



Analytical Validation of UV-Vis Methods for Total Flavonoids, Polyphenols Content, and Antioxidant Capacity in Aqueous *Anemopsis californica* Leaf Extracts

Graciela Sosa-España¹ · Víctor Rodríguez-Orozco¹ · Benjamín Valdez-Salas¹ · Jhonathan Castillo-Saenz¹ · Ernesto Beltran-Partida¹ · Jorge Salvador-Carlos¹ · Roberto Gamboa-Becerra¹

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Abstract

This study aimed to validate UV-Vis spectrophotometric methods for the determination of total flavonoid content (TFC), total polyphenol content (TPC), and total antioxidant capacity (TAC) in aqueous leaf extracts of *Anemopsis californica* obtained by ultrasonic probe-assisted extraction. The methods demonstrated excellent linearity within the 0.00–0.08 mM range, yielding coefficients of determination (R^2) of 0.9995 (TFC), 0.9984 (TPC), and 0.9972 (TAC). The established limits of quantification were exceptionally low (0.0083 mM for TFC, 0.0145 mM for TPC, and 0.0099 mM for TAC), while the accuracy was supported by high recovery rates ranging between 95.73% and 97.36%. Methodological evaluation of the extraction performance determined that a solid to liquid ratio of 0.125 g of leaf material per 10 mL of water provided the optimal analytical response. Under these standardized conditions, initial extract values reached 43.63 mg QE·g⁻¹ (TFC), 16.70 mg GAE·g⁻¹ (TPC), and 17.93 mg GAE·g⁻¹ (TAC). Furthermore, a 4-week refrigerated storage study (4 °C) revealed differential stability profiles, and TPC and TAC remained highly stable, whereas TFC underwent significant degradation, underscoring the lability of flavonoid-associated responses over time. Complementary DPPH assays confirmed relevant radical scavenging activity, which varied notably depending on the plant material source (collected versus commercially leaves). Additionally, preliminary in vitro cytocompatibility assessments showed no evidence of cytotoxicity toward human dermal fibroblasts and J774A.1 macrophages. Overall, this work provides a robust, validated analytical platform for evaluating *A. californica* aqueous extracts, establishing a reliable foundation for future detailed phytochemical profiling and mechanism-oriented biological assays.

Keywords *Anemopsis californica* · Aqueous leaf extracts · Ultrasonic probe-assisted extraction · UV-Vis spectrophotometry · Analytical validation · DPPH radical scavenging activity

Graciela Sosa-España and Víctor Rodríguez-Orozco contributed equally to this work.

Highlights

- Validation of UV-Vis methods for TFC, TPC, and TAC in aqueous *A. californica* leaf extracts.
- Ultrasound-assisted aqueous extraction provided the best analytical response.
- TFC was the least stable parameter during refrigerated storage, whereas TPC and TAC were more stable.
- DPPH activity and preliminary cytocompatibility supported the antioxidant relevance and low cytotoxicity of the aqueous extract.

Extended author information available on the last page of the article

Introduction

Anemopsis californica (Nutt.) Hook. and Arn. (1841), commonly known as yerba mansa or hierba del manso, is the only species of the genus *Anemopsis* within the Saururaceae family. It is distributed in the southwestern USA and northern Mexico, where it grows in humid and saline-alkaline habitats. This ecological adaptation, associated with stoloniferous rhizomes and aerenchyma tissues, distinguishes the species from typical xerophytic plants of arid environments (Medina et al. 2005; Medina-Holguín et al. 2008).

A. californica has long been used in traditional medicine as decoctions, poultices, and washes for the management of skin infections, wounds, respiratory disorders,

digestive complaints, and inflammatory conditions (Mat-tar et al. 2025). Ethnobotanical reports and phytochemical studies suggest that these uses are associated with secondary metabolites present in different plant organs, including phenolic compounds, flavonoids, volatile constituents, and lignans (Medina et al. 2005; Bussey et al. 2014; Medina-Holguín et al. 2008; Kaminski et al. 2010). Previous works have shown relevant antioxidant activity in *A. californica* extracts and significant changes in phenolic composition during storage, indicating that the antioxidant-related fraction of this species deserves further analytical attention (Del-Toro-Sánchez et al. 2015).

Phenolic compounds and flavonoids are among the most relevant plant metabolites because they contribute to antioxidant activity through redox mechanisms, metal chelation, and interactions with oxidative pathways. For this reason, the determination of total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant capacity (TAC) is commonly used as a first analytical approach for the screening and quality assessment of botanical extracts (Halliwell 2012; Kumar and Pandey 2013; Panche et al. 2016; Christodoulou et al. 2022). However, for medicinal plants, the analytical value of these determinations depends not only on the assay itself, but also on the extraction conditions, solvent selection, and validation of the applied method.

In recent years, ultrasound-assisted extraction has gained relevance as an efficient and more sustainable alternative to conventional extraction procedures because it may reduce extraction time, solvent consumption, and energy demand while improving mass transfer from plant matrices (Shen et al. 2023; El Baakili et al. 2023; Solomakou et al. 2024). Recent studies continue to confirm that optimization of ultrasound-based extraction conditions remains an active analytical topic and that extraction variables strongly affect the recovery of phenolics and flavonoids, as well as the antioxidant response of plant extracts (Benmanseur et al. 2025; Nabiyeve et al. 2025). In this context, water is particularly relevant when the purpose is to obtain safer and greener extracts and when the traditional use of the species is associated with aqueous preparations.

For *A. californica*, previous reports have described phytochemical composition and antioxidant-related properties in extracts obtained with organic solvents, especially methanol, and have shown that phenolic and flavonoid contents vary according to plant organ and storage conditions (Del-Toro-Sánchez et al. 2015). Nevertheless, the available information for aqueous leaf extracts remains limited, particularly regarding validated UV-Vis spectrophotometric procedures for the determination of TFC, TPC, and TAC under defined ultrasound-assisted extraction conditions. In addition, although detailed chromatographic profiling would be desirable for molecular characterization, rapid spectrophotometric methods remain useful for preliminary screening, routine

comparison, and quality control of plant extracts when they are properly validated (Way et al. 2020; Michiu et al. 2022; Christodoulou et al. 2022; Knez et al. 2025).

Therefore, the aim of this study was to validate UV-Vis spectrophotometric methods for determining TFC, TPC, and TAC in aqueous leaf extracts of *A. californica* obtained by ultrasonic probe-assisted extraction (Malpica-Acosta et al. 2024), and to evaluate the effect of plant material mass on the analytical response of these parameters. By focusing on an aqueous extraction system, this study sought to generate reproducible analytical data consistent with green chemistry criteria and the traditional use of the species, while providing a methodological basis for future studies involving the comparative solvent evaluation of solvents and detailed phenolic profiling.

Materials and Methods

Plant Material

Leaves of *Anemopsis californica* (Nutt.) Hook. & Arn. were collected in March 2025 at the Instituto de Ingeniería, Universidad Autónoma de Baja California, Mexicali, Mexico (32° 37' 52" N, 115° 26' 40" W). Taxonomic identity was verified using voucher specimen records available in the University of California, Berkeley database (Thieret and McClintock 2012; USDA NRCS 2000). The detailed morphological description of the species was transferred to the "Results and Discussion" section in order to improve the organization of the manuscript.

Reagents

Aluminum chloride, sodium nitrite, sodium hydroxide, and absolute ethanol were used for total flavonoid content (TFC) determination. Quercetin (Sigma-Aldrich, $\geq 95\%$) was used as the external standard for the TFC calibration curve. Folin-Ciocalteu reagent (Sigma-Aldrich, 2 M), sodium carbonate, and gallic acid were used for total polyphenol content (TPC) determination, with gallic acid as the external standard. Total antioxidant capacity (TAC) was determined by the phosphomolybdenum method using ammonium molybdate, sodium phosphate, sulfuric acid, and gallic acid as the reference compound. For the complementary antioxidant assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and methanol were used. For the in vitro cytocompatibility study, human dermal fibroblasts (Lonza, Switzerland), the murine macrophage cell line J774A.1, high-glucose Dulbecco's Modified Eagle Medium (DMEM, 4.5 g L⁻¹), L-glutamine, penicillin-streptomycin-amphotericin B, phosphate-buffered saline (PBS),

fetal bovine serum (FBS), and the Blue Cell Viability Assay Kit were employed.

Preparation of Aqueous Leaf Extracts

Aqueous extracts were prepared by ultrasonic probe-assisted solid-liquid extraction following Jiménez-Morales et al. (2022), with slight modifications as illustrated in Fig. 1. This extraction approach was selected because it reduces extraction time and solvent consumption while maintaining compatibility with an aqueous system, in agreement with green analytical principles and with the traditional use of *A. californica* in water-based preparations. Powdered leaves (0.125, 0.250, 0.375, and 0.500 g) were weighed into centrifuge tubes, and 10 mL of Milli-Q water was added to each tube. Extraction was carried out using an ultrasonic probe at 130 W and 30 kHz for 10 min at 20 °C. After sonication, the extracts were centrifuged at 10,000 rpm for 15 min, and the supernatants were filtered through Whatman No. 1 filter paper. The filtered extracts were transferred to amber vials and stored at 4 °C until analysis.

Moisture Determination

Sample moisture was determined gravimetrically. Porcelain dishes were dried at 120 °C, cooled in a desiccator, and weighed. Approximately 1 g of sample was placed in each dish and dried for 1, 2, 6, and 24 h. After each drying interval, the dishes were cooled in a desiccator and reweighed. Moisture percentage was calculated according to Eq. (1).

$$\text{Moisture (\%)} = [(M_2 - M_1) - (M_3 - M_1)] / (M_2 - M_1) \quad (1)$$

Preliminary Phytochemical Screening

Preliminary phytochemical screening of the aqueous leaf extracts was carried out using conventional qualitative color and precipitation tests. The procedures used for the detection of phenols, flavonoids, alkaloids, saponins, glycosides, sterols, and reducing sugars are summarized in Table 1, together with the corresponding methodological references (Shaikh and Patil 2020; Balamurugan et al. 2019; Saeed et al. 2012; Sengar et al. 2020).

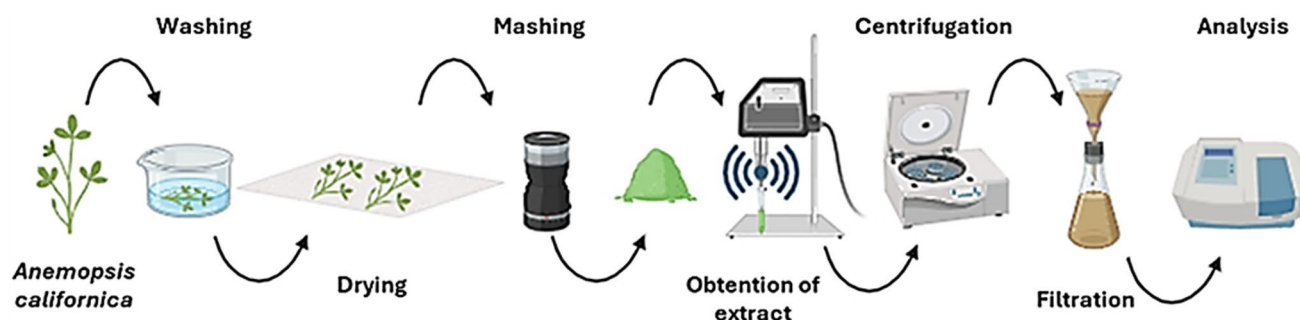


Fig. 1 Outline of *A. californica* aqueous extract preparation assisted by the ultrasonic probe and TFC, TPC, and TAC quantification

Table 1 Qualitative tests used for the preliminary detection of secondary metabolites in aqueous *A. californica* leaf extracts

Phytochemicals	Procedure	Positive results
Phenols	A few drops of 5% ferric chloride (FeCl ₃) are added to 1 mL of the extract	Appearance of a dark green color
Flavonoids	Alkaline reagent test: 1 mL of 2% sodium hydroxide (NaOH) is added to 1 mL of the extract. When a yellowish color appears, a few drops of hydrochloric acid (HCl) are added	Appearance of a yellow color, followed by decoloration
Alkaloids	Wagner's test: a few drops of Wagner's reagent (a mixture of iodine and potassium iodide) are added to 1 mL of the extract	Appearance of a reddish-brown precipitate
Saponins	2.5 mL of water is added to 1 mL of the extract, then it is shaken	Appearance of foam
Glycosides	Borntrager's test: 1 mL of the 10% extract is heated in a water bath at 60–80 °C, and 5 to 10 drops of 10% HCl are added. Then, the mixture is allowed to cool and extracted with chloroform (CHCl ₃) in a 1:1 ratio. NaOH is added dropwise to the organic phase	Appearance of a yellow color
Sterols	Salkowski's test: 1 mL of chloroform (CHCl ₃) and 1 mL of sulfuric acid (H ₂ SO ₄) are added to 1 mL of the extract	Appearance of a yellow layer
Reducing sugars	Benedict's test: the extract (2 mL) is heated with Benedict's reagent (1 mL) in a water bath at 70–80 °C for 2 min	Color changes to green, yellow, or orange

Determination of Total Flavonoid Content (TFC)

TFC was determined using a UV-Vis Thermo Evolution™ 60S spectrophotometer equipped with 1 cm quartz cuvettes. The assay was adapted from Sultana et al. (2024), with slight modifications. A five-point external calibration curve ($n=5$) was prepared from a 0.80 mM quercetin stock solution in the range of 0.00–0.08 mM. For calibration, 2 mL of Milli-Q water and 150 μ L of 5% (w/v) sodium nitrite were added to a 10-mL volumetric flask, followed by the corresponding volume of quercetin standard solution and 400 μ L of ethanol. After 5 min, 150 μ L of 10% (w/v) aluminum chloride was added, and the mixture was allowed to react for a further 5 min. Subsequently, 2 mL of 1 M sodium hydroxide was added, and the reaction mixture was allowed to stand for 15 min before dilution to volume with Milli-Q water. For extract analysis, the standard solution was replaced with 250 μ L of the sample. Absorbance was measured at 415 nm, and results were expressed as mg quercetin equivalents per gram of dry sample ($\text{mg QE} \cdot \text{g}^{-1}$) using Eq. (2) (Amamra et al. 2018; Marques et al. 2013; Velickovic et al. 2014; Chaves et al. 2020).

$$\text{Total flavonoids content (mg QE} \cdot \text{g}^{-1}) = \frac{C_{\text{sample}}(\text{mg/mL}) \times V_{\text{extraction}}(\text{mL})}{m_{\text{sample}}(\text{g})} \quad (2)$$

Determination of Total Polyphenol Content (TPC)

TPC was determined by UV-Vis spectrophotometry according to Rizvi et al. (2023), with slight modifications. A five-point calibration curve ($n=5$) was prepared from a 0.80 mM gallic acid stock solution over the range of 0.00–0.08 mM. Aliquots of standard solution were transferred to 10-mL volumetric flasks, followed by 125 μ L of Folin-Ciocalteu reagent and 935 μ L of 7.5% (w/v) sodium carbonate solution. The final volume was adjusted to 10 mL with Milli-Q water. The mixtures were transferred to test tubes and incubated at 37 °C for 30 min. For extract analysis, 250 μ L of leaf extract was used instead of the standard solution. Absorbance was measured at 720 nm, and results were expressed as mg gallic acid equivalents per gram of dry sample ($\text{mg GAE} \cdot \text{g}^{-1}$) using Eq. (3).

$$\text{Total polyphenols content (mg GAE} \cdot \text{g}^{-1}) = \frac{C(\text{sample}) \times V(\text{extraction})}{m(\text{sample})} \quad (3)$$

Determination of Total Antioxidant Capacity (TAC)

TAC was determined by the phosphomolybdenum reduction method adapted from Hussen and Endalew (2023), with slight modifications. Gallic acid was used as the external standard to construct a five-point calibration curve ($n=5$) in the range of

0.00–0.08 mM. Aliquots of the standard solution were transferred to 10-mL volumetric flasks and mixed with 2 mL of phosphomolybdenum reagent prepared in sulfuric acid, sodium phosphate, and ammonium molybdate. The reaction mixtures were incubated at 90 °C for 90 min and then cooled to room temperature. For extract analysis, the standard solution was replaced with 200 μ L of leaf extract. Absorbance was measured at 700 nm, and TAC was expressed as mg gallic acid equivalents per gram of dry sample ($\text{mg GAE} \cdot \text{g}^{-1}$) using Eq. (4) (Amamra et al. 2018; Marques et al. 2013; Velickovic et al. 2014; Chaves et al. 2020).

$$\text{Total Antioxidant Capacity (mg GAE} \cdot \text{g}^{-1}) = \frac{C(\text{sample}) \times V(\text{extraction})}{m(\text{sample})} \quad (4)$$

Determination of DPPH Radical Scavenging Activity

DPPH radical scavenging activity was determined in aliquots of the aqueous extracts prepared under the optimized extraction conditions. The analysis was performed using the spectrophotometric procedure described by Tzintzun-Camacho et al. (2021), with some modifications. A 0.1 mM DPPH solution was prepared in methanol (96%, v/v). The reaction mixture consisted of 0.1 mL of extract and 2.9 mL of DPPH solution. Samples were incubated at room temperature for 30 min and absorbance was measured at 517 nm, using methanol as the blank. A 0.1 mM DPPH solution without extract was used as the control. Radical scavenging activity was calculated according to Eq. (5).

$$\% \text{DPPH scavenging activity} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100 \quad (5)$$

Preliminary In Vitro Cytocompatibility Assessment

A preliminary in vitro cytocompatibility assessment of *A. californica* extracts was conducted using human dermal fibroblasts and the murine macrophage cell line J774A.1. The cells were cultured separately in complete DMEM containing high-glucose DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin-amphotericin B, and were maintained at 37 °C in a humidified atmosphere with 5% CO₂ until confluence. For cytocompatibility testing, cells were seeded in 96-well plates at a density of 60,000 cells cm^{-2} and incubated for 24 h. Subsequently, the culture medium was replaced, and the cells were exposed to serial dilutions of the aqueous extract for 24 h. Concentrations were evaluated between 1.95 and 31.25 mg/mL. Cell viability was assessed using the metabolic activity method with the Blue Cell Viability Assay, following the methodology described by Mattar

et al. (2025). After exposure to the extract, the treatment medium was removed, and the viability reagent was added directly to the wells at 10% (v/v) in DMEM. The plates were incubated for 4 h under standard culture conditions, and fluorescence was measured at 535/590 nm (excitation/emission) using a Synergy HT microplate reader. Results were normalized relative to untreated control cells, which were defined as 100% viability, while wells containing medium and assay reagent without cells were used as controls.

Analytical Validation

The analytical performance of the UV-Vis methods for TFC, TPC, and TAC determination was evaluated in terms of selectivity, linearity, limits of detection (LOD), limits of quantification (LOQ), precision, accuracy, and robustness, following ICH Q2(R1) criteria (International Conference on Harmonisation 2005). Calibration curves were prepared weekly over four consecutive weeks to evaluate the consistency of the analytical response under the selected assay conditions. Selectivity was assessed by comparing the regression behavior of standards and sample responses and by evaluating the coincidence of the absorbance maxima at the selected wavelengths. Accuracy was evaluated by recovery assays through standard addition to the extract matrix. Precision was assessed as repeatability (intra-day) and intermediate precision (inter-day/inter-week) using replicate measurements performed on different days. Robustness was evaluated by introducing minor deliberate changes in selected analytical conditions.

Statistical Analysis

For extract optimization, the effect of plant material mass (0.125, 0.250, 0.375, and 0.500 g) on TFC, TPC, and TAC responses was analyzed by one-way analysis of variance (ANOVA) at a significance level of 95% ($p < 0.05$), followed by Tukey's honestly significant difference (HSD) test. The desirability function approach was used to identify the mass that simultaneously maximized the analytical responses. Extract stability during storage at 4 °C for 4 weeks was evaluated by periodic determination of TFC, TPC, and TAC, and the data were analyzed by ANOVA and linear regression. For the DPPH assay and cytocompatibility study, results were expressed as mean $\pm$ standard deviation. Cytocompatibility data were analyzed using Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). Differences between groups were evaluated by Student's t test, and statistical significance was established at $p < 0.05$. The analytical validation, extraction optimization, and stability data for TFC, TPC, and TAC were processed using OriginPro® software (OriginLab).

Results and Discussion

Morphological Characteristics and Preliminary Phytochemical Screening of *Anemopsis californica*

The morphological features of *A. californica* are shown in Fig. 2. The collected material exhibited the diagnostic traits commonly described for the species, including a thick stoloniferous rhizome, erect stems, basal leaves with ovate to elliptical morphology, and dense spicate inflorescences subtended by conspicuous white bracts. The relocation of this botanical description to the “Results and Discussion” section improves the continuity of the manuscript by placing the descriptive context immediately before the visual record of the analyzed plant material.

The preliminary phytochemical screening of the aqueous leaf extract is summarized in Table 2. Positive reactions were observed for phenols, flavonoids, alkaloids, saponins, glycosides, sterols, and reducing sugars, indicating that the aqueous extract contains chemically diverse secondary metabolites (Perez-Perez et al. 2017; Basilo et al. 2024). The detection of phenols and flavonoids is particularly relevant because these classes are commonly associated with antioxidant responses and supports the suitability of the extract for subsequent spectrophotometric evaluation. In addition, the broad qualitative profile observed in the aqueous extract is consistent with previous reports describing the occurrence of bioactive metabolites in different organs of *A. californica*, although the present study was not designed for compound-specific identification.

Effect of Plant Material Mass on the Extraction Response

The effect of plant material mass on the analytical response is shown in Fig. 3. Among the evaluated conditions, 0.125 g of powdered leaves in 10 mL of water produced the highest global desirability, whereas the desirability index decreased sharply at 0.250 g and reached zero at 0.375 and 0.500 g. Under the fixed extraction conditions used in this study, these results indicate that the lowest solid load provided the most favorable balance for the simultaneous determination of TFC, TPC, and TAC.

This behavior is analytically plausible because, in ultrasound-assisted extraction, the solvent-to-solid ratio strongly affects cavitation efficiency, solute diffusion, and mass transfer. When plant material increases at constant solvent volume, the available extracting medium may become insufficient to maintain efficient solubilization of the target compounds, especially in aqueous systems. Recent optimization studies on ultrasound-assisted extraction have similarly shown that extraction yield, total

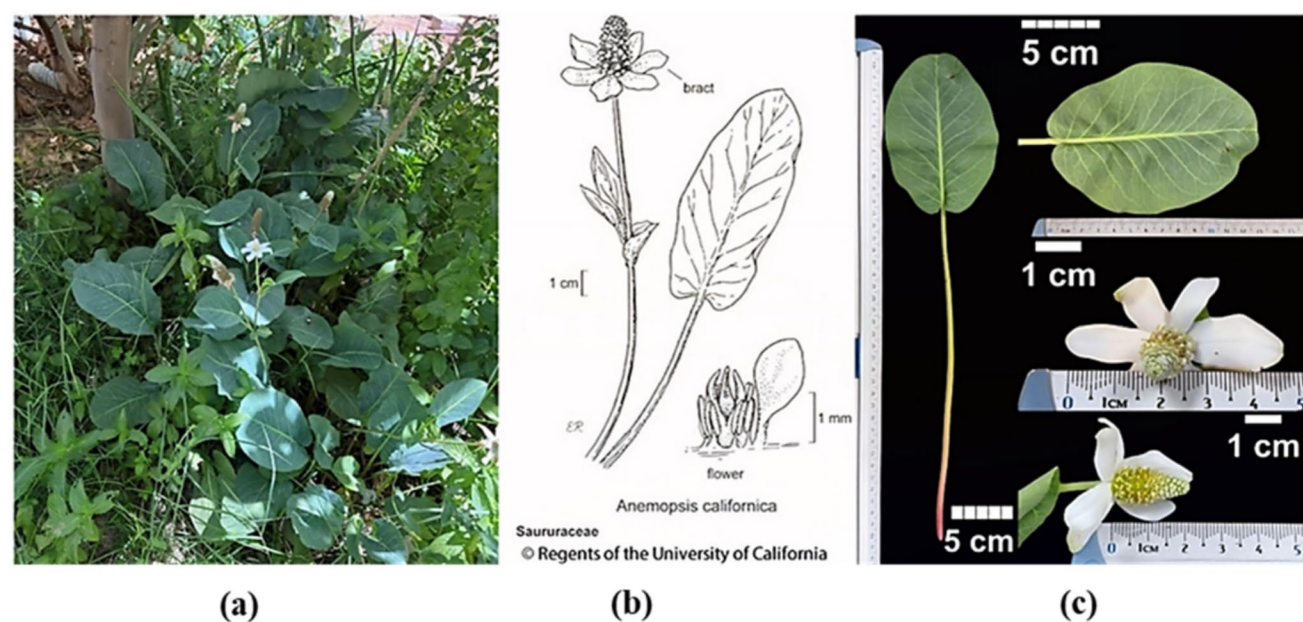


Fig. 2 Morphological characteristics of *Anemopsis californica*: **a** fresh plant in its natural habitat; **b** botanical illustration from the University of California voucher specimen; and **(c)** detailed view of the leaf and inflorescence

Table 2 Preliminary qualitative phytochemical screening of aqueous leaf extracts of *A. californica*

Phytochemicals	Results
Phenols	+
Flavonoids	+
Alkaloids	+
Saponins	+
Glycosides	+
Sterols	+
Reducing sugars	+

phenolic content, total flavonoid content, and antioxidant activity are highly dependent on solvent-to-material ratio and related extraction parameters (El Baakili et al. 2023; Xiang et al. 2024).

A differential trend was also observed among the three analytical responses. TFC and TPC decreased progressively as plant mass increased, whereas TAC showed a more abrupt decline. This suggests that the compounds contributing to the phosphomolybdenum response were particularly sensitive to the reduction in extraction efficiency at higher solid loads. The result should be interpreted within the limits of the selected aqueous ultrasound-assisted system and not as an absolute estimate of the total metabolite content of the

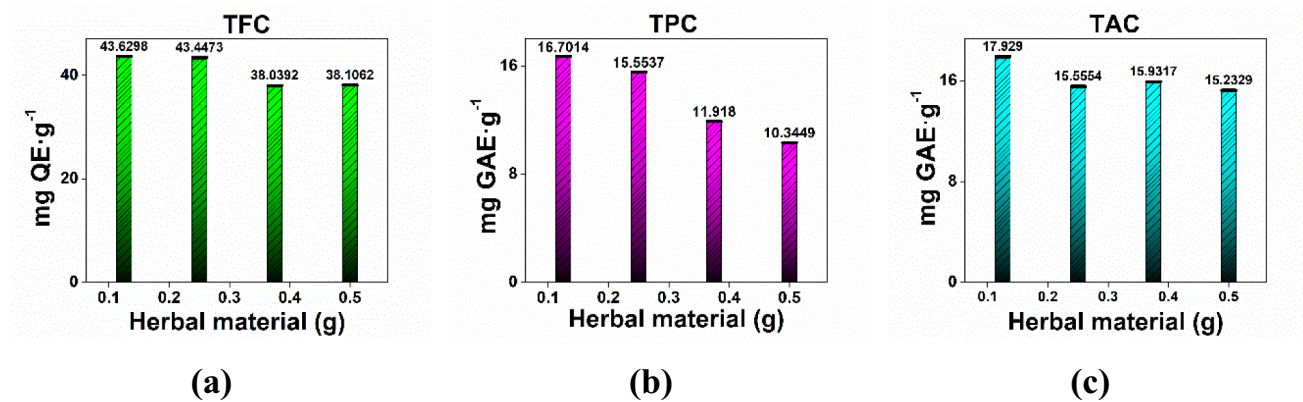


Fig. 3 Effect of plant material mass on the extraction recovery of bioactive compounds: **a** total flavonoid content (mg QE·g⁻¹); **b** total polyphenol content (mg GAE·g⁻¹); and **(c)** total antioxidant capacity (mg GAE·g⁻¹) in aqueous leaf extracts of *A. californica*, as a function of the mass of *A. californica* leaves used for extraction

plant. Rather, it indicates that the selected analytical configuration was most effective at 0.125 g/10 mL for the simultaneous recovery and measurement of the antioxidant-related groups considered here.

Analytical Performance of the UV-Vis Methods

The weekly calibration curves for TFC, TPC, and TAC are presented in Fig. 4, and the corresponding analytical parameters are summarized in Table 3. The three assays showed adequate linearity in the working range of 0.00–0.08 mM, with coefficients of determination of 0.9995 for TFC, 0.9984 for TPC, and 0.9972 for TAC. The limits of detection were 0.0027, 0.0048, and 0.0032 mM for TFC, TPC, and TAC, respectively, whereas the limits of quantification were 0.0083, 0.0145, and 0.0099 mM. Recovery values ranged from 95.73 to 97.36%, supporting the adequacy of the methods for the intended group-level determinations in the aqueous extract matrix. These values are fully consistent with the sensitivity required for the concentrations measured in the present study and support the analytical applicability of the three colorimetric procedures.

Selectivity was supported by the parallelism between linearity and specificity regressions and by the coincidence of absorbance maxima between the standards and the extracts at 415, 720, and 700 nm for TFC, TPC, and TAC, respectively. To address the reviewer's concern more directly, Fig. 4 may remain focused on the weekly calibration curves. This approach preserves the readability of the main text while providing the experimental evidence requested for selectivity assessment.

The relative standard deviations reported in Table 3 indicate acceptable repeatability of the repeated calibration measurements, and the precision and robustness assessments remained within acceptable analytical limits, with RSD values below 5% for the evaluated assays. These results support the reproducibility of the methods under the

Table 3 Analytical validation parameters (linearity, sensitivity, precision, and accuracy) for the spectrophotometric determination of TFC, TPC, and TAC

Parameters	TPC	TFC	TAC
Concentration range (mM)	[0.00–0.08]	[0.00–0.08]	[0.00–0.08]
Coefficient of determination (R^2)	0.9984	0.9995	0.9972
Slope	17.7243	4.8353	7.4814
Relative standard deviations (%)	2.29	1.93	2.06
Intercept	0.0213	0.0118	0.0090
Limit of detection (LOD, mM)	0.0048	0.0027	0.0032
Limit of quantification (LOQ, mM)	0.0145	0.0083	0.0099
Recovery (%)	96.49	97.36	95.73

selected operating conditions. At the same time, the present data should be interpreted as validation of spectrophotometric group assays rather than molecular characterization. Recent reviews continue to recognize UV-Vis methods as useful tools for rapid screening and routine comparison of plant matrices, while emphasizing that assay response is method-dependent and does not replace chromatographic profiling (Li et al. 2024; Knez et al. 2025).

Quantification and Storage Stability of TFC, TPC, and TAC

To avoid ambiguity in the presentation of the quantitative data, Table 4 should be interpreted exclusively as the summary of the 4-week storage study, whereas Table 5 should be used for comparison with the literature based on the first measurement obtained in the present work. This distinction is important because the values discussed below correspond to the initial extract composition, while the averages shown

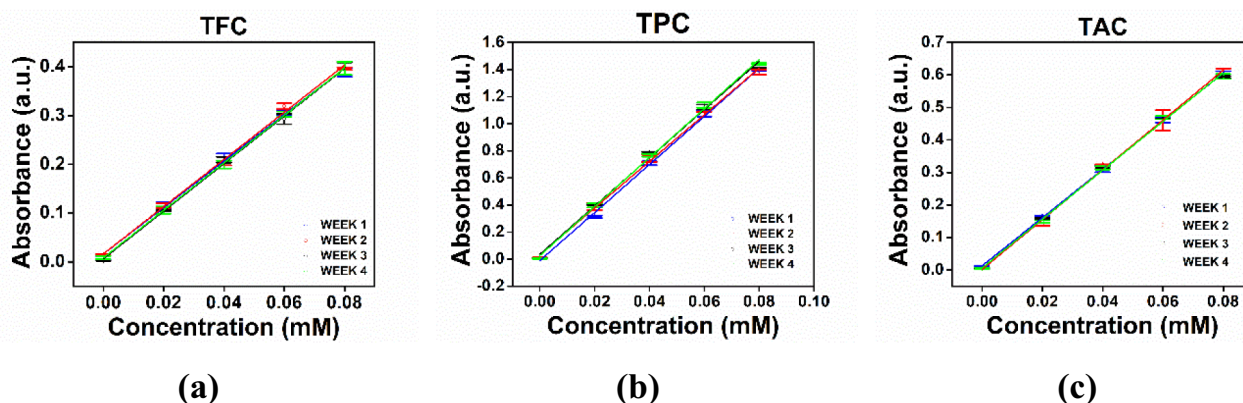


Fig. 4 Calibration curves for the UV-Vis spectrophotometric determination of (a) TFC, b TPC, and c TAC, evaluated over a 4-week period to assess method reproducibility

Table 4 Stability parameters and statistical analysis of TFC, TPC, and TAC in aqueous leaf extracts of *A. californica* over a 4-week storage period

Parameters	TFC [mg QE·g ⁻¹]	TPC [mg GAE·g ⁻¹]	TAC [mg GAE·g ⁻¹]
Average	30.4826 ± 17.15	13.948 ± 2.17	16.0048 ± 1.51
Variance	294.2181	4.7095	2.2885
%CV	56.27	15.56	9.45
ANOVA (<i>p</i> -value)	0.0150	6.2773E-05	6.5138E-06
% of change	-87.49	-29.05	-20.44

Table 5 Comparison of the initial TFC, TPC, and TAC values obtained for *A. californica* aqueous extracts with previously reported literature for the Saururaceae family

Specie	Part of the plant used	Solvent used	TFC [mg QE·g ⁻¹]	TPC [mg GAE·g ⁻¹]	TAC [mg GAE·g ⁻¹]	Ref
<i>Anemopsis californica</i>	Leaves	Methanol	15.8 ± 1.2	27.8 ± 0.9	-	Del-Toro-Sánchez et al. (2015)
<i>Anemopsis californica</i>	Stem	Methanol	23.7 ± 3.1	79.4 ± 3.5	-	Del-Toro-Sánchez et al. (2015)
<i>Anemopsis californica</i>	Rhizomes	Methanol	38.1 ± 1.9	92.3 ± 2.8	-	Del-Toro-Sánchez et al. (2015)
<i>Houttuynia cordata</i>	Leaves	Water	0.81 ± 0.01	4.80 ± 0.01	-	Moorthy et al. (2024)
<i>Saururus chinensis</i>	Root	Water	72.1 ± 0.1	67.1 ± 0.9	-	Alaklabi et al. (2018)
<i>Anemopsis californica</i>	Leaves	Water	30.4826 ± 17.15	13.948 ± 2.17	16.0048 ± 1.51	This work

in Table 4 reflect the stability study and should not be used as the main basis for inter-study comparison.

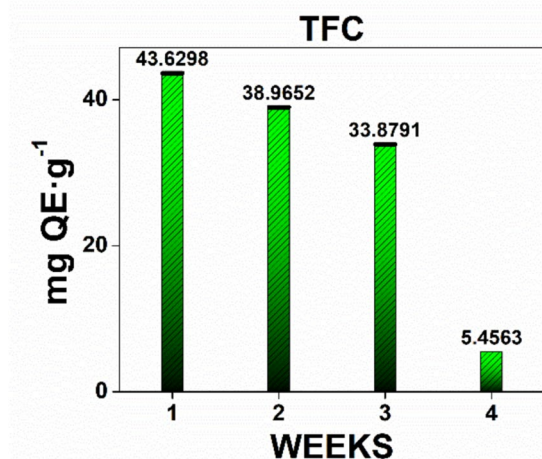
Total Flavonoid Content

The initial TFC of the aqueous leaf extract was 43.63 ± 0.03 mg QE·g⁻¹, and after 4 weeks of storage at 4 °C, it decreased to 5.46 ± 0.03 mg QE·g⁻¹ (Fig. 5), corresponding to an 87.49% reduction. As summarized in Table 4, TFC also showed the highest coefficient of variation among the three

responses, indicating that the flavonoid-related fraction was the least stable under the evaluated storage conditions. This pronounced decline is consistent with the known lability of flavonoids during storage, particularly when oxidation, light exposure, or matrix-dependent instability are involved.

When compared with the methanolic leaf extract reported by Del-Toro-Sánchez et al. (2015), the aqueous ultrasound-assisted extract analyzed here showed a higher initial TFC. This result suggests that, under the specific conditions used in the present work, the aqueous system was effective for recovering the flavonoid fraction measured by the aluminum chloride assay. However, the rapid decline during storage indicates that this initially favorable response did not translate into prolonged stability. This behavior is also consistent with recent studies showing that antioxidant-related plant constituents may exhibit markedly different stability profiles depending on compound class, matrix composition, and assay mechanism (Di Mattia et al. 2024).

Within the Saururaceae family, the initial TFC obtained in the present study was higher than that reported for aqueous leaf extracts of *Houttuynia cordata*, but lower than the value reported for aqueous root extracts of *Saururus chinensis* (Table 5). These differences are expected because flavonoid recovery is affected by species, plant organs, extraction conditions, and solvent polarity. Therefore, the present data supports a relatively strong initial flavonoid response in *A. californica* leaves under the selected aqueous extraction

**Fig. 5** Degradation of total flavonoids in *A. californica* stored for 4 weeks

conditions but also shows that this fraction is the most storage-sensitive of the three analytical responses.

Total Polyphenol Content

The initial TPC was 16.70 ± 0.01 mg GAE·g⁻¹ and decreased to 11.84 ± 0.05 mg GAE·g⁻¹ after 4 weeks at 4 °C (Fig. 6), corresponding to a 29.05% reduction. In contrast with TFC, the decrease in TPC was more moderate, indicating greater stability of the Folin-Ciocalteu-reactive fraction during refrigerated storage.

Compared with the methanolic leaf extract reported by Del-Toro-Sánchez et al. (2015), the present aqueous extract showed a lower initial TPC. This difference is analytically coherent and likely reflects solvent selectivity rather than an inconsistency of the plant matrix. Recent studies continue to show that solvent identity and concentration strongly influence the recovery of phenolic compounds and the associated antioxidant response, even within the same botanical matrix (Xiang et al. 2024). In this context, the lower TPC observed in the present work can be interpreted as a consequence of restricting the system to water, which is consistent with the green analytical rationale of the study but may underextract less water-soluble phenolics.

This point is especially relevant in light of the reviewer's comparison with Del-Toro-Sánchez et al. (2015). The present results suggest that water favored a comparatively stronger response for TFC than for TPC under the selected extraction conditions, whereas methanol or aqueous ethanol mixtures may be more effective when the objective is broader recovery of phenolic subclasses. Thus, the current data not only supports the analytical validity of the aqueous method but also identifies solvent composition as a relevant variable for future studies.

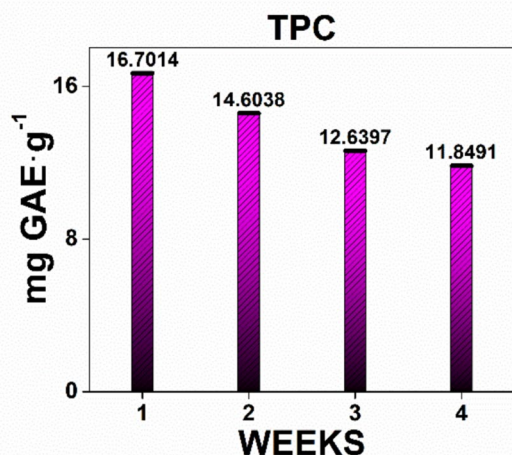


Fig. 6 Degradation of total polyphenols in *A. californica* stored for 4 weeks

Total Antioxidant Capacity

The initial TAC determined by the phosphomolybdenum assay was 17.93 ± 0.32 mg GAE·g⁻¹ and decreased to 14.26 ± 0.03 mg GAE·g⁻¹ after 4 weeks (Fig. 7), corresponding to a 20.44% reduction. According to Table 4, TAC showed the lowest coefficient of variation and the smallest relative loss during storage, indicating that the overall reducing capacity of the extract was more stable than the TFC and TPC responses.

This result is important because it shows that the three analytical responses did not evolve in parallel during storage. TAC remained comparatively more stable even though both TFC and TPC declined, suggesting that the phosphomolybdenum response was sustained by a broader set of reducing constituents than those measured exclusively by the aluminum chloride or Folin-Ciocalteu assays. Recent critical evaluations of spectrophotometric antioxidant methods have emphasized that different assays reflect different reaction mechanisms and should therefore be interpreted as complementary rather than interchangeable (Knez et al. 2025). Likewise, recent stability studies have shown that antioxidant capacity may remain comparatively preserved even when individual phenolic fractions decline, depending on the chemical structure of the compounds and the underlying assay chemistry (Di Mattia et al. 2024).

Accordingly, the lower percentage loss observed for TAC should not be interpreted as a contradiction with the declines in TFC and TPC, but rather as evidence that the aqueous extract retained a measurable global reducing capacity despite compositional changes during storage. This distinction strengthens the analytical interpretation of the storage study and avoids overgeneralization from a single assay.

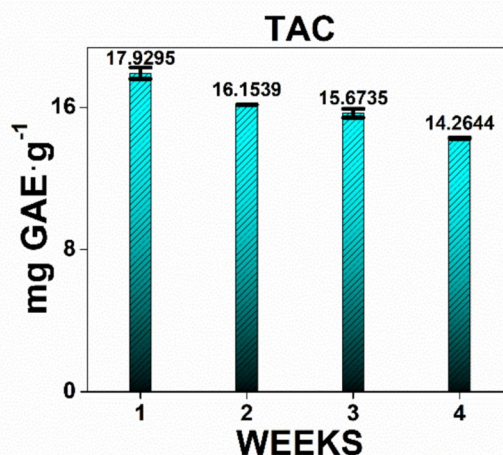


Fig. 7 Degradation of total antioxidant compounds in *A. californica* stored for 4 weeks

Antioxidant Activity Assessed

To strengthen the antioxidant characterization of the aqueous extracts, DPPH radical scavenging activity was determined in extracts prepared under identical conditions from two sources of *A. californica* leaves, collected and commercially leaves acquired from a local herbal market. As shown in Fig. 8, the extracts prepared from collected leaves exhibited substantially higher DPPH radical scavenging activity ($53.41 \pm 0.12\%$) than those prepared from commercial leaves ($29.30 \pm 1.36\%$). Because both sets of extracts were obtained under the same extraction conditions, this difference is most likely associated with differences in the source and pre-processing condition of the plant material. Recent studies have shown that post-harvest treatments, including drying and storage, may significantly affect phenolic composition and antioxidant response, which supports the interpretation that the antioxidant behavior of the present extracts depended not only on the extraction procedure, but also on the condition of the starting material (Gómez-Mejía et al. 2023).

DPPH results should be interpreted as complementary to TAC rather than directly comparable to it, since the DPPH assay evaluates radical scavenging capacity under a specific reaction environment, whereas the phosphomolybdenum method reflects total reducing capacity through a different mechanism. Recent methodological analyses have emphasized that spectrophotometric antioxidant assays do not measure the same antioxidant dimension and therefore should not be treated as equivalent endpoints (Knez et al. 2025). In this context, the DPPH assay provided additional evidence of the presence of redox-active constituents in *A. californica* aqueous leaf extracts and strengthened the antioxidant characterization of the study.

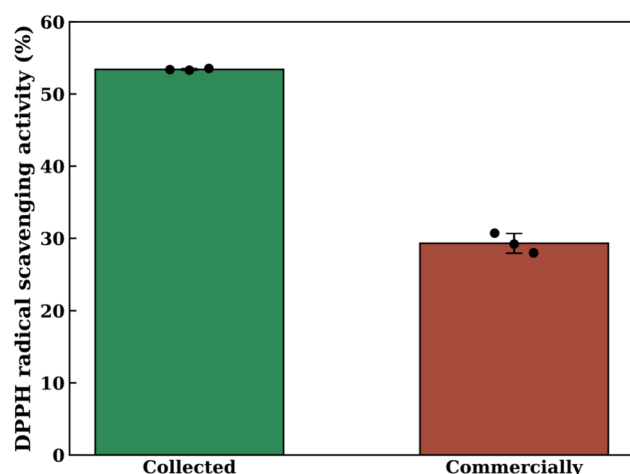


Fig. 8 DPPH radical scavenging activity of aqueous extracts prepared from collected and commercially *Anemopsis californica* leaves

Cytocompatibility of the Aqueous Extract

A preliminary in vitro cytocompatibility assessment was carried out using human dermal fibroblasts and J774A.1 murine macrophages exposed to aqueous *A. californica* leaf extract at concentrations ranging from 0.19 to 3.12 mg mL⁻¹. As shown in Fig. 9, fibroblast viability remained above the 70% threshold in all tested groups and, in several cases, exceeded 100%, whereas macrophage viability values remained close to, but still above, the same threshold. According to ISO 10993-5-based interpretation, treatment is considered cytotoxic when it reduces cell viability by more than 30% relative to the control. Therefore, under the evaluated conditions, the tested aqueous extract did not show evidence of cytotoxicity in either cell model (Gruber and Nickel 2023).

The response observed in fibroblasts is particularly relevant because viability values above 100% may suggest that the extract did not interfere with basal cellular metabolic activity and may even have favored cell proliferation under the tested conditions. In macrophages, viability showed a slight decrease relative to the control, although all values remained within the non-cytotoxic range. This difference between cell types may be related to the intrinsic biological characteristics of macrophages, including their phagocytic activity and their greater sensitivity to external stimuli. Therefore, the present data indicates that the aqueous extract was cytocompatible within the tested concentration range and exposure time.

These findings are consistent with previous reports on *A. californica*. Nguyen et al. (2021) reported that an ethanolic extract of this species did not reduce the viability of human dermal fibroblasts and additionally protected cells against oxidative damage. The same study also showed reduced inflammatory markers in LPS-stimulated

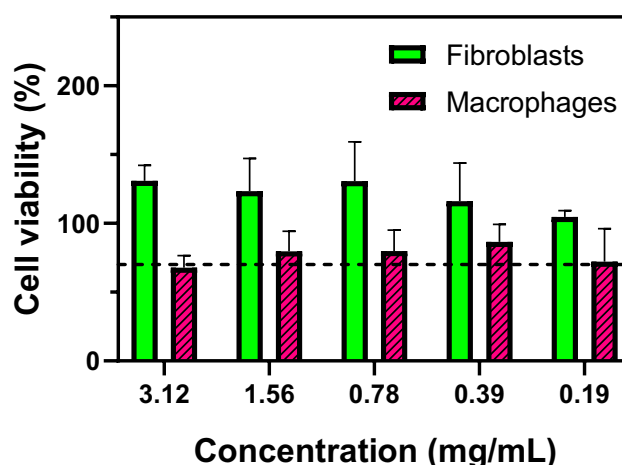


Fig. 9 Cell viability in fibroblasts and macrophages exposed to aqueous leaf extract of *A. californica*

RAW264.7 macrophages, suggesting antioxidant and anti-inflammatory effects mediated through NRF2 activation in fibroblasts and NFATc1 repression in macrophages. However, the present assay was performed only under basal conditions and not in an inflammatory stimulation model; therefore, the current results do not allow direct attribution of anti-inflammatory activity or the establishment of specific molecular mechanisms.

These results are useful as a preliminary complement to the analytical characterization of the extract, but they should be interpreted conservatively. The present essay was performed under basal conditions and was intended only to evaluate short-term cytocompatibility. Therefore, these data do not demonstrate anti-inflammatory activity, wound-healing efficacy, or clinical safety. Their contribution is more limited and more defensible: the analytically characterized aqueous extract did not reduce viability below the accepted cytotoxicity threshold in the two tested cell models. In the context of the manuscript, this subsection should remain secondary to the analytical contribution of the work.

Taken together, the results show that aqueous ultrasound-assisted extraction using 0.125 g of leaf material in 10 mL of water provided the most suitable condition for the simultaneous analytical determination of TFC, TPC, and TAC in *A. californica* leaves. The validated UV-Vis methods showed adequate linearity, sensitivity, accuracy, and reproducibility for rapid group-level analysis, while the storage study demonstrated that TFC, TPC, and TAC do not behave identically over time. The complementary DPPH assay confirmed antioxidant activity by an additional spectrophotometric endpoint, and the preliminary cell-based assay indicated the absence of cytotoxicity under the evaluated conditions. At the same time, the present work remains an analytical and exploratory study; therefore, detailed phenolic profiling and mechanism-oriented biological studies are still required for deeper characterization of the extract.

Conclusion

In this study, UV-Vis spectrophotometric methods were validated for the determination of total flavonoid content, total polyphenol content, and total antioxidant capacity in aqueous leaf extracts of *Anemopsis californica* obtained by ultrasonic probe-assisted extraction. The validated assays showed adequate linearity, sensitivity, accuracy, and reproducibility under the selected analytical conditions, supporting their suitability for routine group-level evaluation of antioxidant-related responses in this plant matrix. Among the evaluated extraction conditions, 0.125 g of leaf material in 10 mL of water provided the most favorable analytical response for the simultaneous determination of TFC, TPC, and TAC.

Under these conditions, the extract showed an initial TFC of 43.63 ± 0.03 mg QE·g⁻¹, a TPC of 16.70 ± 0.01 mg GAE·g⁻¹, and a TAC of 17.93 ± 0.32 mg GAE·g⁻¹. During storage at 4 °C for 4 weeks, the three responses did not behave similarly: TFC showed the greatest decrease, whereas TPC and especially TAC were comparatively more stable. These findings indicate that the antioxidant-related profile of the aqueous extract is affected by storage time and that flavonoid-associated responses are the most labile under the evaluated conditions. The complementary DPPH assay confirmed the radical scavenging activity of the aqueous extracts and showed differences between extracts prepared from collected leaves and those obtained from commercially dried leaves, indicating that the source and pre-processing condition of the plant material influenced the antioxidant response. In addition, the preliminary in vitro cytocompatibility assessment showed no evidence of cytotoxicity toward human dermal fibroblasts or J774A.1 macrophages under the tested conditions, supporting the low cytotoxicity of the evaluated aqueous extract. Overall, the present work provides a validated analytical basis for the study of aqueous *A. californica* leaf extracts and supports their further investigation as a source of antioxidant-related compounds. However, detailed phenolic profiling, comparative solvent studies, and mechanism-oriented biological assays are still required for a deeper characterization of the extract and its potential applications.

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Author Contribution Graciela Sosa-España: Conceptualization, Methodology, Investigation, Writing – original draft, Data curation. Victor Rodriguez-Orozco: Conceptualization, Methodology, Investigation, Writing – original draft, Data Curation. Benjamin Valdez-Salas: Conceptualization, Writing – review and editing, Supervision, Funding acquisition, Project administration. Jhonathan Castillo-Saenz: Writing – original draft, Investigation, Writing – review and editing, Supervision. Ernesto Beltrán-Partida: Writing – review and editing, Supervision, Project administration. Jorge Salomón-Carlos: Review and editing, Data curation, Roberto Gamboa-Becerra: Writing – review and editing, Data curation.

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Data Availability All data are available from the corresponding author upon request.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing interests The authors declare no competing interests.

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Authors and Affiliations

Graciela Sosa-España¹ · Víctor Rodríguez-Orozco¹ · Benjamín Valdez-Salas¹ · Jhonathan Castillo-Saenz¹ · Ernesto Beltran-Partida¹ · Jorge Salvador-Carlos¹ · Roberto Gamboa-Becerra¹

✉ Benjamín Valdez-Salas
benva@uabc.edu.mx

✉ Jhonathan Castillo-Saenz
jhonathan.saenz@uabc.edu.mx

¹ Core Facilities de Química y Materiales Avanzados, Instituto de Ingeniería, Universidad Autónoma de Baja California, Mexicali 21280, Baja California, Mexico