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Original Research Article

Bioactive Compounds and their Antioxidant Activities in the Ethanol Extract from Rice Leaves (*Oryza sativa* L.) of Different Varieties

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ABSTRACT

This article presented a comprehensive analysis of the polyphenol, chlorophyll, tocopherol contents, and antioxidant activities in leaves of three rice varieties (*Oryza sativa* L. *indica*), named IR50404, Huyet Rong, and Nang Thom. The study aimed to investigate the potential antioxidant properties of these rice varieties for applications in the pharmaceutical, cosmetic, and food industries. The leaves of three rice varieties harvested at week 5 were collected and then extracted using ethanol as solvent. The results revealed significant variations in the levels of total polyphenols (ranging from 3.04 to 3.90 mg GAE/g of fresh leaf weight), total chlorophyll (1180-1315 µg/g of fresh leaf weight), and tocopherols (40.14 to 187.18 µg/g of fresh leaf weight) among the three varieties. Furthermore, in antioxidant assays, significant variations were observed among rice varieties in terms of their DPPH and ABTS radical scavenging activities, ranging from 19.03 to 21.66 µmol TE/g leaf fresh weight and from 18.77 to 23.33 µmol TE/g leaf fresh weight, respectively. The IR50404 variety was found to exhibit superior potential in terms of bioactive compounds, DPPH, and ABTS radical scavenging activities compared to the other varieties. These findings suggest that certain rice varieties possess considerable antioxidant activity in their leaves, making them promising candidates for applications in the pharmaceutical, cosmetic, and food industries. The identification of these antioxidant-rich rice varieties paved the way for the development of natural antioxidant formulations and functional food products.

Keywords: Antioxidants, chlorophyll, rice leaves, polyphenols, tocopherols.

Introduction

Rice (*Oryza sativa*) is one of the most important staple crops worldwide, providing a significant source of calories and nutrition for a large portion of the global population. [1] Apart from its grain, rice plants possess other valuable biomass resources that can be harnessed for various applications.[2] One such resource is the young rice leaf biomass, which has gained attention due to its potential for exploitation in different fields. Biological components present in young rice leaves encompass a diverse array of bioactive compounds. These include antioxidants, phenolic compounds, flavonoids, vitamins, minerals, and other phytochemicals.[3] These components contribute to the nutritional value and health benefits associated with young rice leaf biomass.
Polyphenols and chlorophyll are bioactive compounds derived from plants that have attracted attention due to their antioxidant properties. Research done by Luong *et al*. (2022) has demonstrated that ethanol extract from the EOGT96 rice variety, which is rich in polyphenols, results in strong antioxidant activity.[4] Similarly, chlorophyll has also been shown to be associated with antioxidant activity and free radical scavenging activity such as DPPH and ABTS.[5]

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Another specific bioactive compound of interest in young rice leaves is tocopherols. Tocopherols demonstrate robust antioxidant activity, playing a pivotal role in various physiological processes in plants.[6] The content of tocopherols varies among different plant varieties such as rice, providing an opportunity to select and exploit varieties with high tocopherol levels for specific applications. However, currently, there is a lack of research determining the presence of this component in rice leaves.
In the pharmaceutical and nutraceutical industries, the bioactive compounds derived from young rice leaves, including chlorophyll, polyphenol, and tocopherols, can be utilized in the development of functional foods, dietary supplements, and pharmaceutical formulations. These applications have the potential to promote human health, prevent oxidative stress-related diseases, and support overall well-being.[7] The cosmetic industry can benefit from the incorporation of young rice leaf extracts or derivatives into skincare products. The antioxidant properties of vitamin E, along with other bioactive components, offer the potency for anti-aging, moisturizing, and UV-protective properties.[8] Such natural and sustainable alternatives align with the increasing consumer demand for eco-friendly and health-conscious cosmetic options.
Young rice leaf biomass, with its notable content of tocopherols and other beneficial compounds, presents an intriguing potential for application in various industries. By determining the specific content of these compounds in rice leaves, valuable insights can be gained regarding their potential for use in functional foods, dietary supplements, and natural health products. Exploring and harnessing the potential of young rice leaf biomass can contribute to sustainable development, innovation, and the utilization of underutilized resources in the rice industry.[9] Therefore, the study evaluated the content of

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antioxidant compounds in young rice leaves of different varieties to determine their biomass exploitation potential.

Materials and Methods

Plant conditions and extraction

In this study, three rice varieties (*Oryza sativa* L.), namely IR50404, Huyet Rong, and Nang Thom, were utilized. The rice plants were cultivated under natural light conditions, receiving two daily waterings, without the application of fertilizers or pesticides.[10] At 35 days of age, rice leaves were carefully collected for further analysis.

To extract polyphenol and chlorophyll compounds from the rice leaves, a previously established method outlined by Tamprasit *et al.* (2019) was employed.[11] The extraction process involved utilizing 80% ethanol as the solvent. Before extraction, the rice leaves were thoroughly washed and then finely chopped into small pieces. These plant samples were then subjected to sonication in an ethanol solution containing 1% HCl, specifically using a ratio of 10 grams of plant material per 100 milliliters of the solvent. The sonication process lasted for a duration of 30 minutes, ensuring efficient extraction. Following the sonication step, the resultant samples were carefully filtered through Whatman filter paper No.1 to eliminate any solid residues. Subsequently, the solvent was removed from the filtered samples using a rotary evaporator, set at a temperature below 45 °C.

Total polyphenol content

To determine the total phenolic content, the Folin-Ciocalteu method was employed with slight modifications based on established protocols.[3,11] In this procedure, 0.5 mL of the extracted sample was combined with 5 mL of the Folin-Ciocalteu reagent. Following a 5-minute incubation at room temperature, 4 mL of a 1M Na_2CO_3 solution was added to the mixture. Thorough mixing was ensured, and the solution was subsequently incubated in darkness for 90 minutes. After the incubation period, the absorbance of the solution was measured at 725 nm using a spectrophotometer. To determine the total phenolic content, a gallic acid standard curve was employed for reference. The content was expressed as the gallic acid equivalent (GAE) in micrograms per gram of leaf fresh weight.

Total chlorophyll content

The total chlorophyll content was determined using a modified version of the method outlined by Tamprasit *et al.* (2019). [3] In this procedure, 2 mL of the extracted sample was subjected to scanning at 645 nm and 663 nm wavelengths using a spectrophotometer. To calculate the total chlorophyll content, the following equations were utilized:

Total chlorophyll content (mg/g leaf fresh weight) = $[(20.2 \times A645) + (8.02 \times A663)]$

These calculations enabled the determination of the total chlorophyll content, expressed in micrograms per gram of leaf fresh weight.

Antioxidant assays

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays were performed following the methodology outlined by Gumul and Berski (2021) with certain modifications.[12] To assess the DPPH radical scavenging activity, 1.5 mL of the extract being analyzed was combined with 1.5 mL of 0.15 mM DPPH solution dissolved in 95% ethanol. The mixture was thoroughly mixed and kept in darkness at room temperature for a duration of 30 minutes. Following the incubation period, the absorbance of the resulting solution was measured at 517 nm using a spectrophotometer.

To generate (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) $ABTS^+$ radicals, a solution was prepared by combining 5 mL of ABTS stock solution (7.0 mM) with 88 µL of potassium persulfate (148 mM). The resulting mixture was further diluted with sodium acetate buffer (20 mM, pH 4.5) to obtain a working solution. This working solution exhibited an absorbance value of 0.70 ± 0.02 at 734 nm. Next, a reaction mixture was prepared by combining 188 µL of the ABTS working solution with 12 µL of sample dilutions (water was used as a blank). The mixture was allowed to react for 30 minutes at room temperature. Following the incubation period, the absorbance of the reaction mixture was measured at 734 nm using a spectrophotometer.[13]

The antioxidant activity was determined using the Trolox standard curve in the concentration range of 10-60 µM. The results of two antioxidant assays were expressed as micromoles of Trolox equivalents (TE) per gram of leaf fresh weight, indicating the antioxidant activity exhibited by the sample under investigation.

α-Tocopherol identification using High-Performance Liquid Chromatography (HPLC)

The extraction of α-tocopherol from rice leaves was conducted using n-hexane as the solvent, following preparation according to the method described by Konyalıoğlu *et al.* (2005).[14] The subsequent analysis was carried out using HPLC in isocratic mode. During the HPLC run, the separation was achieved by utilizing two solvents, namely solvent A and solvent B. Solvent A consisted of a mixture of acetonitrile, methanol, and tetrahydrofuran in the ratio of 52:40:8 (v/v/v) and was used for the first 15 minutes of the run. Subsequently, pure methanol (solvent B) was employed for the remaining 10 minutes. The utilization of solvent B towards the end of the run facilitated the cleaning and regeneration of the column. The flow rate was set at 2 mL/min, and the temperature of the system was maintained at 25 °C. The eluted α-tocopherol compounds were detected by measuring their fluorescence, with excitation occurring at 295 nm and emission at 340 nm. The quantification of α-tocopherol was achieved by comparing the peak surface areas obtained from the samples with those obtained from pure α-tocopherol standard solutions according to the equation $y = 7.27x - 1.28$ (where x is Amount; y is Area).[15]

Statistical analysis

The data obtained in this study were presented as means ± standard deviations. One-way analysis of variance (ANOVA) was performed to evaluate the significance of differences between groups. Subsequently, Duncan's test procedure was applied to identify statistically significant differences ($p<0.05$). The statistical analysis was conducted using the software Statgraphics Centurion XVIII.

Results and Discussion

α-Tocopherol content in rice leaves

Tocopherols, renowned for its potent antioxidant properties, is a significant biological compound of interest. Consequently, this study aimed to identify and quantify the substances present in the leaves of various rice varieties using HPLC analysis. The outcomes of this analysis are visually represented in Figure 1 and Figure 2, providing a graphical depiction of the results obtained. The results of HPLC analysis revealed that the rice leaves of the IR50404 variety exhibited the highest tocopherol content, accounting for 187.18 µg/g of fresh leaf weight. In comparison, the Nang Thom variety demonstrated a lower tocopherol content at 83.28 µg/g of fresh leaf weight, while the Huyet Rong variety had the lowest content of 41.14 µg/g of fresh leaf weight (Figure 1). These findings provide quantitative evidence of the variation in tocopherol content among studied rice varieties.

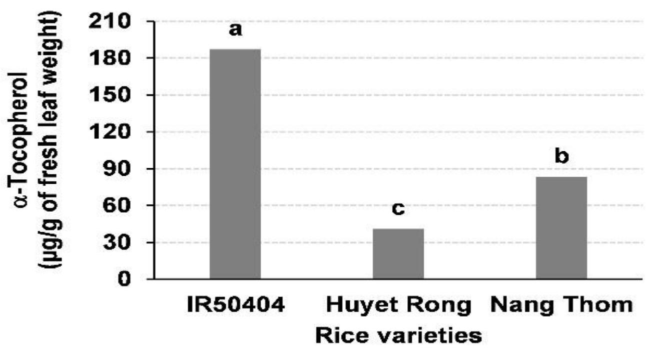

Figure 1: α-Tocopherol content in leaves of different rice varieties

Tocopherols, known for its potent antioxidant properties, is a biologically significant compound with potential health benefits.[7] According to the results, the tocopherol content varied significantly among the rice varieties. These quantitative measurements provided robust evidence of the variations in tocopherol content among the different rice varieties studied. The findings highlighted the genetic and compositional differences within the rice varieties, which could impact the synthesis and accumulation of tocopherols.[16] The variation in tocopherol content can influence the suitability of different leaf biomass extractions for specific applications in industries such as functional foods, dietary supplements, and natural health products. The higher tocopherol content in certain rice varieties, such as IR50404, may make them more desirable for the production of tocopherol-rich products.

Comparing the tocopherol content in rice leaves with other fruits, such as berries, nuts, and seeds, reveals that rice leaves can be a competitive source of tocopherols. For example, fruits like almonds, hazelnuts, and sunflower seeds are known for their high tocopherol content, typically ranging from 150 to 360 µg/g.[6] Interestingly, rice leaves have been found to contain a comparable or even higher concentration of tocopherols in certain instances, ranging from 40.14 to 187.18 µg/g. Exploiting rice leaf biomass for food and cosmetic applications can be a promising avenue considering its potential tocopherol content. Tocopherols are widely used in the food industry as natural antioxidants to extend the shelf life of products and prevent lipid oxidation.[17] Incorporating rice leaf-derived tocopherols into food formulations can enhance their nutritional and functional properties. In the cosmetic industry, tocopherols are valued for their antioxidant and skin-protective properties.[18] They can be utilized in formulations for skincare products, such as creams, lotions, and serums, to provide anti-aging effects and protect the skin from oxidative damage. Rice leaf-derived tocopherols can offer a sustainable and potentially cost-effective source for the development of natural and eco-friendly cosmetic products.[19] However, it is important to note that further research is needed to optimize the extraction and purification methods for tocopherols from rice leaves. Additionally, comprehensive studies on the stability and bioavailability of rice leaf-derived tocopherols in food and cosmetic applications are necessary to ensure their effectiveness and safety.

Total polyphenol and chlorophyll contents in rice leaves

The total polyphenol and chlorophyll contents were determined as part of this work. Polyphenols are a class of bioactive compounds known for their antioxidant properties and potential health benefits.[20] Chlorophyll, on the other hand, is a natural pigment responsible for the green color in plants and plays a vital role in photosynthesis.[21] To assess the total polyphenol and chlorophyll contents, specific analytical methods were employed. The results obtained from these analyses provide valuable insights into the composition and concentration of these compounds in rice leaves under investigation (Table 1).

Based on the results presented in Table 1, it was observed that the total chlorophyll content varied among the different rice varieties. The IR50404 variety exhibited the highest chlorophyll content, measuring at 1315.59 µg/g of fresh leaf weight. In comparison, the Huyet Rong and Nang Thom varieties recorded lower chlorophyll contents at 1180.03 and 1231.38 µg/g of fresh leaf weight, respectively. In addition to chlorophyll, the total polyphenol content was also determined in the tested rice varieties. The IR50404 variety displayed the highest total polyphenol content, measuring at 3.77 mg GAE/g of fresh leaf weight. Following closely, the Nang Thom variety recorded a total polyphenol content at 3.90 mg GAE/g of fresh leaf weight. Conversely, the Huyet Rong variety displayed the lowest total polyphenol content, measuring at 3.04 mg GAE/g of fresh leaf weight. The results of this study provide quantitative evidence of variation in the content of chlorophyll and polyphenols among studied rice varieties.

The study revealed notable variations in both total chlorophyll and polyphenol contents among the different rice varieties. Previous reports have also analyzed these compounds in the leaves of black rice and some other rice varieties.[22,23] Chlorophyll, responsible for the green pigment and photosynthesis in plants, and polyphenols, a class of bioactive compounds with antioxidant properties, both play crucial roles in plant physiology and potentially offer health benefits.[24,25] These variations in polyphenol and chlorophyll contents reflect the different genetic profiles[6] and environmental factors [26] that influence the synthesis and accumulation of these compounds in rice plants. The variations in chlorophyll and polyphenol content can influence the selection of rice varieties for specific applications. For example, rice varieties with higher chlorophyll content might be more desirable for green pigments in food additives or cosmetic industries,[27] while those with higher polyphenol content could be targeted for the production of functional foods or dietary supplements.[28]

Antioxidant activity of rice leaves

The evaluation of DPPH and ABTS radical scavenging activities is a valuable approach for assessing the antioxidant capacity of substances. In this particular study, the focus was on examining the DPPH and ABTS radical scavenging activities of three distinct rice varieties. The results, illustrated in Figure 3, revealed notable variations in DPPH radical scavenging activity among the tested rice varieties. Specifically, the Huyet Rong rice variety exhibited a comparatively lower DPPH radical scavenging activity (19.03 µmol TE/g leaf fresh weight) when compared to the other varieties. Conversely, the IR50404 variety displayed the highest DPPH radical scavenging activity (21.66 µmol TE/g leaf fresh weight). However, it is important to note that the variance in DPPH activity between the IR50404 and Nang Thom varieties was not considered statistically significant (Figure 3).

Table 1: Total content of polyphenols and chlorophyll in rice leaves

Rice varieties	Total chlorophyll (µg/g of fresh leaf weight)	Total polyphenols (mg GAE/ g of fresh leaf weight)
IR50404	1315.59 ± 9.03^{a}	3.77 ± 0.15^{a}
Huyet Rong	1180.03 ± 7.06^{c}	3.04 ± 0.30^{b}
Nang Thom	1231.38 ± 6.12^{b}	3.90 ± 0.11^{a}

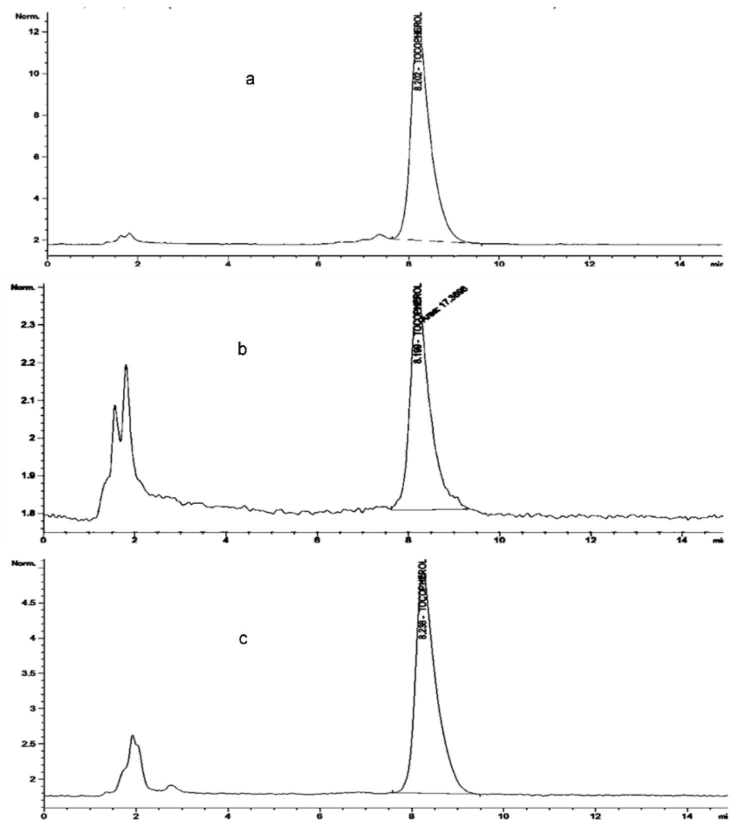

Figure 2: HPLC chromatograms of tocopherols in leaves of rice varieties (a) IR50404, (b) Huyet Rong, and (c) Nang Thom

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A similar trend was observed in the ABTS assay, where the highest ABTS activity was found in the rice leaves of the IR50404 variety, followed by the Nang Thom and Huyet Rong varieties with radical scavenging activity values of 23.33, 21.10, and 18.77 µmol TE/g leaf fresh weight, respectively. In line with our study, previous researchers have also reported the antioxidant activity of rice leaves through their free radical scavenging capability.[3,23] The results of the study revealed significant antioxidant activity in the leaves of the rice varieties IR50404, Nang Thom, and Huyet Rong. This finding highlights the potential of rice leaves as a natural source of antioxidants.

Correlation analysis between antioxidant compounds and antioxidant activity

A correlation analysis was conducted to examine the relationship between antioxidant active substances and antioxidant activity, aiming to comprehend the individual contributions of each antioxidant to the overall antioxidant activity. It is well established that genotypic factors have a substantial impact on antioxidants,[29] consequently influencing the correlation between antioxidants and antioxidant activity (Table 2). The findings indicated a significant association between total polyphenols, total chlorophyll, and tocopherol in the DPPH and ABTS assays. Notably, chlorophyll exhibited the highest positive correlation (DPPH: 0.98**; ABTS: 0.99**), followed by tocopherol and polyphenols.

The results of this analysis revealed a strong positive correlation between antioxidant compounds and antioxidant activity, suggesting that the accumulation of these antioxidants played a pivotal role in enhancing the overall antioxidant activity. Consistent with previous research, the antioxidant properties of rice leaves are gradually influenced by the presence of biologically active components, such as chlorophyll and polyphenols.[22,23] Among the various antioxidant compounds analyzed, chlorophyll exhibited the highest positive correlation with both the DPPH and ABTS assays. This finding suggests that chlorophyll made a significant contribution to the total antioxidant activity. Following chlorophyll, tocopherols and polyphenols also exhibited positive correlations with the DPPH and ABTS assays, indicating their substantial involvement in the overall antioxidant activity. This has also been observed in many plant species.[30]

Table 2: Correlation between antioxidant compounds and antioxidant activity in rice leaves

Antioxidant compounds	Radical scavenging activities	
	DPPH	ABTS
α-Tocopherol	0.96**	0.97**
Chlorophyll	0.98**	0.99**
Polyphenol	0.82**	0.80**

** Significant at $p < 0.05$, respectively using Pearson's correlation analysis

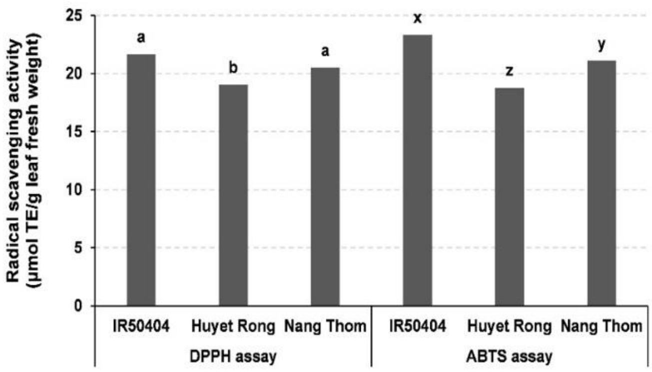

Figure 3: Antioxidant activity of different rice varieties. Different letters at the top of the column in the same assay indicate statistically significant differences at the $p < 0.05$ level

It is worth noting that chlorophyll, being primarily involved in photosynthesis, may have a more indirect relationship with antioxidant activity compared to tocopherols and polyphenols, which are directly associated with antioxidant properties. The positive correlation between chlorophyll and tocopherol content suggests a potential interplay between these compounds, possibly influenced by common regulatory mechanisms.[31] According to recent genetic findings, it has been established that phytyl diphosphate serves as the primary prenyl precursor for tocopherol biosynthesis. The supply of phytyl diphosphate to the tocopherol biosynthetic pathway predominantly occurs through chlorophyll degradation and subsequent phytol phosphorylation steps, as part of a sequential process.[31]

Conclusion

Overall, the observed correlations between tocopherols, chlorophyll, polyphenols, and antioxidant activity suggest that these compounds collectively contribute to the antioxidant capacity of the rice varieties. The potential for exploiting biological compounds, particularly tocopherols, in rice leaves is significant. Their antioxidant properties, coupled with their availability and sustainability, make rice leaf-derived tocopherols attractive for food, cosmetic, and nutraceutical industries. Continued research and development in this area will unlock further opportunities and contribute to the advancement of sustainable and natural product innovation.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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