

Comparative Antioxidant Activity of *Leucaena leucocephala* Leaf Extract and Fractions Using CUPRAC and FRAP Assays

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ABSTRACT

Leucaena leucocephala is widely recognized as a medicinal and nutritional plant due to its abundance of bioactive phytochemicals, particularly phenolics and flavonoids, which play a major role in its antioxidant properties. This study aimed to compare the antioxidant activity of extract and fractions of *L. leucocephala* leaves. The ethanol extraction was performed using the Ultrasound-Assisted Extraction (UAE) method, followed by fractionation with water, ethyl acetate and *n*-hexane. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) were determined using the Folin-Ciocalteu and aluminium chloride colorimetric methods, respectively. Their antioxidant activity was tested using CUPRAC and FRAP assays, along with method validation (accuracy, precision, linearity, limit of detection (LoD), and limit of quantification (LoQ)). The aqueous fraction exhibited the highest percentage yield ($66.85 \pm 0.17\%$). The ethyl acetate fraction exhibited the highest TPC (845.3 ± 1.1 mg GAE/g sample) and TFC (87.23 ± 0.15 mg QE/g sample), followed by ethanol extract, aqueous, and *n*-hexane fractions. The ethyl acetate fraction showed the strongest antioxidant activity using both assays, with EC₅₀ values of 31.76 µg/mL

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(CUPRAC) and 22.93 $\mu\text{g/mL}$ (FRAP), while the *n*-hexane fraction demonstrated the weakest antioxidant activity. Method validation indicated that both CUPRAC and FRAP assays showed acceptable precision and linearity, while only the CUPRAC assay fulfilled all validation requirements. Overall, the CUPRAC assay demonstrated better performance than the FRAP assay. These results confirm the strong antioxidant potential of *L. leucocephala* and emphasize the importance of solvent fractionation in enhancing the recovery of bioactive compounds. The findings support the potential use of *L. leucocephala* as a natural antioxidant, with promising applications in pharmaceutical formulations and nutraceutical product development.

INTRODUCTION

Oxidative stress arises when the production of reactive oxygen species (ROS) exceeds the body's antioxidant defense system's capacity to neutralize them. This imbalance may cause cellular and molecular damage and has been implicated in the progression of numerous chronic diseases, such as cardiovascular disease, diabetes mellitus, cancer, and neurodegenerative disorders [1]. Therefore, the exploration of natural antioxidants derived from plant sources has attracted increasing attention due to their safety, bioavailability, and multifunctional bioactivities [2].

Leucaena leucocephala (Lam.) is a tropical leguminous plant widely distributed in Southeast Asia. Traditionally, its leaves and seeds have been utilized as animal feed and herbal medicine. Recent phytochemical studies have revealed that *L. leucocephala* leaves contain abundant phenolic compounds, flavonoids, tannins, and bioactive peptides, which exhibit strong antioxidant, anti-inflammatory, and antimicrobial activities [3]. These findings suggest that *L. leucocephala* leaves possess strong potential as a natural source of antioxidant compounds.

Various in vitro assays are commonly employed to evaluate antioxidant activity, including DPPH, ABTS, CUPRAC, and FRAP. Among these methods, CUPRAC and FRAP are widely preferred because of their simplicity, sensitivity, cost-effectiveness, and reproducibility. The CUPRAC assay measures the ability of antioxidants to reduce the Cu(II)–neocuproine complex into the Cu(I)–neocuproine complex, whereas the FRAP assay is based on the reduction of the ferric–tripyridyltriazine (Fe^{3+} –TPTZ) complex into the ferrous form (Fe^{2+} –TPTZ) under acidic conditions [1,4]. Although both methods evaluate reducing power, they differ in reaction mechanisms, metal ion systems, and sensitivity toward different classes of antioxidant compounds. Therefore, the combined use of CUPRAC and FRAP assays may provide a more comprehensive evaluation of antioxidant capacity.

In addition, solvent fractionation is an important approach for separating phytochemicals according to their polarity, thereby enabling the enrichment and identification of antioxidant-active constituents. Semi-polar solvents such as ethyl acetate have been reported to efficiently extract polyphenolic compounds with high antioxidant potential [4]. Nevertheless, previous studies on *L. leucocephala* have mainly focused on crude extracts or employed only a single antioxidant assay, resulting in limited comparative information regarding the antioxidant behavior of different solvent fractions across multiple assay systems. Furthermore, the relationship between solvent polarity and antioxidant performance in CUPRAC and FRAP assays has not been thoroughly investigated for *L. leucocephala* leaf fractions.

Therefore, this study aimed to comparatively evaluate the antioxidant activities of different extract and fractions of *L. leucocephala* leaf using CUPRAC and FRAP assays. The novelty of this study lies in the comparative assessment of fractionated extracts using two distinct electron transfer-based antioxidant assays, namely CUPRAC and FRAP to determine the extract and fractions with the highest antioxidant activity and to provide a broader understanding of the antioxidant characteristics of *L. leucocephala* leaves.

EXPERIMENTAL

Plant material and extraction

The leaves of *L. leucocephala* were harvested from trees growing at Unit Ladang, Universiti Teknologi MARA (UiTM) Cawangan Pahang, Kampus Jengka, Pahang, Malaysia. A voucher specimen was deposited at the Herbarium of UiTM Cawangan Pahang, Kampus Jengka. The fresh leaves of *L. leucocephala* were washed, dried at 60 °C for two days and ground using a grinder mill to obtain an average particle size of 0.5 mm and stored in airtight containers. The dried powder was extracted using the Ultrasound-Assisted Extraction (UAE) method with 70% ethanol as the solvent at a solvent-to-sample ratio of 10:1 (mL/g). The extraction mixture was immersed for 72 hours and subjected to ultrasonic treatment at 40 kHz frequency and 200 W power at 30 °C to enhance solvent penetration and facilitate the release of bioactive compounds from the plant matrix. The resulting filtrate was concentrated under reduced pressure using a rotary evaporator to obtain the crude extract [3].

Fractionation

The crude extract was initially dissolved in distilled water and subjected to sequential liquid–liquid partitioning using *n*-hexane and ethyl acetate. The resulting aqueous, ethyl acetate, and *n*-hexane fractions were concentrated under reduced pressure using an evaporator and stored at 4 °C until further analysis [4].

Determination of Total Phenolic Content (TPC)

TPC was determined using the Folin–Ciocalteu method with gallic acid as the standard, prepared in 70% ethanol. A calibration curve was constructed using gallic acid standards (0–500 µg/mL). For analysis, each sample concentration (1.0 mL) was transferred into a test tube, followed by the addition of 0.2 mL Folin–Ciocalteu reagent. The mixture was vortexed and allowed to stand for 3 minutes. Then, 0.2 mL of 7.5% (w/v) sodium carbonate (Na₂CO₃) solution was added, and the final volume was adjusted with distilled water. The reaction mixture was incubated at room temperature in the dark for 30 minutes. Absorbance was measured at 765 nm using a UV–Vis spectrophotometer. All measurements were performed in triplicate (n = 3) [5]. TPC was quantified from the gallic acid calibration curve ($y = mx + c$) and expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE/g sample), using the equation:

$$\text{TPC} = (C \times V) / m$$

where C is the concentration obtained from the calibration curve (mg/mL), V is the extract volume (mL), and m is the mass of the sample (g).

Determination of Total Flavonoid Content (TFC)

TFC was determined using the aluminium chloride colorimetric method with quercetin as the reference standard. A quercetin calibration curve was prepared using a series of standard concentrations (0–500 µg/mL). For analysis, 0.5 mL of sample or series concentration of standard solution was mixed with 0.10 mL of 10% (w/v) AlCl₃ solution, followed by the addition of 0.10 mL of 1 M sodium acetate and 2.8 mL of distilled water. The reaction mixture was incubated at room temperature for 30 minutes to allow complete complex formation. Absorbance was measured at 425 nm using a UV–Vis spectrophotometer. All measurements were performed in triplicate (n = 3). TFC was quantified from the quercetin calibration curve ($y = mx + c$) and expressed as milligrams of quercetin equivalents per gram of sample (mg QE/g sample), using the equation:

$$\text{TFC} = (C \times V) / m$$

where C is the concentration obtained from the calibration curve (mg/mL), V is the extract volume (mL), and m is the mass of the sample (g).

Determination of antioxidant activity

CUPRAC Assay

The CUPRAC assay was carried out according to the procedure outlined in the referenced method [6] with slight modifications. The reaction mixture consisted of 1.0 mL of 10 mM CuCl₂ solution, 1.0 mL of 7.5 mM neocuproine solution, and 1.0 mL of 1 M ammonium acetate buffer (pH 7.0). To this mixture, 1.0 mL of sample or standard solution was added and the final volume was adjusted with distilled water. The mixture was incubated at room temperature for 30 minutes to allow the formation of the Cu(I)–neocuproine complex. Absorbance was measured at 450 nm using a UV–Vis spectrophotometer. All measurements were performed in triplicate (n = 3). Trolox was used as the reference standard, and a calibration curve was prepared using a series of concentrations (0–200 µM Trolox). Antioxidant capacity was expressed as Trolox equivalent antioxidant capacity (TEAC) using the calibration curve ($y = mx + c$). The TEAC value was calculated using the equation:

$$\text{TEAC } (\mu\text{mol TE/g}) = (C \times V) / m$$

where C is the Trolox equivalent concentration obtained from the calibration curve (µmol/L), V is the extract volume (L), and m is the mass of the sample (g).

For consistency with dose–response analysis, percentage reduction activity was also calculated from absorbance values, and an effective concentration (EC₅₀) value was determined from the concentration corresponding to 50% reduction activity.

FRAP Assay

The FRAP assay was conducted according to the procedure established by Benzie and Strain, with modifications as described by [7]. The FRAP working reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM FeCl₃·6H₂O in a ratio of 10:1:1 (v/v/v), respectively. The reagent was incubated at 37 °C for 10 minutes prior to use. For analysis, 1.0 mL of sample or standard solution was mixed with 3.0 mL of FRAP reagent and incubated at 37 °C for 30 minutes in the dark to allow complete reduction of Fe³⁺–TPTZ to Fe²⁺–TPTZ complex. Absorbance was measured at 593 nm using a UV–Vis spectrophotometer. All measurements were performed in triplicate (n = 3). Trolox was used as the reference standard, and a calibration curve was constructed using a series of concentrations (0–200 µM Trolox). Antioxidant capacity was expressed as Trolox equivalent antioxidant capacity (TEAC) based on the calibration curve ($y = mx + c$). The TEAC value was calculated using the equation:

$$\text{TEAC } (\mu\text{mol TE/g}) = (C \times V) / m$$

where C is the Trolox equivalent concentration obtained from the calibration curve (µmol/L), V is the extract volume (L), and m is the mass of the sample (g).

For consistency with dose–response analysis, percentage reduction activity was also calculated from absorbance values, and an effective concentration (EC₅₀) value was determined from the concentration corresponding to 50% reduction activity.

Validation method

Method validation was performed to ensure the reliability and consistency of the CUPRAC and FRAP assays for antioxidant activity evaluation of *L. leucocephala* extract and fractions. Although these assays are mainly used for antioxidant screening, validation was carried out to confirm the reproducibility and linear response of the analytical measurements. Accuracy was evaluated through recovery analysis using Trolox standard solutions, calculated as:

$$\% \text{Recovery} = (C_{\text{found}} / C_{\text{added}}) \times 100$$

where *C_{found}* is the measured concentration after spiking and *C_{added}* is the known added concentration.

Precision was expressed as relative standard deviation (%RSD), where $\%RSD \leq 2\%$ was considered acceptable. Linearity was assessed using calibration curves constructed from Trolox standards and expressed as the coefficient of determination (R^2), with $R^2 \geq 0.99$ indicating good linearity. The limits of detection (LoD) and quantification (LoQ) were determined from the calibration curve using the standard deviation of the response and slope, following $LoD = (3.3\sigma)/S$ and $LoQ = (10\sigma)/S$. These parameters define the lowest detectable and quantifiable concentrations under the analytical conditions.

Statistical analysis

Experimental procedures were conducted in three replicates. Results are expressed as mean values accompanied by standard deviations (SD). Statistical evaluation was performed through one-way ANOVA, followed by Tukey's multiple comparison test, where significance was set at < 0.05 .

RESULTS AND DISCUSSION

The extraction and fractionation processes resulted in different percentage yields for the crude extract and each solvent fraction. The percentage yield reflects the efficiency of solvent extraction and the distribution of phytochemical constituents according to their polarity. Variations in yield may be influenced by the solvent polarity, extraction method, and the solubility of bioactive compounds present in the sample. The percentage yield of the *L. leucocephala* extract and fraction samples is presented in Table 1.

Table 1. Percentage yield of *L. leucocephala* extract and fraction samples

Sample	Percentage (%)
Ethanol Extract	17.13 ± 0.32^c
Aqueous Fraction	66.85 ± 0.17^a
Ethyl acetate Fraction	22.31 ± 0.00^b
<i>n</i> -Hexane Fraction	9.68 ± 0.10^d

Different letters (a–d) indicate significant differences between samples at $p < 0.05$.

Table 1 presents the percentage yield of the *L. leucocephala* crude extract and its respective fractions obtained following the extraction and fractionation processes. The aqueous fraction exhibited the highest percentage yield ($66.85 \pm 0.17\%$), indicating that most compounds present in the sample were highly polar and readily soluble in water. This suggests the abundance of polar phytochemicals such as phenolic acids, glycosides, sugars, and other hydrophilic constituents. The ethyl acetate fraction showed a moderate yield ($22.31 \pm 0.00\%$), suggesting the presence of semi-polar compounds, including certain flavonoids and phenolic constituents, which are more soluble in ethyl acetate. The ethanol extract produced a yield of $17.13 \pm 0.32\%$, demonstrating the effectiveness of ethanol in extracting a broad spectrum of phytochemicals with varying polarities. As a polar solvent, ethanol is widely recognized for its ability to extract bioactive compounds such as phenolics, flavonoids, and other secondary metabolites. In contrast, the *n*-hexane fraction recorded the lowest yield ($9.68 \pm 0.10\%$), indicating that only a small proportion of non-polar compounds, such as lipids, waxes, chlorophylls, and non-polar terpenoids were present in the sample. The variations in extraction yield among the fractions may be attributed to differences in solvent polarity and the solubility characteristics of the phytochemical constituents. Generally, polar solvents tend to extract higher amounts of polar compounds, resulting in greater extraction yields, whereas non-polar solvents selectively extract compounds with limited solubility [8]. Overall, the obtained results demonstrate the effectiveness of the extraction and fractionation procedures in separating phytochemical constituents according to their polarity.

The principle of TPC involves a redox reaction between phenolic constituents and the Folin–Ciocalteu reagent, resulting in the formation of a blue chromophore. The operating time for the reaction between gallic acid and the Folin–Ciocalteu reagent was determined to be 40 minutes. The present result corresponds to the observations documented by [9] in their study, who reported that the reaction reached stability within 21–39 minutes. TPC values were derived from the calibration curve of gallic acid, expressed as $y = 0.0615x - 0.0852$, yielding a coefficient of determination (R^2) of 0.992. A value closer to 1 reflects a stronger correlation and a better linear relationship between the variables. Table 2 shows the TPC in *L. leucocephala* extract and fraction samples.

Table 2. Total phenolic content in *L. leucocephala* extract and fraction samples

Sample	Total Phenolic Content (mg GAE/g sample)
Ethanol Extract	796.40 ± 1.20^b
Aqueous Fraction	655.20 ± 1.10^c
Ethyl acetate Fraction	845.30 ± 1.10^a
<i>n</i> -Hexane Fraction	480.10 ± 1.50^d

Different letters (a–d) indicate significant differences between samples at $p < 0.05$.

The table presents the TPC of different extract and fraction samples, expressed as mg GAE/g sample. Phenolic compounds are important plant metabolites known for their antioxidant properties; therefore, higher TPC values generally indicate greater potential antioxidant activity. Among all samples, the ethyl acetate fraction showed the highest total phenolic content with a value of 845.30 ± 1.10 mg GAE/g sample. This suggests that ethyl acetate was the most effective solvent for extracting phenolic compounds from the sample. The ethanol extract recorded the second highest phenolic content at 796.40 ± 1.20 mg GAE/g sample, followed by the aqueous fraction with 655.20 ± 1.10 mg GAE/g sample. The *n*-hexane fraction exhibited the lowest phenolic content at 480.10 ± 1.50 mg GAE/g sample, indicating that non-polar solvents such as *n*-hexane were less efficient in extracting phenolic compounds. The different superscript letters (a–d) indicate that the values are significantly different ($p < 0.05$) from one another. These findings indicate variations in TPC distribution across the different extract and solvent fractions, likely influenced by the polarity of the extraction solvents and the solubility properties of phenolic compounds [10]. Overall, the results demonstrate that solvent polarity strongly influenced the extraction of phenolic constituents, with moderately polar solvents such as ethyl acetate yielding the highest phenolic recovery.

TFC was determined using the aluminium chloride colorimetric technique. In this assay, $AlCl_3$ reacts with the ortho-dihydroxyl groups present on ring A or ring B of the flavonoid structure to form a labile acid complex, which produces a more intense yellow coloration. The addition of CH_3COONa serves to maintain the measurement within the visible wavelength region and to enhance the stability of the formed complex. The maximum absorption wavelength of quercetin was determined using a UV–Vis spectrophotometer within the range of 370–450 nm [11]. In this study, the peak wavelength was identified at 425 nm, with an absorbance of 0.664. This finding is consistent with previous studies. The TFC was calculated using the calibration curve equation $y = 0.0068x + 0.1198$, with a determination coefficient (R^2) of 0.9949. The correlation coefficient (r) reflects the strength of the linear relationship between the variables; values approaching 1 indicate a stronger correlation. Table 3 shows the TFC in *L. leucocephala* extract and fraction samples.

Table 3. Total flavonoid content in *L. leucocephala* extract and fraction samples

Sample	Total Flavonoid Content (mg QE/g sample)
Ethanol Extract	55.17 ± 0.41 ^b
Aqueous Fraction	33.27 ± 0.25 ^c
Ethyl acetate Fraction	87.23 ± 0.15 ^a
<i>n</i> -Hexane Fraction	21.17 ± 0.22 ^d

Different letters (a–d) indicate significant differences between samples at $p < 0.05$.

The table shows the TFC of different extract and fraction samples, expressed as mg QE/g sample. Flavonoids are a major group of phenolic compounds that contribute significantly to antioxidant activity and other biological properties. Among the tested samples, the ethyl acetate fraction exhibited the highest TFC with a value of 87.23 ± 0.15 mg QE/g sample. This result suggests that ethyl acetate was the most effective solvent for extracting flavonoid compounds from the sample. The ethanol extract showed the second highest TFC at 55.17 ± 0.41 mg QE/g sample, followed by the aqueous fraction with 33.27 ± 0.25 mg QE/g sample. The *n*-hexane fraction recorded the lowest TFC at 21.17 ± 0.22 mg QE/g sample, indicating that non-polar solvents such as *n*-hexane were less suitable for flavonoid extraction. The superscript letters (a–d) indicate that all values are significantly different at $p < 0.05$. This demonstrates that the choice of solvent significantly affected the flavonoid yield. These findings indicate variations in flavonoid distribution across the different solvent fractions, which may be attributed to differences in solvent polarity and the solubility properties of flavonoids. Similar trends have been reported in previous studies. [12] reported that the TFC in the ethanol extract was the highest TFC, followed by ethyl acetate, aqueous, and *n*-hexane fractions. Overall, the findings indicate that moderately polar solvents, particularly ethyl acetate, were more efficient in extracting flavonoid compounds compared to polar aqueous solvents or non-polar *n*-hexane solvents.

Antioxidant activity was evaluated using the CUPRAC and FRAP assays. The antioxidant activity was classified based on values as follows: very strong: $EC_{50} < 50$ µg/mL, strong: $50 \text{ µg/mL} \leq EC_{50} < 100$ µg/mL, moderate: $100 \text{ µg/mL} \leq EC_{50} < 150$ µg/mL, weak: $150 \text{ µg/mL} \leq EC_{50} < 200$ µg/mL, and very weak: $EC_{50} > 200$ µg/mL. A lower EC_{50} value indicates higher antioxidant activity [13]. The antioxidant activity of *L. leucocephala* extract and fraction samples using CUPRAC and FRAP assays is presented in Table 4.

Table 4. Antioxidant activity of *L. leucocephala* extract and fraction samples using the CUPRAC and FRAP assays

Sample	EC_{50} (µg/mL)	EC_{50} (µg/mL)
Quercetin	16.28 ± 1.85 ^a	8.36 ± 0.18 ^a
Ethanol Extract	63.55 ± 1.23 ^c	29.15 ± 0.40 ^c
Aqueous Fraction	75.53 ± 0.73 ^d	39.26 ± 0.12 ^d
Ethyl acetate Fraction	31.76 ± 0.45 ^b	22.93 ± 0.15 ^b
<i>n</i> -Hexane Fraction	105.54 ± 1.28 ^e	88.20 ± 0.20 ^e

Different letters (a–d) indicate significant differences between samples at $p < 0.05$.

For CUPRAC assay, the maximum absorption wavelength was determined by UV–Vis spectrophotometry (400–600 nm), with quercetin showing a peak at 450 nm (absorbance 0.666). This result is consistent with previous studies, although slight differences may occur due to experimental conditions. The optimal reaction time for quercetin with the CUPRAC reagent was 53–55 minutes. Absorbance of the extracts, fractions, and quercetin (as a reference) was measured at 450 nm. The Table 4 presents the antioxidant activity of different extract and fraction samples determined using the CUPRAC assay, expressed as EC_{50} values ($\mu\text{g/mL}$). Based on the classification criteria, the reference compound quercetin exhibited an EC_{50} value of $16.28 \pm 1.85 \mu\text{g/mL}$, which falls under the very strong antioxidant activity category. Similarly, the ethyl acetate fraction showed an EC_{50} value of $31.76 \pm 0.45 \mu\text{g/mL}$, also indicating very strong antioxidant activity. This suggests that the ethyl acetate fraction contains a high concentration of antioxidant compounds, likely due to its higher phenolic and flavonoid contents. The ethanol extract recorded an EC_{50} value of $63.55 \pm 1.23 \mu\text{g/mL}$, while the aqueous fraction showed a value of $75.53 \pm 0.73 \mu\text{g/mL}$. Both samples were categorized as having strong antioxidant activity, as their EC_{50} values ranged between 50 and $100 \mu\text{g/mL}$. Although their antioxidant capacities were lower than the ethyl acetate fraction, they still demonstrated good radical scavenging potential. In contrast, the n-hexane fraction exhibited the highest EC_{50} value of $105.54 \pm 1.28 \mu\text{g/mL}$, which corresponds to moderate antioxidant activity. The weaker activity of the n-hexane fraction may be attributed to its lower phenolic and flavonoid contents, since non-polar solvents are generally less effective in extracting antioxidant compounds. The superscript letters (a–e) indicate that all values were significantly different ($p < 0.05$) from one another. This suggests that polar constituents, particularly phenolics and flavonoids, play a major role in contributing to the antioxidant activity of *L. leucocephala* leaves. These findings are consistent with those reported by [14], who found that ethyl acetate fractions of medicinal plants tend to exhibit higher CUPRAC values due to their enriched polyphenolic content. The pronounced reducing power of the ethyl acetate fraction highlights its effectiveness in donating electrons to neutralize free radicals. Overall, the results suggest that the antioxidant activity measured by the CUPRAC assay is closely related to the phenolic and flavonoid contents of the extracts, with the ethyl acetate fraction showing the most promising antioxidant potential among the tested samples.

For FRAP assay, the maximum wavelength of quercetin obtained in this study was 593 nm, with an absorbance value of 0.666. The reaction time between quercetin and the FRAP reagents was determined to be 44 minutes. In this study, quercetin was selected as the reference standard due to its role as a secondary antioxidant, capable of scavenging free radicals and inhibiting chain reactions [12]. Among all samples, the reference standard, quercetin, exhibited the lowest EC_{50} value of $8.36 \pm 0.18 \mu\text{g/mL}$, indicating the strongest antioxidant activity and classifying it as a very strong antioxidant. Similarly, the ethyl acetate fraction showed a low EC_{50} value of $22.93 \pm 0.15 \mu\text{g/mL}$, demonstrating very strong antioxidant activity among the tested fractions. The ethanol extract also exhibited very strong antioxidant potential with an EC_{50} value of $29.15 \pm 0.40 \mu\text{g/mL}$, followed by the aqueous fraction with $39.26 \pm 0.12 \mu\text{g/mL}$, which was likewise categorized as very strong. In contrast, the n-hexane fraction recorded the highest EC_{50} value of $88.20 \pm 0.20 \mu\text{g/mL}$ indicating weaker antioxidant activity compared to the other samples. However, based on the classification scale, this fraction was categorized as having strong antioxidant activity. The superscript letters (a–e) indicate that all samples were significantly different from each other at $p < 0.05$. Overall, the results suggest that the ethyl acetate fraction possessed the highest antioxidant capacity among the extracts and fractions tested, which may be associated with its higher phenolic and flavonoid contents. Conversely, the lower antioxidant activity observed in the n-hexane fraction may be due to its lower concentration of polar antioxidant compounds. This pattern suggests a strong correlation between phenolic content and reducing power, as phenolic-rich extracts exhibit higher FRAP values, reflecting their effective electron-donating activity. Moreover, [15] highlighted that polyphenolic compounds and antioxidant peptides play a critical role in metal ion reduction mechanisms.

Emphasized that hydroxyl (-OH) groups play a critical role in the stabilization of free radicals. The antioxidant activity of polyphenols and flavonoids is largely determined by the number and position of their hydroxyl groups, with greater substitution enhancing their ability to donate electrons or hydrogen atoms. The strong antioxidant activity observed in the 70% ethanol extract, the ethyl acetate fraction, and the aqueous fraction is likely due to the presence of hydroxyl-rich compounds. Due to their hydrophilic nature, these compounds are more efficiently extracted by polar solvents, resulting in enrichment of phenolics and flavonoids in polar and semi-polar fractions. Conversely, non-polar constituents such as triterpenoids and steroids exhibit comparatively lower antioxidant activity, primarily due to their limited hydroxyl group content. Although these compounds can act as primary antioxidants by interrupting radical chain reactions, their effectiveness is generally reduced. The moderate antioxidant activity of the *n*-hexane fraction may be associated with lipophilic compounds containing long hydrocarbon chains, which can diminish the hydrogen-donating ability of hydroxyl groups. Additionally, interactions or binding with other constituents may hinder electron transfer. The inherently low number of hydroxyl groups in triterpenoids further limits their radical-scavenging activity. Previous studies have shown that *L. leucocephala* leaves are rich in phenolics, flavonoids, and tannins [3], which function as primary antioxidants via hydrogen atom transfer and single-electron transfer mechanisms [6]. The higher antioxidant activity observed in the ethyl acetate fraction suggests that fractionation enhances the selective isolation of bioactive phytochemicals. Similar findings have been reported in other medicinal plants, where semi-polar fractions consistently exhibit superior antioxidant activity [16].

Method validation is a reconfirmation process that ensures an analytical method meets the required standards. The results of method verification can also be used to assess the quality, reliability, and consistency of analytical outcomes. Table 5 shows validation of the CUPRAC and FRAP assays.

Table 5. Validation of the CUPRAC and FRAP Assays

Parameter	Acceptance Range	CUPRAC Assay	FRAP Assay
Accuracy	98% - 102%	100.12%	93.94%
Precision	≤ 2%	1.93%	0.81%
Linearity	$R^2 \geq 0.99$	$= 0.0021x + 0.4694$ $R^2 = 0.99$	$y = 0.0112x + 0.2438$ $R^2 = 0.99$
LoD	-	2.22	2.30
LoQ	-	7.39	7.77

The accuracy results showed that the CUPRAC assay had a recovery of 100.12%, while the FRAP assay had a recovery of 93.94%. The acceptable range for percentage recovery (% Recovery) is 98–102% [17]. Therefore, the CUPRAC assay met the accuracy requirements, whereas the FRAP assay did not meet the required accuracy criteria. This result indicates that the antioxidant activity analysis using the CUPRAC assay for the *L. leucocephala* extract and fractions provides accurate results. In this study, precision was evaluated using Relative Standard Deviation (%RSD). The CUPRAC assay produced a %RSD of 1.93%, while the FRAP assay showed a %RSD of 0.81%. These values comply with the established requirement for good repeatability, which stipulates that %RSD should not exceed 2%. The observed deviations remained within the acceptable range, indicating that the analytical methods used exhibit good repeatability. Furthermore, a lower %RSD value indicates higher analytical precision and suggests that the method is more suitable for the analysis of chemical compounds. The linearity parameter used in this study was the correlation coefficient (R) obtained from linear regression analysis using the equation $y = bx + a$ [17]. The CUPRAC assay produced a regression equation of $y = 0.0021x + 0.4694$ with $R^2 = 0.99$, while the FRAP assay produced $y = 0.0112x + 0.2438$ with $R^2 = 0.99$. A method is considered linear when the correlation

coefficient (R) lies between 0.9 and 1.0. Therefore, both the CUPRAC and FRAP assays met the linearity criteria.

The LoD represents the lowest concentration of an analyte that can be detected, although it may not be quantified with high precision. In this study, the LoD values were 2.22 µg/mL for the CUPRAC assay and 2.30 µg/mL for the FRAP assay, indicating that the analyte can still be detected at these concentrations. However, these values are not suitable for quantitative calculations as they may introduce bias into the analysis. The LoQ refers to the lowest concentration of an analyte that can be measured with acceptable precision and accuracy. In this study, the LoQ values were 7.39 µg/mL for the CUPRAC assay and 7.77 µg/mL for the FRAP assay, representing the minimum concentrations that can be reliably quantified. Analysis of TPC and TFC revealed that the ethyl acetate fraction exhibited the highest values among all tested fractions. These findings are consistent with the antioxidant activity results obtained from the CUPRAC and FRAP assays, which indicated that the ethyl acetate fraction possessed very strong antioxidant activity. Correlation analysis was performed to evaluate the relationship between TFC, TPC, and antioxidant activity in the ethyl acetate fraction. The Spearman correlation test was conducted using IBM SPSS version 25, as the dataset comprised non-parametric variables (TFC and TPC) and ranked antioxidant activity values (CUPRAC and FRAP assays). The results showed a correlation coefficient of -1.000 with a significance value of 0.000. Since the p-value was less than 0.05, the results indicate a statistically significant association between TPC, TFC, and antioxidant activity. A correlation coefficient between 0.80 and 1.00 indicates a very strong correlation.

The negative correlation indicates that higher TPC and TFC are associated with lower EC₅₀ values, reflecting stronger antioxidant activity. This is because phenolic and flavonoid compounds have strong electron-donating abilities, allowing them to effectively neutralize free radicals and inhibit oxidative processes. The ethyl acetate fraction showed the highest TPC and TFC values and the lowest EC₅₀ value, while the *n*-hexane fraction had the lowest TPC and TFC values and the highest EC₅₀ value. This confirms that phenolics and flavonoids play a major role in antioxidant activity. Generally, higher phenolic content corresponds to stronger antioxidant activity due to the presence of hydroxyl (-OH) groups that can stabilize free radicals. These findings are consistent with previous studies, which reported that increased phenolic content is associated with lower EC₅₀ values. Statistical analysis showed that the data were normally distributed, as indicated by the Shapiro-Wilk test ($p > 0.05$). Levene's test further confirmed that the data had equal variance. An independent t-test revealed a significant difference ($p < 0.05$) in EC₅₀ values between the CUPRAC and FRAP assays.

CONCLUSION

Extracts and fractions of *L. leucocephala* demonstrated significant antioxidant activity, as evidenced by both CUPRAC and FRAP assays. Among the fractions, the ethyl acetate fraction exhibited the highest antioxidant activity, likely due to its elevated phenolic and flavonoid contents. These findings underscore the potential of *L. leucocephala* as a valuable natural source of antioxidants, with prospective applications in pharmaceutical formulations, nutraceuticals, and the development of functional foods.

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CONFLICT OF INTEREST STATEMENT

The authors declared that they have no conflicts of interest to disclose.

AUTHORS' CONTRIBUTIONS

Firstca Aulia Rachma conducted the research and wrote the article. The supervisory team, consisting of Agustono Wibowo, Nurain Aziman, Norizan Ahmat, and Iswan Budy Suyub, designed the experimental work and guided the research progress. Neni Kartini Che Mohd Ramli assisted with sample collection.

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