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Fermentation-Mediated Modulation of Nutraceutically Relevant Polyphenolic Compounds and Antioxidant Capacity of Red Spinach (*Amaranthus tricolor* L.) Leaf Extract

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Abstract

Fermentation has emerged as a promising biotechnological approach to enhance the nutraceutical quality of plant-based materials through targeted modulation of bioactive compounds. This study investigated the effects of aerobic and anaerobic fermentation on total phenolic content (TPC), total flavonoid content (TFC), and antioxidant capacity of red spinach (*Amaranthus tricolor* L.) leaf extracts. Aerobic fermentation markedly enhanced the phytochemical profile, yielding a 1.87-fold increase in TPC (121.28 mg GAE/g extract) and a 1.66-fold increase in TFC (897.82 mg QE/g extract) compared with fresh leaves. These compositional improvements translated into superior functional activity, as evidenced by the lowest IC_{50} value (56.07 mg/L) and the highest antioxidant activity index (AAI = 0.713). In contrast, anaerobic fermentation provided limited phenolic enrichment and resulted in substantial flavonoid degradation, leading to inferior antioxidant performance. Strong correlations between polyphenolic contents and antioxidant parameters confirmed that phenolics and flavonoids are key contributors to radical scavenging activity. Overall, the findings demonstrate that aerobic fermentation is an effective, low-cost strategy to improve the nutraceutical value and antioxidant capacity of red spinach leaf extracts, highlighting its potential application in the development of functional foods and nutraceutical ingredients.

Keywords: Red spinach; fermentation; polyphenols; antioxidant activity; nutraceuticals.

INTRODUCTION

Indonesia's tropical climate endows the nation with exceptional biodiversity, particularly in indigenous plant species harboring substantial potential as sources of bioactive compounds for health applications (Elfahmi et al., 2014). The strategic exploitation of plant-derived bioactive constituents has emerged as a critical frontier in nutraceutical science, offering preventive and therapeutic interventions for health maintenance (Newman & Cragg, 2020). Among the most pressing contemporary health challenges is the escalating global burden of degenerative diseases, fundamentally linked to oxidative stress induced by excessive free radical generation (Chaudhary et al., 2023). This urgency has intensified efforts to identify natural antioxidant sources that combine high functional efficacy with optimal safety profiles, positioning such research at the forefront of nutraceutical innovation.

Red spinach (*Amaranthus tricolor* L.), a member of the Amaranthaceae family, represents a widely cultivated leafy vegetable distinguished by its robust antioxidant properties attributable to substantial phenolic and flavonoid concentrations (Spórna-Kucab et al., 2023). Several reports documented total flavonoid content ranging from 4.27 – 17.75 mg QE/g in red spinach leaf extracts via UV–Vis spectrophotometry (Jahan et al., 2022; Permanasari et al., 2024). Moreover, the ethanolic extract exhibited potent antioxidant activities with antioxidant capacity values of 33 – 36 µg g^{-1} TEAC DW (DPPH) and 60 – 68 µg TEAC g^{-1} DW (ABTS), classifications indicating strong to very strong radical scavenging capacity (Sarker et al., 2022). Comprehensive phytochemical profiling has revealed a diverse array of secondary metabolites, including carotenoids, quercetin-O-hexoside, luteolin-7-O-glucoside, luteolin-6-C-hexoside, gallic acid, thereby underscoring red spinach's superior nutraceutical potential (Sarker et al., 2022; Spórna-Kucab et al., 2023).

Despite these promising attributes, a critical limitation exists. Phenolic compounds in plant matrices predominantly occur in bound forms, particularly through glycosidic linkages with polysaccharides, substantially restricting their bioavailability and antioxidant efficacy (Gu et al., 2019). Fermentation has emerged as a sophisticated bioprocessing strategy capable of overcoming this constraint by enhancing the liberation and bioactivity of phytochemicals. Evangelista-Albacea et al. (2025) demonstrated that *Lactiplantibacillus plantarum* -mediated fermentation of Java plum (*Syzygium cumini*) juice dramatically elevated antioxidant activity 1.7 to 4.5-fold higher than 0.1% BHT, a well-known antioxidant. This remarkable enhancement was attributed to microbial enzymatic hydrolysis of polysaccharide-phenolic complexes, liberating bound phenolic compounds into more bioavailable forms with superior antioxidant capacity. During fermentation, microorganisms metabolize carbohydrates while secreting hydrolytic enzymes, including β-glucosidases, cellulases, and esterases, that cleave complex glycosidic and ester bonds, transforming structurally complex phenolics into simpler, more bioactive derivatives (Kumar et al., 2025; Paventi et al., 2025).

These mechanistic insights establish fermentation as a transformative approach for phenolic compound optimization and antioxidant enhancement in plant-based nutraceutical development. However, studies investigating the fermentation-mediated modulation of nutraceutically relevant phenolic compounds and antioxidant capacity in red spinach (*Amaranthus tricolor* L.) leaf extract remain limited. Therefore, this study aims to evaluate the effect of fermentation on the phenolic and flavonoid contents as well as the antioxidant capacity of red spinach leaf extract, with the ultimate goal of strengthening its functional value and supporting its development as a nutraceutical ingredient.

MATERIALS AND METHODS

Chemicals and reagents

The materials used in this study included 96% ethanol (Merck), methanol p.a. (Merck), magnesium powder (Merck), hydrochloric acid (HCl) (Merck), 10% aluminum chloride ($AlCl_3$) solution (Merck), 5% acetic acid solution (Merck), 5% ferric chloride ($FeCl_3$) solution (Merck), Folin–Ciocalteu reagent (Merck), 15% sodium carbonate (Na_2CO_3) solution (Merck), gallic acid standard (Sigma-Aldrich), quercetin standard (Sigma-Aldrich), *Saccharomyces cerevisiae* yeast, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich).

Sample collection and identification

The sample used in this study consisted of red spinach leaves (*Amaranthus tricolor* L.) collected from local Vegetable Garden, Talang Kelapa District, Banyuasin Regency, South Sumatra, Indonesia. The collected plant material was prepared as a herbarium specimen and subjected to botanical identification to confirm the authenticity of the plant species used in this study. Plant determination was conducted at the University of North Sumatra (USU) (No. 1160/MEDA/2025).

Sample preparation

Fresh leaves were subjected to wet sorting to remove foreign matter, impurities, and undesired plant parts, followed by washing under running water and draining. A total of 3 kg of cleaned leaves was weighed and divided into three treatment groups (1 kg per group).

The first group served as the control and consisted of fresh, non-fermented samples. The leaves were directly homogenized using a blender and subsequently extracted using the maceration method.

The second group underwent anaerobic fermentation with yeast. One kilogram of fresh leaves was placed in a fermentation vessel and inoculated with 20 g of *Saccharomyces cerevisiae*. The vessel was covered with a black cloth to create anaerobic conditions and incubated for 3 days at room temperature. After fermentation, the samples were homogenized, dry-sorted, and extracted by maceration.

The third group was subjected to aerobic fermentation without yeast inoculation. One kilogram of fresh leaves was fermented under aerobic conditions following the same procedure as the anaerobic group, except for yeast addition. After fermentation, the samples were homogenized, dry-sorted, and extracted using the maceration method.

Extraction

Extraction was performed using the maceration method with powdered red spinach (*Amaranthus tricolor* L.) leaf simplicia. A total of 200 g of the powdered sample was immersed in 1 L of 96% ethanol and gently stirred to ensure complete solvent contact. The container was tightly closed and stored in a dark place. Maceration was carried out for 72 h, with solvent replacement every 24 h and occasional stirring. The extraction process was repeated twice (remaceration) using the same solvent volume. After maceration, the mixture was filtered to separate the filtrate from the residue. The combined filtrates were re-filtered and subsequently concentrated using a vacuum rotary evaporator with water bath temperature was set at 45 °C until solvent evaporation yielded a viscous crude extract.

Total phenolic content analysis

Total phenolic content was determined using the Folin–Ciocalteu method with gallic acid as the standard as previously descibed by Setiawansyah et al. (2025). A gallic acid stock solution (80 ppm) was prepared in 96% ethanol, and standard solutions (5–80 ppm) were used to construct a calibration curve. For analysis, 0.1 mL of

standard or sample solution was mixed with 0.5 mL of Folin–Ciocalteu reagent, followed by the addition of 2 mL of 15% sodium carbonate (Na_2CO_3). The mixtures were incubated at room temperature for 55 min, and absorbance was measured at 759 nm using a UV–Vis spectrophotometer. Sample solutions were prepared by dissolving 0.8 mg of red spinach leaf extract in 96% ethanol to a final volume of 10 mL. All measurements were performed in triplicate. Total phenolic content was calculated using the linear regression equation obtained from the gallic acid calibration curve (y = ax + b) and expressed as gallic acid equivalents (GAE).

Total flavonoid content analysis

Total flavonoid content was determined using the aluminum chloride ($AlCl_3$) colorimetric method with quercetin as the reference standard. A quercetin stock solution (100 ppm) was prepared in 96% ethanol, and standard solutions (6.25–100 ppm) were used to construct a calibration curve. For analysis, 0.1 mL of standard or sample solution was mixed with 0.5 mL of 10% $AlCl_3$ solution and 2 mL of 5% acetic acid. The mixtures were incubated at room temperature for 15 min, and absorbance was measured at 414 nm using a UV–Vis spectrophotometer. Sample solutions were prepared by dissolving 1 mg of red spinach leaf extract in 96% ethanol to a final volume of 10 mL. All measurements were performed in triplicate. Total flavonoid content was calculated using the linear regression equation obtained from the quercetin calibration curve (y = ax + b) and expressed as quercetin equivalents (QE), according to the method reported by Setiawansyah, Widiyawati, et al. (2025).

Antioxidant assay

Antioxidant activity index of red spinach was assessed using DPPH as previously reported by Setiawansyah, Arsul, et al., (2024). A 1 mL of standard (quercetin: 2 – 10 µg/mL) or red spinach solution (20 – 100 µg/mL) were mixed with 1 mL of DPPH solution (40 µg/mL). The mixtures were incubated at room temperature for 10 min in the dark, and absorbance was measured at 516 nm using a UV–Vis spectrophotometer. All measurements were performed in triplicate. The percentage of DPPH radical scavenging activity was calculated using the following equation:

$$Inhibition\ (\%) = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} x\ 100$$

The IC_{50} value, defined as the concentration required to inhibit 50% of DPPH radicals, was determined from the linear regression equation (y = bx + a) obtained by plotting percentage inhibition against sample concentration. A lower IC_{50} value indicates higher antioxidant activity.

The antioxidant activity was also evaluated using the Antioxidant Activity Index (AAI), which was calculated based on the ratio between the final concentration of DPPH and the IC_{50} value of the sample, according to Scherer & Godoy (2009), using the following equation:

$$AAI = \frac{[\text{DPPH}]}{\text{IC50}}$$

Where [DPPH] represents the final concentration of DPPH (µg/mL) in the reaction mixture, and is the concentration of the sample required to scavenge 50% of DPPH radicals. Based on the AAI values, antioxidant activity was classified as very weak (AAI < 0.5), weak (0.5 ≤ AAI < 1.0), moderate (1.0 ≤ AAI < 2.0), and strong (AAI ≥ 2.0).

Data analysis

All experimental data were obtained from triplicate measurements and are presented as mean ± standard deviation (SD). Data normality was assessed prior to statistical analysis. Differences among groups were analyzed using one-way analysis of variance (ANOVA). When a significant difference was observed ($p < 0.05$), post hoc analysis was performed using Tukey's honestly significant difference (HSD) test to identify pairwise differences between groups. A p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Extract yield

The extraction of red spinach (*Amaranthus tricolor* L.) leaves under different fermentation conditions resulted in variations in both solid extract weight and extraction yield, as presented in Figure 1.

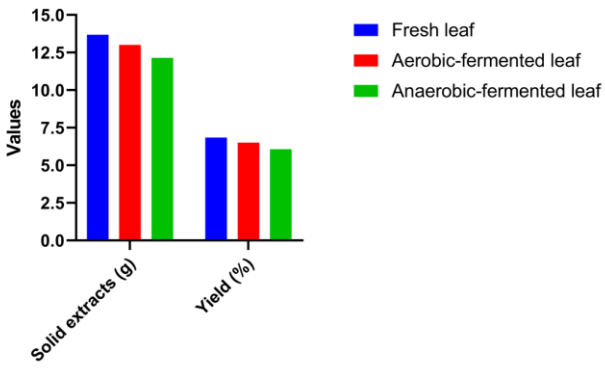

Figure 1. Solid extract weight (g) and extraction yield (%) of red spinach (*Amaranthus tricolor* L.) leaves under different fermentation treatments.

Fresh leaf extract yielded the highest solid extract weight at approximately 13.7 g, followed by aerobic-fermented leaf extract at 13 g, and anaerobic-fermented leaf extract at 12.2 g. This indicates a reduction of approximately 4.4% and 11.7% in solid extract weight for aerobic and anaerobic fermentation, respectively, compared to fresh leaves. For extraction yield, fresh

leaves demonstrated the highest percentage at approximately 6.84%, while aerobic-fermented leaves showed 6.5% and anaerobic-fermented leaves exhibited 6.1%. The fermentation treatments resulted in yield reductions of 4.3% and 11.6% for aerobic and anaerobic conditions, respectively, relative to the fresh leaf control.

Both parameters showed a consistent trend where fresh leaves produced the highest values, followed by aerobic-fermented leaves, with anaerobic-fermented leaves showing the lowest extraction performance. The parallel pattern between solid extract weight and extraction yield suggests a direct relationship between these two parameters across all treatment groups. The observed reductions in both solid extract weight and yield following fermentation treatments indicate that microbial processes during fermentation influence the extractability and recovery of compounds from red spinach leaves.

Total phenolic content

The total phenolic content of red spinach (*Amaranthus tricolor* L.) leaf extracts was determined using the Folin-Ciocalteu method with gallic acid as the standard. The calibration curve for gallic acid (Figure 2A) demonstrated excellent linearity with the equation $Y = 0.006333X + 0.1219$ and a correlation coefficient (R^2) of 0.9268, indicating a strong linear relationship between gallic acid concentration and absorbance values across the tested range of 20-100 µg/mL.

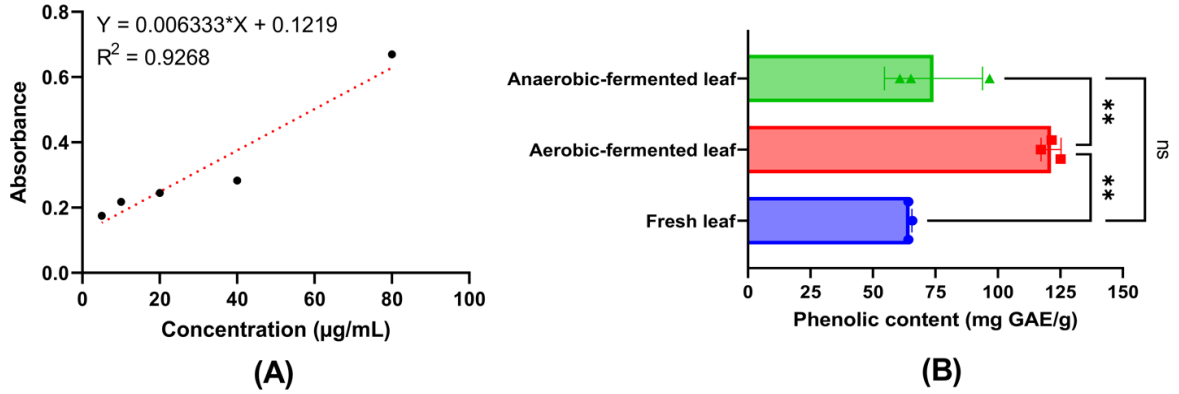

Figure 2. Gallic acid calibration curve and total phenolic content of red spinach (*Amaranthus tricolor* L.) leaf extracts. (A) Standard calibration curve of gallic acid showing the linear relationship between concentration (µg/mL) and absorbance, with regression equation $Y = 0.006333X + 0.1219$ and $R^2 = 0.9268$. (B) Total phenolic content (mg GAE/g) of fresh leaf, aerobic-fermented leaf, and anaerobic-fermented leaf extracts. Data are presented as mean ± standard deviation. Asterisks indicate significant differences between groups (** $p < 0.01$, ns = not significant).

The total phenolic content varied significantly among the different fermentation treatments (Figure 2B). Aerobic-fermented leaf extract exhibited the highest total phenolic content at approximately 121.28 ± 3.97 mg GAE/g, which was significantly different ($p < 0.01$) from both anaerobic-fermented and fresh leaf extracts. Anaerobic-fermented leaf extract showed an intermediate phenolic content of approximately 74.26 ± 19.60 mg GAE/g, while fresh leaf extract displayed the lowest value at 64.74 ± 0.92 mg GAE/g. Statistical analysis revealed highly significant differences ($p < 0.01$) between aerobic-fermented samples and the other two groups. The aerobic fermentation process notably enhanced the total phenolic content, resulting in an impressive 1.87-fold increase compared to fresh leaves, while anaerobic fermentation showed only a modest 1.15-fold increase.

Total flavonoid content

The total flavonoid content of red spinach (*Amaranthus tricolor* L.) leaf extracts was quantified using the aluminum chloride colorimetric method with quercetin serving as the reference standard. The calibration curve for quercetin (Figure 3A) exhibited exceptional linearity across the tested concentration range of 20-100 µg/mL, as evidenced by the regression equation $Y = 0.005194X + 0.1449$ and a remarkably high correlation coefficient (R^2) of 0.9922. This robust linear relationship between quercetin concentration and absorbance values demonstrates the reliability and precision of the analytical method employed for flavonoid quantification in the present study.

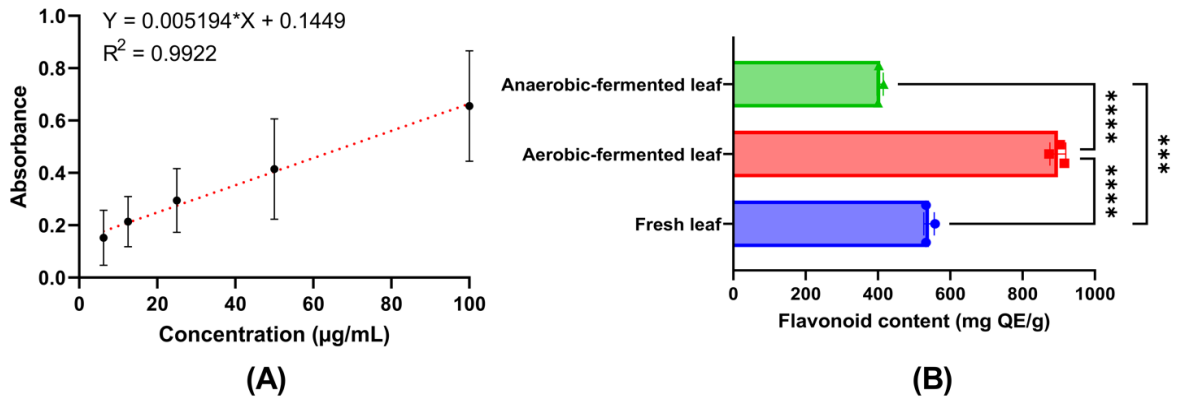

Figure 3. Quercetin calibration curve and total flavonoid content of red spinach (*Amaranthus tricolor* L.) leaf extracts. (A) Standard calibration curve of quercetin demonstrating the linear relationship between concentration (µg/mL) and absorbance, with regression equation Y = 0.005194X + 0.1449 and R^2 = 0.9922. Error bars represent standard deviation of measurements. (B) Total flavonoid content (mg QE/g) of fresh leaf, aerobic-fermented leaf, and anaerobic-fermented leaf extracts. Data are presented as mean ± standard deviation. Asterisks indicate statistically significant differences between groups (**** $p < 0.0001$).

The total flavonoid content revealed profound variations among the different fermentation treatments, with a clear hierarchical pattern emerging across all sample groups (Figure 3B). Aerobic-fermented leaf extract demonstrated the most remarkable flavonoid accumulation at approximately 897.82 ± 17.86 mg QE/g, representing the highest concentration among all treatments. Fresh leaf extract exhibited an intermediate flavonoid content of 541.41 ± 11.79 mg QE/g, while anaerobic-fermented leaf extract showed the lowest concentration at 406.15 ± 6.84 mg QE/g. Comprehensive statistical analysis confirmed highly significant differences ($p < 0.0001$) between all treatment groups, providing strong evidence that each fermentation condition exerted a distinct and measurable effect on flavonoid accumulation in red spinach leaves.

The aerobic fermentation process demonstrated a profound capacity to enhance total flavonoid content, producing an impressive 1.66-fold increase relative to unfermented fresh leaves. Surprisingly, anaerobic fermentation resulted in a decrease in flavonoid content, showing only 0.75-fold (a 25% reduction) of the fresh leaf value, indicating that oxygen-limited conditions may actually degrade or inhibit flavonoid preservation during fermentation.

Antioxidant activity

The antioxidant activity of red spinach (*Amaranthus tricolor* L.) leaf extracts was evaluated using the DPPH radical scavenging assay, which measures the capacity of antioxidant compounds to donate hydrogen atoms or electrons to neutralize free radicals. The relationship between extract concentration and DPPH radical inhibition percentage was examined across multiple concentrations for all treatment groups, with quercetin serving as the positive control (Figure 4A-D). Quercetin, the standard antioxidant compound, exhibited the most potent radical scavenging activity with the steepest concentration-response curve (Figure 4A), demonstrating rapid and efficient DPPH radical neutralization even at lower concentrations. Fresh leaf extract (Figure 4B) showed relatively consistent radical scavenging behavior across triplicates with nearly overlapping regression lines, indicating good reproducibility but moderate antioxidant potency. Aerobic-fermented leaf extract (Figure 4C) demonstrated remarkably uniform and enhanced radical scavenging kinetics with virtually identical regression equations across all replicates, showing steeper slopes than fresh leaves and suggesting superior antioxidant capacity with excellent experimental consistency. Anaerobic-fermented leaf extract (Figure 4D) exhibited highly consistent performance across triplicates, with similar slopes to aerobic-fermented samples but slightly lower y-intercepts.

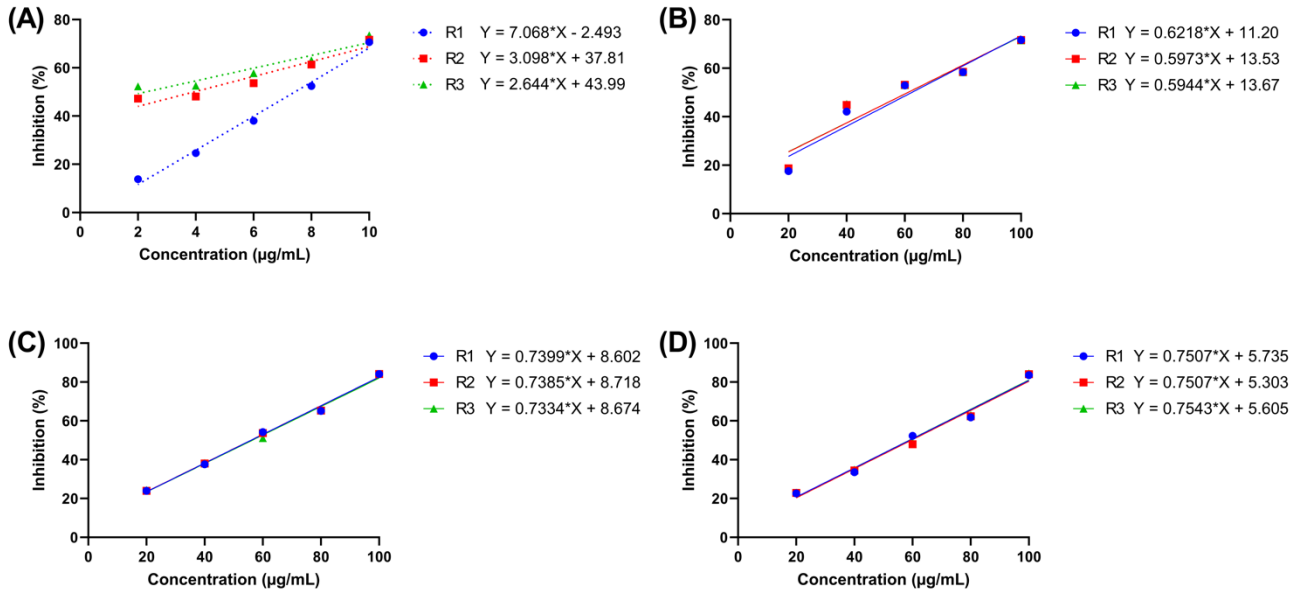

Figure 4. Concentration-dependent DPPH radical scavenging activity of red spinach (*Amaranthus tricolor* L.) leaf extracts. Relationship between extract concentration (µg/mL) and inhibition percentage (%) for (a) quercetin as positive control, (b) fresh leaf extract, (c) aerobic-fermented leaf extract, and (d) anaerobic-fermented leaf extract. R1, R2, and R3 represent three independent replicates with their respective linear regression equations. Data points represent individual measurements with dotted trend lines indicating the concentration-response relationship.

The antioxidant capacity was further quantified through IC_{50} values and AAI calculations, providing complementary metrics for evaluating radical scavenging efficiency (Figure 5). IC_{50} values, representing the concentration required to inhibit 50% of DPPH radicals, showed dramatic differences between treatments (Figure 5A). Quercetin demonstrated exceptional antioxidant potency with an IC_{50} value of approximately 4.5 ± 2.63 µg/mL, which was significantly lower ($p < 0.0001$) than all red spinach leaf extracts.

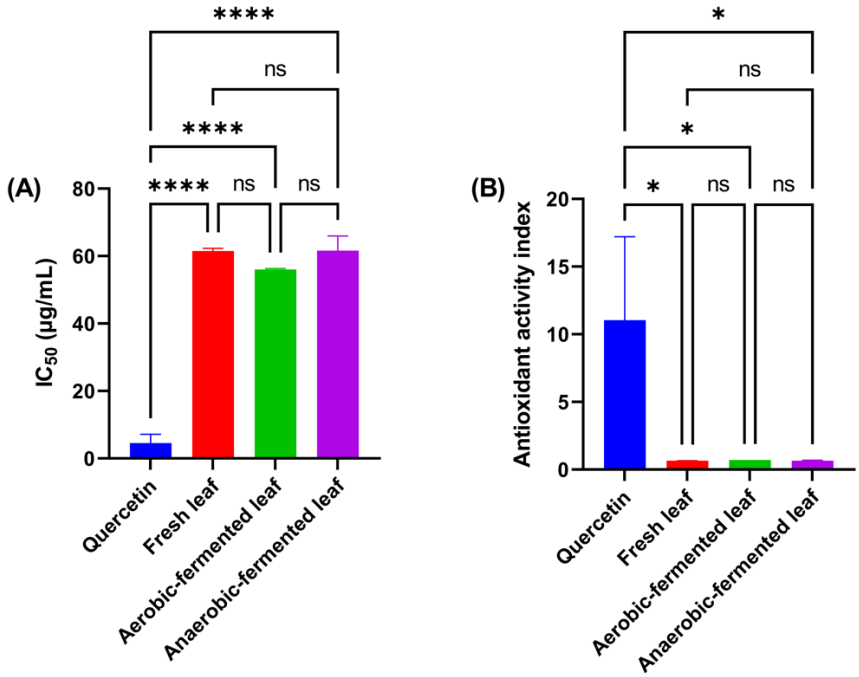

Figure 5. Antioxidant capacity of red spinach (*Amaranthus tricolor* L.) leaf extracts assessed by IC_{50} values and antioxidant activity index (AAI). (a) IC_{50} values (µg/mL) representing the concentration required to inhibit 50% of DPPH radicals. (b) Antioxidant activity index (AAI) calculated as the ratio of final DPPH concentration to IC_{50}. Data are presented as mean ± standard deviation. Asterisks indicate statistically significant differences (**** $p < 0.0001$, * $p < 0.05$, ns = not significant).

Among the experimental samples, aerobic-fermented leaf extract exhibited the lowest IC_{50} value at 56.07 ± 0.25 µg/mL, indicating the strongest antioxidant activity among all leaf treatments. Both fresh leaf extract and anaerobic-fermented leaf extract showed substantially weaker antioxidant capacity, with IC_{50} values of 61.53 ± 0.04 µg/mL and 61.66 ± 0.76 µg/mL, respectively. Statistical analysis revealed highly significant differences ($p < 0.0001$) between quercetin and all leaf extracts. While no significant differences were observed among the three leaf extract treatments in statistical comparison, aerobic-fermented samples showed a numerical improvement of approximately 8.9% in IC_{50} compared to fresh leaves, representing a modest but meaningful enhancement in antioxidant capacity.

The AAI provides an alternative metric that normalizes antioxidant activity relative to DPPH concentration, with values above 2.0 indicating very strong antioxidant activity, 1.0-2.0 indicating strong activity, 0.5-1.0 indicating moderate activity, and below 0.5 indicating weak activity. Quercetin exhibited the highest AAI of 11.05 ± 6.15, confirming its exceptional antioxidant capacity (Figure 5B). Among red spinach leaf extracts, aerobic-fermented samples demonstrated the highest AAI value at 0.713 ± 0.003, categorizing them as having moderate antioxidant activity. In contrast, both fresh leaf extract and anaerobic-fermented leaf extract showed lower AAI values of 0.650 ± 0.043 and 0.651 ± 0.01, respectively. Significant differences ($p < 0.05$) were observed between quercetin and all leaf extracts, while no significant differences were detected among the three leaf extract treatments. Nevertheless, the aerobic-fermented samples showed a 9.7% numerical increase in AAI compared to fresh leaves, paralleling the IC_{50} improvement and confirming the consistent trend of enhanced antioxidant performance following aerobic fermentation.

Pearson correlation analysis was performed to elucidate the relationships between antioxidant capacity metrics (IC_{50} and AAI) and polyphenolic compound concentrations (TFC and TPC) in red spinach leaf extracts (Figure 6). The correlation matrix revealed strong and highly consistent relationships between all measured parameters.

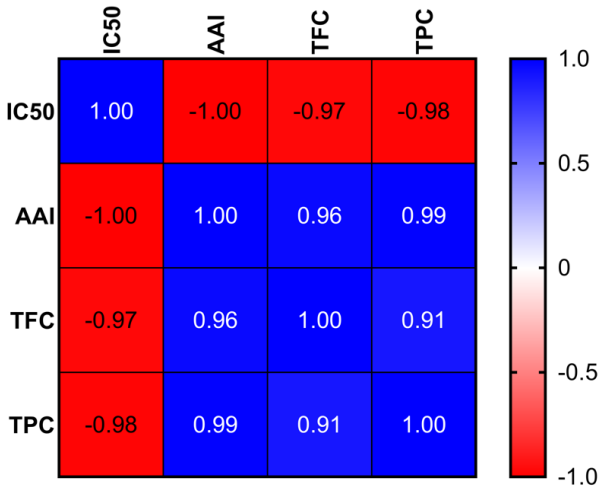

Figure 6. Pearson correlation matrix showing relationships between antioxidant activity parameters and polyphenolic content in red spinach (*Amaranthus tricolor* L.) leaf extracts. The heatmap displays correlation coefficients (r) between IC_{50}, antioxidant activity index (AAI), total flavonoid content (TFC), and total phenolic content (TPC). Blue color indicates positive correlations, red color indicates negative correlations, and color intensity represents correlation strength. Values range from -1.00 (perfect negative correlation) to 1.00 (perfect positive correlation).

IC_{50} values exhibited perfect negative correlations with AAI ($r = -1.00$), as expected from their inverse mathematical relationship, where lower IC_{50} values correspond to higher antioxidant activity. Notably, IC_{50} demonstrated very strong negative correlations with both TFC ($r = -0.97$) and TPC ($r = -0.98$), indicating that higher polyphenolic and flavonoid content was associated with lower IC_{50} values and thus stronger antioxidant capacity. Conversely, AAI showed strong positive correlations with both TFC ($r = 0.96$) and TPC ($r = 0.99$), confirming that increased polyphenolic compound concentrations directly corresponded to enhanced antioxidant activity index values. The relationship between TFC and TPC was also remarkably strong ($r = 0.91$), suggesting that flavonoid accumulation paralleled total phenolic content across different fermentation treatments.

These robust correlations provide compelling evidence that the enhanced polyphenolic and flavonoid content resulting from fermentation treatments directly contributes to the antioxidant capacity of red spinach leaf extracts. The near-perfect correlations observed ($r > 0.90$ for most relationships) suggest that phenolic and flavonoid compounds serve as the primary contributors to the radical scavenging activity in these extracts, with their concentration being a reliable predictor of antioxidant performance. This finding underscores the importance of fermentation as a biotransformation strategy for modulating not only the phytochemical profile but also the functional antioxidant properties of red spinach leaves.

Discussion

The present study demonstrates that aerobic fermentation significantly enhances the phytochemical profile and antioxidant capacity of red spinach (*Amaranthus tricolor* L.) leaves, with profound implications for the development of nutraceutical products with superior bioactive properties. Indeed, the substantial increases in total phenolic content following aerobic fermentation represent one of the most significant findings of this investigation and provide clear evidence that fermentation quality should be assessed based on bioactive compound enrichment rather than simple yield metrics. Aerobic-fermented samples exhibited the highest phenolic content with an impressive 1.87-fold increase over fresh leaves, while anaerobic fermentation achieved only minimal enhancement (1.15-fold increase). This striking difference between aerobic and anaerobic fermentation outcomes suggests that oxygen availability plays a critical role in determining the extent and nature of phenolic compound liberation and accumulation during fermentation. The superior performance of aerobic fermentation indicates that aerobic microorganisms, including various bacteria, yeasts, and molds that thrive under oxygen-rich conditions, possess enzymatic machinery particularly effective at degrading plant cell wall matrices and releasing bound phenolic compounds. Bhanja Dey & Kuhad (2014) reported that aerobic microorganisms can produce a diverse array of extracellular enzymes, including cellulases, hemicellulases, pectinases, and various oxidoreductases, that synergistically degrade complex polysaccharide structures sequestering phenolic compounds in plant tissues, thereby liberating these bioactive molecules and making them more accessible for extraction. Furthermore, aerobic conditions may favor specific biotransformation pathways that convert phenolic glycosides to their corresponding aglycones through β-glucosidase activity, while simultaneously preventing the excessive oxidative degradation that might occur with certain aerobic microorganisms when fermentation parameters are not properly controlled (Xiao et al., 2016). The contrast with anaerobic fermentation, which produced only marginal phenolic enhancement, suggests that lactic acid bacteria and other obligate anaerobes, while effective at producing organic acids and preserving foods, may lack the comprehensive enzymatic toolkit necessary for extensive cell wall degradation and phenolic liberation in leafy vegetable matrices.

Building upon the phenolic enrichment patterns, the total flavonoid content showed even more striking differences between fermentation modes, with aerobic fermentation producing remarkably superior results. Aerobic fermentation achieved the highest flavonoid content (1.66-fold increase) compared to fresh leaves. In stark contrast, anaerobic fermentation resulted in a dramatic decrease (25% loss) of flavonoids. This unexpected degradation of flavonoids under anaerobic conditions represents a critical finding that fundamentally distinguishes the two fermentation approaches. The mechanisms underlying flavonoid enrichment during aerobic fermentation likely involve multiple complementary biochemical pathways. First, aerobic microorganisms can produce specific β-glucosidases (Ahmed et al., 2017; Karageorgou et al., 2022) that efficiently hydrolyze flavonoid glycosides, which are the predominant forms of flavonoids in fresh plant tissues, into their more bioactive and more readily extractable aglycone forms (Day et al., 1998; PHAM & SHAH, 2009; Walle et al., 2005). Second, aerobic fermentation facilitates controlled cell wall disruption by inhibiting aldehyde dehydrogenase via enhancement of nitric oxide production (Stiti et al., 2020; Tsuchiya et al., 2021; Yamamoto et al., 2024), thus releasing intracellular and cell wall-bound flavonoids without causing their degradation. Third, the metabolic diversity of aerobic microbial communities may include organisms capable of biotransforming complex flavonoid into more extractable flavonoid structures (Park et al., 2021). The catastrophic loss of flavonoids under anaerobic conditions, however, reveals important limitations of oxygen-limited fermentation for leafy vegetables. Several factors may explain this degradation: lactic acid bacteria, which dominate anaerobic fermentation, may produce enzymes or metabolites that degrade flavonoid structures (Huang et al., 2024); the highly acidic environment created during lactic acid fermentation may chemically destabilize certain flavonoid compounds (Fuguet et al., 2023; Lin et al., 2008); anaerobic conditions may favor reductive reactions that cleave flavonoid ring structures (Lv et al., 2025; Schink, 2006); or the absence of oxygen may prevent the oxidative coupling reactions necessary for maintaining flavonoid stability in plant matrices (Yang et al., 2022). This fundamental difference in flavonoid preservation represents a critical advantage of aerobic fermentation and directly impacts the functional quality of the resulting extracts.

The antioxidant activity results provide important validation that the phytochemical enhancements achieved through aerobic fermentation translate into meaningful functional improvements. While fermented extracts did not achieve the exceptional antioxidant potency of pure quercetin, the comparison among leaf extracts revealed a clear pattern favoring aerobic fermentation. Aerobic-fermented leaf extract exhibited the lowest IC_{50} value, representing an 8.9% improvement compared to fresh leaves and an even greater improvement compared to anaerobic-fermented samples. Similarly, the AAI results corroborated this superiority, with aerobic-fermented samples achieving the highest value, representing a 9.7% increase over both fresh leaves and anaerobic-fermented samples. Although statistical analysis revealed no significant differences among the three leaf extract treatments due to experimental variability and the relatively modest magnitude of differences, the

consistent numerical superiority of aerobic-fermented samples across both antioxidant metrics, combined with their dramatically higher phytochemical content, provides compelling evidence of functional enhancement. The relationship between phytochemical enrichment and antioxidant activity improvement demonstrates that aerobic fermentation produces not merely higher quantities of phenolic compounds but also a qualitatively superior phytochemical profile optimized for radical scavenging activity. The DPPH assay measures hydrogen atom transfer and single electron transfer mechanisms of antioxidant activity (Bondet et al., 1997), and the consistent improvements observed in aerobic-fermented samples suggest that this fermentation mode generates phenolic compounds with structural features particularly well-suited for these mechanisms, such as optimal hydroxylation patterns, reduced steric hindrance, and appropriate molecular sizes for efficient radical interaction (Nimse & Pal, 2015; Perron & Brumaghim, 2009; Zeb, 2020).

The strong negative correlations observed between IC_{50} values and both total phenolic content ($r = -0.98$) and total flavonoid content ($r = -0.97$) provide compelling statistical evidence that polyphenolic compounds serve as the primary determinants of antioxidant capacity in red spinach leaf extracts. These near-perfect correlations indicate that across all samples, the overall trend of increasing phenolic and flavonoid content directly corresponds with decreasing IC_{50} values, representing stronger antioxidant activity. This phenomenon can be attributed to the increased capacity of phenolic and flavonoid compounds to donate hydrogen atoms and electrons as their concentrations rise, thereby enhancing their effectiveness in neutralizing free radical species (Nowak et al., 2022; Piechocka et al., 2021). The presence of multiple hydroxyl groups within these polyphenolic structures facilitates resonance stabilization of the resulting radical intermediates, which further strengthens their radical-scavenging efficiency and overall antioxidant activity. This finding aligns with previous research demonstrating that various classes of phenolic and flavonoid compounds work synergistically to provide antioxidant protection through complementary mechanisms (Heo et al., 2007; Zhang et al., 2023). These robust correlations validate the functional significance of the phytochemical enhancements achieved through aerobic fermentation, demonstrating that compositional improvements translate reliably into enhanced biological activity. The correlation data thus provide a mechanistic link between the dramatic differences in phytochemical content across fermentation treatments and their respective antioxidant capacities, confirming that aerobic fermentation's superior phytochemical enrichment directly underlies its enhanced functional properties.

From a practical standpoint, the aerobic fermentation-mediated enhancement of both phytochemical content and antioxidant capacity in red spinach leaves presents significant opportunities for developing value-added functional foods and nutraceutical ingredients. Aerobic fermentation emerges from this study as the clear optimal processing strategy due to its superior outcomes across all evaluated parameters. The relatively simple and cost-effective nature of aerobic fermentation technology makes this approach highly accessible for various scales of production. Unlike anaerobic fermentation which requires strict oxygen exclusion and specialized fermentation vessels, aerobic fermentation can be conducted in simple open or loosely covered containers with occasional mixing or stirring to ensure oxygen availability, reducing infrastructure requirements and capital costs. The moderate nature of aerobic fermentation, avoiding both the strict anaerobic conditions required for lactic acid bacteria and the intensive aeration required for some industrial fermentations, makes it particularly well-suited for small and medium-scale processors in developing regions where red spinach is cultivated. The 1.87-fold increase in total phenolic content and 1.66-fold increase in total flavonoid content achieved through aerobic fermentation represent substantial value additions that could justify premium pricing for fermented red spinach products, enable their use as concentrated sources of bioactive compounds in dietary supplements, or support health claims on functional food labels. Moreover, aerobic fermentation may improve the organoleptic properties of red spinach by reducing raw vegetable flavors, developing pleasant fermented notes, and potentially reducing bitterness or astringency through enzymatic modification of certain compounds, thereby increasing consumer acceptability compared to both fresh and anaerobically fermented products (Marco et al., 2017). The antimicrobial compounds and organic acids produced during controlled aerobic fermentation can also contribute to improved microbial stability and extended shelf life, adding food safety benefits beyond the phytochemical enhancements (Okoye et al., 2023; Sharma et al., 2020). The dramatic failure of anaerobic fermentation to enhance flavonoids, indeed causing a 25% loss, represents a critical practical finding that should guide industry away from traditional lactic acid fermentation approaches for leafy vegetables when the goal is nutraceutical value addition rather than simple preservation.

CONCLUSIONS

This study demonstrates that aerobic fermentation is an effective strategy to enhance the nutraceutical quality of red spinach (*Amaranthus tricolor* L.) leaves. Aerobic fermentation significantly increased total phenolic and flavonoid contents, resulting in improved antioxidant capacity, as evidenced by the lowest IC_{50} and highest antioxidant activity index values among treatments. In contrast, anaerobic fermentation provided limited phenolic enhancement and caused substantial flavonoid

degradation, with no improvement in antioxidant activity. Strong correlations between phytochemical levels and antioxidant parameters confirm their functional contribution. Overall, aerobic fermentation represents a simple, cost-effective approach for developing value-added functional food and nutraceutical ingredients from red spinach leaves.

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