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Impact total of harvesting age on phenolic content, flavonoid level, and antioxidant capacity in *Justicia gendarussa* leaves

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Abstract This study evaluated the influence of harvesting age on the total phenolic content, flavonoid levels, and antioxidant properties of *Justicia gendarussa* leaves. Significant differences were observed across different leaf ages (3, 4, 5, and 6 months). The highest phenolic content was recorded in leaves harvested at four months (1.769 mg GAE/g DW), while the six-month-old leaves exhibited the highest concentration of flavonoids (27.505 mg QE/g DW). The antioxidant capacity peaked in the three-month-old leaves (8.1985 µmol TE/g DW) using the DPPH method, while six-month-old leaves displayed the greatest antioxidant potential (12.8684 µmol TE/g DW) measured via the FRAP method. Therefore, the six-month-old leaves were found to have the optimal combination of high flavonoid content and antioxidant activity as determined by the FRAP method.

Keywords: Antioxidant capacity, Harvesting age, *Justicia gendarussa*, Phytochemical

Introduction

Justicia gendarussa Burm f. is a shrub species commonly found in several Asian regions, including Malaysia, Sri Lanka, India, and Indonesia (Ayob *et al.*, 2013). It is taxonomically classified within the kingdom Plantae, division Tracheophyta, class Magnoliopsida, order Lamiales, family Acanthaceae, genus *Justicia*, and species *J. gendarussa* (Murugesan, 2017). This plant typically grows in forested areas and along riverbanks and is often cultivated for its medicinal properties or used as a natural hedge. It flourishes in lowland areas, particularly at elevations ranging from 1 to 500 meters above sea level

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(Sinansari *et al.*, 2018). *J. gendarussa* is characterized by an upright, branched form that can reach approximately one meter in height, with narrow, lance-shaped leaves and small white flowers adorned with pink or purple spots (Yadav *et al.*, 2017). Traditionally, the plant has been utilized to treat a range of health conditions, such as fever, rheumatism, diarrhea, and dysuria (Raghu & Agrawal, 2016).

Oxidative stress, which results from an imbalance between pro-oxidant and antioxidant systems, is a significant factor contributing to the development of inflammatory, metabolic, cardiovascular, degenerative, and cancerous diseases. Preventing these disease processes requires the intervention of exogenous antioxidants, which work alongside endogenous systems to restore and sustain redox balance (Abdel-Daim *et al.*, 2019). While both natural and synthetic exogenous antioxidants are extensively used in modern medicine, growing concerns about the potential toxicological impacts of synthetic antioxidants have increased interest in natural alternatives, such as phenols and flavonoids (Liu *et al.*, 2013). Many plants naturally contain antioxidants that play crucial roles in regulating hormone activities, promoting growth, exhibiting antimicrobial properties, and supporting metabolic functions (Eghbaliferiz and Iranshah, 2016).

J. gendarussa is rich in secondary metabolites that enhance its antioxidant capacity. These bioactive compounds include phenolics, flavonoids, glycosides, terpenoids, tannins, alkaloids, and saponins found within its leaves (Putri *et al.*, 2020). The production of these metabolites is influenced by factors such as plant age, environmental conditions, seasonal changes, and leaf maturity (Ghasemzadeh *et al.*, 2014). However, previous research has yielded inconsistent results regarding the effect of harvesting age on the levels of phenolics and flavonoids in plants. The study was evaluated and compare the total phenolic content, flavonoid concentrations, and antioxidant capacity of *J. gendarussa*leaves harvested at different stages of maturity.

Materials and methods

Planting and harvesting

The planting and harvesting processes were conducted at the Tropical Biopharmaca Research Center, IPB University, located at Cikabayan Gardens Block C on the IPB Campus in Dramaga, Bogor (coordinates: 6°3'49" S, 106°42'57" E) at an altitude of 141 meters. The *Justicia gendarussa* seeds were sourced from the Bogor accession, and propagation was done using stem cuttings. Plants were grouped based on harvest ages of 3, 4, 5, and 6 months. Fertilizers used included manure (20 tons/ha), urea (300 kg/ha), SP-36 (200

kg/ha), and KCl (225 kg/ha). Leaves were harvested according to the predefined age groups from June to September 2022, with samples collected from leaves positioned 10 cm above ground level.

Plant preparation and extraction

Plant preparation followed the protocol outlined by Calvindi *et al.* (2020). Leaves were separated, washed, and dried at 45°C for 24 hours, then ground to a fine powder using an 80-mesh sieve. Ten grams of the leaf powder were mixed with ethanol p.a. (50 mL) and sonicated for 30 minutes. The mixture underwent maceration in the dark at room temperature for 24 hours, followed by filtration to obtain a solution with a concentration of 0.2 g/mL.

Determination of total phenolic (TPC) and flavonoid content (TFC)

The total phenolic and flavonoid contents were determined using colorimetric assays adapted from methods by Arista *et al.* (2023). For phenolics, the Folin-Ciocalteu reagent was used, where the absorbance was measured at 750 nm to quantify phenolic content as mg of gallic acid equivalent (GAE) per gram of dried weight (DW). For flavonoids, the reaction with $AlCl_3$ reagent allowed absorbance measurement at 415 nm, with results expressed as mg of quercetin equivalent (QE) per gram of DW.

Antioxidant capacity assays

The antioxidant activities of the extracts were assessed using two methods: the DPPH free radical scavenging assay and the Ferric Reducing Antioxidant Power (FRAP) assay, based on protocols by Marliani *et al.* (2022) and Wojtunik-Kulesza (2020), respectively. The DPPH method measures the reduction in absorbance at 515 nm after reacting the extract with the DPPH solution, with results expressed as µmol Trolox equivalent (TE) per gram of DW. The FRAP assay evaluates the reduction of ferric to ferrous ions in the presence of antioxidants, with absorbance recorded at 593 nm and results similarly expressed as µmol TE per gram of DW.

Data analysis

Quantitative data were analyzed using Analysis of Variance (ANOVA) at a 95% confidence level ($\alpha = 0.05$) in IBM SPSS version 25, followed by Tukey's HSD test for post hoc comparisons. Correlation analysis between phenolic content, flavonoid levels, and antioxidant capacities was carried out using the

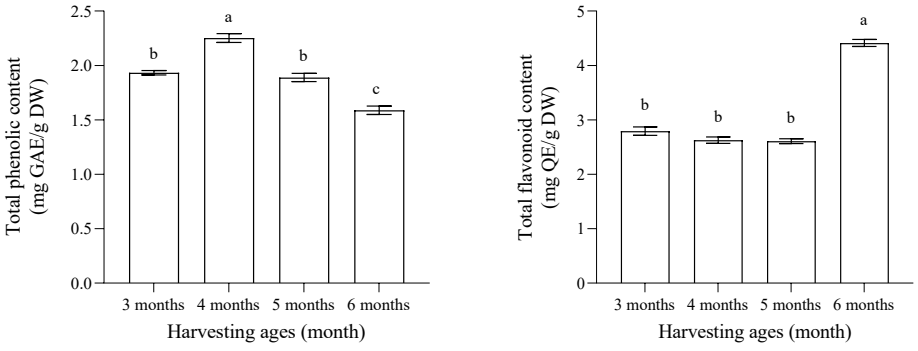
PerformanceAnalytics package in R-Studio version 4.2.2. Hierarchical Clustering Analysis (HCA) for different harvest ages was performed using the MetaboAnalyst 5.0 web server.

Results

Total phenolic and flavonoid content

The findings demonstrated that *J. gendarussa* leaves reached their peak phenolic content (TPC) at four months, yielding 2.252 mg GAE/g DW (Figure 1A). For leaves harvested at 3, 5, and 6 months, the TPC values were 1.934 mg GAE/g DW, 1.889 mg GAE/g DW, and 1.589 mg GAE/g DW, respectively. Conversely, the maximum flavonoid content (TFC) was observed at six months, with a recorded value of 4.413 mg QE/g DW (Figure 1B). Leaves harvested at 3, 4, and 5 months showed TFC values of 2.793 mg QE/g DW, 2.627 mg QE/g DW, and 2.608 mg QE/g DW, respectively.

The ANOVA analysis identified significant variations ($p < 0.05$) in the phenolic and flavonoid content of *J. gendarussa*leaves based on their age at harvest. According to Tukey's HSD test, there were significant differences in total phenolic content between leaves harvested at four months and those collected at six months, while no significant differences were found in flavonoid levels until leaves reached the age of five months. However, leaves harvested at six months exhibited significant differences when compared to those from other harvest ages.



(A) (B)

Figure 1. Bar chart illustrating (A) the total phenolic content and (B) the total flavonoid content of ethanol extracts from *J. gendarussa* leaves. Note: a, b, and c represent Tukey's HSD test results for the differences among various harvest ages

DPPH and FRAP assay

The antioxidant capacity of *J. gendarussa* leaves, as evaluated using the DPPH method, showed the highest activity in leaves harvested at three months, with a recorded value of 10.668 µmol TE/g DW (Figure 2A). This antioxidant potential was observed to decline progressively with leaf aging, where four, five, and six-month-old leaves exhibited reduced activities of 9.024, 8.896, and 9.410 µmol TE/g DW, respectively. On the other hand, the FRAP assay revealed an increasing trend in antioxidant capacity with leaf age, with values measured at 14.100, 14.183, and 14.711 µmol TE/g DW for leaves at three, four, and five months, respectively. The highest antioxidant capacity using the FRAP method was observed in six-month-old leaves, peaking at 16.239 µmol TE/g DW (Figure 2B).

An ANOVA analysis identified significant variations in antioxidant capacity across the different harvesting ages for both assays. Tukey's HSD test further confirmed that the DPPH method indicated a significant disparity in the antioxidant activity between three-month-old leaves and other age groups (Figure 2). Similarly, the FRAP method showed a notable difference for six-month-old leaves, which exhibited superior antioxidant capacity compared to those harvested at other stages.

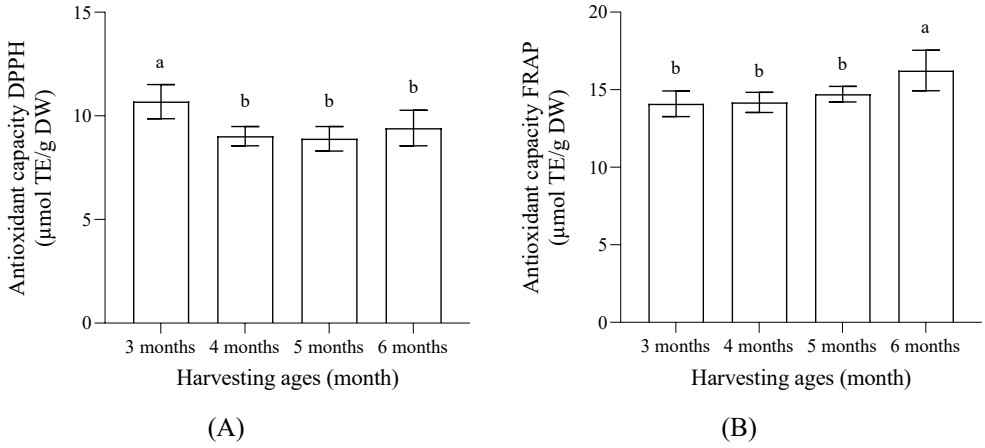


Figure 2. Antioxidant capacity of *J. gendarussa* leaf extracts determined using (A) DPPH and (B) FRAP assays across different harvesting ages. Error bars represent the mean ± standard deviation. Letters (a, b, c) indicate significant differences according to Tukey's HSD test

Correlation analysis of phytochemical content and antioxidant capacity in ethanol extract of J. gendarussa leaves

The analysis of the correlation between phytochemical content and antioxidant capacity in the ethanol extracts of *J. gendarussa* is presented in Figure 3. The results indicated that total phenolic content exhibited a weak negative correlation with antioxidant capacity, as measured by the DPPH assay ($r = -0.14$) and the FRAP method ($r = -0.69$). In contrast, total flavonoid content demonstrated a positive correlation with antioxidant capacity, with correlation coefficients of $r = 0.06$ for the DPPH method and $r = 0.78$ for the FRAP method. Notably, there was a significant inverse relationship between phenolic and flavonoid contents ($r = -0.79$).

Furthermore, the antioxidant capacity determined using the DPPH assay showed a weak negative correlation with that measured by the FRAP method ($r = -0.019$). Both phenolic and flavonoid contents were significantly correlated with antioxidant capacity as measured by the FRAP method, achieving statistical significance at $p < 0.05$ and $p < 0.01$, respectively. Additionally, both antioxidant assays indicated a high level of statistical significance ($p < 0.01$).

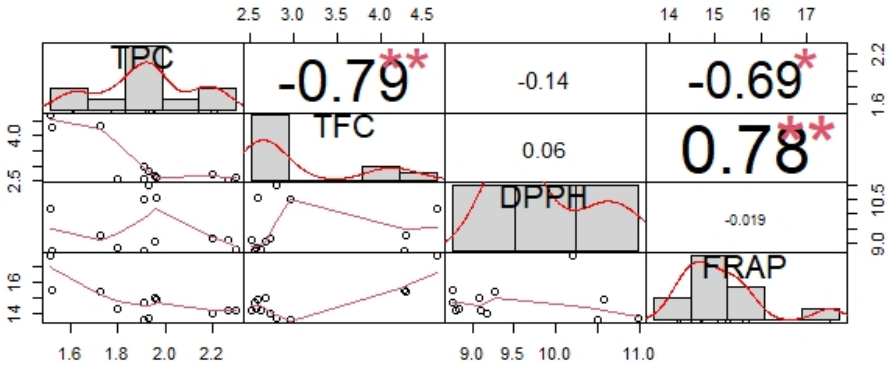


Figure 3. Correlation matrix of phytochemical content and antioxidant capacity in ethanol extract of *J. gendarussa* leaves. * and ** = significance at $p < 0.05$ and 0.01, respectively

***Hierarchical clustering analysis* (HCA)**

The heatmap generated through HCA classified the samples into four distinct groups according to their harvest age: 3, 4, 5, and 6 months (Figure 4). This clustering analysis identified patterns in total phenolic content, flavonoid content, and antioxidant capacity. The color gradient in the heatmap depicts the correlation between harvest age and measured parameters, with red indicating

strong positive correlations (+1) and green indicating strong negative correlations (-1).

Samples harvested at three months showed high antioxidant capacity (measured via DPPH), moderate phenolic levels, but relatively low flavonoid content and FRAP antioxidant activity. In contrast, four-month-old samples were characterized by elevated phenolic levels but displayed reduced levels of flavonoids and antioxidant capacity. The five-month-old group exhibited low concentrations of phenolics and flavonoids, coupled with diminished antioxidant capacity in both the DPPH and FRAP assays. Notably, six-month-old samples were distinguished by high flavonoid content and strong antioxidant capacity via the FRAP method, although they had reduced phenolic levels and lower antioxidant activity in the DPPH assay.

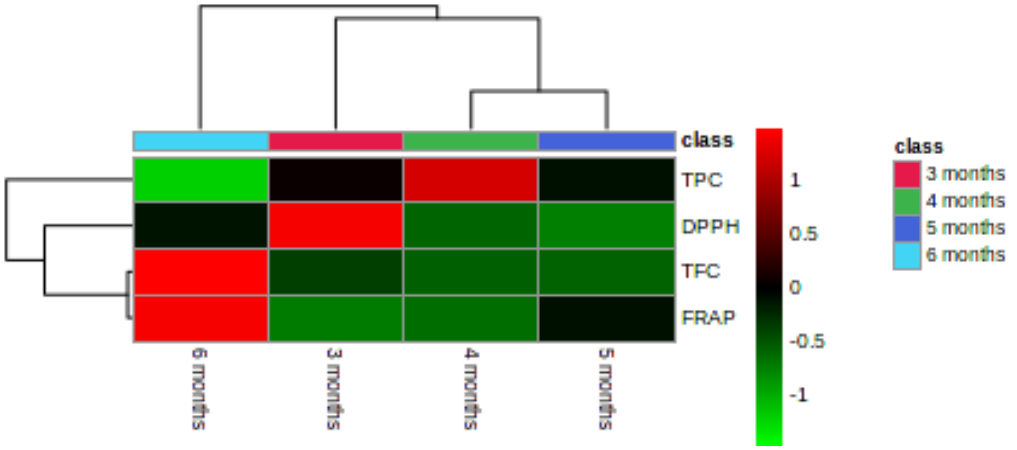


Figure 4. Hierarchical clustering analysis (HCA) of harvest-age groups based on phytochemical content and antioxidant capacity

Discussion

The ethanol extract of *J. gendarussa* leaves harvested at 4 months showed the highest TPC, reaching 2.252 mg GAE/g DW, followed by a decline with increasing leaf age. This decrease may be attributed to reduced biosynthesis rates, the incorporation of phenolic compounds into insoluble cell wall structures, or their transformation into oligomers and polymers (Nurmi *et al*., 1996). In contrast, the total flavonoid content (TFC) remained stable between the ages of 3 to 5 months but increased sharply at 6 months, peaking at 4.413 mg QE/g DW. This trend is consistent with findings in *Moringa oleifera*, where flavonoid levels rose with age to counter oxidative stress (Nobossé *et al*., 2018; Moradi *et al*., 2020). However, in *Orthosiphon aristatus*, older leaves exhibited reduced

flavonoid levels compared to younger ones, possibly due to decreased synthesis during later stages of cell development (Komariah *et al*., 2021).

The antioxidant capacity assessed using the DPPH assay was highest in three-month-old leaves (10.688 µmol TE/g DW) but declined significantly in four-month-old leaves, stabilizing thereafter. This pattern aligns with observations in *Ilex guayusa*, where younger leaves displayed superior DPPH activity, potentially due to a reduction in other antioxidant compounds such as alkaloids with advancing age (Villacís-Chiriboga *et al*., 2017; Achakzai *et al*., 2009). On the other hand, the FRAP assay indicated an increase in antioxidant capacity with leaf maturity, peaking at 16.239 µmol TE/g DW in six-month-old leaves, a trend similar to that seen in green tea leaves, where FRAP capacity increased with age (Lee *et al*., 2014).

Correlation analysis revealed significant interactions among the measured parameters. There was a strong inverse correlation between TPC and TFC ($r = -0.79$, $p < 0.01$), suggesting that a higher TPC is associated with lower TFC in *J. gendarussa* extracts. TPC showed weak negative correlations with DPPH ($r = -0.14$) and moderate negative correlations with FRAP ($r = -0.69$, $p < 0.05$) antioxidant capacity, aligning with previous findings that phenolics may not significantly contribute to reducing power measured by FRAP (Liu *et al*., 2007; Irakli *et al*., 2018). In contrast, TFC exhibited a strong positive correlation with FRAP ($r = 0.78$, $p < 0.01$), supporting the role of flavonoids in electron transfer reactions, which are critical in reducing Fe^{3+} to Fe^{2+} (Youn *et al*., 2018; Kaurinovic and Vastag, 2019).

The hierarchical clustering analysis (HCA) revealed distinct patterns based on harvest age. Six-month-old leaves showed high flavonoid content and FRAP antioxidant capacity, likely driven by increased age-related flavonoid biosynthesis and enzyme activity (Moradi *et al*., 2020). Conversely, three-month-old leaves exhibited strong DPPH antioxidant activity despite low phenolic and flavonoid content, suggesting the influence of other bioactive compounds such as alkaloids, steroids, and saponins (Yadav *et al*., 2017). The clustering also highlighted that four-month-old leaves had high TPC but lower antioxidant capacities, potentially due to specific phenolic profiles influenced by environmental factors (Pavlovic *et al*., 2019). Further research is needed to perform detailed metabolite profiling of *J. gendarussa* leaves across harvest ages to pinpoint compounds contributing to their antioxidant effects.

In summary, the ethanol extracts of *J. gendarussa* leaves demonstrate varying phytochemical and antioxidant profiles depending on harvest age. The highest phenolic content was observed in four-month-old leaves, while six-month-old leaves exhibited the greatest flavonoid content and FRAP antioxidant capacity. Conversely, the strongest DPPH antioxidant activity was recorded in

three-month-old leaves, indicating that different harvest ages optimize different antioxidant potentials. These findings highlight the importance of selecting appropriate harvest times to maximize the bioactive compound content of *J. gendarussa* extracts. Future research focusing on comprehensive metabolite profiling is recommended to further elucidate the specific compounds responsible for the observed antioxidant properties and their potential health benefits.

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