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DETECTION OF SECONDARY METABOLITE OF *MYCENA PELIANTHINA* GROWTH IN VARIOUS LIQUID MEDIUM

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ABSTRACT

Mushrooms which is mostly belong to the phylum Basidiomycota have been recognized as functional foods and a source for the nutraceuticals. Genus Mycena is one of mushrooms, which has been investigated for its secondary metabolites. *Mycena pelianthina* produces pelianthinarubins A, pelianthinarubins B, muscarin, and epimuscarin from alkaloid group. The purposes of this research were to determine the effects of composition of medium and incubation time on production of mycelial biomass of *M. pelianthina* in liquid medium and to know the group of secondary metabolite compounds produced. This research was done experimentally using a Factorial Completely Randomized Design consisted of 12 treatments and three replications. The first factor was medium type, which were Mushroom Complete Medium (MCM), Potato Dextrose Yeast Broth (PDYB), and Yeast Malt Extract Medium (YM). The second factor was the incubation time consisting of 15, 20, 25, and 30 days. The observed main parameters were the dry weight of mycelial biomass and the secondary metabolite groups. The dried weights of mycelia were analyzed using analysis of variance (ANOVA), continued with Duncan test at a 95% confidence level. The best growth of *M. pelianthina* mycelium was produced on PDYB medium at incubation time of 20 days with an average mycelial dry weight of 0.92 g/100 mL. *M. pelianthina* contained secondary metabolite compound groups of alkaloids, terpenoids, and flavonoids.

Keywords: *Alkaloid, flavonoids, liquid medium, mycena pelianthina, secondary metabolite, terpenoids*

ABSTRAK

Jamur pangan merupakan salah satu bahan pangan yang dikenal sebagai pangan fungsional dan sebagai sumber nutrasetikal. Salah satu jamur pangan yang dikenal menghasilkan senyawa aktif sebagai metabolit sekunder adalah jamur *Mycena pelianthina*. Jamur ini dikenal menghasilkan senyawa metabolit sekunder pelianthinarubins A, pelianthinarubins B, muscarin, and epimuscarin dari kelompok alkaloid. Tujuan penelitian ini adalah untuk mengetahui pengaruh medium pertumbuhan dan waktu inkubasi terhadap bobot miselia *M. pelianthina* serta mengetahui golongan senyawa metabolit sekunder yang dihasilkannya. Penelitian ini dilakukan secara eksperimental menggunakan Rancangan Acak Lengkap Faktorial, dengan 12 perlakuan dan tiga kali ulangan. Faktor pertama meliputi jenis medium, yaitu *Mushroom Complete Medium* (MCM), *Potato Dextrose Yeast Broth* (PDYB), dan *Yeast Malt Extract Medium* (YM). Faktor kedua meliputi waktu inkubasi, yaitu 15, 20, 25, dan 30 hari. Bobot kering miselia tertinggi (0,92 g/100 mL) dihasilkan pada medium PDYB dengan waktu inkubasi 20 hari. *M. pelianthina* mengandung senyawa metabolit sekunder golongan alkaloid, terpenoid, dan flavonoid.

Kata kunci: *Alkaloid, flavonoid, medium cair, metabolit sekunder, mycena pelianthina, terpenoid*

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INTRODUCTION

The potential of mushrooms as a functional food and as a source of nutraceutical has been widely studied and developed in several countries. Mushrooms are considered to produce various types of bioactive metabolites. These bioactive metabolites can be either primary metabolites or secondary metabolites (Ekowati *et al*., 2016). Various kinds of bioactive compounds have been isolated and identified, including those from the groups of polysaccharides, proteins, phenols, vitamin B, tocopherols, organic acids and terpenoids (Khatua *et al*., 2013). Some of them have been approved for clinical uses (Ren *et al*., 2014).

Mycena is a large group of Basidiomycetes. Several species belong to this genus have been studied for their bioactivity and the structures of metabolite compounds have been identified. Strobilurin bioactive compounds isolated from *M. galopoda*, *M. atromarginata*, *M. rosella* and *M. vitilis* have antifungal and antibacterial activity (Bäurle & Anke, 1980). Leaianafulvene isolated from *M. leaiana* and tintinnadiol from *M. tintinnabulum* is cytotoxic (Engler *et al.*, 1998). However, research on bioactive compounds produced by *M. pelianthina* has not been widely carried out.

Mushroom growth can be influenced by the composition of medium and incubation time. Liquid medium is widely used for production of mycelial biomass since it has advantages such as a short incubation period, low possibility of contamination, and easy to take of mycelia for further analysis (Ekowati *et al.*, 2011). The composition of the medium can affect microorganisms for producing secondary metabolites. According to Suciatmih (2010), potato dextrose yeast broth is a fermentation medium commonly used for testing secondary metabolites. According to Behera and Gupta (2015), production of mycelial biomass in mushroom complete medium is higher than that of yeast malt extract medium. Mushroom complete medium is a complete medium that is very suitable for mushroom growth because it contains a source of carbon, nitrogen, vitamins, and minerals (Kim *et al*., 2002).

Mushrooms need time to hydrolyze available nutrient sources for growth and secondary metabolite production. The longer incubation time, the mycelium growth increases to a certain extent so that the production of metabolites is also high (Saputra, 2010). Bäurle *et al.* (1982) reported that the best growth of *M. viridimarginata* mycelium is at 25 days incubation on YMG medium.

Secondary metabolite compounds can be detected using TLC (Thin Layer Chromatography). It can separate chemical components, based on the principle of adsorption and partitioning of compounds by the stationary phase (adsorbent) and the mobile phase (eluent). The chemical compounds move up based on the mobile phase because the adsorption of adsorbent is different so that the chemical components can move at different distances based on the level of polarity. The displacement process causes the separation of chemical components (Alen *et al*., 2017). The purpose of this study were to elucidate the best media for production of mycelial biomass of *M. pelianthina* and to identify the compound group of secondary metabolites they produced.

MATERIALS AND METHOD

Pure culture of *M. pelianthina* was obtained from the Laboratory of Mycology and Phytopathology, Faculty of Biology, Jenderal Soedirman University. It was cultured on PDA medium on areas of 90 mm petri dish diameter. A piece of mycelium of 5 mm in diameter was inoculated using a cork borer in that various liquid media. The experimental design used in this research was a Factorial Completely Randomized Design (CRD) consisting of 12 treatments and three replications. The first factor was medium type i.e. Mushroom Complete Medium (MCM), Potato Dextrose Yeast Broth (PDYB), and Yeast Malt Extract Medium (YM). The second factor was the incubation time, namely, 15, 20, 25, and 30 days. The main observed parameters were the dry weight of mycelia and the secondary metabolite groups.

The composition of liquid medium.

The MCM was comprised of 20 g of glucose, 0.46 g of KH_2PO_4, 1 g of K_2HPO_4, 0.5 g of $MgSO_4.7H_2O$, 2 of g peptone, and 2 g of yeast extract (Kim *et al*., 2002). The PDYB was comprised of 200 g of potato, 3 g of yeast extract, 20 g of dextrose, and 1,000 mL of distilled water (Maharani *et al*., 2014). The YM was comprised of 10 g of glucose, 3 g of malt extract, 5 g of peptone, and 5 g of yeast extract (Kim *et al*., 2002).

Cultivation of M. pelianthina mushroom (Elfita *et al*., 2014)

Mycena pelianthina mycelium was inoculated by transferring 5 plugs, respectively (5 mm diameter) from rejuvenating medium into a 250 mL Erlenmeyer flask containing 100 mL of PDYB, MCM, and YM, respectively. Each Erlenmeyer flask was closed with cotton and glued with plastic wrap. The inoculated media were then incubated for 15, 20, 25, 30 days at room temperature using a rotary incubator.

Weight of M. pelianthina mycelia (Irianto *et al*., 2008)

The mycelial cultures that had been incubated for 15, 20, 25, or 30 days were filtered using Whatman papers no. 41, and a vacuum pump was used to accelerate the filtration. The mycelia from each filtered sample were weighed and then dried in an oven at a temperature of 60°C until a constant weight was obtained and the dry weight was recorded.

Determination of pH medium

The pH of medium was determined using Horiba Laqua pH meter (Wagestu et al., 2016).

Extraction of M. pelianthina mycelium (Vamanu, 2013)

Extraction of *M. pelianthina* mycelium was conducted following the procedures shown in the diagram Figure 1.

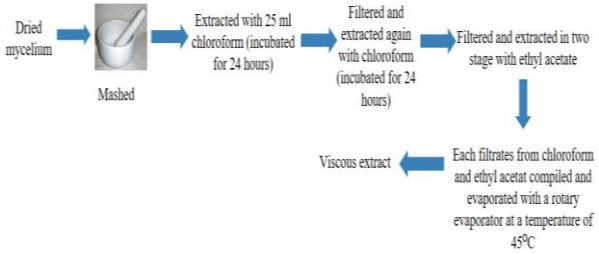

Figure 1. Extraction of *M. pelianthina* mycelia

Production of M. pelianthina filtrate extract (Ekowati *et al*., 2011)

Production of *M. pelianthina* filtrate extract was conducted following the procedures shown in the diagram Figure 2.

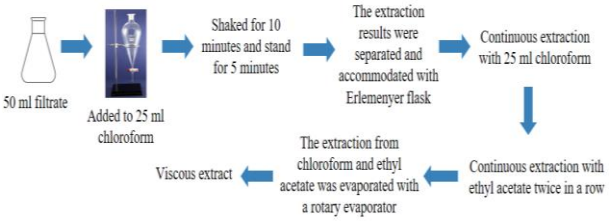

Figure 2. Production of *M. pelianthina* extract mixtures

Identification of secondary metabolite group compounds (Wagner *et al*., 1984)

Identification of alkaloid, terpenoid, and flavonoid were carried out using a silica gel GF254 plate with the mobile phase of dichloromethane: methanol (10:1) mixture. Each eluted spot was calculated by the value of Retardation Factor (Rf) with the following formula.

$$Rf\ (cm) = \frac{\text{Distance moved by the compound}}{\text{Distance moved by the surface of the solvent}} \quad (1)$$

Statistical analysis

Mycelial dry weight data obtained were analyzed using ANOVA at p = 5% and were continued with the Duncan's Multiple Range Test (DMRT) at the p

values. The detection of secondary metabolites was performed descriptively.

RESULTS AND DISCUSSION

Mycena pelianthina mycelial biomass

The growth of *M. pelianthina* mushroom on MCM and YM media continued to increase until 30 days of incubation. The growth of *M. pelianthina* mushroom on PDYB medium increased until 20 days and then decreased during 25 and 30 days (Figure 3). The interaction between the types of media and the incubation time significantly affected the dry weight of mycelia (Table 1).

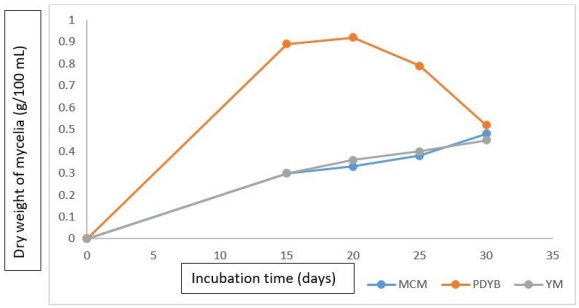

Figure 3. The growth of *M. pelianthina* in mushroom complete medium (MCM), potato dextrose yeast broth (PDYB), and yeast malt extract medium (YM), respectively.

The growths of *M. pelianthina* on MCM and YM, respectively, were in the exponential phase from 15 to 30 days of incubation. The growth of *M. pelianthina* in the MCM and YM, respectively, were not in either the stationary phase nor the death phase because the mushroom was still utilizing nutrients for growth so that mycelial dry weight continued to increase. The growth of *M. pelianthina* on PDYB medium entered the death phase subsequently, during the 25 to 30 days of incubation. According to Setyati *et al.* (2015), the death phase was characterized by a decreasing rate of growth caused by a lack of growth material such as vitamins and mineral elements

PDYB was the best medium for the growth of *M. pelianthina* mycelium regarding the production of mycelial dry weight. According to Teoh & Don (2012), yeast extract, glucose, $MgSO_4.7H_2O$, KH_2PO_4, K_2HPO_4 contained in MCM can increase the mycelial growth. On the other hand, Laurie et al. (2015) reported that potatoes in PDYB medium contain more complete minerals and vitamins. Potatoes contain minerals such as calcium, iron, magnesium, phosphorus, potassium, and zinc as well as vitamins such as thiamine, niacin, riboflavin, and vitamin B6. Based on the research by Behera & Gupta (2015), YM produced a lower mycelial dry weight compared to MCM for the mushroom *Rusula* spp, *Pleurotus sajor-caju*, *Lentinus tuberregium* and *Calocybe indica* because YM has limited mineral elements.

Table 1. Statistical analysis of dry weight mycelium of *M. pelianthina* in MCM, PDYB, YM medium and incubation time

Media	Incubation Time (days)	Dry Weight of Mycelia (g/100 mL)
MCM	15	0.30 [a]
	20	0.33 [a]
	25	0.38 [a]
	30	0.48 [a]
PDYB	15	0.89 [b]
	20	0.92 [b]
	25	0.79 [b]
	30	0.52 [a]
YM	15	0.30 [a]
	20	0.36 [a]
	25	0.40 [a]
	30	0.45 [a]

Note: Numbers with different notation show significantly different at p = 5%

Based on the ANOVA results (Table 2), the interaction between the type of medium and the incubation time had a significant effect on the dry weight gain of mycelia. Mycelial growth can be influenced by several factors such as growth medium, incubation time, pH, temperature, nutrition and several environmental factors (Muthu & Shanmugasundaram, 2015). The growth medium is the most important factor because it contains nutrients needed for mycelial growth. The nutrients needed by microorganisms for growth include carbon, nitrogen, non-metallic elements such as sulfur and phosphorus, metal elements such as Ca, Zn, Na, K, Cu, Mn, Mg, and Fe, vitamins, water,

and energy. Mushrooms require a certain amount of time to break the sources of nutrients down that are available in the medium and then use it up for growing and production of secondary metabolites (Cappucino & Sherman, 2014).

PDYB is the main medium for mushrooms growth. Mushrooms can metabolize starch in potatoes resulting in dissolved sugars that act as sources of carbon and energy as well (Laurie *et al*., 2015). Potato is a complex medium that provides nitrogen, enzymes, vitamins and mineral elements for mushrooms growth. In addition, the yeast extract in the PDYB contained not only organic nitrogen; but also vitamins, minerals, sugar and cofactors. High carbon sources and yeast contain various supporting compounds for growth, enabling efficient mushroom growth (Suciatmih, 2010).

Every type of mushroom required a different suitable medium to grow optimally (Smith & Onions, 1994). Each medium has different nutrient compositions to support the mushroom's needs for growing, but the essential goal is to provide balanced nutrients and at adequate concentrations that can enable the mushroom growing well.

Table 2. Anova of dry weight of *M.pelianthina* mycelia in different growth media and incubation times

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F	Sig
Treatment	11	1.703(a)	0.155	10.314	0.000
Time	3	0.017	0.006	0.380	0.768
Medium	2	1.318	0.659	43.910	0.000
Time × Medium	6	0.368	0.061	4.083	0.006
Error	24	0.360	0.015		
Total	35	2.063			

Note : Sig value < 0.05 indicates that the factorial variables have a significant effect on dry weight gain of mycelia

Based on the results of the study, mushrooms grown on MCM decreased the pH (Table 3). According to Angelia *et al*. (2013), the increase of incubation time lowered the medium pH since more sugars were converted into acids. The organic acids produced by the fungus included pyruvic acid and citric acid. The PDYB and YM, respectively, showed pH increase due to mushroom growth (Table 3). According to Sari (2011), the increased pH was caused by the production of ammonia from nitrogen containing compounds in the growth medium.

Table 3. pH Values of the growth media before and after incubation periods

Medium	Incubation Time (days)	Average	
		Initial pH	Final pH
MCM	15	6	5
	20	6	5
	25	6	5
	30	6	5
PDYB	15	6	7
	20	6	7
	25	6	7
	30	6	7
YM	15	6	6
	20	6	7
	25	6	7
	30	6	7

Identification of secondary metabolite group

To elucidate the class of secondary metabolites the mycelial extracts of *M. pelianthina* obtained from chloroform and ethyl acetate, respectively, were then mixed and tentatively determined through TLC method. The results of the active compounds characterized in *M. pelianthina* mycelial concentrated extracts are presented in Figure 4. Alkaloid, terpenoid and flavonoid groups were detected in each medium and the distribution was different for each extract.

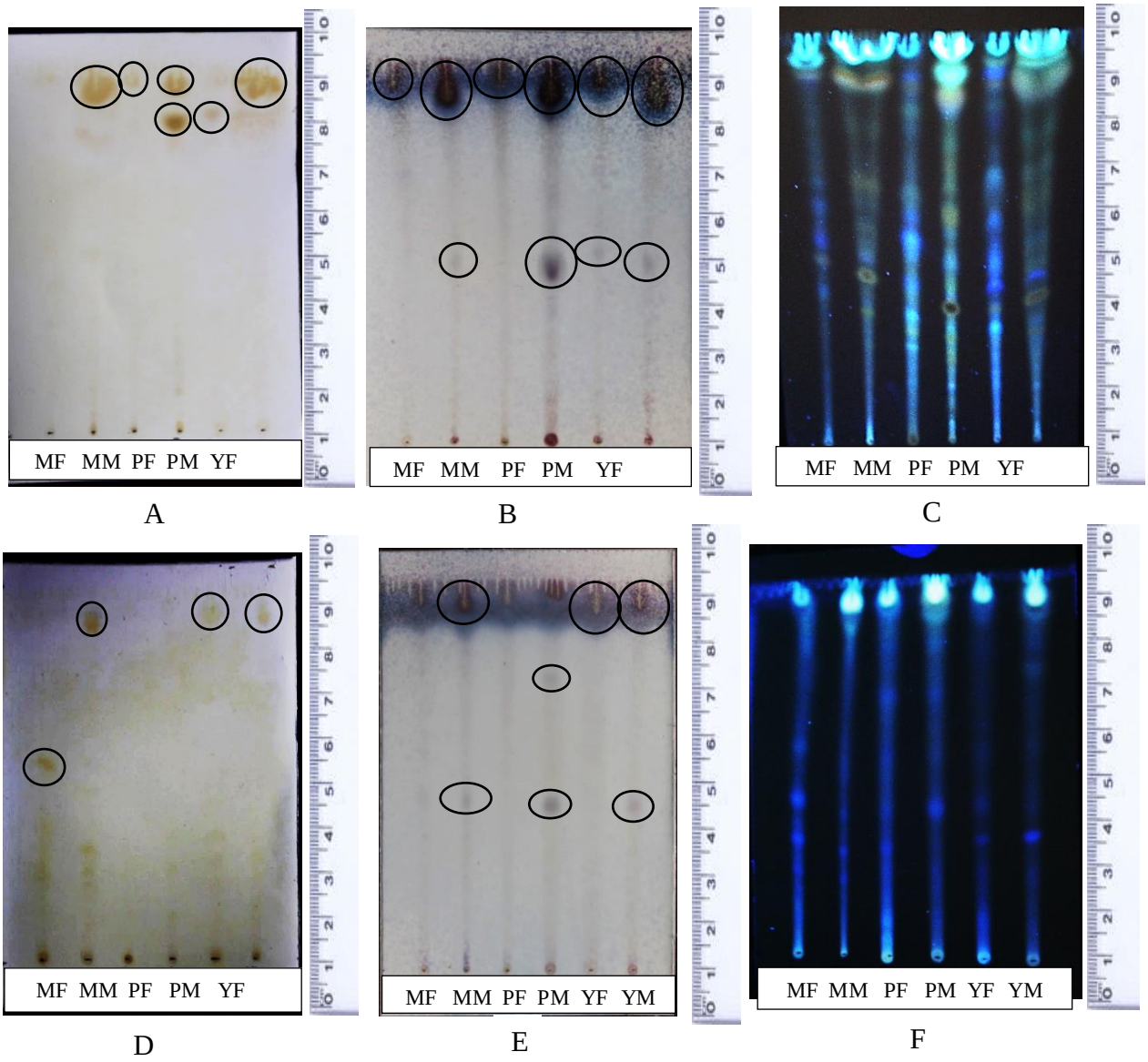

Figure 4. Chloroform extract chromatogram: (A) alkaloid, (B) terpenoid, and (C) flavonoid; ethyl acetate extract chromatogram: (D) alkaloid (E) terpenoid (F) flavonoid

Brownish-yellow spots were formed on the GF254 silica gel plate after being sprayed with Dragendorff reagents (Figure 4.A and 4.D). The Rf values ranged from 0.81 to 0.92 in chloroform elucidation and 0.51 to 0.91 in ethyl acetate eluent. Furthermore, blackish purple spots were formed on the GF254 silica gel plate after sprayed with Vanillin-sulfuric acid reagent (Figure 4.B and 4.E). The Rf values ranged from 0.44 to 0.92 in chloroform mobile phase and 0.41 to 0.91 in ethyl acetate elution. On the other hand, Figures 4.C and 4.F show blue and green spots after sprayed with

$AlCl_3$ reagents observed under 366 nm UV light. The Rf values ranged from 0.12-0.95 when elucidated using chloroform and 0.20-0.94 for ethyl acetate.

The production of bioactive metabolites from microorganisms depends on special adaptations to the environment (Padmavathi *et al*., 2012). The growth medium and incubation conditions play an important role in the production of secondary metabolites. Each observed medium had different compositions. MCM contains the main carbon source of glucose which is more easily metabolized by mushroom. Micronutrients KH_2PO_4, K_2HPO_4, $Na_2HPO_4.12H_2O$, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$ and KCl are important elements for the production of secondary metabolites (Teoh & Don, 2012). PDYB contains a complex carbon source derived from potatoes. YM also contains complex carbon sources derived from maltose. Alkaloids, terpenoids, and flavonoids were detected in PDYB and YM. This was not in accordance with the statement Purwantini *et al*. (2015), that complex carbon sources such as malt extract are more difficult to be metabolized by microorganisms and can result in inhibition of biosynthesis of secondary metabolites. Ramakhrisna & Ravishankar (2011) reported that the production of secondary metabolites as self-defense will increase under stress.

Figure 4 shows trends in accordance with the statement of Suhaenah & Nuryanti (2017), the potential results of the presence of alkaloids are marked with yellow to orange spots after being sprayed with Dragendorff reagent. According to Eva (2007), Rf values for alkaloids ranged from 0.69 to 0.9. Saxena *et al*. (2013) stated that alkaloids have many pharmacological activities including anti-hypertensive, anti-arrhythmic, anti-malaria, and anti-cancer effects.

Vanillin-sulfuric acid reagent is used to detect terpenoid compounds, steroids and essential oil components (Sulistijowati & Gunawan, 2001). Terpenoid compounds on the TLC will change to blue-purple colored spots after sprayed with vanillin-sulfuric acid reagent. Terpenoids are bioactive compounds that can be used as antifungal compounds (Lutfiyanti *et al*., 2012). The toxic nature of terpenoids is known to have the ability to inhibit fungal growth by damaging cell membranes.

Rahayu *et al*. (2015) reported, in similar elution system, spots with Rf values between 0.2–0.75 indicate spots containing flavonoids. On the other hand, Firdiyani *et al*. (2015) stated that flavonoid compounds are non-polar compounds. Flavonoids however have a sugar group which makes it dissolve easily in either polar or semi-polar solvent. According to Prameswari & Widjanarko (2014), flavonoids are polyphenol compounds which have various pharmacological activities such as antioxidants, anti-inflammatory, and anticancer. Overall, type of medium, incubation time and their interactions process are the key factors in increasing mycelia dry weights simultaneously extraction amplified the secondary metabolites' existences.

CONCLUSION

Potato dextrose yeast broth was the best medium for the growth of *Mycena pelianthina*. The highest production of biomass mycelium was 0.92 g/100 mL of the media for 20 days of incubation at room temperature. The extract of *Mycena pelianthina* mycelia contained secondary metabolite compound groups of alkaloid, terpenoid, and flavonoid.

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