International Journal of Agricultural Technology 2022Vol. 18(1):231-240
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Identification of lovastatin analogs-producing *Pleurotus* cultivars in southern Vietnam

Lam, V. N.[1,2], Bui, N. T.[3], Le, T. N.[7], Dang, H. P.[4], Nguyen, V. G. B.[5], Ho, B. T. Q.[3], Dinh, M. H.[6], Nguyen, T. T.[7] and Pham, N. D. H.[8, 9] *

[1]Biotechnology Center of Ho Chi Minh City, HCM City, Vietnam; [2]Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam; [3]Faculty of Biotechnology, Ho Chi Minh City Open University, HCM City, Vietnam; [4]Faculty of Chemistry, University of Science, VNU-HCM, HCM City, Vietnam; [5]Faculty of Pharmacy, University of Medicine and Pharmacy Ho Chi Minh City, Vietnam; [6]Department of Agriculture and Rural Development, HCM City, Vietnam; [7]Institute of Tropical Biology, Vietnam Academy of Science and Technology, HCM City, Vietnam; [8]Applied Biotechnology Institute, HCM City, Vietnam; [9]HUTECH Institute of Applied Sciences, Ho Chi Minh City University of Technology - HUTECH, HCM City, Vietnam.

Lam, V. N., Bui, N. T., Le, T. N., Dang, H. P., Nguyen, V. G. B., Ho, B.T. Q., Dinh, M. H., Nguyen, T. T. and Pham, N.D. H. (2022). Identification of lovastatin analogs-producing *Pleurotus* cultivars in southern Vietnam. International Journal of Agricultural Technology 18(1):231-240.

Abstract *Pleurotus* mushrooms have been widely cultivated worldwide due to their nutritional and medicinal value. Numerous bioactive compounds such as polysaccharides, polyphenols, proteins, and amino acids, have been well documented in its fruiting bodies. However, lovastatin known as an anti-lipidemic medicinal compound produced by several *Pleurotus* strains is neglected. This study aimed to identify local *Pleurotus* strains capable of producing lovastatin analogs collected from southern Vietnam. Accordingly, 31 *Pleurotus* strains were isolated and belonged to 5 morphotypes: blue oyster, golden oyster, abalone, oyster, and phoenix. Lovastatin analogs were detected using a state-of-the-art method quantitative ^{1}H nuclear magnetic resonance (qHNMR). As a result, among 31 strains of *Pleurotus* mushrooms, 4 strains were capable of synthesizing lovastatin analogs. In which, one strain belonged to oyster morphotypes (*Pleurotus ostreatus s. l.*) and 3 strains were phoenix morphotypes (*Pleurotus pulmonarius s. l.*). This is the first to identify the production of lovastatin analogs by phoenix mushrooms.

Keywords: Lovastatin, ^{1}H nuclear magnetic resonance qHNMR, Phoenix mushrooms, *Pleurotus pulmonarius*

Introduction

According to the Food and Agriculture Organization data (FAO), the global production of cultivated mushrooms was about 11 billion tons with 15 billion USD in 2018 (FAOSTAT, 2021). So far, the economic importance of mushrooms has been increased based on their therapeutic and nutritional attributes. Recently, mushrooms were also attributed to support

* **Corresponding Author:** Pham N. D. Hoang.; **Email:** pndhoang@gmail.com

against the Coronavirus disease pandemic (COVID-19) as they can boost the immune system and countermeasures of the superinfection associated with the COVID-19 (Abdul and Aneeqa, 2021).

Pleurotus mushrooms consisting of 207 species (Catalogue of Life, 2021) are the world's third most common cultivated species (Iwona *et al.,* 2018). The global growing and consumption interest of *Pleurotus* mushrooms has been increasing due to their taste, medicinal, and nutritional properties (Mbassi *et al*., 2018). Fruiting bodies of *Pleurotus* spp. have been observed to contain a high content of valuable nutrients, including protein (15.4-28.6%), carbohydrates (61.3-84.1%), and dietary fiber (3-3.33%) (María *et al.,* 2015). On the other hand, the fruiting bodies are not only rich in micro- and macro-elements, such as copper, iron, zinc, and sodium, potassium, magnesium, and phosphorus (FDC, 2019), but also in medicinal compounds such as lovastatin and its analogs (Alarcon *et al*., 2003; Lee *et al*., 2006; Chen *et al*., 2012; Vijay *et al*., 2019).

Lovastatin, one of the secondary metabolites produced by *Pleurotus* mushrooms, is well-known as a β-Hydroxy β-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor (statins). It can lower LDL cholesterol and reduce the risk of cardiovascular disease and associated conditions. This compound might be beneficial in reducing negative impacts on the heart or blood vessels caused by the COVID-19 (Stéphane *et al.*, 2021, WHO, 2021). Several techniques have been used for quantification of lovastatin, such as ultraviolet-visible spectrophotometry (UV-VIS), high-performance liquid chromatography (HPLC) (Chen *et al.*, 2012), and quantitative ^{1}H Nuclear magnetic resonance (qHNMR) (Dirk *et al.*, 2012). A study by Chen *et al.* (2012) showed that freeze-dried fruiting body of Japanese *Pleurotus ostreatus* strains contained a high amount of lovastatin (606.5 mg/kg) by HPLC method. However, Tsiantas *et al.* (2012) showed the constraints of lovastatin quantification in mushrooms. A significant difference in lovastatin content was observed when comparing between UV-VIS and liquid chromatography-mass spectrometry (LC-MS) methods. On the other hand, a simple and direct qHNMR assay has been developed to determine the total statin content in red yeast rice (Dirk *et al.*, 2012). This assay could be applied for quantification of statin content in fruiting bodies of mushrooms.

In this study, to determine the occurrence of lovastatin, we used two assays including thin layer chromatography (TLC), and qHNMR. To identify local *Pleurotus* strains capable of producing lovastatin analogs in southern Vietnam, 31 *Pleurotus* strains were isolated and cultivated from three different resources: nature, markets, and mushroom farms. These selected strains could be helpful for local cultivating implementation and for development of local lovastatin producing *Pleurotus* strains towards increasing their economic values.

Materials and methods

Preparation of fruiting bodies by Pleurotus mushrooms

In this study, the 31 *Pleurotus* strains from a collection at the Applied Biotechnology Institute (Ho Chi Minh City, Vietnam) was cultivated in the same condition (Table 1). All strains were belonged to 5 morphotypes: blue oyster mushroom (*P. ostreatus s. l.*), golden oyster mushroom (*P. citrinopileatus*), abalone mushroom (*P. cystidiosus*), oyster mushroom (*P.ostreatus s. l.*), and phoenix mushroom (*P. pulmonarius s. l.)*.

For cultivation, all *Pleurotus* strains were maintained on potato glucose agar (PDA) (Himedia, Mumbai, India). The primary spawn contained (per spawn) 100 g boiled paddy, 2 g rice bran, 1 g $CaSO_4.2H_2O$ (Himedia, Mumbai, India), and 1 g $CaCO_3$ (Himedia, Mumbai, India). The substrate contains of 79% sawdust, 20% corn bran, 10% $CaSO_4$ (Himedia, Mumbai, India), humidity 65%; sterilized by steam water at 95°C in 4 hours. The substrate was inoculated by mixing with primary spawn at a rate of 5%, filled into polypropylene bags (1,200 g per bag,) and incubated at 25°C in a dark place. Each strain was cultivated by 30 spawns. Once the spawns were fully colonized, they were stimulated to fruit in the condition of 80-90% humidity, 25 – 28°C, 500 – 1,000 lux, 12:12 hours light/dark cycle. The humidity was maintained by frequent spraying of water on the roof and on the floor of cultivating room.

The morphotype of fruit bodies of each strain was detected following the description of Pegler (1986), Corner (1981), Buchanan (1993), Boekhout *et al.* (1990), Segedin *et al.* (1995), and Lechner *et al.* (2004).

Each strain, 30-40 fruiting bodies (300-500 g) from several spawns were harvested at the normal stage for commercial products. All fruiting bodies of each strain were dried at 60 ℃ for 48 hours, homogeneously ground, thoroughly mixed, and stored in desiccant bags at 4°C for further analyses.

Detection of lovastatin and its analogs using thin layer chromatography (TLC)

Dried powder of *Pleurotus* fruit bodies (1 g) was extracted with 10 ml of hexane for 1 hour. A supernatant was then collected.

Thin layer chromatography (TLC) plate was pre-activated in hot air oven at 120 ℃ for 1 hour. Standard lovastatin (STADA®, Germany) and sample were spotted on the pre-activated TLC plate and allowed it to dry. The chromatogram was developed by running in chloroform and acetone (90:10, v/v) mobile phase. For each TLC run, the similar spots and Rf were used to compare among lovastatin standards and samples. Plates were stained with vanillin reagents (Seress *et al.*, 2001).

Table 1. The *Pleurotus* mushrooms collection

No	Code	Origin	Morphotypes	Collected Area
1	ABI-FC-P001	Farm	Blue oyster	Ho Chi Minh City, Vietnam
2	ABI-FC-P004	Market	Golden oyster	Ho Chi Minh City, Vietnam
3	ABI-FC-P008	Farm	Abalone	Binh Dinh Province, Vietnam
4	ABI-FC-P009	Wild	Abalone	Dong Nai Province, Vietnam
5	ABI-FC-P010	Wild	Abalone	Dong Nai Province, Vietnam
6	ABI-FC-P011	Wild	Abalone	Ho Chi Minh City, Vietnam
7	ABI-FC-P014	Farm	Phoenix	Ho Chi Minh City, Vietnam
8	ABI-FC-P016	Farm	Phoenix	Soc Trang Province, Vietnam
9	ABI-FC-P017	Market	Oyster	Ben Tre Province, Vietnam
10	ABI-FC-P019	Farm	Oyster	Can Tho City, Vietnam
11	ABI-FC-P020	Farm	Oyster	Dong Thap Province, Vietnam
12	ABI-FC-P022	Market	Oyster	Ho Chi Minh City, Vietnam
13	ABI-FC-P023	Market	Oyster	Ho Chi Minh City, Vietnam
14	ABI-FC-P024	Farm	Oyster	Dong Nai Province, Vietnam
15	ABI-FC-P038	Farm	Phoenix	Long An Province, Vietnam
16	ABI-FC-P044	Farm	Phoenix	Can Tho City, Vietnam
17	ABI-FC-P047	Market	Phoenix	Ho Chi Minh City, Vietnam
18	ABI-FC-P048	Market	Phoenix	Ho Chi Minh City, Vietnam
19	ABI-FC-P049	Farm	Phoenix	Dong Thap Province, Vietnam
20	ABI-FC-P050	Farm	Phoenix	Dong Thap Province, Vietnam
21	ABI-FC-P052	Farm	Phoenix	Dong Nai Province, Vietnam
22	ABI-FC-P053	Farm	Phoenix	Dong Nai Province, Vietnam
23	ABI-FC-P054	Farm	Phoenix	Binh Duong Province, Vietnam
24	ABI-FC-P055	Market	Phoenix	Ba Ria – Vung Tau Province, Vietnam
25	ABI-FC-P056	Farm	Phoenix	Tay Ninh Province, Vietnam
26	ABI-FC-P057	Farm	Phoenix	Tay Ninh Province, Vietnam
27	ABI-FC-P058	Farm	Phoenix	Tay Ninh Province, Vietnam
28	ABI-FC-P059	Farm	Phoenix	Binh Thuan Province, Vietnam
29	ABI-FC-P060	Farm	Phoenix	Binh Thuan Province, Vietnam
30	ABI-FC-P061	Wild	Phoenix	Lam Dong Province, Vietnam
31	ABI-FC-P062	Wild	Phoenix	Lam Dong Province, Vietnam

Determination of lovastatin and its analogs using qHNMR method

Dried powder of *Pleurotus* fruit bodies (3 g) was refluxed with 10 ml of acetonitrile for 3 hours. The extract was collected by filtering through a 0.45 µm sterile filter (Corning®, Germany) and evaporated by rotary evaporator. The pellet was re-dissolved in chloroform. The mixture was sonicated for 10 minutes and evaporated by rotary evaporator. The pellet was dissolved in 550 µl chloroform-D (CDCl3) (Cambridge Isotope Laboratories, UK) followed by injecting into an NMR instrument. The lovastatin (STADA®, Germany) was employed as the external standard.

The qHNMR spectra were taken on a Bruker Avance III 500 spectrometer (Bruker BioSpin AG, Bangkok, Thailand), number of scans from 5 to 30, receiver gain from 18 to 70, relaxation delay 3.000, temperature 25°C, probe 5 mm PATXI 1H/ D-13C/ 15N Z-GRD Z856901/ 0067, Tetramethylsilane (TMS) as internal standard, and the chemical shifts are expressed in δ values.

Results

TLC assay

TLC results indicated that among 31 samples, four samples (Figure 1, lanes 2 – 5) seemingly appeared similar bands when compared to the standard lovastatin spot (lane 1). It is assumed that these samples might accumulate lovastatin or its analogs. The contrary was true to the remaining samples. Therefore, to confirm this, we further analyze these samples using qHNMR assays.

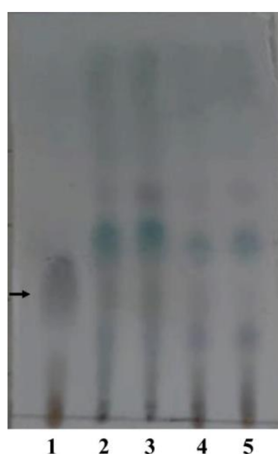

Figure 1. The TLC results. Lane 1: positive control, lane 2-5: Four strains (ABI-FC-P019, ABI-FC-P050, ABI-FC-P058, and ABI-FC-P059, respectively), (chloroform:aceton = 9:1, v/v), the similar spots showed at arrow

qHNMR results

qHNMR-based quantification of lovastatin standard showed the resonance peaks at δ 5.99-5.97 ppm, δ 5.79-5.76 ppm, δ 5.52-5.51 ppm, and δ 5.39-5.37 ppm. We firstly aligned the spectra of 31 samples with the spectrum of lovastatin standard. Data indicated that 4 out of them contained lovastatin analogs. The superimpose spectra of these samples were showed on Figure 2. We further compared the peak of each sample with the standard one and confirmed the appearance of lovastatin analogs in these four samples. The other 27 strains showed the noise resonance peaks and no peaks at δ 5.99-5.37 ppm. Evidently, the spectra of 4 samples ABI-FC-P019, ABI-FC-P050, ABI-FC-P058, and ABI-FC-P059 showed close resonance peaks at δ 5.99-5.37 ppm, especially at δ 5.52-5.51 (Figure 3). Based on these data, we could conclude that the four strains (ABI-FC-P019, ABI-FC-P050, ABI-FC-P058, and ABI-FC-P059) potentially produced lovastatin analogs. Of those, strain ABI-FC-P019 belonged to oyster mushroom morphotype, while the remaining strains ABI-FC-P050, ABI-FC-P058, and ABI-FC-P059 were phoenix mushroom morphotype (Figure 4).

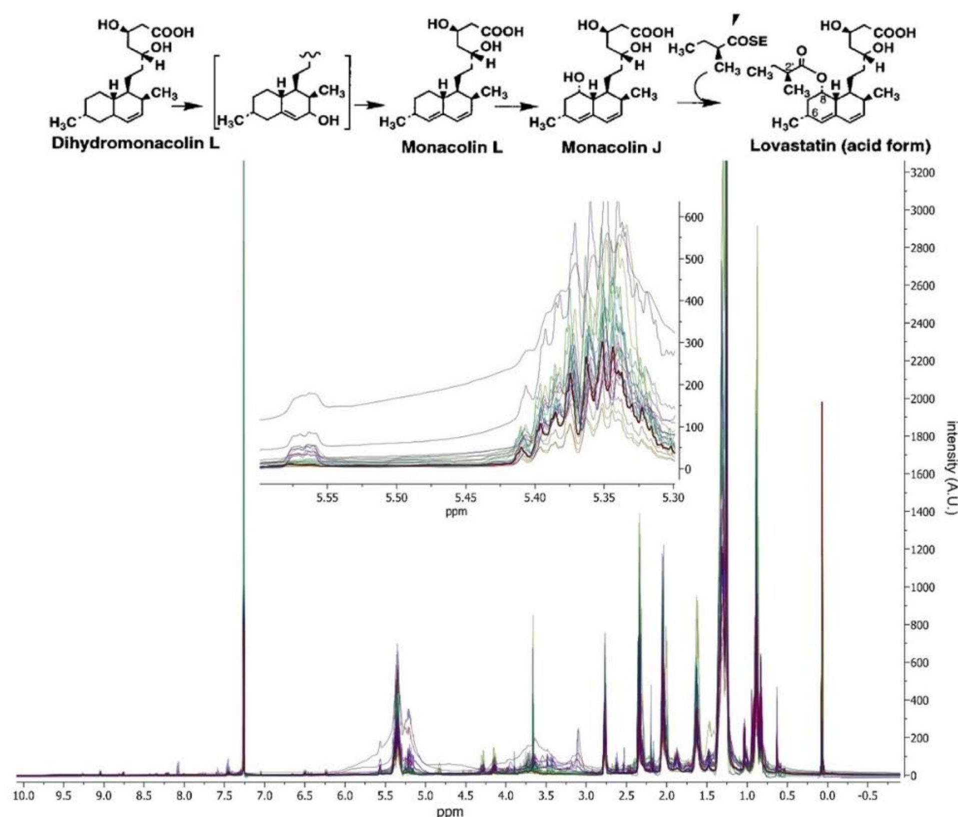

Figure 2. The superimpose spectra qHNMR study of *Pleurotus* spp. samples and lovastatin (500 MHz, $CDCl_3$, 25°C)

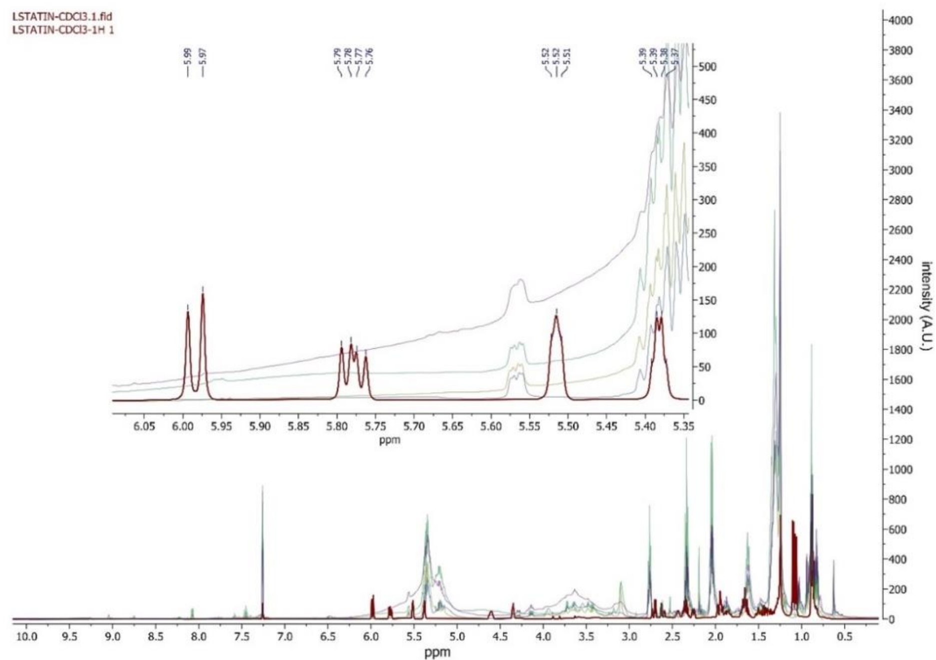

Figure 3. The superimpose spectra of resonance peaks in qHNMR study of 4 samples (ABI-FC-P019, ABI-FC-P050, ABI-FC-P058, and ABI-FC-P059) and lovastatin (500 MHz, $CDCl_3$, 25°C)

Figure 4. The fruiting bodies of (A). *Pleurotus ostreatus s. l.* (ABI-FC-P019), (B), (C), and (D) *Pleurotus pulmonarius s. l.* (ABI-FC-P050, ABI-FC-P058, and ABI-FC-P059, respectively). Scale bar: 1 cm

Discussion

As the best sensitivity and the no interference signal, the resonance peaks at δ 5.37-5.32 ppm were used for lovastatin quantification base on the H6, H4, or overlap of the signals of both protons (Dirk *et al.*, 2012). The disappearance of lovastatin-specific resonance peaks in the samples might not indicate that lovastatin was completely absent in there. Figure 3 showed the qHNMR resonance peaks of integration from all analyzed samples in a comparison with the standard qHNMR resonance peaks of lovastatin. Among 31 samples, four showed the resonance peaks at δ 5.60-5.55 ppm suggesting that these samples could accumulate dihydromonacolin L - a derivative of lovastatin based on the H5 proton (Figure 3). It is possible that since the fruiting bodies by 31 *Pleurotus* spp. were harvested at an early stage for food purposes, the lovastatin biosynthetic pathway was likely incomplete. Dihydromonacolin L was an intermediate. This NMR analysis was also consistent with the preliminary finding on TLC assay (Figure 1).

In fungi, the biosynthesis of secondary metabolites is subject to complex regulation. There are 18 genes involved in the biosynthetic pathway of lovastatin by *Aspergillus terreus* (Javier *et al.*, 2020). In development of new mushroom cultivars, breeders often focus on genes related to yield and quality. However, several genes involved in secondary metabolites biosynthesis such as lovastatin synthesizing genes are neglected. The lack of these genes might result in the absence of lovastatin and its lovastatin derivatives in mushroom.

In this study, four *Pleurotus* spp. strains (ABI-FC-P019, ABI-FC-P050, ABI-FC-P058, and ABI-FC-P059) which could produce lovastatin derivatives were collected from local mushroom farms. The concentration of lovastatin in fruiting bodies varies when the mushroom was cultivated in different conditions. For example, the lovastatin production of *Pleurotus ostreatus* has been demonstrated to be affected by the C:N ratio and it was stimulated when adding organic nitrogen (peptone water or yeast extract) to the substrate (Julio and Sergio, 2005). Lovastatin production of the four *Pleurotus* strains could be improved when the cultivation conditions and media are optimized. The results also showed that both group oyster mushroom type (*Pleurotus ostreatus s. l.*) and phoenix mushroom type (*Pleurotus pulmonarius s. l.*) could be potential strains producing lovastatin analogs. This is the first to identify the production of lovastatin analogs by phoenix mushroom (*Pleurotus pulmonarius s. l.*) groups.

Notwithstanding, lovastatin is commercially produced by filamentous fungi such as *Penicillium*, *Monascus*, and *Aspergillus* (Mohammad *et al.*, 2012). To our knowledge, studies in detection, selection, and breeding for lovastatin-producing *Pleurotus* cultivars are sparse. Therefore, detection and identification of local lovastatin-producing

Pleurotus strains are innovative and significant to improve the economic values of these edible mushrooms in southern Vietnam.

In conclusion, this current study has identified four potentially lovastatin analogs-producing *Pleurotus* spp. strains (ABI-FC-P019, ABI-FC-P050, ABI-FC-P058, and ABI-FC-P059). Dihydromonacolin L could be one of lovastatin analogs secreted by these strains. Our finding could be helpful for breeding plans for lovastatin-producing *Pleurotus* strains in Vietnam. Further studies are required in (i) preparation of the fruiting body of *Pleurotus* mushroom at a later stage for lovastatin determination, (ii). detection of the presence of lovastatin biosynthetic gene cluster on the *Pleurotus* mushroom and (iii) accumulation of them by isolating and mating the monokaryons.

Acknowledgements

We would like to offer a particular thank to Dr. Trang M. TRAN (Faculty of Bioscience Engineering, Ghent University, Belgium) for improving this manuscript.

We would like to give special thanks to DaLat Mushroom company Limited (Cam Ly Street, Mang Lin Village, Ward 7, Da Lat City, Lam Dong Province, Vietnam), and Nguyen Long Joint Stock company (Tran Quy Cap street, Ward 9, Da Lat City, Lam Dong Province, Vietnam) for *Pleurotus* spp. cultivation.

References

Abdul, R. N. and Aneeqa, G. (2021). Different ways to exploit mushrooms: A review. All Life, 14:450-460.

Alarcon, J., Aguila, S., Arancibia-Avila, P., Fuentes, O., Zamorano-Ponce, E. and Hernandez, M. (2003). Production and purification of statins from *Pleurotus ostreatus* (Basidiomycetes) strains. Verlag der Zeitschrift für Naturforschung, 58:62-64.

Buchanan, P. K. (1993). Identification, names and nomenclature of common edible mushrooms. In: Chang ST et al ed. Mushroom biology and mushroom products, Hong Kong, The Chinese University Press, pp.21-32.

Catalogue of Life (2021). Retrieved from https://www.catalogueoflife.org/?taxonKey=6SW5_in Aug 10th, 2021.

Chen, S., Ho, K., Hsieh, Y., Wang, L. and Mau, J. (2012). Contents of lovastatin, γ-aminobutyric acid and ergothioneine in mushroom fruiting bodies and mycelia. LWT - Food Science and Technology, 47:274-278.

Corner, E. J. H. (1981). Beihefte zur Nova Hedwigia, Heft 69: The Agaric Genera: *Lentinus*, *Panus*, and *Pleurotus* with particular reference to Malaysian species, Gantner Verlag Kommanditgesellschaft, J. Cramer, pp.102-111.

Dirk, W. L., Yulia, B. M., Thomas, K., Sigrid, L., Sibylle, M., Matthias, K., Asja, W. and Christian, S. (2012). NMR evaluation of total statin content and HMGCoA reductase inhibition in red yeast rice (*Monascus* spp.) food supplements. Chinese Medicine, 7:8.

Food and Agriculture Organization Corporate Statistical Database (FAOSTAT) (2021). Retrieved from http://www.fao.org/faostat/en/#data in Aug 10th, 2021.

Food Data Central, U.S. Department of Agriculture (2019). Retrieved from https://fdc.nal.usda.gov/fdc-app.html#/food-details/1750345/nutrients_in Aug 10th, 2021.

Iwona, G., Alina, K., Tomasz, S., Marek, S. and Krzysztof, S. (2018). Bioactive compounds and medicinal properties of Oyster mushrooms (*Pleurotus* sp.). Folia Horticulturae, 30:191-201.

Javier, B., Ailed P. and Mar ía, E. B. (2020). New knowledge about the biosynthesis of lovastatin and its production by fermentation of *Aspergillus terreus*. Applied Microbiology and Biotechnology, 104:8979-8998.

Julio, A. and Sergio, A. (2005). Lovastatin Production by *Pleurotus ostreatus*: Effects of the C:N Ratio. Journal of Biosciences, 61:95-8.

Lechner, B. E., Wright, J. E. and Alberto, E. (2004). The genus *Pleurotus* in Argentina. Mycologia, 96:845-858.

Lee, J. W., Lee, S. M., Gwak, K. S., Lee, J. Y. and Choi, I. G. (2006). Screening of edible mushrooms for the production of Lovastatin and its HMG-CoA reductase inhibitory activity. The Korean Journal of Microbiology, 42:83-88.

Mar ía, E. V., Tal ía, H. and Octavio, P. (2015). Edible mushrooms: improving human health and promoting quality life. International Journal of Microbiology, 2015:376-387.

Mbassi J. E. G., Mobou E. Y., Ngome F. A. and Sado K. S. L. (2018). Effect of substrates on nutritional composition and functional properties of *Pleurotus ostreatus*. Current Research in Agricultural Sciences, 5:15-22.

Mohammad, F. J., Juan, B. L., Yin, W. H., Rosfarizan, M., Yong, M. G. and Parisa, S. (2012). Lovastatin production by *Aspergillus terreus* using agro-biomass as substrate in solid state fermentation. BioMed Research International, 2012:11.

Pegler, N. D. (1986). Kew BulletinAdditional Series XII: Agaric flora of Sri Lanka, London, Her Majesty Stationary Office, pp.41-46.

Segedin, P. B., Buchanan, P. K. and Wilkie, J. P. (1995). Studies in the Agaricales of New Zealand: New species, new records and renamed species of *Pleurotus* (Pleurotaceaea). Australian systematic botany, 8:453-482.

Seress, P., Balogh, G., Olah, A. and Cseke, L. (2001). Metabolic controlled fermentation procedure for the manufacture of lovastatin hydroxy acid. U.S. Patent, 6:197-560.

St éphane, O., Jean-Jacques, M., Antonio, G. and Eric, B. (2021). Coronaviruses, cholesterol and statins: Involvement and application for Covid-19. Biochimie, 189:51-64.

Tsiantas, K., Tsiaka, T., Koutrotsios, G., Siapi, E., Zervakis, G. I., Kalogeropoulos, N. and Zoumpoulakis, P. (2021). On the identification and quantification of ergothioneine and lovastatin in various mushroom species: assets and challenges of different analytical approaches. Molecules, 26:1832.

Vijay, V. P., Vinay, K. V. and Amit, P. (2019). Lovastatin: a journey from ascomycetes to basidiomycetes fungi. Journal of Biologically Active Products from Nature, 9:162-178.

World Health Organization (2021). Retrieved from https://www.who.int/health-topics/coronavirus#tab=tab_1 in Aug 10th, 2021.

(Received: 9 September 2021, accepted: 25 December 2021)