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EVALUATION OF ANTIOXIDANT, ANTIBACTERIAL ACTIVITIES, AND EFFECTS ON SELECTED GUT MICROORGANISMS OF YOUNG RICE LEAF POWDER (*Oryza sativa*)

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INTRODUCTION

1. Rationale for the study

Rice (*Oryza sativa*) is a staple crop in many Asian countries, providing food for over half of the global population. Vietnam currently ranks fifth in the world in rice production and consumption, with the Mekong Delta being the key cultivation region.

Recently, numerous studies have explored the nutritional and medicinal value of young cereal leaves such as wheat, barley, and oats. Young leaves are rich in bioactive compounds with antioxidant, anti-inflammatory, detoxifying, anticancer, and antibacterial properties. Wheatgrass powder has been commercialized due to its high content of chlorophyll, polyphenols, amino acids, vitamins, minerals, and enzymes. Emerging research shows that rice leaves have a similar composition to wheatgrass, opening potential applications as a locally sourced material with high biological value.

Young rice leaves contain abundant polyphenols and flavonoids with antioxidant, anti-inflammatory, and anticancer activities. The vegetative tissues of rice also synthesize important bioactive compounds such as amino acids, flavonoids, phenolamides, terpenes, and vitamins. However, in-depth studies on the chemical composition, antibacterial activity, and effects on gut microbiota of young rice leaves remain limited. Moreover, factors affecting their quality, such as rice variety, growth conditions, harvest timing, and processing methods, have not been systematically investigated. Therefore, this study aims to clarify the biological potential of young rice leaves and establish a suitable production process to create high-value products.

2. Research objectives

This dissertation aims to study and develop young rice leaf (YRL) powder from *Oryza sativa* through:

(1) To optimize the harvesting conditions for young rice leaves to obtain the highest content of bioactive compounds.

(2) To establish a production process for bioactive young rice leaf extract powder (BLL) with high biological efficiency and compound stability.

(3) To evaluate the antioxidant, antibacterial activities, and the modulatory effects of BLL on the gut microbiota.

3. Research contents

1/ Determine the optimal rice variety, harvest timing, and shading conditions to enhance bioactive compound accumulation and antioxidant activity.

2/ Develop a production process for YRL powder by optimizing processing steps to preserve bioactive compounds and activities.

3/ Evaluate the antioxidant and antibacterial activities of YRL powder through in vitro assays and investigate its effects on gut microbiota via 16S rRNA analysis and physiological indicators in mice.

4. Scientific and practical significance

The study contributes to the scientific understanding of the antioxidant, antibacterial, and gut microbiota-modulating properties of young rice leaves. It establishes a production process for high-quality YRL powder, supporting the development of natural plant-derived products for functional foods, pharmaceuticals, cosmetics, and enhancing the commercial value of rice cultivation.

Chapter 1. RESEARCH OVERVIEW

1.1. Introduction to rice plants

1.1.1. Origin, classification, and distribution

Rice (*Oryza sativa*), belonging to the *Poaceae* family, originated from South and East Asia and is the most widely cultivated cereal globally. Taxonomically, *O. sativa* comprises two main subspecies: *indica* (distributed in tropical regions, heat-tolerant) and *japonica* (distributed in temperate regions, cold-tolerant). Their distribution is closely associated with ecological characteristics, adaptability, and cultivation practices in different climatic zones.

1.1.2. Rice production and varietal diversity in Vietnam

Vietnam ranks fifth in the world in rice production, with an annual yield of approximately 43–44 million tons of paddy grown on 7 million hectares. The Mekong Delta is the key production area, contributing over 50% of the national yield and most of the exported rice.

Vietnamese rice varieties are highly diverse, including indica, japonica, and local seasonal landraces with good tolerance to drought, salinity, and pests, which play an important role in breeding programs. Beyond its value as a staple food, rice is also a potential biological resource for functional foods, cosmetics, pharmaceuticals, and bioenergy, contributing to a circular economy.

1.1.3. Physiological characteristics and growth stages

Rice plants typically reach 90–120 cm in height (hybrid varieties) or taller in traditional landraces. The leaves consist of sheath, blade, ligule, and auricle, serving as the primary site for photosynthesis. The growth cycle comprises three stages: vegetative, reproductive, and ripening, lasting 3–6 months depending on the variety.

In the Mekong Delta, mainly indica varieties (IR50404, OM, ST, KC) and local landraces (Nang Thom, Tai Nguyen, Nep Tim, Huyet Rong) are cultivated. IR50404 has a short growth duration (85–90 days) but lower

grain quality, while local landraces have longer growth periods, aromatic grains, higher quality, or are rich in anthocyanins (medicinal rice).

1.2.2. Phytochemical compounds in rice leaves

1.2.2.1. Chlorophyll

Rice leaves have high chlorophyll content, comparable to or exceeding wheatgrass but lower than barley leaves. Chlorophyll and chlorophyllin exhibit anticancer, liver-detoxifying, anti-inflammatory, immune-enhancing, blood-cleansing, tissue-regenerating, weight management, and gut microbiota-balancing activities.

1.2.2.2. Polyphenolic compounds

Polyphenols are key secondary metabolites with antioxidant, anti-inflammatory, antibacterial, and cytoprotective properties. Young rice leaves are rich in phenolics and flavonoids, especially anthocyanins in pigmented rice varieties. Polyphenolic composition depends on the variety, harvest stage, and cultivation conditions.

Major compounds include anthocyanins, catechin, epicatechin, EGCG, rutin, protocatechuic acid, vanillic acid, caffeic acid, ferulic acid, luteolin, and kaempferol. These compounds contribute to antioxidant activity, cardiovascular protection, blood glucose regulation, anti-aging, and neuroprotection.

1.3. Factors influencing phytochemical composition

1.3.1. Rice variety

Genetic factors determine the ability to accumulate bioactive compounds. Pigmented rice, especially black rice, contains higher levels of phenolics, flavonoids, and anthocyanins compared to white rice. Varieties tolerant to drought or salinity also tend to accumulate more bioactive compounds.

1.3.2. Growth stage

Young leaves (5–7 days old) have the highest chlorophyll and polyphenol content, which declines as the plant matures. Early harvesting is optimal to obtain leaves rich in bioactive compounds.

1.3.3. Light conditions

Low light promotes chlorophyll accumulation to maintain photosynthesis, whereas strong light stimulates the synthesis of flavonoids and phenolics, enhancing antioxidant activity. Shading reduces flavonoid content, while high light intensity increases secondary metabolite levels.

1.4. Processing methods for rice leaf powder

1.4.1. Solvents used in extraction

Solvent extraction is a common method to obtain antioxidant compounds from plants. The ideal solvent should be safe, volatile, and not cause degradation of bioactive compounds. Ethanol and water are widely used due to their safety and good solubility for flavonoids and polyphenols. Selecting the appropriate solvent type, concentration, and optimal extraction conditions is crucial for ensuring the biological quality of the extract.

1.4.2. Enzyme inactivation methods

Enzyme inactivation, particularly of polyphenol oxidase (PPO), helps prevent oxidation, retain the green color of leaves, and preserve bioactive compounds. Methods include thermal blanching, microwave, ultrasound, or their combinations. Thermal blanching can inhibit PPO but may also reduce chlorophyll content. Combining ultrasound with controlled temperature provides higher efficiency by inhibiting PPO while better preserving chlorophyll and polyphenols. For rice leaves, optimization is required to maximize biological value.

1.4.3. Spray-drying method

Spray-drying is a widely used technique to produce powders, preserving bioactive compounds, improving product stability, and facilitating storage. Maltodextrin (MD) is commonly used as a carrier to protect polyphenols, flavonoids, and anthocyanins from oxidation. Drying efficiency depends on temperature, pressure, feed rate, and MD concentration. Spray-drying is considered a cost-effective and efficient method for producing high-quality young rice leaf powder.

1.5. Gut microbiota and the impact of plant-derived products

The gut microbiota (GM) comprises trillions of bacteria, mainly belonging to *Bacteroidetes* and *Firmicutes*, playing essential roles in nutrient

metabolism, mucosal protection, energy balance, and immune regulation. GM produces short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate, which benefit gut health and help prevent obesity and colorectal cancer.

Plant compounds, particularly polyphenols, exert positive effects on GM by promoting beneficial bacteria (e.g., *Lactobacillus*, *Bifidobacterium*), stimulating SCFA production, inhibiting pathogenic bacteria, improving microbial diversity, and modulating the *Firmicutes*/*Bacteroidetes* ratio.

There is a bidirectional interaction: gut microbes metabolize polyphenols into bioactive metabolites, while polyphenols modulate the structure of GM. Through this mechanism, plant extracts have potential to support digestive health, alleviate metabolic disorders, and enhance immunity.

1.6. Antioxidant activity assays

The antioxidant activity of bioactive compounds is evaluated in vitro based on the single electron transfer (SET) mechanism using common assays such as DPPH, ABTS (TEAC), FRAP, and RP, all of which measure color changes upon reduction of specific substrates by antioxidants.

- DPPH: Measures free radical scavenging capacity against DPPH radicals, reducing the purple color at 517 nm, directly reflecting antioxidant capacity.
- ABTS: Measures the reduction of $ABTS^{+}$• blue-green radicals at 734 nm, expressed as Trolox equivalent antioxidant capacity (TEAC).
- FRAP: Measures the reduction of the Fe^{3+}–TPTZ complex to Fe^{2+}–TPTZ (deep blue color) at 593 nm.
- RP (Reducing Power): Measures the ability to reduce Fe^{3+} to Fe^{2+}, forming Prussian Blue detected at 700 nm.

Combining multiple SET-based assays provides a comprehensive evaluation of the free radical neutralizing and metal-reducing capacity of young rice leaf powder (YRL), clarifying the roles of polyphenols, flavonoids, and chlorophyll.

1.7. Pathogenic microorganisms used in antibacterial and antifungal evaluation

Pseudomonas aeruginosa: A Gram-negative, aerobic bacterium causing burn wound, lung, bloodstream, and urinary infections, resistant to multiple antibiotics, and a major hospital-associated pathogen.

Staphylococcus aureus: A Gram-positive bacterium causing skin, bloodstream, lung, cardiovascular infections, and food poisoning; particularly dangerous are methicillin-resistant strains (MRSA).

Escherichia coli: A Gram-negative bacterium normally residing in the gut but capable of causing enteritis, urinary tract infections, and sepsis; many strains exhibit high virulence and antibiotic resistance.

Candida albicans: An opportunistic yeast residing on skin and mucosa, causing dermatitis, mucosal infections, and systemic fungal infections in immunocompromised individuals, often difficult to treat due to drug resistance.

1.8. Studies on the biological activities of rice leaves in domestic and international contexts

1.8.1. International studies

Research has evaluated the bioactive compound composition (polyphenols, anthocyanins, phenolic acids, volatile fatty acids) in different rice varieties and growth stages. Several studies have demonstrated DNA-protective effects, anti-inflammatory, anticancer properties, and support for managing obesity and diabetes. However, no studies have focused on rice leaf powder products or their impact on gut microbiota.

1.8.2. Domestic studies

There are no direct studies on rice leaves, but many related investigations have optimized extraction, spray-drying, and assessed antioxidant and antibacterial activities from other plant sources (e.g., germinated rice, brown rice, pandan leaves, noni leaves, Peperomia pellucida). Spray-drying techniques, optimal solvent selection, carriers, and processing conditions help preserve polyphenols, chlorophyll, and bioactivities in powdered products.

In summary, young rice leaves (YRL) have potential antioxidant, antibacterial, and health-protective effects due to their richness in polyphenols, flavonoids, and chlorophyll. In vitro assays (DPPH, ABTS,

FRAP, RP) are appropriate for evaluating these activities. Further research on YRL, especially powdered products and their influence on gut microbiota, represents a necessary and novel research direction.

Chapter 2. MATERIALS AND METHODS OF STUDY

2.1. Research materials

2.1.1. Research sites

The study was conducted at the Center for Advanced Science and Technology Application, Can Tho, and Can Tho University of Medicine and Pharmacy.

2.1.2. Research materials

Rice varieties: Six varieties including IR50404 (high-yield), Nang Thom, Tai Nguyen, Huong Lai (aromatic rice), Nep Tim and Huyet Rong (pigmented rice).

Microbial strains: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and the fungus *Candida albicans* (standard ATCC strains).

Experimental animals: Healthy 8-week-old BALB/c mice.

2.2. Research methods

2.2.1. General experimental design

The experiment included the following steps: planting – harvesting leaves – sample processing – biochemical analysis – powder production – bioactivity evaluation.

2.2.2. Experimental layout

Experiment 1: Investigated the effects of rice variety (6 varieties), growth stage (1–6 weeks), and light conditions (natural, 1-layer shading, 2-layer shading) on chlorophyll, polyphenol, soluble fiber content, antioxidant activity (DPPH), and bioactive compound composition analyzed by GC-MS.

Experiment 2: Optimized the production process of young rice leaf (YRL) powder through four steps: enzyme inactivation by blanching, microwave, ultrasound at different durations; extraction with ethanol at 0–80% concentrations and solvent/leaf ratios of 5:1–15:1; spray drying at 110–

160 °C compared to freeze-drying; and powder storage at room temperature, 5 °C, and –18 °C for 6 months. Evaluation criteria included chlorophyll, polyphenol, flavonoid, soluble fiber, and antioxidant capacity.

Experiment 3: Assessed the biological activities of YRL powder, including: 1/ Antioxidant activity via DPPH, ABTS, FRAP, RP assays (compared with vitamin C); 2/ Antibacterial and antifungal activity by agar well diffusion and MIC determination; 3/ Effects of YRL on gut microbiota in mice (16S rRNA analysis).

The experiment used 45 BALB/c mice (8 weeks old), divided into 3 groups: control, YRL150 and YRL300 (150–300 mg/kg/day) for 14 days. Mice were gavaged with YRL dissolved in distilled water, adjusted to body weight. Fresh feces were collected in the morning, pooled by group, stored at −80 °C for analysis of LAB, aerobic bacteria, and microbiota (16S rRNA). Body weight gain, hematology, and small intestine histology were also assessed.

2.3. Analytical methods

2.3.1. Leaf extract preparation: Extracted with 80% ethanol, filtered through Whatman No. 4, rotary evaporated at 45 °C, stored at −18 °C.

2.3.2. Chlorophyll analysis: Extracted with 80% ethanol, UV-Vis spectrophotometry at 645 nm and 663 nm, calculated total, a, and b chlorophyll.

2.3.3. Total polyphenol analysis: Folin–Ciocalteu method, absorbance at 750 nm, expressed as mg GAE/g fresh weight.

2.3.4. Total flavonoid analysis: Using $NaNO_2$, $AlCl_3$, NaOH, spectrophotometry at 510 nm, expressed as quercetin equivalent (QE).

2.3.5. Soluble fiber analysis: Precipitated with cold ethanol, centrifuged, dried, and weighed.

2.3.6. PPO enzyme activity: Pyrocatechol as substrate, absorbance at 420 nm, enzyme activity in IU.

2.3.7. DPPH assay: Reacted with 0.15 mM DPPH solution, absorbance at 517 nm, calculated % inhibition and IC_{50}.

2.3.8. ABTS assay: Standardized $ABTS^{\bullet+}$ solution, 6-minute reaction, absorbance at 734 nm, % inhibition and IC_{50}.

2.3.9. Reducing power (RP): Using $K_3Fe(CN)_6$, absorbance at 700 nm, expressed as IC_{50}.

2.3.10. FRAP assay: FRAP reagent (TPTZ + $FeCl_3$), incubated at 37 °C, absorbance at 593 nm, IC_{50} determination.

2.3.11. GC-MS/MS analysis of compounds: Agilent 7890B–7000C system, spectra matched with NIST-MS and Wiley libraries.

2.3.12. Catechin analysis by HPLC: Alliance E2695 system, C18 column, detection at 280 nm, identification of catechin, EGCG, ECG.

2.3.13. Agar well diffusion: Cultured microbes on LB/SD agar, added extracts, measured inhibition zones (IZ).

2.3.14. MIC determination: Serial dilution in LB/SD broth, OD600/530 nm measurement, recorded minimal inhibitory concentration.

2.3.15. LAB/aerobic bacteria count: Cultured on MRS + $CaCO_3$ agar and LB agar, CFU counted, expressed as $\log_{10}$ CFU/mL.

2.3.16. Culture media: MRS agar for LAB, LB agar for aerobic bacteria, SD agar for C. albicans.

2.3.17. Hematology test: Cardiac puncture blood collection, EDTA anticoagulant, analysis of RBC, WBC, PLT, Hb, HCT, MCV, MCH, RDW.

2.3.18. Small intestine histology: Fixed in 4% paraformaldehyde, dehydrated, paraffin-embedded, 5 μm sections, H&E staining, observed under microscope.

2.3.19. 16S rRNA gene sequencing: DNA extracted using QIAamp kit, PCR of V3–V4 region, Illumina MiSeq sequencing, processed with QIIME2, classified with SILVA, functional prediction with PICRUSt2.

2.3.20. Data processing: Data processed using Excel, ANOVA and Duncan's test ($p<0.05$) with Statgraphics, PCA, clustering, and Spearman correlation analyzed using XLSTAT.

Chapter 3. RESULTS AND DISCUSSION

3.1. Effects of growth stage and light conditions on bioactive compounds and antioxidant activity of young rice leaves

3.1.1. Total chlorophyll content

Chlorophyll content gradually increased from week 1 to week 5 and slightly decreased by week 6. Variety HRO peaked at week 4, while HLA reached the highest at week 3, and NTI, IR504, and TNG peaked at week 5. No shading or single-layer shading maintained higher chlorophyll levels compared to double-layer shading ($p<0.05$). Varieties HLA, IR504, and NTI were more prominent than HRO, NTH, and TNG. The optimal harvest time was weeks 4–5 without shading.

3.1.2. Chlorophyll a/b ratio

The chlorophyll a/b ratio varied by variety and stage, with HRO highest at week 2 and HLA lowest. No significant differences were observed among shading levels, but HRO and TNG tended to have higher ratios than NTI. Double-layer shading produced the highest ratio, followed by no shading, and was lowest with single-layer shading.

3.1.3. Total polyphenol content

Polyphenols increased from week 1 to week 5 and slightly declined at week 6. IR504 had the highest value, followed by TNG and NTH. Full light stimulated polyphenol accumulation, while shading significantly reduced it. IR504 and NTH stood out the most under non-shaded conditions.

3.1.4. Soluble fiber content

Soluble fiber increased sharply until week 4 and then stabilized. Unlike chlorophyll and polyphenols, double-layer shading promoted the highest accumulation of soluble fiber. HRO, NTH, and NTI responded strongly to shading, while TNG showed little variation.

3.1.5. DPPH radical scavenging activity

Antioxidant activity peaked at week 5 and declined thereafter. IR504 and HRO showed the highest values. No shading significantly enhanced

DPPH scavenging activity ($p<0.05$), while double-layer shading markedly reduced it, with HLA being the lowest.

3.1.6. Correlation between compounds and antioxidant activity

Polyphenols showed a strong correlation with DPPH activity, while total chlorophyll was also associated with both polyphenols and DPPH. Conversely, soluble fiber and a/b ratio had no significant correlations. Cluster analysis revealed polyphenols closely linked with DPPH, while chlorophyll clustered with the a/b ratio.

3.1.7. General evaluation

Growth stage and light conditions significantly influenced bioactive compounds and antioxidant activity. Week 5 under non-shaded conditions was optimal for harvesting young rice leaves rich in chlorophyll, polyphenols, and antioxidant activity, particularly in IR504. Strong light stimulated polyphenol accumulation, while mild shading favored soluble fiber accumulation.

3.1.2. Effect of growth stage on the biosynthesis of compounds in young rice leaves

GC-MS analysis of six rice varieties at weeks 1, 3, and 5 revealed clear changes in compound composition. Week 1 was dominated by monoterpenoids, fatty acid esters, and phenols, reflecting initial metabolic activity. Week 3 showed increased meroterpenoids, steroids, fatty acid amines, and straight-chain hydrocarbons—representing the intermediate phase. Week 5 exhibited strong diversification with benzaldehydes, fatty nitriles, triterpenoids, heterocycles, and fatty acid derivatives, indicating physiological maturity.

By variety, IR504 and HRO consistently maintained diterpenoids, phthalate esters, and steroids throughout, but week 5 showed additional characteristic compounds. TNG and NTH increased meroterpenoids, steroids, and heterocycles at later stages. NTI and HLA displayed the richest compound diversity at week 5, with benzaldehydes, cyclic hydrocarbons, fatty amines, and triterpenoids.

In summary, the growth stage strongly affected secondary metabolite biosynthesis in rice leaves, with week 1 representing the initiation phase, week 3 the intermediate phase, and week 5 the maturity phase with the highest compound diversity.

3.1.2.2. Effect of light conditions on compound biosynthesis in rice leaves

GC-MS analysis at week 5 showed that light strongly shaped the metabolic profiles of rice leaves. Under non-shaded conditions, varieties IR504, HLA, HRO, NTH, TNG, and NTI predominantly synthesized diterpenoids, phenols, steroids, meroterpenoids, phthalate esters, fatty alcohols, fatty aldehydes, fatty nitriles, and heterocycles—compounds with high bioactivity. In contrast, double-layer shading promoted lipid synthesis and intermediates such as esters, fatty acid amides, straight-chain hydrocarbons, triterpenoids, terpene ketones, and alkaloids.

CA analysis and dendrograms showed that non-shaded samples clustered distinctly with more homogeneous compound composition, while shaded samples were dispersed and formed separate groups, reflecting adaptive metabolic characteristics under low light.

In conclusion, strong light stimulated the biosynthesis of highly bioactive secondary metabolites, while shading promoted lipid metabolic pathways and adaptive compounds.

3.1.2.3. Evaluation of the effects of growth stage and shading conditions on the biosynthesis of compounds in young rice leaves

GC-MS analysis showed that growth duration and light exposure were two key factors governing the composition of secondary metabolites. Over time, from week 1 to week 5, rice leaves diversified their compound profiles, reflecting increased metabolic demands as the plant developed. Regarding light conditions, full light maintained compound diversity, whereas shading promoted the accumulation of specific compounds for adaptation to low-light environments. Based on these findings, two major metabolic pathways were proposed:

***Chlorophyll–phytol metabolic pathway during growth*

Phytol, the core structural component of chlorophyll, was abundant at all three growth stages. When chlorophyll was hydrolyzed, phytol was reutilized to synthesize vitamin E and fatty acid esters, while also aiding nutrient redistribution and preventing toxic intermediate accumulation. This process ensured sustained photosynthesis, leaf and shoot development, and provided precursors for essential secondary metabolites such as tocopherols, contributing to cell protection and optimized growth efficiency.

***Metabolic differentiation under shading conditions*

Under full light, rice leaves enhanced the synthesis of diterpenoids, steroids, phytosterols, and vitamin E—antioxidant compounds that support photosynthesis and protect cell membranes. In contrast, double-layer shading reduced photosynthetic efficiency, causing the plant to prioritize the accumulation of fatty acid esters, triterpenoids, and brassinosteroids to adjust membrane lipid composition and adapt to low light. Simultaneously, brassinosteroids and auxins increased, promoting leaf and stem elongation to improve light capture.

3.2. Investigation of optimal conditions for young rice leaf powder production

3.2.1. Methods for inactivating polyphenol oxidase (PPO)

3.2.1.1. Effect of enzyme inactivation methods on chlorophyll loss

Chlorophyll loss depended on the method and duration of treatment. Blanching caused the lowest loss (approximately 5–10%), microwaving resulted in higher losses (7–18%), while ultrasonication had the highest, up to 20% after prolonged exposure. Blanching was more effective than microwaving and ultrasonication.

3.2.1.2. Effect of enzyme inactivation methods on polyphenol loss

Blanching caused greater polyphenol loss than other methods (3–15%), whereas microwaving resulted in the lowest loss (<7%) and ultrasonication only increased significantly after 25 minutes. Statistical analysis showed blanching induced higher loss but was not significantly different compared to long microwave or prolonged ultrasound treatment.

3.2.1.3. Effect of treatments on PPO inhibition

Blanching inactivated PPO the fastest and most effectively (>80%), microwaving achieved moderate inhibition (~30–42%), while ultrasonication was the lowest (~20–35%).

3.2.1.4. Overall evaluation

Combined with its sterilizing ability and removal of impurities, blanching at 100°C for 4 minutes was determined optimal, achieving over 80% PPO inactivation while keeping chlorophyll and polyphenol losses minimal throughout the process.

3.2.2. Effect of ethanol ratio and concentration on compound extraction from young rice leaves

3.2.2.1. Effect on chlorophyll extraction

Total chlorophyll content increased with ethanol concentration, peaking at 60–80%, with significant differences compared to other concentrations ($p<0.05$). A ratio of 10:1 was identified as optimal, yielding about 1310–1340 μg/g chlorophyll at 60–80% ethanol.

3.2.2.2. Effect on polyphenol extraction

Ethanol extracted polyphenols more effectively than water ($p<0.05$). At a 5:1 ratio, polyphenol content decreased slightly from 20–60% ethanol, then increased at 80%, while at 10:1 and 15:1 ratios, it increased from 20–60% and slightly decreased at 80%. Polyphenols were highest at 60% ethanol with a 10:1 ratio, equivalent to 15:1 but saving more solvent.

3.2.2.3. Overall evaluation

Ethanol at 60–80% was optimal for extracting chlorophyll and polyphenols, with chlorophyll highest at 80% and polyphenols at 60%. A solvent-to-leaf ratio of 10:1 provided good extraction efficiency and solvent savings, being more suitable than 5:1 (too concentrated) and not less effective than 15:1.

3.2.3. Effects of spray-drying temperature on the quality of young rice leaf powder

3.2.3.1. Effects on chlorophyll, polyphenol content, and DPPH activity

High spray-drying temperatures significantly reduced chlorophyll, polyphenols, flavonoids, and antioxidant activity. At 120–130 °C, these indicators were better retained but decreased sharply when the temperature rose to 140–160 °C. Polyphenols and flavonoids were highest at 120 °C, lower than freeze-drying but still superior compared to higher temperatures. DPPH activity decreased with increasing temperature, peaking under freeze-drying, followed by 120 °C. Soluble fiber content was not significantly affected across conditions.

3.2.3.2. Correlation between compounds and DPPH activity

Spearman analysis showed a strong correlation between polyphenols and flavonoids with DPPH activity, followed by total chlorophyll and chlorophyll b. Cluster analysis confirmed the strongest association between polyphenols and DPPH, consistent with their primary role in free radical scavenging.

3.2.3.3. Overall evaluation

At 120 °C, Spray-dried young rice leaf powder retained the highest levels of chlorophyll, polyphenols, flavonoids, and DPPH activity compared to other temperatures. Spray-drying at 120 °C was deemed optimal, balancing quality, yield, and cost, making it more suitable for industrial-scale production than freeze-drying.

3.2.4. Quality analysis of young rice leaf powder according to food safety standards

No pathogenic microorganisms (*E. coli*, *Coliforms*, *Bacillus cereus*) or molds were detected in the powder, and the total aerobic bacterial count was low (2.2×10^3 CFU/g). Heavy metals (As, Hg, Pb, Cd, Zn) and aflatoxin levels were below detection limits, meeting food safety standards under Decision 46/2007/QĐ-BYT.

3.2.5. Effects of storage temperature and duration on the quality of young rice leaf powder

3.2.5.1. Effects on total chlorophyll content

Chlorophyll gradually decreased over six months of storage. At room temperature (27 ± 2 °C), the decline was fastest, while refrigeration at

5 °C slowed the degradation, and –18 °C maintained the highest chlorophyll content with statistically significant differences ($p < 0.05$).

3.2.5.2. Effects on total polyphenol content

Polyphenols also declined over time, with the slowest reduction at –18 °C, followed by 5 °C, and the fastest at room temperature. After six months, the values at –18 °C remained significantly higher ($p < 0.05$).

3.2.5.3. Effects on soluble fiber content

Soluble fiber showed slight fluctuations (500–545 mg/g) with no significant differences among storage conditions ($p > 0.05$).

3.2.5.4. Effects on DPPH free radical scavenging activity

DPPH activity gradually decreased over six months but was better maintained at 5 °C and –18 °C, while room temperature caused the fastest decline.

3.2.5.5. Overall evaluation of storage temperature and duration

High temperatures accelerated chlorophyll degradation (forming pheophytin) and polyphenol oxidation, reducing antioxidant activity. 5 °C and –18 °C were the most suitable storage conditions, maintaining chlorophyll, polyphenols, and antioxidant capacity of young rice leaf powder over time.

In summary, the recommended production process for young rice leaf powder includes: harvesting IR50404 leaves, blanching for 4 minutes, extracting with 60% ethanol at a solvent-to-leaf ratio of 10:1, spray-drying at 120 °C using 10% maltodextrin, and storing at 5 °C or –18 °C to optimize product quality.

3.3. Investigation of antioxidant activity of young rice leaf powder

3.3.1. HPLC analysis of catechin compounds

HPLC results showed that young rice leaf powder contained three major catechin-group polyphenols: Catechin (1.23 mg/L), Epigallocatechin gallate (EGCG, 0.90 mg/L), and Epicatechin gallate (ECG, 2.59 mg/L).

3.3.2. Investigation of the antioxidant activity of young rice leaf powder

Chlorophyll and polyphenol contents in the powder increased with concentration, peaking at 10% (w/v), while soluble fiber varied insignificantly. The powder demonstrated free radical scavenging activity

(DPPH, ABTS) and ferric reducing ability (FRAP, RP) but was much less potent than vitamin C.

3.3.3. Correlation analysis between chemical composition and antioxidant activity

Spearman analysis revealed a very strong correlation between polyphenols and antioxidant activities (DPPH, ABTS, FRAP, RP), similar to chlorophyll. Cluster analysis confirmed that polyphenols and chlorophyll were the two main compound groups determining the antioxidant capacity of the powder.

3.3.4. Evaluation of the antioxidant potential of young rice leaf powder

The powder contains catechins and their derivatives, contributing to free radical neutralization, metal ion reduction, and cell protection. Although its antioxidant activity is lower than many other plant powders rich in phenolics, the results still demonstrate its potential as a natural antioxidant source. This study provides a basis for developing young rice leaf powder as an ingredient for functional foods and health-protective products.

3.4. Investigation of the antibacterial and antifungal activities of young rice leaf powder

3.4.1. Antibacterial activity against *E. coli*, *P. aeruginosa*, *S. aureus* and antifungal activity against *C. albicans*

Young rice leaf powder (YRL powder) was tested at concentrations ranging from 500 to 8,000 μg/mL using the agar well diffusion method and MIC determination. The results showed that its antibacterial activity was concentration-dependent, with the strongest effect on *S. aureus* (maximum inhibition zone 21.33 mm), followed by *E. coli* (20.24 mm) and *P. aeruginosa* (19.33 mm), while antifungal activity against *C. albicans* was very weak (ZI ≤ 5 mm). MIC values ranged from 3.9–4.8 mg/mL for bacteria and > 8 mg/mL for *C. albicans*, much higher than standard antibiotics like Ampicillin/Metronidazole (MIC < 100 μg/mL). This indicates that YRL powder exhibits only mild antibacterial effects and is almost ineffective against yeast.

3.4.2. Evaluation of antibacterial and antifungal activity of YRL powder

YRL powder showed moderate to strong antibacterial activity on Gram-positive and Gram-negative bacteria at concentrations ≥ 2,000 μg/mL but negligible effects on *C. albicans*. The higher sensitivity of *S. aureus* may be attributed to the more permeable structure of Gram-positive bacteria to phenolic compounds. Catechins (EGCG, ECG) in YRL powder play a key role in bacterial inhibition by disrupting cell membranes and inhibiting intracellular enzymes, but the efficacy is limited due to the requirement for high concentrations. Spray-drying and the use of maltodextrin may reduce the content and diffusion ability of active compounds, leading to higher MIC values.

Overall, YRL powder only mildly inhibits microorganisms, particularly *S. aureus* and *E. coli*, but is not a strong antimicrobial agent. To enhance its effectiveness, extraction optimization, reducing carrier ratios, and combining with membrane permeabilizers or stronger antimicrobial compounds are recommended for potential applications in food preservation or therapeutic support.

3.5. Effects of consuming young rice leaf powder on the gut microbiota in mice

3.5.1. Investigation of the effects of YRL powder on gut microbiota

3.5.1.1. Hematological parameters

After 14 days of supplementation, mice in both 150 mg/kg (PC150) and 300 mg/kg (PC300) groups showed statistically significant increases in RBC, WBC, and PLT compared to the control group (PCDC), suggesting that YRL powder mildly supports hematopoiesis and immune regulation. Notably, WBC and leukocyte subpopulations (NEU, LYM, MON, EOS) increased markedly in the PC300 group, indicating that higher doses activate both innate and adaptive immunity. Conversely, HGB, HCT, MCV, and MCH slightly decreased but remained within physiological limits, indicating no anemia or impairment of oxygen transport. These results demonstrate that YRL powder is safe for hematological functions and may modulate immunity without inducing abnormal inflammation.

3.5.1.2. Small intestine histology

Histological examination of the small intestine (H&E staining) revealed intact epithelial structures in all groups, with regular villi and no signs of inflammation, necrosis, or epithelial loss. This indicates that YRL powder does not damage the intestinal mucosal barrier and meets safety standards for long-term use.

3.5.1.3. Body weight changes

During the 14-day trial, the PC150 group gained less weight than the control (1.5 g vs. 2.0 g), suggesting a potential weight-control effect. In contrast, the PC300 group showed significantly higher weight gain (3.2 g, $p < 0.05$), indicating that a higher dose promotes growth.

3.5.1.4. Counts of LAB and AeB

After 14 days of YRL powder supplementation, the control group (PCDC) maintained low counts of both AeB and LAB. At the dose of 150 mg/kg (PC150), both AeB and LAB increased significantly, with LAB rising several-fold compared to the control ($p < 0.05$), confirming the prebiotic effect of YRL powder by providing fermentable substrates and creating a favorable microenvironment for beneficial bacteria. Conversely, at the higher dose of 300 mg/kg (PC300), AeB remained elevated but LAB counts decreased compared to PC150, although still higher than the control. This may be due to the higher polyphenol concentration mildly inhibiting sensitive LAB strains or causing excessive pH reduction in the gut. This "low-dose optimal – high-dose less effective" phenomenon has been reported in many other prebiotic studies.

3.5.1.5. Composition of gut microbiota

16S rRNA analysis showed that YRL powder modulated gut microbiota diversity and structure in a dose-dependent manner. For alpha diversity indices, PC300 exhibited the highest Ace, Chao1, and Shannon values, reflecting a richer and more balanced microbial community. Beta diversity analysis via Bray-Curtis PCoA revealed that PC300 clustered

distinctly from the control and PC150, indicating a stronger impact of the higher dose on community structure.

At the phylum level, PC300 increased *Firmicutes*—associated with enhanced energy absorption—and *Desulfobacterota*—linked to sulfur metabolism—while reducing *Proteobacteria* and *Bacteroidota*, which are often associated with inflammation and short-term SCFA production. In contrast, PC150 markedly increased *Proteobacteria* compared to the control, indicating a trend toward weight control but potentially with a mild inflammatory risk.

At the family level, PC300 was dominated by *Lachnospiraceae* - a SCFA-producing group (e.g., acetate) that promotes energy absorption and weight gain. PC150, however, favored *Lactobacillaceae*, beneficial bacteria that reduce lipid absorption and help control body weight, though accompanied by an increase in Enterobacteriaceae, a family potentially associated with inflammation.

At the genus level, PC300 enriched *Lachnospiraceae* NK4A136 group, *Ruminococcus*, and *Roseburia*—SCFA producers that enhance energy absorption, glucose homeostasis, and growth promotion. In contrast, PC150 favored *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* - beneficial genera that regulate lipid metabolism, improve immunity, and reduce fat absorption.

Thus, the low dose of 150 mg/kg prioritizes *Lactobacillus*, supporting weight management, whereas the high dose of 300 mg/kg favors *Lachnospiraceae*, promoting energy absorption and weight gain.

3.5.1.6. Predicted functional profiling of gut microbiota

Predicted functional analysis (PICRUSt, KEGG KO, EC, MetaCyc) revealed clear dose-dependent differences. The lower dose (PC150) enhanced pathways for carbohydrate and lipid degradation as well as lactic fermentation, contributing to faster energy expenditure and weight control. Lipid-oxidizing enzymes such as acyl-CoA oxidase and triacylglycerol lipase were strongly expressed, promoting reduced fat accumulation.

Additionally, genes related to catechin metabolism into catechol were upregulated, helping decrease glucose absorption and improve energy metabolism.

Conversely, the higher dose (PC300) prioritized the synthesis of branched-chain amino acids (L-isoleucine, L-valine), which are associated with biomass accumulation and energy storage. This corresponds with the enriched microbiota composition rich in *Lachnospiraceae*, *Ruminococcus*, and *Roseburia* - groups that enhance energy absorption, metabolic efficiency, and weight gain. Meanwhile, the control group (PCDC) maintained basic nucleotide synthesis and genetic stability pathways, reflecting an unaltered baseline microbiota.

In summary, YRL powder is safe in mice, causing no hematological or histological toxicity. The 150 mg/kg dose optimally stimulates LAB, *Lactobacillus*, and *Bifidobacterium*, supporting immunity and weight management through enhanced carbohydrate and lipid degradation. In contrast, the 300 mg/kg dose enriches SCFA-producing bacteria such as *Lachnospiraceae* and *Ruminococcus*, increases microbial diversity, promotes energy absorption, and stimulates growth. This differential effect suggests YRL powder can be flexibly applied for either weight management or growth promotion, depending on the supplementation dose.

CONCLUSION AND RECOMMENDATIONS

Conclusion

This study demonstrated the biological value and application potential of rice leaf powder (RLP) from the IR50404 cultivar. Rice leaves harvested in the 5th week under full light conditions had the highest chlorophyll and polyphenol content, along with strong antioxidant activity. The optimal processing procedure included blanching at 100 °C for 4 minutes, extraction with 60% ethanol (solvent-to-leaf ratio 10:1), and spray drying at 120 °C, which effectively preserved bioactive compounds. The product remained most stable when stored at 5 °C or –18 °C.

Regarding bioactivity, RLP exhibited strong antioxidant capacity (IC_{50} ranging from 36.67–80.9 mg/mL) and inhibited *E. coli*, *P. aeruginosa*, and *S. aureus* at MIC values of 3939–4787 µg/mL, while its antifungal effect against *C. albicans* was weak (MIC > 8000 µg/mL). Component analysis showed that 5-week-old rice leaves were rich in bioactive compounds, suggesting a mechanism of phytol reutilization from chlorophyll degradation for the synthesis of fatty acids, vitamin E, and new chlorophyll, thereby adjusting the plant's adaptive metabolism.

RLP modulated gut microbiota composition in mice in a dose-dependent manner. The 150 mg/kg dose increased *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*, while enhancing gene expression for glucose and lipid degradation and catechin-to-catechol metabolism, supporting weight control and energy expenditure. The 300 mg/kg dose enriched *Lachnospiraceae*, *Ruminococcaceae*, and *Desulfovibrio*, promoting branched-chain amino acid synthesis and lean mass accumulation. These findings confirm that selecting the appropriate dosage determines nutritional and physiological outcomes.

Recommendations

Based on the obtained results, the bioactive extract powder from RLP demonstrates strong potential for application in functional foods, animal feed additives, and sustainable agricultural resource utilization. However, several aspects require further refinement and expansion in subsequent studies.

For the powder production process, it is necessary to evaluate the yield efficiency and technological parameters such as moisture content, particle size, and solubility to optimize the process at an application scale and ensure product stability.

Regarding the biological and probiotic activities, future studies should extend the intervention duration, include pre-treatment microbiota analyses, and adopt individual fecal sampling instead of pooled samples to better capture inter-individual variation. In addition, biochemical and energy

metabolism indices should be analyzed to comprehensively assess the physiological effects of RLP.

Supplementation with RLP at 300 mg/kg showed the potential to improve gut microbiota and promote weight gain in mice, suggesting possible application as a prebiotic feed additive in livestock. However, further studies on farm animals are needed to evaluate energy metabolism, nutrient absorption, and growth performance.

Novel contributions of the dissertation

1/ Developed a technological process for producing young rice leaf powder rich in bioactive compounds by optimizing harvest and processing conditions to preserve beneficial compounds such as polyphenols, flavonoids, and chlorophyll.

2/ Demonstrated the antioxidant, antibacterial, and gut microbiota-regulating activities of YRL powder through in vitro experiments and a mouse model, confirming its potential application in functional foods and digestive health support.

LIST OF THE PUBLICATIONS RELATED TO THE DISSERTATION

1. Nguyen, Thi To Uyen, Nguyen, Phu Tho, Nguyen, Thi Tho, Nguyen, Thi Phuong Thao, and Nguyen, Huu Thanh. (2024) Rice leaves or ricegrass-available biomaterial with potential biological activities for different industrial applications: a review. *Discover Food*. 4 (1), 106.

2. Nguyen, Thi To Uyen, Nguyen, Phu Tho, Nguyen, Thi Tho, Nguyen, Thi Phuong Thao, Dang, Chi Thien, and Nguyen, Huu Thanh. (2024) Phytochemical profile, antioxidant and antibacterial activities of the ethanolic rice (*Oryza sativa*) leaf extract. *Vegetos*. doi.org.10.1007/s42535-024-01142-5.

3. Nguyen, Phu Tho, **Nguyen, Thi To Uyen**, Nguyen, Thi Phuong Thao, and Nguyen, Huu Thanh. (2024) Bioactive compounds and their antioxidant activities in the ethanol extract from rice leaves (*Oryza sativa* L.) of different varieties. *Tropical Journal of Natural Product Research*. 8 (5), 7196–7200.

4. Nguyen, Phu Tho, **Nguyen, Thi To Uyen**, Nguyen, Thi Phuong Thao, Nguyen, Huu Thanh, Dang, Chi Thien, Pham, Minh Nhut, Nguyen, Thi Tho. (2024) Insights into the metabolism of rice leaves (*Oryza sativa* L.) under shade stress by investigating the metabolite profile using gas chromatography-mass spectrometry (GC-MS) analysis. *Russian Journal of Plant Physiology*. 71 (4), 126.

5. Thi-To-Uyen Nguyen, Phu-Tho Nguyen, Minh-Nhut Pham, Thi-Phuong-Thao Nguyen, Thanh-Thai Tran, Huu-Thanh Nguyen, Thi-Tho Nguyen. (2025) Uncovering metabolite changes in rice leaves (*Oryza sativa*) during vegetative stages through GC-MS-based ethanolic extract analysis. *Agriculture and Natural Resources*. 59 (3), 590312.

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7. Nguyễn Thị Tố Uyên, Nguyễn Phú Thọ, Nguyễn Hữu Thanh, Đặng Chí Thiện, and Nguyễn Thị Phương Thảo. (2024) Ảnh hưởng của điều kiện che sáng và thời điểm thu hoạch lá lên hàm lượng chlorophyll, polyphenol và hoạt tính kháng nấm *Candida* của dịch chiết lá lúa (*Oryza sativa* L.). *Tạp chí Dinh dưỡng và Thực phẩm*. 20 (4), 60–71.