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Growth and bioactivity of *Phellinus linteus* in the mushroom farm, at different cultivation times

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Abstract. *Phellinus linteus* (PL) growth depends on environmental conditions such as light intensity, humidity, temperature, and CO_2. This research investigated the growth of PL and their bioactive activities in the PL fruiting body from different locations of Top, Middle, and Bottom zones in the mushroom farm, harvested at different cultivation times, up to 18 months. Results showed that PL weights increased from 9.35 g/fruiting body to 46.89 g/fruiting body at 18 months. PL growths at each location were not significantly different during 12 months, while a bit higher growth of PL at the Bottom zone was observed at 18 months. The temperature during the cultivation was 30-35°C with high humidity (83-100%). The Bottom zone indicated the higher CO_2 than the other two zones. The bioactivity in PL extracts from the cultivation during at 3-18 months showed a non-significant difference in both TPC, and antioxidant activities analyzed by DPPH. PL extract presented an anti-inflammatory effect comparable to the medicine diclofenac. The potential applications of PL extract inhibit cancer; particularly, the low survival rate of cell cancer by the PL extract present its possible use as a functional ingredient. Further studies of their toxicity on normal cells would benefit the application of PL extract.

1 Introduction

Edible mushrooms have been used for food and medicine since ancient times. More than 50 species of mushrooms and their extracts are studied in vitro and animal tests, showing their potential as anticancer products for human [1, 2]. Medicinal mushrooms are popularly consumed in East Asia, especially as traditional medicine [3]. Bioactive compounds in the mushrooms are divided into several groups, including lectins, polysaccharides, phenols, terpenoids, and ceramides [4]. Mushroom extracts contain bioactive compounds, the secondary metabolites, that help maintaining human health [5]. Lion's Mane mushroom (*Hericium erinaceus*) has ergosterol that can be used as nutritional supplements. Ergosterol in the Lion's Mane mushroom can be converted to vitamin D2 [6]. Bamboo mushroom (*Dictyophora indusiata*) provides nutrition and bioactive compounds for health-benefit [7]. Shiitake mushroom (*Lentinula edodes*) contains phenolic compounds, polysaccharides, and sterols that help body functions and health [8].

Phellinus linteus (PL) is a specie of mushrooms belonging to family Hymenochaetaceae, which is indigenous mainly in tropical America, Africa and East Asia, and used as health booster and ancient herbal medicine [9]. In Thailand, PL is commonly found in the northern region [10]. The common name of PL varies by location: "Black Hoof" in Thailand, "Song Gen" in China, "Sanghwang" in Korea, and "Meshimakobu" in Japan [11]. PL fruiting body has a brown or black hard surface. PL generally grows well on perennial trees in the nature, not on annual herbaceous plants. The mushroom indicates various pharmacological activities, including regulating blood glucose, improving blood circulation, being hepatoprotective, and enhancing immunologic function [12]. Moreover, PL also contains antitumor, immuno-modulatory, anti-inflammatory, anti-oxidant, anti-hyperlipidemic and anti-diabetic properties [13]. PL is a good source of various polyphenolic compounds with a diverse pharmacological potential [9]. Twenty-six isolates of *Phellinus* species were found to have medicinal

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functions and utilized along with traditional medicine in Japan, China, and Korea [14].

The important bioactive compounds in PL mycelium and fruiting body are divided into phenylpropanoids, terpenoids, and furans, such as inoscavin A, which has anti-diabetic properties [15]. The antioxidant caffeic acid, hispidin and hispolon was detected in both mycelium and fruiting body [16-18]. Polyphenol compounds present a high potential for industrial and medicinal applications. The content of the secondary metabolites or bioactive compounds in the fruiting body depends on the mushroom growth which are affected by environmental conditions such as light intensity, humidity, temperature, and CO_2 concentration. For spawning, the optimal temperature for each specie is around 25°C, and in the right amount CO_2 concentrations [19]. The effects of cultivation environments vary depending on the mushroom species. Generally, PL can expand their diameter to around 20-30 cm after 100 years cultivation [10]. The optimal relative humidity for PL fruiting bodies is above 96 % during the cultivation [20].

In this research, we have investigated the growth of the PL fruiting body and their bioactive activity at different cultivation times in a semi-close mushroom farm, up to 18 months. Environmental conditions such as temperatures, relative humidity and carbon dioxide concentration were monitored from three locations, Top, Middle, and Bottom in the vertical packs of the mushroom spawns using a IoT system. The PL mushrooms from each location were harvested at different times and measured for their growth and bioactivity. The information obtained will be useful for the management of PL cultivation with the high yield and bioactivity, in PL mushroom farms.

2 Material and methods

2.1 Chemicals and reagents

Ethanol was purchased from RCI Labscan Co., Ltd. (Thailand). Sodium carbonate, gallic acid, Folin-Ciocalteau phenol reagent, methanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH) were bought from Sigma-Aldrich (Chemie GmbH, Steinheim, Germany) while hispolon was purchased from Enzo Life Sciences (USA). All other chemicals and reagents used were analytical grade.

2.2 Mushroom cultivation

One thousand mushroom spawns were prepared, sterilized and inoculated with PL mycelium at the Arunyik Mushroom Farm (Nakornprathom province, Thailand). The inoculated PL mushroom spawns were moved to the Thai Intellectual Learning Center, another mushroom farm, for the PL cultivation at Bangpli (Samutprakarn, Thailand) for 18 months (from February 2021-July 2023). The room was made of wooden structure and slight light intensity could penetrate through the roof. The cultivation was carried out at ambient temperature with water spraying regularly during the day. The environmental conditions consisting of light intensity, CO_2 content, humidity relation, and temperature were monitored during the cultivation using sensors and an IoT system during May 2021-November 2021, corresponding to 5M-11M of the cultivation. The vertical packs for the mushroom spawn consist of 12 bags which were set as 3 zone, Top, Middle, and Bottom zones (4 packs for each zone) as shown in Fig. 1. The mushrooms from each location were randomly harvested at 3, 7, 10, 12, and 18 months of the cultivation, six fruiting bodies for each sampling. The appearance and weights of the PL samples were measured.

2.3 Extraction of PL mushrooms

The fresh PL fruiting bodies harvested at different times were ground into powders by a Blender (7009S, Waring, Japan) and sieved by a 14-mm mesh. The moisture content of PL was determined according to AOAC methods [21]. Mushroom powder (2 g) was added with 130 mL of 95% ethanol as a solvent, and extracted in a Soxhlet Apparatus for 5 h followed the method described by Toopmuang et al. [10]. The solvent in the extract was removed and the extract was concentrated to around 3 mL using a rotary vacuum evaporator (Buchi Labortechnik AG) before drying with nitrogen gas. The samples were kept in amber glass bottles and stored at -20 °C until further analysis. Each dried sample was added with 100 µg/mL of DMSO before further analysis.

2.3.1 Total phenolic compounds

Total phenolic compounds were determined according to Theeraraksakul et al. [22]. Sample (10 µL) was mixed with 40 µL of Folin-Ciocalteu reagent and 200 µL of 75 mg/mL sodium carbonate solution in a 96-wells plate and incubated at room temperature. The absorbances of the samples at 760 nm were measured using a microplate reader (SpectraMax® iD3, Molecular Devices, LLC, CA), and a calibration curve was prepared using gallic acid solution (200-1000 mg/L) as a standard. The results were calculated as micrograms of gallic acid equivalents per gram extracts (GAE mg/g extract).

2.3.2 Antioxidant activity

Free radicals of the PL extract were determined by 1,1-diphenyl-2-picrylhydrazine (DPPH) assay followed Theeraraksakul et al. [22]. The extracts were combined with 190 µL of 2 µg/mL DPPH solution in a 96-wells plate, incubated at room temperature in a dark place for 30 min. The absorbances of the samples were measured at 517 nm using the microplate reader.

2.3.3 Cell viability assay

SiHa-HTB35 ($8x10^3$) and HaCaT ($5x10^3$) cells were seeded into a 96-wells plate. After allowing the cells to attach, the medium was replaced with fresh Dulbecco's Modified Eagle Medium (DMEM) containing various concentrations of each extract (50, 100, and 200 µg/mL). The dimethyl sulfoxide (DMSO) concentration of 0.1% was added in the final solution. After 72 h, cell viability was assessed by adding 10 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution and incubated for an additional 3 h. The resulting formazan crystals were fully dissolved with DMSO. The sample absorbance was measured at 570 nm with a reference wavelength of 630 nm. Cell viability (%) was calculated and presented as a bar graph using a Prism 10 (GraphPad Software Inc).

2.3.4 Antiinflammatory assay

The RAW264.7 cells ($1x10^5$) were seeded into a 96-wells plate for the experiment. After an incubation period of 16-18 h, the cells were pre-treated with the PL extracts (100µg/mL) for 1 h. Diclofenac (Dic) was served as the reference for anti-inflammatory drug control. Subsequently, the pre-treated cells were stimulated with 1 µg/mL lipopolysaccharide (LPS) for 24 h. Unstimulated cells and LPS-stimulated cells were used as negative and positive controls, respectively. At the specified time, the medium was collected for nitric oxide determination using Griess Reagent, and the NO levels were quantified using a calibration curve. Cell viability was assessed as the remaining cells using the MTT method (described earlier), ensuring that cell viability (%) was maintained relative to the LPS-stimulated condition.

2.4 Statistical analysis

All experiments were performed in triplicate and presented as mean ± standard errors. Analysis of variance (ANOVA) was used to analyse statistical significance using SPSS Statistics 26.0 Program (SPSS Inc., Chicago, IL). Differences between mean values were evaluated by one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test.

3 Results and discussion

3.1 Morphology of PL mushrooms harvested at different cultivation times

Fig.1 reveals the PL mushroom spawns cultivated in the mushroom farm at 3, 10 and 12 months. The PL fruiting bodies exhibit a tough, woody, or horseshoe shape. Their caps are generally brown with rough surfaces. The under-view of the mushroom cap shown in Fig. 2 presents a layer-arrangement with light brown to dark brown color, identical to the appearances described by Azeem et al. [12]. It was found that throughout 3-18 months, the weight and shape of the mushrooms increased about 5 times and were significantly different ($p < 0.05$) as shown in Fig. 3.

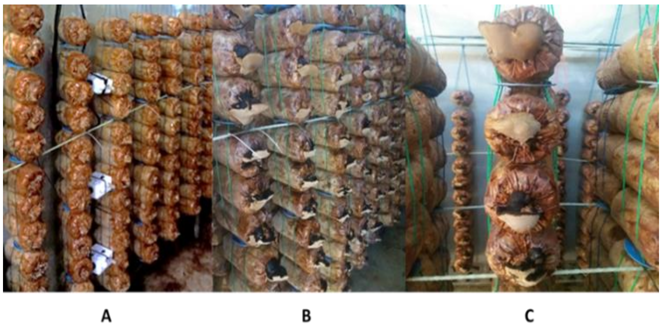

Fig. 1. PL cultivated at the mushroom farm showing the sensors and cameras set at different locations (Top, Middle, and Bottom) in the left picture; (A) 3 months, (B): 10 months, and (C): 12 months.

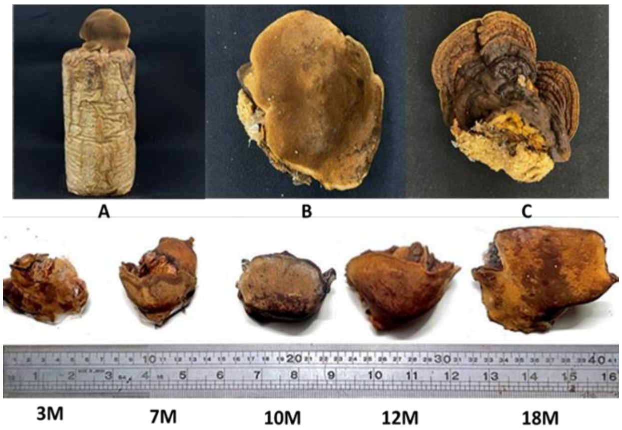

Fig. 2. Morphology of PL cultivated at the mushroom farm; (A): 12 month-PL mushrooms in the spawn; (B): Top view of the fruiting body, and (C): and under-view of the fruiting body. The bottom pictures show the PL fruiting bodies harvested from 3 to 18 months.

The PL growth rate was about 9.35 g/fruiting body, and 23.01g/fruiting body for the first 3, and 7 months, respectively, as shown in Fig. 3. The moisture contents of PL were in the range 38-40 % (wet weight basis, wwb). The PL weights increased from 9.35 g/fruiting body at 3 months to 46.89 g/fruiting body at 18 months. However, their growths at each location were not significantly different during 12 months ($p > 0.05$) while a higher growth trend at the Bottom zone was observed during 12-18 months. The growth of PL mushroom for 18 months in the cultivation room in this study was more than two times the growth of PL on the buried logs for 2 years of incubation, which indicated the highest weight of 19 g of fruiting body [23]. The morphology of the basidiocarps of PL was similar to the data demonstrated by Min et al. [24] that the mushroom has tough, woody and dark brown surface. Cultivation conditions and substrates can significantly affect mushroom growth periods and morphologies as reported for the cultivation of Lion's Mane Mushroom by Tachabenjarong et al. [25].

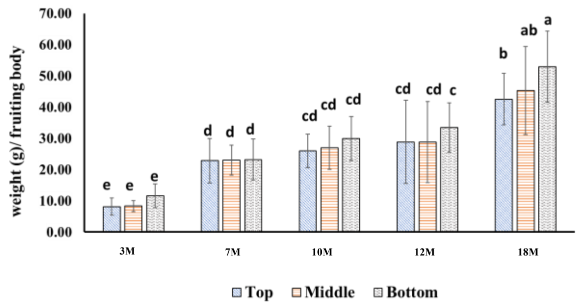

Fig. 3. Weights of PL mushrooms cultivated in the mushroom farm, harvested from each location (Top, Middle, and Bottom zones) at different cultivation times. Different letters in each bar differ significantly ($p < 0.05$).

3.2 Cultivation environments

Environmental factors, including temperature, humidity, light, and CO_2 concentrations, are crucial for mushroom growth [26]. The sensors capturing each environmental condition were placed at the Top, Middle, and Bottom zones as depicted in Fig. 1. Data collection recorded the temperature, humidity, CO_2, and light intensity during the first 5-11 months of the cultivation. Unfortunately, the monitoring system had signal errors after 11 months. However, the data could represent the conditions in the room. The data shows the conditions during the cultivation in the rainy season, from May to November 2021 in Thailand, the initial temperature was 35 °C, and the temperature gradually decreased to 29-30 °C in late May until November as shown in the Fig. 4A, and the initial humidity was 55.6%. The humidity increased to around 100% from late May until June, and gradually decreased from November to 72-75% as shown in Fig. 4B. Each position in the vertical zones indicated non-significant differences in temperature and humidity. In addition, the optimal conditions for the fruiting body of PL growth are 96% relative humidity and 31-35 °C during cultivation [20]. This study was similar to the data demonstrated by Islam et al. [27], that the indoor environment during rainy seasons was optimal for maximum growth of Grey Oyster Mushrooms (*Pleurotus pulmonarius*), with a temperature and humidity range of 27-30ºC, and 80-90% respectively.

Binheam et al. [7] reported that the cultivation of bamboo mushrooms in an open-farm (outdoor) during rainy season yielded the mushroom weights about two times those bamboo mushrooms cultivated in the summer season. Fig. 4C shows that the CO_2 concentration in the cultivation room was around 500 ppm during 3 weeks of May (the 5th month cultivation) and increased in late May, especially in the Bottom zone which showed the highest CO_2 concentration at 1,112 ppm. In June, CO_2 concentrations were as high as 970 ppm, while the Top and Middle zones had lower CO_2 concentrations. Following the results in Fig. 3, the cultivated mushrooms at the Bottom zone started to grow and increase their weight more than the other zones, which might be due to the higher concentration of CO_2 in the Bottom zone as shown in Fig. 4C. This could be explained by the high content of CO_2 released from the mushroom respiration during the forming of fruiting bodies [28]. CO_2 is heavier than oxygen, and commonly accumulates on the ground floor. The production of mushroom fruiting bodies depends on the light intensity, which should be 50-500 lux (lumen) [29]. However, in this research, the initial light was 3 lumens (lm) and increased to 18 lumens (lm) in early June, in the Top zone, which showed the highest light intensity.

In this research, the PL mushroom at the Bottom zone of the vertical packs presents the highest growth at 18 months. The optimal conditions for the PL cultivation to achieve the highest production yield were the high humidity (83-100%), medium temperature (30-35 °C), and slightly light intensity (3-18 lm) while longer cultivation led to a higher weight of the fruiting body. It might be possible to enhance the bioactive compounds in PL by adding some mushroom precursors in the cultivation substrates. For example, Yun et al. [30] added amino acids to the mushroom substrate for

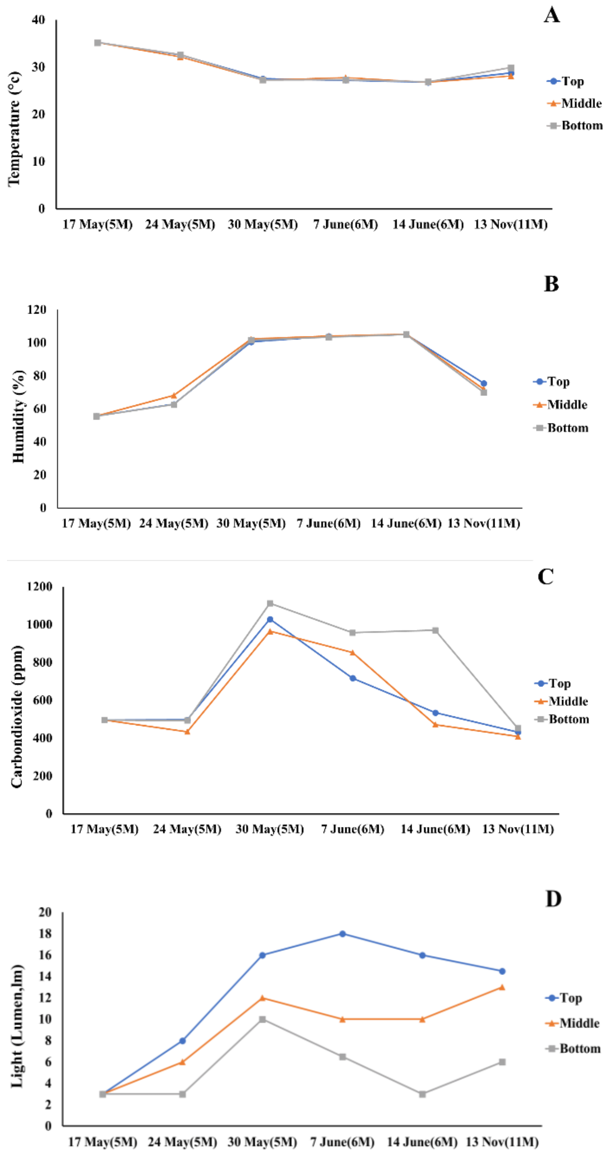

Fig. 4. The temperature (A), humidity (B), CO_2 (C), and light intensity (D) during the cultivation (May 2021-November 2021) corresponding to 5 month-11month (5M-11M) of the cultivation.

Flammulina velutipes cultivation, leading to a higher mushroom yield. In addition, UV radiation exposure caused stress and promoted the production of hispidin and polyphenol compounds by *Inonotus obliquus* [31]. However, in this research, the effects of each environmental condition on the PL growth might not be explained well because there were some variations for environmental factors in the cultivation locations and time since the cultivation room was not a closed system.

3.3 Ethanolic PL extract exhibited Total phenolic compound

Total phenolic content (TPC) in the PL mushroom harvested from different locations and cultivation times are revealed in Fig. 5. TPC of PL mushrooms harvested from the Top zone ranged from 30.42-37.79 mg GAE / g extract for the extracts obtained from different growth periods, showing non-significant differences for their TPC ($p > 0.05$). Some variations were observed from the mushrooms in the Middle zone, in which TPC in the 12-month (39.78 mg GAE /g extract) indicated the highest content similar to the 7M-Top (7-month cultivation in the Top zone, 35.08 mg GAE /g extract), 12M-Top (37.79 GAE /g extract), 3M-Middle (35.48 GAE /g extract), and 18M-Bottom (35.29 GAE /g extract). Interestingly, a more variation in TPC at different cultivation times was revealed in the PL in the Bottom zone. TPC in PL mushroom in the 18M-Bottom exhibited the highest TPC content. In addition, Min et al. [32] reported that PL cultivation on Oak wood logs for 2 years presented a TPC of 19.05 mg GAE/g which is about half of the TPC from 18M-Bottom. Tachabenjarong et al. [25] reported that different growth periods did not affect the increase of TPC in Lion's Mane mushrooms. However, the cultivation of Lion's Mane mushrooms, around 1 month, was much shorter than the PL cultivation in this study. The mushroom farm's different mushroom species and cultivation environments may play a role in the results for total phenolic content during growth periods.

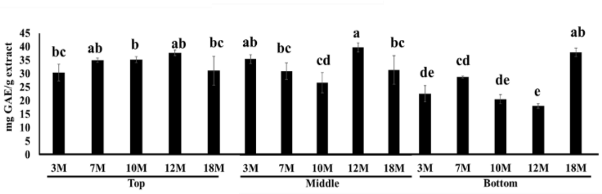

Fig. 5. Total phenolic contents in PL mushroom from harvested during different growth periods Different letters in each bar differ significantly ($p < 0.05$). The experiment was done in triplicate.

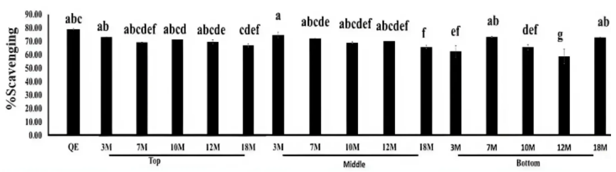

Fig. 6. DPPH radical scavenging assay of PL mushroom harvested at different growth periods, compared with quercetin (QE) Different letters in each bar differ significantly ($p < 0.05$). The experiment was done in triplicate.

3.4 Antioxidant assessment of Ethanolic *Phellinus linteus* extract

Antioxidant activity is used as a primary screening method for evaluating bioactive compounds. A stable artificial free radical molecule, such as DPPH, was utilized to assess antioxidant activity. DPPH acts as a hydrogen donor, which facilitates the conversion of purple DPPH to yellow-colored diphenyl-picryl hydrazine (1,1-diphenyl-2-picrylhydrazine) [33]. As anticipated, the percentage of scavenging activity was positively correlated with total phenolic content. Overall, PL extracts exhibited a higher scavenging activity compared to quercetin (QE) (Fig. 6). Additionally, cultivation time did not affect the scavenging activity in different zones of PL. Enrichment of polyphenol compounds have been reported in *Phellinus sp*. extracts. The presence of flavonoids in mushrooms remains controversial due to the proposed absence of flavonoid biosynthesis-related enzymes [34]. However, controlled cultivation conditions or interactions with neighboring organisms could potentially influence the stimulation of flavonoid biosynthesis. Caffeic acid, inotilone, 4-(3,4-dihydroxyphenyl)-3-buten-2-one and, phellilane H were identified from *Phellinus linteus* as antioxidant compounds that exhibited the activity in a dose-dependent manner [18]. PL extracts demonstrated a protective effect in preventing DNA damage under oxidative stress [35]. Therefore, phenolic content is likely the major component enriched in PL extracts that exhibits antioxidant activity, independent of cultivation time.

3.5 Ethanolic PL extract exhibited cytotoxicity on SiHa cells with mild toxicity on HaCaT cells

Enrichment of phenolic compounds in *Phellinus sp.* has reportedly exerted anticancer activity on various cancer types. Methanolic *Phellinus fastuosus* extract demonstrated anticancer towards A431, MG-63, and Hela with IC50 in the microgram range [36]. Hot ethanol extraction of *Phellinus sp.* diminished cell viability on SiHa cell lines compared to water extraction [37]. In Fig. 7A, PL extracts show a high cytotoxicity against SiHa cell lines. At a screening concentration of 10 µg/mL, the Top zone demonstrated significant anticancer activity, exhibiting a survival rate of less than 40% compared to other location zones, independent of cultivation time. In contrast, the Middle and Bottom zones at 3 months displayed the lowest survival rates, both falling below 20%, with these rates gradually increasing in a cultivation time-dependent manner. Immortalized keratinocyte, HaCaT cells have been used as a cellular model for cytotoxicity assessment and selectivity between normal and cancer cells [38-40]. Interestingly, overall PL extracts showed a mild cytotoxicity (Fig. 7B). PL mushroom extracts in the 12M-Bottom zone, the 10M-Middle zone, and the 18M-Bottom zone at a concentration of 10 mg/ml had 80.96, 67.49, and 66.62% of the highest survival rates in HaCaT cells, respectively. (>50% survival rate) on HaCaT cell lines, although the Bottom zone exhibited

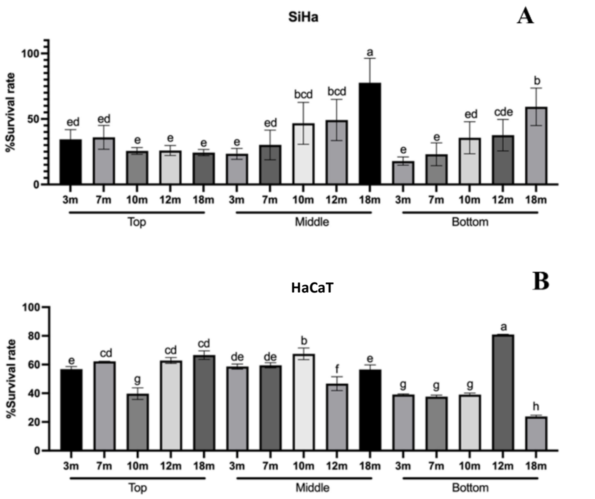

Fig. 7. Anticancer assessment of PL extracts against cancer and normal cells, at a screening concentration of 10 µg/mL in SiHa (A) and HaCaT (B) cell lines was done to assess survival rate by MTT assay. The experiment was done in triplicate.

slightly higher cytotoxicity on these cells. Hispolon, an isolated compound from *Phellinus sp.*, might be a key effector molecule contributing to its anticancer activity. Hispolon-induced human nasopharyngeal carcinoma cells upregulated apoptotic-related proteins through the MAPK pathway [41]. Hispolon demonstrated anti-metastatic effects in HeLa and SiHa cells through an autophagy-dependent mechanism [42]. Therefore, PL extracts exhibited anticancer activity depending on the location zone. Prolonged cultivation periods should be considered to enrich bioactive compounds.

3.6 Ethanolic PL extract attenuated nitric oxide production in LPS-stimulated RAW264.7 cells

Pathogen invasion, viral infection, or exposure to some medicines increased reactive oxygen species accumulation, leading to inflammatory conditions in our body [43-45]. LPS, or lipopolysaccharide, is a gram-negative bacteria cell wall component that has been widely used to mimic inflammation in murine macrophage cells for anti-inflammatory activity assays [35, 46]. Under LPS stimulation, nitric oxide (NO), an immune mediator, was produced from the immune cells to activate the immune response, leading to pro-inflammatory cytokine production [47]. As a result, the LPS-stimulated condition increased nitric oxide production compared to the unstimulated condition. The LPS-stimulated condition led to increased nitric oxide production compared to the unstimulated condition. Diclofenac (Dic), a reference anti-inflammatory reagent, reduced NO production by half compared to the LPS-stimulated condition, as shown in Fig. 8. Interestingly, the Bottom zone of PL extracts significantly ($p < 0.05$) demonstrated anti-inflammation activity by lowering the released NO levels. Notedly, the 3-month Bottom zone elicited an effect similar to Dic by reducing nitric oxide release to 16.67 µmol/L. Additionally, PL mycelium extracts, PLM200, suppressed prostaglandin E2 and NO release, resulting

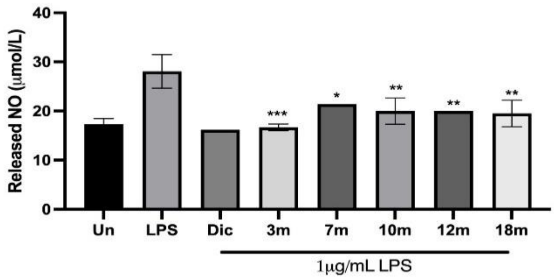

Fig. 8. Released nitric oxide level setermination using Greiss's reagent, RAW264.7 cells was pretreated with each PL extracts (100µg/mL) before LPS stimulation for another 24h. Untreated (Un), LPS, and Dic were used as negative, positive, and inhibitor controls. Asterisk (*) was indiciated as sinificant data.

in reduced pro-inflammatory cytokine production [48]. Diclofenac (Dic) is a well-known COX-2 inhibitor that prevents the conversion of arachidonic acid to prostaglandin E2, thereby reducing pain during inflammation and controlling cancer cell proliferation [49], PL extracts seemingly elicited an inflammatory inhibition mechanism similar to Dic. Hispolon from *Phellinus lonicerinus* exhibited phytoestrogenic properties by suppressing aromatase and COX-2 activities in MCF-7 cells, leading to cell growth arrest [50]. Similar to the data demonstrated by Chang et al. [51], who reported that *Phellinus gilvus* (PG) has strong DPPH free radical scavenging activity and inhibits LPS-induced NO production and COX-2 mRNA expression in RAW264, Hence, PL extract would enrich bioactive compounds containing drug-like properties for alternative medicine applications.

4 Conclusion

Phellinus linteus (PL) mushroom spawns were cultivated for over 18 months, producing fruiting bodies that resemble wood, ranging from light brown to dark brown. Harvesting at different growth stages showed that the highest growth was observed in the Bottom zones at 18 months. PL extracts demonstrated significant antioxidant, anti-inflammatory, and anticancer activities, primarily due to their phenolic content. Non-significant difference of TPC and antioxidant during the cultivation indicated that the mushrooms can be harvested any time from 3-18 months for the bioactive compounds. However, longer cultivation time yield a higher mushroom weight and yield of the TPC and antioxidant. Further investigation is necessary to identify the bioactive chemicals present in PL extracts at various stages of the growing process throughout the cultivation period. It underscores the need for further research in this area, which could potentially lead to the discovery of new bioactive substances and their applications.

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