ASSESSING THE GENOTOXIC AND CYTOTOXIC RESPONSES OF THE H-29 CANCER CELL LINES ON THE ETHANOLIC EXTRACTS OF THE OYSTER MUSHROOM, PLEUROTUS OSTREATUS VAR. FLORIDA

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ABSTRACT: Globally, colorectal cancer is the third most common known today. Lack of cheap, rapid, safe and reliable cancer treatments resulted to the continued pursuit to find natural sources of bioactive compounds that can be cytotoxic or damaging to the DNA of cancer cells. One of the potential resource is the edible oyster mushroom (Pleurotus ostreatus var Florida) reported to have medicinal properties. Since many of the studies did not show specific varieties of the species used for the evaluation specifically on the anti-tumor and anticancer properties of the species, this study was therefore conducted. To determine whether there are cytotoxic and genotoxic effects of the ethanolic extracts of this variety of the white oyster mushroom against HT-29 colon cancer cell lines by damaging the DNA of cancer cells, the Comet assay method was used. This assay made use of differences in the measurements calculated by Open Comet Software of the tail length, tail DNA, tail DNA percent and tail moment in untreated and IC50 ethanolic extract-treated cells. Results of the statistical analysis of the profiles of cells show significant differences between the untreated and treated HT-29 cells. This simply implies that the ethanolic extracts from P. ostreatus var. Florida had induced DNA damage to HT-29 cancer cell lines and that this variety of the species of P. ostreatus is a potential source of bioactive compounds important for the treatment of colorectal cancer.

INTRODUCTION: Colorectal cancer is the third most common cancer globally predicted to increase in the coming years 1,2. In the Philippines, colon cancer surpassed liver cancer in recent statistics making it the leading type of cancer that Filipinos suffer from 3. The applications of chemotherapy and radiation methods are commonly advised but these are also toxic to normal cells leading not only to more human health complications but also to their cancer-inducing properties 4. An efficient organic cancer drug therapy is being sought for thus encouraged researchers to continue to discover possible sources of natural products that are safer, cheaper and more reliable than synthetic drugs. Natural products from plants for example are gaining popularity as alternative ways to combat cancer. It is argued that many species provide reservoirs of natural chemicals without affecting normal cells and causing further complications 5.
Some of these are the edible mushrooms which are considered “the ultimate health food” not only because of their high nutritional value but also for their medicinal and therapeutic applications. Several studies have shown that these groups produce bioactive compounds that are not only excellent antioxidants but also as having a wide range of therapeutic effects. Most compounds act as immune-modulatory, anticarcinogenic, antibacterial and antiviral, anti-hypoglycaemic, antiatherosclerotic and anti-inflammatory agents. It is important to note however that different species of mushrooms vary in terms of the production of these compounds depending on the type of mushroom, substrate applied, cultivation and fruiting conditions, stage of development, age of the fresh mushroom, storage conditions, processing and cooking procedures.

One of the mushroom group of species that is very popularly used as food is the oyster mushroom (Pleurotus spp.) because of its taste, texture and unique aroma. This group is considered as good sources of dietary fiber and other valuable nutrients, antioxidant and other medicinal properties. One of the species of this group Pleurotus ostreatus is reported to contain a number of biologically active compounds. Many of these compounds have therapeutic activities such as modulating the immune system, inhibit tumor growth, anticancer, antitumor, inhibit tumor growth, lower blood lipid concentrations, prevent high blood pressure and atherosclerosis, and have antimicrobial and other activities.

These properties were extensive reviewed by Yashvant et al. What were observed from these studies however, were few studies involving the cytotoxicity and genotoxicity properties of specific varieties or subspecies of the different species of edible mushrooms such as Pleurotus ostreatus Fig. 1. This species has different varieties that are cultured and marketed. Since many studies that were conducted were not specifying what variety of the species were studied, this current study was conducted. The study focused on the Florida variety of P. ostreatus that is cultured and marketed in the Philippines.

The current study specifically assessed the genotoxic and cytotoxic properties of P. ostreatus var. Florida ethanolic extract to be able to understand the basis for many studies on the ethnomedicinal properties of this mushroom specifically on its anticancer properties. To be able to do this, we investigated the DNA damaging activity of the ethanolic extract of the mycelium of the mushroom against HT-29 colon cancer cell lines using comet assay and also evaluate its cytotoxicity using the Presto Blue Assay.

**FIG. 1: WHITE OYSTER MUSHROOM PLEUROTUS OSTREATUS FLORIDA**

**METHODOLOGY:** The protocol followed in this study is summarized in Fig. 2. The fungi samples of Pleurotus ostreatus were collected from a mushroom culture lab in Caloocan City, Philippines, and the fruit bodies were collected before or as the mushroom veils opened. After collection, the fungi samples were air-dried for 24 h, finely cut and 100 g was soaked in 1000 ml of 95% ethanol at room temperature for 48 h. This was then filtered and the ethanol removed through a rotary evaporator. The crude extract was further lyophilized to completely remove water from the sample.

The cellular responses of the HT-29 human colorectal adenocarcinoma cell lines obtained from Research and Biotechnology Division, St. Lukes Medical Center against the ethanolic extract was then evaluated. These cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) added with 10% fetal bovine serum (FBS), Penicillin-Streptomycin 1% (v/v) placed in a tissue culture flask and incubated in 5% CO₂ at 37 °C. A cell count was done with a 1:4 dilution factor. 50 µl of the cell suspension was added with 150 µl trypan blue. Then, this was subjected to a hemocytometer and cell viability was determined.
The cells were then placed onto a 96-well plate and were incubated for 24 h in 5% CO₂ at 37 °C. The layout of the 96-well plate consisted of wells 2 from A to H having cells and the media (with Roswell Park Memorial Institute (RPMI) and 2% Fetal bovine serum (FBS). Wells 3-5 from A to H with cells diluted with the Pleurotus ostreatus extract in a 5-fold 10% DMSO dilution and the media. Wells 6-8 from A to H containing 100% DMSO and the cells diluted in 5-fold. For the negative control, wells 1 from A to H are placed with medium only. The 96-well plate was incubated for 24 h at 37 °C. In order to check the absorbance and IC₅₀, the concentration that kills 50% of the cancer cells, of the wells, a microplate reader (Synergy H4 Hybrid Reader) and a microplate software (Biotek Gen5 Data Analysis Software) were used. Prior to this, the incubated 96-well were washed and aspirated with new media, and placed with PrestoBlue for cell viability testing.


The value of IC₅₀ obtained from the cytotoxicity experiment was used as the concentration of the extract in the genotoxicity. Two sets of flasks of each cell line were subcultured where the first flask was treated with the extract and the other flask was left untreated. The flasks were incubated for 24 h at 36 °C with 5% CO₂. After the incubation period, the cells were subjected to trypsinization to eradicate its adherence to the flasks. The cell suspensions were centrifuged at 1500 rpm for 7 min at room temperature. The supernatant was removed, and the cells (pellet) were washed with RPMI 1640 medium. Centrifugation of the cell suspension at 1500 rpm for 7 min at room temperature was done. The cells were then resuspended with ice-cold 1X PBS. The Comet assay was performed under semi-alkaline conditions based on the protocol of Comet Assay™ Kit from SBYR Green Trevigen Inc.
The working lysis solution was prepared and cooled in Coplin Jar at 4 °C 20 min before the experiment proper. Low melting point agarose (LMA), with its cap loosen, was placed in a beaker with boiling water for 5 min. The bottle was then placed in a 37 °C water bath for 20 min. This part of the experiment was crucial because the temperature of the agarose for the cells was controlled sensitively. The alkaline solution was done by combining 0.6 g of NaOH pellets, 250 µl of 200 mM EDTA, and 49.75 ml distilled H2O. Using the tip of the pipette, 50 µl of the solution was immediately pipette onto the Comet Assay Slide ensuring the whole sample area was covered. The slides were placed in refrigerator for 10 min.

The slides were immersed in the prepared 4 °C lysis solution for 60 min. Slides were then submerged in Alkaline Unwinding Solution at room temperature for 20 min. After lysis and unwinding, the comet slides were placed in an electrophoresis tank filled with TBE buffer. The electrophoresis was set to 21 V for 30 min. Subsequently, the slides were immersed twice in distilled water for 5 min. The final rinse was with 70% ethanol for 5 min. The slides were stored in a storage unit. 100 µl of diluted SYBR® Green was placed onto each well of dried agarose. The slides were then incubated for 24 h. The slides were captured and viewed using fluorescence microscope with magnification of 10x. A minimum of four images were captured, with no overlapping capture areas. The tail length, tail DNA%, and tail moment were analyzed using the Open Comet plugin for the Image J software.

Usable cells were automatically selected from the images Average DNA damage results were calculated from 50 cells selected at random. Calculation for tail moment was used to measure the severity of damage by combining amount of DNA in the tail with the distance of migration. Mean values for each measurement generated standard error of the mean (SEM) and was calculated from the standard deviation. Data were graphically presented as box and whiskers plots, scatter plot and classification matrix to show differences between treatments.

**FIG. 3: COMET ANALYSIS SHOWING THE MEASUREMENT MADE ON TAIL LENGTH AND TAIL MOMENT (A) OVERLAPPING CELLS (B) WERE EXCLUDED FROM THE ANALYSIS**

**RESULTS AND DISCUSSION:** The result of the determination of IC50 (mg/ml) of the *P. ostreatus* ethanolic extract obtained through PrestoBlue assay and used as basis for genotoxicity analysis of HT-29 colon cancer cell lines through Comet Assay is presented in **Fig. 4 and Table 1.** The IC50 value was the inhibitory concentration at which 50 percent of the HT-29 colon cancer adenocarcinoma cell lines were terminated using the *P. ostreatus* extract. The IC50 value was used for the genotoxicity evaluation.

**Table 1: IC50 of the Ethanolic Extract Based on the Result of PrestoBlue Assay**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IC50 (mg/ml)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. ostreatus</em> ethanolic extract</td>
<td>164 mg/ml</td>
<td>610 nm</td>
</tr>
</tbody>
</table>

**FIG. 4: THE GRAPH ABOVE SHOWS THE CORRECTED / NORMALIZED VALUES OF THE ABSORBANCE PER WELL. PLOTTED ON THE GRAPH ARE THE CORRECTED AVERAGE VALUES PER ROW OF EACH SET-UP OF CONCENTRATION**
The Comet assay results to determine if the ethanolic extract could induce DNA damage and fragmentation of HT-29 colon cancer cell lines, the tail length, tail DNA, tail moment measured through the use of the Comet software are shown in Table 2, Fig. 5 and 6.

**TABLE 2. COMET ASSAY SHOWING THE TAIL LENGTH, TAIL DNA, AVERAGE TAIL DNA PERCENT AND TAIL MOMENT BETWEEN TREATED AND UNTREATED HT-29 COLON CANCER ADENOCARCINOMA CELL LINES**

<table>
<thead>
<tr>
<th>Group</th>
<th>Average tail length</th>
<th>Average tail DNA</th>
<th>Average tail DNA%</th>
<th>Average tail moment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture media only (untreated)</td>
<td>16.40</td>
<td>25172.20</td>
<td>22.77</td>
<td>4.23</td>
</tr>
<tr>
<td><em>P. ostreatus</em> ethanolic extract</td>
<td>51.38</td>
<td>39521.86</td>
<td>36.91</td>
<td>20.20</td>
</tr>
</tbody>
</table>

*the blue shaded area indicates the set-up for the fungi extract*

**FIG. 5: IMAGES (a-d) OF TREATED HT-29 COLON CANCER ADENOCARCINOMA CELLS WITH *P. OSTREATUS* FLORIDA ETHANOLIC EXTRACTS (GREY OUTLINES SIGNIFY CELLS THAT HAVE BEEN DECIDED UNUSABLE BY THE SOFTWARE. THOSE WITH RED OUTLINES HAVE BEEN UTILIZED TO CALCULATE THE MEAN DATA. RED NUMBERS TAG THE CELL FOR IDENTIFICATION IN THE RAW DATA. THE ELONGATED CELLS SHOW THAT DAMAGE WAS DONE TO THE HT-29 CELLS BY THE EXTRACT)*

**FIG. 6: IMAGES (a-d) OF UNTREATED HT-29 COLON CANCER ADENOCARCINOMA CELLS (GREY OUTLINES SIGNIFY CELLS THAT HAVE BEEN DECIDED UNUSABLE BY THE SOFTWARE. THOSE WITH RED OUTLINES HAVE BEEN UTILIZED TO CALCULATE THE MEAN DATA. RED NUMBERS TAG THE CELL FOR IDENTIFICATION IN THE RAW DATA. THE ELONGATED CELLS SHOW THAT DAMAGE WAS DONE TO THE HT-29 CELLS BY THE EXTRACT)*
Comparison between treated and untreated HT-29 colon cancer adenocarcinoma cell lines based on tail length, tail DNA, average tail DNA percent and tail moment subjected to CVA analysis show significant differences Table 3. The differences are graphically demonstrated in Fig. 7 and 8.

**TABLE 3: COMPARISON BETWEEN TREATED AND UNTREATED HT-29 COLON CANCER CELL LINE (PILLAI TRACE: 0.5496, P=9.673E-16)**

<table>
<thead>
<tr>
<th></th>
<th>Treated</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated</td>
<td>9.6731E-16</td>
<td>-</td>
</tr>
<tr>
<td>Untreated</td>
<td>-</td>
<td>9.6731E-16</td>
</tr>
</tbody>
</table>

This current study showed similar results generated in a study conducted by Gu et al., the crude oyster extract has a cytotoxic effect on PC-3 cells and the aqueous polysaccharide extract has anti-proliferative and pro-apoptotic effects on HT-29 cells. Mohamed and Mizuno in their study showed the presence of bioactive compounds in P. ostreatus that are responsible not only for the reduction / prevention of hypertension, high cholesterol, anti-atherosclerosis, anti-viral and anti-thrombotic and immunomodulatory, but also anticancer. The polysaccharides, terpenoids, fatty acids, amino acids, steroids, and phenols found in P. ostreatus are considered to be anticancer agents along with its glycoproteins such as lectins which are also responsible for anticancer and immune-stimulating activities.

Lavi and Friesem et al., show the anti-proliferative and pro-apoptotic activities of fractions from P. ostreatus against HT-29 colon cancer cells. Lavin et al., claimed from their study that P. ostreatus aqueous polysaccharide extract exhibited anti-proliferative and pro-apoptotic effect on HT-29 colon cancer cells. Jedinak and Sliva et al., also show in their study that methanolic extracts of P. ostreatus subdued the proliferation of breast cancer (MCF-7, MDA-MB-231) and colon cancer (HT-29, HCT-116) cells, without affecting the normal human epithelial mammary MCF-10A and normal colon FHC cells.

Ekowati et al., showed extracts from P. ostreatus inhibited the growth of Helacyton gartleri cells, making it cytotoxic to cervical cancer cells. The high cytotoxicity of the ethanolic extract against HT-29 colon cancer adenocarcinoma cell lines may also be attributed to the presence of selenium in P. ostreatus. Selenium in mushrooms is reported to be capable of reducing or affecting the metabolic activity of the cells by promoting DNA oxidation that leads to DNA strand breaks and in low concentrations possess anticarcinogenic properties while in higher concentrations may cause death of the cells. These may also explain the results generated in the P. ostreatus var Florida.

While these published reports on this species of mushrooms dwell on the anticancer activities of...
various extracts, it did not specify the exact variety of the species used. While it is argued that many or almost all organisms differ genetically especially on the variations of the production of metabolic compounds produced, the current study which explored if the Florida variety of *P. ostreatus* also show anticancer properties was no different from the abovementioned studies. This means that any variety of *P. ostreatus* will have similar anticancer bioactivity.

CONCLUSION: Results of the study show that the cytotoxic activity of the ethanolic extracts by the *P. ostreatus* var. Florida could explain the induced DNA damage to HT-29 colon cancer cell lines as shown by the differences of the presence of tail DNA, tail length and tail moment between the treated and untreated cells. The percent DNA damage obtained by the *P. ostreatus* var. Florida-treated cells indicate that the extract is highly genotoxic to HT-29 colon cancer cell lines. This study generally confirms previous investigations on *P. ostreatus* tested against other carcinoma cells. This study therefore clearly shows the potential of *P. ostreatus* Florida variety as source of bioactive compounds for use as anticancer agents.

ACKNOWLEDGEMENT: Nil

CONFLICT OF INTEREST: Nil

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6. King TA: Mushrooms, the ultimate health food but little research in U. S to prove it. Mush News 1993; 41: 29-46.


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