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Effect of Aerobic Fermentation on Total Phenolic, Flavonoid, and Antioxidant Activity of *Leucaena leucocephala* (Lam.) de Wit

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ABSTRACT

Introduction: *Leucaena leucocephala (Lam.) de Wit., commonly known as the petai cina plant, contains significant concentrations of bioactive compounds, particularly flavonoids and phenolic compounds, and demonstrates substantial antioxidant properties. These beneficial compounds, however, can be significantly influenced by various processing methods, with fermentation during simplicia preparation being a notable factor.* ***Aims***: *This research investigated the impact of fermentation on three key characteristics of petai cina leaf extract: total flavonoid content, total phenolic content, and antioxidant activity.* ***Methods***: *The study utilized experimental methodology, incorporating both qualitative and quantitative analyses to evaluate these properties. The results revealed that five days fermentation demonstrated the highest value of TFC, TPC, and antioxidant activity.* ***Result***: *The extract yielded a TFC of 279.08 mg QE/g, while TPC measured 282.34 mg GAE/g. The antioxidant activity demonstrated notable potency, with an IC50 and AAI values of 69.66 µg/mL and 0.574.* ***Conclusion***: *These findings conclusively demonstrate that the fermentation process significantly influences the concentration of flavonoids and phenolic compounds, yet unsignificant impact antioxidant activity in the extract. This enhanced understanding of fermentation's effects on bioactive compounds in L. leucocephala provides valuable insights for optimizing processing methods to maximize the plant's therapeutic potential.*

KEYWORDS: *Petai cina, flavonoid content, phenolic content, antioxidant, aerobic fermentation*

INTRODUCTION

The extensive biodiversity of Indonesia's tropical climate has historically provided abundant resources for traditional medicine development. While traditional and herbal medicines maintain global relevance in contemporary healthcare practices, scientific investigation of their bioactive compounds and processing methods remains crucial for optimizing their theraputic potential. In this context, *Leucaena leucacephala* (Lam.) de Wit., com-

monly known as petai cina, has garnered significant attention for its medicinal properties (Grenvilco et al., 2023).

L. leucocephala, a leguminous plant species prevalent in tropical regions including Indonesia, demonstrates considerable therapeutic value, particularly in wound healing and anti-inflammatory applications (Septina et al., 2020). Clinical evidence supports these traditional uses, with research by (Veronica & Dwiastuti, 2022) demonstrating that a 6% (w/v) concentration of *L. leucocephala* leaf extract significantly accelerated excoriation wound healing in experimental models. Phytochemical analysis has identified several bioactive compounds in *L. leucocephala*, including flavonoids, phenolics, saponins, tannins, triterpenoids, and cardiac glicosides (Elbanoby et al., 2024; Zarina et al., 2017). Of particular interest are flavonoids and phenols, secondary metabolites belonging to the polyphenol family, which exhibit notable antioxidant properties. These compounds effectively neutralize free radicals through electron donation, primarily via their hydroxyl groups (Hassanpour & Doroudi, 2023). Research indicates a positive correlation between flavonoid (TFC) and phenolic content (TPC) with antioxidant capacity (Awwalia et al., 2024). Recent investigations by Ahmad Fahrurrozi et al. (2021) utilizing DPPH (2,2-Diphenyl-1-Picrylhidrazyl) assay revealed significant antioxidant activity in *L. leucocephala* leaf ethanol extract, with an IC50 value of 86.309 ppm. However, this activity is influenced by various factors directly related to the alteration of flavonoids and phenolic compound's bioavailability in plants, one of which is fermentation.

METHODS

Materials and Instruments

Chemicals and reagents used in this work were in analytical grade: 96% ethanol provided by Smart Lab (Indonesia), magnesium, HCl, FeCl3, methanol, Follin-Ciocalteu, natrium carbonate, and acetic acid purchased from Merck (Germany), quercetin, gallic acid, and DPPH obtained from Sigma-Aldrich (Singapore).

Several instruments were used in this study, including analytical balance (BEL Engineering, Italy), rotary evaporator (Buchi, Germany), Water bath (Memmert, Germany), and spectrophotometer UV-Vis (Shimadzu, Japan).

Sample Collection and Authentication

Fresh leaves of *L. leucocephala* were harvested from Muara Aman village, Pasemah Air Keruh district, South Sumatera, Indonesia in May 2024. The samples were then botanically authenticated by Angga Dwiartama, PhD., Laboratory of Herbarium Bandungens, School of Life Science and Technology,

Bandung Institute of Technology with identification number of 3294/IT1.C11.2/TA.00/2024, and the specimen voucher was kept at Natural Product Laboratory, Faculty of Pharmacy, Kader Bangsa University, with the voucher code was PC-J01/2024.

Sample Preparation

The sample preparation process was based on the technique previously described by Saputri et al. (2024), begining with comprehensive quality control measures to ensure research-grade materials. Fresh *Leucaena leucocephala* leaves underwent initial wet sorting to eliminate foreign matter, contaminants, and non-essential plant parts. The selected leaves were thoroughly cleansed under running water and drained to remove residual impurities. Following this preliminary preparation, the cleaned leaves were systematically divided into three experimental groups for different processing protocols. The first group served as a control, utilizing fresh simplicia preparation where the cleaned and wet-sorted leaves were immediately processed into a fine consistency using a mechanical blender. The second and third groups were designated for aerobic fermentation studies, employing a controlled environment where the prepared leaves were placed in dedicated fermentation vessels covered with permeable cloth to facilitate aerobic conditions. These groups underwent fermentation periods of three and five days, respectively, allowing for comparative analysis of fermentation duration effects. Upon completion of their respective fermentation periods, the processed leaves underwent a final dry sorting phase to ensure consistent quality. The material from all three experimental groups was then mechanically ground into a fine powder using a blender, yielding standardized samples for subsequent analysis.

Extraction

Following the preparation of the simplicia powder, extraction was performed using 96% ethanol through the maceration method. The *L. leucocephala* powder from all three experimental groups (unfermented, 3-day aerobic fermentation, and 5-day aerobic fermentation) was precisely measured, with 200 grams of each powder being immersed in 1500 mL of 96% ethanol. The maceration process was conducted over a 24-hour period in a light-protected environment, with periodic agitation to ensure thorough extraction. Following this initial extraction, the mixture underwent filtration to separate the residue from the filtrate. To maximize extraction efficiency, the residue underwent two additional maceration cycles using fresh 96% ethanol in equal volumes. The combined filtrates from all maceration cycles were then concentrated using a

rotary evaporator at 45°C, 70 radial per minute until crude extracts were obtained (Utami et al., 2023). The extract yield was calculated using the following formula:

$$\text{Extract yield (\%)} = \frac{\text{Weight of Crude extract}}{\text{Weight of macerated sample}} x\ 100$$

Total Flavonoid Content Analysis

The determination of total flavonoid content follows a modified version of the method established by Setiawansyah et al., (2024). The analytical procedure begins with the precise weighing of a 5 mg sample, which is then dissolved in 50 ml of analytical-grade methanol. For the reaction process, 1 ml of the prepared extract is combined with 1 ml of 10% AlCl3 solution, followed by the addition of 8 ml of 5% acetic acid. The resulting solution is allowed to incubate at room temperature for a 30-minute period, after which the absorbance is measured spectrophotometrically at a wavelength of 412 nm. To ensure analytical accuracy and precision, all measurements are performed in triplicate. The final TFC is then calculated using standardized quantification formula, as follows:

$$\text{TFC} = \frac{\text{c x V x f}}{\text{m}}$$

Where

TFC : Total flavonoid content (mg QE/g)

c : Quercetin equivalence (µg/mL)

V : Total volume of extract (mL)

f : Dilution factor

m : Extract mass (g)

Total Phenolic Content Analysis

The determination of total phenolic content employs a modified version of the analytical methods established by Arsul et al., (2025). This refined protocol incorporates precise measurements and standardized procedures to ensure accurate quantification. The analytical process begins with the preparation of the sample solution, where 5 mg of the specimen was carefully weighed and dissolved in methanol to achieve a final volume of 50 ml. A precise aliquot of 0.1 ml from this stock solution was then transferred to a 10 ml volumetric flask for further analysis. The reaction mixture is prepared by sequentially adding 8 ml of distilled water and 1 ml of Folin-Ciocalteu reagent. The solution was then brought to its final volume of 10 ml through the addition of 20% sodium carbonate (Na_2CO_3) solution. To ensure complete reaction development, the mixture underwent a controlled incubation period of 20 minutes at room temperature. Following incubation, the spectrophotometric analysis was performed at a wavelength of 776 nm using a UV-Vis spectrophotometer. To ensure statistical validity and analytical precision,

all measurements were conducted in triplicate. The final concentration of TPC was calculated using standardized quantification formulas, as follows:

$$TPC = \frac{c \times V \times f}{m}$$

Where

TPC : Total phenolic content (mg GAE/g)

c : Gallic acid equivalence (µg/mL)

V : Total volume of extract (mL)

f : Dilution factor

m : Extract mass (g)

Antioxidant Activity Assay

The assessment of *L. leucocephala* antioxidant capacity employed DPPH methodology, adapting the procedure established by Hadi et al., (2023) with slight modification. The experimental design incorporated a series of five distinct sample concentrations (31.25, 62.5, 125, 250, and 500 µg/mL), each combined in equal proportions with DPPH solution at a concentration of 40 µg/mL. The protocol utilized ethanol and DPPH as control parameters, with quercetin serving as the comparative standard for system calib ration. The analytical procedure required a 25-minute dark incubation phase, followed by spectrophotometric analysis using a Shimadzu UV-Vis instrument at 514 nm wavelength. To ensure robust data quality and statistical significance, all experimental measurements, encompassing both sample and standard analyses, were conducted in triplicate. The quantification of antioxidant activity was determined through the calculation of radical scavenging activity using the following equation:

$$\text{Inhibtion } (\%) = \frac{\text{Abs control} - \text{Abs samples}}{\text{Abs control}} x\ 100$$

The IC_{50} value, representing the concentration required for 50% inhibition, was calculated via linear regression analysis. This quantitative assessment involved constructing a calibration curve that established the relationship between sample concentration and the corresponding inhibition percentage. The antioxidant capacity of *L. leucocephala* was then determined as antioxidant activity index (AAI) based on the formula described by Setiawansyah, Arsul, et al. (2024).

Data Analysis

The data of TFC, TPC, and antioxidant activity are presented as mean ± SD (n=3) and statistically analyzed using One Way ANOVA, Tukey's test in a GraphPad Prism 10.0.1 version.

RESULT AND DISCUSSION

Effect of Aerobic Fermentation on Extract of *L. leucocephala*

The The evaluation of extraction process effectiveness heavily relies on extract yield

Table 1. Extract yield from various post-harvest process

Samples	Sample Dry Weight (g)	Crude Extract Weight (g)	Yield (%)
Fresh leaves	200	16	8.02
Three-day fermentation	200	15.5	7.75
Five-day fermentation	200	14.49	7.45

as a key metric, which helps determining the suitability of methods, solvents, and other parameters for a given sample. A recent study examined petai cina leaf samples using three distinct post-harvest processing approaches while maintaining consistent extraction methods, solvents, and conditions. The findings, presented in Table 1, revealed varying extract yield percentages obtained through maceration with 96% ethanol solvent across the three test samples. The research demonstrated a clear pattern in yield outcomes, with fresh petai cina leaves producing the highest with longer fermentation periods leading to more substantial decreases in extract yield. Interestingly, these empirical results present a contradiction to established theoretical frameworks and previous reports, which generally suggest that fermentation enhances the production of nutrients and various chemical compounds in products, typically leading to increased overall yields. Aerobic fermentation significantly influences the nutritional composition and phytochemical content of natural products through various biochemical processes and microbial activities. During fermentation, protein quality experiences notable enhancement yield at 8.02%. This was followed by a modest decline in samples subjected to three days of fermentation, which yielded 7.75%. The most extensive yield reduction was observed in samples fermented for five days, resulting in a 7.45% yield. These results provide compelling evidence that the post-harvest process significantly impacts the final extract yield. These findings align with contemporary research in the field. Recent studies by both Herlina et al. (2024) and Saputri et al. (2024) highlighted a direct colleration between fermentation duration and yield reduction, through partial protein hydrolysis, improving overall digestibility. Microorganisms involved in the fermentation process can synthesize essential amino acids, thereby elevating the protein quality of the fermented product. Research by Leeuwendaal et al., (2022) has demonstrated substantial increases in free amino acid content during the fermentation of food products, contributing to improved nutritional value. In addition, the carbohydrate profile also undergoes significant modifications during aerobic broken down through enzymatic processes, fermentation. Complex carbohydrates are leading to reduced levels of antinutritional

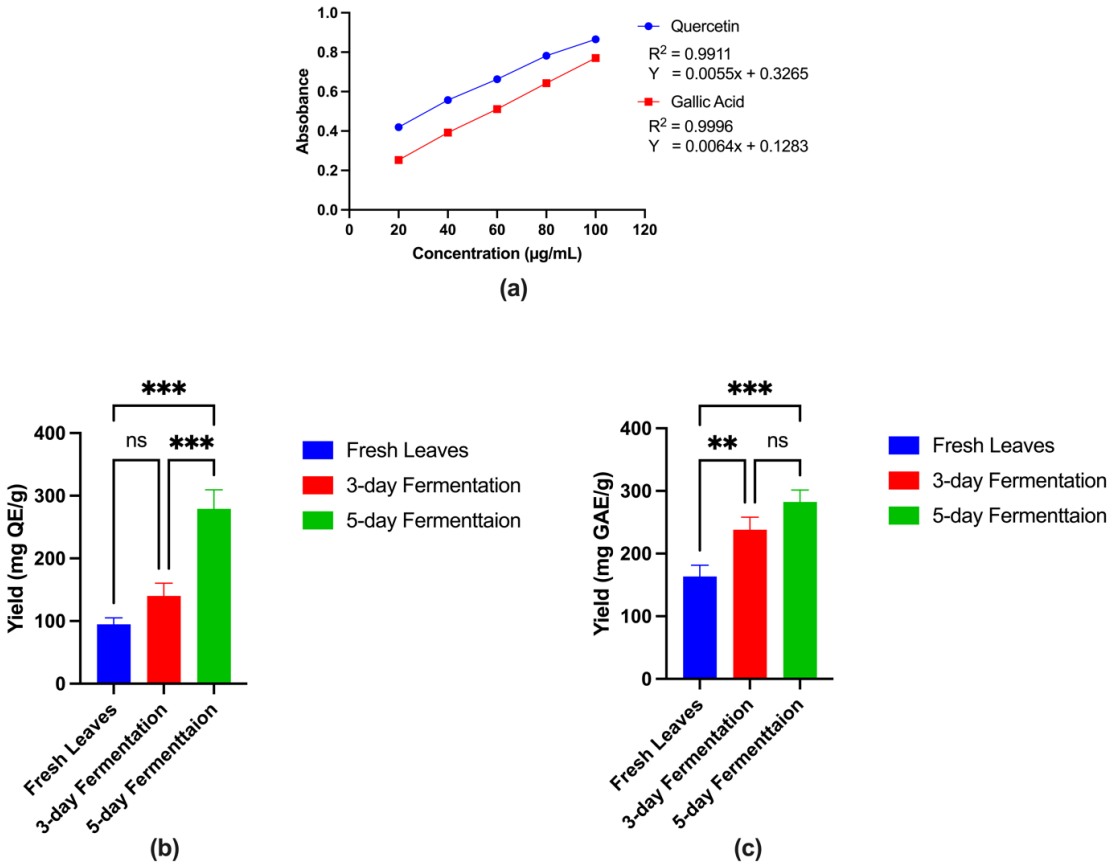

Figure 1. TFC and TPC analysis results of *L. leucocephala* leaf extract from various post-harvest process. (**a**) Standard calibration curve, (**b**) TFC, and (**c**) TPC.

factors such as phytic acid and tannins. This breakdown results in improved starch digestibility and enhanced nutrient bioavailability, as documented by Hur et al. (2014) in their comprehensive study of fermented food products. This discrepancy highlights the complexity of fermentation processes and their effects on extract yields, suggesting the need for further investigation into the specific mechanisms at play.

Effect of Fermentation on TFC and TPC of *L. leucocephala*

Flavonoid and phenols are the main components in *L. leucocephala*, which their bioavailability can be influenced by post-harvest process. In this work, we assessed the effect of post-harvest process, specifically the impact of aerobic fermentation and its duration, on the TFC and TPC of *L. leucocephala* leaves. Figure 1 displays the TFC and TPC of *L. leucocephala* leaf extracts from two distinct fermentation duration in comparison with the untreated *L. leucocephala* as a control. Overall, aerobic fermentation enhanced both the TFC and TPC of *L. leucocephala* leaf extracts, providing the TFC and TPC values higher than the fresh leaf specimen. The TFC of *L. leucocephala* leaf extract, presented in Figu-

re 1b, at three-day fermentation (139.60 mg QE/g) is higher than the fresh leaves (94.84 mg QE/g), and it increased by almost two-fold at five-day fermentation (279.08 mg QE/g). Similarly, the TPC value of three-day fermented sample, depicted in Figure 1c, demonstrated higher value (238.07 mg GAE/g) than the fresh ones (163.59 mg GAE/g). This value slightly elevated to 282.34 mg GAE/g extract at five-day fermentation.

Our research aligns with numerous studies that reported aerobic fermentation as powerful biotechnological approach to enhance bioactive compounds in natural products, particularly phenolics and flavonoids. This process involves the metabolic activities of microorganisms in the presence of oxygen, leading to various biochemical transformations that ultimately increase the bioavailability and concentration of these valuable compounds (Dulf et al., 2016; Martins et al., 2011; Septembre-Malaterre et al., 2018). The enhancement occurs through several interconnected mechanisms that work synergistically to boost the overall phenolic and flavonoid content of natural products. At its core, aerobic fermentation operates through microbial biotransformation, where microbial enzymes break down complex plant cell walls and other structural components. This process releases bound phenolic compounds and make them more accessible. The microorganisms involved, typically including various species of bacteria and fungi, produce enzymes such as cellulases, β-glucosidases, and esterases that cleave chemical bonds holding phenolic compounds in their conjugated forms (Ayar-Sümer et al., 2024; Gulsunoglu-Konuskan & Kilic-Akyilmaz, 2022). Additionally, during fermentation, microorganisms create mild stress conditions that trigger plant cells to produce more secondary metabolites as a defense mechanism. This includes increased synthesis of phenolic compounds and flavonoids. The oxidative stress during fermentation particularly stimulates the phenylpropanoid pathway, which is responsible for flavonoid biosynthesis (Leonard et al., 2021; Yang et al., 2023).

The enhancement of phenolic and flavonoid content through aerobic fermentation involves several specific mechanisms. One of the primary processes is enzymatic deglycosylation, where β-glucosidase activity converts glycosidic forms of phenolics and flavonoids into their respective aglycones (Yan et al., 2016). Research by He et al., (2022) demonstrated that fermentation of soybeans with Aspergillus oryzae increased isoflavone aglycone content by up to 71.3-fold compared to unfermented samples. Furthermore, fermentation enables the

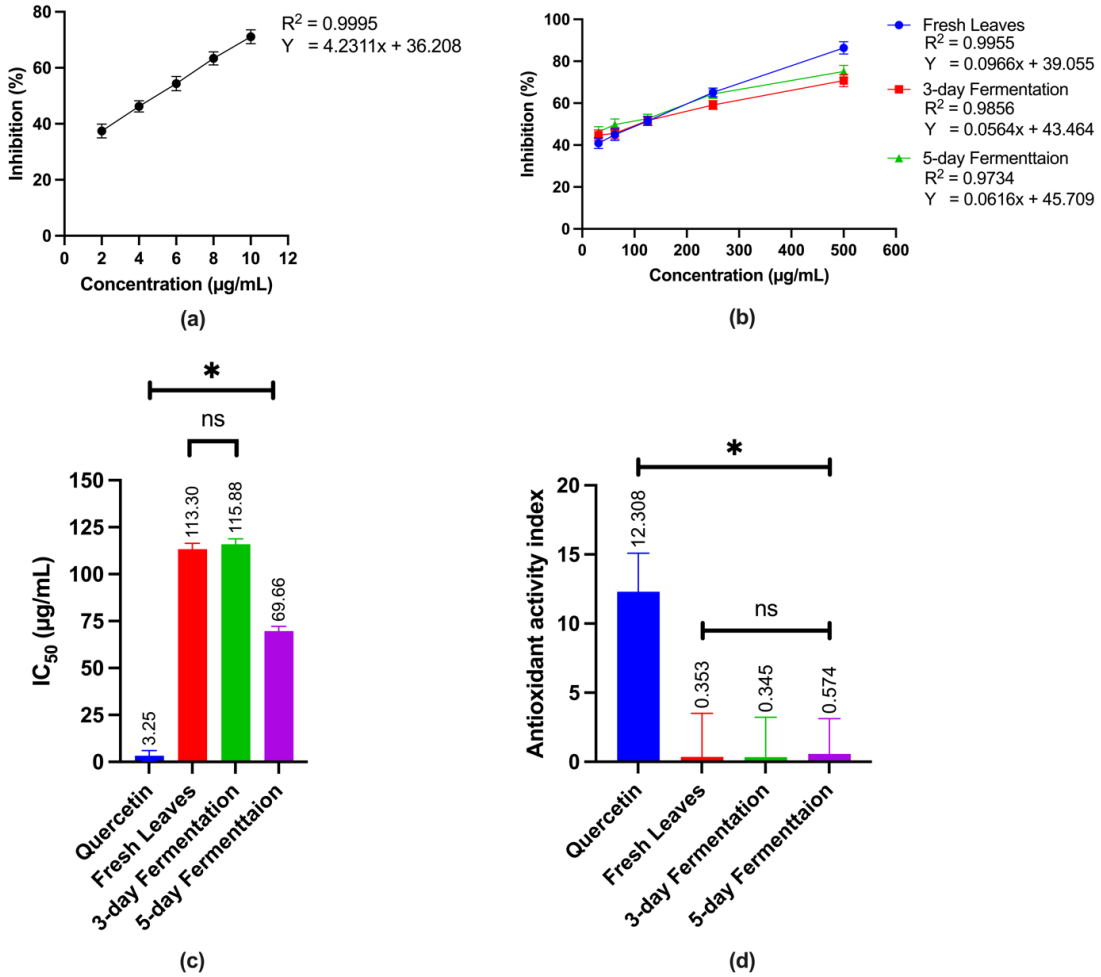

Figure 2. Antioxidant activity of *L. leucocephala* from various post-harvest process in comparison with Quercetin. (a) Standard calibration curve of Quercetin, (b) Standard calibration curve of *L. leucocephala*, (c) IC_{50} values, and (d) Antioxidant activity index values.

release of bound phenolics through the action of carbohydrate-cleaving enzymes and esterases (Saharan et al., 2020; Zhang et al., 2025). These enzymes break down cell wall polysaccharides and ester bonds, liberating previously unavailable phenolic compounds. Studies have shown that this process can increase total phenolic content by 24.56% to 254.93% depending on the substrate and fermentation conditions (Zhang et al., 2025). Another significant mechanism is the de novo synthesis of phenolic compounds during aerobic fermentation. Certain microorganisms can synthesize new phenolic compounds through various metabolic pathways (Gaur & Gänzle, 2023; Huynh et al., 2014). The pH changes during fermentation, typically becoming more acidic, can also enhance the extraction and solubilization of phenolic compounds from plant matrices. This effect is particularly pronounced for flavonoids, which show improved stability and, extra ction efficiency at lower pH values (Adebo et al. 2021; Friedman & Jürgens 2000; Mikulajová et al., 2024; Zhang et al., 2025). The impact of aerobic fermentation on spesific compound classes is particularly

noteworthy. For flavonoids, the process has shown remarkable effectiveness in increasing various subclasses. This includes the conversion of isoflavone glycosides to aglycones, enhanced stability and extraction of anthocyanins, and improved bioavailability of flavanols through structural modifications. Similarly, for phenolic acids, the process increases both free and bound forms through hydrolysis of ester linkages, decarboxylation reactions, and release from complex polyphenol structures. These transformations result in a more diverse and bioavailable profile of phenolic compounds in the fermented product.

Effect of Fermentation on Antioxidant Activity Index of *L. leucocephala*

Antioxidant activity *of L. leucocephala* has been widely reported, yet the impact of fermentation on its antioxidant has never been studied. In the present study, antioxi dant activity of *L. leucocephala* from all samples were tested on DPPH by observing their free-radical scavenging activity. Figure 2 summarizes the antioxidant capacity of three different samples of *L. leucocephala* illustrated with IC_{50} values with quercetin as a control. It can be seen that aerobic fermentation enhanced the overall antioxidant activity of *L. leucocephala*, yet the activity was not comparable to quercetin. Based on the antioxidant activity analysis presented in Figure 2, quercetin demonstrated exceptional antioxidant properties with an IC_{50} value of 3.25 µg/mL, serving as a highly effective positive control. This remarkably low IC_{50} value demonstrates quercetin's capacity to neutralize 50% of free radicals at minimal concentrations, confirming its status as a potent antioxidant compound.

Fresh *L. leucocephala* samples exhibited moderate antioxidant activity with an IC_{50} value of 113.3 µg/mL. While not as potent as quercetin, the fresh samples displayed notable free radical scavenging capacity, likely attributed to their endogenous phenolic compounds and flavonoids. The 3-day fermentation process resulted in a marginal increase in IC_{50} to 115.88 µg/mL, maintaining the moderate antioxidant classification. This slight decrease in antioxidant efficacy compared to fresh samples suggests that the short fermentation period was insufficient to enhance the bioavailability of antioxidant compounds. The brief fermentation duration may not have adequately facilitated the biotransformation of complex compounds into more bioactive derivatives. In contrast, extending the fermentation period to 5 days yielded significantly improved results, with an IC_{50} value of 69.66 µg/mL, elevating the antioxidant activity to the strong category. This marked enhancement suggests that prolonged fermentation effectively promo-

tes the enzymatic breakdown of complex molecules into more potent, low-molecular-weight antioxidant compounds with superior free radical scavenging capabilities. Nevertheless, based on the AAI values shown in Figure 2d, the fermentation process did not demonstrate a significant impact on the antioxidant index of *L. leucocephala*. The AAI values of all fermented samples (day-3 and day-5) were not statistically different (p-value >0.05) from those of fresh leaf samples.

CONCLUSION

The experimental findings demonstrate that aerobic fermentation significantly influences the bioactive compound profile, yet unsignificant antioxidant properties of the samples. The 5-day fermentation period yielded optimal results across all measured parameters. Specifically, this duration pro duced the highest total flavonoid content (279.08 mg QE/g) and total phenolic content (282.34 mg QE/g). Additionally, the 5-day fermented sample exhibited superior antioxidant potential, as evidenced by the lowest IC_{50} value (69.66 µg/mL) among all tested samples. These results collectively suggest that a 5-day aerobic fermentation period represents the optimal duration for enhancing both the concentration of beneficial compounds and their associated antioxidant activity.

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