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RESEARCH ARTICLE

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Artificial Cultivation Characteristics and Bioactive Effects of Novel *Tropicoporus linteus* (Syn. *Phellinus linteus*) Strains HN00K9 and HN6036 in Korea

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ABSTRACT

Phellinus strains were collected from different areas in Korea. Of them, the fast mycelial growing strains were artificially cultivated on the oak logs to produce fruiting body. The varieties, *Phellinus linteus* ASI26099 (Korea Sanghwang) and *P. baumii* PBJS (Jangsoo Sanghwang) were grown under the same conditions as controls. Their cultivating characteristics including mycelial colonization, pinhead formation, and fruiting body formation rate were investigated on the logs. Basidiocarps of *Phellinus* strains HN00K9, HN6036, and ASI26099 were concentrically zonate and shallowly sulcate, and dark chestnut showing typical characteristics of *Tropicoporus linteus* (synonym: *P. linteus, Inonotus linteus, polyporus linteus*), which is distinguishably different to PBJS. HN00K9 showed the highest yield of fruiting body among the mushroom strains. The β-glucan content in fruiting bodies of HN00K9 was 20% higher than those of other strains. Bioactive effects of polysaccharide samples from fruiting bodies of *Phellinus* strains, HN00K9, HN6036, ASI26099, and PBJS were assessed on cell viability and cytokine (IL-6 and TNF-α) inhibition and finally on anticancer to different human cancer cells.

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1. Introduction

A medical mushroom, *Phellinus linteus* (PL) Teng is taxonomically classified into genus *Phellinus*, Hymenochaetcea, Aphyllophorales, Hymenomycetes, Basidiomycota, and a wood decay fungus that grows on different trees including trunk of popular, oak, and mulberry [1]. The mushroom is mainly distributed in tropical America, Africa, and East Asia. PL has been used with traditional oriental medicine in particularly Japan, China, and Korea [2]. PL has been known as the most powerful antitumor mushroom by showing 96.7% inhibition rate of ovary cancer [3]. Ever since, a variety of medical efficacies including anti-inflammatory [4,5], immunomodulatory [6–8], antioxidative [9–11], antimicrobial, and antiviral [4,12,13], as well as anticancer [1,14–16], antidiabetic [17,18], hepatoprotective [19], and neuroprotective [20], have been elucidated from *P. linteus* strains. Polysaccharide fractions isolated from *P. linteus* were found to be related to the increased activity of immune cells such as the production of cytokines by macrophages and B-cells or the increased cytotoxic activity of natural killer cells [6,7]. In addition, the styrylpyrones-class phenolic compounds have been isolated from genus *Phellinus* including *P. linteus* and *P. baumii* and have bioactive functions including antioxidant, anti-inflammation and anticancer [11,21]. *P. linteus* named Sanghwang, Sanghaung, and Meshimakobu mushrooms in Korea, China, and Japan, respectively. Recently, through the taxonomic studies, several species of *Phellinus*, viz., *baumii, linteus*, *vaninii lonicericola*, *lonicerinus,* and *weigelae* were transferred to the genus *Inonotus* or *Sanghuangporus* [2,22]. It was proposed that each *Inonotus* species should be involved in members of *I. linteus* complex, because of indistinguishable basidiocarp shape and the internal transcribed spacer (ITS) rDNA sequences [2,22]. So far, morphological features of fruiting bodies including basidiospore, microstructures, basidiocarp pileus, and pore surface have been proven to be important keys for classification [2,23]. Nevertheless, use of the morphology of fruiting bodies has a limitation in the classification because their characteristics are affected by the natural environmental conditions. Thus, phylogenetic relationships using ITS-rDNA and nLSU sequences have been employed and provided important clues

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in classification of *I. linteus* complex [2,24]. It was proposed that *P. linteus* isolated from tropical America and Africa based on ITS and LSU rDNA sequences and morphological features should be named as *Tropicoporus linteus* [24]. However, scientific names of *P. linteus* strains are still being named differently according to taxonomists based on the classical and molecular methods.

The mycelial materials of PL have mainly used to study their bioactive efficacies in the world, because fruiting bodies of PL are not always available. Artificial cultivation of PL can provide stable samples by supplying fruiting bodies in the same conditions. Therefore, *P. linteus* strains ASI 26099 and ATCC 26710 had been studied for its artificial cultivation to produce fruiting body on the logs of different trees [25,26]. Artificial cultivation conditions, including cultural environments, mycelial colonization, fruiting body, pinhead formation using tree logs, have been studied on each *P. linteus* strain, but, these *P. linteus* strains were found to be inadequate on cultivation due to the long cultivation period of more than 3 years and low fruiting body yield. As the result, *P. linteus* strain that can artificially cultivate the fruiting body with high yield still remained to be not isolated. Alternatively, PBJS (*P. baumii*, Jangsoo Sanghwang) has popularly cultivated with stable yield in Korea. The PBJS showed rapid mycelial growth on the nutrient media compared to the *P. linteus* strains [25,26] and subsequently its mycelia are colonized well on the wood logs and resulted in fruiting body formation with high ratio. Accordingly, it was reasonably considered that the mycelial growth rate is a critical point to artificially cultivate *P. linteus*. *Phellinus* strains have been isolated from different locations in Korea and of them, several strains classified into *P. linteus* based on ITS sequences and of them *P. linteus* HN6036 showed rapid growth rate of mycelium (unpublished data). A *Phellinus* sp. HN00K9 was isolated from mulberry tree in Korea and was classified as novel *P. linteus* based on morphological characteristics of the fruiting body, including spore size, hyphal system, and pore shape and ITS rDNA homology of 98% with those of species [27]. Interestingly, the *P. linteus* strains HN00K9 was shown to exhibit rapid mycelial growth rates similar to that of PBJS.

The aim of present study is to evaluate the cultivation characteristics in the fruiting body production and bioactivities of novel *Tropicoporus linteus* (*P. linteus*) strains HN00K9 and HN6036 that exhibited fast mycelial growth rates. *P. linteus* strains HN00K9 and HN6036, including Sangwhang mushroom varieties, *P. linteus* ASI26099 (Korea Sanghwang) and *P. baumii* PBJS (Jangsoo Sanghwang) were artificially cultivated on the logs under the same conditions and their cultivation characteristics, including pinhead formation, morphologies and productivity of fruiting bodies were investigated. Furthermore, polysaccharide samples extracted from each fruiting body were subjected to assess bioactive effects on cytotoxicity, cytokine production, and anticancer activity

2. Materials and methods

2.1. Phellinus strains and mycelial growth rate

Phellinus strains that show fast mycelial growth rate were used for this study (Table 1). In addition, *T. linteus* ASI26099 (variety, Korea Sangwhang) and *P. baumii* PBJS (variety, Jangsoo sangwhang) were obtained from National Institute of Agricultural Science (NAS), RDA, Korea and used as controls. In addition, *P. linteus* strain HN00K9 has been deposited as a patent strain of KACC93057P in Korea Agricultural Culture Collection (KACC, RDA, Korea). The mycelia of the mushroom species were grown on yeast glucose malt (YGM) mediiu [27] at 25 °C for 20 days.

2.2. Spawn fabrication

Mycelial mat of grown on YGM medium was grounded in sterilized water with homogenizer and used as inoculum for spawn production. For producing sawdust spawn media, the sawdust–rice bran mixture (8:2) was thoroughly mixed with water, put into 850 mL-polyethylene bottles, sterilized at 121 °C for 90 min, and cooled to 20 °C. The wheat grains were soaked in water for a day and sterilized at the condition above mentioned and was added in the bottles, incubated at 25 °C for about 30 days, and then used as grain spawn medium. The mycelial inoculums (25 mL) were inoculated in sawdust and

Table 1. *Phellinus* strains used in this study.

Strains	Sources	[a]Accession No.	rDNA-ITS sequence identity (%)	[b]Mycelial growth rate (cm)
HN00K9	Korea/Chungnam	MT828027	*Phellinus linteus* **SFC970527-1** (97%)	90.0 ± 0.0^{a}
HN6036	Korea/Gyeongbuk	AV839837	*Tropicoporus linteus* SFCC10208 (99%)	79.3 ± 0.3^{b}
ASI26099	Korea/RDA	MT827969	*Tropicoporus linteus* FS6656179 (99%)	67.6 ± 0.1^{c}
PBJS	Korea/RDA	MT827970	*Inonotus baumii* PB0808 (99%)	74.6 ± 0.4^{b}

ASI: National Institute of Agricultural Science, RDA, Korea; HN: Hankyong National University, Korea.
Different letters in the same column indicate significant difference at $p < 0.05$ according to Duncan's multiple range test ($n = 3$).
[a]rDNA-ITS sequence in GenBank.
[b]The mycelial growth rates were measured in 15 days of inoculation.

grain spawn media and incubated for about 30 days. In addition, liquid spawn was prepared by using mycelia cultured in YGM broth for 20 days. The mycelial liquid inoculum (25 mL) was added in the bottles and incubated at 25 °C for about 30 days and then used as spawn for inoculums on the wood logs.

2.3. Log cultivation

The short wood logs (20 cm) with diameter of 12–15 cm were made from *Morus albab* (mulberry), *Quercus acutissima* (oak), and *Betula schmidtii* (birch). The logs were put into polyethylene bags (30 cm × 50 cm), sterilized at approximately 100 °C for 24 h, and then cooled at 20 °C. The spawns were inoculated on the logs and incubated at 22–25 °C for 100 days. The logs with well-established mycelia were scraped with iron brush to remove the skin of the mycelium and placed on the ground covered with small gravel in mushroom houses. Water was supplied by a sprinkler for 30 min a day to provide moisture.

2.4. Scanning electron microscopy (SEM) analysis

The dried *Phellinus* fruiting bodies were cut into 5 × 5 × 5 mm, fixed to the side of the shade to be visible, and then coated in vacuum with white gold-palladium for 60 s. The samples were analyzed using a scanning electron microscope (TM-1000; Hitachi, Tokyo, Japan).

2.5. β-Glucan content

Fruiting bodies from *Phellinus* isolates were powdered to 100 mesh size and each powder sample of 100 mg was used to analyze glucan content. Total α and *β*-glucan were determined by a protocol provided by the Mushroom and Yeast *β*-glucan Assay Kit (Megazyme Int. USA).

2.6. In vitro cytotoxic activity

Direct cytotoxicity of polysaccharide (PS) from purified from fruiting bodies of *Phellinus* strains was assessed. Raw 264.7 cells were evaluated *via* MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Raw 264.7 cells (1×10^6 cells/mL) were transferred into 24 wells. After 12 h of incubation, PS was added to the cells by different concentrations and incubated for 30 min. MTT solution (5 mg/mL) was added in the cells and incubates them for 30 min. The remaining cultural liquid was removed from the wells and 1 mL of dimethyl sulphoxide (DMSO) was added to break the crystal formazan. Afterwards, the absorbency was measured with an ELISA reader (Tecan, Männedorf, Switzerland) at 517 nm after shaking for 5 min. Lipopolysaccharide (LPS) in 1000 ng/mL and saline solution was used as experimental control

2.7. Cytokine analysis

Cytokine activity was tested in Raw 264.7 cells (1×10^6 cells/mL) stimulated with 100 ng/mL of LPS. After incubation for 22 h, the concentrations of serums IL-6 and TNF-α were measured by ELISA method using cytokine ELISA kits (Komabiotech, Seoul, Korea) according to the manufacturer's protocol. TNF-α and IL-6 production in LPS-stimulated RAW264.7 cells by polysaccharides from fruiting bodies of *Phellinus* strains. Cells were incubated with or without LPS (100 ng/mL) in the presence of various doses (12.5, 25, 50, and 100 µg/mL) of PS for 24 h.

2.8. Anticancer activity

Human cancer cells including gastric cancer AGS, prostate cancer DU145, colon cancer HCT-116, and cervical cancer cells were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were maintained in RPMI 1640 medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco-BRL) and 1% anti-biotic (Gibco-BRL) at 37 °C in CO_2 incubator containing 5% CO_2 (Forma Steri-Cycle, Forma Therapeutics, Inc., Watertown, MA, USA). The cancer cells were seeded in 24-well culture plates at the initial cell density of 1 or 2×10^5 cells/mL and were cultured with the varying concentrations of culture plates. Cell morphology was monitored every day. The cell number and viability were assessed at 24 h by MTT assay. After removing MTT reagent, DMSO was added to each well and then absorbance of fomazan solution was read on a microplate reader. Cell growth was then expressed as the percent (%) of viable cells relative to the control reading (100%).

3. Results and discussion

3.1. Mycelial growth

Phellinus isolates have been collected in Korea and of them, eight strains were identified as *P. linteus* by ITS rDNA (data not shown). The *P. linteus* strains including *P. linteus* ASI26099 (variety, Korea Sanghwang) and *P. baumii* PBJS (Jangsu Sanghwang) that were artificially cultivated in Korea were grown on YGM media to assess their mycelial growth rate. Of them, *P. linteus* strains HN00K9 and HN6036 were more rapidly grown on the media

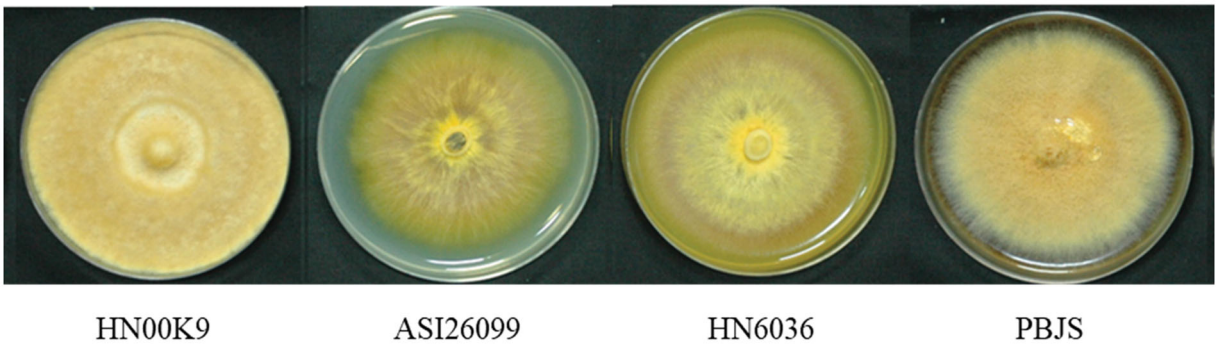

Figure 1. Morphological characteristics of mycelia of *Phellinus* strains grown on yeast malt glucose (YGM) media for 15 days at 25 °C.

when compared to PBJS and *P. linteus* ASI26099 showing mycelial growth diameter of 90.0 mm and 80.0 mm after 15 days of incubation (Table 1). The mycelium of HN00K9 and HN6036 formed a concentric rim, and rich aerial hyphae of golden yellowish color formed uniformly and thickly on the surface of the medium, whereas ASI26099 and PBJS had less aerial hyphae density and formed golden yellowish concentric rim around the central part (Figure 1). It was considered the rapid mycelial growth of HN00K9 and HN6036 can be available to artificially cultivate to produce fruiting body. Thus, the *P. linteus* strains, HN00K9 and HN6036, were used for artificial cultivation of this study, while Sanghwang varieties, ASI26099 and PBJS, were used as controls.

3.2. Mycelial colonization on logs

In the mushroom cultivation, mycelial growth period is required to be about 100 days or longer, causing bacterial and fungal contamination as a major obstacle factor on the mushroom cultivation. Thus, we tested effects of mycelial colonization and contaminations rate on the logs seeded with different spawn types. The grain, sawdust, and liquid spawns were respectively inoculated on each sterilized 100 oak log and the mycelial growth and the contamination rate were investigated in 100 days after inoculation. The spawns, wheat grain and oak sawdust seeded on the logs were contaminated with 12.6 and 17.6%, but the logs seeded with liquid spawn were mostly contaminated more than 95% (data not shown). Therefore, it was confirmed that the grain spawn had the best mycelial growth rate and the less contamination rate on the logs.

The grain spawns from HN00K9, ASI26099, HN6036, and PBJS were inoculated on the oak logs and then fully colonized mycelial rate on the log was investigated after 100 days of incubation. HN00K9 and PBJS were fully grown on the logs with rate of 95% and 96% with high mycelial density, whereas ASI26099 and HN6036 showed low mycelial colonization with 20–25% on the logs (Table 2). Actually, it was revealed ASI26099 and HN6036 needed incubation period of about 120 days for full mycelial colonization more than 80%. It was reported *P. linteus* strain ASI 26099 needs 4 months incubation for full mycelial colonization rate on the logs [25], showing good agreement to result of this study.

Table 2. Mycelial colonization ratio of *Phellinus* strains on the oak logs.

Strains	[a]Mycelial colonization (%)	[b]Mycelial Density
HN00K9	95	+++
ASI26099	20	++
HN6036	25	+
PBJS	96	+++

Rate of full mycerial colonization of *Phellinus* isolates was measured on oak logs in 100 days after inoculation of spawn.
[a]Mycelial colonization of *Phellinus* strains.
[b]Mycelial density: +, poor; ++, mediate compact; +++, compact.

3.3. Cultivation characteristics on the logs

Sanghwang mushroom, *P. linteus* have been known an excellent medicinal mushroom with a variety of bioactivities. Since fruiting bodies of *P. linteus* have been mainly dependent on the wild type mushrooms collected from natural environments, there is a limit to their continuous supply. Thus, artificial cultivation is necessary to stably supply them. Previously, some attempts were made to artificially cultivate the mushrooms [25,26], but failed to achieve success due to low production and long cultural period. This study aimed to find strain capable of artificial cultivation. HN00K9 and HN6036 that showed fast mycelial growth rates on the media were applied to artificial cultivation. PBJS and ASI26099 were included for artificial cultivation as controls for this experiment since their artificial cultivations have already been studied [25].

The oak logs that are fully colonized with mycelial growth derived from HN00K9, ASI26099, HN6036, and PBJS was used to produce fruiting bodies. As first step for cultivation, the mycelia mat on the logs were removed to stimulate fruiting body

formation by iron brush and then they transferred to the mushroom house and cultivated in conditions with 25–35 °C and humidity over 80%. The formation rates of pinhead and fruiting body were investigated on the logs. As shown in Table 3, pinhead of HN00K9 and PBJS formed in 70 days of cultivation on the logs and their pinhead formation rates were 95–96%, whereas ASI 26099 and HN6036 formed pinheads on them after 155 and 140 days of cultivation and their pinhead formation rate were less than 45–55%. Figure 2 shows the pinhead and fruiting body of HN00K9 and PBJS formed on surfaces of oak logs. It was defined that typical fruiting body of Sanghwang mushroom (*Inonotus linteus*) has pileus surface and pore surface [2]. Therefore, whether or not a fruiting body is formed on the log was determined based on the formation of basidiocarp pileus and pore surfaces. HN00K9 initially formed pileate fruiting bodies having pileus surface and pore surface more than 90% on the tested 100 oak logs after 160 days of cultivation, but PBJS did not form the fruiting bodies in the days of cultivation (Figure 2). The fruiting body formation rates of PBJS, ASI26099 and HN6036 exhibited 95% with 28% and 40%, respectively on the tested logs in cultivation period of 295 and 325 days (Table 3). Previously, it was reported that formation rate of pinheads and fruiting bodies from *P. linteus* strains took about a year and two years. [25,26]. On the other hand, the pinhead and fruiting body formation period of HN00K9 was very shortened to 70 and 160 days, so it was evaluated that cultivation was possible with high yield. Artificial cultivation condition of this study was performed with modification of method reported previously [25]. The method burying logs in soil was commonly used for cultivation of *P. linteus* ASI26099 for maintaining proper humidity, but can easily be contaminated with undesirable microbes in soil. If relative humidity over 80% is provided in the mushroom house, pinhead and fruiting bodies were evenly formed on side surfaces of the logs, otherwise, fruiting bodies will form in the log area adjacent to the ground. Currently, mushroom growers in Korea have mostly employed aero-cultivation method using PBJS in which the logs are hung on iron hooks. The method is advantageous to increase productivity of fruiting bodies

Table 3. Formation period and ratio of primordium and fruiting body of *Phellinus* strains on the oak logs.

	Primordium formation		Initial fruiting body formation	
Strains	Periods (days)	100 oak logs (%)	Periods (days)	100 oak logs (%)
HN00K9	70	90	160	90
PBJS	70	92	295	92
ASI26099	155	32	325	28
HN6036	140	46	325	42

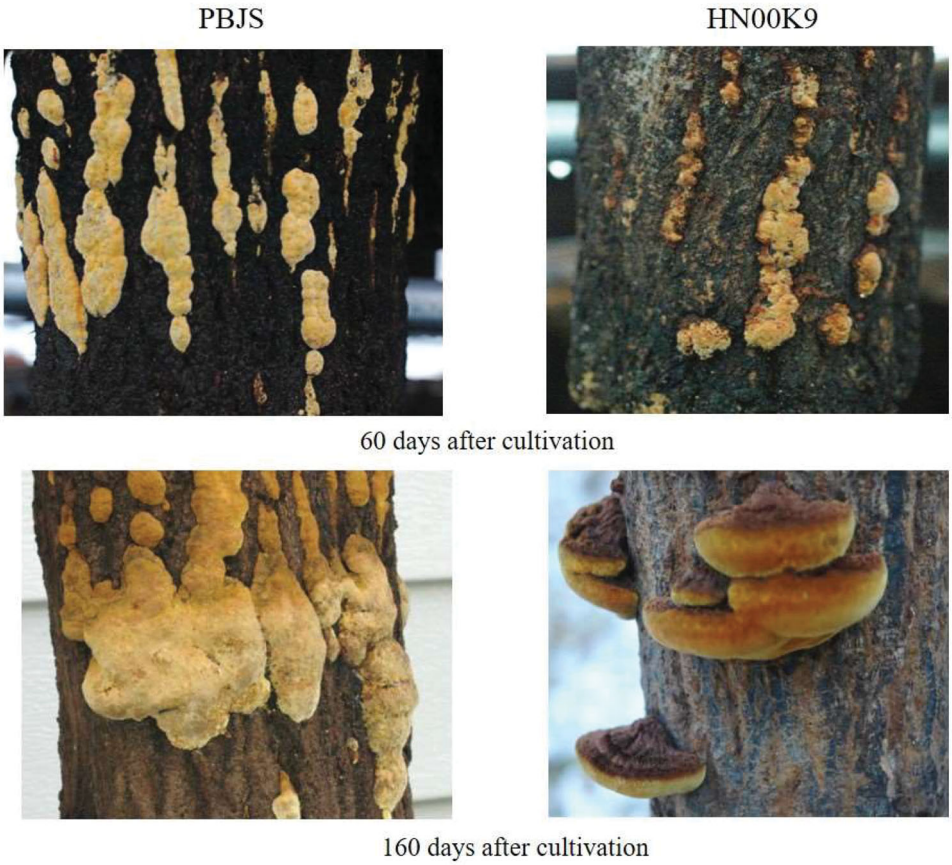

Figure 2. Formation of pinheads and fruiting bodies of *Phellinus* strains HN00K9 and PBJS on oak logs in a mushroom house.

by hanging on them many logs in limited mushroom house space. However, this cultivation method may result in incomplete fruiting bodies that have basidiocarp pileus on bottom surface of the logs without clear pore surface.

In addition, grain spawn of HN00K9 was seeded on different wood logs of *Quercus acutissima, Morus alba,* and *Betula schmidtii* and full mycelial colonization, pinhead and fruiting body formation rates were investigated on the logs. Mycelia of HN00K9 were colonized on the logs of *Quercus acutissima, Morus alba*, and *Betula schmidtii* with 95%, 100%, and 90%, respectively (Table 4). This result suggests that HN00K9 mycelia are significantly able to colonize on the different wood logs, although different primordium and fruiting body formation periods were required on the logs.

3.4. Morphological characteristics of fruiting bodies

Fruiting bodies of *Phellinus* strains, HN00K9, ASI26099, HN6036, and PBJS were observed after 2 years of cultivation under same condition of a mushroom house. As shown in Figure 3, basidiocarp pileus surface of HN00K9, ASI26099, and HN6036 were pileate semicircular, sessile, concentrically zonate, and shallowly sulcate showing typical characteristics of *P. linteus* [25,28], similar to morphological characteristics of *Inonotus sanghuang* reported by Wu et al. (2012). In addition,

Table 4. Effect of different logs on mycelial growth, primordium and fruiting body formation of *Tropicoporus linteus* HN00K9.

Logs from	[a]Mycelial colonization rate (%)	[b]Primordium formation (days)	[c]Fruiting body formation (days)
Quercus acutissima	95	60	155
Morus alba	100	90	180
Betula schmidtii	90	60	155

[a]Full mycerial colonization were observed on different logs after 100 days of spawn inoculation. [b]Primordium and [c]fruiting body formation were investigated on logs. Each 100 log was subjected for the experiment.

Basidiocarp pore surfaces of strains HN00K9, ASI26099, and HN6036 were densely sulcate with brownish yellow or yellowish brown colors, and formed the golden yellowish marginal zone. On the other hand, fruiting body of PBJS showed morphological features with dark yellowish color forming thick marginal zone and was distinctly different to those of the three *P. linteus* strains. The typical genus *Inonotus* refers to the annual basidiocarp and is characterized by the softer basidiocarp with a monomitic hyphal system. Instead, species in *P. baumii-P. linteus* are dimitic and have fairly hard basidiocarps which are generally perennial; hence HN00K9 can morphologically be considered as genus *Phellinus*. However, based on phylogenetic analysis, it has been strongly proposed that the *P. linteus-P. baumii* should be involved in genus *Inonotus* [2,22]. The *I. linteus* complex distributed in Asia is identified as six species. Each species in the *I. linteus* complex is very similar in shape and thus species name should be dependent on its collection hosts; that is, *I. baumii* (on Syringa), *I. lonicericola* (on Lonicera), *I. sanghuang* (mulberry tree, morus), *I. vaninii* (on Populus) and *I. weigelae* sp. nov. (on Weigela). *I. linteus* complex is distributed in boreal, temperate and subtropical to tropical zones. Accordingly, strain HN00K9 in this study that isolated from Korea of temperate zone can be involved into *I. linteus* complex based on morphological characteristics of basidiocarp and phylogenetic relationship by previous reports [2,22]. Nevertheless, Zhou et al. [24] reported that *P. linteus* isolated from tropical zones such as tropical America and Africa should be named as *Tropicoporus linteus* based on ITS and LSU rDNA sequences. However, relying on the molecular analysis without considering the morphological characteristics of the fruiting bodies of *P. linteus* strains, it seems that there is still a great deal of confusion in the nomenclature of *P. linteus* associated with Sanghwang mushroom. Accordingly, it was considered that the mushrooms produced by artificial cultivation in this study can be used as a very useful

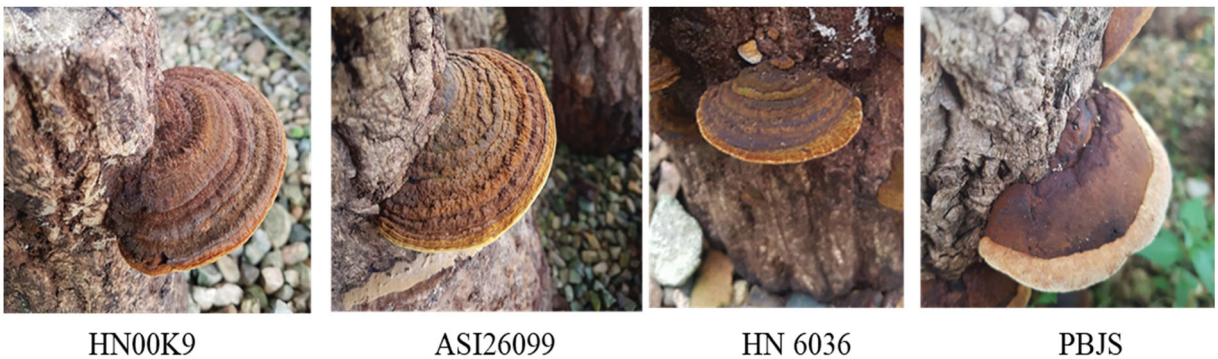

Figure 3. Morphological characteristics of fruiting bodies produced from *Phellinus* strains HN00K9, ASI26099, HN6036, and PBJS formed on the oak logs.

Table 5. Morphological characteristics of fruiting bodies of *Phellinus* isolates formed on the oak logs.

Strains	Horizontal length (cm)	Vertical length (cm)	Thickness (cm)	Dry Weight (g)	No. fruiting body per a log
HN00K9	9.70 ± 2.52^{a}	4.65 ± 1.38^{a}	1.24 ± 0.28^{b}	19.00 ± 7.05^{a}	9.24 ± 2.41^{c}
PBJS	8.32 ± 2.18^{b}	3.27 ± 0.71^{b}	1.91 ± 0.51^{a}	15.52 ± 5.59^{b}	11.14 ± 3.21^{b}
ASI26099	6.16 ± 1.92^{c}	2.9 ± 0.48^{b}	1.92 ± 0.37^{a}	5.08 ± 2.25^{c}	3.25 ± 0.24^{d}
HN6036	4.06 ± 0.93^{d}	2.11 ± 0.51^{c}	0.67 ± 0.18^{c}	5.65 ± 1.89^{c}	20.6 ± 0.91^{a}

The fruiting bodies of each *Phellinus* strain were harvested from 100 oak logs after 2 years of artificial cultivation.
Different letters in the same column indicate significant difference at $p < 0.05$ according to Duncan's multiple range test ($n = 3$).

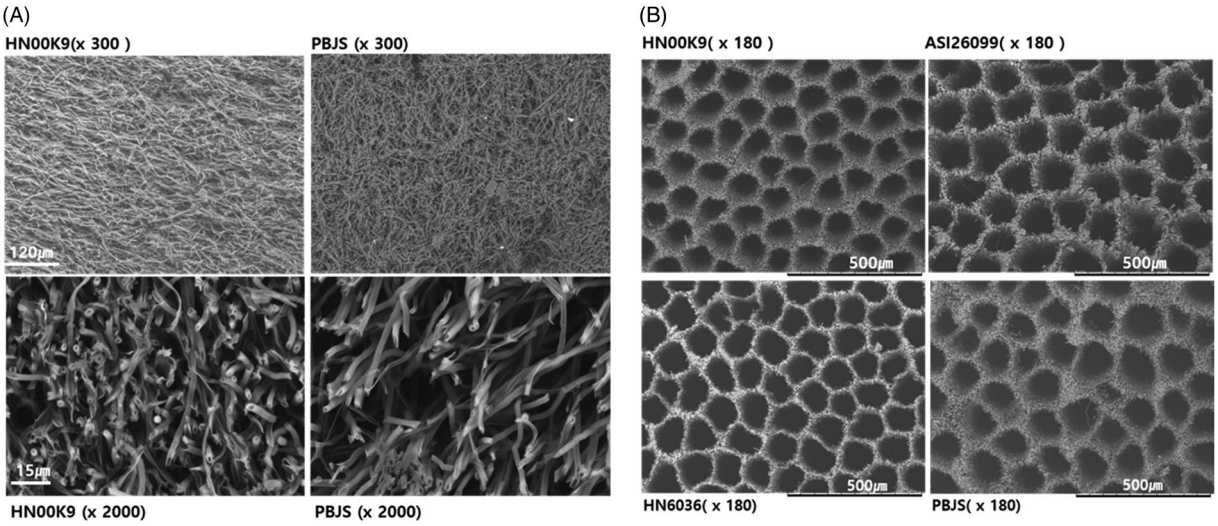

Figure 4. Scanning electron microscopic features of (A) lateral sections and (B) pore surface of basidiocarps derived from *Phellinus* strains HN00K9, ASI26099, HN6036, and PBJS.

material for the morphological identification of Sanghwang mushrooms related to *P. linteus* and *P. baumii* strains.

The morphological features including diameter, thickness, and weight of fruiting bodies of HN00K9, ASI26099, HN6036, and PBJS, which were formed on the oak logs after two years of cultivation under the same mushroom growing conditions, were investigated. As shown in Table 5, the horizontal lengths of HN00K9 and PBJS were 9.7 and 8.3 cm, while ASI 26099 and HN6036 were 6.1 cm and 4.0 cm, respectively. The vertical lengths were 4.6 and 3.2 cm for PLHS and PBJS, and 2.9 and 2.1 cm for ASI 26099 and HN6036, respectively. The thickness of fruiting body was 1.9 cm for PBJS and ASI26099, which was larger than HN00K9 and HN6036. The average weight of one of HN00K9 fruiting bodies was the highest measured at 19 g and the next PBJS was 15 g. On the other hand, ASI26099 and HN6036 weighed 5 g. Consequently, the HN00K9 and PBJS yielded 87.7 g and 62.8 g per an oak log, showing high productivity, while ASI26099 and HN6036 resulted in low yield of 15 g and 30 g, respectively. Until now, *P. linteus* strains, ASI26099 and ATCC26710 has been used for producing fruiting body by artificial cultivation [25,26], but their poor mycelial growth and fruiting body formation rate, and long-term cultivation period were limited factors in producing the fruiting bodies. In this study, experimental cultivation results suggest *P. linteus* HN00K9 is able to stably produce fruiting bodies because of its high yield and fruiting body formation rate on the logs.

It was known that genus *Inonotus* form the annual basidiocarp, monomitic hyphal system, and softer basidiocarps, while *P. baumii* and *P. linteus* are dimitic and have fairly hard basidiocarps which are generally perennial. HN00K9 has stronger hard woody characteristics than PBJS. Thus, lateral sections of fruiting bodies from HN00K9 and PBJS were observed with scanning electron microscopy (SEM). The tiny mycelia-like threads in fruiting body of ASI26099 are densely connected to each other, while PBSJ is loosely (Figure 4(A)). The tissue density of HN00K9 was distinctly compared to PBJS at high magnification (×2000). The result suggests the hardness of HN00K9 fruiting body is contributed to be densely network of the hyphal threads connected to each other in tissue. Figure 4(B) shows SEM photograph of the basidiocarp pores derived from HN00K9, HN6036, ASI26099, and PBJS. Their basidiocarp pores were morphologically circular or angular with similar sizes. In previous study, wild type fruiting body of HN00K9 collected in Korea was hard woody and its basidiocarp pileus surface was concentrically zonate and grooves and had chestnut color, showing typical morphological feature of the reported *P. linteus* strains [27]. In its microscopic observation, the surface pore of basidiocarp was golden yellowish color with circular or

angular and was 5–7 per mm in size, exhibiting morphological characteristics similar to HN00K9 of this study.

In conclusion, morphological features of HN00K9, ASI26099, and HN6036 were resemble to those of previously reported *P. linteus* strains [2,25–28], but was not to that of *P. baumii* PBJS.

3.5. β-Glucan content

Total glucan content in fruiting bodies of HN00K9, PBJS, ASI26099, and HN6036 was respectively measured to be 32.08, 26.62, 24.65, and 23.64 mg per 1 g of fruiting body (Table 6). The content of *β*-glucan accounted for more than 97% of total glucan, and the content of *β*-glucan was the highest in HN00K9 as 31.25 mg/g. It has been reported that the crude polysaccharide purified from the mycelial culture of *P. linteus* stimulated the proliferation of T lymphocytes and the humoral immune function including acting as a polyclonal activator on B cells, as inhibiting tumor growth and metastasis [28,29]. A major polysaccharide component of mushrooms is cell wall derived (1 → 3; 1 → 6)-*β*-D-glucan, the most common biological effect of which is immunomodulation [8,30]. It is known that *β*-glucan has strong antitumor activities but not α-glucan. Consequently, it is natural that the crude polysaccharide would be similar to *β*-glucan, which had a strong effect on the immune responses.

Table 6. β-glucan content in fruiting bodies of *Phellinus* strains.

	Glucan content (mg/1g of fruiting body)		
Strains	Total glucan	α-glucan	β-glucan
HN00K9	32.08 ± 0.51^{a}	0.83 ± 0.01^{a}	31.25 ± 0.52^{a}
PBJS	26.62 ± 2.62^{b}	0.83 ± 0.02^{a}	25.79 ± 2.63^{b}
ASI26099	24.65 ± 2.27^{c}	0.83 ± 0.02^{a}	23.82 ± 2.29^{c}
HN6036	23.64 ± 0.35^{c}	0.82 ± 0.01^{a}	22.82 ± 0.36

The results are represented by the mean ± SD of values obtained from tree replication ($n = 3$). Different letters in the same column indicate significant difference at $p < 0.05$ according to Duncan's multiple range test ($n = 3$).

3.6. Cytotoxicity

The polysaccharide samples (PS) extracted from HN00K9, ASI26099, HN6036, and PBJS were evaluated for the cytotoxicity in LPS-activated RAW 264.7 cell using MTT method. As shown in Figure 5, PS exhibited proliferative activity to the RAW 264.7 cells with a concentration-dependent manner at 125, 250, 500, and 1000 μg/mL without cytotoxicity on the cells. It was confirmed that the derived immune cells were elevated. However, it did not show toxicity, but the cell proliferation in the PBJS treatment group was reduced by 20% in the concentrations. On the other hand, HN6036 treated cell viability was more than 40% less than other strains. Lipopolysaccharide (LPS) is major compound of outer membrane of bacterial strain and act as endotoxin [7], which promotes the secretion of pro-inflammatory related nitric oxide synthase (iNOS).

3.7. Effects of on cytokine production

Proinflammatory cytokines, interleukins (IL-1, IL-2, IL-3, IL-4, and IL-6), tumor necrosis factor alpha (TNF-α), interferon gamma (IFNγ), and granulocyte-macrophage colony-stimulating factor (GM-CSF) are excreted from immune cells like helper T cells and macrophages that induce inflammation [7,31,32]. We examined whether polysaccharides extracted from HN00K9, ASI26099, HN6036, and

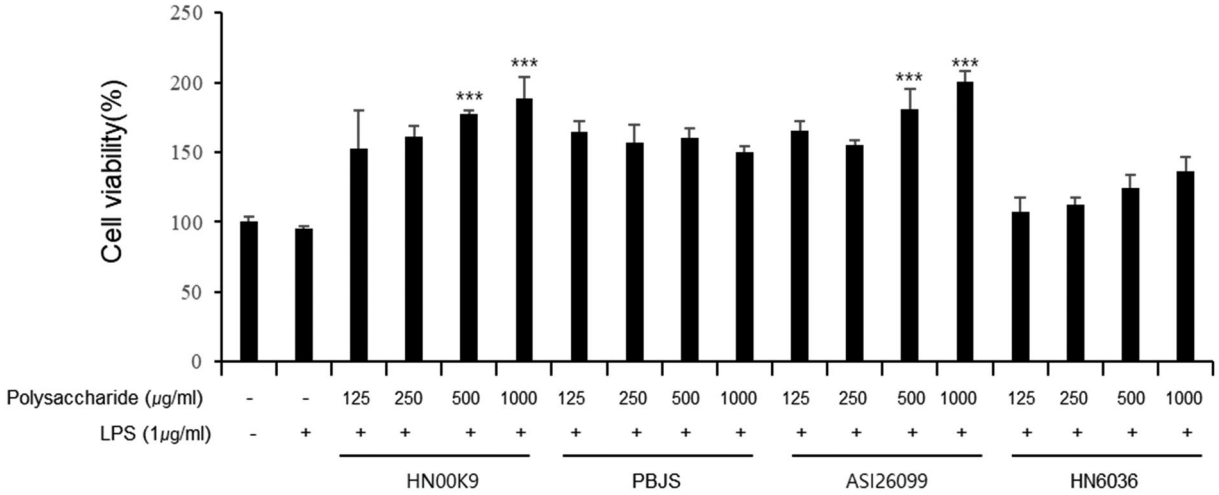

Figure 5. Viability of RAW 264.7 cells in polysaccharide from fruiting bodies of *Inonotus linteus* complex strains HN00K9, ASI 26099, HN6036, and PBJS. The cells were treated with different concentrations of polysaccharides from fruiting bodies for 24 h. LPS activated cell viability was determined by MTT assay. The values are the mean ± SD from three independent experiments. Significant differences between the polysaccharide-treated and untreated groups were analyzed using Student's *t*-test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

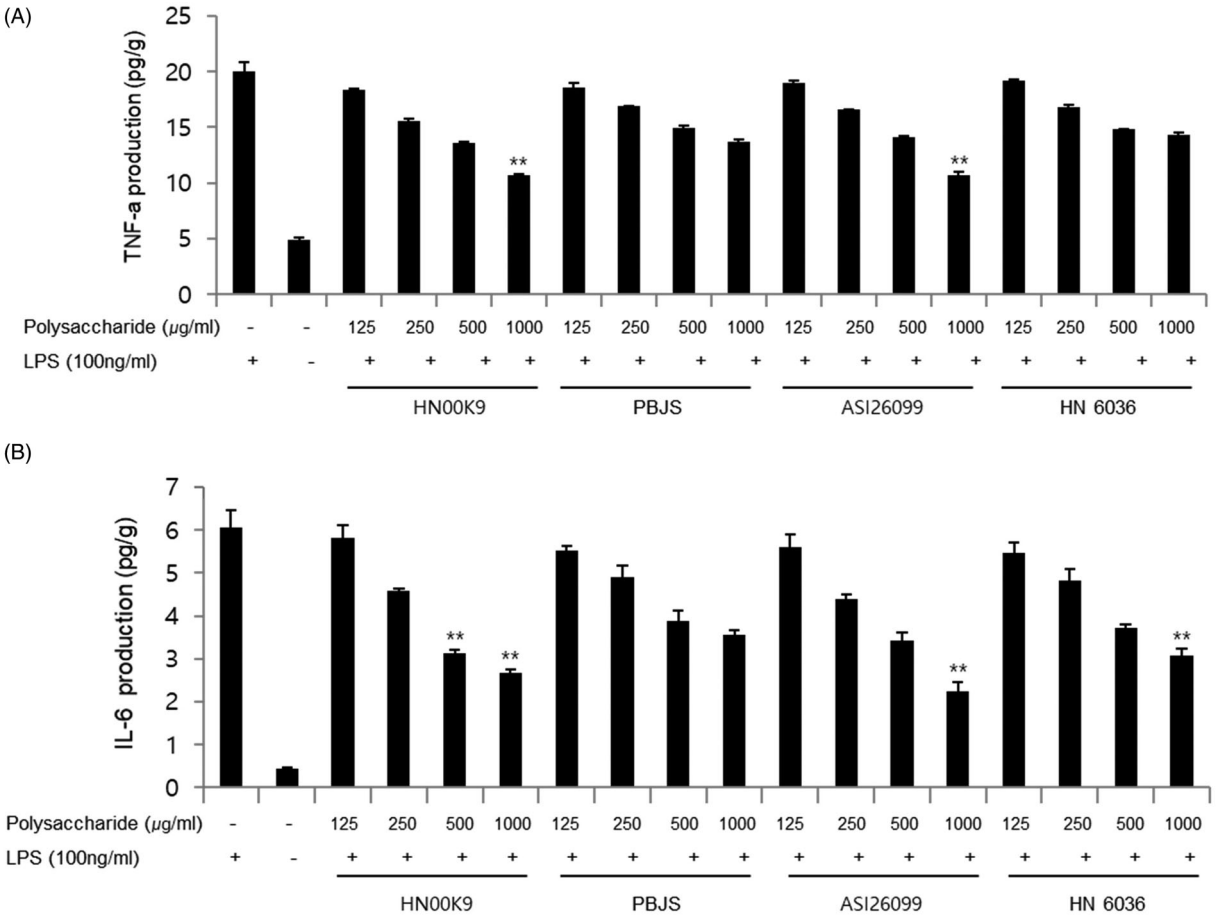

Figure 6. Effect of polysaccharides from fruiting body of *Inonotus linteus* complex strains on the production of TNF-α and IL-6 in RAW 264.7 cells. RAW264.7 cells were treated with different concentrations (125, 250, 500, 1000 μg/mL) of polysaccharides extracted from fruiting bodies of *Phellinus* strains, HN00K9, ASI 26099, HN6036 and PBJS for 24 h under treatment of LPS (100 ng/mL). Each value is expressed as mean ± SD in triplicate experiments. Significant differences between the polysaccharide-treated and untreated groups were analyzed using Student's *t*-test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$).

PBJS inhibit cytokines, IL-6, and TNF-α antigen-antibody reaction using ELISA Kit. As shown in Figure 6(A,B), cytokine production of IL-6 and TNF-α was inhibited by 15–60% in LPS-activated RAW264.7 cells by the polysaccharide samples on concentrations of 250, 500, and 1000 μg/mL. The polysaccharides of HN00K9 and ASI26099 showed a relatively high inhibition rate compared to them of other strains. Treatment with the polysaccharidess decreased the production of the inflammatory mediators, cytokines, IL-6, and TNF-α. The results suggest that the inhibition of inflammation by the polysaccharides is related to the downregulation of proinflammatory cytokines, IL-6 and TNF-α. In the immune response, a large amount of TNF-α is expressed in macrophages against invading pathogens and activates neutrophils and induces production of oxygen free radicals, proteolytic enzymes, IL-1, and IL-6 [1]. Mushroom compounds exert their immune-modulating properties through a variety of molecular mechanisms and TNF-α performs a tumoricidal activity and induces other cells of the innate immune system [1,31]. Therefore, cytokines are the messengers of the immune system and secreted by immune cells, to regulate innate and adaptive immune system.

3.8. Anticancer effects to human cancer cells

Human cancer cells including gastric cancer AGS, prostate cancer DU145, colon cancer HCT-116, and cervical cancer HeLa cells were treated with polysaccharides extracted from fruiting bodies of HN00K9, ASI26099, HN6036, and PBJS at 125, 250, 500, and 1000 μg/mL and cell growth/viability was determined in 12 h by MTT assay. The polysaccharides from each *Phellinus* strains inhibited growth of the cancer cells in a concentration-dependent manner (Table 7). Notably, HN00K9 was higher inhibitory effect of 41–44% at 1000 μg/mL of polysaccharides in the cancer cells (HCT-116, AGS, and DU145) than them of other strains, whereas PBJS showed the highest inhibitory effect in cancer cell HeLa. These results suggest that HN00K9 has a higher anticancer effect (reducing/inhibiting cell growth) in the cancer cells than other *Phellinus* strains.

The polysaccharides from *P. linteus* (PLP) modulate the immune system to fight tumors and other

Table 7. Inhibition of human cancer cells by polysaccharides from fruiting bodies of *Phellinus* strains.

		Inhibition rate (%) on μg/mL			
Cancer cells	Strains	125	250	500	1000
HCT116	AI26099	4.2 ± 7.0^{c}	11.0 ± 5.8^{c}	21.5 ± 5.2^{d}	36.6 ± 5.4^{b}
	HN00K9	16.7 ± 6.1^{a}	22.7 ± 7.2^{a}	30.9 ± 6.7^{b}	41.8 ± 2.1^{a}
	HN6036	17.4 ± 4.8^{a}	22.8 ± 5.5^{a}	38.1 ± 8.8^{a}	41.4 ± 2.5^{a}
	PBJS	11.7 ± 6.9^{b}	21.4 ± 2.4^{b}	28.3 ± 3.6^{c}	36.7 ± 4.3^{b}
AGS	AI26099	5.0 ± 1.1^{d}	$16.0 \pm 7.2^{b,c}$	28.7 ± 3.8^{b}	34.0 ± 7.2^{b}
	HN00K9	9.4 ± 6.5^{c}	14.6 ± 3.4^{c}	24.5 ± 1.7^{c}	40.3 ± 7.0^{a}
	HN6036	$16.4 \pm 6.2^{a,b}$	24.8 ± 8.1^{a}	34.9 ± 5.4^{a}	$39.5 \pm 4.0a^{b}$
	PBJS	19.1 ± 5.0^{a}	20.2 ± 6.0^{b}	24.9 ± 4.1^{c}	25.1 ± 1.0^{c}
DU145	AI26099	26.1 ± 6.1^{d}	38.6 ± 6.4^{a}	38.5 ± 4.8^{b}	40.3 ± 6.5^{b}
	HN00K9	32.6 ± 5.1^{b}	39.4 ± 7.3^{a}	41.9 ± 4.8^{a}	44.8 ± 2.8^{a}
	HN6036	29.6 ± 3.8^{c}	31.6 ± 1.0^{b}	37.8 ± 8.5^{b}	$41.0 \pm 3.1^{a,b}$
	PBJS	41.8 ± 2.0^{a}	$36.2 \pm 7.6^{a,b}$	36.5 ± 8.4^{b}	37.2 ± 6.1^{c}
HeLa	AI26099	2.5 ± 7.6^{d}	5.9 ± 4.4^{c}	6.6 ± 6.1^{c}	8.9 ± 2.8^{b}
	HN00K9	4.6 ± 1.2^{c}	6.3 ± 1.2^{c}	8.8 ± 5.3^{b}	11.9 ± 8.0^{a}
	HN6036	6.5 ± 4.1^{a}	7.1 ± 4.8^{b}	$7.1 \pm 1.2b^{c}$	7.9 ± 2.1^{b}
	PBJS	5.9 ± 2.5^{b}	9.1 ± 6.4^{a}	13.2 ± 3.1^{a}	13.9 ± 5.0^{a}

HCT116: human colon cell; AGS: human stornach cancer; DU145: human proatate cancer; HeLa: human cervix cancer. The results are represented by the mean ± SD of values obtained from tree replication ($n = 3$).
Different letters in the same column indicate significant difference at $p < 0.05$ according to Duncan's multiple range test ($n = 3$).

diseases [28,29,32,33]. These include augmenting the immune system through stimulating lymphocytes, NK cells, and macrophages, enhancing cytokine production, inhibiting proliferation of cancer cells, promoting apoptosis, and blocking angiogenesis, in addition to being cytotoxic to cancer cells [1,31]. It was reported the extract from *P. linteus* exhibited potent anticancer activity, leading to a significant (40–80%) growth reduction in all 10 cancer cells [15]. PLP inhibited tumor growth and reduced the frequency of pulmonary metastasis and is not directly toxic to cancer cells [1]. Apoptosis is fundamental process essential for cancer cell death. Caspase 3 is important in the propagation of apoptotic signaling [5,34]. It has also been known that PLP is able to suppress tumors *in vitro* either indirectly by enhancing the host's immune system, or directly by inducing apoptosis in tumor cells [33].

As a result of this study, it was confirmed that the HN00K9 and HN6036 strains had faster mycelial growth and higher mycelial density than the other strains. The fruiting bodies of HN00k9 and HN6036 formed oak logs showed similar morphological characteristics to Korea Sanghwang (ASI26099), which was consistent with the morphological characteristics typical of the previously reported *P. linteus* strain. So far, the only cultivation study of Sanghwang mushrooms is Korea Sanhwang (ASI26099), but the forming rate of fruiting body is very low, so it has been found that there is no economic feasibility in the mushroom production. Therefore, it is known that Jangsu Sanghwang (PBJS) is mainly being grown in Korea and China. In this study, HN00K9 not only showed comparable production to PBJS, but also showed that bioactivity such as immunity, inflammation, and cancer was superior to other strains in this study. The styrylpyrone-based polyphenol compounds, davallialactone, hispidin, hypholomine B, Interfungin A, and Inoscavin A were identified in fruiting bodies of HN00K9 by HPLC analysis, but not in mycelium-derived extract, showing many differences in fruiting body and mycelium on polyphenol components [35]. The phenolic component extracted from fruiting body of HN00K9 was found to have higher antioxidant activity than other mushrooms on analysis of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbisthiazoline-6-sulfonic acid) (ABTS) radical scavenging activities and the ferric reducing antioxidant power (FRAP) [36]. In addition, fruiting body of HN00K9 derived hypholomine B and davallialactone compounds showed proliferative activity without cytotoxicity over 50% LPS (lipopolysaccharide)-activated RAW264.7cell (data not shown). The compounds inhibited NO production of LPS activated RAW 264.7 cell and the cytokines, IL-6 and TNF-α production of 83–93%, showing their bioactive effects on anti-inflammation and immunity in the cells and further they showed inhibition rate of 42–50% in HCT116 human colon cancer cells (Data not shown).

In conclusion, *T. linteus* (*P. linteus*) HN00K9 was considered to be a novel Sanghwang mushroom variety capable of producing fruiting body with a high yield, and further has a high bioactive effect against anti-oxidation, anti-inflammatory, immune and anti-cancer activities without cytotoxicity. HN00K9, which exhibits the high yield and bioactivity of fruiting bodies, will be able to be used as a new Sanghwang mushroom variety, originated from *P. linteus* strain. This study is the first report of comparative analysis on cultivation characteristics and physiological activity effects using fruiting bodies obtained by simultaneously cultivating *P. linteus* strains and *P. baumii* under the same conditions.

Disclosure statement

No potential conflict of interest was reported by author(s).

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