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Original Research Article

Enzyme-assisted Extraction Optimization of Bioactive Phenolic Components from *Phyllanthus urinaria*

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ABSTRACT

Phyllanthus urinaria is a traditional medicinal plant commonly used in Asian countries due to its rich phenolic content and antioxidant potential. The present study aimed to optimize the extraction method of polyphenols from *P. urinaria* using cellulase-assisted extraction (EAE). Key extraction parameters, including material-to-solvent ratio, extraction time, temperature, pH, and enzyme concentration, were first investigated through single-factor experiments to evaluate polyphenol recovery and antioxidant activity. Response Surface Methodology (RSM) was then employed to determine the optimal conditions, which were established at a solvent-to-material ratio of 1:19, an extraction time of 78 minutes, a temperature of 45.7°C, a pH of 5.02, and an enzyme concentration of 1.6%. Under these conditions, the extract exhibited a total polyphenol content of 17.20 mg/g and antioxidant activity of 15.51 mg/g by DPPH assay, while the quadratic RSM model showed strong predictive reliability (adjusted $R^2 = 0.98$; lack-of-fit, $p > 0.05$). HPLC-QTOF-MS analysis identified 10 major bioactive compounds, confirming the phytochemical richness of the extract. The findings highlight EAE as a sustainable and effective method for recovering functional constituents from *P. urinaria*, with promising applications in functional foods and pharmaceutical product development. Future studies should investigate the bioavailability and absorption mechanisms of the identified compounds to support their therapeutic utilization.

Keywords: *Phyllanthus urinaria*, Cellulase-assisted extraction, Polyphenols, Antioxidant activity, Response surface methodology.

Introduction

Tropical monsoon climate in Vietnam provides an exceptional and highly favorable environment for its rich and diverse flora. The combination of elevated temperatures, abundant rainfall, and distinct wet and dry seasons fosters a thriving ecosystem that supports approximately 12,000 plant species, encompassing 2,500 genera and 300 families. This remarkable biodiversity positions Vietnam as a significant resource for the pharmaceutical, cosmetic, and food technology industries. In recent decades, scientific interest in bioactive compounds derived from plants has intensified, particularly due to their potential roles in disease prevention and treatment.[1]Among the extensive array of plant species in Vietnam, *Phyllanthus urinaria*, commonly called "chamber bitter," has garnered considerable attention for its pharmacological potential. A member of the Euphorbiaceae family, this plant is widely distributed across tropical and subtropical regions and is particularly abundant in Vietnam. Its prominence in traditional medicine, notably within Chinese and Southeast Asian practices, is attributed to its detoxifying and heat-clearing properties. It has been used to treat jaundice, dysentery, and ascites.[2] Modern scientific studies have validated its bioactive potential, emphasizing its antibacterial, anti-inflammatory, and antipyretic effects.[3]

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Compared to other species within the Euphorbiaceae family, *P. urinaria* is distinguished by its abundant polyphenol content and broad therapeutic applications, making it a compelling candidate for further investigation.[4]Despite the increasing interest in this plant, research on optimal methods for extracting bioactive compounds from *P. urinaria* remains limited. Previous studies have employed a range of extraction techniques, including microwave-assisted and ultrasound-assisted methods.[5] However, these approaches often encounter challenges such as high energy consumption, potential degradation of heat-sensitive compounds, and environmental concerns from using organic solvents. In contrast, enzyme-assisted extraction (EAE) presents a promising alternative. This technique utilizes the catalytic activity of enzymes, such as cellulase, to degrade plant cell walls, thereby enhancing the release and yield of target compounds. EAE is characterized by its eco-friendliness, cost efficiency, and reduced processing time.[6, 7]

Polyphenols, the primary focus of this study, are secondary metabolites renowned for their health-promoting properties. These compounds exhibit potent antioxidative, anti-inflammatory, and antimicrobial activities, contributing to the prevention and management of degenerative diseases such as cancer, diabetes, and cardiovascular disorders.[8, 9] Previous research has demonstrated the therapeutic potential of polyphenols extracted from *P. urinaria* in mitigating oxidative stress and reducing inflammation, underscoring their significance in functional food and pharmaceutical applications.[1]

EAE presents several significant advantages over conventional extraction techniques. By breaking down complex structural matrices within plant materials, EAE can substantially improve extraction yields while selectively removing undesirable components. This method typically requires less solvent and shorter extraction times, contributing to a more efficient and environmentally sustainable process. Notably, the mild reaction conditions, such as low temperatures and brief processing durations, help preserve the integrity and bioactivity of sensitive compounds, resulting in high-quality extracts at reduced

operational costs. Recent studies have demonstrated that EAE can enhance both the functional properties and economic feasibility of natural products across various applications.[7, 10]

While microwave-assisted and ultrasonic extraction methods have been investigated for isolating bioactive compounds from *P. urinaria*, no study has focused on cellulase-assisted extraction for this plant. This study is the first to apply cellulase-assisted extraction to *P. urinaria*, offering a novel approach to enhance polyphenol yield and bioactivity retention.

Given the potential of cellulase to degrade plant cell walls and facilitate the release of intracellular bioactives, its application may lead to improved extraction yields and better retention of polyphenol bioactivity.

This study aims to optimize cellulase-assisted extraction of polyphenols from *Phyllanthus urinaria* to maximize total polyphenol content and antioxidant activity, advancing sustainable enzymatic methods for functional natural food development. Enhancing polyphenol recovery supports the creation of health-promoting food products. This work strengthens eco-friendly extraction techniques, addressing global demands for sustainable nutraceuticals.

Materials and Methods

Materials

Folin-Ciocalteu reagent, DPPH (2,2-diphenyl-1-picrylhydrazyl, ≥99.5%, solid), gallic acid (≥96.5%, solid), and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 98%, solid) were obtained from Sigma–Aldrich (Germany). Sodium carbonate (Na_2CO_3), acetic acid, and sodium acetate trihydrate ($CH_3COONa.3H_2O$) were sourced from Sinopharm Chemical Reagent, China. All chemicals were of analytical grade. The enzyme Celluclast® 1.5 L (cellulase, liquid formulation; Novozymes A/S, Denmark) was used. It is a brown liquid with a declared activity of ≥700 EGU/g.

Phyllanthus urinaria plants were collected in February 2024 from Nam Tra My District, Quang Nam Province, Vietnam (15.0069° N, 108.0886° E). The scientific name of the studied species was identified using the comparative morphology method. The voucher specimen, VST-TV022024, was deposited at the Institute of Biotechnology and Food Technology, Industrial University of Ho Chi Minh City. Fresh plants were washed to remove impurities and then dried at 60°C in a hot-air oven (Heidolph Hei-VAP Core, Heidolph Instruments GmbH, Germany) to reduce the moisture content to below 12%, thereby preserving bioactive compounds and minimizing thermal degradation.[11, 12]

The dried material was ground into a fine powder (< 0.5 mm particle size) using a high-speed laboratory grinder (IKA MF 10 Basic, IKA Works, Germany), sieved for consistency, and stored in vacuum-sealed polyethylene bags (DZ-260, Zhejiang Dongfeng Machinery Co., China) which were kept in a dark, ambient environment to safeguard bioactive compounds, particularly polyphenols, from oxidation and photodegradation.

Extraction

The extraction process of polyphenols and antioxidant activity using cellulase-assisted methods was investigated using single-factor experiments. *P. urinaria* powder was mixed with solvent at material-to-solvent ratios ranging from 1:13 to 1:21. Extraction temperatures were set between 40°C and 60°C. The enzyme concentration varied from 0.5% to 2.5%, with pH conditions ranging from 3.5 to 5.5. Enzyme working solutions (0.5%–2.5%) were prepared by diluting the liquid enzyme stock with 50 mM sodium acetate buffer (pH 5.0), stirring gently. Extraction time varied from 20 to 100 minutes. To terminate the enzymatic reaction, the reaction mixture was immersed in a boiling water bath for 1 to 5 minutes, then immediately cooled in an ice bath. After enzymatic treatment, the mixture was centrifuged at 4000 rpm for 10 minutes using a refrigerated centrifuge (Hermle Z326K, Hermle Labortechnik, Germany). The supernatant was filtered through Whatman No. 1 filter paper (0.45 µm; GE Healthcare Life Sciences, UK) under vacuum (KNF Neuberger Laboport, Germany). The resulting extract was analyzed for total polyphenol content (TPC) and antioxidant activity (AA) by the DPPH assay. The most suitable enzymatic extraction conditions were chosen for the next experiments. All experiments were performed in triplicate.

Determination of Total Phenolic Content (TPC)

The samples' total phenolic content (TPC) was determined using the Folin-Ciocalteu colorimetric method, following the procedure described by Thi and Tai (2023).[12] Briefly, an aliquot of 0.1 mL of the extract was combined with 6.9 mL of distilled water and 1.8 mL of Folin-Ciocalteu reagent. Subsequently, 1.2 mL of an aqueous sodium carbonate (Na_2CO_3) solution (15%, w/v) was added. The final volume of the mixture was adjusted to 10 mL with distilled water. The reaction was allowed to proceed for 90 minutes at room temperature under dark conditions. The absorbance of the resulting solution was measured spectrophotometrically at 734 nm using a UV-VIS spectrophotometer (PhotoLab® 6600, Germany) against a reagent blank. A calibration curve was constructed using gallic acid as the standard (R^2=0.9984). The TPC was calculated and expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

DPPH Radical Scavenging Activity Assay

The antioxidant activity (AA) of the extract was evaluated by its 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity, following the procedure of Thi and Tai (2023).[12] Briefly, an aliquot (0.1 mL**)** of the sample extract was mixed with 4.0 mL of a 0.1 mM ethanolic DPPH solution and 0.9 mL of ethanol. The reaction mixture was vortexed and incubated for 30 minutes at room temperature (25 ± 1°C) in the dark. The decrease in absorbance, corresponding to the radical scavenging activity, was measured spectrophotometrically at 517 nm. Quantification was performed using a Trolox standard curve. The curve was prepared by diluting various volumes (0.1-1.0 mL) of a 50 µM Trolox stock solution with ethanol and 4.0 mL of DPPH solution to create a final concentration series ranging from 1 to 10 µM (equivalent to 0.0013-0.0125 mg) in a total reaction volume of 5.0 mL. A reagent blank was prepared similarly but without the addition of Trolox. The antioxidant activity was calculated from the calibration curve and expressed as milligrams of Trolox equivalents per gram of dry weight (mg TE/g DW), reported as the mean ± standard deviation.

High-Performance Liquid Chromatography coupled with Quadrupole Time-of-Flight Mass Spectrometry (HPLC-QTOF-MS) analysis

Chemical composition of the extract was analyzed using HPLC and the QTOF -MS method according to the previous studies.[13] The extract was centrifuged at 8000 rpm for 10 min, filtered through a 0.22 µm PTFE syringe filter, and stored at 4°C. LC-MS analysis was performed using an Agilent 1290 Infinity UHPLC system coupled with a ZORBAX Eclipse Plus C18 column (2.1 × 100 mm, 1.8 µm). The mobile phases were 0.1% formic acid in water (A) and acetonitrile (B), with a gradient elution: 0–2 min (5% B), 2–15 min (to 95% B), 15–17 min (95% B), and 17–20 min (re-equilibration at 5% B). The flow rate was 0.3 mL/min, the injection volume 5 µL, and the column temperature 35°C.

Mass spectrometry was conducted using an Agilent QTOF system with an ESI source in both positive and negative ion modes. Key parameters included: VCap (5.0–5.5 kV ESI^+, 3.5–4.5 kV ESI^-), nebulizer pressure (45–55 psi), drying gas (12 L/min, 350–550°C), and fragmentor voltage (80 V ESI^+, 80–175 V ESI^-). Mass spectra were acquired over m/z 100–1500 with DDA and DIA strategies; collision energies ranged from 15–40 eV or dual fixed energies (e.g., 40/80 eV). Data were acquired using Agilent Q-TOF software (vB.06.01), with internal reference mass calibration ensuring <5 ppm accuracy. Raw data were processed in MassHunter Qualitative Analysis. Compounds were tentatively identified by accurate mass, isotope patterns, MS/MS spectra, and comparison with databases (METLIN, HMDB) and reported *Phyllanthus* constituents.

Experimental design

The Central Composite Design (CCD) methodology was employed to optimize the extraction process of polyphenols and antioxidant activity from *P. urinaria* extract. The relationship between the investigated factors and the response variables was modeled using the Response Surface Methodology (RSM). Five key extraction parameters were examined, including the raw material-to-solvent ratio (X_1), extraction

time (X_2), temperature (X_3), pH (X_4), and enzyme concentration (X_5). The Box-Behnken Design (BBD) was applied to analyze the experimental data.

Based on preliminary single-factor experimental results, the coded levels of the five independent variables were established, as presented in Table 1. The experimental design comprised 46 individual runs, including six center-point replicates, with each experiment conducted in triplicate to ensure statistical reliability. The independent variables were coded according to Equation (1):

$$X = (X_i - X_0)/\Delta X \quad (1)$$

Where X represents the coded variable, X_i is the actual variable, X_0 denotes the central value of the experimental domain, and ΔX corresponds to half the range between the extreme absolute values of the actual variable.

The mathematical model corresponding to the BBD is formulated by Equation (2):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=1+i}^{3} \beta_{ij} X_i X_j + \varepsilon \quad (2)$$

Where Y represents the objective function; **ε** denotes the error term; β_0 is the intercept; and β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interaction coefficients, respectively. These coefficients indicate the linear or nonlinear effects of the independent variables on the response. Objective functions include Y_1: TPC (mg/g) and Y_2: AA (mg/g).

Statistical Analysis

All statistical analyses were performed using Statgraphics Centurion XV to assess the differences among experimental samples. Data analysis and model fitting were performed using JMP® Pro Version 17.0 for optimization analysis, enabling the identification of optimal processing conditions to maximize the TPC and AA Analysis of variance (ANOVA) was conducted to determine significant differences among the samples at a 95% confidence level ($p < 0.05$).

Results and Discussion

The influence of single factors

The solvent-to-material ratio plays a crucial role in the efficiency of extracting bioactive compounds, as it influences the concentration gradient and mass transfer between the plant matrix and the solvent. Diffusion is the primary mechanism governing this process, with solvent availability affecting extraction yield and efficiency.

Figure 1 shows the effect of the solvent-to-material ratios on total polyphenol content (TPC) and antioxidant activity (AA). The results showed that solvent-to-material ratios increased from 1:13 to 1:19, resulting in a rise in polyphenol content from 13.44 to 15.68 mg/g and an improvement in antioxidant activity from 14.17 to 15.02 mg/g. However, the ratio was increased to 1:21, causing a decline in both polyphenol yield (14.28 mg/g) and antioxidant capacity (14.55 mg/g). This trend indicates that while a higher solvent volume initially enhances mass transfer, excessive solvent dilution may reduce extraction efficiency. The limited solvent volume restricts osmotic pressure and solvent diffusion at low solvent-to-material ratios, thereby diminishing extraction efficiency. As the ratio increases, the concentration gradient between intracellular solutes and the solvent is enhanced, facilitating mass transfer and improving yield. However, beyond the optimal ratio, solvent saturation occurs, leading to a plateau in extraction efficiency. This observation is consistent with Fick's second law of diffusion, which posits that the mass transfer rate is proportional to the concentration gradient and the solvent saturation level.[14, 15]

Additionally, excessive solvent volume can introduce higher levels of dissolved oxygen, which may lead to oxidative degradation of polyphenols and a subsequent reduction in antioxidant activity.[16] The polarity of the solvent also plays a crucial role in determining extraction efficiency. Ethanol, a common polyphenol extraction solvent, exhibits optimal solubility within a 50 – 80% concentration range. Deviation from this range can diminish solute interactions and extraction efficiency.[11]

Considering both polyphenol yield and antioxidant activity, a solvent-to-material ratio of 1:19 is identified as optimal, demonstrating statistically significant differences compared to other tested ratios ($p < 0.05$). These findings underscore the importance of optimizing solvent conditions to maximize the recovery of bioactive compounds while minimizing oxidative degradation and solvent waste.[17]

Extraction time plays a critical role in facilitating solvent penetration into plant cell walls and enhancing the solubility of cellular constituents, thereby directly influencing extraction efficiency and kinetics. Variations in extraction duration can lead to significant changes in the yield of bioactive compounds.[18] In the present study, increasing the extraction time from 20 to 80 minutes resulted in a notable rise in TPC values from 13.81 to 16.03 mg/g and AA values from 13.79 to 15.23 mg/g (Figure 2).

However, further extension to 100 minutes led to a slight decline in TPC and AA, reducing to 15.78 mg/g and 14.79 mg/g, respectively. These findings suggest that while extended extraction time can enhance the release of polyphenols, it may also promote their oxidative degradation, thereby reducing yield and bioactivity. EAE facilitates the initial release of polyphenols by disrupting plant cell walls, allowing bioactives to diffuse rapidly into the solvent. Nonetheless, prolonged extraction may expose these compounds to degradation processes, particularly oxidation. Statistical analysis indicated no significant differences in TPC and AA between 80 and 100 minutes, highlighting 80 minutes as the optimal extraction duration to maximize recovery and antioxidant potential while minimizing degradation.

As shown in Figure 3, increasing the extraction temperature from 40°C to 45°C led to a significant enhancement in both total polyphenol content (TPC), from 14.24 to 16.71 mg/g, and antioxidant activity (AA), from 14.78 to 15.44 mg/g. However, further elevation of the temperature beyond 45°C resulted in a sharp decline in TPC (14.3 mg/g) and AA (14.72 mg/g). This trend suggests that moderate heating enhances solubility, enzyme activity, and mass transfer, thereby improving extraction efficiency, while excessive temperatures promote the degradation and oxidation of heat-sensitive polyphenolic compounds.[6, 12]

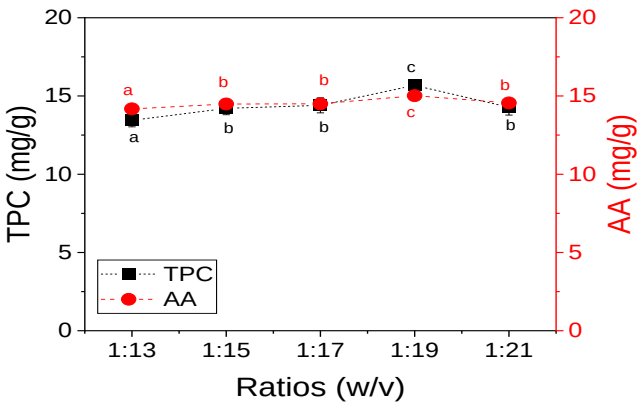

Figure 1: Effect of solvent-to-material ratio on total polyphenol content and antioxidant efficiency.

At lower temperatures, the reduced kinetic energy of both enzyme and substrate molecules limits molecular collisions and the formation of enzyme-substrate complexes, ultimately slowing enzymatic hydrolysis reactions.[19] When temperature increases, several factors contribute to improved extraction efficiency: decreased solvent viscosity, enhanced diffusion rates, and reduced surface tension, all of which facilitate solvent penetration and solute release from the plant matrix.[20, 21] Moreover, elevated temperatures increase molecular motion, promoting more frequent enzyme-substrate interactions and accelerating the breakdown of cell wall structures, which facilitates polyphenol release. Nevertheless, temperatures exceeding the thermal stability threshold of enzymes can induce structural denaturation, particularly at the active site, resulting in diminished catalytic efficiency.[22] In parallel, high temperatures may also degrade thermolabile antioxidant compounds, reducing extract quality.[6]

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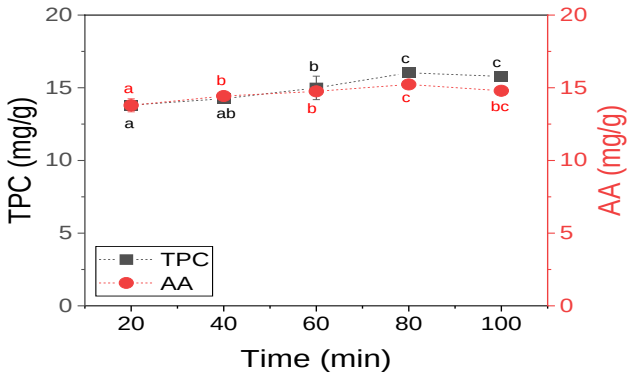

Figure 2: Effect of extraction time on total polyphenol content and antioxidant efficiency.

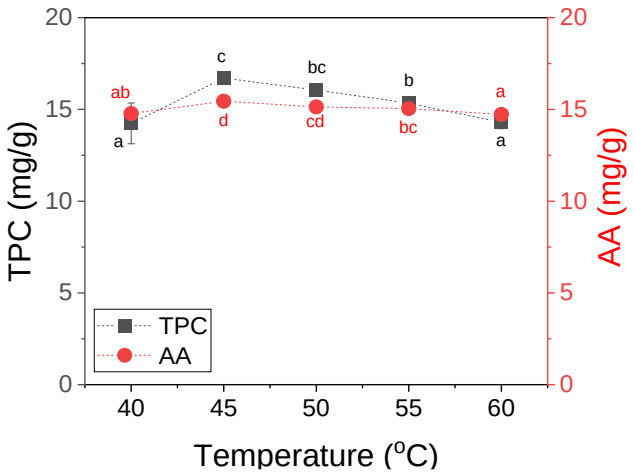

Figure 3: Effect of extraction temperature on total polyphenol content and antioxidant efficiency.

Specifically, temperatures above 60°C have been shown to accelerate polyphenol degradation through oxidation reactions.[8] Compounding this effect, polyphenol oxidase, an enzyme that catalyzes polyphenol oxidation, exhibits optimal activity around 60°C, further diminishing the final polyphenol content in the extract.[19]

Statistical analysis confirmed that extraction at 45°C yielded significantly higher TPC and AA values compared to other tested temperatures ($p < 0.05$). Therefore, 45°C is identified as the optimal extraction temperature, balancing enhanced enzymatic efficiency and mass transfer with minimal thermal degradation of bioactive compounds. pH is a critical factor influencing enzyme activity, polyphenol stability, and extraction efficiency in enzyme-assisted polyphenol extraction.[17, 23] The enzymatic process operates optimally within a specific pH range, affecting both the solubility and oxidative susceptibility of polyphenolic compounds.[24] As shown in Figure 4, the extraction pH was increased from 3.5 to 5.0 resulting in a rise in TPC value from 13.25 to 16.96 mg/g and AA values from 14.57 to 15.51 mg/g. However, the pH was increased to 5.5 led to a decline in TPC from 14.01 mg/g and AA value of 15.05 mg/g. This indicates that moderate acidic condition enhances polyphenol extraction, whereas excessive alkalinity may promote oxidation and degradation of polyphenolic compounds.[25]

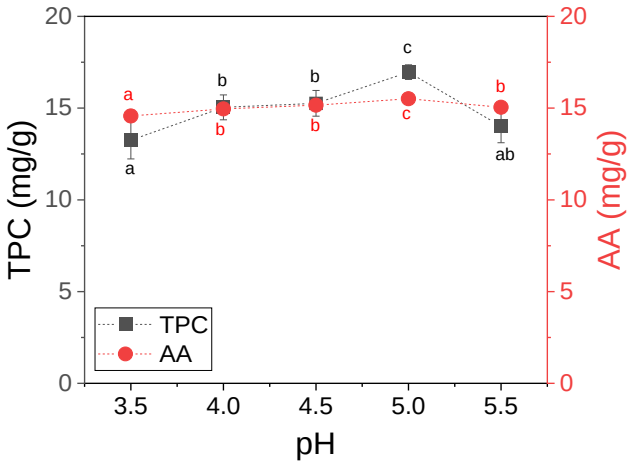

Figure 4: Effect of pH on total polyphenol content and antioxidant efficiency.

Enzyme activity is strongly pH-dependent, and cellulase exhibits optimal catalytic function in the pH range of 4.5–5.5.[26] Within this range, enzymatic hydrolysis effectively disrupts plant cell walls, facilitating the release of polyphenols into the solvent.[27] Additionally, acidic conditions stabilize polyphenolic compounds by inhibiting enzymatic oxidation, particularly by polyphenol oxidase, which becomes more active under neutral or slightly alkaline conditions. At lower pH levels, the integrity of plant cell membranes is reduced, enhancing mass transfer and promoting the diffusion of solutes into the extraction solvent**.** This effect contributes to the higher polyphenol yield observed at pH 5.0. However, beyond this optimal point, increased pH enhances the susceptibility of polyphenols to oxidation, reducing their stability and overall yield.[25] Experimental results indicate that extraction at pH 5.0 yields the highest polyphenol content and antioxidant activity, with statistically significant differences from other pH conditions ($p < 0.05$). Therefore, the optimal condition is identified at pH 5.0.

Enzyme-assisted extraction using cellulase can enhance the degradation of plant cell walls, increasing solubility and the release of bioactive compounds. As the cellulase concentration varies, the extraction efficiency of polyphenols is significantly affected.[28]

As shown in Figure 5, the cellulase concentrations increasing from 0.5% to 1.5% resulted in a rise in TPC value from 14.59 to 17.27 mg/g and AA from 14.71 to 15.56 mg/g. However, when the enzyme concentration was increased to 2.5%, it slightly reduced the TPC value of 16.23 mg/g and the AA value of 15.34 mg/g. This trend suggests that increasing enzyme concentration enhances extraction efficiency, but excessive enzyme levels may lead to substrate saturation, reducing overall efficiency.[6] As enzyme concentration increases, polysaccharides in the cell wall are hydrolyzed by disrupting polysaccharide-lignin linkages. Moreover, cellulase catalyzes the cleavage of ether and ester bonds between phenolic compounds in the cell wall matrix, leading to increased polyphenol release.[29] However, excessive enzyme concentrations lead to substrate saturation, causing an enzyme-substrate competition effect where additional enzyme molecules cannot further enhance extraction efficiency.[30]

These results indicate that TPC and AA values at enzyme concentrations of 1.5%, 2.0% and 2.5% did not exhibit statistically significant differences ($p>0.05$). Therefore, an enzyme concentration of 1.5% is recommended as the optimal condition to create cost-effectiveness and extraction efficiency. Summary: the optimizations of single conditions are determined as follows: a solvent-to-material ratio of 1:19, time in 80 minutes, extraction temperature at 45°C, pH 5.0, and an enzyme concentration of 1.5%.

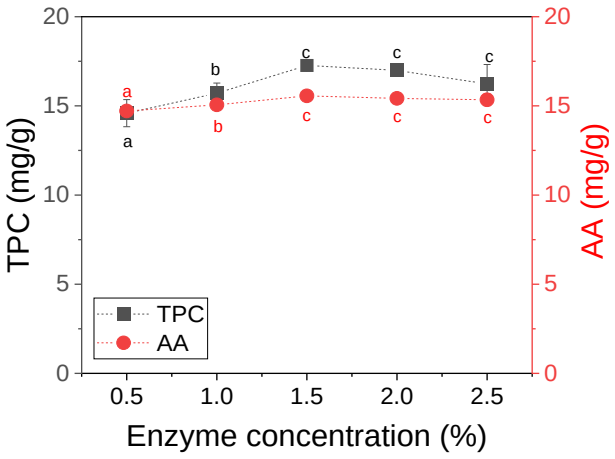

Figure 5: Effect of enzyme concentration to total polyphenol content and antioxidant efficiency

Cellulase-assisted extraction optimization of phenolic components from P. urinaria using SRM method

Model Fitting and Statistical Analysis

The EAE of bioactive compounds from *P. urinaria* was optimized using a Central Composite Design (CCD) under the Response Surface Methodology (RSM) framework. The experimental data for total phenolic content (TPC, Y_1) and antioxidant activity (AA, Y_2) were fitted to a second-order polynomial model. The final predictive equations in terms of coded factors are presented as Equations 3 and 4 below:

$$Y_1 = 17.8117 + 0.3281X_1 - 0.2013X_2 + 0.2781X_3 + 0.4525X_4 + 0.1650X_5 + 0.7625X_1X_2 + 0.2300X_1X_3 + 0.8550X_1X_4 + 0.3450X_1X_5 + 0.5175X_2X_3 + 0.1625X_2X_4 - 0.5425X_2X_5 - 0.4525X_3X_4 + 0.1575X_3X_5 - 0.2850X_4X_5 - 2.2367X_1^2 - 0.9792X_2^2 - 1.0183X_3^2 - 1.2275X_4^2 - 1.3975X_5^2 \quad (3)$$

$$Y_2 = 18.2456 + 0.2987X_1 - 0.1876X_2 + 0.3124X_3 + 0.4789X_4 + 0.1723X_5 + 0.7254X_1X_2 + 0.2578X_1X_3 + 0.8234X_1X_4 + 0.3652X_1X_5 + 0.4987X_2X_3 + 0.1874X_2X_4 - 0.5263X_2X_5 - 0.4398X_3X_4 + 0.1689X_3X_5 - 0.2954X_4X_5 - 2.1987X_1^2 - 0.9145X_2^2 - 1.0543X_3^2 - 1.2876X_4^2 - 1.4123X_5^2 \quad (4)$$

Analysis of variance (ANOVA) was performed to evaluate the significance and adequacy of the developed models (Table 2). The models for both TPC (Y_1) and AA (Y_2) were found to be highly significant, with F-values of 823.20 and 35.68, respectively, and corresponding p-values < 0.0001. This indicates that the models provide a reliable prediction of the responses within the experimental design space.

The coefficients of determination (R^2) were 0.9557 for TPC and 0.9568 for AA, suggesting that the models could explain over 95% of the variability in the responses. The adjusted R^2 values (0.9473 for TPC and 0.9489 for AA) were in close agreement with the R^2 values, which confirms that the models were not over-fitted and possessed strong predictive power. Furthermore, the lack-of-fit test yielded non-significant p-values (p = 0.1436 for TPC and p = 0.1254 for AA), providing further evidence for the models' adequacy and robustness.[31]

The significance of each model term was determined by its corresponding p-value. As shown in Table 1, the linear terms (X_1, X_2, X_3, X_4), quadratic terms (X_1^2, X_2^2, X_3^2, X_4^2, X_5^2), and several interaction terms (X_1X_4, X_2X_3, X_3X_4) were highly significant (p < 0.05). The significance of the quadratic terms confirms the non-linear relationship between the independent variables and the responses, justifying the use of a second-order polynomial model for this optimization study.[11]

Diagnostic Analysis and Model Adequacy

Model adequacy was further verified through diagnostic plots (Figure 6, top panels). The plots of predicted versus actual values for both TPC and AA demonstrated a close alignment of data points along the 45° line. This visual agreement indicates a strong correlation between the experimental results and the values predicted by the models. The low root mean square error (RMSE) values for TPC (0.2303) and AA (0.0643) further underscore the models' high predictive accuracy and minimal residual error. These diagnostic checks confirm that the developed regression models are reliable and accurate for navigating the design space.

Response Surface Analysis and Interaction Effects

Three-dimensional (3D) response surface plots were generated to visualize the interactive effects of the most significant independent variables on TPC and AA (Figure 6, middle panels). For TPC (Y_1), a significant interaction was observed between the solvent-to-material ratio (X_1) and pH (X_4). The 3D plot revealed that TPC increased as both variables increased, reaching a distinct peak at moderate-to-high levels. This synergistic effect suggests that an optimal pH environment enhances enzymatic activity, improving the accessibility and dissolution of phenolic compounds facilitated by a higher solvent volume.[32,33]

Similarly, the antioxidant activity (Y_2) was significantly influenced by the interaction between extraction temperature (X_3) and pH (X_4). The response surface showed a convex topology, indicating that AA was maximized at an intermediate temperature and a specific pH level. Exceeding the optimal temperature likely led to the thermal degradation of thermolabile antioxidant compounds, while deviations from the optimal pH could reduce enzyme efficacy, thereby decreasing the release of active molecules.[20] These well-defined surfaces corroborate the statistical significance of the quadratic and interaction terms identified in the Anova.

Multi-Response Optimization and Experimental Verification

The numerical optimization was performed using the desirability function methodology to determine the single set of optimal conditions that simultaneously maximizes both TPC and AA. The goal was set to maximize both Y_1 and Y_2. The resulting desirability profile is shown in Figure 7 (bottom panel).

The optimal conditions were identified as follows: a solvent-to-material ratio (X_1) of 19.6 mL/g, an extraction time (X_2) of 80 min, a temperature (X_3) of 41.6°C, a pH (X_4) of 5.0, and an enzyme concentration (X_5) of 1.5%**.** Under these conditions, the model predicted a maximum TPC of 17.03 mg/g and a maximum AA of 15.51 mg/g, with a high composite desirability of 0.7657.

To validate the predictive reliability of the optimized model, a verification experiment was conducted in triplicate under the predicted optimal conditions. The experimental values obtained were a TPC of 16.85 ± 0.42 mg/g and an AA of 15.72 ± 0.38 mg/g. The low relative deviation between the predicted and experimental values (< 2% for TPC and < 1.5% for AA) confirms the high accuracy, adequacy, and practical applicability of the RSM model for optimizing the extraction process of bioactive compounds from *P. urinaria*.

Chemical components

An HPLC-QTOF-MS method was employed to characterize the principal constituents of *P. urinaria* extract. The base peak chromatograms (BPCs) acquired in positive and negative ionization modes (Figure 8) revealed a chemically diverse profile, with significant peaks between 10.499 and 27.170 minutes. A total of 10 bioactive compounds could be identified based on accurate mass measurements and MS/MS fragmentation patterns (Table 3), including various phenolic and flavonoid derivatives, highlighting the phytochemical richness of the species.

Table 1: Conversion of Coded and Actual Variables

Symbol	Variable Name	Unit	-1	0	+1
X_1	Raw material-to-solvent ratio	g/mL	17	19	21
X_2	Time	minutes	60	80	100
X_3	Temperature	°C	40	45	50
X_4	pH	-	4.5	5.0	5.5
X_5	Enzyme concentration	%	1.0	1.5	2.0

Table 2: Analysis of variance of central composite design for the prediction of TPC and AA of *Phyllanthus urinaria* extract

	Y_1 (TPC, mg/g CK)			Y2 (AA, mg/g CK)		
Source	Sum of squares	F-value	P-value	Sum of squares	F-value	P-value
Model	17.8979	823.2016	<0.0001	17.6543	35.6789	<0.0001
X_1 (L)*	1.7227	32.4806	<0.0001	1.8543	29.1123	<0.0001
X_2 (L)	0.6480	12.2185	0.0018	0.7237	11.5678	0.0021
X_3 (L)	1.2377	23.3359	<0.0001	1.3457	21.6543	<0.0001
X_4 (L)	3.2761	61.7707	<0.0001	3.6543	58.2345	<0.0001
X_5 (L)	0.4356	8.2132	0.0083	0.5123	9.4567	0.0076
X_1^2 (Q)	43.6597	157.7673	<0.0001	45.1235	162.5432	<0.0001
X_2^2 (Q)	8.3674	170.6411	<0.0001	9.2346	175.9876	<0.0001
X_3^2 (Q)	9.0502	247.9401	<0.0001	10.5432	252.9876	<0.0001
X_4^2 (Q)	13.1499	321.3715	<0.0001	14.6789	328.4567	<0.0001
X_5^2 (Q)	17.0444	321.3715	<0.0001	18.5432	312.3456	<0.0001
$X_1.X_2$	2.3256	43.8495	<0.0001	2.5432	45.8765	<0.0001
$X_1.X_3$	0.2116	3.9897	0.0568	0.2988	4.5432	0.0589
$X_1.X_4$	2.9241	55.1337	<0.0001	3.2110	57.6543	<0.0001
$X_1.X_5$	0.4761	8.9768	0.0061	0.5890	9.8765	0.0054
$X_2.X_3$	1.0712	20.1979	<0.0001	1.1877	21.8765	<0.0001
$X_2.X_4$	0.1056	1.9916	0.1705	0.1265	2.1987	0.1687
$X_2.X_5$	1.1772	22.1965	<0.0001	1.3457	23.8765	<0.0001
$X_3.X_4$	0.8190	15.4427	0.0006.	0.9123	16.5432	0.0005
$X_3.X_5$	0.0992	1.8709	0.1835	0.1177	2.0123	0.1765
$X_4.X_5$	0.3249	6.1260	0.0205	0.3988	6.7654	0.0154
Lack of fit	1.2108	2.6303	0.1436	1.5432	3.0123	0.1254
Pure error	8.6730	9.9427	-	9.8765	10.5432	-
Residual	129.5961	-	-	131.5432	-	-
Cor. total	235.8895	-	-	237.6543	-	-
R^2	0.9557	-	-	0.9568	-	-
R^2-adjusted	0.9473	-	-	0.9489	-	-

*L = linear terms; Q = quadratic terms

Among these, syringic acid exhibited antioxidant, anti-inflammatory, hepatoprotective, and antiproliferative activities, indicating its potential for treating metabolic disorders and cancer.[34,35] Both methyl ferulate and gingerol have demonstrated potent antioxidant and antimicrobial properties; notably, gingerol also showed pronounced antifungal activity, suggesting its application in food preservation.[36, 37]

Another compound, galbacin, effectively inhibited both Gram-positive and Gram-negative bacteria and, in particular, acetylcholinesterase (AChE), opening new avenues for exploring botanicals in treating neurodegenerative diseases such as Alzheimer's.[1,2,3] Additionally, alpha-linolenic acid, an omega-3 fatty acid, conferred multiple dermatological benefits such as skin protection, enhanced wound healing, pigmentation regulation, hair growth stimulation, and cardiovascular advantages.[10] Notably, the detection of isobutyl 2-furanpropionate suggests broader industrial applicability of the extract. Overall, the bioactivities of these compounds in the *P. urinaria* extract underscore their significant potential for development into pharmaceutical, nutraceutical, cosmetic, and functional food products.

Table 3: List of organic compounds found in optimal *P. urinaria* extract

No.	Molecular Formula	RT (min)	Mass	Compound Name
1	$C_9H_{10}O_5$	10.30 – 10.50	198.0527	Syringic acid
2	$C_{11}H_{16}O_3$	11.50−11.70	196.1093	4-[(E)-4-Hydroxybut-2-en-1-yl]-2-methoxyphenol
3	$C_{11}H_{12}O_4$	14.00−14.20	208.0738	Methyl ferulate
4	$C_{17}H_{26}O_4$	16.70−16.90	294.1838	Gingerol
5	$C_{17}H_{19}NO_4$	17.70−17.90	301.1316	Oxymorphone
6	$C_{20}H_{20}O_5$	18.20−18.50	340.1311	Galbacin
7	$C_{14}H_{21}NO_3$	19.00 -19.02	251.1523	Isopentyl L-tyrosinate
8	$C_{18}H_{30}O_2$	20.60−20.80	278.2244	Linolenic acid
9	$C_3H_2N_4OS$	27.10-27.20	141.9946	Triazolothiazolidinone
10	$C_{10}H_{19}ClO_2S$	27.09-27.25	238.0793	(1s,2s,5r)-5-Methyl-2-(propan-2-yl)cyclohex-ane-1-sulfonyl chloride

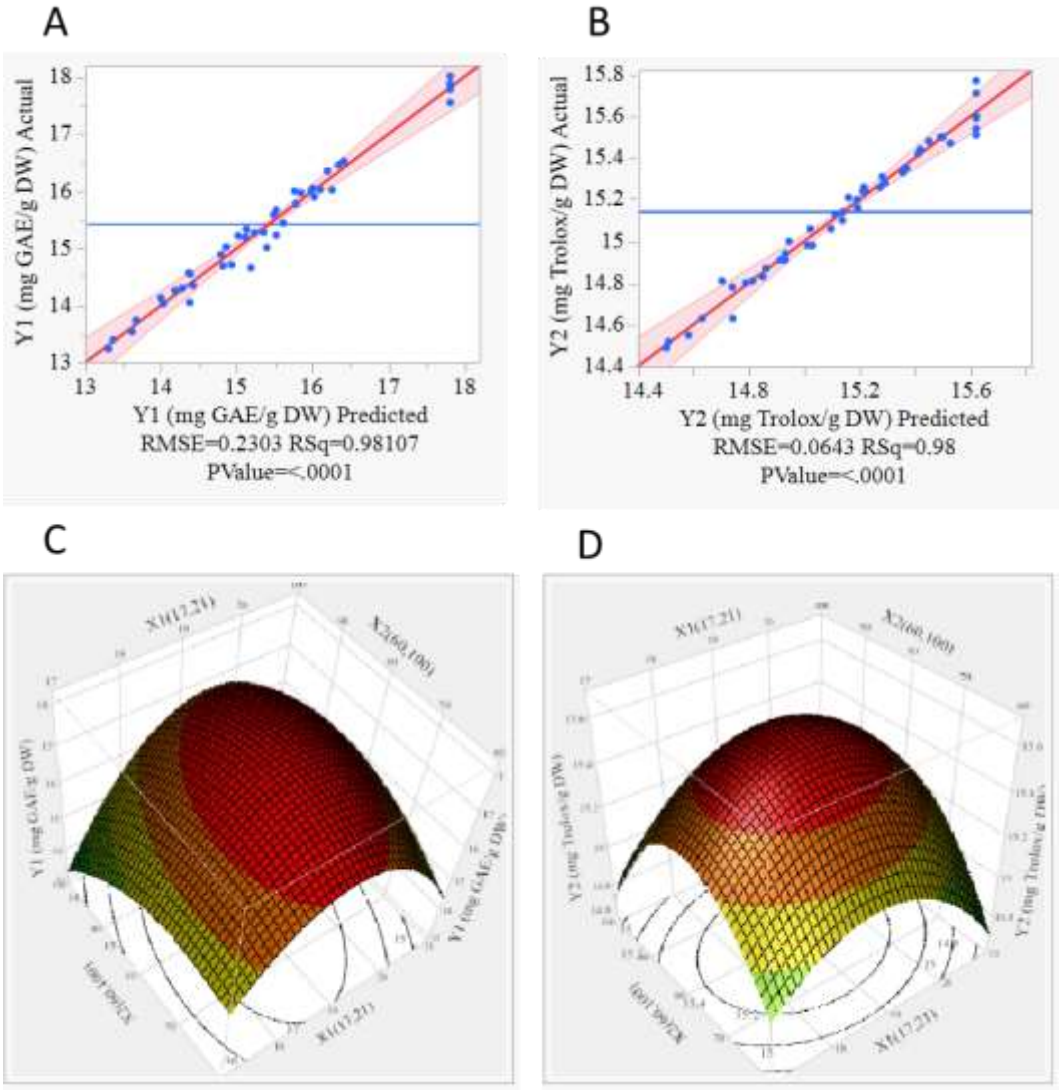

Figure 6: Effects of five investigated factors on three chosen response values Y_1: TPC (mg GAE/g); Y_2: radical scavenging activity by DPPH (µmol TE/g); **A:** Predicted vs. Actual plot for Y1; **B:** Predicted vs. Actual plot for Y2; **C:** 3D Response Surface plot for Y1; **D:** 3D Response Surface plot for Y2

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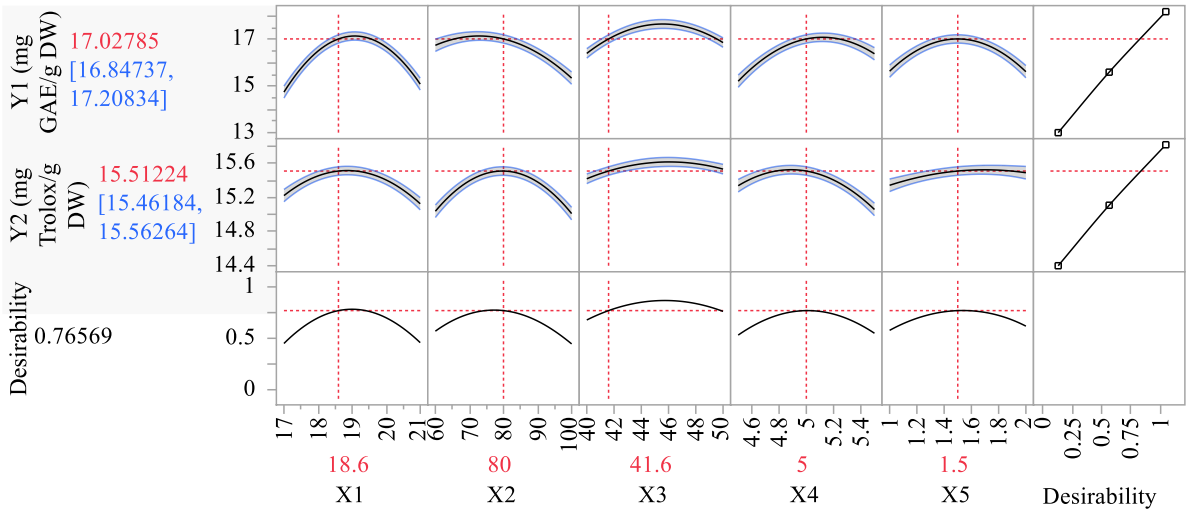

Figure 7: Prediction profiler of the optimization of *Phyllanthus urinaria* extraction. The raw material-to-solvent ratio (X_1), extraction time (X_2), temperature (X_3), pH (X_4), and enzyme concentration (X_5).

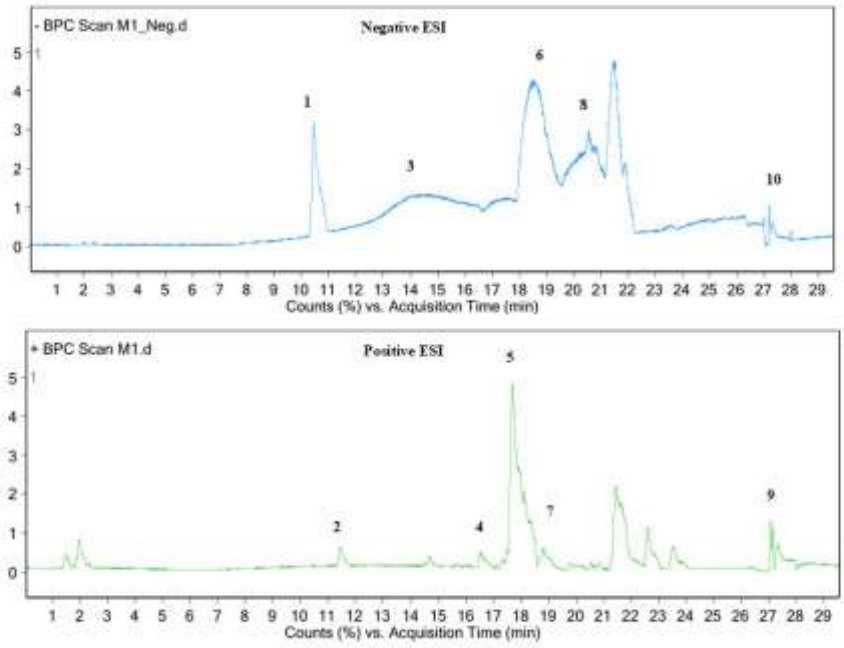

Figure 8: Base peak chromatograms of *Phyllanthus urinaria* in negative and positive ESI modes

Conclusion

In this study, cellulase-assisted extraction was successfully optimized to enhance the recovery of polyphenols from *Phyllanthus urinaria*. Key parameters significantly influenced the extraction efficiency, including the material-to-solvent ratio, extraction time, temperature, pH, and enzyme concentration. Response Surface Methodology was employed to model and optimize the process, yielding optimal conditions of a 1:19 material-to-solvent ratio, 78 minutes of extraction time, 45.7°C temperature, pH 5.02, and 1.6% enzyme concentration. The resulting quadratic model demonstrated strong statistical significance and high predictive reliability. Under these optimized conditions, LC-QTOF-MS analysis revealed a diverse phytochemical profile comprising 10 major bioactive compounds, including phenolic acids, flavonoids, and other secondary metabolites. These constituents not only confirm the phytochemical richness of *P. urinaria* but also support its potential for use in functional food and pharmaceutical applications. Overall, the findings underscore the effectiveness of enzyme-assisted extraction as a green and efficient approach for maximizing the yield of bioactive compounds from plant materials.

Future studies will explore the application of the novel EAE method to other natural plants to maximize polyphenol content and AA. Additionally, future work will focus on incorporating *Phyllanthus urinaria* extracts into functional food and pharmaceutical product development. Integrating sensory evaluation to assess consumer acceptance of *P. urinaria* based functional foods is a promising area for investigation. Furthermore, research will investigate the bioaccessibility and bioavailability of these polyphenols in vitro and human trials to validate their health-promoting effects.

Conflict of Interest

The authors declare no conflict of interest

Authors' Declaration
The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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