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Optimizing Fertilization Strategies for Enhanced Phenolic Content and Antioxidant Activity in *Justicia gendarussa* Burm f.: Synergistic Effects of Manure and NPK Fertilizers

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Abstract: The cultivation of *Justicia gendarussa* Burm f., a medicinal plant with significant pharmacological properties, can be optimized through effective fertilization strategies to enhance its bioactive compounds. This study investigated the effects of different fertilization methods, including manure, NPK (Nitrogen, Phosphorus, and Potassium) fertilizer, and their combinations, on the total phenolic content (TPC) and antioxidant activity (Ferric Reducing Antioxidant Power (FRAP) and 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) methods) of gandarusa leaves and stems. Results showed that T4 (50% manure + 50% NPK) was the most effective treatment, which yielded the highest TPC and FRAP antioxidant activities in both leaves and stems. The highest ABTS antioxidant activity, however, was observed in T3 (NPK). A significant correlation was found between TPC and ABTS antioxidant activity in gandarusa stems, highlighting the role of phenolics in antioxidant properties. This study demonstrates that combining organic and inorganic fertilizers can optimize the medicinal quality of *Justicia gendarussa*, contributing valuable insights to the field of herbal medicine.

Keywords: *Justica gendarussa* Burm f.; organic fertilizer; plant bioactive compounds.

1. Introduction

Justica gendarussa Burm f. is an herbal plant belonging to the Acanthaceae family, commonly referred to as "Nili-Nirgundi." In Indonesia, it is known as "Gandarusa." Gandarusa plants are found in several Asian regions, including Indonesia, Sri Lanka, China, India, and Malaysia [1]. Gandarusa has long been used in traditional medicine in China and India due to its efficacy in treating diseases such as rheumatism, bronchitis, fever, eczema, and jaundice. In traditional Chinese medicine, gandarusa is also utilized for anti-inflammatory treatments and is reported to have an inhibitory effect on platelet aggregation [2]. In Indonesia, particularly in the Papua region, the gandarusa plant is traditionally used as a male contraceptive by reducing sperm count in men and delaying pregnancy in women [3]. The pharmacological effects of gandarusa plants have shown potential for anti-inflammatory, analgesic, antioxidant, hepatoprotective, anti-anxiety, bactericidal, anti-angiogenic, antifungal, anthelmintic, antimicrobial, and anticancer activities [4]. Phytochemical compounds found in these plants play a significant role in their pharmacological properties [5].

Gandarusa leaves contain potassium, flavonoids, justicin, steroids, 0.4% tannins, aromatic amines, iridoids, coumarins, and alkaloids [3]. Additionally, gandarusa leaves are rich in gendarusin A (6,8-di-α-L-arabinopyranosyl-4',5,7-trihydroxyflavone) as the main compound, along with minor compounds such as gendarusin B (6-C-α-L-arabinopyranosyl-4',5,7-trihydroxy-8-C-β-D-cylopyranosyl-flavone), gendarusin C, gendarusin D, and gendarusin E [6]. Besides flavonoids, gandarusa leaves contain phenolics that play critical roles in plant growth regulation, defense against pathogens and predators, pigmentation, UV protection, and nitrogen fixation mechanisms [7]. Studies on the antioxidant activity of gandarusa leaves and stems have shown moderate activity compared to ascorbic acid, gallic acid, and butylated hydroxytoluene (BHT) [5].

The metabolite content in plants has a significant impact on their pharmacological effects. Biotic and abiotic factors influence both primary and secondary metabolite levels. Environmental conditions such as cultivation location, altitude, temperature, sunlight exposure, rainfall intensity, pH, and soil characteristics can affect metabolite composition, as reported by Panico *et al*. [8]. Moreover, the metabolite profile is influenced by the cultivation system, particularly fertilization methods [9]. Basundari *et al*. [10] and Baek *et al*. [11] demonstrated similar findings in *Allium cepa* L. and the Duckweed plant. Previous research on gandarusa has focused on various cultivation methods and their effects on biomass (dry weight) [12]. Studies have also explored the impact of cultivation location and fertilization on secondary metabolites and vegetative growth. However, the influence of fertilization on gandarusa's phytochemical composition remains unexplored. Understanding optimal cultivation practices is crucial for enhancing the production of secondary metabolites, adding value to gandarusa as a traditional medicine. This study aims to investigate the effects of manure, NPK (Nitrogen, Phosphorus, and Potassium), and their combinations on the total phenolic content and antioxidant activity in Gandarusa leaves and stems, as well as to analyze the correlation between phenolic content and antioxidant activity.

2. Materials and Methods

2.1. Plant material and sample preparation.

The gandarusa plant (*J. gendarussa*) was cultivated in the Biopharmaca Cultivation Conservation Unit Garden, Tropical Biopharmaca Research Center, LPPM IPB, Cikabayan Block C Garden, IPB Dramaga Campus, located at 6°3'49" S and 106°42'57" T, with an altitude of 141 meters above sea level. This study employed a randomized complete group design. Plant seedlings were obtained from stem cuttings of 15 cm in length, which were initially cultivated in polybags measuring 10×15 cm for two months before being transferred to larger polybags measuring 25×30 cm for further treatment. The treatments applied to the gandarusa plants included the following: control (no fertilizer, T1), manure (T2), NPK (Nitrogen, Phosphorus, and Potassium) fertilizer (T3), 50% manure combined with 50% NPK fertilizer (T4), 10% manure combined with 50% NPK fertilizer (T5), and 50% manure combined with 10% NPK fertilizer (T6). The NPK doses applied were N (200 kg ha^{-1}), P (100 kg ha^{-1}), and K (150 kg ha^{-1}), while the dose of manure applied was 20 tons ha^{-1}. Fertilizer application rates included 10% and 50% proportions, depending on the treatment. This study consisted of six treatments with three replications, yielding a total of 18 experimental units. The cultivation period lasted for four months before the samples were harvested.

The study utilized stems and leaves from the gandarusa plants. After harvesting, the samples were cleaned under running water to remove dirt and other impurities. The cleaned samples were dried in an oven at 45°C for two days and one night. Once dried, the samples were ground using a grinding machine until they were smooth, resulting in a particle size of 80 mesh.

2.2. Sample extraction.

Samples of stems and leaves of gandarusa plants that had been ground were then extracted using a modified sonication-maceration method based on Asyhar *et al*. [13]. Briefly, 4 grams of simplicia from Gandarusa stems and leaves were dissolved in 20 mL of pro-analytical ethanol solvent, then sonicated using a sonicator (Decon Laboratories, United States) for 30 minutes. This was followed by maceration in the dark at room temperature for 24 hours. The resulting mixture was filtered using filter paper, producing a supernatant. The resulting supernatant was diluted to a concentration of 0.02 g mL ^{1} and subsequently used to analyze total phenolics and antioxidant activities using the FRAP (Ferric Reducing Antioxidant Power) and ABTS (2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid) methods.

2.3. Total phenolic (TPC) assay.

The total phenolic content of gandarusa plants was measured using the Folin-Ciocalteu method, with modifications based on Arista *et al*. [14]. Briefly, 20 µL of Gandarusa ethanol extract (leaves and stems) was added to 100 µL of Folin-Ciocalteu reagent in a 96-well microplate and incubated for 5 minutes. Then, 80 µL of Na_2CO_3 solution (7.5%) was added, followed by incubation in the dark at room temperature for 2 hours. Absorbance was measured using a nano-spectrophotometer (SPECTROstarNano, BMG LABTECH) at a wavelength of 750 nm. The standard solution concentrations used were 50, 75, 100, 150, 200, and 225 ppm. Total phenolic content was expressed as mg GAE (gallic acid equivalent) g^{-1} dry weight (DW).

2.4. Antioxidant assay.

Antioxidant activity was measured using two methods: the FRAP (Ferric Reducing Antioxidant Power) and ABTS (2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid) methods. The FRAP method, based on Arista and Nurcholis [15] with modifications, ensured reliable results. Briefly, 10 µL of gandarusa ethanol extracts (leaves and stems) were separately mixed with 300 µL of FRAP reagent (prepared by mixing acetate buffer pH 3.6 with 10 µM TPTZ solution in 40 µM HCl and 20 µM $FeCl_3$ $6H_2O$ in distilled water) in a v/v/v ratio of 10:1:1. The FRAP reagent was incubated at 37°C for 30 minutes before use. The 96-well microplate containing the sample was then incubated at 37°C for 4 minutes. Absorbance was measured using a microplate reader at a wavelength of 593 nm. The concentrations of the standard solutions used were 100, 200, 400, 500, and 600 µM. The final unit was expressed in µmol TE (Trolox equivalent) g^{-1} dry weight (DW), providing reliable and valid results.

Antioxidant activity was measured using the ABTS method with a microplate reader (Biotek, Winooski, USA), as described by Nurcholis *et al*. [16] with modifications. A total of 20 µL of gandarusa ethanol extracts (leaves and stems) were separately mixed with 180 µL of $ABTS^{*+}$ reagent (prepared by mixing ABTS 7 mM in aquabidest and $K_2S_2O_8$ 2.4 mM in aquabidest) in a 96-well microplate. The 96-well microplate containing the sample was incubated at 30°C for 6 minutes. Absorbance was measured using a microplate reader at a wavelength of 734 nm. The concentrations of the standard solutions used were 100, 200, 300, 400, and 500 µM. The final unit was expressed in µmol TE (Trolox equivalent) g^{-1} dry weight (DW).

2.5. Data analysis.

Statistical analysis of quantitative data was performed using ANOVA one-way at a significance level of $\alpha = 5\%$, followed by Tukey's post hoc test using the Minitab 19 software program (Minitab Inc., State College, Pennsylvania, United States) to determine the effect of treatments on the measured parameters. Regression analysis and correlation between total phenolic content and antioxidant activity in ethanol extracts of gandarusa leaves and stems were evaluated using GraphPad Prism 8 for Windows (GraphPad Software Inc., San Diego, California, USA) Version 8.0.1. Data are presented as mean ± standard error of the mean (SEM).

3. Results and Discussion

The findings of this study highlight the significant influence of fertilizer composition on the total phenolic content (TPC) and antioxidant activity of gandarusa (Justicia gendarussa Burm f.), as well as the correlations between these parameters. The application of 50% manure and 50% NPK (Nitrogen, Phosphorus, and Potassium) fertilizer (T4) emerged as the most effective fertilization strategy for enhancing the TPC and antioxidant capacity (Ferric Reducing Antioxidant Power (FRAP) method) in both leaves and stems. This balanced combination of organic and inorganic fertilizers provides essential macro- and micronutrients, creating optimal conditions for the synthesis of secondary metabolites.

3.1. Total phenolic content (TPC).

The total phenolic content (TPC) of ethanol extracts from the leaves and stems of gandarusa was determined using the Folin–Ciocalteu method. Table 1 summarizes the TPC values observed across different fertilizer treatments after four months of growth. The highest TPC values were observed in plants treated with 50% manure and 50% NPK (Nitrogen, Phosphorus, and Potassium) fertilizer (T4), yielding 7.603 ± 0.352 mg GAE (Gallic Acid Equivalent) g^{-1} DW (Dry Weight) for leaves and 5.214 ± 0.316 mg GAE g^{-1} DW for stems. This was followed by plants treated with 100% NPK fertilizer (T3), which showed 6.987 ± 0.042 mg GAE g^{-1} DW for leaves and 4.497 ± 0.246 mg GAE g^{-1} DW for stems. The lowest TPC values were recorded in the control group (T1), which did not receive any fertilizer treatment. This group yielded 6.255 ± 0.196 mg GAE g^{-1} DW for leaves and 3.709 ± 0.063 mg GAE g^{-1} DW for stems. Among the treatments, plants treated with 10% manure and 90% NPK fertilizer (T5) exhibited the lowest phenolic content in the stems, at 3.376 ± 0.399 mg GAE g^{-1} DW. Statistical analysis revealed significant differences ($p < 0.05$) in TPC values across treatments within the same plant part. Interestingly, no significant differences were observed in TPC between ethanol extracts of leaves and stems for the same treatment group. These findings highlight the potential of balanced fertilizer compositions, particularly T4, in enhancing the phenolic content of gandarusa.

Table 1. Total phenolic content of ethanol extract of gandarusa leaves and stems.

Treatment	Part of the plant	Total phenolic content (mg GAE g^{-1} DW)
T1	Leaves	6.255 ± 0.196a-d
	Stems	3.709 ± 0.063e
T2	Leaves	6.643 ± 0.337abc
	Stems	3.921 ± 0.294e
T3	Leaves	6.987 ± 0.042ab
	Stems	4.497 ± 0.246cde
T4	Leaves	7.603 ± 0.352a
	Stems	5.214 ± 0.316b-e
T5	Leaves	6.320 ± 0.523a-d
	Stems	3.376 ± 0.399e
T6	Leaves	6.532 ± 0.503abc
	Stems	4.058 ± 0.346de

Each value is presented as mean ± standard error mean (SEM); numbers followed by different letters (a-e) showed significant differences ($p<0.05$) in the same column and the same plant parts; treatment T1 (control); T2 (manure); T3 (NPK); T4 (50% manure, 50% NPK); T5 (10% manure, 50% NPK); T6 (50% manure, 10 NPK); GAE, gallic acid equivalent; DW, dry weight.

The treatment of gandrusa plants with manure, NPK, or a combination of both demonstrated that ethanol extracts from leaves consistently exhibited higher total phenolic content than those from stems, with the leaves showing a phenolic content of 33.96 mg g^{-1}. This finding aligns with previous studies, such as those by Kuber [17], which also reported elevated phenolic levels in the leaves of 9.47 ± 0.0216 GAE/g. The difference in phenolic content can be attributed to the higher biosynthesis of secondary metabolites in the leaves, as these tissues are rich in chloroplasts that serve as energy hubs and are pivotal for the production of polyphenols, including phenolic acids and flavonoids [18, 19]. Additionally, the phenylpropanoid pathway, which is highly active in leaf tissues due to the abundance of related biosynthetic genes, further explains the superior phenolic content in the leaves [20]. These structural and metabolic dynamics highlight the potential of gandrusa leaves as a superior source of bioactive phenolic compounds, emphasizing their therapeutic value and warranting further exploration into their applications.

The T4 treatment, consisting of 50% manure and 50% NPK fertilizer, resulted in higher total phenolic content in both leaf and stem extracts of gandrusa compared to other treatments. The production of secondary metabolites in plants is influenced by the availability of essential macroelements (N, P, K) and microelements (Fe, Zn, Mn) in the growing medium. These nutrients play crucial roles as constituents of cellular compartments and as cofactors for enzymes involved in biological reactions, supporting the synthesis of amino acids and proteins [21]. Nitrogen, in particular, significantly affects the accumulation of secondary metabolites. Plants with insufficient nitrogen show reduced biosynthesis of phenolic compounds and terpenoids, while adequate nitrogen availability promotes the production of metabolites such as cyanogenic glucosides and alkaloids. Similarly, Putra *et al*. [22] reported that applying NPK fertilizer at 100 kg ha^{-1} produced the highest total phenolic content in Portulaca grandiflora.

Previous studies have consistently demonstrated the effectiveness of manure treatments in increasing plant phytochemicals and antioxidant activity. For example, using cow, quail, or chicken manure has enhanced the production of essential oils, phenolic compounds, and antioxidants in plants like Lippia dulcis [23]. Organic fertilization with poultry manure has also altered the secondary metabolite content in Morus alba, increasing the concentration of beneficial compounds such as flavonoid polyphenols [24]. Likewise, combining organic vermicompost and inorganic fertilizers has been shown to influence the phytochemical profile of tea plants. In contrast, organic fertilizers have increased the total phenolic content and antioxidant activity in various crops [25]. Using vermicompost derived from livestock manure has further improved Swiss chard's phytochemical content and antioxidant activity [26].

Evidence also highlights the synergistic effect of NPK fertilizers on enhancing the phytochemical and antioxidant properties of plants. Studies on Jatropha zeyheri, Curcuma xanthorrhiza, Orthosiphon stamineus, Cosmos caudatus, and Capsicum annuum confirm that NPK application boosts phenolic and flavonoid content as well as antioxidant activity [27–30]. Furthermore, these treatments enhance the production of polyphenols and flavonoids, which contribute to improved nutritional value and plant health. Combining NPK fertilizers with growth-promoting compost or foliar applications is a promising strategy to optimize phytochemical production, emphasizing the importance of tailored fertilizer applications in improving crop quality and resilience [31].

3.2. Antioxidant activity.

The antioxidant activity of ethanol extracts from the leaves and stems of gandarusa was measured using two methods: FRAP (Ferric Reducing Antioxidant Power) and ABTS (2,2-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid). The results revealed variations in antioxidant capacity across treatments for both methods, as shown in Table 2. The highest FRAP antioxidant activity in gandarusa leaves was observed in plants treated with 50% manure and 50% NPK (Nitrogen, Phosphorus, and Potassium) fertilizer (T4), with a value of 20.736 ± 0.524 µmol TEAC (Trolox Equivalent Antioxidant Capacity) g^{-1} DW (Dry Weight). For the ABTS method, the highest antioxidant activity in leaves was recorded in plants treated with 100% NPK fertilizer (T3), reaching 24.834 ± 0.261 µmol TEAC g^{-1} DW. Conversely, the lowest antioxidant activity in leaves was noted in the FRAP method for plants treated with manure (T2), at 15.915 ± 0.765 µmol TEAC g^{-1} DW, and in the ABTS method for plants treated with 10% manure and 50% NPK fertilizer (T5), at 21.962 ± 0.092 µmol TEAC g^{-1} DW. For stems, the highest FRAP antioxidant activity was recorded in plants treated with 100% NPK fertilizer (T3), at 12.390 ± 0.473 µmol TEAC g^{-1} DW, while the highest ABTS antioxidant activity was found in the control group (T1), with a value of 26.693 ± 0.573 µmol TEAC g^{-1} DW. The lowest antioxidant activity in stems was observed in the manure treatment (T2), with 9.172 ± 1.172 µmol TEAC g^{-1} DW for the FRAP method and 20.806 ± 0.238 µmol TEAC g^{-1} DW for the ABTS method under the 50% manure and 50% NPK fertilizer treatment (T4).

Overall, the antioxidant activity measured using the ABTS method was consistently higher than that measured by the FRAP method for both leaves and stems across all treatments. Statistical analysis revealed significant differences ($p < 0.05$) in the antioxidant activity of the ABTS method for gandarusa stems between the control group (T1) and the treatment with 50% manure and 50% NPK fertilizer (T4). However, no significant differences were observed among treatments for gandarusa leaves using either antioxidant activity measurement method. These findings demonstrate the variability in antioxidant capacity, influenced by fertilizer composition and the method of assessing antioxidant activity.

The chemical composition of compost varies depending on the materials used and the composting process, making it a rich source of essential nutrients for plant growth and development [32]. Compost typically contains macronutrients such as nitrogen (N), phosphorus (P), and potassium (K), which are vital for water regulation, enzyme activation, and disease resistance [33,34]. Nitrogen is particularly essential for plant growth, leaf development, and chlorophyll production [35]. Additionally, compost provides secondary nutrients such as calcium (Ca), necessary for maintaining cell wall stability; magnesium (Mg), a key component of chlorophyll required for photosynthesis; and sulfur (S), which supports enzyme activity and protein synthesis [36,37]. Compost also contains important micronutrients, such as iron (Fe), which is critical for respiration, chlorophyll synthesis, and nucleic acid (DNA) formation, as well as manganese (Mn), involved in photosynthesis and enzyme activation. Other essential elements include copper (Cu), which is vital for reproductive growth and disease resistance; zinc (Zn), required for hormone production and enzyme functions; boron (B), crucial for cell wall stability; and molybdenum (Mo), which supports nitrogen fixation and amino acid synthesis [38,39]. Trace elements such as cobalt (Co), selenium (Se), and iodine (I) further enhance plant nutrition and overall soil health [40]. Composting organic matter, including decomposed plant and animal materials, improves soil structure, enhances water retention, and boosts microbial activity while acting as a source of slowly released nutrients. Compost generally has a neutral to slightly alkaline pH, which balances soil acidity. Its water content is essential for sustaining microbial activity during the composting process. In conclusion, compost enhances the soil's physical, chemical, and biological properties while serving as an excellent nutrient source [41, 42].

Table 2. Antioxidant activity of ethanol extract of gandarusa leaves and stems.

Treatment	Part of the plant	FRAP antioxidant capacity (μmol TEAC g^{-1} DW)	ABTS antioxidant capacity (μmol TEAC g^{-1} DW)
T1	Leaves	17.287 ± 1.065abc	22.355 ± 0.548bc
	Stems	10.659 ± 1.062cde	26.693 ± 0.573a
T2	Leaves	15.915 ± 0.765a-e	22.561 ± 0.508bc
	Stems	9.172 ± 1.172e	25.019 ± 1.252ab
T3	Leaves	19.197 ± 0.469ab	24.834 ± 0.261ab
	Stems	12.390 ± 0.473b-e	25.329 ± 0.351ab
T4	Leaves	20.736 ± 0.524a	24.606 ± 0.291a-c
	Stems	10.813 ± 0.539cde	20.806 ± 0.238c
T5	Leaves	16.864 ± 0.949a-d	21.962 ± 0.092bc
	Stems	10.018 ± 1.124de	21.921 ± 0.209bc
T6	Leaves	17.454 ± 0.224abc	21.983 ± 0.227bc
	Stems	9.326 ± 0.417e	22.561 ± 0.379a-c

Each value is presented as mean ± standard error mean (SEM); numbers followed by different letters (a-c) showed significant differences ($p<0.05$) in the same column and the same plant parts; treatment T1 (control); T2 (manure); T3 (NPK); T4 (50% manure, 50% NPK); T5 (10% manure, 50% NPK); T6 (50% manure, 10 NPK); TEAC, trolox equivalent antioxidant capacity; DW, dry weight; FRAP, Ferric Reducing Antioxidant Power; ABTS, 2,2 azinobis (3-etilbenzotiazolin)-6-asam sulfonate.

3.3. Correlation between phenolic and antioxidant capacities.

The relationship between the total phenolic content (TPC) of ethanol extracts from gandarusa leaves and stems and their antioxidant activity, as measured by the Ferric Reducing Antioxidant Power (FRAP) and (2,2-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid) ABTS methods, was analyzed by performing a Pearson correlation analysis (Figure 1). The results generally indicated a direct relationship between TPC and antioxidant capacity, except in the case of ABTS analysis of gandarusa stems, which displayed an inverse trend.

In ethanol extracts of gandarusa leaves, the correlation coefficients between TPC and antioxidant activity for the FRAP and ABTS methods were 0.4289 and 0.2955, respectively (Figure 1, graphs A and B). These values indicate weak and statistically insignificant correlations. For gandarusa stems, the correlation coefficient between TPC and antioxidant activity, as determined by the FRAP method, was 0.2532 (Figure 1, graph C), indicating a weak and insignificant relationship. However, a significant and strong negative correlation (r = -

0.6829, $p < 0.05$) was observed between TPC and antioxidant activity, as measured by the ABTS method, in Gandarusa stems (Figure 1, graph D).

The data indicate that a significant correlation between TPC and antioxidant activity was observed only in the stem extracts using the ABTS method. This finding suggests that the phenolic compound groups present in gandarusa stems contribute more dominantly to antioxidant activity when measured by the ABTS method than by the FRAP method. In contrast, the weak and insignificant correlation observed in gandarusa leaves may be attributed to the presence of non-phenolic antioxidant compounds, which may play a more prominent role in the antioxidant activity of leaf extracts and potentially exert pharmacological effects as antioxidants. These results highlight the complexity of antioxidant capacity and its dependence on both phenolic and non-phenolic components, as well as the choice of method for measuring antioxidant activity.

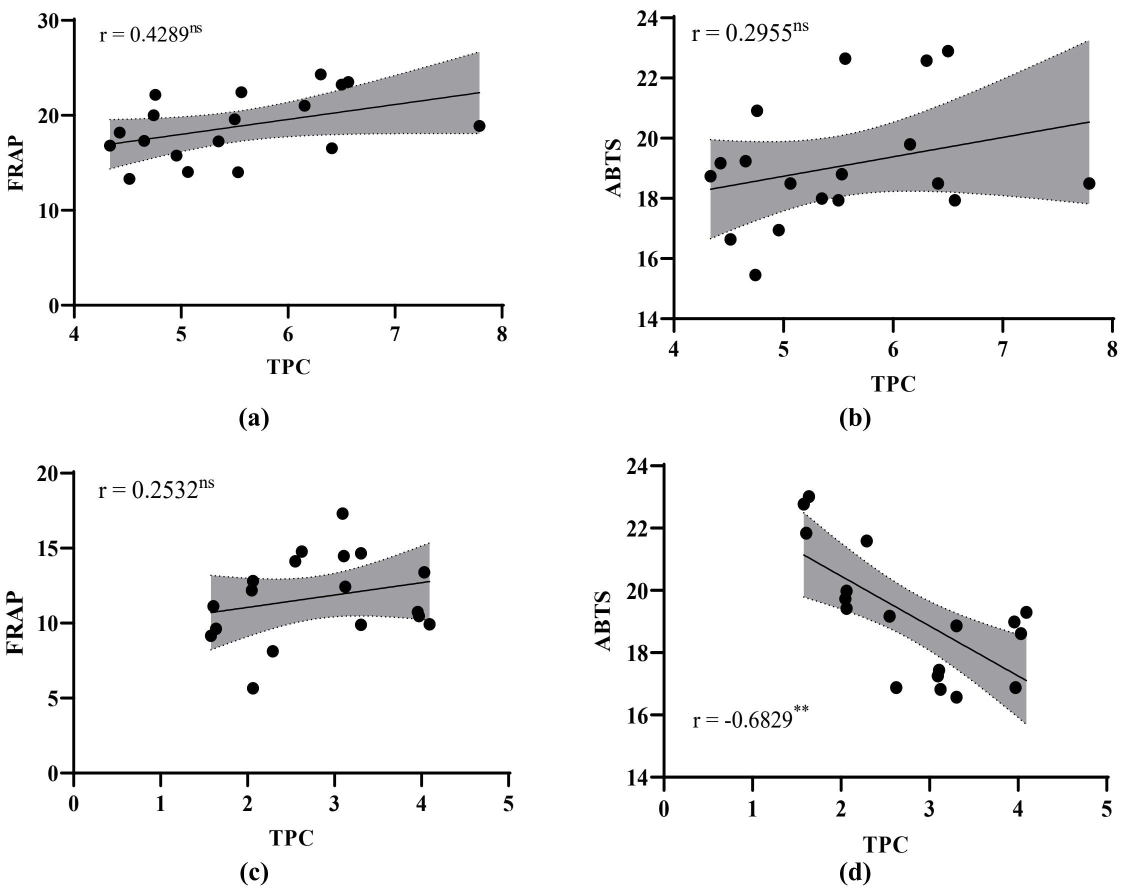

Figure 1. The correlation for total phenolic content (TPC) and antioxidant properties (FRAP, ferric reducing antioxidant power; ABTS, 2,2 azinobis (3-etilbenzotiazolin)-6-asam sulfonate). Ns, not significant level with p-value of 0.05; **, significant level with p-value of 0.05. **(a)** Total phenolic content (TPC) and FRAP of ethanol extract of gandarusa leaves; **(b)** Total phenolic content (TPC) and ABTS of ethanol extract of gandarusa leaves; **(c)** Total phenolic content (TPC) and FRAP of ethanol extract of gandarusa stems; **(d)** Total phenolic content (TPC) and ABTS of ethanol extract of gandarusa stems.

NPK (Nitrogen, Phosphorus, and Potassium) fertilizer is well-known for its ability to enhance the production of secondary metabolite compounds in plants, which play a critical role in protecting plants against pests, diseases, and adverse environmental conditions [43]. Although these compounds are not directly involved in primary plant growth, nitrogen (N), phosphorus (P), and potassium (K) contribute significantly to the biosynthesis of secondary metabolites. Nitrogen is a key component of amino acids, which serve as building blocks for proteins and enzymes necessary for various biosynthetic pathways, including the production of secondary metabolites such as alkaloids, flavonoids, and phenolics [44,45]. Increasing nitrogen levels in the soil enhances enzyme activity and supports protein synthesis, both of which are crucial for secondary metabolite production [46, 47]. Phosphorus plays a vital role in energy transfer and storage through ATP (adenosine triphosphate), which is required for numerous biosynthetic processes, including the synthesis of secondary metabolites [4]. Furthermore, phosphorus is a critical element in forming cell membranes and nucleic acids, both of which are essential for plant growth and secondary metabolite production [48]. Potassium, on the other hand, is indispensable for enzyme activation, protein synthesis, and maintaining osmotic balance in plant cells. By improving photosynthesis efficiency, potassium enhances the transport of photosynthetic products, which supports the synthesis of secondary metabolites. Additionally, potassium regulates stomatal function, optimizing water use efficiency and helping plants adapt to environmental stresses, while promoting the production of defense-related compounds [49, 50].

3.4. Interaction between organic manure and NPK.

The fundamental relationship between organic and inorganic fertilizers (such as NPK) in the best treatment, T4 (50% manure + 50% NPK), shows an improvement in soil chemical quality for plants as well as physiological effects. In terms of soil chemistry, organic manure plays a role in enhancing soil micronutrients (cations and anions) and maintaining soil organic matter content through the mechanism of Cation Exchange Capacity (CEC) and by serving as a source of microorganisms beneficial to plants [51-54]. Additionally, NPK fertilizers serve to supply essential nutrients through root absorption directly and to address deficiencies during active growth phases [54, 55]. From a physiological perspective, the optimal combination of both types of fertilizers can increase the rate of photosynthesis, which, in turn, enhances the production of plant metabolites and biomass [56].

Although both types of fertilizers exhibit good synergy, an imbalance, particularly the excessive use of synthetic fertilizers such as NPK, can inhibit plant growth and cause tissue damage. Moreover, excessive synthetic fertilizers can degrade beneficial biochemical characteristics of the soil by increasing salt content. Therefore, the role of organic fertilizers, such as those derived from animal manure, is often used to neutralize the effects of synthetic fertilizers and can also serve as restorative agents. A proper balance between the two is essential to support optimal plant growth [56, 57].

4. Conclusions

This study demonstrates that combining organic and inorganic fertilizers, specifically 50% manure and 50% NPK (Nitrogen, Phosphorus, and Potassium) (T4), is the most effective strategy for increasing the total phenolic content and antioxidant activity in *J. gendarussa* leaves and stems. Manure improves soil health and nutrient availability, while NPK fertilizers support the biosynthesis of secondary metabolites. The implications of these findings extend beyond improving the phytochemical quality of medicinal plants, as they may also contribute to sustainable agricultural practices and support the development of standardized plant-based phytopharmaceutical products. These findings highlight the importance of balanced fertilization to optimize the medicinal quality of gandarusa. Future research can explore the long-term impact of these strategies on crop productivity and sustainability. Field-scale trials are necessary to evaluate the effectiveness of this fertilizer combination under broader agricultural conditions, along with further studies to assess the stability of the plant's bioactive compounds throughout the growing season.

Author Contributions

Conceptualization, W.N. and S.F.; methodology, L.A.; formal analysis, L.A. and W.N.; investigation, L.A. and W.N.; resources, W.N.; data curation, L.A. and W.N.; writing—original draft preparation, R.A.A., L.A., F.R.M., and W.N.; writing—review and editing, R.A.A., L.A., F.R.M., and W.N.; visualization, R.A.A. and W.N.; supervision, W.N.; project administration, W.N.; funding acquisition, W.N. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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