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Towards profiling differential distribution of bioactive molecules across four phenologies in *Pleurotus djamor* R22

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ABSTRACT

Objective: To document differential distributions of phytomolecules by comparing antioxidant capacities, total phenolic contents, flavonoid contents and the identities of bioactive molecules on four phases of *Pleurotus djamor*.

Methods: Several methods including *in vitro* quantitative methods such as aluminium chloride spectrophotometric for flavonoids, Folin-Ciocalteu assay for phenolics, spectrometric methods for lycopene, β-carotene and carotenoids, and several antioxidant methods, ferric ion reducing antioxidant power assay (FRAP); 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS); and 1,1-diphenyl-2-picrylhydrazyl (DPPH), were used. Besides, liquid chromatography quadrupole time-of-flight mass spectrometry and ChemSpider database for the tentative identification of the compounds was utilized.

Results: The total phenol contents in the 1st and 2nd primordial phases from ethanol and aqueous extracts were (14.369 ± 0.495), (11.470 ± 0.532) and (19.025 ± 0.847) and (14.824 ± 0.890) mg gallic acid equivalent/g dry weight, respectively. The total flavonoid contents ranged from (3.311 ± 0.730) to (14.824 ± 0.890) mg quercetin equivalent/g dry weight. The second primordials phase recorded the highest total carotenoid content [(3.926 ± 0.059) ug/mL dry weight]. The extracts were deprived of lycopene, β-carotene and total carotenoids. However, the primordials reported relatively high contents of all molecules tested. The antioxidant capacity based on FRAP, ABTS and DPPH assays showed that the primordials exhibited more significant capacities in scavenging for these radicals than the mycelia and fruiting phases. The correlation between FRAP, ABTS, DPPH and total phenolic content, total flavonoid content, lycopene, β-carotene were positive and significant ($r > 0.8$, $P < 0.05$). Liquid chromatography quadrupole time-of-flight mass spectrometry analysis identified sixteen compounds using ChemSpide database. Phenolic acids, such as protocatechuic, hydroxycinnamic acid and stilbenes, and the flavonoids belonging to flavones, flavanones, isoflavone and anthocyanins were identified. The peak areas indicated significantly higher contents in the primordials phases for all compounds.

Conclusions: The immature basidiocarps are potent sources of secondary metabolites that should be targeted for *in vivo* validation of their function in such systems.

1. Introduction

In the recent past, there has been increasing interest in the higher basidiomycetes because of their immense added-value properties associated with health promoting phytochemicals[1,2]. For instance, studies showed that edible fungi contain immense pharmacological attributes that play a role in the antioxidative properties[3-5]. Besides, previous studies showed that mushrooms are rich sources of bioactive molecules like β-carotene, lycopene, flavanoids, phenols, which contributes to their unique therapeutic properties such as antioxidants[6,7]. Perhaps the rationale for these was because natural products with high contents of secondary metabolites would scavenge for radicals produced under stress and imbalance metabolism in the body system[8]. Secondary metabolites have been

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shown to reduce burdens of certain cancers as well[9]. Despite these attempts, no study has demonstrated whether the metabolites studied previously changes across physiological states of the basidiomycete development, which may provide leads for their optimization.

Existing studies document that immature spores and mature fruiting possess varying concentrations of the secondary metabolites[10]. Despite these efforts, such studies have not exclusively provided variations of bioactive molecules because most of them used mature fruiting bodies. Besides, most of these studies used commercial internal standards to identify and quantify these bioactive molecules using specialised protocols like ultra performance high performance liquid chromatography[7]. Such processes are likely to miss out on some important molecules whose standards were not incorporated in the process. One perspective recently ascribed is that the quantity of bioactive molecules drops with ageing of fruiting body due to their involvement in defense mechanisms[7,11,12]. To date, no such studies have been reported in higher basidiomycetes, especially the coloured ones, such as pink *Pleurotus djamor* (*P. djamor*), which exhibits observable characteristics as it develops to maturity. There is a need to use alternative protocols that do not need internal standards such as the liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS), which identifies molecules based on their accurate mass and empirical formula. The protocol validates the chemical assays used in the elucidation of essential properties in the sample. Identifying and quantifying the variation of specific flavanoids and polyphenols across phenological phases in mushrooms is an important aspect that would validate their selection in product development, especially for the species with medicinal added-value properties, hence, it is necessary to profile the differential distribution of such molecules in higher basidiomycetes.

2. Materials and methods

2.1. Source of Pleurotus spp. mushroom cultures

The slant cultures of *P. djamor* were shipped from the Plant Pathology Department, Penn State University, Pennsylvania State, USA. A previous method utilized by Zervakis *et al*.[13] was employed for the development of tissue cultures which were maintained at 5 °C using potato dextrose agar.

2.2. Spawn production

The bird millet was used as a carrier material for spawn production with a few modifications in the quantity of calcium carbonate from 10% to 1%[14,15]. The media bottles containing the sterile grains were transferred to the laminar airflow and left to cool. They were prepared for inoculation with pure isolates of *P. djamor* cultures. Each bottle was inoculated with four Petri dishes of fully-grown mycelia. The spawn bottles were incubated at room temperature on the laboratory bench for 14 days for complete colonization.

2.3. Chemicals and reagents

All the chemicals were of analytical grade. The following chemicals were procured for analysis: ferric chloride, ethanol, cold acetone, petroleum ether, silica gel, aluminium chloride, sodium carbonate and Folin-Ciocalteu reagent. Mayer's reagent and acetic acid were also procured. Other reagents included butylated hydroxytolune, alpha tocopherol (TOC), ascorbic acid, L-ascorbic acid (L-AA), and gallic acid. Besides, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic) acid (ABTS), hexane, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and potassium ferricyanide.

2.4. Spawning, inoculation and induction of pinning and sampling

The bird millet was used as the carrier media for the development of spawn using previously described methods[14,15]. The ten-day-old spawn exhibited full colonisation of the carrier media and was utilised for inoculation of the wheat straw substrate bags (500 g/bag), which were prepared under the sterile condition. The spawned bags were incubated in dark room at room temperature to induce the formation of pinheads until they formed their first phase of the primordials. The experiment was designed to ensure collection of basidiocarps across the remaining phases as we previously illustrated[16], summarised in Figure 1.

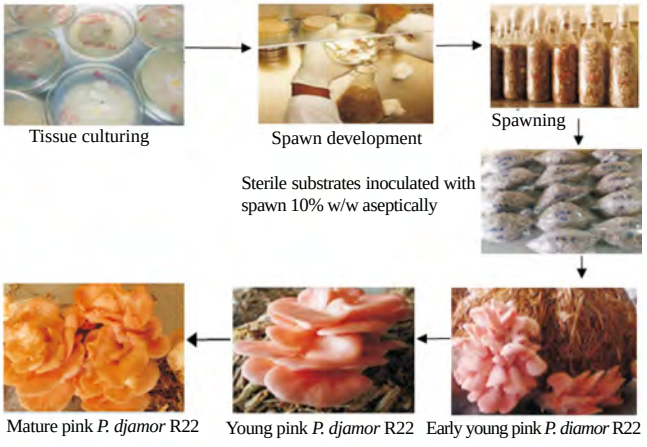

Figure 1. Process of developing pure tissue cultures, spawning, substrate, inoculation and phenological states of basidiomycetes.

2.5. Preparation of sample extracts

The fruiting bodies were prepared by drying at 42 °C for 72 h and milled with an electric blender (Kenwood: BL370, 400 W, 1.6 L, USA). Extraction involved mixing 10 g of the samples in separate extraction solvent (high performance liquid chromatography grade ethanol and water) for 24 h under the shaker (150 r/min) at room temperature. The extracts were filtered on a Whatman paper 12.5 cm and kept at 4 °C before carrying out the determination of the properties of total polyphenols, total flavonoids, ferric ion reducing antioxidant power (FRAP), DPPH, ABTS.

2.6. Total carotenoids content

The previously described method by Shen *et al.*[17] was used for the determination of the total carotenoids content. Fresh samples (10 g) were crushed with 5 g celite 454 in the presence of cold acetone, which was added stepwise under dark condition until extractions were complete. The extracts were cleaned with distilled water with maintaining the integrity of the partitions. Petroleum ether was used for the separation of acetone on the separating funnel. Water was trapped from the funnel using anhydrous sodium sulphate. Each phenological preparation was done three times. The optical density of the extract were measured at 450 nm and calculated using the formula:

Total carotenoids content (ug/mL) = [volume used (50 mL) × absorbance × 3.856]/10 g × 1000

2.7. Determination of β-carotene and lycopene

The previous method by de Rezende Queiroz *et al.*[18] was used for the determination of both the β-carotene and lycopene. This involved shaking the ethanol extracts vigorously after mixing with hexane and acetone (12:8; 20 mL). The content was filtered on a Whatman No. 4 before measuring the absorbance at several wavelengths (663 nm, 505 nm and 453 nm). Each phenological preparation was done three times. The contents were determined using the equations below and expressed as means ± SD:

Lycopene (mg/100 mL) = $-0.0458A_{663} + 0.3720A_{505} - 0.0806A_{453}$

β-carotene (mg/100 mL) = $0.216A_{663} - 0.304A_{505} + 0.452A_{453}$

2.8. ABTS assay

The antioxidant potential ability of coloured edible mushroom to quench and scavenge for ABTS radicals were determined accordingly[19]. Briefly, the samples were mixed with working ABTS solution. The mixture was centrifuged before the determination of the absorbance at 734 nm wavelength. Each phenological preparation was done three times. The method is based on the potential of extracts to reducing the ABTS radicals through the decolouration of the blue green chromophore. The standard used was vitamin E analogue (trolox), therefore results were expressed as Trolox equivalent mg per dry weight.

2.9. FRAP assay

The reduction power of mushroom extracts was determined using the previous method[20]. The process involved designing the concentration gradient of each sample from the four phenologies. Each phenological preparation was done three times. Briefly, the process involved mixing equal volumes of extracts, 1% potassium ferricyanide, and 200 mmol/L sodium phosphate buffer (2.5 mL each). The resulting mixture was incubated at 50 °C for 20 min and added with 2.5 mL of 10% trichloroacetic acid before centrifugation (1200 r/min/10 min) to separate the mixture and pipette the supernatant (5 mL). Equal volumes of deionised water were added to this supernatant before the addition of 0.1% ferric chloride (1 mL) and the absorbance was determined at 700 nm. The positive standards included L-AA, butylated hydroxytoluene (BHT), and TOC. Data obtained were expressed as mean ± SD.

2.10. Total phenolic content

The previous method Folin-Ciocalteau calorimetric assay was utilized[21]. Different concentrations of gallic acid (Sigma-Aldrich, Germany) standard were prepared (0.1 mg/mL–1.0 mg/mL). Briefly, extracts (0.5 mL) were transferred in in a separate tube with a capacity of 10 mL before adding 2 mL ethanol (Sigma-Aldrich, Germany). Specific volume (1.25 mL) of the Folin-Ciocalteu reagent was added to the mixture. The resulting mixture was timed for 5 min before adding 20% aqueous sodium carbonate (6.25 mL), which was mixed vigorously and kept under darkness at room temperature for a duration of 40 min. Each phenological preparation was done three times. One milliliter of each preparation was diluted with 9 mL of ethanol before reading the absorbance at 725 nm. The total amount of phenol was calculated as gallic acid equivalent (GAE) from the curve calibrated with gallic acid as the standard. This was expressed as mg GAE/g dry weight of mushroom material.

2.11. Total flavonoid content

Determination of the total flavonoid content was based on spectrophotometric method using the previously described approach[22]. Briefly, it involved diluting the sample with extraction solvent (1:4 mL) and adding 0.3 mL 5% sodium nitrite. The mixture was mixed vigorously and left for 3 min at room temperature before adding 0.3 mL 10% $AlCl_3$. Two milliliter of 1 mol/L NaOH was added to the resulting mixture and shaken to hasten the reaction for 3 min. The mixture was made to 10 mL by adding 2.4 mL of the extraction solvent. The optical density was measured at 415 nm, and each phenological preparation was done three times. The standard used was quercetin, hence, results were expressed in mean ± SD as mg quercetin/g dry weight.

2.12. DPPH

Fresh solution of DPPH (Sigma-Aldrich, Germany) was prepared in ethanol. DPPH assay was based on the previous assay with a few modifications of the sample volume[23]. A triplicate of the concentration gradient of the extract was designed from all the phenological phases. Four milliliter of the extracts were mixed with 1mL of the 0.1 mmol/L DPPH reagent and incubated for 30 min before measuring the absorbance at 517 nm. Positive standards used included L-AA, TOC, and BHT. The results were expressed as percentage inhibition using the following formula:

Percentage inhibition of DPPH radicals by the extract = $[(A_{blank} - A_{sample}) \times 100]/A_{blank}$.

2.13. Samples for metabolomics profiling

The LC-QTOF-MS analysis involved concentrating the extracts from the four phenological phases in the vacuum to dryness before redissolving the amount gotten in 2 mL liquid chromatography-mass spectrometry grade solvent, which was centrifuged at 7 200 r/min for 5 min as previously described by Wamalwa *et al.*[24]. The 0.5 μL of the extract was injected automatically in the LC-QTOF-MS analyser.

2.13.1. LC-QTOF-MS analysis

The initial stage involved separation of the compound on a chromatograph using waters I-class system (Waters corporation, MA) ACQUITY UPLC. The fitting of this system includes a 2.1 mm × 100 mm, 1.7 μm BEH C18 column (Waters corporation, Ireland). The heating temperature was set at 40 ℃, while the auto-sampling tray was cooled to 15 ℃[24]. The mobile phase consisted of two solvents, water (A) and acetonitrile (B), and each of these were incorporated with 0.01% formic acid. It involved a gradient system previously employed by Wamalwa *et al.*, which consisted of 0 –1.5 min of 10% B; 1.5–2 min of 10%–50% B, 2–6 min of 50%–100% B; 6–9 min of 100% B; 9–10 min of 90%–10% B and 10–12 min of 10% B[24]. A constant flow rate at 0.4 mL/min was utilised. A positive mode utilising full scan MSE was operated after interfacing the waters Xevo QToF-MS with the electrospray ionization. The resolution mode of *m/z* ranging from 0–1 200 was utilised for the acquisition of data. Other conditions for data acquisition included the desolvation temperature of 350 ℃, source temperature of 100 ℃, sampling cone voltage of 40 V, capillary voltage of 0.5 kV, a flow rate of 500 L/h for the nitrogen desolvation and scanning time of 1 s. The argon gas was used as the collision gas for the high energy scan because of its ultrahigh purity (> 99.999%), which was applied in the T-wave using energy ramp ranging from 25–45 eV as the collision energy. The reference compound sampled at an interval of 10 s was leucine enkephalin, $[M+H]^+$ = 556.2766. The calibration of the mass spectrometer had a mass range from 50–1 200 Da that utilised sodium formate (0.5 mmol/L), which was prepared using water/2-propanol (10:90 v/v). Version 4.1 SCN of the MassLynx (Waters Corporation, MA) played a critical role in the acquisition and data processing, which involved generation of elemental composition from every analyte. Mono-isotopic masses were utilised for calculating the potential assignments with a specification tolerant of 10 mg/L deviation taking care of both the even-electron and odd-electron state. The following were the expected types and numbers of atoms: sulfur ⩽ 6, nitrogen ⩽ 6, oxygen ⩽ 50, hydrogen ⩽ 100, and carbon ⩽ 100. The process of identifying compounds was based on their accurate mass and empirical formula, which were used for the prediction of the structure. The online ChemSpider database, fragmentation pattern and previously reported compounds in literature were used in searching for the identity of the compounds.

2.14. Statistical analysis

Data were analyzed for statistical significance using SPSS version 16 software (SPSS Inc, Chicago, USA) based on mean ± SD. The One-way ANOVA was used for the determination of statistical significance based on Tukey honest significant difference test ($P < 0.05$). The relationship between total polyphenolic content, total flavanoids content, lycopene, β-carotene and FRAP, DPPH, ABTS was determined based on Pearson correlation.

3. Results

3.1. Total phenolic content

The Folin-Ciocalteu *in vitro* assay was used in the determination of total polyphenols. Results shown in Tables 1 and 2 indicate that phenolic contents varied across these phenological states in ethanol and water extracts. The primordial phases from both extracts recorded the highest . For instance, (14.369 ± 0.495) mg GAE/g and (19.025 ± 0.847) mg GAE/g for ethanol extract, while water extract reported (12.655 ± 1.019) mg GAE/g and (10.908 ± 1.052) mg GAE/g, respectively (Tables 1 and 2). Although spawn mycelia phase recorded the lowest polyphenolic content, it was not significantly different from that of the mature fruiting body. These observations showed that the primordials exhibited significantly higher phenolics than the mycelia and mature basidiocaps.

Table 1
Total phenolic content and total flavonoid content of *P. djamor* R22 ethanol extracts across phenological states.

Phenological state	Total phenolic content (mg GAE/g dry weight)	Total flavonoid content (mg QE/g dry weight)
SPM	8.211 ± 0.709^{a}	3.311 ± 0.730^{a}
EYFB	14.369 ± 0.495^{b}	11.470 ± 0.532^{c}
YFB	19.025 ± 0.847^{c}	14.824 ± 0.890^{d}
MFB	9.483 ± 0.686^{a}	9.737 ± 0.472^{b}

Values were expressed as mean ± SD; Values with different superscript in the same column denote significant differences ($P < 0.05$).
QE: Quercetin equivalent; SPM: Spawn mycelia phase (10 days); EYFB: Early young fruiting body (2 days after pinning or the 16th day after inoculation of the substrate); YFB: Young fruiting body (3 days after pinning or the 17th day after substrate inoculation); MFB: Mature fruiting body (7 days after pin head formation or the 21st day after inoculation of the substrate).

Table 2
The total phenols and total flavonoid contents for water extracts of pink *P. djamor* across the phenological phases.

Phenological state	Total polyphenols content (mg GAE/g dry weight)	Total flavonoids content (mg QE/g dry weight)
Mycelia	4.708 ± 0.047^{aa}	7.403 ± 1.001^{a}
1st period (EYFB)	10.908 ± 1.052^{bb}	9.612 ± 1.306^{a}
2nd period (YFB)	12.655 ± 1.019^{bb}	8.014 ± 0.703^{a}
3rd period (MFB)	7.317 ± 0.921^{cc}	8.840 ± 0.088^{a}

Values were expressed as mean ± SD, and values with different superscript inthe same column denote significant differences ($P < 0.05$).

3.2. Total flavanoid content

Results from the determination of the total flavanoid content across the four phenological phases indicated that ethanolic extracts of the 2nd phase primordial exhibited a significantly higher value [(14.824

± 0.890) mg/GAE g dry weight; $P < 0.05$] than other phases. However, its comparison with the water extract showed that the variation of flavanoids did not vary significantly (Tables 1 and 2). All the primordial phases reported high values than the fruiting bodies and the spawn mycelium.

3.3. Determination of lycopene, β-carotene and total carotenoids

The second primordial phase recorded the highest β-carotene [(0.137 ± 0.037) μg/g dry weight] and total carotenoid [(3.926 ± 0.059) μg/mL], and the highest lycopene [(0.021 ± 0.006) μg/g dry weight] (Table 3). These secondary metabolites varied across the four phenological states with the primordials exhibiting significantly higher values. The spawn mycelia phase recorded the lowest concentrations of total carotenoids, β-carotene and lycopene. The first phase of primordial recorded the high content of β-carotene and lycopene than fruiting phase (Table 3). The finding suggested that the spawn mycelium exhibited very low antioxidant properties. The concentrations of β-carotene were higher than those of lycopene in all the four phases.

Table 3
Total contents of carotenoids, lycopene and β-carotene of pink *P. djamor*.

Phenological state	Carotenoid (μg/mL)	β-carotene (μg/g dry weight)	Lycopene (μg/g dry weight)
SPM	0.028 ± 0.019^{ac}	0.004 ± 0.001^{a}	0.002 ± 0.001^{aa}
EYFB	2.470 ± 1.004^{bc}	0.112 ± 0.011^{b}	0.017 ± 0.002^{bb}
YFB	3.926 ± 0.059^{ad}	0.137 ± 0.037^{c}	0.021 ± 0.006^{cc}
MFB	3.003 ± 0.001^{bc}	0.023 ± 0.020^{d}	0.014 ± 0.001^{dd}

Values were expressed as mean ± SD and values with different superscripts in the same column denote they are significantly different ($P < 0.05$).

3.4. ABTS assay

The ABTS scavenging properties exhibited a trend of maturity dependency (Table 4). The primordial phases recorded the highest concentration followed by the fruiting body phase while the mycelium recorded a reduced concentration. The ABTS radicals ranged from (1.019 ± 0.004) to (16.209 ± 0.011) and (0.673 ± 0.120) to (31.548 ± 0.307) Trolox equivalent mg/g dry weight (Table 4). The two phases of the primordials recorded significantly higher values than the spawn mycelia phase and the fruiting body. Besides, the correlation between lycopene, β-carotene and tetraethylammonium chloride (TEAC) showed a significant relationship ($r > 0.8$, $P < 0.01$) (Table 5).

Table 4
ABTS assay for *P. djamor* expressed as TEAC (mg Trolox equivalent/g dry weight).

Phenological state	TEAC	
	Water extract	Ethanol extract
SPM	1.019 ± 0.004^{aa}	0.673 ± 0.120^{a}
EYFB	14.633 ± 0.061^{bb}	22.098 ± 1.004^{b}
YFB	16.209 ± 0.011^{cc}	31.548 ± 0.307^{c}
MFB	11.516 ± 0.058^{dd}	13.105 ± 1.622^{d}

Values were expressed as mean ± SD ($n \geqslant 3$), and values with different superscripts in the same column denote significance differences ($P < 0.05$).

Table 5
Pearson correlation between bioactive molecules and FRAP/TEAC/DPPH.

Compound	*r* value		
	FRAP	TEAC	DPPH
Total polyphenols	0.754	0.894	0.914
Total flavonoid	0.828	0.899	0.952
Lycopene	0.915	0.828	0.843
β-carotene	0.941	0.891	0.959

3.5. FRAP

This study demonstrates that the four phenological phases exhibited varied capacities to reduce ferricyanide. Higher reduction power signifies increased potential to reduce ferricyanide. These results show that the two primordial phases (EYFB and YFB) have a better potential in their capacity to reduce ferric ions in comparison to L-AA, TOC and BHT (Table 6). The varying trend in the ability of each phenological phase to reduce ferricyanide in ethanol extract was relatively higher than that in water extracts (Table 6). The power of ethanol extracts ranged from (12.106 ± 1.130) mg/mL to (27.444 ± 2.085) mg/mL for primordials, while its water extract ranged from (9.812 ± 1.473) mg/mL to (19.261 ± 2.180) mg/mL. The reduction power of the first primordials (EYFB) was not significantly different from the fruiting phases (MFB) ($P > 0.05$). Spawn mycelia showed reduced power. The correlation between reducing power and β-carotene, lycopene, total flavanoids content, and total polyphenolic content at 10 mg/mL relates positively ($r > 0.9$, $P < 0.01$) (Table 5).

Table 6
FRAP of coloured edible mushrooms *P. djamor.*

P. djamor		Sample concentrations (mg/mL)			
		10	5	1	0.5
Water extract	SPM	0.431 ± 0.062^{a}	0.168 ± 0.024^{b}	0.027 ± 0.005^{a}	0.011 ± 0.004^{aa}
	EYFB	9.812 ± 1.473^{b}	6.105 ± 0.834^{dd}	1.248 ± 0.091^{c}	0.433 ± 0.126^{cc}
	YFB	19.261 ± 2.180^{c}	10.077 ± 0.982^{cc}	2.853 ± 0.642^{c}	0.740 ± 0.033^{cc}
	MFB	9.307 ± 1.521^{b}	6.290 ± 0.837^{dd}	0.941 ± 0.156^{b}	0.406 ± 0.087^{cc}
Ethanolic extract	SPM	1.423 ± 0.608^{d}	0.914 ± 0.265^{ee}	0.273 ± 0.098^{b}	0.098 ± 0.010^{aa}
	EYFB	12.106 ± 1.130^{b}	7.582 ± 1.404^{dd}	0.815 ± 0.067^{b}	0.332 ± 0.045^{cc}
	YFB	27.444 ± 2.085^{c}	11.491 ± 1.279^{cc}	1.642 ± 0.333^{c}	0.656 ± 0.107^{cc}
	MFB	14.240 ± 1.936^{b}	5.048 ± 0.355^{dd}	0.912 ± 0.108^{b}	0.364 ± 0.129^{cc}
L-AA		26.577 ± 0.651^{c}	12.604 ± 0.444^{cc}	2.171 ± 0.097^{c}	0.893 ± 0.015^{cc}
BHT		32.091 ± 0.384^{e}	14.533 ± 0.294^{cc}	3.824 ± 0.610^{d}	1.679 ± 0.243^{dd}
TOC		29.564 ± 0.705^{e}	13.829 ± 0.333^{cc}	3.065 ± 0.422^{d}	1.634 ± 0.090^{dd}

Values were expressed as mean ± SD, and different superscripts in the same column denote significant difference at $P < 0.05$.

3.6. DPPH radical scavenging properties

The radical scavenging activity of *P. djamor* showed that the properties were dependent on the concentration (Figure 2). The potential of scavenging for the radicals varied across the phases with primordials exhibiting significantly higher values than the mycelia phases and the basidiocarps as compared with the standards utilised. For instance, the first phase of primordial was 82.402% at 10 mg/mL, second primordial was 85.674% at 10 mg/mL, while the mycelia and primordial phases were 73.920% and 70.011% both at 20 mg/mL, respectively, which were compared with TOC (85.694% at 5 mg/mL), L-AA (94.704% at 10 mg/mL), and BHT (89.105% at 5 mg/mL) (Figure 2). There were no significant difference between the mature basidiocarps and the spawn mycelia phases ($P < 0.05$). The finding shows that spawns is the alternative source to mature basidiocarps.

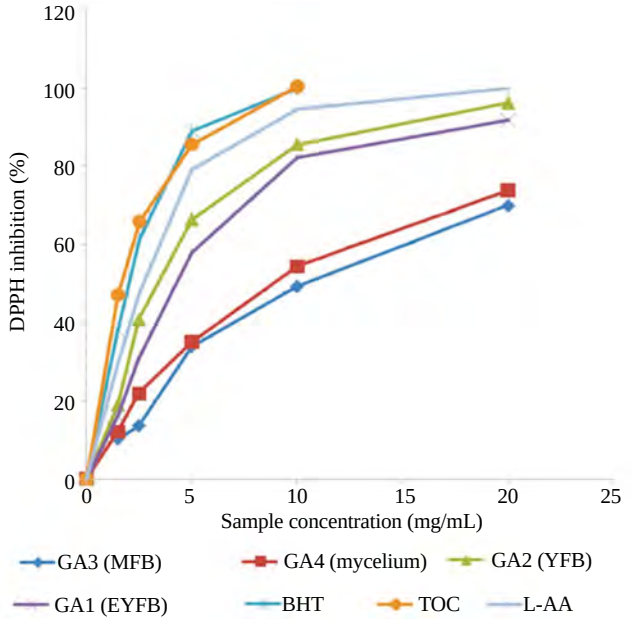

Figure 2. DPPH radical scavenging activity of various phenological states of *P. djamor* R22, L-AA, TOC and BHT.

3.7. Distribution of compounds across phenology

The determination of compounds distribution in the four phonologies shows differential concentrations as illustrated by the peak areas (Table 7). The elemental compositions were used for the acquisition of empirical formula, peak area, percentage confidence, and accurate mass. The concentration difference of each compound was based on the peak area (Table 7). The ChemSpide database was utilised for the identification of compounds based on the empirical formula, accurate mass and literature search from previous studies. The mature basidiocarps were labelled as GA4, young primordials was GA3, early young basidiocarps was regarded GA2, while spawn mycelium was represented by GA1 (Table 7). Similar compounds were identified across the four phases with primordial exhibiting higher concentration. The main flavanoids identified include naringin, biochanin A, hesperetin, kaempherol, loganate, dinoprostone, roseoside, formononetin and resveratrol (Table 7). The polyphenolic acids identified include caffeic acid, 4-phenylbutyric acid, protocatechic acid, gallic acid, homogentisic acid, 4-coumaric acid and ferulic acid,

4. Discussion

Previous studies by Reis *et al.*[3] and Barros *et al.*[7] documented the existence of bioactive molecules in fresh, dried and lyophilized mushrooms. This study introduces a shift in paradigm by contextualizing the previously identified molecules and their differential distribution across four phenological or physiological phases. We designed and developed spawn mycelium that part of it was included for analysis and part of it was used for substrate inoculation to obtain two primordials phases and one mature fruiting body phase.

Phenolics are derived from renewable natural resources, which are characterized as non-enzymatic compounds whose potential in antioxidant and other health promoting properties such as anti-

Table 7
Identity of flavonoids and polyphenolic compounds based on accurate mass and empirical formulas of *P. djamor*.

No.	Retention time	Accurate mass	Confidence (%)	Emprical formula $(M+H)^+$	Identity	Peak area			
						GA1	GA2	GA3	GA4
1	1.10	229.2	99	$C_{14}H_{13}O_3$	Resveratrol	620.25	51 687.90	15 506.40	8 063.31
2	0.61	269.1	95	$C_{16}H_{13}O_4$	Formononetin	931.33	77 610.90	23 283.30	12 107.30
3	0.97	397.6	90	$C_{19}H_{31}O_8$	Roseoside	220.05	18 337.90	5 501.36	2 860.70
4	2.76	195.1	97	$C_{10}H_{10}O_4$	Ferulic acid	3 080.41	25 670.10	77 010.30	40 045.40
5	3.03	353.2	99	$C_{20}H_{33}O_5$	Dinoprostone	104.46	4 502.94	2 611.70	1 358.08
6	2.25	165.1	99	$C_{10}H_{13}O_2$	4-Phenylbutyric acid	1 048.90	45 215.40	26 224.90	13 636.90
7	2.25	165.1	99	$C_9H_9O_3$	4-Coumaric acid	359.74	15 506.40	8 993.69	4 676.71
8	2.51	377.1	99	$C_{16}H_{25}O_{10}$	Loganate	540.17	23 283.30	13 504.30	7 022.23
9	2.63	169.2	99	$C_8H_9O_4$	Homogentisic acid	127.63	5 501.36	3 190.78	1 659.20
10	0.54	171.1	99	$C_7H_7O_5$	Gallic acid	1 786.63	77 010.30	44 666.00	23 226.30
11	0.58	155.1	90	$C_7H_7O_4$	Protocatechic acid	31.34	1350.88	783.51	407.42
12	0.73	181.1	99	$C_9H_9O_4$	Caffeic acid	83.01	13 564.60	2 075.38	1 079.19
13	2.58	287.3	99	$C_{15}H_{11}O_6$	Kaempherol	40.31	704.83	107.83	74.07
14	2.92	303.3	98	$C_{16}H_{15}O_6$	Hesperetin	60.47	1 058.33	161.92	111.19
15	2.76	287.3	90	$C_{16}H_{13}O_5$	Biochanin A	10.53	250.06	38.25	24.39
16	4.57	581.6	90	$C_{27}H_{33}O_{14}$	Naringenin	21.42	3 500.46	535.57	278.49

inflammatory have drawn immense scientific attention[25]. This study illustrates that mushrooms possess phenolic molecules, whose concentrations vary across four phases (Tables 1 and 2). Primordial phases of *P. djamor* R22 recorded the highest values for the polyphenols. Previously, Barros *et al.*[7] ascribed that immature spores from mushrooms are potential sources of secondary metabolites, and our study collaborates such findings. However, other studies suggested that both young and mature mushrooms did possess phenol contents that did not significantly differences ostensibly because they did not utilise coloured species[11,20]. Interestingly, this study show that there was no significant difference in total polyphenolic content levels in mycelium and mature fruiting body in *P. djamor* R22 (P = 0.192). This suggests that spawn mycelium could also be used as a potential source of polyphenols instead of obtaining mature fruiting body as previously demonstrated[25]. The content of polyphenol increases with the growing of the basidiomycete, so that young coloured fruiting bodies have lower values but this increases up to a certain level before beginning to decline as the mushroom ages. Perhaps, a better approach of concentrating the bioactive molecule would involve using fermentation procedure to the mycelia phase. Primordial stages provided a better source for bioactive molecules. The assessment of the correlation between antioxidant capacity and the total polyphenolic content exhibits a positive and significance relationship, which suggests the possibility of phenolic acid contents playing a role in the antioxidant properties.

Findings show that the flavanoid contents varied across the phenologies studied ($P < 0.05$), and the total polyphenolic contents were higher than those of other mushrooms previously reported[26]. Perhaps the best explanation for this observation is the possibilities of the involvement of these compounds in defense mechanism, which reduces their concentrations with age as previously described[1]. The same trend exhibited in the total polyphenolic contents showed significant association between total polyphenolic contents and various assays of the antioxidant capacity (Table 5). Previously, Li *et al.*[11] and Liu *et al.*[20] showed that molecules vary in *Agaricus* mushrooms according to their maturity. Besides, immature spores of *Lactarius piperatus* exhibited high contents of compounds as compared to the fruiting bodies that has attained maturity[7]. Besides, other studies showed that the extraction solvent affects the concentration of secondary metabolites extracted[27-29]. Therefore, using as many solvent as possible was encouraged, which, perhaps, was the shortcoming in our study. Besides, we found a strong and significant correlation between total polyphenolic contents and various assays of antioxidative properties, which corroborates previous findings[26,30].

Edible fungi that exhibit different colouration from the conventional gray and white coloured species have been studied and found to contain molecules such as β-carotene and lycopene[4]. These molecules have been associated with immense health properties in their role to impart antioxidant properties[4]. This work showed that phenological phases of *P. djamor* R22 contains varied concentrations of total β-carotenoids, carotene and lycopene with both phases of primordials exhibiting significantly high concentration. For instance, the second phase of primordial reported (0.262 ± 0.005) μg/g dry weight of β-carotene and (5.632 ± 1.808) μg/mL of total carotenoid while the first phase reported high lycopene of (0.026 ± 0.003) μg/g dry weight. Although the total carotenoid content was significantly higher in these phases, their corresponding β-carotene and lycopene were low, which thereby collaborates the previous findings[4,7].

The potential of the extract to scavenge and decolorize the $ABTS^+$ radicals varied between the phenologies from (1.019 ± 0.004) to (16.209 ± 0.011) and (0.673 ± 0.120) to (31.548 ± 0.307) Trolox equivalent per gram dried weigh of the mushroom during the 2nd primordial phase for water and ethanol extracts in that order. Using the ABTS assay showed that phenological phases exhibited significant antioxidant capacity as compared to both the mature basidiocarp and the mycelia phase. These observations show that age has influences on the deprivation of secondary metabolites, which is responsible for the decolouration of the $ABTS^+$ as well as their relative proportion, which collaborates the previous studies that used other plants[31]. Therefore, the primordials exhibited potent properties in the decolourization of radicals. Besides, a significant correlation was observed between TEAC and the total polyphenol content, total flavonoid content, lycopene and β-carotene (Table 5), which suggested the involvement of these bioactive molecules in the scavenging and decolouration of these radicals.

Our finding demonstrates that phenological phases in the pink edible mushroom have different capacities to reducing ferricyanide for both water and ethanol extracts (Table 6). These properties depended on the concentration of the extract, which exhibited strong and significant correlation coefficient. Generally, ethanol extracts exhibited high potency in the reduction of ferricyanide, but the main observation is that both primordial phases were more potent than both the mycelia phases and the mature phases. The primordials showed relative correlation with the positive standards utilised, justifying their importance in development of nutraceuticals or utilisation for *in vivo* systems to further assess their properties[7]. Similarly, we found statistical significance between the reduction of ferricyanide and contents of flavanoid and polyphenol, which correlated with previous observations[32,33]. Besides, our study found that the primordials correlated strongly and significantly with both β-carotene and lycopene, which was unlike the previous study by Robaszkiewicz *et al.*[4]. Perhaps the different results were because they did design or use the phenological approach. The spawn mycelium phase reported very low concentrations of these molecules. Several studies documented the impact of maturity stage on structural elucidation, chemical compositions and antioxidant properties without defining the stages of the mushrooms[7,34,35]. Our studies agrees with previous studies that reported low concentrations of the compounds in the whole mushrooms as well as pilei and stipes[34]. However, such studies did not assess the concentrations of these molecules in the primordials and mycelia phases. Therefore, we suggest that the better phase for targeting these molecules is at

the primordial stages. It is from this realisation that we designed four phases that can act as a guidance for biotechnology application in mushroom.

The capacity of the extracts to scavenge and quench the free DPPH· radicals was dependent on the concentrations of the extracts. The increase in the concentration of the extract (radical scavengers) leads to a significant decrease in the free radicals. Previous studies found that mushrooms possessed radical scavenging activity that ranged from 0.58–4.58 mg/mL to 0.76–17.00 mg/mL, which corroborates our finding[7,36]. We observed that different phenological phases exhibited different capacities to scavenge for radicals with both levels of primordials having excellent properties than both the mature phases and the mycelia phases. Based on these findings, we conclude that different physiological phases of mushrooms have different capacities to scavenge for free radicals.

The LC-QTOF-MS analysis was set in the positive ionization mode. The identification of the compounds was based on the MS data, retention time, accurate molecular mass and empirical formula, which were used for online searching in the ChemSpider database for their tentative identification to obtain the results (Table 7). Fifteen positive identified molecules belonging to phenolics and flavanoids were identified based on the comparison of the MS data and the ChemSpider database. The identified phenolic acids include hydroxycinnamic acids (ferulic acid, caffeic acid, 4-coumaric acid), hydroxybenzoic acids (gallic acid and protocathechuic acid), stilbenes (resveratrol), terpenoid (loganic acid) and the phenols include reseoside, 4-phenylbutyric acid and homogentisic acid. The distributions of these compounds across the four phenologies differed significantly. For instance, the second phase of the primordials had the highest ferulic acid (77 010.30), 4-coumaric acid (26 224.90) and resveratrol (15 506.40) as compared to the remaining phases. The other identified compounds reported significantly higher levels during the first primordial phase [caffeic acid (13 564.60); gallic acid (77 010.30); protocathechuic acid (1 350.85); loganic acid (23 283.30); reseoside (18 337.90); phenylbutyric acid (45 215.40); and homogentisic acid (5 501.36)] (Table 7). The first phase was also higher than the mature phases with the mycelia phases exhibiting low levels of these molecules. Previously studies identified phenolic compounds such as carotenoids, tocopherols, ascorbic acids, vanillic acids, p-coumaric acid, p-hydroxybenzoic and protocatechuic, which collaborates with our finding[7].

The flavanoids identified belonged to isoflavone (formononetin, biochanin), anthocyanins (ferulic acid, 4-coumaric acid, caffeic acid), flavones (kaempherol), flavanones (naringenin and hesperetin) and flavonol derivatives of aglycones such as kaempherol. The other compounds included dinoprostone. All the flavanoid compounds were significantly higher during the first phase of primordial phases (Table 7). The second phase was also higher than the mature phases while the mycelia phases were deprived of these compounds. As the study unravels, the primordial and early basidiocarps offer significant polyphenols, flavonoids and total antioxidant properties than both spawn mycelium and mature fruiting bodies. This is the first study that developed phenological phases and justifies the variations of health promoting molecules, which plays a significant role in scavenging for the radicals. The first and second primordial phases should be utilised for the *in vivo* systems to document their properties. Our study justifies the need for a paradigm shift in considering the primordial and early basidiocarps as the alternative sources of mushroom products as opposed to the mature fruiting bodies.

Conflict of interest statement

We declare that we have no conflict of interest.

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