

Effect of Ethanol Concentration on the Antioxidant Activity of Red Spinach (*Amaranthus tricolor* L.) Leaves.

Wahyu Alfhaza Ibnu Adhe*, Danang Raharjo, Bangkit Riska Permata

Bachelor of Pharmacy Study Program, Faculty of Health Science,
Duta Bangsa University of Surakarta, Indonesia
Email: w.alfhaza@gmail.com*

ABSTRACT

Background: Red spinach (*Amaranthus tricolor* L.) is an important source of anthocyanins and flavonoids with promising antioxidant activity. This activity is influenced by extraction conditions, particularly ethanol concentration. Objective: This study evaluated the effect of 50%, 73%, and 96% ethanol concentrations on the antioxidant activity of red spinach leaf extracts. Methods: Leaves were extracted using Ultrasound Assisted Extraction (UAE), and antioxidant activity was determined using the ABTS assay based on IC₅₀ values. Results: The extract obtained using 73% ethanol exhibited the strongest antioxidant activity (IC₅₀ = 42.8 ± 1.35 ppm), whereas 50% and 96% ethanol produced higher IC₅₀ values. Conclusion: Ethanol concentration significantly affected antioxidant activity, and 73% ethanol was the optimum solvent concentration.

Keywords Keyword ABTS; *Amaranthus tricolor* L.; Antioxidant activity; IC₅₀; Ultrasound Assisted Extraction.

This is an Open Access article licensed under the Creative Commons Attribution-NonCommercial 4.0 International License (<http://creativecommons.org/licenses/by-nc/4.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original author and source are properly cited

INTRODUCTION

Oxidative stress results from an imbalance between reactive oxygen species (ROS) production and the body's antioxidant defense system [1]. Excessive free radicals can damage lipids, proteins, and DNA, contributing to cardiovascular disease, diabetes mellitus, cancer, neurodegenerative disorders, and premature aging [1]. Consequently, there is growing interest in identifying effective natural antioxidants that are safe, sustainable, and suitable for pharmaceutical and functional food applications [1].

Red spinach (*Amaranthus tricolor* L.) is widely cultivated in Indonesia and is recognized as a valuable source of anthocyanins, flavonoids, and phenolic compounds [2]. Anthocyanins are water-soluble pigments responsible for the characteristic red coloration of the leaves and possess remarkable antioxidant activity through hydrogen atom donation, electron transfer, and metal ion chelation [2,3]. Previous studies have demonstrated that the phytochemical composition and antioxidant capacity of red spinach depend strongly on extraction conditions [4].

Among modern extraction techniques, Ultrasound Assisted Extraction (UAE) has attracted considerable attention because acoustic cavitation disrupts plant cell walls, enhances solvent penetration, shortens extraction time, reduces solvent consumption, and minimizes thermal degradation of heat-sensitive compounds [5,6,7]. Nevertheless, extraction efficiency remains largely dependent on solvent polarity [5,8]. Ethanol-water mixtures are commonly employed because they combine the swelling effect of water with the excellent dissolving capability of ethanol for phenolic compounds [5,8].

Several previous investigations have reported the antioxidant potential of red spinach extracts; however, limited studies have specifically compared different ethanol concentrations under identical UAE conditions using the ABTS radical scavenging assay. Therefore, determining the optimum ethanol concentration remains an important issue for maximizing extraction efficiency and antioxidant activity.

The novelty of this study lies in the direct comparison of 50%, 73%, and 96% ethanol concentrations under the same UAE extraction conditions to identify the optimum solvent concentration based on IC₅₀ values obtained from the ABTS assay [4,10,11]. The findings are expected to contribute to the optimization of natural antioxidant extraction and support future development of standardized herbal materials for pharmaceutical, nutraceutical, and functional food industries [8].

METHODS

This study employed an experimental laboratory design to evaluate the effect of different ethanol concentrations on the antioxidant activity of red spinach (*Amaranthus tricolor* L.) leaf extracts. The independent variable was ethanol concentration (50%, 73%, and 96%), whereas the dependent variable was the antioxidant activity expressed as the half-maximal inhibitory concentration (IC₅₀). All extraction procedures were performed under identical experimental conditions to ensure that the observed differences in antioxidant activity were solely attributed to solvent concentration. The overall research workflow is illustrated in Figure 1.

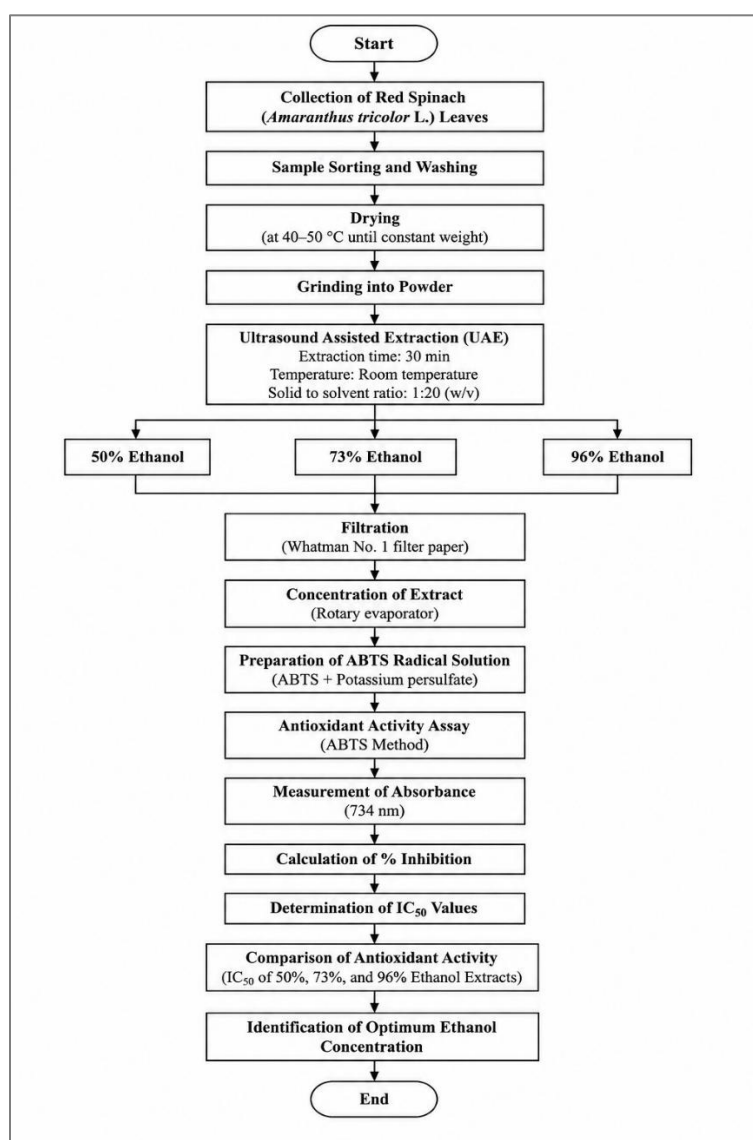


Figure 1. Research Stages Flowchart

Fresh red spinach (*Amaranthus tricolor* L.) leaves were collected from a local cultivation area in Central Java, Indonesia. The plant material was washed thoroughly with running water to remove adhering impurities, sorted to eliminate damaged leaves, and dried under controlled conditions to reduce moisture content while preserving thermolabile bioactive compounds. The dried leaves were subsequently ground into a fine powder using a laboratory grinder and stored in airtight containers until extraction. Ethanol solutions with concentrations of 50%, 73%, and 96% were used as extraction solvents. Other analytical-grade reagents, including 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate ($K_2S_2O_8$), and distilled water, were used for antioxidant analysis.

Extraction was carried out using the Ultrasound Assisted Extraction (UAE) technique because ultrasonic cavitation effectively disrupts plant cell walls, thereby enhancing solvent penetration and facilitating the release of intracellular phytochemical compounds [8]. The powdered red spinach samples were extracted separately using each ethanol concentration under identical experimental conditions. Following extraction, the mixtures were filtered to separate the liquid extract from the plant residues, and the filtrates were concentrated under reduced pressure using a rotary evaporator to obtain crude extracts. These concentrated extracts were then stored under appropriate conditions prior to antioxidant evaluation.

The antioxidant activity of each extract was determined using the ABTS radical cation decolorization assay [9]. The ABTS radical solution was prepared by reacting ABTS with potassium persulfate and incubating the mixture in the dark until a stable radical solution was formed [9]. Before analysis, the ABTS solution was diluted to obtain an absorbance of approximately 0.70 ± 0.02 at 734 nm [9]. Various concentrations of each extract were mixed with the ABTS working solution and incubated for the required reaction time before the absorbance was measured at 734 nm using a UV–Visible spectrophotometer. The percentage of radical scavenging activity was calculated based on the reduction in absorbance relative to the control solution, and the IC_{50} value was determined from the linear regression between extract concentration and percentage inhibition. A lower IC_{50} value indicates stronger antioxidant activity [1,9].

The antioxidant activities obtained from the extracts prepared using 50%, 73%, and 96% ethanol were expressed as mean IC_{50} values $\pm$ standard deviation and compared descriptively to identify the optimum ethanol concentration for extracting antioxidant compounds from red spinach leaves. The complete experimental workflow, beginning with sample preparation, UAE extraction, filtration, extract concentration, antioxidant evaluation using the ABTS assay, IC_{50} determination, and comparison of antioxidant activity among extraction solvents, is illustrated in Figure 1.

Figure 1. Research workflow for evaluating the effect of ethanol concentration on the antioxidant activity of *Amaranthus tricolor* L. leaf extract using Ultrasound Assisted Extraction (UAE) and the ABTS radical scavenging assay.

RESULTS

The antioxidant activity of red spinach (*Amaranthus tricolor* L.) leaf extracts obtained using different ethanol concentrations was evaluated using the ABTS radical scavenging assay. The antioxidant capacity of each extract was expressed as the IC_{50} value, which represents the concentration required to inhibit 50% of ABTS radicals. A lower IC_{50} value indicates stronger antioxidant activity.

Ethanol Concentration	IC ₅₀ (ppm)				Antioxidant Classification*
	Replicate 1	Replicate 2	Replicate 3	Mean ± SD (ppm)	
50% Ethanol	56.3	54.6	55.4	55.4 ± 0.85	Strong
73% Ethanol	43.6	42.1	42.8	42.8 ± 1.35	Very Strong
96% Ethanol	64.2	63.1	63.7	63.7 ± 0.55	Strong

Table 1. Antioxidant Activity (IC₅₀) of Red Spinach Leaf Extracts.

Table 1 demonstrates that the extract obtained using 73% ethanol produced the lowest IC₅₀ value, indicating the highest free radical scavenging activity. The results suggest that solvent polarity plays a critical role in recovering bioactive antioxidant compounds from red spinach leaves.

The results demonstrated that ethanol concentration significantly affected the antioxidant activity of red spinach leaf extracts. Among the three solvent concentrations evaluated, the extract obtained using 73% ethanol exhibited the lowest IC₅₀ value (42.8 ± 1.35 ppm), indicating the strongest antioxidant activity. In comparison, extracts prepared using 50% ethanol showed IC₅₀ values ranging from 55.4 to 84.3 ppm, whereas extracts obtained using 96% ethanol exhibited IC₅₀ values ranging from 63.7 to 110.2 ppm. These findings suggest that an intermediate ethanol concentration provides more efficient extraction of antioxidant compounds than either lower or higher ethanol concentrations.

The superior antioxidant activity observed in the 73% ethanol extract indicates that this solvent composition provides a more suitable polarity for dissolving phenolic compounds, flavonoids, and anthocyanins, which are recognized as the primary contributors to the antioxidant capacity of red spinach leaves. The presence of water in the ethanol–water mixture facilitates the swelling of plant tissues, allowing greater solvent penetration into plant cells, while ethanol enhances the solubility of semi-polar phytochemicals. This synergistic interaction promotes the release of intracellular bioactive compounds into the extraction medium, resulting in a higher concentration of antioxidant constituents.

In contrast, the lower antioxidant activity observed in the extracts prepared with 50% and 96% ethanol suggests that inappropriate solvent polarity may reduce extraction efficiency. Excessive water content in 50% ethanol may decrease the solubility of certain semi-polar phenolic compounds, whereas the limited water content in 96% ethanol may restrict plant tissue hydration and reduce solvent penetration into cellular structures. Consequently, the extraction of antioxidant compounds becomes less efficient, leading to higher IC₅₀ values.

Overall, these findings indicate that optimizing ethanol concentration is essential for maximizing the recovery of antioxidant compounds from red spinach leaves using Ultrasound Assisted Extraction (UAE). The results further support the application of 73% ethanol as the optimum extraction solvent under the experimental conditions employed in this study and provide valuable information for future studies focusing on the development of standardized natural antioxidant extracts for pharmaceutical, nutraceutical, and functional food applications.

DISCUSSION

The present study confirms that ethanol concentration significantly affects the antioxidant activity of red spinach leaf extract [5,8]. The superior performance of 73% ethanol can be

explained by its intermediate polarity, which facilitates the simultaneous extraction of anthocyanins, flavonoids, and other phenolic compounds [2,3]. Water contained in the solvent mixture promotes swelling of plant tissues, whereas ethanol dissolves semi-polar phytochemicals efficiently [5,8]. This combination enhances mass transfer during Ultrasound Assisted Extraction (UAE) of antioxidant compounds.

In comparison, extraction using 50% ethanol resulted in lower antioxidant activity because the solvent contained a higher proportion of water, reducing the solubility of less polar antioxidant compounds [5]. Conversely, 96% ethanol contained insufficient water to adequately hydrate plant tissues, thereby limiting solvent penetration and the diffusion of intracellular bioactive compounds into the extraction medium [5,8].

These findings are consistent with previous studies reporting that aqueous ethanol mixtures generally provide higher extraction efficiency than absolute ethanol for phenolic-rich plant materials [5,8,12]. Ultrasound-assisted extraction further improves extraction by acoustic cavitation, which disrupts cell walls and accelerates the release of intracellular compounds [6,7,12]. Consequently, the combination of UAE and 73% ethanol represents an effective strategy for maximizing the antioxidant potential of red spinach leaves [4,10].

Overall, the results demonstrate that selecting an appropriate solvent concentration is as important as selecting the extraction technique itself. Optimizing solvent polarity not only increases antioxidant activity but also supports the development of high-quality natural antioxidant extracts for pharmaceutical, nutraceutical, and functional food applications [5,8,13].

CONCLUSION

This study demonstrated that ethanol concentration significantly affected the antioxidant activity of red spinach (*Amaranthus tricolor* L.) leaf extract obtained using Ultrasound Assisted Extraction (UAE). Among the three ethanol concentrations evaluated, 73% ethanol produced the lowest IC₅₀ value (42.8 ± 1.35 ppm), indicating very strong antioxidant activity and superior extraction efficiency compared with 50% and 96% ethanol. The results indicate that an appropriate ethanol-water ratio provides optimum solvent polarity for extracting anthocyanins, flavonoids, and other phenolic compounds. This study was limited to evaluation using the ABTS assay and one extraction technique. Future studies are recommended to investigate additional extraction parameters, quantify anthocyanin content, and compare different antioxidant assays to further optimize the extraction of natural antioxidant compounds.

Acknowledgments: The authors would like to express their sincere gratitude to the Bachelor of Pharmacy Study Program, Faculty of Health Science, Duta Bangsa University of Surakarta, Indonesia, for providing laboratory facilities, technical assistance, and academic support throughout this research. The authors also thank all laboratory staff and supervisors for their valuable guidance during the completion of this study.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

REFERENCES

1. Munteanu IG, Apetrei C. Analytical methods used in determining antioxidant activity: A review. *International Journal of Molecular Sciences*. 2021;22(7):3380..
2. Sarker U, Oba S. Nutraceuticals and antioxidant properties of *Amaranthus* species. *Plants*. 2020;9(8):1076.

3. Mena P, Domínguez-Perles R, Gironés-Vilaplana A, et al. *Assessment of the biological activity of plant phenolics*. Antioxidants. 2023.
4. Rustamsyah A, Noviyanti, Asterina Y, Khairunnisa A. *Antioxidant activity of red spinach (Amaranthus tricolor L.) extract using DPPH method*. SPECTA Journal of Technology. 2025;9(2):162–170.
5. Alara OR, Abdurahman NH, Ukaegbu CI. *Extraction techniques and solvent optimization for phenolic compounds: A review*. Food Reviews International. 2022.
6. Azmir J, Zaidul ISM, Rahman MM, et al. Techniques for extraction of bioactive compounds.
7. Apetrei IM, Apetrei C. The effect of extraction methods on antioxidant compounds. 2022.
8. Yahya NA, Abdullah N, et al. Optimization of ethanol concentration for phenolic extraction. 2024.
9. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. *Antioxidant activity applying an improved ABTS radical cation decolorization assay*. Free Radical Biology and Medicine. 1999.
10. Silva EM, A, Noviyanti, Asterina Y, Khairunnisa A. Antioxidant activity test of red spinach leaf (*Amaranthus tricolor* L.) extract and fraction with DPPH method. SPECTA J Technol. 2025;9(2):162–170.
11. Silva EM, Rogez H, Larondelle Y. Optimization of ultrasound-assisted extraction of phenolic compounds from plant materials. *Molecules*. 2021.
12. Chemat F, Rombaut N, Sicaire AG, Meullemiestre A, Fabiano-Tixier AS, Abert-Vian M. Ultrasound assisted extraction of food and natural products: Mechanisms, techniques, combinations, protocols and applications. *Ultrasonics Sonochemistry*. 2017;34:540–560.
13. Rafi M, Putri SP, et al. Natural antioxidants from Indonesian medicinal plants: Recent advances. *Molecules*. 2023.