Chemical Papers (2026) 80:9167–9188
https://doi.org/10.1007/s11696-026-04943-x

ORIGINAL PAPER

# Green ultrasound-assisted extraction of bioactive compounds from *Alternanthera sessilis* leaves

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Received: 23 November 2025 / Accepted: 21 April 2026 / Published online: 11 May 2026
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## Abstract

*Alternanthera sessilis* is a traditional edible and medicinal plant with potential as a source of bioactive compounds. This study provides an initial and innovative assessment of ultrasound-assisted extraction using green solvents, ethanol and palm olein, for the recovery of phytochemicals and antioxidant activity from its leaves. Extraction conditions were evaluated through a one variable at a time approach under 23 experimental conditions, considering ultrasound intensity, ethanol concentration, solvent composition, particle size, temperature and extraction time. The resulting data were further examined using principal component analysis, multiple linear regression, and partial least squares regression. The best extraction performance was obtained with 70% ethanol, ethanol: palm olein (1:1), 60 mesh particle size, 9.5 W $cm^{-2}$, 25 °C and 10 min. Under these conditions, the contents of phenolics (6.42–37.47 mg GAE/g), flavonoids (4.23–40.81 mg CAT/g), tannins (83.85–263.40 mg CAT/g), and saponins (25.89–387.12 mg DIO/g), increased markedly, accompanied by substantial improvements in antioxidant capacity (4.27 to 225.12 µmol TE/g and 25.29 to 232.10 mg GAE/g). All solvents showed environmental performance (AGREE) with scores between 0.63 and 0.54 (palm olein> EtOH: palm olein (1:1)> EtOH). The extracts also showed strong scavenging activity against $H_2O_2$, NO•, and HO• radicals, as well as antimicrobial activity, especially against Gram positive bacteria. Multivariate analysis indicated that ultrasound intensity was the most influential extraction parameter, while the regression models showed good predictive ability. Taken together, these findings indicate that ultrasound assisted extraction combined with green solvents is a rapid and promising strategy for enhancing the recovery of bioactive compounds from *Alternanthera sessilis* leaves, providing a useful foundation for future optimization studies.

**Keywords** Ethanol · Palm olein · Antioxidant capacity · Phenolics · Antimicrobial activity, AGREE

## Introduction

Phytochemicals are naturally occurring compounds synthesized by plants through biosynthetic pathways and are commonly referred to as secondary metabolites. These compounds include phenolics, flavonoids, saponins and carotenoids, and are widely recognized for their diverse biological activities (Abegaz and Kinfe 2019). In the human diet, phytochemicals have attracted increasing attention due to their antioxidant, anti-inflammatory, antimicrobial, and other health-promoting effects (Sauer and Bernstein 2019; Sharma et al. 2021; Wang et al. 2020). Owing to these properties, they are considered promising candidates for the development of nutraceuticals, functional foods, and pharmaceutical products. However, the efficient recovery of phytochemicals from plant matrices strongly depends on the extraction technique employed, which directly influences extraction yield, selectivity, and the preservation of biological activity (Bitwell et al. 2023; Zhang et al. 2018).

Amazonian spinach, also known as monkey ear (*Alternanthera sessilis* L. R.Br.), is a rustic and nutritious PANC (Unconventional Food Plant) with recognized pharmacological potential, has attracted increasing scientific interest. Native to the Amazon region and widely distributed across tropical countries, this species has traditionally been consumed both as a leafy vegetable and as a medicinal herb

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(Ferreira et al. 2021; Ragavan et al. 2023). Phytochemical investigations have revealed a diverse profile of bioactive constituents in its leaves, including phenolic acids, flavonoids, saponins, carotenoids, and condensed tannins. These metabolite classes are frequently associated with several biological activities, such as antihyperglycemic effects, analgesic activity, wound healing, and antioxidant and antimicrobial properties (Hossain et al. 2014; Kota et al. 2017; Muniandy et al. 2018; Saqib and Janbaz 2016). Due to their recognized biological relevance, these compounds represent important targets for extraction and quantification in studies aimed at exploring the functional and pharmacological potential of *Alternanthera sessilis* L. R.Br.

To recover such bioactive compounds, traditional extraction methods such as maceration, Soxhlet extraction, and mechanical agitation have been widely employed. However, these approaches often require large volumes of solvents, long extraction times, and high energy consumption, which may reduce extraction efficiency and raise environmental and economic concerns (Gunathilake and Ranaweera 2016; Kota et al. 2017; Othman et al. 2016; Sun et al. 2025). These limitations have encouraged the development of alternative extraction technologies capable of improving efficiency while reducing environmental impact.

Ultrasound-assisted extraction (UAE) has emerged as a promising alternative technique. UAE relies on acoustic cavitation phenomena that disrupt plant cell walls, enhance solvent penetration, and facilitate mass transfer, thereby improving the release and solubilization of intracellular compounds (Chanioti and Tzia 2017; Panja 2018; Mungwari et al. 2025). Compared with conventional extraction methods, UAE can significantly reduce solvent consumption, shorten extraction times, and increase extraction efficiency (Dzah et al. 2020; Mehmood et al. 2019). Furthermore, its operational flexibility allows compatibility with a wide range of solvents, making UAE particularly attractive for the development of sustainable extraction strategies (Ez Zoubi et al. 2021; Gómez-López et al. 2021; Li et al. 2013).

However, the overall environmental impact and efficiency of extraction processes depend not only on the extraction technology but also on the selection of appropriate solvents. In this context, the concept of green extraction has promoted the use of environmentally friendly solvents capable of replacing conventional organic solvents. Among polar solvents, ethanol (EtOH) and EtOH: water mixtures are widely recognized due to their low toxicity, biodegradability, and acceptance in food and pharmaceutical applications (Prasad et al. 2022).

For non-polar compounds, edible oils have gained increasing attention as alternative green solvents due to their lipophilic nature and compatibility with food systems (Cardoso et al. 2024; Odia et al. 2015; Ong and Goh 2002; Rahimi 2019; Panagiotakopoulos et al. 2023; Ordoñez-Santos et al. 2019; Mba et al. 2015). These oils are considered renewable solvents because of their biodegradability, low toxicity, and ability to dissolve lipophilic bioactive compounds (Yara-Varón et al. 2017). In addition, compared with conventional organic solvents, edible oils can simplify downstream processing by enabling the direct incorporation of extracts into food or nutraceutical formulations (Cardoso et al. 2024).

Among the available edible oils, palm olein offers several practical advantages as an extraction medium. It is the liquid fraction obtained from the physical fractionation of refined palm oil, and is characterized by a balanced composition of saturated and monounsaturated fatty acids, with a relatively low proportion of polyunsaturated fatty acids (Mba et al. 2015; Athanasiadis et al. 2024). This fatty acid profile confers high oxidative stability and suitable physicochemical properties, which are particularly relevant under processing conditions such as ultrasound-assisted extraction, where localized heating and the formation of reactive species may accelerate lipid oxidation.

Although other edible oils, including sunflower, soybean, and olive oil, can also be used as extraction media, those that are particularly rich in polyunsaturated fatty acids, such as soybean and sunflower oil, are more susceptible to oxidation during processing and storage. In contrast, palm olein exhibits higher oxidative stability, which helps maintain solvent integrity and reduces the formation of oxidation products that could promote the degradation of extracted lipophilic bioactive (López-Beceiro et al. 2011; Ong and Goh 2002; Yara-Varón et al. 2017). At the same time, palm olein is widely employed in food systems and combines technological suitability with a neutral or mild sensory impact, which is advantageous for the direct incorporation of oil-based extracts into food and nutraceutical formulations. Moreover, its large-scale production, well-established global supply chain, and economic competitiveness further support its relevance for industrial extraction processes (Mba et al. 2015; Ong and Goh 2002). Taken together, these aspects indicate that palm olein is not only a green solvent, due to its biodegradability and food-grade status, but also a strategically attractive option for developing efficient and industrially viable extraction processes for lipophilic compounds.

However, the effectiveness of extraction systems combining ultrasound and green solvents largely depends on the proper adjustment of operational parameters. In particular, extraction performance is governed by process conditions such as ultrasound intensity, temperature, extraction time, solvent composition, and particle size (Drăghici-Popa et al. 2025; Benedito-Fort et al. 2020; Kumar et al. 2021; Zhang et al. 2023). Ultrasound intensity regulates cavitation strength

and the mechanical disruption of cell walls, whereas temperature and extraction time affect the solubility and diffusion of target compounds in the extraction medium (Liu et al. 2010; Low et al. 2022). Additionally, solvent composition governs the affinity between the solvent and target metabolites, while particle size influences the available surface area for solvent penetration and compound recovery (Martínez-Ramos et al. 2020; Shen et al. 2023). Inadequate operating conditions may therefore lead to inefficient extraction or degradation of sensitive metabolites due to excessive cavitation effects, free radical formation during ultrasonic processing, or thermal degradation of thermolabile compounds caused by elevated temperatures or prolonged extraction times (Ashokkumar et al. 2008; Dzah et al. 2020; Masuda et al. 2015 Sancheti 2017) Therefore, systematic evaluation and modeling of extraction variables are essential for identifying optimal processing conditions and improving the recovery of bioactive compounds.

As an initial step, the influence of individual extraction variables can be investigated using a one-variable-at-a-time (OVAT) approach, which allows the independent effect of each parameter to be assessed (Weremfo et al. 2023). This approach is particularly useful for obtaining preliminary insights into the extraction system and understanding the influence of key operational variables, thereby providing a basis for the design of subsequent optimization strategies. However, this approach does not account for possible interactions between variables, which represents a limitation that should be considered when interpreting the results. Given that extraction processes often involve complex interactions among variables, more comprehensive insights can be obtained through multivariate statistical methods, such as principal component analysis (PCA), multiple linear regression (MLR), and partial least squares regression (PLSR) (Ahmadian-Kouchaksaraie et al. 2016; Ruan 2024; Yu and Yao 2017). These tools enable the identification of relationships between experimental variables and response parameters, improving the interpretation of experimental data and supporting the development of future optimization approaches, such as response surface methodology and Box–Behnken designs.

In addition to extraction efficiency and selectivity, the development of modern extraction processes for phytochemicals must also consider their environmental performance, in line with the principles of green analytical chemistry. In this regard, metric tools such as the Analytical GREEnness calculator (AGREE), which integrates the 12 principles of green analytical chemistry into a single, pictorial score ranging from 0 to 1, have been proposed as a systematic approach to quantify the sustainability of analytical methods, including sample preparation and extraction procedures (Pena-Pereira et al. 2020). By simultaneously accounting for factors such as solvent toxicity, renewability, waste generation, energy consumption, and operator safety, AGREE enables an objective comparison of alternative extraction strategies and supports the selection of greener options without disregarding analytical performance. Within the context of ultrasound-assisted extraction using ethanol, palm olein, and their mixture, the application of AGREE is particularly relevant to assess how the choice of solvent system influences not only the recovery of bioactive compounds but also the overall environmental footprint of the process (Wojnowski et al. 2022).

The main objective of this study was to evaluate ultrasound-assisted extraction using green solvents, specifically palm olein and ethanol, as an innovative strategy for recovering bioactive compounds and antioxidant capacity from *Alternanthera sessilis* L. R. Br. leaves. To achieve this objective, key extraction parameters (ultrasound intensity, solvent composition, particle size, extraction temperature, and extraction time) were systematically varied using a one-variable-at-a-time approach to assess their individual effects on extraction performance. In parallel, multivariate statistical tools, including PCA, MLR, and PLSR, were applied to elucidate the relationships between process variables and the recovery of bioactive compounds and antioxidant responses. In this context, the integration of experimental evaluation and statistical modeling was employed not only to explore the potential of ultrasound-assisted extraction with green solvents as an efficient and sustainable strategy for enhancing the recovery of bioactive compounds from *Alternanthera sessilis* L. R. Br., but also to provide a rational basis for future optimization of the extraction process.

## Methodology

### Chemicals and materials

Refined palm oelin was donated by the company "*Companhia Refinadora da Amazônia*" - CRA (Belém, Brazil). All bacterial strains and the yeast were purchased from Controllab (Rio de Janeiro, Brazil). Sodium carbonate ($Na_2CO_3$; 95%), sodium nitrite ($NaNO_2$; 99%), iron II sulfate heptahydrate ($FeSO_4.7H_2O$; 98%), potassium iodide (KI; 98%), sodium thiosulfate ($Na_2S_2O_3$;98%), sodium nitroprusside ($Na_2[Fe(CN)_5NO]$; 99%), Folin–Ciocalteau reagent, sodium salicylate ($C_7H_5NaO_3$;98%), aluminum chloride ($AlCl_3$; 99%), sodium phosphate ($Na_3PO_4$; 99%), ammonium molybdate ($(NH_4)_6Mo_7O_{24}$; 98%), sodium hydroxide (NaOH; 98%), potassium hydroxide (KOH; 99%), L-ascorbic and vanillin (98%) were purchased from Neon (São Paulo; Brazil). 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2 5 7 8-tetramethylchromane-2-carboxylic

acid (Trolox), gallic acid (99%), catechin (99%), diosgenin (99%), bovine serum albumin (BSA) (98%), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide, RPMI 1640, 3-morpholinopropane sulfonic acid, diclofenac sodium salt, Mueller Hinton Broth, methyl sulfoxide and sabouraud dextrose agar were purchased from Sigma–Aldrich (Steinheim, Germany). Sulfuric acid ($H_2SO_4$), methanol ($CH_4O$), hydrochloric acid (HCl), EtOH ($C_2H_6O$), hydrogen peroxide ($H_2O_2$) was purchased from Synth (São Paulo, Brazil) and their purity was above 98%. Sulfanilamide (98%), ortho-phosphoric acid P.A. and dihydrochloride de N-(1-naftil) ethylenediamine (98%) was purchased from Vetec (São Paulo, Brazil).

## Raw material

Amazonian spinach (*Alternanthera sessilis* L. R.Br.) was purchased from the local market in Belém (Pará, Brazil). Leaves lyophilization was performed using a bench-top lyophilizer (Liotop, model L101, Brazil) at a temperature of -63 °C and a pressure of -65 µHg for 72 h. The dehydrated leaves were stored in metalized packages and kept at–18 ± 2.0 °C for further extractions.

## Single factor experiments

The influence of key parameters in ultrasound-assisted extraction, a systematic one-variable-at-a-time (OVAT) experimental design was employed. In this approach, one parameter was varied while the remaining variables were maintained at constant baseline conditions to isolate the individual effect of each factor. The sequence of experiments was defined considering first the solvent system variables and finally the operating conditions of the extraction process. The factor levels were selected based on ranges commonly reported in the literature for ultrasound-assisted extraction (Anaya-Esparza et al. 2023; Kumar et al. 2021; Yusoff et al. 2022) as well as preliminary tests conducted to ensure feasible operating conditions.

The influence of key factors on both bioactive compound yield and antioxidant capacity was assessed, including EtOH concentration (30, 50, 70, and 96%, v/v), solvent composition (EtOH: palm olein ratios of 1:0, 1:1, and 0:1, v/v), particle size (sieves of 60, 48, 42, and 35 mesh), ultrasound intensity (5.5, 7.9, 9.5, and 12.6 W/cm²), calculated according to the method described by Silva and Saldaña (2020), extraction temperature (25, 35, 45, and 55 °C), and extraction time (5, 10, 20, and 25 min). It is important to emphasize that when the variable being analyzed changes, the other variables remain constant.

A total of 23 OVAT experiments were conducted. The selection of the most favorable operating conditions was based on the maximization of phenolic, flavonoid, condensed tannin, and saponin contents, as well as the antioxidant capacity of the extracts. The experimental data obtained from the OVAT trials were subsequently used for multivariate statistical analysis to evaluate relationships between extraction variables and response parameters.

## Ultrasound-assisted extractions

Ultrasound-assisted extraction was performed using an ultrasonic processor (Qsonica Q700, USA) equipped with a probe transducer operating at 20 kHz and 700 W with a probe diameter of 1.27 cm. The extraction temperature was controlled using a thermostatic bath (Lauda RE 206, Berlin, Germany), and temperature variations caused by ultrasonic irradiation were monitored throughout the process to maintain the selected extraction temperature within ± 3 °C.

Extractions were carried out using 0.1 g of dried sample and 20 mL of solvent under pulsed ultrasound conditions (1 min on/30 s off). At the end of each extraction, the samples were centrifuged (Q222B, Quimis, Brazil) at 1,008 × g for 10 min. After centrifugation phase, the liquid fraction was separated from the solid residue and stored in amber glass containers until further analysis The overall extraction procedure can be summarized as shown in the flow diagram presented in Fig. 1.

The extracts obtained with EtOH, EtOH: palm olein (1:1), and palm olein were analyzed for bioactive compound content and antioxidant capacity. For the EtOH: palm olein (1:1) system, the crude extract was first concentrated using a rotary evaporator (Q344B, Quimis, Brazil) under reduced pressure to completely remove ethanol, yielding an ethanol-free oil-based extract. This ethanol-free fraction and the extract obtained with palm olein as the sole solvent were then processed according to the methodology described by Montedoro et al. (1992), with minor adaptations. Briefly, an aliquot of 100 mg of the oil extract was mixed with 20 mL of methanol and homogenized in a tube shaker (K45-2810, Kasvi, Brazil) at maximum speed for 10 min to transfer bioactive compounds into the methanolic phase, followed by centrifugation at 1,008 × g for 10 min to promote phase separation. The resulting methanolic fraction was used to determine bioactive compound content and antioxidant activity, and all results were expressed on a dry basis to ensure comparability between solvent systems.

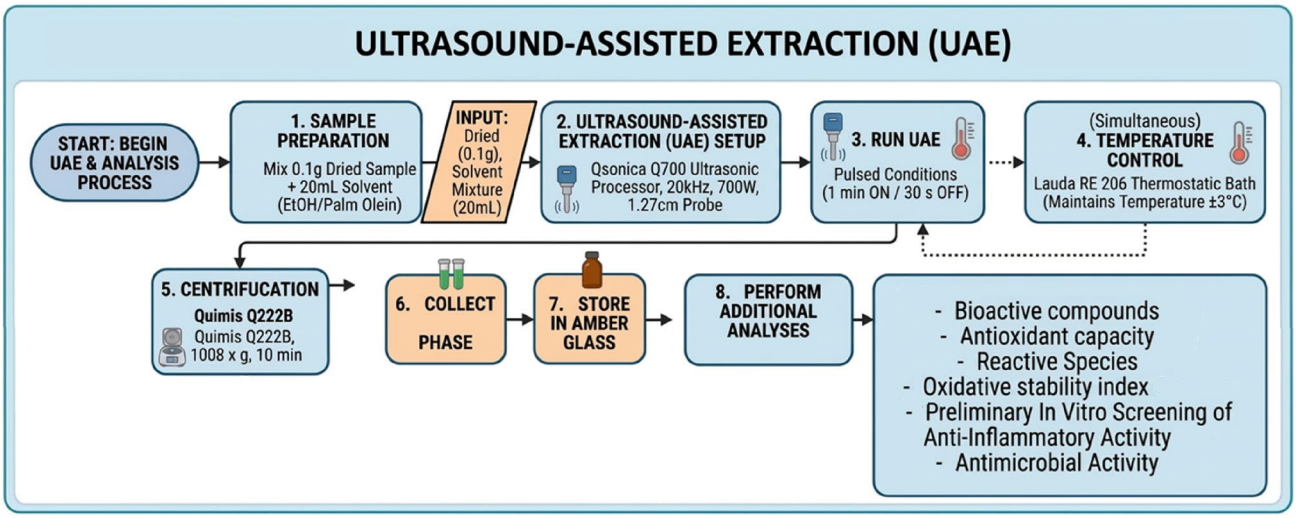



**Fig. 1** - Schematic workflow of ultrasound-assisted extraction

## Bioactive compounds

### Total phenolic content

Total phenolic content was determined using the spectrophotometric method of Singleton et al. (1999). For each assay, 0.5 mL of extract was mixed with 2.5 mL of 10% (v/v) Folin-Ciocalteu reagent and 2.0 mL of 7.5% (m/v) sodium carbonate solution, and incubated for 5 min at 50 °C. Absorbance was measured at 750 nm using a UV-Vis spectrophotometer (NI 1600UV, Nova Instruments, Brazil), and a reagent blank containing extraction solvent was used to correct background absorbance. Total phenolic content was quantified using a gallic acid calibration curve and results expressed as mg gallic acid equivalents per gram of dry leaf (mg GAE/g).

## Total flavonoids content

Total flavonoid content was determined according to Lees and Francis (1972), with modifications. For each assay, 0.25 mL of extract was mixed with 2.72 mL of distilled water and 0.12 mL of 0.5 mol/L sodium nitrite solution. After 5 min, 0.12 mL of 0.3 mol/L aluminum chloride solution was added, and the mixture was allowed to stand for another 5 min. Absorbance was measured at 510 nm, and a reagent blank containing distilled water instead of extract was used to correct background absorbance. Total flavonoid content was quantified using a catechin calibration curve and results expressed as mg catechin equivalents per gram of dry leaf (mg CE/g).

## Total saponins content

Total saponin content was determined according to Hiai et al. (1976). For each assay, 0.5 mL of extract was mixed with 0.5 mL of 8% (m/v) vanillin solution, followed by hydrolysis with 5 mL of 72% (v/v) sulfuric acid, and incubated in a thermostatic bath at 60 °C for 10 min. Absorbance was measured at 520 nm, and a reagent blank containing extraction solvent treated with the same reagents was used to correct background absorbance. Total saponin content was quantified using a diosgenin calibration curve) and expressed as mg diosgenin equivalents per gram of dry leaf (mg DE/g).

### Total condensed tannins content

Condensed tannins were analyzed following Agostini-Costa et al. (1999). For each assay, 1 mL of extract was mixed with 5 mL of vanillin reagent, and absorbance was measured at 490 nm. A reagent blank containing the extraction solvent with vanillin reagent was used to correct background absorbance. Condensed tannin content was quantified using a catechin calibration curve and results expressed as mg catechin equivalents per gram of dry leaf (mg CE/g).

### Antioxidant capacity

To determine the antioxidant capacity, two methods were applied.

### DPPH

The antioxidant capacity using the DPPH radical scavenging assay was determined according to the methodology proposed by Mensor et al. (2001). Briefly, 2.5 mL of the

extract was mixed with 1 mL of DPPH solution (0.3 mmol $L^{-1}$). After 30 min of incubation in the dark, the absorbance was measured at 520 nm. A negative control (DPPH solution without extract) represented the maximum radical concentration, while a reagent blank (extraction solvent without DPPH) was used to correct background absorbance. Antioxidant capacity was quantified using a Trolox standard curve and results expressed as µmol Trolox equivalents per g of dry leaf (µmol TE $g^{-1}$).

### Molybdate assay

Total reducing capacity was determined by the phosphomolybdenum method according to Prieto et al. (1999). For each assay, 0.3 mL of extract was mixed with 3 mL of the reagent solution, heated at 95 °C for 90 min, cooled in an ice bath, and absorbance measured at 695 nm. A reagent blank containing the extraction solvent was used to correct background absorbance. Total reducing capacity was quantified using a gallic acid standard curve and results expressed as mg gallic acid equivalents per gram of dry leaf (mg GAE/g).

### Oxidative stability index (OSI)

It was determined using the Rancimat equipment (Metrohm, 873 Professional Biodiesel Rancimat, Switzerland), according to the Cd 12b-92 method (AOCS, 2004). A sample of 3 g was used, with a temperature of 110 °C and an airflow rate of 10 L/h. Both pure palm olein and the extract obtained under the best extraction condition were selected as samples for this analysis.

### Reactive species

The extract selected for this analysis was the one obtained under the best extraction conditions.

### Hydroxyl radical (HO•) scavenging activity

This analysis was performed according to the methodology by Liu et al. (2010). In a tube, 0.5 mL of $FeSO_4$ (1.5 mmol/L), 0.35 mL of $H_2O_2$ (6 mmol/L), 0.15 mL of sodium salicylate (20 mmol/L), and 1 mL of the extract were added. After incubation for 1 h at 37 °C, the absorbance was measured at 562 nm using a spectrophotometer. A negative control containing all reagents except the extract was used to represent the maximum hydroxyl radical formation. A reagent blank consisting of the extraction solvent and reagents without $H_2O_2$ was used to correct background interference. L-ascorbic acid was used as a positive control.

### Hydrogen peroxide ($H_2O_2$) scavenging activity

This analysis was conducted according to the methodology by Liu et al. (2010). The reaction mixture contained 1 mL of $H_2O_2$ (0.1 mmol/L), 1 mL of the extract, 0.1 mL of ammonium molybdate (3%, m/v), 10 mL of sulfuric acid (2 mol/L), and 7 mL of potassium iodide (1.8 mol/L). The mixture was titrated with 5 mmol/L $Na_2S_2O_3$ until the color disappeared. A negative control containing $H_2O_2$ and all reagents except the extract was used to represent the maximum hydrogen peroxide level. A reagent blank consisting of the extraction solvent and reagents without $H_2O_2$ was used to correct background interference. L-ascorbic acid was used as a positive control.

### Nitric oxide (NO•) radical scavenging activity

This analysis was determined according to the method of Marcocci et al. (1994). Briefly, 1 mL of sodium nitroprusside (100 mmol/L) was added to a tube containing 19 mL of the extract, and it was incubated for 2 h at 25 °C. Finally, 1.5 mL was taken from the tube and mixed with 1.5 mL of the Griess reagent, and the sample was read at a wavelength of 546 nm using a spectrophotometer. A negative control containing sodium nitroprusside without extract was used to represent maximum nitric oxide production, while a reagent blank consisting of the extraction solvent and Griess reagent without sodium nitroprusside was used to correct background absorbance. L-ascorbic acid was used as the positive control.

### Preliminary in vitro screening of anti-inflammatory activity

The assay was performed according to Mizushima and Kobayashi (1968), with modifications based on Saso et al. (1999). The extract selected for this analysis was the one obtained under the best extraction conditions. Briefly, 1 mL of the extract was mixed with 1 mL of a 0.4% albumin solution (m/v). The tubes were shaken and subjected to heating at 70 °C for 30 min. After this step, absorbance was measured at a wavelength of 595 nm. A control containing BSA solution without extract was used to represent 100% protein denaturation, while the reagent blank consisted of the extraction solvent without BSA to correct background absorbance. Diclofenac was used as the positive control.

### Antimicrobial activity

The determination of the antimicrobial activity of the extracts was performed using the microdilution method in a 96-well plate, according to the methodology described by

Ferreira et al. (2015). The extract used in this analysis was the one obtained under the best extraction conditions. The target microorganisms used were *Escherichia coli* (ATCC 19433), *Staphylococcus aureus* (ATCC 25923), *Salmonella typhimurium* (ATCC 14028), *Listeria monocytogenes* (ATCC 19111), *Bacillus cereus* (ATCC 9634), *Pseudomonas aeruginosa* (ATCC 27853), and *Candida albicans* (ATCC 2091). Briefly, in flat-bottom 96-well plates, 25 µL of the extract, 25 µL of culture medium, and 50 µL of the inoculum were added. After the process, the plates were placed on a shaker (JOANLAB VM-600, China) for 25 min at 200 rpm. They were then incubated at 35 °C for 24 h. As indication of the proliferation of microorganisms, 10 µL of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2 Htetrazolium bromide (5 mg/mL) was put into each well, and the plates were incubated for four hours at 35 °C. The absorbance was read using the MTT colorimetric method (reduction of MTT) on a VERSAmax microplate reader (Molecular Devices, EUA) at 570 nm. A negative control containing culture medium, sterile water, and inoculum was used to represent 100% microbial growth. A reagent blank consisting of culture medium and extract without inoculum was used to correct background absorbance. The percentage inhibition at different extract concentrations was calculated based on the optical density relative to the negative control.

### Sustainability analysis

The sustainability of the extraction methods was evaluated using the Analytical GREENness calculator (AGREE), based on the 12 principles of green analytical chemistry (Pena-Pereira et al. 2020). The tool expresses the environmental performance of the method through a pictogram and an overall score ranging from 0 (least sustainable) to 1 (most sustainable).

### Statistical analysis

Principal Component Analysis (PCA) was performed using PAST software version 1.0 to explore the variance and covariance structure of the treatments across the six experimental extraction conditions, in relation to bioactive compound content and antioxidant capacity.

Statistical modeling was conducted in R, applying principal component analysis (PCA), partial least squares regression (PLSR), and multiple linear regression (MLR) to the dataset obtained from the OVAT experimental design. All measurements were performed in triplicate, and average values were used for model development. PCA was employed to explore patterns and relationships among independent variables and bioactive compound concentrations, while PLSR and MLR were used to construct predictive models relating EtOH concentration, particle size, solvent ratio, extraction time, temperature, and ultrasound intensity to phenolics, saponins, flavonoids, and condensed tannins.

For PLSR, the data were pre-scaled, and model performance was evaluated using leave-one-out cross-validation (LOO), which was considered appropriate given the limited dataset ($n=23$); consequently, all available data were used to build the model. The optimal number of principal components and latent variables was determined using the onesigma method, considering variance explained and root mean square error (RMSE). For MLR, regression coefficients were estimated and tested for significance at $p<0.05$. For both PLSR and MLR models, predictive performance was evaluated using the coefficient of determination ($R^2$) and RMSE.

Additionally, model performance was evaluated using the residual predictive deviation (RPD) and range error ratio (RER), which are widely accepted indicators of predictive robustness and practical applicability in regression models (Andrés et al. 2008; Karoui et al. 2006; De Marchi et al. 2011; Jiang et al. 2017). In this study, RPD and RER were calculated using all 23 OVAT experiments, reflecting model performance based on the training dataset.

All assays were performed in triplicate, and the Statistica software (Statsoft Inc., 2006, Tulsa, OK, USA), version 7.0, was used to check for significant differences between assays and to assess the optimal conditions for ultrasonic-assisted extraction. The results were subjected to a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, with a p-value $<0.05$ considered significant.

## Results and discussion

### Influence of EtOH concentration on the extraction process

Table 1 summarizes the OVAT experimental design and the resulting contents of bioactive compounds and antioxidant activity obtained from the extraction process of Amazonian spinach leaves.

EtOH concentration was significantly affected the extraction of bioactive compounds and antioxidant capacity ($p<0.05$), EtOH concentration (70%) produced the highest levels of extracted compounds and antioxidant activity. This behavior is attributed to the intermediate polarity of EtOH: water proportion, which improves solubility and diffusion of bioactive compounds by lowering the dielectric constant relative to pure water (Medina-Torres et al. 2017).

In contrast, high EtOH concentrations (96%) reduced extraction efficiency, likely due to cellular dehydration and

**Table 1** OVAT experimental design and bioactive compounds and antioxidant activity content from Amazonian spinach leaves process extraction

| Run | EtOH (%) | Particle size (mesh) | Solvent ratio* | Extraction time (min) | T (°C) | Ultrasound intensity (W/cm²) | Phenolics** (mg GAE/g) | Flavonoids** (mg CAT/g) | Saponins** (mg DE/g) | Condensed Tannins** (mg CAT/g) | Molybdate** (mg GAE/g) | DPPH** (μmol TE/g) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E1 | 30 | 60 | EtOH | 10 | 25 | 9.5 | $6.42 \pm 0.12^{d}$ | $4.23 \pm 0.17^{d}$ | $25.89 \pm 0.29^{c}$ | $83.85 \pm 0.28^{d}$ | $25.29 \pm 0.26^{d}$ | $4.27 \pm 0.16^{d}$ |
| E2 | 50 | 60 | EtOH | 10 | 25 | 9.5 | $10.02 \pm 0.15^{b}$ | $8.22 \pm 0.24^{c}$ | $36.2 \pm 0.56^{b}$ | $147.37 \pm 4.51^{c}$ | $66.28 \pm 0.32^{c}$ | $8.12 \pm 0.11^{c}$ |
| E3 | 70 | 60 | EtOH | 10 | 25 | 9.5 | $11.45 \pm 0.09^{a}$ | $15.03 \pm 0.2^{a}$ | $89.26 \pm 0.18^{a}$ | $222.37 \pm 8.39^{a}$ | $153.47 \pm 0.20^{a}$ | $13.02 \pm 0.42^{a}$ |
| E4 | 96 | 60 | EtOH | 10 | 25 | 9.5 | $8.62 \pm 0.2^{c}$ | $11.12 \pm 0.26^{b}$ | $89.8 \pm 0.31^{a}$ | $177.34 \pm 5.51^{b}$ | $91.44 \pm 0.88^{b}$ | $10.21 \pm 0.29^{b}$ |
| E5 | 70 | 60 | 1:0 | 10 | 25 | 9.5 | $11.47 \pm 0.18^{b}$ | $15.36 \pm 0.4^{b}$ | $79.46 \pm 0.56^{c}$ | $213.85 \pm 3.14^{b}$ | $152.93 \pm 1.32^{b}$ | $13.20 \pm 0.31^{c}$ |
| E6 | 70 | 60 | 1:1 | 10 | 25 | 9.5 | $35.16 \pm 0.91^{a}$ | $38.93 \pm 1.33^{a}$ | $330.90 \pm 4.33^{a}$ | $251.4 \pm 7.21^{a}$ | $212.49 \pm 1.76^{a}$ | $211.28 \pm 2.62^{a}$ |
| E7 | 70 | 60 | 0:1 | 10 | 25 | 9.5 | $08.27 \pm 1.37^{c}$ | $13.93 \pm 0.57^{c}$ | $271.18 \pm 5.24^{b}$ | $125.36 \pm 9.2^{c}$ | $87.50 \pm 1.81^{c}$ | $67.05 \pm 1.2^{b}$ |
| E8 | 70 | 60 | 1:1 | 10 | 25 | 9.5 | $34.39 \pm 0.98^{a}$ | $38.67 \pm 0.57^{a}$ | $332.48 \pm 3.98^{a}$ | $251.91 \pm 1.39^{a}$ | $214.55 \pm 1.67^{a}$ | $212.33 \pm 1.64^{a}$ |
| E9 | 70 | 48 | 1:1 | 10 | 25 | 9.5 | $22.96 \pm 1.7^{b}$ | $22.17 \pm 0.75^{b}$ | $263.12 \pm 1.25^{b}$ | $158.46 \pm 4.54^{b}$ | $176.12 \pm 1.33^{b}$ | $92.67 \pm 1.64^{b}$ |
| E10 | 70 | 42 | 1:1 | 10 | 25 | 9.5 | $20.02 \pm 0.48^{b}$ | $20.47 \pm 0.81^{b}$ | $147.29 \pm 2.60^{c}$ | $144.18 \pm 3.49^{c}$ | $156.01 \pm 1.44^{c}$ | $82.41 \pm 1.21^{c}$ |
| E11 | 70 | 35 | 1:1 | 10 | 25 | 9.5 | $14.75 \pm 1.04^{c}$ | $11.39 \pm 0.54^{c}$ | $124.79 \pm 5.63^{d}$ | $105.64 \pm 3.01^{d}$ | $134.7 \pm 2.04^{d}$ | $73.52 \pm 0.57^{d}$ |
| E12 | 70 | 60 | 1:1 | 10 | 25 | 5.5 | $19.18 \pm 0.67^{d}$ | $30.65 \pm 0.47^{c}$ | $284.6 \pm 0.76^{c}$ | $154.94 \pm 4.43^{c}$ | $177.24 \pm 1.05^{c}$ | $86.13 \pm 0.6^{d}$ |
| E13 | 70 | 60 | 1:1 | 10 | 25 | 7.9 | $25.98 \pm 0.17^{c}$ | $35.87 \pm 0.2^{b}$ | $306.7 \pm 2.95^{b}$ | $202.48 \pm 5.79^{b}$ | $194.43 \pm 1.09^{b}$ | $168.49 \pm 0.61^{c}$ |
| E14 | 70 | 60 | 1:1 | 10 | 25 | 9.5 | $33.92 \pm 0.39^{a}$ | $38.96 \pm 0.69^{a}$ | $347.02 \pm 1.88^{a}$ | $255.01 \pm 1.77^{a}$ | $213.87 \pm 0.87^{a}$ | $215.72 \pm 2.02^{a}$ |
| E15 | 70 | 60 | 1:1 | 10 | 25 | 12.6 | $30.26 \pm 0.27^{b}$ | $34.72 \pm 1.09^{b}$ | $348.54 \pm 1.39^{a}$ | $256.95 \pm 0.95^{a}$ | $212.27 \pm 1.92^{a}$ | $199.2 \pm 1.2^{b}$ |
| E16 | 70 | 60 | 1:1 | 10 | 25 | 9.5 | $33.87 \pm 0.19^{a}$ | $38.72 \pm 0.81^{a}$ | $386.99 \pm 7.7^{a}$ | $259.91 \pm 1.61^{a}$ | $214.86 \pm 0.56^{a}$ | $220.12 \pm 3.1^{a}$ |
| E17 | 70 | 60 | 1:1 | 10 | 30 | 9.5 | $31.13 \pm 1.04^{b}$ | $18.44 \pm 0.76^{b}$ | $386.69 \pm 3.51^{a}$ | $193.03 \pm 6.06^{b}$ | $189.06 \pm 1.20^{b}$ | $198.12 \pm 1.88^{b}$ |
| E18 | 70 | 60 | 1:1 | 10 | 45 | 9.5 | $29.13 \pm 0.75^{c}$ | $18.1 \pm 0.39^{b}$ | $386.53 \pm 4.12^{a}$ | $157.14 \pm 4.92^{c}$ | $162.60 \pm 1.62^{c}$ | $151.05 \pm 3.12^{c}$ |
| E19 | 70 | 60 | 1:1 | 10 | 55 | 9.5 | $22.34 \pm 0.43^{d}$ | $8.45 \pm 0.46^{c}$ | $385.55 \pm 3.04^{a}$ | $81.42 \pm 3.38^{d}$ | $102.03 \pm 1.78^{d}$ | $90.9 \pm 1.21^{d}$ |
| E20 | 70 | 60 | 1:1 | 5 | 25 | 9.5 | $24.95 \pm 1.33^{b}$ | $22.67 \pm 0.44^{c}$ | $188.22 \pm 2.5^{d}$ | $211.75 \pm 1.72^{b}$ | $145.83 \pm 1.60^{b}$ | $181.20 \pm 2.7^{b}$ |
| E21 | 70 | 60 | 1:1 | 10 | 25 | 9.5 | $37.47 \pm 0.98^{a}$ | $40.81 \pm 0.80^{a}$ | $387.12 \pm 4.46^{a}$ | $263.40 \pm 1.83^{a}$ | $232.1 \pm 5.01^{a}$ | $225.12 \pm 3.1^{a}$ |
| E22 | 70 | 60 | 1:1 | 20 | 25 | 9.5 | $15.36 \pm 0.91^{c}$ | $29.15 \pm 0.57^{b}$ | $334. \pm 2.89^{b}$ | $147.58 \pm 3.81^{c}$ | $112.1 \pm 4.54^{c}$ | $186.54 \pm 5.1^{b}$ |
| E23 | 70 | 60 | 1:1 | 25 | 25 | 9.5 | $7.32 \pm 0.43^{d}$ | $14.57 \pm 0.28^{d}$ | $268.36 \pm 10.2^{c}$ | $118.06 \pm 3.05^{d}$ | $95.12 \pm 1.66^{d}$ | $101.25 \pm 3.25^{c}$ |

*EtOH: Palm olein. Means followed by different letters in the columns indicate a statistically significant difference by the Tukey test ($p \leq 0.05$). **results in dry basis.

structural collapse of plant tissues, which limit solvent penetration and mass transfer (Silva et al. 2018).

The efficiency of ultrasound-assisted extraction observed in this study is consistent with Chemat et al. (2012), indicating that acoustic cavitation enhances plant tissue disruption and promotes the release of intracellular compounds. The results in extraction conditions evaluated here produced higher levels of bioactive compounds when compared with Gunathilake and Ranaweera (2016), Othman et al. (2016) and Kota et al. (2017), which used conventional extraction methods applied to spinach leaves.

### Influence of EtOH: palm olein on extraction

The solvent ratio significantly influenced the extraction of bioactive compounds and antioxidant capacity ($p<0.05$), the EtOH: palm olein (1:1) produced the highest levels of phenolics, flavonoids, condensed tannins, and saponins content, and the greatest antioxidant activity (Table 1).

The improved extraction efficiency can be attributed to the complementary polarity of the solvent system. EtOH, a polar solvent, favors the extraction of phenolic compounds, flavonoids, and tannins, whereas palm olein oil, a non-polar medium, enhances the solubilization of less polar constituents such as saponins (Góral and Wojciechowski 2020). Consequently, the combined solvent system enables the recovery of a broader spectrum of bioactive compounds compared with individual solvents.

During ultrasound-assisted extraction, interactions between the oil and hydroalcoholic phases may promote temporary emulsification, increasing interfacial area and facilitating mass transfer. Cavitation-induced shear forces and microjets disperse immiscible liquids into fine droplets, enhancing solute partitioning and compound release (Delgado-Povedano et al. 2013; Chemat et al. 2012).

Furthermore, the physicochemical properties of ratio solvents, such as viscosity, surface tension, and vapor pressure, can influence cavitation behavior and solvent penetration into plant tissues (Medina-Torres et al. 2017). The EtOH: palm olein (1:1) may provide a favorable balance of these physicochemical properties, which could contribute to the higher extraction yields observed in this study.

### Influence of particle size on extraction

The evaluation of different particle size ranges (35–60 mesh), as presented in Table 1, revealed a statistically significant influence ($p<0.05$) on the levels of bioactive compounds and antioxidant capacity. The highest extraction efficiency was obtained with the 60 mesh particle size, which can be attributed to the larger surface area exposed to ultrasonic irradiation. Smaller particles increase the contact area between the solvent and plant matrix, facilitating mass transfer and enhancing the diffusion of bioactive compounds into the extraction medium. In ultrasound-assisted extraction, reduced particle size may also intensify cavitation effects, promoting more effective disruption of plant cell walls and improving solvent penetration into the matrix. Similar observations were reported by Chanioti and Tzia (2017), who demonstrated that smaller particle sizes improve extraction efficiency by enhancing solvent–plant material interactions and mass transfer processes.

However, excessively fine particles may lead to agglomeration or reduced solvent permeability within the plant matrix, potentially limiting solvent penetration and mass transfer during extraction due to particle aggregation or reduced solvent–solid contact (Hammed et al. 2025) Such effects were not observed under the particle size conditions evaluated in the present study.

## Influence of ultrasound intensity on extraction

Ultrasound intensity significantly influenced ($p<0.05$) the extraction of bioactive compounds and antioxidant capacity (Table 1). Among the evaluated conditions, an intensity of 9.5 W/cm² produced the highest extraction yields. Increased ultrasonic power enhances acoustic cavitation, promoting plant cell disruption and facilitating the release of intracellular metabolites (Al-Dhabi et al. 2017).

However, excessive ultrasonic intensity may negatively affect extraction efficiency. At the highest tested intensity (12.6 W/cm²), a reduction in phenolic and flavonoid levels was observed, while other compounds remained relatively stable. This selective degradation may be associated with the formation of reactive species, such as hydroxyl radicals (•OH), generated during high-intensity ultrasonication, which can oxidize sensitive phytochemicals (Ashokkumar et al. 2008).

Therefore, the 9.5 W/cm² condition appears to provide an effective balance between cavitation efficiency and compound stability, resulting in higher recovery of bioactive compounds while potentially reducing energy consumption.

## Influence of temperature on extraction

Temperature significantly influenced ($p<0.05$) the extraction of bioactive compounds and antioxidant capacity (Table 1). Among the evaluated conditions, 25 °C produced the highest yields of phenolics, flavonoids, and condensed tannins, as well as the greatest antioxidant activity, indicating that lower temperatures favored the preservation of heat-sensitive compounds.

Increasing the temperature resulted in significant reductions ($p<0.05$) in phenolics, flavonoids, and condensed

tannins, suggesting thermal degradation of these metabolites. In contrast, saponins remained relatively stable across the tested temperature range, which may be attributed to their thermal resistance. This stability may be related to the structural characteristics of saponins, which consist of triterpenoid or steroidal aglycones linked to sugar moieties, making them less susceptible to thermal degradation and oxidative reactions compared with phenolic compounds (Das et al. 2022; El Aziz et al. 2019).

Therefore, maintaining the extraction process at 25 °C appears favorable for preserving thermolabile compounds while ensuring efficient extraction. In addition, operating at lower temperatures may reduce thermal energy requirements, potentially improving process efficiency and lowering operational costs.

## Influence of extraction time

Extraction time significantly influenced ($p<0.05$) the recovery of bioactive compounds and antioxidant capacity (Table 1). Among the evaluated conditions (5–25 min), the highest levels of bioactive compounds and antioxidant activity were obtained after 10 min of extraction.

Ultrasound-assisted extraction promotes rapid mass transfer due to cavitation effects, which enhance plant cell disruption and facilitate the release of intracellular metabolites into the solvent (Kumar et al. 2021; Medina-Torres et al. 2017; Shen et al. 2023). Consequently, relatively short extraction times are often sufficient to achieve high extraction efficiency. However, extending the extraction time beyond 10 min resulted in a significant reduction ($p<0.05$) in several bioactive compounds.

This decrease may be attributed to the degradation of thermolabile compounds or oxidative reactions induced by prolonged exposure to ultrasonic irradiation. In addition, extended sonication may increase the likelihood of radical formation and secondary reactions that can affect sensitive phytochemicals. These results suggest that 10 min represents the optimal extraction time, providing efficient compound recovery while minimizing potential degradation. In addition, shorter extraction times may reduce energy consumption, contributing to improved process efficiency and more economical operation.

### Best extraction conditions

According to the OVAT approach, the best extraction parameters for bioactive compounds and antioxidant capacity in Amazonian spinach were identified as follows: a 60-mesh particle size, EtOH (70%), EtOH: palm olein (1:1, v/v) solvent system, an ultrasound intensity of 9.5 W/cm², an operating temperature of 25 °C, and an extraction time of 10 min. This configuration provided three main advantages: (1) improved extraction efficiency through enhanced solvent penetration and controlled cavitation effects, (2) reduced energy demand due to low-temperature processing and shorter extraction time, and (3) improved safety associated with the use of food-grade solvents and the absence of hazardous extraction media.

These extraction conditions substantially increased the recovery of bioactive compounds. Phenolic content increased 5.8-fold, from 6.42 to 37.47 mg GAE/g DW, while flavonoids showed a 9.6-fold increase (4.23 to 40.81 mg CAT/g DW). Condensed tannins increased 3.1-fold, rising from 83.85 to 263.4 mg CAT/g DW. Saponins exhibited the most pronounced increase (14.9-fold), from 25.89 to 387.12 mg DIO/g DW. Antioxidant activity also increased considerably, with DPPH radical scavenging capacity rising 52.7-fold (4.27 to 225.12 µmol TE/g DW) and phosphomolybdate reducing capacity increasing 9.2-fold (25.29 to 232.1 mg GAE/g DW).

These improvements can be attributed to the combined effect of ultrasound-assisted extraction and the use of mixed green solvents, which enhance cell disruption, mass transfer, and the solubilization of both polar and non-polar compounds. As a result, a broader range of bioactive molecules can be efficiently extracted, leading to increased phytochemical content and antioxidant capacity.

The integration of food-grade EtOH with palm olein proved particularly relevant, as it enabled the extraction of compounds with different polarities while maintaining compatibility with food and nutraceutical applications. Overall, the proposed extraction protocol represents an efficient approach for recovering bioactive compounds under mild processing conditions.

### Principal component analysis (PCA)

The effects of different extraction conditions on bioactive compounds and antioxidant capacity are illustrated in Fig. 2.

Figure 2 shows that the first two principal components explain 90.32% of the total variance in the dataset. Principal component 1 (PC1) is mainly associated with the loadings of phenolics, flavonoids, saponins, molybdate, and DPPH, whereas condensed tannins are primarily associated with principal component 2 (PC2).

The score plot in Fig. 2 indicates that extracts located in the same direction as the variable vectors correspond to higher levels of the respective compounds. In this context, E21 is positioned close to the vectors representing phenolics, flavonoids, saponins, molybdate, and DPPH, indicating superior efficiency in recovering multiple classes of bioactive compounds and antioxidant activity. In contrast,

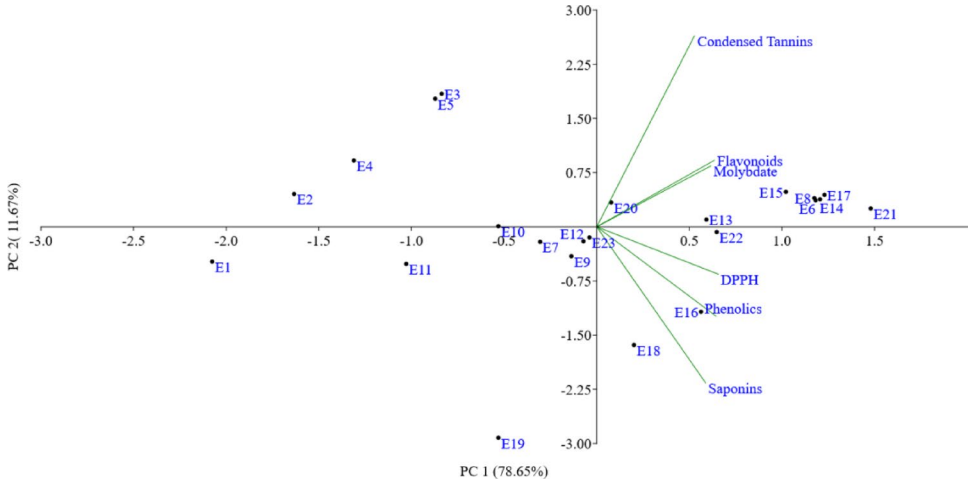



**Fig. 2** PCA evaluation across six extraction stages of Amazonian spinach

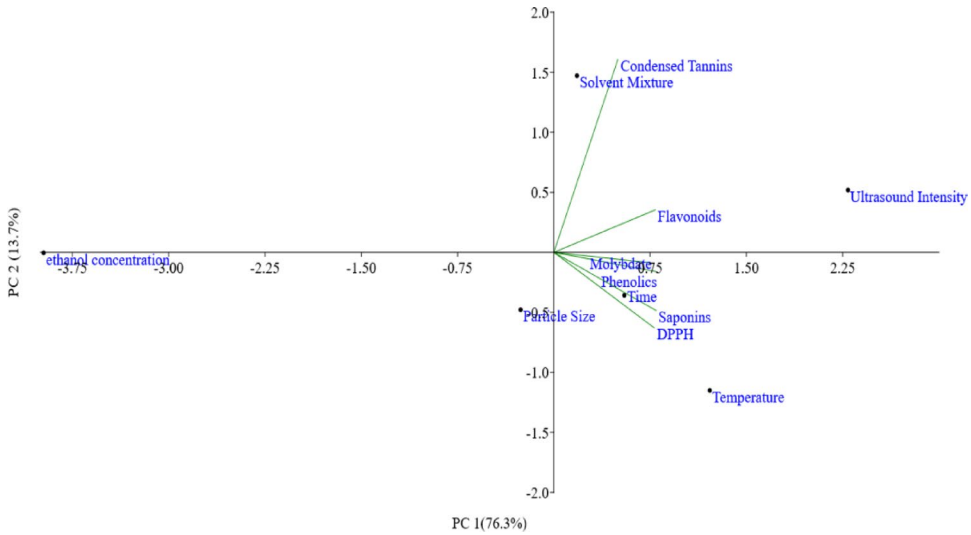



**Fig. 3** PCA of ultrasound extraction parameters showing their influence on bioactive compound yield and antioxidant capacity

extracts such as E1, E2, and E19 are positioned far from these vectors, reflecting lower extraction efficiency under their respective conditions.

Figure 3 further illustrates the individual influence of each extraction parameter on the variance of bioactive compounds and antioxidant capacity.

Based on Fig. 3, PC1 and PC2 together explained 90.0% of the total variance, indicating that the dimensionality of the dataset can be effectively reduced while retaining most of the information.

The weights (Fig. 3) show that phenolics, flavonoids, saponins, molybdate, and DPPH antioxidant capacity are strongly associated with PC1, whereas condensed tannins contributed predominantly to PC2. This suggests that most of the variability in extraction efficiency and antioxidant responses is captured by PC1, while PC2 primarily differentiates the behavior of tannins from the other compounds.

Among the extraction parameters (Fig. 3), ultrasound intensity showed the strongest association with PC1 and was positioned in the same direction as most bioactive compounds and antioxidant responses. This pattern indicates that ultrasound intensity plays a central role in enhancing extraction efficiency, likely by promoting cavitation effects that improve mass transfer and facilitate plant cell disruption.

In contrast, temperature (Fig. 3) was located on the negative side of both PC1 and PC2, suggesting an inverse relationship with bioactive compound levels and antioxidant activity. This behavior may reflect the thermal sensitivity of certain phytochemicals, which can undergo degradation at elevated temperatures.

Extraction time (Fig. 3) showed a moderate association with the group of antioxidant-related variables, indicating that sonication contributes to compound release up to a certain threshold, beyond which prolonged exposure may reduce compound stability.

The solvent mixture (Fig. 3) was strongly associated with PC2 and positioned close to the condensed tannin vector, suggesting that solvent composition exerts a selective influence on the extraction of specific metabolite classes,

particularly tannins. In contrast, EtOH concentration and particle size (Fig. 3) were located on the negative side of PC1, indicating an inverse relationship with the overall extraction of bioactive compounds and antioxidant capacity, suggesting that higher EtOH concentrations and larger particle sizes were associated with lower extraction efficiency. These multivariate relationships are consistent with the experimental results presented in Table 1.

These results highlight the critical importance of systematic optimization of extraction parameters in order to achieve both high efficiency and selective recovery of target metabolites. PCA not only confirmed the dominant influence of ultrasound intensity but also revealed the distinct contributions of solvent composition and temperature to different metabolite groups. While the OVAT approach proved useful for evaluating the individual effects of each parameter, the multivariate perspective provided by PCA enabled a broader understanding of the relationships among extraction variables and response parameters that are not evident in univariate analyses. Taken together, these findings contribute to a more integrative understanding of the influence of extraction parameters on phytochemical recovery, supporting the development of efficient and economically viable UAE processes.

### Evaluation of reactive species scavenging and anti-inflammatory activity

Figure 4 shows the effect of different concentrations of the Amazonian spinach extract on radical scavenging activity and preliminary in vitro anti-inflammatory screening.

Reactive species encompass a broad range of oxidizing molecules with diverse biological properties and functions, ranging from cellular signaling to causing oxidative damage at high concentrations (Sies et al. 2022).

The Amazonian spinach extract demonstrated dose-dependent radical scavenging and preliminary in vitro anti-inflammatory activity, with the highest inhibition observed at 100 µg/mL for both reactive species and inflammatory markers (Fig. 4). The strong correlation between extract concentration and bioactivity suggests that phenolic compounds and saponins are central to neutralizing reactive oxygen and nitrogen species and modulating inflammatory responses, consistent with previous studies showing that higher levels of these bioactive compounds enhance antioxidant capacity and anti-inflammatory effects in plant extracts (Ahmad et al. 2025; Bouzier et al. 2023; Hong et al. 2020; Piao et al. 2023).

Notably, the extract also exhibited strong activity against reactive species, achieving approximately 100% inhibition of $H_2O_2$, followed by around 90% inhibition of HO•, 85% inhibition of NO• at 100 µg/mL and showed approximately 80% inhibition in the preliminary in vitro anti-inflammatory assay at the same concentration. These findings highlight the potential of the extract as a natural source of antioxidants and preliminary anti-inflammatory agents, which could be valuable for functional food or pharmaceutical applications. The results also underscore the importance of carefully chosen extraction conditions to maximize both the yield and activity of bioactive compounds.

### Antimicrobial activity

Table 2 presents the growth inhibition (%) of different microorganisms and the oxidative stability index of the Amazonian spinach leaf extract (E21).

The tested microorganisms include common Gram-positive and Gram-negative bacteria, as well as yeast, which are relevant for food safety and human health. Gram-positive bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus* are known foodborne pathogens, while Gram-negative bacteria like S*almonella typhimurium*, *Escherichia coli*, and *Pseudomonas aeruginosa* are widely used as models for antimicrobial evaluation due to structural

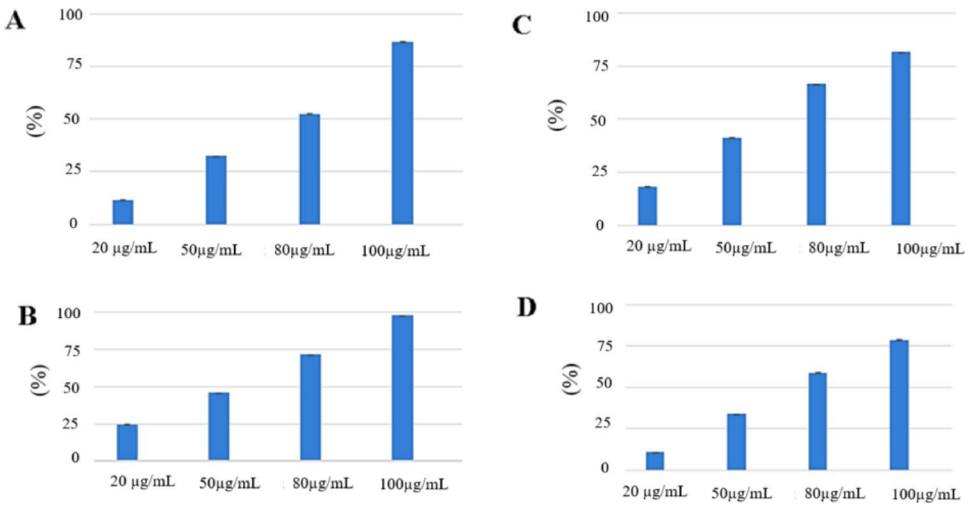



**Fig. 4** Evaluation of HO• scavenging (**A**), $H_2O_2$ scavenging (**B**), NO• scavenging (**C**), and anti-inflammatory capacity (**D**) of the optimized extract

**Table 2** Growth inhibition (%) of different microorganisms and oxidative stability index (OSI) of Amazonian spinach leaf extract (E21)

| Concentration µg/mL | Gram-negative (%) | | | Gram-positive (%) | | | Yeast (%) |
|---|---|---|---|---|---|---|---|
| | Salmonella typhimurium | Escherichia coli | Pseudomonas aeruginosa | Listeria monocytogenes | Bacillus cereus | Staphylococcus aureus | Candida albicans |
| 300 | 82 ± 1.2[a] | 79 ± 1.4[a] | 80 ± 1.8[a] | 90 ± 1.9[a] | 89 ± 1.0[a] | 92 ± 2.3[a] | 68 ± 1.8[a] |
| 100 | 62.1 ± 3.0[b] | 55.4 ± 2.5[b] | 40.4 ± 1.4[b] | 58.5 ± 2.3[b] | 49.1 ± 1.3[b] | 68.4 ± 1.7[b] | 32.7 ± 1.1[b] |
| 1.25 | 5.8 ± 1.1[c] | 4.5 ± 1.0[c] | 3.9 ± 0.9[c] | 6.25 ± 1.1[c] | 2.4 ± 0.8[c] | 9.2 ± 1.1[c] | 3.4 ± 0.6[c] |
| 0.156 | ND | ND | ND | ND | ND | 3.21 ± 0.6[d] | ND |
| OSI (h) | | | | | | | |
| Palm olein | 10.1 ± 0.12[b] | | | | | | |
| Extract | 12.7 ± 0.10[a] | | | | | | |

ND—not detected. Means followed by different letters in the columns indicate a statistically significant difference by the Tukey test ($p \leq 0.05$).

differences (Proft and Baker 2009). *Candida albicans* represents a yeast capable of opportunistic infections, highlighting the extract's potential against both bacterial and fungal microorganisms (Pfaller and Diekema 2007).

The extract exhibited dose-dependent inhibition ($p < 0.05$), with Gram-positive bacteria generally more sensitive than Gram-negative bacteria. Notably, Staphylococcus aureus was inhibited even at the lowest tested concentration (0.156 µg/mL), whereas Gram-negative strains were largely unaffected at this level. *Candida albicans* showed moderate inhibition at higher concentrations (300 µg/mL).

The antimicrobial activity is likely driven by multiple bioactive compounds. Phenolic compounds, including flavonoids, may disrupt cytoplasmic membranes (Tsuchiya and Iinuma 2000), inhibit nucleic acid synthesis (Plaper et al. 2003), and interfere with energy metabolism (Haraguchi et al. 1998). Saponins also contribute due to their amphipathic structure, which facilitates membrane penetration and disruption (Zhao et al. 2018; Góral and Wojciechowski 2020).

Differences in susceptibility between Gram-positive and Gram-negative bacteria are consistent with cell wall structure: Gram-positive bacteria possess a thick peptidoglycan layer, making them more vulnerable, whereas the outer membrane of Gram-negative bacteria, containing lipopolysaccharides, limits compound penetration (Vaou et al. 2021).

The presence of multiple bioactive compounds with complementary mechanisms enhances the extract's overall antimicrobial efficacy. These results support further investigation into synergistic interactions and practical applications, such as food preservation and pharmaceutical formulations, to provide experimental validation of antimicrobial potential.

## Evaluation of oxidative stability

Oxidative stability is an important quality parameter for edible oils because it reflects resistance to oxidation and directly influences shelf life. As shown in Table 2, the addition of the Amazonian spinach leaf extract E21 increased the OSI of palm olein from 10.1 h in the control sample to 12.7 h, corresponding to an improvement of approximately 25.7%. This enhancement in oxidative stability can be attributed to the efficient transfer of bioactive compounds, particularly phenolics and saponins, from the plant matrix to the lipid phase during the ultrasound-assisted extraction process using palm olein as the solvent. The higher concentration of these antioxidant compounds in the enriched oil likely contributes to its greater oxidative protection by scavenging free radicals and interrupting oxidative chain reactions (Blasi and Cossignani 2020; Kang and Kim 2023; Nagarajan and Narayanasamy 2021). These findings are consistent with previous studies showing that plant-derived bioactive compounds can improve the oxidative stability of edible oils and act as natural alternatives to synthetic antioxidants (Blasi and Cossignani 2020; Tundis et al. 2017). Taken together, these results reinforce that ultrasound-assisted extraction using palm olein as the solvent is an effective technological strategy for lipid enrichment with natural compounds, with clear potential for application in the development of food products formulated with naturally derived additives.

## Mathematical modeling

### Statistical assumptions of MLR

The evaluation of statistical assumptions is essential to verify the adequacy of the regression models. In this study, residual autocorrelation, normality, and multicollinearity were assessed using the Durbin–Watson test, Shapiro–Wilk test, and variance inflation factor (VIF), respectively, as shown in Table 3. These results support the validity of the multiple linear regression model and the reliability of the statistical inferences.

Multicollinearity among the independent variables was assessed using the variance inflation factor. The obtained values ranged from 1.01 to 1.08 (Table 3), well below the accepted threshold of 5, indicating low collinearity and

**Table 3** Statistical assumptions of the multiple linear regression model

| Ultrasound intensity (VIF) | Phenolics | Flavonoids | Saponins | Condensed Tannins |
|---|---|---|---|---|
| | 1.01 | 1.01 | 1.02 | 1.01 |
| Temperature (VIF) | 1.05 | 1.05 | 1.04 | 1.05 |
| Extraction time (VIF) | 1.03 | 1.03 | 1.03 | 1.03 |
| EtOH concentration (VIF) | 1.04 | 1.04 | 1.04 | 1.04 |
| Solvent ratio (VIF) | 1.06 | 1.06 | 1.03 | 1.06 |
| Particle size (VIF) | 1.02 | 1.02 | 1.05 | 1.02 |
| Teste Durbin-Watson (p) | 0.74 | 0.674 | 0.074 | 0.252 |
| Teste Shapiro-Wilk (p) | 0.24 | 0.059 | < 0.001 | 0.541 |

Variance inflation factor (VIF).

allowing reliable interpretation of the individual effect of each predictor.

Residual autocorrelation was evaluated using the Durbin–Watson test. The results showed $p > 0.05$ (Table 3), indicating no significant autocorrelation and suggesting that the residuals behaved independently.

Residual normality was assessed by the Shapiro–Wilk test. Most response variables met this assumption, supporting the use of multiple linear regression. However, the residuals for saponin data showed significant deviation from normality and could not be corrected by data transformation. For this reason, multiple linear regression was not considered adequate for this variable, and multivariate approaches such as PLSR were used instead, given their greater robustness in handling non-normality and collinearity among predictors (Hubert and Branden 2003; Guven and Samkar 2019; Mehmood et al. 2012).

Overall, the results indicate that the regression assumptions were satisfied for most datasets, supporting the validity and robustness of the applied models.

## Models performance evaluation

Table 4 presents the regression coefficients obtained by the MLR and PLSR models for the prediction of bioactive compounds.

The analysis of model coefficients (Table 4) showed that the independent variables had distinct effects on the extraction of bioactive compounds. Although the magnitude of PLSR coefficients should not be interpreted as absolute effects, they provide useful information on the relative

**Table 4** Regression coefficients obtained from multiple linear regression (MLR) and partial least squares regression (PLSR) for predicting bioactive compounds

| | Phenolics | | Condensed Tannins | | Flavonoids | | Saponin | Phenolics | Condensed Tannins | Flavonoids | Saponin |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | MLR | | | | | | | PLSR | | | |
| Predict | Estimate | p | Estimate | p | Estimate | p | - | Estimate | Estimate | Estimate | Estimate |
| EtOH concentration | 0.0381 | 0.723 | 1.66 | 0.026 | 0.00715 | 0.022 | - | 0.18 | 13.64 | 1.27 | 16.20 |
| Extraction time | -13.255 | < 0.001 | -6.97 | 0.002 | -0.01459 | 0.080 | - | -4.75 | -31.22 | -2.53 | -6.48 |
| Temperature | -0.1876 | 0.256 | -4.66 | < 0.001 | -0.01841 | < 0.001 | - | -0.72 | -34.72 | -6.08 | 18.62 |
| Ultrasound intensity | 11.868.048 | 0.092 | 15.31 | 0.028 | 0.00518 | 0.845 | - | 1.86 | 16.40 | 0.26 | 11.98 |
| 60 mesh | 153.787 | 0.014 | 125.71 | 0.003 | 0.46489 | 0.006 | - | 4.62 | 74.44 | 8.77 | 86.74 |
| 48 mesh | 82.100 | 0.285 | 52.82 | 0.272 | 0.28924 | 0.157 | - | -0.10 | -14.74 | -1.13 | 8.97 |
| 42 mesh | 52.700 | 0.487 | 38.54 | 0.418 | 0.255459 | 0.209 | - | -1.17 | -20.84 | -1.80 | -40.52 |
| Solvent ratio (1:1) | 202.736 | < 0.001 | 51.12 | 0.015 | 0.48280 | < 0.001 | - | 19.52 | 57.63 | 21.53 | 234.65 |
| Palm olein oil | -15.852 | 0.787 | -54.87 | 0.151 | 0.10534 | 0.496 | - | -3.69 | -29.21 | -2.78 | 33.48 |

importance and direction of the influence of extraction parameters.

For flavonoids in the MLR model, temperature ($p < 0.05$) and extraction time were negatively associated with recovery, whereas particle size reduction (60 mesh), EtOH concentration, and EtOH: palm olein (1:1) significantly increased yields ($p < 0.05$). A similar pattern was observed for phenolics, with extraction time showing the strongest negative effect ($p < 0.001$), while fine particle size and solvent mixtures favored recovery. Condensed tannins showed a more complex response, as nearly all parameters influenced extraction ($p < 0.05$). EtOH concentration, ultrasound intensity, particle size reduction, and solvent mixture had positive effects, whereas temperature and extraction time showed strong negative effects. These results indicate that each class of compounds responds differently to the extraction conditions.

The PLSR results confirmed these patterns and provided additional detail. For phenolics and flavonoids, particle size 60 mesh and solvent mixture (1:1) were the main positive contributors, while extraction time and palm olein oil showed negative effects. EtOH concentration made a smaller positive contribution, supporting its role in adjusting solvent polarity. Saponins showed a distinct behavior, with high positive coefficients for particle size 60 mesh, solvent mixture, and palm olein oil, as well as relevant contributions from ultrasound intensity and temperature. In contrast, condensed tannins remained highly sensitive to time and temperature, with strong negative coefficients, while benefiting from fine particle size, EtOH concentration, solvent mixture, and ultrasound intensity.

Figure 4 shows the variable importance in projection (VIP) scores of the PLSR model, highlighting the relative influence of extraction parameters on the recovery of phenolics, condensed tannins, flavonoids, and saponins from Amazonian spinach leaves. Higher VIP values indicate greater importance of the corresponding extraction variable in explaining the variance of each bioactive compound (Akarachantachote et al. 2014).

The VIP analysis (Fig. 5) provided additional insight into the relative importance of extraction variables across compound classes. EtOH concentration showed a moderate but consistent influence, particularly for tannins and saponins. Particle size reduction to 60 mesh consistently improved recovery, whereas coarser particle sizes had little effect. The solvent mixture was especially important for phenolics and saponins, showing better performance than palm olein oil alone. Extraction time was also relevant, particularly for tannins, while temperature showed a moderate contribution. Ultrasound intensity was an important factor for phenolics and flavonoids, but its influence was less pronounced for saponins.

The comparison between MLR and PLSR revealed similar patterns in the effect of extraction parameters on the recovery of bioactive compounds. Both models identified particle size reduction (60 mesh), EtOH: palm olein (1:1), and EtOH concentration as positive factors, highlighting the importance of increased surface area and solvent polarity balance for improving solubilization and mass transfer. In both approaches, extraction time was negatively associated with phenolic recovery, suggesting that prolonged exposure may favor degradation or polymerization. Ultrasound intensity was also relevant for phenolics and flavonoids in

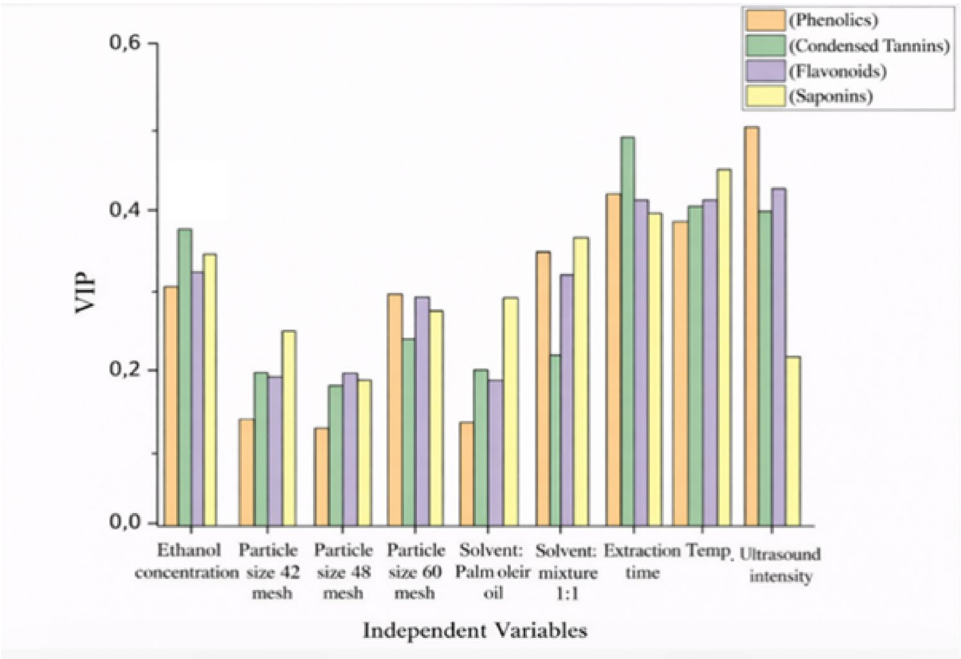



**Fig. 5** Variable Importance in Projection (VIP) scores of the PLSR model, showing the relative influence of extraction parameters on the recovery of bioactive compounds from Amazonian spinach leaves. Temp. (Temperature)

both models. These agreements reinforce the consistency of the results and indicate that the combination of fine particle size, mixed solvents, and controlled extraction conditions improves the recovery of multiple bioactive compounds.

Despite these similarities, the MLR and PLSR models also provided complementary information. MLR coefficients highlighted the negative effects of temperature and longer extraction time on tannin and flavonoid recovery, whereas PLSR indicated that these variables still had moderate overall importance, particularly for tannins, reflecting its ability to capture latent variance. In addition, the effect of solvent mixture appeared more pronounced in MLR, while PLSR distributed its influence more evenly across phenolics and saponins. A further difference was observed for ultrasound intensity: MLR emphasized its positive contribution to tannin recovery, whereas PLSR assigned greater importance to phenolics and flavonoids. These differences highlight the complementary value of both approaches, with MLR providing direct estimates of effect direction and PLSR offering a broader view of variable relevance in a multivariate context.

Together, these findings indicate that ultrasound-assisted extraction benefits from fine particle size, mixed solvent systems, and careful control of time and temperature. The different responses of phenolics, flavonoids, tannins, and saponins show that each compound class responds differently to process conditions, which supports the need for compound-focused extraction strategies. In this context, the combined use of MLR and PLSR strengthens the interpretation of the results and supports the development of efficient extraction protocols.

Table 5 presents the figures of merit of the MLR and PLSR models used for predicting bioactive compounds.

%Y (Variance explained of the dependent variable), MLR (Multiple linear regression), PLSR (Partial least squares regression), LV (latent variables), RPD (residual predictive deviation); RER (range error ratio) and RMSEP (Mean square error of prediction).

The predictive performance of MLR and PLSR models (Table 5) varied according to the bioactive compound analysed. For phenolics, MLR showed the best performance ($R^2 = 0.85$, RMSEP = 4.25, RPD = 2.65, RER = 7.93), outperforming PLSR ($R^2 = 0.79$, RPD = 2.27, RER = 6.70). A similar pattern was observed for flavonoids, with MLR again providing more accurate predictions ($R^2 = 0.79$, RPD = 2.28, RER = 7.02) than PLSR ($R^2 = 0.75$, RPD = 1.96, RER = 6.08). These RPD and RER values indicate that the MLR models are suitable for screening and quality control, whereas the lower values obtained for PLSR suggest more limited predictive performance.

For saponins, the MLR model was not considered valid because the residuals violated the normality assumption. In contrast, PLSR produced a satisfactory predictive model with three latent variables ($R^2 = 0.78$, RMSEP = 56.50, RPD = 2.21, RER = 6.39), confirming its suitability for datasets that do not meet the assumptions required for linear regression.

For condensed tannins, MLR again showed better predictive performance ($R^2 = 0.81$, RMSEP = 24.5, RPD = 2.39, RER = 7.42) than PLSR ($R^2 = 0.78$, RPD = 2.09, RER = 6.50).

From a practical perspective, these RPD values indicate moderate model robustness and acceptable predictive performance for preliminary screening and routine evaluation, although they remain below the threshold for good calibration reliability (RPD > 2.5) and well below the level associated with high analytical accuracy (RPD > 5). In this regard, further refinement of the extraction design using response surface methodology (RSM) or Box–Behnken design may improve model fit and predictive robustness, supporting the development of models with greater quantitative precision. Moreover, the OVAT data obtained in the present study can provide a useful starting point for identifying the most relevant variables and defining appropriate experimental ranges for these subsequent optimization strategies.

## Sustainability assessment

The sustainability of the extraction systems was evaluated using the Analytical GREEnness calculator (AGREE), which integrates the 12 principles of green analytical chemistry into a single pictorial score ranging from 0 (least sustainable) to 1 (most sustainable) (Pena-Pereira et al. 2020). The principles considered by AGREE include: (1) sample preparation, (2) sample amount, (3) location of the analytical

**Table 5** Figures of Merit for MLR and PLSR models of bioactive compounds

| | Models | LV | %Y | $R^2$ | RMSEP | RPD | RER |
|---|---|---|---|---|---|---|---|
| Phenolics | MLR | - | - | 0.85 | 4.25 | 2.65 | 7.93 |
| | PLSR | 3 | 79.95% | 0.79 | 4.16 | 2.27 | 6.7 |
| Flavonoids | MLR | - | - | 0.79 | 5.20 | 2.28 | 7.02 |
| | PLSR | 3 | 73.22% | 0.75 | 6.01 | 1.96 | 6.08 |
| Saponins | MLR | - | - | - | - | - | - |
| | PLSR | 3 | 78.69% | 0.78 | 56.50 | 2.21 | 6.39 |
| Condensed Tannins | MLR | - | - | 0.81 | 24.5 | 2.39 | 7.42 |
| | PLSR | 2 | 76.26% | 0.78 | 27.99 | 2.09 | 6.5 |

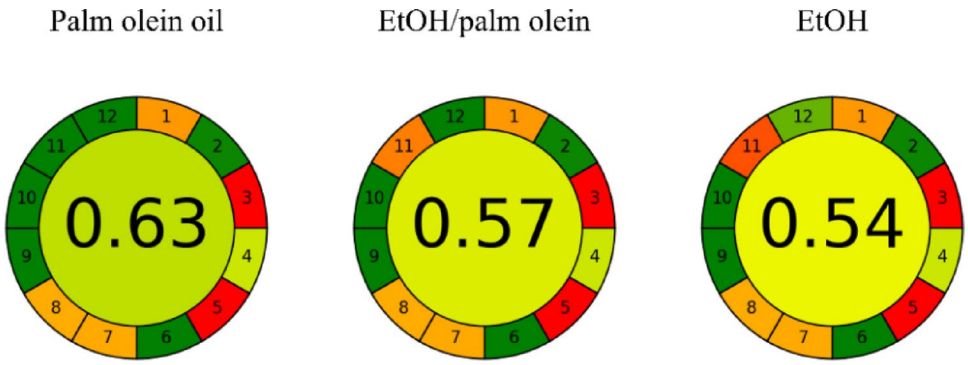



**Fig. 6** Sustainability score of solvents by the AGREE method

device, (4) integration of analytical processes and operations, (5) degree of automation, (6) extent of derivatization, (7) waste generation, (8) analytical throughput (number of samples analyzed per hour), (9) total energy consumption, (10) use of reagents from renewable sources, (11) toxicity of solvents, and (12) operator safety. The AGREE scores obtained for EtOH, EtOH: palm olein (1:1, v/v) and palm olein are shown in Fig. 6, with overall values of 0.54, 0.57 and 0.62, respectively. Thus, palm olein exhibited the highest sustainability score, followed by the mixed solvent system and EtOH alone.

The lower AGREE score observed for EtOH reflects the limitations associated with its use at high concentrations, particularly its flammability, volatility and the need for additional steps for solvent recovery and disposal. Although EtOH is widely recognized as a GRAS (Generally Recognized as Safe) solvent and is extensively used in pharmaceutical and food applications, its use as the main extraction medium increases energy demand and operational complexity, which negatively affects several AGREE criteria related to energy consumption, waste generation and operator safety. These aspects are especially relevant in ultrasound-assisted processes, where solvent evaporation and recovery may require additional equipment and processing time.

In contrast, the extraction process using palm olein achieved the highest greenness score. This performance can be attributed mainly to its low volatility and flammability, its renewable origin and its food-grade status, which contribute positively to criteria associated with solvent toxicity, energy consumption, waste generation and operator safety. In addition, palm olein can be directly used as a carrier in food and nutraceutical formulations, reducing or even eliminating the need for solvent removal and thereby avoiding extra separation and concentration steps. This direct use of the oil phase alleviates several downstream operations, which is reflected in improved scores for the AGREE criteria related to process integration and overall energy consumption.

The intermediate AGREE score obtained for the EtOH: palm olein (1:1) system reflects a balance between the environmental advantages of palm olein and the drawbacks associated with EtOH. This mixed solvent is less sustainable than palm olein alone, but greener than EtOH by itself, while providing clear advantages in terms of extraction efficiency and breadth of extracted compounds, as demonstrated in the previous sections. From the perspective of green analytical chemistry, this system can be seen as a compromise solution, in which a modest increase in environmental burden relative to palm olein is offset by a substantial gain in extraction performance and analytical response.

The AGREE scores obtained in this study are in line with values reported for other green extraction systems. Nascimento et al. (2025) reported intermediate scores around 0.55 for sustainable solvent-based extractions, whereas Pires et al. (2026) obtained lower scores (≈0.51) for methods employing conventional organic solvents such as methanol. In this context, the EtOH and EtOH: palm olein (1:1) systems fall within the range of methods considered moderately green, while palm olein approaches the upper end of this interval, corroborating its potential as a particularly attractive solvent from a sustainability standpoint.

Overall, the sustainability scores for the three solvent systems followed the order EtOH < EtOH: palm olein (1:1) < palm olein, whereas the extraction yields of bioactive compounds showed the opposite trend, EtOH < palm olein < EtOH: palm olein (1:1). This divergence clearly illustrates a trade-off between extraction efficiency and environmental performance. Therefore, the choice of solvent system should be guided by the primary objective of the process. Applications prioritizing maximal recovery of a broad spectrum of bioactive compounds (e.g., high-potency extracts for nutraceuticals) may favor the EtOH: palm olein (1:1) system, despite its intermediate AGREE score. By contrast, processes focused on safety, reduced environmental burden and direct incorporation into lipid matrices (e.g., functional oils and clean-label formulations) may benefit more from palm olein alone, even at the expense of slightly lower extraction yields.

In this context, the integration of AGREE scores with analytical and bioactivity data provides a more comprehensive

basis for solvent selection than either criterion alone. The present findings show that ultrasound-assisted extraction of A. sessilis can be tailored along a continuum ranging from "more efficient but moderately green" (EtOH: palm olein 1:1) to "highly green but moderately efficient" (palm olein), allowing researchers and industry to select conditions that best reconcile extraction performance and environmental impact according to the intended application.

## Conclusions

The combined use of PCA, MLR, and PLSR was effective to describe how extraction conditions affect the recovery of bioactive compounds from *Alternanthera sessilis* leaves.

Among the conditions studied, the best extraction condition was obtained using EtOH: palm olein (1:1, v/v), 60-mesh particle size, ultrasound intensity of 9.5 W/cm², 25 °C, and 10 min with highest extraction yield of bioactive compounds.

Palm olein played a central role both as solvent and as a sustainability driver. In the AGREE assessment, palm olein obtained the highest score (0.62) and increased the oxidative stability index from 10.1 to 12.7 h. Palm olein and EtOH: Palm olein not only improved the yield of bioactive compounds but also enabled the production of a more stable, contributing to more sustainable practices and reducing the need for synthetic antioxidants.

The extract obtained under the best extraction condition showed strong functional properties, with approximately 100% inhibition of $H_2O_2$, about 90% inhibition of HO•, 85% inhibition of NO•, and around 80% inhibition in the preliminary in vitro anti-inflammatory assay at 100 µg/mL, as well as dose-dependent antimicrobial activity, more pronounced against Gram-positive bacteria and with relevant inhibition of Candida albicans. Ultrasound-assisted extraction with EtOH: palm olein (1:1), proved to be an efficient and more sustainable strategy for increasing the yield of bioactive compounds and generating multifunctional extracts, with clear potential for application in food and nutraceutical products aligned with greener industrial practices.

**Acknowledgements** The authors thank PROPESP/UFPA (Office of the Vice Provost for Research and Postgraduate Programs of the Federal University of Para), CNPq (National Council for Scientific and Technological Development, processes, 309876/2016-8, 308396/2018-9 and 313453/2019-5), and CAPES (Coordination for the Improvement of Higher Education Personnel).

**Author contributions** A.F.S.F. - wrote the draft manuscript text, methodology and formal analysis.A.G.M. - contribute with GREEN analysis.L.H.M.S. - financing resources, wrote the main manuscript, correct and contribute in methodology.A.M.C.R. - supervised the work, managed the resources, and conceived the project.

**Funding** The Article Processing Charge (APC) for the publication of this research was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) (ROR identifier: 00x0ma614). The authors thank PROPESP/UFPA (Office of the Vice Provost for Research and Postgraduate Programs of the Universidade Federal do Pará), CNPq (National Council for Scientific and Technological Development, processes no. 307774/2025-2, 305150/2022-7), and CAPES (Coordination for the Improvement of Higher Education Personnel) Funding Declaration 001.

**Data availability** The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Declarations

**Competing interest** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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