THE EFFECT OF DIFFERENT SUBSTRATES ON TRITERPENOIDS AND FATTY ACIDS IN FUNGI OF THE GENUS *Pleurotus*

IVONNE J. NIETO*, CAROLINA CHEGWIN A.

Dr. Ivonne Jeannette Nieto, Chemistry Department, National University of Colombia, Bogotá Campus, Ciudad Universitaria,
Carrera 30 No. 45-03, Edificio 451 Laboratorio 210. Bogotá-Colombia.

(Received: April 18, 2012 - Accepted: October 8, 2012)

ABSTRACT

Three species of cultivated fungi of the genus *Pleurotus*, *P. pulmonarius*, *P. sajor-caju*, and *P. ostreatus*, were studied to determine the effect of different substrates on their production of fatty acids and secondary metabolites. It was discovered that the substrate had a marked influence on fungal composition by promoting the production of a more diverse variety of compounds or by increasing their content. Fungi cultivated on substrates containing cacao shells demonstrated an increased biosynthesis of triterpenoids with greater structural diversity. Their content, however, was less than that obtained using cereal residue substrates.

The species *P. ostreatus* had the highest triterpenoid content, followed by *P. pulmonarius* and, finally, *P. sajor-caju*.

Keywords: Fungi, triterpenoids, fatty acids, *Pleurotus*, substrate.

INTRODUCTION

Mushrooms have greatly varied and important uses throughout the world, such as for food, tonics, and drugs ^{1,2}. Mushrooms have been used in recent decades for the bioconversion of organic waste materials because their large enzymatic pool allows them to develop in many substrates, thus greatly benefiting both humans and the ecosystem. Many mushrooms have properties that allow them to be used as nutraceuticals; that is, they produce compounds that can be extracted for use as antitumoral and hypocholesterolemic agents and as immunological modulators. This fact has awakened the interest of the scientific community in studying the secondary metabolites responsible for these properties. These products are biosynthesized from primary metabolites and are the end products of the metabolism of natural products; therefore, secondary metabolites are restricted to certain organisms. Lipids, a broad group of molecules that includes fatty acids, sterols, sphingolipids, and phospholipids, are the most important metabolic energy source, as they comprise the structural and functional components of biomembranes in different organisms. Unsaturated fatty acids, which belong to this group of compounds, have various positive health effects, such as antioxidant activity, colon cancer inhibition, and cardioprotective effects ³. With respect to compounds of triterpenoid type within sterols found, ergosterol micosterol is of great importance, as it is a synthetic precursor to both vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) with equal potency and are metabolized to form a hormone known as calcitriol. This hormone plays a key role in the metabolism of calcium and phosphorus ⁴. Further beneficial effects for this group of compounds have been reported and include antihyperlipidemic, anti-inflammatory, antioxidant, and both bacterial and fungal growth inhibitory activities ⁵. The use of sterols has been found to cause a hypocholesterolemic effect by competing with cholesterol for absorption by the intestine. In this way, the antiatherosclerotic and anticarcinogenic properties of fungi have been specifically studied in both animal models and cellular systems with very promising results ⁶⁻⁸.

Fungi belonging to the genus *Pleurotus* are the second most highly produced worldwide and represent approximately 25% of the group known as the "big six mushrooms", which are the most widely produced mushrooms, with China as the primary source ⁹. Basidiomycetes can use the greatest number of different substrates for cultivation ¹⁰, which is why they have been the subject of broad research that generally mentions their nutritional quality and the effect of substrate variation on the primary metabolites that are directly related to the nutritional quality ¹¹⁻¹⁵.

Pleurotus cultivation has the advantage of being cultivable in tropical climates, simple to produce, and compatible with organic substrates rich in lignin and cellulose, such as rice straw, sugarcane bagasse, coffee pulp, cocoa waste, and dry grass ¹⁶. It should be highlighted that Colombia, being a tropical and purely agricultural country, generates a wide variety of waste residues that are used for fungi cultivation, which creates a clear need to investigate the influence of the substrate on the secondary metabolites.

In this study, we report the first results relating to the effect of substrate variation on the triterpenoid metabolites and fatty acids present in three

Pleurotus species grown on different substrates. We aim to determine which substrate yields a product that can be used as a natural source for these compounds, which have demonstrated significant bioactivity.

EXPERIMENTAL

Names were given to the fungi used for this research according to their species: **PO** to *P. ostreatus*, **PP** to *P. pulmonarius*, and **PSC** to *P. sajor-caju*. Numbers were assigned based on the substrate used based on the following system:

PO1 was grown using a mixture of 17% bran, 35% sawdust, 3.0% CaCO₃, 43% chips, and 2.0% molasses and was facilitated by the Seeds of the Future Women Association (named AMUSEF according to its Spanish initials). **PO2** was grown using a mixture of 50% sugarcane, 30% beans, and 20% cocoa shells. **PO3** was grown using 16.8% bean stems and pods, 18.6%, maize calyx stems and leaves, 52.2% rice husks, 9.7% rice hulls, and 2.7% CaCO₃. **PP1** was grown using 50% sugarcane bagasse, 25% corn husks, and 25% bean chaff. **PP2** was grown using 50% bagasse, 30% bean chaff, and 20% cocoa shells and was provided by the Association of Fungi Producers of Colombia ASOFUNGICOL. For species *P. sajor-caju*, **PSC1** was cultivated on zoca and **PSC2** was cultivated on a mixture of zoca and coffee pulp. Both of these fungi were provided by the National Center for Coffee Research CENICAFE.

A 10 g sample of each lyophilized and milled fungus was taken and subjected to maceration with 30 ml of methanol for eight days, removing the solvent daily.

The crude methanol extracts were concentrated to dryness on a rotary evaporator followed by an extraction using CH₂Cl₂:H₂O (1:1). Each of these extracts was characterized by GC-MS using a Hewlett Packard 6890 gas chromatograph with the following characteristics: HP5 30 m capillary column with an inside diameter of 0.33 mm and 25 mm thick; helium carrier gas with a flow of 4.5 to 1 ml/min; the 1:10 split mode; the temperature was ramped from 90°C to 300°C at 5°C/min. The gas chromatograph was coupled to a 5973 mass spectrometer with a 70 eV ionization source. The injection volume in all cases was 1 µl with a concentration of 1 mg/ml. The major components for each extract were identified via the analysis of their fragmentation patterns in the mass spectra by comparing our values to the literature¹⁷⁻²¹. The quantity of fatty acids and esters was determined by their relative areas, and the sterol content was measured via the Liebermann-Burchard colorimetric method using a stigmasteryl calibration curve with a concentration range between 0.071 mg/ml and 0.71 mg/23 ml and consisting of nine levels ²².

RESULTS AND DISCUSSION

The chromatographic analysis of the three species was performed individually, and the comparisons were performed afterwards. Two distinct zones were clearly visible in the chromatograms (chromatograms No. 1, 2, and 3): one between 0 and 6 minutes corresponded to fatty acids and esters, and another between 16 and 30 minutes corresponded to triterpenes and triterpenoids.

Table 1 shows the components and concentrations of the fat fraction. This analysis reveals that the composition changes depend on the species, which is the most obvious for the structural variation. *P. ostreatus* had the greatest variation (table 1), followed by *P. pulmonarius* and, finally, *P. sajor-caju*.

Table 1. Fat composition for *P. ostreatus*, *P. pulmonarius*, and *P. sajor-caju* cultivated on different substrates.

Component	<i>Pleurotus species</i> ^a						
	PO1	PO2	PO3	PP1	PP2	PSC1	PSC2
1. Pentadecanoic acid	0.8		0.8	9.4		0.2	0.2
2. Palmitic acid	10.1	9.3	11.0	18.4	18.1	5.1	7.9
3. Linoleic acid	18.2	19.1	18.3		9.7	13.3	13.5
4. Stearic acid			0.7	8.3			
5. Oleic acid				8.0			
6. Methyl hexadecanoate		4.2	0.3		10.1		
7. Ethyl hexadecanoate		2.4				3.6	5.2
8. Methyl 8,11-octadecadienoate		12.5			19.7		
9. Ethyl linoleate		15.5	4.1			10.5	12.2
TOTAL	29.1	63.0	35.2	44.1	57.6	32.7	39.0

^a The amounts are given as percentages calculated based on the total ion chromatogram (TIC) results.

The influence of the substrate on the composition of the studied fractions was clearly shown. This observation is consistent with the chemical composition analysis performed by our research group for the same mushrooms, which found that the highest concentration of fat soluble in petroleum ether corresponded to *P. ostreatus*¹⁵. Such behavior was due to the substrate effects because one of the components was the cocoa shell, which has a relatively high fat content²³. The next most diverse species in terms of the variety of components was *P. pulmonarius*, which also varied in fat composition, with **PP2** possessing the highest content for both the total concentration and the variety of compounds present. Similarly, this variation substrate-dependent and was also cultivated using cocoa shell.

Chromatogram 1. Comparative study of the CH₂Cl₂ extracts from *P. ostreatus* grown on different substrates.

a). PO1 *P. ostreatus* was cultivated using a mixture of 17% bran, 35% sawdust, 3.0% CaCO₃, 43% chips, and 2.0% molasses. **b) PO2** *P. ostreatus* was cultivated using a mixture of 50% sugarcane, 30% beans, and 20% cocoa shells. **c) PO3** *P. ostreatus* was grown using 16.8% bean stems and pods, 18.6% maize calyx stems and leaves, 52.2% rice husks, 9.7% rice hulls, and 2.7% CaCO₃.

Chromatogram 2. Comparative study of the CH₂Cl₂ extracts of *P. pulmonarius* grown on different substrates.

a) PP1 *P. pulmonarius* was cultivated using 50% sugarcane bagasse, 25% corn husks, and 25% bean chaff. **b) PP2** *P. pulmonarius* was cultivated using 50% sugarcane bagasse, 30% bean chaff, and 20% cocoa shells.

The substrate influence was similar for *P. sajor-caju* cultivated on zoca and a zoca-pulp mixture (chromatograms 3a and 3b); i.e., the fat composition was similar in both the quantity and type of compounds, which was fully consistent with their similar substrate compositions. The peak that appeared at a *t_r* of 3.96 min for **PSC2** (chromatogram 3b) corresponds to caffeine incorporated into the fungus from the substrate used for cultivation. This feature had already been reported during other research within this group and is not common to all fungi²⁴.

Chromatogram 3. Comparative study of the CH₂Cl₂ extracts of *P. sajor-caju* cultivated on different substrates.

a) PSC1 was *P. sajor-caju* cultivated using zoca. **b) PSC2** was cultivated using zoca and coffee pulp. **c) PSC3** was cultivated using coffee pulp.

The *P. ostreatus* species had the highest overall fat content. This species had a higher fat content when cultivated on cocoa shell than for the other substrates. The substrates used for **PO1**, **PO3**, and **PP1** favored the production of unsaturated fatty acids rather than esters, and both cocoa and coffee industry wastes produced similar quantities of these compounds, especially for cases

such as **PP2**.

Table 2 summarizes the chromatographic analyses and mass spectral of peaks of interest for the triterpenoid fractions. Table 3 shows the results of the dichloromethane extracts with the sterol percentages calculated for each case. No significant variation was observed in the obtained extract percentage, contrary to what occurred for the sterol content.

Table 2. Triterpenoid compositions of *P. ostreatus*, *P. pulmonarius*, and *P. sajor-caju* grown on different substrates.

Component	Pleurotus ^a Species							
	RT (min)	PO1	PO2	PO3	PP1	PP2	PSC1	PSC2
10. Ergost-2,5,7,9(11),22-pentaene	19.25	3.1	5.3	2.8	2.7	2.7	1.6	1.8
11. Ergost-5,7,9(11),22-tetraen-3 β -ol	22.36	11.3	2.5	0.7		0.9		
12. Ergost-5,7,22-trien-3 β -ol	22.83	0.3	3.5	9.5		5.6		
13. Ergost-7-en-3 β -ol	24.02		0.2					

^a. The amounts are given as percentages calculated based on the TIC results.

- [10] Ergost-2,5,7,9(11),22-pentaene
- [11] Ergost-5,7,9(11),22-tetraen-3 β -ol
- [12] Ergost-5,7,22-trien-3 β -ol
- [13] Ergost-7-en-3 β -ol
- [14] Ergost-5 α ,8 α -epidioxy-6,22-dien-3 β -ol

Table 3. Extract quantity and sterol content obtained from *P. ostreatus*, *P. pulmonarius*, and *P. sajor-caju* grown on different substrates using dichloromethane.

Pleurotus Species	Extract Quantity (%)	Sterol Content (%)
PO1	9.80	17.3
PO2	9.75	15.6
PO3	9.60	18.4
PP1	9.56	9.5
PP2	9.67	13.2
PSC1	9.85	8.7
PSC2	9.34	7.8

Chromatogram 1 shows the profiles for the three studied *P. ostreatus*, and **PO2** showed the greatest triterpenoid structural variations and fat content. However, the triterpenoid structural variations were smaller and their proportions greater for **PO1** and **PO3**, which had very similar chromatographic profiles. This behavior is not easily explained, as the substrates on which these species were cultivated had different compositions and seemed to be characteristic of the species. The results presented for **PO2** indicate that the use of cocoa shells favors the biosynthesis of these compounds; therefore, its inclusion in the substrate would help to increase the production of these fungal compounds, which have been shown to possess extensive medicinal properties^{25, 26}.

Another obvious difference between the three studied *P. ostreatus* groups was their ergosterol content relative to **PO2**, for which the primary component is triterpene [10]. This variation could lead to the use of the culture for substrates **PO1** and **PO3** when the enrichment of ergosterol is desirable, such as for the production of vitamin D₂; the species *P. ostreatus* had the highest production of this vitamin relative to the other basidiomycetes²⁷ upon UV irradiation using 366 nm 254 y wavelengths²⁸.

The effect of substrate variation and the impact of cocoa shells on the triterpenoid biosynthesis were most evident in mushrooms with lower triterpenoid content. Such is the case with *P. pulmonarius*, as indicated by the

chromatographic profile and the data shown in Table 2 and strengthened by the data collected for *P. sajor-caju*. However, in terms of nutritional quality and crop efficiency²⁹, the use of waste from the coffee industry is an excellent alternative. Comparing the profiles of **PSC1** and **PSC2** to **PSC** cultivated using only coffee pulp (chromatogram 3c)¹⁶ indicates that the increased triterpenoid component in *P. sajor-caju* is possibly caused by the nature of the substrate and directly relates to the addition of the pulp in proportions above 50% and not to the species itself.

CONCLUSIONS

Of the mushrooms studied, the *P. ostreatus* species had the highest triterpenoid content, followed by *P. pulmonarius* and *P. sajor-caju*. The substrate directly influenced the composition of the fungus, which indicated that cocoa shells would be an ideal substrate for promoting greater variation in the biosynthesis of these compounds if the objective was to obtain a nutraceutical. If, however, the goal is to obtain a product with high ergosterol content, the use of cereals would be a better alternative. While all of the reported compounds were identified as forming a fat soluble fraction in *Pleurotus*^{4, 6, 30, 31}, this is the first report where this production is assessed by varying the cultivation substrate. The results from this study are consistent with reports that, while not focused on the production of fatty acids and triterpenes, corroborate the observed changes. For example, upon incorporation of cotton seeds or rice husks in the substrate, protein content, free amino acids, and the total lipid content were increased in *P. florida*, while there was a decrease in total dietary fiber and polymeric carbohydrate levels. Both *P. ostreatus* and *P. sajor-caju* have higher ash contents when grown using rice husks rather than banana straw, and *P. sajor-caju* has a higher moisture levels and fiber content when using rice straw¹⁰. Other studies have yielded similar results, although they always focused on changes in the nutritional quality^{12, 32, 33}, biological efficiency,^{29, 34-36} and enzyme production^{37, 38}.

ACKNOWLEDGMENTS

The authors wish to thank the Department of Chemistry and the Research Division of the National University of Colombia in Bogota.

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