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Research article

Ultrasound-assisted extraction of phenolic compounds from ear mushrooms (*Auricularia auricula-judae*): Assessing composition and antioxidant activity during fruiting body development

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Abstract: *Auricularia auricula-judae* (ear mushroom) exhibits significant biological and pharmacological properties, particularly as an antioxidant due to its phenolic compounds. This study introduces a novel ultrasound-assisted extraction technique to quantify phenolic compounds and assess antioxidant activity in ear mushrooms. Key extraction factors, including solvent-to-sample ratio (10:1, 20:1, 30:1 mL/g), pulse duty cycle (0.2, 0.5, 0.8 s^{-1}), and temperature (10, 35, 60 °C), were optimized using a Box–Behnken design and response surface methodology (RSM). Methanol was identified as the most effective solvent, yielding the highest total phenolic content (TPC) and antioxidant activity. The optimal conditions for TPC and 2,2-diphenyl-1-picryl hydrazyl (DPPH) inhibition were determined to be 1 g of sample with 18 mL of methanol at 59 °C and a pulse duty cycle of 0.7 s^{-1}. This

method achieved a recovery rate of 94.85% for TPC and 92.71% for antioxidant inhibition and was validated with high precision (CV < 5%). Application of this method during fruiting body development (7–19 d) revealed that the optimal harvest time for maximum TPC and antioxidant activity was 8 d fruiting age.

Keywords: Box-Behnken design; method development; TPC; antioxidant; validation

1. Introduction

Ear mushroom (*Auricularia auricula-judae*) is one of the most consumed mushrooms in Asia and is valued as both food and medicine. In 2020, Indonesia produced 33,163 tons of mushrooms, while consumption reached 47,753 tons [1]. Renowned as a healthy food and traditional remedy [2], ear mushrooms are rich in essential nutrients, including proteins, carbohydrates, vitamins, minerals, fiber, and essential amino acids [3]. Additionally, ear mushrooms are particularly abundant in bioactive compounds, including phenolic acids [4] that contribute to a range of health benefits [5], such as antioxidant [6,7], antimicrobial [8], anti-inflammatory [9], anticancer, and antidiabetic [10] properties. Their antioxidants play a role in preventing degenerative diseases related to free radicals, which cause cellular damage leading to cardiovascular disease, diabetes, cancer, and neurodegenerative disorders [5]. The significant antioxidant potential of ear mushrooms is primarily due to their rich phenolic compound profile [11]. Therefore, it is crucial to develop reliable methods for their analysis.

Ear mushrooms, like many plants, have a complex matrix structure that requires analytical extraction to detect phenolic compounds accurately. Traditional methods, such as percolation, Soxhlet extraction, and maceration, use large amounts of solvents and require long extraction times (1–3 hours) [12,13]. Additionally, certain phenolic compounds are thermolabile, degrading above 70 °C in open systems [14]. An emerging technology that addresses these challenges is ultrasound-assisted extraction (UAE), which enhances mass transfer and accelerates extraction kinetics through the cavitation effects of ultrasonic waves, offering a promising alternative to traditional methods [15].

The applicability of UAE to recover bioactive compounds from several edible mushrooms has been reported, including phenolic compounds from 15 edible mushrooms from Southern Andalusia and Northern Morocco [16], *Inonotus hispidus* [17], and *Thelephora ganbajun* [18]. However, several factors can influence the efficiency of the UAE, such as the solvent-to-solid ratio, pulse duty cycle, and temperature [19–21]. Therefore, optimizing the extraction conditions to recover phenolic compounds specifically for ear mushrooms is necessary.

In this study, a Box-Behnken design (BBD) was employed to systematically evaluate the effects of various operating factors on the efficiency of the UAE. This experimental design was chosen due to its ability to require fewer experimental units than a complete factorial design, thereby reducing the time, solvent usage, and overall experimental costs [22]. Following this, RSM was utilized to ascertain the optimal conditions for the UAE. The primary focus of this research was the development of a robust UAE method for the extraction of phenolic compounds from mushroom samples, ensuring the maximal antioxidant capacity of the extracts. Furthermore, the optimized UAE method was validated and applied to extract phenolic compounds from ear mushrooms during fruiting body development. By developing an optimized UAE method specifically for ear mushrooms, this study offers a novel and efficient approach to fully harnessing their bioactive potential, setting it apart from previous

research on other mushroom species by focusing on the unique phenolic content and health benefits of ear mushroom.

2. Materials and methods

2.1. Plant material

Ear mushroom samples during fruiting body development (7–19 d) were sourced from a local farmer (Jamal Farming, coordinates: −7.744269045092224, 110.3077243) in the Margoluwih, Seyegan, Sleman region, Yogyakarta, Indonesia (Figure 1). The fresh mushrooms were preserved through freeze-drying. Subsequently, the dried mushrooms were ground using a KLAZ coffee grinder (ACE Hardware, Jakarta, Indonesia) with a cycling pattern of 30 s on and 30 s off, repeated five times. The resulting ground material was further refined by passing it through an 80-mesh screen using a vibratory sieve shaker (Haver Boecker Test Sieve Shaker EML 200 Pure, Ennigerloher, Germany). A homogeneous composite sample, including mushrooms from all stages of development (7 to 19 days), was used to ensure a representative mixture of the entire development process. These composite samples were then stored individually in airtight containers at 4 °C.

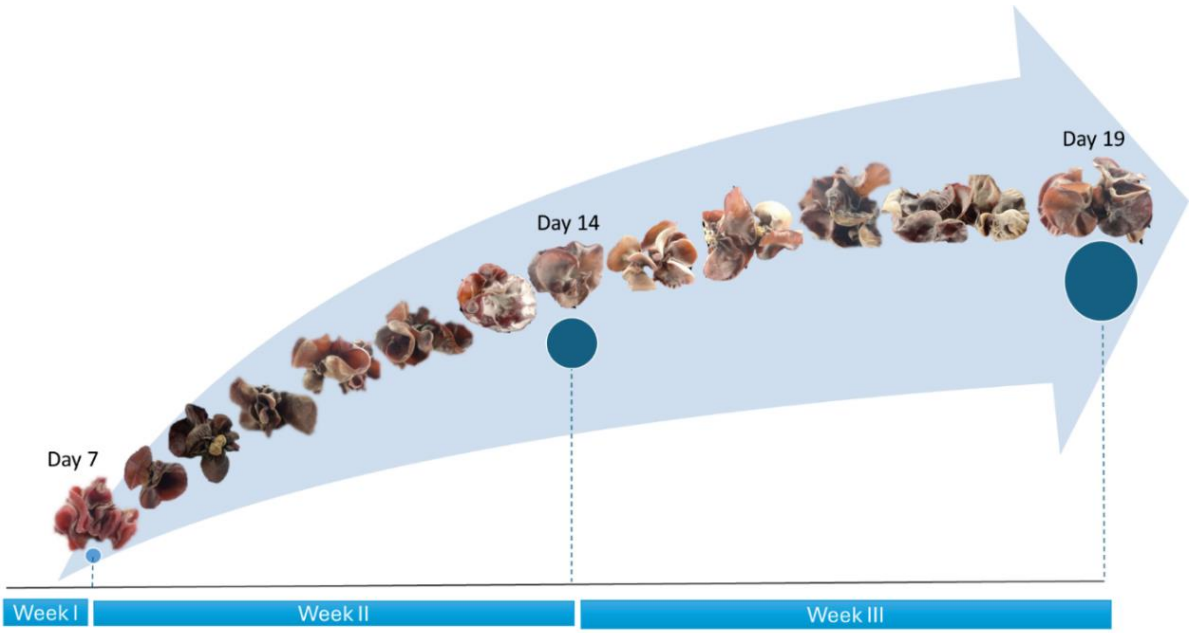

Figure 1. Fruiting body development (7 to 19 d) of ear mushroom.

2.2. Chemical and reagent

The chemicals utilized in this study, including acetonitrile, ethanol, methanol, hexane, ethyl acetate, Folin-Ciocalteu, Na_2CO_3, and 2,2-Diphenyl-1-picryl hydrazyl (DPPH), were procured from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Ultrapure water for the experiments was obtained from Aqua for Injection (Jakarta, Indonesia).

2.3. Ultrasound-assisted extraction

An ultrasonic system, the Sonopuls HD 4200 (20 Hz, 200 W, BANDELIN electronic GmbH & Co KG, Heinrichstrabe, Berlin, Germany), equipped with a TS 104 probe of 4.5 mm diameter, was utilized to facilitate the extraction process. The sample (1 g) was weighed and placed in 30 mL centrifuge tubes. According to the experimental design, a solvent was added to achieve the specified solvent-to-sample ratios (10:1, 20:1, 30:1 mL of solvent per gram of sample). The extraction process was conducted at varying pulse duty cycles (0.2, 0.6, 1.0 s^{-1}) and temperatures (10, 40, 70 °C), controlled by a Frigiterm system (J.P. Selecta, Barcelona, Spain), for 10 minutes. Following extraction, the samples were centrifuged (Thermo Fisher Scientific, Langenselbold, Jerman) at 4000 rpm for 10 minutes. The solvent volume was then adjusted to 25 mL. The extracts were stored in closed vials wrapped in aluminum foil and kept at 4 °C until analysis.

2.4. Total phenolic compound analysis

The total phenolic content in the extracts was quantified using the Folin-Ciocalteu method. A 0.5 mL mushroom extract sample was mixed with 2.5 mL of 10% Folin-Ciocalteu reagent, homogenized, and incubated for 5 minutes. Then, 2 mL of 2% sodium carbonate (Na_2CO_3) was added to form a blue complex. After a 60-minute incubation in the dark, the absorbance was measured at 765 nm using a UV-Vis spectrophotometer (Genesys 10S UV–Vis Spectrophotometer (Thermo Scientific, Massachusetts, USA). A calibration curve was generated using gallic acid standards with concentrations ranging from 0 to 100 mg/L to quantify the phenolic content. The equation derived from the curve was y=0.0114x+0.0406, with a high correlation coefficient R^2 = 0.9772. Additionally, the limits of detection (LOD) and quantification (LOQ) were determined to be 16.63 mg/L and 50.39 mg/L, respectively.

2.5. Antioxidant inhibition

The antioxidant activity was evaluated by assessing the free radical scavenging ability (% RSA) according to the previously described method [23] with minor modifications. Specifically, 0.6 mL of the ear mushroom extract was combined with 3.9 mL of 0.1 mM DPPH reagent and homogenized with vortex. The mixture was then incubated in the dark for 60 min to prevent photodegradation. Subsequently, the absorbance of the solution was measured at 515 nm using a UV-Vis spectrophotometer. The percentage of free radical scavenging activity (% RSA) was calculated by comparing the absorbance value of the DPPH solution reacted with the sample to that of the DPPH solution without the sample (control).

2.6. Experimental design

A Box–Behnken design (BBD) was employed to evaluate the effects of three independent variables: solvent-to-solid ratio (A), pulse duty cycle (B), and temperature (C) on total phenolic compounds and antioxidant activity. The design (Table 1) incorporated three levels for each factor (−1, 0, 1), resulting in 15 experimental conditions, each replicated three times at the central points.

Table 1. BBD measured responses and prediction.

No	*A*	*B*	*C*	Total Phenolic Content (mg GAE/g)		Relative Error (%)	Antioxidant Inhibition (%)		Relative Error (%)
				Observed	Prediction		Observed	Prediction	
1	−1	−1	0	0.0765	0.0749	2.20	36.51	36.90	1.06
2	1	−1	0	0.0404	0.0584	30.96	34.58	35.61	2.91
3	−1	1	0	0.2816	0.2635	6.87	60.62	59.58	1.74
4	1	1	0	0.1807	0.1823	0.90	49.53	49.14	0.79
5	−1	0	−1	0.1412	0.1536	8.03	44.80	45.75	2.09
6	1	0	−1	0.1566	0.1492	4.96	41.57	41.88	0.74
7	−1	0	1	0.2750	0.2824	2.62	63.09	62.78	0.49
8	1	0	1	0.2015	0.1892	6.52	55.88	54.93	1.74
9	0	−1	−1	0.1039	0.0933	11.46	36.19	34.85	3.86
10	0	1	−1	0.2355	0.2413	2.39	53.84	53.92	0.15
11	0	−1	1	0.1752	0.1695	3.40	50.93	50.85	0.16
12	0	1	1	0.3232	0.3339	3.20	66.64	67.99	1.98
13	0	0	0	0.4537	0.3938	15.22	53.52	50.50	5.97
14	0	0	0	0.3375	0.3938	14.29	47.70	50.50	5.54
15	0	0	0	0.3901	0.3938	0.93	50.29	50.50	0.43

Upon completion of the BBD, data analysis was performed using Minitab software (Minitab Ltd., Brandon Curt, UK). The statistical significance of each factor and the goodness of fit of the polynomial model were evaluated through analysis of variance (ANOVA). A second-order polynomial equation incorporating main effects, interactions, and quadratic terms was employed for the analysis.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} A B + \beta_{13} AC + \beta_{23} BC + + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 \quad (1)$$

In this model, Y represents the dependent variable, while A, B, and C denote the independent variables. The coefficients β_0 represent the intercept, β_i the linear terms, β_{ij} the interaction terms, and β_{ii} the quadratic terms. After establishing the response surface equation for the BBD, a multi-response optimization (MRO) technique was employed to simultaneously optimize both total phenolic compounds and antioxidant activity.

2.7. Method validation

The developed UAE method was validated for precision and accuracy. Precision was evaluated using the coefficient of variation (CV, %) at two levels: repeatability and intermediate precision. Repeatability was assessed through nine extractions conducted on the same day, while intermediate precision was evaluated by performing three extractions per day over three consecutive days. To ensure thorough extraction and measure recovery (R, %), the process was repeated for up to four cycles. In the first cycle, the extract (supernatant) was collected post-centrifugation, and the residue was re-extracted with fresh solvent in subsequent cycles. The concentration of phenolic compounds in each extract was quantified. All experiments were performed in triplicate to ensure robustness and reliability.

2.8. Analysis of the phenolic compounds by HPLC-DAD

The ear mushroom extracts were analyzed using high-performance liquid chromatography with a diode array detector (HPLC-DAD) system (Shimadzu Corp., Kyoto, Japan). Phenolic compounds were separated on a C18 reverse-phase column (5 µm, 4.6 × 150 mm) with the column oven maintained at 30 °C. The mobile phases were A (93% water, 5% acetonitrile, 2% acetic acid) and B (10% water, 88% acetonitrile, 2% acetic acid). Elution was performed with a gradient of solvent B: 0 min, 0%; 20 min, 100%; and 25 min, 100%. The flow rate was 1 mL/min. Extracts were concentrated using a rotary evaporator (IKA-Werke GmbH & Co. Kg, Staufen, Jerman) from 25 mL to 5 mL and filtered through a 0.45 µm nylon filter. The injection volume was 20 µL. Chromatographic data were processed with LabSolutions CS software (Shimadzu Corp., Kyoto, Japan). Phenolic compounds were identified by matching retention times and spectra with standards within a 200–400 nm DAD scan range, and quantified at specific maximum wavelengths (260, 280, and 320 nm).

3. Results and discussion

3.1. Solvent screening

The study evaluated the efficacy of five solvents (methanol, ethyl acetate, acetonitrile, hexane, and ethanol) for extracting phenolic compounds and their antioxidant activities from ear mushrooms. Extractions were conducted at midpoint of three factors (40 °C with a pulse duty cycle of 0.6 s^{-1} and a solvent-to-sample ratio of 20:1 mL/g), in triplicate, as shown in Figure 2. Water was excluded due to the high polysaccharide content in ear mushrooms, which can gel under the heat generated during UAE extraction [24,25].

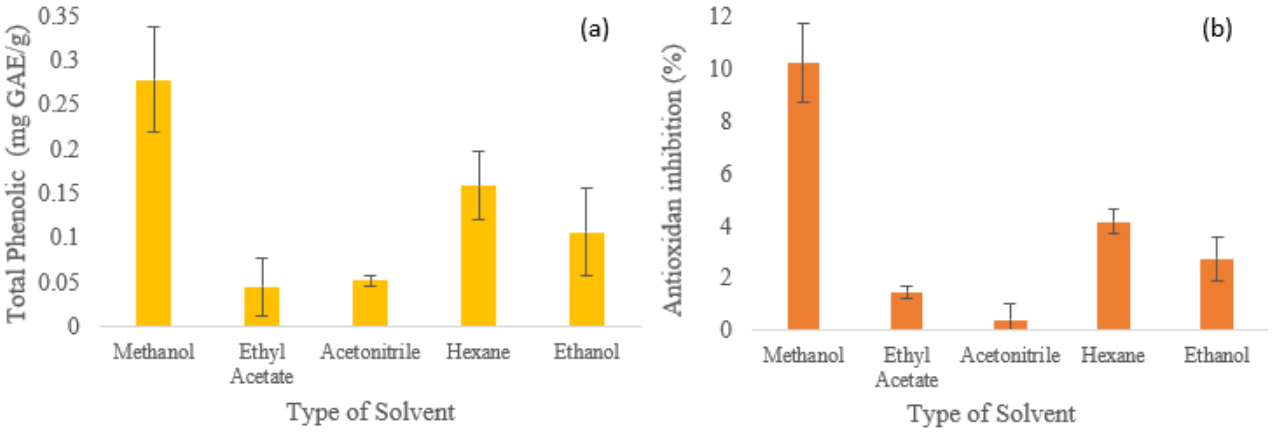

Figure 2. The levels of total phenolic compounds (a) and antioxidant inhibition (b) of the resulting extracts by different solvents.

The findings indicate that methanol is the most effective solvent for extracting phenolic compounds from ear mushrooms, yielding 0.279 ± 0.059 mg GAE/g, followed by hexane, ethanol, acetonitrile, and ethyl acetate. Phenolic compounds exhibit polar characteristics due to their multiple hydroxyl groups, which enhance solubility in polar solvents like methanol. These hydroxyl groups

form hydrogen bonds with the electronegative oxygen atoms in methanol, improving extraction efficiency [26]. The superior performance of methanol can be attributed to its polarity, which is well-suited for extracting a wide range of phenolic compounds that contribute to antioxidant activity [27].

These results align with the observed antioxidant activity, as methanol extracts of ear mushrooms exhibited the highest antioxidant activity. A positive correlation was found between total phenolic content and antioxidant activity, with phenolic-rich extracts demonstrating significantly enhanced free radical scavenging activity [28]. This finding corroborates previous studies on *Adenanthera pavonina* L. bark which also showed the efficacy of methanol in extracting bioactive compounds with strong antioxidant properties [27].

3.2. Optimization of UAE method

BBD-RSM was used to optimize UAE conditions, including solvent-to-solid ratio (*A*), pulse duty cycle (*B*), and temperature (*C*). Fifteen experiments were performed, and analysis of variance (ANOVA) was used to calculate the main, interaction, and quadratic effects of these variables on the levels of total phenolic content and antioxidant activity. The effects of the studied variables were graphically represented in a Pareto chart (Figure 3).

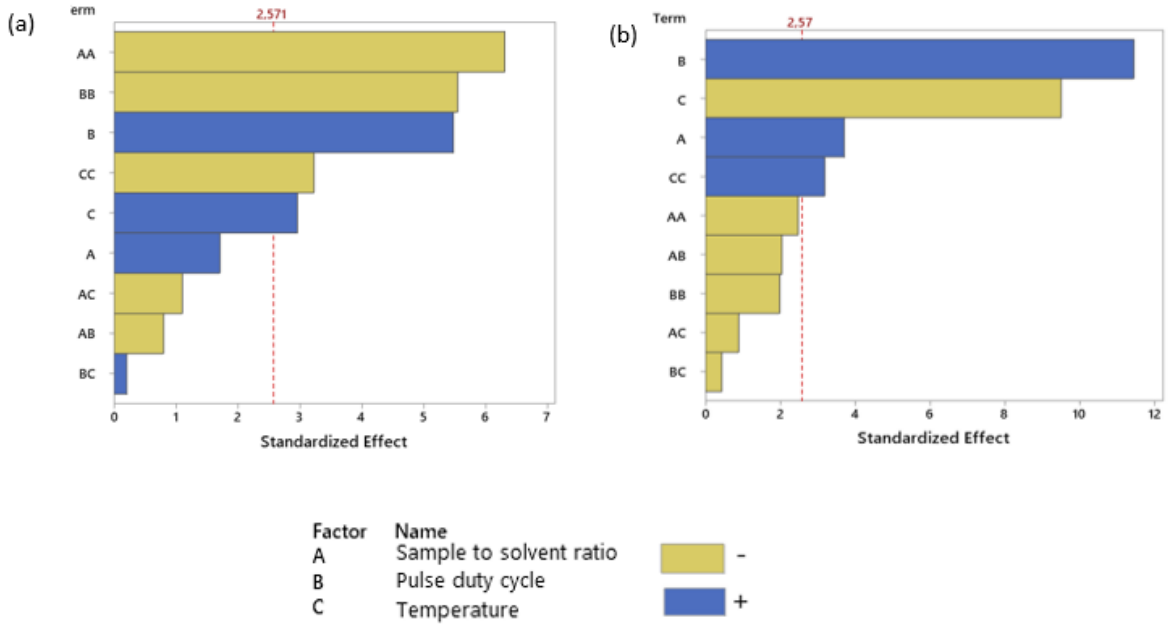

Figure 3. Pareto chart for the standardized effect of the UAE variables on the level of total phenolic compounds (a) and antioxidant inhibition (b). The vertical line across the bars indicates that the limit value informing about the factor has a significant effect at 95% confidence.

ANOVA indicated that all studied variables significantly influenced total phenolic content negatively in their quadratic terms, in the following descending order: solvent-to-sample ratio (AA),

pulse duty cycle (BB), and temperature (CC). However, the main effects of pulse duty cycle (B) and temperature (C) positively impacted the level of total phenolic content. This suggests that while increasing pulse duty cycle and temperature enhances phenolic extraction, excessive increases in these variables may reduce the total phenolic content.

In contrast, the antioxidant activity of the resulting extract was significantly influenced by the pulse duty cycle (B), temperature (C), and solvent-to-sample ratio (A). The pulse duty cycle and solvent-to-sample ratio had positive effects, indicating that higher levels of these variables increased antioxidant activity. Conversely, temperature had a negative effect, suggesting that higher temperatures reduced antioxidant activity. However, an even higher increase in temperature positively impacted antioxidant activity due to the significant positive quadratic effect of temperature (CC).

The pulse duty cycle plays a crucial role in extracting phenolic compounds and enhancing antioxidant activity. Representing the percentage of time ultrasonic energy is applied, it influences extraction efficiency. It is typically expressed as a percentage, illustrating the ratio of the "on" time to the entire cycle duration. Previous studies have shown that antioxidant activity and yields of specific compounds are influenced by the pulse duty cycle, which depends on the intensity and duration of ultrasonic treatment [29]. The duty cycle indirectly affects extraction by altering ultrasonic exposure and temperature [30]. UAE, with the thermal effect of ultrasound, involves the conversion of ultrasonic vibration energy into heat by the medium, with the resultant calorific value dependent on the medium's properties duty of cycle, ultrasonic power, and exposure time [31]. The mechanical effect enhances particle motion and mass transfer, while the predominant cavitation effect involves the formation, vibration, and collapse of microscopic bubbles, releasing significant energy, generating high temperatures and pressures, and creating substantial hydrodynamic shear forces [32]. While it enhances extraction through mechanical and cavitation effects, its quadratic impact negatively affects phenolic extraction due to heat-induced degradation of heat-sensitive phenolic compounds [14,33].

Other results indicated that temperature significantly influenced the extraction of phenolic compounds and antioxidants. While an increase in temperature positively affected the extraction yield of phenolic compounds, it had a detrimental effect on the extraction of antioxidants. The positive effect of increased temperature on phenolic compound extraction can be explained by the fact that higher temperatures improve the solubility and diffusion of phenolic compounds from the mushroom matrix, thus enhancing their extraction efficiency [34]. Increased temperature also reduces the viscosity of the solvent, promoting better penetration into the plant matrix and facilitating the release of bioactive compounds [35].

However, when it comes to antioxidant activity, the negative effect of higher temperatures is likely due to the thermal degradation of thermolabile antioxidant compounds. Phenolic compounds, particularly some flavonoids, are sensitive to high temperatures and can lose their antioxidant properties if exposed to prolonged heating [36,37]. While the extraction of total phenolics may increase with temperature, the antioxidant activity may decline as certain compounds degrade, leading to a reduction in overall antioxidant capacity.

The optimization of UAE for extracting compounds from ear mushrooms involved analyzing the influential coefficients for each variable, their interactions, and quadratic terms to construct second-order polynomial equations. Two predictive equations were established: one for total phenolic compounds (Equation 1) and another for DPPH inhibition under specific experimental conditions (Equation 2).

$$Y_1 = -0.831 + 16.70A + 1.64B + 0.01C - 118.0AA - 1.30BB - 0.0001CC - 1.61AB - 0.03AC + 0.001\,BC \quad (2)$$

$$Y_2 = 8.76 + 409A + 73.3B - 0.0004C - 2560AA - 25.7BB + 0.006CC - 228AB - 1.12AC - 0.07BC \quad (3)$$

Where Y_1 represents the total phenolic compound, Y_2 represents the inhibition of DPPH, and A, B, and C are the studied variables: solvent-to-solid ratio (A), pulse duty cycle (B), and temperature (C).

Table 1 presents the experimental design runs with corresponding measured and predicted values for the responses. The average differences between the measured and predicted values were 7.59% for total phenolic compounds and 1.97% for antioxidant activity. The coefficient of determination (R^2) for the prediction models was 95.83% for TPC and 98.13% for antioxidant activity, indicating a strong fit. The p-values for lack-of-fit in the ANOVA table were 0.931 for TPC and 0.813 for antioxidant activity, suggesting model suitability at a 95% confidence level. Therefore, the model can reliably forecast responses for optimization purposes.

3.3. Optimization conditions and verification

The developed models suggested optimal conditions for each response within the BBD domain. Multi-response optimization identified the most suitable UAE parameters to simultaneously achieve high total phenolic content and antioxidant activity. The optimal extraction conditions for a 1 g sample were 18 mL of methanol at 59 °C, with a pulse duty cycle of 0.7 s^{-1}. The verification experiments yielded a total phenolic content of 0.386 mg GAE/g, deviating by only 0.49% from the predicted value, and an antioxidant inhibition of 59.56% with a deviation of 10.53%.

Deviation close to 10% in antioxidant inhibition may initially appear significant, this variability is consistent with the inherent fluctuations often observed in natural product extractions. Similar studies have reported comparable ranges of variation. For instance, Gebreyohannes et al. [38] demonstrated a maximum scavenging activity of 70.4% in *Auricularia* species, with marginally higher results compared to our study, yet within acceptable error margins. Additionally, Brand-Williams et al. [23] observed variability in DPPH antioxidant activity ranging from 8–12% across replicates, which aligns with the 10.53% deviation observed in our verification experiments .

Given these references and the biological complexity of phenolic compound extraction, the deviation is considered to be within acceptable limits, reflecting the natural variability typical of antioxidant assays in natural matrices. This further confirms that the BBD model, in conjunction with RSM, successfully predicted the optimal UAE conditions for phenolic extraction from ear mushrooms.

3.4. Precision and accuracy

The validation involved analyzing the precision and accuracy of the developed method. Precision was assessed through repeatability and intermediate precision. Repeatability was determined by nine extractions under optimal conditions on the same day, while intermediate precision was measured by performing three extractions daily over three days. The CV was 4.43% for repeatability, 2.21% for intermediate precision of total phenolic compounds, 2.29% for repeatability, and 1.81% for intermediate precision of antioxidant activity. All CV values were below 7%, meeting the AOAC acceptable limit of ± 10% [39], confirming the precision of the UAE method.

The assessment of phenolic compound recovery from ear mushrooms employed multi-cycle UAE to reach complete recovery [19,35]. Table 2 outlines the total phenolic compound level and the corresponding antioxidant activity. The extraction was performed in four cycles. Recovery after the first cycle consistently remained below 5%, making further re-extractions unnecessary. Applying one extraction cycle achieved 97.22% for total phenolic compounds and 92.62% for antioxidant activity. According to AOAC recommendations, these recovery rates (90–107%) are acceptable [39].

Table 2. Accuracy of UAE for phenolic compounds and antioxidant activity from ear mushroom.

Cycle	Total phenolic Content (mg GAE/g)	Antioxidant activity (%)
1	0.755 ± 0.0541	46.601 ± 2.790
2	0.041 ± 0.036	3.712 ± 1.573
3	ND	ND
4	ND	ND

3.5. Real sample

The optimized UAE method was applied to ear mushroom samples at different stages of fruiting body development (days 7–19) to assess its practical utility. Total phenolic content was determined using the Folin-Ciocalteu method, and antioxidant activity was measured via the DPPH radical scavenging assay. The results of total phenolic content and antioxidant inhibition for the different stages of fruiting body development are displayed in Figure 4.

The results showed that ear mushrooms harvested on day 8 had the highest total phenolic content at 0.575 mg GAE/g, while the lowest was on day 16 with 0.233 mg GAE/g, indicating a peak in phenolic content on day 8. Similarly, day 8 mushrooms exhibited the highest antioxidant inhibition at 92.37%, whereas day 16 had the lowest at 47.85%. This suggests that both phenolic content and antioxidant activity are optimal when the mushrooms are harvested on day 8.

Mushrooms harvested fruiting body development phase typically exhibit elevated phenolic content due to increased synthesis and accumulation of secondary metabolites in response to environmental stimuli and physiological needs. This finding is consistent with studies on wild blueberries (*Vaccinium stenophyllum* Steud.), where phenolic content peaked at the immature stage [40]. Enhanced phenolic production helps protect cellular integrity against oxidative stress, maintain structural stability, and prepare for reproduction. Conversely, ear mushrooms harvested at more advanced stages show reduced phenolic content due to metabolite breakdown, decreased metabolic activity, resource allocation for reproduction, accumulation of other compounds, and environmental stress exposure [41,42]. These factors collectively contribute to the observed decline in phenolic compounds in mature mushrooms.

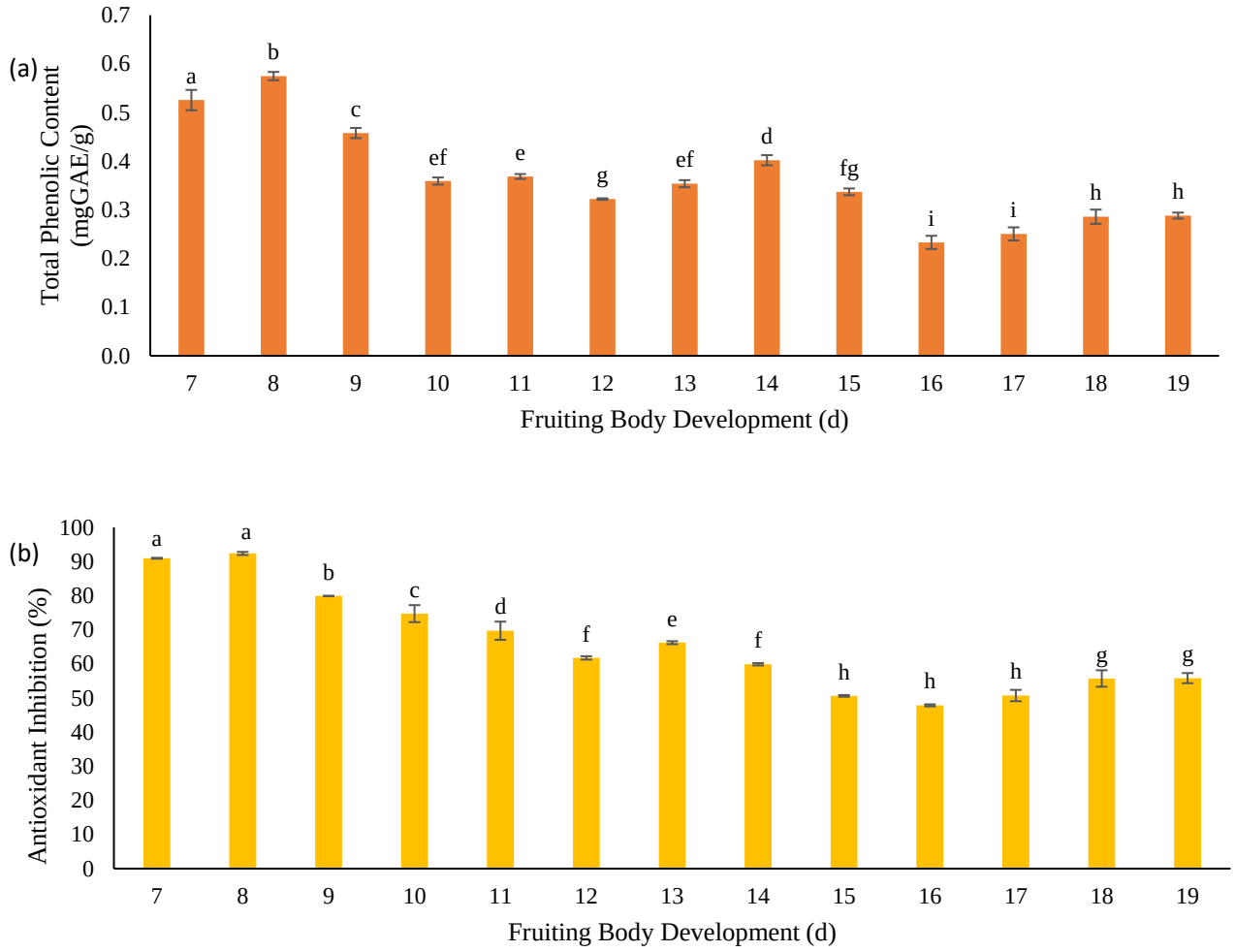

Figure 4. UAE method application for extracting (a) the total phenolic content and (b) the corresponding antioxidant inhibition values for each harvesting day from day 7–19.

To gain a comprehensive understanding of the phenolic compound profile in ear mushrooms at various stages of fruit body development, high-performance liquid chromatography with diode-array detection (HPLC-DAD) was employed to separate individual phenolic compounds (Figure 5). The 30-minute analysis revealed 18 dominant peaks. Peaks appearing within the first 15 minutes (peaks 1–7) corresponded to more polar compounds, while the later peaks (peaks 8–18) indicated the presence of less polar compounds. Tentative identification of the most polar compound suggested a cinnamic acid derivative (peak 1), while a benzoic acid derivative (peak 10) was identified in the mid-chromatogram. This identification was further supported by spectral analysis, which exhibited characteristic patterns consistent with these compounds. For the quantification of phenolic compounds, three distinct wavelengths (i.e., 260 nm, 280 nm, and 320 nm) were selected based on the maximum absorption of the compounds of interest and used during the analysis. These wavelengths allowed for precise determination of the phenolic compounds present. Figure 5 illustrates a representative chromatogram from the HPLC-DAD analysis of the ear mushroom sample on day 7.

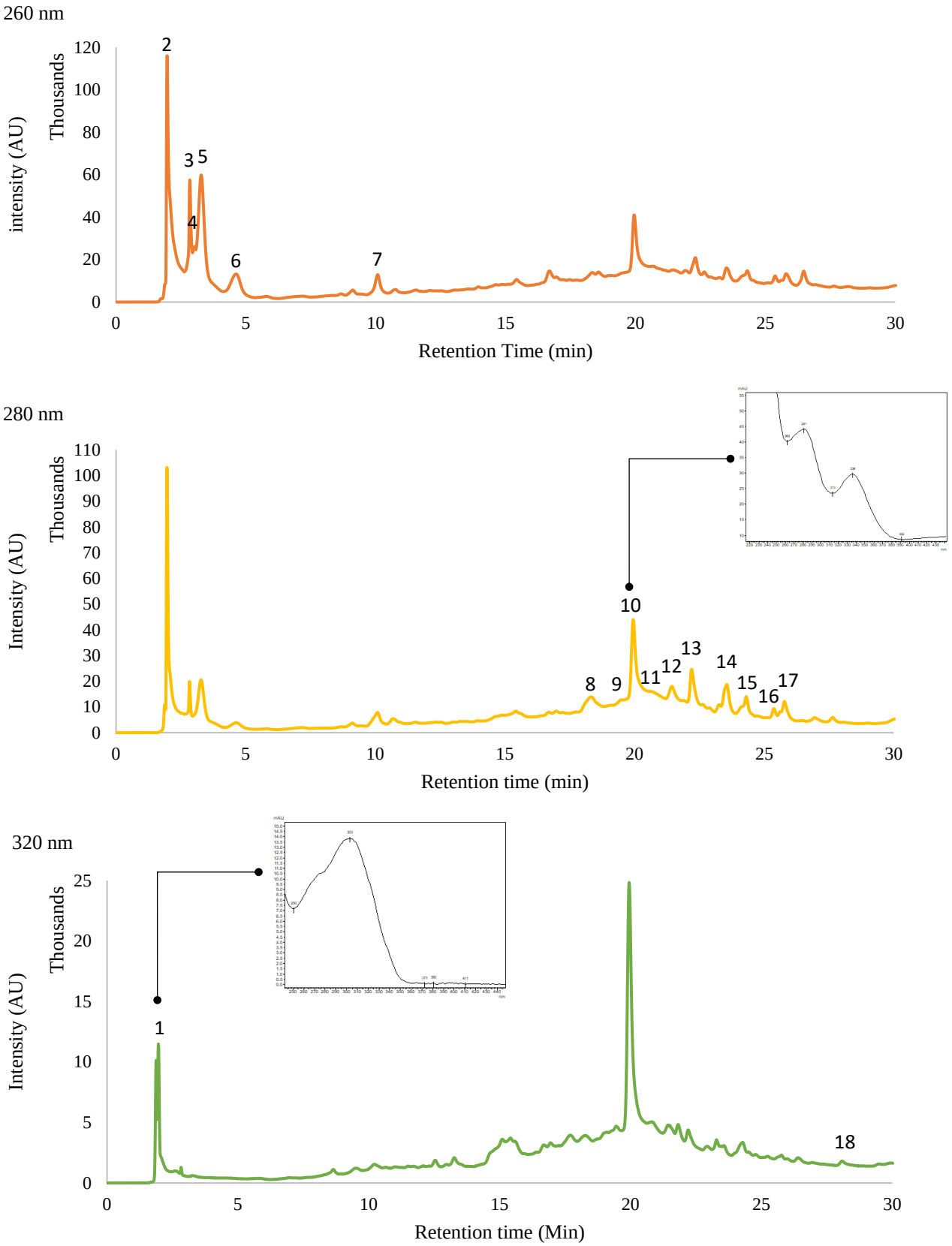

Figure 5. The chromatogram of HPLC-DAD for identifying individual phenolic compounds from ear mushrooms in three different wavelengths based on the maximum absorption.

Although the total phenolic content follows the trend in Figure 4, the phenolic composition fluctuates, exhibiting notable peaks and troughs at different developmental stages. Figure 6 illustrates the proportion of polar and less polar phenolic compounds in ear mushrooms during various stages of fruit body development, from day 7 to day 19, distinguishing between the two groups of phenolic compounds.

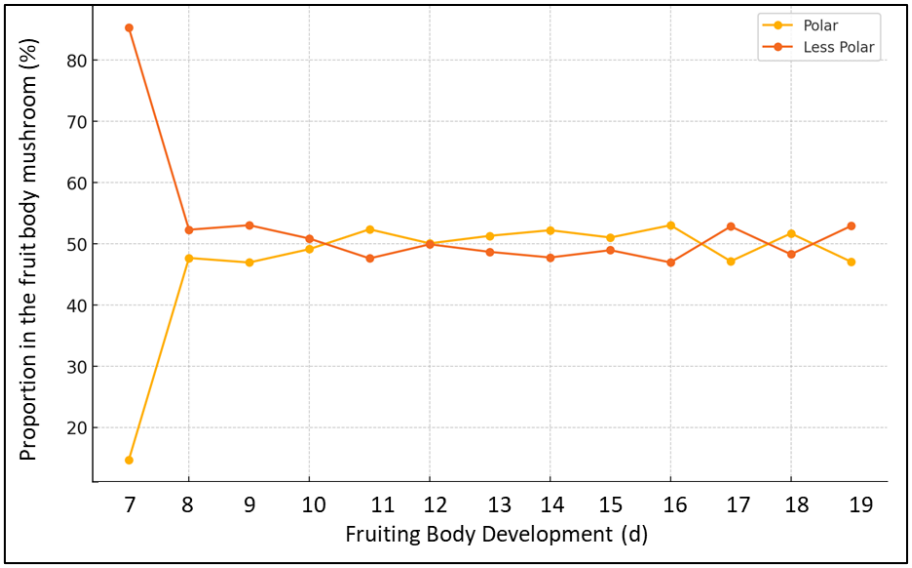

Figure 6. The proportion of polar and less polar phenolic compounds in ear mushrooms during various stages of fruit body development.

There is a general trend where polar phenolic compounds initially increase, stabilize, and then slightly decrease. In contrast, less polar phenolic compounds show a decreasing trend initially and then stabilize towards the later stages. This shift in phenolic composition, from less polar to more polar, suggests an adaptive response to developmental changes and environmental stimuli during the fruiting body development of the mushrooms. This phenomenon highlights how changes in growth conditions or metabolism over time influence the production of specific compounds [42,43].

These findings underscore the importance of optimal harvesting timing to maximize the beneficial phenolic content and antioxidant activity in ear mushrooms. This information is crucial for optimizing the use of ear mushrooms in food and nutraceutical applications, ensuring the highest possible health benefits from these compounds.

4. Conclusions

This study successfully optimized the ultrasonic-assisted extraction of phenolic compounds from ear mushrooms using the BBD. The optimal extraction conditions were determined to be a solvent-to-sample ratio of 1:18, a pulse cycle of 0.7, and an extraction temperature of 59 °C. The method demonstrated high precision and was effectively applied to determine phenolic compounds and DPPH inhibition in ear mushrooms across fruiting body development (7–19 days). The results indicated that the age at which ear mushrooms are harvested significantly affects TPC and antioxidant activity, with the optimal harvest time being 8 days after the mushrooms emerge from the media.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization: W.S. and L.S.C.; Methodology: W.S. and A.B.; Software: A.N. and M.U.H.; Validation: W.S. and L.S.C.; Formal analysis: A.N. and A.B.; Investigation: A.N. and M.U.H.; Resources: A.N., M.U.H. and W.S.; Data Curation: W.S. and V.L.; Writing - Original Draft: A.N., A.B., and M.U.H.; Writing - Review & Editing: W.S. L.S.C., C.X.T., V.L., A.H., and W.S.; Visualization: W.S.; Supervision: W.S. and A.H.; Project administration: L.S.C.; Funding acquisition: W.S. Finally, all authors have read and agreed to the published version of the manuscript.

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