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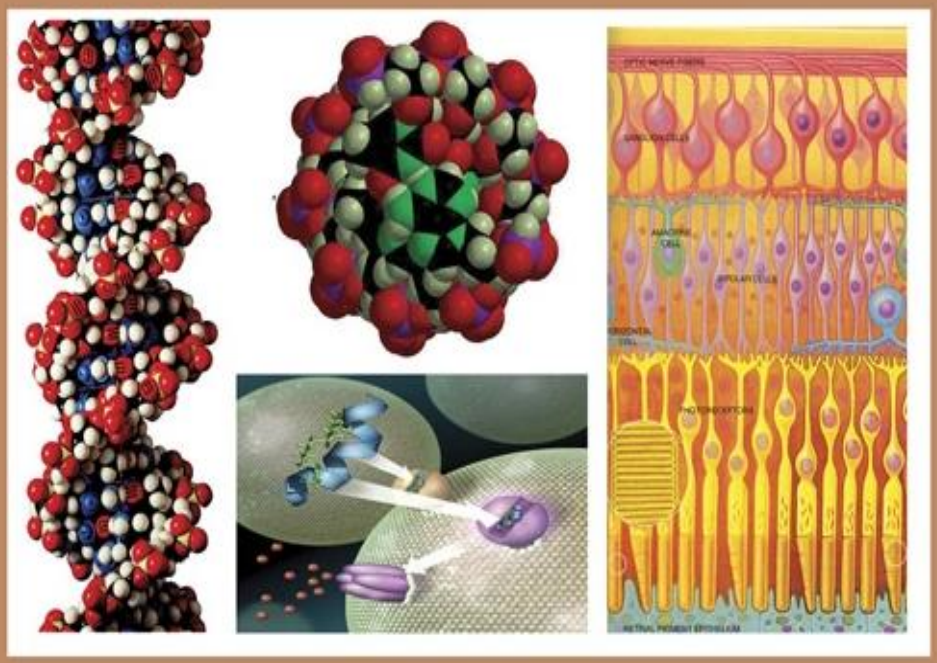

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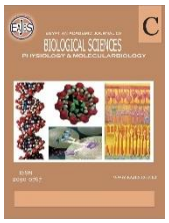

Inonotus obliquus Polysaccharides Inhibited Cellular Growth of NCI-H23 and A549 Lung Cancer Cells Through G0/G1 Cell Cycle Arrest and ROS Mediated Cell Death

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ABSTRACT

Chaga mushroom (*Inonotus obliquus*) has been used for a long time as a folk medicine for treating multiple diseases in several parts of the world without rendering any undesired toxicity. In this study, *I. obliquus* polysaccharides (IOP) were extracted and assessed to determine their anti-tumorigenic potential in human lung cancer cell lines NCI-H23 and A549 using cytotoxicity and apoptosis assays. MTT assay revealed a significant reduction in the cell viability ($p < 0.05$) for NCI-H23 and A549 cell lines exposed to IOP (5-200 µg/mL) in a concentration-dependent manner with IC50 of 100 µg/mL for both cell lines. Cell lines exposed to 50 and 100 µg/mL of IOP were further analyzed. IOP arrested the cancer cell growth at G0/G1 stage that can further implicate an anti-proliferative effect in cancer cells. Intracellular reactive oxygen species (ROS) generation was detected using DCFH-DA dye demonstrated increased levels of ROS generation ($p < 0.05$). Assessment of mitochondrial membrane potential using JC-1 dye exhibited decreased membrane potential, characterized by the low dye-intake, as shown by flow cytometry. In addition, Annexin-V/FITC analysis using flow cytometry demonstrated a significantly increased number of apoptotic cells ($p < 0.05$) in both cancer cell lines in a concentration-dependent manner. These results showed that IOP can induce apoptosis in both tumor cell lines, and therefore might be considered as an effective anti-tumor agent that could be further exploited in clinical settings.

INTRODUCTION

Lung cancer is responsible for significant mortality and low survival rate than any other cancer globally (Gaoe *et al.*, 2020). Therefore there is an intensive search for entirely effective, safe and natural alternative antineoplastic agents, with the ability to protect normal proliferating cells inside the body against the toxic effects of chemotherapy, as a novel approach in fighting cancer.

In addition, there is a great demand for combining chemotherapy with natural bioactive chemo adjuvant agents for potentiating its antitumor effects and diminishing its associated toxic effects (Zhang *et al.,* 2017).

Inonotus obliquus (*I. obliquus*), known as Chaga medicinal mushroom, is traditionally well known for its nutritional and therapeutic potential especially for the treatment of cancer ((Jiang *et al.,* 2019; Wang *et al.,* 2015). It has been used for a long time as a folk medicine for treating multiple diseases in many parts of the world in the form of tea decoctions, extracts, syrup, injections, and aerosols without conferring any unwanted signs of toxicity (Shikov *et al.,* 2014), Moreover, no or low signs of toxicity were shown in treated normal cells *in vitro* (Lemieszek *et al.,* 2011; Eid and Das 2020). Previous studies have reported the medicinal properties of I. obliquus bioactive molecules; such as polysaccharides, polyphenols and terpenoids and their potent anti-tumorigenic activity (Jiang *et al.,* 2019; Gao *et al.,* 2020; Baek *et al.,* 2018; Kuriyama *et al.,* 2013). Despite its beneficial effects and increased usage, the underlying mechanisms for its anti-cancer effects have not been fully understood, particularly in context to the systematic assays of its impact on inducing apoptosis of tumor cells (Kothari *et al.,* 2018; Balandaykin Zmitrovich, 2015; Ning *et al.,* 2014). Moreover, to the best of current knowledge, mechanisms underlying *in vivo* anticancer efficacy of I. obliquus polysaccharides are not fully elucidated.

The use of *in vitro* models became inevitable in most of the research fields to reduce the need of experimental animals (Doke and Dhawale, 2015). NCI-H23 and A549 cell lines are derived from lung cancer patients and represent the major lung cancers affecting the world's population (Aktar *et al*., 2019). In addition, both can cause cancer and demonstrate invasiveness *in vitro* which makes them good candidates for *in vitro* genotoxic studies that could help in elucidating the underlying mechanism for its anticancer activity (Yun *et al*., 2015; Ekwall, 1983). Cytotoxicity tests are of vital importance in detecting basal cytotoxic events in various types of cell lines (Ekwall, 1983). Many tetrazolium salts were developed for applications in cytotoxicity tests using MTT assay (Berridge *et al*., 2005).

Depending on the fact that autonomous proliferation is a hallmark of cancer, cell cycle arrest might be considered as a strong indication of anticancer effect (Bailon-Moscoso *et al*., 2017). Recently, it was demonstrated that anticancer drugs with apoptotic-inducing activity confer their cytotoxic effects through promoting G2/M phase accumulation (Wu *et al*., 2020; da Rocha *et al*., 2020). A 24 h exposure leukemia (K562) cells to 2-(6-(2-thieanisyl)-3(Z)-hexen-1,5-diynyl) alanine (THDA) resulted in G2/M phase arrest and apoptosis Wu *et al*., 2006**,** while morphine reduced the cell viability, growth and colony formation rate of MCF-7 cells, which was associated with cell cycle arrest at the G0/G1 and G2/M phase and apoptosis which was detected using MTT assay, as well as cell cycle and apoptosis assays (Chen *et al*., 2017).

Mitochondrial disturbances often occur earlier before any noticeable apoptotic morphological symptoms are detected and hence, the cytometric detection of dissipation of mitochondrial transmembrane potential**,** is a sensitive marker of early apoptotic events (Hou *et al*., 2018). Most common detection procedures are based on lipofilic cationic probes, J-aggregate fluorochromes, that are readily taken up by live cells and accumulate in mitochondria (Cossarizza and Salvioli, 2001; Castedo *et al.*, 2002; Haughland, 2003). A previous study reported that Chamaejasmine induces apoptosis in human lung adenocarcinoma A549 cells through a ROS-mediated mitochondrial pathway (Yu *et al*., 2011).

Grape proanthocyanidins were found to induce apoptosis in human lung adenocarcinoma A549 *in vitro* and *in vivo* by loss of mitochondrial membrane potential which was detected by JC-1 staining (Singh *et al*., 2011). Flip-flop movement and externalization of phosphatidylserine is a late sign of apoptosis. Annexin V binds phosphatidylserine exposed on the cell surface and is therefore considered a simple tool for detecting apoptosis (Vermes *et al*., 1995). Several previous studies used annexin V for the detection of apoptosis induced by β-Sitosterol, lobaplatin and morusin in A549 cells (Rajavel *et al*., 2018; Zhang *et al*., 2019; Wang *et al*., 2020).

The present study evaluated the anti-cancer potential Chaga mushroom extract by assessing cytotoxicity, cell cycle perturbations, and apoptosis induction; represented by the dissipation of mitochondrial transmembrane potential and Flip-flop movement, externalization of phosphatidylserine and ROS in human cancer cell lines NCI-H23 and A549.

MATERIALS AND METHODS

Extraction of *Inonotus Obliquus* Polysaccharides (IOP):

I. obliquus (50.0 g) was ground to a fine powder and suspended in 500 mL of 95% ethanol in distilled water (v/v) and extracted according to the procedure described by Yue *et al*. (2015) and Liu *et al*. (2019).

Cell Culture, Materials, and Reagents:

NCI-H23 and A549 cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, and Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine, 100 µg/mL penicillin and 100 U/mL of streptomycin. Cells were grown in a humidified incubator at 37°C (95% humidity, 5% CO_2). Other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). All the experiments were performed in triplicates.

Cell Viability Determination by MTT Assay (determination of IC50):

NCI-H23, and A549 cells in the exponential growth phase were collected, washed, trypsinized and suspended in DMEM medium, and seeded at a density of 1×10^4 cells/well (100µL/well) in order to obtain 70% to 80% confluent cultures in 24-well flat-bottomed microtiter plate in appropriate medium. All steps were carried out according to Bridge *et al*. (2005).

Determination of Reactive Oxygen Species (ROS) Using Flow Cytometry:

The cells were plated in 6-well plates with a density of 2 x 105 cells/well for 24 h. Afterward, cells were incubated with the IOP at 50 and 100 µg/mL concentrations for another 24 hrs. After incubation, the cell culture medium was replaced with 2', 7'- dichlorofluorescien diacetate (DCFH-DA) solution (5 µM in cell culture medium) and incubated for 30 min at 37 °C. Cells were then trypsinized and aspirated, followed by flow cytometric analysis under the green fluorescence channel.

Mitochondrial Transmembrane Potential:

In order to assess the degree of intrinsic mitochondria-mediated apoptosis, the mitochondrial transmembrane potential was measured using JC-1 dye ((Molecular Probes, USA) as per manufacturer instructions. The ratio of fluorescence of FITC and PE channels was recorded by flow cytometry using an excitation wavelength of 488 nm in an Attune flow cytometer (Applied Biosystem, USA).

Apoptotic Analysis Using FITC-Annexin-V:

Apoptosis of lung cancer cell lines upon exposure to IOP was assessed using Annexin-V/FITC kit (BD Biosciences, USA) and according to the manufacturer's protocol.

Cell Cycle Analysis by Propidium Iodide-Based Flow Cytometry:

Both cancer cells were seeded separately at around 1×10^4 cells/well in a microplate, incubated with 50 µg/mL concentrations of IOP for 24 hours. Cell cycle analysis through PI staining was

performed according to the method previously described by Davies and Allen (2007), The stained cells were analyzed using an Attune flow cytometer (Applied Biosystem, USA).

Statistical Analysis:

All results in this proposal were expressed as means ± standard deviations. A one-way analysis of variance (ANOVA) test was used to assess the difference between means of groups. For statistically significant outcomes, a Dunnett's post-hoc test was performed to investigate the difference between each group versus the negative control group. A p-value of < 0.05 was considered to reject the null hypothesis, indicating statistical significance. Statistical analysis was carried out using the Statistical Package for Social Sciences version 19.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Cell Viability Determination for IOP-treated NCIH23 and A549 Cells Using MTT Assay:

Lung cancer cell lines (NCI-H23, and A549) in exponential growth phase treated with different concentrations of IOP ranging from 0 (negative control) to 200 µg/mL and their corresponding viability showed that the concentrations starting from 25 µg/mL of the IOP extract significantly affected the percentages of the viability of NCIH23 cells (ANOVA, $F(7,24) = 630.26$, $p < 0.001$, (Fig. 1A) and A549 cells (ANOVA, $F(7,24) = 180.23$, $p < 0.001$, (Fig. 1B) in a dose-dependent manner. Accordingly, the obtained IC50 value was 100 µg/mL as shown in Figure 1C. Although most of the used concentrations significantly affected the viability of both cell lines, an abrupt decrease in cell viability was realized from 50 µg/mL concentration. Based on these findings, 50 and 100 µg/mL concentrations were selected for further testing of the cell lines.

Reactive Oxygen Species (ROS) Analysis:

Cancer cell lines incubated with IOP at 0, 50, and 100 µg/mL were stained with DCFH-DA solution and analyzed by flow cytometry for measuring ROS concentration. The concentration of ROS was significantly increased ($p < 0.05$) upon IOP treatment in both cell lines in a dose-dependent manner (Fig. 2), whereas normal cell line did not show any such effect. A549 cell line appeared to be more severely affected by the Chaga extract exhibiting higher levels of ROS compared to NCIH23. The increased concentration of ROS pertains to the induction of apoptosis. Therefore, the treated cell lines were further subjected to investigation to detect early and/or late markers of apoptosis.

Mitochondrial Transmembrane Potential:

IOP-treated cells were stained with mitochondrial JC-1 dye (Molecular Probes, USA). The ratio of fluorescence from FITC and PE channels was recorded by flow cytometry using an excitation wavelength of 488 nm. A significant reduction in the concentration of JC-dye was observed in the treated cancer cells compared to untreated cells that indicated decreased permeability of mitochondria, which in turn implied the dissipation of mitochondrial membrane potential, particularly loss in the net mitochondrial potential that could be considered as an early sign of apoptosis. The effect of increasing the concentration of IOP extract was almost negligible on the mitochondrial membrane potential in both cell lines (Fig. 3).

Apoptotic Analysis Using FITC-Annexin-V:

IOP-treated cell lines were stained for externalization of phosphatidylserine by the treatment with FITC-Annexin-V and 7-AAD using the Annexin-V apoptosis detection kit (BD Biosciences, USA). Stained phosphatidylserine concentration was analyzed by flow cytometry as an indication of the percentage of apoptotic cells. The obtained results revealed that both cell lines, A549 and NCIH23, showed a highly significant increase ($p < 0.05$) in the percentage of phosphatidylserine-exposing cells in a dose-dependent manner, thereby

demonstrating the induction of apoptosis. In line with the cytotoxicity study, apoptosis was more pronounced in A549 cells compared to NCIH23 cells (Fig. 4, Supplementary Fig 2).

Cell cycle Analysis:

Cell cycle analysis using flow cytometry by measuring the DNA content showed that the IOP-untreated cells/control cells and the cells exposed to IOP depicted selectively different peaks for the G0/G1, S, and G2/M phases; however, the percentage of cells in the G0/G1 in case of both untreated cancer cell lines (71.2% for NCI-H23 and 69.4% for A549) were significantly higher in comparison to IOP-treated cancer cell line (28.1% for NCI-H23, 24.5% for A549) (Fig. 5). This showed that Chaga mushroom polysaccharides arrested the cells mostly at G0/G1, and thus probably checked cell proliferation. This anti-proliferative property of Chaga mushroom polysaccharides could be further utilized in tumor studies.

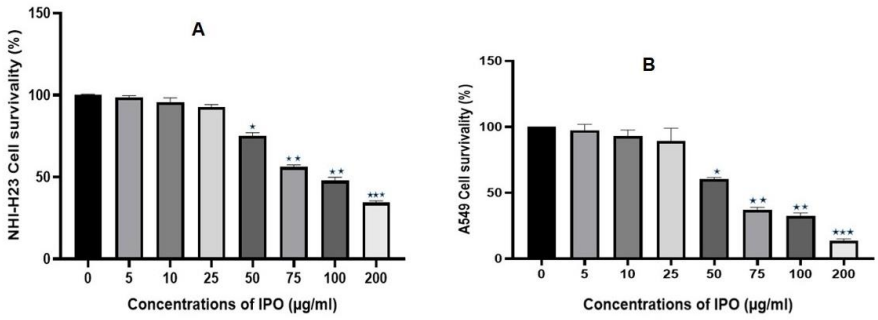

Fig. 1: Analysis of cell viability of NCIH23 cells (A) and A549 cells (B) with different concentrations of the IOP extract. *P<0.05, **P<0.001 and *** P< 0.0001 vs. control (0 µg/mL). (C) IC50 of IOP extract on NCIH23 and A549 cells measured by MTT assay.

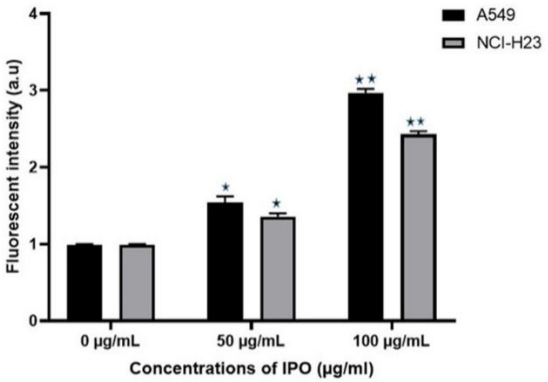

Fig. 2: Analysis of reactive oxygen species concentration of NCIH23 cells and A549 cells with 50 and 100 µg/ml concentrations of the IOP extract. *P<0.05, **P<0.001 vs. control (0µg/mL).

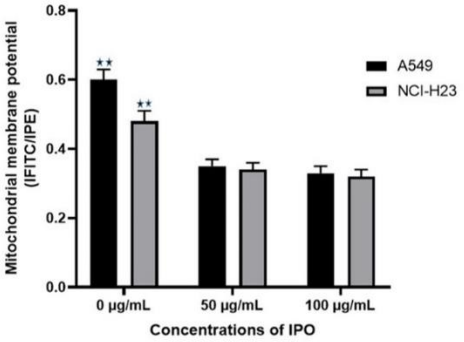

Fig. 3: Analysis of the effect of 50 and 100 µg/mL concentrations of the IOP on the mitochondrial membrane potential of NCIH23 cells (A) and A549cells (B) with *P<0.05, **P<0.001 vs. control.

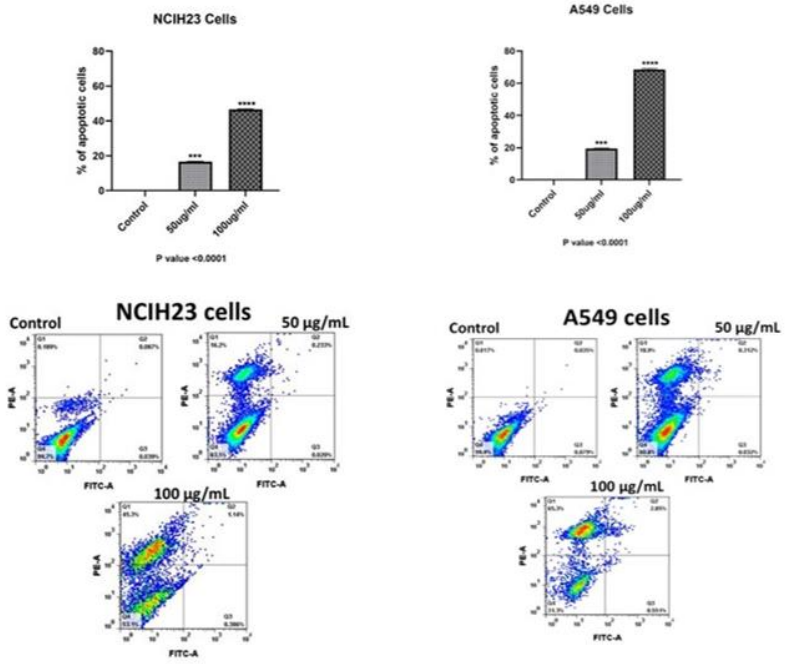

Fig. 4: Analysis of the effect of 50 and 100 µg/ml concentrations of the IOP on phosphatidylserine externalization and percentage of apoptotic cells in NCIH23 cells and A549cells with *P<0.05, **P<0.001 vs. control.

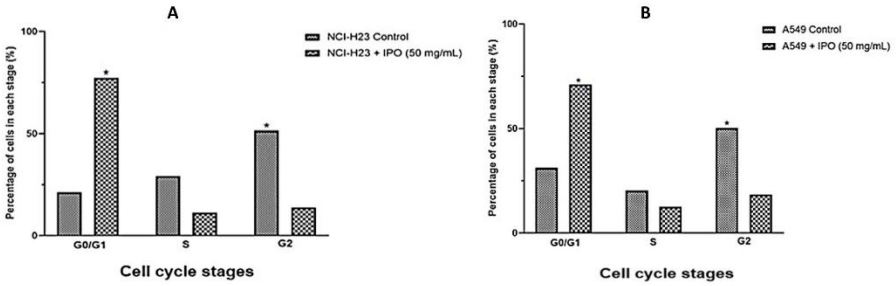

Fig. 5: Cell cycle analysis histogram. The figure shows the percentage of cells after cell cycle analysis upon IOP exposure using flow cytometry in different stages (G0/G1, S and G2, corresponding to 2N, 4N, 2N+2N, respectively, N denotes the ploidy). The percentages of the cell were analysed using one way ANNOVA. The stars on the top of bars represent statistical significance ($p < 0.05$). Most the cells of IOP-treated NCI-H23 (A) and A549 (B) were arrested at G0/G1 stages compared to control cells (Untreated cells), thereby demonstrating the anti-proliferative effect of chaga polysaccharides in cancer cells.

DISCUSSION

The search for effective and natural anti-cancer compounds that can support or replace existing cancer therapeutics became imperative to reduce the morbidity and mortality of cancers (Blagodatski *et al*., 2018). In this regard, chaga mushroom, which has been traditionally used as folk medicine in a plethora of disorders, involving inflammation, tumors and systemic infections could pave a path towards promising natural therapeutics. We assessed the efficacy of IOP extract on the viability of lung cancer metastatic cell lines NCL-H23 and A549 because lung cancer is considered one of the most fatal, high mortality rate cancers. Chaga mushroom polysaccharides didn't show any significant effect on the growth and development of normal cells in our previously conducted investigation (Eid and Das, 2020), so there was no need to test normal cell lines in the current study. In this study, the ethanolic extract of IOP was able to significantly reduce the viability of both cancer cell lines NCI-H23 and A549. Several *in vitro* studies have reported that *I. obliquus* extract in solvents, such as water, ethanol, methanol, and hexane was able to prevent proliferation and metastasis of cancer cells (Song *et al*., 2013; Ma *el al*., 2013; Handa *et al*., 2010; Sun *et al*., 2011; Lee *et al*., 2009; Song *et al*., 2008; Youn *et al*., 2008). In line with our results, previous studies showed that the viability of the A549 cell line was significantly reduced in a dose- and time-dependent manner upon treatment by aqueous chaga extract (Géry *et al*., 2018; Zhao *et al*., 2015; Liu *et al*., 2014; Zhong *et al*., 2011; Chung *et al*., 2010; Mazurkiewicz *et al*., 2010). Antiproliferative activity of Chaga mushroom extract has also been demonstrated in melanoma cells (Youn *et al*, 2009), hepatic cancer cells (Youn *et al*., 2008), sarcoma cells (Chen *et al*., 2007), HeLa cells (Burczyk *et al*., 1996), HCT-116 (Tsai *et al*., 2017) and HT-29 (Lee *et al*., 2009).

The balance between proliferation and apoptosis in cancer cells is essential to determine the process of growth or regression of a tumor inside the host body (Rutkowska *et al*., 2019). In cancer cells, cell cycle mechanisms are deregulated resulting in activation of cell proliferation (Caglar and Biray Avci, 2020). Consequently, some anti-tumor effects might be attributed to the alterations in biochemical mechanisms, such as cell cycle arrest, anti-proliferation, regulation of the immune system, and intrinsic apoptotic pathways (Choudhari *et al*., 2019).

Cell cycle perturbation is a major feature of apoptosis and the most common abnormality in human cancer. Therefore, cell cycle arrest and induction of apoptosis have become the primary target of anti-cancer drugs to prevent cancer cell proliferation (Lee *et al*., 2015). Propidium iodide PI staining and flow cytometry is the most commonly used method to quantitate DNA content in different phases of the cell cycle. Moreover, PI is an intercalating agent that is impermeable to live cells; it can thus distinguish dead cells from live cells (Moore *et al.*, 1998; Crowley *et al*., 2016). Several anticancer agents cause cell cycle arrest and are clinically useful for cancer treatment (Wu *et al*., 2020). Ethanol extract of *I. obliquus* inhibited proliferation and DNA synthesis while inducing G1 arrest in HT-29 cell (Lee *et al*., 2015). Water extract of Chaga treatment induces apoptosis in NCI-H460 lung cancer cells by perturbing cell cycle kinetics at the sub-G1 phase in a dose-dependent manner (Bak *et al*., 2013). In the same trend, Genistein triggered cell cycle arrest in G0/G1 period reduced the cell viability of pancreatic (Bi *et al*., 2018), breast (Fang *et al*., 2016), and esophageal (Gao *et al*., 2020) cancer cells in a dose-dependent manner and, while Amex7 has induced G2/M arrest in HT-29

human colorectal cancer xenografts by regulating cell cycle regulatory proteins (Lee *et al*., 2017).

Previous studies on anti-cancer agents considered generating high levels of intracellular ROS as a sign of induced apoptosis in the cancer cells (Simon et al., 2000; Kowaltowski *et al.,* 2004; Lee *et al.,* 2019; Li *et al.,* 2020). Results of the present study showed that the cells incubated with the IOP at 50 and 100 µg/mL and stained with DCFH-DA exhibited significantly high concentrations of ROS in both cancerous cell lines in a dose-dependent manner in comparison to untreated cells. Therefore, treatment with IOP could disturb the redox balance in NCI-H23 and A549 cells, and hence ROS may be a key mediator, leading to mitochondrial dysfunction, protein oxidation and DNA damage in cancer cells, followed by cell death or apoptosis induced by various anticancer drugs (Xiao *et al.,* 2013; Sahayanathan *et al.,* 2020). Many mushroom polysaccharides have also been shown to promotes production of ROS and induce apoptosis in human cancer cells (Zhang et al., 2020).

In our study, as an early sign of apoptosis, the shifting of the JC-1 peak in cell lines incubated with the IOP at 50 and 100 µg/mL exhibited reduced JC-1 aggregate formation via perturbation of the mitochondrial membrane permeability, which in turn implied the dissipation of mitochondrial membrane potential (Vermes *et al*., 1995). In agreement with our results, a mixture of herbal extracts including chaga induced apoptosis in NCI-H460 lung cancer cells via intrinsic pathways and the dissipation of the mitochondrial membrane potential was recorded using the JC-1 flow cytometry (Bak *et al*., 2013). Similar results were obtained by Bi *et al*. (2018) in pancreatic cancer cell lines treated with genistein.

Membrane phosphatidylserine flip-flop is a sign of induction of apoptosis that can be detected by annexin V-FITC (Petsophonsakul *et al*., 2013). In the present study, cells treated with the IOP extract showed a significant increase in the percentage of phosphatidylserine externalization among tested cells in a dose-dependent manner, thereby demonstrating the induction of apoptosis. In a previous study, a mixture containing chaga extract induced membrane phosphatidylserine flip-flop in NCI-H460 lung cancer cells as a sign of induction of apoptosis detected by annexin V-FITC/PI staining, which correlated with our results (Bak *et al*., 2013).

Conclusion:

Overall, Chaga mushroom can be regarded as a promising, though somewhat understudied medicinal mushroom in spite of its widespread use in folk medicine. The present study proved the antitumorigenic potential of Chaga mushroom against human lung cancer cell lines through cytotoxicity, cell cycle arrest and induction of ROS-mediated apoptosis. The exact mechanisms through which cell cycle arrest and apoptosis are triggered still need to be clarified further, along with *in vivo* investigations in animal models and clinical trials on human patients.

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Conflicts of Interest

No conflict of interest. Submitting authors are responsible for coauthors declaring their interests.

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