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Cytotoxic and Genotoxic Activity of an extract from the mushroom *Lenzites betulina* against K562 leukemia cells

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Abstract: The cytotoxic and genotoxic activity of a water-insoluble polysaccharide extract from the mushroom *Lenzites betulina* against K562 leukemia cells was evaluated in this study. The PrestoBlue™ Assay was used to determine the cytotoxicity level by identifying the IC50, while the comet assay was used to measure the genotoxicity of the extract. Results show that the extract has a low IC50 level of 0.07533 ug/ul, indicating a strong cytotoxic effect. Comet assay values also showed significant differences between treated and untreated cells, indicating possible genotoxic effects. These data provide an initial evaluation of the possibility of the extract as an anticancer agent against K562 leukemia cells.

Key Words: *Lenzites betulina*; cytotoxicity; genotoxicity; K562 cell line

1. INTRODUCTION

Natural products are characterized and isolated for their potential as novel cancer preventive and therapeutic agents. There is increasing evidence for the potential of natural products as inhibitors of various stages of tumorigenesis and associated inflammatory processes, underlining the importance of these products in cancer prevention and therapy (Solowey et.al., 2014). It has been discovered that many mushroom species produce a large number of novel compounds with active biological properties, including anticancer activity. These anti-cancer compounds play crucial roles in the inhibition of progression of cancer cells. Polysaccharides are among the bioactive compounds isolated from different species of mushrooms. These are the best known and most potent mushroom-derived substances with anti-tumor and immunomodulating properties (Patel and Goyal, 2012).

One of the interesting mushrooms which may exhibit anti-cancer capabilities is the species *Lenzites betulina*, more commonly known as the birch mazegill. It gets its name from its stark white gills and its partial affinity to birch trees (Kuo, 2005). Several bioactive compounds have already been isolated from this species of fungi. These include benzoquinone compounds, lignin peroxidases and sterols (Lee et.al., 1996; Yadav et.al., 2010; Liu et.al., 2014).

The determination of cytotoxic and genotoxic levels are important initial steps in characterization of the active compounds in natural products. For in vitro cell culture systems, a compound of interest is considered to be cytotoxic if it interferes with cellular attachment, significantly alters morphology,

adversely affects cell growth rate, or causes cell death (Niles et.al., 2008). The determination of IC50 is one of the most common measures of cytotoxicity. IC50 is defined as half maximal inhibitory concentration, or the concentration of an active compound that produces 50% of its maximal inhibitory effect (Neubig, et.al., 2003). The PrestoBlue™ assay is one of the methods to determine the IC50 of a compound. It compares gradient concentrations of the active compound versus the viability of cells within a certain time of exposure to the compound.

DNA is one of the main targets of anti-cancer therapies because DNA replication is an essential phase of the cell cycle. Many of the agents commonly used to treat cancer patients cause high levels of DNA damage, initiate cell cycle checkpoints, and then leading to cell cycle arrest and/or cell death (Swift and Golsteyn, 2014). This is known as the genotoxic pathway for anti-cancer treatment. Genotoxicity is the property of chemical agents that damages DNA within a cell, eventually causing mutations. The single cell – gel electrophoresis assay (also known as the comet assay) is a simple, rapid and sensitive technique for analyzing and quantifying DNA damage in individual mammalian cells (Ostling & Johanson, 1984). The images obtained from cells looks like "comets" with distinct heads, comprising of intact DNA and tails, consisting of damaged or broken pieces of DNA, hence the name comet assay.

2. METHODOLOGY

2.1 Cell Recovery

K562 leukemia cells were retrieved from liquid nitrogen storage. Cells were thawed, washed and resuspended in complete RPMI 1640 media. The cells were transferred to a tissue culture flask and were stored in a 37°C incubator with 5% CO_2 for 2-3 days. Separate flasks were used assigned for cytotoxicity and genotoxicity.

2.2 Sample Preparation

The dried extract from *Lenzites betulina* was acquired from the Chemistry Department of DLSU-Manila. 80% DMSO in RPMI 1640 was repeatedly added to 100mg of the extract until it was completely dissolved. Serial dilution in RPMI 1640 was then performed to determine the concentration gradients to be used.

2.3 Cytotoxicity Assay

Prior to treatment, spent media in the wells was removed to eliminate unattached cells and debris. 100ul of the serially diluted extracts were placed in the corresponding wells. Three replicates were conducted for each concentration, including blank and untreated wells. The plate was incubated again 37°C incubator with 5% CO_2 for 24 hours.

The cell viability assay was performed following the PrestoBlue™ manufacturer's instructions with a few custom modifications to optimize the assay. After 24 hours of incubation, 10ul of the PrestoBlue™ reagent was added directly to the cells in each well and was incubated again for 10 minutes. The plate was then placed in a fluorescence reader and readings were taken at 560 / 590 excitation and emission wavelengths. Readings after 10minutes, 30 minutes, 1 hour and 2 hours, respectively were taken to observe possible differences in sensitivity. Calculations for IC50 and % cytotoxicity were performed using the GraphPad Prisma™ software.

2.4 Genotoxicity Assay

Extracts were added to the K562 cell cultures and were incubated for 24 hours at 37°C with 5% CO_2. A control set-up with no extract added was also prepared. After 24 hours of incubation, cells were trypsinized to remove adherence to the flasks. Cell suspensions were centrifuged at 1200rpm for 10 minutes at room temperature. The supernatant was removed, then cells were washed with ice-cold 1x PBS. Suspensions were centrifuged again at 1200rpm for 10 minutes at room temperature. Cells were finally resuspended with ice-cold 1X PBS.

The protocol for the comet assay was performed based on the product insert of the CometAssay™ Kit from Trevigen Inc. The working lysis solution was pre-cooled at 4°C 20 minutes before use. The alkaline solution was prepared by combining 0.6g of NaOH pellets, 250µl of 200mM EDTA, and 49.75ml distilled water. Low melting point agarose (LMPA) was melted in a beaker of boiling water for 5 mins, and then allowed to cool in a 37°C water bath for 20 mins. The cell suspension was added to the LMPA in a microcentrifuge tube at a

ratio of 1:10. 50 µl of the resulting suspension was put in the CometSlide™. The slides were placed in a dark container and stored at 4°C for 10 mins. The slides were then immersed in the prechilled lysis solution for 30 mins at 4°C. After lysis, the slides were immersed in the alkaline solution in the dark for 20 minutes at room temperature.

The slides were placed in a Comet Assay ES apparatus containing 1x TBE buffer. The voltage was set at 21 volts, based on the distance between the two electrodes. The slides were subjected to electrophoresis for 30 minutes. The slides were then washed in distilled water, then in 70% ethanol for 5 minutes. The slides were air dried overnight. 100 µl of SYBR Green stain was added on each circle. The slides were viewed using a fluorescence microscope with a fluorescein isothiocyanate (FITC) filter. SYBR Green's maximum excitation and emission are 494nm / 521nm respectively. Comets were analyzed using the OpenComet™ software.

3. RESULTS AND DISCUSSION

The initial volume of RPMI 1640 in which complete dissolution of 100mg of the extract was observed at 10ml. The calculated initial concentration was at 20ug/ul. Three-fold serial dilution was performed and the calculated concentrations are shown in Table 1.

Table 1. Serial dilution concentrations for the *Lenzitus betulina* extract

Concentration (µg/µl)	Dilution (3-fold)	Concentration (µg/µl)	Dilution (3-fold)
20.000	original	0.00914	2187x
6.667	3x	0.00305	6561x
2.222	9x	0.00102	19683x
0.741	27x	0.00034	59049x
0.247	81x	0.00011	177147x
0.082	243x	0.00004	531441x
0.027	729x	0.00001	1594323x

Using the GraphPad Prism software, the readings taken were initially transformed into a logarithmic function of x and then normalized to obtain a standard curve for the IC50. These results are summarized in Table 2. Figure 1 shows the logarithmic graph for % cytotoxicity and the visual representation of the IC50 in the standard dose-response curve. The comparison of results between the four exposure times was calculated to have no significant difference with each other.

Table 2. IC50 and logIC50 results from the different exposure times

	10 mins	30 mins	1 hour	2 hours	Mean
log IC50	-1.169	-1.139	-1.106	-1.083	-1.124
IC50 (ug/ul)	0.06770	0.07269	0.07826	0.08268	0.07533

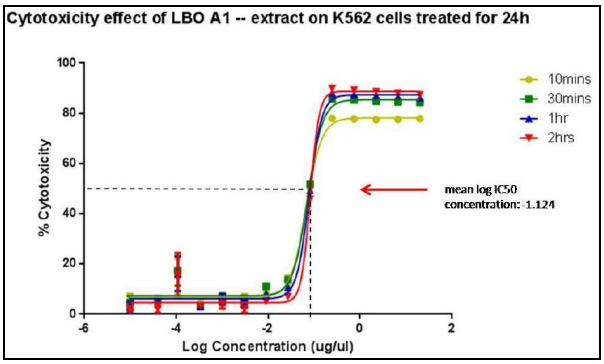

Fig. 1. Logarithmic graph of the cytotoxic effect of *Lenzites betulina* on K562 cells

For the genotoxicity assay, fifty (50) cells from each set-up were captured in images and analyzed. The tail length, % tail DNA and the tail moment were measured. Table 3 presents the results for the two set-ups. Statistical significance was calculated using the two-tailed Mann-Whitney U test. Figure 2 shows representative images from each of the set-ups.

Table 3. Comet assay parameters observed from the treated and control set-ups

	Mean Tail length (pixels)	Mean % DNA in tail	Mean Tail Moment
K562 treated with *Lenzites betulina*	18.86 ± 17.24	7.80 ± 8.53	2.82 ± 4.90
K562 control	14.00 ± 15.82	4.61 ± 7.01	1.69 ± 4.14
Statistical Significance	p value = 0.05238 (NOT SIGNIFICANT at p<0.05)	p value = 0.00736 (SIGNIFICANT at p<0.05)	p value = 0.01596 (SIGNIFICANT at p<0.05)

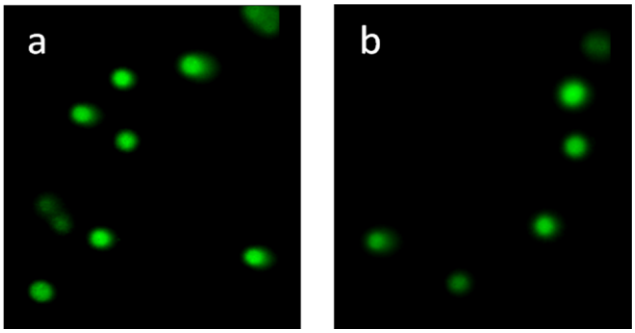

Fig. 2. Representative images of the K562 cells after comet assay. a) set-up treated with *Lenzites betulina* extract; b) control set-up

The cytotoxicity of the water soluble polysaccharide extract from *Lenzites betulina* on K562 cells was determined in this experiment. The IC50 was determined to be 0.07533 ug/ul of the extract. This is a relatively low IC50 value, so this implies that the extract has a strong cytotoxic effect on K562. The PrestoBlue™ assay proved to be an easy and rapid high throughput assay for determining the IC50 of the extract. We were able to assess the cytotoxicity of our extract in a relatively short period of time.

The results of our study showed significant differences in the % DNA in tail and the tail moment between the K562 cells treated by the extract from *Lenzites betulina* at $p<0.05$. However, there was no significant difference in the tail length between the two set-ups at $p<0.05$. Several inferences can be made from the results of our study. The tail length corresponds to the size of DNA fragments that migrated during electrophoresis. The relatively low values of the tail length means that the broken DNA fragments that were produced were large in size, so they were not able to move far away from the cell during electrophoresis. Also, since there was no significant difference from both treatments, it means that the DNA fragments produced in the treated set-up were relatively same in size as spontaneously produced fragments in the control set-up.

In the case of the % DNA in tail, it corresponds to the amount of fragmented DNA in the cell at the time of electrophoresis. The higher the % DNA in tail value, the more fragmented DNA fragments are contained in the cells. Since the %DNA in tail value of the set-up treated with the extract was significantly higher, it can be inferred that the extract produced more fragmented DNA pieces than there normally exists in K562 cells.

The tail moment is the product of the tail length and the %DNA in tail. It is an arbitrary value which describes the overall picture of the DNA fragmentation and damage in cells. In our study, the mean tail moment of the set-up treated with the extract from *Lenzites betulina* was significantly higher than that of the control set-up. This means that the overall DNA damage is higher in the set-up treated with *Lenzites betulina* than in the untreated set-up.

4. CONCLUSIONS

The results of our study showed that the polysaccharide extract from *Lenzites betulina* has significant cytotoxic and genotoxic effect on K562 cells. Even though the tail length values were not significant between set-ups, both the %DNA in tail and the tail moment values were significantly higher in the treated set-up. Since the tail moment gives a better picture of the DNA damage levels produced in the experiment, we can conclude that the extract causes DNA damage in K562 cells. Results of our study support the potential of using *Lenzites betulina* as an anticancer agent.

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