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Sustainable Extraction of Phytochemicals from *Leucaena leucocephala* Leaves: Integrating Laccase Pretreatment with Microwave-Assisted Hydrodistillation

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Leucaena leucocephala leaves are a promising biomass for the extraction of bioactive phytochemicals. However, their complex lignocellulosic matrix limits the extraction process. Thus, this study evaluates a green strategy by incorporating laccase pretreatment with microwave-assisted hydrodistillation (MAHD). *L.leucocephala* leaves were pretreated under optimized conditions (0.55 mg mL^{-1} laccase, pH 4.5, 60 °C, 6 h) and subsequently extracted by MAHD (250 W) across controlled time (0 – 48 min) and temperature (30–70 °C) ranges. Total flavonoid content (TFC) and total coumarin content (TCC) were quantified. In addition, SEM was employed to assess structural changes. Laccase pretreatment has successfully disrupted *L.leucocephala* cell wall and increased its porosity. It also enhances phytochemical recovery if compared to MAHD alone. The results show that TFC was increased by 5.3 % and TCC by 74.5 % in the presence of laccase pretreatment, follow by MAHD. Process optimization identified 36 min and 60 °C as the best MAHD conditions, balancing mass transfer with thermal stability. The integrated approach reduced temperature and residence time relative to conventional extraction, aligning with green-chemistry principles. These findings demonstrate that laccase assisted MAHD extraction improves the recovery of flavonoids and coumarins from *L. leucocephala* and supports the valorization of this fast-growing biomass for nutraceutical and pharmaceutical applications.

Keywords: *Leucaena leucocephala*, enzymatic hydrolysis, laccase, microwave-assisted hydrodistillation, phytochemicals, green chemistry

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The demand for bioactive phytochemicals is increasing due to their diverse applications in pharmaceuticals, nutraceuticals, and functional foods. Phytochemicals are plant-derived compounds classified into primary metabolites, essential for growth, and secondary metabolites, which support defence and adaptation mechanisms [1]. Secondary metabolites such as phenolics, flavonoids, glycosides, coumarins, and terpenoids exhibit significant pharmacological activities, including antioxidant, anti-inflammatory, antidiabetic, and antimicrobial effects [2, 3]. Recent advancements in micro and nanoencapsulation have enhanced their stability and bioavailability for industrial applications [4].

Leucaena leucocephala, a fast-growing leguminous tree, is recognised for its rich phytochemicals. Recent studies have highlighted the antioxidant and antidiabetic potential of this compound, with compounds such as hexadecanoic and oleic acids acting as α-amylase inhibitors [2, 3]. Antimicrobial activity has also been observed against *E. coli* and *S. aureus*, while lupeol isolated from the leaves shows anti-inflammatory and anticancer effects [5, 6]. These findings show the plant's potential as a renewable source of bioactive compounds for therapeutic applications. Other than its phytochemical richness, the extraction of bioactive compounds from *L. leucocephala* is limited by its rigid lignocellulosic matrix. An efficient and sustainable extraction from plant matrices remains challenging, especially for lignocellulosic biomass. The dense network of cellulose, hemicellulose, and lignin limits solvent accessibility and mass transfer, resulting in low extraction efficiency and quantity [7, 8].

The study by Zayed and Samling (2016) provided valuable insights into the phytochemical profile of *Leucaena leucocephala* leaves from Malaysia. They identified bioactive compounds such as squalene, phytol, and other metabolites with notable antioxidant, antimicrobial, and anticancer properties [9]. Even

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though their work discovered the presence of bioactive compounds, it primarily relied on conventional extraction techniques, which are often limited by the complex lignocellulosic structure of plant biomass. This structural rigidity can hinder solvent penetration and mass transfer, resulting in low recovery of phytochemicals. To tackle this issue, the present study aims to enhance extraction efficiency by introducing an enzymatic pretreatment step using laccase from *Trametes versicolor*, before proceeding to microwave-assisted hydrodistillation (MAHD). The enzymatic hydrolysis is expected to selectively degrade lignin and improve cell wall permeability, thereby facilitating the subsequent extraction of phytochemicals through MAHD. Pretreatment is essential to disrupt lignocellulosic matrix. Enzymatic hydrolysis using laccase from *Trametes versicolor* selectively degrades lignin, improving accessibility of flavonoids and coumarins [10]. Previous study demonstrated that combining enzymatic pretreatment with microwave-assisted extraction enhances the mobilization of phenolic compounds more effectively than either technique applied independently [11]. Laccase-mediator systems enhance lignin depolymerization [12] for sustainable phytochemical recovery. This study investigates the integration of laccase pretreatment with microwave-assisted hydrodistillation (MAHD) as a green extraction strategy to enhance the recovery of phytochemicals from *Leucaena leucocephala* leaves, with a specific focus on evaluating whether laccase from *Trametes versicolor* improves total flavonoid content (TFC) and total coumarin content (TCC).

EXPERIMENTAL

Chemicals and Materials

Fresh *Leucaena leucocephala* (Lam.) de Wit (Fabaceae) leaves were collected from trees in the Kuantan area, Malaysia. Distilled water from the UMP Analytical Laboratory was used as the extraction solvent. Laccase from *Trametes versicolor* (≥10 U/mg, CAS 80498-15-3), quercetin hydrate (≥95%, CAS 849061-97-8), aluminium chloride hexahydrate (98%, CAS 7784-13-6), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, CAS 30931-67-0), methanol (CAS 67-56-1), hydrochloric acid (CAS 7647-01-0), coumarin (1,2-benzopyrene, CAS 50-32-8), and lead acetate trihydrate (CAS 6080-56-4) were purchased from Sigma-Aldrich (USA).

Sample Preparation

The collected leaves were washed thoroughly with tap water, oven-dried at 40°C for 24 hours, and ground to a fine powder using a GM200 laboratory blender (Retsch, Germany). The powdered samples were stored at 4 °C in airtight containers until use.

Enzymatic Pretreatment

An ammonium acetate buffer (0.1 M, pH 4.5) was prepared and adjusted with acetic acid. Laccase solution (0.55 mg/mL; 0.8 U/g) was prepared in distilled water and stored at −18 °C. ABTS (0.058 g) was dissolved in 31.9 mL of 0.1 M buffer to prepare a 5 mM mediator solution. For hydrolysis, 5 g of L. leucocephala powder was mixed with 66.7 mL of buffer and 31.9 mL of ABTS solution in a 500 mL Erlenmeyer flask. Laccase solution was added to initiate the reaction. The mixture was incubated at 60 °C with shaking at 200 rpm for 6 hours [13]. After incubation, samples were stored at −18 °C for subsequent extraction.

Microwave-Assisted Hydrodistillation (MAHD)

The enzymatically treated samples (5 g) were combined with 500 mL of distilled water in a 1000 mL round-bottom flask connected to a Clevenger-type apparatus and subjected to MAHD by Milestone Ethos E microwave extraction closed system at 250 W of microwave power. Extractions were performed at temperatures of 30 – 70 °C (10 °C intervals) and times of 0–48 min (12 min intervals). 0 min indicates the immediate measurement. Extracts were filtered, centrifuged (10,000 rpm, 10 min), and then concentrated using a rotary evaporator. The concentrated extracts were diluted in methanol, sonicated at 40 °C for 30 minutes, and stored at 4 °C for further analysis.

Total Flavonoid Content (TFC)

TFC was determined following Alara et al. (2020) [14]. Standard quercetin solutions (0 – 50 µg/mL) were prepared in methanol. Samples or standards (2 mL) were mixed with 2 mL of a 2% $AlCl_3$ solution and incubated at room temperature for 60 minutes. Absorbance was measured at 420 nm using a UV–VIS spectrophotometer [15] (Shimadzu, Japan). Results were expressed as mg quercetin equivalents (QE)/g dry weight based on the calibration curve.

Total Coumarin Content (TCC)

TCC was quantified as described by de Amorim et al. (2012) [16]. Coumarin standards (1–10 µg/mL) were prepared in distilled water. Samples (500 µL) were mixed with 2 mL of distilled water and 500 µL of a 5% lead acetate solution. After stirring, 7 mL of distilled water was added. Aliquots (2 mL) of this mixture were combined with 8 mL of 0.8% HCl and incubated at room temperature for 30 minutes. Absorbance was measured at 320 nm. TCC was expressed as mg coumarin equivalents (CE)/g extract based on the calibration curve.

Statistical Analysis

All data were expressed as the mean of three replicates. Statistical significance was determined using two-way

analysis of variance (ANOVA) performed in OriginPro 2019b, followed by Tukey's post-hoc test to evaluate differences between means at a significance level of $p < 0.05$.

RESULTS AND DISCUSSION

Effect of Laccase Pretreatment on Biomass Structure

The structural changes of *Leucaena leucocephala* leaves subjected to different treatments were analyzed using scanning electron microscopy (SEM) at 3000× magnification (Figures 1a to Figure 1d). The raw, untreated leaves (Figure 1a) displayed a smooth, compact surface with tightly packed epidermal cells and minimal visible pores. This intact morphology reflects the characteristic rigidity of lignocellulosic biomass, where cellulose fibres are encrusted with hemicellulose and lignin, creating a natural barrier to solvent penetration (Wu et al., 2024).

Leaves treated with microwave-assisted hydrodistillation (MAHD) alone (Figure 1b) exhibited minor structural alterations, including slight surface deformation and localized fissures. These effects are likely due to internal heating and pressure gradients generated during microwave irradiation. However, the surface remained largely undisturbed, suggesting that MAHD has limited efficacy in disrupting the lignocellulosic matrix without prior pretreatment. In contrast, leaves undergoing laccase pretreatment (Figure 1c) showed clear morphological changes, including the appearance of pores, cracks, and roughened surfaces. This disruption indicates effective enzymatic degradation of lignin by laccase from *Trametes versicolor*, which selectively oxidizes phenolic units and loosens the cell wall architecture [17, 18]. The enhanced porosity increases solvent accessibility, facilitating the subsequent extraction of phytochemicals [19, 20].

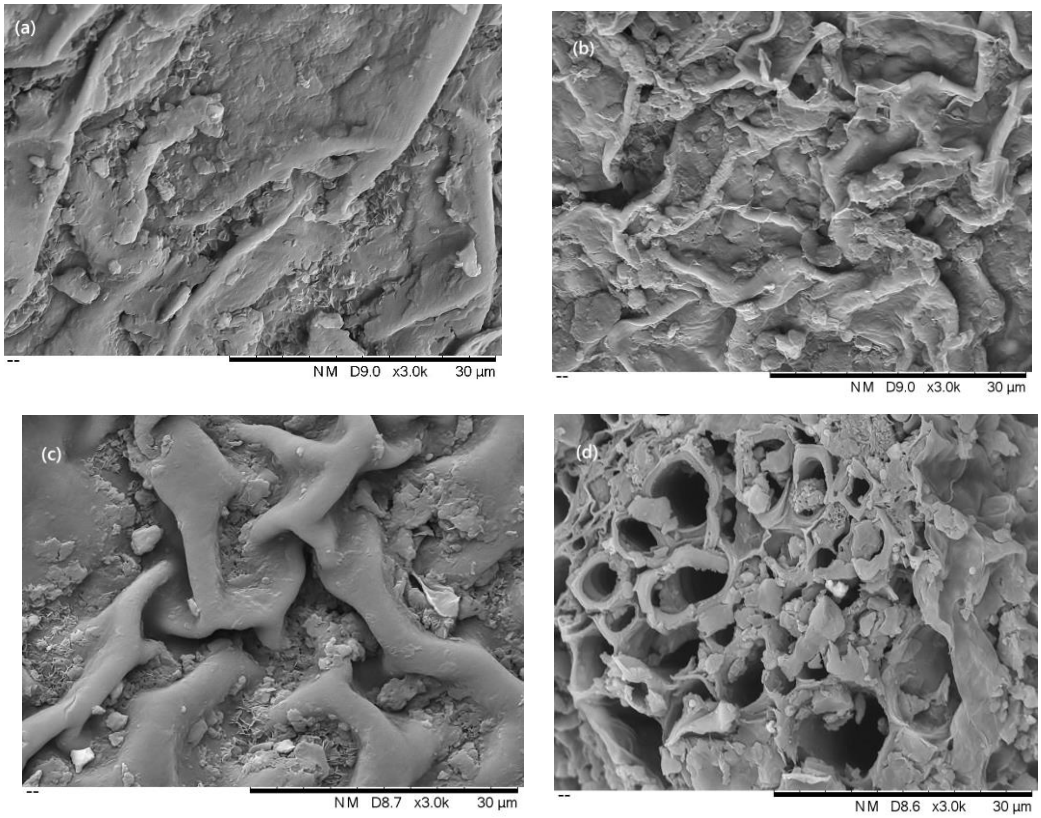

Figure 1. SEM of *Leucaena leucocephala* leaves at 3000× magnification: (a) raw, untreated sample showing an intact and smooth surface; (b) sample treated with microwave-assisted hydrodistillation (MAHD) only, exhibiting minor surface deformation; (c) sample subjected to laccase pretreatment, showing pores and surface cracks indicating lignin degradation; and (d) sample treated with laccase pretreatment followed by MAHD, displaying extensive surface disruption with large pores and collapsed cell walls. Scale bars = 30 µm.

The most extensive structural breakdown was observed in leaves treated with the combined laccase pretreatment and MAHD (Figure 1d). The surface was highly disrupted, with pronounced fissures, large pores, and cell wall collapse. The structural modifications observed in this study align with the findings of Mohd Safaai et al. (2016), who reported that decreasing particle size and increasing pretreatment temperature led to reduced crystallinity and greater surface disruption in *Leucaena leucocephala* [21]. Their SEM analysis revealed more irregular, porous, and damaged structures at smaller particle sizes and higher temperatures, indicating enhanced accessibility of the surface area for cellulose hydrolysis. Similarly, the present SEM results demonstrate that laccase pretreatment induced noticeable surface roughness and pore formation (Figure 1c). In contrast, the combined enzymatic pretreatment and MAHD (Figure 1d) caused extensive disruption of the lignocellulosic matrix. This disrupted morphology, characterised by large pores and collapsed cell walls, is likely due to lignin removal by laccase, which enhances the efficiency of microwave energy absorption and solvent penetration during MAHD. Such structural changes increase surface area and porosity, facilitating the release of bioactive compounds.

Effect of Extraction Time and Temperature

The influence of extraction time and temperature on the total flavonoid content (TFC) and total coumarin content (TCC) of *Leucaena leucocephala* leaves extracted by microwave-assisted hydrodistillation (MAHD) without pretreatment is shown in Figure 2. At different extraction times (0–48 minutes), TFC increased sharply from 31.3 mg QE/g at 0 minutes to a maximum of 66.3 mg QE/g at 36 minutes, after which it decreased with minor fluctuations up to 48 minutes (Figure 2a). This trend suggests that most flavonoids were rapidly released within the initial 12 minutes due to efficient microwave heating, and cause solvent penetration, then cause cell swelling and rupture. The increase in concentration gradients and effective diffusivity [22], facilitate the release of flavonoid. This is consistent with the rapid cell wall disruption reported by Arabian et al. (2022) [23] during microwave heating of *Chlorella vulgaris*. Prolonged extraction beyond 36 minutes did not significantly improve TFC. It even showed a slight decline at 48 minutes, possibly due to the thermal degradation of heat-sensitive flavonoids, as reported recently [24]. As extraction continues, intraparticle diffusion from deeper cell becomes rate-limiting, thus the driving force declines, and the system approaches an asymptotic equilibrium [22]. TCC exhibited a different pattern, rising steadily from 2.5 mg CE/g at 0 min to a peak of 16.8 mg CE/g at 36 min before decreasing to 9.3 mg CE/g at 48 min. The subsequent decline at this time may also reflect thermal degradation [25] or volatilization [26] of coumarins, as reported by Görmez et al. (2022) by using three different oxidation methods.

Temperature had a significant effect on both TFC and TCC (Figure 2b). TFC remained relatively stable (55–57 mg QE/g) across 30–50 °C but peaked at 65.6 mg QE/g at 60 °C, before declining sharply to 57 mg QE/g at 70 °C. TCC exhibited a similar trend, reaching its highest value (13.7 mg CE/g) at 60 °C. The increase in TFC and TCC up to 60 °C suggests improved mass transfer and solubility at moderate temperatures, enhancing phytochemical extraction. However, the decrease at 70 °C could result from thermal degradation of these bioactive compounds, in agreement with the findings of Nie et al. (2019), who reported reduced flavonoid stability at elevated temperatures during microwave-assisted extraction of flavonoids from rosemary [27].

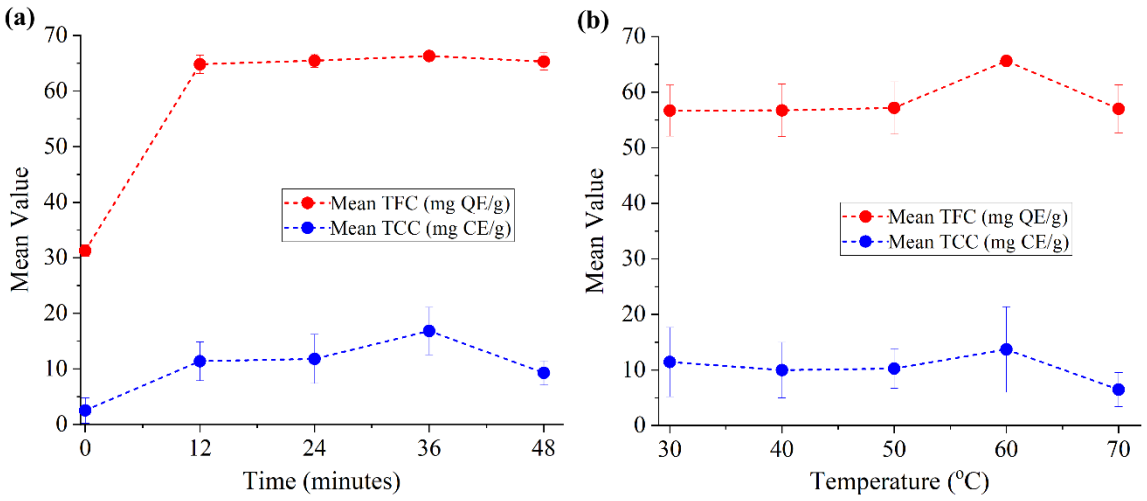

Figure 2. Two-way ANOVA for two independent variables (a) time and (b) temperature for TFC and TCC mean value extracted by MAHD only. The mean values for both TFC and TCC were generated using two-way ANOVA by OriginPro 2019b.

These results highlight the critical role of optimizing MAHD parameters to maximize phytochemical yields while minimizing degradation. Compared to conventional hydrodistillation, MAHD enables rapid heating [28, 29] and efficient extraction [30, 31] at lower temperatures [32, 33] and shorter times [34]. However, the structural rigidity of the lignocellulosic matrix remains a limitation, highlighting the need for effective pretreatment strategies such as enzymatic hydrolysis. Notably, 36 minutes and 60°C were identified as the optimal extraction time and temperature, respectively, for achieving the highest yields of TFC and TCC under MAHD without pretreatment.

The effect of extraction time and temperature on total flavonoid content (TFC) and total coumarin content (TCC) of *Leucaena leucocephala* leaves extracted by microwave-assisted hydrodistillation (MAHD), in the presence and absence of laccase pretreatment, is shown in Figure 3. For the extraction time (Figure 3a), TFC increased sharply within the first 12 minutes for both treatments. In MAHD only, TFC rose from 31.3 mg QE/g to 64.8 mg QE/g at 12 minutes and remained relatively stable up to 36 min before slightly declining at 48 min. A similar trend was observed for TCC, peaking at 16.8 mg CE/g at 36 min before decreasing to 9.3 mg CE/g. In contrast, samples pretreated with laccase consistently showed higher yields across all time points. TFC and TCC reached their maximum values at 36 minutes, with 70 mg QE/g and 65 mg CE/g, respectively. The significant enhancement in phytochemical recovery suggests that laccase pretreatment improved cell wall permeability by degrading lignin and hemicellulose, thereby facilitating solvent penetration and mass transfer during MAHD. This observation aligns with the findings of Šelo et al. (2024), who reported that the extractability of 13 individual phenolic compounds from grape pomace treated with *Trametes versicolor* was improved, suggesting that the enzyme complex, which includes laccase, enhances the extractability of phenolic compounds, which are valuable due to their antioxidant properties [35]. Moreover, Hamidi et al. (2021) reported that MAHD's rapid heating releases intracellular compounds efficiently during the early stages of extraction. However, prolonged exposure can lead to thermal degradation of heat-sensitive phytochemicals [13], explaining the decline observed beyond 36 minutes.

For extraction temperature (Figure 3b), TFC and TCC remained relatively stable between 30–50 °C for MAHD only, with slight increases at 60 °C (65.6 mg QE/g for TFC and 13.7 mg CE/g for TCC). However, at 70 °C, a noticeable decrease was observed, likely due to thermal degradation of flavonoids and coumarins, as noted by Mustafa and Turner (2011) in microwave extractions. In laccase-pretreated samples, phytochemical recovery was significantly higher across all temperatures. TFC peaked at 69.2 mg QE/g and TCC at 54 mg CE/g at 60 °C.

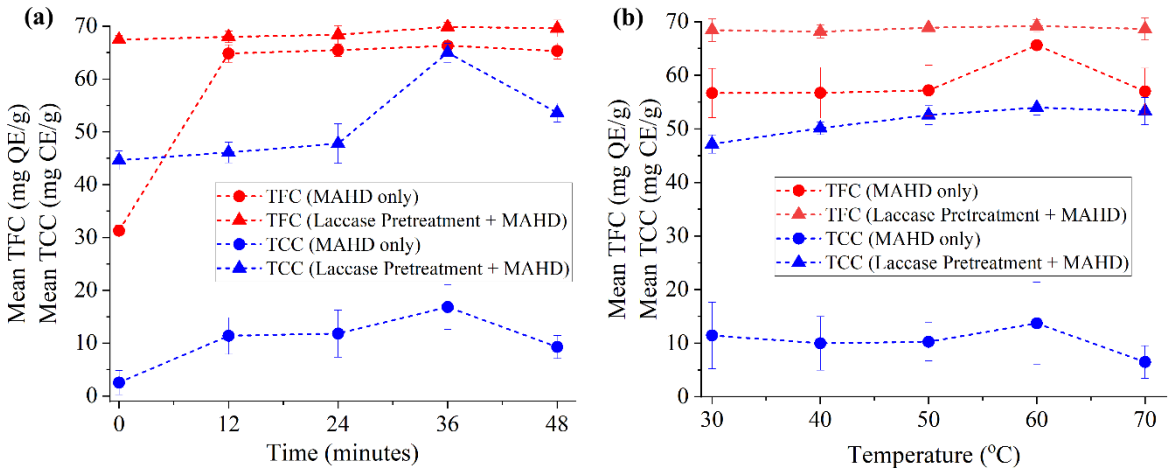

Figure 3. Two-way ANOVA for two independent variables (a) time and (b) temperature by comparing TFC and TCC mean value extracted by MAHD in the presence and absence of laccase pre-treatment. The mean values for both TFC and TCC were generated using two-way ANOVA by OriginPro 2019b.

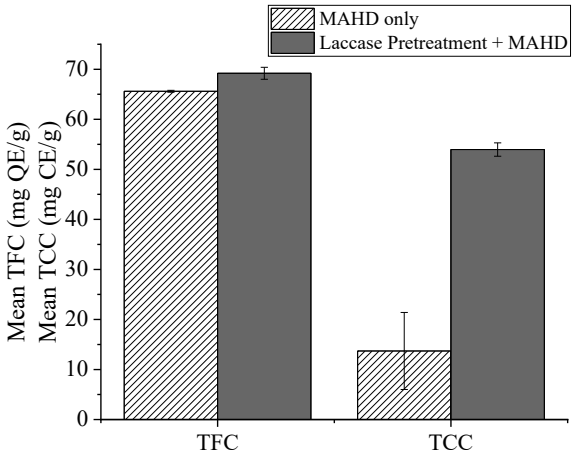

Figure 4. Comparison of TFC and TCC in *Leucaena leucocephala* leaves extracted by microwave-assisted hydrodistillation (MAHD) at 60 °C, with and without laccase pretreatment. The mean values for both TFC and TCC were generated using two-way ANOVA by OriginPro 2019b.

The superior performance of the combined treatment suggests a synergistic effect, where enzymatic pretreatment enhances the susceptibility of biomass to microwave energy, facilitating the release of bound phytochemicals. The comparison of total flavonoid content (TFC) and total coumarin content (TCC) between MAHD only and laccase pretreatment combined with MAHD is presented in Figure 4. Laccase pretreatment resulted in a modest increase of 5.3% in TFC, rising from 66.3 mg QE/g (MAHD only) to 69.8 mg QE/g. In contrast, TCC showed a substantial improvement of 74.5%, increasing from 13.7 mg CE/g to 53.9 mg CE/g. Coumarins shows larger response to laccase pretreatment compared to flavonoids. It is therefore linked to the differences in localization and binding. Most of the flavonoids are vacuolar and readily extractable, whereas coumarins and other phenolic compounds occur in the apoplast and in their insoluble forms, which are linked to lignin or hemicellulose [36]. Coumarins can be extracted only after laccase pretreatment, which increases their porosity. Under moderate MAHD temperature at 60 °C, mass transfer is improved without severe thermolysis, favoring coumarin release while leaving less room to improve accessible flavonoids. This synergistic enhancement is consistent with findings by Brienza et al. (2025), who highlighted the role of enzymatic pretreatment in reducing lignocellulosic recalcitrance and improving extractability of bioactive compounds [37]. The disrupted biomass structure observed in SEM images of pretreated samples (Figure 1d) supports this interpretation, showing large pores and collapsed cell walls that enable efficient mass transfer during MAHD. These results highlight the importance of combining laccase pretreatment with MAHD to achieve higher phytochemical yields while reducing extraction time and temperature, thereby aligning with the principles of green chemistry, which emphasise energy efficiency and sustainability.

CONCLUSION

This study has shown the potential of integrating laccase pretreatment with microwave-assisted hydrodistillation (MAHD) as an efficient and sustainable method for extracting phytochemicals from *Leucaena leucocephala* leaves. Enzymatic pretreatment using laccase from *Trametes versicolor* effectively disrupted the lignocellulosic matrix, as confirmed by SEM analysis showing increased surface porosity and also cell wall degradation. This pretreatment significantly enhanced phytochemical recovery, with total flavonoid content (TFC) and total coumarin content (TCC) increased by 5.3% and 74.5%, respectively, compared to MAHD alone. The results of this investigation show that 36 minutes and 60 °C as optimal conditions for maximizing yields of flavonoids and coumarins, at the same time, is able to minimize energy consumption and thermal degradation.

The findings address a critical research gap in the valorisation of *L. leucocephala* by establishing a green extraction route that supports sustainability and energy-efficiency principles. This information can be used to develop targeted development of standardized botanical extracts that are rich in flavonoids and coumarins for nutraceutical and functional foods. Besides, it can also contribute towards the generation of natural antioxidants and antimicrobial compounds that can be used in cosmetics and food preservation. Interestingly, this process utilized

biomass with reduced thermal footprints and shorter cycle times. The moderate operating parameters (60 °C, 36 min) and aqueous conditions further facilitate the scale-up, process intensification, and regulatory acceptance for clean-label products.

Scientifically, this work advances the field by incorporating microstructural evidence (SEM) with quantitative extraction outcomes (TFC, TCC) to demonstrate the relation between laccase pretreatment and microwave extraction in *L. leucocephala*.

Future studies should focus on scaling up this process to a pilot plant, including the evaluation of economic feasibility and its environmental impact. Furthermore, comprehensive profiling of other bioactive compounds in the extracts is needed, along with their bioactivity assessments. This will be valuable in supporting their therapeutic potential. Interestingly, exploring alternative enzyme systems and pretreatments may enhance extraction efficiency and broaden the application of this approach to other lignocellulosic materials.

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