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Screening of the process parameters for vacuum microwave-assisted extraction of bioactive compounds from amaranth flowers

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ABSTRACT

In this study, a series of trial experiments were carried out to determine the optimal extraction parameter ranges for betalains, phenolic compounds, and antioxidants from amaranth (*Amaranthus caudatus L.)* flowers during the vacuum-microwave extraction (VMAE). VMAE reduces thermal degradation and oxidation risks compared to traditional extraction methods, improving extraction efficiency for thermosensitive and oxygen-sensitive compounds. Since there is no prior study in the literature about optimisation of the extraction conditions of vacuum microwave-assisted extraction of betalains and other bioactive materials in amaranth flowers, this pre-study was essential. The experimental plan was designed according to different ethanol concentrations (20%-40%-60%), extraction periods (5-8-12 min), and pressures (100-450 mmHg). Subsequently, the total betalain content (TBC), total phenolic content (TPC), and total antioxidant capacity (TAC) values were measured. The aim was to identify the minimum and optimal conditions for extracting bioactive compounds using the VMAE method for further optimization studies. The experimental results showed that for the maximization of TBC, the conditions were 40% ethanol concentration, 8 min, and 450 mmHg pressure. Conversely, for the maximisation of TPC and TAC, the conditions were 20% ethanol concentration, 12 min, and 450 mmHg pressure. Scanning Electron Microscopy (SEM) analysis was performed to understand the general effects of VMAE on the morphology of the flower samples. These findings provided valuable insights for the future optimisation of extraction processes from amaranth flowers or similar betalain-containing plants using VMAE, contributing to the enhancement of extraction efficiency and the potential utilization of these bioactive compounds in various food applications.

Keywords: Green Extraction Methods, Nutraceuticals from Amaranth, Antioxidant Capacity, *Amaranthus caudatus L.,* Betalains, Phenolic compounds, Scanning electron microscopy

Introduction

Microwave-assisted extraction (MAE) has gained popularity over conventional extraction methods like maceration and Soxhlet extraction due to its ability to significantly reduce extraction time and solvent usage while enhancing yield and efficiency (Filip et al., 2017). MAE uses microwave energy to rapidly heat the solvent and sample, increasing mass transfer rates and improving bioactive compound extraction. Studies have shown that MAE can extract thermosensitive compounds more effectively than traditional methods by reducing thermal degradation and providing higher yields of phenolics, flavonoids, and other antioxidants (Cardoso-Ugarte et al., 2014). Additionally, MAE's uniform heating reduces the chances of localised overheating, which is common in conventional heating methods, making it a more efficient and controlled extraction process (Gu et al., 2016). This technology aligns with the growing demand for sustainable extraction methods in the food and pharmaceutical industries due to its lower energy consumption and reduced environmental impact (Skenderidis et al., 2021).

Vacuum microwave-assisted extraction (VMAE) builds on the advantages of MAE by incorporating vacuum conditions, which further lower the solvent's boiling point and reduce oxidation and thermal degradation risks (Xiao et al., 2009). The vacuum environment allows extraction at significantly lower temperatures, preserving the integrity of heat-sensitive bioactives, such as antioxidants and pigments, that might otherwise degrade under atmospheric pressure (Hiranvarachat et al., 2015). VMAE enhances solvent penetration and improves mass transfer efficiency, leading to higher extraction rates than atmospheric MAE (Gu et al., 2016). The absence of oxygen under vacuum prevents oxidation, making VMAE particularly advantageous for extracting oxygen-sensitive compounds. This method is recognised for its eco-friendly nature, as it reduces solvent use and energy consumption, making it a preferred choice in applications where the quality of extracted compounds is paramount (Sharma et al., 2023).

The red-coloured tissues of *Amaranthus caudatus* L. flowers offer a compelling alternative to traditional beetroot betalain pigments in the food industry (Govender & Baijnath, 2022; Peter & Gandhi, 2017). Abundant in betacyanins, these pigments have vivid red-violet colours and exceptional stability within specific pH and temperature ranges, surpassing red radish anthocyanins. *Amaranthus* varieties yield higher betacyanin concentrations than beetroot, with dried pigments demonstrating impressive storage stability. Additionally, these betalains exhibit potent antioxidant properties, a trait prevalent across the Amaranthaceae family (Martinez-Lopez et al., 2020). *Amaranthus* betalains hold significant promise as natural colourants for various food products, particularly those requiring low-temperature processing (Sharma et al., 2023). Roriz et al. (2021) investigated the chemical and bioactive properties of *Amaranthus caudatus* L. flowers and optimised the extraction of betalains using ultrasound-assisted techniques. Another study utilised microwave and ultrasound-assisted methods to extract bioactive compounds from the peel of *Opuntia* fruit (*Opuntia engelmannii* cultivar) (Melgar et al., 2017). In research conducted in 2020, microwave-assisted extraction of betacyanin and betaxanthin from *Amaranthus tricolour leaves highlighted their potential as natural food colourants* (Yiğit et al., 2022).

This study aimed to assess the efficacy of VMAE in extracting betalains and other bioactive compounds from amaranth flowers (Amaranthus caudatus L.) and establish a robust methodology, thereby propelling advancements in analytical techniques for natural products and further analysis.

Materials and Methods

Materials

The Eastern Mediterranean Agricultural Research Institute in Adana, Turkiye, kindly provided Amaranthus caudatus L. flower samples. The flowers were harvested between July and August 2023. The samples were stored at 4°C immediately after they were manually separated from the rest of the plant.

Methanol, ethanol, acetic acid, NaOH, gallic acid, and Folin-Ciocalteu were purchased from the Merck Chemical Reagents Company (Darmstadt, Germany). Ammonium acetate, sodium carbonate, KCl, sodium acetate, hydrochloric acid, formic acid, and trolox were purchased from Sigma-Aldrich (Darmstadt, Germany).

Vacuum Microwave-Assisted Extraction (VMAE)

Vacuum microwave-assisted extraction, in which an adjustable pressure range of 100-650 mmHg was used, was carried out while aiming to protect temperature-sensitive components. This allowed for precise control of the extraction temperature based on the solvent used, ensuring optimal conditions for preserving temperature-sensitive compounds like betalains and phenolics. The equipment has a maximum power of 1000 W; during the experiments, a constant of 100% power was applied (IFTECH, Ankara, Türkiye). The independent variables for VMAE conditions were pressure (100 and 450 mmHg), extraction period (5, 8, 12 min), and ethanol ratio (20%, 40%, 60%), which were selected based

on the literature (Melgar et al., 2017; Sharma et al., 2023). After each experiment, the solution was filtered through Whatman No.1 paper, and the supernatant was filtered by a 0.45 µm syringe filter and stored at 4°C for further analysis. The experimental design is presented in Table 1.

Quantifying Phytochemicals

Total betalain content (TBC) was evaluated spectrophotometrically, 535 and 483 nm betacyanin and betaxanthin, respectively, and calculated by Equation (1);

$$TBC\ (mg\frac{betalain}{kg}dr\ ymatter) = \frac{A.df.M_w.V_d.1000}{\varepsilon.L.W_d} \quad (1)$$

where A: absorption value, df: dilution factor, Mw: molecular weight (550 and 308 g/mol for betacyanin and betaxanthin, respectively), Vd: volume of dilution (mL), ε: molar extinction coefficient (60,000 and 48000 L/(mol cm) for betacyanin and betaxanthin, respectively), L: path-length (1 cm) of the cuvette and Wd: the amount of the extracted sample (g) (Castellanos-Santiago & Yahia, 2008).

The Folin–Ciocalteau method was used to determine the amaranth flowers' total phenolic compounds (TPC) (Yiğit et al., 2022). The TPC values of the samples were expressed in mg gallic acid equivalence (GAE)/g dry matter.

The DPPH and CUPRAC radical scavenging assays were used to evaluate the total antioxidant capacity of the amaranth flower extracts spectrophotometrically (TAC_{DPPH} and TAC_{CUPRAC}). Using a UV/VIS spectrophotometer (Thermoscientific, Genesys 10S UV-Vis), the absorbance values for TAC_{DPPH} and TAC_{CUPRAC} were determined to be 517 and 450 nm, respectively (Akdeniz et al., 2018; Apak et al., 2007). The TAC values of the samples were expressed in mmol Trolox equivalence (TE)/kg dry matter for both antioxidant capacity assays.

Scanning Electron Microscopy (SEM) Analysis

The untreated flower and extract samples (extraction conditions: 40% ethanol, 450 mmHg and 12 minutes) samples were identified morphologically. The morphology of the samples was analysed using a scanning electron microscope (SEM) (Tescan, Gaia3, Triglav™, Czech Republic). The VMAE effects on the physical changes in the flower matrix were detected.

Statistical Analysis

Three-way analysis of variance (ANOVA) was applied to all the results from 18 experiments to understand the overall effects of the independent variables. Two-way ANOVA was applied for two experimental groups having 9 experiments having constant pressure (100 mmHg or 450 mmHg) for the efficient representation of the results in the figures visually. Minitab 18 (Pennsylvania, USA) statistical software was used, and Tukey multiple comparison tests were performed to understand the significant differences between the results ($p<0.05$).

Table 1. Experimental design and results*

Run	Ethanol Ratio (%)	Time (min)	Pressure (mmHg)	TBC (mg/g)	TPC (mg GAE/g)	TAC_{DPPH} (mmol TE/ kg)	TAC_{CUPRAC} (mmol TE/ kg)
1	20	5	450	2449.70	11.8	52.86	157.26
2	20	8	450	2299.24	10.81	54.75	162.03
3	20	12	450	2680.94	15.34	65.52	187.81
4	20	5	100	2296.80	9.43	40.1	102.29
5	20	8	100	2704.53	10.69	44.16	130.55
6	20	12	100	2405.94	10.37	43.78	116.63
7	40	5	450	2968.98	13.12	78.99	146.54
8	40	8	450	3382.74	14.7	89.09	183.32
9	40	12	450	2094.22	12.3	75.85	147.23
10	40	5	100	1618.10	7.26	45.29	89.55
11	40	8	100	1884.61	8.63	51.7	104.37
12	40	12	100	1809.44	8.25	45.29	98.97
13	60	5	450	1332.22	8.72	45.56	114.16
14	60	8	450	1680.86	10.91	51.66	132.59
15	60	12	450	2645.01	13.76	64.46	150.14
16	60	5	100	3401.32	2.54	15.6	29.35
17	60	8	100	1476.14	6.73	33.27	78.74
18	60	12	100	3124.00	12.28	56.53	141.06

*Experimental results were given as the mean value of three replications.

Results and Discussion

Total Betalain Content (TBC)

Betalains are water-soluble pigments found in various plants of the Caryophyllales order, such as Amaranthus species. They are appreciated for their vibrant red and yellow hues and their antioxidant properties, making them valuable natural food colourants with potential health benefits. The extraction efficiency of betalains is influenced by factors like solvent concentration, extraction duration, and applied pressure. This study evaluated the Total Betalain Content (TBC) under varying ethanol concentrations, extraction times, and pressures (450 mmHg for Figure 1A and 100 mmHg for Figure 1B), revealing significant variations in TBC depending on these conditions.

In Figure 1A, conducted under 450 mmHg, the highest TBC was observed at 8 minutes with 40% ethanol, suggesting that moderate ethanol concentrations combined with extended extraction times maximise betalain recovery (Wang et al., 2008). At 60% ethanol, TBC was minimised at 8 minutes, indicating that higher ethanol concentrations might not always favour prolonged extraction. Figure 1B, performed at 100 mmHg, further supports these observations; the highest TBC was achieved at 12 minutes with 60% ethanol, underscoring the benefits of lower pressure in enhancing the solubility and stability of thermosensitive compounds (Xiao et al., 2009). Similar findings have been reported in other studies showing that vacuum conditions improve extraction efficiency by preventing the degradation of sensitive compounds (Xiao et al., 2012; Hiranvarachat et al., 2015). These results emphasise optimising solvent concentration, extraction time, and pressure to maximise TBC.

Total Phenolic Compounds (TPC)

Phenolic compounds are a diverse group of secondary metabolites widely distributed in the plant kingdom, known for their antioxidant properties and health benefits. Their extraction efficiency is influenced by factors such as the type of solvent, extraction time, and applied pressure. The total phenolic content (TPC) of extracts from *Amaranthus caudatus* L. flowers was assessed under different conditions, and the results are presented in Figures 2A and 2B. Figure 2A shows TPC results at a constant pressure of 450 mmHg. For a 20% ethanol concentration, the highest TPC was observed at 12 minutes, significantly higher than shorter extraction durations. This indicates that longer extraction times enhance phenolic recovery at this ethanol level, aligning with findings from Lovrić et al. (2017), where microwave-assisted extraction (MAE) enhanced phenolic yields in blackthorn flowers. The ethanol concentration increased to 60%, and the lowest TPC value was obtained at 5 minutes. However, as the extraction time increased, TPC values also increased at this ethanol concentration.

When the pressure decreased to 100 mmHg, Figure 2B showed that TPC results were generally lower than those at 450 mmHg. At 40% ethanol, TPC values at 8 and 12 minutes were not significantly different but were higher than at 5 minutes, demonstrating that medium ethanol concentrations paired with longer extraction times are optimal. Similar trends were observed in other studies, highlighting the benefits of medium ethanol levels under vacuum conditions for phenolic extraction (Nguyen et al., 2020). At a 60% ethanol concentration, TPC peaked at 12 minutes, significantly surpassing all other conditions. This highlights that higher ethanol concentrations combined with longer extraction times greatly improve extraction efficiency due to enhanced solubilisation and solvent penetration into plant tissues, supporting the findings of previous research on the extraction of betalains and phenolics from red beet and Amaranthus species (Cardoso-Ugarte et al., 2014; Li et al., 2015). These results indicate that to maximise TPC, optimal conditions include either 40% ethanol with medium extraction times or 60% ethanol with extended extraction durations (Milutinović et al., 2015).

Total Antioxidant Capacity (TAC_{DPPH})

The antioxidant capacity of the extracts was evaluated using the DPPH radical scavenging method, which measures the ability of antioxidant compounds to neutralise free radicals, thus preventing oxidative stress and related diseases. Figures 3A and 3B present the TAC_{DPPH} results under different extraction conditions, including varying ethanol concentrations, extraction times, and pressures (450 mmHg for Figure 3A and 100 mmHg for Figure 3B). For a 20% ethanol concentration under 450 mmHg, TAC_{DPPH} was initially high but showed no significant increase over time, suggesting that while the initial extraction is efficient, extended durations do not enhance antioxidant capacity, likely due to solvent saturation (Skenderidis et al., 2021). At 40% ethanol, TAC_{DPPH} peaked at 8 minutes, indicating that intermediate ethanol concentrations combined with moderate extraction times are optimal for extracting antioxidant compounds. The highest efficiency was observed at 450 mmHg and 60% ethanol, showing a slight increase over time, reinforcing the benefits of prolonged extraction at higher ethanol concentrations (Wang et al., 2008).

Figure 3B, conducted at 100 mmHg, revealed generally lower TAC values than those at 450 mmHg. At 60% ethanol, TAC reached its maximum at 12 minutes, significantly surpassing

all other conditions, emphasising the combined effect of reduced pressure and extended extraction time in maximising antioxidant yield. Lower ethanol concentrations of 20% and 40% resulted in relatively stable TAC values across different extraction times, illustrating the stabilising effect of reduced pressure on antioxidant compounds (Xiao et al., 2009). The results highlight that 40% ethanol and 450 mmHg pressure generally provide the highest antioxidant capacity, consistent with findings on optimising extraction parameters to enhance antioxidant yields (Nguyen et al., 2020). These insights underline the critical role of careful parameter selection in maximising plant extracts' DPPH radical scavenging activity.

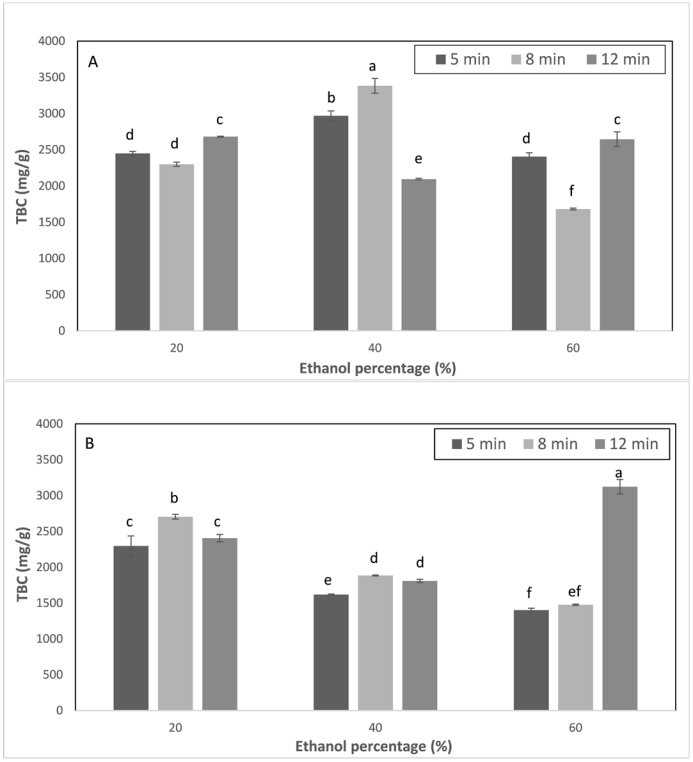

Figure 1. Total betalain content values (TBC) (mg/kg) at different ethanol percentages and extraction periods (A: 450 mmHg, B: 100 mmHg)

Total Antioxidant Capacity (TAC_{CUPRAC})

The antioxidant capacity of the extracts was evaluated using the CUPRAC method, with results shown in Figures 4A and 4B. Statistical analyses revealed significant differences ($p<0.05$) between various extraction conditions, highlighted by Tukey's multiple comparison test. In Figure 4A at 450 mmHg, TAC_{CUPRAC} peaked at 20% ethanol and 12 minutes, significantly higher than shorter extraction times, indicating that extended extraction times enhance antioxidant recovery, especially at moderate ethanol concentrations (Xiao et al., 2012). The highest efficiency at 40% ethanol was noted at 8 minutes, suggesting this combination optimises antioxidant extraction without prolonged exposure. However, at 60% ethanol, TAC_{CUPRAC} values were generally lower but still benefited from increased extraction duration, reflecting the complex interaction between ethanol concentration and time (Hiranvarachat et al., 2015).

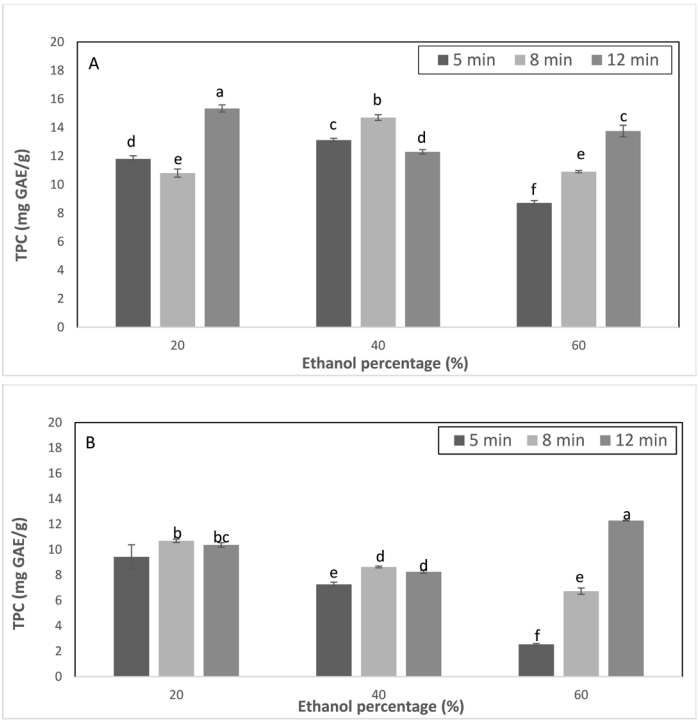

Figure 2. Total phenolic content (TPC) (mg GAE/g) at different ethanol percentages and extraction periods (A: 450 mmHg, B: 100 mmHg)

In Figure 4B, where the pressure was reduced to 100 mmHg, TAC_{CUPRAC} values were generally lower across all ethanol concentrations than 450 mmHg. At 60% ethanol, the highest antioxidant capacity was observed at 12 minutes, significantly outperforming other conditions and demonstrating the synergistic effect of low pressure and extended extraction duration in enhancing antioxidant yield (Lovrić et al., 2017). Lower ethanol concentrations (20% and 40%) exhibited less variability, with relatively stable TAC_{CUPRAC} values across different extraction times, highlighting the stabilising influence of reduced pressure (Wang et al., 2008). These findings suggest that to maximise antioxidant potential, careful optimisation of pressure, ethanol concentration, and extraction time is crucial, particularly when balancing efficiency and compound stability.

Screening of Optimal Ranges for Future Optimization

This study aimed to identify the appropriate ranges of ethanol concentrations, extraction times, and pressures for extracting bioactive compounds from *Amaranthus caudatus* L. flowers using vacuum microwave-assisted extraction (VMAE). The effects on total betalain content (TBC), total phenolic compounds (TPC), and total antioxidant capacity (TAC_{DPPH} and TAC_{CUPRAC}) were evaluated. The findings suggest that 20%, 40%, and 60% ethanol concentrations, 2-12 minutes of extraction time, and 100-450 mmHg pressure effectively extract these compounds. Specifically, the results indicate that for TBC, 40% ethanol concentration at 200 mmHg or 60% ethanol concentration at 100 mmHg yielded the highest extraction efficiencies. For TPC, 40% ethanol concentration at 450 mmHg or 60% ethanol concentration at 100 mmHg were the most effective conditions. TAC_{DPPH} and TAC_{CUPRAC} 40% ethanol concentration at 450 mmHg generally provided the highest antioxidant capacities. These identified ranges provide a foundation for future optimisation studies to fine-tune the extraction conditions and maximise the yield of valuable bioactive compounds from *Amaranthus caudatus* L. flowers.

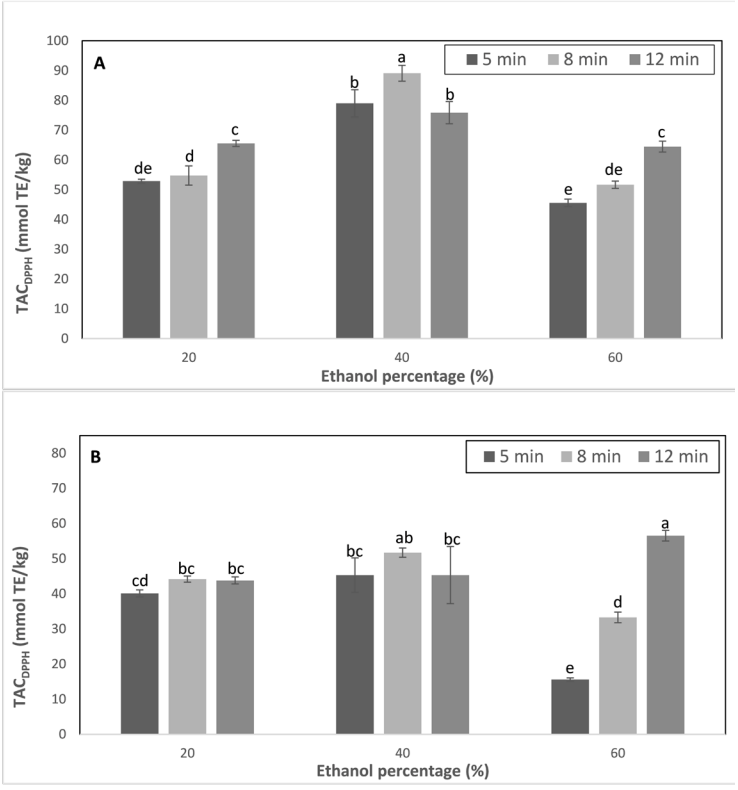

Figure 3. Total antioxidant capacity (TACDPPH) (mmol TE/kg) extraction efficiencies at different ethanol percentages and extraction periods (A: 450 mmHg, B: 100 mmHg)

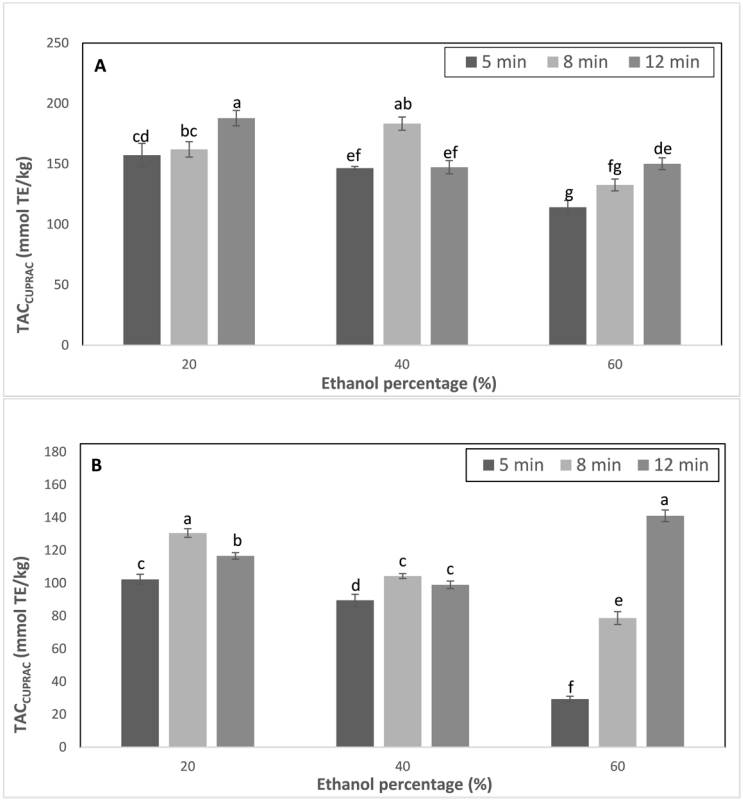

Figure 4. Total antioxidant capacity (TACCUPRAC) (mmol TE/kg) extraction efficiencies at different ethanol percentages and extraction periods (A: 450 mmHg, B: 100 mmHg)

Morphology of Amaranth Samples

The SEM images of the amaranth flowers, both untreated and vacuum microwave-assisted extracted, reveal significant differences in their surface morphology, presence of residues, cell integrity, and porosity. These differences provide insights into the impact of VMAE on plant material. The surface morphology of the untreated amaranth flowers in Figure 5 (a) and Figure (c) appears intact and smooth, with well-preserved cellular structures. The cell walls are clearly defined, and the overall structure is cohesive. Studies on untreated plant cells typically show a well-maintained cellular structure. According to Peter and Gandhi (2017), the natural state of plant cells presents organised and intact structures. On the other hand, the VMAE amaranth flower samples' surfaces in Figures 5 (b) and (d) show significant disruption. The cell walls are broken, and the surfaces are more fractured and porous. The cellular structures are less defined and appear irregular. VMAE often results in significant morphological changes in plant materials. Gu et al. (2016) reported that such extraction methods can disrupt cell walls and create more porous surfaces, enhancing extraction efficiency. Similarly,

Sharma et al. (2023) found that microwave-assisted extraction caused surface roughening and structural damage in plant tissues.

The presence of residues is minimal on the surfaces of the untreated amaranth flowers, with only natural deposits or particles visible. Untreated samples usually have a cleaner appearance with fewer residues. This observation is supported by Filip et al. (2017), who noted the absence of significant residues in untreated plant samples. However, VMAE amaranth samples show visible residues or remnants of the extraction solvent or other chemicals, appearing as white spots or layers on the surfaces. Residues left by the extraction process are common in treated samples. According to Wang et al. (2008), microwave-assisted extraction can leave behind visible residues from the solvents used.

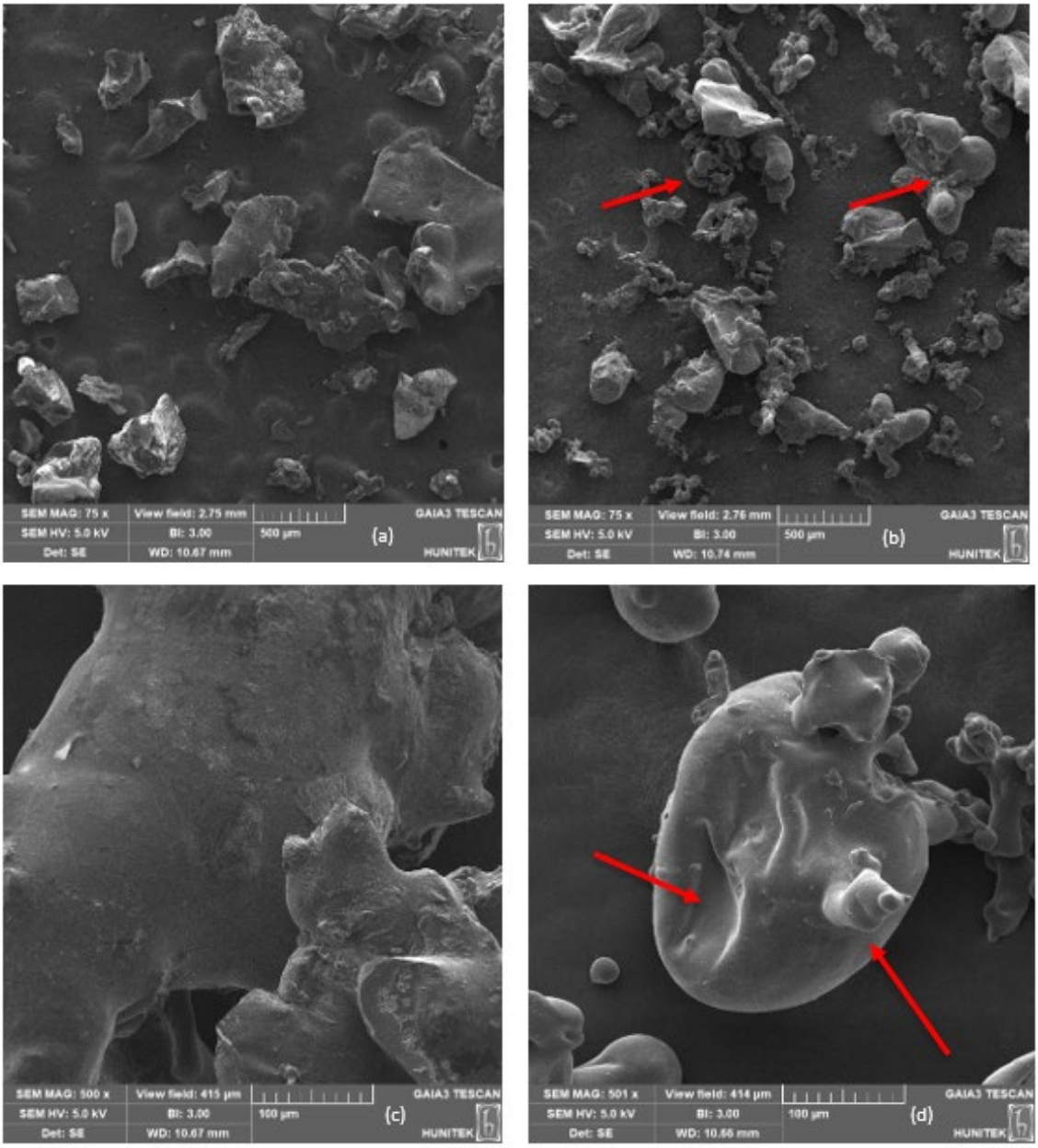

Figure 5. SEM images of amaranth samples, (a) and (c) untreated samples in 75X and 500X magnification, respectively; (b) and (d) VMAE treated samples in 75X and 500X magnification, respectively

Porosity is another critical factor influenced by the extraction process. The natural porosity of the untreated amaranth samples is visible, with pores and spaces distributed evenly throughout the surfaces. Martinez-Lopez et al. (2020) described the consistent natural porosity of untreated plant cells. In the VMAE extracts, porosity has increased due to the extraction process. New pores or enlarged existing ones are visible, providing more surface area for solvent interaction. Thirugnanasambandham and Sivakumar (2017) found that increased porosity is a common result of microwave-assisted extraction.

Amaranth flowers are rich in phenolic compounds and betalains, which can accumulate on the surface and increase electron density, appearing as white stains in SEM images. These chemical compounds exhibit high electron density, indicating prominent white spots in SEM images. The study by Martinez-Lopez et al. (2020) supports this observation, demonstrating that phenolic compounds and betalains in Amaranth flowers contribute to the appearance of white stains due to their high electron density (Martinez-Lopez et al., 2020). Furthermore, studies on vacuum microwave-assisted extraction (VMAE) techniques, such as the research by Wang et al. (2008), observed similar white stains in SEM images attributed to the high density of bioactive compounds like polyphenols. These studies corroborate that dense chemical compounds in plant tissues produce white spots in SEM images.

Conclusion

This work successfully determined optimal process parameter ranges for VMAE of bioactive compounds from Amaranth flowers (Amaranthus caudatus L.). The investigation revealed that 40% ethanol concentration, an 8-minute extraction period, and 450 mmHg pressure were optimal for maximising TBC. On the other hand, the conditions of 20% ethanol concentration, a 12-minute extraction period, and 450 mmHg pressure were the most effective for maximising TPC and TAC. The results show the importance of ethanol concentration, extraction time, and pressure as critical factors influencing the efficiency of VMAE. Therefore, these parameters and the ranges may be used as independent variables in future optimisation studies. SEM analysis revealed some morphological changes in the amaranth flowers after extraction, highlighting the impact of VMAE on cell wall disruption and increased porosity. These findings of this study provide a foundational framework for the future optimisation of VMAE conditions for extracting valuable bioactive compounds from *Amaranthus caudatus* L. flowers and potentially other betalain-rich plants. Further research should focus on refining the identified parameter ranges and exploring the scalability of VMAE for industrial applications.

Compliance with Ethical Standards

Conflict of interest: The author(s) declares that they have no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: The authors declare that this study does not include experiments with human or animal subjects, so ethics committee approval is not required.

Data availability: Data will be made available on request.

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Disclosure: -

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