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Original Research Article

# Optimization of Flavonoid Extraction and Antioxidant Capacity from *Acanthus ilicifolius* Using Microwave-Assisted Extraction and I-Optimal Response Surface Methodology

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ABSTRACT

*Acanthus ilicifolius* is a medicinal plant from the Acanthaceae family commonly found in mangrove forests. Its extract contains antioxidant properties attributed to secondary metabolites, particularly flavonoids. Achieving optimal flavonoid content requires careful consideration of the extraction method. This study focuses on optimizing ethanol concentration and extraction time using I-optimal response surface methodology to maximize total flavonoid content (TFC) and antioxidant capacity in *A. ilicifolius* leaf extracts. The extraction was performed using varying ethanol concentrations and extraction duration. TFC was measured using a nanospectrophotometer, and antioxidant capacity was evaluated through CUPRAC (cupric ion reducing antioxidant capacity) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assays. Statistical analysis identified optimal conditions, with the highest TFC of 0.479 mg GAE/g DW obtained at 69.3% ethanol concentration and an extraction time of 130.2 seconds. Higher ethanol concentrations and longer extraction times generally enhanced flavonoid content and antioxidant activity. The highest antioxidant capacity, as measured by CUPRAC, was achieved at 76.6% ethanol and 138 seconds, yielding 14.149 µmol TE/g DW. The optimized extraction conditions resulted in predicted TFC and antioxidant capacities that closely matched experimental results, demonstrating the accuracy and reliability of the optimization model.

***Keywords:*** *Acanthus ilicifolius*, Flavonoid extraction, Microwave-assisted extraction, Antioxidant capacity.

## Introduction

*Acanthus ilicifolius*, a medicinal plant from the Acanthaceae family, predominantly thrives in mangrove forests. The plant, easily recognized by its spiny leaves, can grow up to 1.5 meters tall. Traditionally, *A. ilicifolius* has been extensively used in treating various ailments, including asthma, diabetes, digestive disorders, hepatitis, rheumatism, skin diseases, and snake bites.[1] Its therapeutic potential is primarily attributed to its rich composition of secondary metabolites such as alkaloids, terpenoids, and flavonoids,[2] which exhibit potent antioxidant activities. Furthermore, other bioactive compounds—including phenolics, glycosides, polyphenols, tannins, and steroids—enhance its free radical-scavenging capacity, thereby solidifying its medicinal value.[3]The plant's relevance is underscored by its potential to neutralize free radicals, a process directly linked to the concentration of bioactive compounds in its extracts.[4]

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However, the natural abundance of these compounds is typically low, necessitating the development of optimized extraction methods to maximize their yield.[5] Among various extraction techniques, microwave-assisted extraction (MAE) has gained prominence due to its ability to enhance extraction efficiency while reducing solvent usage and extraction time.[6] This method utilizes microwave radiation to disrupt plant cell walls, thereby facilitating the rapid release of bioactive compounds with greater efficiency compared to conventional techniques.[7] Optimization of parameters such as solvent concentration and extraction time is critical to ensuring the maximum recovery of target compounds, particularly in medicinal plants.[8-11]

The novelty of this research lies in its application of the I-Optimal Response Surface Methodology (RSM) to optimize flavonoid extraction from *A. ilicifolius*. I-Optimal RSM is a robust statistical approach that identifies optimal experimental conditions with minimal trials, ensuring precise fine-tuning of extraction parameters.[12] This method has been successfully employed to optimize the recovery of various bioactive compounds, including polyphenols (phenolic and flavonoid), alkaloids, and essential oils.[13-21]

This study aims to determine the optimal ethanol concentration and extraction time required to maximize the total flavonoid content (TFC) and antioxidant activity in *A. ilicifolius* extracts. The research combines Microwave-Assisted Extraction (MAE) with I-Optimal Response Surface Methodology (RSM), presenting an efficient strategy for harnessing the plant's antioxidant potential while minimizing resource usage. By integrating advanced statistical modeling with an eco-friendly extraction technique, the study provides a novel framework for the sustainable utilization of medicinal plants.

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## Materials and Methods

*Plant material and sample preparation*

*A. ilicifolius* (voucher number BMK0270102016) leaves were sourced from the Conservation and Cultivation Unit of the Tropical Biopharmaca Research Center, located at IPB University, Bogor, West Java, Indonesia. The geographical coordinates of the collection site are approximately 6°35′16″S, 106°48′05″E. The leaves were carefully selected and subsequently dried in an oven maintained at 45°C for 60 hours. Once dried, the leaves were ground into a fine powder and sieved using a 60-mesh sieve. The powdered samples were then stored at room temperature in airtight containers until further use in extraction processes.

*Extraction procedure*

The extraction was performed using varying ethanol concentrations and extraction times, as determined by an optimal design generated through the Design-Expert software (version 23.1.4, 64-bit, Stat-Ease, Inc., Minneapolis, MN, USA, 2023). The software utilizes I-Optimal Response Surface Methodology in the microwave-assisted extraction method.[22]

A total of 4 g of *A. ilicifolius* leaf powder was placed into 14 separate 100 mL vials, each containing 20 mL of a pre-prepared solvent composition, with each vial clearly labelled. The vials were sealed with plastic wrap and subjected to microwave treatment at a medium-low temperature setting. The specific extraction combinations are provided in Table 1. After the extraction process, the mixtures were filtered to obtain the filtrate. The filtrates were then standardized to a final volume of 10 mL using the same solvent composition used during extraction, resulting in a final concentration of 0.4 g/mL. The extracts were subsequently used to determine the flavonoid content and antioxidant capacity.

**Table 1:** I-Optimal response surface design for time and ethanol concentration in microwave-assisted extraction with total flavonoid content (TFC) and antioxidant capacity of *Acanthus ilicifolius* extracts

| Run | Extraction Parameters | | Responses | | |
|---|---|---|---|---|---|
| | Time (s) | [EtOH] (%) | TFC (mg GAE/g DW) | CUPRAC (µmol TE/g DW) | ABTS (µmol TE/g DW) |
| 1 | 132 | 61.24 | 0.193±0.01 | 7.349±0.08 | 21.313±1.24 |
| 2 | 60 | 65 | 0.099±0.00 | 4.278±0.09 | 11.841±0.97 |
| 3 | 180 | 78 | 0.028±0.00 | 2.023±0.15 | 20.347±0.15 |
| 4 | 130.2 | 69.3 | 0.479±0.02 | 4.327±0.40 | 7.361±0.42 |
| 5 | 180 | 68.91 | 0.020±0.00 | 2.356±0.07 | 3.479±0.10 |
| 6 | 130.2 | 69.3 | 0.337±0.02 | 3.934±0.27 | 8.295±0.24 |
| 7 | 130.2 | 69.3 | 0.422±0.03 | 3.667±0.19 | 12.163±1.04 |
| 8 | 138 | 76.6 | 0.388±0.01 | 14.149±0.09 | 0.807±0.03 |
| 9 | 96 | 80 | 0.449±0.03 | 13.279±0.51 | 1.026±0.05 |
| 10 | 84 | 60 | 0.271±0.02 | 10.084±0.65 | 24.270±0.80 |
| 11 | 180 | 60.6 | 0.163±0.01 | 10.205±0.69 | 23.473±0.40 |
| 12 | 60 | 71.4 | 0.268±0.02 | 12.890±0.35 | 1.138±0.05 |
| 13 | 99 | 73.4 | 0.368±0.03 | 12.890±0.35 | 16.390±0.38 |
| 14 | 96 | 80 | 0.428±0.01 | 12.908±1.08 | 1.205±0.02 |

*Total flavonoid content (TFC)*

The total flavonoid content was measured using a standardized procedure.[15] The analysis involved preparing a 96-well microplate by adding 10 µL of the sample (0.4 g/mL), 10 µL of 10% $AlCl_3$, 10 µL of glacial acetic acid, 50 µL of ethanol, and 120 µL of distilled water. The mixture was incubated in the dark for 30 minutes. After incubation, the absorbance was measured using a nano-spectrophotometer (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany) at 415 nm. The flavonoid content was expressed in quercetin equivalents per gram of dry weight (ng QE/g DW) using a quercetin standard.

*Determination of antioxidant capacity*

*CUPRAC assay*

Antioxidant activity was determined using the Cupric Ion Reducing Antioxidant Capacity (CUPRAC) method. For the assay, 50 µL of the sample (0.4 g/mL) was mixed with 50 µL of 0.01 M $CuCl_2$, 50 µL of 0.0075 M neocuproine, and 50 µL of ammonium acetate buffer (pH 7) in a microplate. The mixture was incubated in the dark at room temperature for 30 minutes, after which the absorbance was measured at 450 nm using a nano-spectrophotometer (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany). The antioxidant capacity was quantified using the Trolox standard and reported as micromoles of Trolox equivalents (TE) per gram of dry weight (µmol TE/g DW).

*ABTS assay*

The ABTS antioxidant capacity was determined using the ABTS method. The ABTS reagent was prepared by mixing 7 mM ABTS with 2.4 mM $K_2S_2O_8$ in a 2:1 ratio. The absorbance of the reagent was adjusted to 0.7 ± 0.02 by dilution with distilled water. For the assay, 20 µL of the sample (0.4 g/mL) was mixed with 180 µL of the ABTS reagent and incubated for 6 minutes in the dark at room temperature. The absorbance was measured at 734 nm using a nano-spectrophotometer (SPECTROstar Nano, BMG LABTECH, Ortenberg, Germany). The antioxidant capacity was quantified using

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the Trolox standard and reported as micromoles of Trolox equivalents (TE) per gram of dry weight (µmol TE/g DW).

*Statistical Analysis*

All data were analysed using Design-Expert software version 23.1.4 (Stat-Ease, Inc., Minneapolis, MN, USA, 2023). The most optimal extraction conditions were selected based on the highest desirability score.

## Results and Discussion

*Optimization of extraction using I-optimal RSM*

The extraction process was optimized to identify the ideal solvent concentration and extraction time that would yield an extract with the highest levels of bioactive compounds. These parameters were selected as key factors to improve the extraction efficiency of flavonoids and antioxidant compounds from *A. ilicifolius*. The I-optimal RSM analysis produced 14 unique formulas that varied in ethanol concentration and extraction time (Table 1). Each combination significantly influenced the total flavonoid content (TFC), which ranged from 0.028 to 0.479 mg GAE/g DW. The antioxidant capacity of the extracts was evaluated using two methods: CUPRAC and ABTS, with results ranging from 2.023 to 14.149 µmol TE/g DW for CUPRAC and 0.807 to 24.270 µmol TE/g DW for ABTS. These findings illustrate the impact of solvent concentration and extraction time on the extraction of bioactive compounds from *A. ilicifolius*.

*Model fitting*

The optimization results were analysed using analysis of variance (ANOVA) to evaluate the accuracy of the model at a 95% confidence level (**Table 2**). The analysis generated a linear model that explained 51.34% ($p > 0.05$) of the variability for TFC, 57.54% ($p < 0.05$) for ABTS, and 22.76% ($p > 0.05$) for CUPRAC. An $R^2$ value above 70% generally indicates a well-fitted model, and values approaching 1.00 suggest that the model is more consistent with the actual data. The adjusted $R^2$ values for TFC, ABTS, and CUPRAC were 0.4161, 0.4904, and 0.0721, respectively, indicating how well the experimental data aligned with theoretical expectations. Adequacy precision, a metric comparing the range of predicted values to the average prediction error, showed that a ratio higher than 4 is adequate for guiding the design space.

**Table 2:** Results of analysis of variance (ANOVA) for response variables in the optimization of ethanol concentration and extraction time for *A. ilicifolius*

| Parameter | TFC | CUPRAC | ABTS |
|---|---|---|---|
| Model Type | Quadratic | Cubic | Cubic |
| F-value | 2.17 | 31.38 | 0.4842 |
| p-value | 0.02783 | 0.0112 | 0.5366 |
| $R^2$ | 0.8445 | 0.9850 | 0.9854 |
| Adjusted $R^2$ | 0.7473 | 0.9514 | 0.9524 |
| Adequacy Precision | 8.3533 | 13.6538 | 14.7022 |

*Effect of ethanol concentration and extraction time on TFC*

The highest total flavonoid content (TFC) was observed at an ethanol concentration of 69.3% and an extraction time of 130.2 seconds, yielding 0.479 mg GAE/g DW. Figure 1 illustrates the contour plot of the quadratic model, predicting the total flavonoid content (TFC) based on variations in extraction time (A) and ethanol concentration (B) during microwave-assisted extraction. The colour gradient in the plot represents TFC, with red indicating the highest concentrations and blue the lowest. The model confirms that an optimal combination of 69.3% ethanol and 130.2 seconds yields the maximum TFC. These conditions were identified as the most effective combination of solvent concentration and extraction time. Theoretically, increasing solvent concentration and extending extraction time generally enhance flavonoid yield.[23] The optimal concentration of 69.3% ethanol is likely due to the polarity of flavonoids in *A. ilicifolius* leaves, which are more soluble in a solvent with matching polarity, such as 69.3% ethanol.[24] Moreover, extraction time is a crucial factor in MAE, as it facilitates the release of bioactive compounds from the plant matrix. If the extraction time is too short, the process may be incomplete, preventing full release of these compounds.[23]

*Effect of solvent concentration and extraction time on antioxidant capacity*

The antioxidant activity of *A. ilicifolius* extracts is significantly influenced by solvent concentration and extraction time, as demonstrated by the highest CUPRAC antioxidant capacity (14.149 µmol TE/g DW) obtained at 76.6% ethanol and 138 seconds extraction time. The choice of solvent is critical, with ethanol and acetone or water-ethanol mixtures often proving the most effective in extracting antioxidant compounds, as supported by previous studies.[3,25,26] Furthermore, their findings emphasize that a water-ethanol mixture can

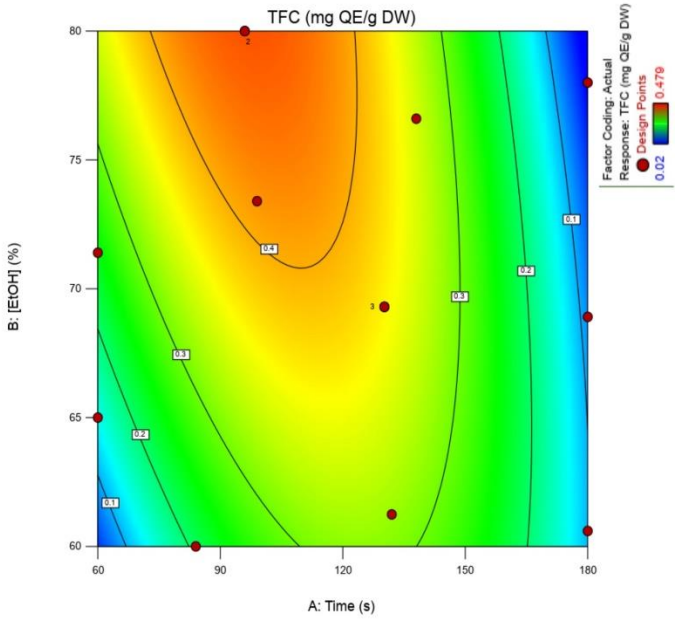


**Figure 1:** Contour plot of the quadratic model predicting total flavonoid content (TFC) based on extraction time (A) and ethanol concentration (B) during microwave-assisted extraction of *A. ilicifolius*.

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enhance the antioxidant properties of *A. ilicifolius*, aligning with our results that ethanol concentration significantly affects antioxidant yield. The cubic model (Figure 2) further supports this by illustrating that ethanol concentration and extraction time significantly influence antioxidant capacity, with optimal conditions of 76.6% ethanol and 138 seconds extraction time yielding the highest activity. These insights underscore the critical role of solvent systems and optimized extraction parameters in harnessing the antioxidant potential of *A. ilicifolius*.

The highest antioxidant activity measured by the ABTS method was observed at an ethanol concentration of 60% and an extraction time of 84 seconds, with a value of 24.270 µmol TE/g DW. Increasing both ethanol concentration and extraction time generally leads to an enhancement in antioxidant activity. This increase in ethanol concentration facilitates the extraction of phenolic compounds, which can inhibit oxidation reactions and exhibit strong antioxidant effects. Furthermore, prolonging the extraction time also positively affects antioxidant activity, as it allows for a greater extraction of phenolic compounds. **Figure 3** provides a contour plot of the cubic model, predicting the antioxidant activity as measured by the ABTS method, with time (A) and ethanol concentration (B) as variables in microwave-assisted extraction optimization. The colour gradient in the figure represents the ABTS antioxidant capacity, with red indicating the highest antioxidant levels and blue indicating the lowest. The model confirms that the optimal conditions for antioxidant activity, measured by ABTS, occur at 60% ethanol and 84 seconds of extraction time.

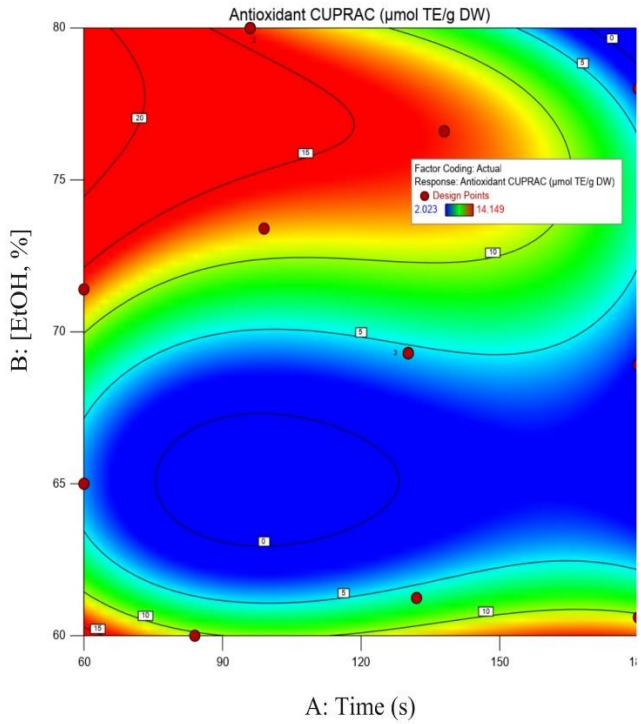


**Figure 2:** Contour plot of the cubic model predicted for antioxidant CUPRAC extraction as a function of time (A) and ethanol concentration (B) in microwave-assisted extraction optimization.

*Optimum formulation and confirmation*

The extraction optimization was carried out using Design Expert version 23.1.4 to optimize the bioactive compounds by adjusting key parameters. These parameters include ethanol concentration and extraction time, both of which were optimized to achieve the best formulation. The most optimal combination was determined based on the desirability value, which ranges from 0 to 1. A desirability value closer to 1 indicates that the combination is more optimal for maximizing total flavonoid content (TFC) and antioxidant capacity in the sample.[25,27] The optimal combination was found to have a desirability value of 0.775, predicting a TFC of 0.406 mg GAE/g DW and antioxidant capacities of 12.923 and 15.243 µmol TE/g DW for the CUPRAC and ABTS methods, respectively.

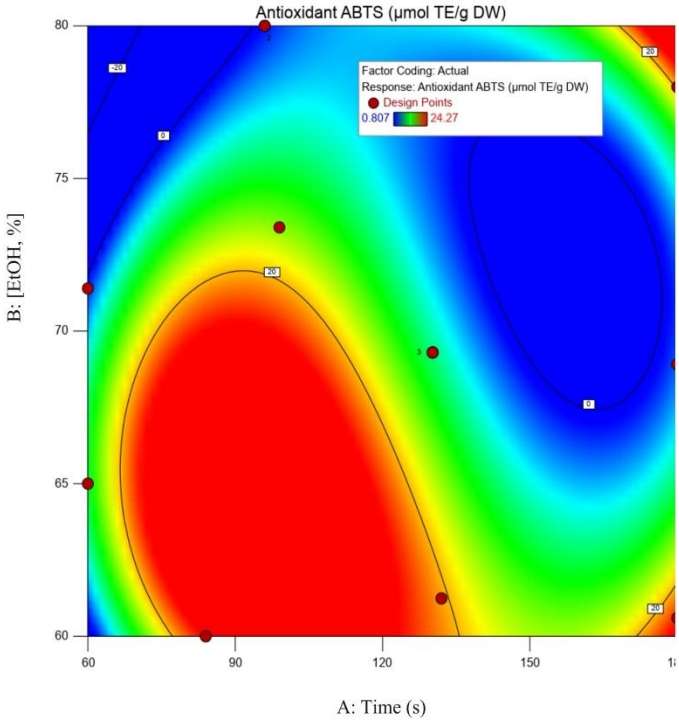


**Figure 3:** Contour plot of the cubic model predicted for antioxidant ABTS extraction as a function of time (A) and ethanol concentration (B) in microwave-assisted extraction optimization.

The optimal combination was further confirmed through prediction interval (PI) analysis. The PI percentage (%PI) represents the range of uncertainty in predicting the model's response, where the desired outcome should fall within the PI range. The 95% PI low represents the lower bound, while the 95% PI high represents the upper bound, both at a 95% confidence level. According to Table 3, the optimal combination for TFC resulted in a PI range of 0.210066 to 0.601381 mg GAE/g DW. For antioxidant capacity, the CUPRAC method showed a PI range of 7.91149 to 22.5732 µmol TE/g DW, while the ABTS method demonstrated a PI range of 9.01153 to 16.8342 µmol TE/g DW. Overall, these results indicate that the optimal combination lies within the prediction intervals, confirming the accuracy of the predictive model.

## Conclusion

This study successfully optimized the extraction of flavonoids and antioxidant capacity from *A. ilicifolius* leaves using microwave-assisted extraction (MAE) combined with I-Optimal Response Surface Methodology. The optimal extraction conditions were determined to be 69.3% ethanol concentration and an extraction time of 130.2 seconds, yielding the highest total flavonoid content (TFC) of 0.479 mg GAE/g DW. Furthermore, the maximum antioxidant capacity measured using the CUPRAC method was achieved at 76.6% ethanol and 138 seconds, with a value of 14.149 µmol TE/g DW, while the ABTS assay revealed the highest antioxidant activity at 60% ethanol and 84 seconds, yielding 24.270 µmol TE/g DW. These results demonstrate that both ethanol concentration and extraction time significantly influence the yield of bioactive compounds from *A. ilicifolius*, providing valuable insights for optimizing the extraction process to maximize antioxidant potential.

**Table 3:** Confirmation in MAE optimization of ethanol concentration and extraction time of *A. ilicifolius* leaves extracts

| Response Variable | Predicted Mean | SD | SE Pred | 95% PI Low | 95% PI High |
|---|---|---|---|---|---|
| TFC | 0.405724 | 0.0775849 | 0.084847 | 0.210066 | 0.601381 |
| CUPRAC | 15.2423 | 2.64037 | 2.64037 | 7.91149 | 22.5732 |
| ABTS | 12.9229 | 1.40876 | 1.40876 | 9.01153 | 16.8342 |

Future research should focus on validating the reproducibility of the optimized conditions at larger scales and investigating the stability of the extracted flavonoids and antioxidants during storage or processing. Additionally, refining solvent combinations and extraction parameters may further enhance the recovery of specific antioxidant compounds while ensuring environmental sustainability. These findings form a robust basis for utilizing *A. ilicifolius* extracts in functional food, supplements, and other health-related applications.

## Conflicts of Interest

The authors declare that they have no conflicts of interest to disclose.

## Authors' Declaration

The authors affirm that the work presented in this article is original and has not been published elsewhere. They further declare that they accept full responsibility for any claims arising from the content of this article.

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