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Antioxidant activity and Cytotoxicity against the Cervical Epithelial Carcinoma (HeLa) Cell Line of Crude *Ganoderma lucidum* mycelial extracts

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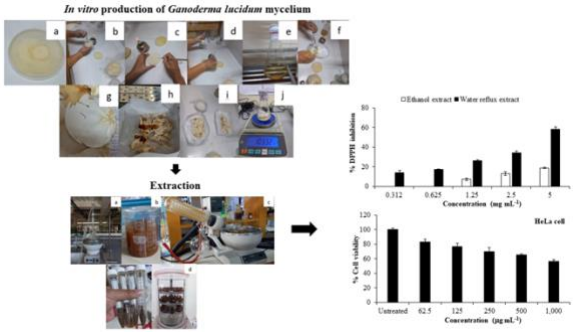
Abstract

This study aimed to investigate the total polysaccharide content, antioxidant activity and cytotoxicity on the Cervical Epithelial Carcinoma (HeLa) cells of the ethanol and water reflux extracts from *Ganoderma lucidum* mycelia. The phenol-sulfuric acid method was used for the standard method of total polysaccharide analysis, the antioxidant was evaluated by DPPH scavenging assay, ABTS assay, and FRAP assay. The MTT assay was used to determine the cytotoxicity of the extracts. The results showed that the contents of total polysaccharide of the ethanol and water reflux extracts were 18.34 ± 0.47 and 207.81 ± 2.45 mg glucose g extract^{-1}, respectively. The assays of antioxidant capacity against DPPH and ABTS demonstrated that the water reflux extract showed a strong radical scavenging effect than the ethanol extract. For the reducing capability of both extracts with FRAP method, they showed the reducing antioxidant activity and possessed concentration dependence by which the water reflux extract had a higher FRAP antioxidant activity than the ethanol extract. The cytotoxic assay of the extracts on normal mammalian cells (Vero cells) revealed that the ethanol extract of *Ganoderma lucidum* mycelia had a cytotoxic effect on normal mammalian cells at 62.5 – 1,000 mg mL^{-1}, whereas the water reflux extract had no cytotoxicity to normal mammalian cells in all tested concentration. Then, the water reflux extract was selected to determine the cytotoxicity against the HeLa cancer cell line. The results indicated that the water reflux extract of *Ganoderma lucidum* mycelia exerted a significant cytotoxic effect on HeLa cancer cell line in possessed concentration dependence. The water reflux extract caused a 56.30% decrease in cervical epithelial carcinoma cell viability with concentration of 1 mg mL^{-1}. Therefore, the results of this research demonstrated that the extract of *Ganoderma lucidum* mycelia provide both antioxidant and anti-cancer activities, which could be used as ingredients pharmaceutical products and functional foods.



Keywords: *Ganoderma lucidum*; Antioxidant activity; Cytotoxicity; Mycelial extracts

1. Introduction

Free radicals are the products of normal natural cellular metabolism, mostly as reactive oxygen species (ROS). Heart disease, neurodegenerative, cancer, and aging can be caused by an excess of ROS [1]. As a result, cellular antioxidant systems are capable of neutralizing free radicals when they are produced. However, the natural antioxidant system is unable to clear them when excessive oxidative stress emerges. In this regard, the discovery of novel natural products with antioxidant molecules have become interesting field of research due to their many benefits such as anti-cancer and anti-inflammation [2]. Antioxidants are essential molecules that have inhibit or quench a free radical-induced oxidative stress. It has been observed that the excessive production of ROS may result in carcinogenesis [3].

Cancer is a major disease burden worldwide. One of the most prevalent cancers found in Thai women is cervical cancer. The Human Papillomavirus (HPV) causes cervical cancer by causing warts in the throat and genital region. Anticancer drugs can cause a variety of side effects in humans, including hair loss, fatigue, and the amount of blood cells was decreased. Natural therapies are an effective tool for inhibiting cancer and reducing the negative effects of anticancer medications [4 – 5]. Several research have shown that natural resources have anticancer activity [6, 7].

Nowadays, mushrooms are becoming more popular due to their bioactivities in cancer prevention, and tumor suppression [8, 9]. *Ganoderma lucidum* (Reishi mushroom) is a medicinal mushroom with pharmacological and nutraceutical properties. Terpenoids, polysaccharides, phenolics, and fatty acids belong to the bioactive substances are found in this mushroom. These compounds have been related to *G. lucidum*'s neuroprotective, anti-diabetic, immunomodulatory, antitumor, antioxidant, hepatoprotective, anti-hypertensive, anti-cancer, and antibacterial properties [10 – 12]. Polysaccharides are the major pharmacologically active compounds in this mushroom [10].

Normally, this mushroom was grown with solid-state fermentation. The fruiting body takes six months to grow and varies in quality. However, submerged fermentation can be produced mushroom mycelia/biomass in a short-time. Furthermore, submerged fermentation enhances chemical extraction and purification, as well as increased biomass output by shortening growing time and decreasing contamination [13]. As a result, mycelia are a low-cost alternative and consistent source to fruit bodies. Mushroom products such as tablets and mushrooms extracts are available on the market owing to the potential health advantages to the human body. Because of restrictions on the use of synthetic antioxidants and anti-cancer, there is an increasing need for natural antioxidants and anti-cancer.

To our knowledge, there have been no research on the anti-cancer activities of *G. lucidum* mycelial extracts on cervical cancer (HeLa) cells was conducted in Thailand. Therefore, this study aimed to investigate both antioxidant activity and cytotoxicity on cervical cancer (HeLa) cells of the ethanol and water reflux extracts obtained from *G. lucidum* mycelia. We demonstrate that crude *G. lucidum* mycelial extracts have the ability to scavenge radicals and exerted a cytotoxic effect on HeLa cancer cells in a concentration-dependent manner.

2. Materials and Methods

Samples and in vitro mycelium production

G. lucidum was procured from Program of Microbiology, Faculty of Science, Ubon Ratchathani Rajabhat University. *G. lucidum* was grown on potato dextrose agar (PDA) for 7 days at 30 °C. Then, they were cultivated in Erlenmeyer flasks with 100 mL of potato dextrose broth (PDB) in each flask and incubated at room temperature for 28 days. Afterward, filtration was used to separate the culture and mycelia, and the mycelia were then dried in the oven.

Extraction

Ethanol extraction (Maceration); The mycelia powder was macerated in 95% ethanol for 24 h (Three times). After extraction, the extracted material was subsequently filtered using Whatman filter paper (No.1). The filtrate solution was evaporated at temperatures below 55 °C, obtaining a concentrated crude ethanol extract of *G. lucidum* mycelial extract.

Water reflux extraction (Hot water-ethanol precipitation); The water reflux extraction was carried out using the material-to-water ratio 1:35. The sample was refluxed for 120 min at 95°C, and then filtered. The filtrate was precipitated by 95% ethanol using the filtrate-to-95% ethanol ratio 1 : 4 for 24 h. After that, the mixture was centrifuged at 5,000 x g for 15 minutes. The precipitate was lyophilized using a freeze dryer to obtain crude water-soluble polysaccharides (Fig. 1).

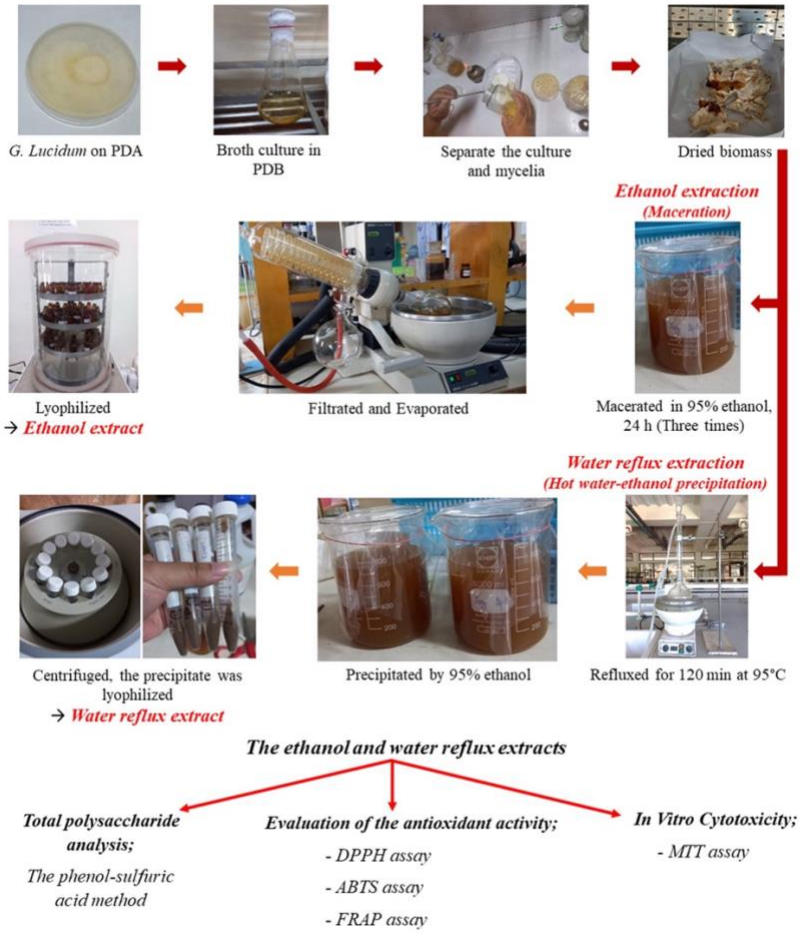


Fig. 1 *In vitro* mycelium production and extraction

Total polysaccharide analysis

The phenol-sulfuric acid method was used to evaluate the total polysaccharide content of *G. lucidum* mycelial extracts using D-glucose as the standard (glucose standard curve). Briefly, 150 µL of sulfuric acid was mixed with 50 µL of extracts, followed by adding 30 µL of 5% phenol. A reaction mixture was reacted for 5 minutes at 90 °C, and the absorbance at 470 nm was determined using a microplate reader. (EZ read 2000, Waterbeach Cambridge, United Kingdom).

DPPH assay

The DPPH assay was performed as described in Xiang et al. [14]. Briefly, 180 µL of 0.1 mM DPPH radical in methanol was added in to 96-well plate with 20 µL of mycelia extract of various concentrations and then incubated for 30 min at room temperature in the dark. The absorbance was performed at 517 nm with microplate reader (EZ read 2000, Waterbeach Cambridge, United Kingdom). The DPPH radical scavenging activity was calculated in a percentage inhibition following equation below:

Evaluation of the antioxidant activity

The percentage of inhibition (%) = $[(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})/ \text{Absorbance}_{\text{control}}] \times 100$

ABTS Scavenging Activity

The ABTS scavenging activity of mycelial extracts was modified from Kim et al. [15]. Briefly, 2.45 mM potassium persulfate and 7 mM ABTS were reacted at a ratio of 1 : 05 to create the ABTS radical solution ($ABTS^{\bullet+}$). The mixture was then stored for 16 h at room temperature in the dark. Then, the absorbance at 734 nm was then adjusted to 0.7 ± 0.02 by diluting the ABTS radical solution with 95% ethanol. The ABTS assay was performed by mixing 180 µL of ABTS radical solution with 20 µL of various concentrations of the extracts in 96-well plate. The mixture was incubated in the dark for 6 minutes, and then the absorbance of the mixture was measured at 743 nm by using microplate reader (EZ read 2000, Waterbeach Cambridge, United Kingdom) using ethanol as a control. The ABTS radical scavenging activity was calculated in a percentage inhibition following equation below:

The percentage of inhibition (%) = $[(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})/ \text{Absorbance}_{\text{control}}] \times 100$

FRAP assay

The method of Benzie and Strain [16] was modified to determine the FRAP assay. A solution of 10 mM TPTZ in 40 mM HCl, 20 mM ferric chloride, and 0.2 M acetate buffer (pH 3.6) was mixed to produce the FRAP reagent at a ratio of 10 :1 :1 (v/v/v). A 25 µL of the extracts was mixed with 175 µL of FRAP reagent. Then, the reaction was incubated for 6 minutes at room temperature. After incubation, the absorbance was measured at 593 nm. The reducing power of the samples was calculated according to the standard curves of $FeSO_4$ (31.25 – 500 mg L^{-1}). The FRAP value was expressed as mg of Fe^{2+} equivalent per g of extract (mg eq. g^{-1}).

In Vitro Cytotoxicity

Vero and Hela cells (1×10^5 cells $well^{-1}$) were seeded in 96-well plates and incubated for 24 h at 37 °C with 5% CO_2 in a humidified incubator. Then, cells were treated with crude extracts with various concentrations (62.5 – 1000 μg mL^{-1}) in complete medium. In addition, as a negative control, cells were treated with complete media. After 24 h' treatment, The MTT assay was used to determine the toxicity [17].

3. Results and Discussion

Total Polysaccharide Content

The main bioactive compounds extracted from *G. lucidum* include terpenoids and polysaccharides. Polysaccharides has an important antioxidant and anti-cancer properties [18]. The phenol-sulfuric acid method is method to determine total carbohydrates in a sample. This method detects virtually all classes of carbohydrates (mono-, di-, oligo-, and polysaccharides). The total polysaccharide content of *G. lucidum* mycelial extracts is presented in Table 1. The results demonstrated the total polysaccharide content in water reflux extract was higher than in ethanol extract. *G. lucidum* extractable metabolite production may vary due to differences in growth medium, solvents and extraction method [10, 13]. The traditional polysaccharide extraction method is water-boiling method, the polar macromolecular compound polysaccharide is dissolved in a polar solvent. For water reflux extraction, after reflux the collected filtrate was precipitated with 95% ethanol, leaving behind impurities and other components that remain soluble in the ethanol-water mixture. The precipitate is crude water-soluble polysaccharides. While the ethanol extraction was using maceration method. Ethanol is a good solvent for a wider range of compounds, it can extract not only the desired polysaccharides but also other chemical compounds mainly fatty acids, alkaloids, saccharides, steroids, amino acids, terpenes, and phenols [19]. Generally, polysaccharides with higher molecular weights are less soluble in ethanol.

Table 1 The percentage yield and total polysaccharide content of *G. lucidum* mycelial extracts

Sample	% Yield of extract	Appearance	Total of Polysaccharide Content (mg glucose g $extract^{-1}$)
Ethanol extract	30.03	Dark brown	18.34 ± 0.47
Water reflux extract	6.56	Dark gray	207.81 ± 2.45

Antioxidant Activity

Three parameters were assessed in order to examine the *in vitro* antioxidant activity of *G. lucidum* mycelial extracts: DPPH and ABTS free radical scavenging abilities, and ferric-reducing antioxidant ability. The inhibition of radical chain reactions is one of the suggested antioxidant mechanisms of polysaccharides. [20]. The principle of both the DPPH and ABTS assays is the reduction of a radical by a hydrogen donor (DPPH) or by an electron donor (ABTS), which results in radical scavenging.

DPPH assay

The DPPH assay is one of the most often used techniques for assessing the antioxidant activity of extracts or compounds. Fig. 2 shows

the free radical scavenging activity of *G. lucidum* mycelial extracts as percentage. In the presence of 5 mg mL^{-1}, water reflux extract (58.14%) showed the highest radical scavenging effect than ethanol extract (18.95%). The results showed that the scavenging activity of extracts increased with increasing the concentration of extracts. This suggests that the mycelial extract has a dose-dependent DPPH scavenging efficacy. The water reflux extract of *G. lucidum* mycelia contained crude water-soluble polysaccharides. They may consist of branched homoglucan (1→3)-β-D-glucan, it is able to exert antioxidant effects [10]. In fact, absolute ethanol and aqueous ethanol have been successfully used to extract phenolic derivative antioxidant compounds [21], including from mushrooms [22]. However, *G. lucidum* represents poor source of phenolic compounds [23]. Similarly, Kalyoncu et al. [24] demonstrated that at 1 mg mL^{-1} concentration, the water mycelial extracts of *G. lucidum* showed the strongest radical scavenging effect (21.51%) followed by ethanol extracts (10.75%) and chloroform extracts (6.83%). In another research, Saltarelli et al. [25] did not find scavenger activity in *G. lucidum* polysaccharide extracted from mycelia.

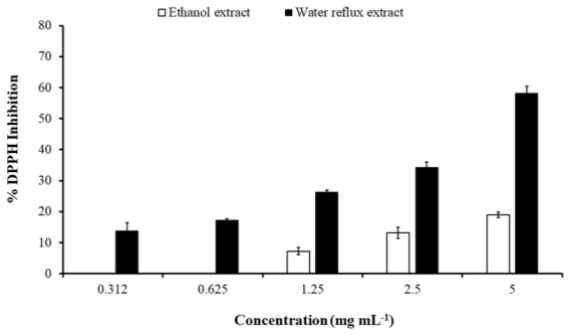


Fig. 2 DPPH• scavenging capacity of *G. lucidum* mycelial extracts at different concentrations.

ABTS assay

The ABTS assays of *G. lucidum* mycelial extracts are presented in Fig. 3. Similar to DPPH assay, water reflux extract of *G. lucidum* mycelia exhibited the higher radical scavenging activity (76.92%) than ethanol extract (19.97%). Kalyoncu et al. [18] found that the water mycelial extracts of *G. lucidum* scavenged 70.71% of $ABTS^{\bullet+}$ radicals at a 1 mg mL^{-1} concentration and, an ethanol extract has a 22.28% of the scavenging effects. The results we obtained are consistent with those reported by Shi et al. [26]. The authors observed that the *G. lucidum* mycelial polysaccharide exhibited approximately 40% ABTS scavenging activity at a concentration of 1 mg mL^{-1}. Additionally, they reported that the activity was concentration-dependent. The active hydroxyl groups of the monosaccharides included in the polysaccharide may be related to the differences in ABTS radical scavenging abilities [27].

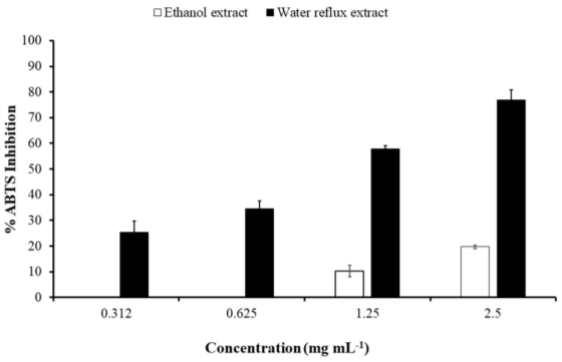


Fig. 3 ABTS Free Radical Scavenging Activity of *G. lucidum* mycelial extracts at different concentrations.

FRAP assay

The reducing power of a polysaccharide refers to its ability to donate electrons to oxidizing agents. This property is often associated with antioxidant activity, as antioxidants also scavenge free radicals by donating electrons. FRAP assay measure the

reducing power of a compound reacting with Fe^{3+}-TPTZ complex (colorless) to blue colored Fe^{2+}-TPTZ in acidic condition, which has absorbance at 593 nm. The results are expressed as milligram equivalents of Fe^{2+} (FRAP value) [28]. The antioxidant activities of mycelial extracts from *G. lucidum* are shown in Fig. 4. The results indicated that they showed the reducing antioxidant activity and possessed concentration dependence with different of FRAP value. Similar results to DPPH and ABTS assay, the water reflux extract had a higher FRAP value (Fig.4B) than the ethanol extract (Fig.4A). Puttaraju et al. [29], and Chirinang and Intarapichet [30] reported that the FRAP values of *Pleurotus sajor-caju* and *P. ostreatus* water extracts were higher than those of their ethanol extracts.

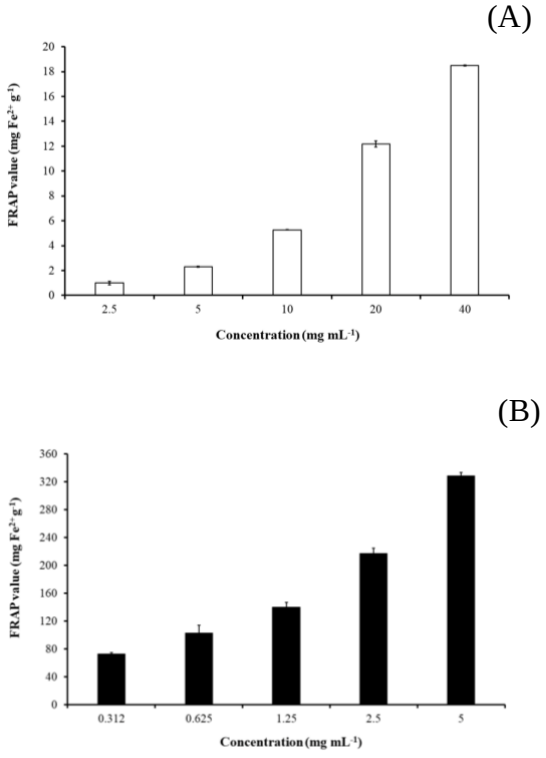


Fig. 4 FRAP activity of *G. lucidum* mycelial extracts (A) Ethanol extract and (B) Water reflux extract at different concentrations.

The antioxidant capacity of polysaccharide complexes and polysaccharides obtained from *G. lucidum* has been established by a number of *in vitro* antioxidant studies [31]. Polysaccharides extracted from *G. lucidum* exert antioxidant activity and protect tissues against the toxicity of reactive oxygen species. Additionally, they maintain the body's oxidative state [32].

Cytotoxicity of Extracts on Vero cells

The ethanol extract and water reflux extract were determined the cytotoxicity on normal mammalian cells (Vero cells) using colorimetric MTT assay. The results demonstrated that the ethanol extract of *G. lucidum* mycelia had cytotoxic effect on Vero cells line at 62.5 – 1,000 mg mL^{-1} (Fig.5). Whereas the water reflux extract of *G. lucidum* mycelia have no cytotoxicity to normal mammalian cells in all tested concentration. Ethanol is a good solvent for a wider range of compounds compared to water, it can extract not only the desired polysaccharides but also other potentially harmful compounds present in the source material, such as alkaloids, terpenes, and phenolic compounds [19]. Some of these extracted compounds may have cytotoxic. But water is a more selective solvent and primarily extracts water-soluble components like polysaccharides, which generally have less toxic potential.

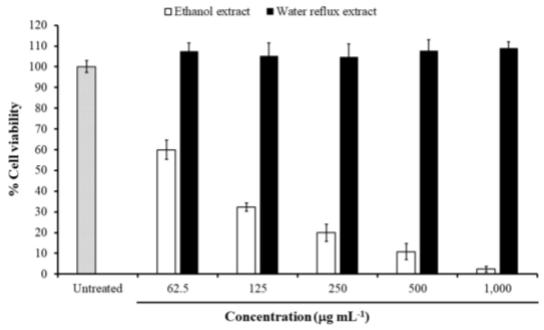


Fig. 5 Cytotoxicity of *G. lucidum* mycelial extracts at different concentrations on Vero cell

Cytotoxicity against Cancer Cell Lines

The water reflux extract was selected to determine the cytotoxicity against HeLa cell line. The cytotoxicity of the water reflux extract of *G. lucidum* mycelia was evaluated, using colorimetric MTT assay. The results demonstrated in Fig.6 in terms of % Cell viability with their concentrations. The water reflux extract of *G. lucidum* mycelia had a strong cytotoxicity and dose-dependent inhibition on HeLa cancer cell line. In particular, the water reflux extract did not cause cytotoxicity in normal cells up to 1,000 μg mL^{-1} indicating a specific anticancer activity (Fig. 5). The cytotoxic effect of the water reflux extract of *G. lucidum* mycelia is related with antioxidant activity and the content of polysaccharides (total sugar content). There are three possible mechanisms that polysaccharides can exert their anti-cancer effects: (i) indirect activity through improving the immune system's ability to recognize and destroy cancer cells; (ii) direct activity through inhibit cancer cell growth and proliferation, induce apoptosis (programmed cell death) in cancer cells; and (iii) acting as a chemotherapy adjuvant [33].

Numerous research groups are investigating into *G. lucidum*, a herb that is widely used as medicine in many oriental countries [34]. Armassa et al. [35] examined the cytotoxic effect in MCF-7 human breast cancer cell line of *Lentinus polychrous* Lev. and *G. lucidum* (Fr.) Karst. They found that the mycelial extract of *G. lucidum* exhibited the higher cytotoxicity on MCF-7 than the extract from *L. polychrous* Lev. mycelia. Two *G. lucidum* extracts from China and Serbia have been investigated for their cytotoxic effects on the MCF-7 cell line and HCT15 colon cancer by Stojkovi´c et al. [36]. The Chinese extract had a cytotoxic effect against MCF-7 cells. In another research, Veljović et al. [23] found that an ethanolic extract showed antiproliferative activity on colon LS174 (colon cancer cell line), HeLa and A549 (lung cancer cell line).

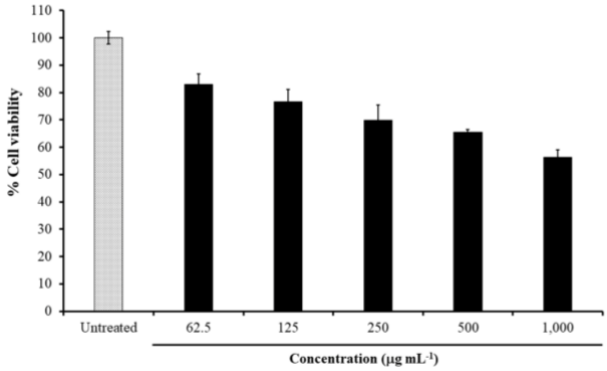


Fig. 6 Cytotoxicity of *Ganoderma lucidum* mycelial extracts at different concentrations on HeLa cell line

4. Conclusion

The results of this study demonstrated that the extracts obtained from *G. lucidum* mycelia has efficient antioxidants activity, and the water reflux extract had a strong cytotoxicity on HeLa cancer cell line. Importantly, it did not show cytotoxicity toward normal cells indicating a specific anticancer activity. Based on the results obtained, it was concluded that the water reflux extracts from *G. lucidum* mycelia may provide a potential application use as an additive ingredient in pharmaceutical and nutraceutical products.

5. Acknowledgement

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