing the effectiveness of the hot water and neutral saline water extracts on the cell viability of three cancer cell lines (A172 glioblastoma, U87 glioblastoma and PAN-C1). Figures 6 and 7 show the effect of hot water and neutral saline water extracts on the viability of the A172 glioblastoma cells, respectively. Figures 8 and 9 show the effect of hot water and neutral saline water extracts on the viability of the U87 glioblastoma cells, respectively. Figures 10 and 11 show the

effect of hot water and neutral saline water extracts on the viability of the PAN-C-1 pancreatic cells, respectively. Error bars show standard errors (n = 8). For both the neutral saline and hot water extracts, the numbers of viable cells were significantly reduced by the increasing doses over a longer treatment period. However, the neutral saline extracts appeared to be less effective in reducing the viability of the cell lines tested. The cell viability test results were similar to that of crystal violet assay. The reduction in cell viability might be due to the different efficiency of the extraction by hot water and neutral saline water, respectively. The reduction in cell viability by *Boletus edulis* extract might be attributable to a variety of organic compounds that have wide spectrum of biological activities. There is a need for chemical analysis to ascertain the presence of beta-glucans in *Boletus edulis* species.

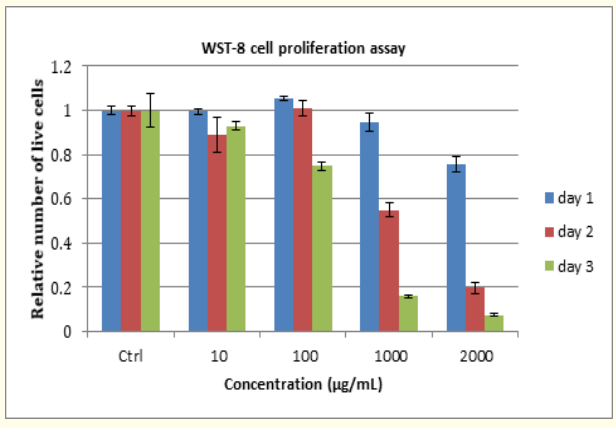

Figure 9: Effect of neutral saline water extracts of *B. edulis* on U87 glioblastoma cells.

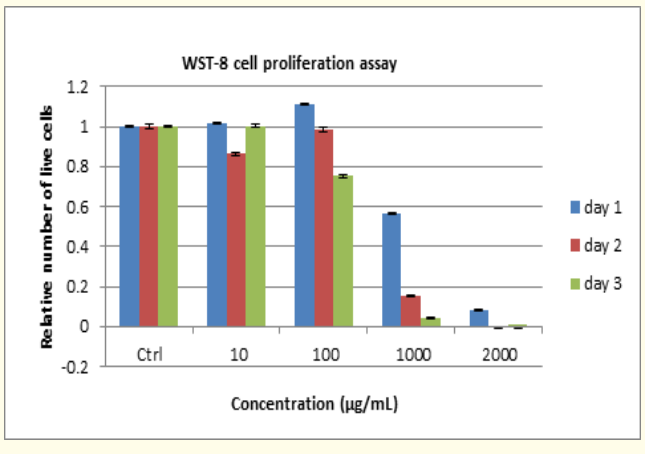

Figure 6: Effect of hot water extract of *B. edulis* on A172 glioblastoma cells.

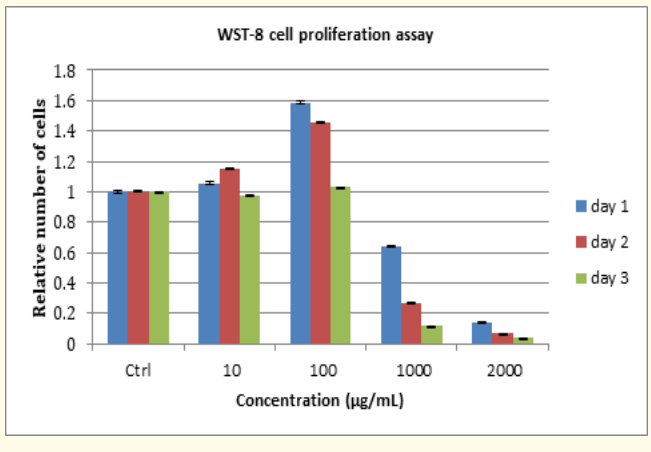

Figure 10: Effect of hot water extracts of *B. edulis* on PAN-C1 pancreatic cells.

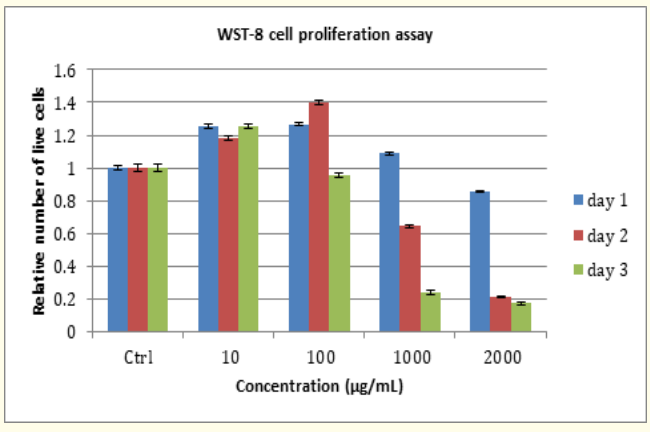

Figure 7: Effect of saline water extract of *B. edulis* on A172 glioblastoma cells.

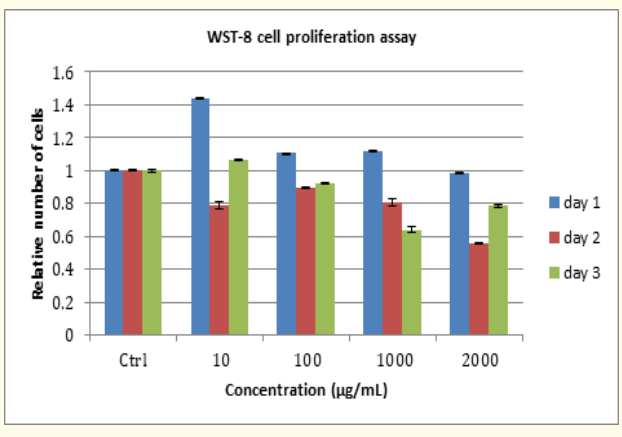

Figure 11: Effect of neutral saline water extracts of *B. edulis* on PAN-C1 pancreatic cells.

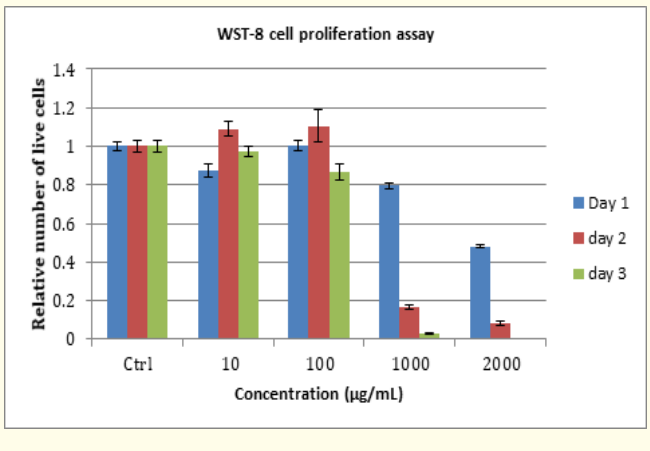

Figure 8: Effect of hot water extracts of *B. edulis* on U87 glioblastoma cells.

In the comparison among three cell proliferation assays on cell viability of Ch157 glioblastoma by *Cantharellus cibarius* extract, similar results were obtained for all assay methods used (Figures 12-14). Error bars show standard errors (n = 6).

To verify that the extracts of these two wild-collected mushrooms played a role in causing cancer cell death (apoptosis), caspase activation assay on Ch157 glioblastoma cells. Caspase activation is the signature of apoptosis. Staurosporine (a known positive activator of caspase) at 6 uM and *Cantharellus cibarius* extract at 2,000 ug/mL were used and compared in the evaluation. Figure 15 shows the results of the assay. *Cantharellus cibarius* extract produced 0.002 activated caspase while Staurosporine produced 0.0028 activated caspase. These results appear to support the hy-

pothesis that the data of the reduced cell viability of this study are likely to account for some of the apoptosis of cancer cells.

Figure 12: WST-8 assay of *C. cibarius* extract on CH157-MN meningioma cells.

Figure 13: Crystal violet assay of *C. cibarius* extract on CH157-MN meningioma cells.

Figure 14: CyQUANT assay of C. cibarius extract on CH157-MN meningioma cells.

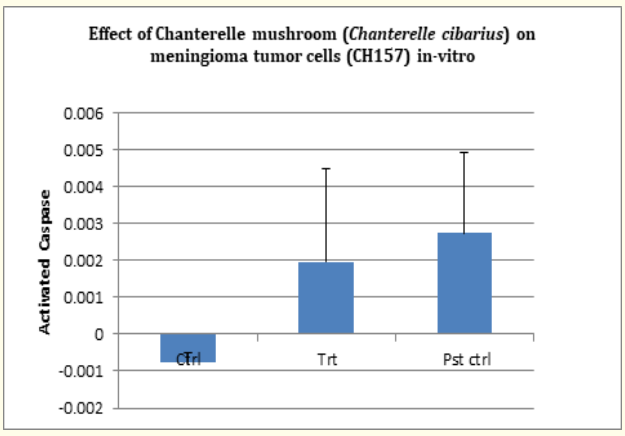

Figure 15: Effect of *C. cibarius* extract and Staurosporine on the Activated Caspase Relative Fluorescence Units. (ctrl: control; Trt: 2,000 ug/ML C. cibarius extract and Pst ctrl = 6uM Staurosporine)

Conclusion

In this study, based on the assays by WST-8, Crystal Violet and CyQUANT, there were some variant results on cancer cell viability treated with the extracts of *Cantharellus cibarius* and *Boletus edulis*. However, all cancer cell lines (U87 glioblastoma, A172 Glioblastoma, CH157-MN meningioma, PAN-C1) and NIH3T3 fibroblast treated with high concentrations of mushroom extracts over a longer period (3 days) of incubation exhibited a significant reduction in the number of viable cells. The results of this study have demonstrated that the extracts of *Cantharellus cibarius* and *Boletus edulis* can reduce cancer cell viability *in vitro*. The demonstrated potency of these mushrooms is probably due to their chemical constituents which have not been determined qualitatively and quantitatively. Their medicinal properties as well as anti-cancer effect on cancer cells remain doubtful and inconclusive. These two mushroom species can currently be used as a culinary food that exhibit some potential health benefits. Further *in vivo* and clinical studies are needed to support such findings.

Acknowledgement

This research work was funded by USDA/NIFA project # 2013-38821-21386.

Bibliography

1. Wasser S. "Medicinal mushrooms as a source of anti-tumor and immunomodulating polysaccharides". *Applied Microbiology and Biotechnology* 60.3 (2002): 258-274.

2. Chang ST and SP Wasser. "The role of culinary-medicinal mushrooms on human welfare with a pyramid model for human health". *International Journal of Medicinal Mushrooms* 14.2 (2012): 95-134.

3. Kolundžić M., *et al*. "Cytotoxic and Antimicrobial Activities of *Cantharellus cibarius* Fr. (Cantarellaceae)". *Journal of Medicinal Food* 20.8 (2017): 790-796.

4. Ribeiro R., *et al*. "Comparative study of phytochemicals and antioxidant potential of wild edible mushroom caps and stipes". *Food Chemistry* 110.1 (2008): 47-56.

5. Cha YJ., *et al*. "Anticancer and Immunopotentiating Activities of Crude Polysaccharides from Pleurotus nebrodensis on Mouse Sarcoma 180". *Mycology* 40.4 (2012): 236-243.

6. CK40: Cell counting kit-8, Technical manual. Dojindo.com.

7. Feoktistova M., *et al*. "Crystal violet assay for determining viability of cultured cells". *Cold Spring Harbor Protocol* 16.4 (2016).

8. Wang J., *et al*. "Antioxidant activity of leaf extracts from different Hibiscus sabdariffa accessions and simultaneous determination of five major antioxidant compounds by LC-Q-TOF-MS". *Molecules* 19.12 (2014): 21226-21238.

9. Kao Chi HJ., *et al*. "Anti-cancer activities of Ganoderma lucidum: Active ingredients and pathways". *Functional Foods in Health and Disease* 3.2 (2013): 48-65.

Volume 2 Issue 5 May 2018